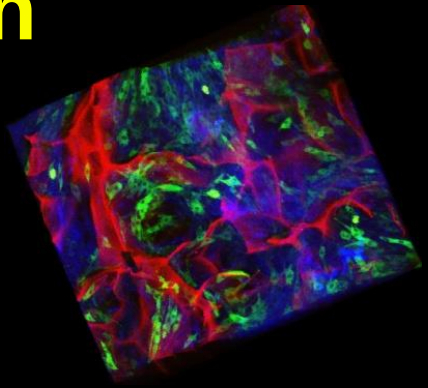
Extended Time-Lapse Multimodal Microscopy for Tracking Cell and Tissue Dynamics in Skin



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University of Illinois at Urbana-Champaign

April 10, 2014

Principles of Fluorescence Techniques
ISS Fluorescence Foundation

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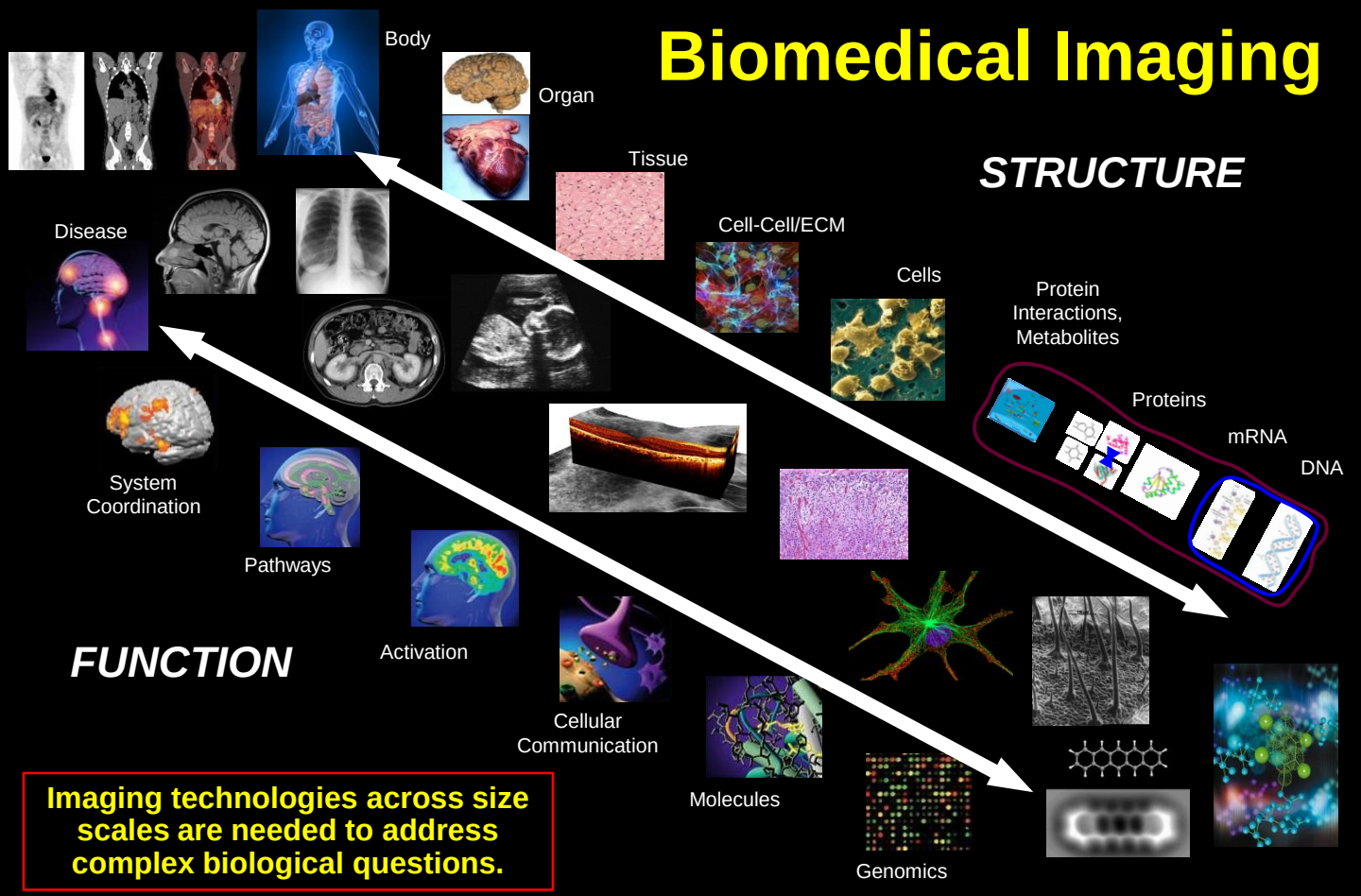
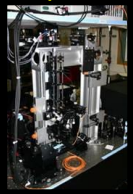
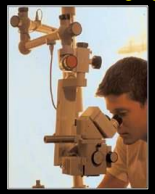
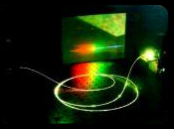
Biomedical Imaging



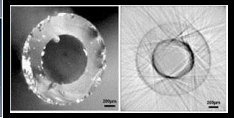
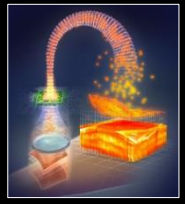
Imaging technologies across size scales are needed to address complex biological questions.

Optical Detection and Imaging Systems

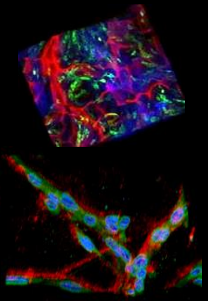
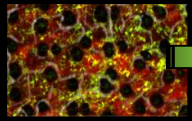
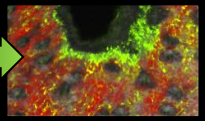





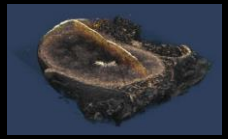
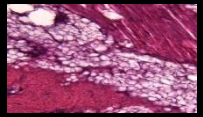
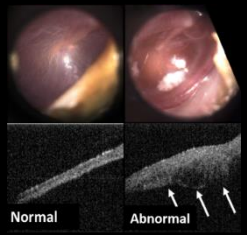
Computational Optical Microscopy

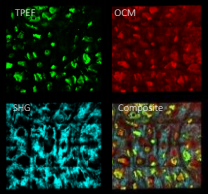
Advanced Microscopy of 3-D Cell Dynamics, Engineered Tissues, Regeneration, and Cancer

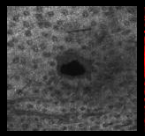
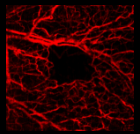
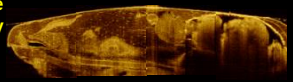
Translational Clinical Imaging Studies

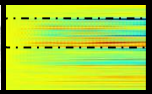
Neurophotonics



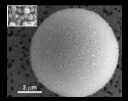
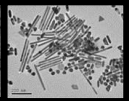
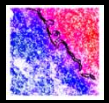
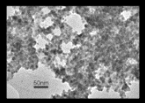
High-Resolution Structural and Functional Optical Coherence Tomography

Optical Elastography Biomechanics



Molecular Imaging Techniques and Contrast Agents

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**

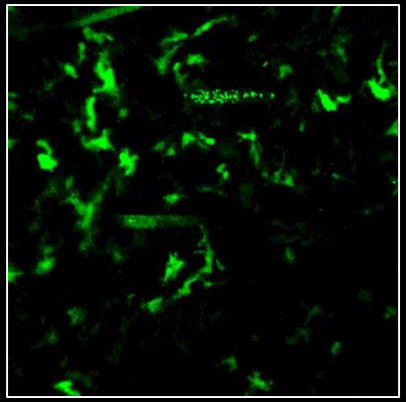
Introduction to Intravital Microscopy in Cell Biology Research

Intravital microscopy

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

What are the current limitations?

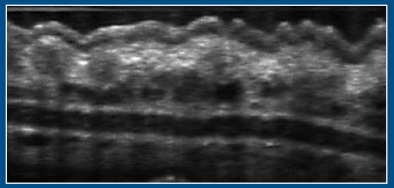
- **Limited contrast**
  - **Minimize the use of contrast agents**
  - **Live tissue has many cellular and extracellular components**
- **Limited time window**
  - **Continuous imaging limited to a few hours**
  - **Difficult to co-register time-lapse data at cellular resolutions**



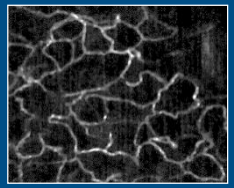
Time-lapse multiphoton microscopy of GFP labeled cells in mouse skin

Integrated optical coherence tomography (OCT), multiphoton microscopy (MPM), and fluorescence lifetime imaging microscopy (FLIM)

OCT

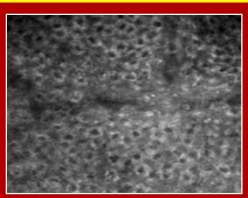


Scattering intensity:
- tissue structure

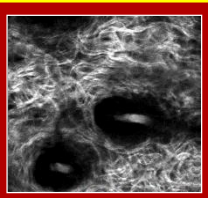


Phase sensitivity:
- vasculature

MPM

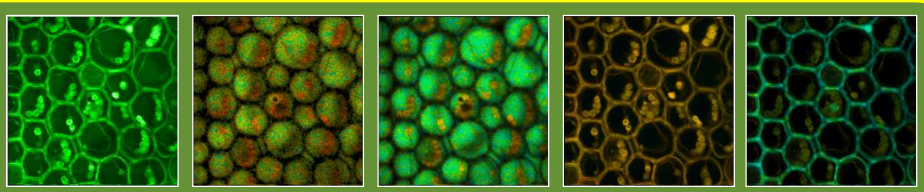


Two photon excited fluorescence:
- endogenous proteins, fluorescent molecules



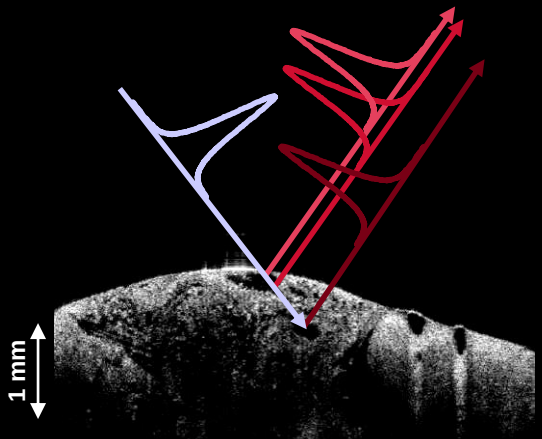
Second harmonic generation:
- collagen structure

FLIM



Fluorescence lifetime:
- metabolic function, molecular dynamics

Optical Coherence Tomography (OCT)



Analogous to ultrasound imaging, but with near-infrared light

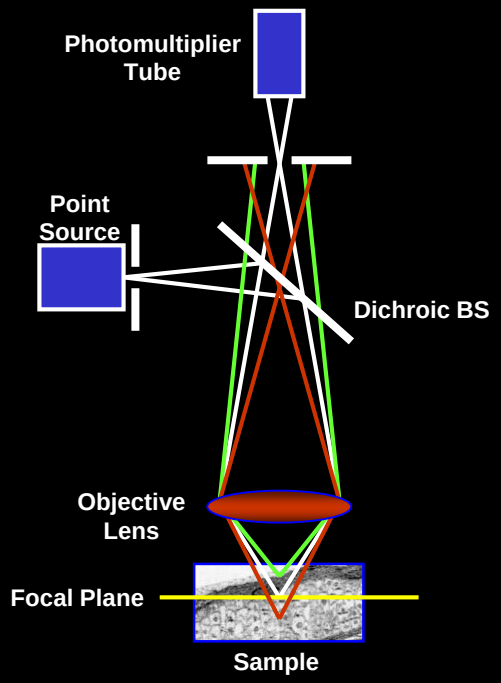
3-D Volume Acquisition

Temporal Acquisition

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

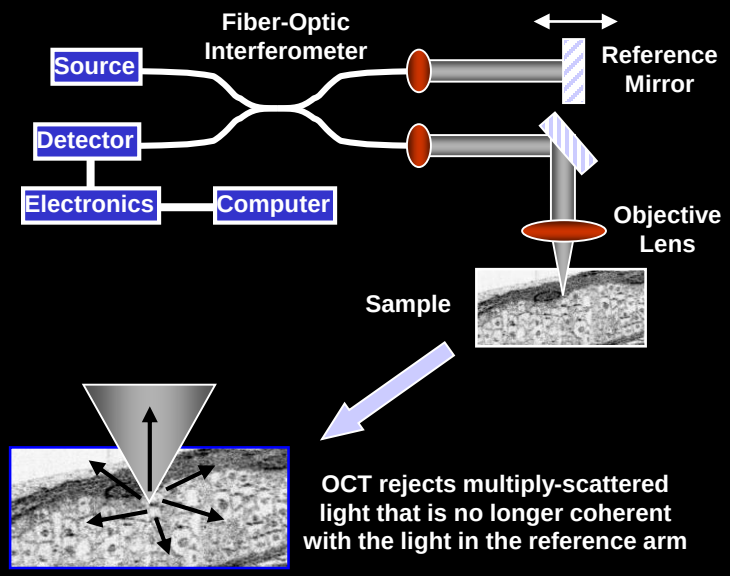
Comparison with Confocal Microscopy

Confocal Microscopy



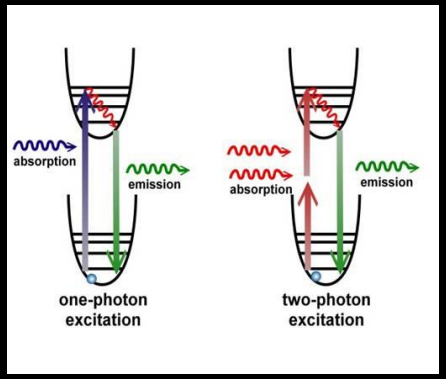
Spatial Rejection of Out-of-Plane Light

Optical Coherence Tomography



Spatial and Coherent Rejection of Out-of-Plane Light

Multi-Photon Microscopy

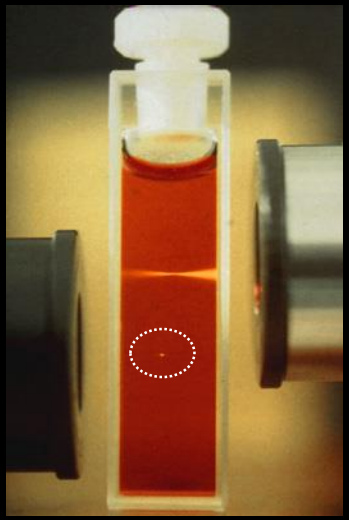


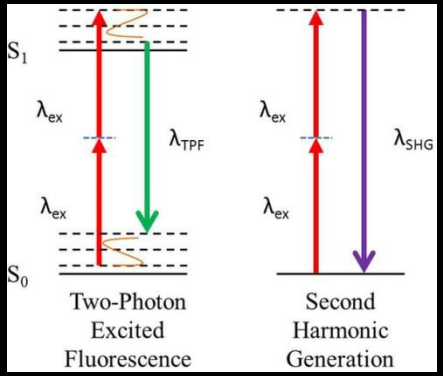
Advantages:

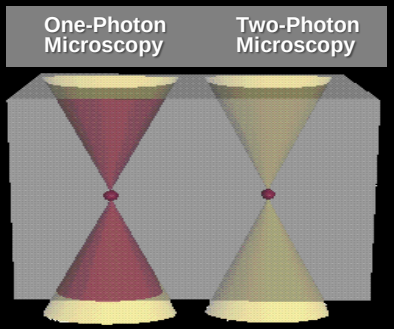
-Longer wavelength excitation
-Deeper penetration
-Less absorption/photodamage
-Less autofluorescence
-Higher resolution
-Easier ex/em separation

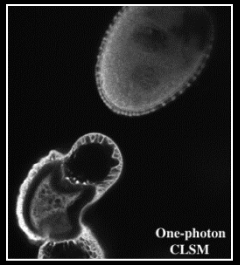
Disadvantages:

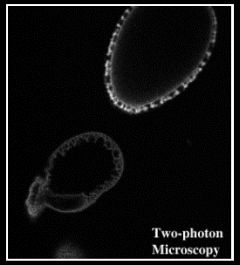
-Complexity
-Expensive laser
-Fewer probes
-Weaker signal











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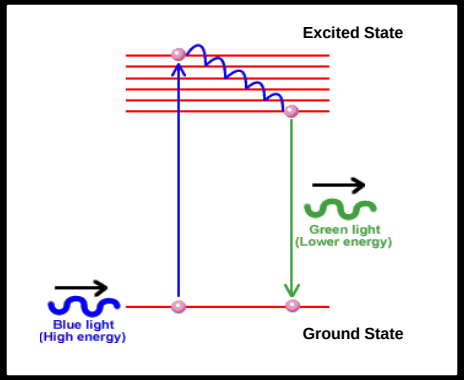
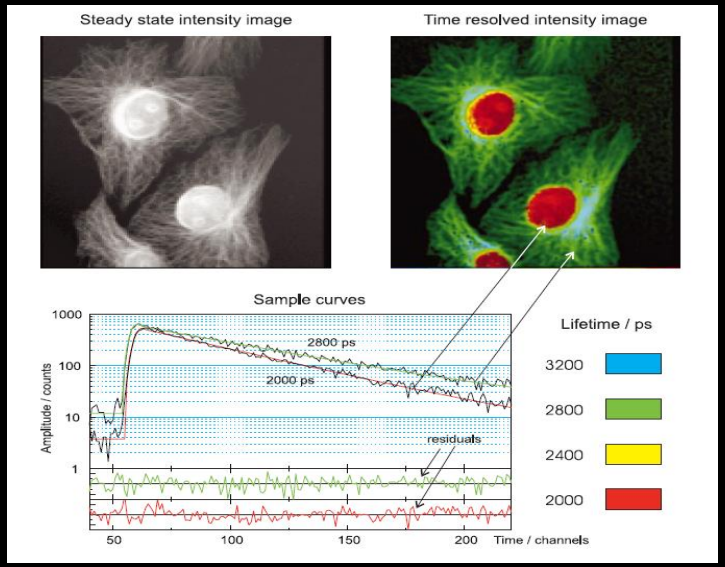
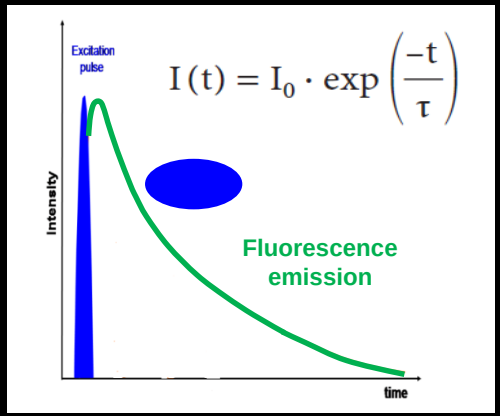
Fluorescence Lifetime Imaging Microscopy (FLIM)







- **Intrinsic properties of isolated molecules**
- **To probe molecular changes in biological environment (pH, ion concentration, protein conformation, protein-protein interaction, etc..)**

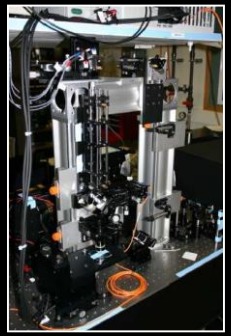
Becker & Hickl GmbH, Berlin, "Lifetime Imaging Techniques for Optical Microscopy"

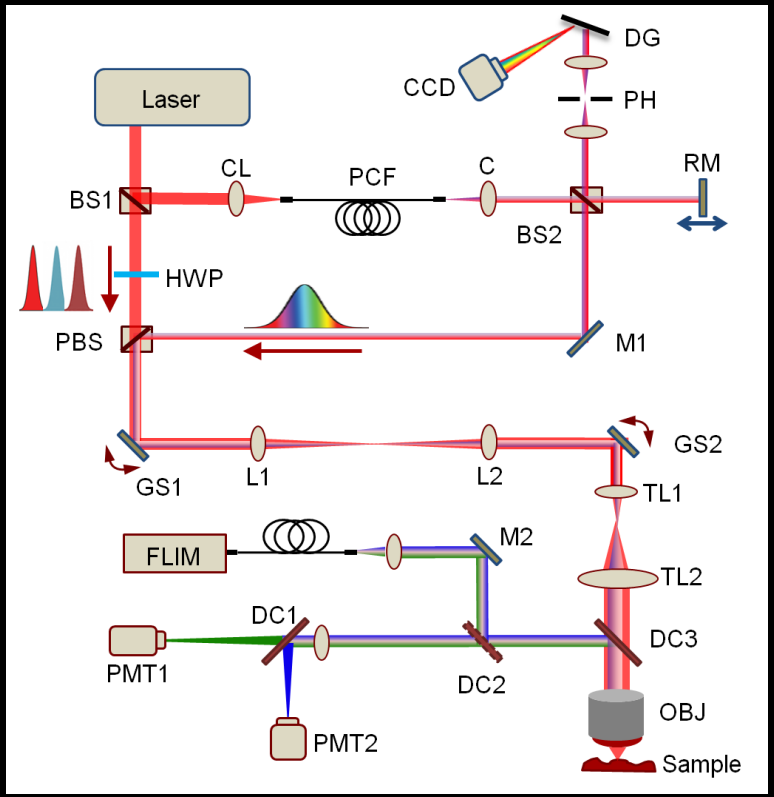
Integrated Microscope Combining MPM, OCM, and FLIM

- **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)

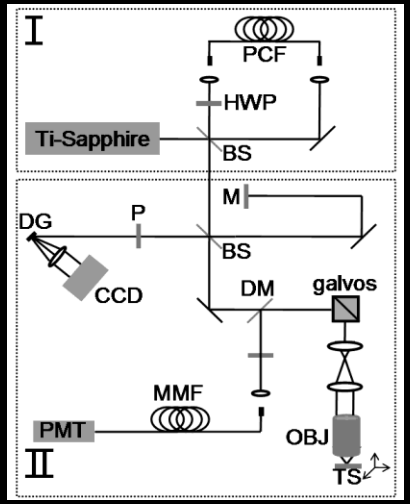
System specifications
- NA: 0.85 (MPM), 0.05 (OCT)
- 920 nm center wavelength
- OCT spectral bandwidth: 80 nm
- CCD max line rate: 32 kHz

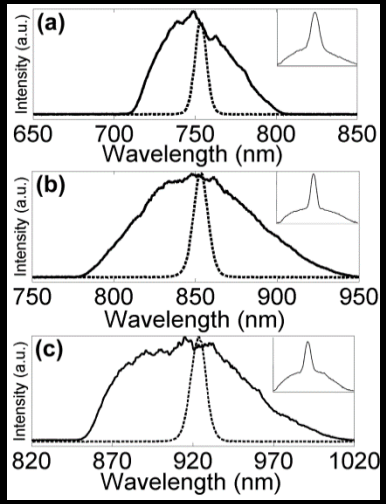


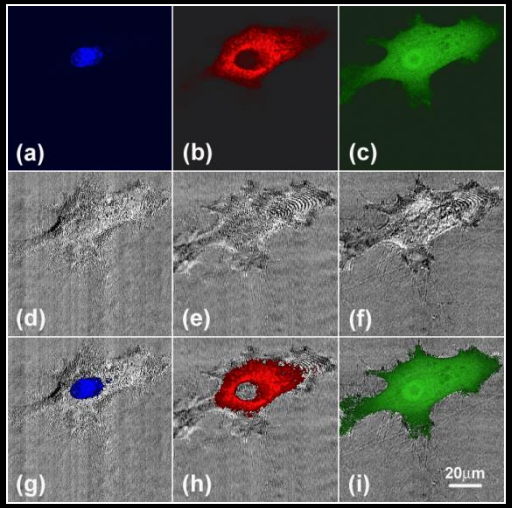


Vinegoni, et. al., Appl. Phys. Lett., 88:053901, 2006
Xu, et. al., Opt. Lett., 31:1079, 2006

Dual-Spectrum Laser Source for Multimodality Microscopy







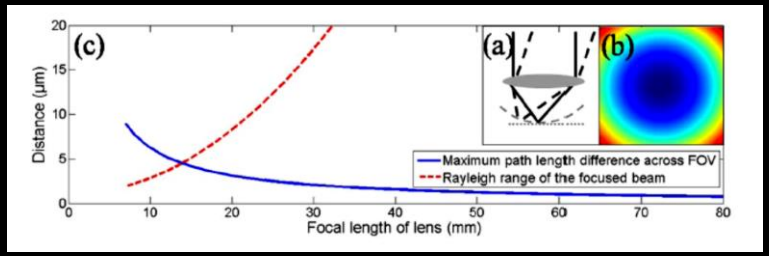
750 nm 850 nm 920 nm

GFP-vinculin fibroblast co-stained with rhodamine and nuclear dye

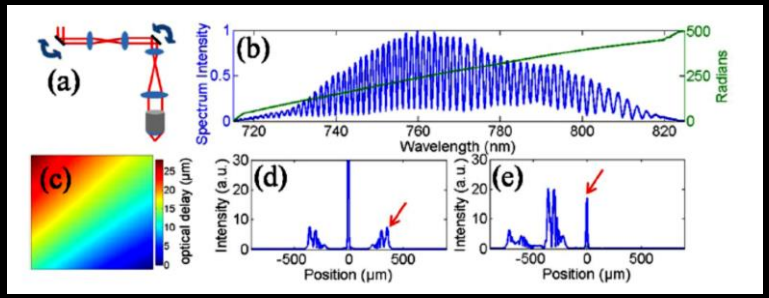
Nonlinear Fiber – 2 m LMA-8 Fiber (Crystal Fibre)
Tunable Ti:sapphire (MaiTai HP, Spectra-Physics)
Noise: Less than 1.1 dBm/Hz difference between pump and broadened beam over 1 Hz to 25 MHz

Graf, et al., J Biomedical Optics, 14:034019, 2009

Coherence Gate Curvature Correction in High Numerical Aperture Optical Coherence Imaging

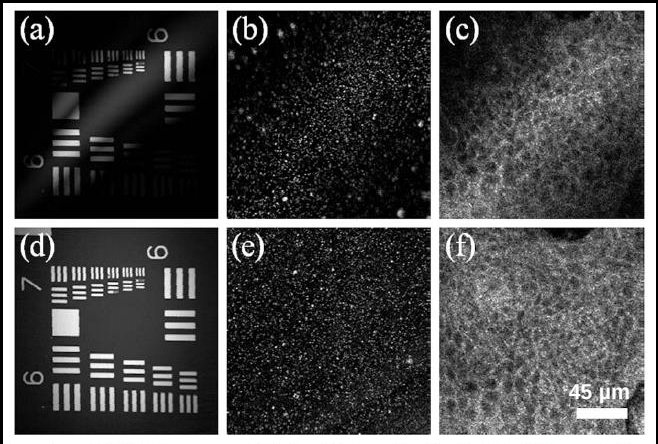


Simplified non-telecentric beam-scanning system
2-D *en face* plane shows curved coherence gate
Maximum pathlength difference across FOV (blue)
Rayleigh range of focused beam (red)



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

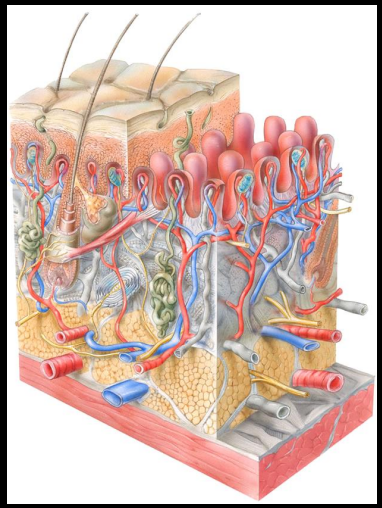
USAF resolution chart | Silicon gel & 50 nm iron-oxide nanoparticles | *In vivo* human epidermis

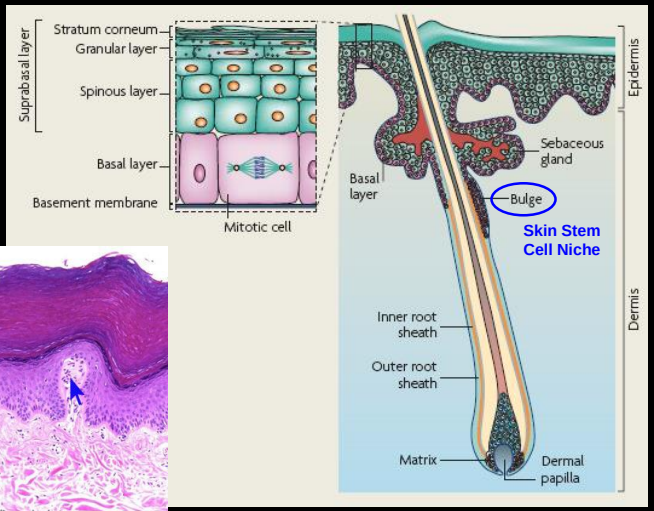


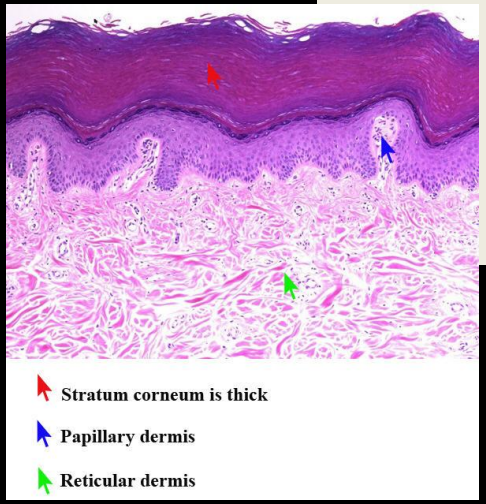
Top Row: Standard imaging
Bottom Row: Corrected curvature

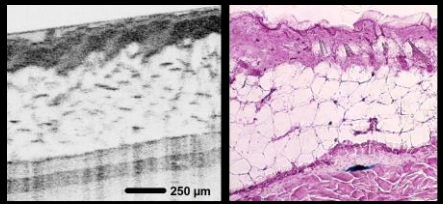
Graf, et al., Opt. Lett., 35:3120, 2010

Architectural Morphology and Histology of Skin

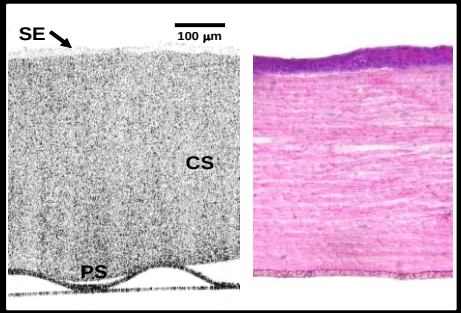






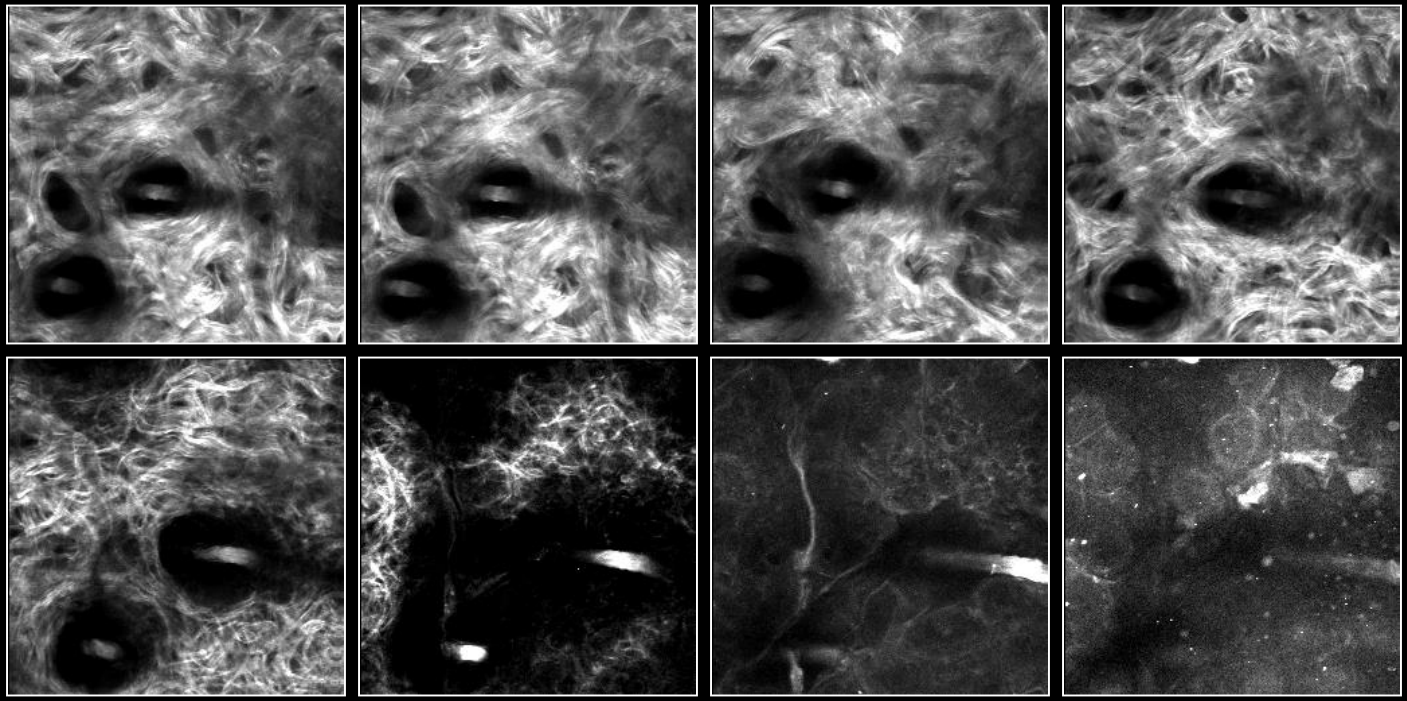


OCT and Histology of Murine Skin



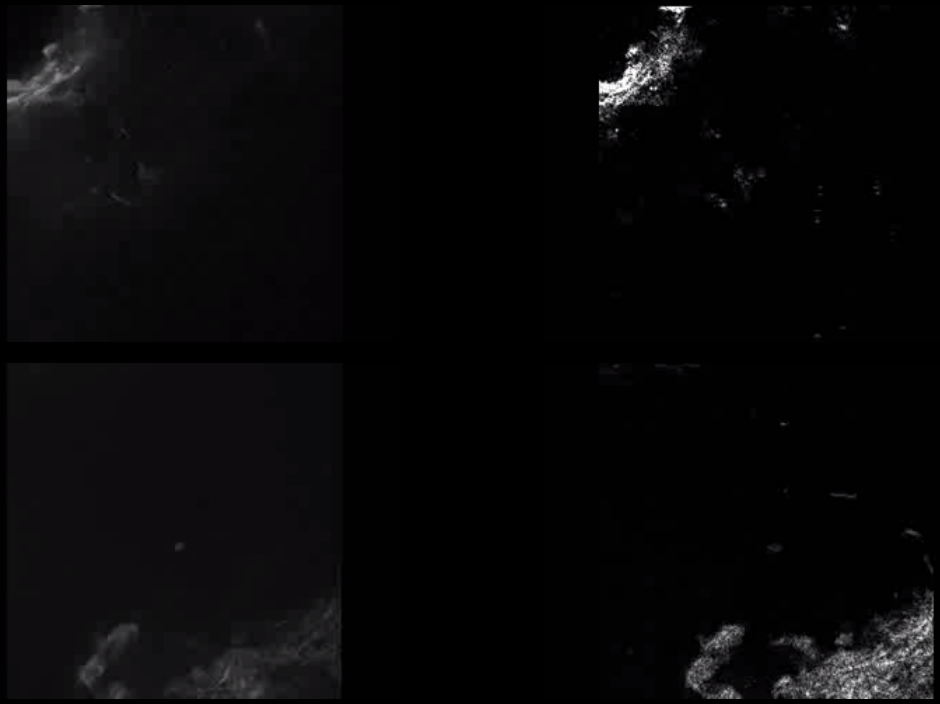
OCT and Histology of Engineered Skin

Multiphoton Microscopy of Hair Follicles in Murine Skin



Multimodality 3-D Imaging of Murine Skin

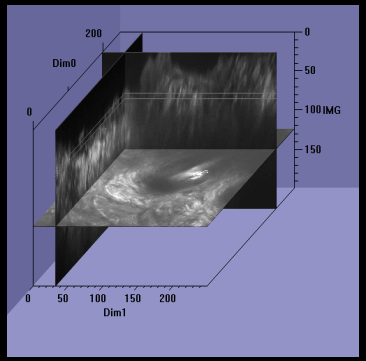
MPM Stacks **OCM Stacks**

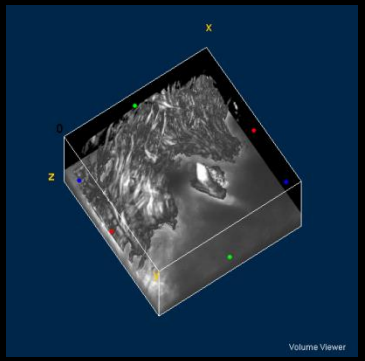


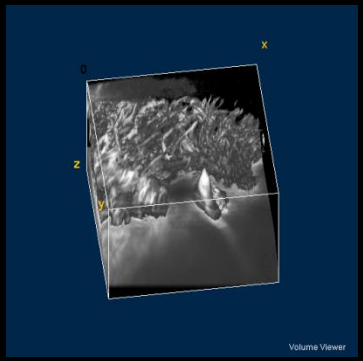
Collagen Network, Single Hair Shaft and Follicle to Depth ~ 150 µm

3-D Multimodality Imaging of Murine Skin

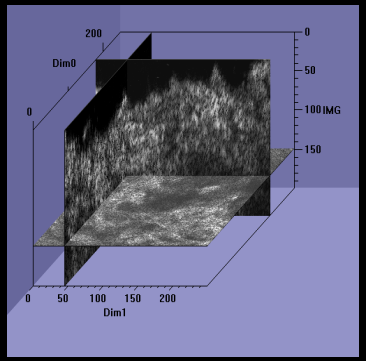
MPM

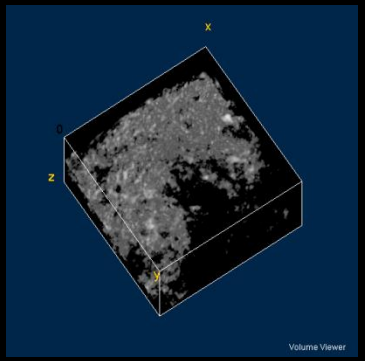


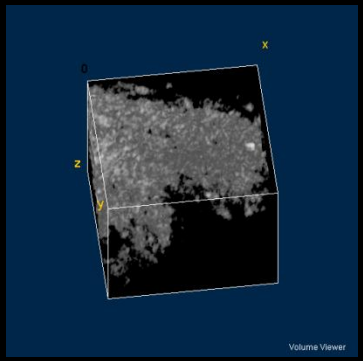




OCM

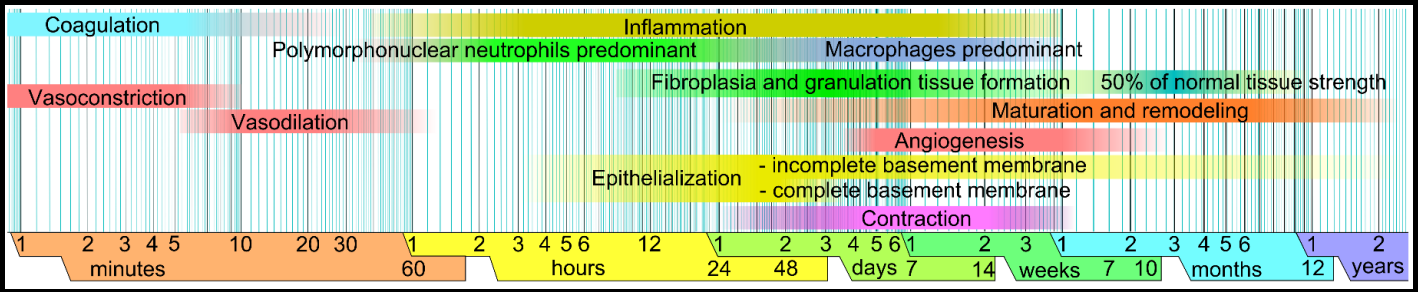






Time-Lapse Multimodal Microscopy of Cutaneous Wound Healing in Mice

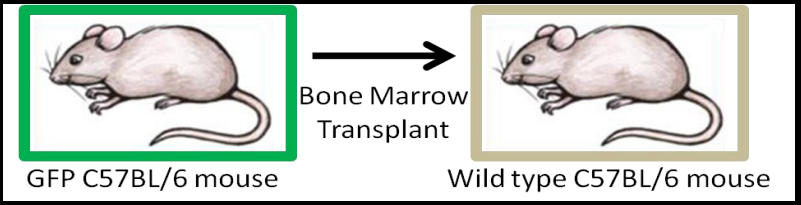
- Bone marrow cells in wound healing
  - Traditionally associated with coagulation and inflammation
  - Recently demonstrated to contribute stem cells
    - Differentiation into keratinocytes, fibroblasts and endothelial cells
    - Poorly understood and inconclusive evidence
- Time-lapse OCT-MPM of wound healing
  - Comprehensive view of wound healing processes
  - Correlation with bone-marrow-derived cell dynamics

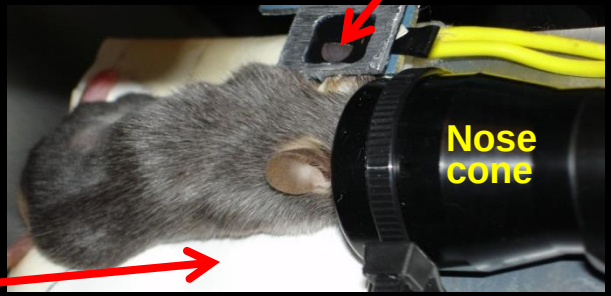


http://en.wikipedia.org/wiki/Wound_healing

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

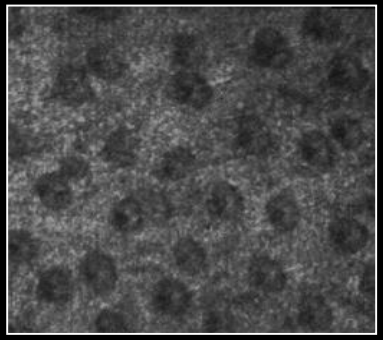




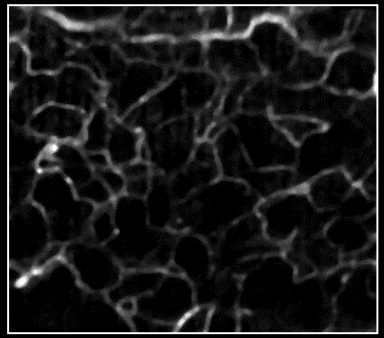
OCT Imaging of GFP Bone Marrow Transplanted Mouse Ear Skin

- 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

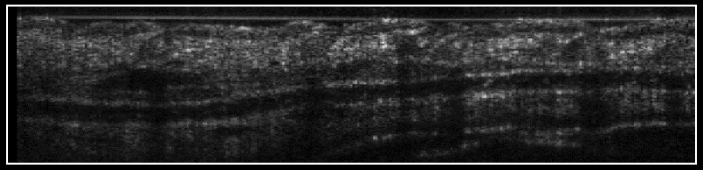
***En face* OCT**



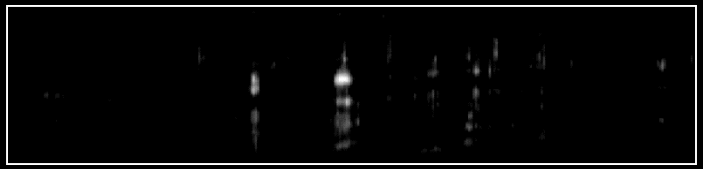
***En face* phase variance**



OCT intensity cross section



Phase variance cross section

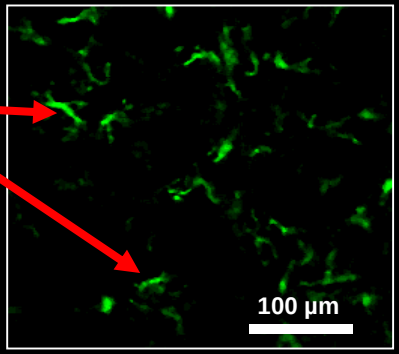


MPM Imaging of GFP Bone Marrow Transplanted Mouse Ear Skin

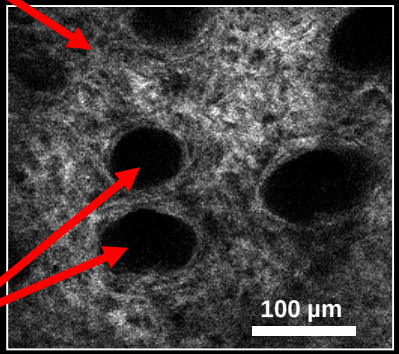
- Two MPM channels
  - GFP fluorescence (bone marrow cells)
    - Autofluorescence from hair is removed using segmentation
  - SHG (collagen fibers in dermis)

- 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

GFP channel

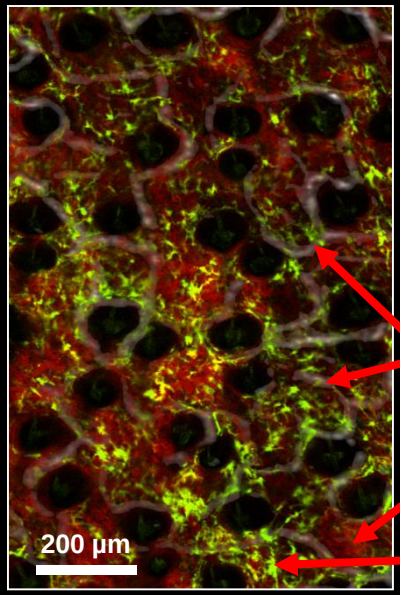


SHG channel



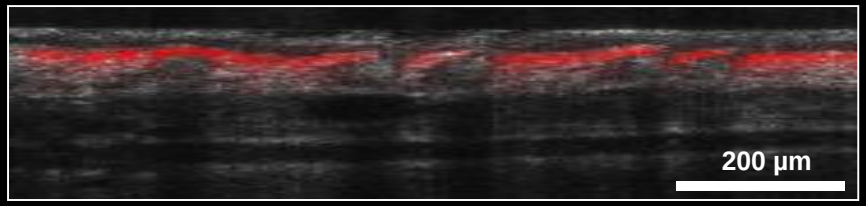
Hair follicles

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



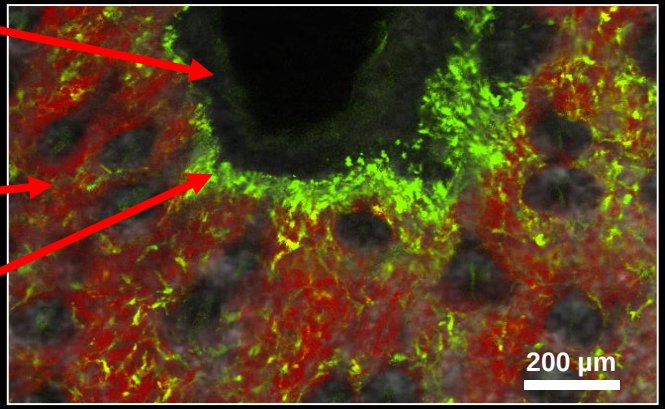
Skin cross section (OCT-SHG overlay)

wound bed

capillaries

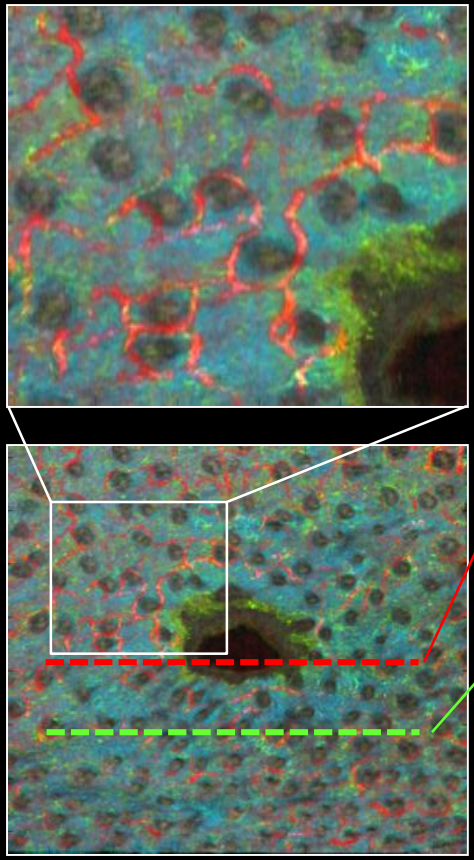
collagen

GFP cells

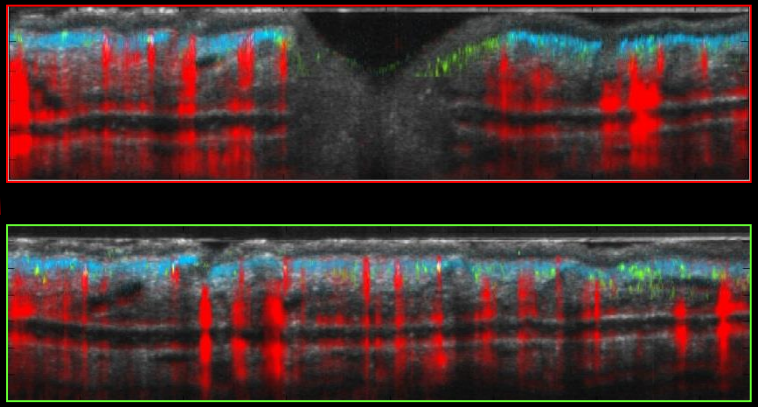


En face view of a skin wound
(OCT intensity-SHG-GFP overlay)

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

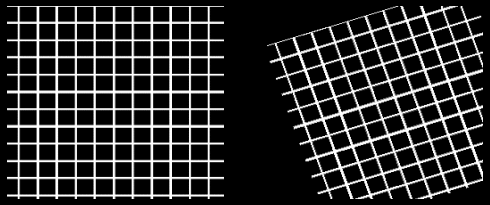
GFP bone-marrow-derived cells

SHG - collagen

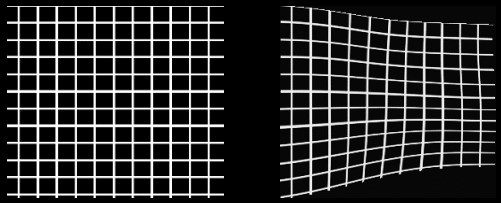
Visualizing Long-Term Dynamic Changes with Intravital Microscopy

- 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)

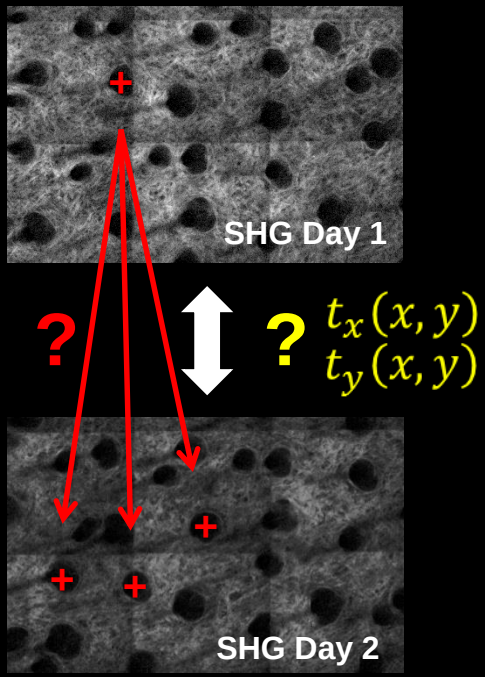


Non-rigid Registration (arbitrary deformation)



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 **correspondence** of the follicles and the image **transformation**



Non-Rigid Image Registration Algorithm based on Hair Follicle Spatial Patterns

Hair follicle detection

- Based on Difference of Gaussian (DOG) filtering and radial symmetry assessment

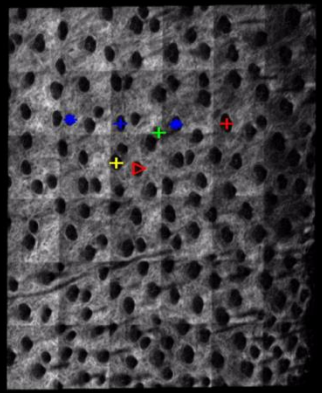
Hair follicle matching

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

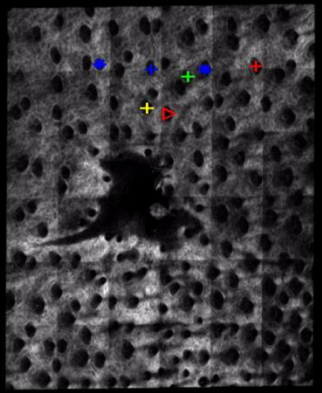
Iterative transformation update and follicle matching update

- Neighborhood metric
- Consistency with the current transformation

SHG Mosaic #0



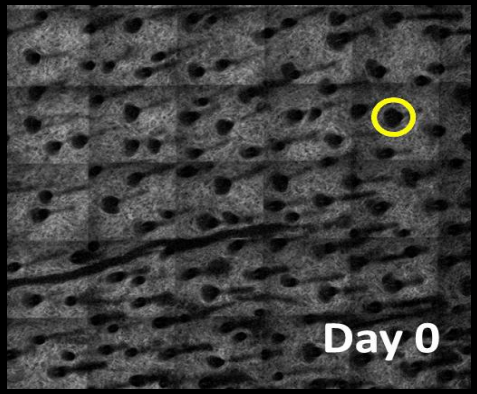
SHG Mosaic #1



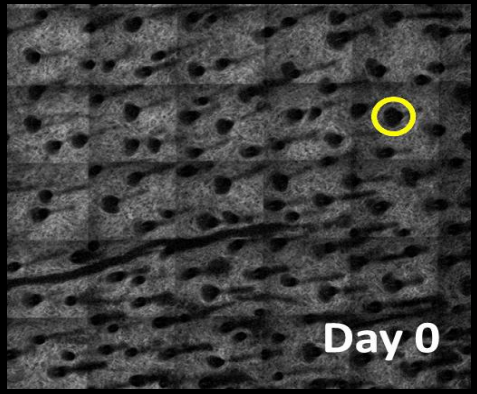
Non-Rigid Image Registration Example: Before and After Skin Wounding

SHG images: day 0 – prior to wound (reference)
day 1 – after wound (target)

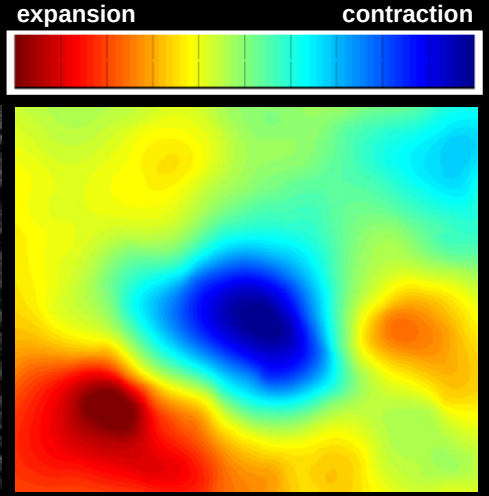
(unregistered)



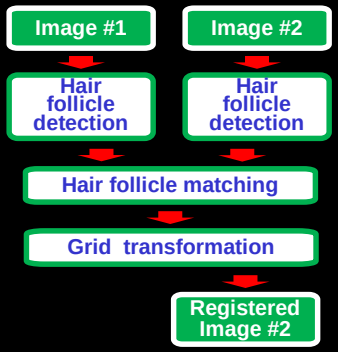
(registered)

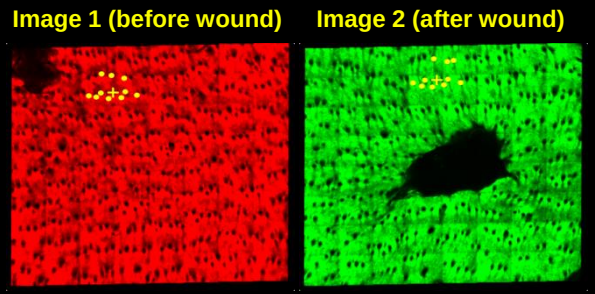


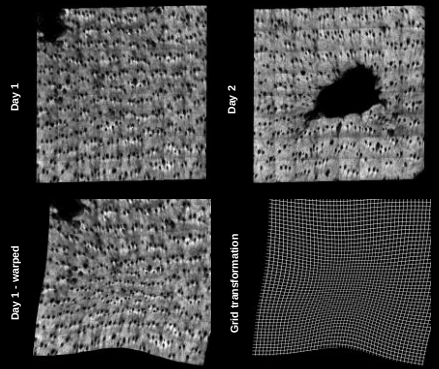
Non-rigid changes

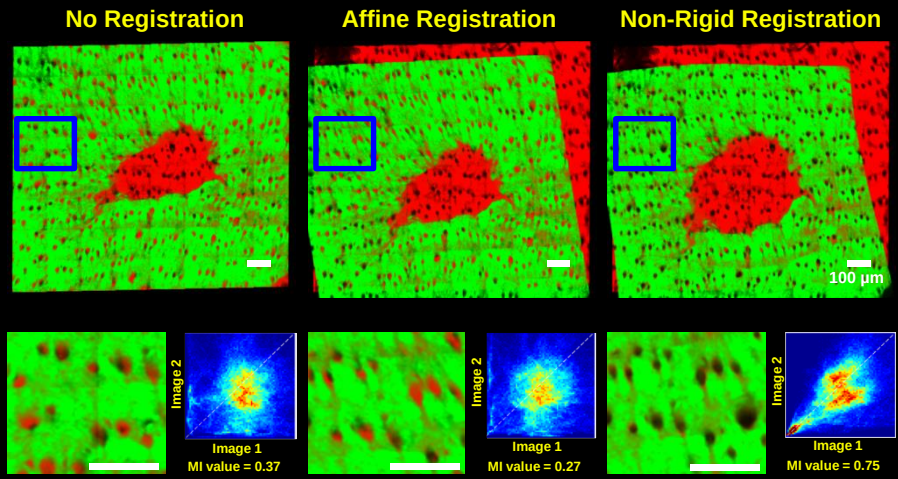


Non-Rigid Image Registration Assessment









Time-Lapse Imaging of Wound Healing

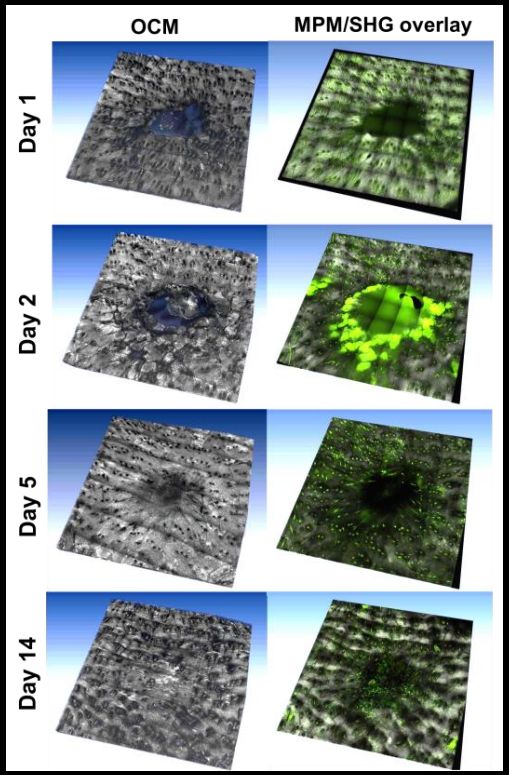
Integrated OCT-MPM and image registration enables multimodal time-lapse imaging of wound healing

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 *in vivo* skin regeneration

Spatially and temporally co-registered 3-D volumes

<u>OCM/OCT:</u> Tissue Structure

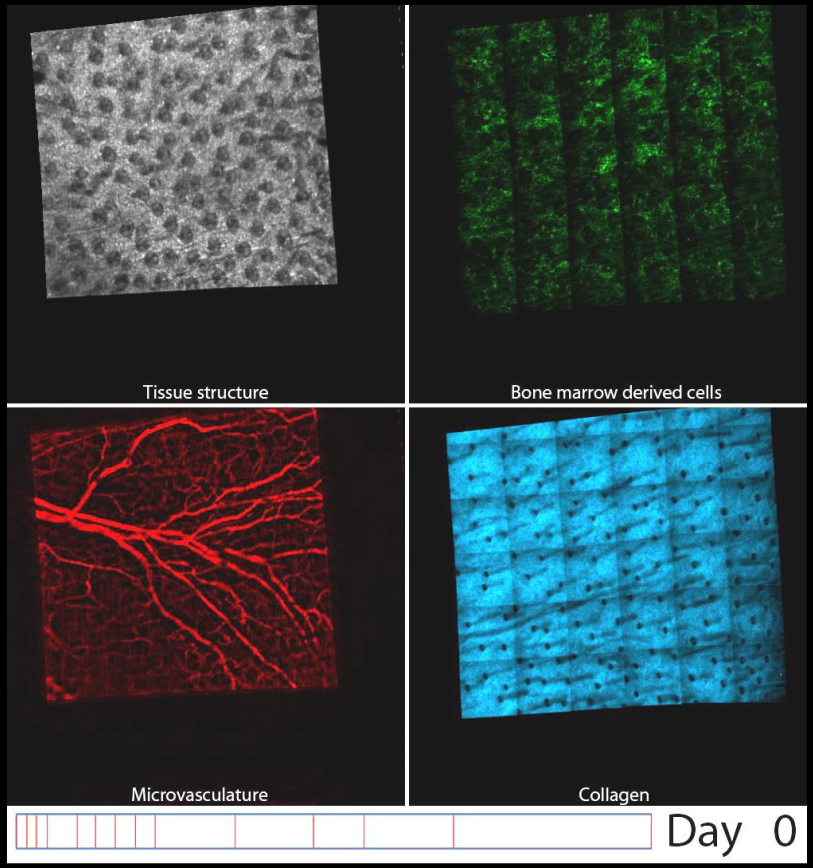
<u>Phase Variance:</u> Microvasculature

<u>TPEF:</u> GFP Bone marrow cells

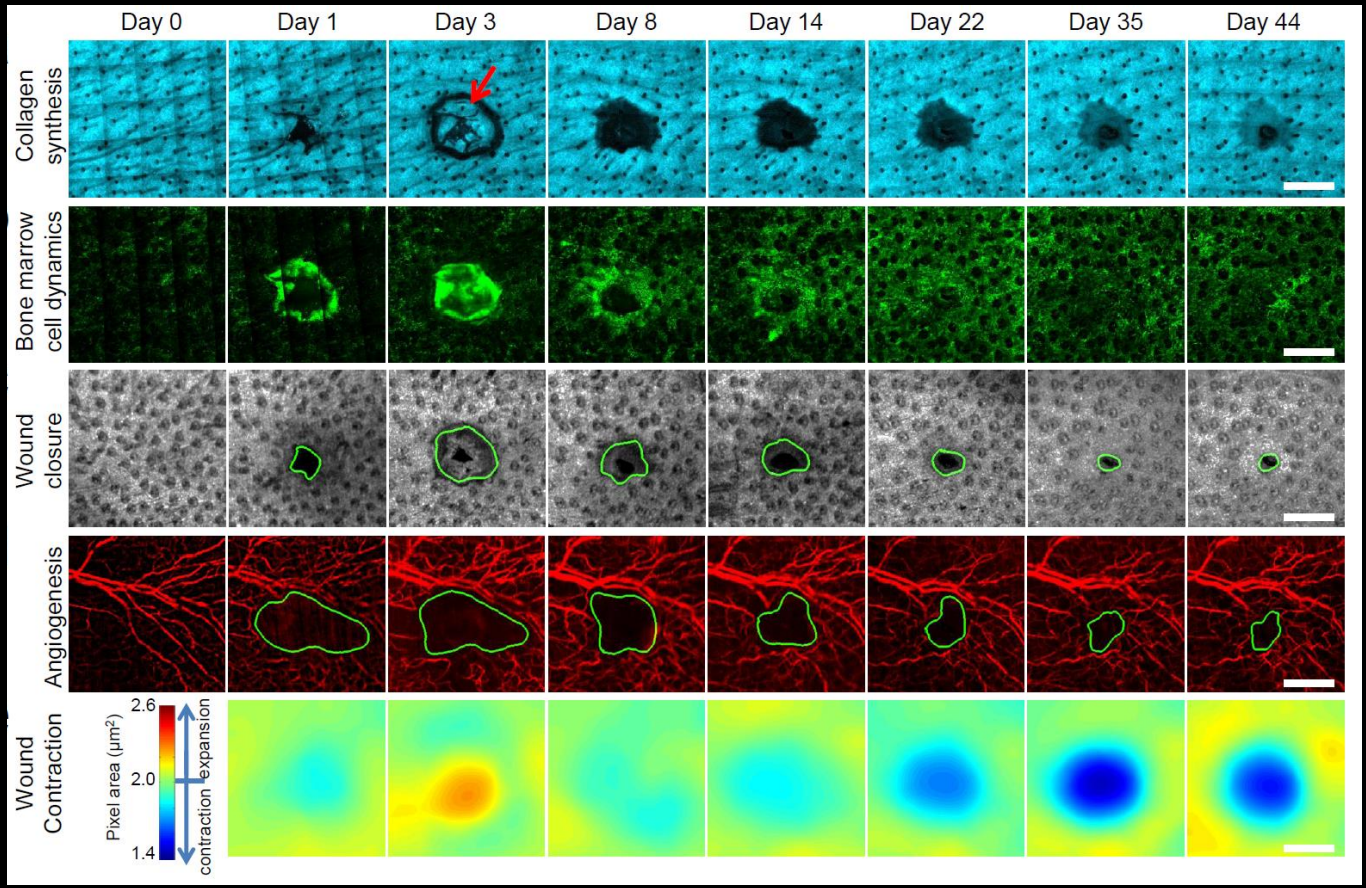
<u>SHG:</u> Collagen

<u>Non-rigid transformation matrix:</u> Biomechanical changes

Time-lapse tracking from minutes to over several months



Graf, *et al.*, Technology, 2013

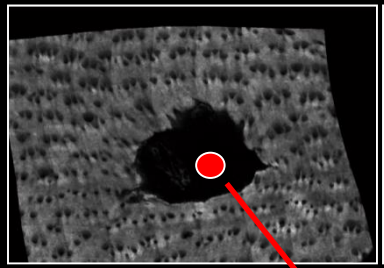


Graf, *et al*., Technology, 2013

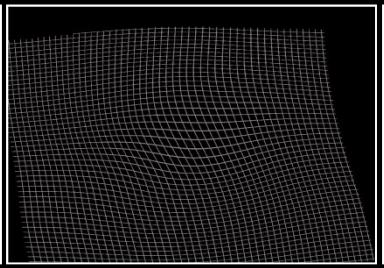
Quantification of Wound Contraction

Registration enables quantification of wound contraction

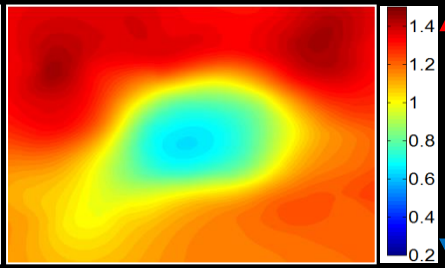
Warped mosaic



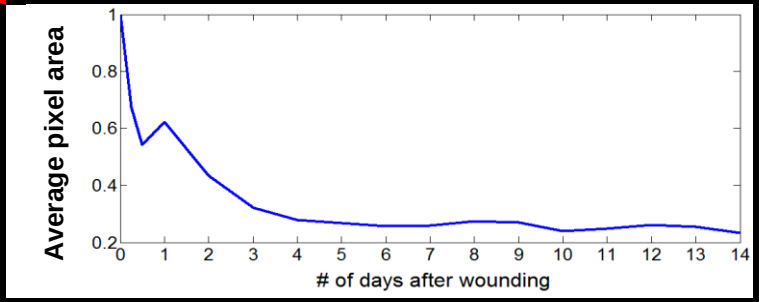
Warped grid



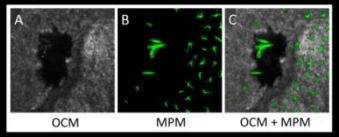
Pixel area of warped mosaic

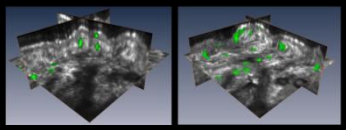


Wound contraction vs. time

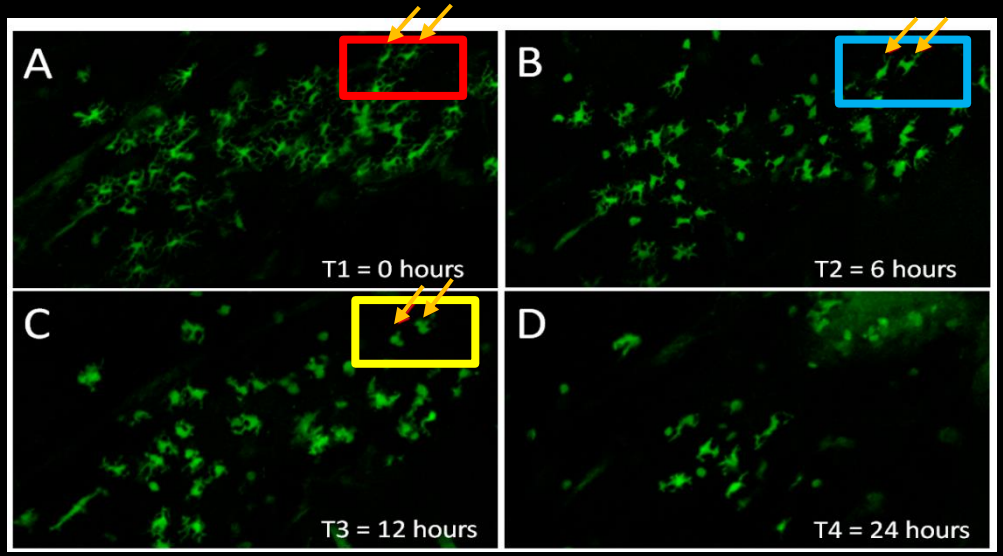


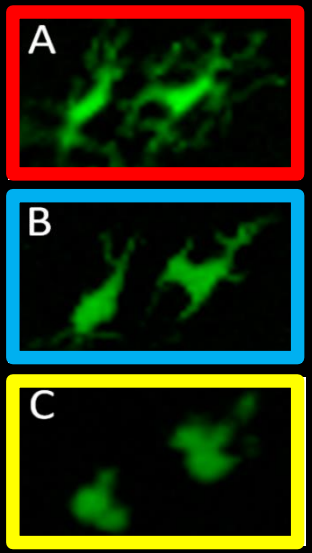
Long-Term Imaging of Single Cells: Visualizing Activation of Langerhans Cells



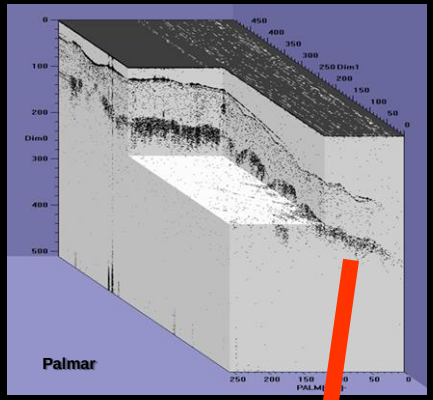


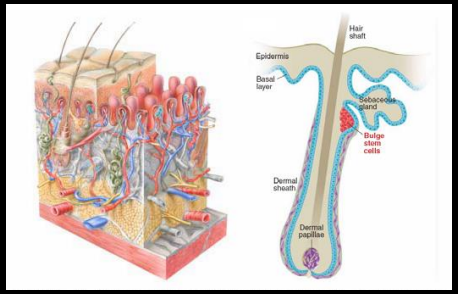
- Langerhans cells: antigen presenting immune cells in the skin
- Following wounding in the vicinity of these cells we observed their activation and migration from the skin





Real-Time OCT of Human Skin





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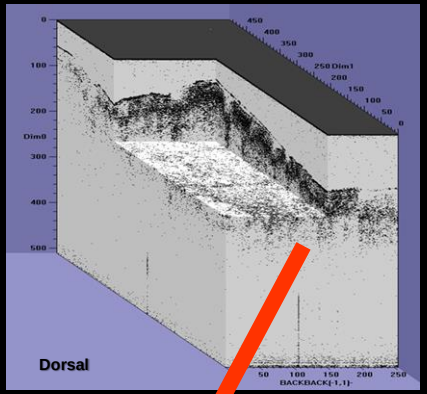
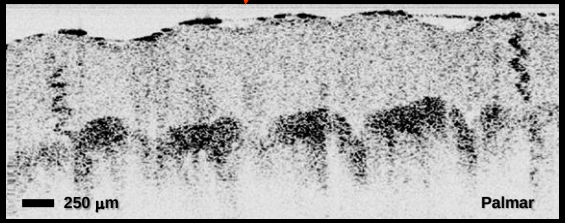
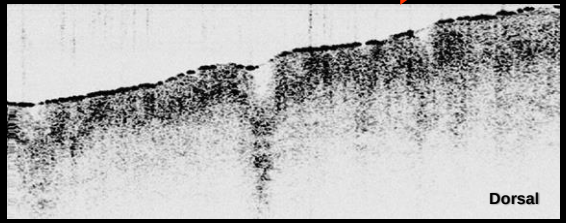
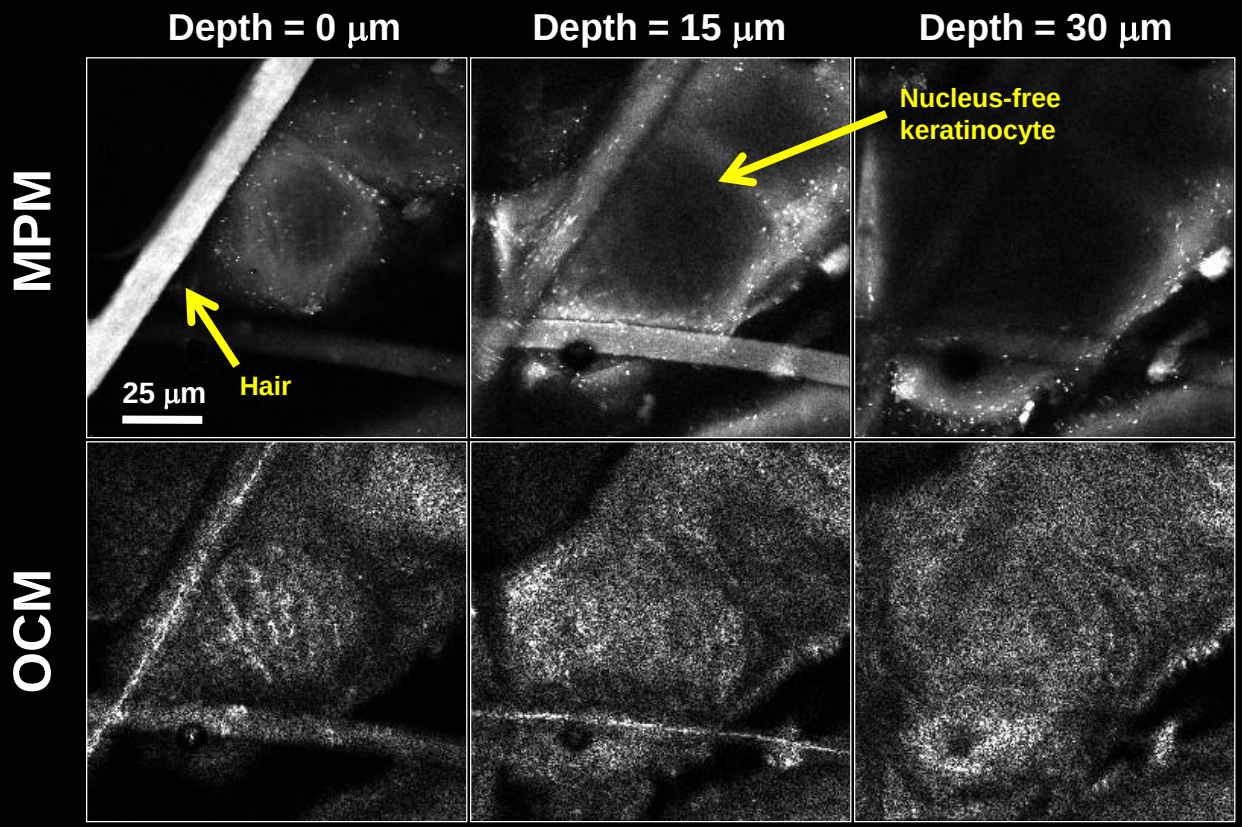




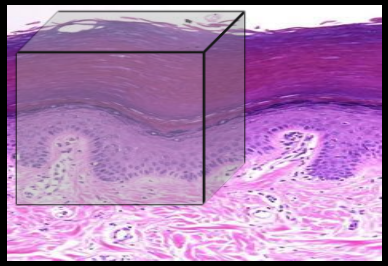


Human – Palmar & Dorsal

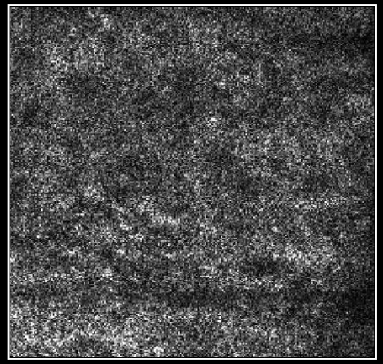
OCM-MPM of *In Vivo* Human Skin - Superficial



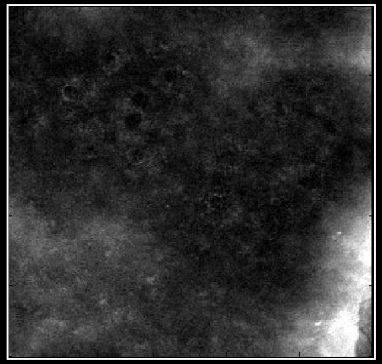
OCM and MPM of *In Vivo* Human Skin Dermal-Epidermal Junction



OCM

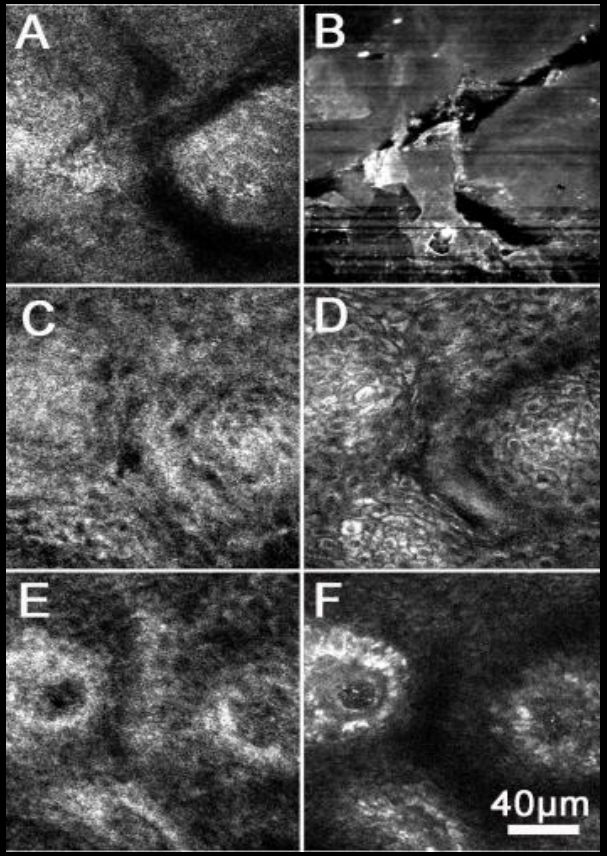


MPM



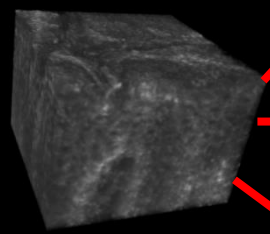
Central tubular microstructures are dermal papillae viewed in *en face* sections

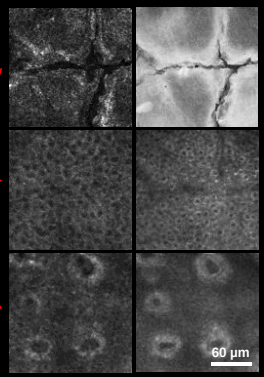
OCM MPM



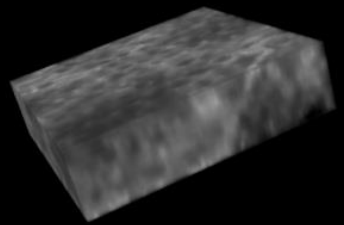
Large-Area Mosaics for Cell Segmentation and Scattering Analysis

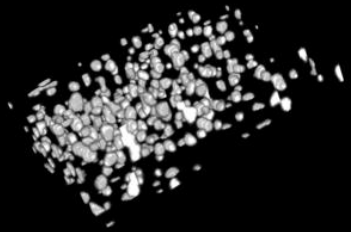
In Vivo Human Skin

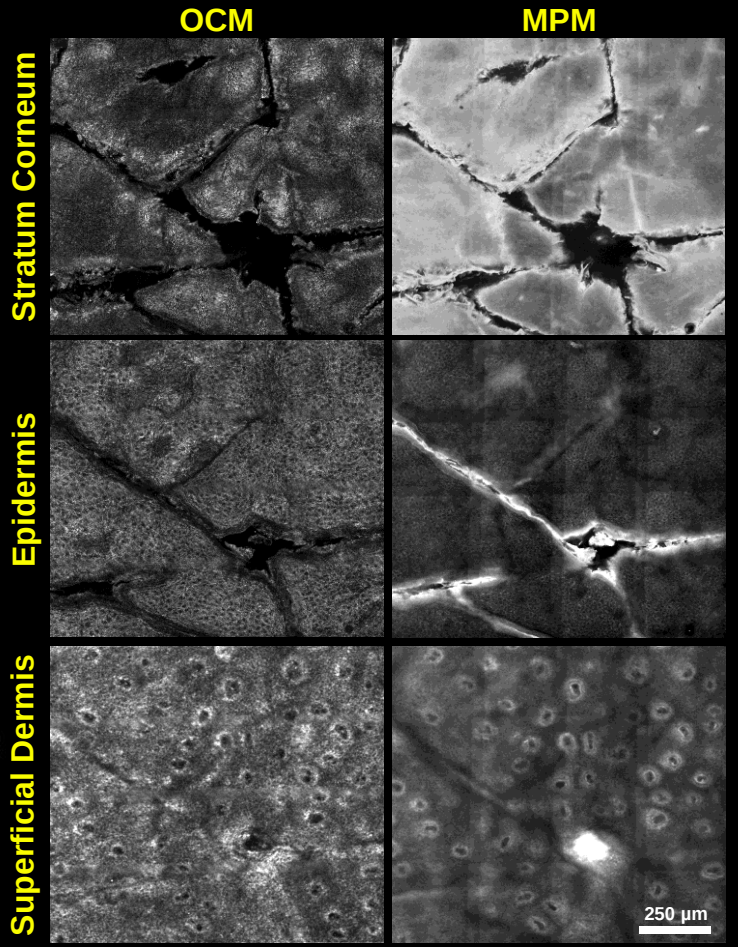




Automated Nuclei Segmentation and Characterization

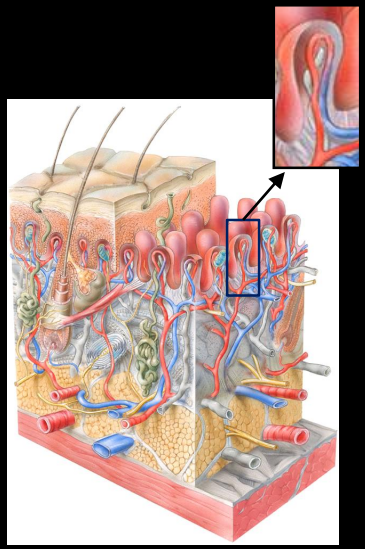


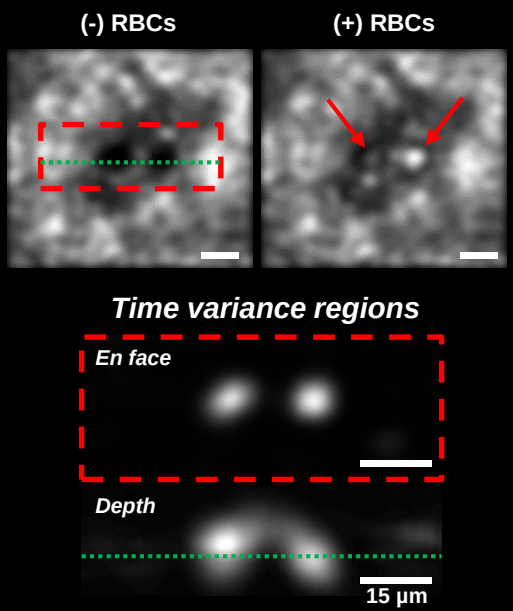


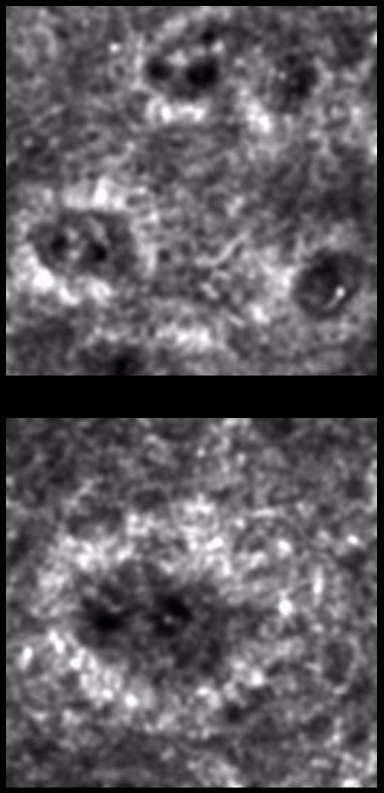


Graf, *et al*., IEEE J. Selected Topics Quant. Elect., 18:1280-1286, 2012

Real-Time Imaging of Capillary Loop Blood Flow







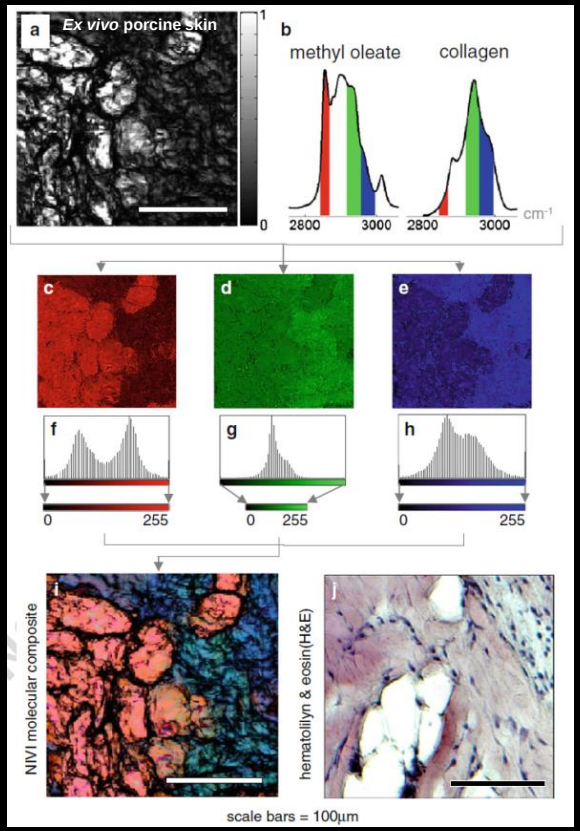
***En face* OCM of *in vivo* human skin**

Graf, *et al.*, IEEE J. Selected Topics Quant. Elect., 18:1280-1286, 2012

Quantitative Molecular Histopathology by Nonlinear Interferometric Vibrational Imaging (NIVI)

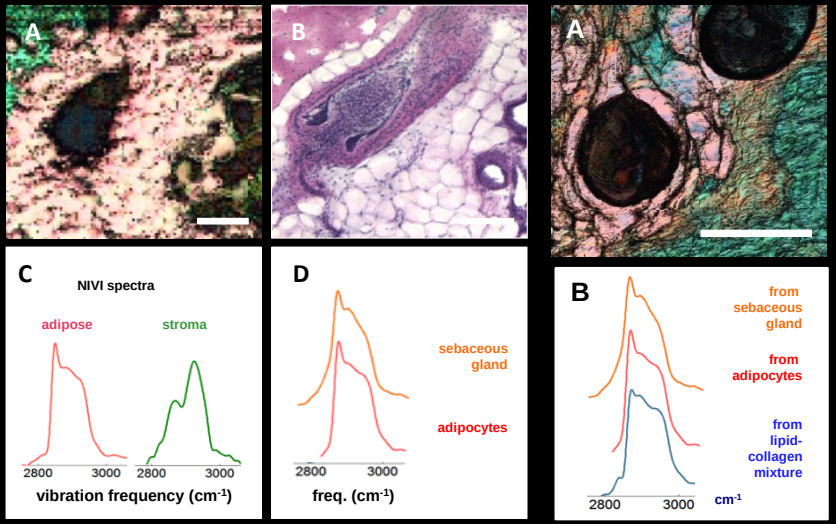
Spectral Decomposition of NIVI Signals from Skin

j	$\Omega_j - \Omega_{j+1}$ (cm^{-1})	Vibration	Contrast?
1	2,842–2,865	CH_2 symmetric	Yes
2	2,865–2,890	CH_2 anti-symmetric	No
3	2,890–2,913	CH_3 symmetric	No
4	2,913–2,955	CH_3 symmetric	Yes
5	2,955–3,000	CH_3 asymmetric	Yes

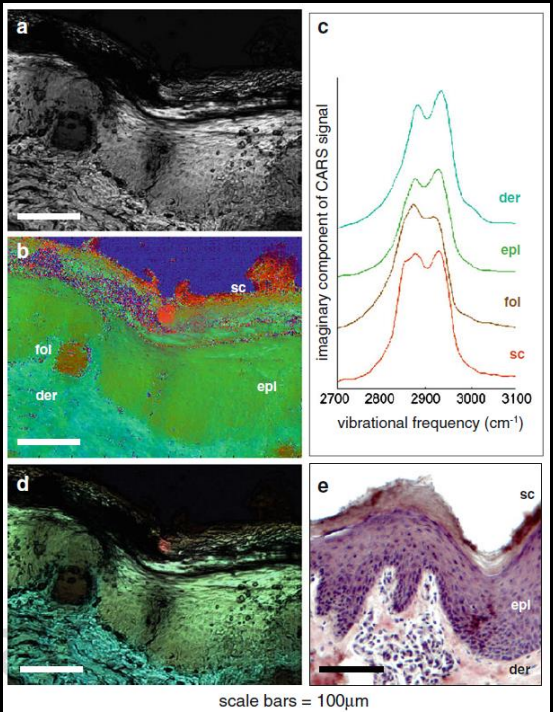


Chowdary, *et al*., Cancer Research 70:9562, 2010
Benalcazar, *et al*., Analytical and Bioanalytical Chemistry, 400:2817, 2011

Spectral Decomposition of NIVI Signals from Skin



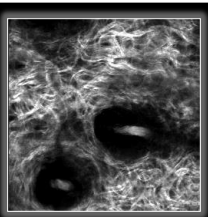
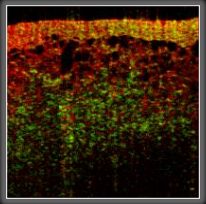
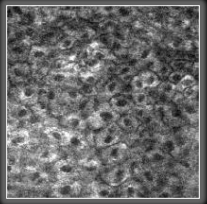
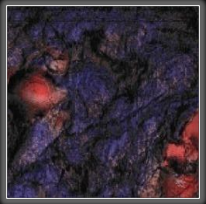
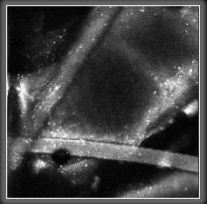
Benalcazar, *et al*., Analytical and Bioanalytical Chemistry, 400:2817, 2011



Conclusions

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

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

*future work

Acknowledgments

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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 Neurophotonics!***
- 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**