Multiphoton microscopy for clinical skin imaging

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Learning Objectives

• Why is multiphoton microscopy (MPM) good for tissue imaging?

• What were the technical challenges related to developing MPM instrumentation for clinical skin imaging and how were they addressed?

• What are the practical challenges related to clinical translation of MPM?

• What are the current technical limitations of the MPM clinical skin imaging and how can they be addressed through further advances of the technology?

• Conclusions
Nonlinear optical microscopy

Light-matter interaction

Light is an electromagnetic wave

\[ P = \varepsilon_0 \left( \chi^{(1)} E + \chi^{(2)} E^2 + \chi^{(3)} E^3 + \ldots \right) \]

- Linear regime (low field intensity)
- Non-linear regime (high-field intensity)
Nonlinear optical microscopy

Light-matter interaction

Light is an electromagnetic wave

\[ P = \varepsilon_0 (\chi^{(1)} E + \chi^{(2)} E^2 + \chi^{(3)} E^3 + \ldots) \]

Second-harmonic generation (SHG)
Two-photon absorption (TPA)

\[ \text{SHG}_{\text{signal}}, \text{TPA}_{\text{signal}} \propto I^2 \]
Why multiphoton/nonlinear optical microscopy?

*Intrinsic 3D resolution*

1-Photon Fluorescence  
(linear optical signal)

2-Photon Fluorescence  
(non-linear optical signal)
Why multiphoton/nonlinear optical microscopy for skin imaging?

Molecular Contrast of Endogenous Molecules

- **Second Harmonic Generation (SHG)**
  - Collagen
  - Myosin

- **Two-photon excited fluorescence (TPEF)**
  - NADH flavins
  - Elastin

- **Two-photon excited fluorescence lifetime**
  - Keratinocytes
  - Pigmented keratinocytes

- **Coherent anti-Stokes Raman Scattering (CARS)**
  - Adipocytes
  - Myelin

- **Pump-probe**
  - Hemoglobin
  - Melanin

- **Third Harmonic Generation (THG)**
  - Skin epithelium
  - Cornea epithelium
Why do we need to have access and study turbid tissue at microscopic scale?

• to understand tissue functionality
• to diagnose diseases non-invasively
• to study and understand potential treatment pathways
• ...
Current standard of care diagnosis method through histopathology involves using conventional optical microscopy.

Clinical diagnosis based on dermoscopy

Biopsy

Sample processing:
- Fixation
- Sectioning
- Staining

Histopathological examination
Conventional versus Multiphoton microscopy

**Focused Illumination (Multiphoton microscope)**

**Widefield Illumination (Conventional microscope)**
Nonlinear optical microscopy

Basic set-up
Technical challenges related to developing MPM for clinical skin imaging

A lab-based technology for a long time...

- large footprint, particularly the light source
- need for stable, flexible patient interface

There are more, but these needed to be addressed first!
Clinical MPM

First commercialized by JenLab, GmbH, Germany


Outstanding engineering +
Key innovation: mechanism for accurate positioning/transmission of a flexible beam

Origins of signals in MPM imaging of skin

- TPEF from keratin in the SC
- TPEF from melanin in basal cells + SHG from collagen in dermal papilla
- TPEF from elastin fibers + SHG from collagen fibers
- TPEF from NADH in epidermal cells

TPEF-Two-Photon Excited Fluorescence
SHG- Second Harmonic Generation
Normal pigmented human skin

Type II sun-exposed forearm
MPM Imaging of Normal Pigmented Human Skin

Type II sun-exposed forearm

MPM en-face view

MPM cross-sectional view

Epidermal cells (TPEF from NADH, keratin, melanin in the cells)

Collagen (SHG)

Elastin fibers (TPEF)

λ_ex = 790 nm; TPEF: ~410-650; SHG: 385-405
Areas of Applications for Non-invasive Clinical Skin Imaging using MPM

Clinical management and research

- Therapy guiding (tumor margin detection BCC) and monitoring (pigmentary skin disorders)
- Non-invasive clinical diagnosis (early detection of melanoma)
- Advance understanding of skin biology, molecular biology of cancer, mechanisms of cancer initiation, etc.
Practical challenges related to clinical translation of MPM

• need to understand the morphological features in live tissue and establish a correlation with histology features (limited literature data!)

Main challenges

Morphological features have a different appearance in MPM and histology images due to:
1) en face/horizontal MPM images vs vertical histology sections
2) origin of images: live vs processed tissue

Example: visualization of melanocytes in human skin

What are melanocytes?
Practical challenges related to clinical translation of MPM

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Example: visualization of melanocytes in human skin

Melanocytes identified based on a processing artifact
Practical challenges related to clinical translation of NLOM

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Main challenges

Morphological features have a different appearance in MPM and histology images due to:
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2) origin of images: live vs processed tissue

How to address them

– research on biopsies, discarded tissue from the skin condition of interest, when available
– work closely with dermatologist collaborators to understand biopsy cut orientation, margin evaluation, etc
– most importantly, work closely with dermatopathologist collaborators to generate a common language to ease communication (scientists need to learn basic dermpath, MDs need to learn the basics about origins of signals in MPM)
Practical challenges related to clinical translation of NLOM

- need to understand the clinical flow and how to integrate smoothly your technology

Main challenges
- For most applications related to *in vivo* clinical skin imaging patients can not be scheduled ahead of time
- Clinicians have a busy, relatively tight schedule for seeing patients

How to address them
- need to work out ways for the scientists/microscopists to be available in clinic
- keep MD collaborators excited and interested in the research

There are more: understand regulatory procedures, how to work with patients, etc.
Non-invasive Clinical Skin Imaging using MPM ~300 patients since 2011

- Melanoma: Balu et al., Cancer Res 74(10) 2688-97 (2014)
- Basal Cell Carcinoma: Balu et al., JAMA Derm 151(10) 1088-74 (2015)
- Keratinocytes metabolism: Balu et al., Biophys J. 104 258-67 (2013)
- Monitoring vitiligo treatment: Balu et al., OSA Technical Digest (2)(2017)
- Scabies: Lentsch et al., JAAD Case Rep, 4 (10), 986 (2018)

More about these in the Application Lecture tomorrow
MPM Technology for Clinical Skin Imaging

Limitations

Small field-of-view

Low scanning speed

> 30 minutes/patient to identify region of interest and scan ~ 1mm²
**Limitations**

*Intensity based contrast only allows access to morphology*

Fluorescence signal from NADH, FAD, keratin, melanin;

Need additional contrast to differentiate these fluorophores: FLIM, spectral detection, etc

**Cost and portability:** current design ~$400K and limited portability

Need to lower cost and enhance portability to facilitate access and studies in more clinics
How can these limitations be addressed through further development advances?

Need for an MPM imaging platform that can image:

1) fast; 2) macroscopically, with 3) microscopic resolution, and 4) enhanced contrast, and be 5) compact, and 6) portable

Previous works in this area do not address all requirements:


Basic description of our proposed MPM instrument and main optical design considerations

- Resonant scanner enables 10 fps (800 x 800 pixels)
- Optimized relay system minimizes scanning aberrations
- Custom-designed beam expander reduces optical aberrations
- Fs fiber laser built into the imaging head for reduced complexity and portability
- Glass window mount is translatable over a ~1 cm² range, allowing large area inspection

fs fiber laser source

Eric Potma
Hideharu Mikami
What are the challenges related to scanning large areas?

Field-of-view (FOV) \( \sim f_{\text{obj}} \times \tan \phi \)

- Large FOV
- Long \( f_{\text{obj}} \)
- Low numerical aperture (NA)
- Low spatial resolution
- Large scanning angles
- Optical aberrations
- Degradation of spatial resolution

\[ \theta = 8.1 \text{ deg} \]
\[ \phi = 4.5 \text{ deg} \]

Olympus 25X 1.05NA (water)

FOV = 800x800 \( \mu \text{m}^2 \)
How do we address these challenges?

Customized Optical Design - Beam expander

![Diagram of beam expander with labels: RMS wavefront error (waves), Incidence angle (deg), 260 mm, Total RMS, Total RMS without defocus, diffraction limit]

How do we address these challenges?

Customized Optical Design-Relay lens system

What are the challenges related to increasing the scanning speed?

<table>
<thead>
<tr>
<th>Galvo-Galvo Scanner</th>
<th>Galvo-Resonant Scanner (4kHz)</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>→ 23 μs</td>
<td>→ 2.4 μs</td>
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<tr>
<td>6s/frame (512x512 pixels)</td>
<td>0.6s/frame (512x512 pixels)</td>
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Pixel dwell time \(\downarrow\) detected photons/signal \(\downarrow\) how do we compensate?
Optimization of signal detection

Sensitive PMTs
Enhancing image information content with neural networks approaches

Training on pairs of quickly and slowly acquired ev-MPM images

Iterative training (300 images) with Unet
Enhancing image information content with neural networks approaches (large scanning areas)

input

prediction

1.2 x 1.0 cm², 64 MPx, 90 s (vs 4 min)

Neural network prediction

Three-fold enhanced acquisition speed!
Maintaining high spatial resolution

Point spread function

**Lateral resolution**
FWHM = 0.5 ± 0.1 μm

**Axial resolution**
FWHM = 3.3 ± 0.5 μm

Goal: Large-scale mapping of superficial skin tissue at high resolution

Excised facial (cheek) skin tissue
Rapid, multiscale MPM ex-vivo imaging of human skin with sub-cellular resolution.
Rapid, multiscale MPM ex vivo imaging of human skin with sub-cellular resolution.
Improving contrast...time correlated single photon counting

Fluorescence photons detected based on their arrival time:

- **0.3-1.7 ns** fluorescence signals from melanin in melanocytes, pigmented keratinocytes
- **0.7-12 ns** fluorescence signals from keratin in keratinocytes and proteins in elastin fibers

Collagen detected through SHG signal
Conclusions

• Multiphoton microscopy (MPM) can visualize/quantify unique features in thick tissues/skin based on intrinsic signals from keratin, melanin, co-enzymes, collagen, elastin, etc...

• Essential elements required for integrating complex imaging technologies, particularly MPM, to clinic:
  1) Continuous push-pull relationship between Technology Development (hardware, contrast, computation, processing and analysis methods) and Clinical Applications (identifying unmet needs and problems worth solving in Medicine, Biology)
  2) Close collaboration with clinicians and pathologists
  3) Infrastructure to allow scientists to be in close proximity to clinic