Non-invasive Microscopy Imaging of Human Skin

Haishan Zeng

Imaging Unit – Integrative Oncology Department
BC Cancer Agency Research Centre

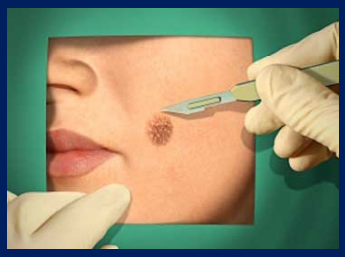
BC Cancer Agency
CARE + RESEARCH
An agency of the Provincial Health Services Authority

UBC Dermatology
Care · Teaching · Research

Vancouver Coastal Health

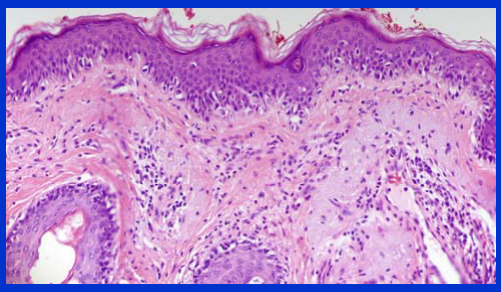
Clinical Diagnosis (optics based already)

- Direct visual inspection under room light
- Dermascope – handheld microscope (10x)
- Wood's Lamp – UV excitation, fluorescence observation by eye
- Followed by invasive biopsy

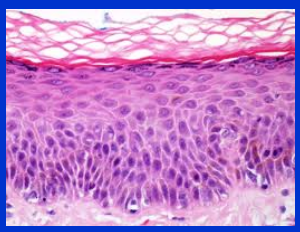


Tissue Processing & Staining

Normal Skin



Malignant Melanoma (Lentigo maligna type) showing single cell spread of melanocytes along the dermal-epidemal junction



http://www.abbeycolor.com/hematoxylin.php
http://www.springer.com/978-3-540-79346-5
http://tissupath.com.au/medical-student-subjects-skin/

Normal Skin (high magnification)

C.F. Heal et al,

Accuracy of clinical diagnosis of skin lesions

British J. of Dermatology, 159: 661–668, 2008

- Skin excisions in 8,694 patients were examined
- PPVs for the clinical diagnoses were:
  - basal cell carcinoma (BCC) 72.7%; (sensitivity=63.9%)
  - squamous cell carcinoma (SCC) 49.4%; (sensitivity=41.1%)
  - cutaneous melanoma (CM) 33.3%; (sensitivity=33.3%)
- PPV is defined as the probability that a person with a clinical diagnosis of a particular skin lesion actually has this skin lesion according to histopathology

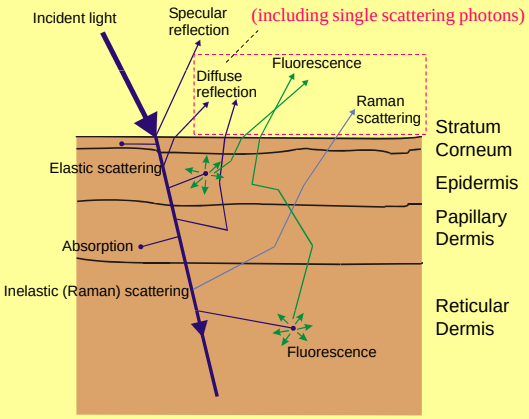
Better non-invasive optical tools are highly desirable

OUTLINE

- Light-Tissue Interactions
- Molecular Processes – "label-free molecular imaging"
- Optical principles & applications
  - Dermascopy
  - Confocal microscopy
  - Optical coherence tomography (OCT)
  - Multiphoton microscopy
  - Coherent anti-Stokes Raman scattering (CARS) microscopy
- Comparison & concluding remarks

Light-Tissue Interaction: Initial Events

- Absorption
  - UV: protein, DNA,
  - Visible: melanin, hemoglobin, bilirubin
  - IR: water
- Elastic Scattering
  - refractive index fluctuations
  - cell membranes, nuclei, collagen
- Inelastic (Raman) Scattering
  - proteins, lipids, DNA/RNA
- Fluorescence
  - tryptophan, porphyrins, collagen, elastin, flavins, NADH, melanin

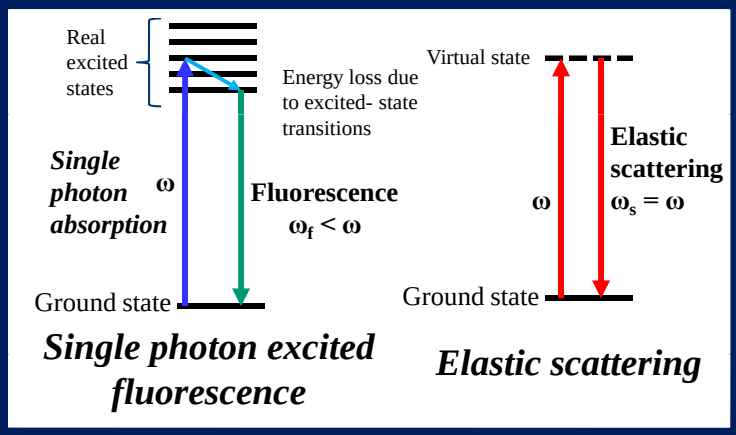


Skin Tissue (Turbid Media)

Molecular Processes

- Single photon excitation – linear process
- Multi-photon excitation – non-linear process
- Inelastic scattering or Raman scattering

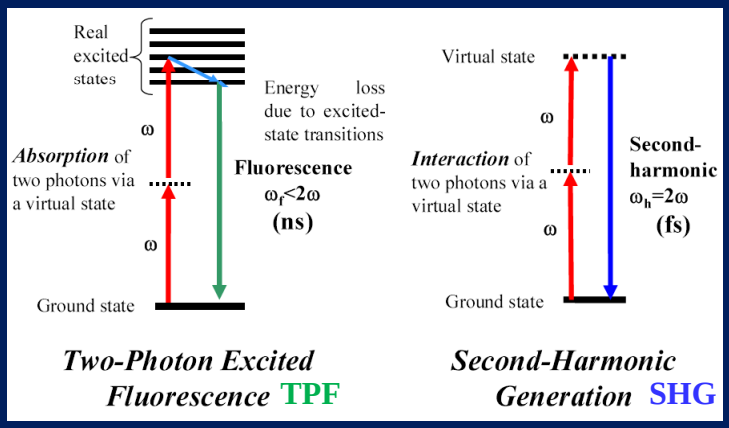
Single Photon Excitation



Fluorescence Confocal Microscopy – FCM
~~(Using exogenous agents)~~

Reflectance Confocal Microscopy – RCM & OCT (Both label free)

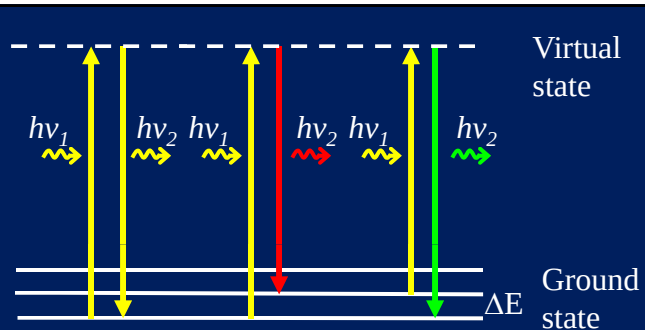
Multi-photon Excitation



Same fluorophores as 1-photon ex.

Non-centrosymmetric structures (e.g. collagen)

Highly spatially localized non-linear interaction → high resolution imaging



Elastic (Rayleigh) Scattering $h\nu_1 = h\nu_2$

Stokes Raman $h\nu_1 > h\nu_2$

Anti-Stokes Raman $h\nu_1 < h\nu_2$

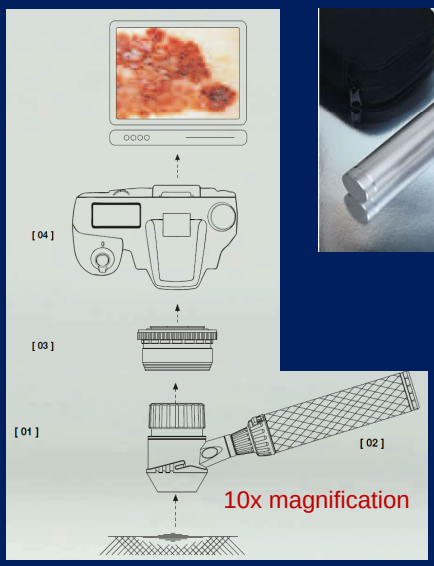
Stokes Raman:
- Raman/normal Raman -> spectroscopy/mapping
- resonance Raman -> spectroscopy/imaging
- surface enhanced Raman (SERS) -> spectroscopy/imaging

Anti-Stokes Raman: Coherent Anti-Stokes Raman Scattering (CARS) Microscopy

OUTLINE

- Light-Tissue Interactions
- Molecular Processes – **"label free molecular imaging"**
- **Optical principles & applications**
  - **Dermascopy**
  - **Reflectance confocal microscopy**
  - **Optical coherence tomography (OCT)**
  - **Multiphoton microscopy**
  - **Coherent anti-Stokes Raman scattering (CARS) microscopy**
- Comparison & concluding remarks

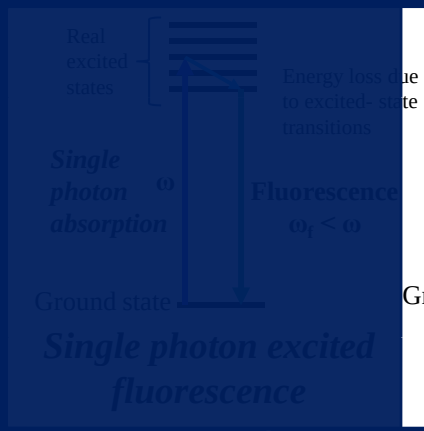
Dermascopy



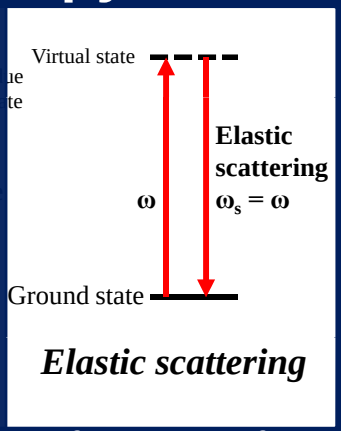
Dermascope/Dermatoscope

- Whole field white light illumination (LED/Halogen Tungsten Lamp)
- Direct visual observation
- Or capture the magnified image with a digital camera
- Signal source: diffuse reflectance
  - Color dominated by absorption chromophores such as melanin and haemoglobin.
  - Scattering affects the brightness

Reflectance Confocal Microscopy

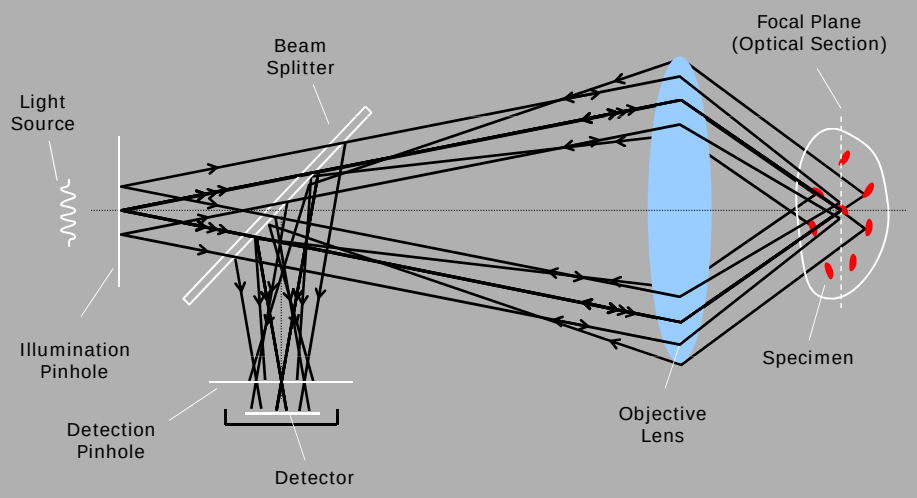


Fluorescence Confocal Microscopy – FCM (Using exogenous agents)



Reflectance Confocal Microscopy – RCM & OCT (Both label free)

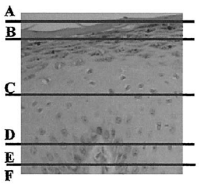
The Principle of Confocal Imaging

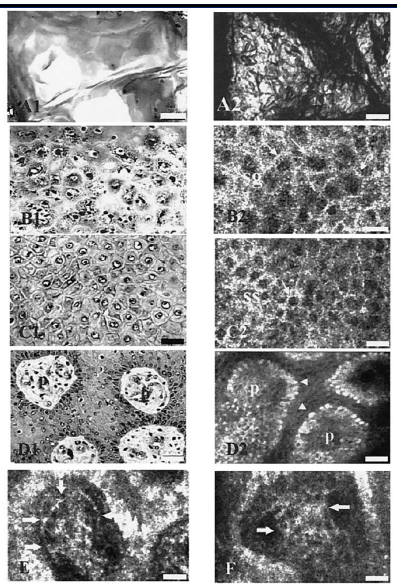


Clinics in Dermatology • *2003;21:359–369*

Confocal Images

Scale Bar: 25 µm

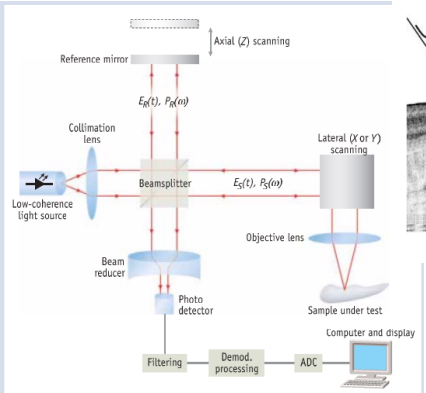




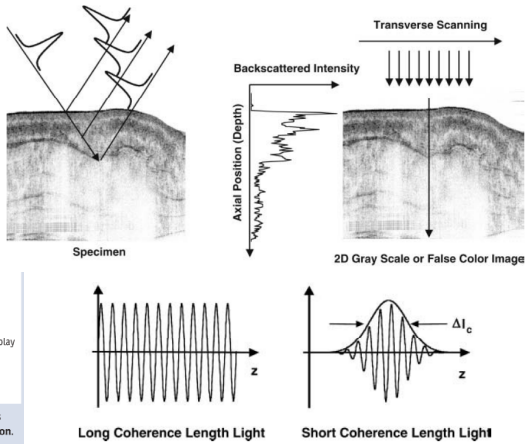
Video rate images
Resolution: 0.5 µm
Imaging depth: 200 µm
Field of view: 250 – 500 µm across

Figure 2. *Correlation between vertical and transverse sections (H&E and in vivo confocal) of normal skin. In confocal images, the corneocytes appear as bright polygonal shapes (A2, arrows), and are of size 10-30 µm. Granular cells (B2, arrows) are regularly seen at depths of 10-15 µm. The dark oval areas correspond to nuclei within the bright cytoplasm. Spinous keratinocytes (C2, arrows) are seen at 20-100 µm below the stratum corneum. Note that basal keratinocytes (D2, arrows), located around of a dermal papillae (p, D), are brighter than keratinocytes of spinosum (ss, C2) and granulosum layers (sg, B2). Blood vessels (arrows, E) and collagen bundles (arrows, F) are also seen (A1, A2, B1, C1, D1, 40×/0.65 NA dry objective lens; B2, C2, D2, E, F, 30×/0.9 NA water immersion objective lens; scale bar, 25 µm). Source: Rajadhyaksha M, González S, Zavislan J, et al. In vivo confocal scanning laser microscopy of human skin II: advances in instrumentation and comparison to histology. J Invest Dermatol 1999;113:293-303. Reprinted by permission of Blackwell Science, Inc.*

Optical Coherence Tomography (OCT)



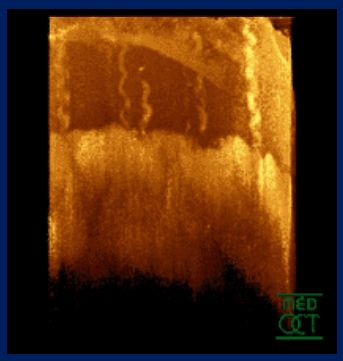
In a typical single-point OCT setup, scanning the light beam on the sample enables noninvasive cross-sectional imaging up to 3 mm in depth with micrometer resolution.



W. Drexler, BioOptics World, January 2008

JG Fujimoto and ME Brezinski, Chapter 13, Biomedical Photonics Handbook, T Vo-Dinh Eds. CRC Press 2003

OCT tomogram of a fingertip

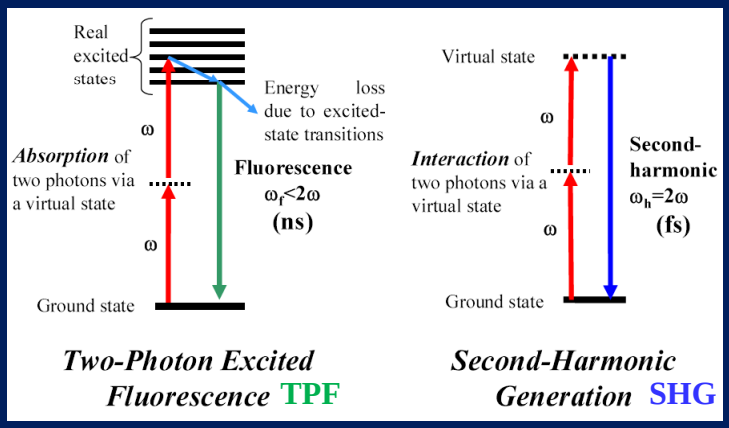


Supplied by the medOCT group, Center of biomedical Engineering and Physics, Medical University Vienna, Austria

Scattering Contrast → High Resolution Imaging

- Reflectance confocal microscopy (RCM)
  - Sub-micron resolution
- Optical coherence tomography (OCT)
  - Low coherence light source + interferometry
  - 10 microns
- Both modalities use single scattering photons to form images

Multi-photon Excitation



Same fluorophores as 1-photon ex.

Non-centrosymmetric structures (e.g. collagen)

Highly spatially localized non-linear interaction → high resolution imaging

Advantages of 2-Photon Excitation

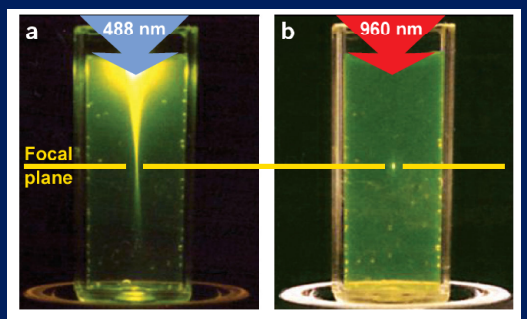
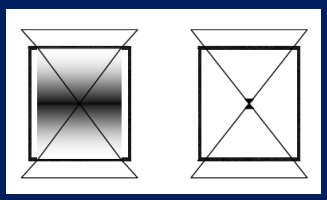
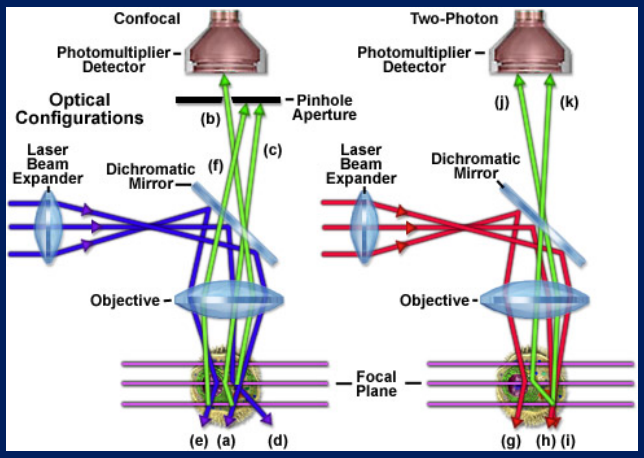


Figure 2 Localization of excitation by two-photon excitation. (**a**) Single-photon excitation of fluorescein by focused 488-nm light (0.16 NA). (**b**) Two-photon excitation using focused (0.16 NA) femtosecond pulses of 960-nm light.



WR Zipfel et al WW Webb, Nature Biotechnology 21, 1369 - 1377 (2003)

Advantages of 2-Photon



1-photon Confocal

2-photon imaging

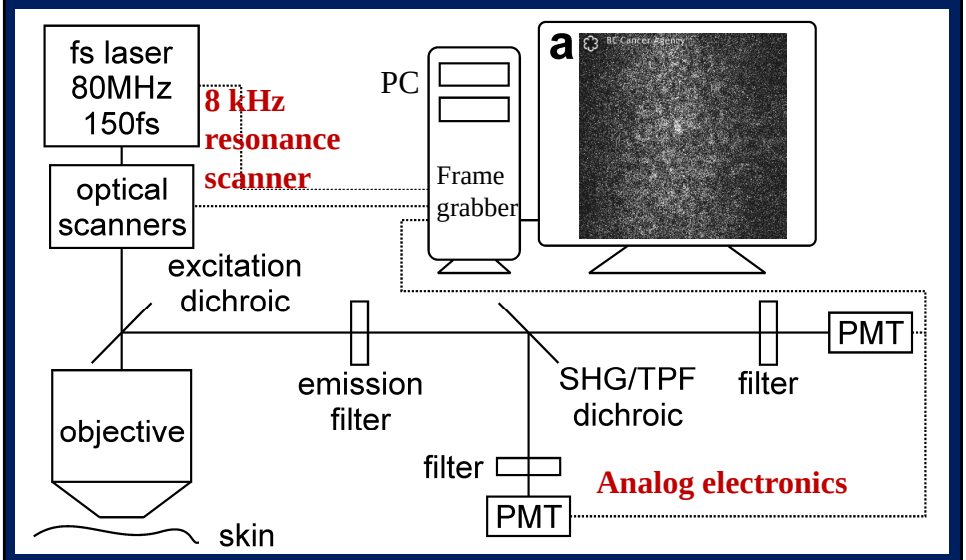
http://www.microscopyu.com

TPF Enabled autofluorescence based microscopy imaging *in vivo*

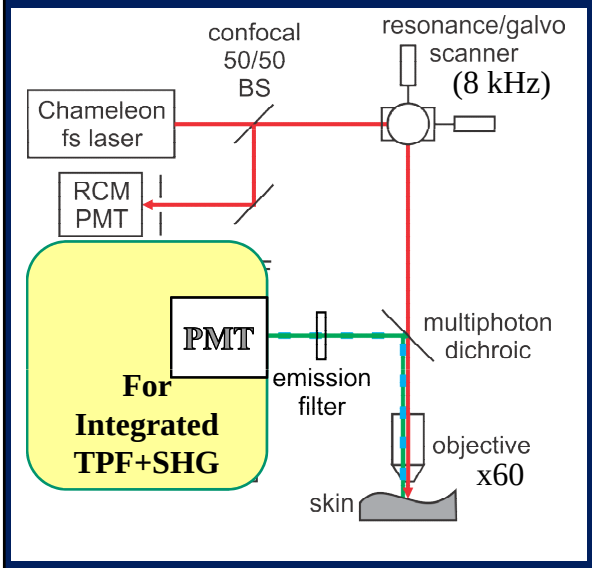
Our Work

- **Limitation of existing MPM systems**
  - Too slow, frame rate: 0.04 to 2 frames/s (fps) = 0.5-24 s/frame, not so practical for clinical use
- **Our Objectives**
  - Achieve video rate/half video rate imaging
    - Decreasing image blurring due to subject movement
    - Imaging a large lesion/multiple lesions in a practical time frame
  - Explore simultaneous RCM/MPM imaging

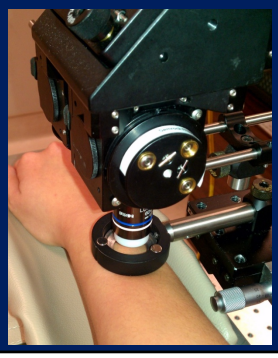
Video Rate *in vivo* Skin MPM System



Simultaneous Confocal and Multiphoton Microscopy

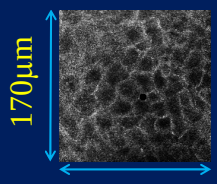


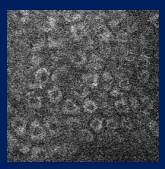
- Co-registered RCM, TPF, and SHG
  or RCM and TPF+SHG
- 27fps (256x256 pixels),
  or 15fps (512x512 pixels)
- Excitation 720nm to 950nm

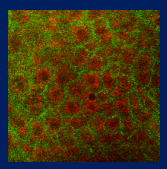


In vivo epidermal imaging at 720nm

Reflectance Confocal (RCM) | TPF | Green = RCM, Red = TPF



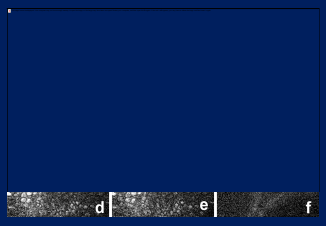




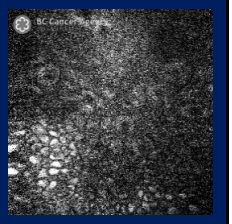
RCM shows cellular membranes and intercellular materials

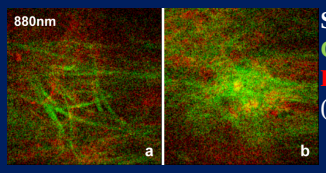
MPM (TPF) shows cytoplasm

Nuclei are dark in both modalities

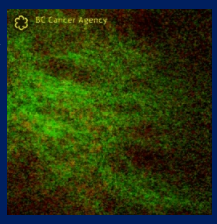


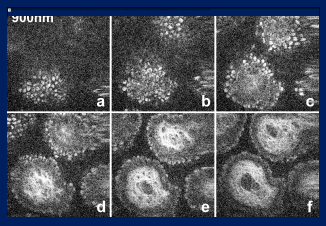
TPF – Two photon excited autofluorescence
(200µm × 200µm)



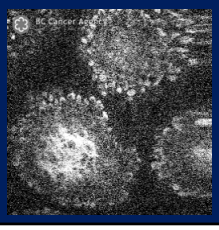


SHG – Second harmonic generation
Green color – SHG collagen fiber
Red color – TPF elastic fiber
(200µm × 200µm)





Integrated TPF+SHG
(200µm × 200µm)

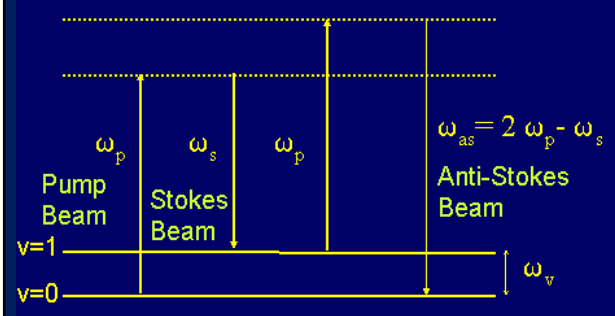


Summary

- **Successfully developed an *in vivo* skin microscopy imaging system capable of**
  - simultaneous RCM, TPF, SHG imaging
  - with perfect co-registration
- **Good contrast, high resolution images comparable to biopsy/histology images**
  - RCM shows cellular membranes and intercellular materials
  - TPF shows cytoplasm and nucleus
  - SGH shows dermal collagen
  - TPF shows dermal elastic fibers

Coherent Anti-Stokes Raman Scattering (CARS)

Energy Diagram



Nonlinear Susceptibility $P_{as} = \chi^{(3)} E_p^2 E_s^*$

$$\chi^{(3)}(\omega_p, \omega_s) = \chi_{nr} + \frac{\chi_R}{(\omega_p - \omega_s - \omega_v) - i\Gamma}$$

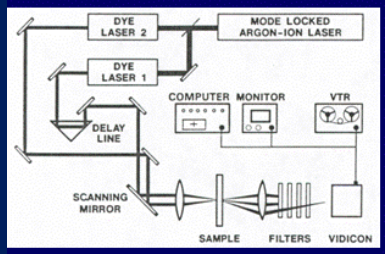
$$I_{as} \propto \left|\chi^{(3)}\right|^2 I_p^2 I_s \left[\frac{\sin(\Delta k \cdot L/2\pi)}{\Delta k \cdot L/2\pi}\right]^2$$

$$\propto n^2$$

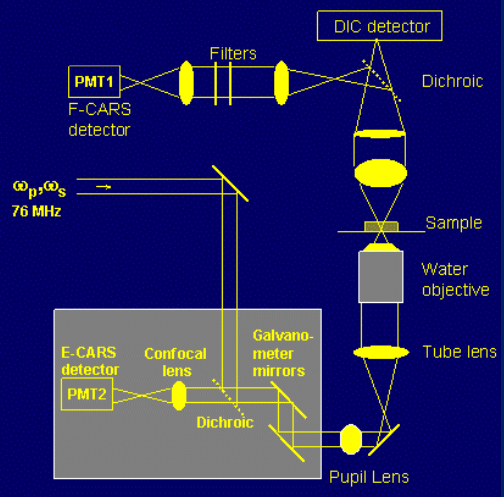
Development of CARS Microscopy

Duncan, Reintjes, Manuccia, *Optics Lett.* 7, 350 (1982)

Zumbusch, Holtom, Xie, *Phys. Rev. Lett.* 82, 4142 (1999)



Noncollinear geometry
picosecond visible lasers



CARS Instrument Need

- Two Lasers at different frequencies ω_p, ω_s
- Picosecond or femtosecond lasers with high peak powers

How to Prove that a Signal is CARS?

- Anti-Stokes frequency at $2\omega_p$-ω_s
- Signal intensity proportional to I_p^2 and I_S
- No signal when ω_p-ω_s is off vibrational resonance

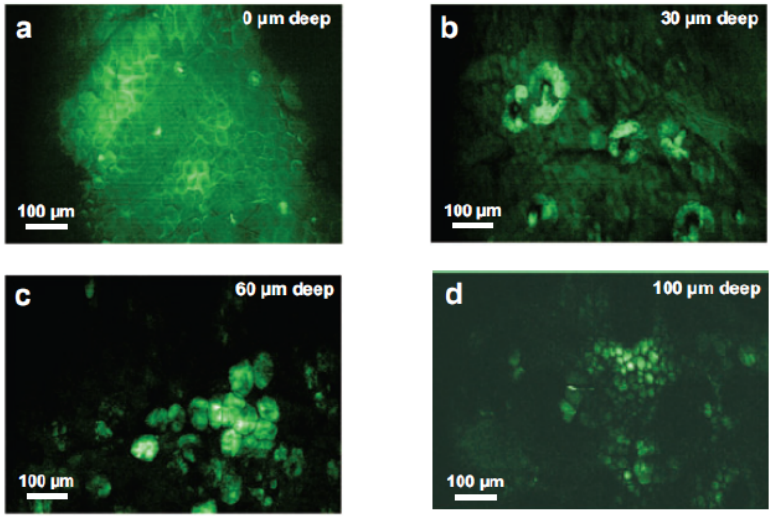


Figure 1. CARS microscopy images of mouse skin at the lipid band (2845 cm^{-1}) *in vivo*. **(a)** The surface of hairless mouse skin imaged at the lipid band. The outlines of the corneocytes are clearly visible due to the lipid-rich intracellular "mortar" of the stratum corneum. **(b)** Sebaceous glands imaged at ~ 30 µm depth. **(c)** Adipocytes at approximately ~ 60 µm depth, in the dermis. **(d)** Subcutaneous fat composed of many small adipocytes, nearly 100 µm deep. Adapted from reference[19].

http://bernstein.harvard.edu/research/cars-why.htm

Advantages of CARS:

•Intrinsic vibrational contrast, label free, high chemical specificity.

•The nonlinear CARS signal is generated only at the focus where the excitation intensities are the highest. This intrinsic sectioning capability eliminates the detection pinhole (just like in MPM).

•CARS microscopy requires only moderate average powers that are easily tolerable by biological samples.

Summary of *in vivo* skin microscopy

- Dermascope imaging
  - Wide field illumination; diffuse reflectance signal imaging
- Confocal imaging
  - Illumination spot, detection spot in confocal arrangement, reject out-of-focus signal
- Optical coherence tomography (OCT)
  - Low coherence light source + interferometer, coherence gating
- Multiphoton imaging
  - Focus fs pulse light to generating non-linear process in tissue, no detection pinhole needed for out-of-focus rejection. Can image, e.g. collagen, elastin, melanin, NADH, keratin, etc.
- CARS microscopy
  - Focus probe beam and stokes beam to generating CARS signal. It is a non-linear process, no detection pinhole needed. Can be tuned to image different vibrational bands/molecules, e.g. lipids.

Comparison of different *in vivo* microscopic imaging modalities

Characteristics / Mode	Origin of imaging contrast	Spatial resolution	Direction of sectioning	Imaging depth
Dermascopy	Diffuse reflectance	tens of microns at the surface	No sectioning capability	sub-mm No depth resolution
OCT	Single scattering	10 microns	Perpendicular	mm
Confocal	Single scattering	sub-micron	Parallel	~200 μm
Multiphoton	Multi-photon fluorescence, SHG, THG	sub-micron	Parallel	Few hundred μm
CARS	Molecular vibration	sub-micron	Parallel	Few hundred μm

Concluding Remarks

- **Different microscopy modalities provide complementary information about skin morphology and chemistry**
- ***In vivo* skin microscopy, now capable of provide non-invasively cellular resolution images with qualities approaching that of histology.**
- **A significant advantage compared to biopsy/histology: Capable of study dynamic biological events non-invasively**

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Edward Yu
Zachary Frehlick
Irene Tong

Canadian Cancer Society
Société canadienne du cancer

CIHR IRSC
Canadian Institutes of Health Research
Instituts de recherche en santé du Canada

CANADIAN DERMATOLOGY FOUNDATION

NSERC CRSNG
Natural Science & Engineering Research Council of Canada

MICHAEL SMITH FOUNDATION FOR HEALTH RESEARCH
Discover. Connect. Engage.

CIHR SKIN RESEARCH TRAINING CENTRE

VGH & UBC HOSPITAL FOUNDATION

The HYDRECS Fund
The BC Hydro Employees' Community Services Fund