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FiberScope: An Optical Fiber Laser Based, Handheld 3D Nonliner Microscope System for *in vivo* Diagnostic Applications in Dermatology and Nanomedicine

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OUTLINE

1. INTRODUCTION TO 3D MICROSCOPY
2. CURRENT USE OF 3D MICROSCOPY IN DERMATOLOGY
3. LABEL FREE IMAGING OF THE SKIN WITH CHEMICAL SELECTIVITY USING NONLINEAR MICROSCOPY (2PEF, SHG, CARS)
4. LASER SOURCES FOR NONLINEAR MICROSCOPY
5. APPLICATIONS OF 3D NONLINEAR MICROSCOPY IN DERMATOLOGY AND NANOMEDICINE
6. FIBERSCOPE: An Optical Fiber Laser Based, Handheld 3D Nonliner Microscope System for *in vivo* Diagnostic Applications in Dermatology and Nanomedicine

3D imaging: optical sectioning required

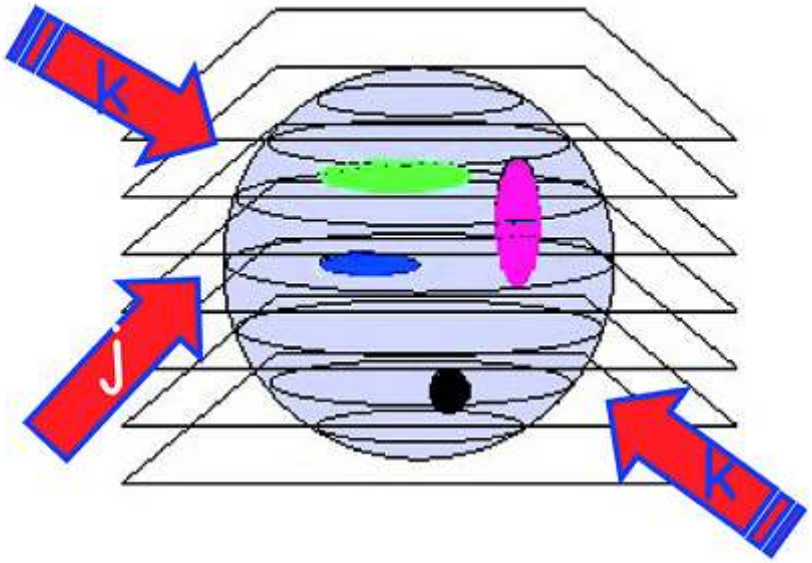
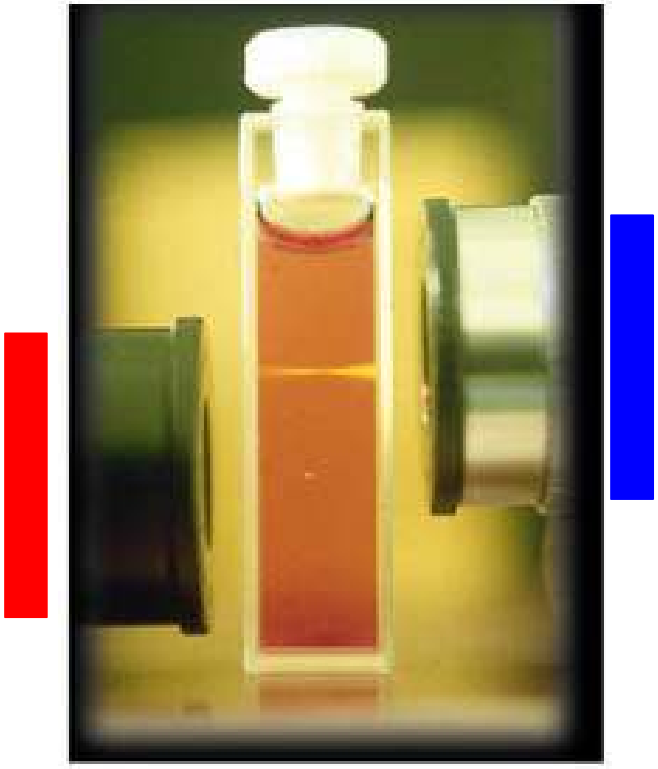


Figure 2
Optical sectioning scheme. A three-dimensional sample can be sketched as a series of optical slices. Let's call slice j the one containing the geometrical focus of the objective and refer to the adjacent planes as k slices. The sample contains a three-dimensional distribution of fluorescently labelled molecules whose intensity distribution is I, slice by slice. The thickness of each optical slice is approximately one half of the axial resolution, say $\approx\lambda/2$.

Optical sectioning in confocal and two-photon excitation fluorescence microscopy



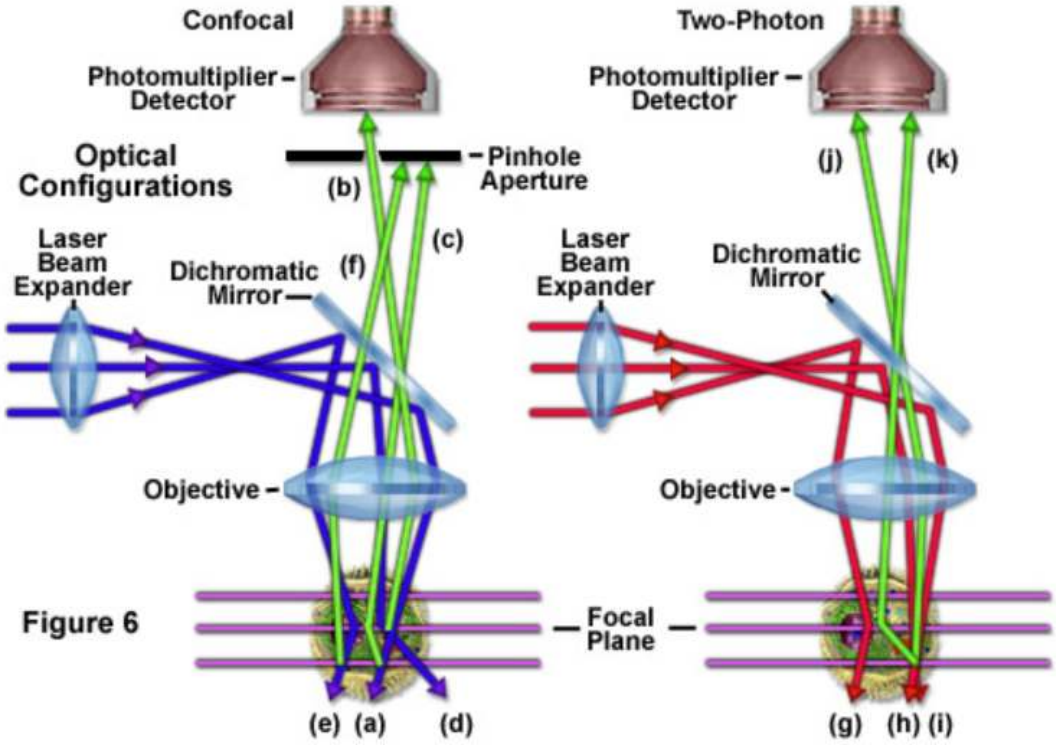
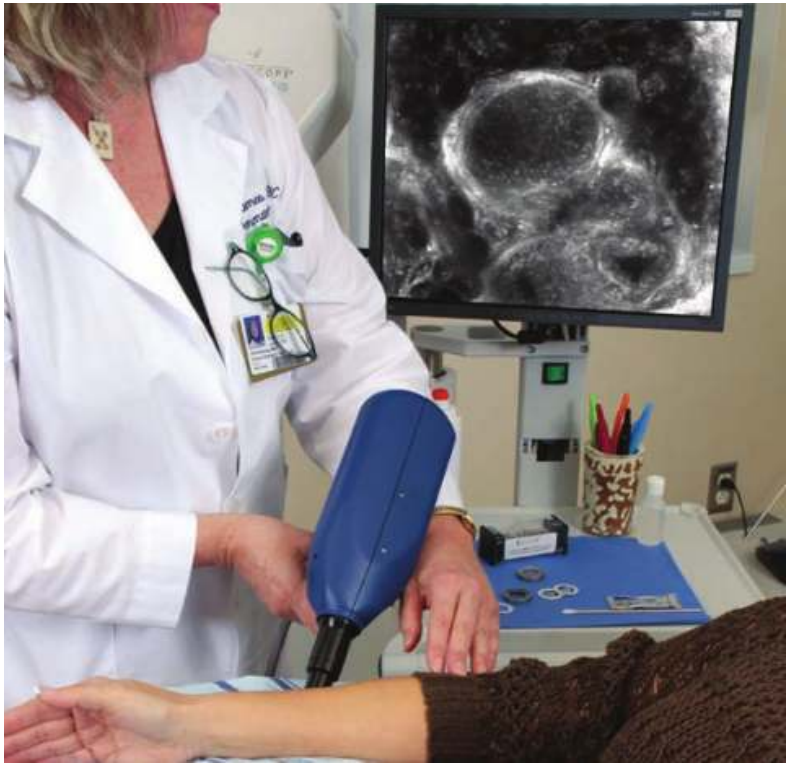


Figure 6

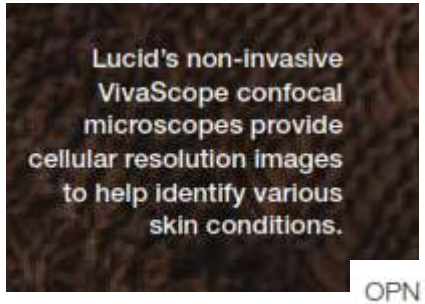
Current use of 3D microscopy in dermatology

VivaScope: Lucid's confocal microscopy

Note: Does not offer chemical selectivity!



The confocal microscope illuminates a portion of skin with the point source of light and detects the reflected light through a pinhole.



OPN November 2007 |

Current use of 3D microscopy in dermatology

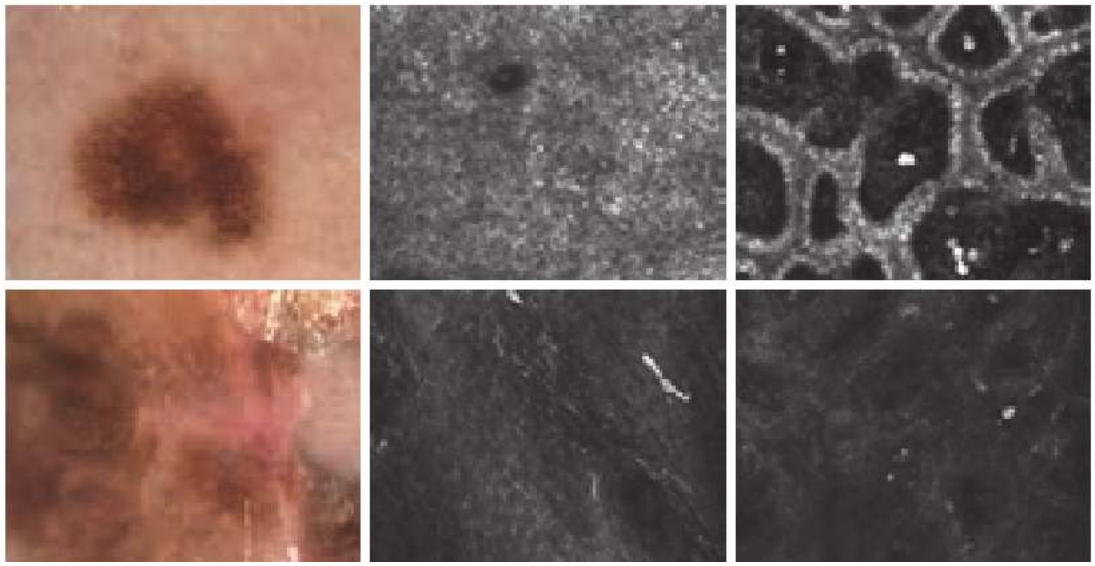
VivaScope: Lucid's confocal microscopy

Possible applications, advantages:

☺ **Precise, non-invasive, *in vivo* determination of tumor borders**

☺ **Immediate pathological results, no need for waiting results from a pathologist.**

Given that this imaging form lets physicians look into tissue *in vivo* without any of the processing that is done in the pathology lab, confocal imaging may well reveal new skin features that cannot be directly correlated to the pathology.



(Top) A benign mole viewed from (left) the skin's surface at visible wavelengths, (center) at a depth of 10 µm and (right) at 161 µm with infrared confocal microscopy. (Bottom) Malignant melanoma at (left) surface level in visible light, (center) at a depth of 35 µm and (right) at 95 µm with confocal microscopy.

Current use of 3D microscopy in dermatology

DermaInspect: JenLab's scanning 2P microscope system

Applications claimed:

Melanoma detection, cosmetic research, drug monitoring, etc.

Laser source: mode-locked Ti:sapphire (Mai Tai, Newport)
Price of the whole imaging system: ~300 kUSD

Semmelweis Egyetem
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Nonlinear microscopy in dermatology

Wigner
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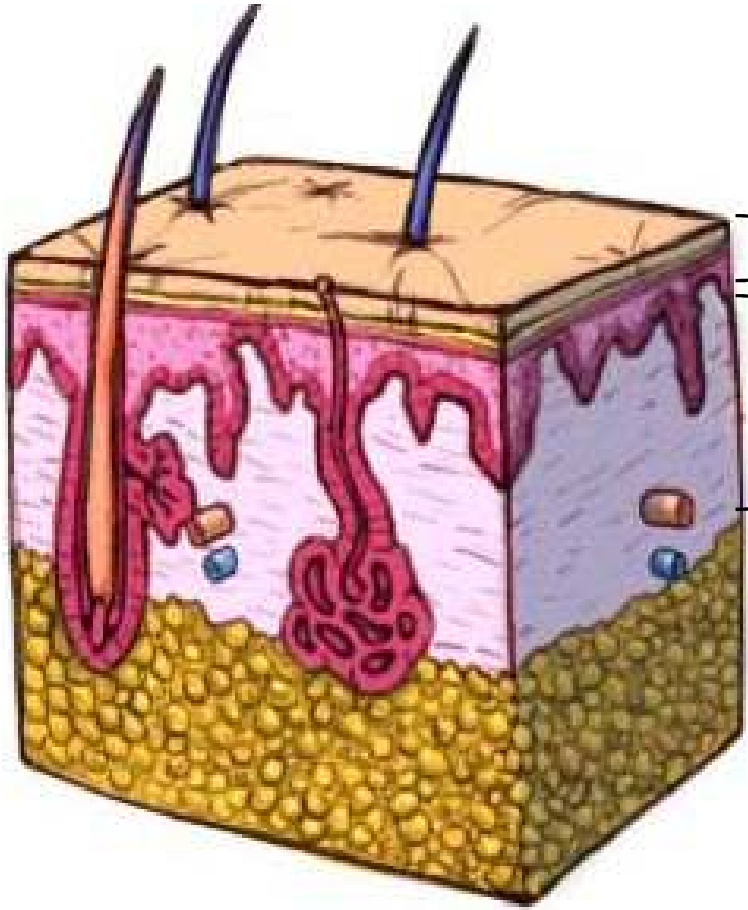
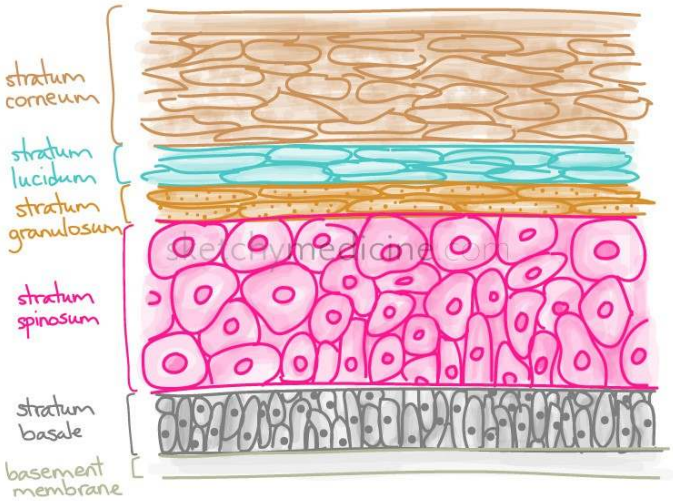
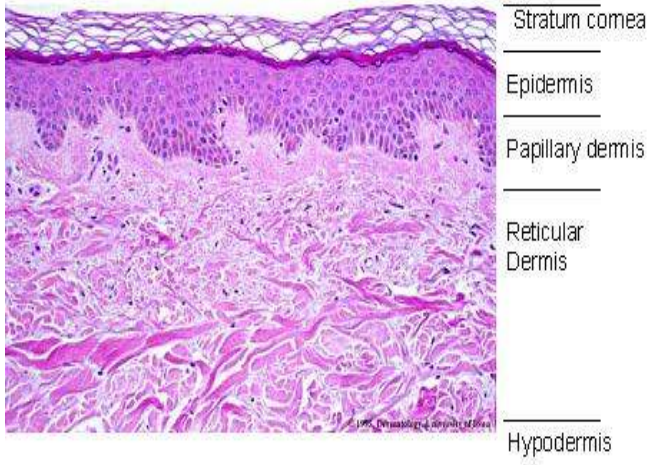


Layers of epidermis



dermis



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In vivo label free imaging of the skin

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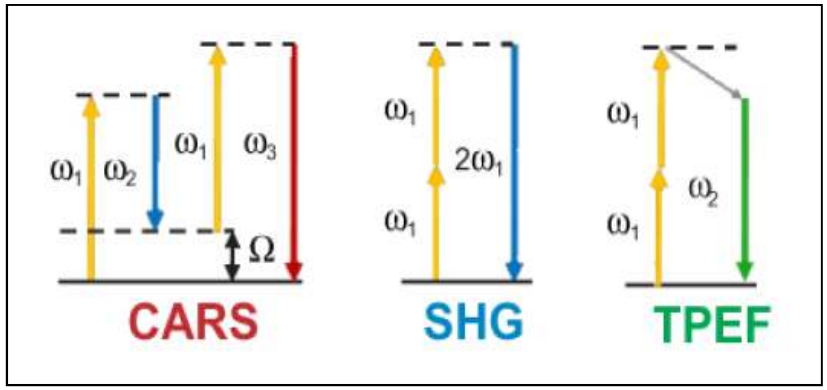
Nonlinear processes applied :

- **TPEF – two photon excitation fluorescence (elastin, keratin, NADH)**
- **SHG – second harmonic generation (collagen)**
- **CARS – coherent anti-Stokes Raman scattering (lipids)**



Endogeneus skin chromophores

Table 1. Endogenous skin chromophores.

Chromophore	Excitation λ_{ex} (nm)	Emission λ (nm)
Fluorescence and SHG		
Retinol (20)	700–830	450
NADH (17,97–100)	340; 690–730	450–470
Vitamin D (20)	< 700	450
Flavins (17)	370, 350; 700–730	430
Melanin (101)	280–450	440, 520, 575
Elastin (101)	300–340; 700–740	420–460
Collagen		
Fluorescence	300–340; 700–740	420–460
SHG (19,101)	720–960 (tunable range of TP laser)	360–480 ($\lambda_{ex}/2$)
CARS	Excitation	Emission ω (cm^{-1})
C-H stretch	See (23,29)	2845 cm^{-1}
Sebaceous glands		2845 cm^{-1}
Adipocytes		2956 cm^{-1}

Hanson et al., **Photochemistry and Photobiology, 2009., (85:33-44)**

NOTE: SEE MOVIE!

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3D imaging of the skin by nonlinear microscopy

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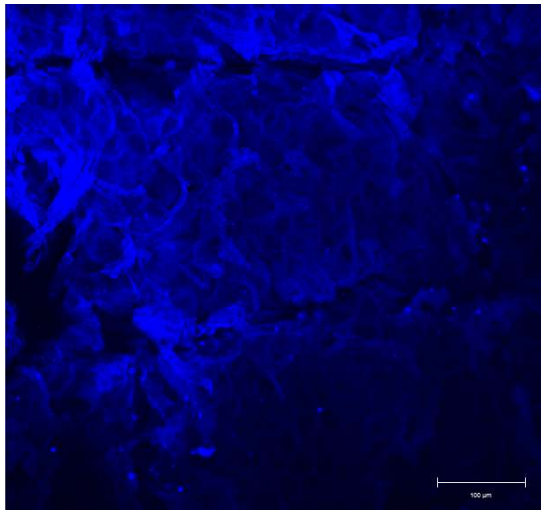
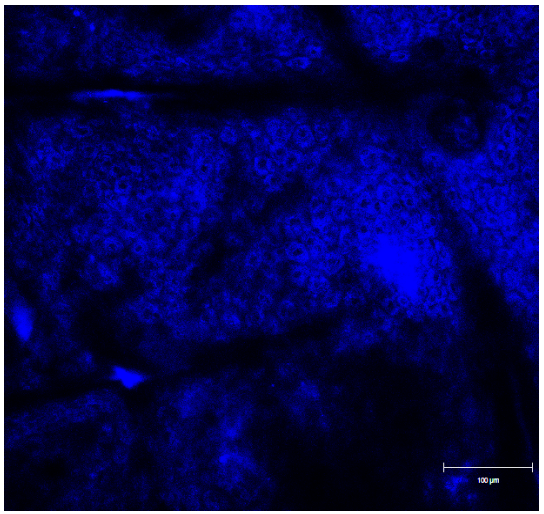
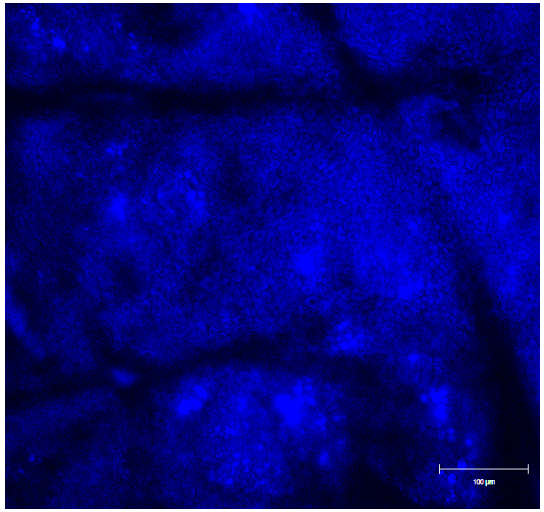
str. corneum: keratin (TPEF)



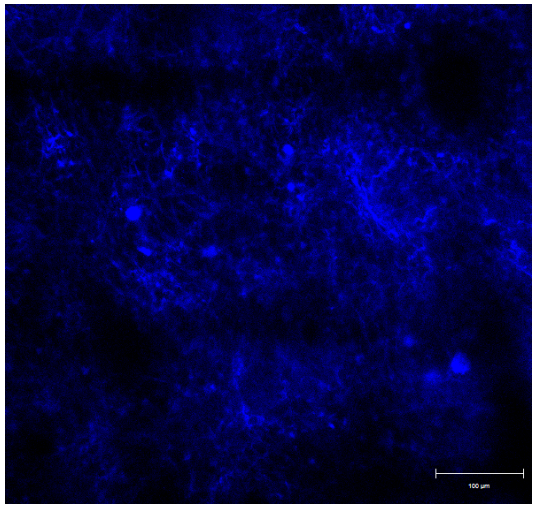
str. spinosum: NADH (TPEF)



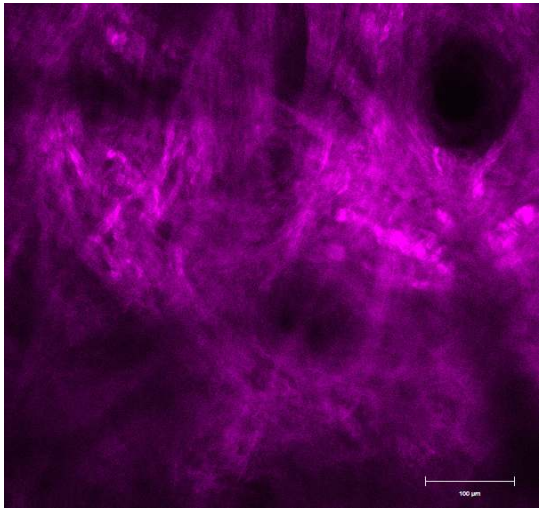
str. basale: NADH (TPEF)



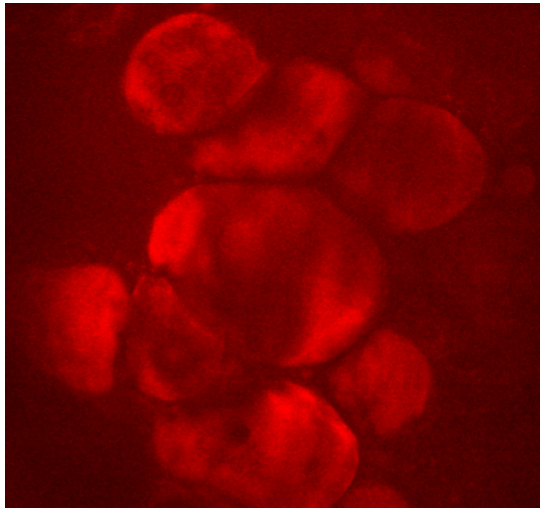
dermis: elasztin (TPEF)



dermis: kollagén (SHG)

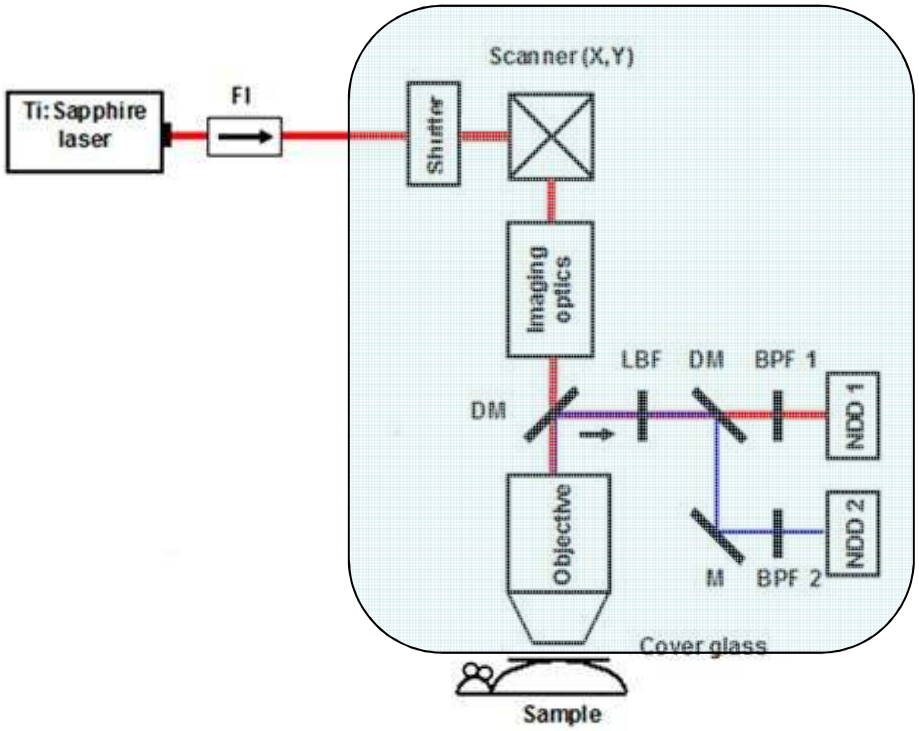


subcutis: adipocytes (CARS)



Main system components of an industry standard, scanning 3D 2PEF microscope

- A broadly tunable, pulsed Ti:sapphire laser
- A laser scanning microscope (e.g, LSM 7MP)



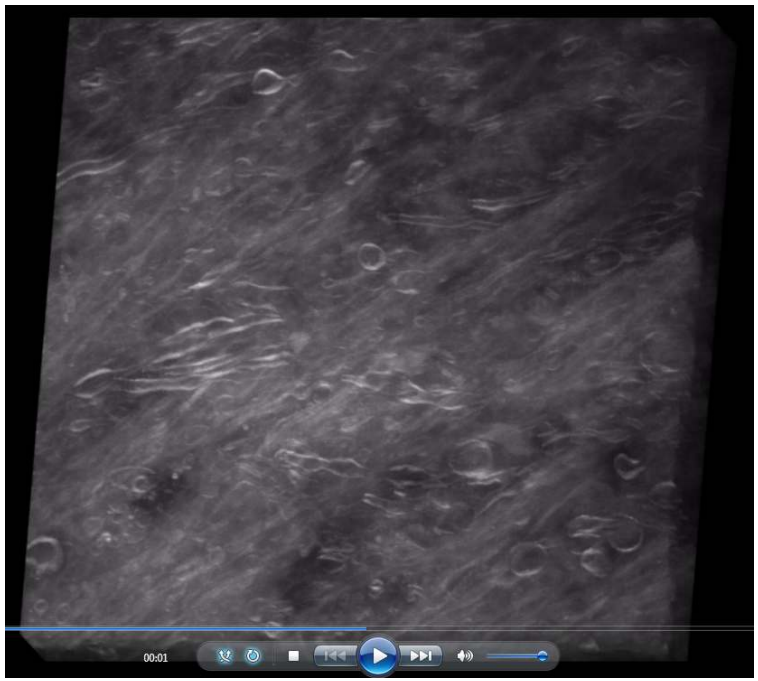
Typical cost of a scanning 3D nonlinear microscope

CARS SYSTEM INSTALLED AT UNIVERSITY OF SZEGED

Related articles

CARS imaging system installed at the University of Szeged, Department of Neurology (Prof. Gábor Tamás lab), June 2014

Photo Gallery

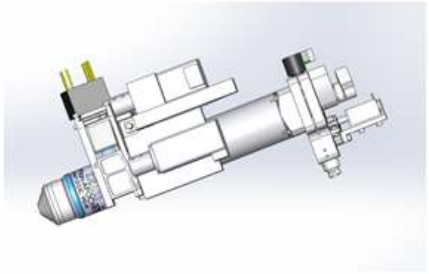


Costs:

- **Tunable, femtosecond pulse Ti:sapphire laser > 100 kUSD**
- **A tabletop scanning 2PEF microscope > 100 kUSD (Carl Zeiss, Nikon, etc.)**
- **Vibration isolated table, climatization required**

Our *FiberScope* development project: R&D targets

- ***Reduce the cost of the pulsed laser applied***
- ***Deliver the ligth for measurements through optical fiber (fiber delivery)***
- ***Small size scanning microscope head (handheld device)***
- ***In vivo 3D measurements: laser safety issues***
- ***Applications in dermatology and nanomedicine***



FemtoFiber + scanning head for confocal/2PF imaging = FiberScope

Project aims

- *Drastically reduce the cost of pulsed laser applied for nonlinear imaging*
- *Fiber delivery, fiber integration*

The solution: mode-locked Yb-fiber laser

Benefits:

- *Small size*
- *Fiber integration is easy*
- *Lower cost*

Drawback:

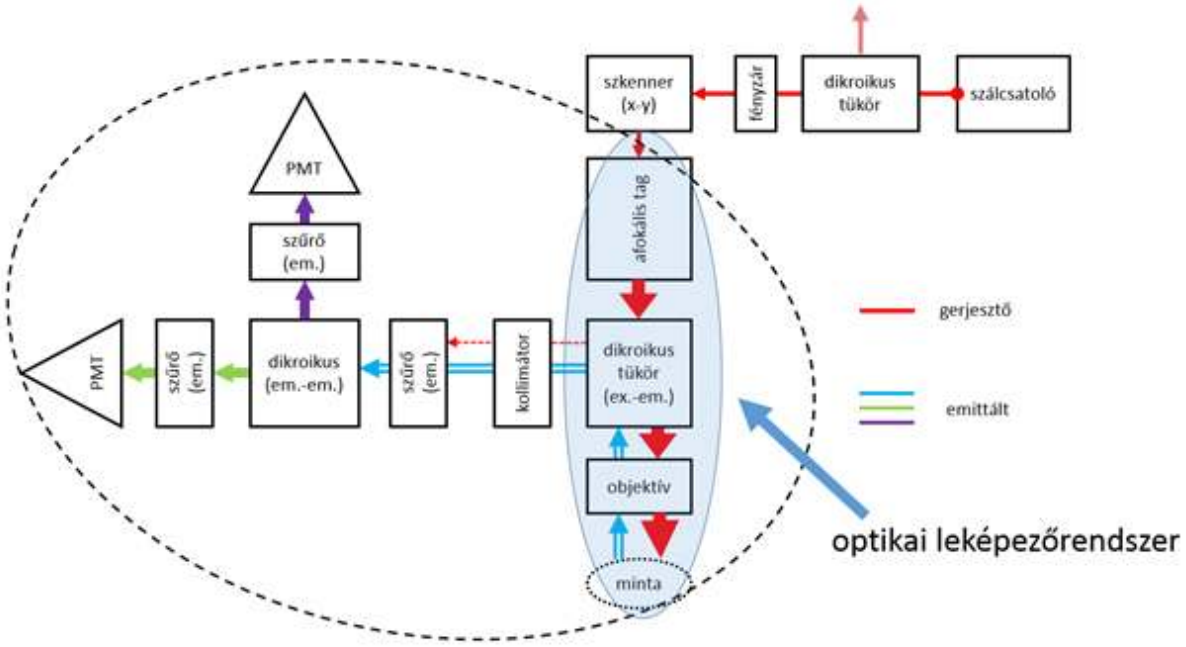
- *Limited tunability (1020-1060 nm)*

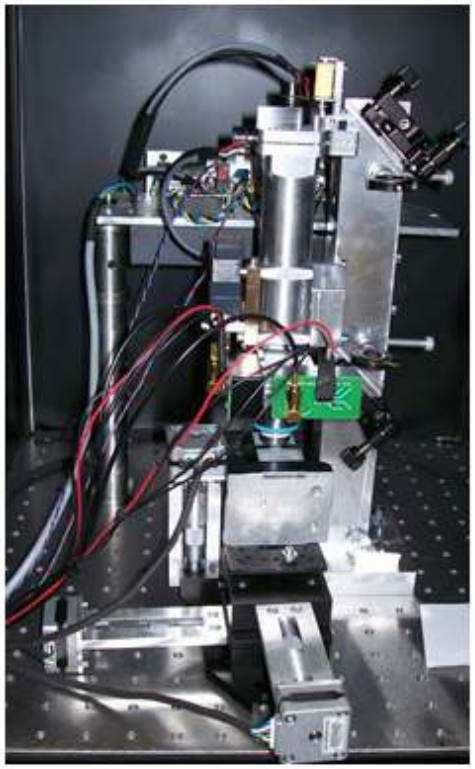
Photo of our prototype, all-fiber Yb-fiber laser in 2009

J. Fekete, A. Cserteg, Szipőcs; **All-fiber, all-normal dispersion ytterbium ring oscillator,** Laser Physics Letters 6, 49-53, 2009

FiberScope nonlinear microscope

Optical design of the scanning microscope head of the *FiberScope*





Csákányi A, Szipőcs R: Fiberscope pásztázó nemlineáris mikroszkóp optikai rendszerének tervezése, Kvantumelektronika 2014, Budapest, 28.11.2014, Paper P64.

FiberScope nonlinear microscope

Photo of the 2MHz Yb-fiber laser system used as a pulsed laser light source of our *FiberScope* device

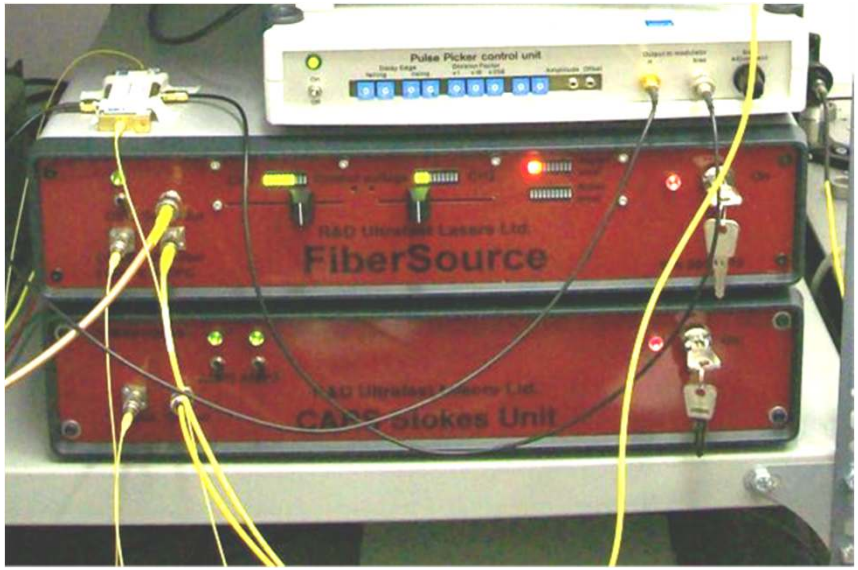
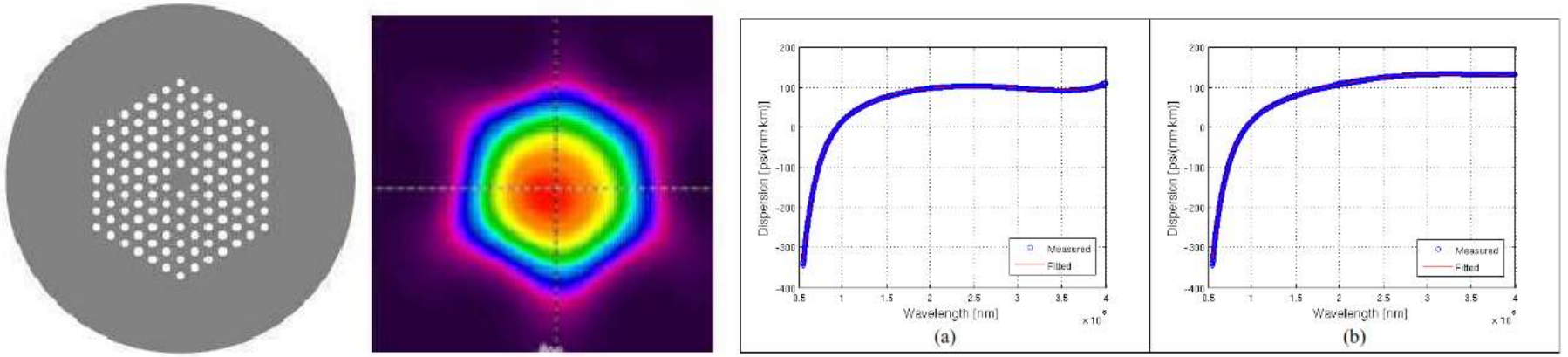


Photo of the scanning microscope head of the *FiberScope* device with plastic covering

Frequency conversion in optical fibers

How to extend the tuning range of an Yb-fiber laser?

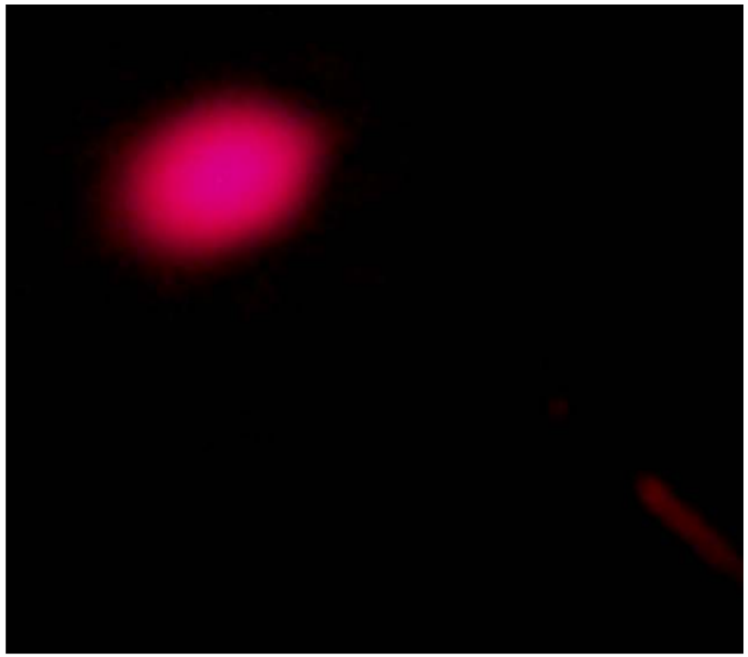
- ***Design and manufacturing of solid core optical fibers with special dispersion properties***
- ***Modelling propagation of optical pulses in these special fibers***
- ***Experimental verification***

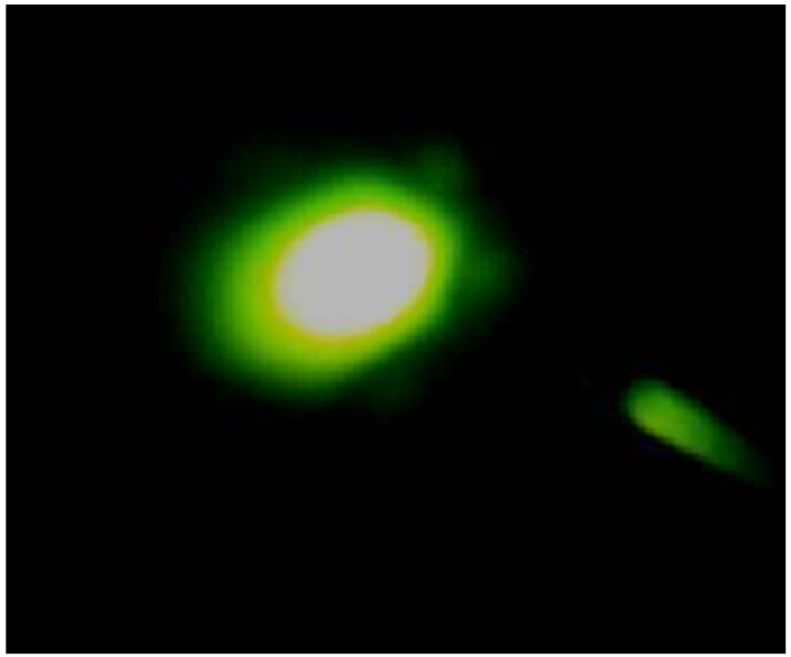


Várallyay Z, Szipőcs R, *Stored Energy, Transmission Group Delay and Mode Field Distortion in Optical Fibers, IEEE JOURNAL OF SELECTED TOPICS IN QUANTUM ELECTRONICS* 20, 0904206/1-6 (2014)

Extending the tuning range of an Yb-fiber laser

- *Higher peak intensities required: reduction of repetition rate and amplification of the pulses are needed*

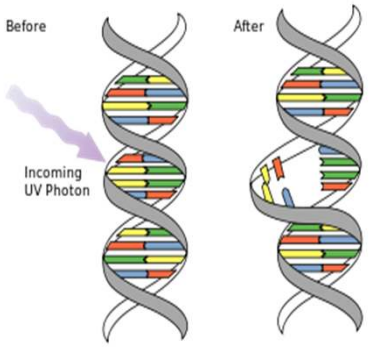


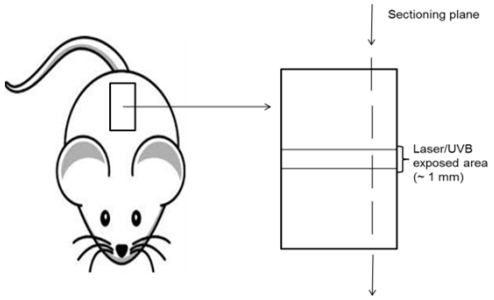


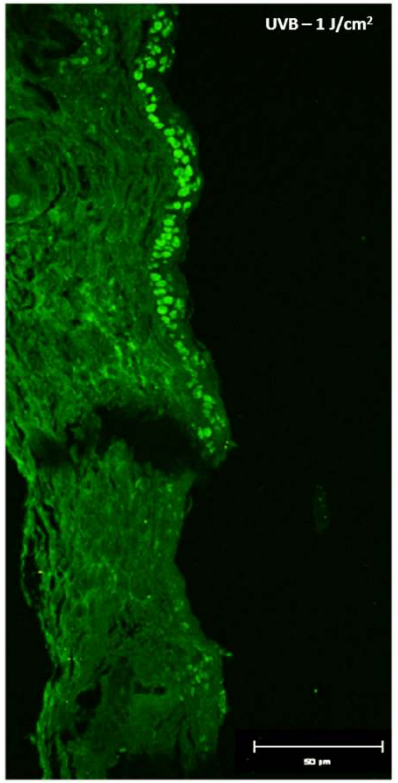
Left: Photo of a red laser beam exiting the photonic crystal fiber being connected to a 2 MHz repetition rate Yb-fiber laser with output power of 200 mW. Right: the same recorded by an IR camera

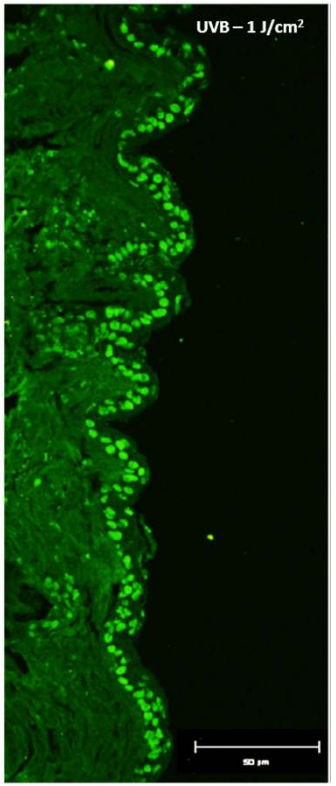
Laser safety issues

- **Possible damage mechanisms: thermal or photochemical**
- **Detection of photochemical damage: like in case of UVB damage**
- **Fluorescent labelling of cyclobutane pyrimidin dimers in DNA**









de Gruijl F.R. and Rebel H., *Early events in UV carcinogenesis-DNA damage, target cells and mutant p53 foci.* Photochem Photobiol, 2008. 84(2): p. 382-7.

F.Fischer, B.Volkmer., S. Puschmann, R. Greinert., W. Breitbart, J. Kiefer, R. Wepf, *Skin imaged by femtosecond laser irradiation: a risk assessment for in vivo applications.* Biophotonics and New Therapy Frontiers, 2006.

Haluszka D, Lorincz K, Banvolgyi A, Gyongyosi N, Kolonics A, Szipocs R, Karpati S, Wikonkal N: *The effect of repair mechanisms on risk of DNA damage during in vivo two-photon skin imaging,* J. OF INVESTIGATIVE DERMATOLOGY 133:(1) p. S219. (2013)

APPLICATIONS OF 3D MIROSCOPY IN DERMATOLOGY

Penetration of FITC through stratum corneum in TGM3/KO and WT mice skin

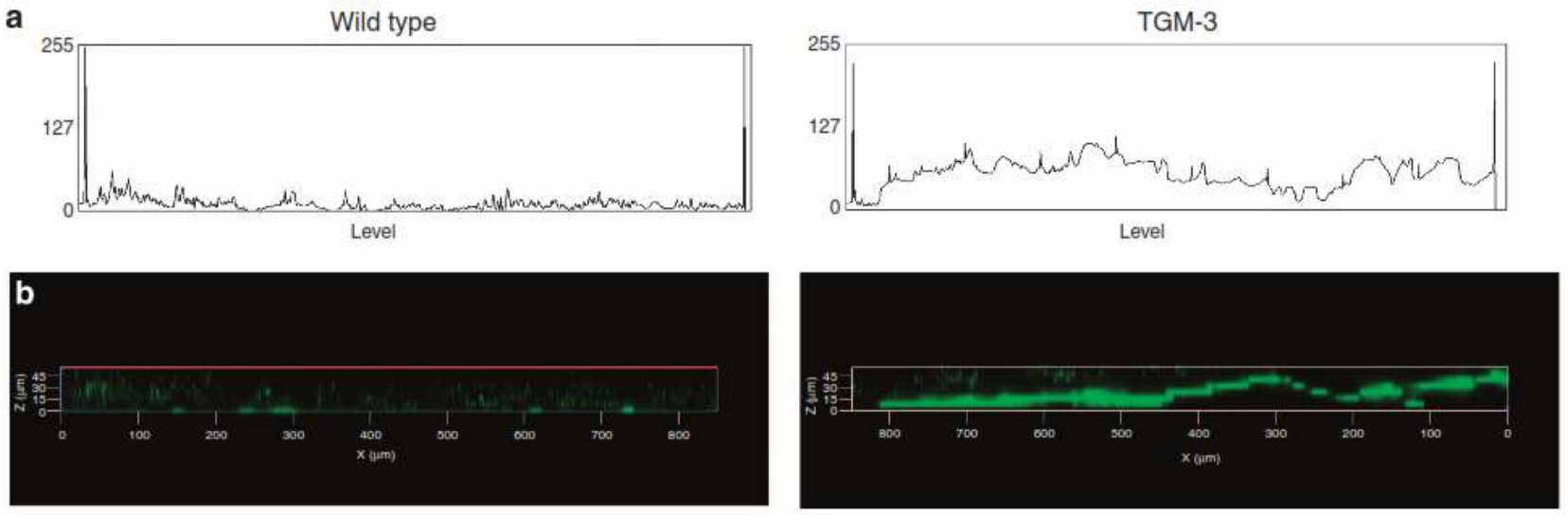
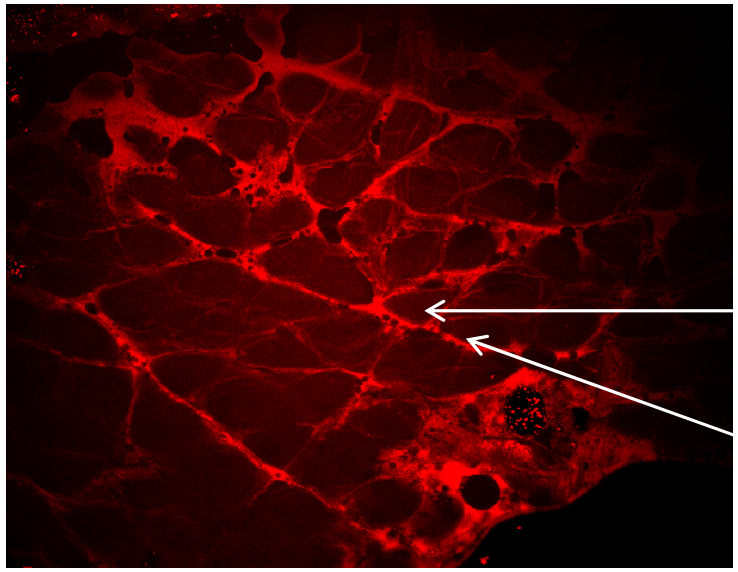


Figure 1. Penetration of FITC through stratum corneum in transglutaminase 3 knockout (TGM3/KO) and wild-type (WT) mice skin *in vivo* by two-photon microscopy. (**a**, **b**) xz-Multitracking sections composed from a stack of xy-optical sections with 8 µm distance were recorded from the skin surface ($Z = 0$ µm) down to the bottom. The representation reveals the frontal penetration profile of FITC in the TGM3/KO skin reaching an average of ~20 µm depth after 30 minutes. A more limited FITC uptake was detected in WT skin both by line analysis (**a**) and by fluorescence imaging (**b**). The line analysis demonstrates the average fluorescence intensity (on vertical axis) measured along 850 µm length of the tissue sample (horizontal axis) (**a**). (**c**, **d**) Three-dimensional images also demonstrated

Bognar P, Nemeth I, Mayer B, Haluszka D, Wikonkal N, Ostorhazi E, John S, Paulsson M, Smyth N, Pasztoi M, Buzas E, Szipocs R, Kolonics A, Temesvari E, Karpati S: Reduced inflammatory threshold indicates skin barrier defect in transglutaminase 3 knockout mice. **J INVEST DERMATOL 134**, 105-111. (2014)

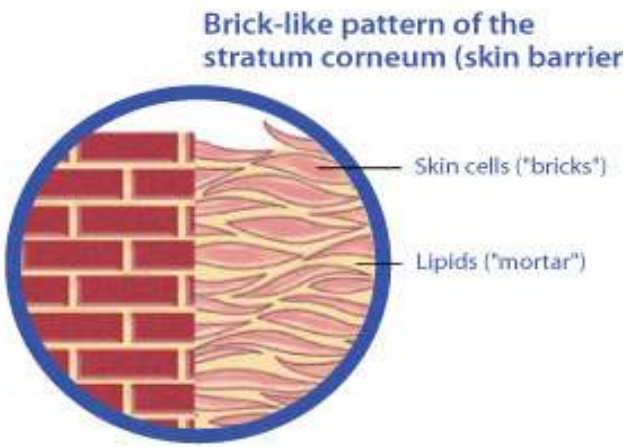
Investigating psoriasis – AF + CARS measurements

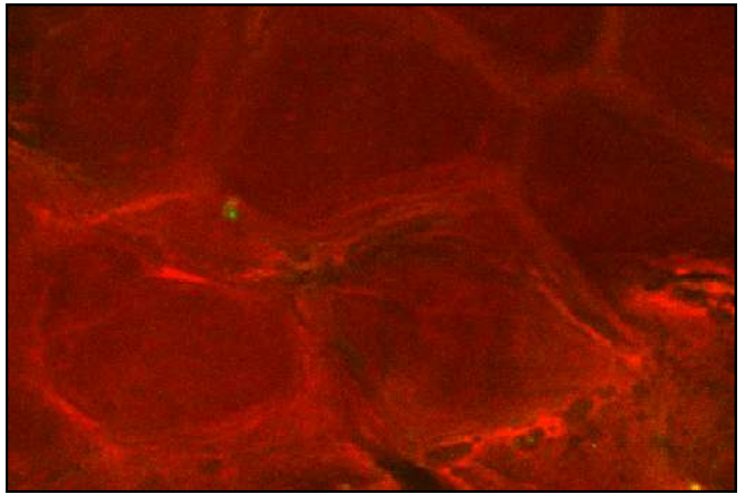


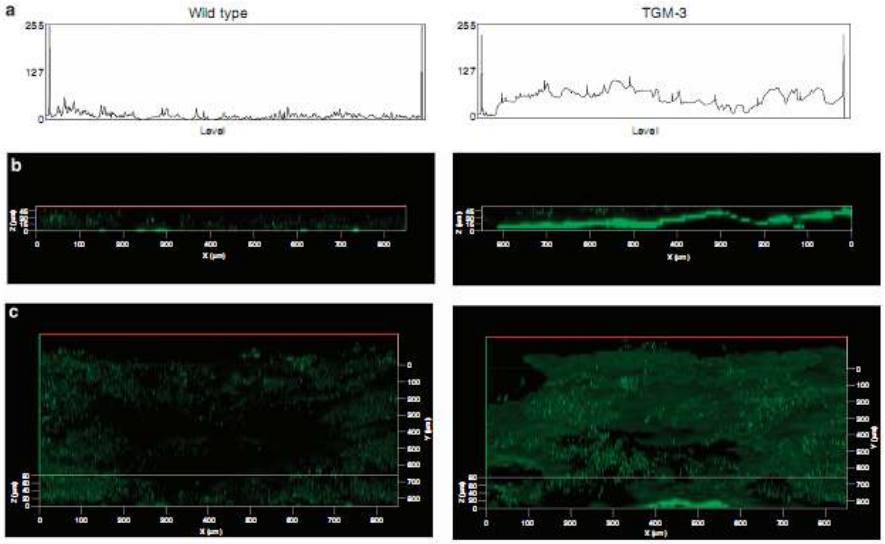
in vivo CARS

keratinocites– „bricks”

lipids – „mortar”







APPLICATIONS OF 3D MIROSCOPY IN DERMATOLOGY

In Vivo Second-Harmonic Generation and Ex Vivo Coherent Anti-Stokes Raman Scattering Microscopy to Study the Effect of Obesity to Fibroblast Cell Function

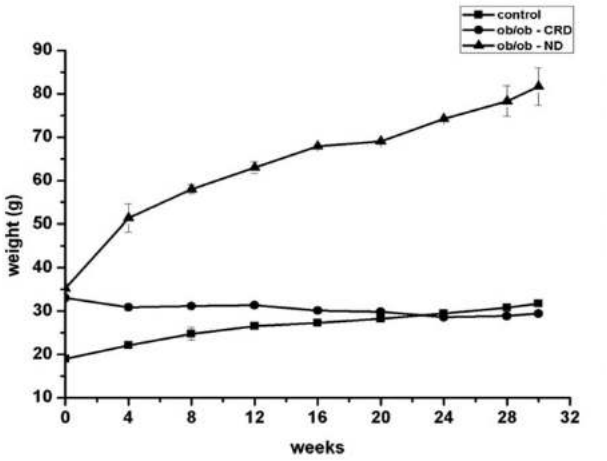


Fig. 2. Results of body weight changes and SHG intensity of dermal collagen measured and followed-up for 30 weeks. **A**: Increase of body weight in groups of mice during 30 weeks. At the beginning of the experiments (week 0) animals in various diet groups were already different; animals with leptin deficiency had higher body weight compared to controls. At week 8 the body weight increase of ob/ob—ND group was approximately twofold higher compared to other groups. The body weight of ob/ob—CRD group stayed relatively constant from week 8. Error bars represent standard deviation (SD) **B**: SHG

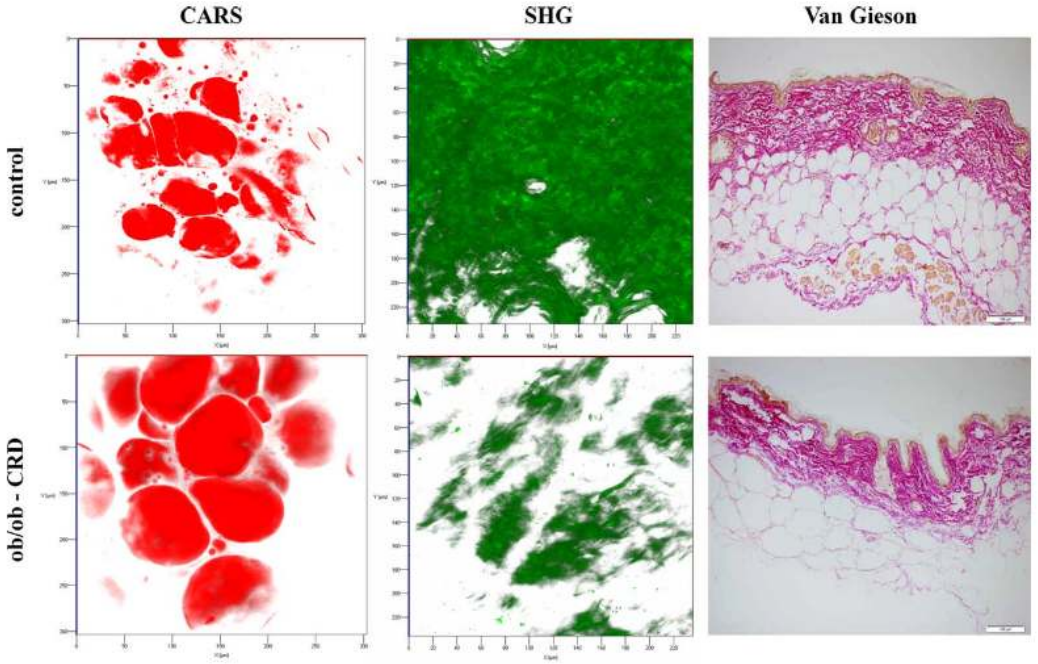


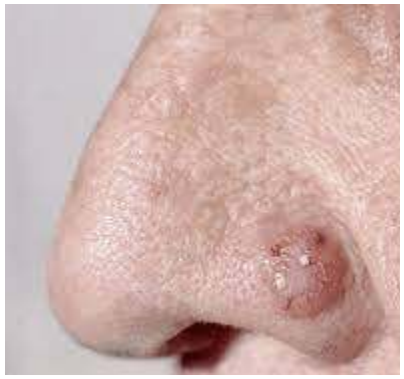
Fig. 4. Comparison of adipocyte sizes by CARS measurement and the collagen morphology, determined by SHG, and *ex vivo* van Gieson staining of control and ob/ob—ND groups. The 3D CARS and SHG images are projected in *z*-direction and imaging area is ~0.6 × 0.6 mm^2. The enlarged adipocytes, damaged collagen fibers and thinner dermis in case of leptin deficient mice are clearly visible, in contrast to control group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

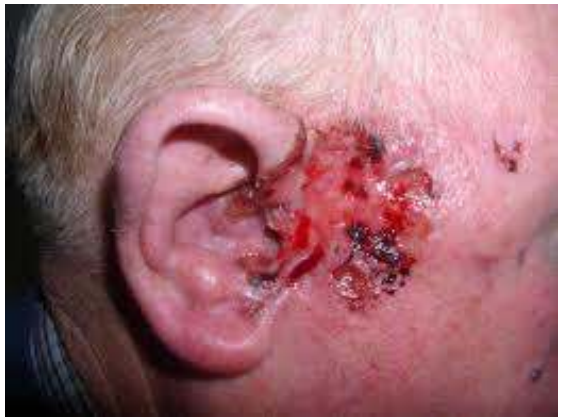
Haluszka, D., Lőrincz, K., Molnár, G., Tamás, G., Kolonics, A., Szipőcs, R., Kárpáti, S. and Wikonkál, N. M: „In vivo second-harmonic generation and ex vivo coherent anti-stokes Raman scattering microscopy to study the effect of obesity to fibroblast cell function using an Yb-fiber laser-based CARS extension unit," **Microsc. Res. Tech. 78,** 823–830 (2015)

APPLICATIONS OF 3D MIROSCOPY IN DERMATOLOGY

Investigation of basalioma

- **Collagen, as marker**
- Ex vivo samples, immediately after surgery





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Skin Research and Technology

Diagnosis of BCC by multiphoton laser tomography

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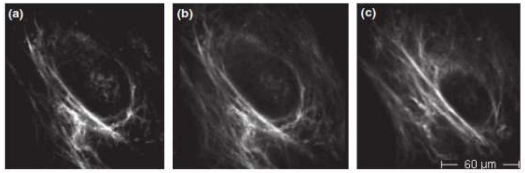


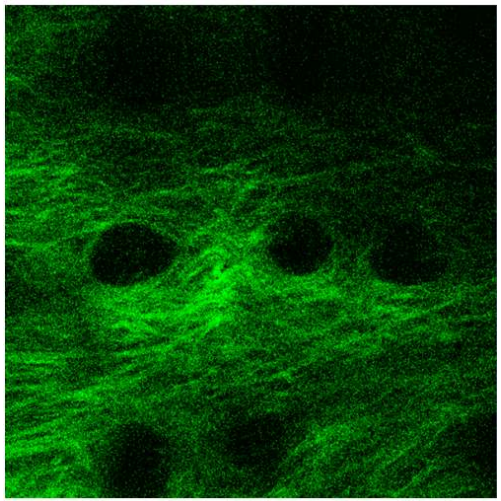
Fig. 4. Basal cell carcinoma. (a) 100 µm depth, excitation wavelength 800 nm. Shifting the wavelength to 800 nm, basaloid cells become less visible; employing an excitation wavelength of 820 nm basaloid cells disappear and it is possible to observe empty spaces surrounded by collagen fibres (phantom island); (b) 100 µm depth; (c) 120 µm depth.

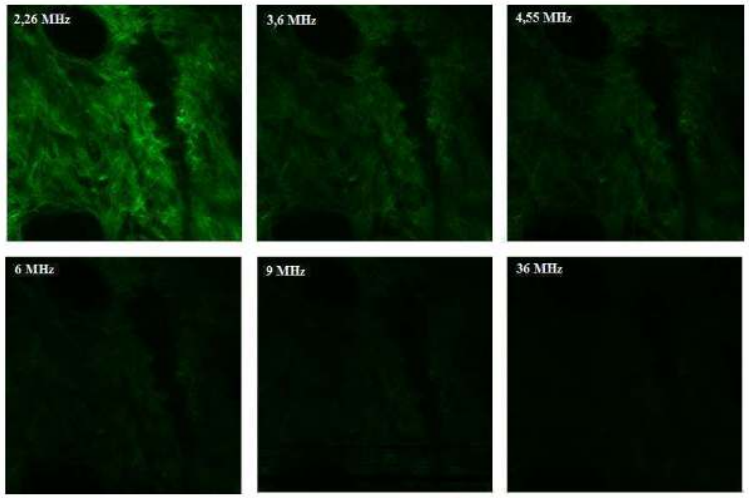
Semmelweis Egyetem
Bőr-, Nemikórtani és Bőronkológiai Klinika

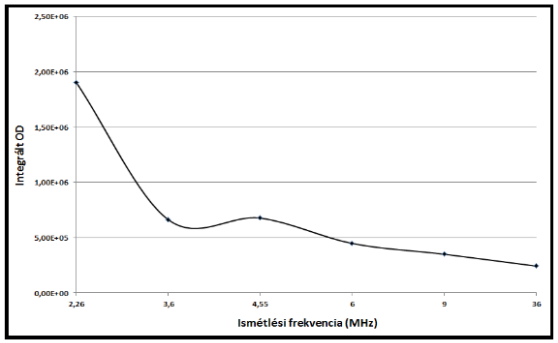
APPLICATIONS OF 3D MIROSCOPY IN DERMATOLOGY

Wigner Szipőcs Group

Using *FiberScope* for collagen detection







SHG intensity of collagen as the funtcion of repetition rate of our Yb-fiber laser (average power: ~5 mW, pixel dwell time: 5 μs)

Safety issues: imaging at 5 mW laser power (power of a laser pointer)

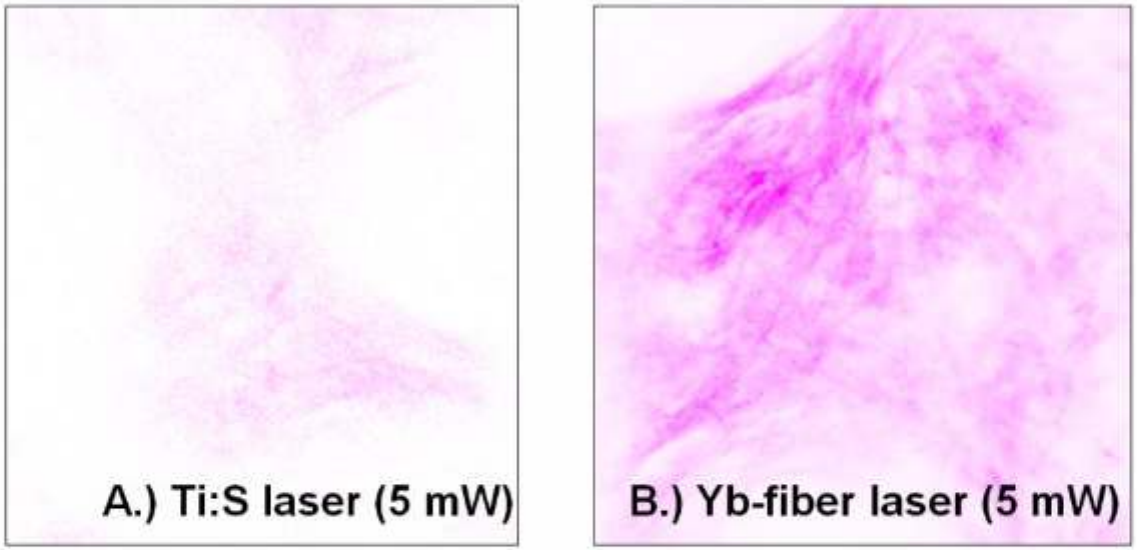


Figure 1. *Comparison of SHG imaging performance of different mode-locked lasers having the same average power of 5 mW (on the sample) for nonlinear microscopy. Ex-vivo murine skin sample, imaging depth: z = 30 µm, same microscope settings. Collagen distribution measured by A) an industry standard, 80 MHz Ti:sapphire laser, and by B) our newly developed Yb-fiber oscillator and amplifier system (with a variable repetition rate).*

APPLICATIONS IN NANOMEDICINE

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Letter to the Editor

In vivo study of targeted nanomedicine delivery into Langerhans cells by multiphoton laser scanning microscopy

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Abstract: Epidermal Langerhans cells (LCs) function as professional antigen-presenting cells of the skin. We investigated the LC-targeting properties of a special mannose–moiety-coated pathogen-like synthetic nanomedicine DermaVir (DV), which is capable to express antigens to induce immune responses and kill HIV-infected cells. Our aim was to use multiphoton laser microscopy (MLM) *in vivo* in order to visualize the uptake of Alexa-labelled DV (AF546-DV) by LCs. Knock-in mice expressing enhanced green fluorescent protein (eGFP) under the control of the langerin gene (CD207) were used to visualize LCs. After 1 h, AF546-DV penetrated the epidermis and entered the eGFP-LCs. The AF546-DV signal was equally distributed inside the LCs. After 9 h, we observed AF546-DV signal accumulation that occurred mainly at the cell body. We demonstrated in live animals that LCs picked up and accumulated the nanoparticles in the cell body.

Key words: eGFP-Langerin knock-in mice – *in vivo* – Langerhans cells – multiphoton laser microscopy – nanomedicine formulation

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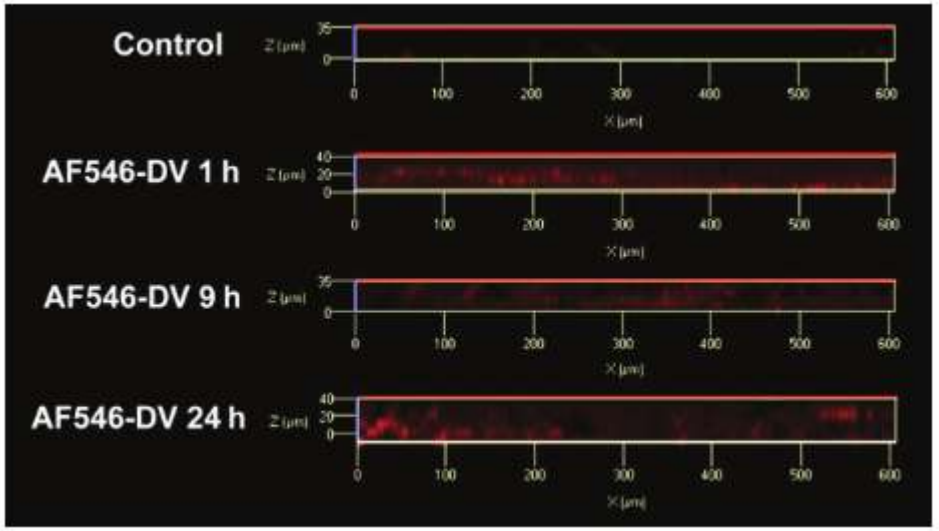


Figure 1. Penetration kinetics of AF546-DV through the stratum corneum in enhanced green fluorescent protein (eGFP)-Langerin knock-in mouse ear *in vivo*. *xz*-Multitracking sections were composed from a stack of *xy*-optical sections with 5 μm distances between the sections. The sections were recorded from the stratum corneum (Z = 0 μm) to the epidermis (Z = 35–40 μm). These representations reveal the penetration profiles of AF546-DV into eGFP-Langerin knock-in mouse skin reaching an average of 20 μm penetration depth underneath the honeycomb-shaped corneocyte layer after 1 h of topical treatment. AF546-DV diffused in the whole depth of the skin after 9 or 24 h despite of the fact that a part of the AF546-DV formula dried on the stratum corneum. Control: intact skin without AF546-DV.

APPLICATIONS IN NANOMEDICINE

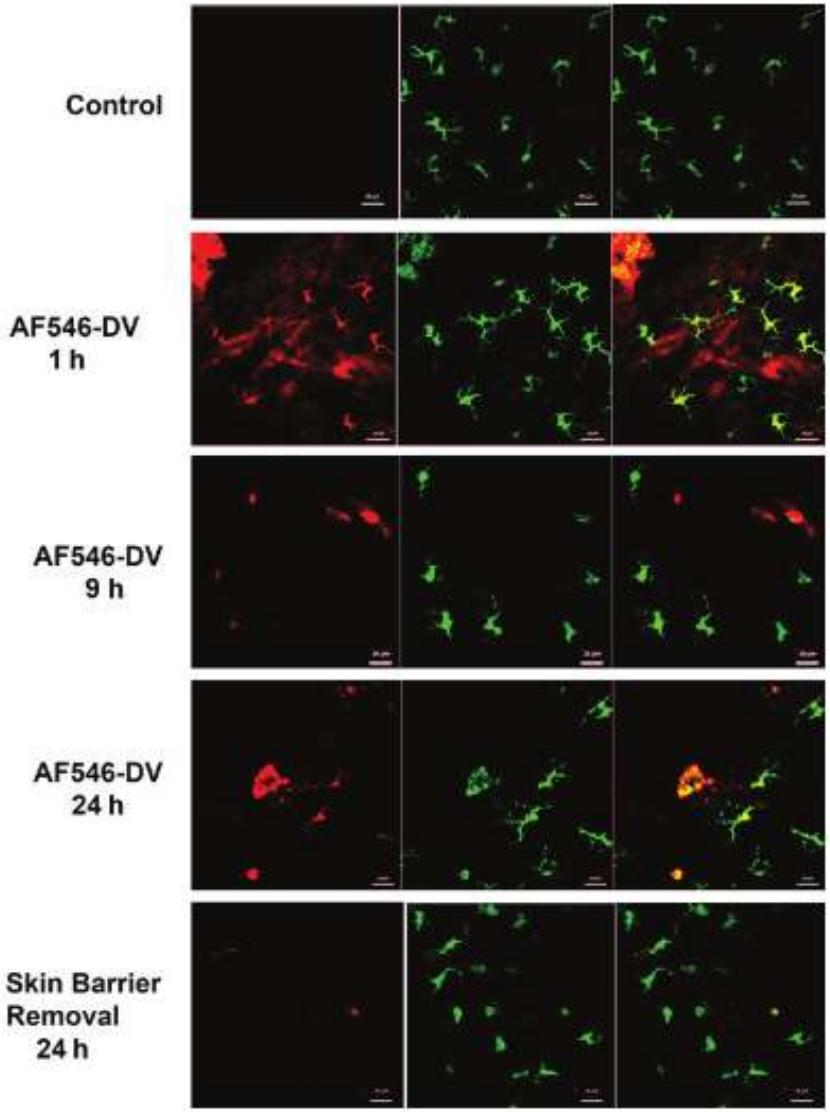
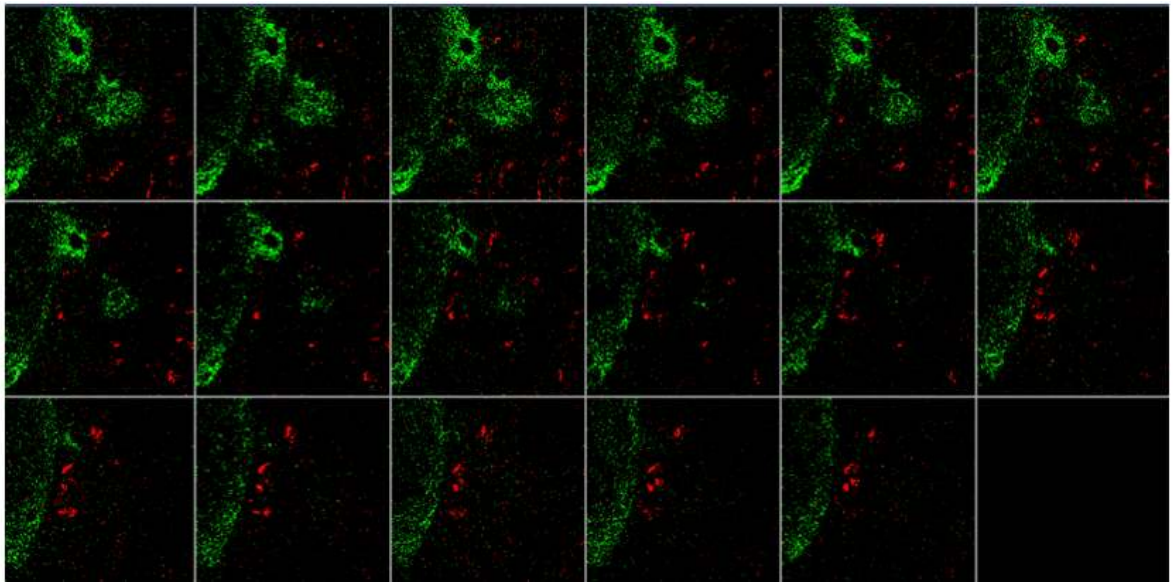


Figure 2. Kinetics of AF546-DV uptake by Langerhans cells (LCs) in eGFP-Langerin knock-in mouse ear *in vivo*. Nearly all LCs had incorporated AF546-DV after 1 h of topical treatment: strong colocalization was detected in both channels [NDD 2 – green/eGFP (middle column) versus NDD 1 – red/AF546-DV (left column)] as presented on the merged pictures (right column). Images of red light emission also revealed that the nanoparticles were distributed homogenously in all parts of the LCs. After 9 h, the intensity of red light emission by AF546-DV decreased significantly and disappeared from the dendrites and concentrated around the nucleus. Intriguingly, after 24 h, the nuclear location as well as a weak signal of AF546 in the dendrites could still be observed. The removal of the stratum corneum resulted in the activation of the vast majority of the LCs characterized by a rounded potato-like shape. The scale bar represents 20 μm.

Using *FiberScope 3D imaging* for Nanomedicine



In vivo **penetration measurement of Alexa-labelled DermaVir nanoparticles (AF546-DV) into the skin of a mice using our FiberScope device using a 2 MHz Yb-laser operating at ~1030 nm after one hour of the treatment. It can be seen that the AF546-DV accumulated in Lagerhans cells.**

SUMMARY

1. A PROTOTYPE OF AN OPTICAL FIBER LASER BASED, HANDHELD 3D NONLINEAR MICROSCOPE SYSTEM NAMED *FiberScope* HAS BEEN DEVELOPED
2. IT IS SUITABLE FOR NON INVASIVE, LABEL FREE, IN VIVO 3D IMAGING OF THE SKIN UP TO A FEW 100 µm THICKNESS
3. APPLICATIONS IN DERMATOLOGY AND NANOMEDICIDE HAS BEEN DEMONSTRATED
4. MODERATE COST OF ~ 50kUSD (compared to DermaInspect)
5. STILL NEEDS FDA APPROVAL FOR IN VIVO HUMAN INVESTIGATIONS
6. MASS PRODUCTION CAN CONSIDERABLE CUT MANUFACTURING COST