Introduction to multiphoton microscopy

Rocco D'Antuono

Advanced Light Microscopy STP

The Francis Crick Institute

London

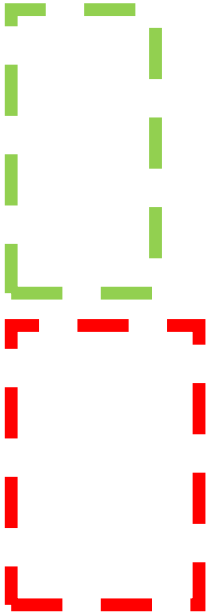
28th Nov 2018

what is in Focus?

Lena: one of the most used sample image for the test of image processing algorithms.

Full story of Lena Soderberg and her fans (and controversies):
IEEE TRANSACTIONS ON IMAGE PROCESSING. VOL. 5. NO. 1. JANUARY 1996
http://www.lenna.org/editor.html

what is in Focus?



Plumes are in FOCUS

Oil lamp is OUT OF FOCUS

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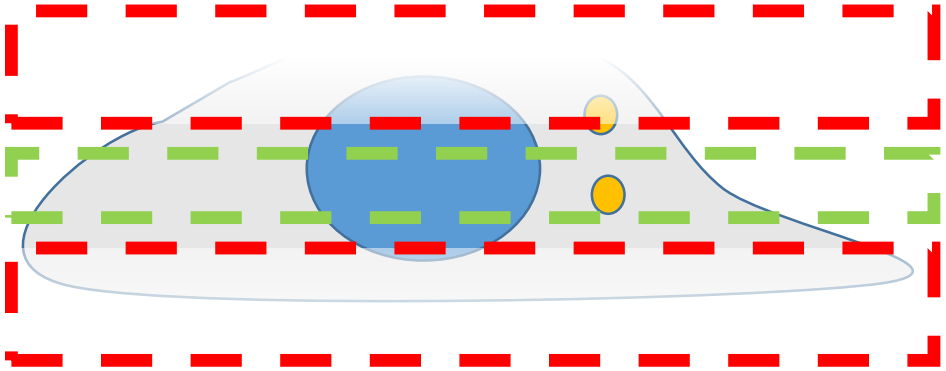
what is in Focus?



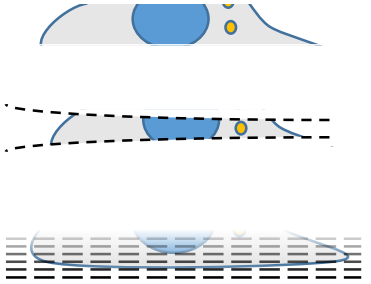
Naïve representation of a cell

Vesicle OUT OF FOCUS

Vesicle in FOCUS

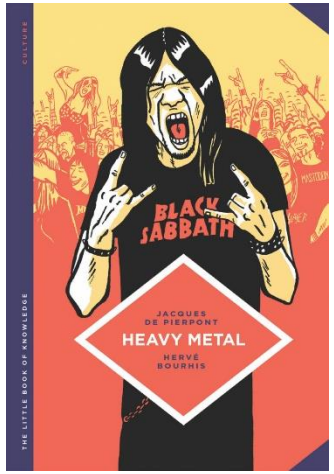
Need for <u>OPTICAL SECTIONING</u>:

- **CONFOCAL**
- **LIGHT SHEET**
- **TIRF**
- **Et al.**

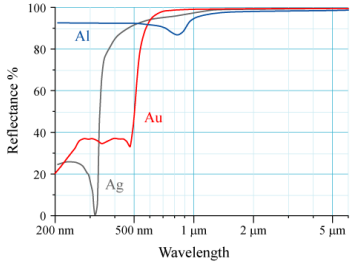


what matters to us?

METAL?



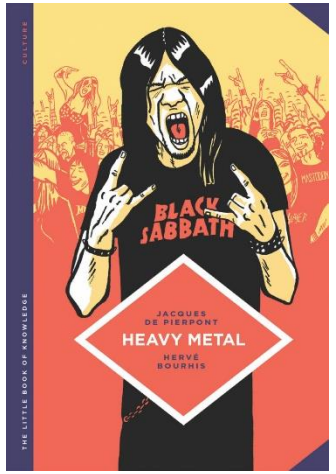
Unluckily not



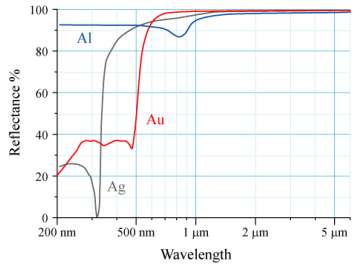
https://en.wikipedia.org/wiki/Optical_coating
#/media/File:Image-Metal-reflectance.png

what matters to us?

METAL?

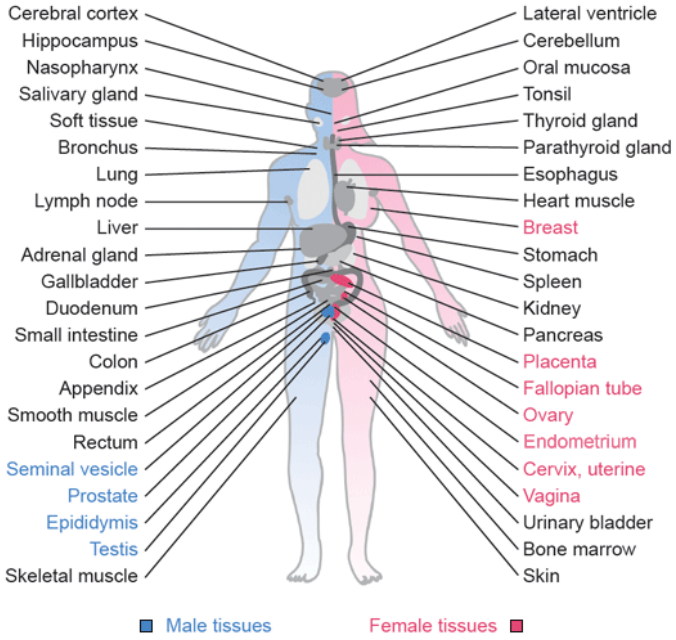


Unluckily not



https://en.wikipedia.org/wiki/Optical_coating#/media/File:Image-Metal-reflectance.png

SOFTER matter: Biological tissues



https://www.proteinatlas.org/learn/dictionary/normal

Need to known optical properties:

- **FLUORESCENCE BASICS**
- **OPTICAL WINDOW ?**
- **TECHNOLOGICAL LIMITS**

Fluorescence Basics

MEMORANDUM

Need to known optical properties:

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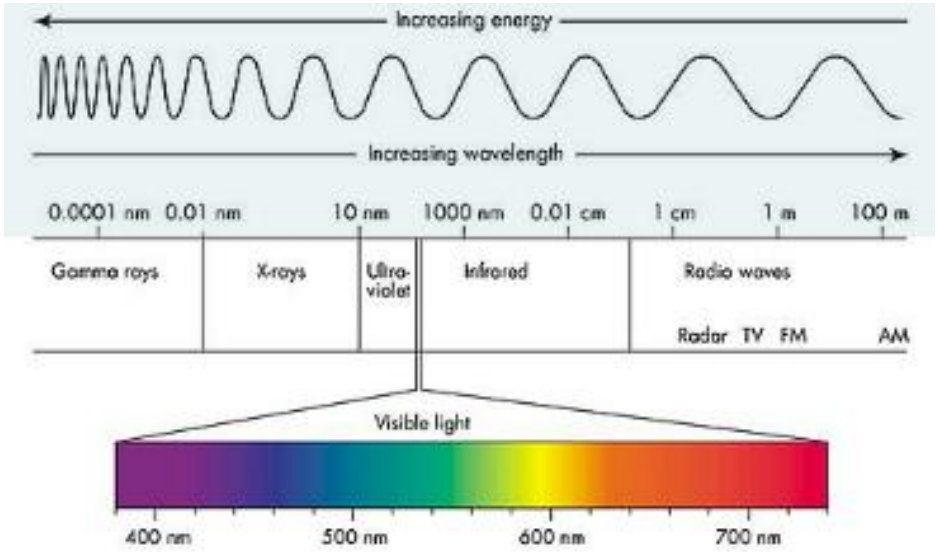
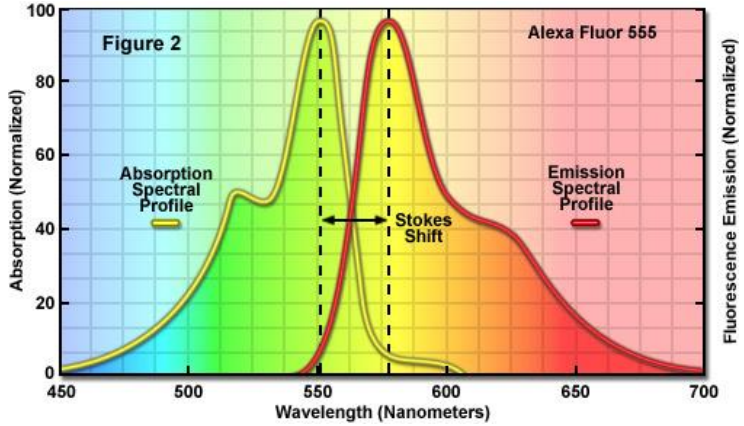


https://www.cyberphysics.co.uk/topics/light/emspect.htm

Electromagnetic radiation: energy and wavelength relationship

$$E = hf = hc / \lambda$$

Fluorescence: Absorption vs Emission



http://zeiss-campus.magnet.fsu.edu/articles/basics/fluorescence.html

MEMORANDUM

Talk about optical properties:

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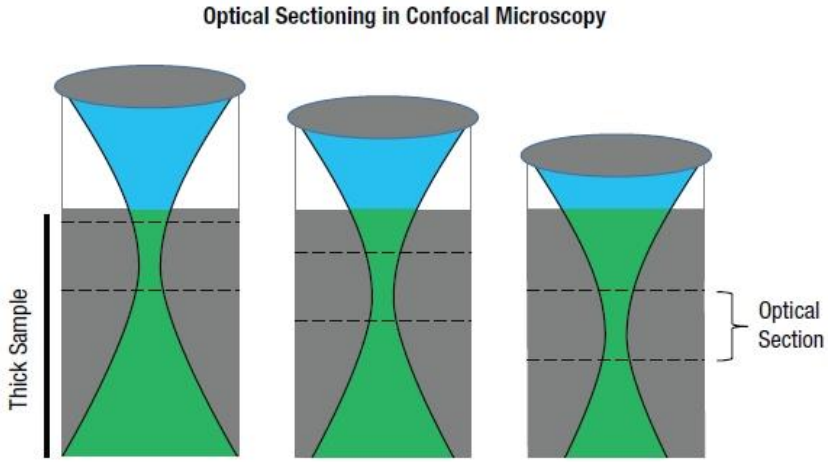
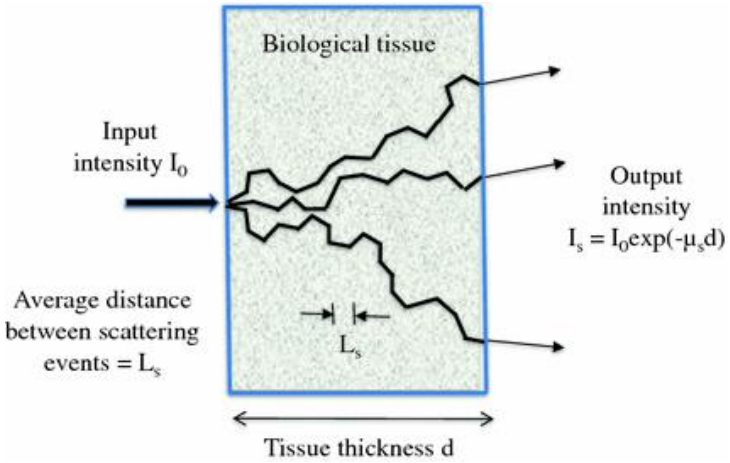
Fluorescence Basics

Visible light through a thick sample



Excitation of fluorescence is NOT specific

Light-Tissue interactions



Light is attenuated

https://www.thorlabs.com/newgrouppage9.cfm?objectgroup_id=10765

Light-Tissue Interactions
Gerd Keiser in "Biophotonics: concepts and applications" -Springer

MEMORANDUM

Talk about optical properties:

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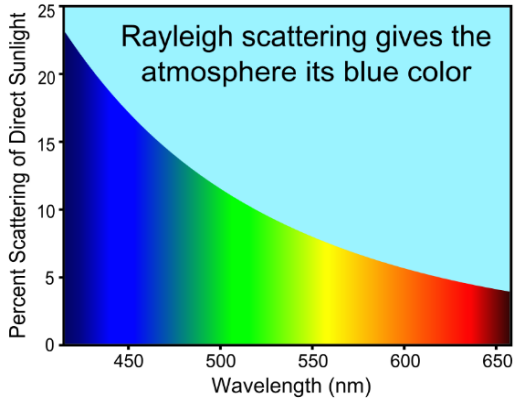
Optical Window?

BUT:

1. Scattering is reduced at longer wavelength



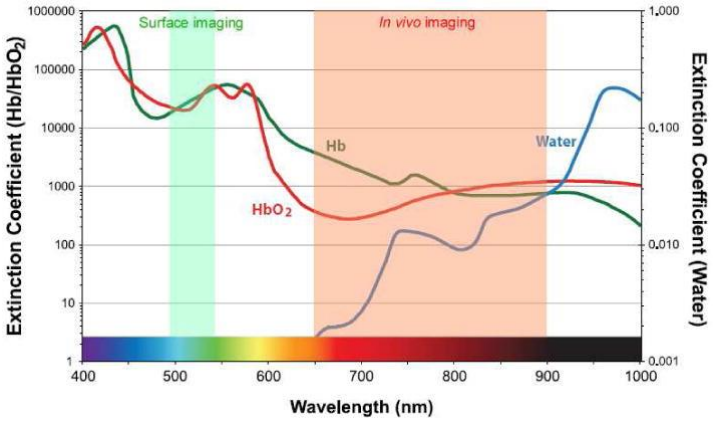
$$I = I_0 \left(\frac{1+\cos^2\theta}{2R^2}\right)\left(\frac{2\pi}{\lambda}\right)^4\left(\frac{n^2-1}{n^2+2}\right)^2\left(\frac{d}{2}\right)^6, \qquad I \propto \frac{1}{\lambda^4}$$

May be there is a chance

https://en.wikipedia.org/wiki/Rayleigh_scattering

"Rayleigh-scattering calculations for the terrestrial atmosphere". A. Bucholtz -Applied Optics 34 (15), 1995

2. Biological tissues show reduced absorption in Near InfraRed



Yes it can work!!

H. Kobayashi - Chem Rev. 2010 May 12; 110(5): 2620–2640.

Optical Window?

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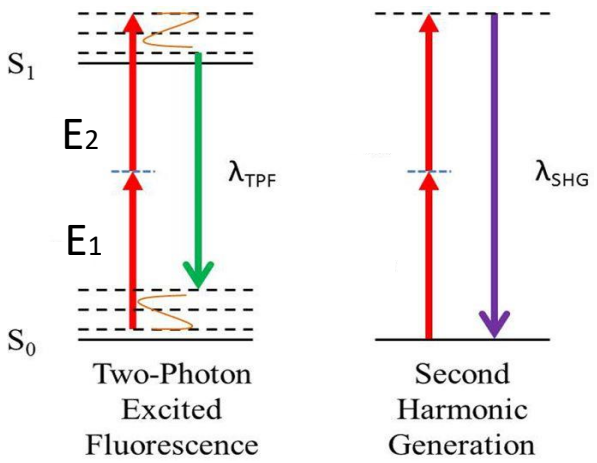
Jablonski diagram for 2-photon excitation

$E_{TOT} = E_1 + E_2$

$\lambda_{TOT} = 2\,\lambda_1$

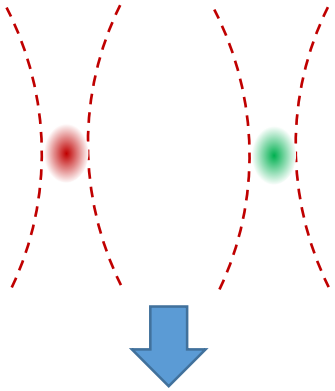


Modified from
Multiphoton microscopy
Adam M. Larson
Nature Photonics **volume5**, page1 (2011)

2-photon excitation:

- **Very rare in nature**
- **Quantum mechanics constraints**

(photons in the same place at the same time*!!)

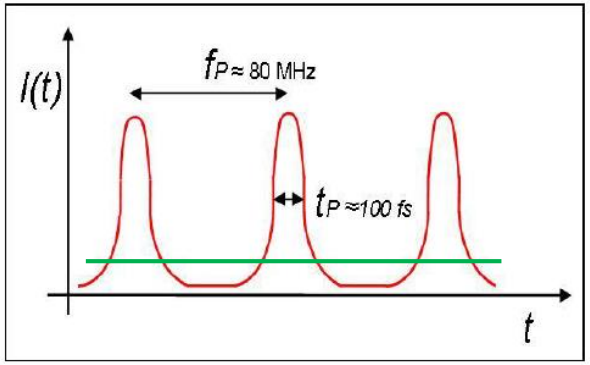


Need special laser to make it:

- **Probable**
- **Excite enough fluorescence!**

*In the limits of Heisenberg uncertainty principle

Pulsed vs Continuous-Wave laser



Continuous-Wave laser: e.g. <P>= 25 mW

Pulsed tunable laser:

e.g. pulse P≈ GW,<P>= 800-2500 mW

Time between following pulses: e.g. **t=12.5ns** !

Energy delivered with individual high energy pulses!

MEMORANDUM

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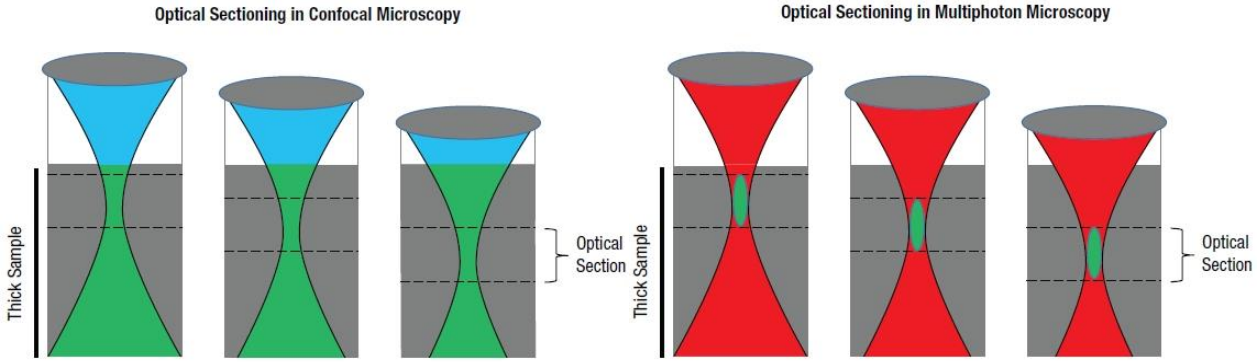
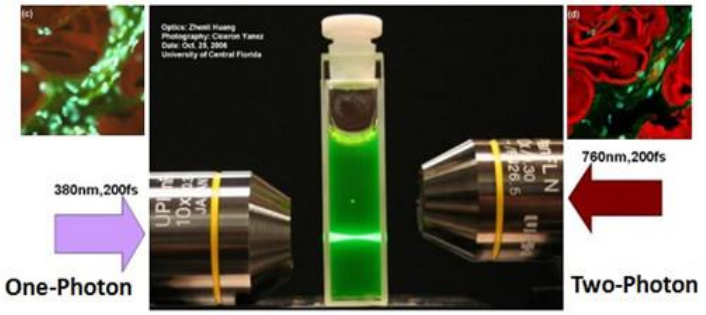
Optical Window?

Excitation volume: confocal vs multiphoton



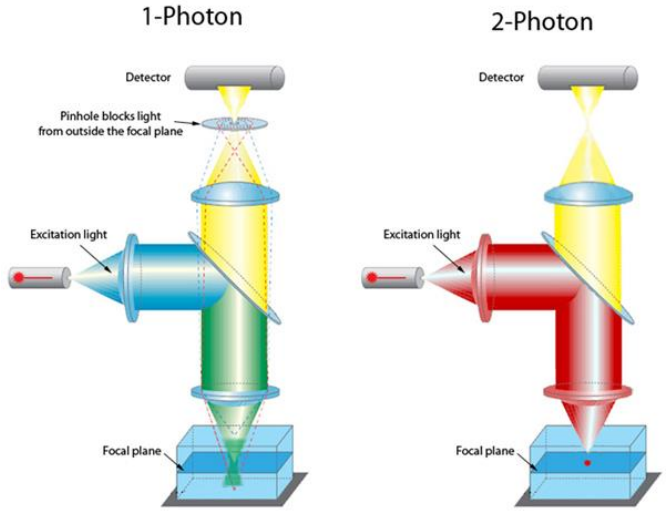
https://www.thorlabs.com/tutorials.cfm



Progress in Chemistry 2017, Vol.29 Issue (10):1215-1227
C. Huang

Really need a pinhole?

- Pin-hole rejects out-of-focus light in confocal microscopy.
- Using a selective excitation **optical sectioning is intrinsic**!!



https://microscopy.duke.edu/introduction-microscopy

Technological limits

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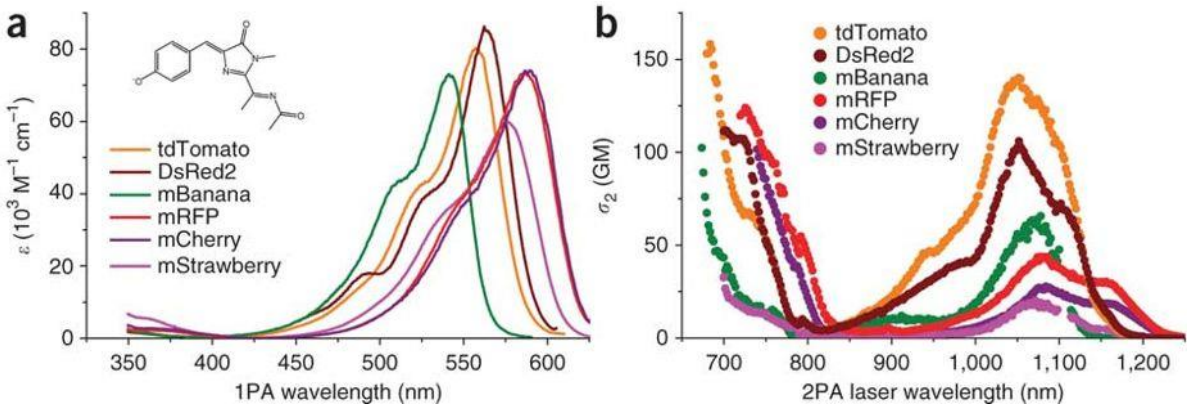
Single vs Two-photon absorption:

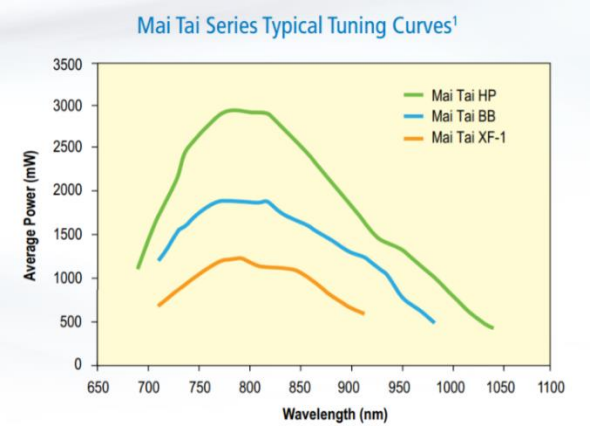
MP excitation may be complex (emission remains the same)



M. Drobizhev, Nature Methods volume 8, pages 393–399 (2011)

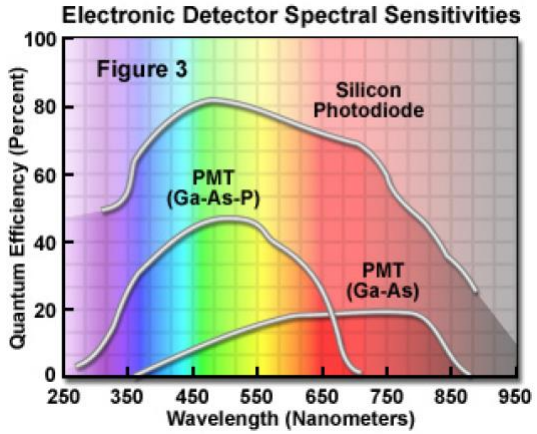
MP Laser tuning curve:

Power is wavelength-dependent



www.spectra-physics.com/

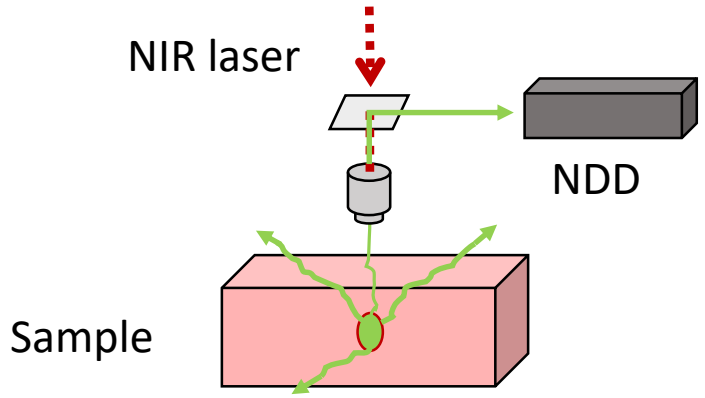
Detectors: not same spectral response



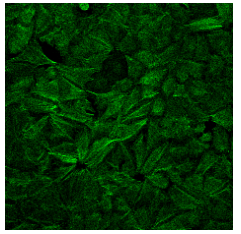
https://www.olympus-lifescience.com/en/microscope-resource/

Deeper means more scattering:

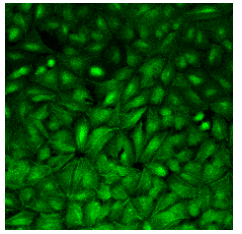
signal must travel backwards



Non-Descanned Detectors (NDD): Light detection more efficient soon after the objective!!



Ch 500-550 nm Descanned @800nm



Ch 500-550 nm NDD @800nm

Technological limits

MEMORANDUM

Talk about optical properties:

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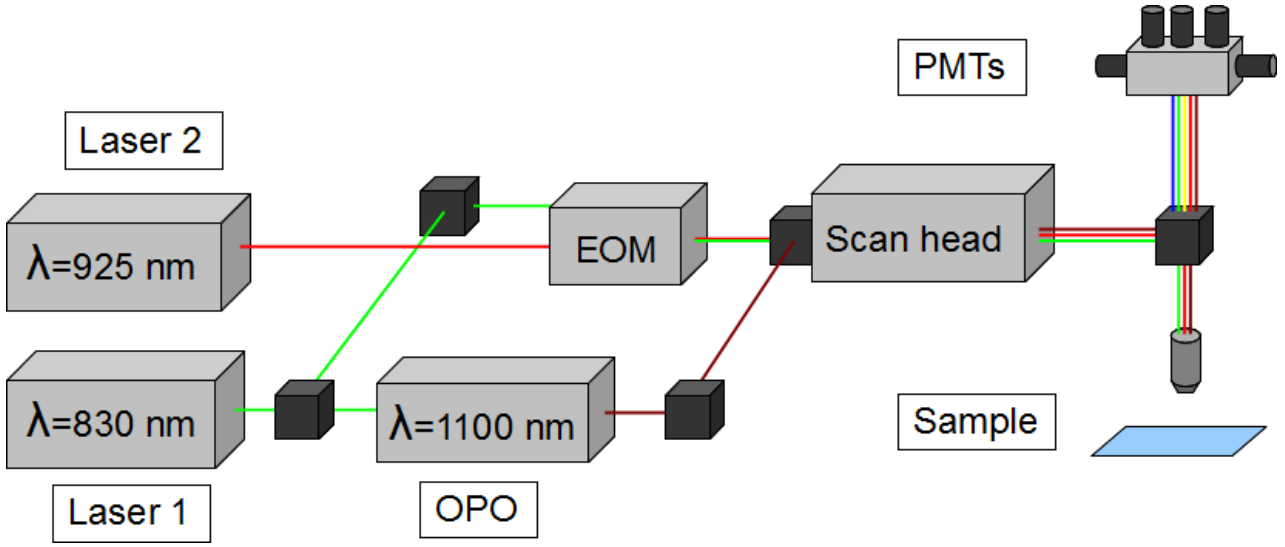
Example of MP microscope: configuration and costs



- 100k MP laser
- 300k Scanner and Microscope
- 15k high spec objective
- 10-15k highly sensitive detector
- 1k Filtercube

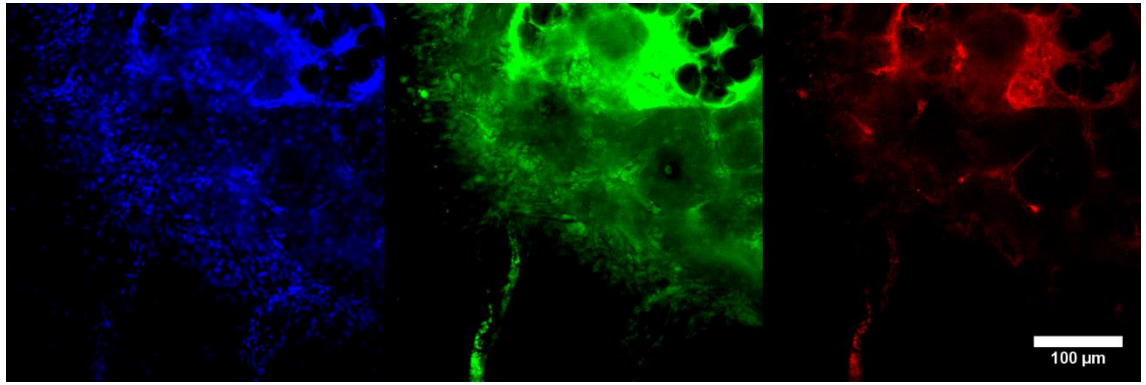
MEMORANDUM

Example of datasets:

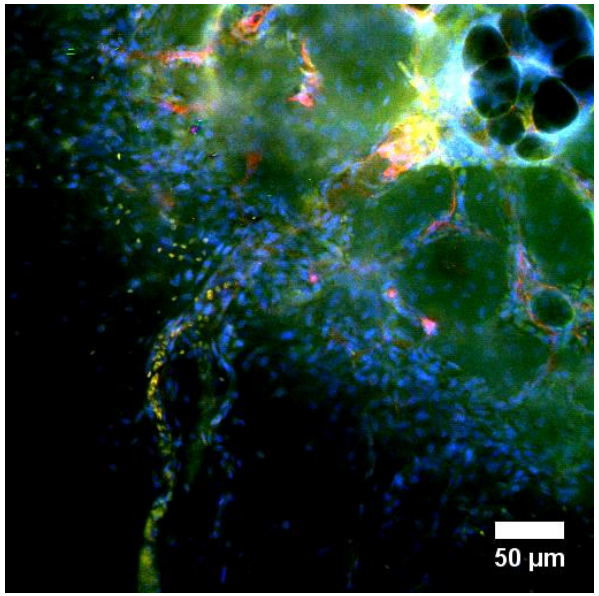
- **Cleared fixed explant imaging**
- **Imaging of the Calvarium bone marrow**

Tuning the laser for specific excitation WL!!

780 nm 860 nm 960 nm

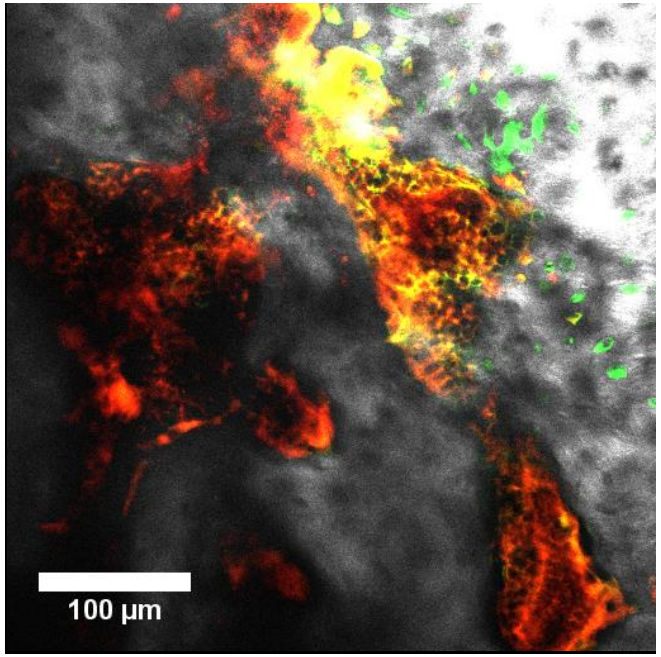


DAPI, GFP (aGFP-AF488) BM precursors, Endomucin-AF555



Patience and courtesy:
Stefania Di Blasio

Single WL for in vivo



Bone Marrow Calvaria:
SHG (white), Qtracker (red), Dextran-TRITC(green)

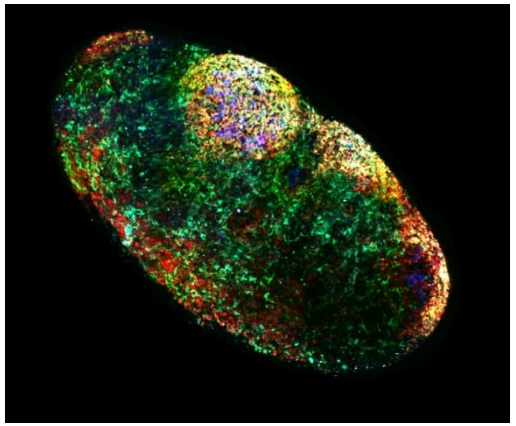
Patience and courtesy:
Diana Passaro

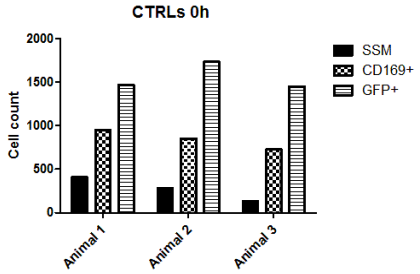
MEMORANDUM

Applications:

- **3D LN reconstruction and cell populations quantification**
- **Gut infection**
- **Lymphocytes tracking**
- **3P intravital longitudinal imaging through skull**

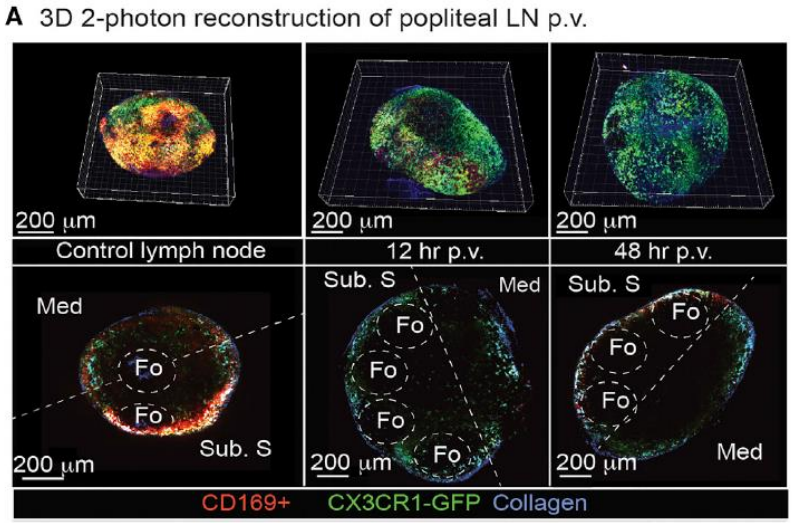
3D Lymph node reconstruction (popliteal, no Clearing)

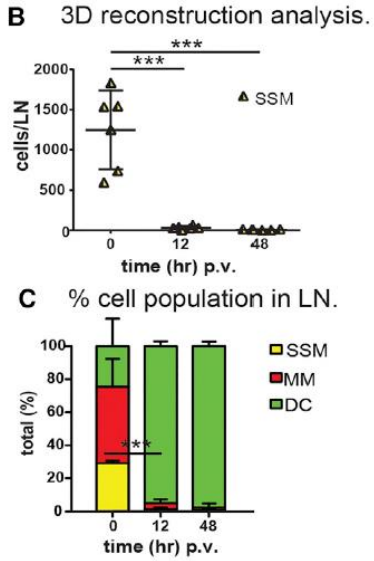




3 x3 x 3 Ch, z-depth of up to 300 um, 16X/0.8 LWD

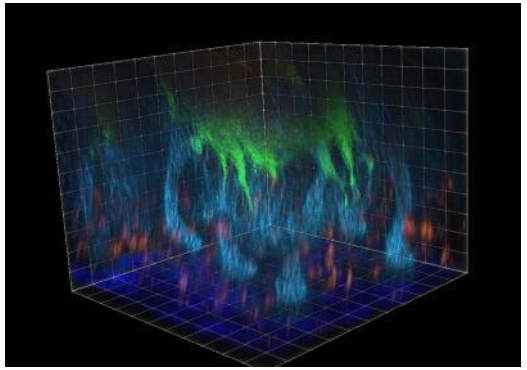
R. D'Antuono, S.F.G. González – NEUBIAS 2018





Chatziandreou et al., 2017, Cell Reports 18, 2427–2440

Gut crypts infection (no Clearing)



E-Cad-mCFP
GFP-tagged S.Tm^{att}
Red autofl.

M. Proietti & L. Peruzza, *Nature Communications* (2019)

MEMORANDUM

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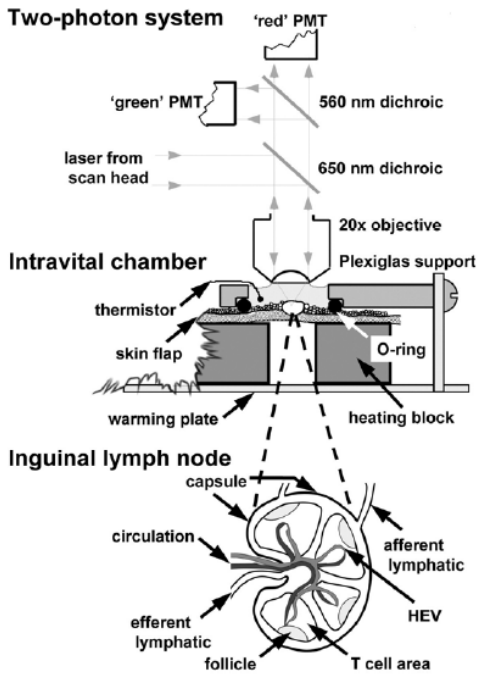
- Gut infection

- **Lymphocytes tracking**

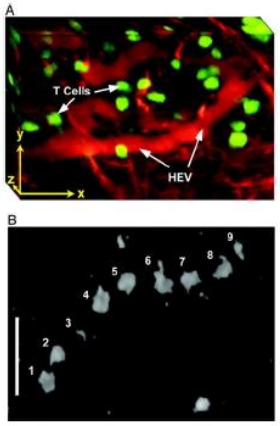
- 3P intravital longitudinal imaging through skull

Intravital imaging of Inguinal Lymph Node and cell tracking



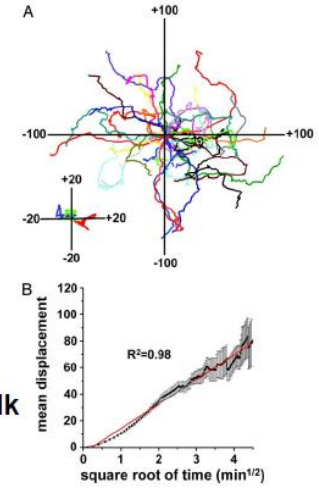
Miller M J et al. PNAS 2003;100:2604-2609

CFSE-labelled naïve T cells
Dextran-TRITC



120 um below surface
30 ms per frame
Scalebar 25 um

Tracking



Random walk theory

T cells speed: e.g. v=20 mm/min

MEMORANDUM

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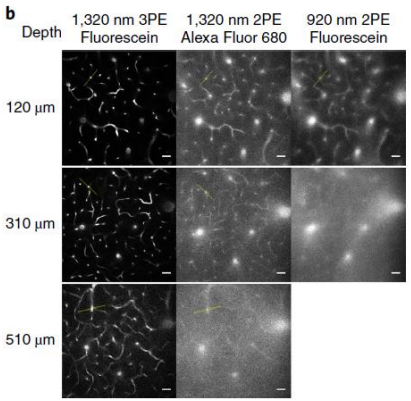
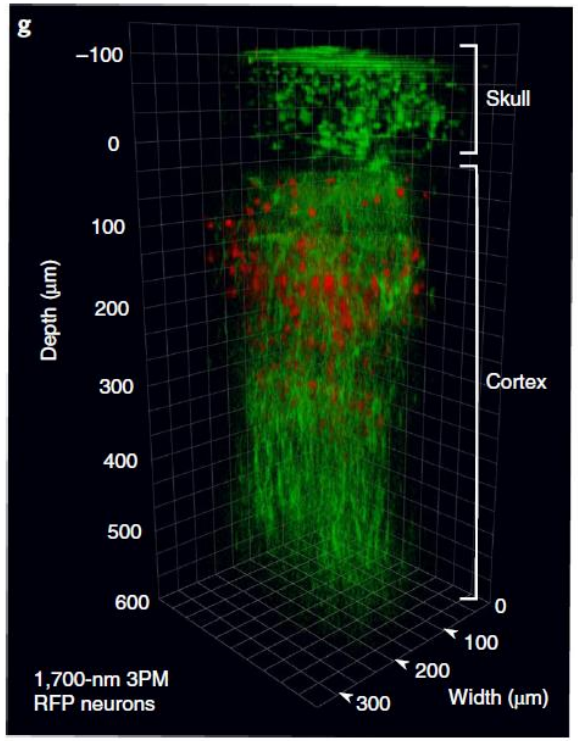
- Lymphocytes tracking
- **3P intravital longitudinal imaging through skull**

Three-photon imaging of mouse brain through the intact skull





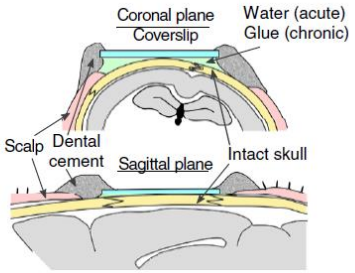
" >500-µm depth, as well as GCaMP6s calcium imaging over weeks in cortical layers 2/3 and 4 in awake mice, with 8.5 frames per second and a field of view spanning hundreds of micrometers."

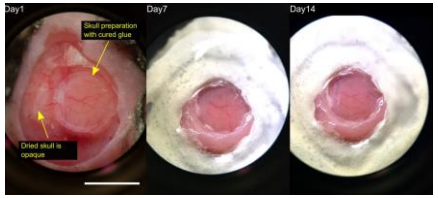
T. Wang, Nature Methods, Vol. 15, October 2018 | 789–792

Bulk solution

Brainbow mouse

a





To solve: window degradation over time. Scalebar 5mm

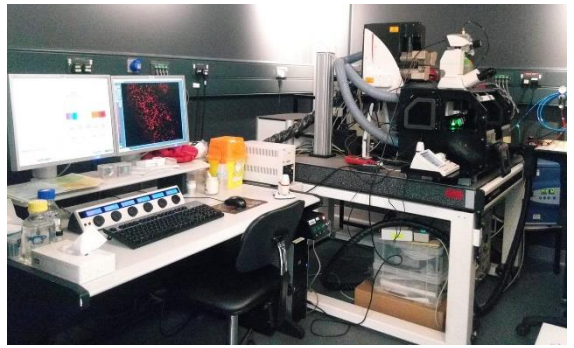
WHERE to do it @ the Crick?

Light Microscopy STP, 5th floor on
Zeiss Invert LSM780 NLO:

- **Confocal** scanner with 405 nm, Argon, 561 nm, 594 nm, 633 nm laser lines
- Standard internal detectors (with spectral unmixing)
- Coherent **Chameleon**

Light Microscopy STP, 3rd floor on
Leica Upright MP SP5:

- **Confocal** scanner with 405 nm, Argon, 561 nm, 594 nm, 633 nm laser lines
- Spectra Physics Mai Tai DeepSee: **690 nm — 1024 nm**
- **20X/1.0 NA IR-corrected LWD**
- **3 HyD + 2 PMT *internal***
- **2 NDD + 2NDD**: 2 PMT Refl. + 2 Trans.
- "Live Data Mode" software module



In-Vivo Imaging STP, -4 floor (B4) on
Zeiss Upright LSM710 NLO:

- **Confocal** scanner with Argon, 561 nm, 594 nm, 633 nm laser lines
- Standard internal detectors (with spectral unmixing)
- Spectra Physics Mai Tai DeepSee: **690 nm — 1024 nm**

www.zeiss.com

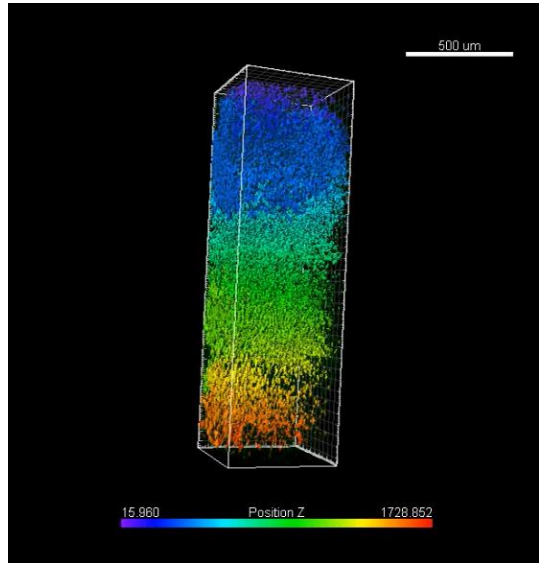
MEMORANDUM

COMING SOON:

- **Intravital Olympus FV-MPE-RS**
- **Multiphoton twin beam Zeiss LSM880**

COMING SOON @ the Crick!!

Cleared brain, Thy1-GFP stained.
Courtesy: Mahesh Karnani, Donald Bell

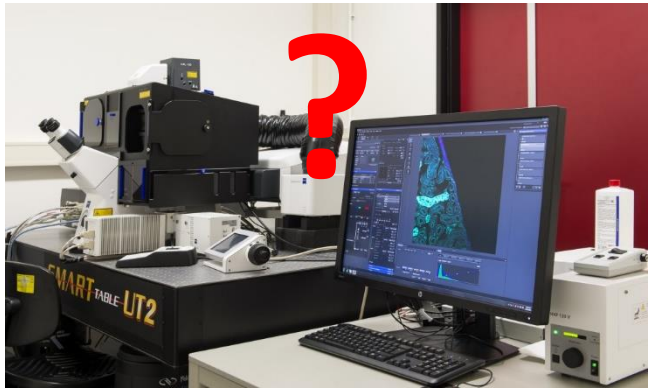


Olympus FVMPE-RS:

- Physiology stand with **large operational volume**
- Prior positioning platform + **piezoelectric** sample holder
- **25X/1.05 NA IR-corrected motCORR LWD** ("TruResolution")
- Spectra Physics **INSIGHT X3 DUAL**/DUALC-OL: **680 nm — 1300 nm + 1045 nm**
- 4 Axes Quadralign **Auto Alignment optics**
- **4 NDD**: 2 PMT + 2 GaAsP
- **Resonant Scanner** (8KHz speed)

Zeiss LSM880 NLO:

- **Visible lasers** lines and standard internal detectors (with spectral unmixing)
- Coherent **Chameleon** + **OPO** with twin beam **pulse matching**
- **4 NDD + 2NDD**: 2 MultiAlkali + 2 GaAsP and 2 MultiAlkali in Trans.



https://www.olympus-lifescience.com/en/laser-scanning/fvmpe-rs/upright-frame/

Spoiler from https://bc-uu.nl/cci/?page_id=814

Acknoledgements:

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Diana Passaro (Bonnet Lab)
Stefania Di Blasio (Malanchi Lab)
In Vivo Imaging STP

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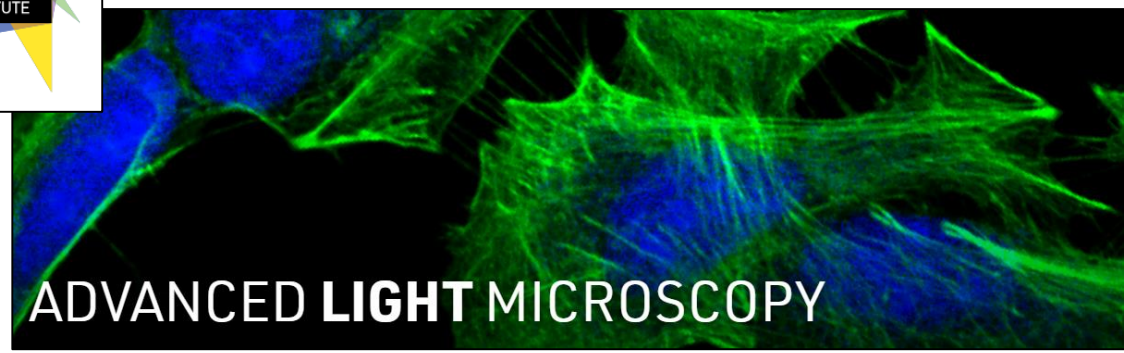
- chat with us at (~~bar~~) Light Microscopy STP, 3rd floor, SW312
- Imaging Help Desk (wed aft twice a month)
- ~~scream while at the microscopes~~