LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Steffen Dietzel
Walter-Brendel-Zentrum für Experimentelle Medizin

and (starting summer 2015)

Core facility bioimaging of the Biomedical Center

Multi-photon Microscopy

Flavors of Multi-Photon-Microscopy

- Two-photon excitation fluorescence (TPEF)
- Second harmonic generation (SHG)
- Third harmonic generation (THG)
- Resonance enhanced THG
- Plasmon induced luminescence
- Coherent Anti-Stokes Raman (CARS)-Microscopy

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

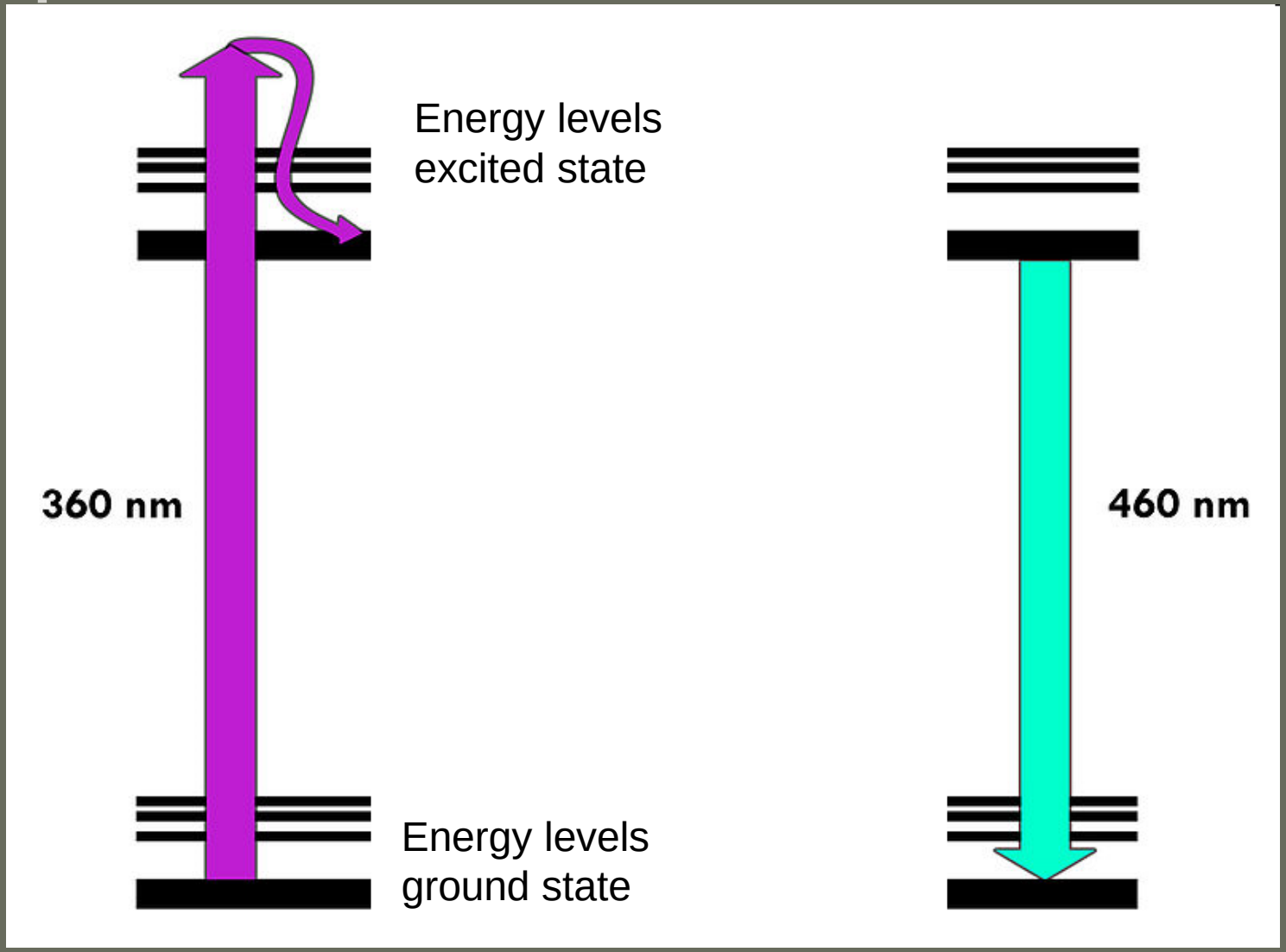
Walter Brendel Zentrum München

BIN LMU Bioimaging Network Munich

Two-photon excitation fluorescence

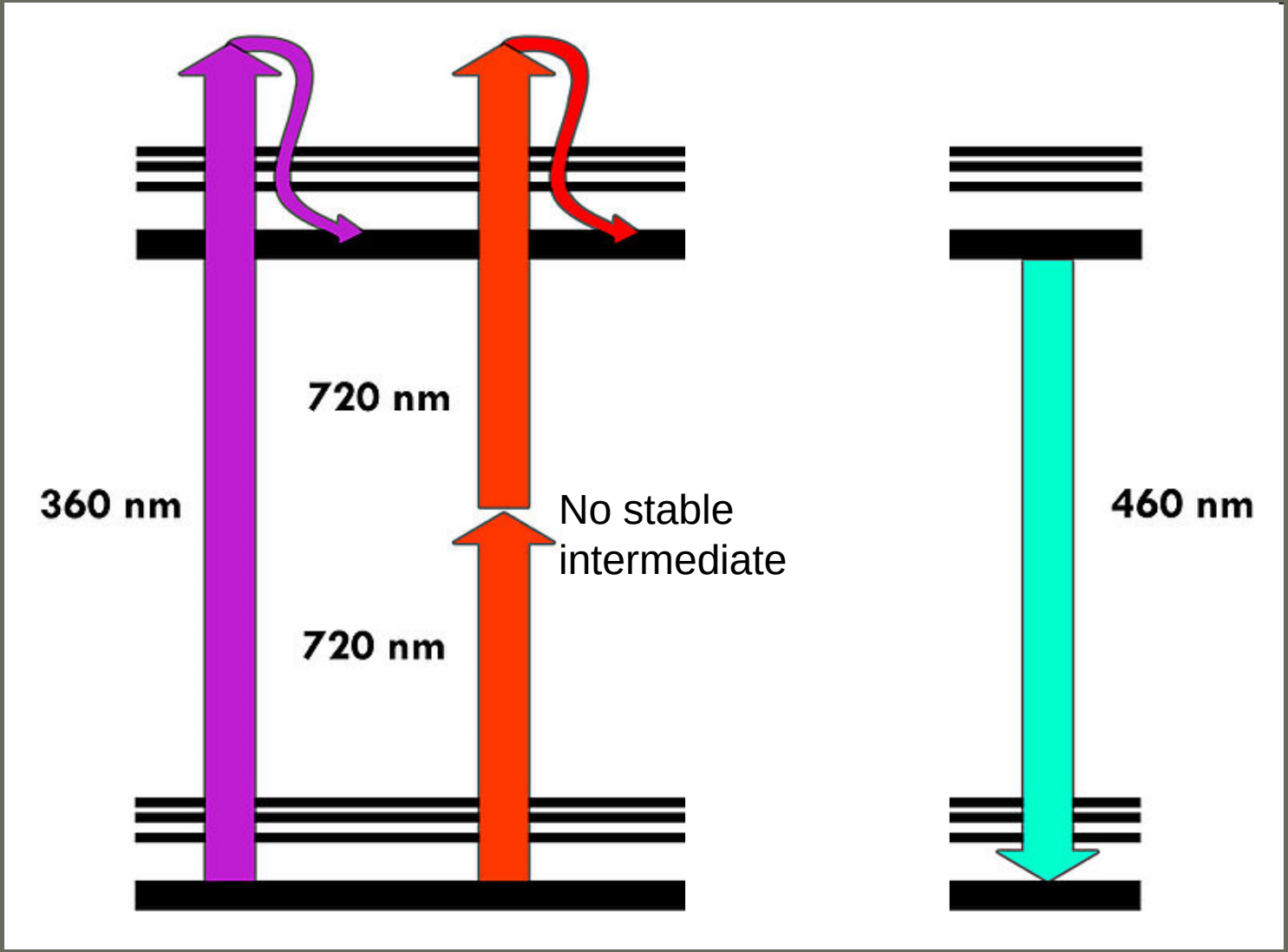
- How
- Why
- What for

1 photon excitation fluorescence



Bildquelle: Multi-photon excitation microscopy. BioMedical Engineering OnLine, 2006, 5:36. DOI:10.1186/1475-925X-5-36.

1+2 photon excitation fluorescence



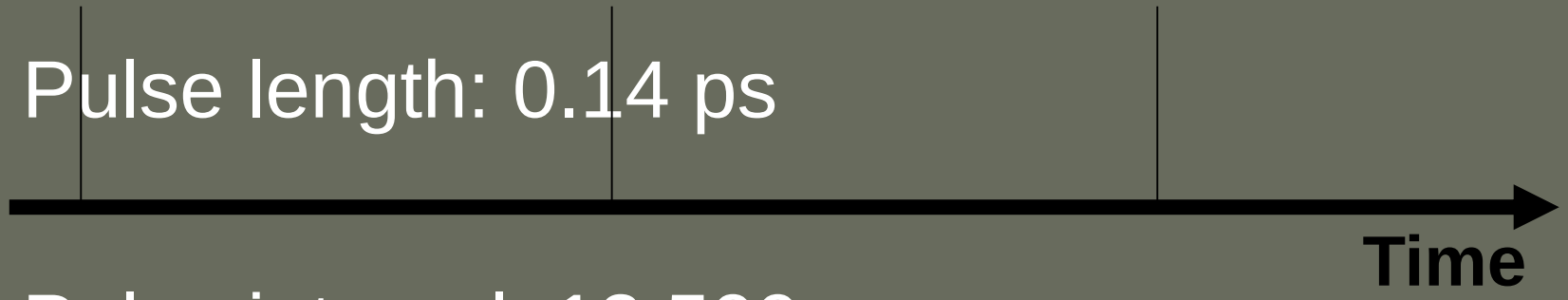
Bildquelle: Multi-photon excitation microscopy. BioMedical Engineering OnLine, 2006, 5:36. DOI:10.1186/1475-925X-5-36.

Two-photon excitation...

- ... only works if two photons arrive at the absorbing electron within 1 attosecond, $1*10^{-18}$s (nano, pico, femto, atto).
- This is statistically very unlikely to happen under normal conditions.
- To generate a high enough photon density, a focused, pulsed laser is required.

Pulsed laser (Chameleon Ultra II)

- Pulse length: 0.14 ps



- Pulse interval: 12 500 ps (= 12.5 ns = 80 MHz)

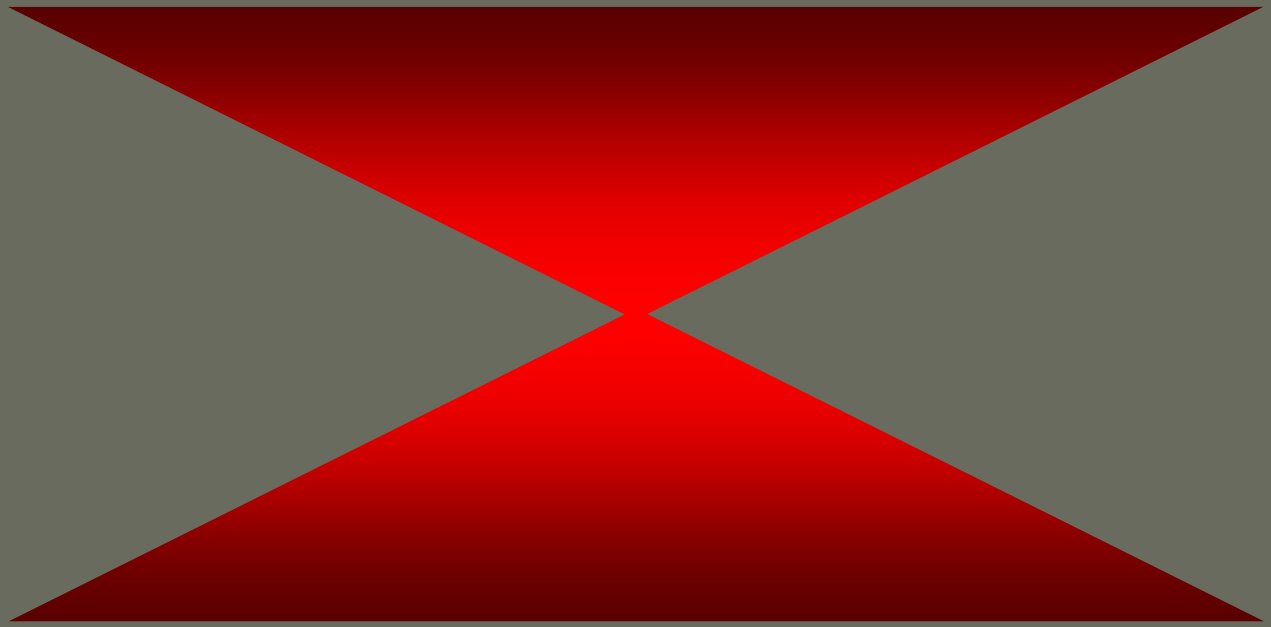
- So, most of the time, the laser is off, only 0.00112 % on.
- About same relation as a 1 second pulse in one day.

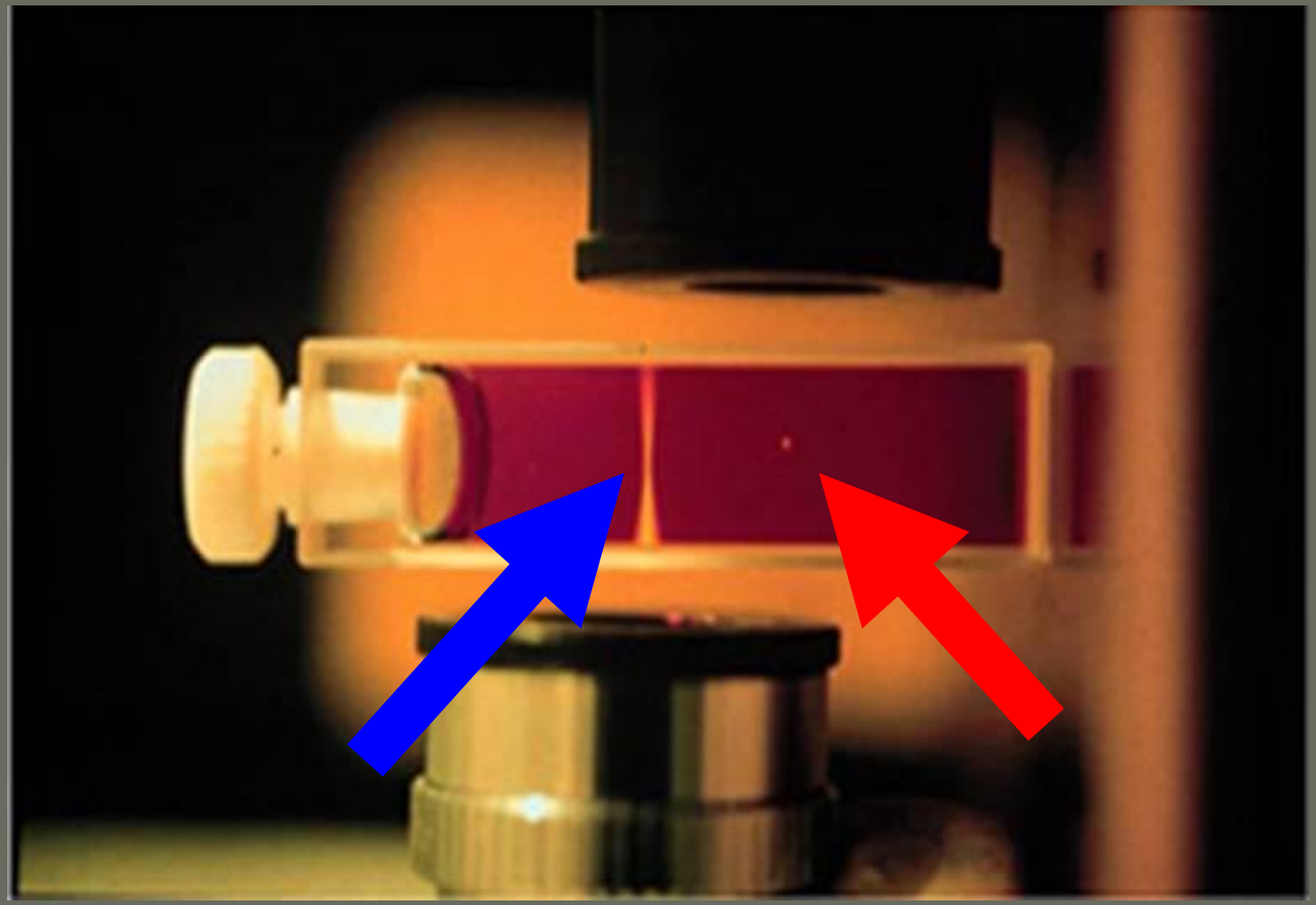
Image formation
(similar to confocal microscope)

- At any given time, fluorescence comes from only one point.
- This point is scanned over the specimen.
- Detectors („PMTs“) record emitted photons from each point, one by one, the computer constructs the image.

It‘s complicated and expensive, so why bother?

- Advantage 1: Excitation and bleaching only at the focal point. Not above and not below.





Advantage 1: No out-of-focus bleaching

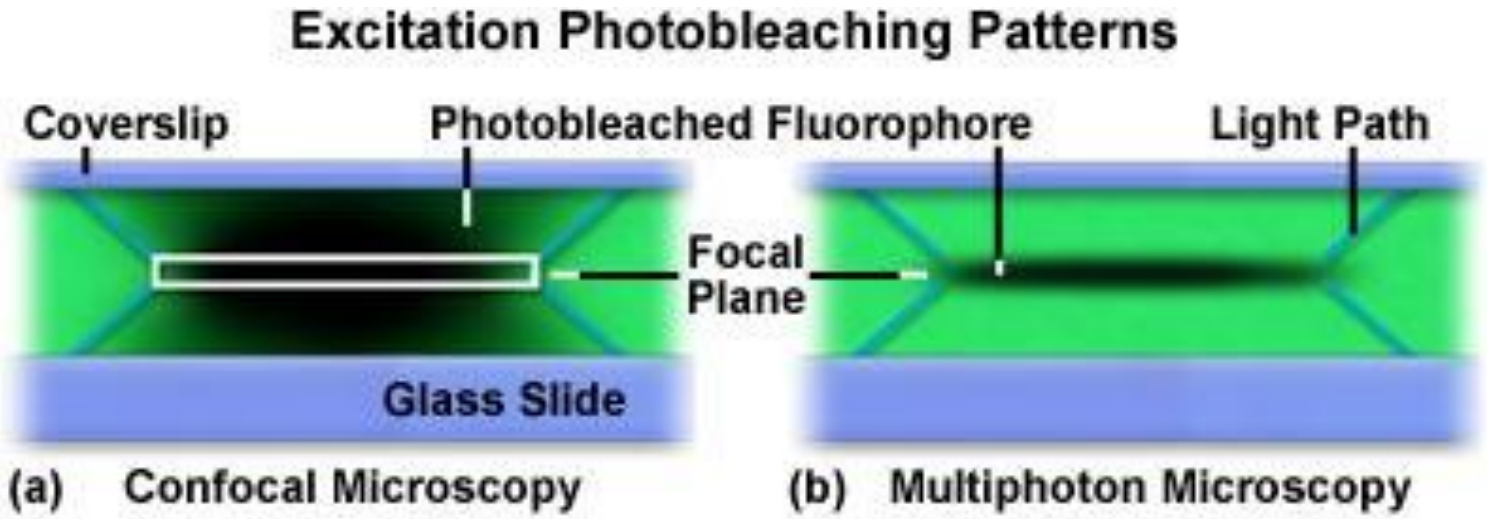


Figure 5

Why bother?

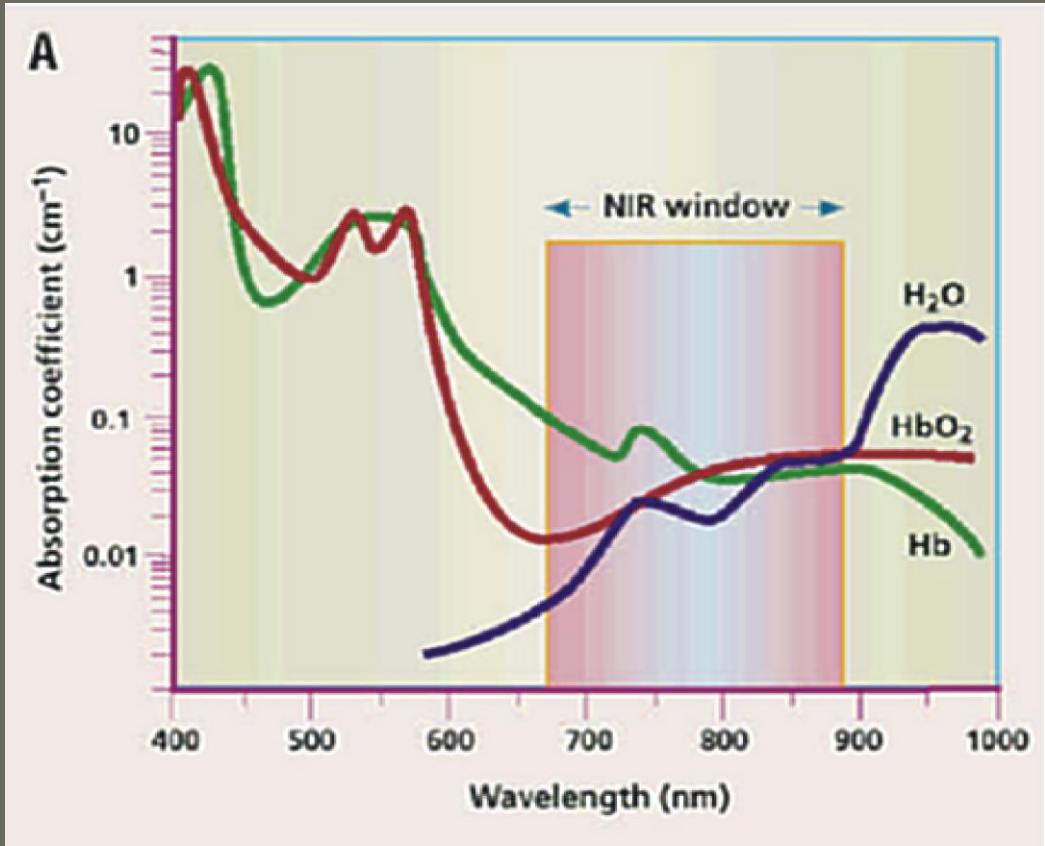
Advantage 2: Deeper penetration, because

- a) scattering decreases with longer wavelengths:
  $1/(nm)^4$ => factor 16 with doubling of λ

Why bother?

Advantage 2: Deeper penetration, because

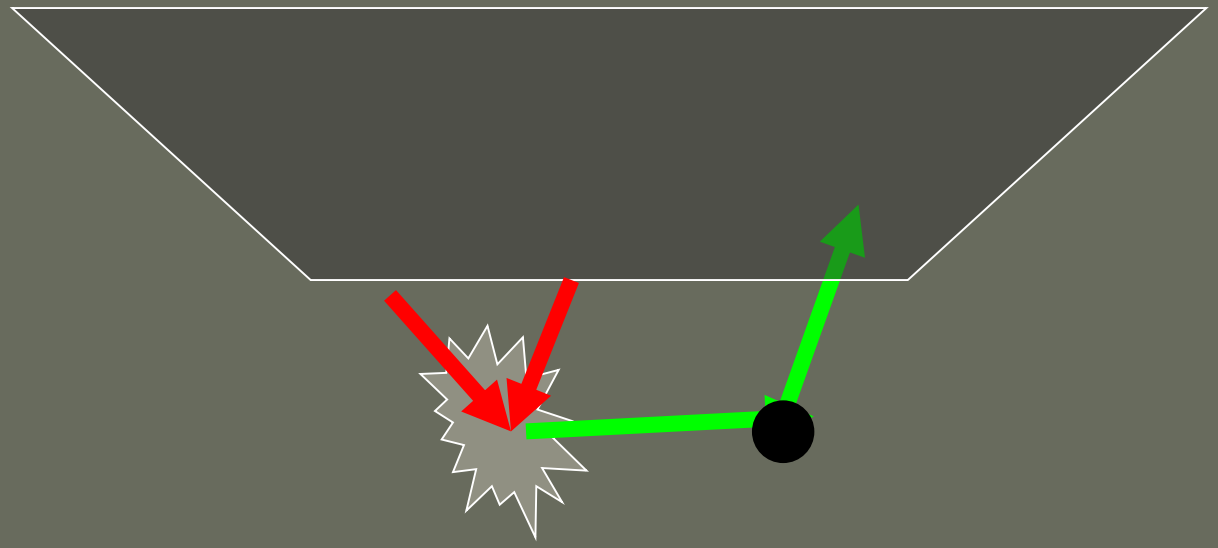
- b) Less absorbtion of tissue in NIR-range



Why bother?

Advantage 3:

- No loss of emitted photons due to pinhole, non-balistic photons contribute to image

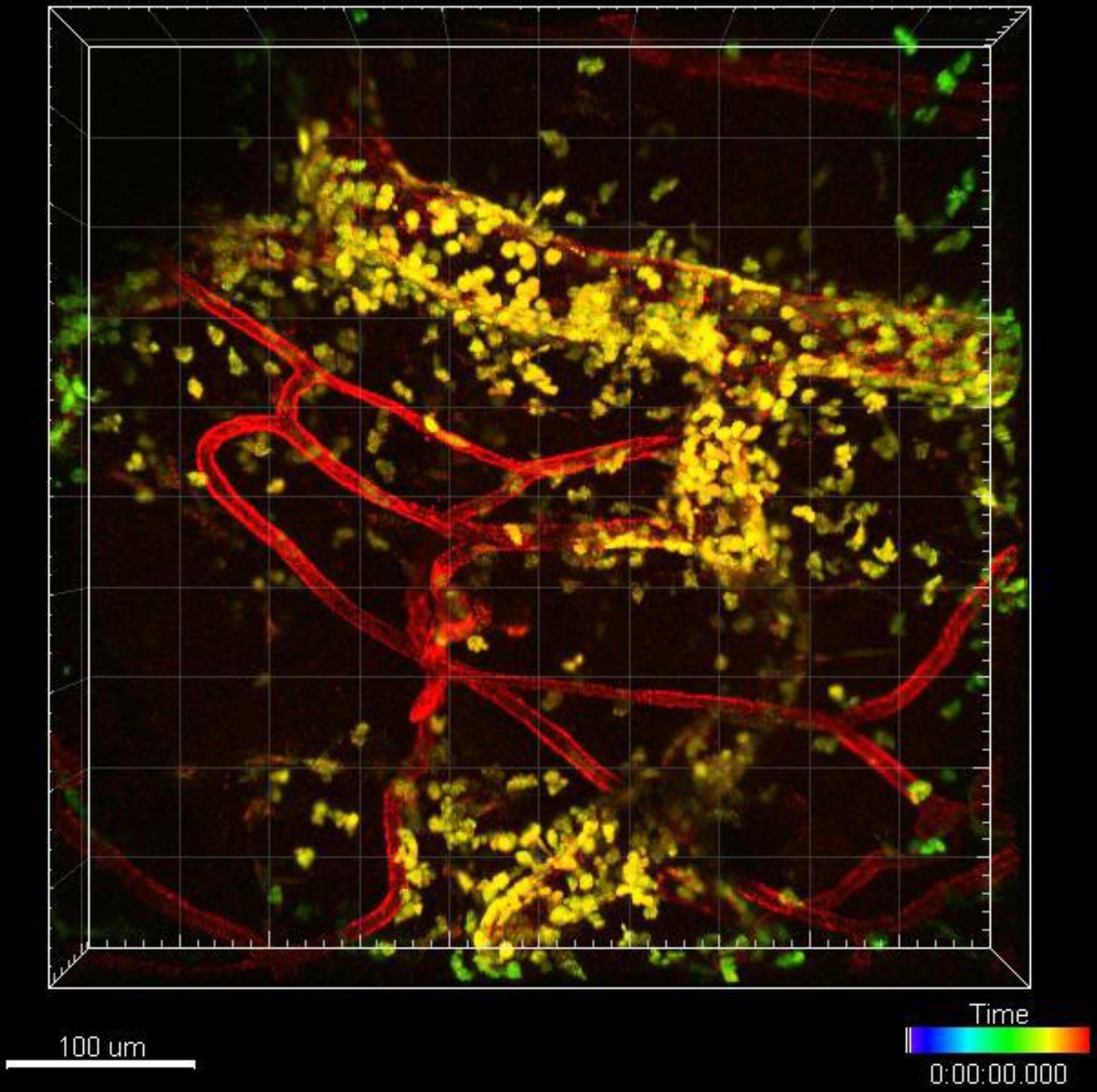


Disadvantages

- Pulsed laser needed, thus
- Expensive
- Light path needs constant maintenance (no glass fiber possible)

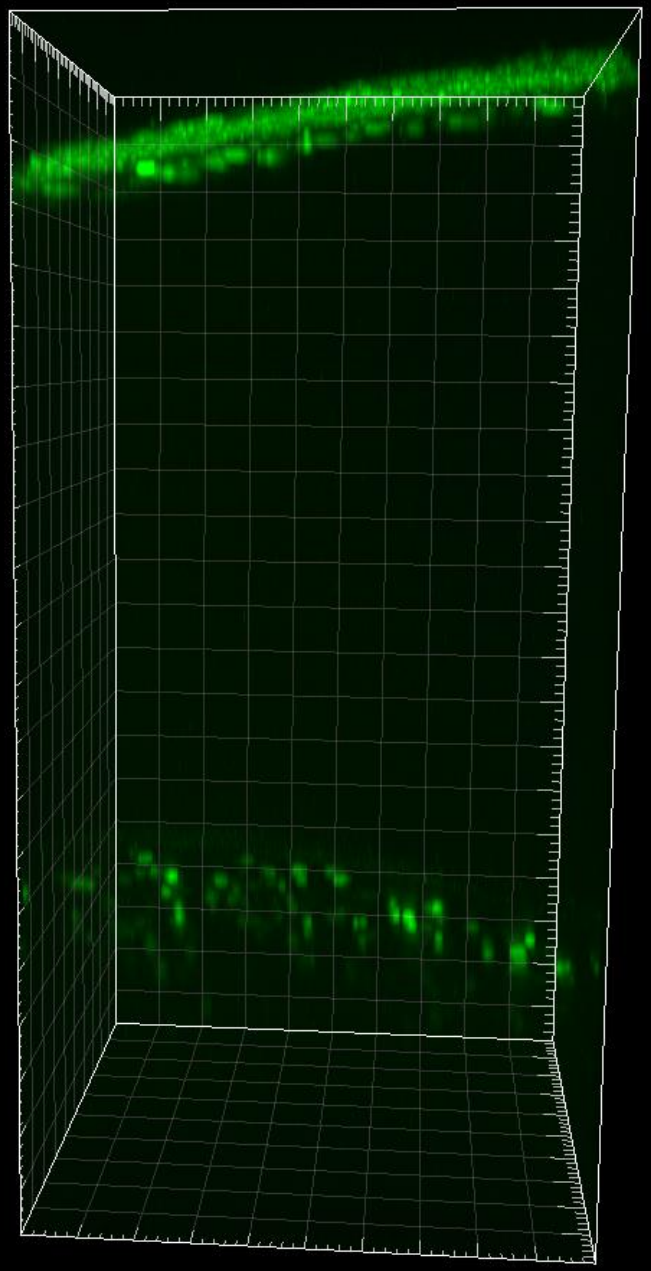
- Separation of neighboring fluorochromes more difficult

Application examples



With
Angela Kurz,
AG Sperandio

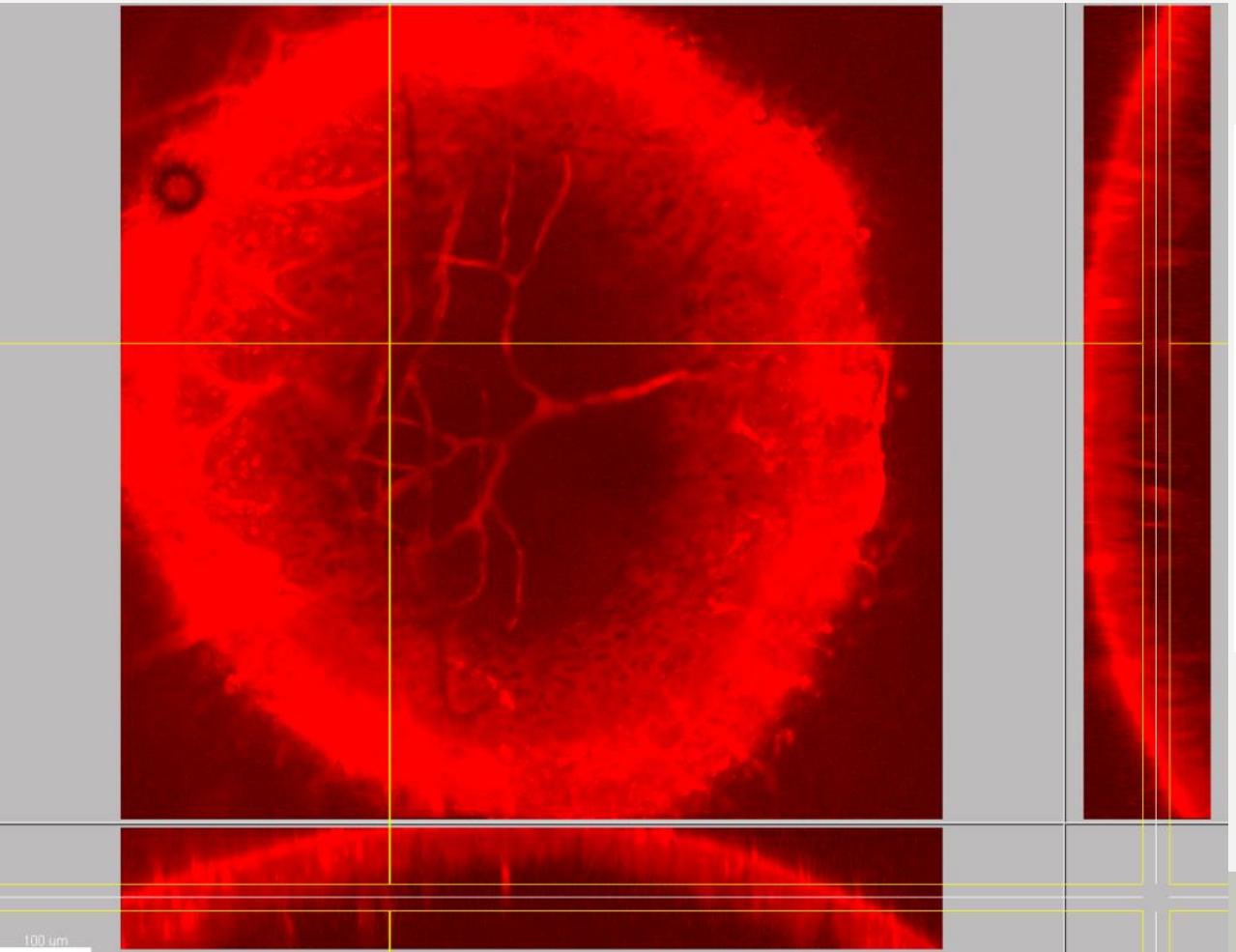
Ly-EGFP mouse embryo, imaged through yolk sac

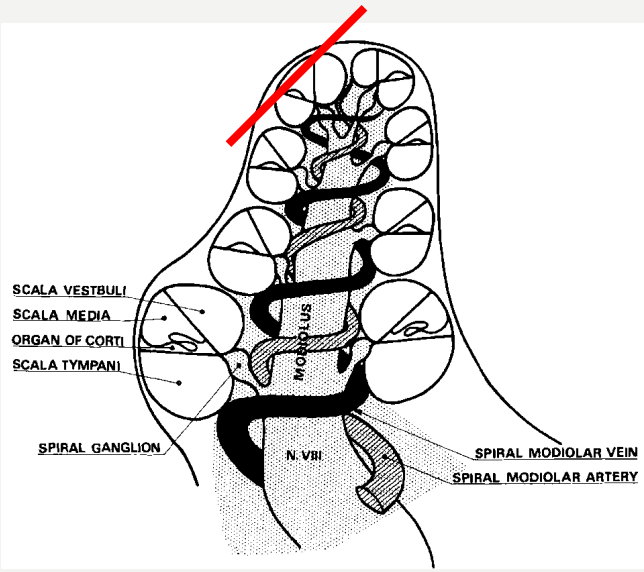


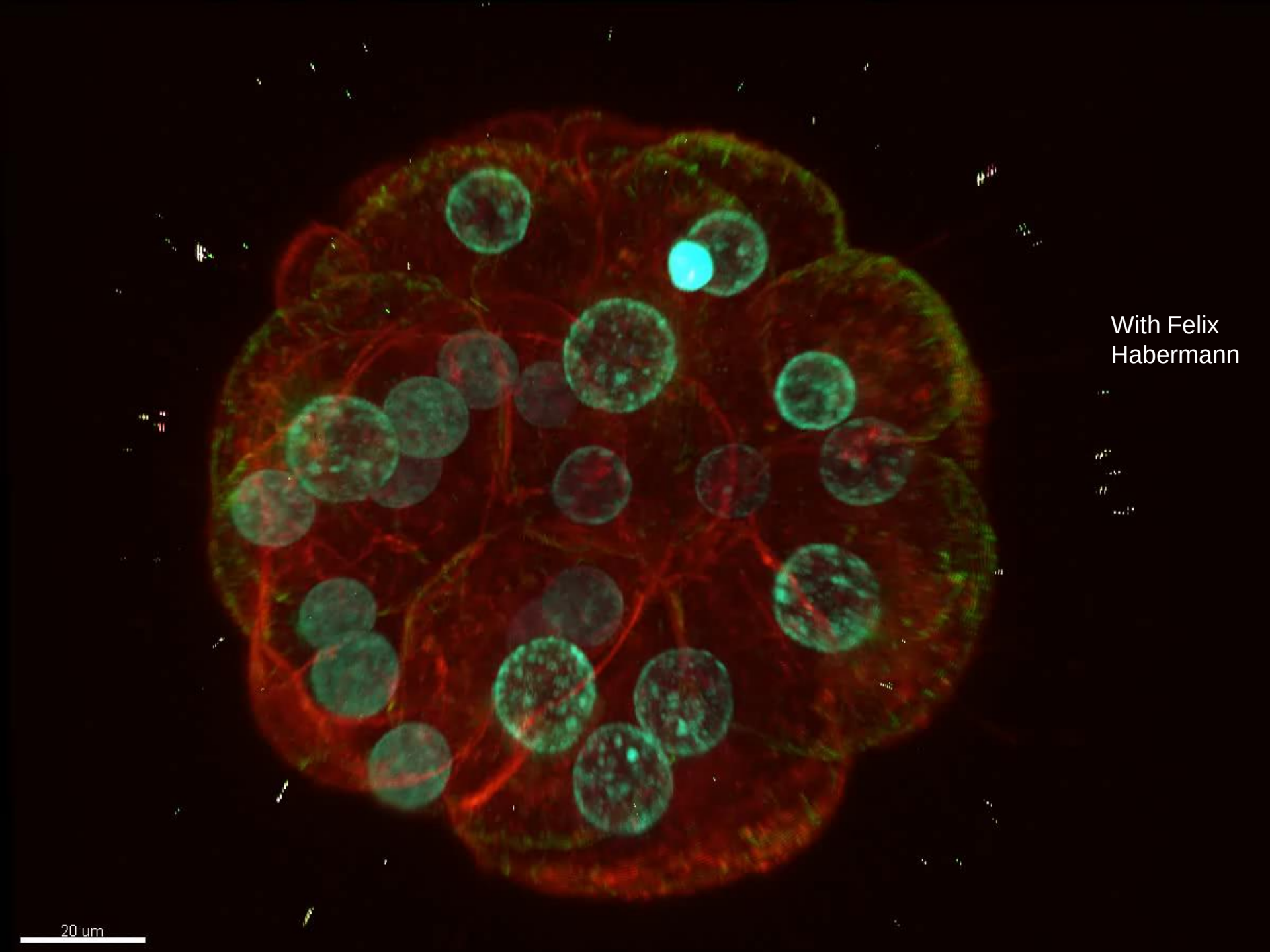
With
Andreas Margraf
AG Sperandio

150 um

Non-invasive visualization of cochlear microcirculation, with Fritz Ihler, Martin Canis





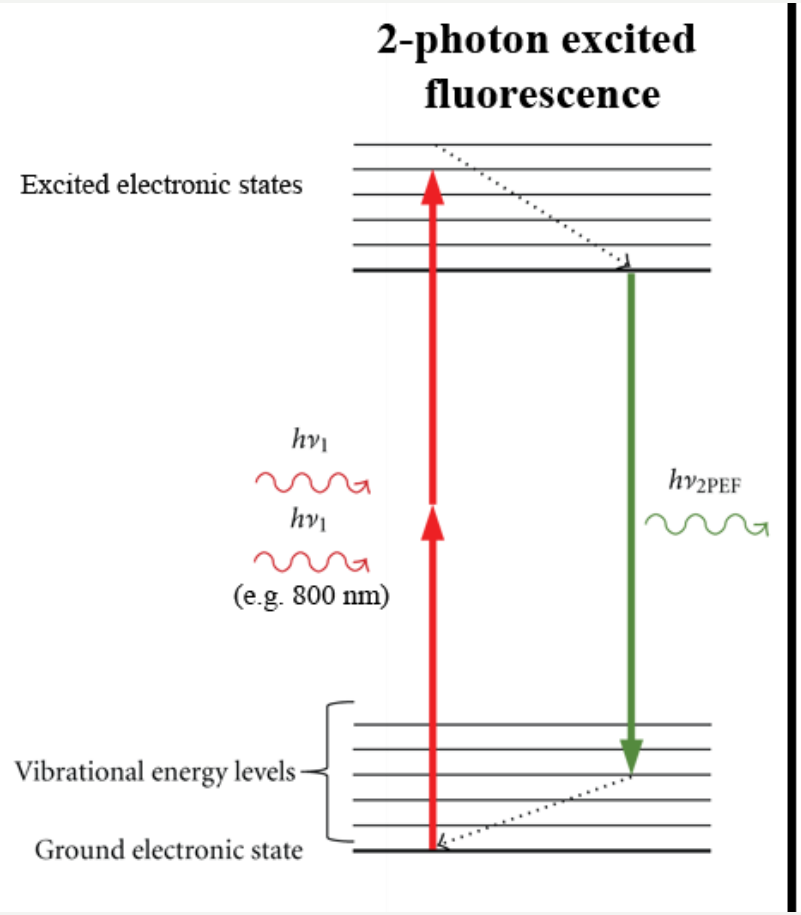

With Felix
Habermann
20 um

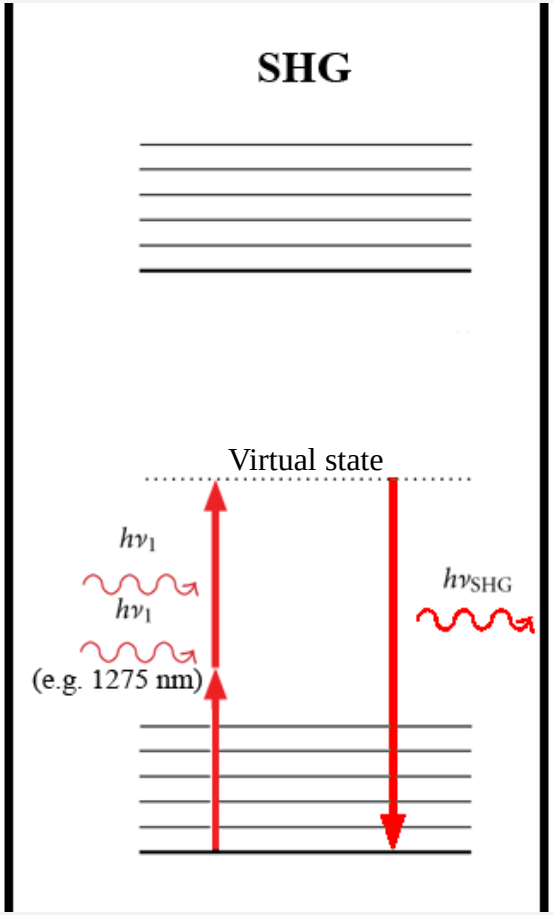
LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

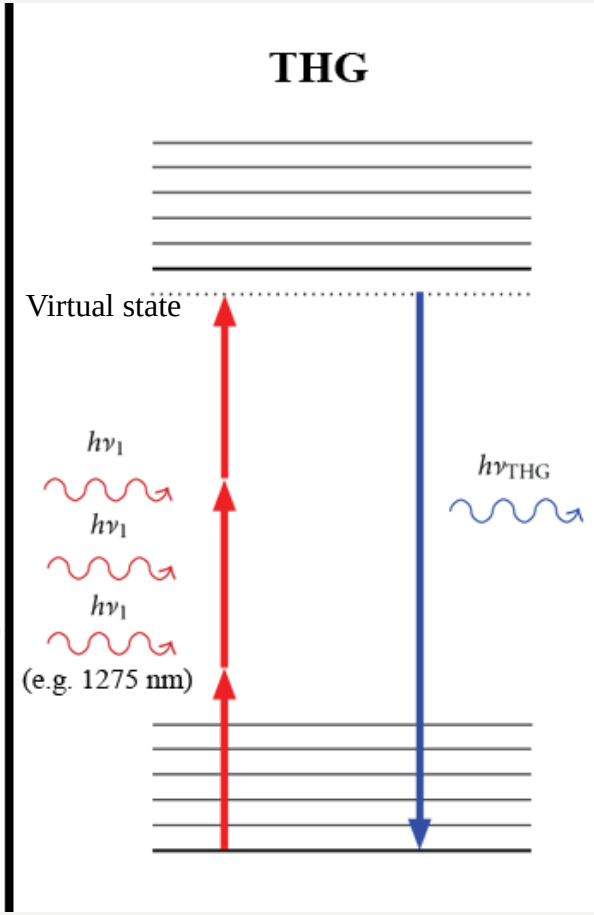
Walter Brendel Zentrum München

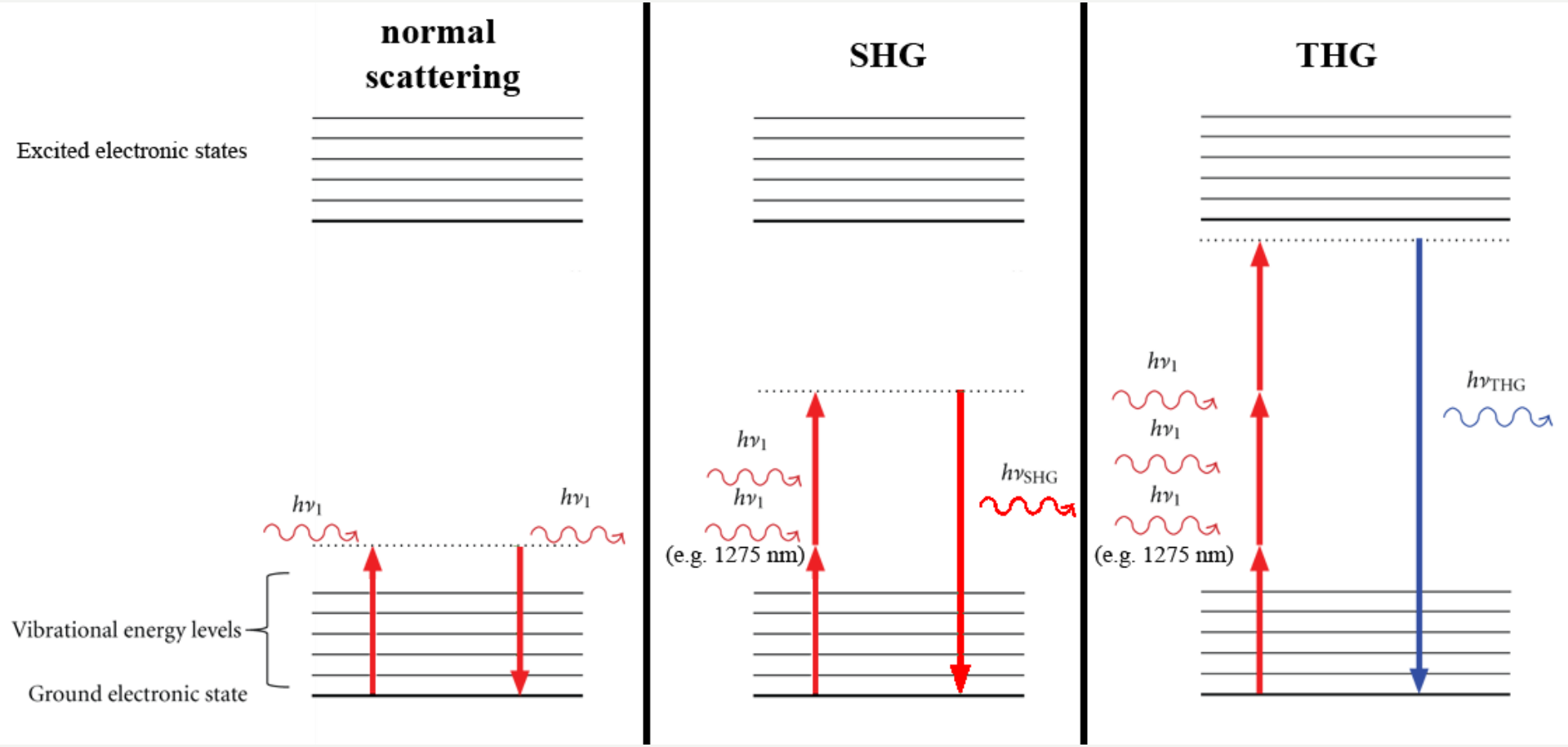
BIN LMU BioImaging Network Munich

Second and Third Harmonic Generation (SHG + THG)


2-photon excited fluorescence
Excited electronic states
$h\nu_1$
$h\nu_1$
(e.g. 800 nm)
$h\nu_{2PEF}$
Vibrational energy levels
Ground electronic state


SHG
Virtual state
$h\nu_1$
$h\nu_1$
(e.g. 1275 nm)
$h\nu_{SHG}$


THG
Virtual state
$h\nu_1$
$h\nu_1$
$h\nu_1$
(e.g. 1275 nm)
$h\nu_{THG}$


normal scattering
SHG
THG
Excited electronic states
Vibrational energy levels
Ground electronic state
hν1
hν1
hν1
hν1
(e.g. 1275 nm)
hνSHG
hν1
hν1
hν1
(e.g. 1275 nm)
hνTHG

SHG

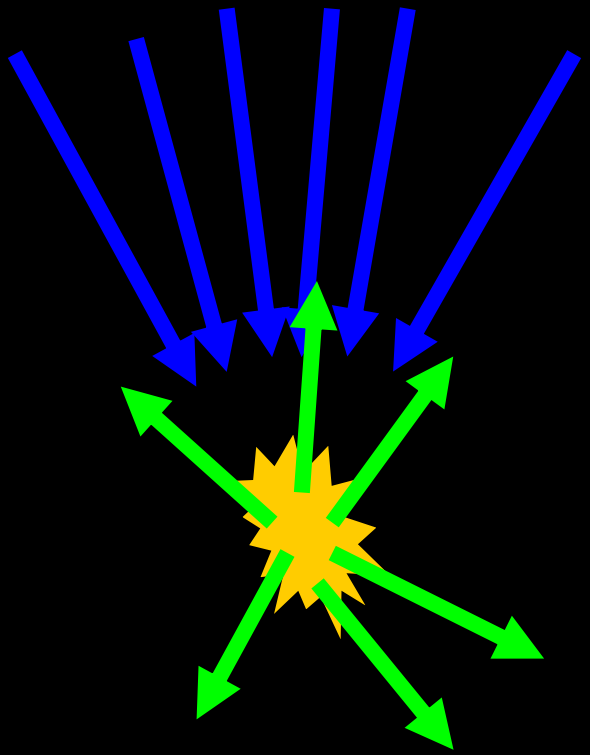
.

Two photons in, one out: energy is constant, thus wavelength is exactly halved: 860 nm→ 430 nm or 1275 nm→ 638 nm

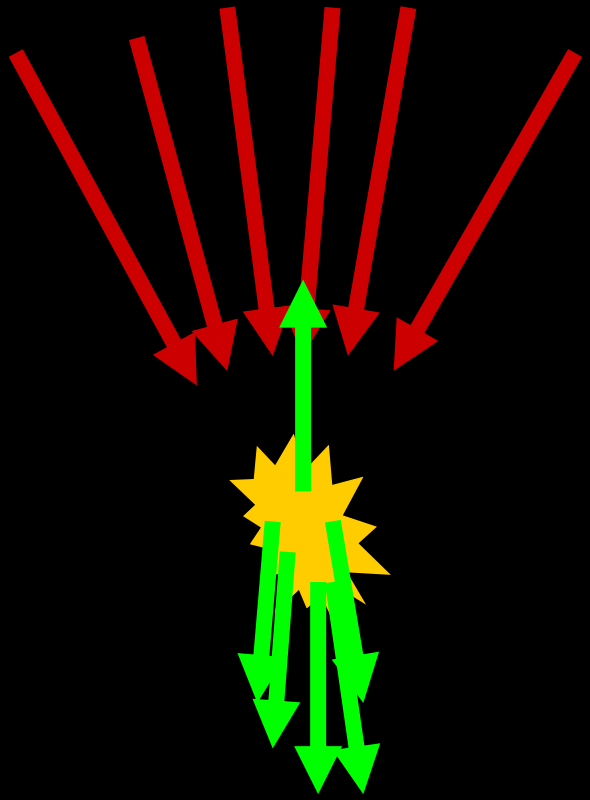
In mammalian soft tissues, generated in collagen fibers and striated muscle myosin (non-centrosymetric, dense substances)

Label-free, 3D

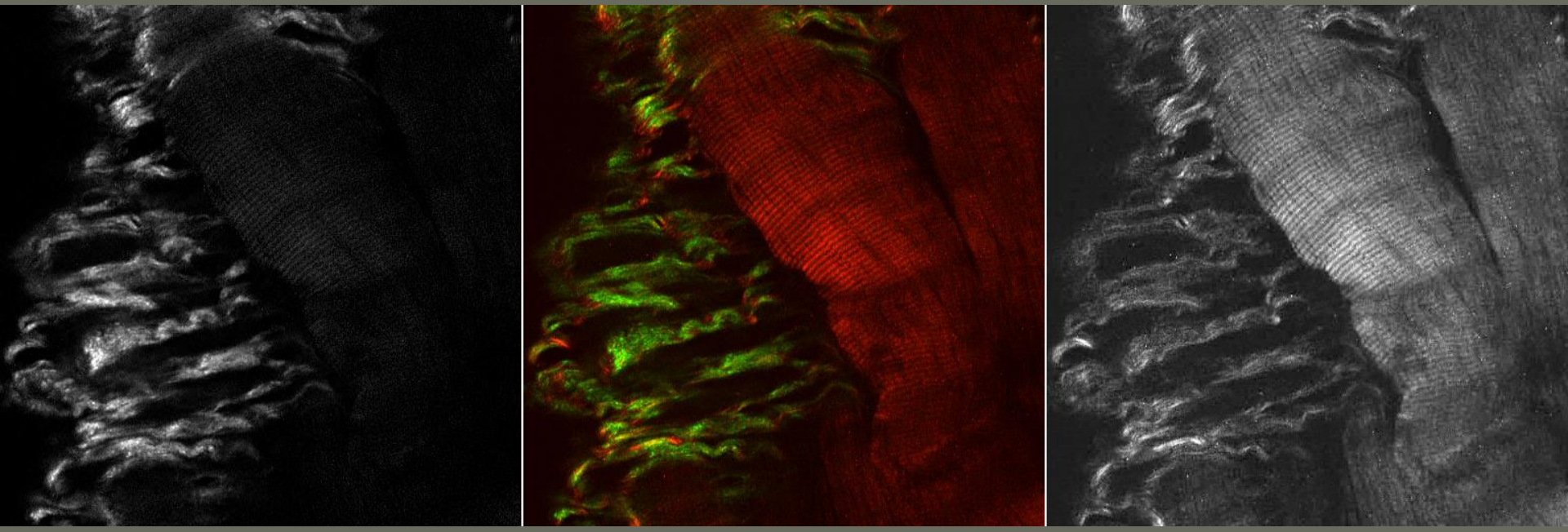
Fluorescence



Higher harmonic generation



Forward and backward SHG are not the same



Backward

Forward
(Detected behind the condenser)

- Collagen and myosin both generate stronger SHG-Signals in forward direction than in backward direction. However, the relative backward component is stronger for collagen than for myosin
- Signals are also polarization dependent.

SHG

Two photons in, one out: energy is constant, thus wavelength is exactly halved: 860 nm→ 430 nm or 1275 nm→ 638 nm

In mammalian soft tissues, generated in collagen fibers and striated muscle myosin (non-centrosymetric, dense substances)

Label-free, 3D

THG

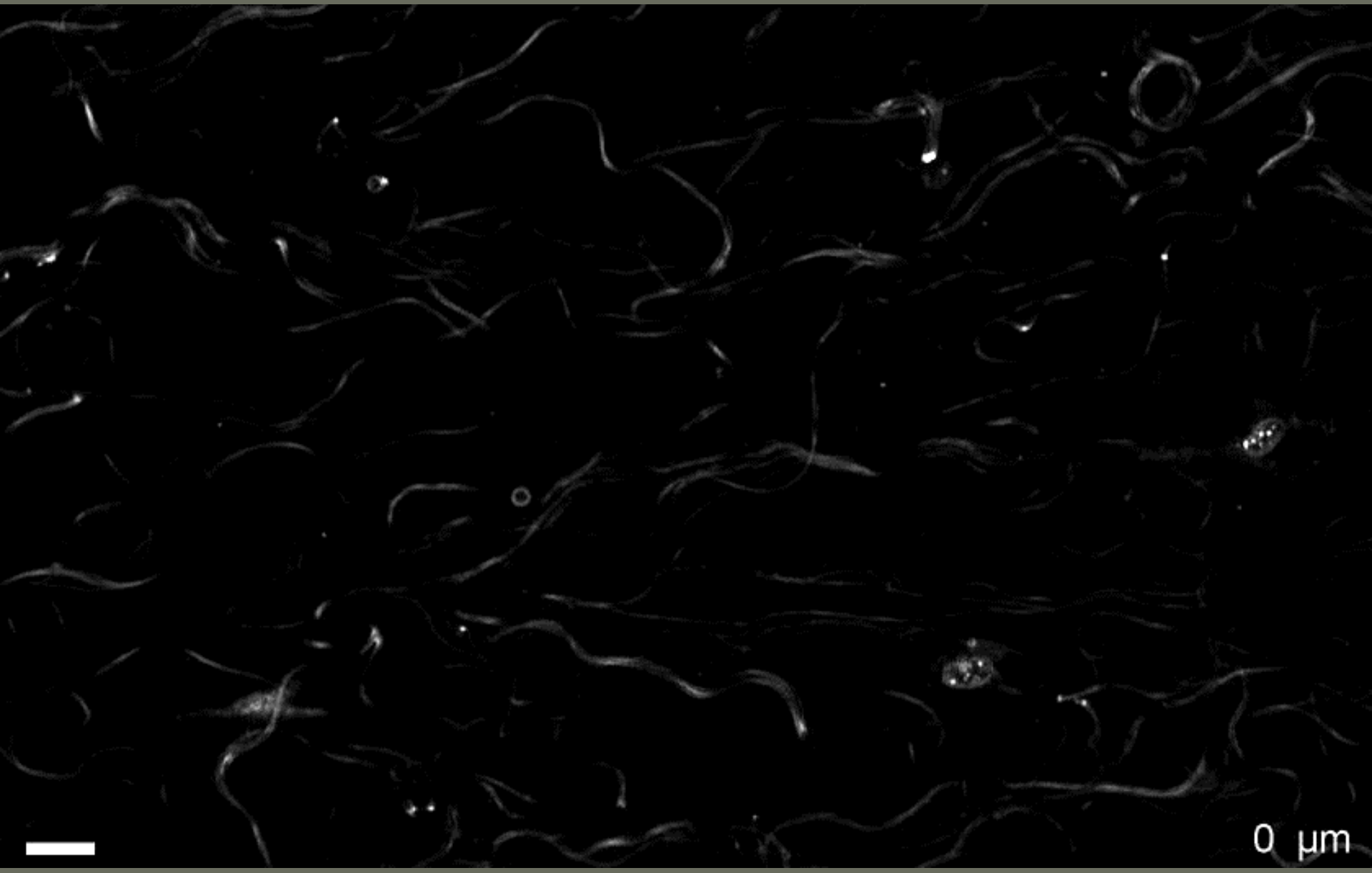
Three photons in, one out: energy is constant, thus wavelength is exactly 1/3: 1275 nm→ 425 nm.

Generated at interfaces, e.g. at membranes or refraction index mismatches

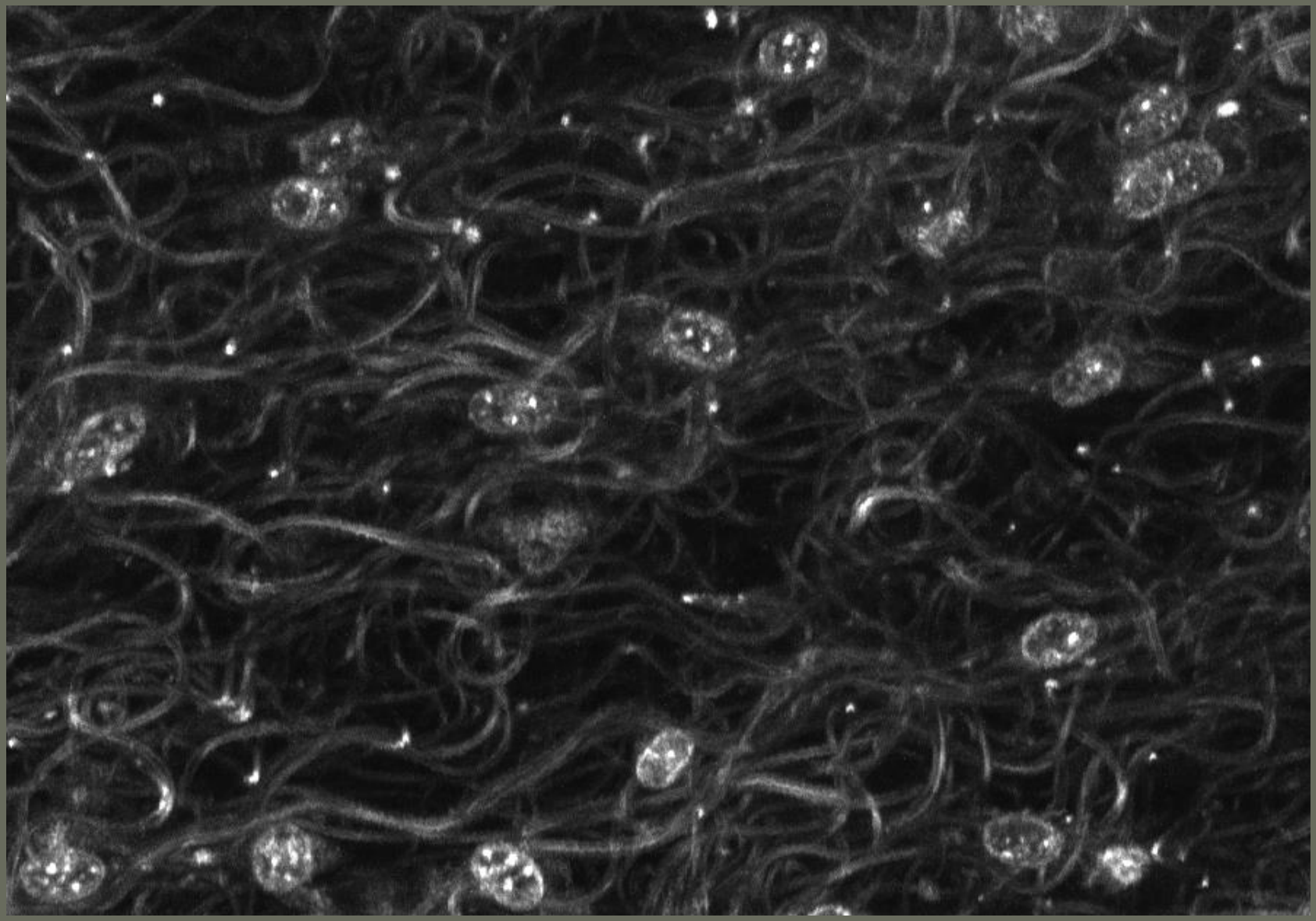
Label-free, 3D

Z-sections

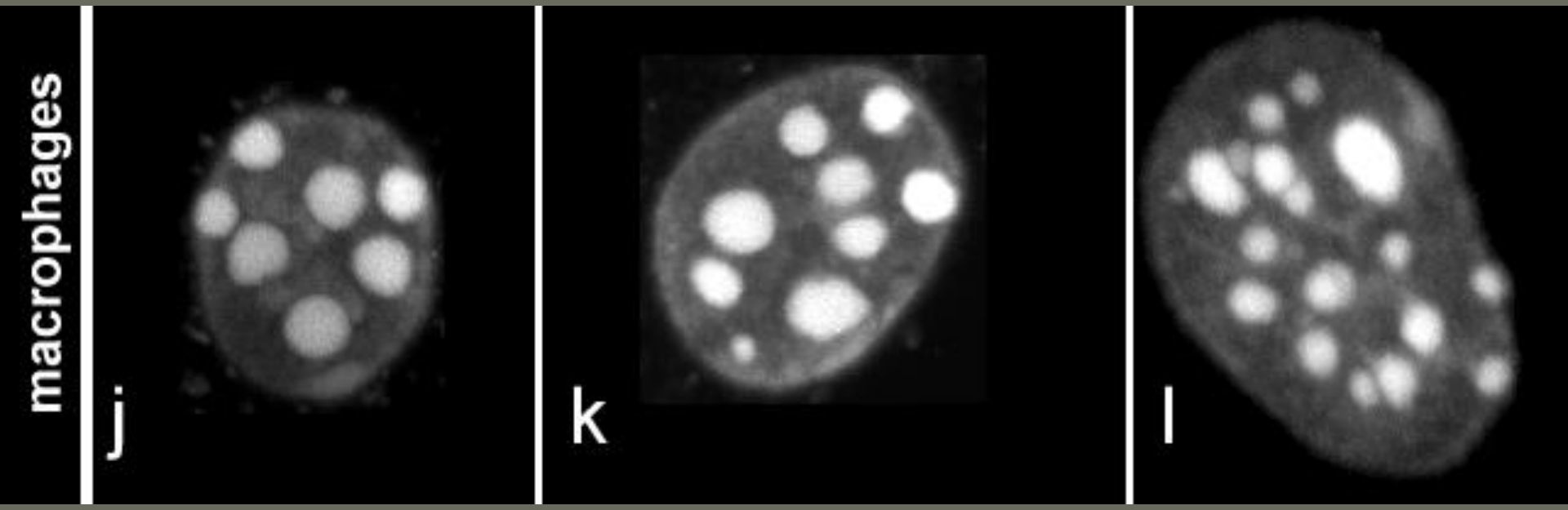
With
Markus Rehberg



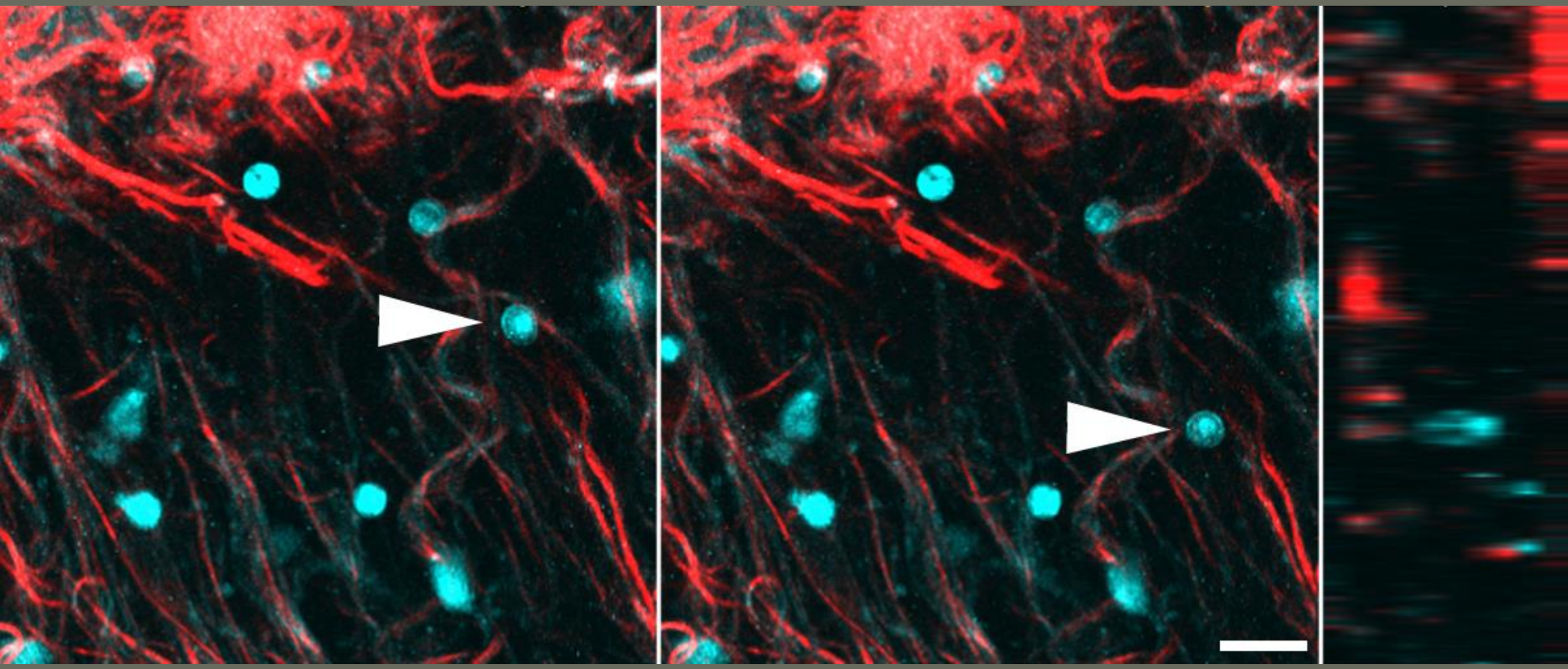
Chromatin structure



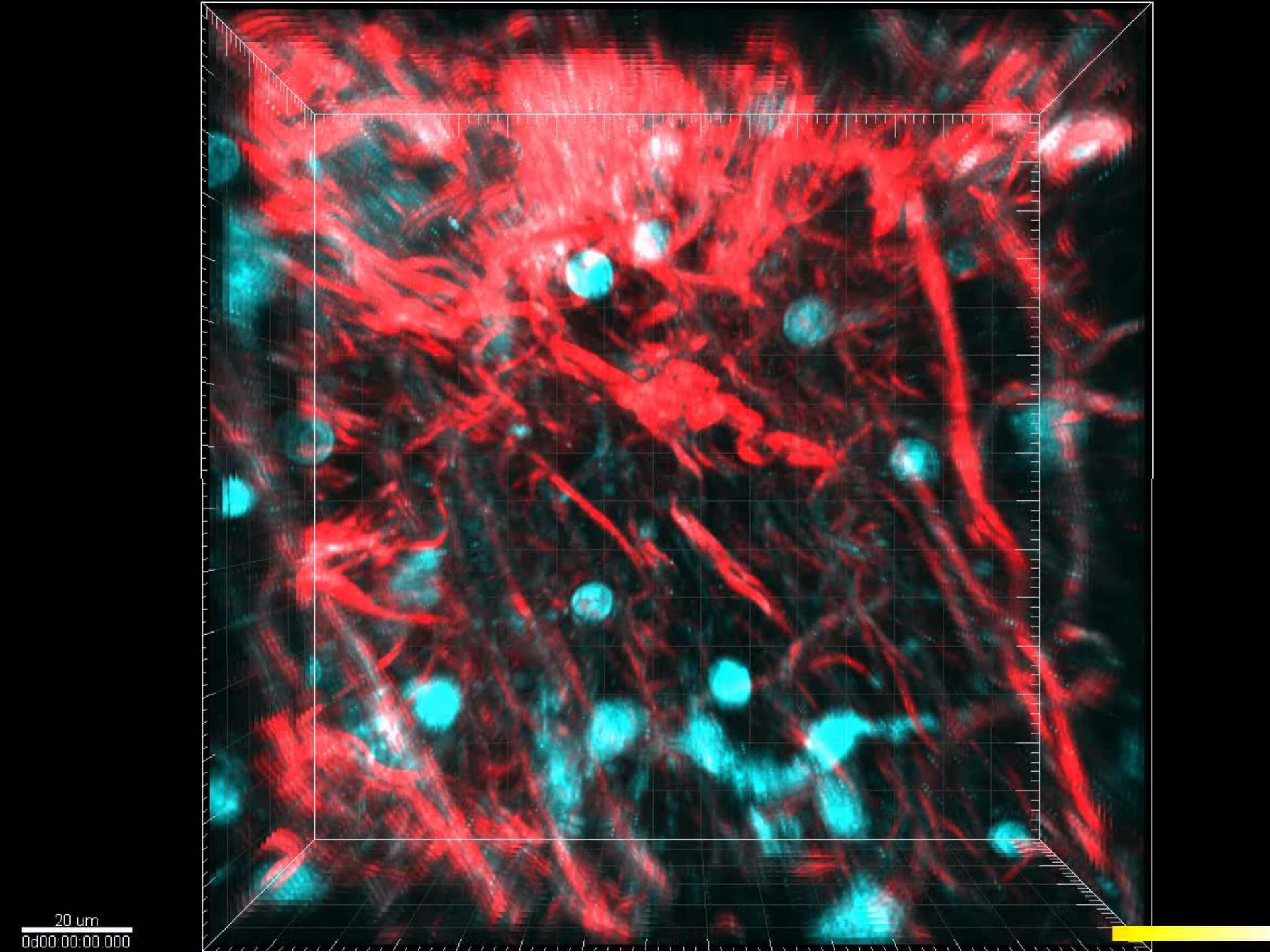
Chromocenters in mouse cell nuclei

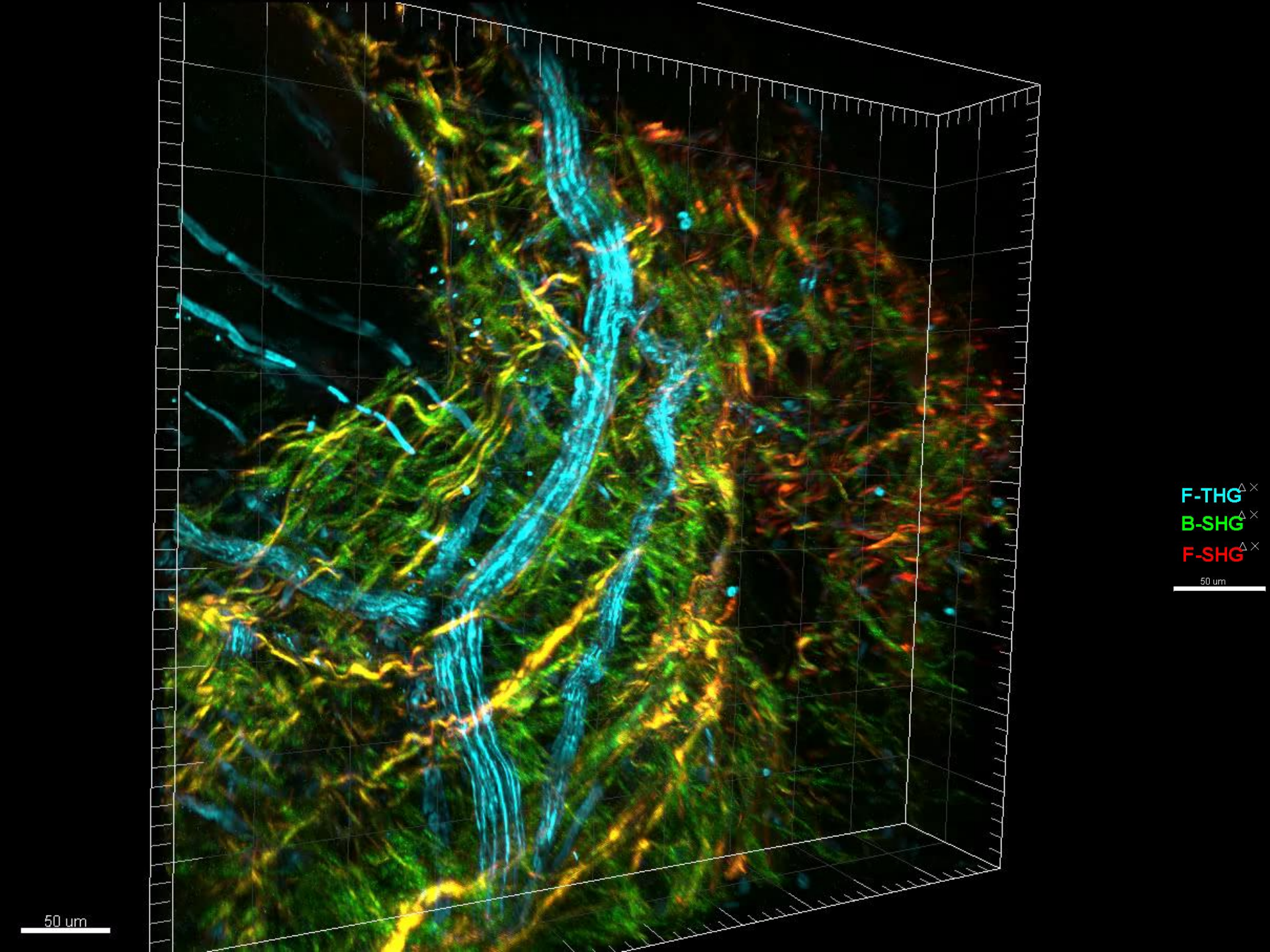


Mayer, R., A. Brero, J. von Hase, T. Schroeder, T. Cremer, and S. Dietzel. 2005. Common themes and cell type specific variations of higher order chromatin arrangements in the mouse. *BMC Cell Biol*. 6:44.

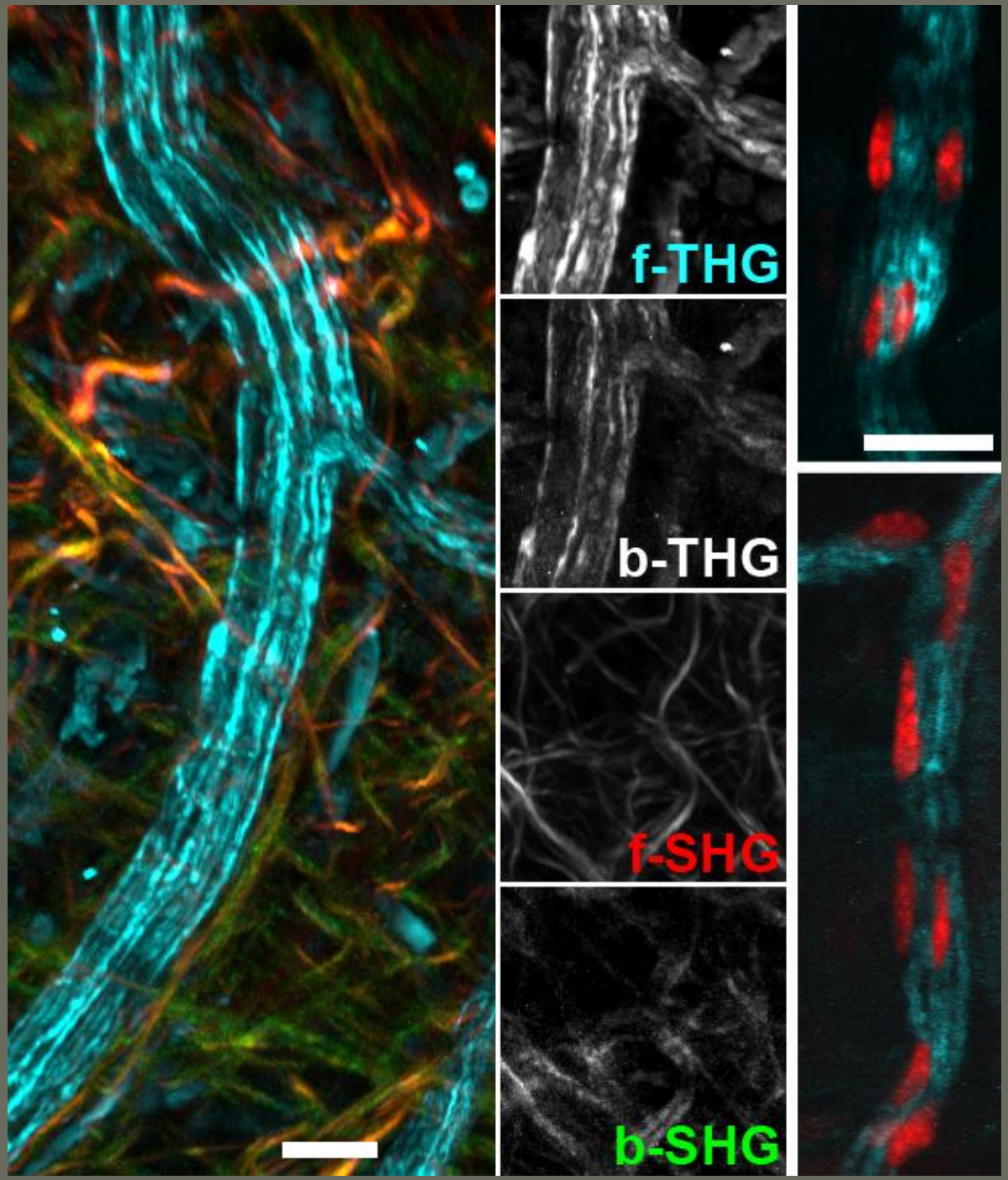


With
Markus Rehberg


20 um
0d00:00:00.000

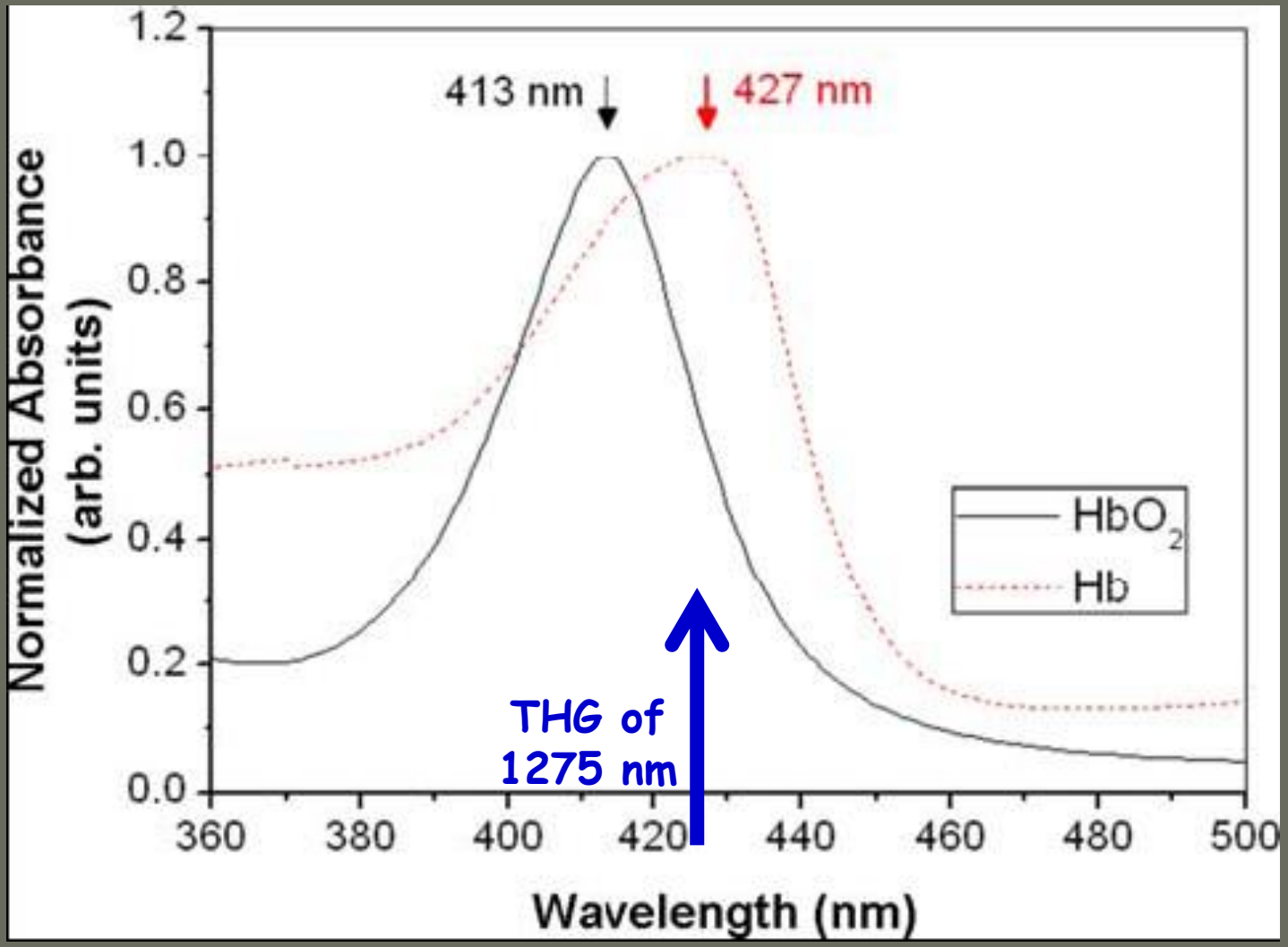

F-THG
B-SHG
F-SHG
50 um
50 um

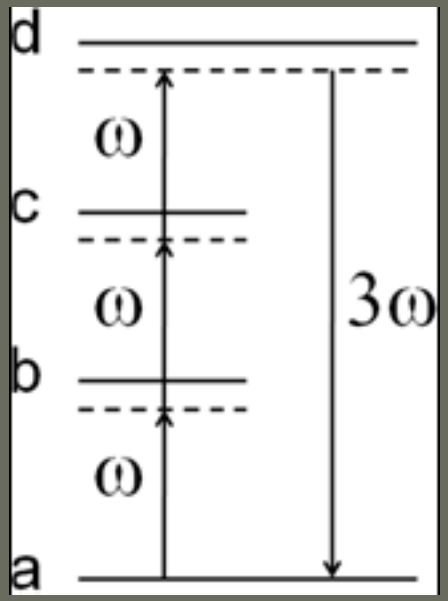
f-THG = b-THG
but
f-SHG ≠ b-SHG



Erythrocytes

Resonance enhancement of THG by hemoglobin

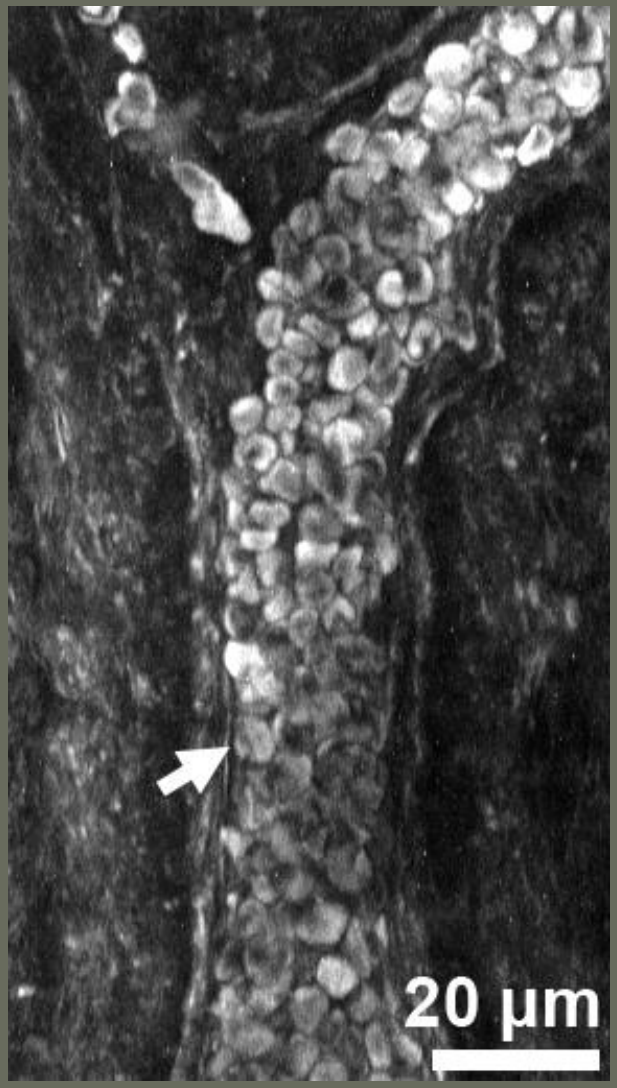


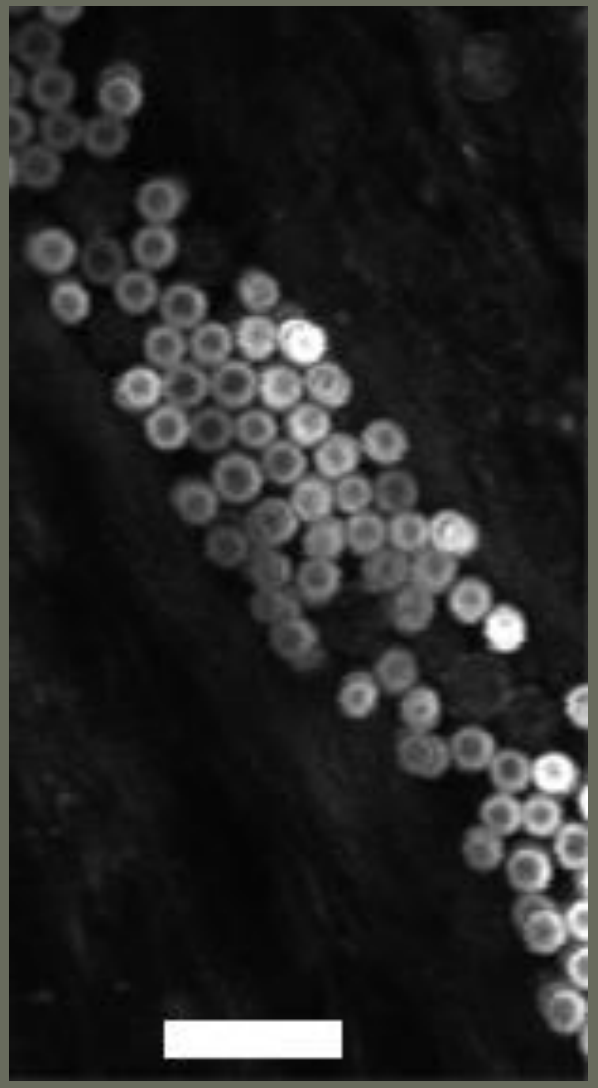


Images from C.-F. Chang, C.-H. Yu, and C.-K. Sun: Multi-photon resonance enhancement of THG. J. Biophotonics 3, No. 10–11, 678–685 (2010) / DOI 10.1002/jbio.201000045

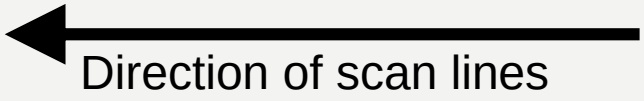
Erythrocytes

Hemoglobin causes resonance enhancement THG



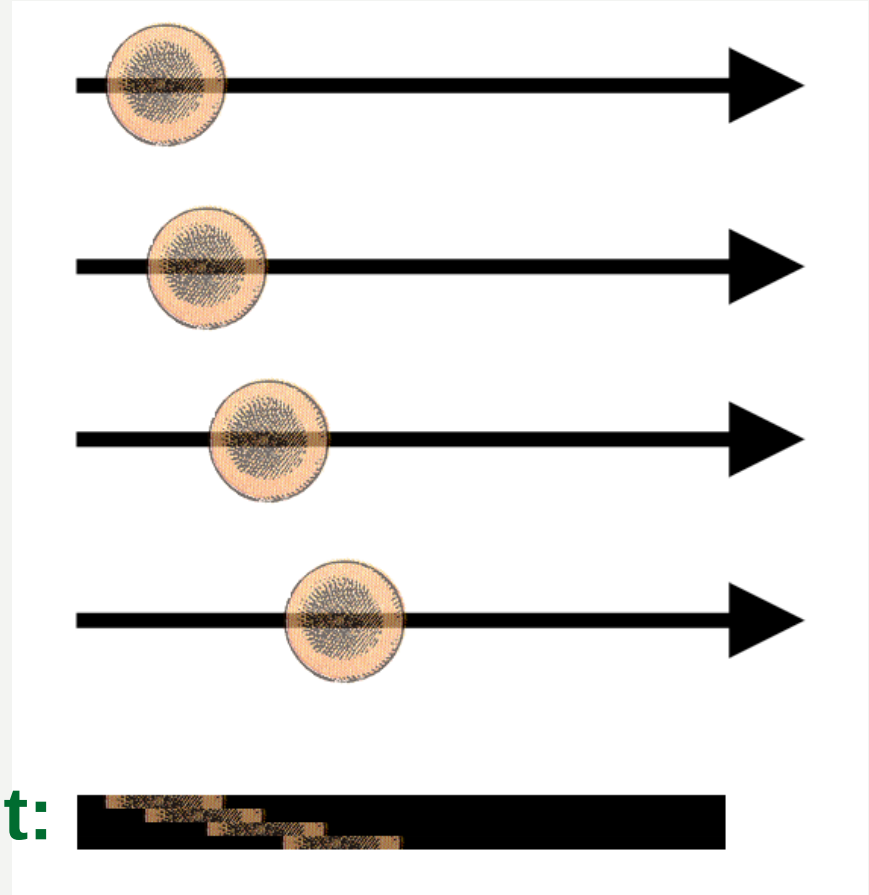


Fast imaging in capillaries (10 fps)



With
Markus Rehberg

Line scan principle

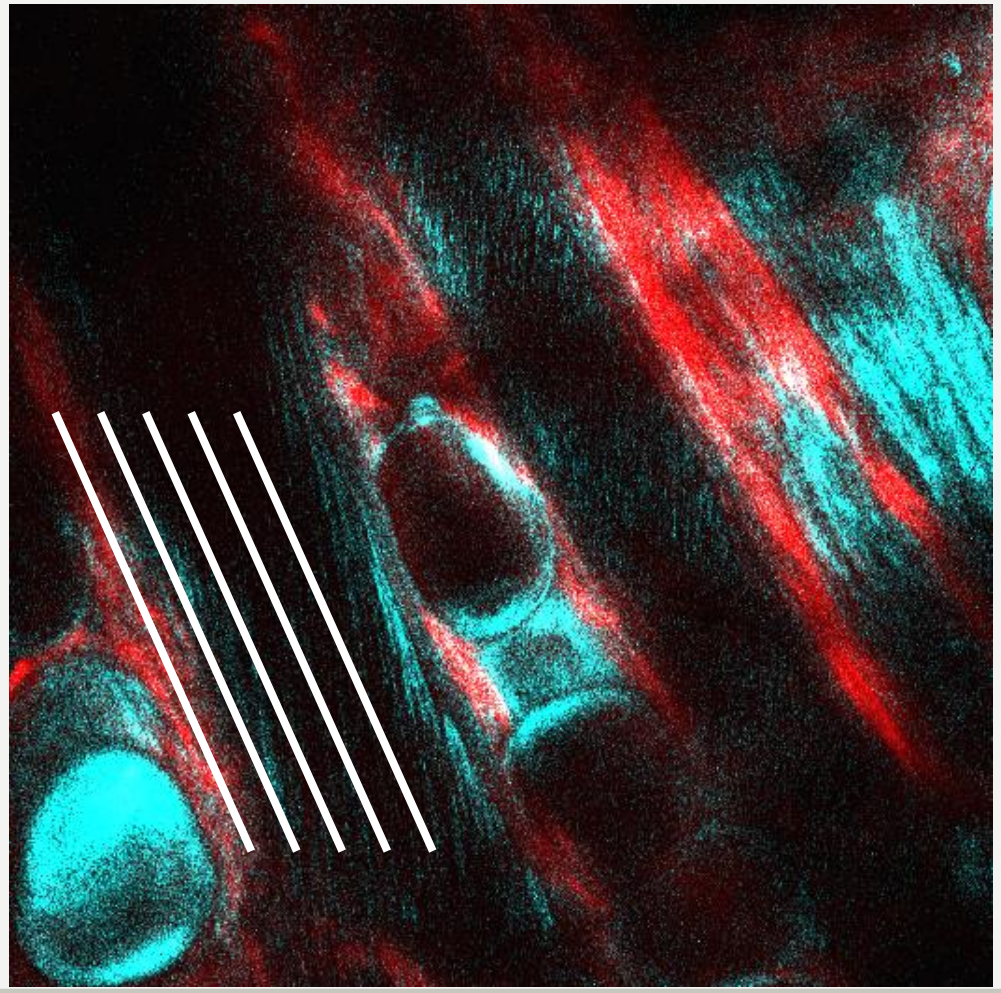


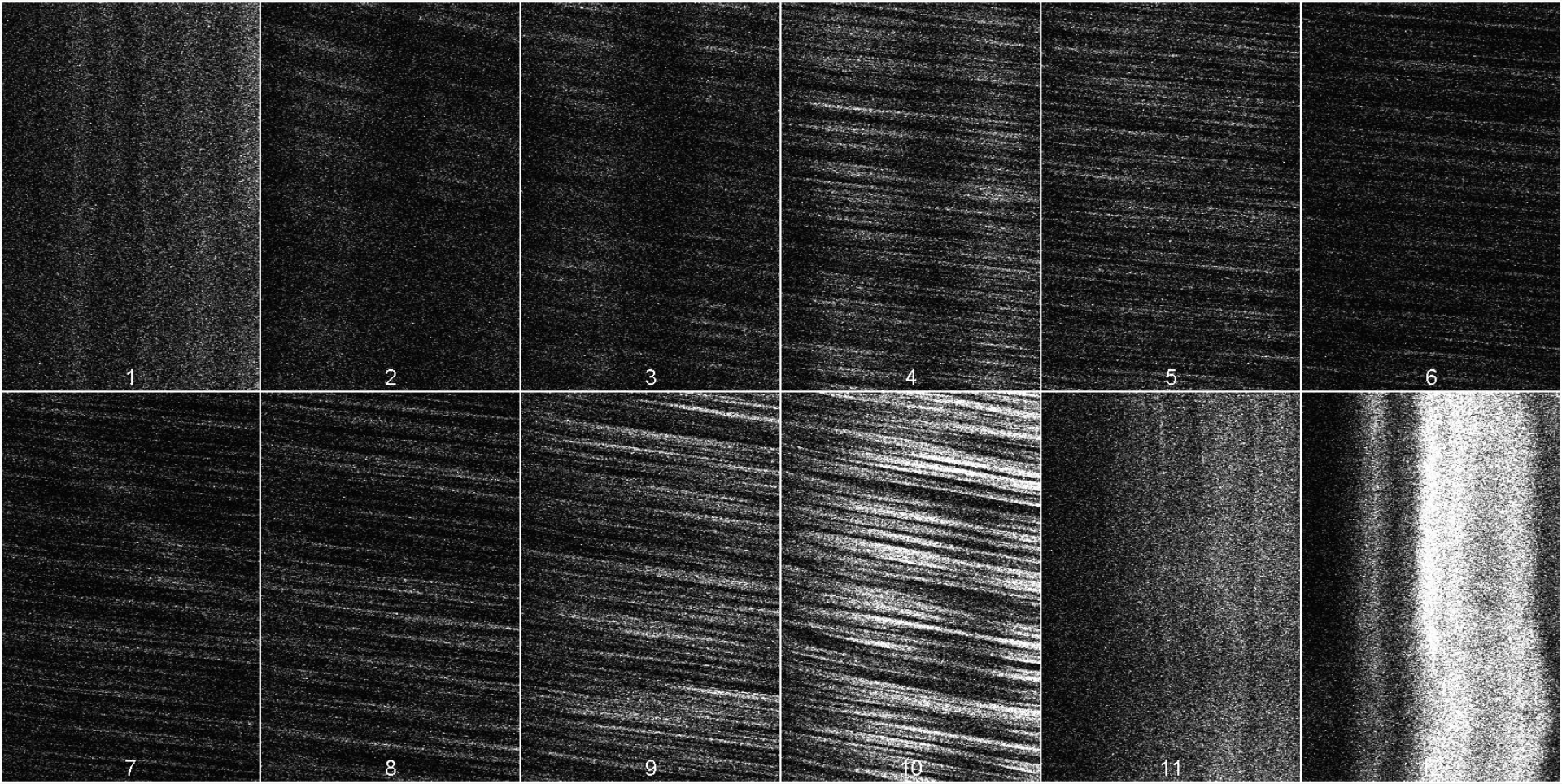
Result:

Line scan in the mouse ear

THG blood flow measurements in the ear allows to measure label-free *and* non-invasively.

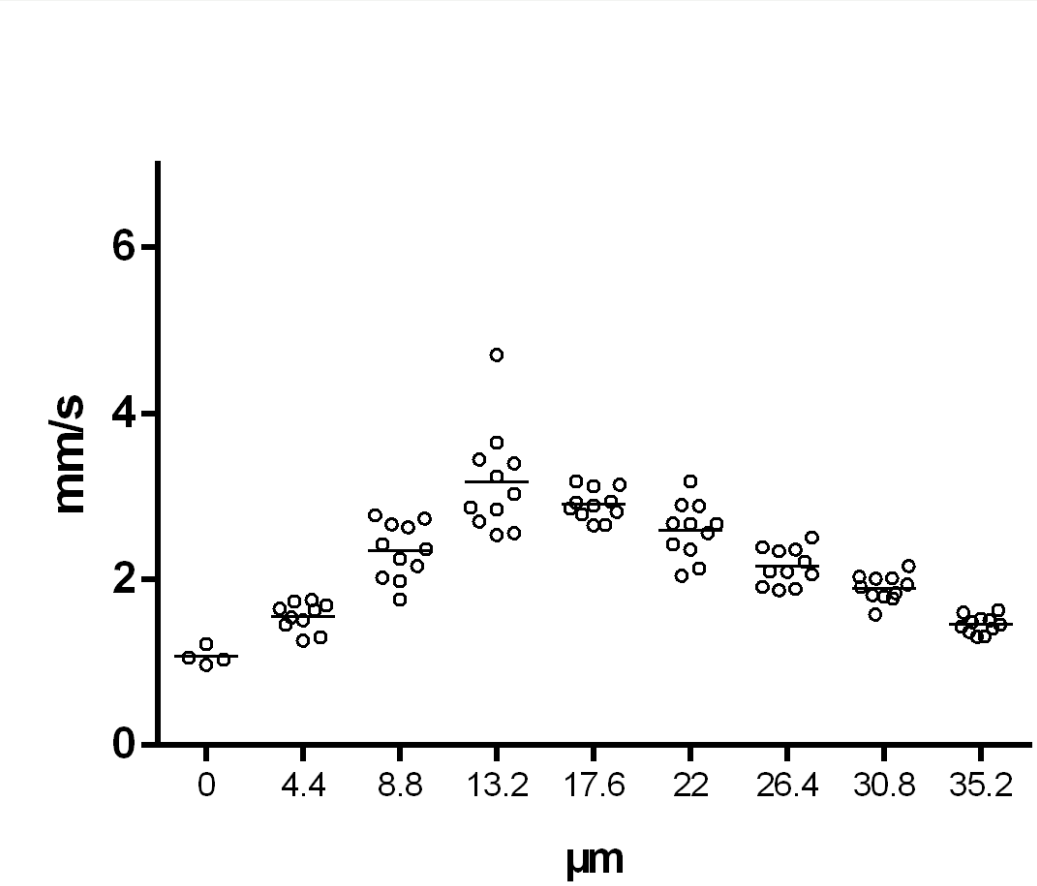
Scans with shifted line



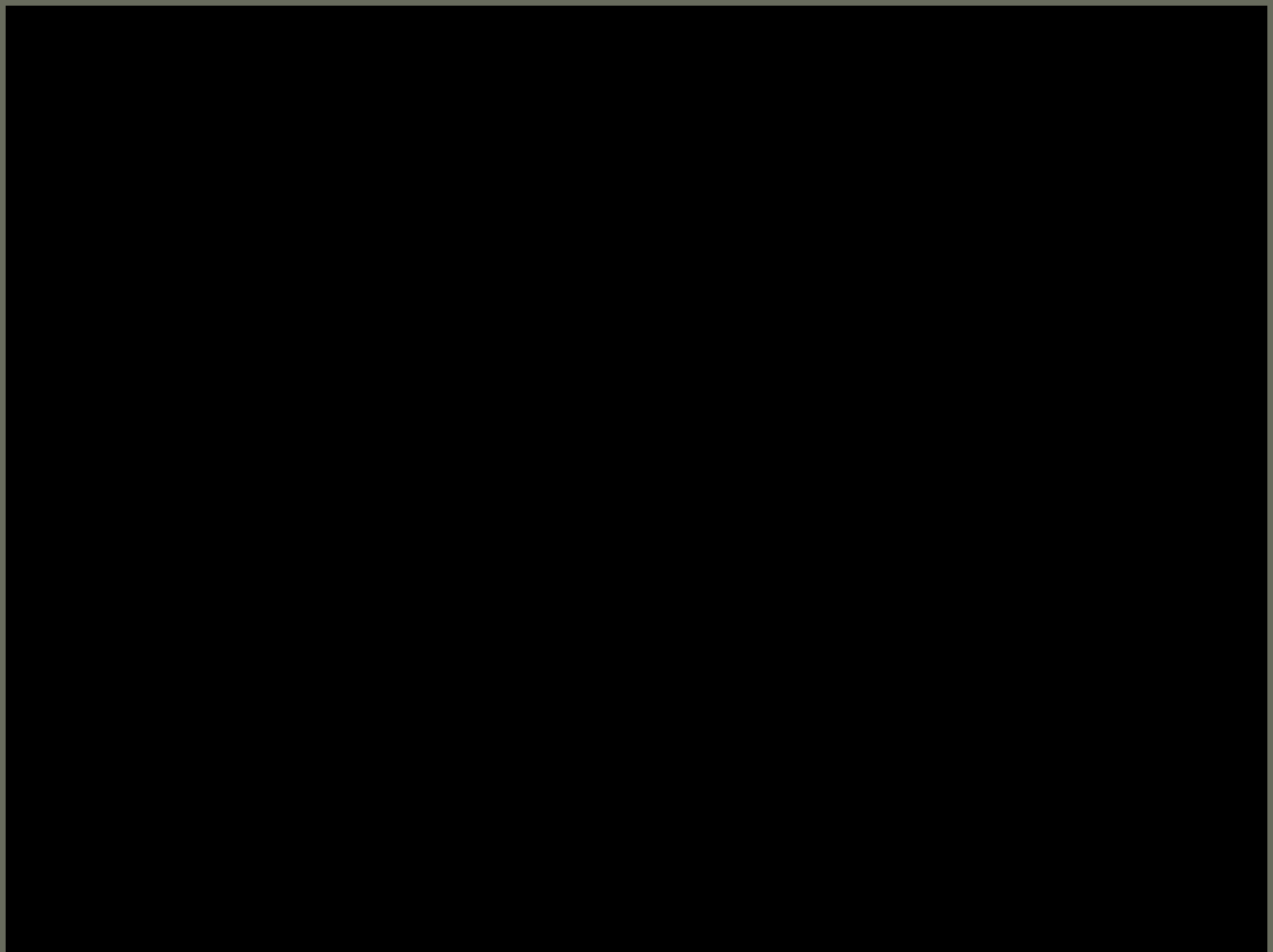


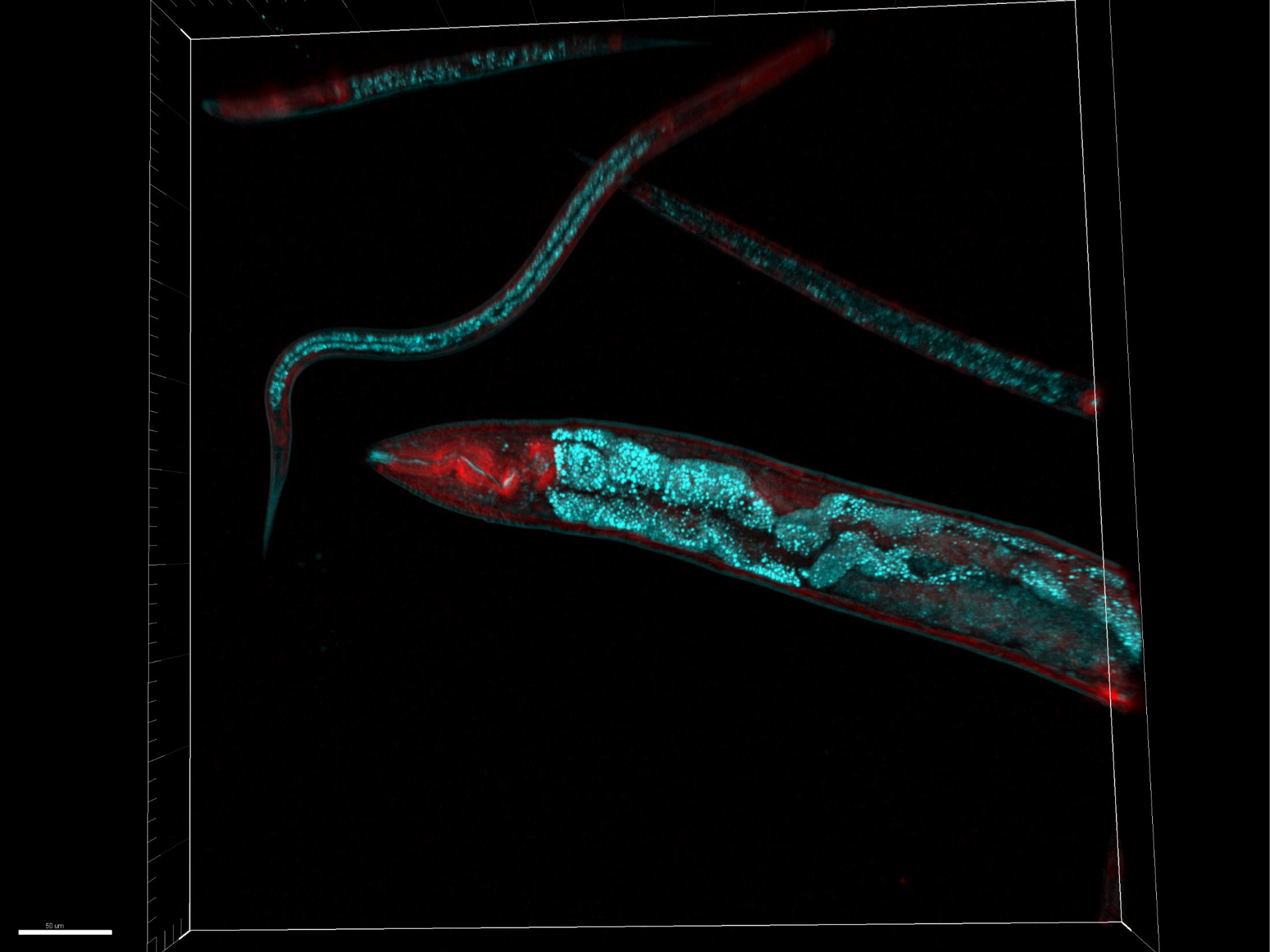
With
Joachim Pircher, AG Pohl

Blood flow in a mouse ear venule



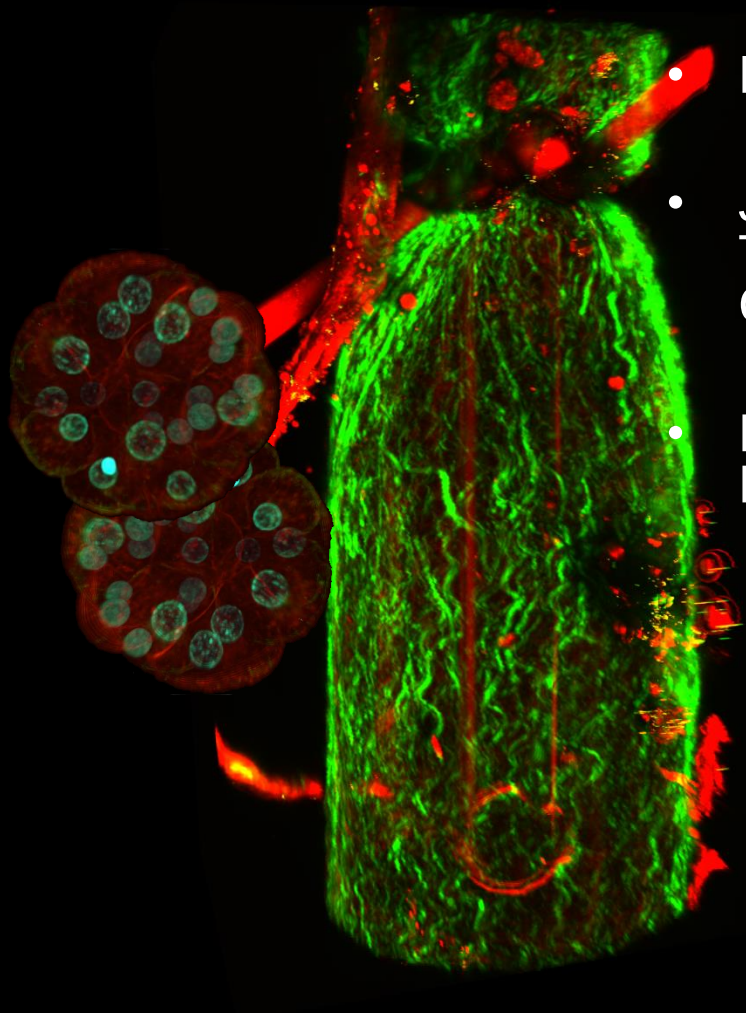
C. elegans




50 um

Collaborators

- Fabian Kellner, Michael Schubert, Ulrich Pohl
- Markus Rehberg, Fritz Krombach
- Angela Kurz, Andreas Margraf Markus Sperandio
- .



- Fritz Ihler, M. Canis
- Jan Horstkotte, Tilman Ziegler, Christian Kupatt, KUM
- Felix Habermann, LMU VetMed