

K. Bukara, S. Jovanić, I. T. Drvenica, A. Stančić, V. Ilić, M. D. Rabasović, D. Pantelić,
B. Jelenković, B. Bugarski, A. J. Krmpot

Hemoglobin imaging using two photon excitation fluorescence microscopy



photonics.ipb.ac.rs



www.ipb.ac.rs



imi.bg.ac.rs



www.tmf.bg.ac.rs

8th Regional Biophysics Conference
RBC 2018, #ReBiCon2018
Zreče, May 16th - 20th 2018



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OUTLINE

Intro: overview and significance of advanced microscopic techniques

Nonlinear Laser Scanning Microscopy (NLSM)

how does it work – underlying physics

what it can be used for – information obtained:

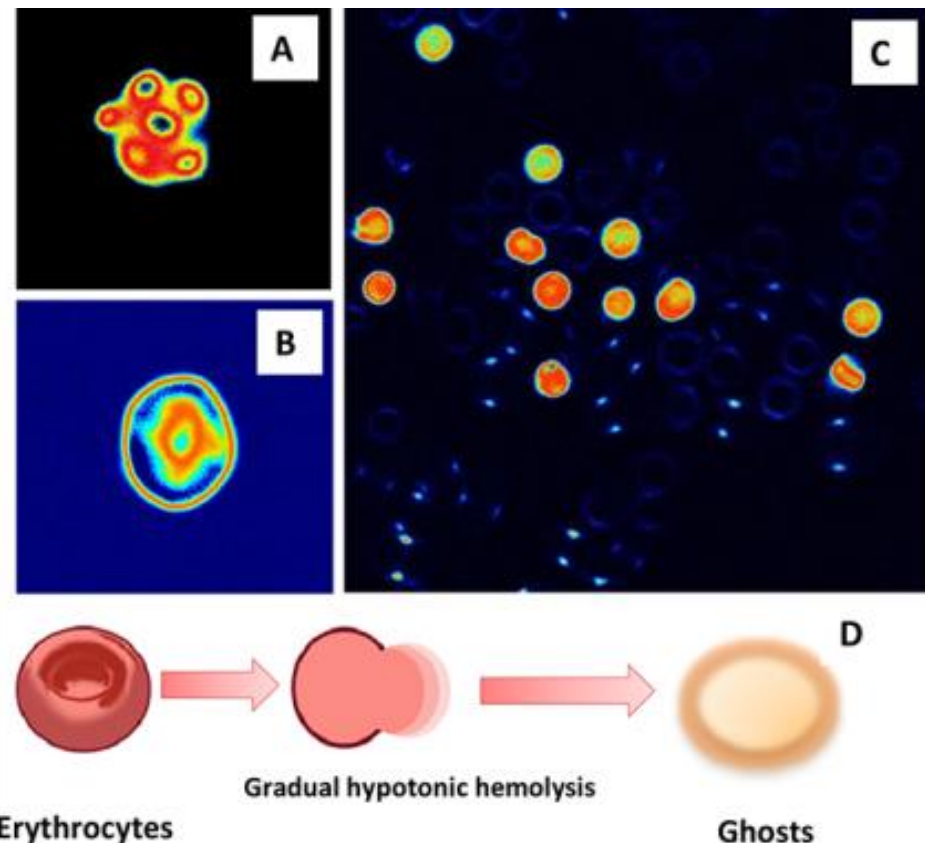
- Photon Excitation Fluorescence - TPEF
- Second and Third harmonic generation – SHG & THG
- Scanning principle and image formation

Motivation, state of the art:

- TPEF properties of hemoglobin
- applied for relevant biomed probl.

Results:

- label and fixation free imaging
- hemoglobin features imaging & erythrocytes morphology
- mapping of residual hemoglobin in erythrocyte's empty membranes (ghosts)



WHY MISROSOCOPY?

MICROSCOPIC TECHNIQUES TODAY

Qualitative (2D, 3D imaging)

- Confocal (Marvin Minsky, 1957) *
- Nonlinear*
- Holographic
- TIRF - Total Internal Reflection
- CARS - Coherent anti-Stokes Raman Scattering(*)

Quantitative :

- Fluorescence Correlation Spectroscopy – FCS
- FLIM - Fluorescence Lifetime Measurements
- FRET - Förster Resonance Energy Transfer

Super resolved:

- STED - Stimulated Emission Depletion *
- STORM - Stochastic Optical Reconstruction Microscopy
- PALM – Photo Activated Localization Microscopy
- SIM - Structured Illumination Microscopy

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- ST **1953 (physics)** : Op

- PA Frits Zernike yate

SIM umi

2008 (chemistry)

Osamu Shimomura,
Martin Chalfie and
Roger Y. Tsien

**discovery and
development of the
green fluorescent
protein, GFP**

copy **2014 (chemistry)**

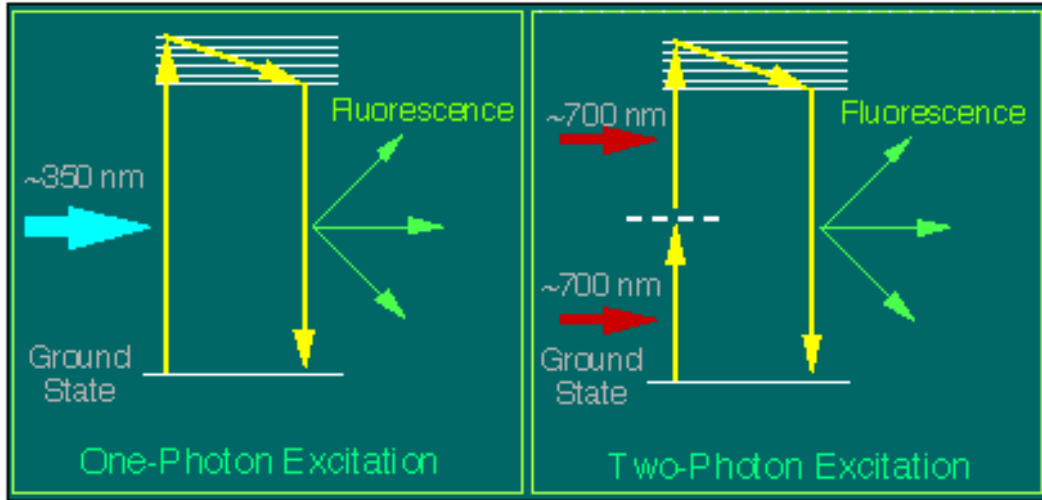
Eric Betzig, William E.
Moerner and Stefan W.
Hell

**development of super-
resolved fluorescence
microscopy**

**phase
contrast
microscopy**

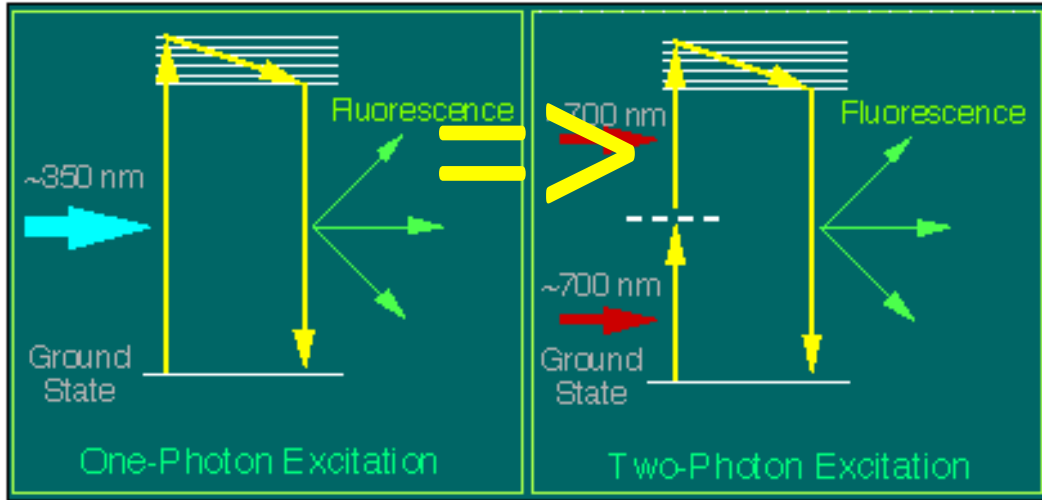


NONLINEAR EFFECTS



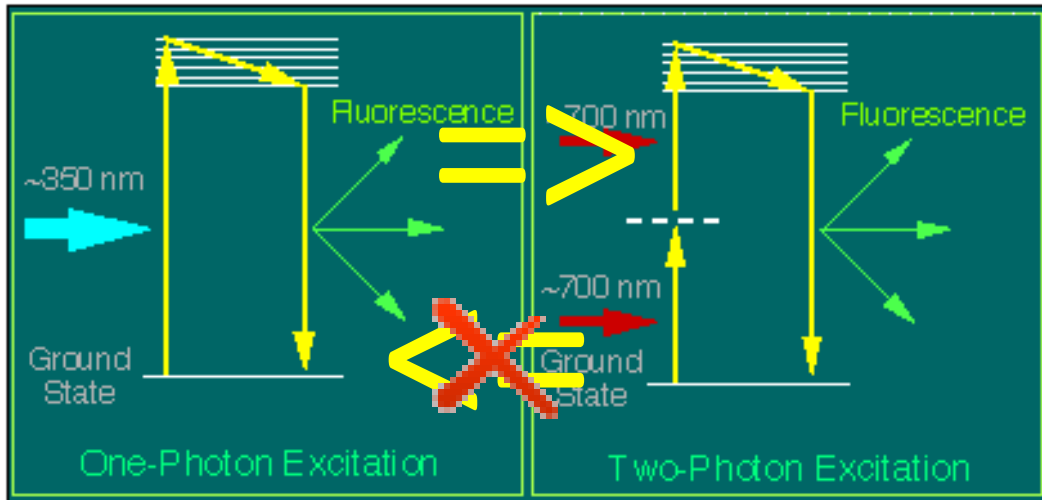
TPEF – Two Photon Excitation
Fluorescence

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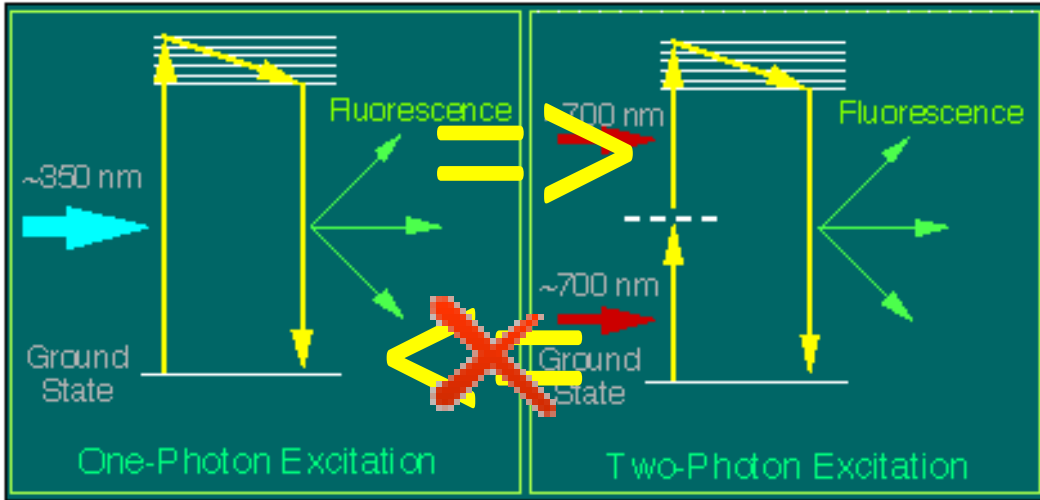
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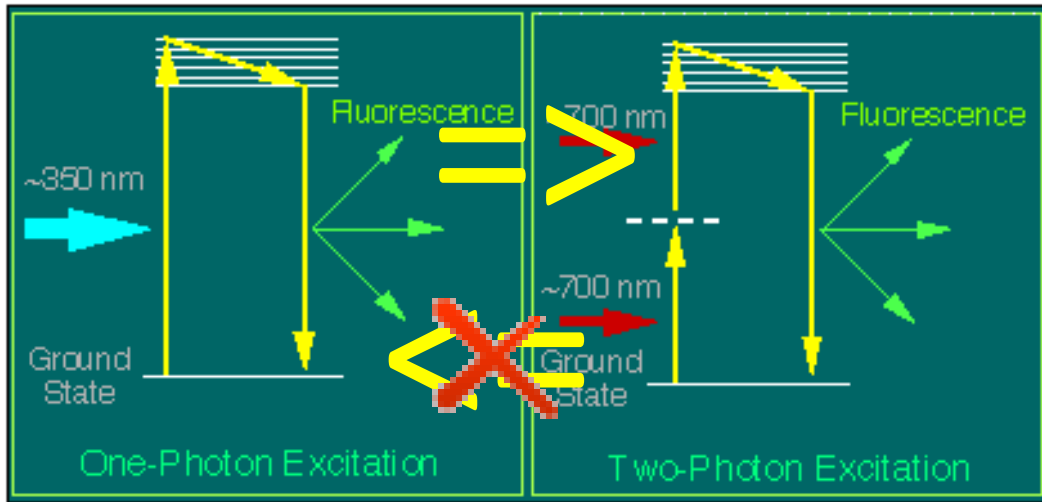
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The probability for multiphoton absorption: $W \sim I^{2(3)}$

I – light intensity [mW/cm^2];

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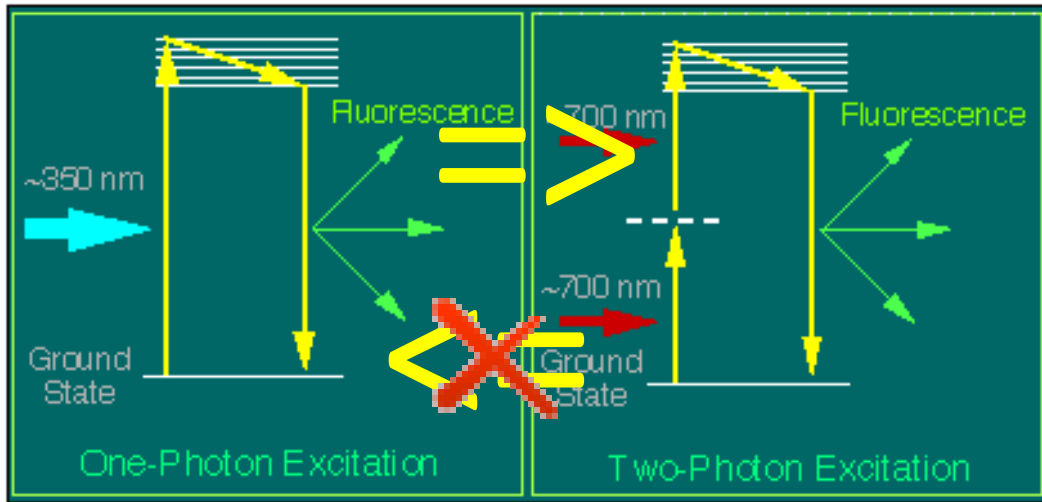
⇒ **High numerical aperture (NA) objectives**

$$d = 1,22 \lambda / \text{NA}$$

d – the laser beam waste in the focus

λ – wavelength

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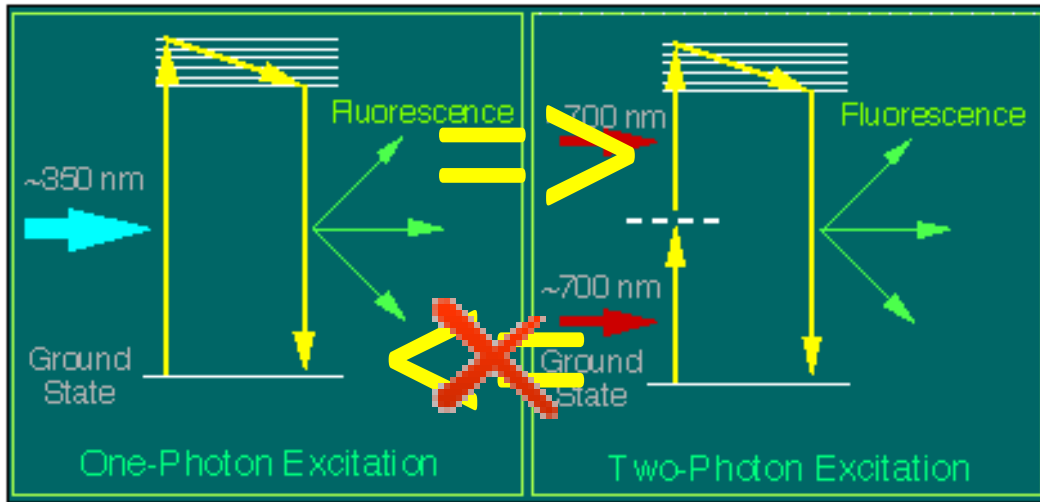
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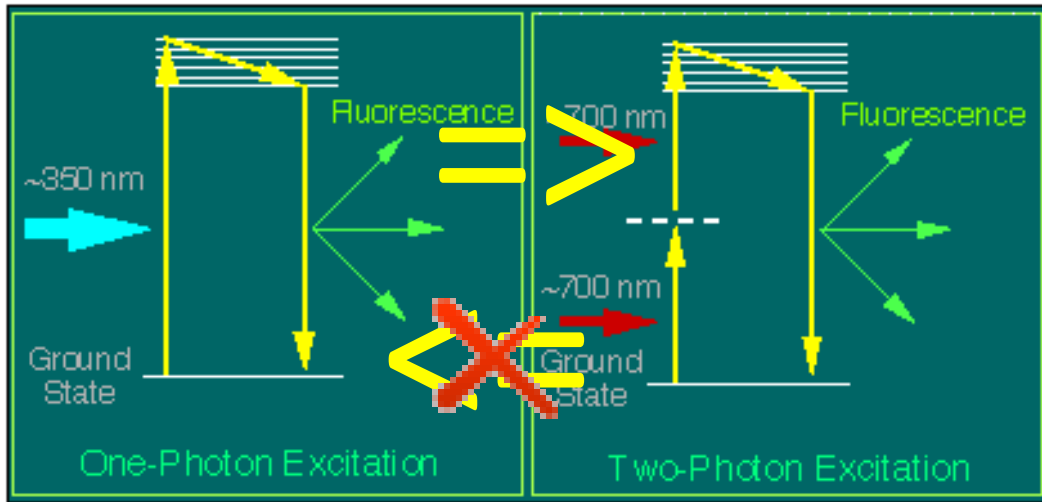
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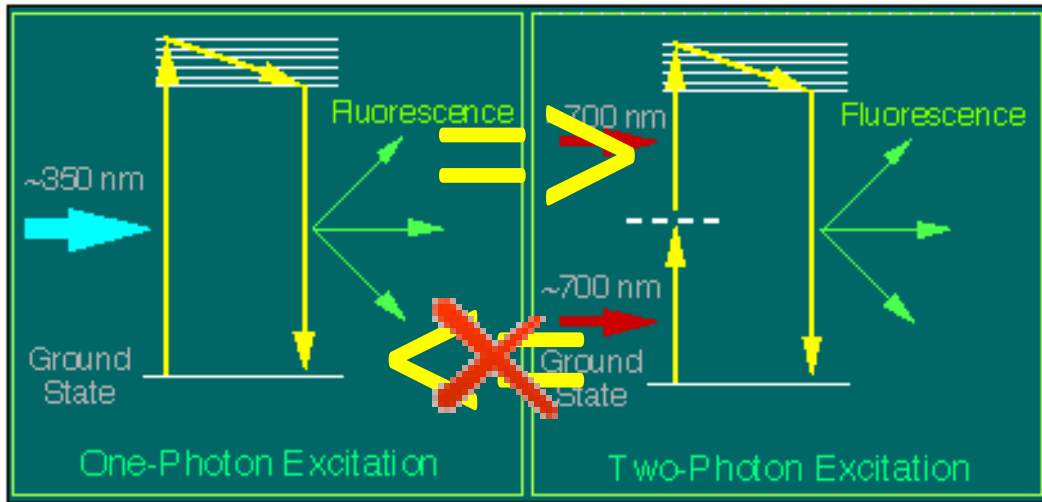
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CW vs. fs lasers

CW He Ne (@ 543nm) = **1mW**

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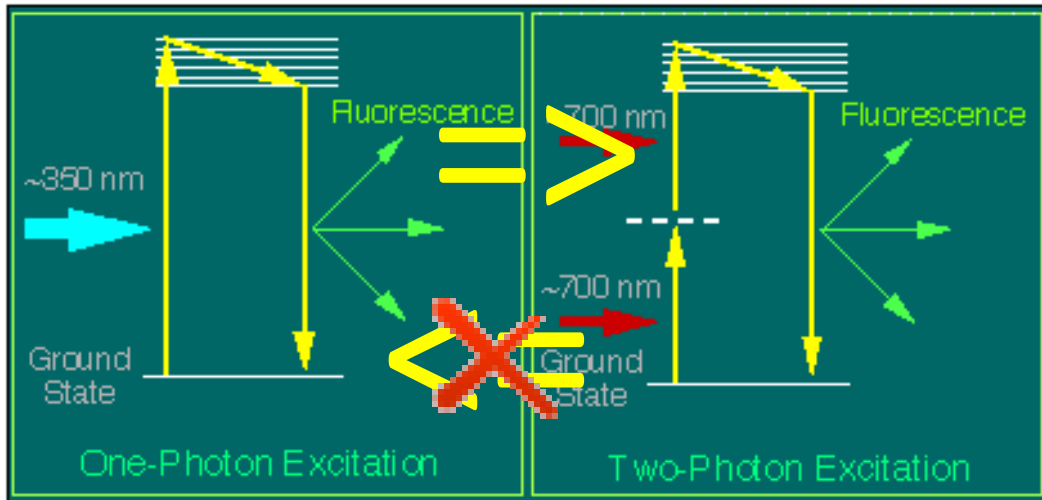
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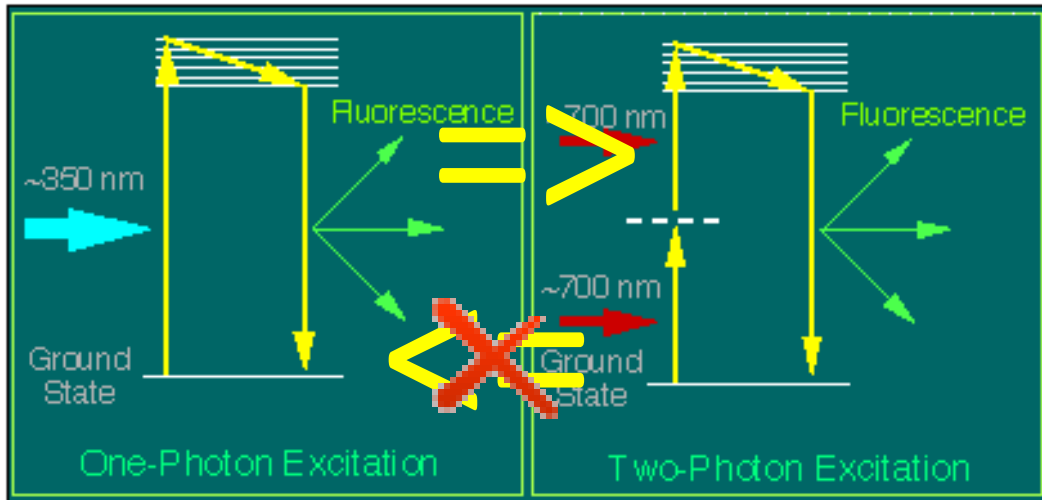
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High power, but low energy!!!

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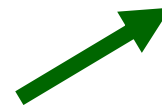
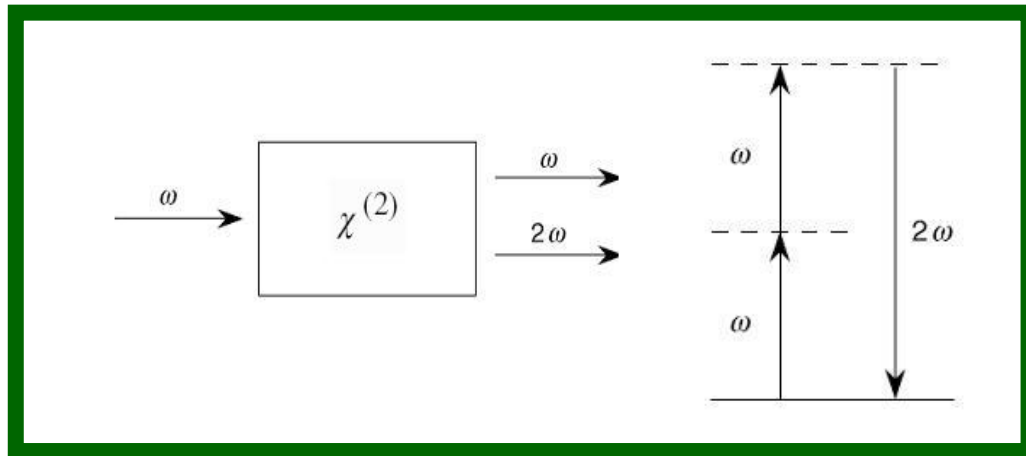
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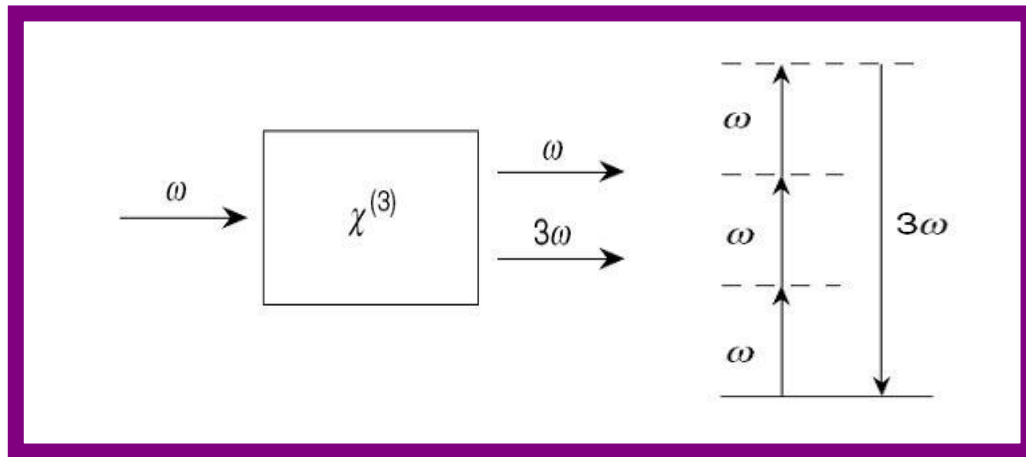
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SECOND AND THIRD HARMONIC GENERATION



$$I_{\text{SHG}} \sim I^2$$



$$I_{\text{THG}} \sim I^3$$

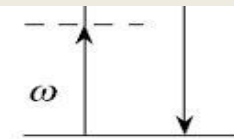
SECOND AND THIRD HARMONIC GENERATION

SHG~ noncentrosymmetric (oriented) structures

- starch, collagen, myosin, crystalized hemoglobin

THG~ refractive index variation

- Various interfaces: nucleus-cytoplasm, cell membrane, organelles etc



$$I_{\text{THG}} \sim I^3$$

SECOND AND THIRD HARMONIC GENERATION

information about the sample – detection the light-matter interaction effects

-(Epi)Fluorescence/Confocal/TPEF microscopy– labeled molecules and/or endogenous fluorescent molecules

-bright-field microscopy – absorption of the light

-phase contrast microscopy – light (e.m. wave) phase shift

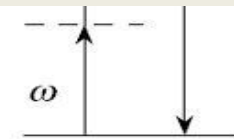
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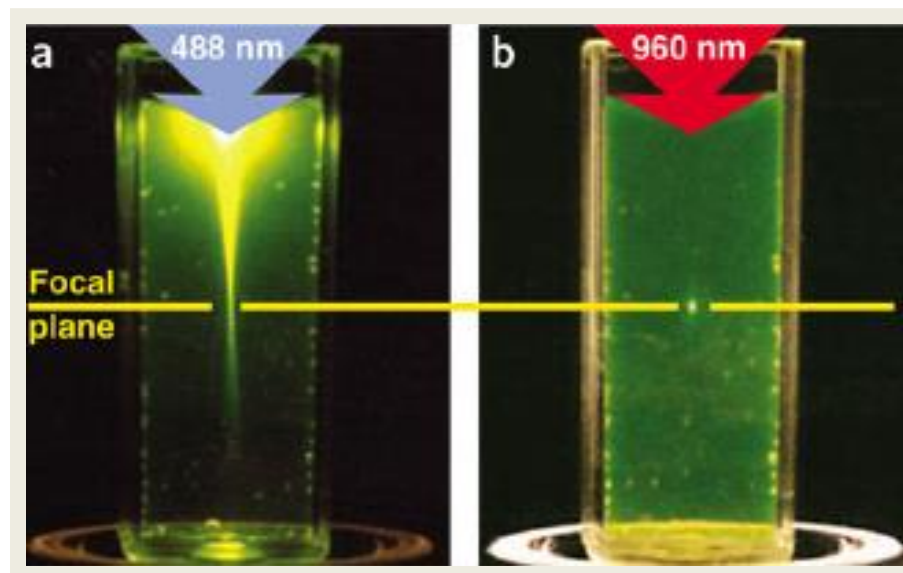
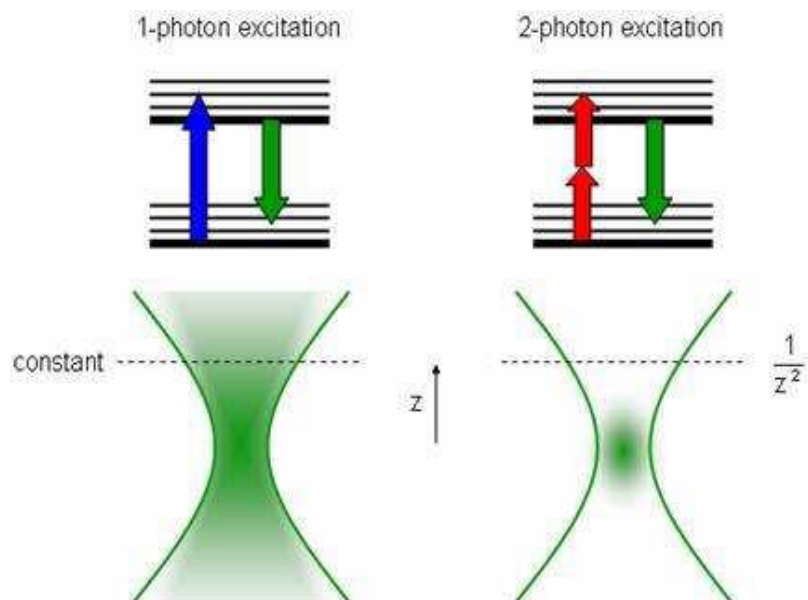
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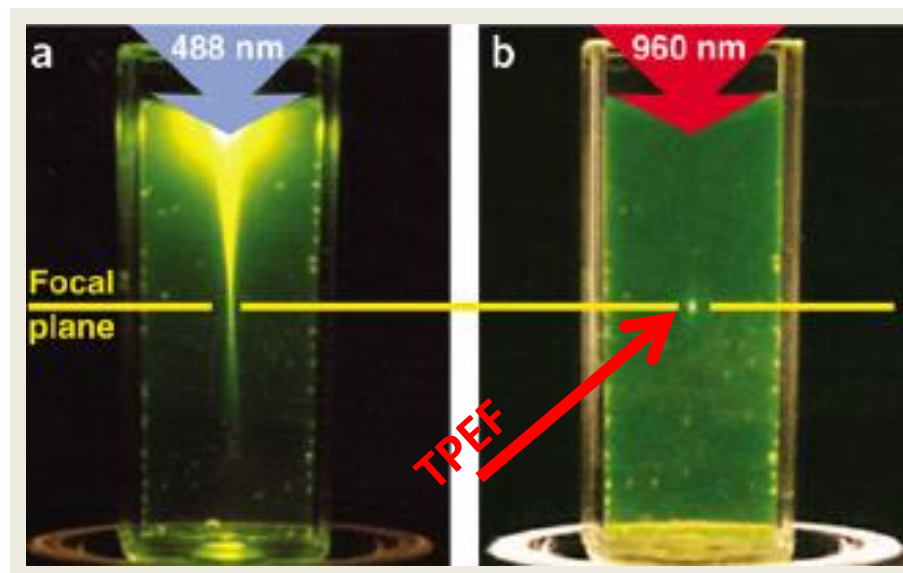
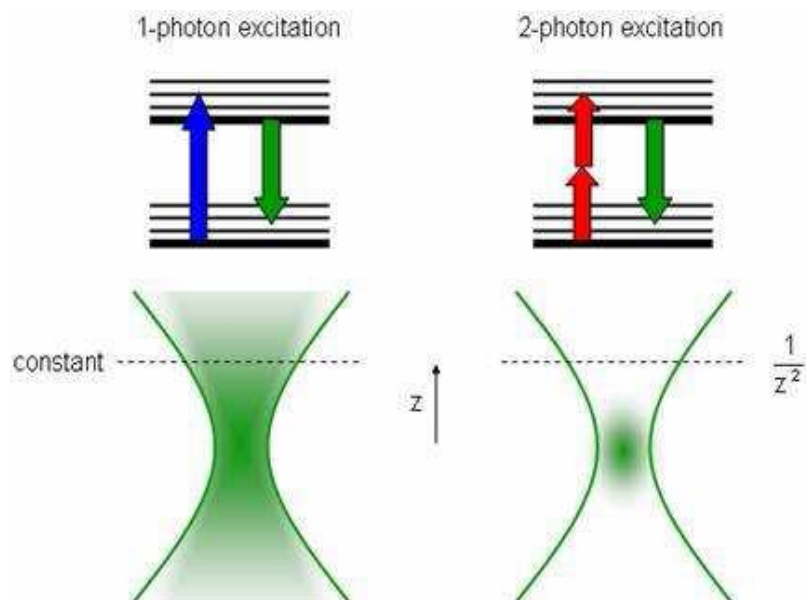


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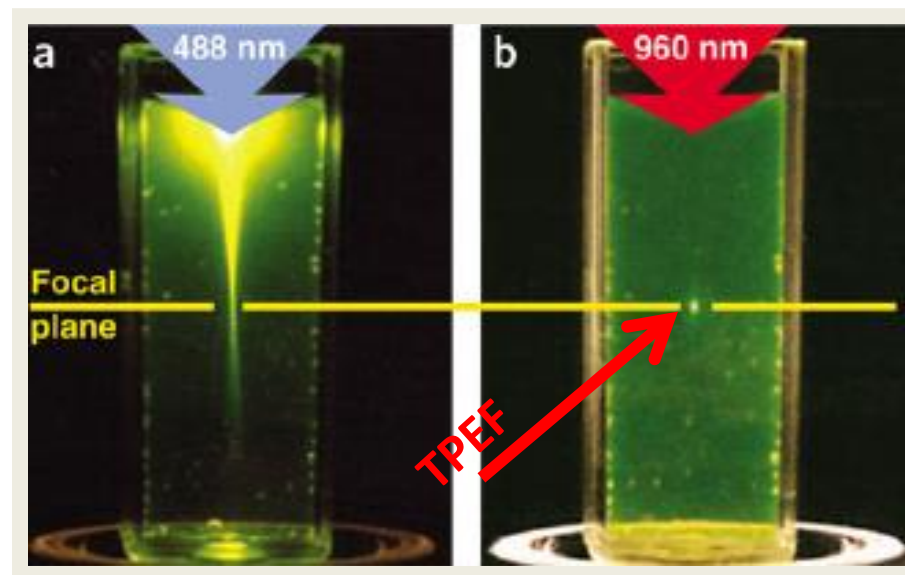
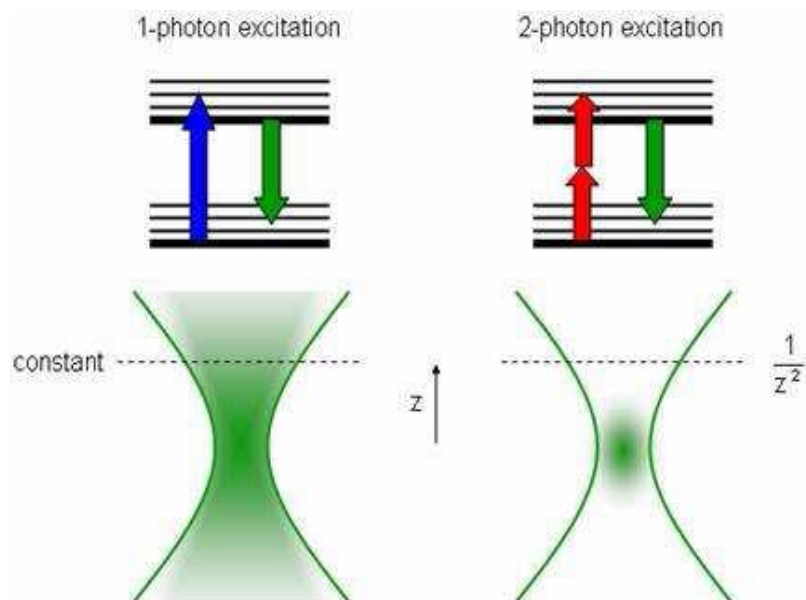
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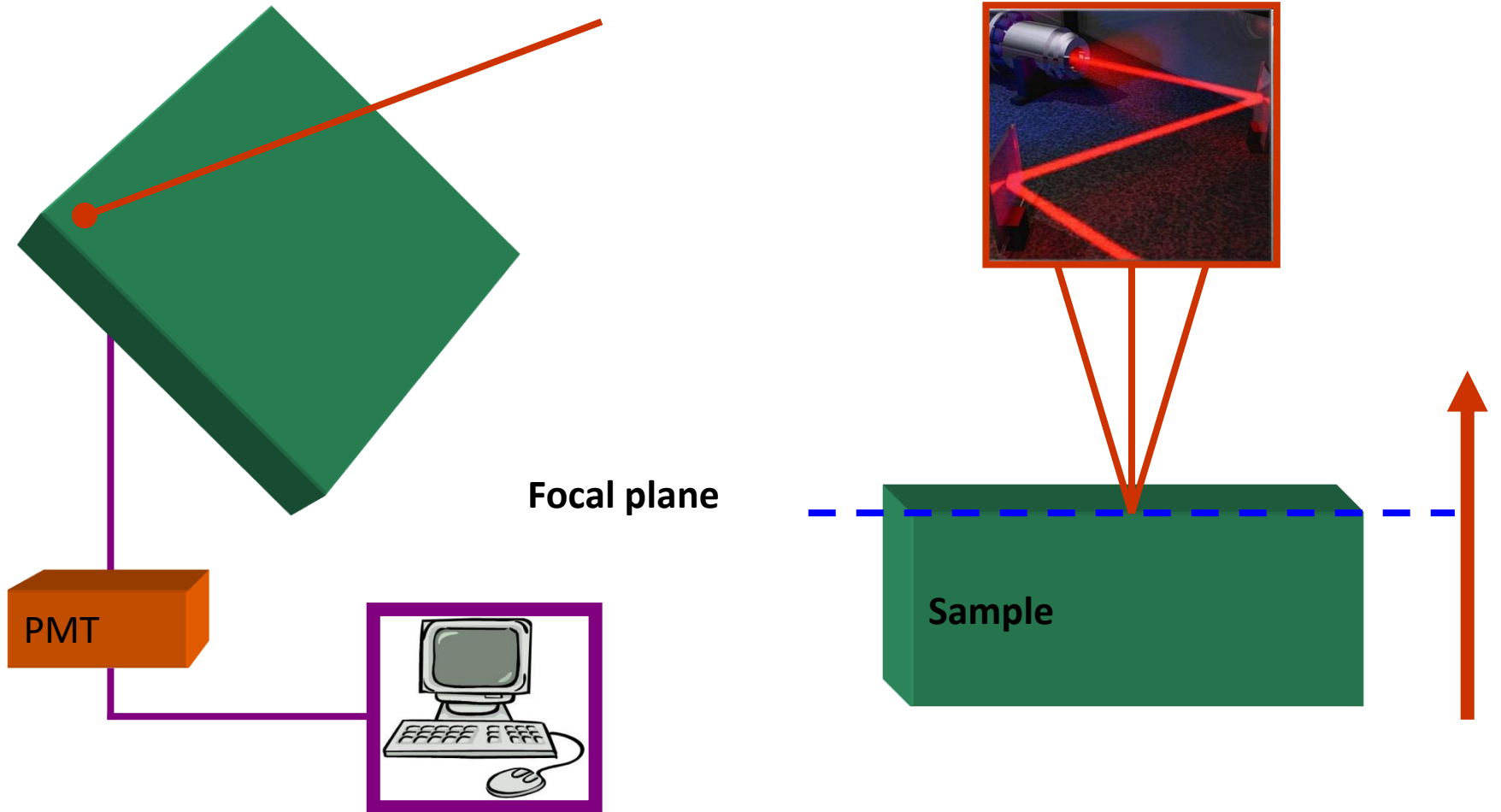


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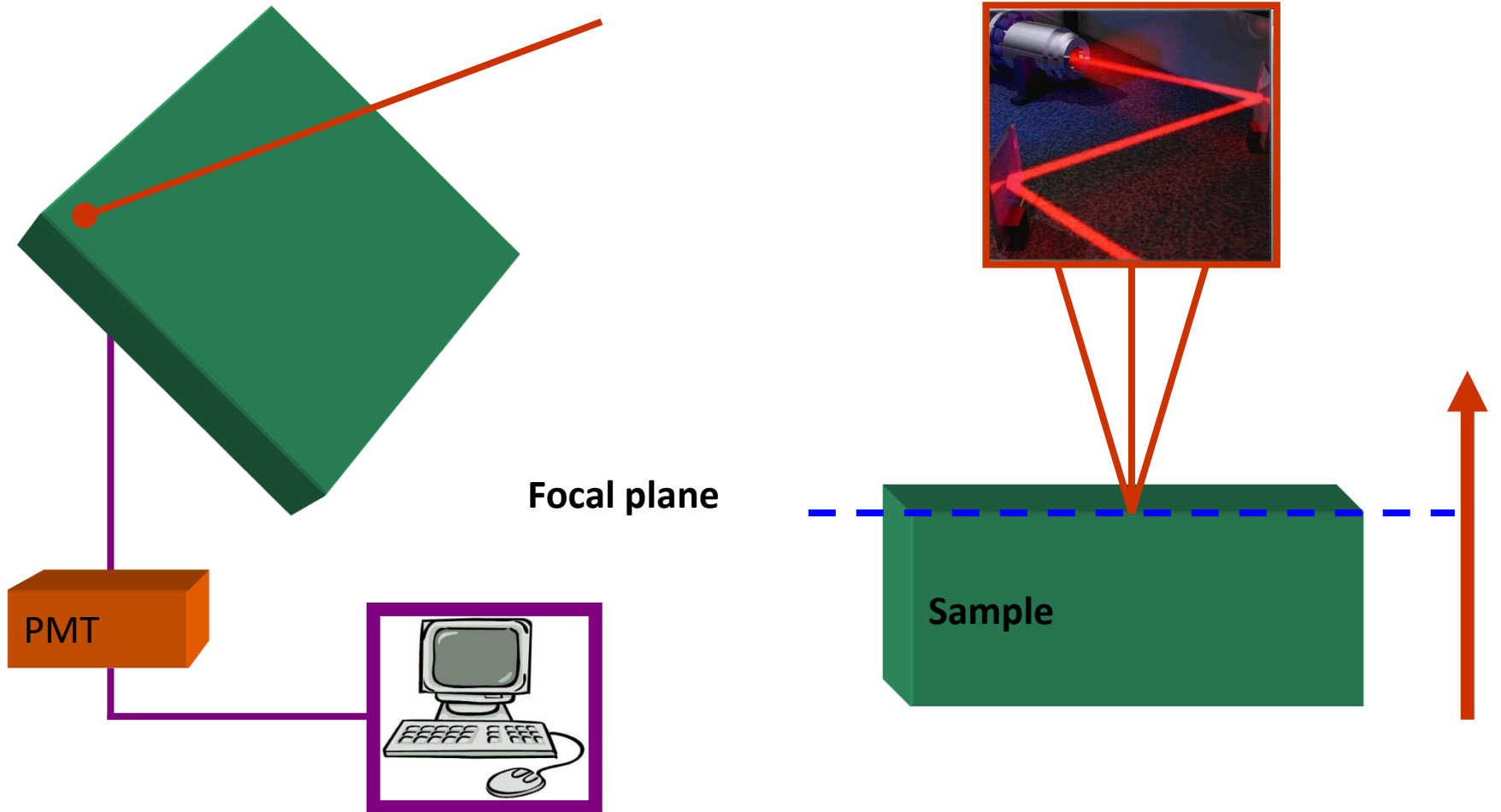


Nonlinear => Intrinsically confocal

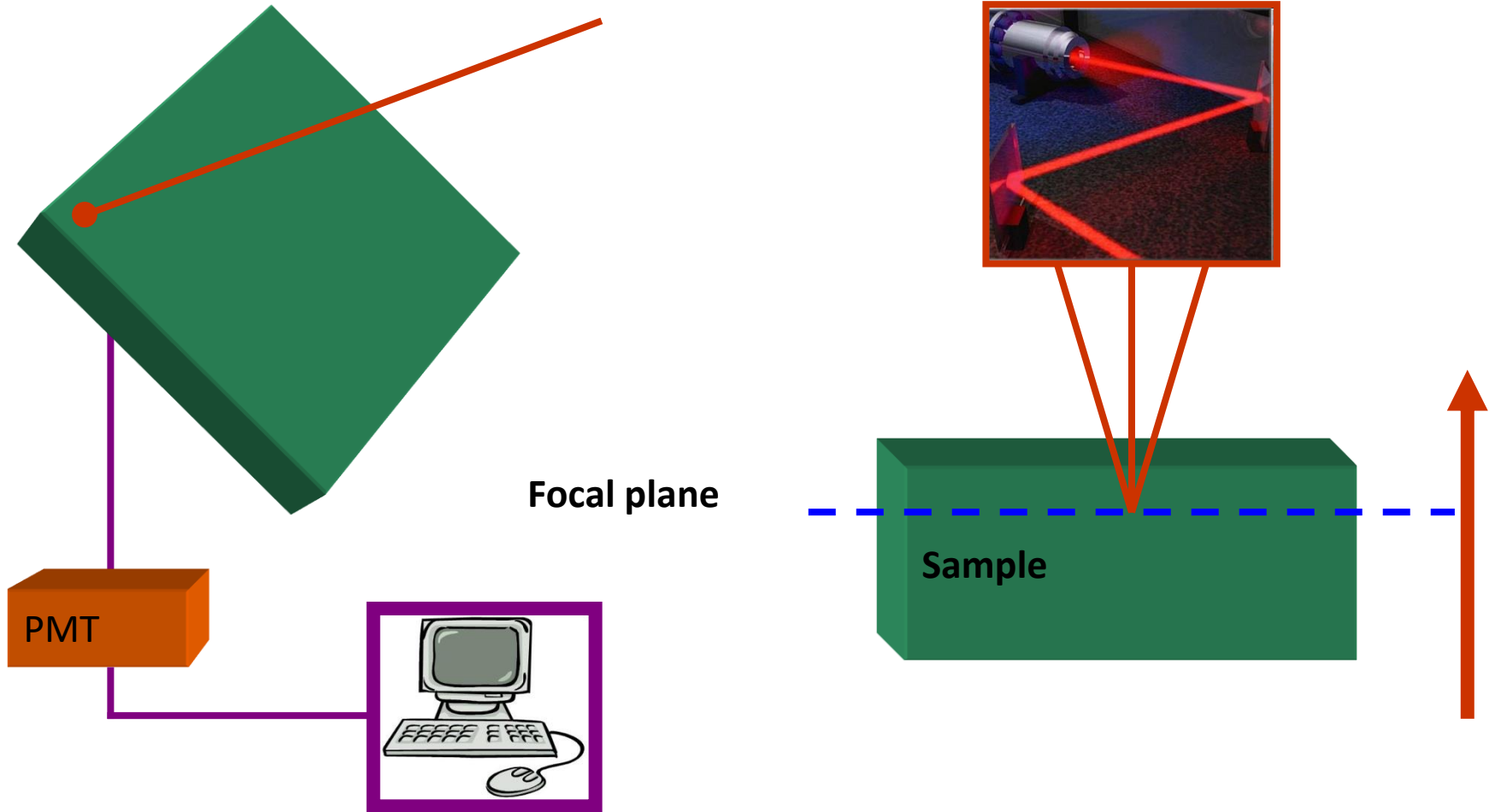
RECORDING PROCESS



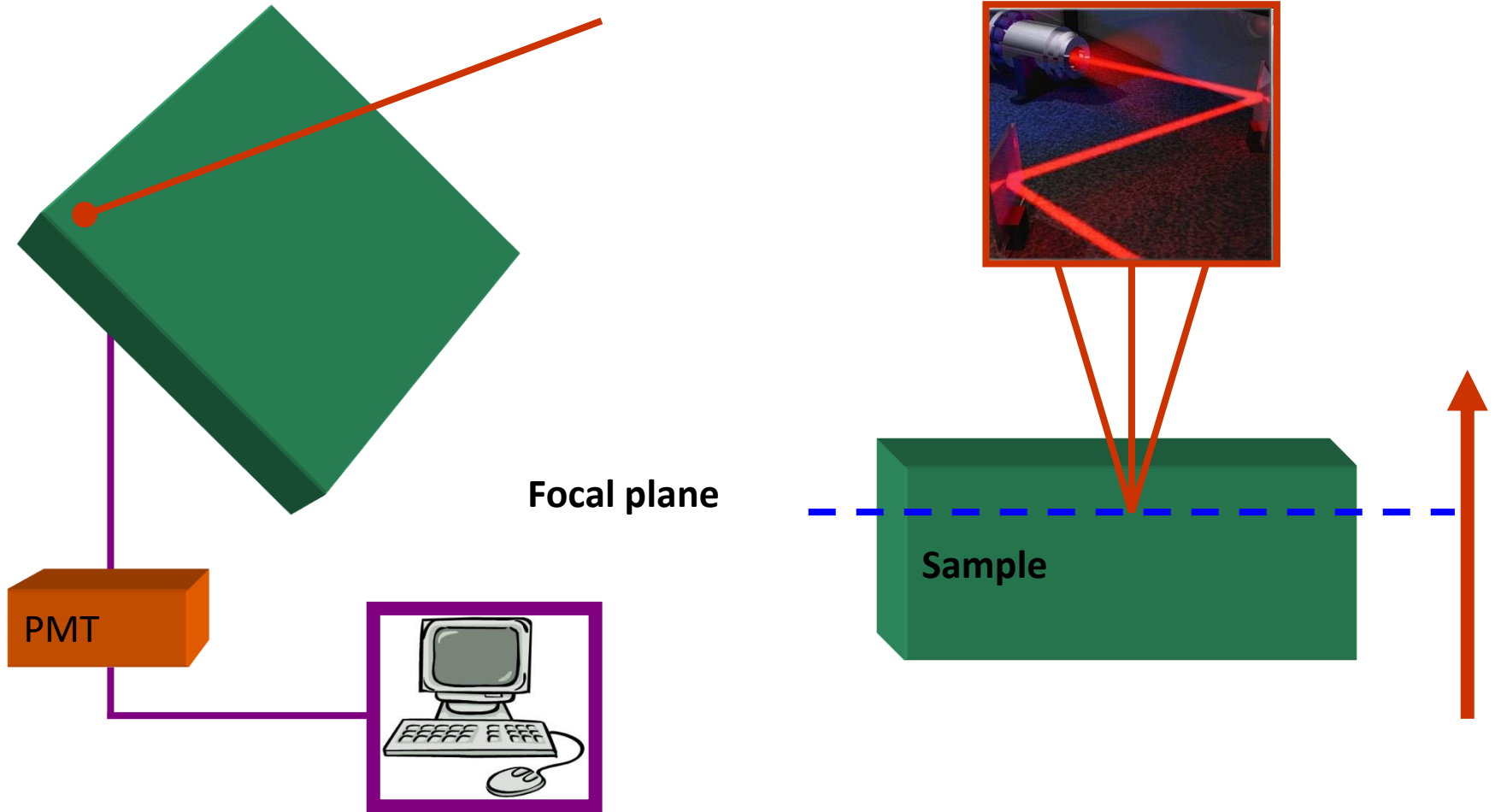
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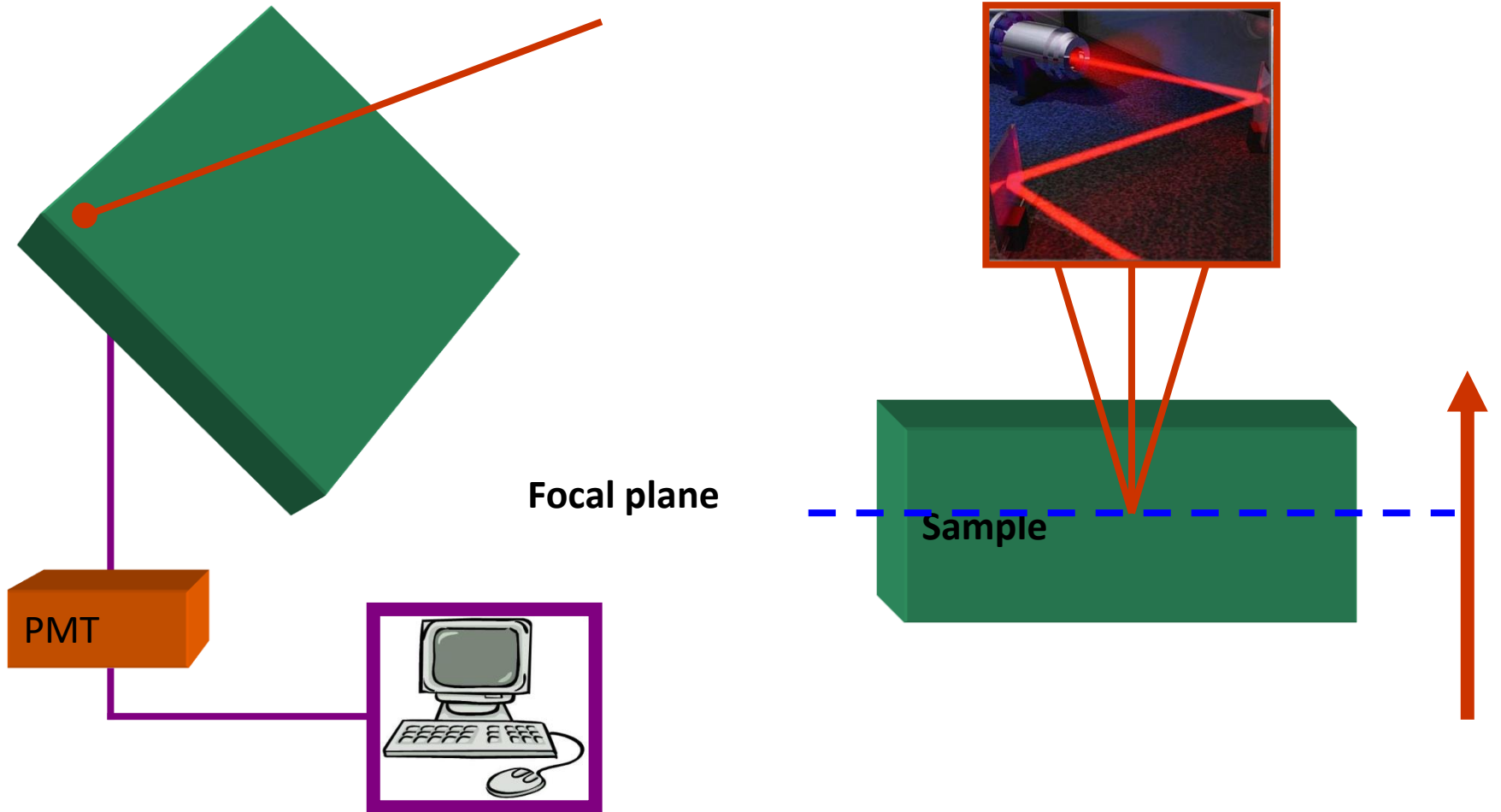
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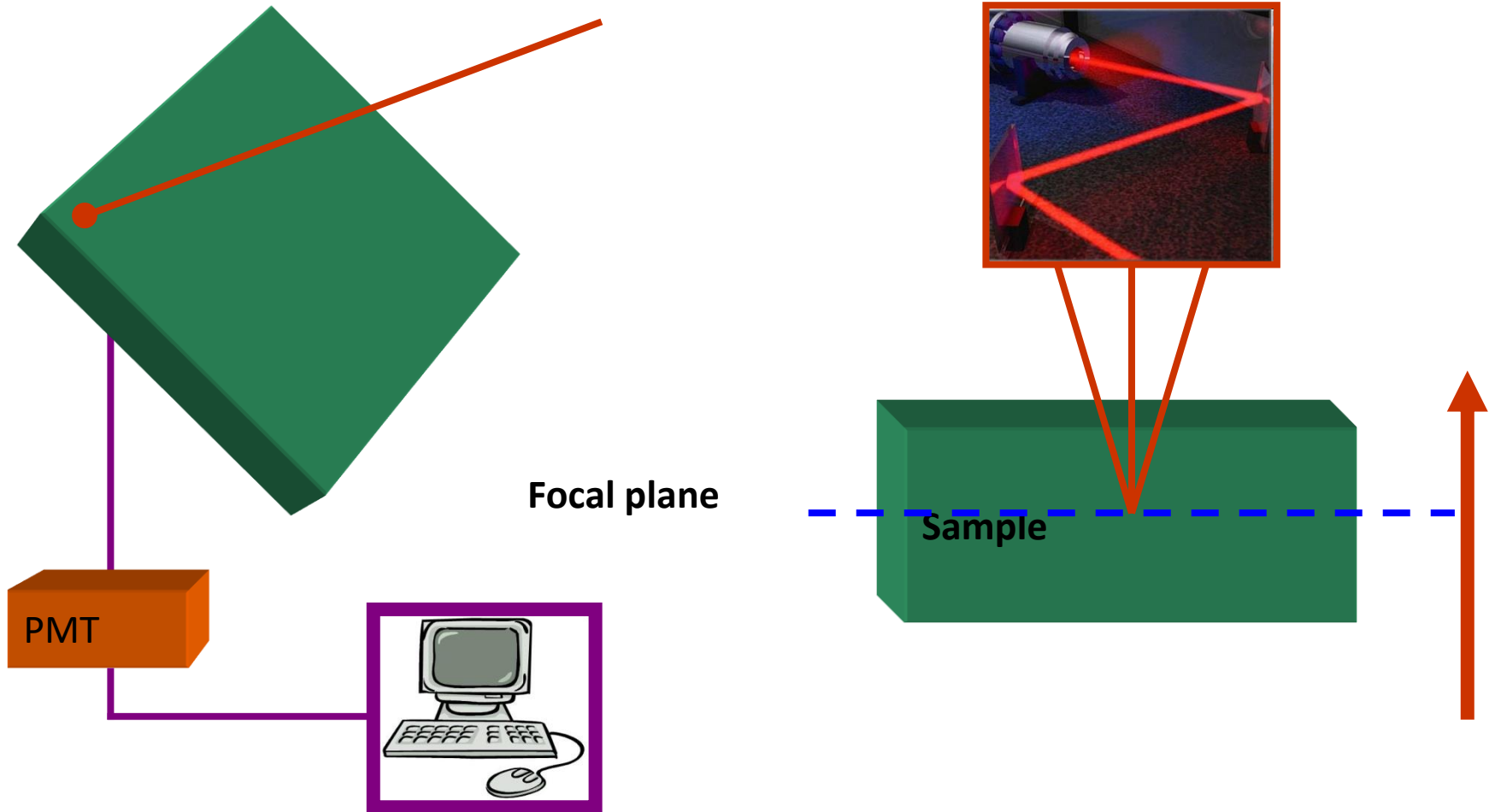
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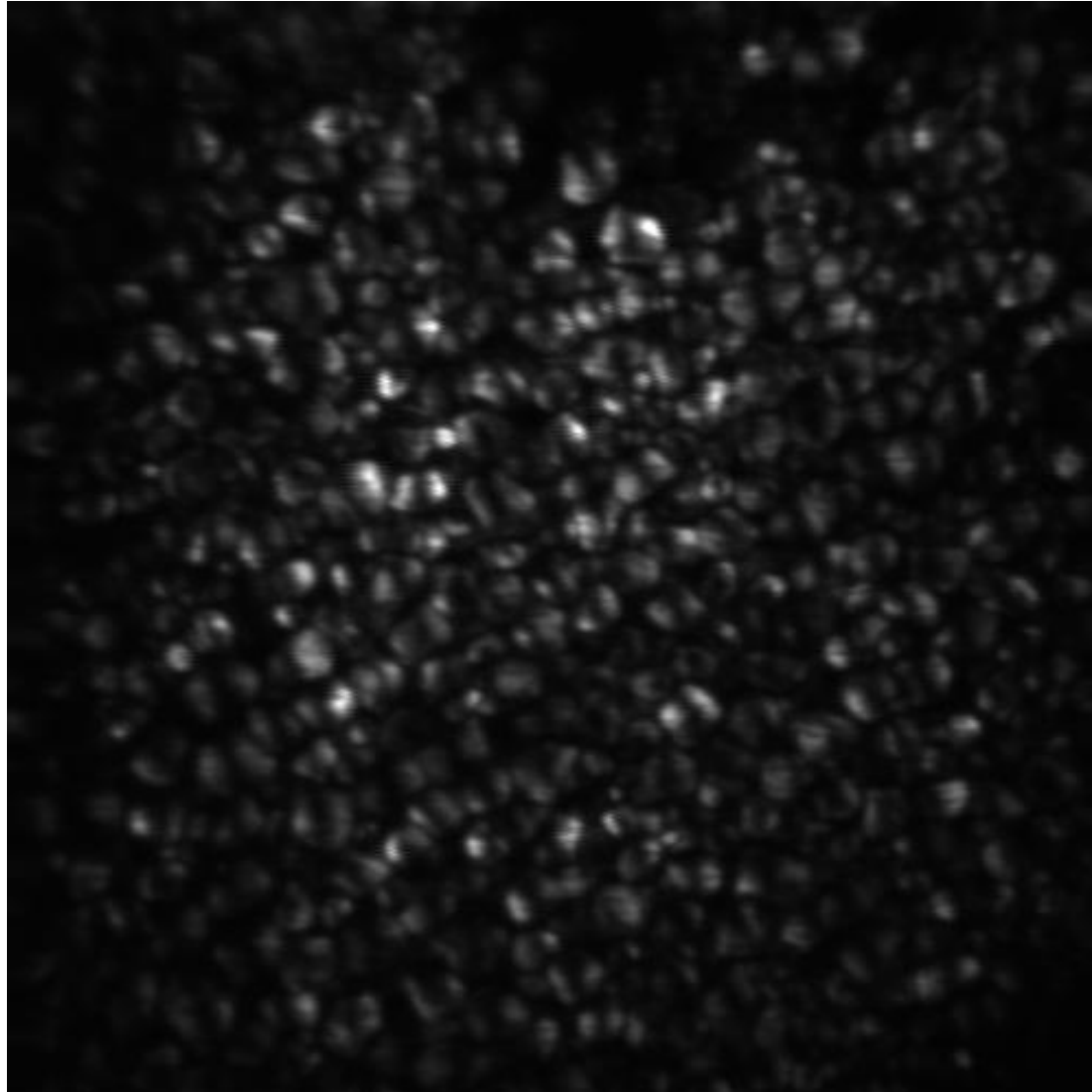


FROM 2D TO 3D IMAGE

```
0,020 0,021 0,022 0,017 0,021 0,020 0,022 0,024 0,025 0,028 0,030
0,017 0,020 0,021 0,020 0,020 0,018 0,018 0,018 0,019 0,020 0,027
0,024 0,016 0,022 0,023 0,021 0,021 0,021 0,022 0,018 0,018 0,017
0,023 0,027 0,026 0,025 0,031 0,030 0,029 0,028 0,025 0,030 0,028
0,024 0,025 0,029 0,035 0,038 0,040 0,040 0,040 0,039 0,037 0,036
0,016 0,024 0,022 0,033 0,036 0,040 0,040 0,037 0,034 0,032 0,030
0,012 0,013 0,017 0,019 0,026 0,025 0,030 0,030 0,030 0,028 0,028
0,010 0,011 0,013 0,016 0,019 0,021 0,021 0,016 0,021 0,019 0,019
0,010 0,011 0,012 0,013 0,014 0,013 0,014 0,013 0,013 0,010 0,012
0,016 0,014 0,013 0,013 0,012 0,015 0,011 0,008 0,011 0,012 0,011
0,017 0,016 0,017 0,015 0,012 0,015 0,015 0,016 0,015 0,016 0,019
0,015 0,014 0,013 0,013 0,013 0,013 0,014 0,016 0,016 0,017 0,015
0,011 0,015 0,019 0,016 0,015 0,017 0,018 0,017 0,013 0,016 0,017
0,012 0,011 0,012 0,014 0,016 0,014 0,015 0,017 0,015 0,021 0,018
0,015 0,014 0,014 0,012 0,010 0,010 0,012 0,012 0,012 0,014 0,012
0,014 0,016 0,016 0,016 0,017 0,014 0,014 0,016 0,015 0,016 0,015
0,018 0,019 0,021 0,020 0,020 0,015 0,018 0,016 0,017 0,016 0,015
0,100 0,124 0,151 0,168 0,143 0,096 0,048 0,018 0,016 0,016 0,018
0,152 0,245 0,352 0,431 0,458 0,440 0,361 0,265 0,178 0,115 0,080
0,287 0,360 0,439 0,471 0,439 0,360 0,248 0,146 0,063 0,035 0,040
0,298 0,368 0,446 0,500 0,532 0,530 0,476 0,381 0,276 0,184 0,122
0,107 0,169 0,255 0,351 0,432 0,462 0,418 0,319 0,188 0,094 0,067
0,048 0,051 0,078 0,120 0,158 0,175 0,168 0,127 0,097 0,068 0,046
0,016 0,016 0,018 0,018 0,021 0,020 0,020 0,020 0,021 0,023 0,032
0,014 0,030 0,078 0,155 0,205 0,203 0,160 0,120 0,083 0,061 0,045
0,226 0,305 0,370 0,401 0,366 0,274 0,153 0,062 0,028 0,025 0,026
0,192 0,258 0,348 0,410 0,442 0,421 0,357 0,273 0,187 0,123 0,079
0,076 0,107 0,147 0,180 0,186 0,152 0,100 0,054 0,031 0,024 0,020
```

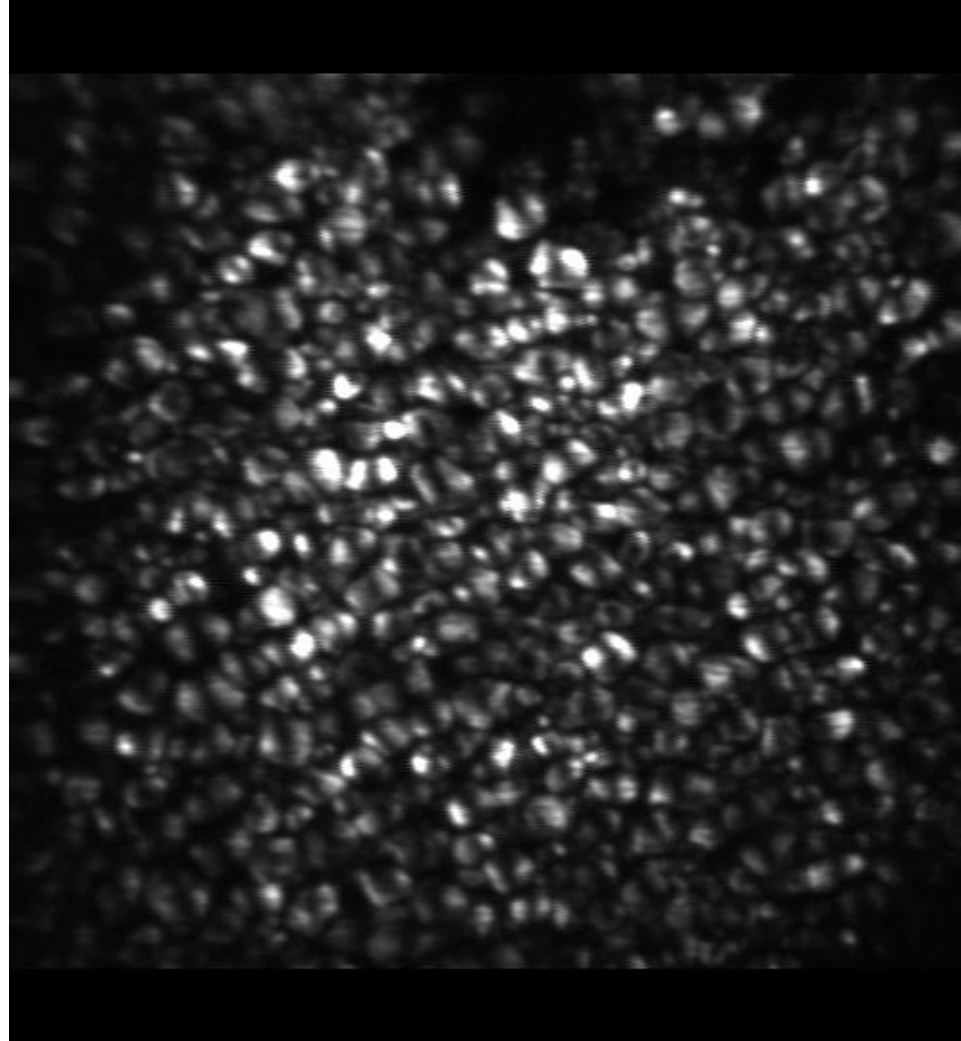
FROM 2D TO 3D IMAGE

Starch grains imaging - SHG signal



FROM 2D TO 3D IMAGE

Starch grains imaging - SHG signal



FROM 2D TO 3D IMAGE

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Why nonlinear laser scanning microscopy (NLSM)?

Because...

- ✓ **Deep penetration into the sample ($\sim 500 \mu\text{m} - 1 \text{mm}$)** ~ intrinsically confocal
- ✓ **High (axial) resolution ($\sim 500 \text{nm}$)**
- ✓ **High contrast images**
- ✓ **3D images (models)**
- ✓ **Reduced/No phototoxicity (TPEF/SHG&THG)** ~ intrinsically confocal
- ✓ **Reduced/No photobleaching (TPEF/SHG&THG)** ~ intrinsically confocal
- ✓ **No need for sample dyeing (label free technique)**
- ✓ **Superior tool for in vivo imaging**
- ✓ **Excitation of molecules that can't be excited by one photon (TPEF)**

Details in:

- R. Carriles, D. N. Schafer et al, *Rev Sci Inst* 80, (2009), 081101
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Why nonlinear laser scanning microscopy (NLSM)?

Because...

- ✓ **Deep penetration into the sample ($\sim 500 \mu\text{m} - 1 \text{mm}$)** ~ intrinsically confocal
- ✓ **High (axial) resolution ($\sim 500 \text{nm}$)**
- ✓ **High contrast images**
- ✓ **3D images (models)**
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Why erythrocytes?

- physiological functions
- vascular disorders (sickle cell disease, malaria)
- **drug vehicles (prolonged and controlled drug delivery systems)**

How to image hemoglobin features (or erythrocytes)?

- phase contrast microscopy (erythrocyte imaging, no chemical selectivity)
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- **Intrinsic two photon excitation fluorescence of hemoglobin** – Sorret
fluorescence band peaked at 438nm
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 - Blood samples - Porcine slaughterhouse blood and human outdated blood
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 - Cyanmethemoglobin assay – spectrophotometry
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Hemoglobin distribution

Two photon absorption of hemoglobin
Omar Clay et al, *J. Chem. Phys.* 126 (2007)

TPEF in hemoglobin properties and proof of the principle
Zheng et al, *Biomed Opt Exp* 71 (2011)

App. on model organism (Zebra fish)
Li et al, *Opt Lett* 36 (2011)

App. on relevant biomed. problem (sickle cell disease)
Vigil et al, *Biomed Opt Exp* 6 (2015)

SHG img of crystalized hemoglobin features

Pilot project at



THG img of erythrocytes
Saytashev et al, *Biomed Opt Exp* 7 (2016)

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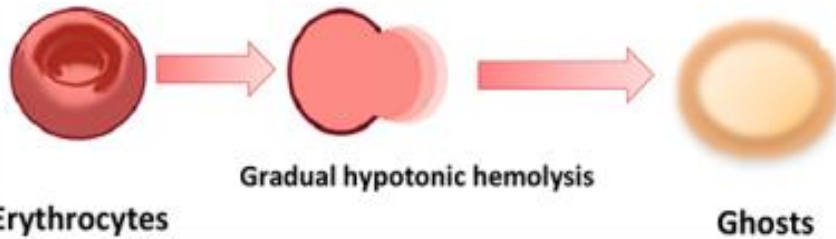
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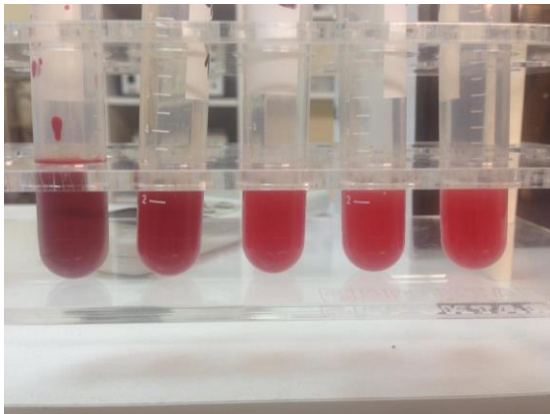
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MOTIVATION & STATE OF THE ART



Empty erythrocyte's membranes (ghosts)
- drug vehicles

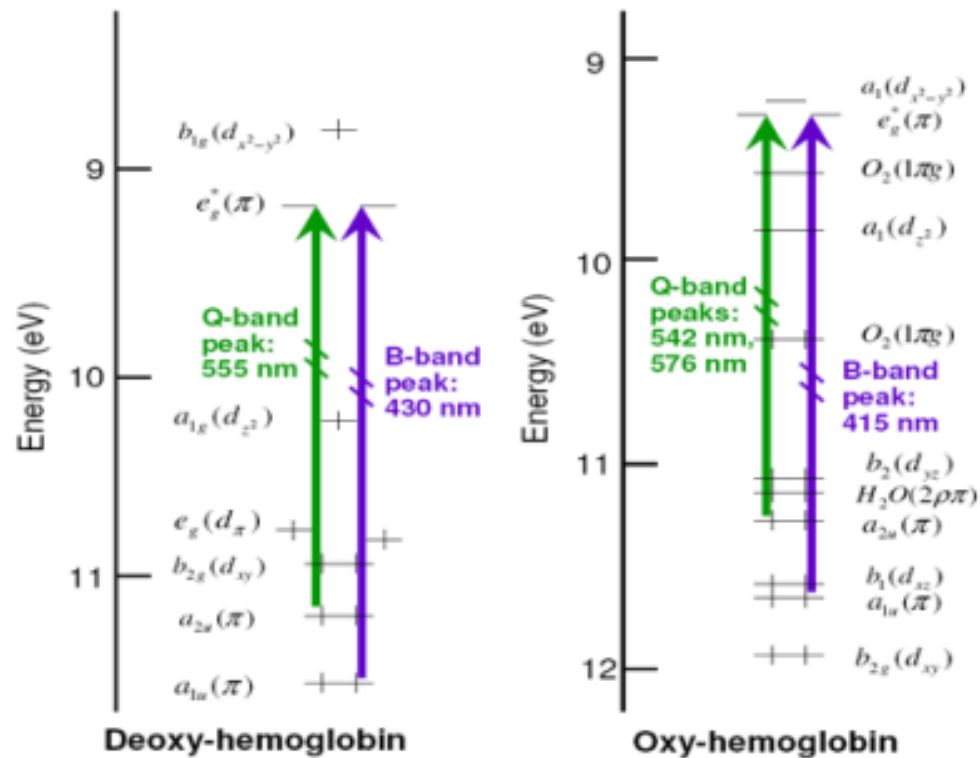
After 4 iterations (rinses) still red



Wei Zheng, Dong Li, Yan Zeng, Yi Luo,
Jianan Y. Qu

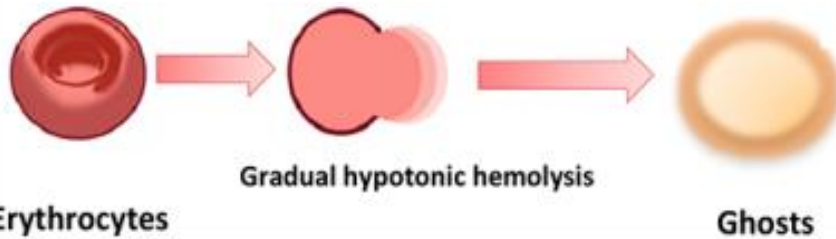
Two-photon excited hemoglobin fluorescence
Biomed Opt Express 2 (2010)

Strong two photon absorption in red-NIR
Soret (B) fluorescence band in VIS (peak@438nm)



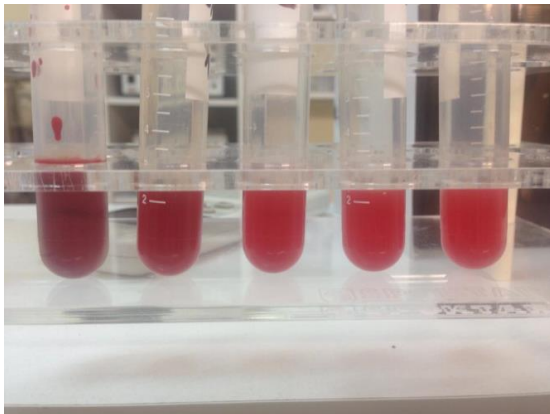
The electronic energy level diagram of hemoglobin

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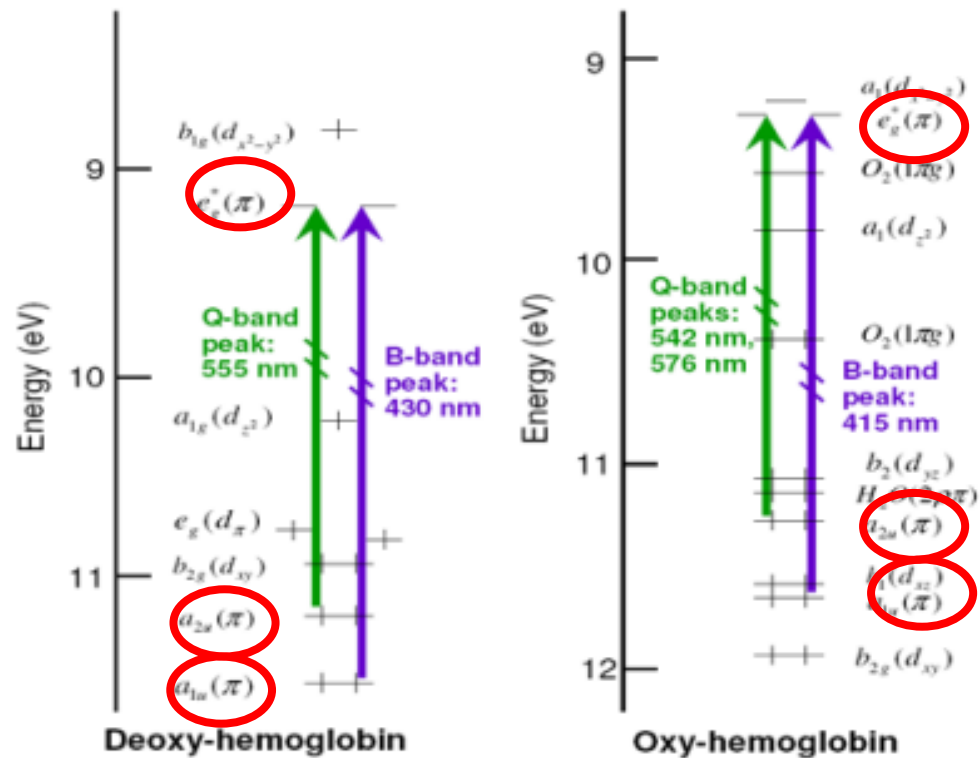
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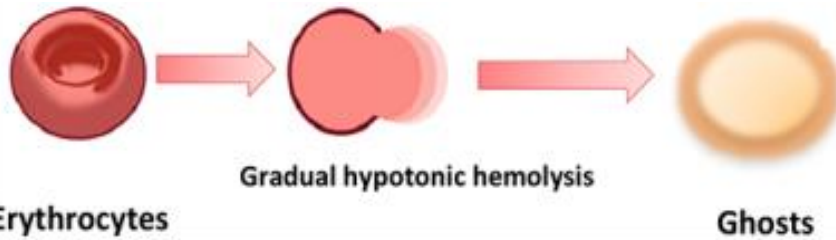
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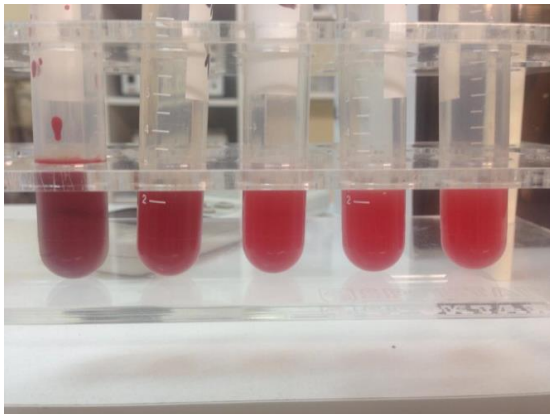
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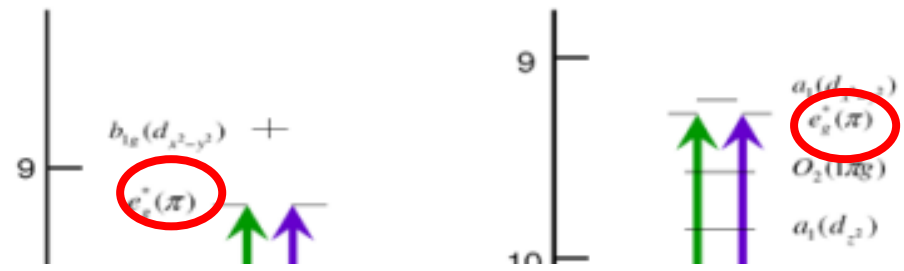
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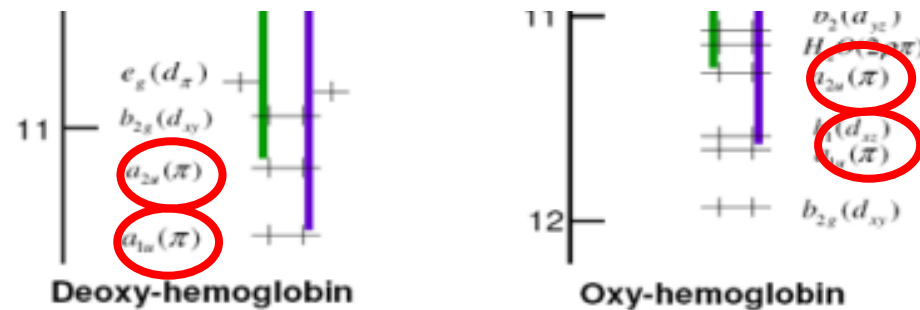
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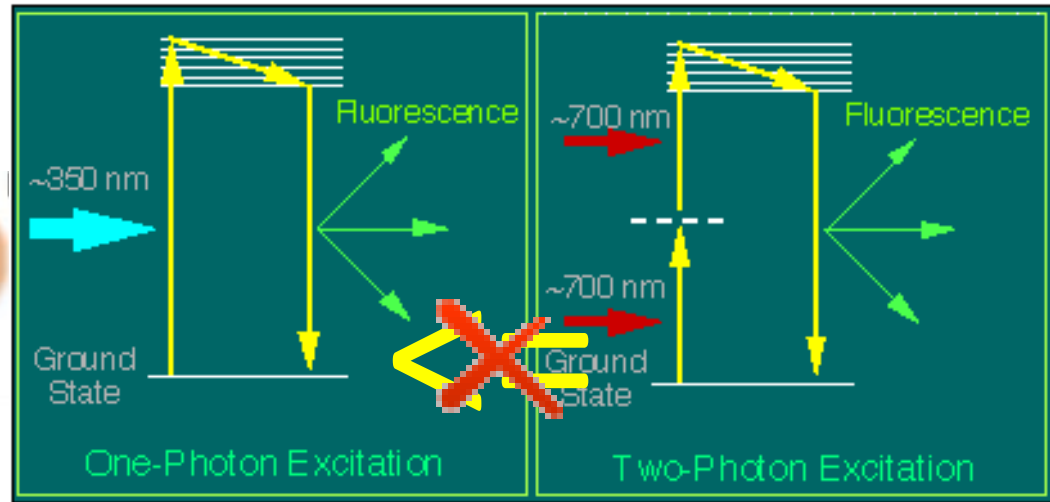
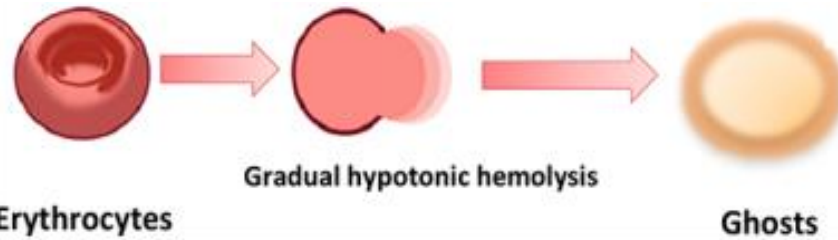


$\pi \rightarrow \pi$ transitions, only by two photon excitation
due to angular momentum conservation
(selection rules)



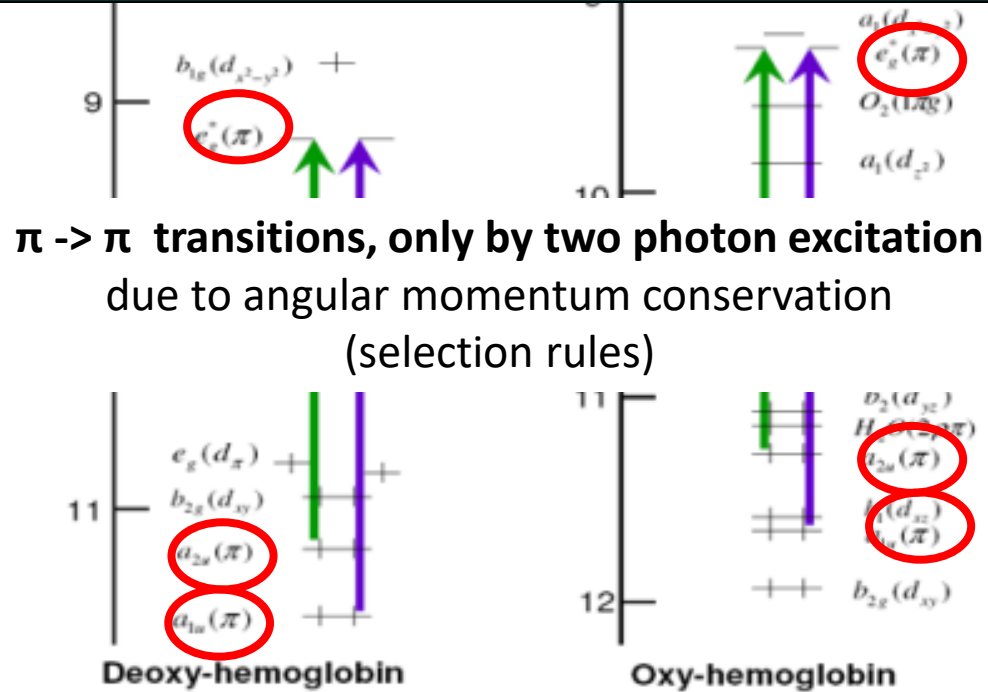
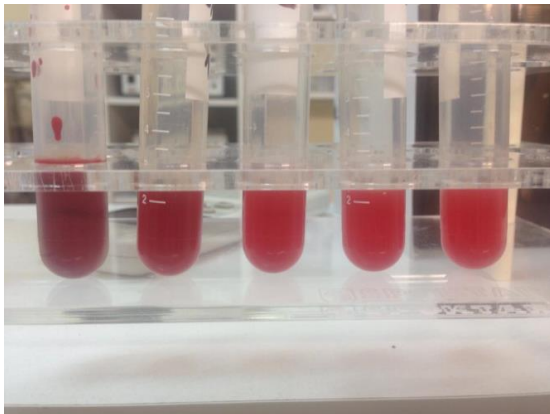
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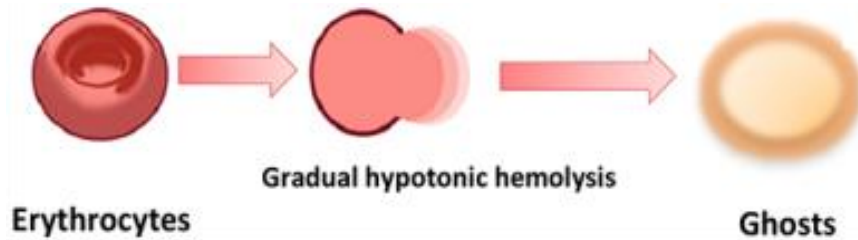
Empty erythrocyte's membranes (ghosts) - drug vehicles

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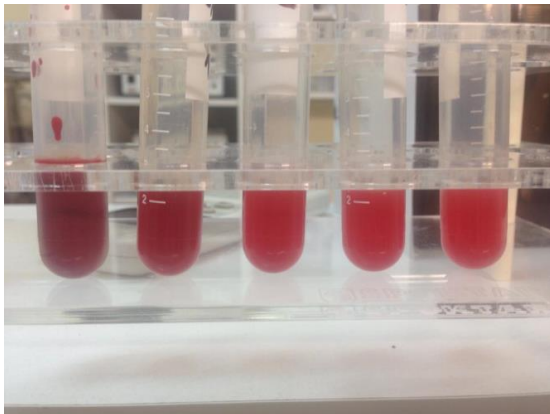
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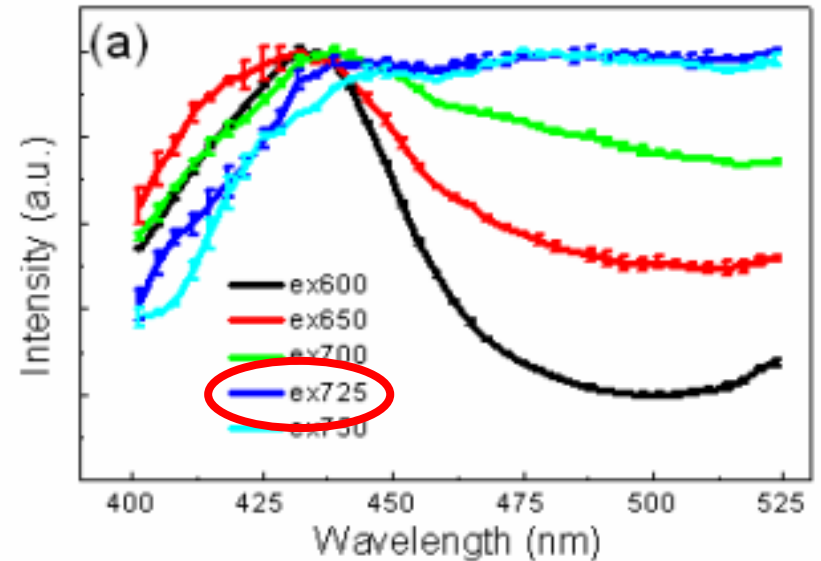
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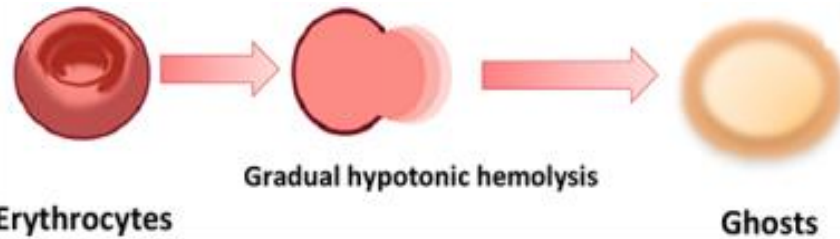
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Two photon excitation spectra

hemoglobin

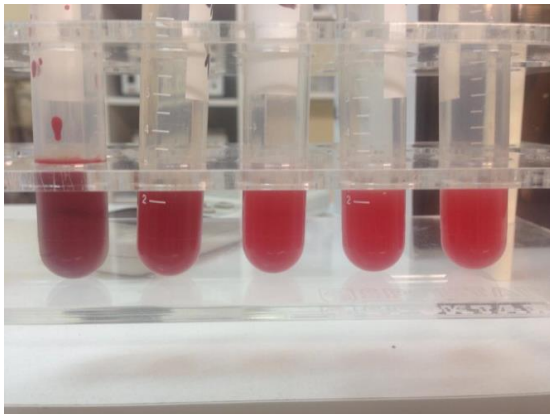


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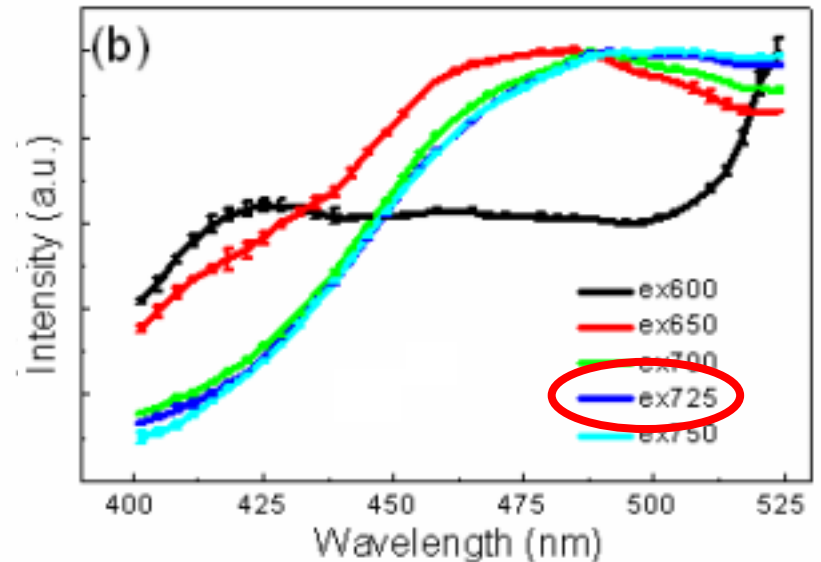
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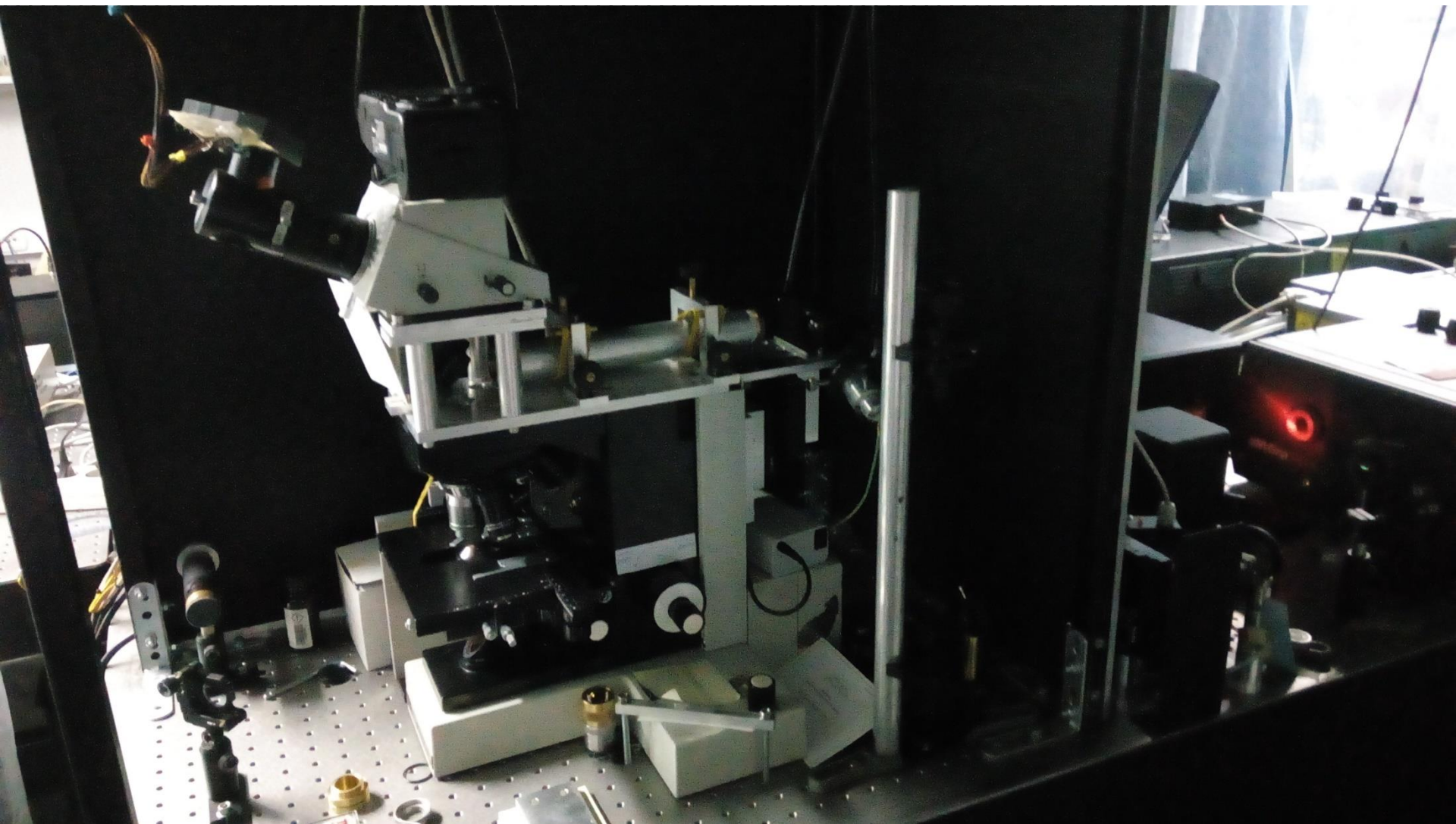
Strong two photon absorption in red-NIR
Soret (B) fluorescence band in VIS (peak@438nm)

Two photon excitation spectra

methemoglobin

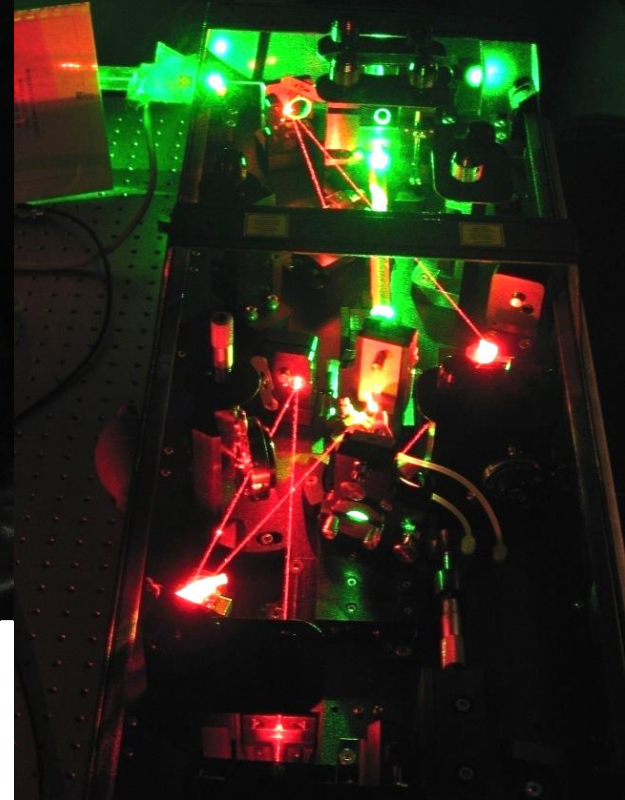
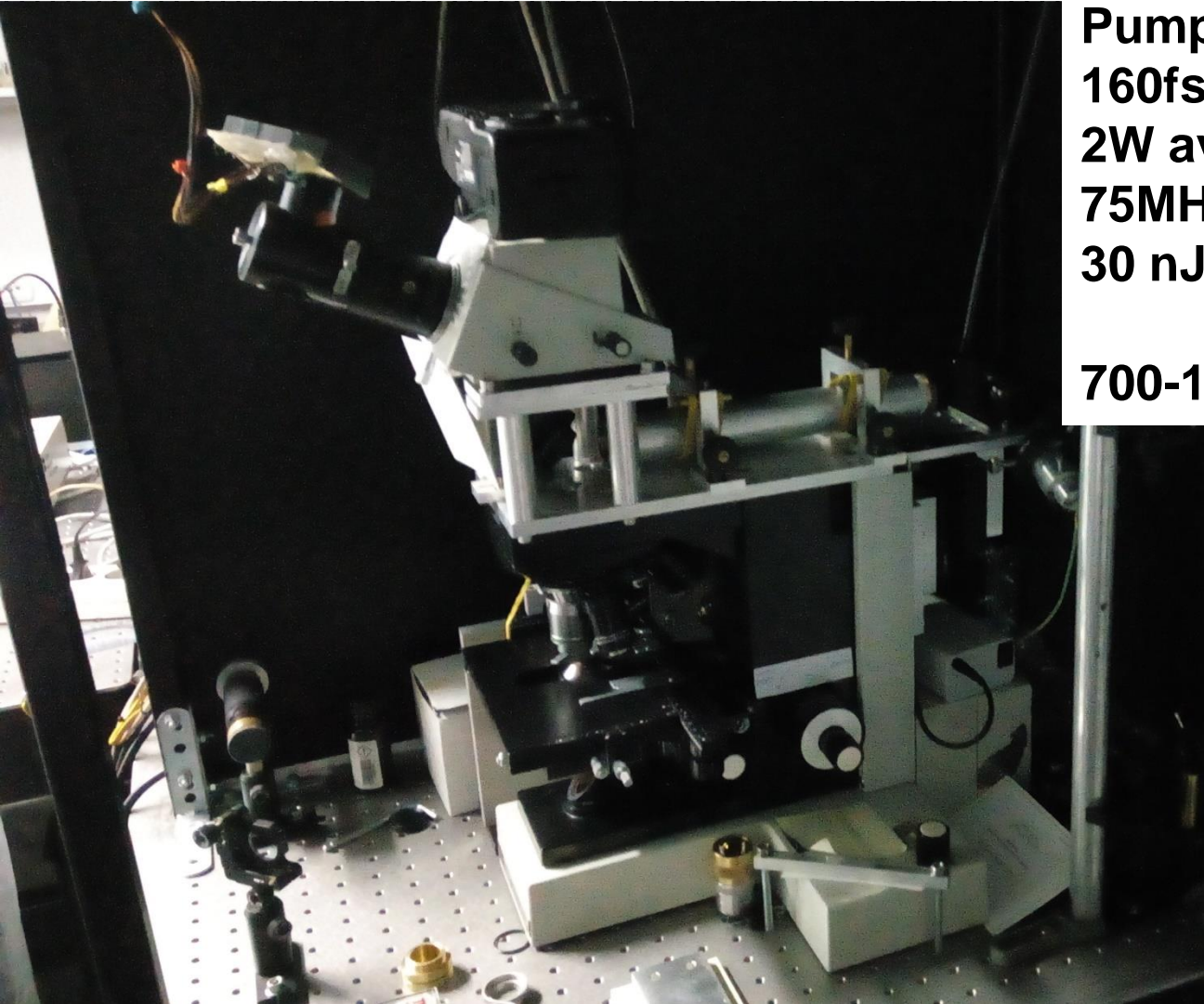


EXPERIMENTAL SETUP

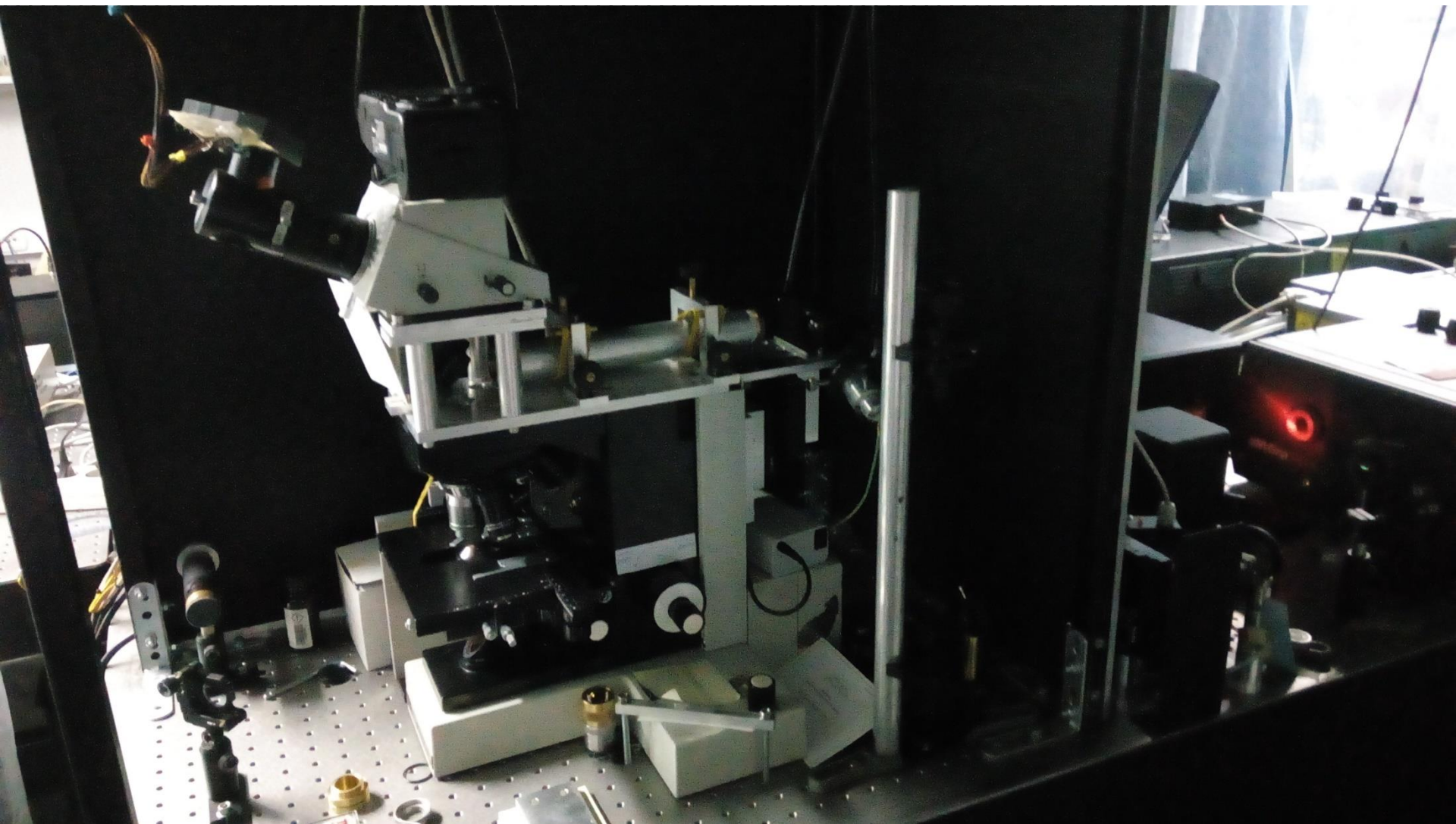


EXPERIMENTAL SETUP

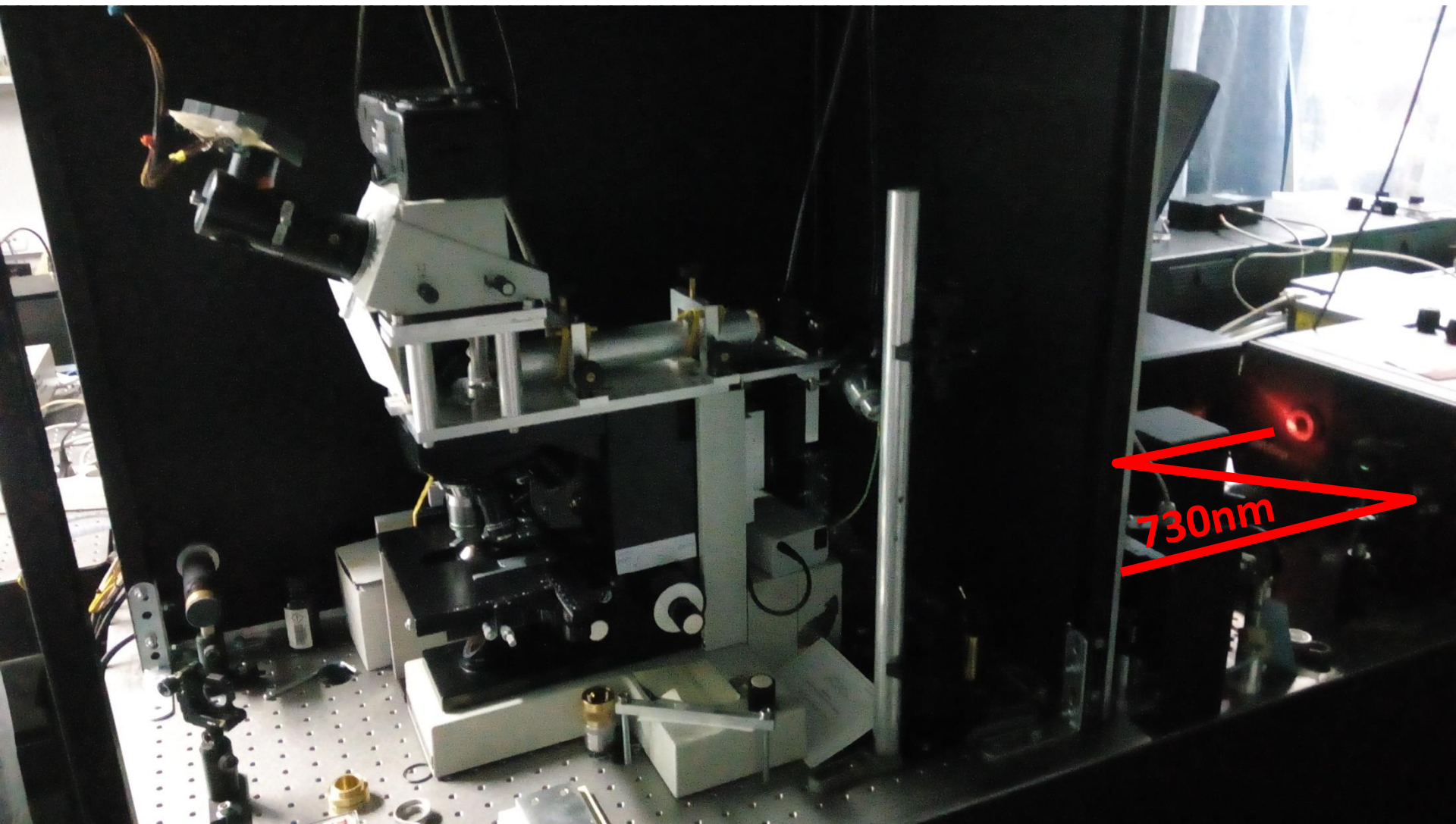
MIRA 900, Coherent Inc.
Ti:Sa Kerr lens mode locked
Pump 10W CW @532nm
160fs pulse duration
2W average power
75MHz repetition rate
30 nJ per pulse
(0.2MW peak power)
700-1000 nm



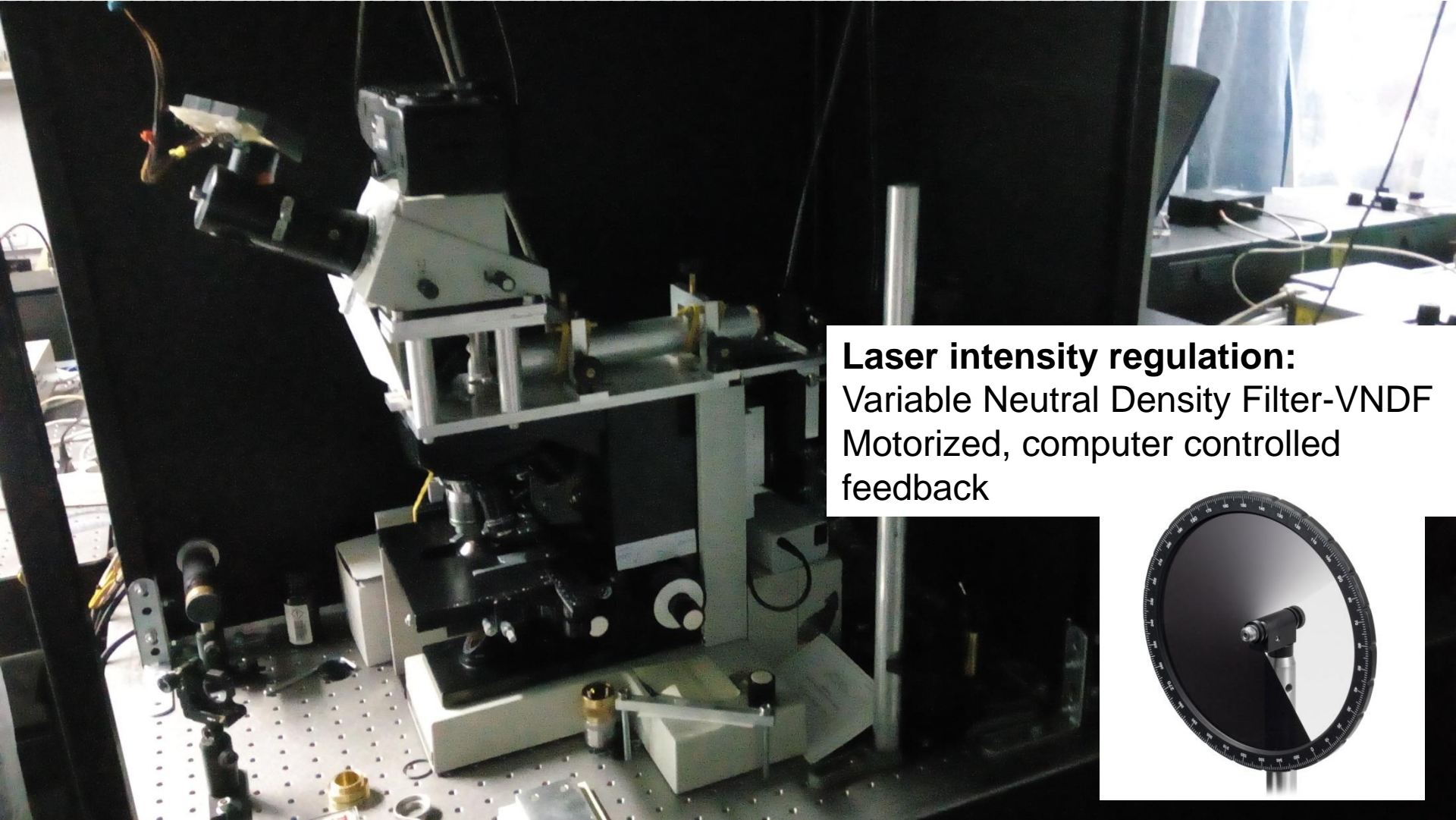
EXPERIMENTAL SETUP



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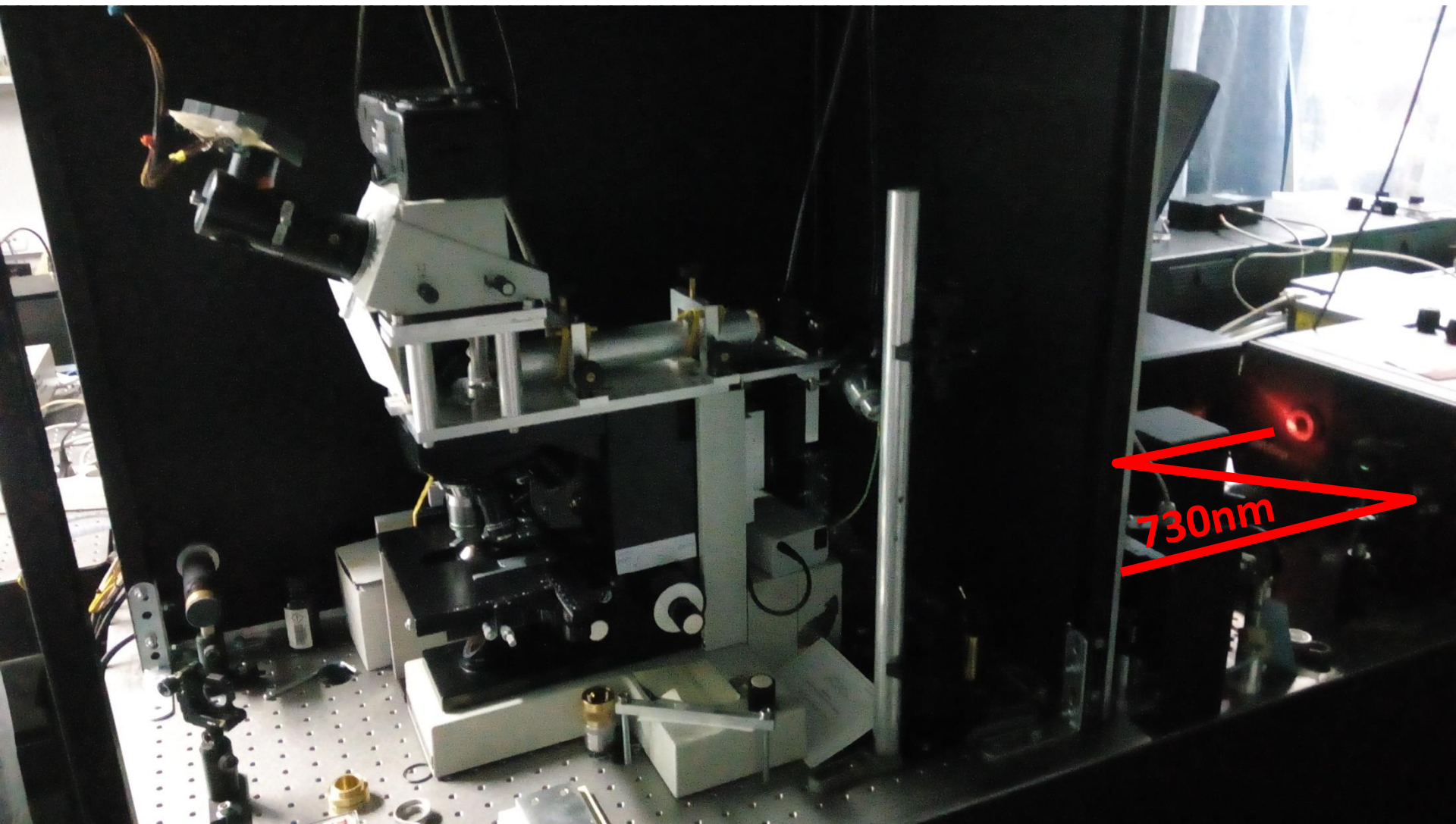
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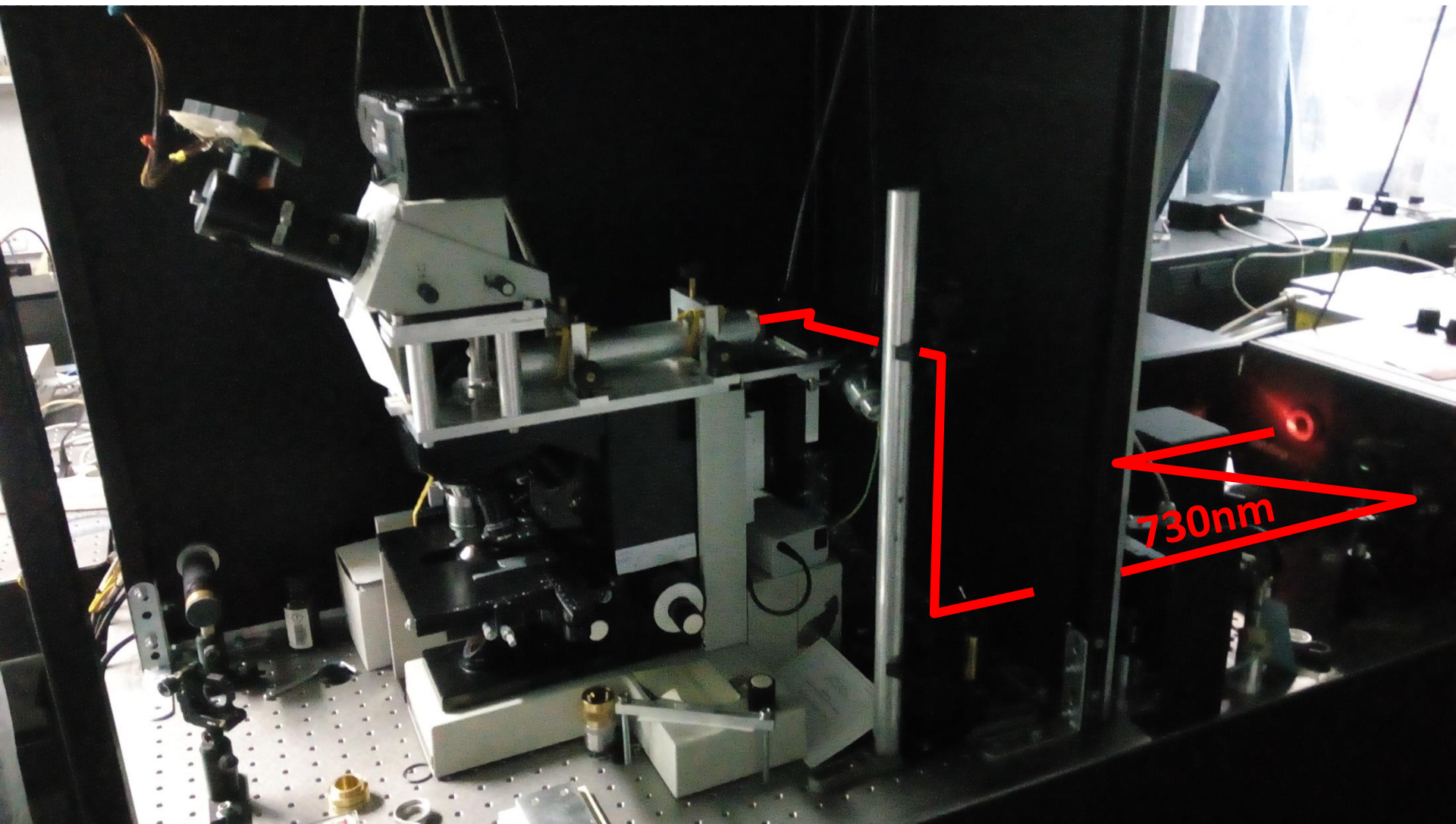
Laser intensity regulation:
Variable Neutral Density Filter-VNDF
Motorized, computer controlled
feedback



EXPERIMENTAL SETUP

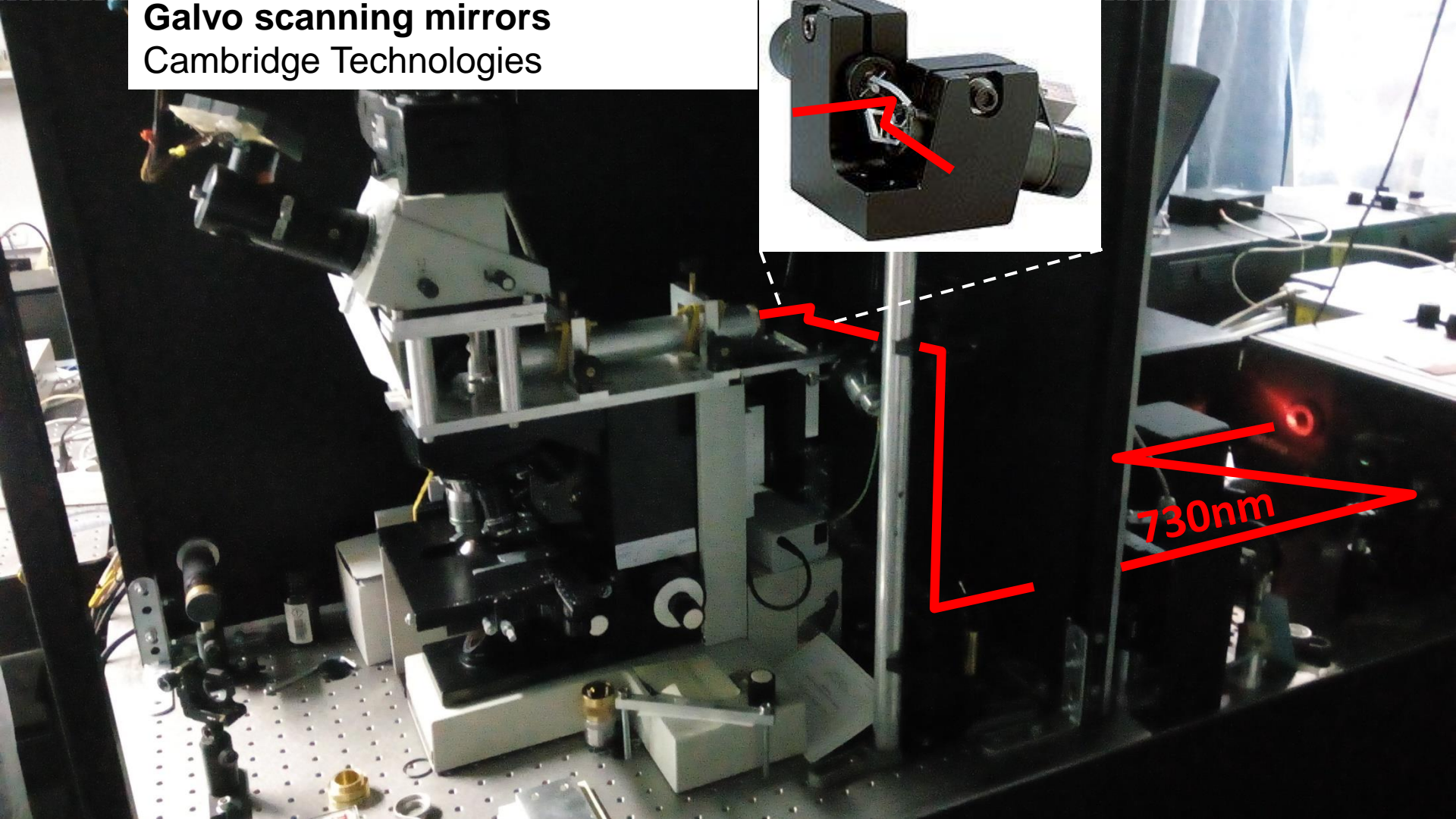


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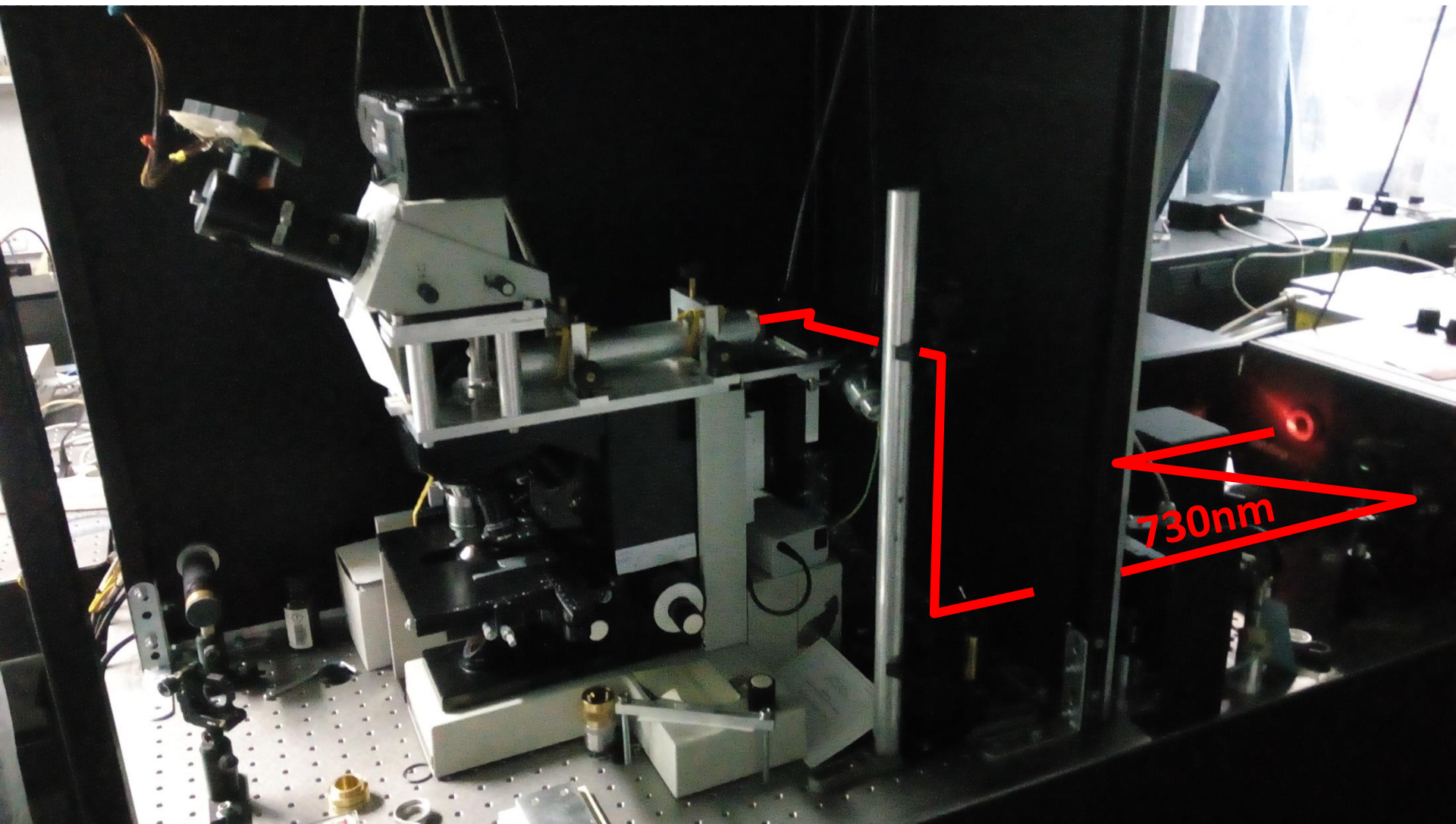


EXPERIMENTAL SETUP

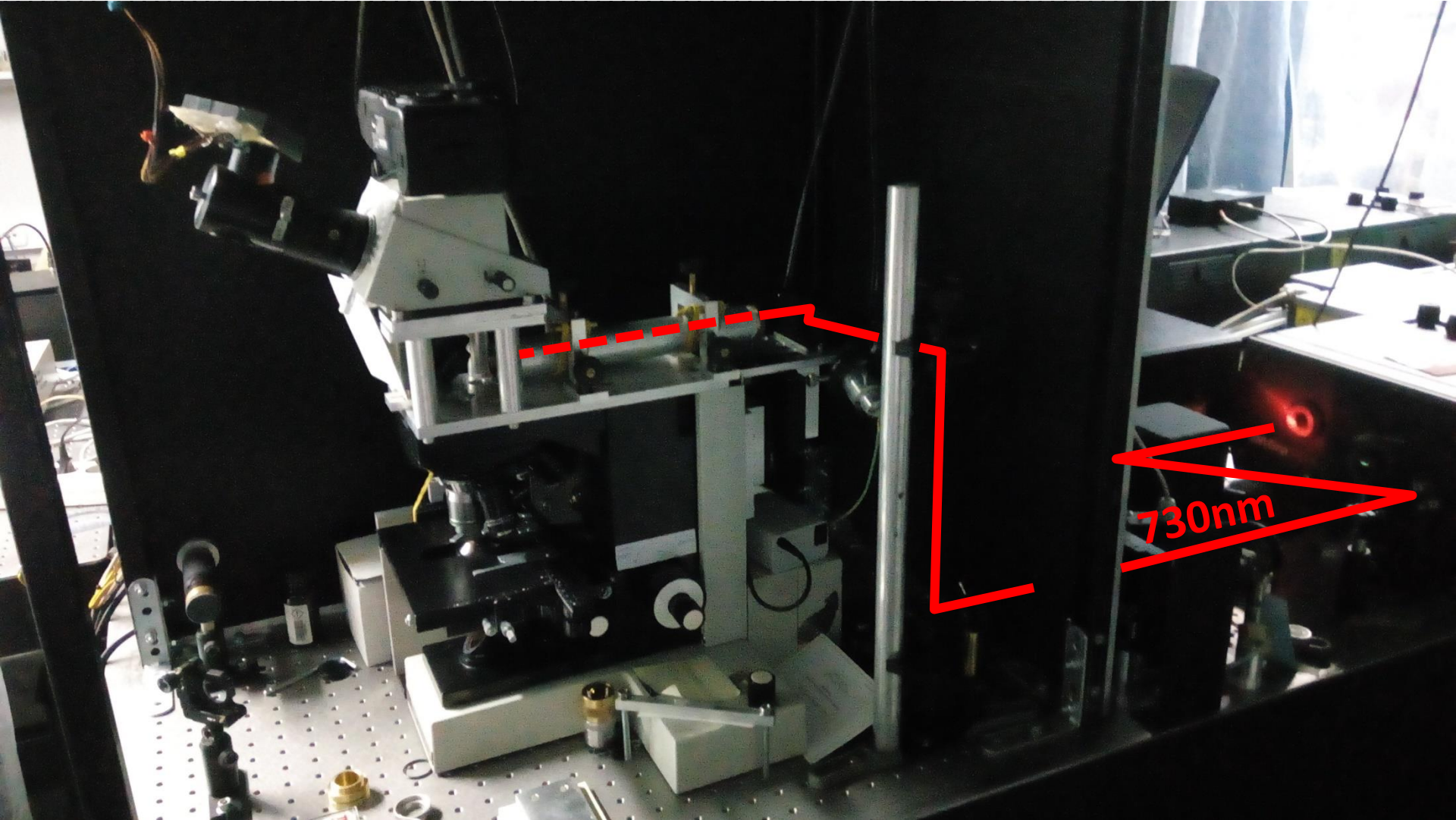
Galvo scanning mirrors
Cambridge Technologies



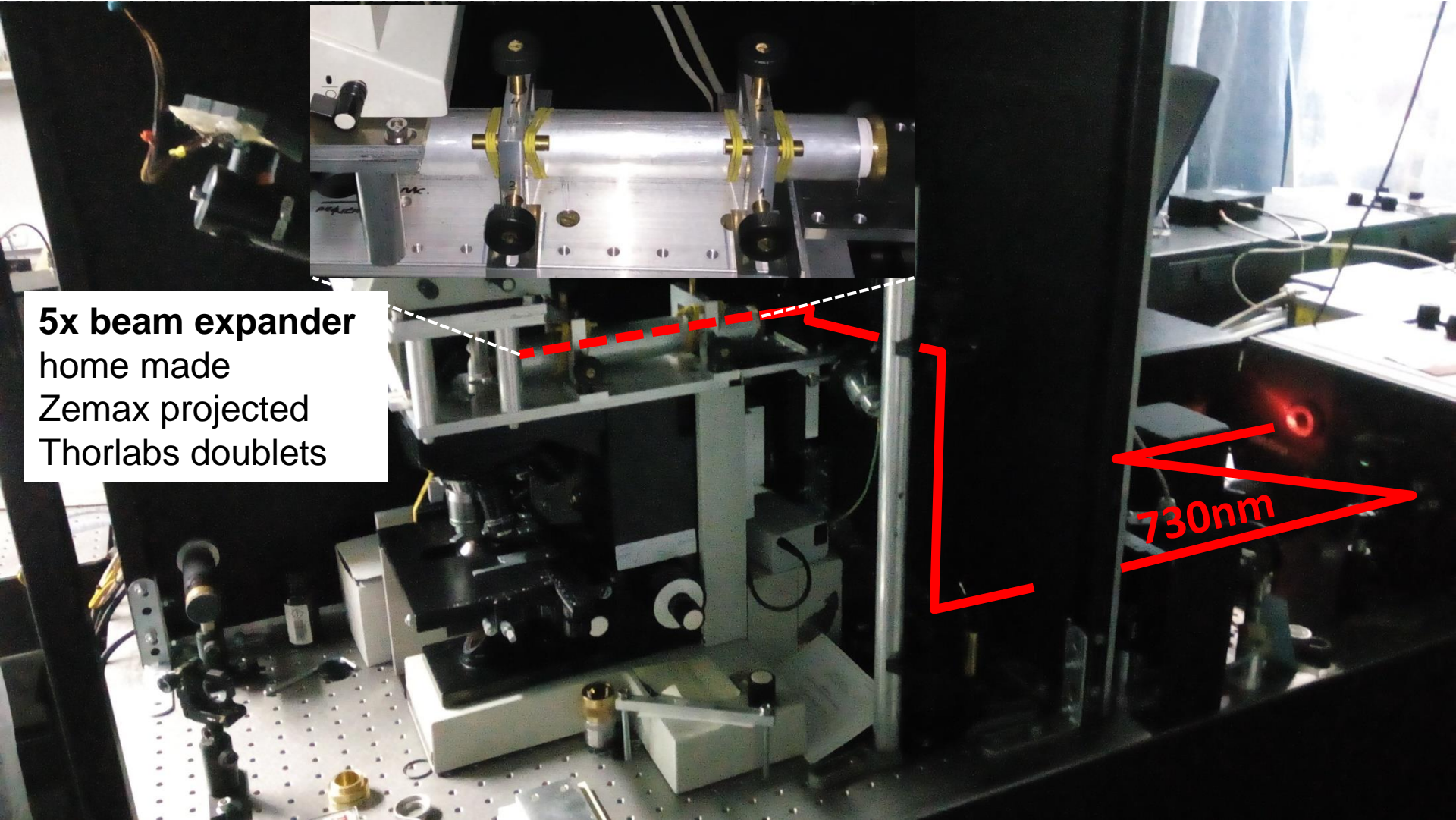
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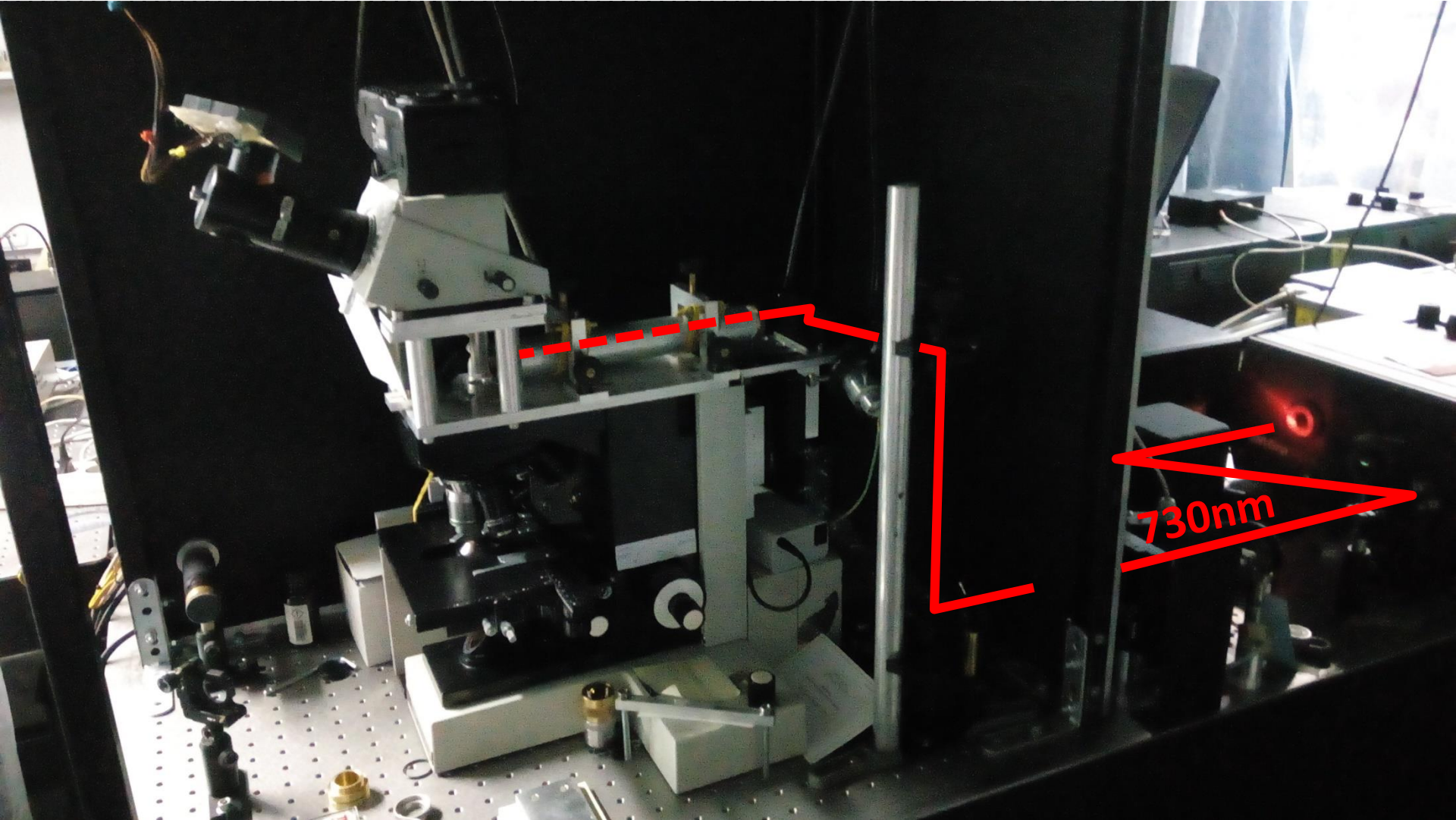
EXPERIMENTAL SETUP



5x beam expander
home made
Zemax projected
Thorlabs doublets

730nm

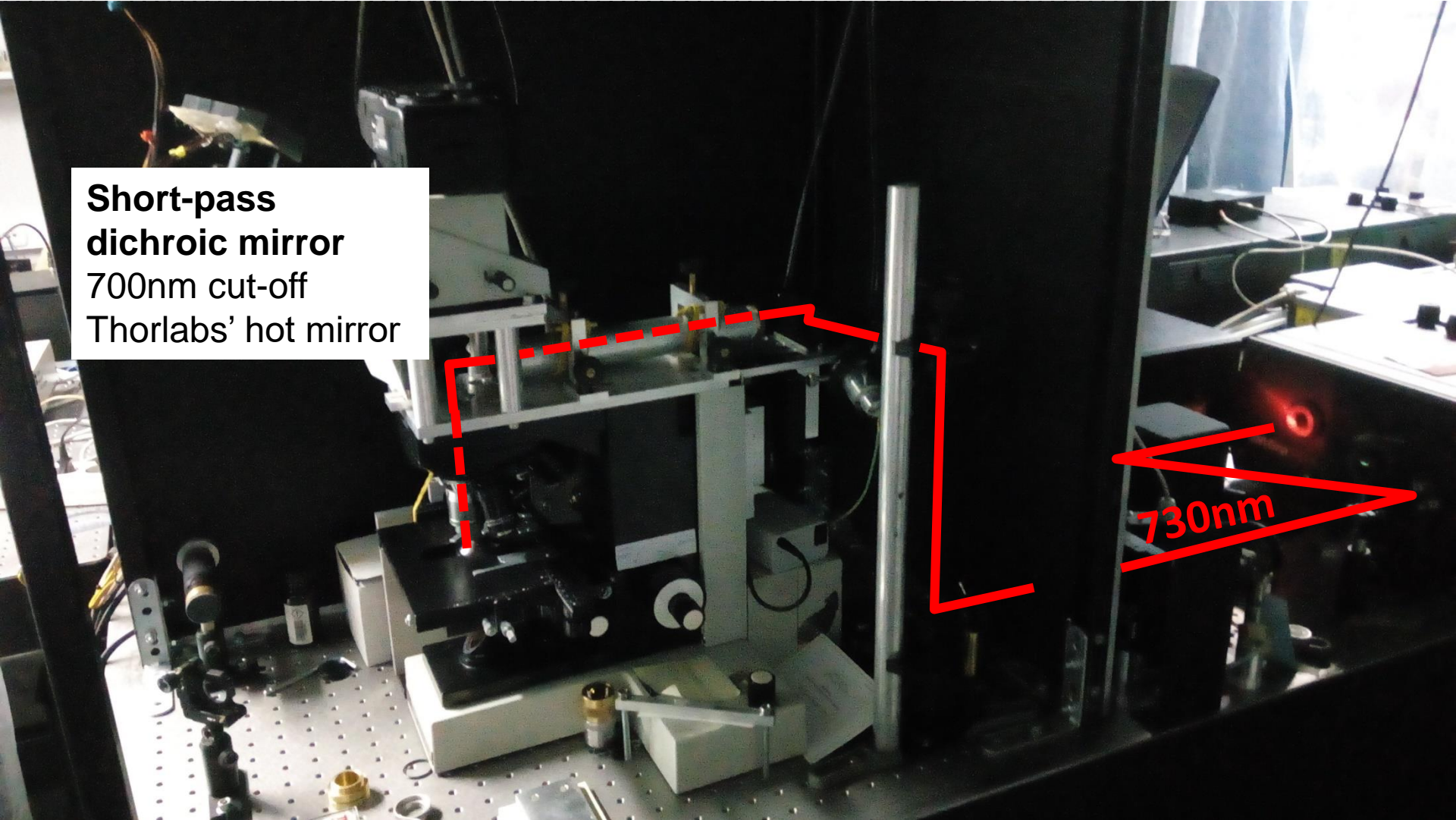
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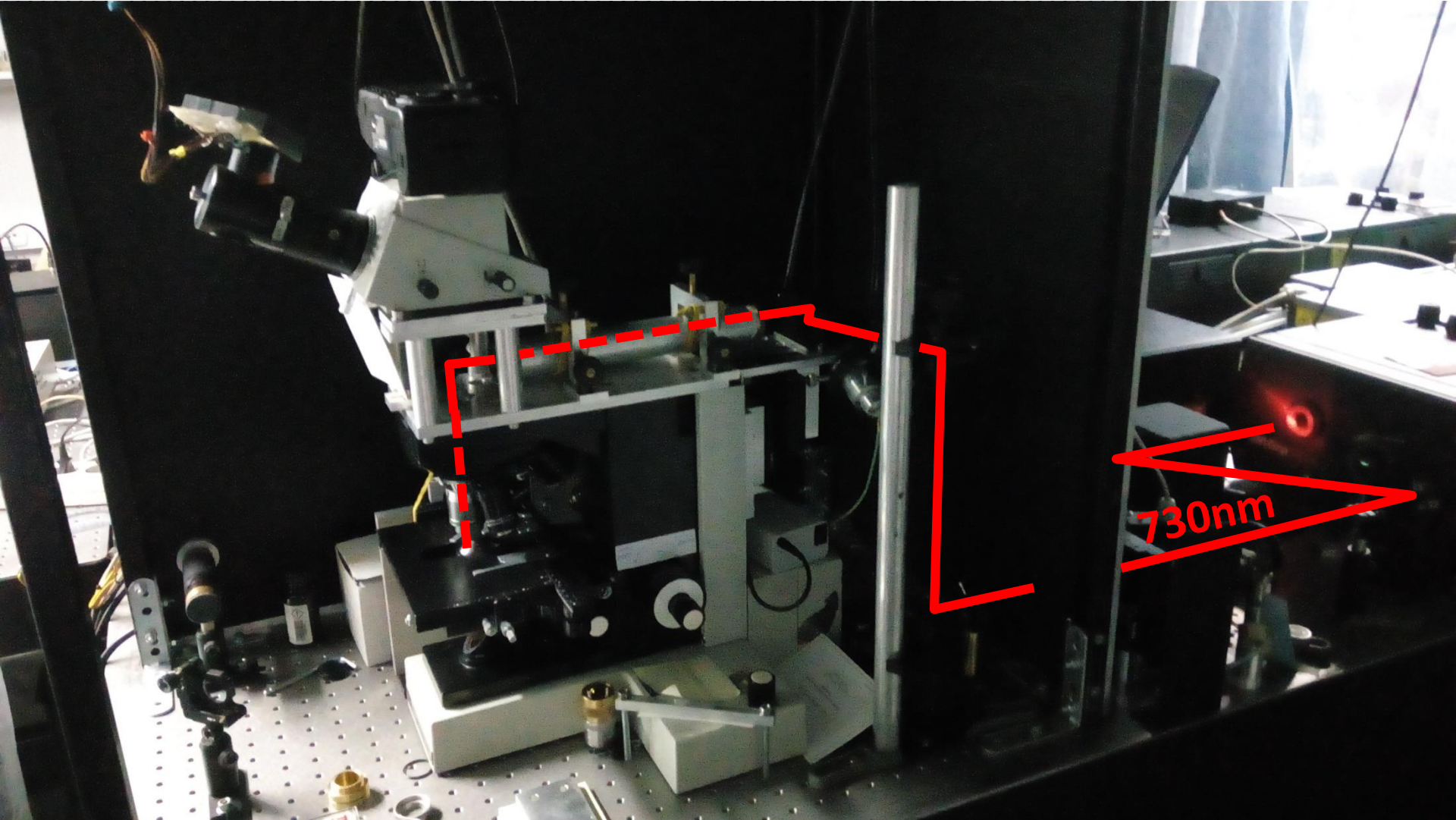
EXPERIMENTAL SETUP

**Short-pass
dichroic mirror**
700nm cut-off
Thorlabs' hot mirror

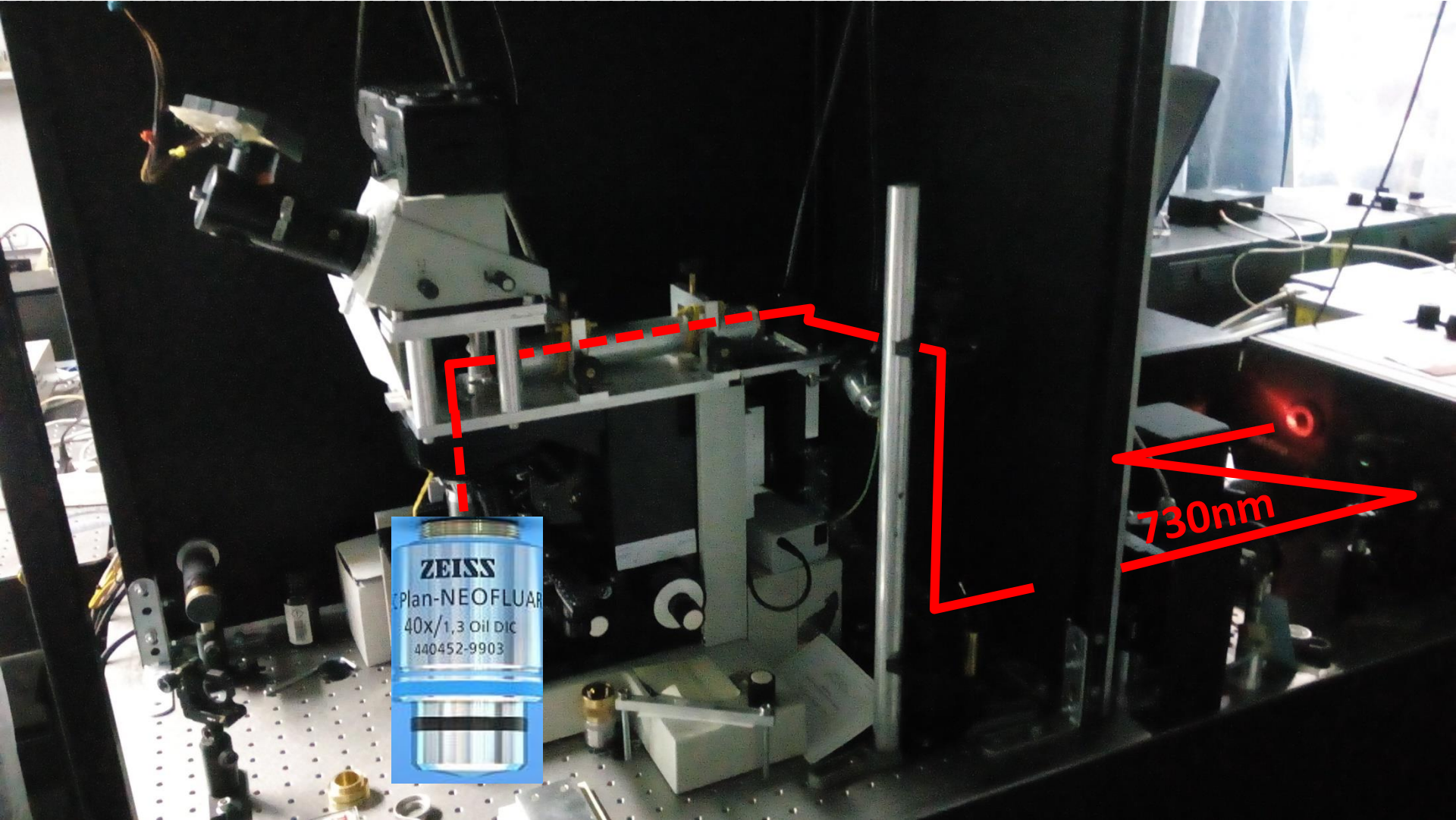
730nm



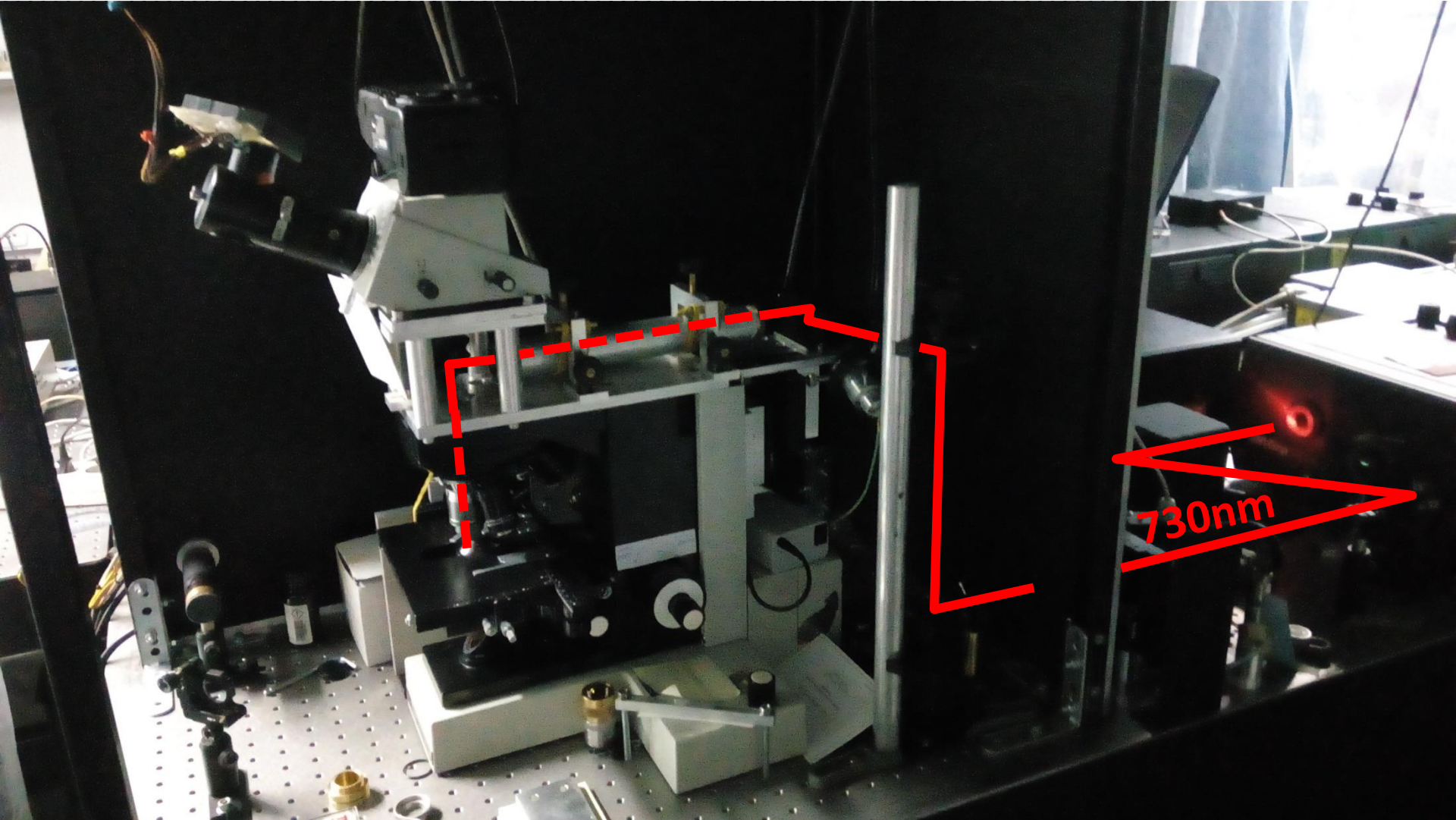
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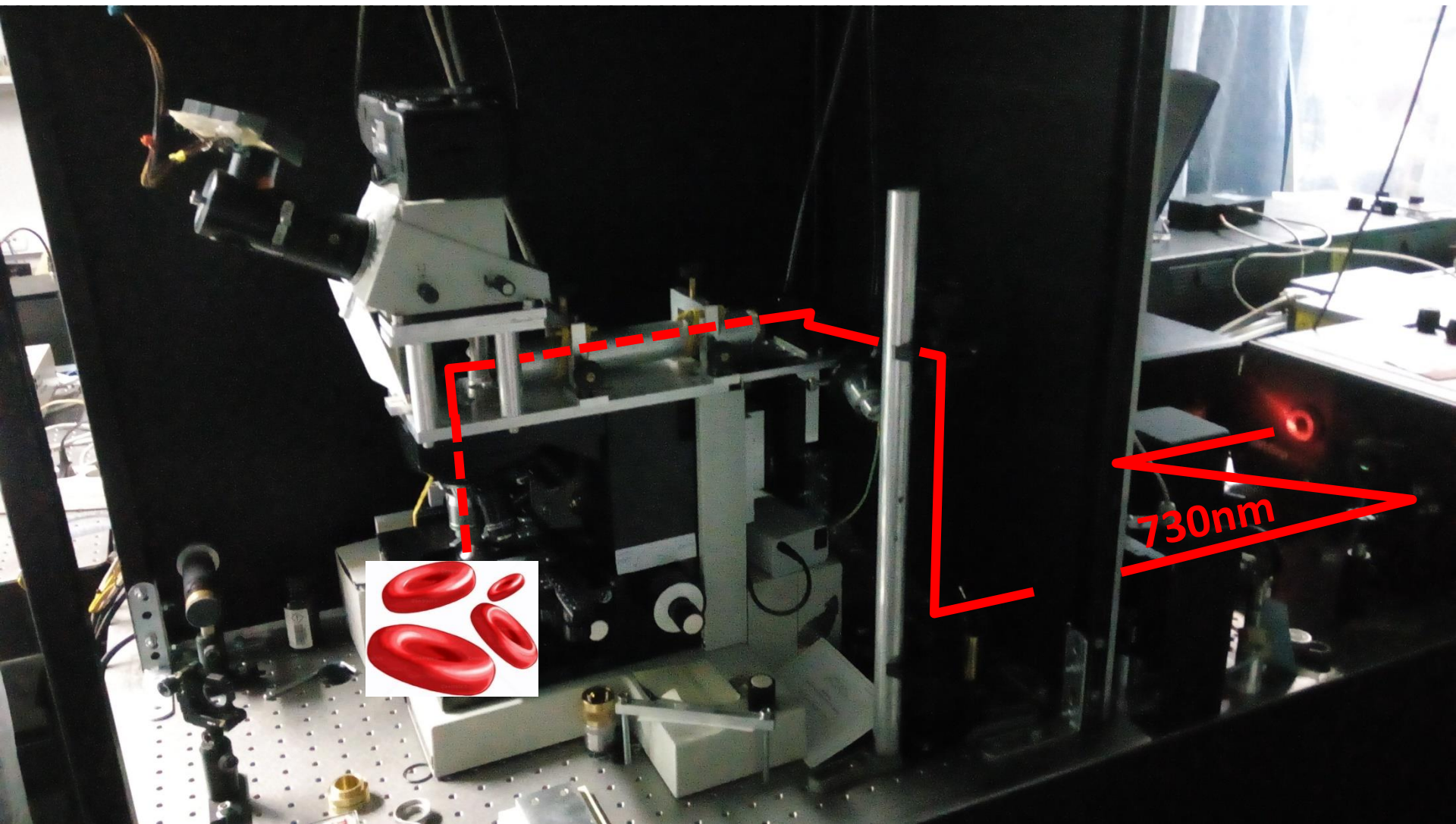
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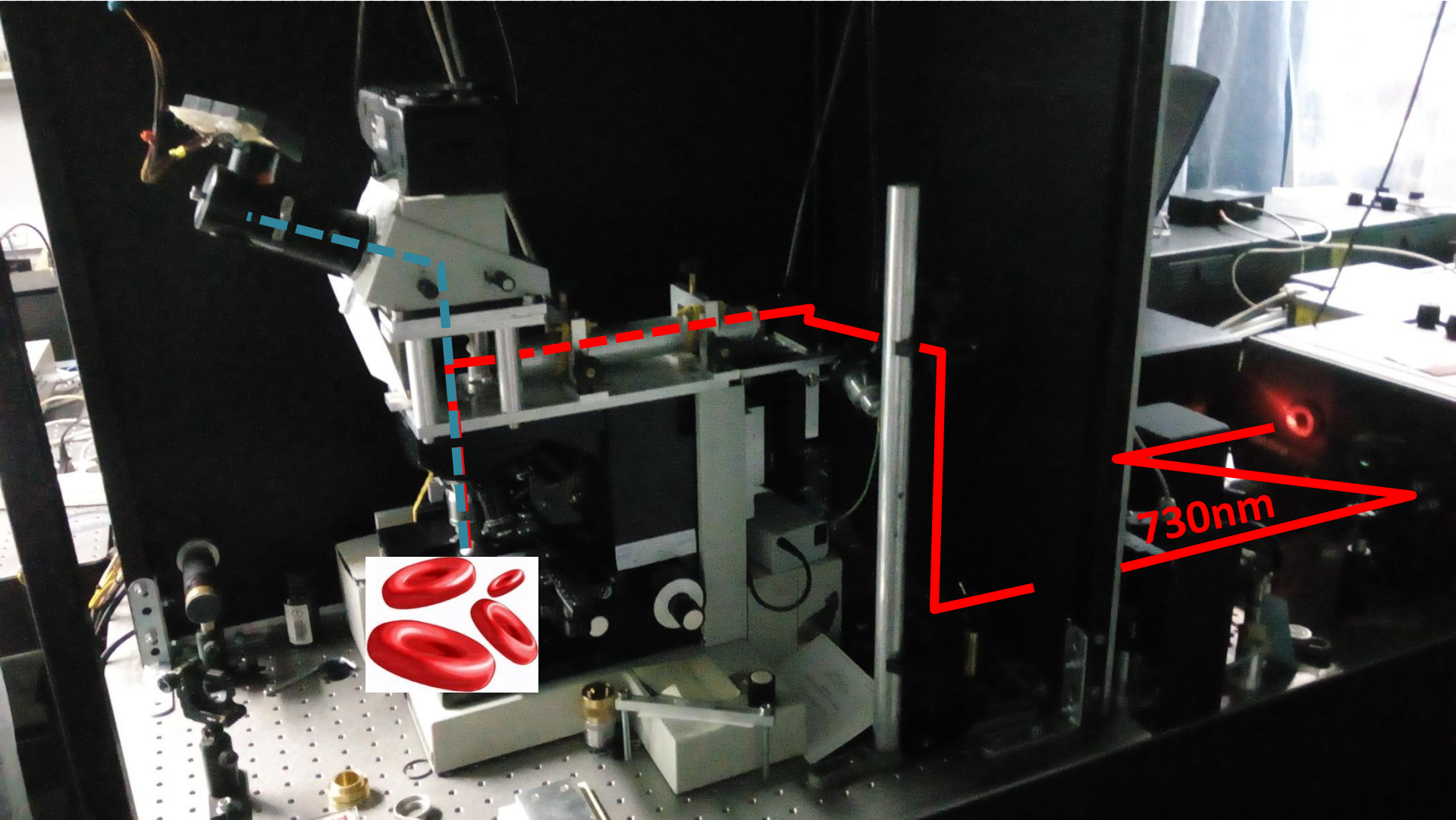
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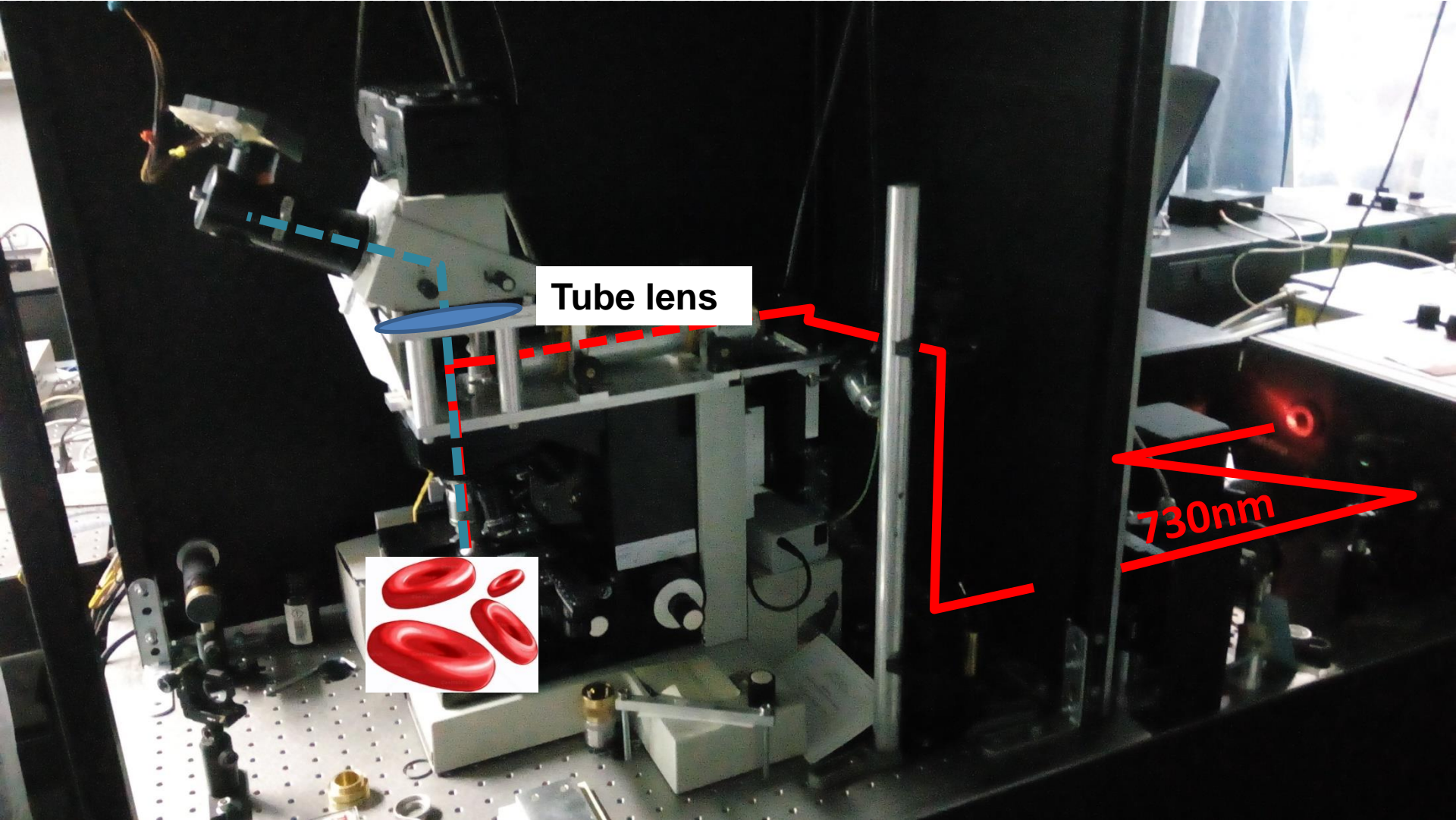
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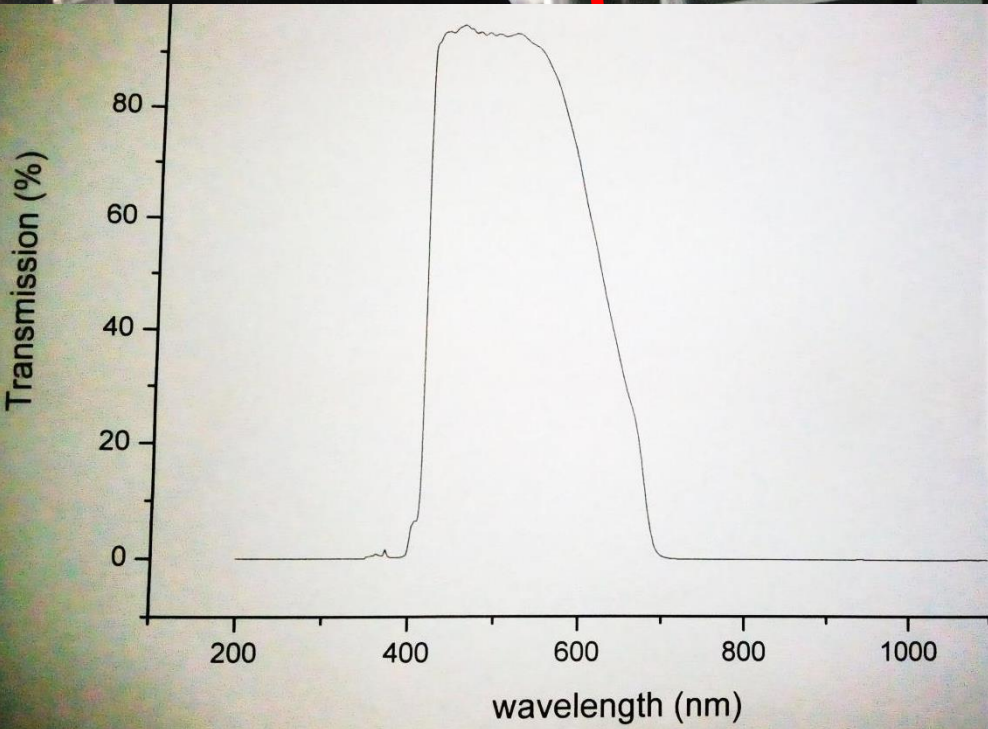
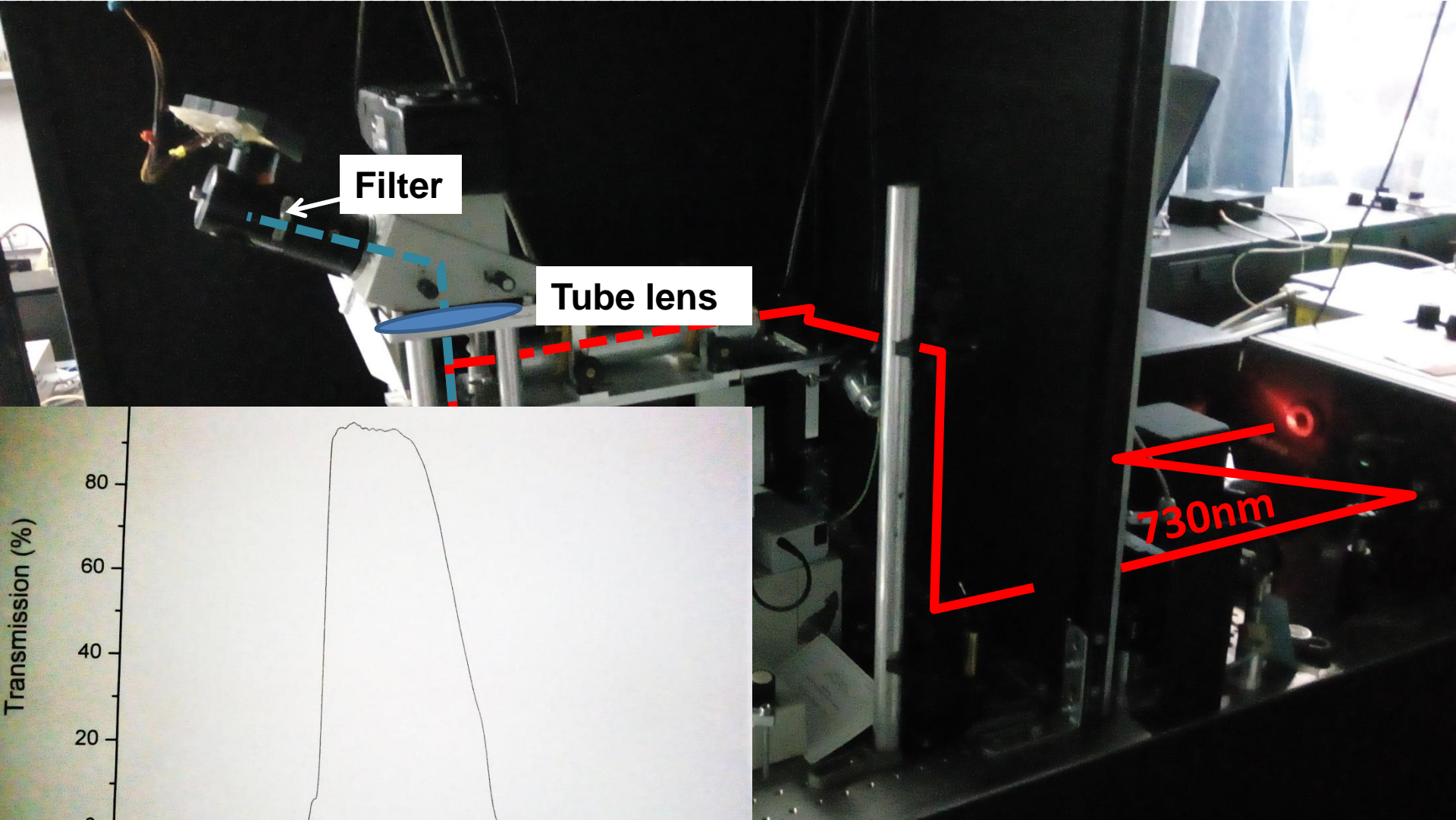
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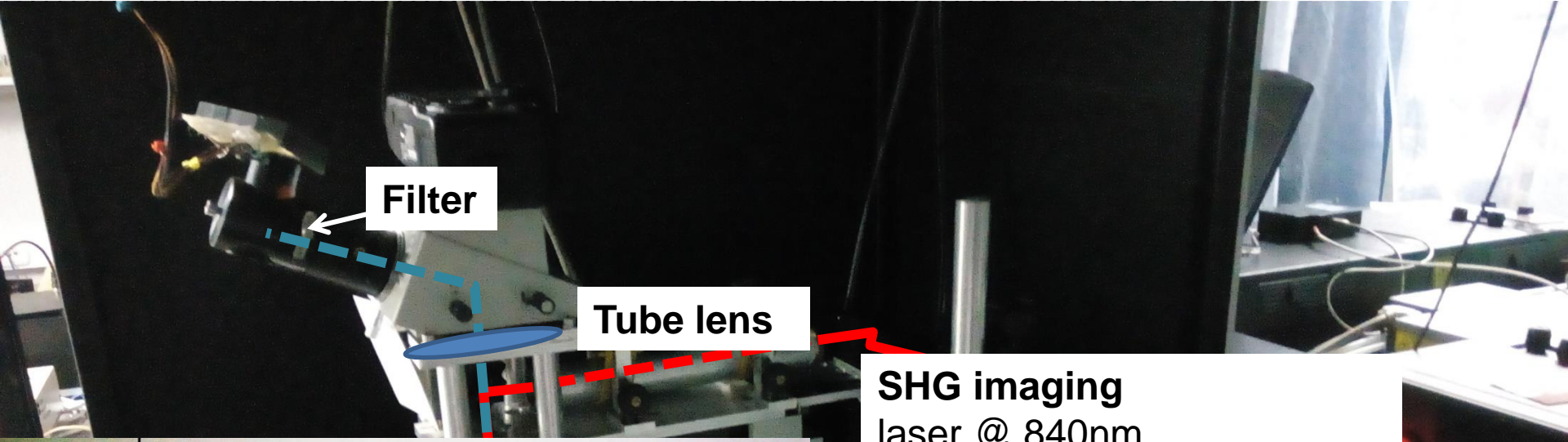
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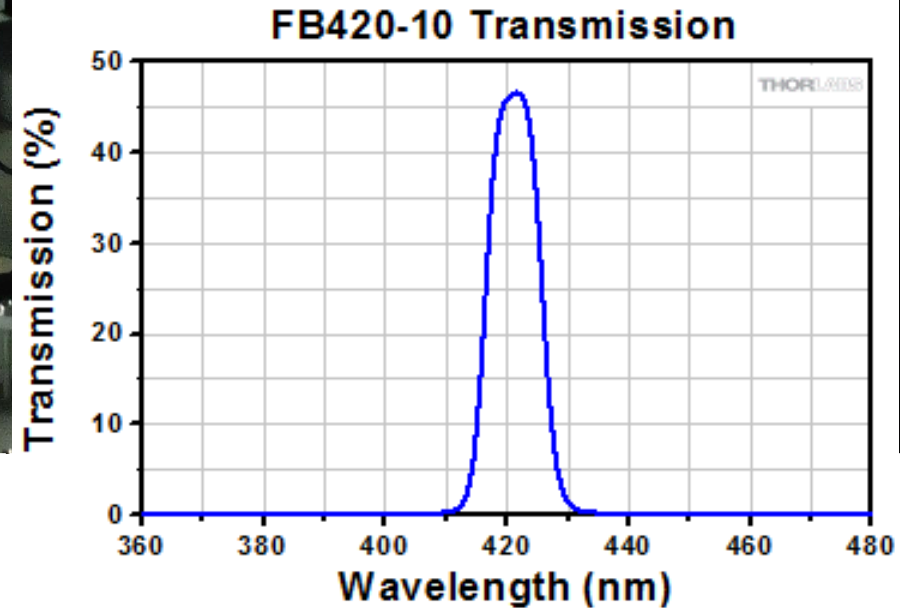
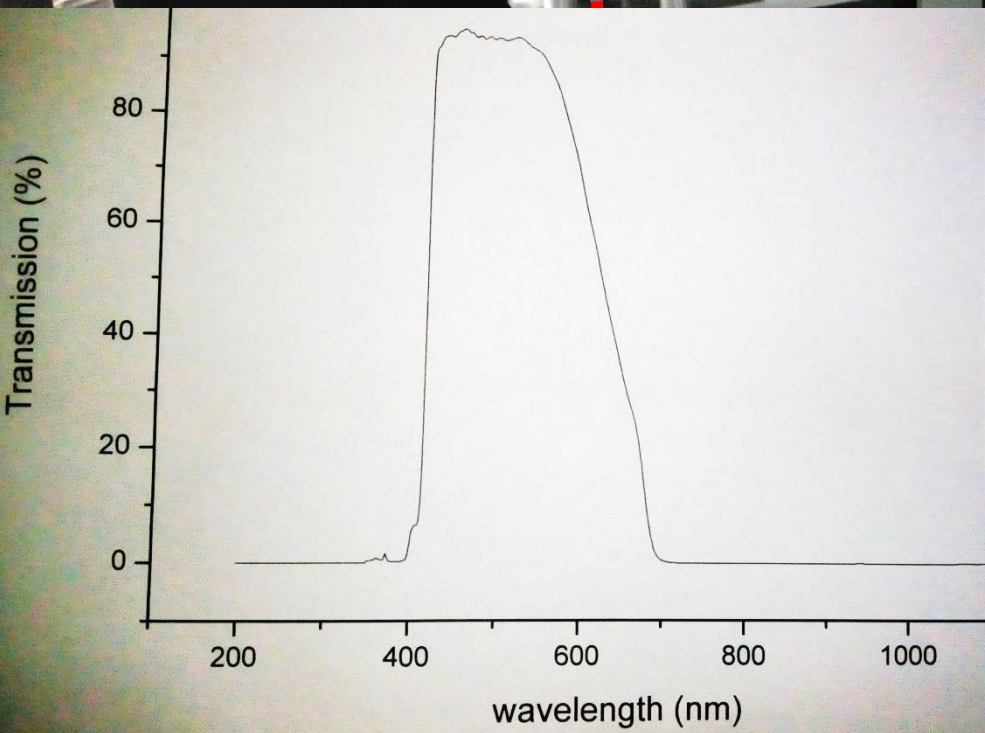
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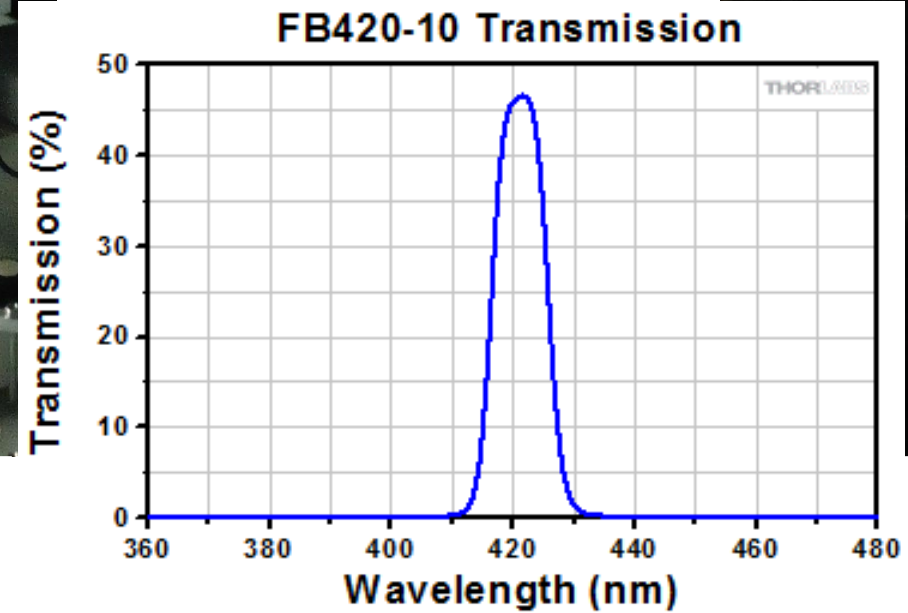
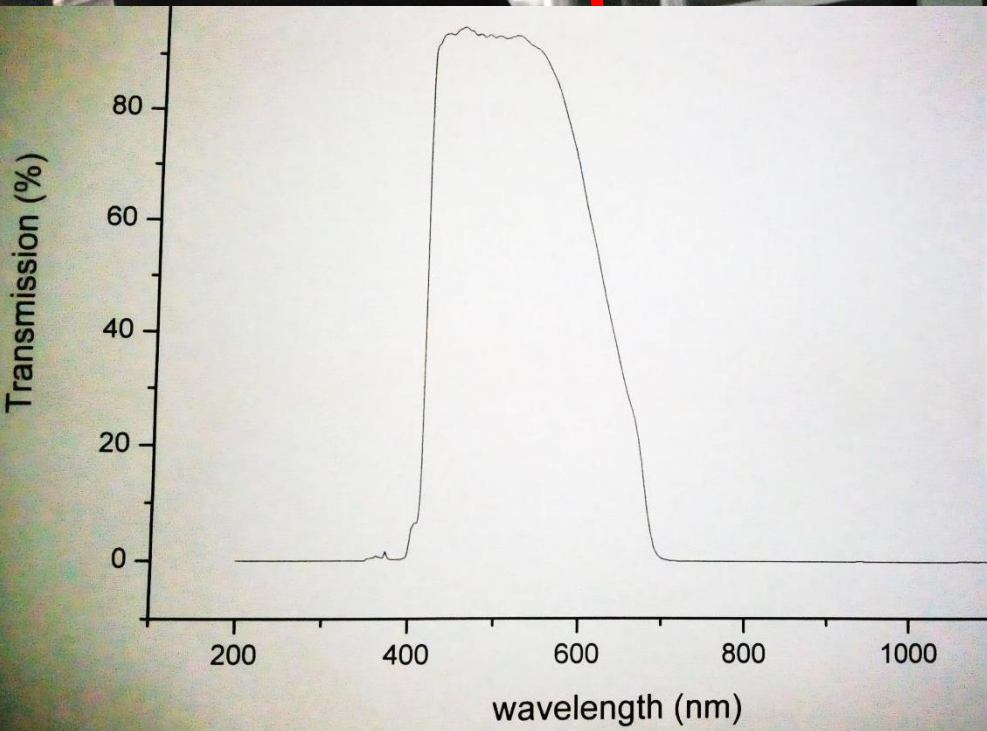
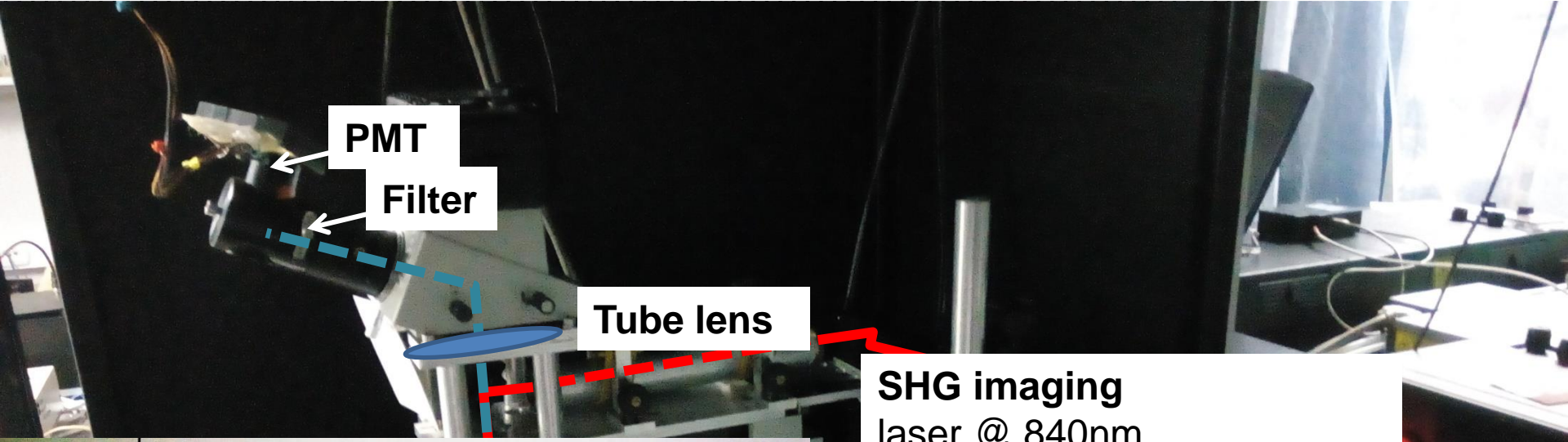
SHG imaging

laser @ 840nm

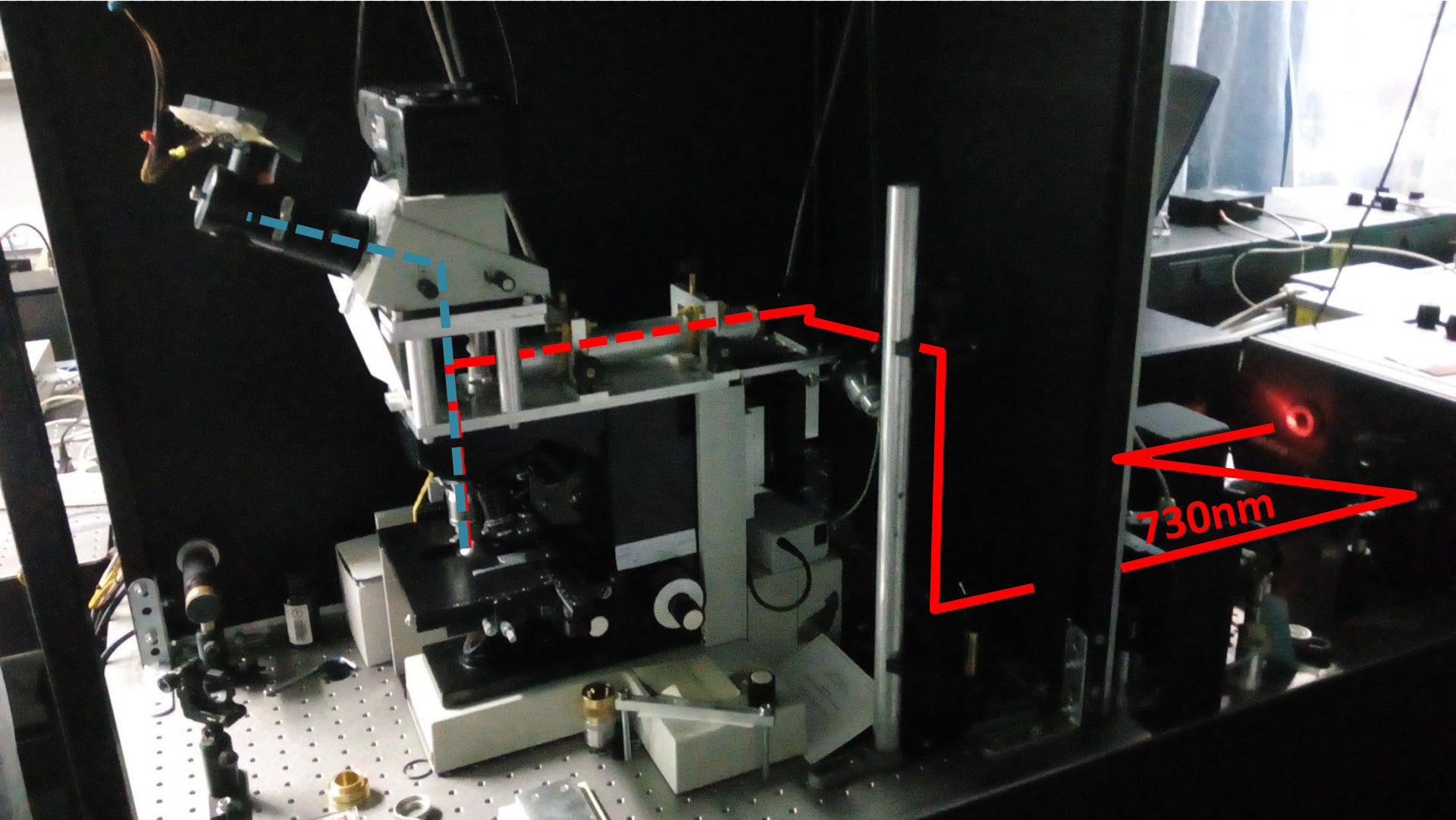
narrow band filter @420nm



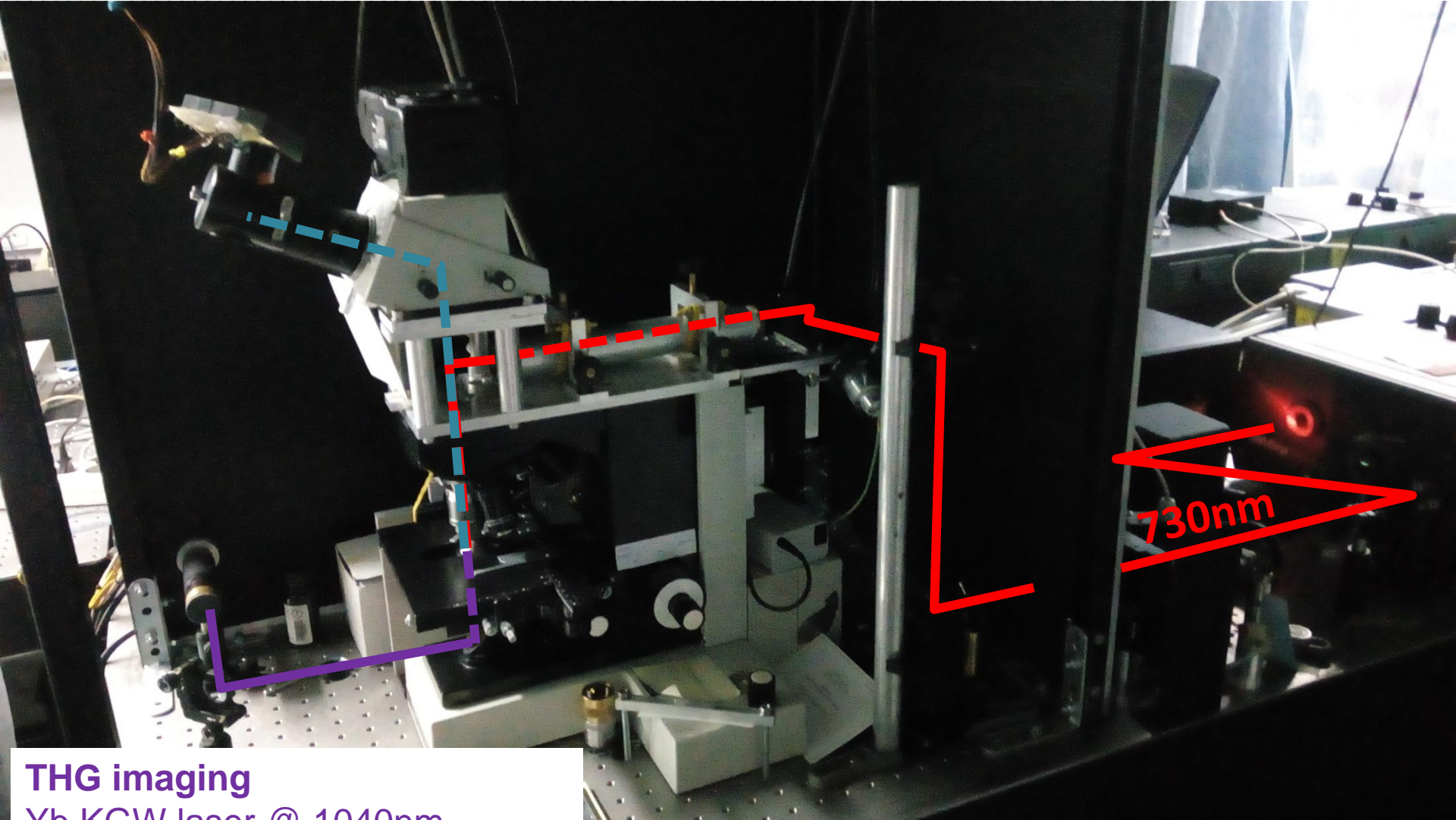
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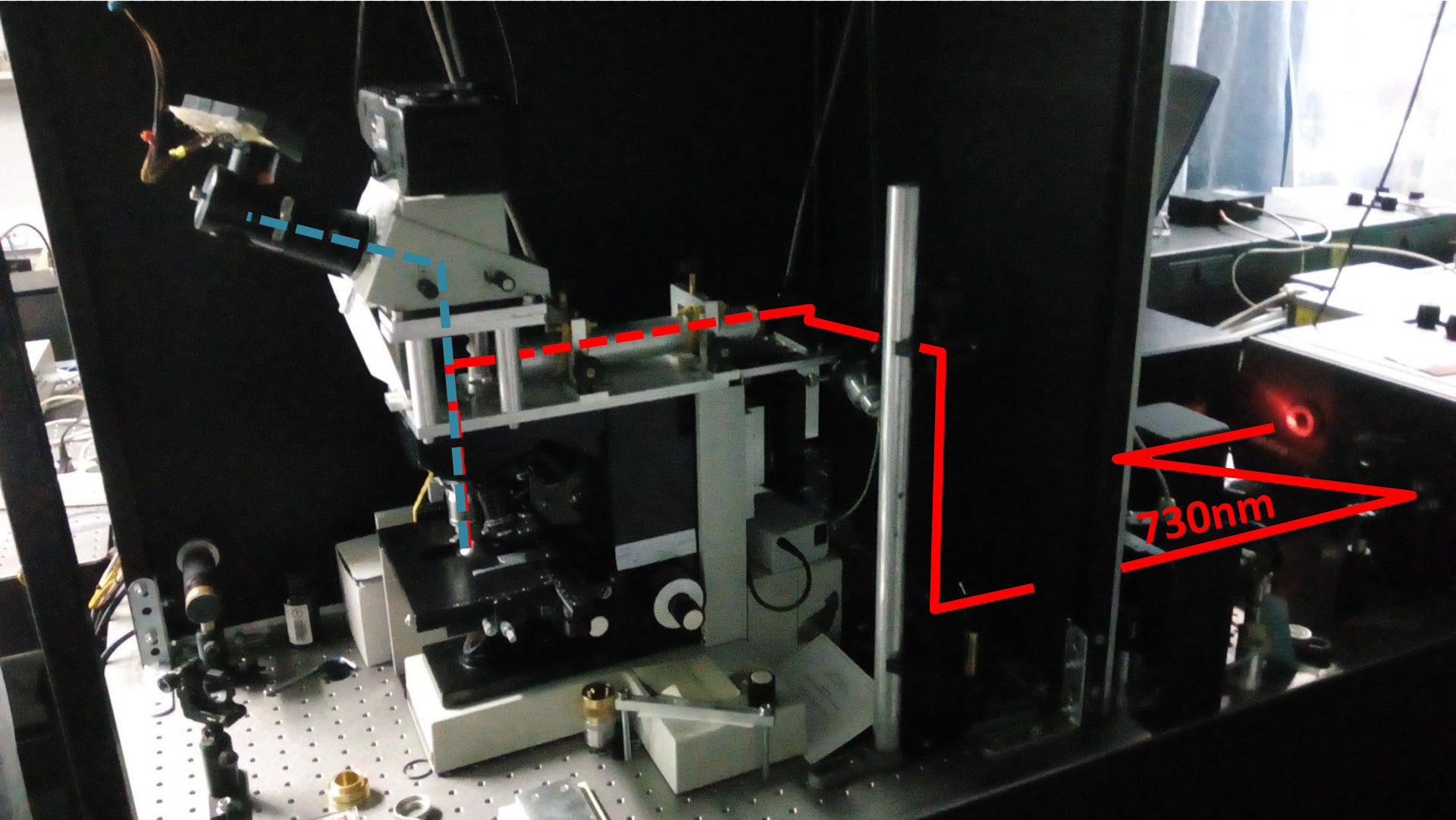
THG imaging

Yb KGW laser @ 1040nm

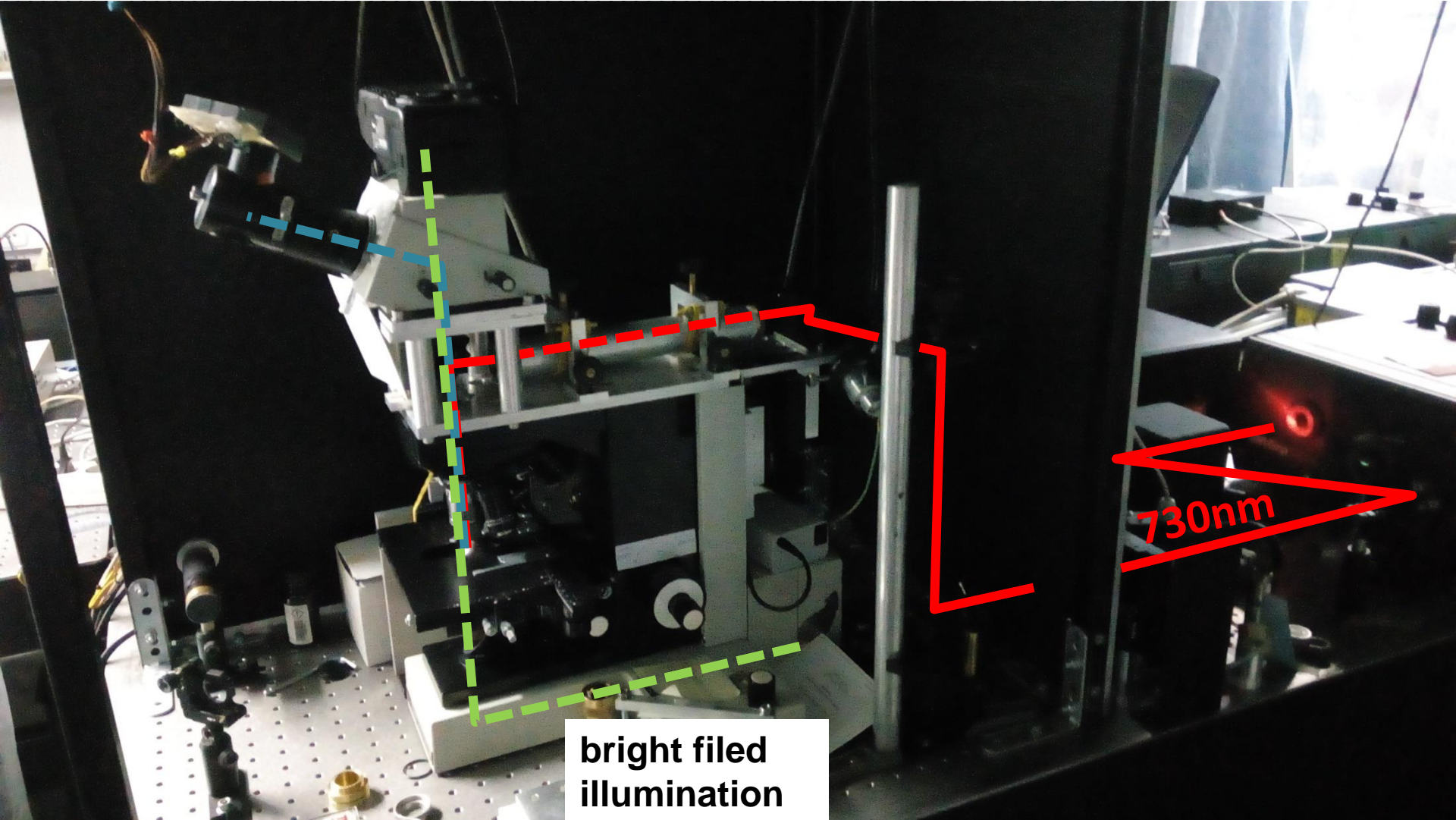
UV filter @ 347nm

single photon PMT (Hamamatsu)

EXPERIMENTAL SETUP



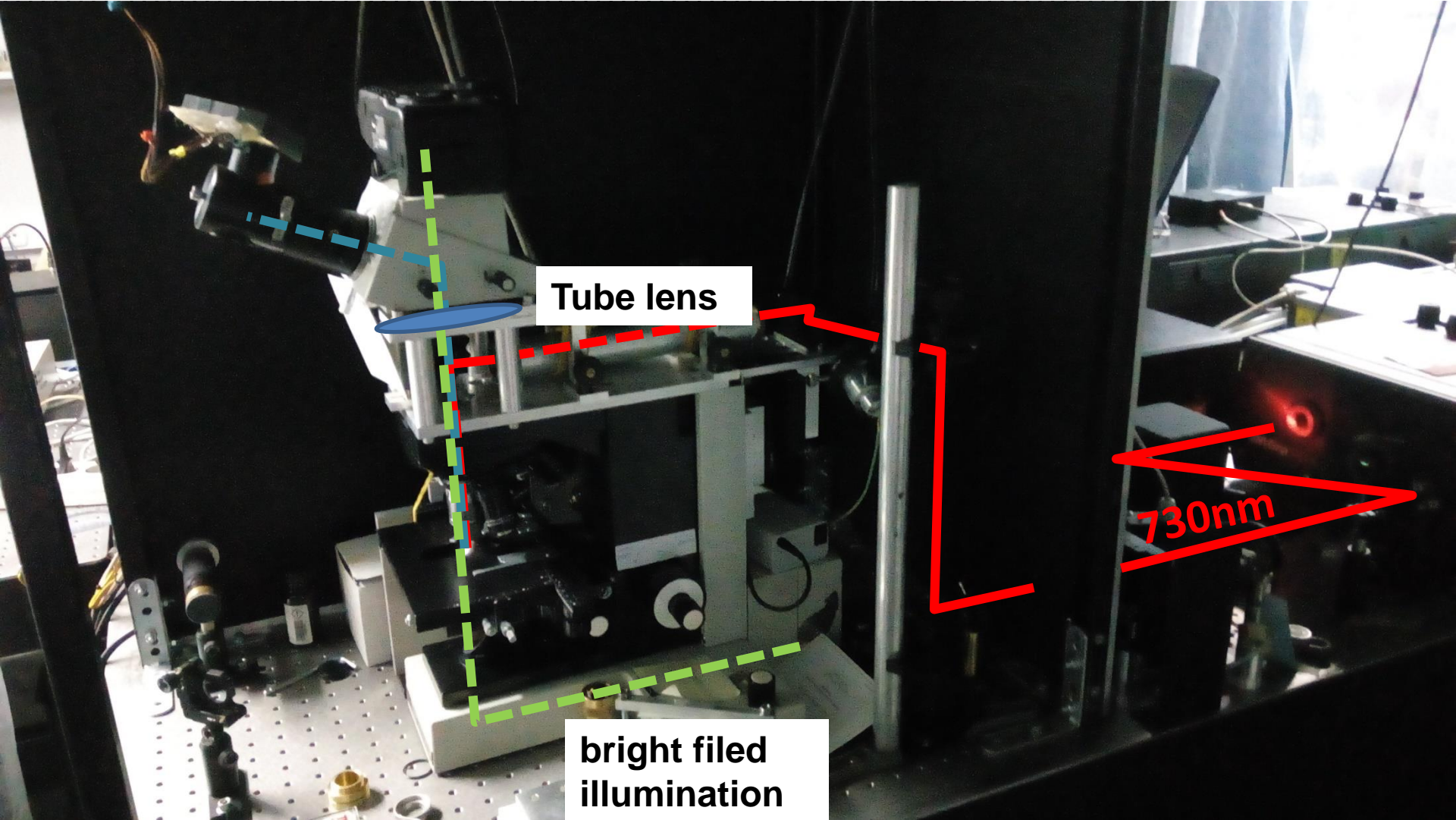
EXPERIMENTAL SETUP



**bright field
illumination**

730nm

EXPERIMENTAL SETUP



Tube lens

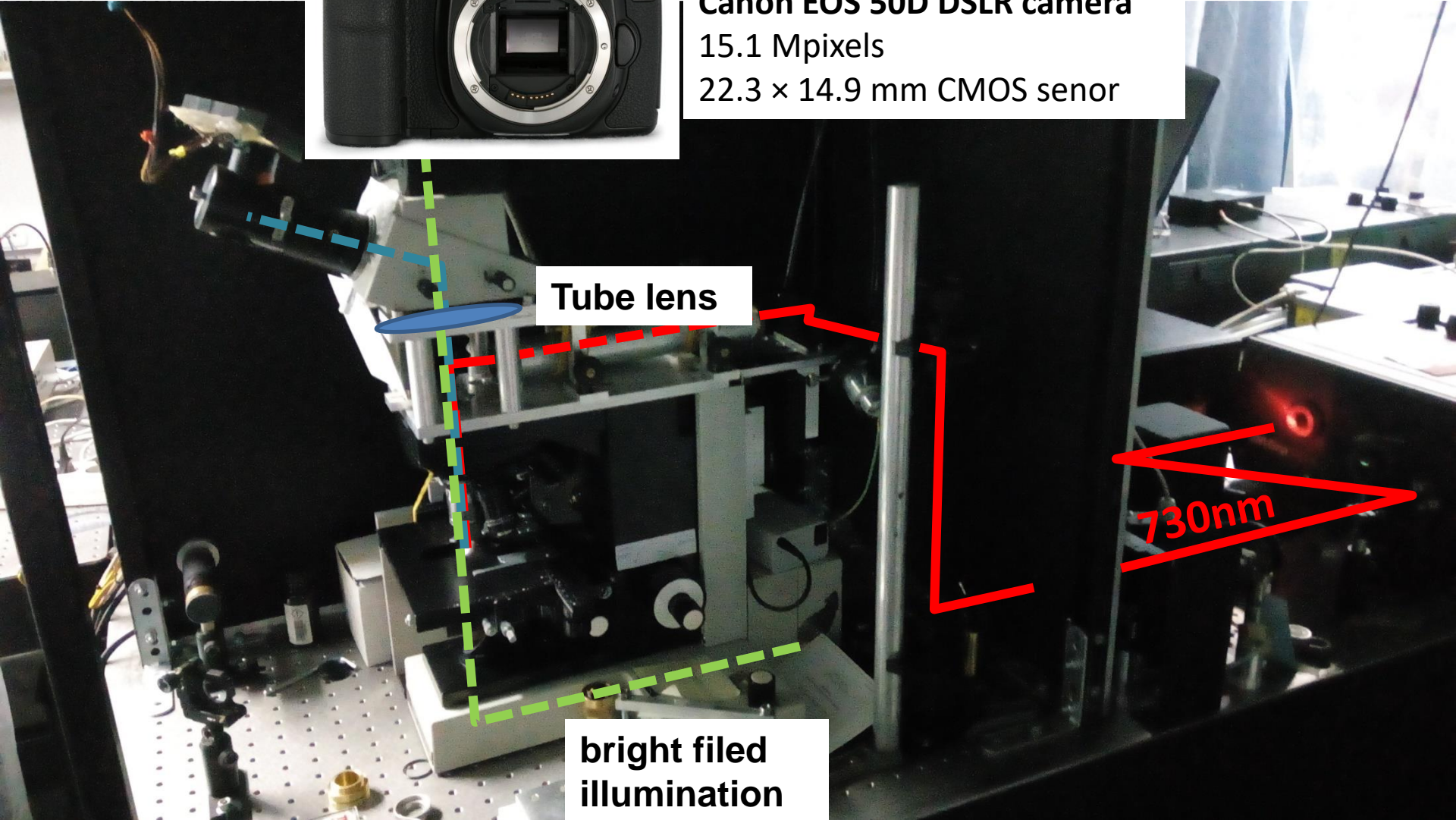
bright filed illumination

730nm

EXPERIMENT



Canon EOS 50D DSLR camera
15.1 Mpixels
22.3 × 14.9 mm CMOS sensor



Tube lens

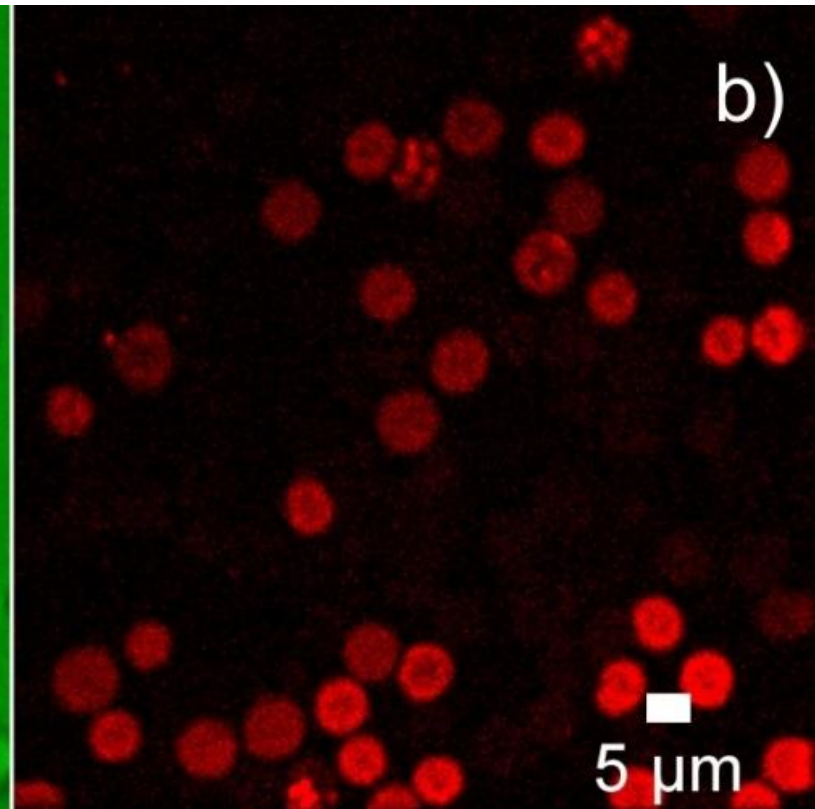
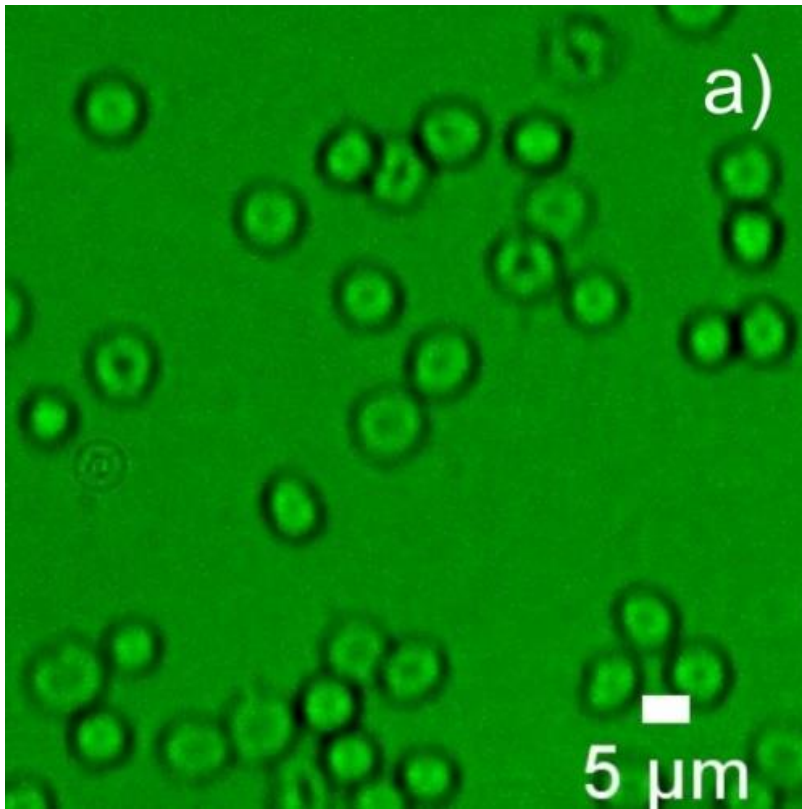
bright field
illumination

730nm

Human erythrocytes imaging

bright field microscopy image

corresponding TPEF image



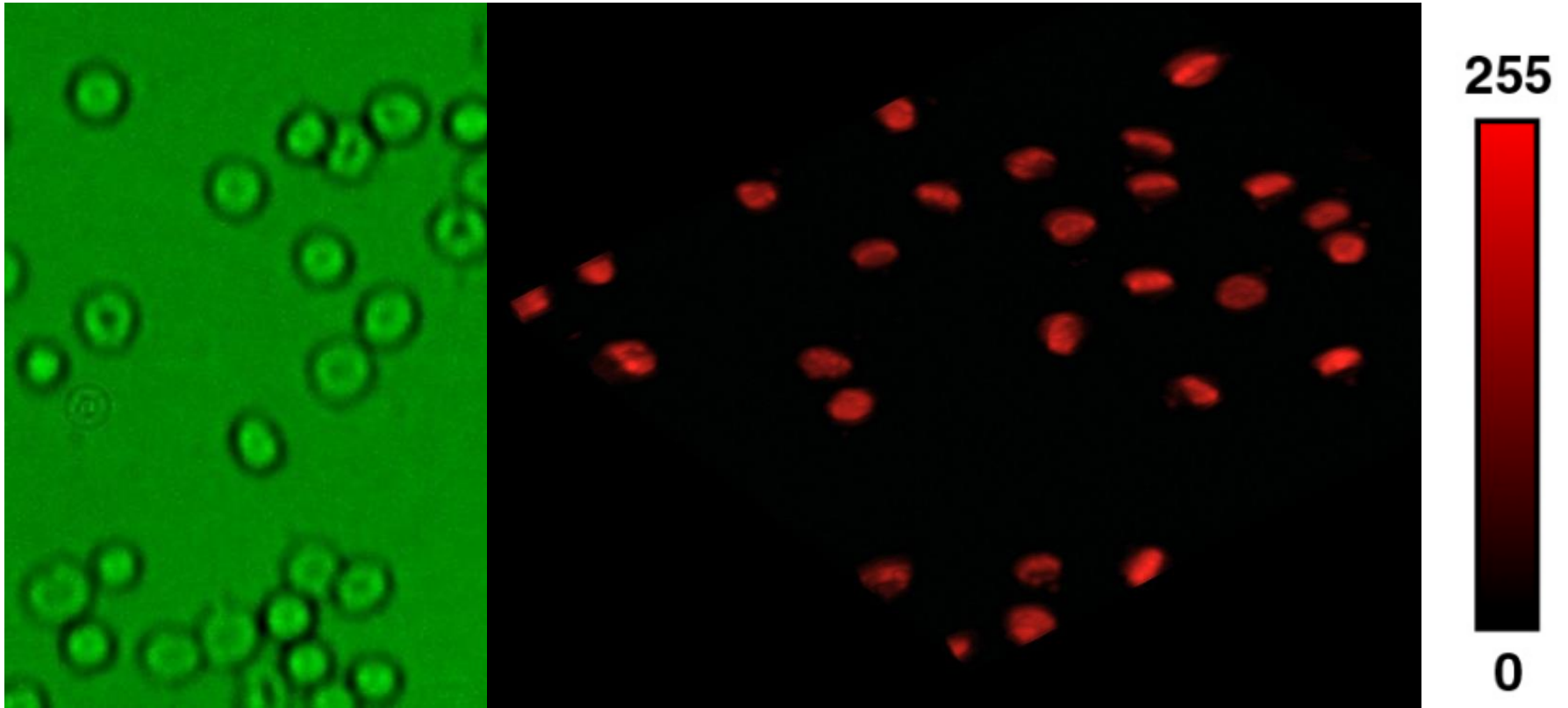
255



0

Digitalized
TPEF signal
intensity

Human erythrocytes imaging

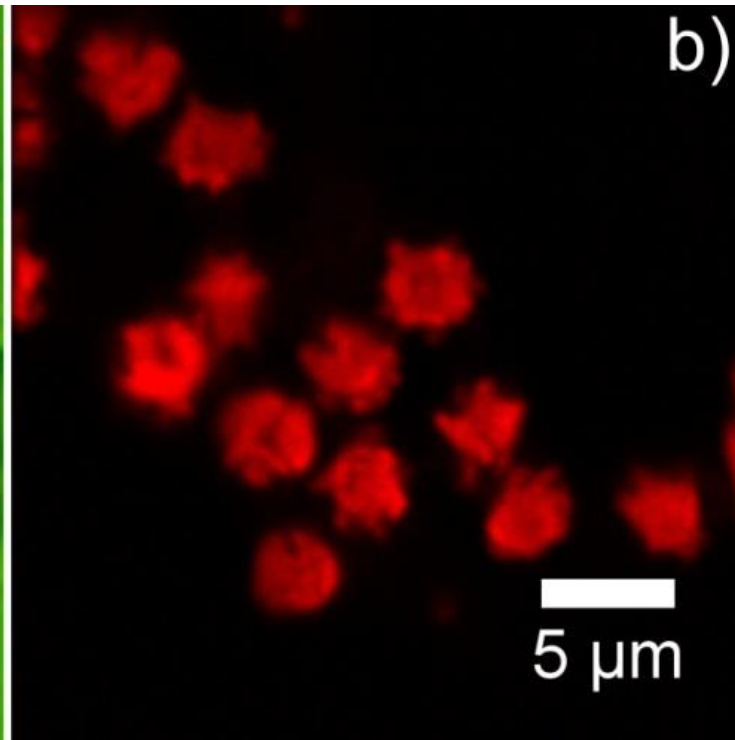
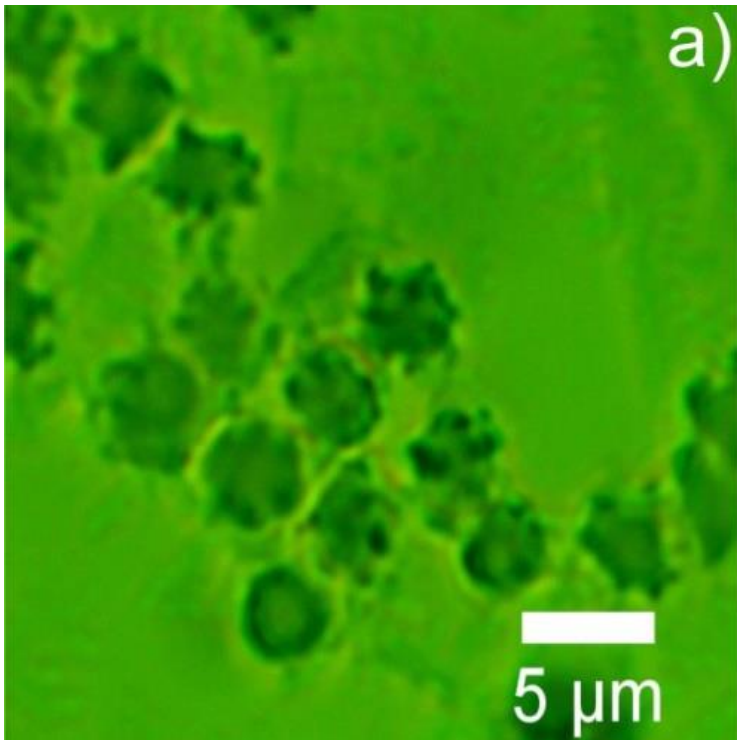


3D model of human erythrocytes

Porcine erythrocytes imaging

bright field microscopy image

corresponding TPEF image revealing
localization of hemoglobin in protrusions



255



0

-TPEF images: 1024x1024 pixels in < 1s

-Averaging: 30 times

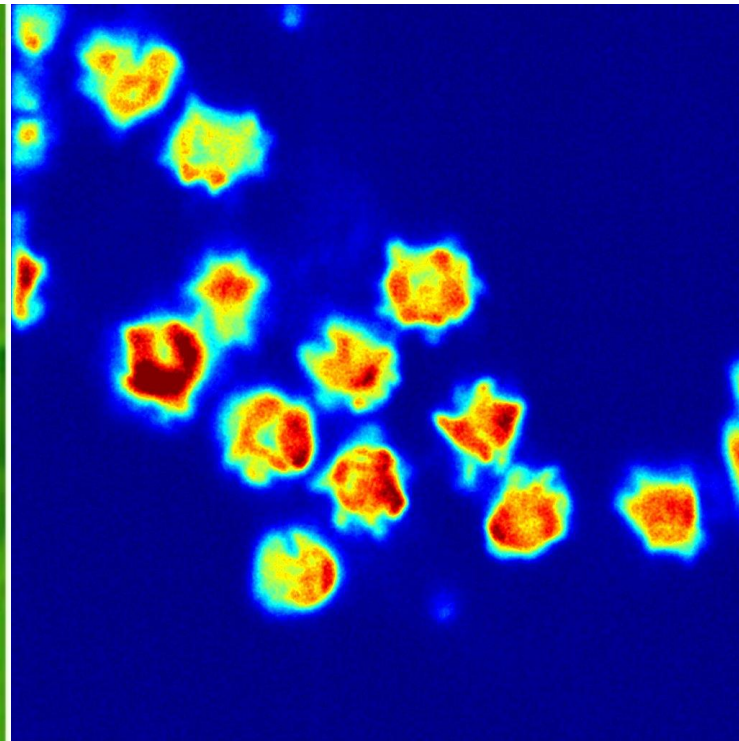
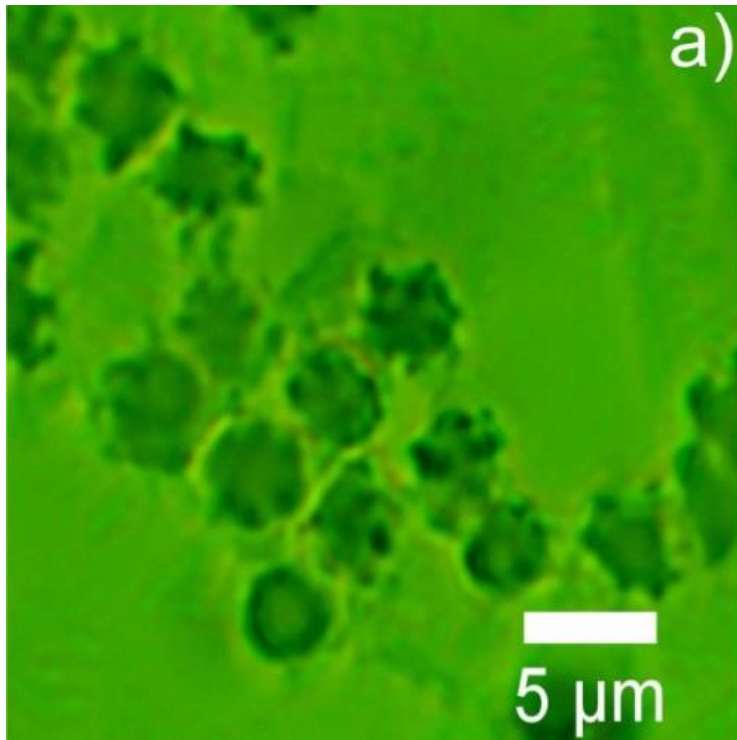
-Lateral resolution \approx 300nm (Point Spread Function)

Digitalized
TPEF signal
intensity

Porcine erythrocytes imaging

bright field microscopy image

corresponding TPEF image revealing
localization of hemoglobin in protrusions



255

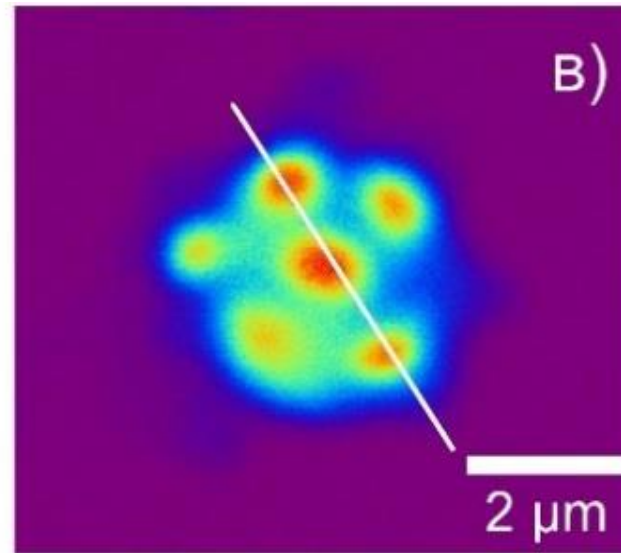
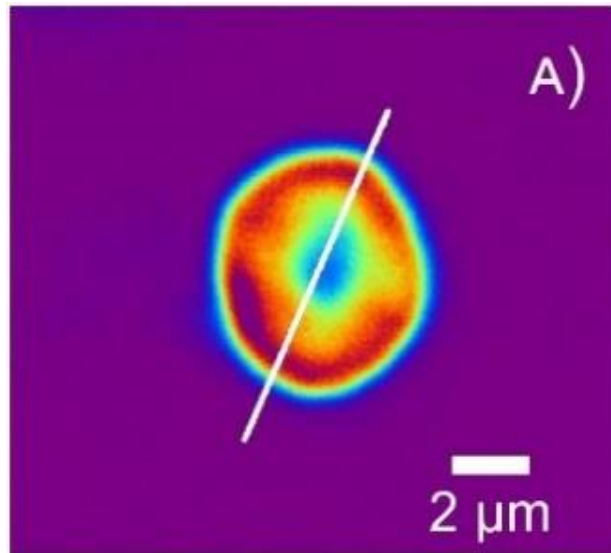


0

- TPEF images: 1024x1024 pixels in $< 1\text{s}$
- Averaging: 30 times
- Lateral resolution $\approx 300\text{nm}$ (Point Spread Function)

Digitalized
TPEF signal
intensity

IMAGING AT SINGLE CELL LEVEL

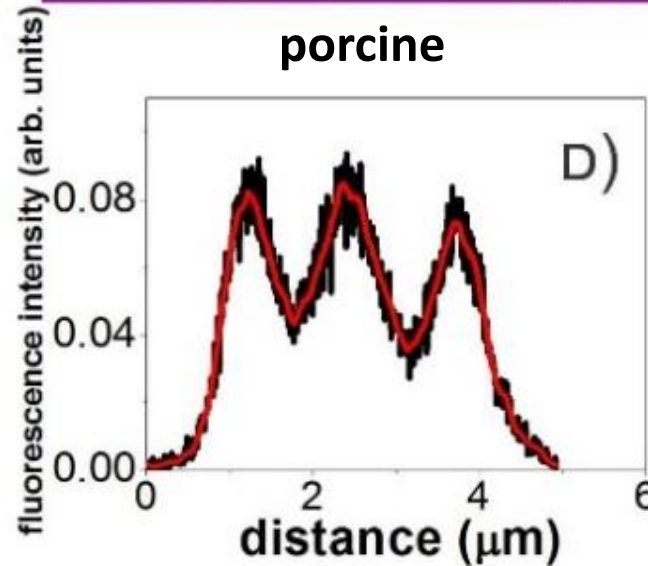
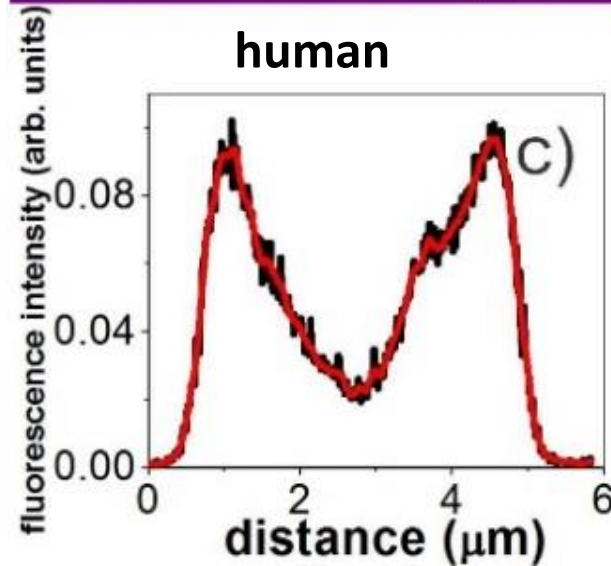


255



0

Digitalized
TPEF signal
intensity



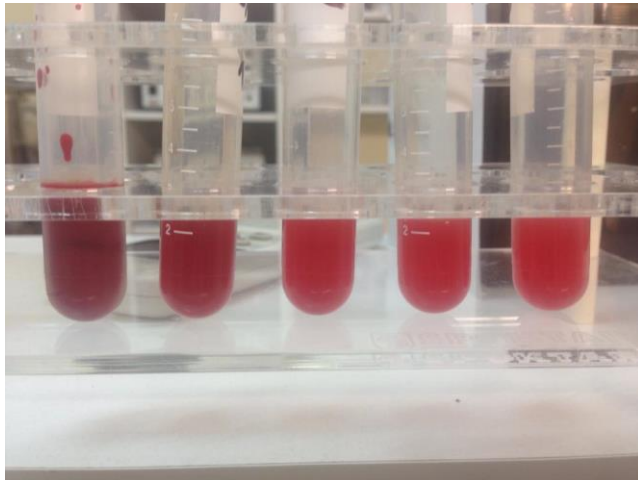
TPEF intensity profile along the white lines shown at the images

- Raw TPEF signal; - adjacent point averaging

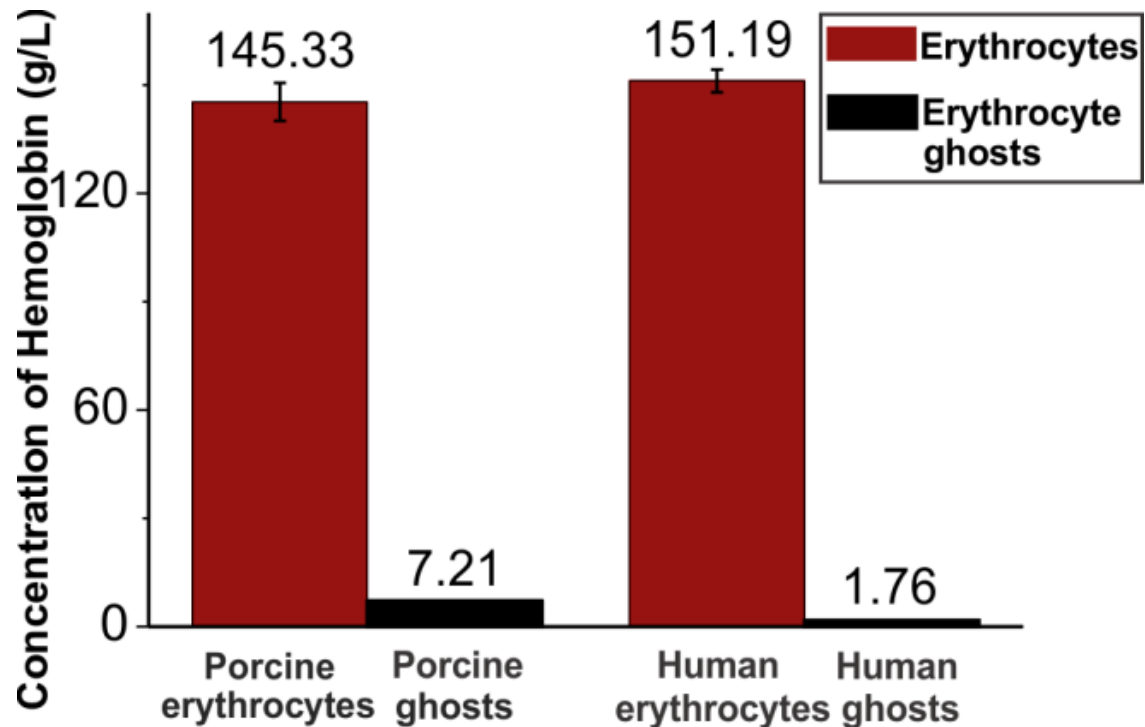
After gradual hypotonic hemolysis

Cyanmethemoglobin assay – spectrophotometry

After 4 iterations (rinses)
still red

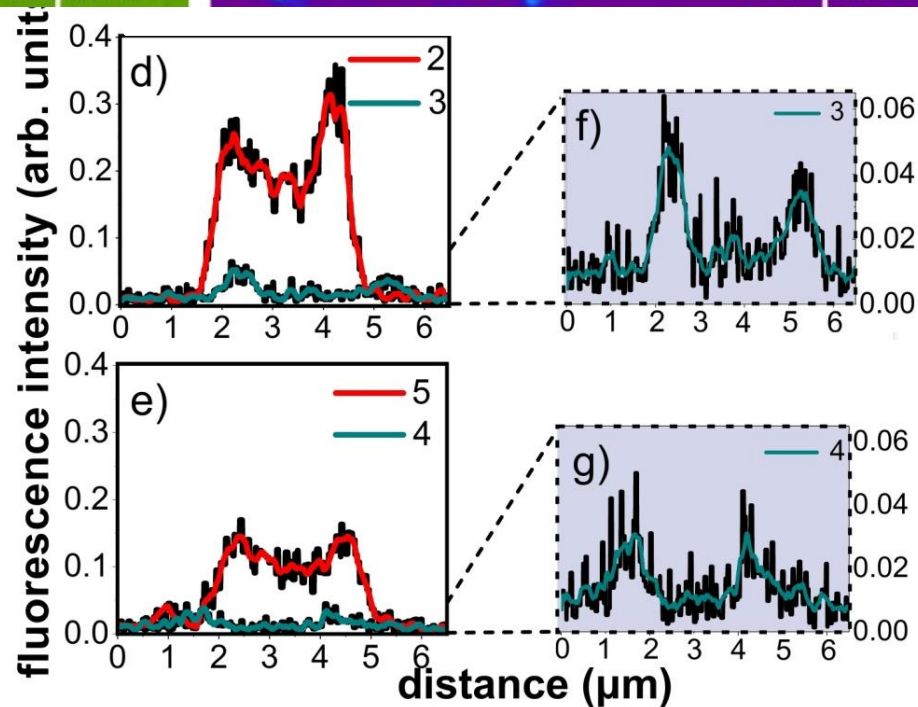
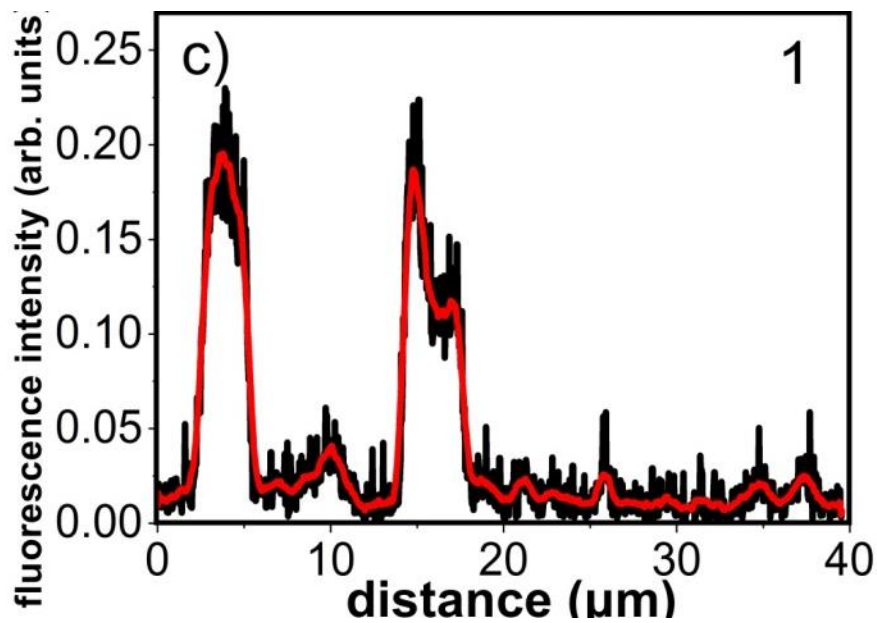
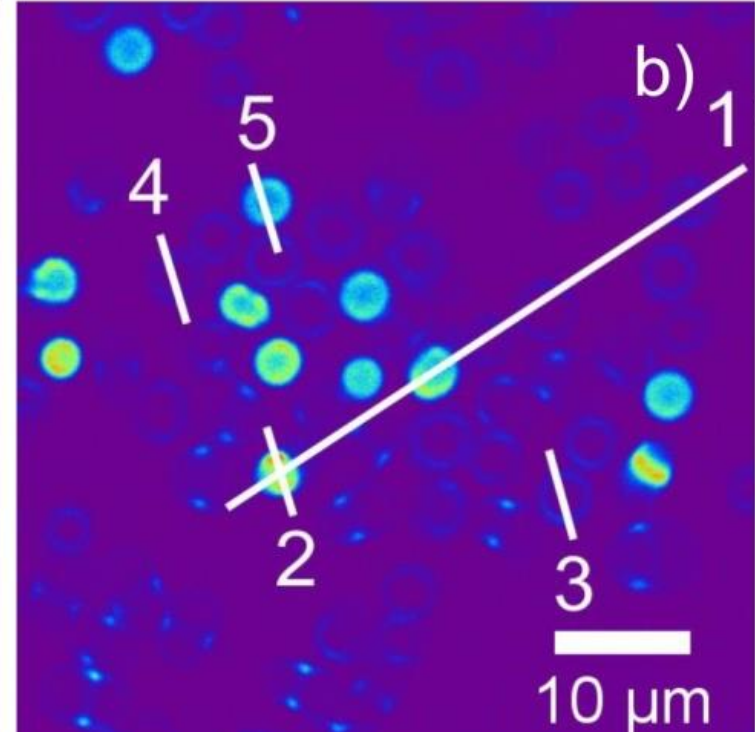
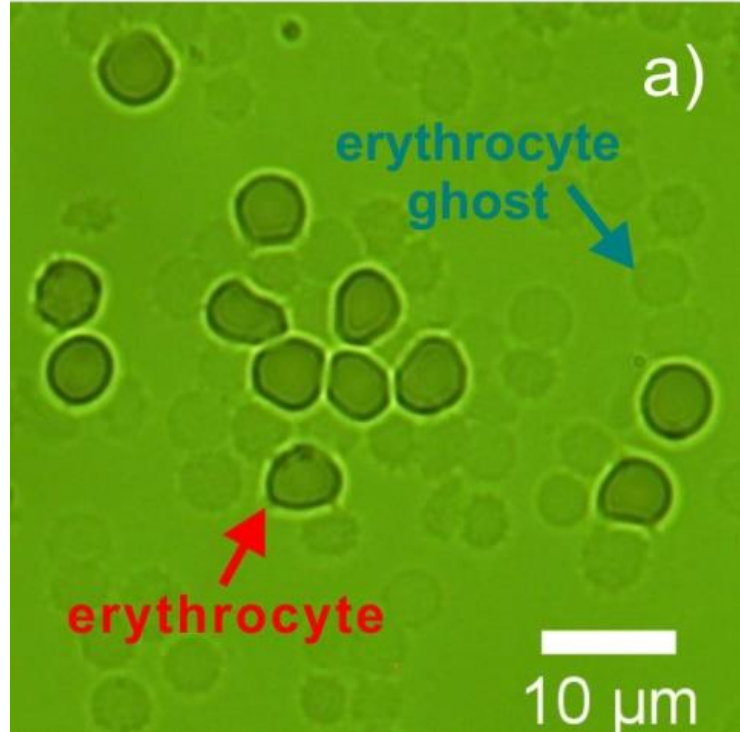


Average content of residual hemoglobin
Distribution/localization unknown

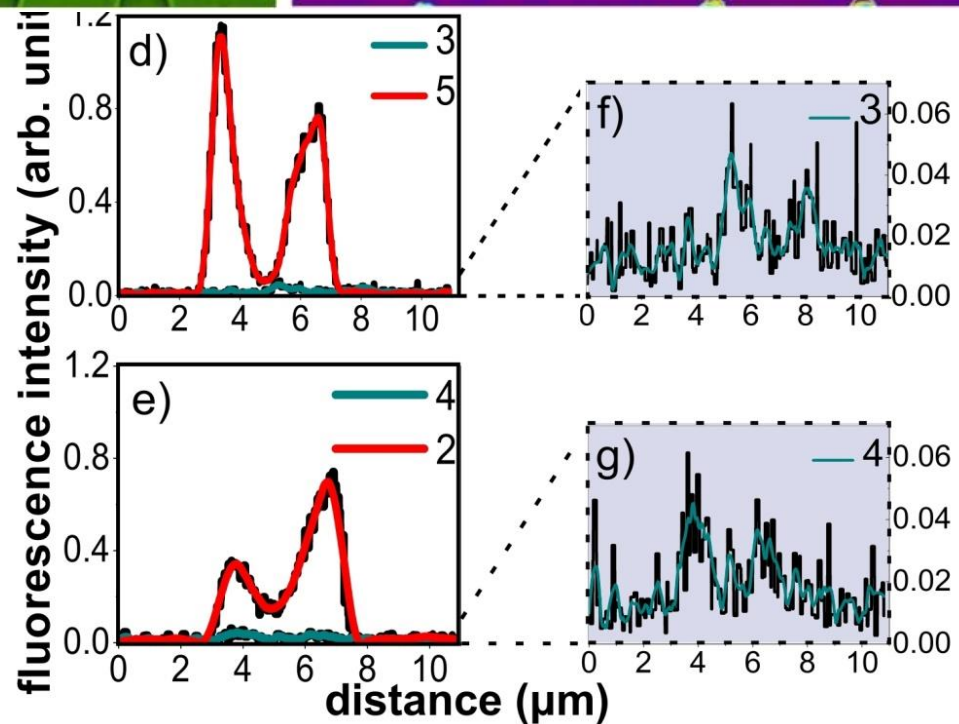
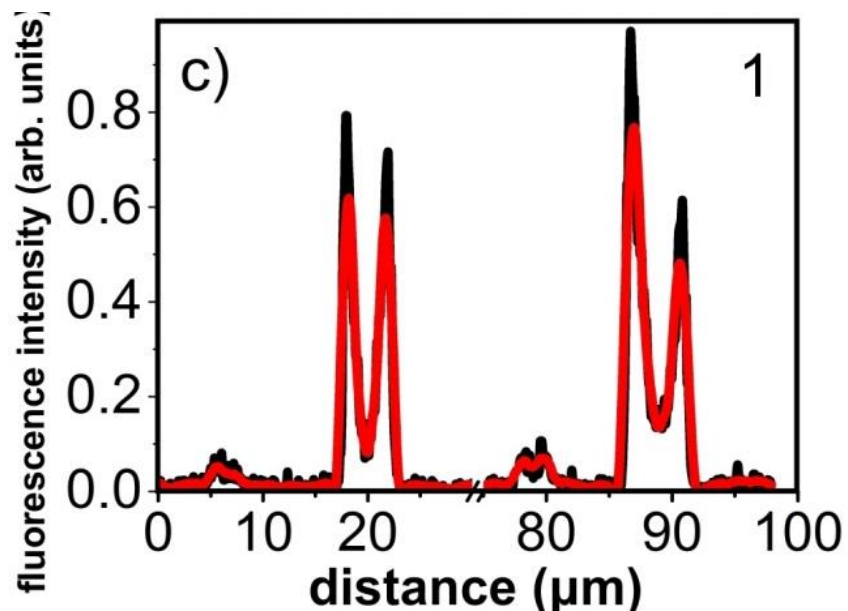
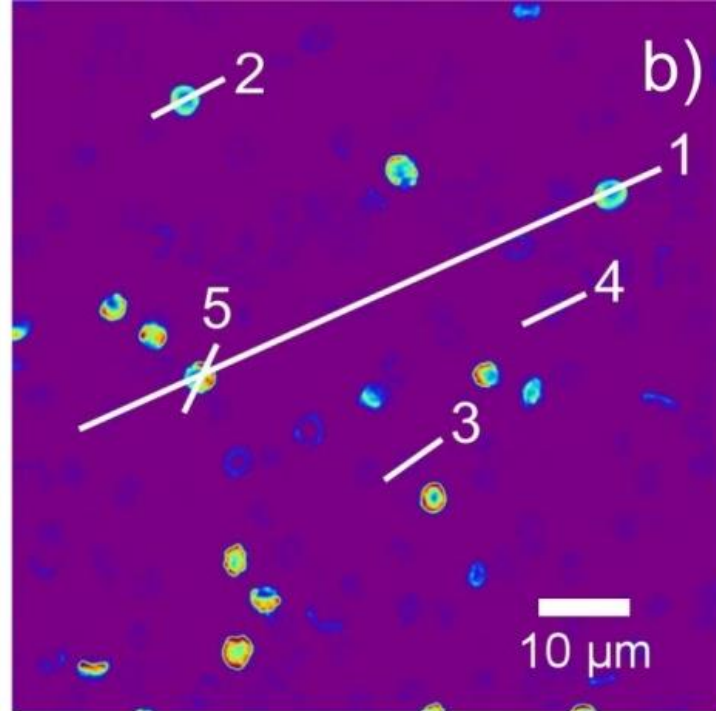
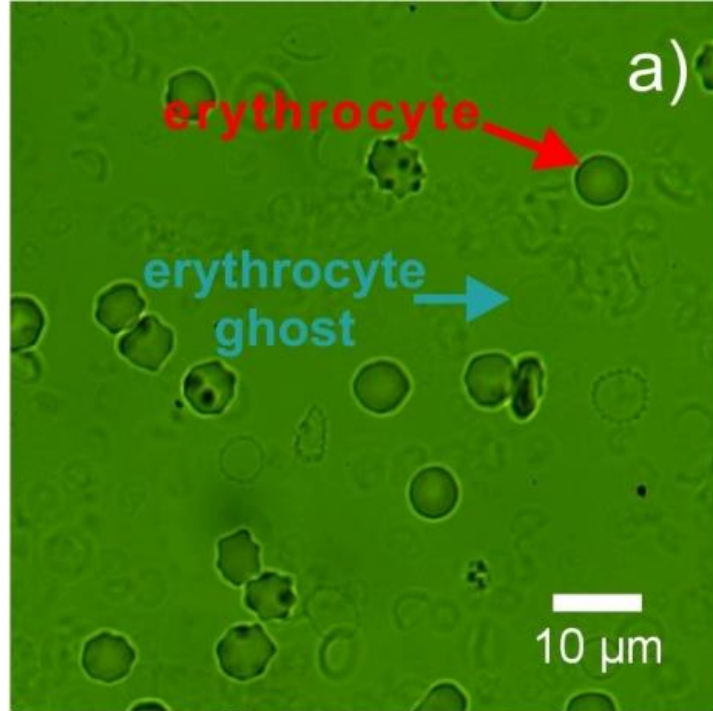


**Mapping
residual
hemoglobin
in
erythrocytes
ghosts

(human)**



**Mapping
residual
hemoglobin
in
erythrocytes
ghosts
(porcine)**

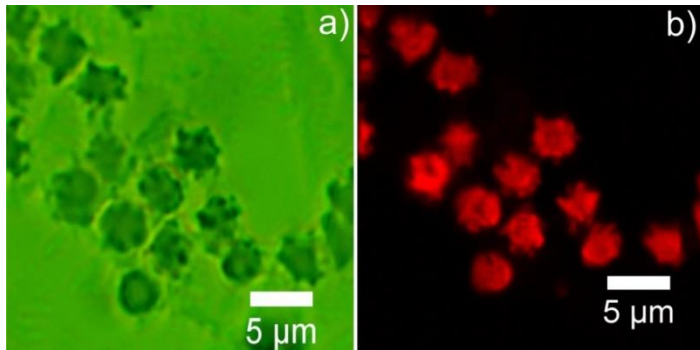


SUMMARY

- Label- and fixation- free visualization of hemoglobin @ 730nm excitation
- TPEF microscopy is applicable for echinocytes and discocytes morphology
- Identification of different pathological and/or non-pathological conditions
- Analysis of spatial distribution (mapping) of hemoglobin in erythrocytes and erythrocyte ghosts, at individual cell level
- Application in material selection in biotechnological processes

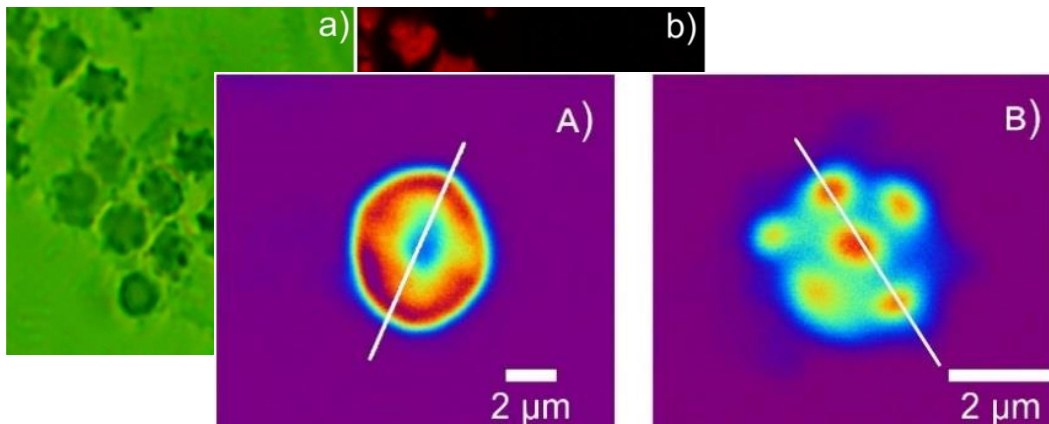
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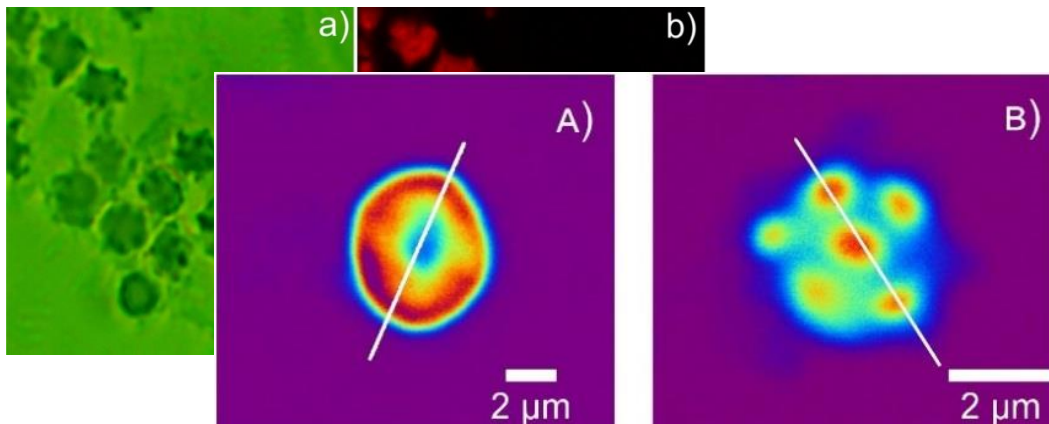
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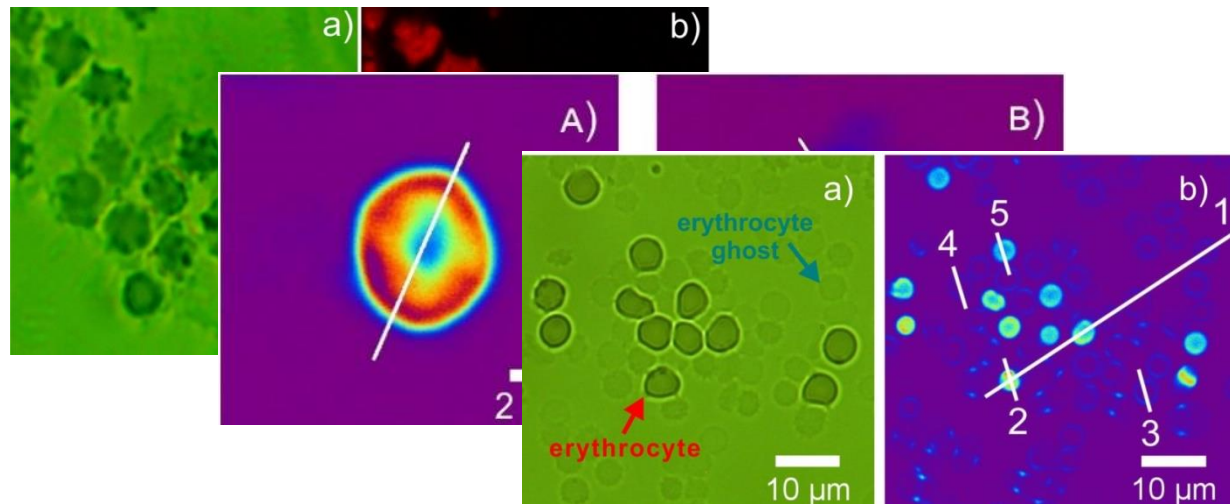
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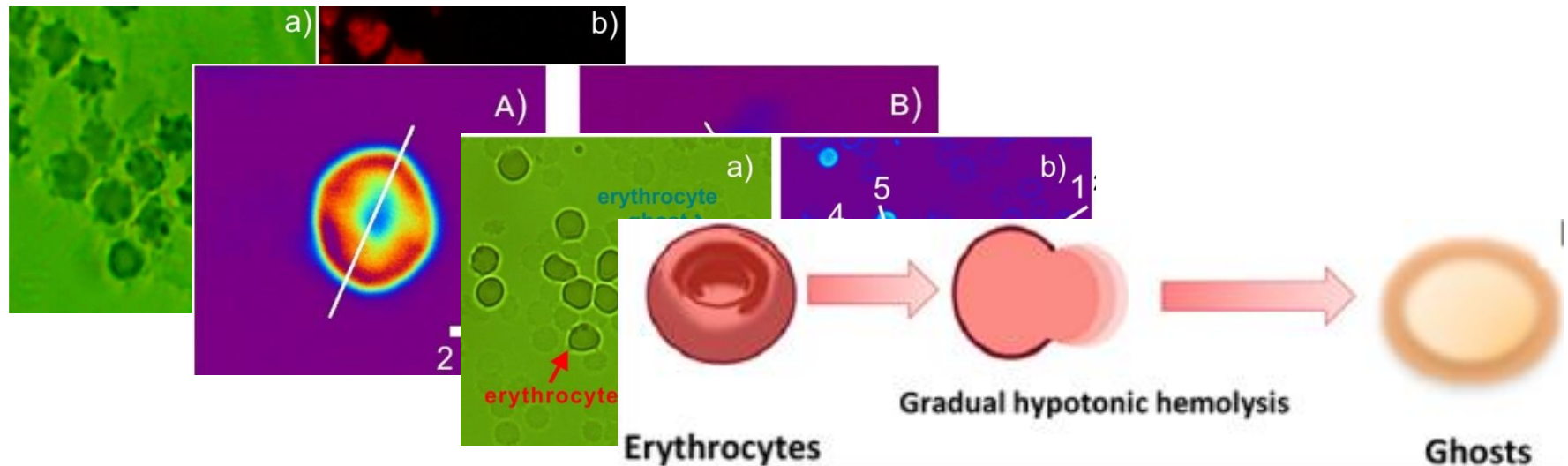
SUMMARY

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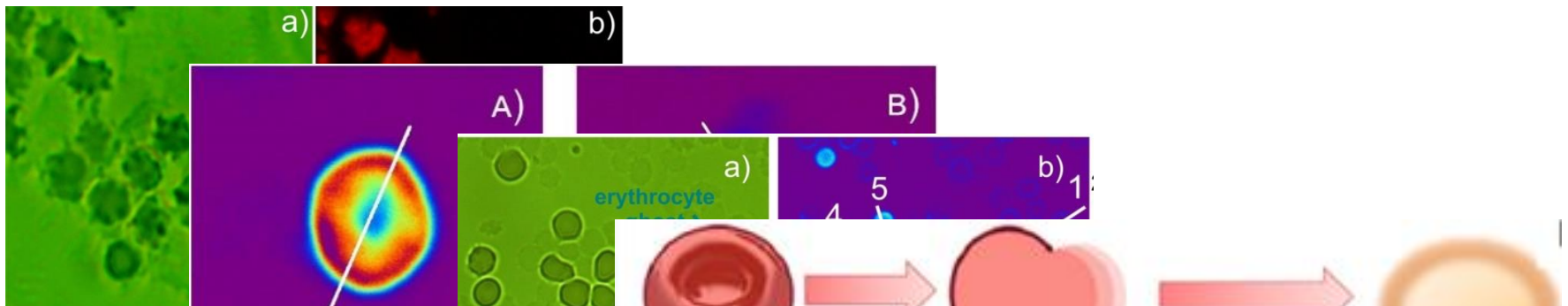
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SUMMARY

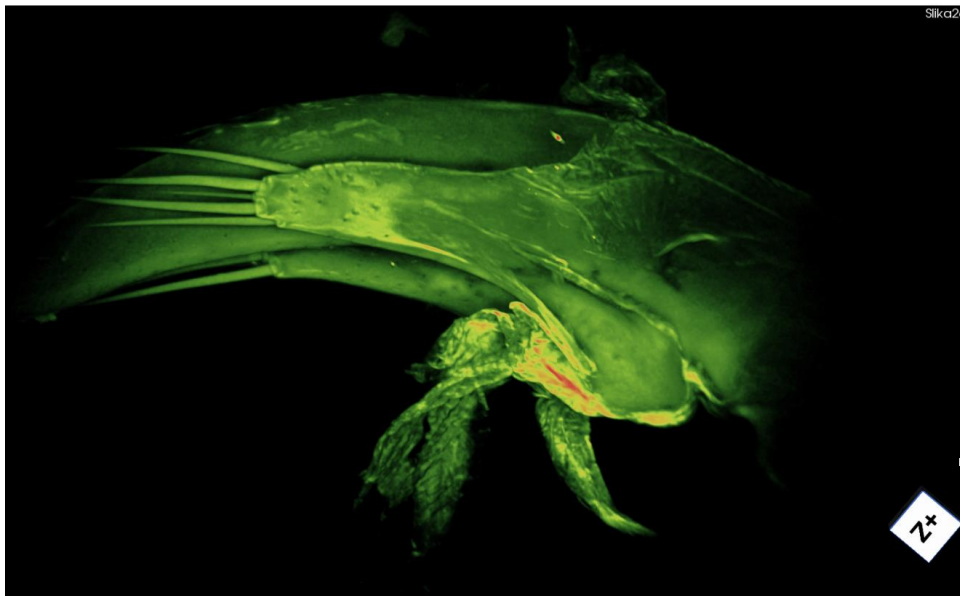
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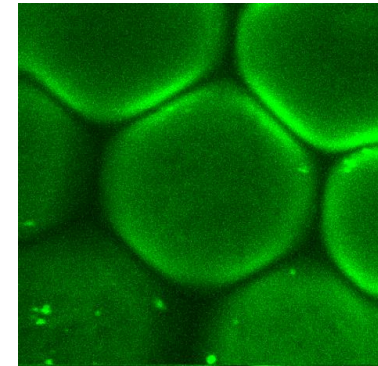
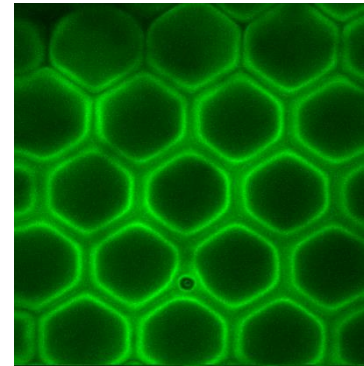
Katarina Bukara, Svetlana Jovanić, Ivana T. Drvenica, Ana Stančić, Vesna Ilić, Mihailo D. Rabasović, Dejan Pantelić, Branislav Jelenković, Branko Bugarski, Aleksandar J. Krmpot, "Mapping of hemoglobin in erythrocytes and erythrocyte ghosts using two photon excitation fluorescence microscopy," *J. Biomed. Opt.* **22**(2), 026003 (2017)

TPEF of chitinous structures

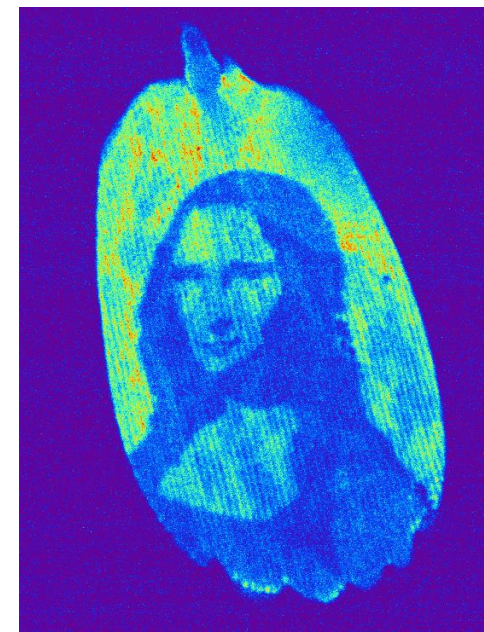
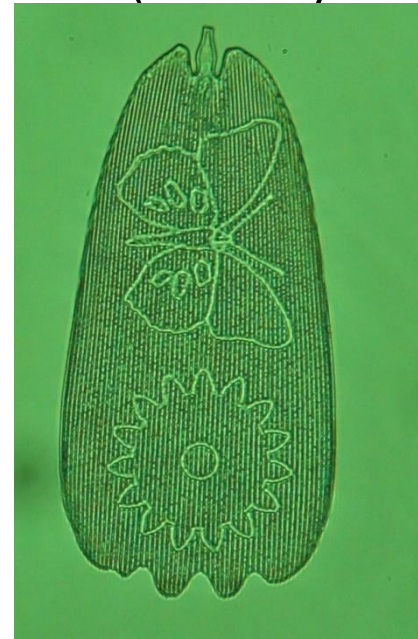
Aedeagus - *Pheggomisetes ninae*
(cave dwelling insect)



In vivo imaging of *Satyrus ferula*
compound eye (ommatidia)



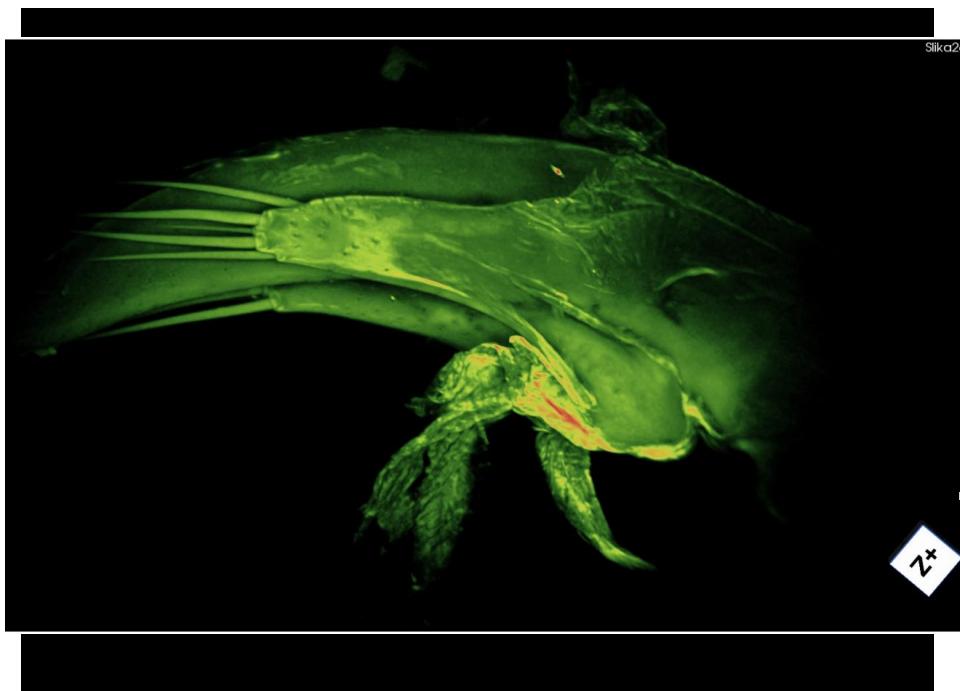
Direct laser writing (and cutting)
of chitinous structures
(butterfly wing scales)



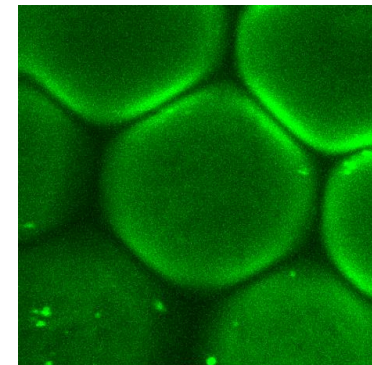
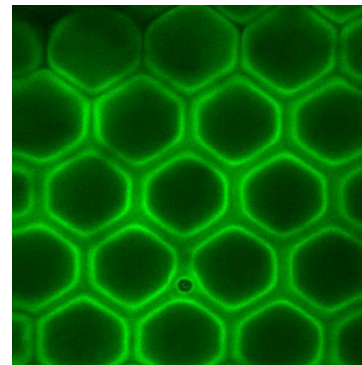
M. D. Rabasović, D. V. Pantelić, B. M. Jelenković,
S. B. Ćurčić, M. S. Rabasović, M. D. Vrbica, V. M.
Lazović, B. P. M. Ćurčić, A. J. Krmpot, *J. Biomed.*
Opt. **20** 016010 (2015)

TPEF of chitinous structures

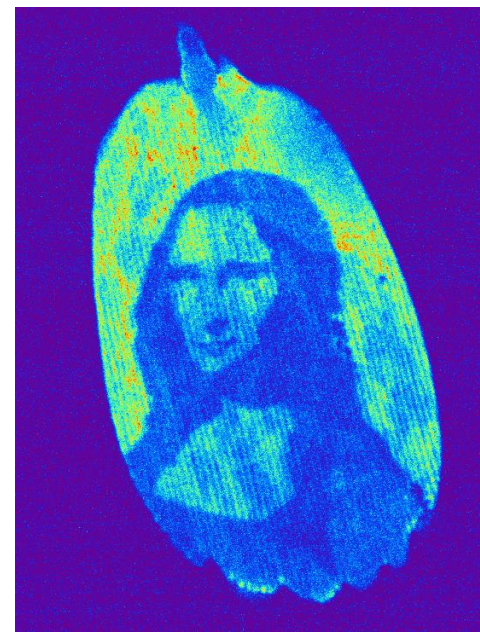
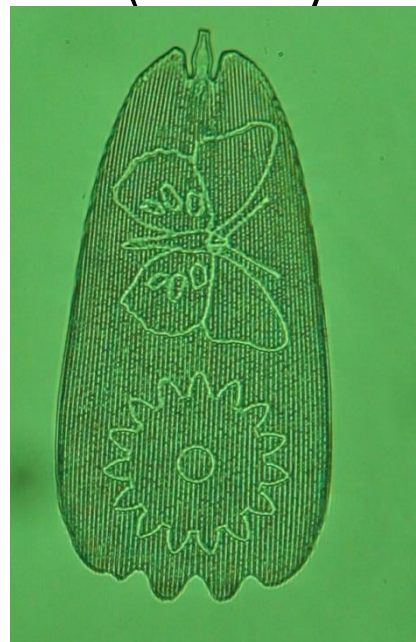
Aedeagus - *Pheggomisetes ninae*
(cave dwelling insect)



In vivo imaging of *Satyrus ferula*
compound eye (ommatidia)



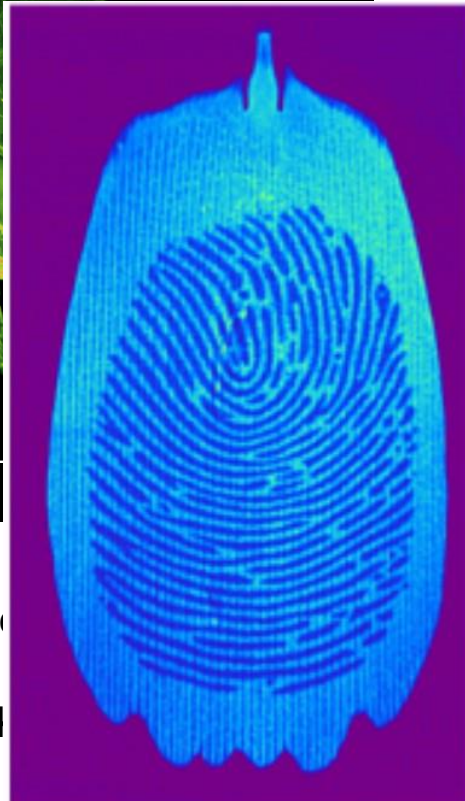
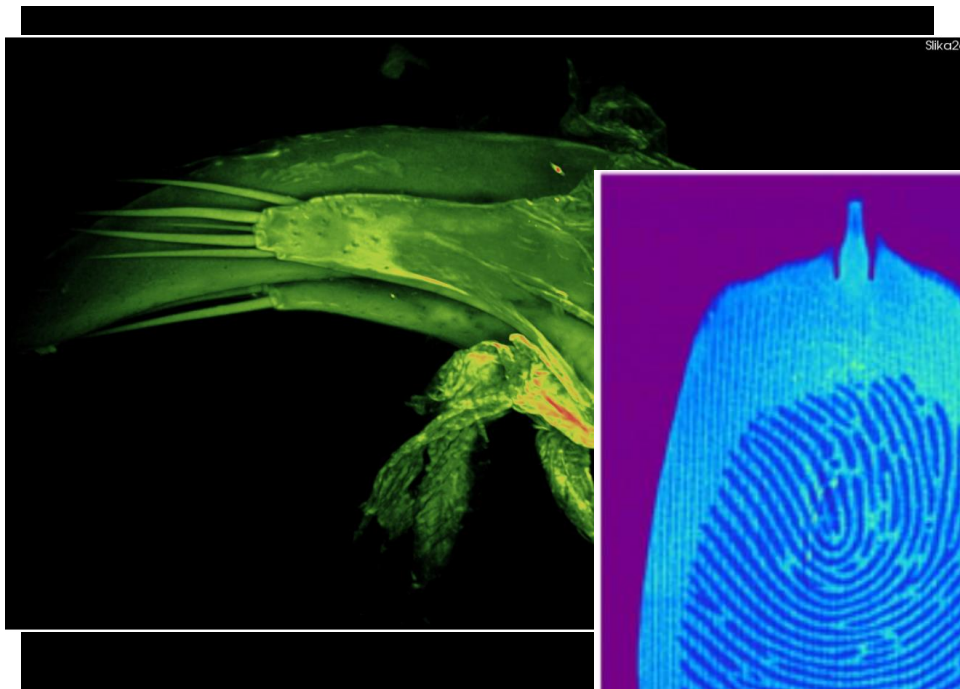
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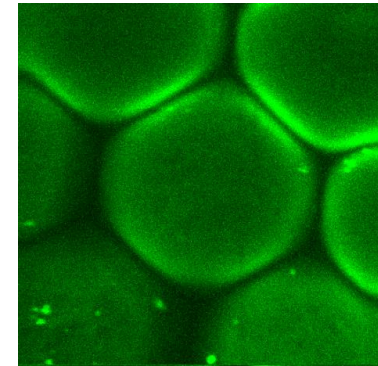
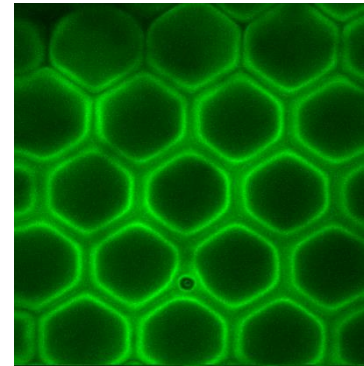
M. D. Rabasović, D. V. Pantelić, B. M. Jelenković,
S. B. Ćurčić, M. S. Rabasović, M. D. Vrbica, V. M.
Lazović, B. P. M. Ćurčić, A. J. Krmpot, *J. Biomed.*
Opt. **20** 016010 (2015)

TPEF of chitinous structures

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(cave dwelling insect)



In vivo imaging of *Satyrus ferula*
compound eye (ommatidia)



Patents (documents security):

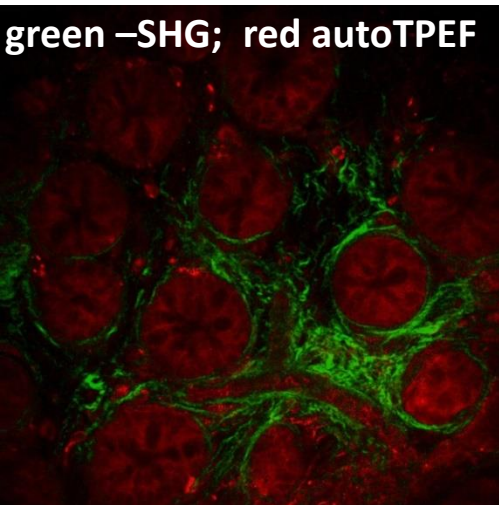
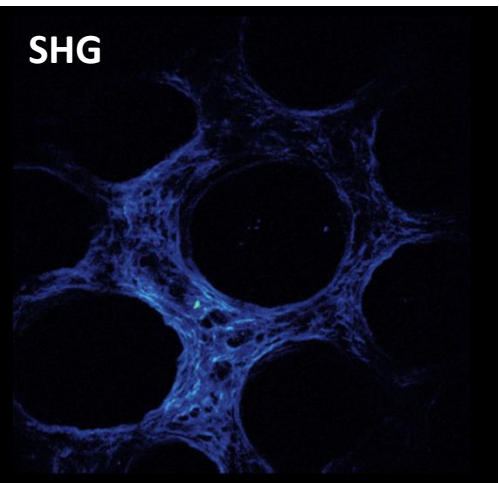
Dejan Pantelic, Mihailo Rabasovic, Aleksandar Krmpot, Vladimir Lazovic, Danica Pavlovic,
Security tag containing a pattern of biological particles - WO2017114570 A1 (2017)

Dejan Pantelic, Mihailo Rabasovic, Aleksandar Krmpot, Vladimir Lazovic, Danica Pavlovic,
Security device individualized with biological particles - WO2017114569 A1 (2017)

Danica Pavlovic, Vladimir Lazovic, Aleksandar Krmpot, Mihailo Rabasovic, Dejan Pantelic,
Security tag with laser-cut particles of biological origin - WO2017114572 A1 (2017)

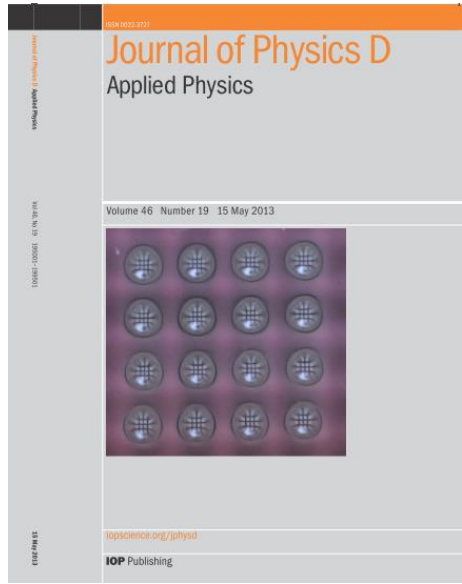
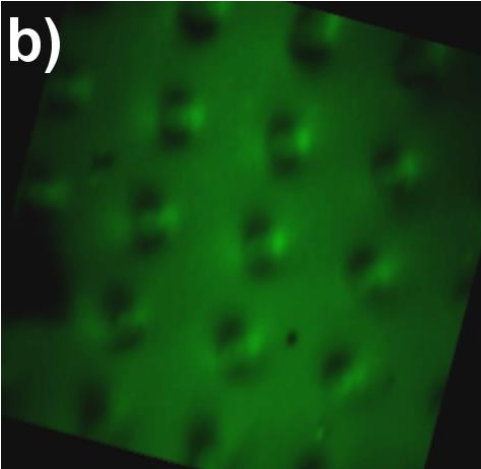
M. D. Rabasović, D. V. Pantelić,
S. B. Ćurčić, M. S. Rabasović,
Lazović, B. P. M. Ćurčić, A. J. I.
Opt. **20** 016010 (2015)

Label free imaging (SHG/TPEF) of human colon



S. Z. Despotovic et al, *Tumor Biology* 39 (2017)

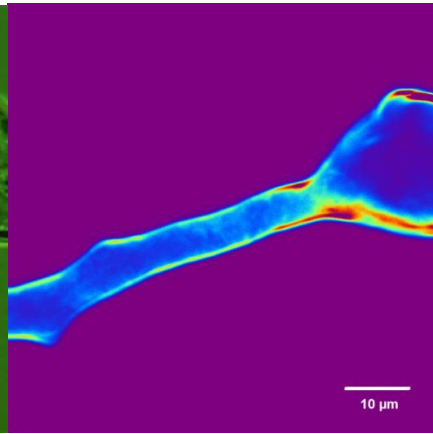
THG imaging of microlenses in soft media



A. J. Krmpot et al, *J. Phys. D: Appl. Phys.* 46 (2013)

Micro surgery of fungi's cell wall

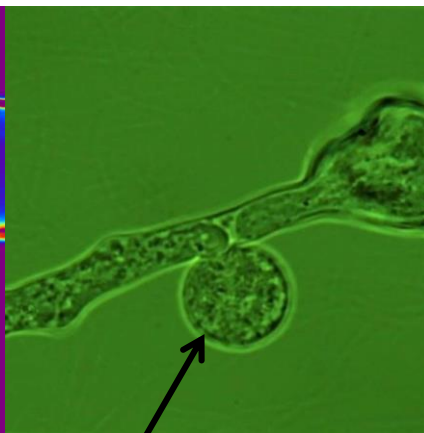
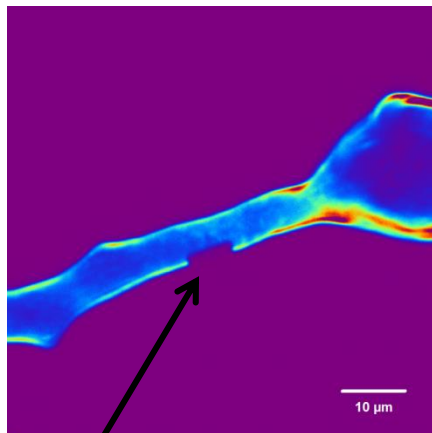
Prior to the surgery



Bright field

TPEF

After the surgery



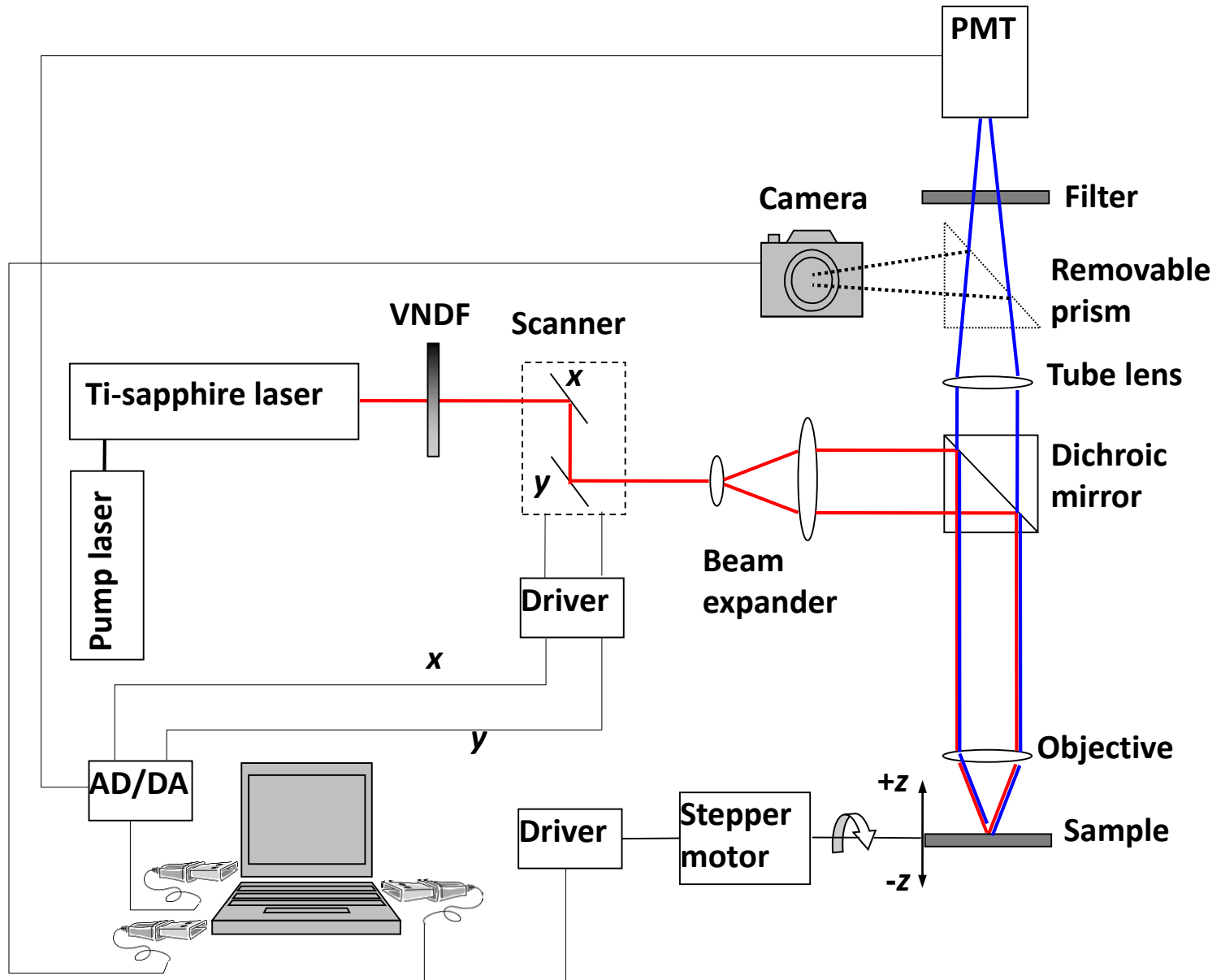
Surgical incision

Released vesicle

**THANK YOU FOR YOUR
ATTENTION!!!**



NLSM EXPERIMENTAL SETUP



IMAGING AT TISSUE LEVEL – TPEF in entomology

Why insects:

- NLM is barely used in entomology (taxonomy)
- Photonics structures at butterfly wings
- Compound eyes of butterflies (in vivo imaging)

Chitin – polysaccharide, a derivative of glucose $(C_8H_{13}O_5N)_n$

- the exoskeleton of arthropods (insects)
- Exhibits strong (single photon) absorption in blue-UV region => auto fluorescence
- Opaque obstacle for confocal microscopy

Pheggomisetes ninae

Endemic species

habitat: caves of south-east Serbia

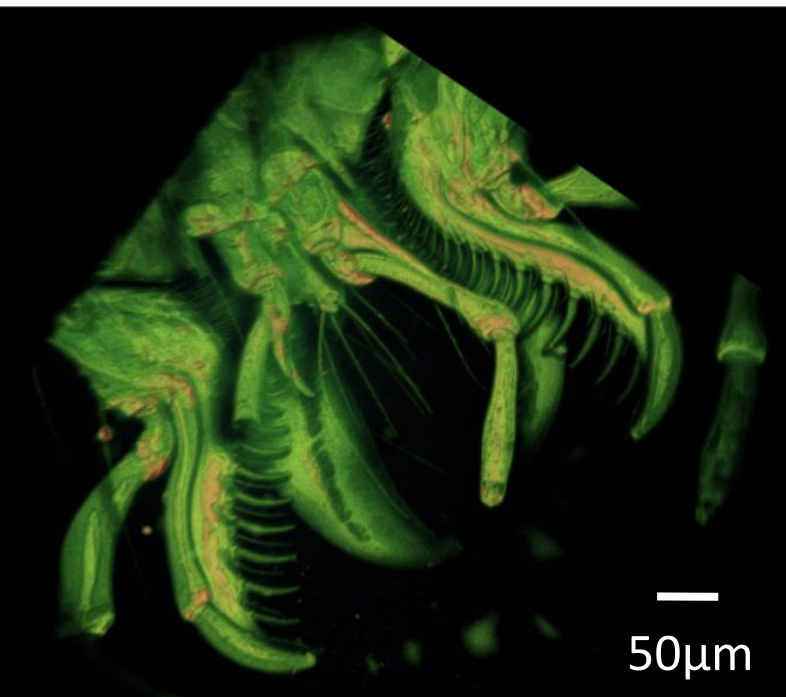
(Srećko Ćurčić, Faculty of biology, University of Belgrade)



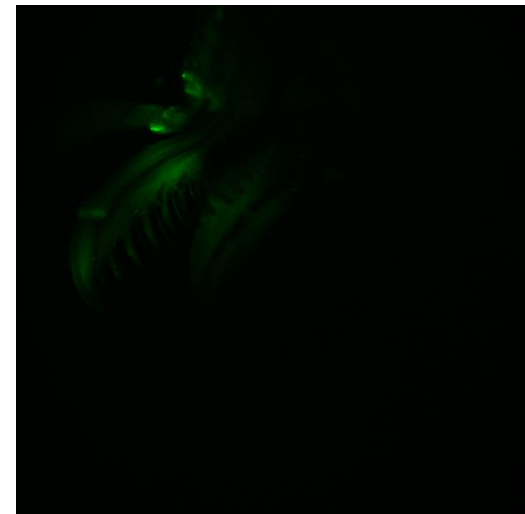
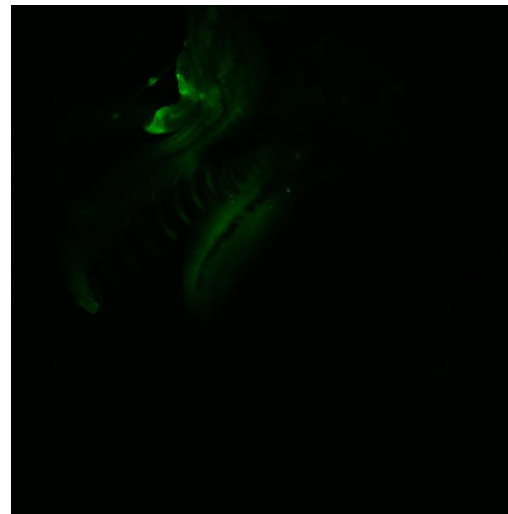
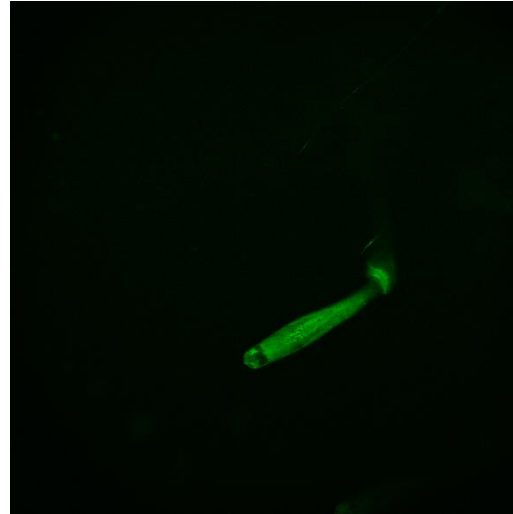
Pheggomisetes ninae - mouthparts

2D slices

3D reconstruction

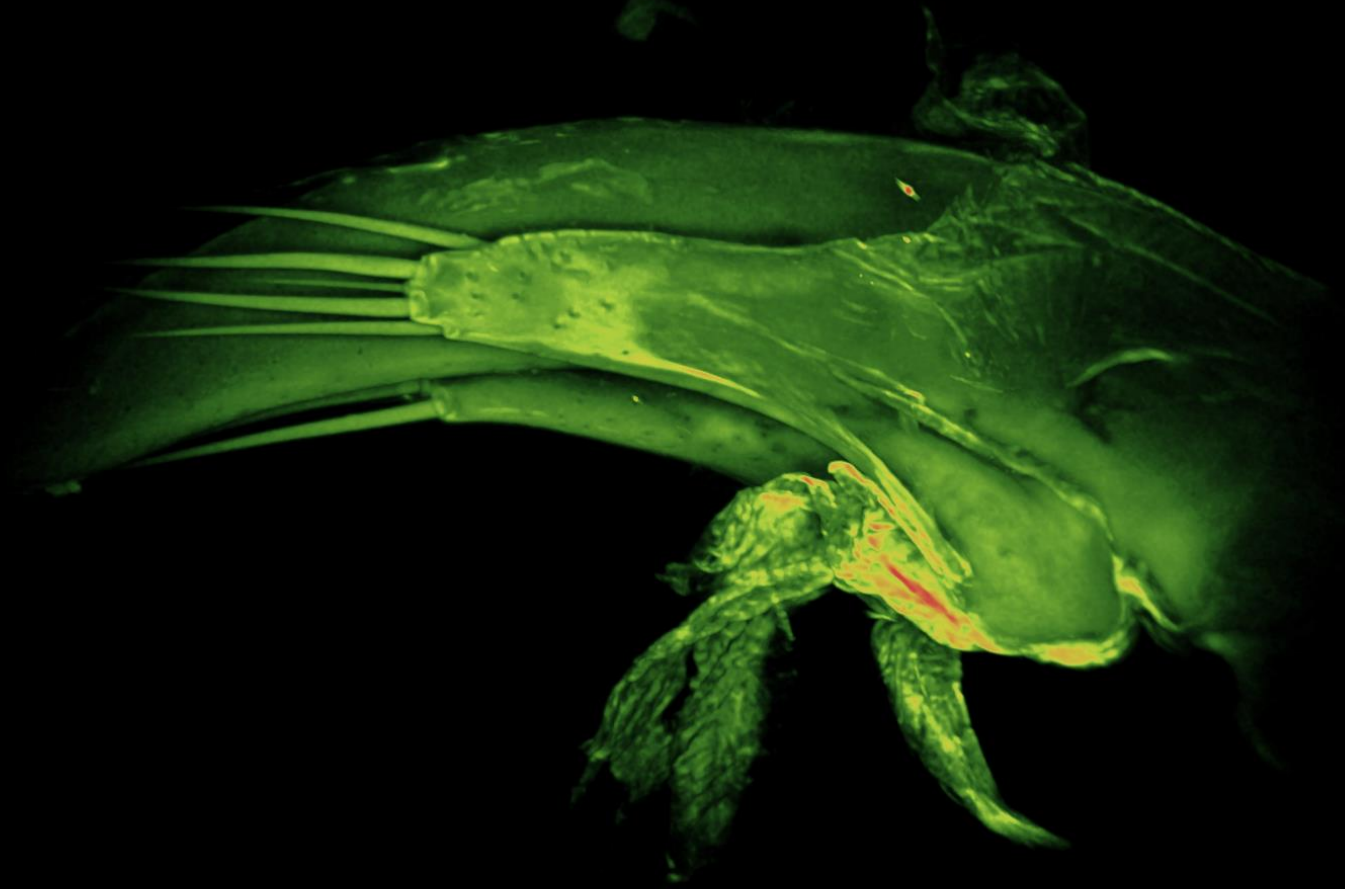


40x, NA 0.65
840nm excitation



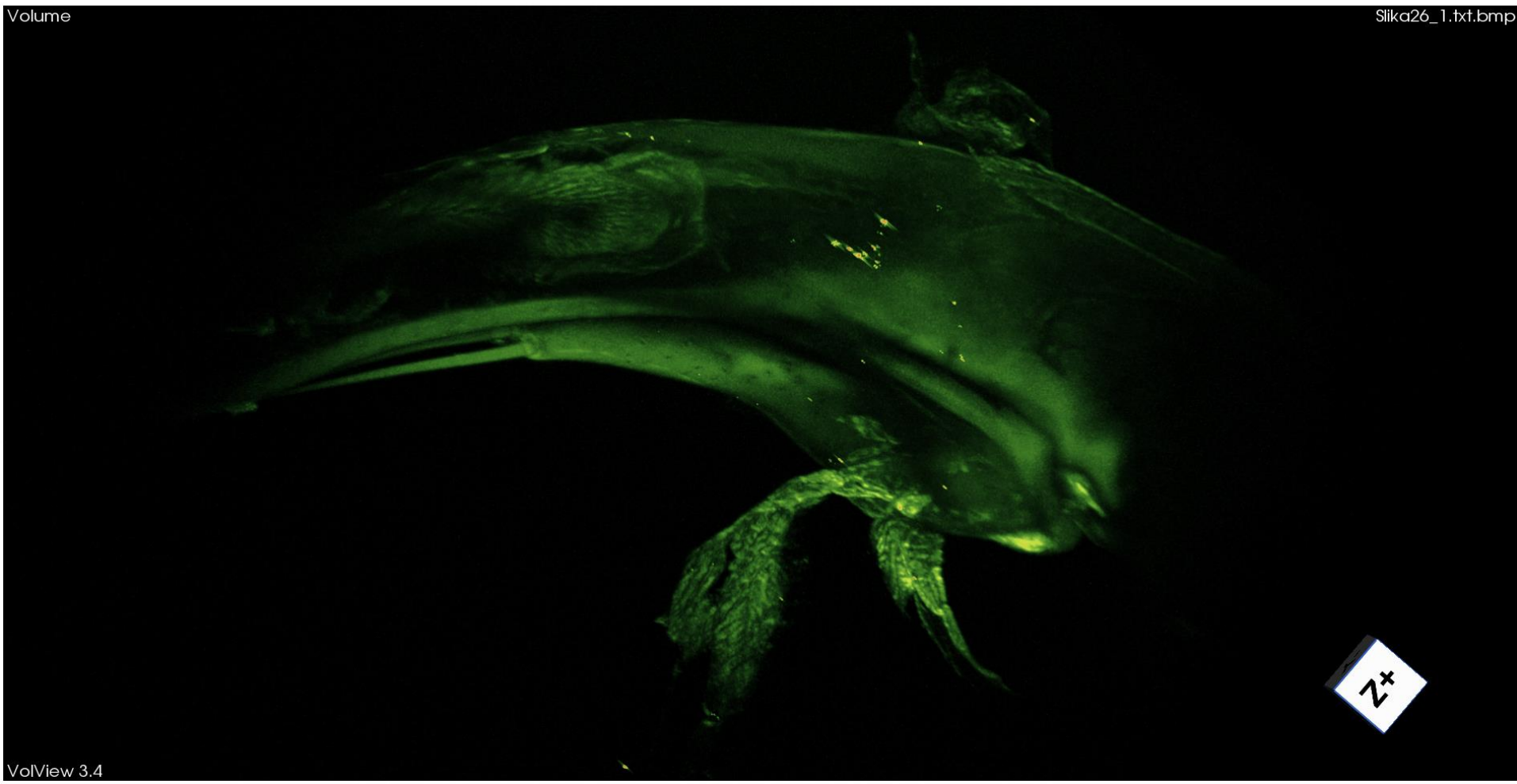
Volume

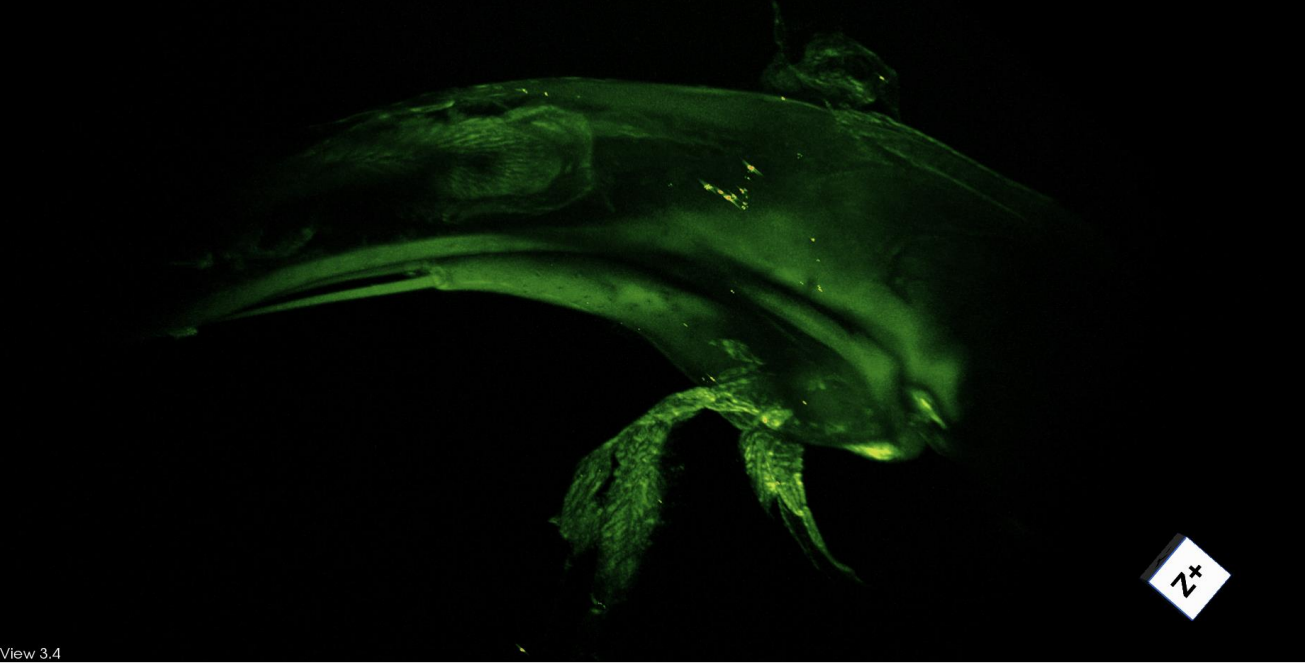
Slika26_1.txt.bmp



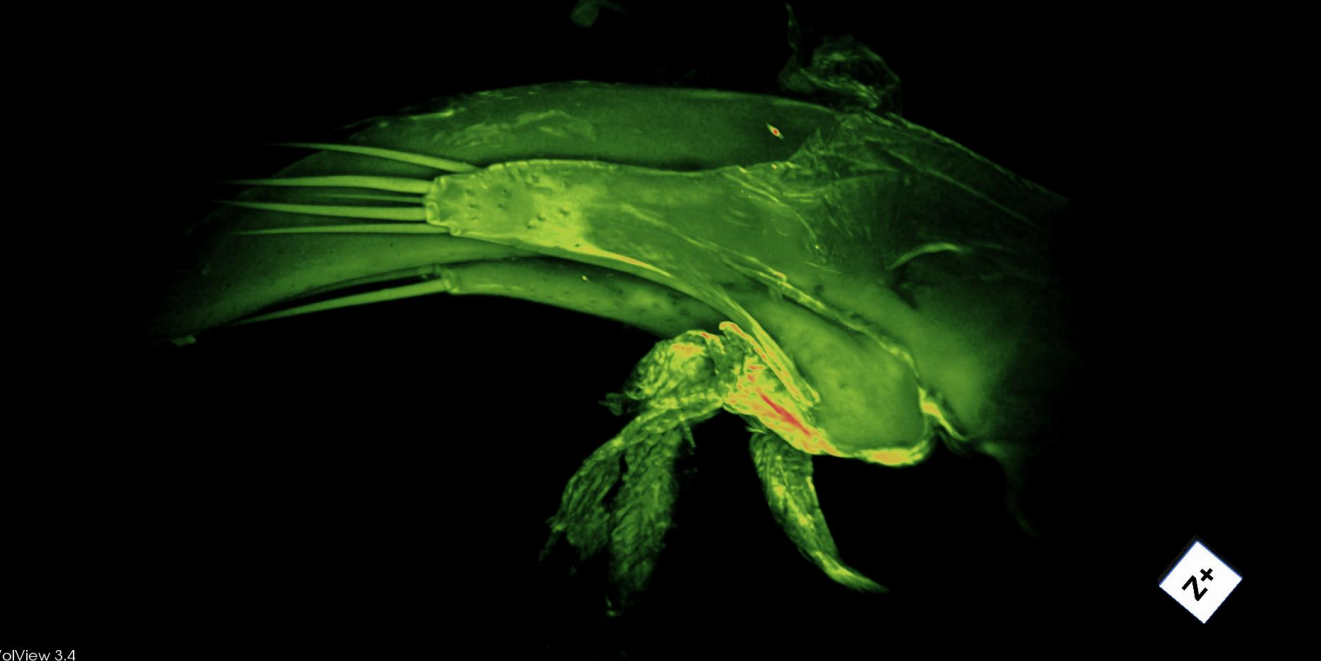
Z+

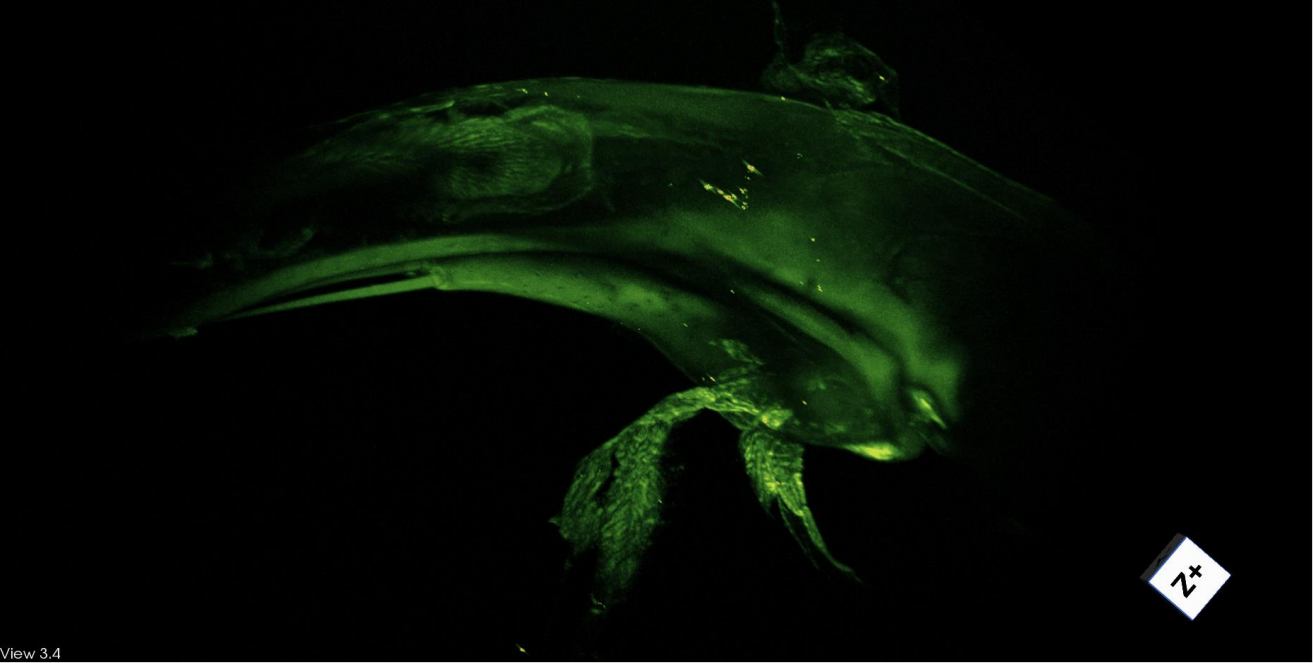
Take out outer slices – inner structure
Deep penetration depth of TPEF microscopy
(chitin – opaque obstacle for confocal microscopy)



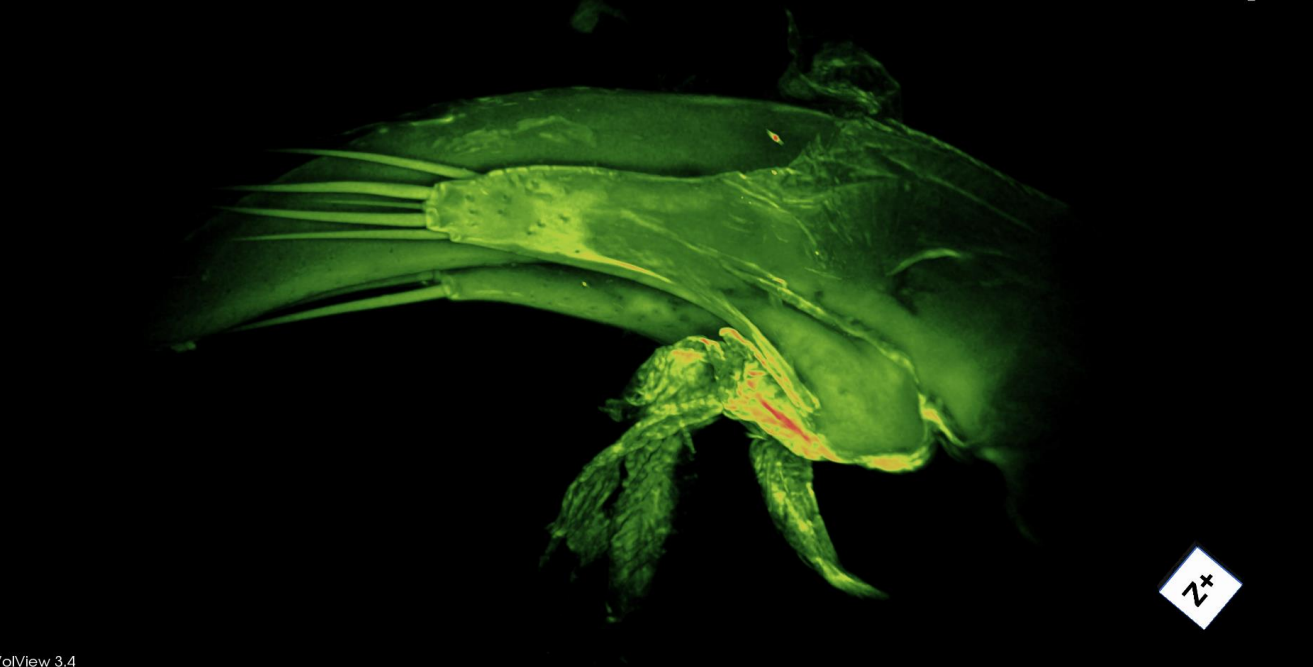
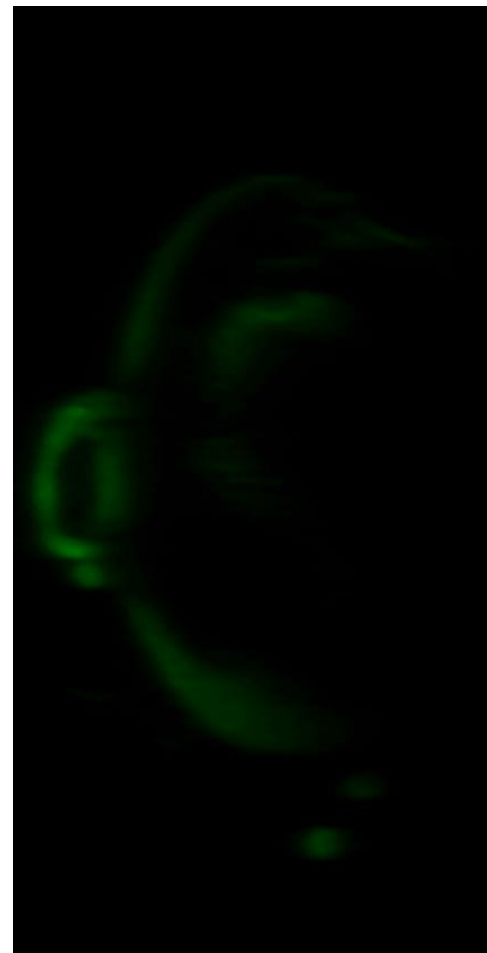


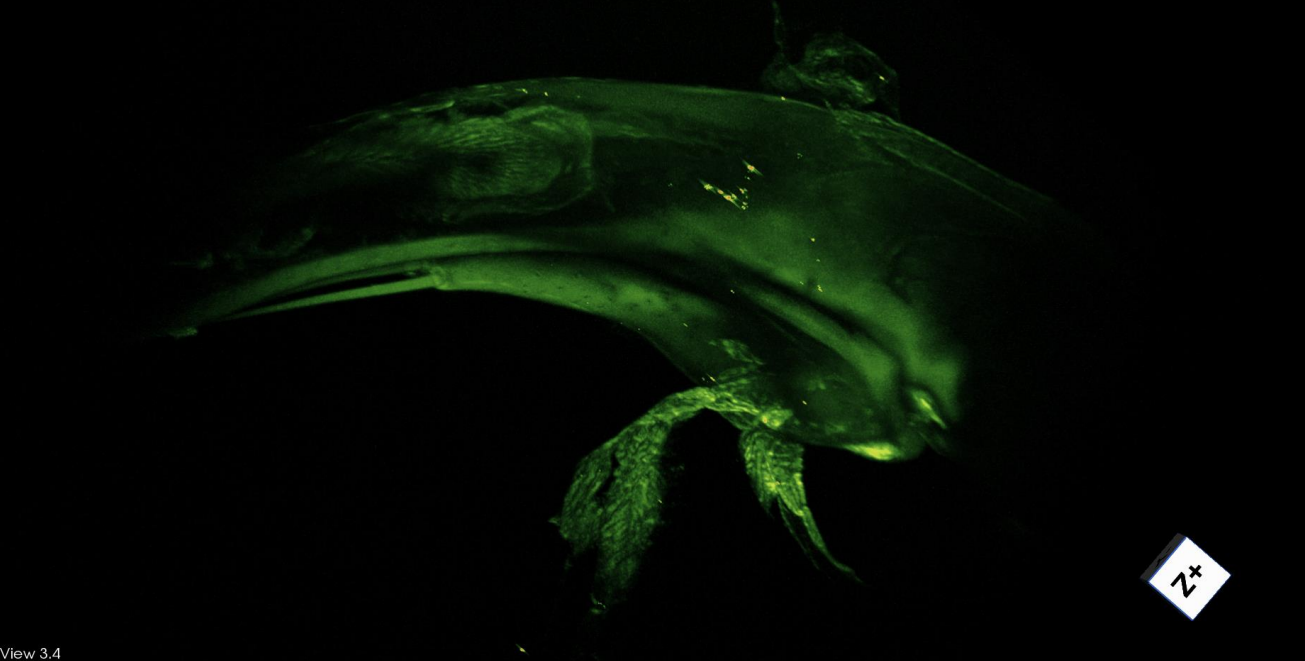
py)



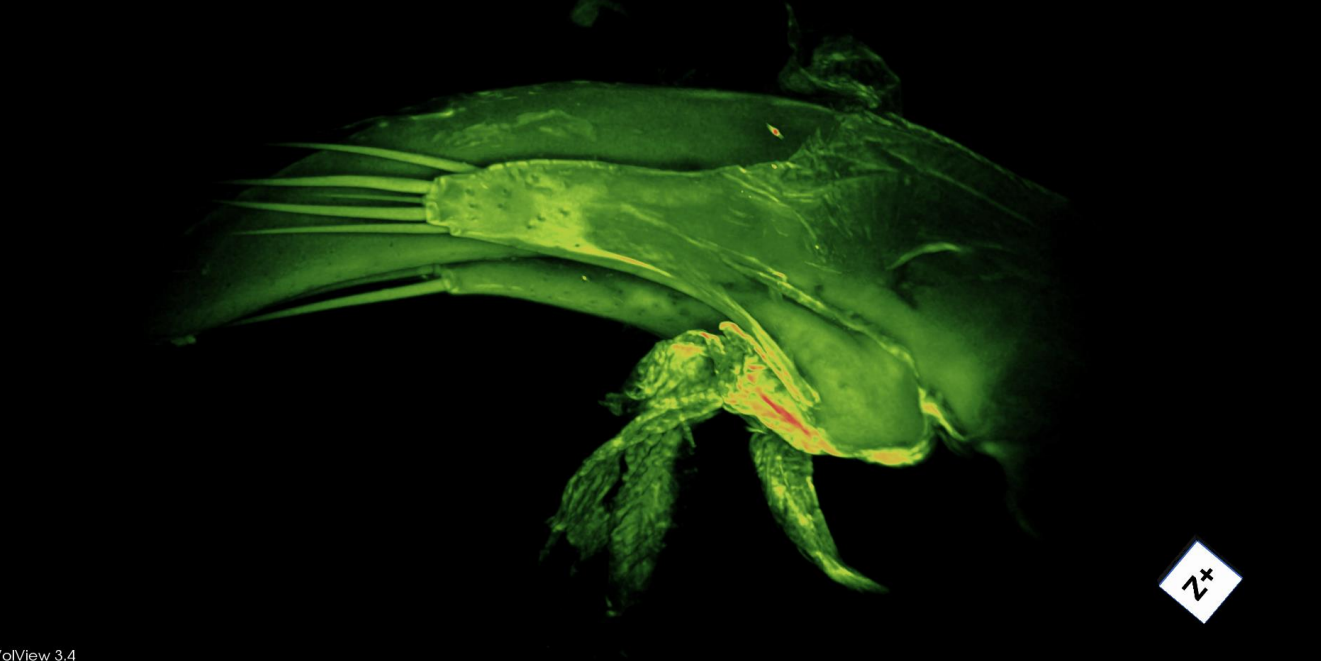


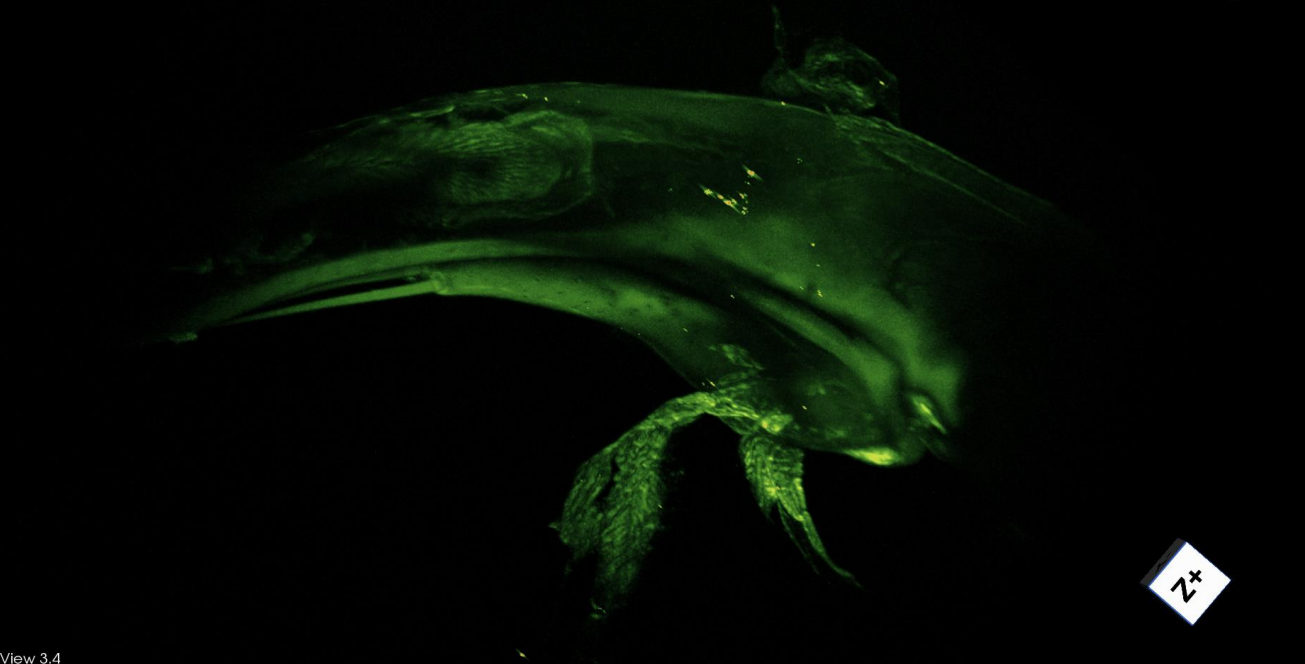
py)



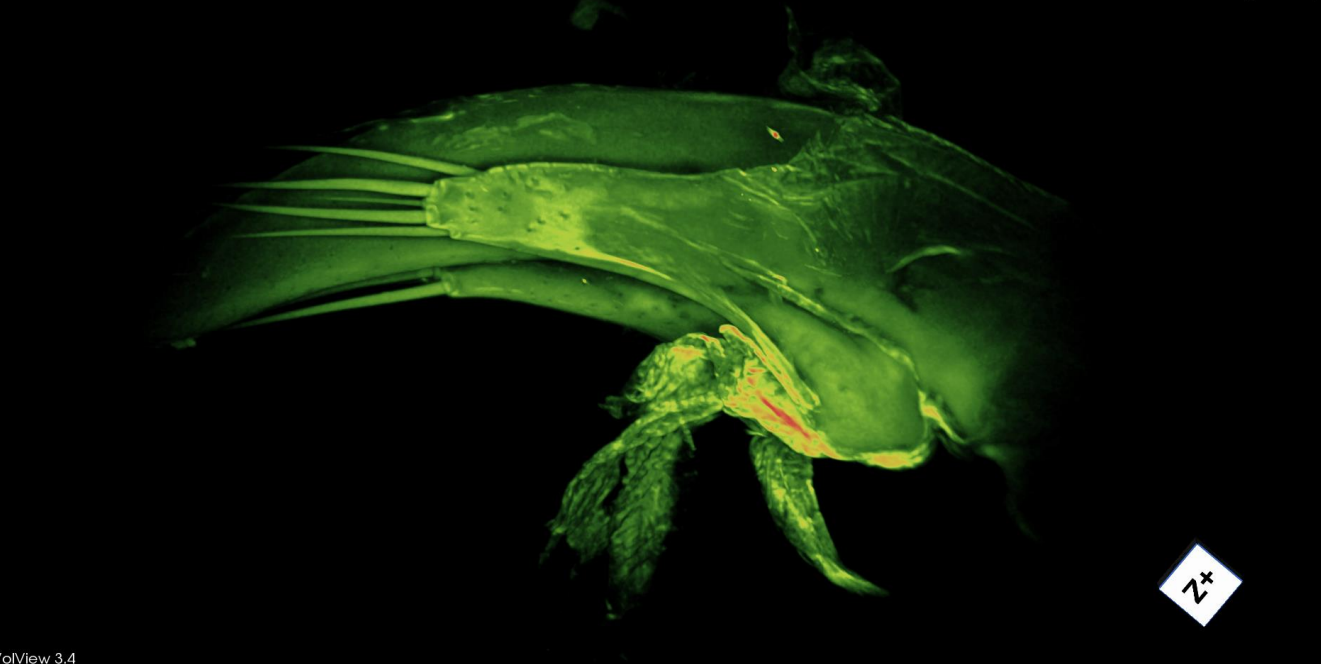
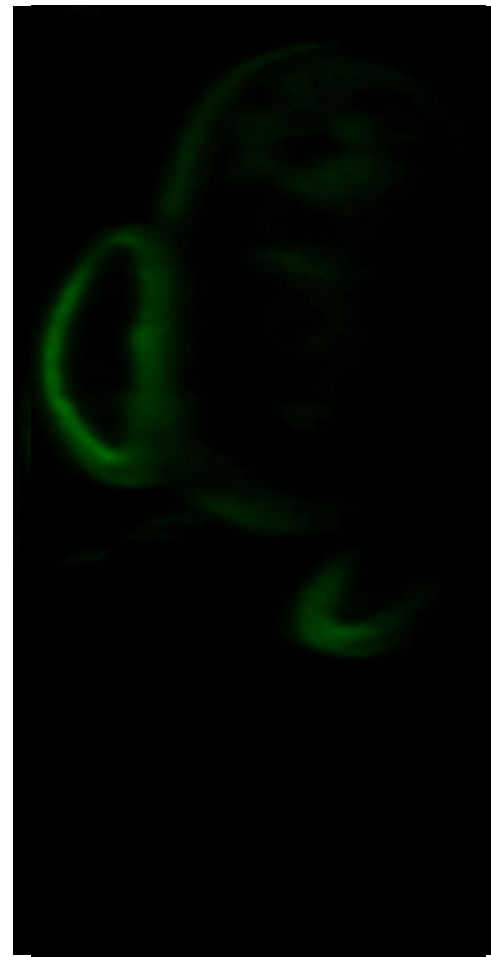


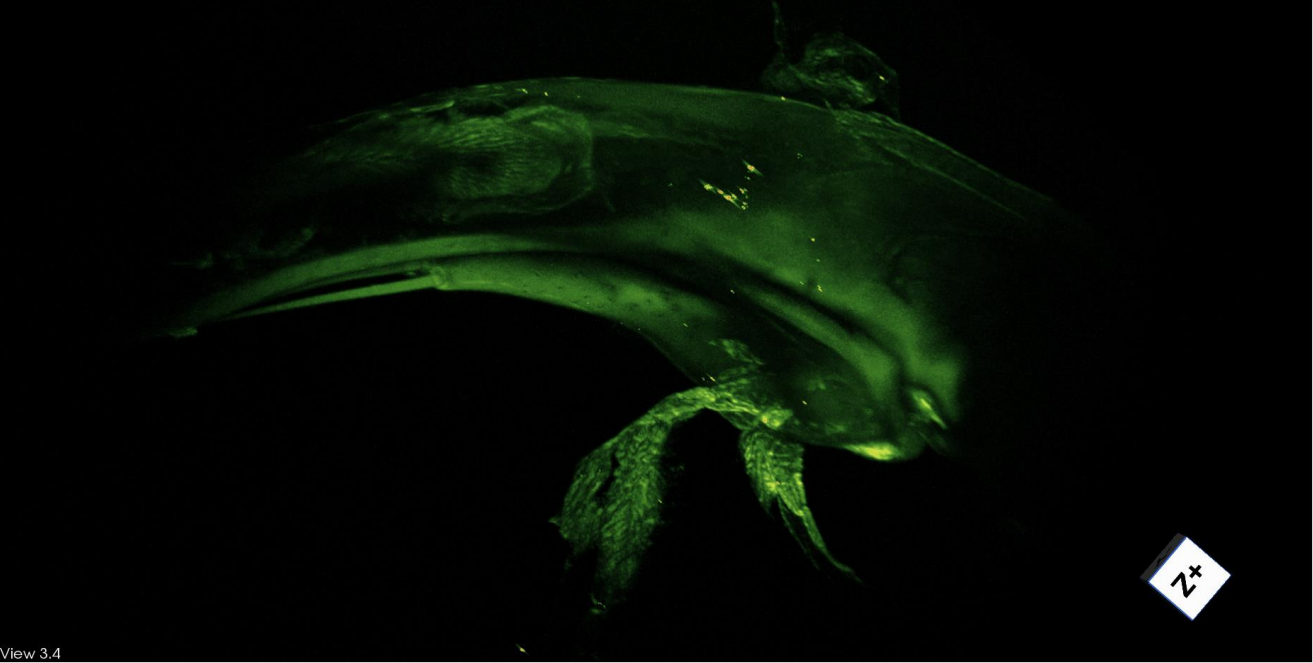
py)



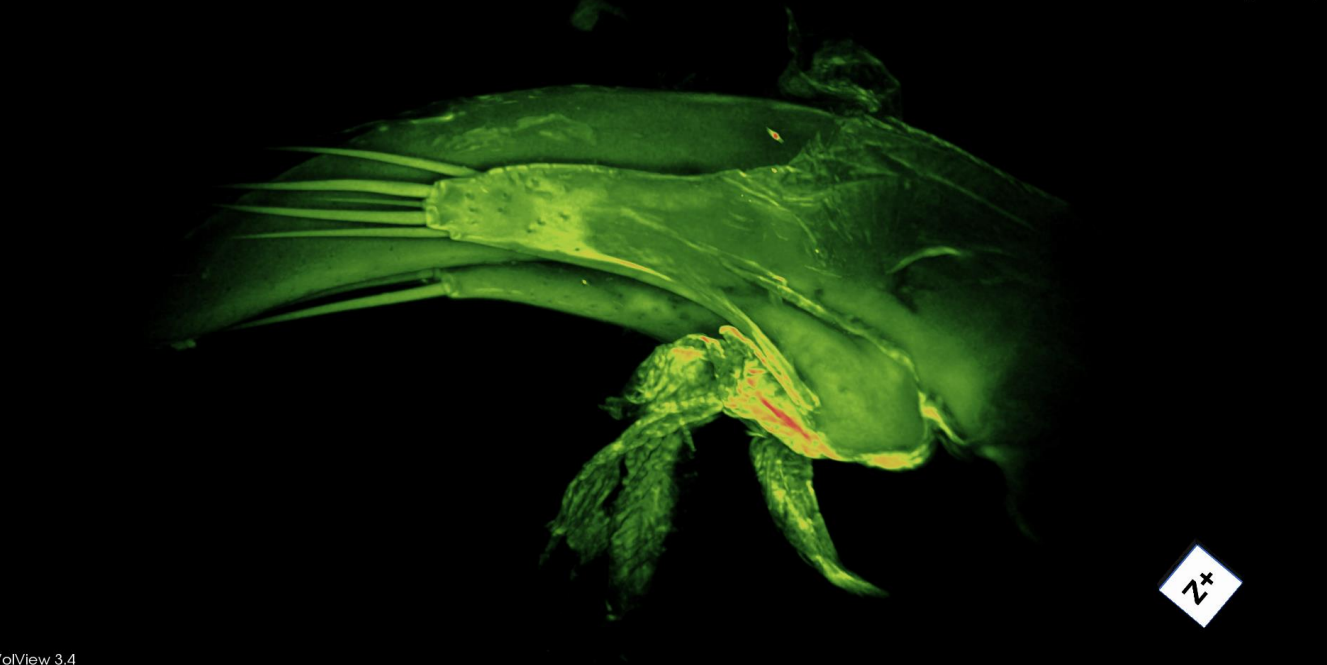


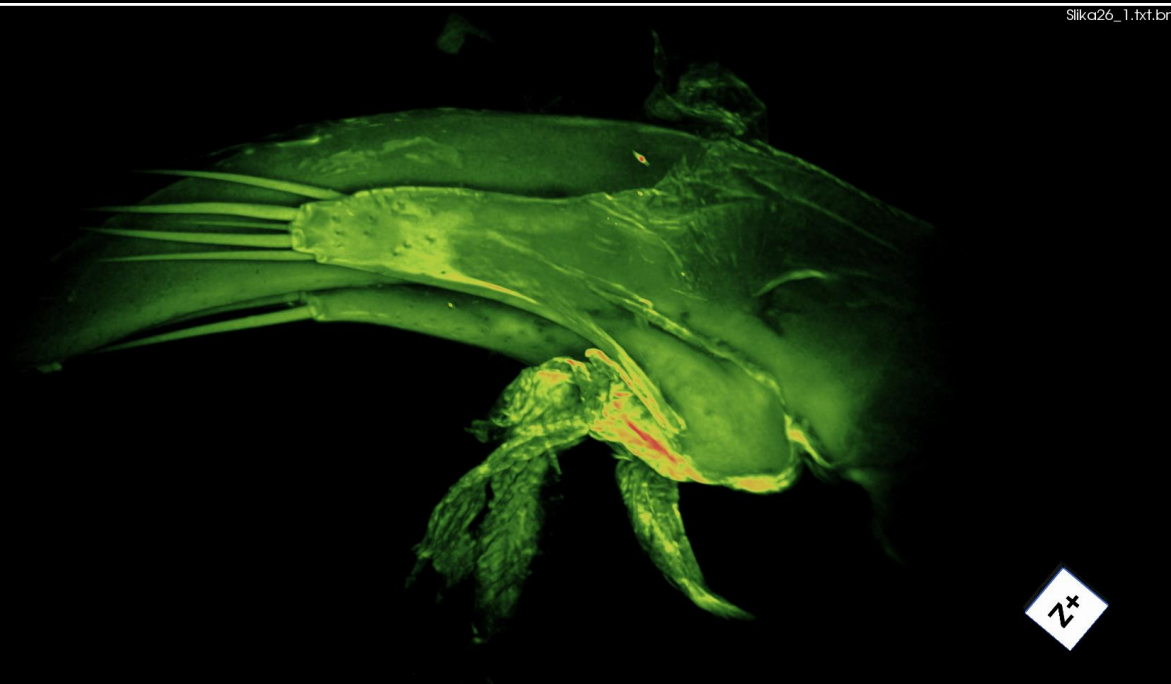
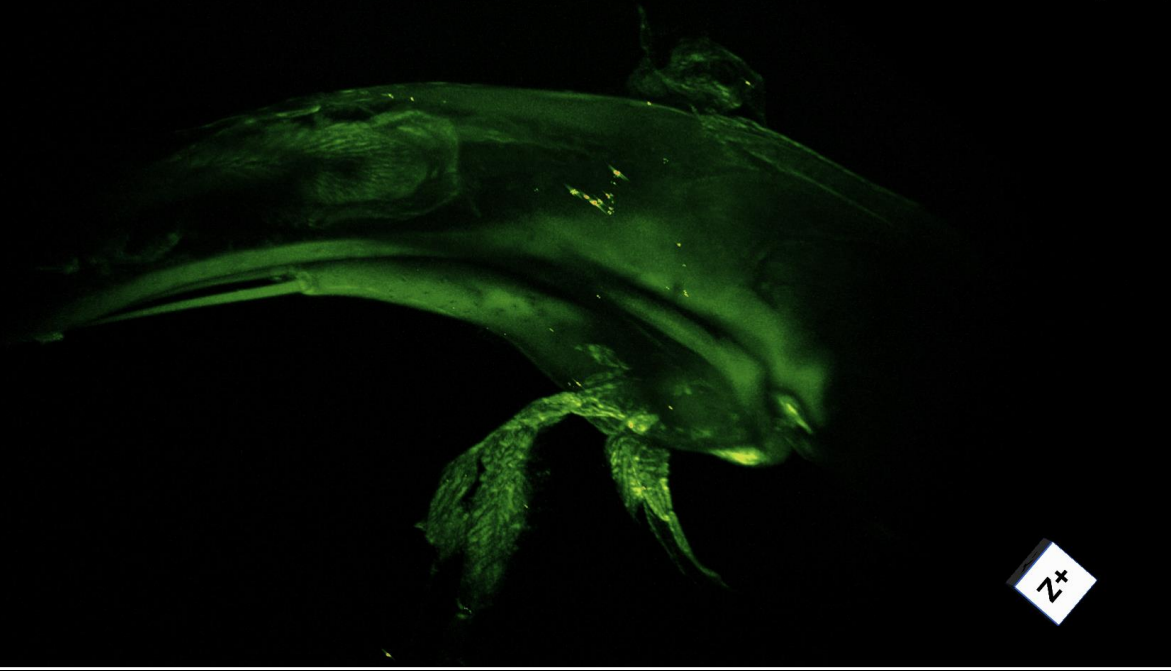
py)





py)



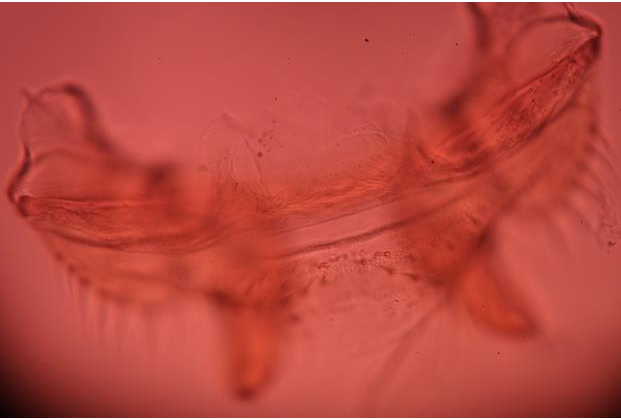


IMAGING AT TISSUE LEVEL – TPEF in entomology

Pheggomisetes ninae

female reproductive organ

Species determination key

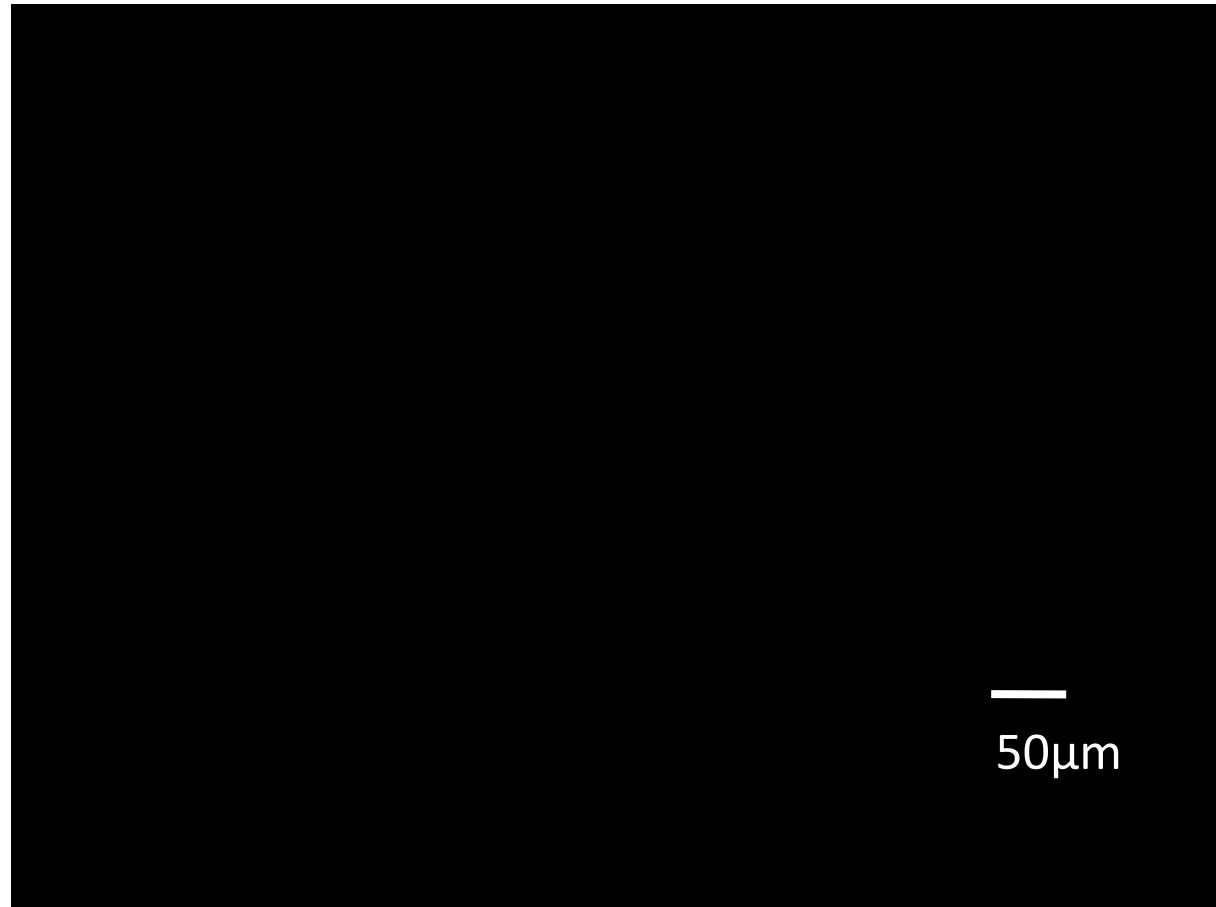
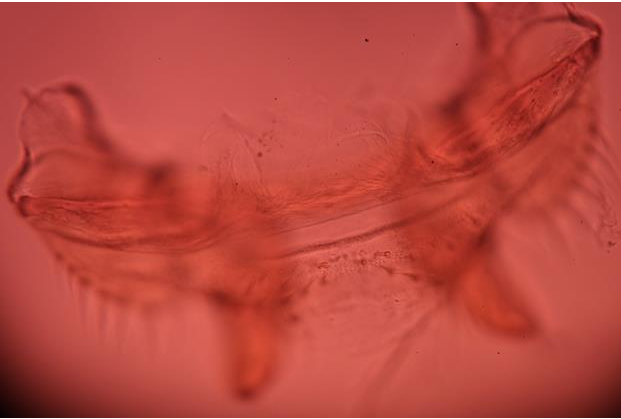


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female reproductive organ

Species determination key

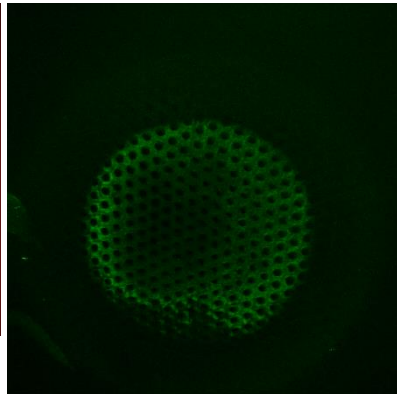
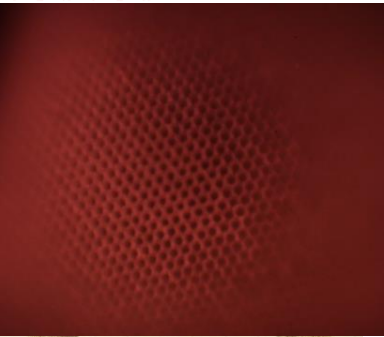


In vivo IMAGING (TPEF)

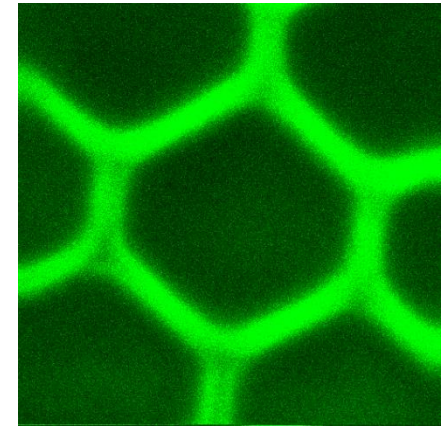
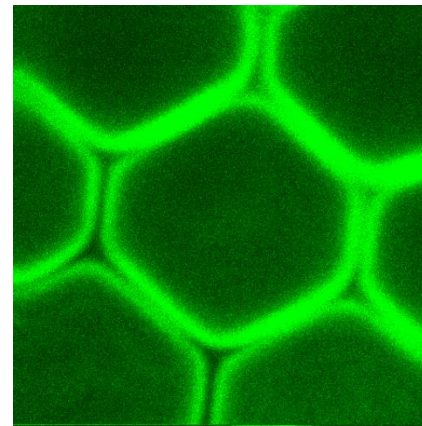
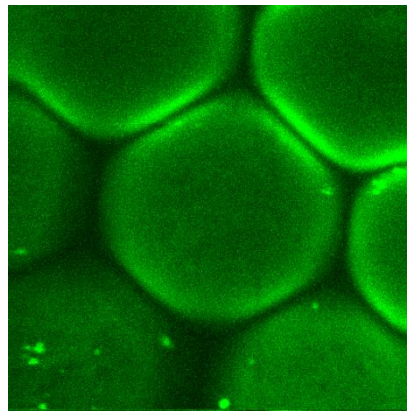
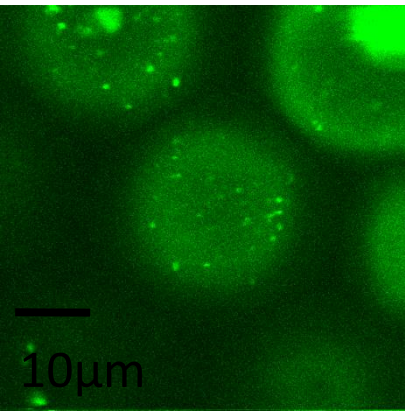
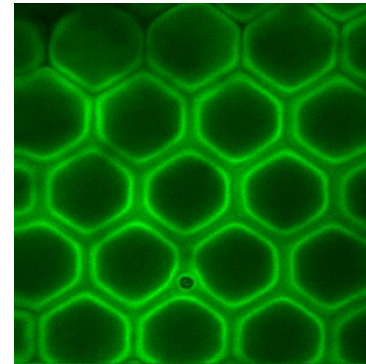
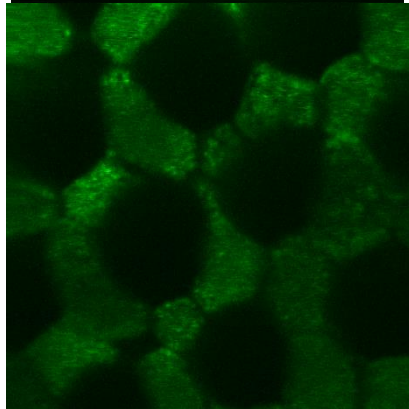
Lepidopterae (Butterflies and moths) Omatidia (Compound eyes)

Satyrus ferula

Habitat: South Europe, Morocco, Asia Minor,
Iran, Kazakhstan, Central Asia, Transbaikal,
West China and the Himalayasuth

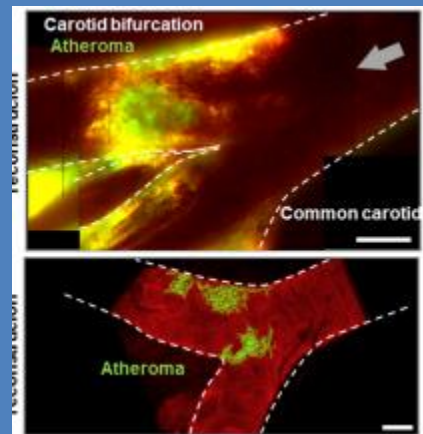


Pieris rapae (Small
cabbage white)



Deep penetration depth

- In vivo imaging of neuronal activity (voltage sensitive dyes)
- High-Resolution Imaging of Intravascular Atherogenic Inflammation in Live Mice (New Methods in Cardiovascular Biology, 2016). Tracking blood flow in carotid artery using TPEF



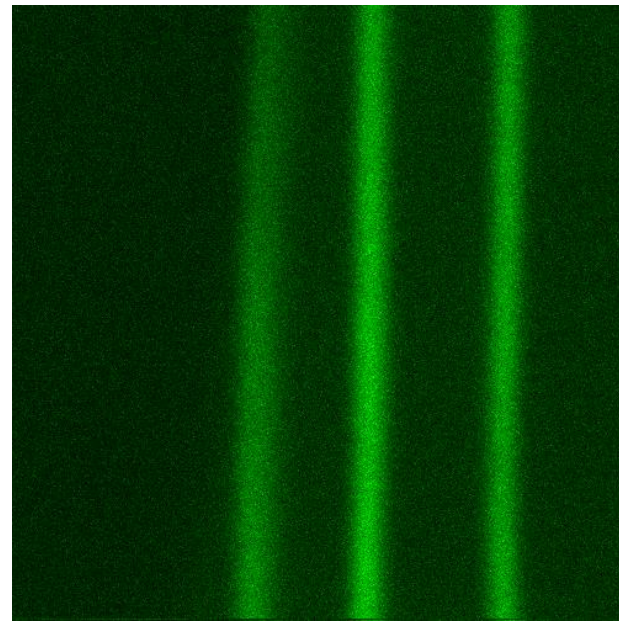
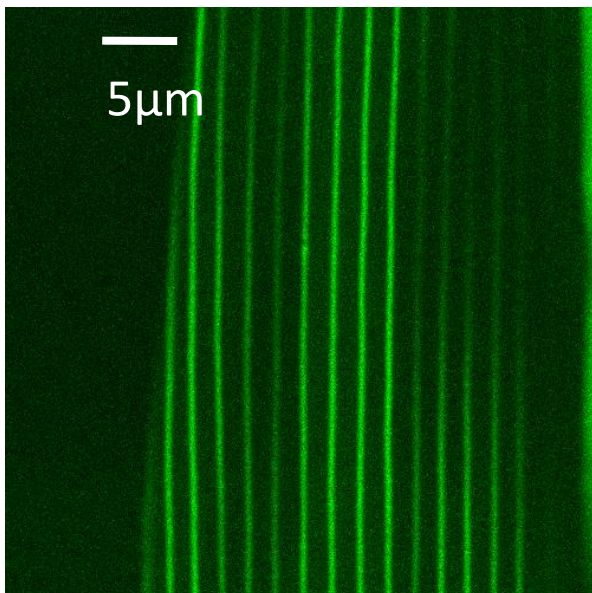
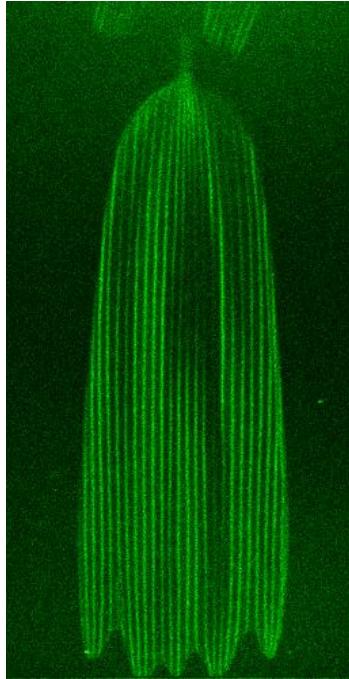
NATURAL PHOTONICS STRUCTURES

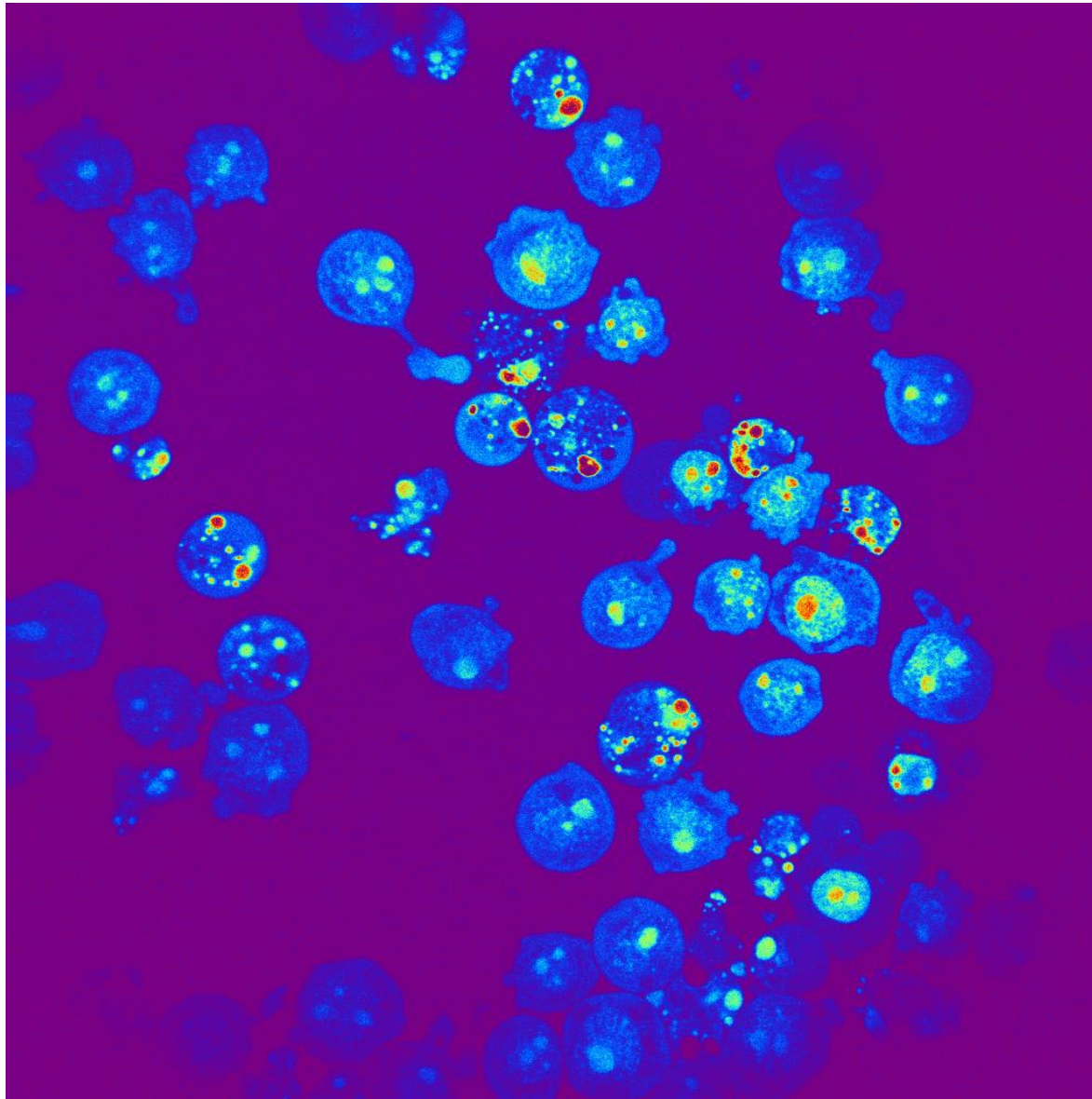
Lepidopterae (Butterflies and moths)

Wing's scales

Diachrisia chrysitis

Habitat: Europe, Caucasus, Russian far east, Siberia



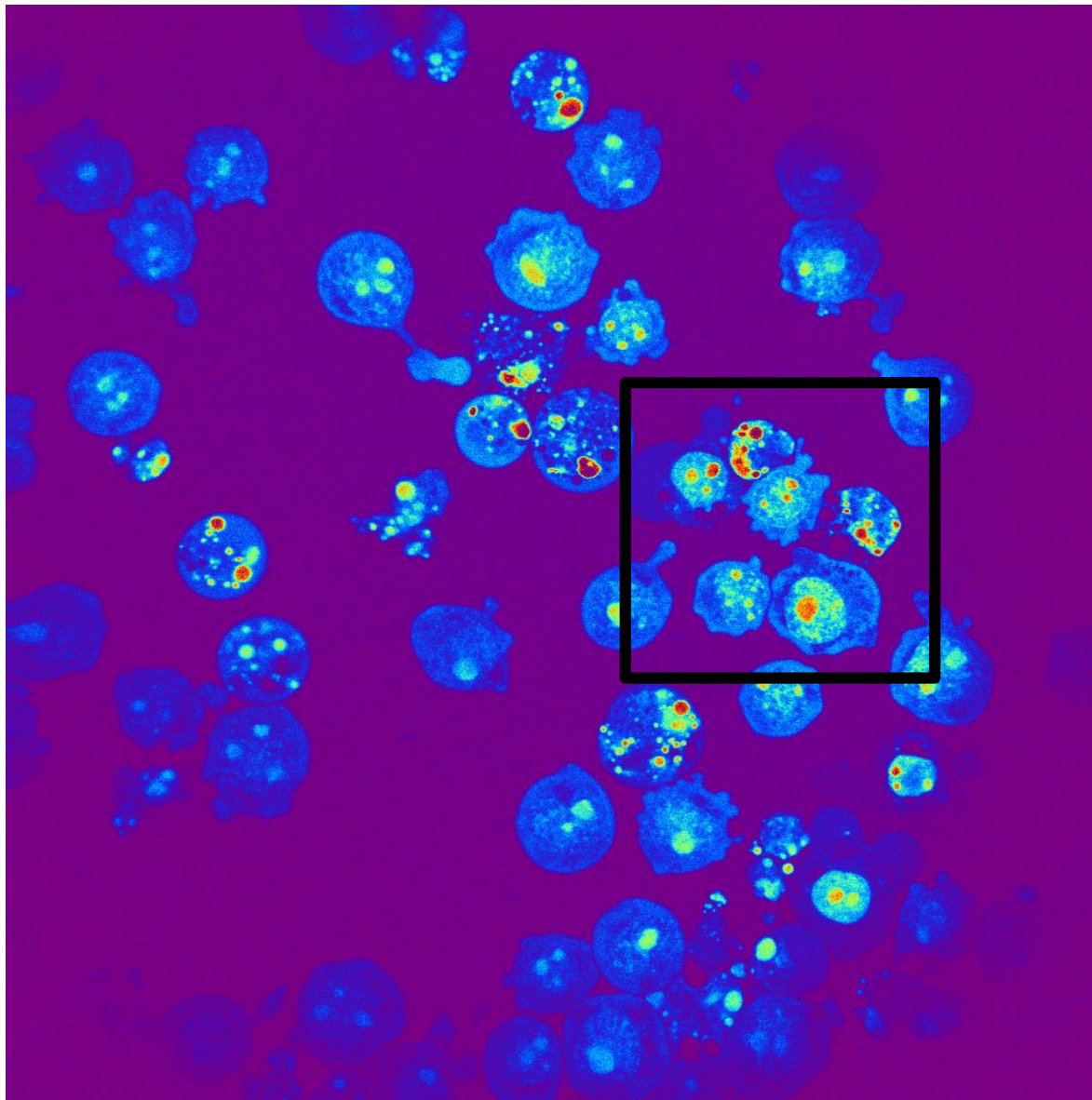


Apoptosis of HL 60 cancer cell

-Staining –
acridine orange

-Reduced
photobleaching =>
prolongued
exposition

Collaboration with Medical
school, University of
Belgrade

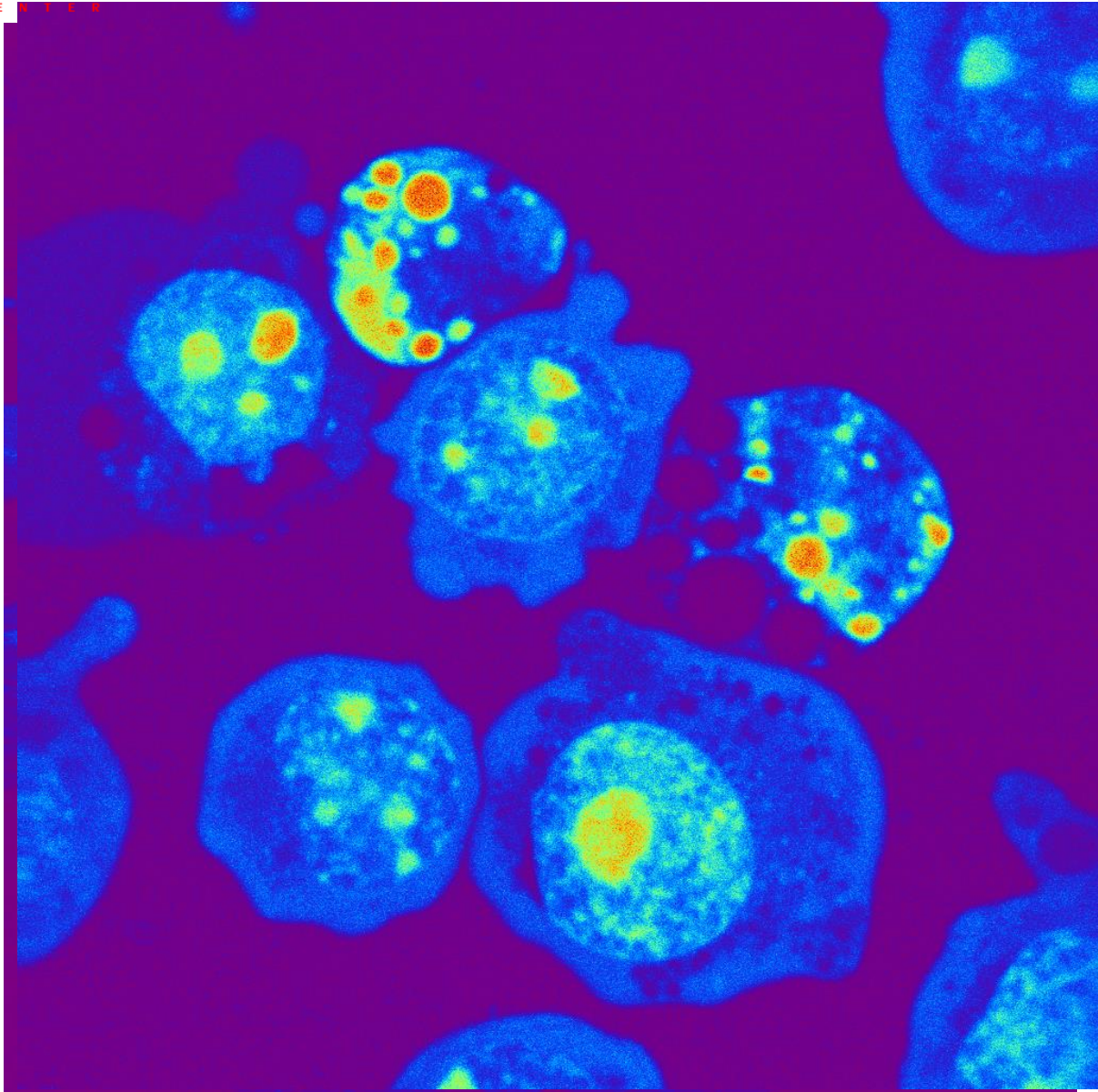


Apoptosis of HL 60 cancer cell

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Apoptosis of HL 60 cancer cell

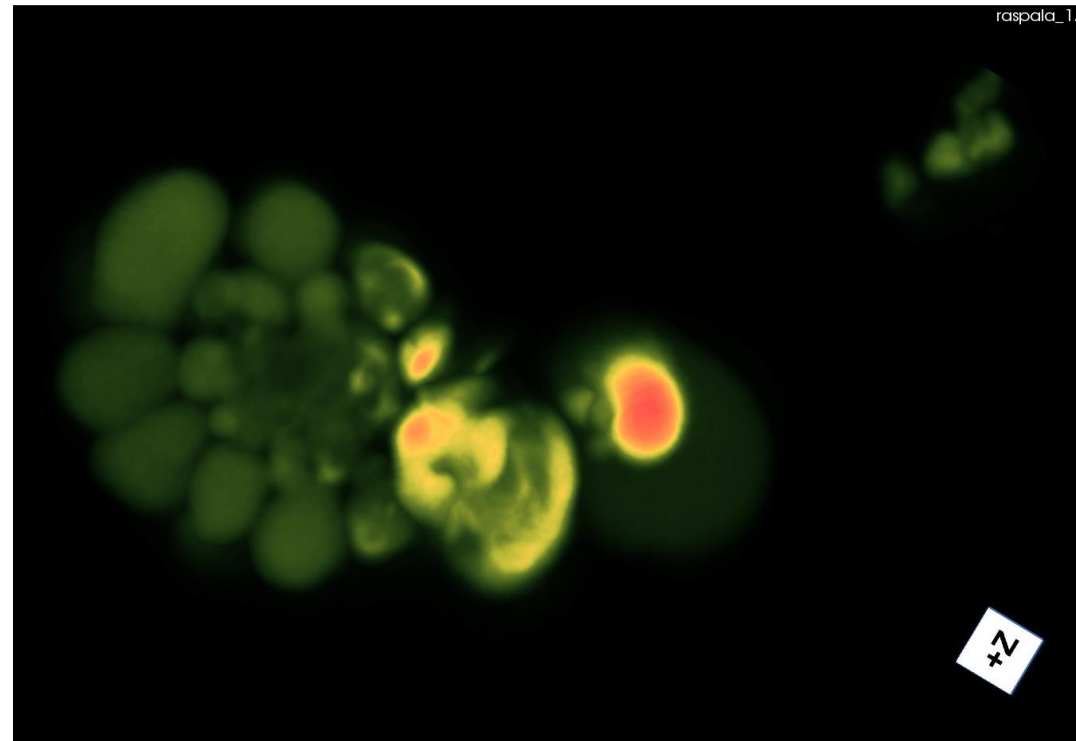
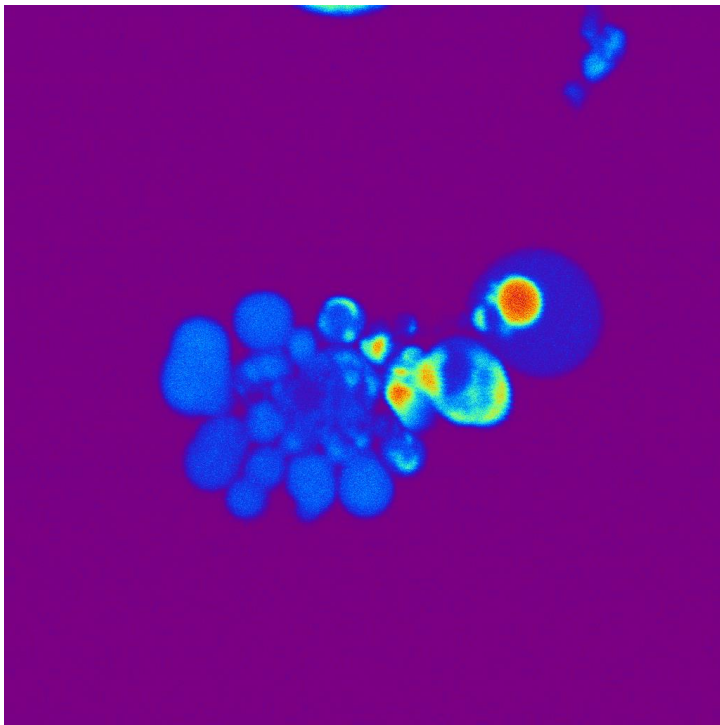
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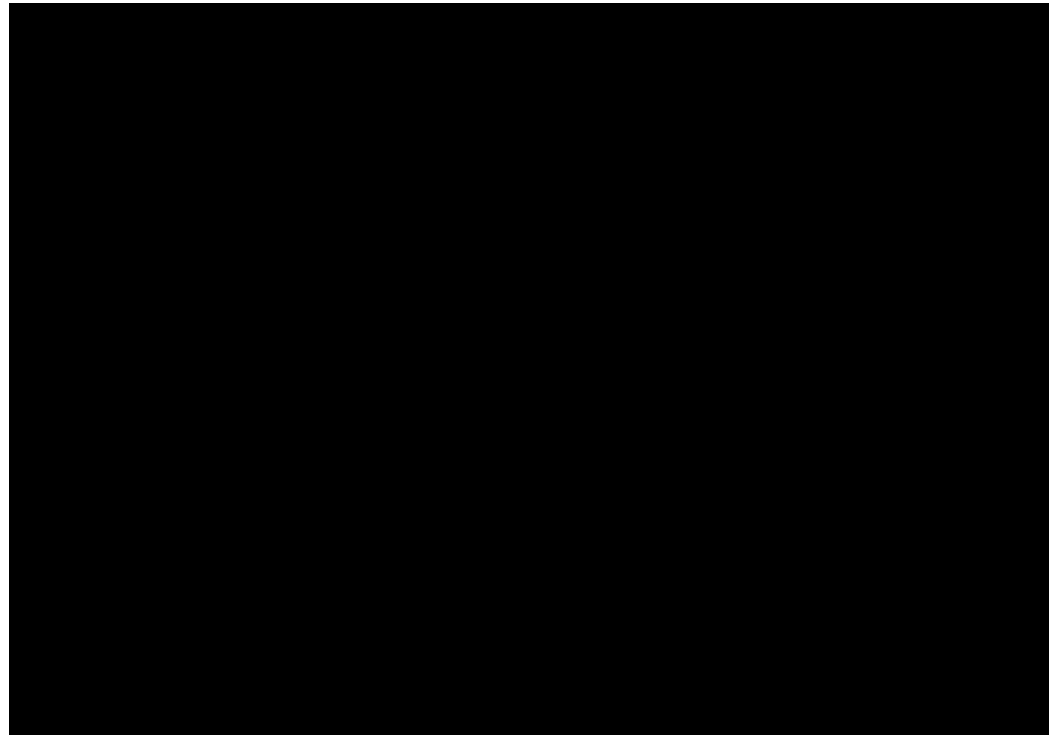
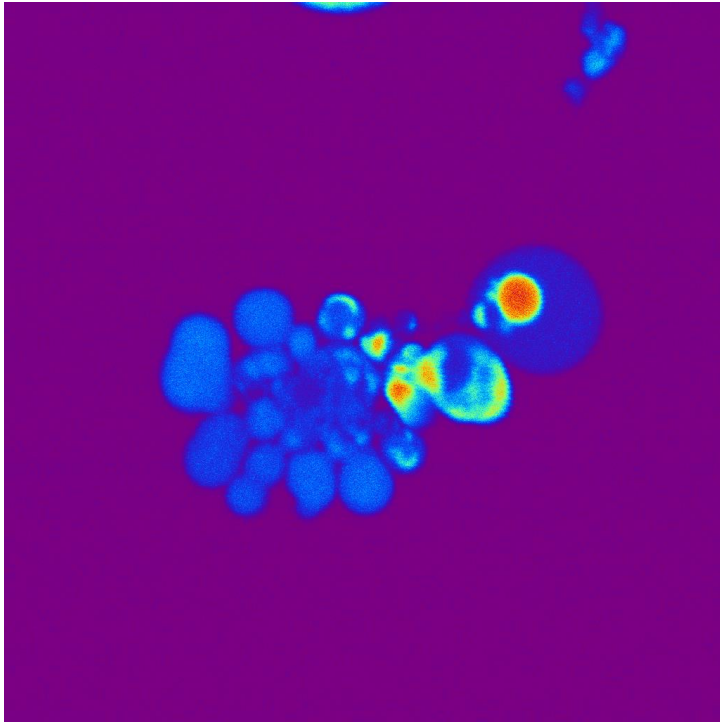
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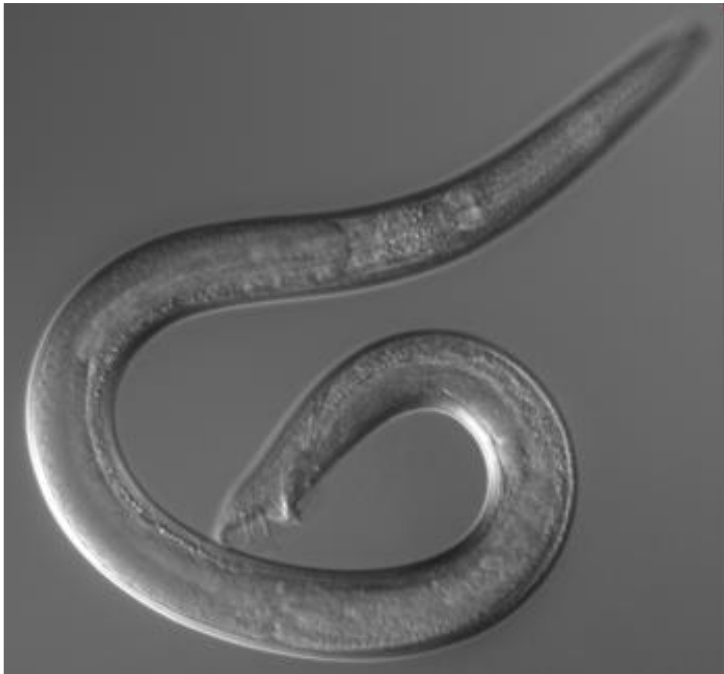


Apoptosis of HL 60 cancer cell

-Staining – acridine orange

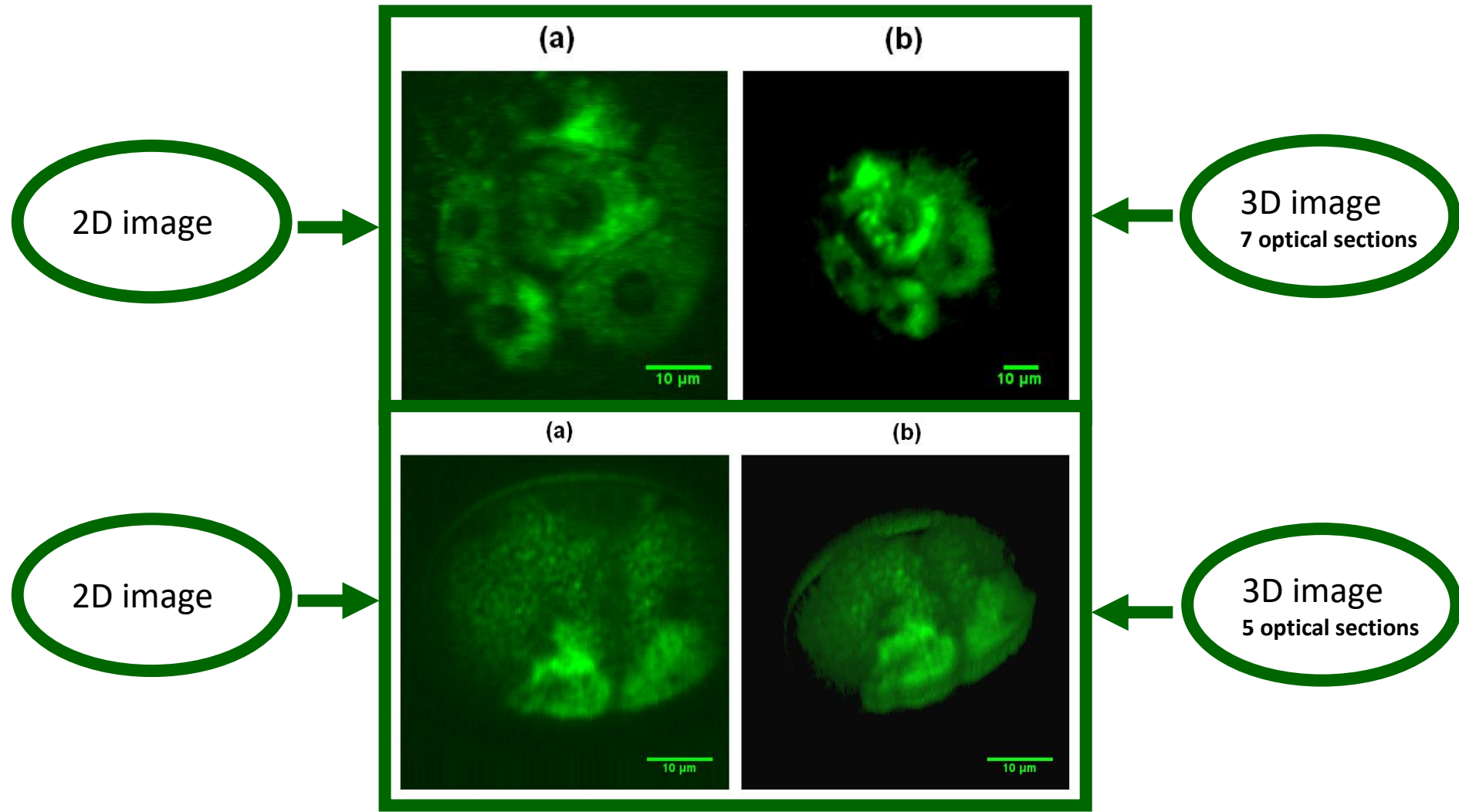


Caenorhabditis Elegans model organism



- micron dimensions, transparent
- well known
- Embryo stadiums: a) bean b) comma c) 1.5-fold d) 2-fold e) 3-fold

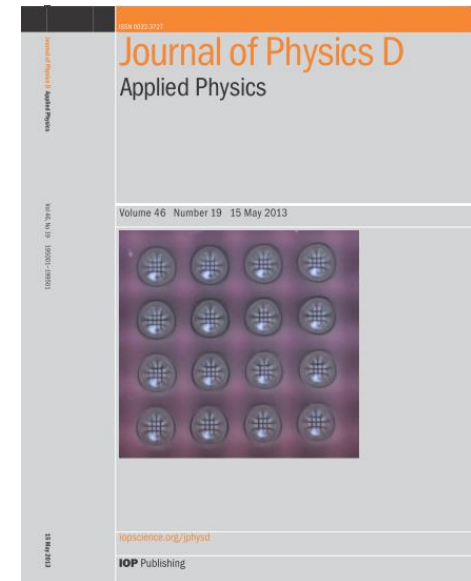
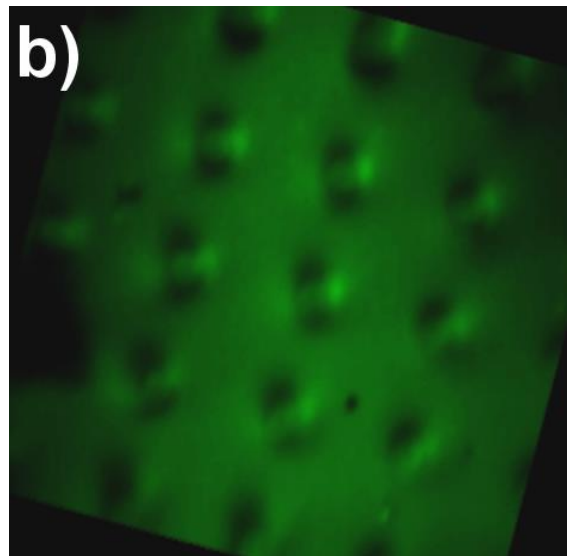
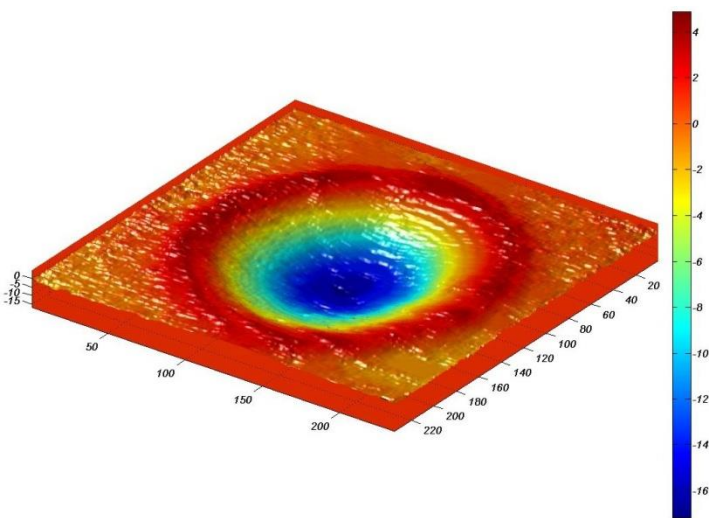
Different stadiums of C.Elegans embriogenesis obtained by THG detection



G. Tservlakis et al, Micron, 2010

Marie Curie Transfer of Knowledge (TOK) Fellowship, **NOLIMBA** (**NO**n **L**inear **I**maging at **M**icroscopic level for **B**iological **A**pplications) project

- THG imaging and characterization of microlenses
- Soft biomaterial development for micro lenses arrays fabrication (Tot'Hema Eosin Sensitized Gelatin – TESG)
- NLM for micro lenses characterization



National:

- Prof. Dr. Pavle Andjus**, Dept for Physiology & Biochemistry, Faculty of Biology, University of Belgrade – advanced microscopic techniques for neurodegenerative diseases studies
- Prof. Dr. Vladimir Trajković**, Medical faculty, University of Belgrade – Laser induced autofagy and NLM application in cancer cells death study
- **Dr. Srećko Ćurčić**, Institute of Zoology, Faculty of Biology, University of Belgrade – NLM in entomology
- Prof. dr. Milica Labudović**, Histology department, Medical Faculty, University of Belgrade– SHG imaging of collagen structures in tissues
- Dr. Aleksandra Divac Rankov, Dr. Aleksandra Nikolić**, Institute for Molecular Genetics and Genetical Engineering, University of Belgrade– NLM microscopy for in vivo Zebra fish imaging
- Dr. Vesna Ilić, Dr. Ivana Kostić**, Technological Faculty, University of Belgrade – label free imaging of erythrocytes

International

- Prof. Dr. Vladana Vukojević and Prof. Dr. Rudolf Rigler**, CMM, Karolinska Institute, Stockholm, Sweden – development of multifocal correlation spectroscopy experiment
- Prof. Dr. Jerker Widengren**, Royal Technical University KTH, Stockholm, Sweden – development of CARS microscopy experiment
- **Prof. Dr. Costas Fotakis, Dr. George Filippidis, Dr. George Tservelakis** (currently at Technische Universität München) IESL FORTH, Heraklion, Greece – THG microscopy in embryogenesis of nematodes, THG microscopy in microlenses characterization
- **Dr. Bojan Resan**, Time-Bandwidth Products AG, Zurich, Switzerland

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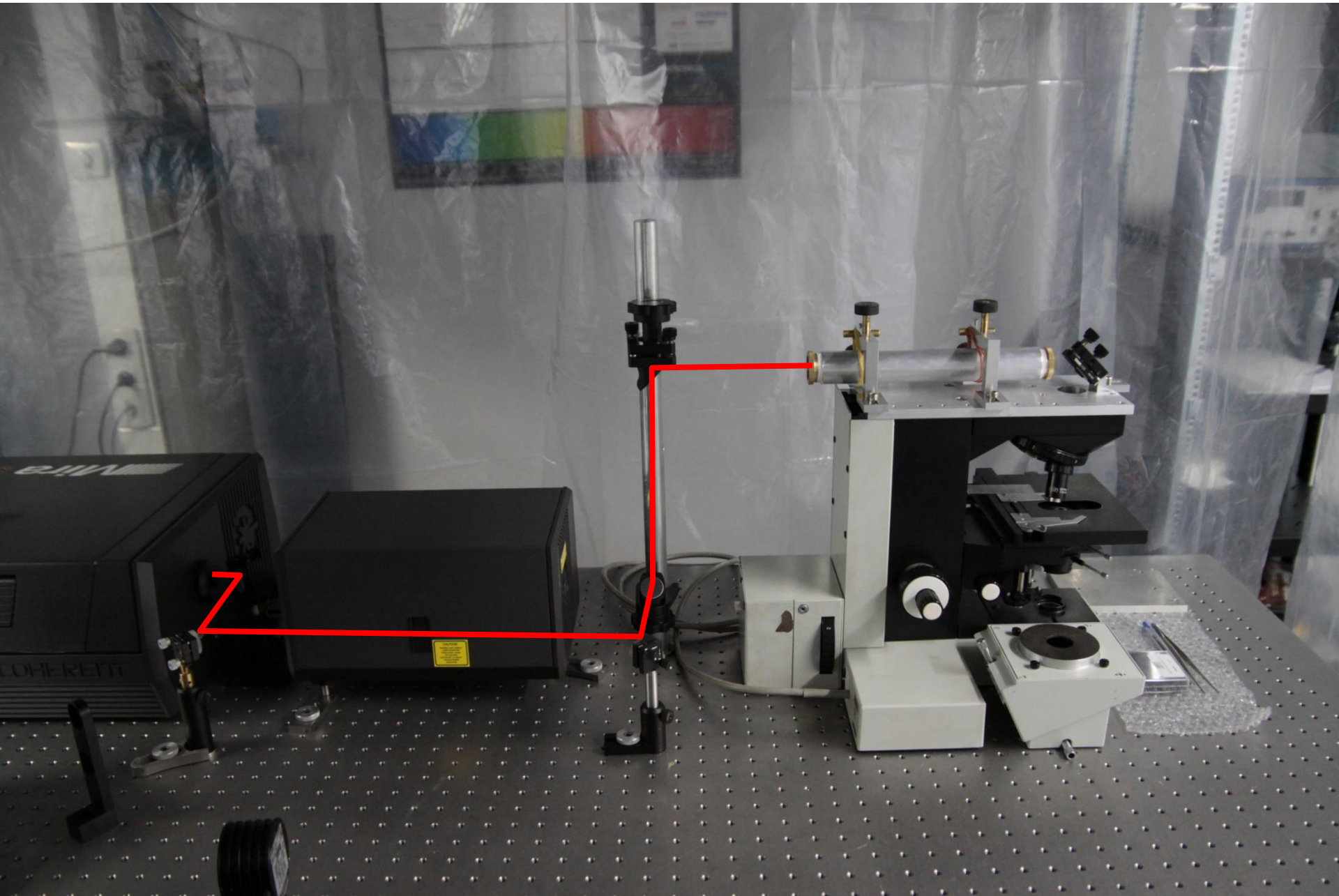
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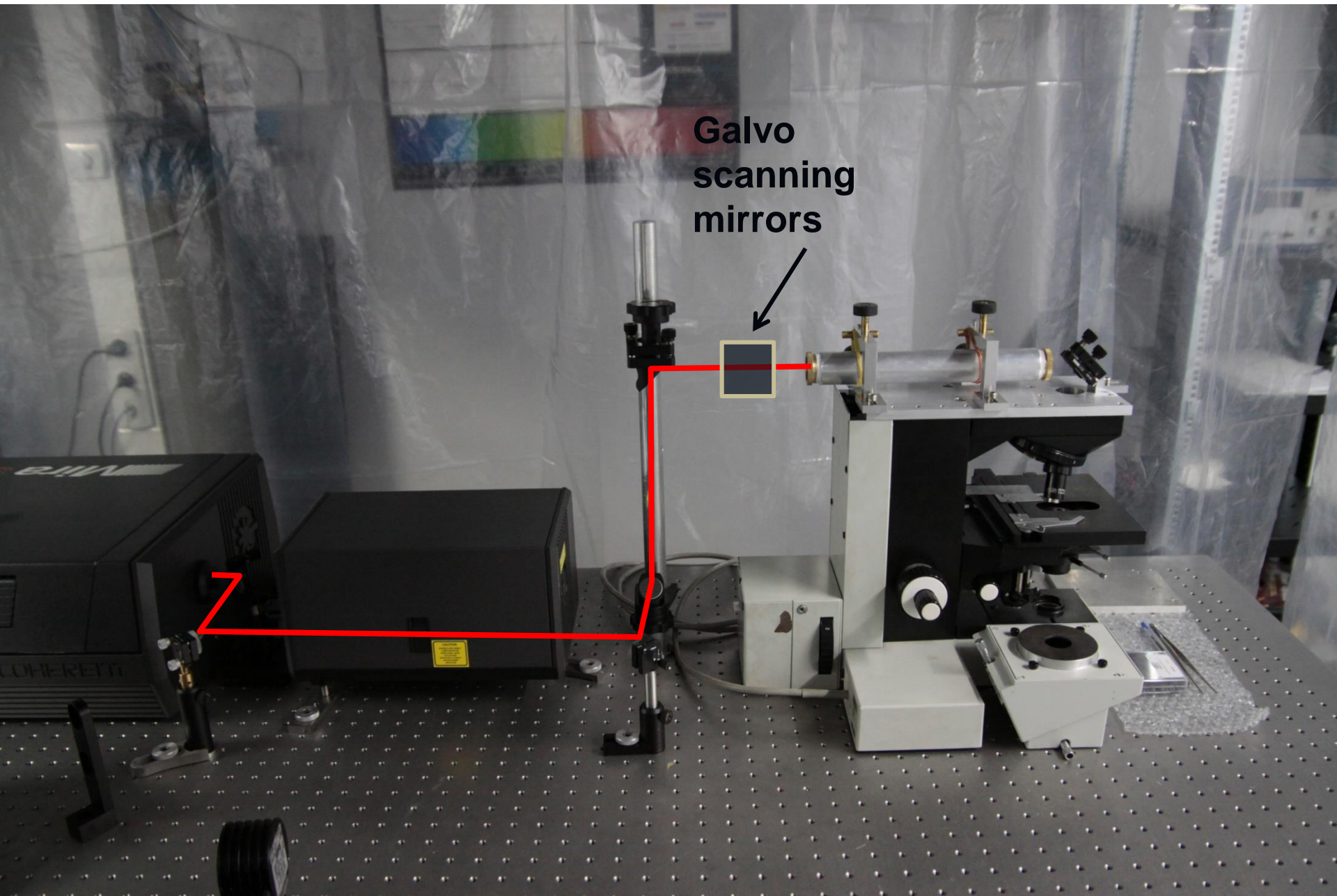
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BUILDING UP THE EXPERIMENT

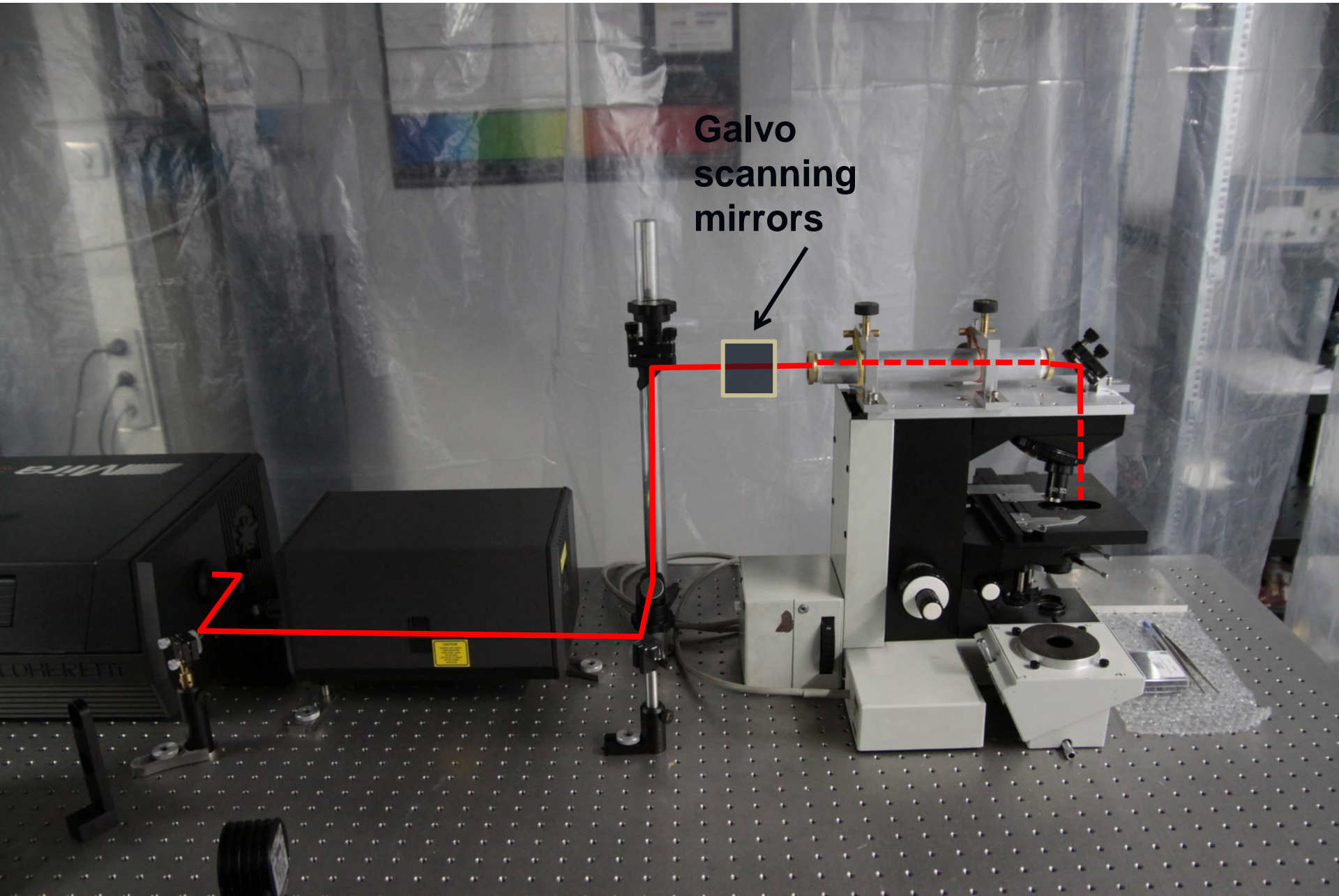


BUILDING UP THE EXPERIMENT



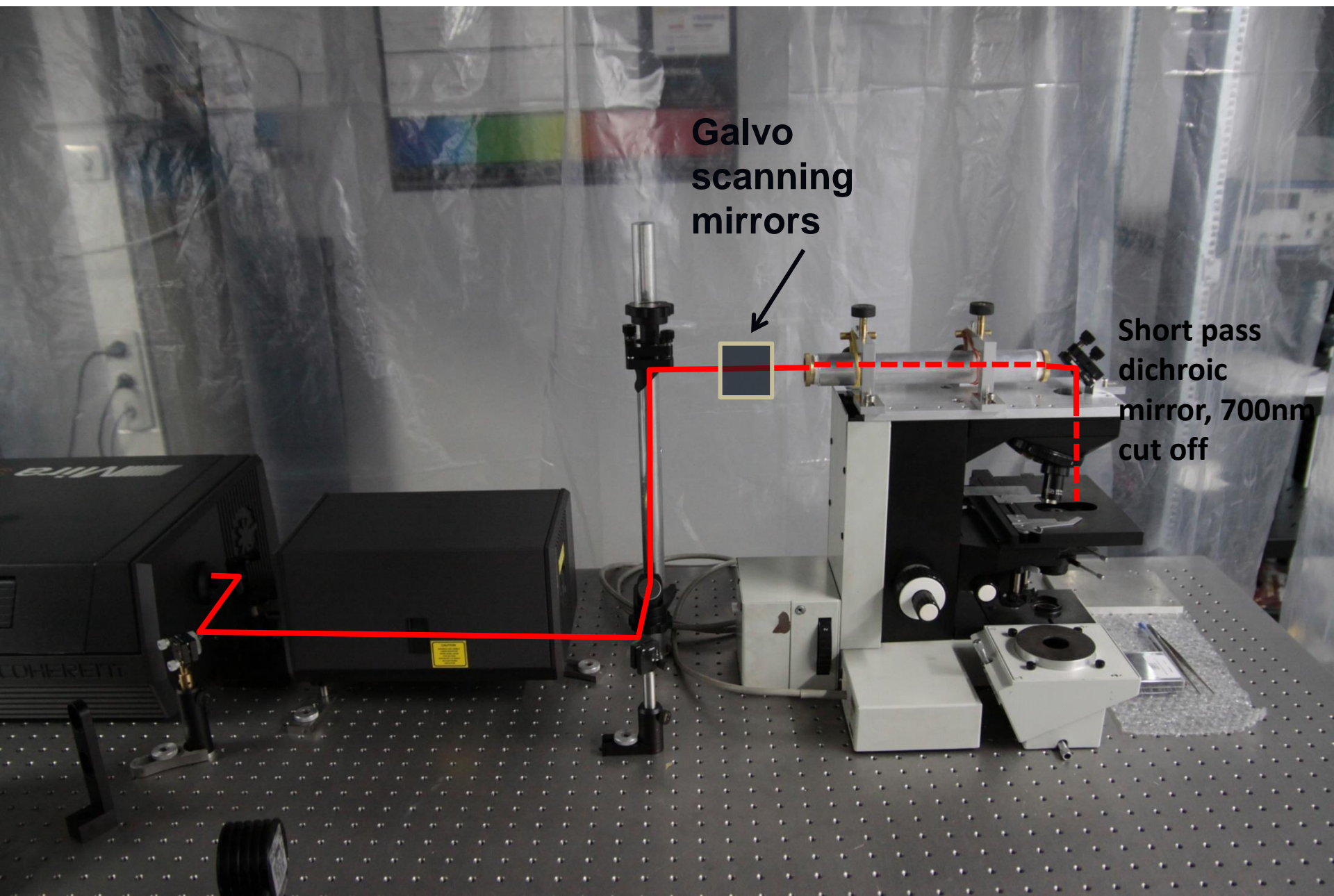
Galvo
scanning
mirrors

BUILDING UP THE EXPERIMENT



Galvo
scanning
mirrors

BUILDING UP THE EXPERIMENT

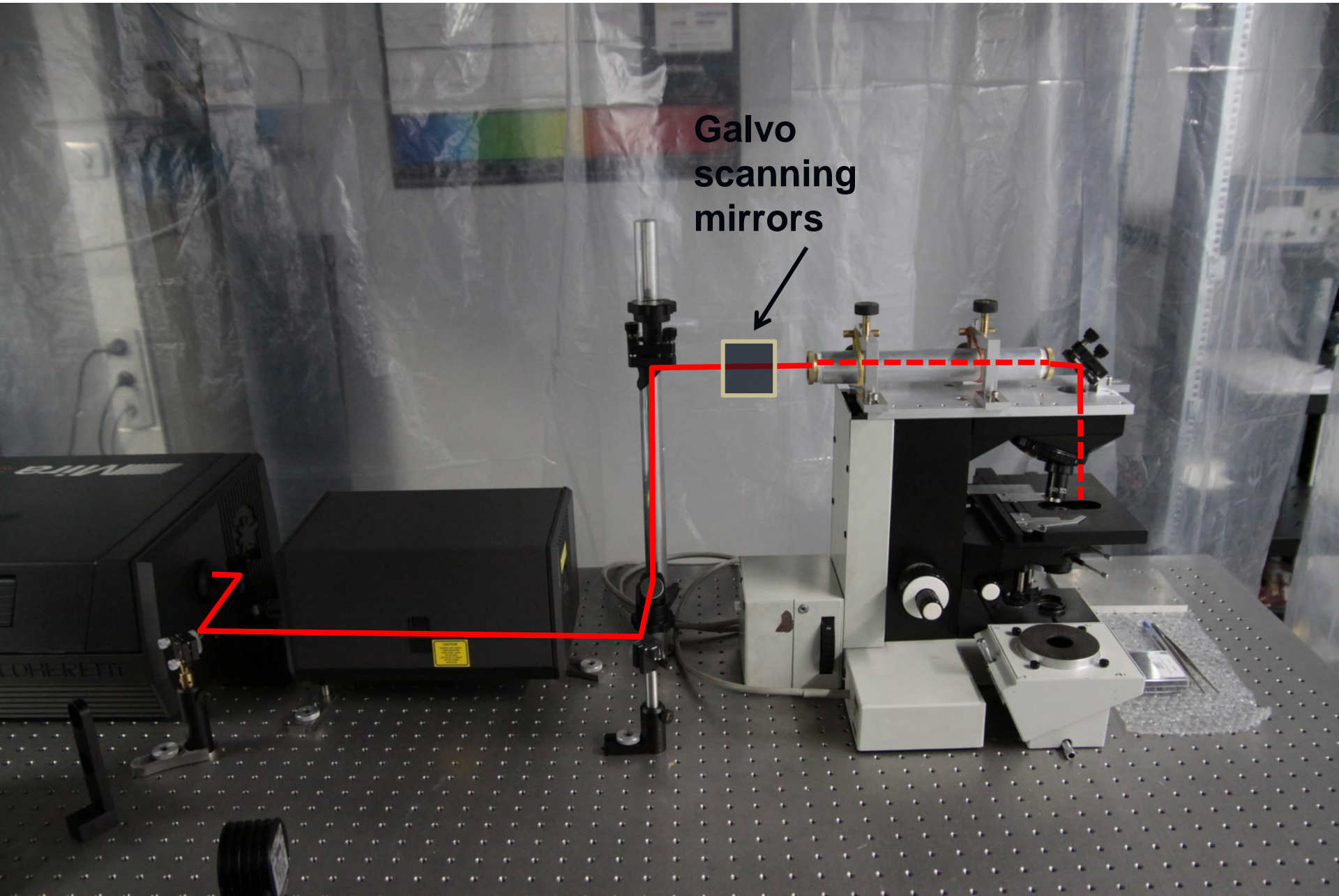


**Galvo
scanning
mirrors**



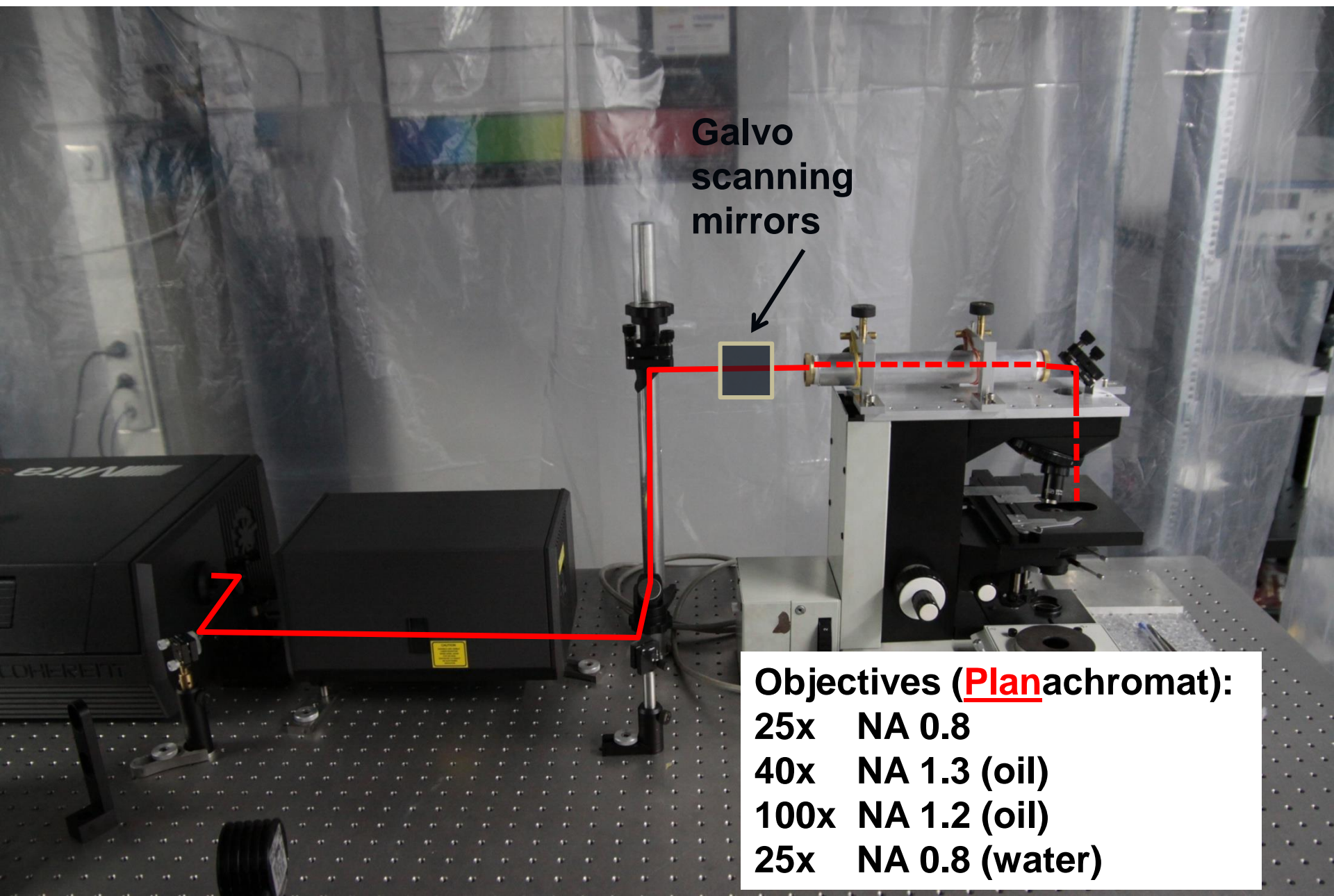
**Short pass
dichroic
mirror, 700nm
cut off**

BUILDING UP THE EXPERIMENT



Galvo
scanning
mirrors

BUILDING UP THE EXPERIMENT



Galvo
scanning
mirrors

Objectives (Planachromat):

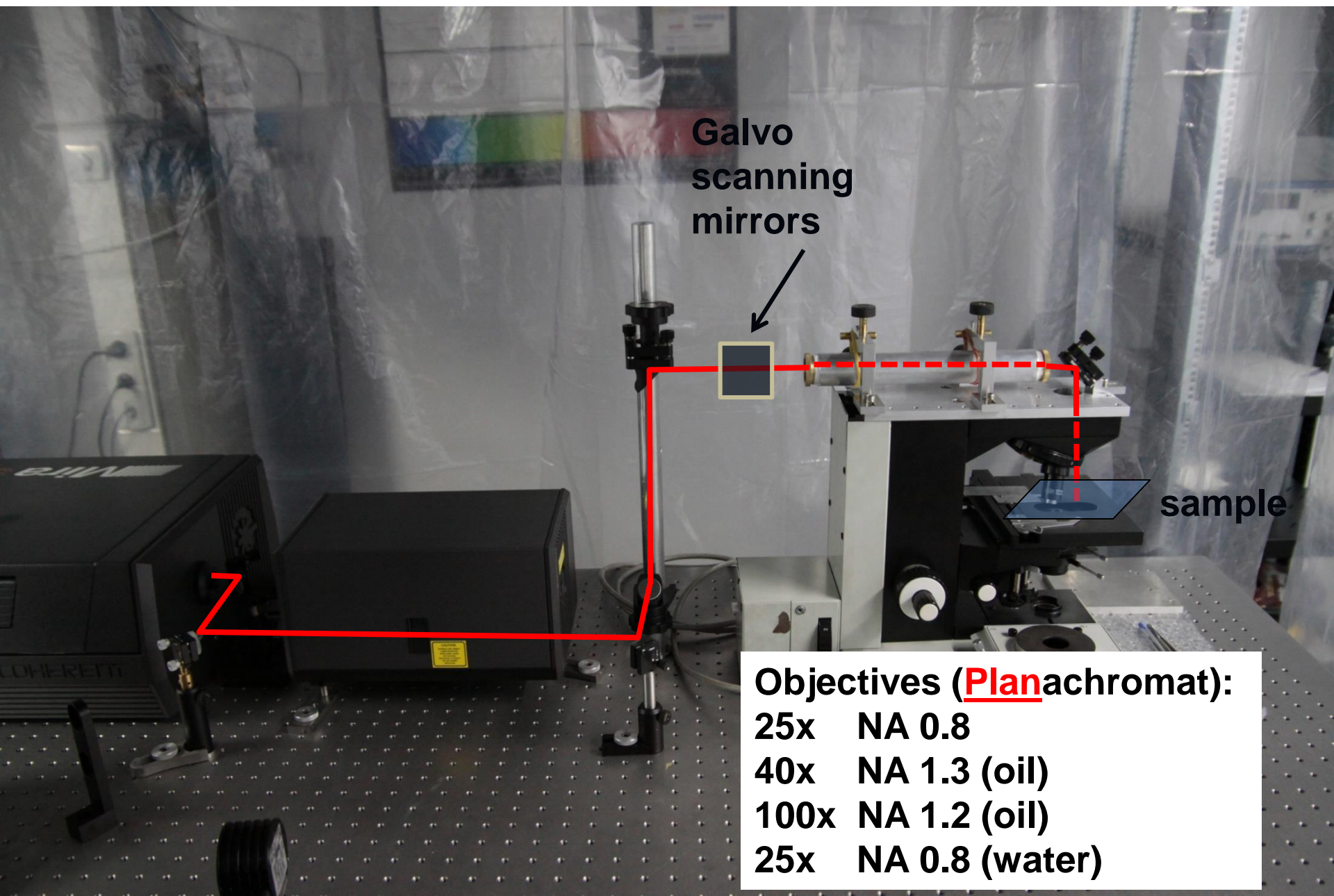
25x NA 0.8

40x NA 1.3 (oil)

100x NA 1.2 (oil)

25x NA 0.8 (water)

BUILDING UP THE EXPERIMENT



Galvo
scanning
mirrors

sample

Objectives (Planachromat):

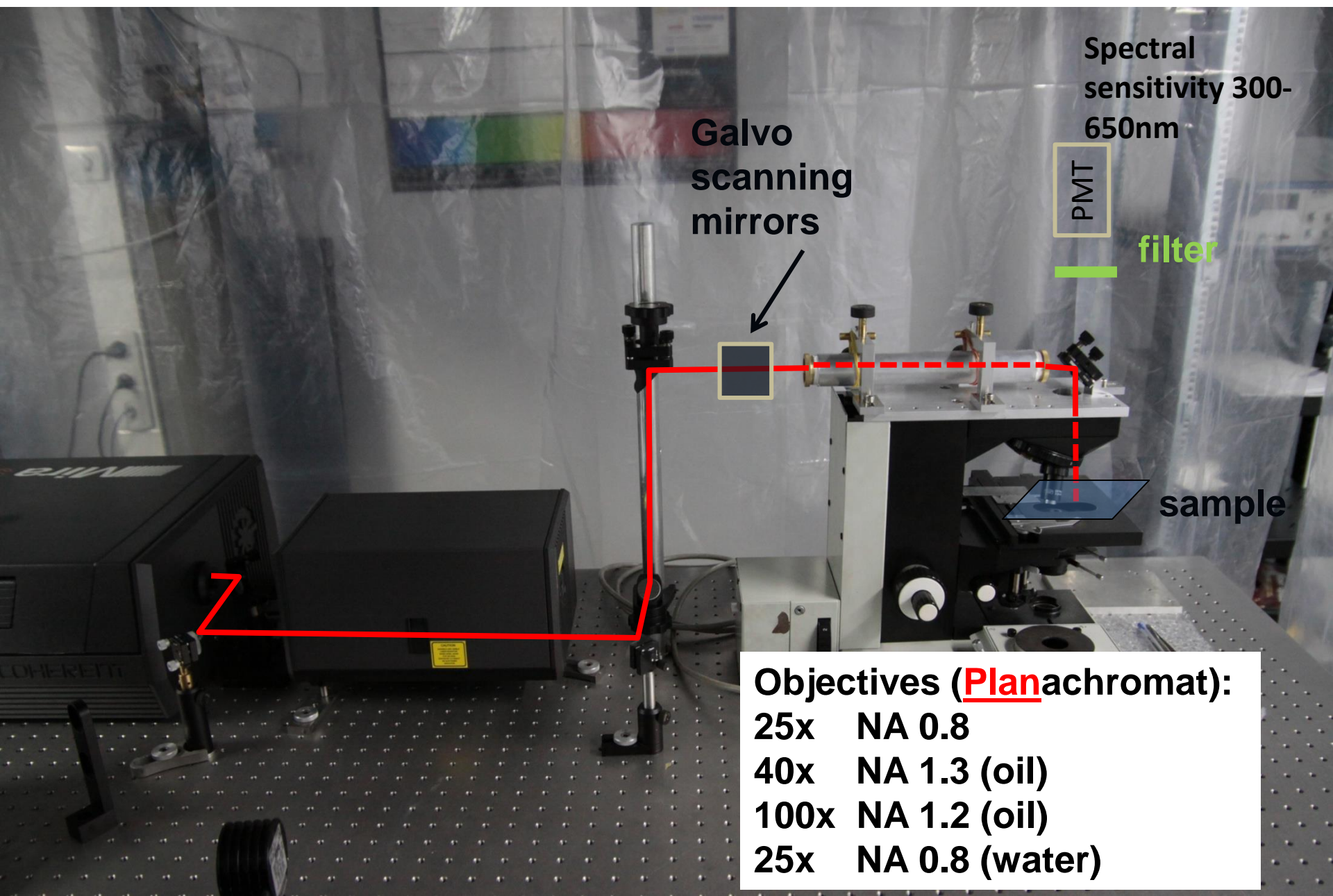
25x NA 0.8

40x NA 1.3 (oil)

100x NA 1.2 (oil)

25x NA 0.8 (water)

BUILDING UP THE EXPERIMENT



Spectral sensitivity 300-650nm

Galvo scanning mirrors

PMT

filter

sample

Objectives (Planachromat):

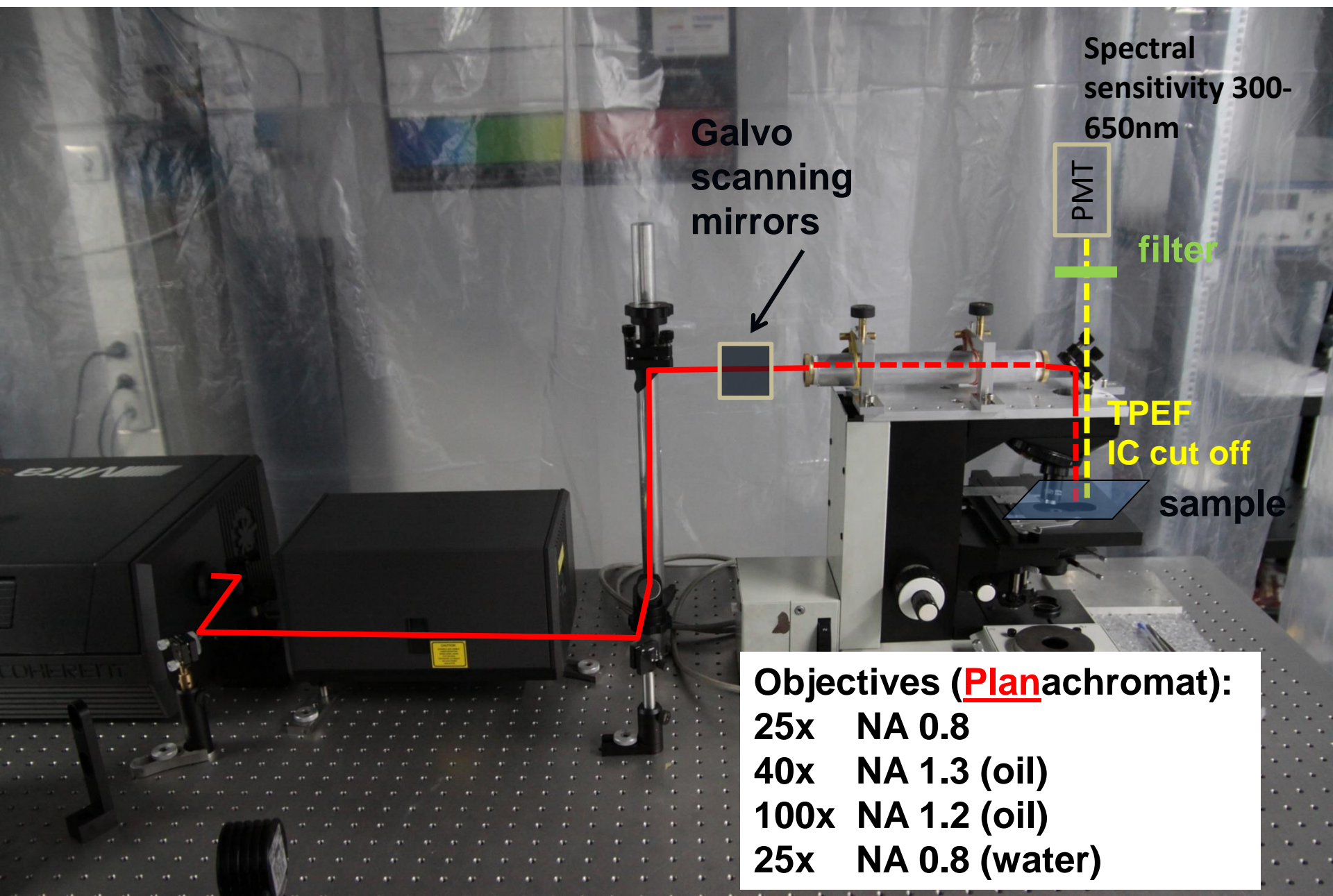
25x NA 0.8

40x NA 1.3 (oil)

100x NA 1.2 (oil)

25x NA 0.8 (water)

BUILDING UP THE EXPERIMENT



Galvo scanning mirrors

Spectral sensitivity 300-650nm

PMT

filter

TPEF

IC cut off

sample

Objectives (Planachromat):

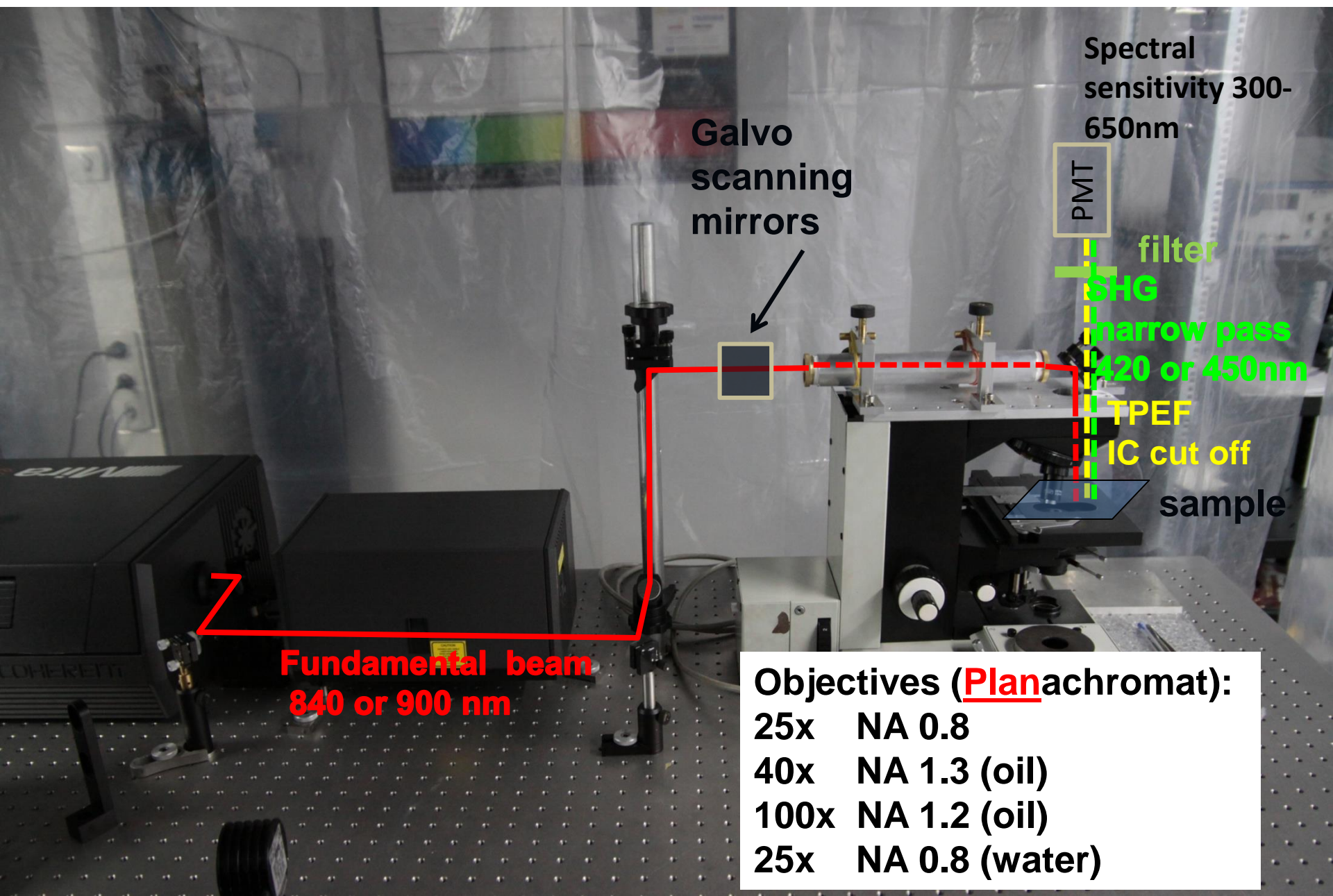
25x NA 0.8

40x NA 1.3 (oil)

100x NA 1.2 (oil)

25x NA 0.8 (water)

BUILDING UP THE EXPERIMENT



Spectral sensitivity 300-650nm

Galvo scanning mirrors

PMT

filter

SHG

narrow pass 420 or 450nm

TPEF

IC cut off

sample

Fundamental beam 840 or 900 nm

Objectives (Planachromat):
25x NA 0.8
40x NA 1.3 (oil)
100x NA 1.2 (oil)
25x NA 0.8 (water)