Imaging Report

Holographic and Nonlinear Microscopy of Astrocyte Cultures and Rat Spinal Cord Sections

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Objectives

Investigating the potential of imaging rat astrocyte cultures and spinal cord sections using:

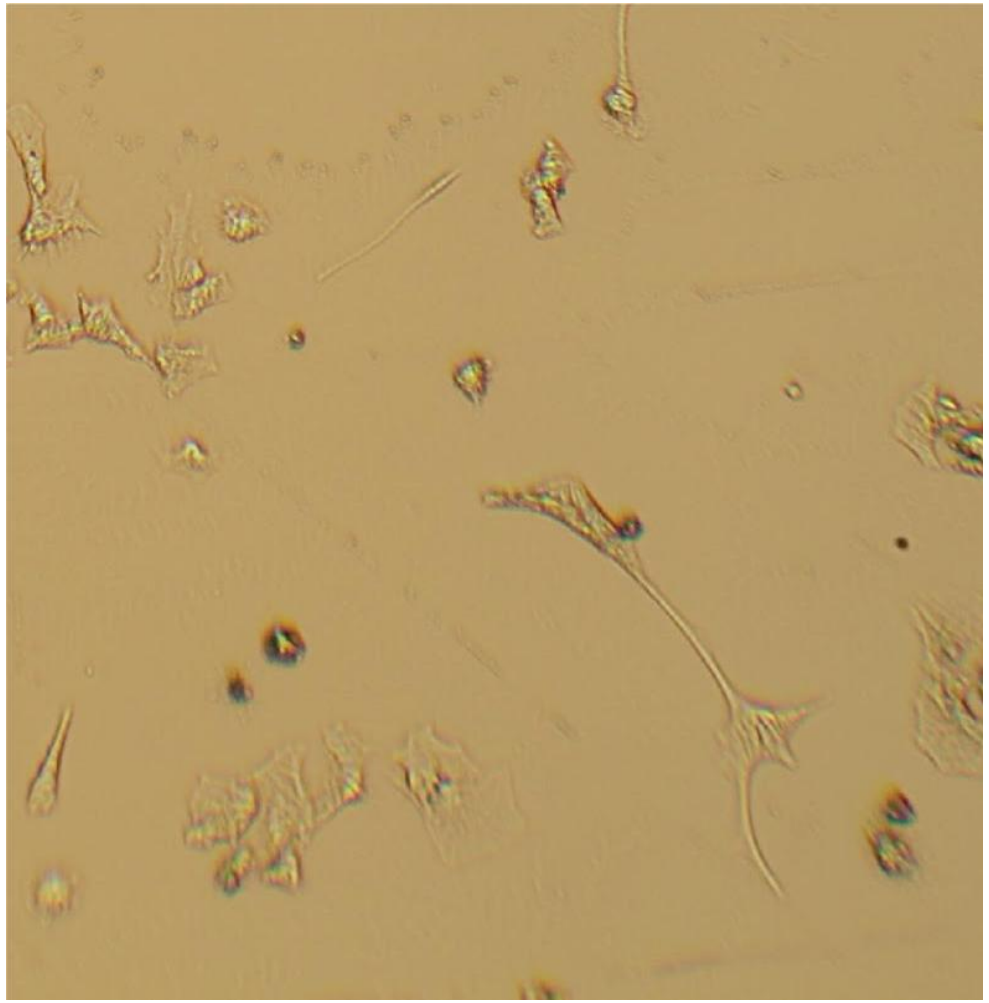
- **Digital Holographic Microscopy**
- **Nonlinear Microscopy** (Two-Photon Excitation Fluorescence –**TPEF**, and Second Harmonic Generation - **SHG**)

Samples:

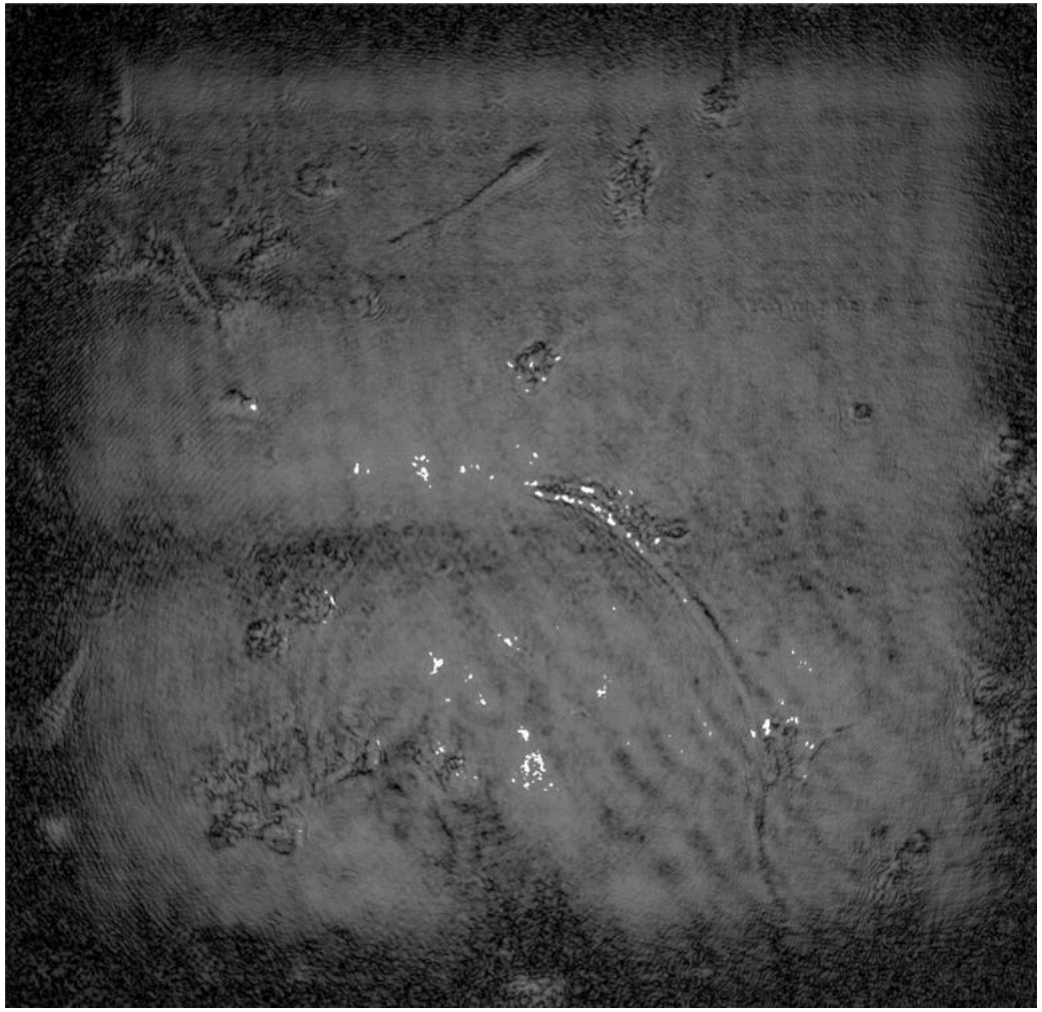
- Fixed culture of astrocytes from rats cortex
- Rat spinal cord cross-sections labeled with fluorophores (AF488, AF555)

Results: Holographic Microscopy

16.7.2025.



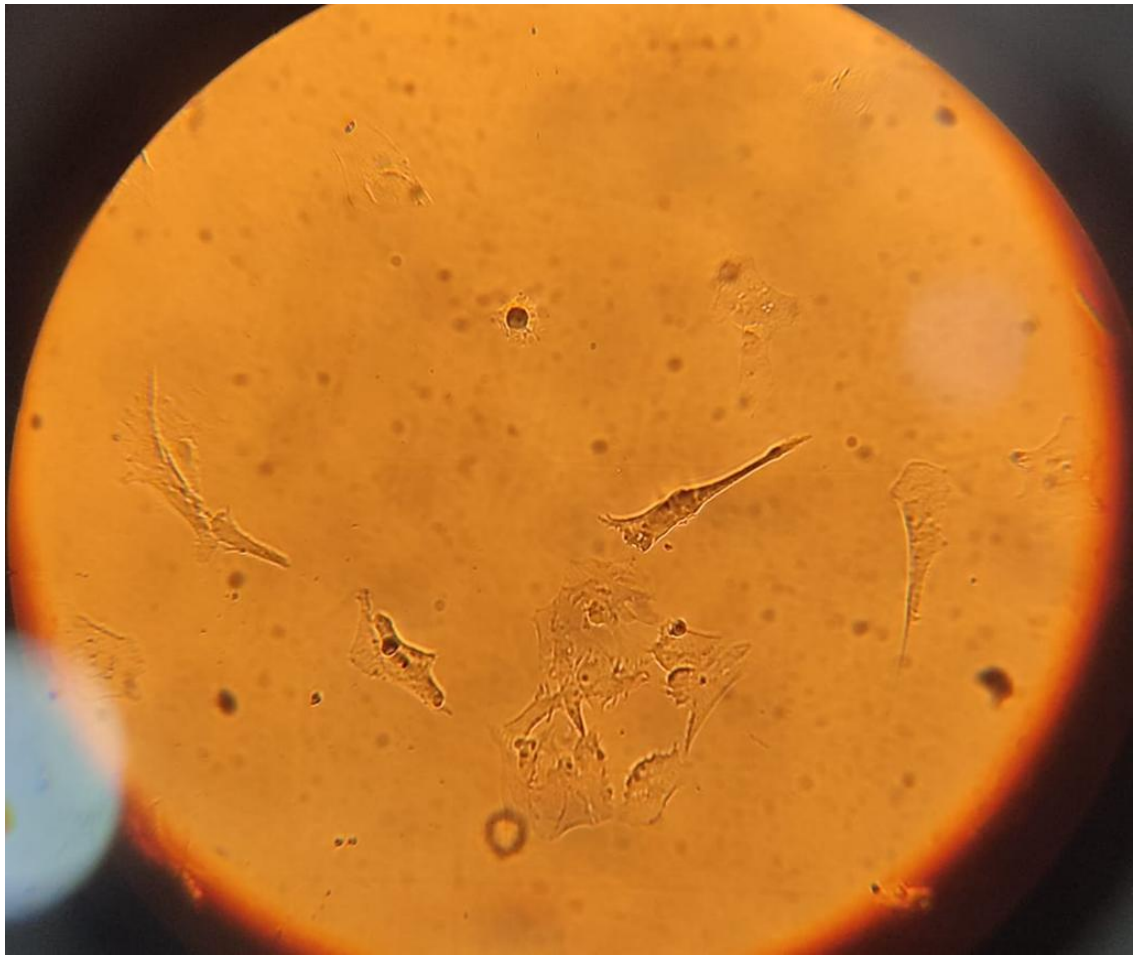
Brightfield image of astrocytes seeded on a glass slide at a density of 10,000 cells/slide



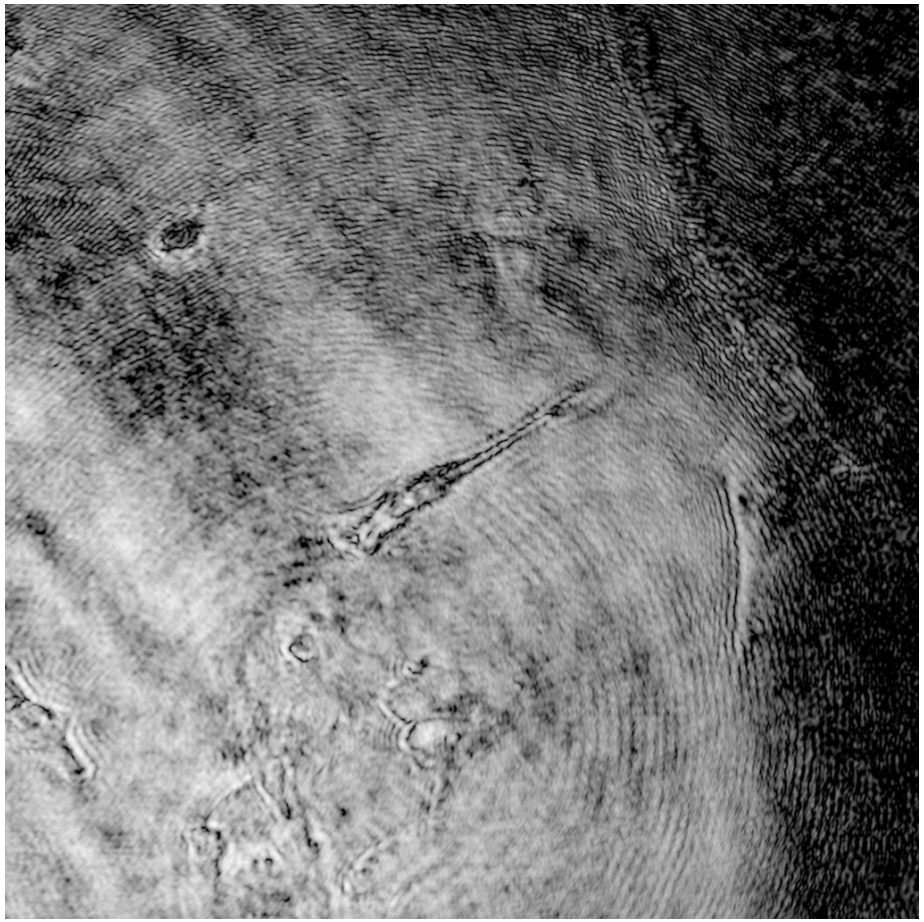
Holographic image (light intensity) imaged with 532 nm laser, 20x objective, ~2 µm resolution. ***Result:*** *Astrocytes are visible but lack sufficient contrast without additional labeling. White granules are artefacts.*

Holographic microcopy

24.7.2025.



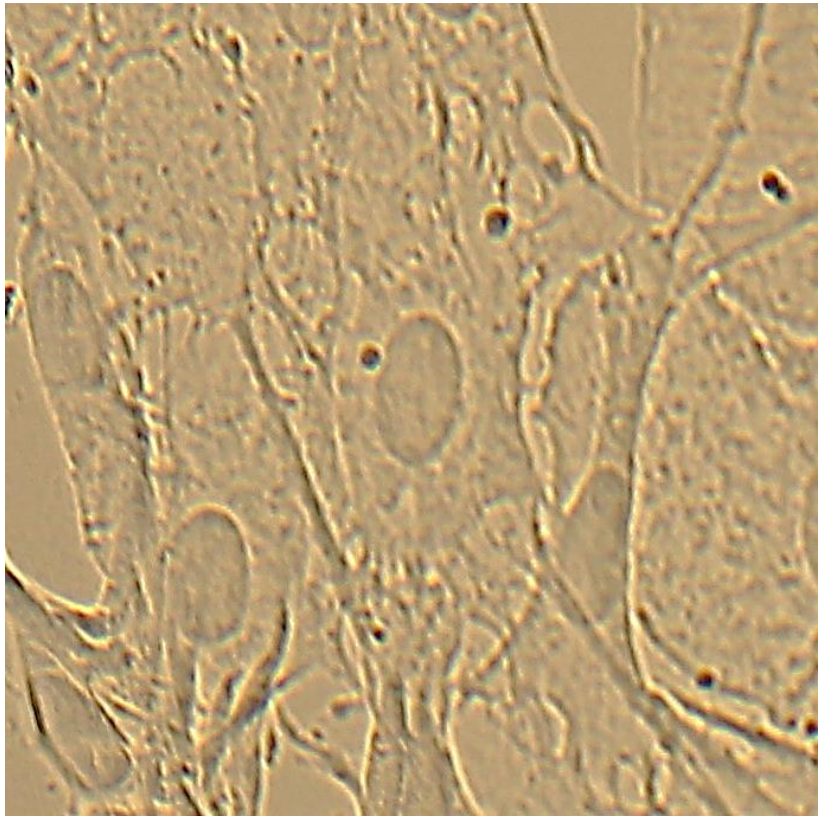
Brightfield image of astrocytes seeded on a glass slide at a density of 10,000 cells/slide



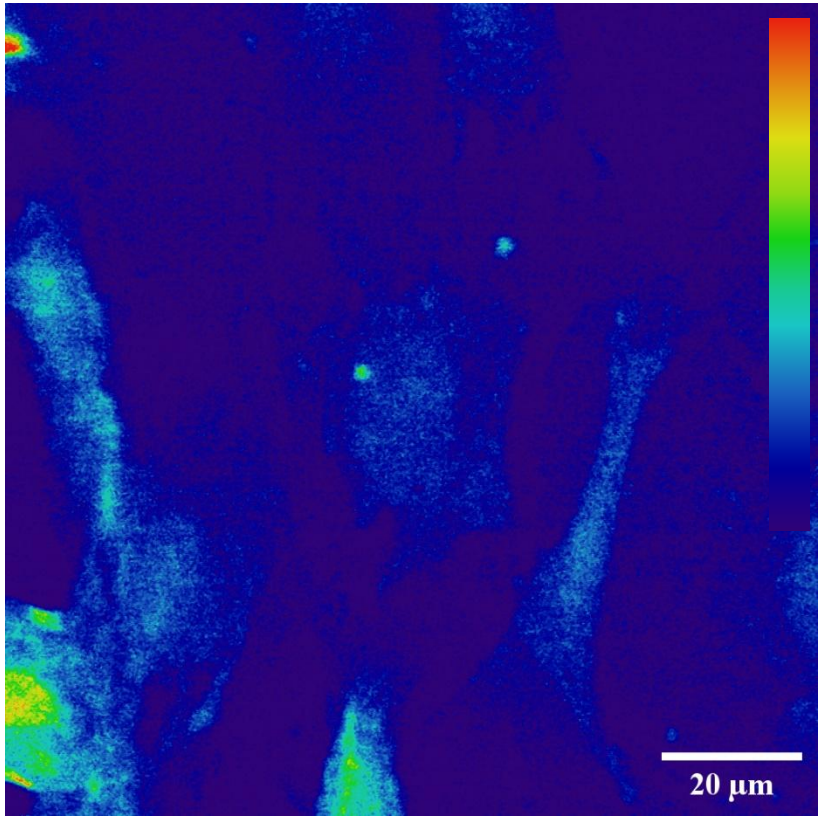
Holographic image (light intensity) imaged with 532 nm laser, 20x objective, ~2 µm resolution.
Result: *Astrocytes are visible but lack sufficient contrast without additional labeling*

Two-photon microscopy (TPEF) and SHG : Astrocytes in the transparent chip

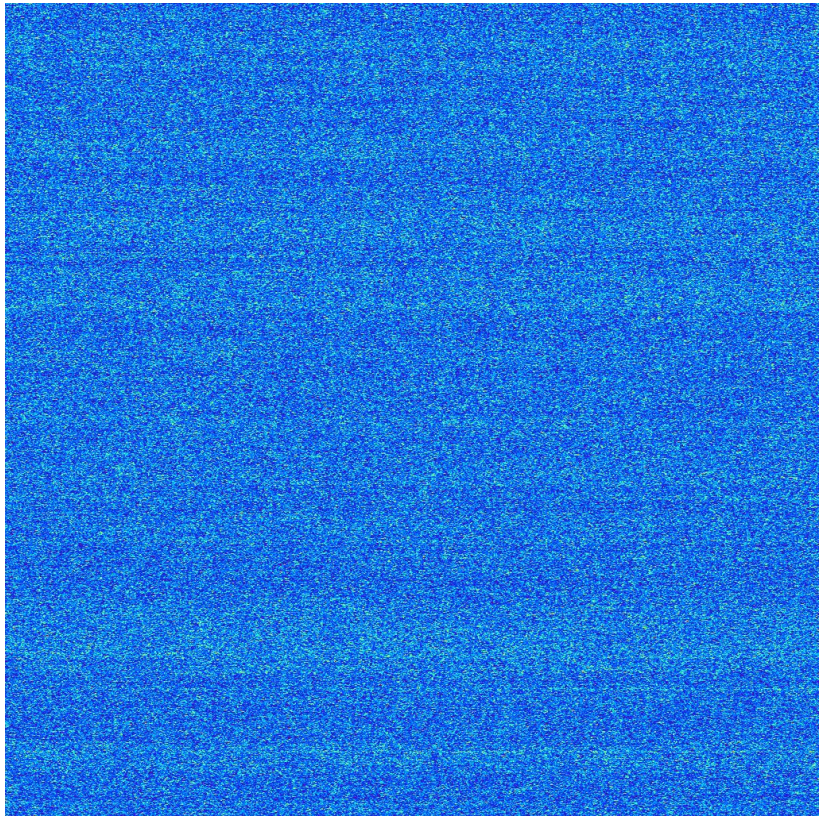
29.05.2025.



Brightfield image of astrocytes seeded on a chip, density 10 000 cells/ml.



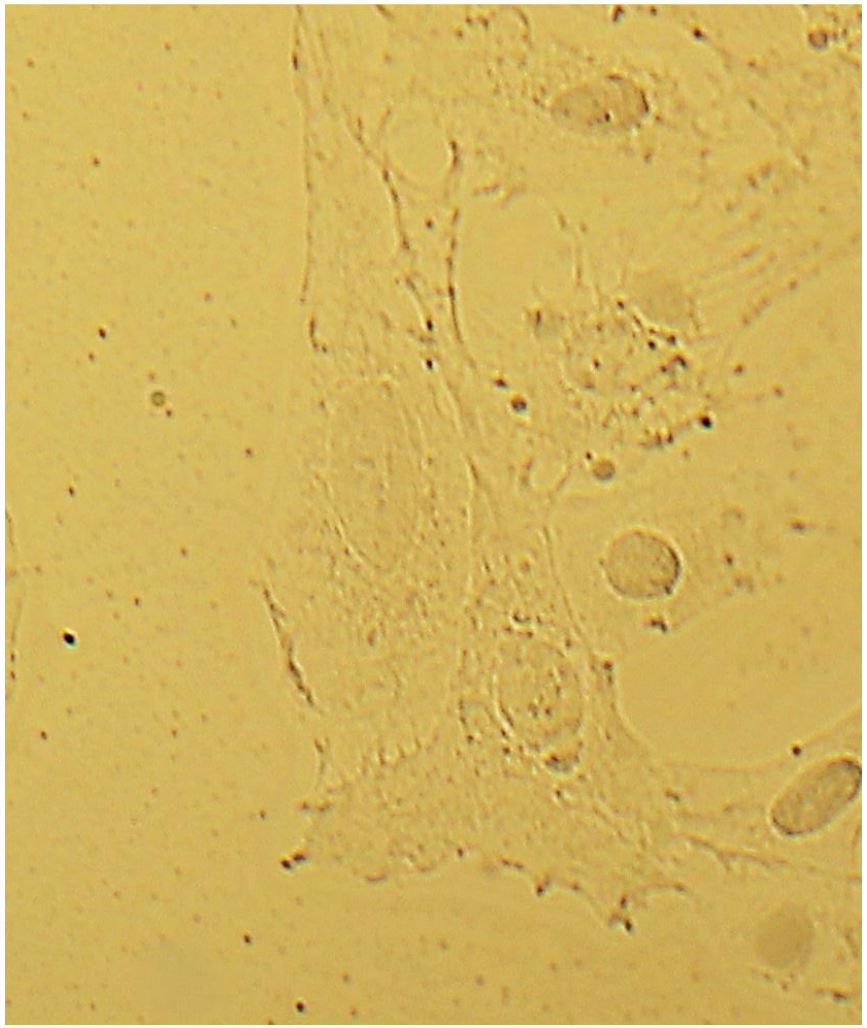
TPEF:
Laser: 800 m
Objective: 20x 0.8 NA
Laser power: 9.7 mW
Filters: 700 short-pass
Result: *Fluorescine diacetate signal (labels live cells, ex. 490 nm, em. 520 nm)*



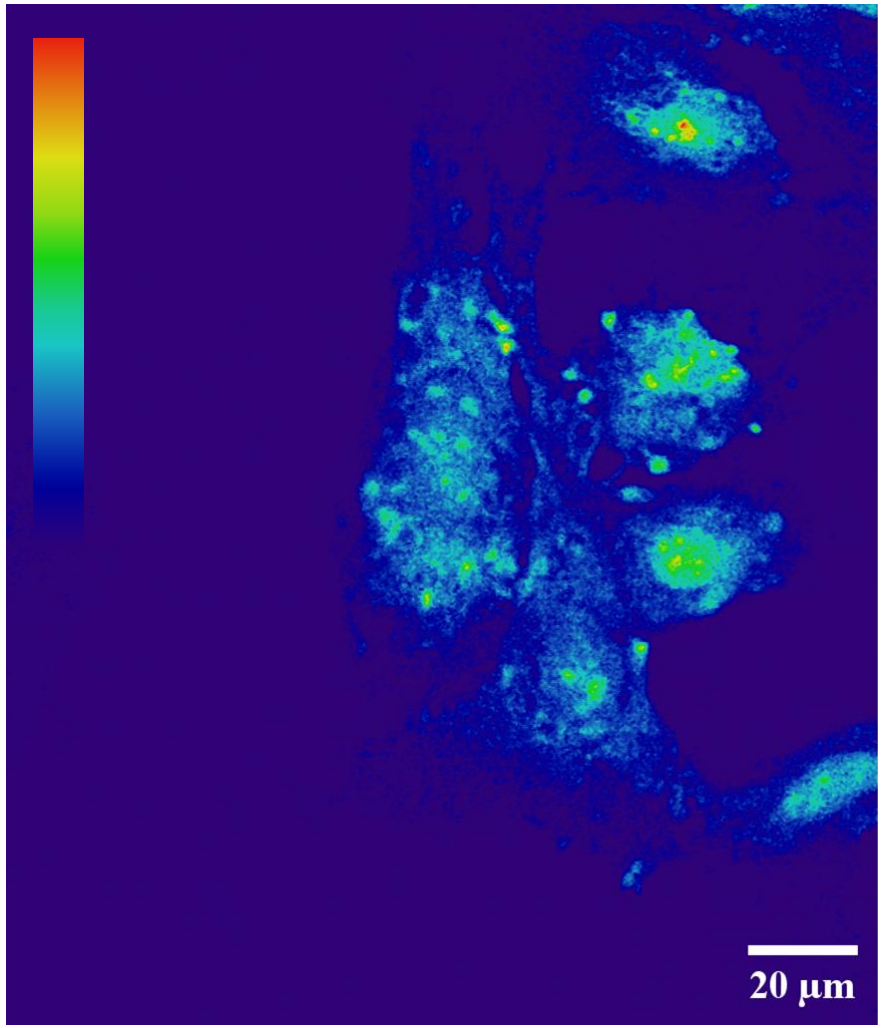
SHG:
Laser: 800 nm, reflection mode
Objective: 20x 0.8 NA
Laser power on sample: 29.4 mW
Filters: 700 short-pass + 400/10 nm
Image size: 115 × 115 µm
Result: *no SHG signal*

Two-photon microscopy
Astrocytes in the transparent chip

29.05.2025.

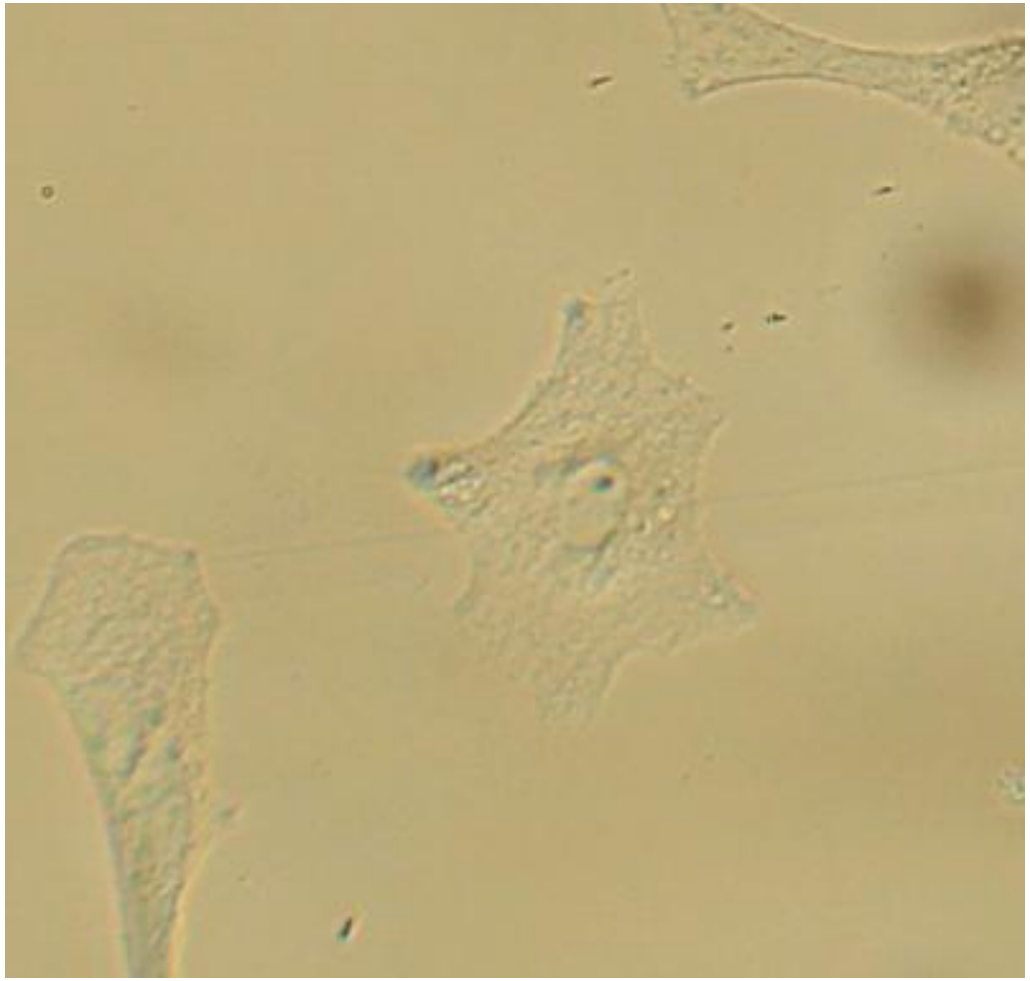


Brightfield image of astrocytes seeded on a chip, density 10 000 cells/ml.

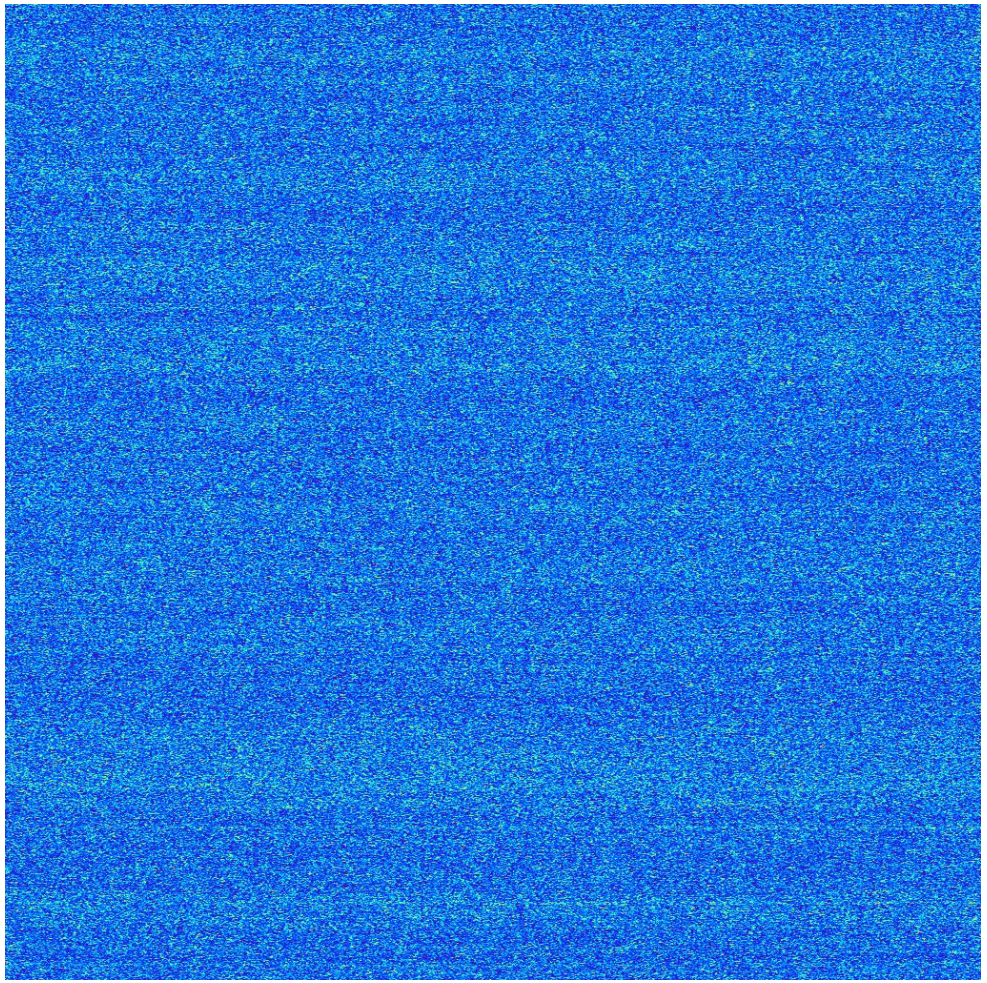


Two-photon excitation: *laser* **730 nm**, *objective* **20x** 0.8 NA, *laser power on the sample* 7.7 mW, *filter* 700sp, *Fluorescine diacetate signal (labels live cells, excitation at 490 nm, emmission at 520 nm)*

SHG - Astrocytes on the slide

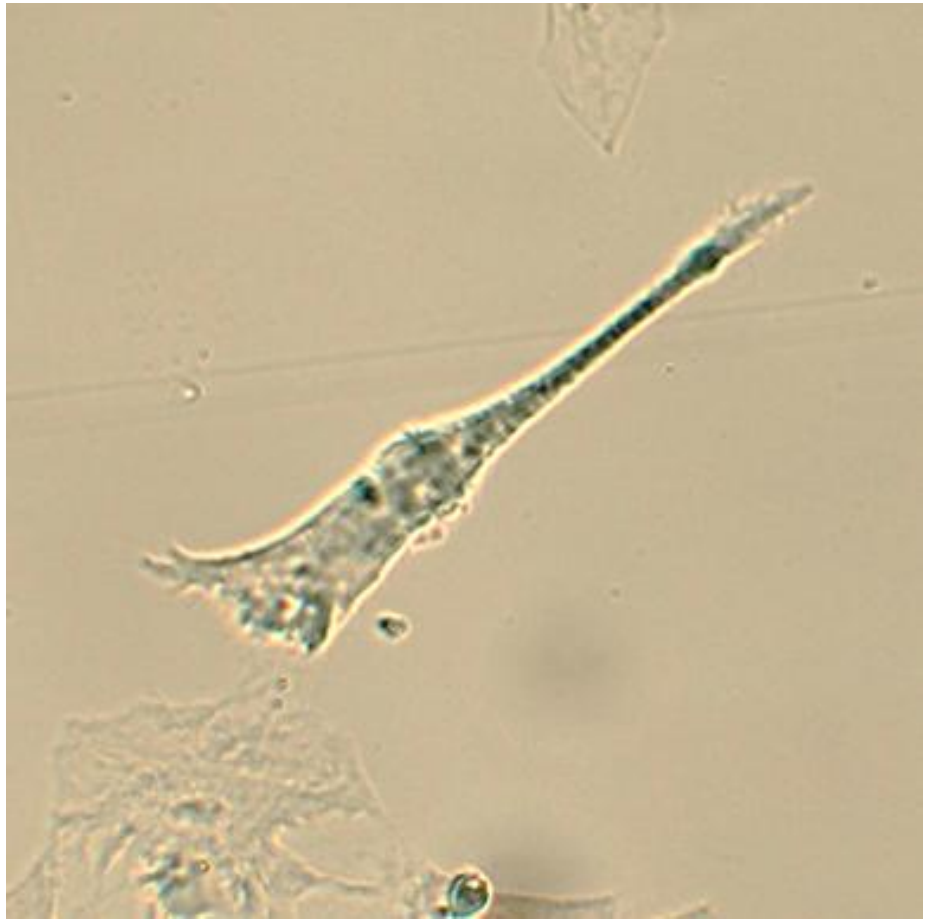


Brightfield image of astrocytes seeded on a g slide at a density of 10,000 cells/slide

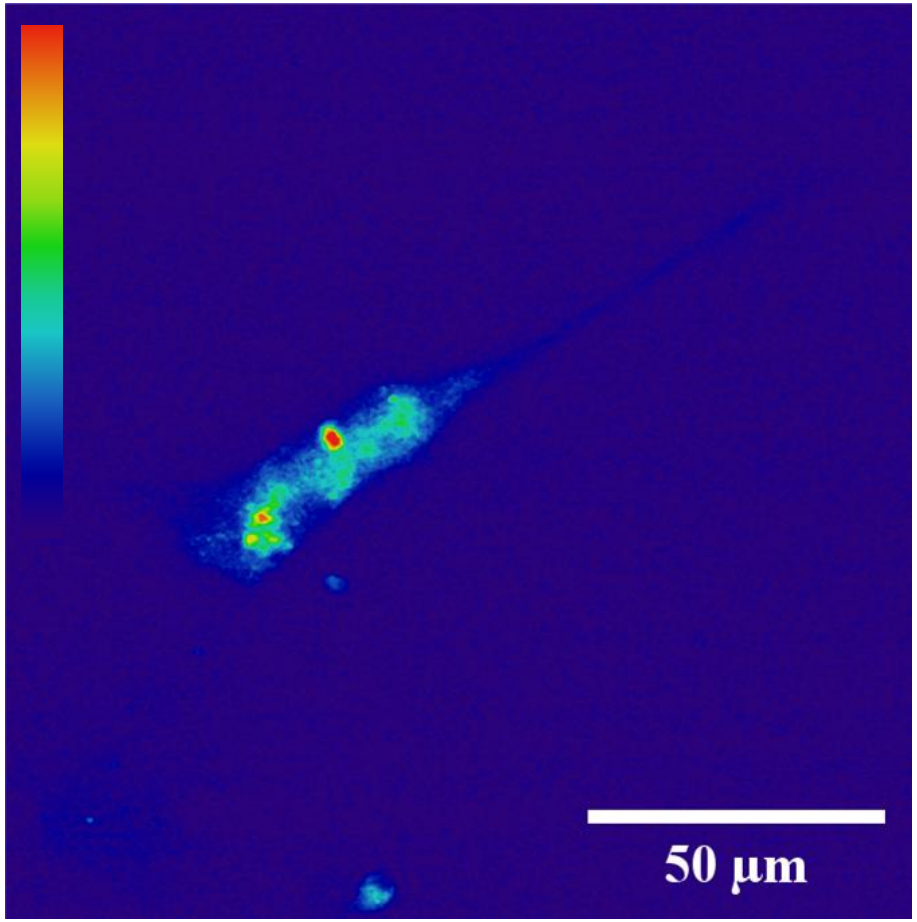


SHG:
Laser: 800 nm, reflection mode,*Objective:* 20x 0.2 NA, *Filters:* 700 short-pass + 400/10 nm,
Result: *no SHG signal*

Two-photon microscopy - astrocyte 1



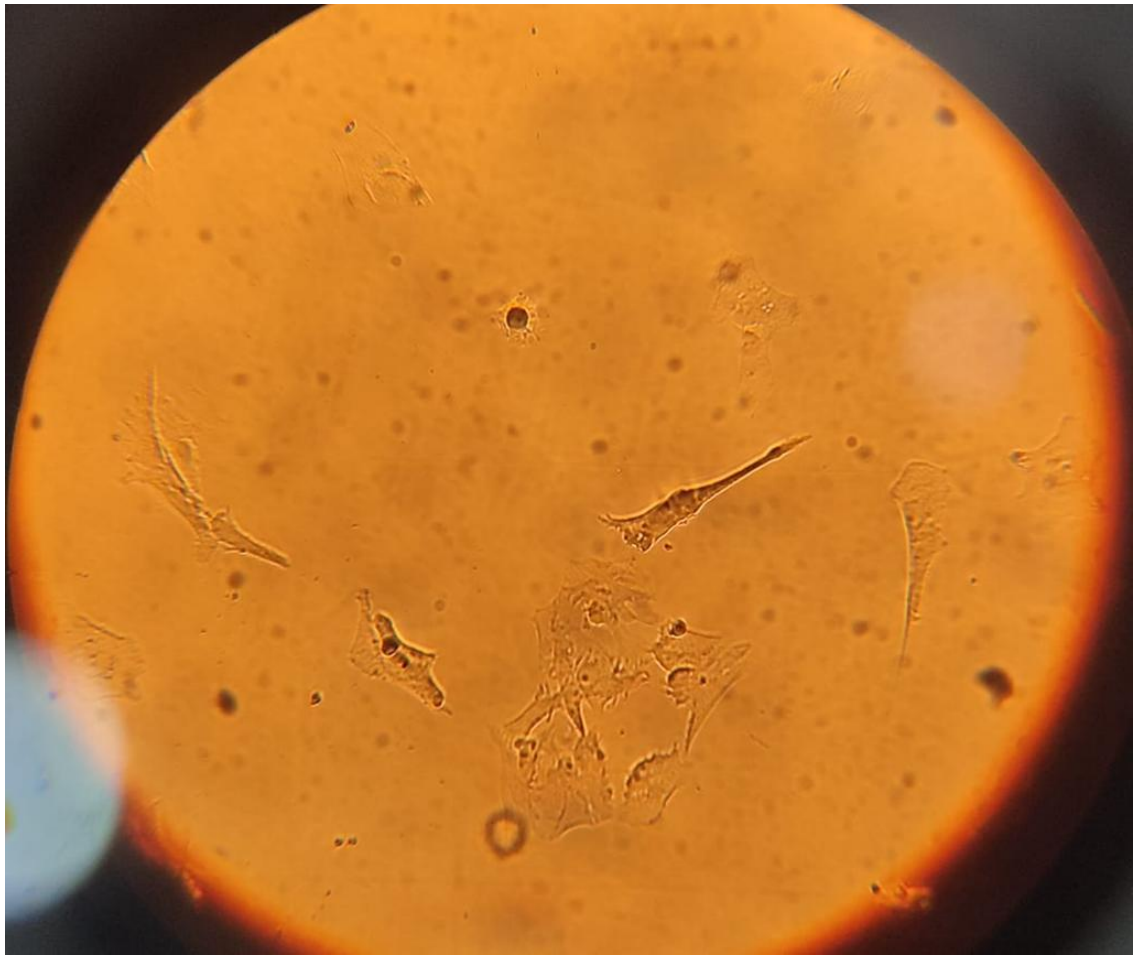
Brightfield image of astrocytes seeded on a glass slide at a density of 10,000 cells/slide



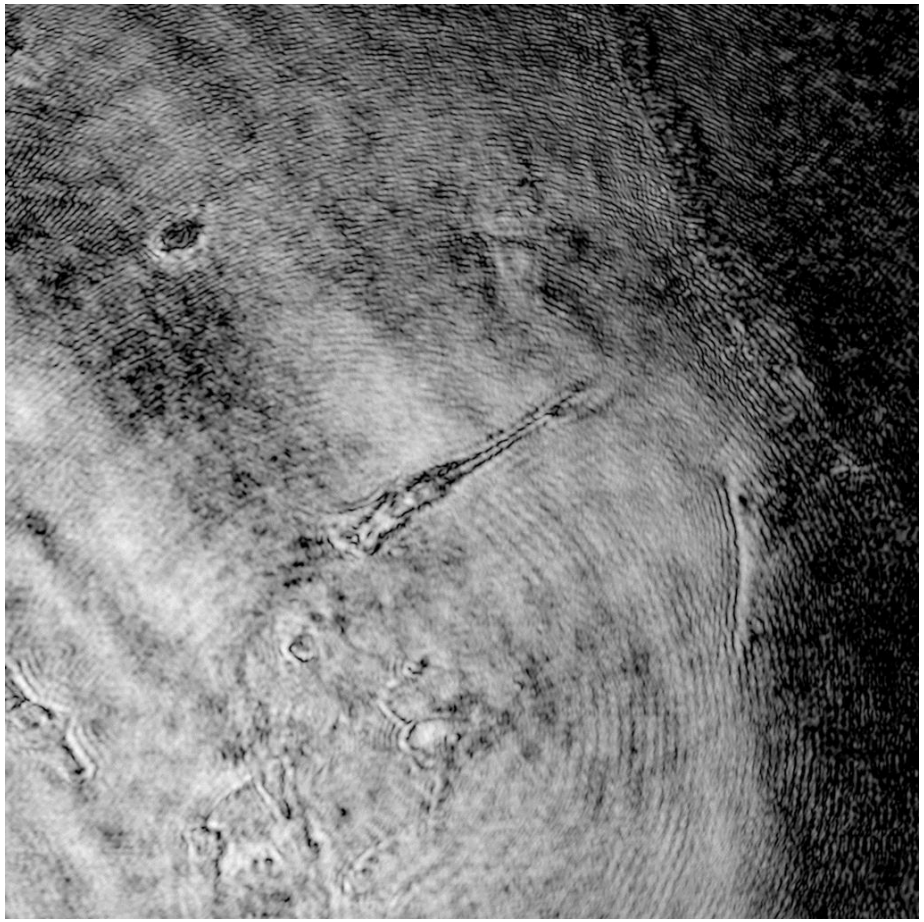
Two-photon excitation: *laser* **730 nm**, *objective* **20x** 0.8 NA, *laser power on the sample* 24.5 mW,
Result: *Autofluorescence signal at 730 nm (possibly from NADH)*

Same astrocyte imaged with holographic microscope

24.7.2025.



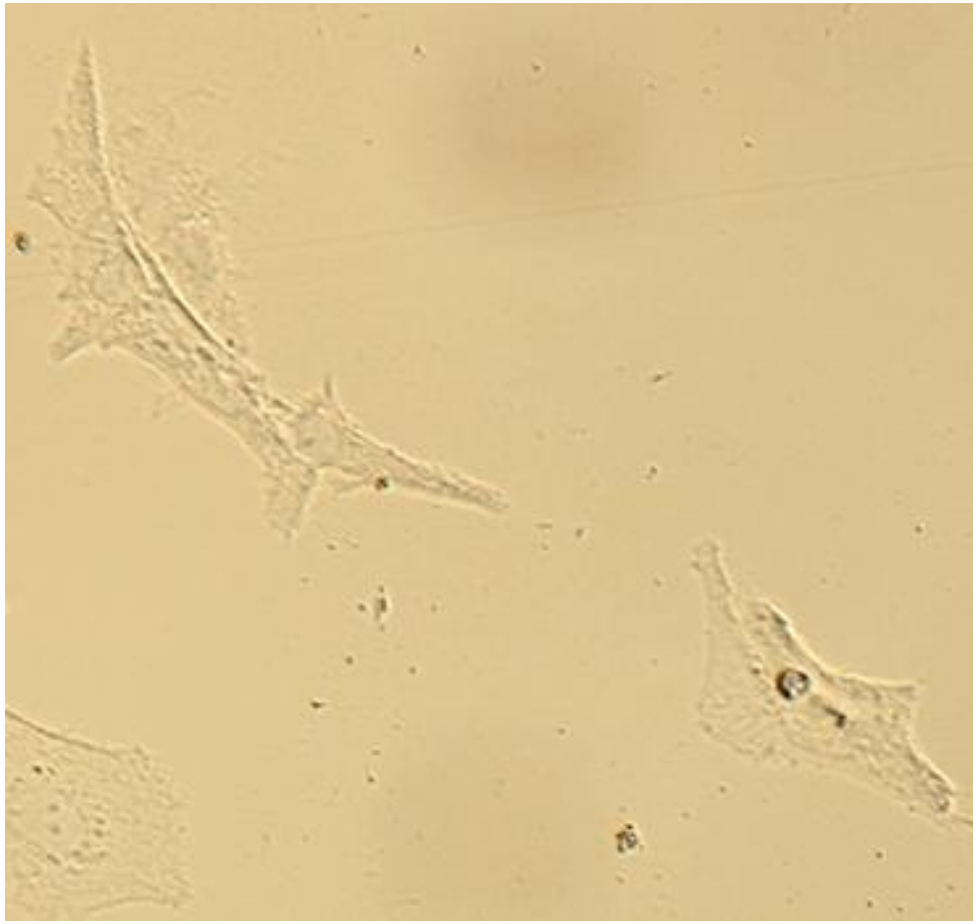
Brightfield image of astrocytes seeded on a glass slide at a density of 10,000 cells/slide



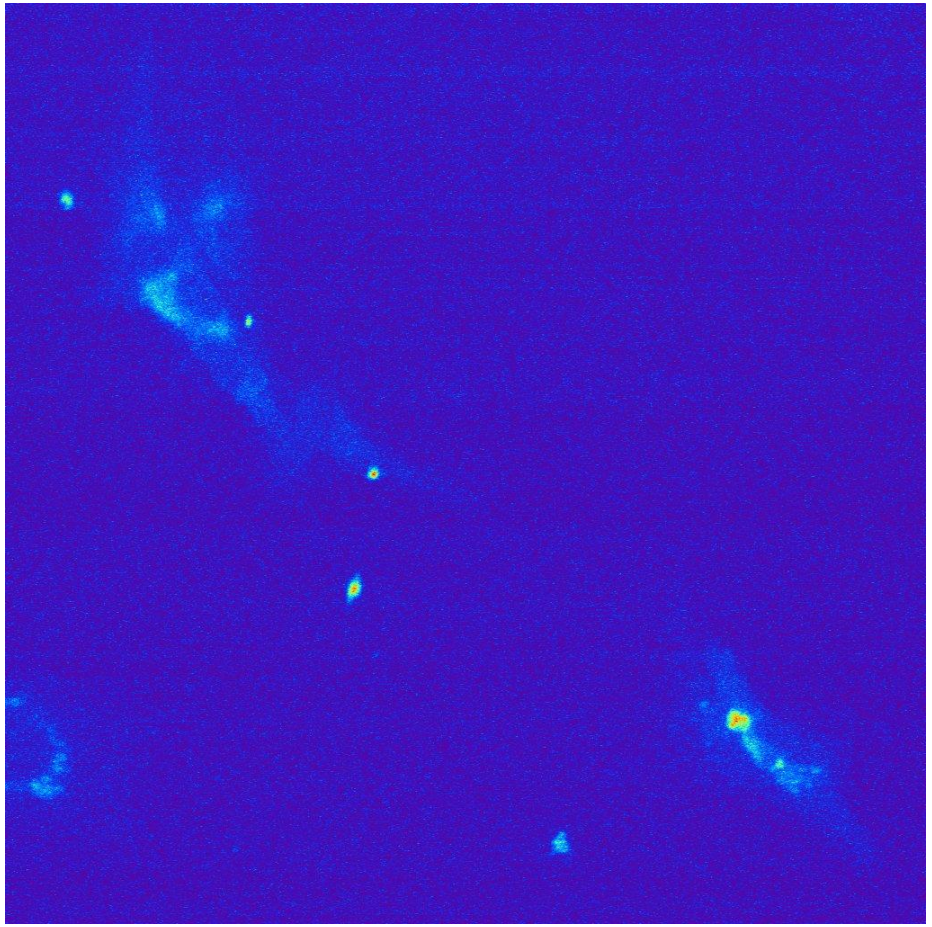
Holographic image (light intensity) imaged with 532 nm laser, 20x objective, ~2 µm resolution.

17.7.2025.

Two-photon microscopy - astrocyte 2



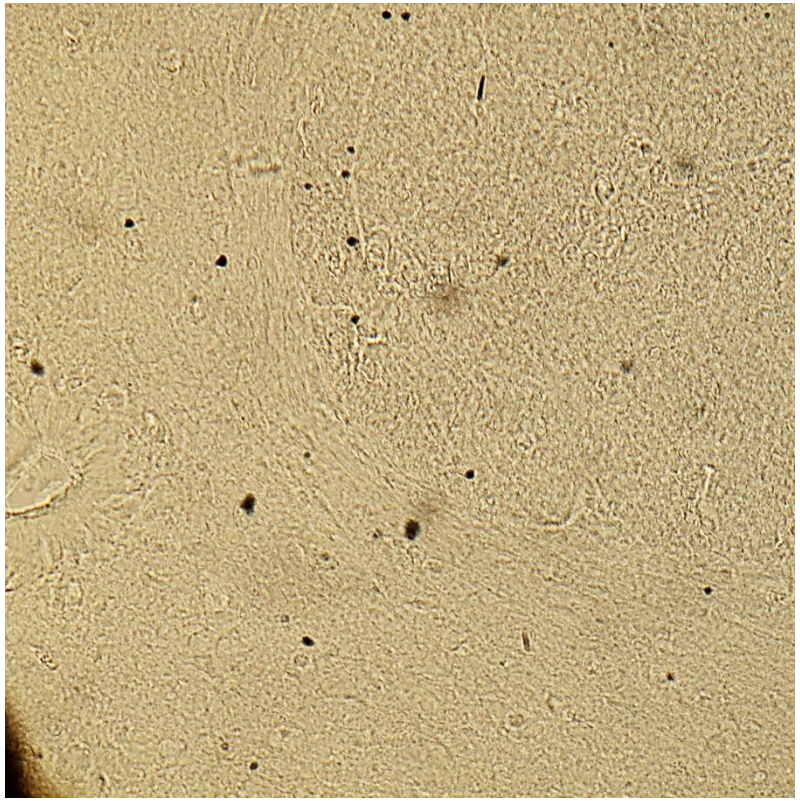
Brightfield image of astrocytes seeded on a glass slide at a density of 10,000 cells/slide



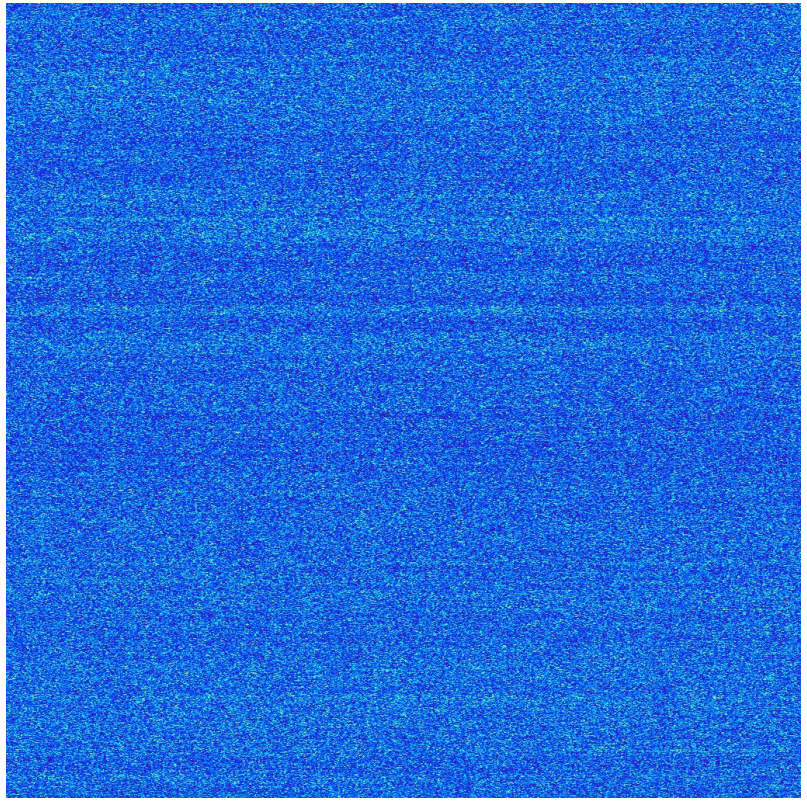
Two-photon excitation: *laser* **730 nm**, *objective* **20x** 0.8 NA, *laser power on the sample* 24.5 mW, ***Result:*** *Autofluorescence signal at 730 nm (possibly from NADH)*

SHG and two-photon microscopy – Spinal cord sections

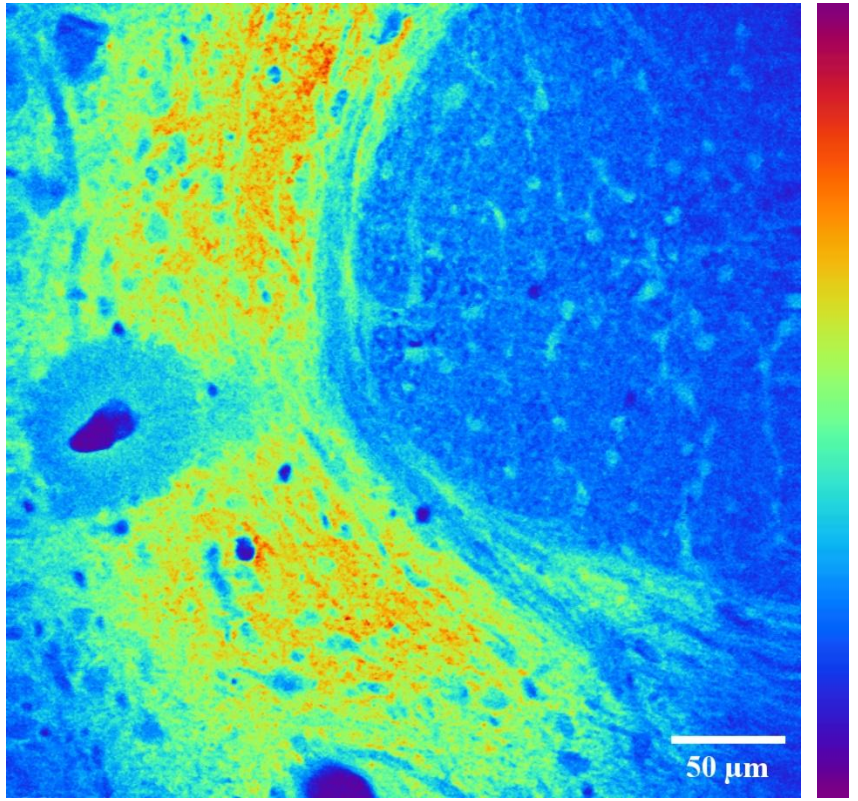
17.7.2025.



Sample: Transverse section of the spinal cord at the lumbar level from an EAE rat model (samples provided by Katarina M.)



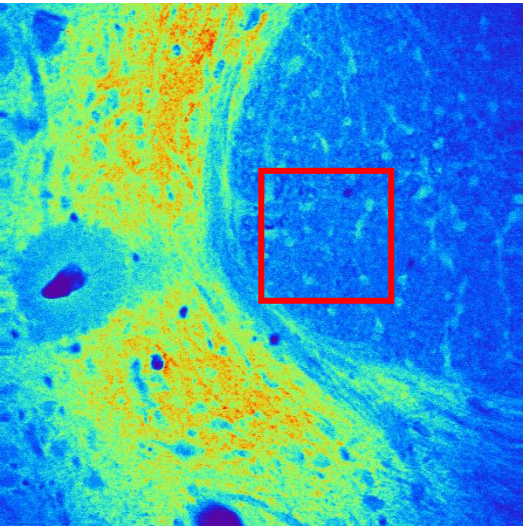
SHG
Laser: 800 nm
Imaging mode: Reflection
Objective: 12.5x, 0.3 NA
Laser power on sample: 35.7 mW
Result: *No SHG signal detected*



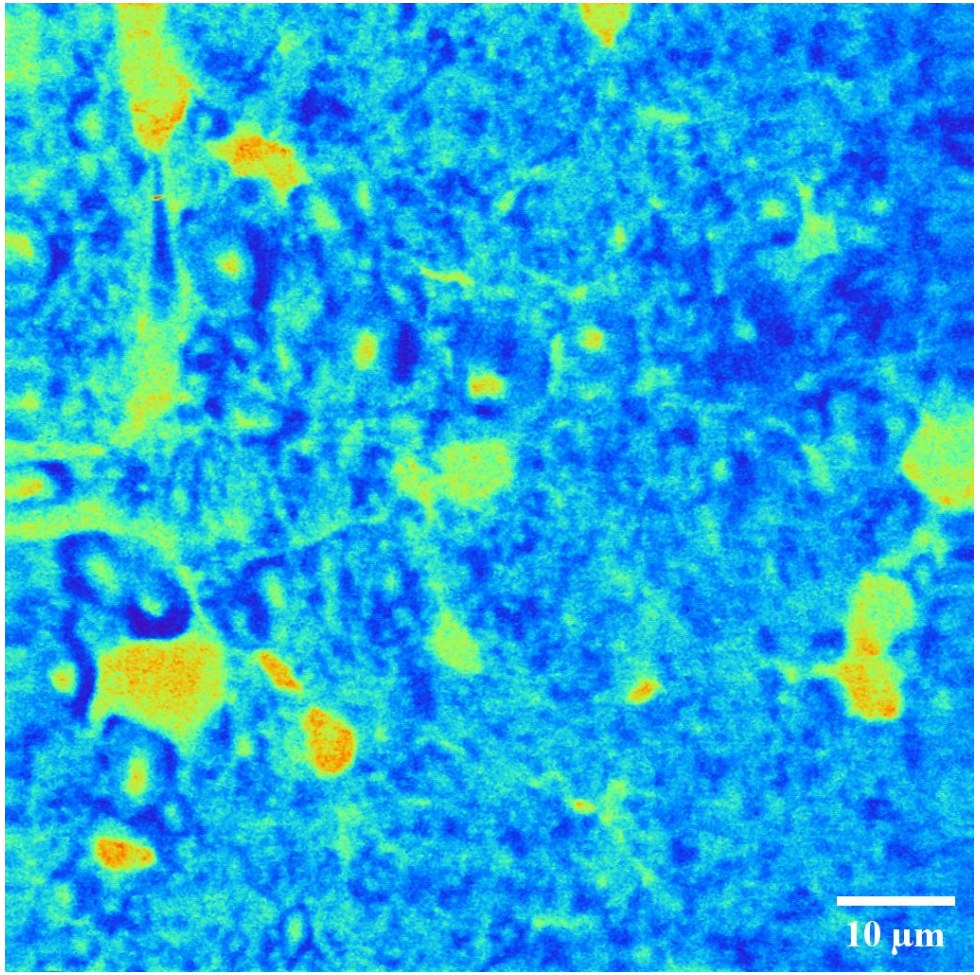
Two-Photon Excitation
Laser: 730 nm
Objective: 20x, 0.8 NA
Result: *autofluorescence and fluorescence*
Image type: Raw image

Two-photon microscopy – Spinal cord section

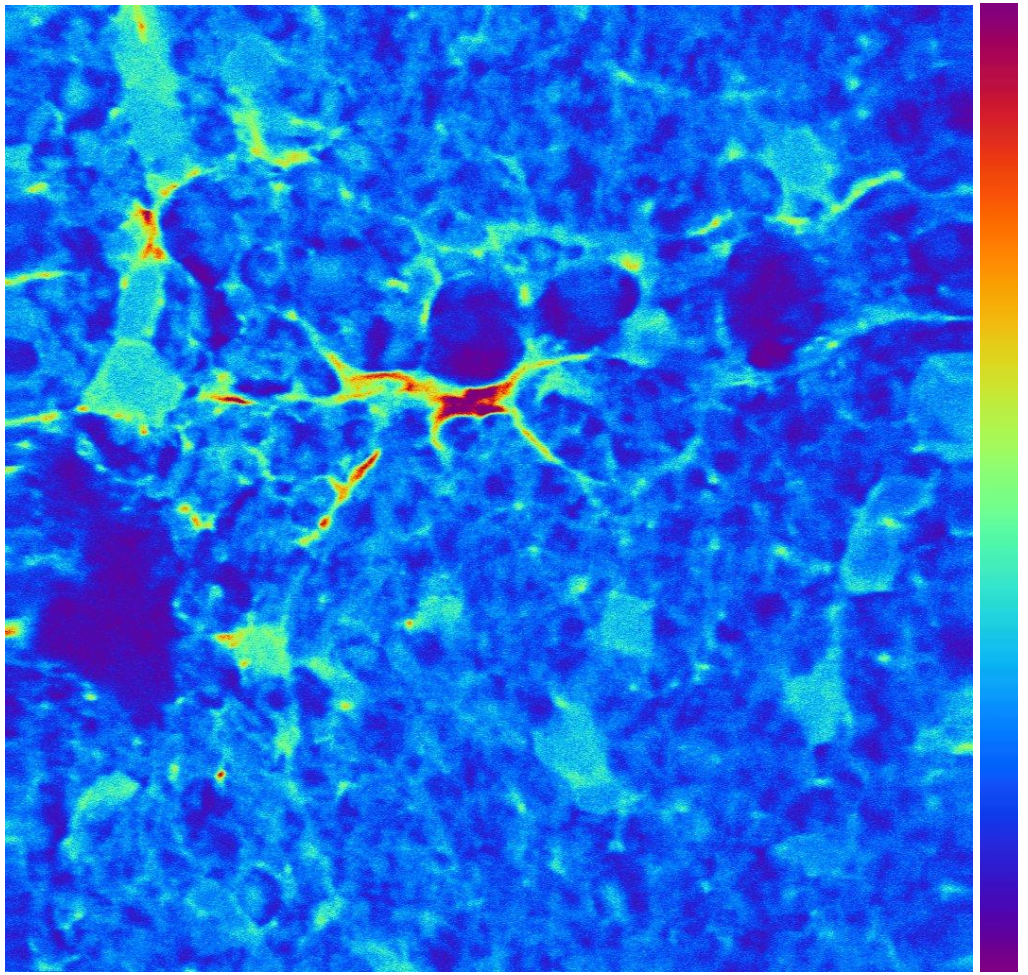
17.7.2025.



Z stack, level 20/33



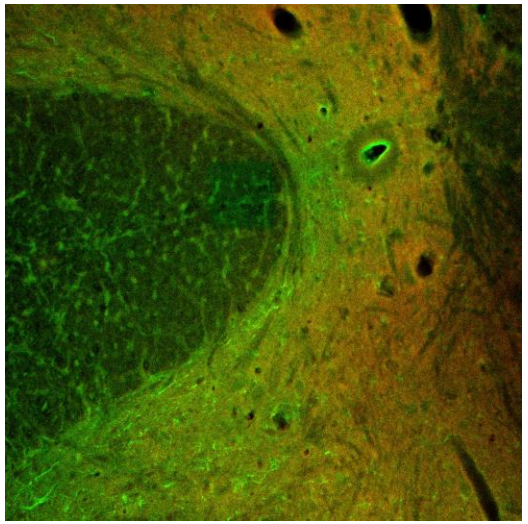
Z stack, level 5/33



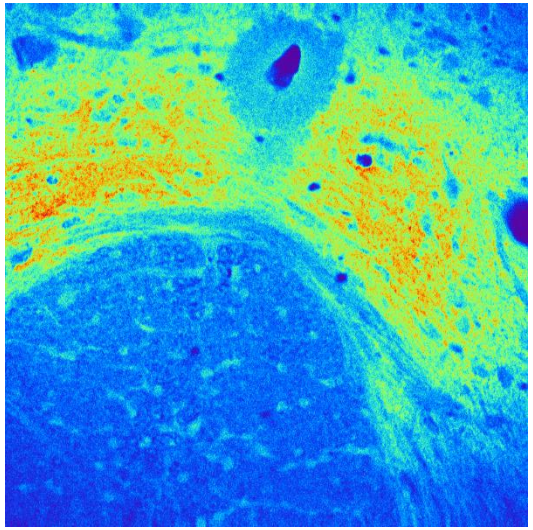
Laser**: 730 nm**, Objective: **40x**, 1.3 NA (oil immersion), **Laser power**: 1.8 mW (middle image, filter: 700sp + 530/43)4.2 mW (right image, filter: 700sp)**, Region**: Gray matter of the spinal cord, **Labeling** :Astrocytes GS (glutamine synthetase) marked with AF 555. Astrocyte-connecting proteins marked with AF488 (connexin 43), ***Result:*** *Detected signal originating from* ***AF 488, (astrocytes marker)*** *and autofluorescence (presumably lipofuscin in motor neurons).*

Two-photon and confocal Microscopy – Astrocytes in the spinal cord

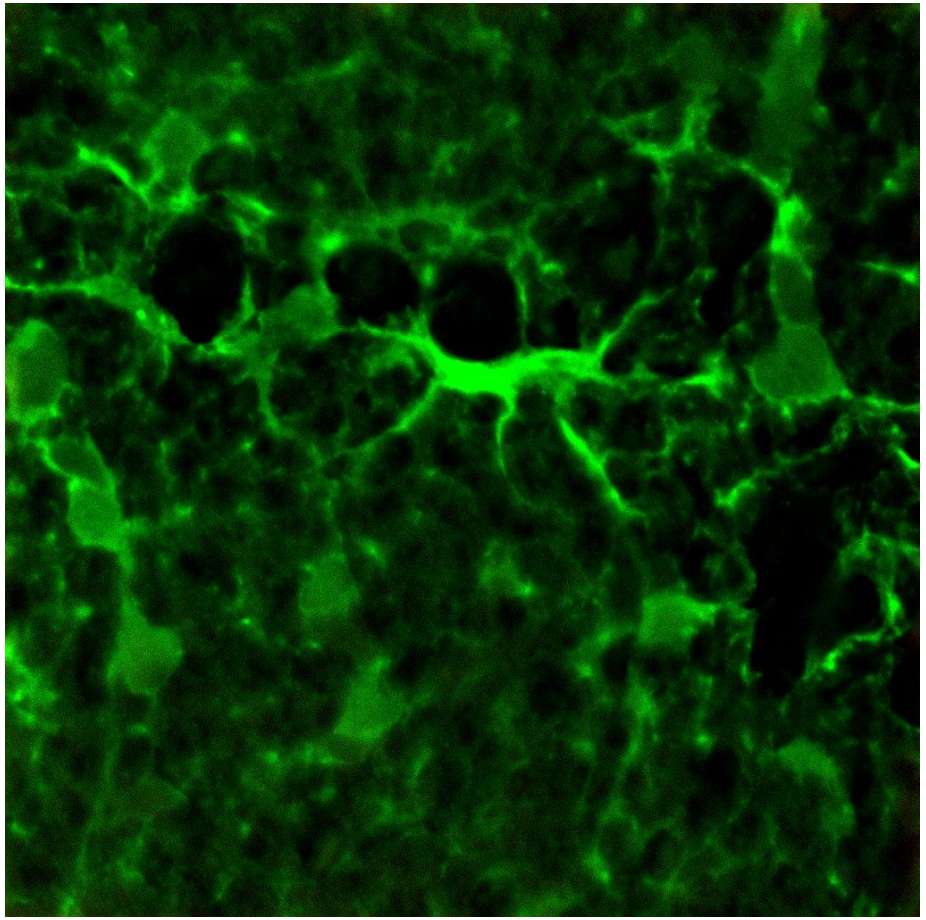
17.7.2025.



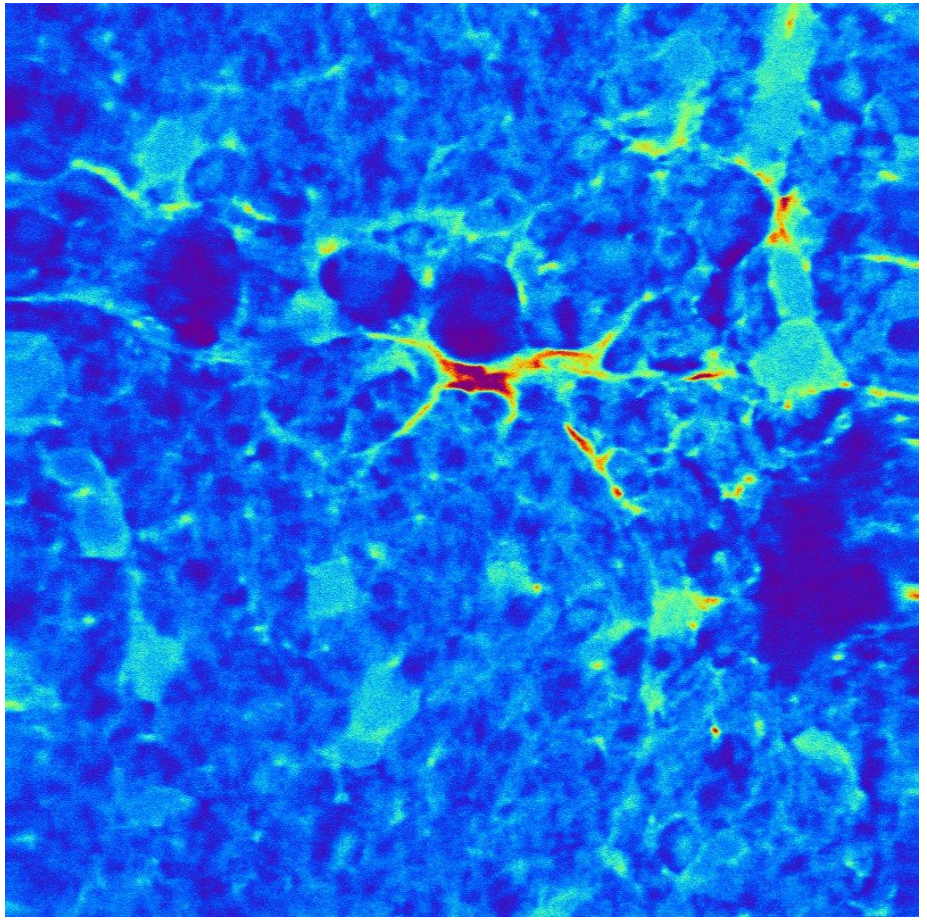
Confocal microscope (objective: 20x)



Two-photon microscope (objective: 20x)



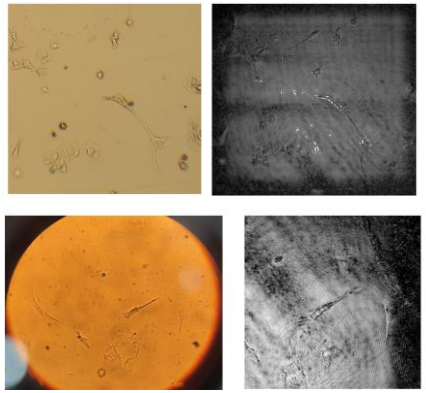
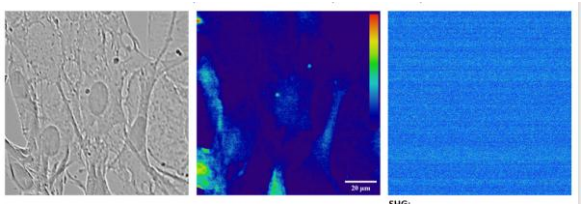
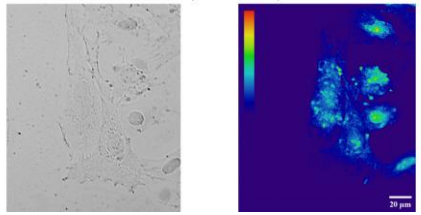
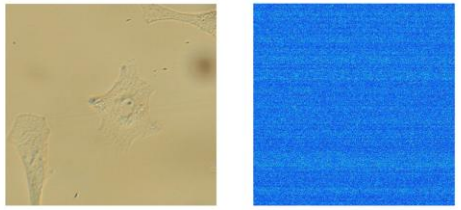
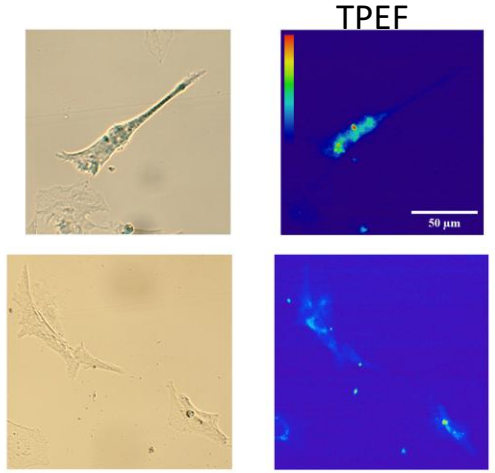
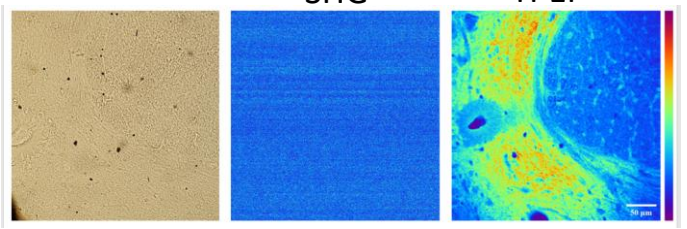
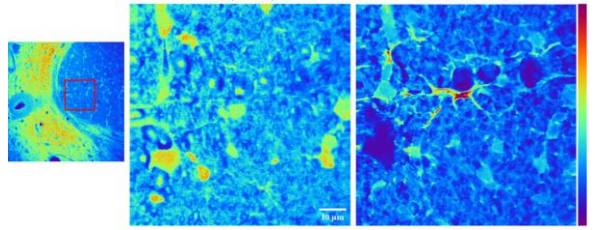
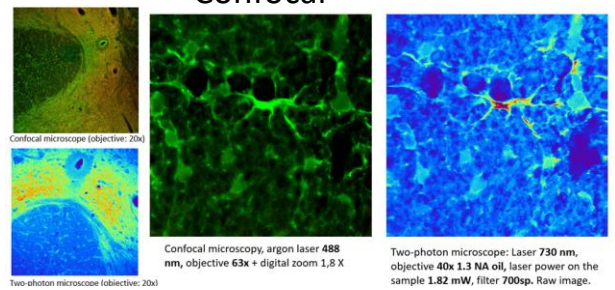
Confocal microscopy, argon laser **488 nm,** objective **63x** + digital zoom 1,8 X



Two-photon microscope: Laser **730 nm**, objective **40x 1.3 NA oil,** laser power on the sample **1.82 mW**, filter **700sp.** Raw image.

Spinal cord gray matter: Astrocytes labeled with AF488 (GS – glutamine synthetase) and astrocyte-associated proteins labeled with AF555 (connexin 43). ***Result:*** *Detected signal from AF488 and autofluorescence (likely lipofuscin)*

Results summary

Holographic microscopy	Nonlinear microscopy		
	Astrocytes, cell culture on the chip	Astrocytes, cell culture on the slide	Astrocytes in the spinal cord
	TPEF SHG  TPEF 	SHG  TPEF 	SHG TPEF  TPEF  Confocal 
Result: *Astrocytes are visible but lack sufficient contrast without additional labeling*	***Result:*** *Fluorescine diacetate signal (labels live cells) is possible to image with TPEF at 730 nm.* ***Result:*** *no SHG signal*	***Result:*** *no SHG signal* ***Result:*** *Autofluorescence signal at 730 nm (possibly from NADH)*	***Result:*** *no SHG signal* ***Result:*** *Detected fluorescence from* ***AF 488, (astrocytes marker)*** *and autofluorescence (presumably lipofuscin in motor neurons).*

Holographic Microscopy:

- Astrocytes are visible but lack sufficient contrast without additional labeling. Cell membranes showed best contrast.

Two-Photon Excitation:

- **Laser at 800 nm** → no signal from either cells or tissue
- **Laser at 730 nm**:
  - Signal observed from:
    - **White matter** (fibrous structures) - **Autofluorescence**, likely from lipofuscin or myelinated axons in spinal cord samples and NADH from astrocytes in culture.
    - **Gray matter** –**Fluorescence** signal from astrocytes marker labeled with Alexa Fluor 488.
  - **Filters used**:
    - 530/43: good signal from white matter
    - LP570: weaker signal

SHG (Second Harmonic Generation):

- Acquired in **reflection mode only**
- Plan to switch to **transmission mode** (requires optical realignment)
- **Possible reasons for failure:**
  - Sectioning plane of a spinal cord tissue.

Assessment and Recommendations by Biologists

Holographic microscopy:

- **Visibility of astrocytes is limited**, but can be improved with additional labeling.

Two-photon microscopy:

- **Tissue autofluorescence** (especially in **white matter**) shows potential for future investigations.

Second harmonic geenration:

- **Imaging was unsuccessful** under current conditions. Repeat with **optimized samples and system** (transmission mode, different wavelengths and sample orientation).

No new biological information was obtained compared to confocal imaging — except for the potential of **label-free** insights via holography and nonlinear microscopy.

Further Steps

Holographic microscopy

- Consider labeling astrocytes with **AF555 fluorophore**, which would selectively absorb 532 nm laser light, potentially making cells appear darker and improve image contrast.

Nonlinear microscopy:

Repeat SHG imaging using:

- Saggital sections of a spinal cord
- Objectives: 20x 0.8 NA (air) or 40x 1.3 NA (oil)
- Excitation wavelengths: 740 nm, 800 nm, 840 nm
- Use transmission mode

Technical details

Method	Wavelength	Objective	Signal	Resolution	Notes
Holography	532 nm	20x	Intensity	~2 µm	Possible use of AF555 fluorophore
SHG	800 nm	20x and 40x	No signal	~300–500 nm	Only tested in reflection mode
Two-Photon	730 nm	20x and 40x	Autofluorescence + Fluorescence	~320 ± 20 nm	Better signal in white matter
Confocal	488 nm, 555 nm	63x	AF488 / AF555	~400–800 nm	Fluorescence confirmation

Credits

Sample preparation and confocal microscopy imaging:
Ana Jakovljević, + samples provided by Katarina Milićević

Holographic microscopy imaging:
Svetlana Savić, Branka Murić, Filip Krajinić, Tanja Pajić, Ana Jakovljević

Nonlinear microscopy imaging:
Tanja Pajić, Ana Jakovljević

Experiment planning and data interpretation:
Branislav Jelenković, Pavle Andjus