BIOCEV

3D CLEM - combining confocal with FIB-SEM

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FACULTY OF SCIENCE
Charles University

CZECH BIOIMAGING
Imaging principles of life

MINISTRY OF EDUCATION,
YOUTH AND SPORTS

Imaging Methods Core Facility at BIOCEV

operated by Faculty of Science, Charles University

CZECH BIOIMAGING — Imaging principles of life

EURO-BIOIMAGING

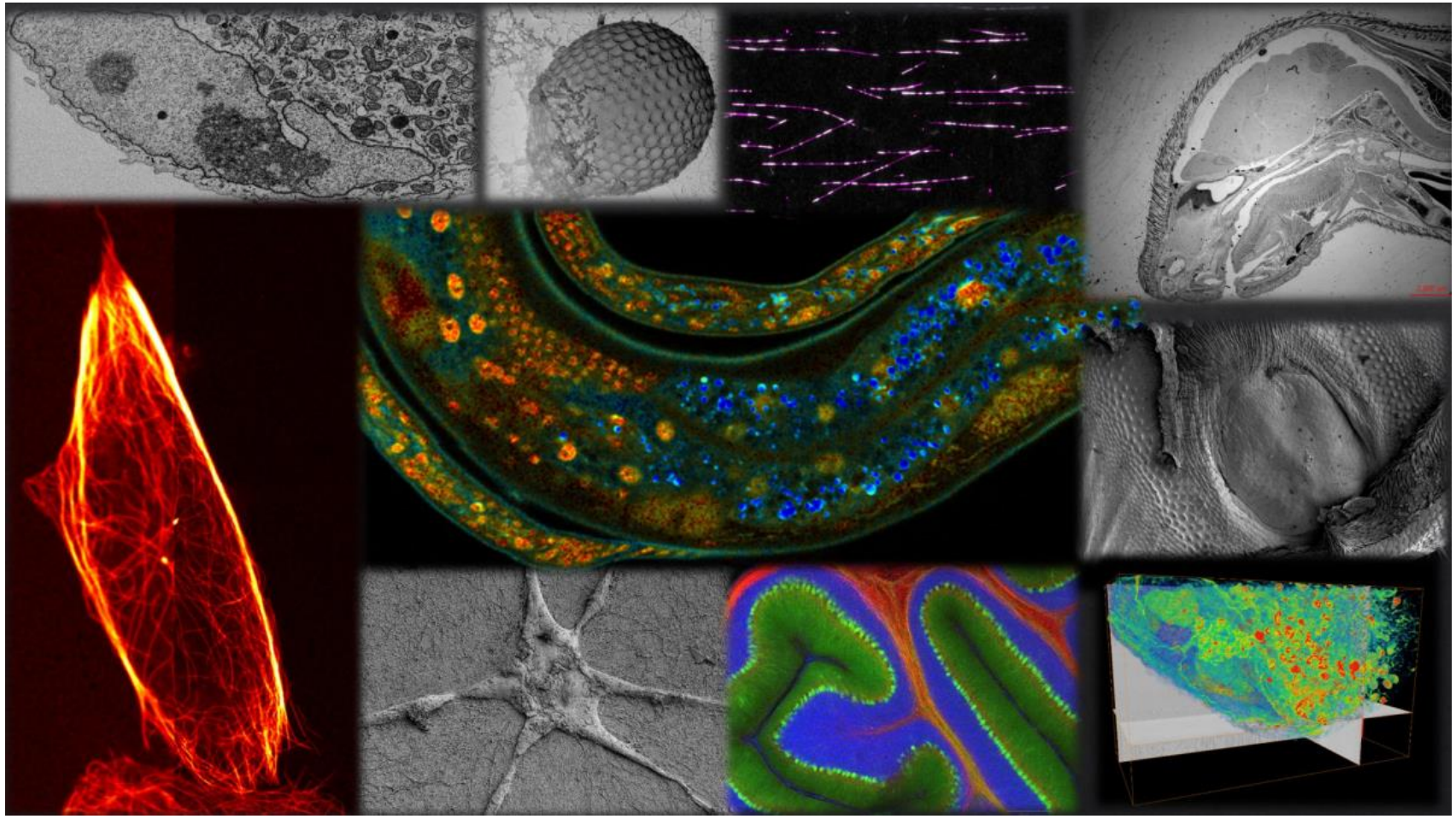
OPEN ACCESS = advanced microscopy for everyone

ONLY users' projects and user inspired methodological projects

1. **Light microscopy**
   - **11 high-end systems**
   - **(mostly)** user operated
2. **Electron microscopy**
   - **FIB-SEM, TEM**
   - **+ sample preparation**
   - **(mostly)** staff operated
3. **Flow cytometry**
   - **2 analyzers + 1 sorter**
   - user and staff operated
4. **Image Data Analysis**
   - **Commercial software + hardware**
   - assistance

http://imcf.natur.cuni.cz

https://twitter.com/IMCF_BIOCEV



The passive translocation mechanism of arginine-rich cell penetrating peptides

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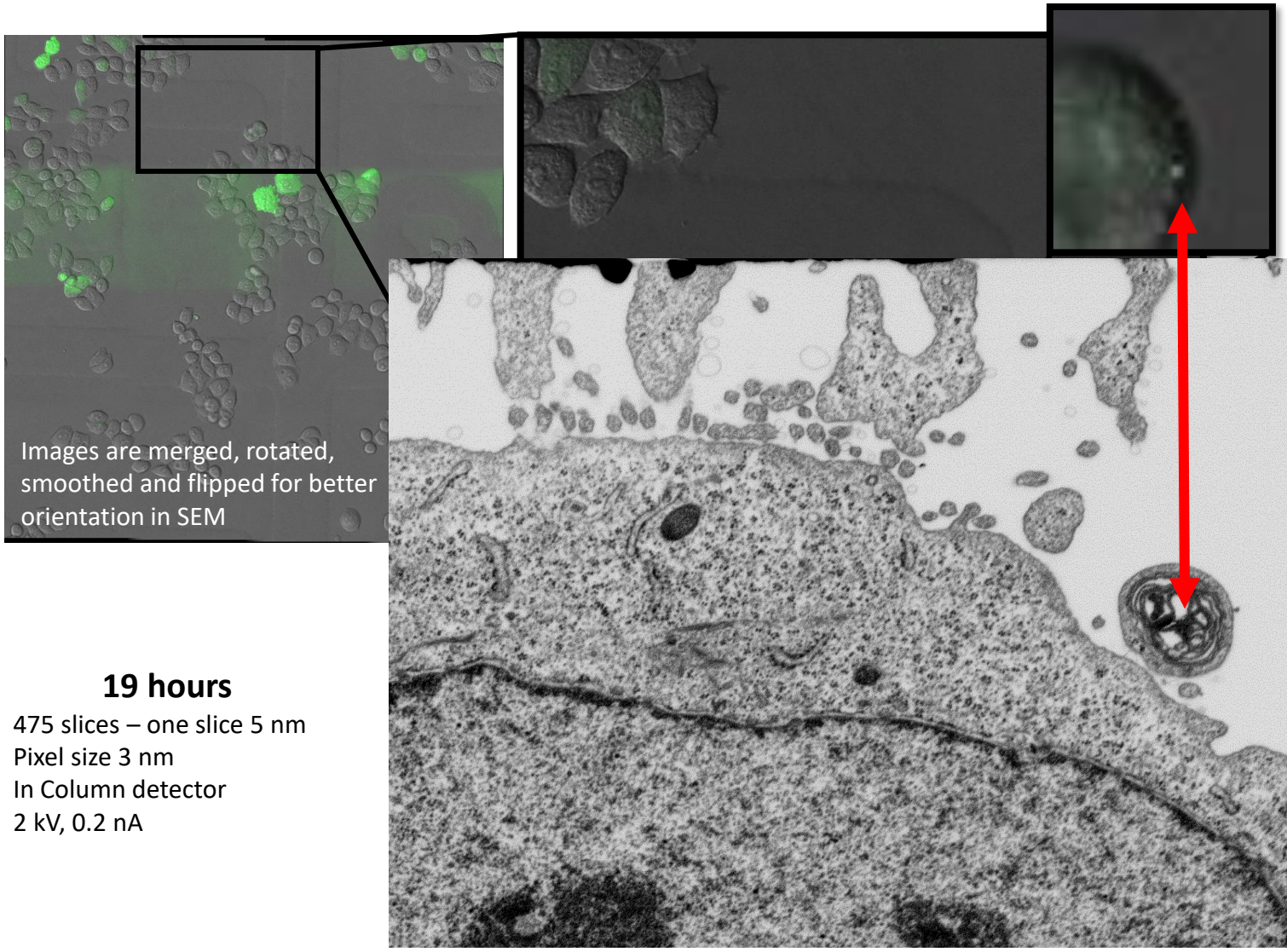
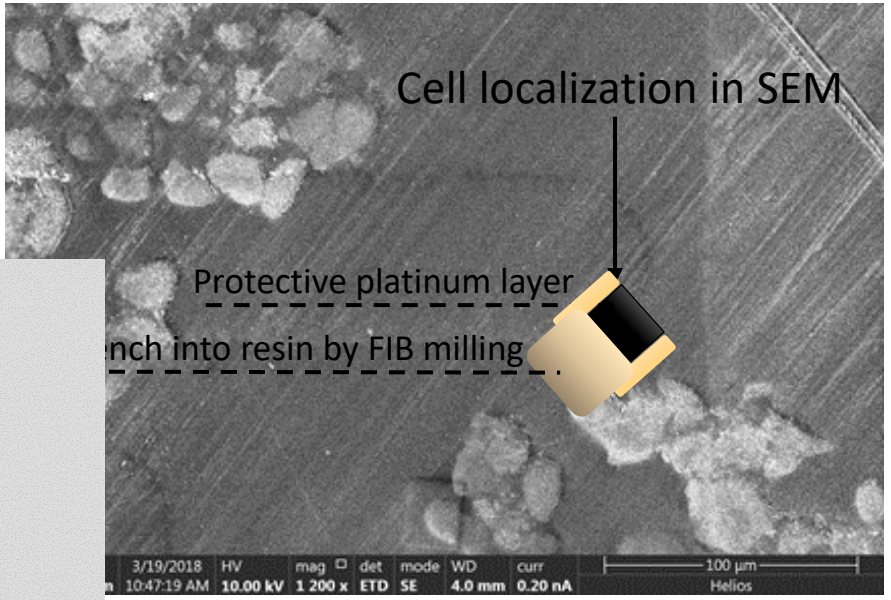


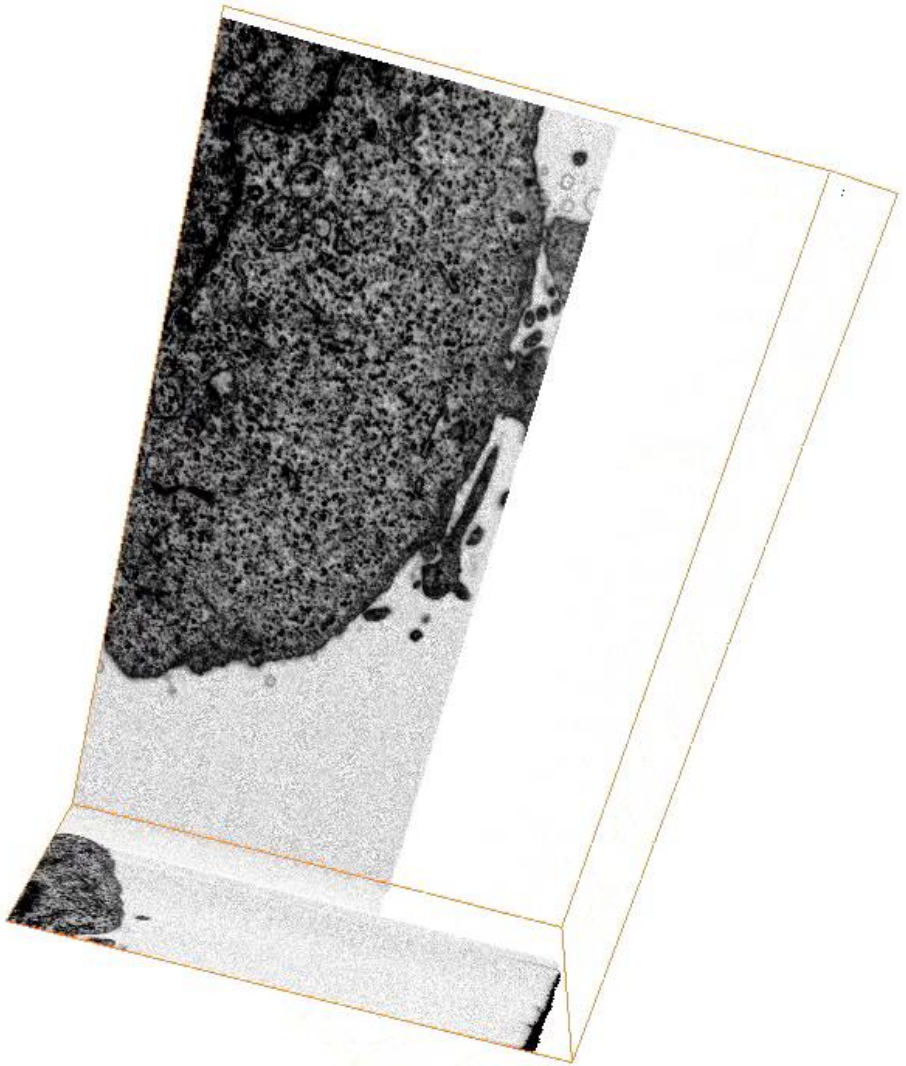


19 hours

- 475 slices – one slice 5 nm
- Pixel size 3 nm
- In Column detector
- 2 kV, 0.2 nA

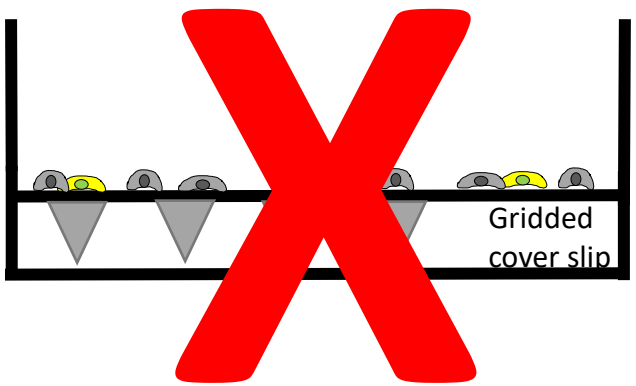
Allolio, C. *et al.* Arginine-rich cell-penetrating peptides induce membrane multilamellarity and subsequently enter via formation of a fusion pore. *Proc. Natl. Acad. Sci.* 201811520 (2018). doi:10.1073/pnas.1811520115

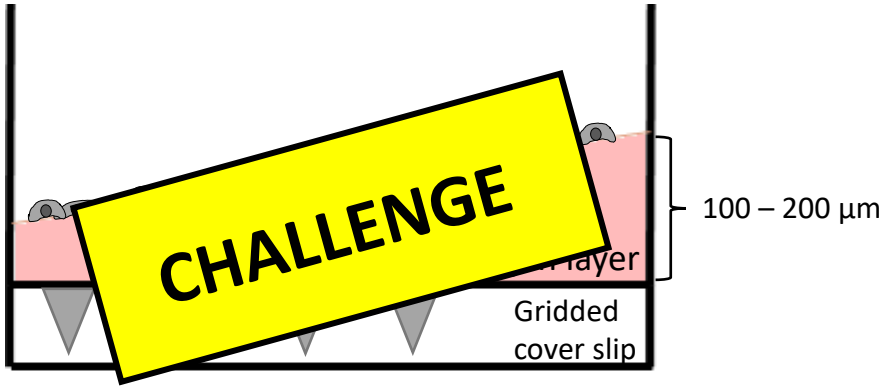
The passive translocation mechanism of arginine-rich cell penetrating peptides



Allolio, C. *et al*. Arginine-rich cell-penetrating peptides induce membrane multilamellarity and subsequently enter via formation of a fusion pore. *Proc. Natl. Acad. Sci.* 201811520 (2018).

When cells are far away from the cover slip……





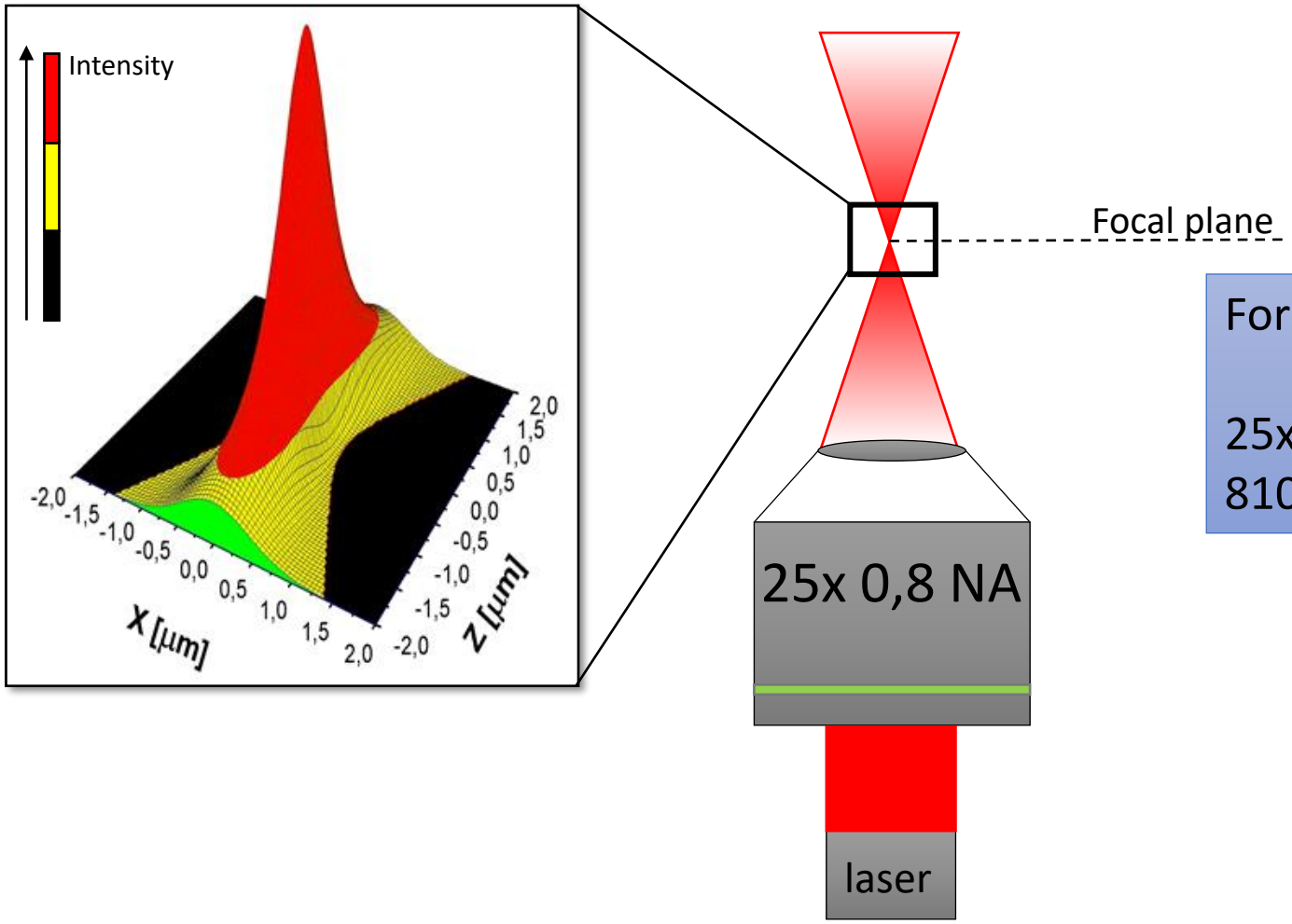
Limitations

- a cell deep inside the resin can not be localized by an overview SEM
- the depth of milled trench for high quality FIB-SEM is limited (standard depth is 5 µm)
- trimming away the resin up to the targeted cell also removes the imprinted grid ☹

The aim – to analyze cancer cell invadopodia ultrastructure in 3D environment and the transport mechanism of lytic enzymes to ECM degradation sites.

Method – CLEM combining fluorescence live cell experiments of cancer cells grown on collagen, expressing genetically labeled matrix metalloproteinases, with FIB-SEM imaging of tumor cells invading the collagen matrix.

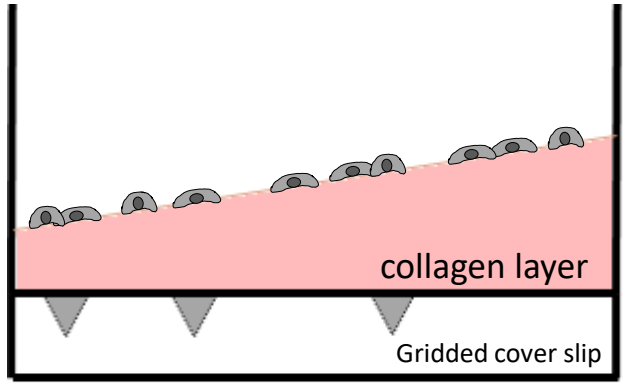
New workflow that extends the pattern from the surface grid deep inside the resin block by using IR laser induced bubbles.



For bubbles burning:

25x WI, 0.8 NA objective
810 nm 2P laser (20-100 mW at sample position)

Cells cultivation on/inside collagen layer in a patterned dish



Live-cell imaging (3D confocal fluorescent and BF images)

Carl Zeiss LSM880 NLO

Sample preparation for FIB-SEM

- Osmium postfixation
- Contrasting with 1% UA
- Dehydration with EtOH
- EPON embedding

Return to confocal microscope

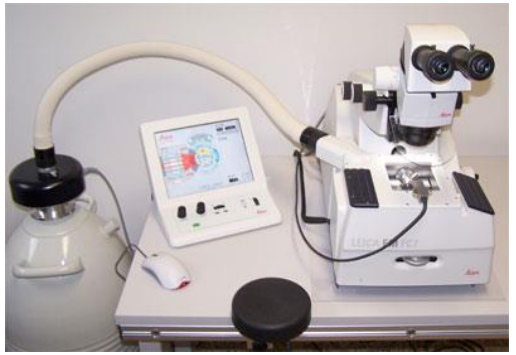
Near-infrared branding (NIRB)

IR laser burning bubbles into a resin block around the selected cell

Carl Zeiss LSM880 NLO

Remove the block from the patterned dish

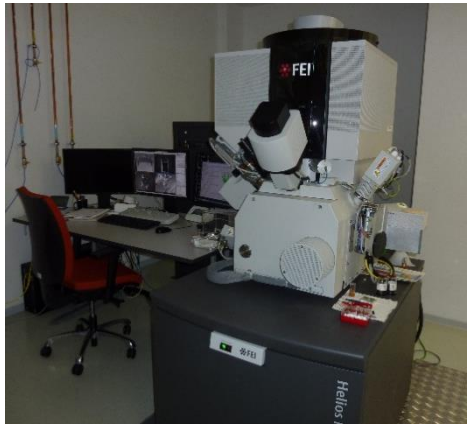
Targeted ultramicrotomy



Ultramicrotome Leica EM UC7

Sputter coating with 25 nm Pt

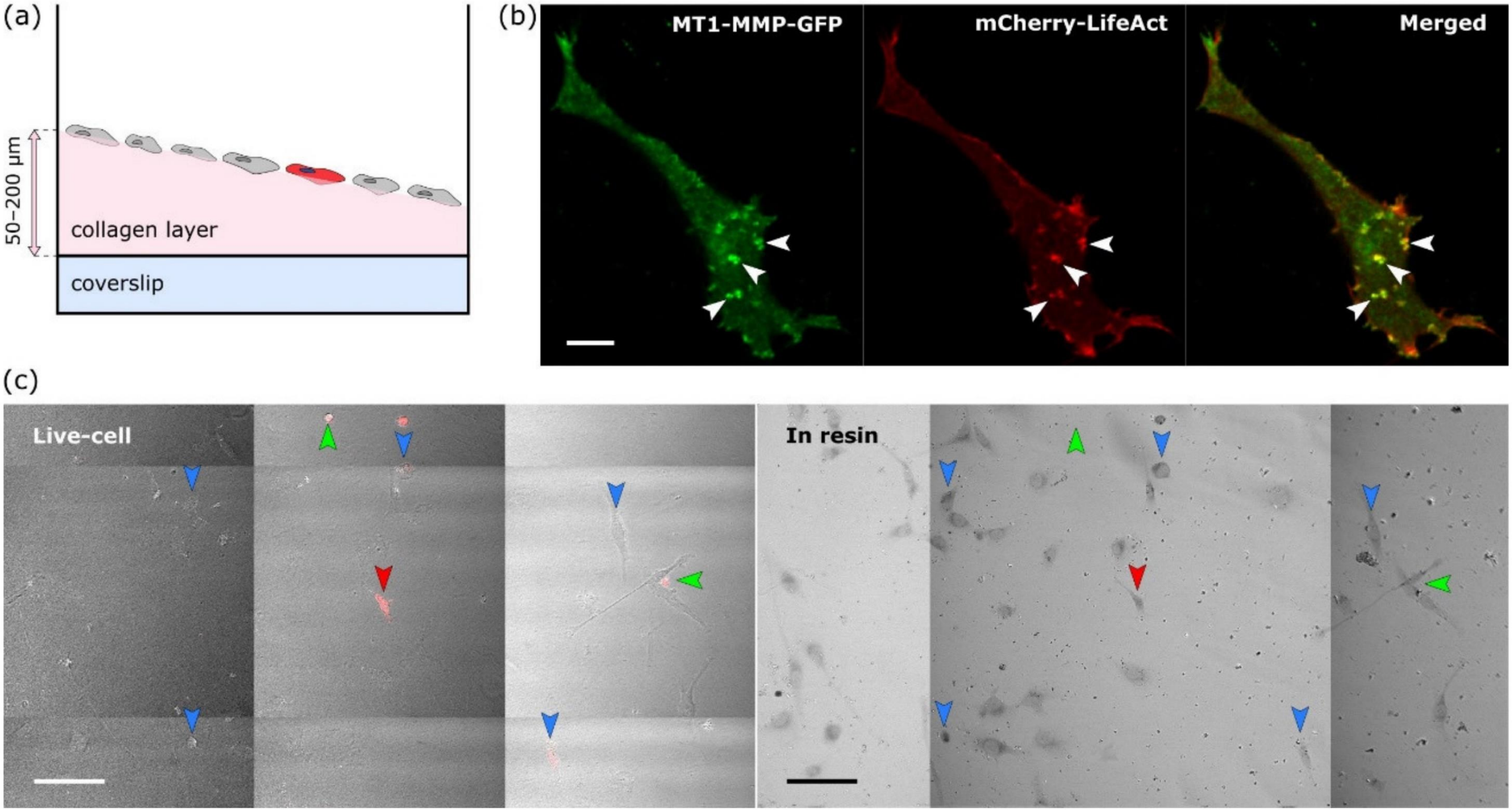
Targeted 3D FIB-SEM



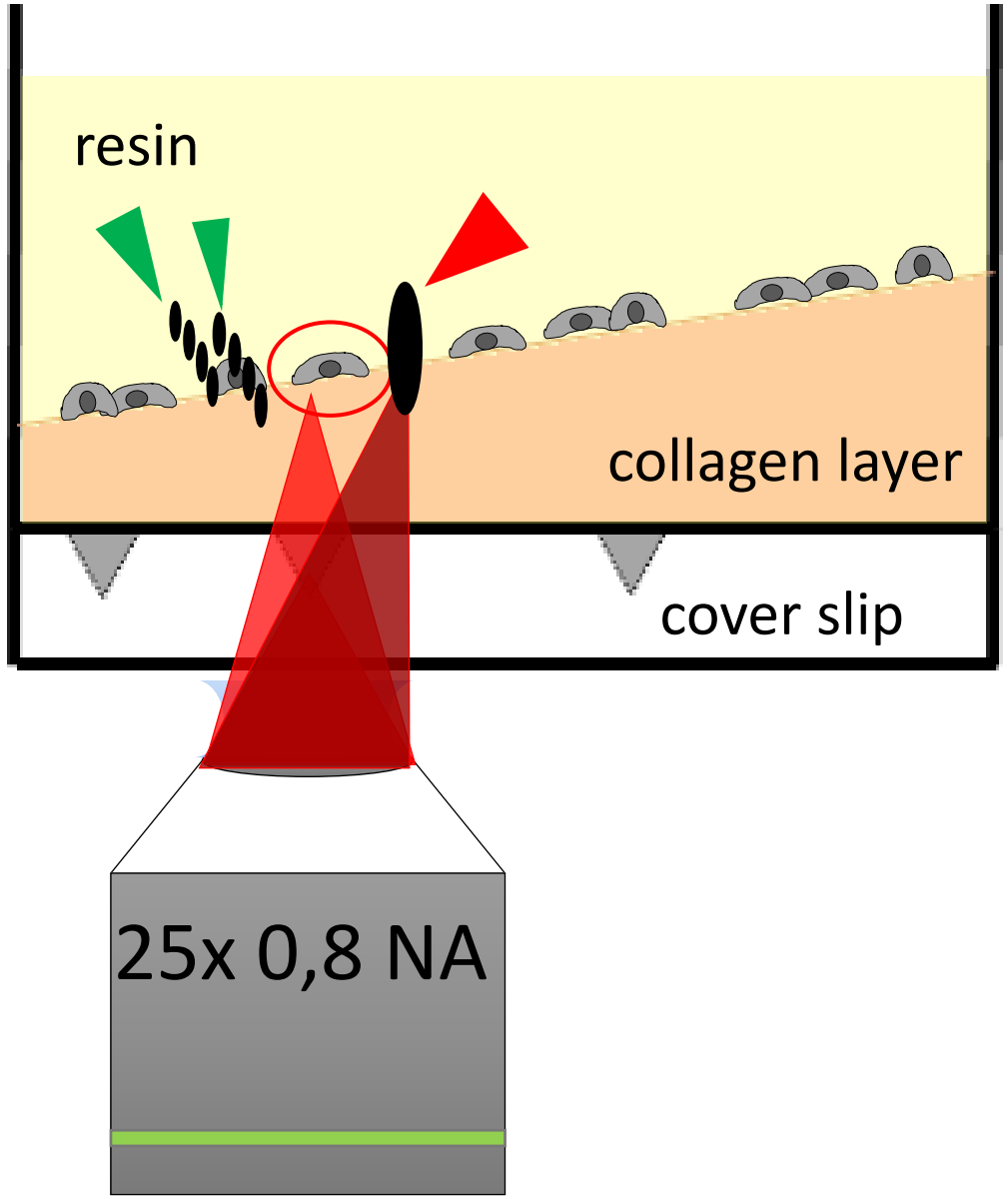
FIB-SEM FEI Helios NanoLab 660 G3

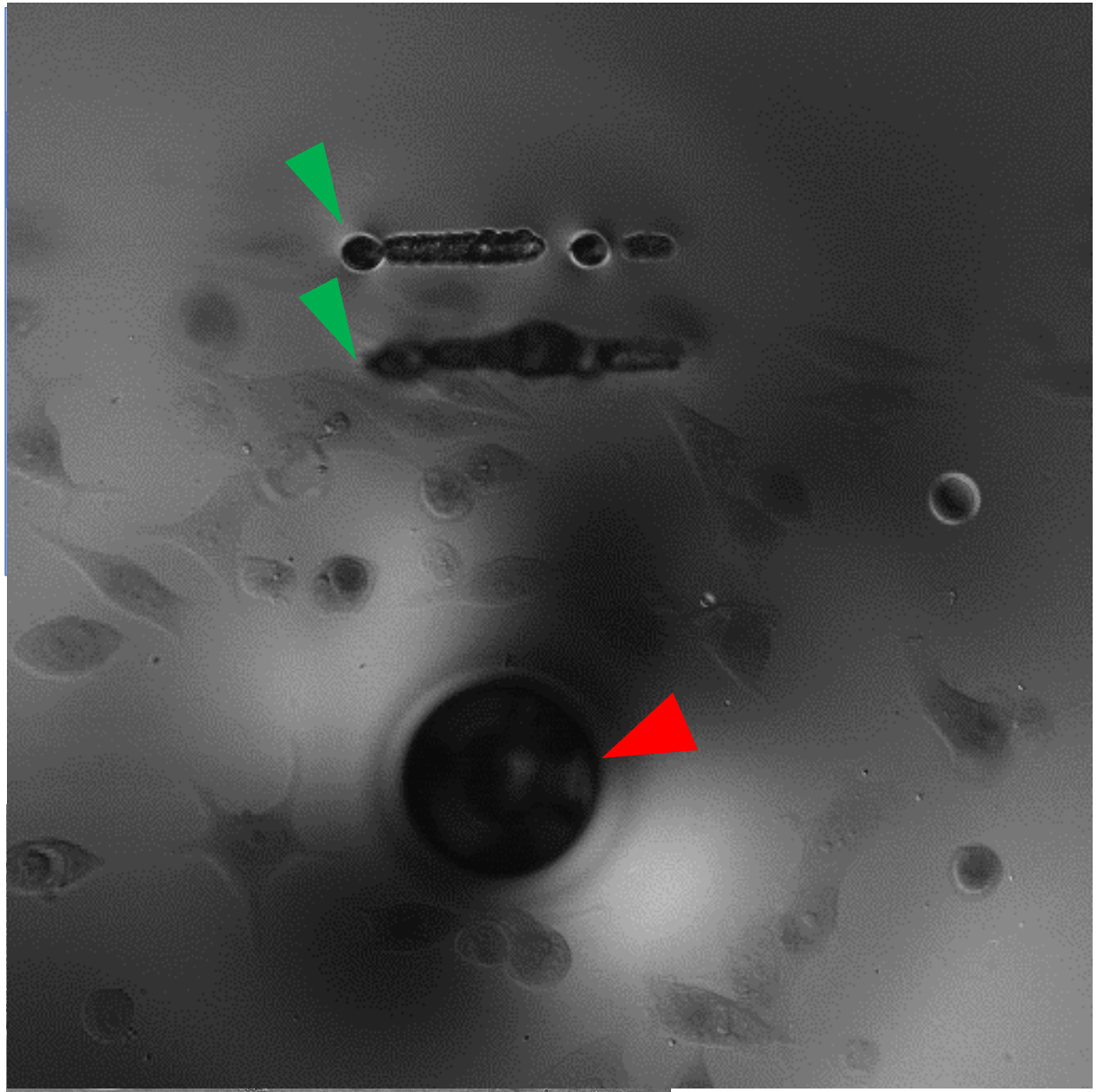
Correlation with 3D fluorescent dataset

Dalecká, M. et al. „Invadopodia Structure in 3D Environment Resolved by Near-Infrared Branding Protocol Combining Correlative Confocal and FIB-SEM Microscopy.“ Int. J. Mol. Sci. **2021**, 22, 7805.


(a)
50–200 µm
collagen layer
coverslip
(b)
MT1-MMP-GFP
mCherry-LifeAct
Merged
(c)
Live-cell
In resin

IR laser burning bubbles into a resin block around the selected cell





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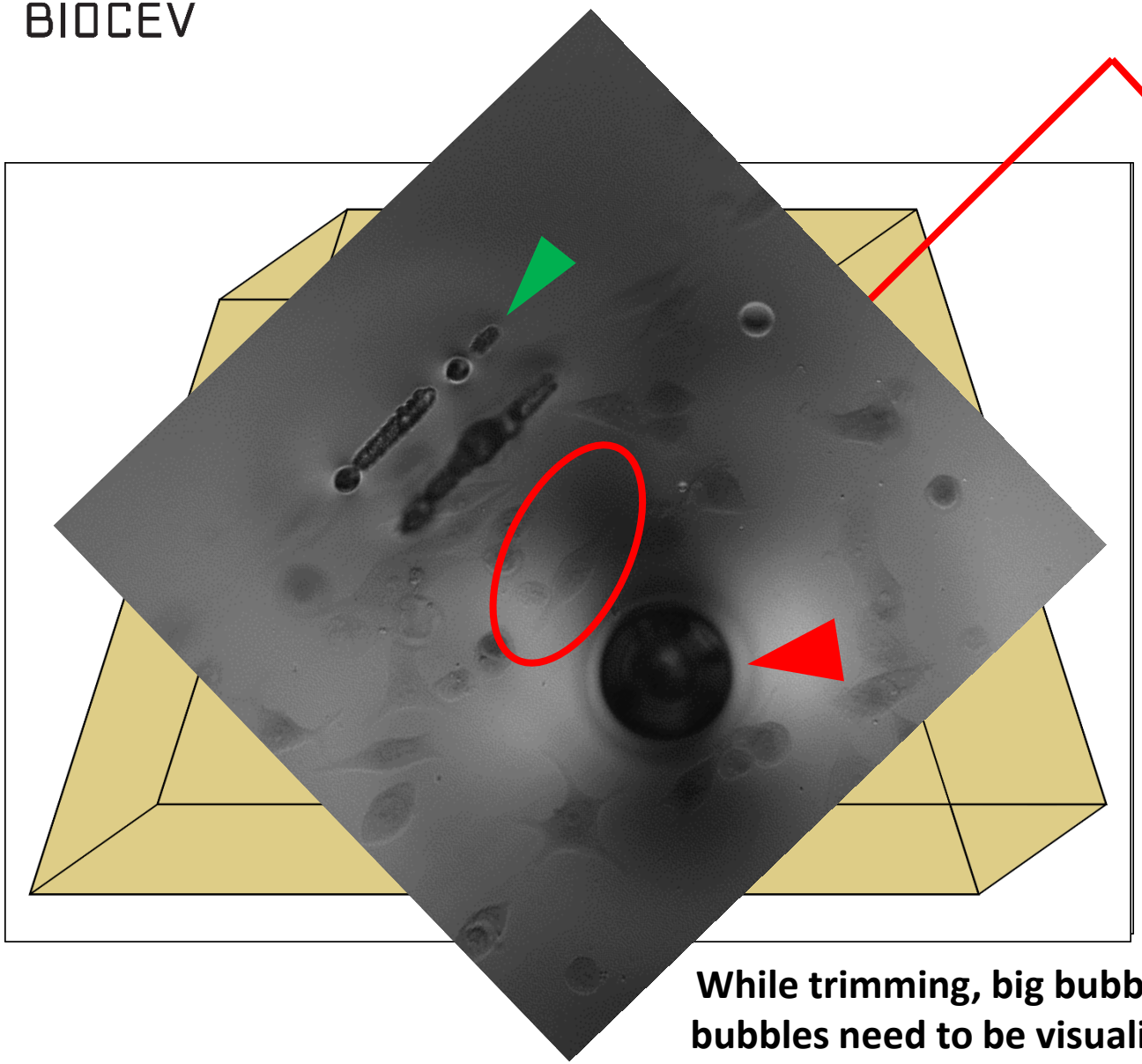
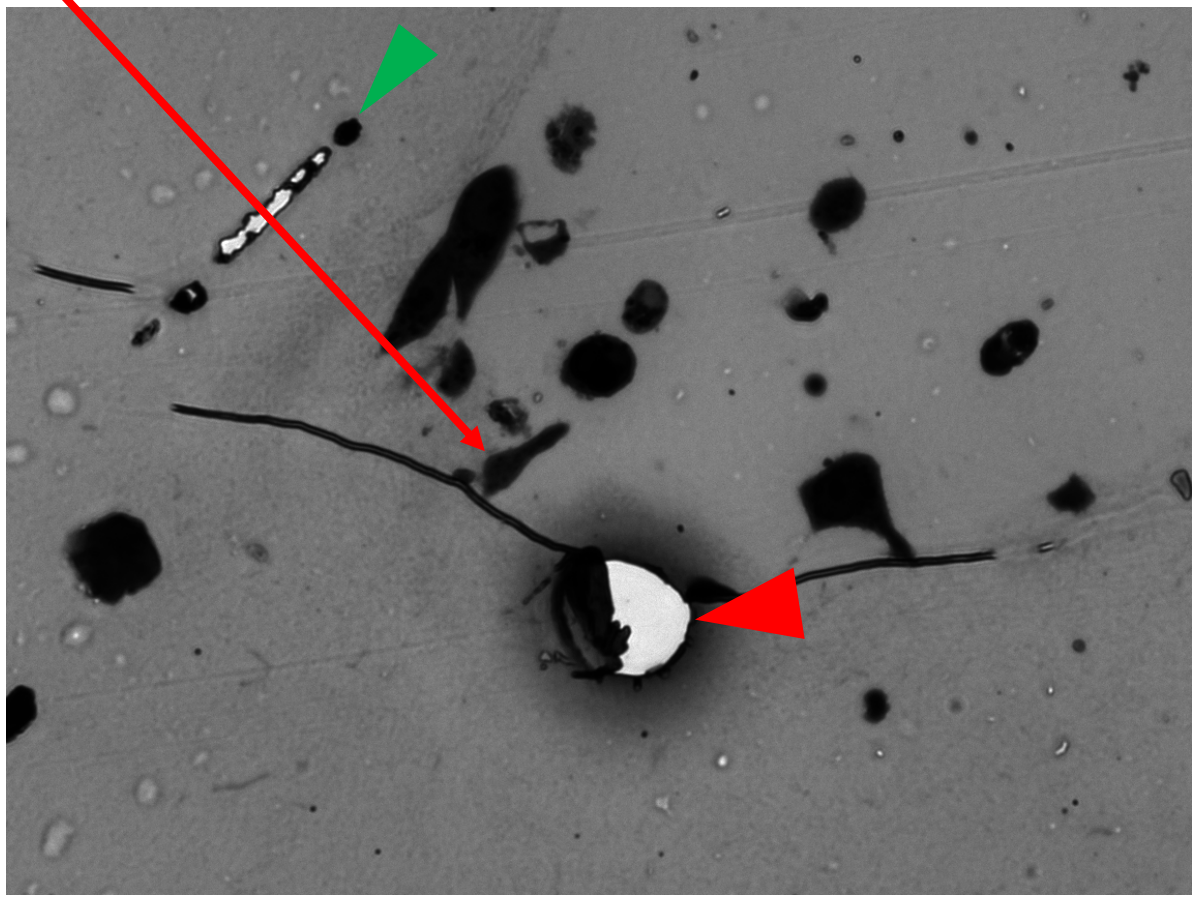
Bubbles assisted cutting through the collagen layer





While trimming, big bubbles are visible with the ultramicrotome optics, cells and line-bubbles need to be visualized by staining sections with toluidine blue.

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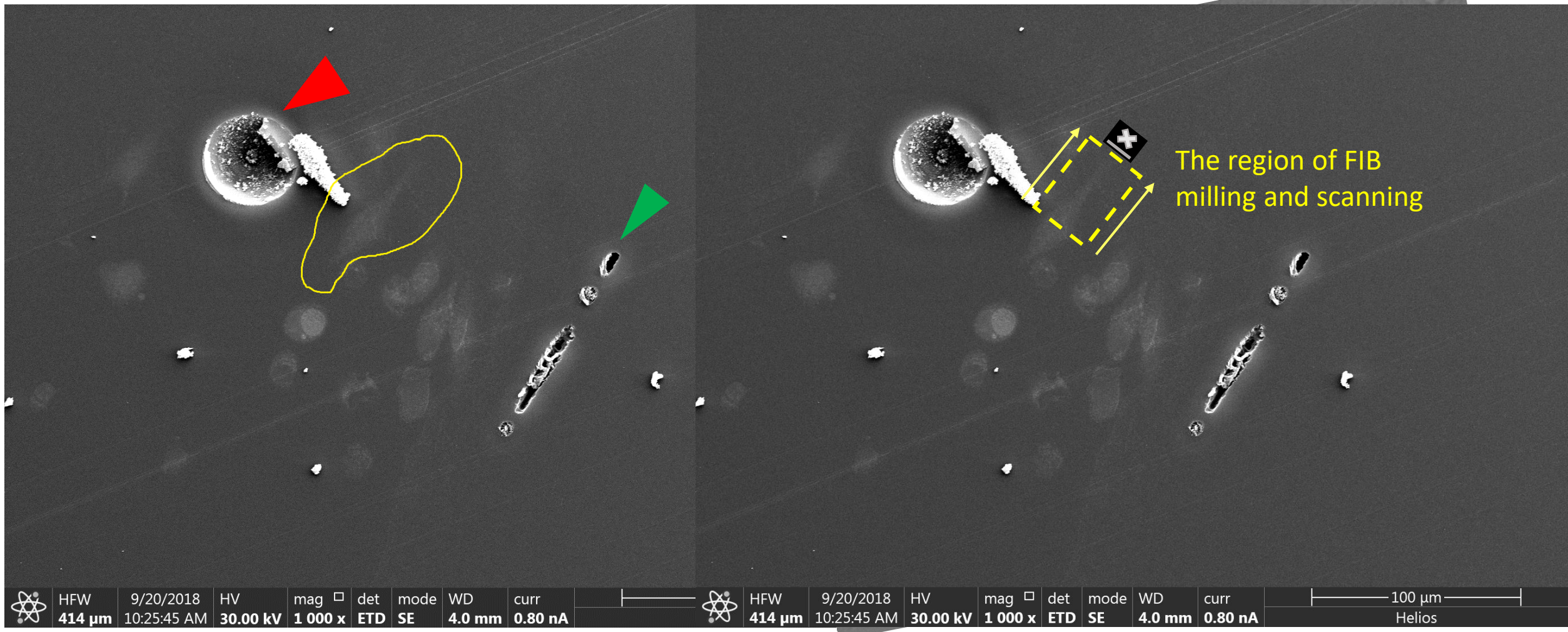
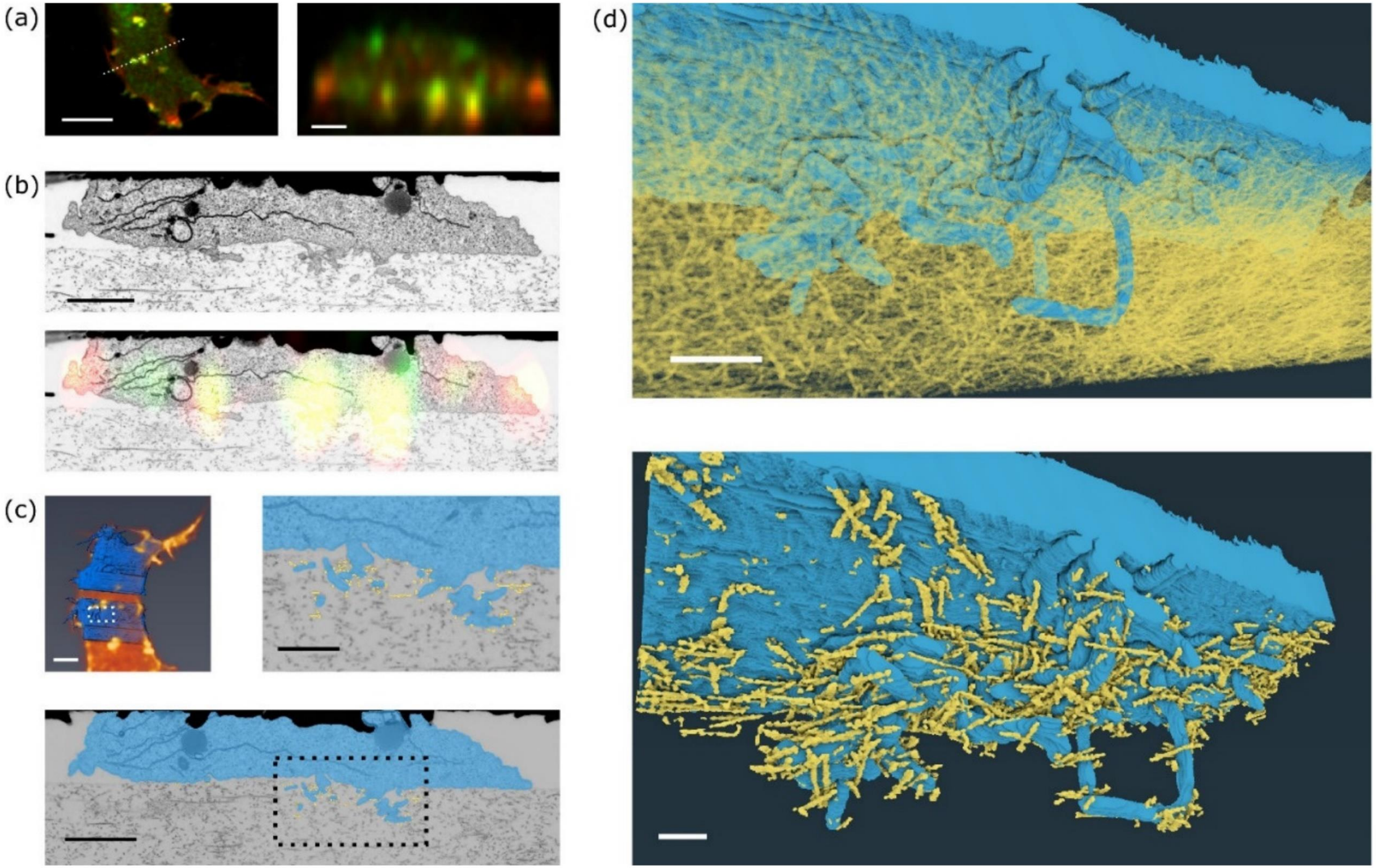
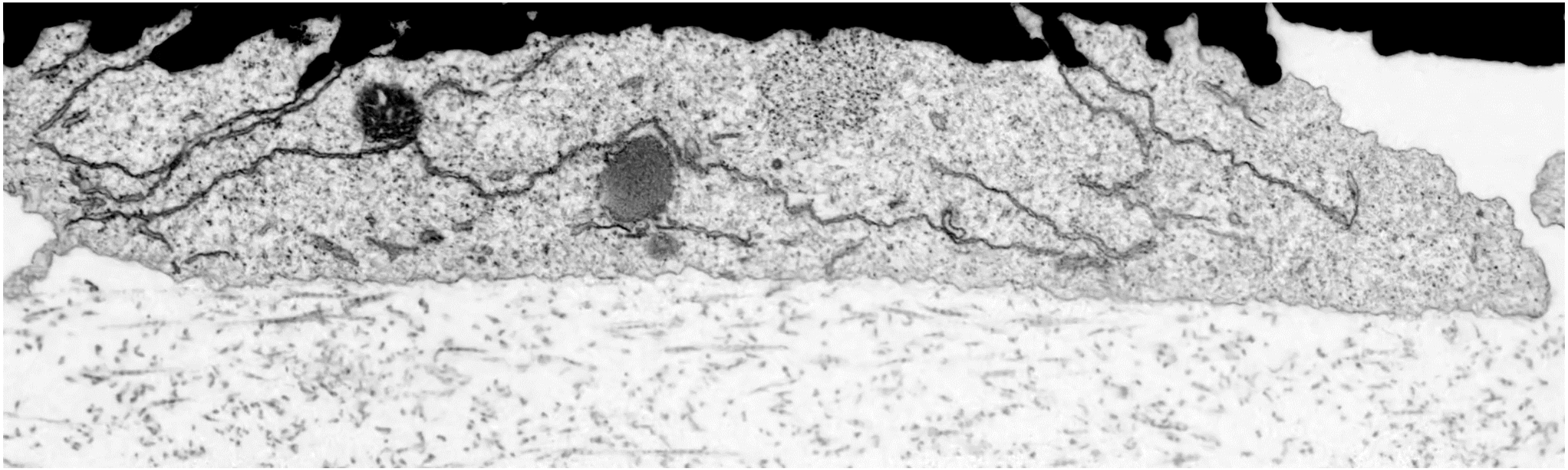
FIB SEM targeting and milling

After finishing trimming, the block was prepared for imaging by FIB-SEM. The sample was mounted on a regular SEM stub using conductive carbon and then coated with 25 nm of platinum




(a)
(b)
(c)
(d)

FIB-SEM movie



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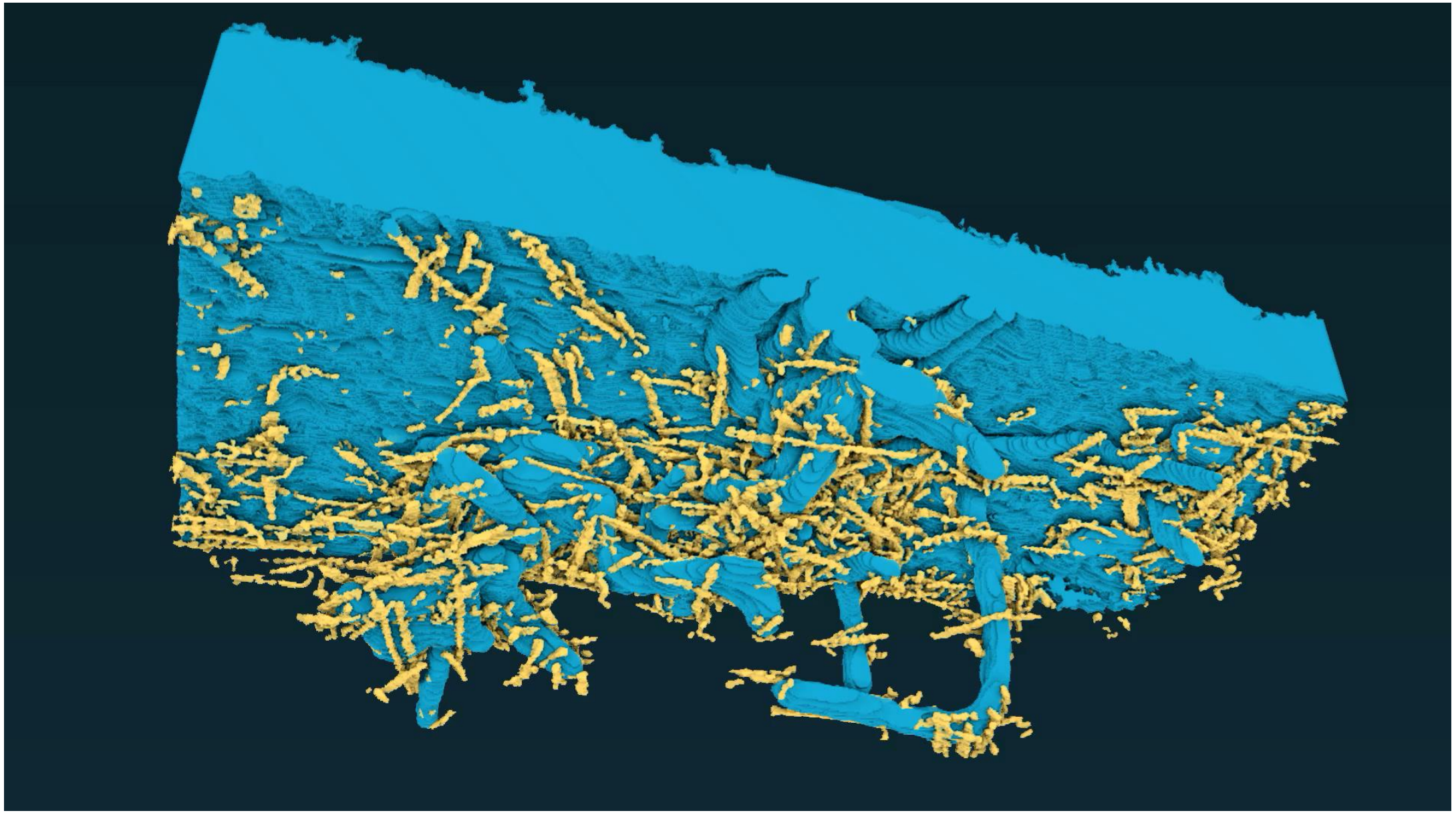
Collagen fibers in a close contact with invadopodia



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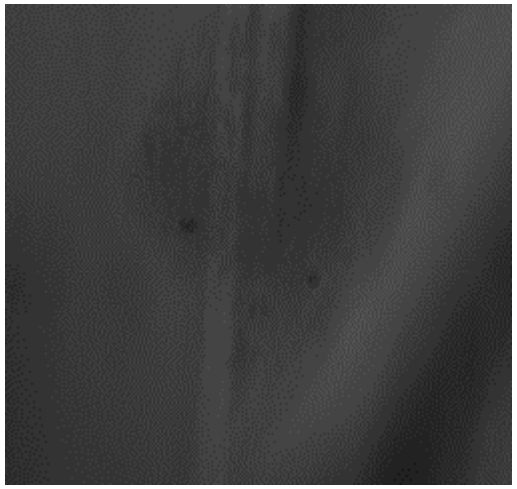
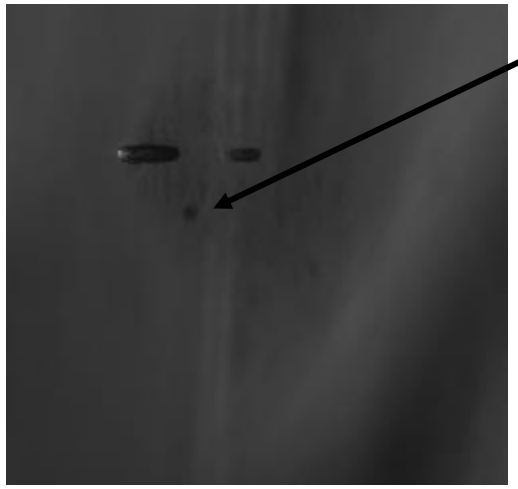
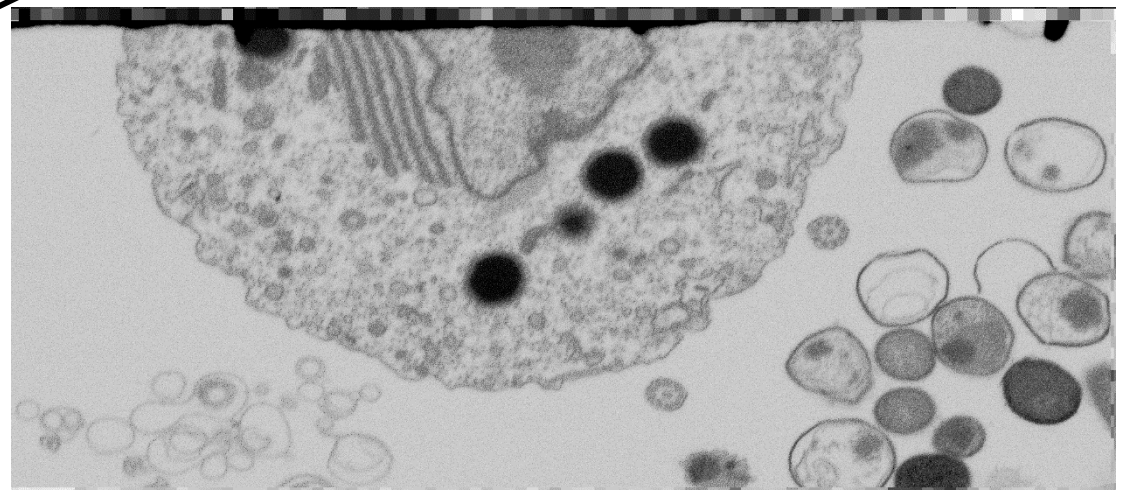
Other applications?

- FIB-SEM targeting of samples which are embedded deep in the volume of the resin

Example: a suspension of bacteria and cells of interest







Acknowledgement

- **ALL our users** (3D-CLEM Ondřej Tolde)
- My IMCF colleagues (http://imcf.natur.cuni.cz/IMCF/team/)



















- To MEYS CR (LM2018129) and ERDF (CZ.02.1.01/0.0/0.0/18_046/0016045)