Multi-scale - correlative SEM imaging

Fluorescence-topography SEM, RT

Live imaging and Fluorescence-vEM, RT

Cryofluorescence-cryoSEM

Cryofluorescence-cryoFIB

Cryo-cathodoluminescence cryoSEM

cryoSEM-EDS

Raman-cryoSEM

Marie Vancová

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Why multiscale….
(multimodal, multidimensional)

Rich multi-dimensional correlative imaging

- all materials are hierarchical in some sense

Philip J Withers and Timothy L Burnett

The Henry Royce Institute, School of Materials, University of Manchester, M13 9PL, United Kingdom

Corresponding author: p.j.withers@manchester.ac.uk
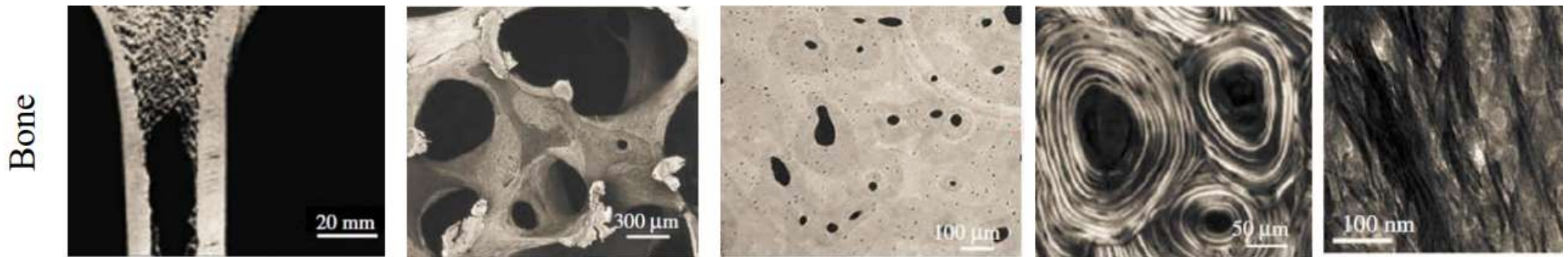
a) an optical micrograph showing the cortical and trabecular bone, b) SEM of the trabecular network, c) micro CT of the Haversian system, d) Lamellar structure in polarized light, e) TEM of collagen fibrils

Why correlative....

Imaging that analyses the same object by at least two different techniques

also at different **scales**, often from biological tissue to the subcellular level with the aim to add information to the selected ROI or to find it.

Fluorescence microscopy

rare or transient phenotypes or specific subpopulations of cells within a complex tissues, dynamics
But: the impossibility of precisely identifying unlabeled structures, resolution

Electron microscopy: add subsequent ultrastructural context but not for screening larger sample areas and is not able to provide any data about cell dynamics.

Influence of viral replication on BBB integrity

Fluorescence-topography SEM, RT

Cells grown on the fibrinogen-coated glass slides

4% formaldehyde 15-30 min

Immunofluorescence

2.5 % glutaraldehyde

OsO4, dehydration, CPD

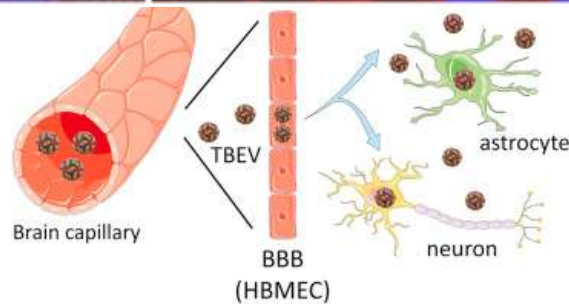Volume changes

Fiducial markers
Lipid droplets(intracelular)

A — TBEV (Hypr) ZO-1 DAPI — 20.0 µm
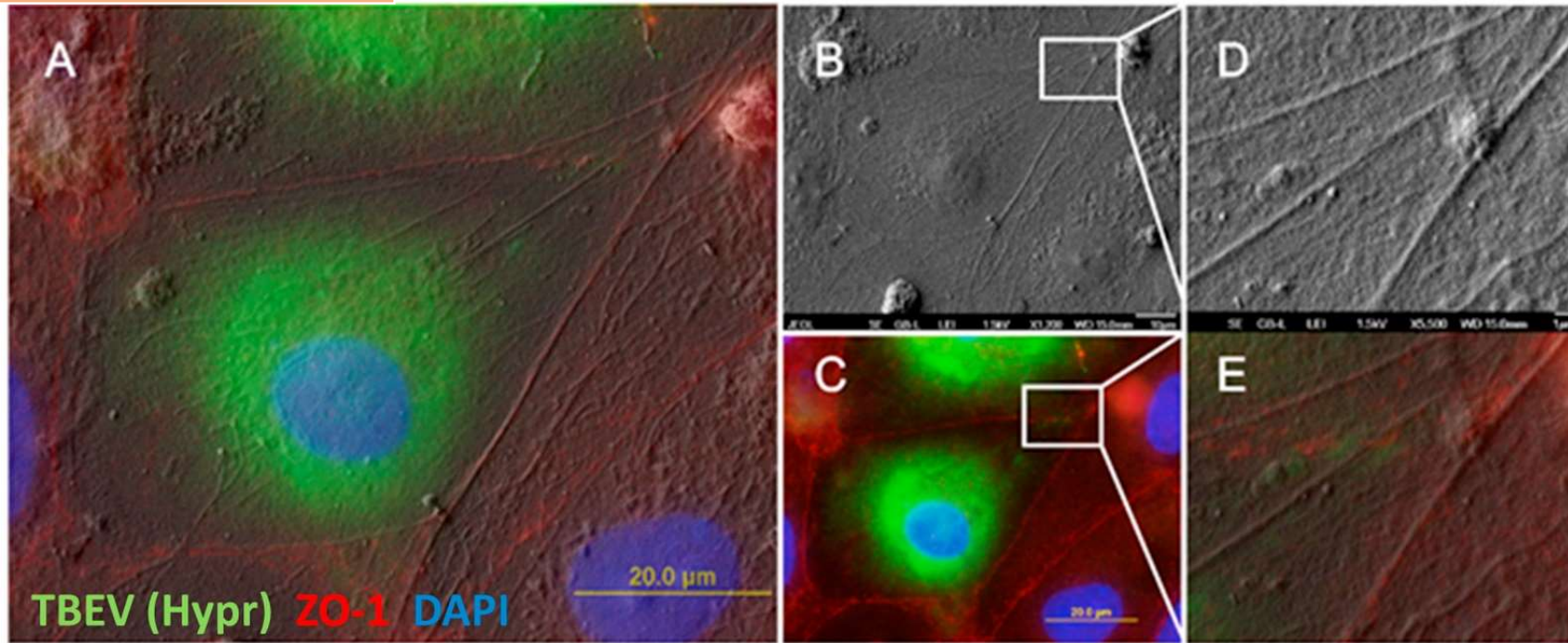
Tick-borne encephalitis virus infects human brain microvascular endothelial cells without compromising blood-brain barrier integrity

Martin Palus [a b c], Marie Vancova [a b], Jana Sirmarova [c], Jana Elsterova [a b c], Jan Perner [a], Daniel Ruzek [a c]
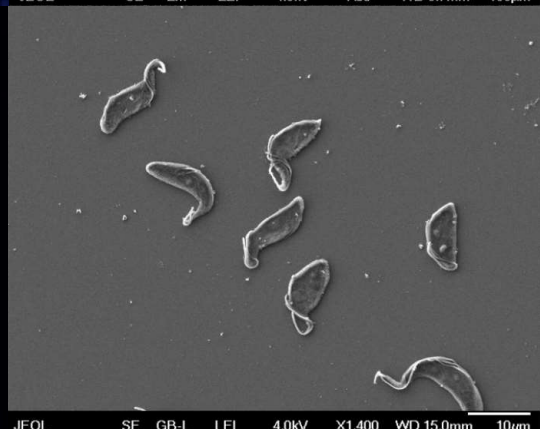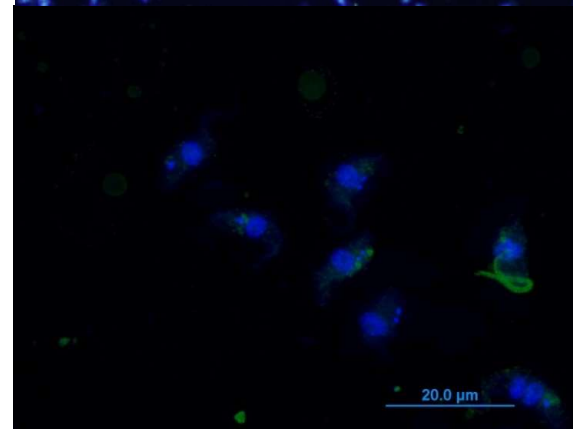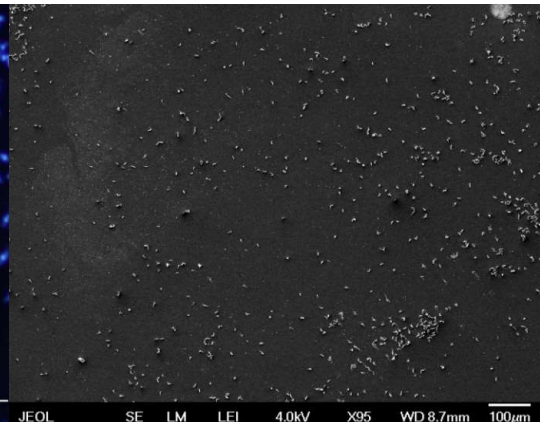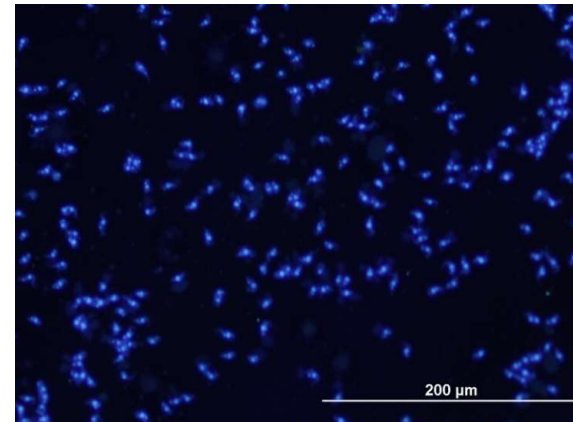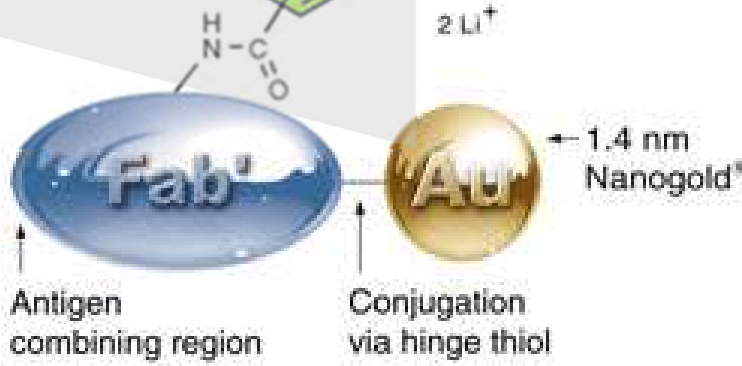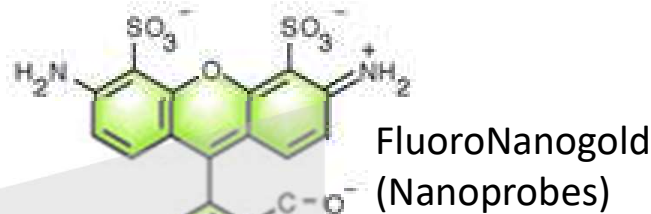
Fluorescence-topography SEM, RT

To find rare events
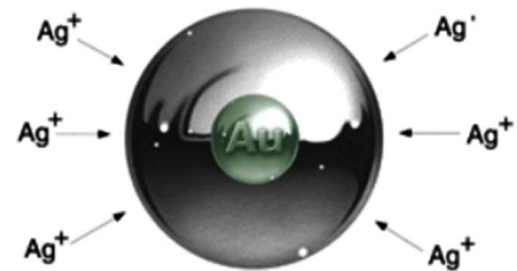
Cells were fixed and attached to poly-L-lysin coated coverslides

Immunofluorescence cell staining

Silver enhancement EM sample prep.

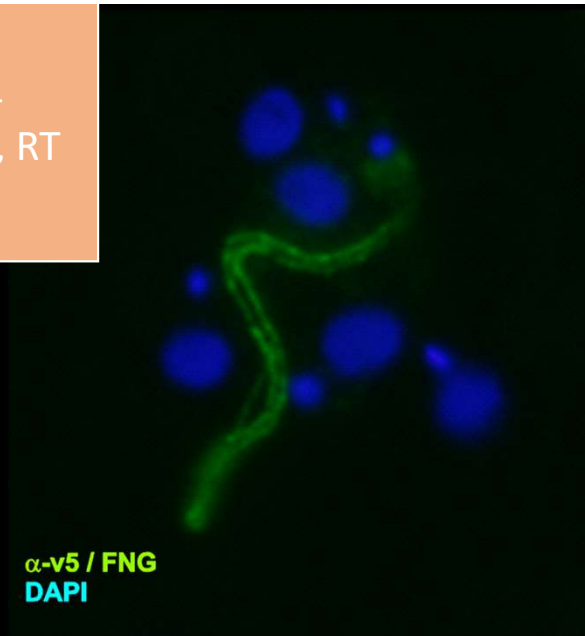BSE 6-10 kV.

FluoroNanogold (Nanoprobes)

$2 Li^+$

1.4 nm Nanogold®

Antigen combining region

Conjugation via hinge thiol

SO_3^- SO_3^-

H_2N NH_2^+

$C-O^-$

O

$N-C=O$

Ag^+ $Ag^.$

Ag^+ Au Ag^+

Ag^+ Ag^+

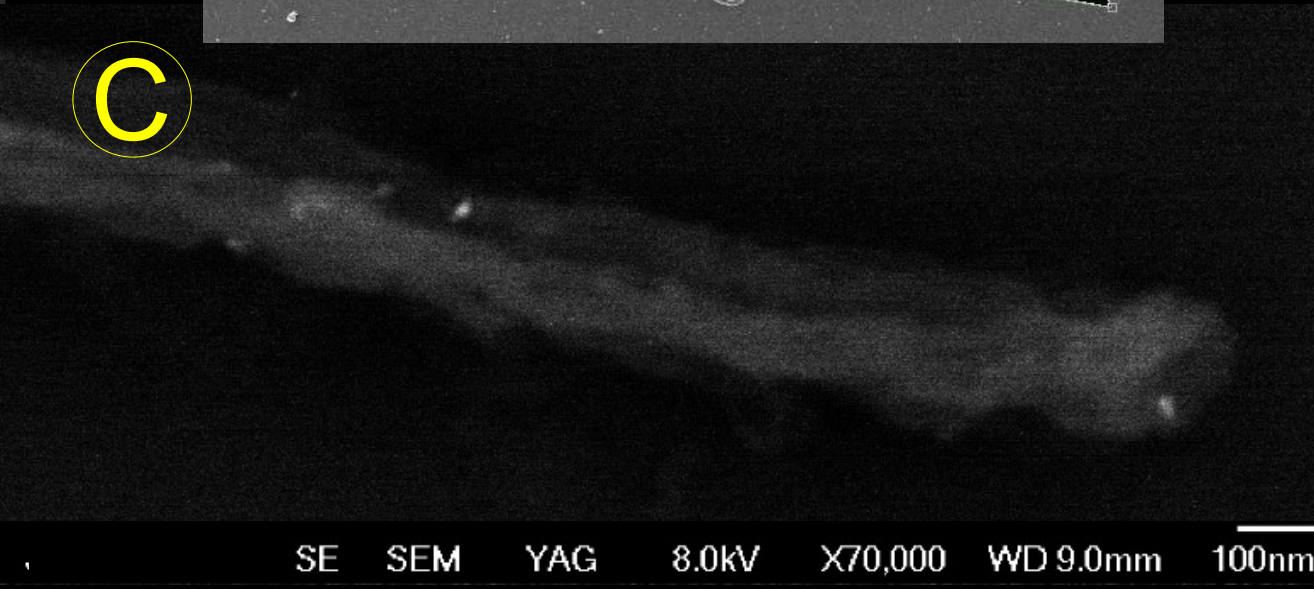Silver/gold enhancement-different dimensions of particles-vs multiple labeling

Fluorescence-topography SEM, RT

α-v5 / FNG
DAPI

B
α-v5 / FNG
DAPI
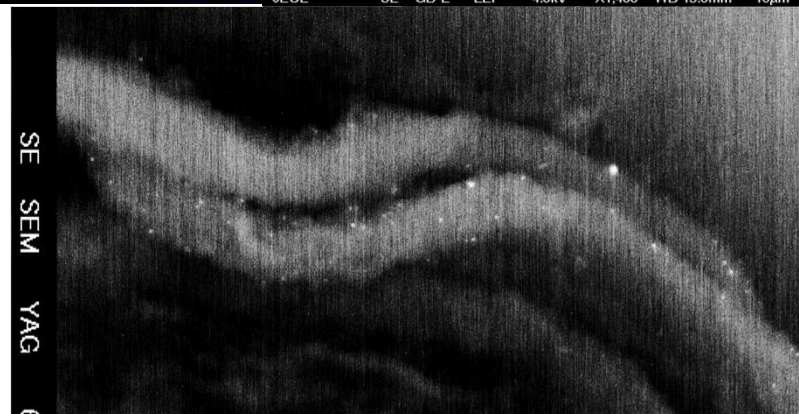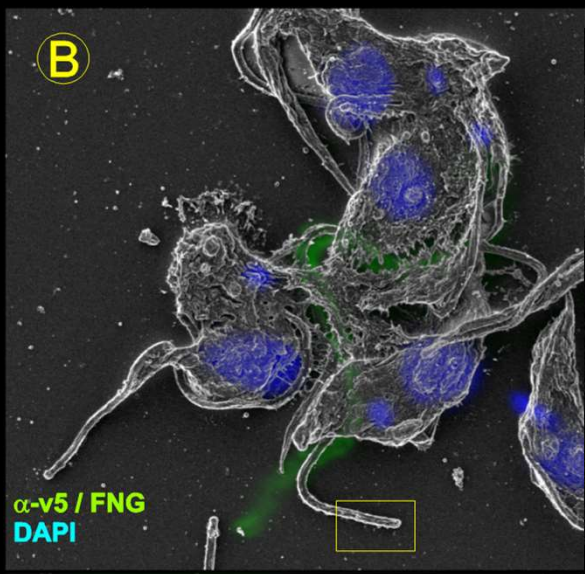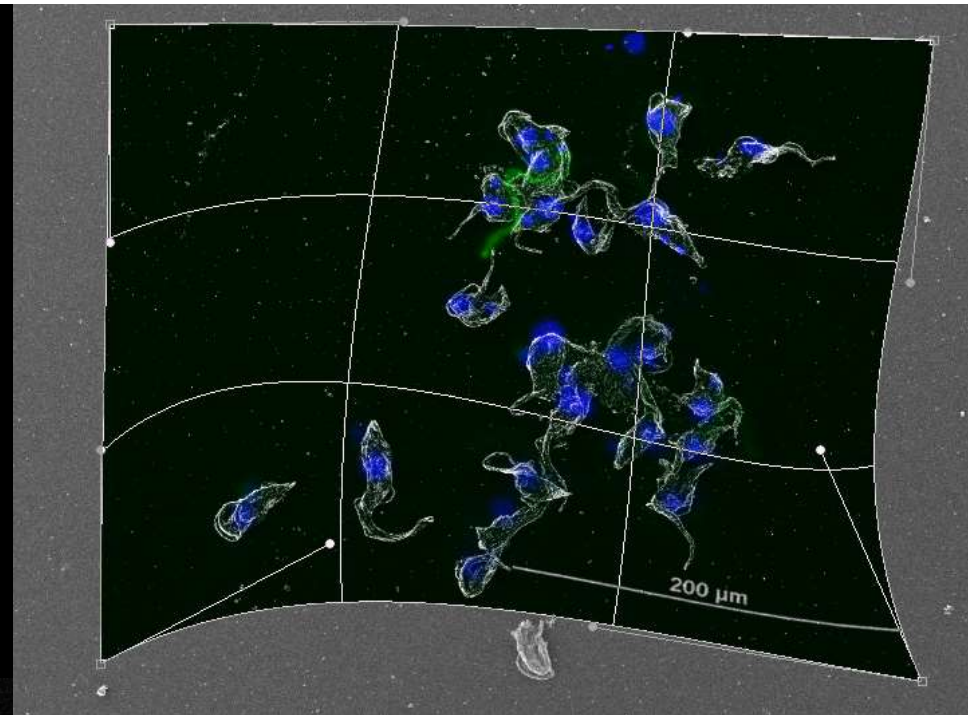
SE GB-H SEI 1.0kV X6,000 WD 9.0mm 1μm

C

200 μm

SE SEM YAG 8.0kV X70,000 WD 9.0mm 100nm

Indium–tin oxide (ITO)-coated glass

- No adverse effects on the physiological behaviour (fibroblasts, cancer cell lines), can be further coated with e.g. type-I collagen to mimic the ECM
- For SEM imaging of uncoated biological samples without charging arfefacts in high vaccum SEM
- Compatible with FL

Advantages of indium–tin oxide-coated glass slides in correlative scanning electron microscopy applications of uncoated cultured cells

H. PLUK*, D.J. STOKES†, B. LICH†, B. WIERINGA* & J. FRANSEN*

*Department of Cell Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
†FEI Company, P.O. Box 80066, 5600 KA Eindhoven, The Netherlands

Shuttle & Find for ZEN Imaging Software

Repositioning Accuracy • < 25 μm (initial accuracy, depending on stage specification) • < 5 μm (using software option for fine calibration)

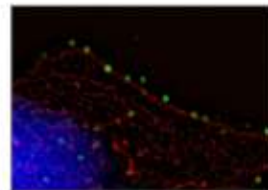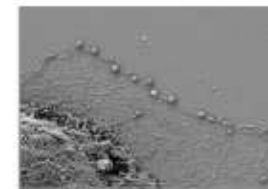| **Sample Preparation**	**Mounting into Correlative Holder**	**Light Microscopy**	**Sample Transfer**	**Electron Microscopy**	**Evaluation & Analysis**
■ Fixation ■ Embedding ■ Labeling	■ Specimen holder for TEM grids ■ Specimen holder for cover glasses ■ Or use any holder with 3 calibration markers	■ Widefield ■ LSM ■ Superresolution	■ Optional: Sample preparation	■ SEM ■ FIB-SEM	■ Correlation ■ Image processing
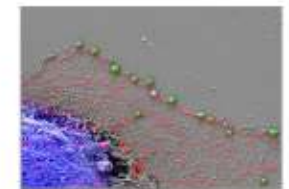
Horiba: nanoGPS navYX™

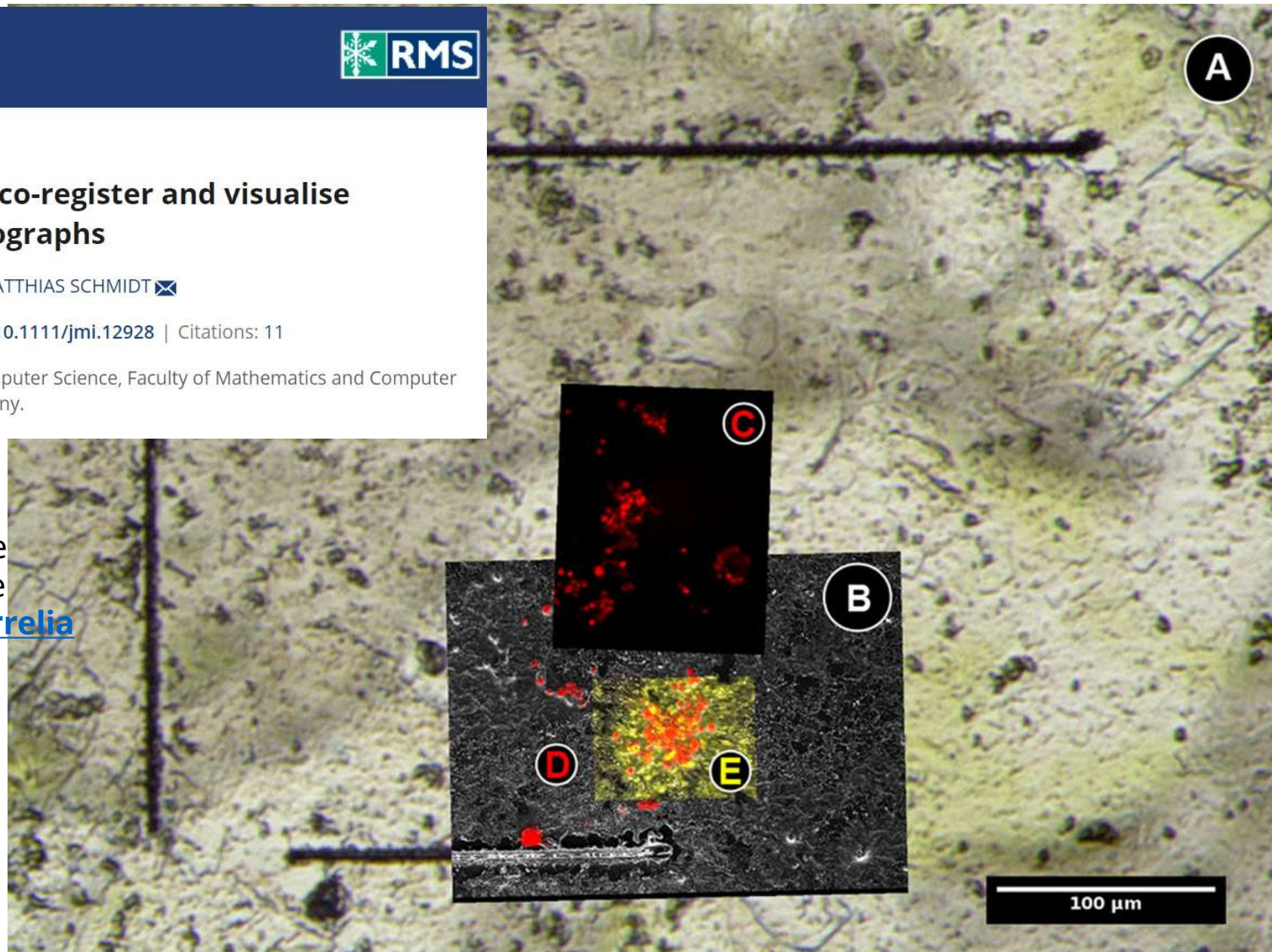|	HORIBA Raman microscope	Optical microscope with high precision XY stage	SEM with regular XY stage	SEM with high precision XY stage
HORIBA Raman microscope	5 - 15 µm	5 - 10 µm	15 - 30 µm	5 - 15 µm

Correlia: an ImageJ plug-in to co-register and visualise multimodal correlative micrographs

FLORENS ROHDE, ULF-DIETRICH BRAUMANN, MATTHIAS SCHMIDT ✉

Correlia is open source software and available from **www.ufz.de/correlia**

100 µm

To find ROI

T62/5a

Borrelia

20 µm

70 µm

1,5 µm

18 x 12 µm

Pixel size : 3 x 3 x 50 nm

250 x 250 µm

1071.4µm

683.1µm

609.4µm

JEOL   SE   LM   LEI   3.0kV   X70   WD 9.8mm   100µm

Fluorescence-vEM, RT

To find ROI

ISIDORe

Pablo Suárez Cortes, Max Planck Institute for Infection Biology, Berlin
Malaria parasites in the mosquito: Imaging the needle in the haystack
 Laboratory of Electron Microscopy, Biology Centre CAS, Budweis, M. Vancová, J. Týč
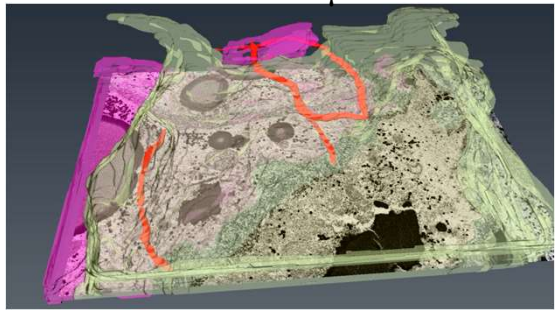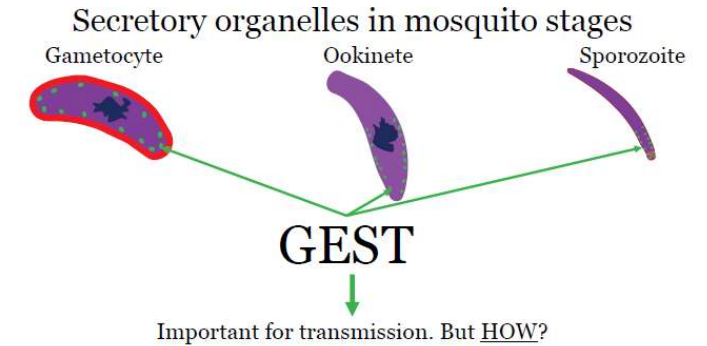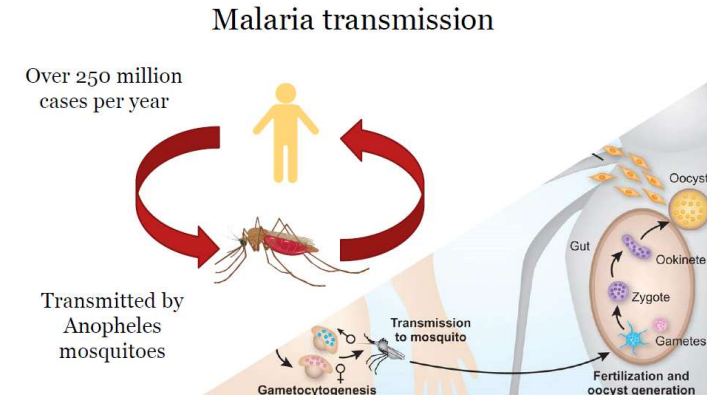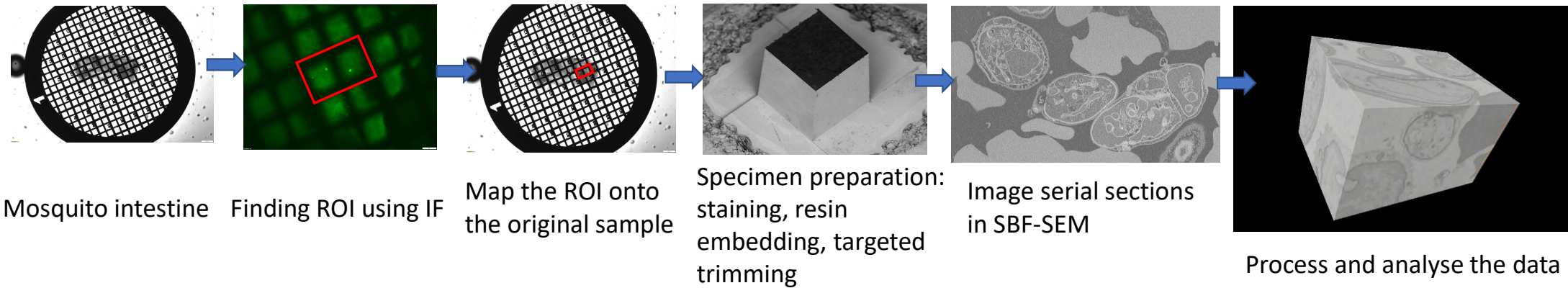
GOAL: Understand role of secretory organelles in mosquito stages for transmission and characterization of ookinete traversal of the midgut
… but at first, we need to find parasites within mosquito tissues

How:

Malaria transmission

Over 250 million cases per year

Transmitted by Anopheles mosquitoes

Secretory organelles in mosquito stages

Gametocyte   Ookinete   Sporozoite

GEST

Important for transmission. But HOW?

Mosquito intestine   Finding ROI using IF   Map the ROI onto the original sample   Specimen preparation: staining, resin embedding, targeted trimming   Image serial sections in SBF-SEM   Process and analyse the data

Live imaging - vEM, RT

# Dynamics: Visualization of ER contact sites with early and late endosomes

Epifluorescence time-lapse movie (dynamic behavior) and **FIB-SEM** (ultrastructural context), **RT.** HeLa cell transfected with LAMP-1-GFP (Lysosomes) and treated with dextran-Alexa568 (endosome-lysosomes)

Images were captured in the GFP channel for a period of 2 minutes at a rate of 0.4 seconds per frame. Images were deconvolved using Softworx 6.5.2 and manually traced using MTrackJ (colored traces). The movie is played back at a rate of 15 frames per second (×6 real time). 3D EM reconstruction and segmentation was performed using IMOD 4.9. Scale bar: 2 μm.

Single organelle dynamics linked to 3D structure by correlative live-cell imaging and 3D electron microscopy
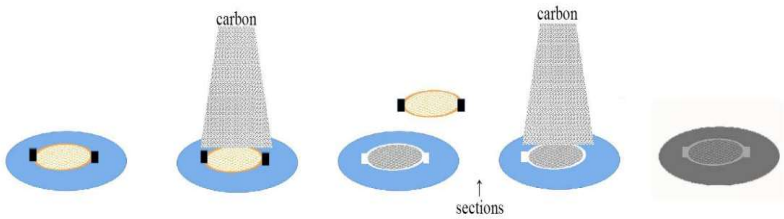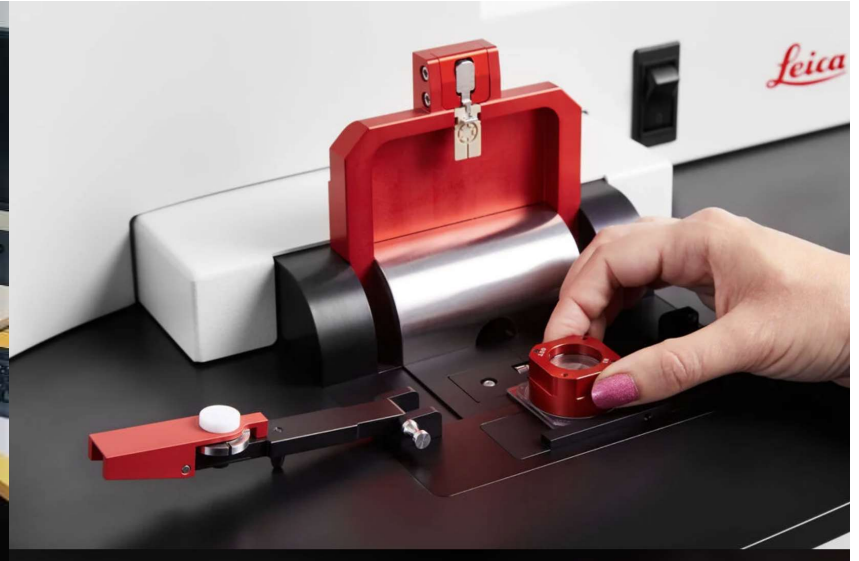
Job Fermie, Nalan Liv, Corlinda ten Brink, Elly G. van Donselaar, Wally H. Müller, Nicole L. Schieber, Yannick Schwab, Hans C. Gerritsen, Judith Klumperman ✉
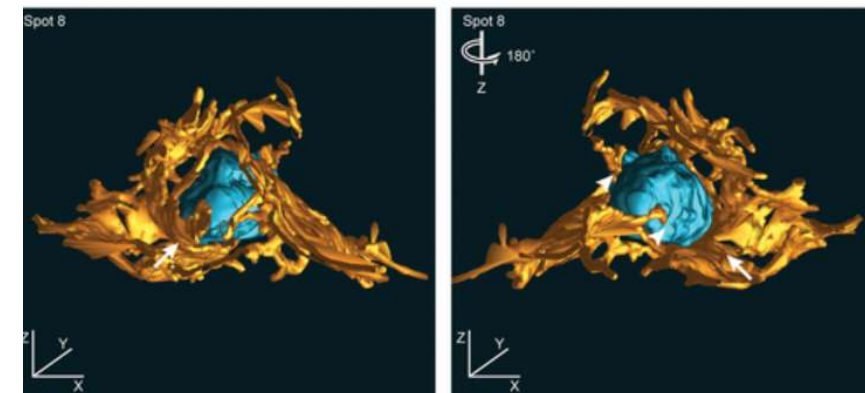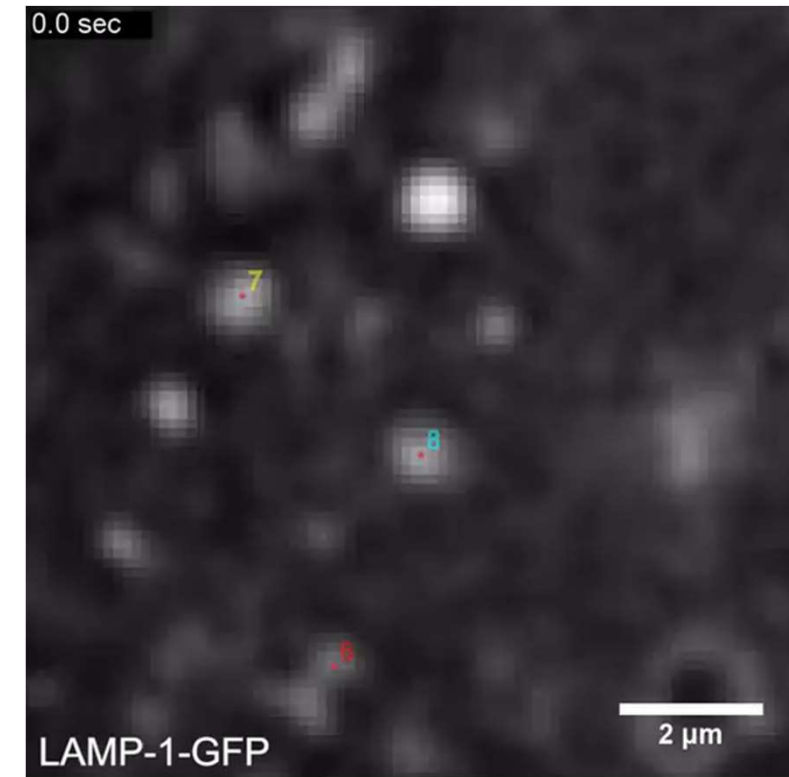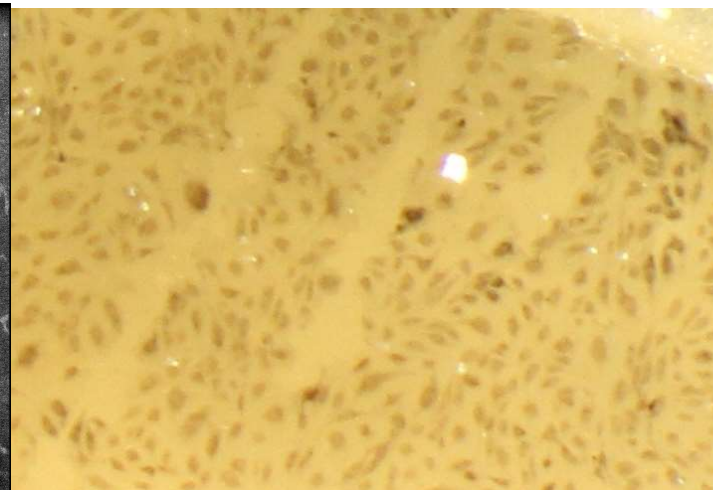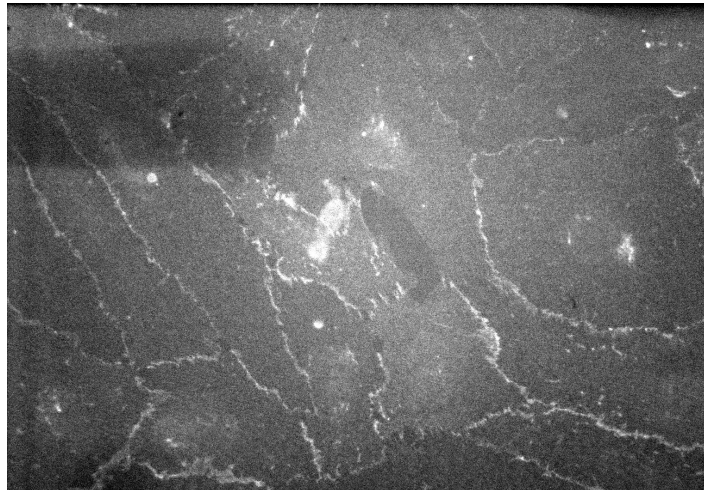
Live imaging - vEM, RT

How Putting Dynamic Live Cell Data into the Ultrastructural Context- Oxygenie + HPF ICE
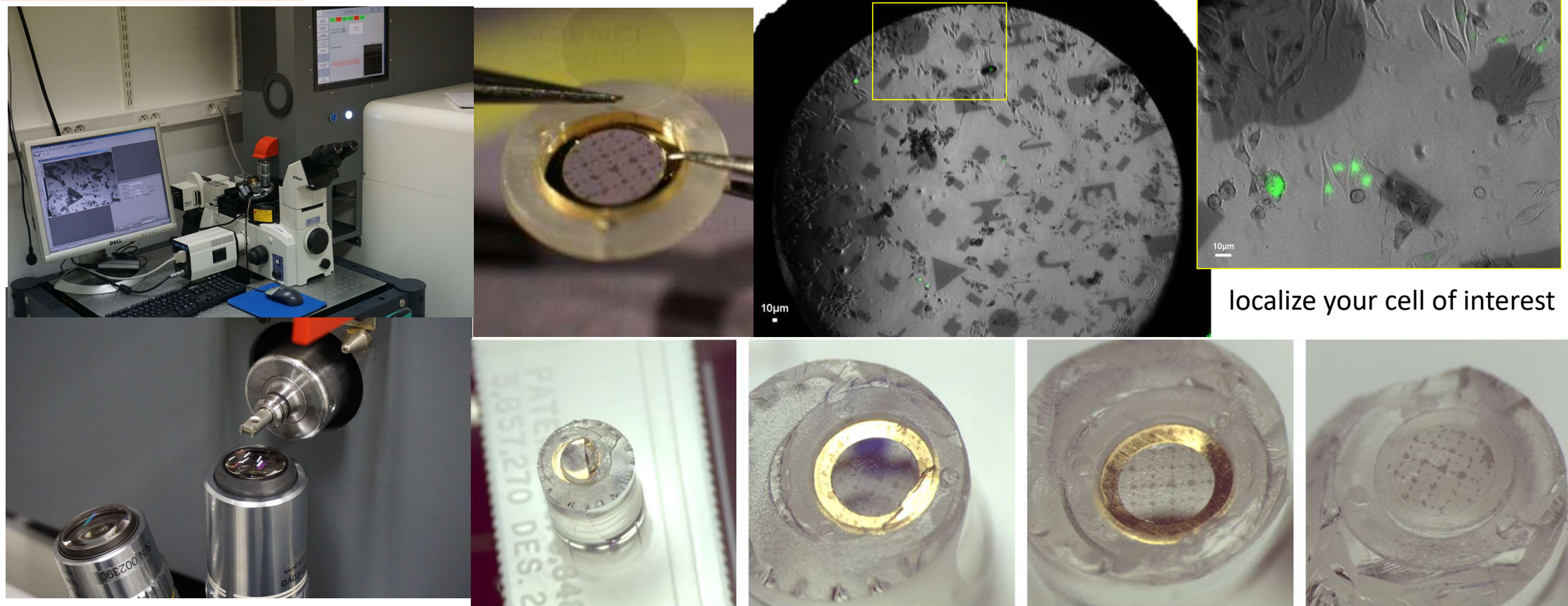
then washing in ethanol and dried at 60°C for 48 h

Live imaging - vEM, RT
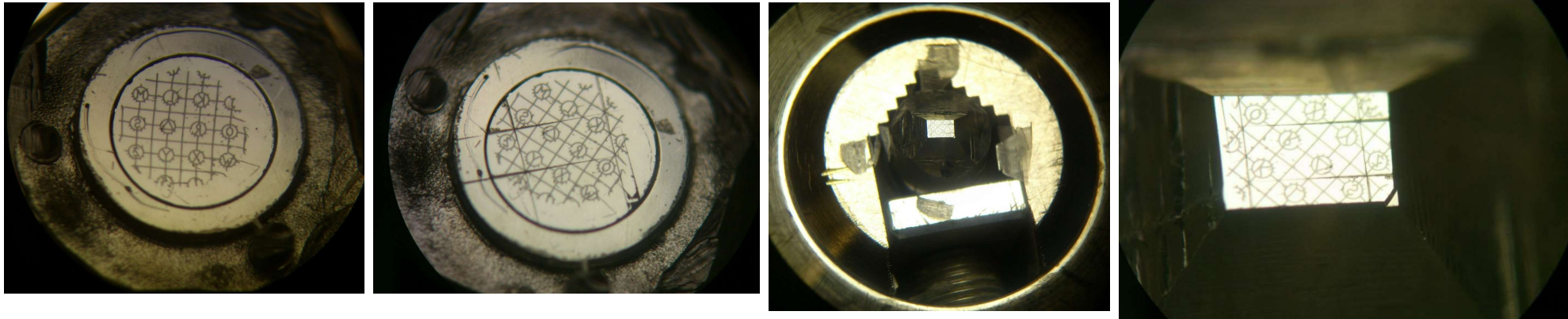
How <u>Putting Dynamic Live Cell Data into the Ultrastructural Context-</u>
HPM010, HPM100, HPF compact 02, From Live to HPF : 1.26 seconds

localize your cell of interest

Removing the CryoCapsule to reach the sample

Extra Long Working

Chapter 6 - HPM live μ for a full CLEM workflow

Xavier Heiligenstein [a], Marit de Beer [b c d], Jérôme Heiligenstein [a], Frédérique Eyraud [e], Laurent Manet [a], Fabrice Schmitt [f], Edwin Lamers [g], Joerg Lindenau [h], Mariska Kea-te Lindert [b c], Jean Salamero [i], Graça Raposo [j k], Nico Sommerdijk [b d], Martin Belle [a], Anat Akiva [b c]

Show more ∨

+ Add to Mendeley   ⛬ Share   ❞ Cite

Get rights and content ↗

Cryofluorescence-cryoSEM

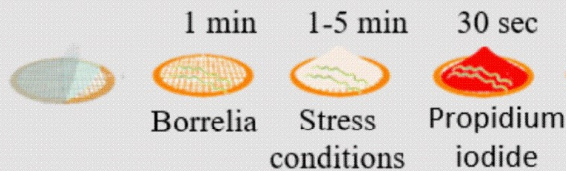How **Putting Dynamic Live Cell Data into the Ultrastructural Context-** Live-dead probes

Pleomorphism and Viability of the Lyme Disease Pathogen *Borrelia burgdorferi* Exposed to Physiological Stress Conditions: A Correlative Cryo-Fluorescence and Cryo-Scanning Electron Microscopy Study
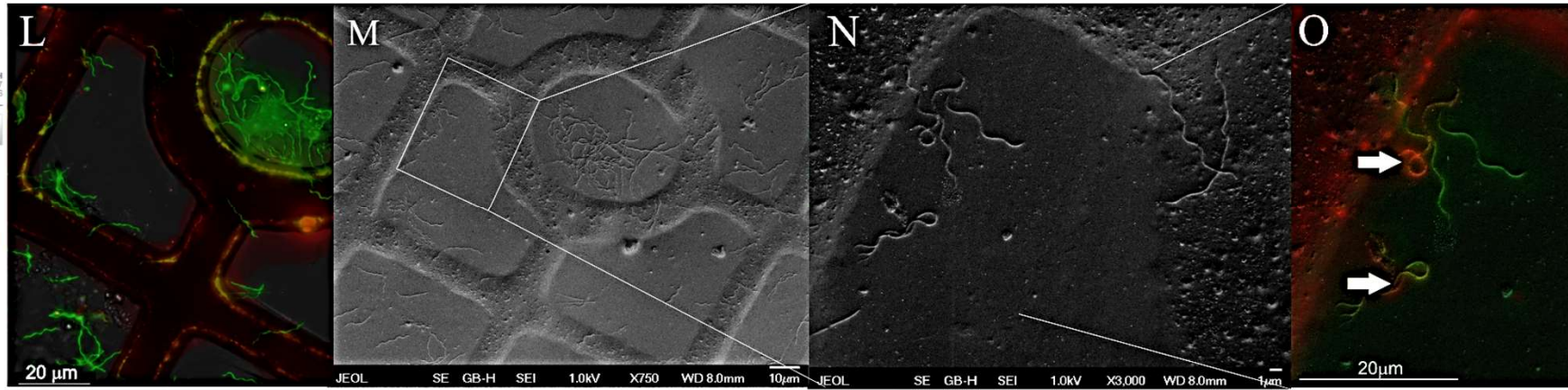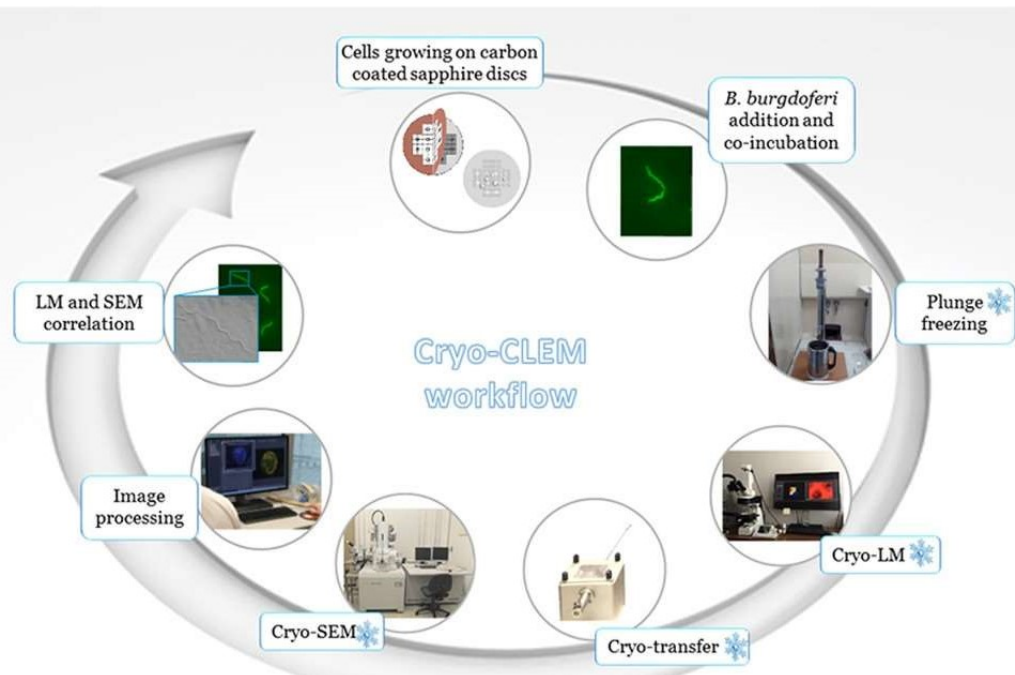
Thickness of the ice!

Ice sublimation was carried out for 10-20 min at a range from -98°C to -95°C under high vacuum. Samples were sputter coated for 10-20 sec with gold.

1 min — Borrelia
1-5 min — Stress conditions
30 sec — Propidium iodide

Cryo-immobilization → Leica EM Cryo CLEM → Cryo-SEM

Cryofluorescence-cryoSEM

Surface interactions at native conditions

Cells growing on carbon coated sapphire discs

B. burgdoferi addition and co-incubation

Plunge freezing ❄

LM and SEM correlation

Cryo-CLEM workflow

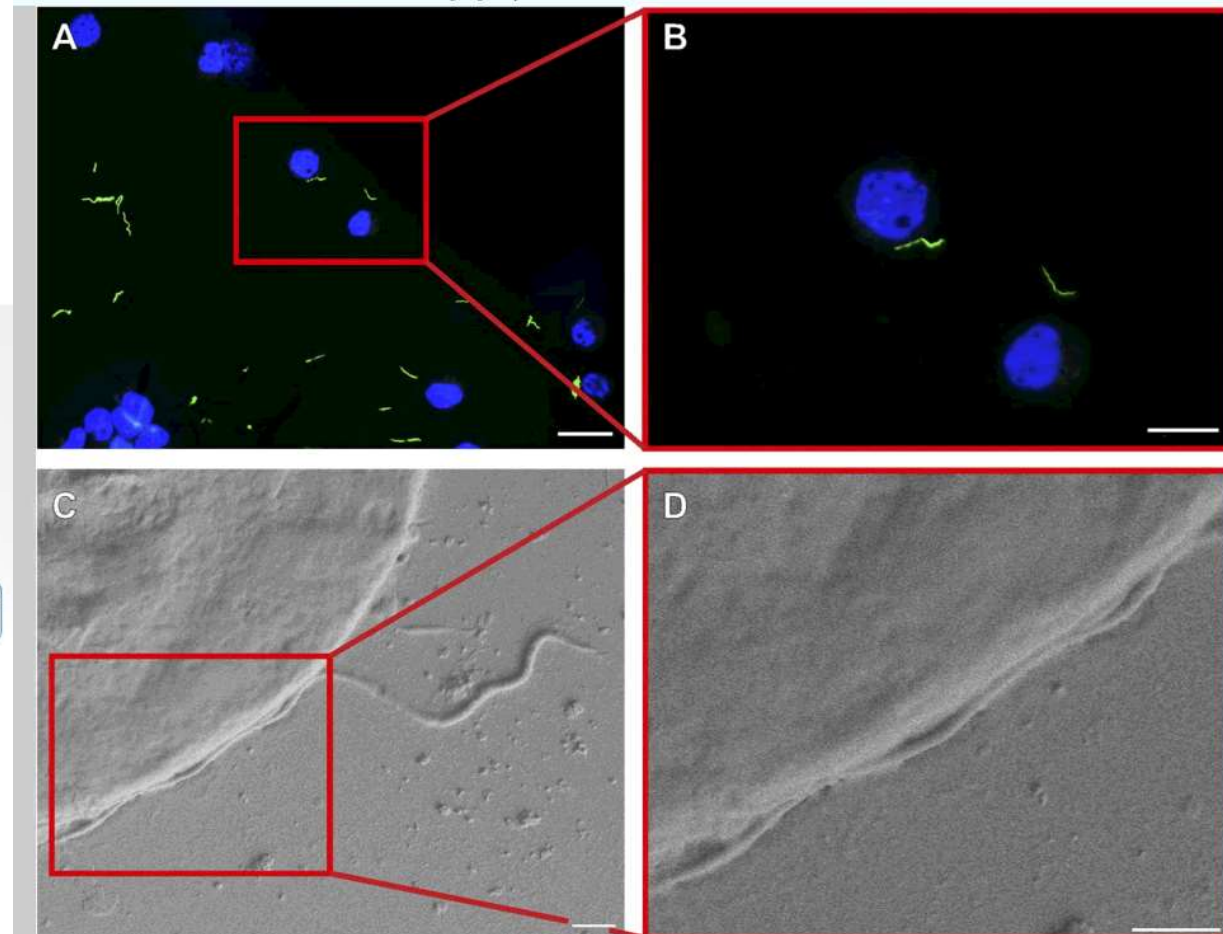Image processing

Cryo-SEM ❄

Cryo-transfer ❄

Cryo-LM ❄

Open Access | Published: 10 December 2015

Correlative cryo-fluorescence and cryo-scanning electron microscopy as a straightforward tool to study host-pathogen interactions

Martin Strnad, Jana Elsterová, Jana Schrenková, Marie Vancová, Ryan O. M. Rego, Libor Grubhoffer & Jana Nebesářová
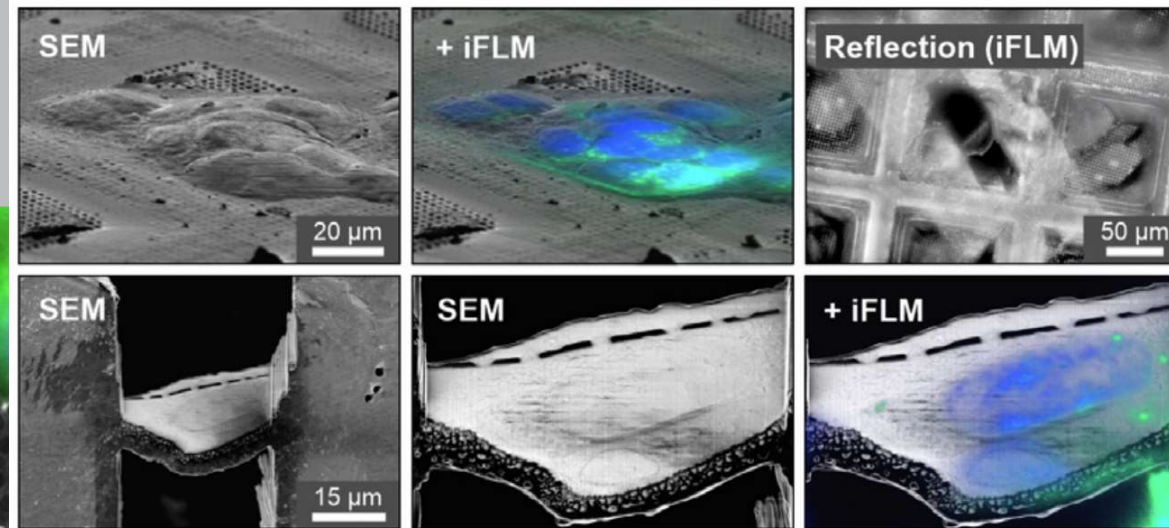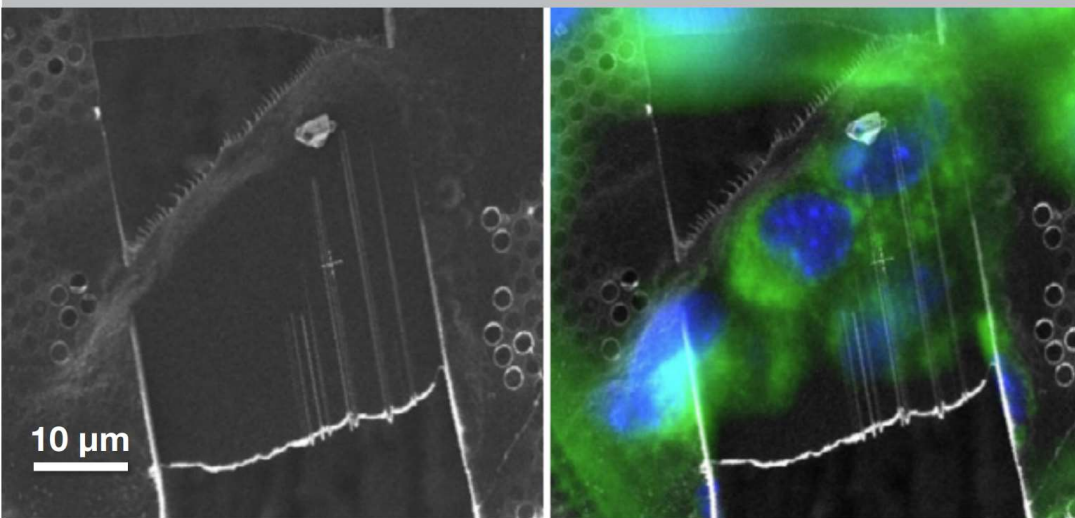
Correlative cryo-fluorescence (A,B) and cryo-scanning electron microscopy (C,D) of *Borrelia burgdorferi*-GFP on the surface of human neuroblastoma cells grown on carbon-coated sapphire discs. A series of images of one particular GFP-tagged spirochete (green) interacting with the cell counterstained with Hoechst 33342 (blue). Images of region of interest from low magnification FM to high magnification SEM.. Scale bars: (**A**) 50 µm, (**B**) 25 µm, (**C,D**) 1 µm.

FL integrated in FIB-SEM, to be sure that target is contained in the final lamella

The iFLM Correlative System module on the Aquilos 2 Cryo-FIB.

Check-back option during and after lamella milling. Correlation with the iFLM Correlative System allows the lamella milling process to be monitored step-by-step to ensure that the target is contained in the final lamella.

10 µm

Cryo-lamella prepared with the Aquilos 2 Cryo-FIB (left). Overlay of SEM image with fluorescence image obtained with the iFLM Correlative System (right).
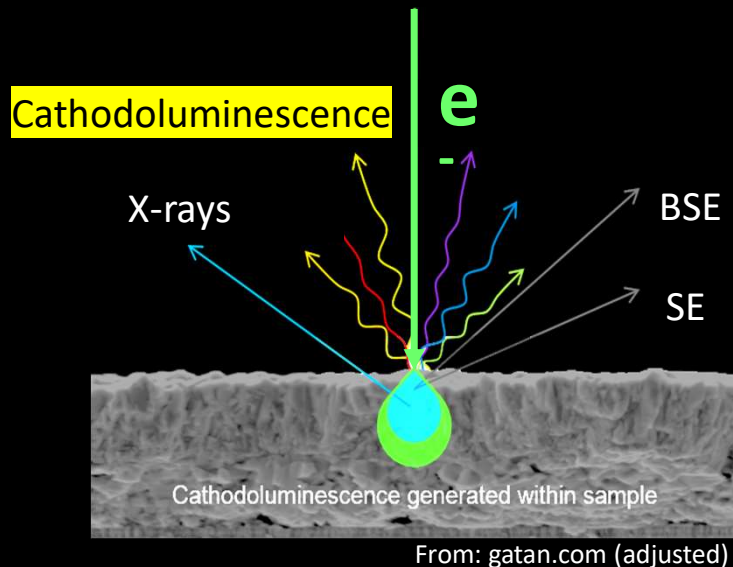
Cryo-lamella preparation with the iFLM Correlative System. **Top row:** Cluster of frozen CHO (Chinese Hamster Ovary) cells on a grid with overlaid fluorescence (blue channel: Hoechst Blue, green channel: Mitotracker). The iFLM Correlative System allows imaging in reflection mode to utilize surface details effectively for correlation. **Bottom row:** Cryo-lamella exhibiting cryo-contrast (secondary electron imaging). The fluorescence overlay highlights labelled subcellular organelles (nucleus and mitochondria) and matches the signal obtained by cryo-contrast very well.

CL-SEM applications in biology

Cathodoluminescence

e
-

X-rays

BSE

SE

Cathodoluminescence generated within sample

From: gatan.com (adjusted)

–easy integration into simultaneous multimodal imaging

– applications in geoscieces, photonic nanostructures,…

– applications in biology : (biofunctionalized) CL nanolabels e.g. nanodiamonds or rare-earth element-doped nanocrystals.

CL-SEM applications in biology

Cryo-cathodoluminescence cryoSEM

Luminescent transition metal complexes as promising CL organelle-specific probes in cryo-SEM

REZOLVE-ER   IRAZOLVE-MITO

fac-[Re(CO)$_3$(1,10-phenanthroline)(4-pyridyltetrazolate)

20 μM - 40 μM
30 min- ON, 34°C
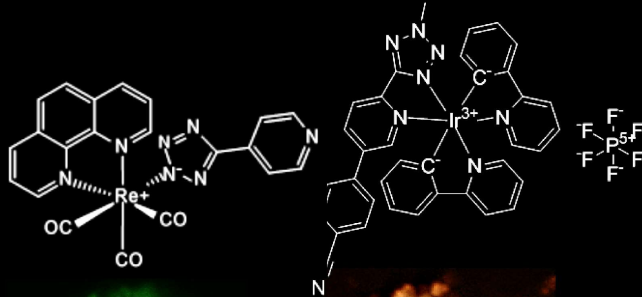
iridium complexed with cyclometalated 2-phenylpyridine and the 5-(5-(4-cyanophen-1-yl)pyrid-2-yl)tetrazolate ligand

In vivo FL
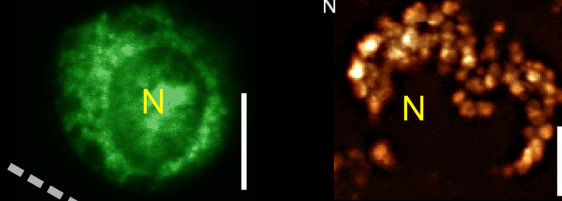
HPF
freeze fracturing
opt. etching and Au-coating

The availability of the probes:

Prof. Sally Plush, University of South Australia
Prof. Max Massi, Curtin University, Perth, Australia

CL

SEM -135°C

Bader CA, et al. DOI: 10.1002/1873-3468.12365

Sorvina A, et al. DOI: 10.1038/s41598-018-24672-w

SE

Marie Vancová,[1,2] Radim Skoupý,[3] Eva Ďurinová,[1,2] Tomáš Bílý,[1,2] Jana Nebesářová,[1,4] Vladislav Krzyžánek,[3] Aleš Kolouch,[5] and Petr Horodyský[5]
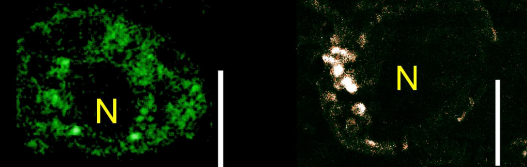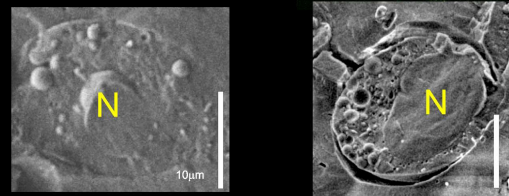
Nucleus, ER, mitochondria

FL a CL imaging of endoplasmic reticulum

Cryo-cathodoluminescence cryoSEM

Cryo CL-SEM

Fluorescence
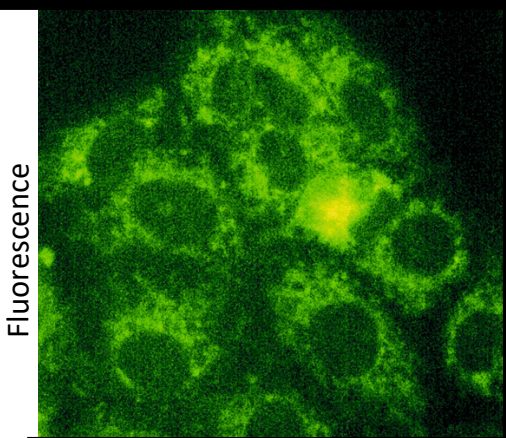
Holographic microscope 3D Cell Explorer (Nanolive)

z-slice RI signal

CL

Intact cell

Freeze -fractured cell

SE

N

N

N

N
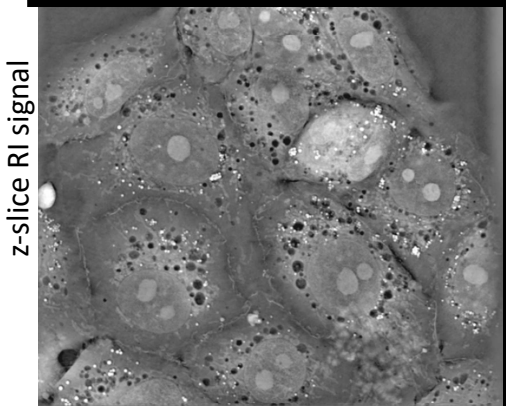
20μm
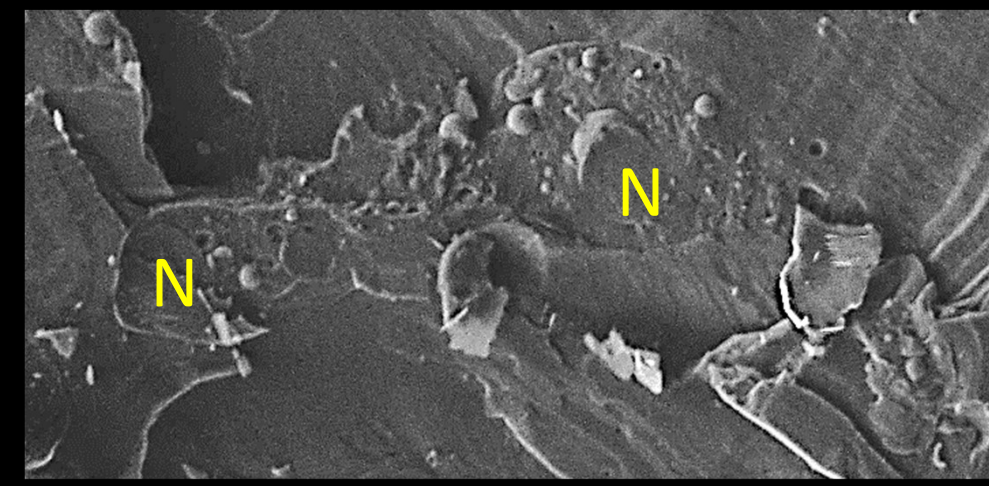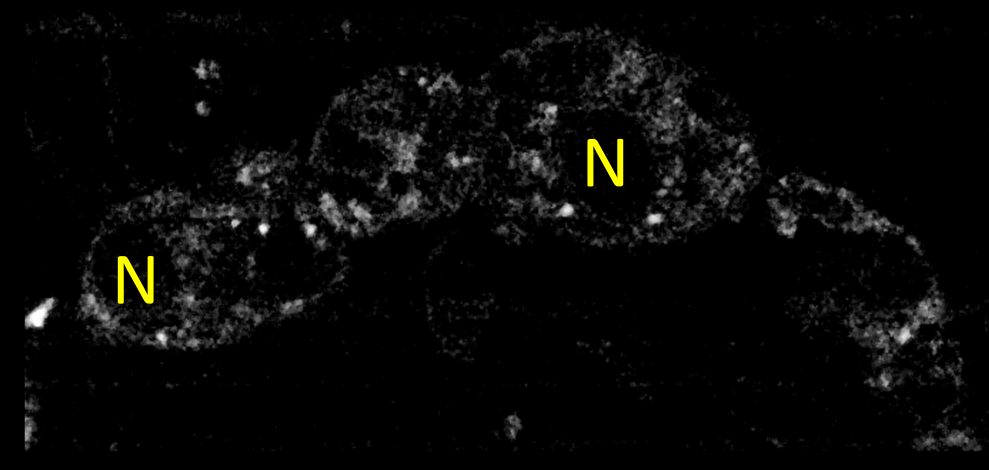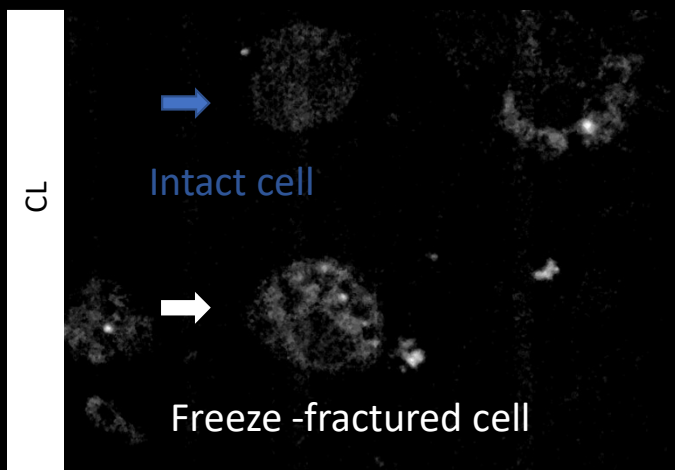
10μm

CryoEDS-cryoSEM

Elemental Mapping
Energy Dispersive X-Ray Spectroscopy

Massive Accumulation of Strontium and Barium in Diplonemid Protists

Jana Pilátová [a,b,c], Daria Tashyreva [a], Jiří Týč [a], Marie Vancová [a,d], Syed Nadeem Hussain Bokhari[e], Radim Skoupý [f], Mariana Klementová [g], Hendrik Küpper [d,e], Peter Mojzeš [c], Julius Lukeš [a,d]

strontium sulfate ($SrSO_4$)

Element	Wt %	Atomic %	Error %
C K	10.17	15.40	8.06
N K	3.90	5.07	8.56
O K	64.99	73.91	6.10
Na K	0.24	0.19	51.92
Mg K	0.89	0.67	7.00
Sr L	5.20	1.08	3.55
P K	0.26	0.15	25.77
Au M	2.60	0.24	10.33
S K	3.20	1.82	4.70
Cl K	0.38	0.19	22.39
Pd L	0.97	0.17	16.40
Ca K	0.53	0.24	23.23
Ba L	6.69	0.89	17.42

Free Draw 1

Element	Atomic %
C	13.1 ± 10.9
N	3.2 ± 11.5
O	69.3 ± 7.7
Sr	7.2 ± 2.8
S	7.2 ± 4.7

Element	Atomic %
C	15.4 ± 8.1
N	5.1 ± 8.6
O	73.9 ± 6.1
Sr	1.1 ± 3.6
S	1.8 ± 4.7
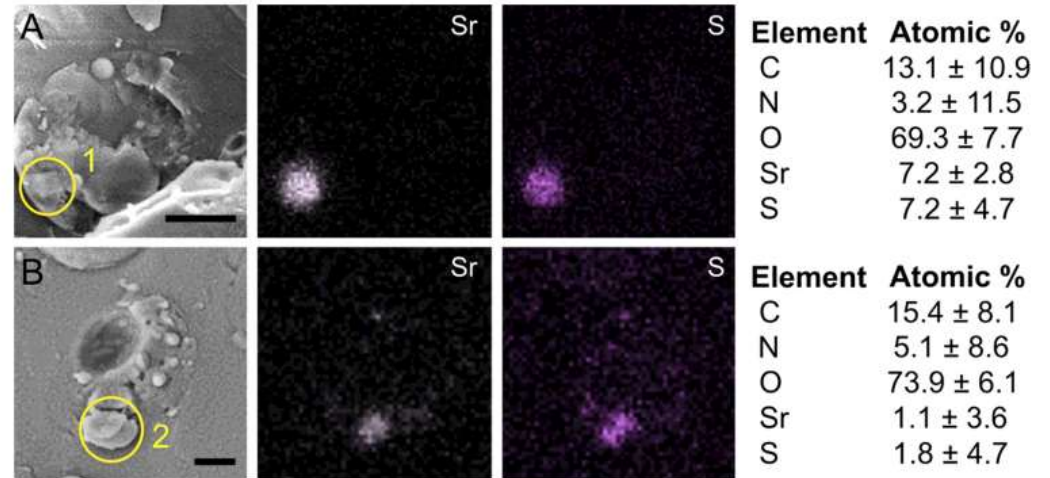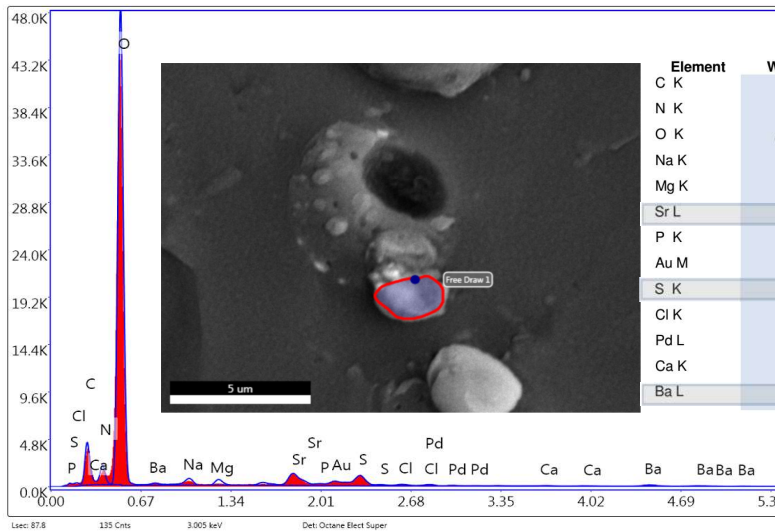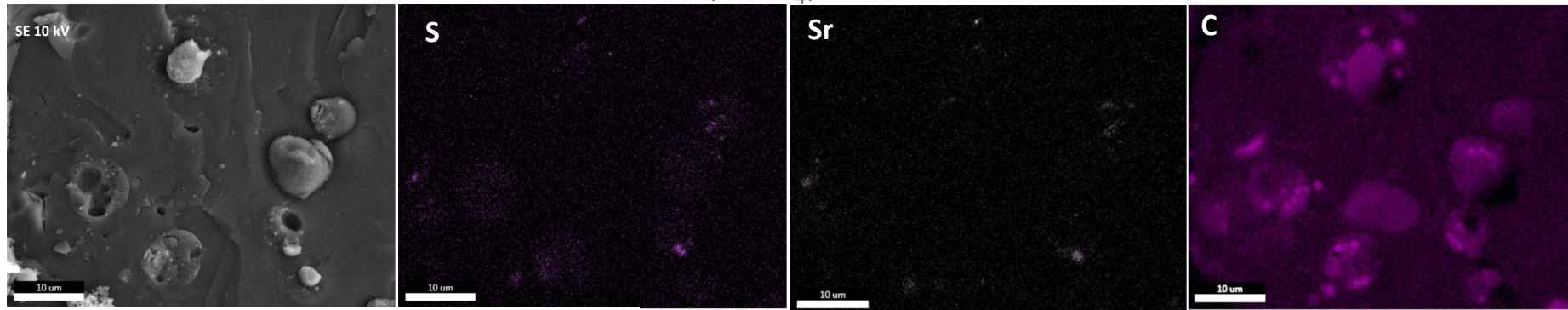
Raman-cryoSEM

chemical mapping, differentiate between modifications and conformations of the same molecule, **hyperspectral imaging**, staining not needed
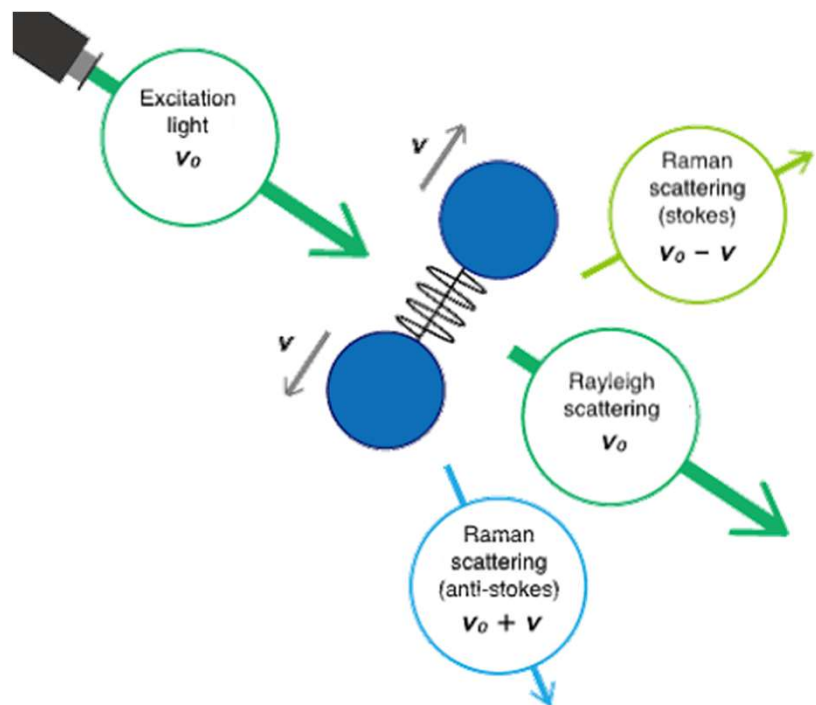
Fig. 1.
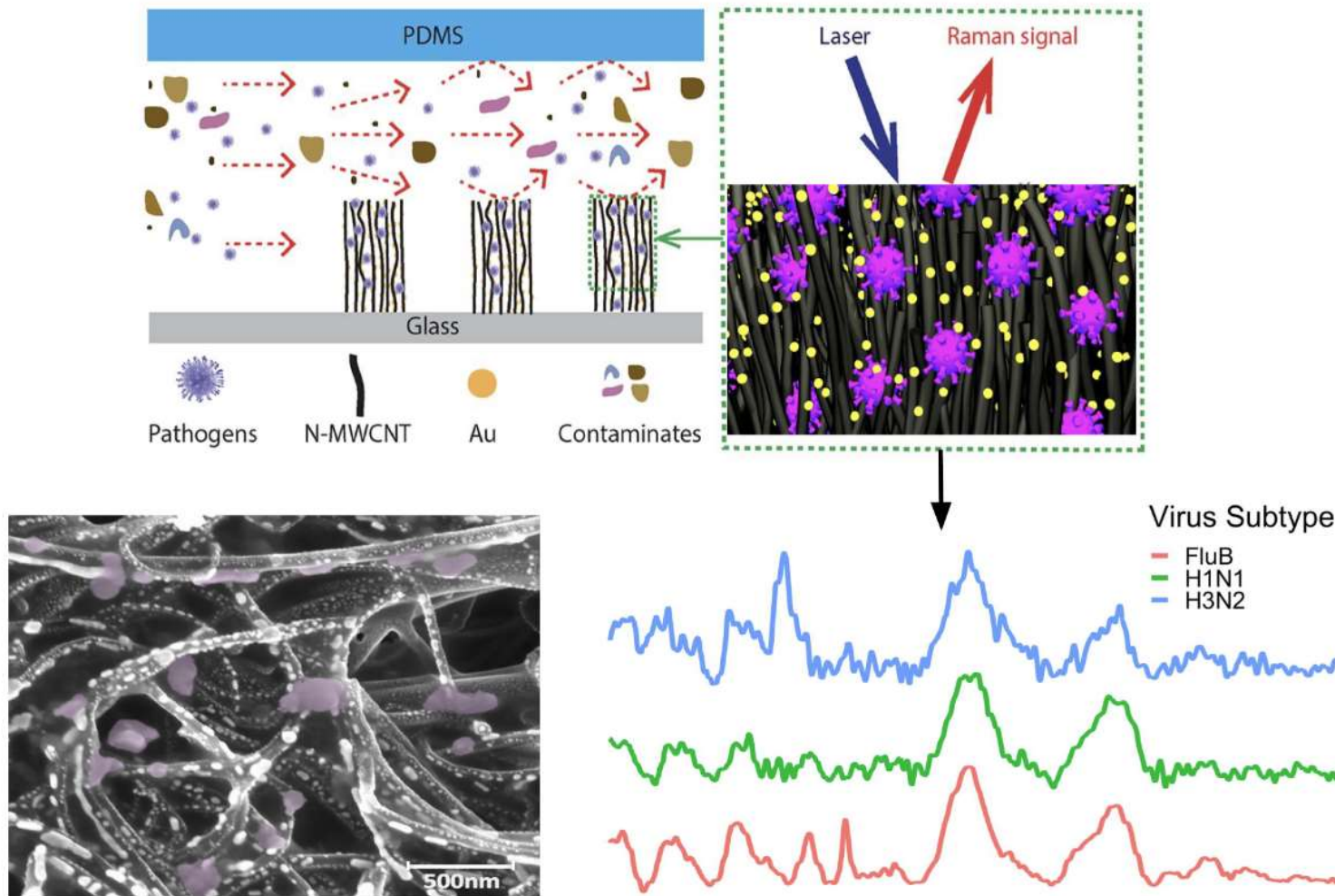
(A) Schematics showing the nitrogen-doped multiwall CNTs device encapsulated in polydimethylsiloxane used to enrich viruses (Top Left). The viruses are enriched between CNTs where the Au nanoparticles are predeposited. Raman spectra are then collected from the virus-enriched samples (Top Right). *A scanning electron microscope image* (Bottom Left) of a sample *shows* CNTs, Au nanoparticles, and *trapped viruses (purple colored).* Raman *spectra from different virus samples* are shown (Bottom Right) (FLUB in red, FLUA H1N1 in green, and FLUA H3N2 in blue).
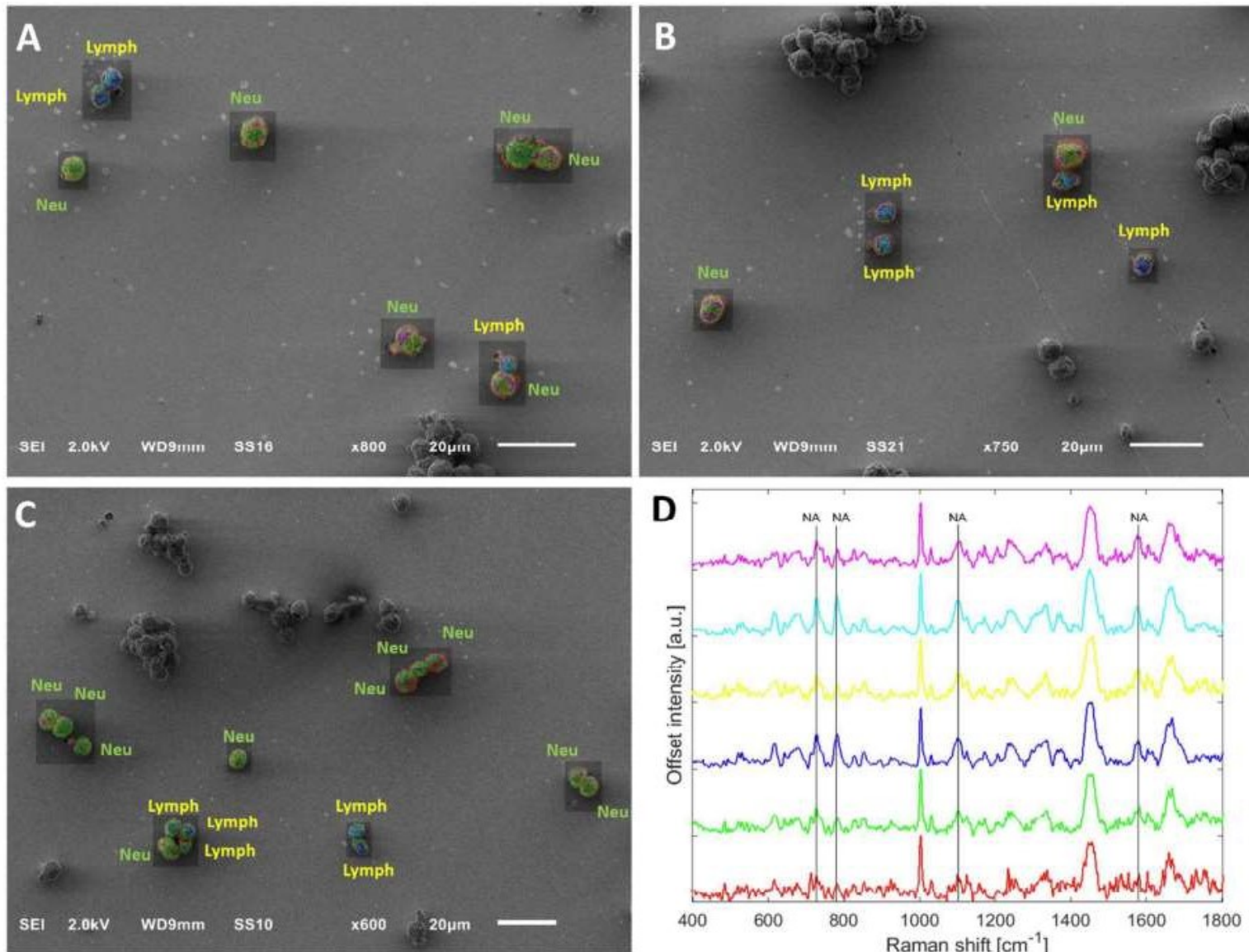
Fig. 6 SEM-Raman overlay of unsorted leukocytes. Panels A, B and C show SEM-Raman image overlays on an unsorted sample of leukocytes where labels have been assigned to cells based on a binary classification performed with HCA. The Raman maps shown on top of the SEM images are obtained after HCA using 6 clusters, whose respective Raman

Leukocyte subpopulations can be identified and differentiated via Raman spectroscopy

Further Raman spectroscopy detected immune cell activation and apoptosis.
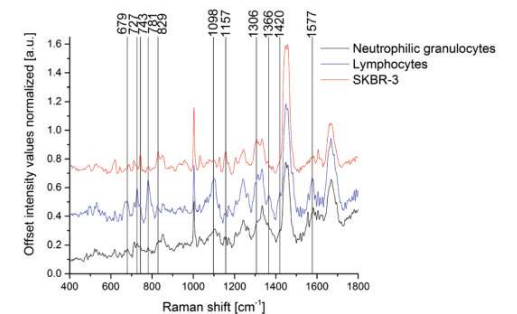
Fig. 4 Comparison of Raman spectra of leukocytes and SKBR-3 cells. The presented spectra are averaged over the measured cells shown in Fig. 3, normalized to one and smoothed using a Savitzky–Golay 7-point filter. Vertical lines are drawn to highlight Raman bands showing specificity for different cell types and their corresponding Raman frequency values are indicated. The 679, 727, and 781 cm^{-1} bands are pronounced for lymphocytes, while SKBR-3 cells show clear peaks at 743 and 1157 cm^{-1}.

Raman imaging

Chemical information can be acquired with a resolution down to 300 nm

Detection Limits: ≥1 wt%; Depth Resolution: Confocal mode 1 – 5 μm

Time (h)	1:15	3:00	6:00	9:00	12:15	13:00	16:00	19:45
Nuclei	1	1	1	1	2	4	cell division	1

Lipid
Starch
PolyP

Figure 7. Raman maps showing the distribution of lipid droplets (red), starch bodies (green) and polyP granules (blue) in the same two innermost cells of eight-celled coenobia as presented in Figure 1C. 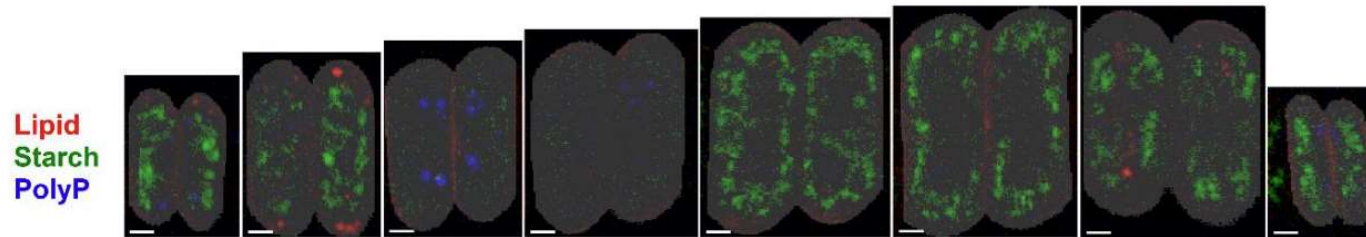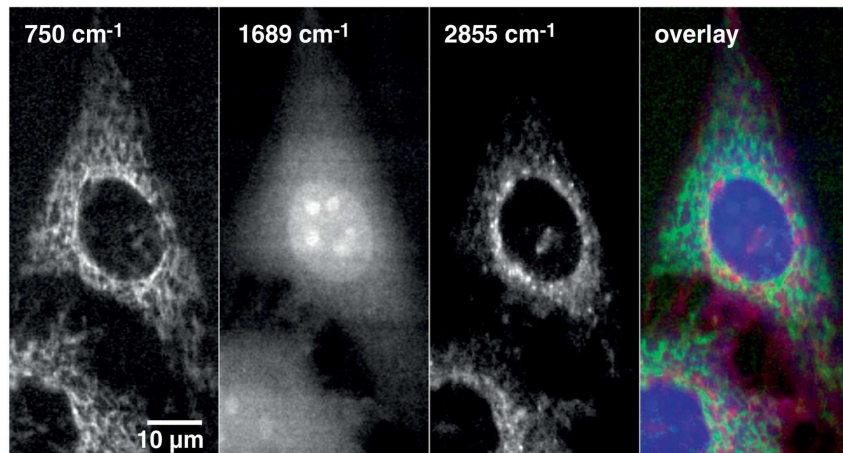The time counted from the beginning of the cell cycle and the number of nuclei determined by DAPI staining of the culture are indicated in the top row. For a given biomolecule, the color scale is the same for all maps. The white bars correspond to 2 μm.

Cells **2021**, *10*(1), 62; https://doi.org/10.3390/cells10010062

750 cm⁻¹   1689 cm⁻¹   2855 cm⁻¹   overlay

10 μm

Color channels: cyto c (G), protein (B), lipid (R)

Current Opinion in Chemical Biology

(a) excitation: 532 nm

605 Cytochrome; 725 Adenine; 753 Cytochrome; 783 Cytosine, Thymine; ~800 Phosphoric acid; 1000 Phenylalanine; 1130 Cytochrome; ~1250 Amide-III; 1314 Cytochrome; 1335 Adenine; 1451 CH₂; 1585 Cytochrome; 1660 C=C, Amide-I; 2850 CH₂; 2935 CH₃

Raman scattering intensity [a.u.] vs Raman shift [cm⁻¹]

A
Organic matter 2800 – 3050 cm⁻¹   Lipids 2851 cm⁻¹
Nuclei 789 cm⁻¹   Heme 754 cm⁻¹   Cluster Analysis image

B
Organic matter 2800 – 3050 cm⁻¹   Lipids 2852 cm⁻¹
Nuclei 787 cm⁻¹   Heme 1372 cm⁻¹   Cluster Analysis image

Colour code:
- Nucleus (green)
- Heme (blue)
- Heme derivatives (red)
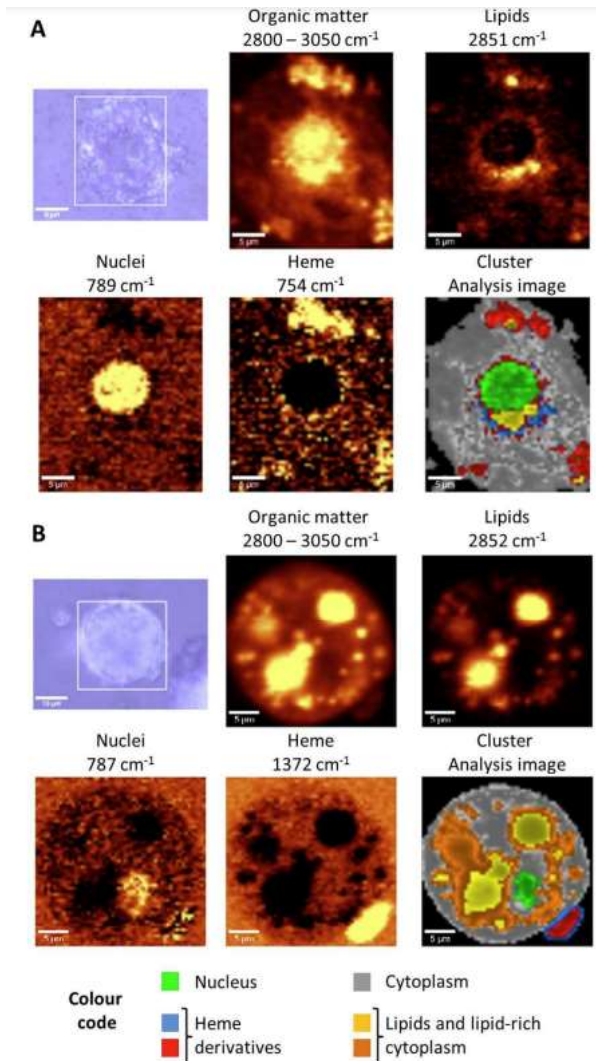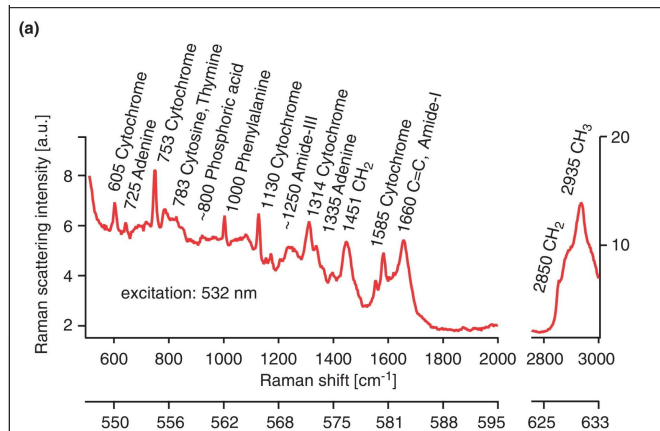- Cytoplasm (grey)
- Lipids and lipid-rich cytoplasm (yellow/orange)

Figure 4. Visual images (×60) of the murine Kupffer cells with Raman images showing distribution of organic matter (integration in the 2800–3050 cm⁻¹ range), lipids (integration of the marker band at approximately 2852 cm⁻¹) and heme (integration of

SEM further provides structural information with high spatial resolution

mt

mt

GA

nucleus
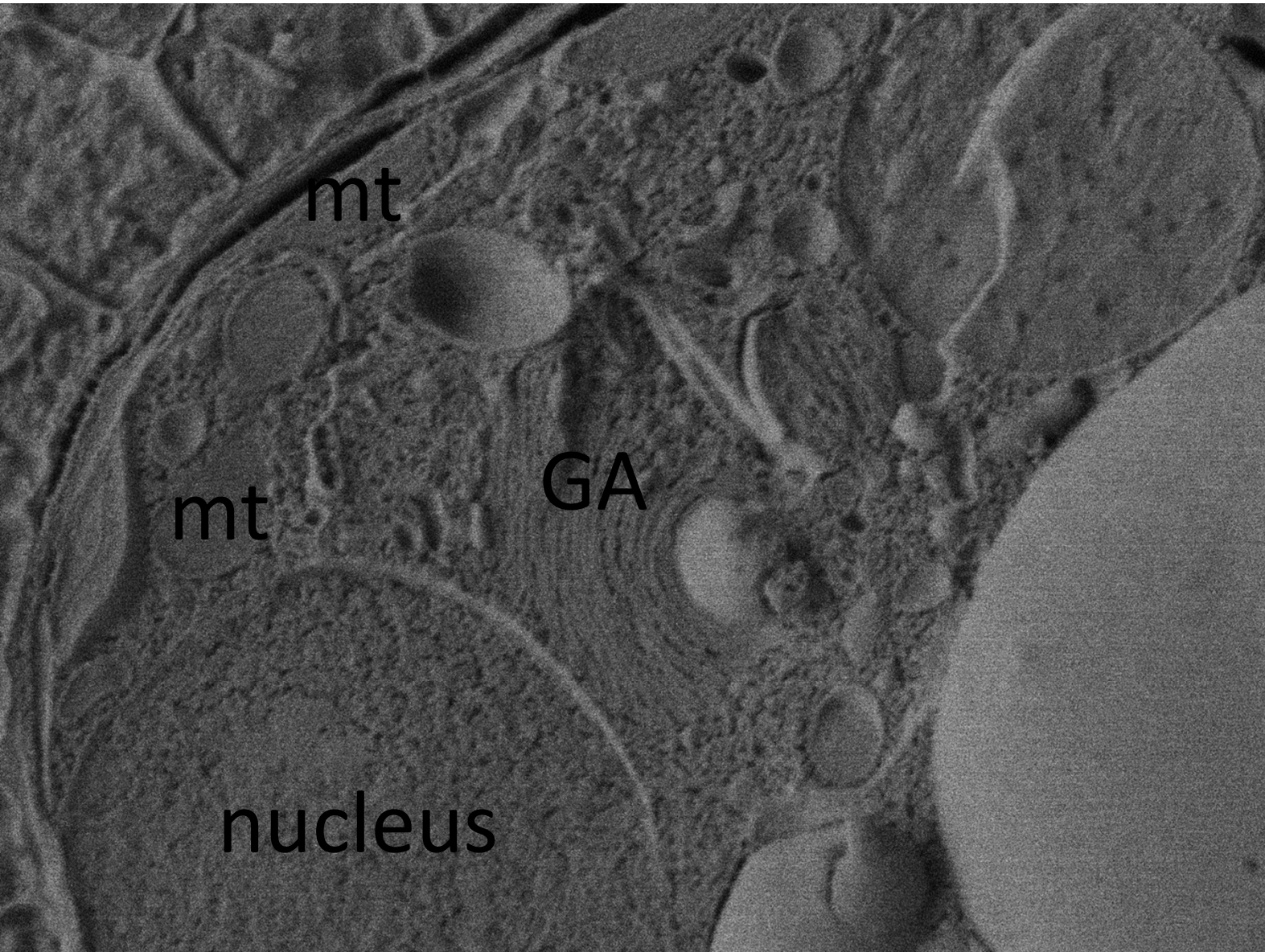
Freeze fracture -160°C
Freeze etching   -98°C
Pt/Pd 30 sec

Hemistasia-clade
 genera
Diplonema
biflagellated
unicellular protist

Galina Prokopchuk
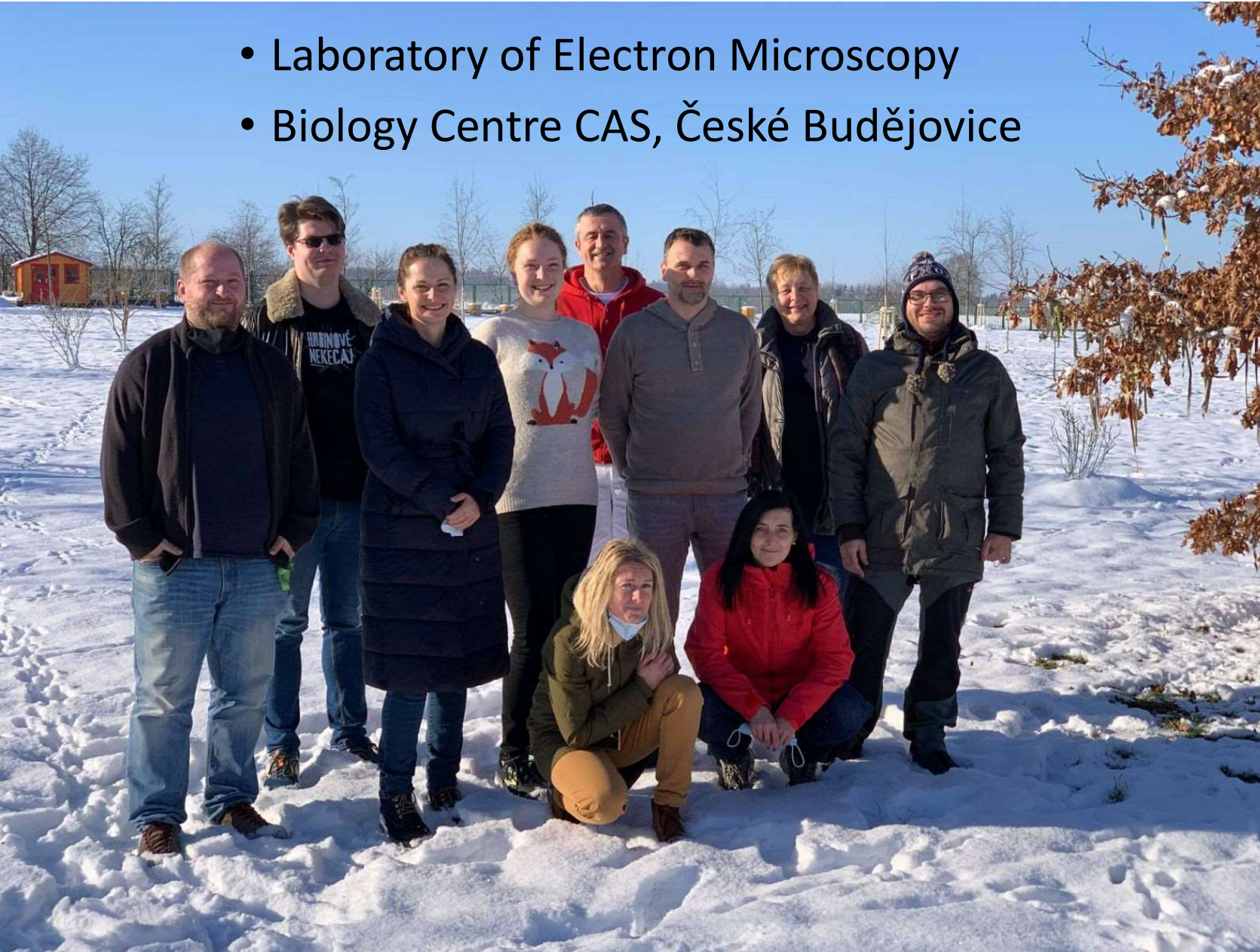
Jiří Vaněček

SE   GB-L   LEI   1.0kV   X18,000   WD 9.0mm   1μm   X8,500   WD 9.0mm   1μm

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