THE MULTIPLE IMMUNOLOCALIZATION METHOD USING AU MARKERS FOR LOW VOLTAGE STEM

František Kitzberger

Biology Centre, CAS, České Budejovice
Faculty of Science, Charles University, Prague
Czech Republic
Frantisek.Kitzberger@paru.cas

PŘÍRODOVĚDECKÁ FAKULTA
Univerzita Karlova

BIOLOGICKÉ CENTRUM AV ČR, v. v. i.

INSTITUTE OF PARASITOLOGY
Biology Centre, ASCR

ThermoFisher SCIENTIFIC

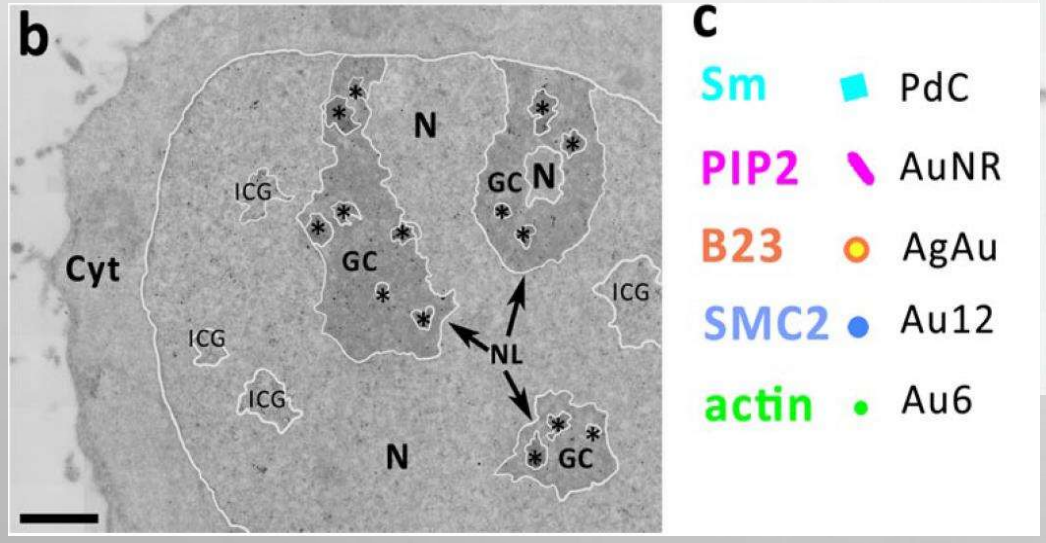
CSMS

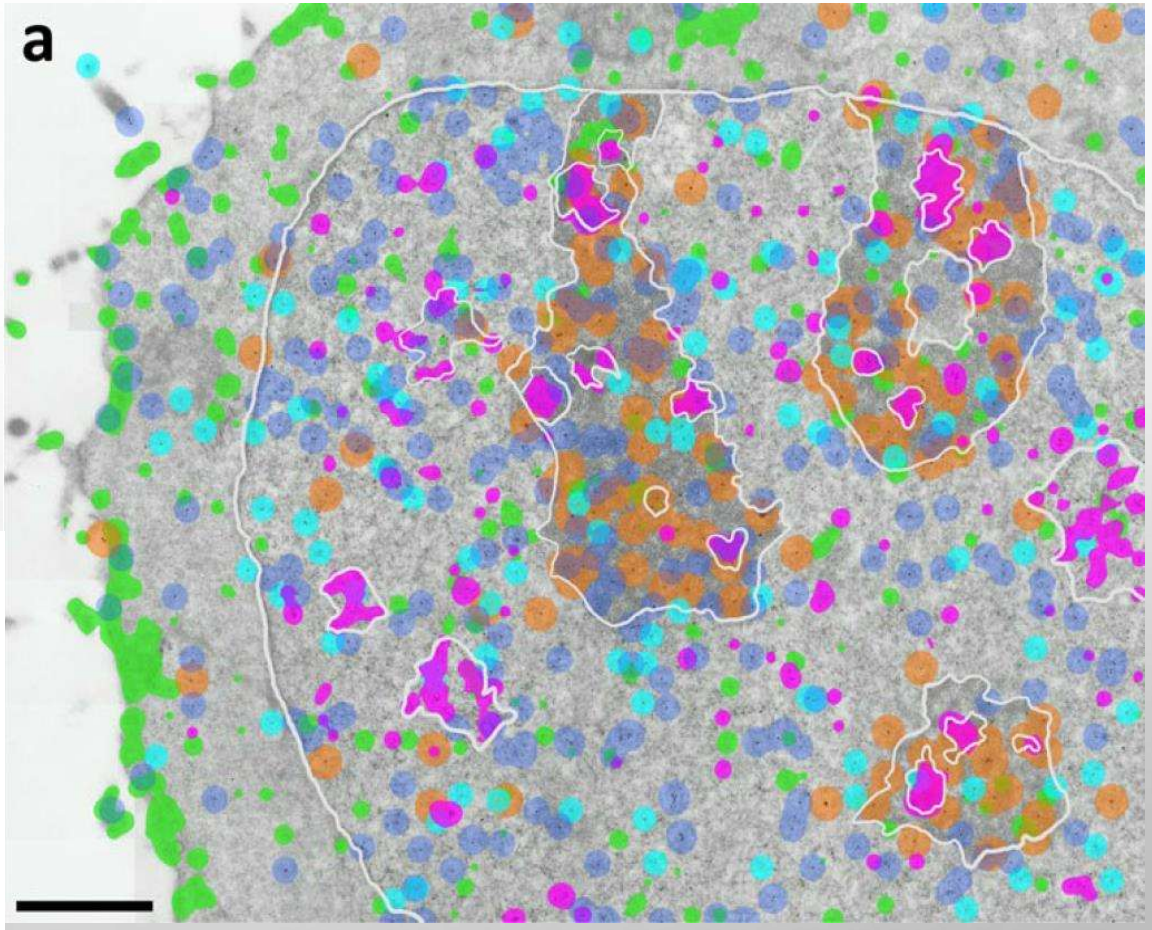
CZECH BIOIMAGING
Imaging principles of life

EURO BIOIMAGING
Image life, discover the future

MULTIPLE IMMUNOLOCALISATION

- MORE ANTIGENS CAN BE DETECTED SIMULTANOUSLY BY USING DIFFERING IMMUNOMARKERS - EG. DIFFERENT DIAMETERS (5 AND 15 NM)
- LIMITATION: NUMBER OF SUITABLE MARKERS.
- IN TEM AU NANOPARTICLES WITH DIFFERENT SHAPES



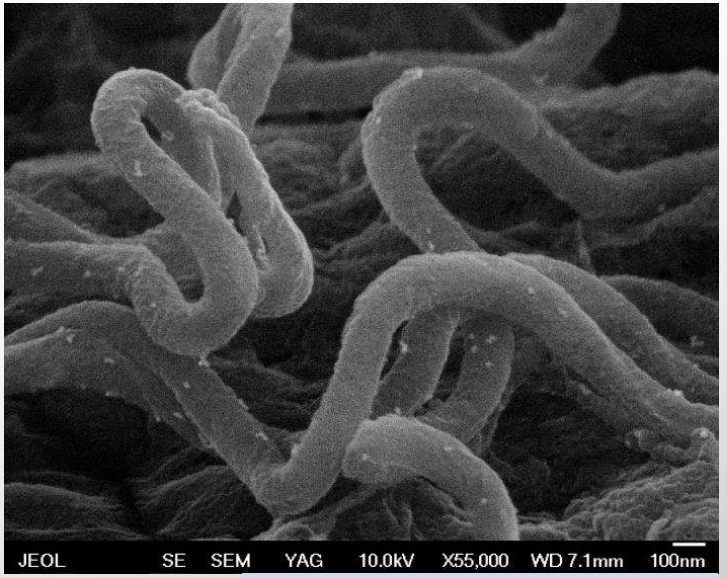


(PhilimonenkoVV et al.2014, Histochem Cell Biol.141(3):241)

MULTIPLE IMMUNOLOCALISATION IN HRSEM

BSE IMAGING FOR THE DETECTION OF IMMUNOLABELING RESULTS:

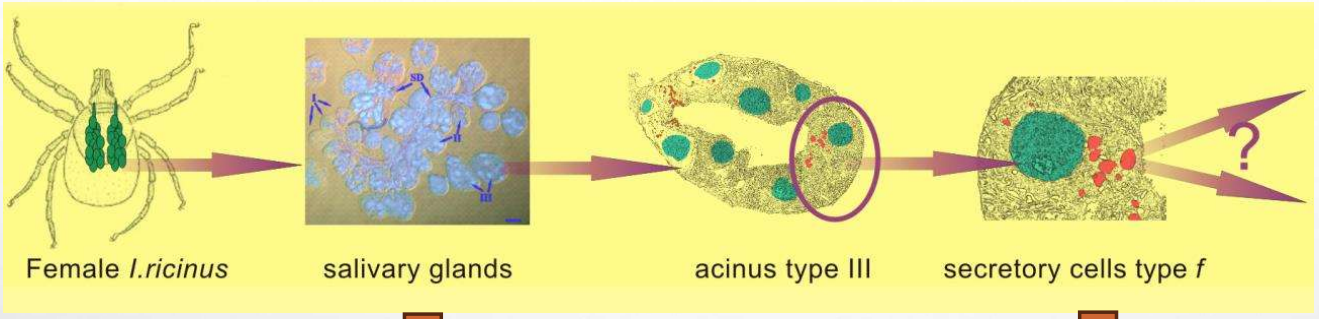
- POSSIBILITY TO WORK WITH LOW VOLTAGES (0,5-5 KV)
- SUFFICIENT RESOLUTION POWER TO DETECT 5 NM METAL NPs
- INTENSITY OF THE BSE SIGNAL DEPENDS ON THE ATOMIC NUMBER Z OF THE NP
- BETTER TOPOGRAPHIC CONTRAST
- LESS SENSITIVE TO THE CONTAMINATION
- LESS SENSITIVE TO THE CHARGING EFFECT



Salivary glands of ticks infected with Borrelia

MULTIPLE IMMUNOLABELING IN HRSEM

- USING NPs OF VARIOUS METALS CONJUGATED WITH SECONDARY ANTIBODIES AS MARKERS



Mannose (cytoplasm)

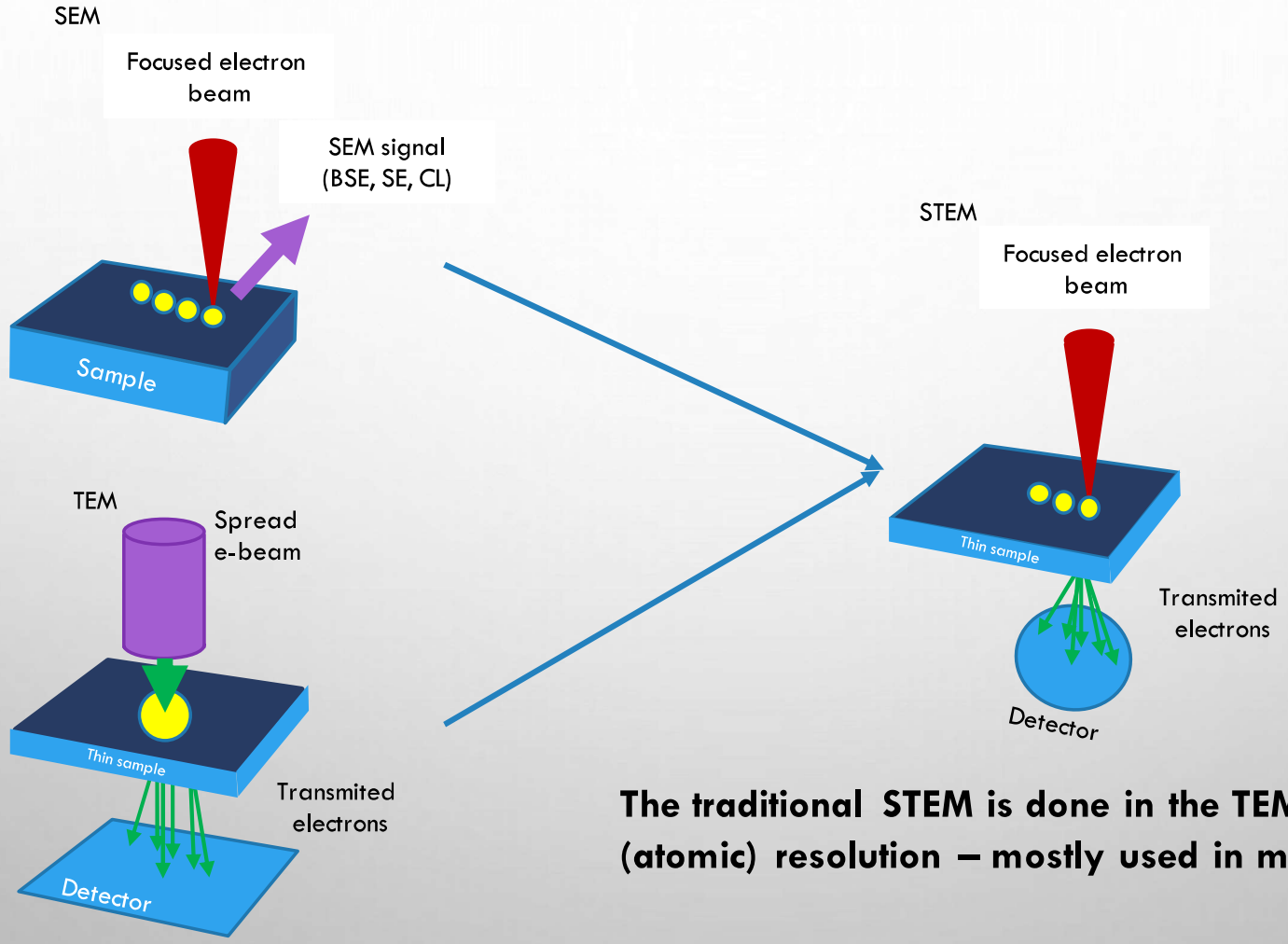
Sm protein (nucleus)

Specimen preparation:
Chemical fixation (4% FA and 0,5% GA)
Dehydration (ethanol, PLT)
Embedding (Lowicryl K4M, UV polymerized, -35˚C)

Immunolabelling:
Primary antibodies
Biotinylated ConA
Human anti Sm
Secondary antibodies
Streptavidin Pd conjugate (9 nm)
Anti-human IgG gold (10 nm)

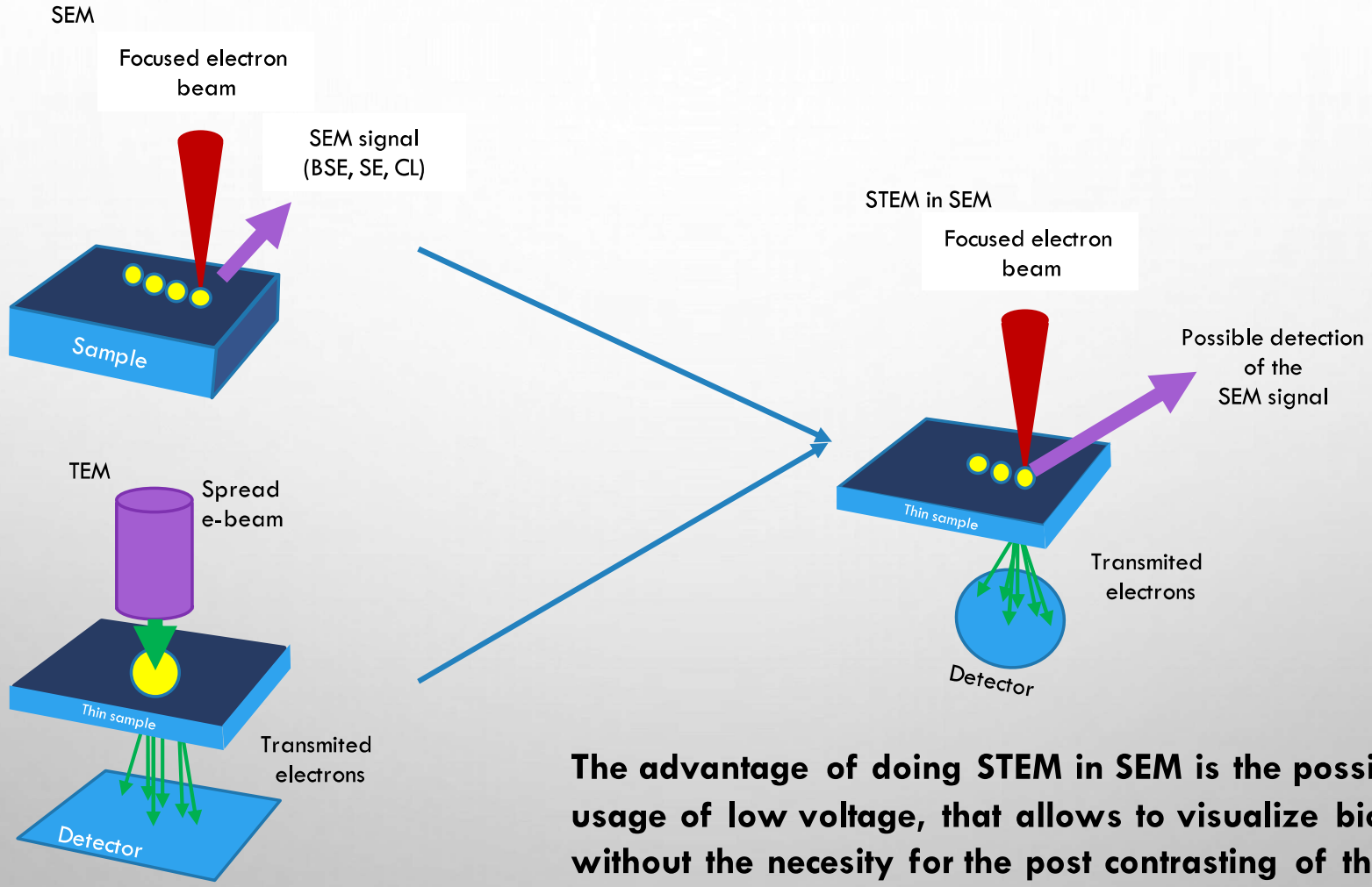
M.Vancova et al.: *Microscopy and Microanalysis*, 2011, 17, 1-7

WHAT IS STEM?



The traditional STEM is done in the TEM and has the best possible (atomic) resolution – mostly used in material sciences

WHAT IS STEM?

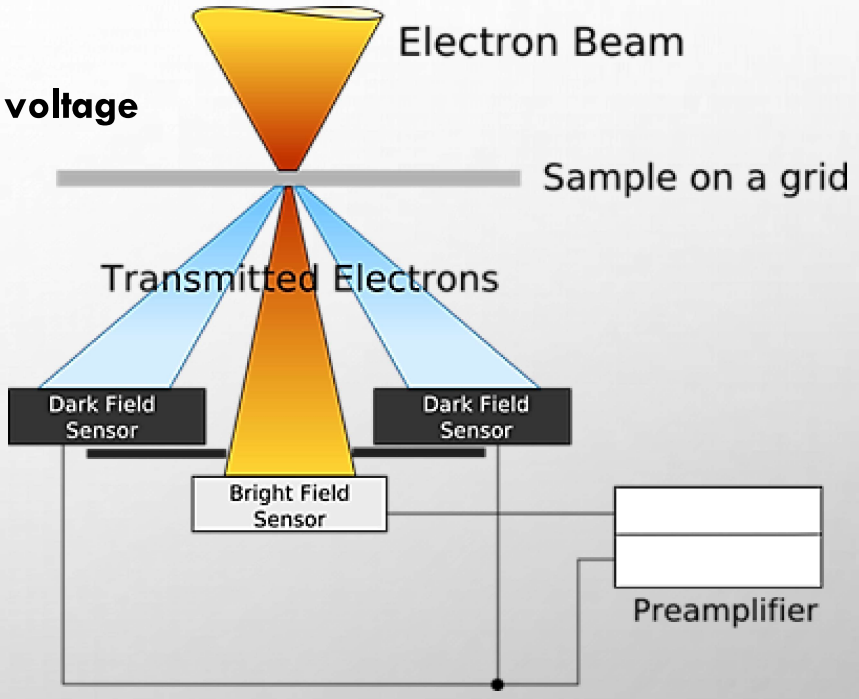


The advantage of doing STEM in SEM is the possibility of usage of low voltage, that allows to visualize bioogical specimens without the necesity for the post contrasting of the sections

MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

STEM in SEM allows:

- **Using low accelerating voltages ranging between 1-30 kV**
- **Visualization of non-contrasted ultrathin sections**
- **Detecting simultaneously BSE and transmitted electrons**
- **Choise the depth of signal origin by selection of accelerating voltage**



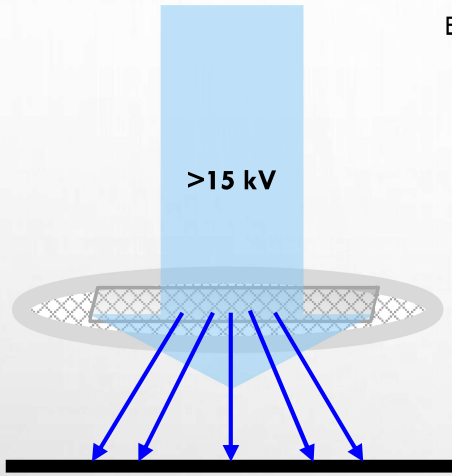
- LABELING ON BOTH SIDES OF THE ULTRATHIN SECTION BY ANTIBODIES CONJUGATED WITH AU-NPS OF DIFFERENT DIAMETERS IN TEM (Pasolli et al, 1994) – USEFUL IF WE HAVE PRIMARY ANTIBODIES OF THE SAME ANIMAL

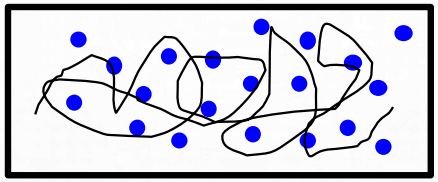
- DETECTION AND DIFFERENTIATION OF AU-NPs ON BOTH SIDES OF THE SECTION BY LOW-VOLTAGE STEM AND BSE IMAGING IN SEM USING TWO ACCELERATING VOLTAGES (Nebesářová et al, 2016)

Ideal workflow...

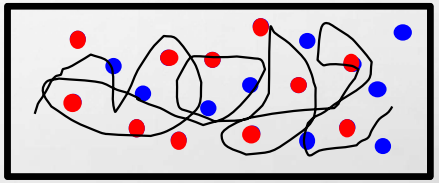
STEM signal

Electrons accelerated by higher accelerating voltage can penetrate section
- We can see all markers and ultrastructure



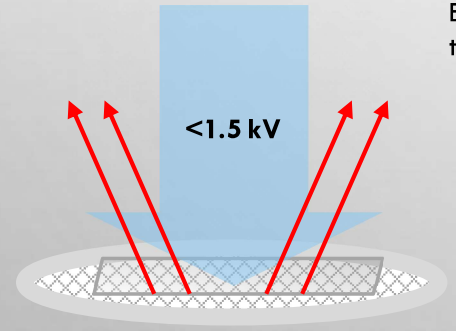


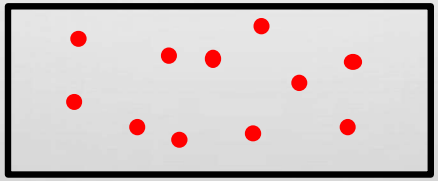
After subtracting the BSE detected markers from the STEM detected ones, we know which markers are on the bottom of the section



BSE signal

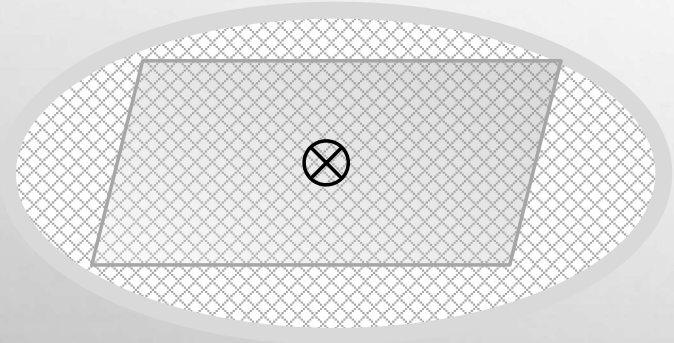
Electrons accelerated by lower voltage do not penetrate the section – we can see only the BSE signal from surface



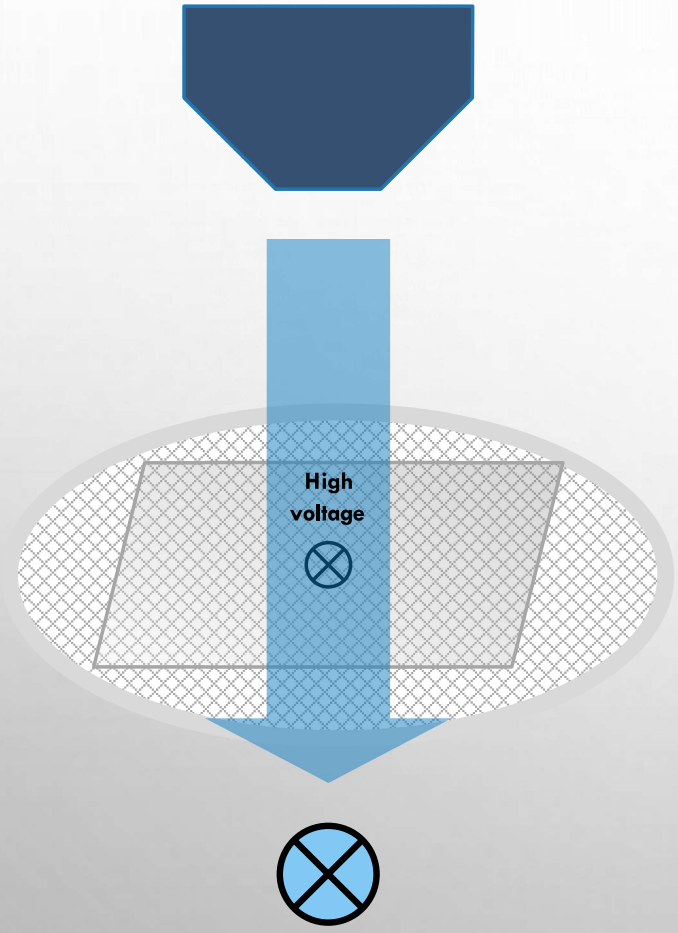


Real workflow – step by step

1) Find the object of interest



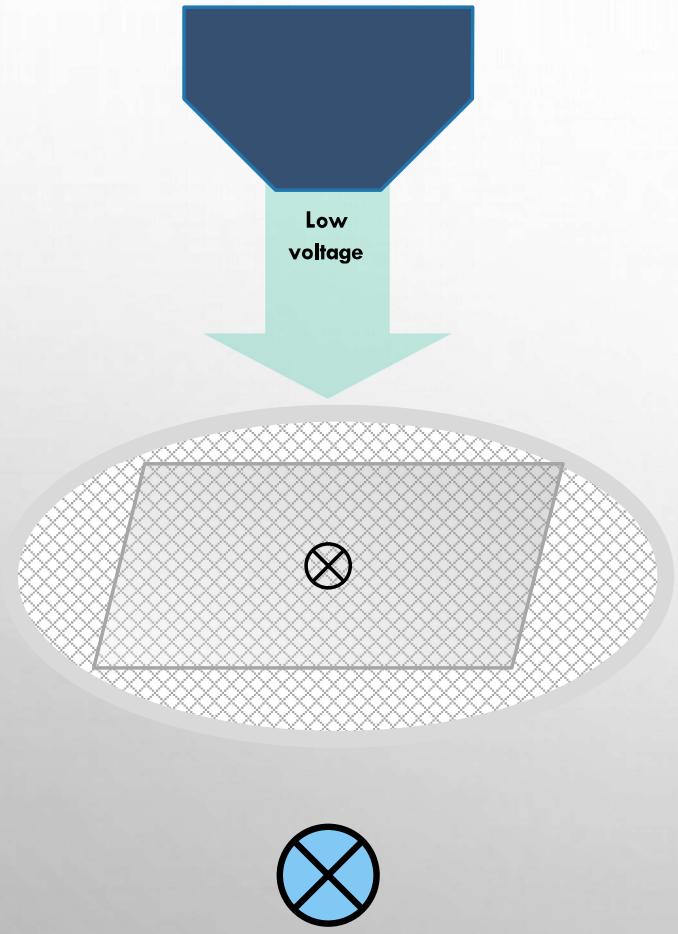
Real workflow – step by step



1) Find the object of interest

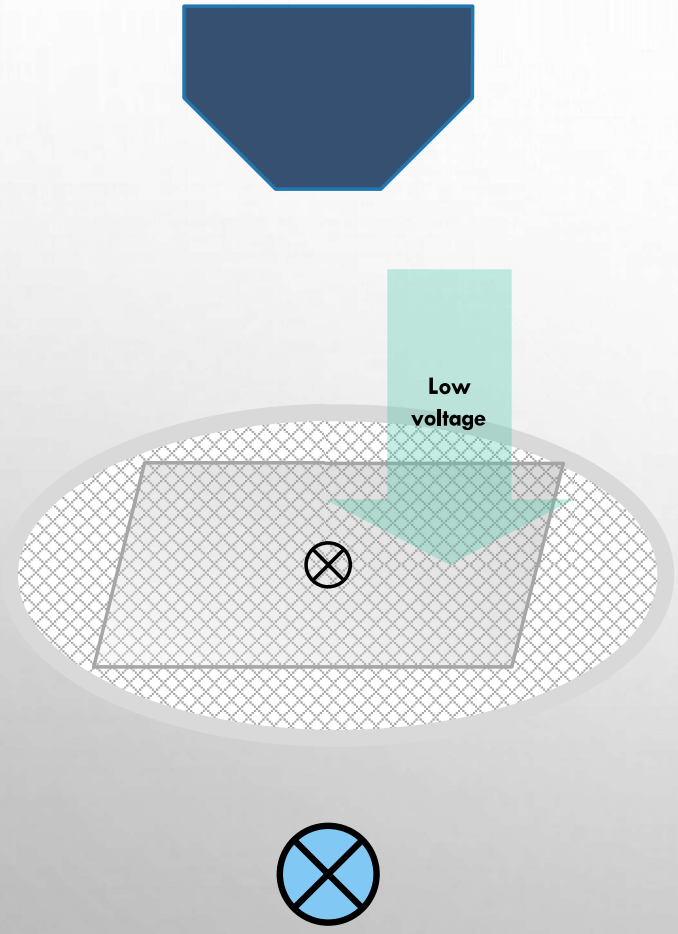
2) Look at it under high accelerating voltage (>15kV) – on STEM
   - you will see everything

Real workflow – step by step



1) Find the object of interest
2) Look at it under high accelerating voltage (>15kV) – on STEM
   - you will see everything
3) Switch to low accelerating voltage (<2kV)

Real workflow – step by step



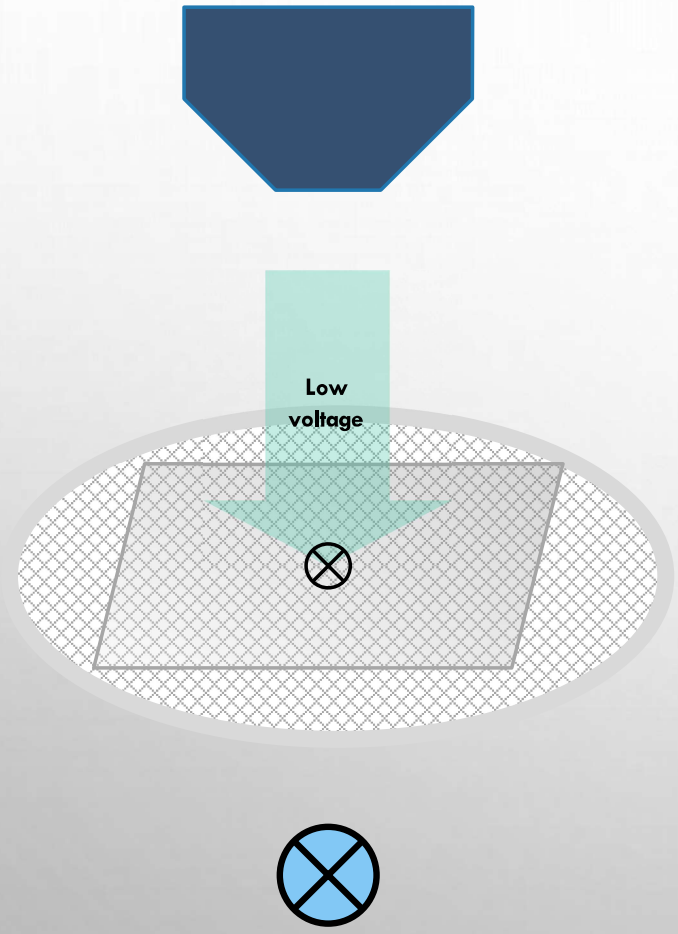
1) [illegible]

2) [illegible]

3) [illegible]

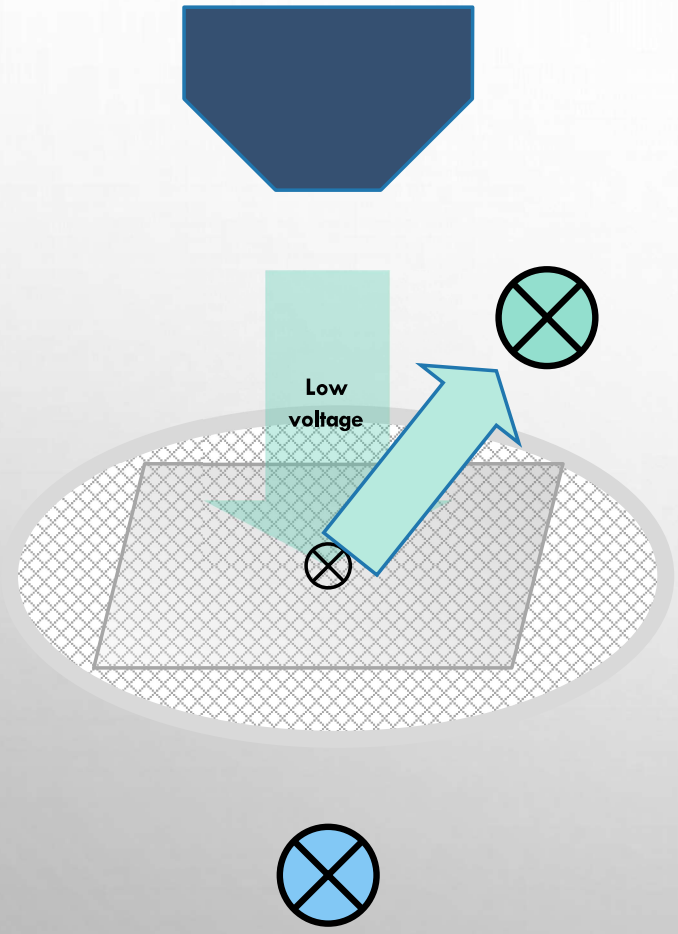
4) Find out that your beam shifted to different position and defocused

Real workflow – step by step



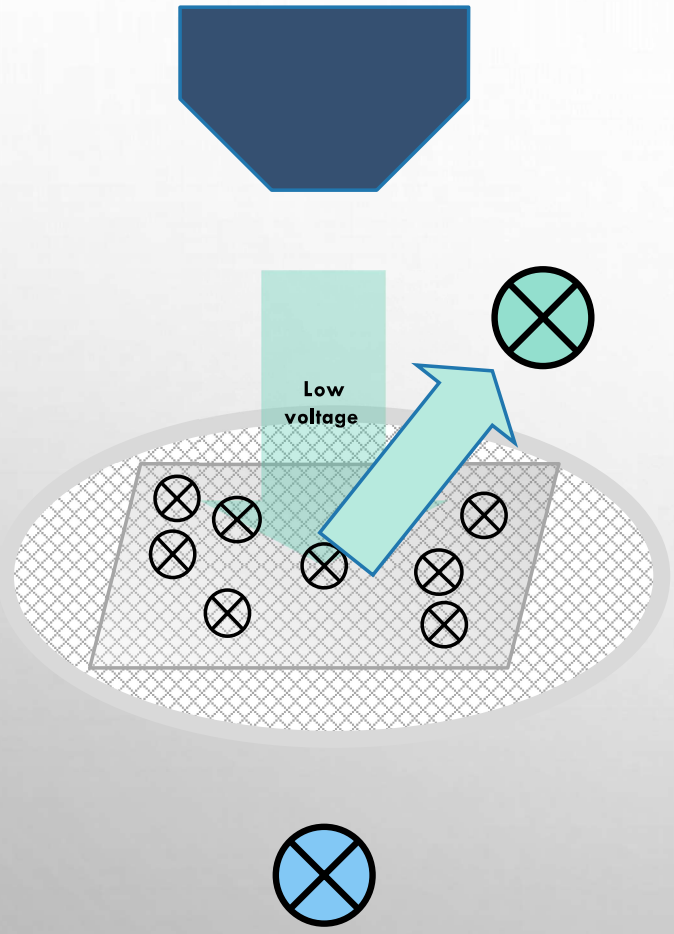
1) Find the object of interest
2) Look at it under high accelerating voltage (>15kV) – on STEM
   - you will see everything
3) Switch to low accelerating voltage (<2kV)
4) Find out that your beam shifted to different position and defocused
5) Refocus the beam and shift it back to the original position

Real workflow – step by step



1) Find the object of interest
2) Look at it under high accelerating voltage (>15kV) – on STEM
   - you will see everything
3) Switch to low accelerating voltage (<2kV)
4) Find out that your beam shifted to different position and defocused
5) Refocus the beam and shift it back to the original position
6) Look at the object of interest on the BSE – see surface

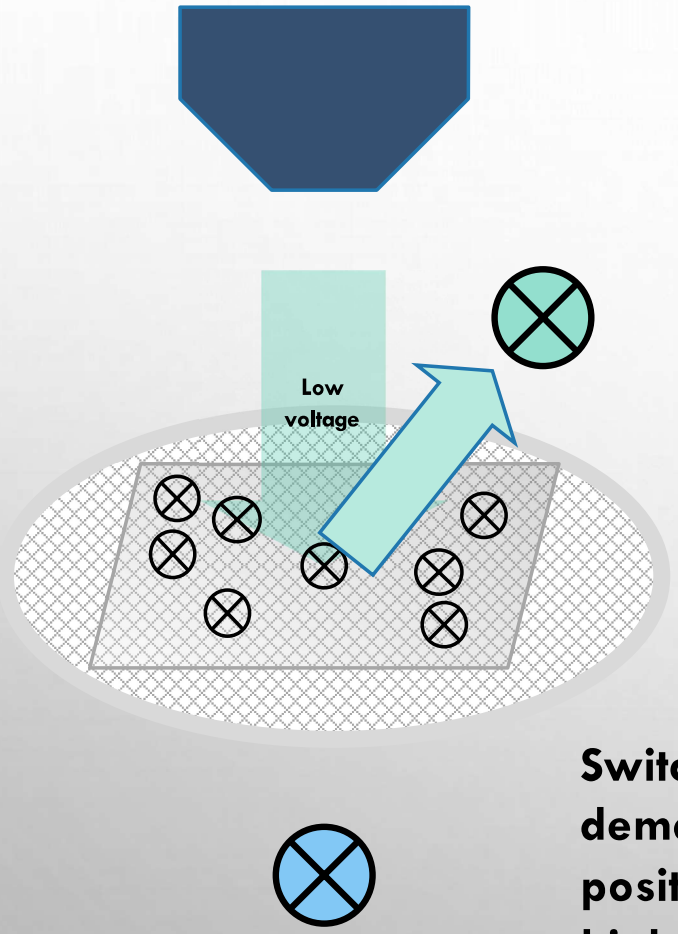
Real workflow – step by step



1) Find the object of interest
2) Look at it under high accelerating voltage (>15kV) – on STEM
   - you will see everything
3) Switch to low accelerating voltage (<2kV)
4) Find out that your beam shifted to different position and defocused
5) Refocus the beam and shift it back to the original position
6) Look at the object of interest on the BSE – see surface
7) Repeat steps 1-6 for each object of interest on each sample you have

x n

Real workflow – step by step



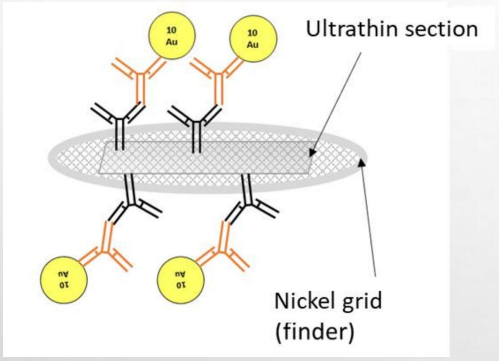
1) Find the object of interest
2) Look at it under high accelerating voltage (>15kV) – on STEM
   - you will see everything
3) Switch to low accelerating voltage (<2kV)
4) Find out that your beam shifted to different position and defocused
5) Refocus the beam and shift it back to the original position
6) Look at the object of interest on the BSE – see surface
7) Repeat steps 1-6 for each object of interest on each sample you have

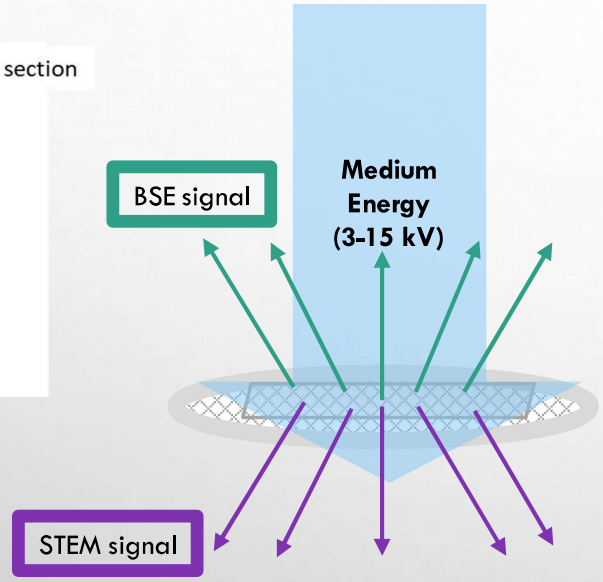
x n

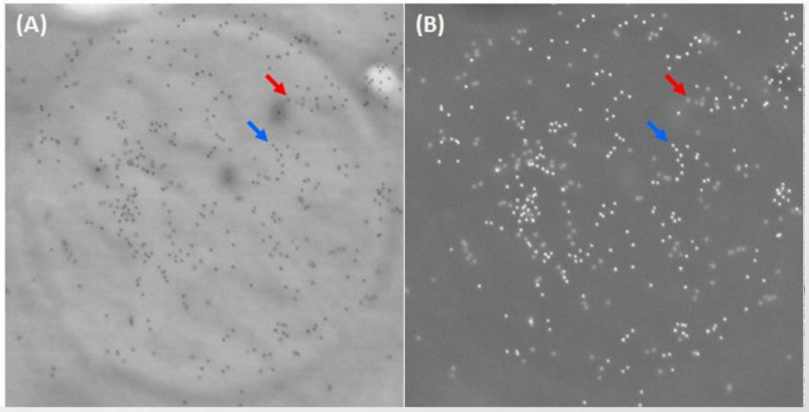
Switching between 2 accelerating voltages is laborious and time demanding due to the constant shifitign of the beam to correct position and the necesity of refocusing. This also may lead to higher radiation damage and formation of contaminations

MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

WORKFLOW OF THE METHOD USING ONLY ONE ACCELERATING VOLTAGE







Dim/blurry – lower nanoparticles,
Bright/sharp – upper nanoparticles

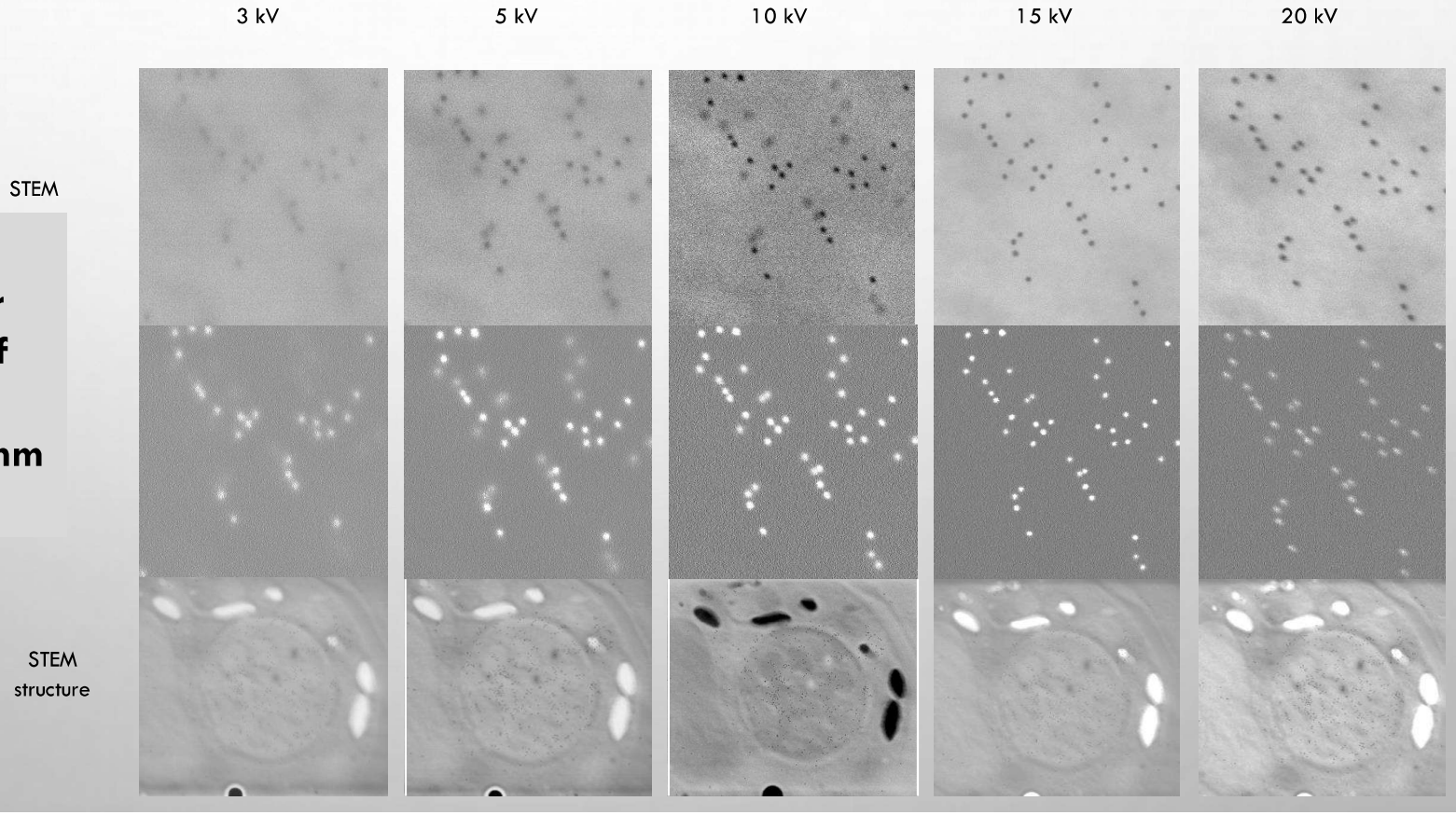
Gold nanoparticles (10 nm) on the nucleus of C. *Velia*, (non-specific reaction) 5kV, 15.68µs, 37 000x, 100 nm section

F. Kitzberger et al, 2023, in press

MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

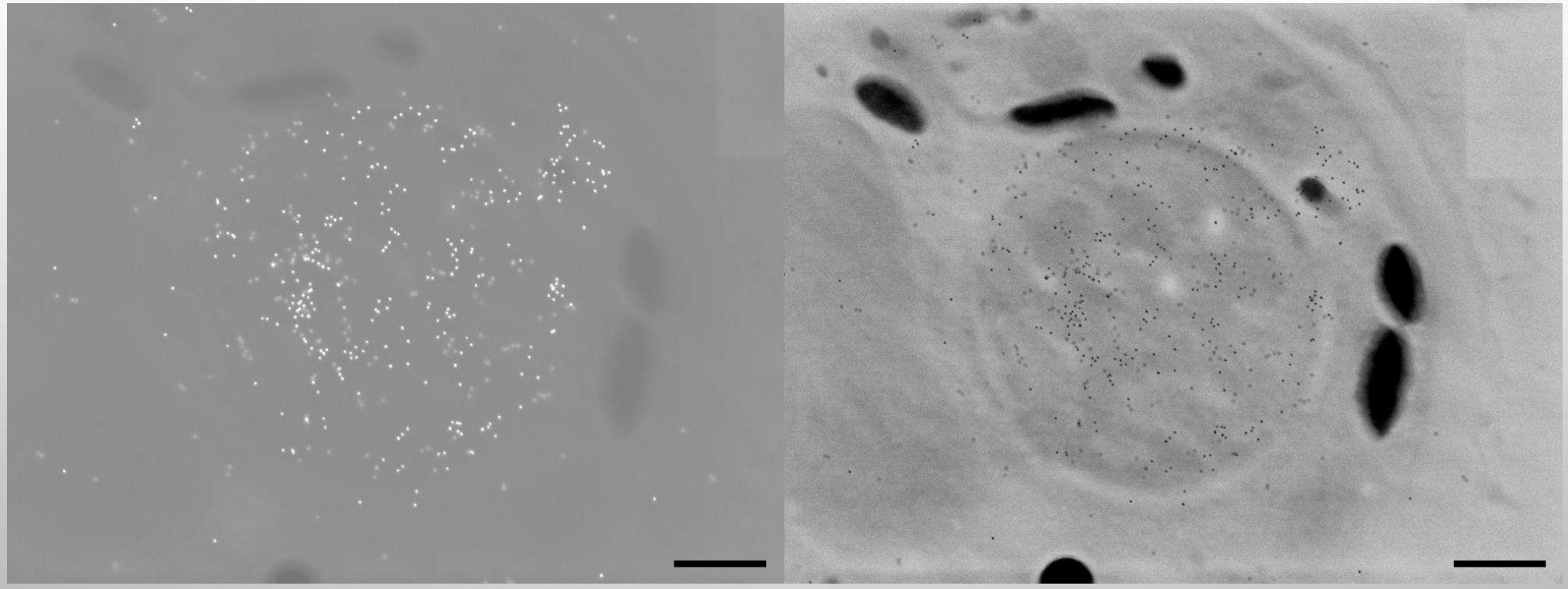
Propper setting of acquisition parameters (accelerating voltage, acquisition time, magnification) allows us to get all necessary information (ultrastructure, differentiate top and bottom NP) from one run – no need to switch the accelerating voltage → no need to refocus and shift the beam back to the proper position – faster and more gentle.

Comparison of different accelerating voltages for simultaneous imaging of the top and bottom nanoparticles on a 100 nm section



MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

THE OPTIMAL ACCELERATING VOLTAGE FOR 100 NM SECTION LAYS BETWEEN 5 AND 10 KV



5kV – BSE

10kV – STEM

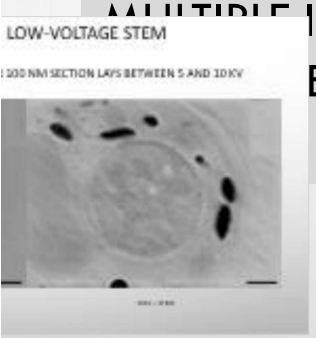
MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

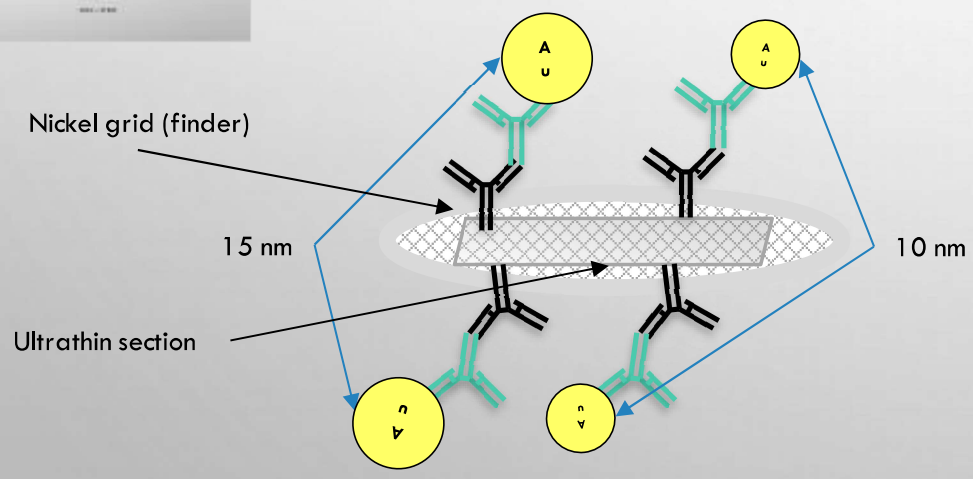
SIMULTANEOUS DETECTION OF TWO Au MARKERS ON BOTH SIDES OF THE SECTION

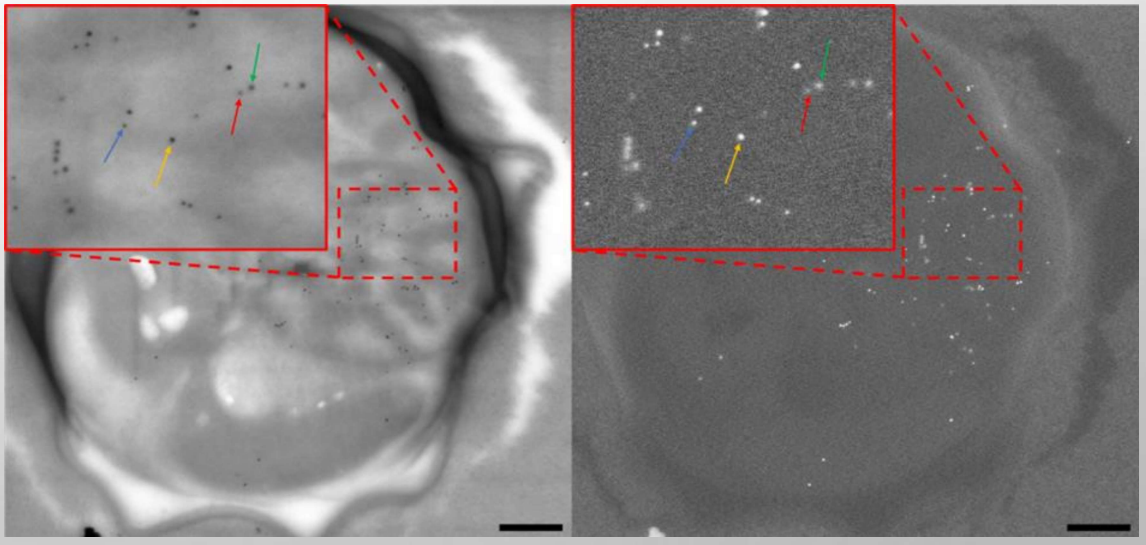
=

MULTIPLE IMMUNOLOCALISATION OF FOUR ANTIGENES OF INTEREST

15 nm top	Big	Bright	Sharp
10 nm top	Small	Bright	Sharp
15 nm bottom	Big	Dim	Blurry
10 nm bottom	Small	Dim	Blurry



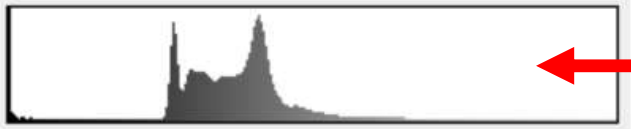




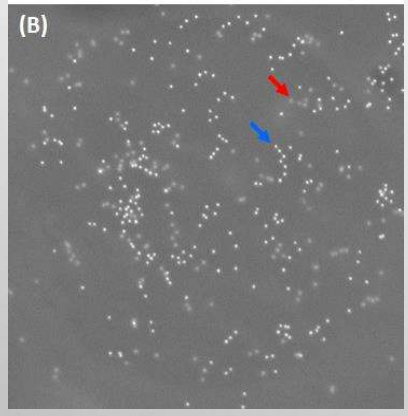
MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

THE ENHANCEMENT OF THE VISUAL DIFFERENCE BETWEEN BOTTOM AND TOP Au NPs WITH THE HELP OF IMAGE PROCESSING

BSE image→Import to ImageJ/FIJI→ Adjust the B/W threshold Adjust the B/W threshold→Upper NPs wil transform to donut shape



B/W threshold histogram

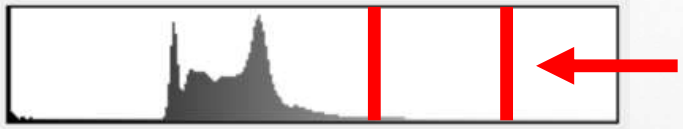


Gold nanoparticles (10 nm) on the nucleus of C. *Velia,* (non-specific reaction) 5kV, 15.68µs, 37 000x, 100 nm section

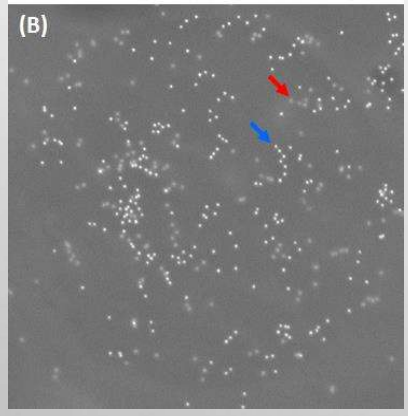
MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

THE ENHANCEMENT OF THE VISUAL DIFFERENCE BETWEEN BOTTOM AND TOP Au NPs WITH THE HELP OF IMAGE PROCESSING

BSE image→Import to ImageJ/FIJI→ Adjust the B/W threshold Adjust the B/W threshold→Upper NPs wil transform to donut shape



adjust the minima and maxima of the B/W threshold

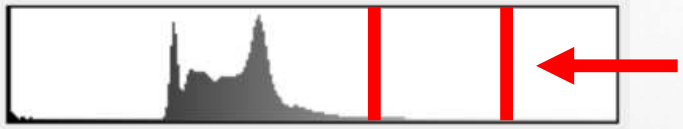


Gold nanoparticles (10 nm) on the nucleus of C. *Velia,* (non-specific reaction) 5kV, 15.68µs, 37 000x, 100 nm section

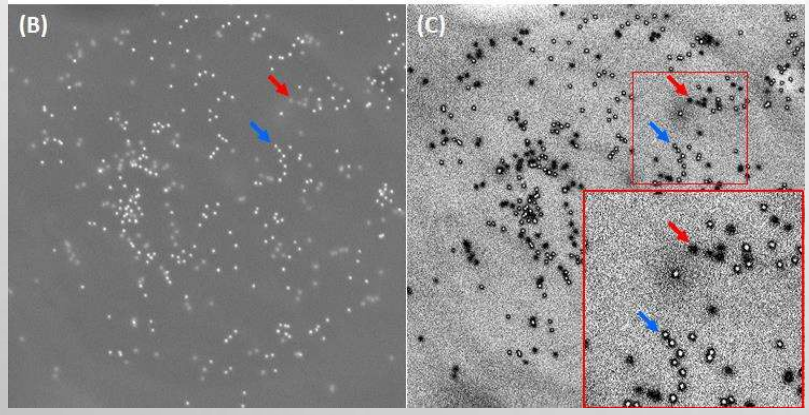
MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

THE ENHANCEMENT OF THE VISUAL DIFFERENCE BETWEEN BOTTOM AND TOP Au NPs WITH THE HELP OF IMAGE PROCESSING

BSE image→Import to ImageJ/FIJI→ Adjust the B/W threshold→Upper NPs wil transform to donut shape Adju



adjust the minima and maxima of the B/W threshold

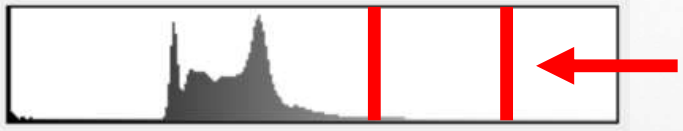


Gold nanoparticles (10 nm) on the nucleus of C. *Velia,* (non-specific reaction) 5kV, 15.68µs, 37 000x, 100 nm section

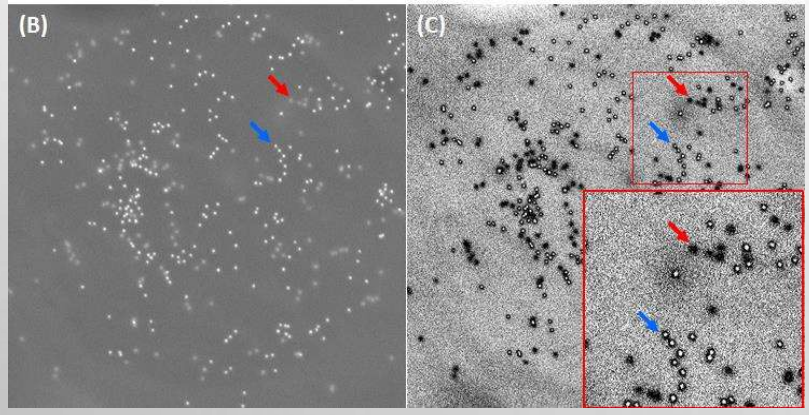
MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

THE ENHANCEMENT OF THE VISUAL DIFFERENCE BETWEEN BOTTOM AND TOP Au NPs WITH THE HELP OF IMAGE PROCESSING

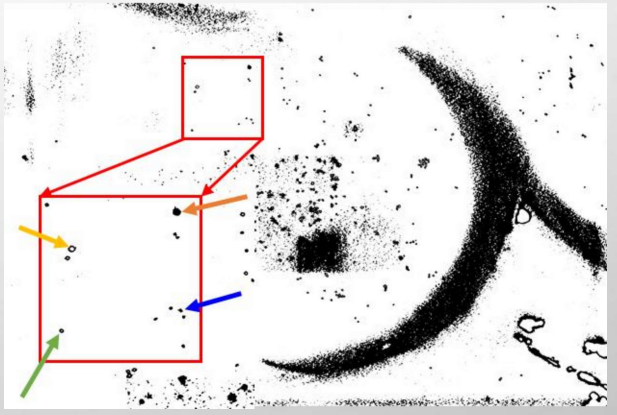
BSE image→Import to ImageJ/FIJI→ Adjust the B/W threshold→Upper NPs wil transform to donut shape Adjust the B



adjust the minima and maxima of the B/W threshold



10 nm NPs, 5kV, 15.68µs, 37 000x, 100 nm section



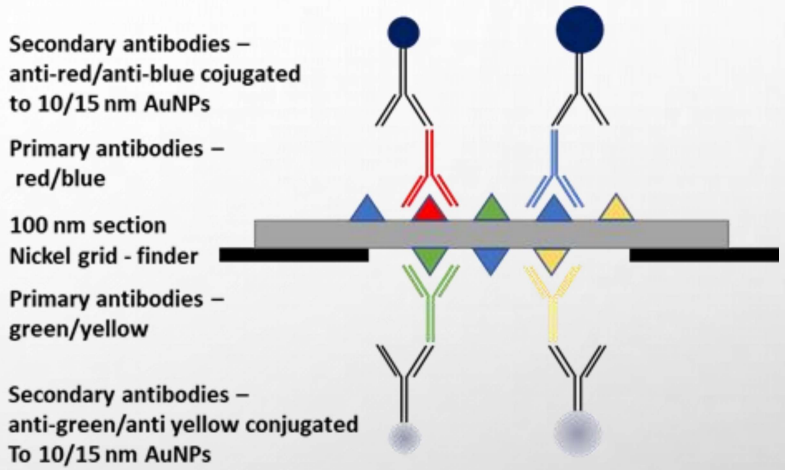
10 a 15 nm NPs, 5kV, 10µs, 20 000x, 100 nm section

This approach works not only for particles of the same diameter, but also for the particles of different diameters; though, each size needs a bit different B/W threshold setting

MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

SUMMARY

- THIS METHOD ALLOWS DOING STRIGHTFORWARD DOUBLE IMMUNO-LOCALIZAZION, EVEN WHEN HAVING PRIMARY ANTIBODIES FROM THE SAME SPECIES – WITH SINGLE ACCELERATING VOLTAGE
- WITH THIS APPROACH THE NUMBER OF LABELLED BIOMOLECULES ARE EFFECTIVELY DOUBLED (2 Au MARKEr SIZES VISUALIZE 4 BIOMOLECULES, 3→6, 4→8…)
- THE VISUAL DIFFERENCE CAN BE FURTHER ENHANCED BY COMMONLY AVAILABLE SOFTWARE



MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

Acknowledgements:
Laboratory of Electron Microscopy in České Budějovice
Marie Vancová
Jana Nebesářová
Jiří Týč
Tomáš Bílý
Petra Masařová
Martina Tesařová
Jiří Vaněček
Jan Langhans
Zdeno Gardian
Eva Ďurinová
Jana Kopecká
Institue of Parasitology:
Shun-Min Yang

BIOLOGICKÉ CENTRUM AV ČR, v. v. i.

INSTITUTE OF PARASITOLOGY Biology Centre, ASCR

ThermoFisher SCIENTIFIC

CSMS

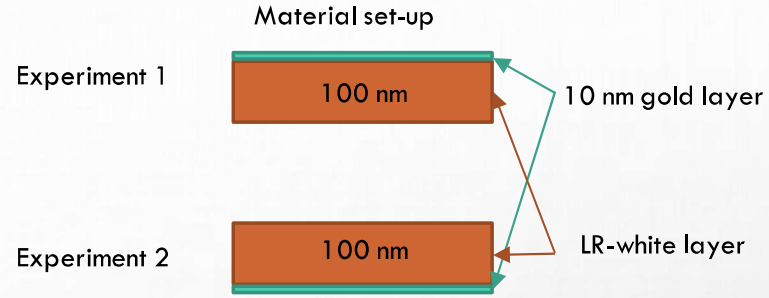
CZECH BIOIMAGING Imaging principles of life

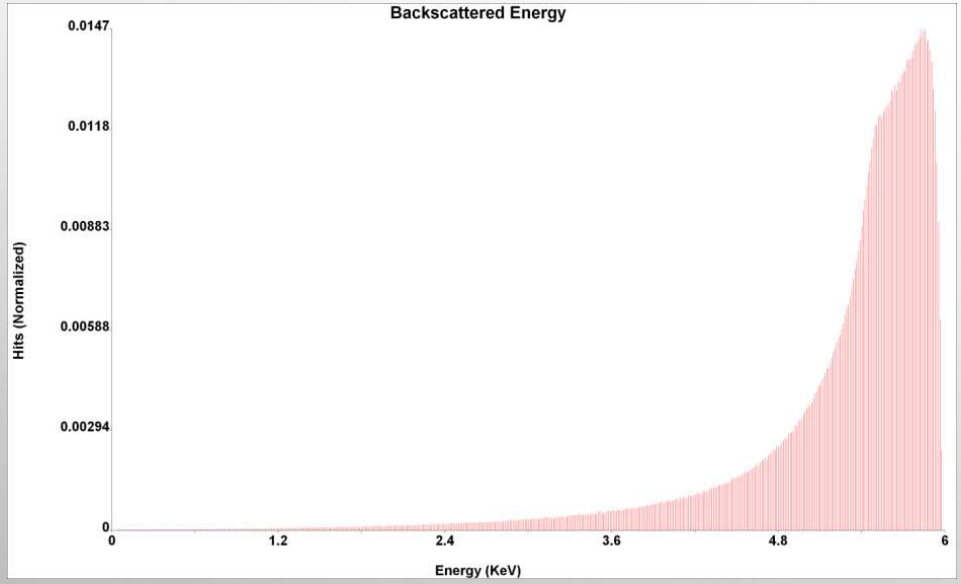
EURO BIOIMAGING Image life, discover the future

THANK YOU FOR YOUR ATTENTION

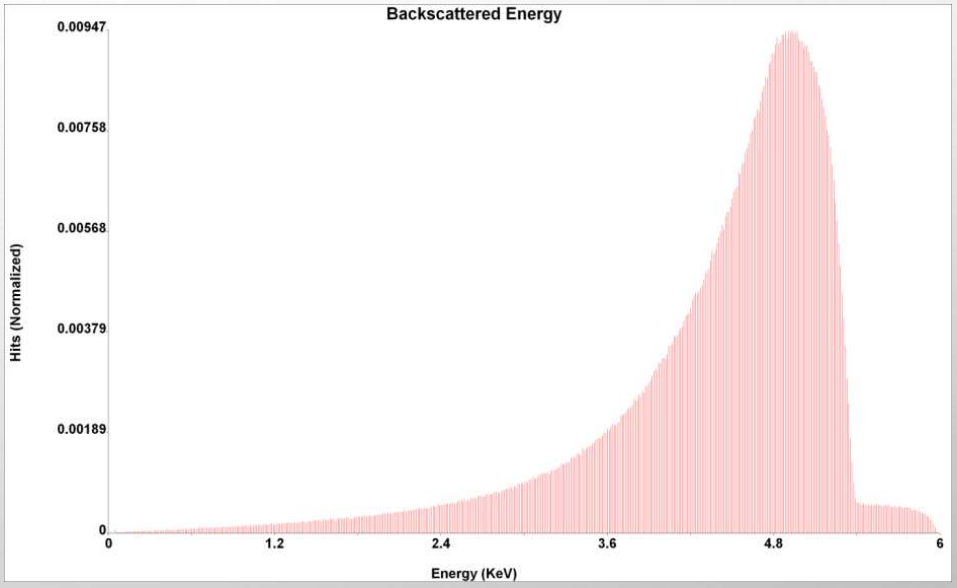
MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

MC SIMULATION OF THE BSE SIGNAL





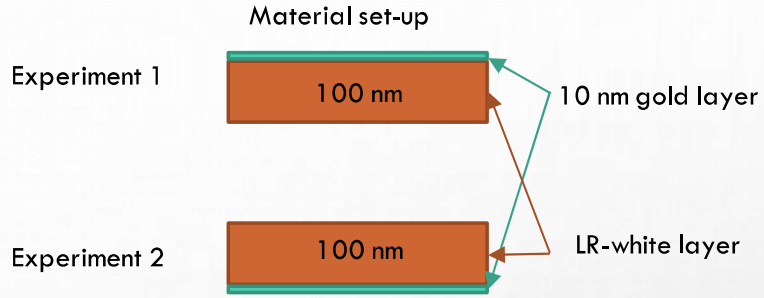
Experiment 1: BSE signal from the gold on top, 6 kV, 10 000 000 electrons

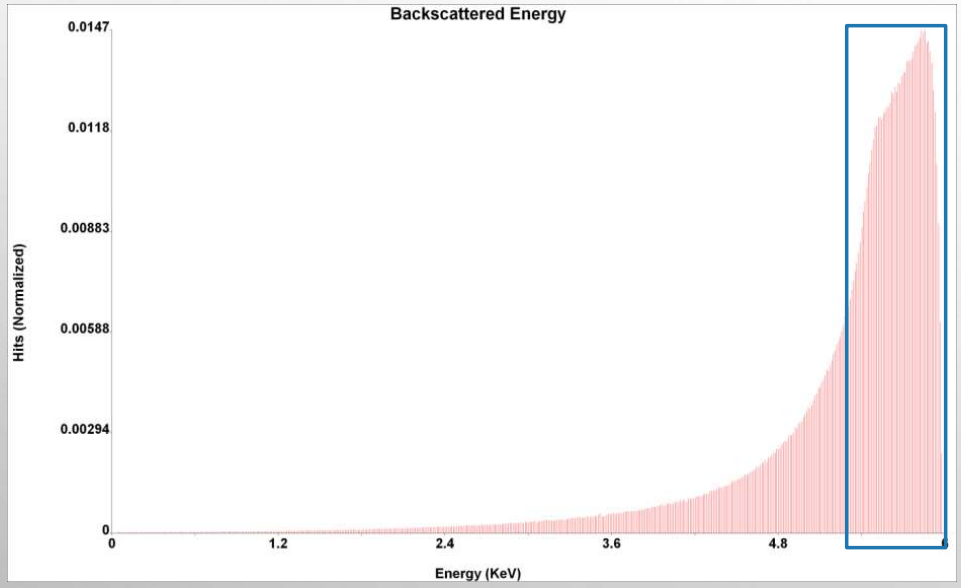


Experiment 2: BSE signal from the gold below the section, 6 kV, 10 000 000 electrons

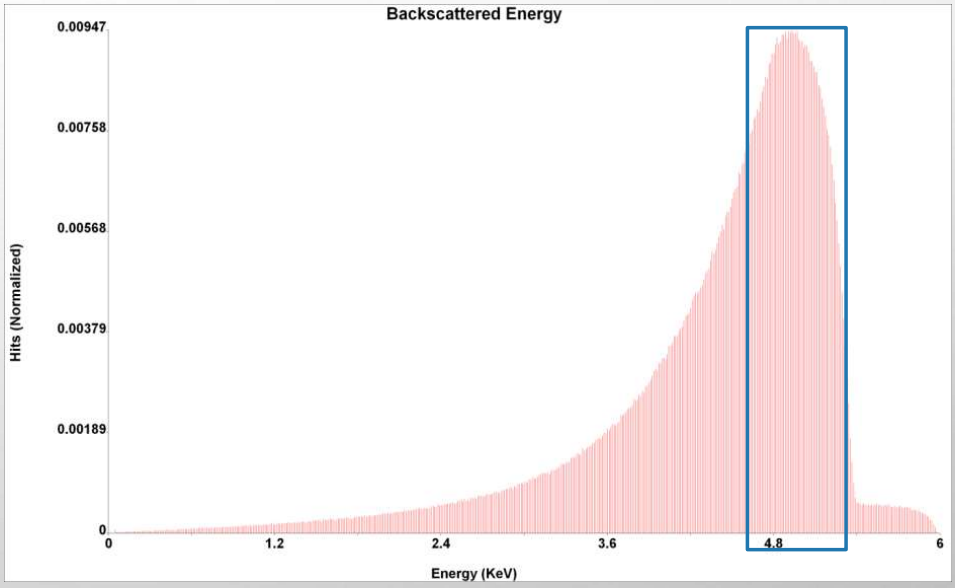
MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

MC SIMULATION OF THE BSE SIGNAL





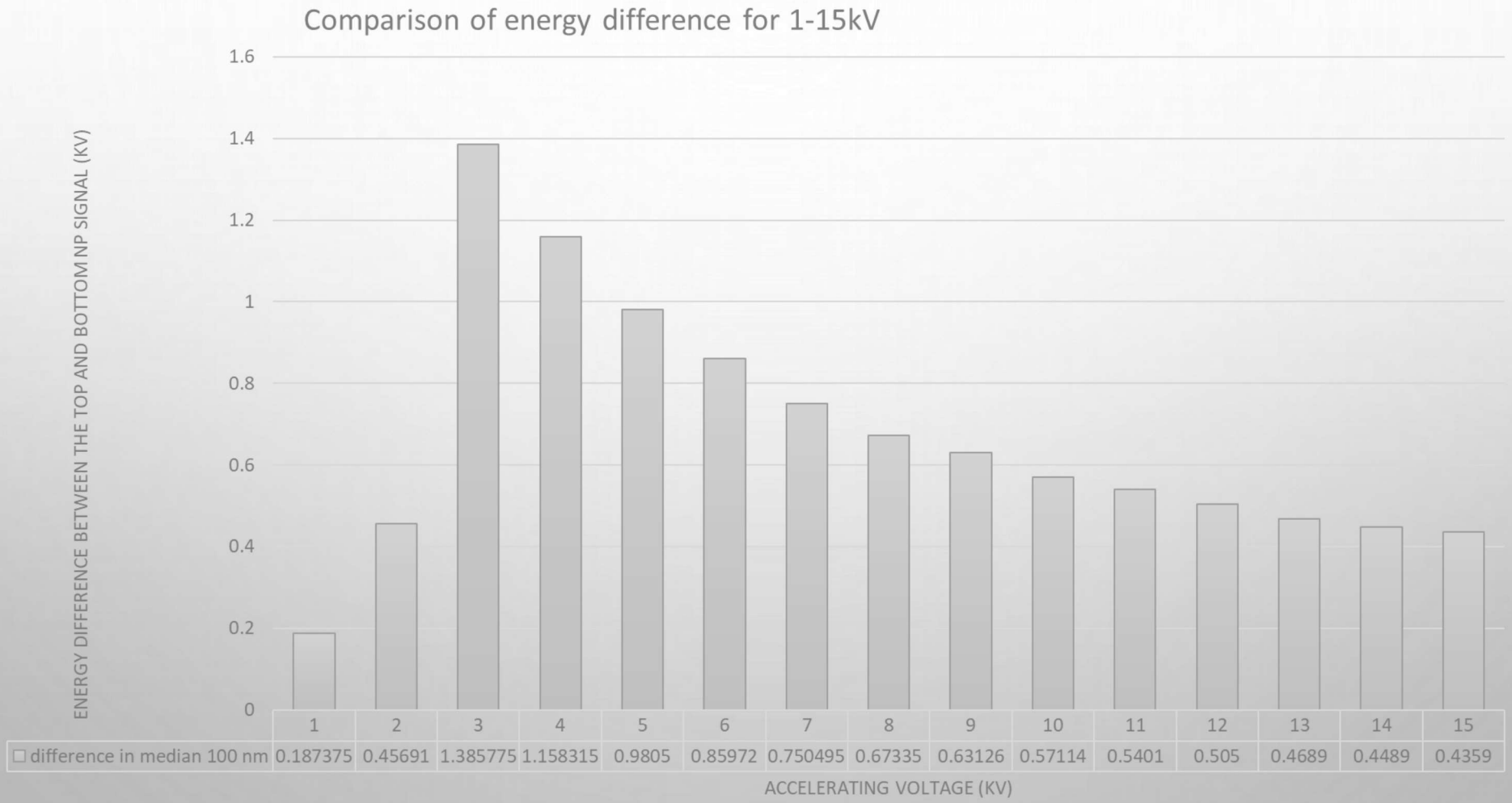
Experiment 1: BSE signal from the gold on top, 6 kV, 10 000 000 electrons



Experiment 2: BSE signal from the gold below the section, 6 kV, 10 000 000 electrons

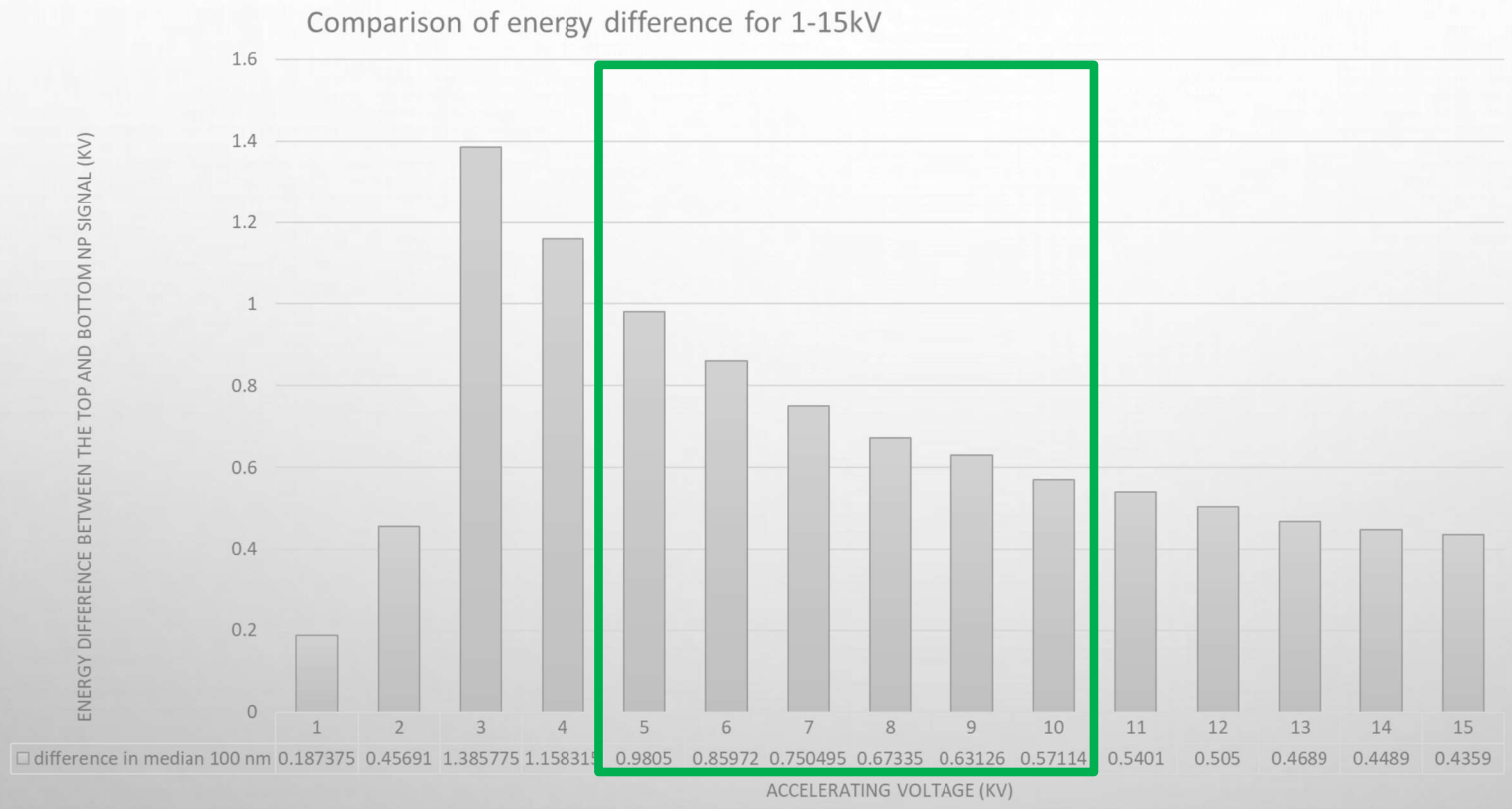
MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

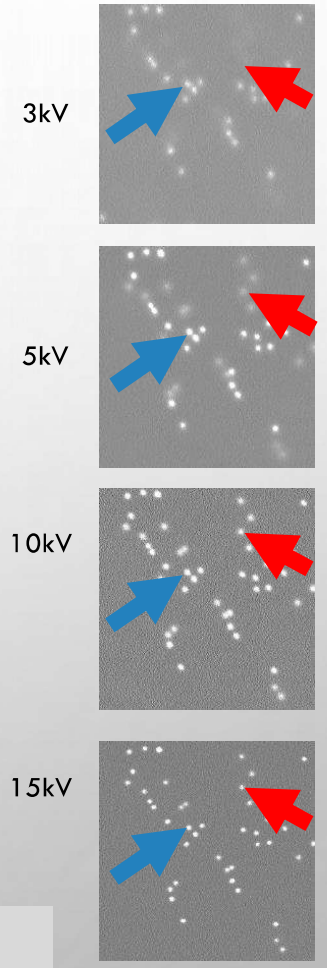
MC SIMULATION OF THE BSE SIGNAL



MULTIPLE IMMUNOLABELING IN LOW-VOLTAGE STEM

MC SIMULATION OF THE BSE SIGNAL





There is a certain energy difference between the BSE signal from the top and bottom NP depending on the accelerating voltage.