

**BIO-444: Advanced Bioengineering Methods Laboratory**

**SEM: Scanning electron microscopy**

**Interfaculty Institute for Bioengineering, School of Engineering (STI)**

**École Polytechnique Fédérale de Lausanne (EPFL), Switzerland**

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The logo of the École Polytechnique Fédérale de Lausanne (EPFL) is displayed in a large, bold, red, sans-serif font. The letters are thick and blocky, with the 'E' and 'F' having a distinctive stepped or 'L-shaped' design.

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## Objectives:

- Understanding scanning electron microscopy (SEM) operation
- Energy-dispersive X-ray spectroscopy (EDX) basics

## Reading & reference material:

- Scanning Electron Microscopy (SEM) Concepts  
(<https://caltech.app.box.com/s/fd1zf5fanzimlpuopwmtai6vqqh5dv8s/file/539167748489>)
- <https://www.thermofisher.com/ch/en/home/global/forms/industrial/tungsten-ceb6-electron-source.html>
- Complementary information:

## Setup:

- ZEISS Crossbeam 550L

## Introduction to electron microscopy

Electron microscopes (EMs) are specialized instruments that can achieve higher resolution than optical microscopes, allowing them to visualize smaller structures. This is because the minimum resolvable distance of an optical system is determined by the wavelength of the incident light and the numerical aperture, as described by the Abbe diffraction limit. Since electrons exhibit wave-like behavior, this same relationship holds true for electron microscopes, but the much shorter wavelength of electrons enables higher theoretical resolution than visible light photons. Thus, a theoretical system that utilizes an electron source of illumination would achieve better resolution than one that uses visible light.

In 1926, Hans Busch developed an electromagnetic lens capable of controlling the path of electrons. The first practical EM was later invented by Max Knoll and Ernst Ruska at TU Berlin in 1931, while the first commercial EM was sold by Siemens. Over time, the theoretical resolution limit of EMs improved, dropping from 10 nm in the late 1930s to 4 nm by 1944. Nowadays, most commercial EMs have a practical resolution limit of around 1 Å, while optical microscopes are limited to approximately 100 nm resolution.

Electron microscopes are generally divided into two main types: transmission electron microscopes (TEMs) and scanning electron microscopes (SEMs).

1. TEMs utilize a high voltage electron beam to illuminate the sample, generating a magnified image of the electrons that pass through the sample onto a fluorescent screen coated with a phosphor or scintillator material. By revealing the inner structure and composition of the sample, TEM images can offer valuable insights.
2. On the other hand, SEMs use a focused electron beam that is scanned across the surface of the sample to produce images. By examining the surface, SEM

images can provide a detailed view of the sample's topography and surface features. SEMs can achieve resolutions in the range of 1 to 10 nm.

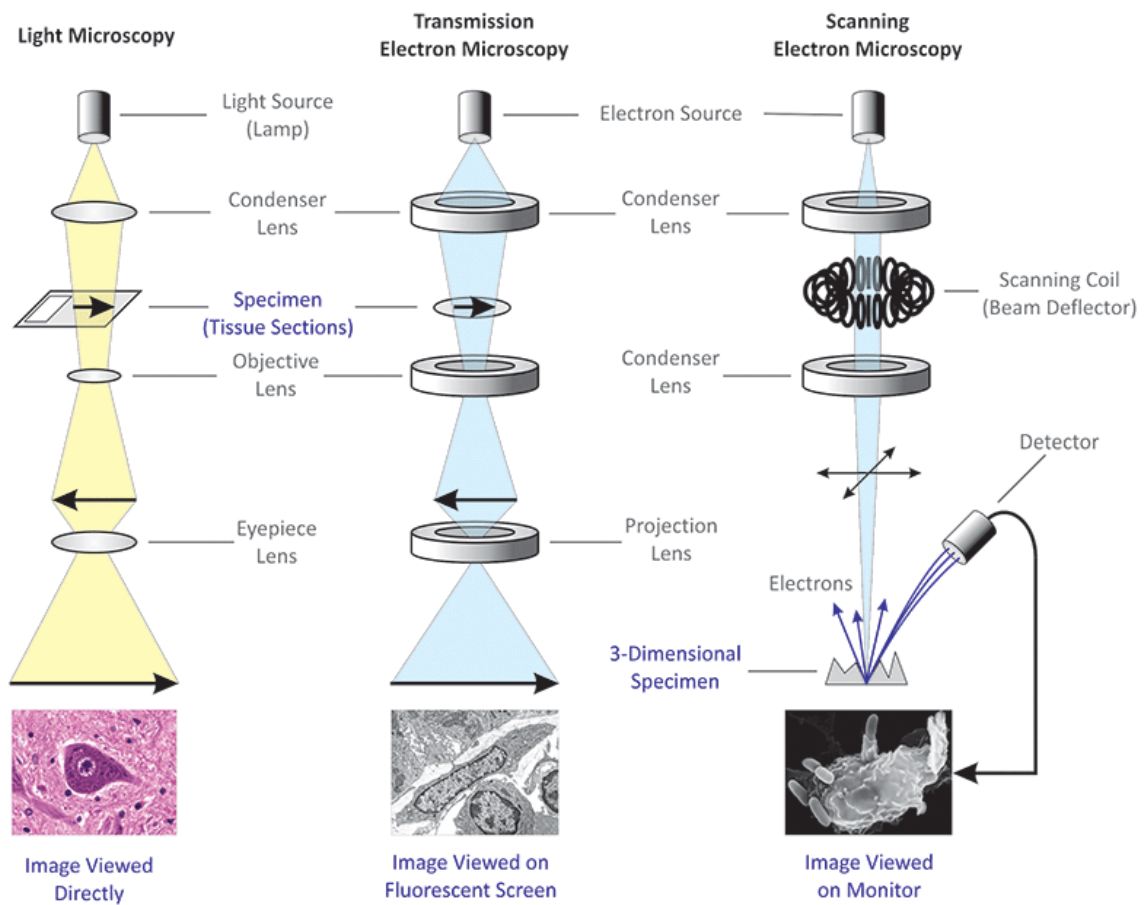
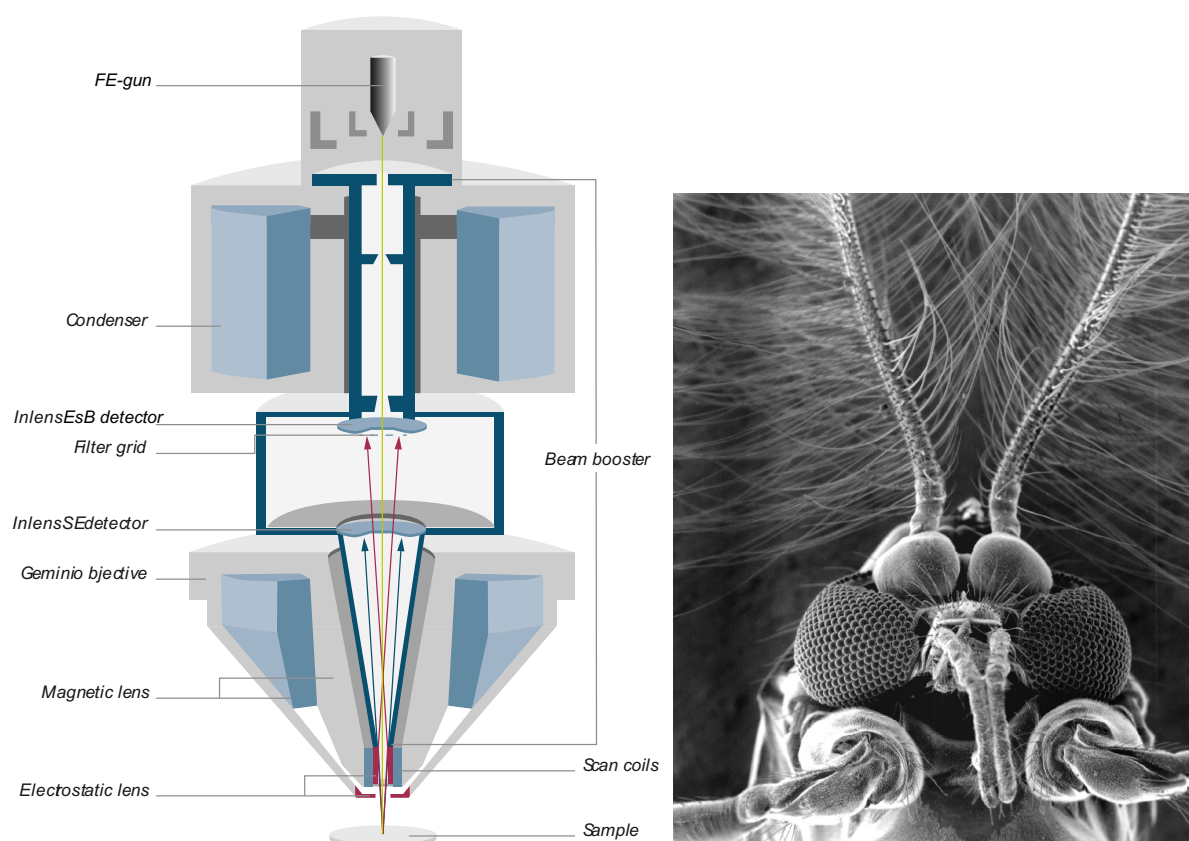


Figure 1. Schematic of an optical microscope, and transmission optical microscope and a scanning electron microscope, and examples of the type of images they can produce (<https://microbiologyinfo.com/differences-between-light-microscope-and-electron-microscope/>).

## Scanning electron microscopy



*Figure 2. Different parts of the scanning electron microscope (left; Zeiss Gemini column and example of a SEM image (right; Louisa Howard, Dartmouth College Electron Microscope Facility), depicting the head of an insect including its compound eyes.*

The SEM uses an electron source to generate electrons that are accelerated with high voltage and controlled with magnetic lenses to scan the surface of a specimen. The electron source can be a heated filament or a field emission tip, which works differently by tunneling electrons out of the tip using a high electric field. The interactions between primary electrons and the sample produce various contrast mechanisms, leading to different types of SEM images. Unlike conventional imaging techniques, SEM generates images by scanning the electron beam over the surface one pixel at a time and then reconstructing the image from the sequence data. Samples can be thick but must be conductive.

While travelling, electrons can be disturbed by gas molecules, resulting in lower quality images, so the SEM requires a vacuum chamber to prevent the interaction of the electrons with the surrounding gas molecules. If electrons encounter gas molecules in like in air, they can collide and fail to reach the sample or become deflected from their path. Additionally, gas molecules can react with the sample and even condense on the surface, lowering the image quality. Thus, the SEM column and sample must always be inside a vacuum chamber, reducing as much as possible gas molecules on the environment to generate high-quality images.

## Basic operating principles

### Electron source



*Figure 3. Different types of EM emitters: thermal emission (left) and field emission (right).*

The first step in an electron microscope is to generate electrons, which can be achieved by extracting them from a conducting material using either heat or an electric field. There are two main types of electron microscopes based on this principle, as shown in Figure 3:

1. Thermal emission microscopes operate like old-style light bulbs, with Lanthanum hexaboride (LaB6) filaments being a common example. In these microscopes, electrons are emitted from the filament by heating it up to high temperatures.
2. Field emission microscopes, on the other hand, use emitters with a very sharp tip. This enables them to extract electrons from a much smaller area compared to thermal emission, resulting in a more powerful microscope. However, fabricating these emitters is more difficult and expensive.

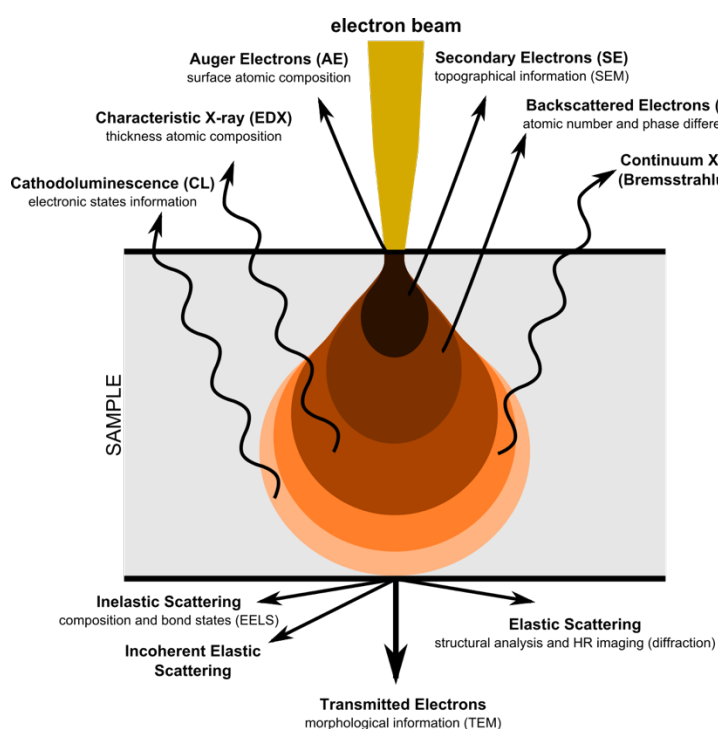
The selection of an electron source for an electron microscope depends on the specific requirements of the microscope and the sample being examined. For high-resolution imaging, a field emission source is typically preferred since it produces a smaller and more stable electron beam. When considering electron source specifications, brightness is an important factor to consider since it relates to the quality of images at high magnification. The beam's brightness increases with the acceleration voltage, with a ten-fold increase in brightness at 10kV compared to 1kV.

Another factor that contributes to image resolution is a small spot size at the electron emitter. The electron beam energy spread, which is the variation in electron energies leaving the source, is also crucial to consider. At low acceleration voltage, when the energy spread is large, chromatic aberration becomes the primary aberration, leading to a less focused beam.

After the electrons are generated, they are accelerated using high voltage, ranging from a few kilovolts to hundreds of kilovolts, depending on the microscope type and application. The electron beam current is controlled by adjusting the current density at the electron source, with current adjustments possible over several orders of magnitude to optimize imaging conditions for specific samples.

Electromagnetic lenses are used in electron microscopes to focus and raster control the electron beam, based on how electrons' charge is affected by magnetic fields. In contrast to optical microscopes, where refractive index is used to (de)focus or collimate the light, electromagnetic lenses deflect the electron beam towards a particular direction or focus it into a specific point.

## Electron - sample interactions



*Figure 4. Interaction volume on the sample, with the different electron-sample interactions.*

Electrons accelerated onto a material result in several interactions with the atoms of the target sample. Accelerated electrons can pass through the sample without interaction (TEM), undergo inelastic scattering (losing energy) and can be elastically scattered (conserving their energy after being deflected). Elastic and inelastic scattering result in different signals that can be used for imaging, quantitative and semi-quantitative information of the target sample and generation of an X-ray source. Typical signals used for imaging include secondary electrons (SE), backscattered electrons (BSE), cathodoluminescence (CL), Auger electrons and characteristic X-rays. Quantitative and semiquantitative analyses of materials as well as element mapping typically utilize characteristic X-rays.

When an electron beam hits the sample's surface, scattering and photon- and X-ray-production develops within a volume (the electron interaction volume, Figure 4), that is dependent on several factors:

- The energy of the incident beam (accelerating voltage) increases the interaction volume, but decreases the elastic scattering (for example, backscattering)(Figure 5).
- The interaction volume decreases as a function of the mean atomic weight (Figure 5).

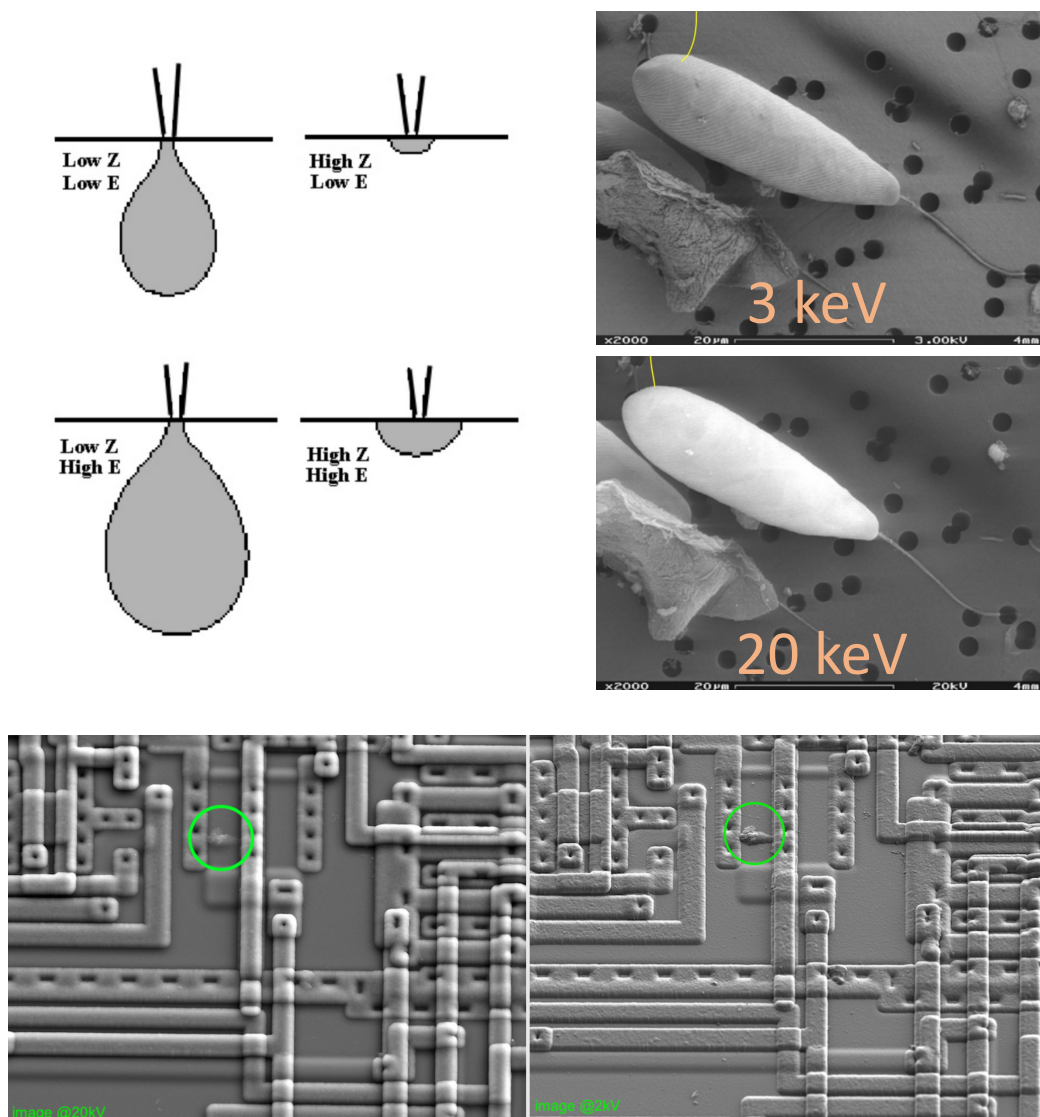
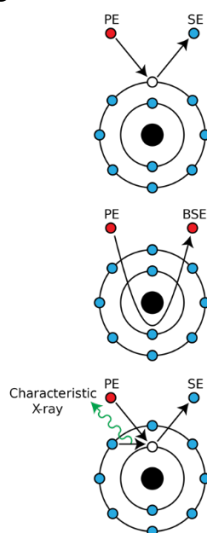


Figure 5. Effects of accelerating voltage: relation of interaction volume for high average atomic weight material under higher and lower accelerating voltage( top left), effect of accelerating voltage on signal generation (top right and bottom)

The interaction volume is what defines the final spatial resolution: the larger the interaction volume is, the lower is the spatial resolution. As the accelerated electrons

interact with the sample, they lose energy and produce a variety of signals that are detected and used to form an image. The primary electrons (PE) emitted from the electron gun follow different paths when they interact with the sample. These interactions produce different contrast mechanisms, resulting in different types of SEM images. SE images based on low energy (around 50 eV) inelastically re-emitted electrons appear 3D due to the SE's low energy and short escape depth, providing surface topography information. BSE images provide material contrast because heavier elements elastically backscatter more electrons than lighter ones, producing a grayscale image. X-ray emission can also provide material contrast through characteristic radiation from different elements, appearing in energy-dispersive X-ray spectroscopy (EDX) images.

SE images are the most used for surface topography, while BSE and EDX images are used for material analysis. The contrast mechanisms of SEM allow for the visualization of fine surface details, enabling high-resolution imaging of complex structures.



*Figure 6. Different paths of the primary electrons (PE) from the electron beam: eject a secondary electron (SE) from the sample (top image); being reflected from the sample (BSE, middle); or excite a SE of the atom inner shell (bottom), important for EDX measurements ([https://en.wikipedia.org/wiki/Scanning\\_electron\\_microscope](https://en.wikipedia.org/wiki/Scanning_electron_microscope)).*

### Image contrast in SEM

SE are collected via a low collection voltage into the SE detector (e.g. Everhart-Thornley detector). The number of SE is dependent on the angle between the surface and the position of the SE detector, which determines the brightness of the image at that position. Thus, SE image includes information about topography. Moreover, SE are strongly affected by surface charge. Large accumulations of surface charges could lead to image artefacts.

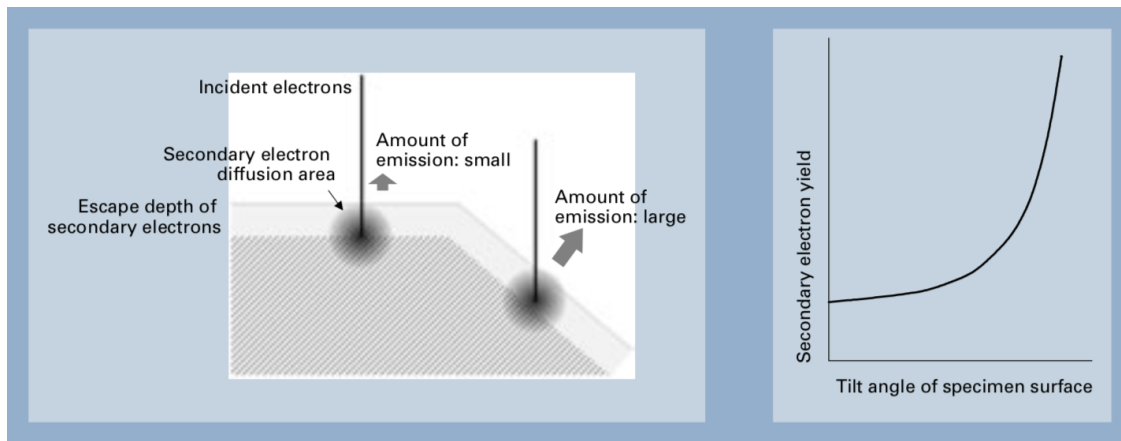


Figure 7. Topographical contrast origin of SE.

The number of secondary electrons that leave the sample and reach the detector depends on the angle and "line of sight" to the detector. As mentioned earlier, this makes the SE image look three dimensional. Furthermore, as the SE detector is placed laterally to the sample, it will detect more SEs emitted towards that specific direction, producing brighter image areas for tilting angles pointing towards the SE detector.

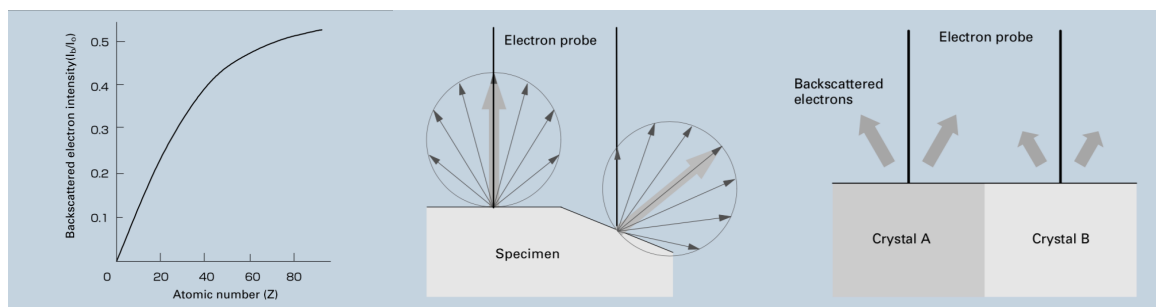
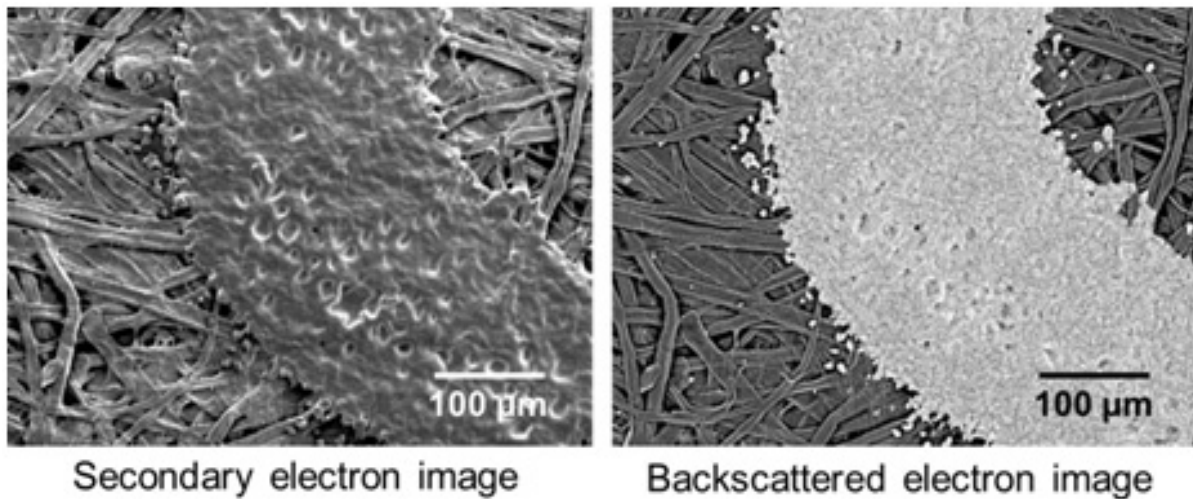


Figure 8. Topographical contrast origin of BSE.

In the case of BSE, the probability of primary electrons to be scattered back increases with higher atomic number ( $Z$ ) of the material. This gives the BSE image material contrast. The angle of the surface also affects the direction in which the BSE are reflected, therefore also producing topography contrast. The back-scattering efficiency also depends on the crystal orientation for crystalline materials (crystallographic contrast). BSE electrons can come from deeper inside the sample. This let them look "underneath" the surface, but it also reduces the resolution.



*Figure 9. Comparison of a secondary electron image (left) and a backscattered electron image (right) of the letter part of a carbon-coated name card (seen at the center of both images). In the backscattered electron image, the letter part appears very bright (compositional contrast) (<https://www.jeol.com/words/semterms/20190129.113542.php>).*

### Electron detection and image formation

The SEM is equipped with detectors that are used to collect the signals produced by the interactions of the primary electrons with the sample. For SE detection, the most common detectors are based on the Everhart-Thornley design, which includes a scintillator inside a Faraday cage in the microscope. To attract the relatively low-energy SE, a low positive voltage is applied to the Faraday cage. The scintillator emits photons when excited by the electrons, and these photons travel through a waveguide to a photocathode. The photocathode converts the photons into electrons using the photoelectric effect, and then amplifies the signal to produce the output. The computer then processes the output signal to generate an image.

For BSE detection, semiconductor-based detectors can be used, located around the pole-piece of the electron gun. Backscattered electrons have a relatively high energy, so no additional acceleration is needed to cause ionization. They can generate electron-hole pairs directly in a semiconductor device. BSE detectors are placed at the top since BSEs are backscattered primarily to the surface normal direction. Larger atoms scatter electrons back more efficiently than light atoms, producing a higher signal. The number of backscattered electrons reaching the detector is proportional to the atomic number, enabling differentiation of materials (phase contrast).

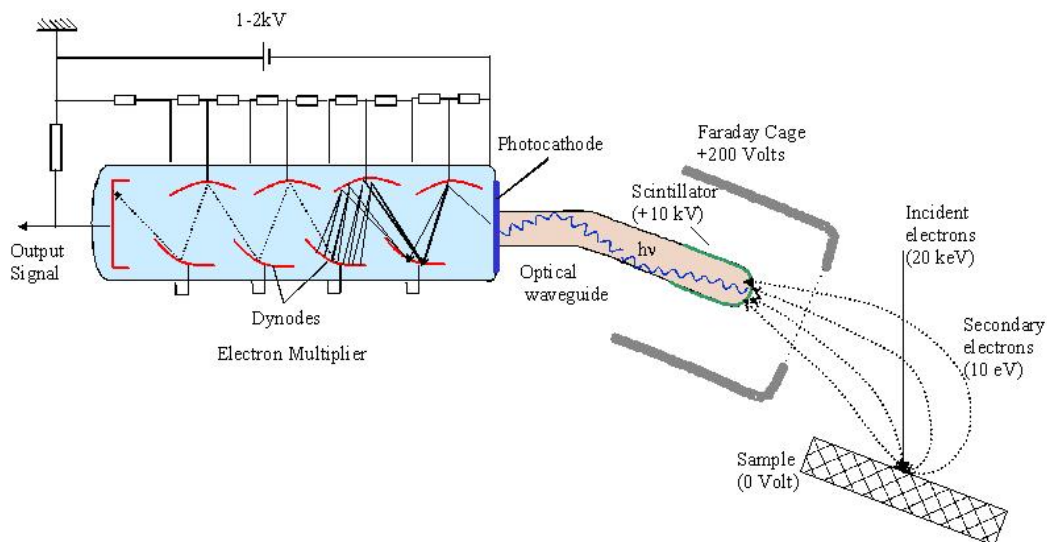


Figure 10. Schematic of the Everhart-Thornley detector  
([https://commons.wikimedia.org/wiki/File:Everhart-Thornley\\_detector.JPG](https://commons.wikimedia.org/wiki/File:Everhart-Thornley_detector.JPG)).

### Effect of measurement parameters on the SEM image formation

The most important parameters to control the electron beam in SEM are the current and the acceleration voltage. Large currents increase the intensity signal on the detector, but they also lead to larger electron beam diameters, thereby decreasing the spatial resolution. Furthermore, high currents can cause sample charging, particularly in materials with low conductivities, producing imaging artefacts. The higher the energy (acceleration voltage) of the electrons is, the deeper into the sample they penetrate. If there is structure underneath the surface, that structure will be superimposed on the surface structure, thereby degrading the resolution. A higher acceleration voltage decreases the magnitude of lens aberrations and therefore results in a smaller spot size, leading to higher spatial resolution. However, the best acceleration voltage strongly depends on the sample and the desired sample properties to be measured.

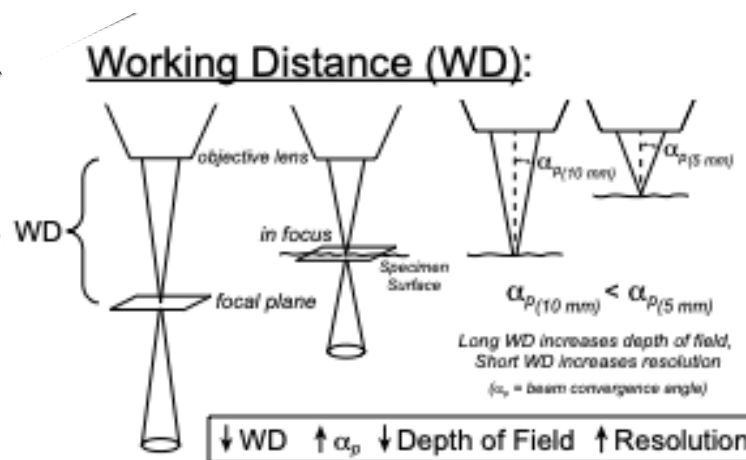


Figure 11. Schematic of the working distance in a SEM.

Another important parameter is the working distance (WD), the distance between the objective lens and the sample. Long WD increases the depth of field, but short WD increases the resolution. The depth of field refers to the thickness of the sample that appears in focus in the SEM image, while the resolution refers to the level of detail that can be seen. In addition, it's important to note the **eucentric point**, which is the position *where the axis of the tilt stage intersects with the optical axis of the electron beam*. This is an important point to find, as tilting the sample away from the eucentric point can cause image distortion due to the sample being out of focus or changing the beam path. By finding the eucentric point, the sample can be tilted without significant image distortion, allowing for more accurate imaging of the 3D structure of the sample.

### Sample requirements and preparation

Prior to imaging, the sample must be firmly fixed to the specimen mount, such that the surface to observe is exposed. The sample should also be vacuum-compatible. Conventional SEM imaging requires electrically conductive samples to prevent the accumulation of electrostatic charge, which can result in imaging artifacts. Non-conductive samples can be coated with a thin layer (a few nm) of conductive material to facilitate SEM imaging. Sputter coating is a widely used method where a conductive material such as gold, platinum, or carbon is deposited onto the surface of the sample using ion bombardment. Another method is carbon coating, which can be achieved by evaporating carbon under vacuum onto the sample. Both methods require specialized equipment, such as a sputter coater or carbon evaporator. A more cost-effective and simple method for conductive coating and to connect them to the specimen holder is the use of conductive paints or adhesive carbon tabs, which can be applied manually. However, these methods may not be suitable for high-resolution imaging, and the coating can interfere with certain types of analysis.

### Interpreting SEM images: artefacts

Artefacts can affect the image quality and interpretation of SEM images. Some common artefacts include:

1. **Charging**: When a sample is exposed to an electron beam, charges can accumulate in the sample if they cannot be dissipated through a conductive path, which can occur in nonconductive samples. This charging phenomenon arises when the number of incident electrons is greater than the number of electrons that can escape the specimen. This leads to the accumulation of a negative charge at the irradiated point, resulting in a large negative potential. This negative potential can distort the incoming electron beam and lead to image distortion and abnormal contrast. Therefore, to mitigate charging effects, it is necessary to coat the sample with a conductive material, use low voltage imaging conditions to minimize the buildup of charges, or reduce the current.

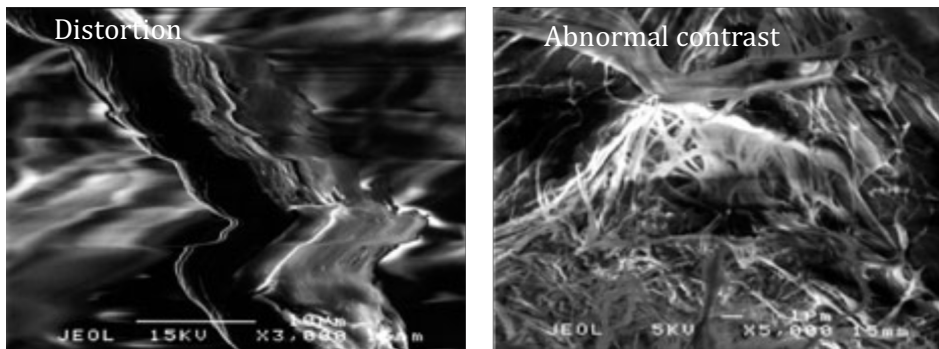


Figure 12 . Charging effects on image quality.

2. **Beam drift**: When the electron beam is not properly focused, it can cause image drift or blurring. This can be corrected by optimizing the beam focus and scan parameters.
3. **Subsurface contrast**: When imaging at high acceleration voltages, the beam can penetrate the surface and interact with the subsurface, producing an image with subsurface contrast. This can be useful for characterizing multilayered or composite materials, but it can also lead to misinterpretation of the sample structure.

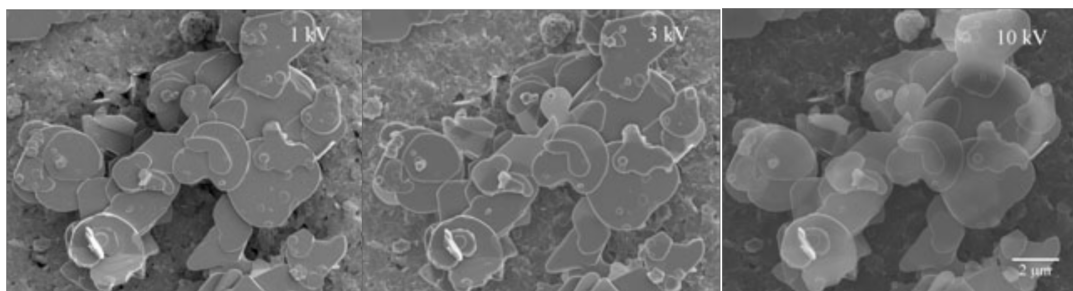
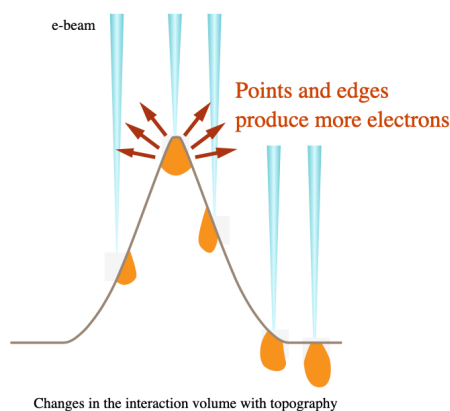


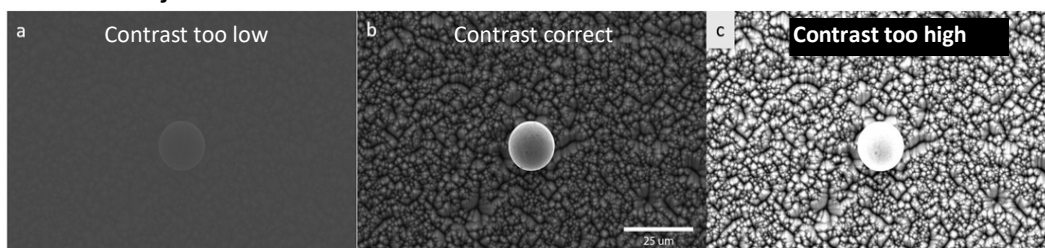
Figure 13. SEM images of a multilayered material at different acceleration voltages.

4. **Edges effects**: Sharp edges appear brighter because SE electrons can be collected from the whole interaction volume.



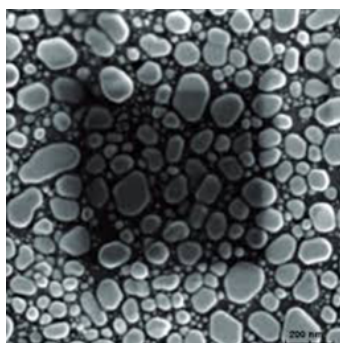
*Figure 14. Effect of the edges on SE emission.*

5. **Contrast and brightness**: The SEM image is a virtual reconstruction of the sample and does therefore not correspond to a real image. The data processing needed to create the sample can affect the clarity of the image. Primarily contrast and brightness are important. Contrast is the number of grayscale intensities that the imaging data is displayed. In the intensity histogram the contrast adjusts the width of the distribution.



*Figure 15. Examples of good (b) and bad (a; c) contrast parameters in image.*

6. **Contamination**: Contamination on the sample surface can produce artefacts that can obscure the image details. Although the SEM is under vacuum, there are always some contaminants in the chamber, for example hydrocarbons. When the electron beam hits these contaminations, the molecules dissociate and form radicals which in turn find other radicals to react with. The dissociated hydrocarbons are re-deposited as C carbon contamination in the area where the SEM beam was rastered.



*Figure 16. Example of beam induced contamination shown on the dark area.*

7. **Astigmatism**: Astigmatism is a common artefact in SEM resulting from misalignment or malfunctioning of the magnetic lenses, which leads to asymmetrical distortion of the electron beam, causing blurring or elongation of sample features. Correcting astigmatism involves precise alignment and adjustment of the magnetic lenses to ensure symmetrical and uniform deflection of the electron beam for optimal imaging.

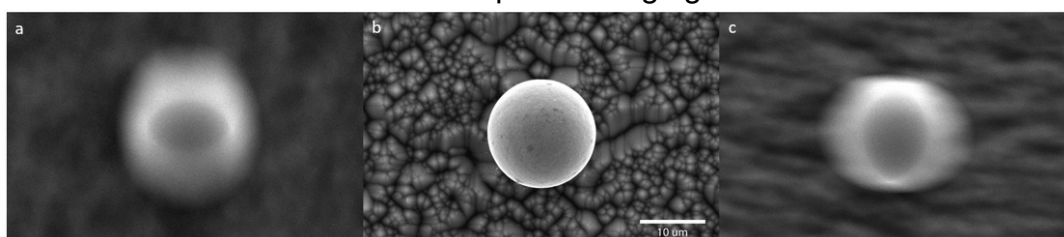


Figure 17. Astigmatism blurs and elongates sample features.

### Energy-dispersive X-ray analysis (EDX)

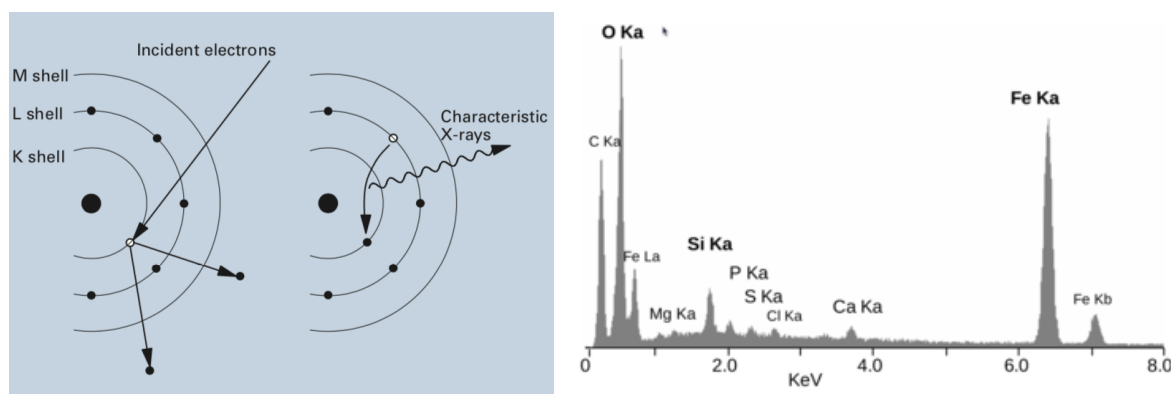


Figure 18. SEs from the inner shell of the atom can be excited and kicked out by PEs, creating a hole that is filled by an electron from an outer higher-energy shell, emitting X-rays characteristic of the specimen atom. On the right, characteristic EDX spectrum revealing the different atom species on the exposed area.

Electron microscopes can generate characteristic X-rays when electrons interact with atoms. A primary electron kicking out an electron from an inner orbital of the atom creates a vacancy that is filled by an electron from an outer orbital, emitting an X-ray photon. The energy spectrum of this X-ray photon is characteristic for the atom, and EDX detectors can record it to generate material maps, which give information about the different atoms presented on a specific area of the sample surface. The characteristic X-rays' energy peaks allow for the identification of the elements present, while the signal intensity provides information about the quantity of the element. EDX analysis can also help detect and identify impurities, inclusions, and other features within a sample. It is possible to acquire a EDX spectra for every pixel of the image and generate material composition maps, identifying the different materials from the EDX spectra (Figure 19). These material maps can help in identifying the chemical composition and distribution of elements in the sample.

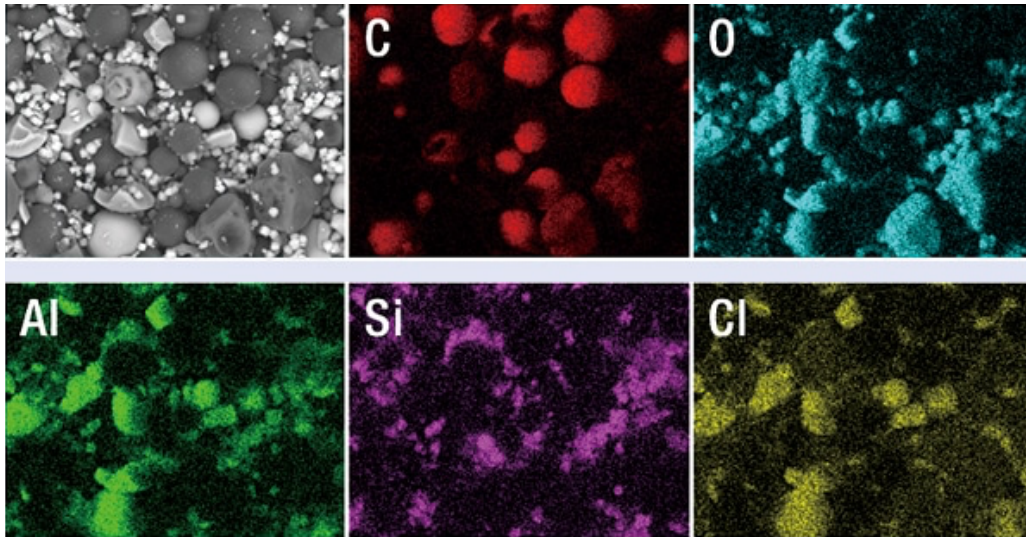


Figure 19. EDX compositional maps from a SEM image (top left): we can distinguish areas with high content of C, O, Al, Si and Cl.

### Practical:

#### SEM imaging of an insect

##### Materials:

Biological specimen to image, aluminium stub, tweezers for stub handling, super glue, silver-containing paint, glass slides

##### Sample mounting and preparation

1. Mount the stub onto a carousel using the special tweezers.



2. Place a small drop of super glue onto the aluminium stub.



3. Fix the sample of interest on the glue such that the surface to observe is exposed. In this case, make sure the eyes and wings are facing upwards as we would like to image the photoreceptors on the retina.



4. Add some silver-containing paint on the bottom of the specimen to increase conductivity.



### Sputtering coating the sample with platinum

5. Open gas valves to vent the sputtering machine. Keep the valve which supplies both the sputtering machine and the SEM open for the duration of the experiment.



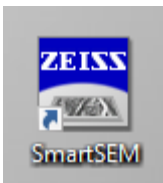
6. Using the CiME sputtering tool, deposit an appropriate thickness (few tens of nm) of Pd/Au on the sample.
7. After the deposition has finished, remove the aluminium stub from the chamber. The specimen should be evenly coated with a layer.



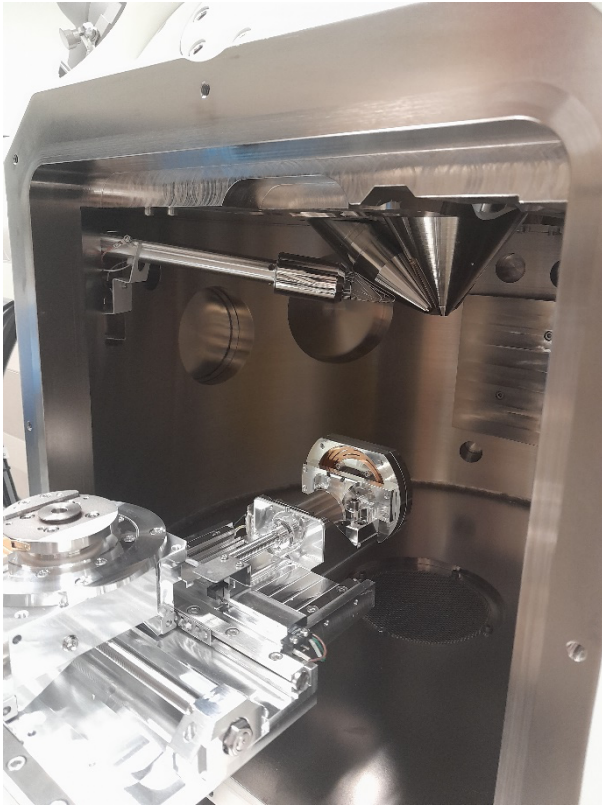
## Imaging biological sample with ZEISS Crossbeam 550

Starting the SEM and loading the sample

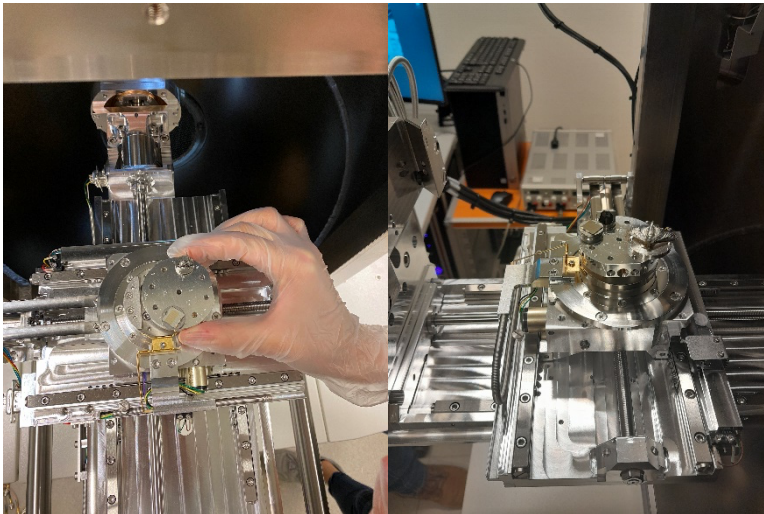
1. Start the SmartSEM user interface via the ZEISS SmartSEM icon on the desktop, and enter the username and password. By logging, the SmartSEM user interface opens and is ready to operate the tool. By default, a TV view inside the specimen chamber is shown.



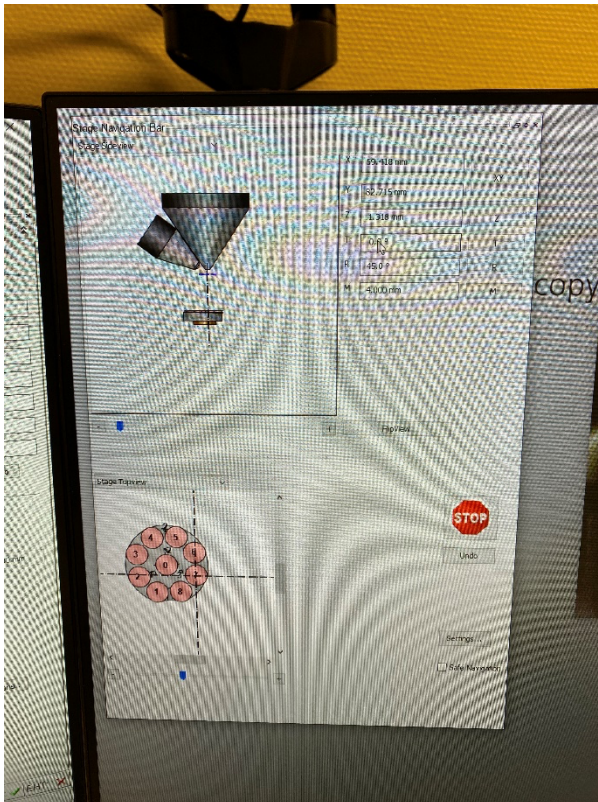
2. To vent the SEM and load the stub onto the carousel, press VENT on the docking panel. Before that, make sure the EHT (high voltage source) is switched off. You will hear a characteristic sound which suggests that the specimen chamber has been vented, and you can open it by pulling the door handle.



3. Slide the multi-pin holder out, and load your sample(s) on one of the holes, remembering the position. Slide the holder in, with the flat of the carousel parallel to the airlock.



4. Press pump, and check that the system vacuum (vacuum tab) drops to low  $10^{-5}$  mbar range.
5. Position the stub under the electron beam by clicking on the same stub on the Stage Navigation dialog.



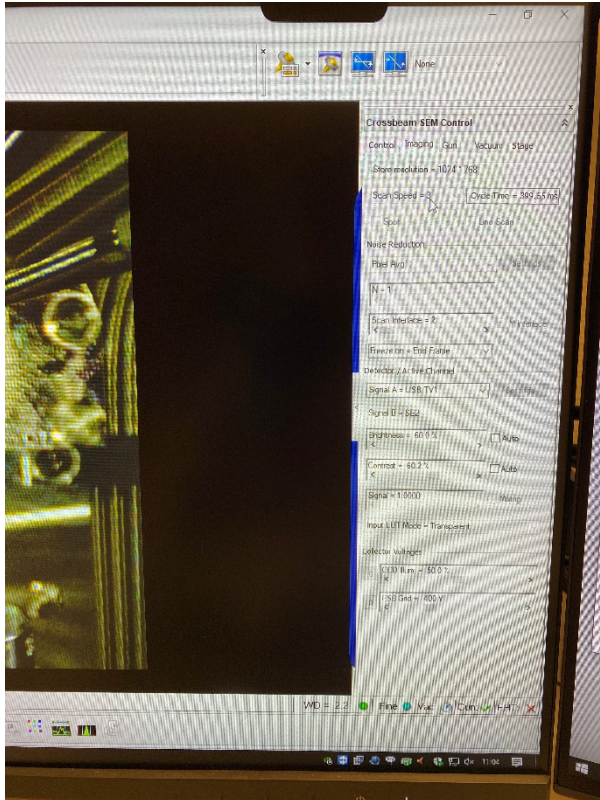
6. Use the dual joystick to carefully move up the stage.



### SEM imaging of biological specimen

7. Switch on the EHT such that the gun starts emitting electrons. To do that, click on the right part of the status bar where the EHT should be next to a red cross.

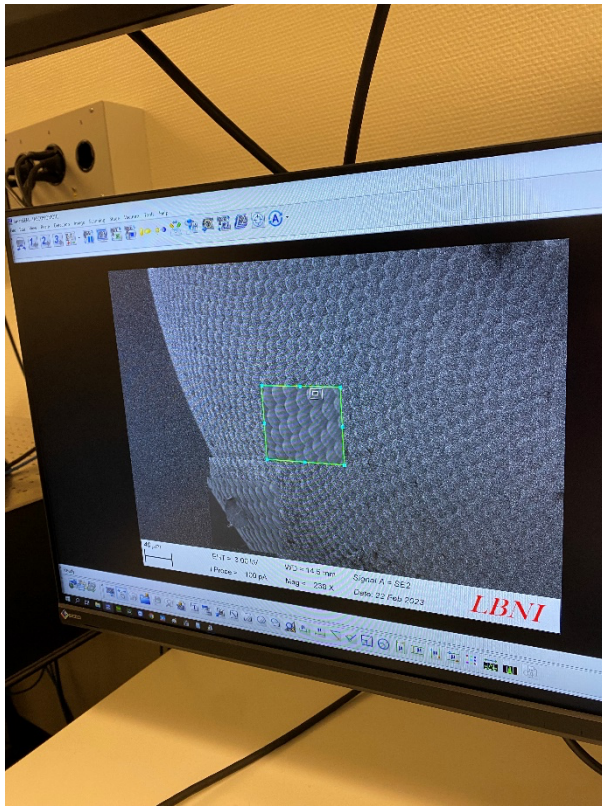
8. Set the probe current to 100 pA
9. In the Detector/ Active Channel section, select **Signal A = SE2** from the **Signal A** drop-down list. The SE detector provides a good SNR even at long working distances.
10. In the Crossbeam SEM Control panel, select the Imaging tab, and select scan speed = 3. This scan speed allows you to acquire images quickly, as the electron beam scans across the specimen.



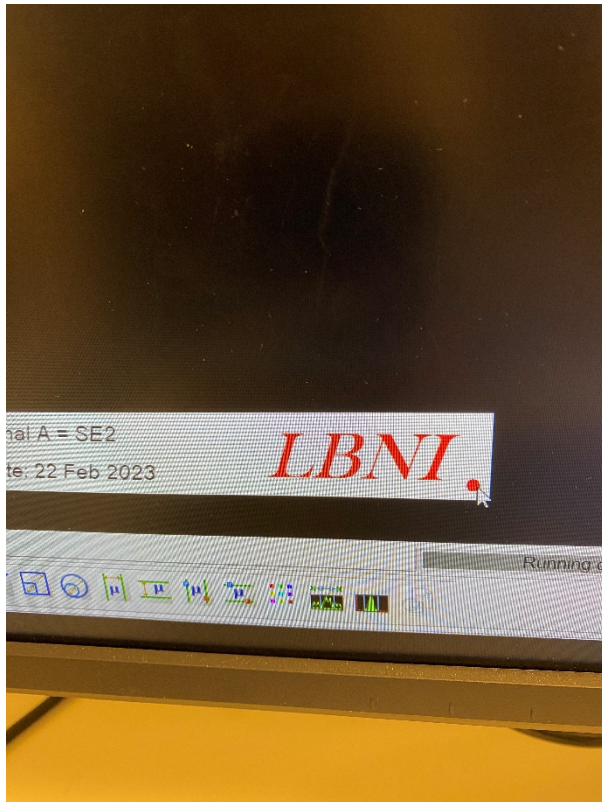
11. You can vary the magnification, and change the focus, contrast, and brightness in the control panel. You can correct for astigmatism through the stigmator x/y knobs, which shape the beam roundness by changing the stigmation deflectors.



12. To optimize the image quality quicker, you can also click the Reduced Raster/ Apertures icon. A small scan frame is displayed, which defines the specimen area to be scanned by the electron beam. You can focus the image in the reduced raster, and then exit the Reduced Raster mode.



13. To take an image, set the scan speed to 7 or 8.
14. Bring the image to focus.
15. Press 'freeze' on the control panel. A red dot on the right bottom of the Image Area indicated that the image is frozen.



16. From the Menu Bar, select File > Save Image.

### To investigate:

- Influence of the different beam and system parameters: current, acceleration voltage and WD.
- Explain the origin of the different image artefacts: charging, drifting, etc.
- Effect of topography on the different detectors: SE lateral, SE top, BSE.

### Task to do after imaging:

- Apply coloring filters (false coloring) to the different parts of the bee using Gimp software.

### EDX analysis of Au patterns on Si coated with Al

Materials:

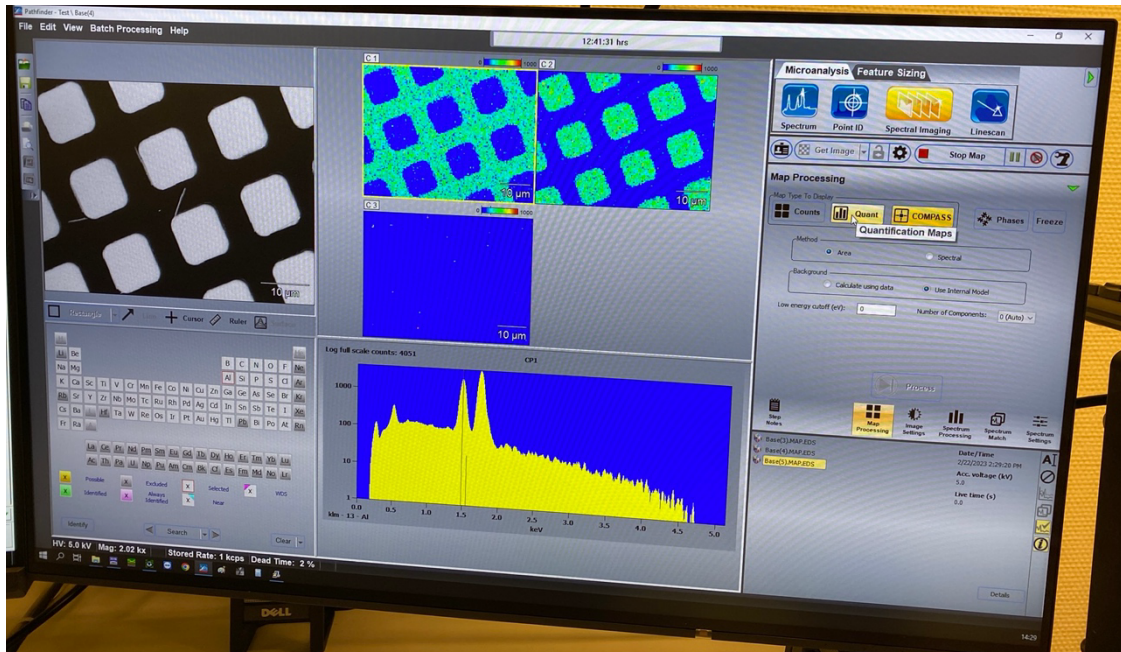
Au patterns on Si coated with Al, aluminium stub

Sample mounting and preparation

1. Mount the sample following the same steps used on the bee sample.
2. Open the Pathfinder software to characterize the different atom species on a patterned sample with different materials.



3. Acquire EDX spectra of the different areas (gold structures and aluminum surface), to compare them and be able to identify the different elements present. Compare the results using acceleration voltages of 5 keV and 20 keV.
4. Do EDX maps of the surface to identify different material phases.



5. Compare spectra with Casino software.

### To investigate:

- Influence of acceleration voltage on the EDX spectra.
- Influence of acceleration voltage on electron penetration depth using Casino software.

### Task to do after imaging:

- Plot the different saved EDX spectra using Python. Identify the energy peaks of the different elements present on the sample.