Phenom's current Delphi SEM and emerging technologies for imaging organic samples

M. van den Berg, 4594711 | R. van Casteren, 4320298 | A. Hoogenboom, 4548094 | O. Kuijpers, 4598415
B. de Leeuw, 4546113 | M. Middelbos, 4577450 | C. Ruegg, 4531906

CONTENTS

I. INTRODUCTION

This report discusses three different ways of making a magnified image of a sample with a very high resolution. The imaging is done with different kinds of electron microscopes. These electrons microscopes operate in quite a different way than light microscopes do.

The main advantage of using an electron microscope lies in the increased resolution. Yet, there are disadvantages to the electron microscope. In order to deal with these disadvantages, different microscopes have been developed. In the rest of the report three different microscopes are discussed, following the individual problems they account for. One of these microscopes is an already existing microscope; the 'Delphi'. The other two electron microscopes that will be discussed are upcoming technologies. These are called 'Cryo-SEM' and 'Environmental-SEM or ESEM'. All of these electron microscopes fall under the same kind of electron microscope: the 'scanning electron microscope' or SEM for short. This is why we will start off with a general explanation of the SEM itself. Subsequently, in chapter III the 'Delphi' will be discussed. Then, an explanation of the cryo-SEM will be added in chapter IV and finally the ESEM will be discussed in chapter V.

II. SCANNING ELECTRON MICROSCOPY

Most people who think of a microscope, think of a optical microscope. These use lenses to magnify a sample. The biggest problem with optical microscopes is that they cannot magnify past a certain point. When a sample is too small there is no way to magnify it using lenses in such a way that the human eye can see it. The SEM was designed to make images of samples that a regular microscope could not image with a magnification range from 20x - 30.000x. The main concept of a SEM is that it shoots a beam of electrons, which are negatively charged particles, on the sample. By analyzing the collisions between the electrons in the beam and the electrons in the atoms of the sample an image can be produced [1].

To produce an electron beam most SEM's use a thermionic cathode gun. This gun heats up a specific material, for example cerium hexaboride (CeB_6) which then shoots out electrons.These electrons are then focused by magnets to form a very precise beam onto the sample. By manipulating these magnets, the electron beam can be directed across the sample to scan the whole surface. [2]

As the focused electron-beam scans across the sample there are a lot of interactions that give off different signals. To create an high resolution image, we must detect/record as many of these signals as possible. Each different signals requires a different detector. [2]

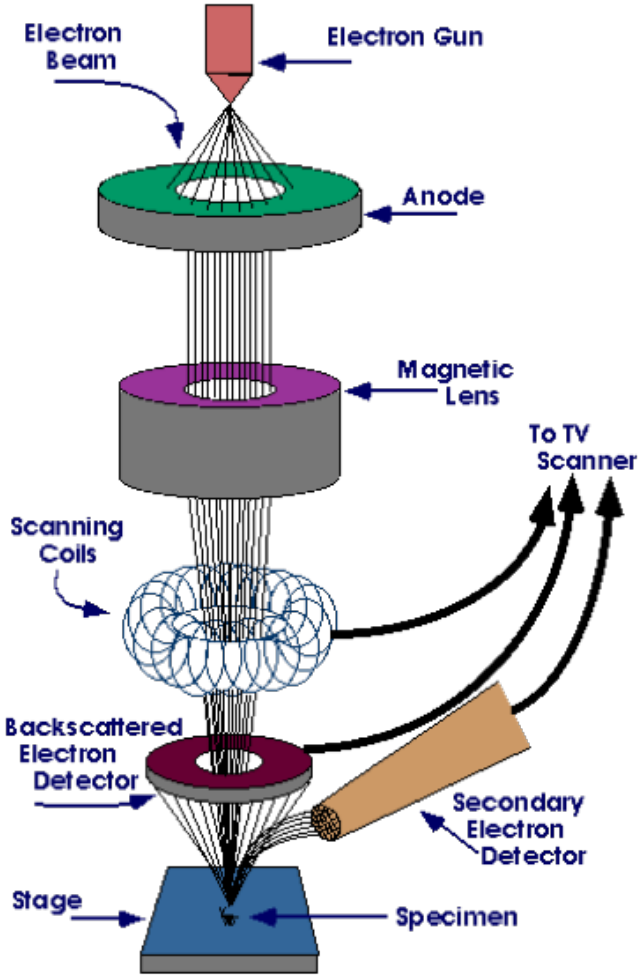


Fig. 1: Schematic components of the SEM [3].

A. Signals

1) Secondary electron emission: When the beam hits the sample, the atoms on the surface of the sample absorb the electron and emit an electron of their own. These electrons are imaged using a ”secondary electron detector”.

2) Backscattering electron emission: Some electrons of your electron beam get reflected by your sample either on the surface or deeper in the sample. These reflected electrons are detected by the backscatter detector. This detector is located on the end of the electron-beam tube because the backscatter electrons tend to reflect directly up off the sample.

3) Auger electrons and x-rays: When the electron-beam hits an atom in the sample, the electron composition of the atoms of the sample can change. If this happens an electron can be released from this atom. This is called a auger electron. Auger electrons can be usedto get information about the surface composition of your sample since the energy released by Auger electrons is characteristic for the material it is released from. Auger electrons are imaged by an Auger electron detector. Instead of auger electrons, it can also happen that x-rays are emitted from the sample. X-rays are electromagnetic radiation and have a characteristic energy for each element. By measuring this characteristic energy an elemental analysis of the sample can be made. X-rays are imaged with an x-ray detector.

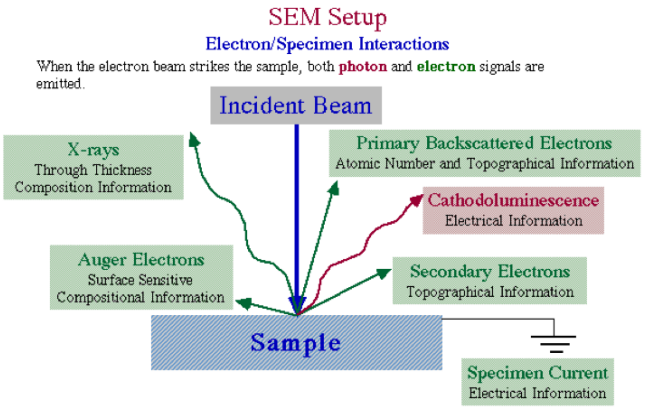


Fig. 2: Different signals coming off a sample. Cathodoluminescence will not be discussed since most SEM’s don’t use these signals [4].

B. Forming an image

Most SEM’s mainly use secondary and backscattered electrons to form an image. This is done by measuring the amount of electron signals on each point on the image relative to all other points. Each points measured is given a gray color depending on the electron intensity. The more electron signals the darker the color. By coloring each point we get a detailed image of our sample.

C. Limitations of signals

Although most of the signals described above are present, not all the signals are generally used. All these signals have limitation in what information they can give us.

1) Backscattered electrons and Secondary electrons: Both these signals can only be used to determine the surface structure but not much more. They don’t give information about the composition of the material. Another limitation is that, because you measure relative energy levels, the images acquired are all in greyscale.

2) Auger electrons: Auger electrons are only emitted on the surface of the sample and they do not have a lot of energy. Because of this they do not give a lot of information. A better way to get information of the composition of the material is x-ray analysis.

3) X-ray emission: Because x-rays are not detected with the detectors that are present in a normal SEM, you’ll need an additional detector to be able to do x-ray analysis. You also can’t use the x-ray’s to get detailed image of your sample.

D. Coating

Before a sample can be imaged it has to be coated. Coating enables or improves the imaging of a sample. There are a lot of different coatings and each is used in different situations.

E. Limitations

A SEM has to operate in a vacuum to work. Many materials exposed to a vacuum turn into gasses and biological samples will be destroyed. The sample chamber is usually around 100 x 40 x 40 mm so bigger samples have to be broken down. As mentioned earlier a SEM is also not able to produce colored images therefore most images are colored artificially. A SEM can also not image through water.

F. Desktop SEM

A regular SEM is a huge, bulky machine (see figure 3). Phenom World has created a solution to this with their Desktop SEM's. A Desktop SEM is a SEM the size of a regular desktop computer. It comes with a screen, keyboard and mouse (see figure 4) The huge advantage in this is that it is a lot cheaper and easier to use than regular SEM's. Phenom also designed their SEM's to be intuitive so there is no training required to use their desktop SEM. The trade off is that, because of the small form factor, a desktop SEM is not as accurate as a regular SEM [2].

Fig. 3: A regular SEM [5]

III. The Delphi

The Delphi (figure 4) is a small and user-friendly desktop microscope, which was released in 2014. The difference with other microscopes of this kind is that it features an additional microscopy technology called fluorescence microscopy (FM). The two integrated technologies are both particularly useful because they have different properties that can work together. For example, the black and white images made using a SEM are of very high resolution. The technique can distinguish between structures up to 14 nanometers in size [6]. This can be very useful for examining all the details in the surface of a sample, however it doesn't tell you anything about the chemical properties of structures in the pictures. Images made with FM have a considerably lower resolution, but do provide very specific information about the chemical composition of the sample. Combining these techniques results in a more relevant image, with which very specific components of a sample can be examined, while keeping track on the general structures.

Fig. 4: The Delphi microscope attached to a monitor [6].

A. Fluorescence Microscopy

The additional technology that the Delphi features besides SEM is called fluorescence microscopy. Specific molecules can be detected due to their fluorescence. These are given different colors and are overlaid with the image produced with the SEM. From the final combined image, it can be seen that there is a difference in composition shown between seemingly identical structures as would be seen on a SEM only image. Fluorescent molecules can be attached to other molecules of interest beforehand in order to show the specific location of them. For example, attaching fluorescent molecules to a bacteria to easily see it's behavior.

Fluorescence is the phenomenon where upon illumination with a specific frequency of light, molecules re-emit some of the received energy as light with lower frequencies [7]. It works as follows, light is shone on a nanostructure such as a molecule. The high energy photon is absorbed by an electron in the structure. It moves to a higher energy state and stays there for a very short time. While it is there some of the energy is dissipated due to vibration of the system. The electron falls back down to its ground state releasing a photon. This photon has lower energy and therefore a longer wavelength. This is also shown in figure 5.

Four different wavelengths of light are emitted by the LED. This light passes through the objective which focuses it on the specimen. Some of the light is absorbed, resulting in fluorescence, while some is reflected. This mixture of light wavelengths travels back through the objective, magnifying it. The light hits the filter cube as shown in figure 6. The light emitted by the specimen is allowed through while the light due to reflection is blocked. The light transmitted hits the sCMOS camera where an image is formed. The light from the different nanostructures are given different colors by the software before the image is displayed.

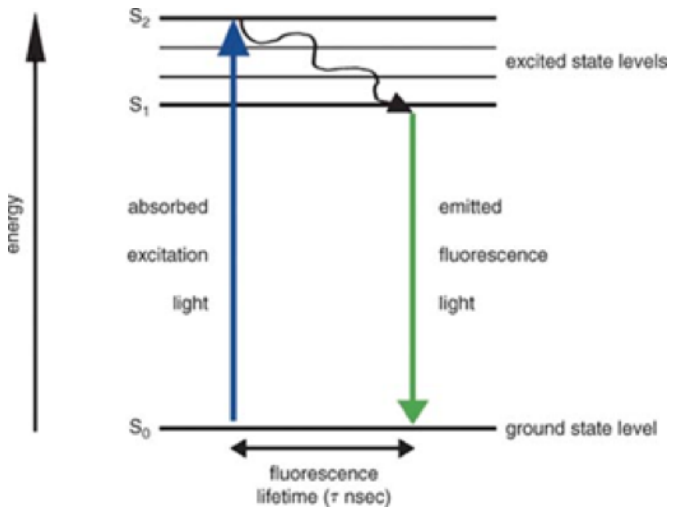


Fig. 5: A Visualization of the fluorescence effect with a Jablonski diagram [8].

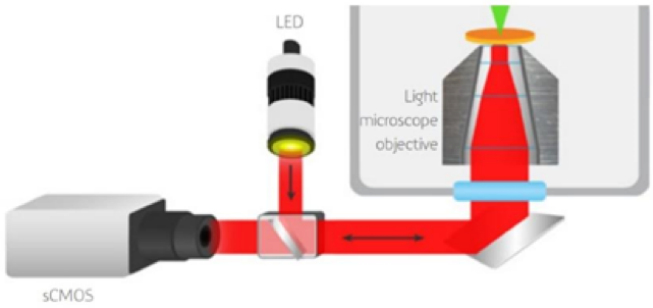


Fig. 6: Schematic representation of the fluorescence microscope from the Delphi [6].

B. Combining the images

The ultimate goal of the Delphi is to merge the images obtained from the SEM and fluorescence microscopy. The new combined image is more valuable because it contains more information about the examined specimen than the individual pictures. Figure 7 shows three pictures that together depict a part of the sample. The image on the left is created using FM techniques only, whereas the black and white image on the right originates from just SEM. Both pictures show information that the other one doesn't have. The image formed by the SEM shows all the detailed structures in the surface of the sample, however the chemical composition is unknown. With the FM image, only the fluorescent parts of the sample are shown. Blending the two pictures results in the image in the centre, which combines the relevant information from both techniques. For example, the large black spot seen only with the SEM actually contains different molecules, one of which is fluorescent, something that would have been unknown without the FM image.

The process of combining two pictures into one is done by the software used by the Delphi. The software is given information about which part of the SEM image corresponds to a which part of the FM image. The required information is gathered by the camera that captures the fluorescence image. It registers the position of the bright dot caused by the electron

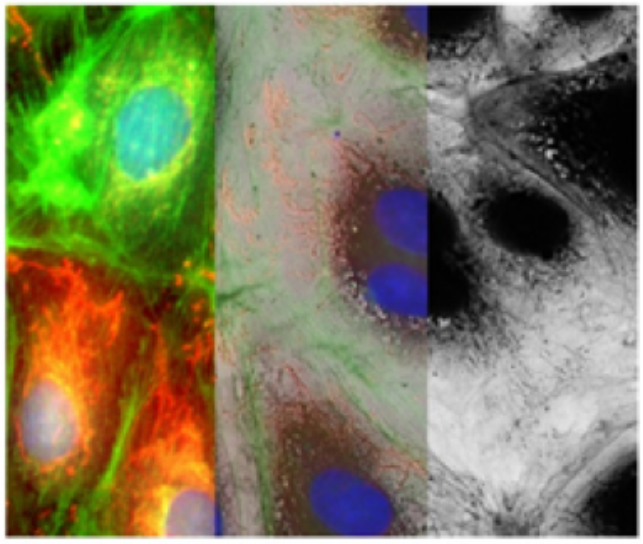


Fig. 7: Image created with the Delphi SEM. On the left: FM image, on the right: EM image of the same sample. The area in the middle shows the result of overlapping the two images [6].

beam hitting the surface of the specimen at a certain time. As a result, every pixel of the FM image now can be matched with a position on the sample's surface where the electron beam shortly interacted. This way the Delphi can guarantee successful, easy and objective image blending.

C. Sample preparation

The sample chamber, where the sample will be placed, is small. The maximum diameter a sample can have is 8mm. Before putting a sample into the SEM, two crucial steps must be taken in order to get the best possible image.

Most importantly, the sample chamber is in near vacuum. In a vacuum there are no particles present in the air and there is no pressure. This is necessary to avoid collisions between the electrons from the electron beam and molecules in the air. The microscope is sealed and a small electrical pump removes all the air. The strong vacuum makes it impossible to use a sample which contains any liquid. This is because at vacuum all liquids boil and evaporate very quickly. If this happens in something organic, like a piece of fruit, the gas produced inside of the sample destroy it.

Another crucial step is making sure that the sample is able to transfer electrons, it has to be electrically conductive. When the sample is not electrically conductive the free electrons which are shot at it will remain on the same point. When all these electrons are concentrated at the same place, a charge will build up. Due to this charged spot more and more incoming electrons will be deviated from their path as they are repelled by the charged spot. This will alter the image. The best solution to this problem is applying a very thin layer of a conducting material, usually a gold-palladium alloy, which can transfer electrons away from the sample [9]. These thin layers have to be applied very precisely and require a separate device called a sputter coater. This is done by atomizing the gold-palladium alloy, which essentially means making a 'cloud' of

metal atoms that evenly coat the surface of the sample. Once this has been completed, the sample can be imaged.

D. Applications

Due to CLEM the composition of the specimen, a mixture for example, can be well shown. For example in a mixture of fat and water, the fat can be given a tag with fluorescence which makes it easy to detect in the image. This is why it's most commonly used in material sciences. The object microscopic structure visualized on the microscope can be used to improve the structure of objects or substances. In the future the Delphi will also find application in education [10].

E. Limitations

Having a small and portable desktop microscope has a downside. Ideally, a microscope is as big as possible since the maximum obtainable resolution increases with the size of the microscope's components. That is why extremely big telescopes are used to observe the outermost places in space, as they need to obtain very high resolutions. Another problem is that the actual act of taking a photo using fluorescence microscopy, alters the composition of your sample. This is called photo bleaching and it occurs when reactive fluorescent molecules, excited by the light of the led, react with molecular oxygen inside the sample. If enough molecules undergo photobleaching, previously highlighted areas will not be visible anymore, causing loss of data.

When taking a photo of specimen using FM, higher intensities of light usually result in better quality pictures. The intensity of the re-emitted light from the specimen is reduced by the microscope's own components, such as light filters and lenses. Since these components are essential for proper functioning of the microscope this limitation is hard to deal with. Using higher quality components will reduce intensity loss to some degree, but is considerably more costly.

IV. Desktop cryo-SEM

As was told in the explanation of the SEM, samples have to be kept in a vacuum in order for the electron beam to be as effective as possible. Yet, there is a large limitation associated with this vacuum; biological samples cannot withstand such harsh environments (very low pressure). In order to be able to image these organic samples, a different technique is required. One possible way of scanning biological samples is the cryo-SEM technique, where the sample is frozen and properly positioned (cryofixated) in order to image it better. A SEM requires a sample to be coated in metal, which means that the SEM is unable to operate with wet or moist samples. By freezing the sample wet or moist samples can be operated with.

A. How a cryo-SEM works

To get a better idea of how a cryo-SEM works, it's helpful to look at figure 8:

As seen in the cross section, the cryo-SEM consists of a preparation area and a scanning area. It's easier to have them connected as one piece, since both the preparation and scanning occur at a high vacuum (an environment that is very close to a pure vacuum).

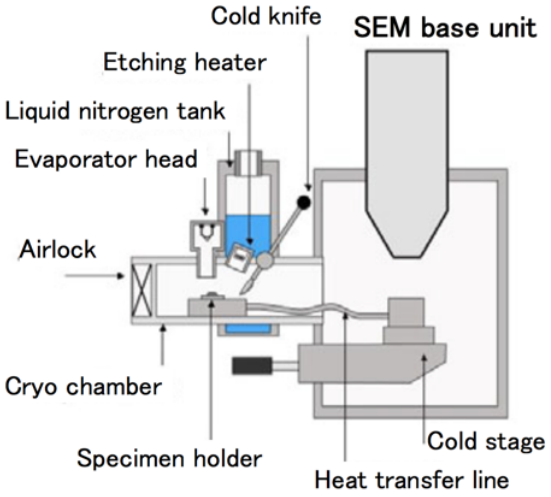


Fig. 8: Cross-sectional view of a typical cryo-SEM setup [11].

The preparation process occurs in 3 places: outside of the chamber for the preliminary freezing, inside the chamber for cutting sample and the etching process, and finally the imaging stage. These steps can be seen in the diagram below:

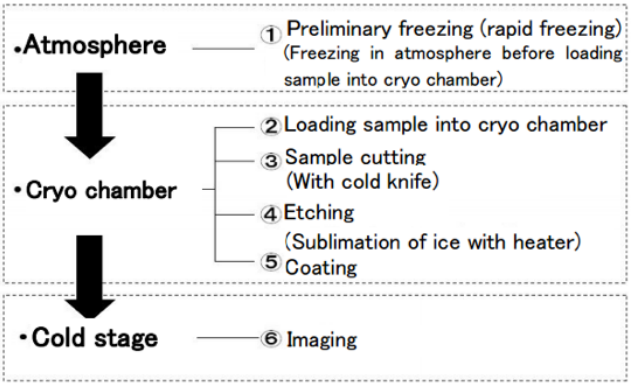


Fig. 9: Steps in the cyro-SEM preparation process [11].

First, the sample is frozen with liquid helium or nitrogen. This is done in order to prevent the sample from collapsing due to the vacuum. The freezing is done as rapidly as possible to prevent the formation of ice crystals which could damage the sample, and to make sure the sample is easier to work with for the following steps. Ideally, as much water as possible is first replaced with glycerol or sucrose, which do not form ice crystals. Once the sample has been fully frozen, it is loaded into the cryo-SEM preparation chamber. There, the sample is cut to the desired size to fit the scanning area. If the researcher wants to view a cross section of the sample, a technique called freeze fracturing is used. The sample is split in half such that internal structures become visible. This can be seen figure 10:

Once the sample is fully cut and split to the ideal dimen-

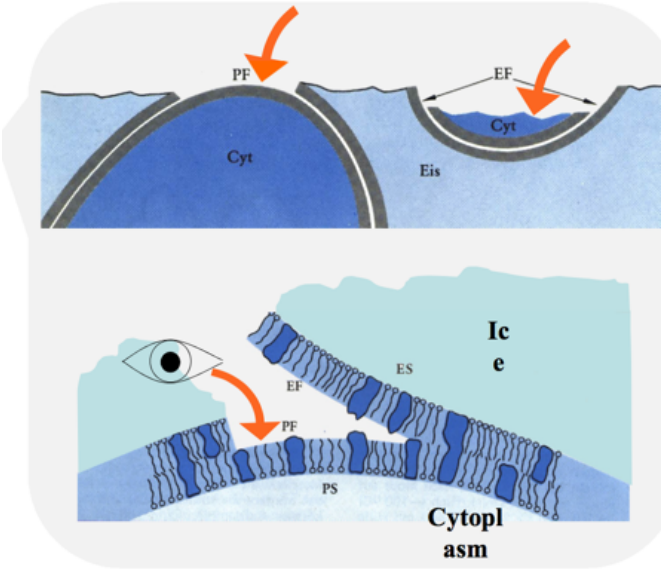
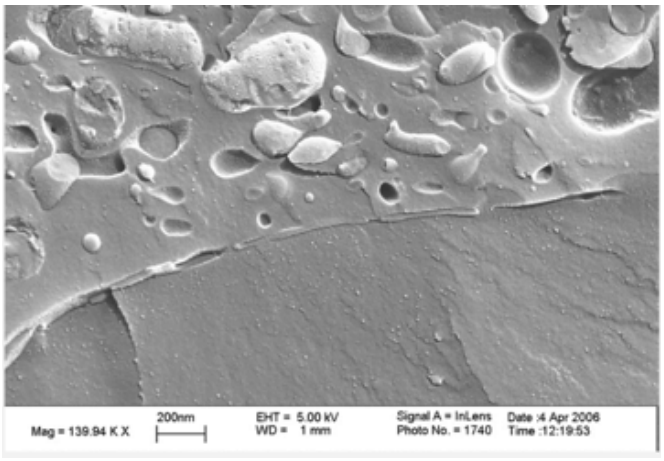


Fig. 10: Freeze fracturing of a sample [12].

sions, the remaining ice is removed through a sublimation process. Sublimation is when ice turns into water vapour without ever reaching the liquid stage. This is crucial for the imaging process, as otherwise the frozen water would block the SEM from imaging critical details. This can be seen in figure 11 below:



After this is complete the sample still needs to be coated to prevent electron buildup.

B. Limitations

Biological samples must be rapidly frozen in order to prevent destructive ice crystals from forming in the structure. There are two main reasons that could cause the sample not to freeze fast enough. Either the sample isn't cooled sufficiently, or the sample is just too big to cool down in a small time period. Different materials absorb and lose heat at different rates, furthermore, the larger and object the more time it takes to heat or cool down. The longer it takes for cooling, the larger the possibility that ice crystals will form. As a result, the sample may not be more than a few micrometers thick for cryofixation to work properly. The liquid substance, surrounding the specimen, has to cool the specimen fast. So there needs to be enough cold liquid available.

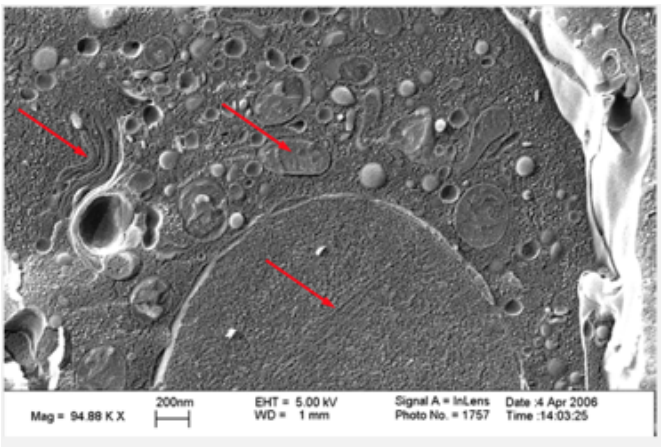


Fig. 11: Photo on the left shows a Vero cell imaged without sublimation, and the image on the top shows the cell with sublimation occurring. Note the increased detail on the top image [12].

There are other reasons why the liquid may not cool the sample enough. When liquid nitrogen is used, its temperature lies just below its boiling temperature. If it absorbs heat from the specimen, the liquid will boil and form a layer between the specimen and the liquid, making it harder for the specimen to give off heat. This is called the leidenfrost effect [13]. For the leidenfrost effect not to take place, the liquid needs to be cooled down even further. To cool the liquid down, an additional device is required. This costs money and time.

C. Applications

In the introduction a brief explanation of the use of the cryo-SEM was included. In most cases the cryo-SEM is used for scanning of biological or organic samples. For this reason the applications mainly fall within the areas of biology and life sciences. A frequently cited article regarding current application of cryo-SEM is the article: Bacteria Viability in Sol-Gel Materials Revisited: Cryo-SEM as a Suitable Tool To Study the Structural Integrity of Encapsulated Bacteria [14]. This article describes the benefits of the use of a cryo-SEM when observing the structure of encapsulated bacteria.

Additionally, cryo-SEM's can also be used to identify emulsions and suspensions in the petroleum industry [15]. Emulsions and suspensions are different kind of mixtures relevant in the oil industry. Ultimately, the technology of desktop cryo-SEM enables high-resolution imagery of organic and biological substances in small, average work spaces for any researcher. Additionally the desktop aspect could reduce company costs of using SEM technology, increasing the availability of this SEM technology for companies and research facilities with smaller budgets.

V. ESEM

The Environmental Scanning Electron Microscope, ESEM figure 13 is in many ways similar to a regular SEM, figure 12.

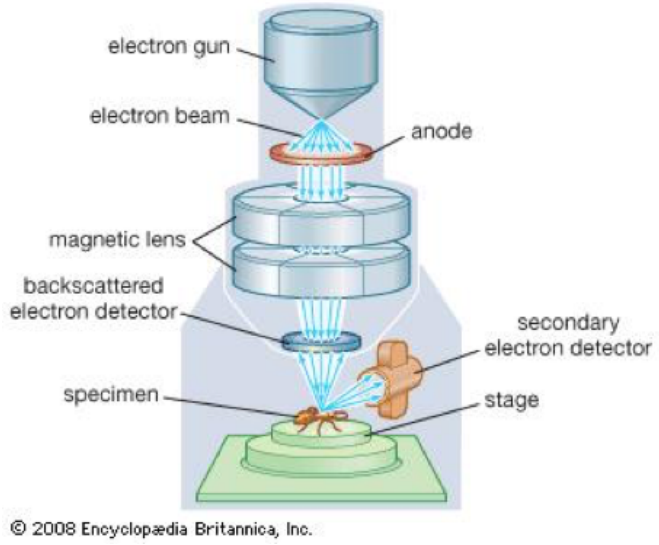


Fig. 12: Simplified Diagram of a SEM [16].

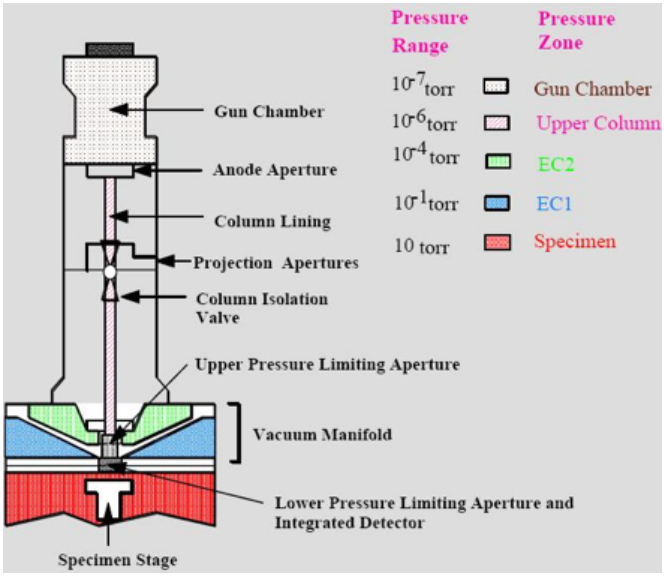


Fig. 13: Cross-Section of an ESEM [17].

The biggest difference is the fact that a SEM operates under a high vacuum condition, while the ESEM works in a high-pressure gaseous environment. The process of imaging in an ESEM does not differ all too much from the imaging in an SEM. In an ESEM, the techniques that are used to create an image are adapted to function in a gas in the sense that the gas itself is able to function as a detector.

In order to examine a specimen, a sample is placed in the gaseous environment. The electron beam is generated as in a SEM. The electrons are accelerated and focused similarly. However, nearing the sample, the pressure is increased. This is done in compartments with a pumping system which stays on during the length of the experiment. Leaving just the last part of the column at high pressure reduces the number of collisions between electrons and air molecules, improving results. However, resolution is obviously lost compared to a SEM in high vacuum [18] [19].

The electron beam carries a negative charge, while the gas surrounding the sample carries a positive charge. This enables the beam to move in a straight line. Once the electrons reach the sample, they bounce back and are detected as secondary or backscattered electrons using a gaseous detection device. This provides us with an image of the sample.

A. Applications of the ESEM

1) Biological applications: The ESEM enables researchers to experiment with biological samples with precision. It is, for example, well known for the imaging of micro-organisms. The images created by the ESEM show all parts of animal and plant cells with great detail. Thus, the ESEM is perfect for organic research on a very small scale.

2) Archaeological applications: The ESEM enables archaeologists to examine magnified images of material on microscopic scale. For example, in the case of the five thousand year old skin of Iceman Ötzi, an ESEM can be used to determine the age of the skin.

For a long time, archaeologists had no other choice than to use magnifying glasses to examine all kinds of materials, including hair, bone, skin, teeth, fingernails, plant remains, fibers, insects and so on. The great advantage of the ESEM is that it provides information on samples without damaging them.

3) Other applications: The ESEM is found to be useful in for instance: Drug research, Virology, Toxicology, Nanometrology and food science [20].

B. Limitations

Although the ESEM does enable researchers to examine all kinds of material, its technology does come with some limitations

The gas present in the ESEM has to have certain properties, otherwise the loss of electrons increases. Researchers have found that the gas used in the ESEM has to be carefully picked out in order to match its density and the pressure in the chamber in a way that does not affect the experiment.

Another disadvantage of the use of an ESEM is the fact that its possible magnification is limited.

Furthermore, problems can occur when dealing with wet samples. If the surface of the sample is wet, contrast issues with the image can arise, meaning that small details are harder to distinguish. This is caused by condensation, or a small layer of water droplets, on the sample. Other than the decreased contrast, the presence of water can confuse the topography of a sample.

REFERENCES

[1] Swapp, S., University of Wyoming, "Scanning Electron Microscopy (SEM)" retrieved on 4 march 2017 from http://serc.carleton.edu/research_education/geochemsheets/techniques/SEM.html.

[2] "How Does an Electron Microscope Basically Work?", The Fellows of Harvard College, Harvard Medical School,http://bsp.med.harvard.edu/node/221. Accessed: 4 march, 2017.

[3] Purdue University, schematic SEM [digital image]. Retrieved on 4 march 2017 from https://www.purdue.edu/ehps/rem/rs/graphics/sem2.gif.

[4] Beam interactions [digital image] retrieved on 4 march 2017 from http://www.hit.ac.il/staff/lapsker/sembeam_interactions.html.

[5] Scanning Electron Microscope [digital image] retrieved on 4 march 2017 from https://nanofabrication.unt.edu/sites/default/files/jeol.jpg.

[6] Phenom-World B.V. (2016) *Delphi specification sheet, Brochure.* Retreived on the 3rd of March 2017, from https://www.phenom-world.com/microscopes/delphi-integrated-clem-microscope/download.

[7] Abramowitz, M., et al. (2012). *Anatomy of the Fluorescence Microscope*. Retrieved 3rd of March 2017, from http://www.olympusmicro.com/primer/techniques/fluorescence/anatomy/fluoromicroanatomy.html

[8] David Lls. (2007, November). Fluorescence Fundamentals [Digital image]. Retrieved March 3, 2017, from https://www.researchgate.net/publication/23236997_Detecting_Protein-Protein_Interactions_In_Vivo_with_FRET_using_Multiphoton_Fluorescence_Lifetime_Imaging_Microscopy_FLIM

[9] Suzuki, E. (2002). "High-resolution scanning electron microscopy of immunogold-labelled cells by the use of thin plasma coating of osmium". Journal of Microscopy. 208 (3): 153?157.m]

[10] Phenom World. (2016). Phenom Markets. Retrieved March 03, 2017, from https://www.phenom-world.com/markets

[11] JEOL. (2011). *Application Data Sheet: Introducing Cryo Scanning Electron Microscopy*. Retrieved from http://www.jeol.co.jp/en/applications/pdf/sm/469_en.pdf

[12] Lin Zhenju, Leica. (2014). *Cryo-immobilization techniques for biology*. Retrieved from https://goo.gl/6vehAX

[13] Eshuis, P., Weele, K. V., Meer, D. V., Lohse, D. (2005, December 15). Granular Leidenfrost Effect: Experiment and Theory of Floating Particle Clusters. Retrieved March 03, 2017, from http://journals.aps.org/prl/abstract/10.1103/PhysRevLett.95.258001

[14] Ferrer, M. L. (2006). Bacteria Viability in Sol?Gel Materials Revisited:? Cryo-SEM as a Suitable Tool To Study the Structural Integrity of Encapsulated Bacteria. Retrieved March 03, 2017, from http://pubs.acs.org/doi/abs/10.1021/cm0522275

[15] Mikula, R. J., Munoz, V. A. (2000, November). Characterization of emulsions and suspensions in the petroleum industry using cryo-SEM and CLSM. Retrieved March 03, 2017, from http://www.sciencedirect.com/science/article/pii/S0927775700005185

[16] Encyclopedia Britannica. (2008). Scanning Electron Microscope [Digital image]. Retrieved March 3, 2017, from https://media1.britannica.com/eb-media/88/113688-004-B14FDB14.jpg

[17] University of Cambridge. (2016). ESEM [Digital image]. Retrieved March 3, 2017, from http://www.phy.cam.ac.uk/research/research-groups-images/bss/images/esem.jpg

[18] Environmental scanning electron microscopy (esem). URL https://xos.com/technologies/sem/environmental-scanning-electron-microscopy/. Accessed on 29-11-2016.

[19] A.Bogner, P.-H.Jouneau, G.Thollet ,D.Basset ,and C.Gauthier. *A history of scanning electron microscopy developments: Towards ?wet-stem? imaging*. 2007.

[20] Ellery Frahm. Scanning Electron Microscopy (SEM): Applications in Archaeology, pages 6487?6495. Springer New York, New York, NY, 2014. ISBN 978-1-4419-0465-2. From http://dx.doi.org/10.1007/978-1-4419-0465-2_341