

# Operation Guide

## Hitachi S-3400N

Variable Pressure Scanning Electron Microscope

with

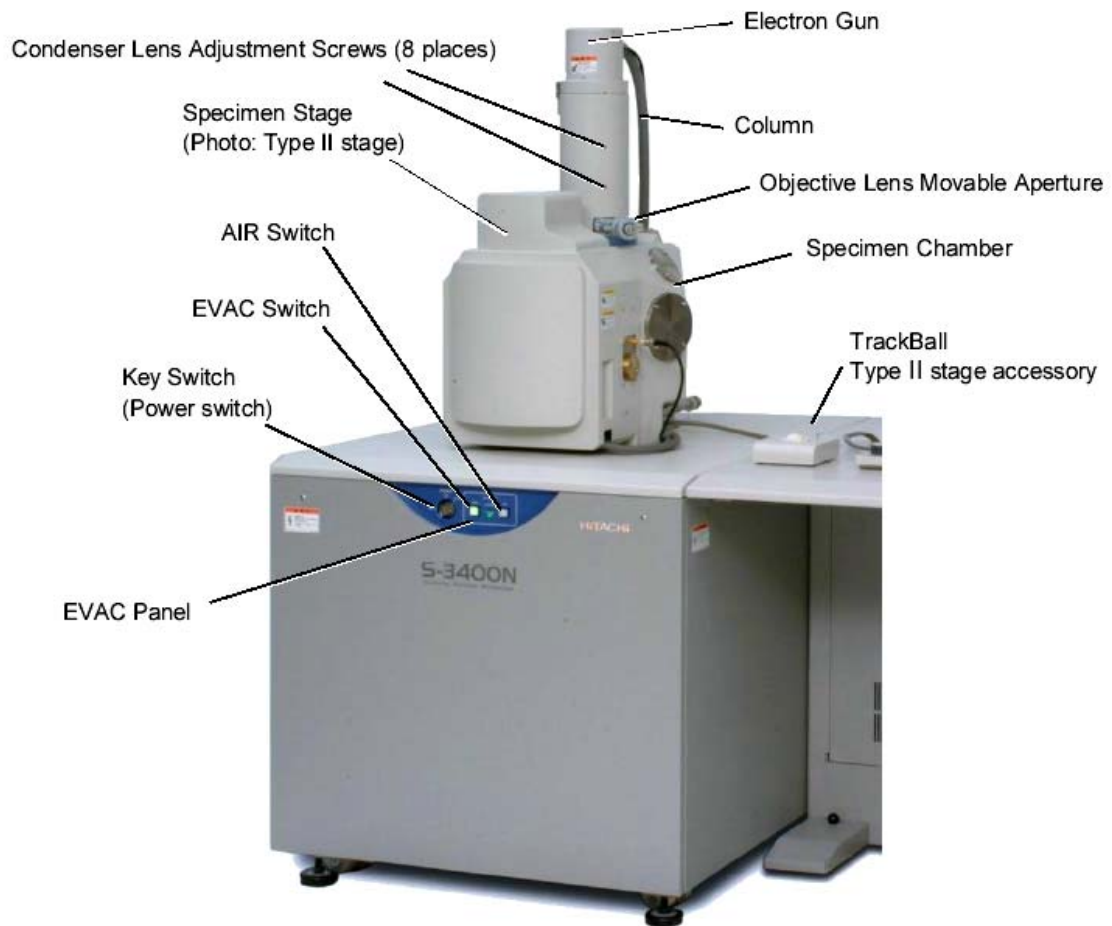
Deben Peltier Coolstage



# Index

<a href="#"><u>Main Unit</u></a>	3
<a href="#"><u>Electron Optical Column</u></a>	4
<a href="#"><u>Starting</u></a>	5
<a href="#"><u>Quick Buttons</u></a>	7
<a href="#"><u>Basic operation</u></a>	8
<a href="#"><u>Shut down</u></a>	8
<a href="#"><u>Advanced operation</u></a>	9
<a href="#"><u>Using the Deben Coolstage</u></a>	10
<a href="#"><u>Operating variables</u></a>	11
<a href="#"><u>Sample preparation</u></a>	12
<a href="#"><u>Sample stubs</u></a>	13
<a href="#"><u>Quantomix</u></a>	13
<a href="#"><u>Useful basics</u></a>	14
<a href="#"><u>Literature</u></a>	16

# 1 Main Unit



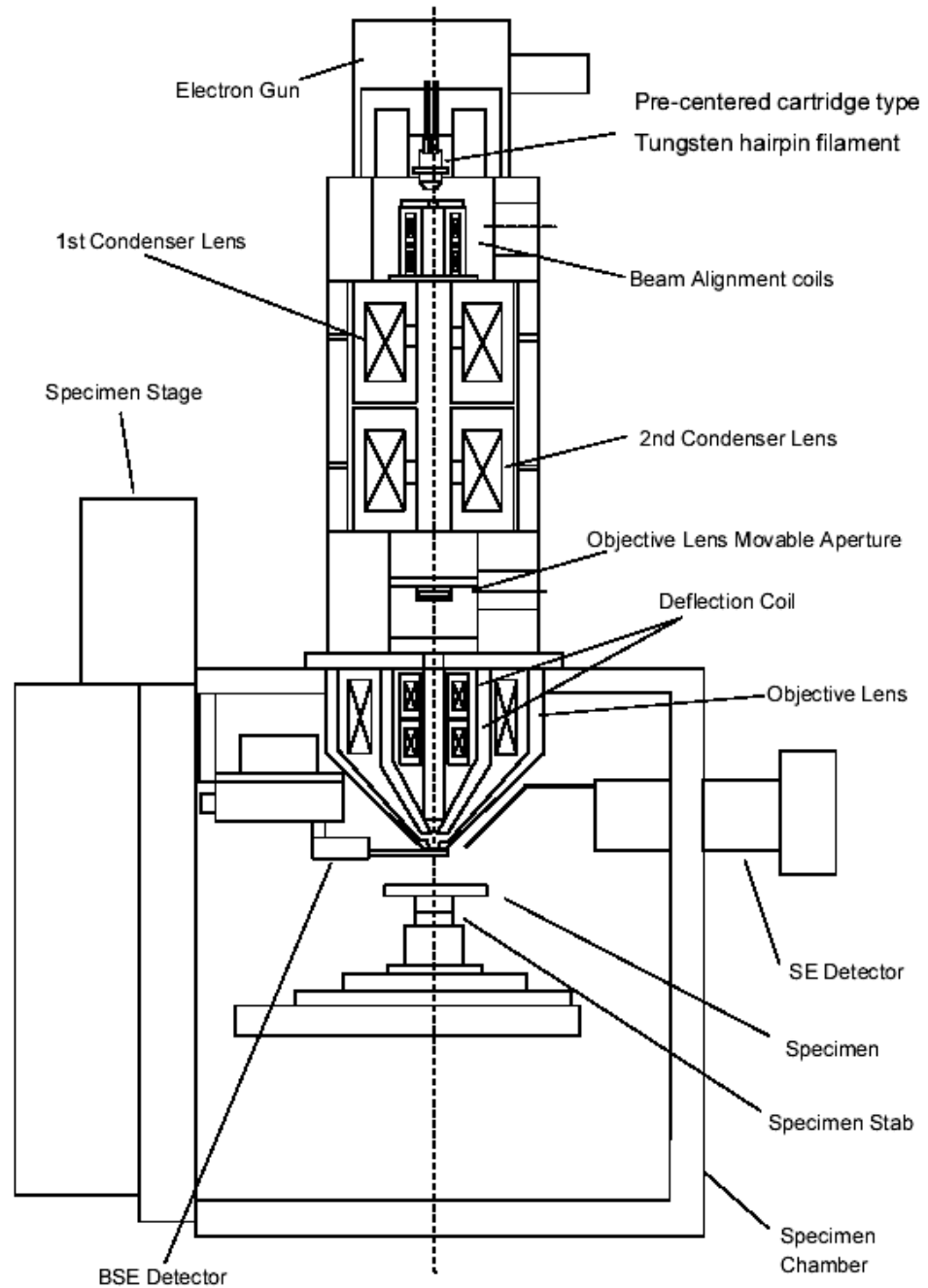
**Fig. 2.1-1 External View of the Column (Type II)**

**NOTICE:** Do not touch the Condenser Lens Adjustment Screws (8 places).  
The position of the condenser lenses adjusted shift, and the first performance might not be obtained.

## The S-3400N main unit

(from Hitachi Instruction Manual: Operation/Maintenance Edition)

## 2 Electron Optical Column



### Sectional view of the S-3400N Column

(from Hitachi Instruction Manual: Operation/Maintenance Edition)

### 3 Starting:

#### **WHEN YOU REACH THE SEM**

- The SEM and computer will be ON
- The SEM Vacuum will be on (EVAC green light )
- Log in with your username and password
- Open the PC-SEM program (no password required for registered logged-in users)
- A dialogue box pops up asking for sample information, which has to be set before continuing with SEM operation

#### **CHECKPOINTS:**

- Make sure the sample stage is at **Z65** home position (upper-right corner, the blue marker before the Home Z65)
- Make sure the High voltage is **off** (upper-left corner, the blue marker before the OFF button, i.e. current = 0)
- Make sure the accelerating voltage (Vacc) is set at 5kV (this will be at the value set by the previous user, and not at a default low Vacc)
- Air the chamber (upper-right corner, **AIR** button)
- Wait for the air in and open the door (a couple of minutes, should hear a bleep sound)

#### **LOADING YOUR SAMPLE**

- Measure the size (height and width) of your sample with the white measuring tool and set it on the pop-up screen. The size is the largest diameter of the sample cross section. Height is the value you just measured using the white tool. When using the coolstage, the height should be measured after inserting the specimen in the stage.
- The system will ask you to confirm these dimensions to make sure no physical damage is done to detectors and lenses inside the chamber.
- Place your sample, and close the door
- Switch on the IR chamber camera to see the sample inside the chamber (ON/OFF button, and POWER button on small screen – only for SEM mode with SE detector -. switch off the camera when going to BSE detector, or when switching on the filament in VP mode.)
- Move mouse to side of screen to allow control panel to pop up with Condition, Image, Utility and Stage Tabs
- Select VP-mode or SEM **NOW** to prevent wet samples to go under high vacuum.
- Evacuate the chamber (upper-right corner, **EVAC** button) Vacuum will be <1Pa in SEM mode, and up to 270Pa in VP-SEM mode

#### **SCOPE OPERATION**

- Wait until the vacuum is ready (the ON button at upper-left corner is activated and turns black; it could take up to several minutes). In VP mode the selected pressure is indicated in Pa
- In Stage screen, click on MultiHolder when using the 4 stub holder, and select stub to be viewed. Click GO to automatically move to that position.

- Click on Stage in control box, and move your specimen to the required height (start with 20mm, click on GO). Wait and check the image on the IR chamber scope (see below).
- Repeat height setting to reach optimal working distance for imaging.
- Select Mouse Button Controls (select Options tab at top of screen) for stage movement, contrast & brightness, astigmatism
- Select proper Voltage conditions under Condition Tab (5-15-30kV are 'sweet spots' with highest emission rates for best resolution)
- Select proper Probe Current (start with value 50, which will allow a wide electron beam to reach the sample. This is equivalent to 'spot size' in other microscopes, and determines the resolution and depth of field - more important at high magnification)
- Select a proper Aperture (2 or 3 for low to medium magnification), externally on the column by turning in the correct aperture. Alignment is done when viewing the sample
  
- Turn ON Beam (click ON, upper left corner). If using BSE, switch off IR camera
- Use TV mode for viewing with SE, and slow scan (3 or 5) for BSE mode or for imaging
- ABCC to adjust image brightness/contrast
- AFC to adjust focus (focus manually on samples with low contrast)
- Adjust working distance once specimen is in focus. Minimum in this system is 5mm. For the multi-sample holder (4 stubs) the working distance will be limited by the height of the highest sample
- Click on 'Detail' in the Condition window to adjust the Emission Current. This is done by slowly adjusting the Gun Bias
- Align aperture mechanically at low magnification for brightest image (turn 2 knobs on column to align bright spot), while using high probe current (50 e.g.)
- Zoom in on area and focus at higher magnification (10 000x), using an appropriate spot size and working distance
- Align aperture electronically
- Correct for astigmatism
- Repeat alignment and astigmatism correction at higher magnification (3-4x working magnification)
- This set of conditions will be saved by the system for your session, even if you move to another set of parameters at a different aperture (which will then also be saved after you optimized conditions)
- Always focus at a higher magnification than your intended image will be taken. Your zoomed down image will be fine-focused for digital enlargement.
- Insert your image details under 'Settings' (3<sup>rd</sup> button from left, or under Image Detail in drop down menu on right)
- Your image is a play-off between beam current (kV), probe current and aperture (1-4) – and further influenced by astigmatism, beam alignment, emission current and contrast/brightness.
- Image: focus at high magnification, check alignment and astigmatism, select size and exposure frame time on upper Quick button (M). Capture 20 to 80 sec.

## Quick buttons



- Freeze: freeze the image after a scan. Click Run to unfreeze again after capturing and image
- TV1/2: to observe instant image = not to be used with BSE detector (in VP or High Vac SEM mode)
- Fast1/2: for fast scan, unstable samples, and with BSE detector
- Slow3/5: slow scan, better resolution, preparation for image capture
- Red1/2: reduced imaging area, for focusing purpose. Use Red3 for BSE detector
- ABCC: auto contrast and brightness; use it whenever you make changes (Mag, kV, etc.), or when starting viewing a new sample
- 640/1280: capture the image using preset conditions (40 or 80 second capture)
- PCI: transfer the captured image to PCI image management program
- SAVE: save the frozen or captured image directly into pre-selected folder

## Image Saving (quick buttons)

- Freeze and Save
- 640/1280 and PCI: pre-settings can be changed by right clicking
  - By frame: integration scan; used for unstable (sensitive to beam) samples
  - By time: line scan; used for stable samples
- For stereo images capture 2 images tilted eucentrically at x degrees. Open both at the same time in Quartz PCI. Under Process tab, select the Stereo Pair command and pick left and right images from the drop down list, as well as the red/green or red/blue stereo image you want. Click on the Align tab on adjust the alignment of the stereo pair with X and Y sliders. Click OK to obtain a new stereo image with the intersecting region of the original images.

## Ending session

- Decrease Vacc gradually to 5kV, and switch off
- Move stage to Home Position Z65
- Air the chamber (not less than 30sec after turning HV off)
- Take out your sample
- Close chamber door
- EVAC the chamber
- Turn off the IR detector (power off)
- Turn off the coolstage if it has been used
- Remove and Clean your stubs, or keep samples in the desiccator

**NEVER Shut down the PC computer controlling the SEM**

## 4 Basic operation

### IR chamber scope

- Push the ON/OFF button on the scope and screen to turn on the IR chamber scope. The chamber scope **cannot** be used during backscatter imaging or X-ray analysis.

### Imaging

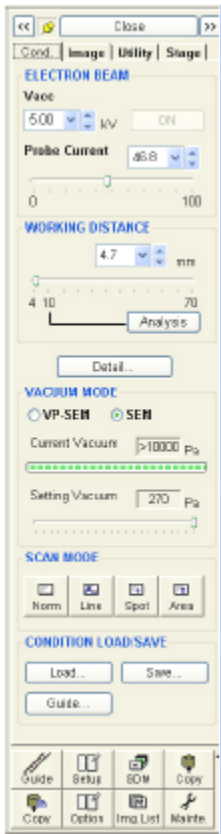
#### Operational panel with knobs and trackball

- Magnification: based on requirements for your sample
- Focus: find the sharpest image
- Astigmatism: find the sharpest edge at high magnification
- Brightness and contrast
- Image shift: move image on the screen by moving the beam (not the sample stage). Only to be used at ultra-high magnification.
- Trackball: moving sample stage

### Control panel and quick buttons

Frequently used functions are all listed on the control panel or by quick buttons.

Description of all functions in the PC-SEM program can be found in the Hitachi Instruction Manual. Listed below are basic ones that are useful to know.



#### Control Panel →Condition Tab

Turn on voltage: 0.1kV~30.0kV; if >25kV, turn on 25 kV first and increase to 30kV by 1kV increment.

High voltage gives better resolution

Low voltage gives better surface detail and reduces surface charges.

Start with 3 – 5kV.

#### → Image Tab

Select Full screen or Small screen, or Dual screen with SE and BSE

#### → Utility Tab

Select Data Entry on image (measuring distance between two points)

#### →Stage Tab

Change the working distance (**WD**): Short WD gives better resolution. Use 5 mm for non-tilt sample.

Rotation: click the eucentric to keep the center while rotation

Tilt: The range of tilt is determined by the sample size

Navigate: select an area to be viewed, and move it to the center

Memory: Indicate coordinates of selected areas to be viewed. Store in memory to be accessed on demand

**DO NOT USE ROTATE AND TILT FUNCTIONS WITH COOLSTAGE**



## 5 Advanced Operation

All basic instructions can be learnt from the SEM-PC menu, or the Hitachi Manual.

**Aperture alignment** Decide on a suitable working distance (e.g. 15-20 mm). Focus at 500x magnification. Adjust X and Y aperture controls manually until brightest 'spot' is obtained. Repeat at higher magnification and suitable working distance and probe current (spot size) by electronic setting adjustment on operational panel

- **Quick Beam Alignment**

- Quick buttons → Align

- Do Auto

- Always check at high mag (>20 000x))

**Image Astigmatism** For high resolution images this alignment is critical, as its effect can be seen as a general loss of image sharpness, to distortion of geometrical features of the object. This can be corrected with the two stigmator controls.

Select a suitable magnification (20 000x, astigmatism is not seen at low magnification)

Select and adjust spot size (probe current) compatible with high resolution

Focus using Focus controls

Click on Align Tab

Refocus using Astigmatism controls

Focus using Focus controls

Repeat to obtain sharpest image.

No 'linearity' of features should be seen when going through focus. When using higher magnification, repeat procedure at higher magnification than intended to photograph image.

### **BSE mode**

Control panel → Image Tab → BSE mode

Switch of IR camera in BSE mode

Can use Dual mode to view BSE image and SE image simultaneously

BSE mode gives better compositional contrast, and is the only mode allowed under variable pressure.

Use higher voltage (15Kv – 30kV) for stronger signal in BSE mode, as these electrons are scattered from deeper layers in the sample

### **VP mode**

Control panel → Cond Tab → VP-SEM mode

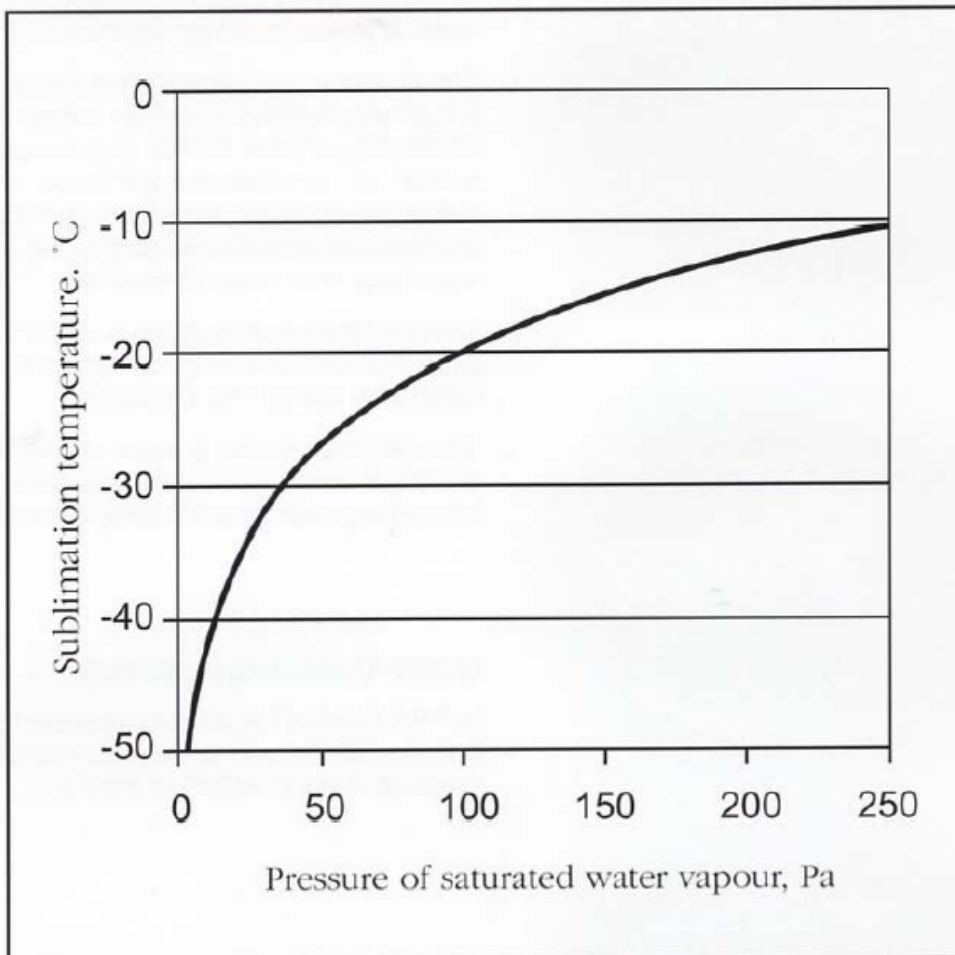
Select VP mode before evacuating the chamber with your sample

The maximum is 270 Pa

VP mode is for hydrated biological samples

## 6 Using the Deben Coolstage

- The MK3 Coolstage is permanently connected to the Hitachi SEM, and will be at ambient temperature, as indicated on the control panel
  - The Peltier cold head is fragile and must be handled with care for optimal performance
  - All functions are controlled from the small keypad controller
  - Switch on the chiller unit 30 minutes before use, allowing time for the cooling water to be chilled to 8C below ambient temperature, which will enable immediate cooling of the specimen to the required temperature, and stabilization of the entire system.
  - Switch ON the coolstage by pressing Auto.
  - Insert your specimen
  - Select your temperature by moving down (or up) with the arrows
  - Measure the size of your sample with the white measuring tool and close chamber door
  - Open PC-SEM program and indicate the size of your sample in the pop-up dialog box
  - Proceed in VP-SEM mode
  - When looking at wet samples, cool the specimen holder before pumping the chamber
  - An initial amount of experimentation is required to optimize timing for your sample
- CONTROL EVAPORATION FOLLOWING THE GRAPH CORRELATING SUBLIMATION TEMPERATURE AND PRESSURE OF SATURATED WATER VAPOUR



**Imaging is done in photo mode** to ensure that no image shift is seen on the final image.

- At the required pre-selected temperature, press photo button and the current through the Peltier device will be fixed. A slight change in temperature (0.1C) may occur
- Press Auto button after taking the photo, to switch SCU back to control temperature.
- DO NOT press while the temperature is still increasing or decreasing, as the Peltier temperature will be fixed at this level, and not reach the desired level.

**Command Summary:**

<b>Off</b>	Switches Peltier device off (cooling or heating)
<b>Auto</b>	Sets controller to control the sample temperature – use up/down keys on controller
<b>Goto</b>	Enter desired temperature. Delete key acts as minus. Values: -25 to +50C
<b>Step</b>	Toggles increment for temp setting, 0.5C is default, can be toggled to 0.1
<b>Up/down</b>	Increase/decrease the set temperature
<b>Cal</b>	Setup & display : (1) displays Peltier device current (2) hides Peltier device current (default) (3) displays heatsink temperature (20-30C) (4) sets sample exchange temp 5-20C (7) sets temp control loop gain (default=6) (8) sets manual mode (operates if Peltier device is switched off first)
<b>Photo</b>	Switches off the closed loop temperature control to keep Peltier current constant
<b>Exch</b>	Sets sample temp to pre-programmed exchange temp
<b>C/F</b>	Changes temp display from degrees Centigrade to Fahrenheit
<b>Del</b>	Cancel or minus key
<b>Enter</b>	Accept

**7. Operating Variables:**

- **Accelerating Voltage (Vacc):** high kV causes (1) High resolution  
(2) More specimen damage  
(3) More specimen charge  
(4) Less surface detail
- **Emission current (Ie):** high current results in (1) High signal  
(2) More specimen damage  
(3) More specimen charge
- **Probe current (spot size):** low current for (1) Higher resolution  
(2) Less signal  
(3) Less specimen damage  
(4) Less specimen charge
- **Final aperture (1to4 )** small aperture for (1) High depth of field  
(2) Low signal levels  
(3) Less specimen charge  
(4) Less specimen damage
- **Working distance (5-65)** short distance for (1) High resolution  
(2) Less depth of field  
(3) Higher magnification

## 8 Sample Preparation

### *General*

- Preferably use gloves when handling samples, stubs and mounts.
- Always clean Al stubs thoroughly before mounting samples – or use new ones.
- Minimize the use of double-stick tape or non-conductive adhesives for sample mounting to avoid outgassing. With the Coolstage samples may be mounted in a drop of water and frozen to the stage
- Before loading the sample, **measure the sample height** using the white measuring tool. This is imperative for safe operation of the SEM, and all buttons are inactive until sample dimensions have been entered in the Hitachi 3400N-software.
- **Make sure the sample height is under the limit to prevent damage to the Backscatter Electron Detector (NNB)**

### *Samples types*

- *Powder samples*  
Disperse a small amount of sample in ethanol and sonicate  
Heat the stub.  
Drop the suspension on the stub. The fast drying of ethanol should spread the powders fairly well on the surface.  
For sample checking (not for high quality images), you can also disperse your sample on the black carbon tapes.
- *Solid pieces*  
Carbon or silver paste is preferred to use for mounting samples for better imaging (compared with tapes)  
Completely dry the sample before loading
- *Biological specimens*  
VP-SEM: NO processing is needed when using VP mode. Samples may be viewed hydrated, unfixed and uncoated at pressures up to 270Pa.  
SEM: samples should be chemically stabilized and dried, and pretreated as follows:
  - fix in 2% Glutaraldehyde (or 2%Glut + 4%Formaldehyde) in 0.1M Cacodylate Buffer pH7.2 (this may need to be adapted for your samples)
  - dehydrate in a graded ethanol series (50-100%)
  - dry sample chemically with HMDS, or critical point dry (CPD) with CO<sub>2</sub>.
  - sputter-coat with Au/Pd (200Å layer) when charging of a specimen occurs at high voltage, or when viewing specimens for long periods of time.

### *Coating*

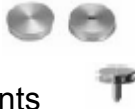
- For best images, and to avoid charging of specimens, it is preferable to gold or carbon coat the sample prior to examination.
- Au coating indeed generates Au particles, whose sizes increase significantly with coating time. Two minutes coating may have particles in the range of 5~20 nm.
- An alternative way to reduce surface charging is to connect an electrical discharge bridge (a copper tape for example) from sample to the grounded sample mount.

## 7 Sample Stubs

Go to <http://www.tedpella.com> OR <http://www.emsdiasum.com/microscopy/default.aspx> for a range of EM supplies

- Common stubs used:

Any M4 thread mounts



Any 1/8" (3.1mm) pin mounts

- For irregular shapes, consult the schematic below before buying appropriate sample holders. Note: larger samples limit both the rotation and the tilt. Stage movement is 10 x 6 cm (depth x width)

## 8 QuantomiX

QuantomiX WetSEM technology can be applied to image and analyse fully hydrated samples in the High Vacuum environment of conventional SEM, using the BSE detector. The QX capsule isolates wet samples from the high vacuum of the microscope chamber, while holding the sample in liquid medium at atmospheric pressure. Visualization is enabled through an electron transparent polyamide membrane which allows structure as well as composition analyses to be applied. A calibration capsule with nanogold particles is convenient for troubleshooting the technique. Experimentation with membrane coatings for cell attachment (gelatin, or poly-lysine), as well as contrasting techniques, is imperative.



For full details, application videos and publication list, see

<http://www.quantomix.com> and

<http://www.emsdiasum.com/microscopy/products/sem/wet/publications.aspx>

## 9 Useful Basics

- **Fundamentals of SEM** In principle the Scanning Electron Microscope allows us to visualize material by the scanning of a fine beam of electrons over the surface of a specimen in synchronism with the spot of the display cathode ray tube (CRT). Electrons can be emitted from a tungsten cathode or via field emission. A detector monitors the intensity of a secondary signal from the specimen (e.g, secondary electrons) and the brightness of the CRT spot is controlled by an amplified version of the detected signal.
- **Processing** For high vacuum, high voltage conditions required in normal SEM, 3D structure is best preserved by chemical fixation and drying followed by critical point drying and sputter-coating with an electrically conductive film (100-200Å). Mounting of the specimen with carbon or silver paint will further decrease charging, and enhance resolution and viewing for longer periods at high voltage
- **VP SEM** Allows visualization hydrated (wet unfixed samples) under high pressure, low vacuum conditions, by using a differentially pumped specimen chamber. Only BSE are allowed under these conditions. The function is obtained by applying a turbo molecular pump and separation valve to the lower part of the column
- **Aperture** The angular aperture is a function of both the physical size of the aperture and the working distance. A smaller aperture is used for increased depth of field – as this also reduces the number of electrons reaching the specimen, an increase in probe current (spot size) will counterbalance such loss of signal. This is mostly applicable to working at lower magnification.
- **Probe current/Spot size** Lower probe current is equivalent to smaller spot size, which means the diameter of the electron beam is smaller than at a higher probe current. The smaller the diameter of the electron beam, the better the potential instrumental resolution. However, a reduction in probe size is accompanied by a reduction in beam current, therefore fewer secondary electrons are emitted, and the image will be noisy. The spot size should be optimized by trial and error. Start with a large spot size and low magnification. Focus at a magnification 2x – 4x the required value, and decrease spot size till the 'noise level' is unchanged. Use slow scan speed as TV scan will always be noisy. The type of specimen also determines the spot size. Delicate samples may demand the use of a smaller than optimal spot size (for that magnification), since such samples may not tolerate the increased electron flux in a large spot. The spot size is not an actual value of only beam diameter, since not only condenser lens settings, but also accelerating voltage, final aperture and working distance may influence this. It becomes a matter of experience, trial and error.
- **Astigmatism** A distorted image that does not perfectly reproduce the shape of the object may be due to astigmatism. It arises when the lens field is not perfectly symmetrical about the optical axis and results in a 'smearing out' of the image when apparently in focus

- **Focusing** As focus setting is unaffected by changes in magnification, focusing is normally done at magnification several times that used for photographing the sample.
- **Secondary Electrons (SE)** are low-energy (<50eV) electrons that originate within a few nanometer from the surface of material entered by a primary electron beam, and are detected to indicate topographical differences. Variation in the incidence angle of the electron beam results in variation in the number of secondary electrons emitted, which are detected as a 3D contrasted image
- **Back Scatter Electrons (BSE)** are high energy electrons originating in the electron beam, that are reflected or back-scattered out of the specimen from deeper levels than secondary electrons. Backscattered electrons also indicate compositional differences, as the brightness of the BSE image increases with atomic number.
- **Imaging/Photographic recording** After obtaining the desired image at the proper magnification,

- **CONSULT THE MANUAL**

- **EXPLORE PC-SEM FOR ADDITIONAL FEATURES AND PCI SOFTWARE**

## 10 Literature

- Bozzola, JJ & Russel, LD. 1999. Electron Microscopy Principles and Techniques for Biologists. 2<sup>nd</sup> Ed, Boston: Jones & Bartlett Publishers
- Chescoe, D. & Goodhew, PJ. 1990. The Operation of Transmission and Scanning Electron Microscopes. Royal Microscopical Society Microscopy Handbooks.
- Gabriel, BL. 1982. Biological Scanning Electron Microscopy. New York: Van Nostrand Reinhold
- Hayat, MA. 1974. Principles and Techniques of Scanning Electron Microscopy Vol1 Biological Applications. New York: Van Nostrand Reinhold
- Hayat, MA. 2000. Principles and Techniques of Electron Microscopy: Biological Applications (4<sup>th</sup> Ed). Cambridge: Cambridge University Press
- The Microscopy ListServer <http://www.microscopy.com/>
- <http://www.tedpella.com>
- <http://www.emsdiasum.com/microscopy/default.aspx>
- <http://www.2spi.com/spihome.html>
- <http://www.quantomix.com>
- <http://www.emsdiasum.com/microscopy/products/sem/wet/publications.aspx>

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