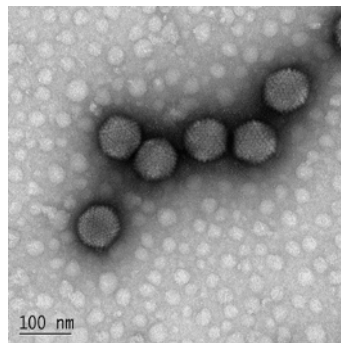
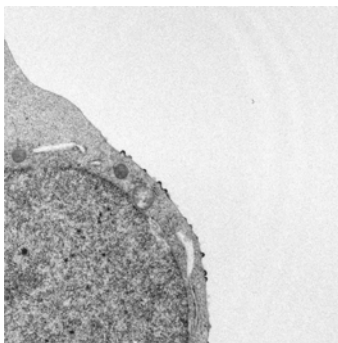
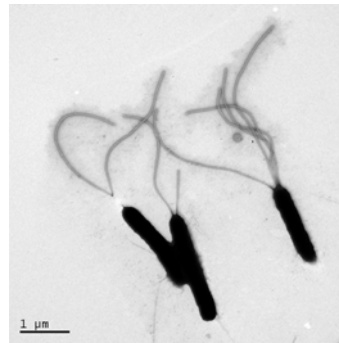
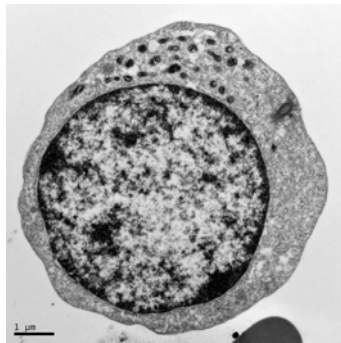


ELECTRON MICROSCOPY PROCEDURES MANUAL

JULY 2010



PROTOCOLS **1**

Specimen Preparation Protocol..... 1

1. Fixation:..... 1

2. Dehydration: 1

3. Embedding:..... 2

4. Sectioning:..... 2

5. Post Staining: 2

Fixing Cells for Electron Microscopy..... 3

Method 1: Scrape Cells with Cell Scraper..... 3

Method 2: Permeabilize Cells from Plate 4

Method 3: Leave on Petri Dish, Permax Chamber Slides or Coverslips 4

Method 4: Transwell Permeable Supports..... 4

LRW Embedding 5

Post-Embedding Immunogold Labeling on LRW Sections of Cells 6

Primary Antibodies: 6

Secondary Antibodies: 6

Dilutions:..... 6

Protocol:..... 6

Negative Staining Procedure 8

Scanning Electron Microscopy Protocol Using HMDS..... 9

OPERATIONS..... **10**

JEOL 1230 TEM Operation..... 10

Signing on: 10

Sample removal and loading: 10

Correcting Z-axis (Height of sample): 11

Centering the Objective aperture (if needed): 11

Using the Gatan Digital Imaging Camera and Software: 12

Shutting Down:	12
Loading the sample holder into the JEOL 1230 microscope.....	13
<i>JEOL 1010 TEM Operation</i>	14
<i>Gatan Digital Camera</i>	15
<i>JEOL JSM-5800 SEM Operation</i>	17
Specimen Loading and Exchange:	17
Observation:	18
Viewing Image and Setting Parameters:	18
Imaging:	21
Save Tiff images using the Orion Imaging System:.....	21
Microscope shut down:	22
ADDITIONAL INFORMATION AND RESOURCES	23
<i>General Electron Microscopy Resources</i>	23
Introduction to Electron Microscopy	23
What are Electron Microscopes?.....	23
Electron Microscopy – Web Course Notes and References.....	23
Transmission Electron Microscopy (TEM) Basics	23
Listserv for Electron Microscopy Information.....	23
<i>Specific Protocol or Technique Resources</i>	24
Binning Explained.....	24
EM info for <i>Caenorhabditis elegans</i>	24
Immunocytochemistry	24
Immunogold labeling	24
Immunogold questions	24
Kohler Illumination.....	24
Negative Staining	25
Optical Image Processing.....	25



PROTOCOLS

SPECIMEN PREPARATION PROTOCOL

1. FIXATION:

Tissue can be fixed by immersion or perfusion. The most commonly used method is immersion. Fixation time is variable, depending on tissue, but usually from 4 hours to overnight at 4 degrees (refrigerator).

- a. Primary Fixative: 1/2 strength Karnovsky's.
 - 2% Paraformaldehyde/2.5% Glutaraldehyde
 - This is buffered with a 0.2M Cacodylate Buffer

Note: Always use the fixative in the fume hood and wear gloves. Glutaraldehyde fixes skin and cacodylate buffer contains arsenic.

- b. 0.1M Cacodylate Buffer wash
- c. Post Fixative: 2% aqueous OsO₄/0.2 M Cacodylate Buffer
 - A 1:1 solution for 2 hours in the refrigerator
- d. 0.1 M Cacodylate Buffer wash

2. DEHYDRATION:

Dehydration with ethanol:

35% ETOH	15 min. and decant (optional)
50% ETOH	15 min. and decant
70% ETOH	15 min. and decant
95% ETOH	15 min. and decant
100% ETOH	15 min. and decant
100% ETOH	15 min. and decant
Propylene Oxide	15 min. and decant
Propylene Oxide	15 min. and decant
1:1 PO/Epon resin	overnight

- The dehydrating times should be adjusted to size and kind of tissue.
- The resin is an Epon 812 recipe

3. EMBEDDING:

Embedding with Epon:

- Next morning, change out to fresh Epon 812 for 1-3 hours.
- Embed (always) in fresh Epon 812
- Polyethylene capsules are placed in a holder and numbered strips of paper are inserted. A drop of fresh Epon 812 is placed in the capsules and the specimen is transferred to the appropriate capsule.
- The “blocks” are cured for 48 hours in a 60 degree oven.

4. SECTIONING:

- 1 micron or thick sections are taken to find the area of interest
- Take ultra thin sections (~70nm) and place on grids

5. POST STAINING:

- Stain grids in uranyl acetate for 2 hrs.
- Stain grids in lead citrate for 5 min.

** This procedure is the basic TEM procedure. Each and every sample that comes to us is evaluated and the procedure is adjusted to make sure the best results will be obtained.

FIXING CELLS FOR ELECTRON MICROSCOPY

METHOD 1: SCRAPE CELLS WITH CELL SCRAPER

****We need 1-5 million cells in order to do our procedure****

Fixation:

Fixative = 1/2 Karnovsky's

- Use fixative (from refrigerator) and warm it up to room temp. or 37 degrees.
- Add fixative to media (1:1) and let it sit for 10 min to fix.
- Take off fix and media (don't let cells dry out) leave a small amount to cover cells and then add pure fix for 30 min - 1 hr.
- Scrape cells, put them into an Eppendorf tube, and spin down at a very low speed (1400 rpm).
- Pipette off fix (Keep a little fix on to not let the cells dry out) and put on new fixative and then resuspend cells.
- Put cells in refrigerator. Overnight with fix or bring them down to the EM lab in fix.

Or

- Wash media off with 0.1M Cacodylate buffer quickly and add ½ Karnovsky's fixative and then scrape after 30min – 1 hr.

Wash:

- Wash cells 3 x 5 minutes in 0.1M Cacodylate buffer.
- Spin down each time.

Post Fixation:

- 1% Osmium Tetroxide in 0.1M Cacodylate buffer for 1 - 2 hr. at 4 degrees in the refrigerator.

Wash:

- Wash 3 x 5 minutes in 0.1M Cacodylate buffer.

Dehydration:

- In graded series of ETOH - 50%, 70%, 95%, 2 x 100% ETOH for 15 min. and 2 x Propylene Oxide for 15 min.

Embed:

- 1:1 PO/Epon resin overnight with Eppendorf tubes capped.

- Next day, spin down cells and uncap Eppendorf tubes.
- Add Fresh 100% Epon resin for 2- 4 hrs.

Polymerize:

- In 60 degree C oven overnight in Eppendorf tubes.

METHOD 2: PERMEABILIZE CELLS FROM PLATE

Fixation: Do as above but permeabilize cells from plate instead of scrape.

METHOD 3: LEAVE ON PETRI DISH, PERMANOX CHAMBER SLIDES OR COVERSGLIPS

Fixation: Do as above but leave cells on the plate in the fixative for 1 hr.

Embed: Change 2x to fresh Epon resin and then invert beam capsules full of slightly hardened resin over relevant areas.

Polymerize: In 60 degree C oven overnight and then pop off beam capsules which contain the cells.

METHOD 4: TRANSWELL PERMEABLE SUPPORTS

Fixation: Fix the attached supports containing cells in plate for 2 hr. or overnight.

Wash, Post fixation, Dehydration: Same as above.

Embed: 100% fresh Epon resin for 1 hr., then punch out supports with an 8mm biopsy punch on wax containing resin. Cut out relevant pieces and flat embed.

Polymerize: in 60 degree C oven overnight.

LRW EMBEDDING

****Note:** Do not use OSO4 fixed tissue or cells

1. Fix tissue or cells in primary fixative.
2. Wash tissue 3X-10 min. in 0.1M cacodylate buffer, pH 7.4.
3. Dehydrate in ethanol:
 - 50% - 1X-5 min. sit in hood/ 2 min. spin for cells
 - 70% - 1X-5 min. sit in hood/ 2 min. spin for cells
 - 95% - 2X-7 min. sit in hood/ 2 min. spin for cells
 - 100% - 2X-7 min. sit in hood/ 2 min. spin for cells
4. Infiltration with LRW:
 - a) Mix LRW Resin with ETOH – 1:1 in a scintillation vial. Put mixture in tissue or cells for 30 min.
 - b) After 30 min., make a 2:1 mixture of LRW to ETOH.
5. 100% LRW Resin – 3X-1 hour and leave overnight in the last change.
6. Embed in Ted Pella flat teflon mold or gelatin capsules. LRW will only polymerize anaerobically, seal tightly.
7. Next:
 - a) Cure in UV light cryo-chamber in 4 degree C walk in refrigerator overnight. Put pans of ice on the bottom of the container.

OR

- b) Polymerize in gelatin capsules in 50 degree C oven overnight.

POST-EMBEDDING IMMUNOGOLD LABELING ON LRW SECTIONS OF CELLS

Example: Cell pellets were fixed for 3 hrs. with 0.025%glut./3% parafo. and then worked up with short dehydration times (10 min starting at 50% ETOH) and polymerized at 4 degrees under UV light for 1.5 days with LRW in gelatin capsules.

PRIMARY ANTIBODIES:

Rabbit Preimmune sera – Investigator supplies

SECONDARY ANTIBODIES:

Goat anti Rabbit 10 nm, IgG (H+L) from EMS – Investigator supplies

DILUTIONS:

- Primary - Preimmune sera – 1:30
- Secondary - 10 nm gold – Straight or 1:25
- Control - No Primary/ Straight gold

PROTOCOL:

****Everything is filtered with a 0.2 micron filter before use and purchased from EMS.**

1. 0.05 M Glycine – 20 min. at room temp.
2. PBS- 3 drops
3. 5% BSA – 45 min. at room temp. (optional)
4. BSA-C/PBS/Tween 20 – 3X5 min drops (PH 7.4)
5. Primary antibody – 1 hr - ? at room temp. or overnight at room temp. or at 4 degrees C in humidified petri dish. (spin Primary in a centrifuge for 5 min on high speed).
6. PBS – 3 drops **OR** Dip into beaker of PBS
7. BSA-C/PBS/Tween 20 – 6X5 min drops (PH 7.4)
8. Secondary – Gold – 1 hr. - ? at room temp. or overnight at 4 degrees C in humidified petri dish (spin Secondary in a centrifuge for 5 min on high speed).
9. BSA-C/PBS/Tween 20 – 6X5 min drops (PH 7.4)

10. PBS 3 drops
11. 2% glut/PBS for 10 min at room temp
12. Warm ddH₂O wash
13. Post-stain with UA for 1 min and Lead citrate for 30 sec.

NEGATIVE STAINING PROCEDURE

1. Use 200 or 300 mesh formvar/carbon coated grids.
2. Place a drop (approx. 25 ul) of specimen on wax plate and put the grid on top of the specimen. Leave for 15 min. to 1 hr.
3. Touch grids to drops of:
 - ½ Karnovsky's Fixative
 - 0.1M Cacodylate Buffer
 - 3 drops d H₂O
4. Touch grids to a drop of U.A and onto a moistened filter paper to draw off excess U.A. The percentage varies with the sample.
5. Dry overnight in a Petri dish, in the desiccators, and view the next day in the microscope.

SCANNING ELECTRON MICROSCOPY PROTOCOL USING HMDS

1. Fix tissue in ½ Karnovsky's Fixative and OsO₄ as usual.
2. Wash tissue in 0.1M Cacodylate Buffer
3. Dehydrate using a series of ethanol washes:
 - 50% Ethanol for 15 – 30 min.
 - 70% Ethanol for 15 – 30 min.
 - 95% Ethanol for 15 – 30 min.
 - 100% Ethanol for 15 – 30 min.
 - 100% Ethanol for 15 – 30 min.
4. Immerse tissue in pure HMDS for 15 min. x 2
5. Just cover the sample with fresh HMDS and leave under the hood until the HMDS evaporates off.
6. Specimens will be ready to mount onto stubs and sputter coated with Au/Pd in the morning or store desiccated until coated.

OPERATIONS

JEOL 1230 TEM OPERATION

The following procedure is a general operating procedure that the majority of users will apply when working on the JEOL TEM. The following should be used as a starting point for your particular work. The settings can be modified to produce the best images of your samples. With that in mind please remember to return the scope to the standard mode of operation for the next user..

SIGNING ON:

1. After signing the paper log, sign on to the Gatan digital camera PC by choosing “Ctrl-Alt-Delete” and entering the password = gatan.
2. Next, you should Login to the JEOL computer. In the right field choose [Login...]. In the center field highlight the “User” field. Backspace to erase previous user’s name and enter emlab. Press “enter”. Highlight [password] and enter your 4-digit password=3333. Hit enter then [Apply].

SAMPLE REMOVAL AND LOADING:

****Please Note: If the 5-grid holder is in the Goniometer, be sure the “Exchange” white dot and the Number 1 (also with a white dot) are aligned before removing.****

1. Center the holder by choosing the Specimen Position [Spc Pos...] button in the left field, below the Spot Size and above Z Control, make sure the Point “ON” is green and click on the center of the grid.
2. Prepare to remove specimen holder by choosing “Monitor” in Right Field (CPU) and select “Vacuum”. All should be @ “Ready” state.
3. Switch Goniometer from “Pump” to “Air”. Nothing should happen.
4. Pull Specimen holder straight out (only green bars should be showing on the PEG) of Goniometer ~7cm until it stops then turn ~75 degrees Counter-Clockwise until it stops. You can now let go and reposition your hand.
5. Pull Specimen holder straight out of Goniometer ~0.5cm until it stops then turn ~15 degrees Counter-Clockwise until it stops. Let go. The square “seat” should be at 12:00. You will hear the Goniometer venting to air and green light will turn off. On the CPU the “Vacuum” SPC field should now read “Air” and will climb to ~245.

6. Gently remove the holder straight out from the Goniometer. If there is resistance wait another ten seconds. Be careful not to touch the holder beyond the screw/collar, as this will introduce grease and dirt into the microscope.

7. Raise clamping mechanism on the specimen holder. Place your grid in the holder, then lower and clamp the mechanism.

****Again Please Note: If you are using the 5-grid specimen holder, be sure the “Exchange” white dot and the Number “1” (also with a white dot) are aligned.****

8. When carefully replacing the specimen holder, align the small screw with opening in side of Gonimeter until seated in Goniometer.

9. With slight pressure pushing specimen holder in Toggle switch from “Air” to “Pump”. You should hear the valve opening and the CPU “vacuum” SPC field should still read “Air” but will drop to about 48-47 (indicator will not change from “Air” to “Ready” but you can go ahead and turn the beam on).

****Note: The pump-out of the Goniometer is on a timed cycle. If the reading does not reach 50, you will have to wait until the next pumping cycle begins. The pressure will rise but should not be more than 200.****

10. Once the SPC on the vacuum monitor reaches below 50 get a good grip on the specimen holder and turn clockwise ~15 degrees until it stops, then allow it to go into the chamber ~0.5cm.

11. Still with a good grip turn clockwise ~75 degrees until it stops and slowly allow it to go into the Goniometer until the square “seat” nestles into the Goniometer at 3:00. Eventually you will do both of these steps as one fluid step. (Please note: indicator will not change from “Air” to “Ready” but you can go ahead and turn the beam on)

12. Select “HT” in the Head field and confirm the HT is at 80 KV and is READY. In the Left or Center Field select the Beam Current and switch it to ON.

CORRECTING Z-AXIS (HEIGHT OF SAMPLE):

1. On the Function box {FB} bring beam to crossover with “Brightness” control. Center beam by pressing {BEAM SHIFT} and using the right mouse button.

2. At around 5000X focus on the sample film or a section itself and select [Z-Control] in the left field of the Display. Select [Auto] in the Center Field. When you have focused on the sample, click [Okay].

3. Refocus image if necessary.

CENTERING THE OBJECTIVE APERTURE (IF NEEDED):

This should be done at the beginning of your session and after changing aperture settings (turn smaller apertures clockwise, larger aperture counterclockwise) or after going from Low Mag back to Mag 1.

1. Move to a clear area on the grid away from grid bars and areas of interest but there must be some film, formvar, carbon, or section to focus the diffraction onto.
2. Bring the centered beam to crossover and select {DIFF} on the Function Box (FB). Focus the spot with diffraction "Focus" knob on {FB} and center the dark ring around the bright beam using the manual knobs on the aperture. **(Do this quickly since a bright beam will burn the fluorescent screen)**
3. Select Mag 1 or 2 and you are ready to begin observations.

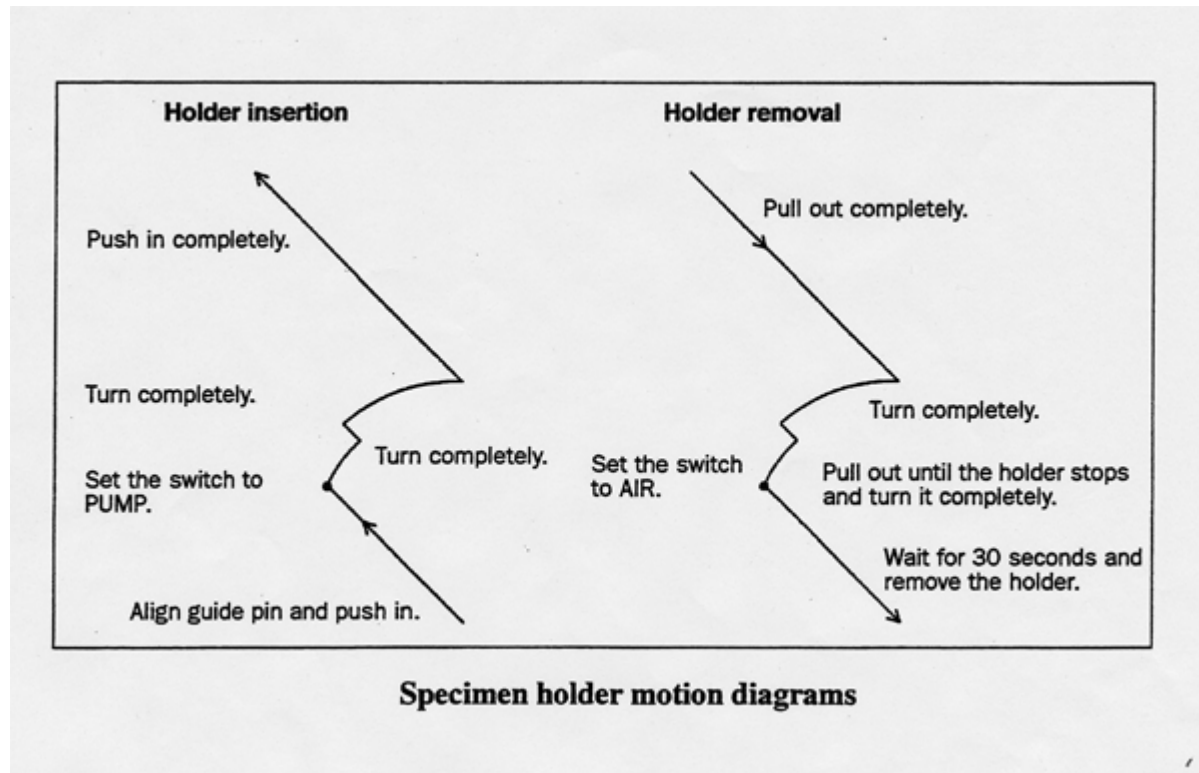
USING THE GATAN DIGITAL IMAGING CAMERA AND SOFTWARE:

1. Focus and center the area of interest.
2. Make sure the Gatan PC screen is on and make a folder on the desktop. Label with username or Hutch account_date. Example: jdoe_011807. No spaces please.
3. Open the Gatan Digital Micrograph application and insert the camera.
4. For the Ultrascan 1000 bottom mounted camera-Lift up the screen by selecting [Screen] on the left microscope field. For the Orius side mounted camera-Make sure illumination is very dim and then insert the camera. Then select [Start View] on the right panel of the Gatan software and you will see a continuous, quick scan of your sample taken @low resolution. Be sure in the "intensity" box that the box is within the green field.
5. If you would like to focus the image switch from the "Search" to the "Focus" mode and manually focus your sample. The focus lupe, when checked, will collect fast/low resolution images making it easier to focus manually. You can also focus on the fluorescent screen of microscope.
6. Now you can select {acquire Image}. You can save the image with micron bar under the File menu and by selecting Save display as. Include the magnification in the file name. At the end you can transfer images to Fred or burn a CD.
7. To go back to viewing your sample on the microscope screen, select {Stop View} and de-select the "Screen" on the microscope for the Ultrascan camera or just take the camera out for the Orius camera.

SHUTTING DOWN:

1. Lower the magnification to ~1000x.
2. Spread the beam maximally. Turn off the beam.
3. Remove your sample from specimen holder.
4. Reinsert sample holder into the microscope.
5. Log off of TEM PC, Gatan PC, and the paper log. Cover fluorescent screen and turn off room lights.

LOADING THE SAMPLE HOLDER INTO THE JEOL 1230 MICROSCOPE



JEOL 1010 TEM OPERATION

1. Log in (note the “start time” from the meter on the scope).
2. Beam current should be ~ 053 with HT button on.
3. Take out specimen holder: pull holder out until it stops, turn towards you (counterclockwise), and pull out slowly (vacuum will pull it in).
4. Open specimen clamps on the specimen holder with the forceps and put grids in. Position #1 is at the tip of the rod.
5. Put back specimen holder: push in until it stops, green light will go on, wait for the green light to go off (~40 sec), turn clockwise and put the holder in very gently (vacuum will try to draw it in).
6. Turn up the brightness and contrast knobs on the side of the TEM screen and go to the main menu – Page 1
 - a. Should be at 80 KV
7. Choose Specimen select #1 position.
8. Turn on filament button: FIL.
9. Focus the black dot on the small screen with the eyepieces off the sample.
10. Set the height of the specimen with the goniometer when measuring is critical.
11. Focus image and center illumination:
 - a. Bring small focus screen into view
 - b. Turn wobbler on by pushing down image X button.
 - c. Push on (16X) button and focus with coarse and fine knob
 - d. Turn wobbler off
 - e. Take small focus screen out of view
 - f. Bring the illumination to a smaller spot with the brightness knob and center with the shift X and Y knobs
 - g. Center image in brackets located on the fluorescent screen.
12. For digital images, see page 15, for the Gatan Digital Camera instructions.
13. When you are finished with your session:
 - a. Turn off the filament button

- b. Take your grids out of the holder
- c. Put the holder back into the scope
- d. Log out (note the "elapsed time" from the filament meter on the scope).
- e. Flip switch to turn off the Gatan Camera.
- f. Turn down brightness and contrast on monitor and turn off the room lights on the scope

GATAN DIGITAL CAMERA

****It is very important never to have the brightness all the way up on the microscope and to insert the camera. This will damage the scintillator!**

1. On the camera box flip the camera switch to on. This is found on top of the microscope to the right. Both camera and shutter switches should be in the up position.
2. Turn on the computer and monitor
 - a. Password = gatan
3. Make a new folder on the desktop by right clicking with the mouse on a blank area on the desktop, point to new, then click folder. Name folder as follows: username or Hutch account_date. Example: jdoe_081905. No spaces please.
4. Allow for communication between the microscope and camera by pushing the PF3 key on the keyboard and then Enter.
5. Double click on Digital Micrograph to bring up the software to run the camera.
6. Prepare a Gain Reference: Note: Need to be at 10,000X mag.
 - a. This will optimize the signal to noise ratio of your image
 - b. Pull the sample holder out to the middle position and turn slightly.
 - c. Turn down the brightness knob so the beam is uniform and set the exposure time to approximately 10 sec., found on microscope menu. Remove small screen from view(optional).
 - d. You may cover the viewing port if the room is bright
 - e. Go to the Camera menu and prepare gain reference
 - f. Insert the camera and answer ok to the prompts
 - g. It will take a few seconds to acquire and a prompt will come up to let you know if it has been successfully acquired.

7. View Sample Area:

- a. Remove the camera and insert the sample into the microscope.
- b. Find an area of interest and focus.
- c. Dim the beam for a 3-5 sec exposure time (on microscope menu) adjusted with brightness knob, remove small screen, and insert the camera.
- d. Click on the "Start View" button to view a continuous image of the sample. This is found on the Camera View menu to the right of the screen.
- e. Use the "Search" mode (this gives the fastest image refresh rate on the monitor).
- f. Adjust the brightness on the intensity bar to the green zone with the brightness knob on the microscope if needed.

8. Adjust Focus (Focus Loupe):

- a. Click on "Focus Loupe". This will allow continuous viewing of the image only from the selected sample area that is marked.
- b. Focus with the microscope focus knobs.
- c. Focus can also be obtained with the focus button under the TEM AutoTune menu (top right of the monitor).

9. Acquire the Final Image:

- a. After focus is set, click the "Start Acquire" button to record the final image. This is found on the Camera Acquire menu to the right of the screen.
- b. Adjust for contrast and brightness using the histogram and sliders.
- c. The image can be enlarged by dragging the corner of the image with the mouse and alt key held down.
- d. When viewing another specimen, stop view and take the camera out.

10. Saving Images:

- a. To save the image as a Full Resolution Tiff Image:
- b. Go to the Custom menu and SaveAsFullRestiff.s
- c. The micron bar will be saved with the image.
- d. Include the magnification of the microscope in the file name.
- e. To save the image in Gatan Digital Format – dm3:

- f. Go to the File menu and Save as
- g. Name it and save as the Gatan Format (*.dm3)
- h. The image can be saved in a Report View with image notes

11. Save Images to Fred:

- a. Follow the sheet on how to map a drive to Fred from a PC.

12. Printing Images:

- a. To print a non-pixelated image choose Report format to see page layout
- b. Click on the image to select it
- c. Under the Control palette (left side of the screen) make sure the print width(W) and height(H) are approximately 7 x 7 inches.
- d. Go to the File menu and Print and select the appropriate options.

JEOL JSM-5800 SEM OPERATION

SPECIMEN LOADING AND EXCHANGE:

To Load Samples:

1. Screw in stubs to holder and load specimen holder on stage.
2. Push **Evac button** on the chamber door, and make sure the door is sealed properly.
3. Wait about 5 minutes for "**HT READY**", located on LCD on front of microscope.

To Exchange Samples:

1. Turn HT off and wait approximately 15 minutes to cool.
2. Select **STAGE** (LF5) menu on right CRT.
3. Click **Init Position** and **Start**.
4. Lower the working distance to approximately 20 with the **T/Z button** and **joystick** to avoid hitting the **Backscattered Detector**. WD is read out on the lower left of microscope on LCD.
5. Push **Vent button** on the chamber door and remove the samples after the beep.

OBSERVATION:

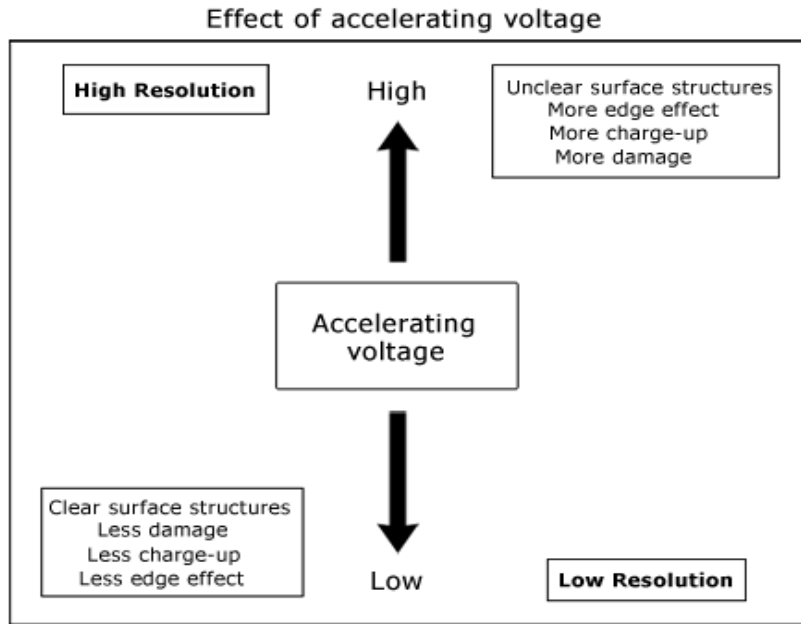
1. Specimen(s) are loaded and **HT button** has been pushed and is ready.
2. Position the first specimen:
3. Select **STAGE** Menu.
4. Click **LOAD**, if not pushed already.
5. Position the curser on the desired stub
6. Click **START**.

VIEWING IMAGE AND SETTING PARAMETERS:

1. **TV2** on
2. "Freeze" L or R off
3. **INST MAG** on.
4. If still no image, manually move stage and adjust brightness, etc, until the specimen is found, then try **AUTO IMAGE** again.
5. The X/Y knob and the joystick will move the sample around.

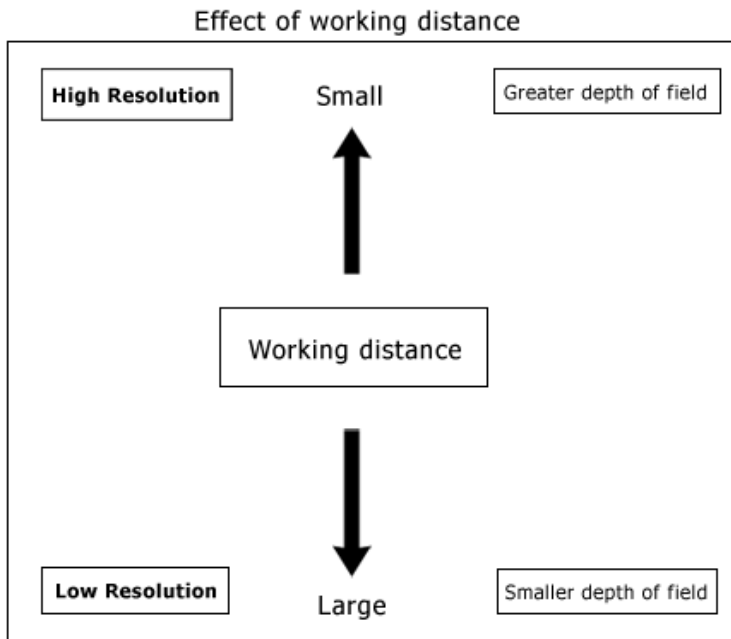
Choose Accelerating Voltage:

1. Press **ACCV** (F2) menu. See Figure. for explanation:



Set Z (WD) and T (tilt) as desired:

1. WD is the distance from the underside of the objective lens to the specimen surface. See Figure for explanation:



Set Spot Size as desired:

1. Spot Size on.
2. Observe LCD graph while turning CRS/Fine knobs.
3. Return to Tone function.

- **NOTES** For Spot Size:

Arrows (>>>) on LCD graph indicate spot size.

(>>>>>> -----) = Spot Size 7.

Spot sizes range from 1 to 24.

<u>Spot</u>	<u>Size</u>	<u>Current</u>	<u>Resolution</u>	<u>Noise</u>
1	Small	Low	High	Most
12	Medium	Medium	Medium	Medium
24	Large	High	Poor	Least

Objective Aperture Size:

- #1 = 20uM (High Resolution)
- **#2 = 30uM (Normal)**
- #3 = 100uM (High Current for EDS)

Centering Objective Aperture:

1. “**OL**” **WOBB** on.
2. Increase Mag. to see image movement.
3. Adjust aperture knobs to minimize movement.
4. “**OL**” **WOBB** off.

Correct Stigmatism:

1. The image is astigmatism-free if it has no unidirectional defocusing when the objective lens is changed to under- or over-focus.
2. This is done by adjusting the two knobs, x and y, of the stigmator with the **STIG** button pushed.

****Note: Set Desired Magnification, Focus, and Stigmatism before taking a photo.****

IMAGING:

- Brightness and Contrast can be set by pushing the Tone button and TV2 two times to bring up the screen for adjustment. Bring the two bars in the middle with the knobs.

SAVE TIFF IMAGES USING THE ORION IMAGING SYSTEM:

1. A folder will be created for you on the desktop with your name and date Ex. Jdoe_012407. Another folder called Export will be in this folder.
2. Click on minimized JEOL Orion 5800.
3. Go to the **File** menu and click on **User Login** and then click on your **name** and **Login**.
4. The **EXT OFF** button (in red) on the left of the screen is the position for the connection with viewing on the SEM. The **EXT ON** button (in blue) and the **L** for live is the position for collecting live images from the SEM.
5. Collection of the image defaults to **1280** resolution and **17** Sec. for scanning speed. If you choose a smaller number the refresh rate becomes faster and the resolution is less. For photography the resolution should be 1280 and **90** sec. (also can do 17 sec.).
6. Correct the brightness and contrast of the image by using the controls on the microscope or the **Line Scan Profile** button (in the image measurement selection) of the Orion image software.
7. In order to save the image it must be frozen. Press the **L** button one time and the image will freeze (**F**) after it scans to the end of the photo. If you press the **L** button 2 times it will freeze (**F**) the image right away.
8. On the bottom of the photo, type in the **KV**, **Mag**, and **WD**. This information will be saved along with the micron bar.
9. Press the **Image File Handler** button (To the right of the **L**, **F** button). Then press the **Save...** button on top right and the **Save Image Data** Menu comes up. Press **OK**.
10. Put this image in the regular folder and not the export folder.
11. On the left side under **File** click the name of the file you just saved and click the **Export** button. **Save Image Data** Menu comes up and press **OK**. Put the image in the **Export** folder. This will export all data on the image.
12. Click on **L** button and **EXT ON** button to switch over to the microscope.
13. Minimize the JEOL Orion 5800 software.
14. Transfer files to your fred account or copy onto CD.

MICROSCOPE SHUT DOWN:

1. HT button should be pushed off
2. Wait approximately **15 min** to take the samples out or ask the EM Staff to do this.
3. Set Mag. to **300,000X** and **SL2**
4. Turn down the screens all the way.

For more information on SEM Operation, please see the handout sheet "Introduction to the Principles of Operation and Applications of the Scanning Electron Microscope and Electron Microprobe" by Vernon E. Robertson, published by JEOL USA INC., Peabody, MA.

ADDITIONAL INFORMATION AND RESOURCES

GENERAL ELECTRON MICROSCOPY RESOURCES

For more information on the topics listed below, please check the websites or publications listed below.

INTRODUCTION TO ELECTRON MICROSCOPY

<http://www.sci.sdsu.edu/emfacility/555class/class1.html>

and

<http://www.amc.anl.gov/> Select the PDF slides/lecture notes by Nestor Zaluzec

WHAT ARE ELECTRON MICROSCOPES?

<http://www.unl.edu/CMRAcfem/em.htm>

ELECTRON MICROSCOPY – WEB COURSE NOTES AND REFERENCES

<http://em-outreach.ucsd.edu/web-course/toc.html>

TRANSMISSION ELECTRON MICROSCOPY (TEM) BASICS

<http://www.matter.org.uk/tem/>

LISTSERV FOR ELECTRON MICROSCOPY INFORMATION

<http://www.microscopy.com>

SPECIFIC PROTOCOL OR TECHNIQUE RESOURCES

For more information on the topics listed below, please check the websites or publications listed below.

BINNING EXPLAINED

<http://www.aecom.yu.edu/aif/instructions/Binning/index.htm>

EM INFO FOR *CAENORHABDITIS ELEGANS*

Electron Microscopy and Three-Dimensional Image Reconstruction.

By David H. Hall, Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461

Methods in Cell Biology, Vol. 48, Ch. 17, pages 395-436.

IMMUNOCYTOCHEMISTRY

<http://www.uiowa.edu/~cemrf/methodology/cytochemistry/index.htm>

IMMUNOGOLD LABELING

For additional information and literature, including company newsletters: <http://www.aurion.nl/>

IMMUNOGOLD QUESTIONS

<http://www.emsdiasum.com> or Electron Microscopy Sciences Catalog 2007-2010

KOHLER ILLUMINATION

<http://www.aecom.yu.edu/aif/instructions/koehler/koehler.htm>

NEGATIVE STAINING

<http://web.uct.ac.za/depts/mmi/stannard/negstain.html>

OPTICAL IMAGE PROCESSING

<http://microscopy.fsu.edu/primer/digitalimaging/russ/index.html>

Course notes by John Russ, Florida State University