Imaging Cryomicrotome User Checklist – adapted from Barlow Scientific Operational Review

Last updated 3-14-14

This checklist is intended as a practical guide for order of operations. Please start by reading the Barlow ICMT User Guide, which provides additional details regarding operational hazards and software functions.

Warnings:

The instrument includes the following hazards:

Freezing surfaces that can cause frostbite throughout the inside of the freezer and during the sample preparation process. Use insulated gloves and appropriate PPE to protect your skin.

Hot surfaces that can burn, particularly the light source

Intense optical radiation from the light source and reflections, including invisible UV and IR.

Sharp surfaces, particularly the knife blade

Automatic motion – the sample inside the instrument moves automatically, and can crush or cut.

Slicing the sample may generate fine aerosols within the freezer. Wear a respirator when cleaning the freezer.

High voltages are required to ignite the lamp and run the motion controller. Do not open these enclosures and be on the lookout for exposed wiring.

Never place your hands inside the instrument when the instrument is moving or is in an automatic mode, such as slicing or acquiring images

General tips:

To avoid damaging the instrument, observe the following rules:

- Do not rapidly toggle the excitation lamp power. The lamp must be left on for a minimum of 3 minutes after being powered on.
- Secure the tissue mounting plate tightly to the tool holder with the 4 grub screws. Loose connections can result in damage to the blade.
- Take care not to run the sample into the blade and when setting the slicing plane.

In general, leave the foam lid and hinged lid closed whenever possible to conserve energy and prevent excessive ice buildup and thawing.

The lid must be closed when imaging - ambient light will affect the image. Remember that ambient light is broad spectrum and does not have to go through the excitation filter.

When not actively using the instrument, prevent the light source from damaging the excitation filters by using the manual shutter to block the light source, or by switching off the light source.

If the program crashes, your last imaging and slicing coordinates can be found in the automatically generated crash log, located in C:\Barlow Scientific\ICM 2010.UCD\exitlog.txt. The coordinates must be manually re-established when restarting the program.

Preparation

☐   Ensure that neoprene, spandex, and foam surface protectors are in place.
- o   Black spandex material covers the motion area. It is allowed to slide on two steel wires that should be secured at either end using snaps. A neoprene lined cutout in the spandex should fit over the tool post.
  - ▪   The front of the neoprene cutout should clear the bottom of the tool post so as not to obstruct installation of the tool holder.

  - ▪   Attach front and rear guide wires via snaps. Rear guide wires attach to snap points on the rear surface of the aluminum chassis. Front wire guides attach to snaps on the top surface. The attachment points for the wire guides are slightly further toward the center of the instrument than the other attachment points.
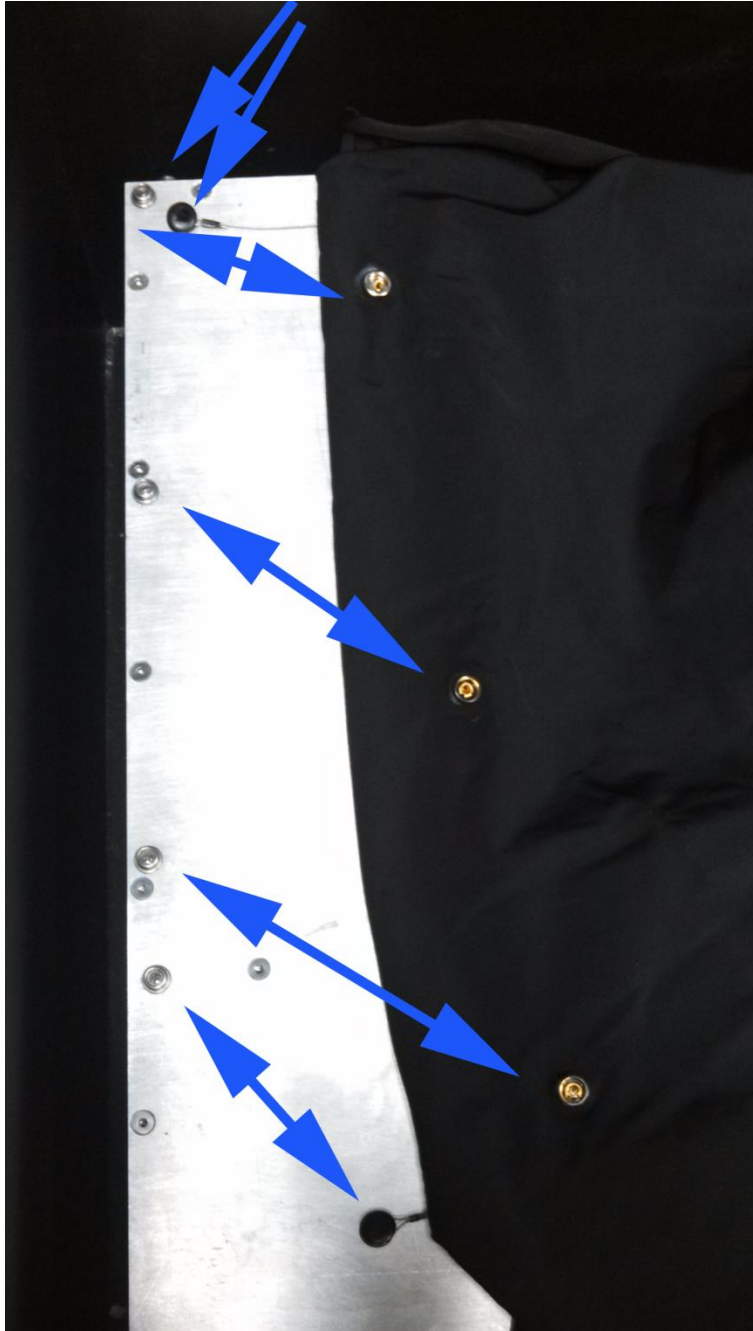
- Attach the spandex to the aluminum frame, 3 snaps per side (in addition to the 2 per side for the guide wires).
-
o A thin neoprene covers the stationary aluminum surface toward the front edge of the instrument. The knife holder should protrude through this cover.
    - Place the neoprene sheet cutout over the blade post, ensuring a snug fit at the bottom.
    - Attach the neoprene sheet to the aluminum frame using snaps: rear-most snaps on right and left attach to points on top of the spandex sheet. In total there are 6 snaps - two on the left, two on the right, and two on the front edge.

- Black foam strips around the back and side edges of the instrument.
  - Tuck in spandex and neoprene first
  - Rear piece of foam may not seat completely.

  -
- Put 3-piece black foam lid into place.

- Close lid.
- Turn Freezer on/Bring down to temperature (requires ~24 hrs)

- Mount sample
  - Embed/mount sample on plate
    - Pour the Base
      - The PVA is poured in two layers: a wide base about ½"-1" deep is used to cover the aluminum sample holder, and then a smaller form is used on top of it to encase the sample.
      - Construct a form of Teflon sheets or non-stick material on the aluminum mounting plate. The form should go between the square protrusions on the mounting plate, and should cover an area at least as large as the field of view of the camera.

Figure 1 - example of a form for pouring the base

      - Cool the mounting plate on dry ice.
      - Chill the 8% PVA/4% Lamp Black mixture as much as possible without freezing.
      - Pour the PVA into the form to create a base layer that extends a minimum of ½" above the top of the mounting plate. Let freeze completely on dry ice or in a -20C freezer. Dry ice will provide faster freezing, resulting in less leakage through the gaps in the form.
      - Remove the form (unless using the same form to embed the sample). The result should be a plateau of black frozen PVA on which you will mount your sample.
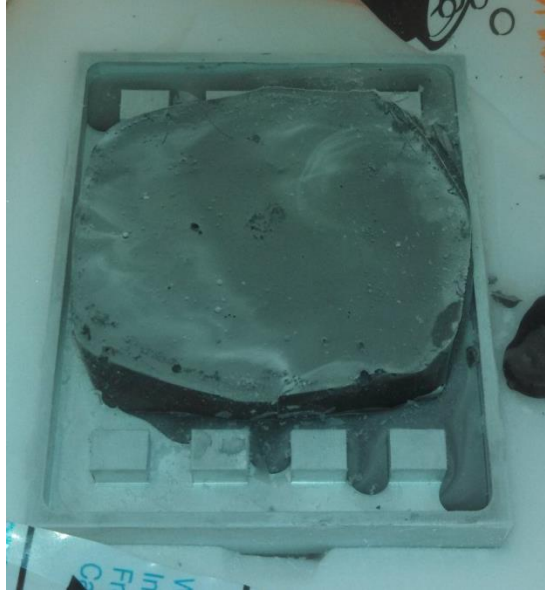
Figure 2 - base layer

- Mount the sample
  - This step is best done on dry ice to facilitate faster freezing.
  - Create a second form that will contain your sample. The form should feature a point or rounded edge in the direction that the blade will approach from for best slicing.
  - If the sample will be adversely affected by thawing, pre-chill it on dry ice and apply several thin layers of PVA/lamp black directly to the sample to create a protective layer. Freeze completely after adding each layer.
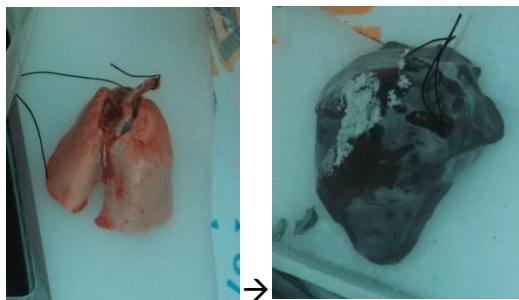

Figure 3 - Protective layers of PVA on sample

  - Use a block of metal soaked in a warm-water bath or a heat gun to melt the top layer of the PVA base before applying the next layer of PVA. Failure to do so will result in a weak seam that will crack during slicing.
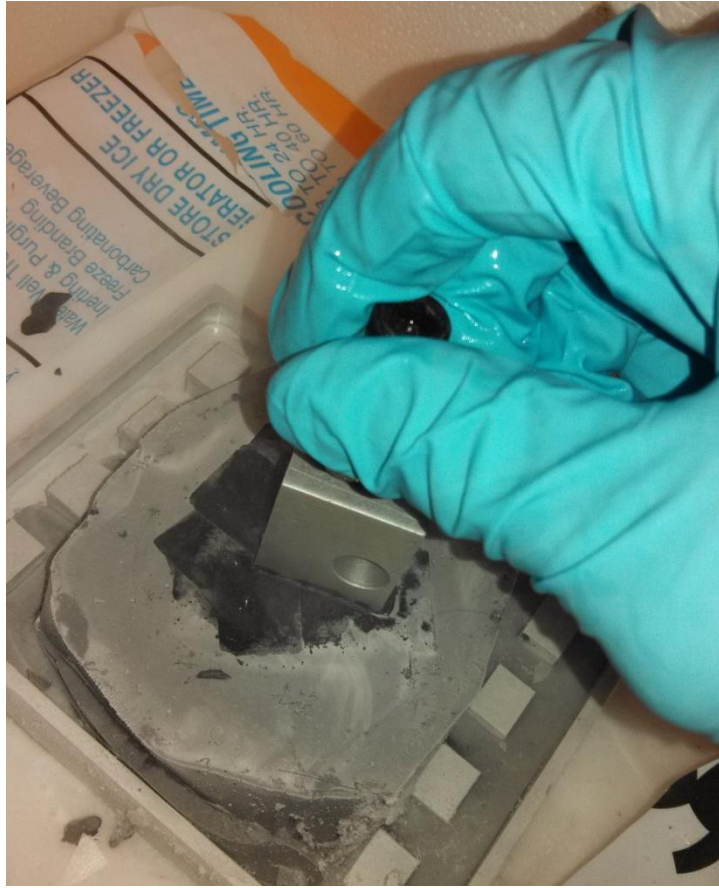
Figure 4 - Using a heated aluminum block to melt the top of the base

- Place the form on the melted top layer and pour about ½" of PVA.
- Once leaks along the bottom of the form have frozen over, insert the sample into the form in the desired orientation and fill the form with PVA until the top of the sample is covered.

- Allow to freeze fully, in a closed container on dry ice if possible.

Figure 5 - Sample embedded in PVA, form still in place. The blade will slice from the right.

- It is often desirable to include a representative sample of the material to be imaged (for example, a spare piece of fluorescent material of interest) at the top of the PVA block. This will allow you to set your imaging parameters in accordance with the fluorescence of the material you wish to image before you slice as far as the actual sample.
- Remove form
- Mount plate on tool holder
  - The blade will travel from the right side of the sample to the left on each pass – keep this in mind when orienting the sample.

- Mount plate using an allen wrench and four 3/16" grub screws. Tighten all 4 screws.
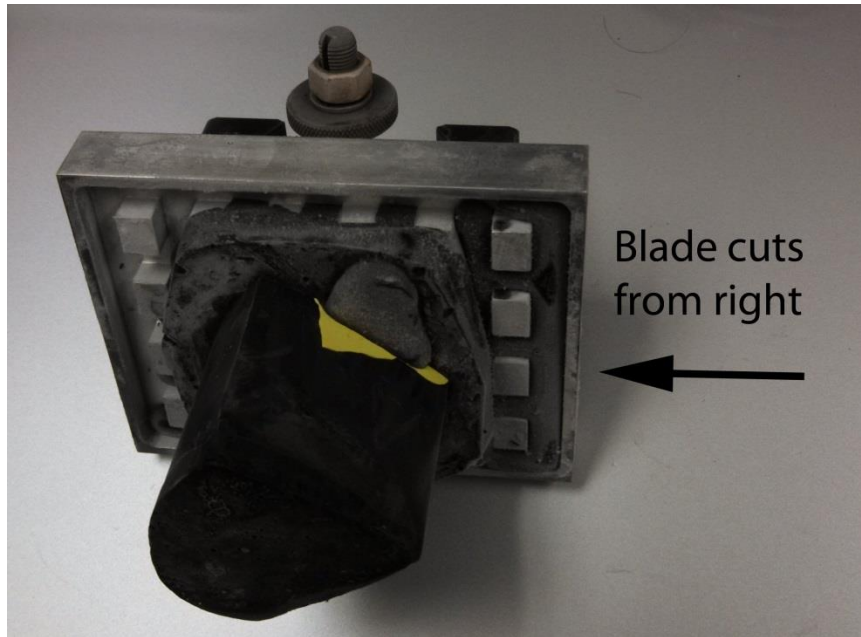

Blade cuts from right

Figure 6 - Sample and mounting plate attached to Tool Holder

- Tighten all 4 screws again – some often become loose when pressure is applied to the neighboring screws.
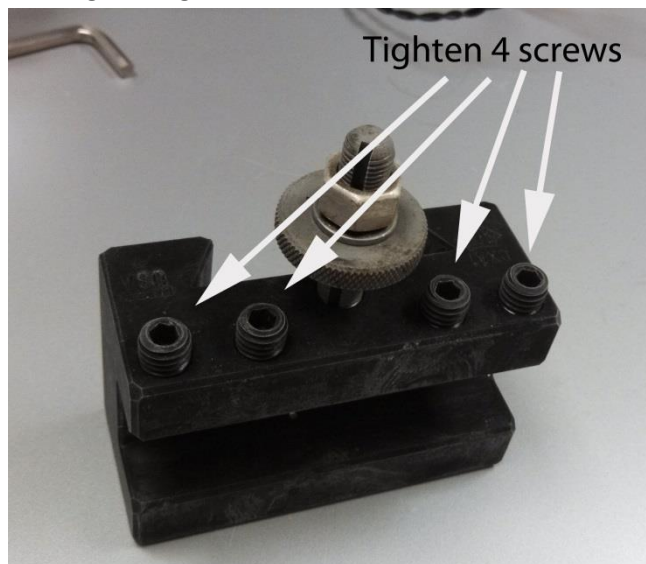

Tighten 4 screws

Figure 7 - Re-tighten these 4 screws

- A loose connection could result in movement of the mounting plate during slicing and damage to the machine or sample.
  o Mount toolholder to toolpost in machine.
    - Freezer should be cold, light source should be off or blocked, orange blade guard should be in place, motor control should be unpowered.

- Insert the aluminum handle (~6" piece of aluminum with a black knob on the end) into the toolpost and turn counter-clockwise ¼ turn to open the toolpost. Slide the tool holder dovetail down onto the toolpost until the aluminum wheel at the top of the tool holder meets the toolpost.
- To adjust the height of the sample, adjust the aluminum wheel.

- Lock the toolpost by turning the handle clockwise (~1/4 turn).
- Remove the handle and store outside of the freezer.
- Test the sample for play or signs of looseness.

Instrument Startup

☐ Warm up Lamp
   - Insert Lamp Shutter
   - Turn on Lamp
      - Switch on power to power strip on floor.
      - Switch on power supply for lamp.
   - Adjust lamp current to 11 unless otherwise instructed.
   - Warm up for 20 minutes before beginning imaging.
   - DO NOT shut down the lamp for a minimum of 3 minutes after powering it on. This can damage the lamp.
☐ Verify ICM Status
   - Ensure that neoprene, spandex, and foam surface protectors are in place.

- Black spandex material covers the motion area. It is allowed to slide on two steel wires that should be secured at either end using snaps. A neoprene lined cutout in the spandex should fit over the tool post.
- Thin neoprene covers for the stationary surface toward the front edge of the instrument. The knife holder should protrude through this cover.
- Black foam strips around the back and side edges of the instrument.
    - Ensure plastic chip tray is in place
    - Ensure that separator panel is in place between camera and light source
    - Verify that knife is well secured and in good condition
        - Check that knife and knife holder are mounted securely
        - Remove the orange protective cover from the knife.
        - Visually inspect for nicks, dullness.
        - DO NOT touch the sharp edge with your hands.
        - Verify that knife is securely attached to post and that post is secured to base.
    - Check for correct optical filter installation
    - Confirm that viewports are clear and free of frost/condensation
        - Clean with lint-free cloth or lens paper if necessary. Do not touch filters directly – clean only viewports (clear glass surfaces). If necessary, clean glass surfaces (not filters themselves) with glass cleaner.
    - Remove camera lens cap
    - Inspect rubber band on camera barrel and replace if necessary.
    - Close all panels and doors on the silver enclosure that houses the lamp and camera.
- Turn on motor controller
        - Both white switches on the side of black motion control box to the 'up' position
- Turn on computer
- Power on filter wheels and camera
        - Switch on power strip on floor (unless already on)
        - Plug in power connectors for 2 filter wheels and camera
- Start ICM Program
        - Execute ICM2010.UCD shortcut on the desktop.
    - The program will start, read the initialization file with the run settings, and send the tissue sample to the home position. Note that this involves automatic movement of the sample. Be sure hands are clear of the interior of the machine!

Instrument Configuration

- Load initialization file if applicable
    - On startup, the program will prompt you as to whether you'd like to load an initialization file or use the default. The initialization file contains saved settings such as filter combinations for imaging. If you have previously saved or edited a *.ini file for this purpose, choose yes, otherwise choose no to load the default settings.
- Confirm that filter wheels have correct assignment (Setup→Filter Wheel)

- o Do not look directly into the light source – wear UV protective eyewear and determine excitation filter position using the light reflected into the freezer.
- o Select Excitation and cycle through the filter wheel settings (0-6). The excitation color should change. If it does not, verify that cycling through *emission* wheel settings changes the *excitation* wheel. If this is the case, the two filter wheel positions are swapped – press the "Swap" button to change them.
- Set Imaging coordinates if sample is visible. Else, set dummy coordinates. (Setup→Sample)
  - o An unfaced (uncut) sample will typically not have a smooth surface to focus on. You must slice until you get a smooth surface to proceed with setting focal plane and to test filter settings. However, the program requires that imaging coordinates be set – move the block to a conservative position near the back left of the instrument and click Set in the Imaging Coordinates region of Setup→Sample.
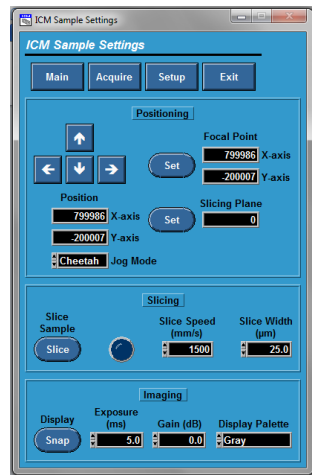
- Create and configure filter combinations and parameters
  - o Applies if desired configuration is known or sample is currently visible. Otherwise, this step may be performed any time before beginning acquisition.
  - o Filter names and save directories(Setup→File)
    - Add, delete, and edit file names and save locations
  - o Filer combinations and exposure parameters (Setup→Imaging)
    - Remove the lamp shutter to allow excitation light to illuminate your sample. Caution: *lamp shutter may be hot!*
    - Filter combinations and parameters can be chosen ahead of time or chosen experimentally if you have samples of the media you are trying to image. This step may be done any time before acquisition. If necessary, perform this step after you have started facing the block and your sample material is showing.
    - Adjust excitation/emission combinations and exposure time, gain.
    - Set increment for each filter combination. For example, an increment of 3 captures an image using this filter combination every third slice.
  - o Save settings if desired for future use

- In Setup panel, click "Save Settings" button. This generates an *.ini file that you can load when the program starts.
- Set slicing plane (Setup→Sample→Set Slicing Plane)
  - Using the arrow keys on the screen or the keyboard, move the sample to within 1 inch in the Y (forward-back direction) and 0.25 inch in the X (left-right direction) of the blade.
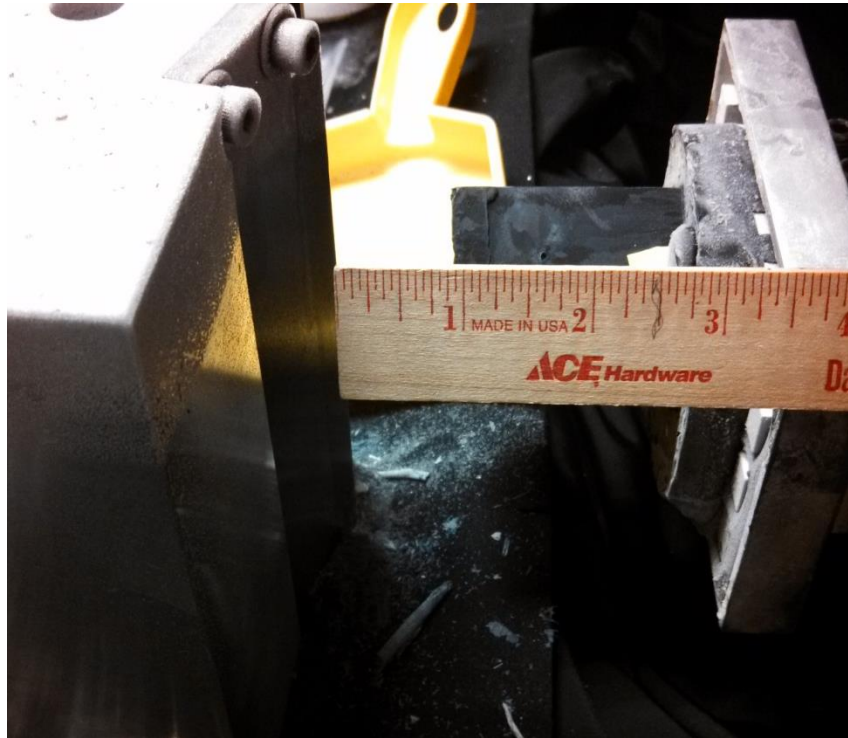

  - Once within this range, switch to a medium jog speed, and position the sample so that the furthest protruding part of it is within 1/8 inch of the blade but DO NOT touch the sample to the blade – running the sample directly into the blade can damage the blade.
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- o Once within 1/8" of the blade, click "Set slicing plane"
- ☐ Slice until the blade engages the block
  - o Set the slice speed to 1500 mm/sec and the cut depth to 150 um unless otherwise directed.
  - o Click "slice" to begin taking the slicing process. The process will continue until you click "stop."
  - o Once the blade starts to remove material from the sample block, click "stop" and adjust slice depth (next step).
- ☐ Set slice depth to 100um and slice sample until there is a flat surface across the front (Setup→Sample→Slice). Reduce cut speed as necessary to achieve good slicing.
  - o Set depth to 100um and click "Slice" to begin. Slicing will continue until you click the "Stop" button.
  - o Stop slicing before you reach your sample, since images are not recorded during the phase.
  - o If you have embedded a piece of "test" material in the block above the sample, slice until the test material is exposed.
- ☐ Attach a small piece of grid paper to the sample surface.
  - ▪ Grid paper is available in the back of the Barlow ICM instructions binder. A piece at least 1cmx1cm is recommended.
  - ▪ A drop of water on the back of the grid paper can be used to freeze the paper to the surface of the sample block.

Flat Surface

Grid Paper

Embeded Sample Material
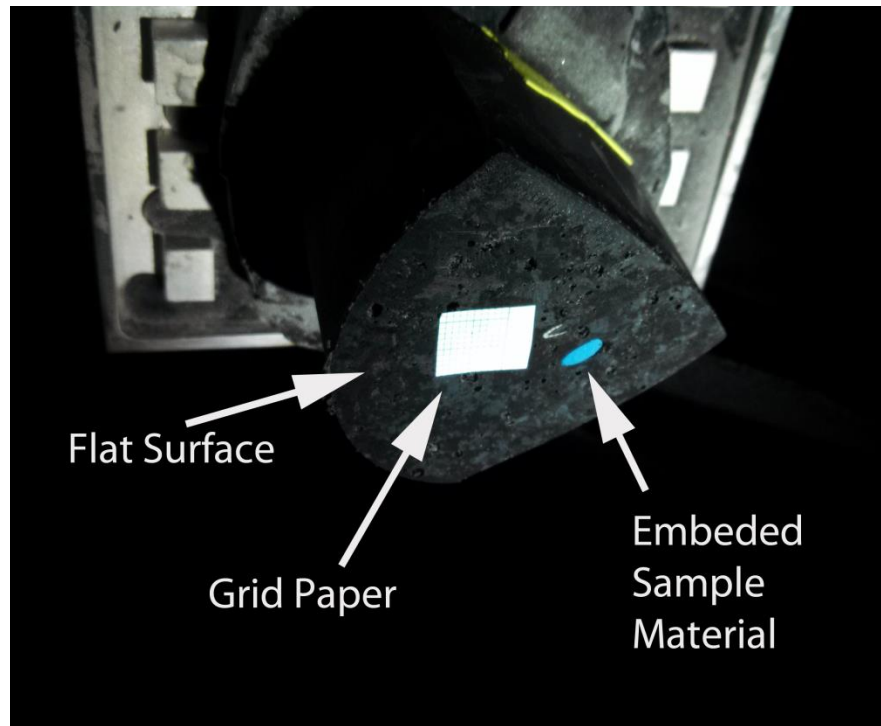
- Position sample in field of view and set focus and magnification by adjusting camera and sample.
  - In Setup→Sample use the arrow keys to position the sample in the X axis (left-right) and position the Y axis at approximately -200,000.
  - Use the camera lens adjustments to set zoom and focus.
    - There are 4 adjustable parameters on the camera, which can be used to achieve the desired focus and level of zoom. The parameters are zoom, focus, distance from camera to subject, and camera extension rings. If possible, camera zoom (large barrel on camera lens) and focus (front ring on camera lens) should be used to achieve the desired results. To adjust the camera, go to Setup→sample and choose exposure and gain parameters that give a viewable image. Then click the "Snap" button to continuously refresh the camera image on the screen as you make adjustments. If necessary, camera distance to the subject can be changed finely by moving the sample forward and backward or coarsely by moving the camera itself forward and backward using the adjustment screw below the camera. To do so, loosen the screw ½ turn while supporting the camera, slide the camera to the desired position, and tighten the screw.
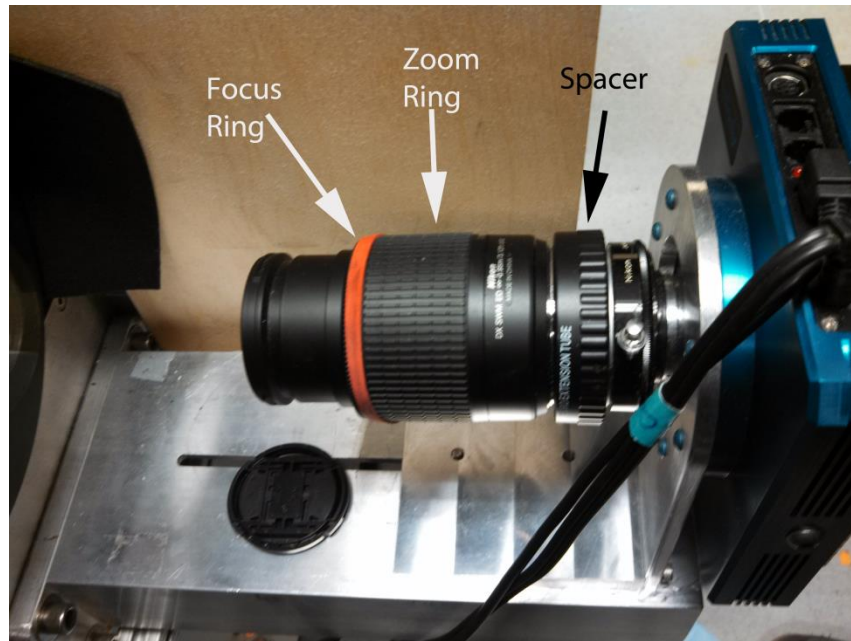
- If these 3 parameters do not give the desired result, change extension rings. The extension rings change the distance between the imaging sensor and the lens (the focal length). A longer focal length results in a more 'macro' shot – more zoom, and a closer focal plane, meaning the sample should be closer to the lens. Adding extension rings has the same effect as turning the zoom barrel so that the lens is extended, zooming in on the sample. Only add extension rings if you are unable to zoom in far enough, or remove extension rings if you cannot zoom out enough.
    - **Depth of field:** The camera does not give us control of aperture. Our only control then of depth of field (the range over which objects will be in focus) is limited to focusing distance, i.e. the distance from the lens to the sample. Placing the camera lens closer to the sample will result in a shallower depth of field. Focal length has negligible effect on depth of field.
    - The rubber band around the camera lens is to prevent accidental movement of your zoom and focus settings.
  - Use the keypad to move the sample in the Y-direction to adjust fine focus.
  - Once proper focus is achieved, set the focal point (Setup→Sample→Set Focal Point)
- Position light beam to illuminate sample surface
  - Remove the lamp shutter if not already removed. Caution: *Lamp shutter may be hot!*
  - Adjust the light beam using the mirror in front of the light source. Fine adjustment is achieved using the two thumb screws on the back of the mirror.
  - Light should illuminate the entire surface of the block.
- Recheck and adjust imaging parameters for each color if necessary
- Double check camera focus at each setting
  - Setup→Imaging, check image at each filter combination
  - The lid must be closed when acquiring images. Ambient light from the room will affect image quality.

- Save an image of the grid paper (Setup→Imaging→Save)
    - This image will allow you to create a size scale later by counting pixels per mm division.
- Slice sample until the fluorescent markers begin to show or the start of the region of interest is reached. Slice depth of approximately 100um is recommended.
- Set slice thickness (Setup→Sample)
    - This is the slice thickness that will be used during image acquisition.
- Double-check the imaging exposure settings
- Triple-check the camera focus.

Image Acquisition

- General note: The lid must be closed when acquiring images. Ambient light from the room will affect image quality.
- Two optional features, "Zamboni" and "Image Check," may be enabled or left off before acquisition begins.

- To enabled Zamboni sprayer, set the slice increment you wish to spray at (Acquire→Zamboni Increment)
    - An Increment of 0 means no spray. An Increment of 3 means it will spray the block face every third slice.
- To enable Image Checking and Notification, click the Image Checking button, select the color number you wish to monitor, and enter your email address in the notification field.
    - The program will check for common imaging errors in the "color" you have selected by looking for slices with no bright spots or slices that are totally saturated, which may indicate an error with the filter wheels. If this is detected, the program will pause and an email will be sent from ucd.icmt.notifier@gmail.com regarding the error.
- Start acquiring images and slicing sample (Acquire→Scan)
    - If you are resuming slicing of a previous sample, set "First slice" to be one greater than the last previous slice number.
    - Set the total number of slices needed to section the entire sample. This will depend on the depth of your sample and the width of your slices. You can stop earlier if you find that the sample has completed.
- Periodically pause (Acquire→Pause) and inspect the sample and brush away shavings from the knife.
    - The period for this will vary, but approximately every 50 slices is a good starting point.
    - "Pause" does not take effect until the current cycle of slicing and imaging is complete. Wait for imaging to finish before opening lid.
- If you need to pause the process and resume later:
    - Click Pause (Acquire→Pause)
    - Wait for the current slice cycle finish
    - Put the orange blade guard in place until you are ready to resume.

- Insert the shutter to block the light source, or turn off the lamp power supply.
- Wrap the sample block in saran wrap
- When max number of slices is reached or sample is finished by visual inspection, stop acquiring (Acquire→Stop)
- Save a Dark-current image for each filter (Acquire→Dark)
  - The camera shutter is closed during this operation. The key parameters here are exposure time and noise.
  - Subtract these dark current images from your images.
    - Requires external software
- Save a white-field image of the fluorescent acrylic sheet
  - Use any filter combination
  - Place a clean fluorescent acrylic sheet in front of the sample.
  - Save an image of the sheet (Setup→Image→Save).
  - Filter the image using a boxcar filter (average value set to 1)
  - Use this image to compensate for non-uniform lighting.
- Save a dark-current image for the white-field image –turn off lamp and cover camera lens
  - Subtract this image from your white-field image.

Shutdown

- Record or save any settings you do not wish to loose in the program, including focal coordinates and imaging parameters
- Double-check to make sure you have a scale image (of grid paper) saved
- Exit the program
- Turn off ICM Motor Control Box
    - Two white switches on the black Motor Control Box to 'down' position.
- Put blade guard on blade.
- Remove fluorescent acrylic sheet from ICM
- Turn off lamp if not already off
- Turn off power to camera and filter wheels
- Collect data from computer, including slice images, dark and light images, grid image, and *.ini file
- Clean ICM
  - If possible, leave the shavings to lyophilize for a day before cleaning to make cleaning easier.
    - Wear appropriate PPE, including gloves and respirator.
    - Use brush and dustpan to collect as many shavings as possible.
    - Dispose of shavings appropriately.
  - Vacuum remaining shavings with shop-vac unless biohazard.
  - Clean shop vac
    - Wipe out and spray container and hose with disinfectant.

- Power down ICM freezer
- Clean and dry ICM
    - Remove foam, neoprene, and spandex surface covers and clean.
    - Position a fan to blow into the ICM while defrosting.
        - This is likely to require 12-24 hours depending on the amount of ice buildup.
    - After the freezer is at room temperature, inspect motion rails and gold encoders for debris or corrosion.
    - Contact CMGI staff for help cleaning the encoders if necessary. Do not touch encoder strips with bare hands. A non-lint cloth and alcohol may be used to gently clean encoders.

Post-Processing Images

- Normalize Images by subtracting the appropriate "dark" image from each image set and then multiplying by a "boxcar filter" to adjust for non-uniform lighting.
    - This can be achieved using user-provided image software or ICMT-provided Matlab code.
    - Matlab code is available on ICMT website or in Appendix 1.
    - Back up your images before normalizing them.
    - Edit the code to include the input and output folders for your image files, your file name root (ie, blue00*.tif), and the location of your dark and brightfield images.

Additional Notes:
The password for the "ICMT User" account on the computer is "Barlow"

Appendix 1: Matlab Code for Image Post-Processing

```matlab
% 3-12-14 Subtract dark value from an image and then multiply by a boxcar
filter
% "Boxcar filter" is an image normalized to 1. The bright field image is used
to create the boxcar filter.
% Each image slice is divided by the boxcar filter to account for
% non-uniform light.

%"12 bit" images (max value 4095) are converted to 16 bit while working
%with them to ensure that we don't exceed the maximum value for 12 bit, since
it is stored in the 16 bit format.
%They are then converted back before saving.
% CDW
clear all
close all
clc

%starting with 12 bit images?  if yes, 1, if no, 2.
startWith12Bit = 1;

%% File handling
inputPath = 'c:\Image Post Processing\Input'; %files to be processed
outputPath = 'c:\Image Post Processing\Output'; %where adjusted files will be
written
fileRoot = 'bl0*.tif'; %the file root of all images you wish to process.  For
example, if all of your files are named GR0###.tif, where ### are varying
numbers, enter 'GR0*.tif';

% Dark and bright images (should not be in same directory as stack images)
bright = imread('c:\Image Post Processing\brightfield_1.tif'); %12 or 16 bit
dark = imread('c:\Image Post Processing\dark-bl01.tif'); %12 or 16 bit

%create output directory if necessary
fullFileName = fullfile(outputPath, 'testout.tif');
if exist(outputPath, 'dir')
%     warning('....')
else
    mkdir(outputPath);
end

%% Create Boxcar Filter from bright image
%convert bright image to floating point
brightfloat = double(bright);

%compute average value of bright image (12 or 16 bit)
% (bright image can be used in 12 bit, since it's just going to be
% normalized to 1.  Result will be same whether we use 12 or 16)

brightmean = mean2(bright);

%create boxcar filter with average value 1.  Boxcar has decimal values
boxcar = brightfloat/brightmean;
```

```matlab
%convert dark image to 16 bit if necessary
if(startWith12Bit)
    dark = immultiply(dark, 16);
end

%% Start batching through files
filelist = dir([inputPath filesep fileRoot]);
fileNames = {filelist.name}'

cd(inputPath);

for i=1:length(fileNames)
    %read a 12 or 16 bit image
    currentImage = imread(fileNames{i});

    %convert to 16 bit if necessary
    if(startWith12Bit)
        currentImage = immultiply(currentImage, 16);
    end

    %subtract dark from current image
    darkSubtr= imsubtract(currentImage,dark);

    %divide 16 bit slice image by boxcar filter
    adjusted16 = uint16(imdivide(double(darkSubtr), boxcar));

    %convert from 16 bit to 12 bit by dropping last 4 bits
    if (startWith12Bit)
        modifiedImage = bitshift(adjusted16,-4);
%         imshow(adjusted12);
    else
        modifiedImage = adjusted16;
%         imshow(adjusted16);
    end

    %write output file
    output_name = fullfile(outputPath, filelist(i).name)
    imwrite(modifiedImage,output_name);
end
```