DEPAR FFPE TISSUE MEASUREMENT PROTOCOL FOR ROUND-ROBIN EXPERIMENT
VERSION 5.0
SUMMARY

Created: 06/06/16

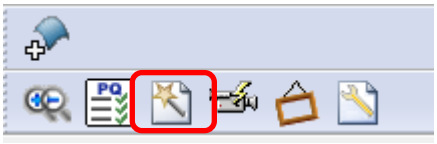
Contents

A. Initial imaging, selection of areas and annotating regions of Interest from H&E sections (performed by CENTRE 1)

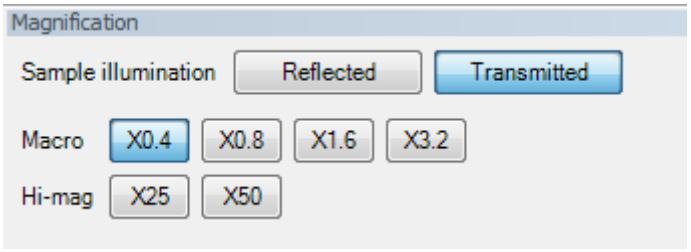
Macro and HiRes montage images of H&E slides

a. Open lid on the 802 by pushing 'Locked' button

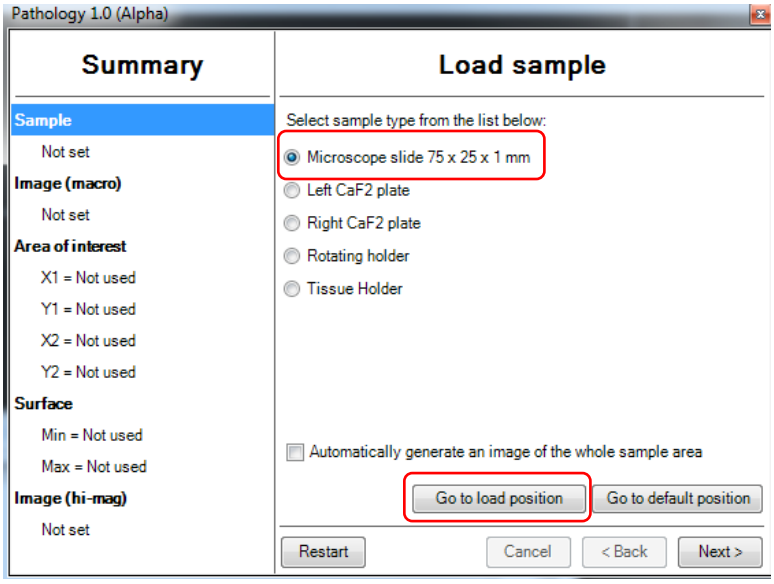
b. Open Wire, click on the 'pathology workflow' button



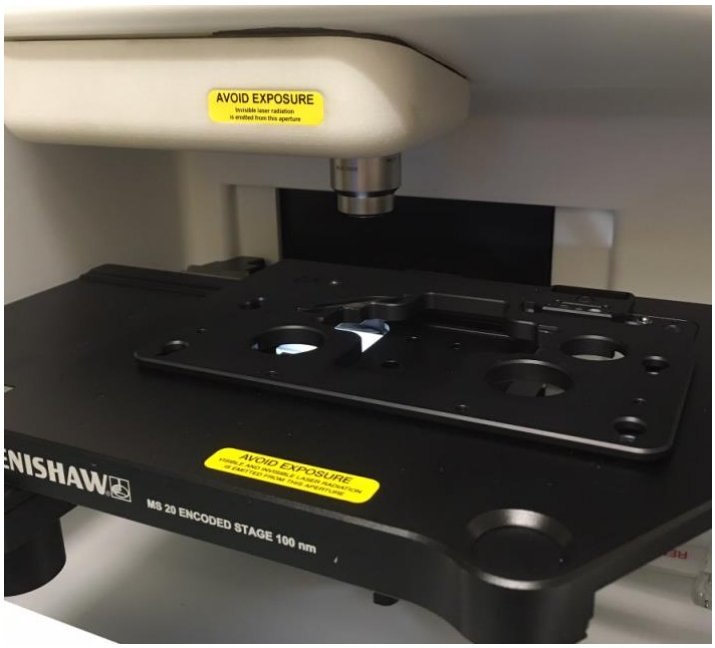
Make sure sample illumination is to 'transmitted' and macro X0.4 is selected on the magnification panel



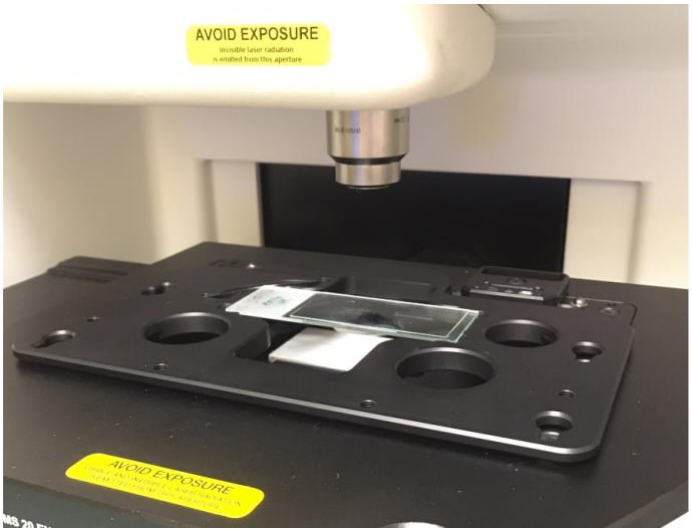
c. Click on the 'microscope slide 75 x 25 x 1 mm' tab and click on 'Go to Load position' button on the panel



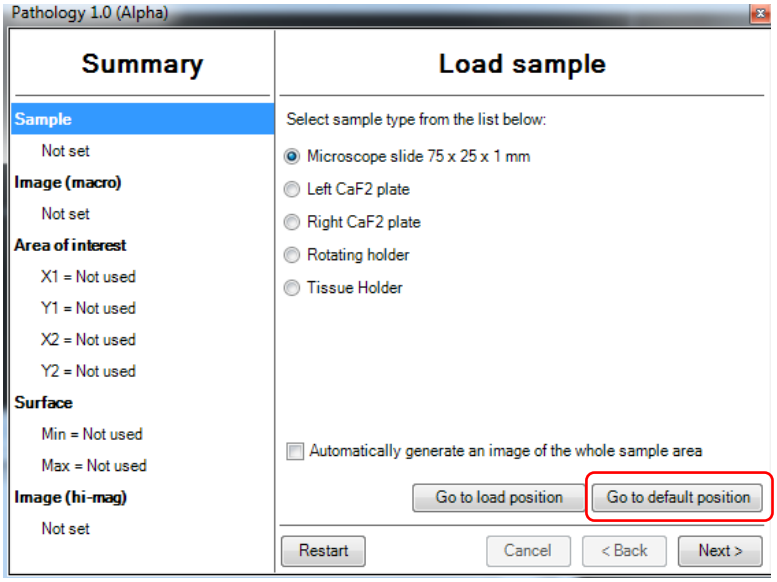
The stage will move down and to loading position

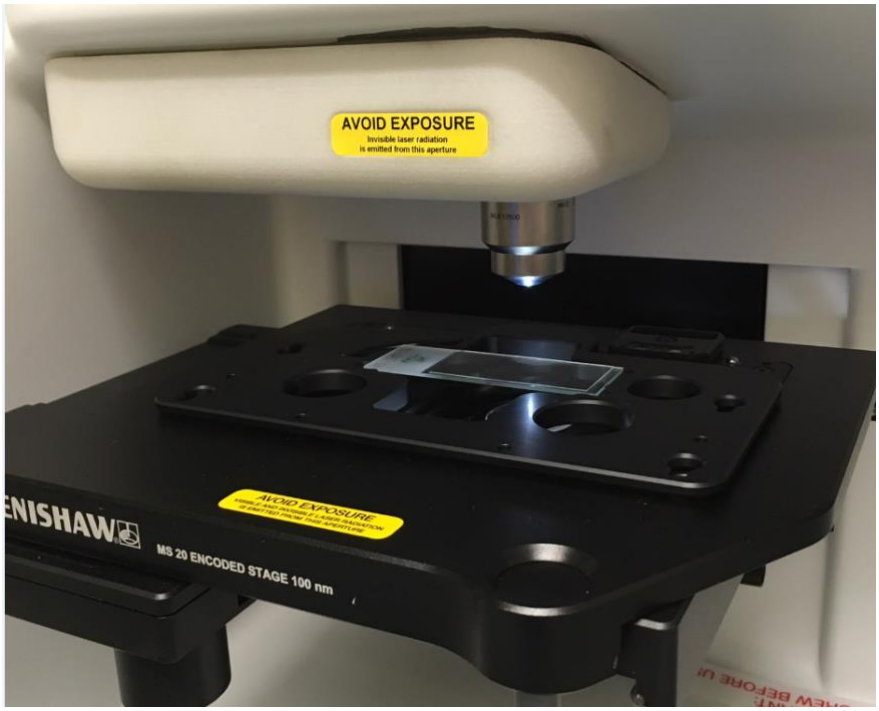


d. Mount H&E slide into glass slide holder and load tray

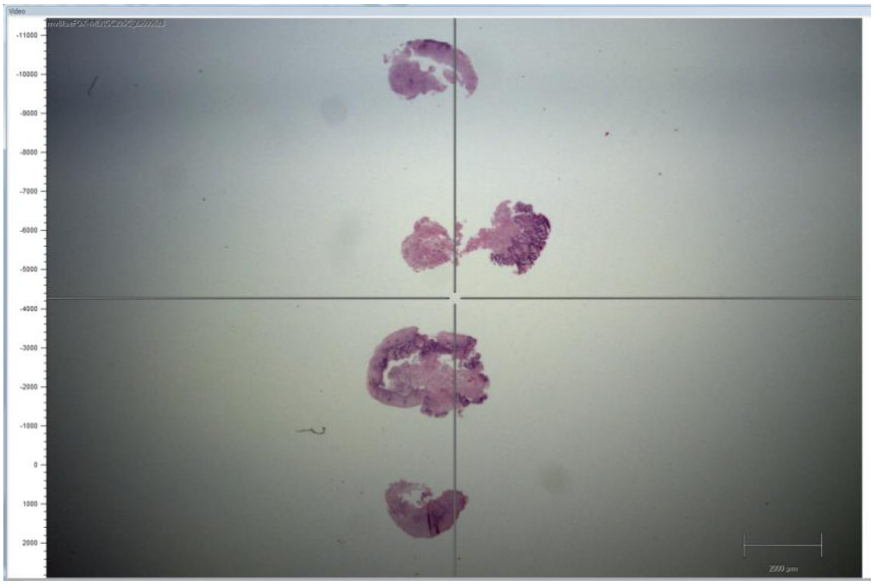


e. Click 'Go to default position' to set stage position and height to default position for microscope glass slides.

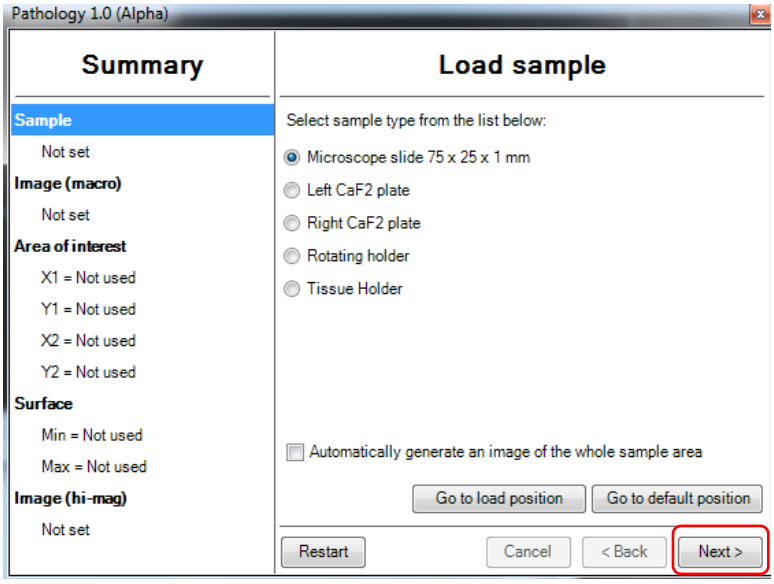




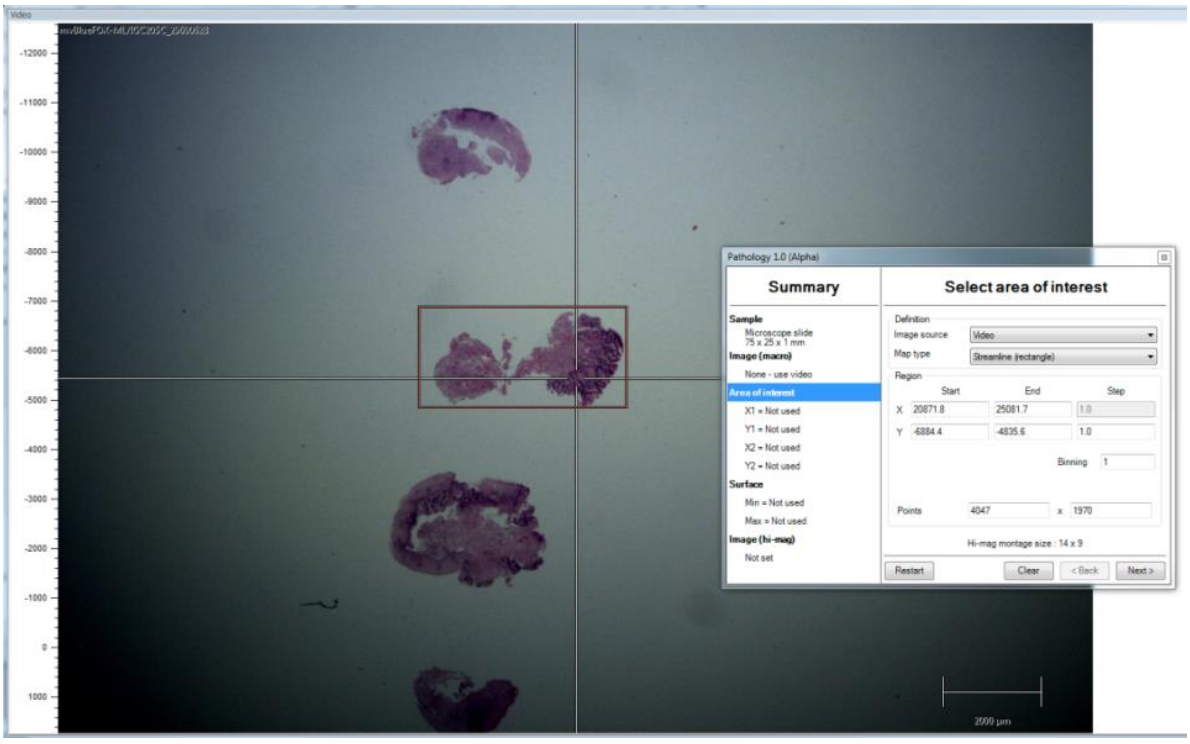
f. When the sections of interest are in view on the live video, switch to HiRes x25 and focus on one of the sections of interest. Once a section is in focus, switch back to macro 0.4x and centre the field of view on the live video using trackball or clicking on the live video window
g. Create single snap image of the low res image using: Live video -> Snap -> Single
h. Save the live macro image as ID_Path_Instit_HE_macro.png using Snipping tool OR Save the image by right clicking over it: Save to -> With Axes
i. Export the surface file using: Export -> Surface file -> save as ID_Path_Instit_HE_macro.srf)



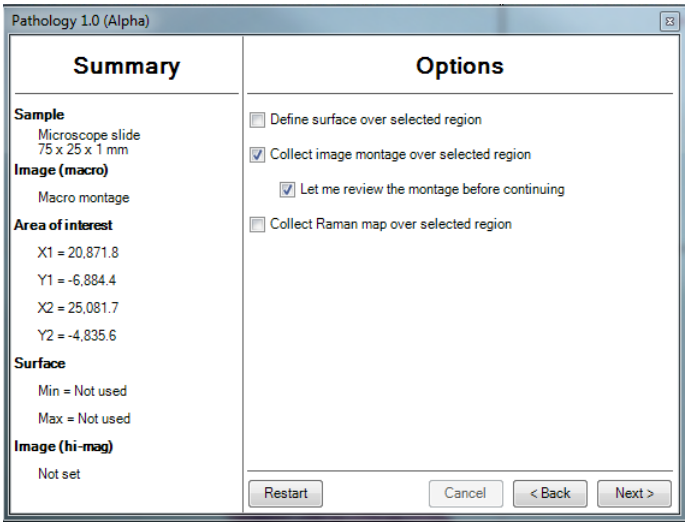
j. Click Next



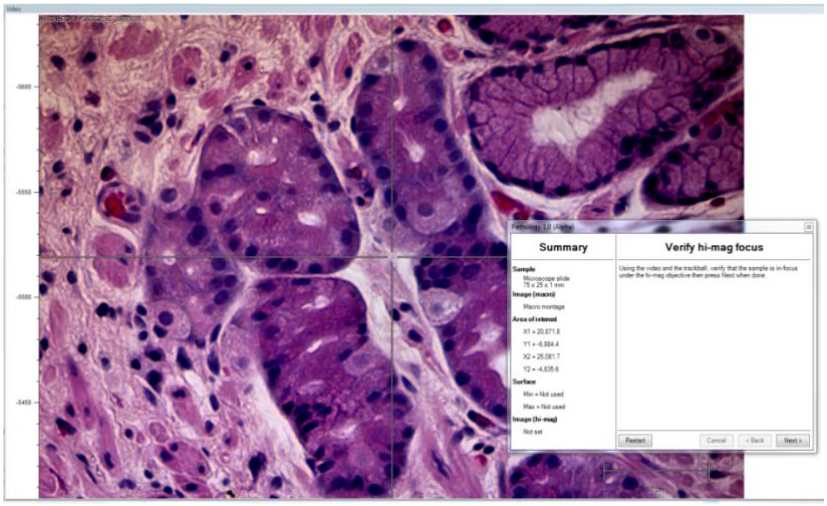
k. Select region on the live video image of the first section to image and click next



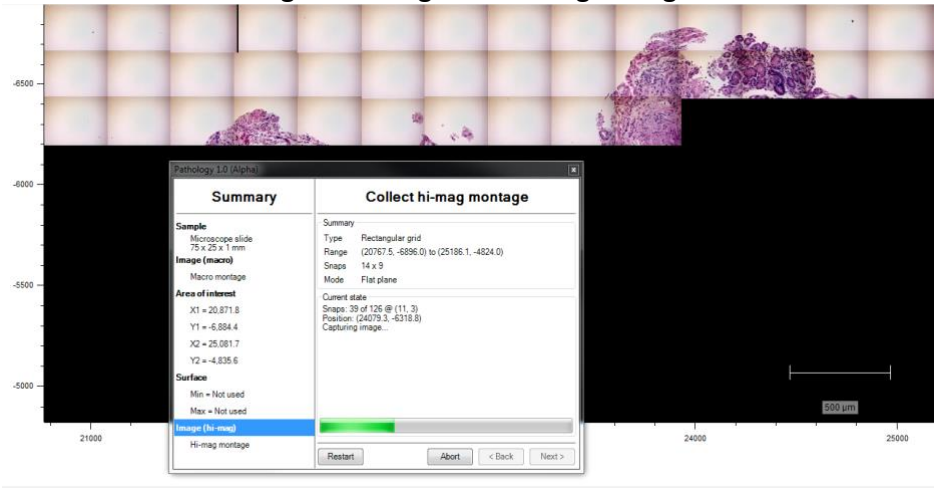
l. Deselect ‘define surface’ and ‘collect Raman map’ and click next



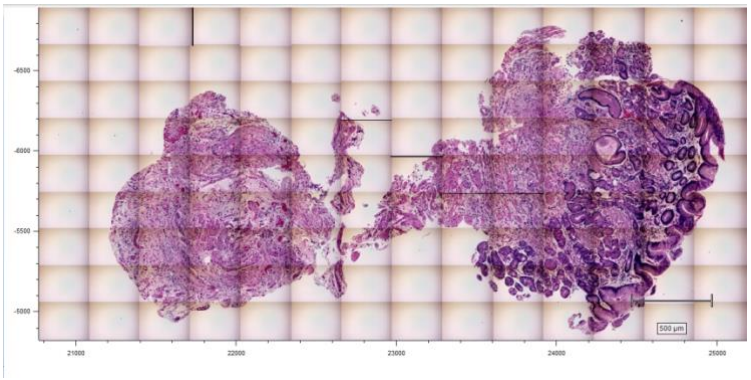
m. The workflow will prompt the user to verify focus of section at x25 magnification (you might have to switch between macro and x25 to get representative area). Click next



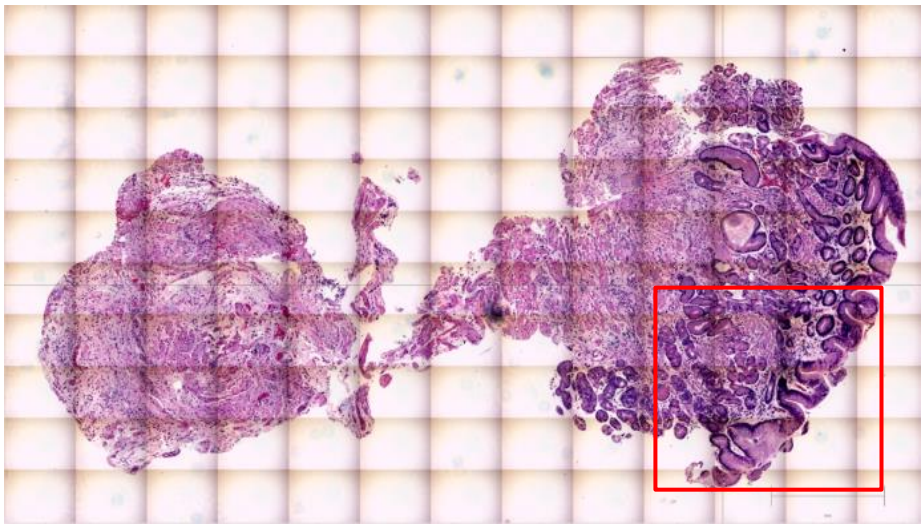
n. The workflow will begin creating the montage image of the selected section



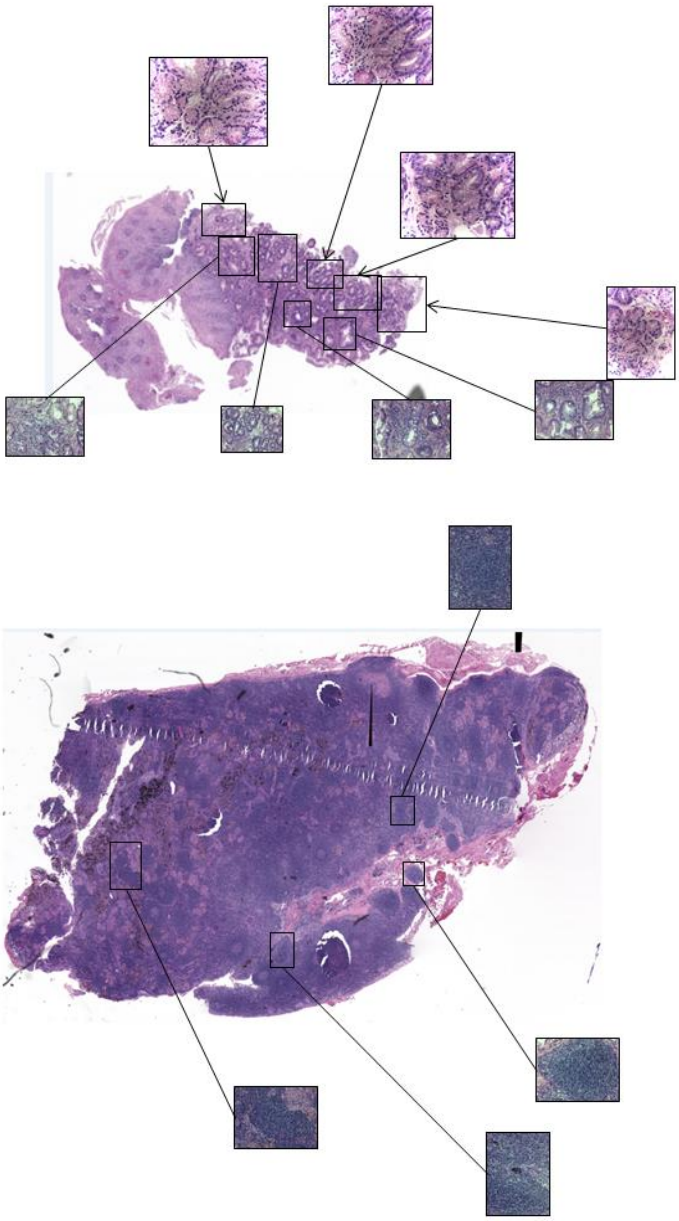
o. Once the montage has been created, Screenshot and save the image using Snipping tool (and Export -> Surface file -> save as ID_Path_Instit_HE_section.srf)



p. Using the x25 in live viewer you can annotate this representative hiRes image with areas of interest for Raman mapping

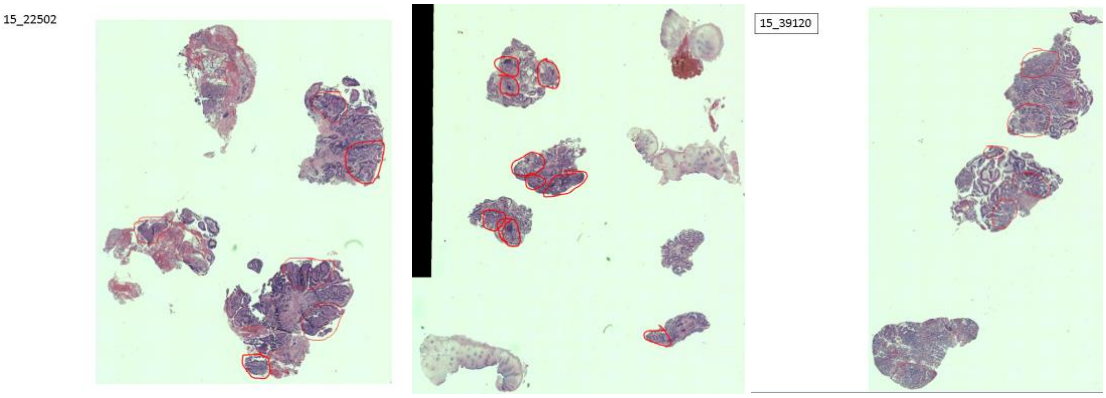


(Performed using 802)

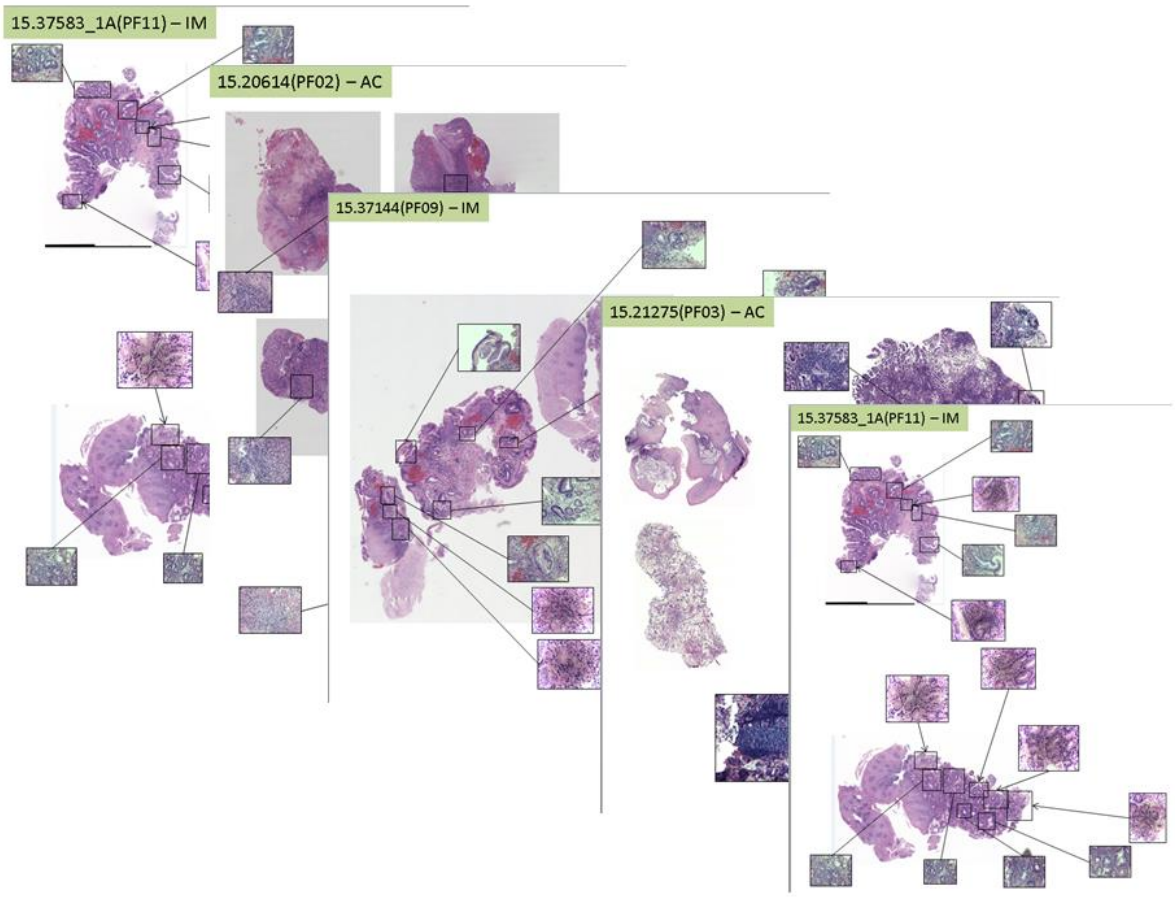


(these representative images and annotations were performed using CENTRE 1's external digital microscopes and 10x and 20x objectives: top is IM and bottom is AC)

q. Repeat this process for different areas of this section as well as regions of interest on other sections on this slide (and Export -> Surface file -> save as ID_Path_Instit_HE_section.srf)
r. Eventually you will build up a collect of representative images from slide samples



s. CENTRE 1 will compile these representatives images, annotations and Hi-mag images into a PowerPoint document for other sites to use and reference for selecting areas of interest on the unstained sections
t. CENTRE 1 will use these images, annotations and original H&E glass slide to get confirmation of pathology diagnosis of selected areas by first the consultant histopathologist and then blinded second opinion by other pathologists



(these representatives and annotations were performed using CENTRE 1's external digital microscopes)

B. Receipt of tissue samples and Raman Spectroscopic Measurements at CENTRE 1 and remote sites

For the remote sites, on delivery of samples, remove samples (in the slide holders) from the bubble-wrap packaging and store at room temperature for future Raman measurements. **Measure samples within a week of receipt to prevent degradation of sample quality.**

Print out digital data which has been emailed (by CENTRE 1) to the sites detailing the regions of interest selected from tissue sections on each slide.

The users at each site will select corresponding map regions at 10um pixel resolution (select 10YBin binning) resulting in ~2000 spectral points per map [not exceeding 6000 spectra points per tissue sample slide]. Each spectral point will be acquired for **6s** resulting in ~30-60 mins mapping time for each region map depending on size. See following section

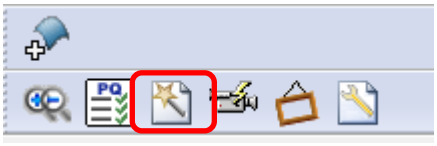
CENTRE 1 will determine the order of preference for the relevant regions on a slide so that all sites are measuring the same regions from each slide. These will be annotated on the PowerPoint slides with the H&E and unstained sections. Users are asked to acquire at least 4000 spectra points in total for each section (~1000-2000 spectra per map).

C. Imaging and selection of areas of Interest from unstained sections using H&E annotated images (performed by all)

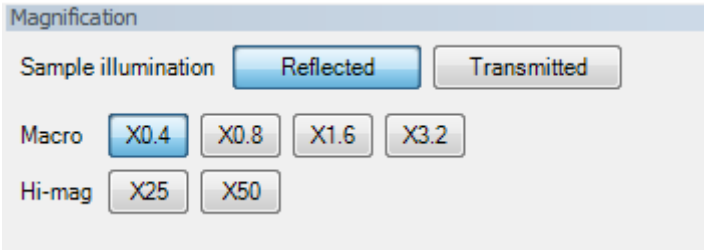
On morning of tissue measurements, perform external standard measurements using protocol: STANDARDS MEASUREMENT PROTOCOL_v2_withAppendix_190516

a. Open lid on 802 by pushing 'Locked' button

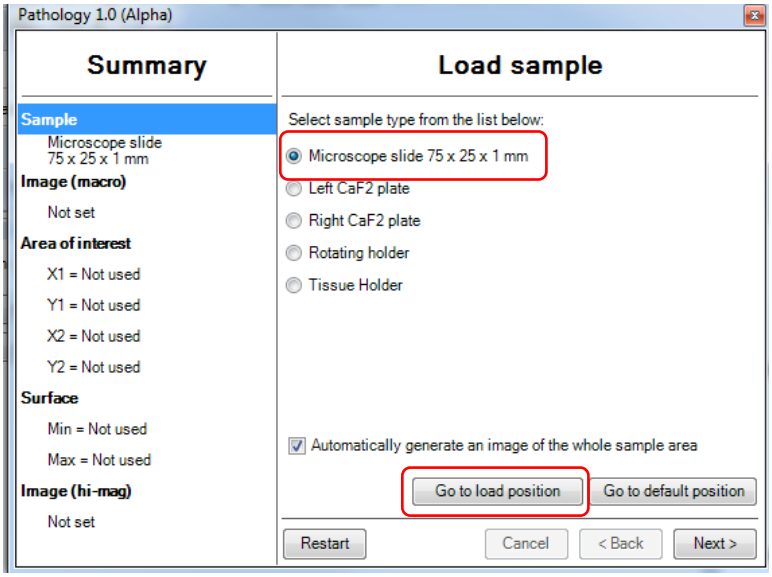
b. Open Wire, click on the 'pathology workflow' button



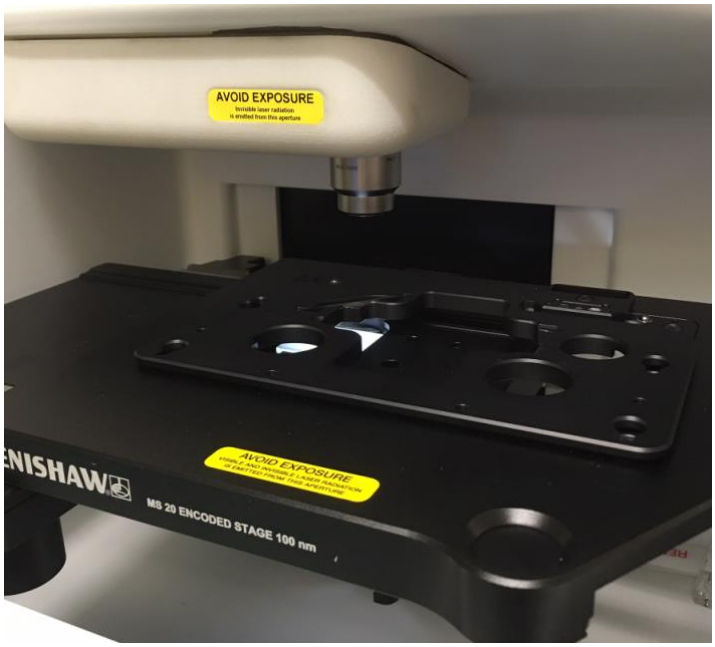
c. Make sure sample illumination is to 'Reflected' and macro X0.4 is selected on the magnification panel



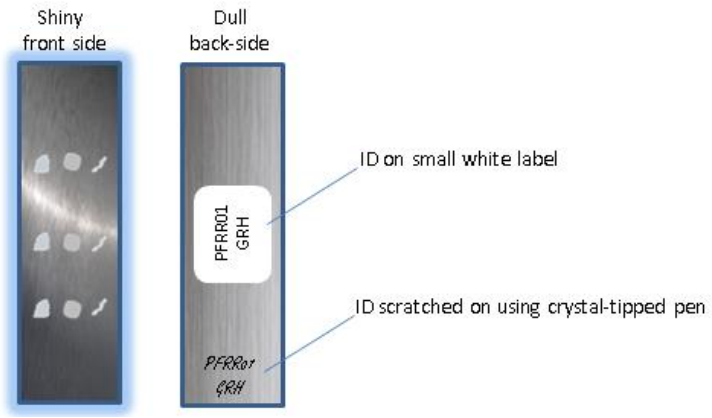
d. Check 'automatically generate an image', click on the 'microscope slide 75 x 25 x 1 mm' tab and click on 'Go to Load position' button on the panel

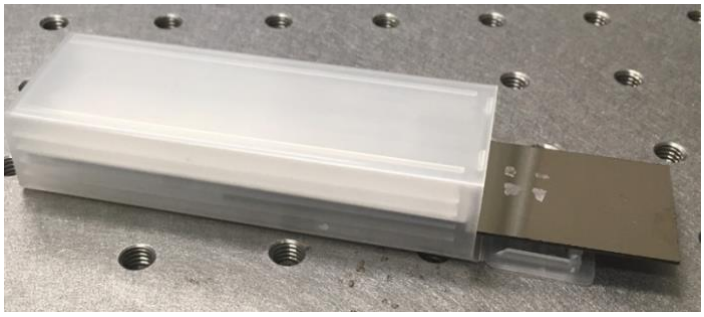


The stage will move down and to loading position



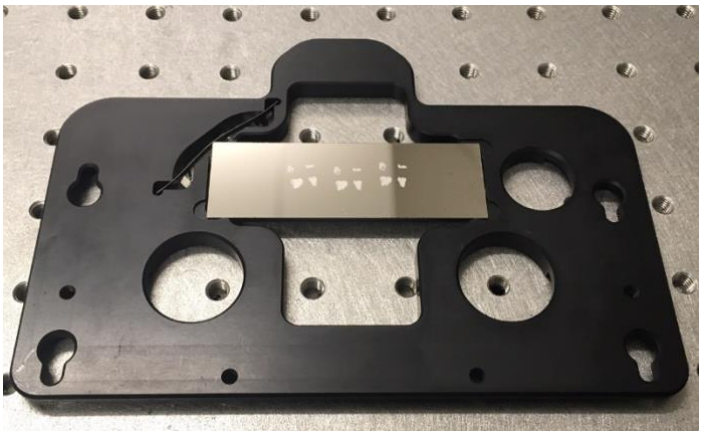
e. Take out sample slide from slide holder. Identification of the slide will be written/etched on the opposite side of the stainless steel slide (duller side) using a diamond crystal pen. The ID will also be clearly written on a white sticker on the reverse side of the slide.

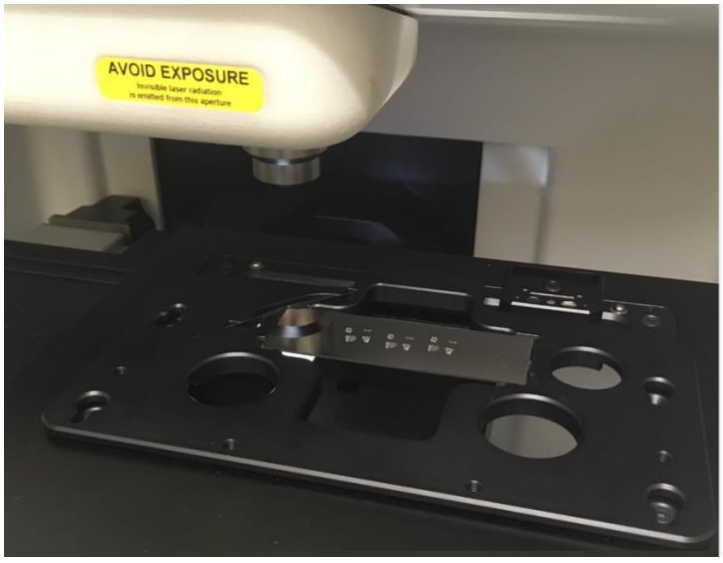




f. Make sure to close slide holder to prevent dust particles from getting on the other slides in the holder

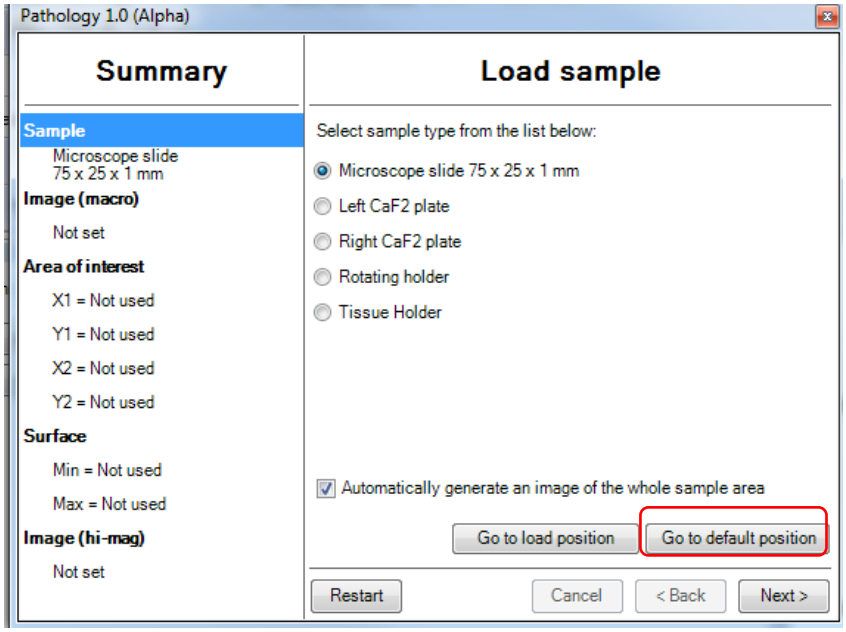
g. Carefully place the stainless steel slide into the ‘microscope slide’ mounting tray shiny side up making sure to orientate the slide similar to orientation of H&E section image

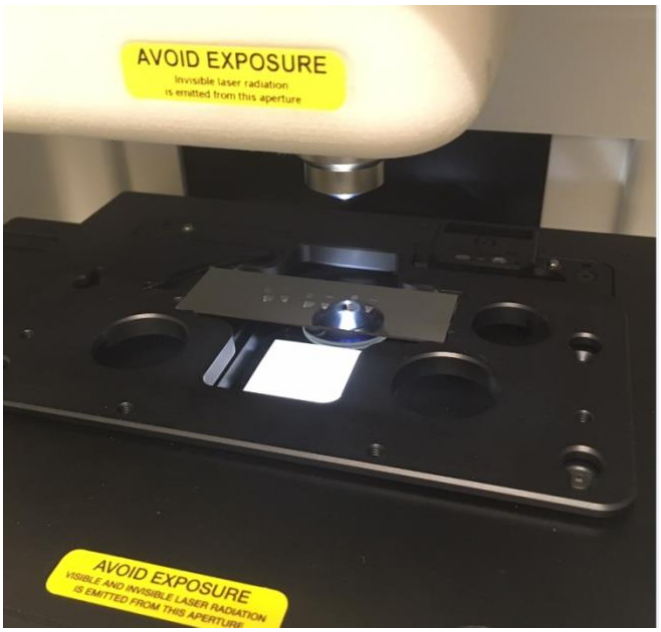




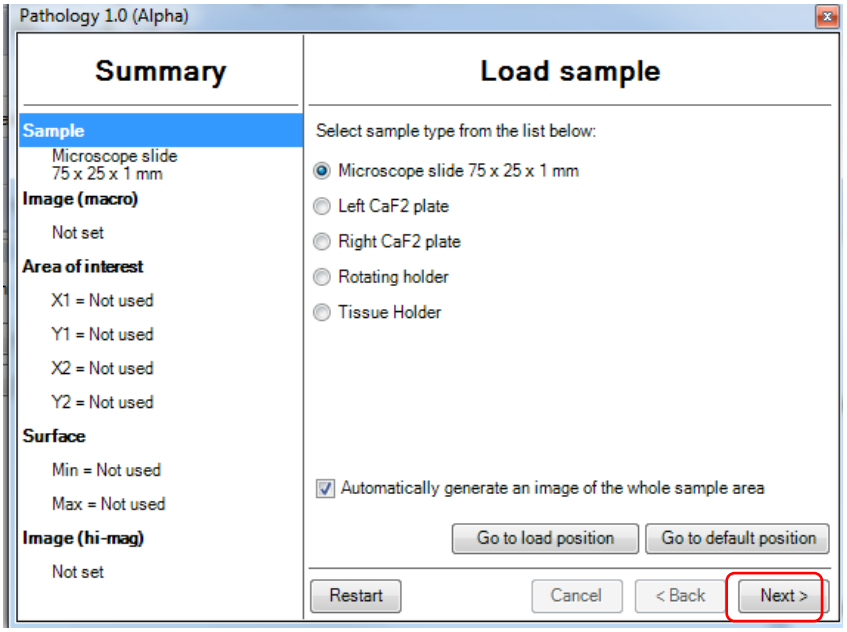
h. Click ‘Go to default position’ to stage position and height to default position for microscope slides.

i. Close lid

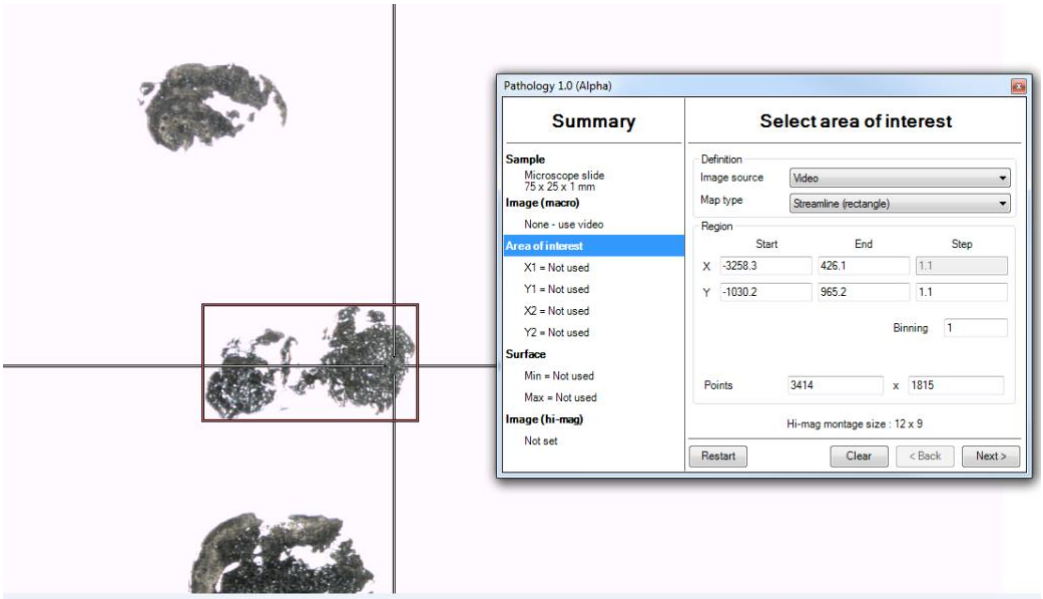




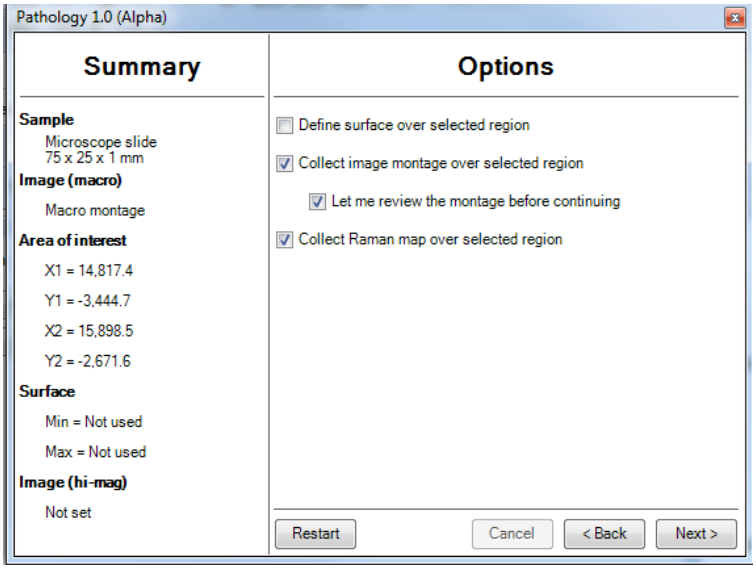
j. Screenshot and save the live macro image using Snipping tool (and Export -> Surface file -> save as ID_Path_Instit_US_macro.srf). Click Next



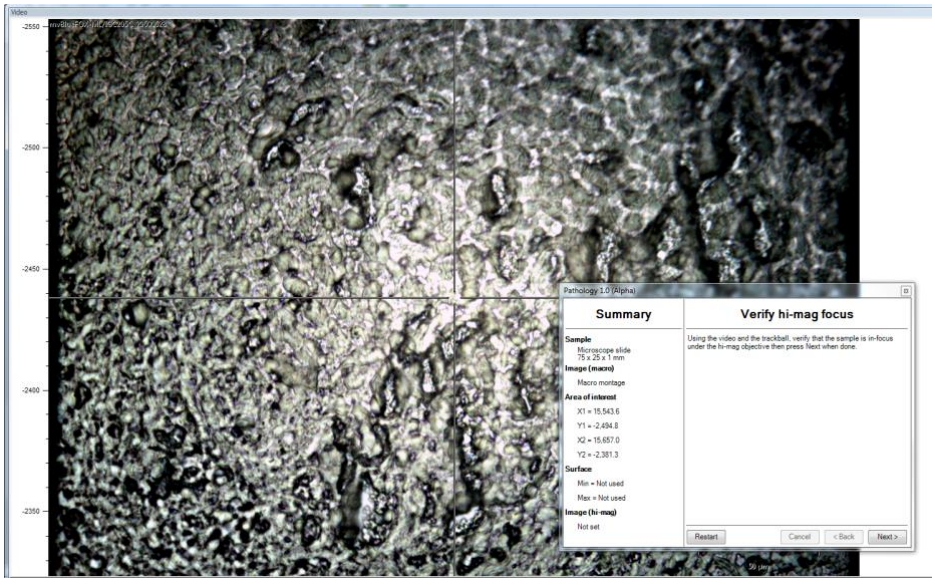
k. Using the annotated H&E images supplied by CENTRE 1, select the correct tissue section(s) to be used for Raman imaging on the newly generated macro montage.



l. Deselect ‘define surface’ and click next



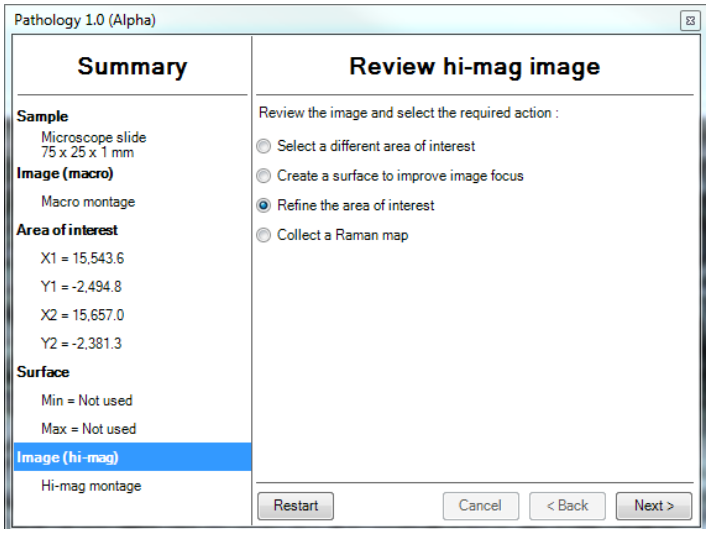
m. The workflow will prompt the user to verify focus of section at x25 magnification (you might have to switch between macro and x25 and move to different position to get representative area).

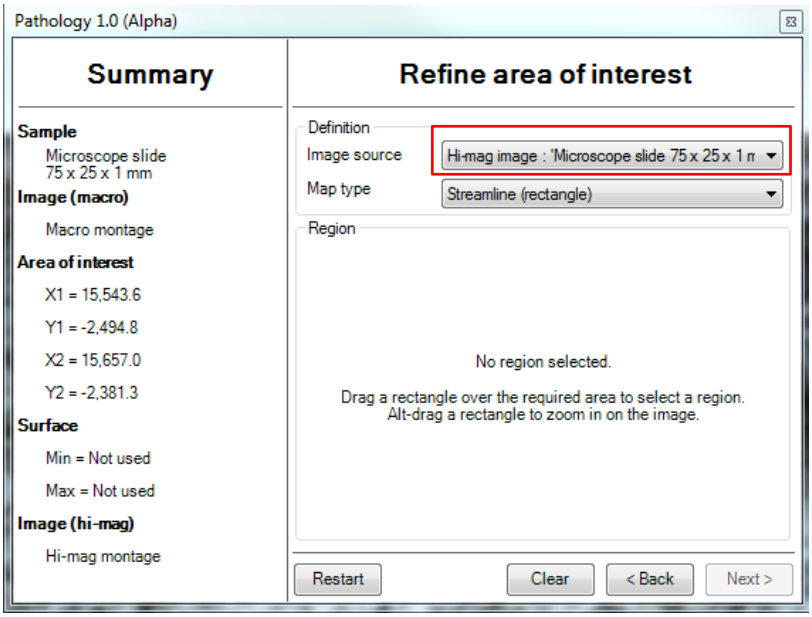


n. Click next
o. The workflow will begin creating the montage image of the selected section
p. Once the montage has been created, Screenshot and save the image using Snipping tool (and Export -> Surface file -> save as ID_Path_Instit_US_section.srf)

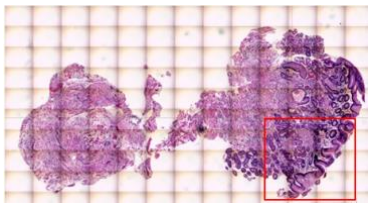
Using Pathology workflow sub-module

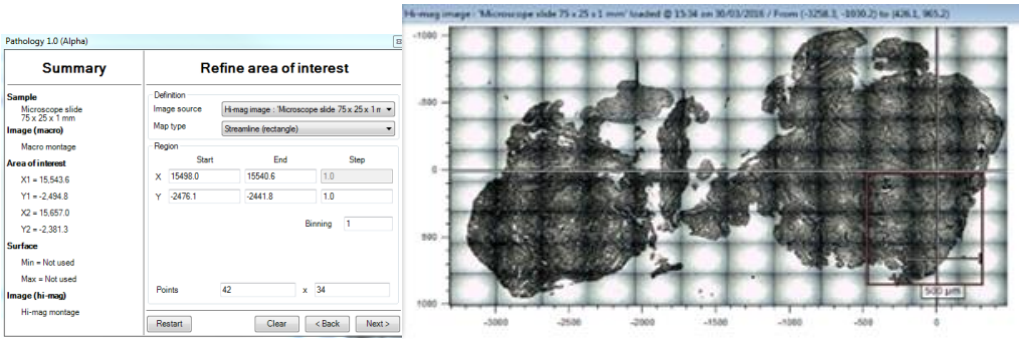
a. Once montage is completed, select 'refine the area of interest' from the next panel to redefine the area to be used for Raman mapping **FROM THE Hi-MAG IMAGE**. Click Next

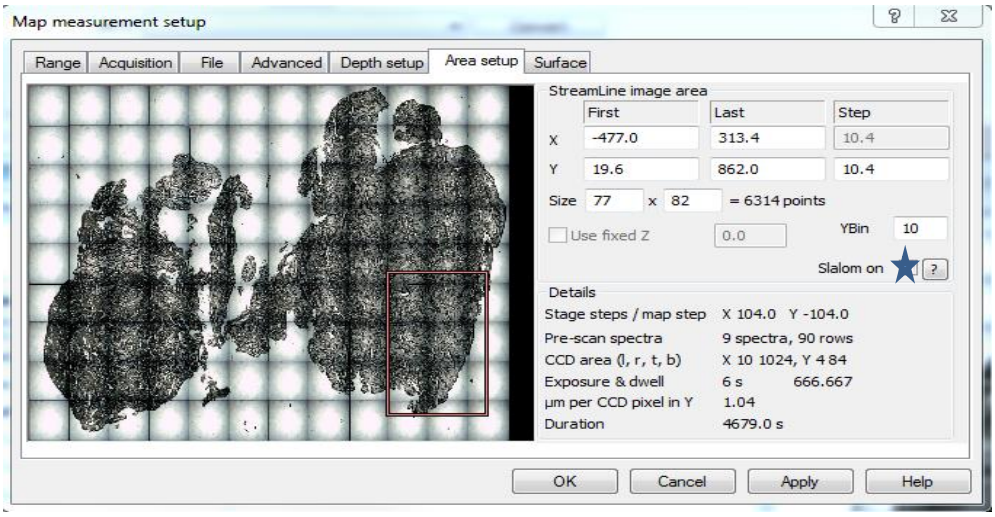




b. Using again the annotated H&E images supplied by CENTRE 1, select requested regions (ordered determined from CENTRE 1) on the live video image of the first section to image and click next



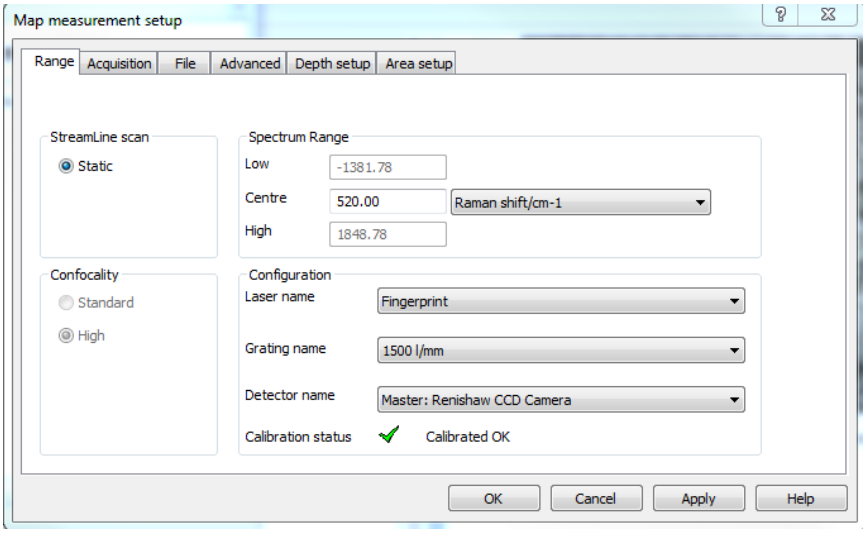




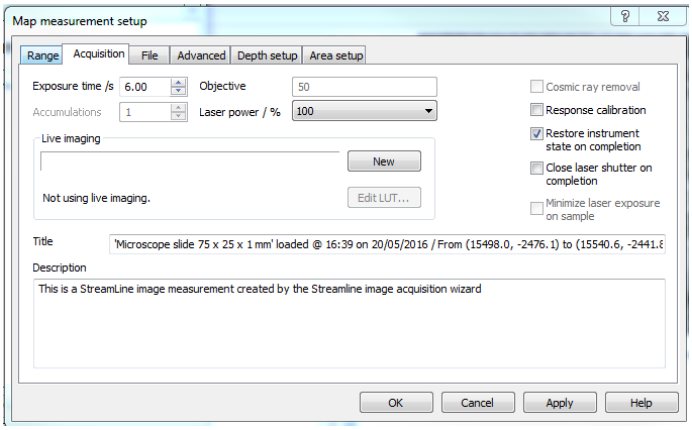
c. **Optional**: Using the x25 in live viewer you can collect and save x25 images from area of interest.

d. **IMPORTANT**: Switch to x25 magnification, focus position at area of interest on section and note down the Z focus position

e. Clicking next will bring up the map measurement setup, click Range tab and set Laser name to fingerprint region

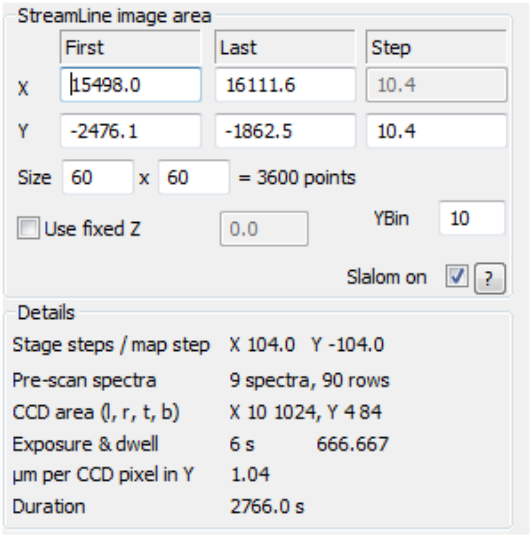


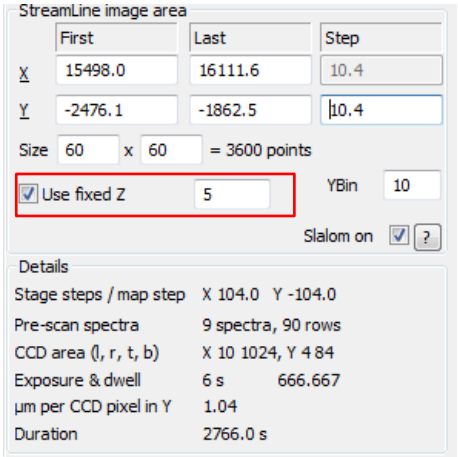
f. Click acquisition tab and set exposure time to 6s.



g. **Click area setup tab and make sure YBin is set to 10 (step size should default to ~10.4um for X and Y) and check slalom on.**

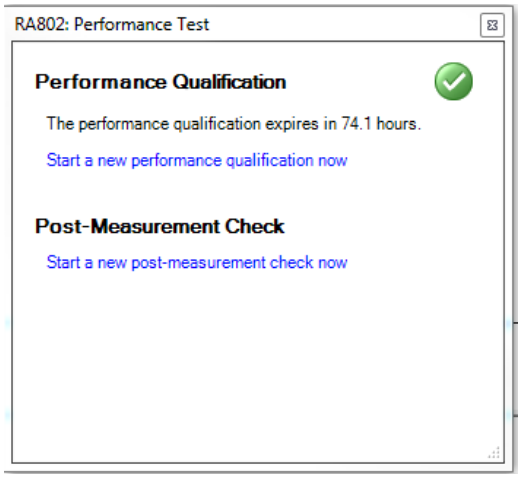
h. **Check the 'Use fixed Z' checkbox and enter the value noted from step d**





i. Click apply and then OK

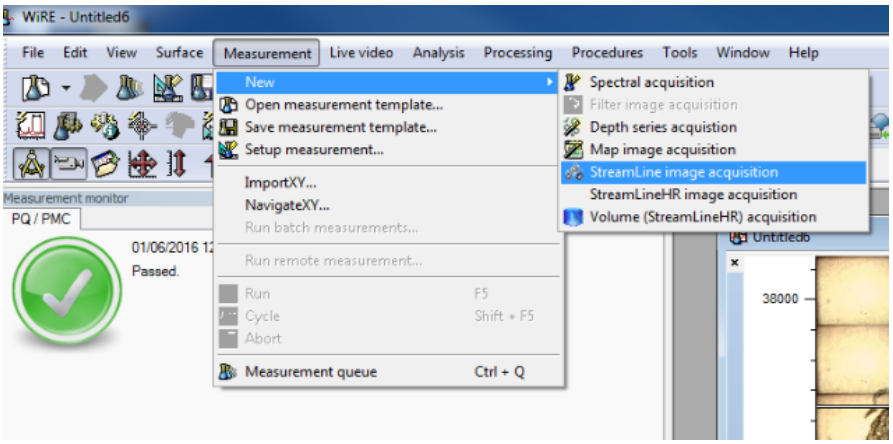
j. Run a PQ before starting Raman map measurement and ensure that PQ passes.



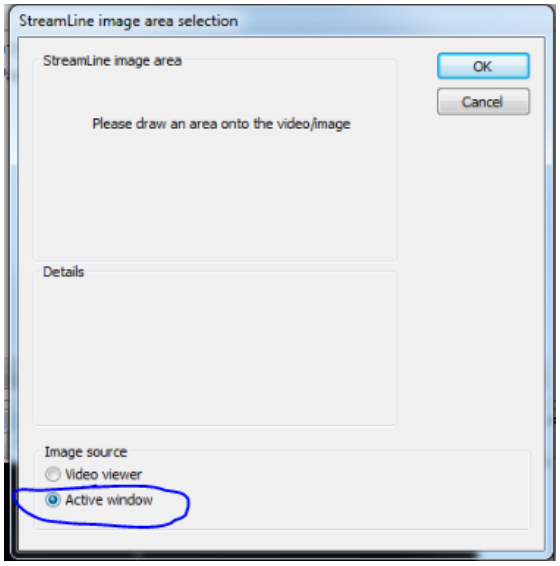
k. Click Run measurement to begin Raman map measurement
l. When map measurement is complete, run a PMC to make sure there is no shift in wavenumber and spectral intensity from PQ has occurred

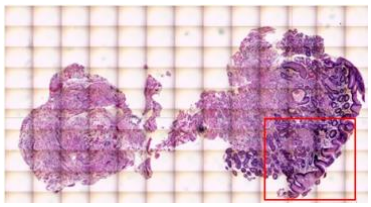
To create additional Raman maps

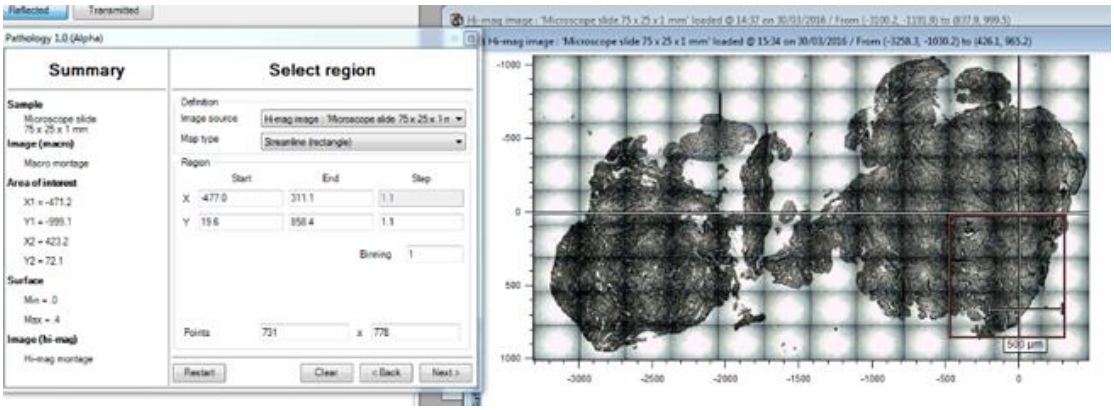
a. **IMPORTANT**: Switch to x25 magnification, focus position at area of interest on section and note down the Z focus position
b. To begin setting up Raman map from existing Hi-Res image, the user will need to make sure the correct montage is selected from the Window menu
c. User will click on Measurement->New->Streamline image acquisition



d. Using again the annotated H&E images supplied by CENTRE 1, select requested regions (ordered determined from CENTRE 1) on the **FROM THE Hi-MAG IMAGE** just created
e. NB: remember to select 'Active Window' as the image source in the panel)

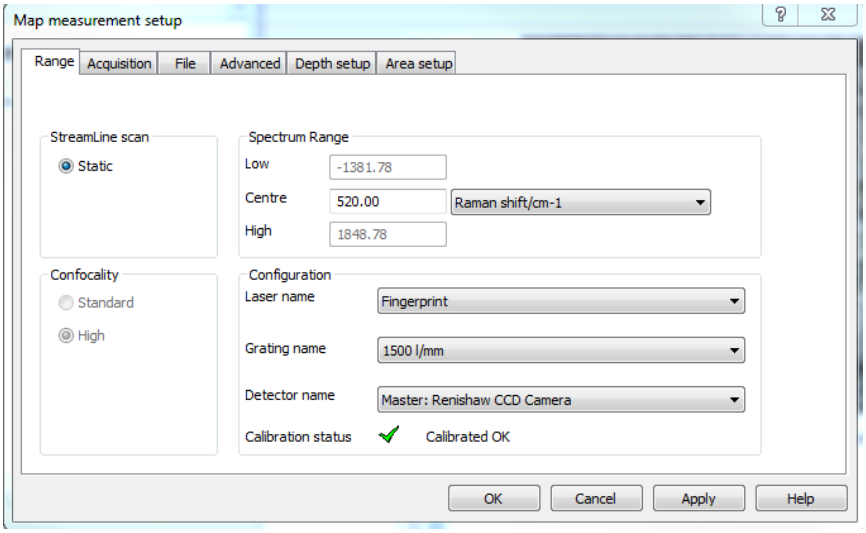




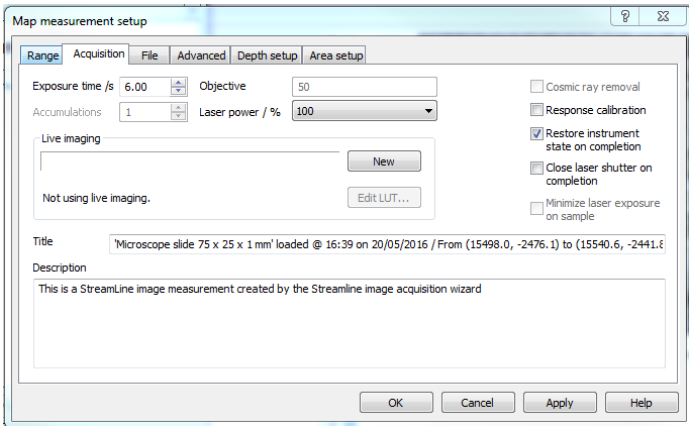


f. **Optional**: Using the x25 in live viewer you can collect and save x25 images from area of interest.

g. **Clicking next will bring up the map measurement setup, click Range tab and set Laser name to fingerprint region**

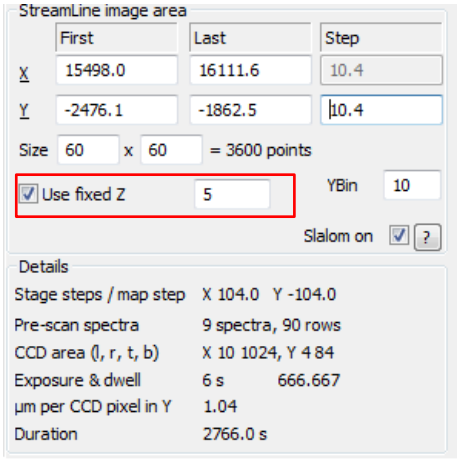


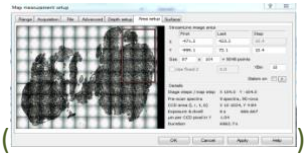
h. Click acquisition tab and set exposure time to 6s



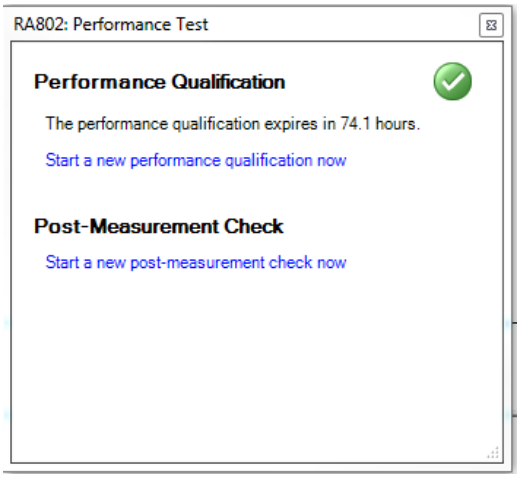
i. **Click area 'Area setup tab' and make sure YBin is set to 10 (step size should default to ~10.4um for X and Y) and check sialon on.**

j. **Check the 'Use fixed Z' checkbox and enter the value noted from step b**



The map parameters of each of the Raman maps can be saved/captured using the Snip-tool windows function to grab the Area setup panel ()

k. Click apply and then OK
l. Run a PQ before starting Raman map measurement and ensure that PQ passes.

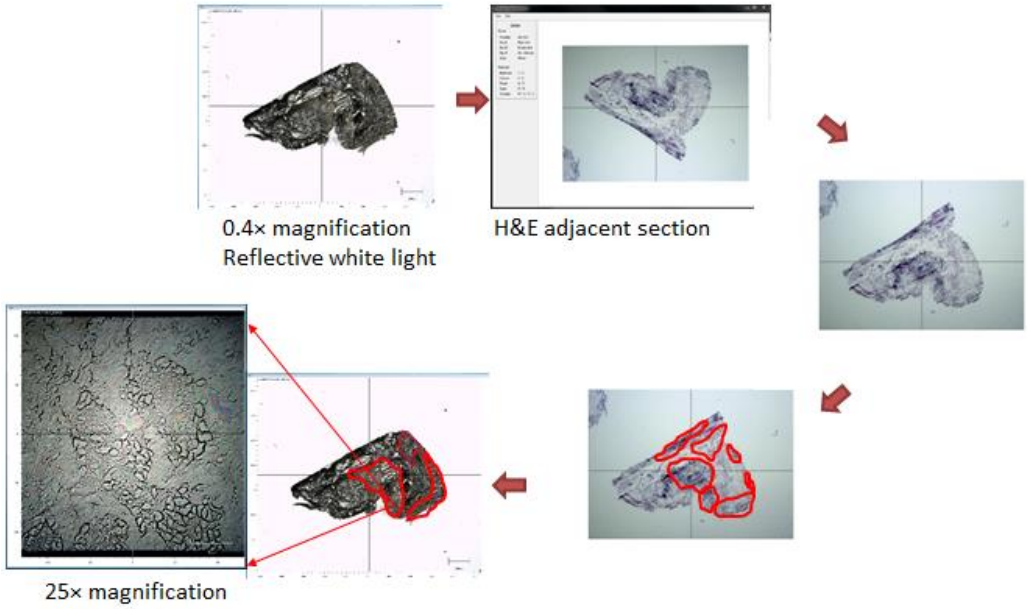


m. Click Run measurement to begin Raman map measurement
n. When map measurement is complete, run a PMC to make sure the no shift in wavenumber and spectral intensity from PQ has occurred

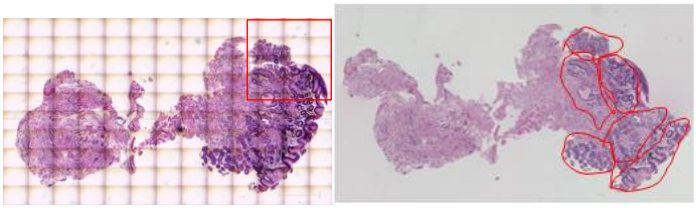
D. Raman Measurement collection

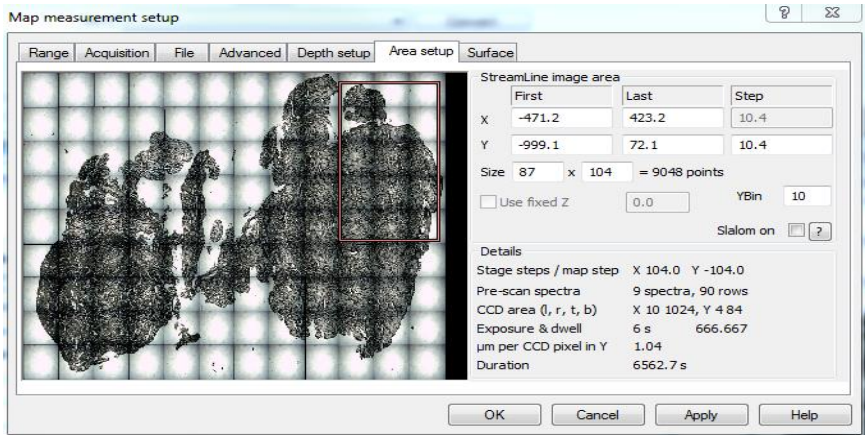
a. Repeat above process for the other areas on the section and slide references from the annotated H&E images

Eventually you will have a collection of Raman maps from the selected areas:

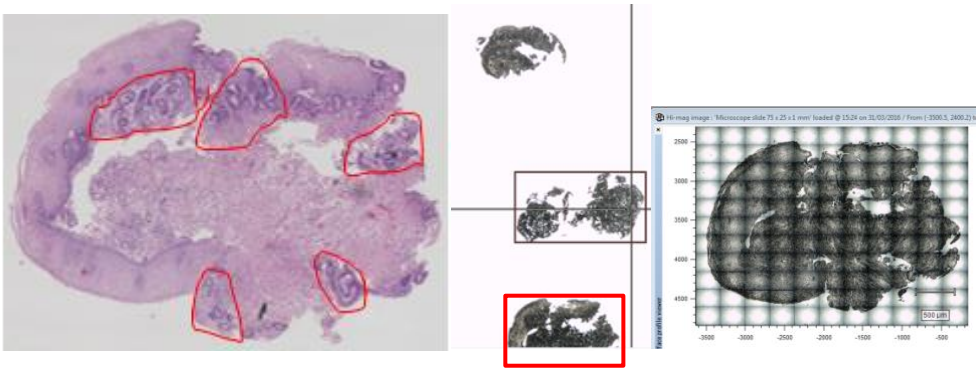


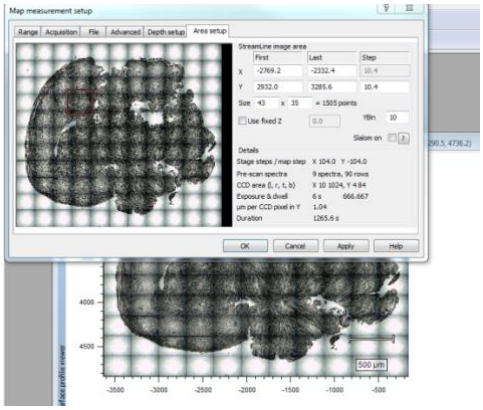
E.g. different region on section



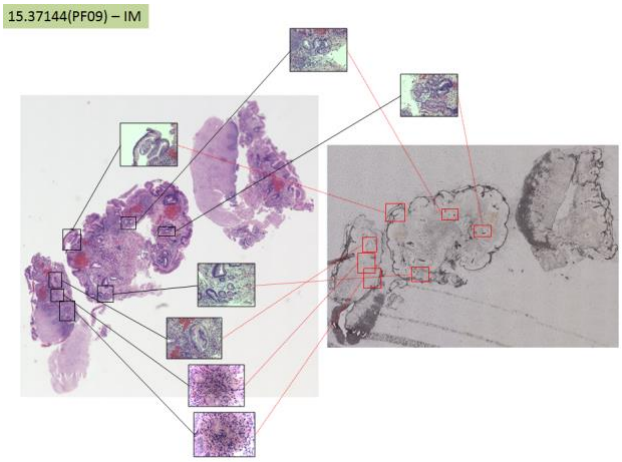


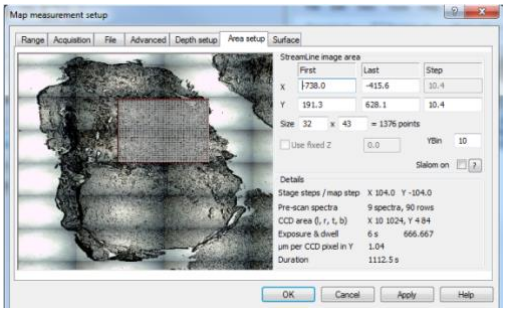
E.g. different section on slide

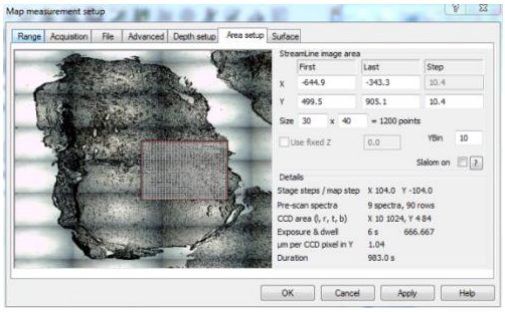


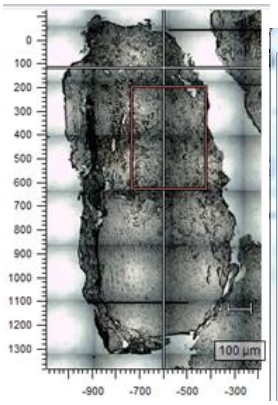


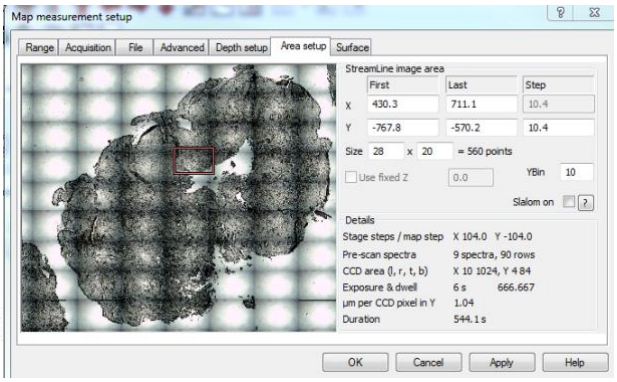
b. Remember to shutdown Wire before starting a new setup of Raman measurements due to memory leak issues with current version
c. Repeat this process (Imaging and selection of areas of Interest from unstained sections using H&E annotated images) for the other tissue slides using the annotated H&E images




Map measurement setup
Range
Acquisition
File
Advanced
Depth setup
Area setup
Surface
StreamLine image area
First
Last
Step
X
Y
Size
Use fixed Z
YBin
Slalom on
Details
Stage steps / map step
Pre-scan spectra
CCD area (l, r, t, b)
Exposure & dwell
µm per CCD pixel in Y
Duration
OK
Cancel
Apply
Help


Map measurement setup
Range
Acquisition
File
Advanced
Depth setup
Area setup
Surface
StreamLine image area
First
Last
Step
X
Y
Size
Use fixed Z
YBin
Slalom on
Details
Stage steps / map step
Pre-scan spectra
CCD area (l, r, t, b)
Exposure & dwell
µm per CCD pixel in Y
Duration
OK
Cancel
Apply
Help


0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
-900
-700
-500
-300
100 µm


Map measurement setup
Range
Acquisition
File
Advanced
Depth setup
Area setup
Surface
StreamLine image area
First
Last
Step
X
430.3
711.1
10.4
Y
-767.8
-570.2
10.4
Size
28
x
20
= 560 points
Use fixed Z
0.0
YBin
10
Slalom on
Details
Stage steps / map step
X 104.0 Y -104.0
Pre-scan spectra
9 spectra, 90 rows
CCD area (l, r, t, b)
X 10 1024, Y 4 84
Exposure & dwell
6 s
666.667
µm per CCD pixel in Y
1.04
Duration
544.1 s
OK
Cancel
Apply
Help