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TM007 - White light image capture, montaging and Surface generation

WiRE™ 5

This document aims to show the WiRE™ 5 user how to collect and save white light images and montages from the microscope camera, and set-up the Surface option.

Note that montaging can be performed using LiveTrack. This is explained in the LiveTrack training module TM033.

This training module covers the collection of:

1. **Single images** - Capture of optical video images

2. **Manual montages** - Capture of multiple manual video images to quickly and easily define mapping regions over very large areas

3. **Automated montages** - Capture of multiple automated video images to easily define mapping regions over a variety of areas spatially connected to the white light view of the sample

4. **Surfaces** - Capture of multiple manual video images with XYZ positions used to define a 3D sample surface for subsequent image or data capture

5. **Surface and montage** - Capture of multiple manual video images with XYZ positions used to define a 3D sample surface together with multiple automated video images for subsequent data capture (i.e. options 3 and 4 together)

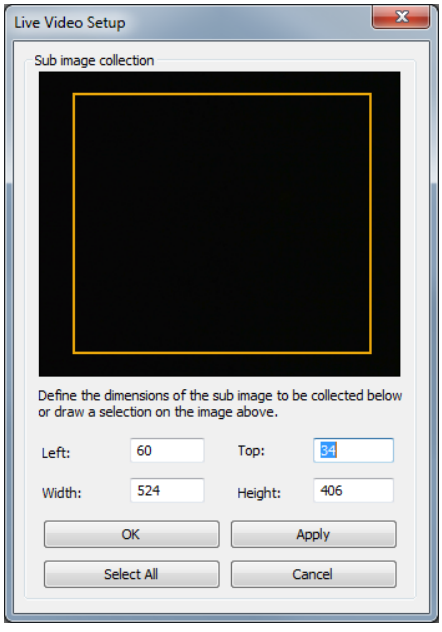
Defining the field of view used for image capture

The captured area can be reduced by selecting **Live Video...Snap...Set-up**. This new area is then used for single or montage image capture only.

The black region represents the entire video area. Use the mouse to draw a region within this. The region within the orange box will be used for single or montage video image collection.

Use **Select All** to collect from the entirety of the video area.

Reducing the area can help to reduce any montage combining features resulting from illumination uniformity – this is somewhat dependant on the sample surface reflectivity to white light.



When capturing video images the objective selected in the sample review must match the physical objective focussed on the sample.

1. White light optical image capture (XY)

A single image can be captured using the **Live Video…Snap…Single** option or the Snap video toolbar button ( ). A Still Image Viewer will appear with the video camera view captured. Right click in the Still Image viewer to add/remove crosshairs, axes and scale bar.

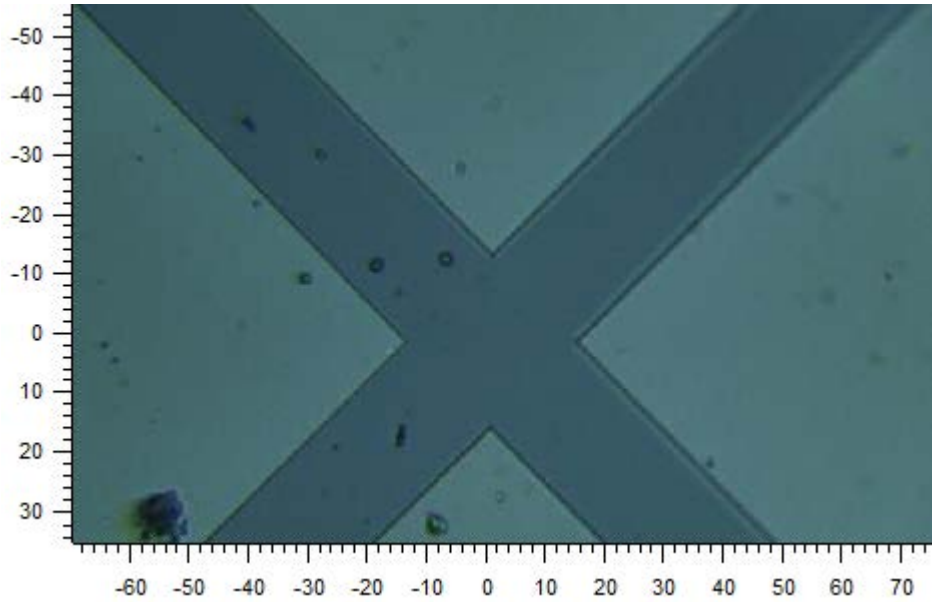
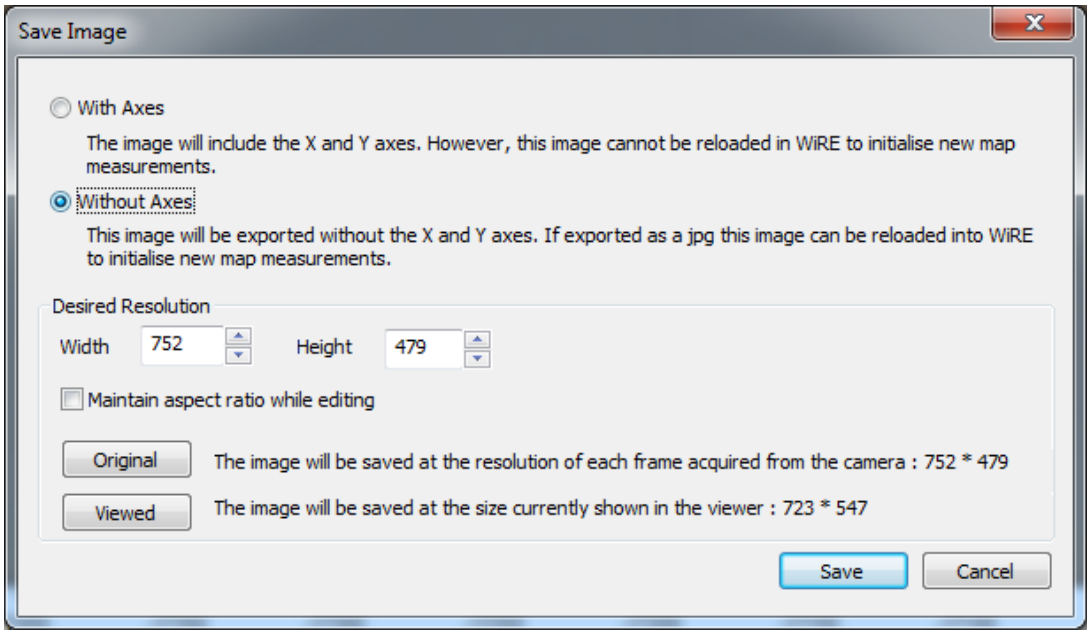


Figure 1. Example single white light image (50× objective)

Right click and select **Save to** to save the image as a bmp or jpg.



- Saving with axes is typically used to present in a report, where specific axes information is part of the image.
- Saving without axes enables the image to be used to define Raman data collection areas (jpg only) or used to remove background artefacts from white light montages. A variety of resolution options are presented including custom and native resolution.

2. Multiple manual white light image capture (XY montaging):

Quickly and easily define mapping regions over very large areas

Repeat the process described in 1, moving the sample on the motorised stage in XY to produce an area partially filled with white light images. The sample can be either moved freely using the track ball, or in a more grid-like manner using the XYZ stage control (typing spatial co-ordinates to move the sample known distances one axis at a time).

As many or as few images as desired can be added by the user. This process is useful where a large area is required to define the mapping area, without the need to collect a large number of images over the entire area (e.g. a large area on a sample with no meaningful white light viewable features).

The resulting montage will have black regions where no image capture has occurred (Figure 2).

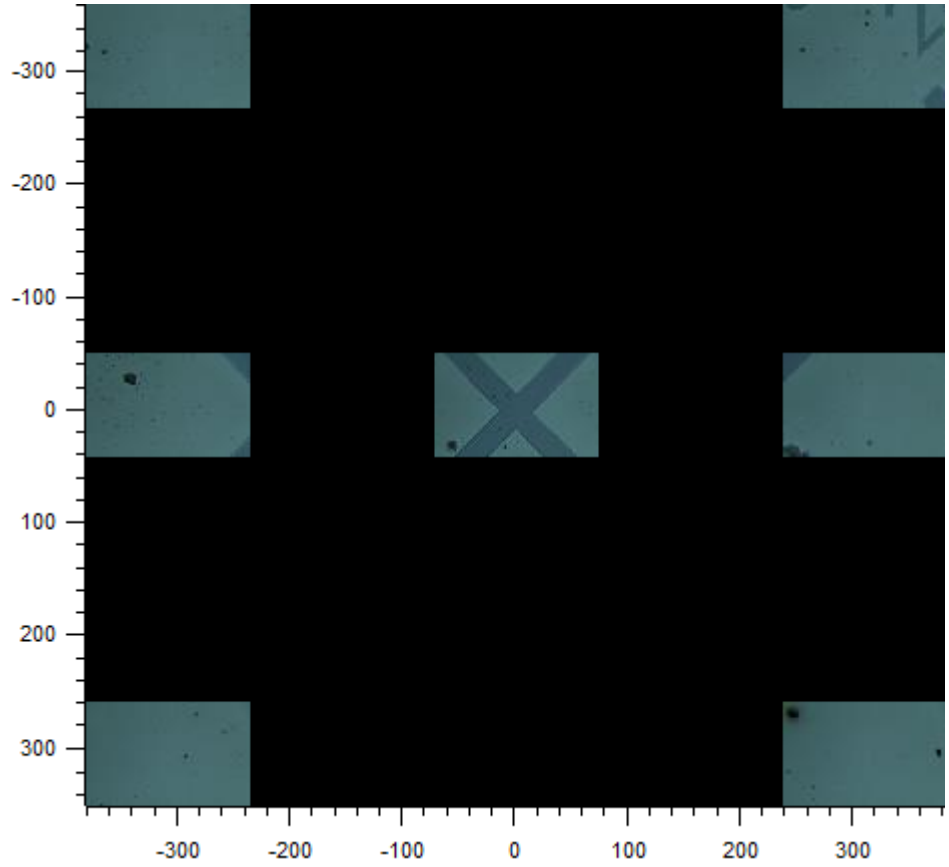


Figure 2. Example manual white light montage (multiple 50× objective images)

3. Multiple automated white light image capture (XY montaging)

Easily define mapping regions over a variety of areas with a complete white light montage

When performing imaging experiments over large areas it is often desirable to be able to compare the white light image of the sample area imaged with the Raman data. Where the Raman image is greater in size than the field of view of the white light image, a montage of these images can be created. It is also easier to define the image area from the montage.

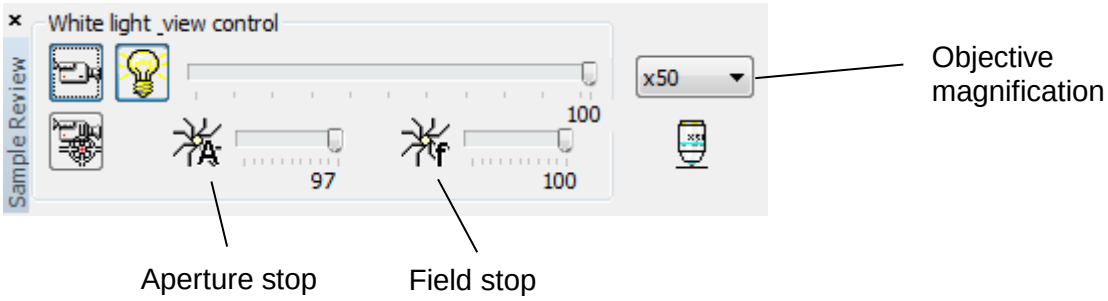
1. Focus on the sample with the objective to be used for the montaging

   Note this can be different to the objective to be used for collecting the Raman data but the sample should be flat for both if the image and collected Raman date are to be in-focus.

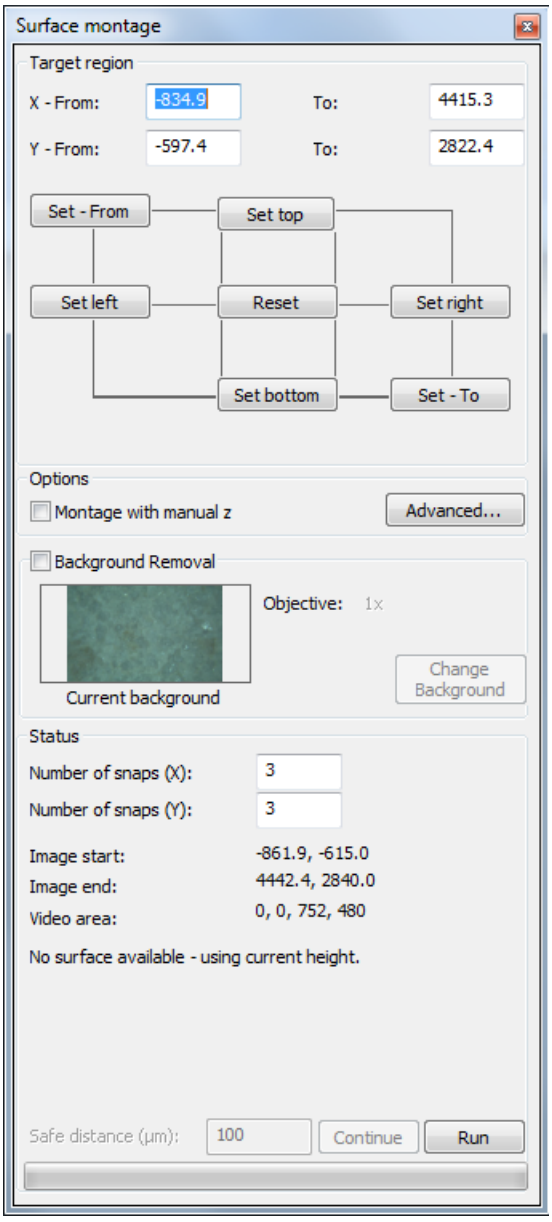
2. Zero the co-ordinates using the Set origin button ( ).

3. Set the correct objective in the Sample Review (this is reflected in the scale bar of the Video viewer).

4. Ensure the video displays the desired brightness and contrast to enable a uniform joining of the images (this will be dependent on sample type). A more seamless montage is often collected by opening the aperture stop and or field stop of the microscope. This is mounted on the Leica microscope for non-Reflex models and is accessed in the Sample Review tool on Reflex models.



5. Select **Live Video…Snap…montage** or the toolbar button ( )

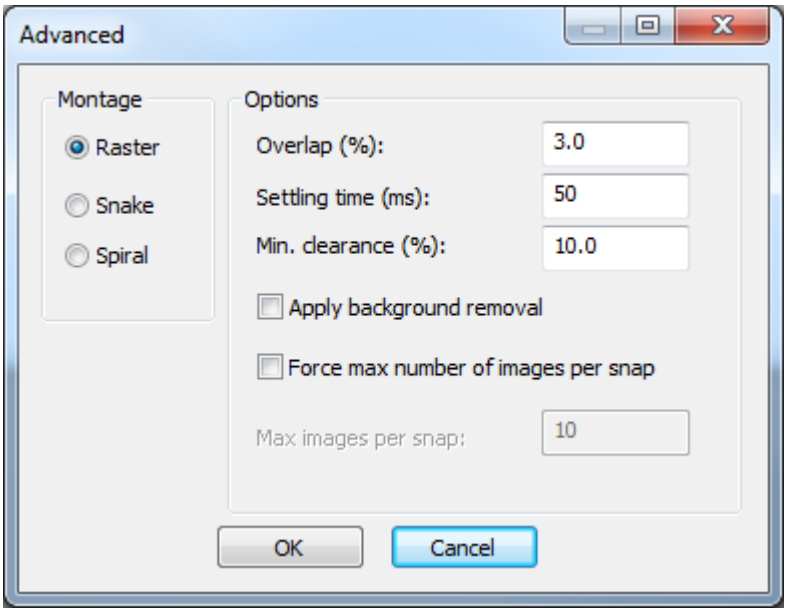


Several options are available in this dialogue:

- X and Y start values are in micrometres taken from the current motorised stage position.
- The user specifies the area in micrometres to collect the montage from. The X and Y distance of a single video image can be determined using the axes of the video viewer.
- The **Set-From** and **Set-To** buttons can be used to define the entire rectangular montage collection area. Move the sample stage to the extreme top left and bottom right points, the button automatically enters the co-ordinates at these points. This method is used where sample features are visible at these extreme points.

- The **Set top**, **Set bottom, Set left** and **Set right** buttons can be used to define the entire rectangular montage collection area when the top left and bottom right positional regions show no useful sample features. Move the sample stage to the extreme points, the button automatically enters the co-ordinates at these points. This method is extremely convenient for samples which are no rectangular in shape (e.g. circular pharmaceutical tablets).
- The **Number of snaps** can be manually changed if a fast means of producing a montage based on the current video view size is wanted.
- **Montage with manual Z** enables the user to focus each montage image in Z (using the trackball) to also generate a Surface.
- The ability to automatically perform **Background removal** using a pre-defined white light image.
- The dialogue also confirms if the montage will be collected using a pre-defined Surface or fixed Z height.

Several options are available within **Advanced**:



- The background removal option applies a post processing operation to flat field the completed montage. This can help remove any effects resulting from uneven illumination on the sample.

  This process is applied immediately on completion of the montage, it is also available to be applied anytime after the montage has completed (from the Image view right click menu).

  Overlap, Settling time, and minimum clearance can all be adjusted.

6. Select **Run** and the system will start the collection of the montage, automatically moving the stage and adding new frames to the image that appears in the Still Image viewer. The image will auto scale to fill the Image viewer size as new frames are added.

   This image can be saved in the same way a single image with the same option being available.

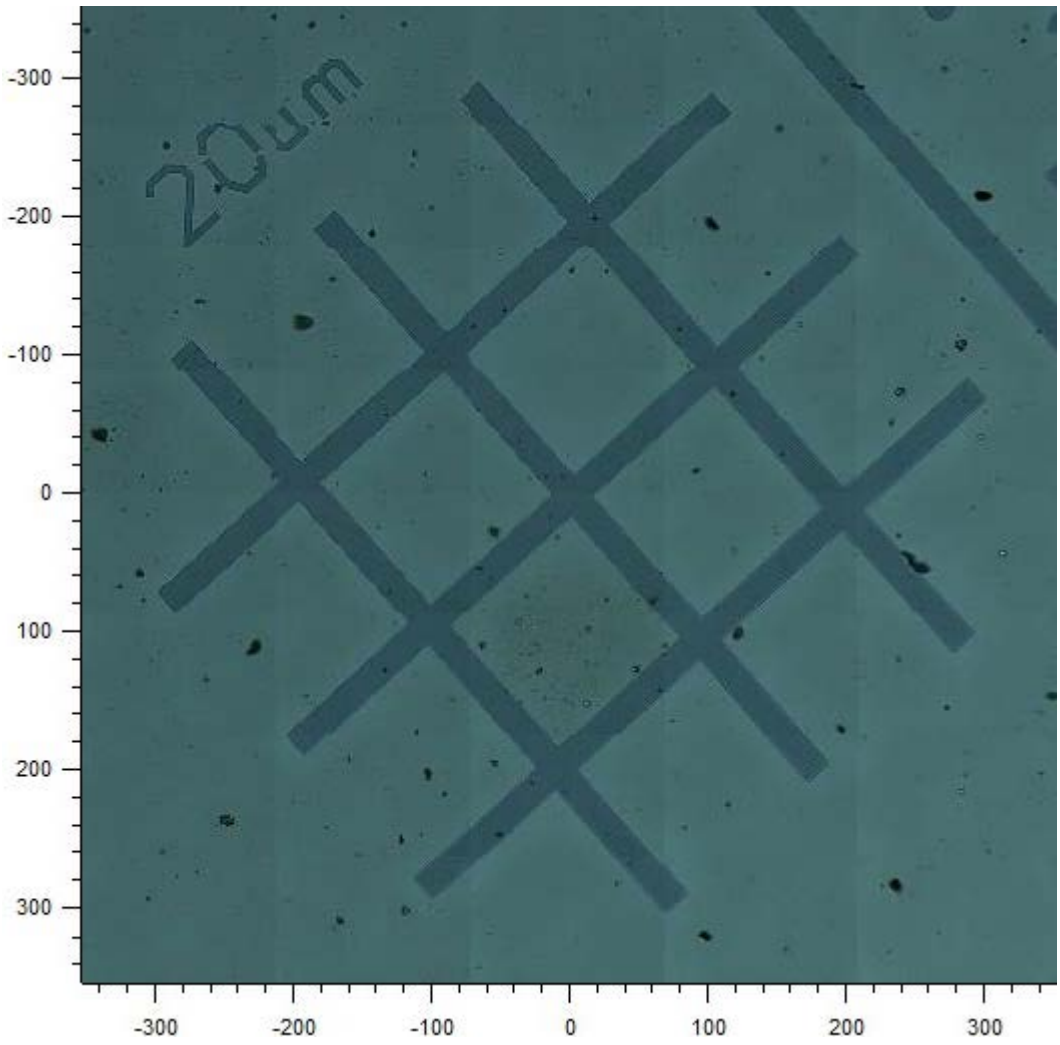


Figure 3. Automated white light montage (forty five 50× objective images)

   Multiple montages can be generated by adding a new Window (**New…Window**) after completing the first montage. The co-ordinates **should not** be reset between montages. This allows data collection from the different montages to be queued.

7. Perform background correction of the montage, if necessary.
   a. Collect the montage
   b. Right click on the montage and select **Background removal setup**

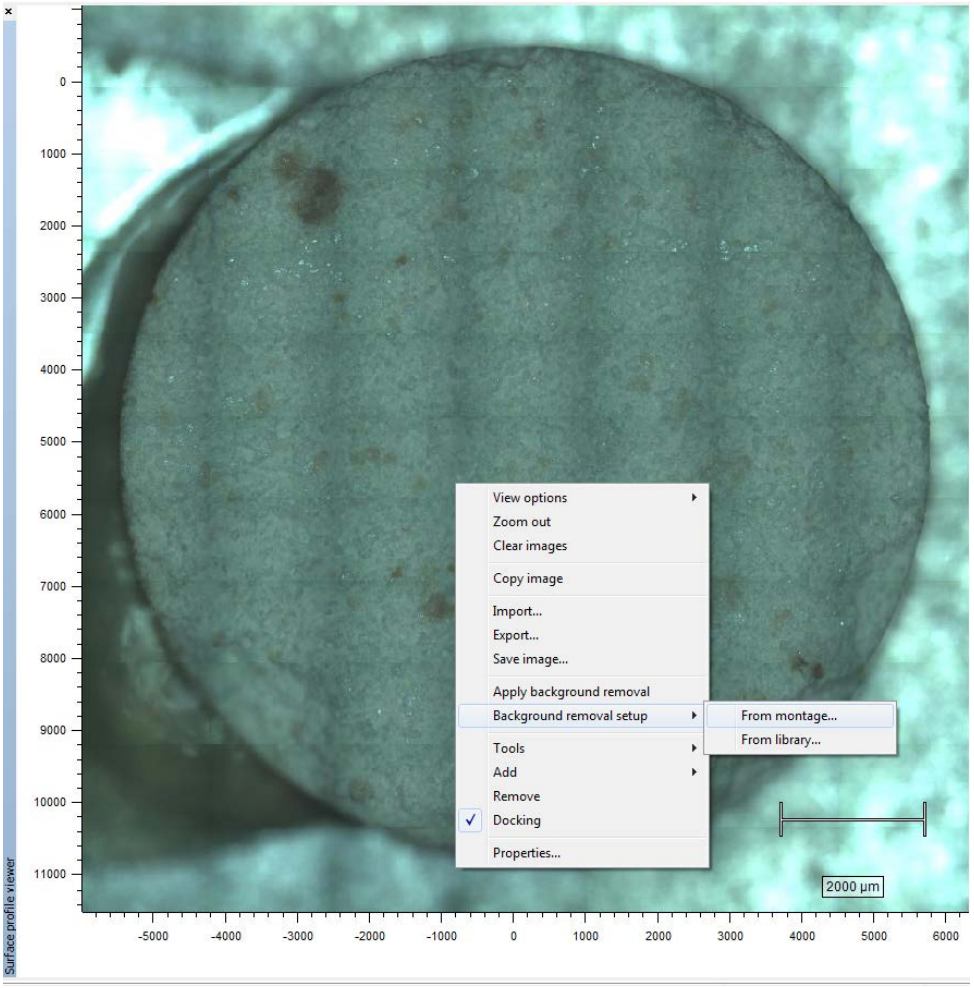


Figure 4. White light montage with background removal dialogue

c. Select **From montage…** to define the image to be used for correction from the montage. This option works optimally where variation within the image is relatively low.

   Select From library… to define the image to be used for correction from pre-existing images saved to the library.

d. If using the 'From montage' option, draw an area over the montage which includes the key regions which are to be used for correction, creating an average image of these individual images.

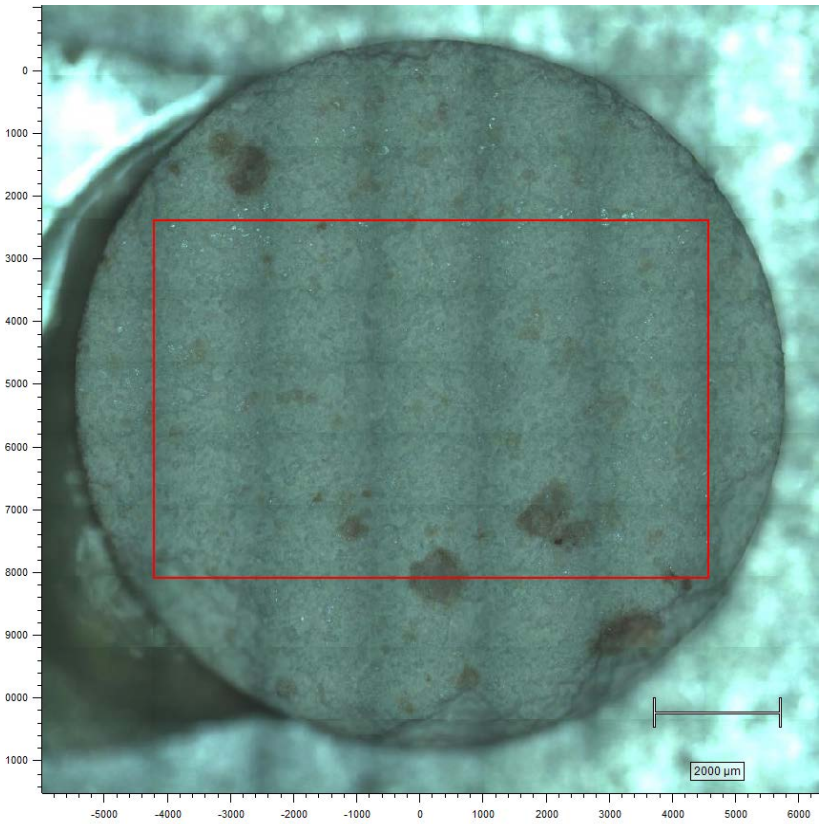


Figure 5. White light montage with defined region indicating images to be used to generate average image for background removal

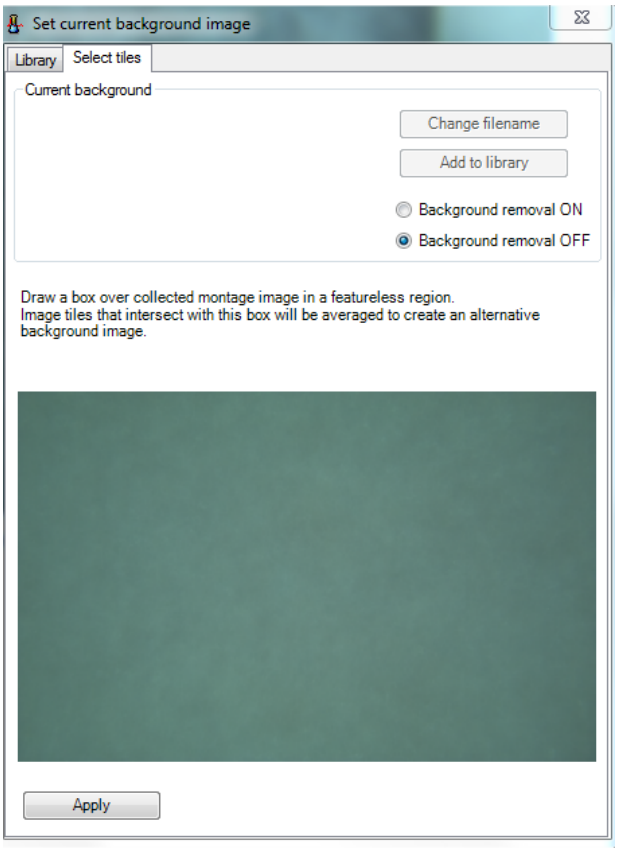


Figure 6. Set background dialogue showing average image

e. Change radial option to **Background correction on**. The image is now corrected and the correction can be toggled on and off from the dialogue or from the **Apply background removal** context menu option.

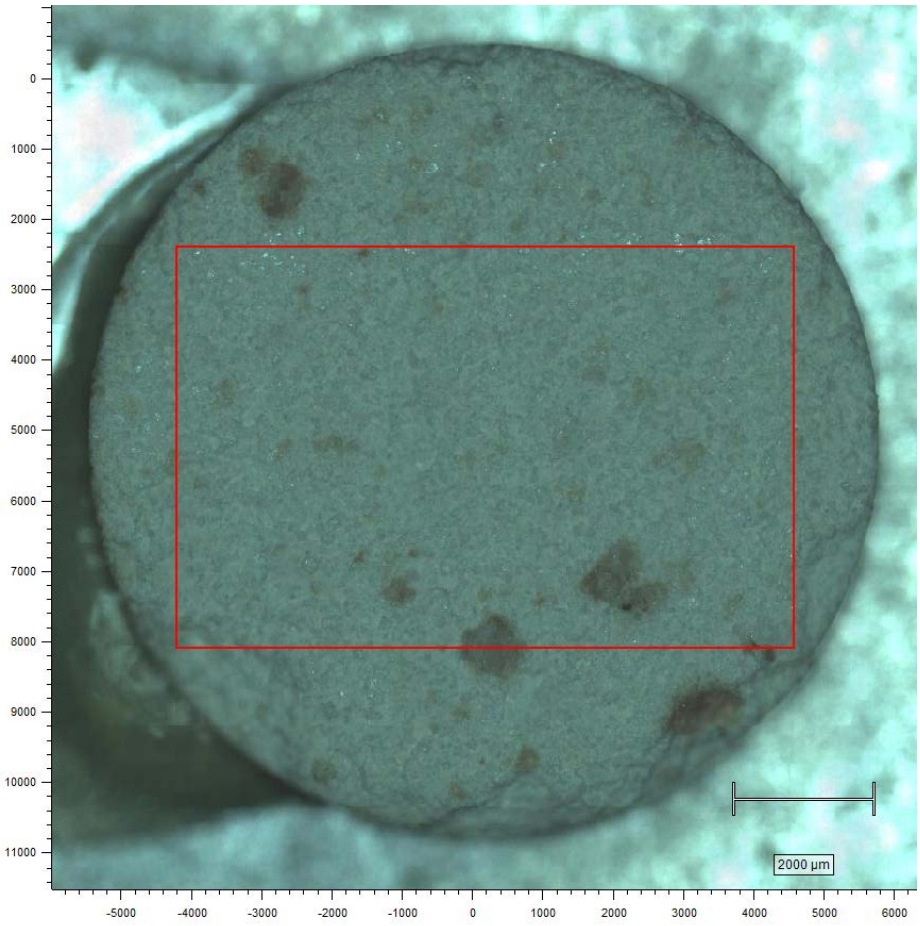


Figure 7. Background corrected montage

4. Multiple manual white light image capture (XYZ Surface):

Used to ensure data collection or image capture occurs with the sample in focus

The Surface option enables the user to manually define 'in-focus' positions for samples which are not level or flat. This process can enable:

- Large in-focus white light montages to be generated based on the defined surface
- Mapping data to be collected with automatic Z change based on the defined surface

Initially a surface must be generated.

Generating a Surface

Specific objective properties (working distance (WD), depth of field (DoF) and diameter (D)) are automatically added to WiRE on installation, for standard objectives (5×, 10×, 20×, 50×, 50×L, 100×). Other objectives need to have this information added manually if they are to be used with the Surface option (see Appendix 1 for this procedure).

1. Position the sample on the microscope stage. The sample should be appropriately constrained so it is unable to move during Surface generation.

2. Select Surface....New ().

3. Set the correct objective in the sample review.
   The surface should be generated using the same objective that will be used for data collection. Lower magnification objectives, whilst having a larger field of view, will not enable the focus to be accurately set for higher magnification objectives normally used for data collection.

4. Determine the area the surface will be collected over and set the XYZ origin if desired.

   Note: Setting the origin on a discrete and easily recognisable sample point can enable any generated Surfaces to be saved and re-used, even if the sample is removed from the stage.

5. Navigate around your sample, modifying the focus and select add new point ().

   Move the sample in XY using either:
   - the XYZ stage control by typing values and selecting **Go to**
   - the high speed encoded stage (HSES) trackball

   Adjust the focus using the HSES trackball (**DO NOT USE THE FOCUS WHEELS ON THE MICROSCOPE)**

6. Adding points (images) will build up the Surface. The images are shown in the **Image** mode, the Surface is shown in the **Surface** mode. These modes are accessed from the right click menu. You can also show the points defining the surface from this menu.

A double left click on either the Image or Surface view (when the cross hairs are active) will move the sample to that point in XYZ. If this point is not in focus, focus the sample and add a new point.

Image points form the 3D surface using triangulation (linear interpolation). Producing a Surface for samples which are inherently flat but not level is therefore fast and easy. A large number of Raman samples consist of such a form. Where samples have more complex variations in sample height a greater number of points need to be added.

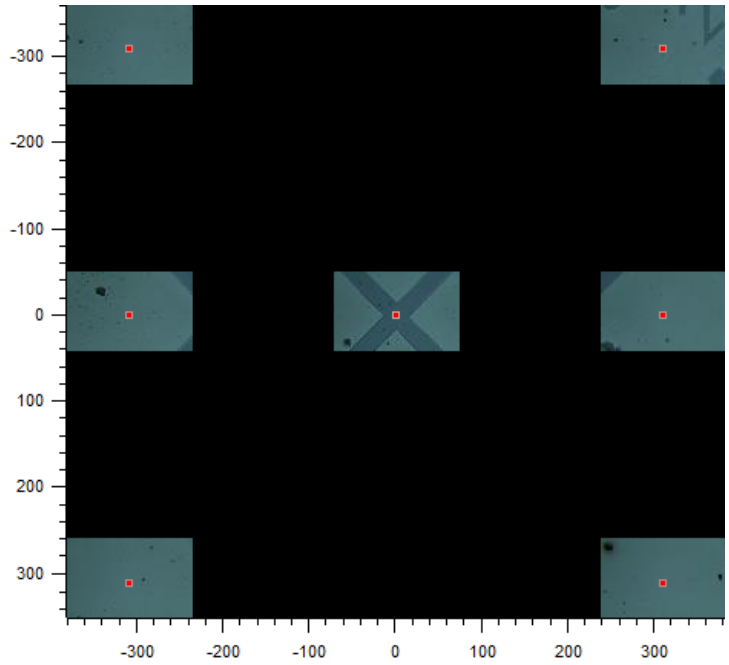


Figure 8. Image view of Surface with points (red)

In addition to the simple image view control, the image view can be changed in the following way from the right click menu:

- Show points (View options...Show data points)
- Show region available for defining the map area (Properties...Image tab...Surface edge)
- Clear images
- Select points
- Remove points

Double click on the image to move the sample to the selected surface XYZ point when the cross hairs are active.

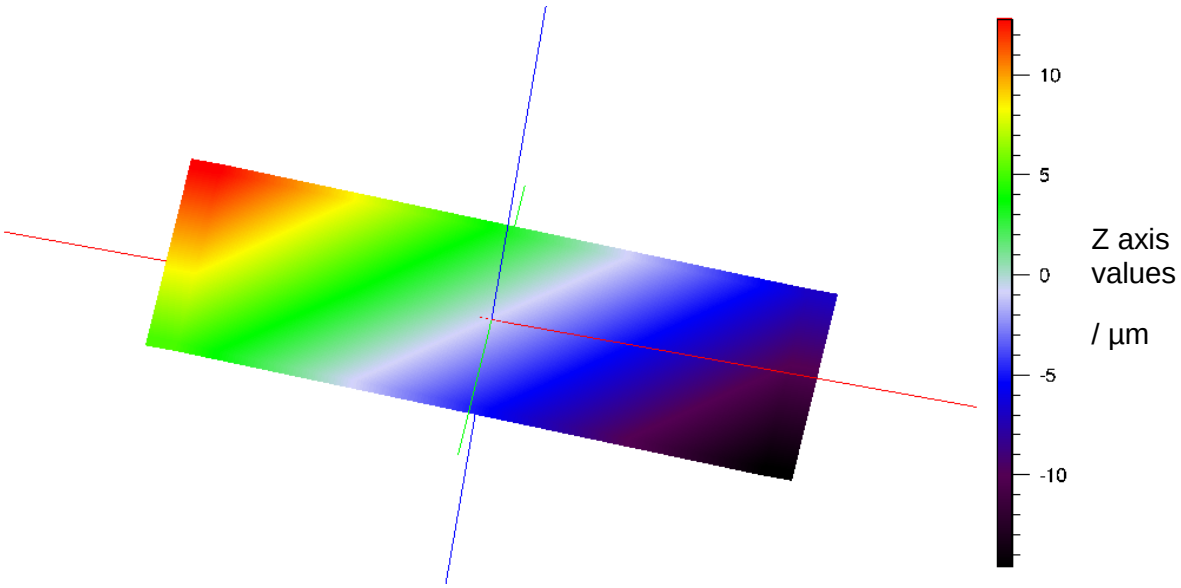


Figure 9. Surface view of Surface (rainbow LUT)

The Surface view can be changed in the following way from the right click menu:

- Reset view
- LUT colour and contrast (View options...Show LUT)
- Top down view (View options...Top down)
- Interpolation options for points beyond the defined Surface (**Constant** – default, or **Linear**)

Double click on the Surface to move the sample to the selected surface XYZ point when the cross hairs are active.

Using the Surface to collect mapping data

The minimum spectral acquisition time which can be used with Surface to ensure correct sample focus is dependent on the rate of XY motion relative to the rate of Z motion. The rate of XY motion is dependent on the spectral acquisition time and X and Y step size. This relationship also varies with the mapping method.

If the Z surface position cannot be reached in the available time during mapping data collection, the time is not delayed and the appropriate focus position may not be reached. This is most likely to occur where the acquisition time is very short or the rate of Z change is very high.

The following example demonstrates map data collection on an X angled sample which is inherently flat.

- StreamLineHR example

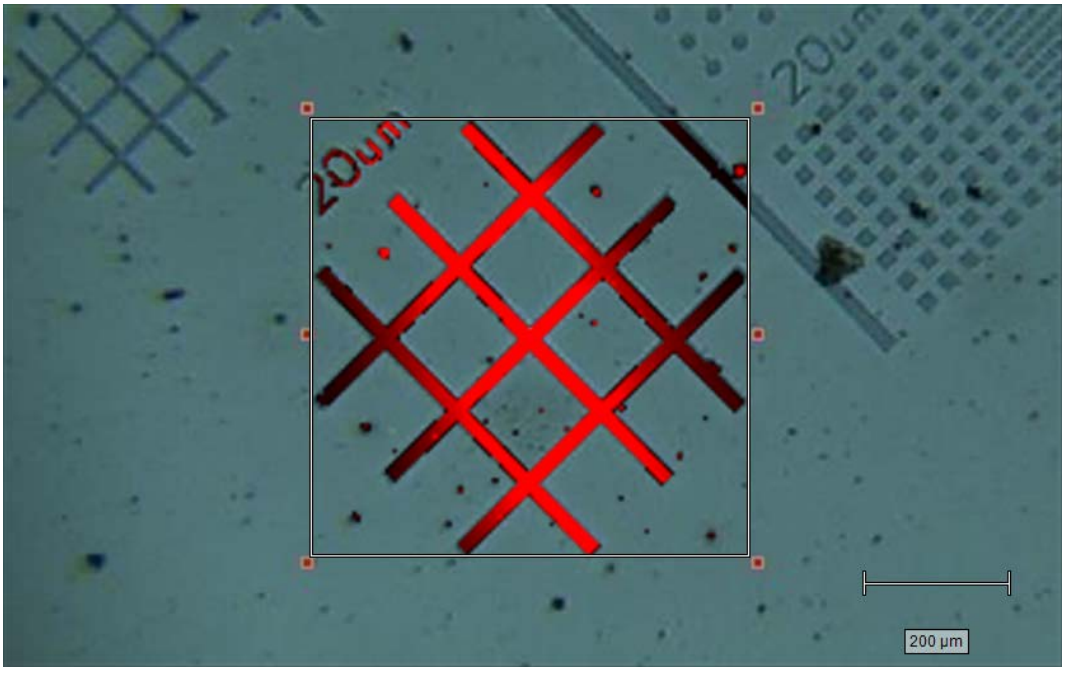


Figure 10. Raman image of angled grid with no surface correction (5 µm step size, 50× objective).

The bright image regions are in focus, darker regions are out of focus. (The centre of the sample is in focus, moving away from the centre in X causes the sample to go out of focus)

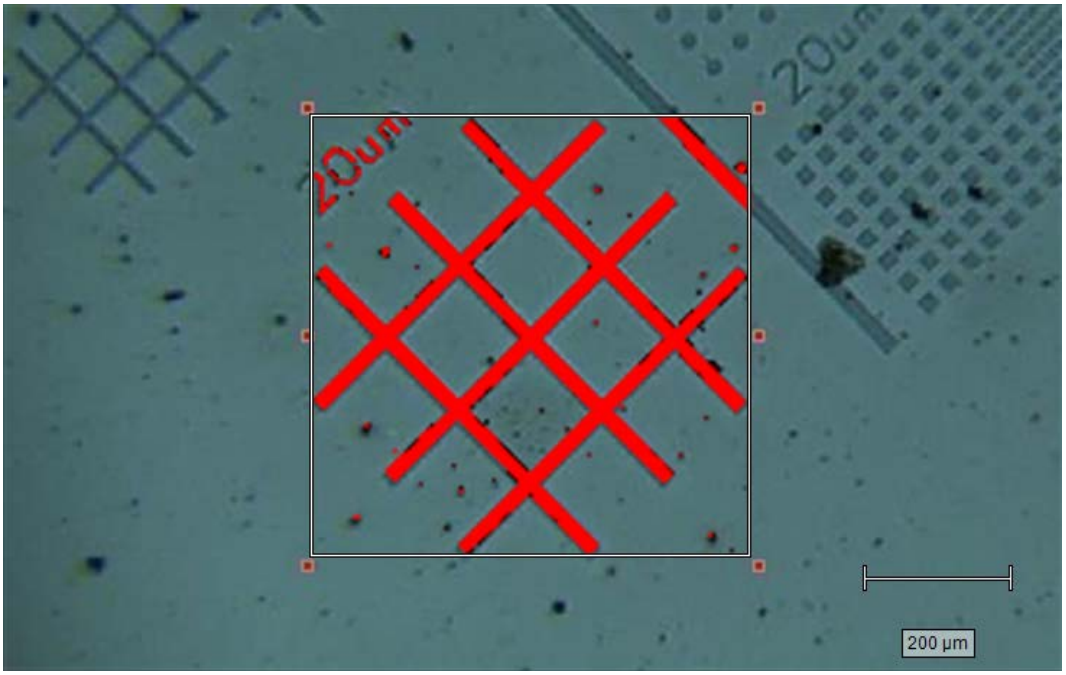


Figure 11. Raman image of angled grid with surface correction (5 µm step size, 50× objective).

The image regions are all in focus, producing no contrast resulting from changes in signal level.

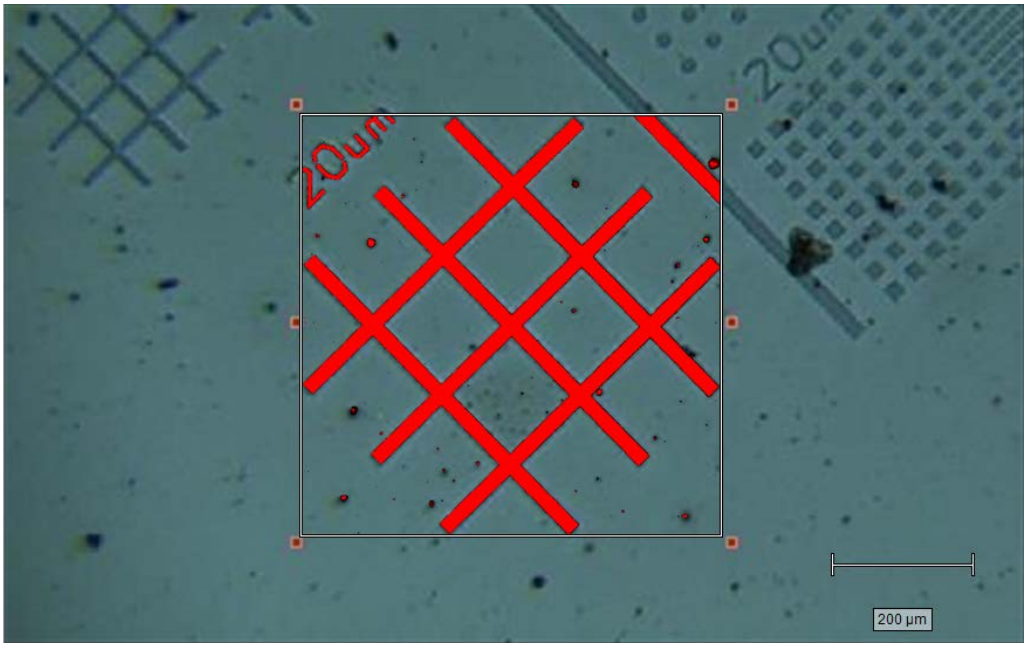


Figure 12. Raman image of angled grid with surface correction (1 µm step size, 50× objective).

The image regions are all in focus, producing no contrast resulting from changes in signal level. The image is sharper as the spatial resolution is higher.

- StreamLine imaging example

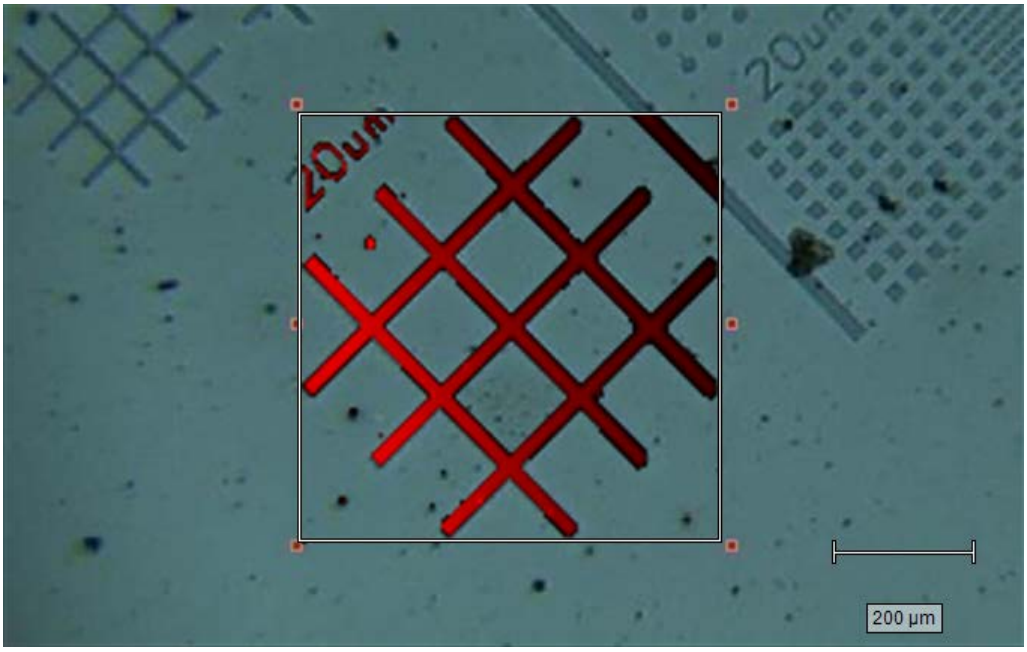


Figure 13. Raman image of angled grid with no surface correction (5.2 µm step size, 50× objective).

The bright image regions are in focus, darker regions are out of focus. (The left side of the image is in-focus and the sample is going further out of focus as X increases)

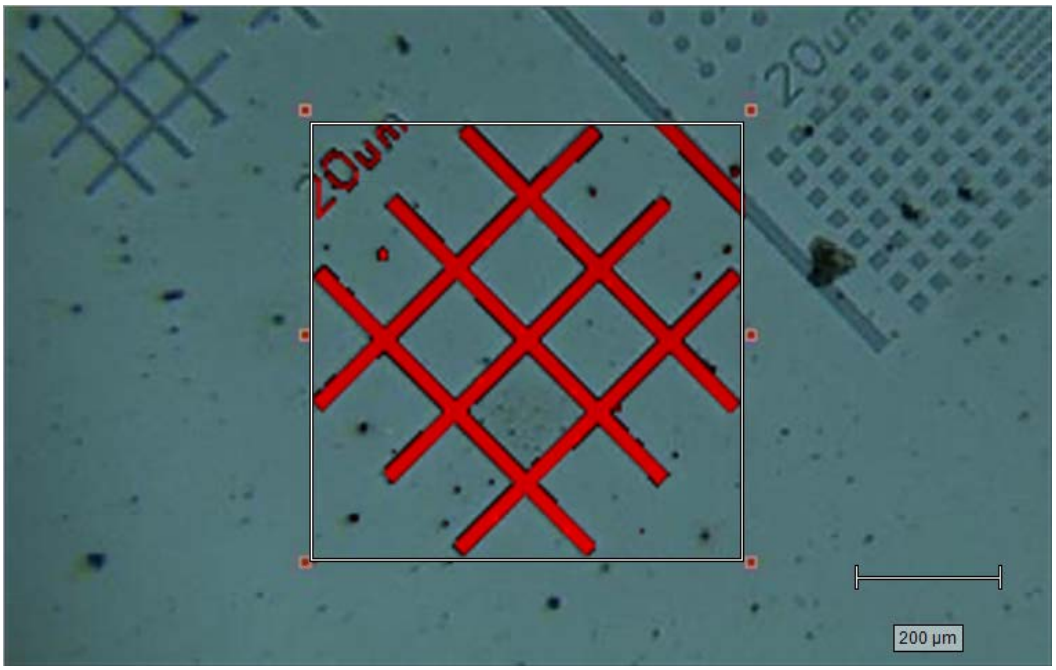


Figure 14. Raman image of angled grid with surface correction (5.2 µm step size, 50× objective).

The image regions are all in focus, producing no contrast resulting from changes in signal level. The right side of the image is now as sharp as the left side of the image and less blurry than before.

Controlling the sample height prior to data collection

The Z position of the sample can be manually defined separately in the area setup tab of the measurement (**Use fixed Z**). This option is defaulted to off meaning data collection will commence at the current Z position, unless Surface has been used. Tick the **Use fixed Z** box to force a defined Z sample position for map data collection.

On map data completion the sample is moved to the first data collection point in XY, and Z if a Surface has been used (regardless of whether the **Restore instrument state on completion** box has been ticked on the Acquisition tab of the measurement setup). Therefore, thought needs to be given to the Z position of the sample which will be potentially used for any future queued data collection. This is particularly the case if queued data collection consists of a mixture of Surface and non-Surface measurements.

Extracting Surface information from collected map data

From collected data, the Surface can be 'extracted' so that it can be viewed and manipulated in the same way as when originally produced. This also allows any associated white light montages to be back ground corrected post data collection. To extract the Surface, Select the **data** tab of the navigator, then expand the **Measurement configuration** node. Right click on the map information (**Surface-map type**) and select **Extract surface**. The Surface will then be loaded into a separate Window.

5. Using the Surface to generate 'in-focus' montages

Useful where mapping data can be spatially connected to the variable focus white light image

The collected Surface can be used to collect in-focus white light montages. The Surface is generated using the objective to be used for data collection. This is usually of high magnification and produces a small field of view within the video. Where the total area for the montage is also relatively small this does not pose a problem. As the total area increases the number of images also increases. Therefore it is often desirable to use a lower magnification which has a larger field of view to significantly reduce the number of images and make the montaging much faster.

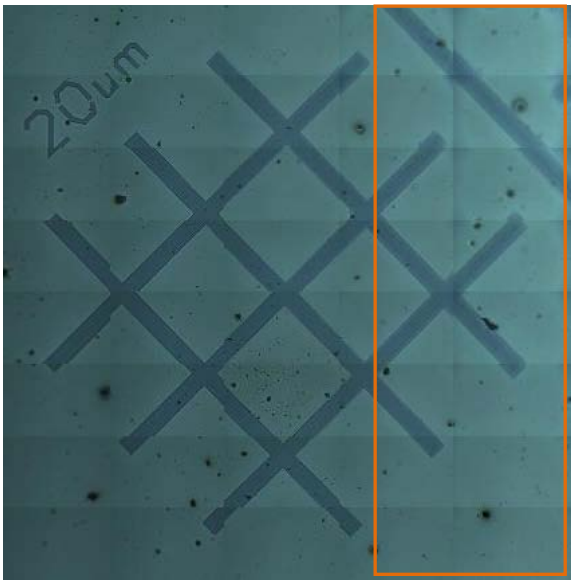
- Ensure the Window containing the Surface is selected
- Change the objective to a lower magnification, if desired
- Select the correct microscope objective, if changed
- Select **Live Video…Snap…montage** or the toolbar button ()

- Enter the X and Y values over which the montage will be collected (typically the same as the Surface).
- Select **Run**

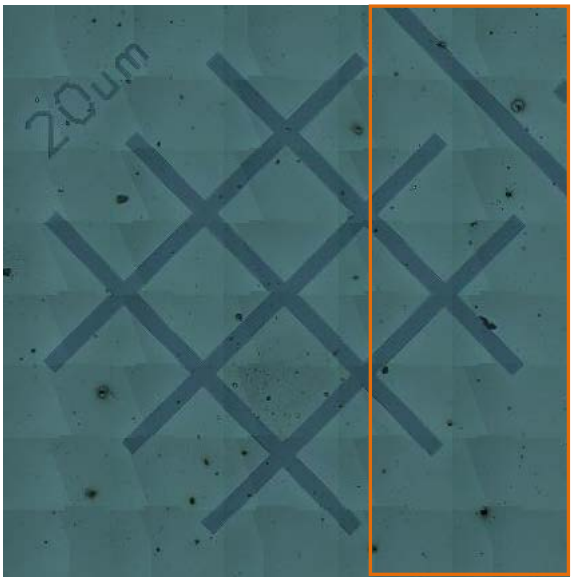
The depth of field information for the objective is used to ensure any single video image is in focus over the entire field of view. This is automatically determined and where the focus changes over the field of view multiple images are collected at different Z positions. The in-focus regions of the combined Z stack are then used together in the final montage.

The result will be a montage collected using one objective, but the Surface generated with another. Remember to collect the Surface first, then the montage.

Example using Surface to generate an in-focus montage using the same objective



50× montage over non-level sample



50× montage over non-level sample using Surface

Figure 15. Original montage compared with in focus montage. Note how the original montage goes out of focus on the right hand side whereas the Surface montage is in focus over the entire area (orange box).

Example using Surface to generate an in-focus montage using different objectives

For very large areas a lower magnification will provide a faster montage whilst using the accuracy of the Surface defined using the data collection objective. The lower magnification is less sensitive to focus changes, but will still benefit from the Surface information

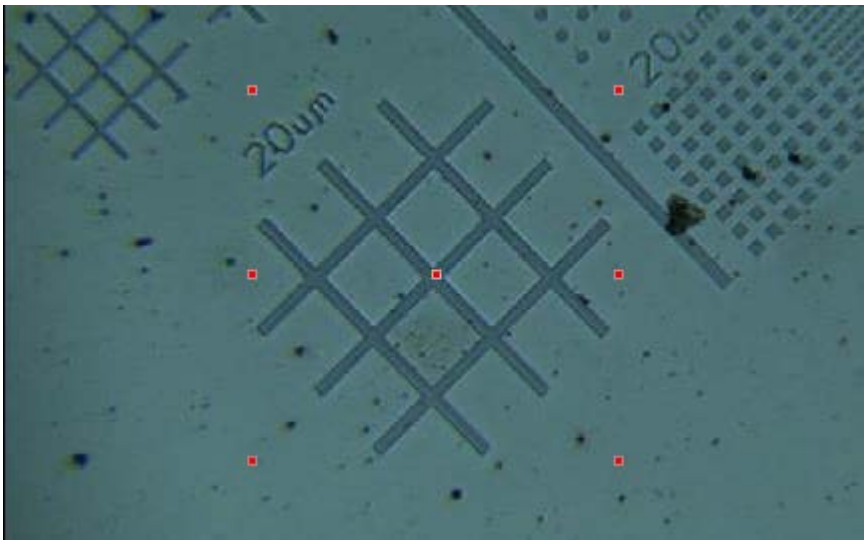


Figure 16. 5× montage over non-level sample using 50× Surface

Now only 2 images are used to enable white light visualisation over a large area whilst ensuring data collection occurs at the accuracy of the 50× Surface. Red points show the location of the 50× Surface points.

The clear images option can be used to remove white light images added to the Surface during generation, to then be replaced by either of the methods above.

To collect a montage separate to the Surface (but on the same sample, or over the same area) add a new Window first before selecting new montage.

Appendix 1

Adding objectives to enable their use with Surface

New objectives are added using System configuration (**System configuration, Podule tab.....objectives**). Additional to the new objective name and magnification, the working distance (WD), depth of field (DoF) and diameter (D) values need to be entered.

- WD is a value provided by the objective supplier (mm)
- DoF is the distance the sample can travel before it becomes out of focus (e.g. 10 µm means +/- 5 µm from the optimum focus point)
- D is not the main objective diameter, but is calculated as the lens radius added to the WD (mm). This ensures gradients of up to 45 degrees can be safely analysed