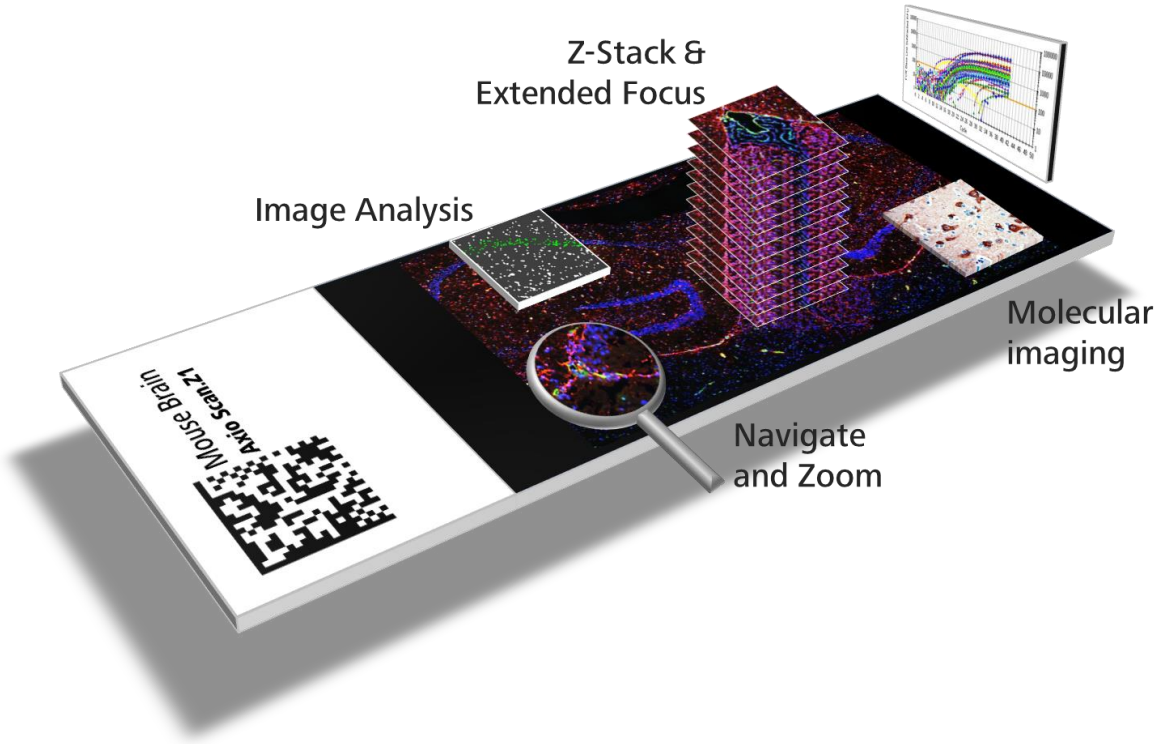


Application Guide

Axio Scan.Z1 v1.1

We make it visible.

A Basic and Quick guide to Axio Scan.Z1

March 2014

OJC

Contents:

1 Purpose of this document

The Axio Scan.Z1 Application Guide is aimed at users with a basic knowledge of the scanner. The document provides an explanation of the principles of scanning and therefore the starting point for scan profile optimisation.

The Application Guide is not an all-encompassing guide to system features. For this information users should refer to the ZEN Slide Scan System Guide also published by Carl Zeiss.

System functions may be referred to with the assumption that the reader already knows how to find the relevant software control in the graphical user interface or GUI.

After reading this guide you should have a much clearer understanding of the scan profile set-up process and crucially, the impact of these settings on your final scan quality.

This document is edition 1 and subsequent revisions will be issued subsequent to changes in the scanner or suggestions from readers.

2 Making a new global profile

This means creating a profile that is not specific to just one slide but can be applied to all slides if needed. So-called global profiles are created from the left-hand side of the ZEN scan software. Next to the drop-down menu for **Default Scan Profile** is a small drop-down menu with a gear or cog/sprocket icon. Selecting this icon allows you to create a new profile. Enter a name into the dialogue box and click the **save** button with a diskette icon on it. This now launches the **Advanced Profile Wizard**. It helps to think of this process as more like a step-by-step work flow as opposed to a typical software wizard.

In total the **Advanced Profile Wizard** has six main steps, each with different settings inside. Understanding the impact of these settings on the outcome of a scan is the key to successful use of the scanner.

So let's begin with step 1 of the process.

2.1 1 Global Data

What is global data? Global data is information about the profile, such as **profile name** and **description**. It is also the point at which you can define if the profile scans in **brightfield** e.g. histological staining or in **fluorescence** e.g. immunofluorescence.

The most important point here is to create global profiles in a judicious way. This means using a defined nomenclature that you can interpret in order to know what the profile is created to do.

For example you can create a profile to scan histology with the 40x lens so a sensible name is **40x BF Haematoxylin Staining**. This seems logical but in practice, managing names carefully avoids creating too many profiles which can cause confusion.

For this document choose a profile name like **20x BF** (brightfield), description is not critical at this point. Select **Brightfield** as the **Profile Type** and select **Tissue** as the **TD Recognition Type** (TD stands for tissue detection). You can now click next.

2.2 2 Label Scan Settings

In this edition of the Application Guide we will not discuss bar-code reading or OCR text recognition. This means that this step in the profile creation workflow is not critical. The only consideration is to make sure that all of the **slide thumbnail** is imaged for the slide preview.

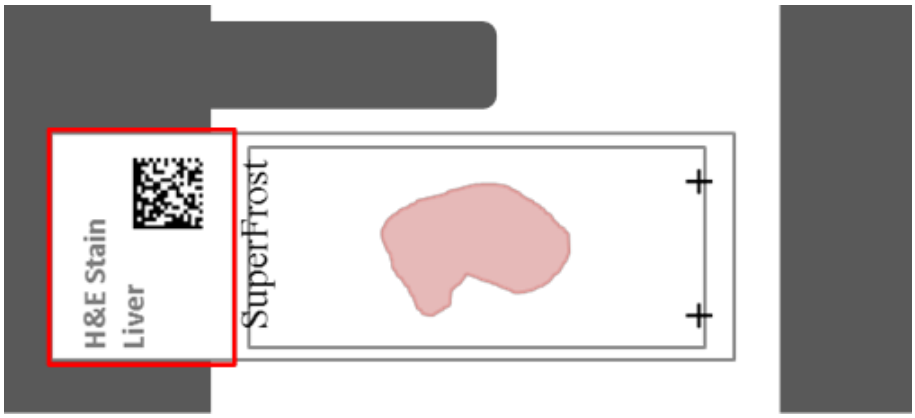


Figure 1 Correct position of thumbnail region

You can see from figure 1 that the **thumbnail region (red box)** should not cut off any text or identifying marks. It can also be a cosmetic advantage to ensure that parts of the specimen region are not included in the thumbnail region.

A detailed description of all features and settings for Label Scanning is included in the official software guide for ZEN Scan.

2.3 3 Preview Scan Settings

It is now in the process that the choices made have a direct impact on the outcome of the scan process.

The critical point to remember here is that the position of the red rectangle is fundamental to the success of the next step **Tissue Detection Settings**. Meaning that positioning of this rectangle is far from arbitrary.

The red rectangle defines the space on the slide that will be used by the Axio Scan.Z1 to detect the edges of the tissue section. This **tissue detection** and the maths behind it, is dependent upon the image information contained inside the red rectangle.

Figure 2 (below) shows the suggested correct position for the **preview scan region** (red rectangle).

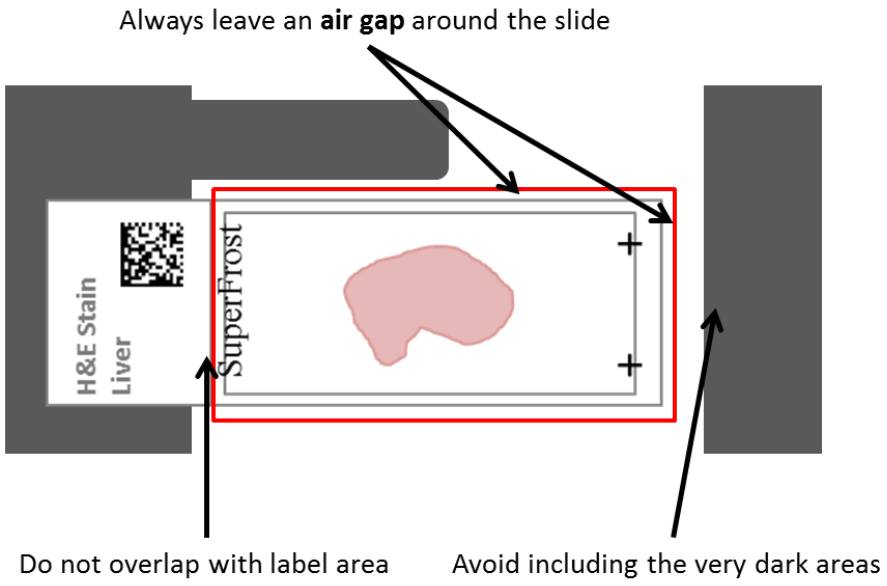


Figure 2 The **correct** position for the red rectangle

Figure 3 (below) shows three examples of **preview scan region** positions that could result in sub-optimal tissue detection outcomes in step 4 of this process.

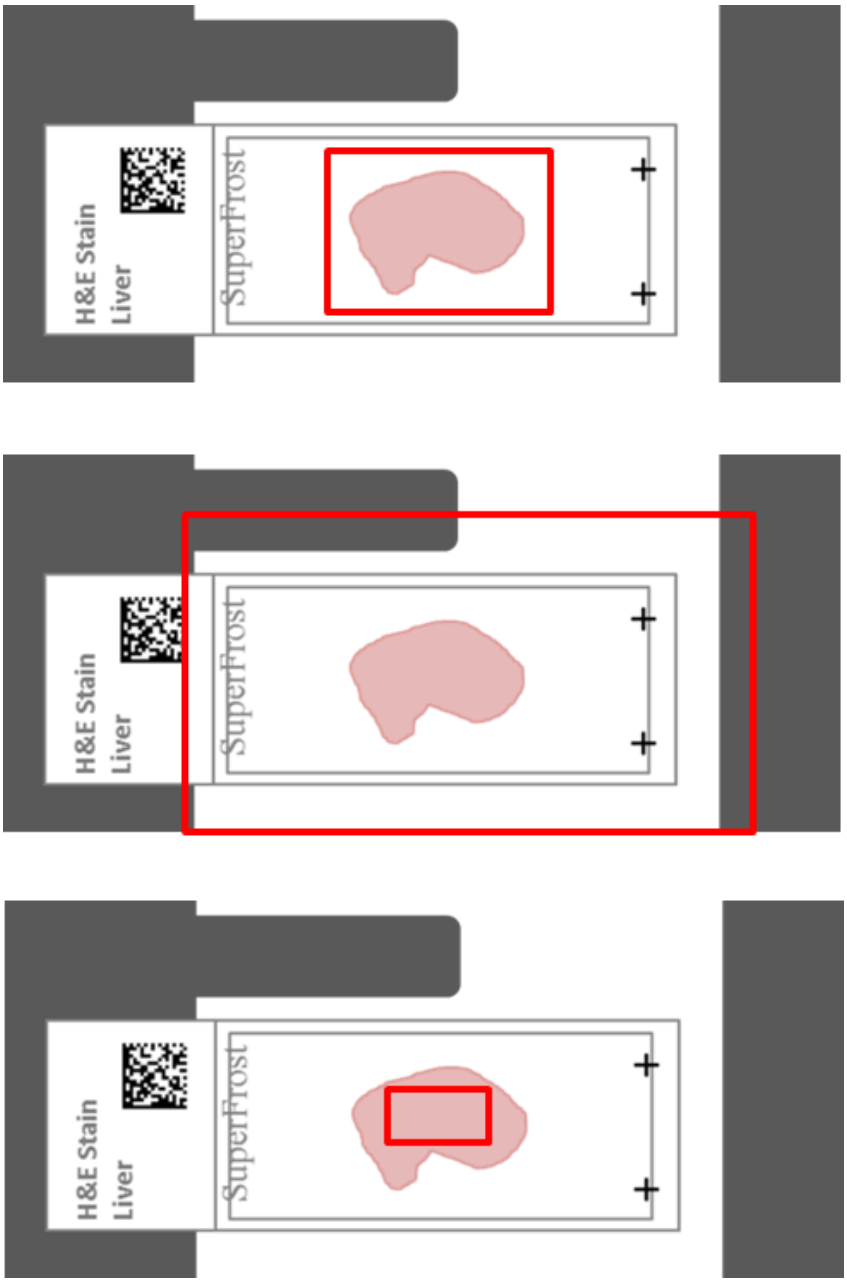


Figure 3 Less than optimal positions for **preview scan region**

Of course, in some cases the selections shown in figure 3 could give perfectly acceptable scans. The problem would be that other settings such as **air border dilation** and **max elongation** (both discussed in 2.4) can be adversely affected by the rectangle positions shown above.

2.4 4 Tissue Detection Settings

Having defined the region within which the tissue detection will be measured, it is now necessary to perform the actual **tissue detection** (TD). Figure 4 (below) shows the actual dialog window for tissue detection in the ZEN scan software.

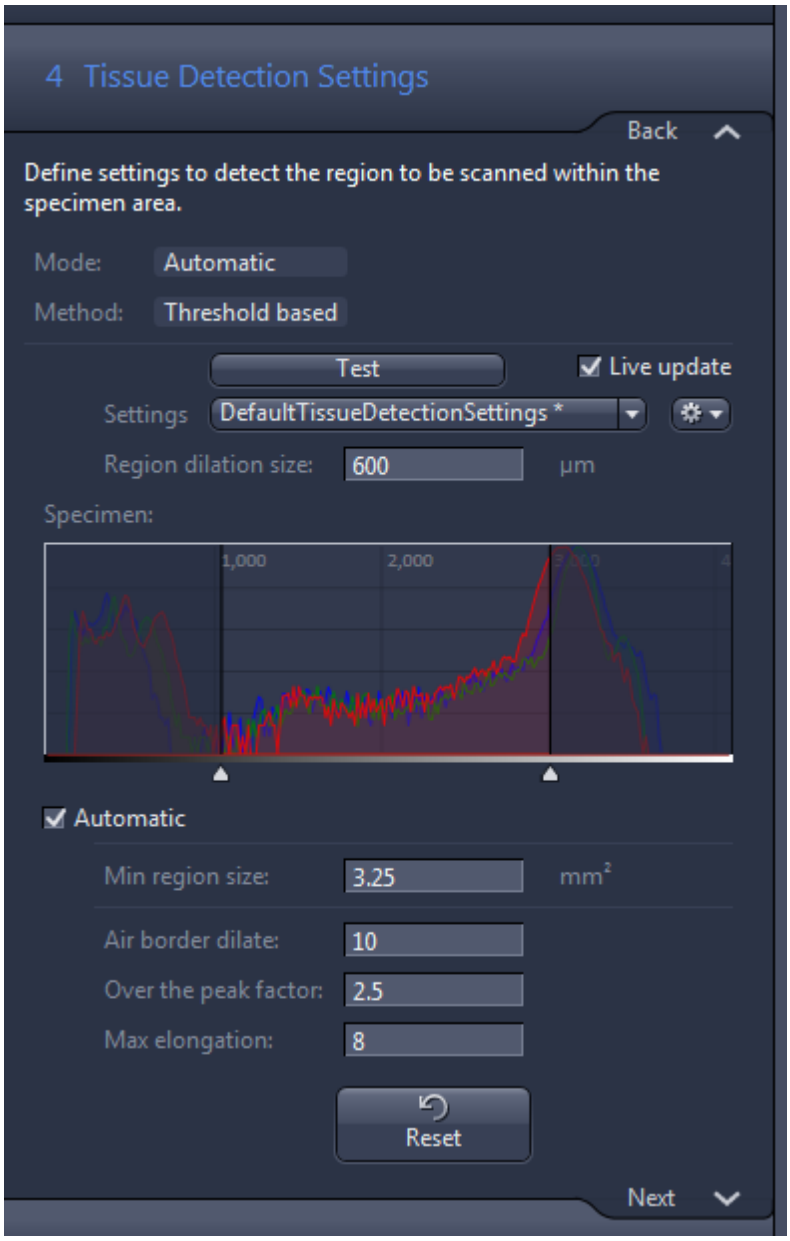


Figure 4 Tissue Detection dialog window

As can be seen in figure 4 (above), the principle component of tissue detection is the **preview image histogram**. This section will explain how to get the best results for difficult specimens using these settings.

2.4.1 Air Border Dilation - explained

In figure it can be seen that the **air border dilate** default setting is 10 pixels. Understanding this setting can save a lot of time as it is possible to remove the large majority of coverslip edge artefacts using this setting.

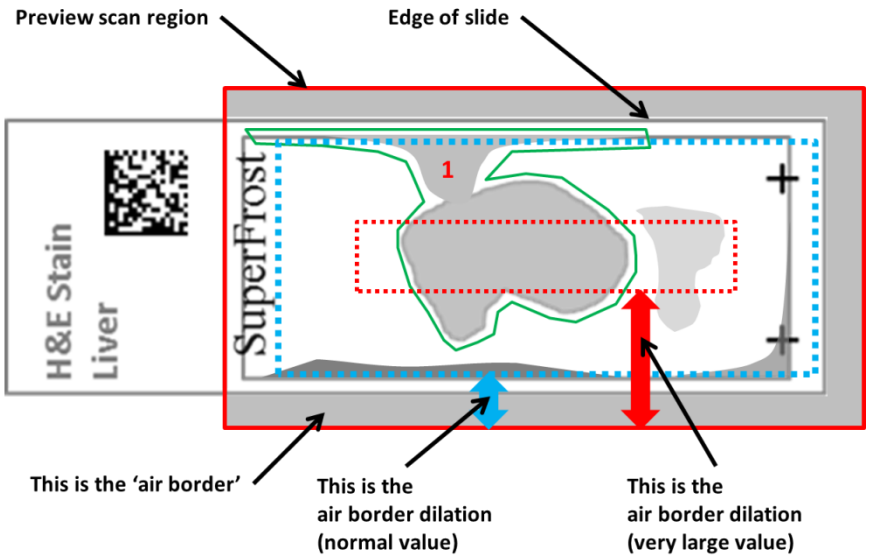


Figure 5 Schematic description of air border dilate setting

In figure five (above) you can see the red rectangle representing the region defined in step 3 of the process (section 2.3). Air border dilation creates an additional rectangle **inside** the preview scan region, by an amount defined by the setting value (e.g. 10 pixels). Put simply, larger values will create a smaller rectangle that is further into the preview scan region. The most practical use of **air border dilation** is to remove shadows and other artefacts that originate from the coverslip edge.

Caution should be taken to not use a larger dilation than necessary because if the interior rectangle touches part of the tissue section then it will be excluded from the scan. An example of this would be the red-dashed rectangle.

2.4.2 Max (maximum) Elongation - explained

Another way to exclude unwanted objects present in the preview scan image is to select according **aspect ratio** X axis dimension divided by Y axis dimension. In Axio Scan.Z1 this is called **elongation**.

Most long and thin artefacts are related in some way to the edge of the coverslip so this is another method that can be used to remove unwanted shadows (as was the case in section 2.4.1).

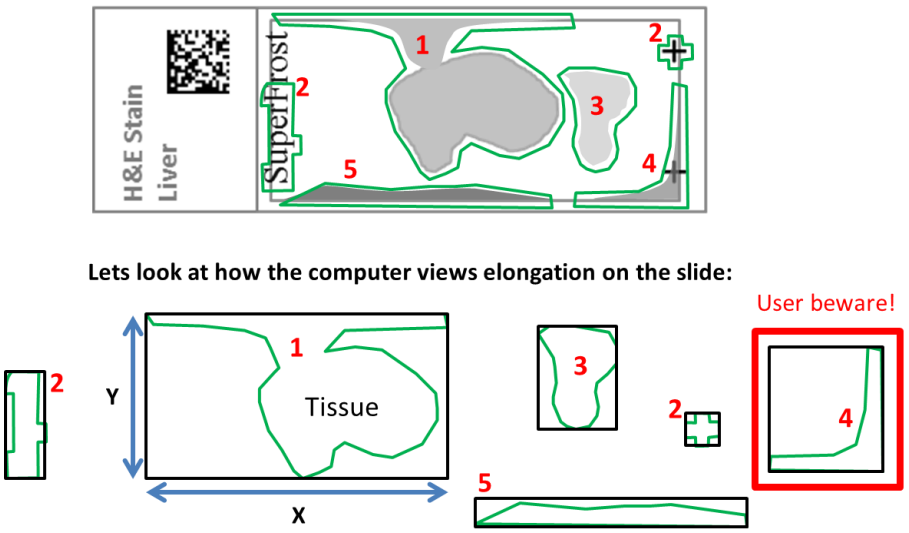


Figure 6 Classification of objects according to **elongation**

It is possible to see in figure six how different detected objects can be classified according to elongation. It is easy to see how object 5 could be excluded as its elongation is very different to the tissue section. The challenge is to recognise that object 4 actually has an elongation less than the tissue section itself, even though it is a coverslip shadow.

Objects can be clicked on to show the **bounding box** shape as shown in figure 6. You can then decide on the right exclusion value.

Max elongation is recommended for use where the object you are trying to remove is like object 5.

2.4.3 Region dilation size - considerations

Region dilation size is perhaps the most frequently adjusted parameter in profile set-up. However one should take care when using this setting as it is not as basic at it seems.

Perhaps the most important guide is not to use an unnecessarily wide or large dilation. This is because it is possible that the Axio Scan.Z1 could then place a focus point in the empty space around the tissue (in the dilated area). It is recommended that all focus points are placed inside the **tissue section** region.

It can also happen that two over dilated objects can touch each other and be detected as one object as shown in figure seven (below).

Figure 7 Touching objects due to over dilated regions

Over-dilation can be a particular problem if the tissue section is of a similar grey level intensity to a neighbouring artefact that you don't want to scan.

On the other hand, it is possible to under dilate an object which means it is possible that some tiles around the edges of the tissue section are clipped out and not scanned.

In general **region dilation size** should not be set below 100 to ensure no clipping occurs.

In some cases over dilation is used to deliberately scan an area well outside of the detected tissue section, for example to include the fatty tissue around a section of very intensely stained tumour in breast histopathology.

2.4.4 The image histogram – what it means

Objects in the preview image are included or excluded from being scanned by applying two thresholds (upper and lower) to the histogram. Any pixels outside of the threshold limits will not be imaged by Axio Scan.Z1.

In ZEN, you can switch off logarithmic scaling at any time by right-clicking in the histogram and deselecting it.

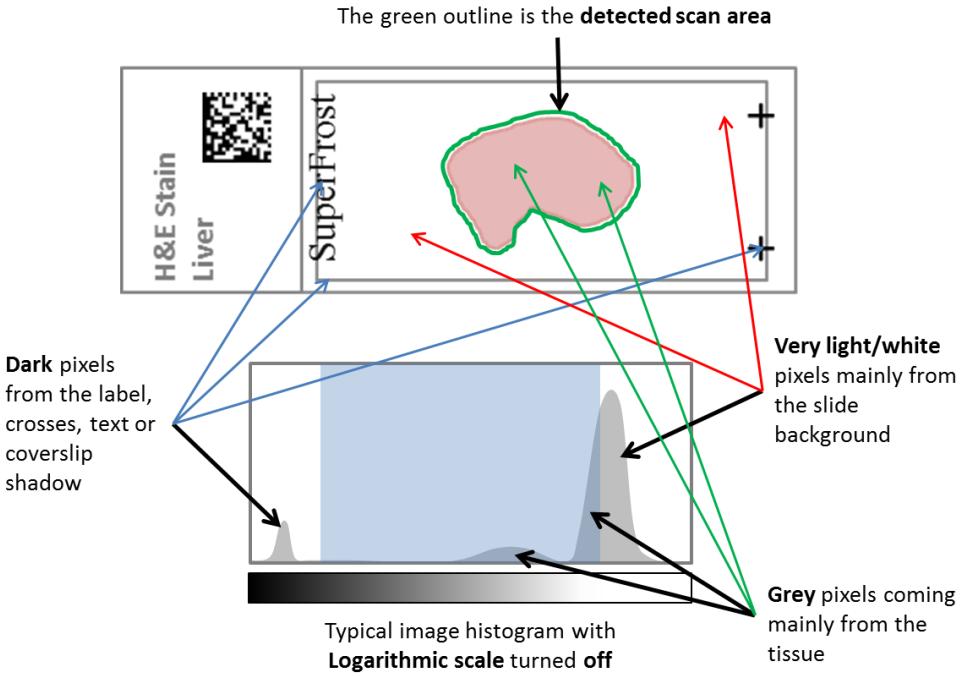


Figure 8 The relationship between preview image and histogram

Figure 8 (above) shows a typical histogram for the image above. The aim is to set the thresholds so that only the tissue section is detected and shown with a green line around it. In figure 8 you can see that the very light pixels of the slide background and the very dark pixels of any coverslip edges or text are removed. They are not inside the **blue** window.

In most cases, simple adjustment of the threshold values will produce a clean selection (green line) of the area want to scan. However in some cases **tissue detection** can require a combination of the methods described so far in section 2.4.X.

Let's now look at a more challenging situation and see how a successful segmentation can be achieved.

2.4.5 Tissue detection strategy on a real-world sample

Making well prepared microscope slides for histology is technically demanding and requires experience to perfect. In many research projects there simply is not enough time to reach a level of perfection whereby segmentation of the tissue section is really simple. In these **real-world** scenarios you need a **flexible scanner** and a sensible strategy.

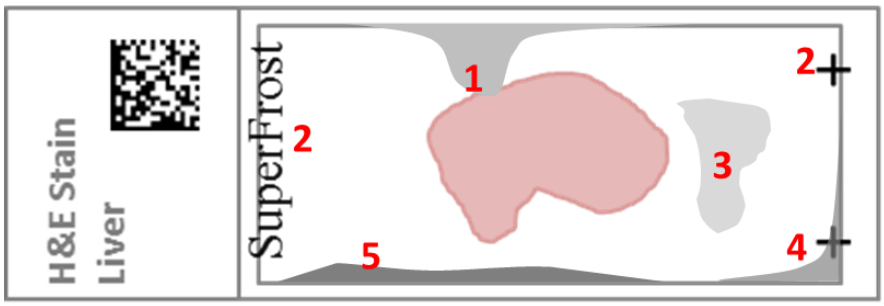


Figure 9 Real-world specimen slide with artefacts

In figure 9 you can see 5 typical artefacts that can easily occur in a sample but that you would prefer not to waste time scanning. Below are the 5 artefacts listed:

1. Shading region that invades and touches the tissue section area
2. Text or locating crosses on the slide
3. A fully included shading area e.g. bubble or back ground stain
4. A corner L-shaped coverslip shading
5. A side edge coverslip shading

Let's look at exactly how flexible **Axio Scan.Z1** is and how to remove these artefacts.

The strategy that follows assumes that the user is making a **new global profile** and is using the **default settings** shown in figure 4 as a starting point.

You should **turn off** the **automatic** setting by unchecking the tick box.

We will not discuss the **over the peak** factor as it relates only to the automatic setting.

2.4.6 Get the histogram segmentation right

It is very difficult to compensate for a poor histogram segmentation using the other settings in the **tissue detection** process. This means that it is advisable to invest more time in perfecting the right thresholds for the specimen. A useful first step is to see the slide how the computer sees the slide:

Effectively in grey scale – not colour

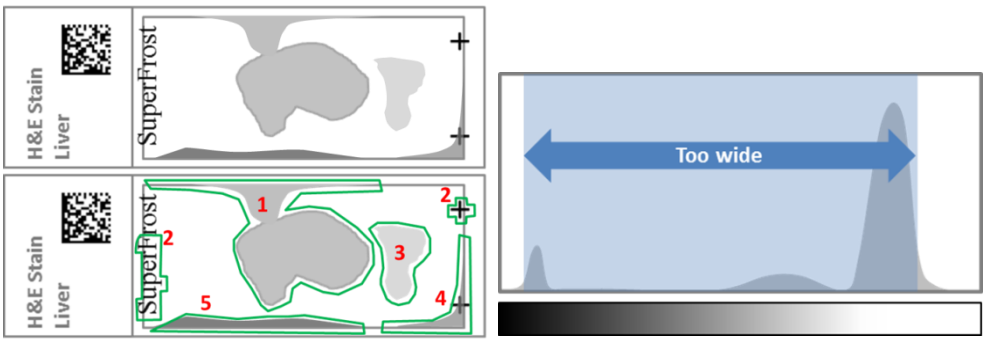


Figure 10 When the histogram threshold is too wide

When you imagine the slide in grey scale you can start to see which artefacts are **lighter** or **darker** than the tissue section – and which are the same!

As shown in figure 10, if you set the histogram threshold to be too wide, then all pixels are included and therefore all artefacts are detected.

The big question is **what do you adjust and how much?**

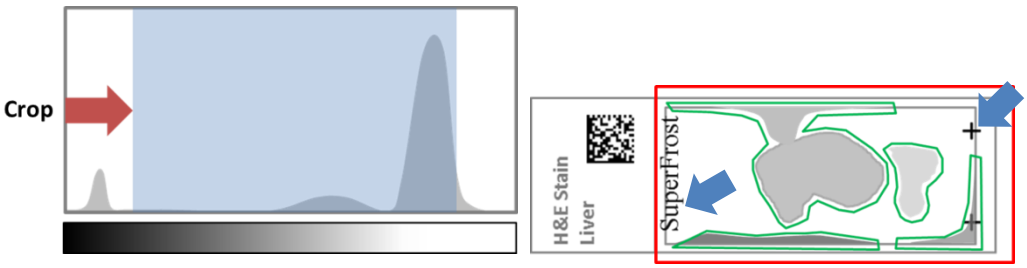


Figure 11 Cropping out the very dark pixels

Cropping from the **left side** to remove the **dark pixels** (smaller of the two sharp peaks) is a good first step. It is unusual for tissue sections to have pixel values in this region unless the staining is unusually intense (e.g. blood or bone marrow smears). Artefacts labelled 2 (text and cross) are now excluded from the scan (blue arrows).

Having removed the very dark pixels you can now focus on thresholding the rest of the image histogram as shown below.

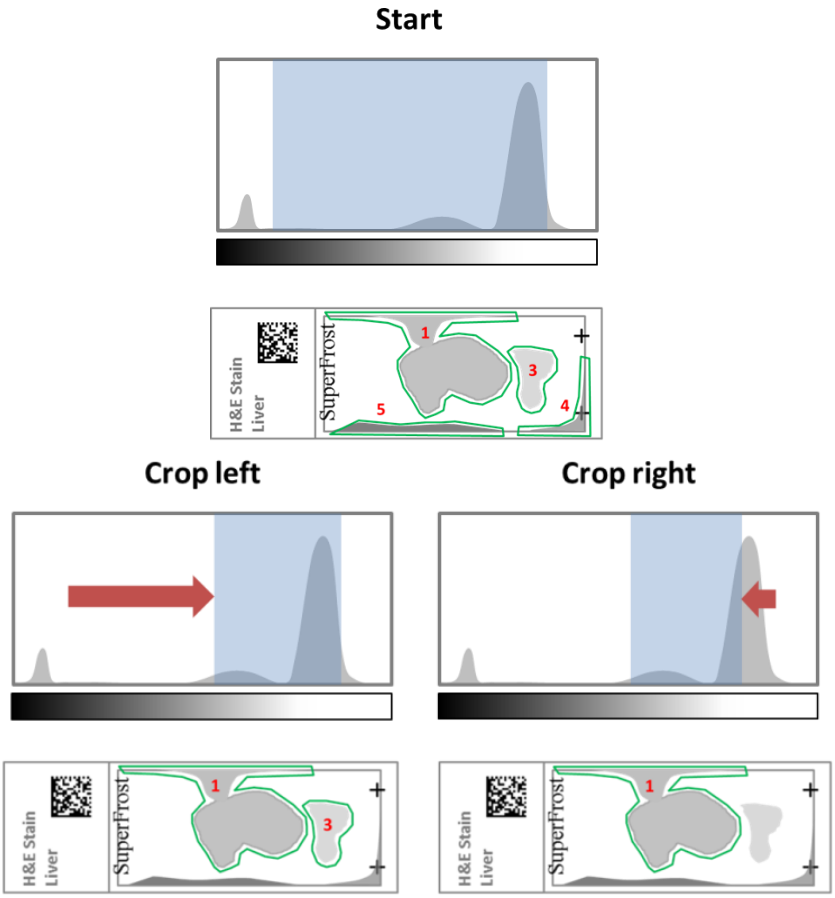


Figure 12 Crop left and crop right

When trying to set the threshold to find your tissue section it is critical to make small subtle adjustments. If you do not crop far enough in from the right side (light end) you could select the entire background which will be immediately excluded as it will touch or cross the **air border dilation box** shown in section 2.4.1.

As can be seen in figure 12 (above), shading artefact number 1 remains even after thresholding, air border dilation and region dilation settings. In this scenario object 1 both touches the tissue section and crucially, has the same grey level (intensity).

This can happen when scanning faintly stained tissue sections for example immunohistochemical stains on frozen tissue. To solve this problem without manual or interactive selection, the remaining option is to **adjust the preview camera**.

2.4.7 Preview camera adjustment

It is possible to adjust either the preview camera exposure time or the illumination intensity. It may be possible to generate a difference in grey level intensity between the tissue section and artefact 1 by increasing or decreasing the **illumination intensity** a little.

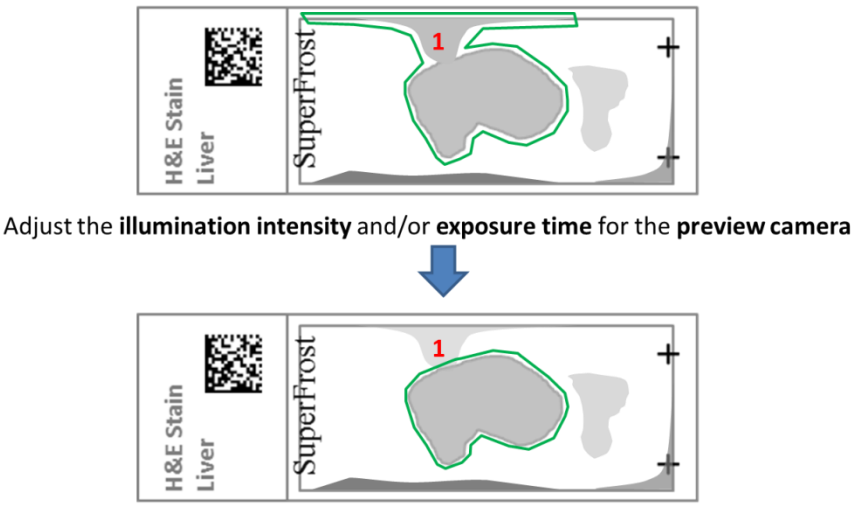


Figure 13 Possible effect of adjusting preview illumination and camera

In many cases, if you increase the illumination intensity a little, then the shading will become more transparent than the tissue section. This should then allow you to adjust the **histogram threshold** again to isolate the tissue section.

2.4.8 Summary

There are many approaches to tissue detection depending on the sample being scanned. There are no right or wrong methods as long as the end result is satisfactory to you.

The key consideration: if your profile is designed to scan multiple slides then your tissue detection settings should be wide enough to work on all slides. In practice this means trying a few trial slides with your new profile.

You can save your tissue detection settings and use them in other profiles. This is especially useful if they are very specialised and took a long time to configure.

2.5 5 Focus map settings

Having made a successful detection of your tissue on the slide it is now necessary to set-up the all-important focus map settings.

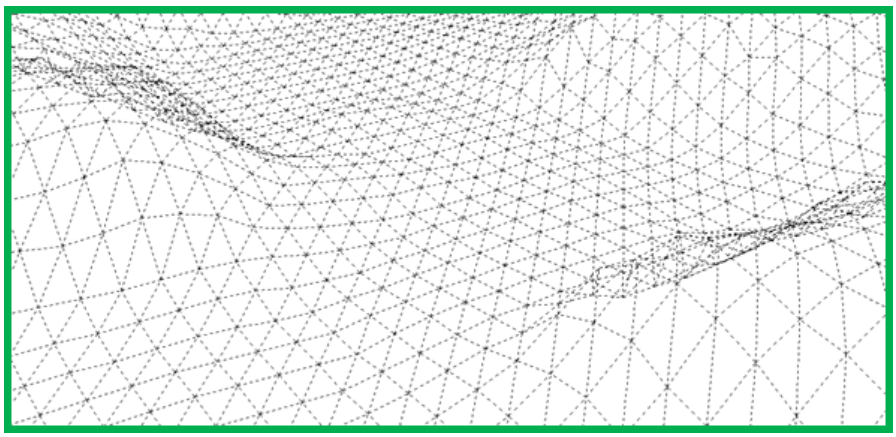


Figure 14 Representation of a focus map

The **focus map** is simply a set of **X Y and Z coordinates** that represent the surface of the detected tissue section. While the slide is being scanned the focus position will change continually, following the **contours** of the focus map. Each connecting point in the focus map is created as a result of either a **course** or **fine autofocus** process.

The user of Axio Scan.Z1 defines the following:

1. The lens used for either course or fine focus
2. The 2D layout of focus points
3. The autofocus range in course and fine
4. The autofocus interval or step size in Z
5. The algorithm used to calculate autofocus

All of these 5 points are important when successfully creating a the focus map.

2.5.1 COURSE and the FINE focus

Axio Scan.Z1 uses a **two-step** focusing method to give the best results. To understand how and why; we need to look at a the microscope slide and specimen in terms of **flatness**.

There are **two** types of flatness that are important for Axio Scan.Z1:

- **Macro-flatness** the overall tilt of the sample and/or slide
- **Micro-flatness** the small focus variations within the tissue section

As a general rule: **course focus** corrects for **macro-flatness** and **fine focus** corrects for **micro-flatness**.

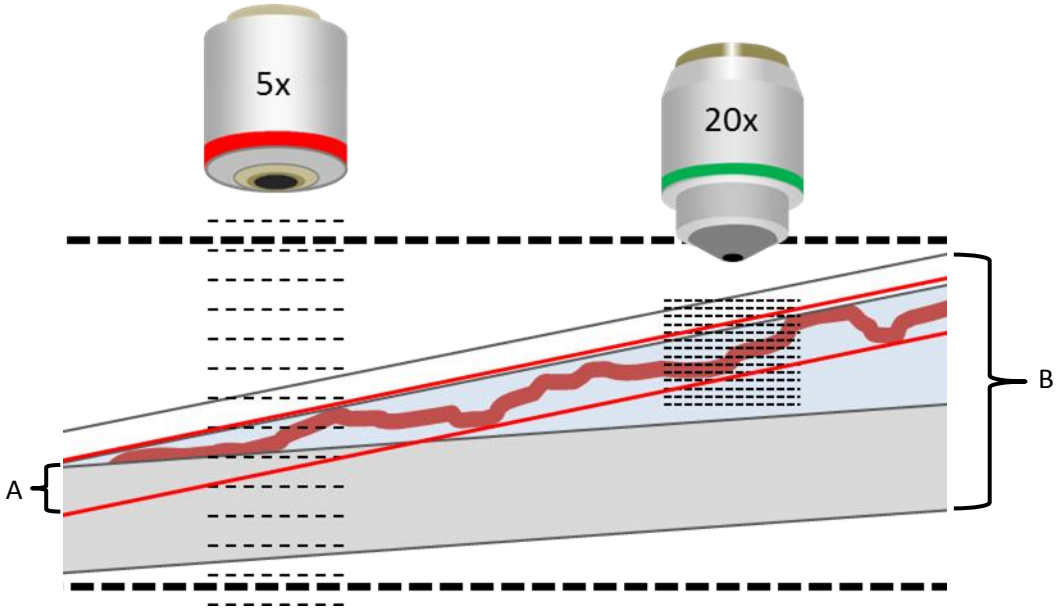


Figure 15 Schematic showing macro and micro flatness

In figure 15 (above) you can see the **macro-flatness** variation as the range between the two heavy black dotted lines (B). Tilt in the slide, cover slip, and mountant thickness, all contribute to **macro-flatness**. The specimen **micro-flatness** is shown as variations in tissue level between the two solid red lines (A).

Both course and fine objectives work together to map the specimen focus levels.

2.5.2 Setting course focus range

One of the first tasks for focus is to define the focus range for the course focus objective lens. Let's assume this is a 5x lens. The size of the course focus range will depend on how **macro-flat** the specimen is.

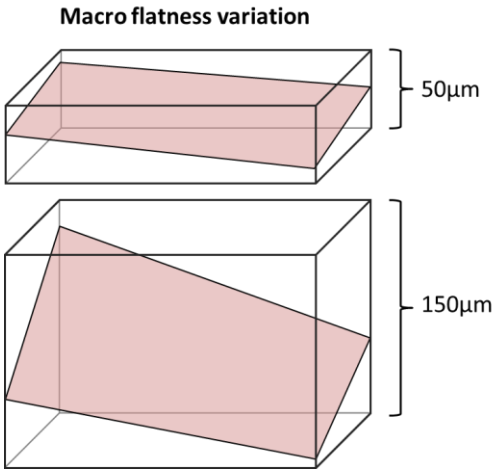


Figure 16 Different macro-flatness

As you can see from figure 16, a specimen that is more **macro-flat** will require a more shallow (less deep) course focus range than a specimen that is much less **macro-flat**.

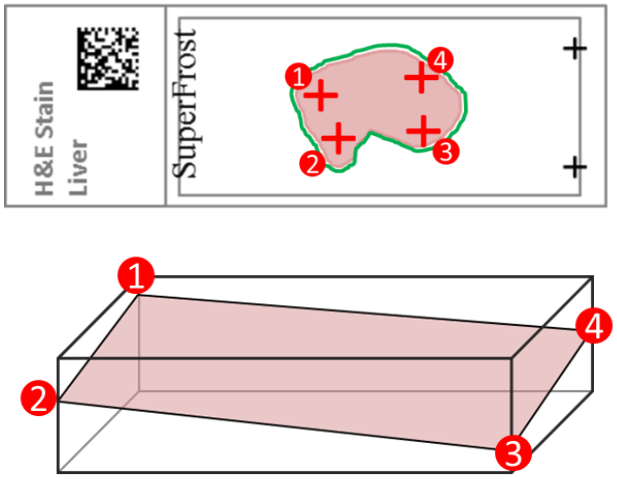


Figure 17 Measuring macro-flatness

Macro-flatness can be measured by choosing a number of points of the sample (number depends on size of section) and running an autofocus at each point using a focus range of 1000um to start with. When you write down the **Z position** for each point, you will quickly see how **macro-flat** the sample is, and therefore what your optimum **course focus range** should be.

2.5.3 Difference between course and fine focus range

A very important point is the difference between the **course** and **fine** focus range setup. As described in the previous section, **course** focus can be defined using actual Z position values for the top and bottom of the range. In ZEN:

Top of Z axis range = **SET LAST button**

Bottom of the Z axis range = **SET FIRST button**

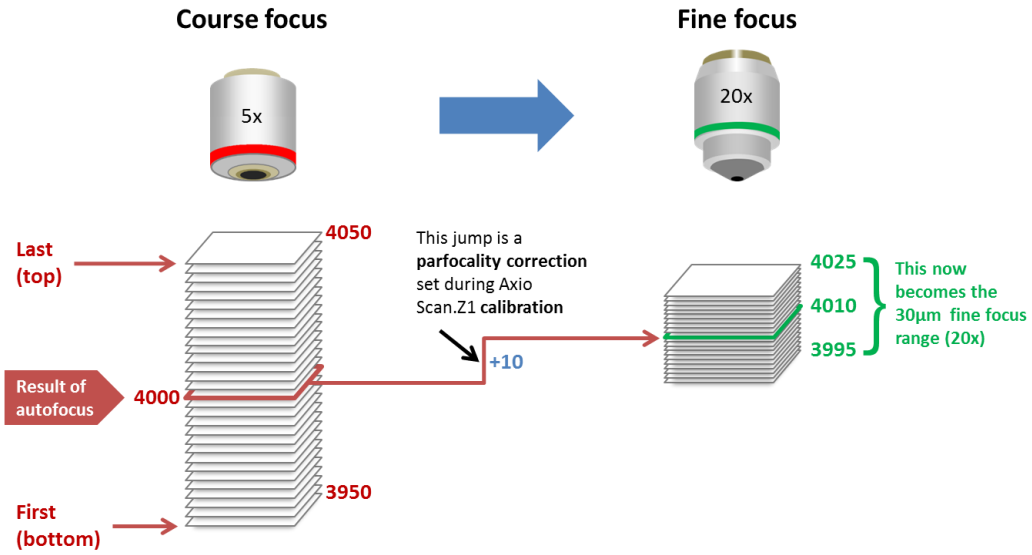


Figure 18 Relationship between course and fine focus range

Figure 18 shows **course** focus as a range of points between two defined values. **Fine focus** is different. Fine focus is a range that is not defined by a top and bottom, but by the position of its **middle** or **centre**. The range is then set by defining a travel distance, for example 30um. The result is a range that is 15um above and 15 micron below the centre point. In figure 18 this is shown in green as the range of 30 centred around 4010um.

So to define a focus position, this is what happens:

1. 5x lens runs an autofocus over the defined range and sets a point of best focus (4000um).
2. Axio Scan.Z1 changes to 20x fine focus and corrects for parfocality difference by applying an offset, in this case **+10um** (4000 + 10 = 4010).
3. The 20x defined range of 30um is then set around the new centre focus value of 4010 by +15 and -15, to give the full range of 30um.
4. **Micro-flatness** variations can then vary by +/- 15um and be successfully mapped for the scanning process.

2.5.4 Course and fine focus combine to map contours

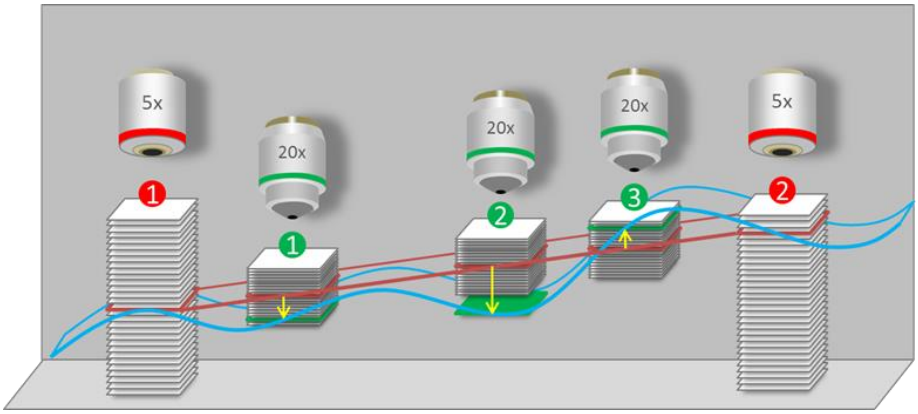


Figure 19 Course and fine focus mapping

In the diagram above (figure 19) there are two course focus points measured by the 5x, shown as red points 1 and 2. The course focus map creates a gradient between these two points. Each fine focus point has its centre position automatically placed on the red course focus gradient. An accurate autofocus point measurement will then be attempted for each fine focus position. If the blue line representing the tissue **micro-flatness** varies by less than 15um in either direction, an autofocus point will be generated.

Green fine focus point number 2 will fail because for that position, the micro-flatness variation has a point of focus **outside** the fine focus range. The Axio Scan.Z1will continue to map the specimen with point 2 excluded.

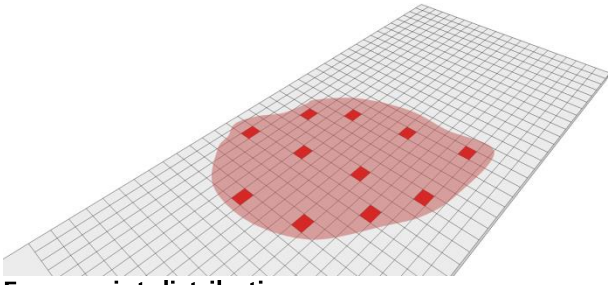
There are TWO options here.

1. **Enlarge the range of the fine focus 20x**
    a. Pro: the focus for this location will be calculated
    b. Con: all of the fine focus points will take longer to measure

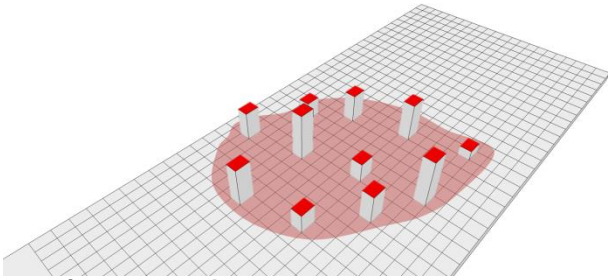
2. **Increase the number of course focus points**
    a. Pro: will give a more accurate fine focus centre point
    b. Con: Cannot guarantee the location of the new points

Option 2 is preferred in most cases as scan times can be dramatically increased by having a fine focus range that is unnecessarily large for most fine focus points.

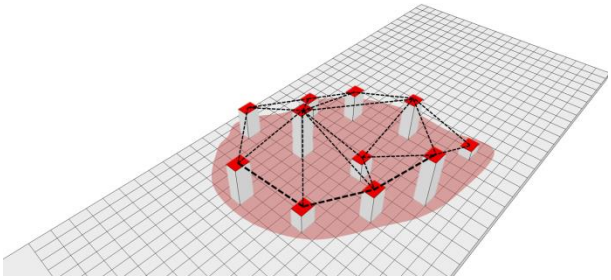
2.5.5 Course focus map in 3D



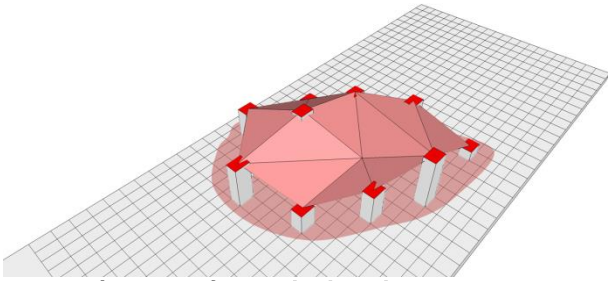
Focus point distribution



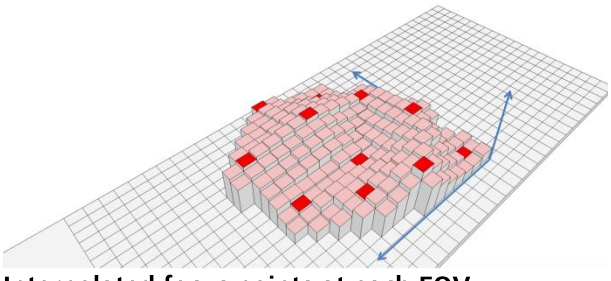
Autofocus at each point



Grid-lines between points



Course focus surface calculated



Interpolated focus points at each FOV

2.5.6 Autofocus step size (interval)

Autofocus step size or interval is the distance between each focus point as the Axio Scan.Z1 scans through the Z axis to find the best focus. The size of the interval determines the accuracy of the autofocus result.

If the interval is too big the autofocus will not be accurate or reliable, producing different values for the same point two times in a row.

If the interval is too small the autofocus speed will be very slow and the focus mapping step in the scan will take a much longer time.

The optimal step size is one that closely matches the depth of focus (DOF) for the lens being used.

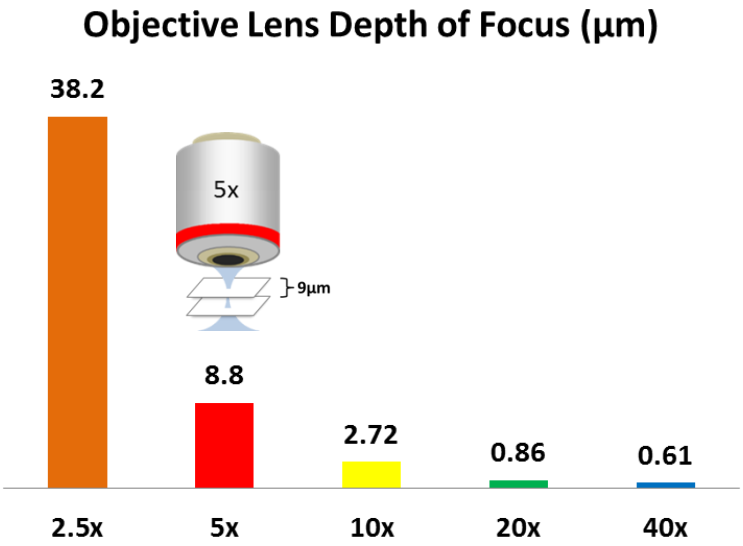


Figure 20 Objective lens depth of field (theoretical)

In practice, good results for autofocus can be achieved when multiplying the above DOFs by approximately two, so that 0.86 for 20x becomes 2.0 microns.

Finding the optimal step size is one of the most important factors in determining the speed of the whole slide scanning process.

Most scan protocols can be improved by changing the autofocus step size either to speed up autofocus or make it more accurate.

2.5.7 Camera settings for focusing

Any user of Axio Scan.Z1 will notice that it is possible to use camera and illumination settings for focusing that are different to scanning.

It is important not to neglect the imaging settings for focus map creation. Autofocus algorithms work on the basis of contrast between pixels. If the specimen is not correctly imaged (illumination and camera) then it becomes harder for the algorithm to detect the correct focus level for the sample.

If the autofocus algorithm also requires colour information then it is necessary to ensure the correct white balance or colour temperature.

In short, it is recommended to take equal care over focus image setup as would be taken for actual scanning image setup.

2.5.8 Useful data for objective lenses

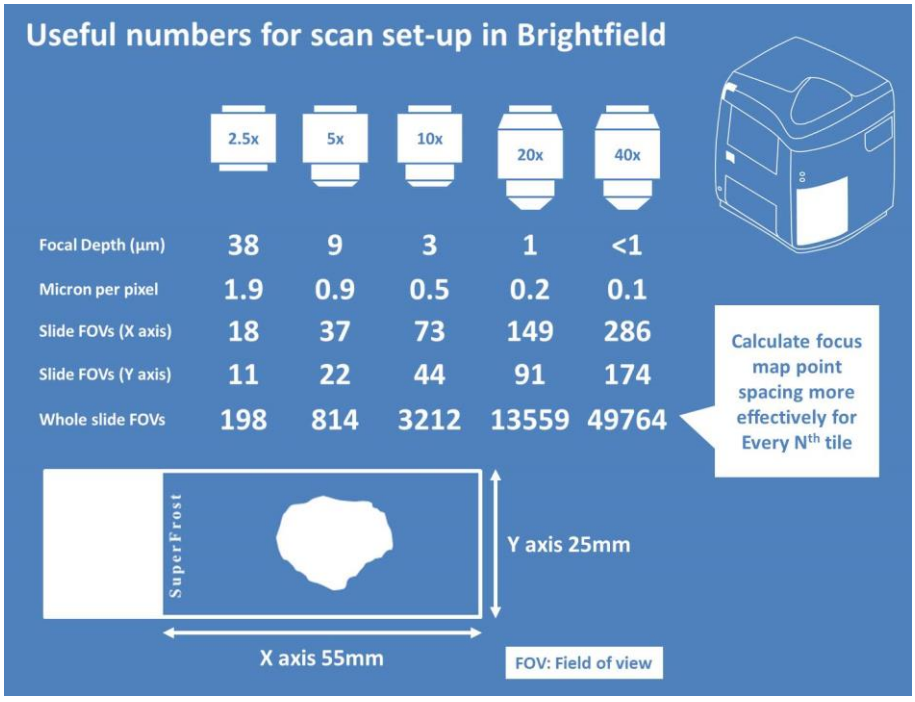


Figure 21 Data for Axio Scan.Z1 objectives lenses

Use the information in figure 21 to estimate the number of fields of view (FOV) that a tissue section covers on the slide. This can help when assigning values for focus map point distribution density.

2.5.9 Focus point distribution strategy

Focus point distribution strategy is used to place the focus points for course and fine focus. There are literally thousands of possible combinations of settings which can make this selection quite daunting. However a simplification of the process will be described by explaining the important strategies and how to choose the correct values for variables.

What is the right focus point strategy for my sample?

1. **Small tissue sections** like mouse testis or TMA cores do not usually require a large number of focus points in either course or fine.

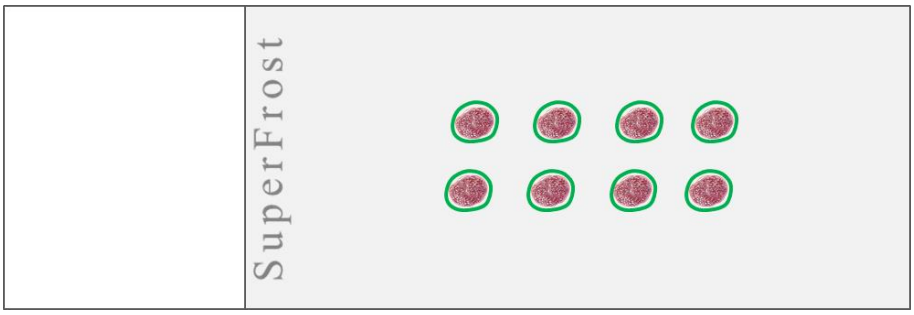


Figure 22 Small section – mouse testis

Due to their small size and probable **good macro-flatness** either **centre of gravity** or **number of points** can be used for **course focus**. If number of points is the method chosen, use a value of 4 for the number of points. Centre of gravity will always use just 1 point per section.

Fine focus is more flexible because at 20x each section in figure 22 covers about 100 FOVs (10 x 10). A good strategy here would be to use **every N^{th} tile** with N = 2/3. A value of N higher than 3 will probably result in too few focus points for a good focus map. **Number of points** could also deliver a good result here with **5-6 points** per section.

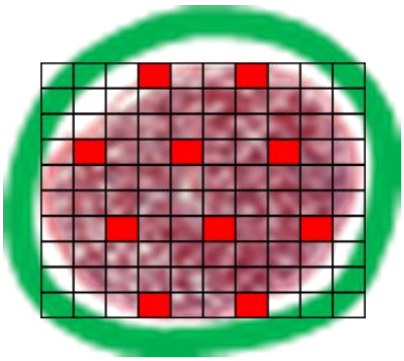


Figure 23 Focus points with N = 3 at 20x for **every Nth tile**

NB: All suggestions are dependent on sample preparation and with poor preparation even small tissue sections can be very un-flat requiring more focus points.

2. **Medium tissue sections** such as mouse brains unsurprisingly need more focus points in course and fine focus.

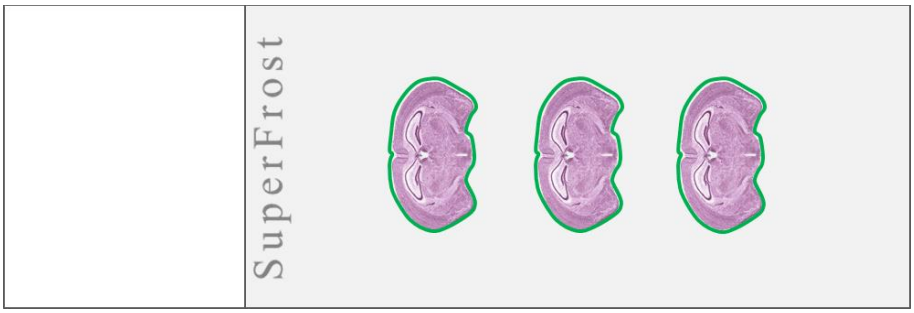


Figure 24 Medium size sections – mouse brains

If your specimen preparation has good **macro-flatness** then it is possible to make the **course focus** using the **number of points** strategy. For a mouse brain section, the number of points should be about **6-8** points. For **less macro-flat** preparations it is recommended to use **every Nth tile** for both course and fine focus.

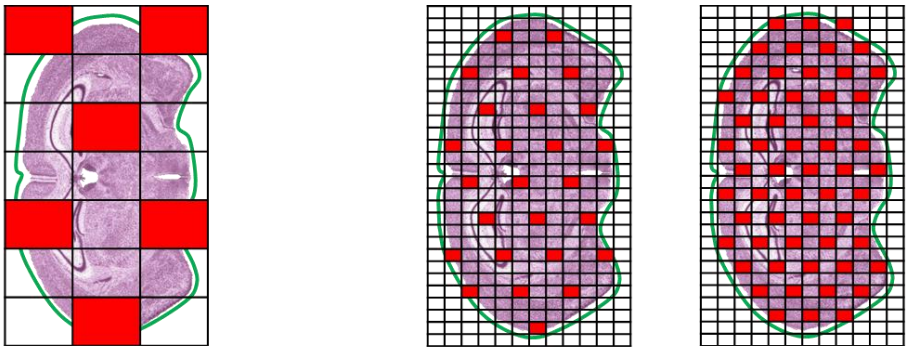


Figure 25 Course and fine point distribution using **every Nth tile**

You can see in figure 25 (above) that for the 5x course focus, N=2 gives you six points and at 20x, N=3 gives 26 points. For a slide of average flatness, this strategy would probably yield good results.

In both cases it is possible to increase the number of focus points to make the focus map more sensitive. At 5x **N=1** is the same as **every tile** and would produce **21 focus points**.

Be careful when using low values of N for fine focus. As you can see from figure 25, by changing N=3 to N=2 you add an **extra 31** focus points. This will make the scan process slow down and should be avoided unless absolutely necessary.

NB: Some trial and error is always needed to fine the right compromise between speed and focus quality. As always, the flatter the sample the better.

3. **Large tissue sections** pose a new challenge as they cover a much larger area of the slide, sometimes all of the space under the coverslip.

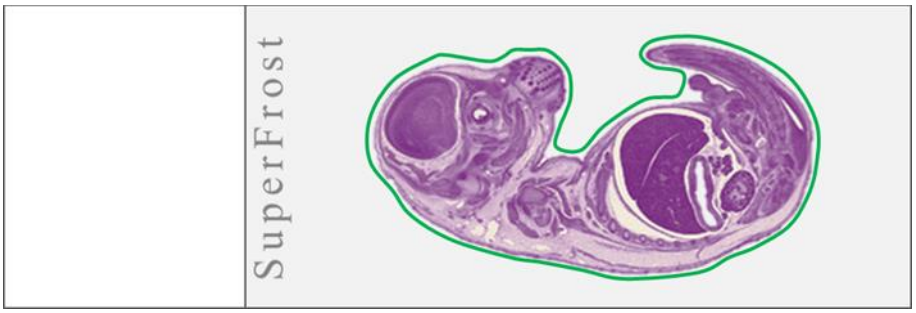


Figure 26 Large tissue sections – whole mouse embryo

Once a single section exceeds 2cm^2 it becomes important to ensure the focus map compensates for **macro-flatness** and this is achieved with **course focus.** As a rule: if you get the course focus right for a large section, the fine focus will improve dramatically. Refer back to section 2.5.2 for a description of how to measure macro-flatness.

The guidelines for **course focus** strategy with large sections are generally the same as those for medium sized sections – **you just need more points** to cover the larger tissue area.

However the **onion skin** strategy is useful for large sections under fine focus. **Onion skin** basically generates focus points along concentric rings within the tissue section. These means the point distribution follows the shape of the section producing a point layout with better coverage using less points than **every Nth tile.**

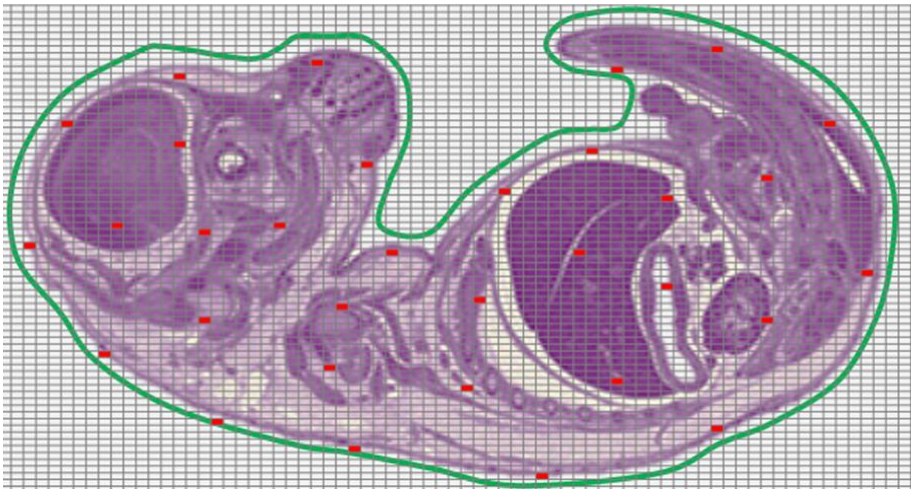


Figure 27 Mouse embryo with **onion skin** distribution at 20x (32 points)

Density is set 0.2 for the scan shown in figure 27. This means the spacing of the concentric ring layout used to position points is **20%** of the width of the slide in the Y direction. Setting a low figure such as 0.1 and increasing the maximum number of points would result in a focus map with more densely positioned points.

2.5.10 Autofocus (sharpness) algorithm

Axio Scan.Z1 uses a number of different **algorithms** for autofocusing. For the majority of profiles (especially in brightfield) you will use the **Basic** autofocus method.

1. **Basic** – this is the most commonly used and fastest method for autofocus. It relies on average contrast detection to determine sharp focus. It is fast because it groups camera pixels into clusters of 4 (2x2) which is a kind of binning, in order to measure focus.
2. **Best** – this method is identical to **basic** apart from it also measures the image **without clustering**. The disadvantage of this is that best is slightly slower than basic. If you want to focus on very fine detail at 40x then use **best** for fine focusing.
3. **HG2^8** – this is a more sophisticated algorithm used for specific detection of small intensely stained objects in the focal plane, such as nuclei. This method uses a weighting to choose focus levels with regions of small dense focus like nuclei. Basic and best to not do this. Use HG2^8 for immunohistochemistry but do to use it for **course focus** as dirt and dust will be prioritised by this method.
4. **FFT** – Fast Fourier Transform or FFT is a specialised algorithm developed to focus **ring aperture contrast** or RAC images. FFT should not be used for any other type of focus task.

Please refer back to section 2.5.6 for information regarding step size and autofocus. The key point is that all autofocus methods work best when there is a detectable difference between one focus level and another.

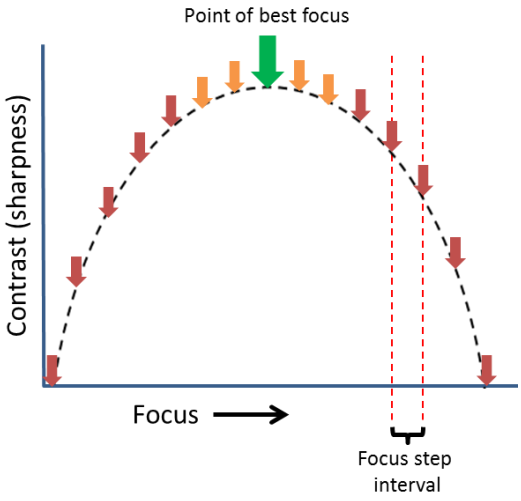


Figure 28 Graph of focus curve calculation

Best focus is calculated from a curve calculation using contrast measurements at each Z axis interval point. The smaller the interval distance the more points on the curve and the more accurate the result.

2.6 6 Scan settings

The final part of the profile setup is the selection of settings for actual scanning of the microscope slide. This means defining:

- Objective lens for scanning
- Illumination, Exposure and white balance
- Z-stack and EDF
- Image format and compression
- Stitching options

In this section we will go through each setting individually with practical recommendations.

2.6.1 Objective lens for scanning

A common misconception is that **fine focus** lens selection automatically selects the lens for **scanning**. It doesn't. It is necessary to select the scanning lens independently. This is an advantage because it is possible to scan with a different lens than either the course or fine focus lens.

For example, **5x Course focus, 40x Fine focus, 20x Scanning**

The setup shown above is possible because Axio Scan.Z1 has very reliable parfocality correction from 40x to 20x AND, the 40x lens is a better tool for autofocus calculation than the 20x – due to its better Z-axis (axial) resolution.

Parfocality correction is shown in figure 18, page 21.

2.6.2 Illumination, Exposure and White Balance

Axio Scan.Z1 uses a white light VID LED illumination system. Two variables called Exposure, Flash Duration and Flash Intensity can be set automatically using the **measure** button. If you want to set white balance at the same time then click **measure and white balance**.

But be aware that if the current stage position is **in the tissue** then the white balance function should not be used.

TIP: If you have very dark or intensely stained regions in your section, you should perform a **measure** function over that region in order to have the right camera scanning settings to image the detail in that region.

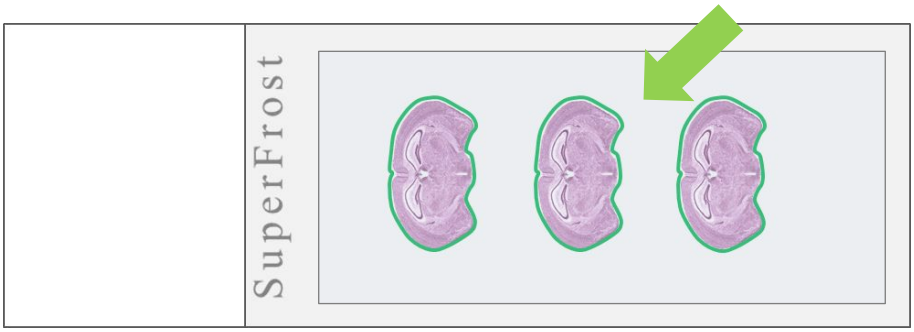


Figure 29 Position for measure and white balance

If a white balance measurement is taken, make sure that you position the stage in a place where there is no tissue but is under the coverslip. This is because all glass between the camera and the sample will influence the result of the white balance.

For a full description of illumination, exposure and white balance, please refer to the ZEN Slidescan Software Guide (2012 SP2).

2.6.3 Z-stack and EDF

Some specimens will have a **micro-flatness** that is so variable and unflat that they require the capture of several different focal plans to capture the right information.

In general, for **brightfield** specimens, the tissue section or sample thickness will not exceed 10 microns. Most paraffin sections are considerably thinner, at 2-3 microns.

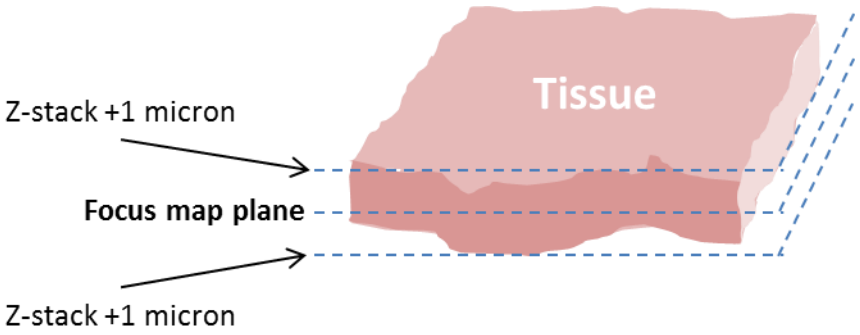


Figure 30 A three micron Z-stack of brightfield tissue section

As can be seen in figure 30, creating a **3 slice** Z-stack with 1.00µm interval gives good coverage of the tissue section thickness.

Once a Z-stack has been acquired the user will be able to scroll through the different focal planes when viewing the digital slide. This gives the effect of moving up and down in focus, as if looking through a microscope in real time.

If an **extended depth of focus** or EDF is required then it is advised to acquire more slices with smaller interval. EDF will create a flat 2D image from a Z-stack by calculating the best focus regions from each slice image. EDF works best when the user **clips** the Z-stack down, to reduce the amount of out-of-focus slices at the top and bottom of the Z-stack.

NB: Creating digital slides with Z-stacks uses a lot of disk space and generates large files. A two slice Z-stack digital slide will be twice the size of a single plane version.

2.6.4 Image format and compression

Default settings on the Axio Scan.Z1 are as follows:

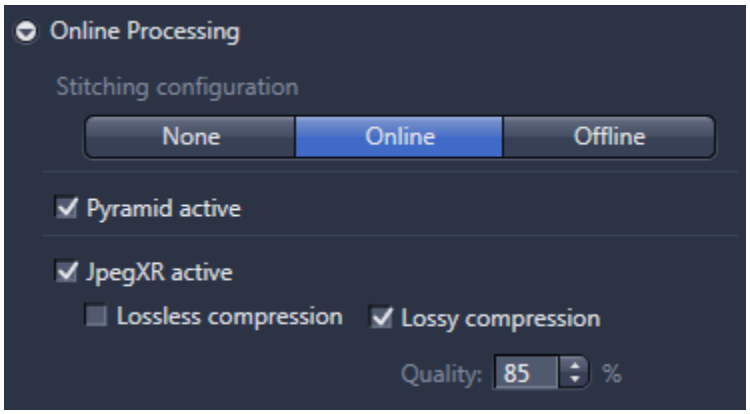


Figure 31 Default settings for image processing

In general, **Pyramid** is always active as this creates the dynamic zoom levels that make a digital slide what it is. JpegXR is the compression method that can be applied to Axio Scan.Z1 digitial slides. Lossy is usually active as it creates files of a manageable size on disk without losing significant resolution. **85%** is recommended as a standard quality setting.

All Axio Scan.Z1 slides are produced in the Zeiss .CZI format, JpegXR is just the compression method.

2.6.5 Stitching options

Axio Scan.Z1 has three choices for stitching.

None as the means that the tiles will be scanned and stored in position but will not be stitched together. The slide can still be opened and navigated but it will be apparent at higher zoom levels, that the tiles are not stitched. **Tiles can be stitched at a later date.**

Online stitching is the default setting and means the scanner compiles and stitches acquired tiles during the scan process. At the end of the scan the digital slide will be fully stitched.

Offline stitching is required if you have a specimen which is not easily stitched online. By stitching offline, the scanner waits until all tiles are acquired before stitching. This means the stitching algorithm has more information to use for the calculation and the results can be improved. However this process can be slower than stitching online.