Method of Procedure

Nanoro Microscope Focus Stacking

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Illustration

Focus stacking consists of combining multiple images taken at different lens heights i.e. focussing on the same part of a sample, but at different depths.

Introduction

This document describes the procedure to follow for creating a focus stacked image using the Nanoro microscope software. It also contains further information explaining focus stacking to help the user make informed choices about what values to use for the focus stacking parameters, where to enter them, and how to enter them efficiently.

An overview of the procedure to follow is provided immediately, for easy access to those having done this before and just seeking a reminder.

Please read on when viewing the document for the first time, or if a fuller explanation is required.

Focus Stacking Procedure

1. Locate the item to be imaged by exploring with the microscope.
2. Focus slightly above the top of the item and note the value of the Z axis reading.
3. Focus slightly below the bottom of the item and again note the value of the Z-axis.
4. Subtract the two values to determine the appropriate Z distance over which to image.
5. Set the image spacing to slightly below the lens depth of field (the default value).
6. Calculate the count of steps the lens must make moving between images.
7. Re-adjust the figures as required to be in synchronisation (distance = spacing *step count)
8. Click OK in the RUN tab to perform the scan.

What is Focus Stacking?

We have all seen images where part of a picture is in focus and part is blurred, due to focussing.

This happens on microscopes as well as ordinary cameras.

A solution is to take multiple images, e.g. one where the foreground is in focus and another where the background is in focus. Then, to combine these images to produce a new image which shows both the foreground and background in focus at the same time. In practice, multiple images may have to be taken with a microscope as the **depth of field** can be extremely small.

There are two related terms, which are often confused: depth of field and depth of focus. Depth of field is the relevant term for focus stacking.

- Depth of field: how far can a point on the viewed sample be moved in total (either raised or lowered, from one limit to the other) and remain in focus.
- Depth of focus: how far can the camera sensor be moved, and the image remain in focus. This is an issue only once when screwing the camera onto the microscope during manufacture or assembly.

The process of combining images with different parts in focus is known as focus stacking. The aim is to create one all-in-focus image.

Suppose for example a sample to be imaged is 40 µm deep and the depth of field is 10 µm. Then we could take 5 images, and we should have layers with everything sharp in at least one of the images.

1. 0,
2. 10 µm,
3. 20 µm,
4. 30 µm,
5. 40 µm,

The software which performs the focus stacking works best if images go into focus and back out again. For this reason, it would be better to take 7 images, at depth:

1. -10 µm
2. 0,
3. 10 µm,
4. 20 µm,
5. 30 µm,
6. 40 µm,
7. 50 µm.

Also, since 10 µm is the ***estimated*** depth of field, (with emphasis on the word estimated,) using a shorter spacing between images may produce sharper images and reduces the probability that something is missed, in the sense of not sharp in any of the layers.

So instead of moving 6 times 10 µm for the images, more and smaller steps could be used.

Here is an example which covers the range 0 - 40 µm in steps of 8 µm. Note that the 8 µm movements are smaller than the 10 µm depth of field and that the entire range is covered with a little excess at each end.

1. -8 µm
2. 0,
3. 8 µm,
4. 16 µm,
5. 24 µm,
6. 32 µm,
7. 40 µm,
8. 48 µm.

This example would involve capturing 8 images by moving 7 times 8 µm, or 56 µm in total, from -8 µm to 48 µm.

The Scan Dialog Z Tab

Spacing (Image Spacing and Depth of field)

Lenses and their depth of field are related. The higher the magnification the shorter the depth of field as illustrated below with some examples.

Lens	Medium	Magnification	Numerical Aperture	Working Distance	Depth of Field
Nikon MRP70101	Air	10x	0.25	7 mm	11.8 µm
Nikon MRP70401	Air	40x	0.65	0.65 mm	1.7 µm
Nikon	Air	100x	0.90	0.26 mm	0.88 µm
Nikon MRP71900	Oil	100x	1.25	0.23 mm	0.695 µm

Depth of field is calculated using the method described here:

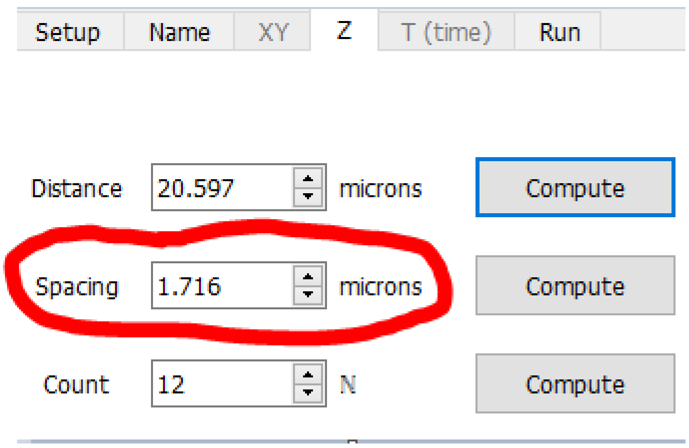
https://www.translatorscafe.com/unit-converter/en-US/calculator/depth-of-field/#dof-scheme-bigger

The working distance in the table above is the distance between the lens and the sample being imaged, when the sample is in focus.

The working distance gives us an idea how far the lens will be from the sample i.e., from damaging the lens, sample, or both. Extreme care must be used to avoid contact between lens and sample.

The depth of field estimate gives us an idea of maximum distance we should move the Z axis between images. For that reason, figures lower than the depth of field are recommended to be used for image spacing.

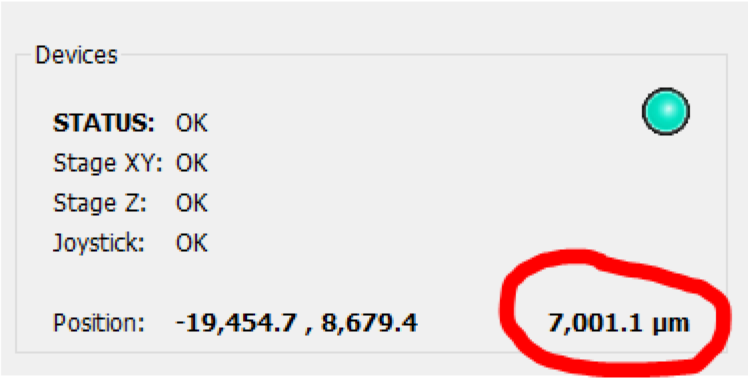
Image spacing is entered in the Z tab of the scan choice dialog box.



The default value for the spacing is a depth of field estimation calculated for the lens in use, and should normally only be lowered, not raised from the default.

Distance (Z Distance)

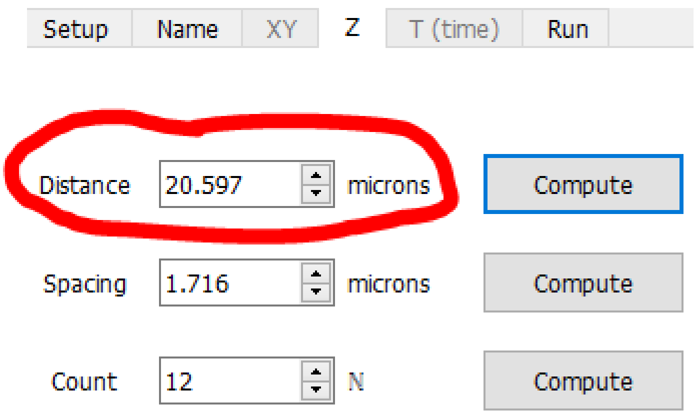
The nominal height of the lens is displayed in the Nanoro software Device Panel.



These values merely represent the range of travel of the Z stage, with 0 meaning the Z stage is halfway between its upper and lower limits.

To determine the distance the Z stage must move for focus stacking, note the Z stage height when focussed on the highest point required for the image stack and at the lowest point, and subtract the two.

The distance required to be scanned to create the Z stack (i.e. series of images to be focus stacked) is entered in the Z tab of scanning dialog box.



The Z stage should move the distance from slightly above the top of the item to slightly below the bottom of the item.

Count

The Z tab of the scanning dialog box includes a third so called "spin box" where a count of the number of steps the Z stage makes can be entered.

The step count is one less than the number of images taken, e.g., to capture three images, only two steps are required.

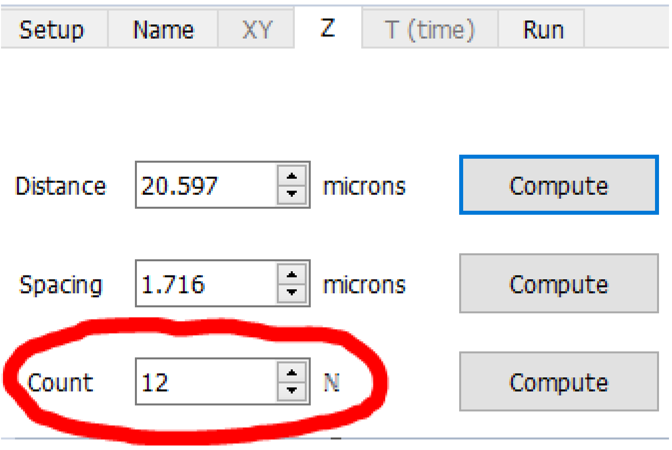
The Distance, Spacing and Count boxes are not independent. The distance covered will be the count times the spacing. **There are only two independent variables.**

The three spin boxes are provided as a matter of convenience for entering the data. Depending on the situation, the user may want to vary any of the three parameters. It is quite normal to adjust all three when setting up a focus stacking operation.

Previously, we have described how the scan distance and is defined by the sample, and the maximum spacing is determined by the lens.

This appears to give us the step count, by simple division, e.g. to scan 54 µm with a 12 µm spacing we need to move the Z stage 4.5 times, capturing 5.5 images. Clearly that's not possible as the step count and image counts are integers. **The number of images captured is one more than the step count.**

So in practice, we might choose to move 5 times instead of 4.5. We could then either increase the distance the Z stage moves or lower the vertical spacing between the image capture heights.



The Compute Buttons

Since there are just two independent variables, but three values which can be adjusted, it is possible to compute any value from the other two. For convenience, buttons are provided which perform the calculation and enter the result automatically.

For example with a count of 3 and a spacing of 2 µm, clicking on the **Distance** Compute button would enter a distance of 6 µm.

Similarly, with a distance of 6 µm and a count of 3, clicking on the **Spacing** Compute button would enter a spacing of 2 µm.

Clicking on the Count Compute button enters the result of the division (distance / spacing) rounded to the nearest whole number.

Note that after clicking on either the **Distance** Compute or **Spacing** Compute button, the three variables should be in synchronization `distance = spacing * count`.

Note that after clicking the Count Compute button, the three variables may be out of synchronization due to integer rounding. Recomputing the distance or spacing should remedy the situation.

The Run Tab

The Run tab will be disabled if the Z parameters do not make sense. Recomputing the distance or spacing should rectify synchronisation of the three parameters and reenable it.

Defaults and Limits

Count 1 - 99

The count can be adjusted in the range 0-99, allowing up to 100 images to be captured. This is a huge number for the focus stacking software, and it is recommended to use significantly smaller numbers. The default is simply 1 as this needs to be calculated from the actual scanning distance and spacing.

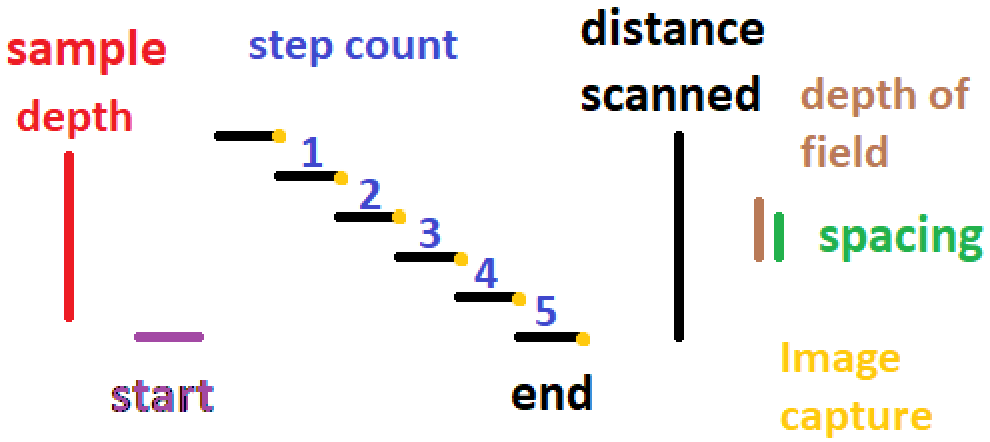
Spacing

The default value for the spacing is a calculated estimate of the depth of field for the chosen lens. As explained earlier, this value should only be decreased. It may however be possible to increase the value. The depth of field depends for example whether wet or dry lenses are used. The default value is the estimated depth of field for a dry lens and the maximum is that for a similar oil immersion lens. The minimum is 1/10 the estimated depth of field.

Distance

The default distance is the same as the default spacing. The maximum distance is the maximum count * maximum spacing, to accommodate those values.

Physical movement of the Z Stage



To prevent damage to the lens, focus stacking only takes place above the location the user has placed the lens.

Images are currently captured in a downward sequence.

Thus, if the step count were 5 and the spacing were 1 µm, the Z stage would move up 5 µm for the first image, then down 1 µm for the second, down a second 1 µm step for the third image, etc. until it had moved down 5 steps of 1 µm, making a total of 5 µm for the sixth and final image.

At this point, the lens would have returned to its original position.

The illustration above shows that

1. the **distance scanned** should be greater than the imaged **sample depth**
2. the **initial point** of focus should be slightly below the sample
3. **number of images** is one more than the **step count**
4. the distance scanned is the count times the spacing
5. the **image spacing** should be no larger than the **depth of field**
6. the capture sequence is from top to bottom (high to low).
7. The lens stays above its initial position during the whole scan process.

