**Confocal Analysis of Materials using Nikon C2 confocal and NIS Software**

For 488nm Reflection imaging, insert the A1-C2 488/10 Reflection cube into Position A within the C2 Detector Box. **Please remember to reinsert the “Pos 1” cube at the end of your session!!**

1. **Open the NIS-Software and select the Reflection-Only** Optical Configuration.This will permit 488nm Reflection imaging. Reflection/fluorescence imaging is also possible. See Melinda for the configuration.
2. **Optimize and acquire an XYZ image series** through the thickness of your sample.
3. Be sure that the Prior Z-drive controller is set to “**Engaged**”.
4. Select the **ND Acquisition** window.
5. Select **Z**.
6. Select the first icon on the left to **Define By Top Bottom.**
7. Begin scanning and set **Top** and **Bottom** boundaries of the Z-depth range.

Set **Bottom** boundary by turning the focus knob towards you and locating the lower boundary. Click **Bottom**.

Set **Top** boundary by turning the focus knob away from you and locating the upper boundary. Click **Top**.

Note: Top and Bottom boundaries may also be located by using the up/down arrow buttons in the **XYZ Navigation** window.

1. Enter **Step** interval or number of **Steps**.

To set the Z-step interval to Nyquist, click on the number (**Suggested Step Size**) that is displayed between the **Step** and # of **Steps** options.

1. Select **Run Now**.
2. To stop the scan early and **SAVE DATA**, select **Finish**.

To stop the scan early and discard the data, select **Abort**.

**Note: Top = highest stage focus position = deeper into the sample**

**Bottom = lowest stage focus position = surface of the sample**

 **XYZ series may be acquired:**

**Top to Bottom: first image (0 depth) would be located deep within the sample.**

**Bottom to Top: first image (0 depth) would be located at the surface of the sample.**

**XYZ orientation may be important if a Z-Profile is generated for depth analysis.**

Z Intensity Correction – Continuously adjusts HV detector setting during XYZ acquisition. Helps to maintain an even intensity distribution throughout the thickness of the sample.

1. Open the **Z Intensity Correction** menu - Right click on the desktop and select **Acquisition Controls/Z Intensity Correction**.
2. While continuously imaging, focus to the bottom (focus down) of the specimen and optimize the REFL HV setting. Within the Z Intensity Correction panel, click the blue **Add New** (+) button to mark the intensity for this Z plane.
3. Focus a little deeper into the specimen (focus up), optimize the REFL HV setting, and mark the intensity by clicking the Add New button.
4. Repeat step c) until the top of the specimen is reached. It is not necessary to save these settings, but do not close the Z Intensity Correction window.
5. From the **ND Acquisition/Z Series** window, begin the XYZ image series by selecting **Run Z Correct** (instead of “Run Now”). The REFL HV setting will gradually change as the specimen is imaged through the Z thickness.
6. For any single XY image plane, a **3D plot of the single XY image plane** can be shown with the intensity of each XY pixel represented in height (Z axis). Select **Measure/Intensity Surface Plot** for the 3D display.
7. From the XYZ image series, several 3D viewing options are available including:

**\*Maximum Intensity Projection**

**\*Volume View (Max Intensity and Depth Coded)**

**\*Orthogonal View (Slices View)**

For each of these three viewing options, 2D (XY) measurements are possible. For height/depth (Z) analysis, the EDF option (see below) may be more useful.

1. **Maximum Intensity Projection** measurement options:

**Show Profile** will permit XY length/distance measurements.

**Measure/Manual Measurements** will permit XY length/distance, area, radius, angle measurements.

1. **Orthogonal View** (2D XY, XZ and YZ views) measurement options:

Using the **Show Profile or Measure/Manual Measurement**, intensity versus depth

information can be collected from the XZ or YZ image, but first a new document must be generated from the XZ or YZ orthogonal view.

**Right Click on XZ or YZ image**, and then select either:

**Create New Document** = single 2D XZ or YZ image

 **Switch Axes and Create New Document** = 3D XZ-Y or YZ-X image series

Both the XY and Z measurements are correct (calibrated) within the XZ and YZ images. However, if the Z-step is greater than the XY pixel spacing, then the Z-step will be interpolated so that the X:Y:Z is 1:1:1. In this case, the data will reflect more Z data points than were actually collected.

The **Show Profile** option can be used to measure the intensity versus Z-depth. The **Show Graticule** option can provide reference marks for measurements within the image. The **Horizontal** or **Vertical** line measurement options within the Measure/Manual Measurement option can aid in measuring the **depth of a structure from the surface of the specimen.**

1. **Volume View** display options: Maximum Intensity and Depth Coded

**Crop** the image in Z to limit the depth. For a Depth Coded image, cropping in Z will have

the effect of creating more colors through a smaller Z range.

Under Volume Options, use the **Z-Zoom** to magnify the image in depth.

Under Volume Options, use the **Z LUT** to change the color scale (LUT) at different Z

depths. For example, if one optical Z section is very bright but image planes above and below are much darker, then the Z LUT could be used to brighten the regions above and below the brighter image plane without affecting the bright image plane itself. Alternatively, the Z LUT could be used to remove background from particular depths without affecting the entire Z-depth of the sample.

**To crop in XY:**

Select **Image / Crop**.

Optimize the size and location of the red box on the image.

**To crop in Z:**

At the bottom of the XYZ series, press the **Shift** key, **left click** the mouse and **drag** to the right in order to select the desired range of images.

The selected buttons will turn from a blue color to a green color.

Right click on the selected buttons to “Keep Selected Frames” or “Delete Selected Frames”.

**EDF (Extended Depth of Focus)**

This option creates a single 2D focused image by selecting the in-focus regions from each optical Z-plane. For more information on the EDF options, go to the **Help** menu and search for **EDF Step by Step.**

1. **Optimize and acquire an XYZ image series** through the thickness of your sample.

The Z Intensity Correction option, which continuously adjusts HV detector setting during XYZ acquisition, may be necessary to maintain an even intensity distribution throughout the thickness of the sample. For more information on the Z Intensity Correction option, see above.

1. From the EDF menu, select **Align Sequence.** This option aligns each image in the Z series so that the EDF option performs properly. Small shifts within the image series that may have occurred during the series acquisition are corrected. The resulting EDF image may appear sharper.
2. From the EDF menu, select **Create Focused Image**. This option will create and display the EDF 2D image. If the raw XYZ data file is saved at this point, the software will provide the option to save (associate) the EDF image with the raw XYZ ND2 data set. This will provide a quick toggle between the XYZ image series and the EDF image from a single file. **This will not alter your XYZ raw data series.**
3. Alternatively, the **Create EDF Focused Document** option can be selected to create a separate window for the EDF image, disassociating the EDF image from the raw XYZ ND2 data set. The single 2D EDF image may then be saved as its own ND2 file.
4. The EDF image may be displayed in three different views:

**Gray scale view**

**3D Surface view**

**3D Anaglyph view** (stereo view, requiring red/blue stereo glasses)

1. **3D Surface View option** - creates a 3D view of the 2D EDF image, with the 3rd dimension (height) being the associated Z-depth.

Use the **LUT** window within the Acquisition Parameters to rescale the image. The

Gamma option can be used to increase the background within the image.

Select **Z-Zoom** to increase the height (thickness) of the 3D display.

Select **Z First Frame Lowest/Highest** option to flip the image.

Select **Surface Grid** to add a 3D topographic grid to the surface of specimen.

**VRML** – exports the data to Virtual Reality Modeling Language. Two files are saved – WRL file with the 3D information and JPG with the 2D texture. A VRML viewer will be needed to view the images. Both the WRL and the JPG files are required, so these two files should be grouped together.

Use Movie Maker to create an AVI movie of the image rotation.

1. Measurement options using the **Gray Scale** view.

The **Show Z-Profile** option will measure Height versus Distance for the designated line. In addition, while using the Show Z-Profile option on the Gray Scale view, the line will also appear simultaneously in the 3D Surface View, showing the position of the line through the Z height (depth).

1. **Orthogonal View** – Show Z Profile

Once the EDF image has been created, display of the Orthogonal View will now provide the option of showing the EDF Z-Profile on the XZ and YZ cross-sectional image displays. From the Orthogonal View, right click on the image and select **Show EDF Z Profile**.

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