

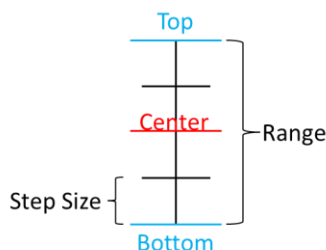
GUIDE TO ACQUIRING Z-STACKS USING METAPRESS® 5.x

The screenshots in this guide are from the ImageXpress Micro, but the same process can be used for the ImageXpress Ultra.

Process for acquiring z-stacks

For some imaging assays, the user may want to image samples more than one focal plane, such as tissue sections, spheroids, or cells grown in a matrix (agar, collagen, etc.). For these assays, MetaXpress can acquire images in a z-series through a sample and either “collapse” the z-series into a single composite image or save all the images as a Z-stack, a single file containing multiple tiff images of each Z-step, for 3D reconstruction. The general process for acquiring Z-stacks requires the user to determine several components. **Note:** Z-plane is defined as a specific Z-position or Z-height.

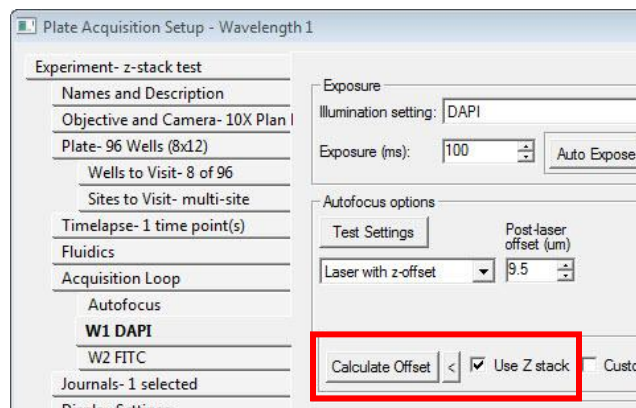
- Top Z-plane (z-position)
- Bottom Z-plane
- Z-range
- Center of Z-range
- Step size

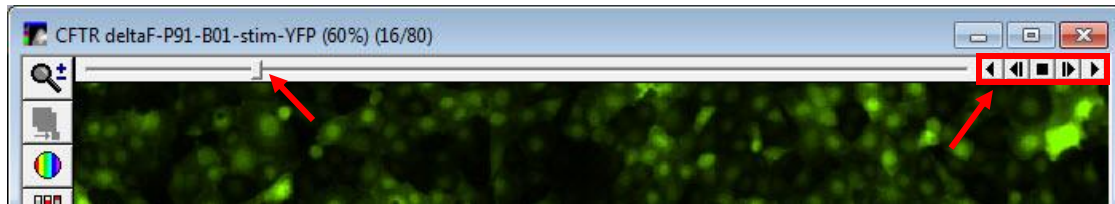


Determine top and bottom z-planes

To find the top and bottom of the z-range, we will use the Calculate Offset function on the W1 tab. This function will use a default range and step size appropriate for the objective being used. These default settings can be changed, if necessary. You want to find the z-positions at which the sample is slightly out of focus above and below the sample.

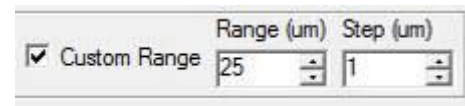
1. Place your sample in the instrument and select an appropriate location to image.
2. In MetaXpress, open the Plate Acquisition Setup dialog and select the first wavelength (W1) tab.
3. Check the box for “Use Z stack.”
4. Click on the Calculate Offset button. The system will collect a z-series of images using the default range and step size values.



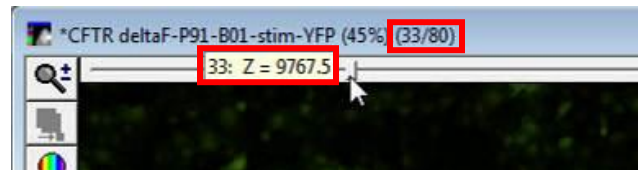


5. In the resulting image window, play through the images to see if the system focused all the way through the sample. If not, then you will need to use a custom range and/or step size. To do this:

- Take note of the default Range and Step values
- Check the box for Custom Range
- Enter a range larger than the default value.
- Set the step size. Use the default value if you want to “collapse” the Z-stack into a composite image. If you want to perform 3D reconstruction, then you can use a step size larger than the default.
- Click on the Calculate Offset button again
- Repeat 5a-e until you have a z-stack that focuses all the way through the sample.



6. Move toward the left through the Z-stack images until the sample is slightly out of focus. Make note of the step number and/or z-height value. This is the bottom z-plane.



7. Move toward the right through the Z-stack images until the sample is slightly out of focus. Make note of the step number and/or z-height value. This is the top z-plane.

Note: You may need to perform these steps in several wells to make sure you are covering an appropriate range for all samples in the plate.

Determine center of Z-range

When acquiring Z-stacks during plate acquisition, MetaXpress will first move to the center of the Z-range, then drop down half of the range to the Bottom Z-plane (diagram on page 1). The software will then acquire images through the entire range, ending at the Top Z-plane.

8. Use the following equation to determine the center of the Z-range (using either step numbers or Z-height values):

$$\text{Center} = \text{Bottom} + [(\text{Top} - \text{Bottom})/2]$$

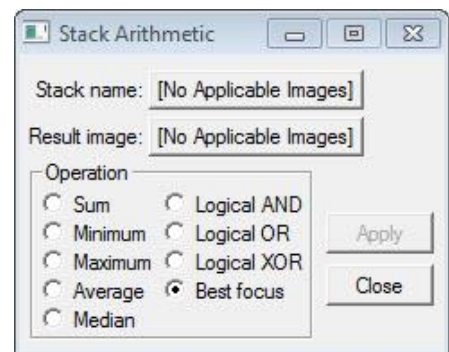
9. In the Z-Stack image window, move to the Center plane.
10. Select OK in the instruction window. This will set the Center Z-plane as the post-laser offset.



Determine the composite algorithm

Using the process outlined here, MetaXpress will always collapse the resulting Z-stack into a composite image. If you do not need the composite images, then you can simply ignore them. However, if you do want to create composite images, then there are several processing algorithms to choose from. For transmitted light (brightfield) images, the Minimum (minimum projection) algorithm is recommended. For fluorescence images, the two main algorithms are Best Focus and Maximum (maximum projection). The user must determine which of these two algorithms works best for their samples.

11. Press the Calculate Offset button again to acquire a Z-stack all the way through the sample.
12. Under the Process Menu, select Stack Arithmetic....
13. For Stack name, select the Z-stack window.
14. Select the radio button to the left of Maximum and press the Apply button. A result image named Maximum will appear.



15. Select the radio button to the left of Best Focus and press the Apply button. A result image named Best Focus will appear.
16. Compare the two images and determine which works best for your samples.

Note: If you want to acquire both fluorescence and transmitted light channels in the same experiment, you will need a custom journal. Contact Technical Support to discuss this.

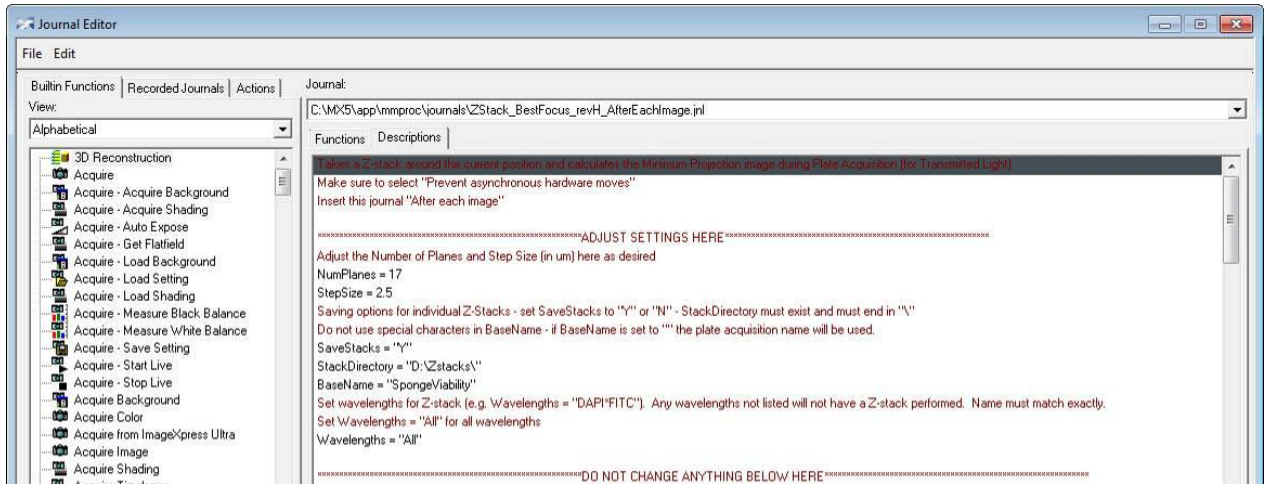
Configure journals for Z-series acquisition

During plate acquisition, MetaXpress uses journals to acquire the Z-Stacks, create the composite images, and save the Z-stacks to a directory (optional).

17. Under the Journal menu, select Edit Journal....

18. Navigate to the C:\MX5\app\mmproc\journals directory.

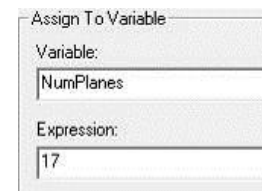
19. Select and open the journal that uses the appropriate composite algorithm for your samples. For example, to use the best focus algorithm, select the journal named “ZStack_BestFocus_revH_AfterEachImage.JNL.” **Note:** Revision H is the newest version as of 9/15/2014. If you want to save Z-stacks to a directory, then do not use revision B.



20. Under the journal section labeled “ADJUST SETTINGS HERE,” enter the determined values for NumPlanes (number of steps), StepSize, SaveStacks, StackDirectory, BaseName, and Wavelengths. Be sure to note the directions in red font for these settings.

21. To enter number of steps, do the following.

- Highlight the NumPlanes line in the journal.
- At the bottom of the Journal Editor, enter your step size into the box below “Expression.”

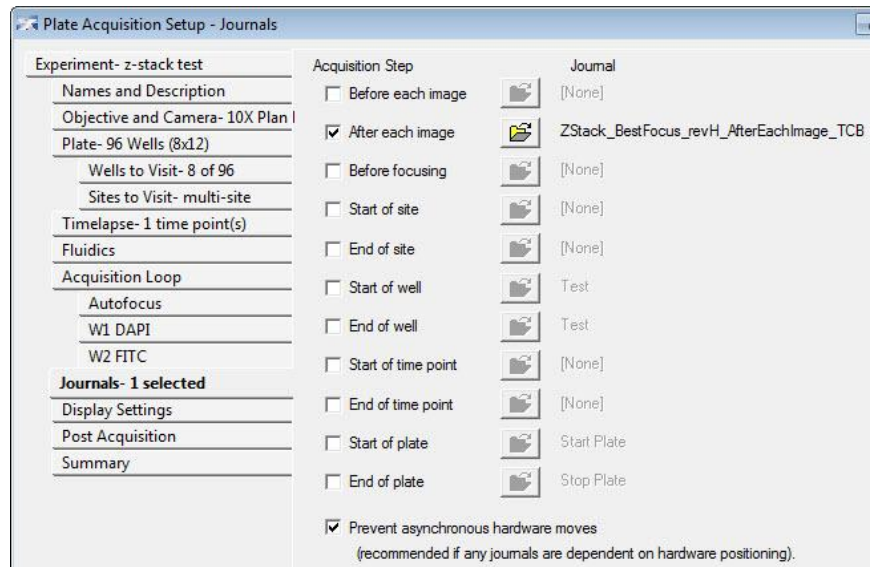


22. Once all of the values have been entered, click on the Save button in the bottom left of the Journal Editor. **Note:** If several users are going to be doing Z-series acquisition, you can use Save As... to save your own copy of the journal with a different name.

23. In the Plate Acquisition Setup dialog, go to the Journals tab. Check the box to the left of “After each image.”

24. Click on the folder icon to the right “After each image” and navigate to the directory containing the Z-stack journals. Select the journal you just saved.

25. Be sure to check the box at the bottom of the tab next to “Prevent asynchronous hardware moves.”



Running Z-stack acquisition

To run the entire plate, select the Summary tab of the Plate Acquisition Setup dialog and press the Acquire Plate button.

To test the setting prior to running the whole plate, go to the Wells to Visit tab and select only a couple of wells, then on the Summary tab, press Acquire Plate.

Recommendations for acquiring Z-series:

- For wavelengths 2-7, set Z-offset from W1 = 0. This will ensure that each wavelength acquires the same z-range.
- Current Z-stack journals can be found on the MetaXpress user forum or on the Molecular Devices Knowledge Base.

Alternate process to configure journals for z-series acquisition

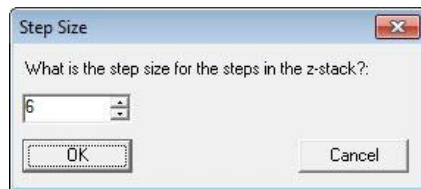
Once you have determined the number of steps, step size, and center of range as described above, you can use these values without directly editing the Z-stack journals.

1. Under the Journal Menu, select Run Journal....
2. Select the journal titled "Z-Stack Variables_revB.JNL." You can find this journal on the MetaXpress user forum (<http://metamorph.moleculardevices.com/forum/showthread.php?tid=317&highlight=z-stack>). This journal will step you through the process of entering all variables.

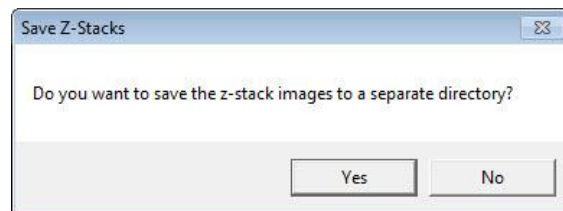
3. Enter the number of steps in the Z-range.



4. Enter the step size.



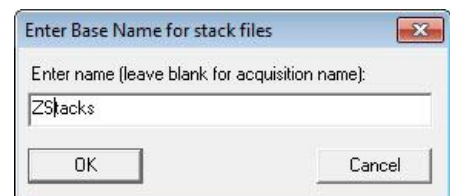
5. Do you want to save the Z-stacks to a directory?



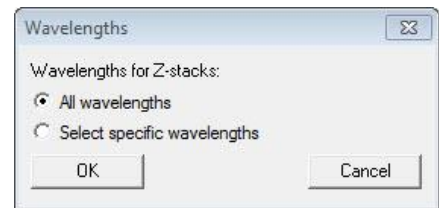
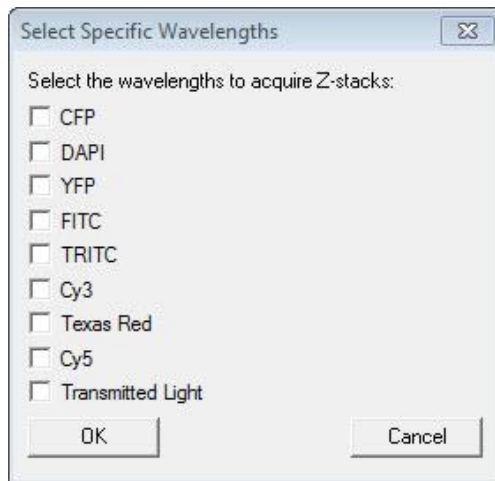
- a. If yes, then navigate to the directory you want to use.



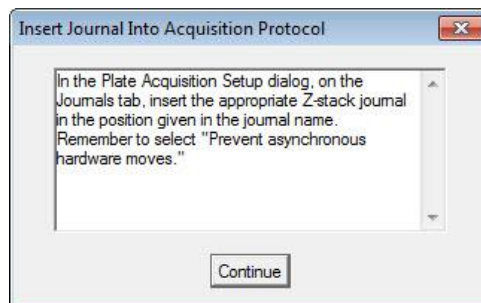
- b. Type in the base name for the Z-stacks. This can be the same as the Experiment Base Name used on the Names and Description tab of Plate Acquisition Setup. The software will automatically append well name, site number, and wavelength number.



6. Select which wavelengths to acquire Z-stacks. If all channels, then select “All wavelengths.” Otherwise choose “Select specific wavelengths,” then place a check mark next to each wavelength you want to acquire Z-stacks in.



7. The final screen gives instructions for placing the appropriate journal on the Journals tab. **Note:** *If you are using this method, you **MUST** use the Z-stack journals with names ending in “DoNotEdit.” These journals can be downloaded from the forum link above.*



There are a few downsides to using this approach:

- These exact values will be used for acquiring all Z-stacks until this process is run again to change the values.
- These values are stored in memory while MetaXpress is open. Once you close MetaXpress, all values are erased. In order to use them again, you must re-run this process before starting your image acquisition, otherwise you will get an error.
- As currently configured, this process does not allow multiple users to save their own values to be loaded with the rest of their acquisition settings.