MetaXpress® 6 Software Guide

Setting up a Z Series Acquisition
(Option to Save All Z Series Images with Projection)

MOLECULAR DEVICES
UNLEASH YOUR BRILLIANCE™

Date Revised 06/24/15 Version A

Chapter Purpose

The purpose of this chapter is to guide the user through setting up an acquisition with Z Series. This includes selecting objectives, plates, wavelengths, focal position, and optimizing Z steps.

Acquiring timelapse images with Z Series will NOT be covered in this chapter. Refer to corresponding chapter for this process.

Setting Up a Z Series Acquisition

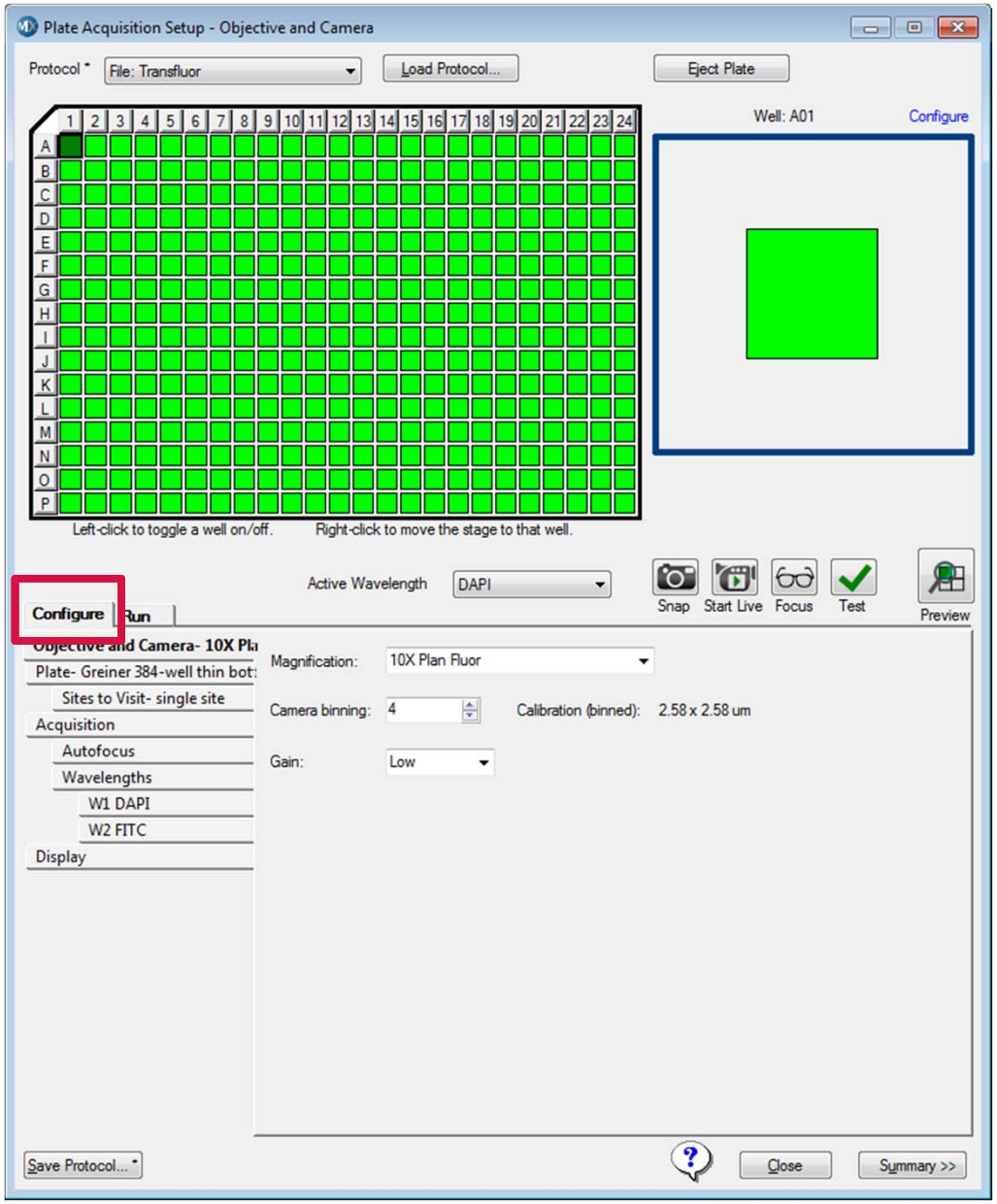
1. Open **Plate Acquisition Setup**

   - In the main toolbar click on

   OR

   - Under the **Screening** menu, select **Plate Acquisition Setup**

2. Select the **Configure** tab

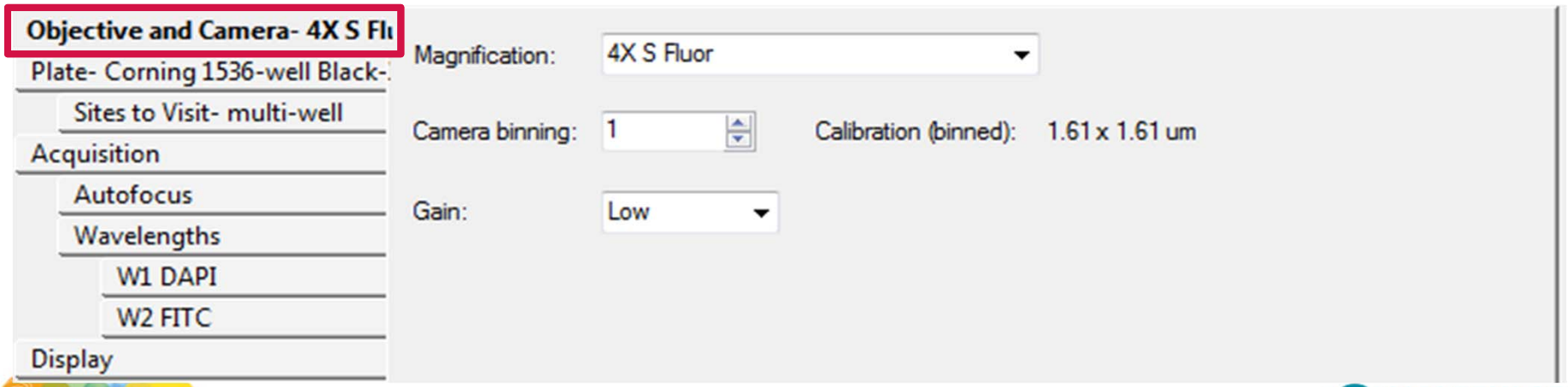


Setting Up a Z Series Acquisition

3. Select the **Objective and Camera** tab

4. Select the appropriate **Magnification** from the drop-down menu
   - You may need to adjust the correction collar of the objective; refer to the Main Taskbar to do this.
   - Select **Camera Binning** (Refer to section on binning for guidance)
   - Pixel size is automatically calculated based on magnification and binning
   - Set Camera Binning to **1** to acquire unbinned images – maximum resolution

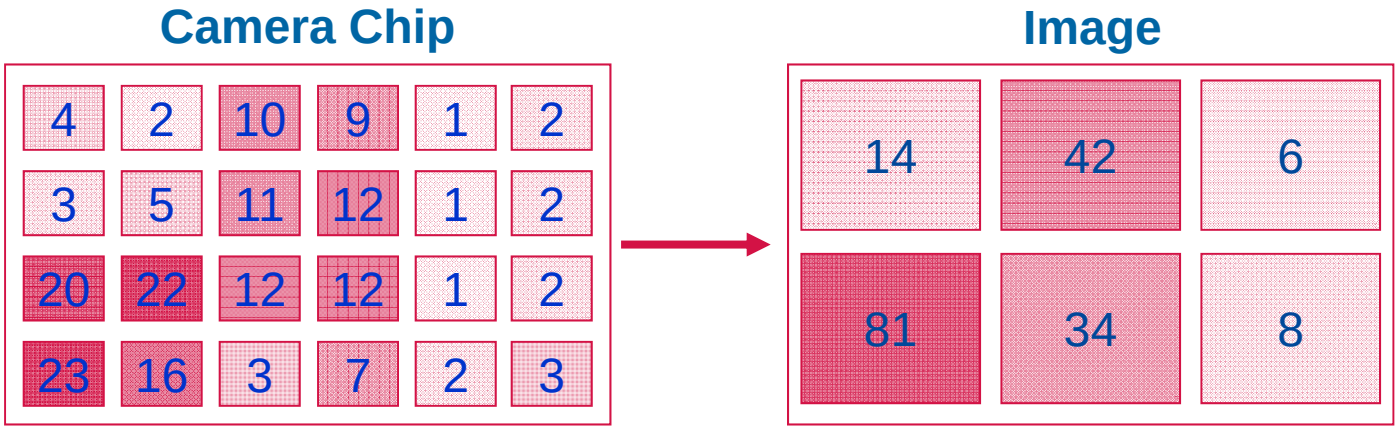
5. If the **Gain** option appears, it is suggested to start with gain set to **Low**



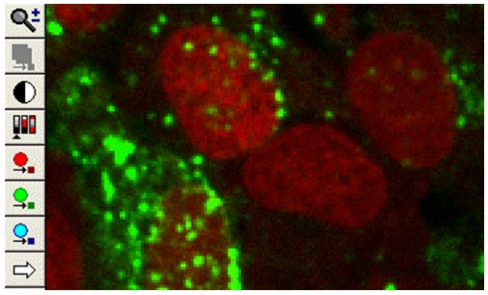
What is Binning?

Combining groups of pixels into a single pixel during image acquisition

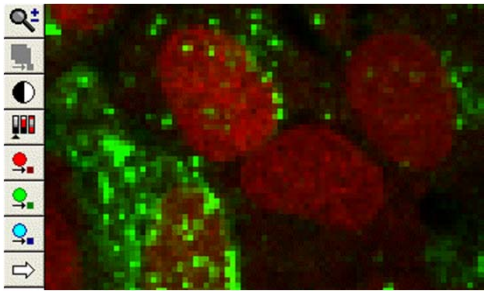
Example of 2x2 Binning



Each pixel records an intensity



4 Pixels are summed to make one larger pixel



Why Bin?

Brighter pixels

- The resultant pixel is brighter than any of the 4 component pixels

Save Space

- 2x2 binning reduces file size 4-fold

Increase Speed

- Faster image saving
- Faster image analysis

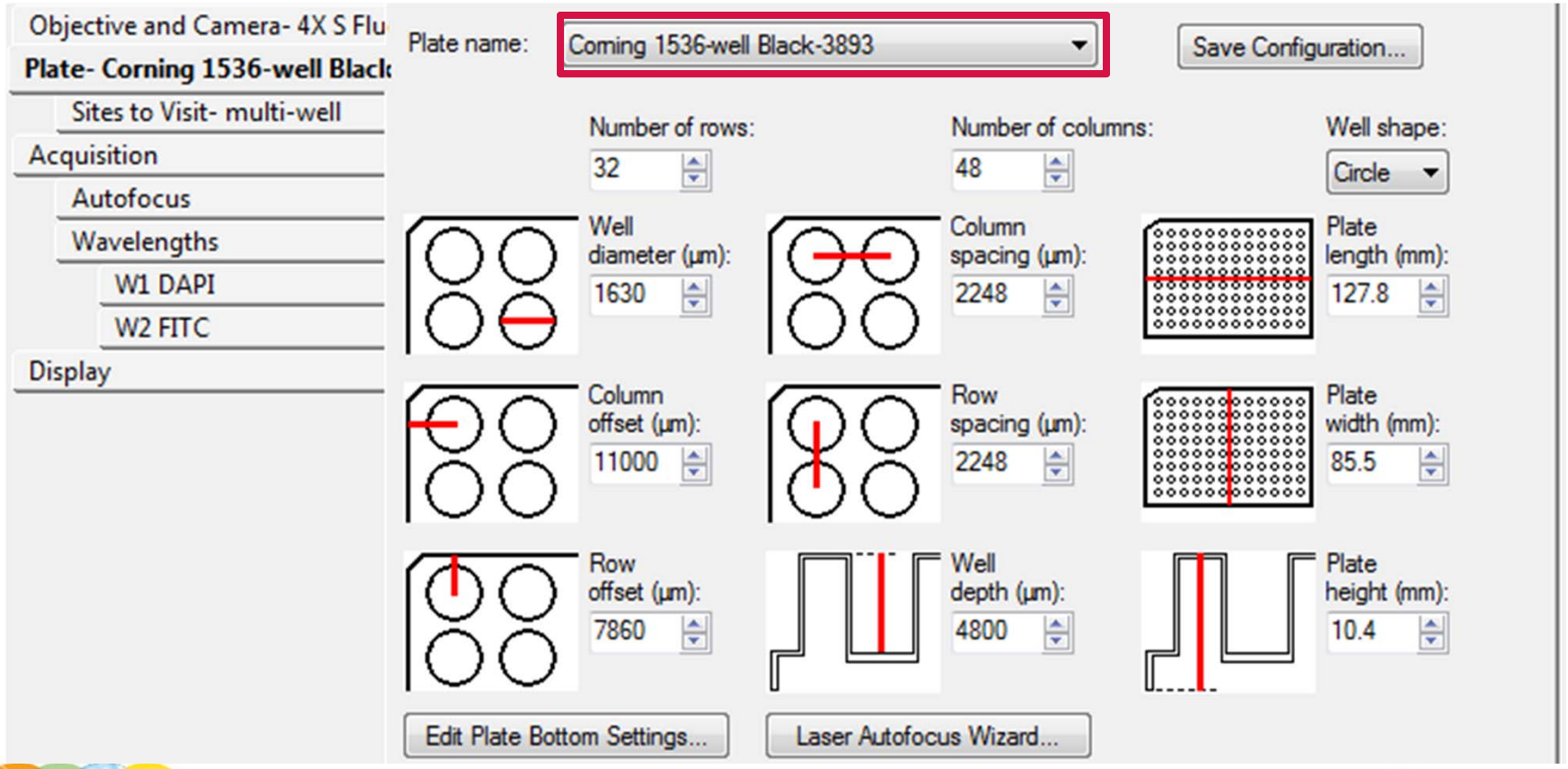
When to Bin

- You do not need to see intricate sub-cellular detail
- Cell counting
- Scoring cells positive or negative for fluorescent markers
- Measuring whole cell intensity

Setting Up a Z Series Acquisition

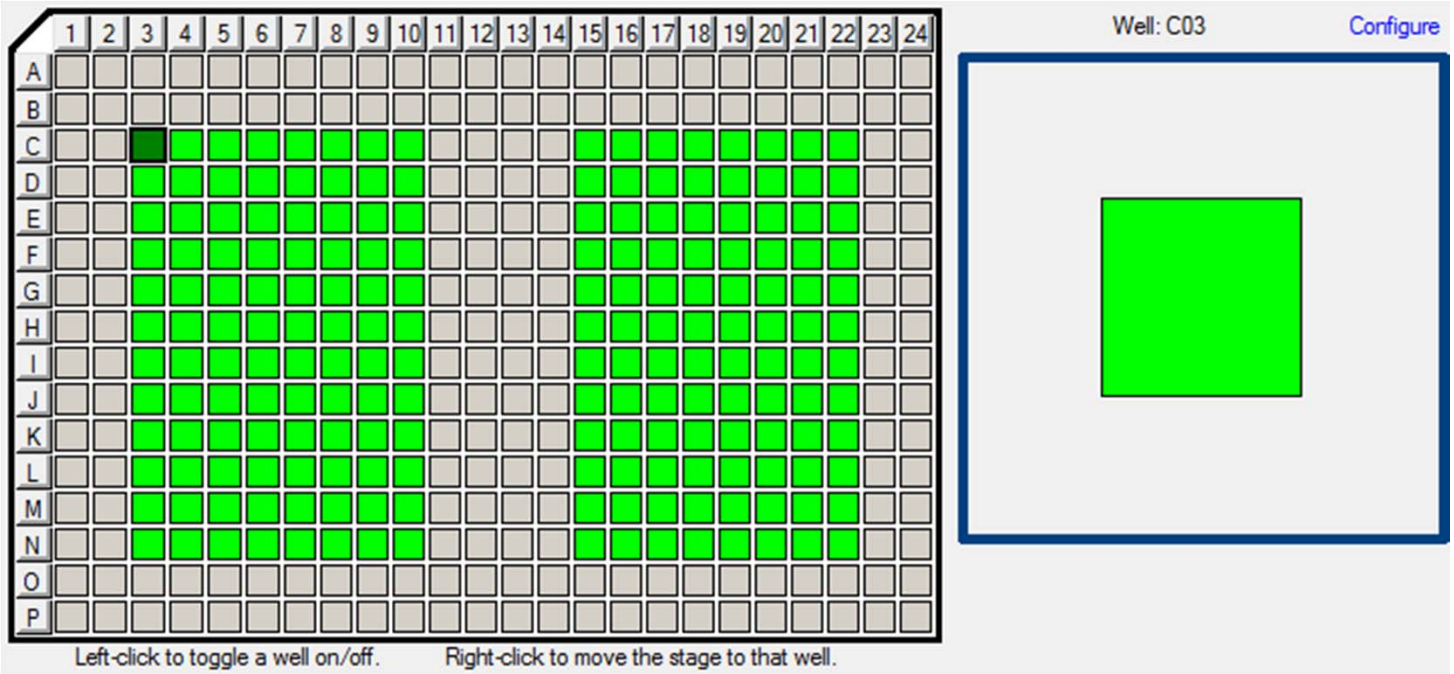
6. Select the **Plate** tab

7. Select the appropriate **Plate Type** from the drop-down menu



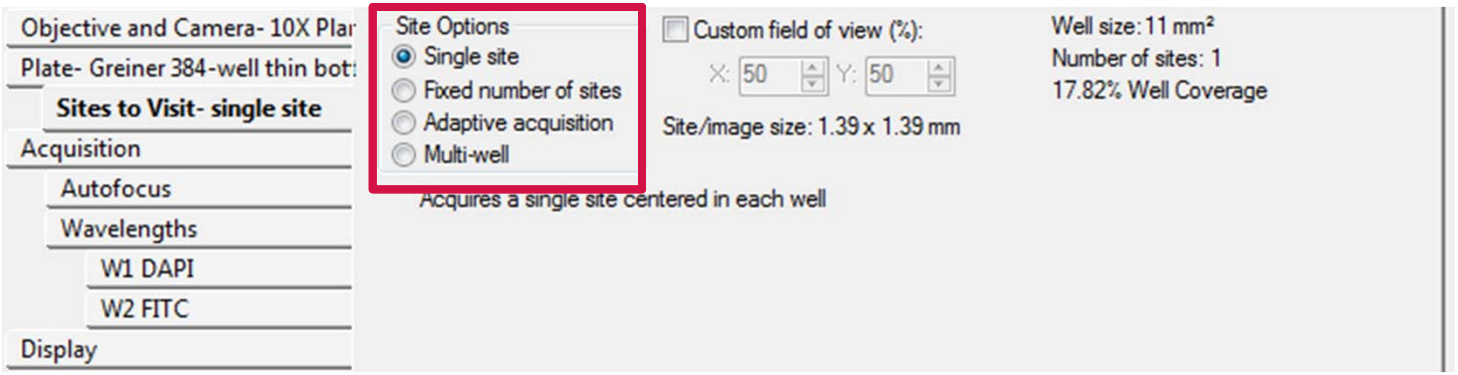
Setting Up a Z Series Acquisition

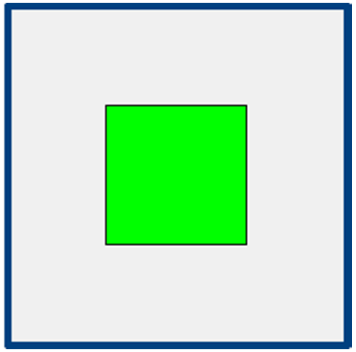
8. In the **Plate Section**, select the wells you would like to acquire
   - Left click and drag mouse to select wells (selections do not need to be contiguous)
   - Click on “All” (top left corner), row letters, column numbers, or individual wells
   - Gray wells are deactivated, green wells are activated and will be imaged
   - Right click on a well to move the stage to that position (well turns dark green)



Setting Up a Z Series Acquisition

9. Select the **Sites to Visit** tab
   - Select **Single Site** to acquire one site in the middle of the well
   - To acquire a single site elsewhere in the well, refer to the section on setting up multiple sites

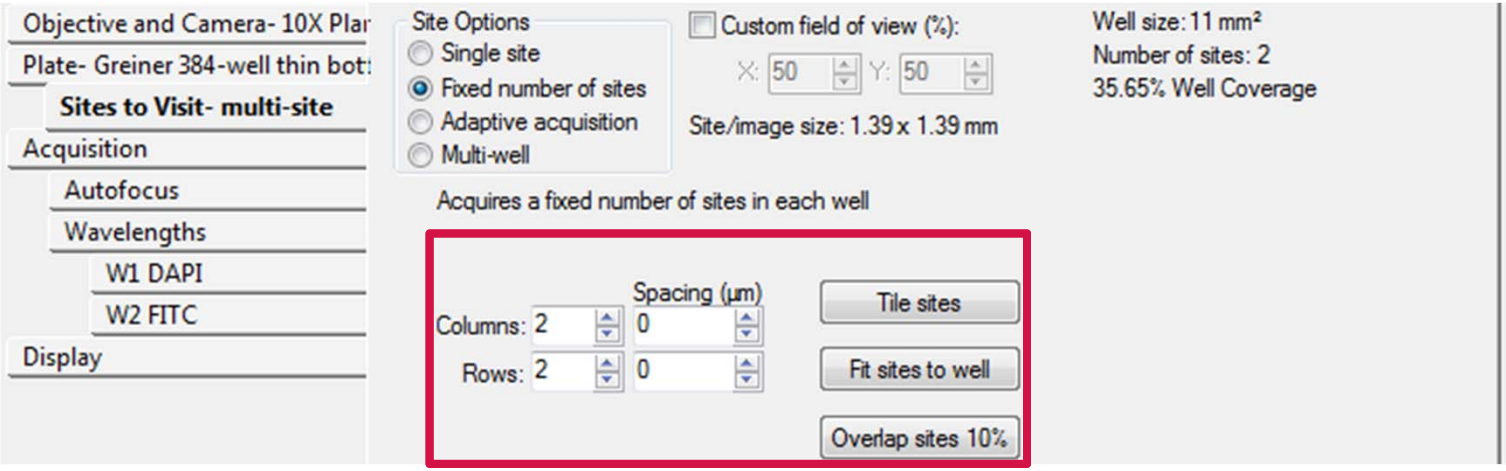




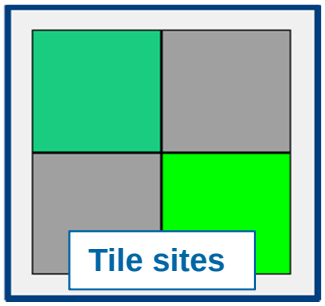
Setting Up a Z Series Acquisition

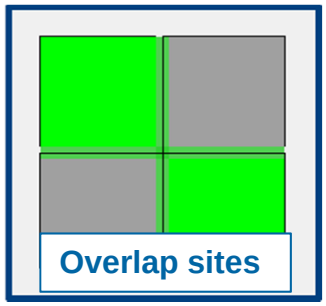
9. On the **Sites to Visit** tab
   - Select **Fixed number of sites** to acquire multiple sites
   - Build site grid by specifying number of Columns and Rows
   - Spacing defines the x-y spacing between sites

NOTE Left clicking on site selects (green) or deselects (gray) for imaging. Right click moves stage to that position (dark green)



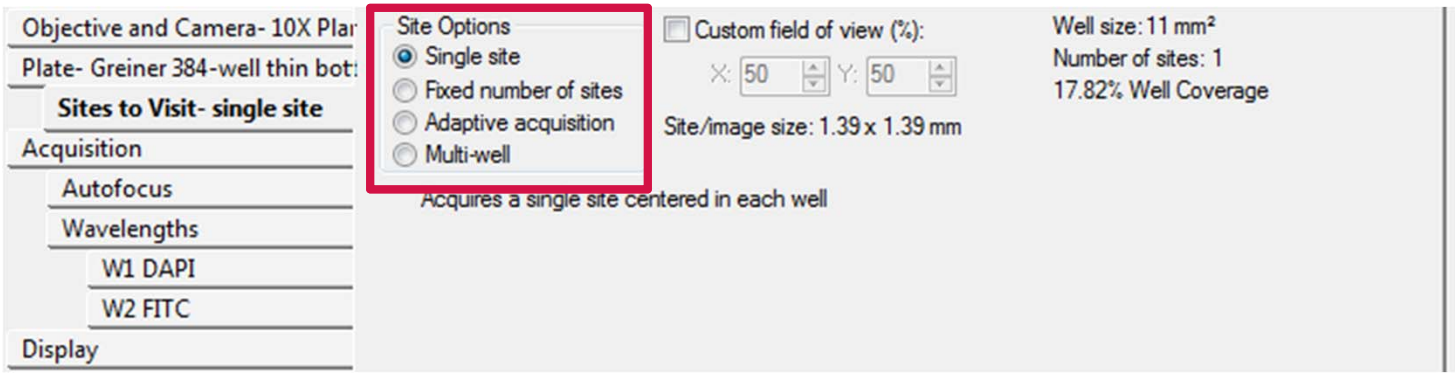
- **Tile sites** places sites edge to edge
- **Fit sites to well** spreads sites to well edge
- **Overlap sites 10%** overlaps edges of sites for stitching





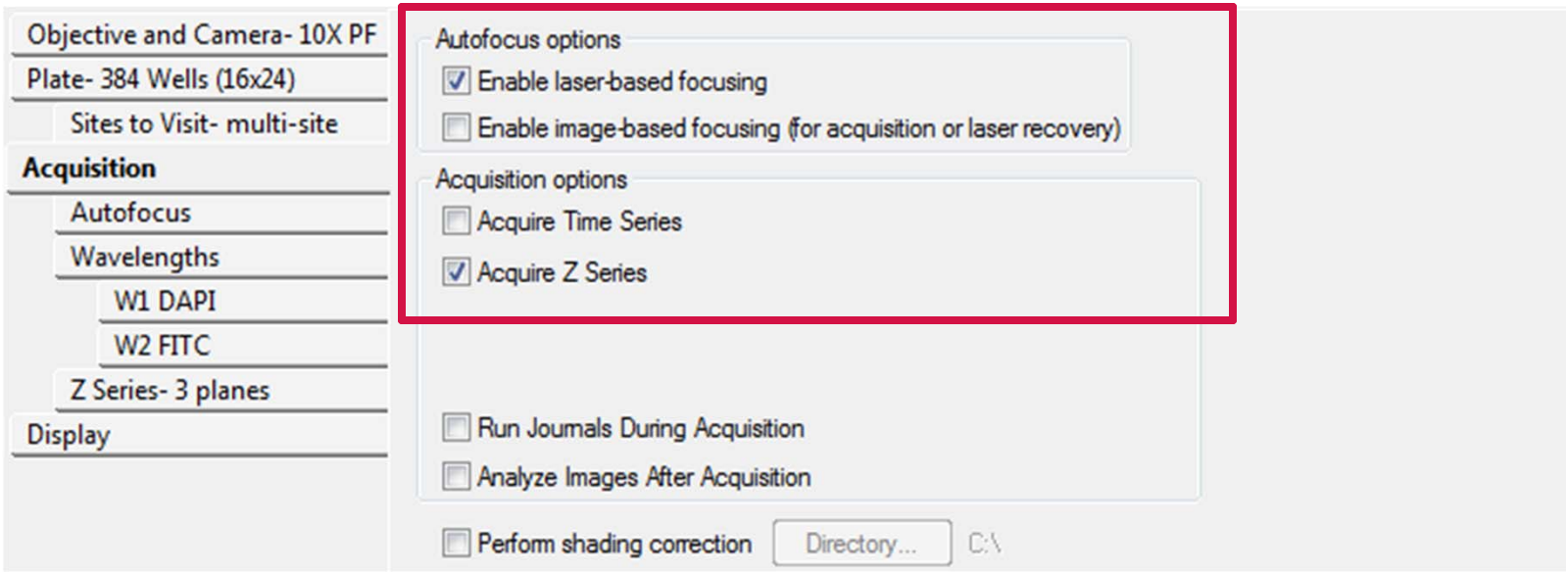
Setting Up a Z Series Acquisition

9. On the **Sites to Visit** tab
   - Refer to corresponding chapters for details on **Adaptive acquisition** and **Multi-well** options



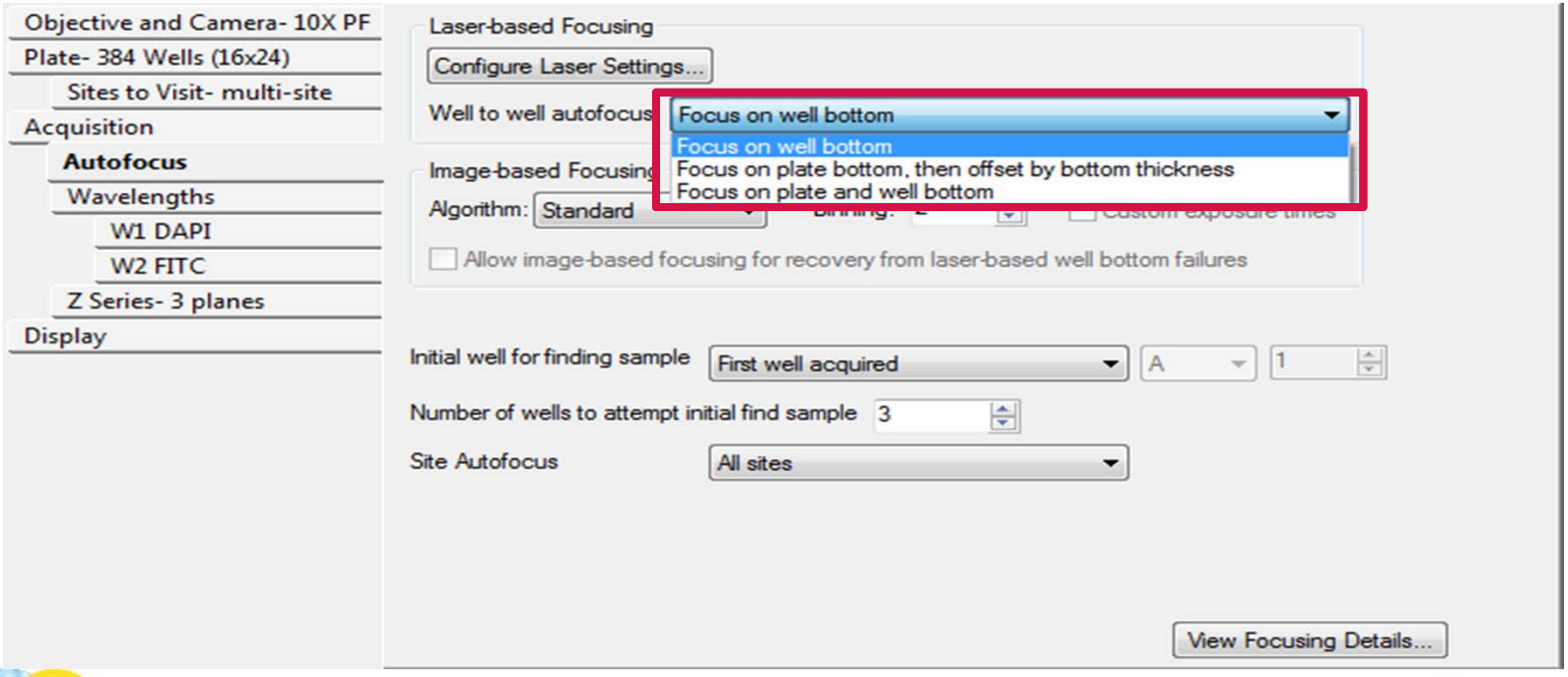
Setting Up a Z Series Acquisition

10. Select the **Acquisition** tab
    - Always **Enable laser-based focusing**
    - For certain samples it may be necessary to **Enable image based focusing** (recommended if samples are in different focal planes or using u-bottom plates)
    - Disable **Acquire Time Series**
    - Enable **Acquire Z Series**
    - Optionally, enable **Perform shading correction**



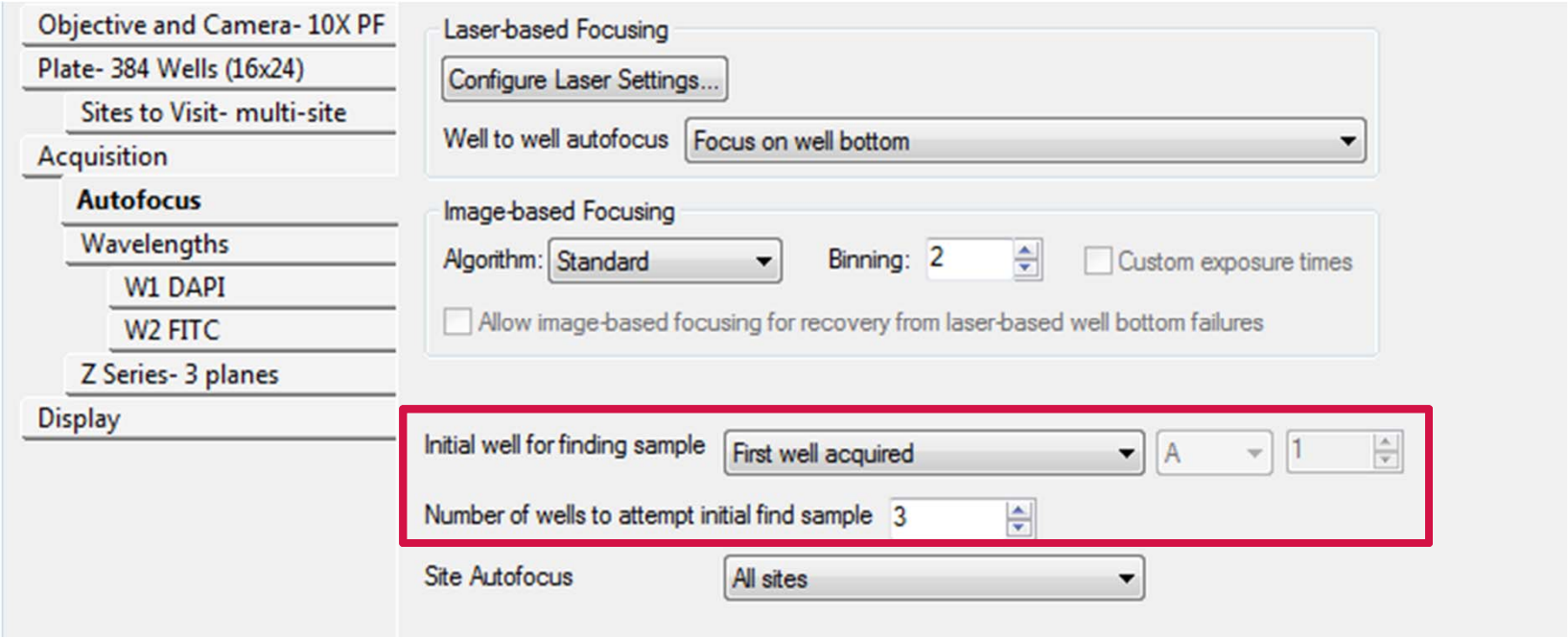
Setting Up a Z Series Acquisition

11. Select the **Autofocus** tab
    - Select the appropriate option for **Well to well autofocus** from the drop-down menu:
      - **Focus on well bottom:** most scenarios using 10X and higher objective
      - **Focus on plate bottom then offset by bottom thickness:** for low magnification objectives (2X, 4X), thin plates, or microscope slide/coverslip.
      - **Focus on plate and well bottom:** for warped plates (plate bottom variation is more than half the optical thickness)



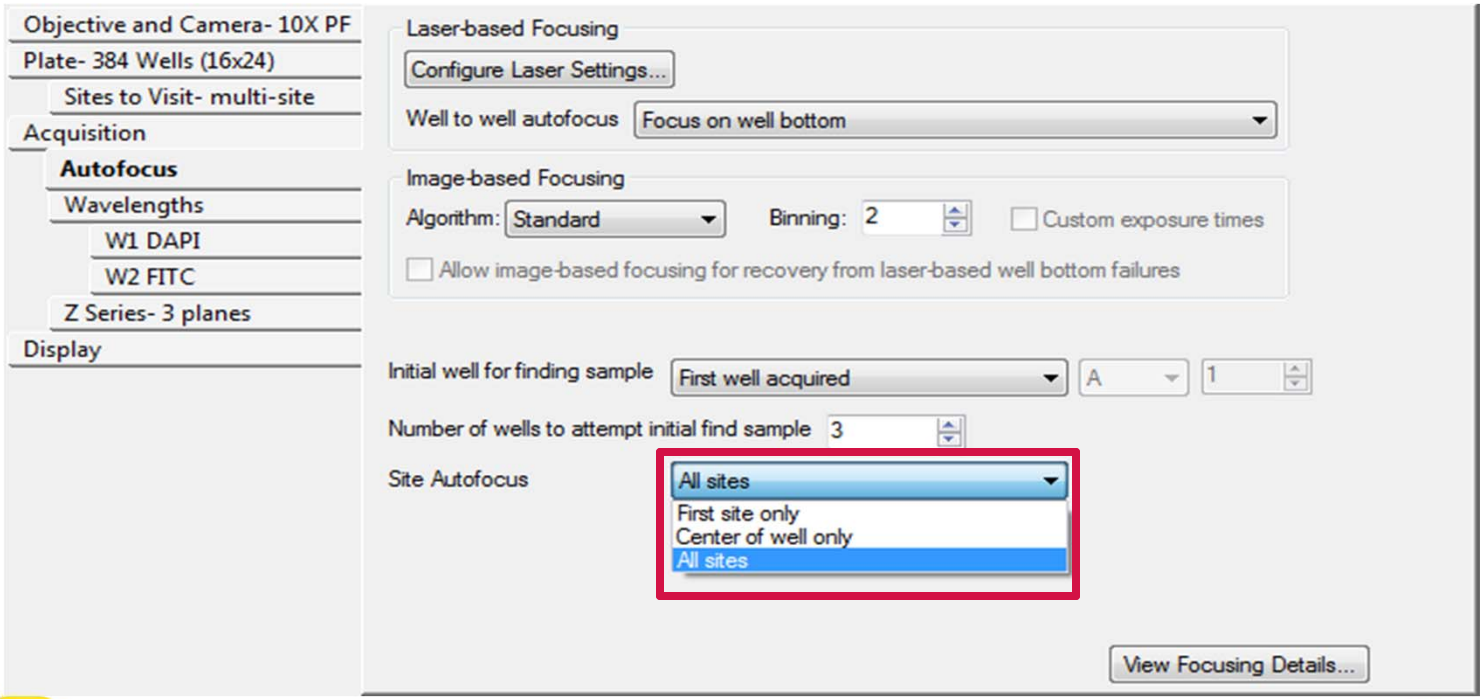
Setting Up a Z Series Acquisition

11. On the **Autofocus** tab
    - Set **Initial well for finding sample** to **First well acquired**
      - This serves as a check to verify a plate is loaded
      - Only disable for very specific applications (i.e., oil immersion objectives)
    - Set **Number of wells to attempt initial find sample** to **3**



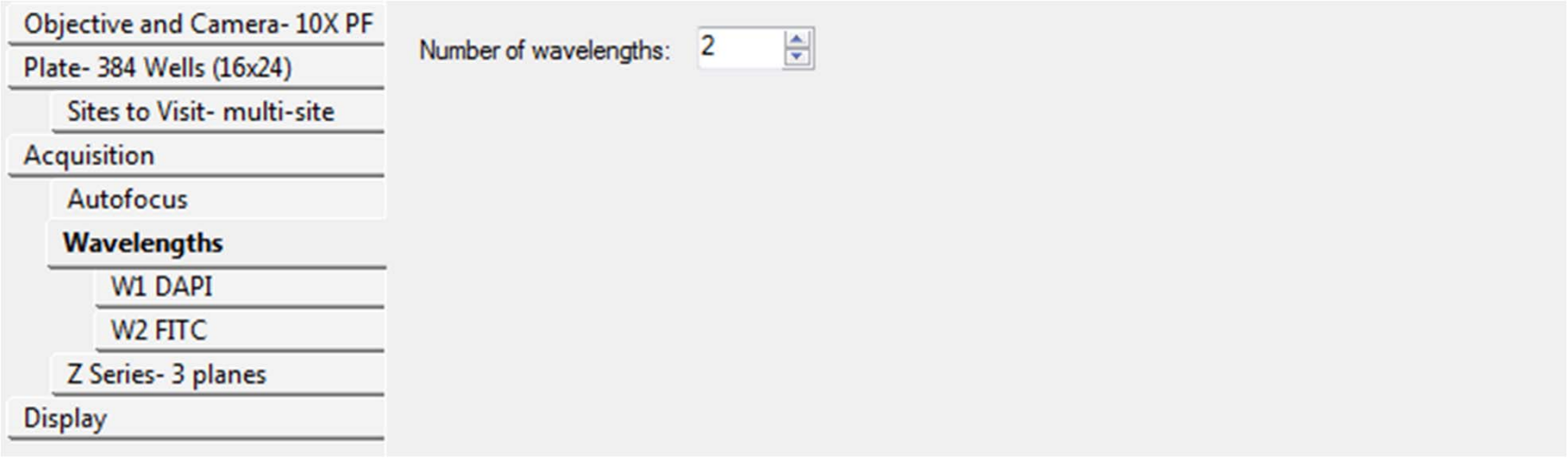
Setting Up a Z Series Acquisition

11. On the **Autofocus** tab
    - Select the appropriate option for **Site Autofocus** from the drop down menu
      - Select **First site only** or **Center of well** only for faster acquisition at lower magnification or with high quality, flat plates
      - Select **All sites** for greater focusing accuracy (recommended)



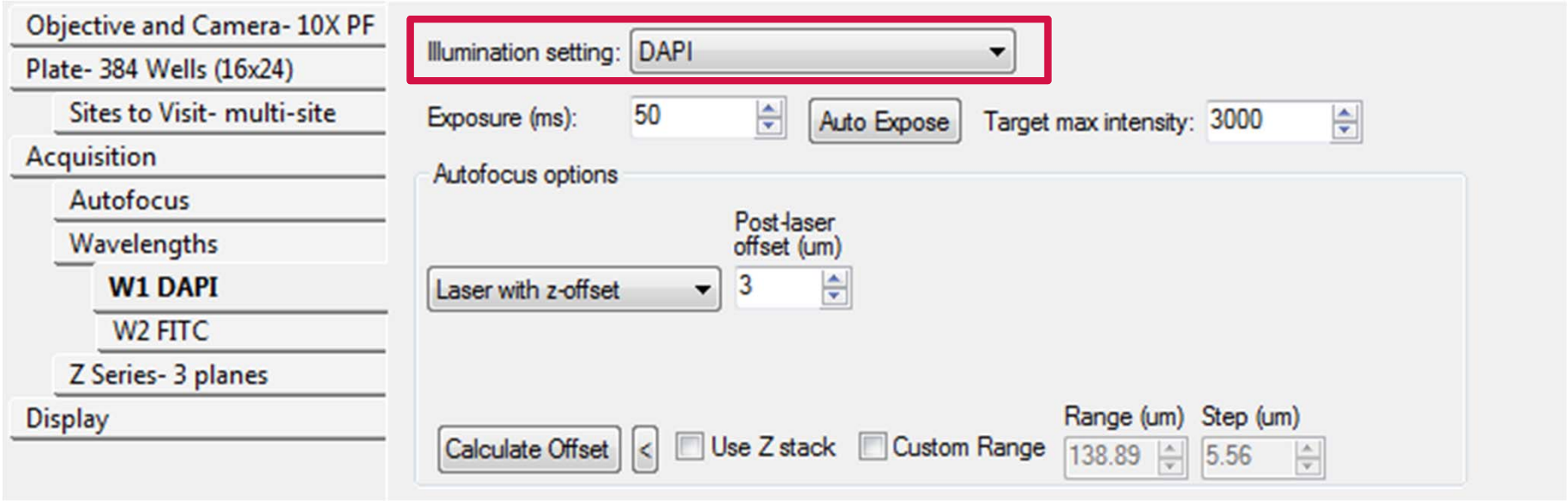
Setting Up a Z Series Acquisition

12. Select the **Wavelengths** tab
    - Enter the number of wavelengths or channels that wish to acquire
      - A separate **W** tab will appear below for each channel
      - You can enter up to 8 wavelengths



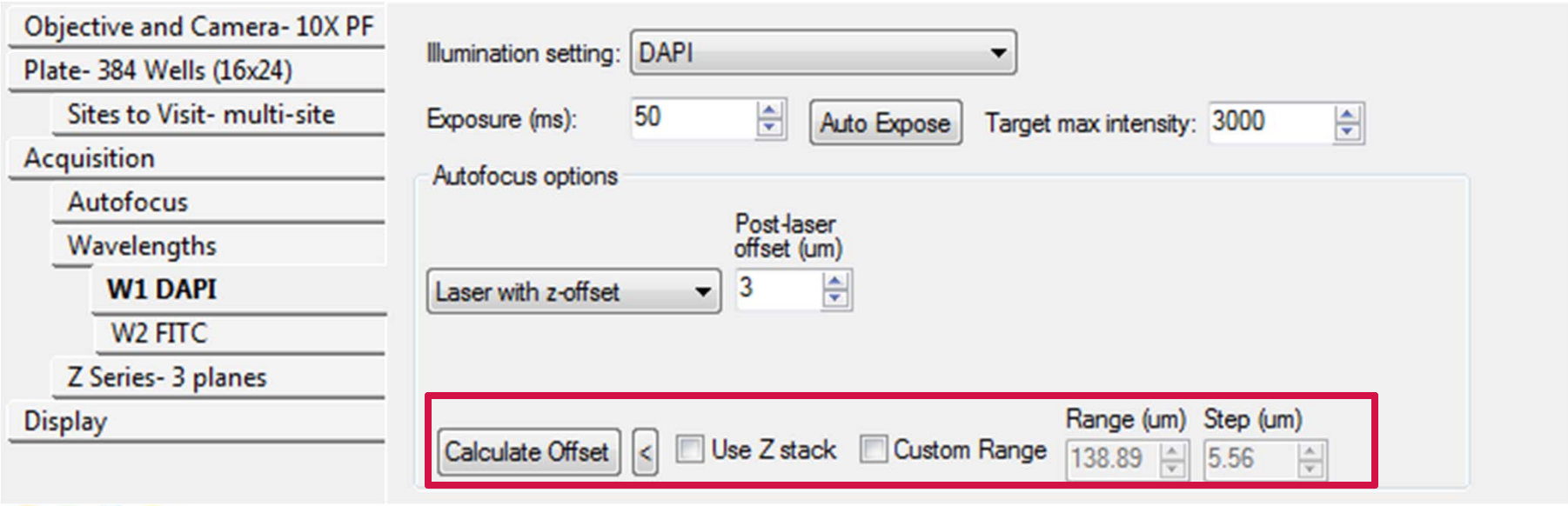
Setting Up a Z Series Acquisition

13. Select the **W1** tab
    - Select desired **Illumination Setting** from the drop-down menu
    - Right-click to select a site/well that should show the highest signal for the wavelength chosen in the plate map

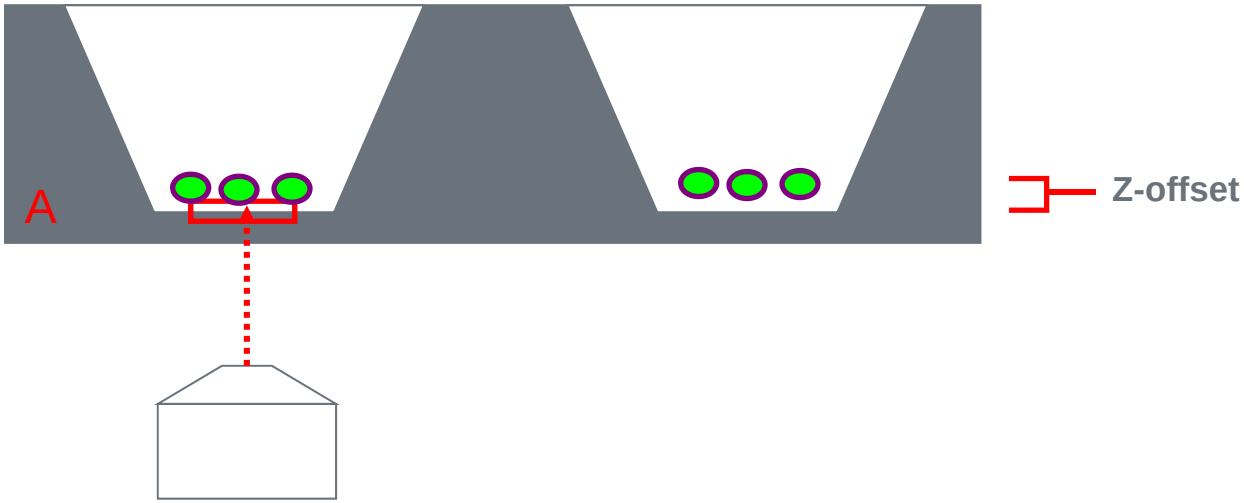


Setting Up a Z Series Acquisition

13. On the **W1** tab
    - Click on the **Calculate offset** button to perform an automatic routine for finding the best focal position (post-laser offset value)
      - Enable **Use Z Stack** for an interactive option to select the focus position. The software will acquire a Z stack of images and allow you to select the most in-focus image.
      - Enable **Custom Range** to specify a custom range and step size for the focus search
    - For Z Series acquisition, Molecular Devices recommends to set the **Post-laser offset** to find the approximate middle of the sample



What is a Post- Laser Offset?



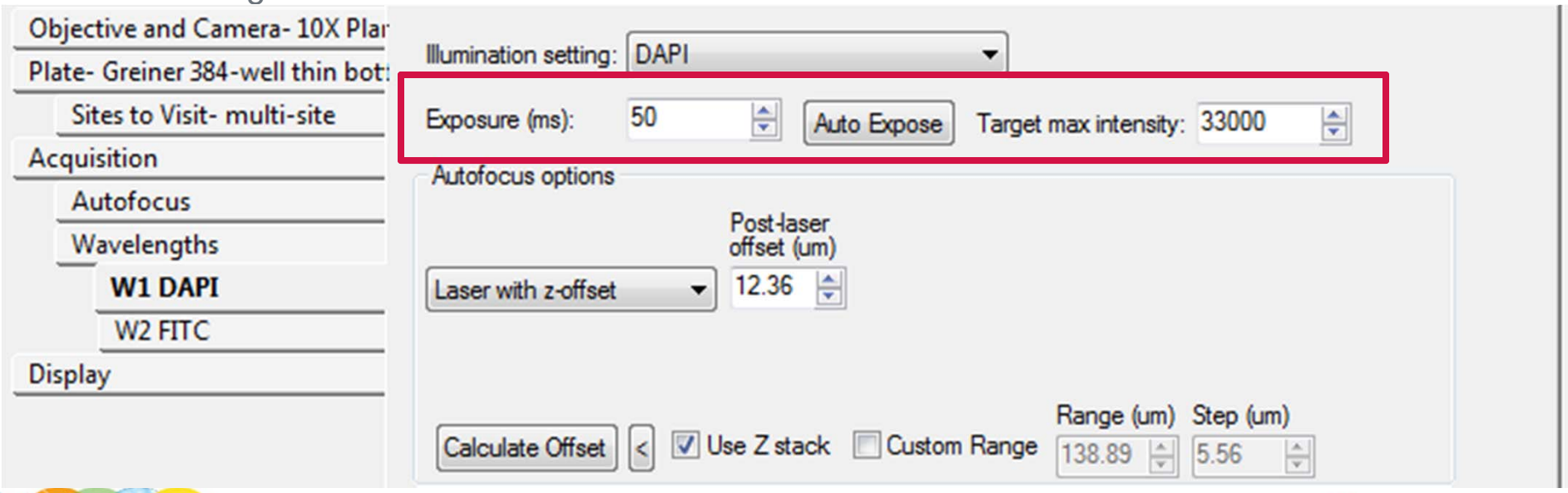
Post-laser offset is the Z distance between the bottom of the well and the sample

- Laser autofocus routine finds the well bottom, NOT the biological sample of interest
- You may need to empirically determine the offset (or distance) between the well bottom and the sample
- Very wavelength dependent (chromatic aberration)
- Offset can be positive or negative
- Molecular Devices recommends checking multiple wells for consistency

Setting Up a Z Series Acquisition

13. On the **W1** tab
    - Enter an **Exposure** time and click the **Focus** 

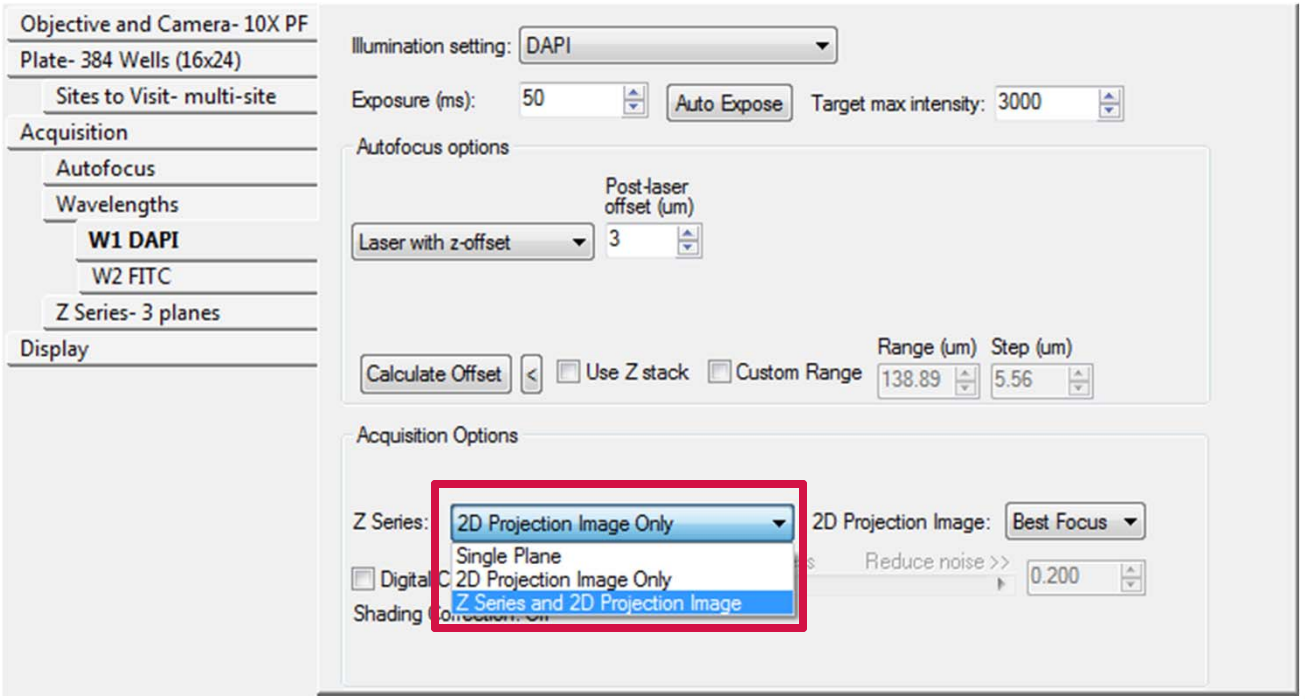
 button
      - Evaluate the image for pixel intensities (bit range)
      - Optionally, click on the **Auto Expose** button to determine exposure automatically (i.e. avoid saturation or very dim signal)
      - Set **Target max intensity** between 33000-45000 for a 16-bit camera (2000-3000 for 12-bit camera). The auto expose routine will attempt to attain this value for the brightest pixel in the image.
      - Molecular Devices recommends checking exposure times for both positive and negative control wells



Setting Up a Z Series Acquisition

14. On the **W1** tab
    - Under **Acquisition Options**, select the appropriate option for saving Z Series
        - **Single Plane**: only the image taken at the Post-laser offset will be saved
        - **2D Projection Image Only**: only the 2D Projection image will be saved
        - **Z Series and 2D Projection Image**: Every Z Series plane as well as the 2D projection image will be saved

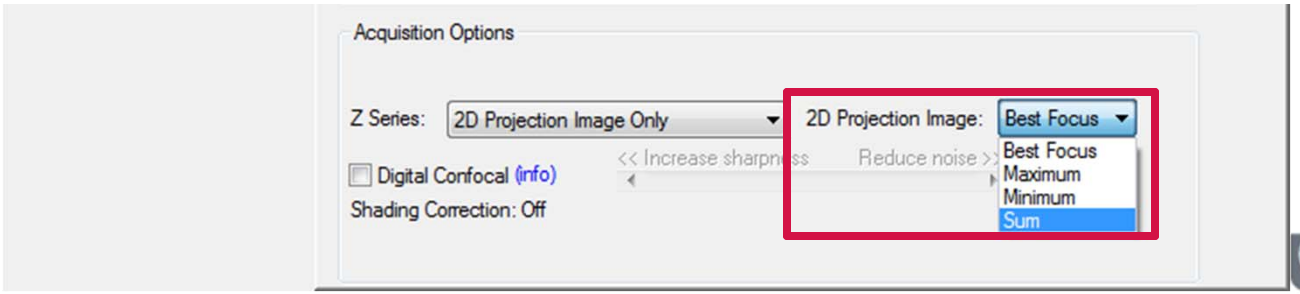
NOTE The above options will be available on each W tab. It is not necessary to acquire and save images the same way for each wavelength



Setting Up a Z Series Acquisition

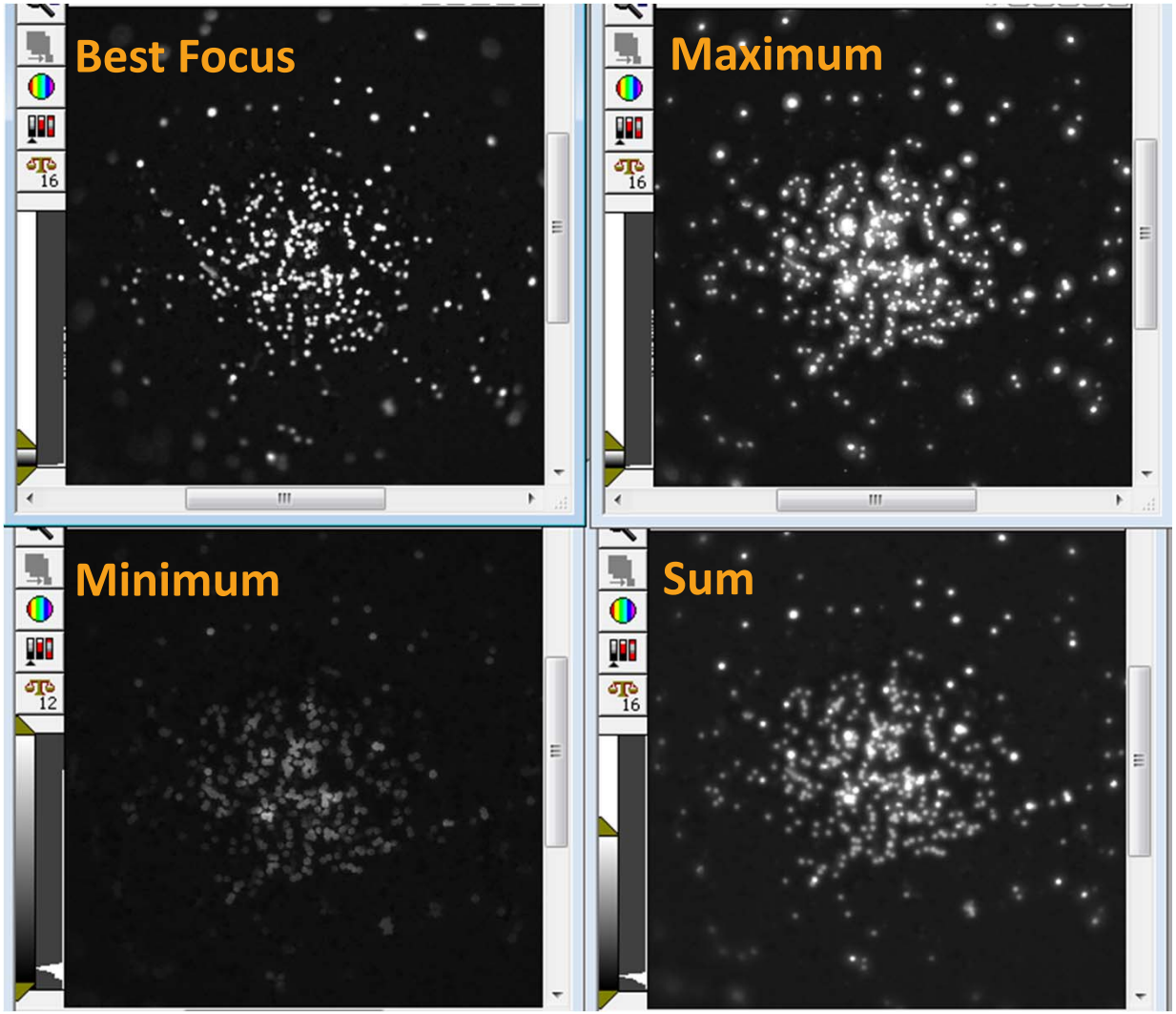
15. On the **W1** tab
    - Under **Acquisition Options**, select the appropriate option for **2D Projection Image**
        - **Best Focus**: estimates the regions of best focus in an image stack to within one-tenth pixel accuracy along Z. Two resolution grid sizes are used to enhance the criterion of focus through the stack (use only for counting or scoring, not for comparing pixel intensities)
        - **Maximum**: For each corresponding pixel position in the images, the Maximum operation finds the pixel that has the highest intensity value out of all the values in all the planes, and outputs this value to the new image (not recommended for samples with high background)
        - **Minimum**: For each corresponding pixel position in the images, the Minimum operation finds the pixel that has the lowest intensity value out of all values in all the planes, and outputs this value to the new image (often used with Transmitted light)
        - **Sum**: For each corresponding pixel position, the Sum operation adds the intensities of the pixels in the stack planes, and outputs this value to the new image. This operation is useful for combining images

    NOTE The above options will be available on each W tab. It is not necessary to apply the same projection image to each wavelength



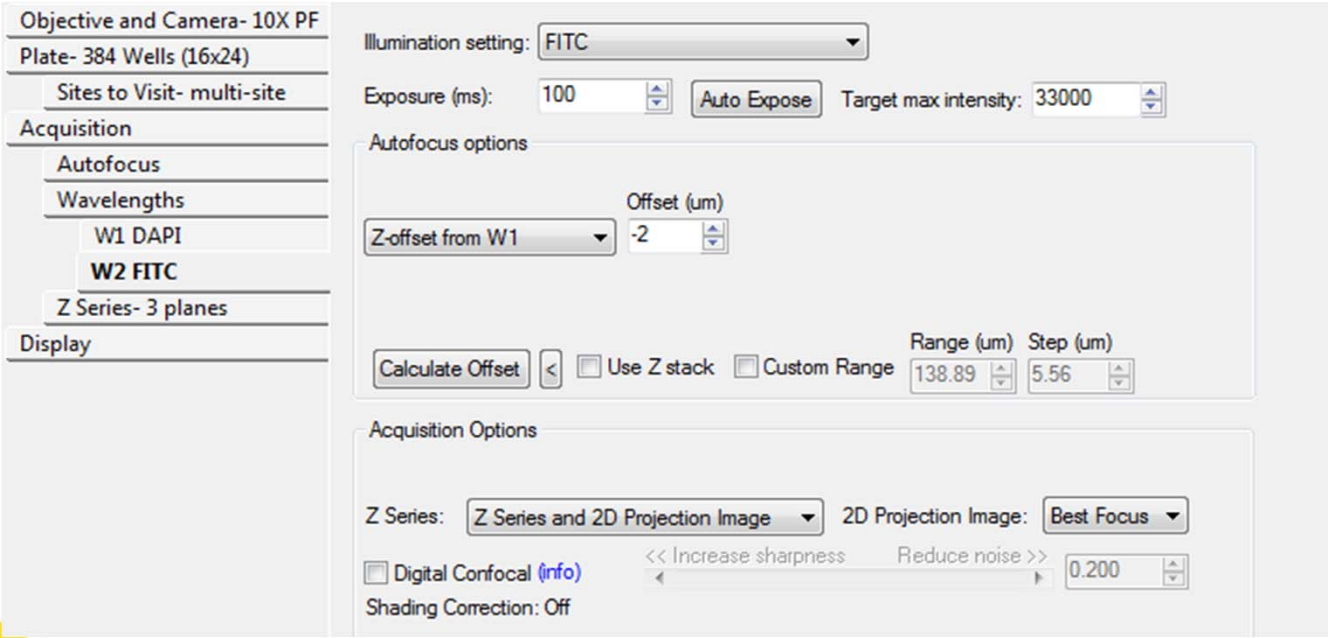
2D Projection Images

- These are examples of 2D Projections generated from the same stack of images
- The optimal choice for 2D projection will depend on your sample type and the analysis goal



Setting Up a Z Series Acquisition

16. Select the **W2** tab (and subsequent W tabs)
    - Select desired **Illumination Setting** from the drop-down menu
    - Right-click to select a site/well that should contain the highest signal for the wavelength chosen in the plate map
    - Calculate **Focus offset**
    - Determine **Exposure** time
    - Determine **Acquisition Options** for Z Series: images to save and 2D projection algorithm



Setting Up a Z Series Acquisition

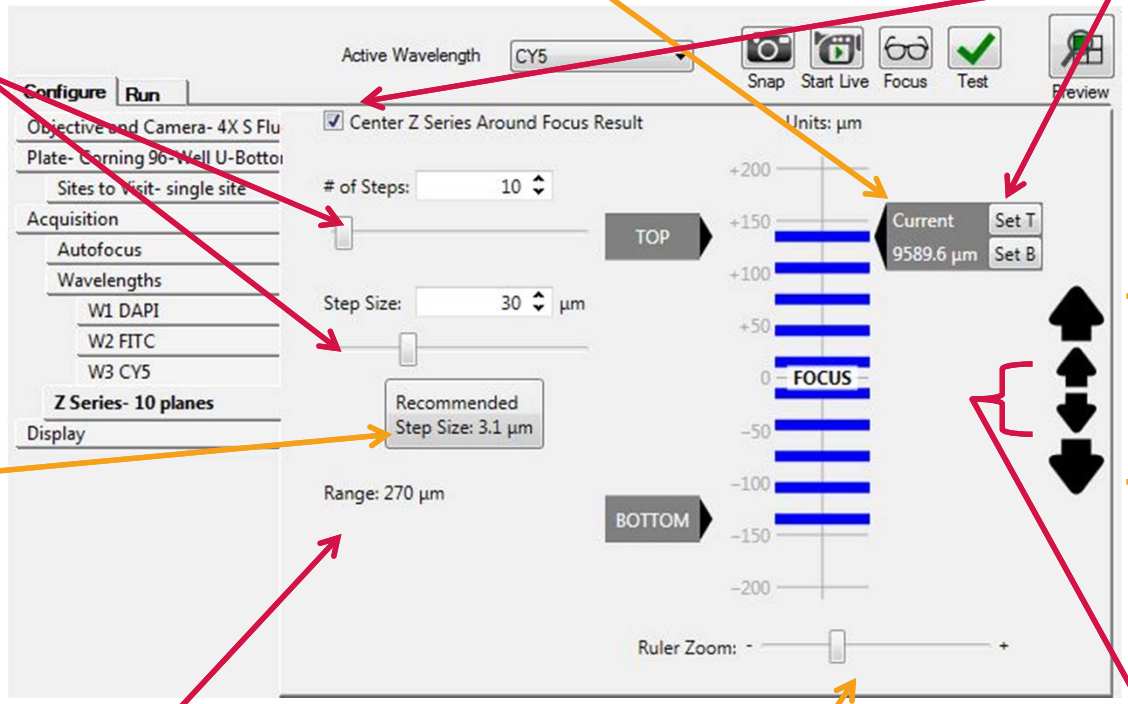
17. Select the **Z Series** tab
    - Enter **# of Steps**: # of Z steps acquired
    - Enter **Step Size**: spacing (µm) between each Z step
    - **Center Z Series Around Focus result**:
        - If checked, # of Steps and Step Size will be center around the post-laser offset configured on each **W** tab.
        - If unchecked, you will need to set the **Top** and **Bottom** Z positions. These Z positions are the distances (see ruler) from the post-laser focus offset

The Z Series Tab

Displays current Z position after clicking on the **Focus**, **Test**, or **Preview** buttons

Use buttons to set Top and Bottom when **Center Z Series Around Focus Result** is unchecked. These are the Z heights that will be used at every site/well from the focus result

Use slider bars to adjust or manually enter values



Click these icons to ove Z stage based on Step Size

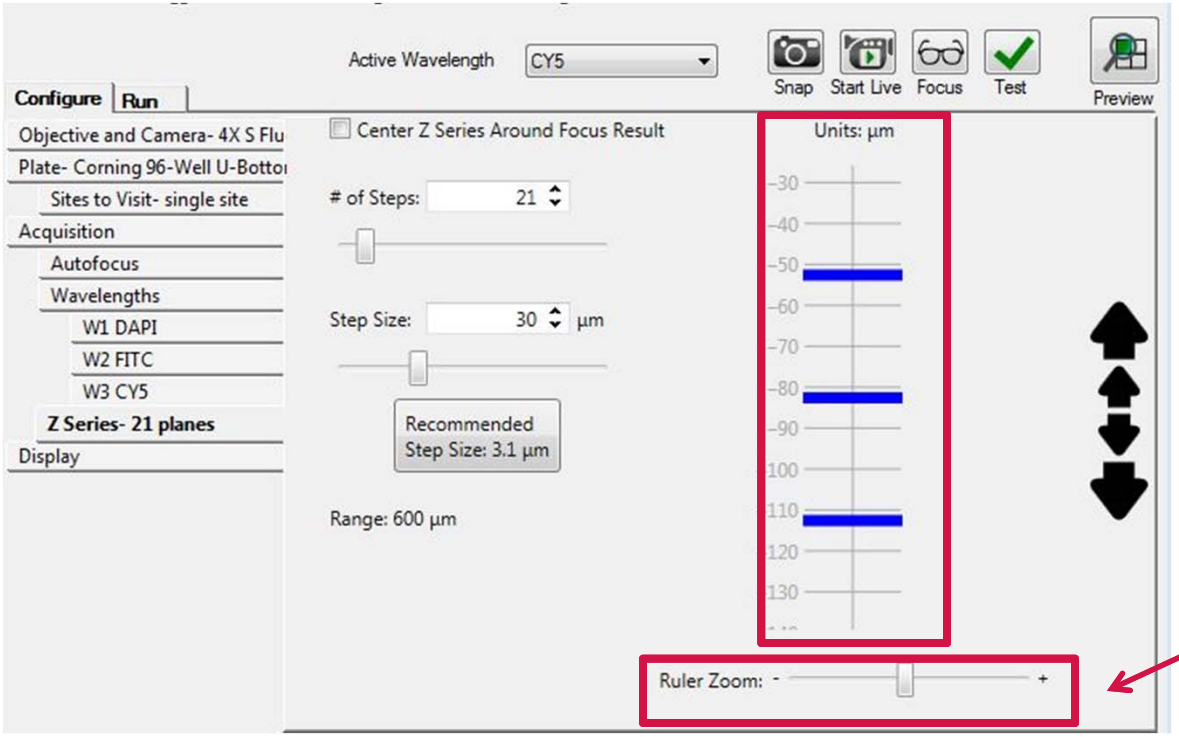
Click here to set step size to recommended value based on objective properties

Indicates range of Z series with current settings

Adjust slider to zoom in and out on ruler

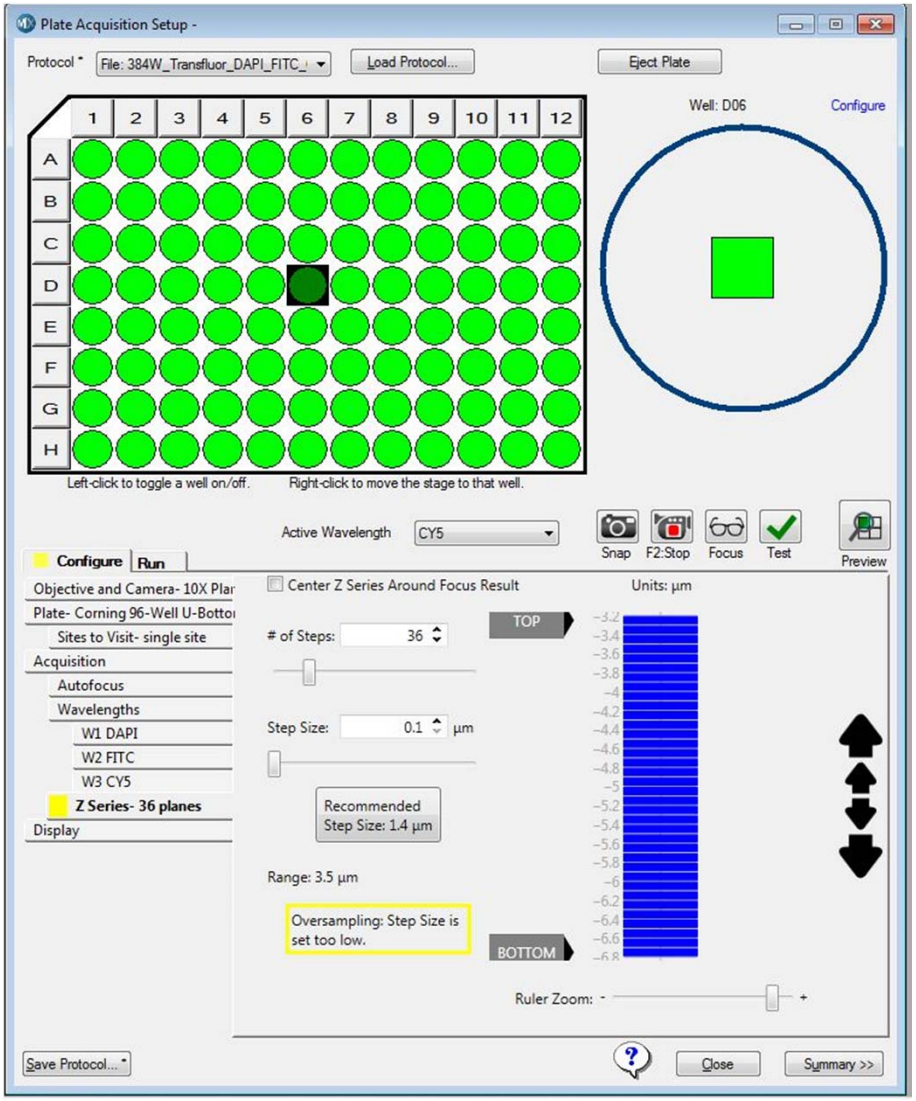
Click these icons to cmve half the focal distance of the objective

Z Series Tab – Missing Current Position Indicator



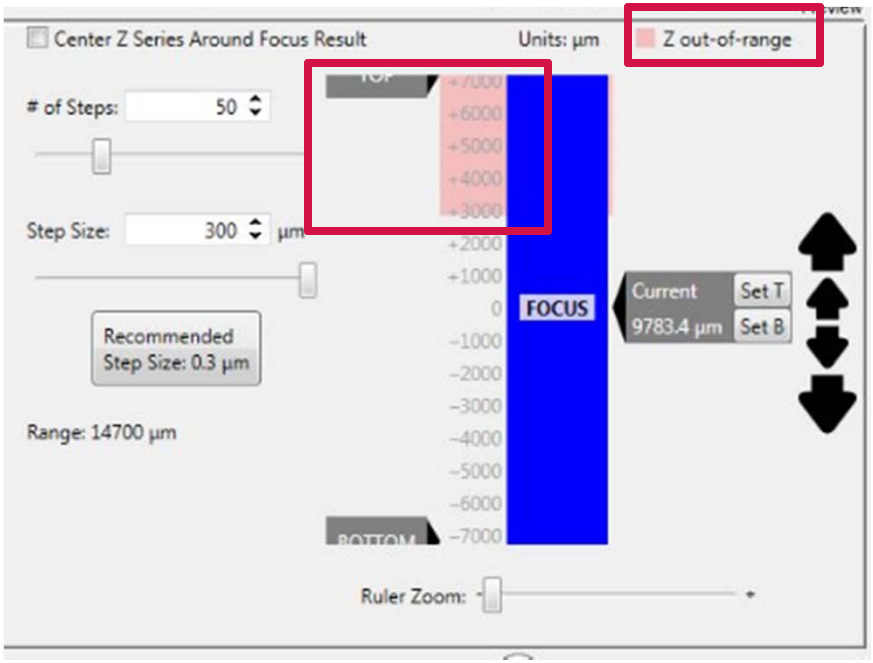
If after clicking the **Focus, Test,** or **Preview** buttons, the current position indicator does not appear, adjust the **Ruler Zoom** to the left

Z Series Tab – Oversampling Message



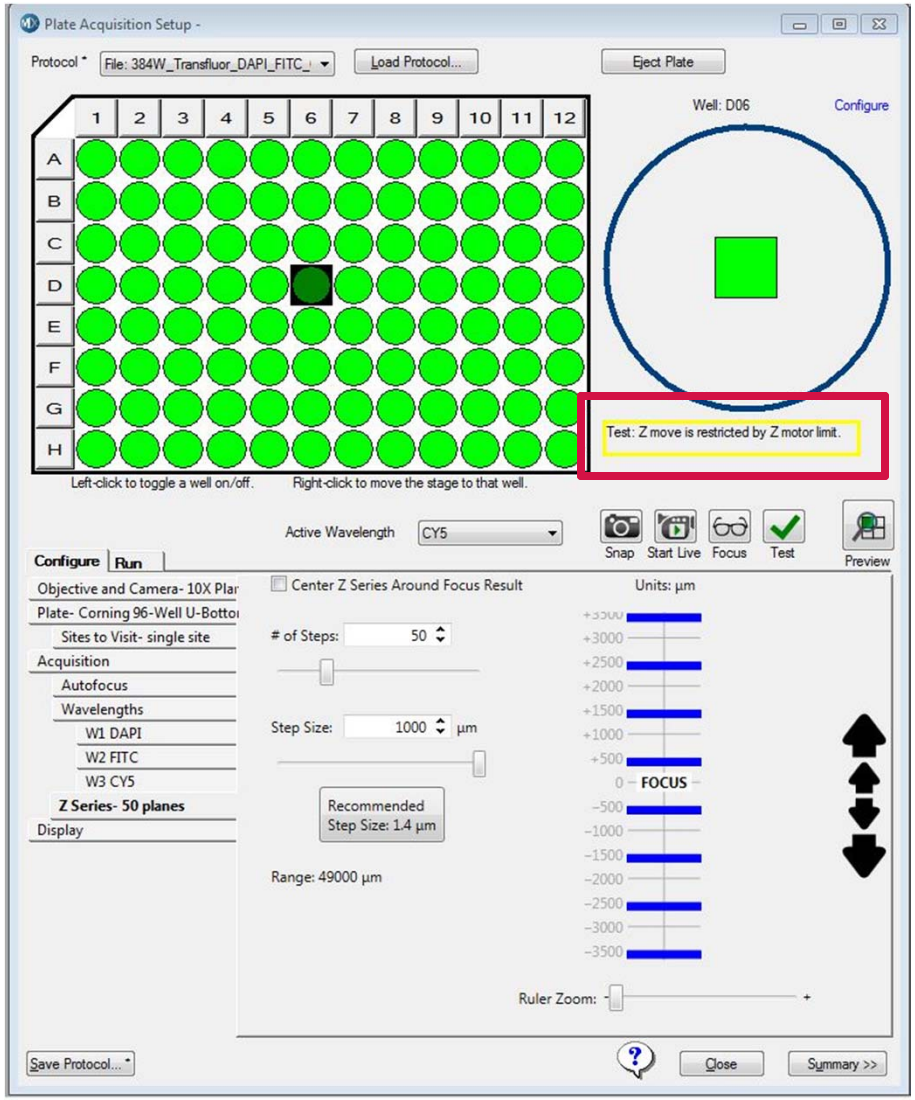
A yellow box will appear on the **Configure** and **Z Series** tabs when step size chosen is too small (oversampling) based on objective properties. Increase step size to get rid of the message.

Z Series Tab – Z Out of Range



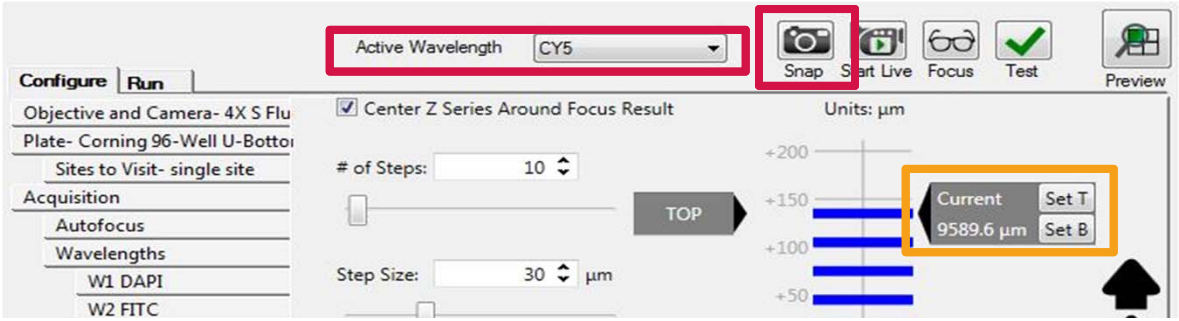
If Step Size and/or # of Steps results in the Z-series being outside of the physical range of the objective, a pink overlay will appear. Reduce the **# of steps**, adjust **Step Size** or set z-series to a position to below the pink area to get rid of the error

Z Series Tab – Z Motor Limit



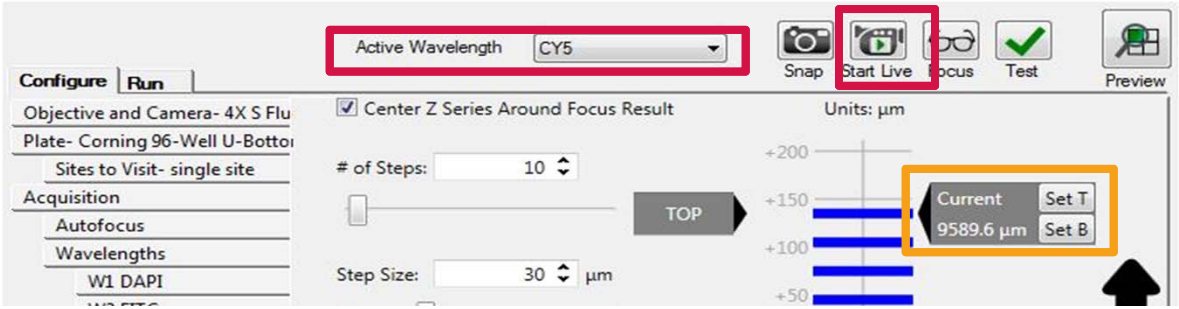
An error message will appear if you click the **Test button** while a Z out of range condition exists. Adjust # **of steps**, **Step size** or Z top position to remove the error message.

Optimizing Z Series Acquisition . . . Snap Method



17. On the **Z Series** tab
    - Click on the **Recommended Step Size** button (or adjust manually)
    - Click on the **Focus** button to display the current position indicator
    - To adjust the Z height:
        - Drag the current position indicator using your mouse and click **Snap** to take an image at the new Z position
        - Click the small and large arrow buttons, then click **Snap** to take an image
    - Adjust # **of Steps** to cover the range desired, or
    - If **Center Z Series around Focus result** is deselected**,** set the Top and Bottom Z positions
    - Click on the **Test** button to verify settings
    - Test the settings for each wavelength by changing the **active wavelength** from the drop down menu and clicking **Test.** You may also click on the **Preview button** (only 2D projection image is shown for all wavelengths).

Optimizing Z Series Acquisition . . . Live Method



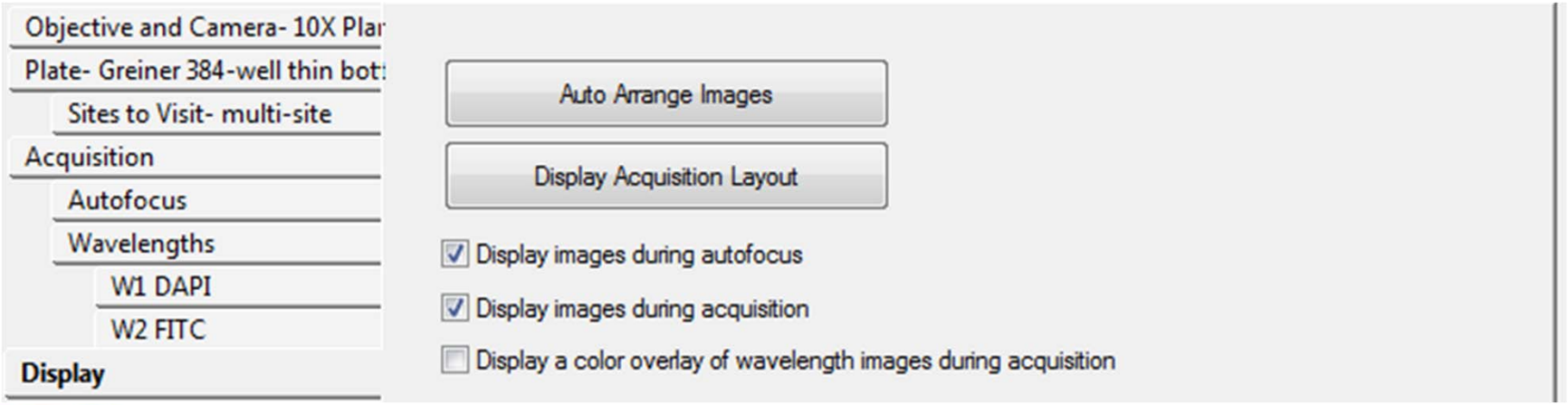
17. On the **Z Series** tab
    - Click on the **Recommended Step Size** button (or adjust manually)
    - Click on the **Focus** button to display the current position indicator
    - Click the **Start Live** button

    NOTE In this method, the sample is constantly exposed to light which can lead to phototoxicity or photobleaching

    - To move the Z Stage:
        - Drag the current position indicator using your mouse
        - Click the small and large arrow buttons
    - Adjust # **of Steps** to cover the range desired, or
    - If **Center Z Series around Focus result is deselected,** set the Top and Bottom Z positions
    - Click on the **Test** button to verify settings
    - Test the settings for each wavelength by changing the **active wavelength** from the drop down menu and clicking **Test.** You may also click on the **Preview button** (only 2D projection image is shown for all wavelengths).

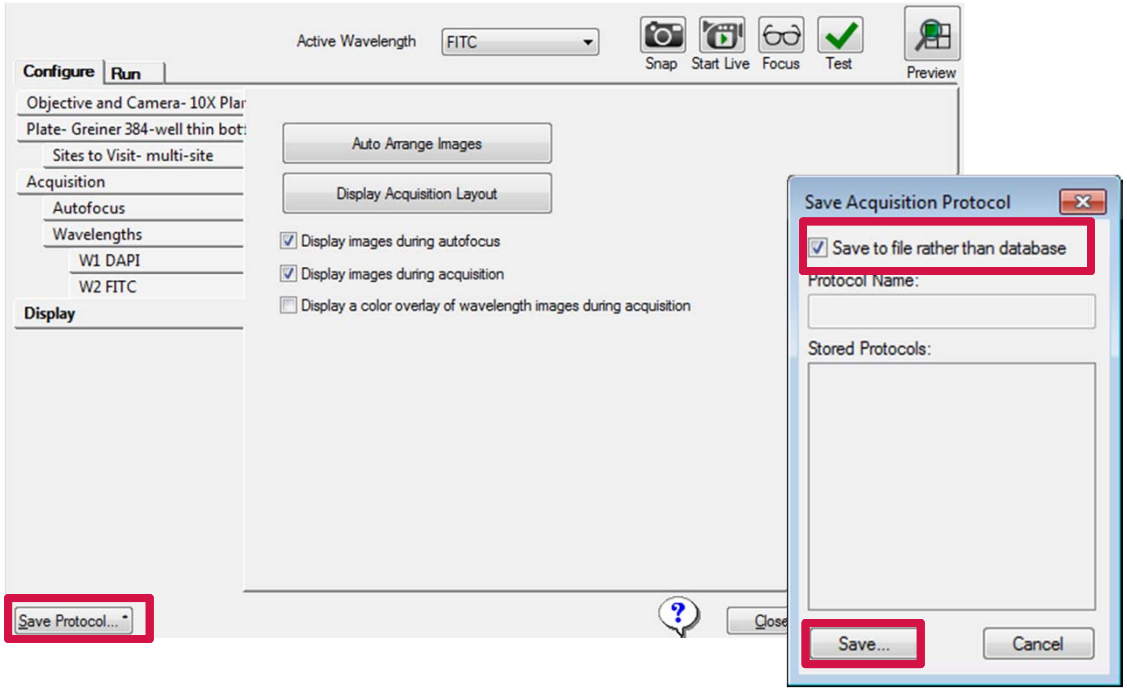
Setting Up a Z Series Acquisition

18. Select the **Display** tab to configure:
    - **Auto Arrange Images**: Software automatically determines the arrangement and size of images shown in MetaXpress
    - **Click on Display Acquisition Layout**: Manually configure how the images will look during acquisition (position, size, scaling, monochrome or color)
    - **Display images during autofocus** should be checked to help with finding post-laser offset
    - **Display images during acquisition** displays images according to the settings determined using Auto Arrange Images or Display Acquisition Layout
    - **Display a color overlay of wavelength images during acquisition**: Will create a color composite of the first 3 wavelengths selected



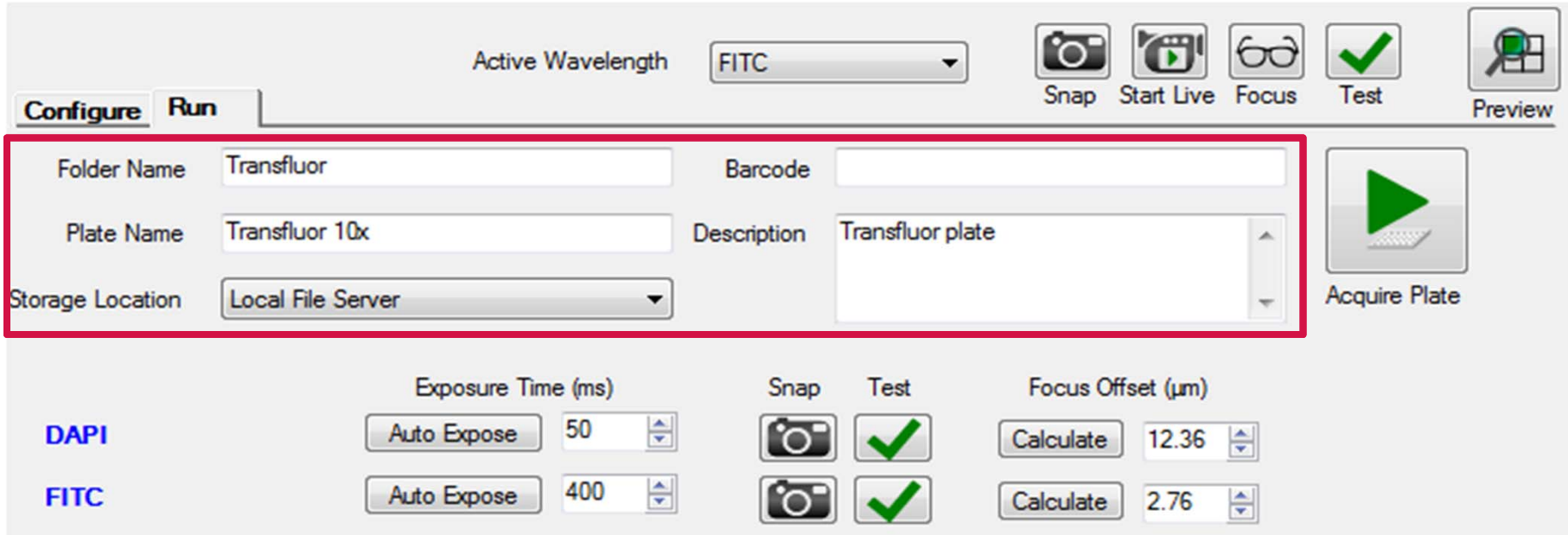
Setting Up a Z Series Acquisition

19. Click on the **Save Protocol** button at the bottom of the **Plate Acquisition Setup** dialog
    - A star on the **Save Protocol** button indicates there are unsaved changes to the protocol
    - Molecular Devices recommends to save settings to a file rather than the database
    - Click on **Save** button, name the protocol, and navigate to the directory where you want to save the file (.hts)



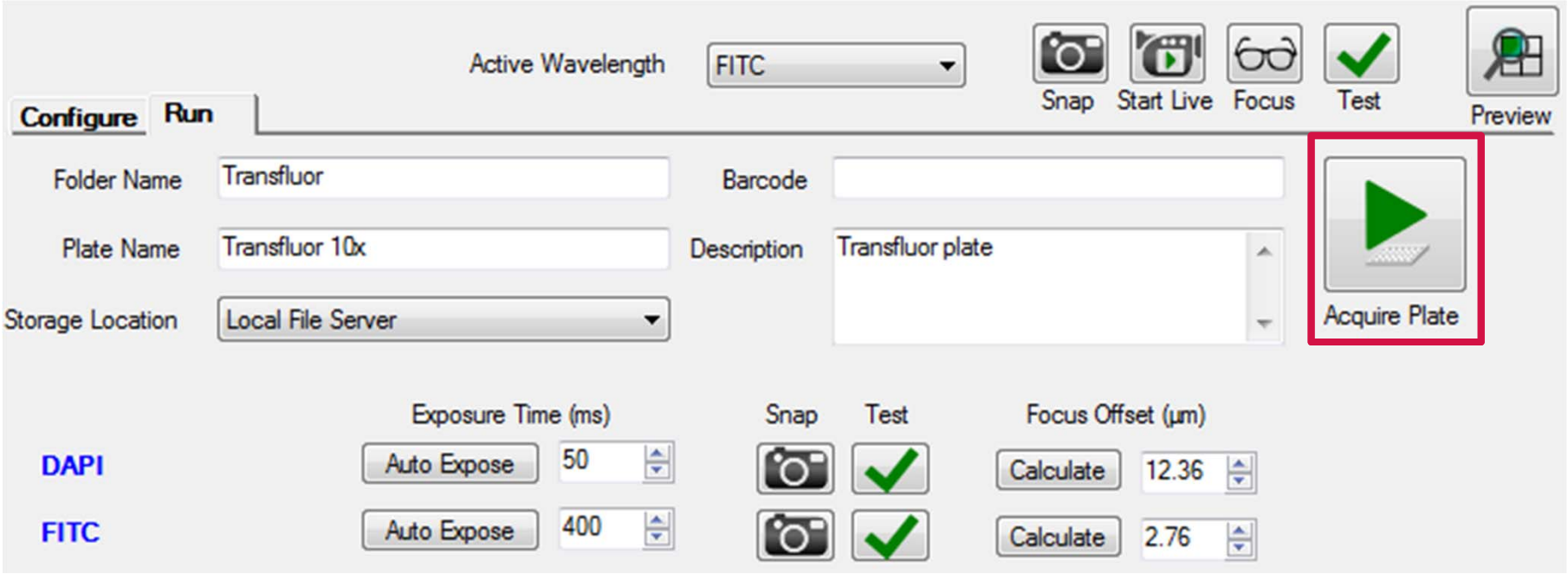
Setting Up a Z Series Acquisition

20. Select the **Run** tab and enter:
    - **Folder Name**: folder to organize your plates in (i.e. project or PI)
    - **Plate Name**: the name of the plate to be imaged (i.e. specific experiment)
    - **Barcode** (optional): manually enter the plate barcode
    - **Storage Location**: select where you want images to be stored (there may only be one choice)
    - **Description**: enter any identifying information you would like to store with the plate



Setting Up a Z Series Acquisition

21. Click on the **Acquire Plate** button to begin acquisition of the plate



Support Resources

- F1 / HELP within MetaXpress® Software
- Support and Knowledge Base: http://mdc.custhelp.com/
- User Forum: http://metamorph.moleculardevices.com/forum/
- Request Support: http://mdc.custhelp.com/app/ask
- Technical Support can also be reached by telephone:
  - 1 (800) 635-5577
  - Select options for Tech Support → Cellular Imaging Products → ImageXpress Instruments

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