

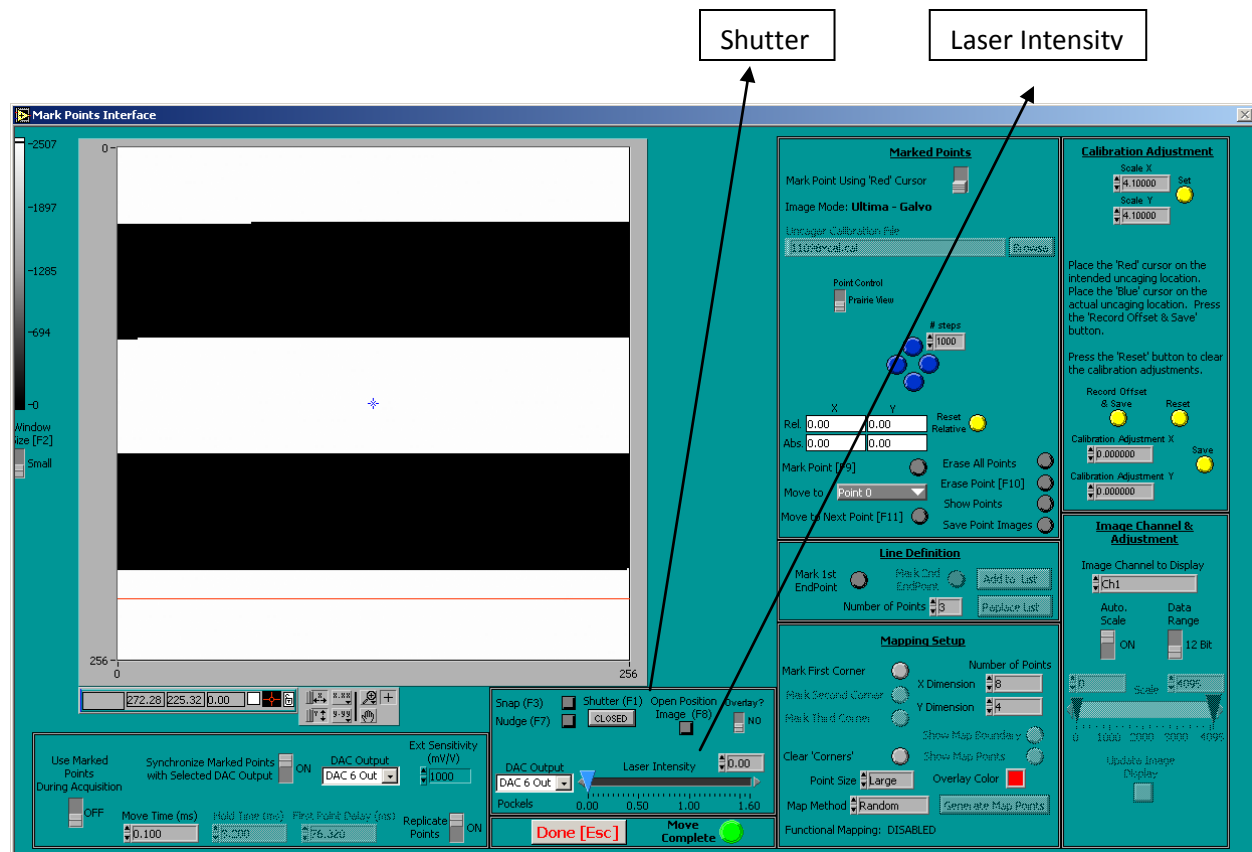
# Point Calibration

July 3, 2012

The purpose of the Point Calibration process is to generate a 'map' of voltages (for galvos) or motor positions of the pointing device to the voltages or pixels of the reference image device. Depending upon the system configuration, the reference image device could be a galvo generated image, an AOD/galvo generated image, a resonant galvo generated image, or a camera generated image. The camera generated image could come from an SFC (Swept Field Confocal) or a stand-alone camera.

There are two possible methods for performing the Point Calibration. In the first method, an image of the uncaging point is used. The second method requires that the operator 'burn' a pattern on a suitable sample and then perform the calibration using this burn pattern as the calibration reference. If the first method is not possible for a given system configuration, then the second method must be used.

To determine which method can be used, start with the system configuration. For a standard dual galvo set Ultima, there is usually a 'Spot' detector installed as one of the imaging channels. If the system includes a 'Confocal' module, then the Spot detector is not installed. To view a 'Spot' image, turn on the 'Spot' channel in an image window, start a 'Live Scan', then in the 'Mark Points' dialog in TriggerSync click on the 'Shutter' button to open the shutter and increase the 'Laser Intensity' value until an image of the uncaging 'Spot' is seen in the image window. The following image shows the location of the 'Shutter' and 'Laser Intensity' controls. The Live Scan can be stopped now.



When using a camera as the reference image device (either stand-alone or as part of an SFC), there is no 'Spot' detector. The camera can be thought of as a Spot detector however with regards to the Point Calibration process. To actually view the location of the uncaging laser with the camera requires a sample that can fluoresce with just a small 'spot' where the uncaging laser is currently hitting the sample. Often times, a solid color 'Chroma' slide will work for this process. To try and image the uncaging spot, place the appropriate slide on the microscope. Start a 'Live Scan', then in the 'Mark Points' dialog in TriggerSync click on the 'Shutter' button to open the shutter and increase the 'Laser Intensity' value until an image of the uncaging 'Spot' is seen in the image window. You might have to experiment with different slides. Also, these slides are very sensitive to laser power so at one value, there might be a 'spot' image but at just a slightly higher laser value, the sample might 'burn' immediately and generate no fluorescent signal. The Live Scan can be stopped now.

For a galvo based reference image, set the image resolution to at least 512x512. For a camera reference image, be sure that the 'Binning' value for the camera is set to 1x1 before starting the calibration process.

If a 'Spot' image was able to be achieved, then the first Point Calibration method (Point Calibration using a 'spot' image) may be used. Otherwise, skip ahead to the 'Point Calibration using a 'Burn' Pattern' section.

### Point Calibration using a 'Spot' image


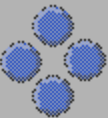
Click on the TriggerSync menu option 'Points -> Calibrate Points...'. The 'Mark Points' dialog will be automatically closed if it is open, and the 'Uncaging Point Calibration' dialog will appear.

**Pinhole Photolysis Calibration**

## Uncaging Point Calibration

**Step 1: Specify Calibration File Name**

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**Move Complete**



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**Step 2.1: Calibration Method**

**Step 2.2:** Acquire an image of the "spot" to calibrate. Using the mouse, left click on the "spot" in the 'Original Image' window. Adjust the 'Tolerance' and/or 'Connectivity' value to create a single continuous image of the "spot" in the 'Processed Image' window.

This factor is applied to the "spot" size to indicate the range of the "spot" area to use when filtering out false spots (0.0 - 1000.0):

Number of Frames to Average:

**Step 3:** Using the 'Blue' buttons above, move the "spot" to the upper left hand corner of the image. Do not have the "spot" touch or extend beyond the edge of the image.

**Step 4:** Using the 'Blue' buttons above, move the "spot" to the upper right hand corner of the image. Do not have the "spot" touch or extend beyond the edge of the image.

**Step 5:** Using the 'Blue' buttons above, move the "spot" to the lower right hand corner of the image. Do not have the "spot" touch or extend beyond the edge of the image.

**Step 6: Perform the Calibration.**

Number of Points:

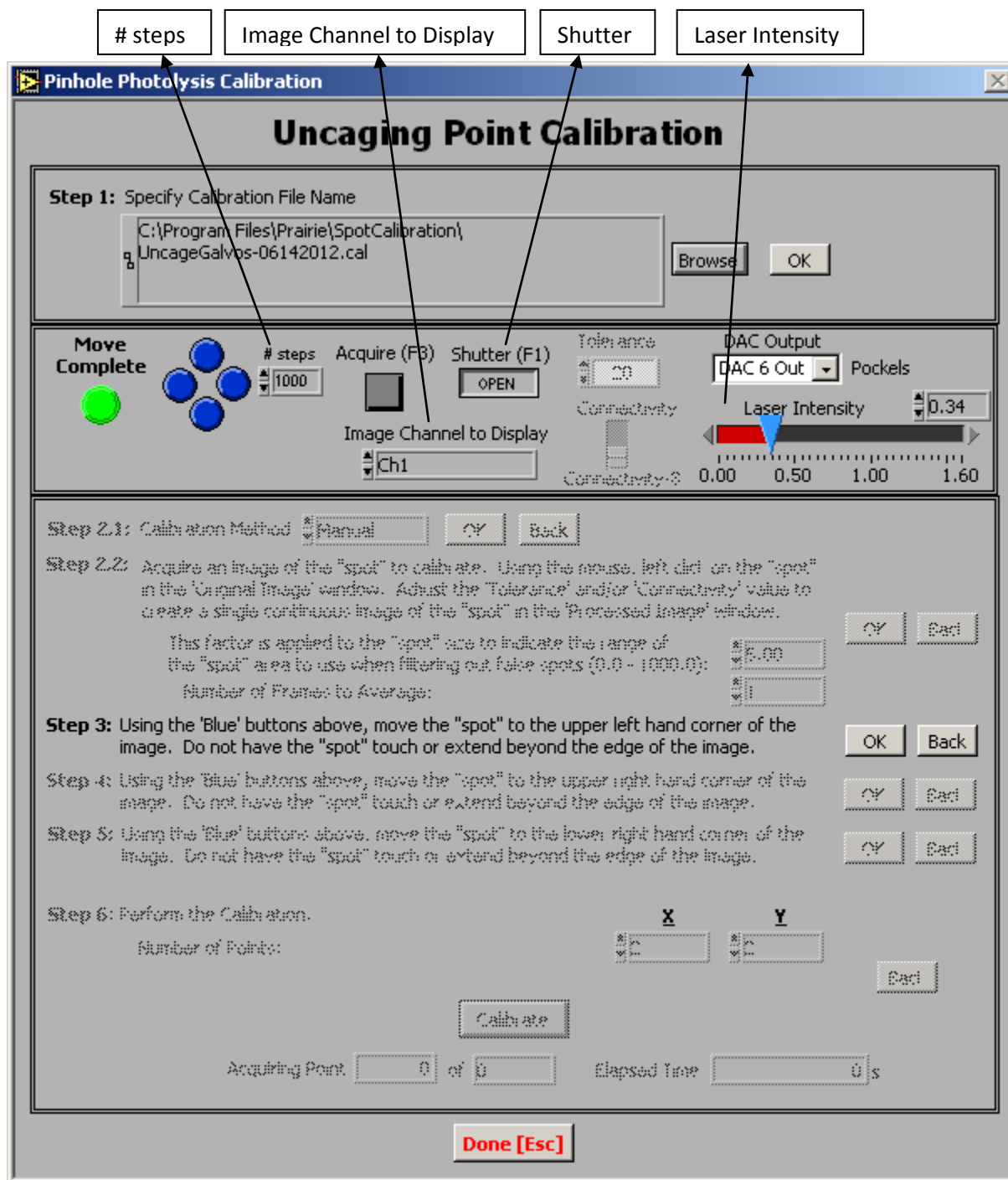
Acquiring Point:  of  Elapsed Time:  s

For 'Step 1' click on the 'Browse' button and navigate to 'C:\Program Files\Prairie\Spot Calibration' and enter a calibration file name (e.g. 'UncageGalvos-06142012'). Click 'OK' button to advance to step 2.

For 'Step 2' select the 'Manual' option and click on the 'OK' button to advance to step 3.

For 'Step 3', start by opening the 'Shutter' and adjusting the 'Laser Intensity' to the previously determined values to generate the 'Spot' image. Set the value for '# steps' to 1000. Select the proper

channel that the 'spot' image is being acquired in within Prairie View with the 'Image Channel to Display' control. The following image shows the location of these controls.



Click the 'Acquire' button to collect a 'spot' image. The image will be displayed in a window labeled 'Upper Left Hand Corner Image'.

You'll be using the four 'blue' round buttons located next to the '# steps' field to physically move the 'spot' image around the field of view. To continue with step three, use the four blue buttons to move the 'spot' image from its current location towards the upper left hand corner of the display window.

**It is important to note that if the movement of the 'spot' image does not match the particular 'blue' button that is pressed, the calibration process will not work. So, when the 'left blue' button is pressed, the 'spot' image should move left in the image window. When the 'right blue' button is pressed, the 'spot' image should move right. When the 'up blue' button is pressed, the 'spot' image should move up. When the 'down blue' button is pressed, the 'spot' image should move down. If this is not the case, terminate the calibration process and contact Prairie Technologies. Explain the situation and someone will help correct the software settings. After the settings are corrected, the calibration procedure may continue.**

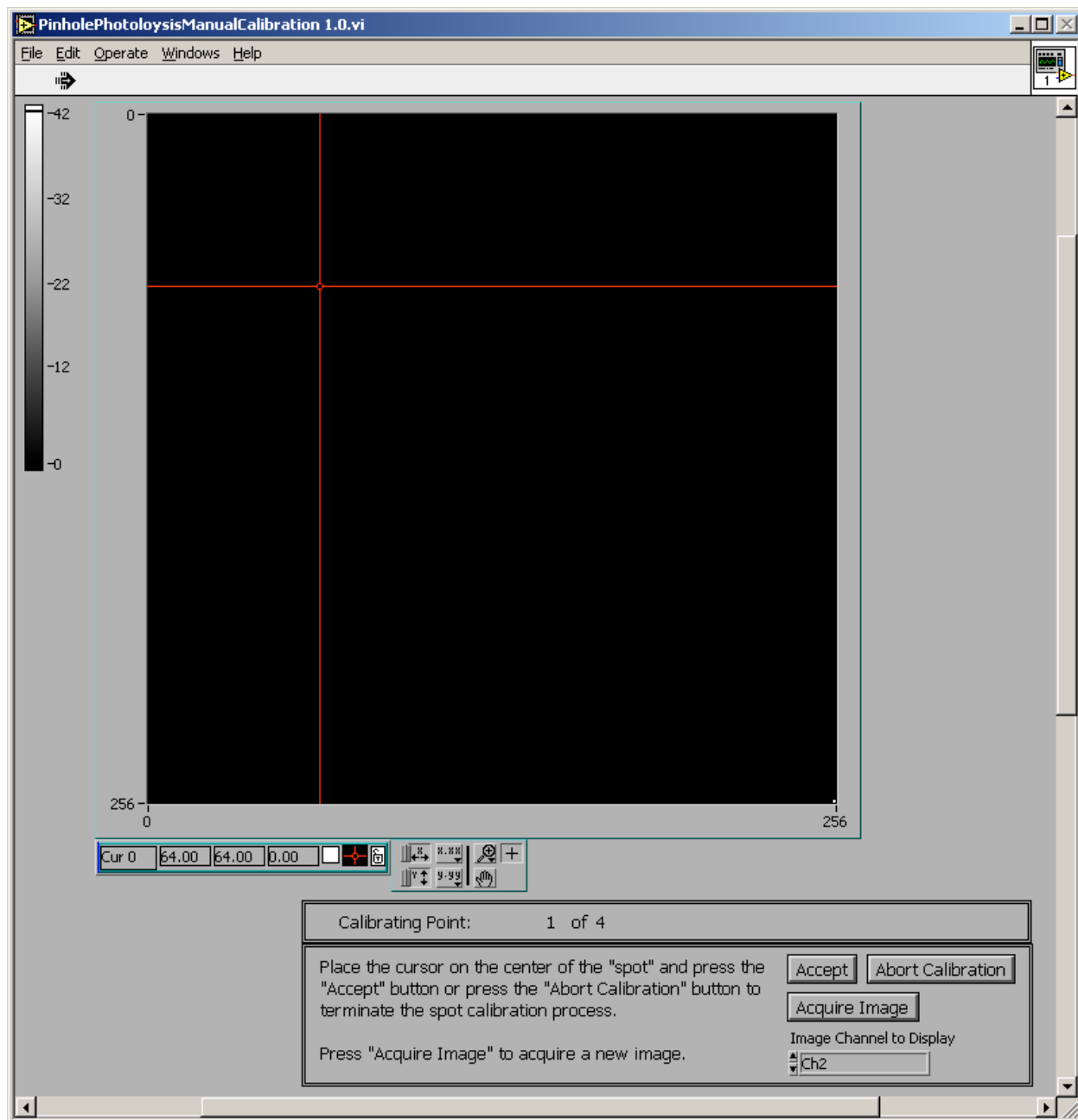
After every couple of steps with the 'blue' buttons, press the 'Acquire' button to update the image in the 'Upper Left Hand Corner Image' window. Based upon various optical considerations, you will not be able to position the 'spot' image in the very corner, nor should you try. Once you have the 'spot' image towards the upper left hand corner, click on the 'OK' button.

For 'Step 4', use the 'blue' buttons (now only the two horizontally opposed buttons will be active) to move the 'spot' image towards the upper right hand corner of the field of view. You'll notice that the image window is now labeled 'Upper Right Hand Corner Image'. Once you have the 'spot' image towards the upper right hand corner, click on the 'OK' button.

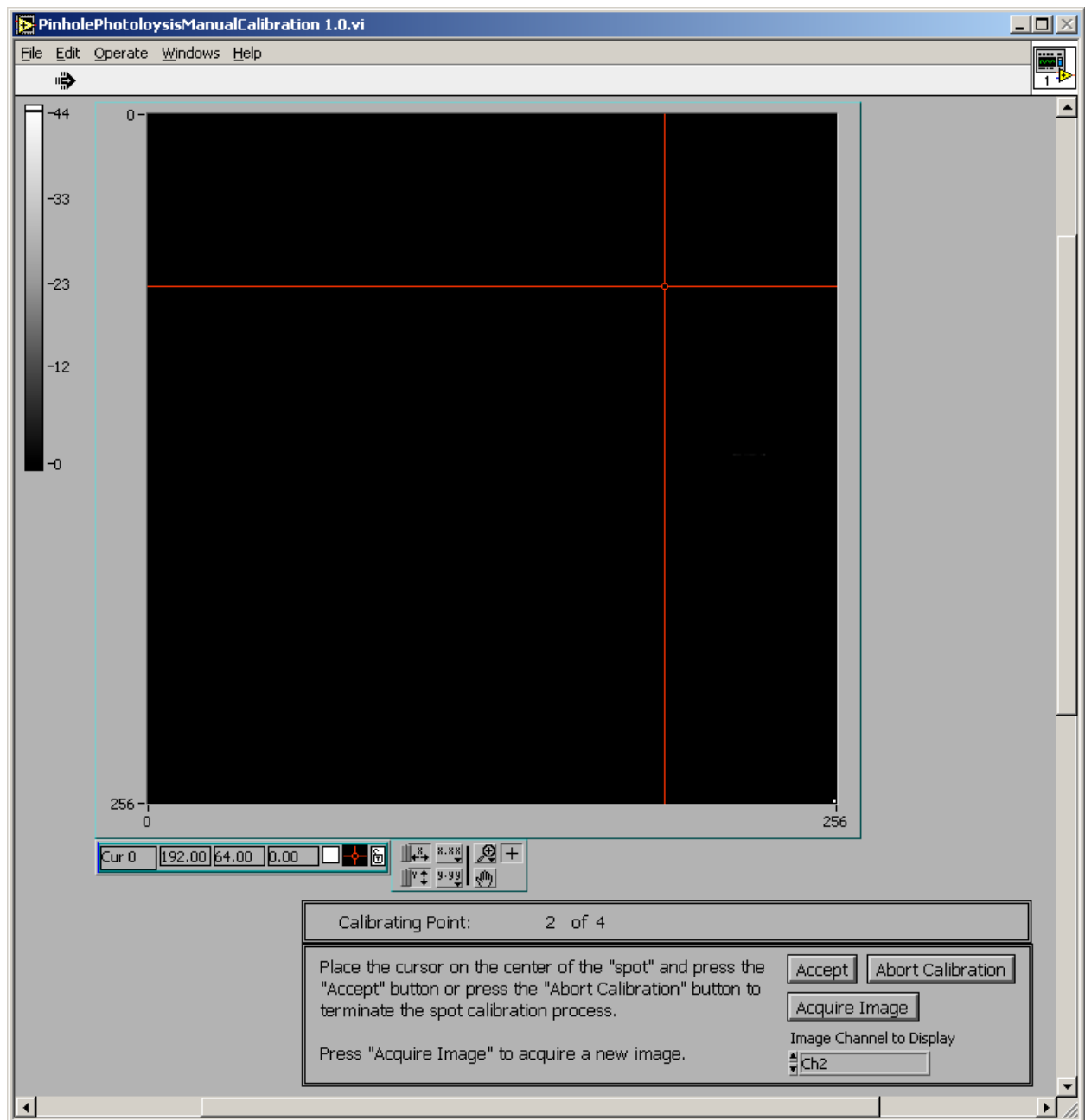
For 'Step 5', use the 'blue' buttons (now only the two vertically opposed buttons will be active) to move the 'spot' image towards the lower right hand corner of the field of view. You'll notice that the image window is now labeled 'Lower Right Hand Corner Image'. Once you have the 'spot' image towards the lower right hand corner, click on the 'OK' button.

For 'Step 6' specify the number of points for the calibration pattern in the X and Y dimensions. The more points you specify the more accurate the calibration process will be, but with the trade-off that the calibration will take longer to perform. If you specify '2' for both X and Y then you'll only be calibrating the four corners of the bounding rectangle defined in steps 3 through 5. Enter the desired values. If a 'Live Scan' is in progress in Prairie View, stop the 'Live Scan' before proceeding. Press the 'Calibrate' button to start the actual calibration process.

When the calibration process is started, the galvos will be positioned to the first point in the upper left hand corner and an image will be automatically acquired. A new dialog will be displayed labeled 'PinholePhotolysisManualCalibration 1.0.vi'. There is a text display in this dialog that states 'Calibrating Point: 1 of 4'. This will help keep you informed of how many points are left in the calibration process. Move the 'red' cursor so that it is centered on the 'spot' image. Even though there is no 'spot' image in the following illustration, assume that the cursor is positioned over the 'spot' image.



When the cursor is properly positioned, press the 'Accept' button. The galvos will be automatically positioned to the next location in the calibration order and the same dialog will appear. Notice that the text display now reads 'Calibrating Point: 2 of 4'.



When the cursor is properly positioned, press the 'Accept' button. The galvos will be automatically positioned to the next location in the calibration order and the same dialog will appear. Notice that the text display now reads 'Calibrating Point: 3 of 4'.

When the cursor is properly positioned, press the 'Accept' button. The galvos will be automatically positioned to the next location in the calibration order and the same dialog will appear. Notice that the text display now reads 'Calibrating Point: 4 of 4'.

After the last point has been calibrated, the 'PinholePhotolysisManualCalibration 1.0.vi' dialog will be automatically closed.

Click on the 'Done' button at the bottom of the uncaging calibration dialog to close it.

Skip ahead to the 'Using the Point Calibration File' section.

### **Point Calibration using a 'Burn' Pattern**

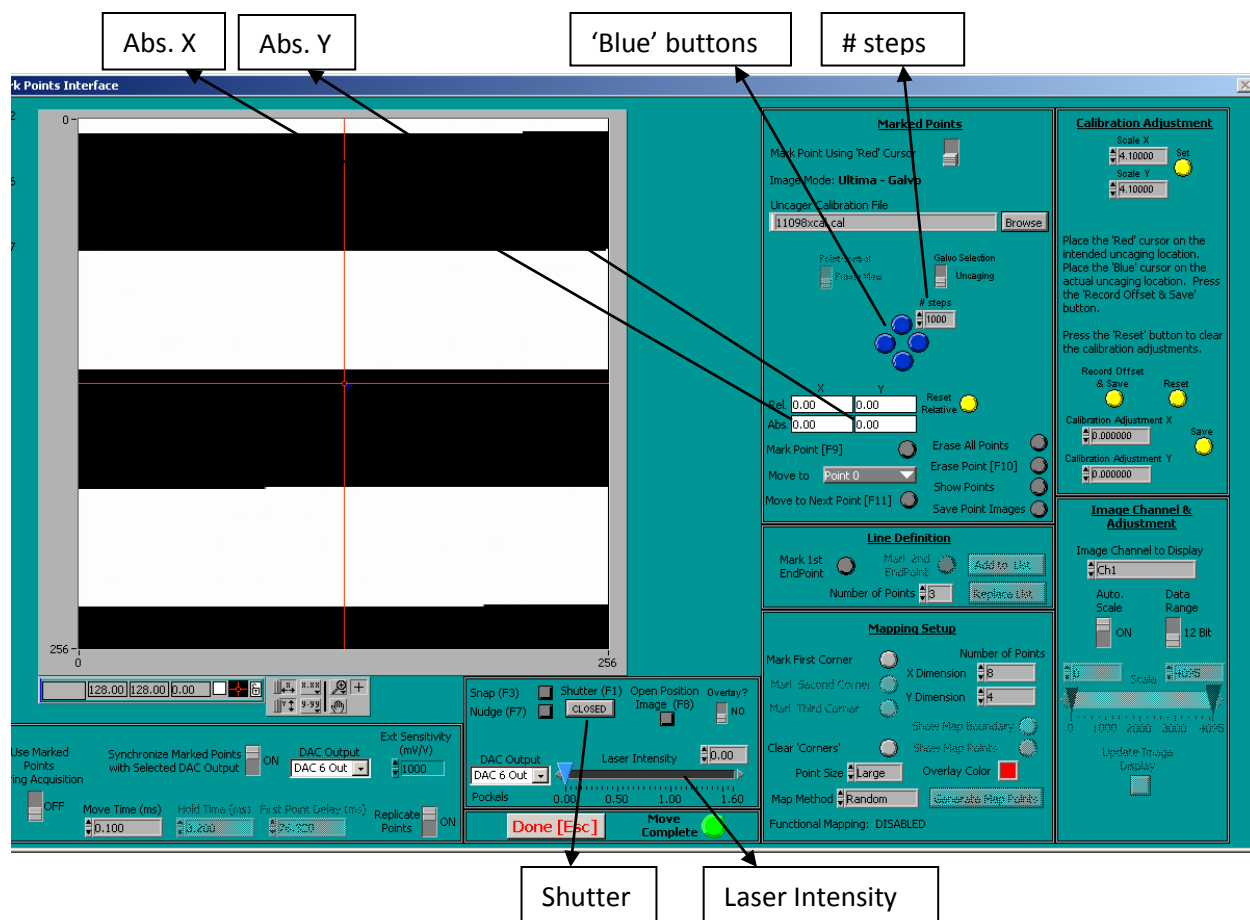
If it is not possible to view an image of the 'spot' for uncaging calibration purposes we are forced to use another method. This method relies upon 'burning' a series of holes into a sample, and then using this hole pattern as the calibration reference.

The sample needs to cover the entire field of view, should fluoresce under one of the available imaging wavelengths, and when exposed to the 'uncaging' laser, small holes should appear in the sample. A good sample would be to take a clean slide, then using a red 'Sharpie' marker, color in a solid area of it. Sometimes it helps to first epoxy a small o-ring to the slide so that if you are calibrating with a 'dipping' lens, the water will not run off of the slide.

Place the slide on the microscope and adjust your parameters in Prairie View so that you can see an image of the 'marker' slide (the specifics of this step are dependent upon the reference image source).

The first thing that needs to be determined is how far the uncaging galvos may be moved and still remains in the field of view. To do this, start by making sure that the uncaging galvos are parked at the 0.00 voltage value for both X and Y. Open the 'Mark Points' dialog and look at the displayed values for 'Abs. X' and 'Abs. Y'. The values displayed for 'Abs. X' and 'Abs. Y' is in volts. The value entered for '# steps' is in millivolts. The following image shows the locations of the relevant controls and displays.





If the values for 'Abs. X' is not 0.00, then enter the same value as 'Abs. X' in the '# steps' field by converting it to millivolts. For example, if the value of 'Abs. X' is 1.23, then enter 1230 in '# steps'. Now press either the 'left' or 'right' 'blue' button until the value displayed in 'Abs. X' is 0.00.

If the values for 'Abs. Y' is not 0.00, then enter the same value as 'Abs. Y' in the '# steps' field by converting it to millivolts. For example, if the value of 'Abs. Y' is 0.64, then enter 640 in '# steps'. Now press either the 'up' or 'down' 'blue' button until the value displayed in 'Abs. X' is 0.00.

Increase the value for the 'Laser Intensity' and then briefly open/close the 'Shutter'. Acquire a new image and see if there is a 'hole' burned into the sample. If there is no hole, increase the laser power and/or keep the shutter open longer and repeat the process until a 'hole' is burned into the sample.

Set the value for '# steps' to 1000 and press the 'left blue' button. Open/close the shutter again to burn a hole at this new location. Acquire a new image and see where the new hole appears. Press the 'up blue' button. Open/close the shutter again to burn a hole at this new location. Acquire a new image and see where the new hole appears. If everything is properly set up, then the second hole should've shown up just to the left of the first hole and the third hole should've shown up just above the second hole. If this is what happened, then we can continue. If either or both the second and third holes did not appear where expected, contact Prairie Technologies. Explain the situation and someone will help correct the software settings. After the settings are corrected, the calibration procedure may continue.

Click on the 'down blue' button and then press the 'left blue' button a couple of times. Acquire a new image. Is the 'hole' getting close to the left edge of the field of view. Keep moving it and burning a new hole to get it near the edge (but not right on the edge, I'd keep about 5% to 10% of the image width as a buffer). Look at the 'Abs. X' value, this will tell you the number of volts available for the galvos based upon the width of the field of view of the reference image. Remember this value as 'field of view X'.

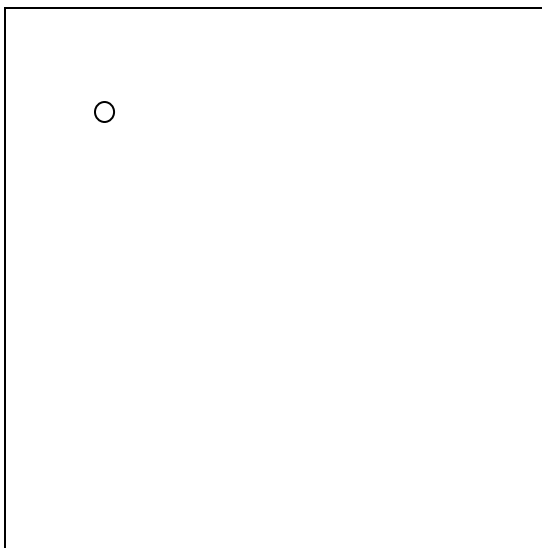
Press the 'up blue' button a couple of times. Acquire a new image. Is the 'hole' getting close to the top edge of the field of view. Keep moving it and burning a new hole to get it near the edge (but not right on the edge, I'd keep about 5% to 10% of the image width as a buffer). Look at the 'Abs. Y' value, this will tell you the number of volts available for the galvos based upon the height of the field of view of the reference image. Remember this value as 'field of view Y'.

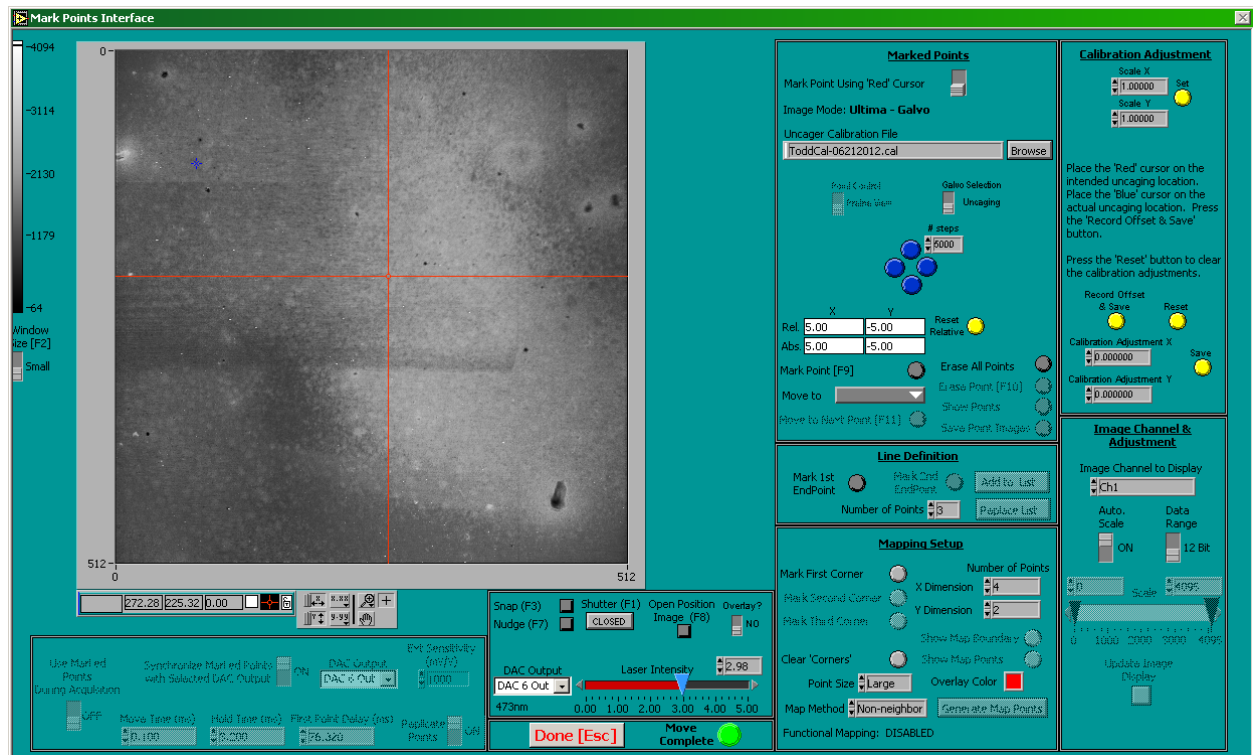
Move the sample to a clean field of view that doesn't include any of the former 'holes'.

Using the 'blue' buttons, move the galvos until the 'Abs. X' and 'Abs.Y' values are 0.00.

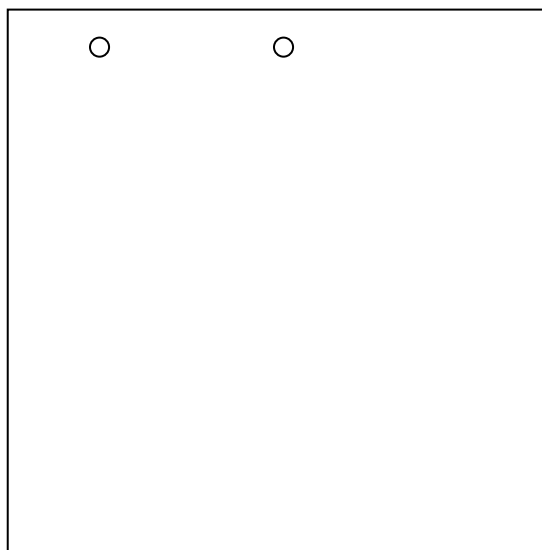
To perform a calibration using the 'Burn' or 'Hole' pattern it is best to make the 'holes' at locations (i.e. voltages values for 'Abs. X' and 'Abs. Y') that are easily reproduced. A typical calibration using this method might be performed using a 3x3 grid of holes. Are the values for 'field of view X' and 'field of view Y' the same? If not, choose the smaller of the two values. Enter this value for '# steps' and be sure to convert it from volts to millivolts (e.g. 'field of view X = 'field of view Y' = 5.00, so enter 5000 for '# steps').

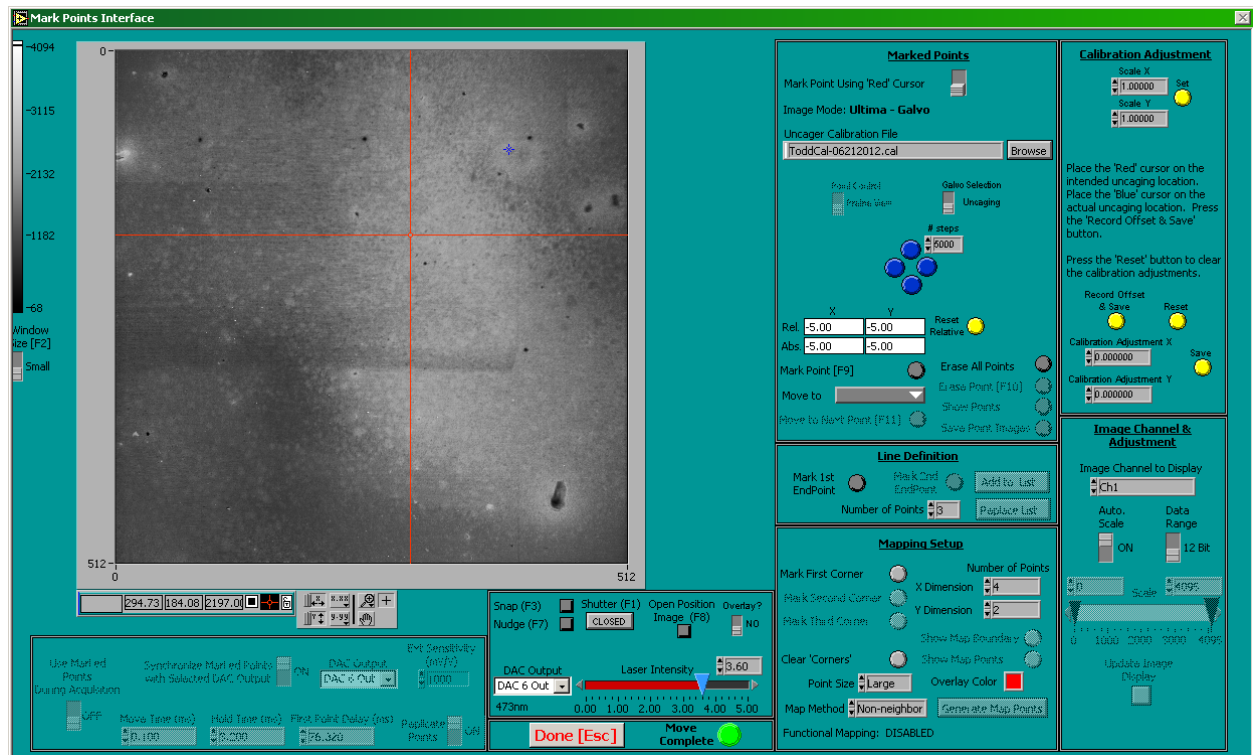
Press the 'left blue' button once and the 'up blue' button once. This should place the galvos on the index of the upper left hand corner point. Record the 'Abs. X' and 'Abs. Y' values for this location as the 'upper left hand corner'. Burn a 'hole' at this location. Acquire a new image and confirm that a 'hole' is present in the desired location. The following illustration provides an example.



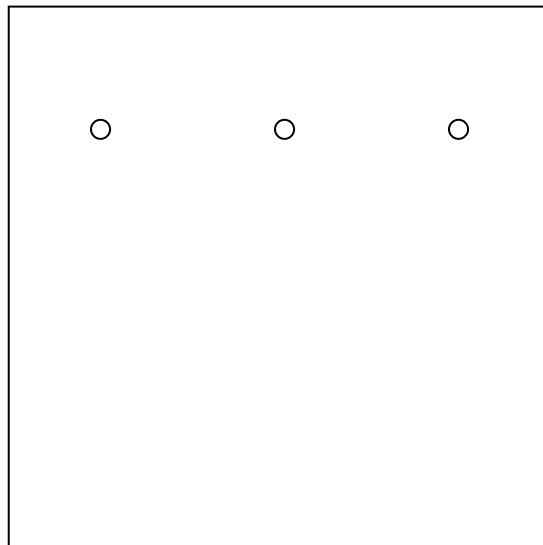


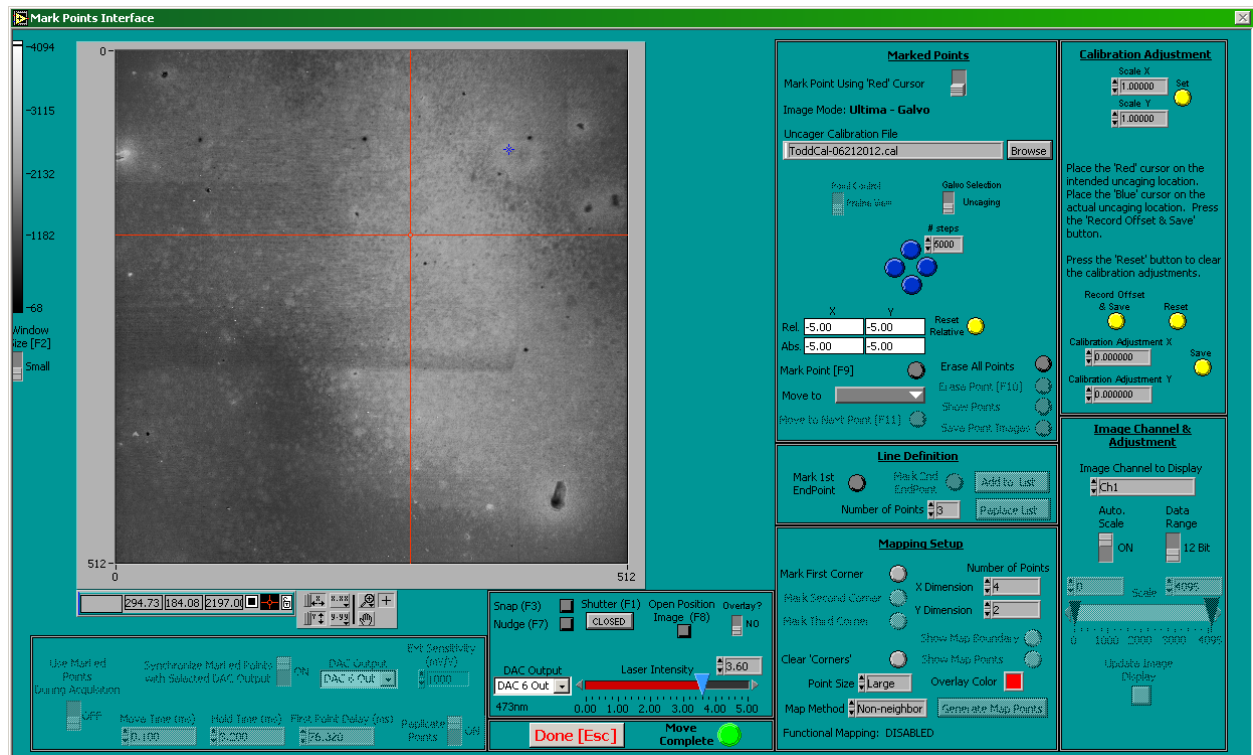
Press the 'right blue' button once. Burn a 'hole' at this location. Acquire a new image and confirm that a 'hole' is present in the desired location. The following illustration provides an example.



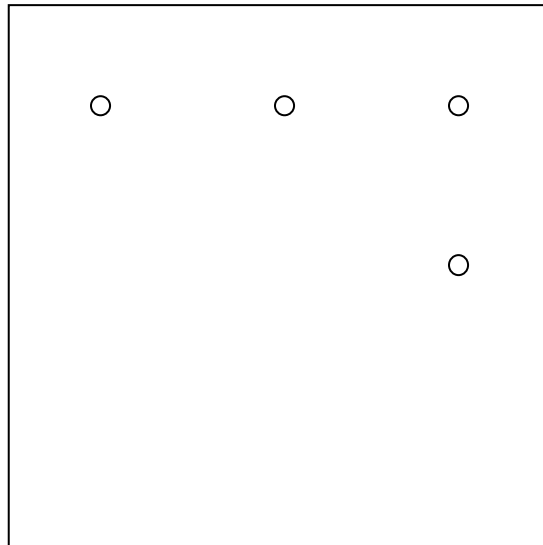


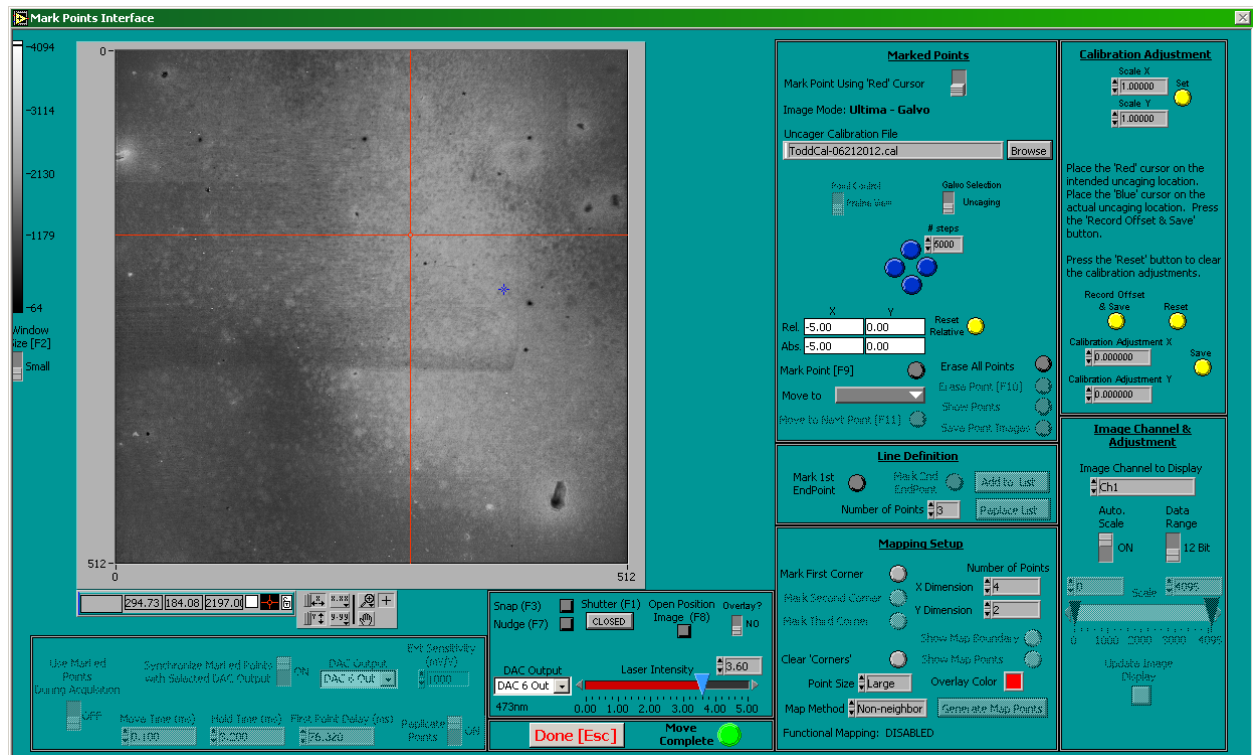
Press the 'right blue' button once. Record the 'Abs. X' and 'Abs. Y' values for this location as the 'upper right hand corner'. Burn a 'hole' at this location. Acquire a new image and confirm that a 'hole' is present in the desired location. The following illustration provides an example.



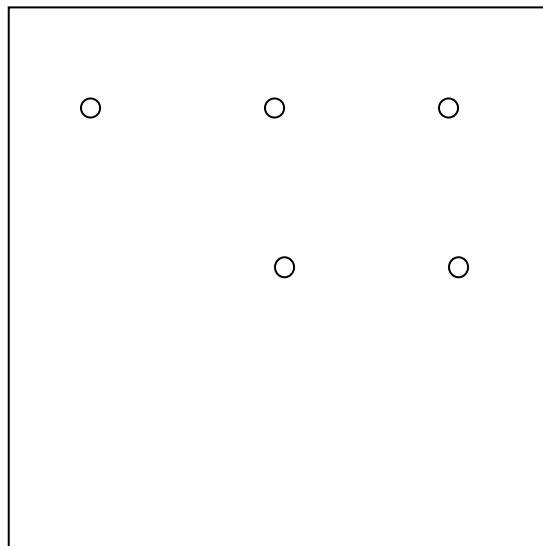


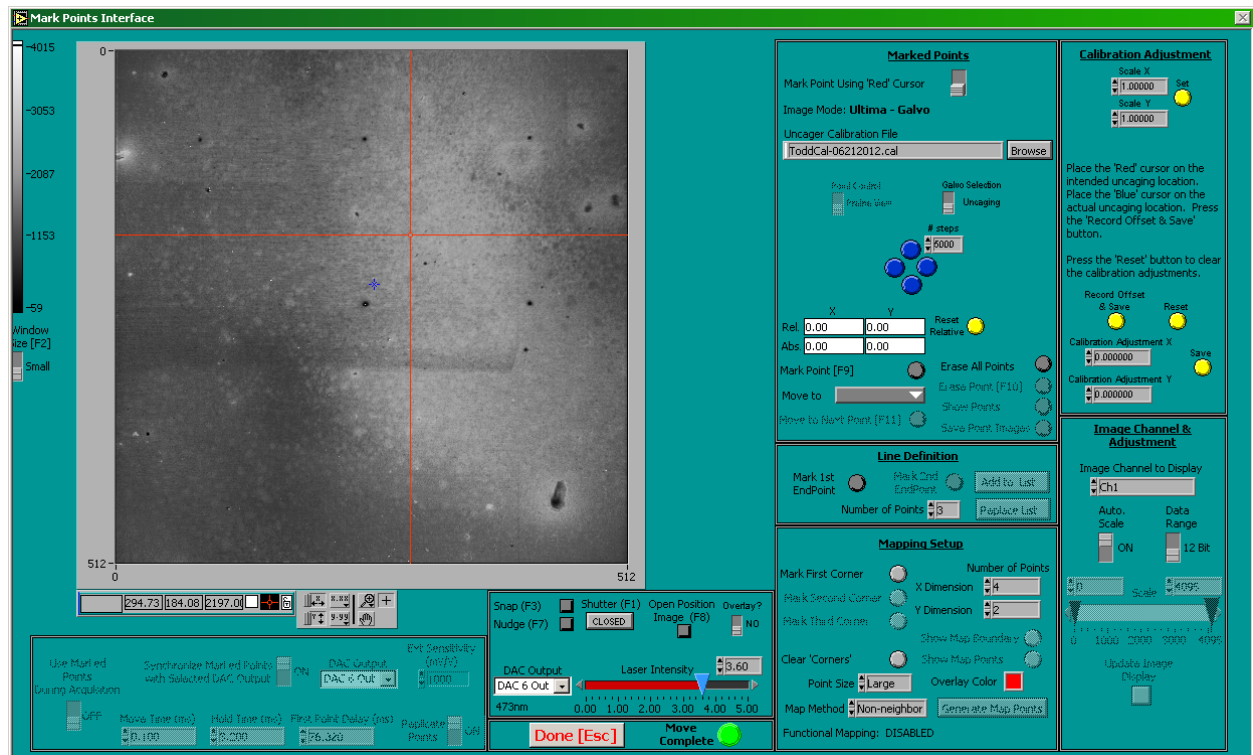
Press the 'down blue' button once. Burn a 'hole' at this location. Acquire a new image and confirm that a 'hole' is present in the desired location. The following illustration provides an example.



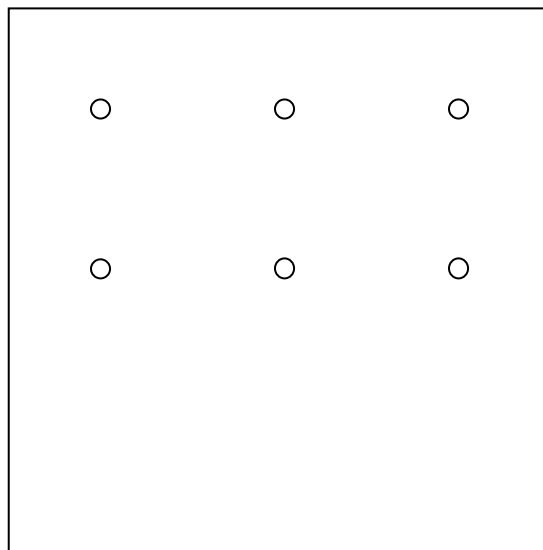


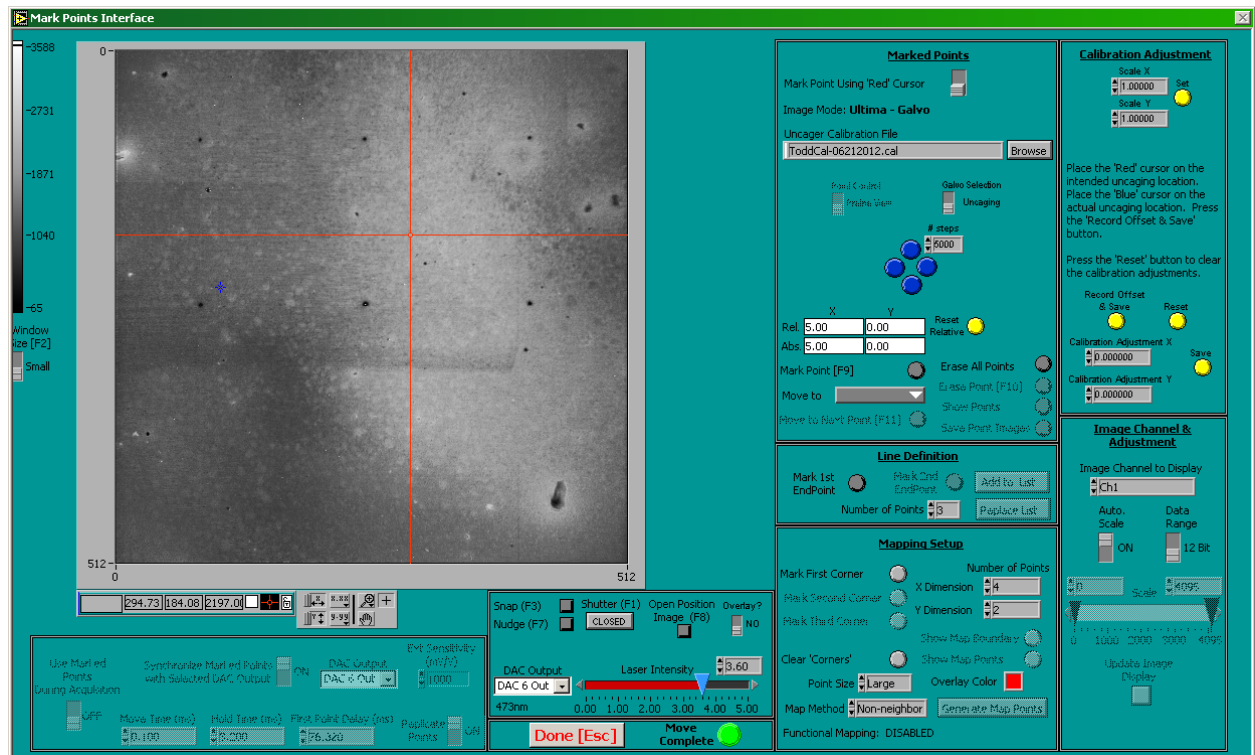
Press the 'left blue' button once. Burn a 'hole' at this location. Acquire a new image and confirm that a 'hole' is present in the desired location. The following illustration provides an example.



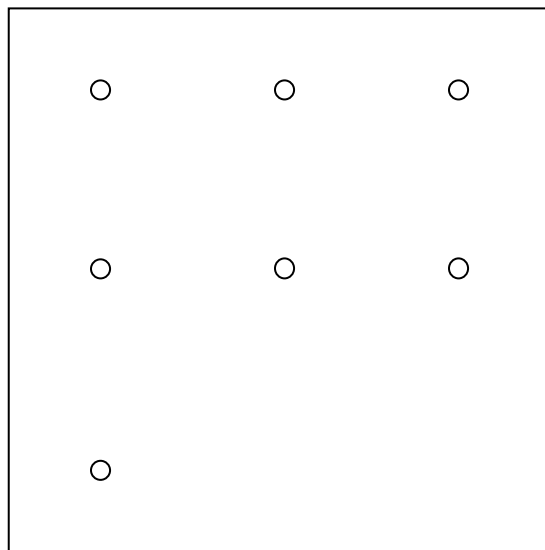


Press the 'left blue' button once. Burn a 'hole' at this location. Acquire a new image and confirm that a 'hole' is present in the desired location. The following illustration provides an example.

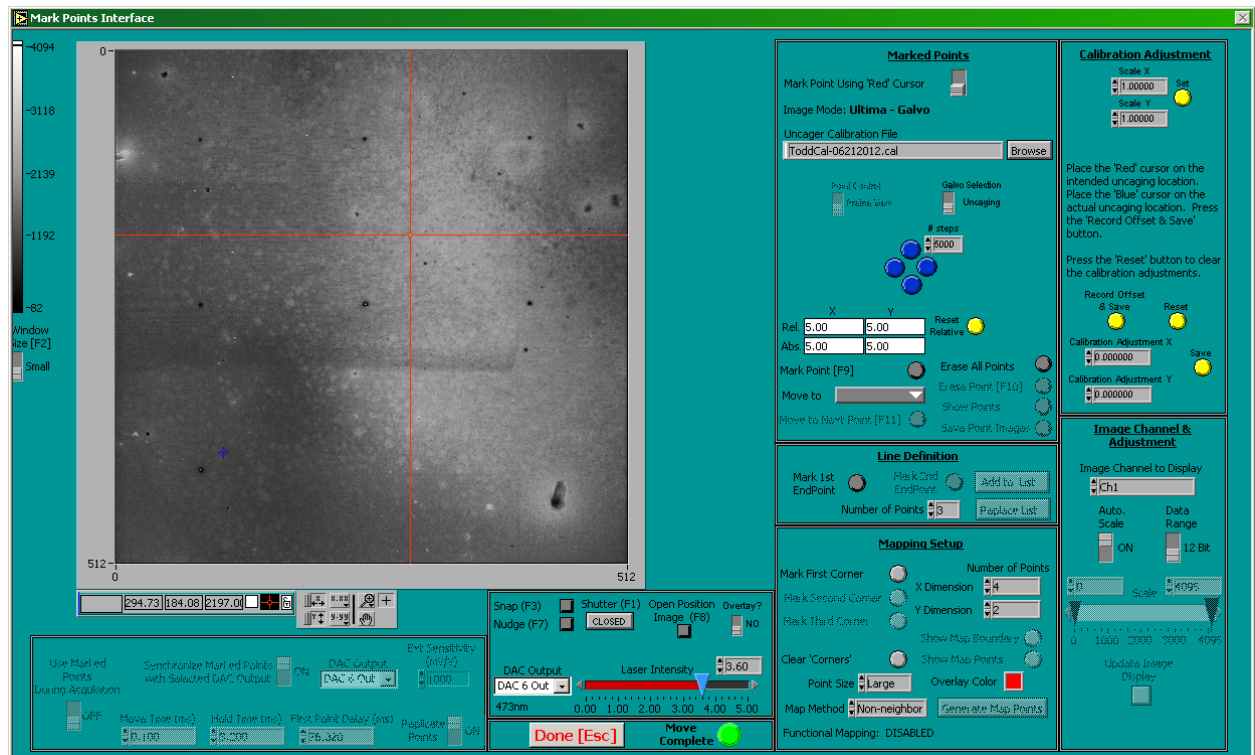




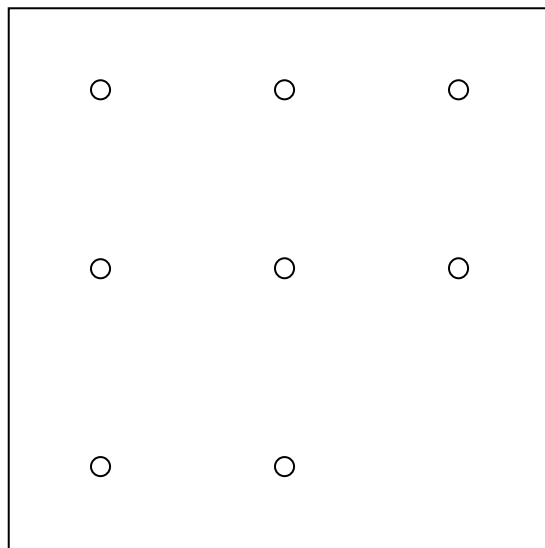
Press the 'down blue' button once. Burn a 'hole' at this location. Acquire a new image and confirm that a 'hole' is present in the desired location. The following illustration provides an example.

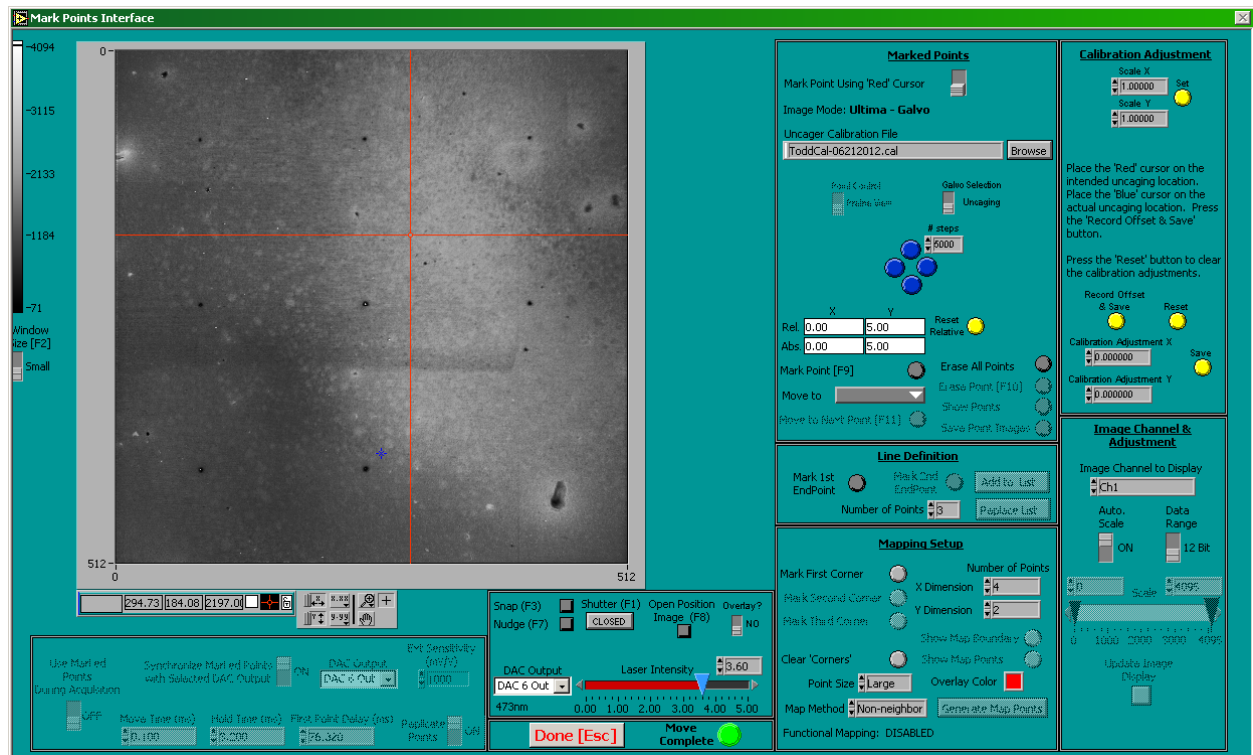




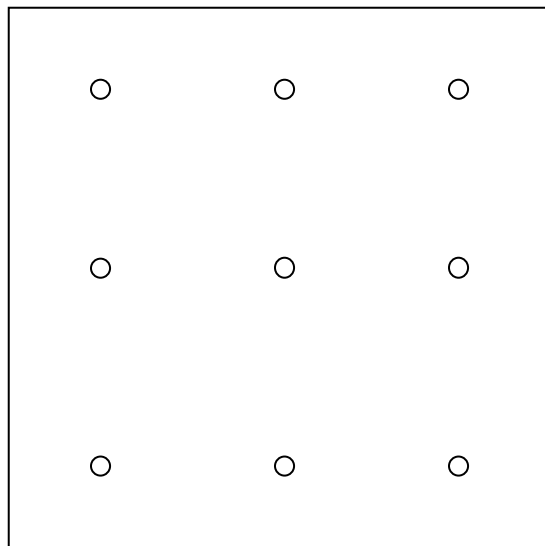


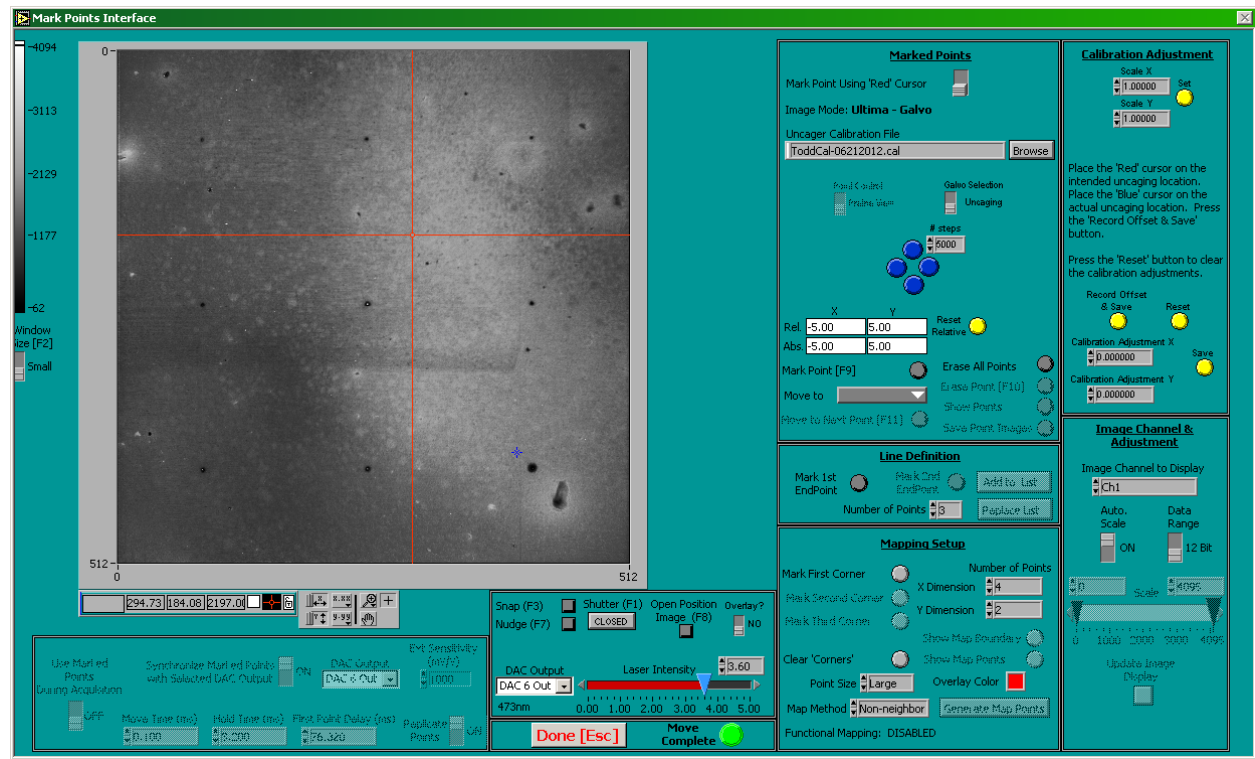
Press the 'right blue' button once. Burn a 'hole' at this location. Acquire a new image and confirm that a 'hole' is present in the desired location. The following illustration provides an example.





Press the 'right blue' button once. Record the 'Abs. X' and 'Abs. Y' values for this location as the 'lower right hand corner'. Burn a 'hole' at this location. Acquire a new image and confirm that a 'hole' is present in the desired location. The following illustration provides an example.





In practice it doesn't matter what order that these 'holes' are made on the sample. What is important is recording the values for the 'Abs. X' and 'Abs. Y' values for the 'upper left hand corner', 'upper right hand corner' and 'lower right hand corner'.


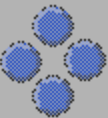
Click on the TriggerSync menu option 'Points -> Calibrate Points...'. The 'Mark Points' dialog will be automatically closed if it is open, and the 'Uncaging Point Calibration' dialog will appear.

**Pinhole Photolysis Calibration**

## Uncaging Point Calibration

**Step 1: Specify Calibration File Name**

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**Move Complete**



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**Step 2.1: Calibration Method**

**Step 2.2:** Acquire an image of the "spot" to calibrate. Using the mouse, left click on the "spot" in the 'Original Image' window. Adjust the 'Tolerance' and/or 'Connectivity' value to create a single continuous image of the "spot" in the 'Processed Image' window.

This factor is applied to the "spot" size to indicate the range of the "spot" area to use when filtering out false spots (0.0 - 1000.0):

Number of Frames to Average:

**Step 3:** Using the 'Blue' buttons above, move the "spot" to the upper left hand corner of the image. Do not have the "spot" touch or extend beyond the edge of the image.

**Step 4:** Using the 'Blue' buttons above, move the "spot" to the upper right hand corner of the image. Do not have the "spot" touch or extend beyond the edge of the image.

**Step 5:** Using the 'Blue' buttons above, move the "spot" to the lower right hand corner of the image. Do not have the "spot" touch or extend beyond the edge of the image.

**Step 6: Perform the Calibration.**

Number of Points:

Acquiring Point:  of  Elapsed Time:  s

For 'Step 1' click on the 'Browse' button and navigate to 'C:\Program Files\Prairie\Spot Calibration' and enter a calibration file name (e.g. 'UncageGalvos-06142012'). Click 'OK' button to advance to step 2.

For 'Step 2' select the 'Manual' option and click on the 'OK' button to advance to step 3.

For 'Step 3', leave the value for '# steps' at the value used when the 'holes' were being made in the sample. Select the proper channel that the 'hole' image is being acquired in within Prairie View with the 'Image Channel to Display' control. The following image shows the location of these controls.

**Pinhole Photolysis Calibration**

## Uncaging Point Calibration

**Step 1: Specify Calibration File Name**

C:\Program Files\Prairie\SpotCalibration\UncageGalvos-06142012.cal

Browse OK

**Move Complete**

# steps 1000

Acquire (F8) Shutter (F1) OPEN

Tolerance 20

DAC Output DAC 6 Out Pockels

Connectivity 0.34

Laser Intensity 0.00 0.50 1.00 1.60

Image Channel to Display Ch1

**Step 2.1: Calibration Method** Manual OK Back

**Step 2.2:** Acquire an image of the "spot" to calibrate. Using the mouse, left click on the "spot" in the 'Original Image' window. Adjust the 'Tolerance' and/or 'Connectivity' value to create a single continuous image of the "spot" in the 'Processed Image' window.

This factor is applied to the "spot" size to indicate the range of the "spot" area to use when filtering out false spots (0.0 - 1000.0): 5.00 OK Back

Number of Frames to Average: 1

**Step 3:** Using the 'Blue' buttons above, move the "spot" to the upper left hand corner of the image. Do not have the "spot" touch or extend beyond the edge of the image. OK Back

**Step 4:** Using the 'Blue' buttons above, move the "spot" to the upper right hand corner of the image. Do not have the "spot" touch or extend beyond the edge of the image. OK Back

**Step 5:** Using the 'Blue' buttons above, move the "spot" to the lower right hand corner of the image. Do not have the "spot" touch or extend beyond the edge of the image. OK Back

**Step 6: Perform the Calibration.**

Number of Points: X Y

Calibrate

Acquiring Point 0 of 0 Elapsed Time 0 s

**Done [Esc]**

Click the 'Acquire' button to collect a 'spot' image. The image will be displayed in a window labeled 'Upper Left Hand Corner Image'.

You'll be using the four 'blue' round buttons located next to the '# steps' field to physically move the galvos around the field of view. To continue with step three, use the four blue buttons to move the galvo from its current location to the exact same voltage for 'Abs. X' and 'Abs. Y' that you recorded for the 'upper left hand corner' during the process of burning the 'holes'. Since the 'Mark Points' dialog cannot be open while the calibration dialog is open, look at the 'Abs. X' and 'Abs. Y' located in the main TriggerSync window along the right hand side just below the 'blue' buttons.

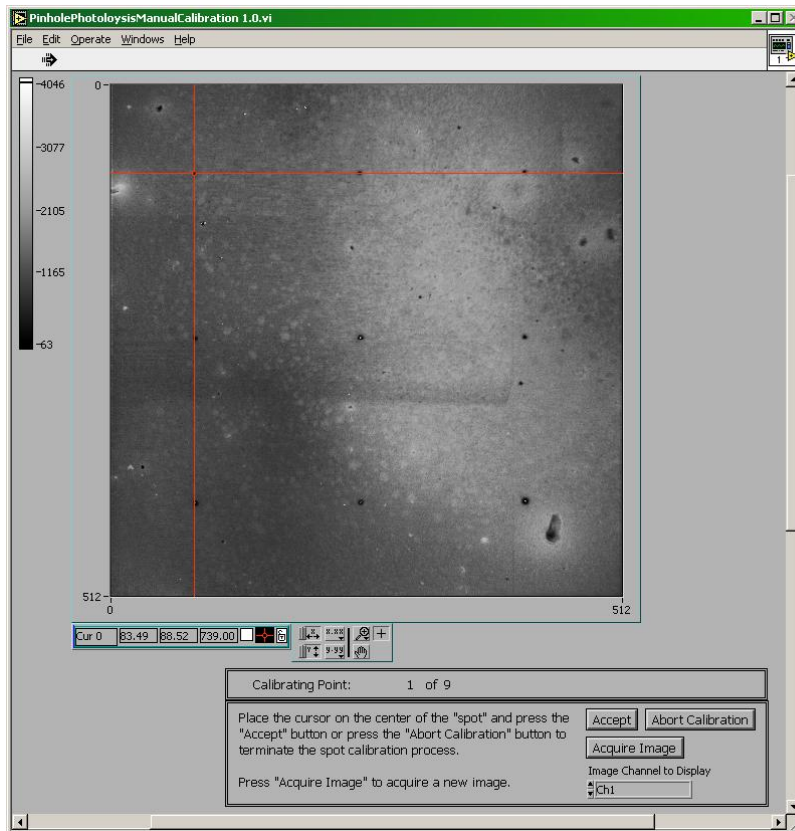
Once you have the 'Abs. X' and 'Abs. Y' values matching those for the 'upper left hand corner', click on the 'OK' button.

For 'Step 4', use the 'blue' buttons (now only the two horizontally opposed buttons will be active) to move the galvos to the exact same voltage for 'Abs. X' and 'Abs. Y' that were recorded for the 'upper right hand corner' during the process of burning the 'holes'. Once you have the 'Abs. X' and 'Abs. Y' values matching those for the 'upper right hand corner', click on the 'OK' button.

For 'Step 5', use the 'blue' buttons (now only the two vertically opposed buttons will be active) to move the galvos to the exact same voltage for 'Abs. X' and 'Abs. Y' that were recorded for the 'lower right hand corner' during the process of burning the 'holes'. Once you have the 'Abs. X' and 'Abs. Y' values matching those for the 'lower right hand corner', click on the 'OK' button.

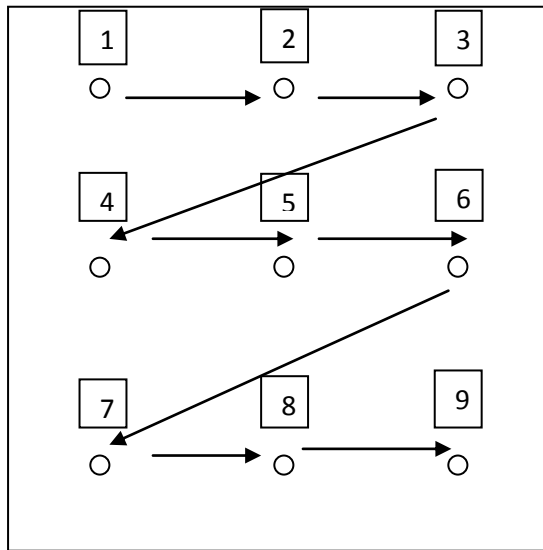
For 'Step 6' specify the number of points for the calibration pattern in the X and Y dimensions. These values should match the number of 'holes' made in each dimension (e.g. 3 for x and 3 for y).

When the calibration process is started, the galvos will be positioned to the first point in the upper left hand corner and an image will be automatically acquired. A new dialog will be displayed labeled 'PinholePhotolysisManualCalibration 1.0.vi'. There is a text display in this dialog that states 'Calibrating Point: 1 of 9'. This will help keep you informed of how many points are left in the calibration process. Move the 'red' cursor so that it is centered on the 'hole' in the upper left hand corner of the image. Even though there is no 'spot' image in the following illustration, assume that the cursor is positioned over the 'spot' image.

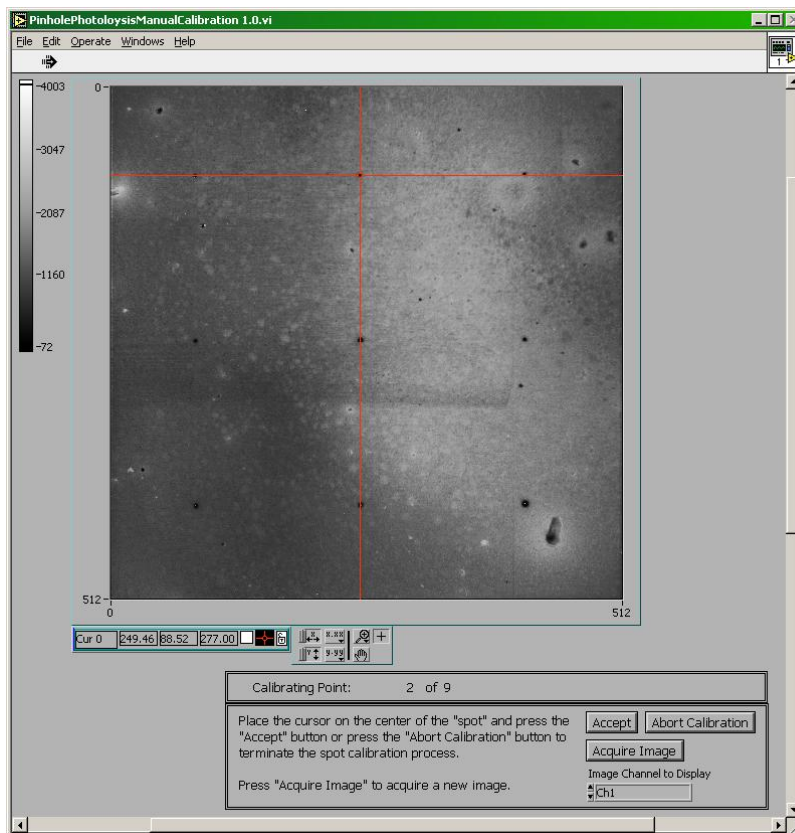


When the cursor is properly positioned, press the 'Accept' button. The galvos will be automatically positioned to the next location in the calibration order and the same dialog will appear. Notice that the text display now reads 'Calibrating Point: 2 of 9'.

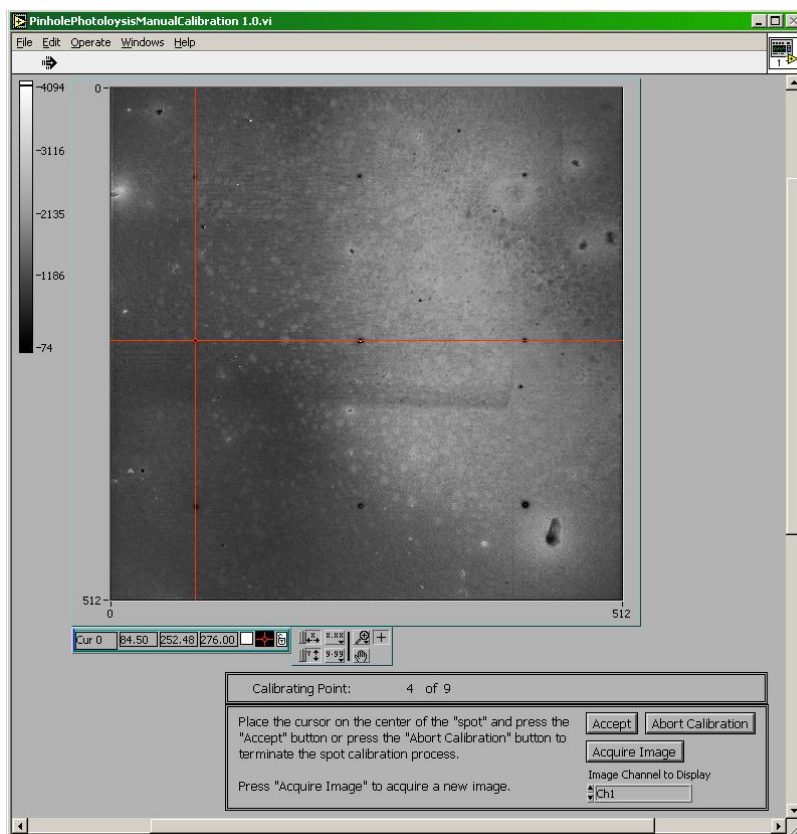
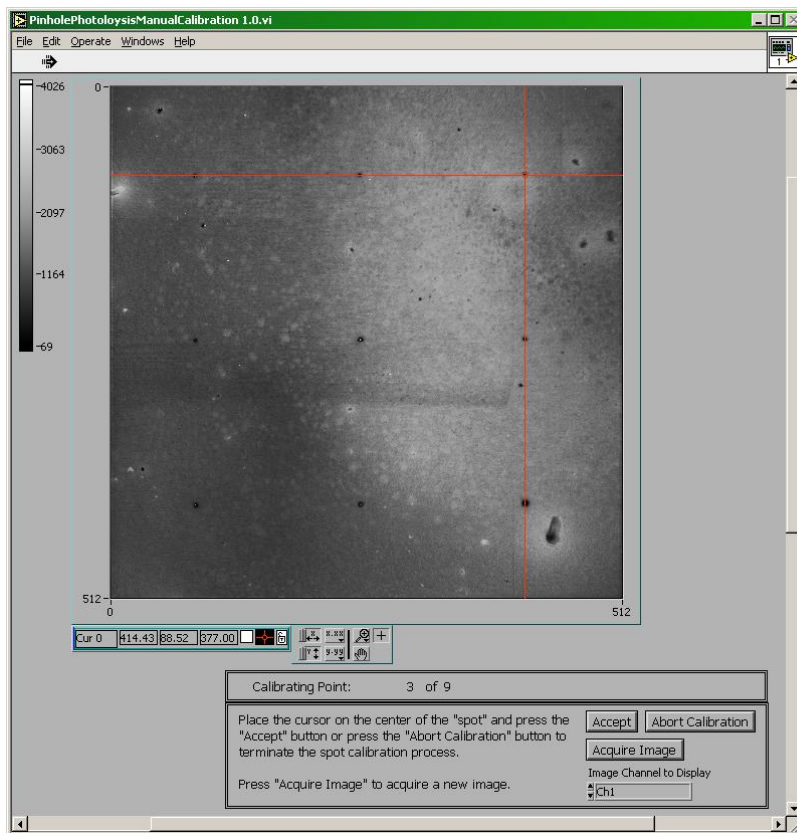
As the calibration process iterates through each of the 'points' all you need to do is move the 'red' cursor to be centered over the correct 'hole' in the image. The order that must be used is always the same, regardless of the number of 'holes'. Always start with the upper left hand corner, then with each successive point, move the red cursor to the next hole to the left in the same row. When the end of the row is reached, move down one row and start at the far left side. Then proceed to the right across that row until the end of the row is reached. When the end of the row is reached, move down one row and start at the far left side, and so on. The following graphic defines the point order.

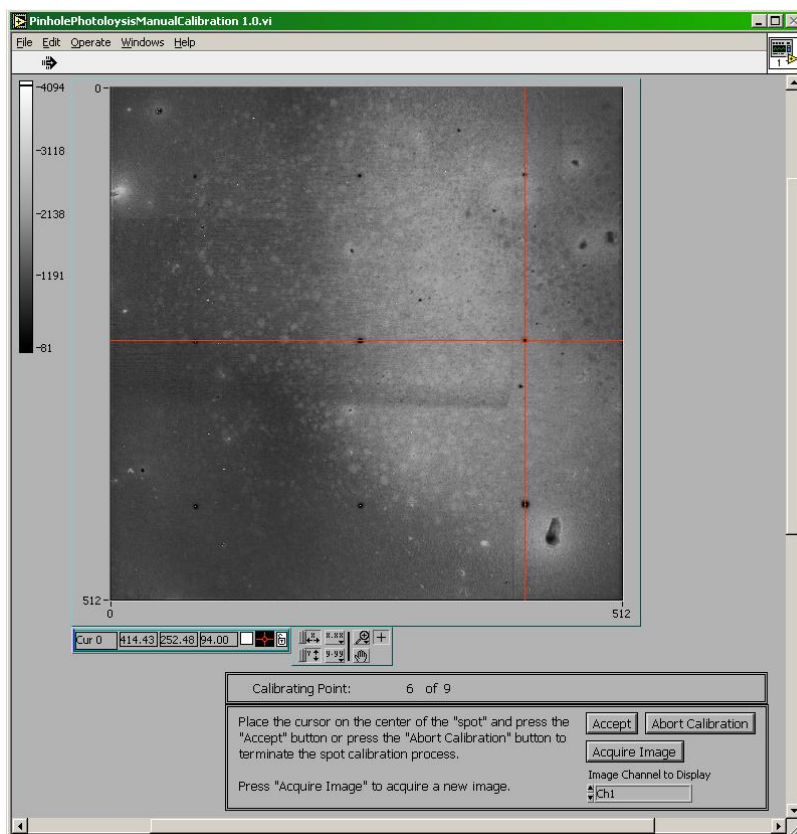
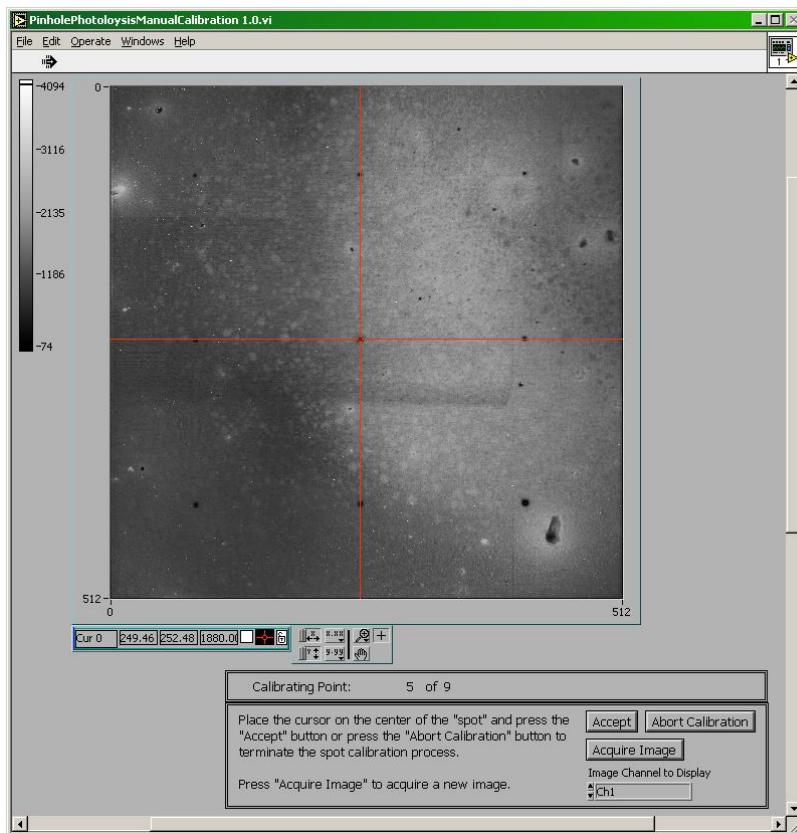


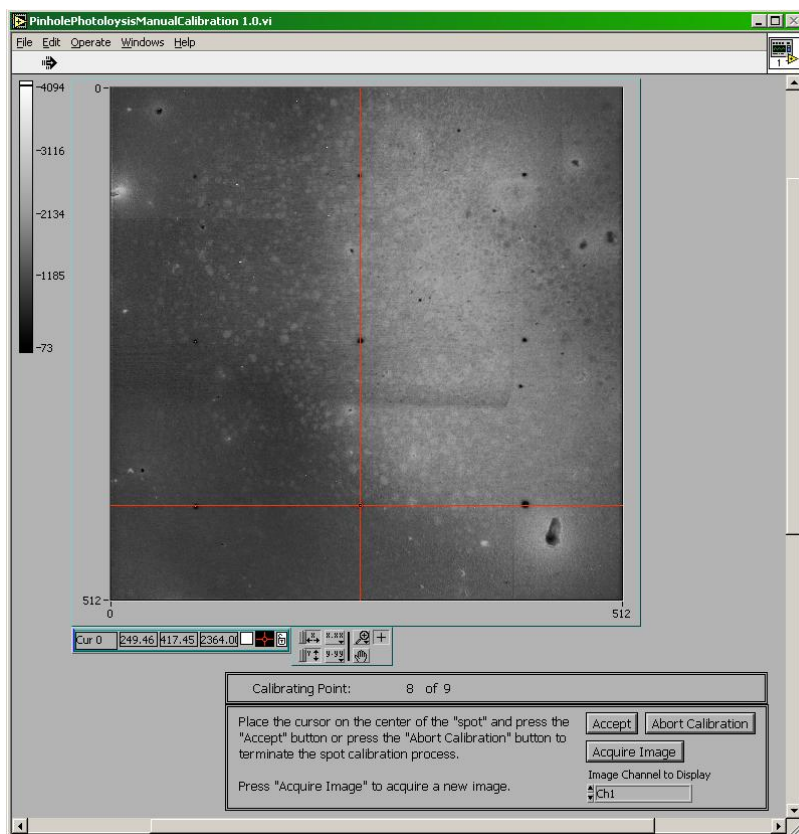
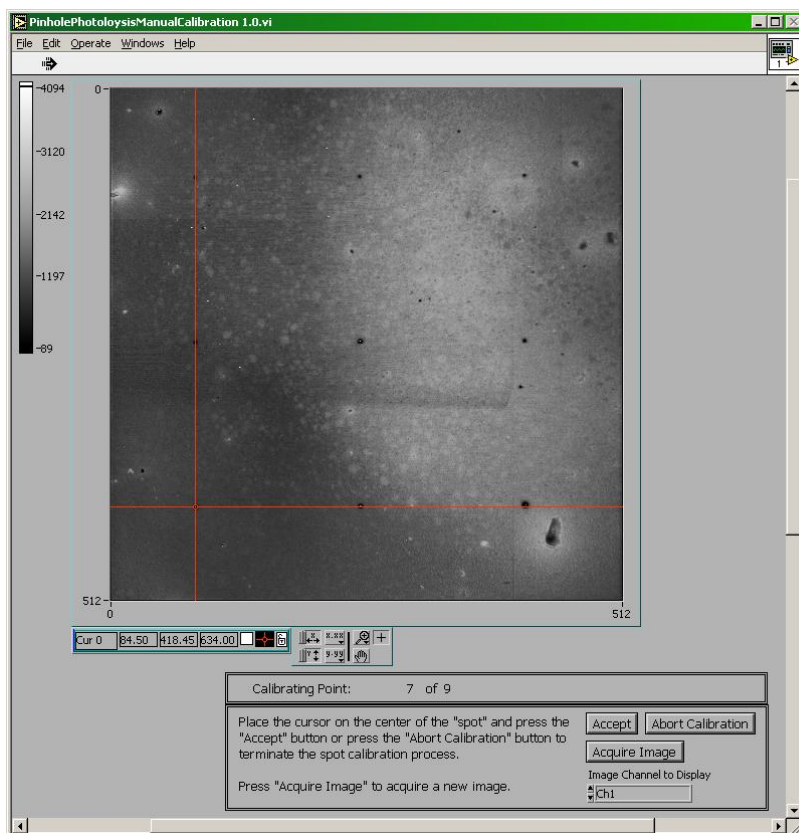
The following screenshots show the calibration window at points 2 through 9.

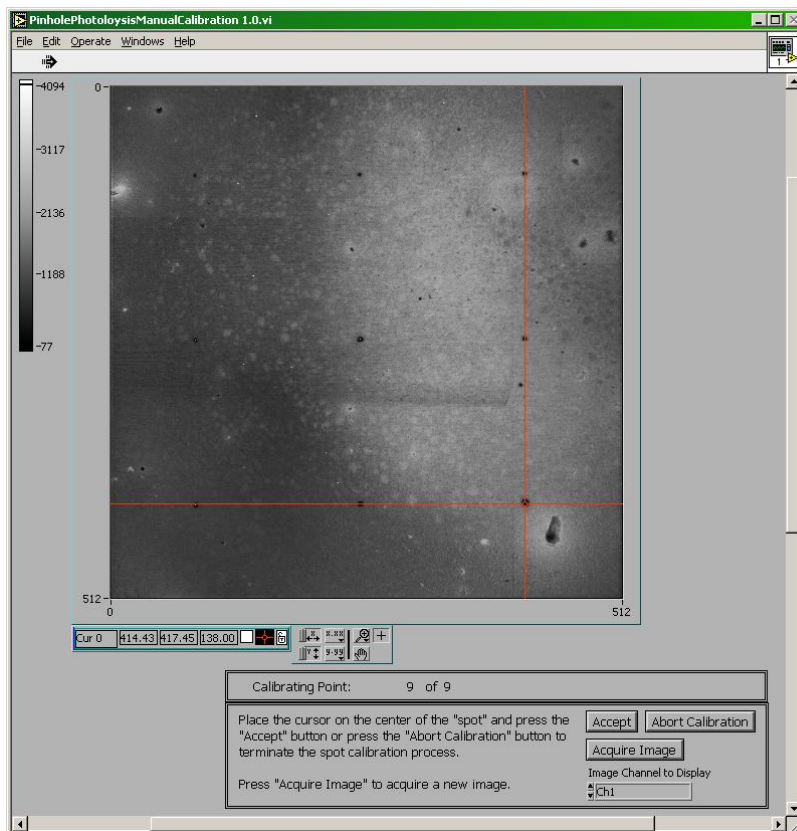










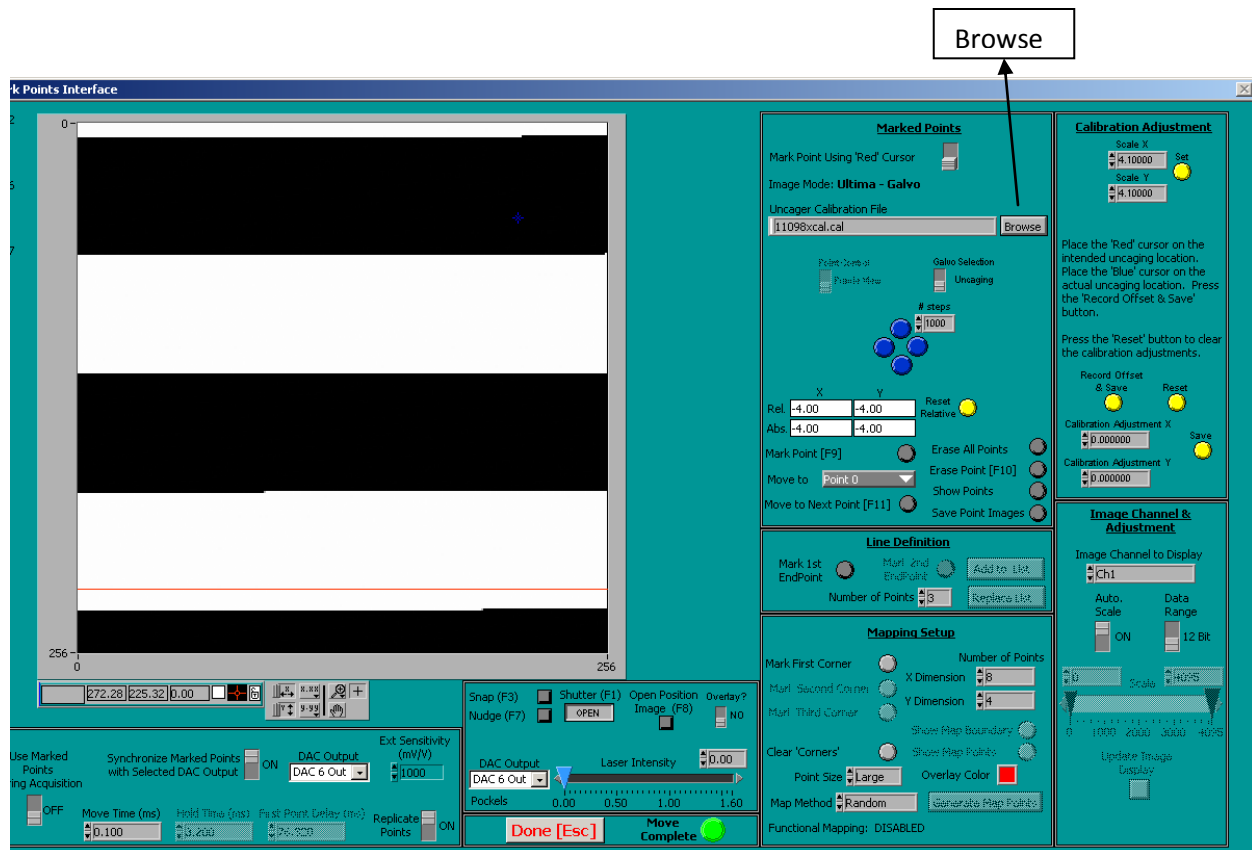


After the last point has been calibrated, the 'PinholePhotolysisManualCalibration 1.0.vi' dialog will be automatically closed.

Click on the 'Done' button at the bottom of the uncaging calibration dialog to close it.

### **Using the Point Calibration File**

Open the 'Mark Points' dialog. Click on the 'Browse' button as identified below.



This will bring up a dialog labeled 'Select a Calibration File'. Navigate to 'C:\Program Files\Prairie\Spot Calibration' and select the file that you just created by either double-clicking on the file or selecting it and pressing the 'Open' button. Some people have reported that the calibration file doesn't always load correctly the first time so it might be a good habit to simply repeat this process a second time to avoid any problems with the calibration information not actually loading.