Calibrating galvo and piezo

I realized that the calibration of the scanners using the oscilloscope, although accurate and comprehensive is not for everyone. The oscilloscope trigger is not easy to set unless you know how to operate it.

Therefore, I decided to take a different approach which does not require the oscilloscope or knowledge of electronics to calibrate the response of the galvos and piezo. The method is based on selecting a single bright isolated particle and then moving the scanner in all directions to prove the scales and the time response. I prepared a sample which contains embedded bright fluorescence beads in plastic for this purpose.

There are two parts of the calibration software that you need to be aware. One is used to determine the PSF along the 3 axis and the other is used to calibrate each scale.

Measurement of the 3D PSF

The PSF is whatever it is. This procedure is used to check the z-scanner factor used so that the pixels in the x-y-z directions have comparable sizes. The pixel size in the x-y directions is determined by the objective magnification and the angular motion of the galvos, while in the z-direction it is determined only by physical the motion of the piezo.

SimFCS stores a calibration factor for the x-y displacement. You must use the Ronchi ruler to calibrate this factor. The procedure requires that you enter the size of the image (as determined by the Ronchi ruler) obtained by setting the voltage at 8000mV using an objective 40x. Once this factor is calibrated, SimFCS will scale the size of the pixel when you change the objective and the zoom voltage. Typical values for our scanners are 72um/8000mV using 40x objective. Of course, this factor changes from instrument to instrument. This factor is stored in the parameter screen under the scanner page.

For the z-piezo, we use the calibration factor given by the factory. For example, for the Piezo in the Olympus FV1000, this factor is 25um/10000mV. You must enter this value in the parameter screen of SimFCS in the scanner page. For the PI piezo this factor is 100um/10000mV.

Example of calculation of the z-scanner factor

If we use a zoom of 10000mV, the pixel size in the z direction will be 25um/256=0.0977 um and in the x-y directions will be 72um*(10000/8000)/256=0.350um. To match the pixel size in the xy and z directions we should use a z-scanner factor of z-scanner factor=0.350um/0.0.977um=3.58. I generally round this number to 4. For this reason, the default value for the z-scanning factor in the Olympus FV1000 is set to 4. In other instruments could be very different.

Considerations about the PSF and the z-displacement

Note that the z-scanning factor acts on the electronics so that in 3D there is a similar pixel size in the 3 directions. However, the PSF is generally larger in the z-direction by at least a factor of 3 in the Olympus
(with pinhole of 1 Airy unit) and about 5 in the 2-photon excitation microscopes. So a spherical particle will always appear oblong in the 3D grid, although the relative position of particles in space is correct.

**Performing the PSF calibration**

Select a small, bright isolated particle in the plastic sample. Possibly use an air objective. If you are using a water objective, make sure that the sample is rigidly fixed with respect to the stage. If the particle is large, the PSF will appear larger than the diffraction limited spot expected, but the algorithm will work fine.

Set the parameter scanner page as follows. This example is for the Olympus microscope. For other system these values can vary.

![Parameter scanner page](image)

Go to the acquire image and select the objective magnification you are using. Set the zoom to 2000mV.

Go to the track page of SimFCS. Get an image. Point to the bead and go to the calibration page. Press PSF only and then check the start checkbox.
Calibration of the galvo and piezo time response

At high speed, the galvo and the piezo cannot follow the command voltage, but the response is reduced in amplitude and delayed in phase. To compensate for the delay and amplitude response, we calculate how much we have to “anticipate” the waveform so that when is sent to driver will elicit a response which is at the proper phase. Since the amplitude is also reduced, we amplify the amplitude so that the effective radius of the orbit is what it is expected.

At long scanning periods, the delay and the amplitude reductions are minimal. In my experience, only below a period of 32ms for the piezo and 2 ms for the galvo unit, there is a significant delay and attenuation. The calibration of the galvo and piezo should be done only once when the instrument is installed or something in the hardware is changed. It should not be done every day!

Note that the piezo driver could be damaged if operated at high frequency. The calibration for the piezo amplitude is only allowed for periods of 8196us and longer. Also the galvo drivers can be damaged for periods shorter than 1024 us. The computer will not automatically calibrate for period values that can damage to galvos.
The calibration algorithm assumes that a single bright isolated particle is at the center of the screen (or you can click on the particle to indicate where it is). Then the scanner is moved at position -16 for the x and the radius of the orbit is changed to 16. If there is no delay or the radius is not attenuated, the particle should appear with a phase=0 in the orbit and the “blip” caused by the particle should be as large as possible. If there is a delay, the particle will appear at a different position, as schematically shown in the figure below.

Since the position along the orbit can be calculated, the correction for the y-axis phase can be calculated. Then the radius of the orbit is increased. If the intensity decreases, then the particle was at the right distance, otherwise a maximum will be detected and this position is used to calculate the attenuation factor and the corresponding increase in amplitude needed to bring the orbit on the particle.

The same operation is repeated for the 3 axis. This algorithm calibrates the phase and amplitude along the 3 axes. It is automatic and it seems to work very well with small bright particles.

Finally, there is one more calibration to be done in regard to the phase of the change between the high and low orbit for 3D tracking. This calibration is needed because there could be a delay between the command to switch from high to low orbit and when this movement actually occurs. To determine this time, we consider the z-modulation. It should be maximal if the change in z occurs at the proper time as schematically shown below.
In this figure the green line is the x-waveform, the yellow line is the y-waveform and the blue line is the z-waveform. The z-waveform should change when the x-waveform is at the maximum (at 0 degrees along the orbit). However, due to the piezo delays, the switch could occur at a different angle. The algorithm scans every position of the z-waveform and monitors the corresponding value of the modZ variable. The angle that gives the maximum value of modZ is the angle that should be used. This is shown schematically in the figure below.

![Graph showing waveforms and angle determination]

The maximum of the ModZ value occurs at 324 degrees in this case.

When finished with the calibration, you can select to store the new calibration value or use the previous calibration. Remember that you need to set the checkmark in the system parameter screen if you want to permanently store the calibration. Please, do so only if the calibration is correct.