

Temperature Calibration

Acquire a thermometer and take the temperature from inside the mini-tweezers. In the host program have the window for the oscilloscopes open and scroll down to the A_Temp and B_Temp lines which show the temperature of traps A and B. Enable centering in the program and zoom in to enlarge the detail of the two lines. Their readings should be close to the thermometer's temperature, or else need adjusting. This can be done by opening the ItCalibration file and edit the temperature fudge factors.

Force Calibration

Here we present three ways to calibrate our force sensors, first by using a fluid-drag test force and second by using the known properties of light momentum. It is good to use both methods and compare results. Agreement confirms the proper operation of the momentum sensors and strongly suggests your calibration will be immune to changes in bead size, bead composition, buffer composition, laser power, and focal quality. Also the momentum method gives individual force calibration factors for the two traps A and B whereas the Stokes test only checks the combined force from 2 traps. However the light method does require extra equipment and effort. The last (3rd) method is to check the overstretching force of a known sequence of dsDNA in a buffer of known salt concentration.

Stokes' Law Calibration

First calibrate the temperature sensors (see above). Then fill the chamber with distilled water, which has a known viscosity versus temperature. The drag test force will be proportional to the fluid viscosity and the bead radius, according to Stokes' law. Zero the PSDs with an empty trap. Note the Iris/Sum ratios for both beams with an empty trap. Next get some uniform polystyrene beads around 2 – 3 μm in diameter. You will need to know the bead size by some independent means, such the manufacturer's data or a photograph of many beads from the distribution by using a calibrated electron microscope (TEM). This test will also work with silica beads but polystyrene comes in narrower size distributions. Next trap a bead in both beam foci. The beam deflections in the PSD window should deviate by equal and opposite amounts, indicating some beam mis-alignment. Now turn AUTOALIGN on and the beam deflections in the PSD window should travel together to become near zero. Note the new Iris/Sum ratios. If these ratios are higher than those noted earlier with an empty trap, then adjust the inter-focal distance by turning the worm gear clockwise (CW) on the Objective Slider Mount (use a 5/64" hex driver). If these ratios were lower than with an empty trap, then turn the worm gear CCW. Stop when the current average of the Iris/Sum readings agrees with the previous (empty) average within a 1% allowable error. Turn AUTOALIGN off. Then under menu bar "Tools" select "Stokes Test". On the new window, observe 3 spots that bounce up and down due to thermal forces: Xforce=green, Yforce=yellow, Zforce=blue. Select the button "ZeroForce" and the 3 spots will become evenly spaced. Select "Move Motors XYZ" and move the fluid chamber with the mouse or joystick. Adjust the Motor Speed slider to give maximum velocity with the mouse. Observe the spots as they form scatter patterns. Forces are plotted vertically and motor velocities are plotted horizontal. The slope of the plots represents the drag coefficient of the bead. Freeze the pattern with the *F* key. Rotate the pattern with the *Q* and *E* keys until the patterns appear most horizontal. The apparent bead size (from Stokes' law) is read in the lower left corner of the test window. If the bead size matches the nominal size from the bead manufacturer, then all is well. If all is not well, consider that the calibration factors, namely trapAForceSensitivity[x,y,z] and trapBForceSensitivity[x,y,z], may need to be changed.

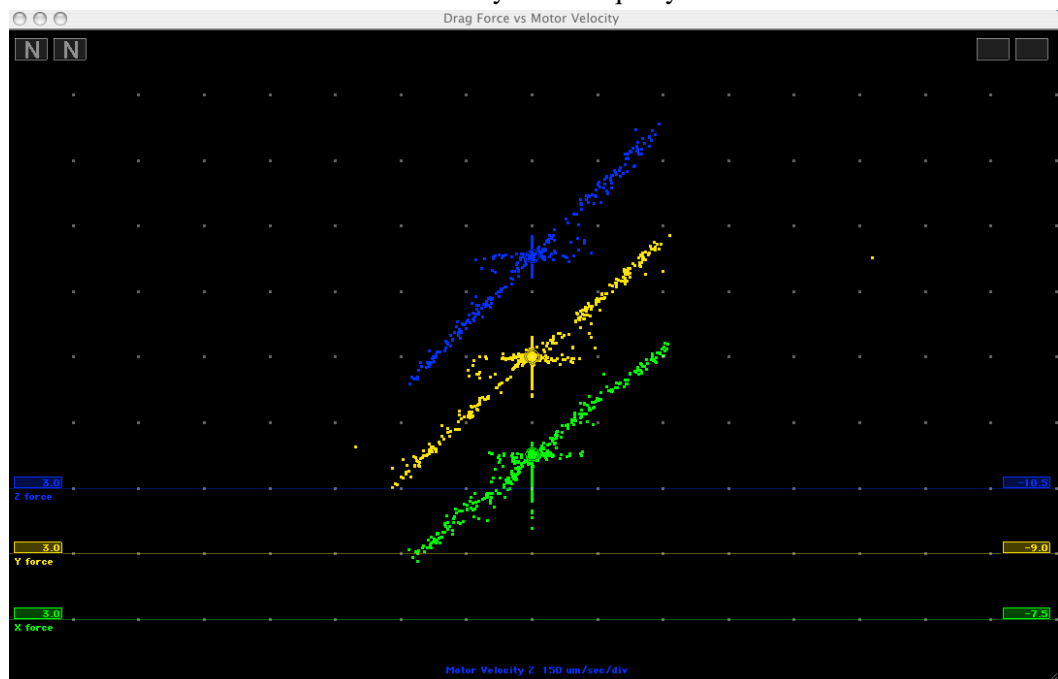
Cautions: The inter-focal distance, as set by the iris/sum method, will affect the X,Y calibration factors slightly (~10%) but the Z factor will change more.

The temperature sensors must be calibrated (see above) since the water viscosity used in the Stokes diameter calculation is corrected for ambient temperature.

The buffer viscosity must be close to water. PEG or Glycerol increase the viscosity of buffers greatly. Bead sizes vary within a batch so try several beads and average their apparent sizes before accepting or changing a calibration factor.

Dirt or long DNA hanging off a bead will increase its drag.

Note that the total force used in Stokes' law is computed from 2 detector signals (A and B) multiplied by 2 sensitivities: trapAforceSensitivity and trapBForceSensitivity. These factors can be assumed to be the same only as long as the optics and detectors are identical. Typical variations in trap sensitivities are on the order of +/-10%, but unfortunately the Stokes test does not tell us anything about differences between the two detectors because the force load is not necessarily shared equally between the 2 beams.



Stoke Test Window shows 3 axes of force versus velocity.

Light Momentum Calibration

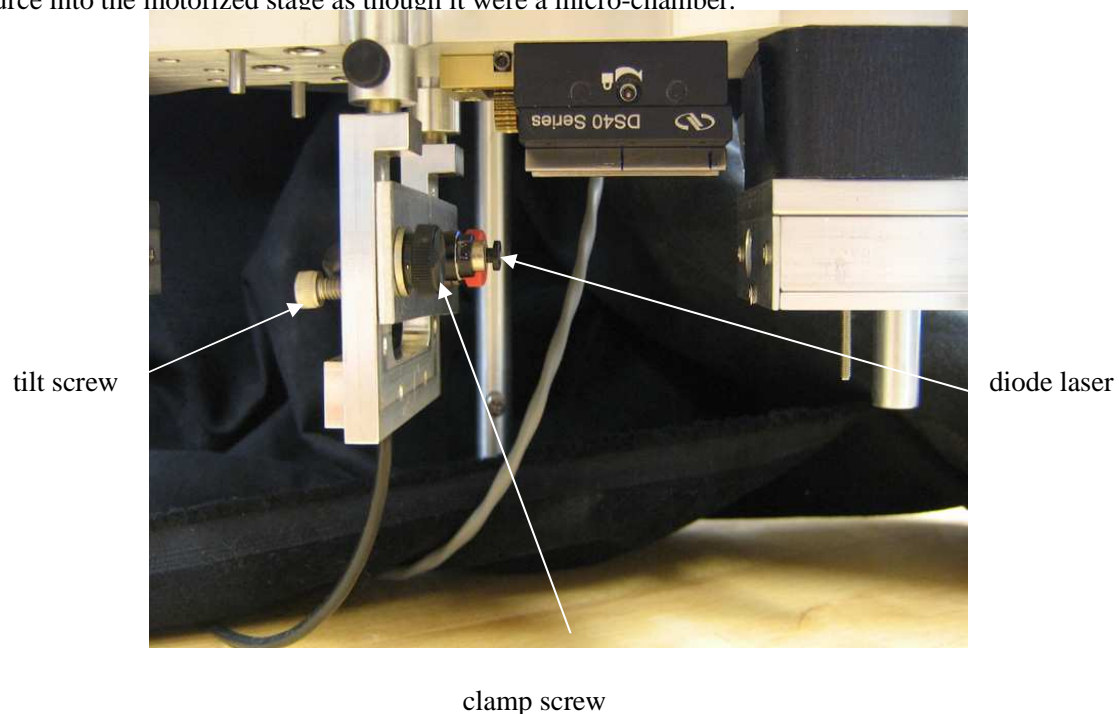
Changes in light momentum flux are detected in the Force Sensor Assembly, which comprises two silicon photodiodes, the PSD detector and the IRIS detector. The dimensions and power sensitivity of these detectors can be used to directly compute their force calibration factors. See *Smith, Cui, Bustamante Method in Enzymology 361 (2002)* and US Patent 7,133,132 for an explanation of Light Momentum Flux force sensors.

The **Raster Test** measures characteristics of both photo diodes as follows:

- (1) Test variation of PSD light sensitivity over its surface area. Detects interference fringes in the optics leading to detectors.
- (2) Measure dimensions of PSD and magnification of relay lens system
- (3) Test transmission profile of Bullseye attenuator
- (4) Check coincidence of Bullseye center and PSD detector center

The raster test requires an auxiliary diode laser and special mount called the *Raster-Test Light Source*.

First, remove both objective lenses and their mounting brackets and set them aside. Before starting the test, explore the X-Y motor limits by moving the stage with the mouse and observing the red-LED limit indicators on the *Motor-Control card*. Position the X and Y motors in their *Motor Clamps* such that they still barely (lightly) touch the stage when their lead screws are retracted to the limit. Next advance the screws until the X and Y motors are near the centers of their ranges. Then insert the Raster-Test Light Source into the motorized stage as though it were a micro-chamber.



The Raster Tester Light Source includes a diode laser which is collimated and masked to give a narrow pencil of light which enters the Prism Box input aperture. To align the beam, first point the laser toward the same side as the CCD camera and adjust the angle of the beam with 8-32 Tilt Screws inserted in the

Chamber Holder Frame until a spot of light appears near the center of the CCD camera. Next, adjust the Raster Test Light Source beam offset position (not the tilt angle) by moving the Chamber Holder Frame up/down in the motorized stage Chamber Legs and by adjusting the clamp screws on the Raster Tester Holster. Use the Infrared Indicator Card to put the beam near the center of the prism-box aperture. Alternately, it is more accurate to insert an [Aperture Iris Button](#) into the aperture and read the PSD Sum in order to place the beam exactly on the center of the prism box aperture. However be sure to remove the aperture iris button before starting the raster test.

Code for controlling the miniTweezers instrument during the raster test scan is shown in the Software section under “Raster Test”. Basically, it causes the motorized stage to move in an X-Y raster pattern covering a 6 mm x 6 mm area. Since that beam passes through the plane which would be at the Back Focal Plane (BFP) of the objective lens, and since we know the X-Y coordinates of that beam at the BFP from the stage motor encoders, we can map the position response of the PSD detector versus the position of any ray in the BFP.

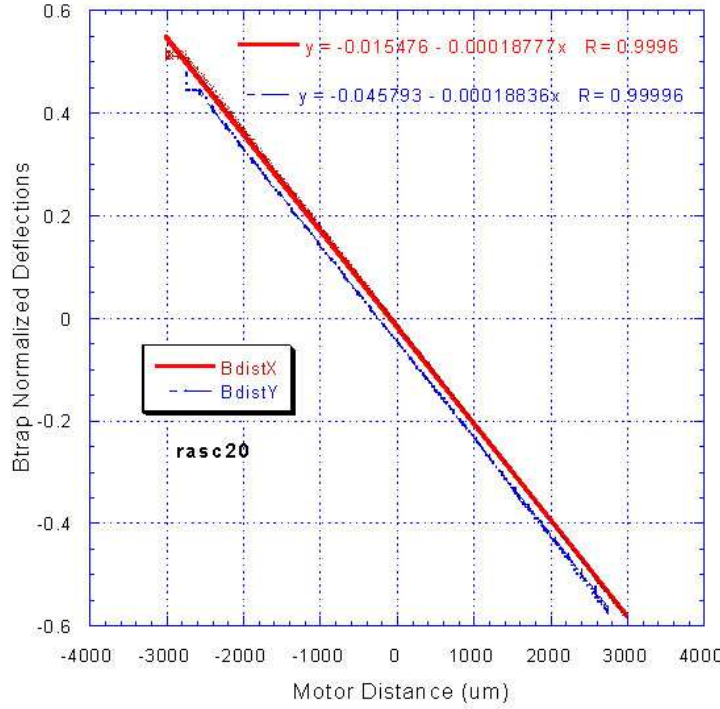
It is necessary to record certain channels of data during the raster test, and it is most convenient to have a preference file set aside for such tests. An example PREF file for raster test is given in the Software section under “Raster Test”. The host program loads the PREF file when it starts so you would need to replace your usual preference file in your user directory with the Raster Preference file and rename it “PREF”. This raster pref file also contains preset locations for the center and 4 extreme corners of the 6mm square pattern. These GOTO locations are numbered 1 through 5. First be sure to reset the motor PIC (under Tools in Host menu) in order to zero the motor positions at the center of the aperture. Stage position#1 will then indicate (0,0,0). Try GOTO positions 2 thru 5 and check the PSD Sum with the laser running at all 4 corner positions. If the sum drops near zero, then the beam has fallen outside the aperture at some corner of the raster pattern and therefore the center position (GOTO 1) needs adjustment. If the motor stops before reaching a corner (it should travel +/-3000 um in X and Y), check the motor-limit lights to see if any are lit. If one is lit, then choose a different part of the motor range for that axis and adjust the light source position screws to compensate so as to keep the beam in the aperture center at motor position#1 = (0,0,0).

Next, open a data file from the Host menu bar “Files” or by pressing “u”. Then under “Tools”, run “Raster X-Y Motor”. The raster pattern will take 5-10 minutes to complete. When the motors stop, close the data file by pressing “u” a second time. If the motors start to repeat the same positions, then stop the raster test manually by selecting “Raster X-Y Motor” a second time and close the data file.

Raster Test analysis:

To determine PSD dimensions and relay lens magnification, use a plotting program such as Kaleidograph, Origin or Excel to import the tab-delimited text from the raster test file. Determine which way the laser was pointing by observing the TrapASum and trapBSum columns. Delete columns for the unused side such as all columns for “B” trap. Create two new “distance” columns by dividing the deflections by the sums. For instance, $A_{distX} = A_PsdX / A_PsdSum$ and $A_{distY} = A_PsdY / A_PsdSum$. Plot the lightlever distance A_{distX} versus Motor_X and A_{distY} versus Motor_Y. Fit the plots to a straight lines and note the slopes. The inverse of the slope is the effective half-width of the PSD in microns, assuming the motor position was recorded in microns. The “effective” width includes the relay lens magnification so that the detector behaves as though it were relocated at the BFP but with a different width than its

native width. For the case shown in the figure below, the native half-width of the trapB DL-10 detector ($r=5$ mm) has been magnified to an effective 5.33 mm in X and 5.31 mm in Y, which are given by the inverse of the respective slopes. These effective radii would be used in the light-momentum force calibration equation below as r_{BX} and r_{BY} .



Power sensitivity test

Our PSD preamps give 3 output signals: ΔX , ΔY and SUM

$$\Delta X = \Psi \sum W_i x_i / r_D$$

$$\Delta Y = \Psi \sum W_i y_i / r_D$$

$$SUM = \Psi \sum W_i$$

where the summation is over all rays of light i hitting the PSD, each ray with a power W_i in watts and each ray falling a distance (x_i, y_i) from the center of the PSD which has a half-width r_D . The last equation suggests the power sensitivity can be measured by observing the SUM at a known total power hitting the detector. More useful however is the effective power sensitivity Ψ which is the ratio of the SUM to the power at the trap focus, assuming all light is collected by the output objective, attenuated by the optics and projected on the PSD. However we cannot know the power at the focus, we must infer it from 2 power-meter measurements by assuming the attenuation in each objective is identical.

To make such an estimate, first install a microchamber, fill it with water and add water to the lenses. Turn on both lasers and bring the system to a focus so we see the focal spots together at the center of the CCD. Nothing should be in the chamber except clean water. Turn off both lasers and remove the beam blocks between prism boxes and objectives. Turn on one laser and place a thin Power Meter probe between the prism box and objective lens. Take readings on both sides where the beam goes into the input objective and where it comes out of the output objective. The power at the trap is the geometric

mean of those 2 powers, that is, $W_{\text{trap}} = \text{sqrt}(W_{\text{in}} * W_{\text{out}})$. The effective power sensitivity is given by $\Psi = \text{SUM}/W_{\text{trap}}$. Make the measurement for both PSD detectors by running one laser at a time.

Transverse light force calibration: The Raster Test and Power Sensitivity Test provide values to calibrate light-momentum forces that are transverse to optic axis.

$$F_x = \text{trapAForceSensitivity}[x] * \Delta X_A + \text{trapBForceSensitivity}[x] * \Delta X_B$$

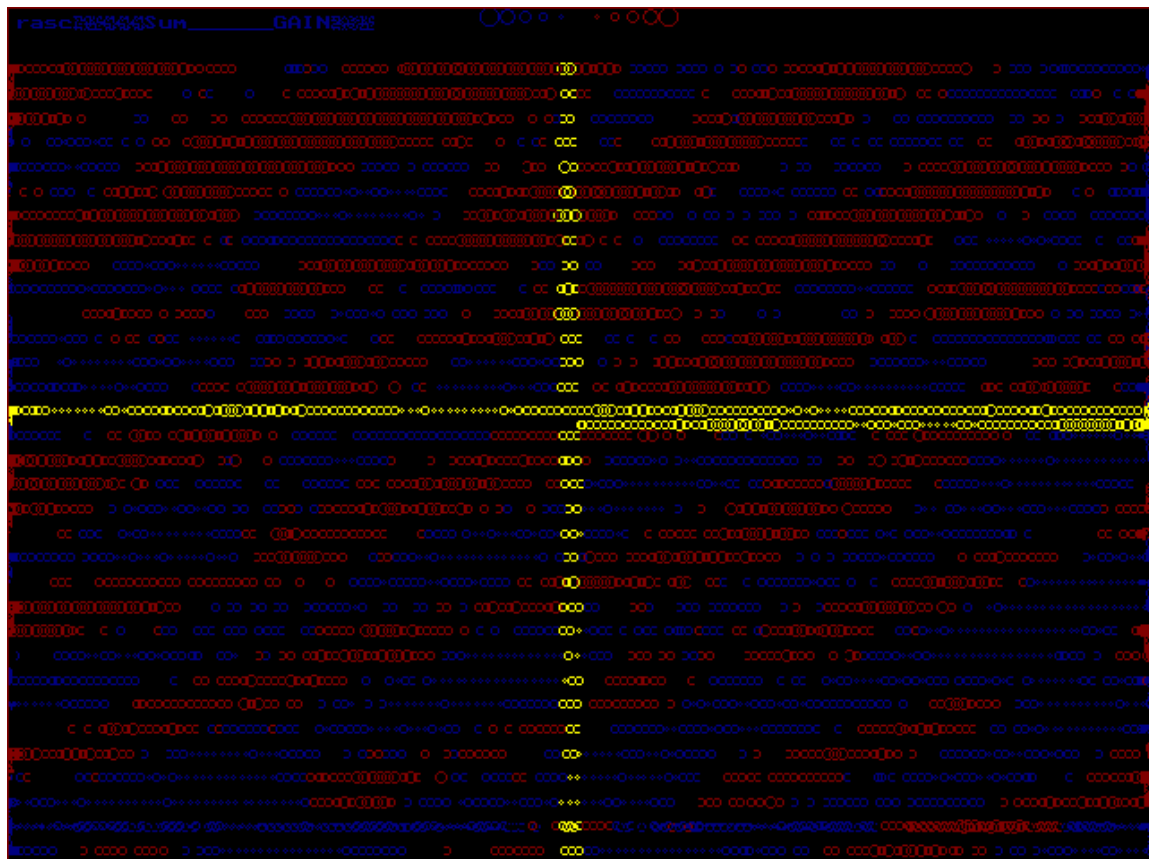
$$F_y = \text{trapAForceSensitivity}[y] * \Delta Y_A + \text{trapBForceSensitivity}[y] * \Delta Y_B$$

where (L is the focal length of the objective lens, typically 3.00 mm)

$$\text{trapAForceSensitivity}[x] = r_{AX}/(c\Psi_AL) \quad \text{trapAForceSensitivity}[y] = r_{AY}/(c\Psi_AL)$$

$$\text{trapBForceSensitivity}[x] = r_{BX}/(c\Psi_BL) \quad \text{trapBForceSensitivity}[y] = r_{BY}/(c\Psi_BL)$$

Some problems might be caused by interfering light paths leading from the trap to the PSD detectors, causing sensitivity fringes in the PSD detectors. Small close-spaced fringes are not a problem because the light pattern from the back-focal-plane of the trap gets averaged over many such fringes. However wide fringes, caused by nearly flat windows and prisms, will mean that when the light pattern is deflected to one side (say upward) it hits a high-transmission fringe but when it is deflected in the opposite direction (say downward) it hits a low-transmission region. Thus the calibration sensitivity might be affected by how far the light is deflected (by the force magnitude) and by the direction of that force. Indeed some of the worst fringes occur because the glass window on the front of the PSD makes interference fringes. You can remove the glass window from the PSDs except for those machines which will need to work in a very humid climate, where the silicon might become wet. See “How to remove PSD Glass Cover” under Documents.



These data were generated by the Raster Test and displayed by the “Raster Test Visualize” program in Software section. The yellow cross indicates the center axes $x=0$ and $y=0$. The red/blue stripes indicate +/- 4% variations in intensity.

Longitudinal Light Force

Force acting on the traps in a longitudinal direction (along the optic axis) is detected by the so-called “Iris Detectors”. Their outputs are proportional to the concentration of light power near the axis, that is, the cosine projection of the ray powers along the optic axis. Thus these detectors use the Bullseye filter to weight the rays according to their distance from the axis and the detector outputs are just the resulting light power hitting the silicon diode times the diode sensitivity. The instrument calculates the Z-force by subtracting the two weighted Iris readings as follows

$$F_Z = \text{trapAForceSensitivity}[z] * Z_A - \text{trapBForceSensitivity}[z] * Z_B + \text{nullOffset}$$

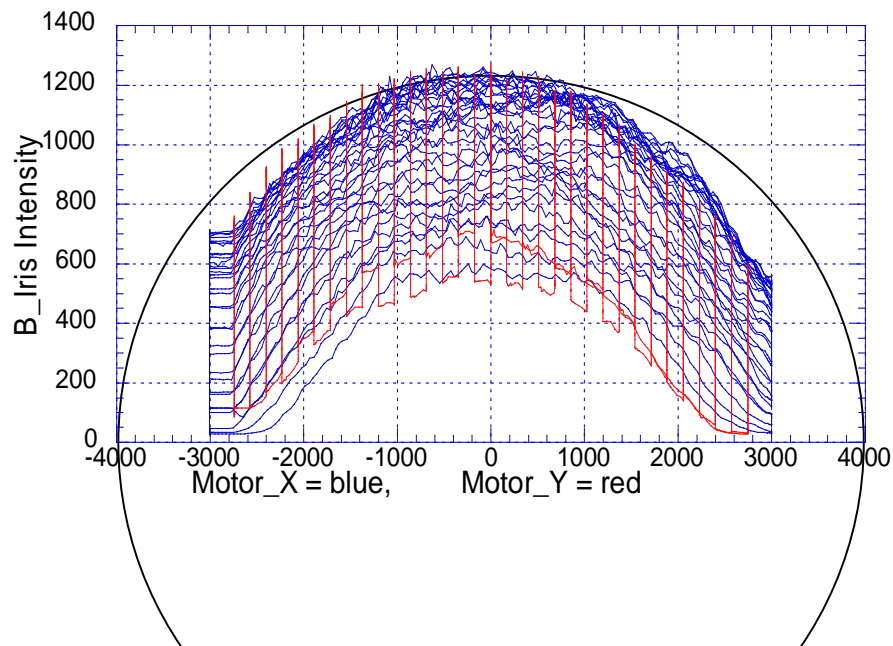
where the nullOffset is measured experimentally for known zero force. The sensitivity values are given by:

$$\text{trapAForceSensitivity}[z] = n_{\text{water}} / (c\Phi_A)$$

$$\text{trapBForceSensitivity}[z] = n_{\text{water}} / (c\Phi_B)$$

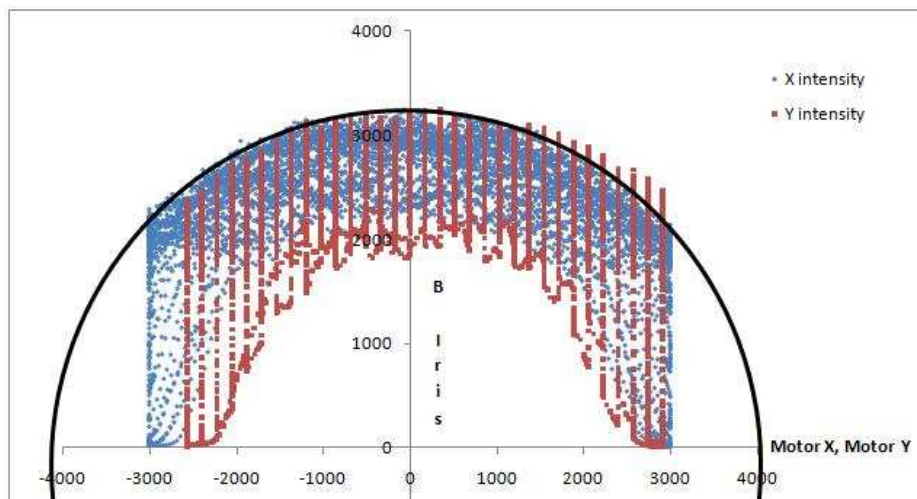
where Φ is the Iris Power Sensitivity = counts/watt from the iris detector. This value is measured by observing the Iris output for known power at the traps but the Iris detectors should have a clear mylar film to simulate a Bullseye with no attenuation except the Freznel reflection off the film (~8%). The Iris output may be observed in the Oscilloscope window or else calculated from the PSD SUM multiplied by the Iris/SUM as indicated in the PSD window.

The raster test gives a valuable check on the shape of the light transmission of the *Bulleye Filter*. In order to properly score the light momentum flux along the optic axis and derive the Z-axis force, the bullseye filter needs to have a semi-circular transmission profile with a radius of nf where n is the refractive index of water and f is the objective focal length. For the Olympus 60X objective $f=3\text{mm}$ and so the bullseye should have an effective radius (referenced to the BFP) of 4 mm.



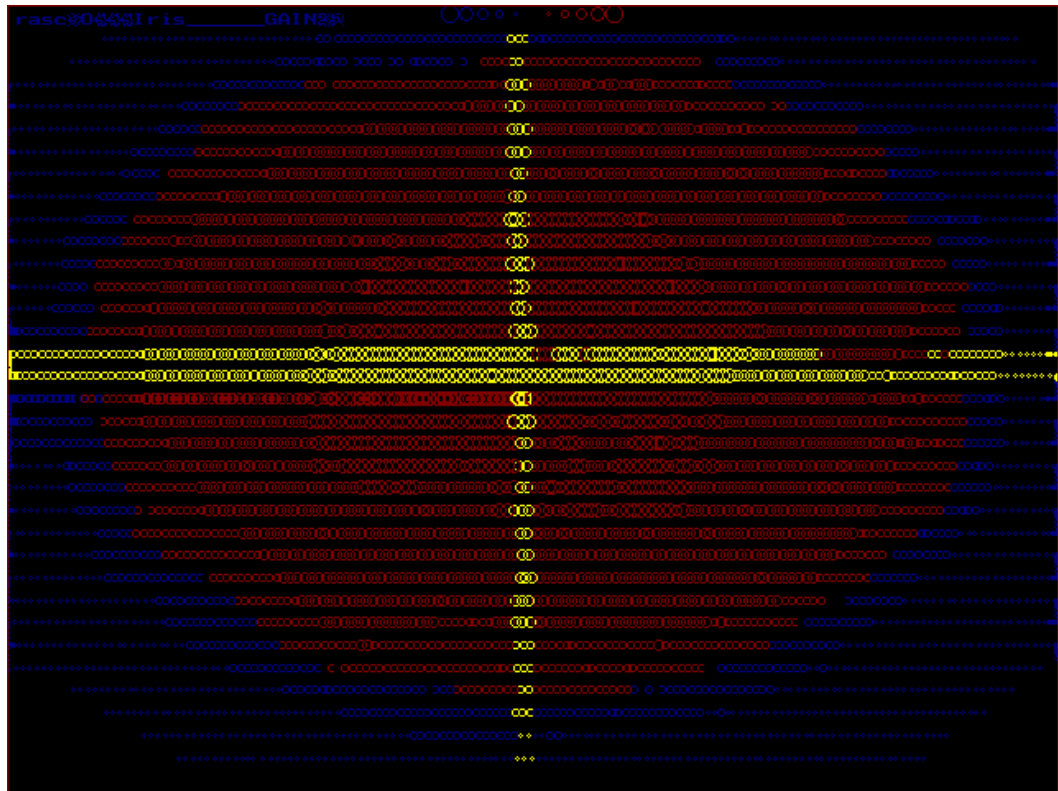
This figure above shows a plot of Iris Detector signal versus X-motor position and Y-motor position for the standard Bullseye filter. Comparing the profile with a circle indicates the filter has too rapid a variation of transmission with radius. Such a filter gives a signal for the Z-axis force that is too large when using equations shown above for $\text{trapAForceSensitivity}[z]$ and $\text{trapBForceSensitivity}[z]$.

This next figure below shows a filter which has been adjusted to 45% brightening using an eraser tool set to 45% opacity with 8mm diameter. This brightening will depend on the laser printer. Examples of brightened filters are provided on the same page as the normal Bullseye Filter, 8mm.



The raster test can also be used to check alignment between the centers of the Iris detector bullseye filter and the PSD transverse force detector. Here below is output from the raster-test visualizing program (see Software section) showing the relative position of the bullseye transmission pattern (red ellipse) and the PSD neutral positions ($\Delta X=0$ and $\Delta Y=0$). If the transfer function is not centered, then try adjusting the position of the iris detector circuit board with attached detector and bullseye filter. To shift the board, remove the flathead screws and replace with round head screws of the

same size with washers. Shift the screws in the circuit-board holes and retest until the patterns are centered.



Overstretching DNA Force check

A useful test to determine if the force calibration is correct is to measure the overstretching force of dsDNA. To make this test, use a piece of dsDNA with roughly 50% AT base pairs and 50% CG base pairs, such as lambda-phage DNA. Fill the chamber with buffer that has 500 mM NaCl and 10 mM Tris pH 7.5 and 1 mM EDTA. Fish the DNA between 2 beads, one on the pipette and another in the trap. Use a large (3-4 μm) bead in the trap. Pull the molecule and plot stretch response in the Force-Position window. Observe the height of overstretching plateau half way between the start and end of the plateau. That force should read between 65 and 70 pN. To check for direction sensitivity in force measurements, pull the same DNA molecule to the right and then left and then upward or down and compare the measured overstretching forces (see below). It is best to turn off any autoalign feedback and pull a long molecule like lambda with the motors, not the piezos. Yes, do use autoalign briefly to make the traps coincident before you start pulling, but turn it off when you pull to high force with the motors. Plot graphs of MotorX vs. ForceX and also MotorY vs. ForceY and compare forces at the middle of the overstretch transitions.

Light-Lever Distance Calibration

Light-levers are used to detect changes in trap positions of the two beams. We have an independent light lever for each trap. To calibrate the distance readings we compare changes in the normalized light-lever readings (channels = A distanceX, A distanceY, B distanceX, B distanceY) with changes in the motorized-stage shaft encoders (MotorX and MotorY). Here the trap positions are locked to the motor positions by running constant-force feedback on both traps which are placed inside of a single bead which is sucked onto the end of a pipette. That pipette, in turn, is fixed to the stage and moved a known distance by the motors.

To run the test, install a fluid chamber with a pipette. Center the traps and capture a single bead, between 3 and 4 microns in diameter in both traps. Then autoalign the traps and zero the force. Next suck that bead onto a pipette and move the pipette to give lowest possible force (under 10 pN). Next, under “Windows” in the menu bar, open the “Motor/Lever” window. Observe new window and check that the feedback mode in shows “F” for both traps (see picture below) which stands for constant-force feedback. Jog the pipette up and down and right and left by pushing the arrow keys. Note that the traps follow the bead over a limited range and patterns develop in the new window as shown in figure below. The response should be fairly linear until the Fiber Wigglers cannot follow the motors beyond their ~10-um ranges. Always reverse the motor direction when this limit is reached or you will lose the feedback lock. Hysteresis in the patterns is due to backlash in the motor actuator gears. The slope of the linear regions should be negative one (45 degrees) given that the window has equal length scales per division on the X and Y axes of the graph (vertical scale in nanometers, horizontal in microns). If the straight regions are not at 45 degrees, adjust the calibration factors TrapAleverSensitivity[X,Y,Z] and TrapBleverSensitivity[X,Y,Z] to make the proper slopes. Note the Z-axis sensitivity is always zero (does not matter) because the light levers only measure distances in the X and Y directions. Typical values for both X and Y sensitivities are 8500. Start with those values but check and refine them values with the Motor/Lever window slope test.

