CRISP Autofocus

Instruction Manual

Applied Scientific Instrumentation, Inc.

29391 West Enid Road

Eugene, OR 97402-9533 USA

Phone: (800) 706-2284

(541) 461-8181

Fax: (541) 461-4018

Web: www.ASIimaging.com

E-mail: Support@ASIimaging.com

Table of Contents

CRISP Continuous Autofocus System

The Continuous Reflection Interface Sampling and Positioning (CRISP) system provides for a very high level of focus stability, allowing a specimen to remain accurately focused for hours at a time with drift <0.1 μm. The system compensates for focus changes caused by temperature variations as well as mechanical drifts of the microscope mechanisms. The CRISP system promises to be a solution to focus drifts that plague time-lapse experiments at high magnification. The CRISP system uses a pupil obscuration method to determine focus from reflective surfaces. The control system allows adjustment of the focal lock position, relative to a nearby surface, once the system is locked. The unit is a C-mount device, that can be placed at the C-mount port. Usually it is used in conjunction with the a dual C-mount Splitter (DCMS) so both the CRISP unit and a data recording camera can share the same microscope photoport.

System Overview

The CRISP system consists of optical, electronic, and mechanical components. The optical system injects IRLED light into the microscope, captures the beam reflected from the specimen slide or cover slip, and routes the reflected beam onto a position-sensitive detector (PSD). The signal from the PSD is conditioned by an amplifier circuit in the MS2000 controller and used as the feedback signal for Z-axis control. The MS-2000 Z-axis controller changes the focal position of the microscope either with a servomotor or with a PZ-2000 piezo Z-axis stage.

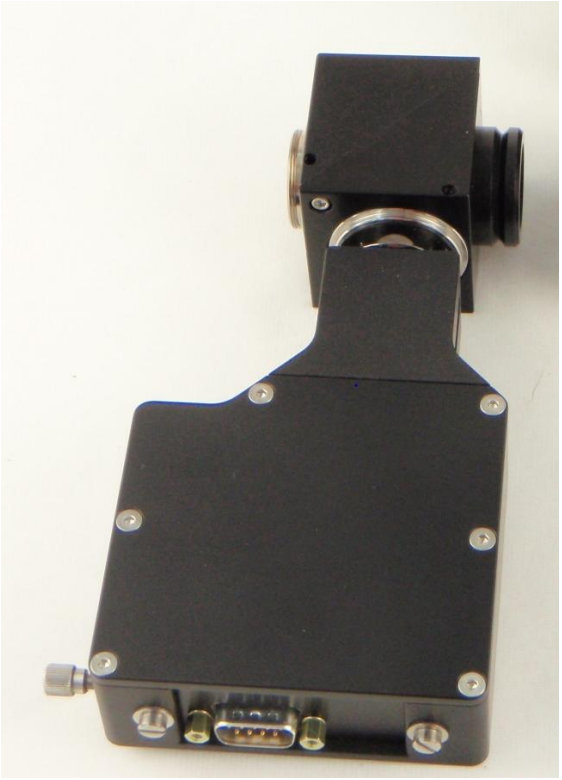


Figure 1: CRISP with DCMS photo-port splitter.

As shown in Figure 2, a dichroic beam splitter that reflects light from the IR LED and passes visible light to the camera is used to couple the CRISP unit to the system at the C-mount photo-port.

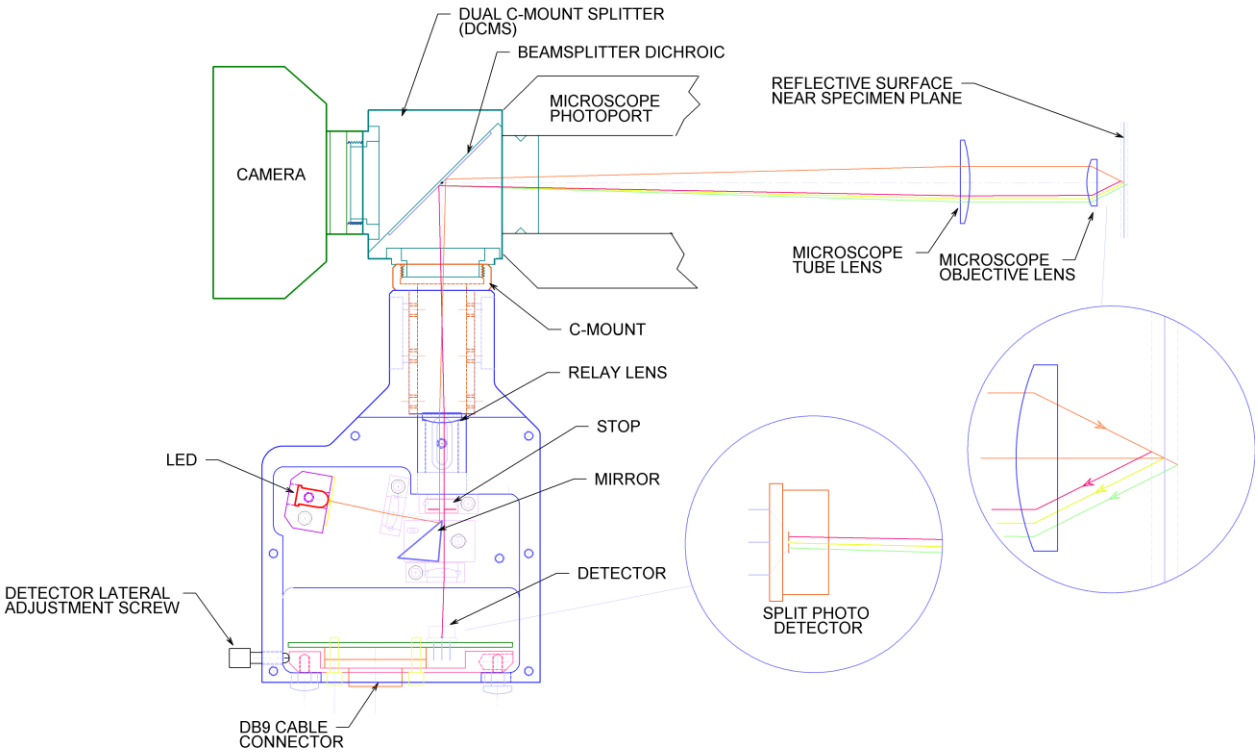


Figure 2: Schematic diagram of CRISP optical system

Fluorescent Filter Considerations

The CRISP system commonly utilizes an 850nm LED that is projected onto the sample. Proper arrangement of the light filters in the microscope is necessary for the system to function properly. A dichroic beam splitter that reflects the IR light is used in the dual C-mount splitter (DCMS). No other filters can be in the path to the objective that block the IR light. An emission filter that blocks the IR LED should be placed in front of the camera and can be located in the C-mount fitting of the DCMS for the camera. Fluorescence dichorics need to have a "window" in the IR to pass the CRISP LED. See the list of commercial filter sets that work with CRISP below.

The long C-mount adapter on the Olympus IX-71 or BX scopes permits the use of both a filter wheel and the CRISP unit in the provided space. This allows use of specific emission filters in conjunction with either a multi-band dichrioc with an IR pass band, or with a single excitation wavelength and a long pass dichroic in the scope.

Some configurations provide an easier solution to the filter problem. If a spinning disk confocal unit attached to the C-mount port is used for fluorescent microscopy, the filter cube is located in the confocal head and not in the microscope. In this case the CRISP mounted on the DCMS will work fine and not be impeded by any fluorescence filters in the microscope.

It may be possible to place the CRISP in the excitation path or to find an alternative location between the objective and the microscope's filter cube to insert the CRISP coupling beam splitter. Although these solutions are perhaps better optically, they probably require customization for the particular case. Contact ASI for details

LED Characteristics and Filters

Several LED wavelengths are available that will provide good performance with the CRISP system. Usually the unit is supplied with an IR LED with 780nm peak wavelength. The table below shows other LEDs that can be supplied, along with the suggested dichroic beam splitter and blocking filters. With sufficient spectral distance between the LED wavelength and the dichroic and camera block cut-off wavelength, a cleanup filter for the LED may not be required. The detector in the CRISP unit begins to lose sensitivity after about 1000nm limiting the maximum useable wavelength to about 1050nm.

LED Part Number	LED Color (nm)	FWHM (nm)	FW to 2% wings	Short Pass Dichroic Beam Splitter		Short Pass Camera Block Filter		Band Pass LED Cleanup Filter	
				cutoff (nm)	Part Number	cutoff (nm)	Part Number	Cutoff (nm)	Part Number
VLCS5830	625	18	580-660	600	69216	600	84710	628/32	84087
L660-06	660	20	615-700	600	69216	600	84710	650/50	84774
L700-06	700	30	650-740	650	69217	650	64330	700/50	84775
L735-06	735	30	680-780	700	69218	700	64331	750/50	84776
L740-06	740	30	685-785	700	69218	700	64331	750/50	84776
L780-06	780	30	710-830	750	69219	750	64332	800/50	84777
TSHG8200	830	40	750-900	750	69219	750	64332		
TSHG5210	850	40	790-930	800	69220	800	64333	850/50	84778
TSFF5210	870	40	810-950	800	69220	800	64333		
TSHF5210	890	40	830-970	850	69221	850	64334	900/50	84779
L940-06	940	50	840-1040	900	69222	900	64335	950/50	84780
L970-06	970	50	910-1070	900	69222	900	64335		
L1050-06	1050	50	950-1130	1064/80	NFD01-1064	1000	64337	1050/50	85881

Commercial Filter Sets Suitable for CRISP

For fluorescent applications, choosing the correct filters is important. Matching the best CRISP LED color with the filter set will give the best results.

Single band sets with single edge long pass dichroic beam splitters

There are many filter sets from several manufacturers that have a single edge, long pass dichroic. Usually the emission filter provides the data pass-band in the region just above the dichroic edge. Frequently the dichroic will continue to pass light well above the emission filter pass-band. Wavelengths above the emission filter pass-band, where the dichroic is still transmitting, provide the ideal wavelength region for CRISP. Specify the CRISP LED color to be

as far in the red/IR as possible away from the emission band, yet still where the dichroic has good transmission (and the emission filter has good blocking for the CRISP LED). Often the fluorescence emission filter can be placed directly in front of the camera in the DCMS C-mount splitter where it will serve to block the CRISP IR LED from the camera.

Contact ASI with your filter specifications for further guidance.

Multi-band filter sets that will work with CRISP

Frequently the dichroic beam splitter on multi-band filter sets has limited transmission outside the data-channel color bands. Nevertheless, there are several multi-band commercial filter sets that can be used with CRISP. One interesting filter set is the Semrock five-band with the dichroic filter characteristics below.

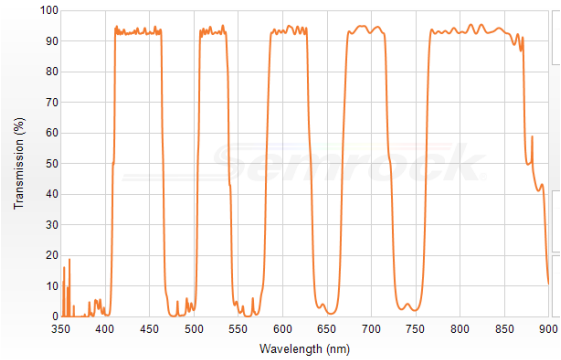


Figure 3: Semrock Dichroic FF408/504/581/667/762-Di01

This dichroic is used with either individual emitters and exciters for each band, or with individual exciters only as a Pinkle set. The upper transmission band of the dichroic is perfect for the standard 780nm CRISP IR LED. Used in this way, this filter set can be installed in the microscope's filter cube in the usual manner. A 750nm IR block is place in the DCMS splitter camera C-mount to block the upper band from the camera.

Another Semrock multi band set, LF405/488/561/635-A-000, has an extended region for the red band that would pass IR light. The pass band above 700nm is open, allowing easy operation with a 780nm IR LED for CRISP. In this case, the emission filter would be installed in the DCMS splitter C-mount in front of the camera and would act as the IR block for the CRISP LED.

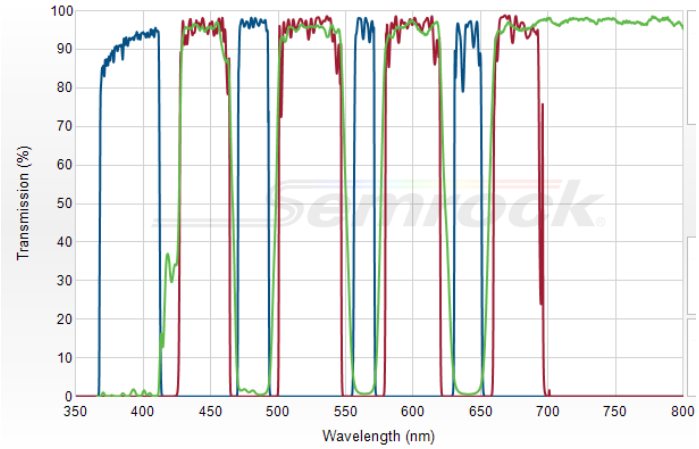


Figure 4: Semrock LF405/488/561/635-A-000

To determine the correct filter set for your application, first check the filters you have to see if there is a pass band in the IR. If not, consider alternatives that have such a pass band. Listed below are several filter sets from major filter manufacturers that will work with CRISP. Some of them require special non-standard LED color.

Semrock multi-band filter sets that will work with CRISP

LF405/488/594-A-000
Uses Di01-R405/488/594 dichroic which passes 780 to 800 IR LED
Uses a multiband emitter that can be placed in the camera's DCMS C-mount

LF405/488/532/635-4x-A-000
Uses Di01-R405/488/532/635 dichroic which passes 780 to 820 IR LED

LF442/514/561-3X-A-000
Uses Di01-R442/514/561 dichroic which passes 780 to 830 IR LED
Uses a multiband emission filter that can be placed in the camera's DCMS C-mount
Uses a multiband emitter that can be placed in the camera's DCMS C-mount

LF488/561-2x-B-000
LF488/561-A-000
Uses Di01-R488/561 dichroic which passes 780 to 830 IR LED
Uses a multiband emission filter that can be placed in the camera's DCMS C-mount

DA/FI/TR/Cy5/Cy7-5x-A-000
Uses FF408/504/581/667/762-Di01 dichroic with passes 780 to 850 IR LED
This five band set has the top band situated perfectly for CRISP
Uses a multiband emission filter with pass band in IR so can be used in microscope filter cube.

FRET - GFP/RFP –C-000

Uses FF 495-Di03 dichroic which passes 780 to 850 IR LED
Requires switched emission filter before camera for two channels

FRET-CFP/YFP-C-000
Uses FF458-Di02 dichroic which passes 780 to 850 IR LED
Requires switched emission filter before camera for two channels

Chroma multiband filter sets that will work with CRISP

59004 FITC/TRITC –ET
59204 FITC/TRITC
Uses 59004bs dichroic with available pass band at 740nm – specify 740nm LED for CRISP.
Uses a multiband emission filter that can be placed in the camera's DCMS C-mount

59017 ECFP/EYFP – ET
59217 ECFP/EYFP
Uses 59017bs dichroic with available pass band at 650nm – specify 660nm LED for CRISP.
Uses a multiband emission filter that can be placed in the camera's DCMS C-mount

69000 DAPI/FITC/TRITC
69300 DAPI/FITC/TRITC
Uses 69000bs dichroic with available pass band at 700nm – specify 700nm LED for CRISP.
Uses a multiband emission filter that can be placed in the camera's DCMS C-mount

69008 ECFP/EYFP/mCherry
69308 ECFP/EYFP/mCherry
Uses 69008bs dichroic with available pass band at 735nm – specify 735nm LED for CRISP
Uses a multiband emission filter that can be placed in the camera's DCMS C-mount

88000v2 DAPI/FITC/TEXAS RED/Cy5
Uses 88100bs dichroic with available pass band at 830nm – specify 830nm LED for CRISP
Uses a multiband emission filter that can be placed in the camera's DCMS C-mount
This set will also work for CRISP in the microscope's filter cube if the Cy5 channel is used for CRISP – Specify 700nm LED for CRISP for this application, and place 650nm SP block in front of camera.

Contact ASI or your filter supplier if you have further questions.

LED Power and Eye Safety

The CRISP system uses an IR LED to illuminate the sample and provide a reflected beam that is used to determine focus. Although relatively bright IR LEDs are used in the CRISP unit, the distributed nature of the LED source, masking of the LED, reduction in aperture and reduce duty cycle combine to make the CRISP light source eye-safe. Never-the-less, please do not stare into

the CRISP C-mount when the unit is powered up. IR LED sources do not generate visible radiation, so prolonged exposure is possible and should be avoided.

The maximum measured average power for a typical CRISP unit at the C-mount is less than 100µW (typically about 70 µW) with the LED set to 100% intensity and the internal aperture stop open fully. The CRISP LED mask appears to be about 1.0 mm × 6.8 mm in the image plane. The brightest part of the LED emitter depends slightly on the LED used and the exact focus, but is about 1.0 mm square at the image plane. Based upon the objective used, you can use these numbers to calculate typical maximum intensity of IR illumination at the sample. However, be aware that many objectives will not pass the full aperture at the CRISP aperture stop, so the number you get this way will be a maximum.

For example, a 60× objective will expose some parts of the sample to a maximum of about 0.36W/mm^2 of IR radiation.

Total Power / Area = 100µW / (1/60 mm × 1/60 mm) = 0.36 W/mm^2

You can reduce the radiative power at the sample by using a lower LED intensity and/or reducing the internal aperture stop.

Installation

Install the Z-axis drive or PZ-2000 stage as described in its manual. Become familiar with the functions of the Z-axis focus control system before installing the CRISP optics.

The CRISP device is designed to be used at a camera C-Mount location. In order to accommodate both data recording camera and the CRISP unit on the microscope photo-port, a dual C-mount splitter, such as the ASI DCMS is used. The DCMS is normally equipped with the appropriate dichroic beam splitter to reflect the CRISP IR LED light into the microscope while allowing the visible light to the camera.

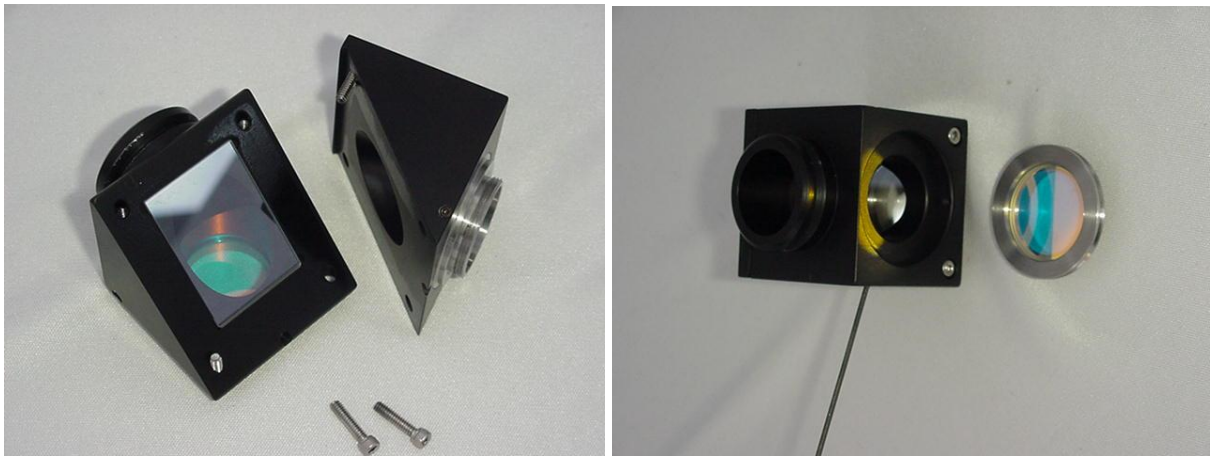


Figure 5: C-mount Splitter (DCMS) contains dichroic mirror and blocking filter.

There should also be a blocking filter on the camera C-mount to keep LED light out of the camera.

Mount the CRISP unit on the reflected port of the DCMS.

Mount the camera on the "straight-through" port of the DCMS.

Connect the DB9 cable from the CRISP unit to the labeled connector on the back of the MS2000 control unit.

Theory of Operation

The CRISP autofocus device uses a conventional pupil obscuration method for determining focal position. The device projects light from a small aperture into the specimen plane, but restricts the projected light to only one half of the optical system's pupil or optical aperture. As a result, light reflected from the specimen appears to move laterally as the focal position is changed. The light reflected from the specimen is focused on a split photodiode detector so that the lateral motion of the reflected light can be detected and used as a feedback signal for the automated focus device.

Sample Considerations

There are several classes of samples that are common in microscopy and present very different challenges for focus systems. CRISP relies on reflected light from the sample to detect focus

position. Often the reflected light comes from small refractive index discontinuities at sample surfaces. The amount of light reflected at a dielectric interface is given by

$R = (n_1 - n_2)^2 / (n_1 + n_2)^2$

where n_1 and n_2 are the refractive indexes of the adjoining dielectric materials. The table below shows the refractive index of several optical materials and the magnitude of the reflection expected at various interfaces between materials. You will note that reflections from an air interface are around 4%, whereas reflections from a water interface is about 1/10 as much. This makes for "easy" and "difficult" focus applications.

The problem can get even harder when trying to discriminate between light coming from two closely spaced interfaces, for example, the two sides of a cover slip, or the variable spacing between a cover slip and a slide.

Table 1: Reflection Intensity from a Dielectric Interface

Material	Refractive index @ 800nm	Reflectance at interface (%)		
		Air	Water	Glass
Air	1.000	—	2.0	4.3
Water	1.329	2.0	—	0.46
Immersion Oil	1.518	4.2	0.45	0.0003
Glycerol	1.473			0.03
Glass (typical)	1.523	4.3	0.46	—
Plastic (Polystyrene)	1.575	5.0	0.72	0.03
Plastic (PMMA acrylic)	1.483	3.8	0.30	0.02
Fused Silica	1.453	3.4	0.20	0.05

Photodiode Displacement Signal

The heart of the focus system is the split photodiode displacement sensor. The difference in intensity of the light falling on the two halves of the detector is used to determine the relative position of the reflected light beam. As focus position changes, the lateral position of the reflected beam will shift. The difference of the two signals from the split photodiode is a measure of the relative focal position.

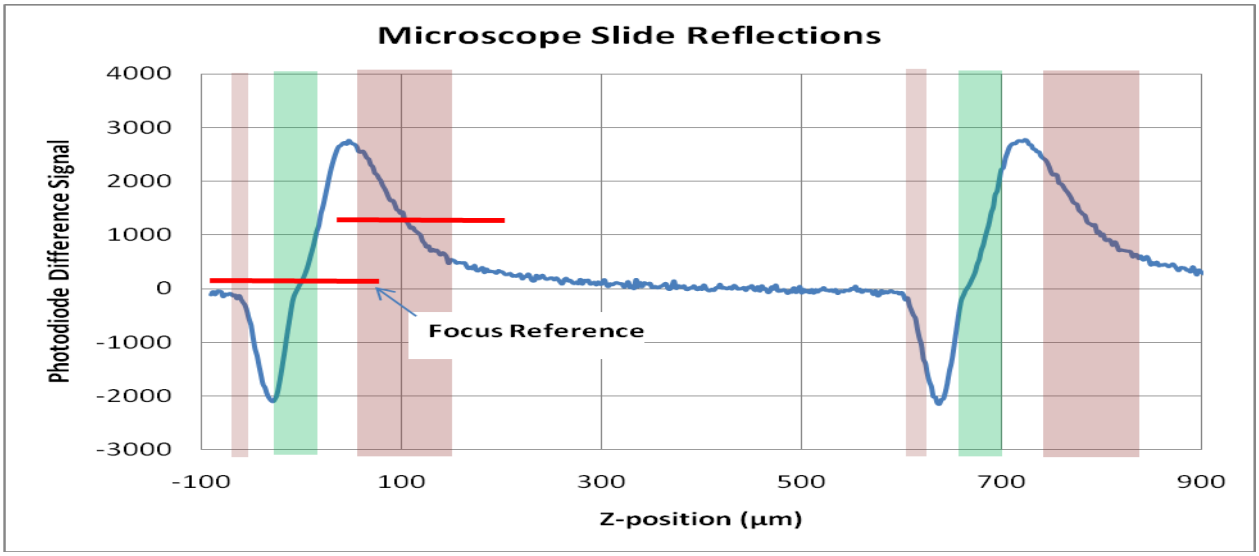


Figure 6: Photo detector difference signal for a scan through a microscope slide.

The figure above shows the difference signal from the photodiode pair as the focus is scanned through a standard microscope slide. You will notice two green shaded zones corresponding to the front and back surface of the slide. Any region with a large slope can be used to lock onto focus. Most often we are interested in viewing right at the reflective surfaces or very near them. A reference signal level, one of those marked by the two red lines, is used to specify the desired focal position. Any deviation from the reference is an error signal that will direct the stage back toward focus. Changing the focus reference allows you to adjust focus within the shaded regions. Once the system is locked, the MS2000 control knob adjusts the focus reference level, thereby effecting focus changes on the locked system.

The brown shaded regions have opposite slope compared to the regions near the surface. If, for some reason, you wished to use one of those places to lock focus, the servo calibration would need to be done again, and you would expect to get a negative calibration value.

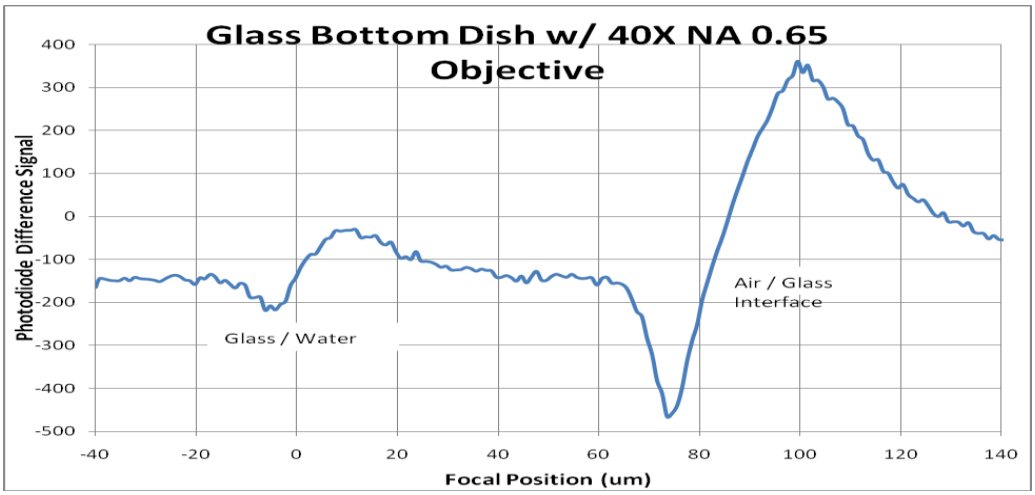


Figure 7: Reflections from a glass bottomed Petri dish.

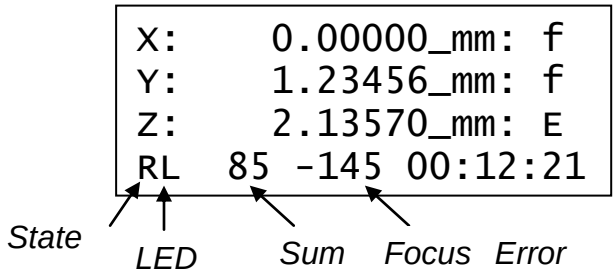
A common typical sample is a glass-bottomed Petri dish with a water sample. Here we can easily see the two reflections again, but note that the glass/water reflection is much less intense that the air/glass reflection.

Control of the CRISP system

To use the LCD display, ensure that the display-mode DIP switches 1 and 2 located on the back of the controller are in the UP position. The MS-2000 controller provides an easy means to turn on and off the CRISP LED as well as to initiate the focus lock. The LCD display shows the status of the system. The figure below shows the typical display.

LCD Display

On the MS2000 controller, the bottom line of the LCD display shows information about the photo-detector signals and CRISP system state.



The meaning of the quantitative information on the display changes depending up on the system state. The first character is the CRISP system state, described in the table below. The next character is **L** if the LED is turned on, otherwise blank. In most states, the photodiode *Sum* signal is next, followed by the *Focus Error* signal. In the **Dither** state, the *change* in focus error over the `cal_range` is displayed instead of the focus error.

Button Actions

The @ button is used to manually control the CRISP system. The duration of the button press determines the action.

Function	Button
Advance to next focus state	Press @ briefly and release.
Back to Previous state or Advance to Calibration state	Press @ >3 sec. and release.
Set Focus Offset to zero from **READY** state	Press @ >10 sec. and release.
Save parameters to flash from **IDLE** state	

CRISP System States

Activating and calibrating the CRISP system is done moving to the next CRISP state using the @ button on the controller and pressing it for various durations as shown in the "Next State" and "Previous State" columns in the table below.

(You can use the serial command **`LK F=<decimal number>`** as shown in column two on the table below to directly force a CRISP system state. For example, to set the CRISP state to **Balance**, issue the serial command **`LK F=66.`** Use with care, as out-of-sequence events are not necessarily handled smoothly.)

Table 2: CRISP system control states

State Character on LCD	"LK F" ASCII code	State Name	Next State (@ short)	Previous State (@ long)	Comment
I	**79 (O)**	**Idle**	**R**	**G**	LED is tuned off going from **Ready** to **Idle**
R	**85 (U)**	**Ready**	**K (D)**	**I**	LED ON - @ button locks
D		**Dim**	**(R)**	**I**	Low returned light signal (prevents **Ready** state)
K	**83 (S)**	**Lock**	**R(F)**	**R**	@ button unlocks
F		**In Focus**	**R(K)**	**R**	@ button unlocks
N		**Inhibit**	**R**	**I**	Low returned signal (unlocks system)
E		**Error**		**R**	Usually Out-of-Range Error
G	**72 (H)**	**loG_cal**	**R**	**1**	Initiate basic Log-Amp Calibration
	67 (C)	**gain_Cal**	**(2,3,B,f)**		Initiate Servo-Gain Calibration
f (g,h,i,j)	**102 (f)**	**Dither**	**R**	**R**	Dither Z for optical adjustments
† **c**	**97 (a)**	**Curve**	**(R)**		Generate focus curve data
† **B**	**66 (B)**	**Balance**	**R**		Display shows signal from each half of detector. Use to balance optics.
† **l**	**108 (l)**	**Set Offset**	**(R)**		Resets focus offset to zero for preset focal postion.

† States can only be initiated with LK F=code command.

CRISP Operations

The following guide assumes that the default CRISP parameter settings are adequate and will provide an adequate focus lock with many objectives and sample types. Focus on your sample.

Quick Start Instructions

1) Press @ button for 3 seconds to achieve reflectivity calibration. Verify that LCD shows at least 2.0 dB SNR on the LCD display and that the status indicators on the Left side of the LCD show GL, indicating the Log Amp calibration is complete and the LED is on.
2) Press @ button for 3 seconds to initiate a gain calibration and Z-axis focus dither. After a few seconds it should be apparent that the focus system is moving rapidly back and forth a small distance. The number in the middle on the LCD status line indicates the magnitude of the focus error change over the dither range.

hL 75 145 00:12:21

3) Adjust the detector lateral adjustment screw on the CRISP unit for maximum absolute value. Motion of the detector will give large temporary values, so pause after changing the adjustment to observe the reading. For best performance you would like to have a value >50 with only modest fluctuations. When you have discovered the best spot for the detector…
4) Press @ button briefly to advance to the **READY** state. You can verify that you have a good calibration by changing the focus of the sample and observing the change of the *Focus Error* value. You should see *Focus Error* respond proportionally to the change in focus, going positive in one direction and negative in the other.
5) Press @ button briefly to advance to the **Lock** state. If the focus is not perfect, you can use the knob on the controller to change the lock reference and hence the focus. If the lock state is "nervous" or "sluggish", see details below for how to adjust the loop gain and averaging for more desirable behavior.
6) Press @ button briefly to unlock and return to the **READY** state. Subsequently you can just use a quick-press of @ to toggle the focus lock on and off.

For optimum performance, please refer to the more detailed instructions below.

Engaging the LOCK for Normal Operation

In addition to the quick start instructions above…

If you have calibrated the system, but then perhaps changed samples or significantly disturbed the system, you may find that the focus-error shown on the LCD is nowhere near zero when in the **Ready** state prior to locking. If you try to lock, the system could easily run away. Instead reset the offset by holding down the @ button for >10s first. When you release the button, the *Focus Error* numbers should fluctuate about zero, and the transition to the lock state should be smooth.

Once the **Lock** is engaged, the Z-axis control knob on the controller can be used to manually adjust the reference lock value. This allows manual focus adjustment of the locked system.

To unlock the system, again, a short-press of the @ button to will do it, returning to the **Ready** state.

When the **Lock** is engaged, any commanded move to the focus axis will fail and will generate a **COD 47** error.

Saving Calibration and Offsets

Once you are satisfied with the focus performance and adjustments, you can save the calibration parameters to the controller so that in the future you don't have to go through the entire calibration procedure again. Merely back out of the **READY** state, to the **IDLE** state, with a long-press (3 sec.) of the @ button. In the **IDLE** state, hold down the @ button for >10 seconds to save settings to flash memory.

Now, as long as you stay with the same sample preps and objective lens, you should not need to go through the first three steps above. When you power on the controller, advance from the **IDLE** state to the **READY** state with a brief press of the @ button. A brief press again, and the system is locked.

Calibration Details

Different samples and objective lenses can result in dramatically different levels of signal of returned light and different sensitivity of the detector to focus error. For this reason, there are two "single button" calibration steps that need to be done before the system is ready to use.

Log-Amp Calibration

Before calibration, choose your objective and focus on your sample.

This calibration step is initiated from the **Idle** state by a long press (3 sec.) of the @ button.

The log amplifier range offset is adjusted so that the light level on the photodiode is approximately 75% of full scale. The LCD display shows a signal-to-noise number that is the signal level on the photodiode compared to when the LED is turned off. For best results, it is good to have SNR > 4.0 dB. If you have low levels, be sure your sample is in focus, and increase the LED intensity using the **UL X=*n*%** command. Default LED level is 50%.

Focus Sensitivity Calibration and Detector Lateral Adjustment

Before this step, first focus on the sample and perform the Log-Amp Calibration described above. This calibration step can be initiated from the **loG_CAL** complete state (**G**) by long-press (3 sec.) of the @ button, or with the serial command **LR Y=NA**, where NA is the numerical aperture of the objective you are using. Using the serial command with the correct numerical aperture will allow the system to use an optimal distance for the focus moves it needs to make. The default is NA=0.65 which generates move distances suitable for a wide range of objectives, if not ideal. This calibration step moves the focus up and down a few microns to determine the focus sensitivity of

the system and then proceeds to the **Dither** state where the focus is continuously moved back and forth a small amount.

Focus Dither for Optical Adjustments

In the **Dither** state the focus is changed by up and down by the `cal_range` amount. The difference in focus error signal is displayed on the LCD. The system will remain in the dither state, moving the focus up and down, until commanded to turn off. During the dither, the LCD *Focus Error* number shows the change in focus signal from the top to bottom of the dithered focus move. Now you can make changes to the optical alignment while maximizing the *Focus Error* number.

Slowly adjust the detector lateral adjustment screw for a maximum absolute *Focus Error* value. Large negative numbers are just as good as large positive numbers for obtaining a lock. When you make any optical adjustments using the **Dither** function you should keep an eye on the *Sum* indicator on the LCD display as well. If the signal level on either detector half gets out of range for amplifiers, the *Sum* will read either 0 or 100 for saturated low or high levels respectively. You may find that best *Focus Error* reading results in a lower or higher *Sum* signal that you started with. If the *Sum* signal is outside the range 60-80 it is best to redo the log-amp calibration step.

When satisfied that the focus slope is the best possible, a short press of the @ button will cause the controller to return the stage to the initial position, check and set the error offset to zero, and leave the system in the **Ready** state.

Parameters used with the CRISP system

The serial commands give the user access to several parameters used with the CRISP system. Advanced users may find that they have a need to change particular settings from the default values for specific purposes.

`cal_range` Sets the distance the stage moves gain calibrations, dither moves, and focus curve generation. This can be set directly using the **`LR F=`**`cal_range` command, or indirectly using the **`LR Y=`**NA command where `cal_range` = 1.5µm/NA^2 and NA is the numerical aperture of the objective lens used.

`cal_gain` Sets the relative gain of the detector system to the focus motor. Higher numbers represent less overall loop gain. This number is set by the focus sensitivity calibration. The value can be queried or set with the **`LR X`** command. Users are encouraged to use the **`KA`** command to change the relative gain rather the **`LR X`**, although either parameter can be used to similar effect.

`lock_range` Specifies a maximum range of travel from the point of lock at which point the controller will disable the lock function and halt motion. This prevents runaway conditions from damaging objective lenses and sample. Set with the **`LR Z=`**`lock_range` command.

`lock_offset` This parameter is an signed integer number representing the focus error on the detector that corresponds with the desired focus point. The `lock_offset` is set upon calibration, changes when the control wheel is turned when locked, and can be reset to the

value that will cause no change in position when the @ button is pressed for >10s in the **Ready** state. The user can directly read and write this value with the **LK Z** command.

`LED_Intensity` The LED light level can be controlled with this parameter. The default value of 50% is adequate for many applications. Improved signal to-noise can be obtained using more light. Set with the **UL X=**`LED_Intensity` command.

`Log_Amp_AGC` This parameter is set automatically during the log amp calibration step. A digital potentiometer is set such that the signal on the photodiode fills, but does not saturate, the ADC converter input range. This number will increase with higher `LED_Intensity` and more reflective samples.

The values of some of the parameters that are set during calibration will be sent to the serial port if the verbose mode **VB X=16** is set.

Optical Adjustment

The CRISP unit is pre-adjusted at the factory, and should not need major adjustments. However, this guide will allow anyone to test and check the proper adjustment. The recording camera is helpful for adjusting the primary mirror position. In order to see the illumination light, any blocking filter in front of the camera needs to be removed.

Adjusting the Relay Lens position

The relay lens should be set to the center of its range. There is usually no reason to change this.

Adjusting Position of the LED Light Source

Focus on a glass slide with a 10X or 20X objective so that a typical glass/air reflected beam is obtained. Remove the transmitted light and obtain an image of the reflected light on the camera. (be sure the L LED indicator is showing on the LCD display) Be sure the mirror is intercepting the beam. (You may wish to slide the mirror as far away from the LED holder as possible to ensure adequate light entering the microscope.) Loosen the Adjusting screw on the LED holder and move and twist the holder so that the image of the LED slit is in the center of the camera sensor. Focus slightly deeper until the LED element comes in view and then twist the LED holder so that the active element is showing near the center of the slit. Tighten the screw to hold the LED in place.

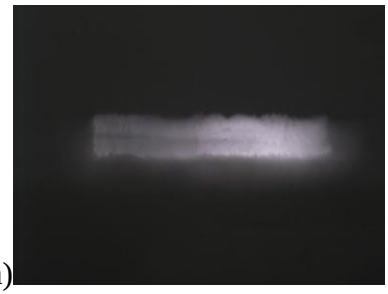
a)  b)  c) 

Figure 8: Reflection from glass slide of a) LED exit slit and b) focused deeper, the LED emitter, when the LED holder is properly aligned by moving c) LED holder.

Adjusting the Primary Mirror

The most critical alignment in the CRISP device is the primary mirror that injects the LED light into half of the optical aperture and allows light from the other half of the optical aperture to reach the photo-detector. It is important that this mirror reflect the light exactly onto the optical axis and that the edge of the mirror exactly stop at the centerline of the system as well. The best approach for adjusting the mirror position is to maximize the error signal received when in **Dither** mode. First center the photo-detector board so it is in the middle of its travel range. Use the mirror adjusting screw to move the mirror back and forth.

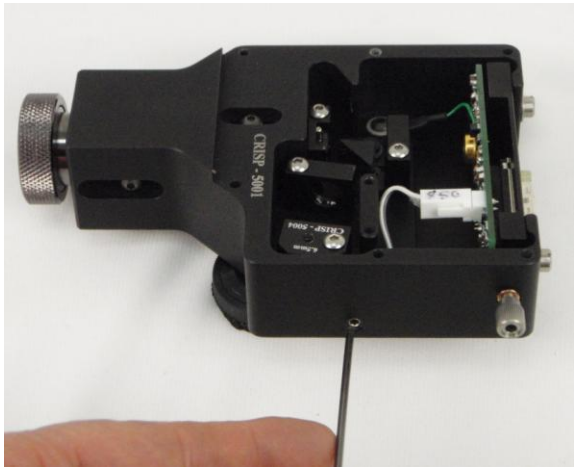


Figure 9: Mirror Adjusting Screw

Advanced Techniques

A common problem is that the CRISP system will be able to hold focus best at a location that is not in the center of the best focus range for the sample. This can typically happen because the reference cover-slip is slightly in front of the region where the sample is best in focus. Often, adjustment of the lateral position of the photo-detector will be enough to get acceptable operation. For more extreme cases, the solution is to move the entire CRISP unit further back from the C-mount using the sliding C-mount built into the CRISP body. The distance, D, to move the back depends upon the objective magnification, M, and the required depth of focus change, δ, in media of refractive index n. The dependence is:

$$D = \delta / n \, M^{2}$$

For high magnification objectives, small focal plane changes can require substantial extension at the C-mount. Looking 5µm deeper into a water sample with a 100X objective would require about a 38mm extension. There may be the desire to focus slightly above the interface on biological samples, so the CRISP C-mount can be pulled back up to 29mm (see photo). This

might be handy, especially with 100X objectives. If longer extension is required, additional extension tubes could be employed.

The C-mount extension lengths recommended for various objectives to optimize the focus range of the system within the sample are included in the table below.

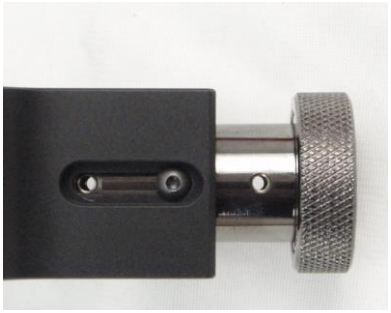


Figure 10: C-mount extension.

Table 3: Focus Properties for Typical Objectives

Microscope Objective	Relative EPI Light Gathering Power	Objective Pupil Diameter (mm)	Depth of Field (µm)	C-Mount Extension Length (mm)	Typical Focus Range (µm) (w.r.t. glass/water surface in 150 µm glass-bottom dish)	Typical Capture Range (µm)
100X NA 1.25	2.4	4.5	0.43	14-29	+/-3	> +/- 10
60X NA 1.4	10.7	8.4	0.34	0-15	+/-2.5	> +/- 8
40X NA 1.3	17.8	10.7	0.40	0-15	+/-3	> +/- 20
40X NA 0.7	1.5	6.3	1.4	0-15	+/-10	> +/- 20
20X NA 0.75	7.9	15.0	1.2	0	+/-10	> +/- 20
20X NA 0.4	0.64	7.2	4.2	0	+/-25 *	> +/- 50
10X NA 0.25	0.39	9.0	10.8	0	+/-50 *	> +/- 100

*Glass/air interface provides focus signal while focused on glass/water interface for low NA objectives.

The focus range of the system is largely determined by the numerical aperture of the objective used. Once the reflective interface is well outside the depth of focus (DOF) of the objective, little useful light is returned to the detector and it is difficult to capture focus.

The amount of light available for CRISP depends not only on the type of reflective interface, but also on the light gathering ability of the objective lens. CRISP both illuminates and collects through the objective, so the relative brightness goes as NA^4/M^2. Examples are shown in Table 3.

With low power, low numerical aperture objectives, the light reflected from the air/glass interface will begin to significantly contribute to the focus signal. If this is desirable (the glass is flat and uniform), then an extension tube will enhance the focus signal from the air/glass interface when focused on the glass/water interface. If you wish only to look at the glass/water interface, there are various tricks you can play to enhance the separation between the light obtained from the two closely space interfaces. You can preferentially detect the deeper reflection by the position of the detector. Turn the detector lateral adjustment screw as far counter-clockwise as you can and still obtain a good dither response magnitude with non-zero signal on both detector halves. 20X objectives seem to be about the most difficult to separate the glass/water signal from a nearby much larger glass/air interface. Tricks might include using thicker glass bottom dishes or coverslips so the interface is further away, or just maintaining a very clean and uniform glass/air surface so what contamination of signal from that surface that there is will not affect the overall focus position.

Using the Iris LED Beam Stop

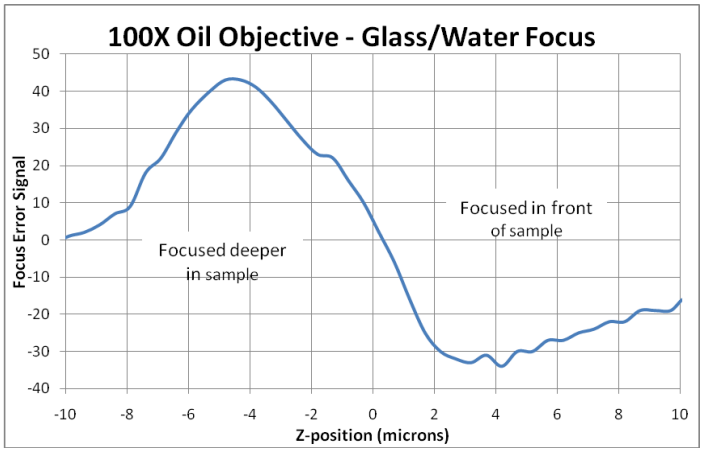
The internal iris in the CRISP unit can be used to improve the returned beam quality by reducing the amount of stray LED light that cannot be accepted by the objective aperture. The

system is shipped with the iris all the way open so as to be able to accommodate high brightness objectives. Use the dither calibration to optimize the iris size. You may discover that the *Focus Error* signal remains relatively constant as you decrease the aperture, but he *Sum* signal decrease, reflecting the decrease in background light level. Decrease the aperture size until the *Focus Error* signal begins to decrease, and then redo the log-amp calibration.

Using Focus Curve Generation for Optimizing Adjustments

Sometimes it is instructive to plot the focus error response so you can asses exactly the useable lock range and capture range of the system. To do this, first lock focus and adjust the lock optimally. Unlock to the **Ready** state and press the Zero button to set the Z-in-focus position to zero. Then issue the command **`LK F=97`** to initiate the focus curve generation. Below is the serial output from such a run. The third and fourth columns are the Z-position in microns, and the relative focus error respectively.

```
LK F=97
:A a
T:     0   -10.4     0
T:    50   -10.4    -1
T:   100    -9.9     1
T:   150    -9.4     2
T:   200    -8.9     4
T:   250    -8.4     7
T:   300    -7.9     9
T:   350    -7.4    18
T:   400    -6.9    22
T:   450    -6.4    29
T:   500    -5.9    35
T:   550    -5.3    40
T:   600    -4.8    43
T:   650    -4.3    43 +Peak
T:   700    -3.8    41
T:   750    -3.3    37
T:   800    -2.8    32
T:   850    -2.3    27
T:   900    -1.8    23
T:   950    -1.3    22
T:  1000    -0.8    16
T:  1050    -0.3    10
T:  1100     0.2     2 Focus
T:  1150     0.7    -6
T:  1200     1.2   -16
T:  1250     1.7   -25
T:  1300     2.2   -30
T:  1350     2.7   -32
T:  1400     3.2   -33
T:  1450     3.7   -31
T:  1500     4.2   -34 -Peak
T:  1550     4.7   -30
T:  1600     5.2   -30
T:  1650     5.7   -27
T:  1700     6.2   -27
T:  1750     6.7   -25
T:  1800     7.2   -24
T:  1850     7.7   -22
T:  1900     8.2   -22
T:  1950     8.7   -19
T:  2000     9.2   -19
T:  2050     9.7   -19
T:  2100    10.1   -16
```



Cut and paste into Excel to plot the numbers. You can see the strong slope near the surface (Z=0) that provides the focus feedback. For a focus at the interface, the *capture range* includes any Z-position that has the *correct polarity* of focus error signal to bring the stage back to the lock point. In this case the capture range extends to about 10µm into the sample and even further before the surface. The useful *lock range* depth into the sample is any region of the focus error curve with the *correct slope* – allowing for a little capture range beyond the lock point. In this case beyond about -3.5µm (into the sample) it would be hard to hold lock since it is very close to the focus peak.

Focus Variation Reduction by Averaging

In many instances, the focus lock mechanism is used to hold the subject in focus for long periods of time. Dynamic performance is secondary, and stable focus on a weak interface may be more important. In these cases, turning on averaging of the error correction signal can improve performance. Use the "**RT F=***n*" command to average 2^n samples. Beware that excessive averaging adds a time delay that can effect stability. If oscillation occurs, either reduce the number of averaged samples or reduce the loop gain. Using n = 5 to 9 with reduced loop gain (**KA** 1 to 5) can significantly smooth out marginal focus situations and hold the focus very still even on weak interfaces where the purpose of the device is mainly thermal drift compensation.

Dynamic Performance Optimization

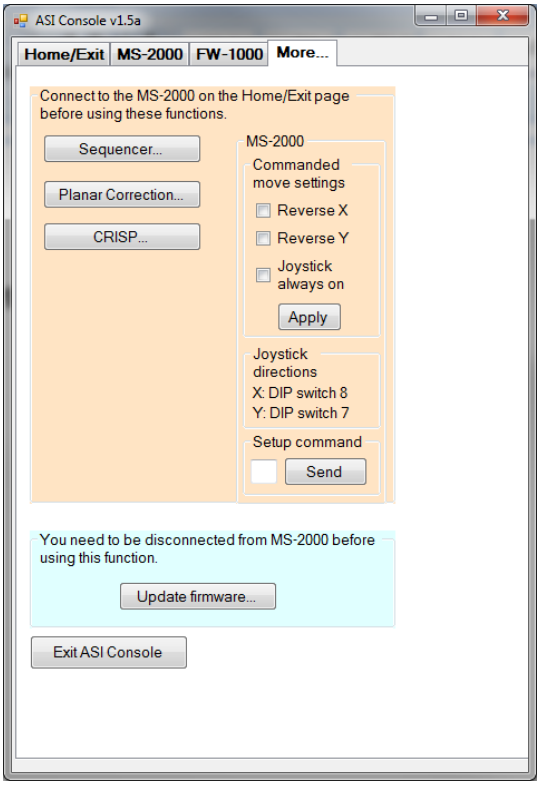
For application of automated image acquisition, the speed of the autofocus can limit the throughput of the system. In these instances, it is important that the focus respond quickly to error conditions that change as the stage moves in XY. Default loop gain established by the calibration procedure results in modest speed and moderate stability. To push the speed, there is a separate variable set by "**KA Z=***m*" that is used as a gain multiplier. The default KA value is 10. Usually the system will be stable increasing the value to 20 or higher, with significant speed improvement, but increasing **KA** above 10 is best used only when you have a strong reflection with low noise variation. Averaging should be turned off (**RT F=0**) when higher gain is used since the time delay associated with the average can introduce instability. There is no substitute for a strong reflected signal if you need to focus quickly and accurately.

Troubleshooting Steps

If you cannot get sufficient *Focus Error* difference in the **Dither** state, check these troubleshooting steps.

1) Verify that the electronics are working. Using the CRISP control in ASI console…
   a) Set LED Intensity to 0%. Apply.
   b) Click Step 1 Log Amp Calibration. Verify that the AGC number returned is less than ten. If so, that indicates that the LED can be controlled, and the amplifier has good noise performance. If the AGC number is >10, contact ASI.
   c) Set LED Intensity to ~70%. Apply. Save. Be sure you can get AGC value >35.
2) Verify that you have an appropriate sample that is in focus. Prepared fixed samples usually will not return a good reflected beam because of the index matching mounting media. Choose a simple glass/air interface if you are having trouble before graduating to glass/water interfaces.
3) Verify that the IR light can reach the objective. Remove filter cubes if unsure of their properties.
4) Flat glass windows and prism surfaces can reflect more IR light than is coming from the desired interface. Be sure you are using a 100% photo port without intervening prisms.

ASI Console support for CRISP



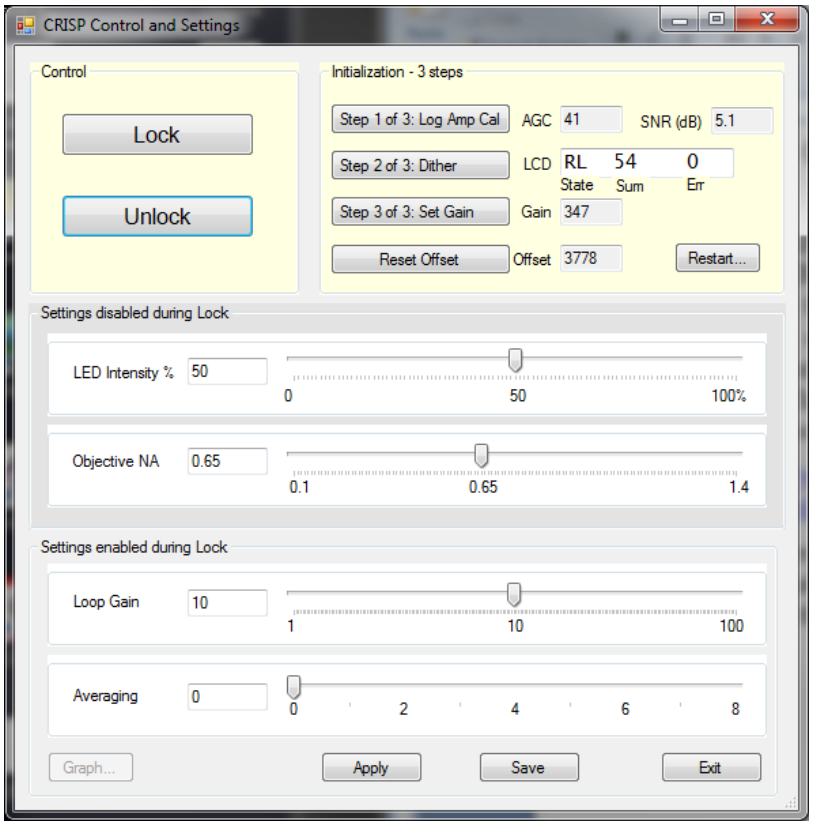
The **ASI Console** program has built-in support for the CRISP unit that makes it easy to setup and calibrate the CRISP unit. Using the **ASI Console** program eliminates the need to learn all of the special button presses to accomplish the calibration steps. The **ASI Console** program is available on the ASI web site at:

http://www.asiimaging.com/support/downloads/asi-console/ .

In operation, the CRISP control is found on the MORE tab. Clicking on the CRISP button will bring up the main CRISP control panel.

The main initialization steps are presented with three buttons. Lock and Unlock functions are provided as buttons as well. Set-up parameters are presented at the bottom of the CRISP window as sliders for setting LED intensity, Objective Numerical Aperture (used to determine the range of calibration moves), relative Loop gain and signal averaging.

Once you have calibrated the system with the three steps indicated, you may wish to obtain a plot of the focus curve. The Graph… button will generate the focus curve. The z-depth of the focus range for the graph is determined by the Objective NA setting – smaller NA, longer travel.



Computer Control of the CRISP System

The focus controller responds to several commands dedicated to controlling the feedback system. Please see the MS-2000 Programming Manual for further information about using serial commands.

Command: LOCK

Shortcut: LK

Format: LK [X] [Y] [Z=*lock_offset*] [F=*code*]

Function: The LOCK command without any arguments X, Y, or Z advances to the next focus state just as would a short-press of the @ button.

`LK X?` returns the single character indicating the current focus state as described in the table on page 2 of this manual.

`LK Y?` returns the present value of the focus error which is also shown on the LCD display.

`LK Z?` returns the present value of the focus error *lock_offset*. The offset is automatically determined during calibration and is modified when the command wheel on the controller is used to focus a locked system. The offset is also reset with a >10 sec. press of the @ button. A particular value of *lock_offset* may be set using **`LK Z=`** *lock_offset*.

`LK F=`*`code`* will unconditionally set the focus state. ***`code`*** is the ASCII decimal equivalent for the 'state' character that is displayed on the LCD. For example, to unconditionally enter the '**`B`**' state the command would be **`LK F=66`**. Not all states are best entered directly. See the system state table for the appropriate ASCII code to enter a particular state gracefully.

Reply: "**`:A`**" is returned upon receipt of the command.

Example: **`LK X?`**

`:A W` shows the system is in the WAIT state.

Command: UNLOCK

Shortcut: UL

Format: UL [X=*LED_Intensity*][Z=*Rel_LK_knob_spd*][F=*focus_index*]

Function: Without arguments, this command unlocks the servo from the focus system and returns control to encoder feedback from the Z-axis drive. The focus error *offset* is not changed.

The *LED_Intensity* may be set from 0 to 100 (%) of full power using the X argument. Default is 99.

Rel_LK_knob_spd (default 2) controls the sensitivity of the control focus knob when the system is locked. This will vary depending on the calibration factor that the system finds, so don't be alarmed if you find large sensitivity differences with conditions.

If the controller can handle more than one Z-axis focus device, you can specify the *focus_index* to select which one is active for the CRISP system. Save the parameter change (**`SS Z`**) and reset the controller.

Reply: "**`:A`**" is returned upon receipt of the command.

Command: LOCKRG

Shortcut: LR

Format: LR [X=*cal_gain*] [Y= *objective lens NA*] [Z=*lock_range*] [F=*cal_range*]

Function: The **`LOCKRG`** command allows the user to control of several system variables. The X parameter, *cal_gain*, is the gain variable normally obtained from running the calibration sequence. Although not recommended, it can be changed with this command, but it will be reset upon running the calibration sequence.

The Y sets the *cal_range* focus depth appropriately for a given objective's numerical aperture.

The Z parameter controls the maximum excursion of the stage before the system generates an error condition and unlocks. The value *lock_range* is in units of millimeters. The default value is 1.0 mm.

The F parameter controls the excursion of the stage when going through the calibration sequence. The default value for *cal_range* is 0.005 mm.

Reply: "**`:A`**" is returned upon receipt of the command.

Query: **`LR Z?`** returns the lock range.

```
A: Z = 0.050          (for example)
```

Command: KADC

Shortcut: KA

Format: KA [X=*n*] [Y=*n*] [Z=*n*] [F=*n*]

Function: Adjusts a gain multiplier in the CRISP servo loop where *n* is a signed integer.

Reply: "**:A**" is returned upon receipt of the command.

Query: **KA Z?** returns the current value.

:A Z=10 (for example)

Command: RT

Shortcut: RT

Format: RT [X=*report_time*] [Y=*pulse_length*] [Z=*delay_time*] [F=*num_aves*]

Function: The X argument Sets the time interval between report events when using *IN0_mode* = 5, TTL triggered serial interface asynchronous reporting. The *report_time* value has an acceptable range from 20 to 32700 milliseconds. The default value is 200 ms.

The Y argument sets the length of the TTL output pulse when using any *OUT0_mode* that generates a TTL pulse.

The Z argument sets the post-move delay time for sequenced arrays.

The F argument sets *num_aves*, the power-of-two exponent for the number of samples to be averaged. Used with the CRISP system.

Reply: "**:A**" is returned upon receipt of the command.

Command: AFLIM

Format: AFLIM [X=*Log_amp_AGC*] [Y=*LED_intesity_pot*]

Function: Use this command to directly read and write values (0 to 255) to the CRISP electronics digital potentiometers. (Not recommended for use with host software.)

Command: EXTRA

Format: EXTRA [X?] [Y?]

Function: X? Provides the CRISP bottom line string as is shown on the LCD display.

Y? Returns the SNR value shown on the LCD after log amp calibration.