

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 $<0.1 \mu\text{m}$. 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.

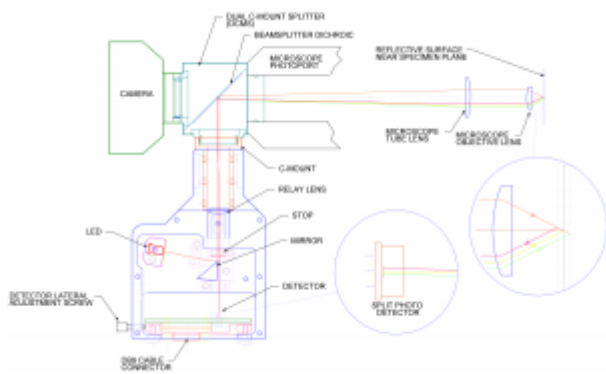


Figure 2: Schematic diagram of CRISP optical system

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 dichroic 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)	Typ. LED Power @50mA (mW)	FW HM (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	10	18	580-660	600	69216	600	84710	628/32	84087
L660-06	660	3	20	615-700	600	69216	600	84710	650/50	84774
L700-06	700	13	30	650-740	650	69217	650	84712	700/50	84775
L720-06	720	13	30	670-760	700	69218	700	84712	725/50	86943
L735-06	735	18	30	680-780	700	69218	700	84714	725/50	86943
L740-06	740	18	30	685-785	700	69218	700	84714	750/50	84776
L780-06	780	20	30	710-830	750	69219	750	64332	775/50	86944
TSHG8200	830	25	40	750-900	750	69219	750	64332	825/50	86945
TSHG5210	850	27	40	790-930	800	69220	800	64333	850/50	84778
TSFF5210	870	23	40	810-950	800	69220	800	64333	875/50	86946
TSHF5210	890	23	40	830-970	850	69221	850	64334	900/50	84779
L940-06	940	17	50	840-1040	900	69222	900	64335	950/50	84780
L970-06	970	5.5	50	910-1070	900	69222	900	64335	975/50	86948
L1050-06	1050	2.5	50	950-1130	1000	86695	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.

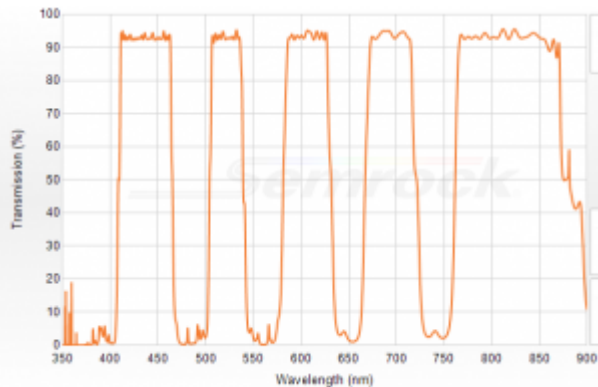


Figure 3: Semrock Dichroic FF408/504/581/667/762-Di01

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.

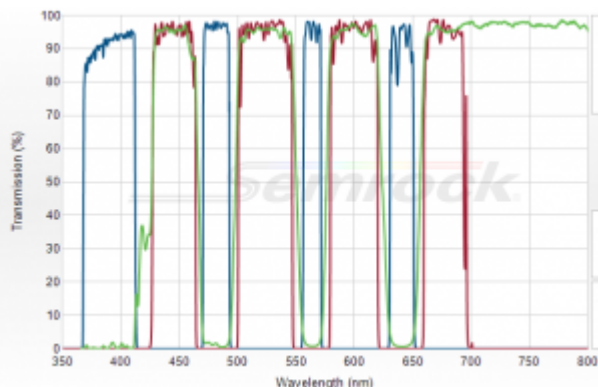


Figure 4: Semrock LF405/488/561/635-A-000

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 100 μ W (typically about 70 μ W) 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 \times 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 \times objective will expose some parts of the sample to a maximum of about 0.36W/mm² of IR radiation.

$$\begin{equation} \frac{\text{Total Power}}{\text{Area}} = \frac{100\mu\text{W}}{1/60 \text{ mm} \times 1/60 \text{ mm}} = 0.36 \text{ W/mm}^2 \end{equation}$$

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.



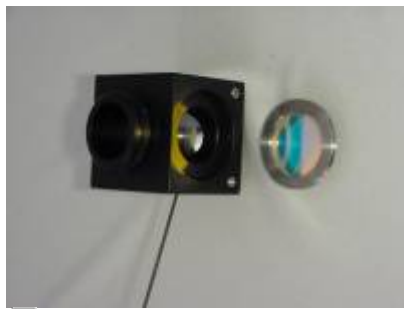


Figure 5: C-mount Splitter (DCMS) contains dichroic mirror and blocking filter

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.

CRISP Cable Connection

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

Pin-outs for this cable are shown below.

CRISP DB-9 Connector		
PIN	SIGNAL	INFORMATION
1	PD_CH1	Photodiode Channel 1
2	N.C.	Not Connected
3	GND	Ground
4	LED	LED control voltage
5	N.C.	Not Connected
6	PD_CH2	Photodiode Channel 2
7	+5V	+5V power
8	N.C.	Not Connected
9	N.C.	Not Connected

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

$$R = \frac{(n_1 - n_2)^2}{(n_1 + n_2)^2}$$

where n1 and n2 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.

Please note that prepared samples made with index matching mounting media will not work with the CRISP system. It is a common problem for new users to try to get CRISP to work on such samples and fail completely. Don't bother! A good test sample for oil objectives is just a fingerprint on a cover-slip-bottom dish with water in it.

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
PDMS	1.410	2.9	0.09	0.43
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.

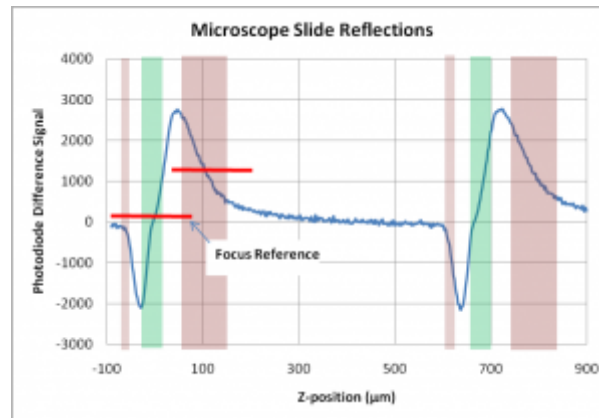


Figure 6: Photo detector difference signal for a scan through a microscope slide.

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.

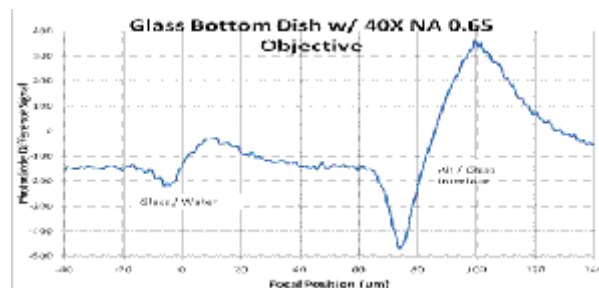


Figure 7: Reflections from a glass bottomed Petri dish.

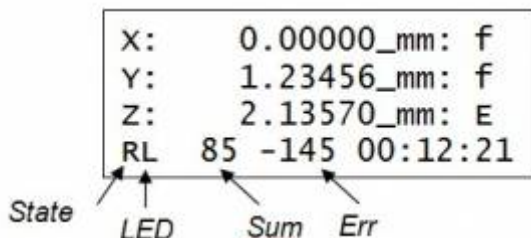
A common typical sample is a glass-bottomed Petri dish with a water sample. Here we can easily see the two reflections again, but note that the glass/water reflection is much less intense than the air/glass reflection.

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 Err signal. In the Dither state, the Err signal is the change in focus error as the focus is moved over the cal_range. In any other state, the Err number is the relative 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

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.)

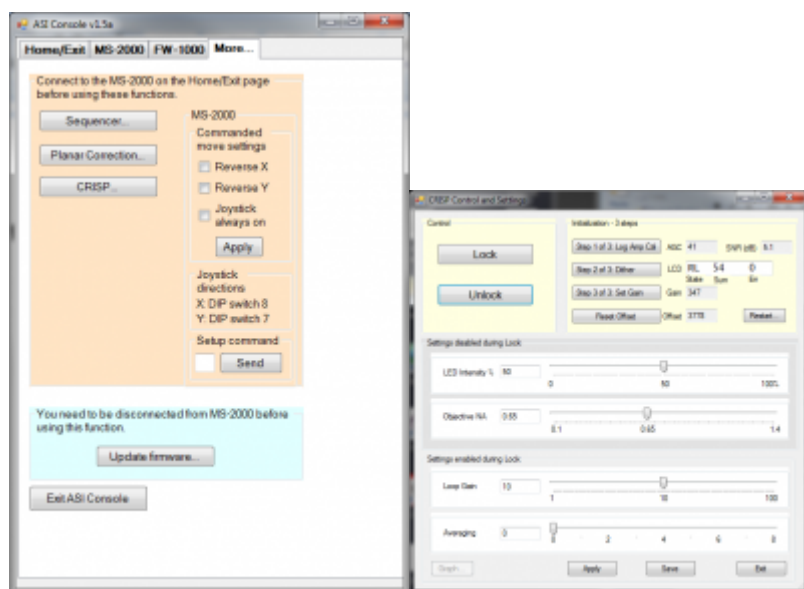
Table 2: CRISP system control states

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

State Character on LCD	LK F ASCII code	State Name	Next State (@ short)	Previous State (@ long)	Comment
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(k)	R	@ button unlocks
k		Locking	(F)	R	Seeking In Focus precision
F		In Focus	R	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.
†o	111 (o)	Set Offset	(R)		Resets focus offset to zero for present focal position.

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

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.

After 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.

Once the system is basically working, the Loop Gain slider is the easiest way to optimize the performance. If you have plenty of signal (dither Err number > 200) you can probably increase the Loop Gain to obtain faster focus and tighter focus position. If the system is marginally unstable, reduce the Loop Gain and it will become more stable.

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 Using ASI_Console

1. Download and install ASI_Console from the ASI website:
<http://www.asiimaging.com/support/downloads/asi-console/>
2. Using ASI_Console, connect to the MS2000 controller and navigate to the CRISP control panel via the More... page.
3. Follow the three step initialization and calibration procedure in the CRISP control window. Use the Lateral adjustment thumb screw to maximize the ERR signal for Step 2 dither.

Quick Start Instructions Using Controller Only

- 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.
- Press @ button for 3 seconds to initiate the 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

- Adjust the detector lateral adjustment screw on the CRISP unit for maximum absolute value of the focus error change. 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...

- 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 Err value. You should see Err respond proportionally to the change in focus, going positive in one direction and negative in the other.
- 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.
- 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 Err 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 Err 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 Err number.

Slowly adjust the detector lateral adjustment screw for a maximum absolute Err 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 Err reading results in a lower or higher Sum signal that you started with. If the Sum signal is outside the range 50-80 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 $\text{Cal Range} = \frac{1.5\mu\text{m}}{\text{NA}^2}$ where 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.
in_focus_mm	This parameter sets the point when the status changes from Locking (K or k) to In_Focus (F). Specified in the AFLIM Z=in_focus_mm command, using a smaller value will increase the time to achieve the level of precision desired for the state switch.

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.

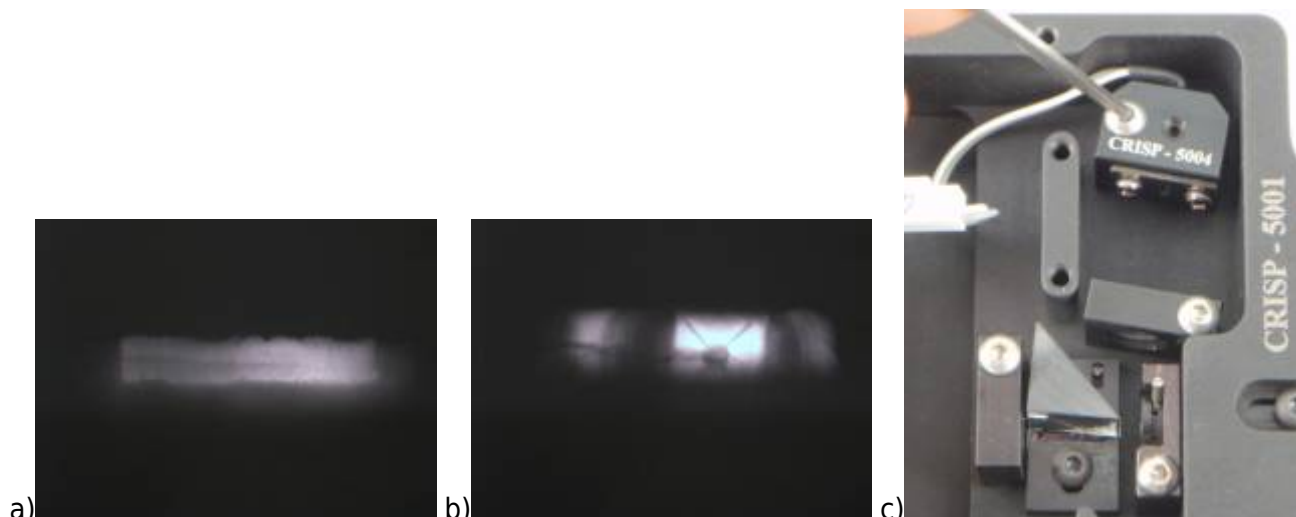


Figure 8: Reflection from glass slide of a) LED exit slit and b) focused deeper, the LED emitter, when the LED holder is properly aligned by moving c) LED holder.

Sometimes you will notice an unfocused glare from the LED in the camera. This can be due to light reflecting from within the microscope, often directly from the front surface of the tube lens or other windows in the light path. Adjusting the position of the LED slightly off center or with a small tilt can reduce this glare and significantly improve the performance of the CRISP unit.

Adjusting the Primary Mirror



Figure 9: Mirror Adjusting Screw

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.

One 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.

You should also find that when the detector and LED are aligned so that you are getting a reasonable dither ERR, you can adjust the mirror position to maximize the Sum signal. However, a high dither Err number is most important for proper operation, so be sure you can return to a good Err number if you use the Sum as a guide to the mirror adjustment. In general, you will need to adjust the Lateral offset adjustment thumb screw as you change the mirror position to maintain maximum dither ERR.

With the mirror properly adjusted, you should observe a strong lateral movement of the LED mask image in the camera as the focus is changed back and forth.

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:

$$D = \frac{\delta}{n} M^2$$
 For high magnification objectives, small focal plane changes can require substantial extension at the C-mount. Looking $5\mu\text{m}$ 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.



Figure 10: C-mount extension.

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.

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
10XNA 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 NA⁴/M². 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 Err signal remains relatively constant as you decrease the aperture, but the Sum signal decrease, reflecting the decrease in background light level. Decrease the aperture size until the Err signal begins to decrease, and then redo the log-amp calibration.

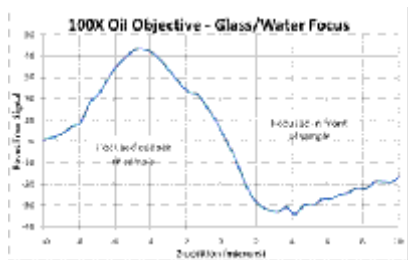
Using Focus Curve Generation for Optimizing Adjustments

Sometimes it is instructive to plot the focus error response so you can assess 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

 This feature is only available on MS2000 and RM2000 controllers



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 2n 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 Err difference in the Dither state, check these troubleshooting steps.

- Verify that the electronics are working. Using the CRISP control in ASI console...
- Set LED Intensity to 0%. Apply.
- 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.
- Set LED Intensity to ~70%. Apply. Save. Be sure you can get AGC value >35.
- 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.
- Verify that the IR light can reach the objective. Remove filter cubes if unsure of their properties.
- 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.
- If problems persist, achieve and image of the CRISP LED on the system camera by removing the IR block from the optical path. Usually there is sufficient light that leaks through the dichroic to be able to see the CRISP LED light on the camera when there is no other illumination. Verify that the CRISP LED light is present at the sample and go through the steps described in the Optical Adjustment section of this manual.

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:AFLIM \(AL\)](#) 2018/02/07 22:15
- [Command:EXTRA](#) 2016/09/07 22:03
- [Command:KADC \(KA\)](#) 2019/04/18 23:33
- [Command:LOCK \(LK\)](#) 2019/05/26 00:11
- [Command:LOCKRG \(LR\)](#) 2019/05/29 12:14
- [Command:RTIME \(RT\)](#) 2019/05/25 03:32
- [Command:TTL](#) 2019/05/25 22:32
- [Command:UNLOCK \(UL\)](#) 2018/06/04 22:00

TTL Control of the CRISP focus lock

TTL input function (TTL X=9)

In some instances it may be desirable to be able to turn on/off the CRISP lock using a TTL signal. Imaging software that does not support CRISP explicitly may support controller TTL I/O which can

provide a simple method to synchronize the autofocus function with other imaging functions.

The MS2000 TTL Input can be programmed for several functions. Use TTL X=9 to select the CRISP control function. (See the TTL command section in the MS2000 Programming manual for more details.)

Use ASI_Console, the @ button interface, or explicit serial commands to calibrate and set the CRISP system to the READY state (R on the LCD display). Once in the READY state, and with TTL X=9 selected, a logic HIGH on the TTL IN BNC connector will engage the CRISP lock. The lock will be maintained until the TTL IN signal goes to a logic LOW, at which point the system will unlock and return to the READY state.

TTL output function (TTL Y=11, 12)

You can also use TTL control to determine when the system is in focus. Use TTL Y=11 to have the controller send a TTL output pulse after any commanded move, providing CRISP is in the 'F' state. The controller will wait for the CRISP 'F' state after the move is completed before sending a pulse. Firmware version 9.2i+, the controller STATUS ('B' / 'N') will follow both the stage move and the CRISP transition to the 'F' stage to get the 'N' state when TTL Y=11 is set. The TTL output can be used to directly monitor if CRISP is in the 'F' state or not using TTL Y=12. TTL OUT0 is high when CRISP is 'F' state, low otherwise.

Serial polling of the CRISP focus lock

The TTL control discussed above can eliminate polling delays, but sometimes it is easier to implement serial polling. When XY stage motion and focus lock all need to be coordinated with image capture, the TTL Y=11 function will also enable the global controller busy state to include a successful focus condition before the 'N' Not Busy state is allowed. Polling the controller for busy status using '/' can provide a simple and efficient method of knowing if all stage axes and focus are ready. In the TTL Y=11 mode, there are a few pathological conditions that can develop. If a move is executed but no focus system is activated, then the 'B' state will remain indefinitely. The set-up command TTL Y=11, will reset the status to 'N' regardless of the stage or focus status. Initiating a move will change the status to 'B' where it will remain until both the move is completed and CRISP focus state is 'F', in-focus.

For any other TTL Y=n state (n≠11) the global status command will only respond to the XY stage busy status. If knowledge of both XY stage and CRISP functionality are desired, the individual axes status command RB X, RB Y, and the CRISP state LK X? can all be used to poll for the desired information.

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