

PureFocus 850 | Manual

Laser Autofocus System
V 2.5



Thank you for purchasing this product from Prior Scientific – we are confident it will be a reliable and useful addition to your microscope system. Please take the time to read and understand this manual before using this product – it contains not only important operating instructions but also vital safety information. Use this product only as specified in this manual. If you wish to use it differently, contact Prior Scientific beforehand.

Please do not hesitate to contact us with any comments or questions regarding this product.

Contents

| | |
|--|----|
| Section 1: Important Safety Information | 5 |
| 1.1 Important Safety Information | 5 |
| Section 2: Product Description | 6 |
| 2.1 What is the PureFocus850? | 6 |
| 2.2 Working Principle | 6 |
| 2.3 Typical sample types | 8 |
| Section 3: Hardware Installation | 9 |
| 3.1 Types of microscope system | 9 |
| 3.2 Types of Illumination | 9 |
| 3.2.1 Transmitted light/brightfield illumination | 9 |
| 3.2.2 Reflected light/epi illumination and fluorescence | 9 |
| 3.2.3 Phase contrast and DIC | 9 |
| 3.3 Unpacking your system | 9 |
| 3.4 Installing your PF850 mounting kit | 11 |
| 3.5 Installing the PF850 | 11 |
| 3.5.1 Installing the PF850 head | 11 |
| 3.5.2 Connecting the PF850 controller | 11 |
| 3.5.3 Pre-alignment checklist | 12 |
| 3.6 PF850 Controller Guide | 12 |
| 3.6.1 Controller Modes | 12 |
| 3.6.2 Display Indicators | 13 |
| Section 4: Optical Alignment | 13 |
| 4.1 Aligning the PF850 with the optical path | 14 |
| 4.2 Laser setup considerations | 17 |
| 4.2.1 Biological samples | 17 |
| 4.2.2 Samples requiring reflected light | 17 |
| 4.3 Setting the sensor (pinhole) centre | 17 |
| 4.3.1 Sensor centre setup for slice mode systems | 17 |
| 4.3.2 Sensor centre setup for line mode systems | 18 |
| 4.3.3 Laser Setup Troubleshooting | 20 |
| 4.4 Creating signal imbalance | 21 |
| 4.5 Removing background signal | 23 |
| Section 5: Focusing System Setup | 24 |

| | |
|--|----|
| 5.1 Stepper motor focusing system | 25 |
| 5.2 Piezo nanopositioning focusing system | 25 |
| Section 6: Autofocus Parameter Setup | 26 |
| 6.1 Installation Review | 26 |
| 6.2 Objective selection | 27 |
| 6.2.1 Focusing on the sample | 27 |
| 6.3 Setting the offset | 28 |
| 6.3.1 Automated offset discovery | 28 |
| 6.3.2 Manual offset discovery | 30 |
| 6.3.3 Checking error swing | 32 |
| 6.4 Checking for back reflections | 32 |
| 6.5 Setting the focus recovery speed | 34 |
| 6.5.1 Stepper motor control | 35 |
| 6.5.2 Piezo focusing system control | 35 |
| 6.5.3 D-gain | 37 |
| 6.6 Fine offset adjustments | 37 |
| 6.7 Saving offset values | 37 |
| 6.8 Parameter setup for remaining objectives | 37 |
| 6.9 Saving your settings | 38 |
| 6.10 Flags | 38 |
| Section 7: Advanced features | 38 |
| 7.1 Non-typical sample types | 38 |
| 7.2 Signal to noise | 38 |
| 7.2.1 Optimising signal to noise | 39 |
| 7.2.2 Signal to noise considerations | 41 |
| 7.3 Enhanced focus control via Focus Flag | 42 |
| 7.3.1 Focus Stability | 42 |
| 7.3.2 Low depth of focus objectives | 42 |
| 7.3.3 Two-factor focus verification | 43 |
| 7.3.4 Testing focus recovery time | 43 |
| 7.4 Sample detection via Sample Flag | 43 |
| 7.5 Using multiple offsets | 44 |
| 7.6 Software limits via Range Flag | 44 |
| Section 8: OEM features | 45 |
| 8.1 Interface selection via interface flag | 45 |
| 8.2 Focus search | 47 |
| 8.2.1 Search and Lock | 47 |
| 8.2.2 Fast Capture | 48 |
| 8.3 Measure mode | 48 |
| Section 9: ASCII command sets | 49 |

| | |
|--|-----------|
| 9.1 Signal settings commands | 49 |
| 9.2 Focus signal commands | 50 |
| 9.3 Servo settings commands | 50 |
| 9.4 Flag settings commands | 51 |
| 9.5 Objective parameters commands | 52 |
| 9.6 Digipot settings commands | 53 |
| 9.7 Focus commands | 53 |
| 9.8 System commands | 55 |
| 9.9 Advanced commands | 56 |
| 9.10 Error Codes | 57 |
| Section 10: Troubleshooting | 58 |
| 10.1 No laser line emitted visible on the target | 58 |
| 10.2 Laser signal is too high or too low in setup mode | 58 |
| 10.3 Additional peaks are visible in setup mode | 59 |
| 10.4 Background signal in setup mode is high or uneven | 59 |
| 10.5 No or imbalanced error value swing | 62 |
| 10.5.1 No error value swing | 62 |
| 10.5.2 Imbalanced error swing | 63 |
| 10.6 Focus recovery is too fast or focus unstable | 63 |
| 10.7 Focus recovery is too slow | 63 |
| 10.8 Focus recovery does not occur despite a good error value swing | 63 |
| 10.9 Performance is good but the flags are inactive | 63 |
| 10.10 Focus locks when my sample is not in focus | 64 |
| 10.11 A suitable offset cannot be calculated for an objective | 65 |
| 10.11.1 The offset is outside the range specified for my magnification | 65 |
| 10.11.2 Insufficient offset range | 66 |
| 10.12 Z Scan | 68 |
| Section 11: Spare parts, repairs and returns | 69 |
| Section 12: Controller Z connector Pinout | 70 |
| Appendix 1: Windows USB Driver Update | 71 |

Section 1: Important Safety Information

1.1 Important Safety Information

Class 1 laser product, laser wavelength 850 nm, laser output < 0.77 mW

CLASSIFIED TO BS EN 60825-1:2014

It is important to follow these safety warnings to avoid potential injury or damage. Please read and understand these warnings, operating instructions and specifications before using the PureFocus850. If you have any questions do not hesitate to contact Prior Scientific. If you intend to use this unit in a manner not specified by Prior in this manual, contact Prior Scientific beforehand.

SAVE THIS MANUAL AS IT CONTAINS IMPORTANT INFORMATION AND INSTRUCTIONS.

Before using the system, please follow and adhere to all warnings, safety and operating instructions located either on the product or in this User's Manual.

- Do not expose the product to water or moisture.
- Do not expose the product to extreme hot or cold temperatures.
- Do not expose the product to open flames.
- Do not allow objects to fall on or liquids to spill on the product.
- Do not touch the glass plate fitted between the circular dovetail and the top plate. Any dust, dirt, fingerprints will cause degradation of image quality.
- Do not poke inside the open aperture in the base plate of the unit. There are delicate optical components which are easily damaged if touched.

WARNING. This unit emits non-visible laser radiation at 850 nm from the dichroic aperture as indicated by a warning label on the unit. The total output power is below the class 1 emission limit of 770uW and is therefore eye-safe. However, staring into the aperture should still be avoided.

The supplied mains adaptor must always be used with an earthed mains socket. The equipment should be positioned in such a way that the mains switch, power supply and system power switch are easily accessible.

If the equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired.

Only the exterior of this product should be cleaned using a damp lint-free cloth. If internal contamination is suspected, please contact your supplier for advice.

DANGER. Under no circumstances unscrew the lid of the unit. Disassembly of the unit will void the warranty. This product does not contain consumer serviceable components. Service and Repair should be performed by authorised service centres only.

Use only the proper type of power supply cord set (provided with the system) for this unit. Failure to do so could instantly destroy the electronics and laser diode. The unit requires 24 VDC at 2 Amperes.

Always switch off the unit using the on/off switch SW1 or unplug the PSU (CON3) when plugging/unplugging the stepper motor (CON4) or DIGIPOT (CON2). It is safe to plug/unplug the USB connector (CON1) with the unit powered.

Keep this manual in a safe place as it contains important safety information and operating instructions.

Section 2: Product Description

2.1 What is the PureFocus850?

The Prior Scientific PureFocus850 is an advanced, integrated, unit comprising of an IR laser diode, precision optical components, detector and signal processing electronics with on-board micro controller. The system allows optimum visual focus to be found and maintained on a microscope system for a range of different sample types, microscope objectives and imaging methods.

The PureFocus system allows powerful automated autofocus functionality to be added to existing microscope systems by installing the unit into the infinity space (between objective and tube lens). The system has been designed to fit on many popular microscopes using infinity corrected optics, both upright and inverted types, using the relevant mounting kit. The PureFocus controller outputs signals suitable for controlling piezo or motor focus drives and is compatible with Prior piezo actuators and Prior stepper motors, by simply attaching to the fine focus knob of the microscope.

With the laser autofocus system the user has the ability to work with a range of sample types with a reflective surface, including permanently mounted glass slides, live specimens in aqueous solution, metallurgical, semiconductors and other samples with multiple reflective layers. The system can also work with plastic vessels such as well plates.

PureFocus works with both epi and transmitted illumination, and can be used for fluorescence applications with the 850 nm source being outside of most fluorescence bands.

A fully standalone system gives the end user the option of using the PureFocus controller with digipot, display and buttons allowing all basic functionality options without the need for a host PC. The inbuilt signal processing electronics generates focus correction information internally every 1 ms allowing for fast focus capture and tight closed loop action. For more advanced functionality PureFocus can be fully remote controlled via USB communication, using our ASCII commands set.

2.2 Working Principle

The PureFocus operates using a sensor with multiple pixels. Half the aperture of a collimated laser beam is blocked via a knife edge and directed into the back of the microscope objective. The laser light is focussed to a line on a reflective surface at the sample and then reflected back through the objective and directed towards the sensor forming a corresponding line on the sensor at the centre point. Due to half the aperture of the laser being blocked, motion of the reflective sample up or down causes this line to move either left or right on the sensor, giving information to automatically control the focus of the microscope and keep the sample in focus (Fig. 1).

Due to the nature of the line sensor there is freedom to choose what range of pixels either side of the centre point are used in calculating a focus error signal. This allows the rejection of reflections from spurious reflective surfaces and offers great flexibility when dealing with various different samples.

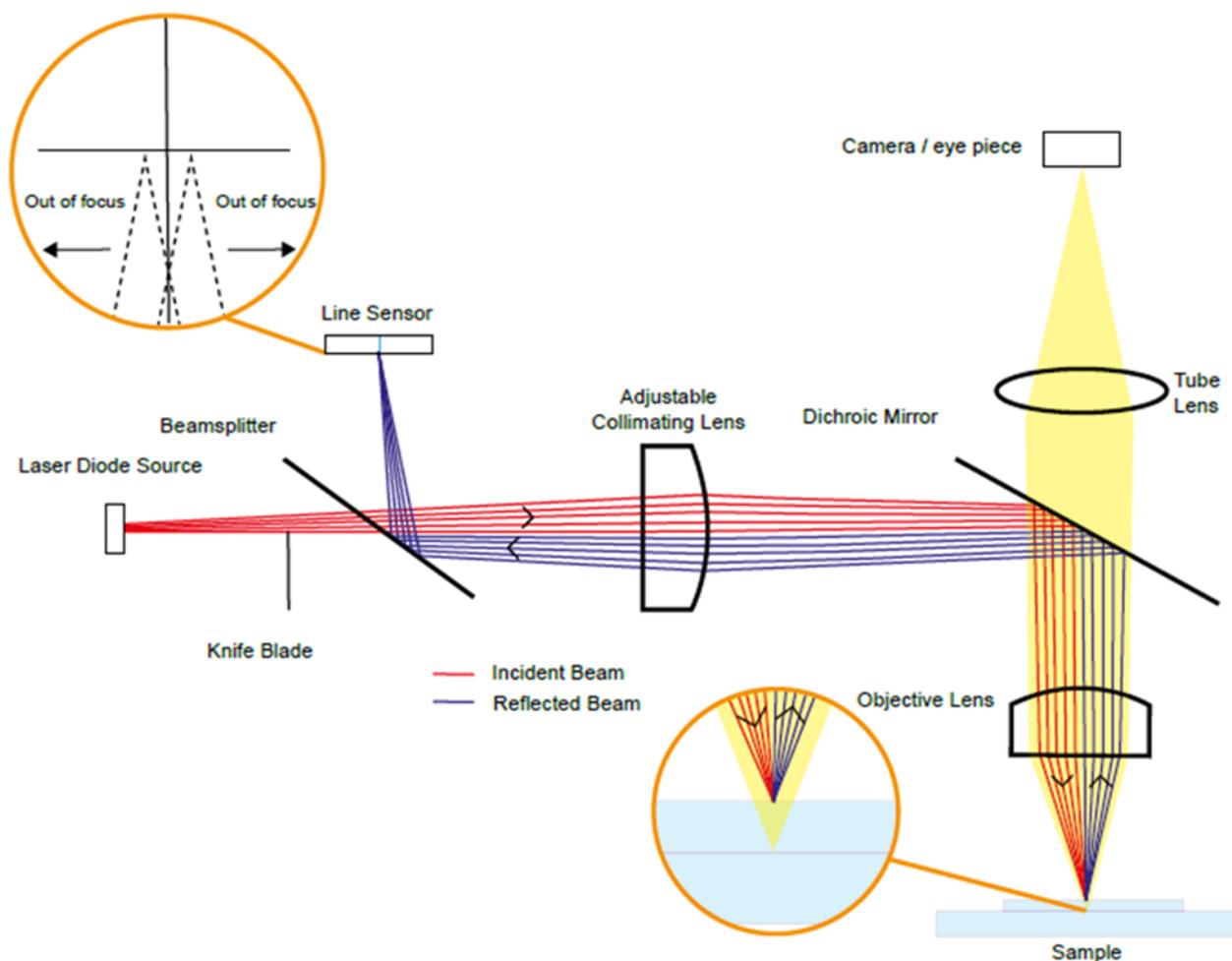


Figure 1: Principle of operation

The addition of adjustable laser collimation also allows the reflective surface, used by PureFocus for holding focus, to be located at a different plane to the microscope's imaging plane, where the specimen resides. This allows for continuously variable offsets to be added. This is especially useful when dealing with biological samples where the specimen will reside under a coverslip, which has a reflective top surface (Fig. 2).

To form a focus error signal firstly the system sums the pixel values to the left and right of a defined centre pixel on the sensor, these summations are named A and B respectively. The number of pixels that are summed either side of the centre to generate A and B can be chosen. The command PINHOLE allows the centre point and width to be set. The sensor has 1500 pixels so the maximum possible width is 750 pixels, for a centre point in the exact centre of the sensor at pixel 750.

The position signal is then computed as

$$POS = (A-B)/(A+B)$$

which is a signal that nominally swings between -1 and +1. A target value is subtracted from the position signal to form an error signal

$$ERROR = TARGET - POS = TARGET - (A-B)/(A+B)$$

This signal is fed into a PID controller and the output of this controller is sent either to a Prior stepper motor or to an analogue voltage for driving an external piezo controller.

PureFocus also computes two further values from the line sensor, C and D, which aid in operation. C is simply the value of the centre pixel. D is the summation of pixels across an arbitrary section of the sensor, which is settable to any range of pixels. This D value is useful for detecting if the system is focussed to the correct interface when working with samples that contain more than one reflective surface.

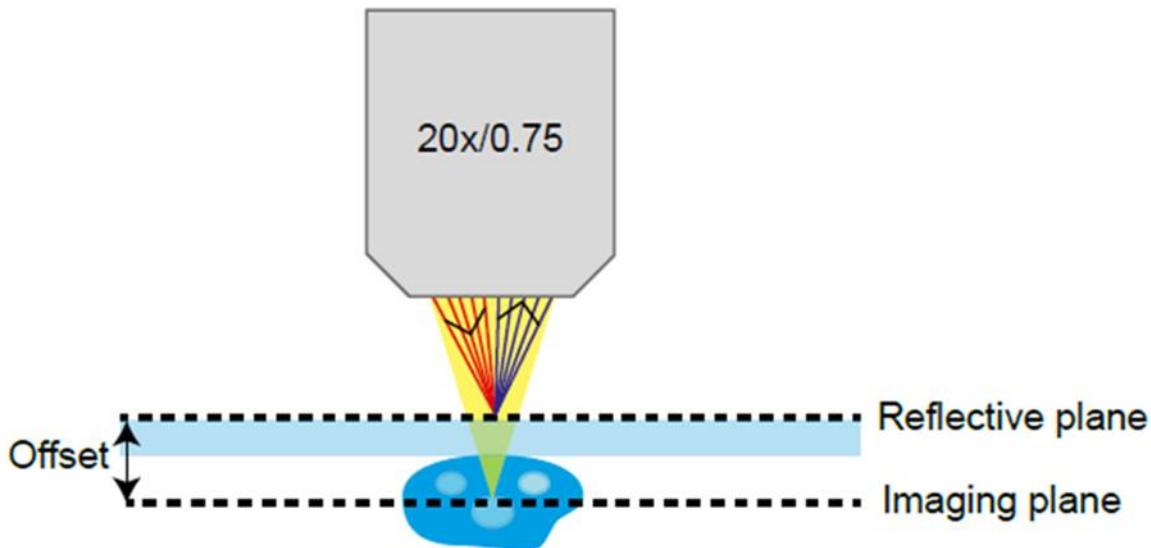


Figure 2: Offset principle

2.3 Typical sample types

The PureFocus850 is designed with these common sample types in mind:

- Permanent fixed biological slides with a 1.5 thickness coverslip
- Cell culture dishes/wellplates
- Wafer samples e.g. semiconductor
- Flat metallic samples

Other sample types are discussed in section 7.1.

Section 3: Hardware Installation

3.1 Types of microscope system

PureFocus850 works on a range of different microscopes which use infinity corrected optics. The PureFocus head sits in the infinity space of the optical path between the objective and tube lens.

On inverted microscopes where the objective moves and the sample is fixed the PureFocus head sits directly behind the objective nosepiece moving with the objective nosepiece as it changes focus height, this is achieved using a mounting kit for the particular microscope being used.

On upright microscopes where the objective is fixed and the sample moves the PureFocus head can sit at any position in the infinity space of the optical path, remaining fixed to the body of the microscope using a dovetail kit for the particular microscope being used.

3.2 Types of Illumination

3.2.1 Transmitted light/brightfield illumination

When working with transmitted light illumination, PureFocus could be susceptible to illumination light reaching its sensor. In this case it is better to set the illumination light source to the lowest brightness that still gives an acceptable image at the camera/eye pieces.

3.2.2 Reflected light/epi illumination and fluorescence

For reflected light illumination and fluorescent applications it is beneficial to position the PureFocus head below beam splitters and fluorescence filter cubes which could attenuate or prevent the 850 nm PureFocus light reaching the sample.

3.2.3 Phase contrast and DIC

PureFocus can work with a range of phase contrast objectives. The polarisation optics in DIC microscopes must be positioned after the PureFocus head to be compatible.

3.3 Unpacking your system

All standard PureFocus850 systems contains the following equipment:

| Part Number | Description |
|-------------|---------------------------------|
| PF185(M) | PureFocus850 Head (Line Mode) |
| PF100* | PureFocus850 Controller |
| PF400 | Head-controller connector cable |
| H407 | Controller power supply |
| W3045 | USB cable |

*Contact Prior to control the Purefocus head with OEM controller.

The following items will need to be purchased separately to PF850(M). Each system will require a target, setup slide and setup camera for optical alignment of the PureFocus850 infrared laser beam with the microscope. Each target has a range of thread sizes in order to be compatible with a variety of microscope nosepieces.

| | |
|-------|---|
| PF200 | Alignment target for RMS, M26 and M32 threads |
| PF201 | Alignment target for M25, M27 and M32 threads |
| PF300 | PureFocus850 Setup Camera |
| PF209 | PureFocus850 Setup Slide |

If purchased alongside a piezo nano-positioning system, a specialist cable will be provided in order to connect the system to the PureFocus850 controller.

| | |
|-------|---------------------------------|
| PF404 | BNC to 15-pin D-connector cable |
|-------|---------------------------------|

Different microscopes require different mounting kits in order to install the PureFocus850.

| Part Number | Description |
|-------------|--|
| LF335 | Flange set for Olympus upright microscopes |
| LF320 | Flange set for Nikon upright microscopes |
| LF310 | Flange set for Leica upright microscopes (contact Prior) |
| LF312 | Flange set for Leica upright microscopes (contact Prior) |
| LF341 | Flange set for Zeiss upright microscopes (contact Prior) |
| PF202 | Mounting kit for Olympus IX73 (2-deck) |
| PF208 | Mounting kit for Olympus IX73 (2-deck) |
| PF214 | Mounting kit for Olympus IX73 (1-deck) |
| PF203 | Mounting kit for Olympus IX71 |
| PF213 | Mounting kit for Olympus IX81 |
| PF211 | Mounting kit for Nikon Ti |
| PF212 | Mounting kit for Nikon Ti2 |
| PF210 | Mounting kit for Olympus BX41/43/51/53/63 |

If the PureFocus850 mounting kits above are not suitable for your microscope, please contact Prior Scientific.

3.4 Installing your PF850 mounting kit

Please follow the dedicated guides for each mounting kit for full instructions, available at <https://www.prior.com/download-category/help-sheets-installation-instructions>.

3.5 Installing the PF850

3.5.1 Installing the PF850 head

The mounting kit installation guides cover the specifics of how and where to mount the various components directly onto the PF850 head and onto the microscope.

Special considerations should be made depending on the imaging technique and if you have purchased a line mode system.

| Factor | Action |
|----------------------------|--|
| Fluorescence imaging | Mount the PF850 head between the nosepiece and the beam splitter/filter cubes. Additional mounting brackets may be required. |
| Phase contrast/DIC imaging | Mount the PF850 head between the nosepiece and any DIC optics. Phase objectives are compatible with the PF850. |
| Line mode | For upright microscopes, mount the PF850 at 30-60 degrees with respect to the arm it is mounted on. This maximises the effectiveness of the line mode focus-recovery algorithm. For inverted microscopes, the orientation of the PF850 is fixed, meaning the sample should be oriented so that any linear features are viewed at 30-60 degrees with respect to the orientation of the PF850 head |

3.5.2 Connecting the PF850 controller

Once installed, connect the head to the controller via the PF400 cable.

Connect the controller to the mains using the H407 power supply.

Connect the focus system to the 15-pin D-connector on the PureFocus850 controller. Use the PF404 cable to link BNC outputs from piezo nano-positioning systems to this connector.

Connect the controller to the PC via the USB cable provided (Fig. 3).



Figure 3: Controller and head connections

3.5.3 Pre-alignment checklist

Download the PureFocus850 GUI application (<https://www.prior.com/download-category/software>) and help sheet (<https://www.prior.com/download-category/help-sheets-installation-instructions>).

Insert the alignment target into the nosepiece.

Insert a dustcap into the nosepiece if available. If a suitable dustcap is unavailable, leave an empty position on the nosepiece.

Insert your required objectives into the remaining positions on nosepiece.

Turn the blade screw clockwise until it reaches the limit. It is set close to the limit during factory processing. Do not force the screw.

Switch on the controller and start the PureFocus850 GUI. Reset the PureFocus to factory defaults by clicking Parameters > Factory Reset. This will close the GUI.

3.6 PF850 Controller Guide

The controller complements the GUI by allowing manual input of a number of instructions. This allows the PF850 to be used independently from computer control during day-to-day operation, once setup is complete.

3.6.1 Controller Modes

The controller can be toggled into various modes using the Servo On/Off and Offset/Focus buttons. There are four mode combinations, which are indicated on the right hand side of the controller display.

The Servo On/Off button activates or deactivates the servo, which is responsible for holding the sample in focus. This changes the controller display; SRV indicates servo activation and MAN indicates servo inactivity. Whilst in SRV mode, the digipot cannot be used to control the focusing mechanism as the PureFocus850 repositioning algorithm is actively controlling it.

Offset/Focus button allows control of the focusing mechanism or the PureFocus offset lens mechanics using the digipot. Which piece of hardware is being controlled is indicated by the controller display; FOC indicates control of the focusing mechanism and OFF indicates control of the offset lens mechanics.

See below for the functionality of these mode combinations.

| Mode combination | Functionality |
|------------------|---|
| MAN FOC | The servo is inactive and the digipot controls the focus mechanism. |
| SRV FOC | The servo is active and the PF850 is automatically focusing on the sample. <i>Moving the digipot has no effect and no moves will occur on servo deactivation.</i> |
| MAN OFF | The servo is inactive and the digipot controls the offset lens mechanics. Focus position cannot be changed in this mode. This mode is primarily used during manual setup. |
| SRV OFF | The servo is active and the digipot controls the offset lens mechanics. Moving the offset lens will cause a change in focus position in real time, as the servo causes the focus motor to move a distance corresponding to the increase or decrease in the magnitude of the offset. |

The function button currently is not used. It may be utilised for OEM applications.

3.6.2 Display Indicators

The Obj Select button changes the parameters used to control the servo to match the objective that is currently in use. Repeatedly pressing this button loads the parameters for objectives 1 to 6 with reference to the GUI, and will display OBJ1-OBJ6 corresponding to which settings are currently in use.

When the PureFocus850 is holding focus in SRV mode, this is indicated by the appearance of the letter F, which appears next to the objective number indicator. This indicator is dependent on the focus flag discussed later in this document (see advanced features).

If no sample is detected by the PureFocus850 (in any mode), this is indicated by the appearance of the letter NS next to the objective number indicator. This overwrites the 'F' focus indicator even if the sample is in focus. This is dependent on the sample flag discussed later in this document (see advanced features).

Section 4: Optical Alignment

The PF850 is mounted in the infinity space of the microscope. In order perform correctly it needs to be aligned with the optical path. First, the refractive and reflective optical elements of the PF850 head must be aligned with the microscope objectives. The focal point of the laser on the sensor must then

be determined, half of the laser blocked in order to generate signal imbalance either side of focus, and the average background illumination reaching the PureFocus850 sensor calculated. Optical alignment of the system is only required once.

4.1 Aligning the PF850 with the optical path

Rotate or move the nosepiece to place the alignment target into the optical path.

Place the PF300 alignment camera onto the microscope stage using an appropriate sample holder. Connect the camera to the PC via the USB, using the 1.8-metre extension lead if required. Move microscope stage to manoeuvre the camera close to the centre of the alignment target (Fig. 4).

Open the ‘camera’ application, select USB Camera 2.0, and then click start.

Start the PureFocus850 GUI. Go to the offset submenu and select ‘Go to Limit’. The offset lens will move in the PureFocus850 head.



Figure 4: Positioning the setup camera (PF300) and target (PF200/1)

A pink laser signal should be visible on the camera. Ensure the centre of the target is in the centre of the field of view. Set the PureFocus850 controller into MAN OFF mode and rotate the digipot to extend the laser signal to the approximate diameter of the first visible ring of the target.

Open the laser submenu in the PureFocus850 GUI and adjust the laser power to ensure the signal is clearly visible but not reflecting off the target. Typical values for this are 400-1000, however this varies between systems and values outside of this range are acceptable if required.

To set the alignment correctly, the setup lens, 45 degree and 0 degree screws are required (Fig. 5).

Using a flathead screwdriver, rotate the setup screw to reduce the spread to the laser signal to a minimum (Fig. 6).

Using the 45° screw, adjust the lateral position of the laser line so that it aligns over the centre of the

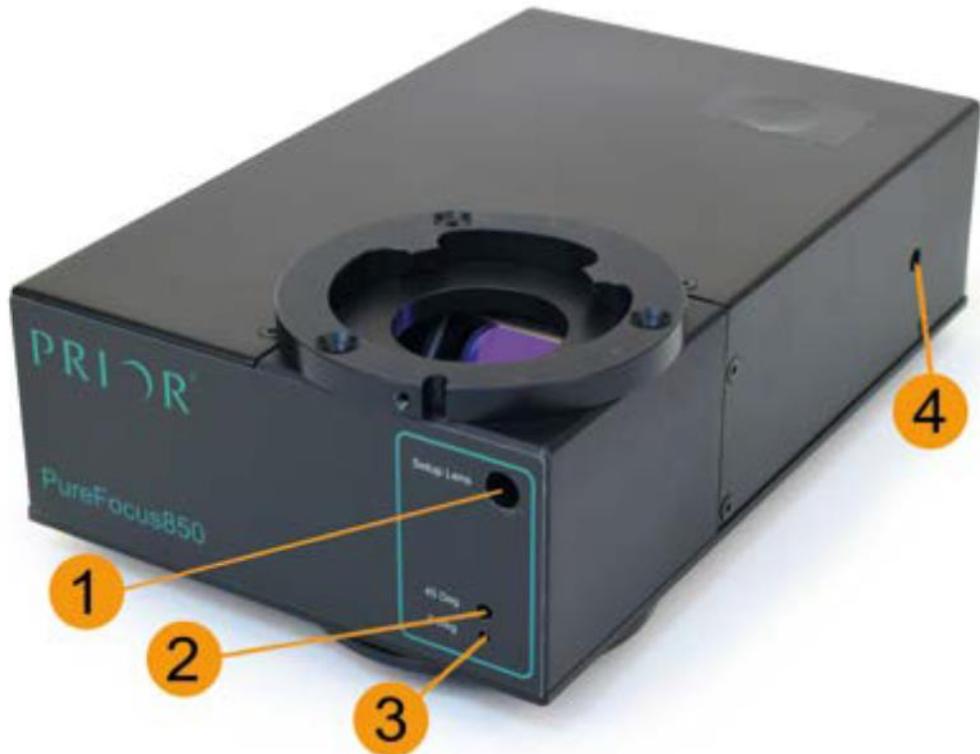


Figure 5: Setup (1), 45 degree (2), 0 degree (3) and blade (4) adjustment screws

target (Fig. 7).

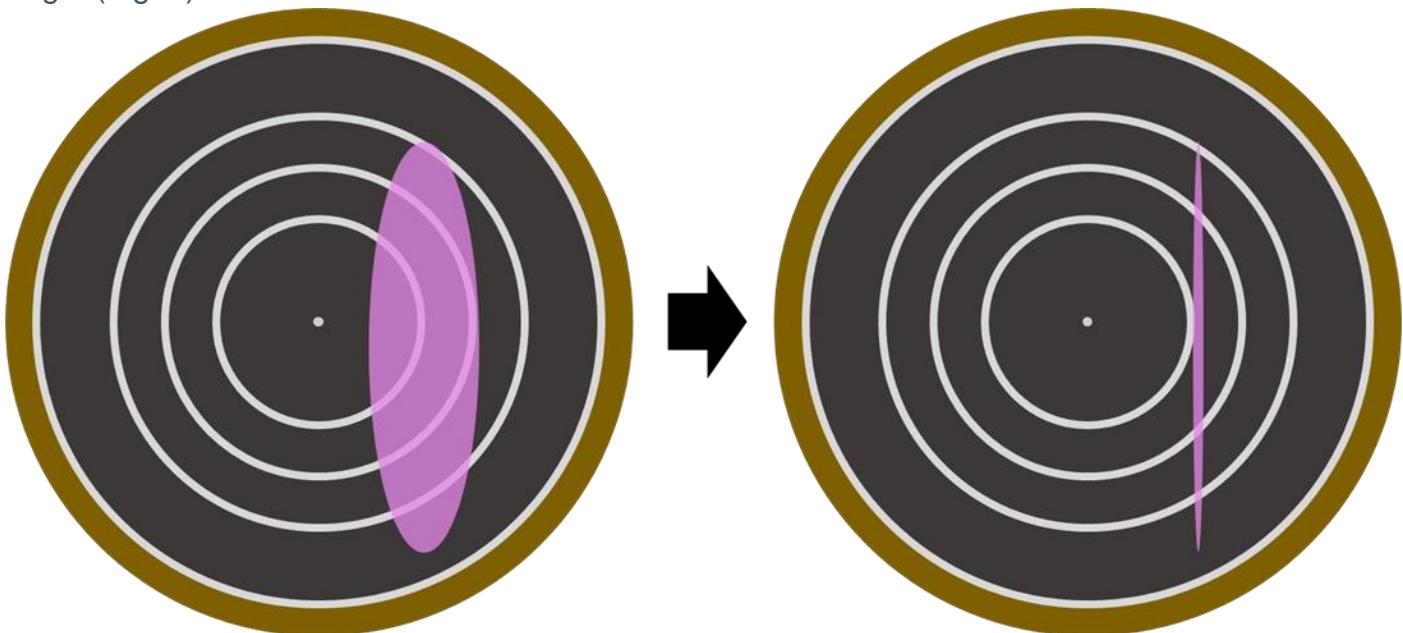


Figure 6: Focus the beam on the target using the setup screw

Using the 0° screw adjust the position of the laser line so that equal halves of the line are on either side of the centre of the target. Adjust the length of the line using the digipot if necessary.

Make any final adjustments to both the 45° and 0° screws to ensure optimal alignment of the laser line

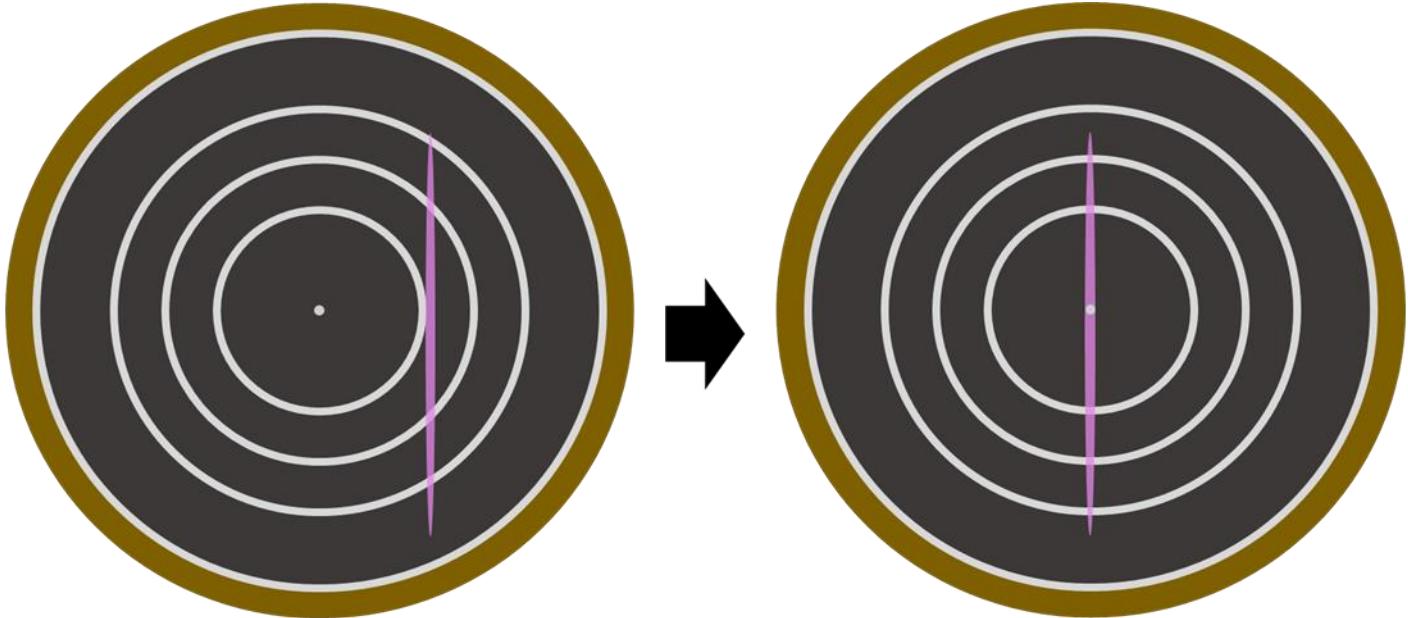


Figure 7: Align the laser with the centre of the target using the 45 degree adjustment

with the target. Once completed, remove the camera from the microscope stage and the alignment target from the nosepiece.

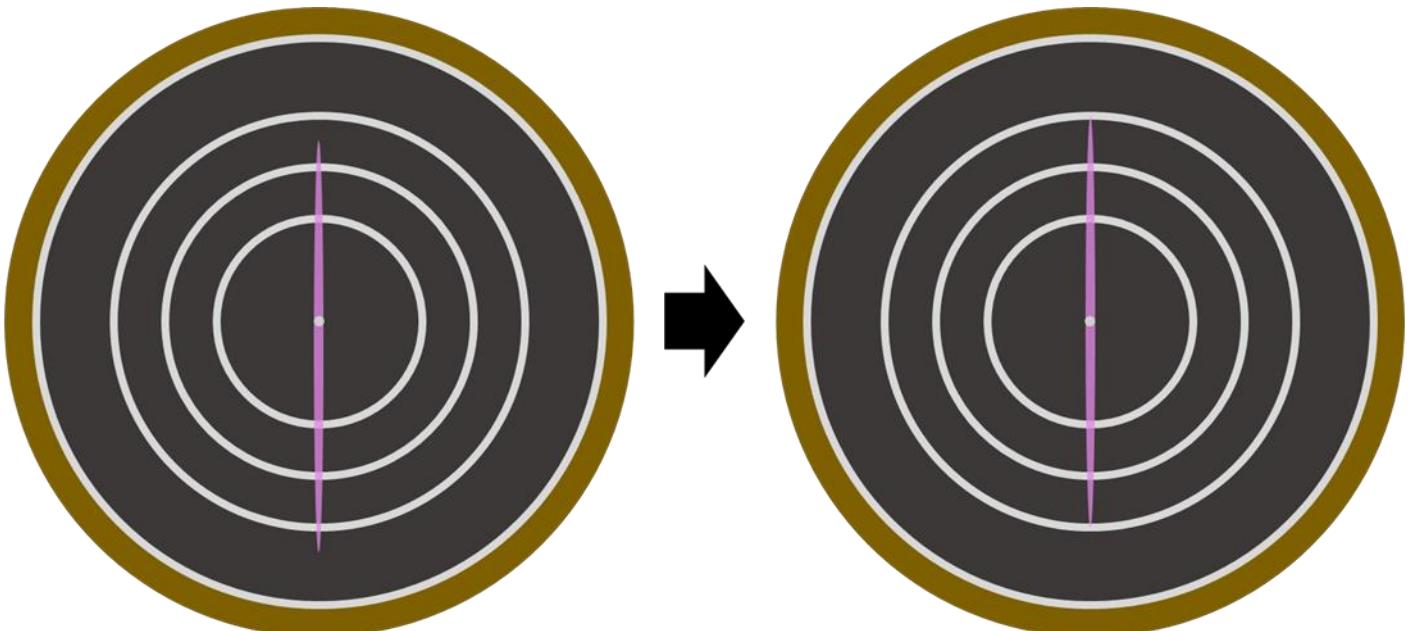


Figure 8: Balance the laser line equally across the centre of the target using the 0 degree adjustment

4.2 Laser setup considerations

Before setting up the laser to allow autofocus function, consider the nature of your microscope system. The PF209 setup slide used for this procedure has a number of different options. Please note that Line mode systems (PF850M) have a slightly different procedure than spot mode systems.

4.2.1 Biological samples

Use the coverslip sample, which most closely mimics permanent slides or petri dish samples. Transmitted light only (diascopic) microscope systems can only be setup using the coverslip sample. Alternatively, the mirror sample can be used if sufficient ambient light is available to illuminate the sample. If using a reflected light technique (e.g. fluorescence) for the majority of imaging, the mirror sample can be used. The sample provided on the PF209 also may not fluoresce at the required wavelength.

4.2.2 Samples requiring reflected light

Use the mirror sample, which is the simplest reflective sample. This sample closely mimics wafers. When using biological samples on microscopes without diascopic illumination, Prior Scientific recommends this sample for ease of use. Please note that large increases in laser power may be required when setting up the samples you intend to image.

4.3 Setting the sensor (pinhole) centre

Please refer to section 4.3.1 for slice mode systems (PF850) and section 4.3.2 for line mode systems (PF850M).

4.3.1 Sensor centre setup for slice mode systems

In the PureFocus850 GUI, go to the offset submenu and click ‘Go to Factory Home’. The offset lens will move. Go to the laser submenu and increase the value to 100. Click set.

Go to the mode submenu and ensure slice mode is selected.

Set the PureFocus controller to MAN FOC mode.

Move an objective into the light path. Prior Scientific recommends a 10x objective for this procedure due to the working distance relative to higher magnifications, but any objective of higher or lower magnification can be used.

Place the PF209 onto the stage and focus on either the coverslip sample or the mirror sample. Use the digipot, in MAN FOC mode, to achieve focus.

Go to the setup submenu, which will open a graph that indicates the laser intensity over the PureFocus850 sensor. Adjust focus until a peak appears in the signal. Make this peak as sharp as possible. It is optimal for the peak to fill almost the entire y-axis of the graph, which can usually be achieved by increasing or decreasing the laser power. See section 4.3.3 for full details.

Click the Auto Set Centre button, and click ‘Yes’ in the following pop-up box. This will set the centre of the PureFocus850 sensor, which is used to positively verify whether the sample is in focus (Fig. 8).

The blue line, which indicates the pinhole centre, will move to the middle of the peak. Half of the total signal will be on the left side of the line, and other half will be on the right.

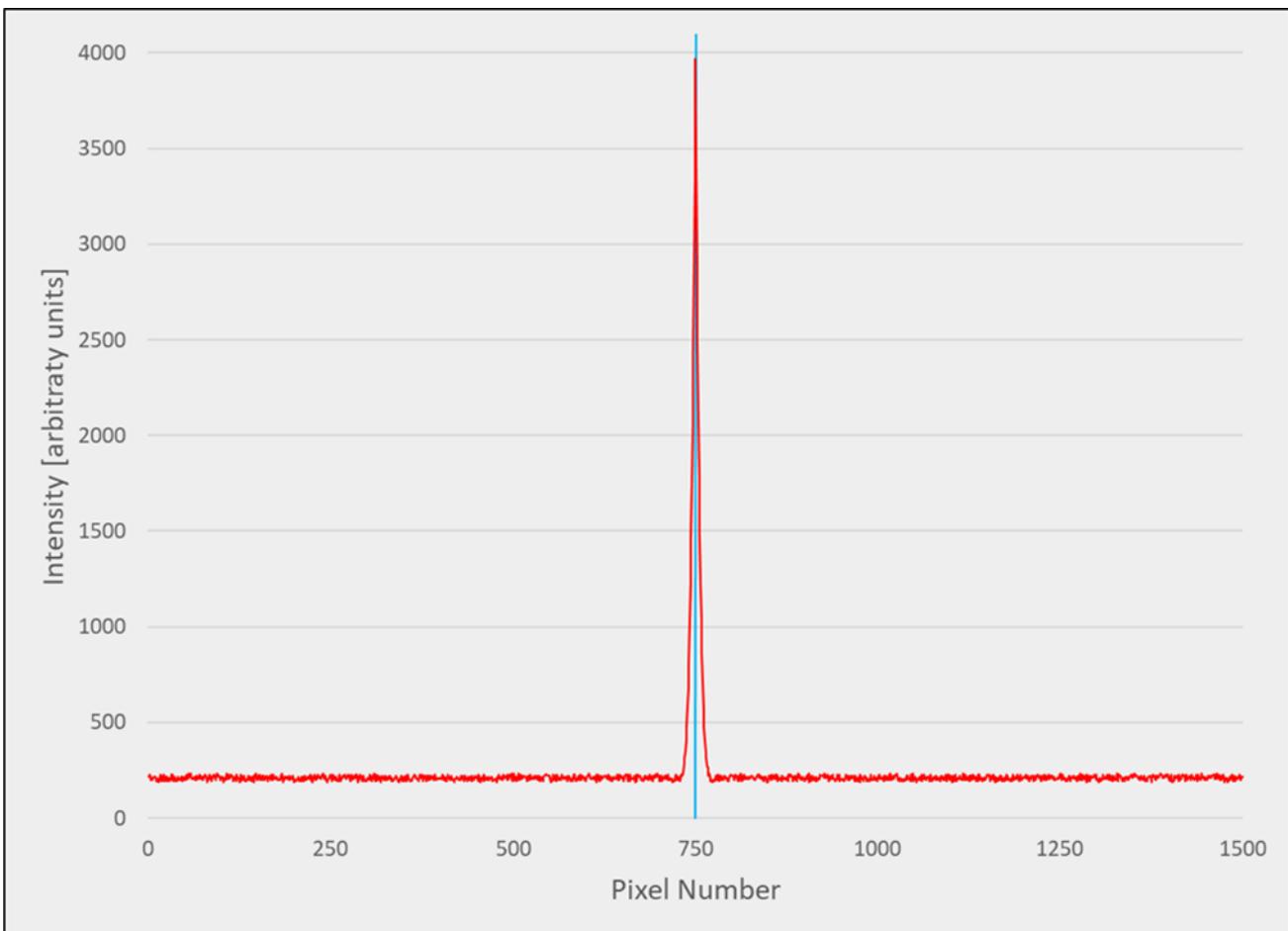


Figure 8: Aligning the PF850 sensor with the optical path by defining the pinhole centre

4.3.2 Sensor centre setup for line mode systems

In the PureFocus850 GUI, go to the offset submenu and click ‘Go to Factory Home’. The offset lens will move. Go to the laser submenu and increase the value to 100. Click set.

Go to the mode submenu and ensure line mode is selected.

Set the PureFocus controller to MAN FOC mode.

Move an objective into the light path. Prior Scientific recommends the use of the highest magnification objective that provides a clear peak maximum close to the top of the setup graph. If a low magnification objective is used, further adjustments may be necessary when setting up individual objectives. *It is strongly advised to maximise the size of the peak using the 45 degree adjustment, then reduce the laser power.*

Place the PF209 onto the stage and focus on either the coverslip sample or the mirror sample. Use the digipot, in MAN FOC mode, to achieve focus.

Click Setup, which will open a graph that indicates the laser intensity over the PureFocus850 sensor. Adjust focus until a peak appears in the signal. At this stage, the peak may be very small or may not be visible at all, even at high laser power.

If the peak is visible, slightly adjust the position of the 45° screw to increase the size of the peak (Fig. 9).

If the peak is not visible at all, go to the mode menu and temporarily put the system into slice mode. This will amplify the signal by a factor of 10, which may bring the peak above the noise. If the peak is still not visible, adjust the focus to identify the peak. Adjust the 45° screw to increase the size of the peak. Remember to switch back into line mode before continuing. If necessary, adjust the 45° further to maximise the size of the peak. After maximising the size of the peak, if the peak crosses the top of the graph reduce the laser power until the top of the peak is visible (Fig. 10).

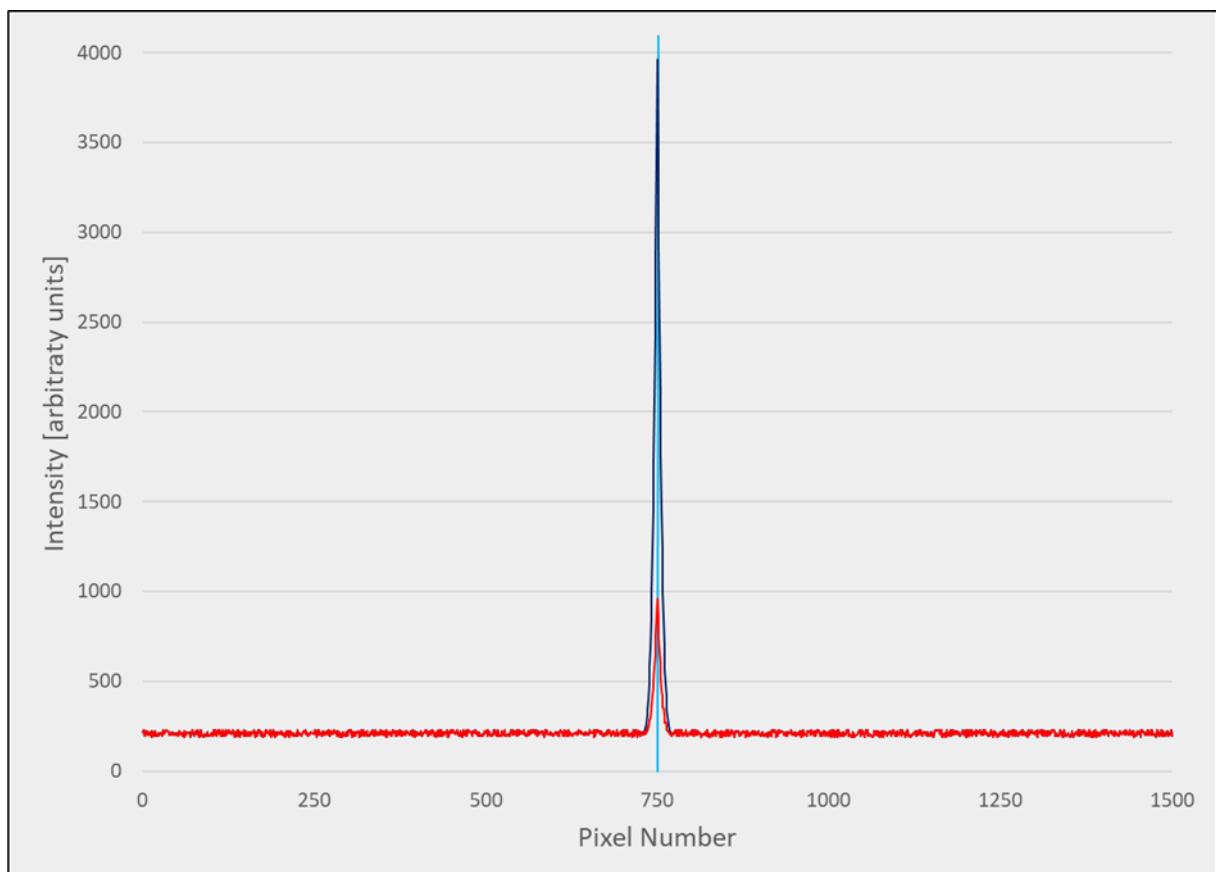


Figure 9: A weak signal (red) can be increased (dark blue) by using the 45 degree adjustment during setup of a line mode system

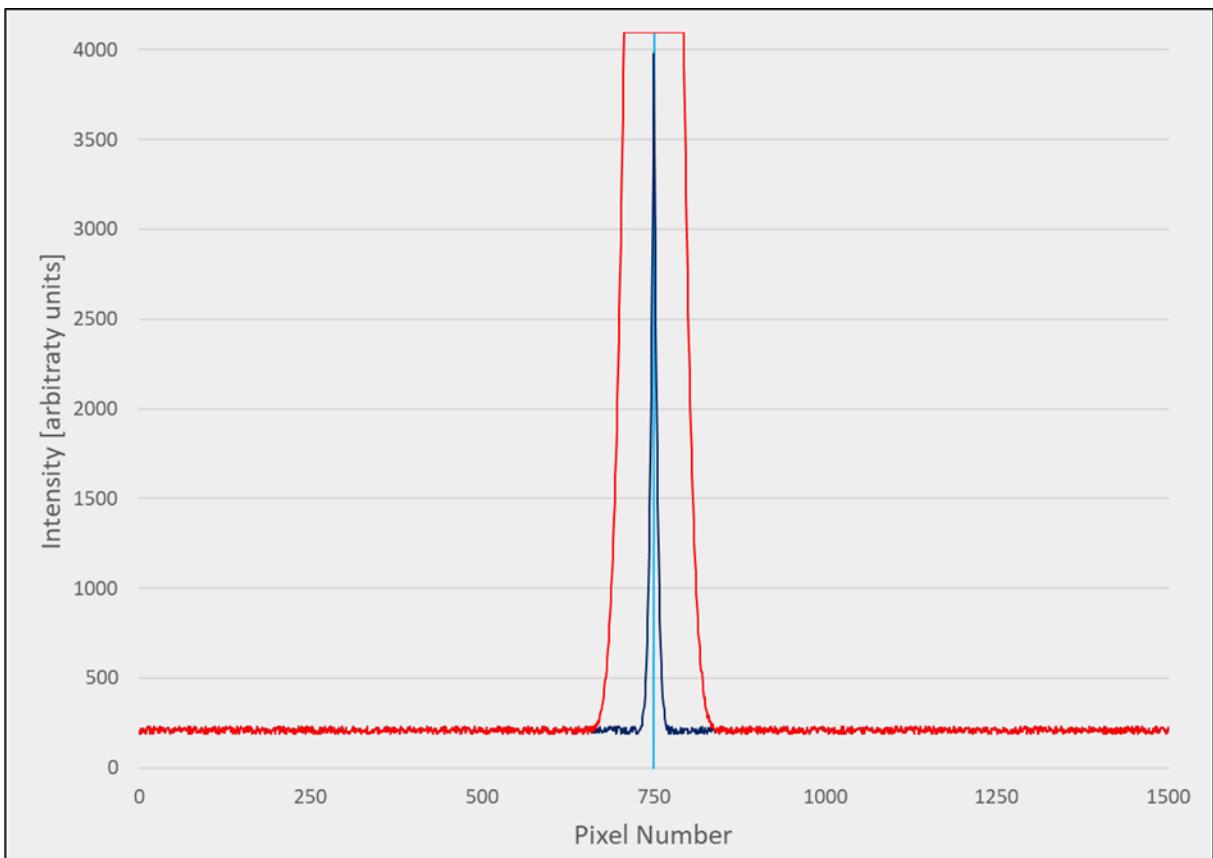


Figure 10: An oversaturated signal (red) can be decreased (dark blue) by reducing the laser power

Make this peak as sharp as possible by rotating the digipot. It is optimal for the peak to fill almost the entire y-axis of the graph. See section 4.3.3 for full details.

Click the Auto Set Centre button, and click ‘Yes’ in the following pop-up box. This will set the centre of the PureFocus850 sensor, which is used to positively verify whether the sample is in focus.

Check that a peak is visible with each of the other objectives, before returning to the highest magnification objective.

4.3.3 Laser Setup Troubleshooting

The appearance of the peak does not necessarily correlate with sample focus. It is normal if the sample is out of focus whilst the peak is present.

If the signal is a flat line with no features and no features appear following refocusing, increase the laser power in 500 unit steps and see if any features appear.

If the signal is very noisy and no peak is visible, decrease the laser power in 500 unit steps and see if any features are retained.

If the peak is short, increase the laser power in 100 unit steps until it reaches the optimal height.

If the peak goes over the top of the y-axis of the graph, reduce the laser power in 100 unit steps until it reaches the optimal height.

If there are multiple features on the graph, which have intensities above the baseline noise, this can affect the Auto Set Centre function. If the calculated centre of the sensor does not correlate with the peak, go to the pinhole submenu and reduce the pinhole width in order to exclude these features. Please refer to the section 7.2.1 for further guidance on this topic.

(Line Mode only) If you are having trouble with the 45° adjustment step with your highest magnification objective, switch to the next highest magnification objective. Note the extra care will be required when configuring the highest magnification objective with your sample later in the procedure.

4.4 Creating signal imbalance

Ensure the controller is in MAN FOC mode.

Ensure the setup submenu graph is open.

Rotate the digipot in such a way that the single peak splits into a broad signal with peaks on either side. The direction of rotation is system dependent. If the signal does not split, and just becomes broader and less intense, rotate the digipot in the opposite direction. Increase the laser power such that the split peak fills as much of the vertical axis as possible

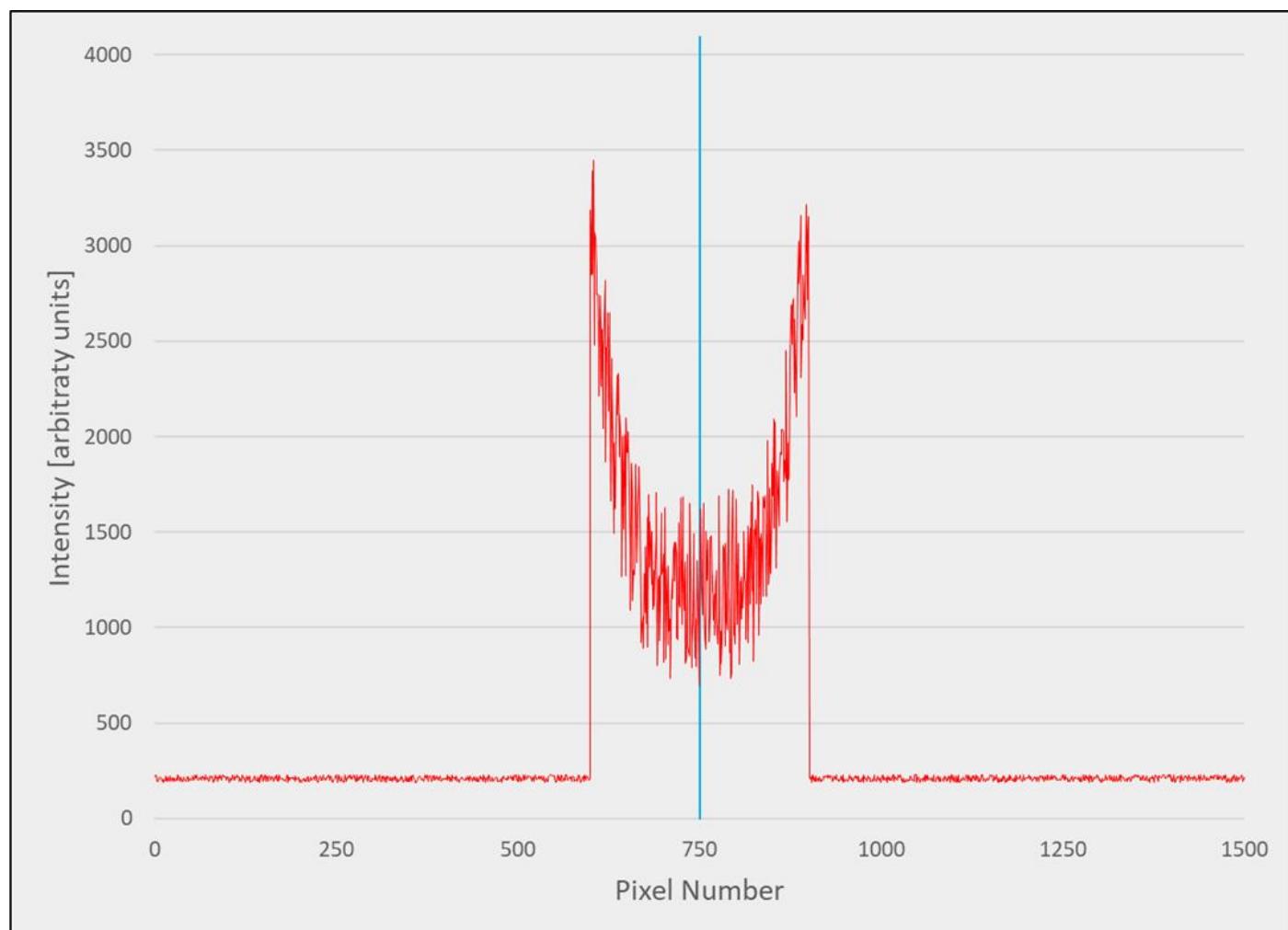


Figure 11: The split peaks signal morphology. The actual shape of the signal can differ significantly from the above, with one or both peaks not being visible, or a third peak present at the pinhole centre. In all cases however a significant amount of signal is present across the central 200-300 pixels of the sensor.

Rotating anti-clockwise, adjust the blade screw on the side of the PureFocus Head until the blade blocks the left half of the peak, up to the sensor centre that was set up in the previous step (Fig. 12). Note that the blade will not remove any background signal. The blade screw has considerable tolerance built in so expect to undertake a large number of screw rotations during this step.

Once the blade is suitably positioned, take the opportunity to adjust the focus and observe the remaining signal move from one side of the pinhole centre to the other. If this doesn't occur, refer to Section 10: Troubleshooting.

For biological samples, it is recommended to turn the blade screw one additional rotation in order to maximise signal swing.

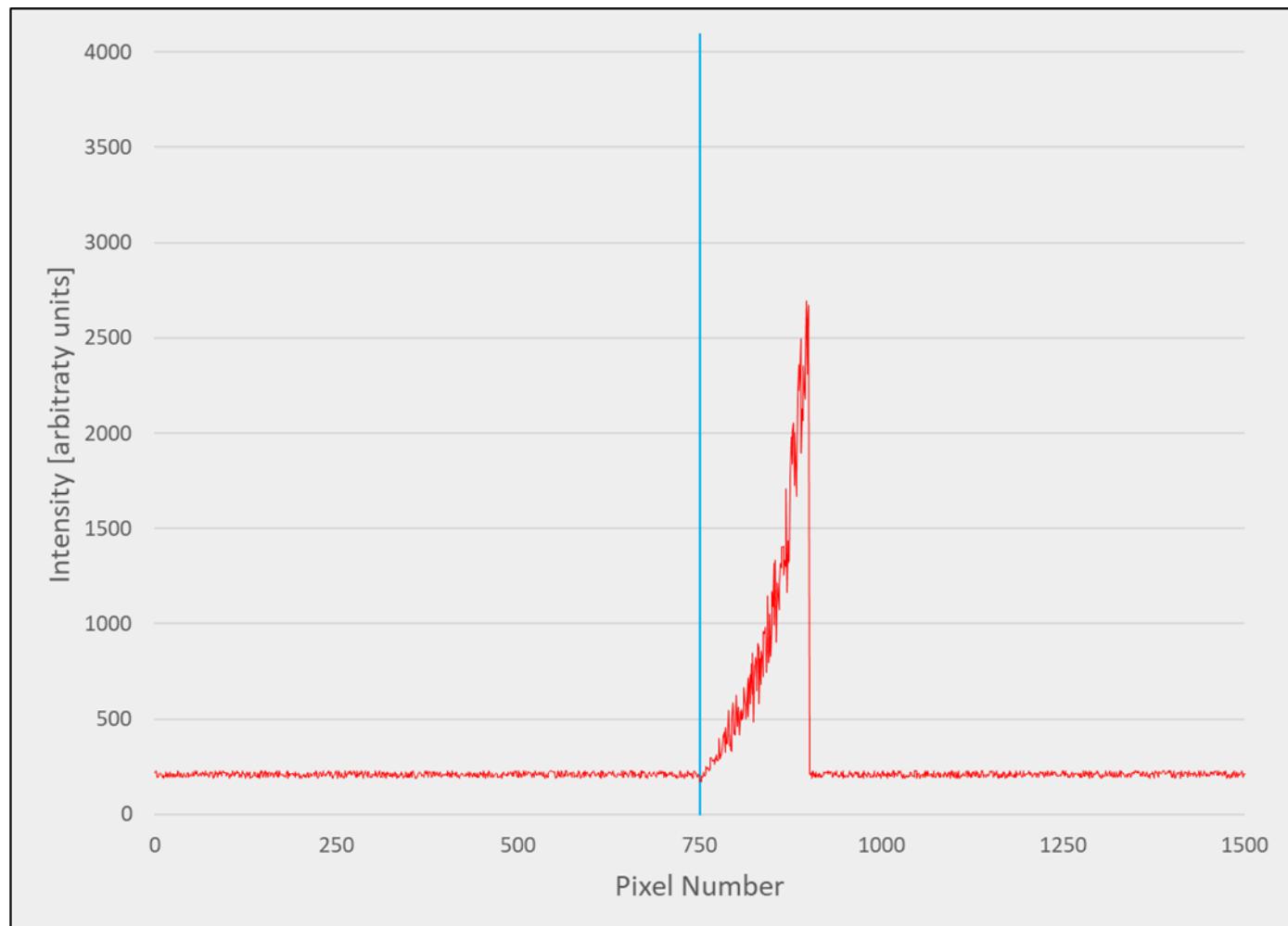


Figure 12: Signal morphology after positioning the blade into the light path. One additional rotation of the blade screw is recommended for biological samples such as slides and cell culture dishes.

4.5 Removing background signal

Move the dustcap into the light path, leaving any illumination and the PureFocus850 switched on. The signal above the background will disappear.

Click Measure Average Background. Click ‘Yes’ in the following pop-up to apply this background setting to all objectives. Signal below the background threshold will be removed from the setup graph (Fig. 13).

Go to the parameters submenu and click save to controller. This will save the default sensor centre and background to the controller.

This concludes the Optical Alignment section. This procedure only needs to be performed once per system installation.

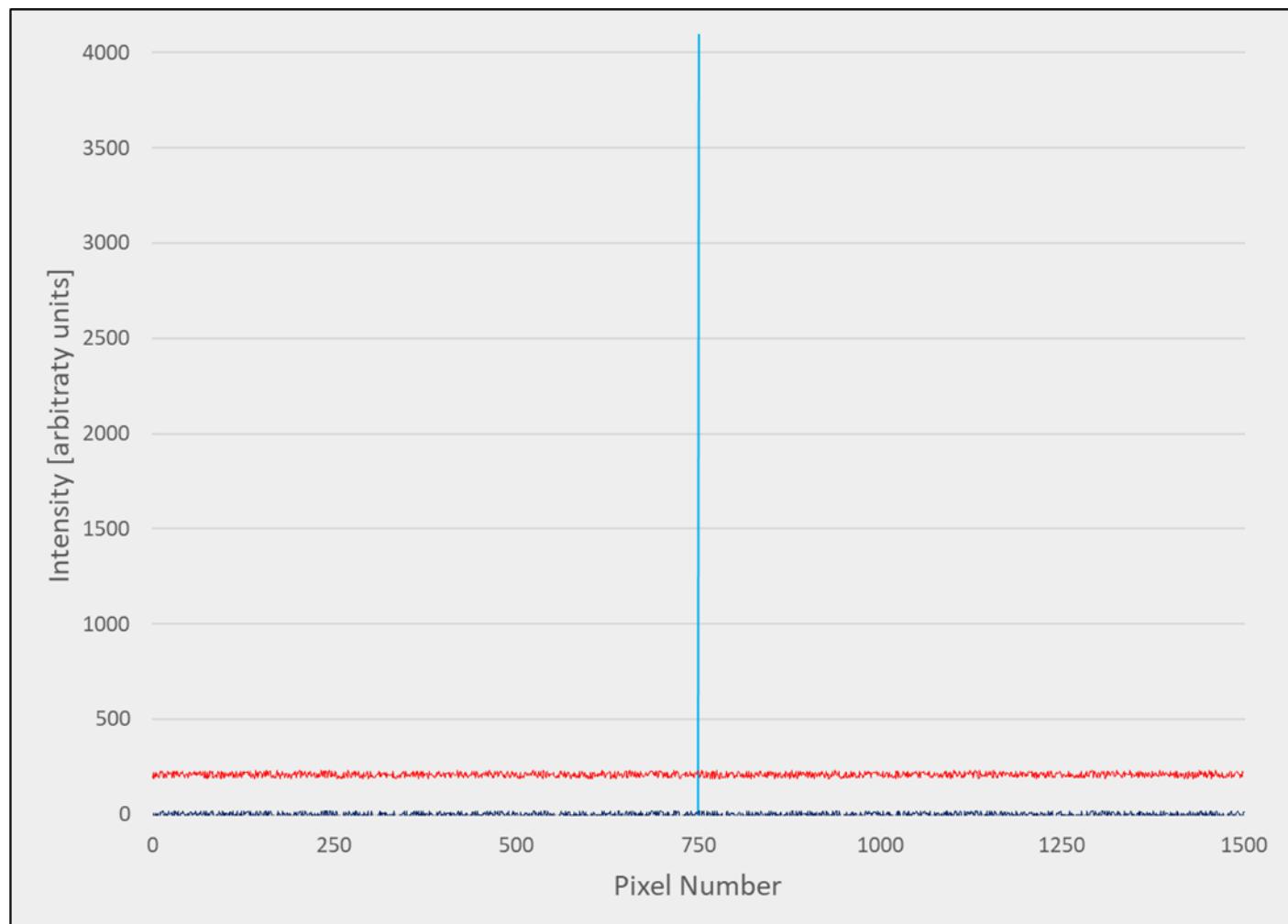


Figure 13: ‘Average background and set’ removes the universal background (red) detected by the sensor in the absence of any reflected 850 nm light from the sample. After setting the background the signal on the sensor will be reduced to almost zero (dark blue).

Section 5: Focusing System Setup

If the focusing system is not already connected to the PureFocus850 controller, close the PureFocus850 GUI and switch off the controller. Plug the focusing system into the 15-pin connector. Piezo nanopositioning systems require the PF404 BNC to 15-pin D-connector cable. Switch the controller back on, and restart the GUI. The parameters set during optical alignment do not need to be recalculated if they were saved to the controller as mentioned in section 4.5.

WARNING: this step is essential for operation of the PureFocus850. If the focus direction is set incorrectly this can create a positive feedback loop. This can cause a system crash which may damage your objective or sample. Please read sections 5.1 and 5.2 carefully.

5.1 Stepper motor focusing system

Prior Scientific focus motor systems should be installed on the left hand focus knob when possible.

Move the lowest magnification, longest working distance objective into the optical path. Go to the mode submenu and select the ‘Stepper’ option.

Enter 100 into the step size box in the Z-position field in the GUI. Click ‘step up’ and check the direction the objective moves relative to the sample. If movement is too small to be visible, increase this value up in 100-unit steps until the movement can be seen. Depending on the direction of movement, change settings in the PID or mode submenus. *The table below is a guide to expected settings but please note that the settings actually required may be different.*

| Microscope type | Initial Objective Movement | Required settings | |
|-----------------|----------------------------|-------------------|--------------|
| Upright | Towards sample | PID | Positive |
| | | Mode | Inverted |
| Upright | Away from sample | PID | Negative |
| | | Mode | Non-inverted |
| Inverted | Away from sample | PID | Negative |
| | | Mode | Non-inverted |
| Inverted | Towards sample | PID | Positive |
| | | Mode | Inverted |

The aim of this procedure is to configure your system such that clicking ‘step up’ causes the objective to move away from the sample, which sets feedback in the correct direction.

5.2 Piezo nanopositioning focusing system

Switch on the Purefocus controller and piezo nanopositioning focusing system controller.

Move the lowest magnification, longest working distance objective into the optical path.

Go to the mode submenu and select the ‘piezo’ option. Click the piezo travel option in the mode submenu and set the range of the piezo to match the specified range of the nanopositioning system you are using. This will set an appropriate output limit for your system (see section 6.5.2 for more details).

Move the coarse focus of the microscope in such a way that the sample is in focus when the piezo stage is at the middle of its travel range. For example, a 400µm stage should be positioned at ~200µm.

Enter 100 into the step size box in the Z-position field in the GUI. Click ‘step up’ and check the direction the objective moves relative to the sample. If movement is too small to be visible, increase this value up in 100-unit steps until the movement can be seen. Depending on the direction of movement,

change settings in the PID or mode submenus. *The table below is a guide to expected settings but please note that the settings actually required may be different.*

| Nanopositioning system type | Initial Objective Movement | Suggested settings | |
|-----------------------------|-------------------------------------|--------------------|--------------|
| Objective positioner | Away from sample | PID | Positive |
| | | Mode | Inverted |
| Objective positioner | Towards sample | PID | Negative |
| | | Mode | Non-inverted |
| Z-stage | Sample moves away from objective | PID | Positive |
| | | Mode | Inverted |
| Z-stage | Sample moves towards from objective | PID | Negative |
| | | Mode | Non-inverted |

The aim of this procedure is to configure your system such that clicking ‘step up’ causes the distance between the sample and objective to increase.

Section 6: Autofocus Parameter Setup

6.1 Installation Review

Sections 3, 4 and 5 are only required during the initial system setup and should only be performed once per installation. This concludes the configuration of the PureFocus850 with the microscope. The following sections guide the user through setting parameters specific to their samples and objectives, and therefore these parameters may require adjustment if the samples and objectives used with the PureFocus850 change.

Complete the following autofocus parameter setup first using the PF209 setup slide in order to ensure user familiarity with the various controls. Once the offset value has been saved and PureFocus850 servo has been successfully activated using the setup slide, review and adjust the parameters in line with your samples by repeating the procedure. The below table shows which element of the setup slide is best used as a starting point for various sample types.

| User sample | PF209 element |
|-----------------------|---------------|
| Permanent fixed slide | Coverslip |
| Cell culture dish | Coverslip |

| | |
|--------------------------|---------------|
| Plain wafer | Mirror |
| Semiconductor wafer | Metallic disc |
| General metallic samples | Metallic disc |

6.2 Objective selection

Go to the parameters submenu and click New Objective set.

In the following pop-up window, select an option from the list that most closely correlates with the objective, focusing mechanism and sample you are using. Each option contains a number of pre-set values, which give a good starting point for configuring each objective. 'Fixed slide' options are used for biological samples containing a glass layer in front of the sample e.g. a coverslip, and 'materials' options are used for materials samples e.g. a semiconductor wafer. Refer to the examples below.

| User Objective/Focusing Mechanism /Sample | Suggested Objective Option |
|---|---------------------------------------|
| 10x/Stepper motor/fixed slide | 10x 0.17 apo 0.40 fixed slide |
| 10x/Stepper motor/wafer | 10x M achro 0.25 materials |
| 20x/Stepper motor/fixed slide | 20x 0.17 apo 0.75 fixed slide |
| 20x/Stepper motor/cell culture dish | 20x 0.17 apo 0.75 fixed slide |
| 100x/Piezo nosepiece/wafer | 100x M achro 0.90 materials - OP400 |
| 10x/Piezo stage/semiconductor wafer | 10x M achro 0.25 materials - NZ100 |
| 10x/Piezo stage/96-well plate | 10x 0.17 apo 0.40 fixed slide - NZ100 |

Following selection of an objective option, there is an opportunity to rename the objectives with their actual specifications or with a name that will be recognisable to the user. Changing the name will not change any of the pre-set values.

Please note once the new objective set has been selected and renamed your objectives, the settings for those objective sets needs to be saved to the controller and exported into a file before selecting a new objective set. If not the settings for the current objectives set will all be lost when creating a new objective set.

6.2.1 Focusing on the sample

Prior Scientific recommends using a 5x, 10x or 20x objective when setting up an objective for the first time, although any objective can be used. Once steps 6.3-6.7 have been understood, those steps will be used to set-up all objectives in your imaging system.

Set the PureFocus850 controller into MAN FOC mode.

If using a piezo-based focusing system, bring focusing system to the midpoint of its travel range using the digipot on the controller or the step up/step down buttons in the GUI, then use the microscope's coarse focusing system to focus on the sample

Bring the sample into focus using the microscope's focusing system. Do not use any secondary focusing systems with a limited travel range e.g. a piezo stage. If using a stepper motor to control the PureFocus850, use the digipot to achieve this. Alternatively, use the manual focus knobs or any internal motorised focusing system inherent to the microscope.

6.3 Setting the offset

The offset between the reflective plane that the PureFocus850 detects and the focal plane of interest needs to be defined for each objective. Materials samples typically have an offset close to zero but the optimal performance is achieved by following the below procedure. Typical offset values are shown for both biological and materials samples at various magnifications in the table below. Achieving values outside of the given range may be as a result of identification of an inappropriate reflected signal. Refer to Section 10: Troubleshooting if required. Please note the below values are considered typical but other values may also be viable.

| Magnification | Biological sample using the glass/air interface with 1.5 standard coverslip | Materials sample |
|---------------|---|------------------|
| 5x | 5000 - 20000 | -13000 - 10000 |
| 10x | 10000 - 40000 | -13000 - 10000 |
| 20x | 40000 - 100000 | -13000 - 20000 |
| 40x | 100000 - 250000 | -13000 - 20000 |
| 60x | 250000 - 450000 | -13000 - 30000 |
| 100x | 50000 - 200000* | -13000 - 50000 |

*When using immersion objectives and the glass/sample interface. Non-immersion 100x objectives do not have sufficient offset to use the glass/air interface.

The PureFocus850 allows both automated and manual approaches to offset discovery. If automated discovery is not successful, attempt manual discovery.

6.3.1 Automated offset discovery

Go to the offset submenu and click Auto Find.

In the subsequent pop-up window, type a speed setting. Prior Scientific advises using a speed of 50% initially.

Click okay, and the offset calculation will begin. The calculation can be stopped at any time by clicking the – button in the offsets section of the main GUI interface. Ensure the offset value has returned to zero before attempting the calculation again.

If the offset calculation is unsuccessful, reduce the speed to 20% or lower and attempt the calculation again.

Click on Setup to open the setup graph. A peak should be visible at the pinhole centre. If the peak maximum crosses the top of the y-axis of the graph, reduce the laser power until the peak maximum is just visible. If the peak is very small, increase the laser power until the peak maximum is at least half way up the y-axis. The size of the peak scales with to the laser power and inversely proportional to the diameter of the back aperture of the objective, which often correlates with its magnification.

Make a note of the calculated offset value and compare to the table at the start of section 6.3.

If the peak maximum remains low even at maximum laser power, it may still be useable if it is still significantly above any background signal. Proceed to step 6.3.3. Note however that higher magnification objectives may not allow enough laser signal to operate the PureFocus850. If low peak maximums are already apparent at 5-20x magnification, or no peak can be identified, refer to Section 10: Troubleshooting.

6.3.2 Manual offset discovery

Check the offsets section of the main GUI interface to ensure the offset value is set to zero. If not zero, go to Offsets, then click 'Go to Factory Home'.

Click on Setup to open the setup graph. For materials samples, where the offset should be very close to zero, a peak should either be visible in the pinhole centre or slightly offset from it.

For biological samples, a diffuse peak either will appear offset from the pinhole, diffuse or may not be visible at all, particularly when using a high magnification objective. Examples of this are shown below.

Set the PureFocus850 controller into MAN OFF mode.

Rotate the digipot on the controller so that the offset value becomes positive. The peak will shift towards the centre of the pinhole (Fig. 14). Adjust the position of the peak carefully until

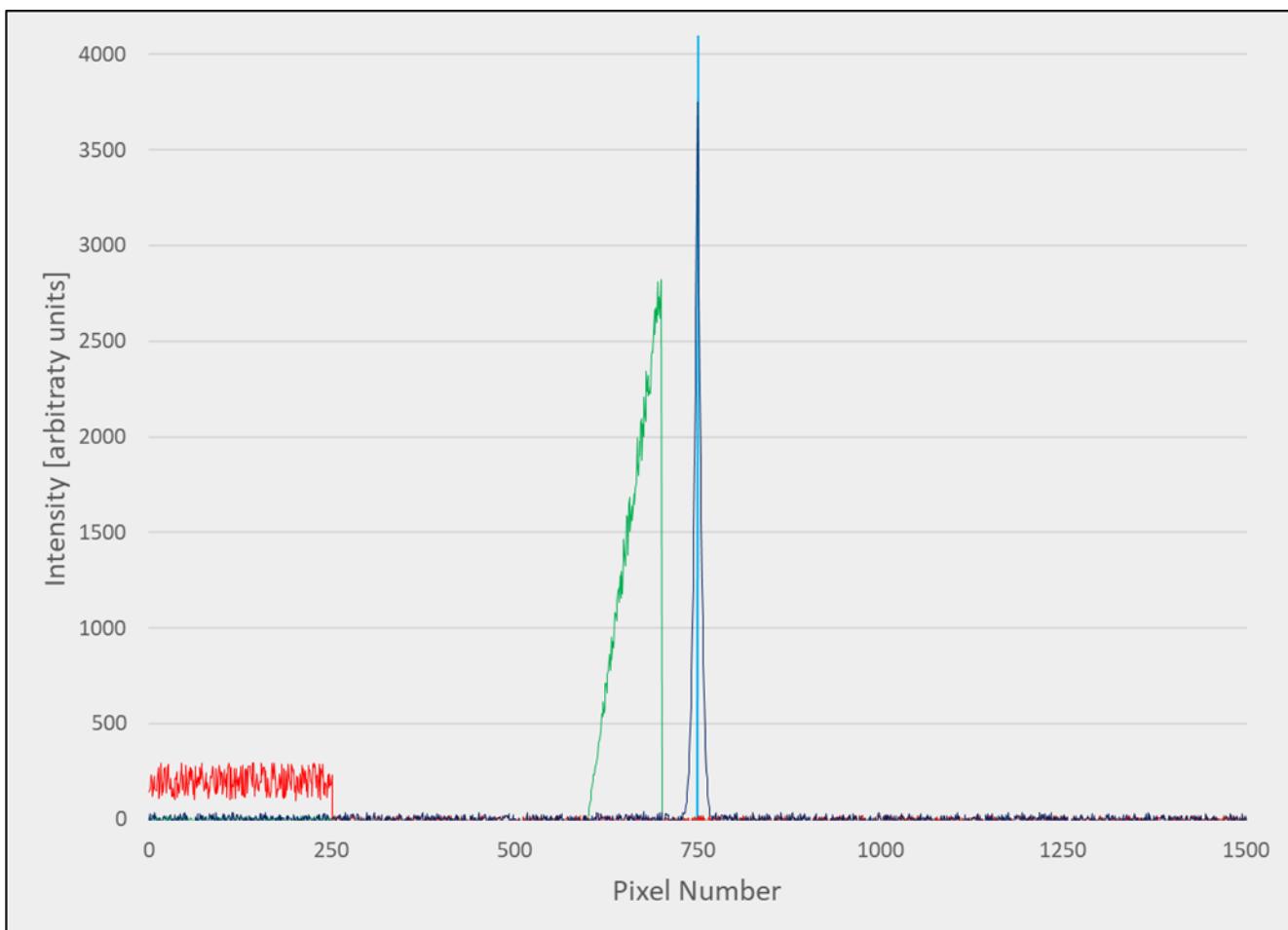


Figure 14: While in MAN OFF mode, the digipot controls the position of the offset lens. Typically, when the offset value is 0 and using a biological sample, the peak appears on the left of the pinhole centre. At low magnifications, the peak will be close to the pinhole centre (green), at moderate magnification the peak may just be visible on the left hand side of the setup graph (red) and high magnification the peak may not be visible at all. Moving the offset lens through its positive range will align the peak with the pinhole centre (dark blue).

its maximum aligns with the pinhole centre. When using high magnification objectives with biological samples expect to rotate the digipot a large number of rotations. *Signal morphology can be extremely variable. The below examples are representative examples.*

In rare cases, a sample may require a negative offset (Fig. 15). This is where the focal plane of interest sits above the main reflective interface in the sample. The PureFocus850 has a limited negative offset range that can be used if required.

By aligning the peak maximum with the pinhole centre, the required offset value has been calculated. If the peak maximum crosses the top of the y-axis of the graph, reduce the laser power until the peak maximum is just visible. If the peak is very small, increase the laser power until the peak maximum is at least half way up the y-axis.

Make a note of the calculated offset value and compare to the table at the start of section 6.3.

If the peak maximum remains low even at maximum laser power, it may still be useable if it is still significantly above any background signal. Proceed to step 6.3.3. Note however that higher magnification objectives than the one currently in use may not allow enough laser signal to

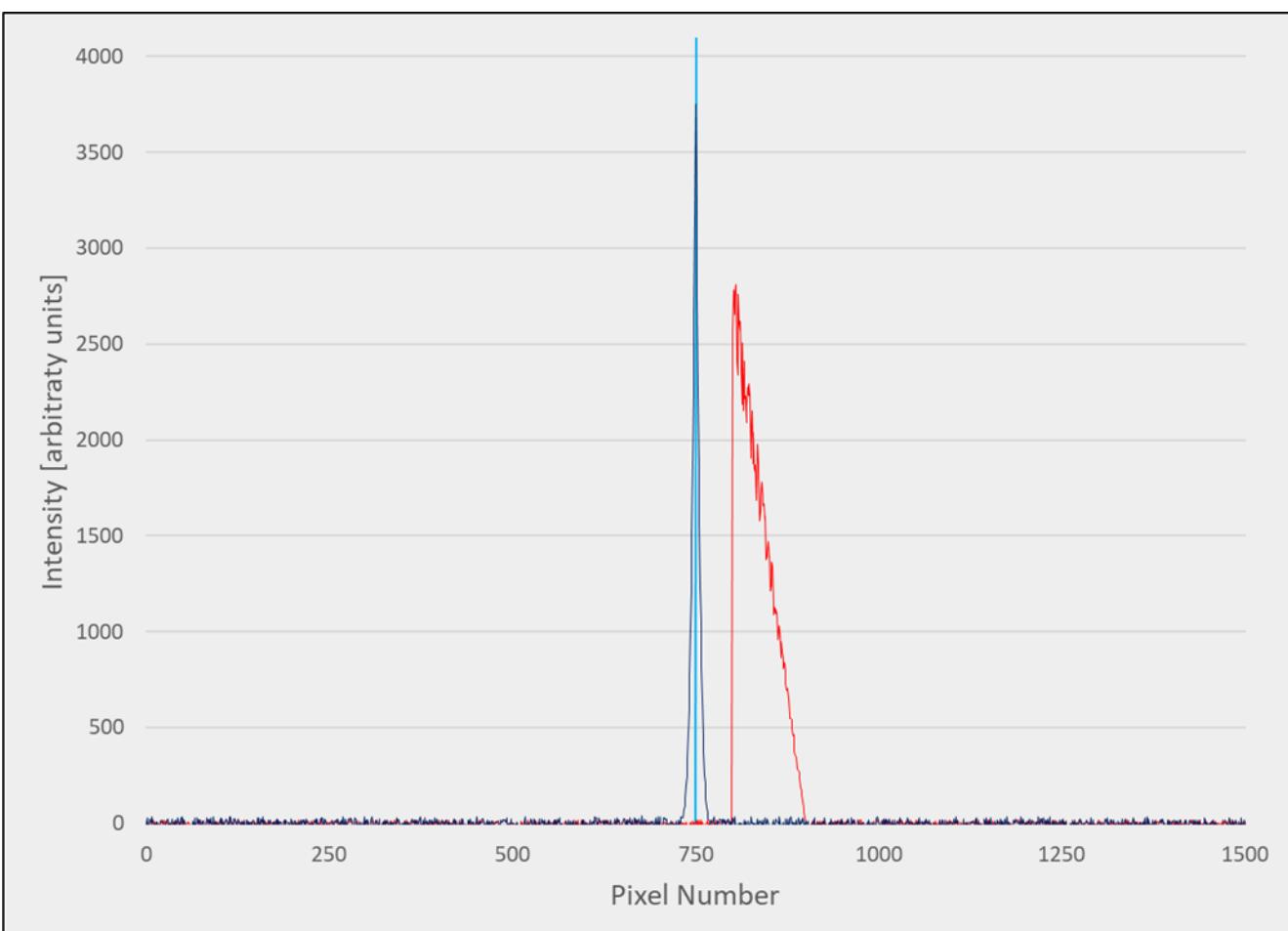


Figure 15: A sample requiring a negative offset will display a peak on the right hand side of the graph (red) when the offset value is 0. While in MAN OFF mode, the peak can be aligned with the pinhole centre (dark blue) by rotating the digipot.

operate the PureFocus850. If low peak maxima are already apparent at 5-20x magnification, or no peak can be identified, refer to Section 10: Troubleshooting.

6.3.3 Checking error swing

Error swing is essential for operation of the PureFocus850. The speed and magnitude of swing contribute to the focus recovery range and focus stability. Error swing is indicated by the green bars in the centre of the main interface of the PureFocus850 GUI.

Set the PureFocus controller into MAN FOC mode.

Rotate the digipot clockwise to bring the sample out of focus. The error bar will swing towards +1 or -1, with the error signal value indicated to the right. Make a note of the maximum error value. Rotate the signal anticlockwise, passing through focus and putting the sample out of focus again. The error bar will swing in the opposite direction. Similarly, make a note of the maximum error signal and the rate at which error signal accumulates. Finally bring the sample back into focus.

The optimal outcome of this procedure is even swing which accumulates at equivalent rates either side of focus, with a maximum error value between +/- 0.75 and +/- 1 on each side. Error values lower than this can be still be used, however, with error signal maxima being proportional to the signal-to-noise ratio and typically inversely proportional to objective magnification. Error maxima may also be slightly imbalanced, with an imbalance of <0.2 considered acceptable.

Refer to Section 6.4 and Section 10: Troubleshooting section if any of the following scenarios occur:

- Significant imbalance of error signal maxima either side of focus (>0.25)
- No visible error swing either side of focus
- Extremely rapid increase of error value, followed by an immediate decrease to zero, over a small Z-axis movement.

6.4 Checking for back reflections

Reflections from other reflective surfaces, such as lenses inside the objective, may produce unwanted signals on the PureFocus850 sensor. It is important to minimise the impact of these signals on the error swing.

Remove the sample and turn off all illumination sources. The error signal is now derived solely from the reflection of the PureFocus850 laser from components of the optical path.

Go to the Pinhole submenu in the PureFocus850 GUI and check the Average Pixel Value. This value should be less than 100 and greater than zero.

If the value is greater than 100, click on Setup and review the setup graph.

If the signal is spread across a number of pixels, click on Laser and reduce the laser power until the Average Pixel Value remains consistently below 100 (Fig. 16).

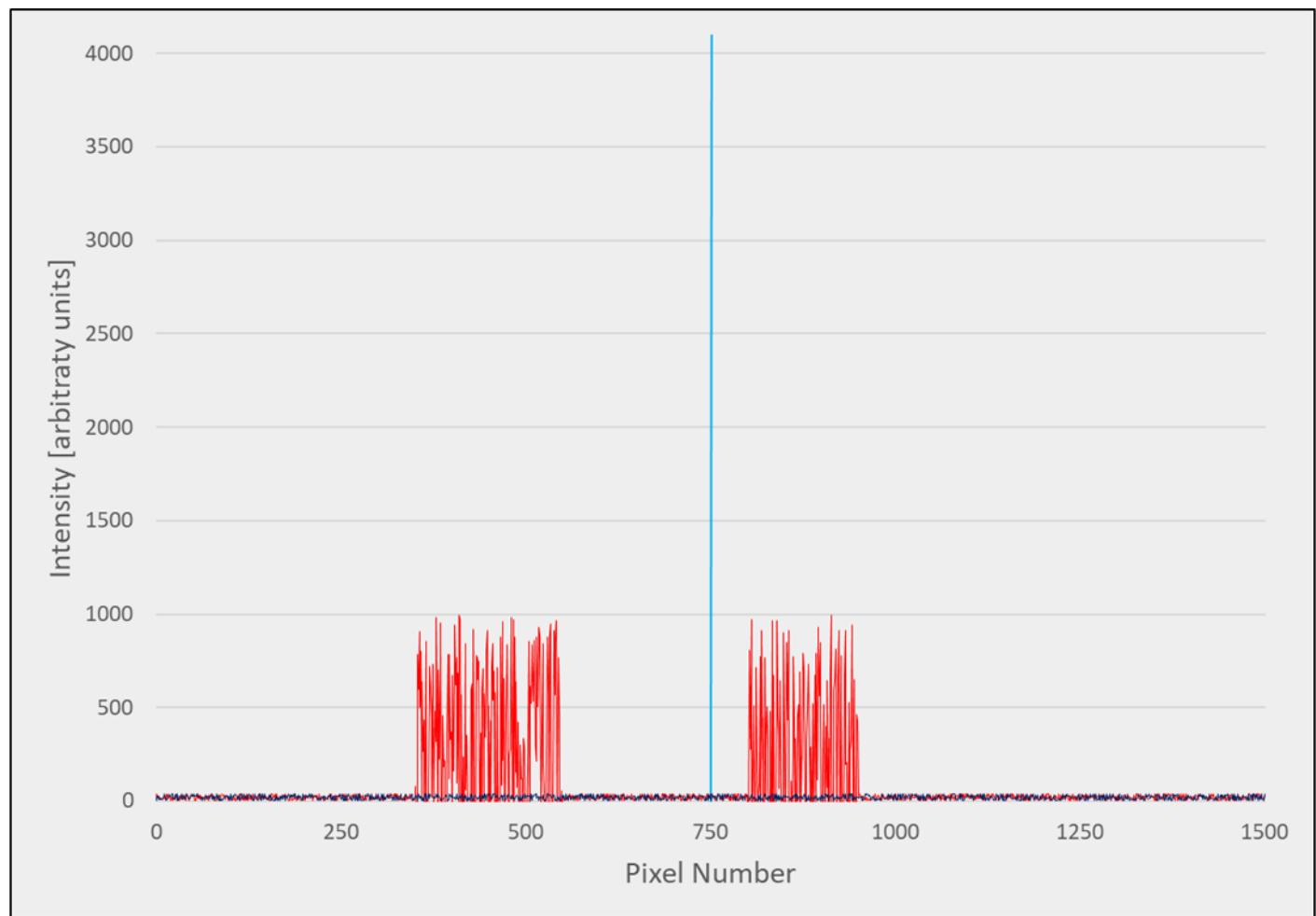


Figure 16: Back reflection signal morphologies differ significantly, but always appear above the background signal baseline and have a fixed position on the sensor (red). Reducing laser power will reduce their intensity (dark blue).

If the signal is localised to a small number of pixels, often in the form of a large peak located away from the pinhole centre, reduce the pinhole half width in the pinhole submenu such that the signal is removed from the pinhole boundaries. This will exclude this signal from the error value calculation (Fig. 17).

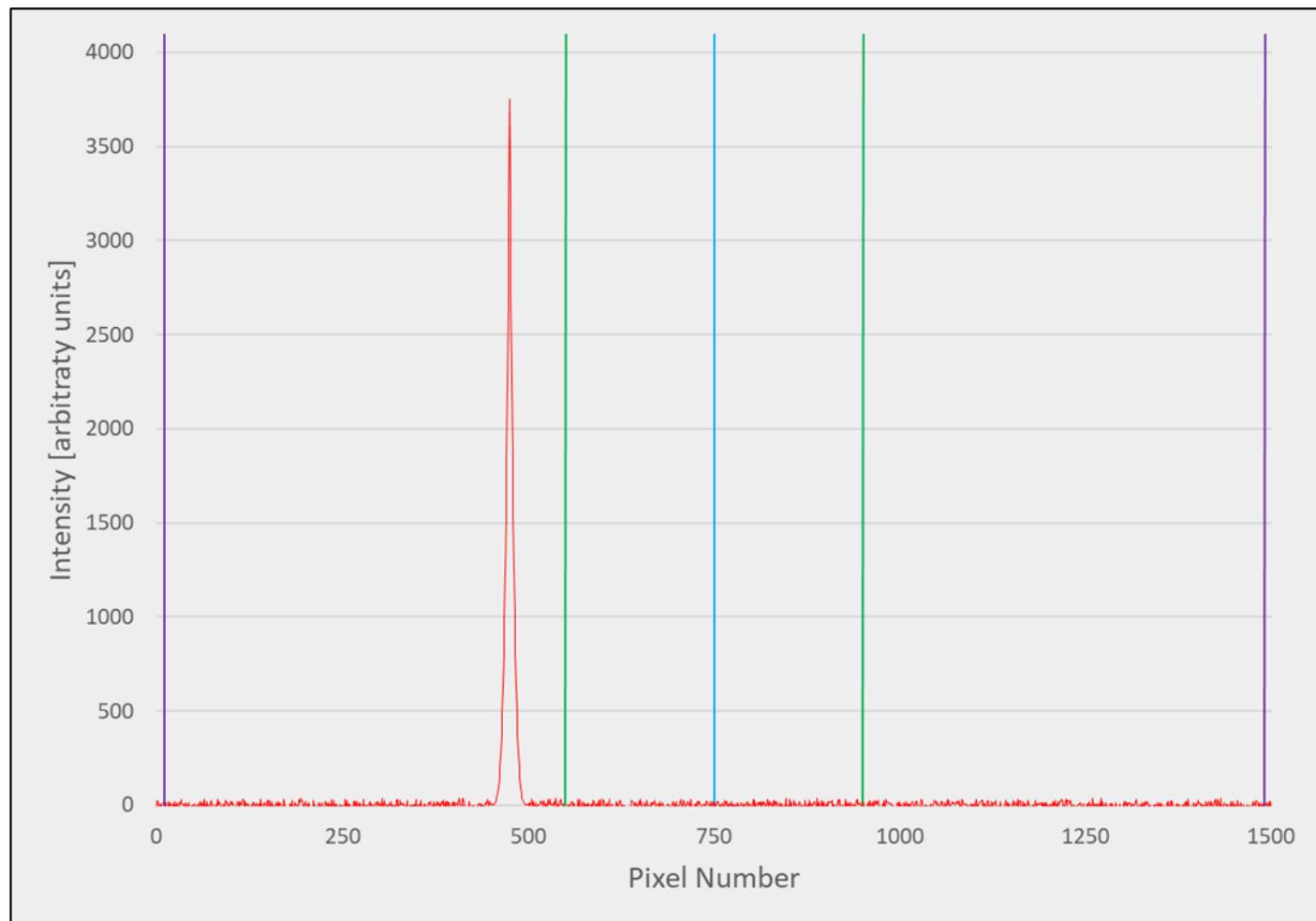


Figure 17: Significant back reflections can occur that cannot be eliminated by adjusting the laser power (red). In this case the pinhole width can be reduced in order to exclude the signal from the autofocus position calculation. The wide pinhole (purple, half width = 750), will not exclude the back reflection. Reducing the half width to 200 (green), in contrast, will exclude the back reflection.

6.5 Setting the focus recovery speed

Only attempt the following procedure after checking the error swing and minimising the effects of any back reflections.

The objective pre-set values loaded during section 6.2 contain PID values. PID is a common control loop mechanism that allows the PureFocus850 to maintain focus on the sample. The magnitude of the proportional (P), integral (I) and derivative (D) gains define the rate at which the PureFocus850 recovers sample focus and focus stability.

Whilst the pre-set values will be approximately correct for a given sample type and objective, it likely these values will need to be changed to achieve optimal performance.

For access to all PID settings, click on the PID submenu in the main PureFocus GUI.

6.5.1 Stepper motor control

Stepper motor systems can be used effectively using suitable P-gain values.

Bring the sample into focus. The error value should be close to zero.

Put the controller into SRV mode by pressing the Servo on/off button on the controller.

If the image is unstable, reduce the P gain by a factor of 10. If the image is very unstable, switch the controller into MAN mode immediately and reduce the P-gain by a factor of 10, before switching the controller into SRV mode again. If required, repeatedly reduce the P gain by a factor of two until the image becomes stable.

If focus on the sample cannot be held stably with any parameters, refer to Section 10: Troubleshooting.

When the image is stable, switch the controller into MAN FOC mode. Bring the sample out of focus to a positive error value close to the maximum achieved in section 6.3.3.

Switch the controller into SRV mode, paying close attention to the rate at which focus on the sample is recovered.

If the rate of recovery is fast, and focus is stable, then the current P-gain value is appropriate. The P-gain can be adjusted +/- 10% to ensure it is optimal. Proceed to the next section.

If the rate of recovery is slow, and the focus is stable, the the P-gain value can be increased. Move the sample back out of focus (in MAN FOC mode). increase the value in by 10% and then reactivate SRV mode. Repeat this until the rate of recovery is fast, with the focus remaining stable.

If the rate of recovery is fast, but focus is unstable, then the P-gain must be reduced. Decrease the P-gain in 10% reductions until stable focus can be achieved. Move the sample back out of focus (in MAN FOC mode) and the reactivate SRV mode to check the speed of recovery. Find the P-gain value which gives focus stability at the maximum rate of focus recovery.

Once a suitable P-gain has been found, return to MAN FOC mode. Bring the sample out of focus to a negative error value close to the maximum achieved in section 6.3.3. Switch the controller into SRV mode to validate the P-gain setting.

For high magnification objectives, fast focus recovery speeds may not be attainable due to the generally lower signal-to-noise ratio causing focus instability. Find the best compromise between focus stability and focus recovery.

6.5.2 Piezo focusing system control

Piezo focusing systems require the use of the P-gain and I-gain. I-gain should be set to be 100x greater than P-gain. Attempting to use piezo system without I-gain will result in poor performance.

Objective pre-set values for piezo control also have a defined output limit. This will limit the distance the piezo can travel. If set to low the piezo system will have a limited travel range,

potentially preventing focus recovery. If set too high the piezo system will repeatedly attempt to drive over its limits.

Ensure the output limit is set to less than the open loop travel range of the piezo system. Refer to section 5.2 in order to specify the travel range, which should automatically update the travel range. For example, a system with an open loop travel range of 450 µm and a closed loop travel range of 400 µm, an output limit of ~4190000 is set in the pre-set objective values. The first three digits give the output limit in microns; 400 < 419 < 450. Refer to the manual of your piezo system to identify these travel ranges.

Bring the sample into focus. The error value should be close to zero, and the piezo focusing system should be close to its midpoint of travel.

Put the controller into SRV mode by pressing the Servo on/off button on the controller.

If the image is unstable, reduce the P-gain and I-gain by a factor of 10. If the image is very unstable, switch the controller into MAN mode immediately and reduce the P-gain and I-gain by a factor of 10, before switching the controller into SRV mode again. If required, repeatedly reduce the P-gain and I-gain by a factor of two until the image becomes stable.

If focus on the sample cannot be held stably with any parameters, refer to Section 10: Troubleshooting.

When the image is stable, switch the controller into MAN FOC mode. Bring the sample out of focus to a positive error value close to the maximum achieved in section 6.3.3.

Switch the controller into SRV mode, paying close attention to the rate at which focus on the sample is recovered.

If the rate of recovery is fast, and focus is stable, then the current P-gain and I –gain values are appropriate. Both gains can be adjusted +/- 10% to ensure it is optimal. Proceed to the section section.

If the rate of recovery is slow, and the focus is stable, the the P-gain and I-gain value can be increased. Move the sample back out of focus (in MAN FOC mode). Increase the value in by 10% and then reactivate SRV mode. Repeat this until the rate of recovery is fast, with the focus remaining stable.

If the rate of recovery is fast, but focus is unstable, then the P-gain and I-gain must be reduced. Decrease the P-gain and I-gain in 10% reductions until stable focus can be achieved. Move the sample back out of focus (in MAN FOC mode) and the reactivate SRV mode to check the speed of recovery. Find the P-gain and I-gain values that give focus stability at the maximum rate of focus recovery.

Once suitable P-gain and I-gain have been found, return to MAN FOC mode. Bring the sample out of focus to a negative error value close to the maximum achieved in section 6.3.3. Switch the controller into SRV mode to validate the P-and I-gain settings.

For high magnification objectives, fast focus recovery speeds may not be attainable due to the generally lower signal-to-noise ratio causing focus instability. Find the best compromise between focus stability and focus recovery.

6.5.3 D-gain

Derivative (D) gain is not strictly required for any focus systems to achieve near-optimal performance. Prior Scientific strongly advise that only users with experience of PID control mechanisms attempt to use this value.

6.6 Fine offset adjustments

Offsets values calculated using the auto-find function or by a manual setup are likely to be close to the optimal. These values can be further refined if required by the following procedure.

Switch the controller into SRV OFF mode.

Moving the digipot will now move the offset lens. Whilst the servo is active, it will compensate for this movement to keep the error value close to zero, causing the focal plane to change.

Adjust the offset position until the sample is perfectly in focus.

6.7 Saving offset values

A default offset value must be set for each objective. This value will be loaded automatically when that objective is selected.

Go to the offsets submenu in the PureFocus850 GUI.

Click Set, and then click Default.

The default offset value, in the offset section of the main PureFocus GUI, will change to match the current offset value below it. It is important to save a default offset after setting up each objective.

6.8 Parameter setup for remaining objectives

Switch the controller into MAN FOC mode.

Click on the name of the next objective to be set up.

Repeat steps 6.3-6.7 for the remaining objectives in your imaging system.

If you wish to add another objective that was not part of your original objective set, click on an empty slot in the objectives list and then follow the same procedure. No pre-set values will be set. It is therefore imperative to pay special attention to the following parameters: laser power, PID, offset value, background and output limit. Initial PID values should be set with reference to other objectives in the set, with P-gain and I-gain being proportional to objective magnification.

6.9 Saving your settings

Go to the parameters submenu and click save to controller. The objective settings will now be saved to the controller memory and will be retained when the controller is power cycled.

Resetting the controller will delete these settings.

In order to generate a permanent record of the objective settings, go to the parameters submenu and click export to file. This will open a dialogue box that can be used to rename any objectives, if desired. Click OK, and then save the parameters into a desired location on the PC. These settings can be recalled at any time, for example following a controller reset or if multiple users of the PureFocus850 wish to use their own parameters for different sample types.

6.10 Flags

There are four performance indicators, or flags, displayed on the left hand side of the GUI. These are not essential for operation but can be used to indicate acquisition of focus, sample presence, focus locking at the correct interface, or if the PF850 is within an allowed travel range. These are discussed in more detail in Section 7 and Section 8.

Section 7: Advanced features

The features in this section are not strictly required for operation of the PureFocus850. They can however be used to enhance performance, enable the use of non-traditional samples, or as triggers in a customised imaging protocol for OEM applications.

7.1 Non-typical sample types

The PureFocus850 is designed with these common sample types in mind:

- Permanent fixed biological slides with a 1.5 thickness coverslip
- Cell culture dishes
- Wafer samples e.g. semiconductor
- Flat metallic samples

Other sample types, however, reflect infrared light and therefore can be used. Challenges that must be overcome with these samples include weak reflectance, light scattering or noise, focus instability or multiple reflective planes. The following sections detail how the advanced features can be used to overcome these challenges.

7.2 Signal to noise

Focus correction is dependent on the error value, which is generated by an imbalance of reflected signal on each side of the PureFocus850 sensor. The error value must be close to zero when the sample is in focus and increase linearly to +1 or -1 either side of focus (see section 6.3-6.4 for more details). This is achieved by a good signal to noise ratio.

Assuming that an offset value has been found (see section 6.3) The following table indicates scenarios which may indicate a poor signal to noise ratio.

| Problem | Possible Cause |
|---|---|
| Error value does not increase significantly either side of focus | High background |
| Error value does not swing evenly either side of focus | Imbalanced background Signal from another light path element |
| Error value increases rapidly and then decreases to zero rapidly either side of focus | Low signal |

7.2.1 Optimising signal to noise

Bring the sample into focus. If you have set up an offset previously, the error value will be close to zero. If you have not set up an offset before, refer to section 6.3.2.

Click on Setup in the main PureFocus850 GUI. A signal should be present at the pinhole centre.

Click on Laser in the main PureFocus850 GUI.

Review the setup graph. Adjust the laser power so that the peak is as large as possible and any other signals are small (Fig. 18).

Click on Pinhole in the main PureFocus850 GUI.

If any signal, other than the signal at the pinhole centre, appear to be significantly stronger than the other background signal after the previous step, these signals need to be excluded from the error value calculation. Decrease the pinhole half width setting such that this signal is outside of the bounds of the pair of blue lines which appear on the setup graph.

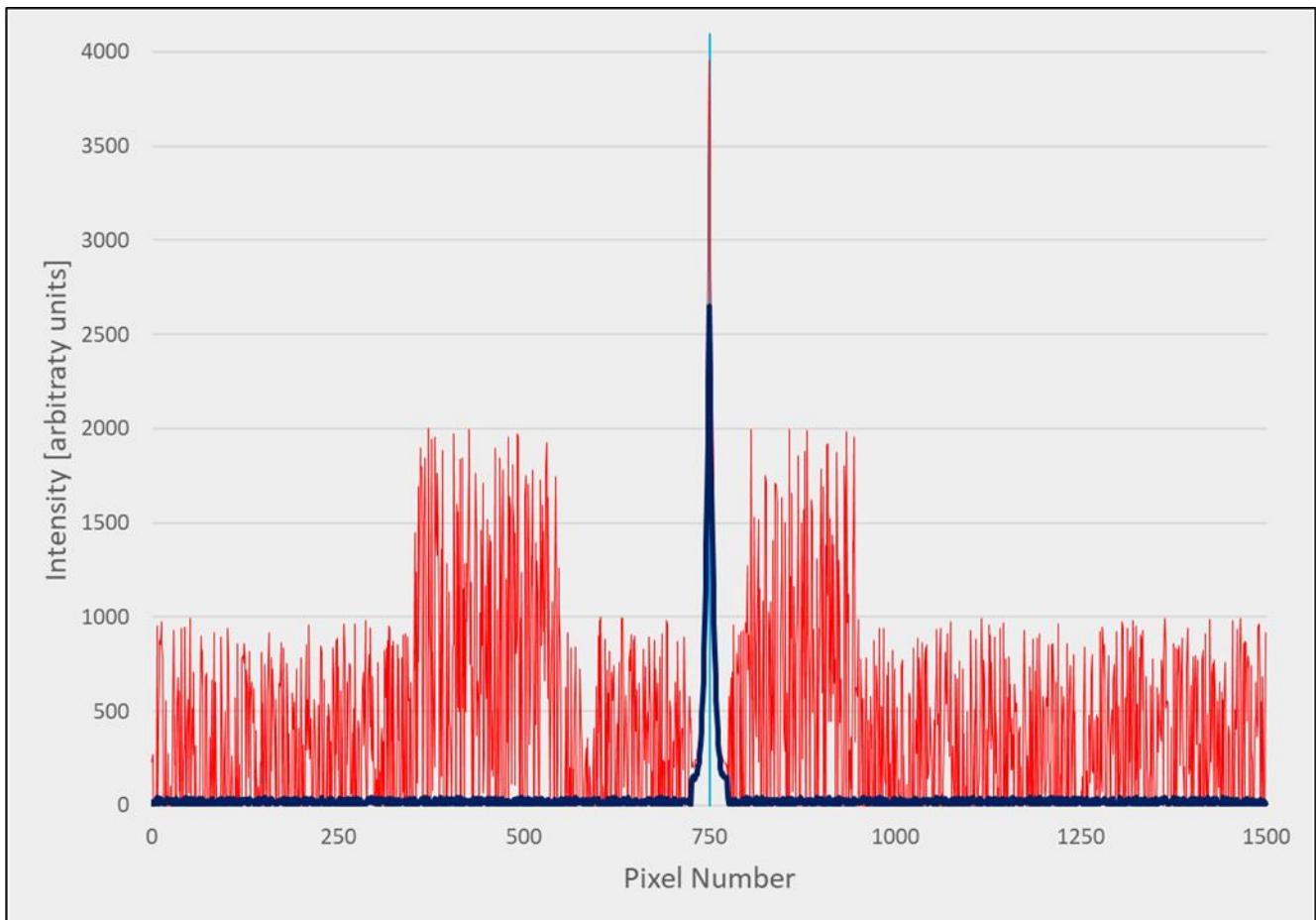


Figure 18: Signal-to-noise ratio is critical for generating error signal swing. Even with a strong signal, excessive laser power can generate additional unwanted background signal on the sensor (red). By reducing the laser power, the background can be reduced or eliminated, making the signal from the sample much more dominant and generating better error signal swing (dark blue). The relative size of the sample-generated peak to the background, rather than the absolute size of the peak, is more important.

Repeat for each objective in the objective set.

Individual objectives, especially if they use a different imaging method or light source, may also exhibit excess background when compared to others in the objective set.

Mouse over the background signal to determine an approximate value, which will be displayed as the Y-coordinate underneath the setup graph.

Increase the average background signal to eliminate the background signal from the setup graph. Click 'No' when asked whether the new background should be made the default for all objectives.

Any additional signal that is still present can be excluded from the error value calculation by changing the pinhole half width (Fig. 19).

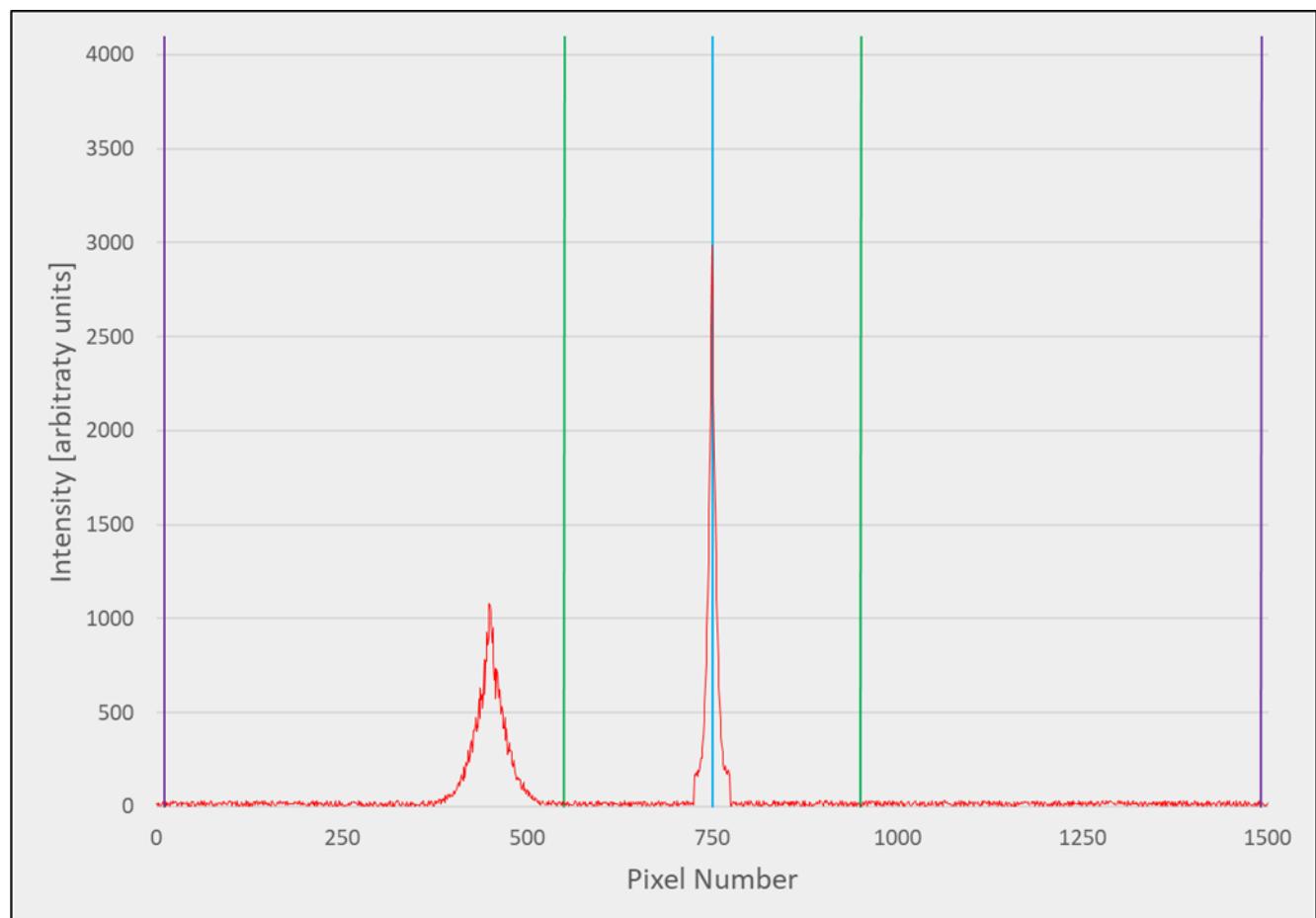


Figure 19: If multiple reflective layers are present in the sample, additional unwanted signals produced by the sample can appear on the sensor. With a wide pinhole (purple), these signals are used in autofocus position calculation and can lead to sub-optimal focus recovery speed and accuracy. The additional signal on the left of the central peak can be excluded by reducing the pinhole width (green).

7.2.2 Signal to noise considerations

Laser power and pinhole width have opposite effects on PureFocus850 performance. Raising the laser power increases focus recovery speed, but increases background signal. Decreasing the width of the pinhole helps to remove unwanted signals from the error value calculation, but can also limit the focus recapture range if set too narrow. The aim of section 7 is finding the best compromise between the two, to generate a balanced error swing.

Increasing the average background value to remove background is a valid approach to enhancing signal to noise, however excluding too much of the useful signal reflected from the sample can cause focus instability. Average background values should be set so they just eliminate the majority of background signals. Laser power and pinhole width can then be used to refine the signal morphology to ensure the error value calculation is optimised.

7.3 Enhanced focus control via Focus Flag

7.3.1 Focus Stability

Focus stability can be enhanced using the Focus Flag parameters. This can allow higher P-gain and I-gain settings to be used with higher magnification objectives which may otherwise cause visible oscillations when acquiring images. It can also increase focus stability for samples which exhibit poor low signal to noise.

If, after having following the instructions in Section 6 and checking the section 10: Troubleshooting, the focus is still unstable, the interrupt function can be used to turn off the autofocus servo (without switching the controller into MAN mode) when the focus flag is active. By default, the focus flag is true when the error value is <0.05.

Bring the sample into focus, ideally by switching the controller into SRV mode.

Open the Flags submenu in the main PureFocus850 GUI.

Click ‘On’ in the focus flag section of the Flags submenu.

If focus stability is still insufficient after switching on the interrupt function, the error parameter can be increased to inactivate the servo at a higher error value.

Switch the controller into MAN FOC mode.

Slowly adjust the focus on the sample using the controller digipot. Check the error value displayed on the main PureFocus850 GUI. Determine the maximum error value which can be allowed whilst also maintaining focus on the sample. Increase the value into the error parameter field in the Focus Flag submenu to this value. This will depend on the depth of focus of the objective.

7.3.2 Low depth of focus objectives

For low depth of focus objectives, an error parameter value of 0.05 may be insufficient to keep the sample in focus with the interrupt function switched on. Signal complexity can also lead to focus oscillation between two adjacent focal planes. In these cases, the focal plane must be specified stringently.

Bring the sample into focus, ideally by switching the controller into SRV mode.

Open the Flags submenu in the main PureFocus850 GUI.

Turn on the interrupt function by clicking ‘On’ in the focus flag section of the Flags submenu.

Switch the controller into MAN FOC mode.

Slowly adjust the focus on the sample using the controller digipot. Check the error value displayed on the main PureFocus850 GUI.

Determine the maximum error value that can be allowed whilst also maintaining focus on the sample.

Decrease the value into the error parameter field in the Focus Flag submenu to this value. This will depend on the depth of focus of the objective.

7.3.3 Two-factor focus verification

The focal plane can also be defined, in addition to the error signal threshold, using the signal magnitude at the pinhole centre, to provide even greater control.

Bring the sample into focus, ideally by switching the controller into SRV mode.

Open the Pinhole submenu in the main PureFocus850 GUI. Click on setup to open the setup graph.

Check the value at the pinhole centre in the pinhole submenu. Enter this value plus 10% into the 'Higher' threshold box in the Focus Flag area of the Flags submenu.

Check the pixel value of the highest intensity background signal inside the pinhole on the setup graph. Set the 'Lower' threshold box to be this value plus 100. If no significant background signals are observable, set the 'Lower' threshold to 500. This threshold can be increased to increase focal plane specification.

WARNING: The higher and lower thresholds can override the error signal control of focus position, which can lead to focus being held in undesirable positions if the signal is complex.

7.3.4 Testing focus recovery time

The timing function can be used to test focus recapture speed or optimise PID settings It requires that the focus flag be set correctly.

Switch the controller into MAN FOC mode. Open the Timing dialogue box, which can be found on the main GUI taskbar

Enter a time window (in milliseconds) that the focus flag must be true (green) for stable focus to have been achieved.

Move the sample a suitable distance away from focus. Increasing the distance will increase the focus recapture time.

Switch the controller into MAN SRV mode. Focus will be recaptured, and the time taken to refocus will be displayed in the Timing dialogue box. The time includes both the refocusing time and the time window that the focus flag much be true.

7.4 Sample detection via Sample Flag

The sample flag can be used to switch off the autofocus servo if no sample is present. This is a useful safety feature for all users, but can also be used to enable rapid exchange of samples in a higher throughput environment.

Open the Flags submenu in the main PureFocus850 GUI.

Switch the controller into SRV mode.

Scan across the sample by moving the XY stage of the microscope. Review the A+B value displayed in the main PureFocus850 GUI. Determine the minimum A+B value produced by the sample. Enter this value minus 5% into the ‘Lower’ field in the sample flag area of the Flags submenu. When the A+B value is greater than the value in this field, the sample flag will be active.

Switch the controller into MAN mode.

Move off the sample onto an area of the sample holder. Check that the A+B value is significantly smaller than the ‘Lower’ field value.

Turn on the inhibit function. This will deactivate the autofocus servo (without switching the controller into MAN mode) whenever the sample flag is not active.

Move the sample back under the objective. Switch the controller into SRV mode and scan the sample, ensuring that focus is maintained. If the sample flag deactivates (the flag is showing in green on the main PureFocus850 GUI) decrease that ‘Lower’ field value by an appropriate amount.

In SRV mode, move onto an area of the sample holder. Verify that the sample flag deactivates.

7.5 Using multiple offsets

Samples may contain multiple focal planes of interest. Multiple offsets can be saved per objective to allow focus to be held on different planes.

Set the default offset as described in Section 6.

Switch the controller into SRV OFF mode.

Rotate the digipot on the controller. The focal plane will change as the autofocus corrects for the movement of the offset lens.

Once a new focal plane has been selected, click Offset in the main PureFocus850 GUI. Click Set, and select ‘1’. Repeat for any further focal planes, using the next available number in the list. Up to five offsets can be set per objective.

Whilst in SRV mode, these focal planes can be moved through dynamically. Click Offset, followed by Go to and select the number of the desired focal plane.

7.6 Software limits via Range Flag

The range flag can be used to switch off the autofocus servo outside of a certain Z-position range. This is a useful safety feature for all users, but can also be used to maintain focus on certain focal planes at high magnification without refocusing onto other planes, and to enable rapid exchange of samples.

Switch the controller into MAN FOC mode. Bring the sample into focus. Open the Range dialogue box which can be found on the main GUI taskbar.

Tick the box to turn on the range flag.

Enter positive and negative movement limits. The values entered relate to the maximum allowed positive and negative change in Z-position from the Z-position at which the PureFocus 850 servo was switched on. Limits can either be narrow to ensure fidelity to a small depth of field, or wide to simply protect the objective.

Click Set.

Switch the controller into MAN SRV mode. The range flag should appear green.

Take note of the Z-position shown in the main GUI. If this value moves outside the range limits set, the range flag will deactivate (turn grey) and the autofocus servo will also deactivate.

Note that the servo will reactivate if the error signal dictates that the PF850 drive the focus back into the defined range.

To turn off the range limits, untick the box in the range dialogue box *and click set*.

Section 8: OEM features

The PureFocus850 GUI has a number of features which can be used by customers who are writing their own software to initiate specific behaviours. These features are not intended for use outside of this context.

8.1 Interface selection via interface flag

Samples such as cell culture dishes or LCD screens may contain multiple reflective interfaces. Similarly, immersion objectives may produce complex signals with multiple interfaces. Any of these interfaces can be used by the PureFocus850 assuming the error value is zero at a given offset value.

The signal reflected at each of these interfaces can be used, via the interface flag, by software developers to trigger unique scanning behaviour in the PureFocus850.

In the following example two interfaces will be shown, and whilst further interfaces may be present in practise, the principles remain the same.

Click setup to open the setup graph. Switch the controller into MAN FOC mode.

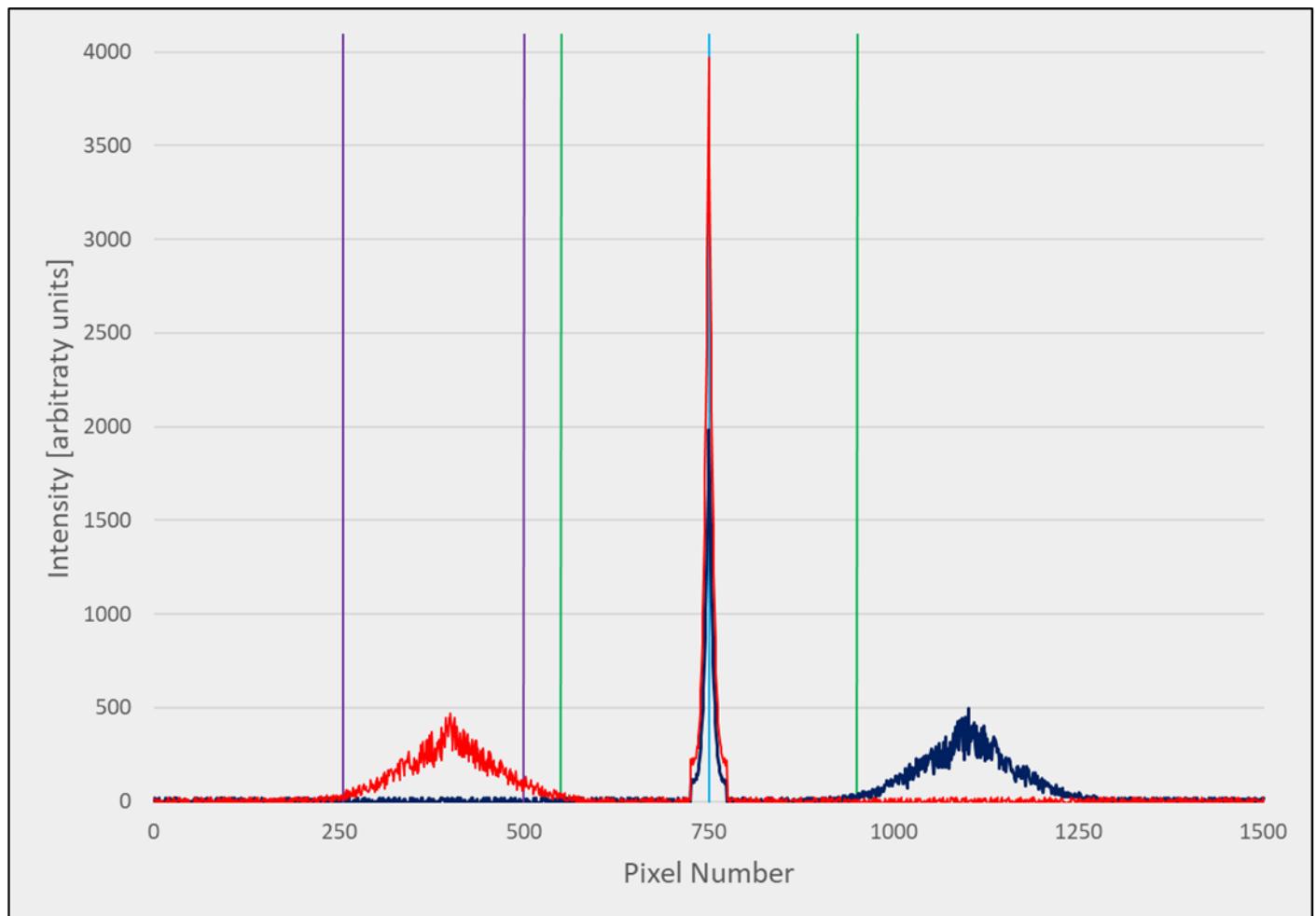


Figure 20: For samples with multiple interfaces, diffuse secondary peaks can be derived from the sample. In the above example, the red signal represents when the sample is in focus, and the dark blue signal indicates a different focus position where the PF850 is focus locked at another reflective interface and the sample is not in focus. These can be excluded from autofocus position calculation by narrowing the pinhole width (green), so in both cases the error value is 0. The interface flag calculates the total amount of signal present in an area of the sensor, defined by a start and end pixel (purple), and determines if it is between the upper and lower thresholds. In this example the thresholds are 50000 and 20000 respectively. In the dark blue example, very little signal is detected between the start and end pixel, so the interface flag is inactive. In the red example, a significant signal is detected, so the interface flag is active. If focus becomes locked such that the error value is 0, but the interface flag is false (dark blue), this can be used as a trigger to initiate a move to the correct interface (red).

Use the digipot to adjust the focus on the sample. Observe each time a peak is visible across the pinhole centre. Each of these peaks is a distinct interface in the sample. It is advantageous to use from the strongest peak in the sample as focus capture range and focus stability will be enhanced, however this is not mandatory.

Follow the objective parameter set up guide (section 6) using the manual offset discovery method in section 6.3.2. Adjust the offset position until the chosen peak is positioned at the pinhole centre.

Close the setup graph and switch the controller into SRV mode. Verify that the sample is held stably in focus. Refer to section 6.5 if the focus is unstable.

Reopen the setup graph. Observe the signal morphology, identifying major features which are significantly more intense than any background signal. Ensure the controller is in MAN FOC mode and check these features move when focus is adjusted. Ensure that these features change significantly when a different peak is at the pinhole centre.

Move the sample back into focus.

Open the flags submenu. Identify the pixel positions where this major feature is present by mousing over the signal on the setup graph. The pixel positions are displayed underneath the graph. Enter the desired start and end pixels into the appropriate boxes in the interface section of the flags submenu. It is possible to use the signal inside the pinhole itself as a 'major feature' if the peaks are significantly different in size (Fig. 20).

The D value, shown in the interface section, will have increased significantly. This is the total signal present between the pixels specified.

Adjust the focus on the sample. Observe the maximum and minimum D values when the error value is between the error signal maxima (see section 6.3.3 for full details). Set these as the upper and lower threshold values in the interface section of the flags submenu.

If the above procedure has been followed correctly the correct interface flag, present on the main PureFocus GUI, will be active (green) when chosen peak is at or close to the pinhole centre, and inactive when the chosen peak is far from the pinhole centre or a different peak is close to the pinhole centre.

The activity of this flag can now be used to trigger specific behaviour in customised pieces of software.

8.2 Focus search

The focus and sample flags can be used to increase the range and speed of successful focus recapture. Refer to sections 7.3 and 7.4 for full details on how to setup these flags. Note that the corresponding interrupt and inhibit functions are not required to be active for these OEM features. These features are not currently available for use with piezo focusing mechanisms.

8.2.1 Search and Lock

This can be used when the sample flag is false and PureFocus850 is unable to capture focus. The routine will carry out a ramp of the Z-axis across a specified peak to peak (pk to pk) range and at a specified speed. If the SAMPLE flag becomes true at any point during this ramp the ramp will stop and the focus servo will be activated, pulling the sample into focus.

Ensure the sample flag is set up correctly

Open the Search submenu. Refer to the Search section of the submenu.

Set the pk to pk range for the scan. The value refers to the maximum Z-axis distance will move during the scan. Refer to the Z position field in the main PureFocus850 GUI. The default setting, 10, gives a peak-to-peak range of 1 micron.

WARNING: When the search begins, the focus will move half the peak-to-peak range relative to the starting position, and then scan through the starting position for the full peak-to-peak range specified. Take care to ensure that a crash does not occur in the peak-to-peak range.

Set the maximum speed for the scan. Lower speeds will increase the chance of a successful focus search but decrease the overall speed of the scan. Higher speeds will make the scan run more quickly, but focus search may be unsuccessful if the sample flag threshold is high.

Click 'search and lock'. The PureFocus850 will adjust focus until the sample flag is active (green), then activate the servo and begin to autofocus.

If the search is unsuccessful, consider increasing the peak-to-peak range or decreasing the focus speed.

8.2.2 Fast Capture

Fast capture can be used when the sample flag is true but the sample is far from focus. In this scenario, the focus error signal may be falling off and driving the sample to focus may be slow, even with the maximum stable gain settings. This routine will drive the Z-axis towards focus at a specified speed, with the direction specified by the current sign of the error signal. When the sign of the error signal changes PureFocus detects it has just passed through focus, ending the routine and activating the focus servo.

Ensure the sample flag is active, either by bringing the sample closer to focus or by making the sample detection threshold less stringent. Ensure that the sample detection threshold is appropriate for your sample.

WARNING: Ensure the focus flag is set up correctly. If it is not set this procedure may result in a crash.

Open the Search submenu. Refer to the Fast Capture section of the submenu.

Set the maximum speed for the scan. Lower speeds will increase the chance of a successful focus recapture but decrease the overall speed of the scan. Higher speeds will make the scan run more quickly, but focus recapture may be unsuccessful if the focus flag settings are stringent.

Click 'capture'. The PureFocus850 will adjust focus until the error value sign changes, then activate the servo and begin to autofocus.

If the fast capture is unsuccessful, refer to section 7.3 to refine the focus flag settings.

8.3 Measure mode

For users wanting to generate control loops directly from the positional error signal (see POS command in section 9.2), measure mode can be accessed via the mode menu in the main GUI. This mode differs from the stepper and piezo modes as it does not use the PID output (see OUTPUT command in section 9.2).

POS refers to the error signal indicated by the green bar in the centre of the main GUI and operates between a value of -1 and +1. These signals are calibrated with respect to the DAC output pins (see section 12) such that POS = -1 at 0V and POS = 1 at 10V. When the sample is nominally in focus, and POS = 0, the DAC output would be 5V.

Section 9: ASCII command sets

The PureFocus850 communicates with a baud rate of 460800, 8 data bits, no parity, 1 stop bit, no flow control. All commands/responses are terminated by <CR> character (ASCII 0x0D).

9.1 Signal settings commands

| Command | Response | Description |
|------------------------|------------------------|--|
| PINHOLE | Centre, width | Returns pinhole centre and width |
| PINHOLE, c, w | 0 | Sets regions A and B to symmetric regions with equal numbers of pixels (w) either side of a centre pixel (c) for current objective. See also REGION command. |
| LASER | LASER, n | Returns laser power for current selected objective. n=range 0 to 4095 |
| LASER,N | 0 | Sets laser power in range 0 to 4095 for current objective |
| BACKGROUND, a, b, c ,d | BACKGROUND, a, b, c ,d | Sets the single pixel background levels for the 4 user regions A,B,C & D for the current objective. a, b, c and d are integers between 0 and 4095 |
| BACKGROUND | BACKGROUND, a, b, c, d | Returns the single pixel background levels for the current objective. a,b,c and d are integers between 0 and 4095. |

9.2 Focus signal commands

| | | |
|---------|------------------|--|
| ABCD | A, B, C, D, I, S | Returns region summations A,B,C and D range 0 to 6142500 (4095 x 1500) I = FOCUS state [0 1] S = SAMPLE state [0 1] |
| POS | f | Returns current position signal. $f = (A-B)/(A+B)$ |
| OUTPUT | f | Returns current PID output signal f |
| ERROR | f | Returns current error signal. $f = \text{POS-TARGET}$ |
| TEST, t | 0 | Begins test mode t: 0=disabled 1=continuously prints TARGET,INPUT,ERROR,OUTPUT to the terminal window. 2=continuously prints A,B,C,D to the terminal window |

9.3 Servo settings commands

| | | |
|------------------|---|--|
| SERVO | b | Returns servo state b: 0=off 1=on |
| SERVO,b | 0 | Set servo state b: 0=off 1=on NOTE: servo can only be enabled when AUTO=2 |
| KP | n | Returns proportional gain n for current objective |
| KP,n | 0 | Sets proportional gain value n for current objective |
| KI | n | Returns integral gain n for current objective |
| KI,n | 0 | Sets integral gain value n for current objective |
| KD | n | Returns differential gain n for current objective |
| KD,n | 0 | Sets differential gain value n for current objective |
| TARGET | 0 | Sets servo set point to current error signal value for current objective. |
| TARGET,f | 0 | Sets servo set point to value f. Where $-1 \leq f \leq 1$ |
| TARGET,? | f | Returns target position for current objective |
| SERVODIR | n | Returns sign used for servo PID loop, independent of ZD: n=-1 or 1 |
| SERVODIR,n | 0 | Set sign used for servo PID loop, independent of ZD: n=-1 or 1 |
| OUTLIM, min, max | 0 | Sets minimum and maximum allowed limits for OUTPUT (output of the PID) for the current objective |

| | | |
|--------|---------|--|
| OUTLIM | min,max | Returns the minimum and maximum allowed limits for OUTPUT. |
|--------|---------|--|

9.4 Flag settings commands

| | | |
|--------------|---|--|
| SAMPLE | b | Returns current sample state b: 0=sample not detected 1=sample detected when A+B>SAMPLEL |
| SAMPLEL | I | Returns the low threshold I for determining the sample state for the current objective |
| SAMPLEL,n | 0 | Sets the low threshold n for determining the sample state for the current objective |
| FOCUS | b | Returns current focus state b: 0=not in focus, 1=in focus when FOCUSL<C<FOCUSH and ERROR<FOUSR |
| FOCUSL | n | Returns the low threshold n for determining the focus state for the current objective |
| FOCUSL ,n | 0 | Sets the low threshold l for determining the focus state for the current objective |
| FOCUSH | n | Returns the high threshold n for determining the focus state for the current objective |
| FOCUSH,n | 0 | Sets the high threshold h for determining the focus state for the current objective |
| FOUSR | f | Returns the range threshold f for determining the focus state for the current objective |
| FOUSR,f | 0 | Sets the range threshold f for determining the focus state for the current objective |
| IFP,n | 0 | Used for checking focus recovery time. Sets focus period to n milliseconds where Focus = 1 (see focus flag commands) |
| IFP | n | Returns focus period n ms. |
| TTIF | n | Returns time to achieve in focus n in ms |
| INTERFACE | b | Returns current interface state b: 0=wrong interface 1=correct interface when INTERFACEL<D<INTERFACEH |
| INTERFACEH | n | Returns the high threshold n for determining the interface state for the current objective |
| INTERFACEH,n | 0 | sets the high threshold n for determining the interface state for the current objective |
| INTERFACEL | n | returns the low threshold n for determining the interface state for the current objective |
| INTERFACEL,n | 0 | sets the low threshold n for determining the interface state for the current objective |

| | | |
|---------------------|---------|--|
| INHIBIT | b | Returns the servo inhibit status b: 0=servo inhibit off 1=servo inhibit on |
| INHIBIT,i | 0 | Sets the servo inhibit status |
| FOCUSI | b | Returns the servo interrupt status b: 0=servo interrupt off 1=servo interrupt on |
| FOCUSI,b | 0 | Sets the servo interrupt status (see above) |
| SERVOLIMIT, a, p, m | 0 | Sets software limits on servo position (stepper only): a = flag activation status (0=inactive, 1=active) p = maximum positive movement distance (p*current user unit) m = maximum negative movement distance (m* current user unit) |
| SERVOLIMIT | a, p, m | Returns parameters associated with servo limits. |
| SERVOINLIMIT | b | Returns servo within limit state b: 0=out of active servo range 1=within active servo range |

9.5 Objective parameters commands

| | | |
|-------|---|---|
| OBJ,n | 0 | Loads parameters that are saved for objective n (n=1 to 6) |
| OBJ | n | Returns current objective n (n=1 to 6) |
| LIST | 0 Example: OBJ,1 KP,2000.10 KD,0.00 KI,0.00 LASER,0 TARGET, 0 FOCUSL,2000 FOCUSH,4095 FOCUSR,0.02 INTERFACEH,0 INTERFACEL,0 OUTLIM,-100000,100000 SAMPLEL,50000 ABON,0 PINHOLE,750,750 REGION,D,0,0 BACKGROUND,0,0,0,0 LENSSO,1,0 LENSSO,2,0 | Lists all parameters that are set for the current objective. Note this list may change in subsequent releases as new features are added. User must read each <CR> terminated line up to and including "END" |

| | | |
|--|---|--|
| | LENSSO,3,0 LENSSO,4,0 LENSSO,5,0 SERVOLIMIT,1,2,1 END | |
|--|---|--|

9.6 Digipot settings commands

| | | |
|------------|---|--|
| LENS | n | Returns current function of the digipot control n: 0=focus motor/piezo 1=offset lens |
| OF | n | Return speed scaling of digipot for focus control. n= 1-100% |
| OF,n | 0 | Set speed scaling of digipot for focus control. n= 1-100% |
| OFL | n | Return speed scaling of digipot for offset lens control. n= 1-100% |
| OFL,n | 0 | Set speed scaling of digipot for offset lens control n= 1-100% |
| LENSH | 0 | Sends the offset lens to its home position |
| LENSV,n | 0 | Moves offset lens at velocity n steps/s |
| LENSP | p | Returns current offset lens position in steps (there are 25600 steps/mm) |
| LENS\$ | b | Returns offset lens motion status b: 0=idle 1=moving |
| LENSACC,a | 0 | Set acceleration of offset lens |
| LENSVEL,v | 0 | Set maximum velocity of offset lens |
| LENSG,p | 0 | Move offset lens to position p in steps |
| LENSGO,n | 0 | Move offset lens to stored offset lens position n for current objective n=1 to 5 |
| LENSSO,n | 0 | Save current offset lens position to stored offset lens position n n=1 to 5 |
| LENSSO,n,p | 0 | Save position p as stored offset lens. Position p in microsteps Offset n=1 to 5 |

9.7 Focus commands

| | | |
|-----|---|---|
| UPR | n | Reports the microns/revolution n of the focus drive in stepper mode (default is 100) or the full travel range (microns) in piezo mode |
|-----|---|---|

| | | |
|---------|---|--|
| UPR,n | 0 | Sets the microns/revolution n of the focus drive in stepper mode or the full travel range in piezo mode. |
| \$ | n | Returns focus motion status: n=0, idle n=4, moving |
| PZ | n | Returns current focus position in user units. Default user units are 100 nm steps. See SSZ. |
| PZ,n | 0 | Sets current focus position to n user units. Default user units are 100 nm steps. See SSZ. NOTE: only available in stepper control. |
| VZ,f | 0 | Moves focus at velocity f microns/s. NOTE: only available in stepper control |
| U | R | Move focus up by C steps |
| D | R | Move focus down C steps |
| C | n | Returns default step size for U and D commands in user units. Default user units are 100 nm. See SSZ |
| C,n | 0 | Sets default step size for U and D in user units. Default user units are 100 nm. See SSZ |
| V,n | R | Move focus to position n in multiples of user units. Default user unit is 100nm |
| Z | 0 | Set current focus position to zero |
| SMZ | n | Return maximum focus speed n in 1-100% range, NOTE: SMZ/SAZ not relevant for Piezo drives. |
| SMZ,n | 0 | Set maximum focus speed n in 1-100% range. |
| SAZ | n | Return maximum focus acceleration n in 1-100% range. |
| SAZ,n | 0 | Set maximum focus acceleration n in 1-100% range. |
| SMZ,U | n | Return maximum focus speed n in microns/s |
| SMZ,n,U | 0 | Set maximum focus speed n in microns/s |
| SAZ,U | n | Return maximum focus acceleration n in microns/s/s |
| SAZ,n,U | 0 | Set maximum focus acceleration n in microns/s/s |

| | | |
|---------|---|---|
| SSZ | n | Returns number of microsteps per user unit for focus. Default value is 50, giving default user units of 100 nm when UPR=100u. When using stepper motor, the smallest step size is when SSZ=1. Resulting in theoretical step size of UPR/50000 microns. Actual user units are therefore restricted to multiples of this base step size. This also applies to piezo positions when using the V,U and D commands. Although total position range is limited to 0-UPR, and as the piezo DAC output is 12bits the theoretical smallest step is UPR/4096 microns. Consider using the PIEZO command directly for piezo systems and convert positions in your application. |
| SSZ,s | 0 | Sets number of microsteps per user unit for focus (see above) |
| ZD | d | Returns sign of focus drive direction: d=-1 or 1 |
| ZD,d | 0 | Set sign of focus drive direction: d=-1 or 1 |
| LMT | h | Reports active limit switches h in hex string format: n=00, no limits active n=10, positive Z limit active n=20, negative Z limit active |
| PIEZO,n | 0 | Sets the raw piezo DAC output when in piezo mode. n is an integer between 0-4095 equating to 0-10V on piezo analogue output |
| PIEZO | p | Returns current raw piezo DAC output n when in piezo mode. |

9.8 System commands

| | | |
|---------|---|--|
| DATE | Prior Scientific Instruments OptiScan LF Version 1.10 compiled Sep 22 2021 15:19:20 | Returns two <CR> terminated lines indicating product, version and build date. |
| SERIAL | n | Returns serial number n of controller. |
| RESET | 0 | Resets unit and sets all parameters to default. |
| RESTART | 0 | Restarts unit, saved parameters maintained. |
| FLAG,h | 0 | Sets general-purpose 32-bit user flag h in hex. Reset to zero on power cycle, restart or reset. e.g. FLAG,1234FACE |

| | | |
|------------|-----|--|
| FLAG | h | Returns general-purpose 32-bit integer value in hex format. |
| SAVE | 0 | Save all current parameters to flash backup. May take several seconds. |
| CONFIG,m,s | 0 | Sets focus control and sensor mode: m='S' for Stepper drive m='P' for Piezo drive m='H' for measure – error is output to DAC. s='S' for slice mode s='L' for line mode e.g. "CONFIG,P,S" |
| CONFIG | m,s | Returns focus control and sensor mode (see above) |
| KBDLOCK | b | Returns the lock status of controller keypad: 0=unlocked. 1=locked. Lock status is indicated on the controller display. OBJ = unlocked. DIS = locked |
| KBDLOCK, b | 0 | Sets the lock status b of controller keypad: 0=unlock keypad. 1=lock keypad |

9.9 Advanced commands

| | | |
|-------------|---|--|
| @, start, n | Example: to read the entire line sensor array Send : @,0,1500 Receive multiple data: @,064,064070082..<CR> @,064,07908506C..<CR> @,064,07A06606D..<CR> ... @,028,12412D12D..<CR> | Returns n pixels worth of raw line sensor values from start to start+n-1. Each line consists of three comma separated parts: 1: @ 2: decimal number of pixels in part 3 3rd series of bytes, each 3 bytes are 0-FFF hex pixel values Not available in AUTO2 mode. |
| REGION,r | s, f | Returns start and finish pixel for region r = A,B,C or D s,f = integers in range 0 to 1499 |

| | | |
|--------------|------------|---|
| REGION,r,s,f | 0 | <p>Set region start and finish pixels. e.g. REGION,D,840,841</p> <p>Note: although regions A,B & C can be set via this command usually the PINHOLE is used for this.</p> |
| AUTO, n | AUTO, n | <p>Sets current auto update state n: 0=disabled. A REFRESH command is required to update ABCD data. 1= auto refresh enabled but controller not updated in real time. 2=auto refresh enabled and head continuously sends ABCD data to controller in real time. This is the default mode and is required for using the servo.</p> |
| AUTO | n | Returns current auto update state. |
| REFRESH | REFRESH, 0 | Request the controller to update the ABCD values when in AUTO,0 mode. |
| EXPOSURE, n | 0 | Sets the line sensor exposure time n in microseconds. |
| EXPOSURE | n | Returns the line sensor exposure time n in microseconds. |

9.10 Error Codes

| | |
|------|-------------------------|
| E,2 | Not idle |
| E,3 | No drive |
| E,4 | String parse |
| E,5 | Command not found |
| E,8 | Value out of range |
| E,10 | Argument 1 out of range |
| E,11 | Argument 2 out of range |
| E,12 | Argument 3 out of range |
| E,13 | Argument 4 out of range |
| E,14 | Argument 5 out of range |
| E,15 | Argument 6 out of range |

Section 10: Troubleshooting

10.1 No laser line emitted visible on the target

Open the main PureFocus850 GUI. The laser power is set to zero by default during installation. Increase the laser power to 500 and the laser line will become visible. Increase the laser power further if necessary.

If there is still no laser present, adjust the microscope focus such that the entire PF200/PF201 target can be seen. Adjust the PF300 camera focus to bring the target into focus. The laser may now be visible at this point

If the laser is still not present, use a hex key to sweep the 45° control for up to three turns both clockwise and anticlockwise. Repeat with the 0° control. The laser line should come into view.

PureFocus850 systems are shipped with the blade screw fully unwound. If an error has caused the blade to be fully wound, no laser line will be present. Turn the screw clockwise until resistance can be felt.

If the above steps do not allow the laser line to be viewed, power off the PureFocus850 system, remove the PureFocus850 head from the microscope, and remove the knife edge from the beam path.

WARNING: The following procedure will cause the laser to be emitted up towards the user. Do not look into the exit aperture of the PureFocus850 head or stare at the laser line visible on the tissue paper. Do not undertake this procedure if you are not able to work safely.

Carefully place the head on a tabletop with the laser exit aperture side facing up, place a piece of thin tissue paper across the exit aperture.

Turn on the PureFocus system, open the GUI and set the laser power to 4000 and darken the room.

Open the alignment camera software and holding the alignment camera adjust focus to view the tissue paper. If the laser line is visible on the paper adjust the 0 degree and 45 degree dichroic adjustments to centre it on the exit aperture of the PureFocus head.

Power off the system, reinstall the head and continue with the installation procedure.

If the laser line is still not present, contact Prior Scientific.

10.2 Laser signal is too high or too low in setup mode

If the laser signal is too high or too low, use the laser power setting to reduce or increase the signal as appropriate.

For line mode systems, if low signal intensities are present with low magnification objectives and further higher magnifications are due to be calibrated, ensure that the 45° mirror is optimally positioned. As described in section 4.3.2, using a high magnification during the alignment procedure can help to avoid this effect.

Ensure your system is in the correct mode. Slice mode systems (PF850) should be used in slice mode, and line mode systems should be used in line mode (PF850M) under normal circumstances.

Line mode systems that are being used with samples which do not reflect a high amount of signal can be used in slice mode to increase the amount of signal used for positional recalculation. Note that this will also increase the background signal. It will not affect the line mode functionality of the system, however.

If a peak cannot be detected during line mode setup, even at maximum laser power, switch into slice mode. This will allow the peak to be seen more clearly when adjusting the 45° mirror. Set the PureFocus850 back into line mode once the peak has been identified.

10.3 Additional peaks are visible in setup mode

Additional peaks are a common feature in certain sample types e.g. cell culture dishes. They can also be features of certain objectives or other components in the light path.

Use the pinhole half width function and laser power to eliminate these peaks from the error value recalculation. See section 7.2 for full details on optimising signal to noise.

10.4 Background signal in setup mode is high or uneven

Decrease the laser power to minimise the background level.

If this results in the peak used for error value calculation becoming <25% the height of the setup graph, the average background can be increased either for the entire objective set or just for a single objective. The laser power can then be increased such that only the peak is visible on the graph. Note this will impact focus stability; see to section 7.3 for methods to improve focus stability.

Illumination can also create significant amounts of background signal if it has a high infrared component, which produces a signal morphology similar to the below. Where possible, decrease illumination power and increase camera exposure time. Similar to the above, the average background can also be increased to eliminate this background signal (Fig. 21).

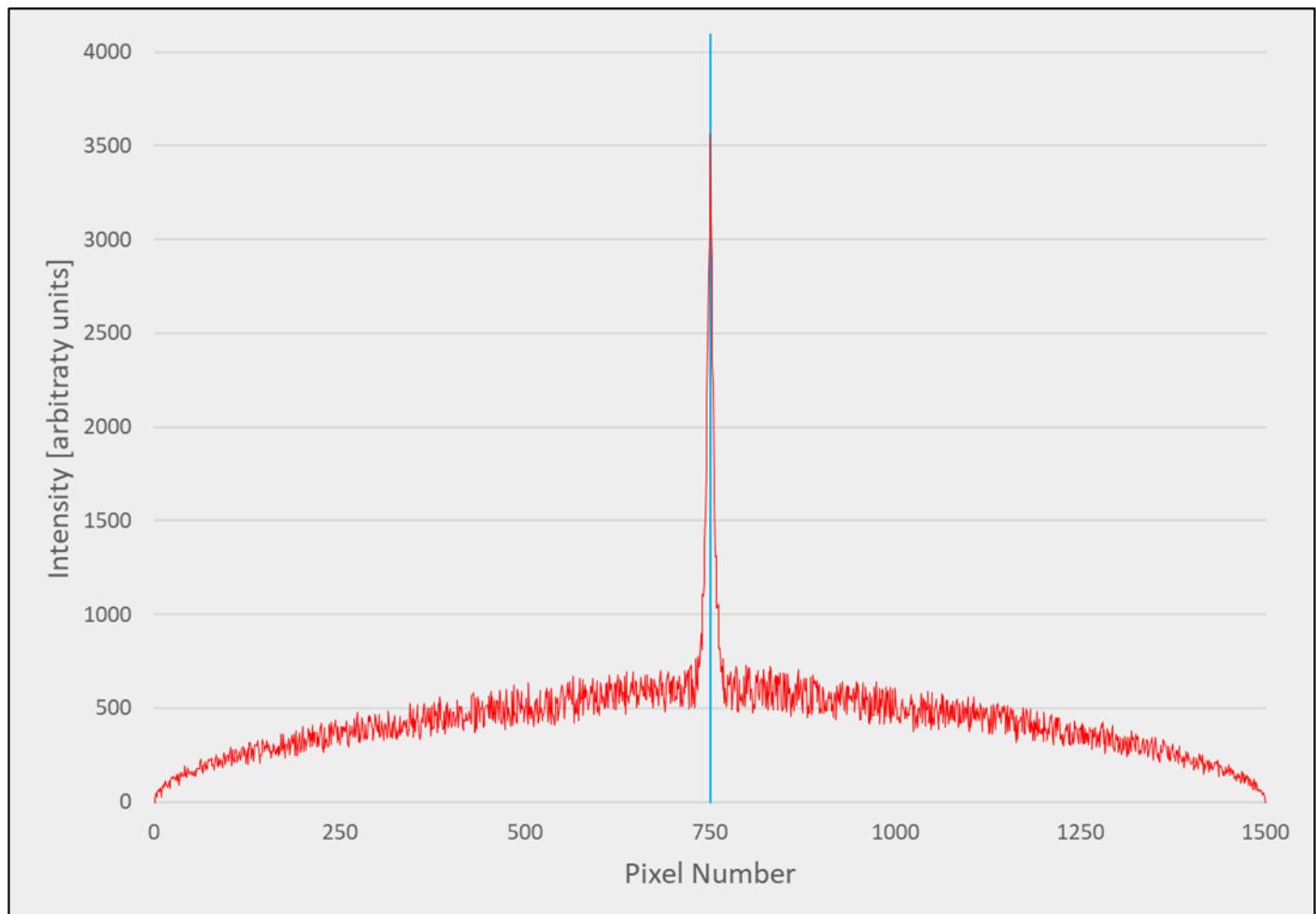


Figure 21: Excessive background signals produced by illumination typically have this morphology. To reduce this effect, first reduce the laser power of the PF850. Then reduce the illumination intensity and increase the exposure time of the camera. Note that the central peak (derived from the sample) is still significantly above the background. If some background remains adjust the background threshold to improve the signal to noise, as described in section 7.2.

If the background is high but skewed to one side of the sensor as below, it is highly likely that an element of the illumination pathway is misaligned (often the condenser in transmitted light systems) (Fig. 22). Check the setup manual for your microscope.

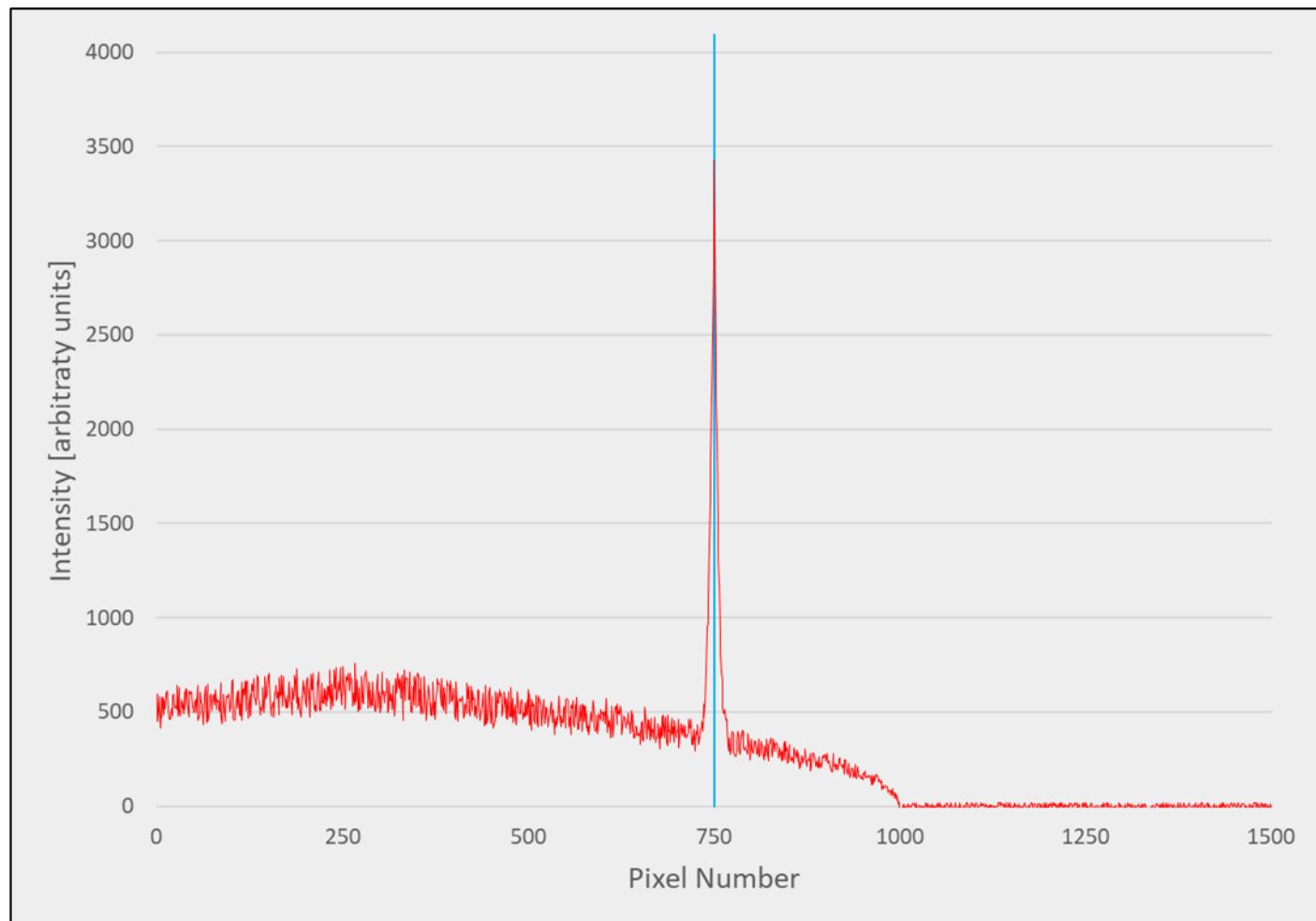


Figure 22: Additional background caused by illumination can also appear skewed on the sensor, appearing more concentrated on the left or right side. This is often caused by a misaligned component in the optical path, such as the condenser in transmitted light microscopes.

If the signal appears to be bouncing up and down, reflected laser light may be returning directly to the sensor and/or laser.

Click Setup to open the setup graph

Attempt to deliberately misalign the dichroic in one axis by making very small adjustments to 45° control. When making this adjustment, observe the sensor signal in Setup Mode to confirm that the signal size is not compromised.

10.5 No or imbalanced error value swing

10.5.1 No error value swing

If the error value is always close to zero it is likely that the laser power is set too low, or the background setting is too high. Ensure that an average background has been set and it is appropriate for your objective (see section 4.5) for details. Increase the laser power if required.

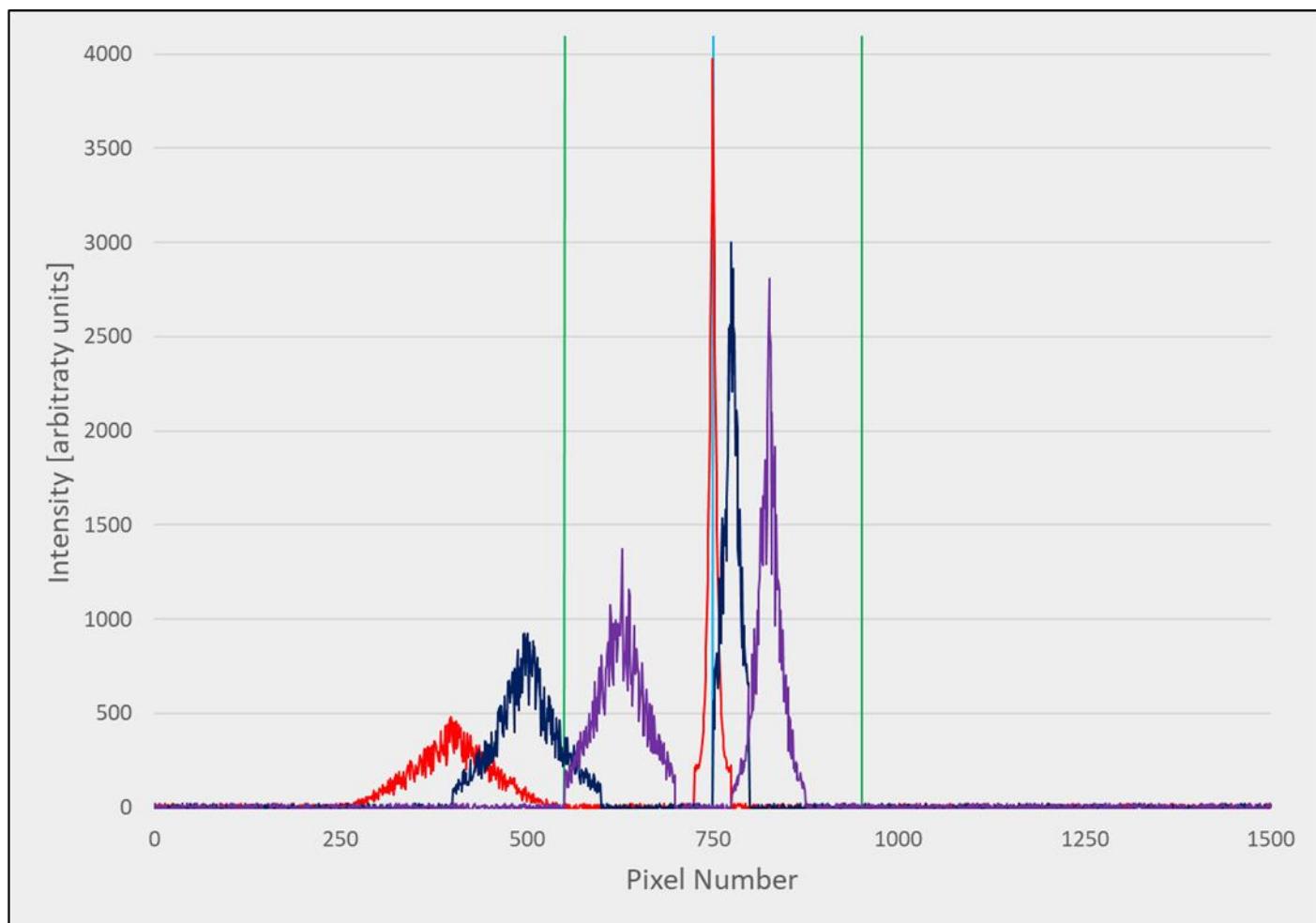


Figure 23: In this example two reflective planes are visible on the sensor. The red signal morphology is representative of when the sample is in focus. the primary interface gives a strong peak at the pinhole centre, and the secondary interface gives a second diffuse peak on the left. To exclude the signal coming from the second peak from error signal calculation, the pinhole (green) has been narrowed. When focusing down, below the desired focal plane, the signal morphology changes, and the secondary peak appears within the pinhole. Initially, the error signal will increase in magnitude, as the primary peak moves left away from the pinhole centre, and only a small amount of the secondary peak is present in the pinhole (dark blue). Moving further, the entire secondary peak moves into the pinhole, and increases in intensity, whilst the primary peak decreases in intensity, subsequently resulting in a reduction in the error signal (purple).

Alternatively, there may be multiple signals from optical path components that happen to produce an error value close to or equal to zero. Use the pinhole function to eliminate these from the error value calculation; see section 7.2.

If the error value is close to +1 or -1, it is likely that a reflection from an optical path component is present on the sensor.

If there is no error swing after calculating an offset, it is likely that one of the above situations is true. The peak identified during offset calculation is not derived from the sample, and therefore cannot be used by the PureFocus850.

10.5.2 Imbalanced error swing

Imbalanced error swing is most often caused by an additional signal caused by a reflection within the light path

For samples with two interfaces that are close together, for example very thin tissue sections, the error signal may appear to increase initially and then decrease again when moving the focus in one direction, but not the other. This is caused by the signal from the second interface crossing the pinhole centre, and tends to occur when the objective moves towards the sample.

When the error value is at its maximum when moving in this direction, set the pinhole width such that it just touches the signal on the right hand side of the setup graph. This will produce a more continuous error signal increase when focusing in this direction (Fig. 23).

10.6 Focus recovery is too fast or focus unstable

PID values are too high. Refer to section 6.5.

Alternatively, the signal morphology may be complex close to the pinhole centre when the sample is in focus. Refer to section 7.2 to increase signal to noise. Section 7.3 is also useful to stabilise focus at a specific focal plane.

10.7 Focus recovery is too slow

PID values may be too low. Refer to section 6.5.

Fast recovery times may not be achievable depending on objective magnification or signal complexity. OEM users should refer to section 8.2.

10.8 Focus recovery does not occur despite a good error value swing

If the sample flag is inactive (not green), check that the inhibit function is switched off; see section 7.4.

Low PID values can result in very poor focus recapture rates that appear to result in no focus recovery. Increase the PID values, referring to section 6.5.

The PureFocus850 servo is deactivated whilst the setup graph is open. Ensure the setup graph is closed, and that the controller is in SRV mode.

10.9 Performance is good but the flags are inactive

For many users, flag setup is not required and it will not impact performance of the PureFocus850. See sections 7.3, 7.4, 7.6 and 8.1 to set up the focus, sample, range and interface flags respectively.

10.10 Focus locks when my sample is not in focus

If the sample is close to focus, switch the controller into SRV OFF mode. Use the digipot to adjust the focus of the sample. Once optimal focus has been achieved, ensure the offset is saved by going to the offsets submenu, set and then select either default or 1-4.

Once an objective has been set up successfully, but is not performing correctly after setting up another objective in the set, check that a default offset has been set for the former objective. Refer to section 6.7.

If your signal morphology is complex, the PureFocus850 may be using the incorrect reflection from

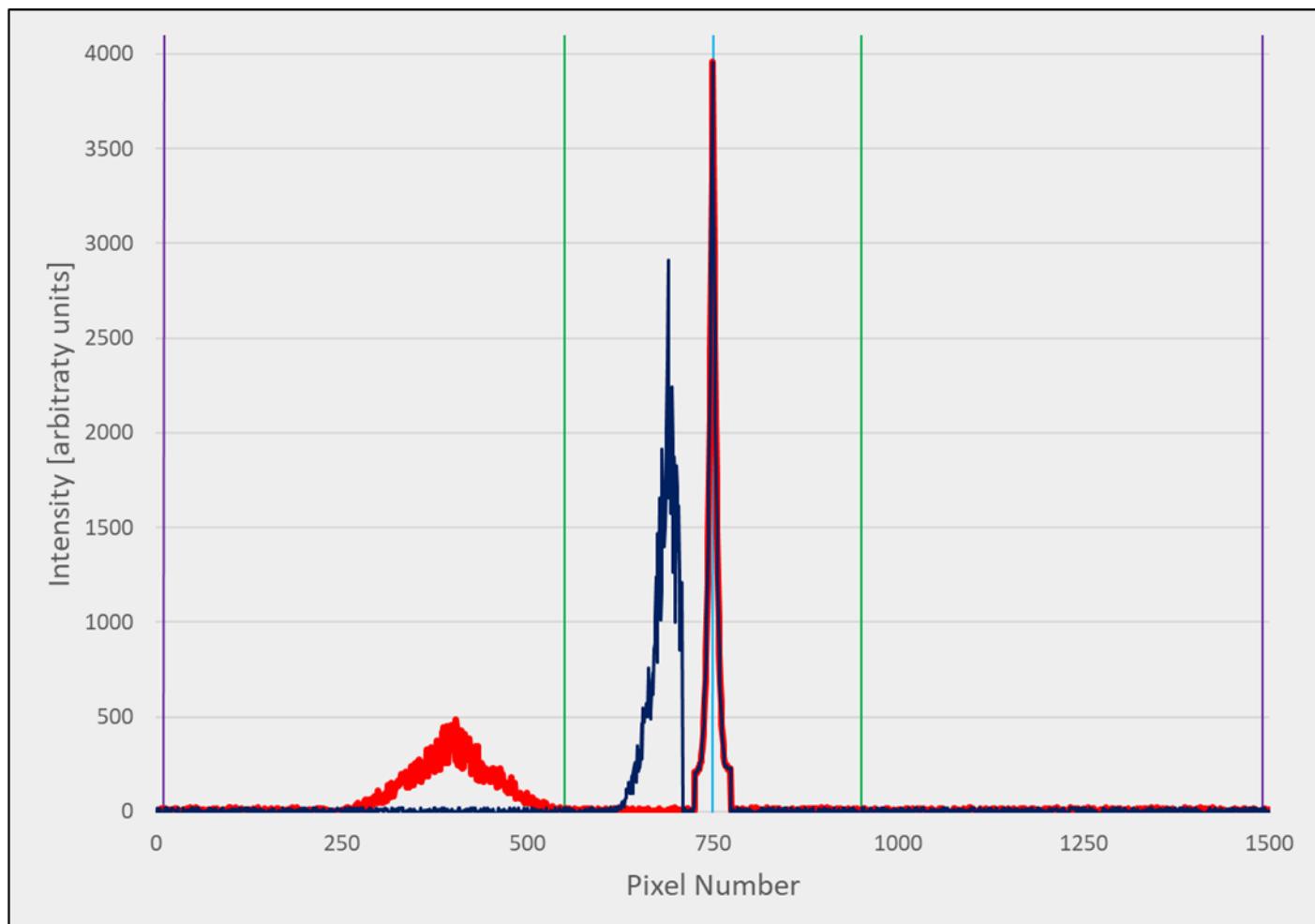


Figure 24: Where two sample-derived reflections appear on the sensor, but are distinct and 250 or more pixels apart (red), reducing the pinhole size (green) to exclude the second peak from the error signal calculation is the best approach. If the two signal are close together however (dark blue), reducing the pinhole size is too restrictive in terms of how far from focus the PF850 will recover from, and will often lead to other problems with error swing as described in figure 23. It is better to leave the pinhole wide (purple) consider these peaks as one continuous signal and adjust the offset position to compensate whilst in SRV OFF mode. The focus flag signal intensity thresholds at the pinhole centre can be set to define the ‘in focus’ state, and the interrupt function used to stabilise focus once it has been achieved.

the sample to offset from. There are a number of options for dealing with this scenario.

Check the setup graph. If the PureFocus preferentially uses the signal morphology caused by this reflection, set the controller into SRV OFF mode and adjust the offset value to move the sample back into focus.

If the PureFocus variably uses multiple reflections, and the focus locks on different positions reduce the size of the pinhole to allow the signal from one reflection to dominate the signal used for error signal calculation.

If the reflections are very close together, widen the pinhole to improve error swing, and then refer to section 7.3 to enhance focus stability at one of the reflections (Fig. 24).

All users can use the interface flag to identify when focus is locked around the incorrect interface; see section 8.1 for details. Most users can use this as an indicator to manually move the sample close to optimal focus, activate the servo, and begin their tile scan or imaging routine. OEM users can use this flag to trigger other behaviours, using the search functions in section 8.2 or customised protocols. Section 9 contains a number of ASCII commands which can be used for the latter.

10.11 A suitable offset cannot be calculated for an objective

10.11.1 The offset is outside the range specified for my magnification

If the offset calculated by the Autofind function is not within the range specified in section 6.3, the signal being used to calculate the offset may not be generated by the sample.

Set the controller into MAN FOC mode. Bring the sample into focus and check that the error value is close to zero. Then adjust the focus up and down, checking that the error value changes to approximately +1/-1. If a good error value swing can be achieved, then it is safe to proceed with setting up the objective set.

If the error value does not change either side of focus, it is likely that the signal used to calculate the offset is produced by another reflection in the optical path. This is likely to be the case when a high offset value (>450000) is calculated for a low magnification objective.

Open the setup graph whilst the sample is in focus. Set the controller into MAN OFF mode. Using the digipot, move the offset lens into the range specified in the table below.

| Magnification | Biological sample with outer interface of 0.15 standard coverslip | Materials sample |
|---------------|---|------------------|
| 5x | 5000 - 20000 | -13000 - 10000 |
| 10x | 10000 - 40000 | -13000 - 10000 |
| 20x | 40000 - 100000 | -13000 - 20000 |
| 40x | 100000 - 250000 | -13000 - 20000 |
| 60x | 250000 - 450000 | -13000 - 30000 |
| 100x | 50000 - 200000* | -13000 - 50000 |

**When using immersion objectives and the glass/sample interface; non-immersion 100x objectives do not have sufficient offset to use the glass/air interface.

Determine if there are any small peaks in this range. Increase the laser power to increase the size of the peak. Continue to adjust the offset lens until the peak is aligned with the pinhole centre, then check the error value swing as above. Pairs of peaks, caused by interfaces which are close together, can be the cause of identification of an inappropriate offset. If imbalanced error swing is now a problem, refer to section 10.5 for further information.

10.11.2 Insufficient offset range

High magnification objectives will approach the positive offset limit when used with standard thickness coverslips. Thicker coverslips may therefore prevent the use of these objectives with the PureFocus850.

The PureFocus850 has a limited negative offset range (typical maximum = -13,000). Certain materials objectives, or samples which have their focal plane of interest above their first reflective layer, use this range. In some cases, the offset limit is reached before the peak generated by the reflective layer crosses the centre of the pinhole.

First, ensure your microscope objectives are parfocal. Lack of parfocality can unnecessarily cause this effect.

Select the objective requiring the negative offset in the GUI. Alternatively, if the sample is causing this effect save the current objective set to the controller and export the parameters to file; see section 6.9. Go to the parameters submenu and create a new objective set.

Open the setup graph. Bring the sample into focus, and observe the signal on the setup graph.

Set the controller into MAN OFF mode. Use the digipot to adjust the offset position such that the peak is as close to the centre of the graph as possible. The peak should be sharp with minimal imbalance of signal on each side of the peak maximum. The challenge this presents is that an error swing cannot be achieved either side of the current pinhole centre.

Manually set the pinhole centre. Select the appropriate pixel by mousing over the peak maximum and viewing the coordinate displayed beneath the graph. Type the pixel number into the box and click ‘set pinhole centre’. The vertical blue pinhole centre line will now be aligned with the peak. Typical values for the pinhole centre are between 300 and 500, but others are acceptable. Lower values can be used but this restricts the size of the PureFocus850 sensor significantly (Fig. 25).

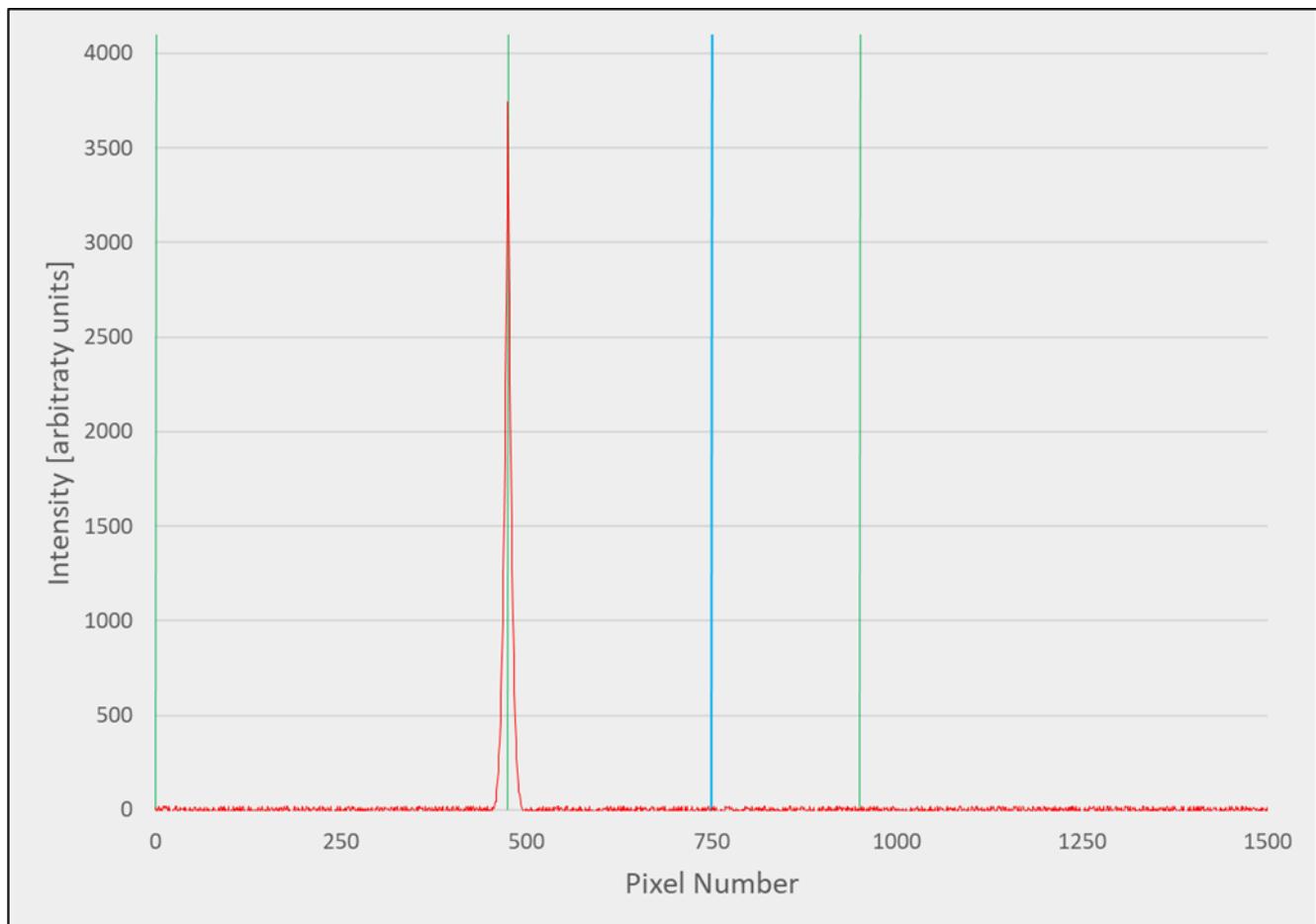


Figure 25: Objectives or samples sometimes require the use of the negative offset range of the PF850. If the negative offset required is less than -13000, typical pinhole centres (700-800) cannot be used (blue) as the signal from the sample will never cross the pinhole centre. A new pinhole centre can be set (green) to allow error signal swing either side of 0. Pinhole centre values are typically set between 300 and 500. The pinhole width is automatically reduced to compensate for the reduction in the number of useable pixels on the sensor on the left hand side of the graph.

Review section 6.3.2 and continue to set up the objective set. Any higher magnification objectives are also likely to use this new pinhole centre.

Two objective sets may need to be used; the pinhole centre is applied to all objectives within the same set.

10.12 Z Scan

The Z Scan submenu can be used to interrogate signal properties at a range of Z-positions. These properties are output to a log file, which can be sent to Prior Scientific for analysis.

Section 11: Spare parts, repairs and returns

The PureFocus850 contains no user serviceable parts. Attempting to repair or disassemble the unit will void the warranty and likely damage the unit. If the unit is not working, first contact either your distributor or Prior Scientific directly. If you wish, or are advised to, return your unit, a RMA number must first be obtained by filling in a returns form located on the prior.com website. We will then send you an RMA number which should be added to a decontamination form again located on the Prior Scientific website. Do not attempt to return this unit before such a number is obtained.

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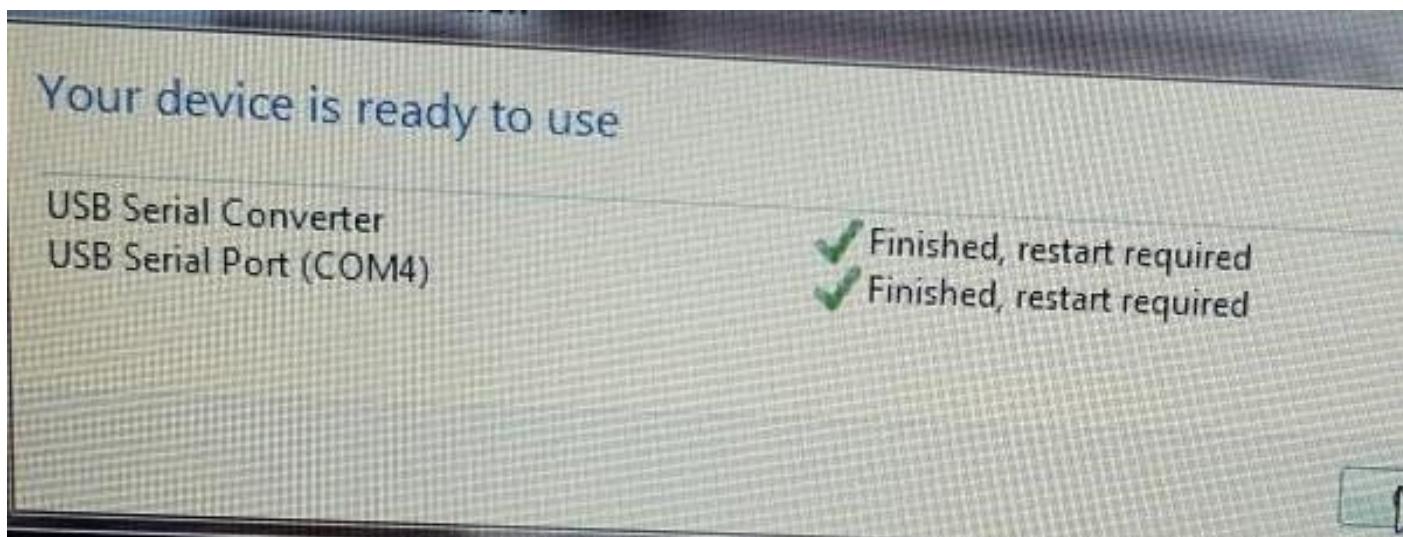
Section 12: Controller Z connector Pinout

The 15 way D connector on the PureFocus850 controller allows connection to either a stepper motor or a piezo actuator system which can accept an analogue 0-10V input. Pin connections are as follows:

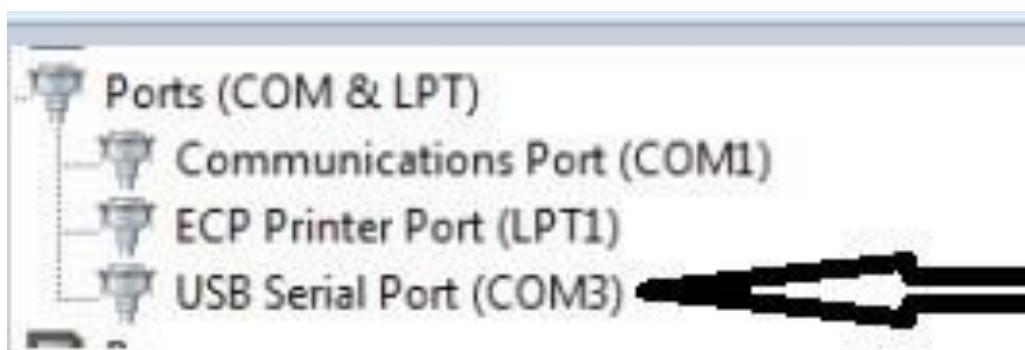
| Pin Number | Function | Comments |
|------------|----------------------|--|
| 1 | Motor Phase A+ | Minimum motor current rating = 1A. |
| 9 | Motor Phase A- | |
| 2 | Motor Phase B+ | |
| 10 | Motor Phase B- | |
| 3 | Limit Switch +ve | Limit switches should be wired “normally open” |
| 11 | Limit Switch –ve | |
| 4 | Limit Switch Common | |
| 12 | Piezo Ground Return | |
| 15 | Piezo Output (0-10V) | |

Appendix 1: Windows USB Driver Update

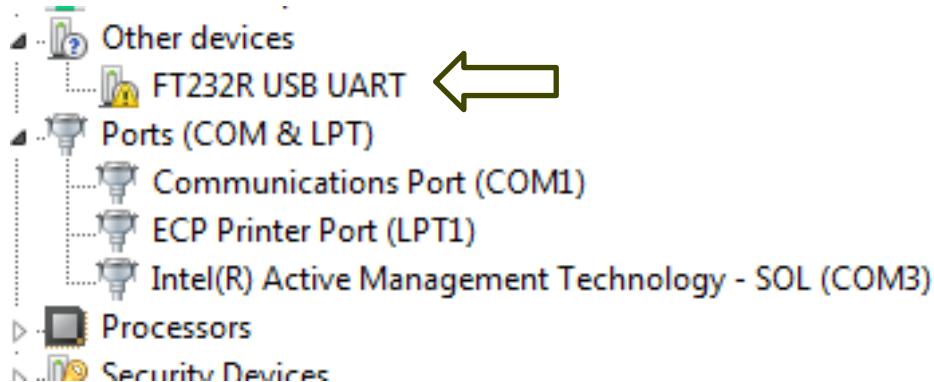
Recently the PureFocus850 controller has had an upgrade/change to the USB connection hardware. The company FTDI Ltd. manufactures the hardware and constantly updates the associated Windows USB Driver. The new USB hardware and interface driver is Windows Hardware Quality Labs (WHQL) tested and will automatically download the latest drivers when connected to a PC with internet access. The Driver Software Installation Window shown below will alert the user that the USB driver installation is complete and the Port is ready to use.



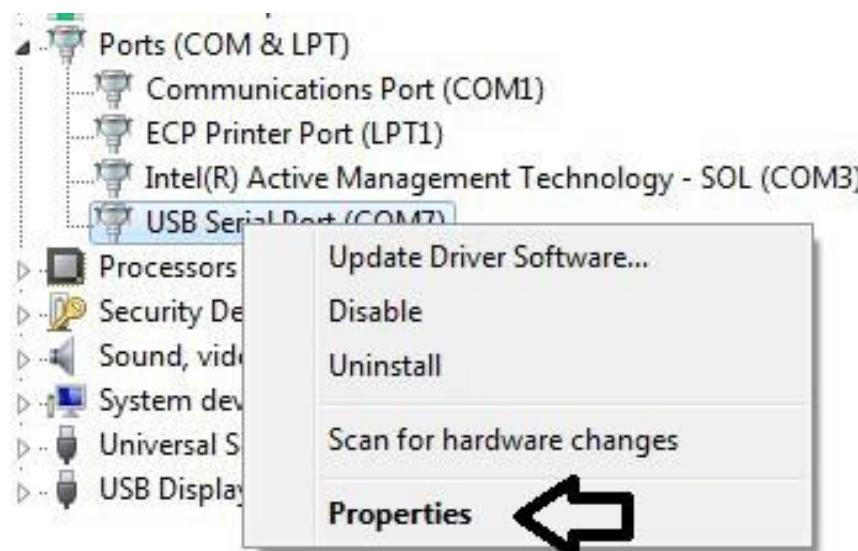
When a PC is not connected to the internet and an older or no FTDI driver is installed onto the PC there can be problems communicating via USB. The driver can appear to have installed correctly and yet not communicate. The image below is from the Device Manager of a PC where the PF850 FTDI USB driver has set up COM3 on the PC, but USB communications is still unavailable.



Alternatively, on some installs the USB connection can show up under "Other devices" as FT232R USB UART



Right clicking on the PF850 USB port in Device Manager will allow you to click on the Properties menu.



In the Properties window, click on the Driver tab and view the Driver Date: If it is older than 2016 or if the Driver Provider is not listed as FTDI, see the image below, a new driver needs to be installed. If the PC does not have internet access the files can be loaded onto the PC via a USB Stick



It is recommended to uninstall the existing FTDI drivers. There is an uninstaller tool that can be accessed from

<http://www.ftdichip.com/Support/Utilities.htm#CDMUninstaller>

From the website download the CDM Uninstaller as shown below

M Uninstaller 1.4 - Windows Device Driver Uninstaller

Uninstaller is a free application that can selectively remove Windows device drivers from the user's system as specified by the device Vendor ID and Product ID. The URL for the command line version is available [here](#) and the readme for the GUI version can be viewed [here](#). The applications come as a zipped executable that needs to be extracted prior to running. Please refer to the readme for running the application..

[CDM Uninstaller \(command line version + GUI version\)](#)

M Uninstaller 1.4 - Windows Device Driver Uninstaller

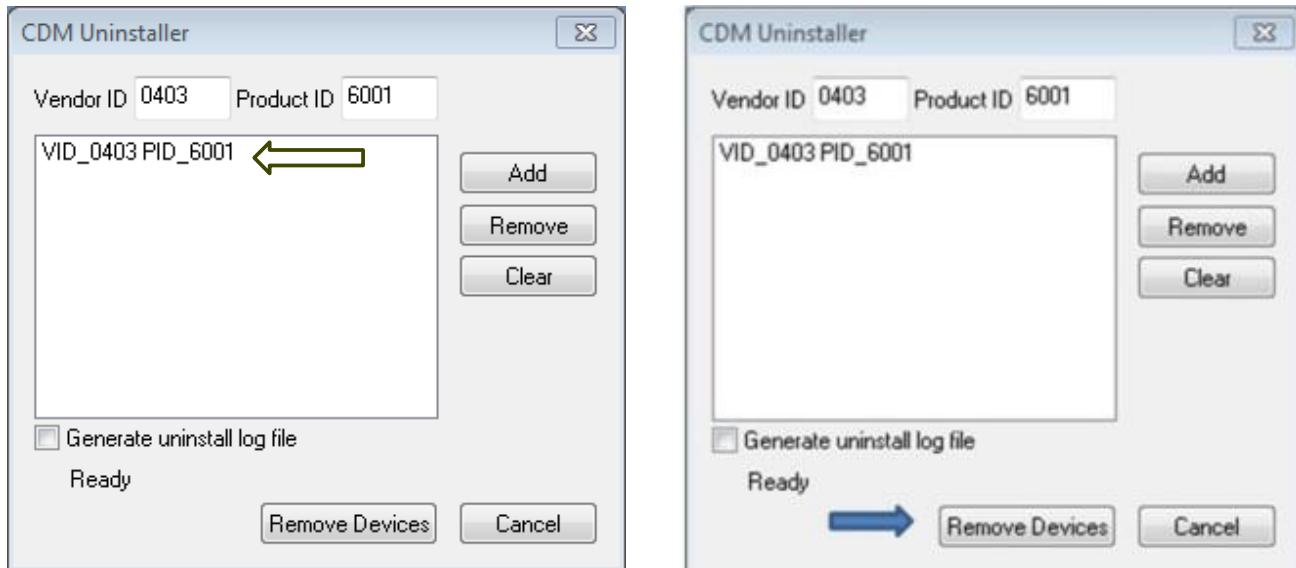
Uninstaller is a free application that can selectively remove Windows device drivers from the user's system as specified by the device Vendor ID and Product ID. The URL for the command line version is available [here](#) and the readme for the GUI version can be viewed [here](#). The applications come as a zipped executable that needs to be extracted prior to running. Please refer to the readme for running the application..

[CDM Uninstaller \(command line version + GUI version\)](#)

Extract all the files and run the CDMUninstallaerGUI.

| | | | |
|----------------------|-------------------|-------------|--------|
| ● CDMUninstaller | 3/28/2017 5:24 PM | Application | 563 KB |
| ● CDMUninstallaerGUI | 3/28/2017 5:24 PM | Application | 644 KB |

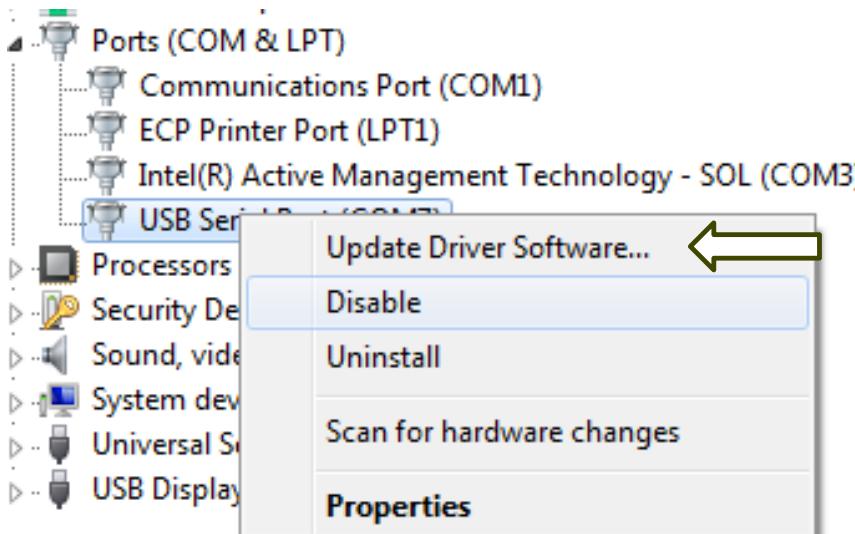
Click the Add button on the right and the Vendor ID and Product ID will be populated inside the blank area.



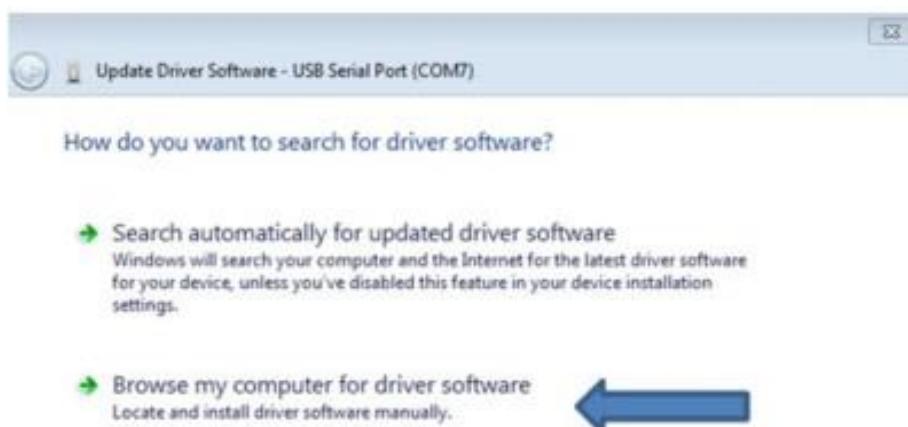
Download the latest USB Driver version from <http://www.ftdichip.com/Drivers/VCP.htm> Download the proper 32- or 64-bit driver as shown below.

Extract all of the files to the desktop or other download folder.

In Device Manager right click on the PF850 USB and choose Update Driver Software



Choose Browse my computer for driver software



Browse to the location of the saved Driver and click Next

Windows will then update the Driver using the downloaded files. A successful installation will show a new date in the USB properties as shown below.

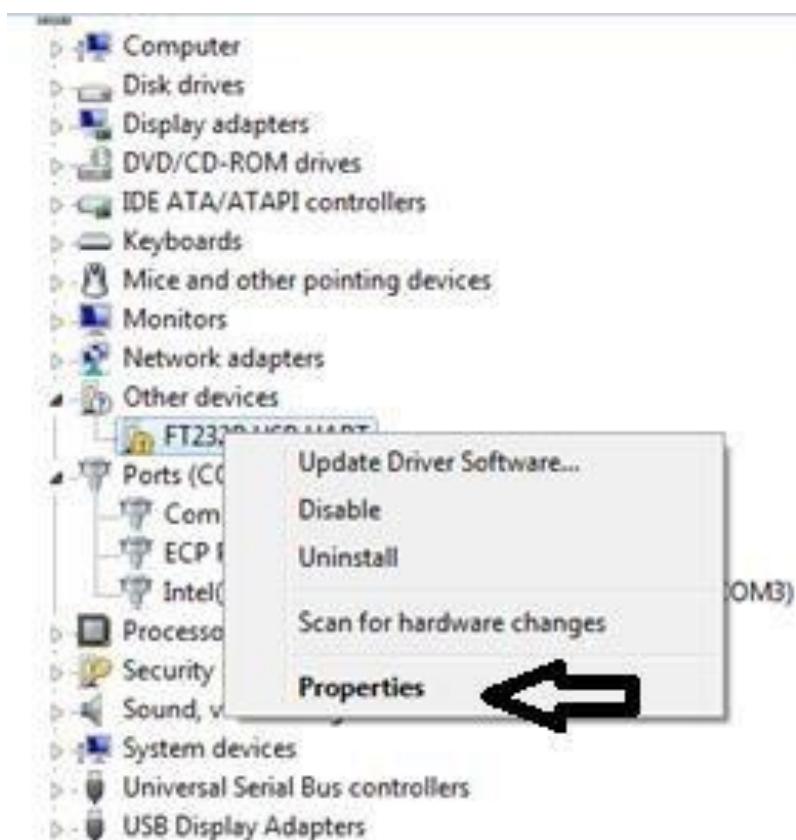


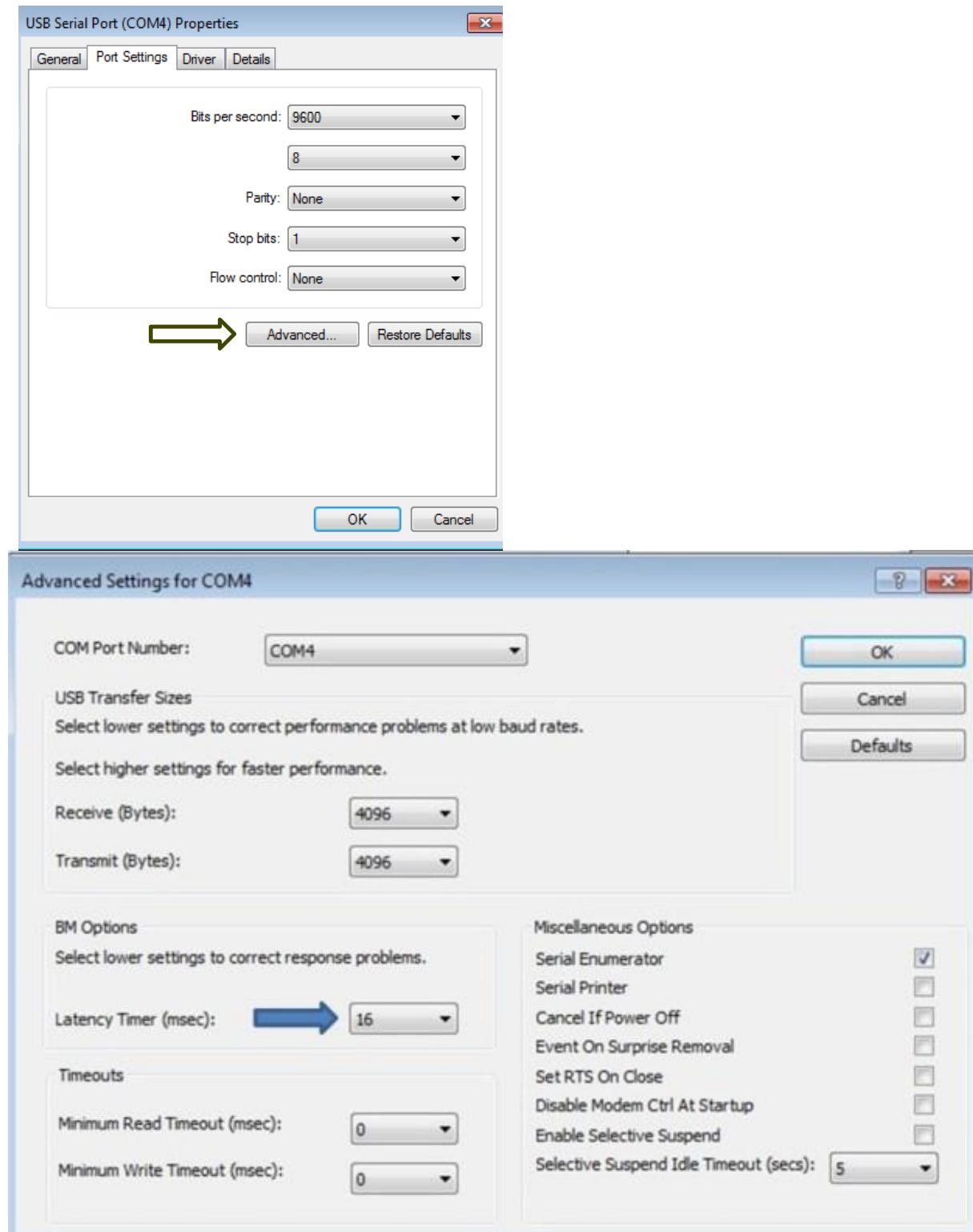
If at this point a COM port has not been assigned to your USB connection, retry the “Update Driver”

More to PRIOR than meets the eye

Version issue No. - 2.0 For more information visit www.prior.com 40 41

Software”, as in some instances Windows will not assign a proper COM port the first time the driver is updated. At this point it is advisable to adjust the USB latency timer from the standard 16ms down to 2ms. Right click on the new COM port and choose Properties. From the Port Settings tab, click on Advanced. In the Advanced tab, change the Latency Timer from 16 to 2.





Click OK twice to save the Latency Timer change.