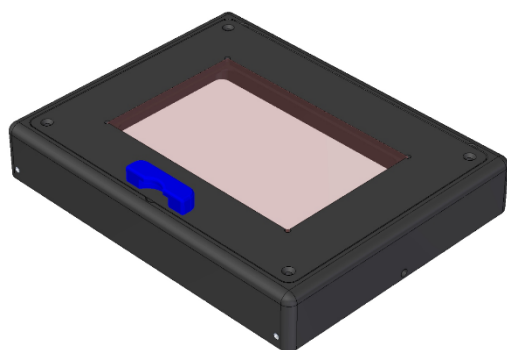


OptoTIRF – User Guide

Safety precautions:

The OptoTIRF motorised TIRF (STORM/PALM) and widefield condenser is designed to be used with an FC/PC or FC/APC single-mode, or small core ($\leq 100\mu\text{m}$) multi-mode fibre-optic and appropriate laser launch or pigtailed laser. Please check your delivery note for which connector is fitted (this can be changed retrospectively if needed).

In TIRF experiments the output laser beam is collimated by the microscope objective lens so an **eyepiece interlock** and an **interlocked cover** over the sample holder are essential for safe operation. These are an integral part of the Cairn MultiLine LaserBank System and will be included with any complete solution from Cairn. If you are self-integrating, or working with one of our resellers, then please ensure that appropriate safety measures have been taken. If you are in **any** doubt, then please call us on 00 44 1795 590140 or email tech@cairn-research.co.uk for advice.



Cairn Laser safety interlock cover for 96-well plate

In addition to the safe configuration of both the microscope and the laser launch we have provided several additional secondary safety features within the OptoTIRF.

- (i) A digital **TTL output** BNC which should be connected to TTL “shutter” input(s) of the laser source. In order for this BNC to give a high signal to enable the laser(s) the following conditions must be met:
 - a. The lid of the OptoTIRF condenser unit must be on, so as to close the safety microswitch
 - b. The “Shutter” command must be activated using a COM command or the remote.

- c. The **TTL input** BNC must be at logic high, or disconnected if not being used.
- (ii) A microswitch to ensure that shutter line is overridden to **TTL off** AND the **bypass mirror is inserted into the light path** when the lid is removed. The Bypass mirror can also be used by software as an additional mechanical shutter, but this is relatively slow, is not failsafe and should not be used as an alternative to other safety interlocks.
- (iii) An interlock connection which breaks a connection with a relay when the OptoTIRF condenser lid is removed.

These features will help to make the system safe, but are **not sufficient** on their own.

Before installation the following points should be considered:

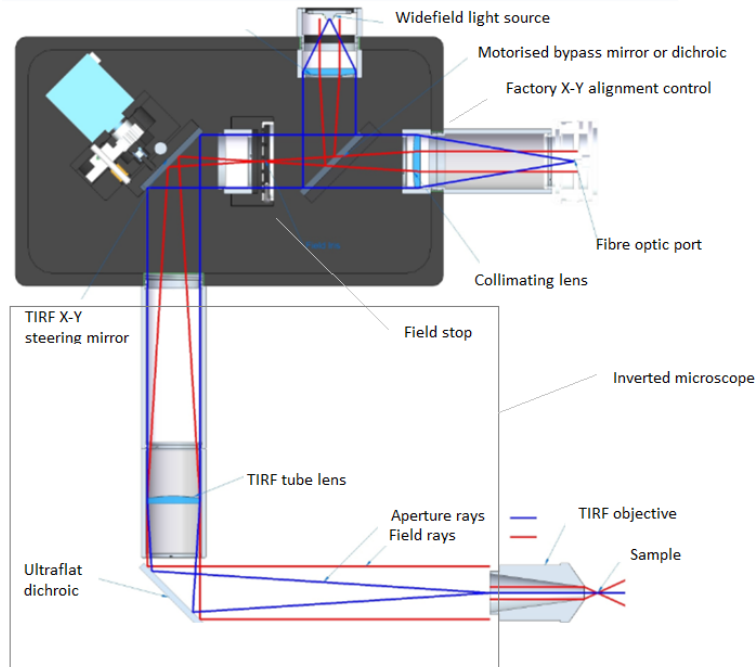
- (i) You will need the following:
 - a. Inverted microscope frame with TIRF objective
 - b. Laser source with fibre optic output
 - c. Drilled AV table, or alternative stable workbench
 - d. Scientific camera, software and PC
 - e. An ultra-flat TIRF dichroic beamsplitter with appropriate cleanup exciter (if needed) and emitter installed in the microscope filter carousel. We recommend and supply the Chroma range, but Semrock also offer high quality dichroics.
- (ii) Check that the OptoTIRF fibre connector, as specified on the delivery note matches the fibre from your lasers source (FC, APC or SMA)
- (iii) The OptoTIRF is shipped with a transit screw to prevent the bypass mirror from moving. This screw should be removed.
- (iv) The unit is factory aligned. Please do **not** make any mechanical or electronic adjustments until the condenser unit is connected and centered on the microscope port.
- (v) For long-term and reliable alignment, the TIRF condenser will need to be clamped into position on the support legs provided. If your antivibration table does not have drilled mounting holes, then please consider purchasing a magnetic clamp or equivalent to hold it rigidly in place.
- (vi) The microscope should be screwed down to an antivibration table and a plumb point taken from directly above the tip of the objective to the ceiling or top of enclosure and clearly marked.
- (vii) Throughout this guide and other documentation XY refers to radial positioning of the laser spot whereas Z refers to the focusing of the spot in the objective back aperture. Compass points North, South, East and West are used to refer to the direction that the laser beam leaves the objective lens as displayed on the remote handset display.
- (viii) The instructions refer to the use of a plumb line and white paper for checking alignment of the OptoTIRF. This is **only** necessary if suitable fluorescence laser targets are not available. We would strongly recommend that the system integrator has both a back aperture target and “distant” target for ensuring that the collimated beam is pointing straight through the centre of the objective.

Introduction

The OptoTIRF is a simple and inexpensive motorised TIRF illuminator designed to offer the following:

- (i) Compatibility with single-mode and small multimode fibres for TIRF, TIRM, PALM / STORM and oblique illumination.
- (ii) Automated TIRF and widefield illumination using a single microscope port.
- (iii) Motorised positioning of the laser spot anywhere in the back focal plane to allow the researcher to select the best TIRF position for each sample / wavelength / required depth.
- (iv) Free choice of microscope. Can be adapted to any inverted microscope which allows free optical access to the dichroic cube and objective lens (ie. has no intermediary lenses)
- (v) There are 8 TIRF presets and 1 widefield bypass preset. There is also a full COM command set for more expansive control of the system. The intention behind the presets is for them to be used:
 - a. For small radial differences to set different critical angles for different laser wavelengths or penetration depths.
 - b. To select different positions for the laser spot to experimentally reduce artefacts.
 - c. To illuminate from several different spots during an exposure or with sequential averaged exposures to improve field uniformity.
 - d. To allow precise centering of widefield illumination from the bypass port.
 - e. To allow setting to be saved for different dichroic mirrors or with different focus points for different magnification objectives.
- (vi) Software independence. All setup can be performed using the remote handset, terminal window, or standalone Cairn software so the imaging software does not require drivers. Having set up the required parameters for multi wavelength / depth TIRF, oblique illumination, and widefield bypass the host imaging software can access these presets using simple RS232/COM commands through the supplied USB (virtual COM) port, or an auxiliary digital interface. Conceptually the TIRF condenser can be treated as a multi-channel illuminator.
- (vii) Large field of view. The beam is expanded to fill a large iris field stop which can be set for various sCMOS or CCD sensor sizes.
- (viii) Motorised focusing of TIRF tube lens; especially useful if using different sample holders or different magnification TIRF objectives.

Optical schematic (flattened optical path for illustrative purposes)



The OptoTIRF comprises of a fibre collimator and refocusing “tube” lens to focus a magnified spot of the fibre tip onto the objective back aperture. Precise alignment is achieved by a combination of translation of the fibre tip relative to the collimator lens and by a motorised tip-tilt mirror prior to the refocusing lens. A second, widefield, source can share this lightpath using a motorised mirror/dichroic that can swing into the lightpath at 45 degrees. A manual iris field stop is provided primarily to define the illumination field of the widefield source. The refocusing “tube” lens is located inside the microscope frame and is focused in z using an additional motor drive. The overall optical design is telecentric and is specifically designed to be accessible and customizable / tweakable by expert users.

Parts list

Details will vary and should be checked off against your delivery note, but the system broadly comprises of:

- (i) A direct mount to the back of your inverted microscope. All mounts will comprise of a lens tube, a mechanical mount to the fix to the microscope and a cage connector to rigidly fix the OptoTIRF condenser onto the microscope mount. Optically the OptoTIRF interfaces directly with the microscope dichroic cube and objective; any epicondenser assembly must be removed prior to installation. Couplings are available for various microscopes including the:
 - a. Nikon Ti, TE2000
 - b. Olympus IX3, IX2
 - c. Leica DMI8
 - d. Zeiss Axiovert
 - e. ASI RAMM frame
- (ii) The main OptoTIRF condenser unit with output to microscope and inputs to laser fibre and widefield LED or light guide.
- (iii) OptoTIRF power supply
- (iv) OptoTIRF controller.
- (v) Joystick.
- (vi) Cables:
 - a. Mains input (kettle lead)
 - b. High current D connector to link power supply with controller
 - c. 25-way D connector to link controller with TIRF condenser motors
 - d. 9-way D connector to link controller with TIRF condenser sensors
 - e. USB for COM control / software integration
 - f. 2 BNC leads for laser control
 - g. 3-pin slave interlock cable (must be short circuited to function) and engineer override key (if applicable)
 - h. DIN connector lead to connect controller to joystick remote

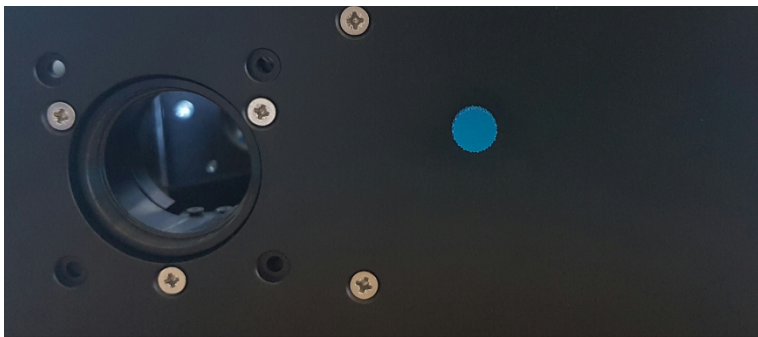
Mounting to microscope (please follow all standard laser safety procedures)

The OptoTIRF is factory aligned to produce a centered spot at the back of the microscope objective. Initial alignment should be carried out as follows:

- (i) Mount the TIRF epicondenser to the back of the microscope frame using the coupling provided.



- (ii) Set the support legs so that the TIRF condenser appears to be level and centered in all planes on a visual inspection.
- (iii) Ensure that the bypass transport screw is removed and that the entire assembly is free to slide in and out of the back of the microscope so that the z position of the TIRF tube lens can be set coarsely.



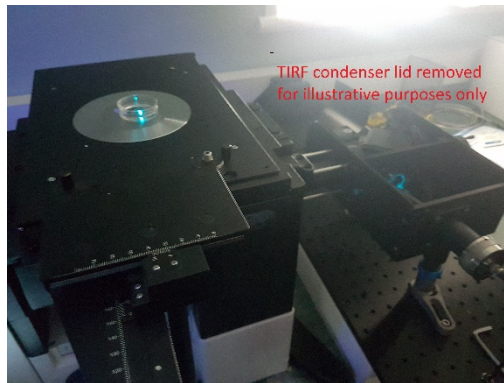
Blue transport screw should be removed

- (iv) Connect a laser source to the OptoTIRF and power up the controller and joystick.
- (v) Run a factory reset either from the remote menu selecting **Options > Full Factory Reset**, or using the COM command **"RESET"**
- (vi) Select any of the positions from the menu (see menu section below) and switch on the laser. Please control the laser using both its normal control method, and also the OptoTIRF BNC out as this will ensure that the laser is switched off when the motor is moving. There is a BNC shutter input on the OptoTIRF controller so that a digital TTL shutter line can be logic ANDed with the TIRF safety line.
- (vii) Move the objective nosepiece to an empty position, cover with a piece of paper or objective back focal plane target, and switch on the laser.

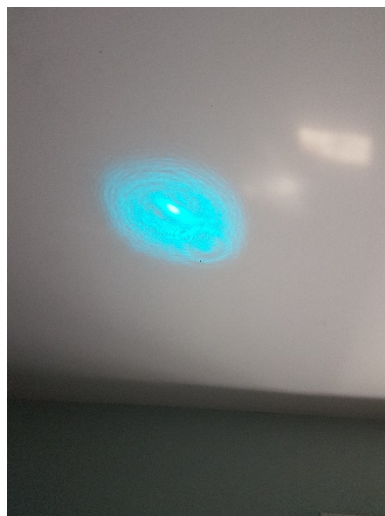
- (viii) Make physical adjustments to the X, Y, Z position of the TIRF condenser so that the laser spot is small and close to the centre of the objective aperture



- (ix) Switch off the laser, swing in the TIRF objective and focus the TIRF objective lens on a sample dish using transmitted illumination ensuring that there is a defined clear area on the coverslip with no sample. A pen mark will usually suffice. Do not make any further adjustments to the microscope focus and make a note of the settings.
- (x) Move the stage to a clear area of the coverslip / chamber, cover the sample with a piece of white paper and turn on the laser. Gradually move the paper away and adjust the position of the OptoTIRF condenser in X, Y and Z so that light is pointing as close to vertical as possible.



- (xi) Once sure that the laser is pointing up then remove the paper, fold back the transmitted light pillar and adjust the z position of the OptoTIRF to get the smallest possible spot on the ceiling.



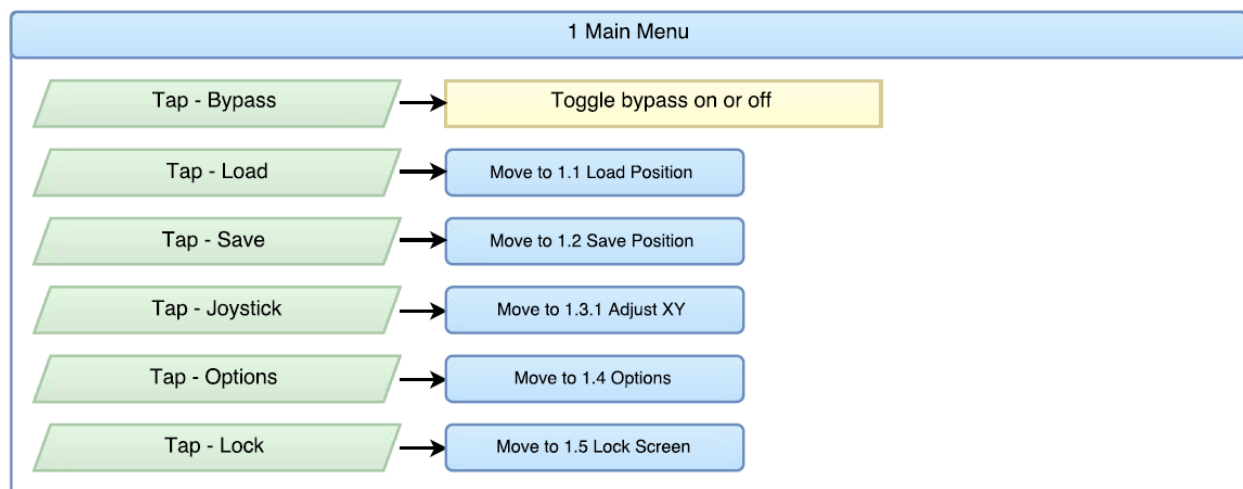
Roughly aligned TIRF beam

- (xii) Adjust the OptoTIRF X,Y to centre the spot on the plumbed target / distant fluorescence target (see preinstallation instructions)
- (xiii) Make further adjustments to the X,Y,Z position of the OptoTIRF unit to get the best possible spot as close to the centre of the target as possible and then rigidly fix the OptoTIRF to the AV table to ensure stable operation.
- (xiv) If it is not possible to get a small laser spot close to the target without adjusting the OptoTIRF electronically then please check with an alternative dichroic cube or reseal the dichroic mirror. If this doesn't correct the problem, then please consult with Cairn as it is best not to use the electronic adjustments to correct for an incorrect starting alignment.

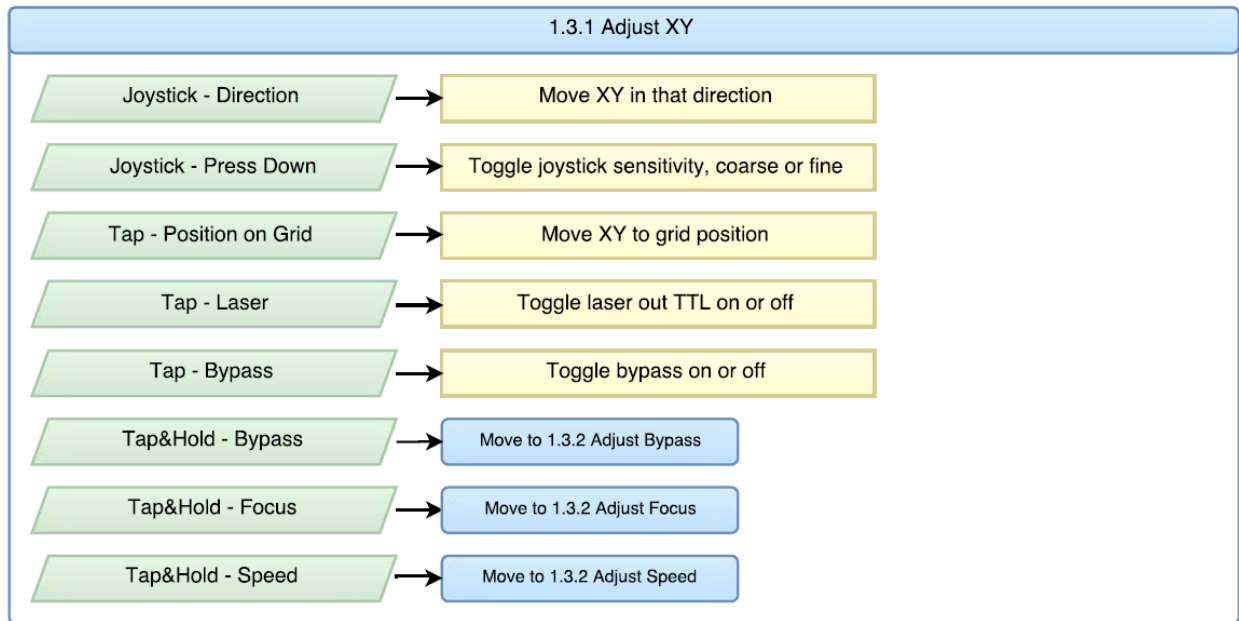
Exploring the remote control functions

Leaving the sample holder in a clear position it is useful to now explore the remote control menu structure.

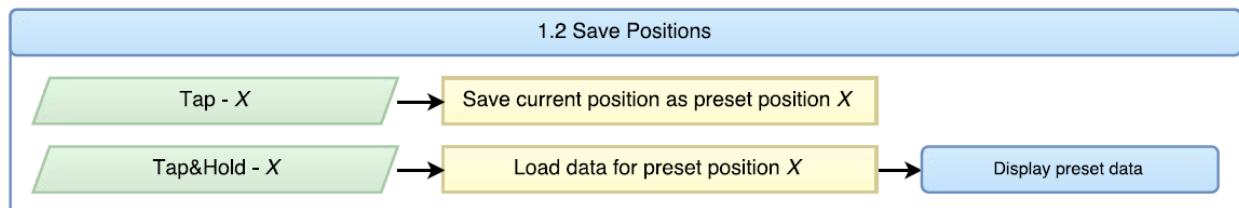
- (i) From the **Main menu** select **Joystick**



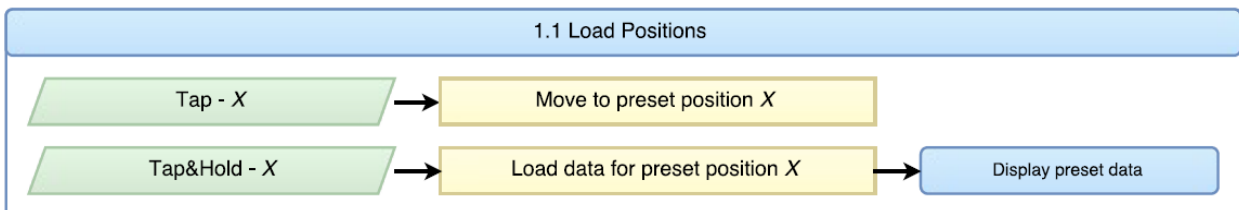
- (ii) From the **Adjust X Y** menu tap and hold **Focus** and make any further adjustments to the TIRF tube lens position to reduce the spot size on the ceiling. It should be possible to create astigmatism in both directions and this should help to find the best focus. Once the best focus has been found please be sure to “approach” it by moving the slider up (for the final tweak), as this is the direction of movement during start up calibration and this will ensure the best reproducibility. Next return the spot back to the plumbed mark on the ceiling (dead centre) and set the concentric circles on the remote : **Options > Set Centre Guide**



- (iii) Save this focus position to Preset 1 from the **Save Positions** menu. Alternatively, use the COM command "**SAVE Z**". This saves the current focus, Z, for all preset positions including bypass and saves having to resave Z for each position. "**Save XY**" is another useful global COM command to ensure that all presets and bypass mode start off centered.

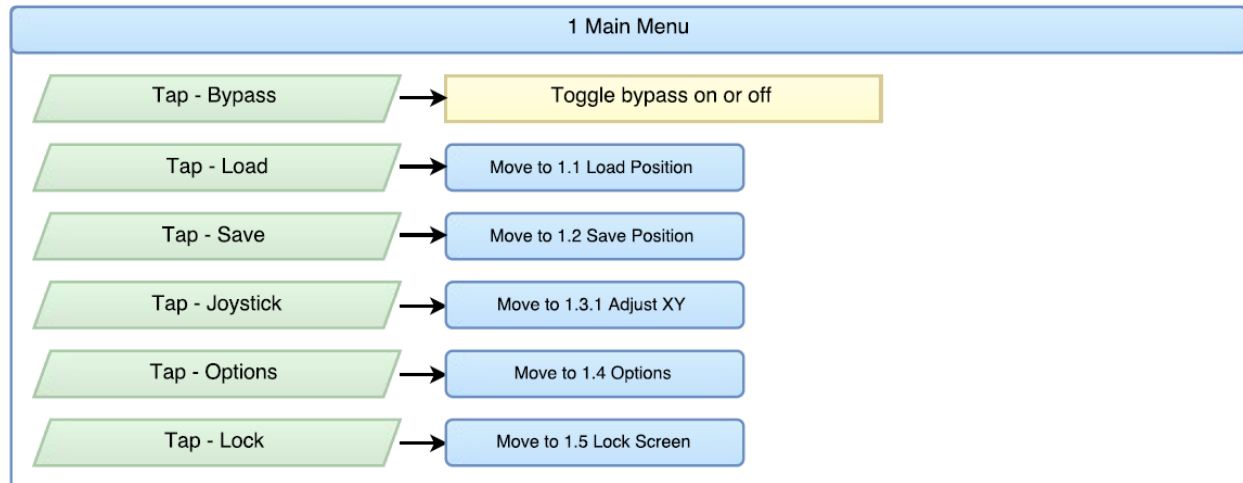


- (iv) Position a piece of paper approximately 10cm above sample holder and return to **Adjust X Y** menu. Move the joystick around and view the effect on the laser spot at different joystick sensitivities. At the lowest sensitivity with minimal pressure it should be possible to make tiny movements to the position of the spot. Store different X Y positions to different Presets from the **Save Positions** menu and switch between presets from the **Load Positions** menu.

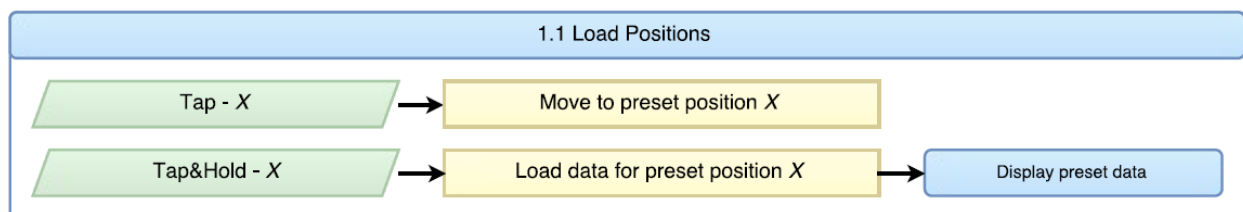


A Flowchart of menu options is included in Appendix 1, but the following hints may be helpful:

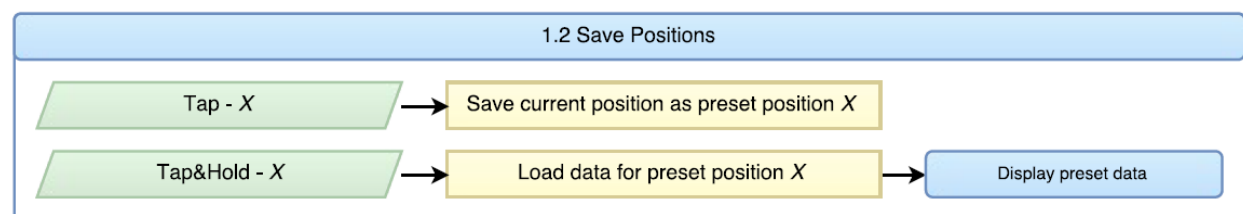
- (i) **Main Menu** – the widefield **bypass** can be accessed directly from this menu. Please ensure that the bypass mirror moves in and out of the lightpath on successive toggles. This menu gives access to all other menus and also allows the screen to be locked. It also displays the current preset; green circle for bypass, red circle with number for presets 1-8 and red circle with a dash for a position that is as yet unsaved.



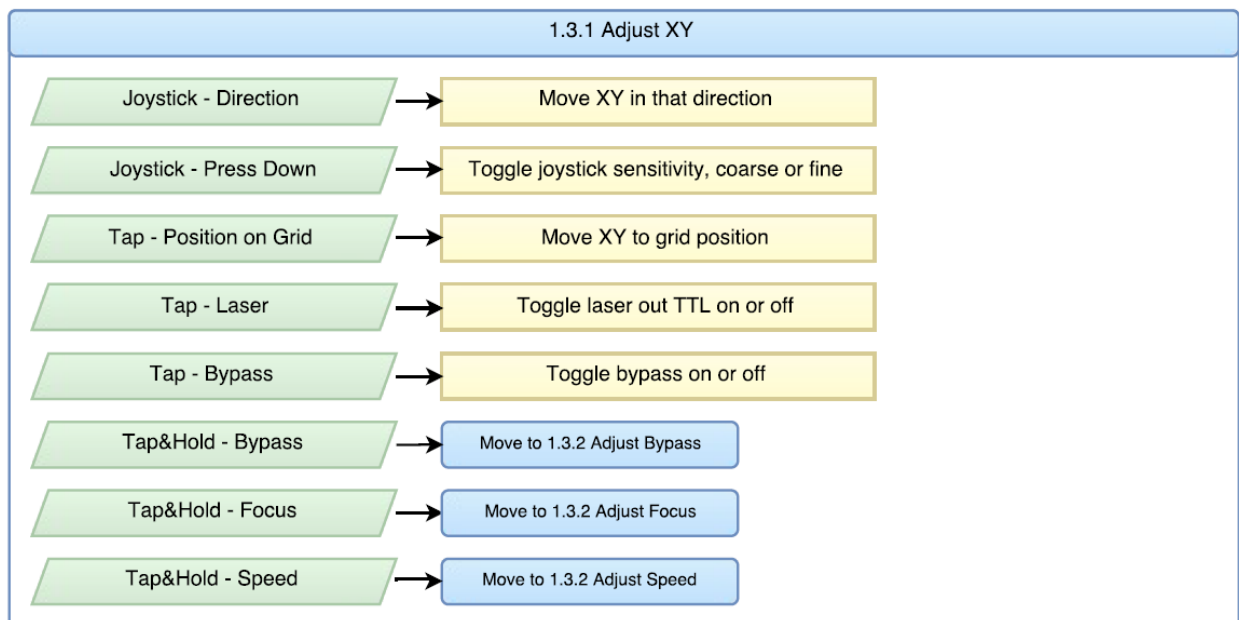
- (ii) **Load Positions** menu – moves between preset positions and also recalls the full list of settings for reference. Please note that all settings are saved to the numbered Preset, not just the XY position of the laser spot.



- (iii) **Save Positions** menu – equivalent to the Load menu for saving settings



- (iv) The **Adjust (XY)** menu is central to the operation of the system. Being careful to use suitable eye protection it is useful to get an impression of where the spot on the display is relative to the back focal plane. The laser spot can be moved in the back aperture by touching the display or by moving the joystick. There are concentric circles on the display that are calibrated to be coaxial with the objective so with a little practice it should become easy to use the remote display panel and the camera image to achieve widefield, oblique and TIRF illumination whilst the safety cover is over the objective. At this stage it would also be advisable that the master laser safety shutter line is routed through the OptoTIRF control unit so that the system can automatically switch off whilst the XY mirrors are moving from preset to preset (unless using the "**CYCLE**" COM function that will cycle through the presets without turning off the laser). When tweaking X_Y angles with the joystick or COM the laser shutter stays on as this is assumed to be a set up phase. At this stage please also ensure that the widefield bypass control brings in the bypass mirror at approximately 45 degrees, alignment of the widefield source is discussed in the following section. It is also possible to adjust the motor movement speed, but we hope to optimize this in the factory settings and would only suggest altering this if attempting to average several TIRF illumination points in a single exposure.

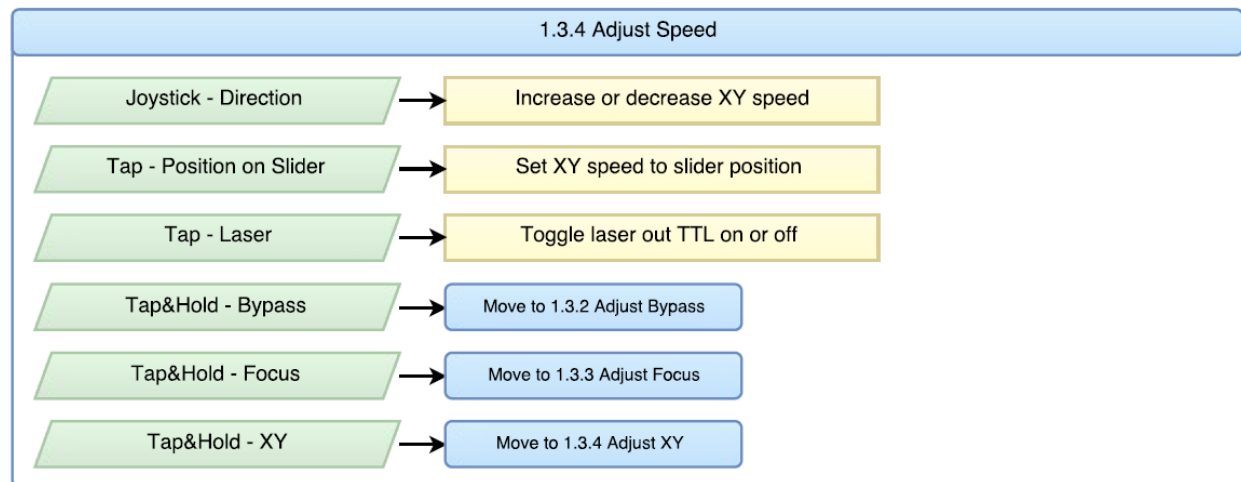
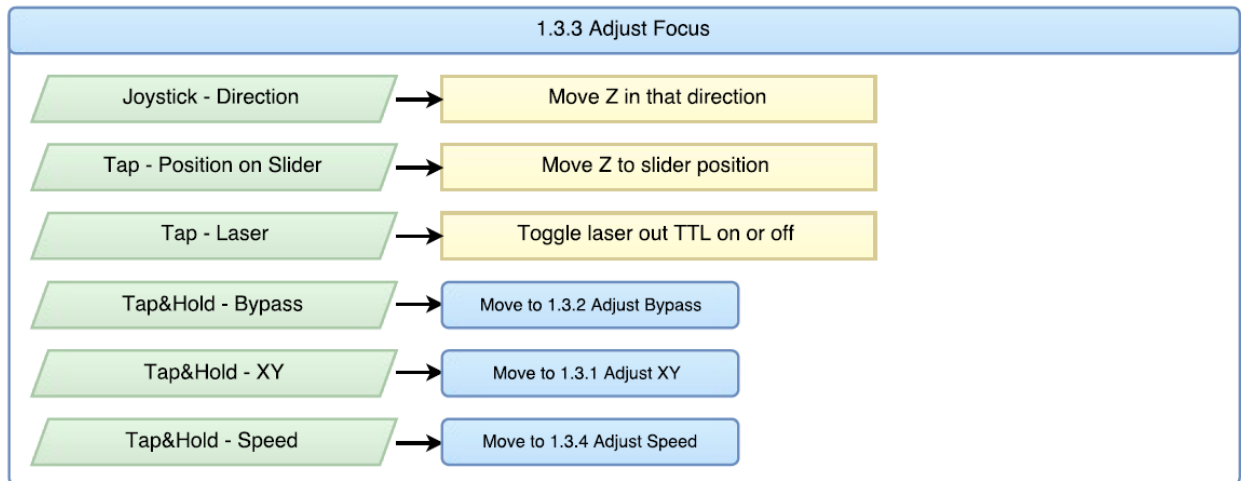


- (v) **Adjust Speed and Focus** menus are called from the main **Adjust** menu, but are likely to be used far less often. If the mounting of the system initially is carried out with great care, then it may be that the focus doesn't need any adjustment. For additional control, as an alternative to moving the TIRF tube lens, there is a standard mechanical

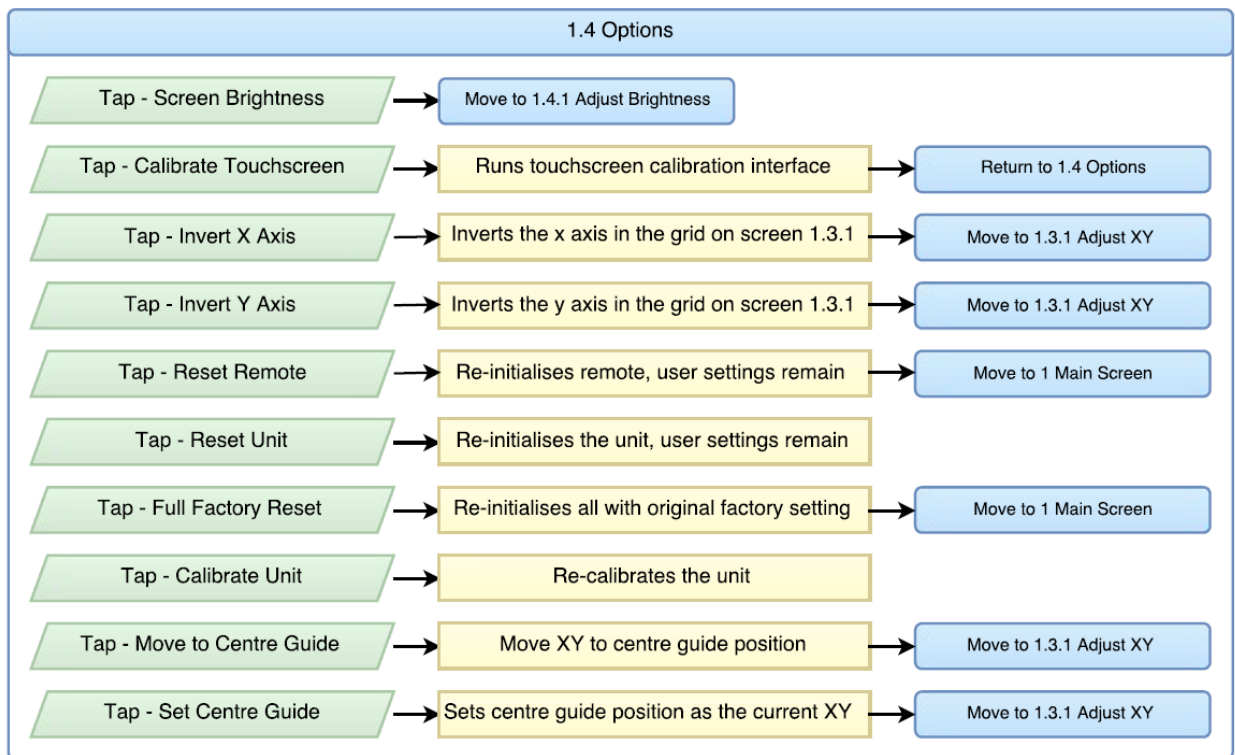
rotary focus control on the fibre mount, however this is factory set to produce a well collimated beam so only very small adjustments should be made.



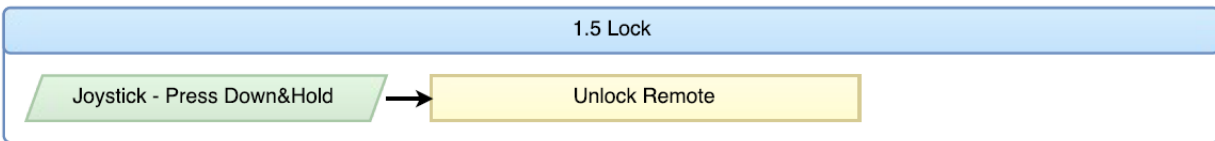
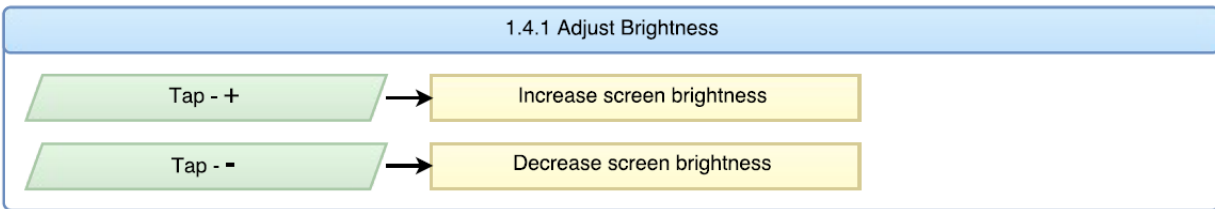
Factory collimated so only use for final tweaks



- (vi) The **Options** menu and equivalent COM commands allow the OptoTIRF to be configured so as to be convenient for your experiments.
- Screen brightness** and **Calibrate touchscreen** should be self-explanatory
 - Inverting** the axis may be necessary to ensure that the visual representation of the touchscreen represents what is actually going on with the laser. For safety reasons we would recommend that the screen is set to reflect the direction of the beam rather than the position of the spot on the back aperture i.e moving the spot on the screen to the North East sends the beam to the rear right of the objective
 - Reset remote or unit** should be used if communication is lost.
 - Factory reset** should be avoided if possible after initial set up!
 - Recalibrate** goes through start up procedure without needing to switch off the unit. This could be used if something has got stuck and the motors “know” where they are.
 - Centre guides** are useful for having working out where the beam is in relation to the objective. **Set Centre Guide** may be required from time to time especially if anything is knocked. A plumb line or reference centered spot will be needed as described earlier in this guide.



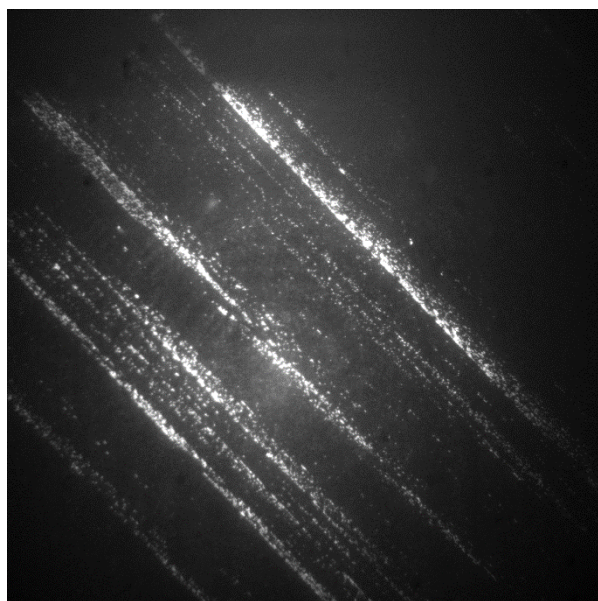
(vii) The **Brightness** and **Lock** menus require no further explanation.



Generating TIRF images

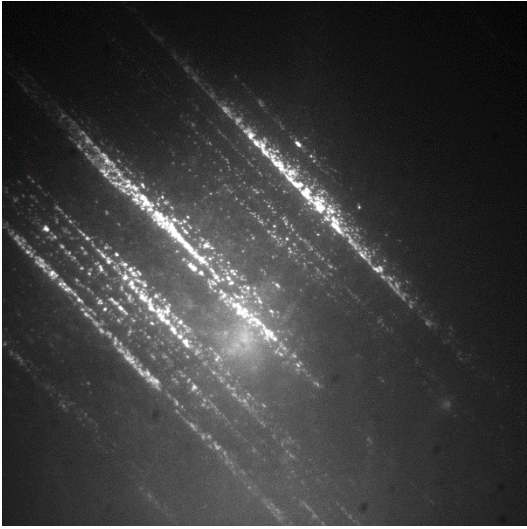
To test the OptoTIRF system we would recommend using the remote control unit and a familiar sample so as to be sure when Total Internal Reflectance is achieved. If a suitable biological sample is not available, then a fluorescence marker pen mark on the coverslip base of a chamber will work instead and is easier in many respects. After the pen mark has been left for a few minutes to dry, a little water should be added. This is a reliable sample as the distinct patterns on the coverslip will be visible in TIRF / oblique / widefield, but any dye in solution will swamp the signal until the critical angle is reached making the widefield image unusable in practice.

Taking all due laser safety precautions with a blue laser at a low power setting, focus on the sample using the TIRF objective lens and scientific camera with the laser spot centered. Gradually reduce the illumination angle by adjusting the Y motor using the joystick to steer the beam to point towards the rear of the microscope (compass point **North** on the remote). Before the beam disappears (i.e. misses the back aperture of the objective completely) then TIRF should be reached and the image should approximate that below.

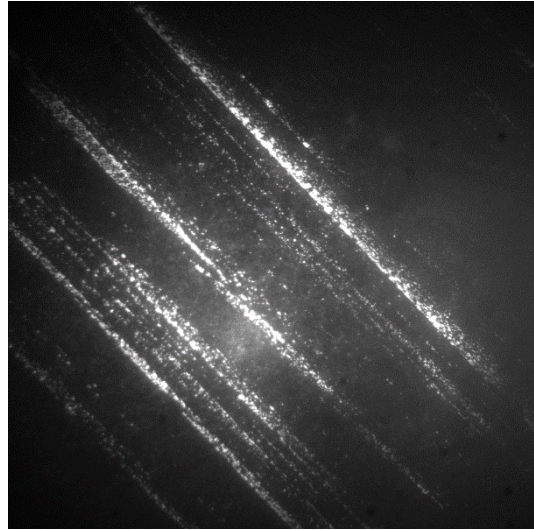


*TIRF image of marker pen with the illumination spot orientated to the **North** ↑ of the remote handset*

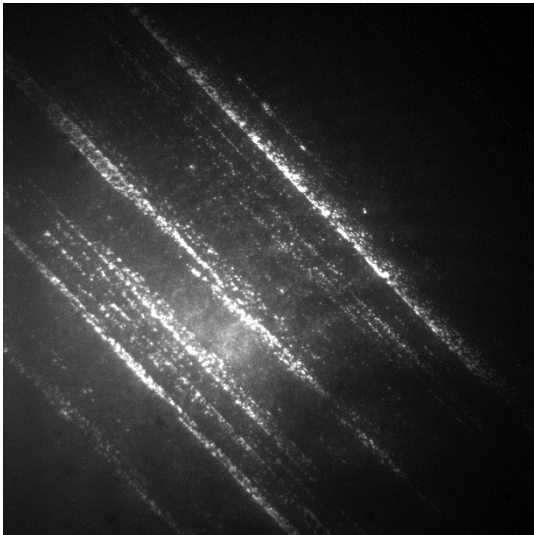
Store this TIRF point as Preset 1 and, referring to the position of spot on the remote display, set equivalent spots in the South, East and West orientations (take great care when positioning the beam South as the laser beam will point towards the front of the microscope). These 3 additional points can be stored as Preset 2,3 and 4 with Preset 5 stored so as to give a widefield image. Images below have been auto-ranged to use the available dynamic range, but in practice intensities will vary depending on the precise angle and penetration depth. The non-adjusted images are displayed as a montage below.



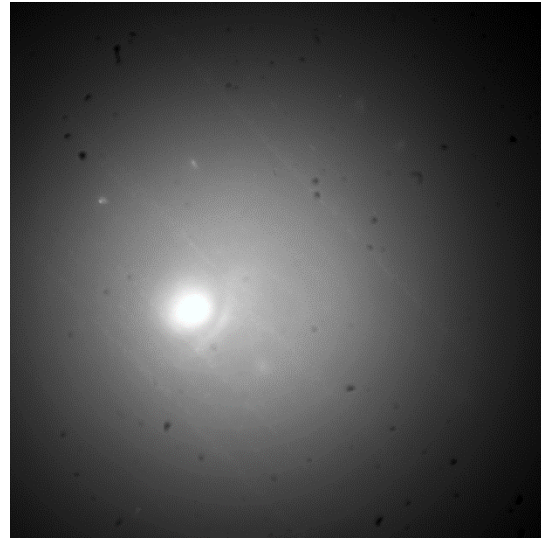
South ↓



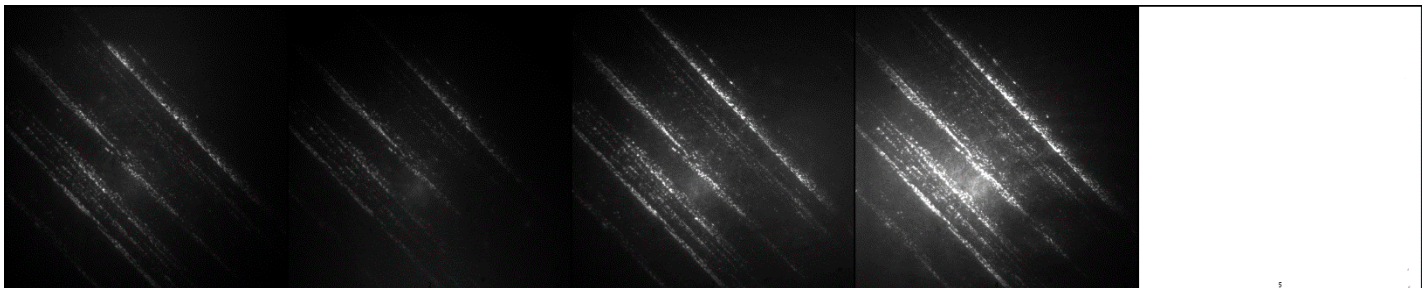
East →



West ←



When the laser spot is anywhere close to the centre of the objective then the structure of the penstroke will be completely masked by out of focus fluorescence as shown above.



N

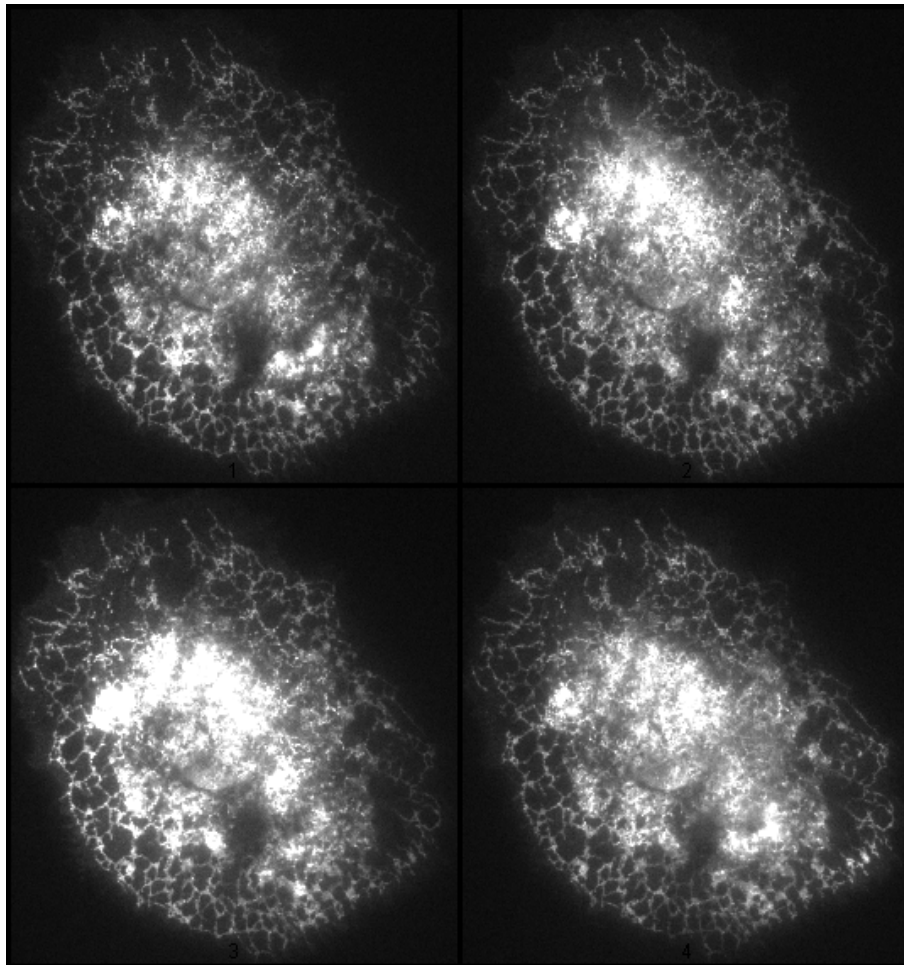
S

E

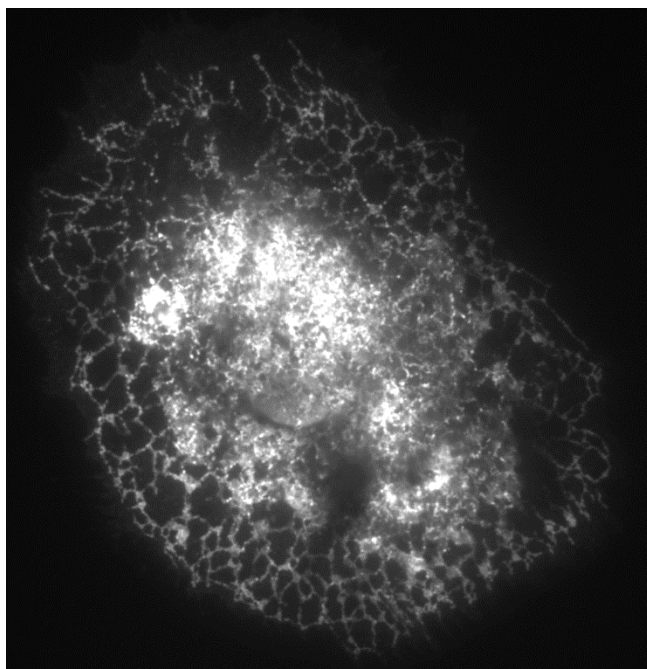
W

Widefield

The advantages of averaging or integrating the images from several preset illumination angles can be better seen on Hela cells below:

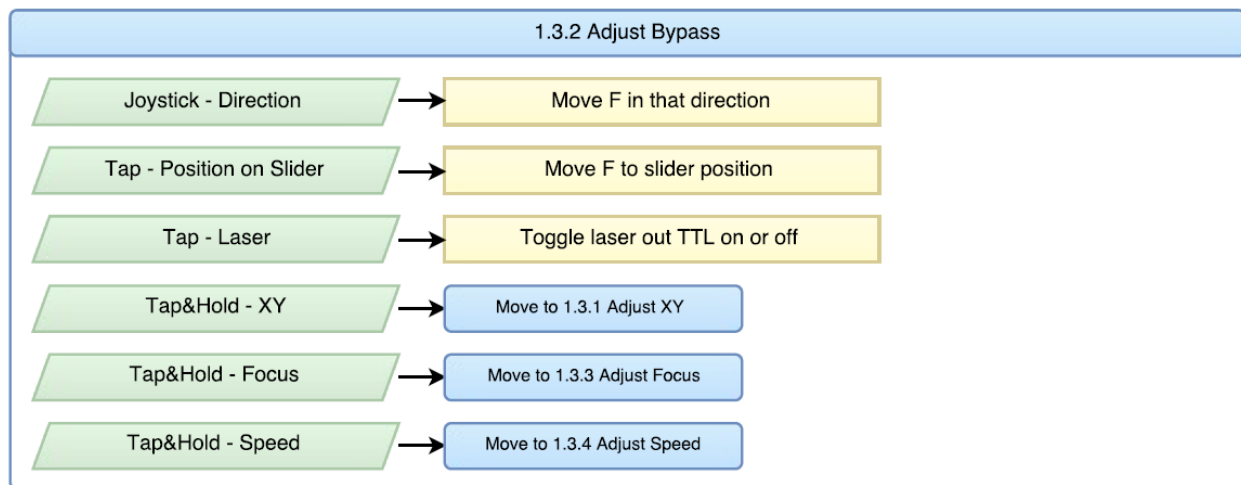


*Images taken
with
illumination
from 4
positions
around
circumference
of objective*



*Image averaged / integrated across
several preset positions helps to
prevent artefacts.*

Setting up widefield bypass



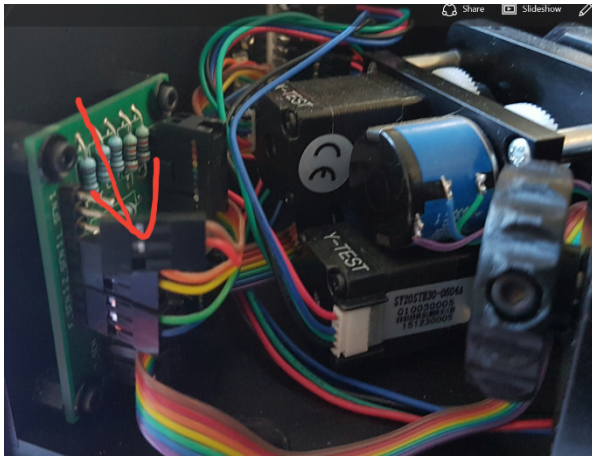
The OptoTIRF has a motorised bypass mirror that can be configured with a dichroic mirror or polarising beamsplitter for simultaneous WF/TIRF imaging, or with a fully reflective mirror for increased wavelength flexibility. Please check your delivery note and ensure that the chosen widefield illuminator is of suitable wavelength. This could be a fibre-optic, liquid light guide or a direct LED and should be plugged into the bypass port either using a condenser lens in the OptoTIRF or external to the unit so as to produce a collimated beam. Please ensure that the collimation is not duplicated as this really won't work! To align the widefield source for Kohler Illumination then follow the following procedure:

- (i) Select **Bypass** from the remote menu. Please ensure that the values for this have not been changed from factory settings.
- (ii) Adjust the axial position of the emitter relative to the condenser lens to produce a focused image of the emitter at the back focal plane of the objective. Some nosepieces will have an alignment graticule mounted, but if not then thin white paper over the aperture works well.
- (iii) If the image is centred then go to stage (vi)
- (iv) If not then Tap&Hold the **Bypass** control to centre the emitter image in one axis.
- (v) Use the X_Y Adjust to centre the emitter image in the other axis.
- (vi) Store the Bypass position in memory using the COM command "**SAVE B**", or store the settings to an unused preset e.g. Preset 8.
- (vii) Put a good fluorescence sample on the microscope, illuminate with bypass fluorescence and confirm that it produce good Kohler illumination.

Checking the field stop

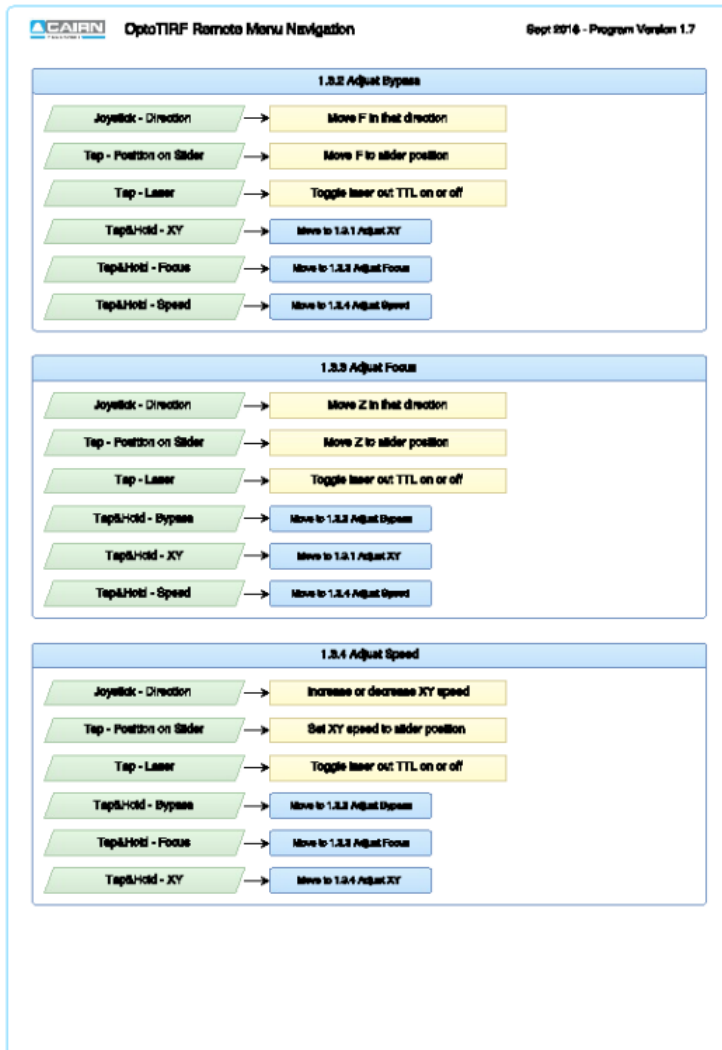
The field stop is factory aligned to slightly overfill a 13.2x13.2mm sensor (sCMOS / Ixon 888 EM CCD). If this needs adjusting, then please follow the following procedure:

- (i) Remove the lid of the OptoTIRF condenser box, first removing the four screws.
- (ii) Unplug the safety microswitch.




- (iii) Ensure that the OptoTIRF is still in Bypass position by selecting the appropriate preset or bypass position if using COM commands.
- (iv) Close the field stop until it becomes visible on the camera.
- (v) Open it up to the required size. If it is not well centered and this is important for your experiments then please let us know. The centration is factory set, but if we find that this varies too much from scope to scope then we could add X_Y adjustments.
- (vi) The fieldstop can be slid backwards and forwards on the three mounting rails to produce a sharp focus.
- (vii) Replace microswitch cable and lid.

Appendix 1 – remote control menu structure



Appendix 2 – COM commands

 Tomas Parrado Aug 2016			
Command	Argument	Num.	Description
SAVE	P	α	Saves the current setting for X, Y, Z, F and L to preset, P, position α . Where α can be a number from 1 to 8.
	XY		Saves the current position for X and Y for all preset positions including bypass. Useful for globally centring your unit.
	Z		Saves the current focus, Z, for all preset positions including bypass. Useful for globally focusing your unit.
	B		Saves the current setting for X, Y, Z, F and L to the bypass preset. Note bypass mirror must be in the path.
RESET			Restores unit to factory settings and wipes any saved presets.
CALI			Forces the unit to re-calibrate all it's motors and then returns to it's previous position.
CYCLE		α, β	Cycle through the preset positions from 1 to α . Where α can be a number from 2 to 8. The number of cycles is governed by β . Where β can be a number from 0 to 99. $\beta = 0$ is a special case where the cycle runs continuously until another command is received. Note, the remote is disabled during this feature.
REPORT	P	α	Returns the saved setting for X, Y, Z, F and L for the preset, P, position α . Where α can be a number from 0 to 9. Number 9 will report the current settings whether the unit is currently at a saved preset position or not.
	B		Returns the saved setting for X, Y, Z, F and L for the preset bypass, B, position.
	POT	α	Returns internal analogue absolute readings of the current X and Y positions. α is optional, and adjusts the number of readings given.
	IN	α	Returns the state of all the inputs. α is optional, and adjusts the number of readings given.
	OUT	α	Returns the state of all the outputs. α is optional, and adjusts the number of readings given.
	BYPASS		Returns the approximate speed of moving the bypass mirror, B.

The Arduino project is an open-source electronics platform. If you would like a copy of the program to modify or archive please contact our support team at tech@cairn-research.co.uk.

Program: OboTIRF MainUnit V1.7