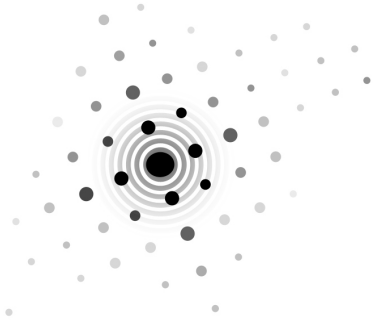
Live-SR spinning disk confocal with TIRF CBIS user manual



March 30, 2025

Contents

Prefatory note

Welcome to the Live-SR spinning disk confocal with TIRF system at CBIS. The latest version of this manual can be downloaded at `https://www.dbs.nus.edu.sg/2021/03/15/live-sr-spinning-disk-confocal-tirf/`. Please email me with queries, corrections and prospective research projects.

I am grateful to Amritha Bhat, Emma Feng Yu, Goh Wah Ing, Iong Ying, Divyanshu Mahajan, Pathoor Nithin, Phua Siew Cheng, Tan Yuan Lee and Tong Yan for their assistance and discussions relating to the operation of the microscope.

Lin Yangchen
yangchen@nus.edu.sg

1 Switch on the system

1.1 Start-up procedure

For incubation see §1.2 for additional steps to be done in advance.

Turn on the three main overhead plugs and boot the computer in any order. Wait until the lights on the back of the cameras extinguish before launching MetaMorph software, otherwise the software may fail to connect to the cameras.

Check that the magnification knob at lower right on the front of the microscope is rotated to `1X` pointing to the right, otherwise the scale information in the images will be wrong.

Lasers stable and ready to use 5 min after turning on main system plugs.

1.2 Incubation

You have a choice of enclosure or stage-top heating or both, the former offering greater stability for long-term imaging. Enclosure heating should be turned on at least 6 h before your experiment, and stage-top heating at least 30 min before. Don't turn on humidity and CO_2 until you are ready to mount your sample, otherwise water vapour and CO_2 will dissipate through the big hole at the bottom of the stage.

If you wish to heat the objective, attach the objective heater by wrapping the heating band around the objective and securing it with the accompanying velcro strap. Remember to never change objective while the heater is attached.

Check that there is enough water in the humidifier bottle and the water isn't cloudy. If cloudy, inform facility staff. Top up with purified water if necessary and tighten the cap securely.

For enclosure heating, turn on the `Heater` plug on the overhead socket rack.

For stage-top heating, first insert the stage insert you'll be using and place the glass cover on it. Then turn on the `Okolab T` plug on the overhead socket rack.

Switch on the system

Turn on the Okolab touch screen on the table by pressing the button at the lower right corner of the screen. If necessary adjust the set values. Ignore the flow rate setting as it is controlled separately (see below).

In `Settings` → `Temp` → `Control Mode`, choose `Sample` or `Chamber`. For experiments requiring very accurate sample temperature, choose `Sample` and fully immerse the tip of the green fine-gauge thermocouple in the sample medium.[1]

The system can also perform temperature cycling. Go to `Settings` → `Temp` → `Cycle`.

If you are using the objective heater, go to `Settings` → `Temp` → `Objective Heater` and select `Enabled`. You can either use the factory calibration for temperature offset, which assumes that the room temperature is 23 °C, or calibrate it to your specific conditions and the objective you're using. If you're doing the latter, mount a dummy sample with the same mount type and medium you'll be using for the actual sample, and replace the glass cover. If using an immersion objective, make sure the immersion fluid is in contact with the dummy sample. If you chose `Sample` in `Control Mode`, immerse the thermocouple in the medium. Otherwise you can leave the thermocouple in the air. We recommend turning on the humidity controller (overhead plug Okolab H) now and letting the humidity stabilize before calibrating, as water vapour will change the heat capacity of the atmosphere in the chamber. The humidity reading is displayed on the Okolab touch screen.

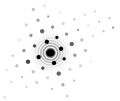
About 15 min before your experiment, mount your sample (§2.2) and replace the glass cover. If you are going to use transmitted light, check that the glass cover is clean. Turn on the CO_2 tank and the overhead CO_2 plug. Turn on the Okolab H (humidity) plug if it's not on yet. The humidity reading is displayed on the Okolab touch screen while the CO_2 reading is shown on the gas mixer located on the right side of the overhead rack.

On the LCI box under the workstation monitors, press `set` and then the arrows to adjust CO_2 percentage. You can use 6% instead of 5% if the colour of your medium indicates that there isn't enough CO_2. Turn the knob at lower right to set flow rate as indicated by the ball in the scale. For single or double dishes, set it to 70 cc min^{-1}. For multi-well plates, set it to 100.

data logger?

[1] The heat capacity of water is very different from that of air.

For the full Okolab manual see `https://www.oko-lab.com/images/okaImages/PDF-files/H301-T-UNIT-BL-PLUS_Manual.pdf`



2 Sample handling

2.1 Sample preparation guidelines

For live cells, consider staining nuclei with far-red dyes like SiR-DNA or DRAQ5 instead of Hoechst or DAPI, as 405 nm excitation is bad for the cells. Note that DRAQ5 is leaky so if doing segmentation adjust your algorithm accordingly (see Schwendy *et al.* [1]).

If colocalization, make sure labeling density is high enough.

Stage inserts and spacers are available for a variety of sample holders such as 35 mm Petri dish, chambered coverslip, standard microscope slide and multiple-well plate. But you may wish to check before starting your project that your holder can fit on the microscope.

Use nº 1.5 coverslip if sample is contacting coverslip, or nº 1 coverslip if there is some space between the sample and the coverslip due to mounting medium. If coverslip thickness is critical for sensitive applications like FCS (§9), use a micrometer screw gauge to measure the thickness or use high-precision coverslips such as those manufactured by MARIENFELD or ZEISS to 170 $\pm$ 5 μm.

Use glass surfaces to mount your sample if using DIC (§3.1.3).

to measure accurate incubation temperature, immerse the tip of the green wire in the culture medium as water has a different specific heat capacity from air.

See §2.2.1 for additional requirements if you plan to use focus drift correction.

See the sections on specific microscopical techniques for additional sample preparation requirements.

2.1.1 Fluorescent microspheres

for experimental PSF and colocalization calibration

Use 200 nm TetraSpeck™ microspheres for high-NA 20× objectives and 100 nm microspheres for objectives of NA > 1.2.

Sonicate microspheres for 10 min at room temperature, then dilute 1:50 with 100% ethanol and vortex to mix. Spread 5 μl of the working suspension across a nº 1.5 coverslip using pipette tip in circular motion and air dry. Oil-mount on microscope slide and seal with nail varnish. Use within a few hours.

2.2 Mount and focus sample

The switch for the enclosure lighting is to the right of the enclosure.

Warning: minimize external forces on the piezo unit on the stage, especially asymmetric forces.

Select objective in MetaMorph. To change objective without the software, you can use the microscope's remote touch screen or rotate the turret physically, but if acquiring images, make sure you also select the objective in MetaMorph for correct metadata. Check that the objective is clean. Objective specs in Table 2.1.

procedure for changing and calibrating objectives

If you are using an objective with a correction collar, you may wish to adjust the collar to the correct coverslip thickness, sample depth and temperature before putting in the stage insert, although it is still possible to do it after. The lower scale corrects for sample depth. On the 20× objective, the leftmost graduation is for depth 0, and each subsequent graduation is for a 0.25 mm increase in depth.

Apply the correct immersion liquid if applicable. Double check the label on the container of immersion liquid, as containers for different liquids may have very similar labels. For the water-immersion objective you can use Immersol which is less prone to evaporation.

If you are using an oil-immersion objective with an ibidi® chambered coverslip with polymer base, use only the compatible oils listed at `https://ibidi.com/content/551-immersion-oils-compatible-with-ibidi-labware-products`, otherwise the coverslip may dissolve and start leaking.

Table 2.1. Objectives.

Mag	NA	Imm.	WD (mm)	Coverslip	Correction	DIC*	Phase*
10×	0.30	air	15.2	1.2	plan fluor	none	Ph1
20×	0.95	water	0.95	0.11–0.23	apo	N2	none
40×	0.95	air	0.25–0.17	0.11–0.23	plan apo	N2	none
40×	‡	oil					
60×	1.2	water	0.28–0.31		plan apo *VC*		none
60×	1.40	oil	0.13	0.17	plan apo	N2	none
100×	1.45	oil	0.13	0.17	plan apo λ		none
100×	1.49†	oil			apo	N2	none

*The DIC and Phase codes are for condenser turret position.
†Available on special request for TIRF (§5) only. This objective has poor field flatness for blue.
‡Available on request. Gives higher-resolution images than the 40× air objective but gives less precise stage position recall due to surface tension of the immersion fluid.

Mount your sample on the appropriate stage insert, then place the insert on the stage. Refrain from placing the empty insert on the stage and then placing the sample on the insert, as this makes you more likely to press on the piezo unit.

If liquid sample spills onto the objective turret, soak it up with C-fold towels and report it immediately.

Select the light path in MetaMorph (see relevant section for your illumination mode).

Focus the sample using the focus knobs on the microscope or the knob on the right side of the joystick console. If necessary, fine-tune the objective correction collar while observing the image visually.

If you are not confident of your manual focusing skills, you can check the z-position display at the front of the microscope. It should be somewhere between 2500 and 2900 for slides. The closest approach to the sample is set at 3500 to prevent long objectives that are not in the light path from hitting the underside of the stage. If you need to go beyond the limit, ask facility staff.

Move the stage xy using the joystick. Twist the knob on the joystick to adjust sensitivity.

2.2.1 Focus drift correction

Perfect Focus System PFS is useful for time lapses. Correction on millisecond scale. Requires nº 1.5 glass coverslip.

PFS may not work with:

- coverslips thicker than nº 1.5
- plastic dishes
- fixed samples in high-refractive index mountant[2]
- thick or light-scattering samples
- samples or immersion media containing bubbles

First focus the sample using the normal focus knobs.

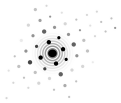
Check that the NIR dichroic mirror lever on the right of the nosepiece is swung in.

In the `Stage` tab, click `Start Continuous Focusing`, or press the `ON` button above `PFS` on the front bottom of the microscope. The microscope will use the NIR light to find the coverslip surface adjacent to the sample. If this succeeds, the `ON` button will light up continuously in green. If it blinks, it's not working. If using plastic dish with glued-on glass coverslip, check that the FOV hasn't moved to where the glass and plastic overlap.

The main focusing knobs are now disabled. Use the PFS offset wheel to fine-tune focus on the sample. The wheel goes in the opposite direction from the main focus knobs. Rotating it clockwise will move the objective closer to the sample. Press the blue button to get fine or coarse focusing. When done, press the `MEMORY` button above `PFS` on the front bottom of the microscope to save the position. The button will light. A different position can be saved for each objective. Do not touch the offset wheel thereafter, otherwise the focus will go off.

When replacing sample, first press the `ON` button to stop PFS. When finished using PFS, press the `ON` button to stop it, and leave the NIR dichroic mirror in the light path.

[2] but will work with PBS or non-hardening mountants like VECTASHIELD® H-1000.



3 Non-laser illumination modes

3.1 Transmitted light

in front of light source at top of arm: filter handles all in

brightness control

Click `Trans` button in MetaMorph and click the shutter icon to open the shutter. If necessary, press `EYE` button on the front of the microscope to direct the light path through the eyepieces. If you see nothing, check the eyepiece shutter knob below the eyepieces.

control brightness using the console slider.

Micrographs can be acquired sequentially with fluorescence channels using multi-dimensional acquisition (§6).

3.1.1 Transmitted Köhler illumination

The condenser has been immobilized at the Köhler position to prevent it from smashing the glass cover of the stage-top incubator. If you need the condenser in a different position, ask facility staff for assistance.

- Focus the sample.
- Stop down the field diaphragm and focus it with the condenser focusing knob.
- Center the field diaphragm using the condenser centering knobs.
- Open the field diaphragm to just beyond the ocular or camera field of view.
- Swing in the Bertrand lens and focus on the objective back focal plane with the knob on the right.
- Adjust the aperture diaphragm for good contrast with minimal diffraction. Usually the diaphragm aperture should be about 80% of the objective aperture.
- Swing the Bertrand lens out of the light path.

3.1.2 Transmitted polarized light

Slide polarizer above condenser into light path.

Rotate condenser turret Nomarski prisms out of light path. Remove objective DIC prism.

Click `DIC Eyes` or `DIC Camera` in MetaMorph to rotate the analyzer into the light path.

Rotate polarizer to crossed position (what angle/click stop)

Swing the Bertrand lens into the light path and focus it with the knob on the right. Stop down aperture diaphragm until view of back focal plane is dark.

For observing conoscopic interference figures:

- Cross the polarizers.
- Select objective of highest practical magnification and NA.
- Only one homogeneous area of sample should be in view. If necessary, stop down field diaphragm to achieve this.
- Open aperture diaphragm fully.
- Increase illumination brightness to maximum.
- Swing in the Bertrand lens and focus it with the knob on the right.

3.1.3 Transmitted DIC

Ask facility staff for the objective Nomarski prisms. Insert the correct prism for the objective.

Slide polarizer above condenser into light path. Rotate polarizer to adjust DIC effect.

Turn condenser turret to correct position (see Table 2.1).

Click `DIC Eyes` or `DIC Camera` in MetaMorph. If no light comes through the stage, try clicking the shutter icon.

DIC not recommended in the same experiment as fluorescence at high magnification because the Nomarski prism interferes with the fluorescence image.

3.1.4 Phase contrast

Turn condenser turret to correct position (see Table 2.1).

What button in MetaMorph?

Swing the Bertrand lens into the light path and focus it with the knob on the right.

center the phase ring [how]

3.2 Episcopic

Flip lever behind microscope to 45° position.

Click `Lumencor Intensity` button to adjust brightness.

3.2.1 Widefield epifluorescence

Select desired channel in MetaMorph. We use a quad-band emission filter so the filter cube turret does not move.

You can also try the more complicated oblique dark field (§5.2) to reduce out-of-focus light and photodamage.

3.2.2 Direct reflected light

Köhler illumination

- Focus the specimen.

- Open episcopic field diaphragm (left side of stand at the back) to just beyond field of view.
- Swing in the Bertrand lens and focus it with the knob on the right.
- Center the lamp if necessary.
- Stop down the episcopic aperture diaphragm (right side of stand at the back). If doing polarization microscopy, stop the aperture diaphragm down until the conoscopic view is dark.

3.2.3 Reflection contrast microscopy

[2–4]

First, illuminate using only one wavelength. Then block the direct reflections using emission filter that blocks same wavelength. Use oblique illumination (decenter which diaphragm) to reduce direct reflections. The interference between cell and coverslip will be of modified wavelength and will therefore pass through the filter and into the eyepiece.

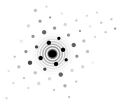


Table 4.1. Effective pinhole sizes.

Magnification	NA	Pinhole (AU)
10×		
20×	0.95	
40×	1.30	
60×	1.40	1.8
100×	1.45	
100×	1.49	

Emission wavelength 525 nm. Information compiled from Oreopoulos *et al.* [5] and . Pinhole size can be estimated for any objective at `http://www.iscopecalc.com`.

4 Confocal system configuration

4.1 Spinning disk

Our Yokogawa CSU-W1 Nipkow disk has relatively large 50 μm pinholes optimized for higher magnifications and weaker signals. See Table 4.1 for effective pinhole sizes for different objectives. The 500 μm pinhole spacing reduces pinhole crosstalk for thick samples. microlens array disk for greater transmission efficiency.

One revolution of the disk scans the entire field of view three times. If the frames scanned during exposure are not a whole number, stripes may appear. To avoid this, our disk is set to its maximal rotation speed of 4000 rpm so any stripes will not be visible unless the exposure time is very short. If you need to adjust rotation speed, you can change it at `Devices` → `Configure Illumination` but do not save changes.

Spinning disks are susceptible to light fall-off toward the image periphery. You may wish to take a reference image for shading correction (§11). Use a chroma slide, or if that is unavailable, find an empty region in your sample, increase laser power to get a signal and check that the intensity throughout the FOV is above the camera offset (§4.3).

For more on spinning disk confocal microscopy see OREOPOULOS *et al.* [5].

Table 4.2. Measured laser power values.

Wavelength (nm)	Type	Power* (mW cm^{-2}) confocal	TIRF†
405			
488	DPSS		
514			
561	DPSS		
640	diode		
647	ytterbium-doped fibre		

*Maximal power at stage. [which objective?]
†Incidence angle set to 0°.

4.2 Laser excitation and emission

Click `ModularSpinning` at top left if the confocal laser control window isn't open.

See Table 4.2 for available laser wavelengths and maximal available laser power at stage. In the software, percentage power is adjusted in different windows for different imaging modes; see respective sections. For 647 nm laser used for SMLM only, see §10.

Schematic of lasers, dichroics, emission filters and cameras in Fig. 4.1. Preset fluorescence channels are available. You can configure your own (including reflectance confocal) at `Devices` → `Configure Illumination`. Do not overwrite the existing presets! Rename and click add instead.

4.3 Cameras

Pair of Photometrics 95B back-illuminated scientific CMOS. 1200 × 1200 pixels, pixel size 11 μm, FOV 19 mm, bias offset 100. 40 fps at full frame in 16-bit, 80 fps in 12-bit.[3] what fps when cropped? how to select bit depth

[3] To reach these frame rates you have to stream data to RAM during acquisition (§7).

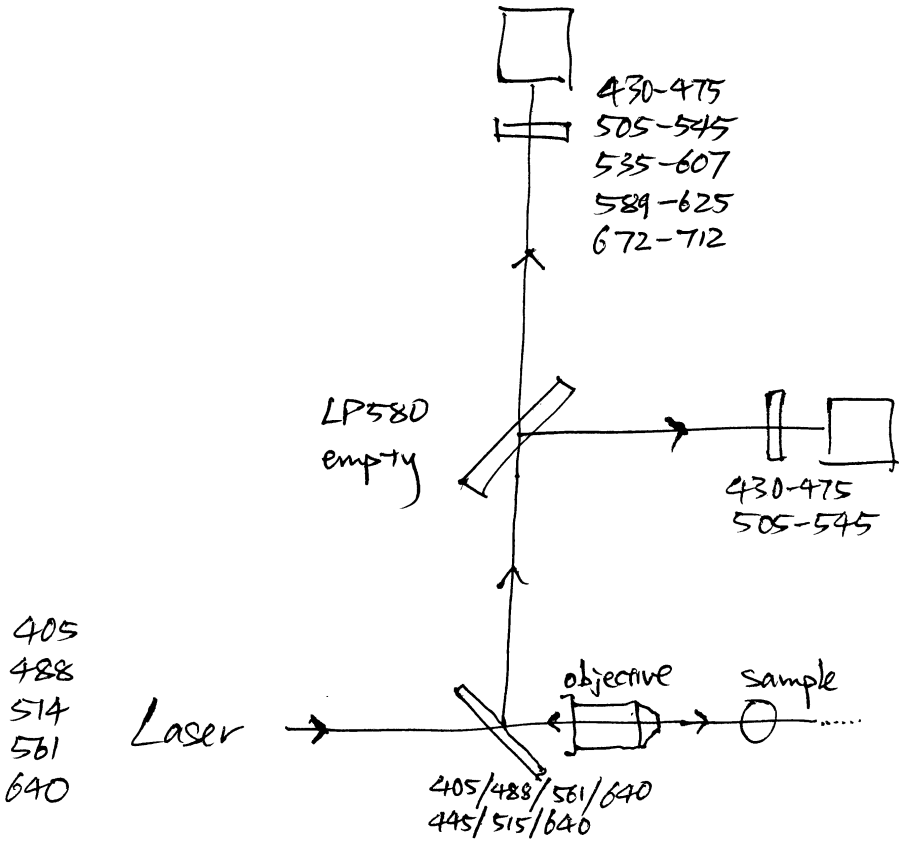


Figure 4.1. Schematic. Excludes 647 nm laser for SMLM. Which excitation dichroic better at passing just 640 nm?

If you are doing super-resolution microscopy or FCS (§9) or measuring low signal intensities at small spatial scale, you may wish to maximize accuracy by calibrating the camera sensor for intrinsic pixel-to-pixel variations [6].

Any two channels can be simultaneously acquired except if one of the channels is YFP, as in this case the optimal filter combination is unavailable.

When imaging YFP and Cy5 in the same experiment, select the Cy5 channel that is grouped together with the YFP button so the same dichroic is used, as changing the dichroic is relatively slow.

Click `Prime95B` or `Prime95B-2` button (whichever works) on the left in the main window if you have selected a single-channel preset, or `Prime95B Dual` if you have selected a dual-channel preset.

Click `Acquire` in MetaMorph main menu to access full camera controls. This gives you more control over camera settings and ROI than the multi-dimensional acquisition window (§6) but has no autosave.

Check that the camera sensor temperature is −10 °C or colder. The temperature is shown at lower left of the `Acquire` window.

Click `Show Live` to see live view or `Acquire` button to capture an image.

To set the ROI from the `Acquire` window, check `Enable Custom Field of View` and adjust the width and height. You can't move the ROI around, so move the sample using the joystick instead. This ROI will be applied in multi-dimensional acquisition (§6) as well.

4.4 Live-SR super-resolution

Gataca Live-SR optically demodulated analogue structured illumination [7] available for confocal imaging.

doubles lateral resolution. z-resolution enhanced 30% [what step size? Emma to revert].

will break diffraction limit only with the 100× objective. However, you can use it with a low-power objective to improve resolution if a higher-NA objective isn't available.

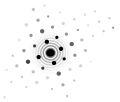
magnification increased 1.7 times, FOV accordingly smaller. First find your imaging area without Live-SR.

Axial super-resolution can also be achieved with scanning angle interference microscopy (§5.3).

click `LiveSR` button to open the `LiveSR-3D` window. In the `Main` tab, toggle switch to on. Click `Snap` to get a fully processed super-resolution image to assess the result and check exposure. weaker signal, about 2.5 to 3 times photon

number needed. Exposure time can be increased by this factor but not laser power, as fluorescence response to excitation is nonlinear. The `Live` button in the `LiveSR-3D` window is of limited utility and causes more photodamage.

To perform batch processing on existing images, first go to the `Set Up` tab in the `LiveSR-3D` window and choose whether to process `.nd` or `.tif` files. You can uncheck `Batch Background` to get faster processing, but you won't be able to use MetaMorph while the job is running.



5 TIRF-based configurations

Our TIRF has the standard configuration with a relatively large illumination FOV that covers the entire camera FOV, as contrasted with specialized TIRF for SMLM.

See Table 4.2 for TIRF laser power measurements.

5.1 Total internal reflection

Flip lever behind microscope to 90° `iLas` position. The lever can be accessed by either reaching behind the enclosure or reaching into the enclosure and out through the small door at the back of the enclosure.

Select the 100×/1.49 TIRF objective. Adjust correction collar. Add immersion oil.

sample should be in aqueous medium with nº 1.5 glass coverslip.

Check all enclosure doors closed so lasers can fire. Warning: the microscope arm at the top and the small sliding port at the right for the enclosure lighting cable are not interlocked.

See §4.2 for laser excitation and emission configurations and §4.3 for camera settings.

Click `Modular` button at top left if control bar on right side of window is missing. Click the double arrow above `360 TIRF` to expand the TIRF control window.

Click the double concentric circle icon in the control bar to access the imaging settings.

Select channel combination at upper left and add or remove channels at the bottom. Channels prefixed `SINGLE`, when used alongside those prefixed `TIRF`, enable you to acquire the same colour at two different incidence angles sequentially. `DUAL` channels are for simultaneous acquisition at the same angle, while `COMBI` is for sequential acquisition which can be at different angles.

For each channel, check that the selected FOV alignment file is the default [what name] or select your own if you have one (see §5.1.1 for doing your own alignment).

At upper right, select `Ellipse`, `Arc` or `Point` to set the amount of spin. A spinning laser traces a ring for more homogeneous illumination and less interference artifact.

Adjust laser power (in the TIRF control window) and incidence angle. The penetration depth is shown [fix wrong display] as you drag the circle. You can adjust the angle separately for each laser to get the same depth, but in this case they can't be fired simultaneously.

Start live view and focus sample (§2.2).

If you wish to check that the laser is aligned properly, lower the laser power, push back the arm of the microscope and observe the laser on the piece of white paper pasted overhead. If the alignment is unsatisfactory, inform facility staff.

5.1.1 Field-of-view alignment

The illumination axis and maximal incidence angle have already been aligned to the camera FOV and you can usually just use it. If you wish to align it yourself, click the gear icon in the main control bar to bring up the alignment window. In the lower part of the window, click one of the boxes with a `+` sign. Name it.

Click the button with a `+` sign at upper left corner of the preview diagram. A cross numbered `1` appears in the diagram. Drag it towards the left while observing the live view of the sample until the signal disappears. To fine-tune the position, click the two small arrowheads that have appeared on either side of the corner button. You may wish to click a constant number, say 10 times, beyond signal disappearance in each direction to ensure maximal utilization of TIRF.

Move on to the three remaining buttons to similarly adjust crosses `2`, `3` and `4` demarcating respectively the right, bottom and top of the TIRF ring in the diagram.

Finally, click the green box with a tick to store the alignment for this session. You can also click the floppy disk icon to save file for future recall.

5.2 Oblique dark field (HiLo)

configure microscope for TIRF (§5.1) and adjust incidence angle steeper than TIRF so the laser passes through the coverslip but illuminates the sample at a very shallow angle. Essentially widefield with less out-of-focus light, clearer image and less photodamage

5.3 Scanning angle interference microscopy

A form of structured illumination microscopy. Interference between oblique incident laser excitation and its reflection off an opaque sample substrate yields structured illumination which varies with the angle of incidence [8]. nanometer axial resolution

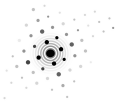
grow cells on pieces of silicon wafer mirror with transparent silicon oxide spacer layer of thickness $\geq$ 500 nm to maximize interference contrast [8]. Thickness of oxide layer has to be measured precisely for each sample, such as by ellipsometry [9].

first configure microscope for TIRF (§5.1).

linearly polarized excitation perpendicular to incidence plane [select analyzer (is it in the correct azimuth) in excitation filter wheel?]

Click the icon that looks like Θ at upper right of the control bar. Set incidence angles to scan through. It will sync to the camera exposure settings to obtain one image per angle.

Analyze data using `Saim` plugin [9] in Fiji



6 Multi-dimensional acquisition

6.1 Preliminary settings

If you don't need DIC, remove the objective Nomarski prism if it is in the light path, as it will reduce illumination power and distort the image and PSF.

Check all enclosure doors closed so lasers can fire. Warning: the microscope arm at the top and the small sliding port at the right for the enclosure lighting cable are not interlocked.

MetaMorph: `Apps` in top menu → `Multi Dimensional Acquisition`. If not doing multiple channels, z-stack or time lapse, use the `Acquire` window (§4.3) for more control of camera settings and ROI.

`Main` tab. Select time lapse, z-stack *etc.* as necessary. `Load State`: choose `CSU` for confocal spinning disk, `TIRF` for TIRF. [confirm file path]

`Saving` tab. Choose folder to autosave. Use `E` drive, unless you are doing high-speed acquisition, FCS (§9) or SMLM (§10), in which case use the `D` drive which is smaller but is a faster SSD. Type in a sensible file name prefix without spaces or special characters, otherwise you may run into problems with image processing later.

`Stream` tab. See high-speed acquisition (§7).

`Journal` tab: design complicated custom acquisition pipelines

`Display` tab: enabling `Show acquired images` will slow down the acquisition slightly.

6.2 Channel settings

`Wavelengths` tab

For sequential acquisition, set `Number of Wavelengths` to number of excitation wavelengths.

In the subtabs that appear, select channel(s) prefixed `CSU` or `TIRF` as required. `TIRF` channels should match those in the TIRF control window.

For dual-channel simultaneous acquisition, do one of the following:

- assign two `Wavelengths` to each simultaneous pair of channels and select the same dual channel in both subtabs. The channels will be acquired into separate tiff files/layers.
- assign one `Wavelength` to each simultaneous pair of channels. The channels will be montaged side by side in the tiff. The left side is the signal from the longer excitation wavelength. The montage can be split later (see §11.3). [is this faster? if so, add to high-speed section]

Subtabs `W1`, `W2` and so on will be acquired in that order. You may wish to acquire in descending order of wavelength, as emission caused by shorter excitation wavelengths can cause excitation, crosstalk and bleaching at longer wavelengths. [seq in data reversed?]

Select `Trans` channel for transmitted light. Set up the diascopic illumination (§3.1) and, if necessary, DIC (§3.1.3). If not using DIC, check that the objective Nomarski prism is out of the light path and the condenser turret is in the `A` (empty) position.

For each channel subtab:

`Gain`. 12-bit has more gain and faster readout than 16-bit. Without streaming (§7), the maximal frame rate at 16-bit is 5 FPS. If you select 12-bit, it gives you three choices with different amounts of readout noise. 12-bit has more than enough dynamic range for most samples.

`Digitizer`.

`Exposure`

Two channels being acquired simultaneously can have different exposure times.

If you are using Live-SR (§4.4), adjust the exposure accordingly.

To double check or modify the components in the light path, go to `Devices` → `Configure Illumination` and select the channel of interest. Warning: if you change anything, do not save!

Click `Prime95B` button on the left in the main window if you are doing sequential imaging, or `Prime95B Dual` for simultaneous imaging. [what if doing a combination of both?]

To start camera live view, click the camera icon with black triangular arrow. Image window will close when live view is stopped.

You can also snap one image (camera icon) which will remain open for examination and minimize photodamage. It will not be saved unless you click save button. If using dual camera mode, click `Prime95B Dual` button before snapping, otherwise you may get a black frame.

Adjust exposure, and adjust laser power in the confocal laser control window. Click `ModularSpinning` at top left if the window isn't open. High laser power causes more photodamage and bleaching than long exposure. But avoid exposures > 500 ms as the camera's hot pixels will become obvious. For live cells, SNR should be at least 1.5 (this is the bare minimum and will be very noisy).

Adjust histogram display range by dragging the triangular sliders along left edge of image window. Click the button above the histogram to turn auto scaling on or off. When auto scaling is on, intensity changes can be deceptive.

When you mouse over the pixels, the pixel intensity is displayed at lower left of MetaMorph window. how to display saturated pixels

click circular icon with rainbow stripes to change LUT.

6.3 Z-stacks

We recommend using the piezo stage motor which is much faster and has less mechanical backlash than the objective turret. In the `Main` tab, check `Use Dual Z Motors` to engage the piezo. Click the `Configure` button and check that `Stage Z1` is set to `Ti Z` and `Z series` is set to `MCC Z`.

In the `Wavelengths` subtabs (§6.2), make sure `Z series with the wavelength` is checked for each channel in which you require a stack, otherwise it will just acquire one slice. If you are acquiring a `Trans` channel you may wish to uncheck `Z series` for this channel if you aren't doing EDF.

It is better to define z-stack with Live-SR (§4.4) turned off.

First focus the sample using the focus knobs. Then go to the `Z Series` tab and find the top and bottom of the z-stack by clicking the up and down buttons of `Current Position`.

Two ways to define stack:

- Check `Range Around Current`, focus on the sample and enter the required range in μm (depends on cell line and mountant; try 5–10 μm for live cells, or focus through the volume of interest and check the stage position display). This must be used for multiple stage positions (§6.5) but is not ideal for samples in which the structure of interest is not equidistant to the top and bottom of the sample.
- Uncheck `Range Around Current` and click `Set Top To Current` and `Set Bottom To Current` to set the limits. Turn off focus drift correction (§2.2.1) while doing this.

If collecting experimental PSF or doing deconvolution, make sure stack covers all out-of-focus light at both ends.

Set `Step Size` in μm. Make sure the correct objective is selected in MetaMorph if you changed the objective manually or using the microscope's touch screen, otherwise the `Recommended Step Size` shown lower down in the window will be wrong. The `Recommended Step Size` is based on 2× [or 2.3?] Nyquist sampling. For optimal deconvolution, use smaller values as recommended by `https://svi.nl/NyquistCalculator`.

If acquiring multiple channels, select `Acquire wavelength set at each Z` for accurate colocalization and `Acquire Z series for one wavelength at a time` for speed. For simultaneous imaging you must select `Acquire wavelength set at each Z`.

Before starting acquisition, move the turret to the lower z-limit to avoid the mechanical backlash that may occur if the stage moves itself there at the start of the acquisition. [does this still apply with piezo?]

6.4 Regions of interest

For finer control, set ROI in the main camera control window (§4.3). Otherwise use the buttons at the bottom of the MDA window.

[multi-roi within camera FOV]

If doing colocalization, use the centre of the FOV where chromatic aberration is most highly corrected. Use beads (§2.1.1) to calibrate and correct shift.

6.5 Multiple stage positions and well plates

`Main` tab: check `Multiple Stage Positions`.

If using focus drift correction (§2.2.1), activate it first and it will adjust itself to each position. If the PFS `ON` button starts blinking because the focus position of the new stage position is too far from the previous, use the normal focus knobs to focus until the button lights up continuously again, and then fine-tune with the PFS offset wheel.

If using immersion objectives, minimize the distances between stage positions to avoid loss of contact. Do not let the immersion fluid drip onto the turret.

Move the stage to a desired position and focus the sample using the focus knobs (not the piezo buttons). In the `Stage` tab, edit `Position Label` and click `+`. The z position is stored as well as x and y. Move the stage to another position and repeat. To overwrite an existing position, select it and click `+`.

For well plates and other situations in which the same set of stage positions is needed over multiple sessions, you can `Load` spreadsheet [format? how to set up?]

6.6 Tiling

6.7 Time lapse

Estimate the acquisition time of each time point from the exposure time, number of slices and number of channels. If you have multiple stage positions, factor in the time for the stage to move between positions.

In the `Timelapse` tab, set a sufficient time interval, as a time point can begin acquisition only when the previous one is finished.

You can acquire different channels at different time points by adjusting settings in `Wavelengths` tab

[lateral drift correction for time lapse?]

6.8 Experiment overview

If you are using Live-SR (§4.4), click `Setup MDA` in the Live-SR window if you wish to enable on-the-fly super-resolution processing during acquisition (§6). This may slow down the acquisition [but gives sample time to recover from photobleaching?], so you may want to click `Batch Process` later instead.

real-time denoising to reduce effect of photon shot noise?

real-time particle tracking?

`Save State` in `Main` tab. Does not save laser power settings.

Check the camera sensor temperatures (§4.3).

Check that the enclosure lights are off, unless you need them to sustain photosynthesis during the experiment.

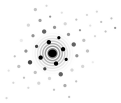
A software bug may generate a black frame at the beginning of the acquisition. To prevent this, click the snap button until no black frame appears in the camera window and you see the sample image. If using dual camera mode, click

`Prime95B Dual` before starting the experiment, otherwise acquisition may not start.

Click `Acquire` to run the experiment.

To close all images, click the ⨯ icon.

If you used Live-SR (§4.4) with live processing, the processing may still be going on after the acquisition has finished and the files have appeared in the folder, especially if you were acquiring stacks. Give it some time before opening the files, otherwise you may see blank images.



7 High-speed acquisition

For applications such as FCS (§9), SMLM (§10), Ca^{2+} imaging. Also useful for capturing 3D dynamics, since z-stacks consume precious time.

If acquiring 2D time lapse of a single channel or single pair of simultaneous channels, first open the `Acquire` window and set the exposure time, ROI and other camera settings (§4.3).

Then go to `Acquire` → `Stream Acquisition`. In the `Acquire` tab, set the number of frames and whether to stream to RAM or to hard disk. Streaming to RAM is faster but check whether there's sufficient RAM for your requirements as indicated below the setting. After acquisition you have to right-click on the image window and save to hard disk. If streaming to hard disk, set the save location to the faster solid-state `D` drive. Try not to stream to hard disk if you are acquiring a large number of frames, as it will save each frame as a separate file.

7.1 Multi-dimensional streaming

Open the `Multi Dimensional Acquisition` window (§6).

In the `Main` tab, check `Stream`.

If using simultaneous channel pairs, assign one `Wavelength` to each pair (§6.2) to get the side-by-side montaged tiff which streams faster.

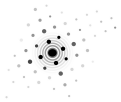
`Acquire Z series for one wavelength at a time`. Note that using PFS (§2.2.1) during an acquisition involving z-stacks will increase acquisition time as the PFS will turn off while the microscope acquires a stack and turn back on after that.

[can multiple stage positions be done?]

In the `Display` tab, uncheck `Show acquired images`.

In the `Stream` tab:

- Check the stream boxes for all applicable data dimensions. If acquiring sequential channels or multiple pairs of simultaneous channels, do not `Stream Multiple Wavelengths`. Otherwise, filter movements between channels will be captured in the continuously streamed images.
- Set `Stream Exposure Time` which will override the exposure settings in the individual `Wavelengths` subtabs (§6.2).
- Set `Stream To: RAM`.



8 Fluorescence recovery after photobleaching

8.1 FRAP setup

Can be done with TIRF (§5.1) or spinning disk (§4.1). Can combine with z-stack but no 3D ROI.

Can use Live-SR (§4.4)? Will FRAP mirror in light path cause colour shift?

In the `Multi Dimensional Acquisition` window (§6), select a `FRAP` channel for the imaging (not bleaching) part.

Click `Modular` button at top left if control bar on right side of window is missing. Click the double arrow above `TARGET` to expand the FRAP control window.

8.2 FRAP alignment

The FRAP laser scanner should be aligned to the camera FOV. Click the gear icon in the FRAP control bar. You can load the existing alignment by clicking the folder icon and choosing the appropriate FRAP alignment file for `CSU` or `TIRF`.

If you are doing your own FRAP alignment, use a scratched Chroma® slide or a slide with highlighter ink drawn on it, or find an empty region in your sample. Start live view in the main software. Click the gear icon in the FRAP control bar. In the FRAP control window, click the power button to turn on the FRAP laser. Focus the laser spot using the focus knobs. Click and drag the top left and bottom right corners of the preview diagram until the laser spot is just inside the respective corners of the camera live view. Click the pencil-and-ruler button at lower right. When done, stop live and turn off the FRAP laser. Click the save icon to save.

8.3 Define bleaching regions

Using camera live view, find the area of interest in your sample. [then stop live or not?]

Click the lightning icon in the control bar. Select point or circular area to bleach. If circle, set diameter. [relation to roi?]

Click the live button in the FRAP control window. Test alignment accuracy by clicking somewhere on image and checking that bleached spot is centered to crosshairs. Stop live.

Draw regions of any combination of shapes. Click the plus sign to add. Set `Thick.` (line thickness) for line ROIs. Right-click to copy and paste regions. Click the small save icon at bottom left of the box to save. To load previously saved ROI set, go to `Regions` → `Load Regions` in the main MetaMorph menu.

Set `Rep.` for bleaching time. Adjust FRAP laser power in the FRAP control window. The bleaching wavelength can be different from the imaging wavelength. Bleaching too long or with excessive laser power may cause an area larger than that defined to be bleached. [click live in the FRAP control window?] Click the small lightning icon at bottom right of the box to trial bleach and check visually.

8.4 FRAP acquisition regime

Click the play icon in the control bar.

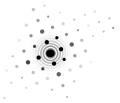
Select `FLIP` to repeat FRAP cycles.

Set time lapse duration and interval for pre- and post-bleach image acquisition. Click box with plus sign to add time lapse sequence with different settings.

When done, click the play icon in the FRAP control window to transfer settings to the `Multi Dimensional Acquisition` window (§6).

[how to bleach and track fast-moving structures using streaming]

Fiji simFRAP plugin [10] for calculating diffusion coefficient



9 Fluorescence correlation spectroscopy

imaging FCS using TIRF. spinning or stationary?

change BNC cable connection

first configure microscope for TIRF (§5.1).

`Stream` tab [ref earlier sect?] and save to the fast SSD D drive

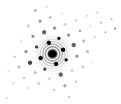
For one-time PSF calibration see Bag *et al.* [11].

See SANKARAN *et al.* [12] for guidelines on obtaining accurate and precise camera-based FCS measurements. Note that the SCMOS cameras on this microscope are noisier than the EMCCD often used for FCS.

You may wish to calibrate the camera sensor for intrinsic pixel-to-pixel variations [6], especially for spatial flow analysis.

can use `ImFCS` plugin? get instructions from luxendo manual

TIRF-based FCCS [13] using simultaneous detection by two cameras?



10 Single-molecule localization microscopy

Special training is required for doing SMLM on this instrument. Please contact the facility managers.

such as STORM or DNA-PAINT

sample prep: need high-enough labeling density otherwise linear structures may be broken

Insert the Thorlabs mirror cube (this will block the normal TIRF lasers) and turn on the `647` plug [how much time for laser to warm up?]. Press the power button on the red Thorlabs controller so that the green LED lights up. Launch the GUI-VFL software, set the power and click `On`. See Table 4.2 for measured power at stage.

Configure system for TIRF (§5.1). Select the `TIRF Cy5` channel and set the power of the 640 nm TIRF laser to 0.

Press the `ENABLE` button on the red Thorlabs controller so that its green LED lights up. Click `Activate` in the laser software window. The `emission` dot in the software turns blue and the 647 nm high-power laser is now illuminating the sample. If you change laser power, click `Activate` again. If the laser spot is not centered in the full-chip view (which can be assessed more easily at a steeper laser incidence angle), reach behind without looking at the laser and jiggle the mirror cube until it is. To cut the laser from the sample, press `ENABLE` again. Minimize exposure of the sample to the laser, as prolonged irradiation at high power will damage both the sample and the optics.

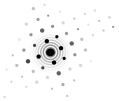
Field-of-view alignment as for TIRF (§5.1.1).

Define ROI using camera control window (§4.3).

Use high-speed acquisition (§7).

Fiji `ThunderSTORM` [14] or other plugins [15]. Fiji `HAWK` plugin (`https://www.coxphysics.com`) for pre-processing to get rid of artificial sharpening artifacts (does not work with 3D-STORM).

After use, turn off the 647 plug and remove the Thorlabs mirror cube to return the light path to normal configuration.



11 Image processing

11.1 MetaMorph

Image metadata can be viewed by clicking the blue circular icon with the letter `i`.

To quickly review data on the microscope workstation, we recommend using Imaris (§11.2) which requires fewer steps and is less confusing.

If you just want to see the channels overlaid, you can go to `Display` → `Color Combine` in MetaMorph.

To review data in more detail in MetaMorph, click the icon that shows a computer monitor with three axes to open the `Review Multi Dimensional Data` window.

Click `Select Base File`. In the pop-up dialogue, browse to your data and click `View`. Select the channels and time points you want: click top left corner of spreadsheet to select everything, or click individual cells.

In the `Display` tab, you can check or uncheck `Wavelength Composite` (which overlays the channels). In the `Z Projection` tab, check `Z Projection` and choose the type of projection, which slices to subset *etc.*

You can also examine the z-dimension with `Stack` → `View Orthogonal Planes`.

For shading correction of spinning disk images, go to `Process` → `Arithmetic`. Select your data as `Source image 1` and the correction file as `Source 2`. Select `Divide` [how to determine constants]

tile stitching?

11.2 Imaris

First launch Imaris File Converter to convert the data to Imaris format.

The Imaris on the microscope workstation only allows you to view (orthogonal, 3D etc.) and export the data. You cannot do analysis or animations.

[steps]

11.3 ImageJ (Fiji)

If your images were acquired in the dual-channel side-by-side format, you can split and rearrange them into normal multichannel stacks using the script available at `https://github.com/linyangchen/ImageJ-Fiji-scripts/blob/main/split_MetaMorph_dual_stacks.ijm`

Drag the `.nd` file into Fiji. As the `Bio-Formats` plugin may assign LUT of the wrong colour(s) when opening `.nd` files, open in greyscale and reassign the LUT yourself.

shading correction: `Shading Corrector` plugin

tile stitching: `Image Stitching` plugins (`https://imagej.net/plugins/image-stitching`)

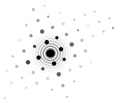
colocalization: `BIOP JACoP` plugin

particle tracking: `TrackMate` plugin [16] which can do 3D tracking

Refer to sections on specific imaging methods for specialized processing.

11.4 Data transfer

Portable drives are not allowed to be connected to the workstation. Please transfer via Internet to the CBIS server (CBIS users only) or other servers within two weeks of your imaging session.



12 Clean up and switch off

Stop PFS if it is running (§2.2.1).

Remove sample. Snip off and properly discard tip of green thermocouple if you immersed it in culture medium.

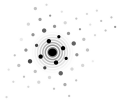
clean objective front elements and stage insert if necessary. Do not unscrew objectives.

Turn off the enclosure lights.

Check the PPMS schedule. If you are the last user for the day, shut down the entire system. Otherwise, log out of PPMS.

If applicable, turn off the incubation plugs and CO_2 tank unless the next user has requested for it to remain on. To cool system to room temperature faster, open all enclosure doors and push back the microscope arm.

Sign the log book.



Troubleshooting

No laser

- check that all enclosure doors are closed.
- try nudging the laser power slider in the software.
- check physical USB connection at the back of CPU
- exit MetaMorph. Run InstaCal and check that `Board# 0 - USB-3103 (79)` is shown

No camera

- Run PVCam and look for error message at bottom left

Displayed ROI doesn't correspond to setting in MDA

- check ROI setting in main camera control window (§4.3)

Image looks blur

- check binning setting

Missing software controls

- restart software

Literature cited

[1] Schwendy, M. *et al.* (2019). Automated cell segmentation in FIJI® using the DRAQ5 nuclear dye. *BMC Bioinformatics* 20:39.

[2] Verschueren, H. (1985). Interference reflection microscopy in cell biology: methodology and applications. *J. Cell Sci.* 75:279–301.

[3] Pluta, M. (1989). *Advanced Light Microscopy vol. 2 Specialized Methods.* Elsevier.

[4] Filler, T. J. & Peuker, E. T. (2000). Reflection contrast microscopy (RCM): a forgotten technique? *J. Pathol.* 190:635–638.

[5] Oreopoulos, J. *et al.* (2014). Spinning-disk confocal microscopy: present technology and future trends. In: Waters, J. C. & Wittman, T., eds. *Methods in Cell Biology.* Vol. 123. Elsevier pp. 153–175.

[6] Diekmann, R. *et al.* (2022). Photon-free (s)CMOS camera characterization for artifact reduction in high- and super-resolution microscopy. *Nat. Commun.* 13:3362.

[7] York, A. G. *et al.* (2013). Instant super-resolution imaging in live cells and embryos via analog image processing. *Nat. Methods* 10(11):1122–1126.

[8] Paszek, M. J. *et al.* (2012). Scanning angle interference microscopy reveals cell dynamics at the nanoscale. *Nat. Methods* 9(8):825–827.

[9] Carbone, C. B. *et al.* (2016). An acquisition and analysis pipeline for scanning angle interference microscopy. *Nat. Methods* 13(11):897–898.

[10] Blumenthal, D. *et al.* (2015). Universal approach to FRAP analysis of arbitrary bleaching patterns. *Sci. Rep.* 5:11655.

[11] Bag, N. *et al.* (2012). Calibration and limits of camera-based fluorescence correlation spectroscopy: a supported lipid bilayer study. *Chemphyschem* 13(11):2784–2794.

[12] Sankaran, J. *et al.* (2013). Accuracy and precision in camera-based fluorescence correlation spectroscopy measurements. *Anal. Chem.* 85:3948–3954.

[13] Sankaran, J. *et al.* (2009). Diffusion, transport, and cell membrane organization investigated by imaging fluorescence cross-correlation spectroscopy. *Biophys. J.* 97(9):2630–2639.

[14] Ovesný, M. *et al.* (2014). ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging. *Bioinformatics* 30(16):2389–2390.

[15] Linde, S. van de (2019). Single-molecule localization microscopy analysis with ImageJ. *J. Phys. D: Appl. Phys.* 52:203002.

[16] Ershov, D. *et al.* (2022). TrackMate 7: integrating state-of-the-art segmentation algorithms into tracking pipelines. *Nat. Methods* 19:829–832.