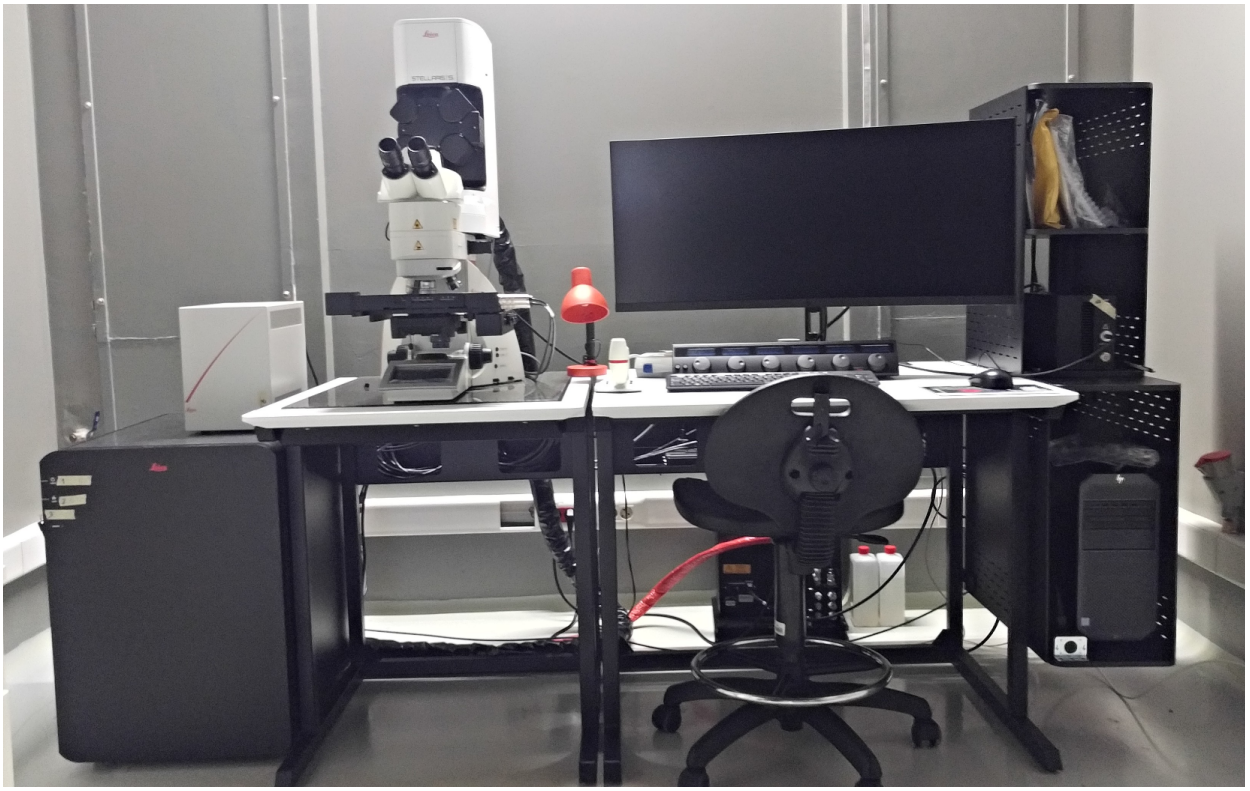
Leica Stellaris5 (upright) confocal - SOP/User's guide



Author(s): Donald F, ...

Date: v2021.09.**

Purpose: "This guide describes how to use the AIF and the Leica Stellaris5 confocal upright microscope"

Contents

Safety

This system does NOT operate with lasers; however it uses an intense light source for fluorescence excitation - Colibri7 - which is a potential radiation hazard (*LED radiation in 400 nm to 700 nm range! LED risk group 1 under DIN EN 62471:2009)*
Avoid exposure or staring directly at the LED light source!

The surface of the Colibri module can also get hot.

The microscope's motorised stage & objective turret could cause personal injury or damage samples (and project glass shards) when misused. Crushing hazard on the motorized stage

For more information / emergencies please contact the facility staff:

From 9AM to 5PM: **x4533,** after 5PM and weekends: **+351 214 464 539**

Email contact: **aif@igc.gulbenkian.pt**

Equipment location, description & technical specs

Location: UIC Room 2 (0B03), Bartolomeu Dias Wing
URL:
Manufacturer/model: Leica DMI6 (upright) + Stellaris5 scanhead
Year Installed: 2021 **Funding**: ERC
Detectors: 2x power HyD S detectors, spectral. + 1 T- PMT (transmitted light)
Keywords: Widefield, ApoTome, optical sectioning, tile & stitching, upright mic., high throughput

The Leica Stellaris 5 system is mounted on a Leica DM6 upright microscope, equipped with 405 nm, 448 nm, 488 nm, 561 nm and 638 nm laser lines, and with two of Leica's new Power HyD S spectral detectors. The Stellaris5 is equipped also to perform FRET (CFP-YFP AB or SE), FRAP, and Lightning ("Super-Resolution").

LED fluorescence illuminator + filters (eyepieces only)

Excitation lines	Abs/relative power*	Use for:
"405" (DAPI)	20 W.cm^{-2} / 133%	DAPI, Hoescht
GFP (blue)	15 W.cm^{-2} / 100%	GFP, FITC, Alexa/ATTO488, DyLight green
N1 (green)	5 W.cm^{-2} /33%	TRITC, Alexa/ATTO550-568, Cy

Note: * Absolute radiance / relative power to 488nm line. Values apply only to the single band filters

Objectives (vales in µm, unless noted otherwise)

Objectives*	NA*	imm.	WD (mm)	FOV (mm)	Theoretical res. / @Nyquist	Pixel size RGB cam.	.. with Flash cam. / +Optovar*	DIC?
10x PL APO CS2	0.4	air	2.5	2.5	1.12/ 0.45	0.44	0.650/ 0.406	N/A
10x Apo L W	0.3	water	3.6	2.5	1.12/ 0.45	0.44	0.650/ 0.406	N/A
20x PlanApo	0.8	air	25	1.25	0.42/ 0.17	0.22	0.325/ 0.203	N/A
63x PlanApo	1.4	oil	0.19	0.397	0.24/ 0.10	0.069	0.103/ 0.064	Y
optional (ask staff)								
10x Apo L W	0.3	water	3.6	2.5	1.12/ 0.45	0.44	0.650/ 0.406	N/A
40x PLAPO CS2	1.3	oil						
40x W Apo L	0.8	water	3.3					

Notes*: NA = Numerical Aperture. For more information, follow this link. (2) Theoretical resolution for each objective might differ from the actual pixel size of acquired image, calculated as 0.61*550nm/NA.
Nyquist = max resolution with sampling at 2.5x the theoretical resolution; must be used for images to be deconvolved.
Optovar allows "extra" 1.6x magnification but only on the Hamamatsu Flash; 2nd pixel size is for the extra magnif.
Obj. Zeiss refs: 2.5x0.085 ON420320-9902-000; 10x0.3 ON420340-9900-000; 20x0.8 ON420650-9902-000 20x0.75 ON440649 1101 957; 40x1.3 ON420462-9900-000; 63x ON420782-9900-000; 100x ON420792-9900-000

Lasers installed:

405 DMOD - 405 nm (software intensity control)	Solid State; 448, 488, 561, 638 nm (laser power controlled by AOTF)

General rules of usage, user prerequisites and equipment booking

Before using the facility, new users must show that they have a clear understanding of the principles of light microscopy, fluorescence and widefield vs optical sectioning (e.g. confocal). The facility provides on-demand training, as well as regular workshops where these topics are addressed. You can also find more information online here https://www.microscopyu.com/, including highly recommended tutorials.

Before starting a project to use this system, you have to make an appointment with the facility head to learn more about the usage of the system, including all the conditions explained in this document. Booking is done online via Agendo (http://igc.agendo.science). Users must first register and get training from facility staff. The general facility usage terms should be consulted here https://my.agendo.science/terms/facility/. Booking for and using this system implies that the researcher and Lab PI have read and accepted these terms. Usage will only be allowed and booking only be possible after training has been completed. When a user is ready to use a microscope independently, will be decided case to case, by the staff responsible for training.
Please also read the "where are the rules...", "intellectual contributions", and "negligent use" appended to this SOP!

Usage charges: Usage is charged hourly. Max 3h per session per user, up to 12h max per week:

IGC researchers (internal)	9€/h
Academia (external)	12€/h
Industry (external)	24€/h

Disclaimer: These are indicative prices at the time of writing this SOP/guide and may be updated afterwards. Always consult in Agendo the most up-to-date price of each equipment by clicking on its picture or the (i) next to the calendar title

Suggestion for Description in "Materials and Methods"

Images were acquired on a Zeiss Imager Z2/ApoTome.2 system, equipped with a Hamamatsu Orca Flash 4.0 v2 CMOS camera [/ Axiocam 105 color camera], using the 63x 1.4NA Oil immersion objective, DAPI + CY5 fluorescence filter-sets and DIC optics. Serial sections were acquired every ** µms in ApoTome mode with 5 phase images and processed for bleach correction with Zeiss's ZEN v3.0.

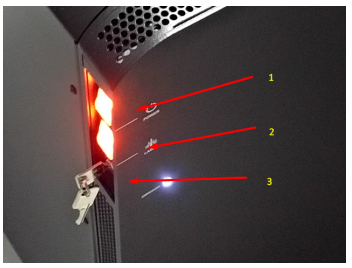
Required "Acknowledgments"

We thank the technical support of IGC's Advanced Imaging Facility, which is supported by Portuguese funding ref# PPBI-POCI-01-0145-FEDER-022122, co-financed by Lisboa Regional Operational Programme (Lisboa 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER) and Fundação para a Ciência e a Tecnologia (FCT, Portugal).

Stellaris Startup

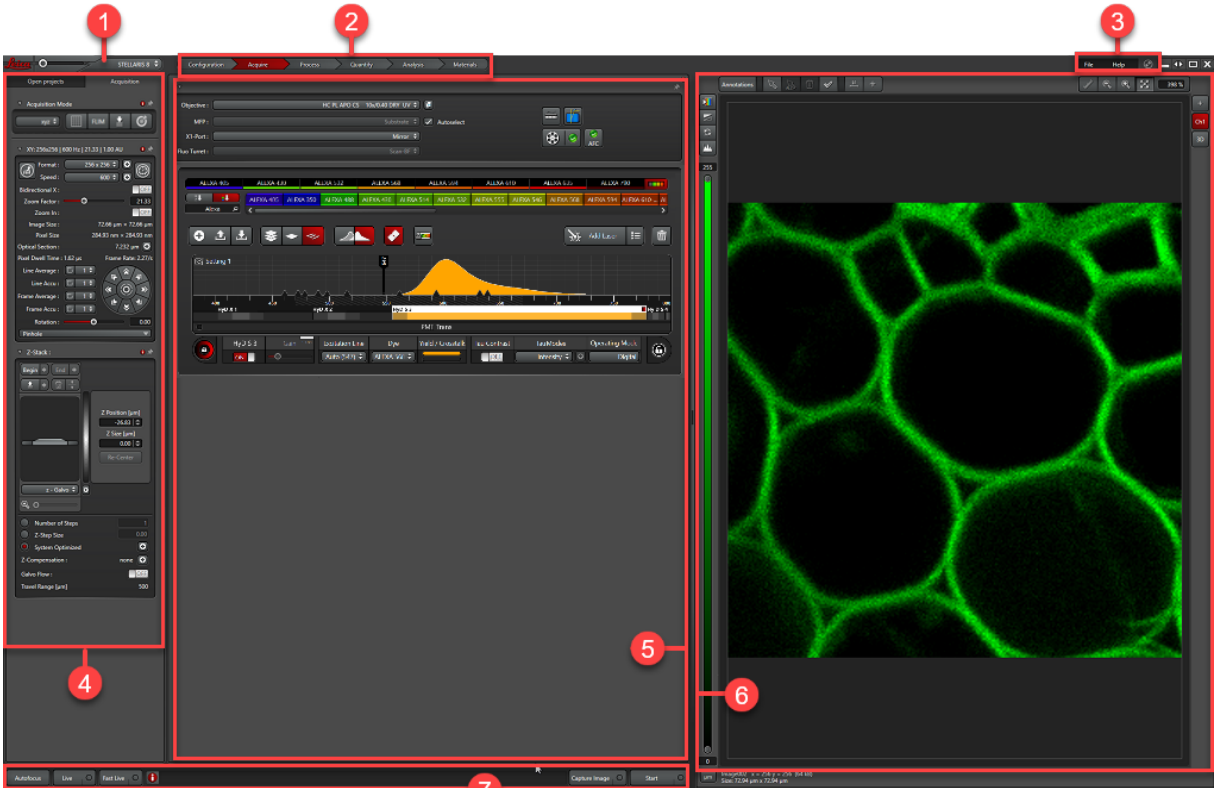
The computer can be turned on or off regardless of whether the microscope is on, and if you need to use it to transfer files or something similar, the microscope does not need to be on as well.

1) Turn buttons (1) (2) and the Key switch (3) on the large black cabinet, to the left of the microscope. The mic. and its components detect when power is ON and begin to turn them on in sequence. Wait a few moments for all to start.
2) Turn on the Computer (if not on already!).
3) Log on to the computer using your Agendo credentials
4) Ensure that the stage is lowered from the objectives and free to move, then start LAS X control software.
5) Ask staff if it is necessary to exchange the stage insert (glass or universal)



LAS X

When you start LAS X, you will have to accept the start-up settings. To operate the microscope, 'Machine' should be in the top selection box, and 'DM6' should be in the bottom selection box.



1&4) Scanner parameters (XY) and Z stack controls. **2)** Workflow steps **3)** Help & support **5)** Beam path parameters & Channel settings **6)** Image viewer **7)** Acquisition toolbar.

Stellaris Shutdown

1) Please lower the stage to the lowest point before removing your sample, and allow cleaning of oil from the objectives.
    a) Confirm that your datasets (*.lif projects) are all saved.
2) If there is a user after you leave the system on, and check with staff. Transfer data.
3) If a user is not coming in the next 30min, turn off the lasers via software in LAS X and proceed to transferring data to server. You may close LAS-X, or leave it on.
4) If you are the last user of the day, shutdown LAS X and turn off the components:
    a) After LAS-X is closed (and ONLY AFTER!) turn off the laser key (the white emission led should go out), and then the power buttons on the large cabinet to the left of the microscope. Shutting down LAS X may take 1-2 minutes!
    b) The computer can be left on for transferring files.

Acquiring images in the Stellaris5 (under construction)

Acquiring images in a confocal is a complex procedure, first time users are required to have at least two supervised training sessions with the AIF staff to work with the system independently. If you need assistance don't forget to press "with assistance" when booking this microscope.

Tips:

To quickly setup a new experiment we recommend using the "dye assistant", which allows the user to interactively configure the best detector settings for every combination of fluorophores.

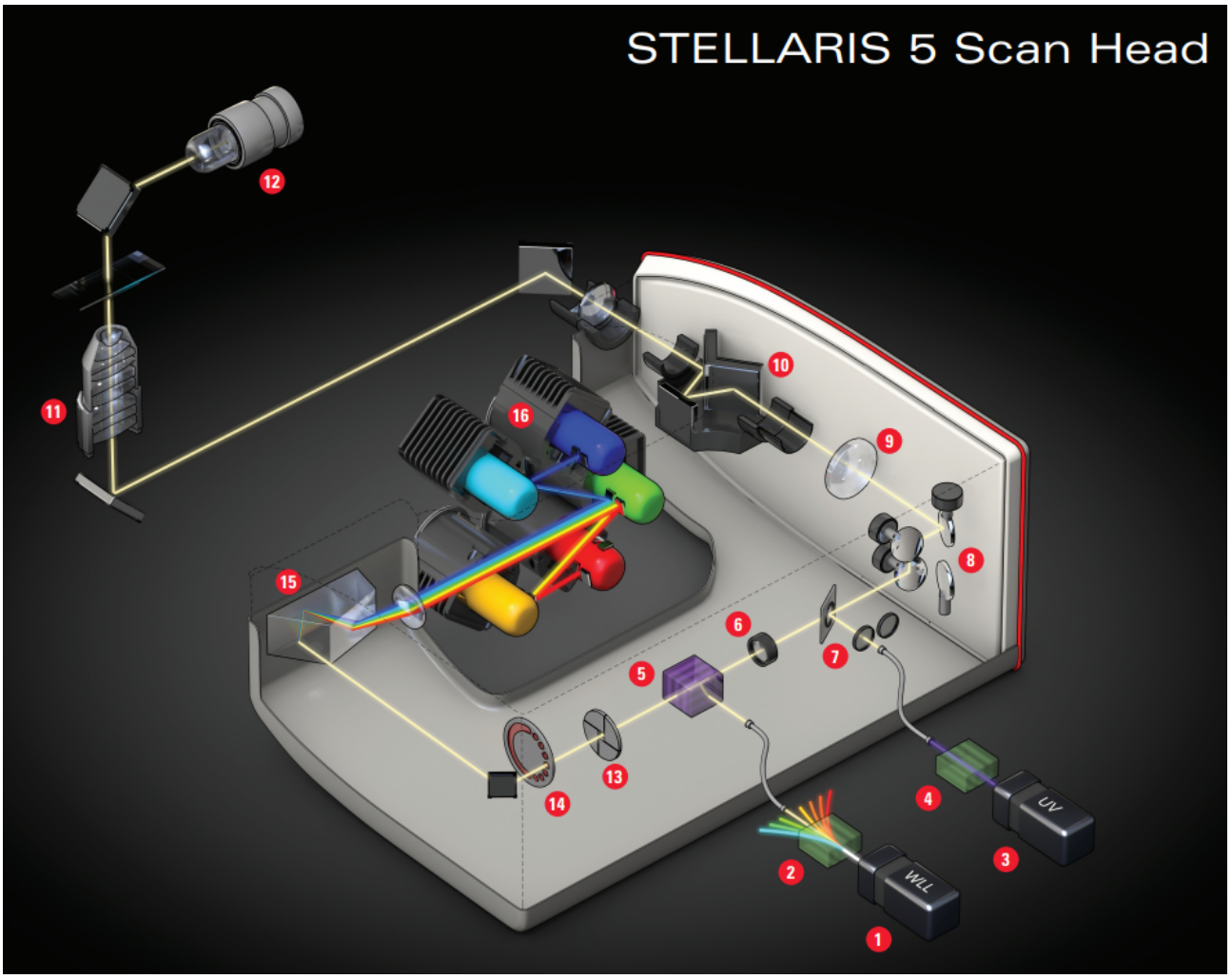
Operation in Lightning mode (Super-resolution) requires switching to a different mode of the LAS-X software

FRET and FRAP modules are also accessible as additional modules of LAS-X.

Note: one of the major changes of this version of LAS-X is the multi-position acquisition. Consult with the staff to learn how to use this new "Mosaic" feature. This allows the setting up of automated acquisition of multiple positions in full auto mode.

Please be aware of the laser lines installed on this system, which limits the number of fluorophores the microscope can image, and should inform the preparation of your sample.

If your experiment requires quantification, the system takes about 5 minutes from startup for the lasers to normalize.



1. White light laser (WLL: 485 nm - 685 nm) and up to five visible line lasers
2. Acousto-optical tunable filter (AOTF)
3. Ultraviolet (UV: 405 nm) laser *
4. AOTF or direct modulation (DMOD)
5. Acousto-optical beam splitter (AOBS), other options available
6. FRAP Booster *
7. Ultraviolet (UV: 405 nm) laser incoupling with CS2 UV optics
8. FOV Scanner with Tandem Scanner option
9. Scan optics with HIVISR
10. Scan field rotation *
11. Objective lens (different options available)
12. Transmitted light detection
13. Square confocal pinhole
14. Fluorifier disc *
15. Prism-based dispersion
16. SP Detection with up to five Power HyD S detectors

*optional

The Stellaris5 scanhead and general operation concepts: There are 5 lasers (1 “UV” [3] + 4 visible [1]) and two Power HYD S detectors on the Stellaris5. Light being received from the sample is reflected to the detectors along a range of wavelengths via a set of mirrors. These mirrors can receive a narrow or wide band of wavelengths, but they cannot change their relationship to one another. HyD1 is always on the violet, lower wavelength, side of HyD2. HyD1 can receive red-spectrum signals, but HyD2 cannot switch places and receive the more violet-spectrum of light at the same time.

There is a general tradeoff between the amount of time it takes for an image to capture, and its resolution, e.g. number of pixels, and fidelity, i.e. how ‘good’ it looks. Longer times allow for higher resolutions and improved fidelity, while shorter times means limitations to both. 200hz is the recommended for a good SNR, but 400 hz is the most frequently used when acquisition time is a

limitation. The Stellaris5 is capable of 600Hz also, without compromising the acquisition field (ie, without introducing Zoom). All speeds above 600Hz will require a Zoo, ie, limited area of acquisition.

A confocal microscope operates by scanning a laser along each ‘pixel’ of an x-y image, and then detecting the amount of emitted light that comes back. A 3D scan does this by selectively imaging consecutive x-y focal planes. The amount of light is the ‘brightness of that pixel.’ The faster the laser scans, the less time it has to illuminate and detect light. In the LAS X software, the speed is given in hertz, higher numbers are faster while lower numbers are slower. In general, values for speed will be between 100 and 1000, with further improvements in image quality achieved by accumulating or averaging multiple passes across the specimen.

Operating the LAS X software.

Before you begin to image, you should try to get to your focus plane using the oculars.

The easiest way to begin to use the LAS X software is to search through the Fluorophores tab and drag and drop the desired Fluorophore from the tab to the detector in the spectrum graph, either HyD1 or HyD2. To remove a setting from a detector, simply select the detector and hit the ‘delete’ key.

The “live” preview

Most experiments require imaging more than one fluorophore, and this can be accomplished one of two ways. The first is to image on two different passes of the laser, with different settings for each. In the LAS X, you can add ‘Settings’ to the experiment which will add a new pass to the laser.

When you have more than one setting, you should change the setting operation to ‘frame-by-frame’ instead of the default ‘line-by-line.’ Many parameters cannot be changed in ‘line-by-line’ mode, while they can be changed in the ‘frame-by-frame’ and ‘volume-by-volume’ mode. HyD detector mirrors to cover different wavelengths, and differences in scanning frequency and whether you are accumulating or averaging over multiple passes. In general, ‘frame-by-frame’ mode is the best compromise between the fastest but limited ‘line-by-line,’ and the slowest ‘volume-by-volume’ mode.

The microscope has two detectors, and it is possible to detect two fluorophores at the same time on the same setting. Be aware that these two fluorophores should be separated in the spectrum from each other to minimize cross talk between the two channels. The LAS X software will optimize the settings based on what you give it, and will provide settings for two Fluorophores close to each other on the spectrum. It is up to you to try to space out your fluorophores, and be aware that many common fluorophores, especially on the violet to green part of the spectrum, will emit some light when scanned with the ultraviolet laser (405).

The delete key is useful for turning off laser lines manually.

If you are imaging two Fluorophores and definitely if you are imaging three Fluorophores, you may need to use different 'settings.' These are a separate suite of settings determining a separate scan of the area being imaged.

You can use the setting '-simulator' to open pictures and do other work while the microscope is OFF.

"Photon counting" *vs* "analog" modes

(in construction)

Lightning Notes

The Lightning tab, separate from the Stellaris 5, applies a near-real-time deconvolution of the image you are taking. As a result, any image taken in Lightning mode will produce two images, the first is the raw image, and the second is the deconvolved image.

The deconvolution process works the best if you input the immersion media of your sample. Please have this information at hand before you start your Lightning imaging.

Lightning further offers a slider that algorithmically calculates the tradeoff between speed of acquisition and resolution of the resulting image. You can turn off this slider and apply your own settings if you wish. Even in this case, the image will be deconvolved, and LAS X will still produce two images, the raw image, and the deconvolved one.

According to Leica, the deconvolved image should have the same amount of intensity as the original image. So it should be quantifiable.

A note on dynamic range and image bit-depth:

The Lightning algorithm biases towards the dynamic range found in an 8-bit image. In most cases the settings along the slider it recommends will keep the maximum value in the image below 255. In some cases, for example if you increase the laser intensity, you can find the maximum value of your image above 255 and you may want to acquire in 12-bit, i.e. 4,096 shades. To change the bit-depth of the image, you need to go to 'configuration' tab and it is in the 'Hardware' settings.

Regardless of the input bit-depth, the deconvolution process will produce a 16-bit image. This will increase the size of the image.

The deconvolution process does not work well if part of your image is too bright, represented by pixels at the brightest range of your image's bit-depth. If you do find this, you can reduce the laser's intensity, increase the bit-depth, or reduce the amount of time the image is scanned.

Saving and retrieval of bioimage datasets

- All data acquired using this system should be saved inside the “D:\Users_data” folder, and we recommend the following subfolders structure:

  D: \ Users_data \ lab \ researcher \ AAAA-mm-dd \

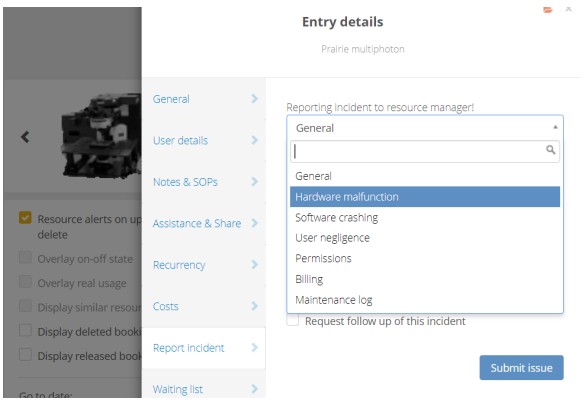
  Where “*lab*” = title of lab or PI, “*researcher*” = name of researcher, “*AAAA-mm-dd*” = date of the experiment or imaging session (year and month 1st!).

- Bioimage data will be kept in this workstation for a maximum of 1 month, after which all data will be automatically deleted. We recommend retrieving your data at the end of every session - note that transferring several Gbs may take a while, so try to account for that, so the next user is not affected.

- Datasets can be temporarily transferred and stored for processing and analysis into the facility’s temporary storage space, accessible at \\uic-nas\ (this is faster than transferring to the IGC server). Datasets can be kept in this temporary storage for a maximum of 6 months (or less if our storage space eventually runs out!), after which they will be automatically deleted. This is only temporary storage, and the facility does NOT guarantee the integrity or backups of the datasets in it. For that service, please contact the IT unit.

Troubleshooting (under construction)

You need to report any problem/incident, whenever possible, directly to facility staff before leaving the facility.
After reporting it in person, the incident should also be logged into the agendo system so that it is taken care of timely, the Login into Agendo, select your current booking in the system's calendar, select "report incident" and then provide the necessary information so we can adequately track this problem. Give us details, and request a follow up if necessary.



Cannot start "live" because of "interlock" error?

The laser switch key is not ON. Check again that the small white LED "emission" is lit.

Where are the core facility's terms of usage/code of conduct?

The imaging facility follows the general terms of usage for facilities, which can be consulted here: https://my.agendo.science/terms/facility/ (you must login to be able to access these rules).

The IGC's "code of conduct" also contains relevant information about the expected conduct at the facility: http://lists.igc.gulbenkian.pt/pipermail/research/attachments/20190708/03f8a111/attachment.pdf

Below you can find more information that pertains specifically to the conduct and terms of usage that should be observed at the Imaging Facility, including how to avoid negligent use of equipment and how to recognize intellectual contributions from facility staff.

In order to receive training on this system the user must 1st login in agendo as ask for permission by clicking on the button (top row of agendo's webpage). Training normally requires several sessions for optimization and for the user to get accustomed to the system.

How to avoid negligent use of the imaging facility?

Any negligent use of this system has to be promptly reported (by users and staff) to the facility head or staff, which will evaluate whether the user should be denied future booking and use. Agendo allows reporting "incidents" that should be used when such problems are detected.

Examples of what may be considered negligent use:

- Forgetting to turn off the system or critical components such as fluorescent lamps & lasers, when no one else is booked after you (check calendar or ask staff before leaving),
- Failure to clean or report dirty optics or system malfunction to staff (see instructions below)
- Repeatedly arriving late, skipping or deleting booked sessions without providing an explanation; using equipment without, or past, the time booked without informing the staff.
- Repeatedly booking on behalf of others.
- Attempting to use the system without training and previous authorisation from staff
- Attempting to alter the characteristics or functionality of the system, or parts of it, without previous training AND consent of the staff (*eg* removing/replacing objectives)
- Installing any software or connecting external disks/USB sticks to the workstations
- Making use of the workstation and internet access for purposes other than checking protocols, system booking, acquiring, processing/analysing & transferring image datasets.
- Falsification of bioimage results or purposeful misrepresentation of bioimaging data.

- Filling the hard drive with data and failing to provide notice to the facility staff, when it can compromise the work of the next user. Note that transferring Gbs of data may take hours.

How to cite the AIF in "Acknowledgments"?

We thank the technical support of IGC's Advanced Imaging Facility, which is supported by Portuguese funding ref# PPBI-POCI-01-0145-FEDER-022122, co-financed by Lisboa Regional Operational Programme (Lisboa 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER) and Fundação para a Ciência e a Tecnologia (FCT, Portugal).

Please do NOT forget to also mention the staff who helped with the acquisition or analysis of data and make sure to read the "what is an intellectual contribution" section below.

What may constitute an intellectual contribution from facility staff?

Granting equipment access, training and providing consumables, *per se*, DO NOT warrant co -authorship in publications from any of the facility staff.

Support from staff with acquisition of raw datasets (image data acquired "as is", without pre-processing), opening and converting to other file generic formats, basic process & analysis procedures (eg, contrast enhancement, 2D measurements, basic segmentation and particle analysis) also DO NOT constitute a significant intellectual contribution that would warrant co-authorship in publications from staff. Help with deconvolution and other basic forms of data restoration or pre-processing are also not considered significant intellectual contributions.

The following may constitute an intellectual contribution, and should be discussed in advance with the facility head and staff member providing the support:

- Implementation of novel (or uncommon/untested) protocols for sample preparation, image acquisition, processing and/or analysis.
- Custom-building of hardware or software
- Obtaining a substantial amount of the images used in a publication (or the ones presented).
- Complex image feature enhancement or extraction, 3D/4D image segmentation/tracking, complex measurements (eg, those not available in ImageJ's "particle analysis" or "set measurements")
- Preparation of manuscript plates/figures or text (even if later they are altered by the researchers) - note that suggestions for Material & Methods are available at the website.
- Preparation of complex macros/scripts for automation of processing & analysis
- Performing statistical tests on bioimage derived data
- Preparation of complex 3D renderings & animations/illustrations
- Processing, segmenting and analysis of large volumes of data
- Implementation of tools not common in facility or for which the staff are not trained

In these cases, the facility staff should be consulted before publication to make sure the methods/results are properly described and acknowledged. When co-authorship is warranted, the senior author should obtain the agreement of the imaging staff member regarding his/her contribution and the contents of the publication and be allowed to edit the relevant portions of the

work being submitted to publication. When deemed necessary, the Facility users' committee will be consulted to provide assistance in evaluating significance of intellectual contributions.

Payment of imaging services is always due, even if staff carried work that warrants co-authorship.

Data Management Plan & FAIR Bioimage Data guidelines

DMP requirements for H2020 funding explained here:
https://ec.europa.eu/research/participants/docs/h2020-funding-guide/cross-cutting-issues/open-access-data-management/data-management_en.htm

- Practical help preparing a DMP can be found here: https://ds-wizard.org/

Types and formats of bioimage data generated: This system acquires image datasets in the *.tif file format, with each acquired dataset stored in a separate folder with multiple *.tif files, one per channel, per optical slice, per time-point, and per position (order of file naming is XYCZT, ie, NOT the default im ImageJ-FIJI which is XYZCT!); a separate metadata file is also saved in *.html format. These files can be read and exported by the open-source software ImageJ/FIJI and the Bioformats plugin, confirmed > version 6.0 and also with Huygens and Imaris.

Expected size of datasets? A typical dataset is ~2 MB per channel, per Z slice, per time-point and per position at normal resolution sampling (12bit gray level sampling) and full field of view.

FAIR data? Learn more about principles of FAIR Bioimage data here: https://osf.io/fh47u/

Repositories (to make data "findable"): To make datasets available/deposited online, we suggest the following (some may require a pre-publication). DOIs are provided:

- IDR - Image data resource, https://idr.openmicroscopy.org/ (cells or tissues)
- Bioimage Archive - https://www.ebi.ac.uk/bioimage-archive/ (anything, new!)
- Zenodo - https://zenodo.org/ (any scientific data, even w/o publication).

Considerations before submitting: https://idr.openmicroscopy.org/about/submission.html

Compress datasets for local storage?

The only safe way to save "raw" data and the associated metadata is to use the original format of the equipment, in this case Leica's "LIF" format. In most cases ImageJ 's Bio-Formats can extract the metadata and allow saving as a text file. If "raw" datasets are not the final format for local storage, this procedure should be considered.

Where to find guidelines about storage of data and recording to the electronic lab book?