

Zeiss LSM 880 with Airyscan - Users' Guide

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Last Update - June 2020

Purpose: Zeiss LSM 880 is an inverted confocal laser scanning microscope with Airyscan, a new detector featuring a 32 channel GaAsP-PMT area detector. The Airyscan detector brings improvements in spatial resolution and signal-to-noise ratio in comparison with conventional confocal microscopy. This allows resolving structures up to 140 nm (XY) and 350 nm (Z) even in thick samples. The fast module for the Airyscan offers a compromise between perfect optical sections and live cell imaging experiments. The Fast Airyscan mode reduces photo-bleaching and allows acquisition speeds of up to 19 frames per second at 512 x 512-pixel image resolution.

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Safety instructions and contacts

Safety instructions and first aid procedures

All lasers are connected via shielded fibres to their respective microscope systems. Lasers are activated by key switches and via software control. Beam paths are shielded except for a fixed open beam between objective and specimen. All systems are equipped with remote safety interlocks and integrated beam-splitters that prevent stray laser light if the user switches from confocal observation to eyepiece observation.

- Never look directly into the laser beam or a reflection of the beam.
- Never interfere with the beam path.
- Never change a specimen during scanning.
- Never change objectives during scanning.
- Never change filters during scanning.

Lens cleaning solution is toxic.

- Use gloves to avoid skin contact (if contact occurs wash thoroughly with water).
- Avoid spillages when cleaning objectives.

Specimens should be sealed before observation under the microscope and handled with care using the appropriate level of protection.

- Leaky or cracked samples must not be examined.
- Any spillages should be cleaned up immediately and the area swabbed with 95% alcohol.
- Glass slides should be disposed of in the contaminated sharps bin – broken fragments of glass should be brushed on to paper and disposed of in a similar fashion.

All biological samples and hazardous waste must be appropriately disposed of by the user. Potential risks associated with the waste material should be addressed by the user in conformity with ITQB-NOVA rules.



ITQB-NOVA – Bacterial Imaging Cluster



Contacts

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Equipment location, description and technical specifications

Location: Room 5.01 (Bacterial Imaging Cluster), 5th floor, Instituto de Tecnologia Química e Biológica António Xavier (ITQB NOVA)

Manufacturer: Zeiss

Model: Zeiss LSM 880

Year Installed: 2019

Equipment Specifications

- Axio Observer 7 SP Inverted Microscope Stand
- Motorized Stage 130 x 100 STEP
- Stage Controller XY STEP SMC 2009
- Z-Piezo WSB 500
- Motorized Condenser w/ six positions (w/ DIC)
- Definite Focus 2 w/ ACR (Automatic Component Recognition) motorized nosepiece
- Eyepieces: PL 10x/23 Br. Foc. (High eyepoint and Focusing)

Laser Lines

- Diode - 405 nm (maximum power: 30 mW)
- Multi-line Argon - 458/488/514 nm (maximum power: 25 mW)
- DPSS - 561 nm (maximum power: 20 mW)
- 633 nm (maximum power: 5 mW)

Incident Light

- Fluorescence (HXP 120 V illuminator (w/ 200 W mercury short-arc reflector lamp))

Detectors

- 2 Photo-Multiplier Tube (PMT)
- 1 Gallium Arsenide Phosphide (GaAsP)
- 1 Airyscan (32 GaAsP)
- 1 Transmitted Photo-Multiplier Tube (T-PMT)

Transmitted Light Techniques

- Differential interference contrast (DIC)
- Phase Contrast
- Bright Field

Potential Advanced Techniques

- Super-Resolution microscopy
- Photobleaching *
- FRAP *
- FRET *
- Spectral unmixing *
- Lambda scanning *
- FLIM (please contact Mariana Pinho, mgpinho@itqb.unl.pt)

* For instructions on how to use these techniques please refer to the Advanced User's Guide available at the [BIC website](#) and contact the responsible people.

Objectives

Magnification¹	10x	20x	63x
<i>ZEISS System</i>	EC Plan-NEOFLUAR	Plan-APOCHROMAT	Plan-APOCHROMAT
<i>Class</i>	Colour and Chromatic Correction	Colour and Chromatic Correction	Colour and Chromatic Correction
<i>Numerical Aperture (NA)²</i>	0.3	0.8	1.4
<i>Immersion</i>	Air	Air	Oil
<i>Free Working Distance (mm)</i>	5.2	0.55	0.19
<i>Cover Glass (mm)</i>	0.17	0.17	0.17
<i>Contrasting Methods</i>	-	Differential Interference Contrast (DIC II)	Differential Interference Contrast (DIC III)
<i>Lateral Resolution (XY) @525 nm (µm)</i>	0.7	0.2625	0.15
<i>Nyquist Sampling (XY) @525 nm (µm)³</i>	0.28	0.105	0.06
<i>Zoom to Nyquist Sampling (XY) @525 nm with 512x512 scan format</i>	Number of field/(magnification*zoom)	=	Nyquist sampling
<i>Axial Resolution (Z) @525 nm (µm)</i>	$\frac{0.735}{0.09}$	$\frac{1.4 \times 0.525 \times 1.338}{0.64}$	$\frac{1.4 \times 0.525 \times 1.515}{1.96}$
<i>Nyquist Sampling (Z) @525 nm (µm)³</i>	3.2667	0.6146	0.2273

¹Notice that the microscope is equipped with two Eyepieces: HC Plan S 10x/23 Br. M

² NA - Numerical Aperture. For more information, follow this [link](#).

³ $Nyquist\ Sampling = \frac{r_{lateral\ or\ axial}}{2.5}$

- Remember to choose the proper calibration if doing montages/size measurements.

- The pixel size in microns of your picture is saved automatically (if you've chosen the correct calibration). Use [LOCI](#) to recognize it with ImageJ.

- The pixel size in microns of your picture is saved automatically when using Zen. Use [Bio-formats](#) to recognize it with ImageJ/Fiji (already installed in the Fiji package).

Filter-cubes for Eye-piece visualization (Locate Tab)

Filter Cube	Excitation Filter	Dichromatic Mirror	Emission Filter	Emission Colour
DAPI	G 365	395	BP 445/50	Blue
GFP	BP 470/40	495	BP 525/50	Green
RFP	BP 565/30	585	BP 620/60	Red

BP – Band Pass filter

Confocal Main Beam Splitter (MBS) Dichroic Mirrors

Visible Light

Position	Wavelength(s)	Example Fluorophore	Notes
1	458	CFP	
2	458/514	CFP/YFP	
3	458/561	CFP/RFP	
4	None	-	-
5	488	GFP	
6	488/561	GFP/RFP	
7	None	-	-
8	488/561/633	GFP/RFP/Cy5	
9	T80/R20	-	For confocal reflection microscopy
10	Plate	-	Use when not using Visible Light lasers

Invisible Light

Position	Wavelength(s)	Example Fluorophore	Notes
1	Plate	-	Use when not using invisible Light lasers
2	None	-	-
3	405	DAPI	
4	445	-	No corresponding Laser
5	440/532	-	Used for FLIM system
6	None	-	-
7	None	-	-
8	None	-	-
9	T80/R20	-	For confocal reflection microscopy
10	None	-	-

Emission Filters for Airyscan Modes

Position	Wavelengths	Emission Colors	Optimal Fluorophores
2	BP 420-480 + BP 495-550	Blue + Green to Yellow	DAPI + GFP, FITC, YFP
3	BP 420-480 + BP 495-620	Blue + Green to Red	DAPI + GFP, FITC, YFP, Rhodamine, RFP
4	BP 465-505 + LP 525	Cyan + From Yellow	CFP + YFP, Rhodamine, Cy5
5	BP 495-550 + LP 570	Green to Yellow + From Red	GFP, FITC, YFP + RFP, Cy5
6	BP 570-620 + LP 645	Red + Far Red	RFP + Cy5

BP – Band Pass filter

LP – Long Pass filter

Additional Components Specifications

Computer

- Type: Desktop
- Brand: HP®
- Model: HP 840
- Features: 2x Intel® Xeon® E5-2623 v3 3.0 GHz (quad-core); 64GB DDR4-2133 MHz ECC RAM (12 free slots); 4x 2TB 4 TB RAID 10; NVIDIA Quadro K2200 4GB
- OS: Windows 7 Ultimate Embedded x64
- Available Software: **Zeiss Zen 2.3**

Monitor

- Brand: HP®
- Model: DreamColor Z32x
- Diagonal: 32”
- Native Resolution: 4K UHD (3840 x 2160 @ 60 Hz)

Zen 2.3 (black edition)

- The ZEN 2.3 (black edition) can be equipped with several modules, applications and licenses. This guideline is strictly specific for the **ZEN 2.3 (black edition)** version available on the microscope's computer.

Suggestion for description in “Materials and Methods”

Confocal Z-series stacks (step size 200 nm) were acquired on a Zeiss LSM 880 point scanning confocal microscope using the Airyscan detector, a 63x Plan-Apochromat 1.4NA DIC oil immersion objective (Zeiss) and the 405 nm, 488 nm and 561 nm laser lines. The Zeiss Zen 2.3 (black edition) software was used to control the microscope, adjust spectral detection for the emission of 4',6-diamidino-2-phenylindole (DAPI), Green Fluorescent Protein (GFP) and tetramethylrhodamine (TMR) SNAP-tag fluorochromes and for processing of the Airyscan raw images.

Confocal Z-series stacks and tile regions with multiple positions were acquired on a Zeiss LSM 880 point scanning confocal microscope using photomultiplier tube detectors (PMTs) and a gallium arsenide phosphide (GaAsP) detector, a 63x Plan-Apochromat 1.4NA DIC oil immersion objective (Zeiss) and the 405 nm, 488 nm and 561 nm laser lines. The Zeiss Zen 2.3 (black edition) software was used to control the microscope and adjust spectral detection for the emission of 4',6-diamidino-2-phenylindole (DAPI), Green Fluorescent Protein (GFP) and tetramethylrhodamine (TMR) SNAP-tag fluorochromes.

Images were acquired on a Zeiss LSM 880 point scanning confocal microscope controlled with the Zeiss Zen 2.3 (black edition) software, using a 63x Plan-Apochromat 1.4NA DIC oil immersion objective (Zeiss), the fluorescence filter sets GFP + TX2 and differential interference contrast (DIC) optics.

Information to be added to the “acknowledgements” section

This work was partially supported by PPBI - Portuguese Platform of Biolmaging (PPBI-POCI-01-0145-FEDER-022122) co-funded by national funds from OE - "Orçamento de Estado" and by european funds from FEDER - "Fundo Europeu de Desenvolvimento Regional".

User pre-requisites, booking and general rules

- The equipment cannot be used without official training.
- Before your first use contact Mariana Ferreira to schedule training.
- New users must have a clear understanding of the principles of light microscopy, how fluorescence and more specifically confocal microscopy work.
- This equipment operates on class III lasers
- Save all data into your own storage device as we routinely clean the computer's data
- Register your utilization in the logbook
- Airyscan raw data reconstruction must be done in the microscope computer (book slots accordingly). For all downstream image analysis image analysis a free software license is available for ZEN lite at ZEISS [website](#). Alternatively, you can use [FIJI](#).
- Contact the responsible people in case of any doubt or any issue with the equipment.

Booking is done online at the [ITQB intranet](#). Please mind the following rules when booking the microscope:

- Priority booking of afternoon slots is given to users with live cell experiments that require growth in the same day. If you have fixed or time insensitive samples, please try to book other times;
- Maximum 2 slots per user per week (one slot = one morning or one afternoon);
- These rules are applicable until the previous Friday. If you need more than two slots in a week and they are still available on the previous Friday, at 4pm, feel free to book them.

Instrument startup procedure

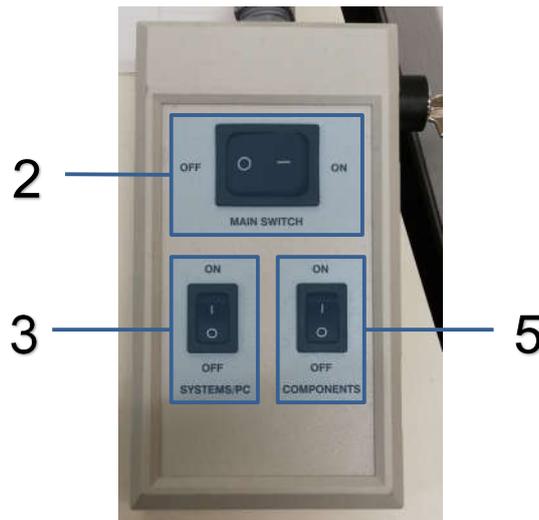
System Overview



- | | |
|---------------------|--------------------|
| 1- Confocal PC | 7- FLIM PC |
| 2- Confocal Monitor | 8- FLIM Monitor |
| 3- Microscope Body | 9- FLIM Laser |
| 4- Confocal Module | 10- FLIM Detectors |
| 5- Airyscan Module | |
| 6- Confocal Lasers | |

Step by step startup

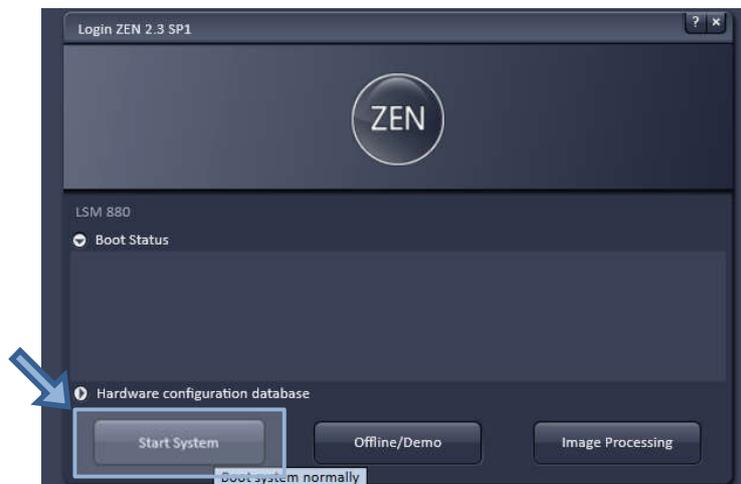
1. Take the dust cover off;
2. Turn on the **Main Switch** and wait a few seconds (button 2);
3. Turn on the **Systems/PC Switch** (button 3);
4. Start up the **Computer** and wait until you see the Windows start up screen;
5. Turn on the **Components Switch** (button 5);



6. Wait for everything to finish initializing;
7. Open **Zen Black** on the computer;



8. Choose **Start System** and wait for the initialization to finish.



In case of any errors follow the shutdown procedure and then start it back up.
If this does not fix the errors, please contact the people responsible for the equipment!

Advanced Techniques

This guide is for advanced users who require functions beyond those expanded upon the Basic Users' Guide for this equipment available at the [BIC website](#). If you intend to use any of the techniques in this guide, please contact the responsible people beforehand for support.

Bleaching

Fluorophore bleaching is the permanent loss of the ability to fluoresce due to photon-induced damage and alterations. This concept is the basis for many experiments such as FRAP (Fluorescence Recovery After Photobleaching) and Acceptor Bleaching FRET (Förster Resonance Energy Transfer).

This is one of the default multidimensional acquisition options available on this equipment, when selected it will also activate the required Regions and Time Series functions.

Bleaching Tool

- **Start bleaching after # scans** – How many images to acquire before the bleaching step. Recommend at least 3 to have a good idea of original fluorescence intensity.
- **Repeat Bleach after # scans** – For repetitive bleaching experiments choose how many images to acquire before repeating the bleaching step.
- **Iterations** – How many times should the laser pass on the ROI during the bleach step.
- **Different scan speed** - Allows the use of a different scan speed from the acquisition scan speed for the bleach step increasing its effectiveness. Slower scan speeds can use fewer iterations and vice-versa.
- **Safe bleach for GaAsP** – Select when using the GaAsP detector to protect it from excessive fluorescence intensity.
- **Different Z position** – Allows you to choose a different Z position for the bleach.
- **Zoom Bleach** – Faster bleach step but the bleached ROI is less accurate.
- **Different settings for different ROIs** – This will allow the use of different lasers to bleach different ROIs in the same bleach step.
- **Laser** – Select the laser line for the bleach step and the laser power should be set to 100%.



Regions Tool

Use the Regions tool to select one or multiple ROI (Region of Interest) in the scan area to bleach and/or analyze. For example, select multiple nuclei some for the bleaching experiments and others to record the natural bleaching caused by the scanning process.

Time Series

The Time Series tab will control how many images will be acquired in total and the acquisition interval between each image. This needs to be set to at a minimum of 3 for successful before and after bleach images, but higher numbers are recommended.

FRAP

To perform Fluorescence Recuperation After Photobleaching (FRAP) assays on our system you must adjust the Bleaching setting to acquire enough before and after pictures to allow observation of the phenomenon. FRAP time is very variable, it can take from a few milliseconds to minutes or even hours, so it is very important to adjust your settings accordingly. Data from the experiments can then be analyzed using external software such as ImageJ/FIJI.

Acceptor Bleaching FRET

Förster Resonance Energy Transfer (FRET) is a mechanism describing energy transfer between two light sensitive molecules (chromophore). Therefore, a donor chromophore in its excited state may transfer energy to a close enough acceptor chromophore with the efficiency being inversely proportional to the distance between them.

In biology FRET measurements are utilized to determine if two fluorophores are within a certain distance of each other by exciting the donor fluorophore whose photon emissions then should excite the acceptor fluorophore so the fluorescent signal detected is the acceptor's.

Acceptor Bleaching FRET involves bleaching the acceptor fluorophores to increase the donor fluorophore emission.

Lambda Mode Acquisition

The Lambda mode is chosen for imaging heavily overlapping emission signals. When choosing this mode by activating the Lambda mode button display changes to show the currently active laser line(s) and the detection range of the detector. With this imaging mode, it is possible to acquire an intensity image displaying the intensity of the fluorochrome(s) within a spectral bandwidth of 10 nm. According to the detection range that is set for the detector, a specific number of such images is acquired which is called a Lambda stack. These image data provide the intensity information within the selected detection range for each pixel. Therefore, the data allow deducing an emission spectrum for each pixel corresponding to the emission spectrum of a specific dye. This calculation is done by linear unmixing (see section Unmixing View) using the acquired Lambda stack and allows to clearly separate even heavily overlapping emission signals. The spectral resolution is set in the Range column.

Online Fingerprinting

The imaging mode Online Fingerprinting is based on the acquisition of a Lambda stack. It requires using already available emission spectra from the dyes which are used as markers in the imaged specimen. The choice of the emission spectra and the setting of the detection range for the QUASAR detector are defined in the loaded configuration or using the Imaging Setup tool. Within the Use column the spectra of the dyes for unmixing are chosen /added. The detection range is equal for all dyes. When changing it for one dye it will be changed for all other dyes.

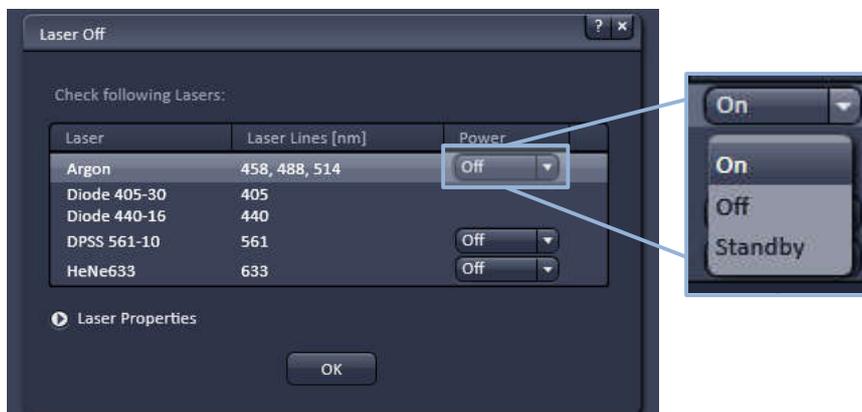
All other tools are controlled as described for the Channels mode. For best results, the settings for Online Fingerprinting should match the settings of the Lambda Mode when the dyes were acquired for defining the reference spectra.

Linear Unmixing

The Linear Unmixing processing tool permits extracting the emission of single fluorescence dyes (e.g. GFP only, YFP only etc.) from strongly overlapping multi-fluorescence data acquired in multi-channel images and so-called "Lambda stacks" (only available in LSM imaging mode). In brief, with the knowledge of the spectral characteristic of individual components within a multi-component sample, even heavily overlapping individual spectral characteristics can be mathematically extracted from an experimental multi-channel data. This method is a strictly pixel by-pixel image analysis procedure. Experimentally, fluorescence spectra of mono-labeled samples are acquired and stored in the Spectra Database as an external reference. Then a multi-channel image or Lambda stack (available only in LSM imaging mode) from the multi-labeled sample is acquired. Finally, the individual components are mathematically extracted using the information from the reference spectra. Up to ten different reference signals can be fed into the least-square-fit based algorithm to produce a 10-channel multi-fluorescence stack without any partial overlap between the channels.

Shutdown procedure

1. Save your images in Users folder in the D drive;
2. Close Zen Black;
3. A window to save any unsaved images/profiles should appear if there are any, **DO NOT overwrite other users' experiment profiles!**
4. A Laser Off window appear:
 - a. If someone is using the system in the next **4 hours** leave the Argon laser in **Standby** mode and turn all other lasers off;
 - b. Otherwise turn all lasers **Off**.



5. If turned **Off**, it will take the **Argon Laser** at least **5 minutes** to cool (fans turn off);
6. Shutdown the computer and then wait for the **Argon Laser** fans to turn off;
7. **AFTER** the Argon Laser cools down:
 - a. Turn off the **Components Switch**;
 - b. Turn off the **Systems/PC Switch**;
 - c. Turn off the **Main Switch**;
8. Clean the objective you used
9. Put the dust cover back on.
10. Take out everything you brought (pipettes, slides, etc...)

ATTENTION

Turning the switches off before the Argon Laser module is done cooling can damage the laser!