Instruction LSM 880 2024-03-25 all.docx 2024-03-25

Confocal LSM 880

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Introduction

The Zeiss LSM 880 with Airyscan is a new generation confocal instrument for more sensitivity, faster linear scanning and super-resolution (x1.7 improvement) imaging.

The system features a cooled GaAsP detector and two detectors that complement the spectral working range enabling flexible imaging strategies, including molecular dynamics, recording fluorescence at single photon signal levels and sequential spectral acquisition followed by unmiximg of overlapped emission signals.

With the Airyscan detector the system achieves resolution enhancement by use of a radial array of 32 GaAsp detectors which utilizes light that otherwise would be rejected by the confocal pinhole, increasing signal to noise ratio by 4 to 8 times which is utilized to further speed up scanning, get higher sensitivity or increase resolution in all 3 dimensions.

Hardware

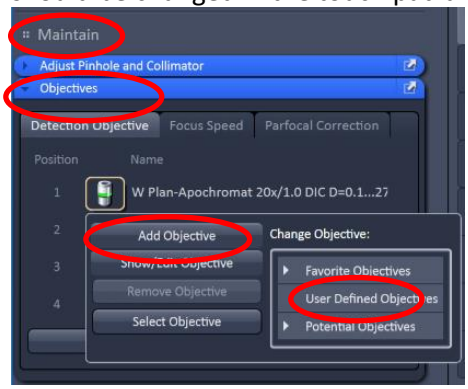
The microscope:

The LSM 880 is attached to an upright fixed stage microscope (Axio Examiner Z1). The stage carrier can achieve total clearance of 80mm for experimentation on small animals. The stage can move in the XY direction with the help of a joystick. Toggle [F1] for high and low speed.

Objectives:

Position	Objective lens	Magnification	NA	coverslip	Working distance	Immersion	Parfocal length
2 (option)	Plan Apochromat	x10	0.45	0.17 mm	2 mm	Air	45 mm
2	LD LCI Plan Apochromat	X25	0.8	0.17 mm	0.55 mm	Oil/Glycerin/Water	45 mm
3	Plan Apochromat	x20	0.8	0.17 mm	0.55 mm	Air	45 mm
1 (bombila)	W Plan Apochromat	x20	1.0	0	1.8 mm	Water dipping	75 mm
4+ adaptor	W Plan Apochromat	x40	1.0	0	2.5 mm	Water dipping	45 mm
4+ adaptor	Plan Apochromat	x63	1.4	0.17	0.19 mm	Oil	45 mm

Only BCF staff are allowed to replace objectives. In case of objective replacement the objective setting should be changed in the touch pad and in the software.



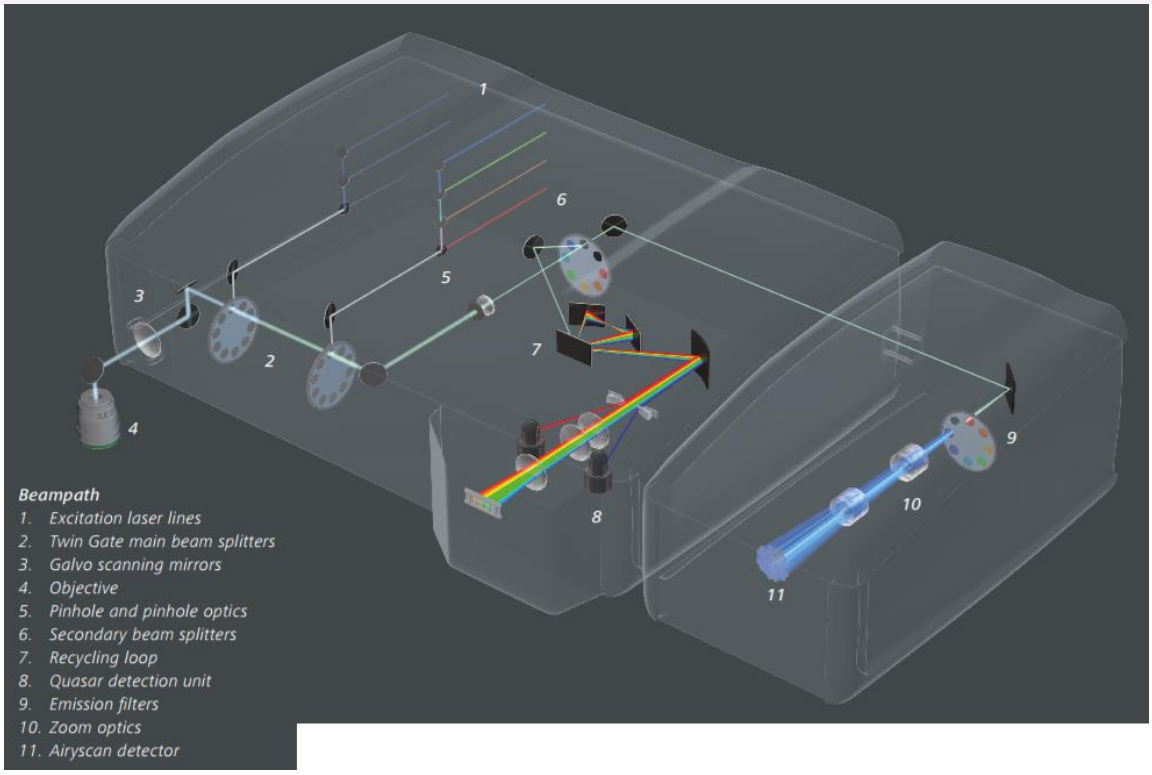
Filter turret

Position	Filter set	Excitation filter	Beam splitter	Emission filter	Fluorophore family
1	None (laser path)				
2	FS38	BP 470/40	FT 495	BP 525/50	Cy2,GFP
3	FS43	BP 545/25	FT 570	BP 605/70	Cy3,Al555
4	FS49	G 365	FT 395	BP 445/50	DAPI,BFP
5	DIC				

Laser lines

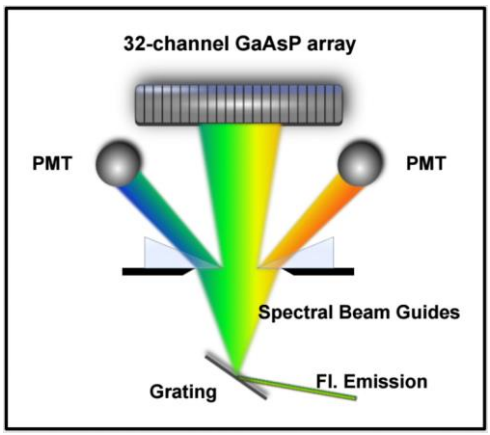
Laser	Line
HeNe 633	633
DPSS 561-10	561
Multiline Argon	458, 488, 514
Diode 405-30	405

Light Path

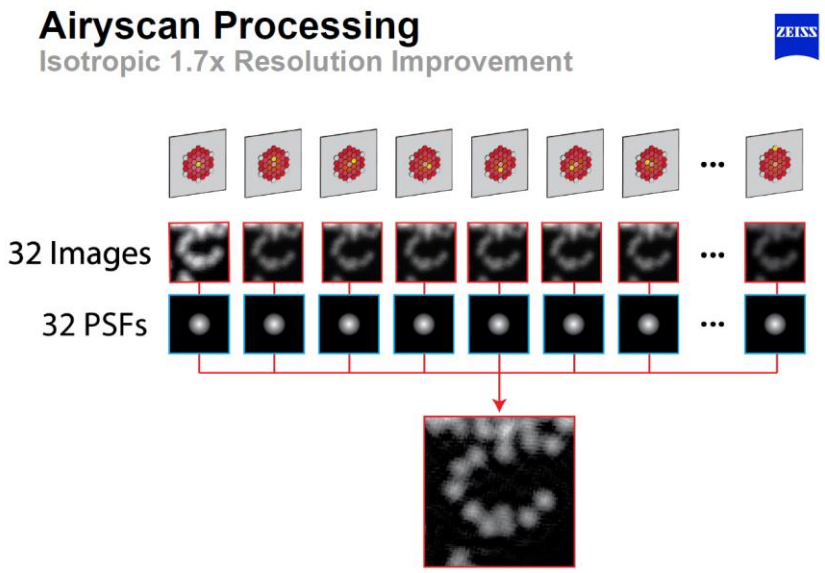


1. The lasers come aligned from the manufacturer; all 4 lasers are attached to an optic cable to enter the confocal scan head (1). The lasers are divided to two main groups: the visible lines (multi Argon, 633nm, 561nm) and the invisible line (405nm); each group has its own path. The 405 laser line is directly controlled by voltage and has a collimator which helps to adjust the beam focus to bring it to the same position with the visible lines. The visible lines are controlled by AOTF.
2. Each group of lasers has a main dichroic mirror wheel, selected in the software. The lasers hit the mirror at an angle of 10° which helps to get more "clean" rays. Note that the chosen mirror has to be suitable to the laser line (all laser light rays are reflected to the specimen).
3. In this unique scanning mirror model the scanning mirror galvanometers (galvos) (3) are cooled by a special cooling liquid. Cooling down the galvos allows more rapid scanning without damage by heating because of movement.
4. The light is focused on a specific focal plane by the objective (4). The emission from the specimen passes through the objective and is directed to the main dichroic mirrors (2) which transmit only the emission light and reflect all laser rays.
5. The light is directed to the confocal pinhole (5); only light from the lens focal plan will pass through the pinhole. In addition, the pinhole aperture has a critical role in the resolution of the system. Closing the pinhole will narrow the detection PSF and contribute to a signal with higher localization precision. The smaller the pinhole becomes, the higher the resolution will be (which scales linearly with the pinhole diameter). However, gain in resolution by closing the pinhole is accompanied by reduction in detection efficiencies, which scale with the pinhole area, and result in images with poor signal-to-noise ratios (SNR).
6. The second filter\mirror wheel is a junction between the confocal and the Airyscan (6). For confocal a mirror should be selected in the software, for Airyscan a plate should be selected in the software or a filter can be selected which divides the light between the confocal and the Airyscan.
7. The light is dispersed by a diffraction grating and reaches the Quasar detection unit (8).
8. The Quasar detection unit is based on a filter-free system that guides the desired wavelength range to the target detector using adjustable optical wedges and slider light stops. The detector

operates such that the tips of the wedges serve as one detection border while the light stop sliders act as the other. By configuring these elements to create custom spectra, any longpass, bandpass, or shortpass filter strategy can be achieved without the need for traditional dichromatic mirrors or emission filters. In the system there are 3 detectors:
Ch1 detector - a Multi-Anode Photo-Multiplier tube (MA-PMT) mostly used for the Blue channels (DAPI…)
Ch2 detector - an MA-PMT mostly used for the NIR channel (Cy5…)
ChS detector – a 32 channel GaAsP PMT used for green-red and for spectral applications.

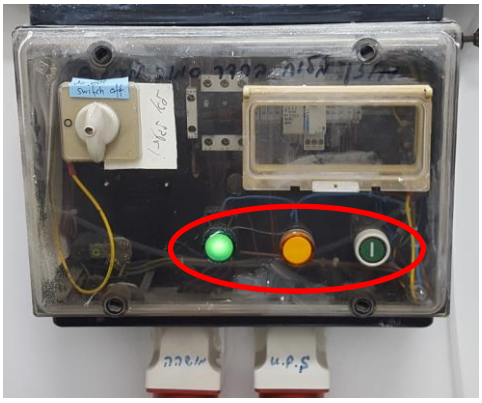


9. **The Airyscan unit** - by inserting a plate in the second filter wheel (6) the light will reach the Airyscan unit. In this scanning mode the pinhole (5) (in most cases) will lose its role (will be twice as big as the detection Airy unit). The light will pass a third filter wheel (these filters are double emission filters).
   The light is projected to the Airyscan detector by adaptive zoom optics (2 elements) (10).
   The light is detected by a special GaAsP detector which is an array of 32 hexagonal elements (11).
   With this special light path arrangement there is no light discrimination by the pinhole which leads to better SNR, the zoom optics image up to 4 Airy units (AU) on the detector diameter as the diameter is composed of a maximum of 6 detectors. The improvement in the resolution is a combination of several techniques:
   - Each hexagonal element is equal to 0.2AU which gives better resolution then 1AU collection. (the total detection area is equivalent to a 1.25AU pinhole setting)
   - Except of the central hexagonal detector each of the external circuit hexagonal detectors is displaced in regard to the optical axis or the illumination beam. The displacement leads to narrowing of the effective PSF. As the overlap decreases with larger displacements, the width of the resulting Airy disk gets even slightly narrower. You see an emitter being excited with a displaced pinhole, the highest probability of its location is within the narrow overlap between the illumination and detection PSFs and consequently it can be localized with higher precision.
     Further resolution gain is achieved by the use of a Wiener filter-based deconvolution step during image reconstruction.



Start up

- Make sure the room temperature is 22-24°C **(turn on the Air Condition)**
- Make sure the green light is on in the main power supply on the wall; if there is no light (after electricity failure only) wait until the orange light appears and push the green button.



- In the main switch box turn on the following switches 1 → 2 → 3 **(do not touch the key)**

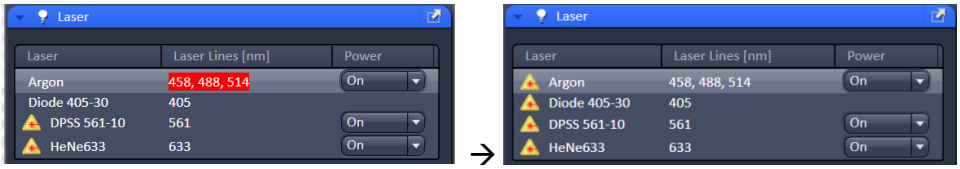
- Turn on the computer and log in
  User name: **multilabs**
  Password: **123456**

Software

- Log into **BookitLab** and activate your reservation to start **Black Zen 2.3** 

- To acquire images click **Start System**

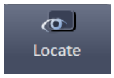
- In the **Acquisition** tab → **Laser** tab
  Switch on the lasers you need for your experiment; the Argon laser should be turned on first on Standby and then ON. Before scanning you will have to wait 5' minutes until the laser stabilizes, its status should be **ready** and power **optimal** in the laser properties section.



Sample mounting and viewing

Insert the slide or dish in the stage holder.

Manually choose the proper objective, and rotate the objective knob until the objective is fully lowered. If there is no objective perfectly aligned when you open the system (switch 2), the objectives will not be recognized by the microscope.

In the software go back to **Locate Main Tool Tab**. 

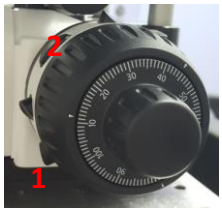
On the microscope's right side VIS will be selected automatically (or you can change the shutter position manually) 

Choose the proper filter set and open the TL\RL shutter:

- **Via the microscope**:

  Focus wheel buttons:

Position	Back buttons (1)	Flat button (2)	Front buttons (3)
Left	Turret filter scroll	RL shutter	(unassigned)
Right	Turret filter scroll	TL shutter	Halogen brightness



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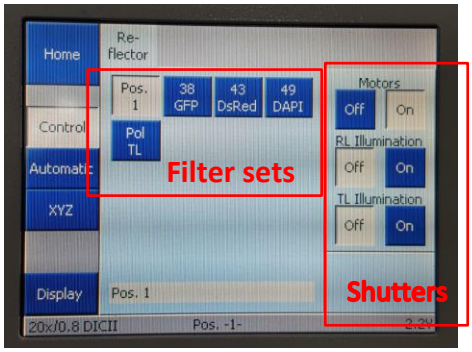
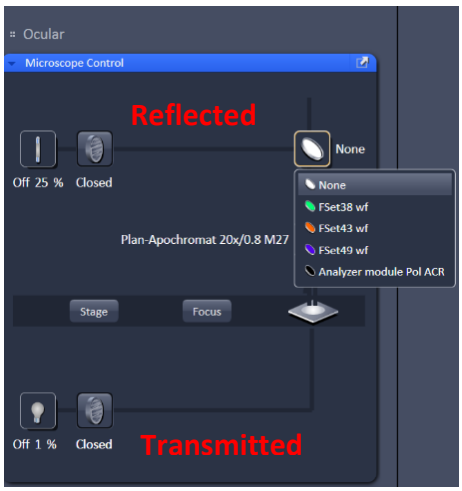
- **Via the touch pad**:

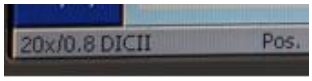
  Select the turret filter and open the appropriate shutter (TL/ RL)



- **Via the software**:
  Select a filter set from the list and switch on the Reflected or Transmitted light shutter in the light path schema

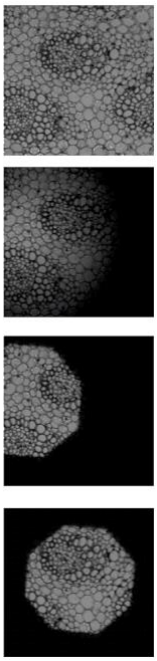


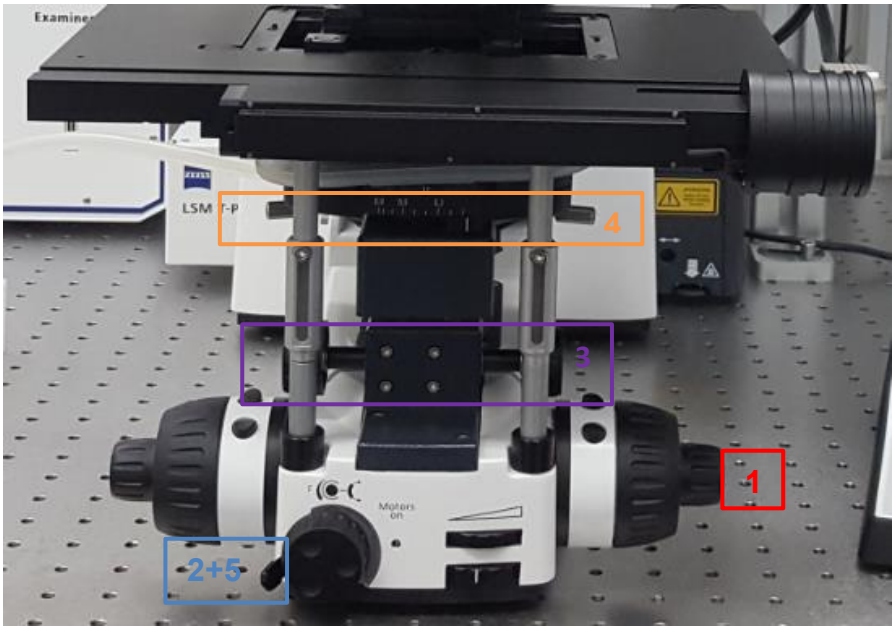
- To set the Transmitted light:
  Click the transmitted light lamp icon, click ON and set desired brightness
  (or via the microscope).
  Set the filter turret position to DIC and the condenser below the stage to II or III according to the objective lens (see objective lens info on touchpad).



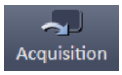
If you acquire transmitted light you will have to set up Kohler illumination:

1. Bring the sample into focus.
2. Close the field diaphragm until you can see at least one edge.
3. Adjust the condenser height until the edges of the diaphragm image are crisp.
4. Center the diaphragm image using the two centering screws.
5. Open the field diaphragm, just until the image fills the field of view.

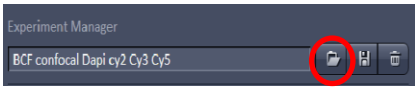


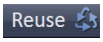


LSM Channel mode confocal settings

- **Configuration**:
  - In the software go to **Acquisition Main Tool Tab** 

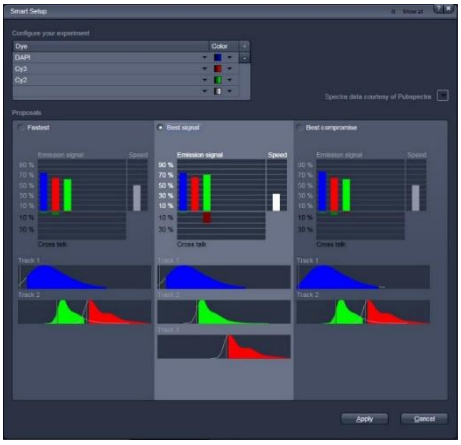
.
  - Choose one:
    - Load a saved configuration from the **Experiment Manager**

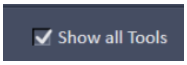


    - Reuse from image: open an image and below the image click 

    - **New setup**: choose 

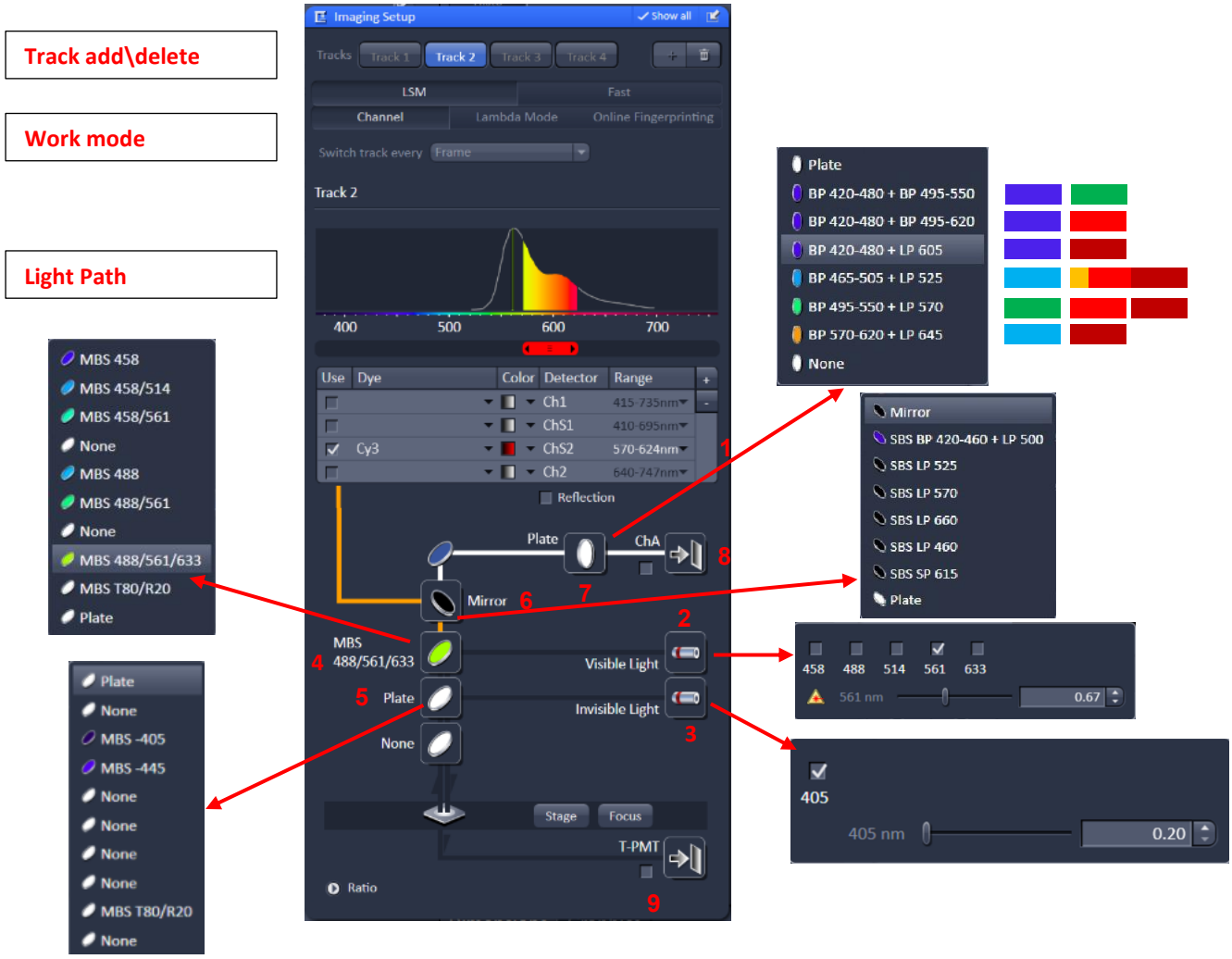
, choose the desired dyes from the list and choose the Best Signal and click **Apply**



  - Select **show all tool** 

Imaging Setup Tab

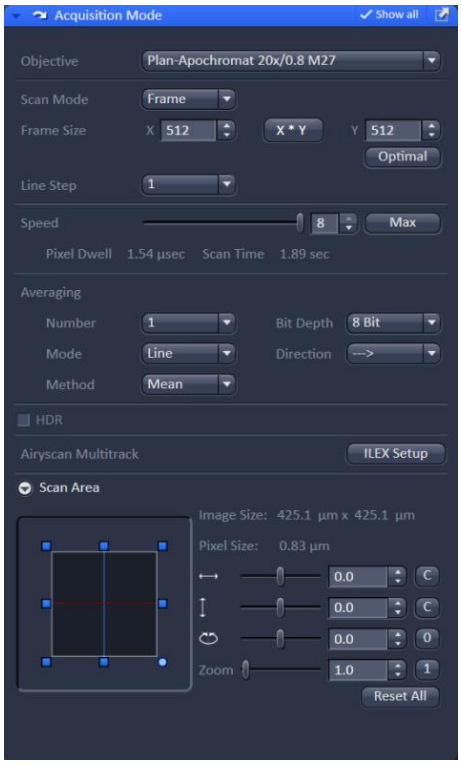
Note: make sure that the chosen configuration is correct and suits the fluorophores in your experiment, make changes if needed; it is highly recommended to check all fluorophore spectra in a Spectra viewer e.g., [ThermoFisher] or [Chroma]



1- PMT selection & emission range
2- Visible laser lines
3- Invisible laser line
4- Visible laser dichroic mirror filter wheel
5- Invisible laser dichroic mirror filter wheel
6- Filter wheel between confocal and Airyscan
7- Airyscan filter wheel
8- Airy scan detector (activation)
9- T-PMT activation to collect the transmitted light image

Parameter setting:

Acquisition Mode Tab



- **Scan mode**: frame (in most cases); line scan mode can be useful for physiology experiments.
- **Frame size**: 512 X 512 for setting parameters, press Optimal for acquisition
- **Speed** 8 (or more) for setting parameters, usually 6-7 for acquisition
- **Averaging**:

  Method: **Mean** (in most cases); in case of low signal with low noise **Sum** method may be useful

  Number: 1 during parameter settings, higher number in actual acquisition

  Mode: **Frame** for fixed sample, for fast moving live cells and in case of unstable dye **Line** mode will be more suitable

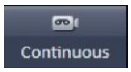
  Bit Depth: defines the dynamic range of the image, choose **8bit/12 bit** according to whether you will need image analysis or not.

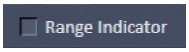
  Direction: **Meander** scanning is fine and faster in most cases.
- **Scan area**: slight nudge, zoom and rotation of the field of view

Channel Tab

- To set the correct parameters for your experiment, in **Acquisition** tab:

  Frame size: 512 x 512
  Scanning speed: 8
  Averaging number: 1

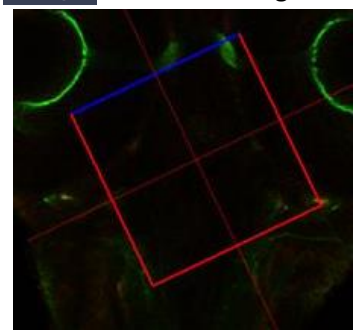
- Check and select only the shortest wavelength track; press "**1AU**" and start scanning in continuous mode 

- Check Range indicator 

(below the image in the **Dimensions** tab).
- Set your image parameters to avoid saturation (saturation=red pixels in the image) and the correct black level (black= blue pixels in the image)
  - Set the two MA-PMT gains to 500-700
    Set the GaAsP gain between 700-850
  - Set the Laser %; it is recommended to use the lowest % as possible and to increase gain
  - In case of low signal with high laser and high gain you can increase Digital Gain (avoid digital gain more than 2).
  - Set the offset level until you reach the PMT threshold (blue pixels will appear in the image while scanning in range indicator mode).
- Stop the scan
- For each additional track:
  - Check and select track;
  - Set pinhole to the same section (µm) as the first track
  - Set its parameters as above

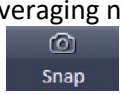
Single image acquisition

- In **Acquisition mode** tab select the appropriate Zoom or crop and zoom in via the **crop tool** Crop below the image in the Dimensions tab

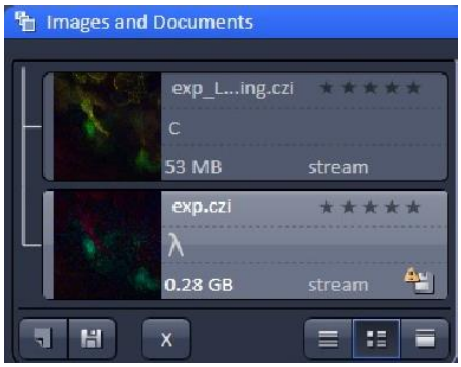


- Check all tracks in the **Channel** tab, select the shortest wavelength track and scan in continuous mode one frame to confirm field of view.
- In the **Acquisition mode** tab:

  Frame size: press optimal
  Scanning speed: 6
  Averaging number: 2-4 (according to the noise in the image)

- press Snap 

  Save the image – press  in the **Images and Documents** tab at the right of the software window and save in the directory: **D:/Users' data/PI/MM-YYYY/User/YYYY-MM-DD**



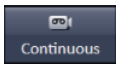
Multidimensional acquisition

Z stack

- In the **multidimensional acquisition panel** below the start buttons check **Z stack**: a new tab will appear under the **multidimensional acquisition** section.
- Press First/Last
- In the **Acquisition mode** tab:

  Frame size: 512 X 512

Scanning speed: 8 or more
Averaging number: 1

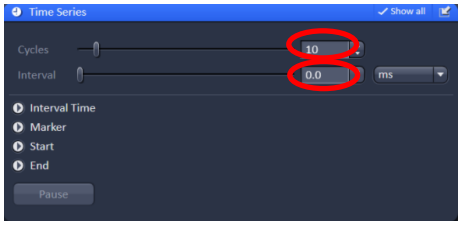
- With one channel checked, press 

to scan
- Define the signal boundaries by focusing at one boundary of the stack and pressing **Set First** and focusing towards the second boundary and pressing **Set Last**.
- Set interval to Optimal (according to the Nyquist criterion)

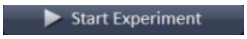
- In the **Acquisition mode** tab:
  Frame size: Press optimal
  Scanning speed: 7
  Averaging number: 2 or 4
- In **Imaging Setup** tab:
  Set Switch track every every Z-stack (which is faster)
- Press ▶ Start Experiment
- Save the Image as soon as it is acquired
  Note: there are more advanced Z stack options in this tab, such as brightness correction over Z in case of thick specimens.

Time series

- In the **multidimensional acquisition** section below the start buttons check **Time series**; a new tab will appear under the **Multidimensional acquisition** section.
- Define number of cycles and interval
  Note: avoid high laser intensity**, especially** in case of live cell imaging



- In the **Acquisition mode** tab:
  Frame size: Press optimal
  In case of big frame size with long Scan Time it is recommended to increase the zoom and set a new frame size with less pixels by pressing optimal again after selecting all channels.
  Scanning speed: 7-8
  Averaging number: 1

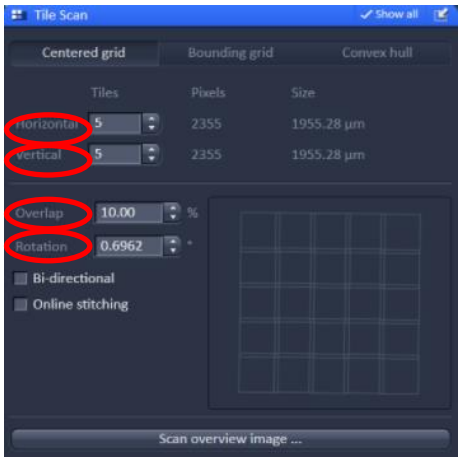
- Press 

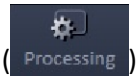
- Save the Image

Tile Scan

- In the multidimensional acquisition panel below the start buttons check **Tile scan**; a new tab will appear under the multidimensional section.
- Define the number of tiles
- Define the overlap % - recommended: 8%-15%
- Calibrate slide rotation: In the menus choose **Macros → Tile scan rotation**
  In the popup window press **Calibrate** and wait until the process is finished; press **close.**

The rotation angle will populate the relevant textbox at the tile window.



Note: Do not check **Online stitching**; stitching with options is configured at the Processing Main Tool Tab (

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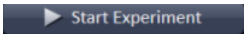
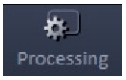
- In the **Acquisition mode** tab:

Frame size:	Press optimal
Zoom:	at least 1.3 zoom to reduce shading
Scanning speed:	7
Averaging number:	2

- Press 

- Save the Image
- **Stitching: Processing Main Tool Tab** 

→ Stitch → select your input image → press Apply.

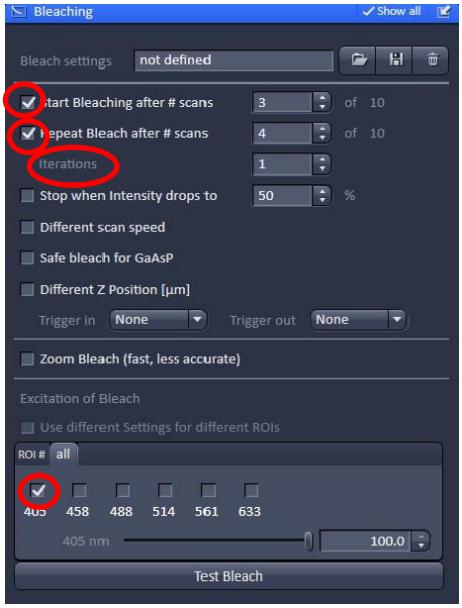
  In case of uneven illumination the stitching process can be done in ZEN blue (File → Send to Zen – Blue edition) with shading correction options.

How to set shading reference:

Acquire for each channel a time series of about 20 consecutive images of a field of view of the appropriate Chroma slide deeper than the surface at the same settings as the experiment.
In Zen Blue (not lite):
Processing → time series → gliding average → averaging length → 20 →set input → apply
Processing → Smooth → lowpass filter → input → kernel size big (X≈15 and Y≈15) → apply
Processing → Stitching →new output →set input → fuse tiles → shading → reference → set input 2 → apply

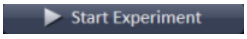
Regions and Bleaching

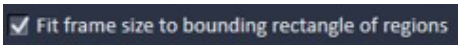
- In the multidimensional tab options check **Bleaching**; the **Bleaching**, **Time series** and **Regions** tabs will appear under the **multidimensional acquisition** section.
- In the **Bleaching** tab define:
  After how many scans to start bleaching
  After how many scans to repeat
  Number of iterations
  Define the bleaching laser and its intensity
  More settings can be defined in the dialog.



- **ROI**
  After a preliminary scan, in the **Regions** tab choose one of the ROI tools and draw on the acquired image.
  Define which process will be performed in the ROI: Acquisition/Bleach/Analysis.

Time series

- In the **Acquisition mode** tab:
  Frame size: Press optimal
  Scanning speed: 7 or more
  Averaging number: 1-2
- Press 

- Save the Image
- The intensity data from the **ROI** can be viewed in the **ROI mean** tab in the image window
  Note: ROIs can be defined in other types of experiments with or without time lapse and can also be used as a tool for zooming in: 

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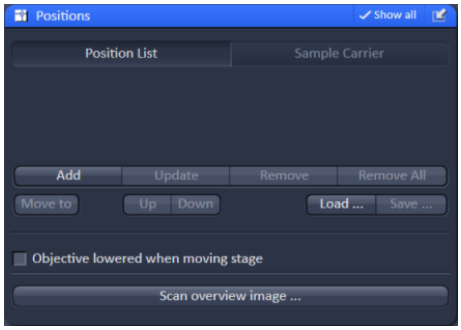
Positions

- A positions list can be added to the experiment in which all of the acquisitions settings will be acquires (channels, Z stack, tiles, and time series).
- The Positions option can be a useful tool in managing a large tile and for quick slide scanning (via the ocular) with small magnification before moving to high magnification with small field of view.

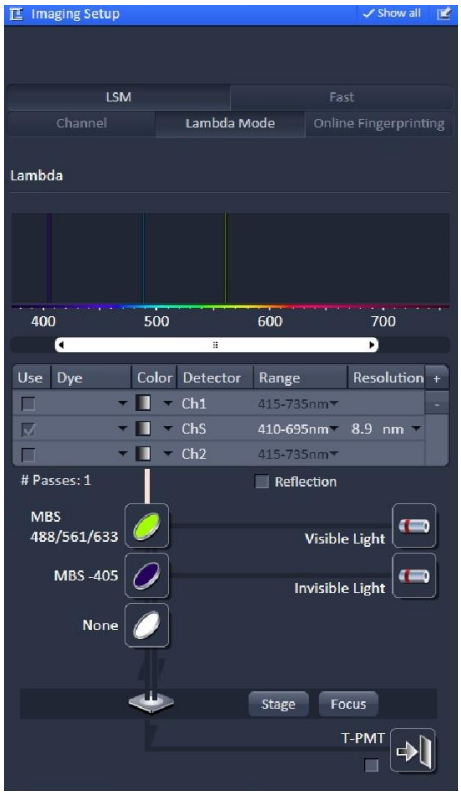


- Add a position at the current stage center by pressing **Add**
- Move to a position in the list by pressing **Move to**
- A position can also be added with the positions tool below the image in the **dimensions tab**: press on the **positions** button and click on a point in the image.

Note you can use multiple options in the multidimensional scanning for example Z stack & tile, time series, bleaching, ROI & Z stack, etc.

Lambda mode

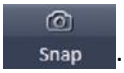
- **Configuration**
  - In the **Imaging setup** tab press **Lambda Mode.**
  - Choose the laser lines in the expected spectrum of your experiment.
  - Choose the dichroic mirrors that suit the laser lines.
  - Choose the entire emission spectrum of all dyes.
  - Define the resolution of the spectral GaAsP PMT – e.g., 8.9nm step size



- **Acquisition of lambda stack**
  - **In the Acquisition mode** tab set:

    Frame size: 512x512
    Scanning speed: 8 or more
    Averaging number: 1
  - Start scanning in **continuous** mode
  - In the **Channels** tab adjust the pinhole, laser intensity, gain and offset.
    Use the gallery view (vertical tab to the left of the image container) of the lambda image to balance the detector gain, offset, scan speed and laser powers to create a well-balanced image.
    It is important to eliminate any saturated and underexposed pixels in the image to ensure proper unmixing
    Select range indicator palette (below the image) to view underexposed pixels (in blue) and saturated pixels (in red). Adjust imaging parameters to rid image of blue and red pixels.
  - Acquire a single plane image:
  - In the Acquisition mode tab:

    Frame size: Press optimal
    Scanning speed: 6-7
    Averaging number: 2-4

    Press 

    .
    Save the image.

Lambda unmixing

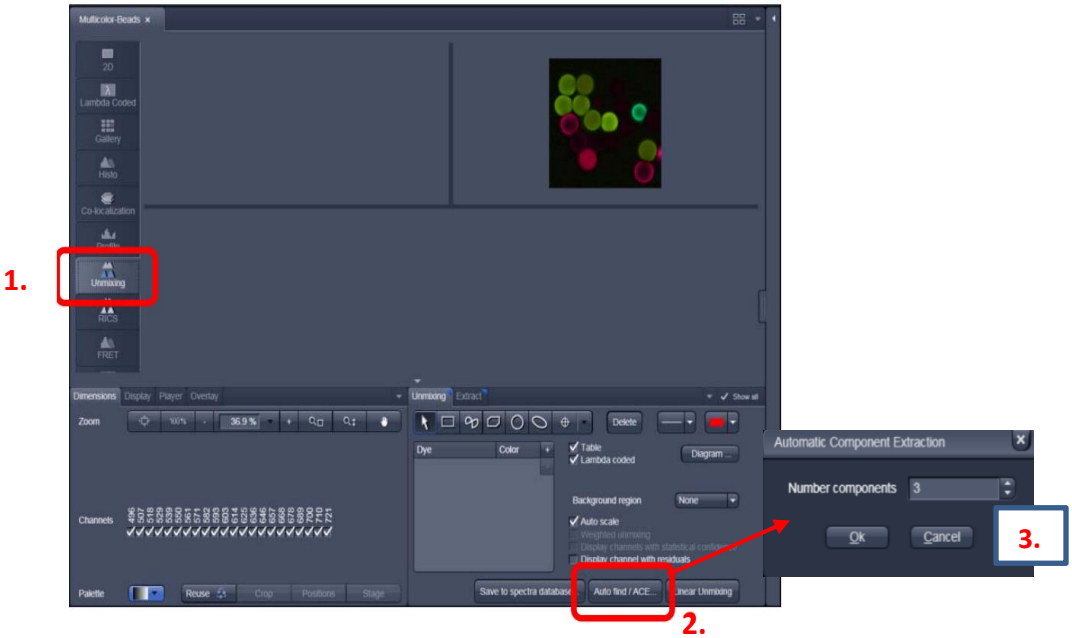
- Define Spectra to use for Unmixing
  When performing an unmixing experiment, a reference spectrum from each individual fluorophore is needed.
  There are two different ways to acquire the reference spectra:
  1. Automatic Component Extraction (ACE).
  2. Individual measurements with biological controls.

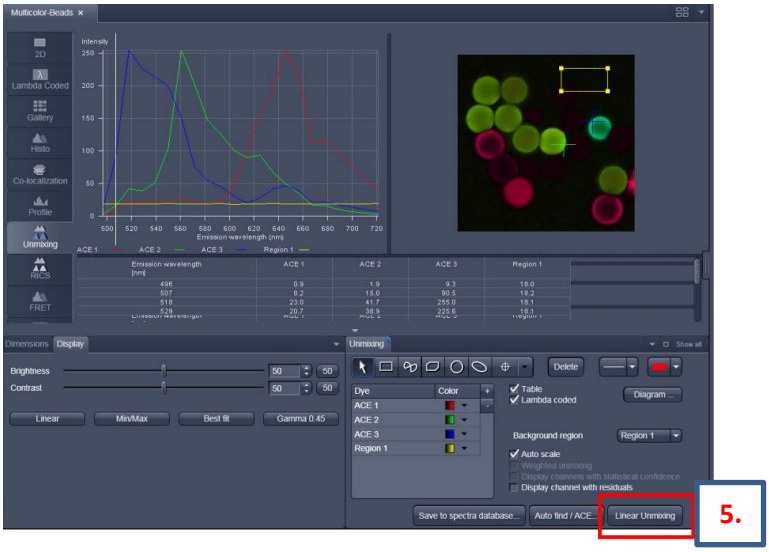
1. <u>Automatic Component Extraction (ACE)</u>

   The automatic component extraction function, ACE, will automatically try to detect the individual spectrum of each component in the image. This function will only work if each component is not completely co-localized with any other components (i.e., there are pixels in the image with only one fluorophore present).

   After the components have been automatically identified with ACE, the image may be unmixed.

   1. Go to the Unmixing vertical tab to the left of the image container
   2. Press the Auto find/ACE button
   3. Select number of components
   4. Identify background ROI using selection tools if needed.
   5. Unmix image





The resulting unmixed image will be created in a new image tab with the suffix "_unmixed". The unmixed Image contains a channel for each component with a reference spectrum

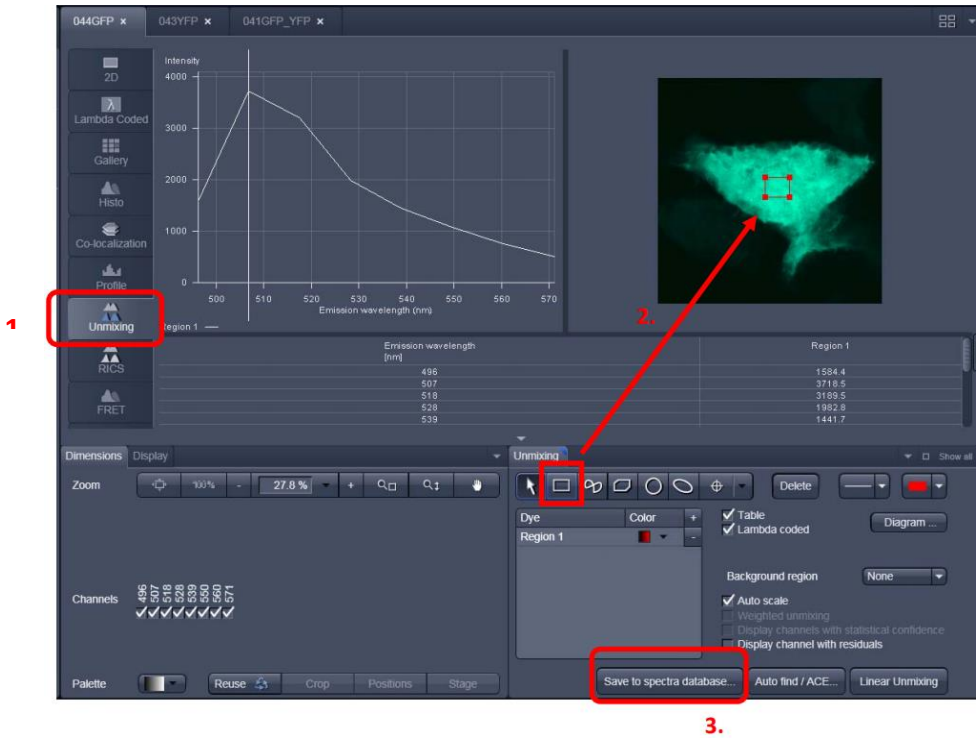
2. <u>Individual measurements with biological controls</u>

   In many cases, creating biological controls with only one reference spectrum per control is required. Typically this is required when the fluorophores to be used have a high degree of spectral emission overlap as well as a high degree of overlap in excitation spectrum and if the sample has a high degree of colocalization between the individual components (i.e., samples with GFP and YFP).

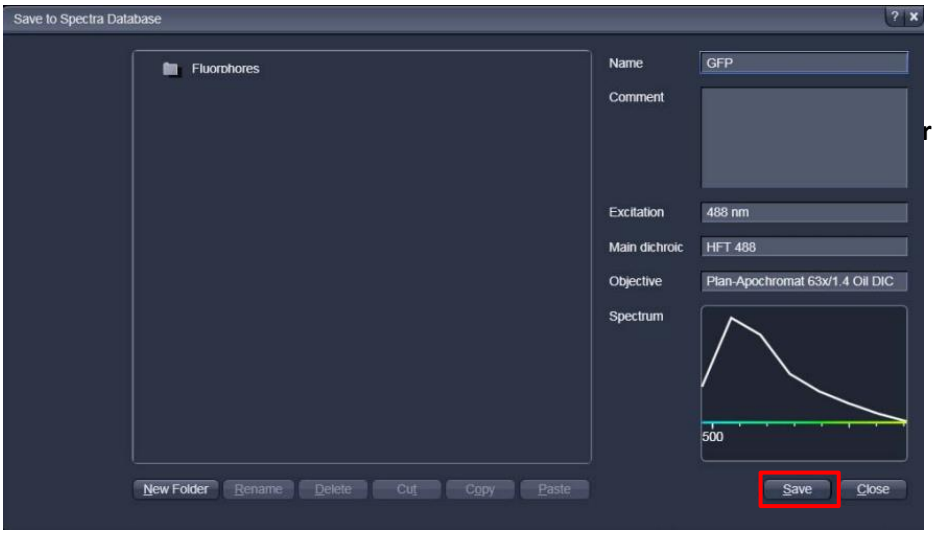
   In this scenario, the individual reference spectra must be collected and saved for use with the data of interest. As with ACE, it is important to acquire data without any underexposed or saturated pixels.

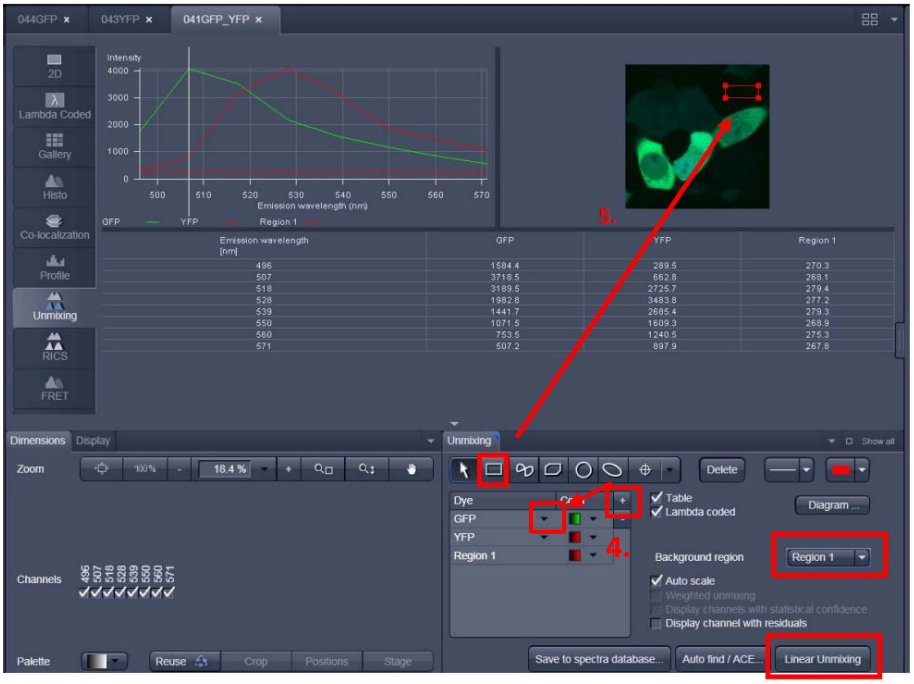
   Note: It is important that the reference spectrum and data are collected over the same wavelength range image sampling parameters.

   1. Go to the unmixing tab on the left side of the image container.
   2. Select an ROI shape at the unmixing tab below the image and mark on the image.
   3. Save reference spectra.
   4. Repeat for all reference spectra
   5. Load reference spectra
   6. Identify background ROI using selection tools if needed
   7. Press **Linear umnixing**



r unmixing


Save to Spectra Database
Fluorphores
Name
GFP
Comment
Excitation
488 nm
Main dichroic
HFT 488
Objective
Plan-Apochromat 63x/1.4 Oil DIC
Spectrum
500
New Folder
Rename
Delete
Cut
Copy
Paste
Save
Close

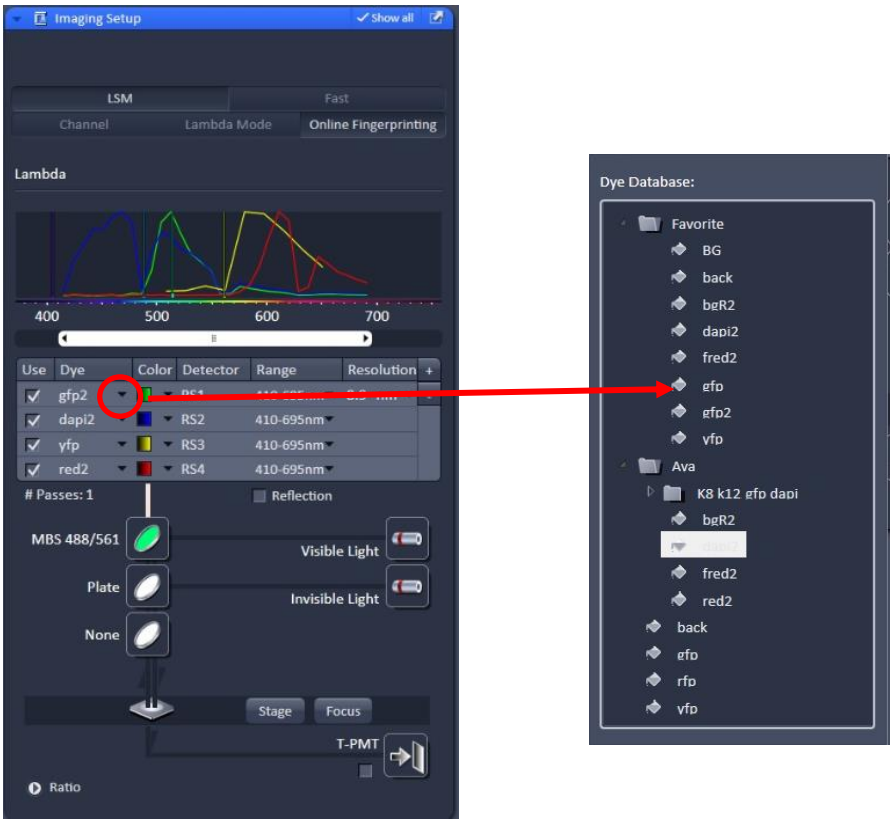

044GFP
043YFP
041GFP_YFP
2D
Lambda Coded
Gallery
Histo
Co-localization
Profile
Unmixing
RICS
FRET
Intensity
Emission wavelength (nm)
GFP
YFP
Region 1
Emission wavelength [nm]
5.
Dimensions
Display
Unmixing
Zoom
Delete
Dye
GFP
YFP
Region 1
4.
Table
Lambda coded
Diagram ...
Background region
Region 1
Auto scale
Weighted unmixing
Display channels with statistical confidence
Display channel with residuals
Channels
Palette
Reuse
Crop
Positions
Stage
Save to spectra database...
Auto find / ACE...
Linear Unmixing
5.
6.

Online Fingerprinting

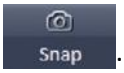
The imaging mode **Online Fingerprinting** is doing online spectral unmixing and is based on prior acquisition of a Lambda stack and using previously saved emission spectra from fluorophores which are used as markers in the imaged specimen. The choice of the emission spectra and the settings of the detection range for the QUASAR detector are defined in a loaded configuration or using the **Imaging Setup** tab.

The detection parameters (**Channels** tab→gain/offset/digital gain) are equal for all fluorophores. When changing them for one fluorophore, they will be changed for all (except laser intensity).
For best results, the settings for **Online fingerprinting** should match the settings of the Lambda mode when the fluorophores were acquired for defining the reference spectra.

- **Configuration**
  - In the **Imaging setup** tab choose **Online fingerprinting**
  - Choose the laser lines that suit the fluorophores in the experiment.
  - Choose the dichroic mirrors that suit the laser lines.
  - Choose the entire emission spectrum of all fluorophores.
  - Define the resolution of the spectral GaAsP PMT, e.g., step size 8.9nm
  - Add the reference spectra from the **Dye database**



  - Set the fluorophore parameters in the **Channels** tab (as described above).
  - Set the scanning parameters in the **Acquisition** tab (as described above)
  - Acquire a single plane image:

    Press 

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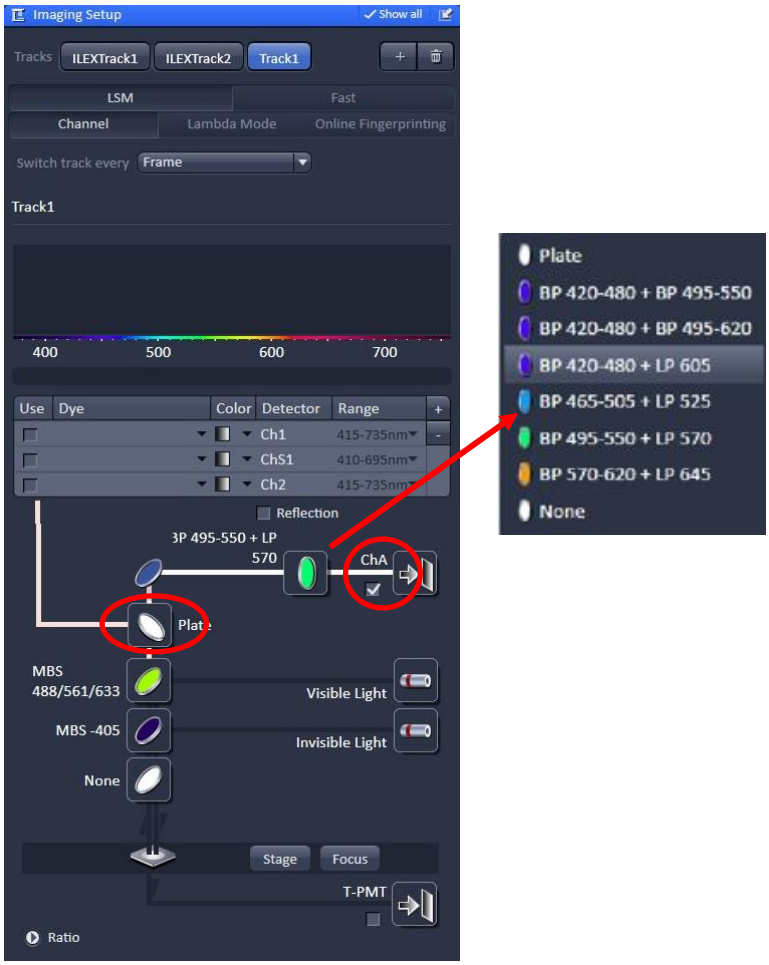
LSM Channel mode Airyscan

- **Configuration**
  - In the imaging setup tab in **channel** mode choose the Airyscan detector
    The mirror in the filter wheel between the Airyscan and the other PMTs will change to a plate



    Note: the transmitted light detection (T-PMT) cannot be conjugated to the Airyscan.
    If needed this can be added to a dedicated channel via **Experiment designer** tool.
  - For single channel acquisition select suitable Airyscan filter, laser line and dichroic mirror.
  - For two track acquisition:
    Acquisition mode tab → **Airyscan multi track** → choose **ILEX setup**

    Choose a filter from the Airyscan filter list that will suit the two fluorophores, press OK
    In **Imaging setup** tab → **Switch track every** tab → choose **Line**
    Choose for each ILEX track the suitable laser line
    Choose dichroic mirrors that will suit the laser lines (for visible choose one that fits both fluorophores, no changing - and one for invisible light if applicable).
    Note: the components selection will be the same for both tracks (except the laser line)
  - For multi-channel acquisition, in **Imaging setup** tab → **Switch track every** tab → choose **Frame** mode; this option will let changes in hardware to be made between tracks so it will

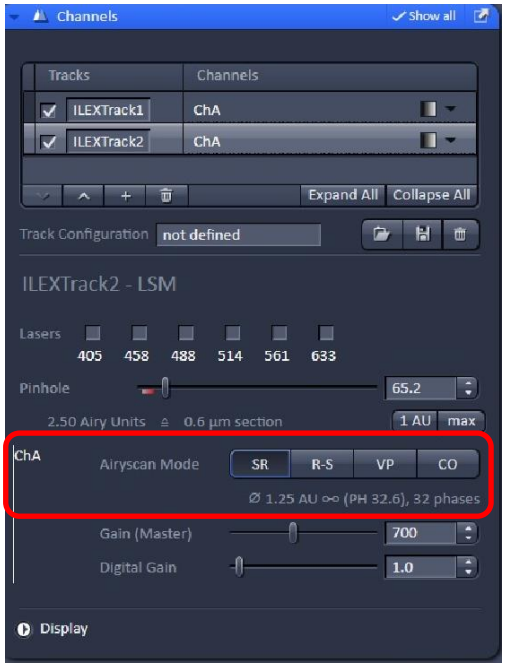
be suitable to the fluorophores (filter, laser). A channel can be added to an existing ILEX setup

- In the **Channels** tab:
  Select the Airy scan mode
  1. **SR:** in this mode there is a detection of 1.25 AU in the Airyscan detector; each element is equal to 0.2AU. The Confocal pinhole is opened to 2.5 AU (x2 than detection). In this mode the resolution improves by a factor of 1.7.
  2. **R-S:** in this mode there is a compromise between resolution & signal (more signal less resolution) the detection is of 2 AU and each element is equal to 0.3 AU. The Confocal pinhole is opened to 4 AU (x2 than detection). In this mode the resolution improves by a factor of 1.4.
  3. **VP:** in this mode there is a detection of 3AU in the Airyscan detector. The confocal pinhole must be opened to 6 AU (x2 than detection). In this mode you detect more signal and can mimic digitally the confocal effect post-acquisition with a virtual pinhole slider (under the image) and choose the AU level which is best for the experiment.
  4. **CO:** in this mode the pinhole can be used as in a regular confocal and there is binning of the 32 elements of the Airyscan detector which now acts as one detector unit. This mode can be useful in a low signal experiments because it has x5-x6 better SNR.

     Note: The pinhole and zoom sliders must be above the red zone in any condition.

     Note: The system automatically sets the pinhole aperture according to the mode selected (except CO). It is best to keep the recommended setting.

     Note: You MUST click optimal every time you change airyscan mode.



- **Acquisition**

  In the **Acquisition tab**

  Set the Zoom slider above the red zone.

  For best results scan with high zoom e.g., 50µm x 50µm

  - Set the acquisition parameters:

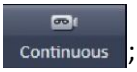
    Frame size: 512x512

    Speed rate: high

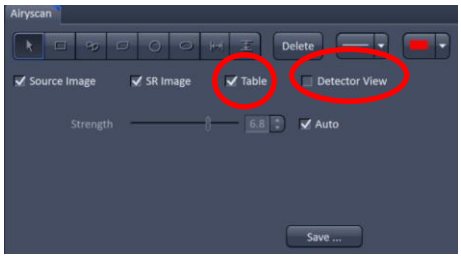
    Averaging number: 1

    Bit depth: 8 bit (at higher dynamic range larger files will be created)

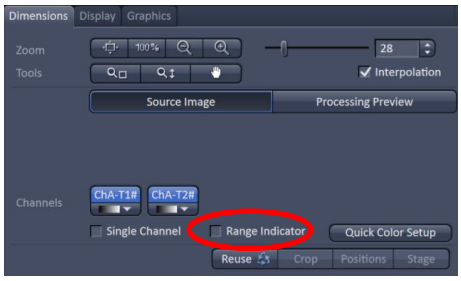
    Direction: in most cases choose meander (<-->) (Airyscan process is slow)

  - Start scan in continuous mode 

; the detector element activity can be viewed during scanning by checking **detector view** and/or **Table** in the Airyscan tab (below the image)



In the image window set the source image to range indicator (below the image)



  - In the **channels** tab set the channels parameters (for each channel)–

    Laser intensity

    Gain: 750-850

    Digital gain: 1-2

    Note: The offset is set automatically by the system; for best processing (after reassignment of data from all 32 elements in the detector and a deconvolution) avoid any saturated pixels in the source image (red pixels in range indicator mode).

  - Stop scan 

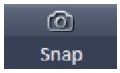
  - **To acquire single image** –

    Set the acquisition parameters-

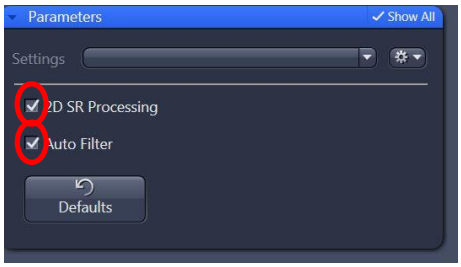
    Frame size: press optimal

    Speed rate: Recommended to choose 1.5-2µsec pixel dwell time

    Averaging number: 2

  - press the **Snap** tab 

- Save the file: Save the image – press  in the **Images and Documents** section (right hand side of the software window). The file includes the source image and the preview of the processed image; for saving the processed image DO NOT press save in the Airyscan tab (below the image). For an enhanced deconvolution algorithm of Airyscan 2D datasets open the saved source image in Zen Blue (on the same computer), do Airyscan Processing, select the input file, check Auto filter and 2D SR Processing in Parameters and press **Apply**.



Note: In case of unsatisfactory deconvolution result, the strength can be changed manually. Uncheck Auto strength and change its level until a satisfactory result is obtained before saving the image.

- **Multidimensional acquisition**

  More dimensions can be added to the acquisition (Z-stack, tile etc.)

  Check the required option

  Change the respective settings as described above.

  To start the acquisition press **Start Experiment**

  Save the file.

  Save the processed image:

  Note: The saving option in the Airyscan tab below the image will save only a 2D image in the current view.

  To save the processed 3D image:

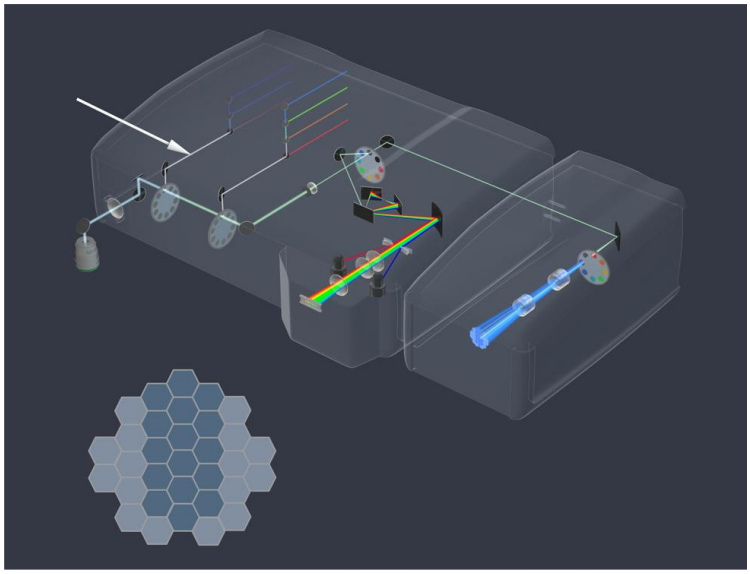
Go to Processing tab → Adjust → Airyscan processing → [select input image] → method parameters √ auto deconvolution 3D → apply

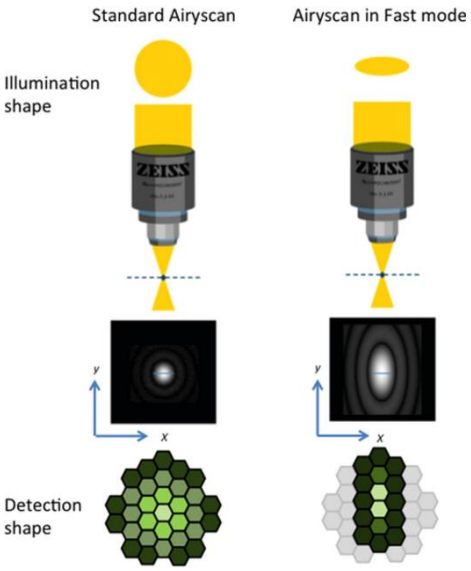
Note: In case of unsatisfactory deconvolution result the strength can be changed manually. Uncheck Auto strength in **Method Parameters** and change its level until a satisfactory result is obtained before saving the image.

Airyscan Fast mode

The Fast Module shapes the illumination beam into a short line, instead of a round spot, and uses only the center detectors to image the beam. This creates simultaneous, parallel illumination and detection of a line within the sample, but still with an improvement in resolution. The final image has 1.5X improved resolution although it is non-isometric in XY, and a 4X improvement in signal-to-noise ratio. Since the laser beam is spread out across a larger area, Fast Mode allows for gentler imaging, which reduces photobleaching and phototoxicity with 4X faster scanning compared to standard confocal or Airyscan microscopy.

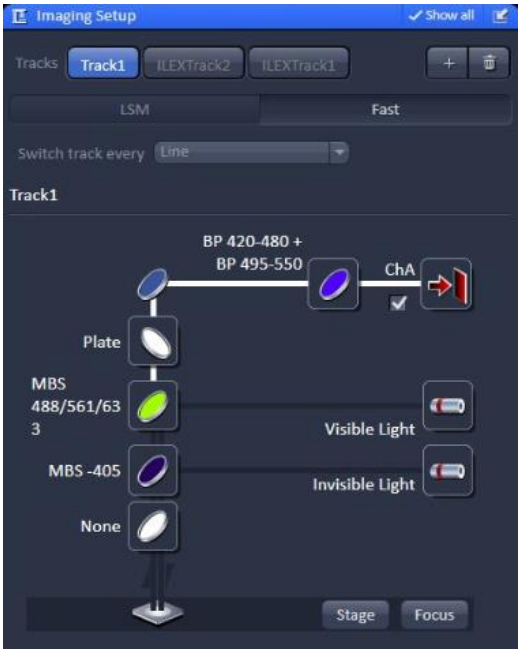
The laser beam is shaped before it enters the objective lens back aperture. If the laser beam is narrowed in its y-axis while its size in the x-direction remains unchanged before entering the objective lens, the resulting excitation beam is stretched into an ellipse along the y-axis. In LSM 880 the beam shaping is performed by using slit apertures positioned in the excitation path of the scanhead. Different slit sizes serve different objective lenses. The appropriate width slit is positioned in the laser beam path. The resulting fluorescence for each 4-pixel column is collected by the Airyscan utilizing 16 detector elements of the Airyscan detector's center where three horizontal detector elements cover 0.9 AU and up to 6 vertical elements cover 1.65 AU of the emission Airy disk. As a result, each detector element acts as an individual pinhole with a diameter of about 0.3 AU.




Standard Airyscan
Airyscan in Fast mode
Illumination shape
Detection shape
ZEISS
ZEISS
y
x
y
x

Configuration

- In the **Imaging setup** tab press **Fast**

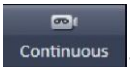


Note: the transmitted light detection (T-PMT) cannot be added to simultaneous acquisition with the Airyscan. If needed it can be added via the experiment designer tool.

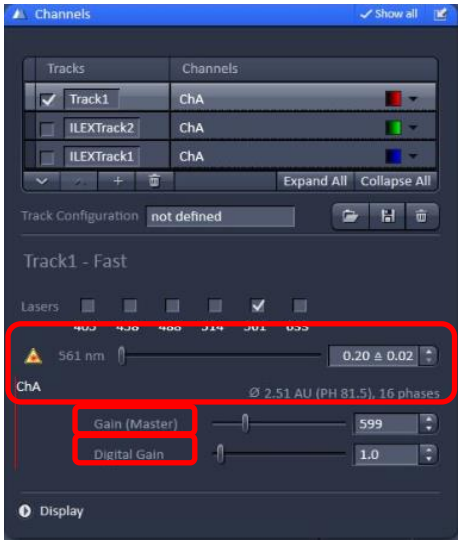
- For single channel acquisition select a suitable Airyscan filter, select laser line and suitable dichroic mirror.
- For multi-channel acquisition –In **Imaging setup** tab add tracks → press + → choose **Switch track every** → **Frame**.

- **Acquisition**
  - Choose the suitable sampling the system automatically will adjust the frame size according to the selected zoom and sampling. The sampling (frame format) has greater importance than in conventional confocal acquisition, since only certain sampling values will match the correct detection elements on the Airyscan detector. Choosing one of the predefined sampling steps is the most convenient way for maximum acquisition speed and imaging quality.
  - **Choose zoom and press sampling option, there is no flexibility to manually change the frame size.**
    1. **SR-** sets the sampling for super resolution imaging with Fast at 2x Nyquist sampling the acquisition is 4x faster than with conventional Airyscan SR acquisition.
    2. **Opt-** sets the sampling for optimal confocal imaging with Fast at 1x Nyquist sampling.
    3. **Flx-** sets the sampling for flexible confocal imaging with 0.7x Nyquist sampling. **The Opt and Flx sampling can be combined by pressing +button. In this case' slower speeds will automatically use 1x sampling and faster speeds the 0.7x sampling. The confocality is still very good.**

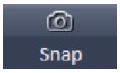
4. **FS-** sets the sampling for fastest speed imaging with Fast at 0.5x Nyquist sampling. This allows highest frame rates of up to 200fps with sufficient resolution for good image quality.
5. **Sampling slider-** can be used for further degrees of freedom in speed selection. Any desired sampling value can be selected

- **Speed-** the speed selection is operated with the **speed fps** slider**.** The slider automatically adjusts to the speeds possible by the selected sampling. For requiring a higher speed range the sampling needs to be adjusted (less Nyquist) or zooming in will allow to choose higher sampling for required speeds.
- In the **Acquisition Mode** tab choose the suitable zoom**.**
  Averaging number- 1
  Averaging mode- choose frame\line (for live with fast activity experiment line mode will be more suitable)
  Direction – choose meander scanning for faster acquisition.
- Start scan in continuous mode 

. In the image window set the source image to range indicator (below the image)
  In the **Channels** tab set the channel parameters (for each channel)–
  Laser intensity is shown as 2 values the first value is the laser before beam shaping; the second value is the intensity after extension of the beam.
  Gain: 750-850
  Digital gain: 1-2
  Note: The offset is automatically set by the system. For a good processed image (after reassignment of the data from all 32 elements in the detector and deconvolution) avoid any saturated pixels in the source image (red pixels in range indicator mode).
  Pinhole**:** the pinhole slider is not available and the value of the detection is indicated on the window

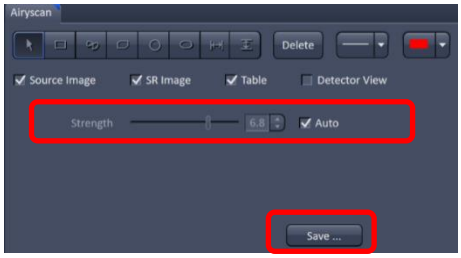


- Stop scan 

- **To acquire single image** –
  Set the acquisition parameters:
  Averaging number- choose the required number according to the experiment goal.
  Averaging mode: choose frame\line (for live with fast activity experiment line mode will be more suitable)
  Direction: choose meander scanning for faster acquisition.
- Press the **Snap** tab 

- Save the file: Save the image – press  in the **Images and Documents** tab (right hand side of the software window). The file includes the source image and the preview of the processed image; for saving the processed image press **Save** in the Airyscan tab below the image.

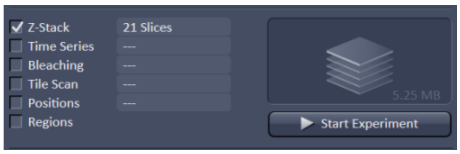
  Note: in case of unsatisfactory deconvolution result, the strength can be changed manually. Uncheck Auto strength and change its level until a satisfactory result is obtained before saving the image.



- **Multidimensional acquisition**

  More dimensions can be added to the acquisition (Z stack, tile etc.)

  Check the required option



  Change the setting in the selected option window (as described above).

  To start the acquisition press **Start Experiment**

  Save the file.

  Save the processed image:

  Note: The saving option in the Airyscan tab below the image will save only a 2D image in the current view.

  To save the processed 3D image:

  Processing tab → Adjust → Airyscan processing → [select input image] → method parameters √ auto deconvolution 3D → apply

  Note: In case of unsatisfactory deconvolution result the strength can be changed m anually. Uncheck Auto strength in **Method Parameters** and change its level until a satisfactory result is obtained before saving the image.

  Note:

  Tile - the overlap should be 10%-15% because of the change in the beam shape, zoom should be 2

  FRAP (or bleaching experiment) - the laser intensity will not be as powerful as in the other modes; for better bleaching define more iterations in the bleaching tab

  ROI - there is no free hand drawing option.

  Rotation – the rotation option in the **Acquisition Mode tab** → scan area isn't available.

  To start the acquisition press **Start Experiment**

Linear unmixing

Samples:

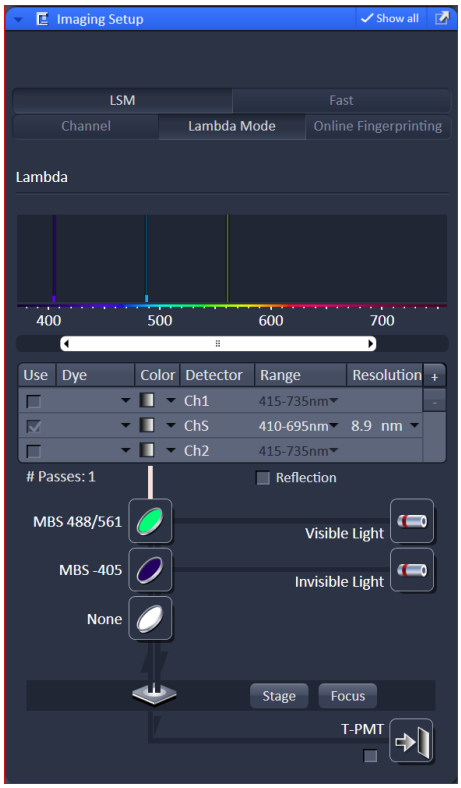
- Each fluorophore by itself.
- Unstained cells/tissue for possible autofluorescence which counts as a fluorophore if present.
  If you do not see autofluorescence, do not count as fluorophore. Discard.
- Stained (mix).

In **Imaging setup tab** set:

LSM → **Lambda mode** → **ChS** as wide as possible (e.g., for DAPI to Cy3: 410-695, 8.9nm).

If you have Cy5.5 check also Ch2 for range up to 760 concomitantly.

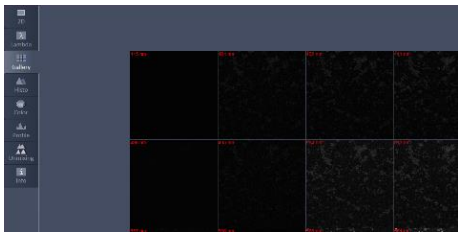
Set relevant lasers and dichroic mirrors.



Use the mix sample first to get an impression of the final spectra and roughly set lasers, gain etc. so that the channels are more or less balanced.

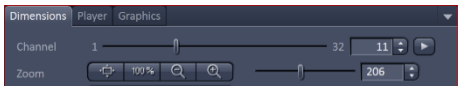
Focus on the sample and in fast acquisition settings (512 pixels, speed 8, averaging 1) press **Continuous**.

In **Gallery vertical tab** you can see all channels, but small.



Press **show text** to show wavelength of each channel

To focus precisely, go to **2D vertical tab** and in **dimensions tab** below the image choose a strong channel to focus.



Click palette below to enable range indicator (toggles, no need to choose from list).

(In **Channels** there is only one gain/offset.)

Approximately optimize lasers and gain by using the 2D (for precision) and the Gallery views to attain no saturation and minimal offset.

Place reference sample of first fluorophore.

Focus (using a big view of a strong channel), optimize lasers and gain/offset.

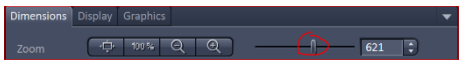
Set optimal pixel size, speed 6, averaging 4-8, bit depth 12.

The SNR should be excellent, grainy signal will not produce reliable results. No saturation.

Snap.

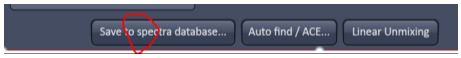
Save.

In **unmixing vertical tab** zoom in using the zoom slider in the **dimensions tab** below

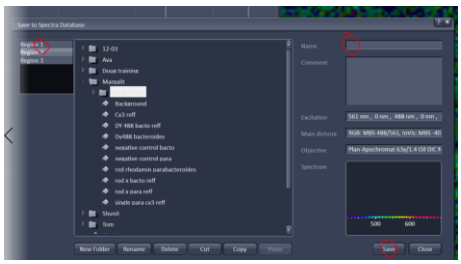


Using an ROI shape from the **unmixing tab** (below the image) choose an ROI (representative and not very small) of the fluorophore avoiding background.

Save to spectra database.



Choose region from the list, choose folder, give name, save.



Repeat for each fluorophore and for autofluorescence if present.

If you cannot find focus in a sample, use the transmitted channel by temporarily enabling it in **imaging setup** and disabling before final acquisition.

Always keep the same components: lasers, dichroic mirrors, channels, objectives, zoom.

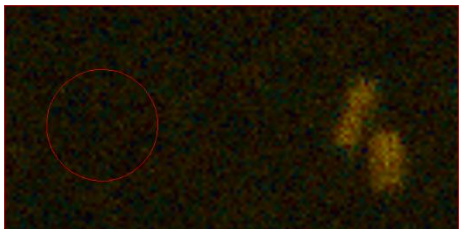
Place sample of mix.

Focus, set optimal pixel size, speed 6, averaging 4-8, bit depth 12.

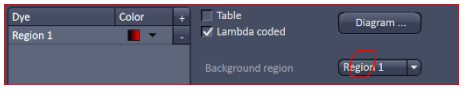
Snap.

Save.

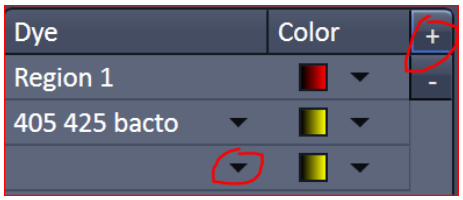
In **unmixing vertical tab** set ROI of pure background.



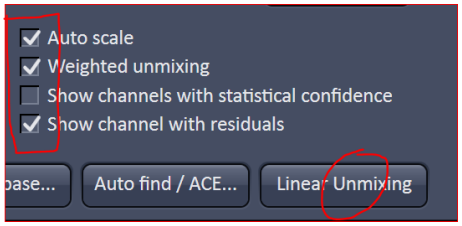
Set this as background



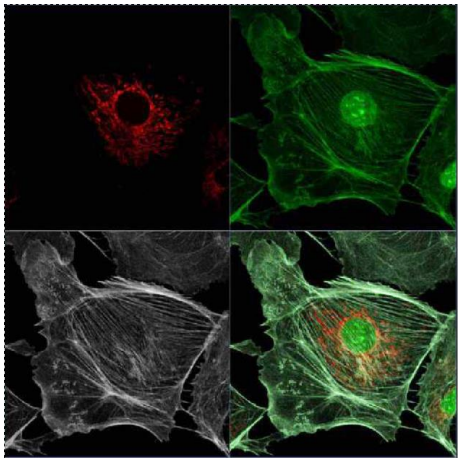
Press + and add all other saved spectra.



Select Auto scale, Weighted unmixing, Show channels with residuals and press **Linear unmixing**.



The “channel with residuals”, the pixel by pixel difference between the best spectral fit based on the reference spectra and the original spectrum acquired from the specimen, should be minimal. If the residual is high in specific structures, then the respective reference spectrum has not been acquired properly or a spectrum is missing.



Shut down

Before you finish please make sure whether the next user is coming.

If yes:

- Close the ZEN software
- Log off your reservation at BookItLab
- Clean oil immersion objectives used with lens tissue dipped in petroleum ether, twice
- Clean water dipping objectives by dipping in DDW (e.g., in 35mm dish)
- Leave nosepiece at 10x or if there is no 10x leave at 20x objective lens
- Center the stage
- Leave surfaces clean

If no user is coming in the next two hours, **in this order**:

- Turn off the lasers in the software
- Close the ZEN software
- Log off your reservation in BookItLab
- Switch off (3) → (2) in the switch box
- **Wait until the Lasos fan shuts off and only then switch off → (1)**
- Cover microscope
- Turn off air condition

  Do not turn computer off unless you are the last user before the weekend.

  Please do not use any type of USB drive to copy your data.