

G Sonntag Oct/2019

Zeiss LSM 980 – Basic User Notes

Airyscan

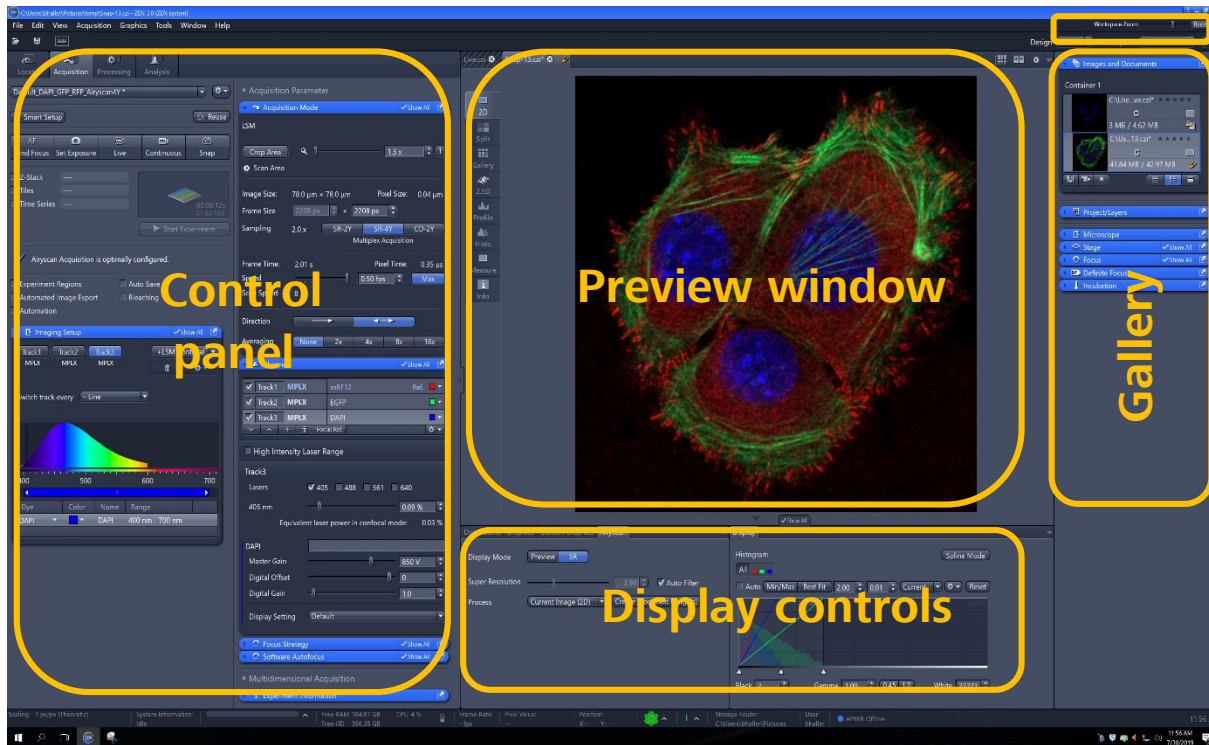


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1. Turning on:

Follow the startup guide specific for our machine



Note: At the top of the control panel select **Show all tools** to show all functionality:
From the drop down menu select **View > Show all (global)** or tick **Show all** in each tab as needed

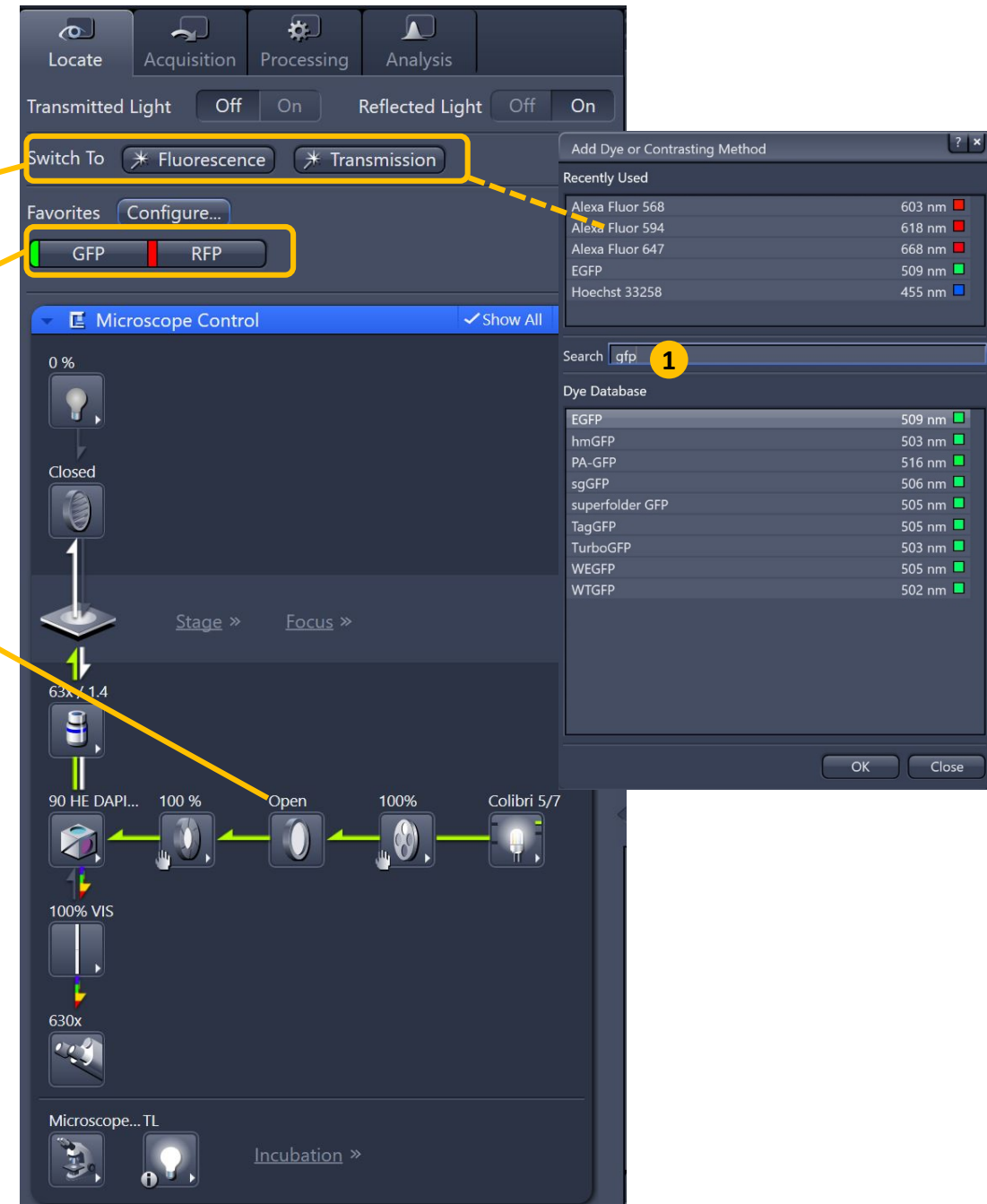
2. Locate tab: To view in Microscope:

Use pre-set configuration tabs to view sample in microscope

Switch To configures your light path for visual observation. You only need to select the dye or contrasting method (1)

To create buttons: **Tools > Hardware settings**; select light source and filter combinations; open/close shutters as required in beam path; save configuration and create shortcut buttons using **Configure**

Select light source and filter combinations manually as needed



3. Acquisition tab:

Templates: Drop down menu to select a pre-defined experiment Set-up

Smart Setup: Wizard to set up experiments from scratch

Sample Navigator: Wizard to quickly find regions of interest

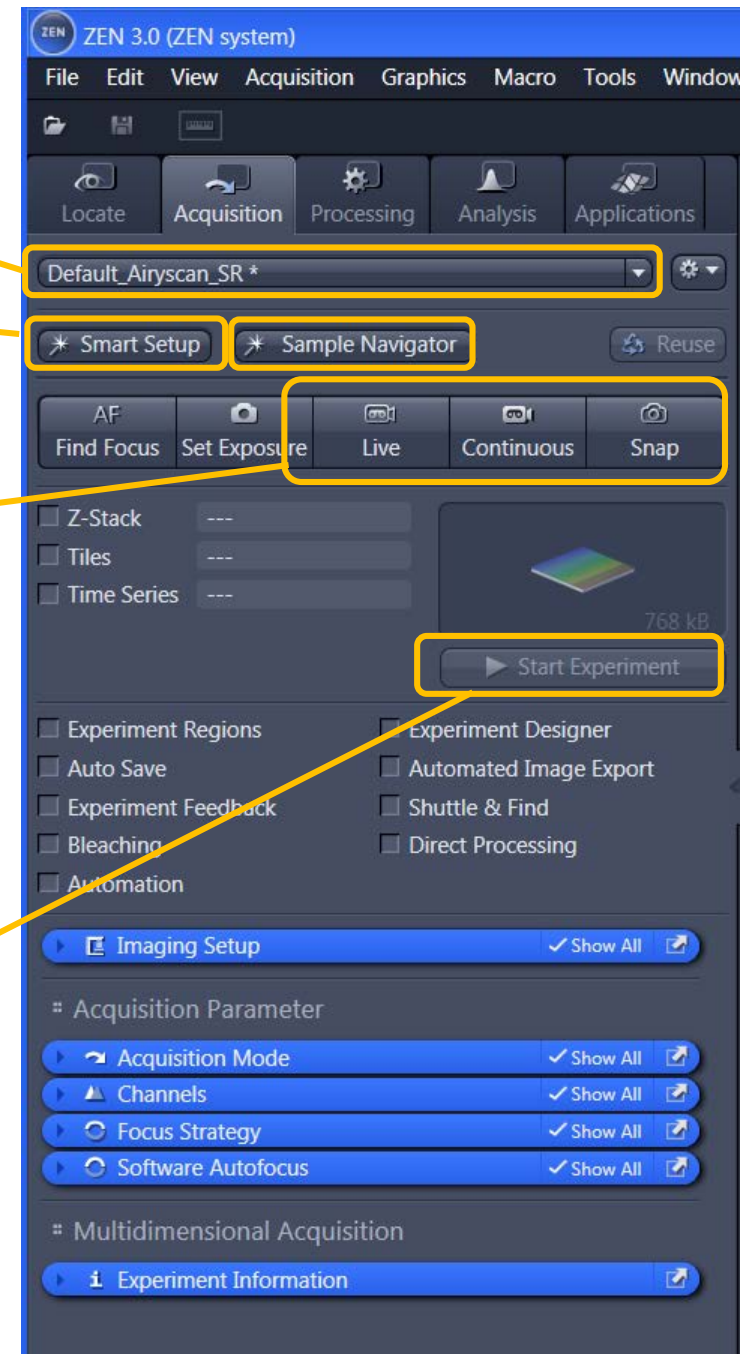
Imaging Mode:

Live: Fast 512x512 – no averaging - only the highlighted track. You can switch between tracks while Live is running by selecting the track in the Channels panel

Continuous: Runs continuous acquisition as defined in **Acquisition Mode** tab

Snap: Takes a snapshot of all ticked tracks/ channels as defined in **Channels/ Acquisition Mode** tab

Start Experiment: Starts multidimensional acquisition (z, time series, tiles, bleaching etc) as defined in **Acquisition Mode** tab



4. Smart Set up / Imaging Set up:



LSM Mode

Dye selection

Multiple tracks = sequential

Allows changing hardware/
specific filters between frames
Acquires longer wavelengths
first

Smartest = minimal hardware
changes for maximal speed with
minimal bleed through

Single Track Set up:

Note: If dyes are too similar,
Fastest may also come up
with multiple tracks



4.1 Imaging Set: (without Smart Set up)

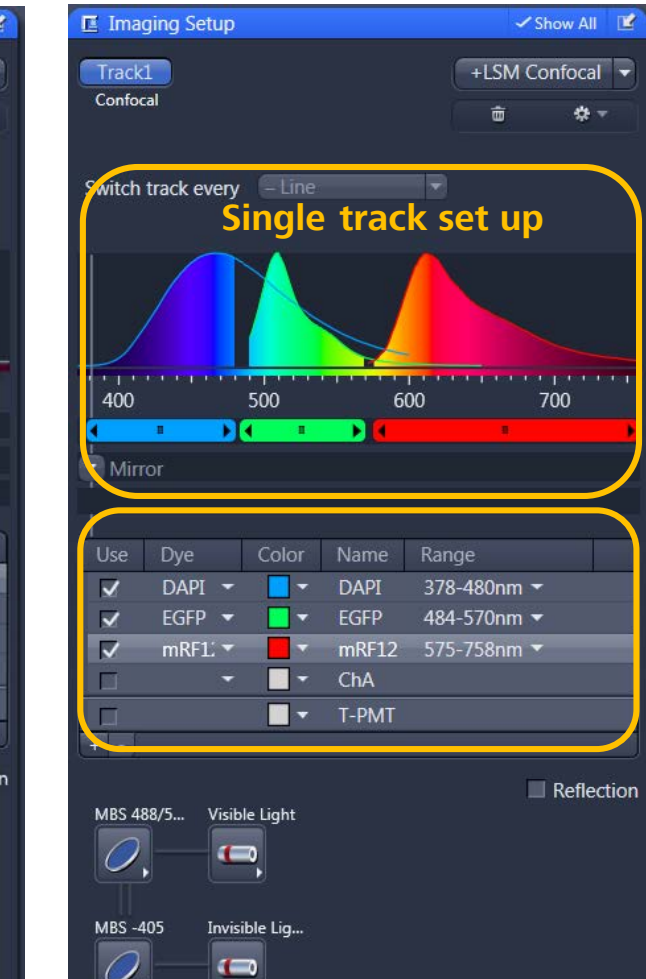
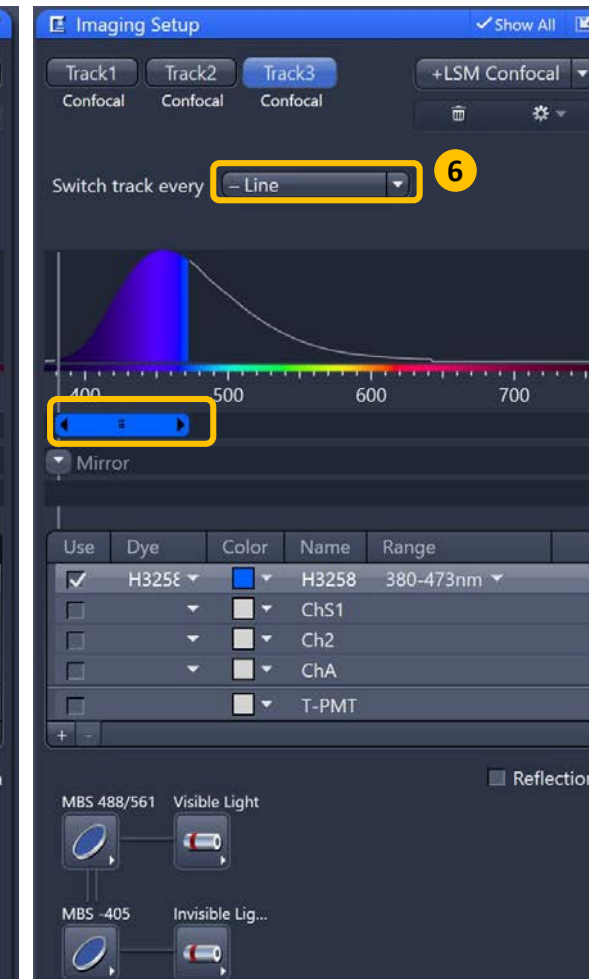
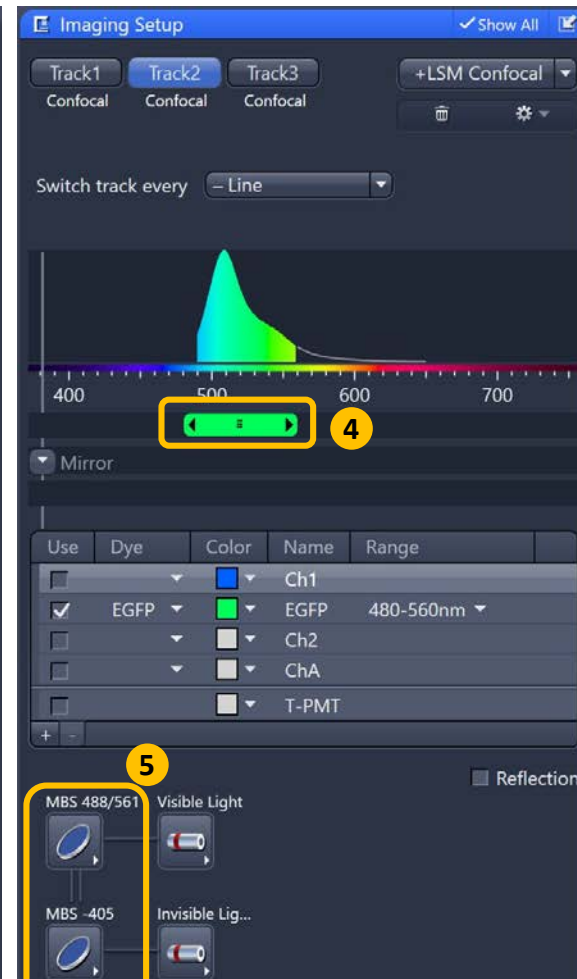
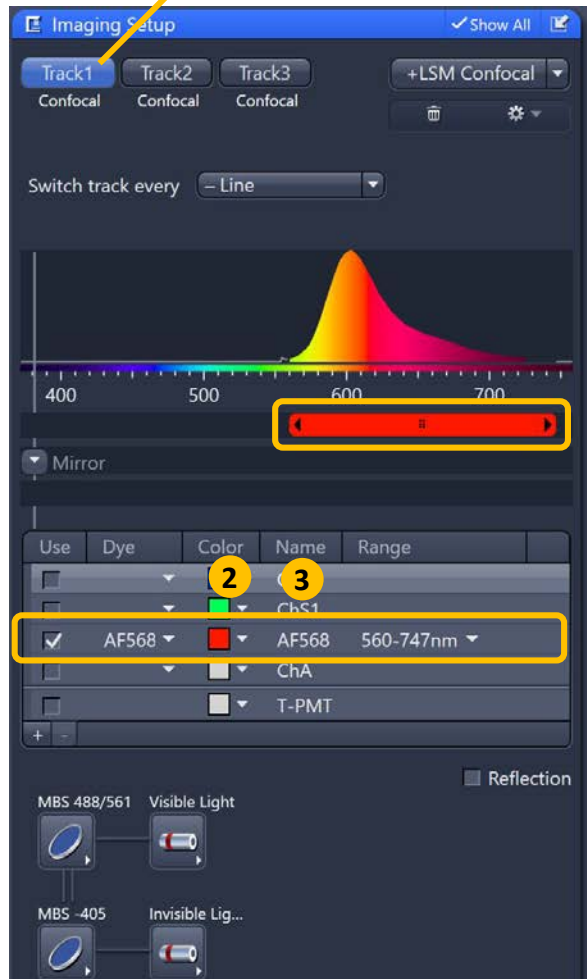
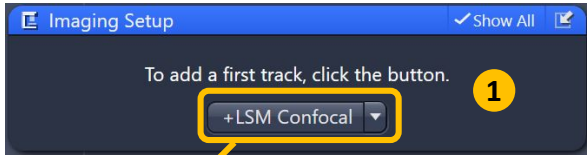
Imaging Set up tab:

Select **Show All**, add LSM Confocal track (1)

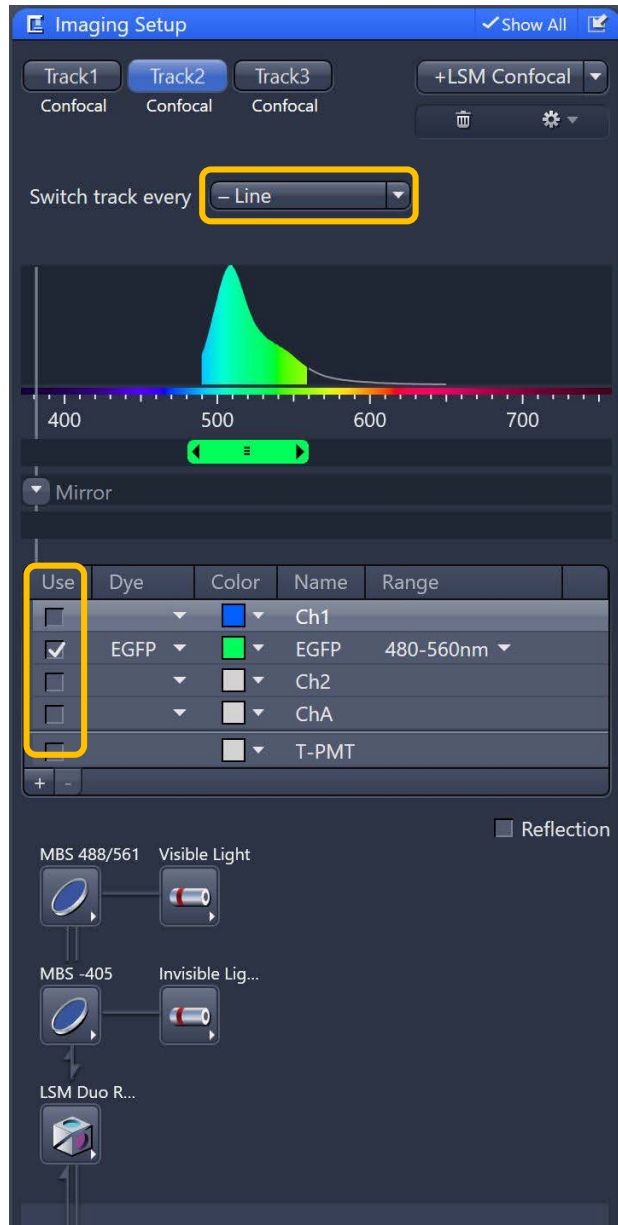
Tick your detectors, LUT colours (2) and choose fluorophores (3) from drop down menus
Adjust detection range (4) to collect appropriate wavelengths, adjust MBS if needed (5)

Note: Adjusting all detector ranges at this point will reduce movement between tracks

Specify Line / Frame / Frame fast (Line and Frame fast require the same hardware between all tracks (i.e. same position detection range) (6)

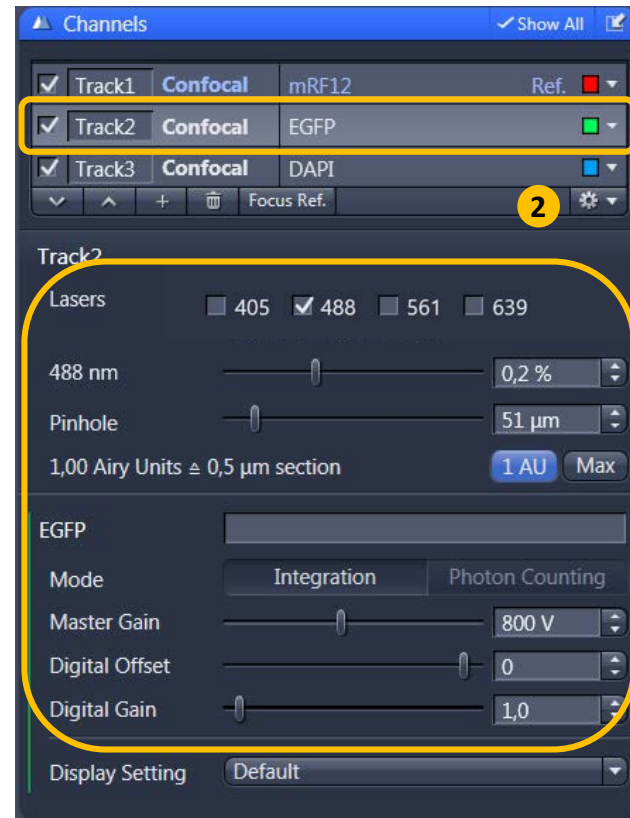


5. Optimizing Acquisition: Imaging Set up and Channels:



In **Channels**; click on the track you want to adjust so it becomes highlighted. The chosen track will then be shown in the **Imaging Set up** tab. For ease, untick the other tracks. Click **Live** or **Continuous** and focus your sample.

In switch track every **Frame** mode adjust each track or channel one at a time (Laser Power; Pinhole; Gain), ensuring use of the full dynamic range without saturating signal (see next slide).



Adjust

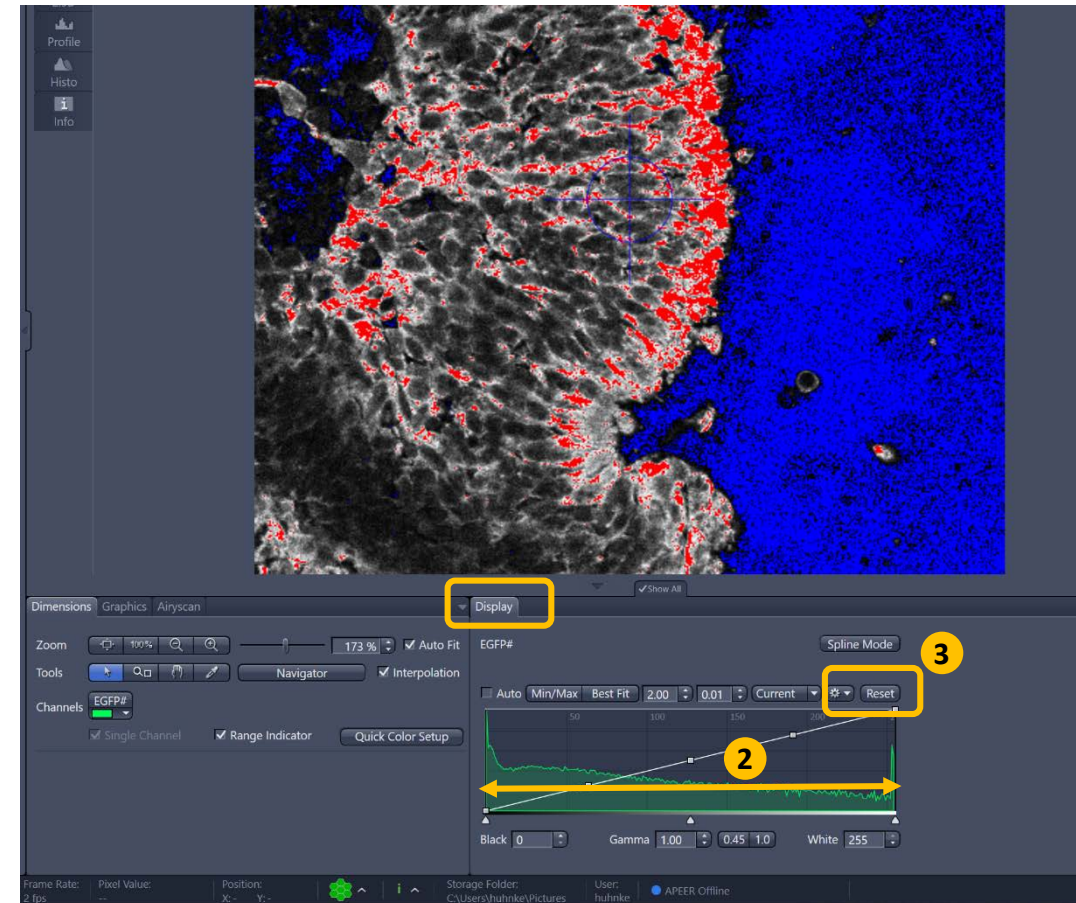
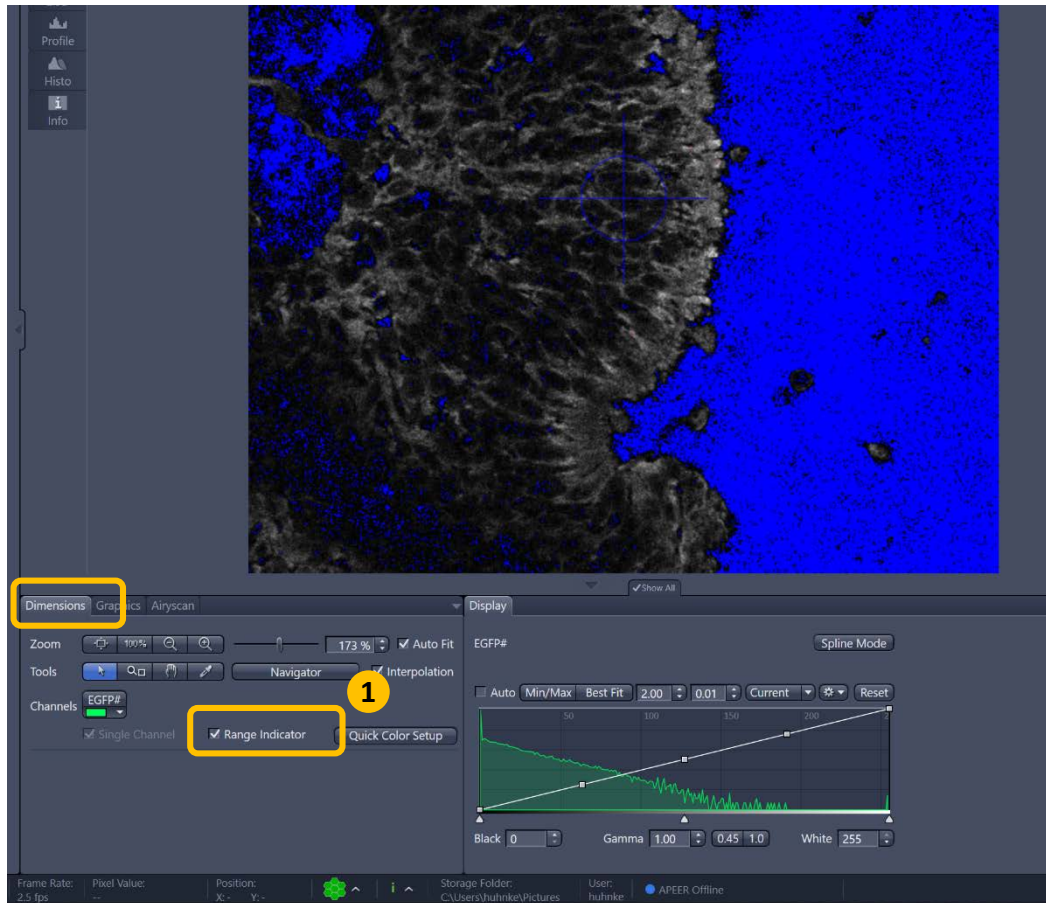
Laser power, Pinhole (1AU for confocal/ section thickness Master Gain (650-900). Short warm up period for some lasers are indicated in red (1)

Note: Either Ctrl+click on the tracks, or Select All in the cog wheel (2), this way you can see all the lasers/gains in the same window without having to cycle through the individual tracks

5. Optimizing Acquisition: Imaging Set up and Channels:

Saturation: In Live or Continuous mode tick the **Range indicator** (1) and aim to fill enough of the dynamic range (2) suitable for acquisition / analysis.

Note: do this with display **RESET** (3) or it will appear saturated when it isn't.



6. Define Acquisition mode:

Acquisition Mode: Recommended settings for optimal image quality:

Scan area:

Shift area in X and Y

Rotate field

Zoom (crop area): Zoom < 1 is possible but not recommended for quantitative imaging or tiling

Zoom 1.0 = minimum recommended zoom for confocal

Reset Scan Area – quick reset button

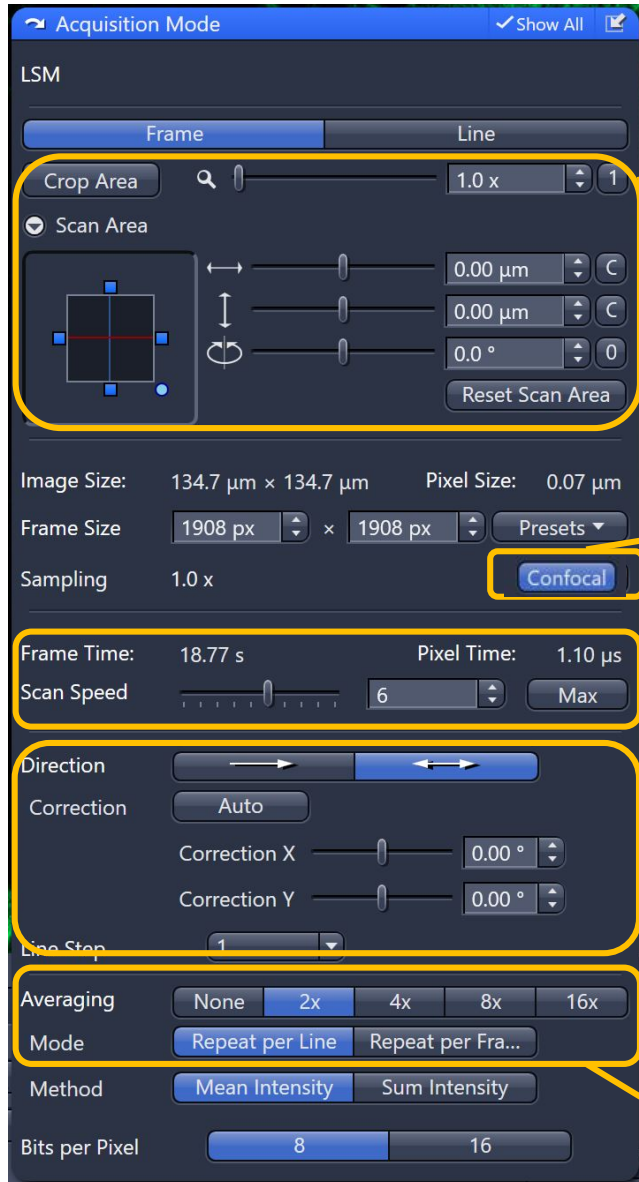
Define **Frame Size**. **Confocal** selects pixel size to satisfy Nyquist criteria. Increases acquisition time if large field of view. If **Confocal** is highlighted (active) all related parameters correspond each other to allow for best confocal quality

Scan speed and **pixel dwell time**: choose scan speed with a pixel dwell time of 1 μs – 2 μs for optimal signal quality. You can highlight the **Max** scan speed so it will always use the fastest possible scan speed whenever you change the zoom and number of pixels

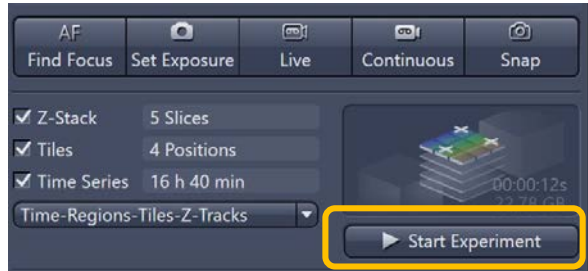
Uni / **Bidirectional** scanning. Scans on return, halving the Frame time / scan time. Bidirectional may need aligning. Correct using the Auto button, or manually (X slider). If scan area rotated, you may need to adjust the Y slider as well.

Note: Check bidirectional alignment with a single track: if not aligned, the image will appear distorted / double vision. If using two tracks with line wise switching, misaligned images will each image appear perfect but may be pixel shifted. Bidirectional is easier to align when not rotated.

Averaging – reduces noise. Averaging can use line / frame mode



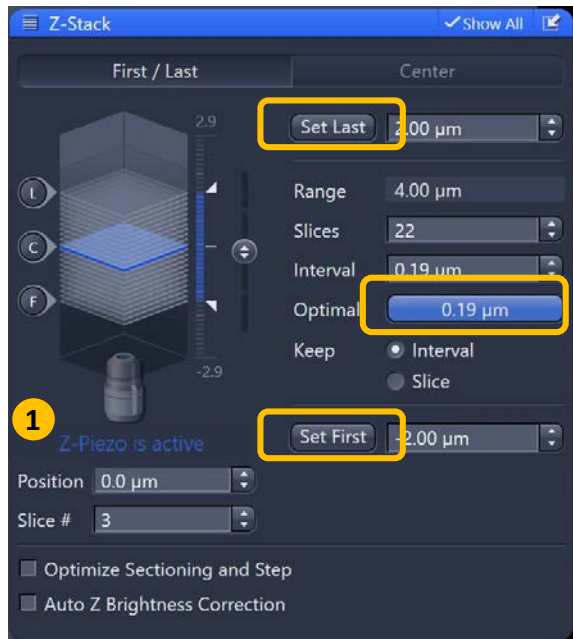
7. Multidimensional Acquisition:



A combination of functionalities can be used to create a multidimensional experiment. Make sure there is enough space on the hard drive to save your data. Set up each tab, then **Start Experiment** to run acquisition.

Experiment / data size can be checked in the info tab and above the **Start Experiment** button.

8. Z-Stack:



Setting a Z stack: Use Live (for fast live view)

First/ Last: Focus to the bottom of your required stack and click **Set Last**. Then find the top and **Set First**.

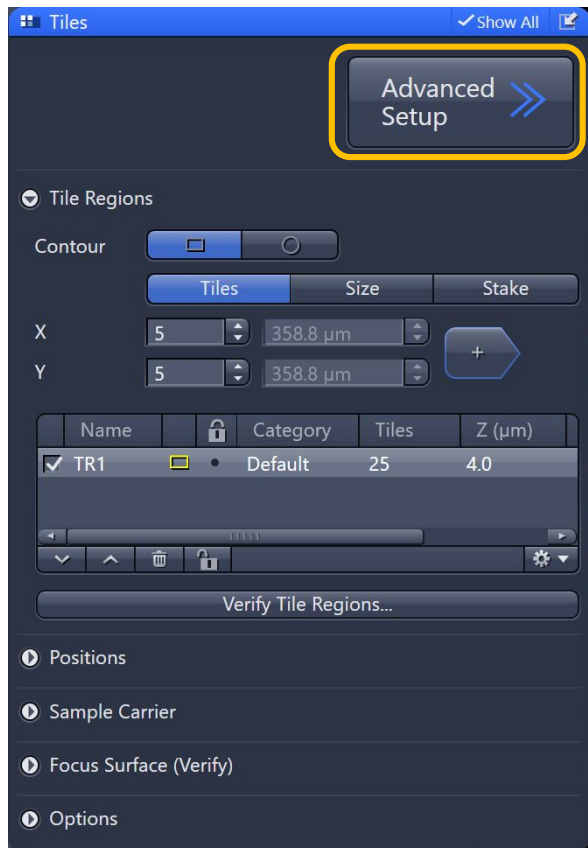
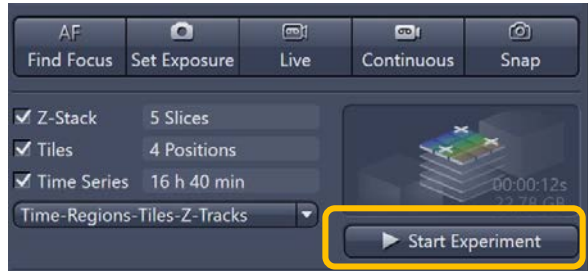
Check the total range, slices and interval. Clicking **Optimal** will select appropriate Nyquist settings for 3D imaging. If the Optimal button is highlighted, z step will automatically be adjusted when changing PH size, objective and wavelength. Start Experiment. Save your data.

Centre: Focus your specimen. Click **Centre**, then input range and adjust slices / interval / offset as needed.

If your system comes with a z-piezo insert, z-stacks smaller than the z-piezo's travel range will get operated by this device (1), indicated by „z-piezo is active“.

Note: For multiposition Z stacks: Each single XYZ position will refer to the center of the stack performed at these position. Interval / number of slices will stay the same for all positions needed.

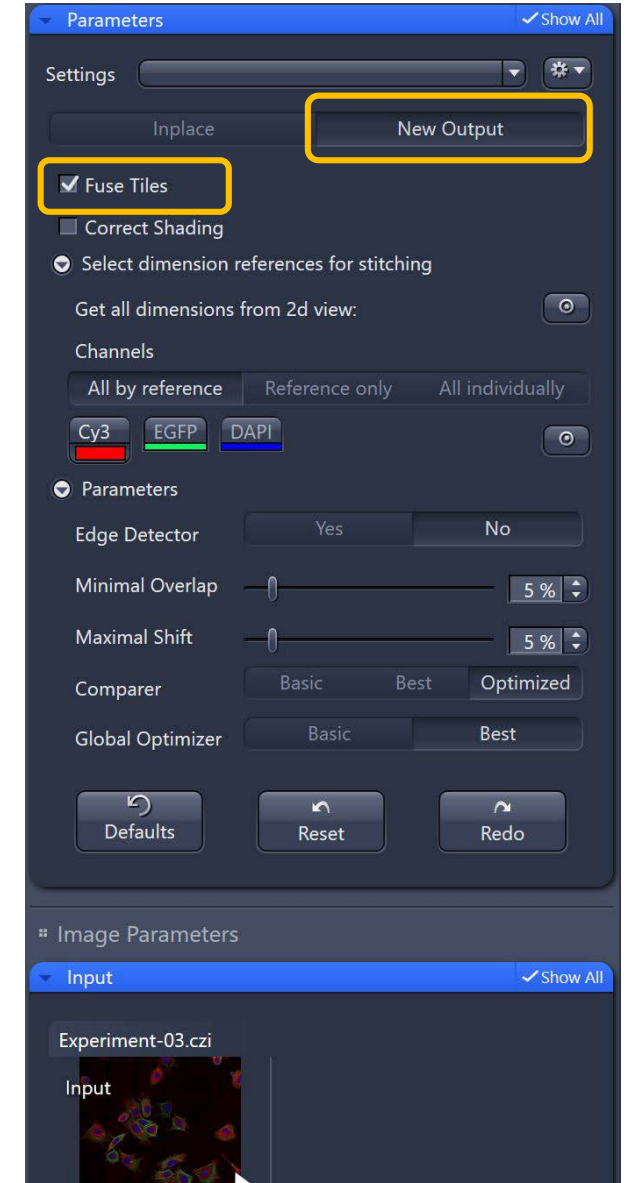
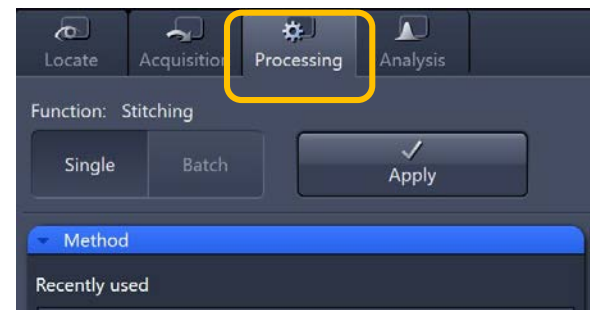
9. Tiles: Tile Regions



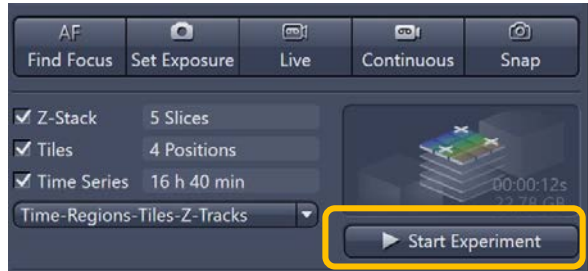
Tick tiles in experiment window and expand the **Tiles** tab. Add **Tile Regions** using the Advanced Set up. **Live** in a separate container may get used to set the best focus position. Set the number of **Tiles** in **XY** or use **Stake** tool. Multiple Tile Regions can be defined for your experiment. Start experiment. Save your data. Ensure you check there is sufficient space on the hard drive to save your data.

Stitching a tiled scan:

Go to the processing tab. In the Method tab list, select Geometric/Stitching. Choose the data you want to stitch. Tick **New Output** and **Fuse Tiles** and set the proper parameters. Then Apply. Save the stitched image – it will have the extension `_stitched.czi*`



9. Tiles: Positions

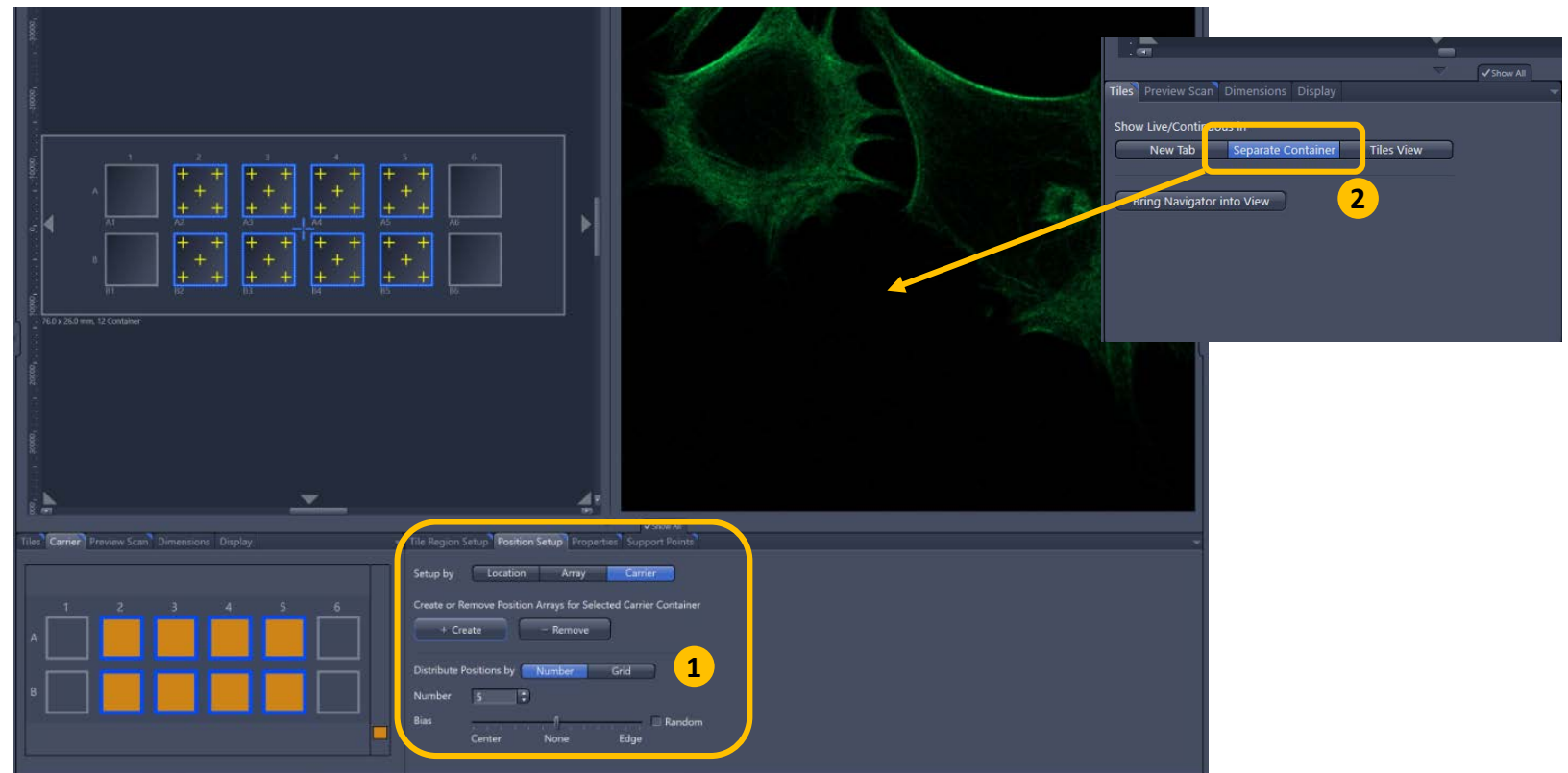


Tick tiles in experiment window and expand the **Tiles** tab. Add **Positions** using the Advanced Set up: this can also be used to map carrier templates.

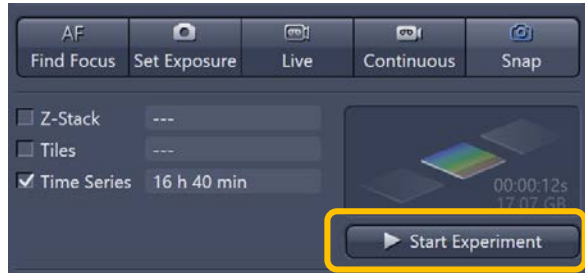
Live (2) in a separate container helps to set the best focus position. Use **Position Set up** (1) to define a certain distribution pattern of positions.

Start experiment. Save your data. Ensure you check there is sufficient space on the hard drive to save your data.

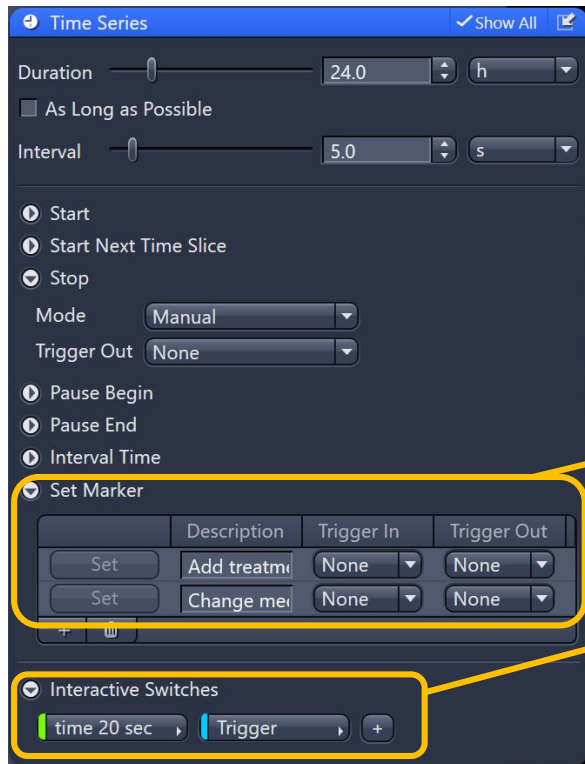
Make sure you use an appropriate focus strategy to maintain focus in all positions/ time points



10. Time Series:



Tick time series in experiment window and expand the **Time Series** tab. Set the number of cycles (**Duration**) or the duration and **Interval**. Start experiment. Save your data. Ensure you check there is sufficient space on the hard drive to save your data.



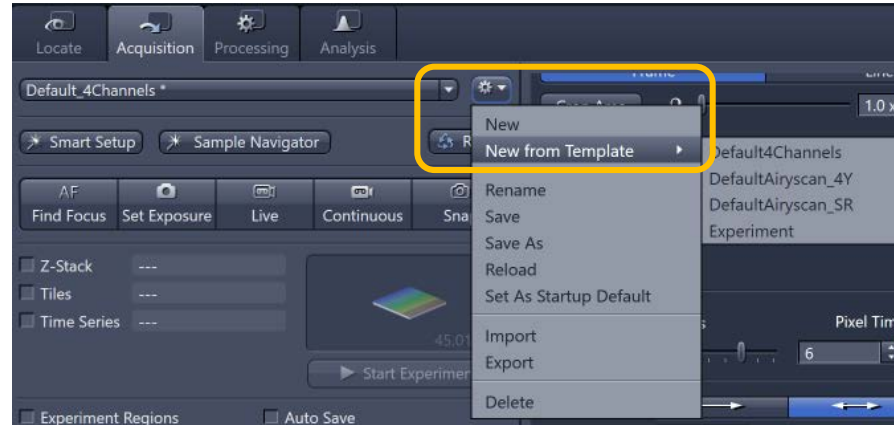
Note: Markers and **Switches** can also be added with TTL pulses coming from 3rd party hardware (electro-physiology, pumps etc) if there's trigger box installed on the system. Or pressing the "Set" marker button during an experiment can send a TTL pulse to a 3rd party equipment.

Show all: You can add **Markers** during your experiment, or change certain acquisition parameters. These will appear as colour-coded square in the gallery format at the specific time points.

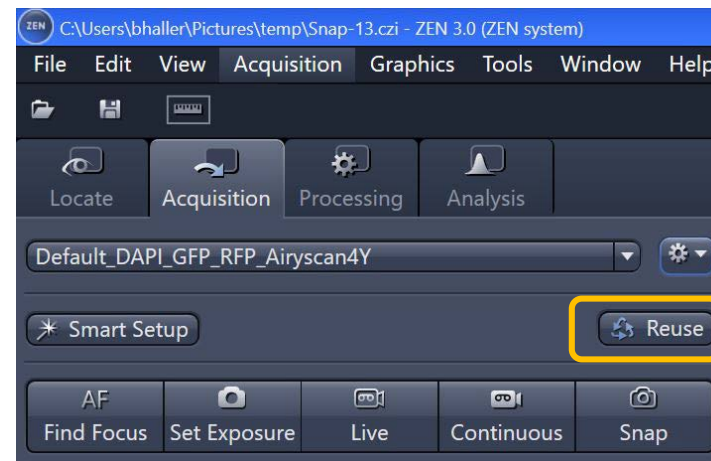
Interactive Switches: You can create a shortcut button to acquire at different time intervals. E.g., longer intervals to monitor sample, then click button for shorter intervals.

11. Re-using Templates:

Note: To protect some default experiment templates from overwriting save a copy under Public documents > Carl Zeiss > ZEN > Templates > Experiment Set up. Users only have access via copying one of those templates.



Note: For comparable data acquisition you can open an image already acquired and use the **Re-Use**-option to apply exactly the same settings and parameters for the next experiment.



12. Crop Area, Stage tool and Navigation with Live:

Both of these options allow to adjust the position and orientation of the following scan.

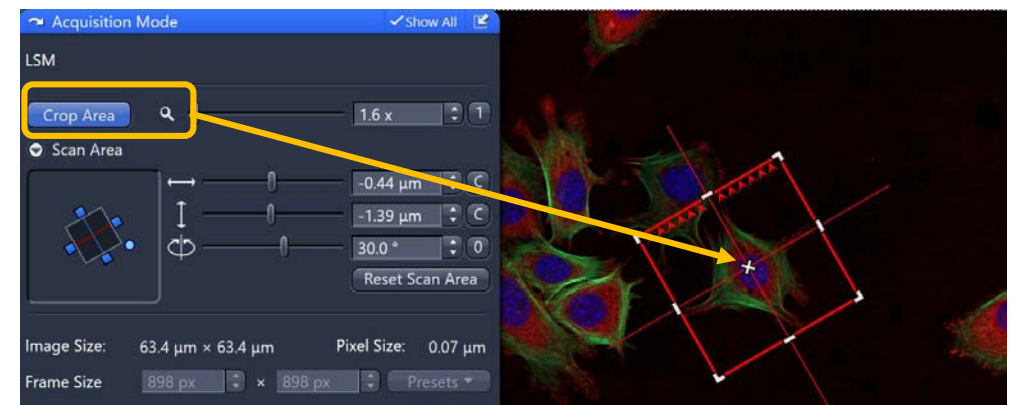
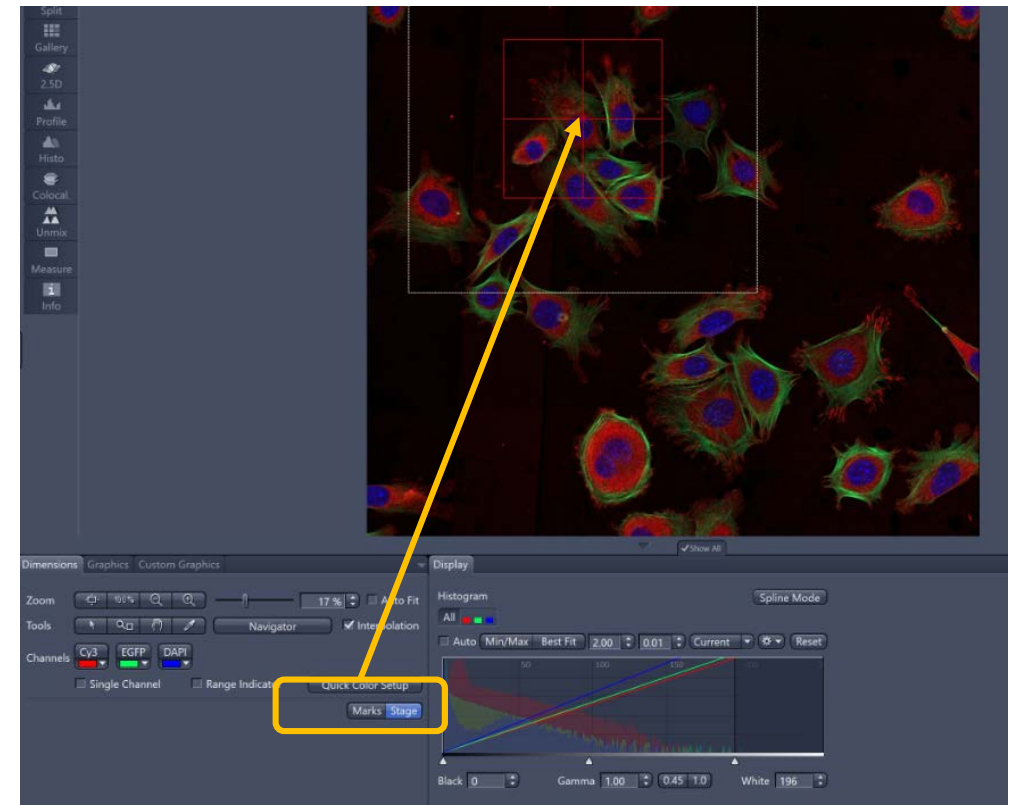
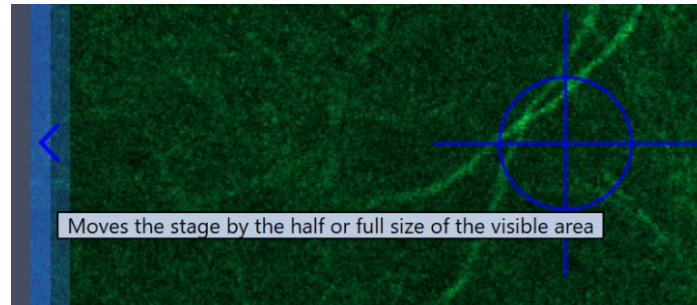
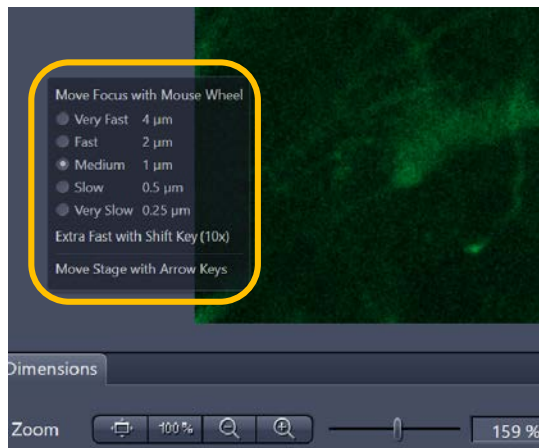
Crop Area defines the zoom, XY position and rotates the field for the next scan.

Stage tool: You can use overview scans to position the stage for the next scan.

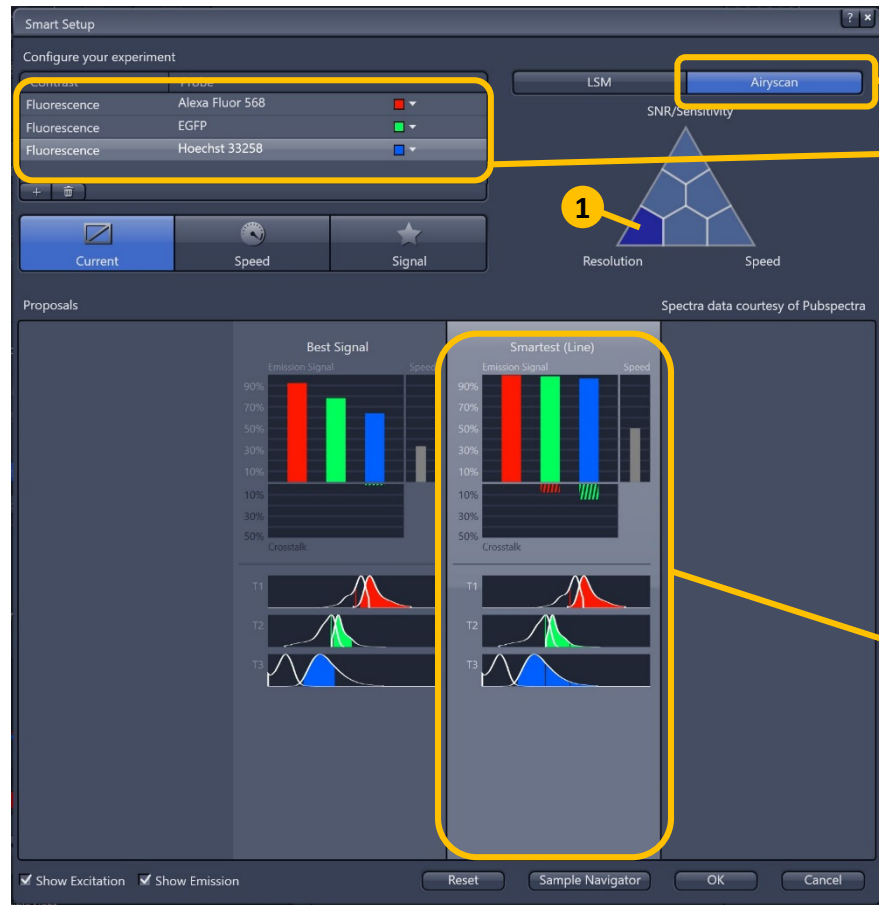
Ctrl + Mouse wheel: Fast access for focussing in Live and Continuous

XY-Navigation: Double click in Live image centers that position. Click at the outer edges of Live image moves stage in that direction.

Note: Best to center the stage first, then crop and rotate to ensure image is acquired *centrally aligned* on the objective.



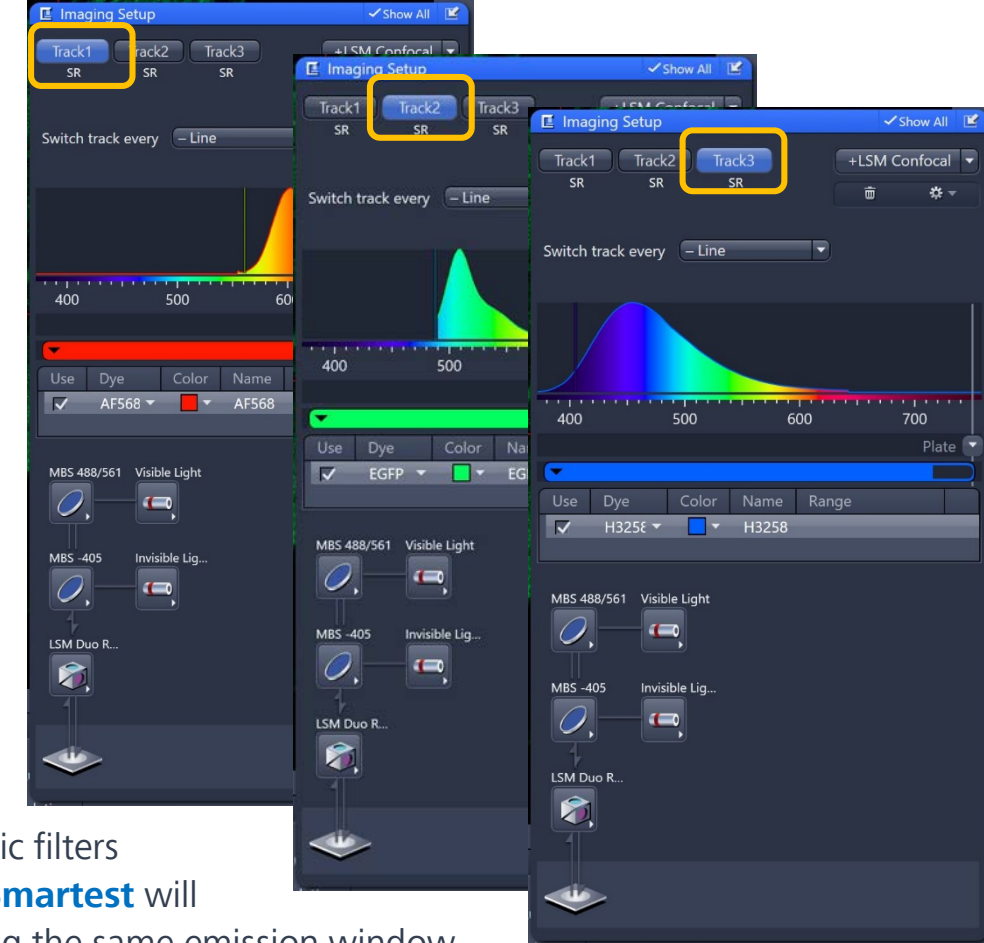
13. Airyscan: SR Mode



Airyscan Mode

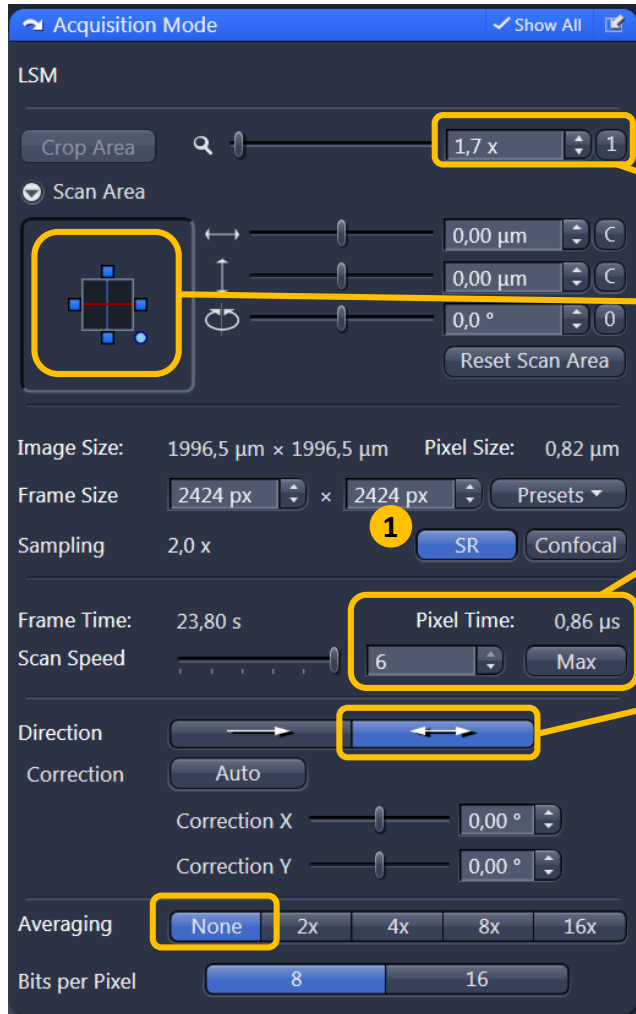
Dye selection

Best Signal will try to use specific filters (so Frame-sequential), whereas **Smartest** will attempt to be faster by measuring the same emission window for all tracks (may not be possible with some fluorophore combinations, in which case it will do a frame-wise multitrack).



SR Mode: (1) Maximum gain in resolution and SNR; Pinhole default 0.2 AU; Total detection area 1.25 AU

14. Optimizing Airyscan: Acquisition Mode and Channel Set up:



Acquisition Mode: Recommended settings for optimal image quality:

Crop Area (Zoom) > 1.7x

Scan Area: stage centered

Maximum Scan Speed

Bidirectional scan and No Averaging

Note: If **SR** mode (1) is active all relevant parameters are optimized for super resolution imaging (2x Nyquist criterion). If **Confocal** mode is active Airyscan is used in confocal mode with improved SNR.

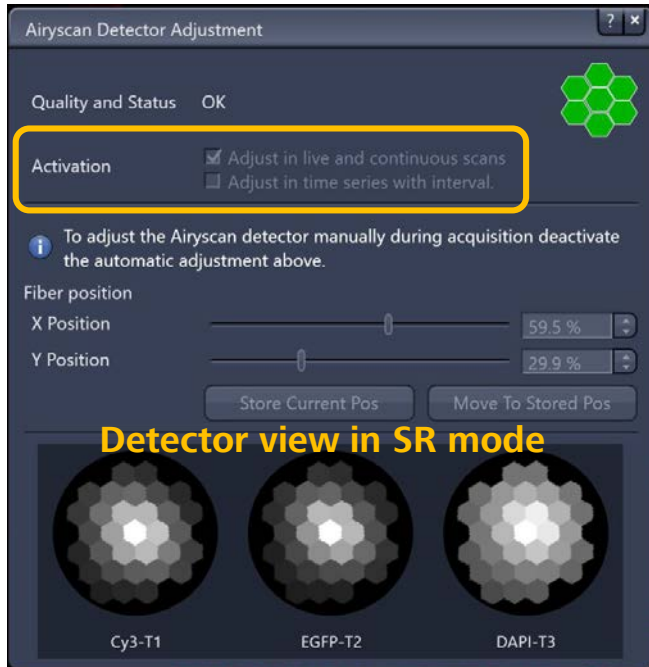
Channel Set up:

Adjust laser power (2) and Master Gain (3) (700 – 900)

Note: With Airyscan you do **NOT** need to fill the dynamic range fully



15. Airyscan: Alignment



Airyscan Detector Adjustment: Open from Tool bar

Default Setting: Adjust in live and continuous scans

Note: Airyscan detector adjustment may again be needed after changes in your light path: crop area, scan area, objective, hardware changes like VSD settings.

In line mode and frame fast mode the detector alignment is the same for all your channels. To perform detector alignment properly you need a sample focussed.

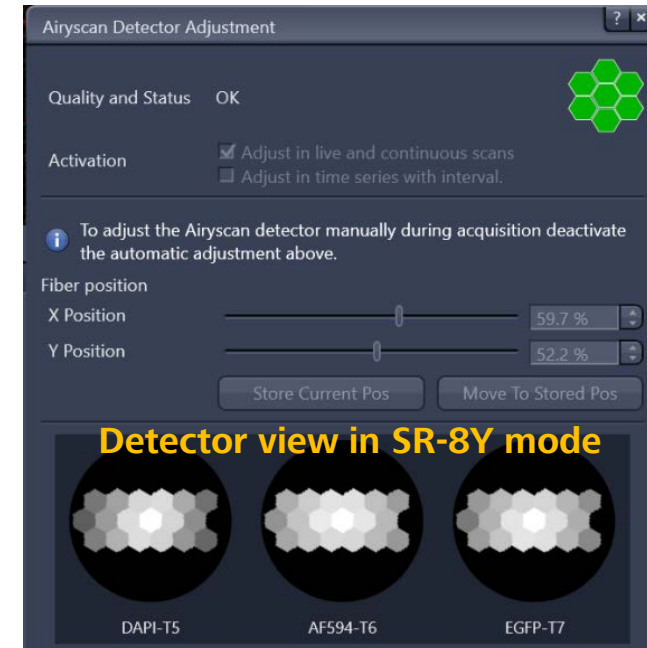
If the detector alignment is not OK (1) check again with Live or Continuous

Alignment tricks:

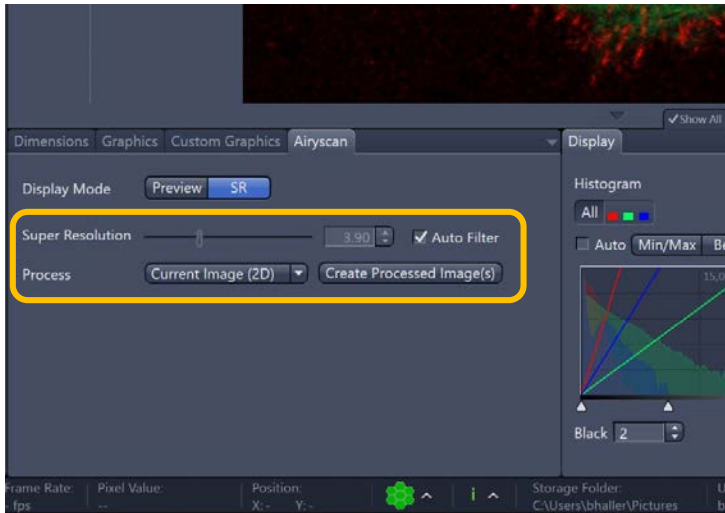
- Refocus specimen
- Increase gain (temporarily)



1



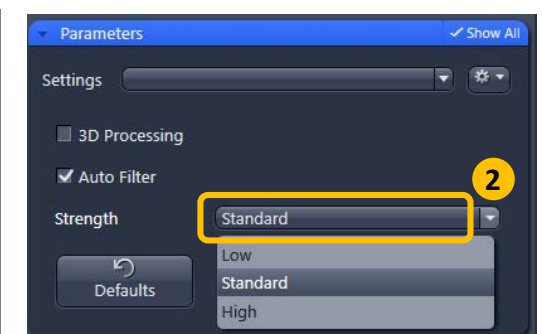
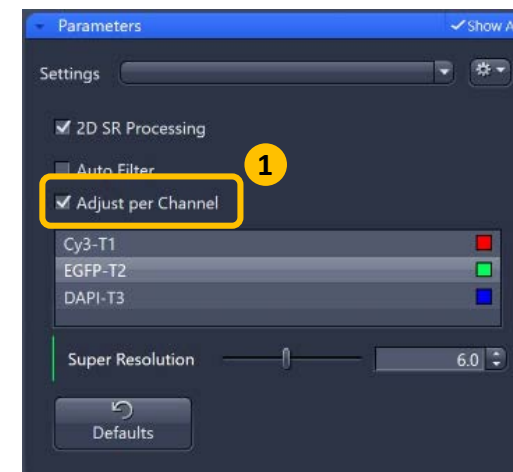
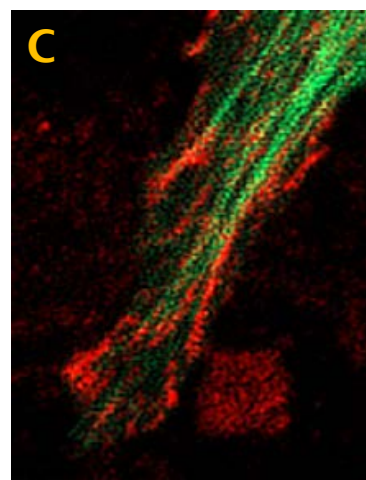
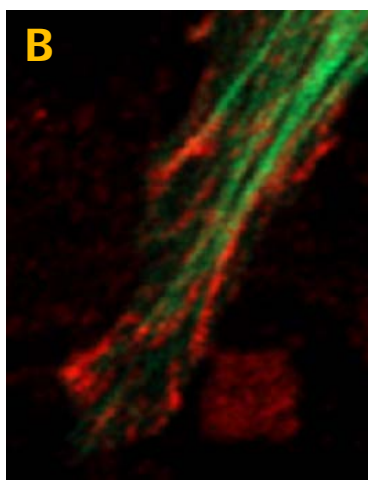
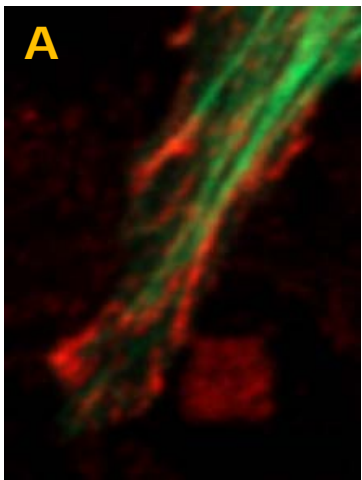
16. Airyscan: Pixel Re-assignment and Deconvolution



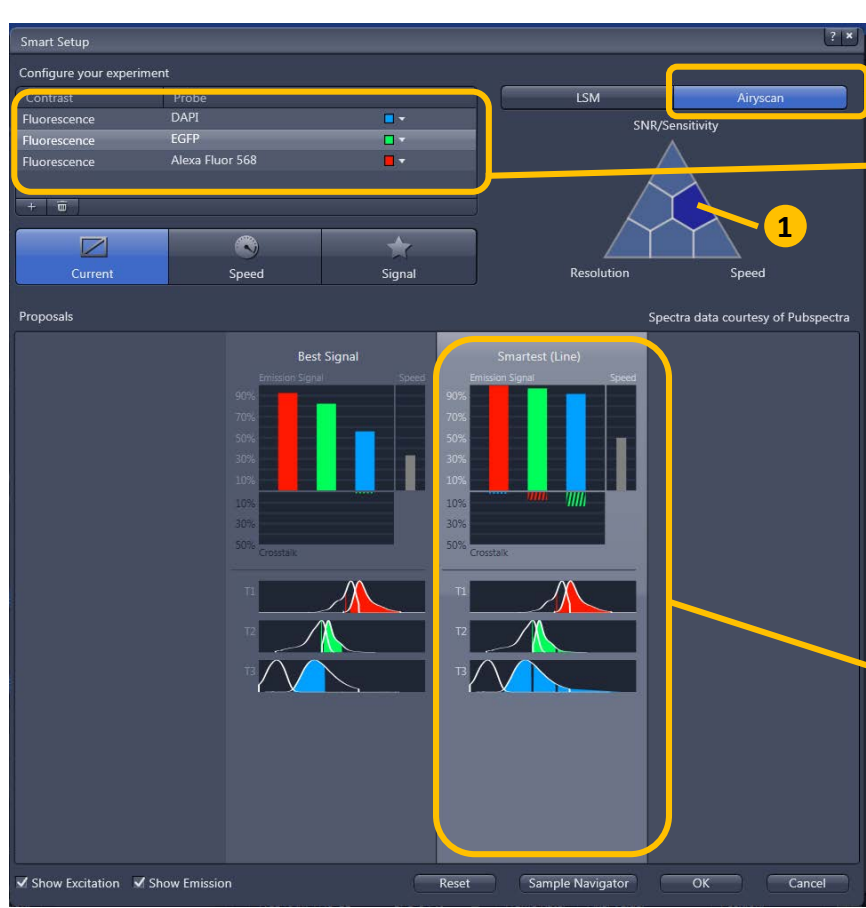
The SR Image represents the pixel re-assigned image + Deconvolution (Wiener filtering). You can adjust the strength of the filter by deselecting auto, and adjusting slider.

- A) If the filter strength of the deconvolution step is set to 1, the image will be pixel re-assignment and weak filtering. (1.4x LSM resolution)
- B) Clicking Auto will add a safe deconvolution with conservative settings, as calculated from the SNR of your image. The resolution improvement is up to 2x in 3D and 2D-SR modes
- C) Over-deconvolved can create „structured noise“.

Note: Airyscan processing should be applied before any other processing options like stitching, MPIs, unmixing. **Processing** tab allows to adjust processing settings for channels individually (1). In the **Processing** tab, you can also choose between 3 **Auto** modes (2). Low is conservative and High is aggressive.



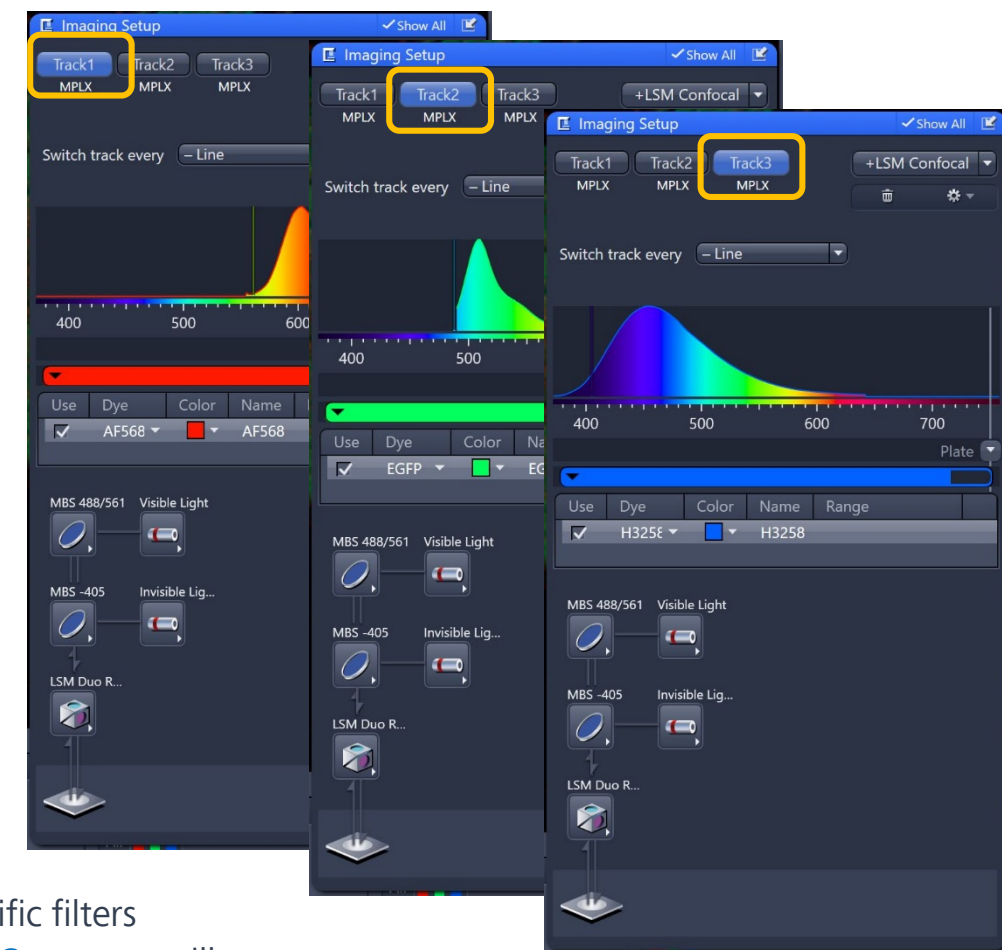
17. Airyscan Multiplexing: SR-4Y and SR-8Y Mode



Airyscan Mode

Dye selection

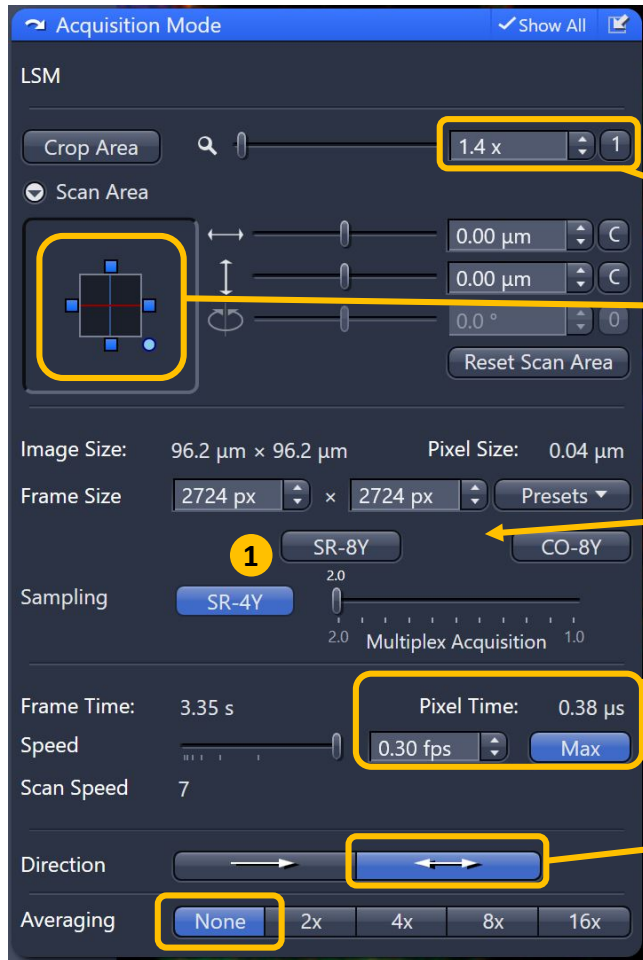
Best Signal will try to use specific filters (so Frame-sequential), whereas **Smartest** will attempt to be faster by measuring the same emission window for all tracks (may not be possible with some fluorophore combinations, in which case it will do a frame-wise multitrack).



SR-4Y and SR-8Y Mode: (1) Increased resolution and SNR; Pinhole default 0.2 AU; Total detection area 1.25 AU

Note: SR-8Y Mode uses excitation beamshaping over y-dimension to allow for 8x parallelization.

18. Optimizing Airyscan in SR-4Y and SR-8Y Mode: Acquisition Mode and Channel Set up:



Acquisition Mode: Recommended settings for optimal image quality:

Crop Area (Zoom) > 1.4x

Scan Area: stage centered



Maximum Scan Speed (SR-4Y mode: 25 fps and SR-8Y mode: 47.5 fps with 512 x 512 pixels)

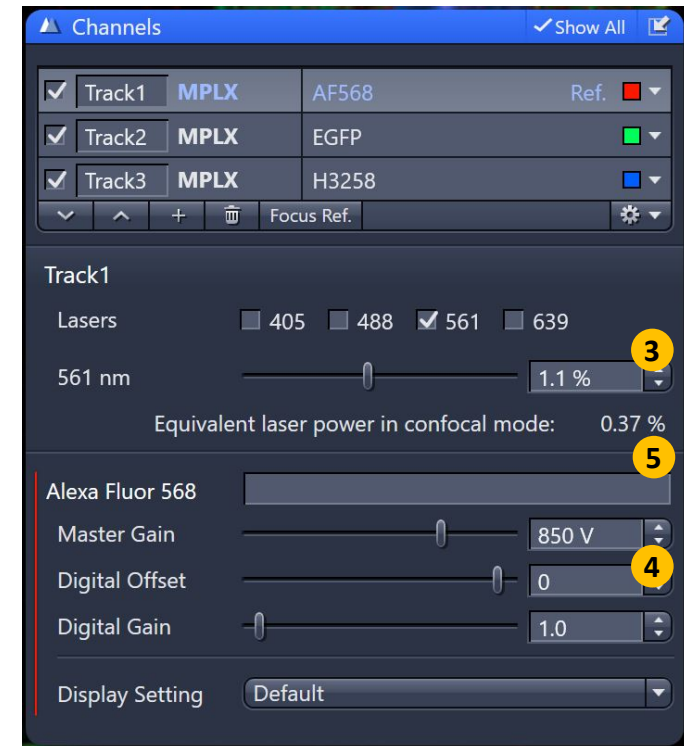
Bidirectional scan and No Averaging

Note: If **SR-4Y** and **SR-8Y** mode (1) is active all relevant parameters are optimized for super resolution imaging (2x Nyquist criterion). If **Confocal CO-8Y** mode (2) is active Airyscan is used in confocal mode (1x Nyquist criterion) with improved SNR.

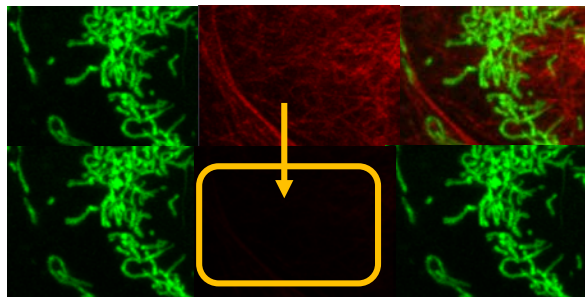
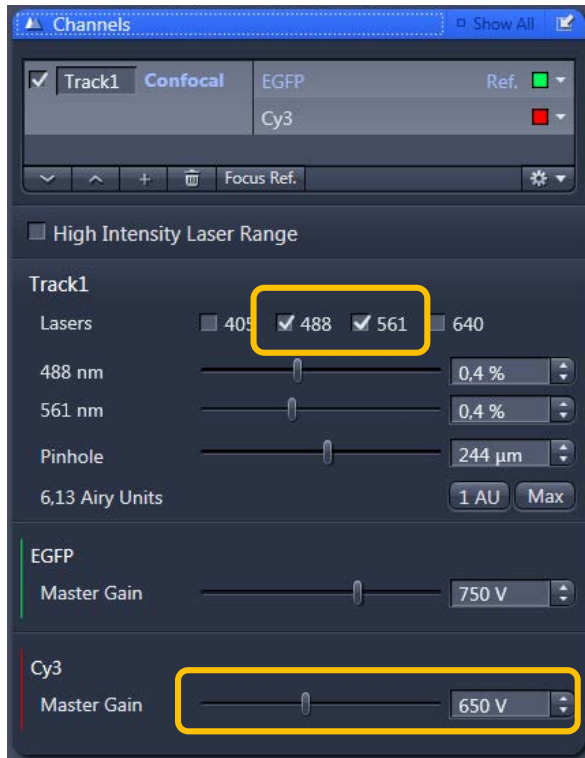
Channel Set up:

Adjust laser power (3) and Master Gain (4) (700 – 900) (5) Shows the equivalent laser power compared to confocal mode.

Note: With Airyscan you do not need to fill the dynamic range fully



I. Experimental Notes

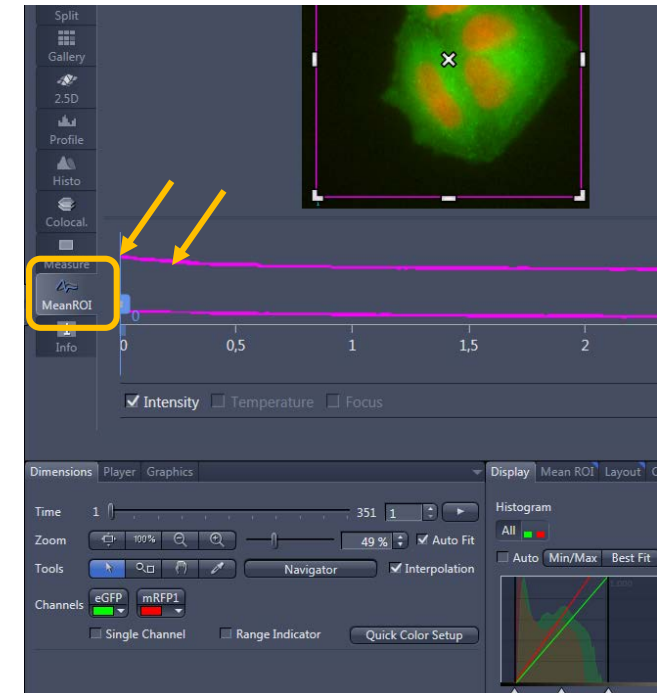


Note: Optimize dynamic range on your brightest treatment / time-point to avoid saturation in part of your data set. Remember to put your strongest fluophore on your weakest signal.

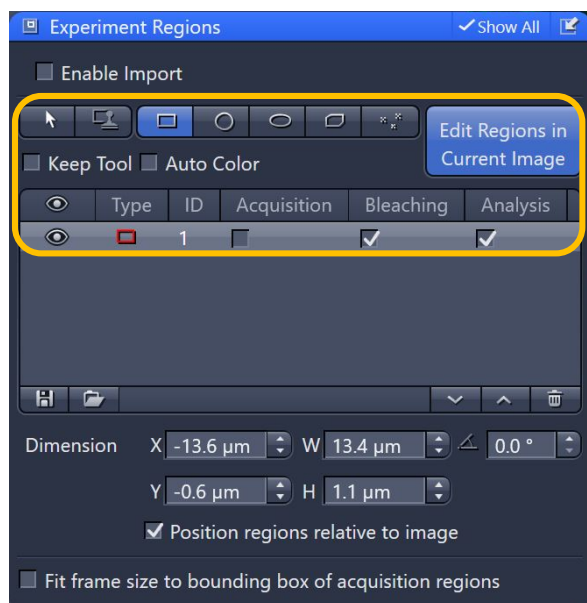
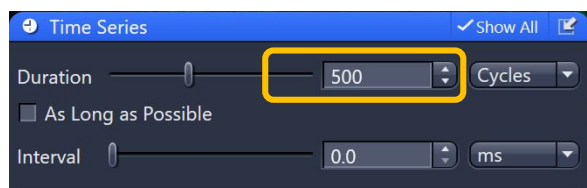
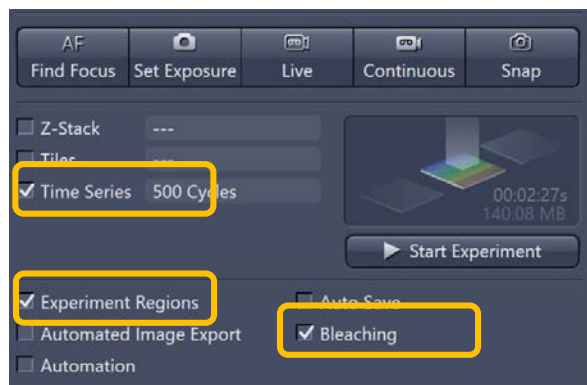
a. Checking and reducing Cross-talk in tracks with multiple channels: Deselect the laser with the longest wavelength – all signal should disappear in that channel. Remaining signal may be cross talk. Reduce either the laser power from the first channel, AND / OR reduce the gain of the second channel. Do not adjust these values when adding the second channel back in. If cross-talk cannot be removed by adjusting gain/laser power, then you may want to use a different imaging strategy (separate tracks).

b. Checking for Photobleaching: Optimize acquisition, select **Time Series**. E.g. 500 cycles at 0.0 ms interval. Click Start Experiment. In Image display, select **MeanROI** tab. Create an ROI region in the image. The ROI intensity versus Time plot will appear for each channel. Check for decrease in intensity with time at a set gain as the gain will affect the apparent bleaching (e.g. 600 for confocal). If a decrease (bleaching) is evident, decrease the laser power until the signal levels out. If no bleaching, you could increase your laser. Stop experiment and adjust gain to fill dynamic range (only after appropriate laser power is determined).

Note: This only helps assess photobleaching of the fluorophores. It does not assess photodamage or phototoxicity in the actual sample. The gain value affects apparent photobleaching. The higher the gain, the greater the apparent photobleaching. Hence advising one gain value while assessing the laser.



II. Time series, Bleaching and Regions (FRAP):



FRAP (Fluorescence Recovery After Photobleaching) can be monitored using **Bleaching**, which should get used in combination with **Time Series** and **Regions**. Intensity profiles for each ROI can be viewed once you have started the experiment.

Create regions across your samples in the **Experiment Regions** tab. Reference and Background regions can be added before or after (if added before, ensure bleaching is unticked for these regions). Create a continuous scanning mode in the **Time Series** tab. E.g. 500 cycles, 0.0 interval. Check your **Acquisition Mode** settings whether your frame rate is high enough for the experiment to perform.

Timed Bleaching:

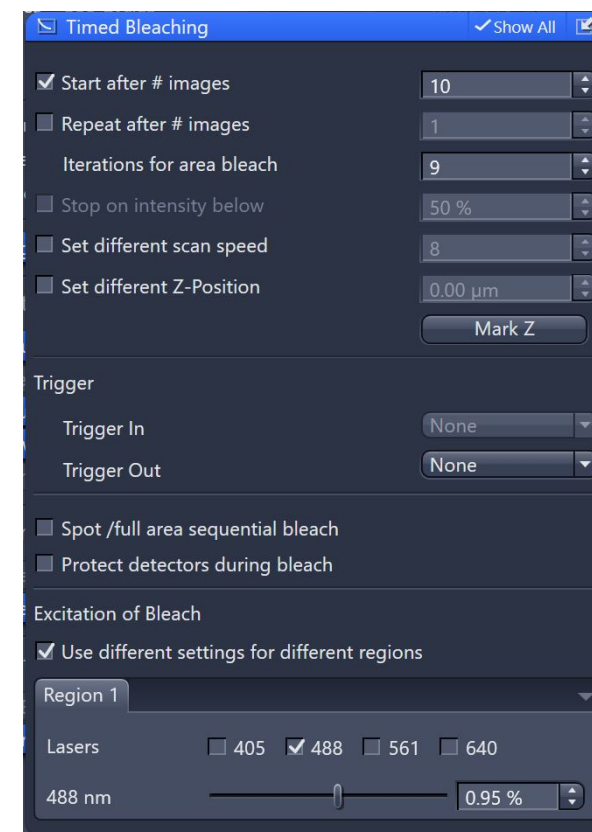
Start after # images: Creates baseline

Repeat after # images: Sets interval between bleaching

Iterations: Total number of scans performed for bleaching of selected region (Frame)

Stop on intensity below: In repeat bleaching, bleaching will stop at the specific intensity

Set different scan speed: Determines pixel dwell time during bleaching event. Bleaching efficiency is increased with increased dwell time. To reduce irreversible photodamage, this can be combined with iterations.



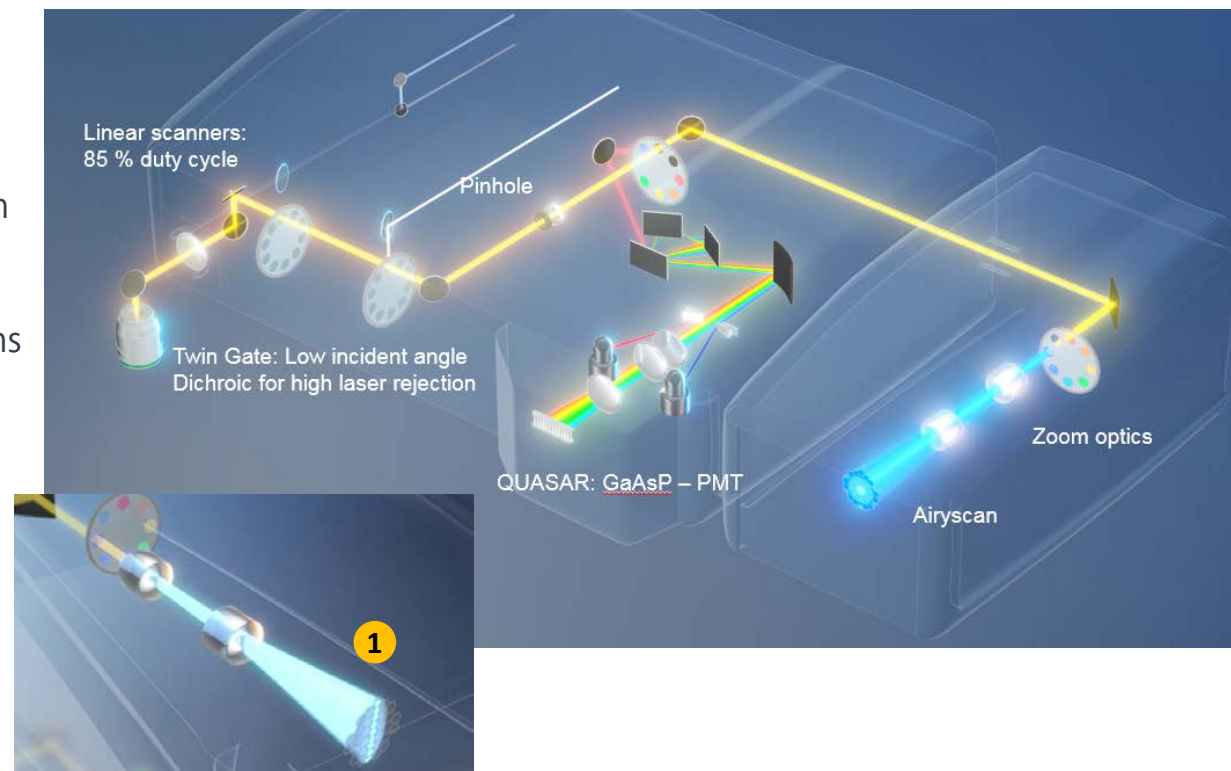
Note: To speed bleaching, create ROIs within a small Y range. In normal mode (not zoom bleach) the scanners scan the whole width (but start at the topmost ROI and stop at the bottom-most ROI).

III. Beampath of LSM 980 and Airyscan 2:

Classic confocal laser scanning microscopes use point illumination to scan the sample sequentially. The microscope optics transform each point to an extended Airy disk (Airy pattern). A pinhole then spatially limits this Airy disk to block out-of-focus light from reaching the detector. Closing the pinhole gives higher resolution, but at the price of detecting fewer photons – and these photons cannot be brought back by e.g. deconvolution.

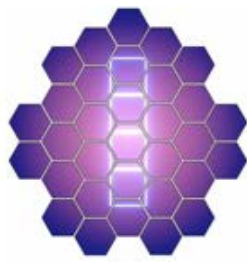
Airyscan 2 is an area detector with 32 concentrically arranged detection elements. This allows you to acquire more of the Airy disk at once. The confocal pinhole itself remains open and does not block light, thus more photons are collected. This produces much greater light efficiency while imaging. Airyscan 2 gives you a unique combination of gentle super resolution imaging and high sensitivity.

With Airyscan 2, the excitation beam can also be shaped to allow the excitation and detection of up to 8 pixels in parallel (1).



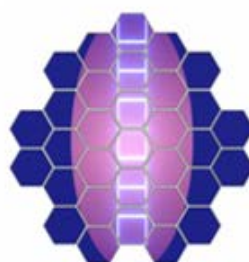
SR Mode

120 x 120 x 350
4.7 fps



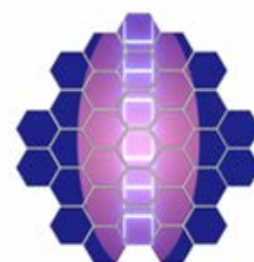
SR-4Y Mode

140 x 140 x 450
25 fps



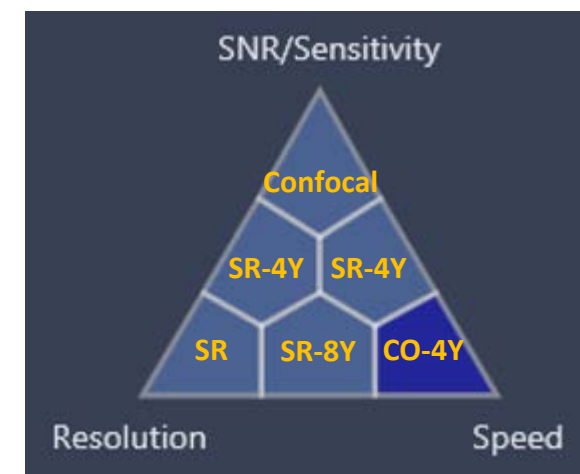
SR-8Y Mode

120 x 160 x 450
47.5 fps



CO-8Y Mode

180 x 120 x 550
34.4 fps



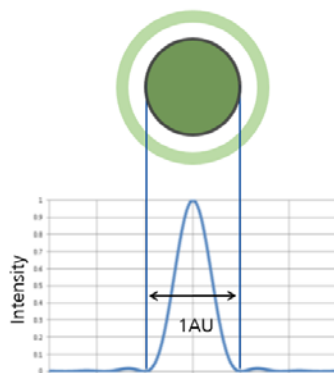
Resolution X x Y x Z in nm and Max **Frame rate** 512 x 512 pixel

Representation of **Airyscan Mode** in **Smart Set up**

IV. Basic Principle of Airyscanning:

Classic Airyscanning

Superresolution and Sensitivity in a Nut Shell



If a point emitter is imaged with an optical instrument, the light is distributed into a pattern: **PSF**

Airy disc: **1 AU** is the diameter of the zero order maximum

A pinhole can narrow the detection PSF, resulting in 1,4x better resolution than wide field

Closing the pinhole increases resolution but reduces detection efficiency

$$\text{Effective PSF} = \text{PSF}_{\text{illumination}} \times \text{PSF}_{\text{detection}}$$

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8/19/2019

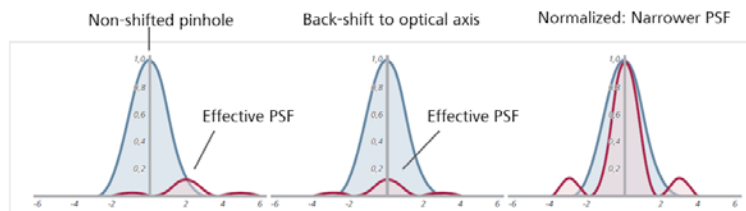
Classic Airyscanning

Displaced Pinhole



"Displaced" pinhole / "delocalized" pinhole

A displaced pinhole would get significantly less signal since a point source at the center of the excitation PSF will not be optimally detected. And vice versa.



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Classic Airyscanning

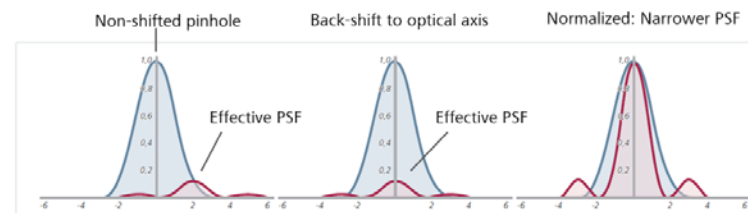
Displaced Pinhole



"Displaced" pinhole / "delocalized" pinhole

Captured by displaced pinhole is a proportion of higher frequencies

$$\text{Effective PSF} = \text{PSF}_{\text{illumination}} \times \text{PSF}_{\text{detection}}$$

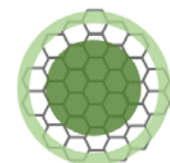


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Classic Airyscanning

Displaced Pinholes: Aisyscan



32 pinholes : Aisyscan; 31 of them "displaced"

Exact shift of effective PSF is known

Pixel Reassignment (Sheppard sum) ends up with

- increased signal
- better signal to noise (SNR)
- sharper images: disproportionately higher amounts of better localized emitters

Major part in resolution gain is achieved by better SNR:
Pixel dwell time of 0.30 μs ...0.6 μs !

DCV: Wiener filtering – better than confocal images + DCV

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IV. Basic Principle of Airyscanning:

Classic Airyscanning

Scanning the Airy Disc

*White paper; Sept 2014

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Classic Airyscanning

Sheppard Summing and Pixel Reassignment

...His areas of research are in optics, microscopy and imaging, including confocal and multiphoton microscopy, diffraction, 3D imaging and reconstruction, super resolution, beam propagation, and pulse propagation...

*Data: Wikipedia

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9/30/2019

Confocal Laser Scanning

We always talk about Airy Discs

...Among other accomplishments he was the first to write a full theoretical treatment in 1835 explaining an optical phenomenon known today as the Airy Disc. 180 years later ZEISS honours his legacy by introducing a new Technology Note detailing Airyscan detector.

1. Mirror
2. Emission filter
3. Zoom optics
4. Airy disc
5. Airyscan detector

Sir George Biddell Airy
*1801 + 1892
Famous British mathematician and astronomer

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8/20/2019

Airyscanning

Wiener Filter

For signal processing, the Wiener filter is ... published in 1942 as a classified document. Its purpose is to **reduce the amount of noise present in a signal by comparison with an estimate of the desired noiseless signal**. Wiener developed the filter at the Radiation Laboratory at MIT to predict the position of German bombers from radar reflections. It is necessary to predict the future, because by the time the shell reaches the vicinity of the target, the target has moved, and may have changed direction slightly. ...

The unmanned V1's were particularly easy to model, and on a good day, American guns fitted with Wiener filters would shoot down 99 out of 100 V1's as they entered Britain from the English channel, on their way to London.

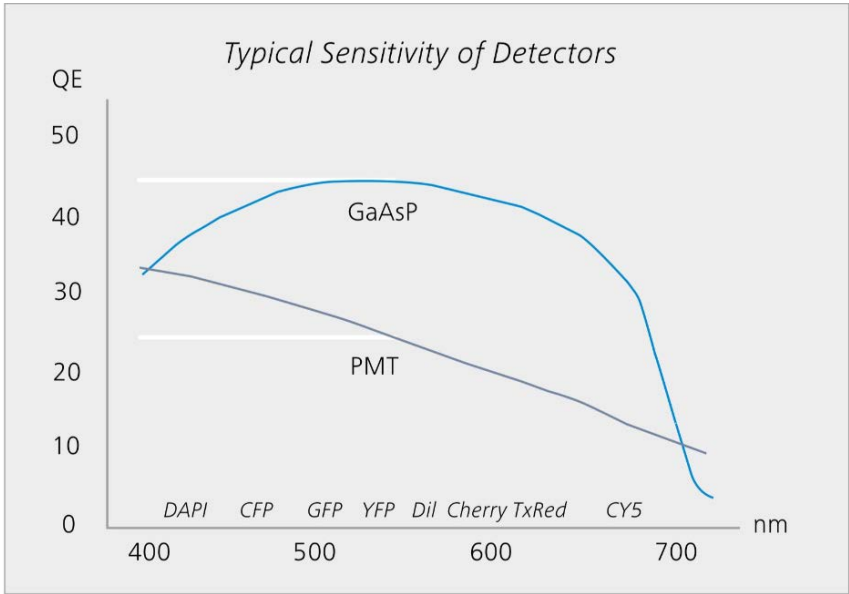
What emerged was a mathematical theory of great generality—a theory for predicting the future as best one can on the basis of incomplete information about the past. It was a statistical theory that included applications that did not, strictly speaking, predict the future, but only tried to remove noise.

Prof. Norbert Wiener
*1894 + 1964
Famous American mathematician and philosopher

Carl Zeiss Microscopy GmbH

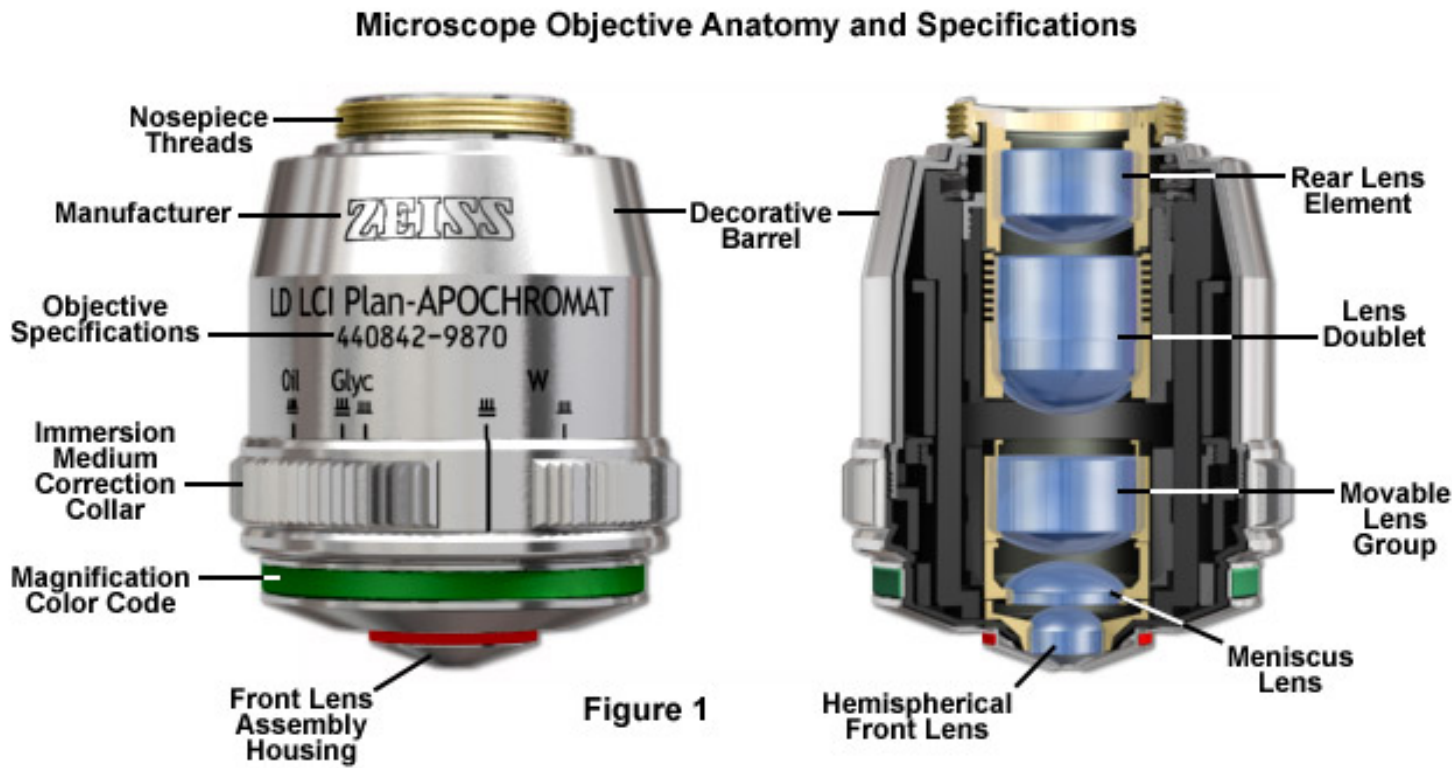
9/3/2019

V. Typical Sensitivity of Detectors:



PMT: Photomultiplier tube
GaAsP: Gallium arsenide - gallium phosphide alloy

VI. Objective Anatomy and Specifications:



VII. Optical Equipment:



EC Plan-NEOFLUAR 2.5x/0.085 (WD=8.8mm) (sample navigation)

Plan-APOCHROMAT 10x/0.45 (WD=2.1mm)

Plan-APOCHROMAT 20x/0.8 (WD=0.55mm)

***LD LCI Plan-Apochromat 25x/0.8 Imm Corr DIC** for water, silicone oil, glycerine or oil immersion (D=0-0.17mm) (WD=0.57mm at D=0.17mm)

***LD LCI Plan-Apochromat 40x/1.2 Imm Corr DIC** for water, silicon oil or glycerine immersion (CD=0.15-0.19mm) (FWD=0.41mm at CG=0.17mm)

***C Plan-Apochromat 63x/1.4 Oil DIC** (WD=0.14mm), UV-VIS-IR

Calibration objective LSM

*) objective with Strehl ratio > 0.9 for super resolution with Airyscan 2

Strehl Ratio

The Definition of Perfect Optics.



The **Strehl ratio** is a measure of the quality of optical image formation, originally proposed by **Karl Strehl (1864-1940)** after whom the term is named. Used variously in situations where optical resolution is compromised due to lens aberrations or due to imaging through the turbulent atmosphere, the **Strehl ratio** has a value between 0 and 1, with an unaberrated optical system attaining the value of unity.

Strehl of 1 equals perfect optics

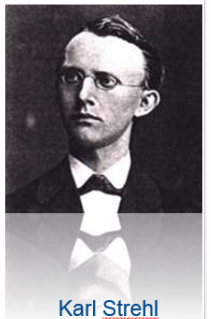
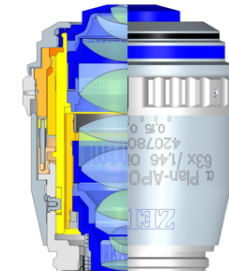
Strehl of > 0.9 allow to go for super resolution

Practically Strehl values > 0.9 are difficult to achieve!

Strehl of 0.8 means aberrations of $\Lambda/4$. This leads to refraction limited images

$$S = |\langle e^{i\phi} \rangle|^2 = |\langle e^{i2\pi\delta/\lambda} \rangle|^2$$

(Untersuchung eines Mikroskopobjektives, Z. wiss. Instrumentenkd., 25, p. 3-10, 1905)



Karl Strehl

VIII. How it all began:

How it all began...

Jena in 1848. Carl Zeiss (1816-1888)



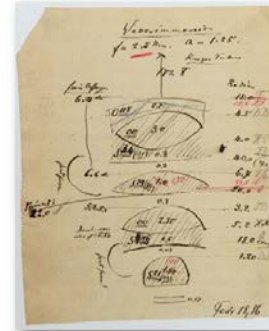
Karl Zeiss, Friedrich Wölff, August Reibsch, Wilhelm Weber, Hermann Pöppel, Gustav Reibsch.
Optische Werkstatt von 1848.

Ernst Abbe

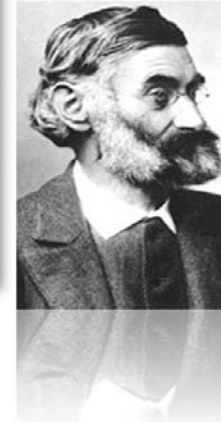
Brilliant Physicist, Social Reformer, ZEISS CEO (1840-1905)



$$d = \frac{\lambda}{2 \sin \alpha}$$



APOCHROMAT NA 1.30
Homogeneous immersion

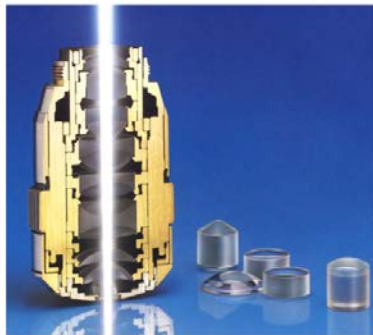


„Glastechnisches Laboratorium Schott & Genossen“

Otto Schott (1851-1935).



SCHOTT
glass made of ideas



Jena

Optics Workshop



~1900



~1921