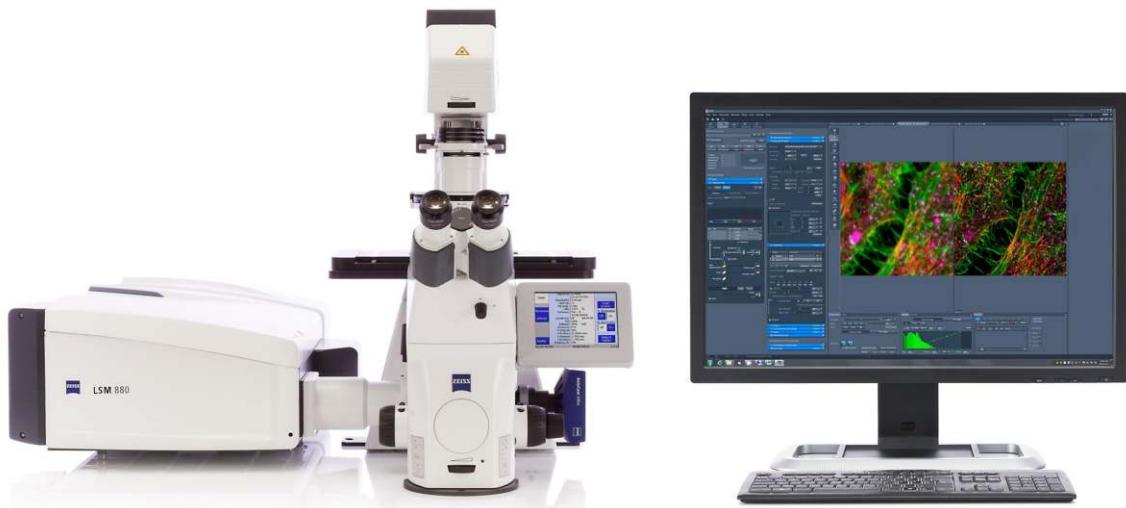


Zeiss Confocal Microscope - LSM880



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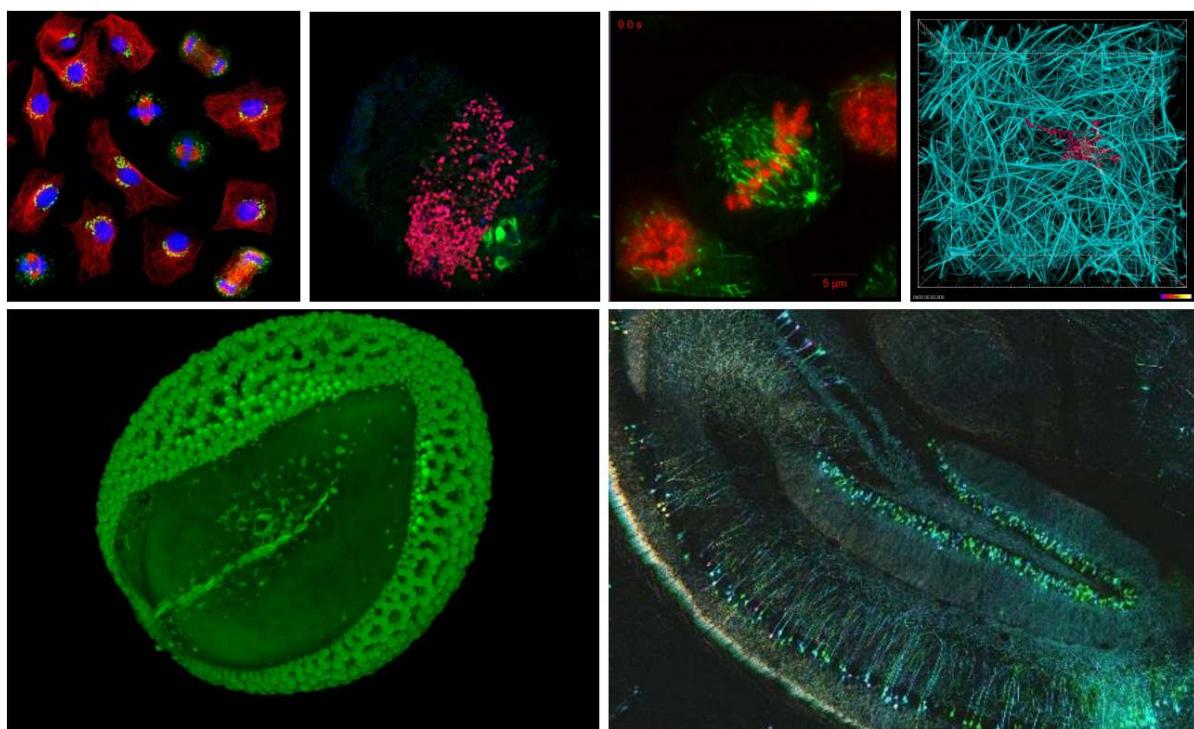
2.5 Tile Scan

2.6 Airyscan Imaging

Introduction

1.1 Applications of Confocal Microscope

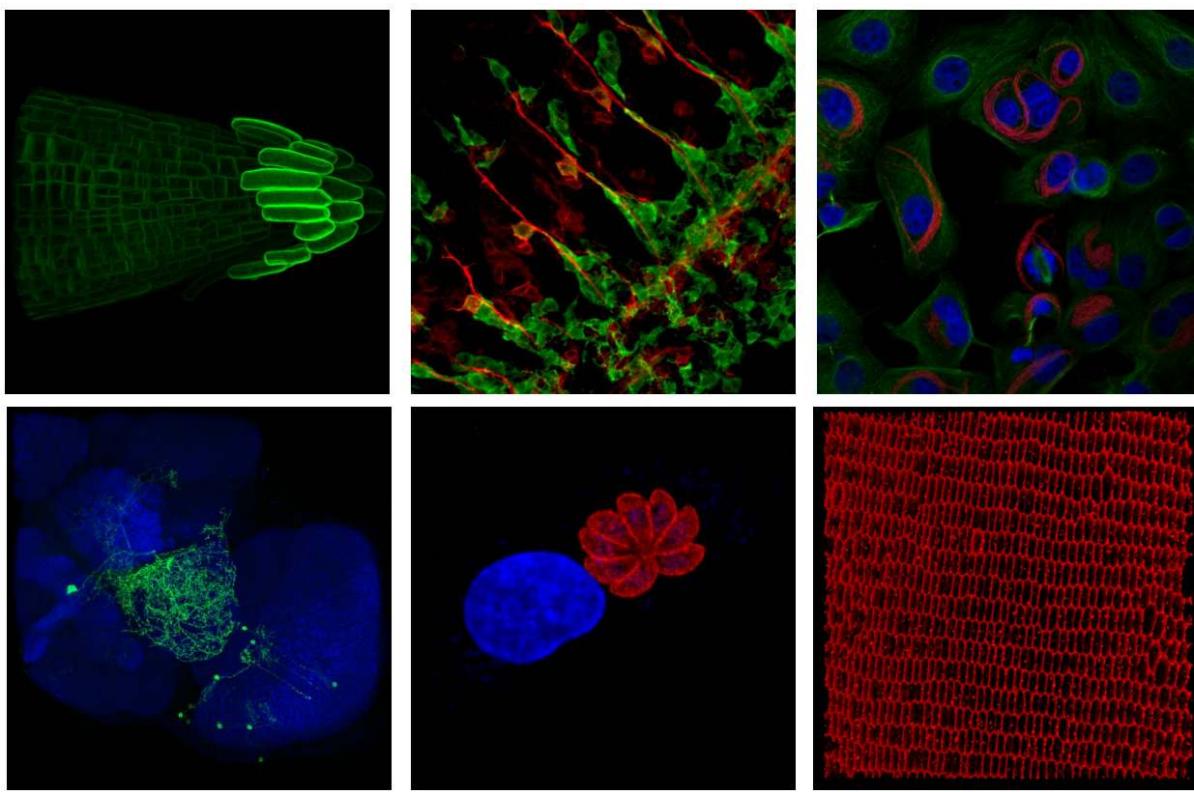
As a high quality, multi-dimensions, large view-field imaging technique, confocal microscope is widely used in almost all field of Biological and Medical Research, including Agricultural Research, Alzheimer, Cancer, Cell Science, Biochemistry, Botany, Immunology, Developmental Biology, Ecology, Epidemiological, Diseases, Evolutionary Biology, Food design, Genetics, HIV, Material Quality Control, Material Sciences, Medicine, Membrane Research, Neurobiology , Parasitology, Pharmacology, Physics, Plant Biology, Proteomics, Signal Transduction, Virology...



Images of Basic Functions

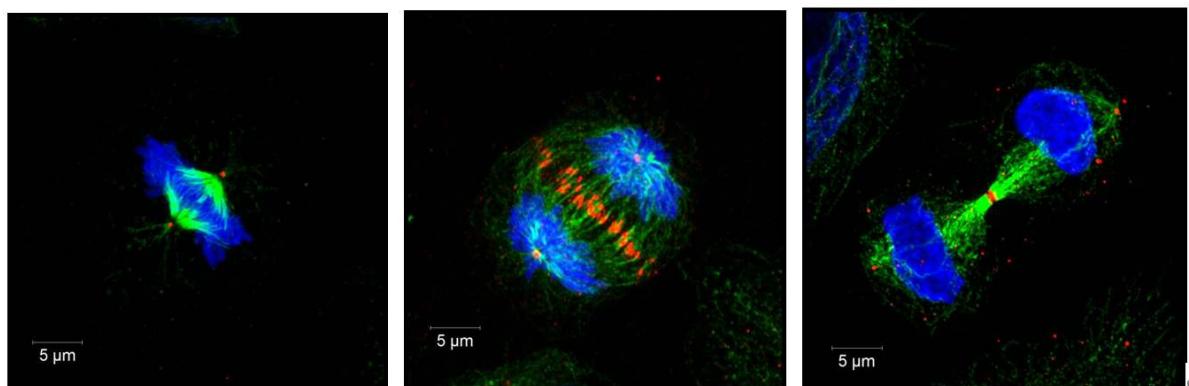
2D Fluorescent Images

High quality images of single or multiple channels

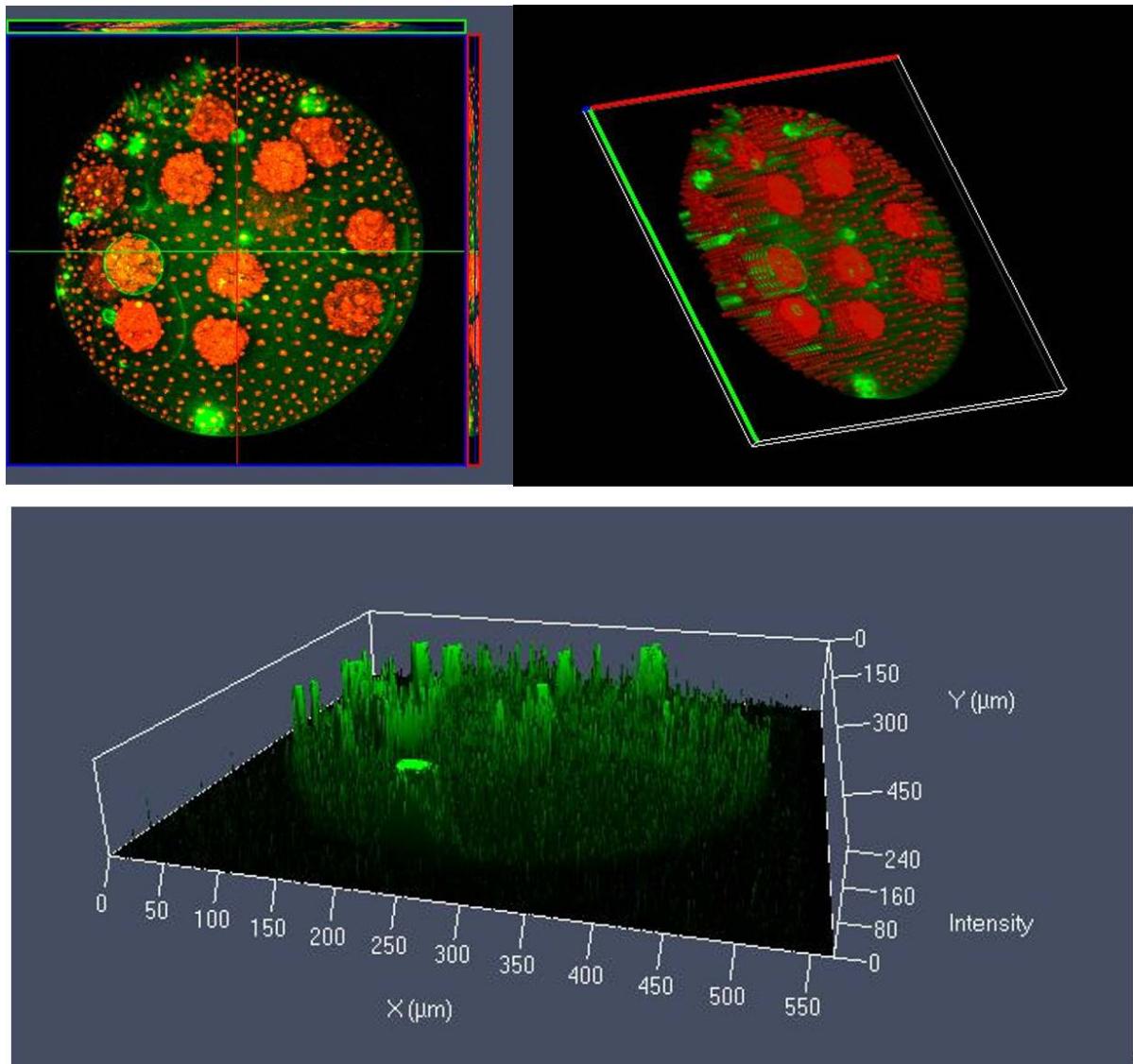


Z-stack

Images at different focal depths (Z - planes) to reconstruct a three - dimensional representation of the sample

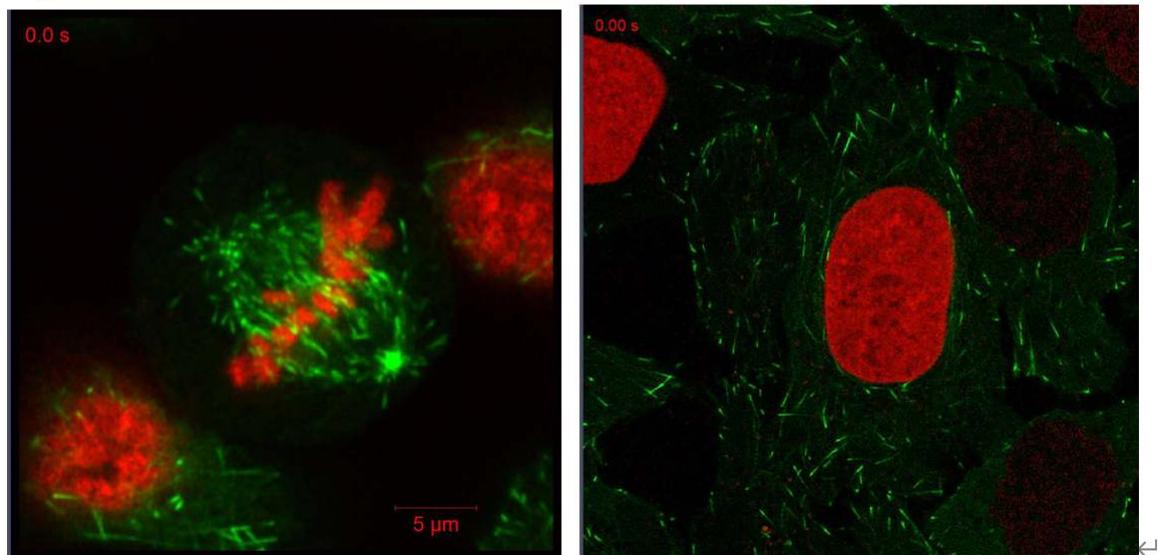


Ortho Function: X-Z, Y-Z cross section



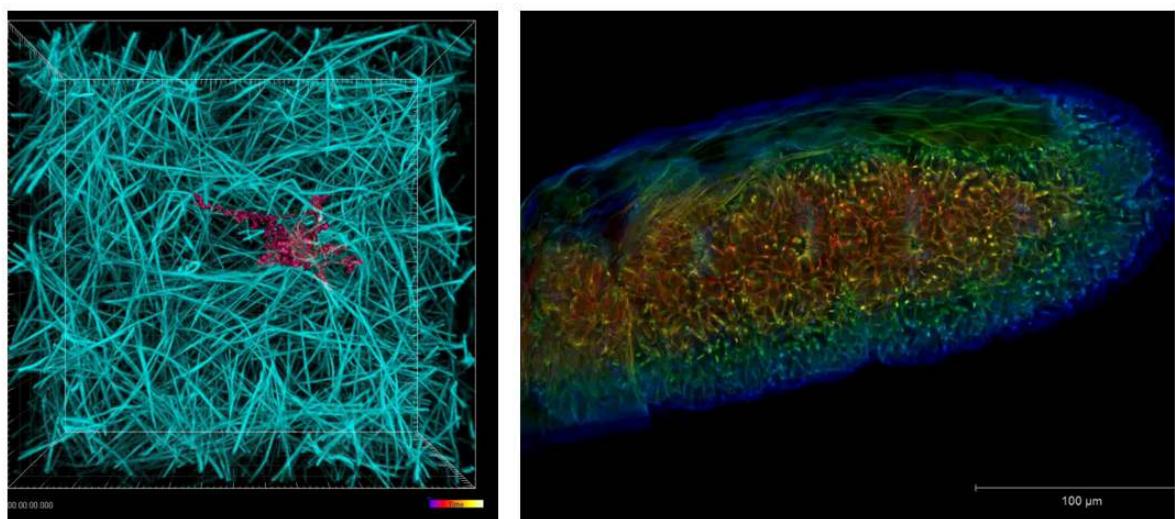
Time series

Sequential images over time to observe dynamic processes in biological samples



4D dynamic structure

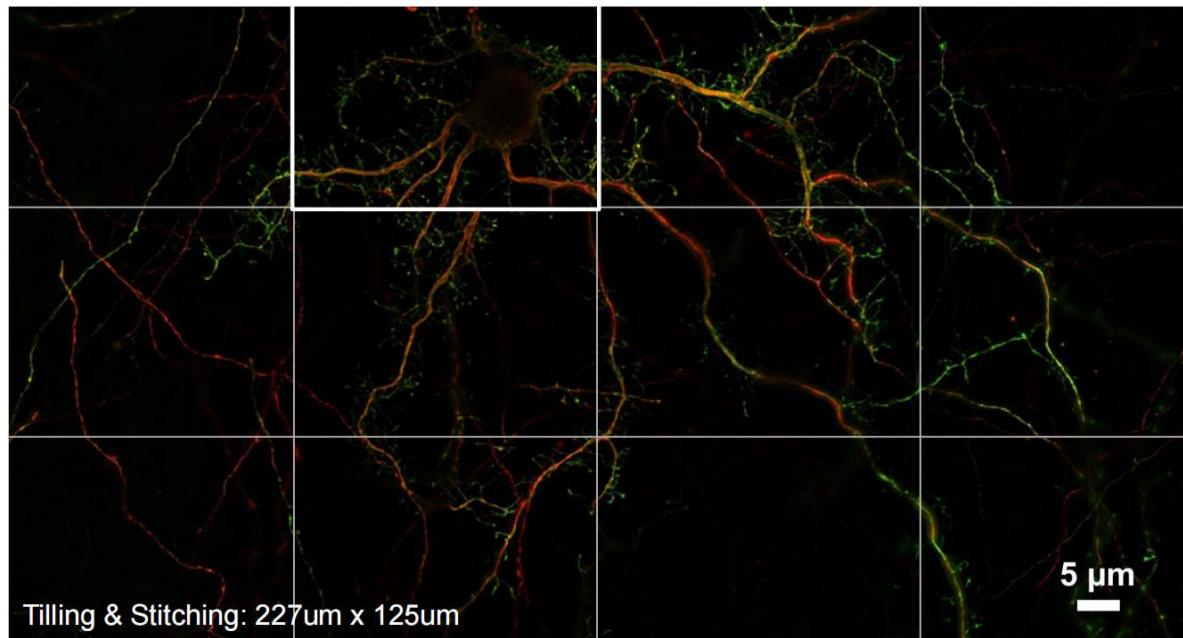
A three - dimensional structure that changes over time, allowing the observation of biological processes in both space and time



Tile scan

multiple adjacent images that are stitched together to create a large - field - of - view, high - resolution

mosaic of a large sample



Examples of basic analysis

Measurement and analysis: Intensity

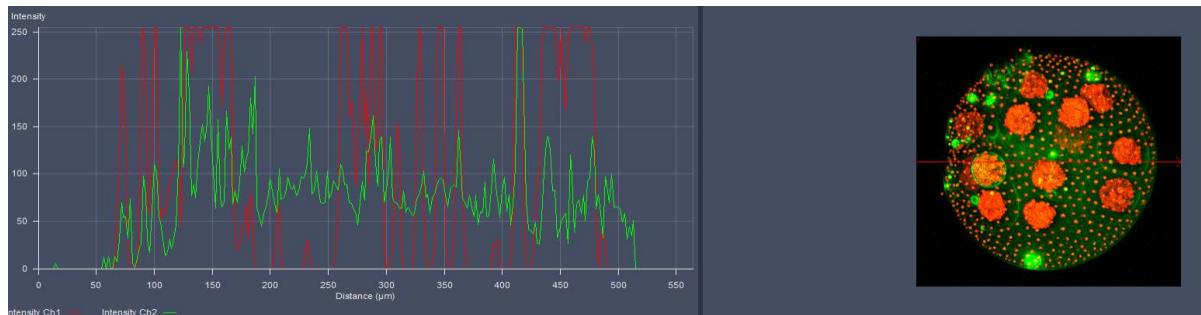
quantifies and evaluates the intensity distribution of fluorescent signals, enabling analysis of signal

strength, distribution patterns, and changes in biological samples



Profile

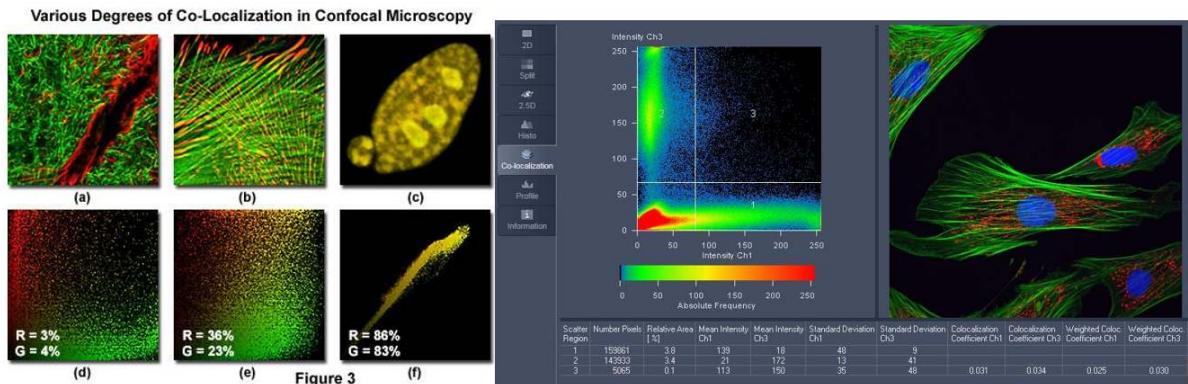
Analyze intensity distribution of specific markers, when using medicine to stimulate bioplast or suspension cells



co-localization

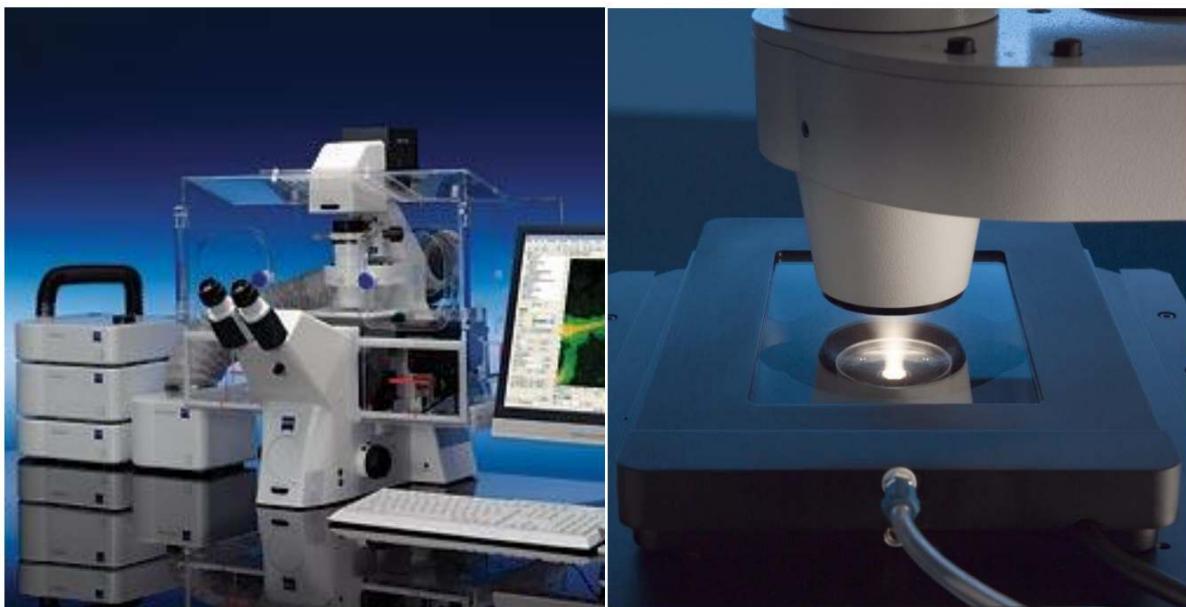
determines the spatial overlap between different fluorescently labeled structures or molecules within

a sample, indicating potential interactions or associations



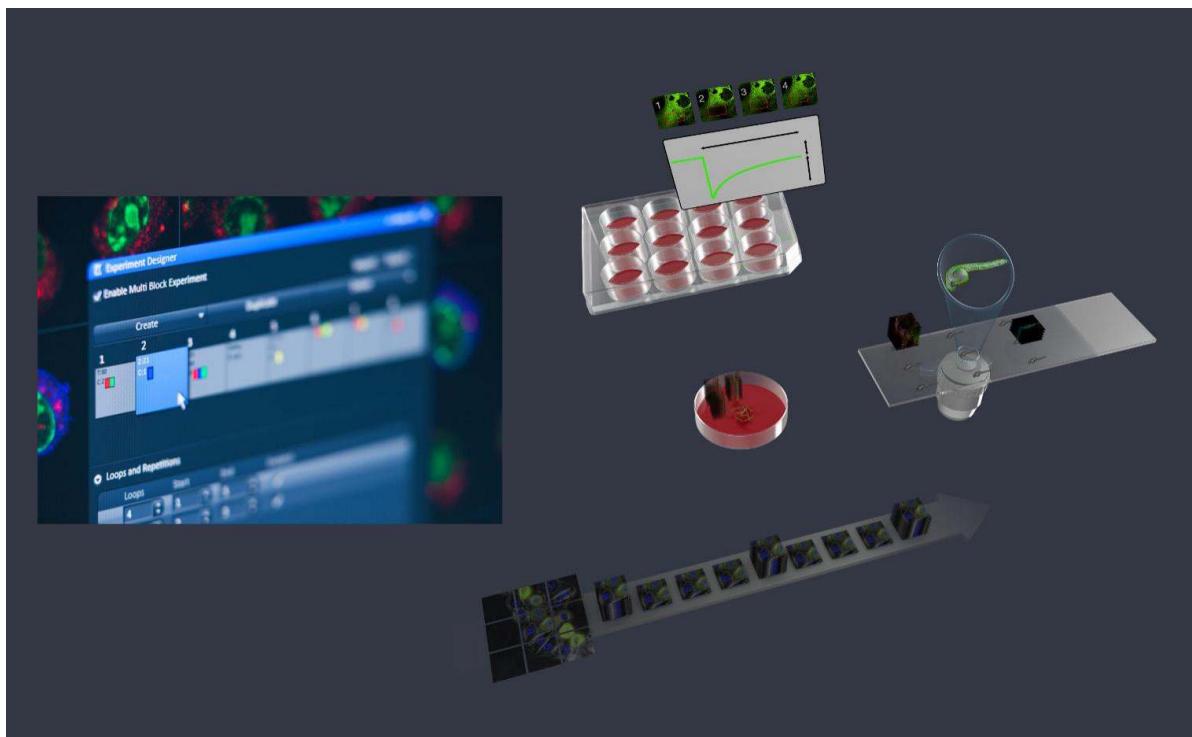
Expanding Function: Live cell Incubator

Culture and imaging of live cells



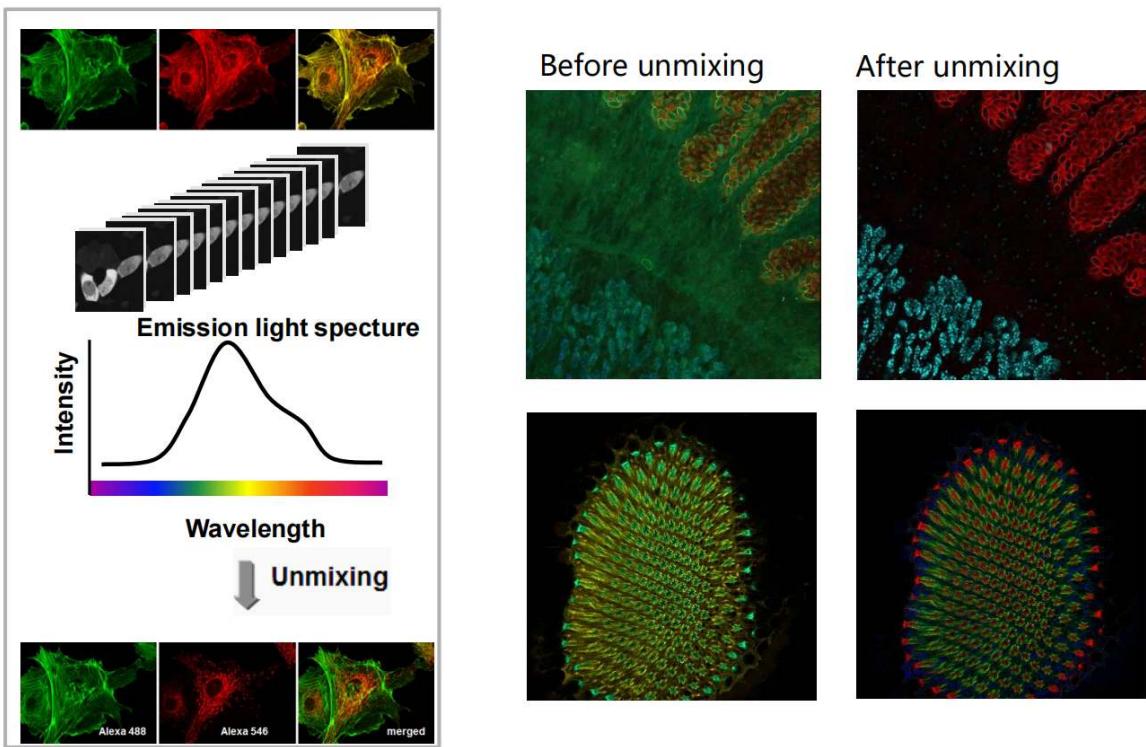
Experiment designer

6D long term live cell imaging



Linear unmixing

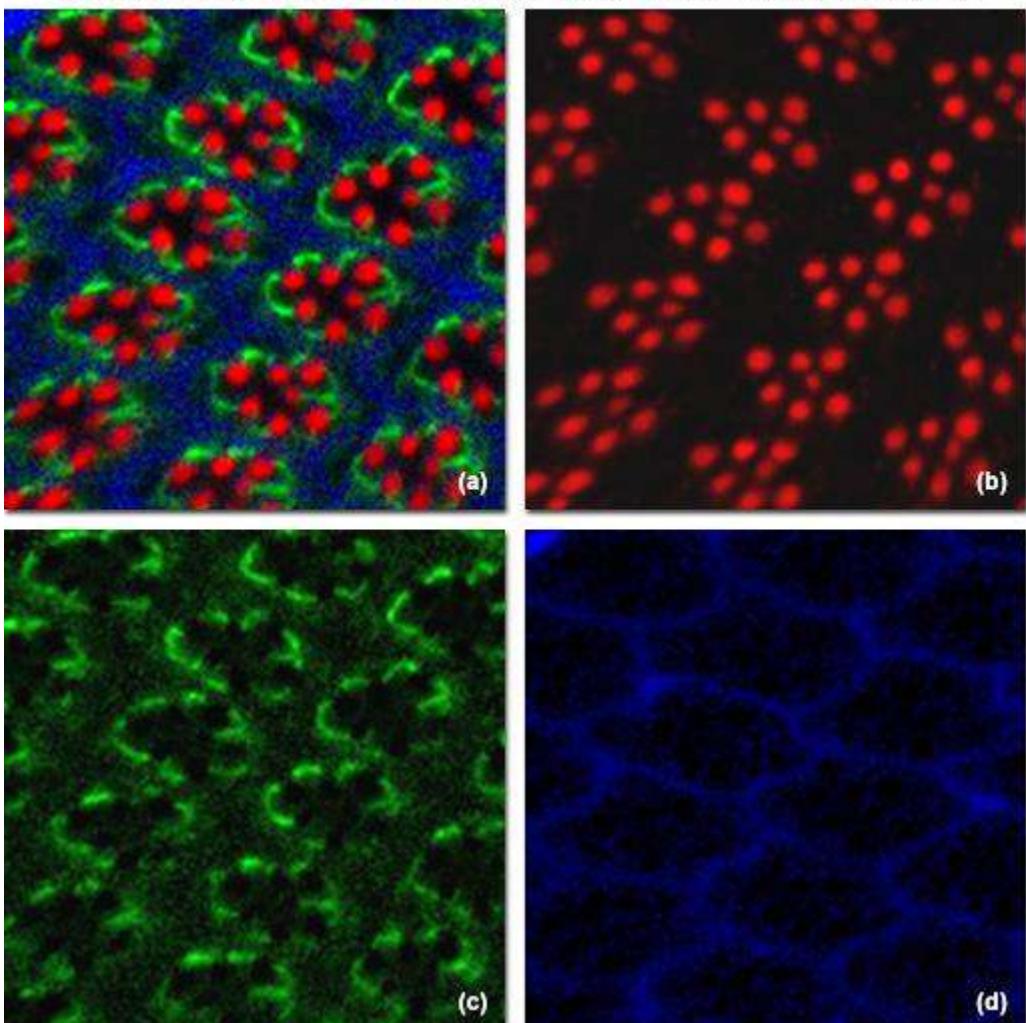
Minimize the influence of fluorescence cross-talk to the greatest extent



Spectral splitting

Remove spontaneous fluorescence

Spectral Imaging and Linear Unmixing to Remove Autofluorescence



FRET (Fluorescence Resonance Energy Transfer)

FRET refers to a non-radiative, dipole-dipole pairing process that occurs when two fluorescent groups meet certain conditions. Through this process, energy is transferred from the excited-state fluorescent donor to the fluorescent receptor at a very close wavelength.

The conditions for FRET to occur:

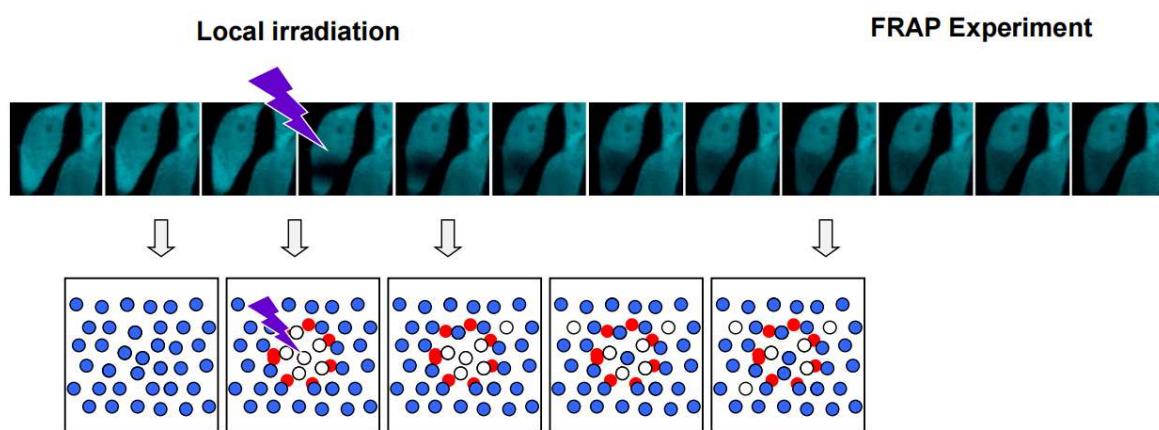
- The distance between the donor and the acceptor is within 2-10 nm
- The emission wavelength of the donor is consistent with the excitation wavelength of the acceptor
- The polarity of the donor and the acceptor is the same
- The fluorescence lifetimes of the donor and the acceptor are sufficient

Common FRET combinations:

CFP/YFP
CFP/dsRED
BFP/GFP
GFP/dsRED
YFP/dsRED
Cy3/Cy5
Alexa488/Alexa555
Alexa488/Cy3
FITC/TRITC
YFP/TRITC
YFP/ Cy3

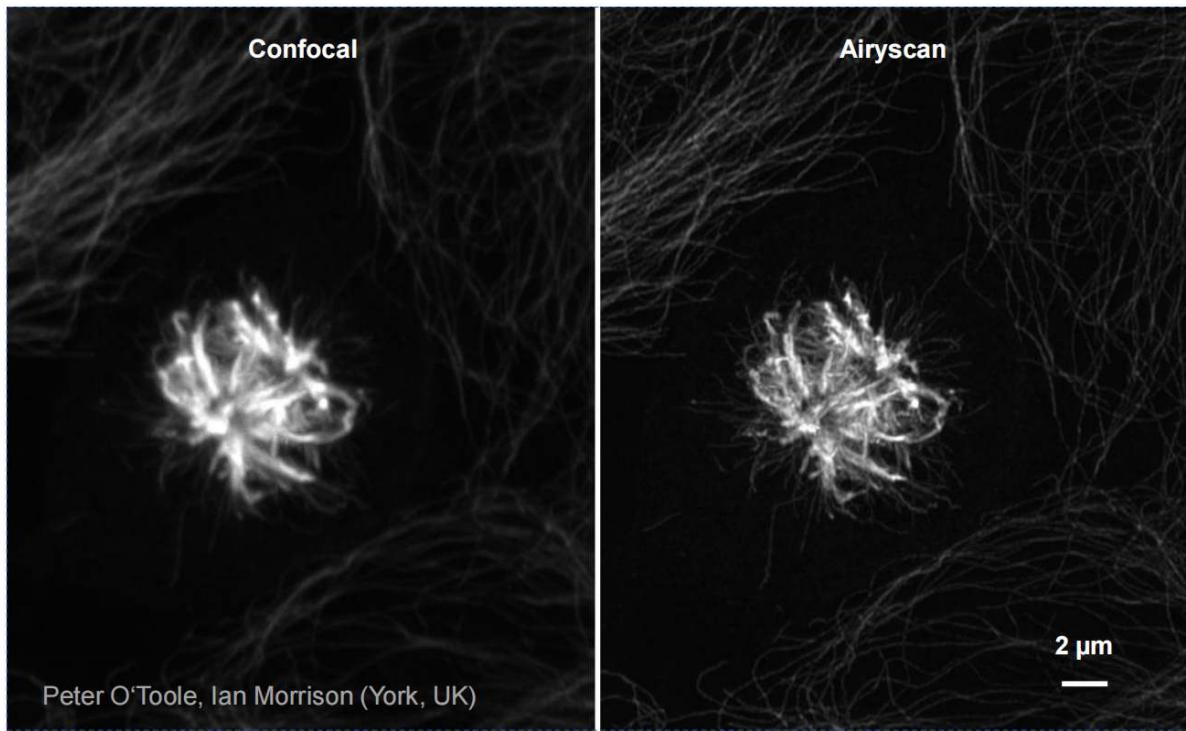
FRAP (Fluorescence Recovery After Photobleaching)

Quantify the mobility of molecules

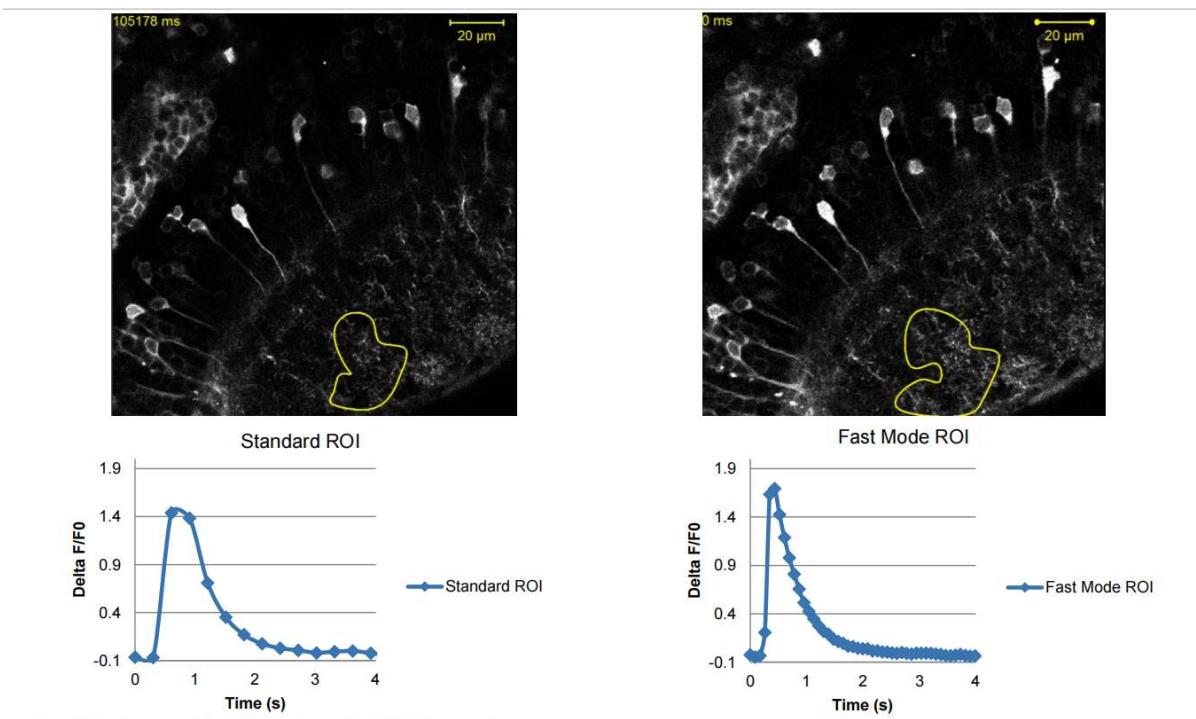


Super Resolution Microscopy

Airyscan improves resolution in lateral and axial direction



Fast Airyscan



Summary: LSM880 with Fast Airyscan Configuration

Hardware:

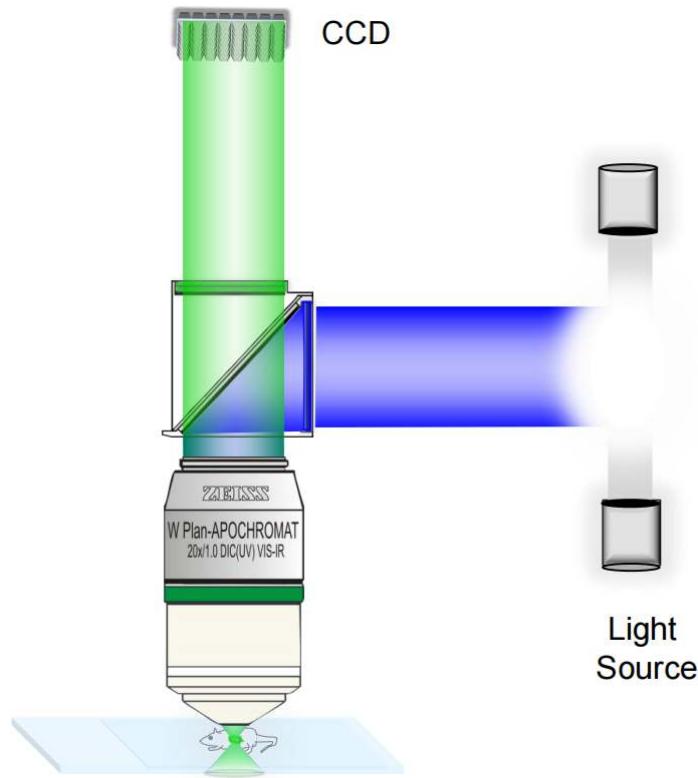
Axio Observer 7—— Inverted Microscopy with scanning stage
 4 Lasers—— 405, Argon (458/488/514), 561 , 633
 3 Detectors—— 2 PMT + 1 GaAsP
 Objectives ——10x/0.45, 20x/0.8, 40x/1.4 oil, 63x/1.4 oil, 100x/1.4 oil

Software:

Tile and Position
Experiment Designer
FRET
FRAP

1.2 Principle of Confocal Microscope

structure



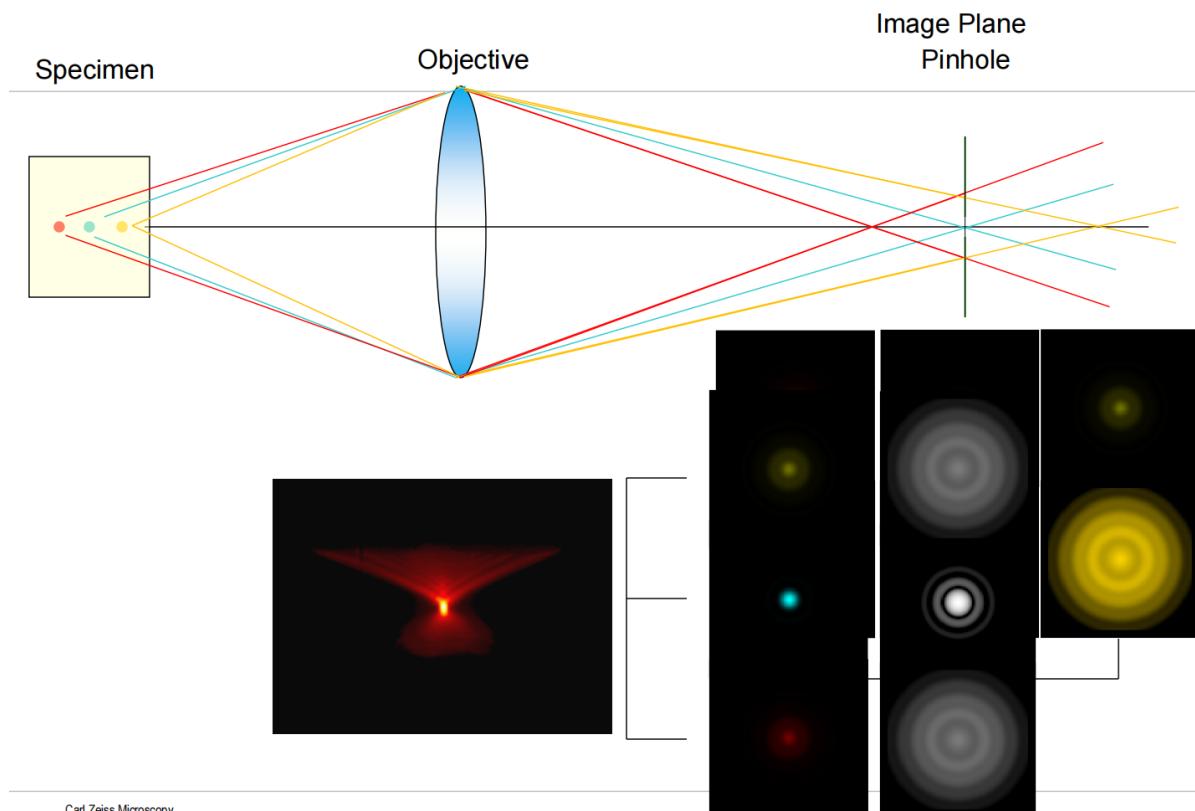
Light Source: Located on the right, it provides the illumination needed for imaging. In fluorescence microscopy, this is often a laser or arc lamp that excites fluorescent markers in the specimen.

Objective Lens: Positioned at the bottom, it focuses the light onto the specimen. The objective lens is crucial for magnification and resolution. The label "W Plan-APOCHROMAT 20x/1.0 DIC(UV) VIS-IR" indicates a high - quality, apochromatic lens designed for use across a range of wavelengths (UV to infrared) and with a high numerical aperture (1.0), which enhances light - gathering ability and resolution.

Beam Splitter/Filter: The diagonal component within the green structure (possibly a filter cube or beam splitter assembly) selectively reflects and transmits light of different wavelengths. It directs excitation light from the source to the specimen and allows emitted fluorescent light (of a different wavelength) to pass through to the detector.

CCD Camera: Located at the top, it's the detector that captures the light emitted from the specimen. CCD (Charge - Coupled Device) cameras are sensitive detectors commonly used in fluorescence microscopy to convert light into electrical signals, which are then processed into images. In confocal microscopy, a photomultiplier tube (PMT) is often used as the detector instead of a CCD camera for its higher sensitivity and faster response, but the specific setup can vary.

imaging principle



Carl Zeiss Microscopy

Confocal microscopy is an optical imaging technique that uses a pinhole to eliminate out-of-focus light, resulting in high-resolution images. Here's a detailed look:

Point Illumination: The specimen is illuminated by a light source, often a laser, focused to a tiny spot. This minimizes the illuminated area at any given time reduces scattered light and increases resolution.

Objective Lens: The objective lens focuses the light onto the specimen. It also collects the emitted light (e.g., fluorescent light) and directs it toward the detector.

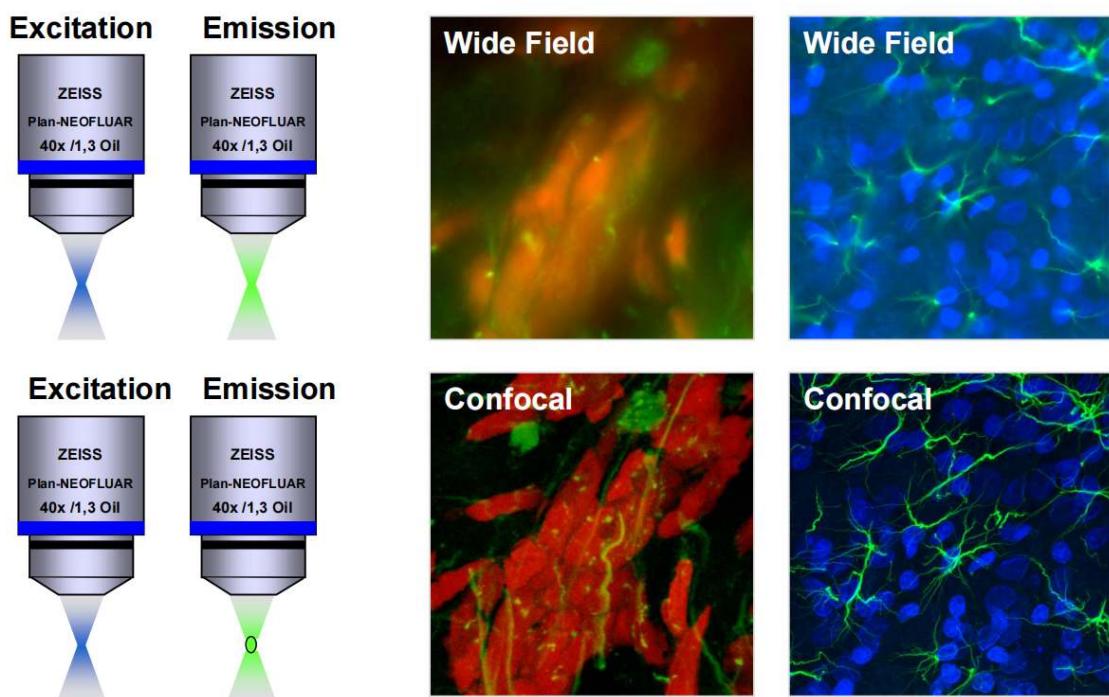
Pinhole Detector: After the emitted light passes through the objective lens, it goes through a pinhole before reaching the detector. The pinhole blocks out-of-focus light from above and below the focal plane, ensuring that only light from the focal point is detected. This is key to the confocal microscope's ability to produce clear, high-contrast images with minimal background noise.

Scanning Mechanism: To create a full image, the microscope scans the specimen either by moving the specimen or using mirrors to redirect the light beam. The detector records the intensity of the emitted light at each point, and this data is used to construct a 2D image of the specimen's focal plane.

Optical Sectioning: By adjusting the focus of the microscope and repeating the scanning process, multiple images (optical sections) from different depths within the specimen can be obtained. These sections can then be combined using specialized software to create a 3D representation of the specimen.

Compare to wild-field microscope

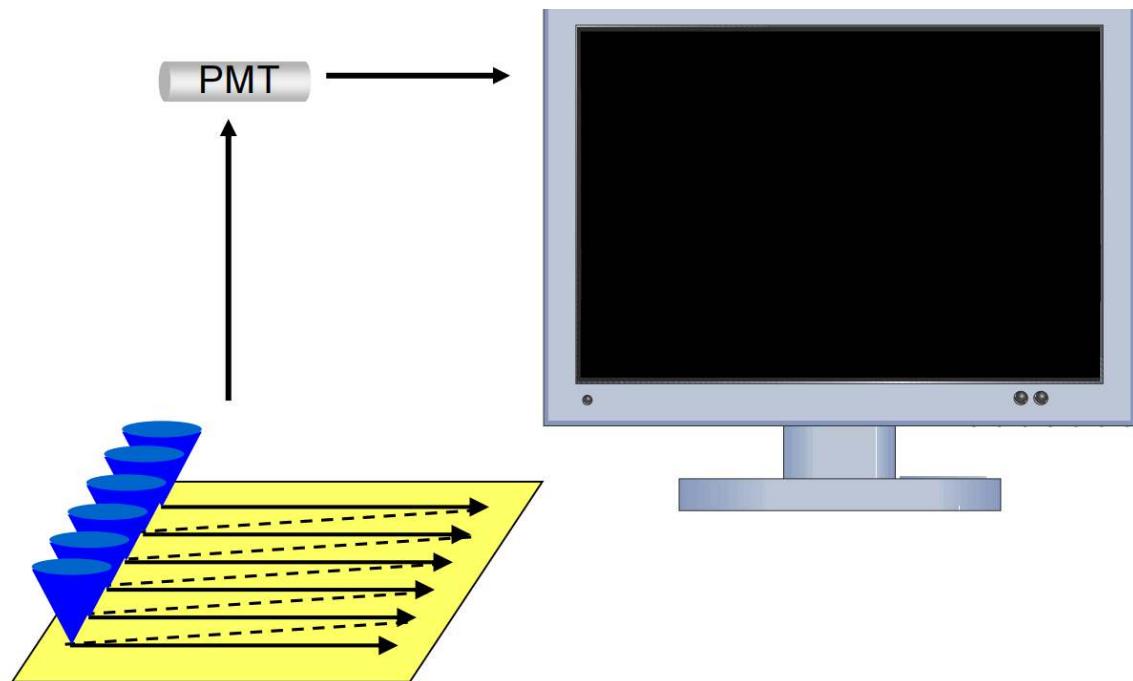
The advantage of confocal microscopy detecting only in - focus information lies in its principle. It uses a focused light source to illuminate a tiny spot on the specimen. Fluorescent light from this spot passes through an objective lens and a pinhole detector. The pinhole blocks out - of - focus light from above and below the focal plane, ensuring only light from the focal point is detected. By scanning the specimen and adjusting the focal depth, multiple in - focus images at different depths can be obtained and combined to create a 3D image. This principle helps eliminate out - of - focus light, improving image resolution and clarity. It allows researchers to acquire high - contrast images with reduced background noise. Also, it enables optical sectioning, making it possible to study specific depths within thick specimens and understand the spatial relationships within them through detailed 3D representations



Scanning Strategy

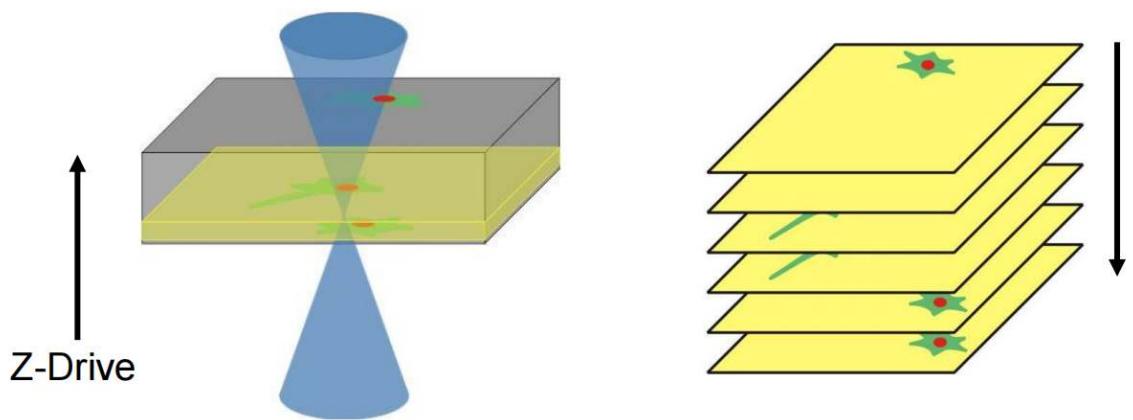
In X-Y plane

Ponit – Line - Frame



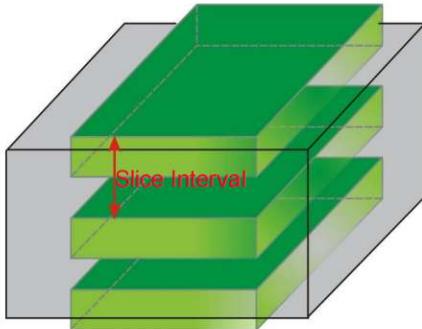
In Z-Stack

Frame by Frame

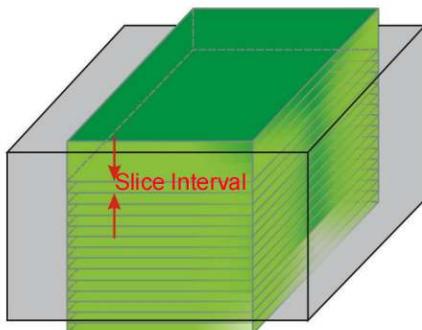


Optical Slice Thickness

Overlap between Optical Slices

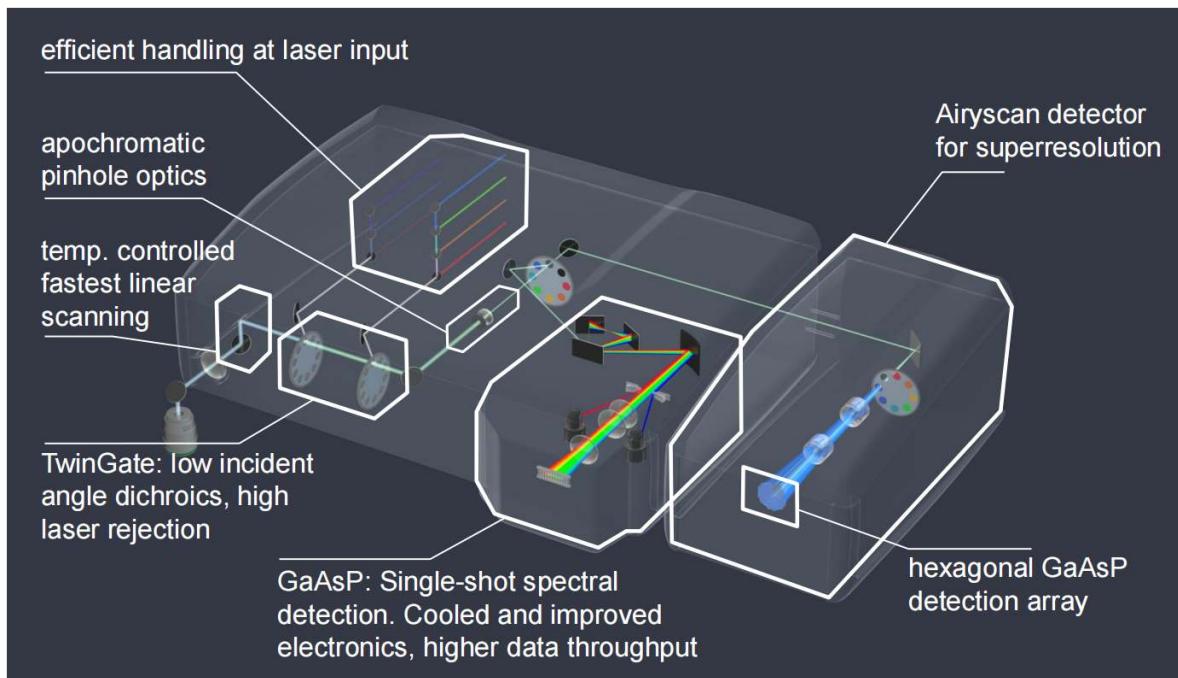


With this setting, the object structures between the slices cannot be detected.



At very small intervals a lot of additional data without additional information is generated.

1.3 Light path components of LSM880



The light path starts with efficient handling at the laser input, ensuring optimal laser light management for fluorescence excitation. The system uses apoachromatic pinhole optics to correct chromatic aberration, enhancing image quality and resolution. The temp - controlled fastest linear scanning mechanism allows for rapid, stable image acquisition. The TwinGate component, featuring low - incident - angle dichroics and high laser rejection, minimizes noise by reducing unwanted laser reflection. The GaAsP detector enables single - shot spectral detection with cooled and improved electronics for higher data throughput. Its hexagonal GaAsP detection array efficiently collects light, improving spatial resolution and sensitivity. Finally, the Airyscan detector

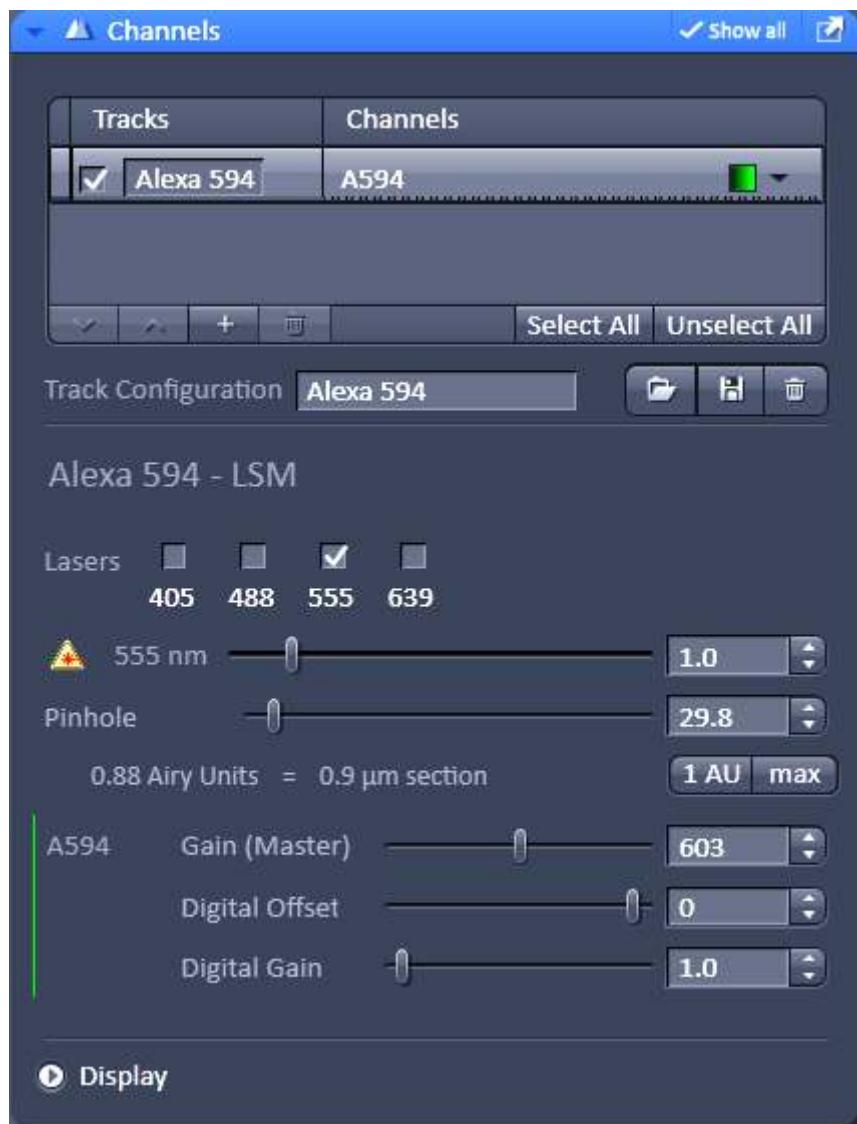
provides super - resolution imaging beyond the diffraction limit, making the LSM880 suitable for high - resolution and fast - scanning 3D imaging applications.

The main tools are:

Laser power

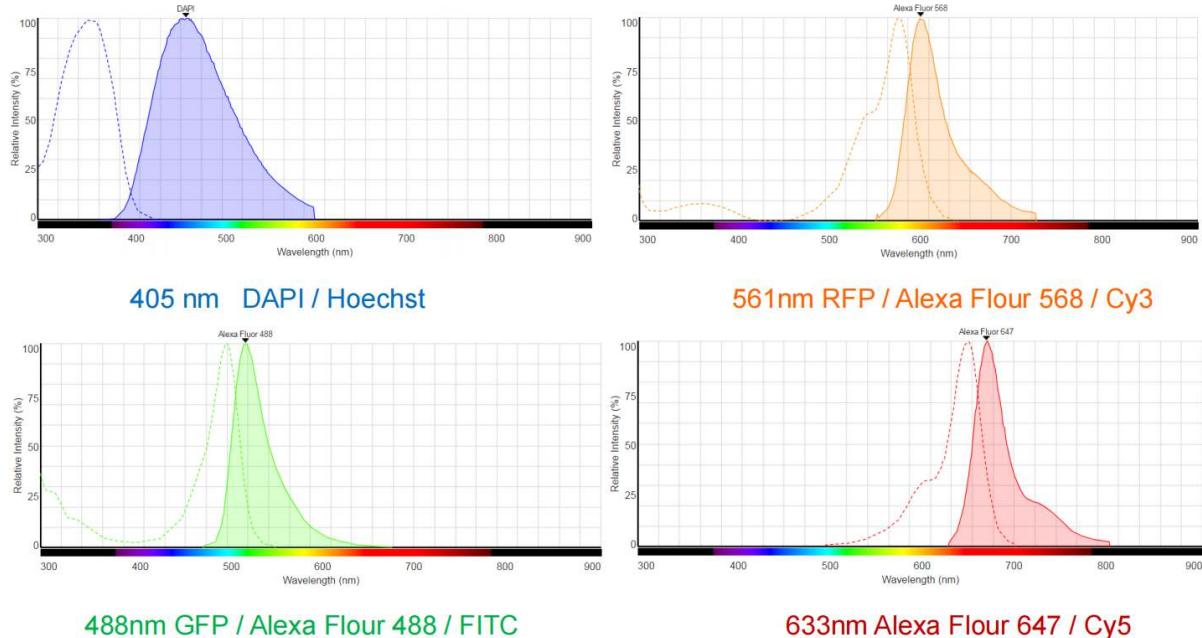
Pinhole

PMT (Gain)



Laser

The laser color should match the fluorophore and high laser power bleached the sample signal.



Sample:

Cultured mammalian cell expressing GFP (live cell)

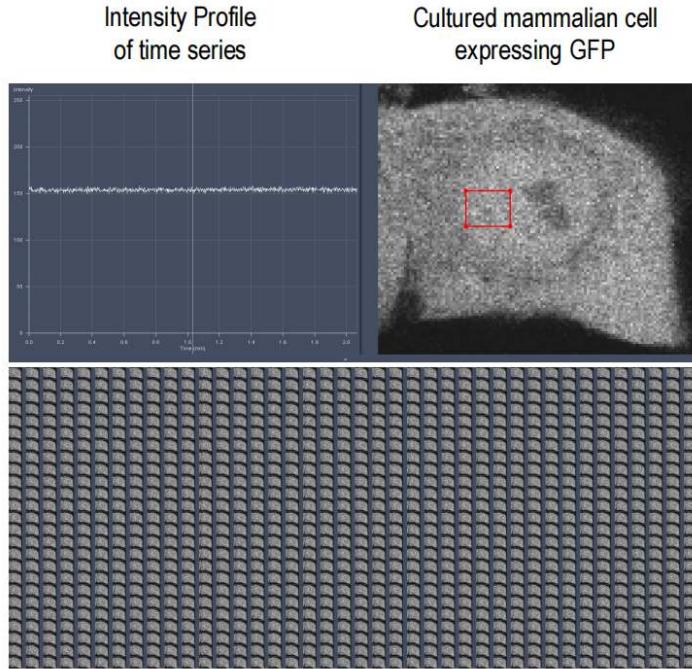
Imaging Strategy:

Time series of 1000 images acquired at low laser power but relatively high gain setting.

(excitation at 488 nm with just 0.5% of the max. power).

Result:

Hardly any photobleaching !

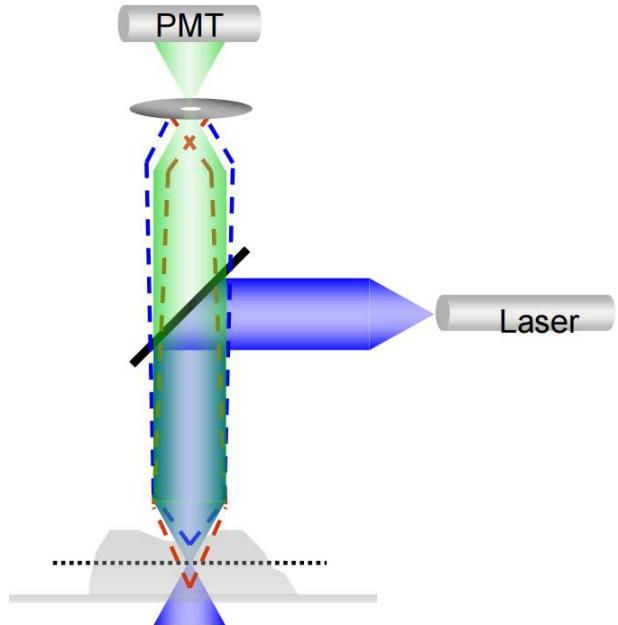


Pinhole

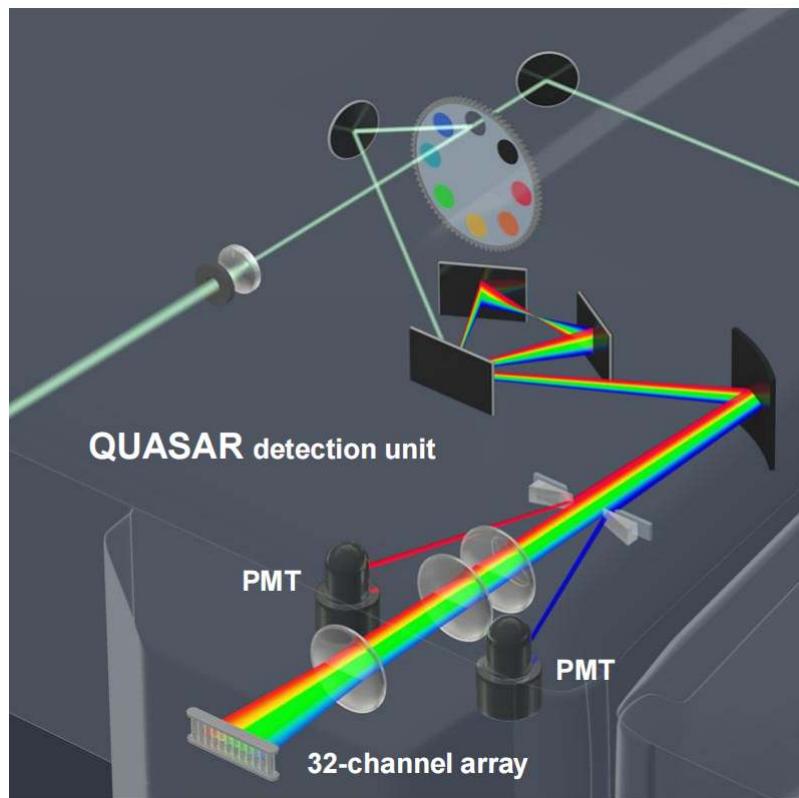
The larger the pinhole, the more light pass through, the more out of focus signal.

The larger the pinhole, the thicker the optic section, the better Z axis resolution.

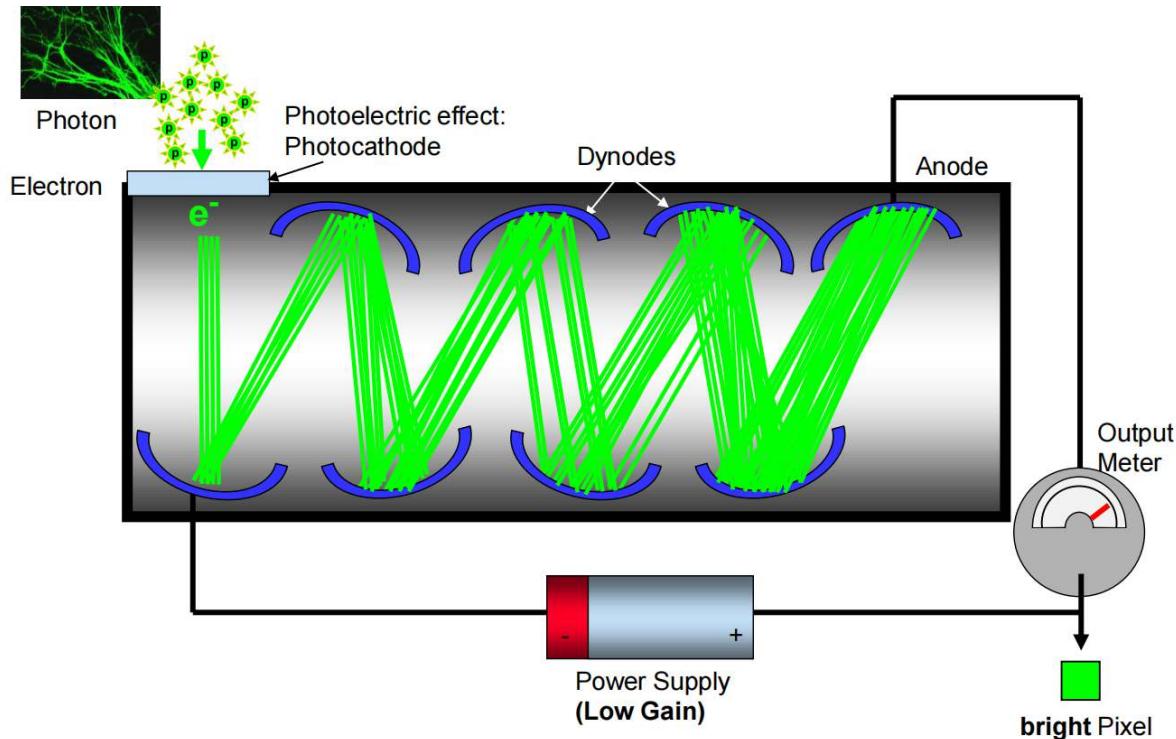
Generally, the pinhole is 1AU.



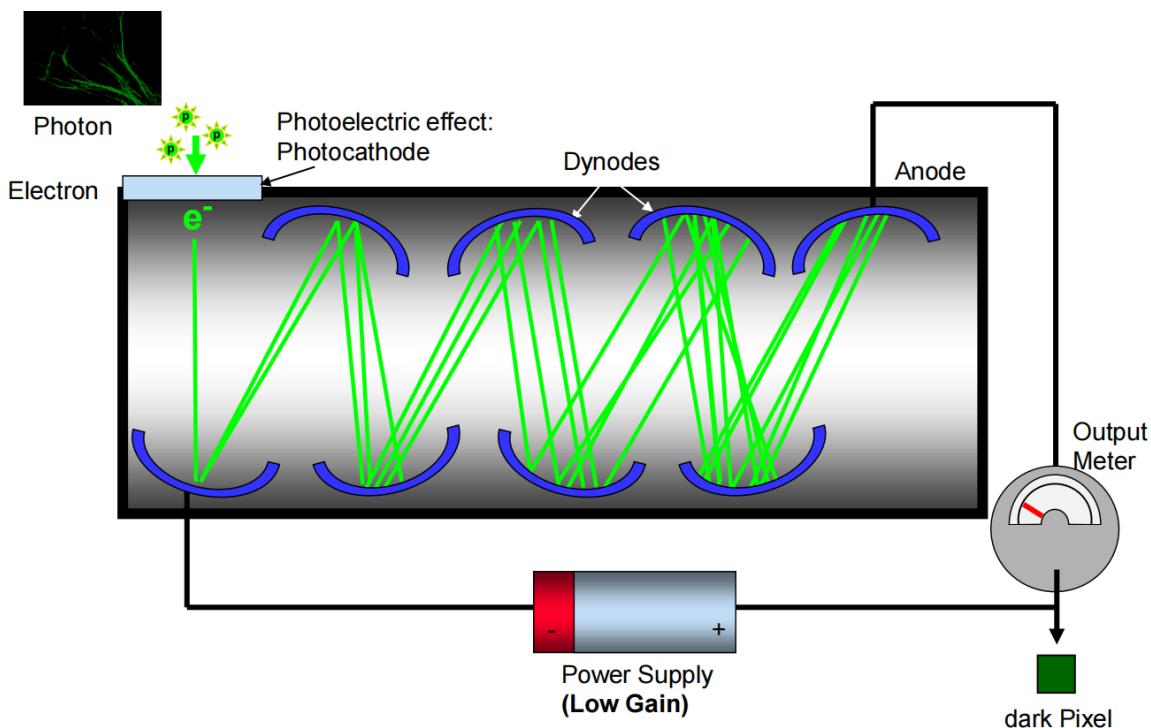
PMT Detector/ Photomultiplier Tube



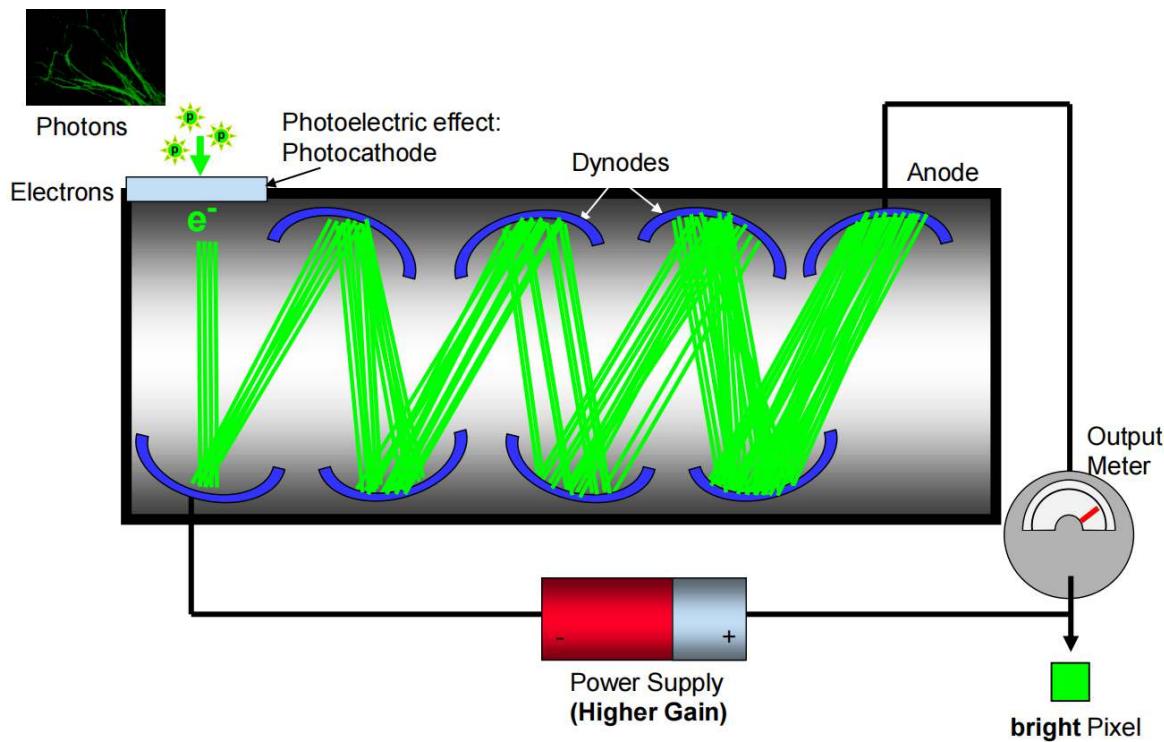
Assuming a bright sample



Assuming a dark sample

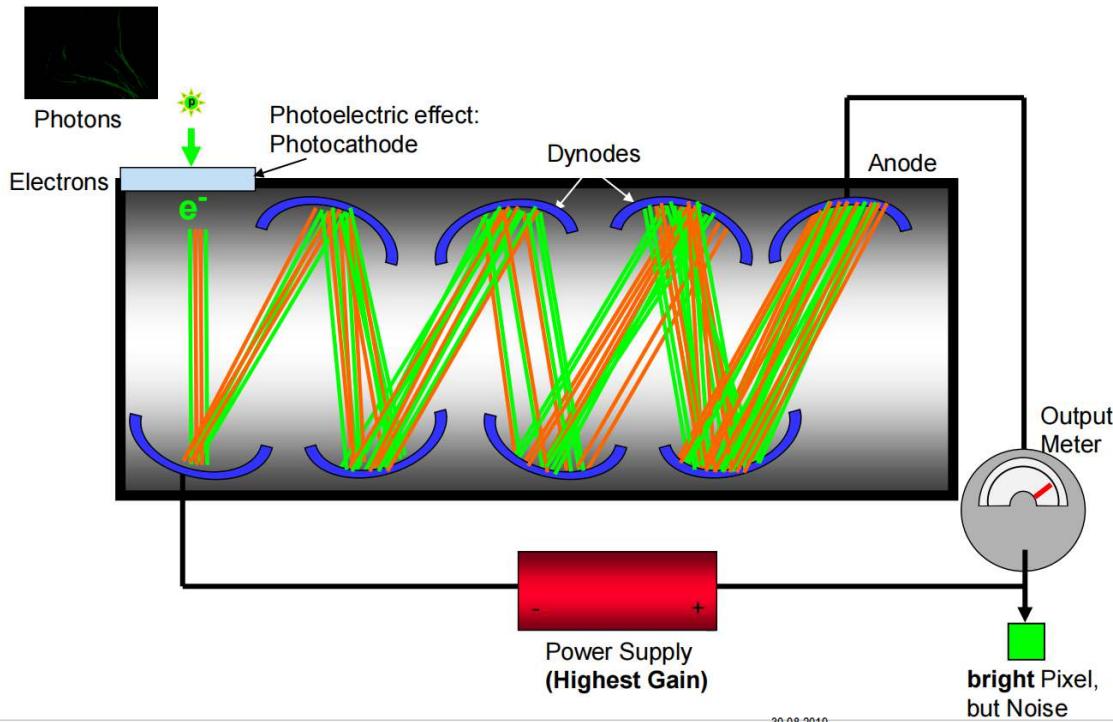


Increase a dark sample's signal with more Gain



Assume a really dimm sample --Extreme Gain values result in Noise

Red lines indicate electrons that were generated but have not come from the photons within the sample.



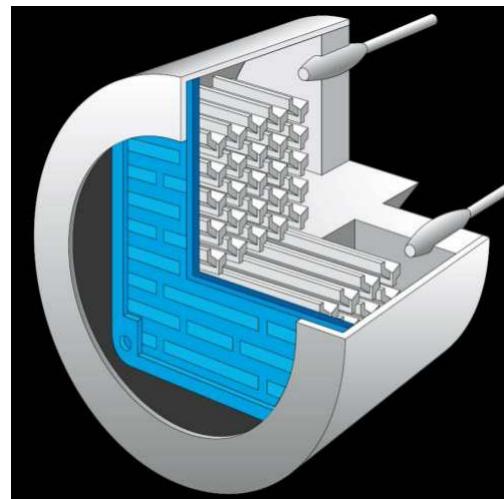
GaAsP Detector

GaAsP (Gallium Arsenide Phosphide) is a semiconductor material with ideal characteristics for converting photons into electrical signals.

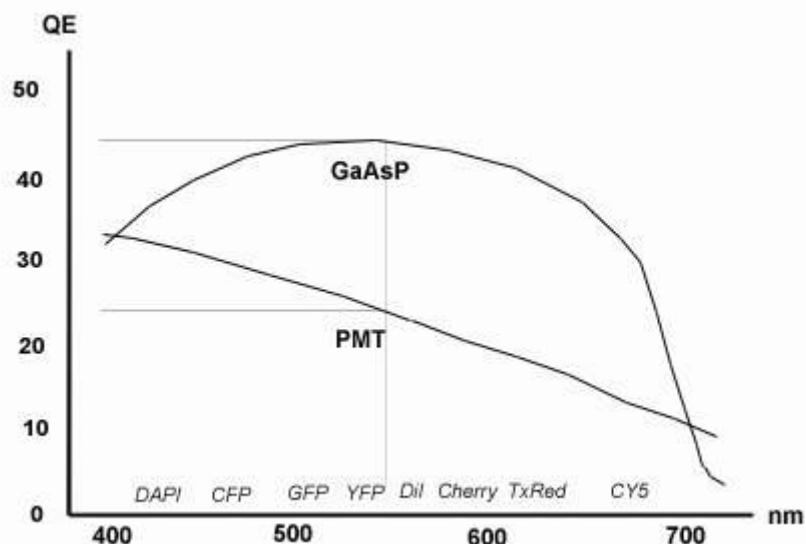
Benefits of GaAsP detectors:

Two times better Quantum efficiency than PMTs (resulting in higher sensitivity, better image quality, and higher acquisition speed). GaAsP detectors can be operated in integration mode as well as in photon counting mode.

schematic illustration:

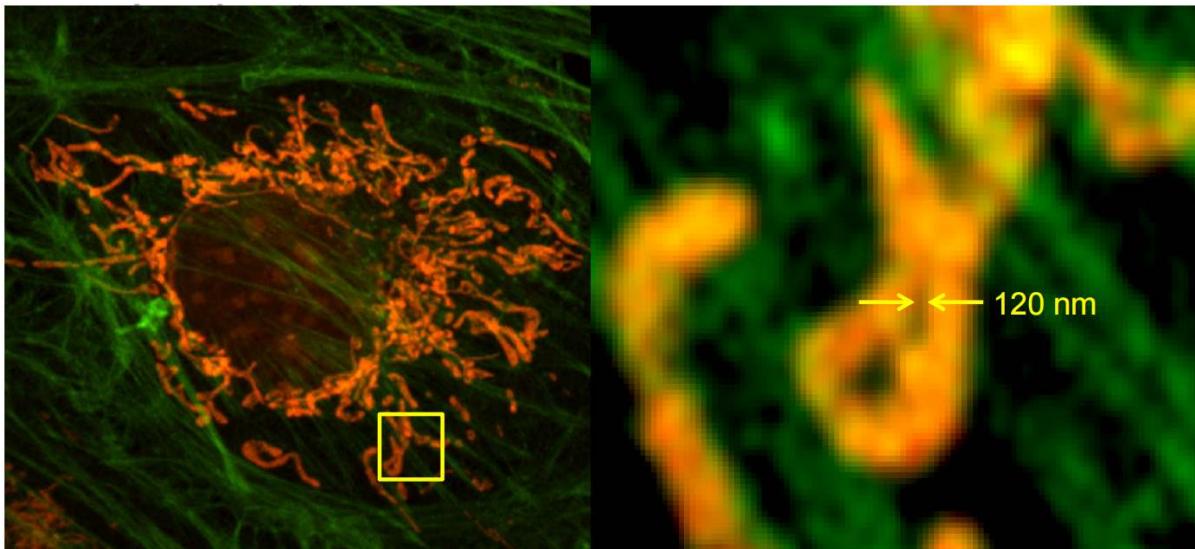


typical sensitivity comparasion



Airyscan: Resolution, SNR, and Flexibility

Use Airyscan just as an additional detector. Scan speeds, ROIs, field orientations, laser wavelengths, multitracking, z-stacks, time series etc. are setup as always. sample handling and fluorophore selection don't need rethinking.



1.4 How to acquire a high quality image

evaluation:

Intensity

SNR

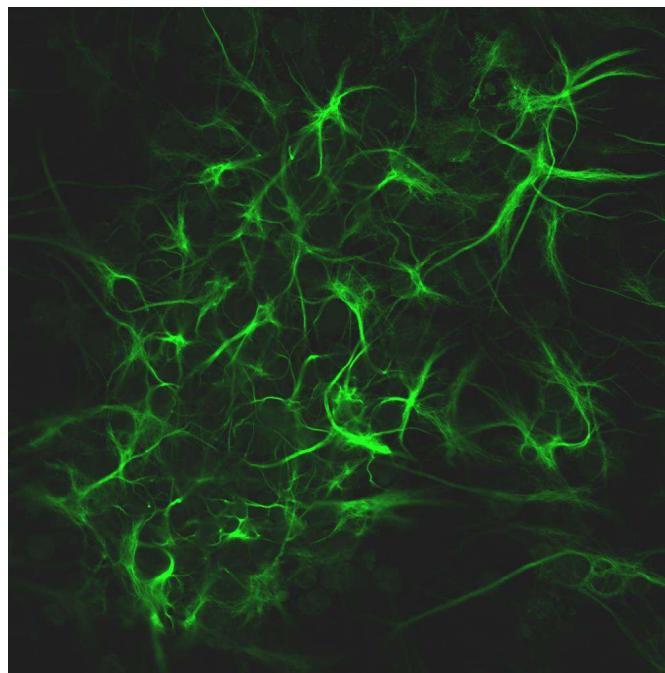
Resolution

set up the optimal values

Illumination:

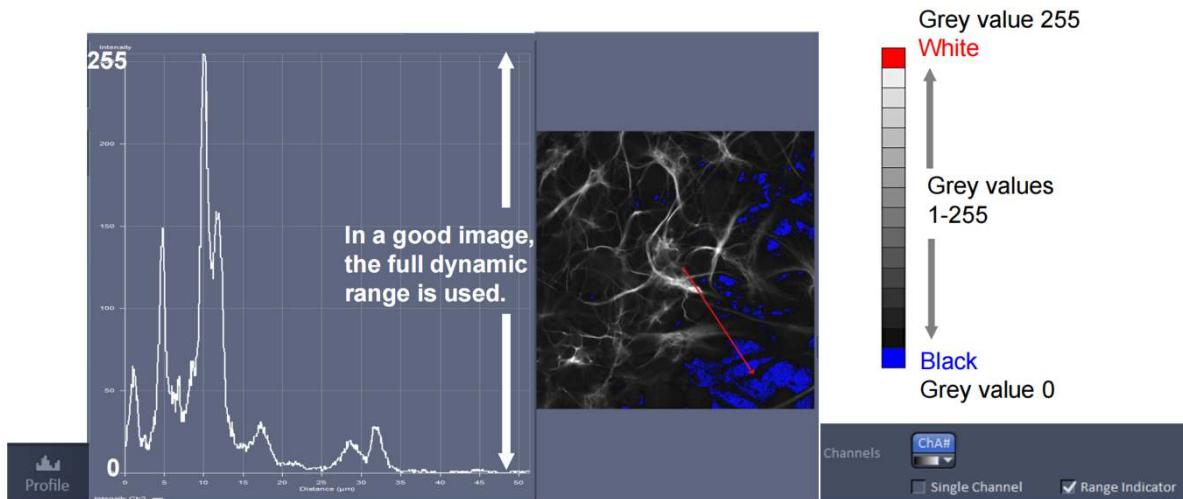
not too bright, not too dimm

Example as followed:

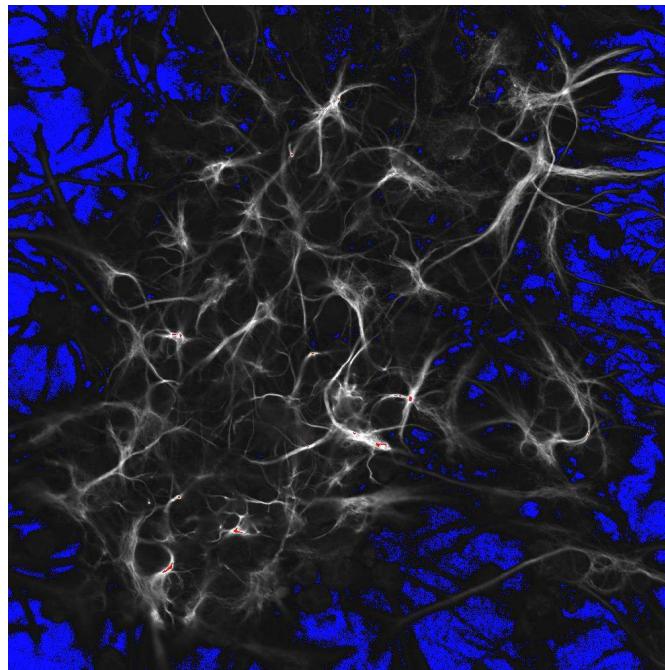


Range Indicator:

Look-up table Range Indicator (8bit). Profile= gives measurements of grey values along a line



The example changed to



Scanning Strategies

to avoid noises : Speed and Averaging

To decrease the effect of noise, more photons (signal) must be collected:

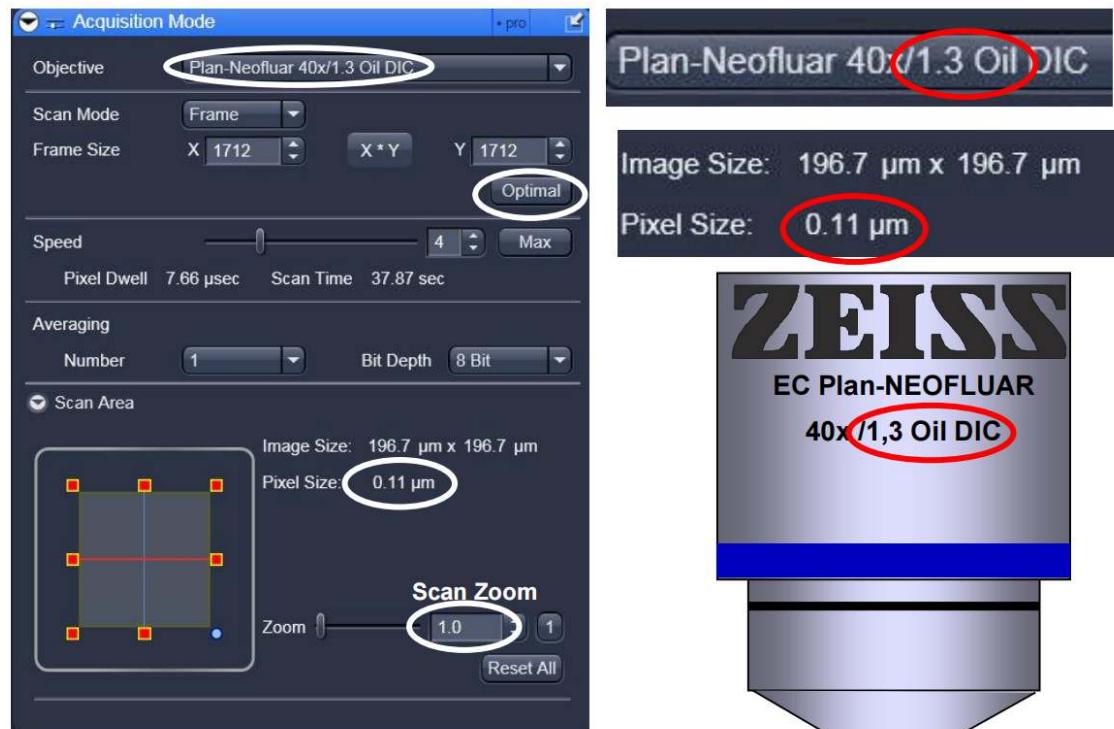
1. Slower Scan Speed

2. Averaging

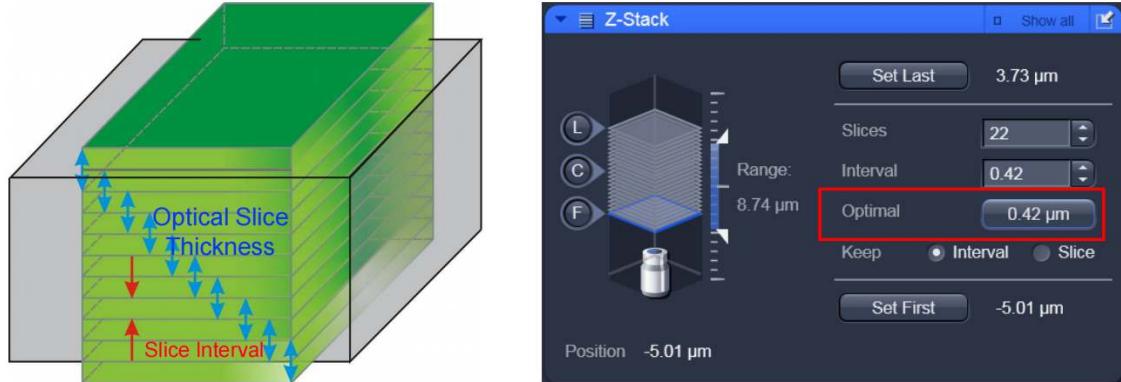
Scan the image x-times and take the average signal for each pixel-> addition of photons from several scanning runs.

Resolution

Information given in the Software



Optical Slice Thickness



The optimal overlap is fulfilled at "Nyquist" or "Sampling Theorem" conditions.

→ Sampling frequency (slice interval) must be the double of the information frequency (zresolution or optical slice thickness).

To achieve these conditions just press Optimal Interval in Z-Stack dialog. Then, the slices overlap by half of their thickness (no missing information @ minimal number of sections).

Operation

2.1 Startup and Shutdown of the System

Start the system

1. Turn on the voltage stabilizer:
 - a. Switch on the main power supply at the rear of the voltage stabilizer.
 - b. Switch on the power supply on the front panel of the voltage stabilizer.
 - c. Ensure that the voltage stabilizer displays 220V.



2. Turn on the system stabilizer:

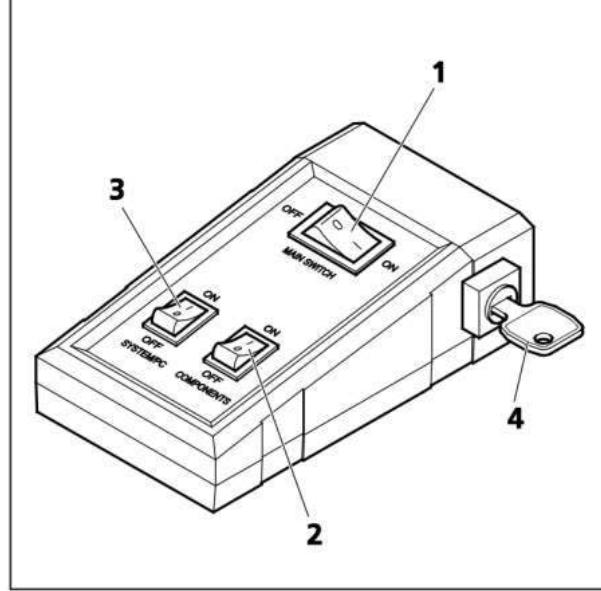
Before powering on the entire system, ensure that the key (Figure 4) is in the "ON" position;

- a. Turn on the "MAIN SWITCH" (Figure 1);
- b. Turn on the "SYSTEM/PC" (Figure 3), then start the computer and log in to the system;
- c. Turn on the "COMPONENTS" (Figure 2);
- d. Turn on the metal halide lamp.

Within 30 minutes, do not switch on and off repeatedly!

- e. Launch the ZEN software (black).

- f. Click "start system"

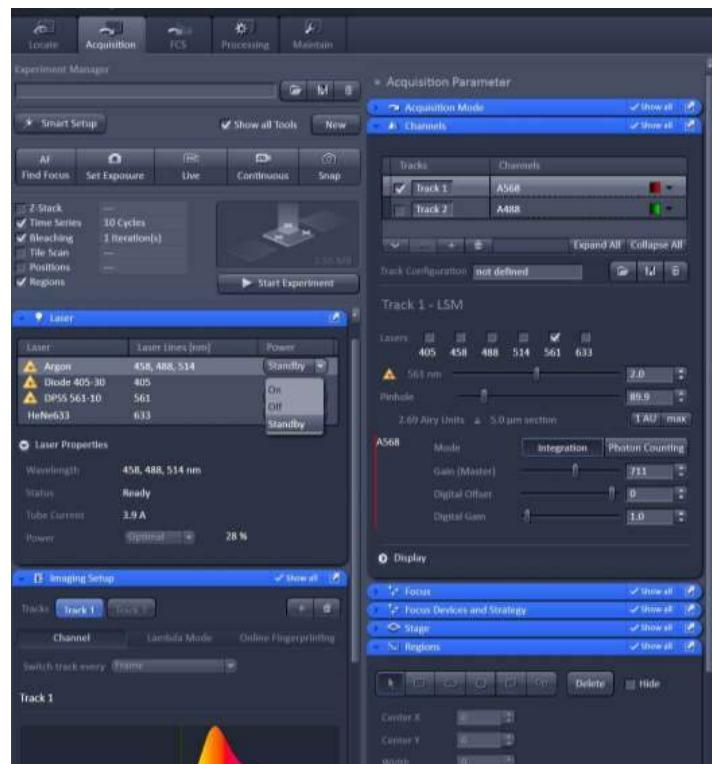


- 1** Main switch ON/OFF
- 2** COMPONENTS switch ON/OFF
- 3** SYSTEM PC switch ON/OFF
- 4** Key switch



3. If you need to use 458, 488, 514, you need to turn on the Argon laser:

- In the "Argon" tab under "Laser", select "Standby" and wait for the laser to warm up;
- After warming up, when the "status" shows "Ready", select "On";
- Then you can start confocal imaging.

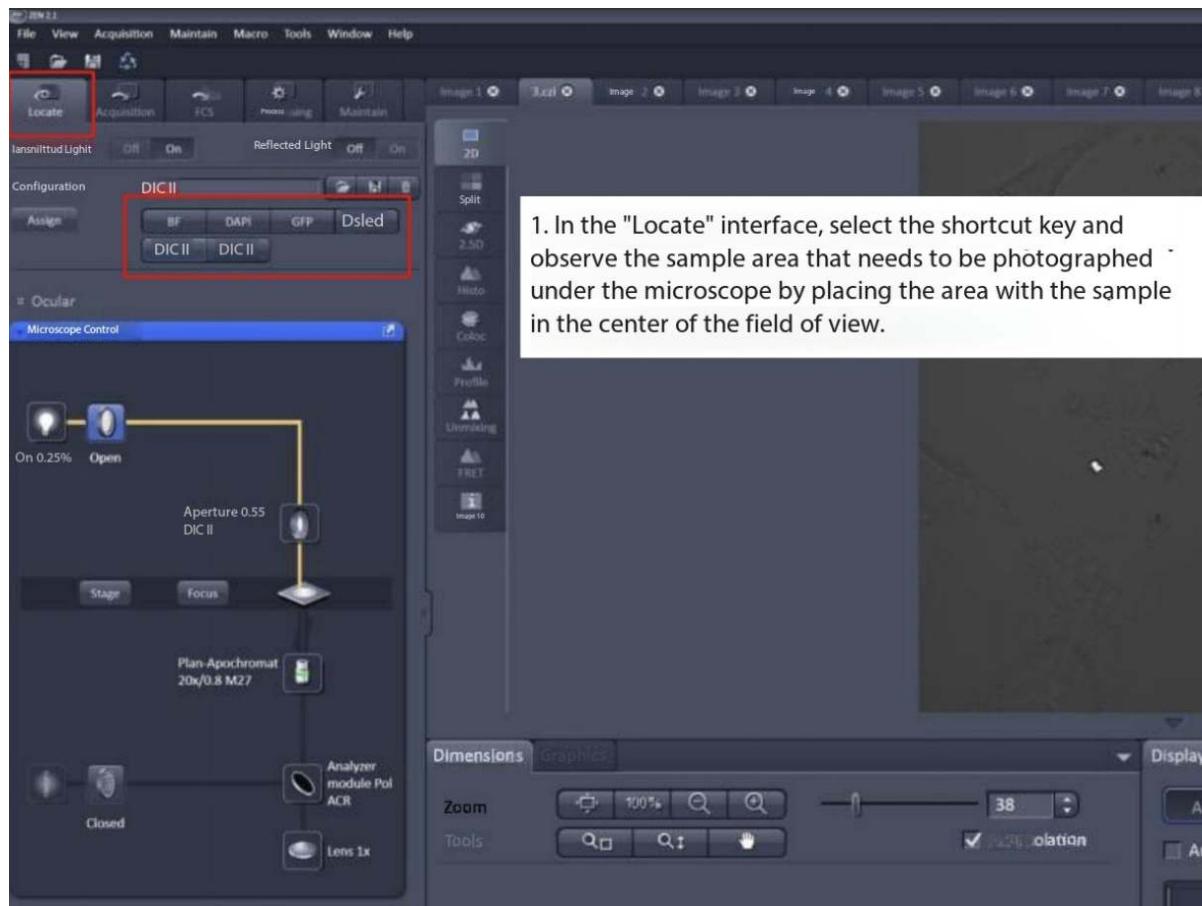


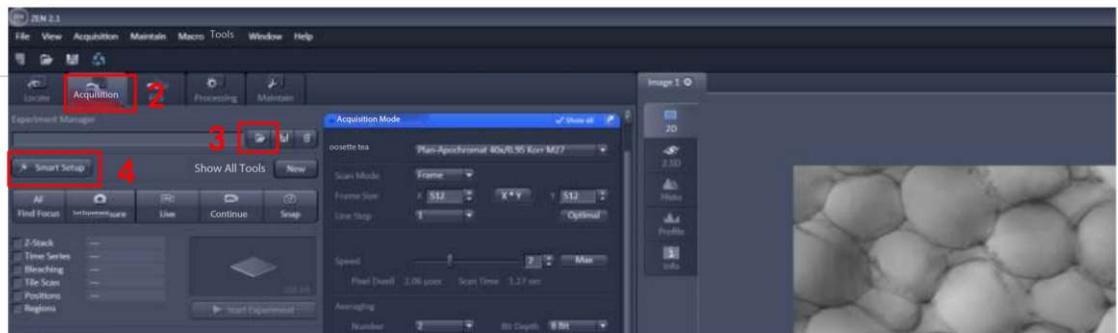
Shutdown of the System

The shutdown procedure is essentially the reverse of the startup procedure:

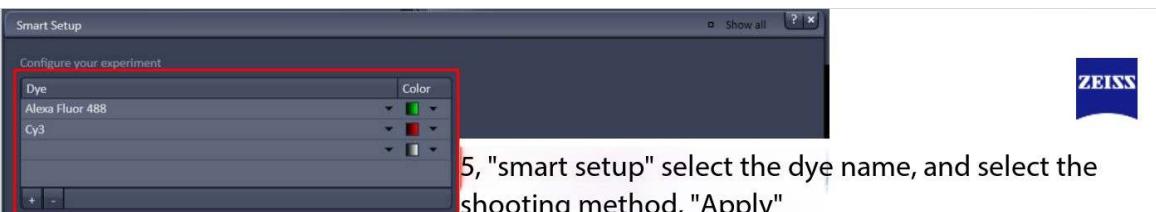
1. Turn off the metal halide lamp;
2. Turn off the laser: select "Off" in "Power" to switch off the laser;
3. Close the ZEN software;
4. Shut down the computer;
5. Wait about 5 minutes for the laser to cool down after shutting down the computer. The Argon laser's fan will stop;
6. Turn off "COMPONENTS", "SYSTEM/PC", and "MAIN SWITCH" in sequence;
7. Turn off the voltage stabilizer.

2.2 Acquiring Multi-Channel images

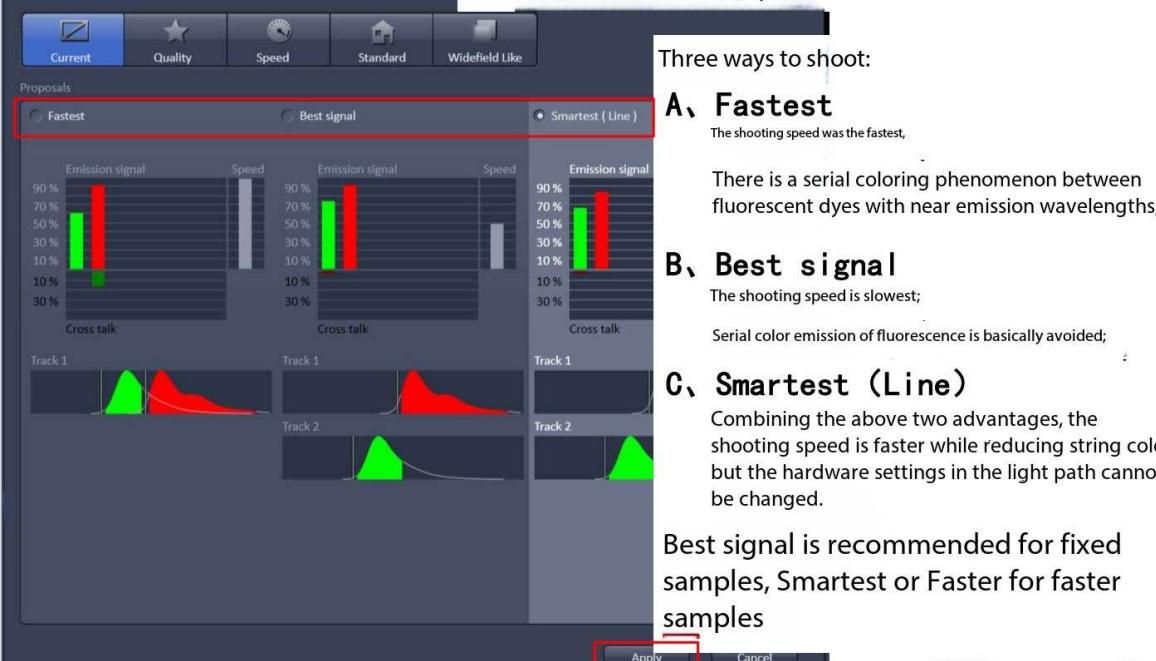




2. Enter the "Acquisition" interface;
3. Select previously saved light path settings ☐ "channel" and "acquisition mode" settings
4. Or create a new light path setup ☐ "Smart Setup"



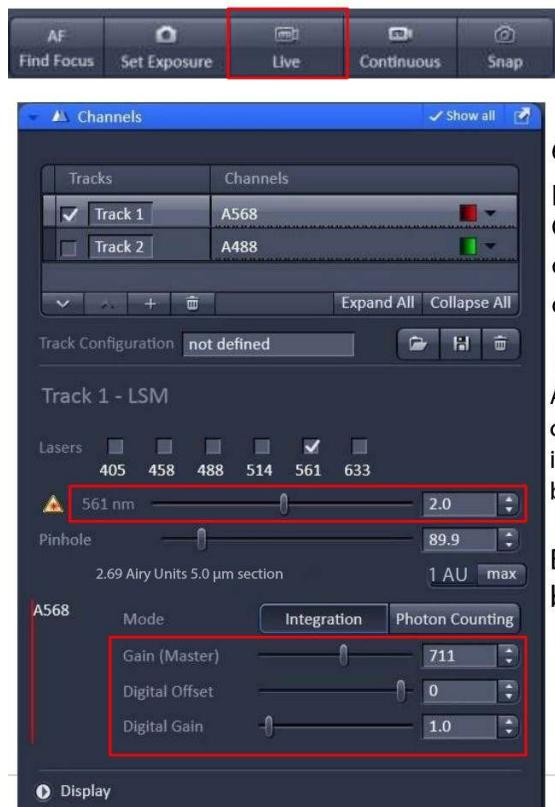
5, "smart setup" select the dye name, and select the shooting method, "Apply"



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Channels



6. In "live", set the laser intensity "Laser" and pinhole size "Pinhole" under Channels, detector "Gain" value, as well as "digital gain" or "digital offset"; each track is set separately, so only select one track at a time and highlight that track;

A, "Pinhole" is generally set to 1 AU. Increasing the Pinhole can increase image brightness but also adds non-focal plane information; reducing the Pinhole increases depth of field but decreases image brightness;

B, "Gain" and "Digital Gain" can increase image brightness but also increase background noise.



Set up principles to ensure that the image does not overexpose and try to reduce background noise:

Select "range indicator" under live mode;
Can show exposure level;

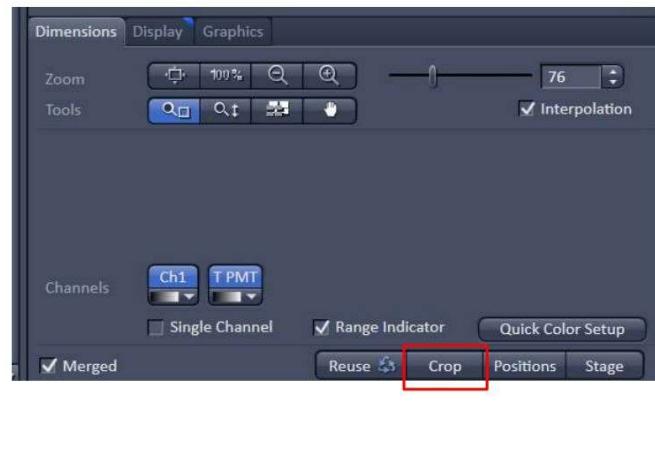
2016-05-15

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Acquisition Mode



7. The following parameters are mainly set in Acquisition Mode:
A, select the scan area by scan area or select the scan area by "crop" under the image window;



B Set speed: The slower the scanning speed, the better the signal-to-noise ratio, but more light bleaching occurs;
C Averaging: Increasing the averaging times can reduce noise, but will increase scanning time;
D Direction: Bidirectional scanning can reduce scan time;
E Frame Size: Generally choose 512x512 or 1024x1024, the larger the image, the longer the scanning time;

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2016-05-15

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Acquiring Multi-Channel images



The screenshot shows the ZEN 2.1 software interface for acquiring multi-channel images. In the top right, there are several buttons: AF, Find Focus, Set Exposure, Live, Continuous, and Snap, with the Snap button highlighted by a red box. On the left, the 'Channels' panel is open, showing two tracks: Track 1 (A568) and Track 2 (A488), both with checkboxes checked. Below this, the 'Track 1 - LSM' configuration panel is visible, showing settings for lasers (405, 458, 488, 514, 561, 633 nm), pinhole size (561 nm at 2.0), and exposure parameters (Gain (Master) at 711, Digital Offset at 0, Digital Gain at 1.0). To the right is a multi-channel fluorescence image showing red and green signal overlays.

8. Select the track you need to image, click "Snap"; get an multi-channel image.

Experiment Manager and Reuse

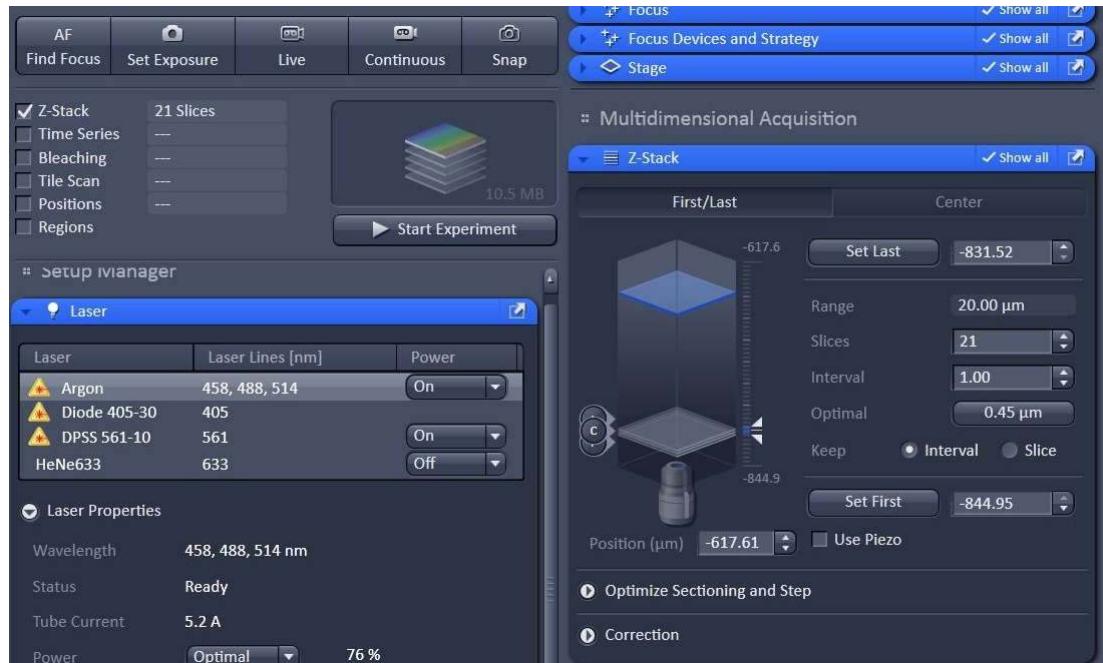


The screenshot shows the ZEN 2.1 software interface focusing on the Experiment Manager and a display panel. In the top right, the 'Experiment Manager' window is open, with its toolbar showing a folder icon and a reuse icon, both highlighted by a red box. Below it, the 'Smart Setup' panel has a 'Reuse' button highlighted by a red box. At the bottom, the 'Display' panel is active, showing various controls like zoom (100%), interpolation (76), and a 'Reuse' button highlighted by a red box. The overall interface is designed for managing and reusing experimental parameters.

The Experiment Manager can be used to save the parameters for shooting or open saved images (czi format), and call the previously shot parameter settings through "Reuse".

2.3 Z-stack image

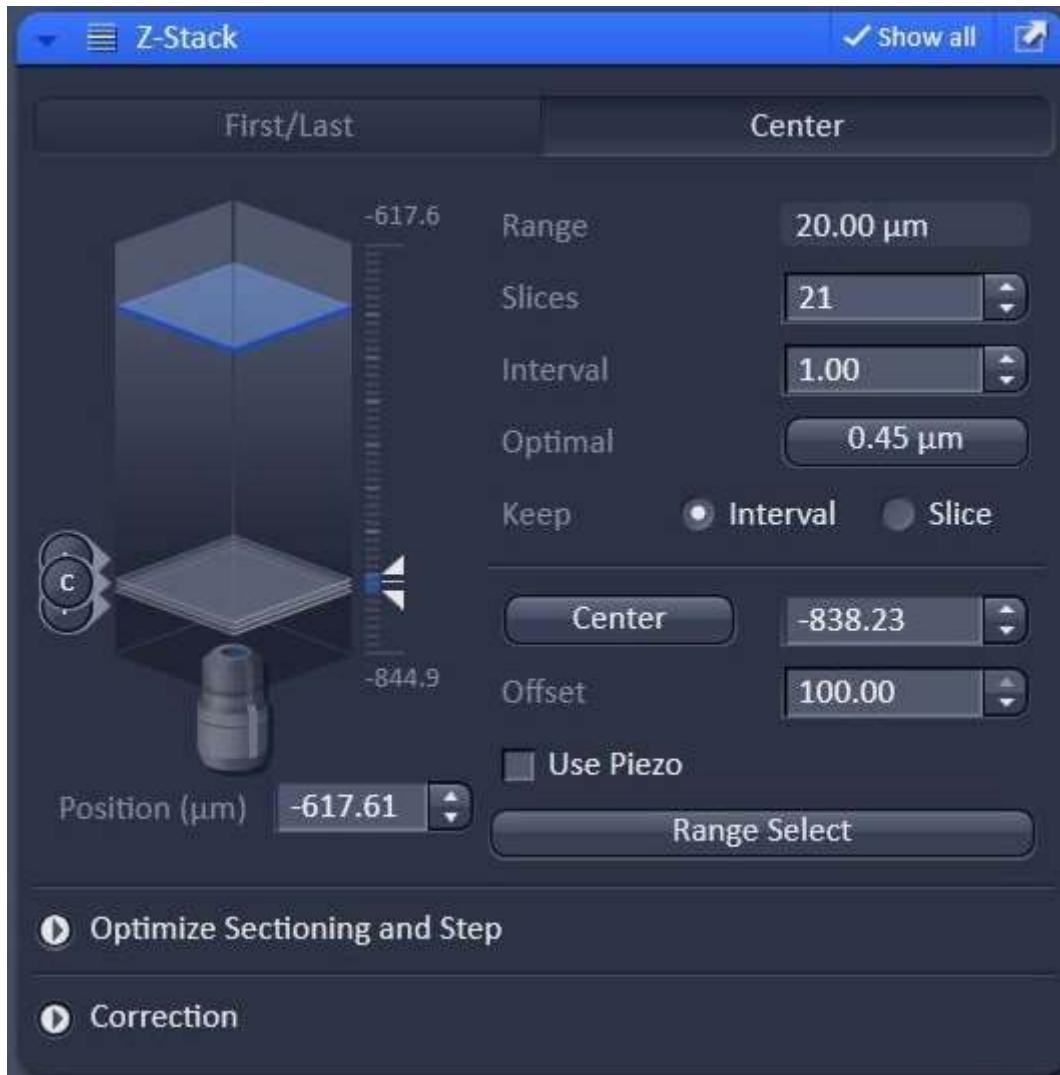
First/last



Select "Z-stack";

1. Under "Live", adjust the focus to set the scan range with "Set First" and "Set Last";
2. Click "Optimal" to set the appropriate interval;
3. Click "Start Experiment".

Center

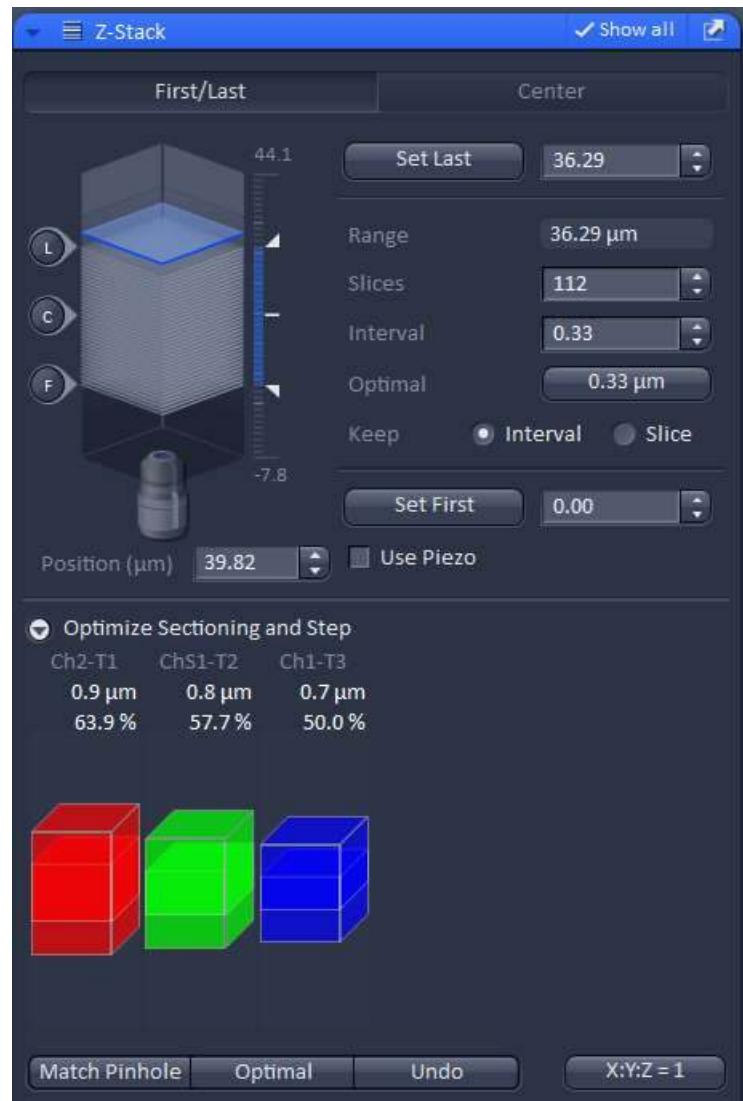


1. In "center" mode, select the midpoint under "Live", click "Center", set the number of "Slices", and click "Optimal";
2. Click "Start Experiment".

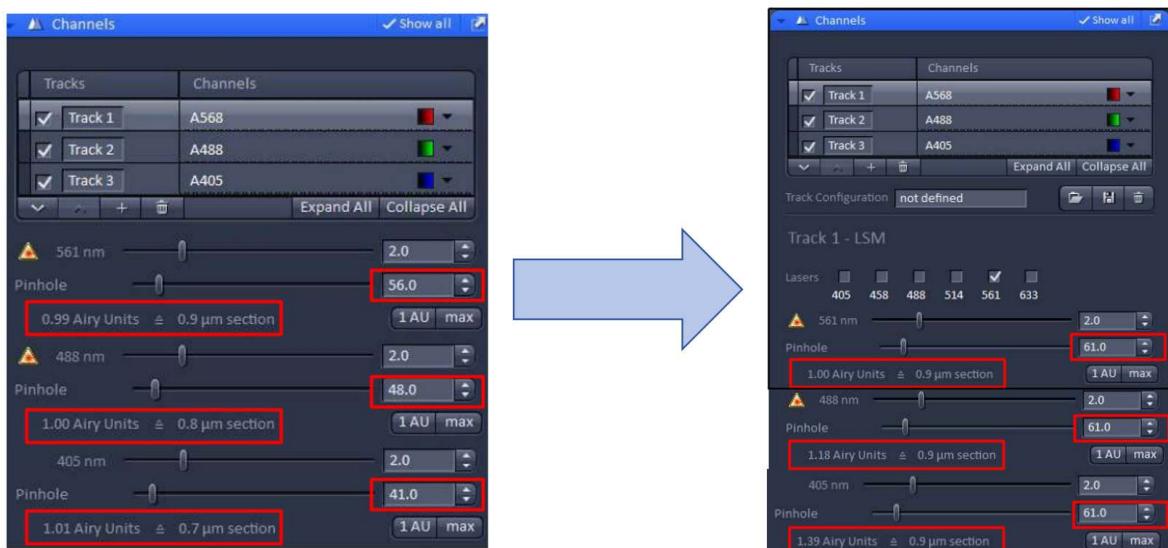
Match Pinhole

For multi-channel fluorescence Z-stack imaging, inconsistent optical section thickness can be an issue:

1. You can click "Match Pinhole" to automatically adjust pinholes across different tracks for similar optical section thickness;
However, this method may reduce the pinhole size for longer wavelengths, which can be detrimental to imaging weak fluorescence.



2. Adjusting the pinhole size manually can ensure consistent optical section thickness while preserving fluorescence intensity.



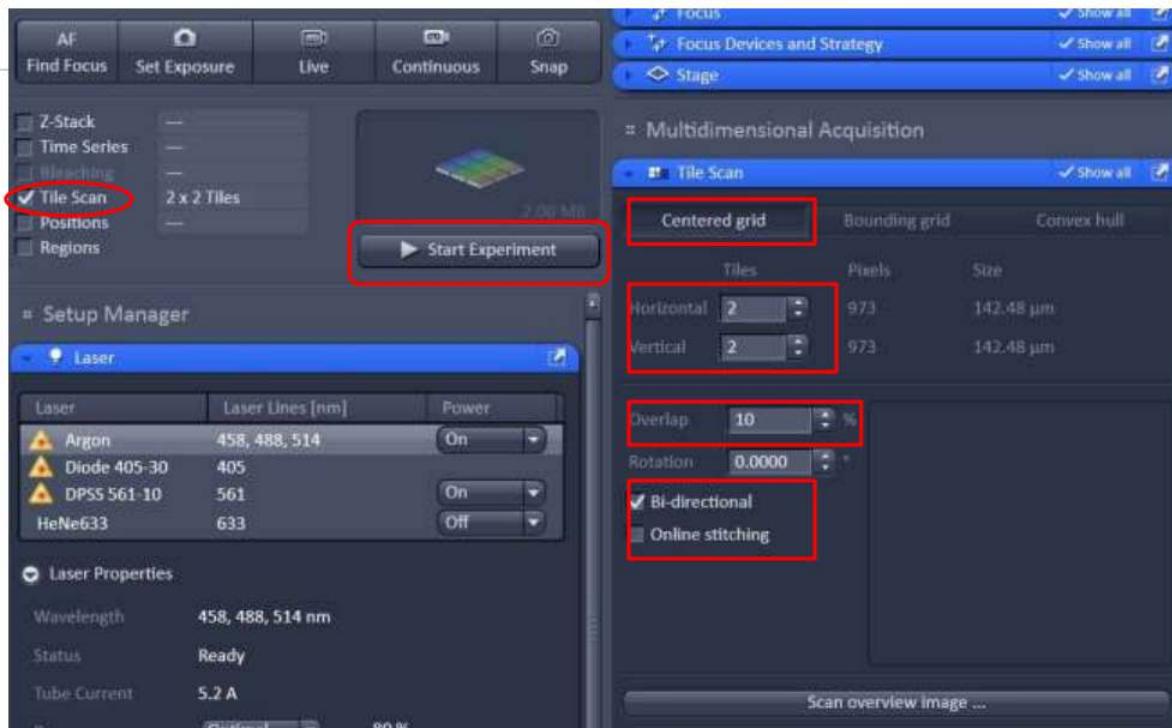
2.4 Time Series image



1. Select "Time Series";
2. Set the number of cycles for the imaging process;
3. Define the interval between cycles, which includes the acquisition time of the previous cycle;
4. Click "Start Experiment".

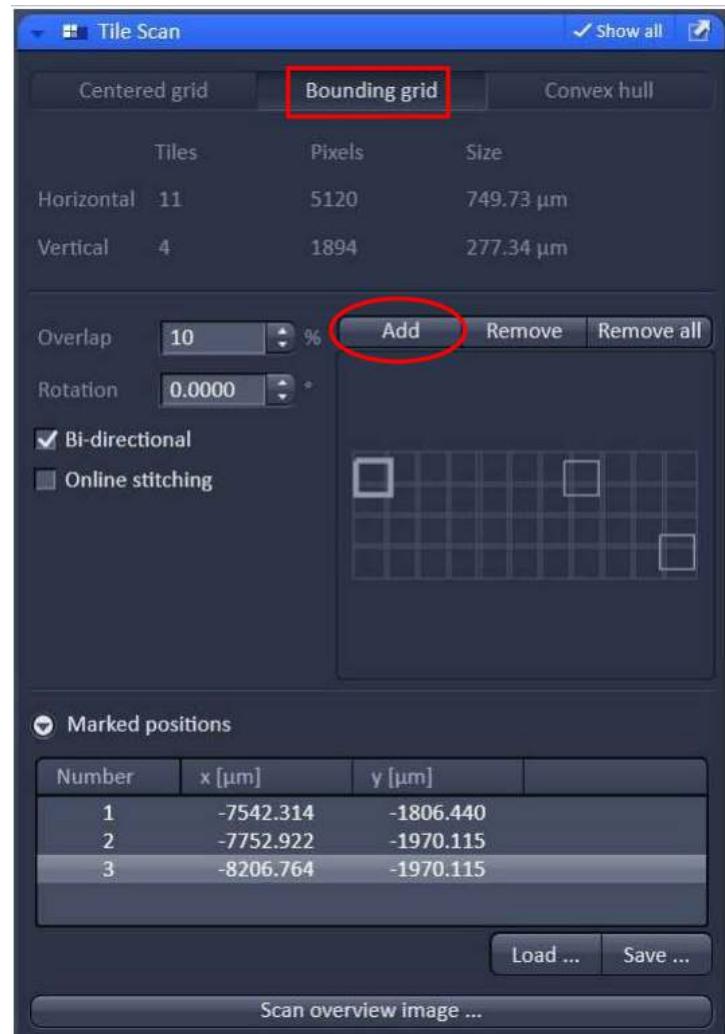
2.5 Tile Scan

common



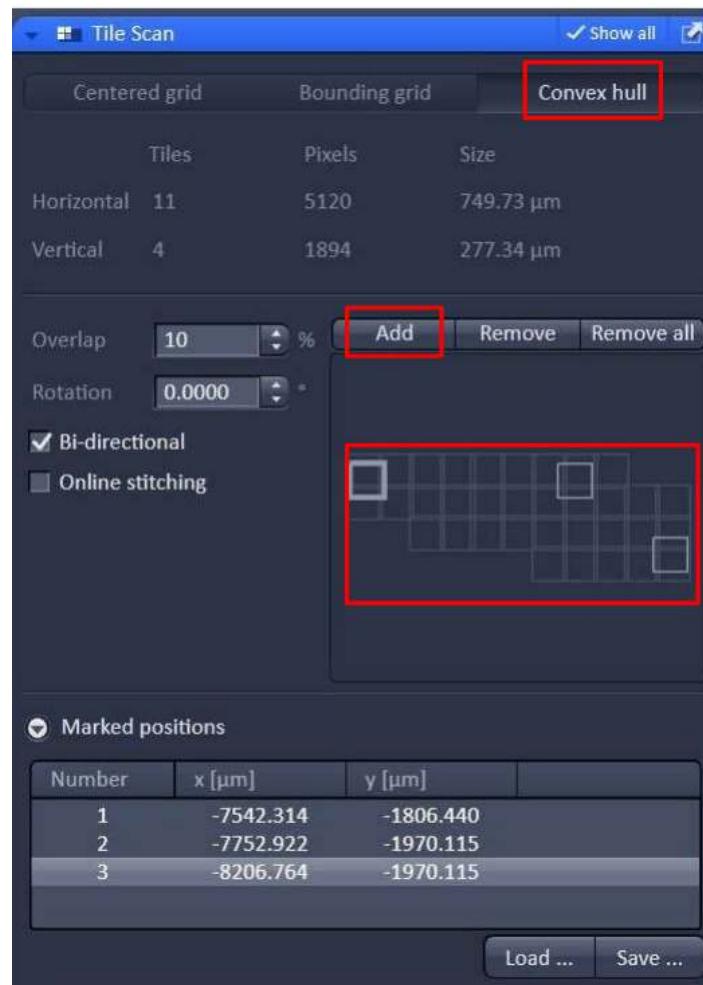
1. Select "Tile Scan";
2. In the "Centered grid" mode, set up the tiling scan centered on the current field of view:
 - A. Define the "Horizontal" and "Vertical" scanning ranges;
 - B. Adjust the "overlap" between tiles, choose "Bi - directional" scanning if needed, and enable "Online stitching" for post - acquisition image stitching;
3. Click "Start Experiment".

Bounding grid



1. In "Bounding grid" mode, move the stage to the edge of the image area, click "Add" to mark the boundary. The software will define the tiling area automatically.
2. Click "Start Experiment".

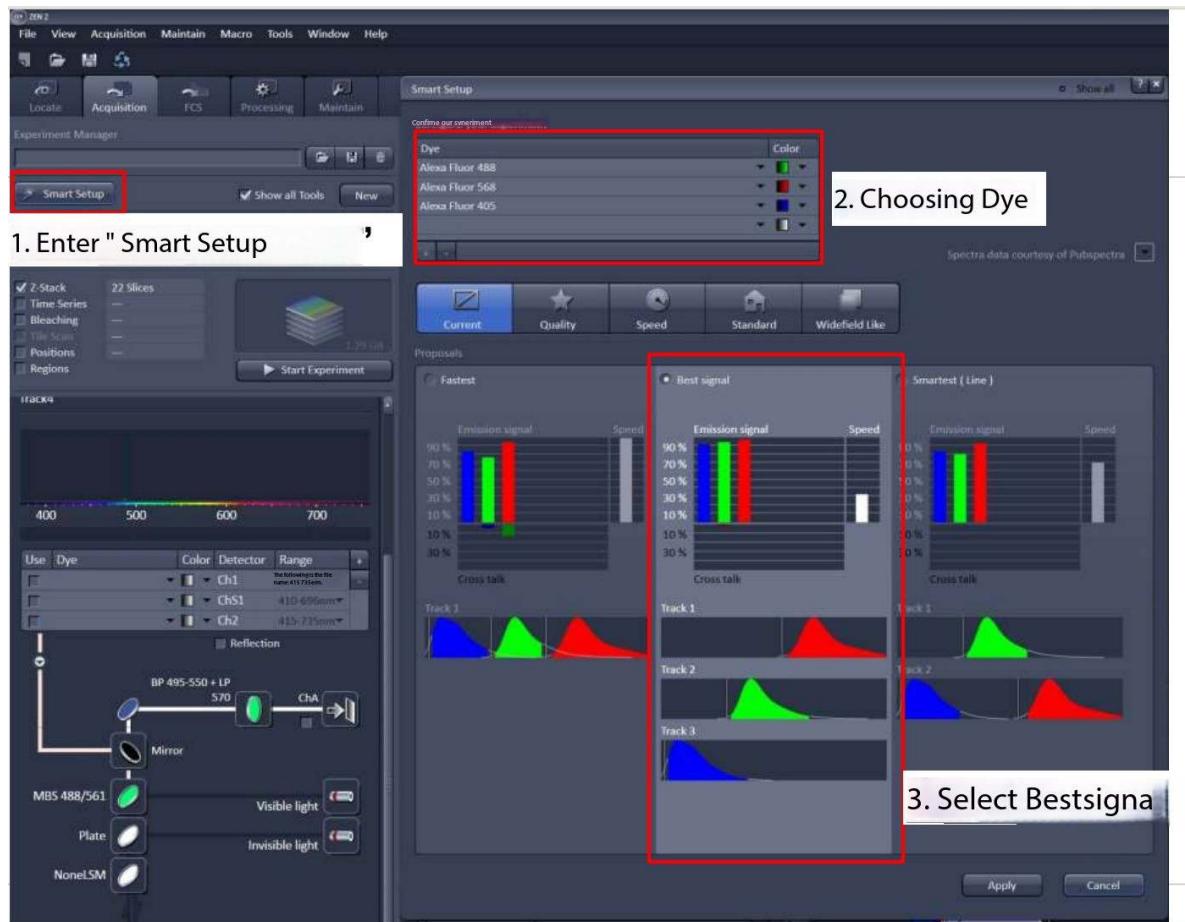
Convex hull



"Convex hull" works similarly to "Bounding grid":

1. move the stage to the edge of the desired tiling area, click "Add" to set boundary points, and the software will auto - define the tiling area, which closely follows the selected points unlike "Bounding grid";
2. Click "Start Experiment".

2.6 Airyscan Imaging





4. Channel has added a good track

Track 1

400 500 600 700

Use	Dye	Color	Detector	Range
<input type="checkbox"/>			Ch1	415-735nm
<input type="checkbox"/>			ChS1	410-696nm
<input checked="" type="checkbox"/>	Alexa Fluor 568		Ch2	568-712nm

BP 570-620 + LP 645 ChA Mirror

Lasers: 405, 458, 488, 514, 561, 633
561 nm: 2.0
Pinhole: 90.1
1.48 Airy Units, 1.3 μm section, 1 AU max

A568 Mode: Integration, Photon Counting
Gain (Master): 750
Digital Offset: 0
Digital Gain: 1.0

Display: Focus, Focus Devices and Strategy, Stage

Multidimensional Acquisition: Information On Experiment, Auto Save

5. Set the Airyscan light path here



Track 1

400 500 600 700

Use	Dye	Color	Detector	Range
<input type="checkbox"/>			Ch1	415-735nm
<input type="checkbox"/>			ChS1	410-696nm
<input type="checkbox"/>	Alexa Fluor 568		Channel 2	568-712nm

645 ChA



7. The software will automatically close other detectors and prompt "airyscan cannot be used with other detectors" in the bottom right corner.

6. Select ChA detector (i.e., Airyscan detector)

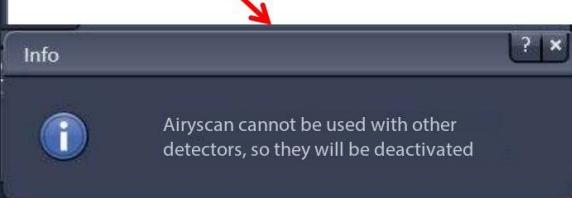
MBS 458/561

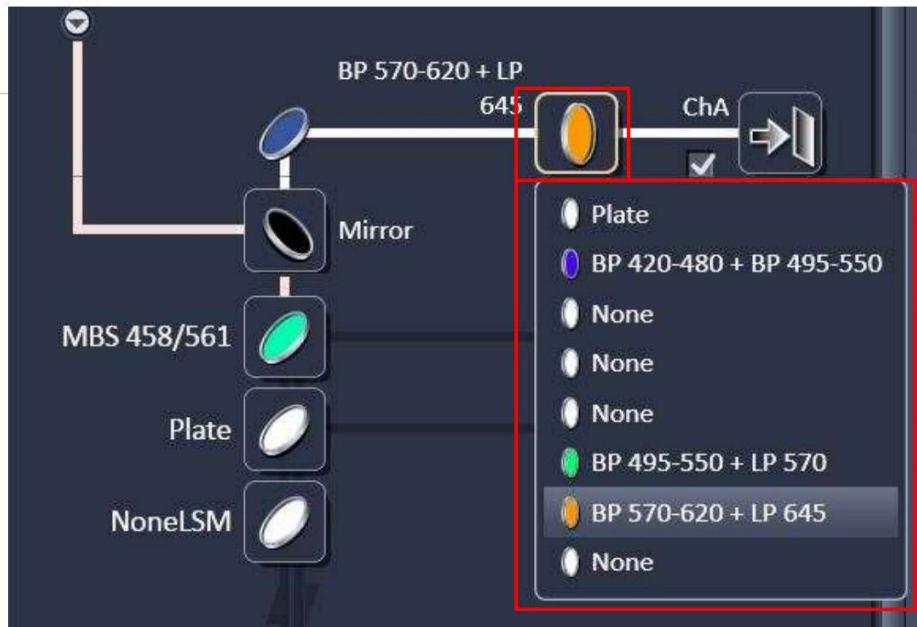
Visible light

Plate

Invisible light

None LSM

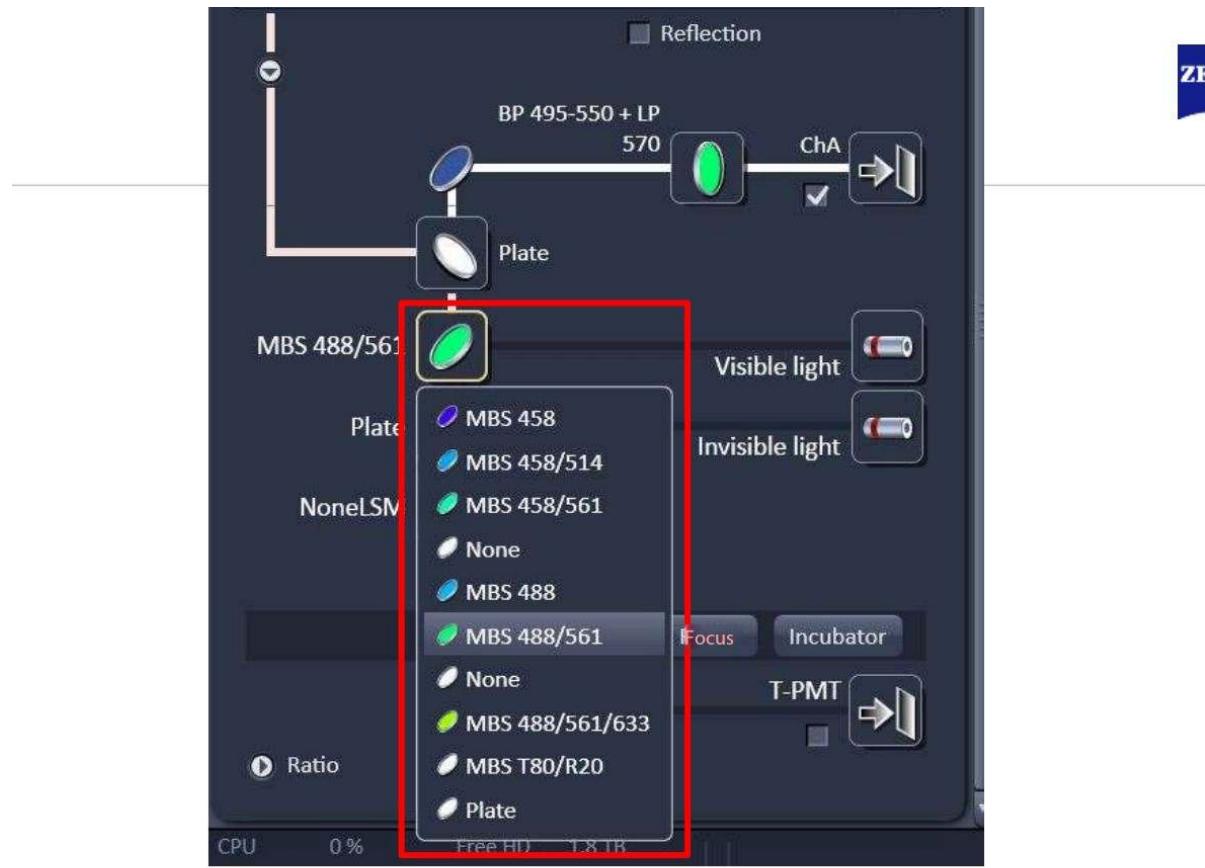




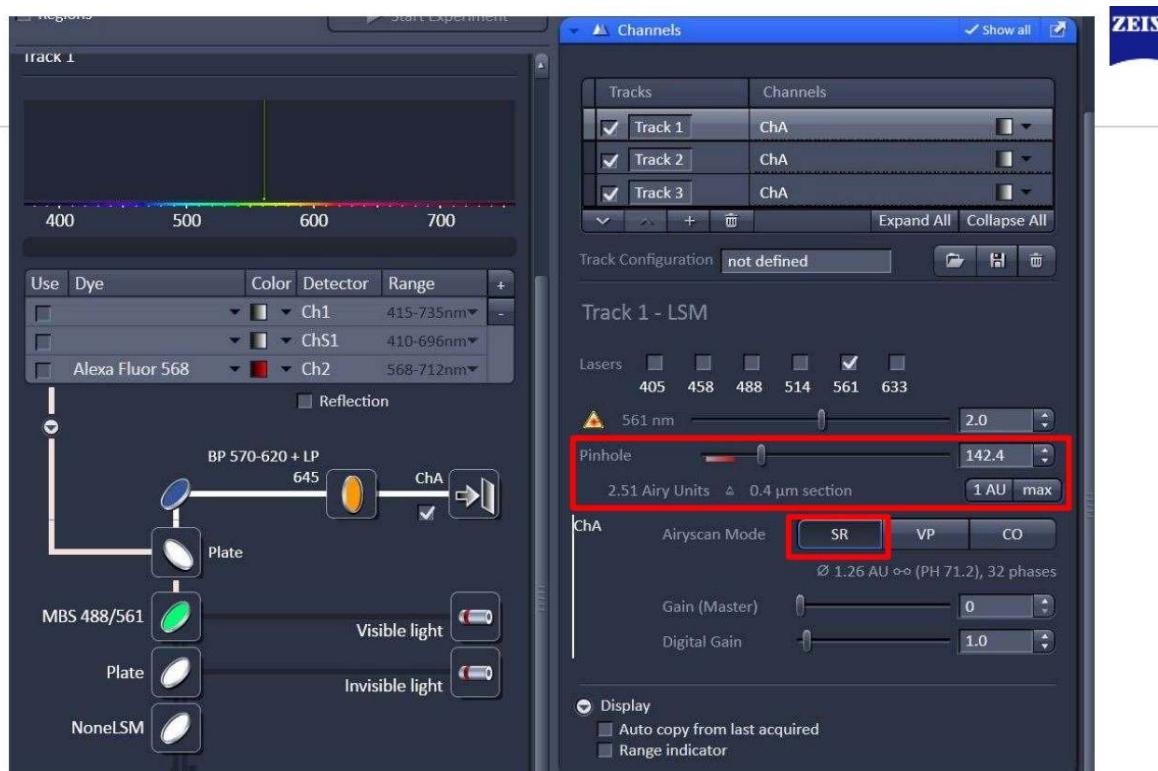
8. Choose appropriate filters, such as BP420-480 representing blue wavelength band, BP495-550 representing green wavelength band, and BP570-620 representing red wavelength band.



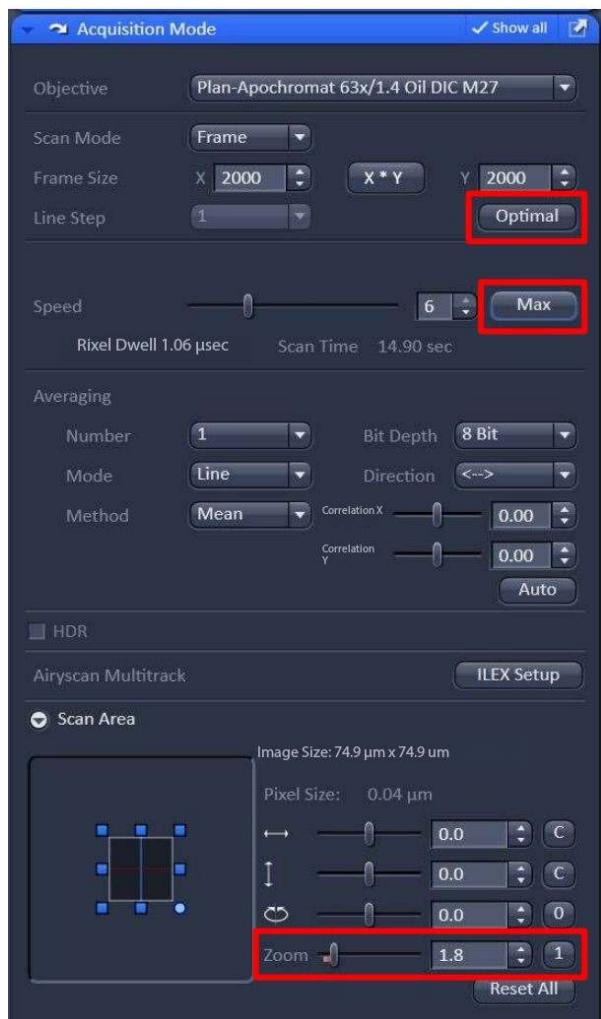
9, Secondary color separation mirror (SBS) select "Plate"



10. If multi-color fluorescence imaging is used, you can choose multiple laser reflection main dichroic mirrors (MBS). As shown in the figure, MBS can simultaneously reflect 488 and 561, reducing the need for MBS conversion.



11. do not change the size of the pinhole, Airyscan Mode is confirmed to be "SR" mode

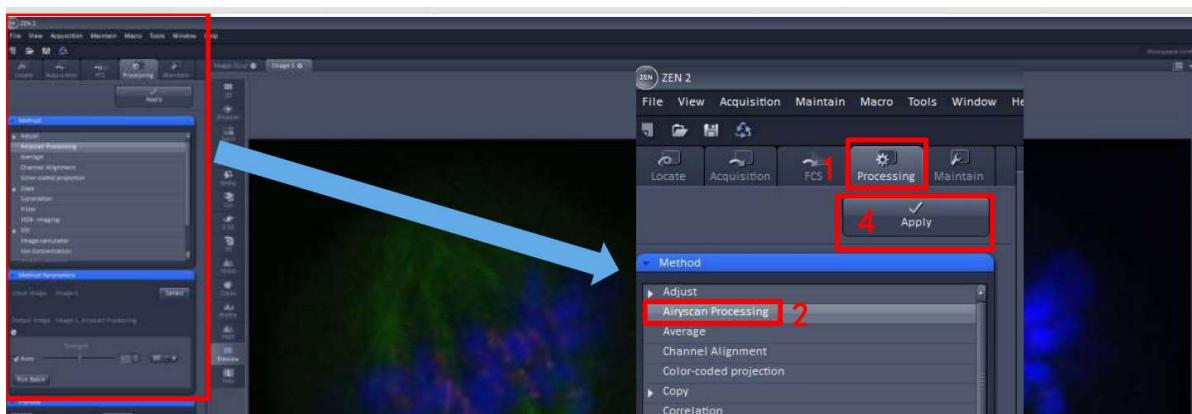
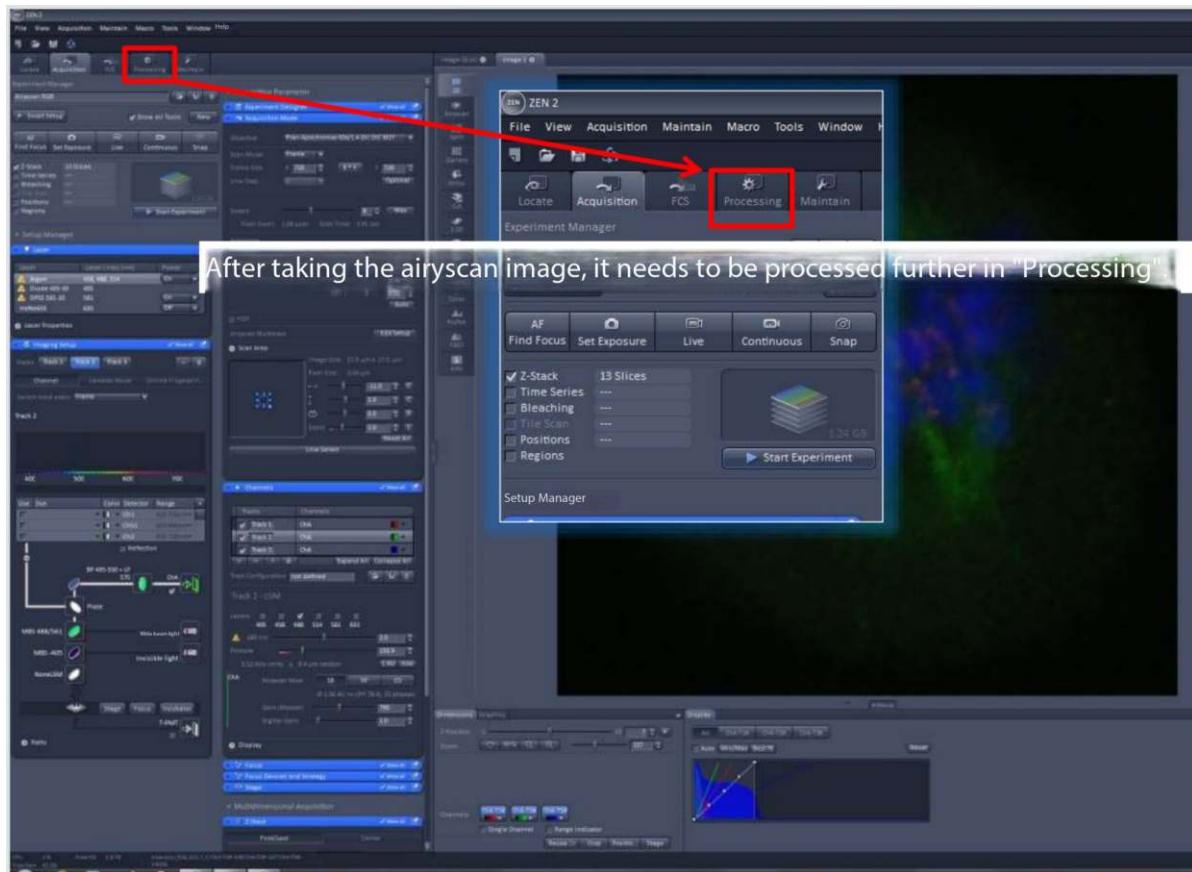


13. Frame Size Select "optimal"

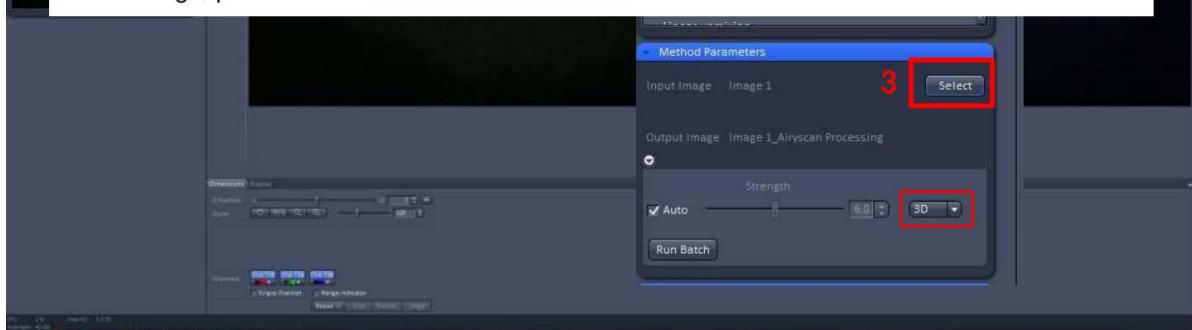
14. Speed select "Max"

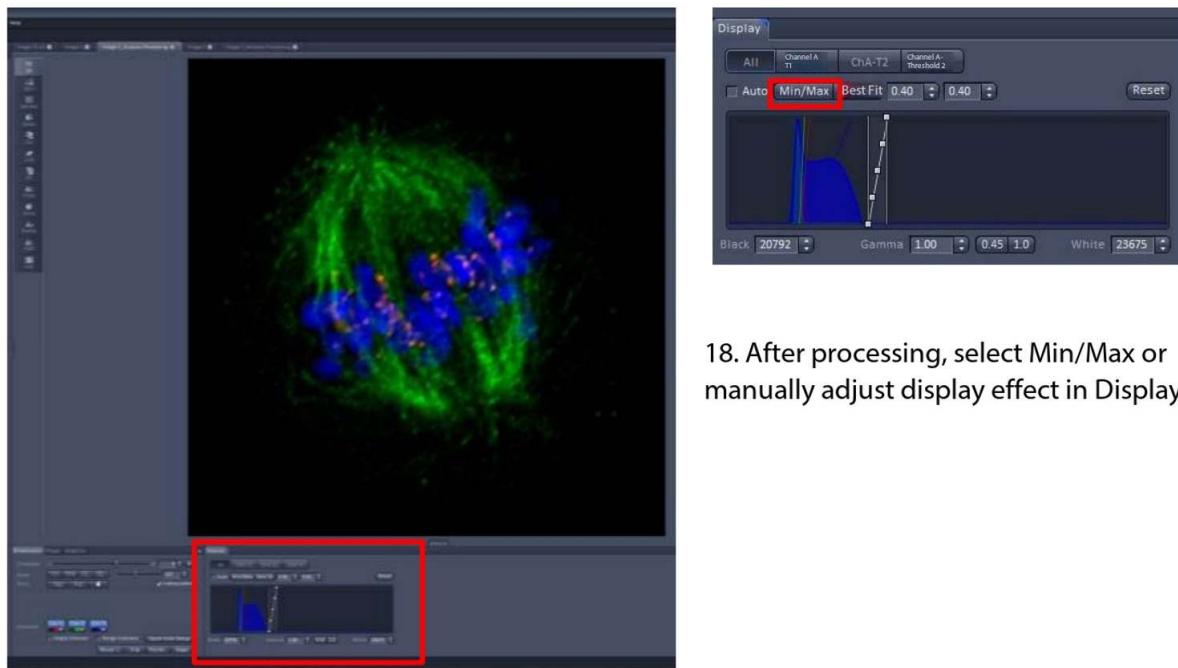


15. Then you can take two-dimensional image shots (Snap) or multi-dimensional image shots.



17. Follow "Processing Airyscan Processing Select Apply" for image processing. If it is a z-stack image, please select "3D"





18. After processing, select Min/Max or manually adjust display effect in Display.