

The Zeiss AiryScan System, Confocal Four.

Overview.

The Zeiss AiryScan module is a segmented, radially stacked GaASP detector and collector system designed to subsample the airy disk of a point emission light source. Deconvolution of the subsample data within the ZEN software then yields a degree of resolution enhancement. Zeiss claim a potential resolution of down to 140nm radially and 400nm axially. We have measured 125nm radial resolution (63x objective) whilst imaging fluorescent beads at IMB.

The AiryScan module contains emission and beam splitting filters permitting its use in place of and in conjunction with standard confocal hardware.

The module can be operated in several modes. In its default, Super Resolution (SR) Mode, it can capture an enhanced resolution image of a single or pair of simultaneously imaged fluorophores in a single channel. This can be combined into a multitrack capture performed in concert with the standard PMT channels.

In Confocal (CO) Mode it can function as a standard resolution and sensitivity detector, again either on its own or in concert with the standard PMT channels.

In R-S Mode the detector maximizes signal to noise ratio at the expense of resolution and confocality; a compromise potentially useful for capturing dim, dynamic processes.

Finally, in VP Mode, the apparent pinhole size and hence confocal sectioning of a sample can be manipulated post image capture, facilitating examination of optical sectioning effects.

Of these, SR and CO modes are likely to be most widely used, and will be discussed below. Training in use of R-S and VP modes is available by request, as a separate tutorial session.

Note: the AiryScan module is **NOT** supported by Smart Setup. Lightpath settings must be configured manually. **Note also**, that while the airyscan module may be configured to capture images in concert with the standard confocal detectors, this is only possible for sequential captures. Simultaneous imaging with the standard detectors is not possible.

Super Resolution Mode.

Basic SR image capture.

Start ZEN. Ensure ZEN starts without errors.

On the initial “Locate” screen, select a dedicated SR objective.

Note, one SR objective is installed by default; a 63x C Plan-Apochromat. This is NOT the same as the standard 63x objective, and should not be used for general imaging. Special 30 and 37°C corrected immersion oils are available for use with the SR 63x C Plan-Apochromat. A SR 40x C-Apochromat water immersion objective is also available, by request.

N.B. The special SR objectives are **very** expensive and their super resolution capabilities will be ruined by the slightest damage. Use with **EXTREME CARE**.

The ordinary objectives can be used for SR work, but resolution will not meet Zeiss claims. There may also be settings indicated acceptable within the software but in practice fail to produce an image, produce an image with artifacts, or render the software or microscope system unstable. This is not recommended.

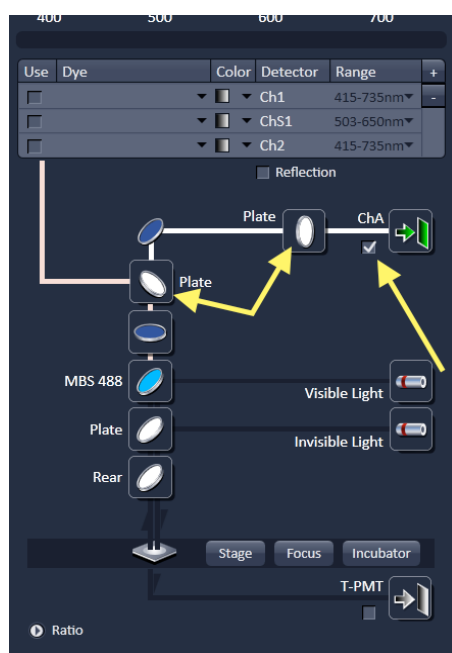
Open the ZEN “Acquisition” Screen, and click “Show all tools”.

Expand the “Imaging Setup” panel, and click “Show all”.

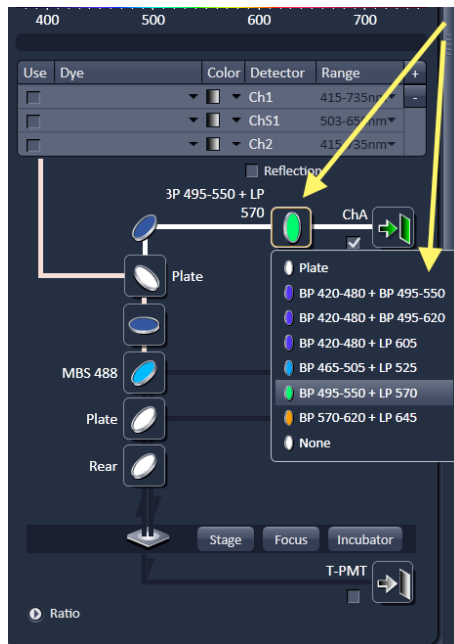
Select an excitation laser and set the laser power as normal.

Set the Main Beam Splitter to reflect the excitation laser to the sample, as normal.

Select the AiryScan Module, “ChA”, as the imaging detector. Selecting ChA turns off the default PMT selection and replaces the mirror in the Secondary Beam Splitter position with a plate (which lets light through to the AiryScan).



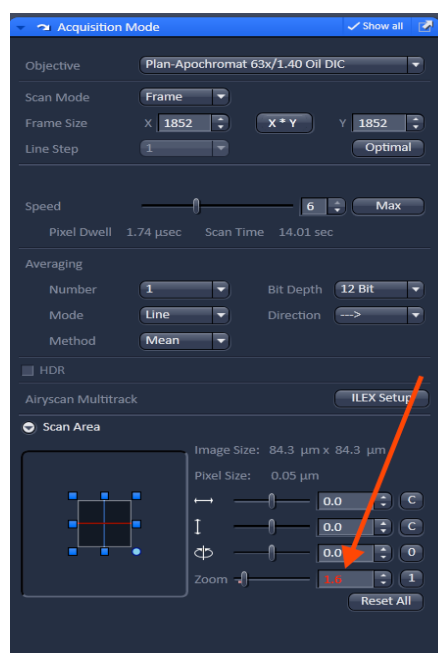
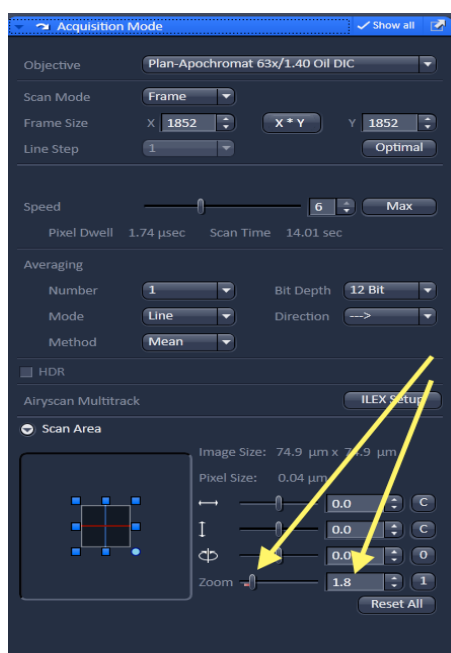
Select an appropriate emission filter for the AiryScan channel. Note, most of these are dual pass filters. Select the one most suitable not only for detection of your fluorophore, but also for rejection of unwanted emissions.



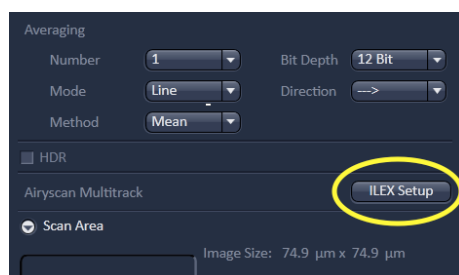
Set the AiryScan channel colour, if desired.

Expand the “Acquisition Mode” panel.

Set the optical zoom (“Scan Area”) to a level acceptable to the AiryScan module. This varies with the objective and allows the image of the airy disk to be scaled to the detector array. The slider should be to the right of the red zone and the zoom value displayed in white text. Too small a zoom value will display in red. Observe the minimum zoom requirements; ? with the 40x and 1.8 with the 63x objective.



At this time note the AiryScan Multitrack ILEX Setup button. This is for purely SR multitrack imaging and requires a slightly different workflow. This will be covered in a separate section, below.

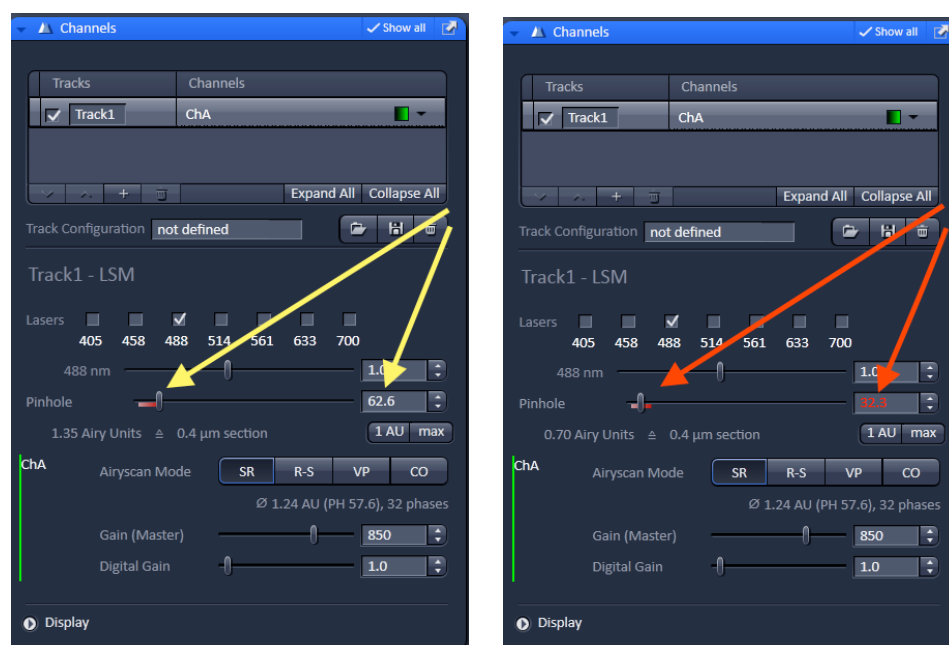


Set the capture Bit Depth and Scan Direction. The default for the AiryScan is an 8 bit, bidirectional scan. Twelve bit and single direction capture can be used, if desired.

Set the image Frame Size by clicking the “Optimal” button. This ensures acceptable image detail. Setting the frame size automatically sets the scan speed.

Expand the “Channels” panel.

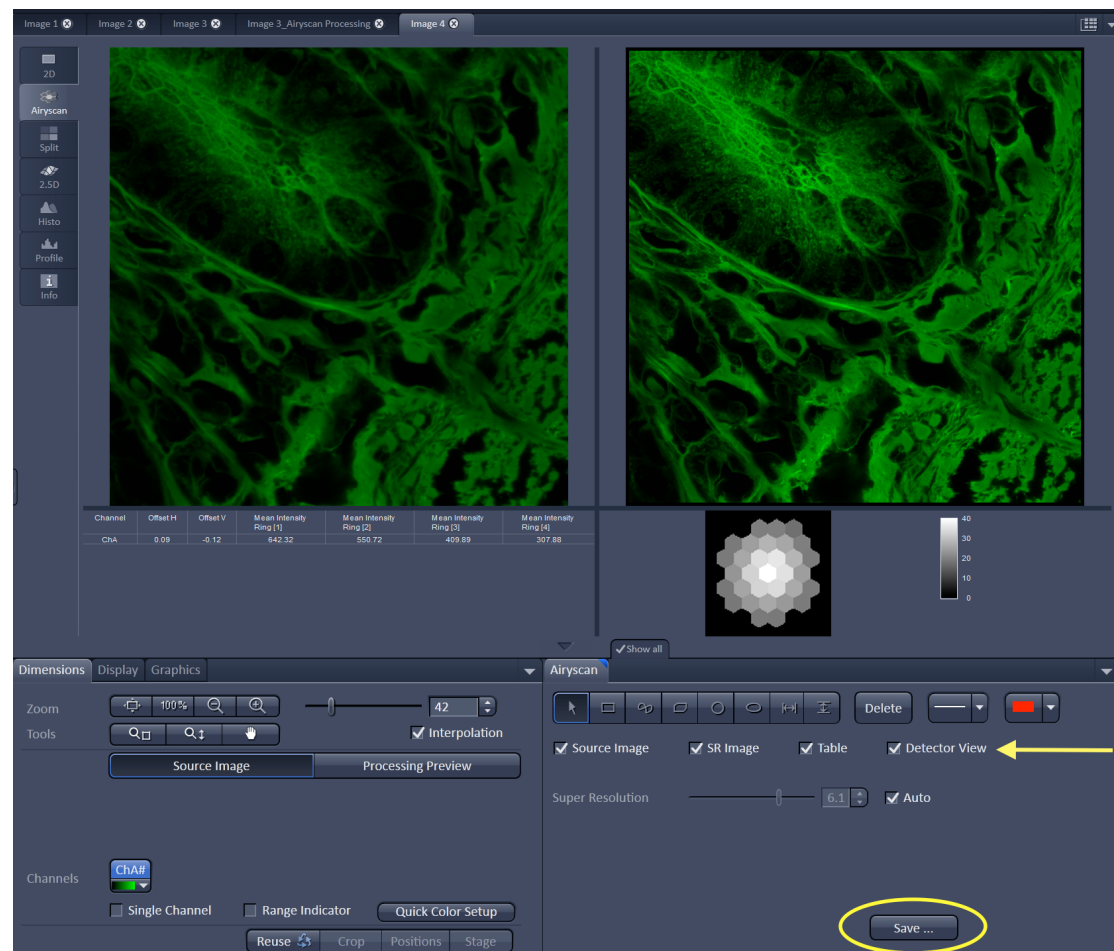
Set the pinhole size to a value acceptable to the AiryScan. In SR mode, the default value loaded by the system will be considerably larger than 1 Airy unit. This is required for transmission of sufficient Airy disk information. Zeiss recommend that the default values be used. Note that while the default will be greater than the minimum size acceptable, closing the pinhole can prevent optimization of Airy disk alignment on the detector, and hence yield substandard images. Should adjustment be desired, the slider must be positioned to the right of the red zone, and the value displayed in white text. Too small a value will display in red.



Set and optimize the exposure. You must use the range indicator to ensure there are no saturated (overexposed) pixels. Note the AiryScan does not present an adjustment for offset (black level).

From the tabs on the top left side of the image display, select “Airyscan”.

Select (tick) the “Source Image”, “SR Image”, “Table” and “Detector View” (if available) check boxes. The image window should resemble this:

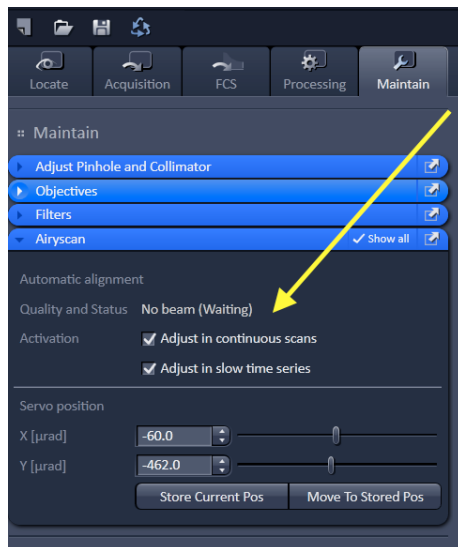


Clicking “Save” in the AiryScan image window (circled above) will save the processed, 2D SR image.

Notice the arrangement of hexagonal elements representing the AiryScan detector. This should be brightest in the centre, falling off evenly toward the edges (as above). It should not appear markedly unbalanced.

If markedly unbalanced, proceed as follows:

Go to the “Maintain” window and expand the “AiryScan” panel. We recommend you undock this panel (ie, drag it out from the panel stack), so that the alignment process may be monitored. Ensure that both the “Adjust in Continuous Scans” and “Adjust in Slow Time Series” boxes are checked.



Return to the “Acquisition” window and click on “Continuous Scan”. Alignment may take 30 seconds or more. The “AiryScan” panel you have undocked will show “Quality and Status” messages such as “No Beam (Waiting)” “Analyzing” and “Good (Waiting)”. The latter, displayed in green, indicates correct alignment. The Detector View display should update to show a more balanced illumination. Close the “AiryScan” panel (this will return to the “Maintain” window).

N.B. Alignment requires a bright sample with fine detail. The scan length necessary to optimize the alignment **WILL** bleach your sample. Therefore, use an unimportant area of your sample for the alignment process.

Imaging Multiple Fluorophores in SR Mode.

The AiryScan module is a single channel device. With its dual band emission filters and an appropriate choice of a multi laser dichroic, simultaneous capture of two or more fluorophores within the single AiryScan channel is technically possible. However, this is of no practical value.

ILEX Fast Laser Switching, Concurrent Capture.

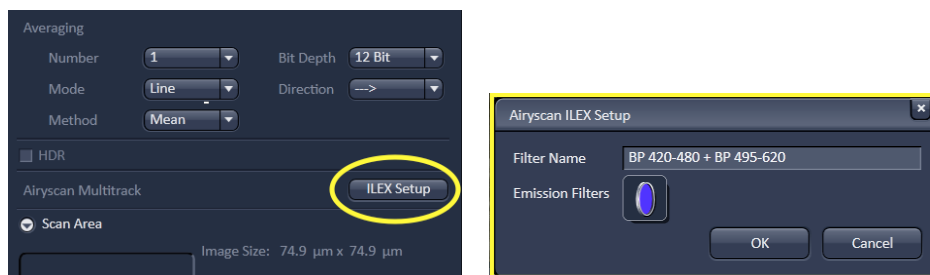
The dual band emission filters do however permit a concurrent capture of two fluorophores, with a rapid, line by line switching of laser illumination and detector read out.

Set up as follows:

Expand the “Imaging Setup” panel, and select “ChA”, as the imaging detector. Do not set up any other light path parameters at this time.

Open the “Acquisition Mode” panel, and click the “ILEX Setup” button. A popup dialog will appear with a filter name text box and a filter selection button. You will recognise the filter choices displayed by pressing the filter selection button as the AiryScan dual

band emission filters. Select the one most appropriate to the two fluorophores you wish to image. The filter name text box will populate. Click OK to apply the selection and close the dialog.



You will note that two capture tracks “ILEX Track 1” and “ILEX Track 2” have been established on the “Imaging Setup” panel. Selecting one of these at a time, set up the excitation lasers, laser powers and dichroic mirrors suitable for your fluorophores.

On the “Acquisition Mode” panel, set up the Zoom and Frame Size as before. Note that the scan direction has been set to bidirectional (<->), Bit Depth to 8 bit, and Averaging to “2”, by default. Other values for these parameters may be set, if desired.

The “Channels” panel will show the two ILEX tracks. Note that the panel “Show all” option is selected by default.

Set and optimize the exposure for each channel separately, and set channel colours (if desired).

Click “Snap”, as normal, to capture the two channel raw image. The “Split Channels” tab on the left of the image display panel permits viewing of the individual channels (and observation of the concurrent capture). Selection of the “AiryScan” tab will display an overlay view of the two channels as a raw image, and a processed preview.

NB: in order to save the processed view the two channel image must first be manually processed.

Go to the “Processing” screen.

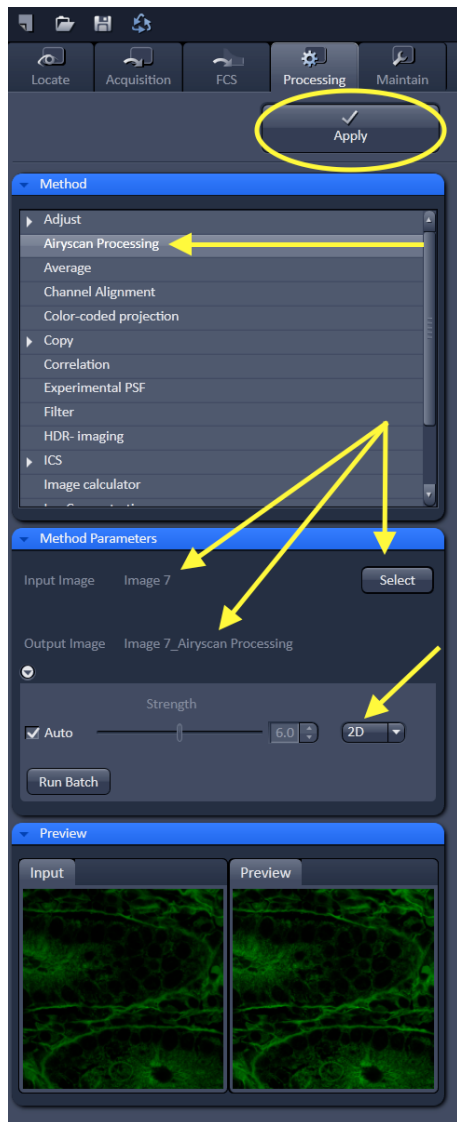
Select “AiryScan Processing” from the “Method” panel.

The “Method Parameters” panel will appear, with the text “Input Image” and “Output Image”.

Click on the “Select” button. The name of the raw image should appear beside “Input Image” and a Zeiss selected derivative of this beside “Output Image”. Ensure this is correct.

Ensure the “2D” option is selected.

Click the “Apply” button at the top of the processing panel lists.



A new image is generated. This is the completed SR multitrack and may now be saved. This image can be viewed and manipulated as as any normal dual channel image.

Multitrack (Sequential) Capture.

A sequentially captured SR multitrack with more than two channels may be captured. Set up is analogous to a conventional sequential multitrack, with each track configured as an AiryScan “ChA” SR capture. Note that careful, systematic setup and optimization of each channel, individually, is important to ensure a trouble free capture. The option “Switch track every...” **MUST** be set to **frame**.

The raw image must be processed to yield the SR multitrack, as described for ILEX captures.

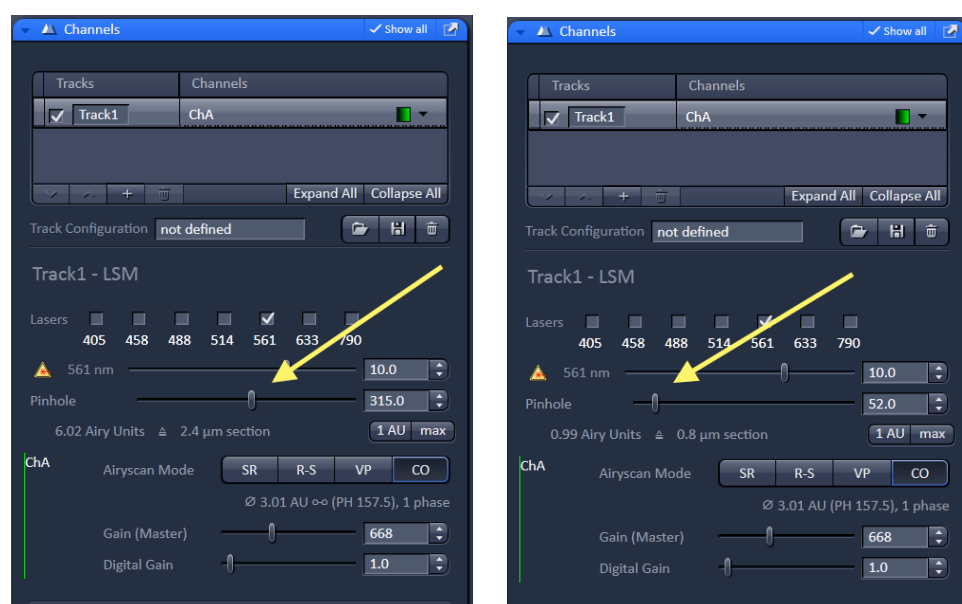
Use in Conjunction with the System's Standard Detectors.

Sequential multichannel imaging with the AiryScan detector functioning as one of the multichannel set is possible. Again, this is analogous to conventional multichannel configuration. Simultaneous capture with the conventional detectors is not possible.

CO Mode.

Selection of Confocal (CO) Mode permits the AiryScan module to function as a single channel, standard resolution detector. This may be used on its own, in concert with the standard system PMT channels in sequential multitrack captures, or as a sequential multitrack with each track configured as an AiryScan "ChA" CO capture. Configuration is analogous to that described for SR multitracks above.

Note that the CO track will be set by default to a large pinhole size. This may be closed to the more normal 1AU to improve confocal sectioning.



Note: Simultaneous capture with the conventional detectors is not possible.

Z – Stacks.

Z-Stacks are configured following set up of the desired SR or CO capture, in the same manner as they are established in standard confocal imaging.

Z stacked SR images must be processed via the "Processing" Screen, "AiryScan" and "Method Parameters" panels described above, with the "3D" option selected.