Resolution Benchmarking Platform For Laser Scanning Confocal and STED Microscopy*

*Images from other microscope techniques can be submitted, though their compatibility may not have been validated and optimized yet. **Spinning Disk Confocal** images should be classified as "Other"

About: The Resolution Benchmarking Platform is a community resource hosted by Advanced Optical Microscopy Facility at the University Health Network, Toronto, Canada. This platform allows microscopists to measure and compare the resolution of their microscopes with measurements from other instruments around the world by submitting images to OMERO. Access to the platform is **free** and open to any member of the global imaging community, though registration is required to participate. Please refer to the FAQ pages at the end of this document for more information.

Platform Workflow

1. Register each microscope

Link to Registration Form

Registration collects metadata about your microscope,
Assigns each microscope a unique instrument ID,
And grants users access to OMERO and OneDrive.

2. Record image pairs

Use a commercially available benchmarking sample (page 3)
Follow acquisition protocol (page 2, with examples provided pages 9-19)

3. Submit images to OMERO image repository via OneDrive

Upload images to the OneDrive folder. Link provided after registration. Add metadata to the excel sheet (page 6)
The RBP team will transfer these to OMERO.
Login to OMERO with credentials provided, **view comments and results as key-values.** Instructions (page 5)

Images submitted to OneDrive will be queued to be processed and measured using OMERO and FIJI. Measurements and metadata are exported to the dashboard.

4. Visit interactive Dashboard to "benchmark" resolution

Compare your results to results from the imaging community.
Dashboard Tip Sheet (Page 9)
Results will reach the dashboard 1-3 days after submission.
Visit: aomf.ca/#benchmarking

Image Pair Acquisition Protocol

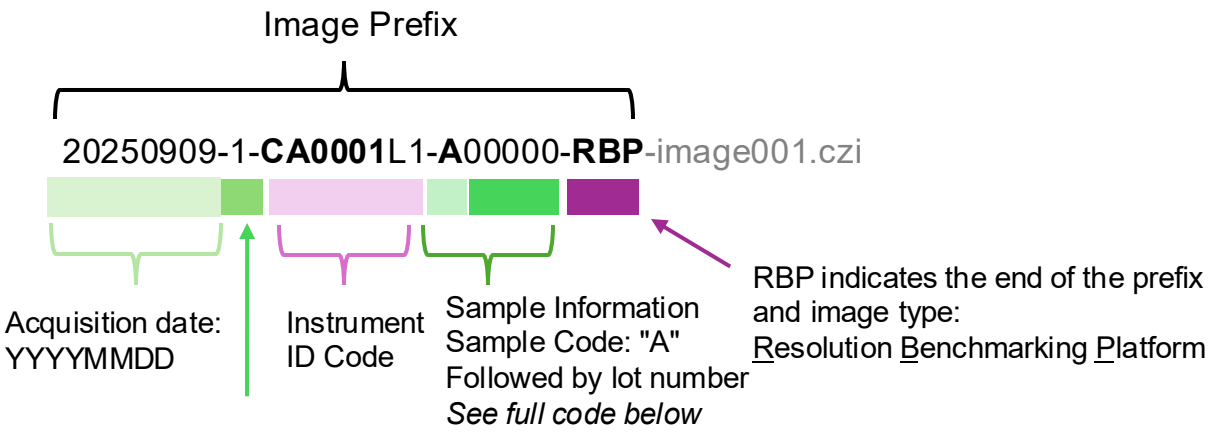
- **Acquire an image pair (two identical images) for 3-5 fields of view**
  - Each image in the pair must be acquired with the same parameters.
  - **Acquire pair as C-stack or T-stack**: duplicate channels (AF488 and AF488),or time series with no delay
  - Take 3-5 image pairs (replicates) in new fields of view.
  - **All replicates should have the same data-point-number.** (File name instructions page 3)
- Minimum image size: 512x512px, | Maximum: 2048x2048px
  - *Ideally 1024x1024 or 2048x2048px.*
  - *If using small fields of view, take more replicates.*
  - *Bit depth: Use your standard setting. Intensities in 8-bit images should fill the dynamic range.*
- Pixel size: 43 nm confocal, 11 nm STED
  - *Sizes above assume ~1.4 NA objective, and AF488 confocal or STAR RED STED*
  - *Use approximately Nyquist sampling according to SVI:* **https://svi.nl/Nyquist-Calculator**
  - *Pixel size must be: > expected resolution/5, < expected resolution x 2*
- Pinhole: 1 Airy Unit at emission wavelength
  - Emissions: 520 nm for AF488, 660 nm for STAR RED
- No saturated pixels! *Do not submit images with visible stripes*
- Optimize for high signal-to-noise

  Example parameters. These vary by microscope, but the settings below may be a helpful starting point!
  - *Set laser power relatively high (~1% bleaching between frames) but do not saturate*
  - *700-3000 ns pixel dwell time (not including accumulations/averaging)*
  - *4 line averages (>8 accumulations STED)*
  - *1.0 sec exposure for camera-based systems (spinning disk or widefield)*
  - *No bi-directional scanning*
  - *Spectral detectors should collect 500nm – 600nm, or narrower to avoid mitochondrial signal.*
- Minimize time between acquiring each image of the pair
  - *Line sequential preferred*
  - *Frame sequential <0.08 fps (12.5 s/frame)*
- Do not process the image (no deconvolution)
- Image name format: Include date, instrument ID, sample code and info
  - *(Optional)* Also include objective info and "TEST" or "STED" if applicable (page 4, 6)
    - Example: 20250909-**1**-CA0001L2-N54321-RBP.lif Image001
    - Example: 20250909-**2**-CA0001Z1-A12345-RBP.lif TEST063x1.40na-001
  - All replicates should have the same **data-point-number**, listed after the date.
  - See page 4 for tips on how to add additional information in the filename.
- Original image file type
  - Examples: .czi, .lif, .nd, .obf etc.
  - Recommended max file size 60 MB
  - Original files preserve metadata. Exported .tif or ome.tif files will also work (make sure they retain pixel calibration
  - Submit additional metadata in excel spreadsheet (see page 5-6)
  - Each image file should contain the FRC image pair as a multidimensional image. Merge channels and time points as needed before uploading.

Image file name guidelines and recommended samples

Image name should include:

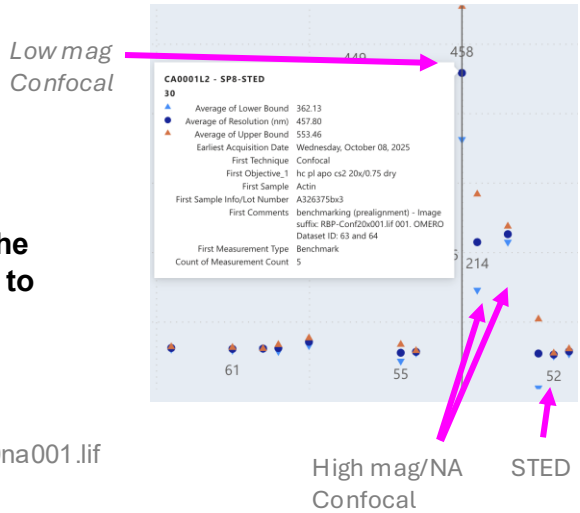
Date, data-point-number, microscope ID, sample code and lot number in the image prefix:



Data-point-number
The *average* of images with the same date/data-point-number/instrument ID combination will be plotted as one data point in the "Per Instrument" plots on the PowerBI Dashboard.

Sample Code	Sample Type	Sample Description
A	Actin*	Image of Phalloidin AF488 from FluoCells™ Prepared Slide #1 (BPAE cells with MitoTracker™ RedCMXRos, **Alexa Fluor™ 488 Phalloidin**, and DAPI) Catalog number F36924
N	NPC-ring*	Image of NPC Ring STAR RED from Abberior Cells **NPC-Ring STAR RED**, NPC-center STAR ORANGE, Item number IG2COLOR-4021
S	Spheres/Beads	Include the diameter in nanometers and excitation wavelength in the image name. Ex. "Sd100ex488"
X	Other	Please describe sample in comments in the Key-Value spreadsheet. A short identifier can be included after the sample code within the filename. Ex. "XtubSTARG"

Image file name tips



Data-Point-Numbers separate measurements from the same day on the same instrument that are expected to have different resolutions.

- 20260205-**1**-CA0001L2-A00000-RBP-020x0.80na001.lif
- 20260205-**2**-CA0001L2-A00000-RBP-063x1.40na001.lif
- 20260205-**3**-CA0001L2-A00000-RBP-100x1.40na001.lif
- 20260205-**4**-CA0001L2-A00000-RBP-100STED100x1.40na001.lif

- Add the data-point-number just after the date.
- Data point numbers necessary to plot measurements as different datapoints in the dashboard per instrument view when they are submitted for the same instrument, same day.
- The example filenames above show three confocal benchmarks on different objectives and a STED benchmark. Without unique data-point-numbers, all 4 conditions are averaged into one data point.

Instead of adding information in the Specify Key-Value excel sheet (page 6), certain metadata values can be defined in the image filename directly.

Define imaging technique as STED

If submitting a STED image, you may include the term STED (all CAPS) in the filename.
This will over-ride the "Confocal" default defined at the time of registration.
- Example:
- …-RBP-**STED**.obf

Define an image pair as a "TEST" (instead of a "Benchmark")

By default, all images are considered "Benchmarking" images as opposed to "Test" images.
Images can be re-defined as "Test" images by including TEST in the image filename to the right of "RBP"
…-RBP-TESTimage001.nd
Test images should be used to optimize your benchmarking conditions. They are not included in the best or average resolution calculation for the microscope. A "comment" key-value (page 6) will need to be added to specify the test conditions. Brief descriptions can be added to the image suffix (for example "0.5AU" to indicate a narrowed pinhole). Test measurements submitted on the same day as benchmarking measurements must have different data-point-numbers.

Define the objective magnification and NA in the image filename

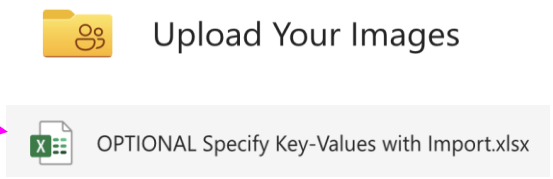
The default values for objective magnification and NA are 000x and 0.00NA
These properties can be defined in the image name by including them in the filename using the following format:
- [0-9][0-9][0-9]**X**[0-9].[0-9][0-9]**NA**
- Examples:
  - *005x0.25na*
  - *060x1.40na*
  - *100x*
  - *020x*

The magnification can be defined without the NA. This information is not case sensitive.

Upload images to OneDrive: RBP-PUBLIC

Add information about your images as Key-Values in Excel sheet in the Upload Your Images folder (Page 6)

AOMF Staff will transfer your images to OMERO



Finding your images in OMERO

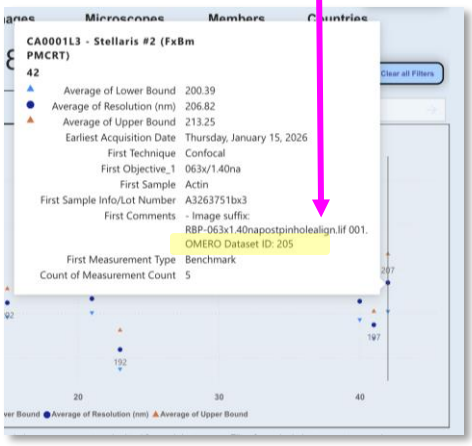
The OMERO web client can be used to view, but not to upload images. Images submitted to OneDrive will be checked to make sure they are safe to upload to OMERO and then uploaded by AOMF Staff.

Our platform organizes images into:

- "Groups" - Containing images submitted for each year.
- "Projects" - Named with the image Dataset ID
- "Datasets" - Containing **Images** or **FRC curves**

Log into the web client: https://omero.uhn.ca/omero/
Find your images:

Hover over data points in the PowerBI Dashboard to find the OMERO Dataset ID



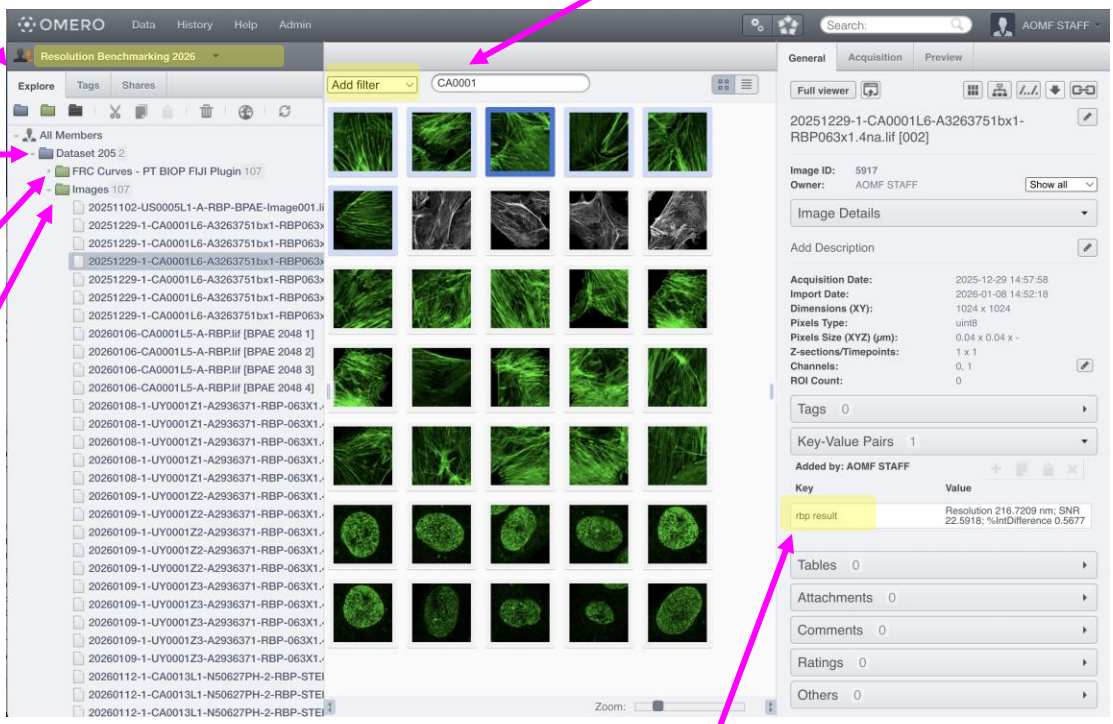
Click here to change the group, and view "All Members"

Filter for *your* data by searching "name" your group ID

Project
Named with Image Dataset ID

Curves

Images



View results for each image

Specify key-value pairs using MS Excel template in OneDrive

Information about your images can be specified as Key-Values that will be added to images in OMERO. Use the excel sheet "Specify Key-Values" in the Upload Your Images folder in OneDrive.

(Recommended) Enter information about the **OBJECTIVE LENS** 1

Example: "63x/1.4 NA oil" or "HC PL APO 63x/1.4 NA OIL"

- List in this format, [mag]**x/**[0.00] NA
- Adding this information here if the objective information is not already included in the filename, or the metadata.
  - See page 4, instructions for including objective info in filename.
  - Original file formats should retain objective information in metadata.

(Optional) Add information about the images as a **COMMENT.** This can be viewed with the data in the Per Instrument plots in the PowerBI Dashboard 2

Example: "Pre-service visit, 100% depletion."

- *Images of samples other than the recommended benchmarking slides, can be described here. Ex. "Tubulin GFP, GFP Nanobody STAR RED"*
- *Images captured using parameters that differ from the protocol should be noted here.*
- *Images using a technique other than Laser Scanning Confocal or STED, can be described. Examples, "Tau STED"*

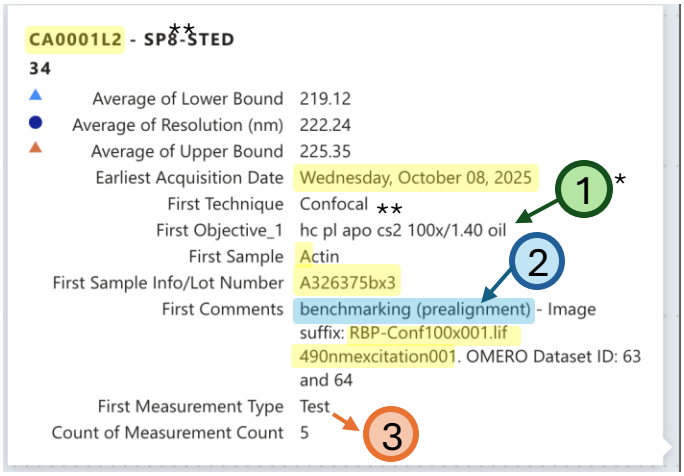
(Optional) Indicate if the image is a "**TEST**" instead of a "benchmark." 3

Enter "FALSE" in the benchmark column

- "Benchmarking" results should aim to be a best result.
- "Test" results are used to test how variables affect resolution.
- Both test and benchmarking results are plotted in the "Per Instrument" plots.
- Only benchmarking results appear in the "Best Results" and "Average" plots.
- **Default:** Images are considered benchmarking images unless specified by a key-value in the Excel template or in the filename.

(Optional) Indicate if the images were captured with non-default **"TECHNIQUE"**

- Indicate which microscopy technique was used to capture the images if different from the default.
- **Default:** This is defined during registration for each microscope. Confirm values in the List of Registered Instruments
- STED images can be identified in their filenames (see page 4)



Hover over data points in the PowerBI Dashboard to find information about the images. This information is provided by the filename, registration form, and Excel template in OneDrive

Information provided in the filename

** Provided at registration

* Provided in Excel, or metadata

Frequently Asked Questions

How is image resolution calculated?

*The platform uses a technique called **Fourier Ring Correlation (FRC)** by implementing the FRC plugin by PTBIOP in FIJI. The values we report are determined using a fixed one-seventh cut-off. FRC measures the correlation between two near identical images to separate image features from noise. Desired signals and structures in the image are reproducibly acquired between the two images. Noise should be random and therefore poorly correlated between two acquisitions.*

Why do I need to submit an image pair?

FRC, the technique we use to calculate resolution (see above) requires two images to measure the correlation between them.

Do I need to use one of the recommended benchmarking samples?

No. We accept and process images from other sample types. However, you will not be able to confidently compare your results to results from other microscopes. The recommended samples yield high quality (good resolution) and reproducible results. They are commercially available and easy to obtain.

Is the resolution calculated the best I can expect?

No. There are several definitions of resolution. FRC calculation is one particular definition. The FRC calculation also represents an average of the whole image, smaller regions may be higher resolution. Resolution depends on sample type and other samples may perform better than our recommended samples. We encourage users to submit higher-resolution samples to the platform, and please include relevant information in the comments (structure, labeling technique, dye, etc.) Resolution can also be improved by post-acquisition processing. In general, processed images should not be submitted to the platform as these processing steps remove noise, which interferes with FRC. If you want to try submitting processed images, you should also submit the pre-processed images.

Additional questions? henrietta.bennett@uhn.ca

Frequently Asked Questions

What do I do with the results from my microscope?

Compare your measurements with those from the community. Determine if your microscope is achieving reasonable resolution. Regular submission of measurements over time can be used to track the stability of your system, and the effects of incidents, upgrades, or service interventions.

How do I improve my resolution?

If you feel your resolution should be better, work with members of the imaging community, including your company representatives, to evaluate whether the acquisition parameters or hardware can be adjusted.

Who sees the data I submit to the platform?

Everyone! Data in the dashboard is public. Additionally, all registered participants in the resolution benchmarking platform can also see images and original metadata in OMERO. This includes facilities, labs, and companies.

How is the data used?

The data is used by members of the imaging community (including yourself!) to compare the practical resolution achieved by all participating microscopes. Platform data will also be published in academic articles. All participants, including commercial participants, have agreed not use the data for marketing purposes that target other groups and individuals.

Can I edit the information submitted with my images?

Yes. Metadata and comments can be edited in OMERO at any time. It may take some time for those updates to appear on the dashboard. Contact the RBP team to request edits to your group, microscope, and contact information.

What are the future directions for this project?

As the platform grows, we hope to add more microscopy techniques and to improve the current workflow, including how resolution is calculated and which samples are used. Suggestions for improvements and expansions to the platform can be made by contacting the RBP team.

Tips for using the Power BI Dashboard for Resolution Benchmarking

Last updated February 5, 2026

Visit the dashboard at: AOMF | UHN

This dashboard refreshes daily at 12:30pm Eastern (Toronto time)

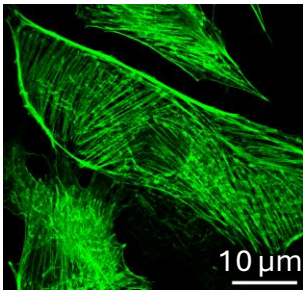
- **View the dashboard full-screen,** use double-ended arrow in bottom right corner.
- To view the list of Countries, Members, or Instruments as a table, right click next to the title for each list to the left of the plot space.
- To view the data in each plot as a table, right click on the plot area.
- To find your results, perform a **keyword filter** for part of your group name or instrument name. The group or instrument IDs can also be a keywords.
- To clear keyword filters, click the eraser icon in the top-right corner of the filter bar.
- **Can't find your data?** After you submit images to OneDrive, they still have to be processed and exported to the dashboard. This may take 1-3 days.
- **Still can't find your data?** It may have warnings attached to it. Measurements with warnings are excluded from the **Best Results** and **Average** plot. Go to the **Per Instrument** tab and use the Instrument search bar above the plot space to filter for the keyword "warning" to see plots that contain all the measurements that contain warnings. Note that images from the same session can contain different warnings (for example: one image pair may be saturated and another image pair may have a suspicious curve). These will be grouped together in the plot. To evaluate the types of warnings attached to each image, check their key-value pairs in OMERO.

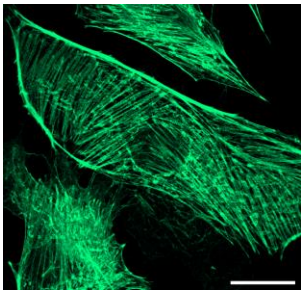
Current Image acquisition recommendations (Confocal):

FluoCells™ Prepared Slide #1 (BPAE cells with MitoTracker™ Red CMXRos, **Alexa Fluor™ 488 Phalloidin**, and DAPI)
Catalog number F36924

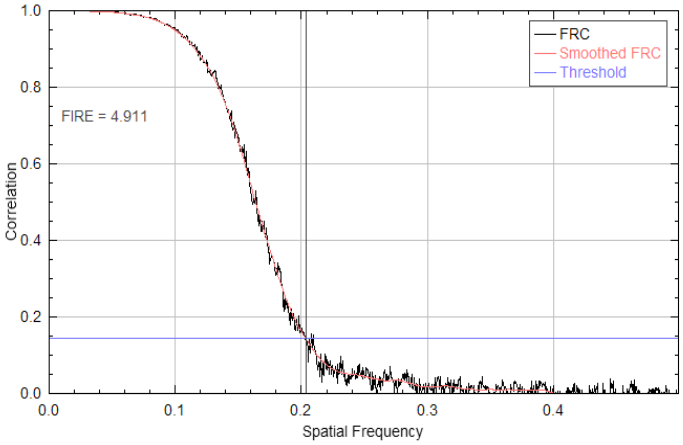
<u>Example AOMF "Standard FRC conditions" - developed on Stellaris Confocal</u>

BPAE slide: Alexa Fluor™ 488 Phalloidin
488 nm Excitation
Detection ~500-600nm
63x 1.4 NA Objective
1 Airy unit pinhole
45 nm pixel size
2048x2048 or 1024x1024 pixels
700 ns pixel dwell time
4 Line Averages
Line sequential scanning --> **2 "near identical" frames for correlation**
No saturation (<0.1%)
FRC Threshold: 1/7, 3-Sigma





FRC Resolution: 220 nm



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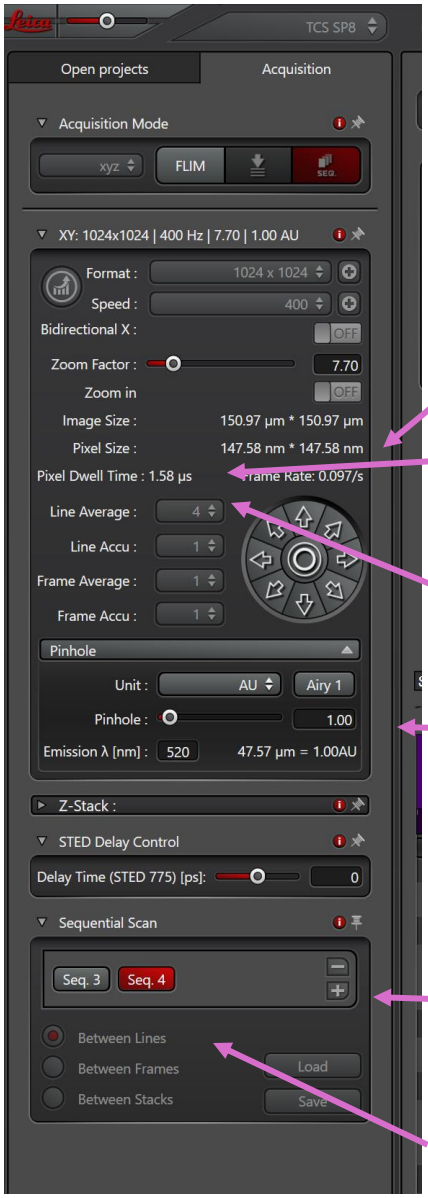
UHN

Leica SP8 Image Acquisition Set-Up
For capturing image pairs (confocal)



Pixel size (use Nyquist, 43nm for NA 1.4, AF488)
Adjusted by image size and zoom.

Pixel dwell time (use 0.7-3 *µs)*
Adjusted by scan speed.
400Hz recommended

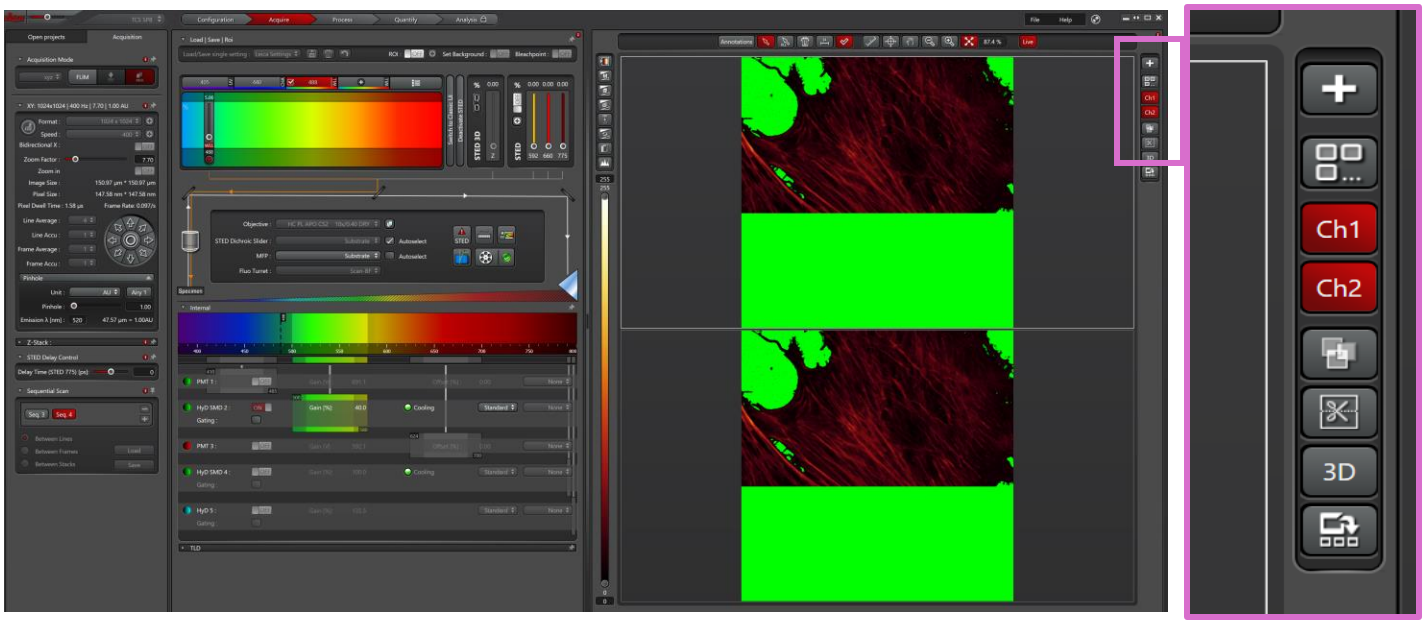
4 Line averages

Pinhole 1 Airy Unit, emission 520nm
(for benchmarking with AF488)

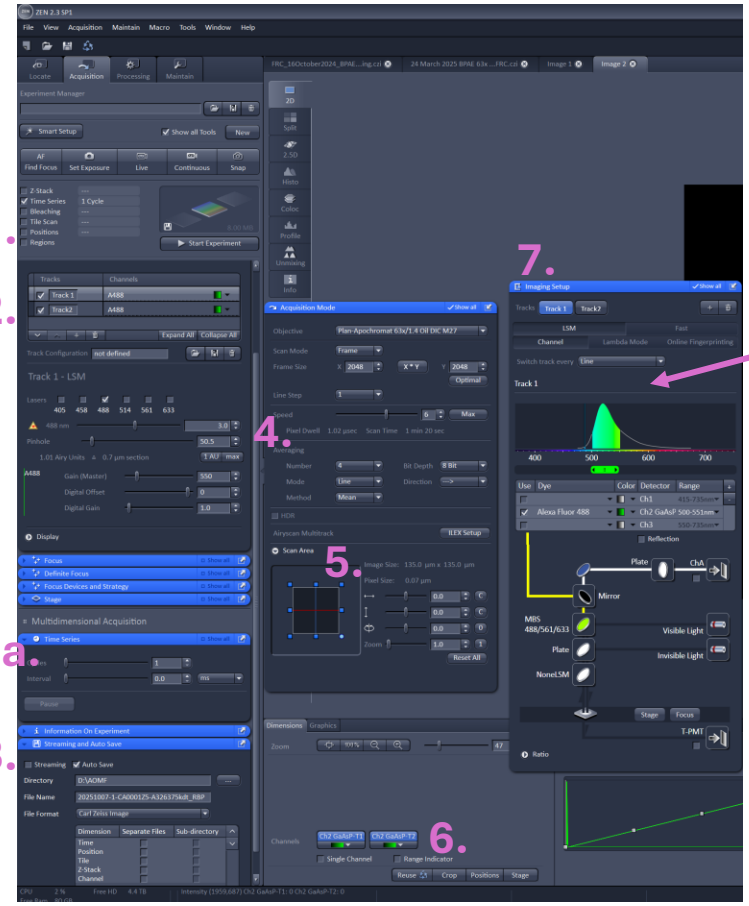
(+) will duplicate the setting
Set up excitation and detection for one channel.
The (+) to perfectly duplicate it.

Line Sequential

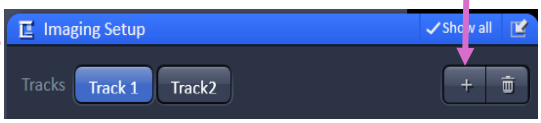
FRC image pair
acquired as 2 Channels

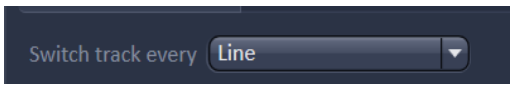


Zeiss LSM880 Image Acquisition Set-Up
For capturing image pairs (confocal)

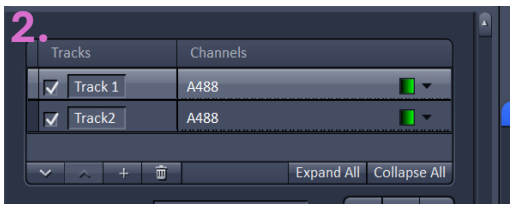


(7) **Select (+) to add a new track**
Adjust the laser and detector so that Track 2 is identical to Track 1.

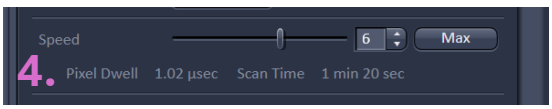




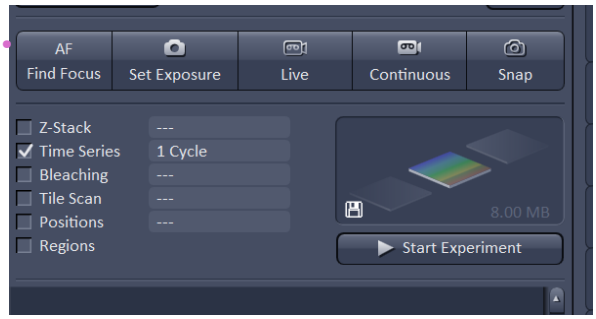
Use line sequential acquisition

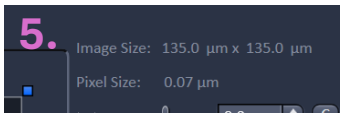


(2 and 6)
FRC image pair acquired as 2 Channels

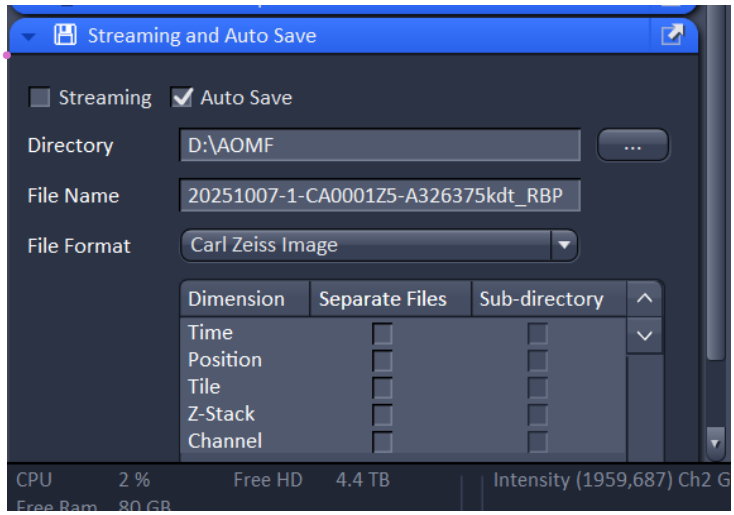


(4) Pixel dwell time
(use 0.7-2 *µs)*
Adjusted by scan speed.





(5) Pixel size (use Nyquist, 43nm for NA 1.4, AF488)
Adjusted by image size and zoom.

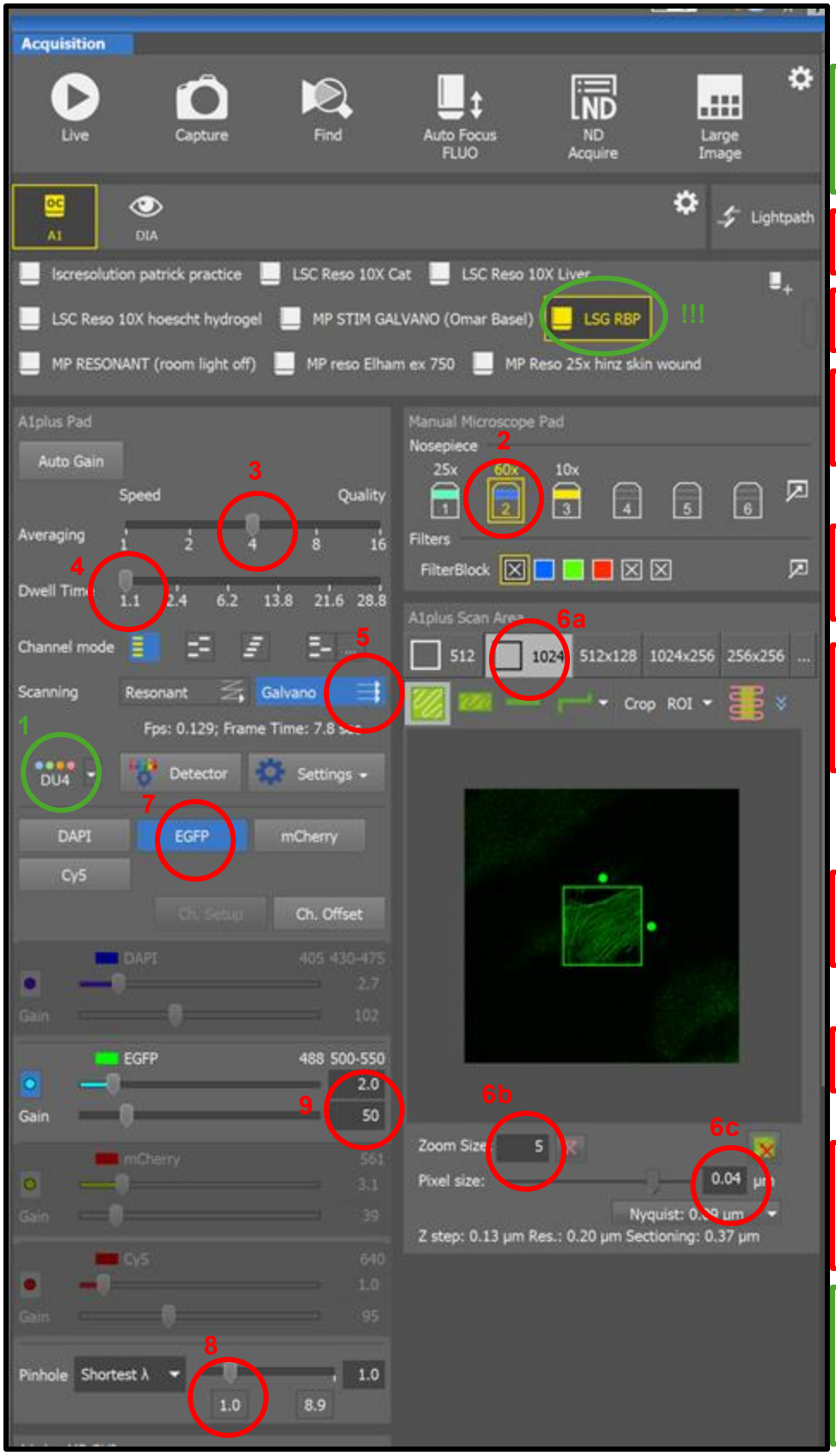


Saving images

Set up a time series (1) of one cycle. Use "Start Experiment" to acquire and autosave image

(3) **Autosave** images with with the required **filename** format that includes Date, Session, Instrument ID, and Sample information.

Nikon A1R Image Acquisition Set-Up
For capturing image pairs (confocal)



Galvano Mode
(Filter based system)

1. To view your light path click DU4 (see last page)

2. Select correct lens

3. Set averaging to 4

4. Set dwell time to 1.1msec = 1,000usec

5. Scan in single direction

6 (a,b,c). Set scan area zoom to achieve pixel size of 40nm

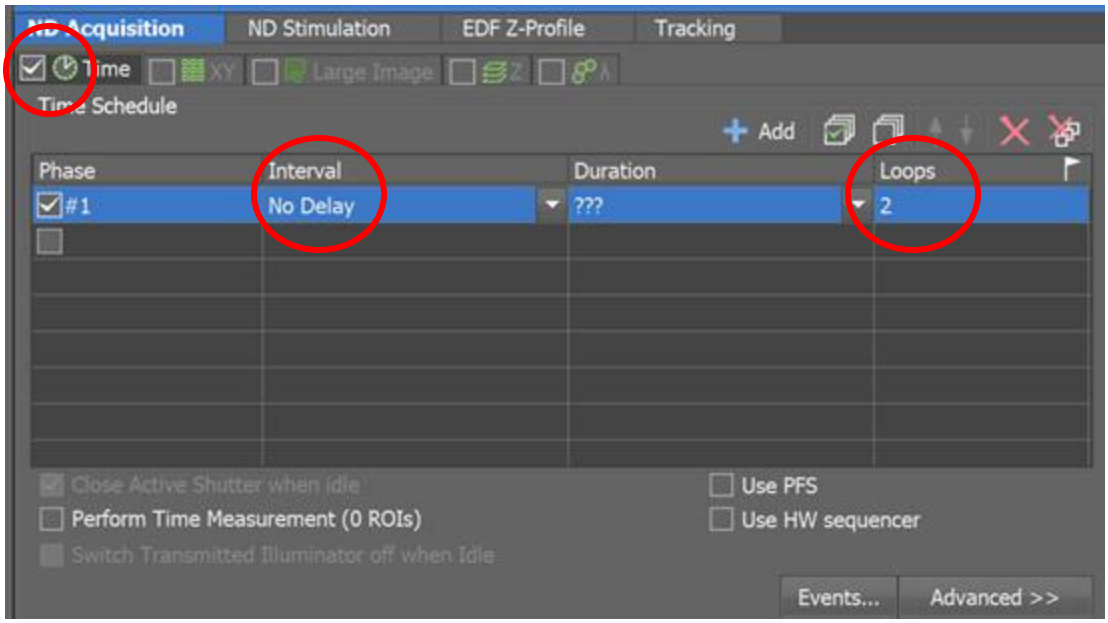
7. Select appropriate channel

8. Set pinhole to 1 AU

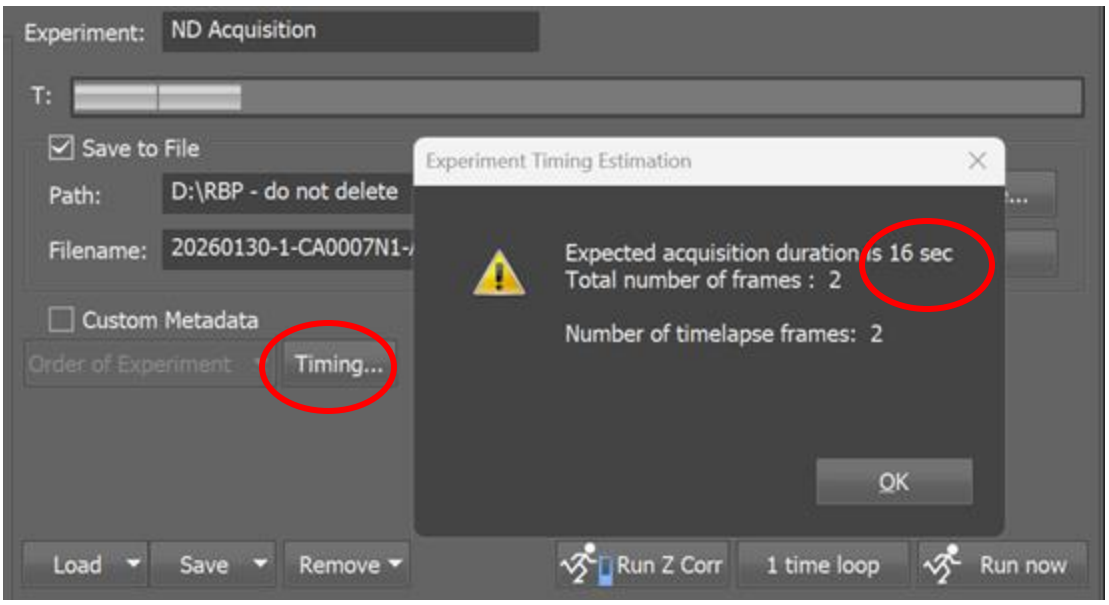
9. Set laser power and gain to achieve optimal SNR without saturation

!!! When parameters 1-9 are set you can save this as an optical configuration.

10. Under ND Acquisition, check “Time”. Set parameters to no delay, 2 loops

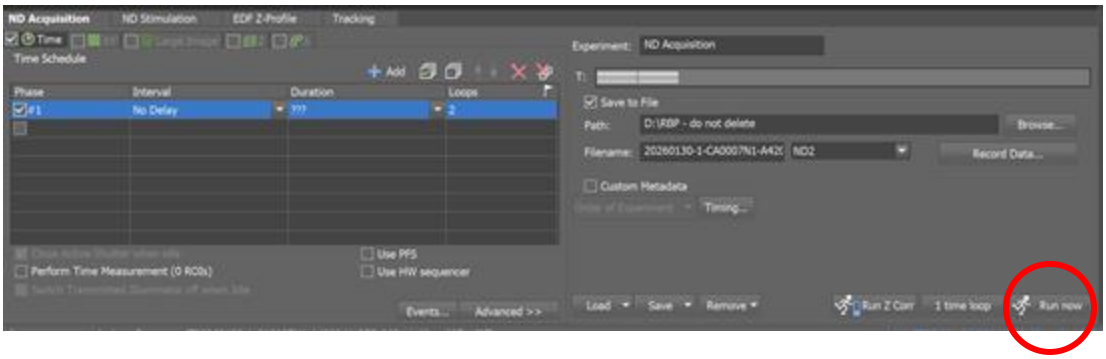


11. Under Experiment, click Timing. Check that each frame acquisition does not exceed 12.5 sec

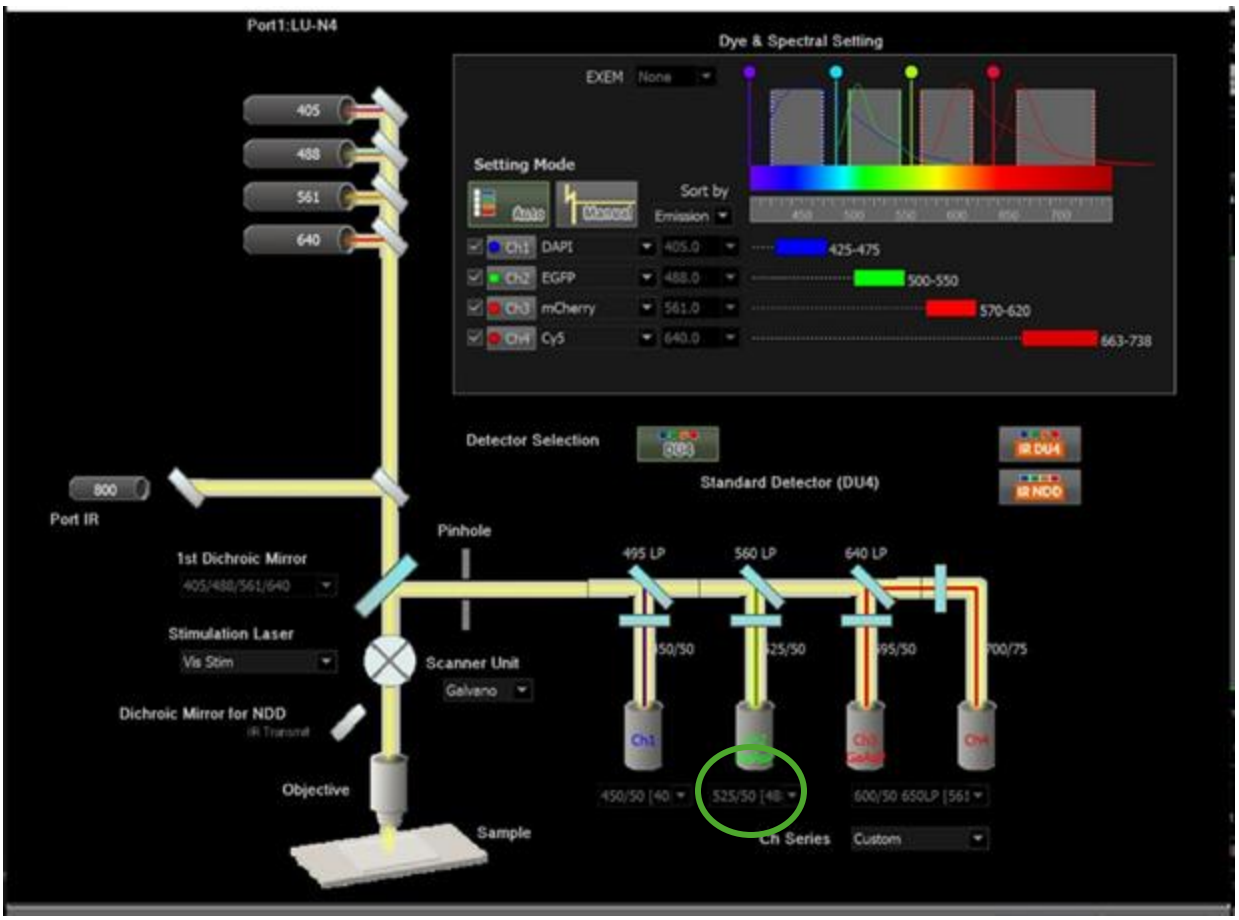


8 sec per frame. OK!

12. Click “Run now”



Light Path: This is a filter based system. For AF488 we are collecting 525/50 nm emission.



Example - Nikon A1R - Laser Scanning Reso Mode (Filter based system)

Notes:
Resonant mode

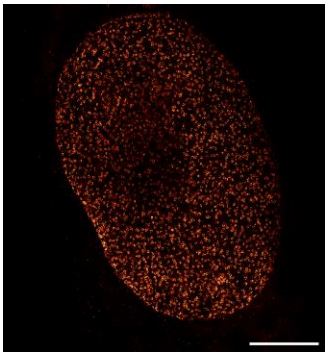
- Fixed dwell time
- Fixed size scan areas
- This leads to discreet selections of pixel size, 30nm or 50nm
- As the dwell time is so low, recommend to use max averaging of 16 to minimize noise as much as possible

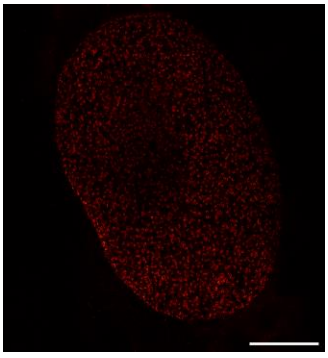
Current Image acquisition recommendations (STED):

STED standard: Abberior Cells: **NPC-Ring STAR RED**, NPC-center STAR ORANGE, **Item number IG2COLOR-4021**

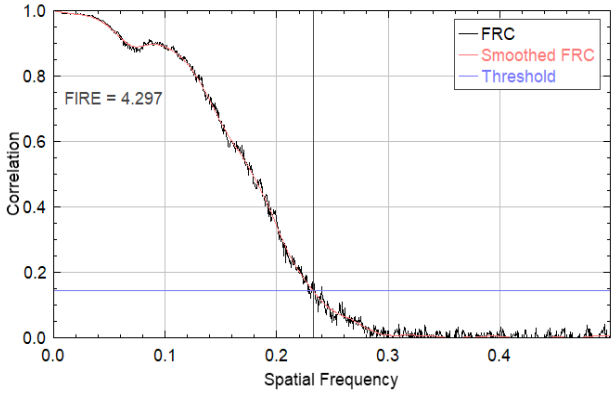
Example AOMF "Standard FRC conditions" - Developed on LEICA SP8 STED

Abberior slide: NPC ring subunits, STAR RED
633 nm Excitation (~50 mWatt)
100x 1.4 NA STED Objective
1 Airy unit pinhole (for 660 nm)
11 nm pixel size
2048x2048 or 1024x1024 pixels
787 ns pixel dwell time
8 Line Accumulations (aim for ~20-25 cts/px in brightest areas)
Detection: 638-770 nm
Photon counting mode
Gating: 0.5-6 ns
Line sequential scanning --> **2 "near identical" frames for correlation**
Test a range of depletion power settings ex. 0% 20% 40% 60% 80% 100% *(actual power TBD)*
Images with less than the optimal depeletion power should be defined as TEST images in the filename, or using the Excel template.
Find sample with no depletion/confocal mode, small image format for speed and reducing bleaching.
FRC Threshold: 1/7 or 3-Sigma





FRC Resolution: 45.4 nm



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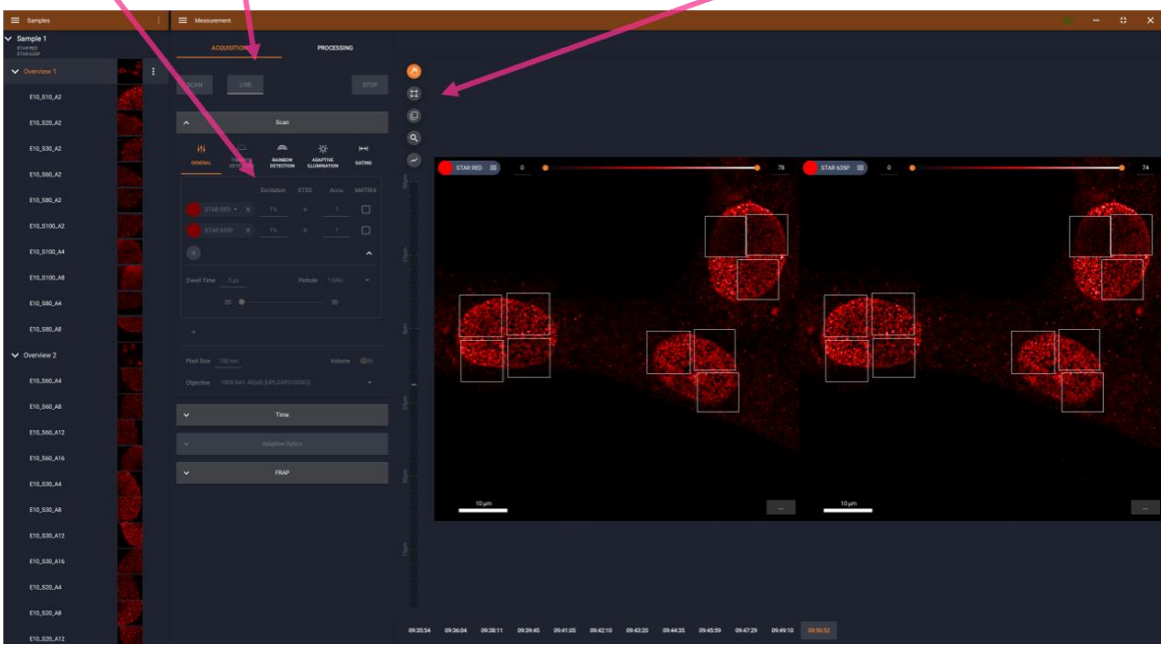
Abberior Image Acquisition Set-Up in Lightbox
For capturing image pairs (STED)

Set up excitation power output for each channel **(same excitation power for each channel)**

Click live to preview image and find ROI

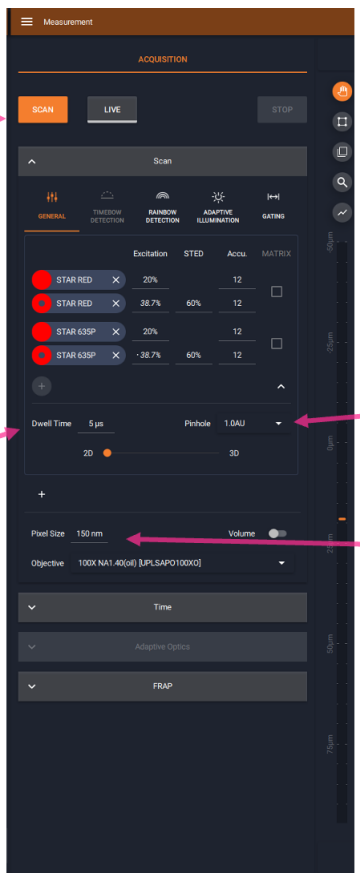
Select the box icon to size your ROI (minimum 3 µm x 3 µm, max 12 µm x 12 µm)



<u>To acquire FRC image pair</u>

If available, use 2 dyes with identical settings (STAR RED and STAR 635P)
Otherwise, one dye time series no delay

Click scan to acquire image



Set up excitation and depletion laser power output and accumulation for each channel for the STED laser **(same power for each channel)**

Pinhole 1 Airy Unit for 660 nm

Pixel dwell time 700-2000 ns

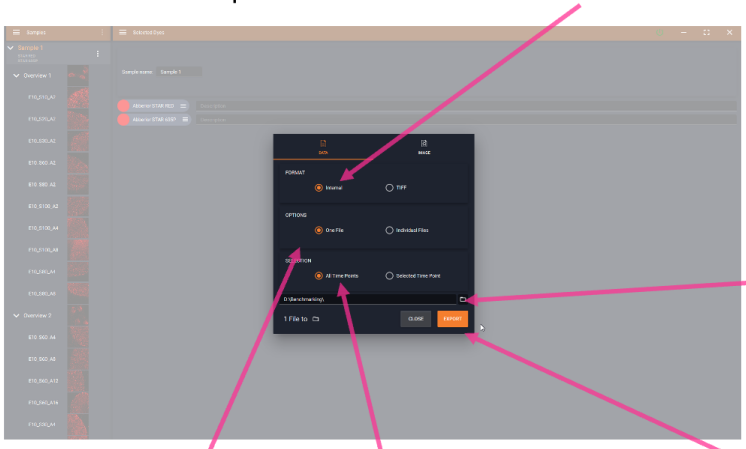
Pixel size (use Nyquist, 11 nm for NA 1.4, STAR RED)

Line sequential is automatically set as the default acquisition mode in iMSPECTOR (not available in Lightbox alone)

Abberior Image Acquisition Set-Up in Lightbox
For capturing image pairs (STED)

To save an image, click on the 3 dots then click export

Select internal for the file format (.obf file)



Double click the image name to edit

Click here to select the folder to save the data file in

Once all the saving parameters are set up, click export

Select one file

Select all time points if there is no time series acquisition

** Individual .obf files with **date, session number, instrument ID, and sample information** should be created for each different condition. Session numbers should be the same for replicates of the same condition

Save one .obf file for each session!

The average results for all the images in a single .obf will be reported.

Before saving the .obf file: delete any images that are not to be used for benchmarking resolutions such as:

- Delete incomplete scans
- Overview images
- Low-res image previews

Limit the size of the .obf file!

If it is too large, the original file will not be uploaded to OMERO.

Before uploading to OneDrive:

- If the image pairs are stored as separate channels (ex. STAR RED and STAR 635P), these will need to be merged into a single multi-channel image for each FOV. *A macro for merging channels in FIJI is provided*
- obf-tif-FRCpair-ChMergeExporter-2025Nov4