Resolution Benchmarking Platform For Confocal and STED Microscopy*

Pilot Phase – September-December 2025

*Images from other microscope techniques can be submitted, though their compatibility may not have been validated and optimized yet.

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 9-14)

3. Submit images to OMERO image repository via OneDrive

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

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

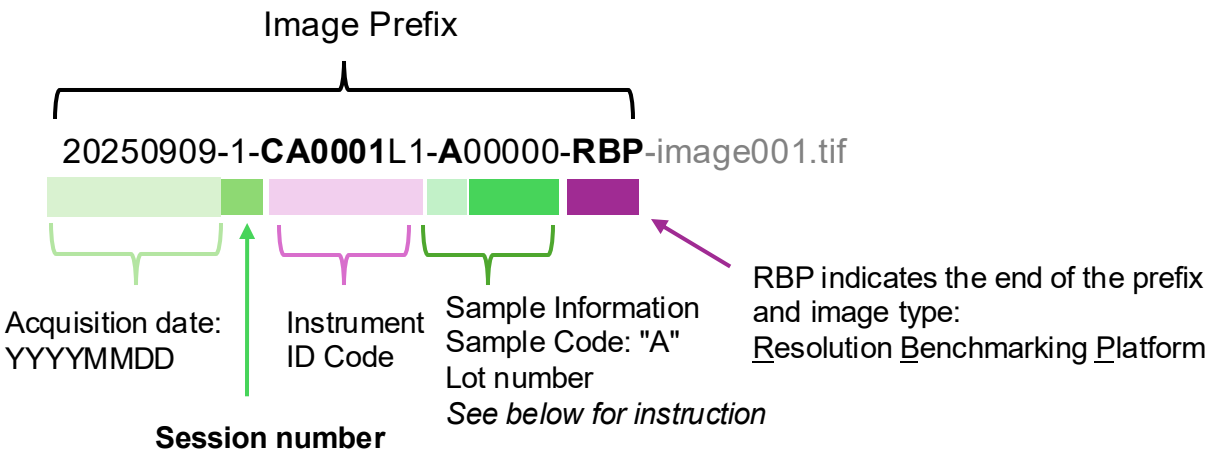
- **Acquire an image pair (two identical images) for 3-5 fields of view**
  - Each image in the pair must be acquired with the same parameters.
  - **Acquire pair as C-stack or T-stack**: duplicate channels (AF488 and AF488),or time series with no delay
  - Take 3-5 image pairs (replicates) in new fields of view.
  - **All replicates should have the same session number.** (File name instructions page 3)
- Minimum image size: > 256x256px, (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*2*
- Pinhole: 1 Airy Unit at emission wavelength
  - Emissions: 520 nm for AF488, 660 nm for STAR RED
- No saturated pixels!
- 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 (spinning disk or widefield)*
  - *No bi-directional scanning*
  - *Spectral detectors should collect 500nm – 600nm 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 "EXP" or "STED" if applicable (page 4)
    - Example: 20250909-**1**-CA0001L2-N54321-RBP.lif - Image001.tif
    - Example: 20250909-2-CA0001Z1-A12345-RBP.lif - EXP063x1.40na-001.tif
  - All replicates should have the same session number, listed after the date.
  - See page 4 for tips on how to add additional information in the filename.
- Original image file type
  - Original files preserve metadata. Exported .tif files will also work.
  - Examples: .czi, .lif, .nd, .obf etc.
  - Recommended max file size 30 MB
  - Add additional metadata as key-value pairs in OMERO (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, session, microscope ID, sample code and lot number or other identifier in the image prefix:



Session number
All images within a "session" are considered replicates. The *average* of these replicates will be plotted in the dashboard. This number also indicates the order in which measurements on the same day will be plotted in the "Per Instrument" view.

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 on OMERO. A short identifier can be included after the sample code. Ex. "XtubSTARG"

Image file name tips

Instead of defining key-value pairs in OMERO (page 6), which can take some time to populate in the dashboard, certain metadata values can be defined in the image filename directly.

Define an imaging technique as STED
If submitting a STED image,you may include the termSTED (all CAPS) in the filename. This will over-ride the default defined at the time of registration.

- Example:
- …-RBP-**STED**image001.tif

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.tif
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. Test measurements submitted on the same day as benchmarking measurements must have different session numbers.

Define the objective magnification and NA in the image filename
The default values for objective magnification and NA are 000x and 000NA
These properties can be defined in the image name by including them in the filename using the following format:

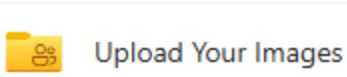
- [0-9][0-9][0-9]**X**[0-9].[0-9][0-9]**NA**
- Examples:
    - *005x0.25na*
    - *063x1.40na*
    - *100x*
    - *020x*

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

Original filename or altname?
If an altname has been defined, these image properties will be read from the altname rather than the original filename.

Upload images to OneDrive

RBP-PUBLIC



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 the AOMF Staff.

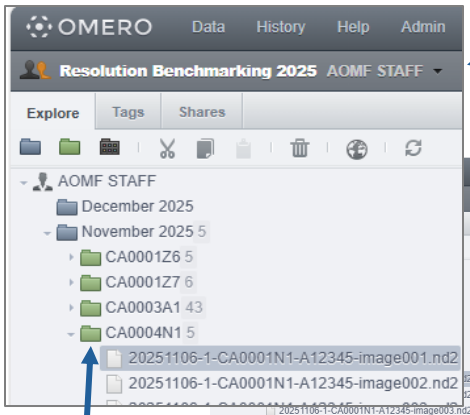
Our platform organizes images into:

- "Groups" - Containing all images submitted for a given year.
- "Projects" - Month of image submission.
- "Datasets" - Submitted images or FRC curves

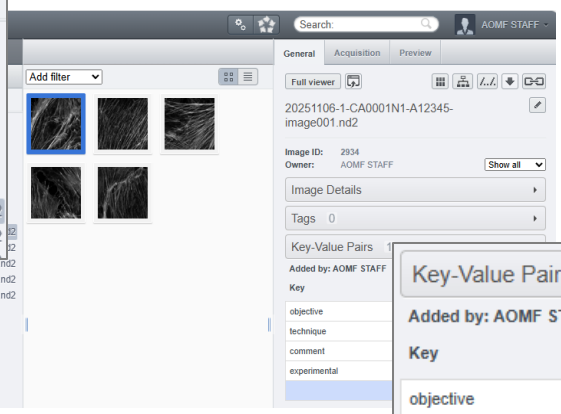
Log into the web client: https://omero.uhn.ca/omero/

Find your images and add key values: Click on an image, or collection of images. In the righthand menu, on the "General" tab, open the "Key-Value Pairs" container.

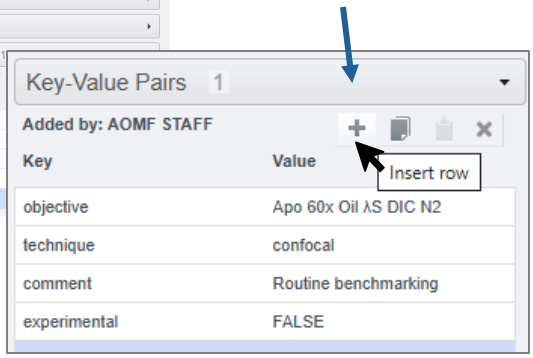
Key-values can be entered for one or more images at a time.
Key-values can also be copied to other images from the same dataset using the copy and paste tools in the key-value container.



1. Select the correct "Group" (year). Update displayed member to "All Members" or "AOMF STAFF" to find images



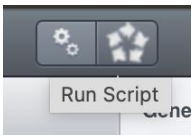
3. Add key-value pairs (see next page)



2. Find the "Dataset" for your microscope

Add key-value pairs in OMERO

Key-Values can be added to images directly in OMERO or imported from a .csv file. Use Run Script / annotation_scripts / export to csv... to create a template. Add new "Keys" as columns. Then run "import from csv..."



"Keys" are case sensitive
Please use **lower case**
"Values" are case insensitive

- Add a Key-Value pair called **"objective"**
  - Key = objective
  - Example: Value = "63x/1.4 NA oil" or "HC PL APO 63x/1.4 NA OIL"
  - The magnification and NA must be listed in this format, [mag]**x/**[0.00] NA
  - **Default:** objective - "000x/000 NA"
  - Objective mag and NA can also be identified in the filename (see page 4)

- *(Optional)* Add a Key-Value pair called **"comment"** with any information you want to view with the data in the dashboard.
  - Key = comment
  - All comments should appear in one **Value**. *Examples:*
    - Value = "Pre-service visit, 100% depletion"
    - *If you submit images of a sample or technique other than the recommended benchmarking samples, you can describe this sample here. Ex. "Tubulin GFP, GFP Nanobody STAR RED"*
  - **Default:** comment - "none"

- *(Optional)* Add a Key-Value pair called **"benchmark"** to indicate if the image is a "test image" or a "benchmark."
  - Key = "benchmark"
  - Value = "TRUE" or "FALSE"
  - A "benchmarking" result 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" tab of the dashboard. Only benchmarking results appear in the "Best Results" and "Average" tabs.
  - **Default:** benchmark- "TRUE" - assumes image is a benchmarking image.

- *(Optional)* Add a Key-Value pair called **"technique"**
  - Use this key to indicate which microscopy technique was used to capture the images if different from the default.
    - Key = technique
    - Value = confocal, STED or other*
    - *Specify which technique in the comment key
  - **Default** technique - This is defined during registration for each microscope
  - STED images can be identified in their filenames (see page 4)

- *(Optional)* Add a Key-Value pair called **"altname"** if the original image title does not contain the correct format or information.

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 December 3, 2025

Visit the dashboard at: AOMF | UHN

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

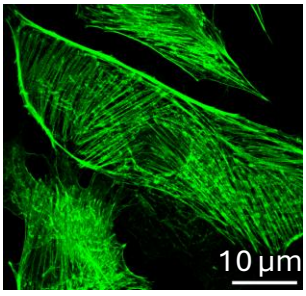
- **View the dashboard full-screen,** use double-ended arrow in bottom right corner.
- To view the list of Countries, Members, or Instruments as a table, right click next to the title for each list to the left of the plot space.
- To view the data in each plot as a table, right click on the plot area.
- 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.

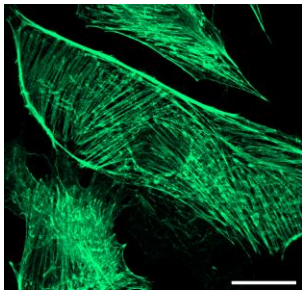
Current Image acquisition recommendations (Confocal):

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

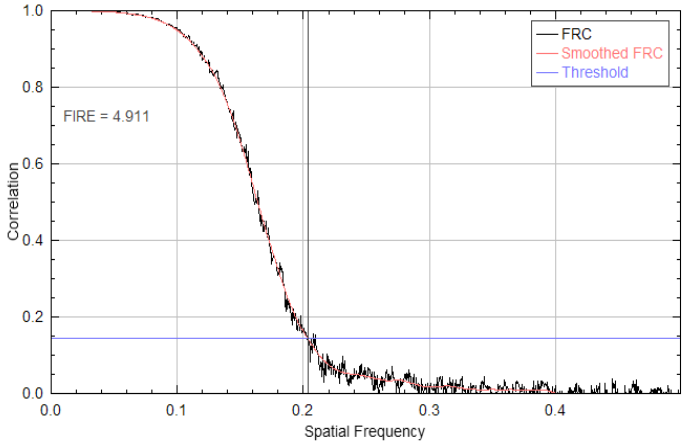
<u>Example AOMF "Standard FRC conditions" - developed on Stellaris Confocal</u>

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 (~Twice Nyquist)
2048x2048 or 1024x1024 pixels ***<u>must be power of 2</u>***
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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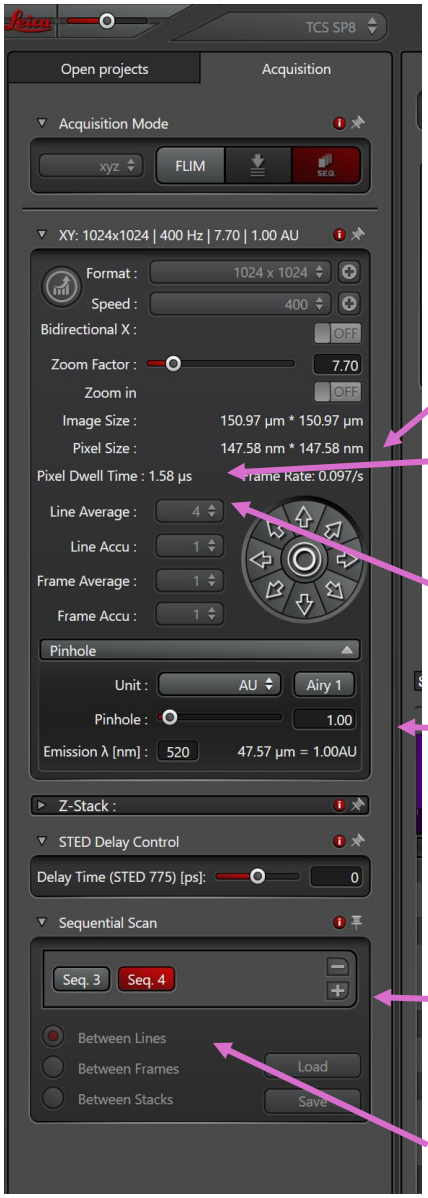
Leica SP8 Image Acquisition Set-Up
For capturing image pairs (confocal)



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

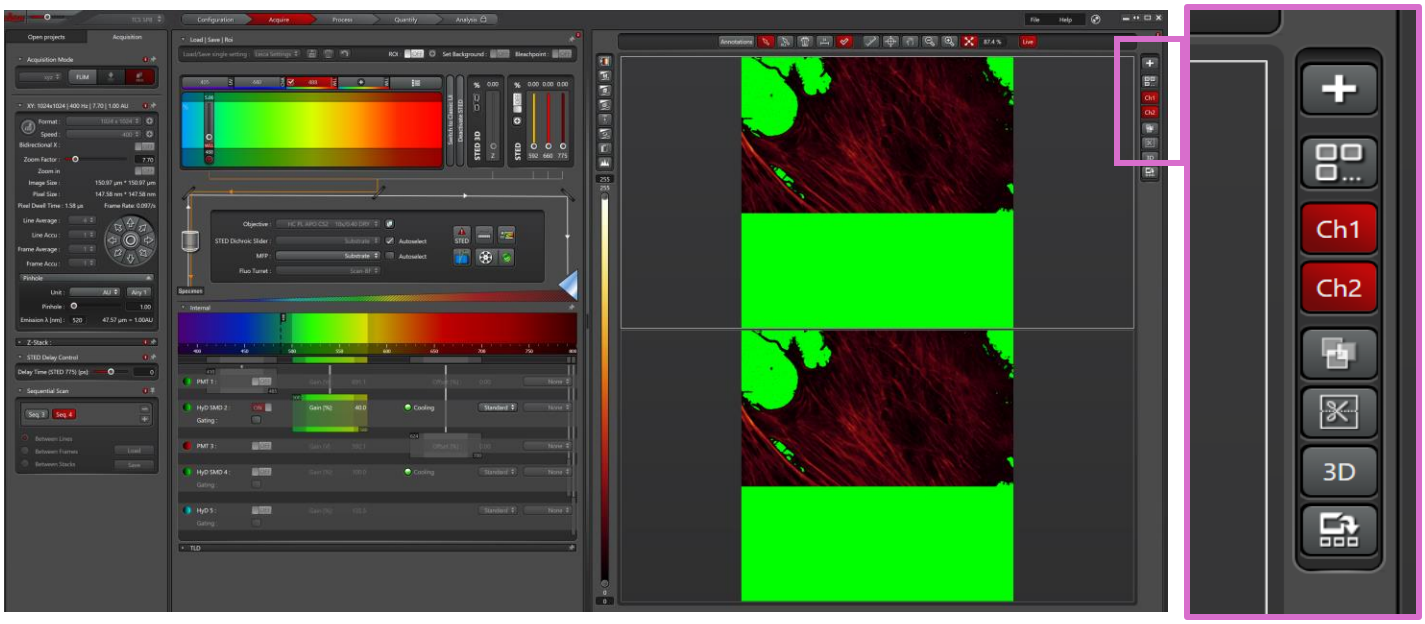
4 Line averages

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

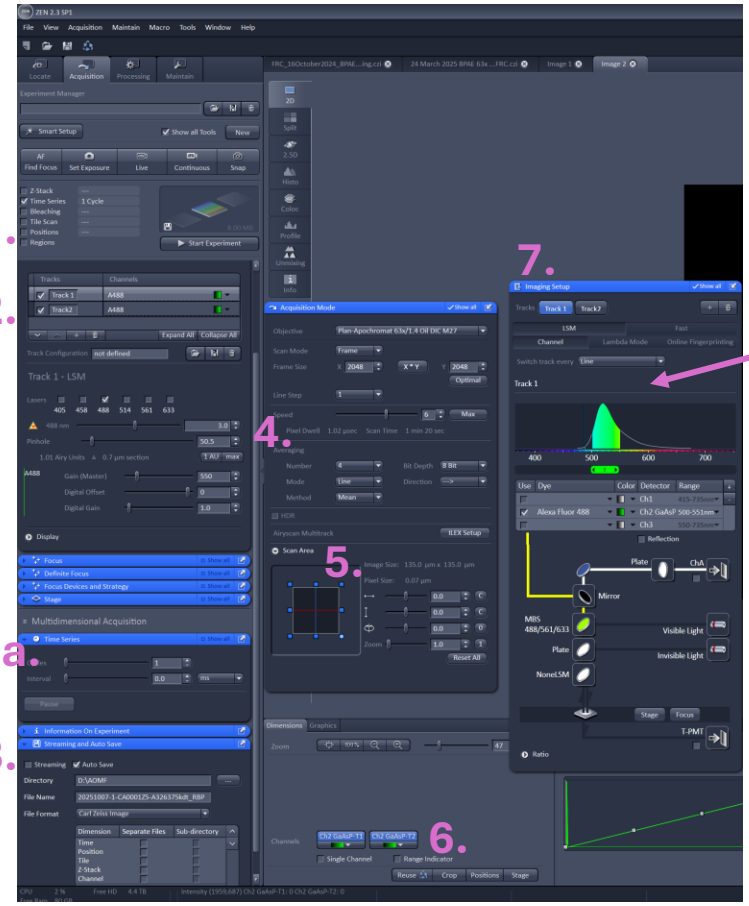
(+) will duplicate the setting
Set up excitation and detection for one channel.
The (+) to perfectly duplicate it.

Line Sequential

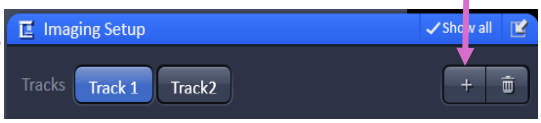
FRC image pair
acquired as 2 Channels

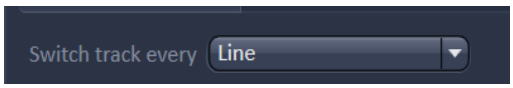


Zeiss LSM880 Image Acquisition Set-Up
For capturing image pairs (confocal)

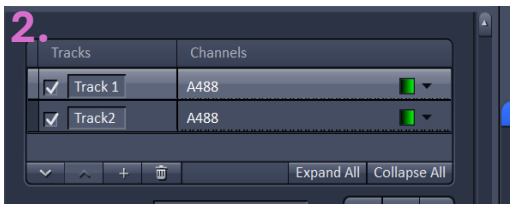


(7) **Select (+) to add a new track**
Adjust the laser and detector so that Track 2 is identical to Track 1.

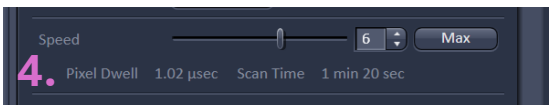




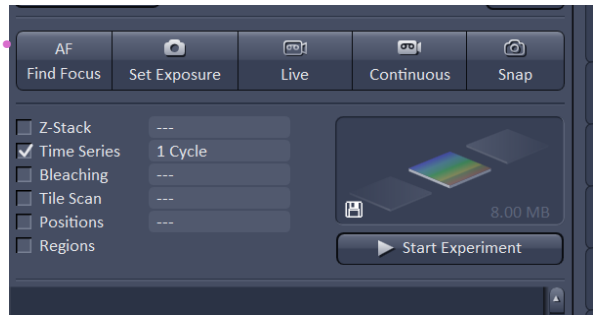
Use line sequential acquisition

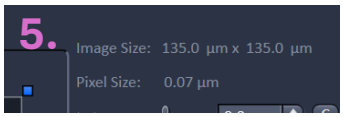


(2 and 6)
FRC image pair acquired as 2 Channels

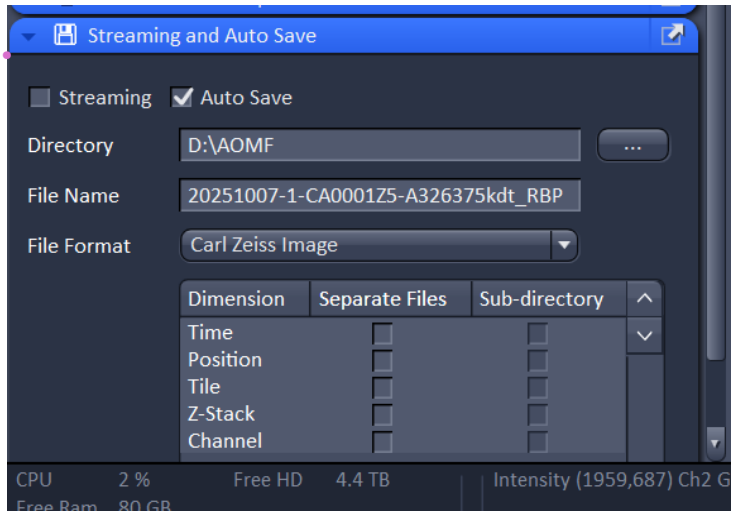


(4) Pixel dwell time
(use 0.7-2 *µs)*
Adjusted by scan speed.





(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

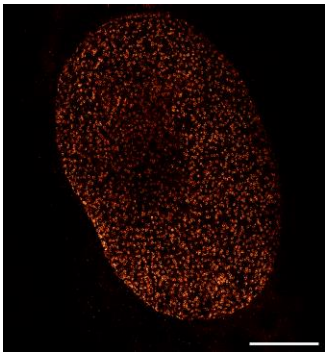
(3) **Autosave** images with with the required **filename** format that includes Date, Session, Instrument ID, and Sample information.

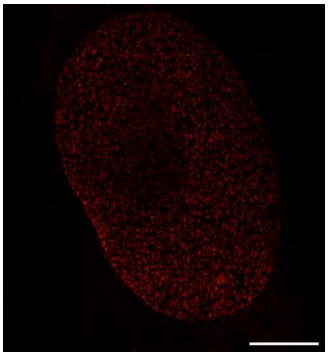
Current Image acquisition recommendations (STED):

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

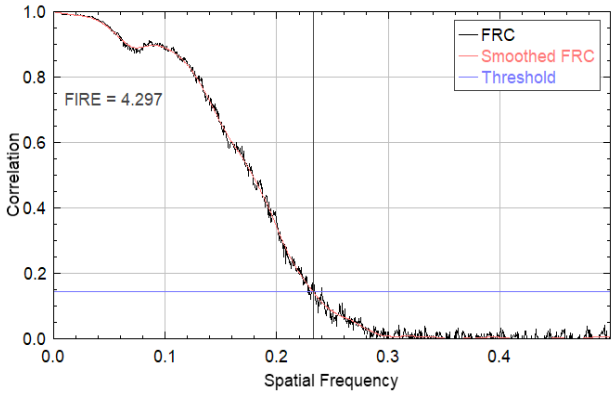
Example AOMF "Standard FRC conditions" - Developed on LEICA SP8 STED

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 (~Nyquist assuming 25 nm resolution)
2048x2048 or 1024x1024 pixels ***must be power of 2***
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)*
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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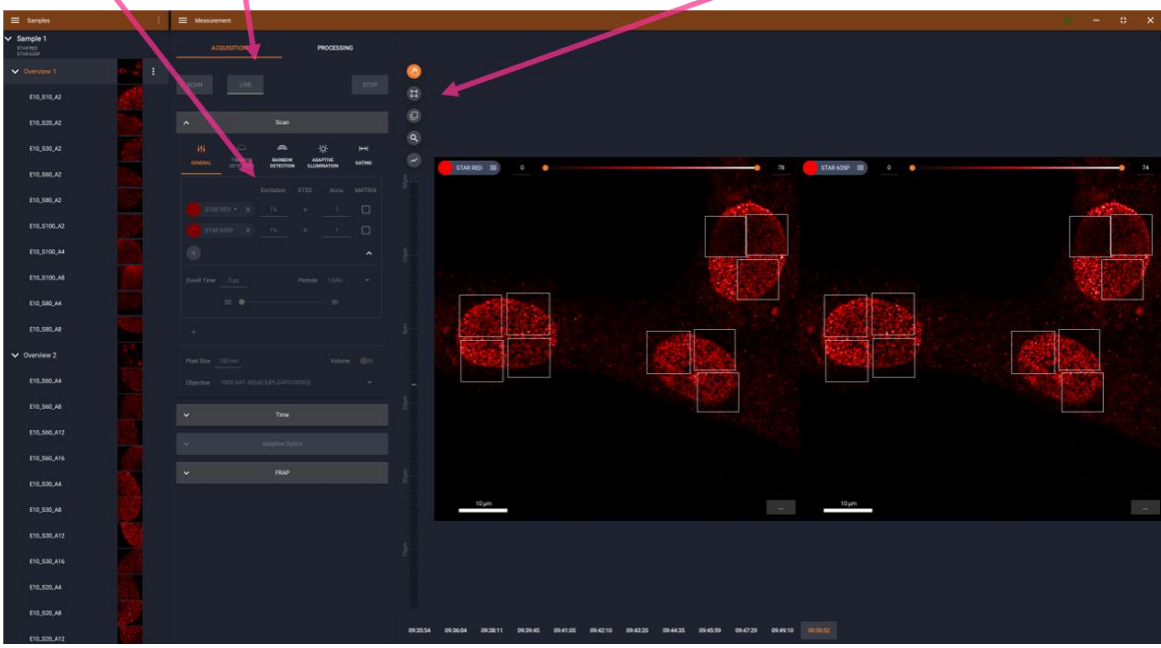
Abberior Image Acquisition Set-Up in Lightbox
For capturing image pairs (STED)

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

Click live to preview image and find ROI

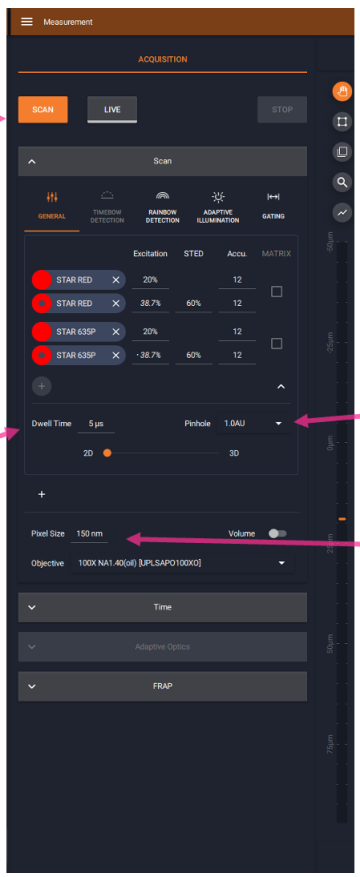
Select the box icon to size your ROI (minimum 3 µm x 3 µm, max 12 µm x 12 µm)



<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

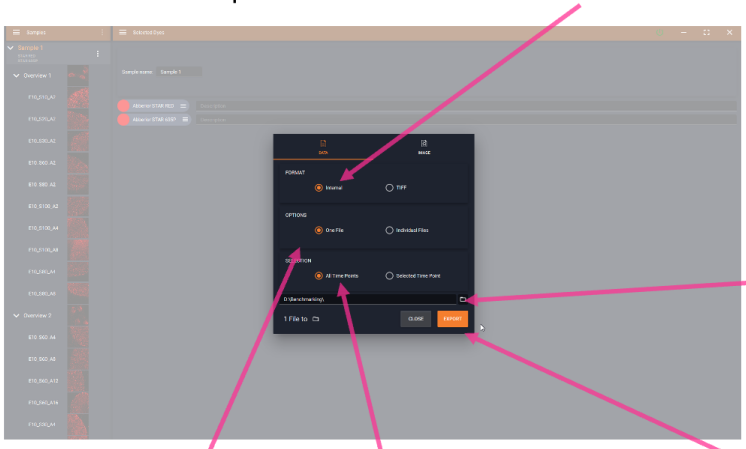
Pixel size (use Nyquist, 11 nm for NA 1.4, STAR RED)

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