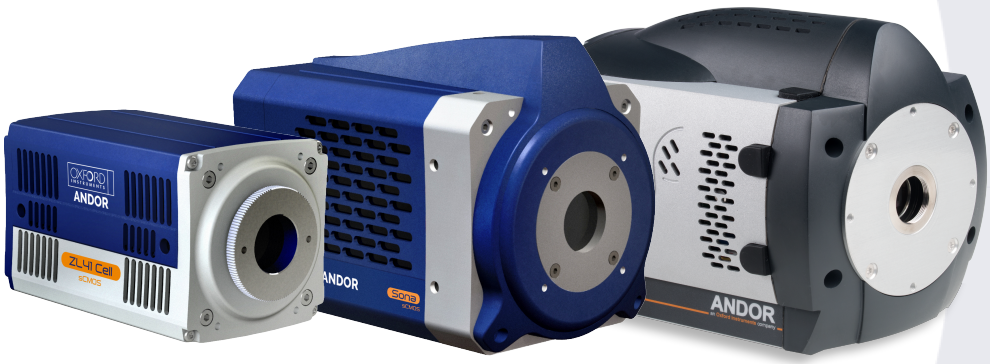
SRRF Stream Plus

1.2 rev 05 April 2024



User Guide

For models: iXon EMCCD, Sona, Zyla and ZL41 sCMOS

Section 1 - Introduction

1.1 About this Guide

This guide is applicable to SRRF-Stream+, a camera-based real-time super-resolution imaging technique that is based on the "SRRF" (Super Resolution Radial Fluctuations) method of Gustafson et al. 2016. SRRF-Stream and SRRF-Stream+ are exclusive for use on compatible Andor cameras. Using SRRF-Stream or SRRF-Stream+ to obtain super-resolved images is very easy, but with some extra guidance it can help you get the best possible image quality from your datasets. This guide includes how to use the available settings to adjust the image along with some troubleshooting and other helpful information.

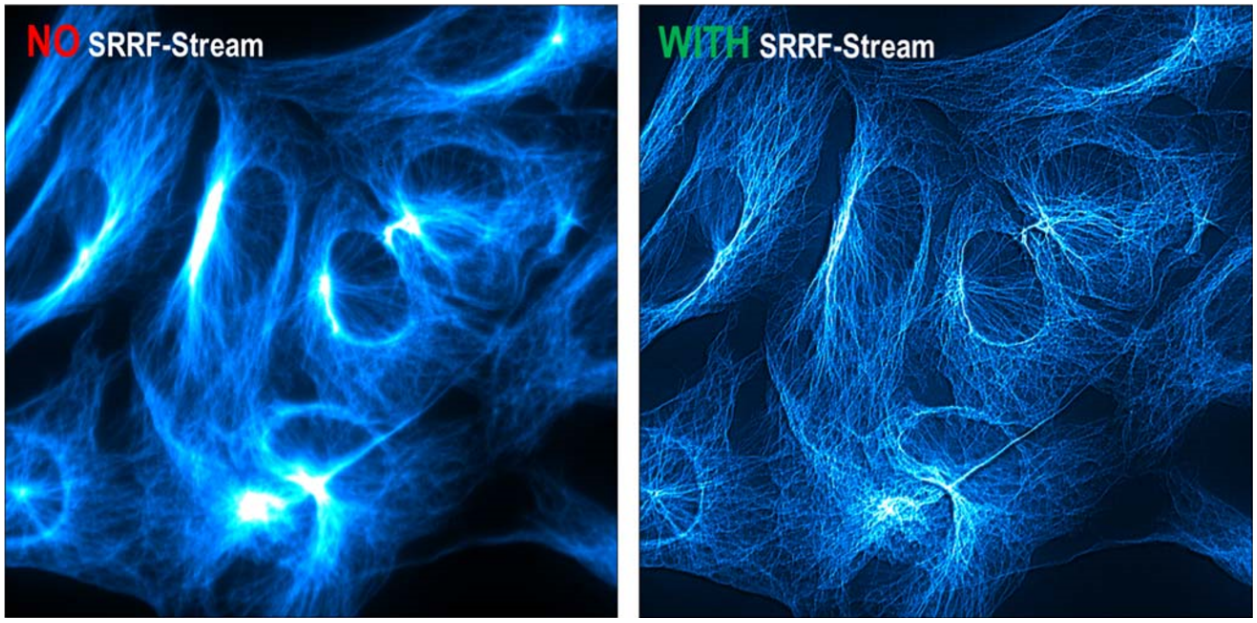


Figure 1:Microtubule structure in fluorescently labelled BPAE cells (Fluocells, ThermoFisher), comparing a widefield image with and without use of SRRF-Stream. The full 1024 x 1024 pixel field of view of the iXon Ultra 888 camera was used. A x63 high-NA objective was used, with further 2x magnification and 560 nm illumination. 100 raw 'input' images were recorded for every resultant super-resolution image. For a fair comparison without SRRF-Stream, 100 standard widefield images were recorded and then averaged.

1.2 Learning more about SRRF-Stream

In brief, SRRF-Stream determines localised intensity gradients around fluorescence emitters (labels) in images of short exposure, and correlates the fluctuations over time across a number of frames, typically between 10 and 100. This enables a super-resolved image to be constructed from this image data. Since this is data intensive, the processing power of a GPU is used. To help understand the concept of SRRF, and how to use it to its full potential the following articles are recommended:

Publication by Culley et al., 2018: SRRF: Universal live-cell super-resolution microscopy

The white paper from Oxford Instruments Andor on SRRF-Stream Technical Note

1.3 Terminology

SRRF-Stream

Released in Summer 2017, SRRF-Stream is the initial release of the software based on the "SRRF" (Super Resolution Radial Fluctuations) method of Gustafson et al. 2016.

SRRF-Stream+

Is the new and improved implementation of SRRF, released in Autumn 2020 and supersedes all earlier versions of SRRF-Stream. SRRF-Stream+ provides improved image quality over the original version of SRRF-Stream (by improved radiality processing that previously resulted in artefacts in some image datasets).

Note
This guide is for users of SRRF-Stream+

1.4 Revision History

Version	Released	Description
1.0	29 January 2021	Initial release.
1.1	10 November 2022	Updates for 1.18.0.0 including Zyla 4.2 PLUS and ZL41 Cell 4.2 support. Further information on best use of EMCCD and sCMOS.
1.2	05 April 2024	Updated sections throughout for new release 1.19.

1.5 Updates to the Manual

Changes are periodically made to the product, and these will be incorporated into new editions of the manual. Please check for new releases of the manual at: andor.oxinst.com/downloads. If you find an issue in this manual, please contact your customer support representative with a description of the issue.

Section 2 - SRRF Stream+ Requirements

Please read the following requirements and recommendations to ensure SRRF-Stream+ works to its full potential.

2.1 Camera Compatibility and SRRF-Stream+ licences

The following Andor cameras are certified for running SRRF-Stream+:

- iXon Ultra EMCCD models 897 and 888
- iXon Life EMCCD: models 897 and 888
- Sona back-illuminated sCMOS: models 4.2B-11, 2.0B-11 and 4.2B-6
- Zyla 4.2 PLUS sCMOS cameras
- ZL41 Cell 4.2 sCMOS cameras

Notes
• **iXon Ultra and Life 888 models not optimized for SRRF-Stream+ at time of ordering need optimized. Refer to the iXon Upgrade technical note** • **SRRF-Stream and SRRF-Stream+ Licenses are linked to a specific camera serial number. Therefore, it is not possible to run SRRF-Stream or SRRF-Stream+ on another camera that it was not originally linked to. For systems with 2 cameras, each camera will need a SRRF-Stream or SRRF-Stream+ license.** • **If you have lost a SRRF-Stream+ .lic file please contact your Andor Support representative.** • **All Sona camera series supported in SRRF-Stream+ from version 1.16.0.0 onwards.** • **Zyla 4.2 PLUS cameras from SRRF-Stream+ version 1.18.0.0 onwards. Note camera serial number must be checked with Andor support to ensure compatibility.** • **All ZL41 Cell 4.2 sCMOS only in SRRF-Stream+ version 1.18.0.0 onwards.** • **Previous Andor iXon, sCMOS or other EMCCD or sCMOS cameras from other camera manufacturers cannot run SRRF-Stream.**

2.2 GPU Compatibility

SRRF-Stream and SRRF-Stream+ are a software-based approach to super-resolution so the GPU on a graphics card is used. It is important to running SRRF-Stream acquisition and processing efficiently for optimum speeds and must have sufficient operating memory. Suitability should be confirmed when ordering SRRF-Stream, however here are some guidelines:

- Nvidia GPU card
- Compute Capability v3.5 or above
- 4 GB or greater on-board GPU RAM for iXon EMCCD. 8 GB or greater for Sona Zyla 4,2 PLUS or ZL41 Cell sCMOS cameras - these cameras have larger sensor sizes and therefore require more RAM.

Note
Andor have tested 'mid-range' cards such as the GTX 1070, the Quadro P4000, Quadro RTX4000 on the iXon EMCCD cameras and found that, with SRRF-Stream, this level of card can process data much faster than the rate of camera data acquisition. New models are continually released that offer higher performance at lower costs, please refer to GPU benchmarking tools using these cards as a reference against a potential new graphics card. If you have any further queries on this topic please contact your local Andor representative and they can assist if required.

2.3 Microscope Compatibility

Most conventional microscopes and modalities such as widefield, confocal and TIRF should be compatible with the SRRF method. However more specifically the objective compatibility is fundamental to obtaining quality images with SRRF. The resolution of the microscope can be described by that of the objective lens according to the following relationship:

Resolution = l/2NA

It is critical for SRRF-Stream to work that a suitable objective is used matched to the pixel size of the imaging detector to meet Nyquist sampling criteria. This provides good spatial information into the SRRF algorithm which helps determine and localise intensity information. A high NA objective will help provide a higher resolution. A large pixel will capture a good signal- but will not be able to oversample the image and maintain resolution at lower magnifications and objective NAs.

While good quality images can be obtained for objective lens and camera combinations that are approaching Nyquist, the appearance of artefacts is often linked to not meeting this requirement.

2.4 Fluorophore Compatibility

One of the benefits of the "SRRF" technique is its applicability to live cell imaging even over extended durations, this comes from being compatible with conventional fluorophores at low illumination intensities. Other localisation based super-resolution microscopy techniques such as STORM or PALM require specialized photoswitchable or photoconvertible fluorophores coupled to high illumination intensities. High intensity laser illumination will lead to most fluorophores being in their dark ground state i.e. a low emission of photons making it easier to localize emission of individual fluorophores. If low intensity LED illumination was used to better suit live cell imaging, many molecules would be in their emission state within the same frame and this fluorophore overlap would greatly reduce the effectiveness of these approaches (Culley et al 2018).

However, the detection process of SRRF is different as a gradient map is built up in each frame using all of the emission information present, and this is correlated over time over e.g 100 frames. This means that the SRRF detection stage will function effectively even when a large portion of the fluorophores are emitting within each frame. This is what makes the SRRF and SRRF-Stream+ suited to use at low illumination intensities and thus for live cell imaging.

Even standard fluorophores will exhibit some degree of fluctuations in their emission. The timeframes involved in these fluctuations can be as short as microseconds, to tens of milliseconds for some organic dyes. Fluorophores may therefore respond slightly differently to settings used for SRRF-Stream+ e.g. exposure times and varying illumination levels. Overall however, good results are possible with most normal fluorophores such as those based on e.g Alexafluor series, and for GFP which has relatively stable emission characteristics. Autofluorescence of plant tissues has also been shown to work with SRRF imaging approaches (Donaldson LA, 2022).

The most important aspect of the flurophore is the brightness. The stronger the signal emitted and the larger the difference between this and the background, the more rich intensity information is present. SRRF-Stream+ will be able to provide more dynamic and detailed image with a good input signal. If the fluorophore emits only a weak signal the final image is likely to be poor with little separation between the noise background and the real signal.

The image Look Up Table (LUT) can be used to view the count levels of the image. Guidelines for recommendations on what a suitable counts level is for SRRF-Stream+ is provided in "How to use the Look Up Table to Optimise Exposure" on page 23.

No special preparation is needed, however, as with normal imaging, well-prepared specimens that provide a low background will provide better results. A poorly prepared sample should not be expected to provide a high-quality image. This becomes even more important to super-resolution, such as SRRF-Stream+ as you push past the limits of diffraction.

Section 3 - SRRF Stream Users Only

For users of "SRRF-Stream" it is recommended to update to the latest version of SRRF-Stream+. This will provide improvements in image quality. Read the SRRF-Stream+ technical note to find out more.

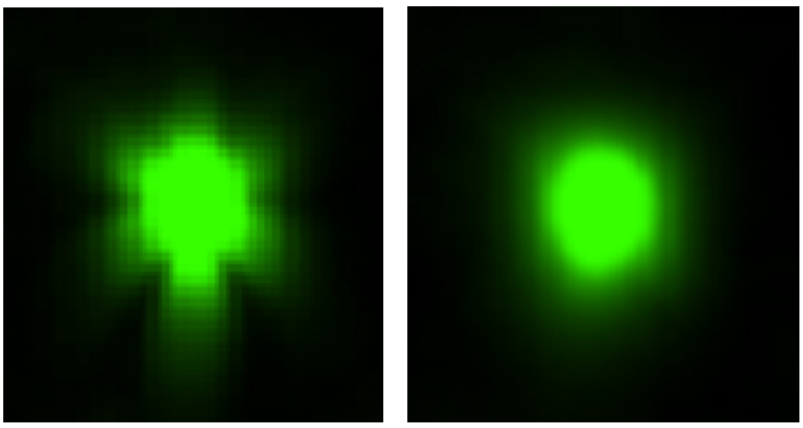


Figure 2:SRRF-Stream+ (right) eliminates artefacts that could be found in some image datasets with SRRF-Stream (left) by increasing axes of radiality from 6 to 24.

3.1 Identifying SRRF-Stream Version

Identifying what SRRF library version you have can be done from the Properties>Details of the installed file SRRF_STREAM.dll.

- For Micro-Manager it is usually located in C:\Program Files\Micro-Manager-1.4
- For SDK it is usually located C:\Program Files\Andor SRRF Stream\lib\x64

SRRF-Stream+:

- 1.19.0.0 Latest release of SRRF-Stream+. Sentinel licensing verification updated.
- 1.18.0.0 Includes support for ZL41 Cell and Zyla 4.2Plus models. Updated GPU support. Improved handling of small point structures.
- 1.16.1.0 SRRF-Stream+ Includes support for Sona sCMOS. Radiality axes increased from 6 to 24 to eliminate Star artefacts and enhance image quality.

SRRF-Stream: Versions prior to 1.16.1 e.g. 1.12 are the original SRRF-Stream algorithm.

3.2 How to upgrade from SRRF-Stream to SRRF-Stream+

The upgrade to the latest version of SRRF-Stream+ is available free by contacting your local Andor product support team. A simple upgrade utility will update the SRRF library files without affecting your licence.

Section 4 - SRRF-Stream+ Settings

In this section we cover each of the settings and how they may impact images taken using SRRF-Stream+. The settings discussed are common to Micro-manager, SDK and Fusion software. The default settings are very good starting points for most image datasets, but you can adjust the settings to suit different data sets, or to suit what you are trying to determine from your data.

Note
The sample preparation, and microscope set-up should always be considered and optimized. If not, then the quality of information feeding into the SRRF-Stream+ processing will not allow SRRF to work properly and poor-quality images may be obtained.

Andor sCMOS Camera-SRRF Status	Ready
Andor sCMOS Camera-SRRF | Enable	Enabled
Andor sCMOS Camera-SRRF | Interpolation Type	Catmull-Rom
Andor sCMOS Camera-SRRF | Number of Frames per Time ...	100
Andor sCMOS Camera-SRRF | Radiality Magnification	4
Andor sCMOS Camera-SRRF | Radiality Temporal Analysis	Mean
Andor sCMOS Camera-SRRF | Ring Radius	1
Andor sCMOS Camera-SRRF | Save Original Data | Option	None
Andor sCMOS Camera-SRRF | Save Original Data | Path	C:\Users\CZC944D9KS\Documents
Andor sCMOS Camera-SRRF | Version Information	1.16.0.1
Andor sCMOS Camera-Sensitivity/DynamicRange	High dynamic range (16-bit)

Figure 3:Micro-Manager settings for SRRF-Stream.

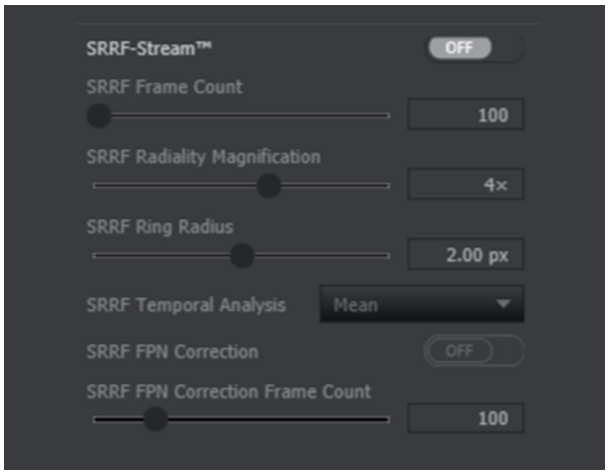


Figure 4:Fusion settings for SRRF-Stream.

4.1 Enable SRRF-Stream+

When SRRF-Stream+ has been installed and a licence successfully detected for the current camera options for SRRF-Stream+ will be available. SRRF-Stream+ can simply be switched on and off using this option.

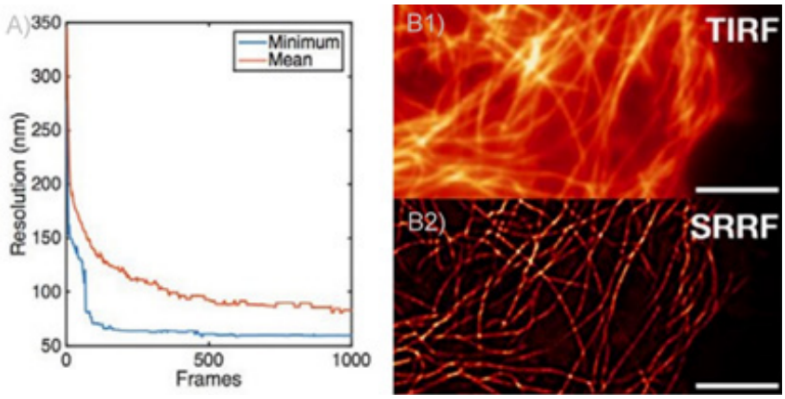
4.2 Number of Frames per Time point

This setting is an important parameter in setting the balance between resolution and time per image (frame rate). SRRF-Stream+ uses a series of image frames to build the super-resolved image so more frames will mean more temporal fluctuation data is being used to construct the image. With most data sets, increasing past 100 frames will only improve the image resolution with a diminishing return, yet increasing time per image.

Example:

100 frames x 10 ms exposure = 1 frame per second

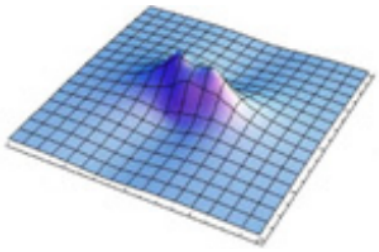
500 frames x 10 ms exposure = 0.2 frames per second



For robust or fixed samples, in which you are not looking at live cells, you could of course increase the number of frames used to maximise resolution. However, it is normally best to focus on ensuring a good signal to background by extending the exposure time, rather than increasing the number of frames. i.e. 10 good signal to noise frames is better than 100 low signal to noise frames (for the same overall effective exposure).

4.3 Interpolation Type

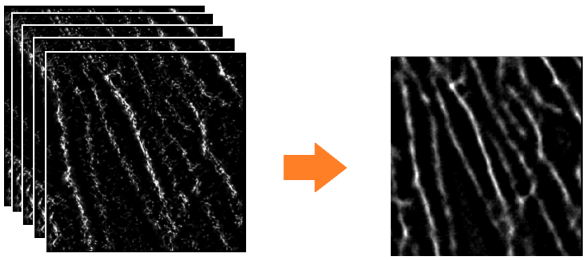
This determines the method for how sub-pixel values are calculated from the intensity gradient fields that are calculated within each frame.



- **Catmull-Rom** – default. This method typically provides the sharpest and smoothest images and it is recommended to use this mode.
- **Fast B-Spline**. This alternative calculation is faster, results in a less well resolved image than default method. This interpolation method, however, is much faster, and can provide speed-boost of an extra ~50%. Therefore, it is an option if you are trying to maximize speed from your imaging.

4.4 Radiality Temporal Analysis

This setting concerns how the final image is processed during the radial transformations stage of SRRF processing as the frame are used frames used to build a final super-resolved image.



Mean – (default)

This method calculates the final image as an average intensity calculation of the radiality transforms of each acquired frame in the frame-burst. This method is recommended for use for most samples. Such as punctate, filamentous or other high contrast features and thin sections. It will provide a slightly higher resolution than MIP.

MIP

This method calculates the final image as a maximum intensity projection of the radiality transforms of acquired frame in the frame-burst. This method works best for thicker samples, or when there may be areas of diffuse or higher background. It can also help with fainter samples. The final resolution may be slightly lower than Mean and it may be unable to resolve finer networks e.g. actin or mitochondria.

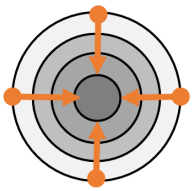
4.5 Radiality Magnification

The radiality magnification upscales the data to higher resolution to help with precision during processing of the image information. For example, the default value of 4 will result in a 4096 x 4096 image from the full sensor of the iXon 888 which has a real pixel resolution of 1024x1024.

Higher values will provide a smoother final output image, but the effective resolution would only be marginally better from moving from x4 to x6. However, this will place more load on the GPU and therefore result in longer processing times and larger file sizes. Since resolution is largely determined by the ring radius, this mode would be recommended to keep in the default x4 setting. If you are trying to push for the highest quality image e.g. for publication for a sample with optimal conditions then this setting could be increased to x6. Note that this will increase GPU processing load and thus time, and double the file size.

4.6 Ring Radius

The Ring Radius is used in the calculation of the radiality at each co-ordinate in the image. SRRF calculates the intensity gradients in a ring surrounding each co-ordinate. What is optimal for your image will depend on the optical set-up and the image data. The smaller the ring radius the tighter this localisation will be and thus the better the resolution.



- **To increase resolution** - reduce the ring radius from default value- but adjusting too far it can make the data look too noisy/overprocessed. A setting of 1.0 would provide the highest resolution.
- **To reduce noise** - you can increase ring radius at the expense of a reduction in resolution. This can be useful for noiser datasets. A setting of 2.5 could be used.

4.7 Fixed Pattern Noise Correction (Fusion and SDK Software only)

Fusion software and SDK features an additional function to correct for Fixed Pattern Noise (FPN). This function was initially developed to allow for correction of FPN that may have affected image quality when running SRRF-Stream+ on sCMOS cameras. It remains as an option in Fusion and SDK, however it should not be required for Sona and supported Zyla or ZL41 Cell sCMOS cameras due to the very low FPN present. Enabling the function results in the following: a pre-acquisition (dark) frame-burst of a user defined number of frames is taken, and the result is averaged. The value is then subtracted from each acquired frame passed to the SRRF algorithm thus correcting for any FPN that may be present. This additional processing will result in slower imaging speeds.

For most datasets, if the information in this guide is followed there should be no need to use FPN correction. Note also that the number of FPN frames used does not need to match that of the number of SRRF acquisition frames.

If the signal is weak and the dynamic range is poor it could be used. If it is used it is only necessary to use 1-5 frames of FPN correction. There is no benefit to higher levels of precision since the correction being applied is such a small number of counts.

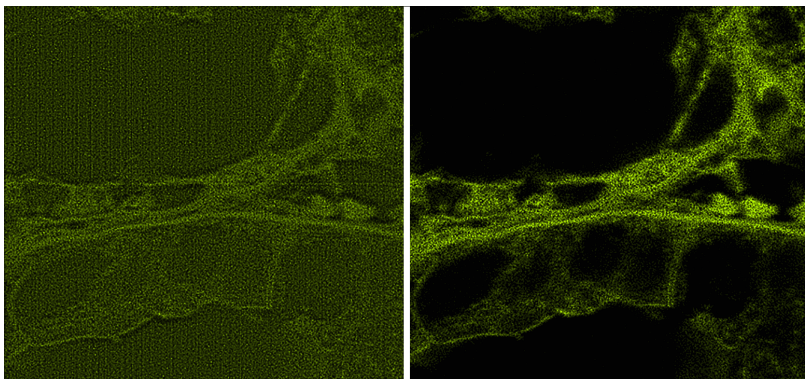


Figure 5:Illustrates the fixed pattern noise found using earlier generation sCMOS cameras under certain imaging conditions. Left SRRF image showing Fixed pattern noise. Right Image with fixed pattern noise correction applied.

4.8 Save Original Data

For Micro-manager, an option is available to save original data. By default, this is turned off since high volumes of data can be generated very quickly using SRRF-Stream+. The file location path is also displayed.

4.9 Camera settings

Aside from the SRRF specific settings there are a number of camera settings that can impact images produced using SRRF-Stream+

4.10 Exposure

The exposure time is the key factor that you can use to control the signal to noise from the specimen during the imaging experiment. It is therefore important to set this to a suitable level.

As a general guide:

- Shorter exposures permit faster imaging, however if the signal level is insufficient, the input data will be poor in intensity variation data. The corresponding image would be low in dynamic range, lacking low level details and prone to background noise.
- Longer exposures provide a larger signal, rich in intensity information. These conditions will provide the most dynamic and detailed image. However, extended exposures will increase overall processing time and effective exposure.

Samples will vary widely in terms of what labels have been used, their relative brightness, their stability and the abundance of labels and background. However, it is possible to give guidance on what is a suitable signal level, and how to set exposure to achieve this in a practical way for a given specimen.

A good starting point is 100 milliseconds.

For commercial samples that are widely available, guidance for typical exposures (widefield-EPI):

- FluoCells™ Prepared Slide #1 (BPAE cells) Invitrogen.
- MitoTracker™ Red CMXRos: 150 ms
- Alexa Fluor™ 488 Phalloidin: 100 ms
- DAPI: 100 ms
- FluoCells™ Prepared Slide #3 (mouse kidney section) Invitrogen
- Alexa Fluor™ 488 WGA: 60 ms
- Alexa Fluor™ 568 Phalloidin: 50 ms
- DAPI: 80 ms

4.10.1 How to use the Look Up Table to Optimise Exposure

Different samples will have different signal levels and therefore may require longer or shorter exposures than the commercial samples examples used above. A practical way to quickly assess this for your sample is by using the Look Up Table (LUT) for the specific image channel(s) and looking at the number of counts present. A guide is shown below:

Table 1:Optimise exposure settings.

Max counts in channel	Comments	Actions
<100	Insufficient signal level and poor dynamic range. Image may have poor separation between background and signal, and may have strong background patterning.	Exposure should be increased to boost signal to noise to >400 counts
100-349	Low signal range and low dynamic range. The upper end of this range can be suitable for some samples if speed is the priority.	Exposure should be increased to boost signal to noise to >400 counts.
350-500	Moderate signal level and moderate dynamic range. Should provide good results for most quality samples.	Exposure could be increased to obtain highest dynamic range at the expense of increased processing time.
>500	High Signal level and high dynamic range. Will provide the most detailed dynamic images.	May be possible to reduce exposure to allow for faster speeds and reduced photobleaching.

4.11 EM gain (EMCCD cameras only)

iXon EMCCD cameras can use EM gain to boost low level signals well above the noise floor and achieve a high SNR even at very short exposures. Thus, if the signal levels are very low it is possible to increase EM gain and boost the signal without the need to extend the exposures which would make imaging slower, and reduce the fluctuation data used by SRRF-Stream+ during temporal analysis.

A suitable EM gain range is x4 that of the noise listed for the mode being used. This data is present in the performance sheet. This will typically be between x200 and x300 EM Gain. If this is set to a much lower Gain the EMCCD camera will not be providing the sensitivity it is capable of. Setting the EM Gain in excess of >500 will not provide any further benefit and will reduce the overall dynamic range of the camera. Using EM gain at these levels will not cause any issues such as EM Gain ageing, or other issues that were sometimes misreported by other sources.

Due to the extreme sensitivity of the iXon EMCCD camera, with some samples exposures times as low as 10 milliseconds can be used.

4.12 Pixel Size

Different camera models have different pixel sizes and it is important to consider how this impacts imaging, and indeed for SRRF-Stream+.

Table 2:Sensor pixel and sensor size parameters of Andor EMCCD and sCMOS cameras.

Camera Model	Pixel Size (µm)	Pixel Area (μm^2)	Native Resolution	Sensor Diagonal (mm)
iXon Ultra 897 & Life 897	16	256	512x512	11.6
iXon Ultra 888 & Life 888	13	169	1024x1024	18.8
Sona 4.2B-11	11	121	2048x2048	32
Sona 2.0B-11	11	121	1400x1400	22
Sona 4.2B-6 / Zyla 4.2 PLUS / ZL41 Cell	6.5	39	2048x2048	18.8

- A larger pixel can collect more light and thus help provide a higher signal to noise ratio.
- A smaller pixel can provide better oversampling of an image and thus more easily maintain the resolution of the optical system. This is also a benefit for SRRF-Stream+ as this provides better spatial information for SRRF-Stream+ to work with.

Therefore, there is some compromise between having a small pixel to meet Nyquist criteria and it being able to collect sufficient signal to generate an image with a high signal to noise ratio.

The pixels of EMCCD cameras are relatively large, meaning that it is difficult to achieve Nyquist criteria for typical objectives at less than 100x magnification without using additional optovars/magnification. sCMOS cameras with the smaller pixel more readily meet Nyquist at a range of magnifications. By using additional magnification such as 1.5x or 2x, it is possible to reduce the effective pixel size and improve oversampling of the image at the expense of reduced field of view.

This topic is further covered in the section "Camera matching examples".

4.13 Camera ROI (Region Of Interest)

SRRF-Stream+ can be set to run at the full sensor resolution, or this can be reduced to increase the frame rate possible.

Setting a smaller ROI is also useful for testing camera settings since it allows images to be adjusted more quickly with a faster response. It is therefore useful to start at lower ROIs and increase these to gain a wider field of view once initial settings are established.

4.14 Binning

Binning is the process by which pixels are grouped together to form "Superpixels" and the signal can be boosted in doing so i.e. 2 x 2 binning groups 4 pixels together.

For EMCCD cameras binning should be avoided since the large native pixel size makes it difficult to meet Nyquist and binning would be detrimental to imaging with SRRF-Stream+.

For sCMOS cameras, this again would not be a general recommendation since SRRF-Stream+ benefits from better sampling of image data. However, with the Sona 4.2B-6, Zyla 4.2 PLUS and ZL41 Cell models that have a much smaller 6.5 µm pixel, this could be considered as an option if the image is not providing a sufficient signal at higher magnifications.

Section 5 - Optimising SRRF-Stream+

5.1 Camera and Objective Matching combinations

As discussed at the beginning of this user guide, meeting Nyquist is fundamental to obtaining good results with SRRF-Stream+. The following table outlines some example objective lens and additional magnification combinations and the effect that this will have on meeting Nyquist.

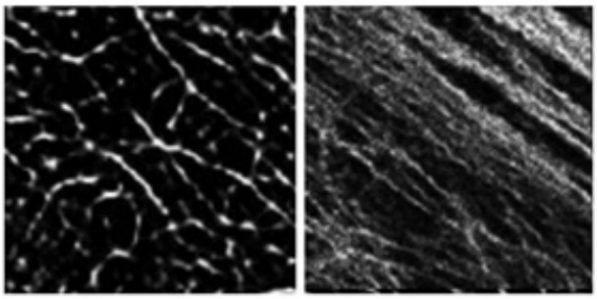


Figure 6:Left undersampled image as shown using SRRF-Stream+, compared to same image (right) corrected to meet Nyquist using suitable objective and coupler magnification.

Since EMCCD cameras have a large pixel size, optimized for photon collection efficiency this is important in getting the best that these cameras are capable of.

Table 3:Example combinations of objectives and magnifier couplers for EMCCD cameras

EMCCD (Ultra/Life) Camera Model	Region of Interest	Pixel Size (µm)	Objective Magnification	NA	Coupler Mag (x)	Total Magnification (x)	Effective Pixel Size in Image in nm (pixel size /total magnification)	Nyquist Sampling (resolution limit /pixel size)
iXon 897	512x512	16	100	1.49	1	100	160	1.5
	512x512	16	100	1.49	1.5	150	106.7	2.3
	512x512	16	60	1.4	1.5	90	178	1.5
	512x512	16	60	1.4	2	120	133	2.0
	2048x2048	11	60	1.4	2	120	91.7	2.9
iXon 888	1024x1024	13	100	1.49	1	100	130	1.9
	1024x1024	13	100	1.49	1.5	150	86.7	2.8
	1024x1024	13	60	1.4	1.5	90	144	1.8
	1024x1024	13	60	1.4	2	120	108.3	2.4

From this, you can see that additional magnification is often required, especially when working at 60x magnification for achieving, or approaching Nyquist of 2 (strictly this is 2.3). For typical 40x objectives it would not be possible to meet Nyquist without adding a much higher magnification mag coupler.

Table 4:Example combinations of objectives and magnifier couplers for sCMOS cameras

Sona Camera Model	Region of Interest	Pixel Size (µm)	Objective Magnification (x)	NA	Coupler Mag (x)	Total Magnification (x)	Effective Pixel Size in Image in nm (pixel size /total magnification)	Nyquist Sampling (Resolution limit /pixel size)
Sona 4.2B-11	2048x2048	11	100	1.49	1	100	110.0	2.2
	2048x2048	11	100	1.49	1.5	150	73.3	3.4

Sona Camera Model	Region of Interest	Pixel Size (µm)	Objective Mag-nification (x)	NA	Coupler Mag (x)	Total Mag-nification (x)	Effective Pixel Size in Image in nm (pixel size /total mag-nification)	Nyquist Sampling (Res-olution limit /pixel size)
	2048x204-8	11	60	1.4	1	60	183	1.5
	2048x204-8	11	60	1.4	1.5	90	122	2.2
	2048x204-8	11	60	1.4	2	120	91.7	2.9
Sona 4.2B-6 / Zyla 4.2 PLUS / ZL41 Cell	2048x204-8	6.5	100	1.49	1	100	65	3.8
	2048x204-8	6.5	60	1.4	1	60	108	3.6
	2048x204-8	6.5	40	0.9	1	40	162.5	2.5
	2048x204-8	6.5	40	0.9	1.5	60	108	3.7

The smaller pixels of these sCMOS cameras can be seen to be advantageous in meeting Nyquist without additional magnification across a wider range of objectives. It would still be recommended that for the sCMOS cameras, a high NA 100x or 60x objective will give the best results. Note that a high NA 100x objective will give the highest resolution, but an 60x objective can be brighter and have a wider field of view. Different scenarios may mean either a 60x of 100x objectives may be more suitable.

5.2 Resolution Expectations from SRRF-Stream+

Since the aim of using this method is to boost the resolution and see greater information than previously possible– what kind of resolution improvements can be expected from this technique is a basic question. There are many different variables in imaging setup, the type of fluorophores and the settings, therefore the achievable resolution will vary. However, to give some expectations of what should be possible we can indicate the following:

- Typically 2 – 6 times better than the original data.
- Original data may have maximal lateral resolution in the range 200-300 nm.
- In the original SRRF publications it was reported that resolutions down to 50 nm could be achieved under optimal conditions using the SRRF technique.
- SRRF-Stream+ users, that have published in the period 2018-2022, reported resolution within the region 98 nm to 150 nm.
- This compares favourably with other super resolutions such as SIM and allows for resolution of many subcellular structures, organelles and processes at low illumination intensities.

5.3 Other factors that affect resolution

Labelling density

Sparser labelling will help allow for higher resolution whereas denser labelling will reduce resolution – some examples:

- high-intensity laser-illuminated widefield epifluorescence will tend to generate a low-density data set
- low-intensity LED-illuminated widefield epifluorescence will tend to generate a high-density data set
- laser-scanning confocal, will tend to generate a high-density data set

Addressing Vibration

Localization based super resolution is sensitive to vibration within the optical system. Vibrations may potentially cause additional inaccuracy in localization of photon emission, and this is also true for SRRF-Stream+. Therefore, it is important to ensure that the microscope and camera are securely mounted using mounting feet on the camera, optical table and other sources of vibration are addressed e.g. filter wheels, illumination with cooling fans, motors etc.

One source of vibration is the fan used when air cooling. Andor cameras have been designed to have exceptionally low levels of vibration, so this should not be an issue for most set-ups, however it should be considered. Mounting feet can help minimize vibration when side port mounted, while fan speeds can be set to lower speeds. Alternatively, water cooling is highly effective at eliminating vibration for critical measurements.

Note that the standard Zyla or ZL41 Cell camera is air cooled only and cannot be connected for water cooled operation. The water cooled option of the Zyla must be specified at time of ordering if measurements are vibration sensitive and for example resolution must be pushed for SRRF-Stream+ operation.

5.4 Achieving Optimal Resolution

Assuming we have the microscope and camera matched and setup as well as possible, we can start to consider adjusting settings available for SRRF-Stream+ to maximise the resolution.

To get the best resolution we can adjust the following settings:

- Number of Frames per Time point (default=100) increase to improve resolution, decrease for higher speeds.
- Interpolation Type - use default catmull ROM.
- Ring Radius (default 2.0) decrease the ring radius to improve resolution. If image becomes "overprocessed" put the ring radius back towards default value.
- Radiality Temporal Analysis: Mean for most datasets, using MIP in the case of sparse datasets and can work well for the sCMOS cameras.
- Radiality Magnification: (default 4). Increasing this to x6 may provide some incremental improvement in resolution providing the graphics card can handle the extra processing workload.
- Binning: in general pixel binning should be avoided since this reduces localization accuracy, and thus ultimate resolution that can be achieved.

5.5 Notes on EMCCD Cameras

Start with exposures of 100 milliseconds. If signal level is relatively high, the exposure could be reduced as low as 10 ms for bright samples in combination with increasing the number of frames. Increasing EM gain may also help if the initial EM gain was not enough to boost the signal well above the noise floor e.g. if EM gain was set too low, and the signal is very weak, boosting this in the region of 150 to 500 will improve the Signal to Noise and thus the image quality. (Note that the correct EM gain should be set at 4-5x the read noise value for the mode of operation. This can be found in the performance sheet).

The higher sensitivity of the iXon EMCCD cameras make them well suited for live cell dynamics since the camera can collect a suitable high SNR image at short exposures. This will permit 5- 10 fps over small fields of view. The downside of this sensor format and technology is the small sensor area giving a smaller area of a sample being imaged. The large pixel size also requires use at high NA 100x objectives and optical matching to achieve Nyquist.

5.6 Notes on sCMOS Cameras

Start with exposures of 100 milliseconds. If signal level is relatively high, this could be reduced in combination with increasing the number of frames. Extending exposures to 150-200 ms may help with very weak samples, along with a smaller number of frames e.g. 10-25.

For the supported sCMOS cameras, users report that SRRF-Stream+ is well suited to delivering very detailed images over a large area, especially at 60x. However the sensitivity of sCMOS cameras is not as high as EMCCD cameras. For SRRF-Stream this means they are not as well suited to imaging dynamic processes that require very short exposures.

5.7 Achieving the Fastest Speeds

The other aspect of SRRF-Stream+ is visualization of dynamic cellular processes with improved clarity. In making these adjustments there may be some compromises to resolution or field of view - these will be indicated with each setting.

SRRF-Stream+ Settings For High Speeds

- Number of Frames per Time point (default=100) decrease to 50 frames to improve imaging speeds, without significant drop in resolution (depending on dataset).
- Interpolation Type- (catmull ROM). Change to Fast Spline B to increase speeds by a factor of 2. This will make image less well resolved.
- Radiality Magnification: keep at the default value 4.
- Camera ROI: Reduce the camera ROI. This setting will reduce the field of view, but as with normal imaging, running at a small ROI will allow significantly increased speeds.

5.8 What Speeds are possible with SRRF-Stream+?

The following tables shows tested speeds using SRRF-Stream+ for a range of ROI and Frames for the iXon Ultra 888 Camera using a Quadro RTX 4000 graphics card.

Note
Speeds will vary depending on GPU used, exposures, no of frames and Camera array size.

Table 5:Time Taken for Single SRRF-Stream+ Image. Note these times are from a user perspective using Fusion, including all overheads and acquisition time, they are not processing times.

iXon 888 ROI Size	Number of SRRF-Stream+ frames	Time taken to acquire single SRRF-Stream+ image (s)
1024X1024	50	2.56
512X512	50	1.57
256X256	50	0.99
128X128	50	0.53
1024X1024	100	4.51
512X512	100	2.52
256X256	100	1.77
128X128	100	1.05
Camera Settings: Exposure time 1 ms, Overlap ON, Vertical Shift set to minimum with Finite Burst Protocol.		

Table 6:Frame rates possible for generation of continuous "Live SRRF-Stream+" images

Standard ROI Mode			
iXon 888 ROI Size	**Camera Frame rate - Standard ROI (fps)**	**SRRF-Stream+ Frame rate - 100 input frames (fps)**	**SRRF-Stream+ Frame rate - 50 input frames (fps)**
512 x 512	50	0.5	1.0
256 x 256	95	1.0	1.9
128 x 128	171	1.7	3.4
Crop Mode ROI Mode			
iXon 888 ROI Size	**Camera Frame rate - Crop Mode ROI (fps)**	**SRRF-Stream+ Frame rate - 100 input frames (fps)**	**SRRF-Stream+ Frame rate - 50 input frames (fps)**
512 x 512	78	0.8	1.6
256 x 256	251	2.5	5.0
128 x 128	697	7	14

Section 6 - Troubleshooting

This section covers some possible issues and how you may resolve them.

Issue	Possible Cause	Solution
Cannot see SRRF-Stream+ Settings in device settings	No valid license detected	• Check .lic file is matched to camera and present in directory location shown in Quickstart guide
SRRF-Stream+ only works on one camera	There is only one license	• Each camera needs a license to run SRRF-Stream+
	SRRF-Stream+ library not installed	• Repeat SRRF-Stream+ Installation procedure. If issues remain contact Andor product support.
Cannot see SRRF-Stream+ image	SRRF-Stream+ LUT is different to original image and not visible	• Adjust image histogram or scaling to see SRRF image
	Issue with Microscope or camera lightpath/settings	• Check imaging without SRRF-Stream+ and ensure correct filters and lightpath from illumination source.
Image obtained is poor quality	Objective and camera combination do not meet Nyquist criteria for imaging	• Check that camera pixel size meets Nyquist for objective used. Add additional magnification, or use suitable objective. • Check that camera binning is not used i.e. set at 1x1. • Check ring radius value is not set too low. Use default value.
	Low quality image information available for SRRF to work effectively	• Check firstly that non-SRRF image generates an acceptable image.

Issue	Possible Cause	Solution
		• Then move to default SRRF settings, adjusting as per described in this guide.
SRRF-Stream+ applied to all channels of a protocol (Fusion users)	SRRF-Stream+ is enabled for all channels in a protocol.	• SRRF-Stream+ can not be applied to specific channels when imaged as part of a protocol.
Star artefacts present in image	SRRF-Stream+ is previous SRRF-Stream+ version as SRRF-Stream+ eliminates this type of artefact.	• Check SRRF-Stream+ version is 1.16.1 or greater. If not use upgrade process. • If SRRF version is 1.16.1 or greater, most likely cause is objective and pixel size do not allow Nyquist criteria to be met.
Shadow artefacts present around high contrast features	SRRF-Stream+ has calculated that there is no photon emission in that area.	• This effect is aesthetic since there is no presence of fluorescence emitter in that area. • Adjust image scaling to reduce visible effect.
iXon 888 model shows vertical line artefacts in some images	iXon 888 models not optimized for SRRF-Stream+ at time of ordering may show low level lines in image that are detected by SRRF-Stream+ but not apparent in normal imaging.	• Optimization of iXon Ultra and Life models is only guaranteed to eliminate this effect if ordered at initial ordering of the camera. • Later upgrade will minimize the effect as much as possible, but not always eliminate the effect.
Image in confocal stack has apparent high value pixels extending through-	Low level patterning present in each image frame may become apparent when displayed in a 3D volume of stacked images.	• Enable FPN correction in Fusion SRRF-stream settings to correct for pattern noise effects.

Issue	Possible Cause	Solution
out the image volume.		
Imaging speeds are very slow	Current settings selected are limited by GPU processing capability.	• Check GPU and memory requirements are met. • Note that sCMOS cameras have large sensor sizes that create a high load on GPU at full frame.
	Current settings selected result in slow frame rates	• Check exposure and images per time point. • Each image should take exposure x no of images per i.e. 10 ms x 100 frames takes 1 second per super resolved image. • Check that Radiality setting is set at 4 as higher values tax the GPU
Imaging speeds are insufficient for process being studied	Settings result in insufficient frame rate.	• Refer to guidelines for improving imaging speed. e.g. reduction of ROI used will have significant increase in speeds.
Live imaging shows lag when making adjustments	Each SRRF image is generated from a sequence of frames as defined in settings e.g. 100 frames used per super resolved image.	• To increase response, reduce ROI used and/or no of frames per image. • These parameters can be changed back once imaging settings have been confirmed suitable.
'Donut' artefacts present in image	Library needs to be updated to 1.18	• Check SRRF-Stream+ version is 1.18 or greater. If not use upgrade process detailed here "How to upgrade from SRRF-Stream

Issue	Possible Cause	Solution
		to SRRF-Stream+" on page 14.

Section 7 - SRRF-Stream Publications

Since the original version of SRRF-Stream was released there have been many publications using this technique. The following selected publications provide some examples of how SRRF-Stream has been used.

1. Gustafsson, N., Culley, S., Ashdown, G. et al. (2016) Fast live-cell conventional fluorophore nanoscopy with ImageJ through super-resolution radial fluctuations. Nat Commun 7, 12471 (2016). doi.org/10.1038/ncomms12471
2. Culley S, Tosheva KL, Matos Pereira P, Henriques R. SRRF: Universal live-cell super-resolution microscopy. Int J Biochem Cell Biol. 101:74-79 (2018). doi:10.1016/j.biocel.2018.05.014
3. Browne M and Mullan A (2020). Andor Technical Note: 'SRRF-Stream': Real-Time Super-Resolution in a Camera SRRF-Stream: Real-Time Super-Resolution in a Camera (oxinst.com)
4. Cooper J et al. 2019. Real Time Multi-modal Super-Resolution Microscopy through Super-Resolution Radial Fluctuations (SRRF-Stream Real time multi-modal super-resolution microscopy through Super-Resolution Radial Fluctuations (SRRF-Stream) (spiedigitallibrary.org)
5. Florindo C (2020). Super-Resolution Microscopy Accessible to All. Super-Resolution Microscopy Accessible to All- Oxford Instruments (oxinst.com)
6. Mullan A (2022). SRRF-Stream+ User Guide. srrf-stream-plus-user-guide.pdf (oxinst.com)
7. Huokko T, et al. 2021 Probing the biogenesis pathway and dynamics of thylakoid membranes. Probing the biogenesis pathway and dynamics of thylakoid membranes | Nature Communications
8. Shimizu S et al 2021 Class II phosphatidylinositol 3-kinase-C2a is essential for Notch signaling by regulating the endocytosis of γ-secretase in endothelial cells nature.com/articles/s41598-021-84548-4
9. Castillo-Badillo J A et al. 2020 SRRF-Stream Imaging of Optogenetically Controlled Furrow Formation Shows Localized and Coordinated Endocytosis and Exocytosis Mediating Membrane Remodeling: pubs.acs.org/doi/abs/10.1021/acssynbio.9b00521

10. Aung K T et al. 2019 The class II phosphoinositide 3-kinases PI3K-C2a and PI3K-C2β differentially regulate clathrin-dependent pinocytosis in human vascular endothelial cells: link.springer.com/article/10.1007/s12576-018-0644-2
11. Chang M et al. 2019 F-actin dynamics transform filopodial bridges into intercellular nanotubes capable of distant cell communication: biorxiv.org/content/early/2018/08/30/405340.abstract
12. Meyerink J G et al. 2019 Transparent Titanium Dioxide Nanotubes: Processing, Characterization, and Application in Establishing Cellular Response Mechanisms biorxiv.org/content/early/2018/05/25/330712.abstract
13. Sarker M A K et al. 2019 Class II PI3Ks a and β Are Required for Rho-Dependent Uterine Smooth Muscle Contraction and Parturition in Mice: academic.oup.com/endo/article/160/1/235/5193468
14. Oh J Y et al. 2019 FRBM-17BETA-Estradiol protects mesenchymal stem cells against high glucose-induced mitochondrial oxidants production: sciencedirect.com/science/article/pii/S0891584918313601
15. Choi G E et al. 2019 Cell Death Dis-Glucocorticoid-mediated ER-mitochondria contacts reduce AMPA receptor and mitochondria trafficking into cell terminus via microtubule destabilization: nature.com/articles/s41419-018-1172-y
16. Lee H K et al. 2019 Cell Death Differ-BICD1 mediates HIF1ÑÞ nuclear translocation in mesenchymal stem cells during hypoxia adaptation: nature.com/articles/s41418-018-0241-1