

# Super Resolution Spinning Disk Confocal Microscopy

## Introduction

Spinning disk confocal microscopy is a versatile and widely used imaging technique in biology due to its ability to perform fast, 3D imaging of live cells. Recently, techniques have been created that combine the high resolution of super resolution fluorescence microscopy with the simplicity and optical sectioning capability of spinning disk confocal, resulting in a spinning disk system capable of a twofold resolution improvement over the diffraction limit.

Confocal microscopy uses optical sectioning to take multiple, thin, 2-dimensional slices of a sample which are used to construct a 3-dimensional image. This process removes the out-of-focus light from other planes, giving a high contrast image which is often further processed to improve image quality. Compared to other optical sectioning techniques, spinning disk confocal microscopy is high-speed, high-sensitivity and simple to implement. However, resolution is limited by the diffraction limit of light which typically prevents detailed imaging of structures below ~200 nm.

Super-resolution fluorescence microscopy allows for structures to be resolved beyond the diffraction limit, which is of substantial benefit to researchers investigating processes that occur at this level. There are many super resolution techniques currently available (i.e. STED, SIM, STORM, PALM) and they have different capabilities to suit different applications. However, the ability to increase spatial resolution with these advanced techniques comes at the cost of technical complexity, dye restrictions, and a typically slow imaging speed.

Combining super resolution with spinning disk confocal overcomes many of the issues of both techniques to create a powerful new technique for live cell imaging that promises high sensitivity, resolution, and speed.

## Confocal Surpasses the Diffraction Limit

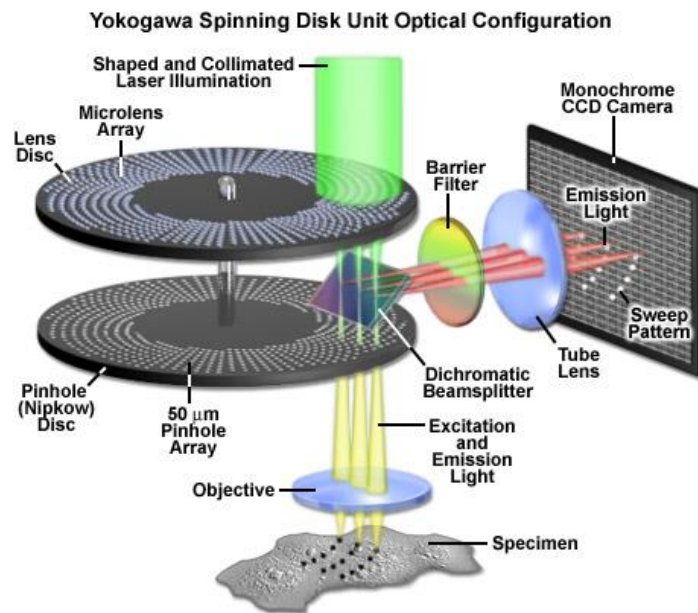
It has been known for a long time that confocal microscopy could surpass the diffraction limit, as proposed by Sheppard (1988) and implemented by Mueller and Enderlein (2010) with image scanning microscopy (ISM).

The reason for this, as shown in Figure 1, is that in confocal microscopy there are two point spread functions (PSFs); the PSF for the illuminating optics at the sample plane (excitation) and the PSF for the imaging optics at the pinhole plane (detection). An image is generated from the combination of the illuminating point spread function and the imaging point spread function so narrowing either can provide increased resolution.

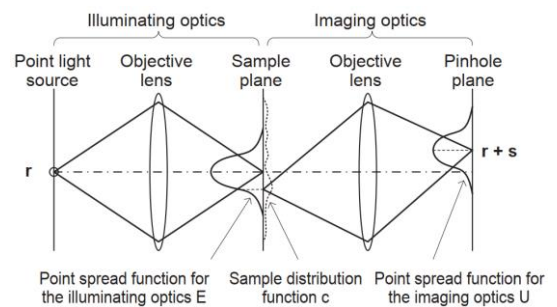
The excitation PSF is diffraction limited but there are options available to manipulate the detection PSF. For example, the confocal like super resolution method STED/RESOLFT narrows the emission PSF by suppressing fluorescence emission off the central axis of the excitation PSF (Roobala *et al.* 2018).

In the same way, narrowing the pinhole of a confocal microscope or focusing the emission PSF leads to increased resolution in the image. Unfortunately, although the theoretical maximum resolution of a confocal microscope is  $\sqrt{2}$  times the diffraction limit, this is only when using an infinitely small pinhole. However, there is a way to overcome this.

A



B



**Figure 1:** Illustration of the (A) Yokogawa spinning disk system. The top disk with microlenses focuses the laser light to the imaging pinhole of the bottom disk to increase light throughput. As the disks spin, imaging of the sample through the pinholes fills the field of view of the camera.

(B) Optical system. For simplicity, the illuminating and imaging optics are drawn separately. The PSF for the illuminating optics has its peak at  $r$  whereas the PSF for the imaging optics has its peak at  $r + s$ .

(A) Adapted from Zeiss, Introduction to Spinning Disk Confocal Microscopy.

(B) Adapted from Azuma and Kei (2017).

## Photon Reassignment

The principle used by Sheppard (1988) to increase resolution with ISM can also be applied to spinning disk confocal microscopy using a technique called photon reassignment.

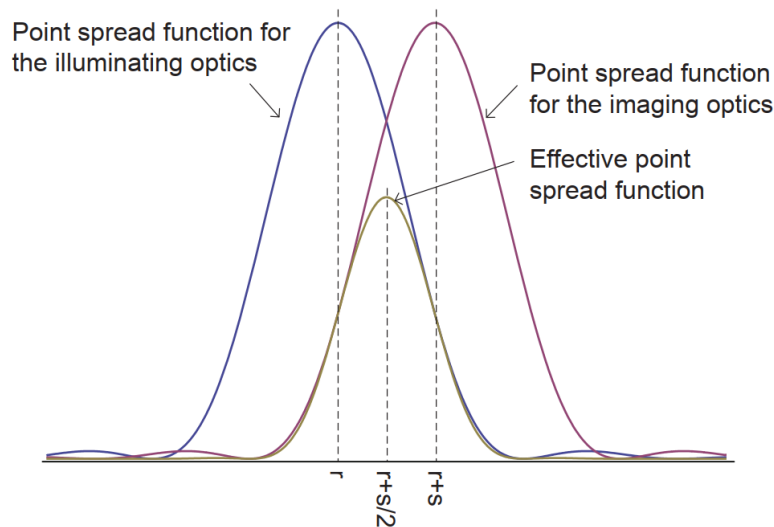
As shown in Figure 1B, the PSF for the illuminating optics has its peak at  $r$  whereas the PSF for the imaging optics has its peak at  $r + s$ , which means that the PSFs do not perfectly overlap. The PSFs are shown more clearly in Figure 2.

The problem with this is that the image on a conventional spinning disk confocal microscope is

imaged at the position  $r + s$ , when the actual image is most probably coming from the position  $r+s/2$  - halfway between both PSFs. This results in a reduction in spatial resolution.

Ideally, the imaging point should be shifted from  $r + s$  to  $r + s/2$ . The image formed at  $r+s/2$  has a narrower width than either excitation or detection PSF, giving an increased resolution improvement of  $\sqrt{2}$  fold. This process makes the resolution nearly equal to that of an ideal confocal microscope in which the pinhole has been reduced to an infinitely small size.

Following this up with deconvolution would make it theoretically possible to get twofold higher spatial resolution than the diffraction limit (Azuma and Kei, 2015).



**Figure 2:** Effective point spread function of a spinning disk confocal microscope.  
Adapted from Azuma and Kei (2017).

## Applying Photon Reassignment to Confocal Microscopy

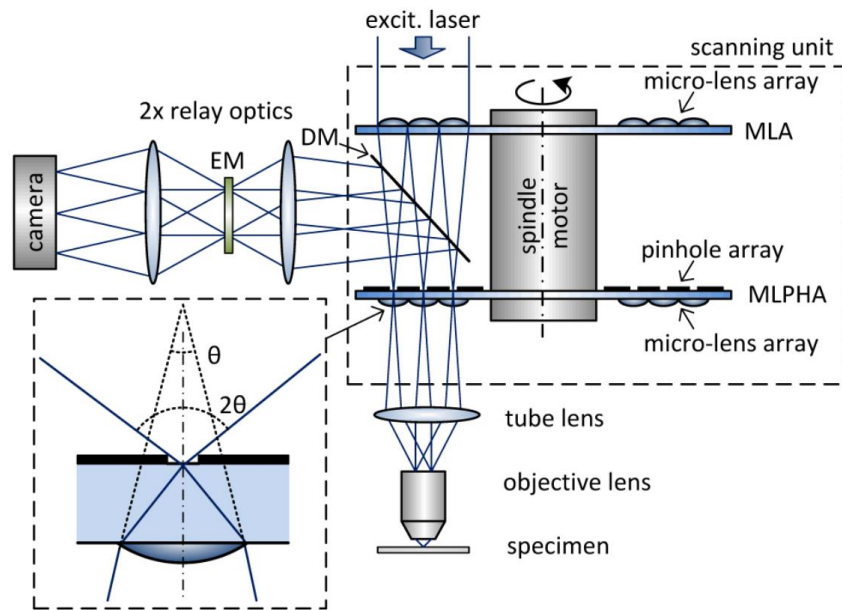
While presented theoretically, photon reassignment was first applied to single point (Sheppard *et al.*, 2013) and array point scanning confocal systems (York *et al.*, 2013). The application of photon reassignment to a spinning disk confocal was envisioned by Shroff and York (US Patent, awarded 2017) and achieved optically with a relatively simple modification to the bottom pinhole array disk of the Yokogawa confocal spinning disk system (Figure 1A) by Azuma and Kei (2015).

An array of microlenses is placed on the lower side of the bottom disk (Figure 3) which doubles the convergence angle of light emitted from the sample (see inset of Figure 3). The emission light coming from the sample is refracted by the microlenses to diverge at a cone angle of  $2\theta$  which, because the size of focus is approximately inversely proportional to the cone angle if the angle is small, contracts the focus twofold. This optically reassigns emitted photons to where they most likely originated from.

This has the effect of increasing resolution by a factor of  $\sqrt{2}$ , which can then be further improved with deconvolution to give a twofold increase over diffraction limited resolution (Figure 4).

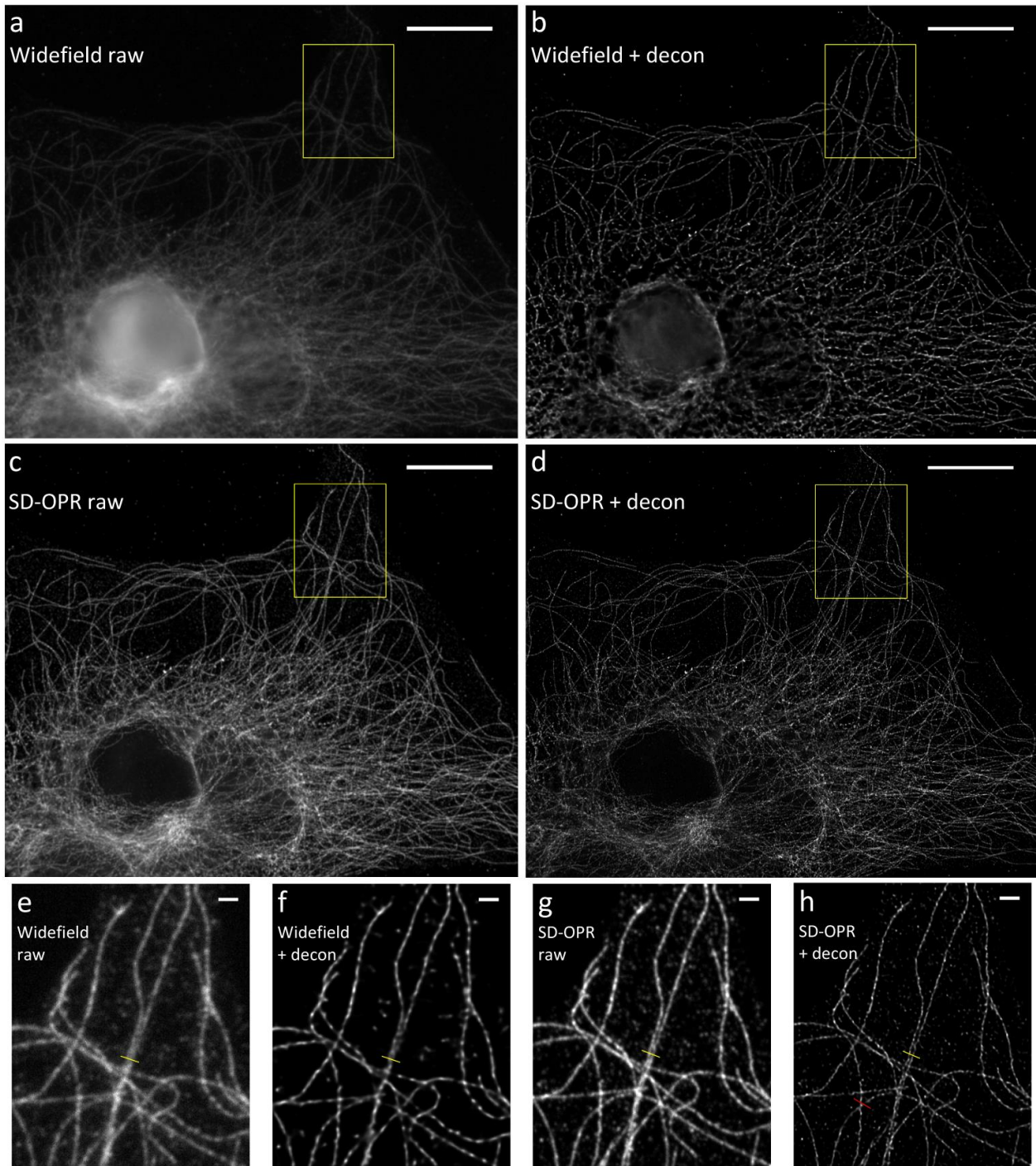
With this system, the speed and optical sectioning advantages of a spinning disk system are maintained but are now combined with super resolution imaging. By rotating the disks at 4000 rpm, super resolution images can be captured live at up to 200 fps using any fluorescent dye, overcoming many of the drawbacks of conventional super resolution techniques.

This system is currently manufactured by Yokogawa as the SoRa (Super Resolution via Optical Re-assignment) and sold by many companies offering microscope systems.



**Figure 3:** Simplified optical path of the Yokogawa SoRa spinning disk confocal scan head. The key difference is the addition of microlenses to the previously un-lensed bottom, pinhole array disk.

As noted in the inset at bottom left, the microlensed pinhole array allows light coming from up to twice the angle to pass through the pinhole than the pinhole would otherwise allow. Coupled with the relay lens system after the dichroic, the contraction of the emission increases resolution by 1.4-fold in the captured image. Adapted from Azuma and Kei (2017).



**Figure 4:** Comparison of microtubules with widefield, widefield + deconvolution, super resolution spinning disk (SD-OPR) and super resolution spinning disk + deconvolution. Deconvolved images were obtained by applying 3D deconvolution using Huygens. The scale bars represent 10  $\mu\text{m}$ . Adapted from Azuma and Kei (2015).

## Super Resolution Sampling

When performing super resolution imaging, the camera needs to be able to accurately sample the image. If the effective pixel size of the camera is too large there will be no advantage to performing super resolution imaging because the camera won't be able to sample it.

The ability of the camera to sample the image is entirely dependent on the size of the pixel and by how much it's magnified. For example, if the maximum resolution possible with the microscope optics is 250 nm, the effective pixel size needs to be lower than this.

Typically, when performing any type of imaging, users should aim to sample at Nyquist. Nyquist sampling allows for the maximum resolution of the microscope to be sampled by the camera. To calculate the effective pixel size needed for Nyquist sampling, the following equation should be used:

$$\text{Camera resolution} = \frac{\text{Pixel Size}}{\text{Objective Magnification}} \times 2.3$$

For example, typical diffraction limited imaging allows for a maximum resolution of ~250 nm. A 6.5  $\mu\text{m}$  pixel camera would, therefore, need 60x magnification for Nyquist sampling.

$$\frac{6.5 \mu\text{m}}{60\text{x}} \times 2.3 = 250 \text{ nm}$$

This is made more complicated with super resolution spinning disk because the resolution limit is now ~120 nm. Additionally, deconvolution usually requires a certain amount of oversampling. For this reason, the Yokogawa SoRa is usually used with a 60x objective + 4x magnification changer or a 100x objective + 2.8x magnification changer to reach 240x or 280x total magnification, respectively.

## Cameras For Super Resolution Spinning Disk

Cameras that maximize sensitivity and speed are typically used for super resolution spinning disk which means that back-illuminated CMOS cameras are generally preferred.

Typical cameras used with super resolution spinning disk systems have either a 6.5  $\mu\text{m}$  or 11  $\mu\text{m}$  pixel size to be used with 240x or 280x magnification, respectively. This allows for Nyquist sampling at 62 nm and 90 nm, either of which are suitable for oversampling from 120 nm.

The smaller pixel size of a 6.5  $\mu\text{m}$  pixel camera may give slightly improved resolution but at the cost of reduced sensitivity, detecting 2.2x fewer photons than an 11  $\mu\text{m}$  pixel camera. The 11  $\mu\text{m}$  pixel camera greatly improves contrast and allows for exposure times to be reduced while still oversampling enough for effective deconvolution.

## Summary

In any confocal instrument, optical contraction of the PSF for the imaging optics provides a mechanism to increase resolution beyond the diffraction limit. The Yokogawa SoRa is currently the most widely used super resolution spinning disk solution. It provides an increase in resolution through optical photon reassignment via a microlens array on the underside of the bottom disk. Confocal imaging followed by deconvolution results in a two-fold increase in resolution over the diffraction limit.

The choice of camera for super resolution spinning disk is also important to make sure that the image is sampled correctly with high enough contrast for effective post-processing and analysis. The high sensitivity of an 11  $\mu\text{m}$  pixel camera will give the highest contrast while still oversampling enough for effective deconvolution and super resolution imaging. A 6.5  $\mu\text{m}$  pixel camera may give slightly improved resolution but at the cost of reduced sensitivity which may necessitate longer exposure times.

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