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Advantages of Re-scan confocal microscopy vs PMT-based confocal systems and its benefits for deconvolution

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Introduction

Confocal microscopy is broadly applied in the laboratories and helps scientists make new discoveries. However it has certain limitations regarding resolution and sensitivity. On one hand, the resolution depends on the diffraction limit and therefore subcellular components or microorganisms cannot be resolved. On the other hand, the sensitivity of the most common confocal detectors, like PMTs, is relatively low.

In many applications, deconvolution is used to further improve the resolution but the results are sub-optimal when the image has noise and low contrast.

Re-scan confocal microscopy (RCM) is a new super-resolution technique that improves resolution and sensitivity, overcoming the limitations of traditional confocal systems (De Luca *et al.* 2013).

Here we demonstrate that RCM technology generates images with higher resolution and contrast than PMT-based confocal systems, leading to much better results, especially when using deconvolution.

Results

RCM generates images with higher resolution and contrast than standard confocal systems

The synaptonemal complex (SC) is a large proteinaceous structure present in the spermatocytes that holds together homologous chromosomes during meiosis. Visualization of this structure is challenging due to the short distance between chromatids, which is under the diffraction limit.

Mouse spermatocytes stained with SYCP3 (Alexa-488) were imaged using a standard PMT-based confocal system with the recommended pinhole aperture of 1 Airy Unit (AU) (Fig. 1A). In this case, the resolution was not enough to properly resolve the chromosomes. In order to increase resolution, the pinhole was closed to 0.3 AU (Fig. 1B), achieving 170nm. Closing down the pinhole means that less emission light is reaching the detector, and the contrast of the image was lower. Laser intensity was increased to compensate for the loss of emitted light but it also led to more noise (Fig. 1B) (Table 1).

Raw images obtained with RCM showed better resolution (170nm) and signal to noise ratio than PMT-based confocal images (Fig. 1C).

The features in the background observed in Fig. 1B were lost with RCM (Fig. 1C) and less laser intensity was required to obtain an image with high contrast (Table 1).

High contrast RCM images allow for optimal deconvolution

In order to resolve the SC structure of the spermatocytes deconvolution was performed in the 3 raw images obtained in Fig. 1A, B and C. Only with RCM deconvolved images the SC structure was properly differentiated (Fig. 1 D, E, F).

The distance between the chromatids was then measured as 120nm (Fig. 2).

> **"RCM generates images with higher resolution and contrast than PMT-based confocal systems"**

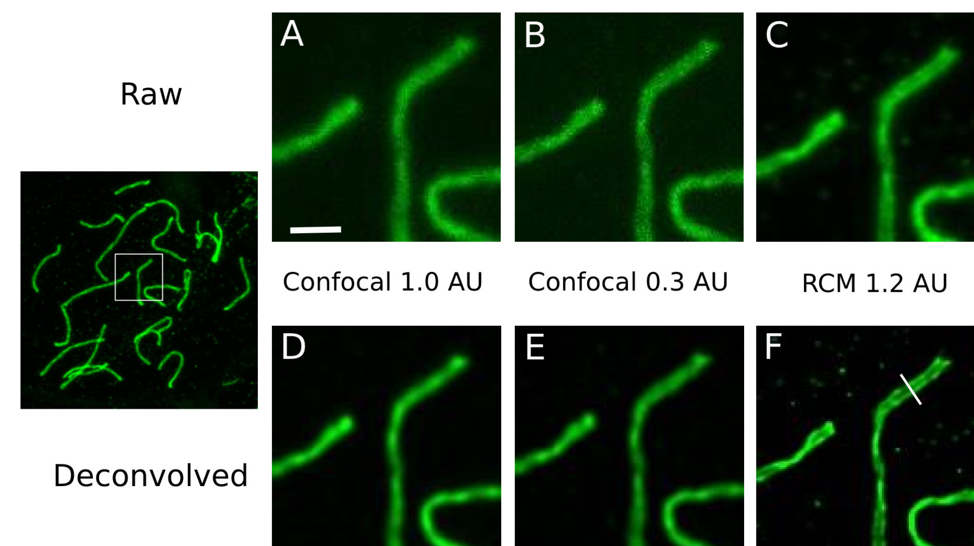

Figure 1. Comparison of synaptonemal complex images obtained with standard-confocal vs RCM.
Left panel image shows an overview of the sample. A, B and C are raw images obtained from the same area in the sample using the indicated imaging systems and conditions. D, E and F show the same images after performing deconvolution with a calculated PSF. Scale bar 1µm.

Confocal 1.0 AU	Confocal 0.3 AU	RCM
4.29 µW	12.5 µW	3.0 µW

Table 1. Laser intensity at the sample plane.
The laser power measured with a Thorlabs S170C sensor in the three different experimental conditions.

Materials & Methods

Fixed mouse spermatocytes chromosomes preparation were kindly provided by A. Agostinho (Advanced Light Microscopy Facility, Science for Life Laboratory). Samples were stained for SYCP3 and labeled with Alexa-488. Chromosomes were imaged with RCM1 coupled to a Nikon TiE microscope utilizing a Plan Apo 1.45NA oil objective (Nikon Instruments). The signal was detected using the RCM1 and a Hamamatsu Orca Flash 4 V3 camera.

Regular confocal imaging was performed on a PMT-based confocal microscope. The HyD detector was used to have a higher quantum efficiency.

The zoom level was set to get a 43nm pixel size in the image plane (same as RCM), and a z-stack was made with a 150nm slice interval for proper deconvolution.

The laser power was measured with a Thorlabs S170C sensor, using oil immersion to capture all the light rays.

All images were deconvolved using SVI Huygens with a measured point spread function.

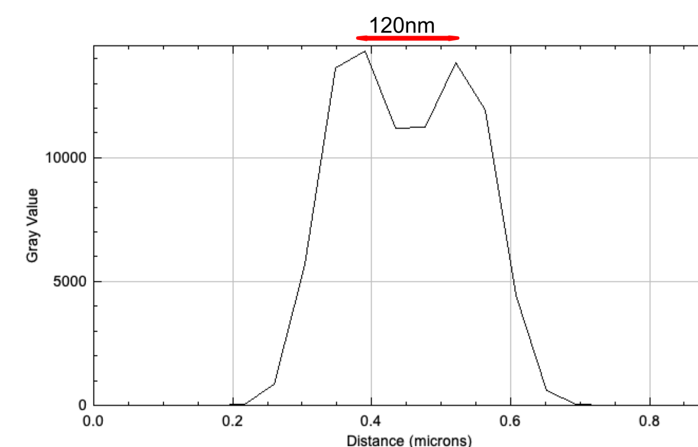

Figure 2. Graph showing the distance between chromatids measured in image 1F.
RCM deconvolved image presented a resolution of 120nm which allowed to properly visualize the synaptonemal complex.

Conclusions

These results demonstrate that RCM has higher resolution and sensitivity than PMT-based confocal systems and allows using lower laser power for better results. Thanks to the re-scanning step, resolution is 40% improved without closing the pinhole. The pinhole within RCM is 1.2 AU which means that more emitted light is passing through it.

In contrast to other single-point scanning confocal microscopes, RCM uses the most sensitive detector to date, the sCMOS camera. This translates into a much higher sensitivity and signal to noise ratio in the raw image. The high sensitivity of the RCM system allows to decrease the laser power to a minimum, reducing bleaching of fluorophores and phototoxicity.

Deconvolution algorithms are sensitive to excess noise in the image. Therefore, we were not able to resolve the ultrastructure of the SC after deconvolving the image in Fig. 1B (Confocal 0.30AU). However, despite having the same raw resolution, deconvolution of RCM image did showed the structure of the SC well defined due to the much higher contrast of the raw image.

RCM technology opens the door to super-resolution imaging with low laser power and high sensitivity. Live cell imaging is an area which will benefit most from this combination as cells are sensitive to the phototoxicity.

RCM

- ✓ 40% improved resolution
- ✓ 4 times more contrast
- ✓ Better results after deconvolution (120nm)

References

Giulia MR de Luca, Ronald MP Breedijk, Rick AJ Brandt, Christiaan HC Zeelenberg, Babette E de Jong, Wendy Timmermans, Leila Nahidi Azar, Ron A Hoebe, Sjoerd Stallinga, and Erik MM Manders. "Re-scan confocal microscopy: scanning twice for better resolution". *Biomed Opt Express* 2013. DOI 10.1364/BOE.4.002644

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