Re-Scan Confocal Microscopy

A Simple Trick for Better Resolution and Sensitivity

As we all know, the most important property of the confocal microscope is optical sectioning (axial resolution). A bit forgotten fact is that confocal microscopy has the potential of lateral super-resolution (better resolution than Abbe’s Law dictates). This lateral super-resolution can only be obtained by sacrificing precious fluorescence signal and thus by getting noisy images. This problem has now been solved by a simple re-design of the optics: Re-scan Confocal Microscopy (RCM).

Why Confocal Microscopes Should Give Super-Resolution

A confocal microscope is a point scanning technique, which means that a focused laser beam is directed into the sample in a scanning fashion. The light emitted by the fluorescent molecules in the sample is directed towards a small pinhole. The most important function of the pinhole is that it filters out all the out-of-focus light from the sample leading to the high axial resolution (optical sectioning) which is essential for 3D-imaging.

An additional function of the pinhole is that it potentially improves the latteral resolution. However, lateral resolution improvement can only be achieved with a very small pinhole which leads to a low signal-to-noise ratio of the image. In brief, confocal microscopy images can be made sharper but on costs a lot of extra noise.

This is why most biologists are of the opinion “Better less sharp than more noisy” and therefore decide to use a relatively large pinhole. In this way they sacrifice resolution for signal-to-noise ratio (SNR). In the next paragraphs we will explain how a simple modification of the confocal microscope, according to the re-scan principle (RCM), can provide sharper images with improved SNR.

The Inspiration: Image Scanning Microscopy

The new development of the Re-scan Confocal Microscope (RCM) started during the PhD project of Giulia De Luca (sponsored by the Dutch Technology Foundation (STW)) in the research group “Innovative Microscopy Lab” of Erik Manders at the University of Amsterdam. Giulia was inspired by the work of Prof. Jörg Enderlein
Collin Sheppard who made clear that the use of a pinhole in confocal microscopy has a fundamental problem: Light that passes the pinhole is detected very well, but the spatial distribution of this light is completely lost. Sheppard described that this irreversible loss of information leads to reduced resolution and Enderlein described a method to restore this information by using a camera behind the pinhole instead of a point-detector. This technique, Image Scanning Microscopy (ISM), uses a mathematical algorithm on multiple small pinhole-images (sometimes more than 1,000,000 mini-images) to reconstruct the image of the object with an improved resolution.

The Invention of RCM

The basic idea of Re-scan Confocal Microscopy (RCM) is to let the optics do the work of the computer [1, 4]. This means that grabbing numerous mini-images, image processing and calculations that were needed in the ISM microscope are now simply performed by lenses, mirrors and a camera. With this optical on-the-fly reconstruction approach, super-resolution images can be takes without any image processing. The heart of the optical modification of the good old confocal microscope is an additional re-scanning unit (two galvo-driven orthogonal mirrors) that re-directs the light from the pinhole to a sensitive camera. With a simple optical trick, super-resolution is obtained while the optical sectioning is maintained.

The Optical Trick of RCM
The RCM consists of a standard confocal microscope with an additional re-scanning detection unit. The movements of the scanner (in the confocal unit) and the re-scanner (in the detection unit) are highly synchronized, meaning that they move with identical frequency, phase and angular amplitude. So, while the focused laser beam “reads” the sample, the focused emission beam “writes” the image of the sample on a camera chip. Since we have a diffraction limited optical system, we are “writing” the image on the camera with a blurry spot according to the point-spread-function (PSF) of the optical system. In fact the limitation to the resolution is the thickness of our optical pencil (the PSF) that “writes” the image. To “write” an image with improved resolution our optical pencil should be sharpened.

Unfortunately, Abbe’s Law disallows to sharpen the pencil; the PSF is simply defined by the NA of the optics. Result: on the camera a confocal image can be seen, including its sectioning properties, but still without extra resolution. To get super-resolution in RCM only a simple optical trick is needed.

The optical trick is based on the fact that the magnification of the image can be changed by changing the amplitude of the re-scanner. By making the amplitude larger, the image on the camera chip will be larger. Abbe forbids to sharpen the pencil, but it is not forbidden to make a larger picture with the same pencil! It can simply make the image larger by doubling the angular amplitude of the re-scanner. In this way, the size of the image “written” on the camera chip is doubled and it still “writes” with the same pencil and therefore the final image becomes relatively sharper. Unfortunately, such resolution improvement has a price. Since the “optical pencil” is moving too fast over the image plane of the camera, some motion blur is introduced. With some mathematics it can be proven that, by doubling the angular amplitude of the re-scanner, the image will be $\sqrt{2}$ ($\approx 1.4$) times more blurry, but the image is two times larger. So it ends up with an image that is 1.4 times sharper ($=2/\sqrt{2} = \sqrt{2} \approx 1.4$). This is the key idea to resolution improvement in RCM.

**Prove for 170 nm Resolution without Image Processing**

After the development of this basic idea, Giulia De Luca, Ronald Breedijk and Erik Manders started to sketch optical configurations and building the first prototype on the optical table. After two months hard work in the optical lab, they obtained their first images. And indeed, by doubling the sweep factor, the optical resolution dropped from 240 nm to 170 nm; exactly a factor of $\sqrt{2}$ as was promised by the theory.

**RCM in Biological Applications**
After this proof of principle, a smaller prototype was constructed. Biomedical students from the university and guests from all over the world started to use and test the instrument [2, 3, 5]. During their experiments it became more and more clear that the improved resolution of 170 nm is not the only advantage of the RCM microscope. Also its four times higher signal-to-noise ratio (SNR) made that the images were crisper. For biologists this better SNR turned out to be even more important since their fluorescent samples are often very dim and they do not want to waste any photon.

The RCM has been modified for standard multi-color imaging and many other modalities have been explored. In the dual-color mode the RCM was used for measurement of FRET in apoptotic cells. Ratiometric pH measurements were performed by dual-excitation of pHluorine, with light of 405 nm and 488 nm. But most students used the RCM microscope to image their samples labeled with a red fluorophore (e.g. mCherry of Alexa 561), a green one (e.g. GFP of Alexa 488) and DAPI. For them it was important that the microscope gave them optical sectioning, better resolution and a lower signal-to-noise. Moreover, they found it very pleasant that the RCM was easy to use due to the lag of complicated options.

**Future Developments in RCM**

At the moment the RCM technology is commercially available and is spreading around the world. Meanwhile, in the group of Erik Manders and Ronald Breedijk at the University of Amsterdam, research on the implementation of new ideas is continued. At the moment they focus on deep-tissue imaging with RCM. Here, near infra-red (NIR) is used to penetrate deep into tissue in the single-photon excitation mode. This method may be a good alternative for two-photon excitation. Moreover, loss of resolution due to the long wavelength in NIR will be compensated by improved resolution of RCM. Furthermore, the group is now finishing the software drivers and plug-ins in Micro-Manager for the interfacing the RCM microscope. For the developers of RCM it is important to make to technology available according to the “by-researchers-for-researcher” principle. Manders: “It is really fun to see that researchers on the other side of the world are now using our RCM technology”.

**References**


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