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Vision Technology for Science

Technical Note

IMAGE SCANNING MICROSCOPY – SUPER RESOLUTION FOR ALL

For over a decade, super-resolution imaging in fluorescence based light-microscopy, has become an important tool for life science research. These techniques allow for features below the traditional diffraction limit of light-microscopy (as defined by Abbe's Law) to be resolved.

These methods include but are no way limited to; Single Molecule Localisation Microscopy (SMLM), Structured Illumination Microscopy (SIM), STimulated Emission Depletion Microscopy (STED), Lattice Light Sheet Microscopy….

However, these techniques have some significant complications when compared to traditional confocal microscopes, such as; requirement for specific fluorescent probes, increased levels of photo-bleaching, low temporal resolution, introduction of image artefacts, requirement for specific sample prep, requirement for specific imaging knowledge, instability and thus decreased repeatability.

These complications have often resulted in super-resolution imaging to be the sole enterprise of microscopy and imaging experts. However, this has changed with the immergence of a far simpler super-resolution imaging modality, which is able to reach all corners of imaging within the life sciences. Such techniques can be categorised as forms of Image Scanning Microscopy (ISM).

Image Scanning Microscopy

Image Scanning Microscopy was first hypothesised by Colin Sheppard in 1988; '*Super-resolution in confocal imaging'*, Optik;

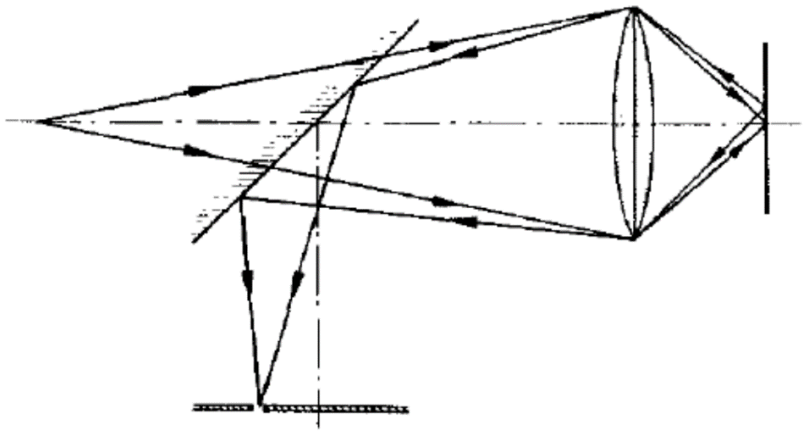

Fig. 1. Schematic diagram of the optical system.

This paper describes scanning a sample as per a regular point-scanning confocal microscope, however, the emission signal is detected via a series of displaced point-detectors and then re-assigned to facilitate the recovery of an image which has up to 2x the optical resolution of a conventional confocal microscope.

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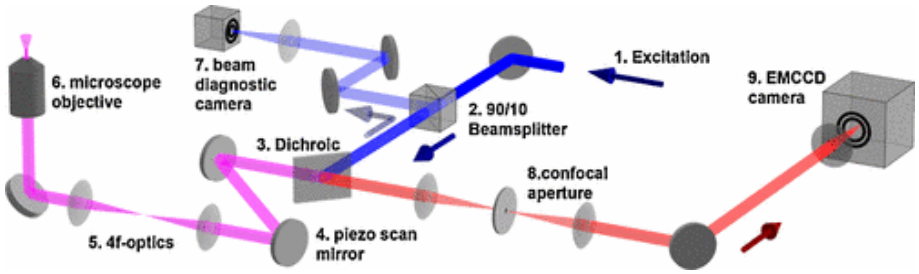
More than a decade after this technique was first theorised, it was implemented (in one form or another) with modern imaging technology, in what were independent but parallel developments by Muller and Enderlein in Physical Review Letters DOI: doi.org/10.1103/PhysRevLett.104.198101, and Carl Zeiss in the development of the Airy Scan system.

These developments (as will be discussed below) can be grouped as digital implementations of Image Scanning Microscopy as they require the image information detected through the displaced point-detectors to be digitally re-assigned so an image of super-resolution can be recovered.

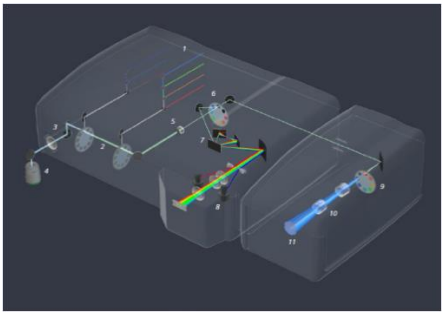
Digital Implememtation of Image Scanning Microscopy

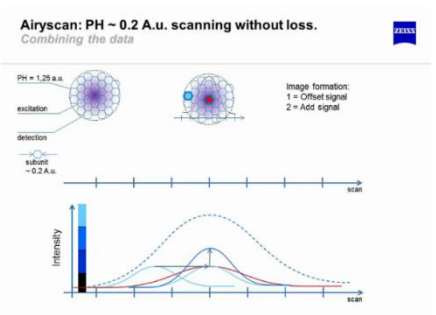
In Muller and Enderlein's implementation, they developed a regular point-scanning confocal microscope, but rather than detect onto a Photo Multiplier Tool (PMT), an EMCCD camera is used. Each pixel of the EMCCD camera is considered a displaced point-detector and if a camera image is taken at each scan position, the image information collected on each pixel is re-assigned to recover an image of super resolution.



However, this is relatively slow as a camera image needs to be taken at each scan position, this process can be parallelised by scanning multiple points at a time, as per MSIM (York et al; 'Resolution doubling in live, multicellular organisms via multifocal structured illumination microscopy', Nat Methods 9, 749–754 (2012). https://doi.org/10.1038/nmeth.2025), and as per VisiTech International Patent(s); US17/049595, EP2520965.

However, the first commercial implementation of this technique was the Zeiss Airyscan. The





Airyscan, in its purest form, is a regular point scanning confocal, whereby the standard detector is replaced with a fast array detector. This enables the image at each scan position to be read significantly quicker than with a CCD camera as described above. The image of super resolution can then be restored with minimal impact to image acquisition speed when compared to a regular confocal microscope, what's more, no specific fluorophore is required and the operation is simple.

Without the requirement for specific fluorophores, no requirement for image reconstruction and hence no risk of image artefacts, a simple and understood optical path and simple operation which is as per a regular confocal microscope, the Digital Implementation of Image Scanning Microscopy brought Super-Resolution Imaging to all corners of imaging within the life-sciences.

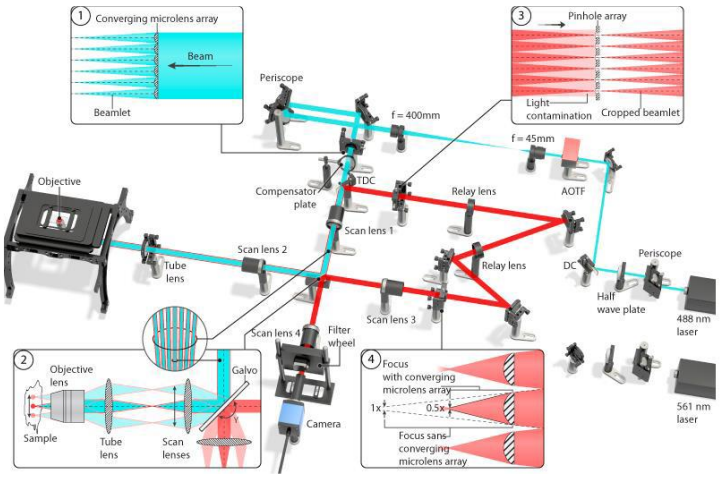
Further commercial implementations have been introduced since the Airyscan, such as the Re-Scan Confocal from Confocal.nl or the NSPARC originally developed by the Vicidomini Lab in IIT Genoa (https://vicidominilab.github.io/publications/) and commercially released by Nikon.

However, that's by no means where the story of Image Scanning Microscopy ends. These digital implementations of ISM, although revolutionary in bringing super-resolution imaging to the life science masses, still suffered some drawbacks due to heavy post-processing and non-optimal nature of the scanning technique for live-cell imaging… which is where the story of the Analogue Implementation of Image Scanning Microscopy begins.

The Analogue Implementations of Image Scanning Microscopy (as will be described below) have no requirements for post-processing, generating the super-resolution image in real-time, and can adopt multi-point scanning techniques for high-speed, live-cell imaging applications.

Analogue Implementation of Image Scanning Microscopy

As mentioned above, the analogue implementation of Image Scanning Microscope allows for the super-resolution image to be generated in real-time, through the real-time re-assignment of the image information.

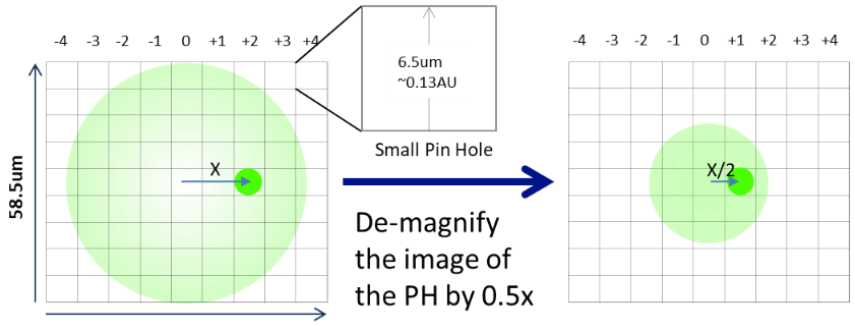


This was a further development of the MSIM technique described above and was developed by York et al (York, A., Chandris, P., Nogare, D. *et al.* Instant super-resolution imaging in live cells and embryos via analog image processing. *Nat Methods* **10**, 1122–1126 https://doi.org/10.1038/nmeth.2687), utilising a scan Architecture developed by VisiTech International (Patents; EP1852724, EP1467235, US 17049595).

The re-assignment discussed in the Digital Implementation is now performed by a single optical element in the emission path (µLens array), which re-assigns the image information in real-time

without the requirement for any post-processing. This allows for a truly parallelised, array scanning architecture, also enabling high-speed, live-cell imaging.

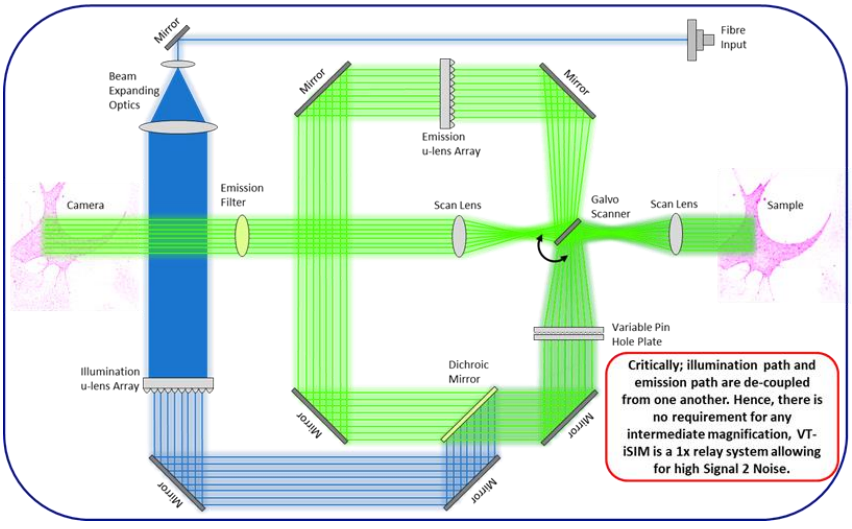
The first commercial implementation of an instrument featuring Analogue, Image Scanning Microscopy, was the VT-iSIM from VisiTech International, released in 2015.



As described above, the analogue implementation utilises a µLens in a near-conjugate plane of the emission path to re-assign the image information by shrinking the image of each individual Point Spread Function (PSF) of the image by 2x and projecting onto a camera where by the re-scanning onto the camera, and integration of the camera, forms the image of super-resolution in real-time.

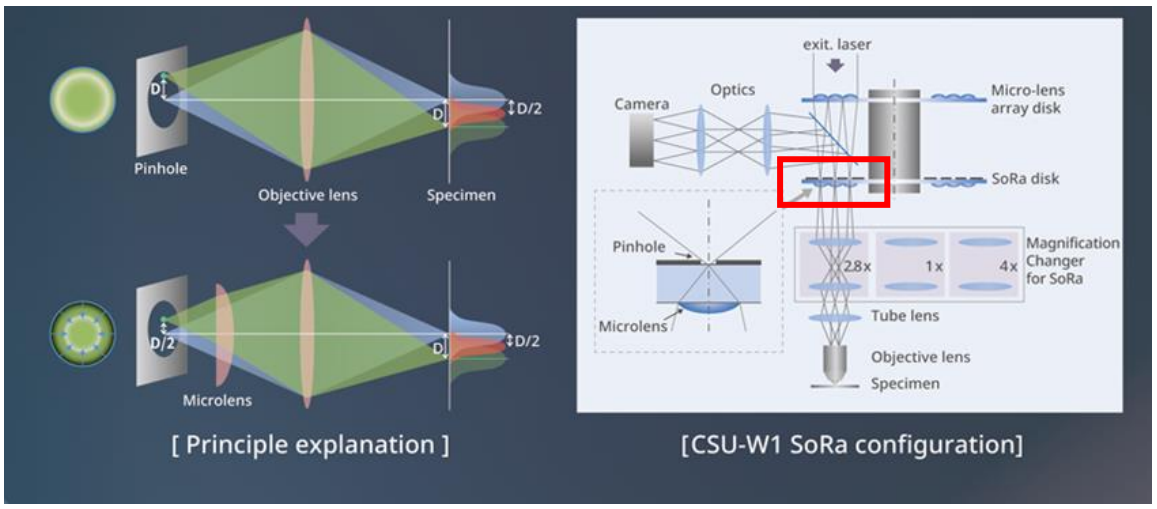
In turn therefore, we can utilise an array scanner scanning at 1,000Hz to scan multiple points at the sample plane, de-scan the emission and pass through a Pin-Hole array to remove out of focus light, and finally use a µLens array in a near conjugate plane to the pin-hole array to re-assign the image information from each PSF of the emission, and finally re-scan onto the camera whilst the camera is integrating, to create an image of super-resolution without the requirement for ANY post-processing.

What should also be noted, is that as the VT-iSIM has the emission µLens array (the re-assignment optic) in the emission path only, the system operates as a simple 1x relay and there is no requirement for intermediary mag. Hence, live-cell imaging applications can be taken from an existing array scanning confocal (such as a Spinning Disk), placed on the VT-iSIM and without any loss of temporal resolution, or without any decrease in cell-viability, the resolution on all axis (X, Y & Z) can be doubled.



Obviously, this technique has since been adopted by other developers of array scanning confocal, not least the Spinning Disk from Yokogawa. However, due to the nature of their scan architecture (i.e., two disks rotating at 10,000rpm), the only location the re-

assignment optic could go into the light path (that was in a near conjugate plane to the pin hole array), was between the pin hole array and the sample plane, as shown below;



Such an implementation has two significant draw-backs;

1. The input NA is reduced and the PH's appear much smaller at the sample plane, hence intermediary mag has to be introduced between the scanner and the microscope. This results in the image being magnified up to 4x on the camera chip, resulting in up to 16x the amount of pixels per unit area and hence 16x the read noise. As read noise is dominant in live cell imaging applications, this results in a ~10x reduction in S2N when compared to the VT-iSIM implementation, and
2. µLens arrays are designed to collect light, whether that's for telecoms, camera's or microscope based applications. Placing the re-assignment µLens in-between the pin holes and the sample, results in out-of-focus light being collected by the µLens' and focused through the pin holes, resulting in an even further increase in the background signal.

However, such implementations operate as a simple camera based imaging system; simply focus a fluorescent sample, set laser power and camera exposure and collect super-resolution images.

Summary

In summary therefore; Image Scanning Microscopy (ISM) has delivered Super-Resolution Imaging to the research masses, due to their simple operation and limited requirements for advanced sample preparation. I would be so bold to say, that ISM is now the most common form of Super-Resolution Imaging adopted by life science laboratories globally… and their numbers are still increasing.