Introduction to SIM and iSIM

Introduction

One of the goals of biological microscopy is to observe and analyze biological processes and structures on the sub-cellular scale. However, the size of the smallest structures that can be observed is set by the diffraction limit of light, meaning no detail can be resolved smaller than around 250 nm. Overcoming this barrier is one of the most active subjects of investigation in modern microscopy and as a result, there are many techniques for super-resolution microscopy.

Many of these techniques require lengthy multi-frame acquisitions (such as PALM/STORM/DNA-PAINT) or rastering of high powered lasers (STED), meaning they require significant post-processing and are largely unsuitable for imaging live cells.

Structured Illumination Microscopy (SIM) and the high-speed alternative instant SIM (iSIM), can halve the resolution limit of conventional light microscopy. However, unlike other super-resolution methods, iSIM can capture images at up to 100Hz, and with a greater degree of optical sectioning (rejection of out-of-focus light). This enables iSIM to generate 3D + time super-resolved images deep into biological structures, all while using conventional fluorescent dyes and molecules. This opens up the possibility of observing sub-diffraction limited dynamic processes within cells and tissues that may previously not have been possible to visualize.

A resolution comparison of iSIM to other live-cell imaging techniques is shown in Figure 1.

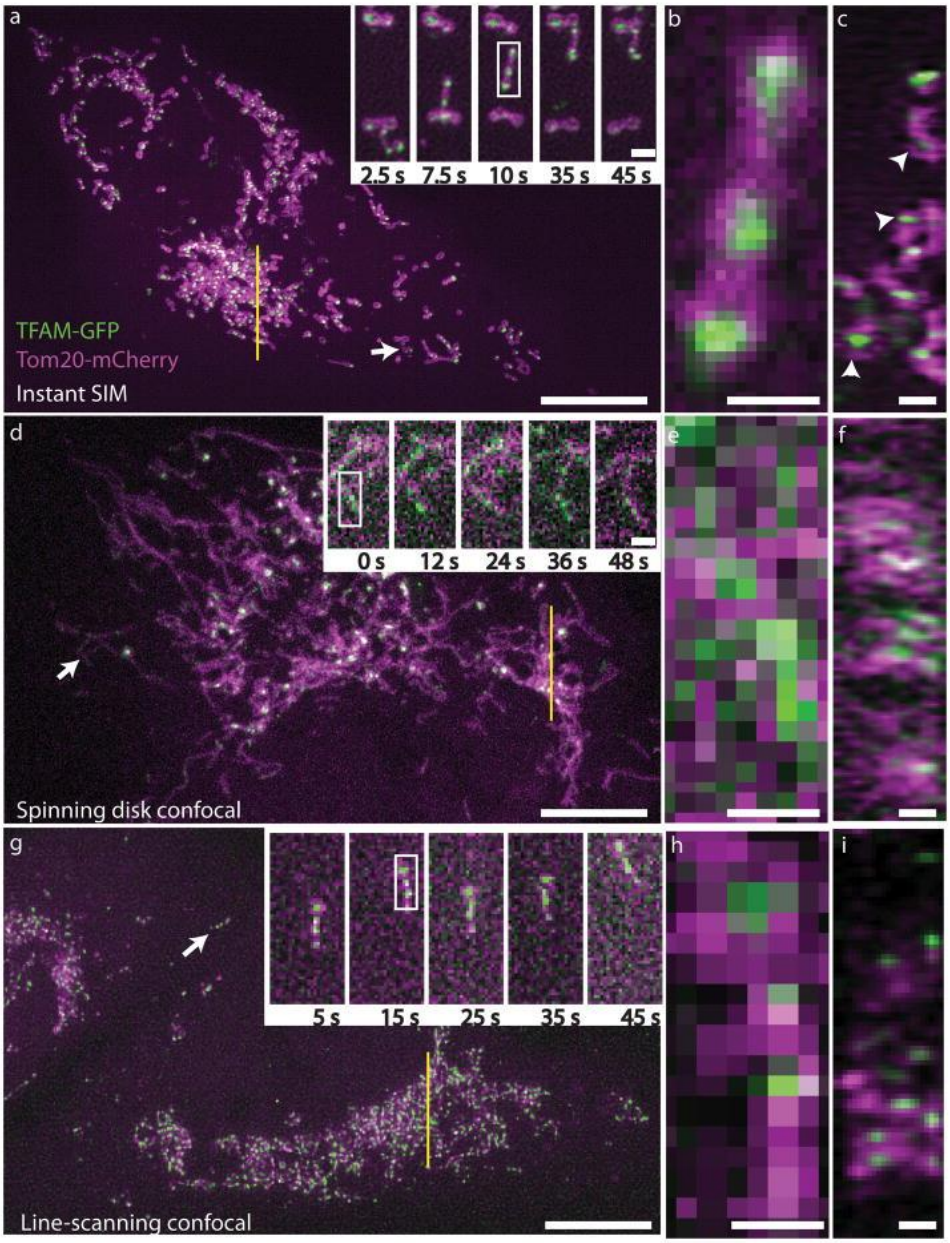

Figure 1: Live MRL-TR transformed human lung fibroblasts expressing TFAM-GFP (green) and Tom20-mCherry (purple).
Images **a-c** are captured using the iSIM system, **d-f** with a spinning disk confocal microscope, and **g-I** with a line-scanning confocal microscope.
a, d, g: Maximum intensity projections (XY) of 3 µm thick volumes are shown, with sub regions (white arrows) at indicated timepoints shown in higher magnification insets.
b, e, h: Higher magnification of mitochondria highlighted by white rectangles in **a, d, g** insets. Scalebars: 0.5 µm.
c, f, i: Axial (ZY) view of ~270 nm thick slices (yellow lines) from **a, d, g,** with scale bars 1 µm.

The resolution challenge of this sample is to resolve the absence of Tom20-mCherry in internal voids with the mitochondria. This is only achieved with iSIM indicated in **c.** by white arrows.

Image and caption adapted from York *et al.*, 2013.

Structured Illumination Microscopy (SIM)

The ability of SIM to achieve its resolution increase relies on the principle of Moiré patterns – an effect frequently utilized for optical illusions.

An intuitive understanding of how Moiré patterns can be used to reveal information of a higher spatial frequency can be gained from Figure 2. As the two fine (high spatial frequency) grids are overlaid in the rightmost image, the interference reveals a grid of a larger size (lower spatial frequency), that contains information about the two smaller grids.

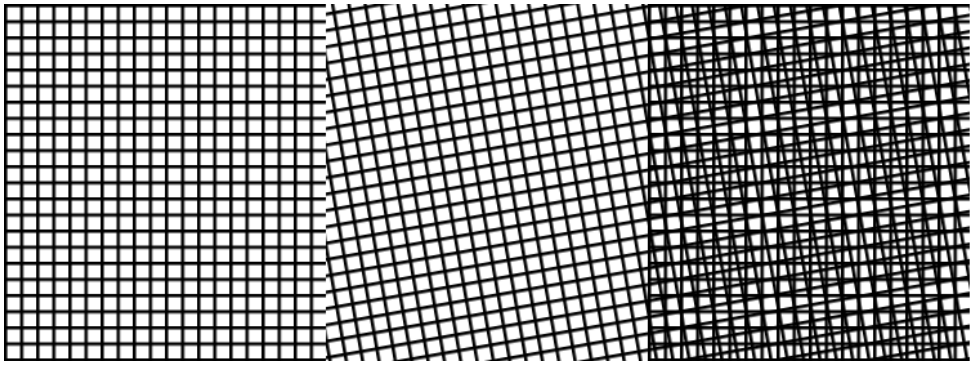

Figure 2: Moiré pattern formed through the interference of two fine grids overlaid at an angle relative to each other.
Adapted from Wikimedia Commons.

By applying illumination patterned with lines of sinusoidally-varying brightness, structures in the sample interfere with the grid and reveal additional spatial information. Through acquiring multiple images, shifting and rotating that pattern to cover every visible area of the sample, a single super-resolution image can be reconstructed, achieving double the spatial resolution that the optical system is capable of.

This process is understandably slow as it requires the digital combination and summation of multiple images but it's considerably faster than super-resolution localization microscopy techniques.

iSIM allows for even greater speed increases, on the order of 100x better time resolution than super-resolution localization microscopy techniques. When considering the addition of processing time, acquisitions can be created on the order of 10,000 times faster. iSIM also allows for 10x deeper sample penetration compared to other SIM-based approaches, and with better optical sectioning than a spinning disk confocal.

iSIM achieves this by building on multifocal SIM (MSIM), a technique which combines pinhole-based optical sectioning and structured illumination to create a confocal super-resolved image. Instead of performing the combination and summation of multiple images digitally, iSIM achieves its speed by performing this summation optically through the use of micro-optics arrays and galvo-scanning mirrors.

iSIM: How Live Super-resolved Images Are Obtained

iSIM expands on a variant of SIM called 'multifocal SIM' that uses multiple sparse individual points of light, rather than lines (Figure 3).

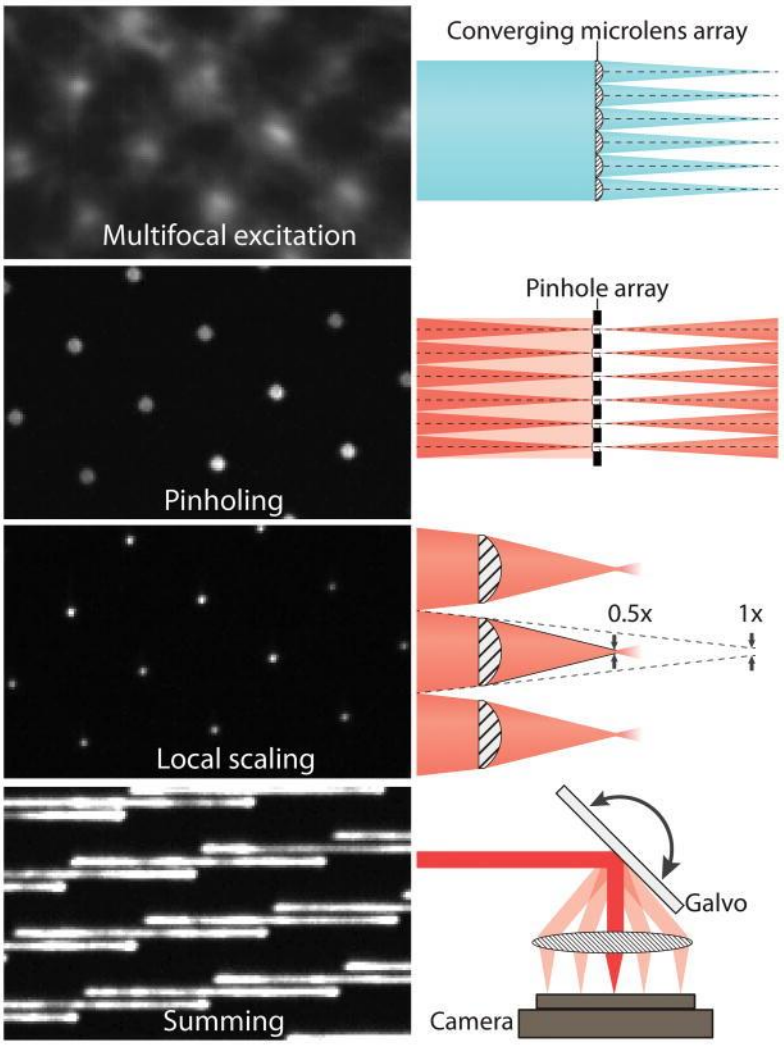

Figure 3: A converging microlens array is used to produce a multifocal excitation. After exciting the sample, out-of-focus fluorescence is rejected with a pinhole array that is matched to the microlens array. A 2x local contraction of each pinholed fluorescence emission is achieved with the aid of a second, matched microlens array. A galvo serves to raster multifocal excitation and sum multifocal emission, producing a super-resolution image during each camera exposure (for clarity, only a partial galvo scan is shown in this figure).
Left Raw data corresponding to each of these steps.
Right Cartoon representation.
Taken from York *et al.*, 2013;

The focal points are formed with microlenses and are combined with the use of pinholes on the emission path which provide optical sectioning (rejection of out of focus light). Next, a microlens array provides 2x optical contraction of the

image from each illumination focus point individually. To image the entire sample, a galvanometric mirror translates the excitation pattern across the sample.

Where iSIM differs from its predecessor MSIM is that the summing of images from multiple mirror positions is done optically rather than in software, improving speed and reducing the contribution of camera noise. As the pattern scans across the sample the emission light is simultaneously translated across the camera and due to the ongoing exposure of the camera during scanning, effectively sums the contributions from the points along the scan.

The final step is to perform deconvolution on the acquired image to reduce the contribution of the point spread function (PSF) of the optical system. Deconvolution is very powerful with iSIM, due to the small PSF and good optical sectioning. This leads to the overall 2x increase in resolution.

The process of building an iSIM system is thoroughly detailed in Curd *et al.*, 2015.

iSIM Applications

The endoplasmic reticulum (ER) moves and grows very rapidly, with the formation and growth of new ER tubules occurring on timescales of the order of 100 ms, which would be blurred with other SIM implementations, yet not resolvable spatially without a super-resolution technique. Figure 4 shows that iSIM is capable of capturing this challenging phenomenon very clearly.

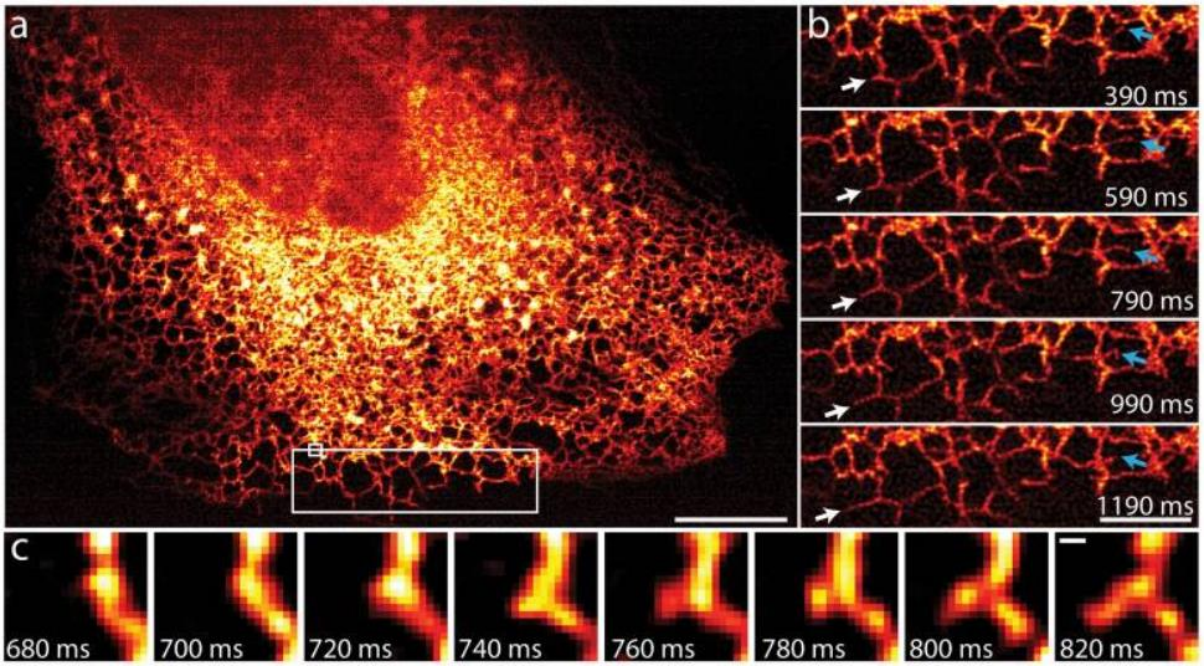

Figure 4: Instant SIM reveals endoplasmic reticulum (ER) dynamics at 100Hz.
a: First image in 200 timepoint series, showing ER labelled with GFP-Sec61A within MRL- TR transformed human lung fibroblasts. Data were acquired at the coverslip surface. Scalebar: 10 µm.
b: Higher magnification view of the large white rectangle in a. White arrows mark growth of an ER tubule, blue arrows indicate remodelling of an ER tubule. Scalebar: 5 µm.
c: Higher magnification view of the small white rectangle in a, indicating formation of a new tubule within 140 ms. Scale bar: 200 nm.
Image and caption adapted from York *et al.*, 2013.

iSIM was also successfully used to resolve the structures of red blood cells moving with the blood vessels of a living 3-day-old zebrafish, without motion blur. Figure 5 demonstrates the capability of iSIM to image moving structures non-invasively within living subjects.

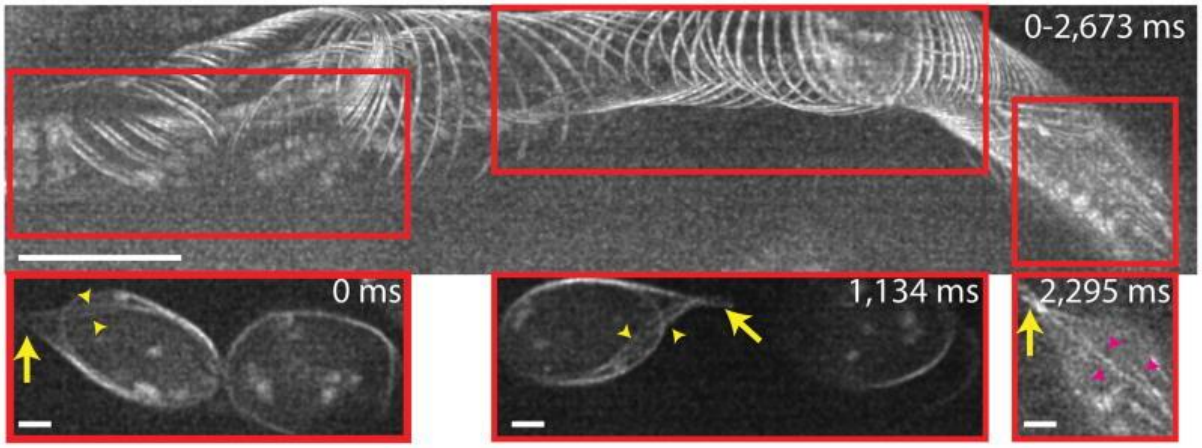

Figure 5: *Top* Maximum intensity projection across 100 2D frames (spanning 2673 ms), highlighting GFP labeled microtubules in blood cells located within cranial vessels, 20 µm into a 3 day old zebrafish embryo. Direction of motion is from left to right. Sharp cell boundaries indicate the absence of motion blur. Scale bar: 10 µm. *Bottom* Spatial regions corresponding to the red boxes in the top panel at indicated times. **Yellow arrows:** 'Tail' structure at the end of the same cell; **Yellow arrowheads:** Microtubules feeding into tail; **Magenta arrowheads:** microtubules present inside the cell. Scalebars: 2 µm. Image and caption from York *et al.*, 2013.

Cameras for SIM and iSIM

SIM and iSIM are well suited to the large field of view, high speed and smaller pixels present on sCMOS devices which excel at imaging live cells at high spatial and temporal resolutions.

Back-illuminated CMOS cameras should also be a strong consideration as they provide the extra sensitivity necessary to lower exposure times and thereby increase acquisition speed. The clean, pattern-free backgrounds present on back-illuminated CMOS cameras are also of great value for SIM and iSIM which rely on creating patterns to achieve super-resolution imaging.

References

Curd, A., Cleasby, A., Makowska, K., York, A., Shroff, H., & Peckham, M. (2015). **Construction of an instant structured illumination microscope**. *Methods*, *88*, 37–47. https://doi.org/10.1016/j.ymeth.2015.07.012

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