# Live Cell Super-Resolution Imaging with N-SIM

**Christopher B. O'Connell**
Nikon Instruments Inc., Melville, NY 11747
coconnell@nikon.net



## Introduction

The ability to visualize the distributions of specific proteins with a light microscope and fluorescent probes is largely responsible for our current understanding of cellular structure. A major limitation of this approach arises from the blurring effects of diffraction, which decreases resolution and limits the ability to obtain information at the nanoscale. There has been a tremendous drive to develop optical and computational methods that improve the resolution of the light microscope, and structured illumination microscopy (SIM) is one solution. This method uses patterned illumination to double both lateral and axial resolution. Nikon's N-SIM is a commercial system that integrates the most desirable features of light microscopy, specific labeling of molecules, and live cell imaging, with structured illumination. This provides the ability to achieve super resolution suitable for a range of biological applications.

At the heart of the system is the N-SIM illuminator, which is based on technology originally developed by Gustafsson and colleagues [1] and licensed from The University of California, San Francisco. This is integrated with a Nikon Ti-E PFS inverted research microscope and a highly sensitive, back-illuminated, electron multiplying charge coupled device (EMCCD, Model DU-897, Andor Technology) (Figure 1). N-SIM reconstructs super-resolution fluorescence images from structured illumination information of the fluorophores in a sample. Several modalities are standard, including 2D-SIM [1], 3D-SIM [2], TIRF-SIM [3], and multi-channel SIM [4]. In particular, the speed with which N-SIM can acquire images in these modes is a major development that makes the system well suited to live cell super-resolution imaging applications.

## Principles of N-SIM Operation

The range of spatial frequencies transmitted by a microscope defines the limit of resolution. Light rays diffracted by the smallest structures have the highest spatial frequencies and require higher numerical apertures (NA) to be collected. With standard objectives, cover glass, and immersion oil, the modulation transfer function (MTF) cutoff results in a limit of resolution ~200–250 nm in $x$ and ~800 nm in $z$. The principle of SIM is to shift high spatial frequency information, which would normally not be collected, down to a frequency that falls within the NA of the objective. This is accomplished using a striped illumination pattern to intentionally introduce optical artifacts called moiré fringes (Figure 2A). These fringes are a manifestation of the higher spatial frequencies. Higher-resolution information is now, therefore, contained within the raw images. The pattern is applied to the sample over three angles and shifted laterally in three or more phases. These steps are necessary to accurately reconstruct a super-resolution image [1, 2]. Nikon's NIS Elements software is able to extract information regarding all of the spatial frequencies present in the images and recombines it into an extended-resolution image. The effects of patterned illumination are clearly seen by examination of the Fourier transforms from conventional widefield and N-SIM images (Figure 2B). The Fourier transform is a reasonable proxy for the modulation transfer function of the microscope; information transferred at higher frequencies is indicated as intensity further from the FFT center. From this, we have an effective means of evaluating the increased frequency response of the system. The diameter of the N-SIM Fourier transform is approximately double, demonstrating that N-SIM provides a two-fold improvement of resolution, which can be extended into all three dimensions.

Three modes of operation of N-SIM allow the system to be fine-tuned for a particular application. The TIRF and 2D-SIM modes use a two-dimensional striped illumination pattern (Figure 2C, left). This is generated by passing the beam through a diffraction grating. Only the resulting +1 and −1 orders are allowed to pass and interfere in the sample plane to generate the pattern. Use of TIRF mode limits penetration depth [3] but gives improved contrast and the highest resolution possible in the N-SIM system (to 85 nm, depending on wavelength). 2D-SIM mode is suitable for thin to moderately thick samples (~3–5 μm thickness). 3D-SIM mode operates using a slightly different principle. Here, the addition of the 0 order diffracted beam results in a three-dimensional, honeycombed illumination pattern that allows the contribution of out-of-focus light to be taken into consideration [2] (Figure 2C, right). This modality is best suited for thicker samples, which typically have higher levels of background fluorescence. Switching from the 100× 1.49 NA objective lens to the 60× 1.27 NA water immersion lens allows the patterned illumination to be projected deeper into samples, up to 20 μm.

## Applications of N-SIM

Using N-SIM, a demonstration has been made of the improved resolution resulting from structured illumination

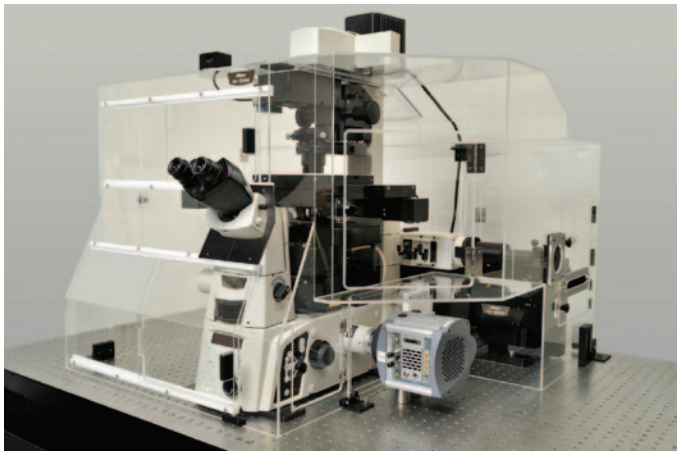

**Figure 1:** The N-SIM super-resolution microscopy system built around a Nikon Ti-E inverted microscope.

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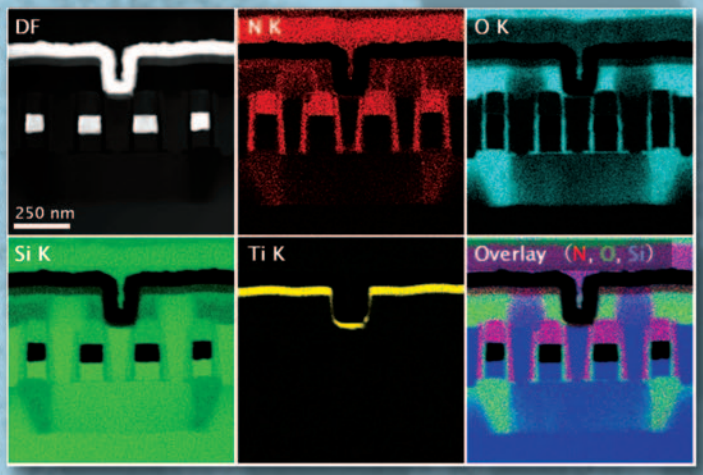


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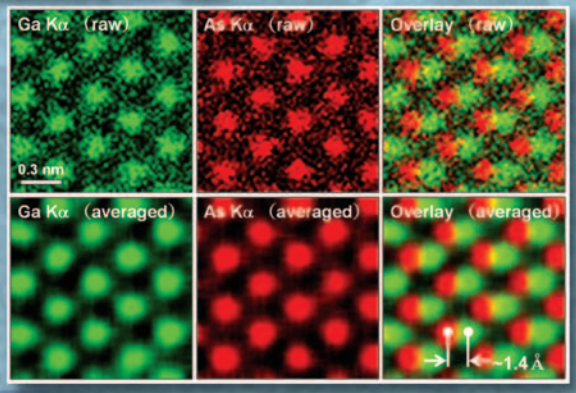


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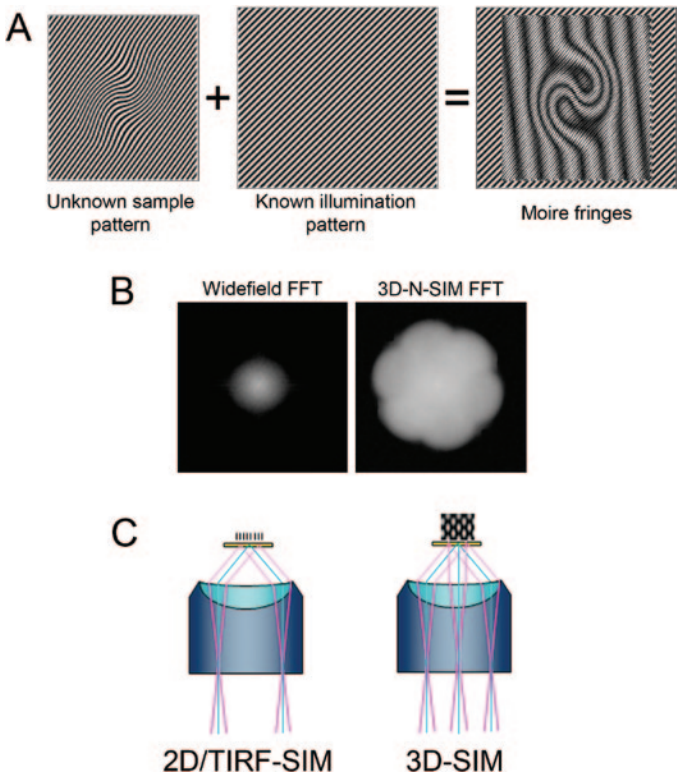


**Figure 2:** Illumination pattern and extension of the frequency response. (A) A sample with unknown structure, including a mix of spatial frequencies, is overlayed with a known, striped illumination pattern generated by the N-SIM illuminator. The resulting constructive and destructive interference results in the appearance of moiré fringes. These fringes contain the higher spatial frequencies shifted down to a frequency below the MTF cutoff and are, therefore, collected. (B) Fast Fourier transforms (FFTs) of images collected using conventional widefield fluorescence and 3D-N-SIM. Transforms were made using ImageJ software. After applying the pattern over a number of phases and angles, an enlarged reciprocal space is constructed based on the additional information present from the moiré fringes [1, 3]. The larger FFT of N-SIM is a result of the additional, high spatial frequency information. (C) 2D and TIRF SIM use a two-beam illumination pattern to generate a 2D illumination pattern, while 3D SIM adds an additional beam to make a 3D pattern. Images in (A): Copyright 2005 *National Academy of Sciences, U.S.A.*

by imaging structures that are just below the diffraction limit of a conventional fluorescence microscope [5]. Kinetochores, the organelles connecting chromosomes to the microtubule-based spindle during cell division, typically appear as round or oval spots with no substructure. Still, it is clear from electron microscopy that these ~200 nm long organelles are very heterogeneous in their morphology. This substructure is revealed for the first time with optical microscopy by N-SIM structured illumination (Figure 3A), opening the door to live cell studies seeking to probe the structural rearrangements within this organelle.

The relative arrangement of two or more proteins is of particular interest in many areas of biological research. The combination of multi-channel imaging with the two-fold improvement in resolution provides the opportunity to gain new insight into cellular structure. N-SIM imaging of differentiated mouse tracheal cells reveals a hole in the distribution of Chibby (Figure 3B), a protein found at the base of cilia [7]. The diameter of these holes, as measured in N-SIM reconstructions, is often below the diffraction limit (120–250 nm) of a conventional microscope (Figure 3C-D). The N-SIM microscope supports super-resolution imaging of multiple fluorophores through sequential acquisition. Seven laser lines are available, ranging from 405–640 nm, to give extensive flexibility for multi-channel imaging.

Most biological processes are dynamic, and their examination benefits greatly from analysis of living cells. Structured illumination is the super-resolution method best suited for this application. It can be used with standard fluorescent labels and requires no special imaging conditions or buffers.

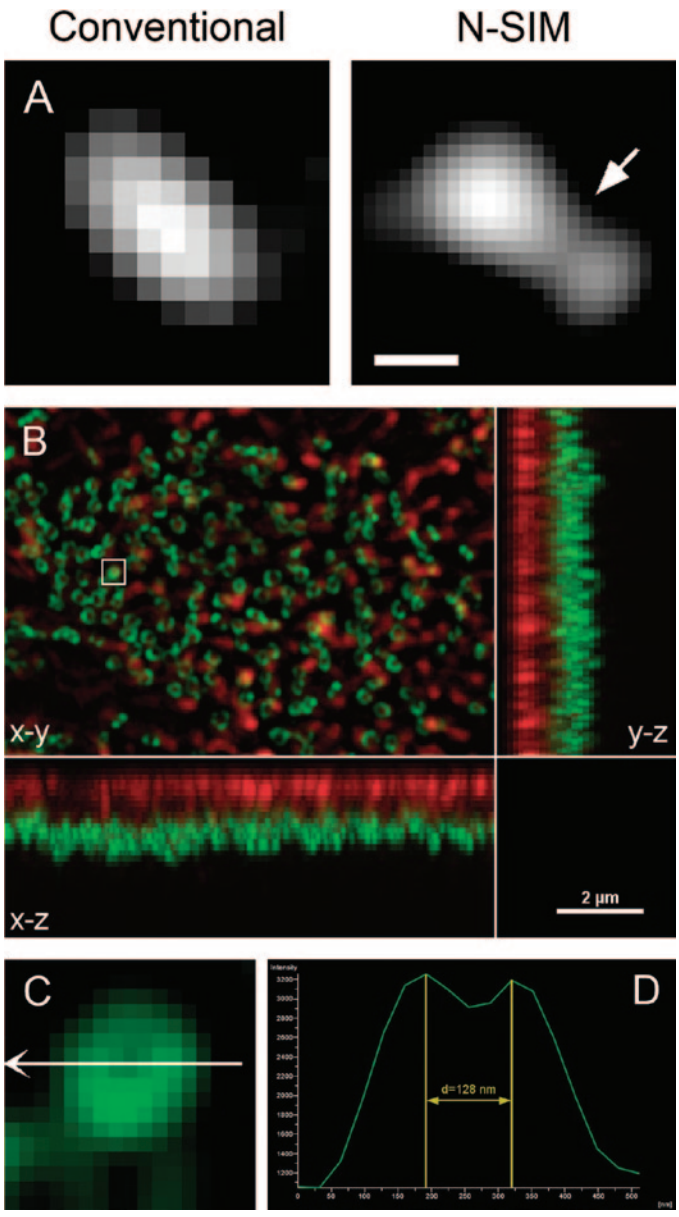


**Figure 3:** Applications of N-SIM. (A) A single human kinetochore labeled via immunofluorescence is shown with conventional optics and N-SIM. Note the substructure present in the super-resolution image (arrow) [6]. (B) Differentiated mouse tracheal cells grown on filter supports, fixed, and stained with primary antibodies to label Chibby (green) or acetylated alpha-tubulin (red). Maximum intensity *x–y*, *x–z*, and *y–z* projections are shown. (C) Enlargement of the Chibby ring from the boxed region in (B). (D) A line profile through the ring (arrow in C) demonstrates a peak–to-peak distance of 128 nm. This is well below the diffraction limit of a conventional microscope. Sample provided by Ken-Ichi Takemaru, Stony Brook University. Bar in (A), 0.25 µm.

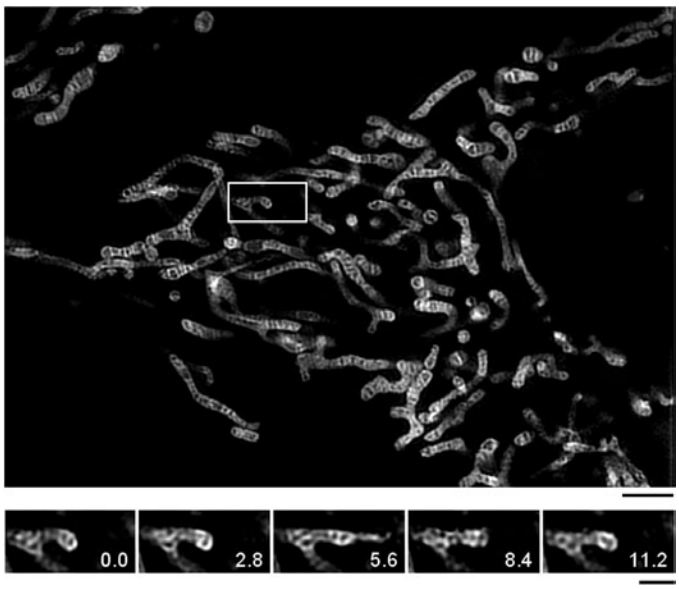


**Figure 4:** Live cell super-resolution imaging using N-SIM. HeLa cell labeled with MitoTracker Red, imaged using single-layer 3D-N-SIM at 561 nm. An enlargement of the inset is presented in the bottom panel, showing a time-lapse series of mitochondrial dynamics. A membrane protrusion is observed to extend at 5.6 seconds. Each frame is taken from a reconstruction generated from fifteen images collected for a given interval. The cristae, or internal folds, are now apparent because of the increased resolution of this system. Time shown is seconds. Scale bar for top image, 2.5 µm. Scale bar for bottom panel, 1 µm.

However, two important considerations are the required temporal resolution and the speed of acquisition. Images of the illumination pattern as it is applied in all of the angles and phases must be collected to fully reconstruct reciprocal space. For the TIRF-SIM and 2D-SIM modes, three angles and three phases are collected [1, 3] for a total of nine images per *z* plane. The N-SIM illuminator rapidly rotates and translates the pattern, such that all nine images can maximally be acquired in 600 ms. This rate is sufficient for imaging all but the most rapid biological processes. For thicker specimens, single-layer 3D-SIM provides a good compromise that can remove out-of-focus light while still acquiring images at a rate supporting live cell work. In this mode, a super-resolution reconstruction is made from a total of fifteen images encompassing three angles and five phases. Use of the single-layer 3D-SIM mode can be advantageous because it requires only a single *z* plane to reconstruct the super-resolution image, instead of the three *z* slices required with methods that depend on information from the planes above and below. All fifteen images can be acquired in approximately one second with this modality. An example of live cell imaging with the N-SIM system is shown in Figure 4. These time-lapse data were acquired using the single-layer 3D mode of a HeLa cell stained with MitoTracker Red (Invitrogen). Of particular note is the clear visualization of mitochondrial christae, which previously could only be seen using electron microscopy.

## Conclusions

Structured illumination and the N-SIM system are powerful tools that double the resolution of the light microscope. The range of labels, multiple modalities, and live cell imaging capability preserve the best features of classic fluorescence microscopy approaches while adding super-resolution capabilities. The modularity of the components is such that N-SIM can be integrated onto the same microscope with confocal imaging and stochastic optical reconstruction microscopy (N-STORM) for additional functionality. Such a range of modalities will facilitate correlation of structures across scales, an area of great importance to the biomedical imaging community.

## References

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https://doi.org/10.1017/S1551929512000375 Published online by Cambridge University Press