N-STORM with DNA-PAINT for Reliable Multicolor & 3D Single Molecule Localization Imaging

New DNA-PAINT immunolabeling reagents available from Ultivue Inc. enables photobleach-free single molecule localization imaging using the Nikon N-STORM 4.0 super-resolution microscope. Multicolor and 3D imaging with a depth of more than 5 µm is demonstrated here.

What is DNA-PAINT?

The past decade has seen the introduction of super-resolution STORM[1] and similar single molecule localization microscopies for nanoscale imaging. These techniques generally rely upon the photochemical manipulation of fluorophores to induce long-lived non-fluorescent 'dark' states, allowing the emission profiles of non-overlapping single emitters to be identified. In contrast, Point Accumulation for Imaging in Nanoscale Topography (**PAINT**) is a set of techniques that utilizes short-lived reversible binding of fluorophores to localize single molecules[2]. The advantage of this approach is that it virtually negates the effects of photobleaching as fatigued fluorophores are continuously replaced, allowing each molecule of interest to be sampled a theoretically unlimited number of times.

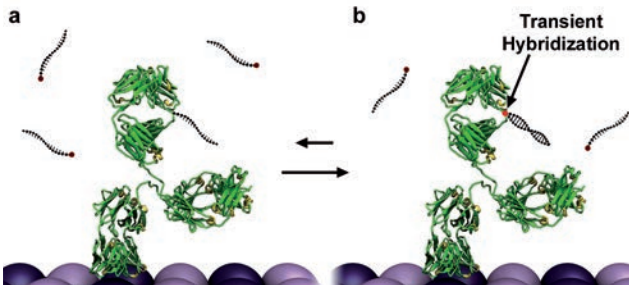

Figure 1. Illustration of the DNA-PAINT Concept. Imager strands reversibly bind to docking strands on the secondary antibody with short on-times. Although fluorescence is excited from imager strands free in solution, immobilization of a fluorophore allows for a much greater amount of signal to be integrated when average binding on-time is synced to camera exposure, usually about 25-40 milliseconds per frame.

DNA-PAINT[3] is a variation of PAINT utilizing conventional primary-secondary antibody immunolabeling techniques. However, instead of a fluorophore, the secondary antibody is conjugated to short single-stranded DNA oligomers known as **docking strands**. The imaging buffer contains complementary oligomers, known as **imager strands**, labeled with fluorophore. Imager strands demonstrate binding on-times in the tens of milliseconds, during which time the observed fluorescence is greatly enhanced over the background as the fluorophore becomes temporarily immobilized (Figure 1), ideal for single molecule localization.

Complete DNA-PAINT reagent kits, including secondary antibodies, imager strands, and buffers, are commercially available from Ultivue Inc. (www.ultivue.com), providing a convenient and consistent solution.

N-STORM 4.0 for PAINT Imaging

DNA-PAINT can be performed on the Nikon N-STORM 4.0 without alteration to the microscope, requiring only minor changes to standard immunolabeling protocols. DNA-PAINT kits are provided with both goat anti-mouse and goat anti-rabbit secondary antibodies, each with a unique docking strand sequence. This allows for the application of imager strands labeled with different fluorophores for simultaneous imaging, or with the same fluorophore for sequential imaging (Exchange-PAINT[3]). Note that using different imager strands with the same fluorophore requires washing one set out before labeling with the other.

Multi-Color DNA-PAINT

Here we demonstrate wash-free simultaneous two-color DNA-PAINT imaging with spectrally distinct fluorophores. Up to three color channels are available on the N-STORM 4.0, allowing for imaging up to three colors, or imaging of two

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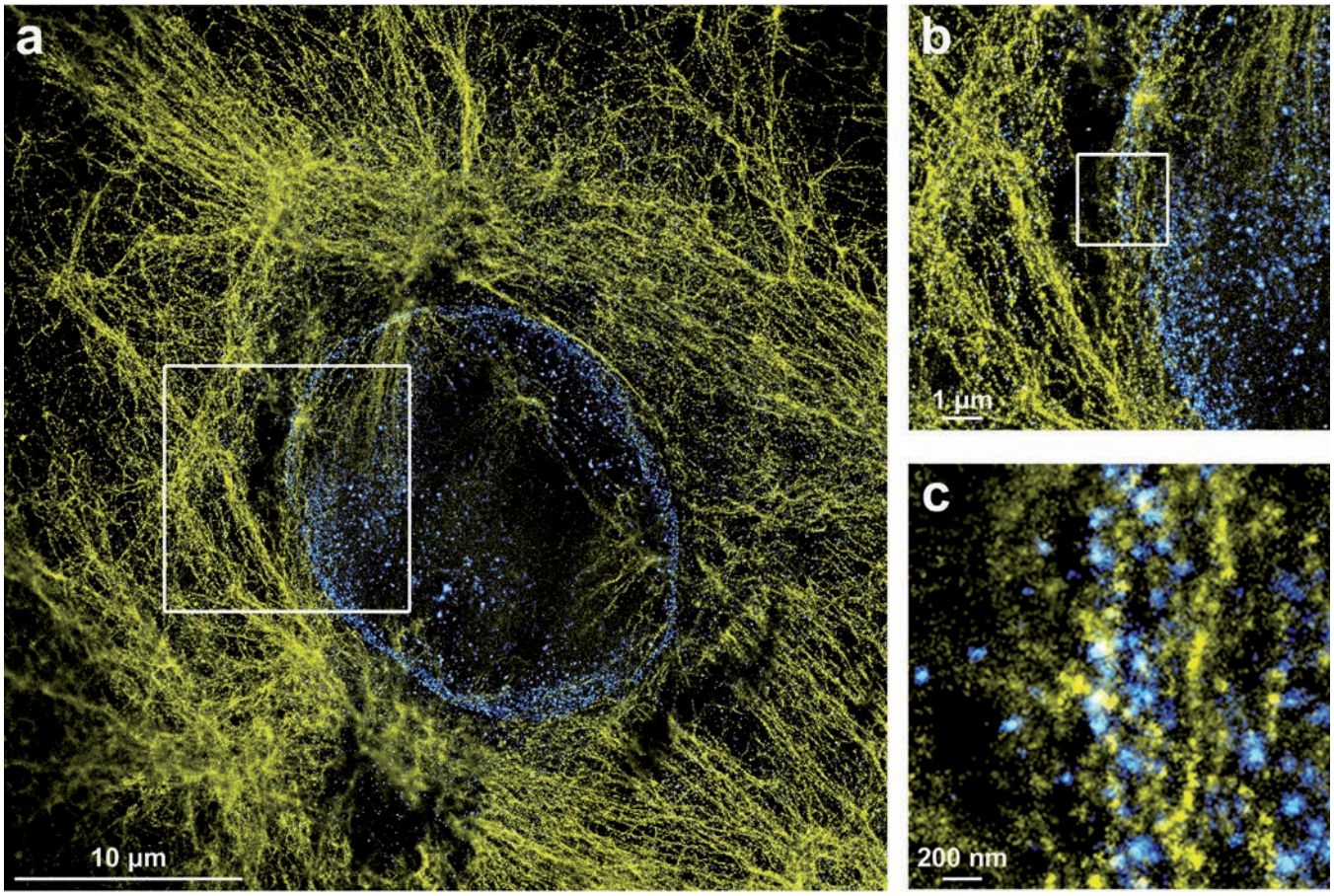

Figure 2. Two-color DNA-PAINT of Vimentin (yellow) and LaminB1 (cyan). This two color single plane DNA-PAINT image was acquired using the Nikon N-STORM 4.0 with oblique illumination. Imaging was performed at a rate of 40 ms per frame for both channels. CV-1 cells were stained with mouse anti-vimentin and rabbit anti-lamin B1 antibodies. Goat anti-mouse and anti-rabbit secondary antibodies conjugated to docking strands were provided by Ultivue Inc. Corresponding imager strands conjugated to red fluorophore ($564_{ex}/590_{em}$, vimentin) and far-red fluorophore ($646_{ex}/664_{em}$, lamin B1) were added to imaging buffer at a concentration of 1 nM. Fluorescence was excited using 561nm and 647 nm laser lines and detected using a Hamamatsu ORCA-Flash 4.0 V2 sCMOS camera. Drift correction was performed using by tracking fiducial markers (0.04 µm green fluorescent FluoSpheres®) excited by a 488 nm laser line. More than 40 million localizations are represented in this reconstruction. (a) Whole image (256 x 256 pixels) (b) zoomed-in region denoted by the box in (a). (c) zoomed-in region denoted by box in (b).

colors with the third color channel for tracking fiducial markers for drift correction, as in Figure 2.

Multi-color DNA-PAINT has several inherent advantages over multi-color dSTORM. DNA-PAINT probes exhibit similar brightness in all tested color channels – providing consistent resolution performance in each channel and competitive with Alexa Fluor 647, the current standard in the field. Multi-color dSTORM typically relies upon fluorophores with markedly reduced performance for imaging in the green (e.g. Atto 488) and red (e.g. Cy3B) channels. Localization precision when using DNA-PAINT is dependent on the binding kinetics of the imager strand and the brightness of the fluorescent probe, not requiring specialized and short-lived (1-2 hours) reducing/oxidizing buffers. Imager strands are simply diluted in the provided salt buffer – with the resultant imaging buffer good for up to several days at room temperature and with very little drop-off in the number of molecules localized in each frame during that time.

3D DNA-PAINT

The N-STORM 4.0 provides a proprietary z-stepping mechanism for single molecule imaging using Nikon's Perfect Focus System. Single molecule z-stacks 5+ µm thick can be acquired with high quality. Enhanced signal to noise and background suppression is provided through a specialized combination of TIRF and oblique illumination, where the angle of illumination changes with imaging depth, allowing for more selective excitation of the z plane of interest. Drift correction for the data in Figure 3 was performed using image-based auto-correlation analysis.

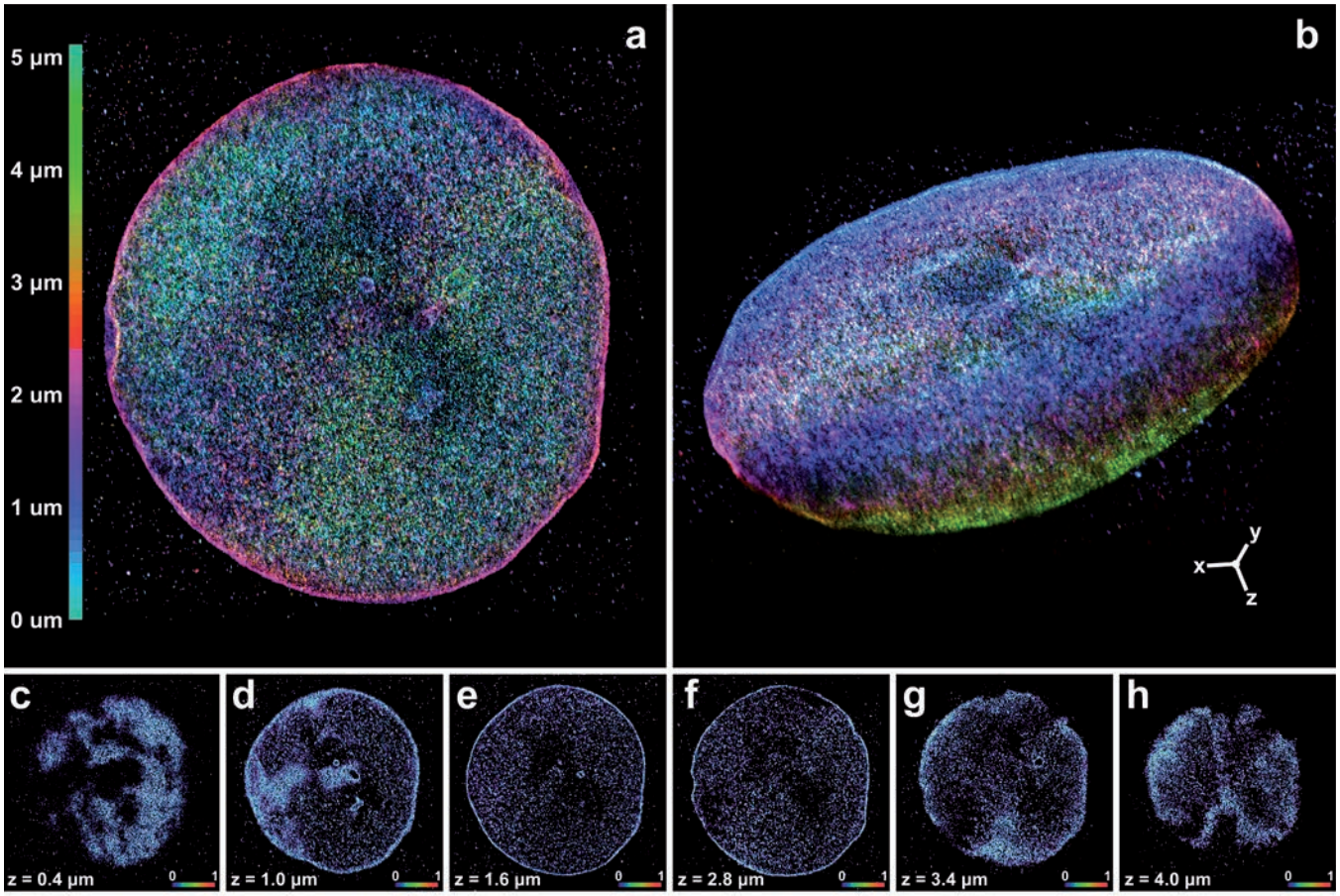

Figure 3. 3D DNA-PAINT image of the nuclear lamina protein lamin A/C in a fixed CV-1 cell acquired using the Nikon N-STORM 4.0 system. This 5.1 um deep z-stack was acquired using 3D astigmatism-based single molecule localization and z-stepping using Nikon's Perfect Focus System in combination with variable-angle oblique illumination for high signal to noise imaging. Imaging was performed at a rate of 30 ms per frame, 15,000 frames per z plane, and 51 z planes acquired at 100 nm intervals. Lamin A/C primary antibodies were labeled using goat anti-rabbit secondary antibodies conjugated to docking strands. Fluorescence was excited using a 647 nm laser line and detected by a Hamamatsu ORCA-Flash 4.0 V2 sCMOS camera. Drift correction was performed using image-based auto-correlation. More than 3.8 million localizations are represented in the reconstruction. (a) 2D image with color-coded z position. (b) 3D volume rendering. (c-h) Representative individual z planes, depth denoted in bottom left of each panel.

While z-stacking can be performed using conventional STORM labels and buffers on the N-STORM 4.0, the DNA-PAINT approach provides improved performance as the user no longer has to balance photobleaching (occurring throughout the sample volume) with the acquisition period for each z position.

Outlook

DNA-PAINT probes from Ultivue Inc. provide a convenient and standardized method for performing high quality 3D and multicolor single molecule imaging on Nikon's N-STORM 4.0 system, and with conventional Immunolabeling techniques. Combination of DNA-PAINT probes with other dyes validated for PAINT imaging allows for potential three-color single molecule imaging, and in 3D. We also expect the future development of a wider variety of DNA-PAINT probes to continue to expand the number of potential applications for this technique.

References

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2. Sharonov, A. and Hochstrasser, R.M. Wide-field subdiffraction imaging by accumulated binding of diffusing probes. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 18911-18916 (2006).
3. Jungmann, R., Avendano, M.S., Woehrstein, J.B., *et al.* Multiplexed 3D cellular super-resolution imaging with DNA-PAINT and Exchange-PAINT. *Nature Methods* **11**, 313-318 (2014).