Quantitative Cluster Analysis Applications for N-STORM

Optical super-resolution techniques such as Stochastic Optical Reconstruction Microscopy[1] (STORM) enable the visualization of sub-cellular structures below the diffraction limit, and with high molecular specificity. In this application note we highlight the work of Ricci *et al.*[2], who have applied STORM microscopy towards probing the distribution of nucleosomes on chromatin fibers, helping to elucidate mechanisms of chromatin folding, gene expression, and pluripotency.

Using N-STORM to quantify the nano-scale distribution of nucleosomes in intact cell nuclei

The classic view of nucleosome organization is relatively simple: a regular pattern of nucleosomes and histone H1 along a stretch of DNA and forming a fiber about 10 nm in diameter and following the 'beads on a string' model. The 10 nm fiber is thought to fold into a fiber about 30 nm in diameter, and to further condense into increasingly higher-ordered structures. However, Ricci *et al.*[2] have applied both single and multi-color STORM imaging to show that nucleosomes organize into heterogeneous nanodomains (**Fig. 1c**), termed 'clutches' in reference to the variably sized egg clutches of birds. Clutch size, composition, and organization are highly variable within and between populations of different cell types.

In this work Nikon's N-STORM system is demonstrated with ~20 nm lateral resolution, an order of magnitude higher than dictated by the diffraction limit, and thus theoretically capable of resolving 30 nm wide chromatin fibers. However, such fibers have been difficult to identify using conventional optical and electron microscopy techniques. Ricci *et al.*[2] calls the strict existence of the 30 nm fiber into question. Conventional optical microscopies cannot resolve such nano-scale features, while electron microscopies

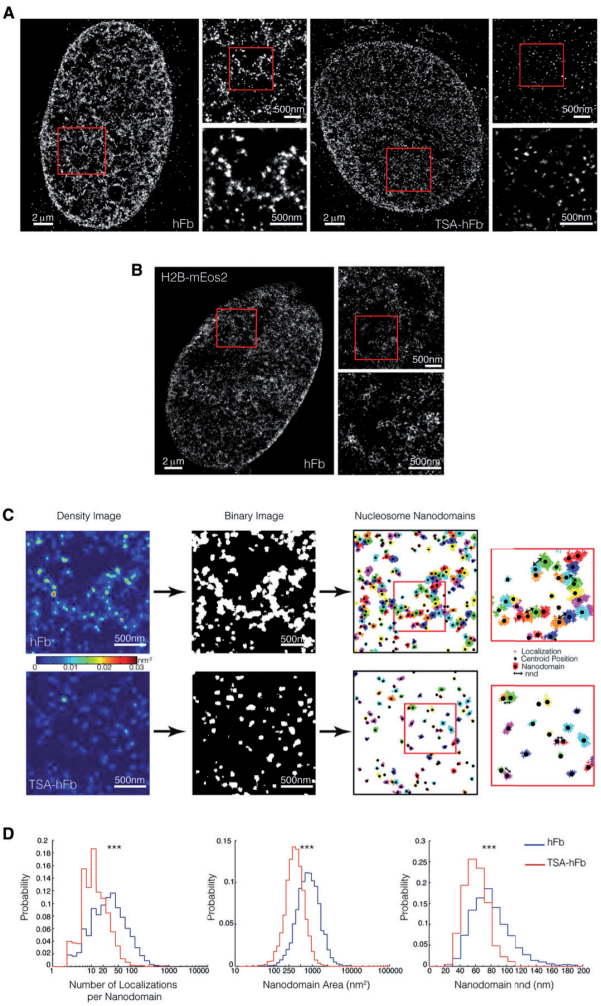

Figure 1. Using N-STORM for cluster analysis of nucleosome clutches. (**a**) Representative single-color 2D STORM image of untreated human fibroblast (hFb) nuclei (left) and Trichostatin A (TSA) treated hFb nuclei (right), both labeled with Alexa Fluor 647. TSA is a histone deacetylase inhibitor and its application results in decondensation of chromatin throughout the genome. Thus untreated nuclei have larger and more densely clustered clutches than treated nuclei. (**b**) Representative live-cell STORM image of H2B-mEos2 in hFb nuclei. (**c**) Cluster analysis workflow for identifying nucleosome clutches and nearest neighbor distances from STORM localization data. Far left is an H2B localization heat map (localizations/nm2), which is converted into a binary image (left), a mask for efficient identification and quantification of clutches using appropriate cluster analysis algorithms (right). (**d**) Probability distributions showing that untreated hFbs tend towards greater number of localizations per nanodomain, larger nanodomain area, and longer nanodomain nnds. Nikon's new N-STORM 4.0 features cluster analysis and distance measurement tools. Figure recreated with permission from ref. 2.

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lack the necessary molecular specificity and require harsh sample preparation techniques. Thus STORM is able to fill a unique imaging niche, bridging the gap between resolution and molecular specificity. The work of Ricci *et al.*[2] marks the first time the organization of nucleosomes in intact nuclei has been resolved.

Using STORM to quantify nucleosome distribution in pluripotent cells

Pluripotent mouse embryonic stem cells (mESCs) were chosen as a model system for evaluating nucleosome clutch size, density, and distribution under several different experimental conditions:

- Treatment with cytokine leukemia inhibitory factor (sLif), a differentiation inhibitor (**Fig 2a, b)**.
- Treatment with inhibitors of Mek and Gsk3 kinases (2iLif), which keeps the cells in a ground state of pluripotency (**Fig. 2c**).
- A Tcf3 double knockout line (mESC$^{Tcf3-/-}$). Tcf3 is an effector of the Wnt/β-Catenin pathway, which helps maintain ground state pluripotency (**Fig. 2d**).
- mESCs differentiated into neural progenitor cells (NPCs), (**Fig. 2e**).
- An H1 'triple knockout': mESCs with deletion of three H1 isoforms (mESC^{H1tKO}), (**Fig. 2f**).

As expected, the number of localizations per nanodomain and their nnds are lower in pluripotent mESCs with respect to mNPCs. Additionally, Ricci *et al.*[2] demonstrate high anti-correlation between nucleosome clutch size and pluripotency in human induced pluripotent stem cells (hiPSCs) using established gene card technology to score pluripotency using five markers: Nanog, TRA1-60, SSEA4, Oct4, and Sox2.

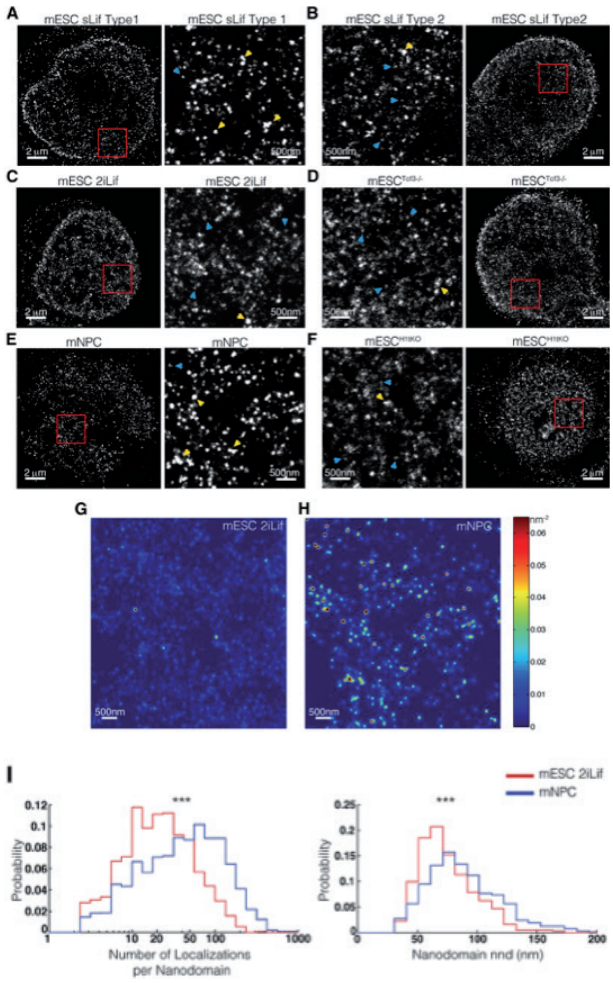

Figure 2. STORM images of H2B distribution in mESCs under several different conditions. mESCs cultured in sLif with low Nanog expression (**a**) and high Nanog expression (**b**). (**c**) mESCs cultured in 2iLif. (**d**) mESCs with Tcf3 double knockout. (**e**) mNPCs derived from mESCs. (**f**) mESCs with H1 triple knockout. (**g**) Heatmap comparing nucleosome density of mESCs culture in 2iLif with differentiated mNPCs (**h**), clearly showing denser clusters with more localizations in the latter. (**i**) Comparison of the number of localizations per nanodomain and nanodomain nnd between mESC 2iLif and mNPC. The more differentiated cell line, mNPC, tends toward greater number of localizations and nanodomain nnd. Figure recreated with permission from ref. 2.

Multicolor STORM imaging of H2B with H1 and RNA Polymerase II in a variety of cell lines

2-channel STORM imaging with Nikon's N-STORM system revealed that RNA Polymerase II preferentially associates with small clutches while histone H1 is enriched in large clutches (**Fig. 3**). This is consistent with the expectation that smaller and less compacted clutches are more open to the binding of transcription factors and polymerases such as PolII. H1 is important in nucleosome compaction and is enriched in heterochromatin. Furthermore H1 is found to be most abundant in the nuclear periphery, where heterochromatin is most enriched.

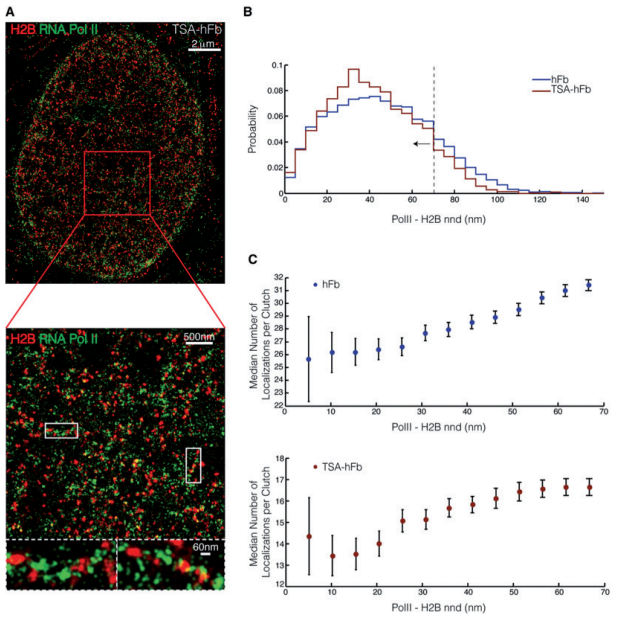

Figure 3. Preferential association of RNA Polymerase II (PolII) with smaller and more disperse nucleosome nanodomains. (**a**) 2-channel STORM image of histone H2B, tandem-labeled with Alexa Fluor 405 and 647, and RNA Polymerase II, labeled with Cy3-Alexa Fluor 647, in human fibroblasts (hFbs) treated with (**b**) Distribution of nearest neighbor distances (nnds) between H2B and PolII in both treated and untreated hFbs, showing TSA-treated cells having slightly smaller nnds. (**c**) Number of single molecule localizations per clutch as a function of nnd for untreated hFbs, showing larger clutch sizes with increasing H2B-PolII nnds. (**d**) Similar results are obtained with TSA-treated hFbs. Figure recreated with permission from ref. 2.

Conclusions

Nikon's N-STORM platform has proven to be a powerful platform for the visualization of multi-protein complexes, as demonstrated by Ricci *et al*. Nikon's new N-STORM 4.0 includes integrated analysis tools for cluster analysis and distance measurements, as well as improved 3D and speed performance. To learn more about Nikon's super-resolution solutions, please visit our website (www.nikoninstruments.com/sr).

1. Rust, M.J., Bates, M. & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **3**, 793–795 (2006).

2. Ricci, M.A., Manzo, C., Garcia-Parajo, M.F., Lakadamyali, M. & Cosma, M.P. Chromatin Fibers Are Formed by Heterogeneous Groups of Nucleosomes In Vivo. *Cell* **160**, 1145-1158 (2015).