



Unlocking sub-nanometer spatial precision: Automated 2D and 3D RESI mapping with Aplo Flow

SUMMARY

Studying critical biological processes such as biomarker interactions, receptor organization, or antibody binding requires super-resolution microscopy capable of nanoscale spatial resolution. However, executing multi-cycle, multiplexed experiments like DNA-PAINT and RESI manually is labor-intensive and introduces variability.

ONi's Aplo Flow and Aplo Scope integrate fluidics with automated super-resolution imaging to enable automated, complex multiplexed imaging workflows. This platform unlocks reproducible, high-throughput imaging at nanometer precision. Here we show:

- Automated and reproducible fluidics reduce active hands-on time and ensure consistent data quality across multiple super-resolution imaging cycles
- Sub-nanometer 2D and 3D RESI imaging can resolve 10 nm-spaced Nup96 subunit pairs and reconstruct the 3D double-ring scaffold of the nuclear pore complex
- Platform flexibility through custom fluidic protocols, adaptable 3D optics, and compatibility with third-party analysis tools.

INTRODUCTION

DNA-PAINT (DNA Point Accumulation in Nanoscale Topography) has transformed super-resolution imaging through its high multiplexing capacity and quantitative precision, enabling target visualization at sub-20 nm resolution. As a Single-Molecule Localization Microscopy (SMLM) technique, DNA-PAINT relies on transient, reversible binding between short fluorescent "imager" strands and target-bound "docking" strands to generate stochastic single-molecule blinking events¹.

The ability to discern two separate targets, known as spatial resolution, can be further enhanced down to the Ångström scale (1 Å = 0.1 nm) using Resolution Enhancement by Sequential Imaging (RESI). By sequentially imaging sparse target subsets, RESI achieves single-protein resolution in intact cells using standard fluorescence microscopy hardware and reagents². This approach is particularly powerful for resolving dense target clusters or studying oligomerization states, such as elucidating the molecular mechanisms of monoclonal therapeutic antibodies in cancer immunotherapy³.

However, multi-cycle workflows like DNA-PAINT and RESI traditionally depend on repetitive manual fluid exchanges between imaging cycles, increasing operational complexity and reducing experimental reproducibility. ONi's Aplo Flow fluidics module automates these workflows in direct sync with the Aplo Scope super-resolution microscope. By executing multi-lane, multi-position runs as an uninterrupted process, the integrated system eliminates manual intervention, ensuring consistent, high-plex datasets across positions while maintaining full flexibility to adjust experimental parameters.

In this application note, we demonstrate how the automated Aplo Scope and Aplo Flow ecosystem streamlines multi-cycle DNA-PAINT and RESI workflows to achieve high-precision 2D and 3D structural mapping of nuclear pore complexes. By automating liquid handling across multi-lane flow chips, the integrated platform eliminates manual intervention, preserving sample stability to yield sub-nanometer localization precision (0.86 nm in 2D, 0.97 nm in 3D). We showcase clear resolution of 10 nm-spaced Nup96 subunit pairs in 2D and the complete double-ring scaffold architecture in 3D, highlighting how seamless synchronization between automated hardware and open-source analysis software delivers highly reproducible single-protein datasets at scale.

METHODS

Sample preparation

Previously described U2OS cells expressing eGFP-NupP96⁴ were seeded into a 4-lane ONI Cell Flow Chip at a concentration of 50,000–150,000 cells/mL. The inlet/outlet and the membrane reservoirs were filled with complete McCoy media (cMcCoy, McCoy basal medium, 10% FBS, 1% Pen/Streptomycin). After 24–48 hours incubation at 37°C, cells were fixed with 2.4% paraformaldehyde for 30 minutes at room temperature (RT) and washed three times with PBS. Fixed cells were permeabilized and blocked to prevent non-specific binding using Ab incubation buffer (Massive Photonics) with 0.25% Triton-X100 for 90 minutes at RT. To stain eGFP-Nup96, cells were incubated overnight at 4°C with 50 nM antiGFP-sdAb docker using the MASSIVE-RESI ANTI-GFP kit (Massive Photonics). Cells were then washed three times with 1 × Wash buffer and incubated with gold nanoparticles for 10 min at RT as fiducials for image alignment, and post-fixed with 2.4% paraformaldehyde for 15 min at RT.

Automated image acquisition and liquid handling

Imaging and liquid exchange for DNA-PAINT and RESI were performed using an ONI Aplo Scope super-resolution microscope with the Aplo Flow liquid handling module, including the pipettor accommodating the input reagents and a Fluidics Driver to enable fluid handling, temperature control, valve switching, and waste management, all automated within smart routines in the Aplo Flow SDK script (Figure 1). The chip was engaged with the fluidics tether, mounted on the Aplo Scope

stage, and primed with 1 mL of imaging buffer across all four lanes. Regions of interest (ROIs) were identified by looking at the GFP signal, and their x, y, and z positions were entered in the Aplo Flow SDK script together with the acquisition parameters (details below).

For each cycle of RESI imaging, lanes were washed with 500 µL wash buffer twice, followed by sequential delivery of 150 µL imaging buffer, then 150 µL imaging buffer containing the imager strand with PCA, PCD, and Trolox supplement. Imager strand concentrations were 400 pM for cycle F1, 300 pM for cycles F2–F4. Following RESI image acquisition, a pooled mixture of all imagers (100 pM each, 400 pM total) was introduced for single-cycle DNA-PAINT imaging.

For image acquisition, illumination was delivered using a 561 nm laser (100 W/cm²) in HILO mode (63–64°) with a 595/44 nm emission filter. RESI images were acquired at 150 ms exposure for 10,000 frames per sequence, while DNA-PAINT acquired 40,000 frames at 150 ms exposure. For 3D astigmatic localization, the standard Aplo Scope objective was swapped for a UAPON100X (Olympus) oil objective, and the collar correction adjusted according to the calibration parameters.

Data analysis

Data processing was performed using Picasso (Localize, Filter, Render) and a custom Python script. Single-molecule localizations were identified in Picasso-Localize (net gradient = 2,000, MLE-integrated Gaussian fit) and filtered ($-400 \text{ nm} < z < 400 \text{ nm}$ and $0.5 \text{ px} < \sigma_x/\sigma_y < 2.5 \text{ px}$). Filtered localization results of sequential images were loaded in Picasso-Render for drift correction using the AIM algorithm anchored by gold nanoparticle fiducials, as well as cycle alignment, RESI analysis, and visualization.

Nuclear Pore Complexes (NPCs, 30–50 initial picks, expanded via ‘pick similar’) were constrained to the central field of view (250–650 px in x, y) to maximize accurate axial localization. RESI analysis was performed in Picasso-Render. For each imaging cycle, localizations were grouped into clusters using a search radius of 12 nm laterally and 25 nm axially. Clusters containing at least 10 localizations were retained, and each was collapsed to a single RESI localization at the mean position of its constituent localizations. Molecular averaging-based images of NPCs were

obtained using a custom script based on LocMoFit model-free averaging⁵. A total of 606 NPCs were averaged for 2D imaging, and 1735 NPCs for 3D NPC structure reconstruction.

Subunit separation within Nup96 pairs was quantified by analyzing localization clusters along lateral (maximum variance via PCA) and axial (z) axes. Lateral separation was determined via a two-component Gaussian mixture model. For axial separation, localizations assigned to each lateral subpopulation were independently fitted with single Gaussians to calculate peak-to-peak distances.

RESULTS

Simplified, automated RESI workflows using Aplo Flow and Aplo Scope

Executing sequential or multiplexed SMLM experiments such as DNA-PAINT and RESI requires precise, repetitive liquid exchanges interspersed with high-frame-rate optical imaging.

In traditional manual workflows, each liquid exchange requires physical handling of the sample microfluidics, introducing a risk of focus drift, cross-lane contamination, and operator-induced variability across imaging cycles.

To address these challenges, we integrated ONI's Aplo Flow microfluidic automation ecosystem with the Aplo Scope super-resolution microscope for full automation of image acquisition and liquid handling, after manual sample preparation (Figure 1). The integrated hardware-software architecture allows complex multi-cycle RESI and DNA-PAINT sequences to be configured and executed as a single, fully automated run. The workflow steps include: 1. Cell Flow Chip mounting, fluidics connection, and chip lane priming; 2. Regions of Interest (ROI) and parameter definition using Aplo Flow SDK; 3. Automated sequential fluidics for each RESI cycle (F1-F4); 4. Image acquisition with Aplo Scope immediately after fluid exchange.

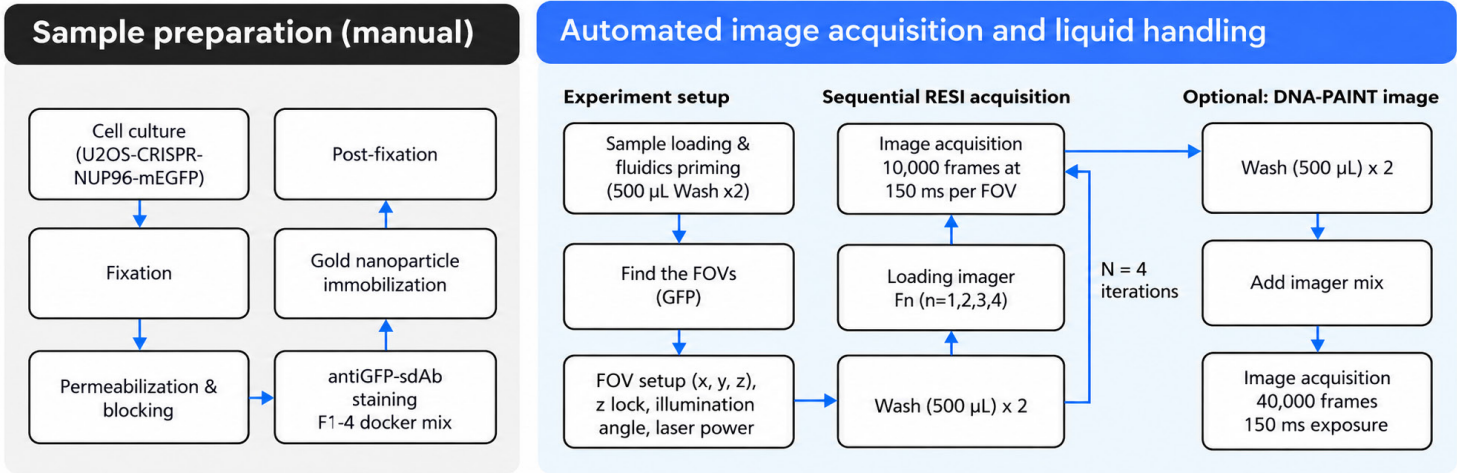


Figure 1 | Schematic of the automated image acquisition and liquid-handling workflow steps using Aplo Flow and Aplo Scope, including optimized experimental setup, sequential RESI acquisition, and a final (optional) DNA-PAINT image acquisition step.

Sub-nanometer 2D RESI imaging resolves nuclear pore complex subunits

To evaluate the resolving power of our automated workflow, we mapped the 2D spatial arrangement of endogenously tagged eGFP-Nup96 in U2OS cells (Figure 2). Using Aplo Flow, four sequential RESI cycles (F1-F4) were executed automatically within the same region of interest, with each cycle recording sparse target subsets. This resulted in a high-resolution RESI image with a median localization precision of 0.86 nm after the alignment and combination of localizations across all four cycles (Figures 2A-2F).

We compared this RESI composite to conventional single-cycle DNA-PAINT, showing a marked gain in spatial resolution and sharply defined, individual Nup96 subunits (Figures 2G and 2H). To quantify structural fidelity, model-free molecular averaging (in-house script written based on LocMoFit's algorithm⁵) was performed across 606 individual nuclear pore complexes (Figure 2I). The resulting average clearly resolved the characteristic eight-fold symmetry of the outer NPC ring, distinctly separating the paired Nup96 protein clusters. These results highlight how automated RESI reliably yields structural detail in dense cellular assemblies with sub-nanometer precision.

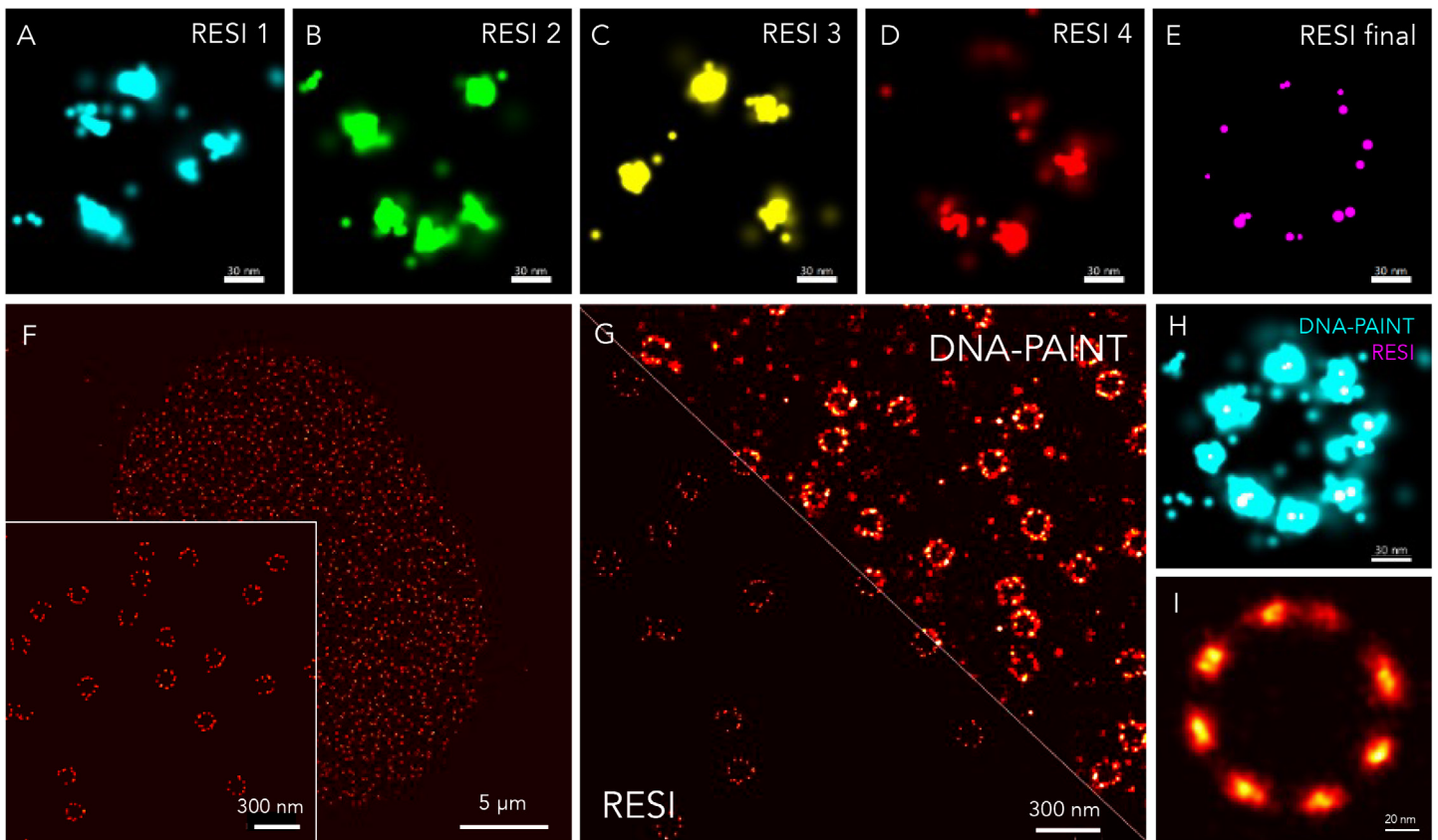


Figure 2 | High-accuracy 2D RESI imaging and molecular averaging of Nup96 nuclear pore complexes (NPCs). (A-D) Individual sequential RESI cycle reconstructions (F1-F4) showing sparse target subsets localized with 0.86 nm precision. (E) Reconstructed 4-cycle RESI image. (F) Resulting RESI image of NPCs at different scales. (G) Comparison of DNA-PAINT and RESI imaging of NPCs. (H) Overlay comparison of DNA-PAINT (green) versus RESI (magenta), demonstrating enhanced subunit separation with RESI. (I) Molecular averaging of 606 NPCs resolving the eight-fold ring architecture and paired Nup96 subunit pairs.

3D astigmatic RESI resolves nanoscale axial subunit architecture

To extend multi-cycle automated workflows into three dimensions (3D), Aplo Scope allows for flexible optical optimization. The system features an integrated astigmatic cylindrical lens and supports swapping to application-specific objectives. In addition, output data formats are designed for open compatibility with third-party analysis tools like Picasso and LocMoFit. We applied this setup to map the 3D organization of single eGFP-Nup96 complexes (Figure 3A).

Comparing 3D reconstructions of a single NPC highlighted the dramatic resolution gain of RESI over conventional DNA-PAINT (Figure 3A). While DNA-PAINT provided broad localization clusters (median localization precision: 4.93 nm lateral, 10.19 nm axial), 3D RESI improved precision to sub-nanometer lateral

(0.97 nm) and sub-2-nanometer axial (1.85 nm) precision. In the top-down (x,y) view, 3D RESI distinctly resolved adjacent Nup96 subunit pairs separated by 10 nm, while the axial (x,z) cross-section clearly separated the nuclear and cytoplasmic ring planes.

To evaluate overall structural consistency, 3D model-free molecular averaging was performed across 1735 individual NPCs (Figure 3B). The resulting 3D density maps, displayed in top-down (x,y), side (x,z), and tilted perspectives, reconstructed the complete double-ring architecture of the nuclear pore scaffold with nanoscale fidelity. These findings demonstrate that combining automated fluidics with flexible 3D astigmatic RESI delivers isotropic, single-protein resolution across complex cellular structures.

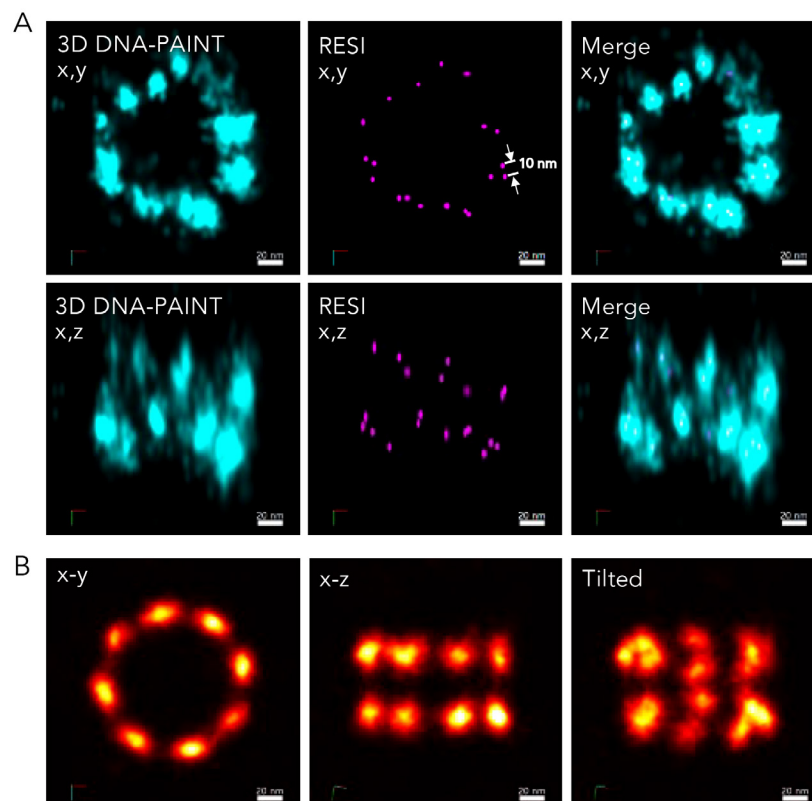


Figure 3 | 3D astigmatic RESI imaging and structural reconstruction of eGFP-Nup96 nuclear pore complexes. (A) Single-NPC 3D imaging comparison in x,y (top row) and x,z (bottom row) projections for DNA-PAINT (left), RESI (middle), and merged overlay (right). RESI achieves 0.97 nm lateral and 1.85 nm axial localization precision, resolving a 10 nm subunit separation in x,y. (B) 3D molecular averaging of 1,735 NPCs showing top-down (x,y), axial cross-section (x,z), and tilted 3D perspective views.

CONCLUSION

By integrating ONI's Aplo Flow microfluidic module directly with the Aplo Scope super-resolution microscope, complex multi-cycle imaging protocols like DNA-PAINT and RESI now run as uninterrupted processes to deliver unmatched spatial resolution with reproducible and scalable results. Through high-precision 2D and 3D NPC mapping, we showed the integrated Aplo Flow and Aplo Scope platform achieves sub-nanometer localization precision and clearly resolves 10 nm subunit separations. This automated routine dramatically reduces active hands-on time while establishing high cycle-to-cycle reproducibility across

multi-lane flow chips. Furthermore, software flexibility allows seamless integration with third-party tools for robust downstream analysis.

Ultimately, combining liquid handling automation with super-resolution microscopy removes the primary friction points of multi-cycle SMLM. The Aplo platform provides robust, scalable, and fully automated workflows, empowering researchers to explore cellular structures in depth and uncover critical insights in drug delivery, biomarker discovery, and antibody therapeutics.

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