

# Custom LNP ligand detection with the LNP Profiler kit and AutoLNP: a mouse case study

## SUMMARY

Critical lipid nanoparticle (LNP) characterization metrics to verify engineering efficiency and functionality include particle size, cargo-loaded fraction, and ligand engineering.

The ONi Application Kit™: LNP Profiler with AutoLNP allows whole-population analysis across thousands of particles in minutes with single-particle sensitivity.

The LNP Profiler workflow is designed to target human or humanized ligands, here we show how to adapt this workflow to study various ligand components, enabling:

- Custom ligand detection alongside a PanLNP marker and cargo detection
- Ligand positivity using conventional, non-spontaneously blinking fluorophores
- Accurate sizing, morphology assessment, and cargo quantification on a single particle basis.

## INTRODUCTION

Lipid nanoparticles (LNPs) are advanced delivery systems that encapsulate therapeutic agents and mRNA to protect them from degradation and facilitate their entry into target cells. The clinical efficacy of LNPs hinges on their precise engineering, where manufacturers must balance particle size and structural stability with high encapsulation efficiency and targeted ligand composition to ensure transport to the target region and reliable cellular uptake. While various complementary platforms exist for LNP researchers to assess these metrics (e.g., DLS, western blot, spectrophotometry), many rely on bulk readouts and often have inherent limits of resolution that may lead to sample averaging or limited analysis of a subset of particles.

Characterizing LNP properties at the single particle level is non-trivial due to their small size (30-200 nm). In addition, LNP sample heterogeneity means that differences in size, morphology, ligand density, cargo-loaded fraction, and molecular differences can be overlooked in bulk measurements. Single-molecule localization microscopy (SMLM) can uniquely address these gaps by enabling multi-channel fluorescence imaging with sub-20 nm resolution, simultaneously assessing biomarker distributions, size, morphology, encapsulation efficiency, and loaded fraction within a single measurement. In addition, SMLM can enable imaging of LNPs within cells, supporting studies of uptake mechanisms<sup>1</sup> and direct correlation of structural metrics with functional outcomes<sup>2-3</sup>.

ONi's LNP Profiler Kit with AutoLNP uses SMLM to provide whole-population analysis across thousands of particles in minutes, relaying readouts for individual particles, including sizing, morphology, cargo encapsulation, and surface ligand quantification. This end-to-end assay, powered by ONi's SMLM-based platform, is designed to allow rapid quantification of key, clinically relevant sample information across a broad range of samples containing human ligands, without the need to optimize reagents or protocols for each unique sample. However, many LNP components are of interest to formulation scientists, including alternative species of surface ligand that may not be detectable using the standard LNP Profiler Kit protocol.

In this application note, we describe an adaptation of the standard LNP Profiler Kit with AutoLNP to detect a mouse IgG ligand on the surface of four samples engineered with varying ligand levels. These studies were performed using a widely available commercial antibody conjugated with a conventional, non-spontaneously blinking fluorophore, AlexaFluor™-555, along with basic adaptations of the AutoLNP acquisition and analysis workflow. We show successful detection of mouse ligand and a correlation between percentage positivity and ligand concentration used during formulation. Overall, we show that the the LNP Profiler with AutoLNP workflow can easily be adapted to study a wide range of LNP ligands.

## METHODS

### Sample preparation

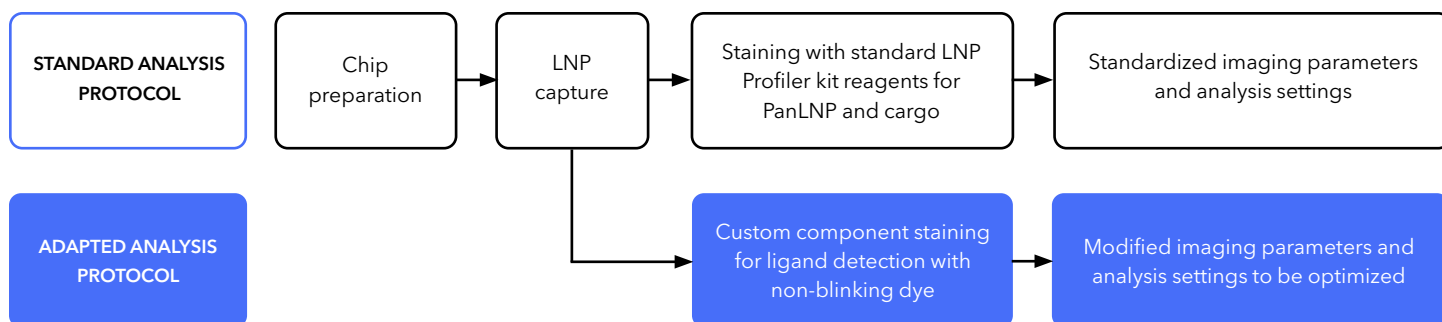
MC3/Cholesterol/DSPC/DMG-PEG/DSPE-PEG-DBCO based LNPs were generated containing varying levels of Dibenzocyclooctyne (DBCO) encapsulating polyA (Phosphorex LLC). Four LNP samples were formulated, with approximate final mouse monoclonal antibody concentrations of 0, 0.058, 0.161, and 0.337 mg/mL, respectively. The standard LNP Profiler protocol was adapted as follows (see Figure 1). After capture on an ONI LNP Assay Chip, LNPs were labelled with a cocktail of the standard PanLNP stain provided in the LNP Profiler kit together with an AlexaFluor™-555-conjugated donkey anti-mouse IgG antibody (Thermo Fisher Scientific a32773) at a final concentration of 4 µg/mL, 2 µg/mL, or 0.5 µg/mL. A 45-minute incubation was performed at room temperature, followed by washing and cargo staining as per the LNP Profiler user guide.

### Image acquisition

Imaging was performed using an ONI Nanoimager utilizing AutoLNP acquisition App within ONI's CODI platform. The ligand detection channel was acquired with 500 frames at 20 ms exposure/frame at 18 mW of 561 power at the sample. The PanLNP SMLM acquisition was performed with the 640 nm laser at 60 mW power at the sample for 2500 frames and a 20 ms exposure/frame. For nucleic acid cargo detection, standard diffraction-limited imaging was performed with the 488 nm laser at 0.15 mW power at the sample, with 200 frames at 100 ms exposure captured. All imaging was performed with a 100X, 1.45NA objective using Total Internal Reflection Fluorescence (TIRF) illumination, with illumination angle being determined using the autoTIRF algorithm in CODI for each acquisition. A minimum of 3 fields of view (FOV) per condition were collected. All imaging was performed at 32° C.

### Data analysis

Quantification was performed using the AI LNP Profiling analysis workflow on ONI's CODI platform. This analysis consists of four steps: drift correction, SMLM filtering for individual channels, machine-learning-based clustering applied to the PanLNP localizations, and a localization-based ligand positivity tool and an AI-based counting and abundance tool for cargo. Positivity for the ligand channel was calculated with a minimum of 3 localizations (min locs), as previously described in the LNP Profiler user guide. Note that some optimization of the imaging and analysis settings is required. Contact ONI for assistance.



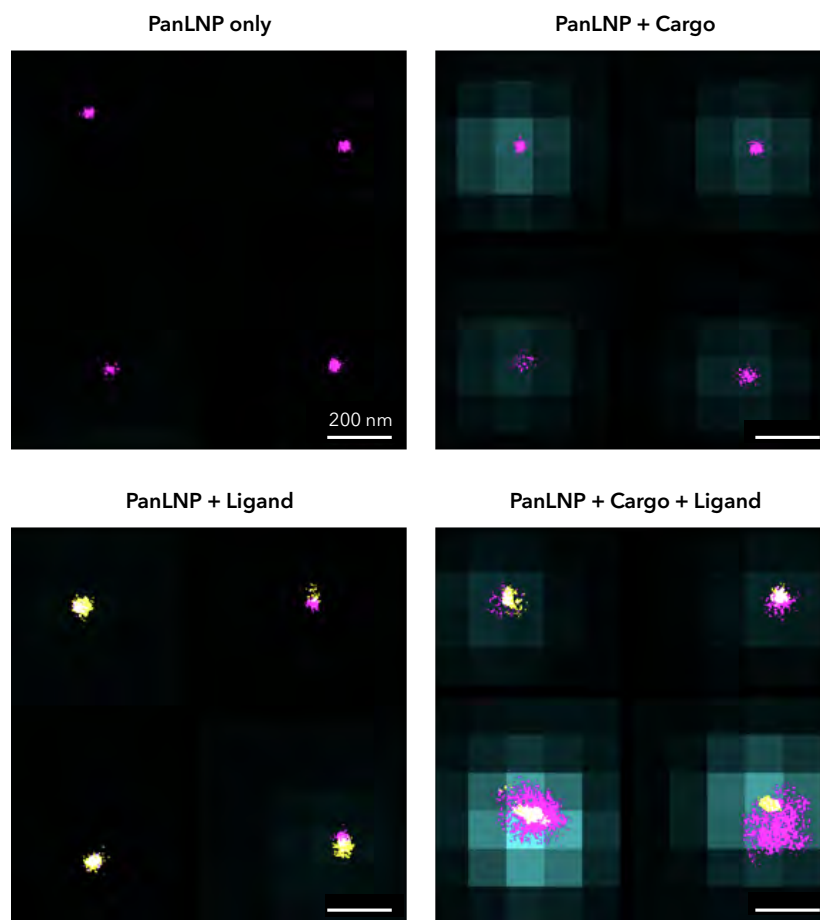
**Figure 1** | Schematic of the standard (white boxes) and adapted LNP Profiler with Auto LNP protocol (blue boxes) for custom ligand detection.

## RESULTS

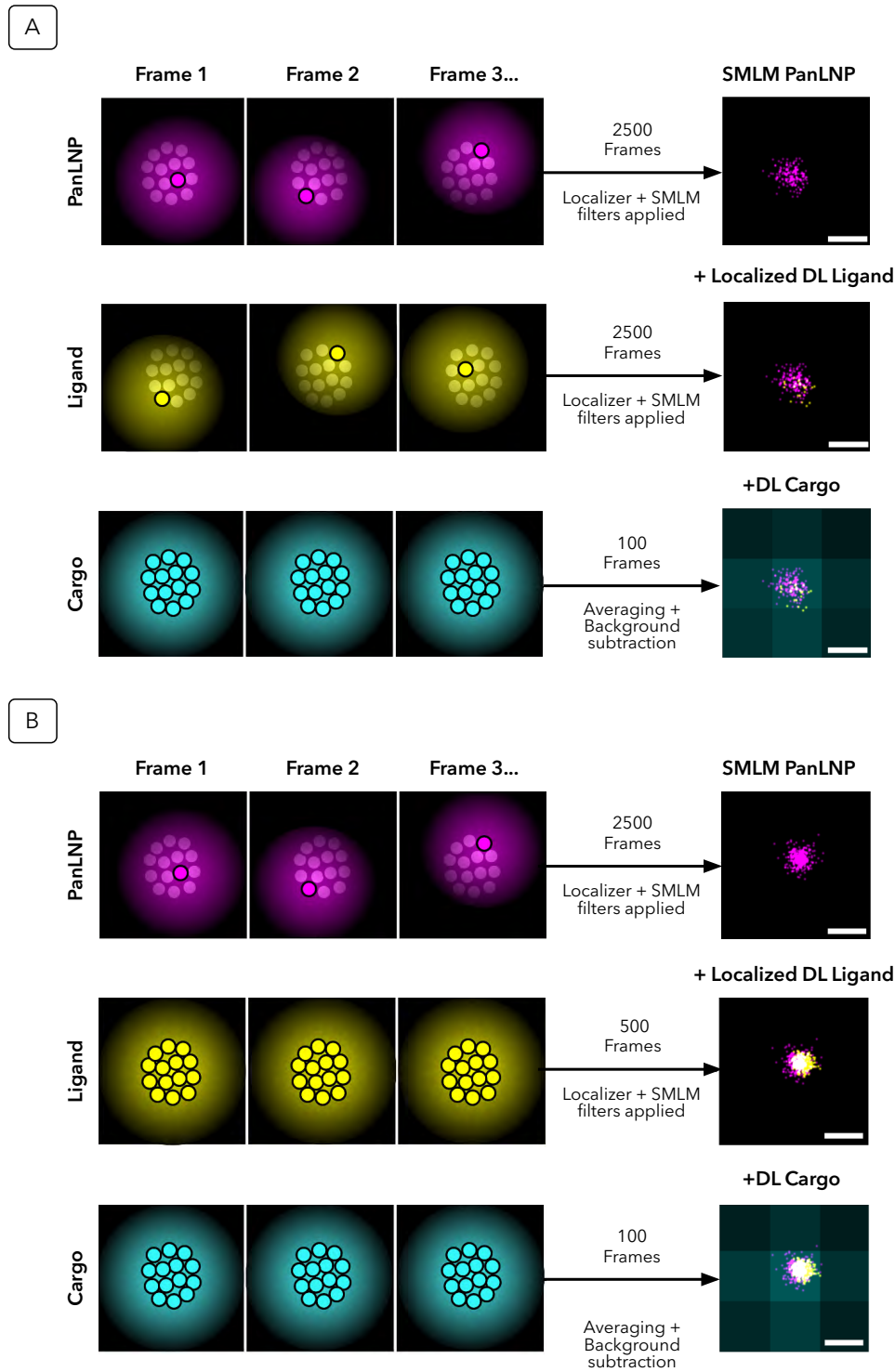
### Detection of a mouse ligand using LNP Profiler with AutoLNP and a conventional fluorophore AF555

To assess if custom LNP components may be detected using an adapted staining workflow, LNP samples with varying levels of mouse IgG (formulated and provided by Phosphorex LLC) were characterized to determine the presence or absence of ligand, together with the PanLNP stain and cargo detection. To that end, ligand detection was performed using a commercially available anti-mouse IgG antibody conjugated to AF555. Unlike the spontaneously blinking fluorophores used for SMLM the conventional fluorophore AF555 emits continuously, resulting in the ligand channel signal appearing as a small cluster of localizations within the center of the LNPs, with a clear colocalization between the ligand signal and the PanLNP stain (Figure 2). Utilizing this workflow, the software can clearly classify LNP's in to sub-groups based on presence/absence of Cargo and Ligand.

Unlike the spontaneously blinking fluorophores used for super-resolution imaging, which require 2500 frames of imaging to localize proteins in a spatially accurate manner (Figure 3A), here, the continuous emission of AF555 was captured over 500 frames as localizations that overlay on the super-resolved PanLNP (Figure 3B). While no spatial information is determined for the ligand signal due to the dye's non-blinking nature, colocalization of the ligand signal with the PanLNP-based detection is clear. Thus, ligand positivity determination is possible with no adaptation of the standard localization counting-based analysis pipeline.



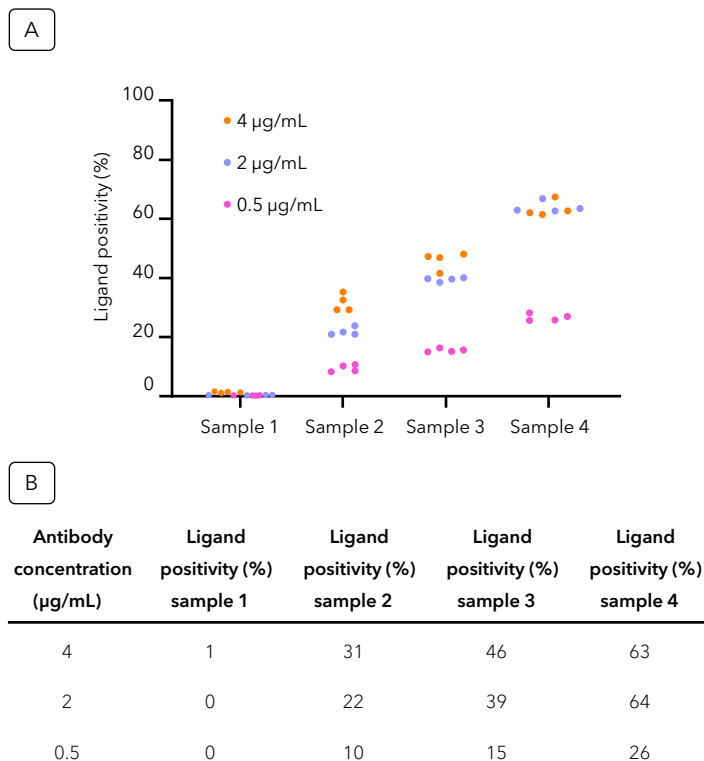
**Figure 2 |** Representative examples of the AutoLNP output report showing LNPs captured and imaged using the adapted LNP Profiler Kit and AutoLNP. LNP examples of clusters grouped by positivity readout show PanLNP localizations (magenta), mouse IgG ligand localizations (yellow), and the DL nucleic acid cargo (cyan). Scale bars = 200 nm.



**Figure 3 |** Multichannel image reconstruction schematic. A) For the standard AutoLNP workflow, spontaneously blinking fluorophores were used for PanLNP (top row) and ligand (middle row) detection, while a non-blinking continuously emitting dye was used for cargo detection (bottom row). B) In the described protocol modification, a non-blinking continuously emitting fluorophore replaced the standard ligand detection reagent (middle row) with a resulting non-blinking localization based image (“+localized non-blinking”). The spontaneously-blinking dyes are represented by a single circle marking a blinking event (= 1 dye emitting) and the PSF represented as a diffused colored circle (in A top and middle rows, in B top row only). For the non-blinking dye, the figure illustrates that all dyes are emitting with the corresponding PSF highlighted as a diffused colored circle (in A, bottom row, and in B, middle and bottom rows). Scale bars = 100 nm.

### Antibody titration to optimize custom ligand detection

To find the optimal condition for custom ligand positivity detection, the LNP samples containing four distinct amounts of mouse IgG ligand were stained using three AF555-conjugated anti-mouse IgG antibody concentrations, namely 0.5 µg/mL, 2 µg/mL, and 4 µg/mL. An increase in total ligand positivity was observed as the final mouse antibody concentration on the LNP increased from 0 to 0.058, 0.161, and 0.337 mg/mL for samples 1, 2, 3, and 4, respectively (Figure 4). The highest IgG ligand positivity was observed in samples 2 and 3 at the 4 µg/mL antibody concentration (Figure 4B). Sample 4, the highest ligand concentration formulation, showed equivalent positivity values for both 2 µg/mL and 4 µg/mL and much lower readout with 0.5 µg/mL, while sample 1 (the negative control) showed a small increase from 0% to 1% positivity with the highest antibody amount compared to the other three. Taken together, these data suggest that 2-4 µg/mL of this antibody is the optimal range for ligand detection.

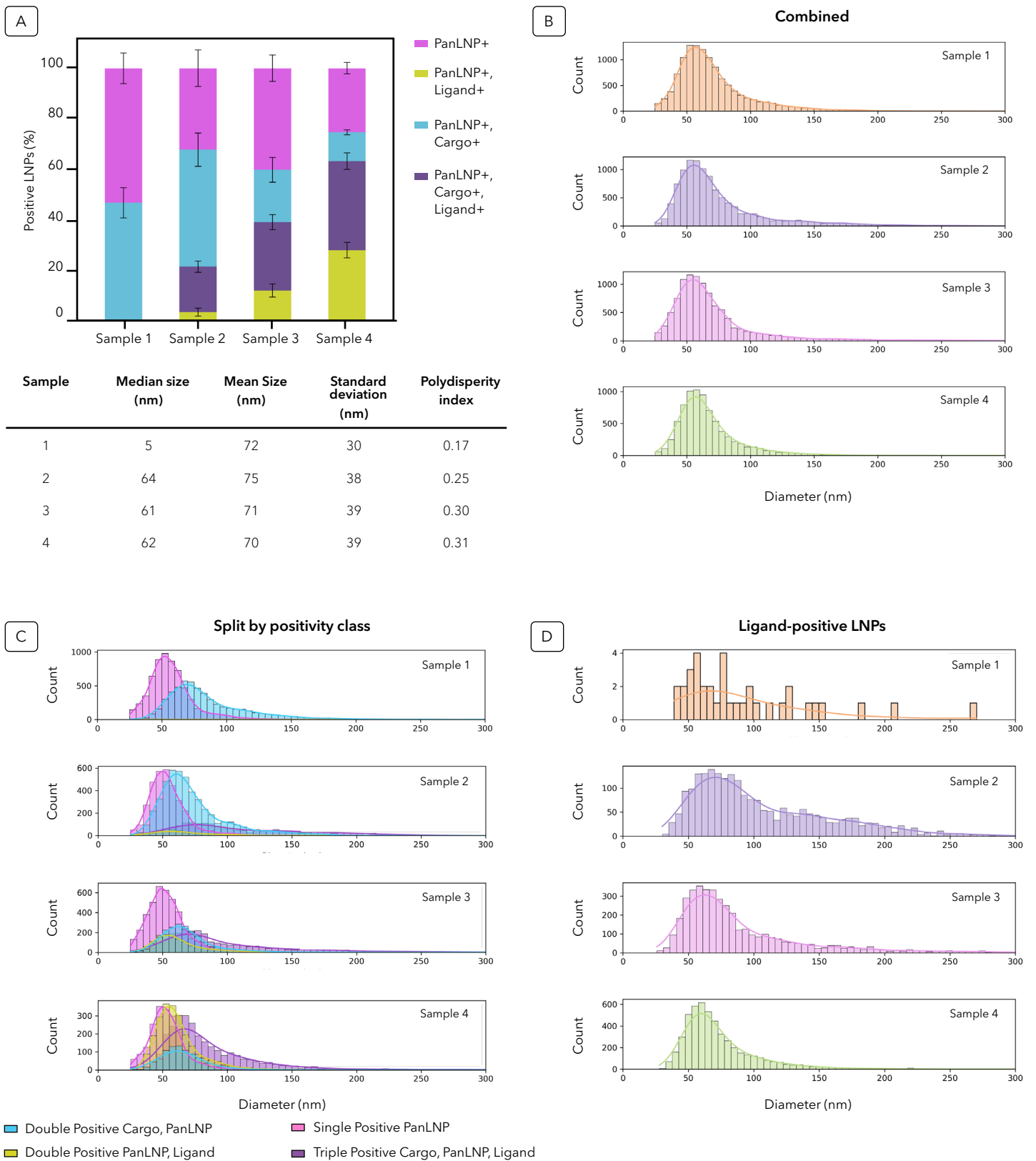


**Figure 4 |** Custom ligand detection was assessed using the four LNP samples engineered with varying ligand amounts, analyzed using three different AF555-conjugated anti-mouse IgG antibody concentrations. A) Graph showing the percentage of ligand positivity per sample, split by antibody concentration. Pink = 0.5 µg/mL, blue = 2 µg/mL, orange = 4 µg/mL. Each dot is a single field of view (FOV) acquired. B) Ligand positivity across all FOVs per antibody concentration per sample.

### Multimeric LNP sizing, morphology and positivity assessment with AI LNP Profiling

The LNP Profiler Kit with AutoLNP enables multimetric LNP sizing and morphology assessment from the whole LNP population down to individual particles, alongside cargo encapsulation and surface ligand quantification. The data obtained from the standard AI LNP Profiling workflow confirmed the increase in ligand positivity observed during titration of the anti-mouse IgG, with 0%, 22%, 39%, and 64% total ligand positivity at an antibody concentration of 2 µg/mL for samples 1 to 4, respectively (Figure 5A). This was observed for both cargo-negative (0%, 4%, 12% and 29% ligand positivity in samples 1-4, respectively) and cargo-positive LNPs (0%, 18%, 27%, and 35% for samples 1-4, respectively).

Further data processing using the AI LNP Profiling analysis outputs (CSV files) and ONI’s LNP Axis (visit [oni.bio/protocols/lnp-axis/](https://oni.bio/protocols/lnp-axis/)) multiparametric plotting tool showed that when looking at combined histograms for all LNP classifications, all four samples had a similar size distribution, with peaks between 50 and 60 nm and similar average sizes across samples (Figure 5B). Histograms split by particle classification revealed a slight tendency for LNP sizes to be larger in ligand-positive vs ligand-negative LNPs at the highest ligand density (sample 4) (Figure 5C). Similarly, the negative control (sample 1) showed a lower polydispersity index (PDI), indicating that incorporating the surface ligand slightly reduces particle-size uniformity. Across all four samples, cargo-positive particles tend to be larger than cargo-negative particles. As sample ligand density increases from sample 1 to 4, the portion of ligand-positive LNPs increase, with their size centered around 60 nm (Figure 5D).



**Figure 5** | Analysis output from the LNP Axis tool (2  $\mu\text{g}/\text{mL}$  anti-mouse IgG antibody concentration) showing: A) The normalized positivity plots per sample, where magenta = PanLNP only, cyan = PanLNP + Cargo, yellow = PanLNP + Ligand, purple = PanLNP + Cargo + Ligand positive LNPs. The table shows the bulk particle-size statistics for each sample. B-D) Size distribution histograms of combined particles per sample (B), split by classification (C) and ligand positive only (D). For panels C and D, colors do not correlate with positivity and simply represent different samples.

## CONCLUSIONS

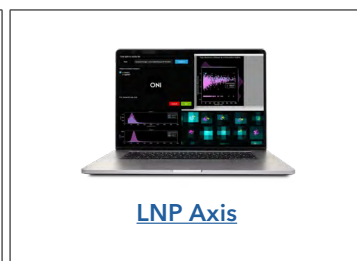
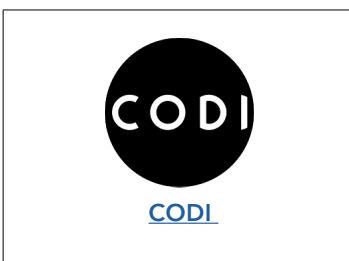
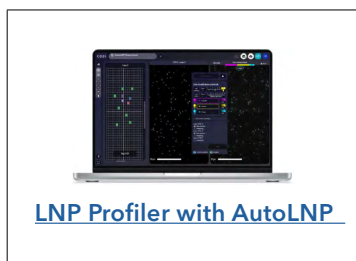
Characterizing LNPs is crucial for assessing formulation efficacy, quality, and batch-to-batch consistency. The combination of throughput, resolution, and quantitative multi-parameter readout in a single platform distinguishes ONI's SMLM-based LNP Profiler with AutoLNP workflow from other nanoparticle characterization methods. This study exemplifies a simple way to tailor the existing protocol, allowing for non-human ligand detection and positivity quantification using a readily available commercial antibody. Further, this approach can enable LNP researchers to rapidly screen other LNP components of interest with the appropriate protocol changes (contact ONI, if interested).

This example can be particularly useful when antibody labeling is not feasible, either because the target antigen is not accessible on the LNP surface or because no compatible antibodies are available. In such cases, pre-labeling with widely available fluorophores, such as AF555, may enable investigation of these targets when combined with minimal adaptation of the analysis parameters used for the standard LNP Profiler's SMLM ligand detection. While some optimization of staining and imaging parameters may be required by the user, the downstream analysis can be relatively straightforward.

## REFERENCES

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