

CLiC platform enables single-particle multi-parametric characterization of LNP and payloads

Problem: Lipid nanoparticles (LNP) have progressed rapidly from experimental carriers of nanomedicines to clinically validated drug products^{1,2,3}. Despite this success, development and manufacturing workflows still rely on measurements that collapse complex nanoparticle populations into single average values. A single LNP formulation often contains a mixture of particles that differ in size, internal structure, lipid organization, and nucleic acid content. Some particles carry multiple mRNA molecules, others only one, and some none. These subpopulations can coexist within the same batch yet exhibit distinct biological behaviors that influence delivery efficiency, stability, immunotoxicity, and therapeutic response^{4,5}.

Standard analytical tools—including dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and bulk fluorescence assays—are not designed to resolve this diversity. They report ensemble averages that obscure particle-to-particle variability and provide little insight into how formulation parameters shape internal organization. As a result, formulation optimization, scale-up, and comparability assessments remain largely empirical, limiting the ability to rationally engineer next-generation nanomedicine therapeutics.

Solution: To address this gap, a single-particle analytical framework has been developed that directly interrogates individual mRNA-LNPs while they remain freely suspended in solution. This employs Convex Lens-induced Confinement (CLiC) microscopy⁶—a technology developed by Dr Sabrina Leslie and exclusively licensed to ScopeSys Inc.—to image individual LNPs, which was mounted on the stage of a Nikon Ti2 inverted microscope platform. Rather than inferring properties from population averages, this approach measures thousands of particles one at a time, capturing the full distribution of physical and compositional states within a formulation, which is enhanced by the large field of view of the Nikon Ti2 system.

As shown in **Figure 1**, by transiently confining nanoparticles in micron-scale wells, individual LNPs can be tracked for extended durations without immobilization or surface attachment⁷, focus maintained by the Nikon Perfect Focus System (PFS). This enables simultaneous extraction of hydrodynamic size, lipid-associated fluorescence, and cargo presence from each particle. Alternating excitation of spectrally distinct probes further allows separation of direct fluorescence from energy-transfer effects (e.g., FRET), yielding quantitative readouts of both payload copy number and lipid-cargo organization. The result is a high-throughput, in-solution method that transforms LNP characterization from ensemble averages to particle-resolved analytics. This framework reveals hidden subpopulations, links formulation chemistry to internal structure, and provides actionable metrics for formulation design, quality control, and process development.

Key technical features of CLiC platform:

Nanoscale Confinement: Molecules are restricted to a thin volume, reducing out-of-plane diffusion.

Enhanced Signal-to-Noise: The shallow imaging depth provides ~20x better background rejection than Total Internal Reflection Fluorescence (TIRF) microscopy.

Extended Observation Time: Freely diffusing molecules can be observed ~10,000x longer than with confocal fluorescence correlation spectroscopy. (trajectories > 10x longer than those of NTA)

No Surface Immobilization: Avoids artifacts from tethering molecules to surfaces, preserving native dynamics.

Discern mechanism-of-action, in out-of-equilibrium conditions: Resolve the time courses of several species of molecules while they are interacting. This includes the ability to exchange reagents and environmental conditions during observations.

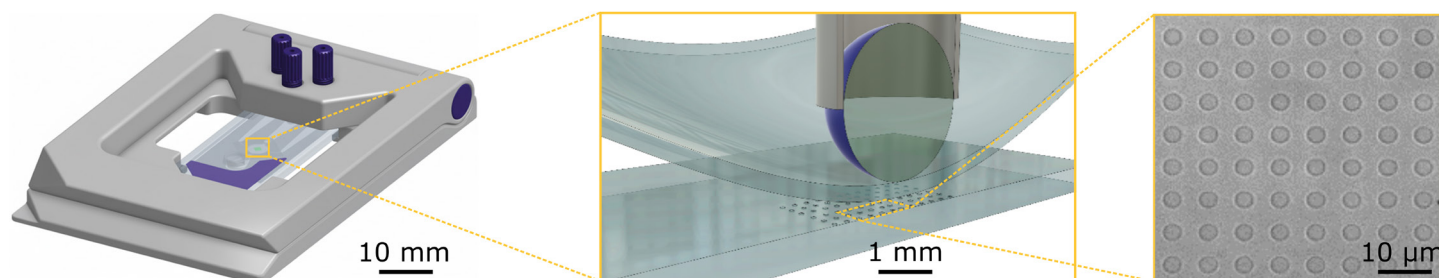


Figure 1. *Left Panel:* CLiC interface with consumable that easily fits onto the stage of the Nikon Ti2 for the quantitative, real-time, characterization of biomaterials such as molecules (DNA, RNA), particles (LNPs, EVs) and live cells. *Middle Panel:* Proprietary CLiC technology operation where either through mechanical or pneumatic pressure the top glass is deflected to isolate and confine single diffusing biomaterials to the focal plane of the microscope. *Right Panel:* Microwells within the CLiC consumable where individual particles can be imaged for extended periods of time under biological conditions. This physical confinement approach enables extended observation time in solution, allowing for accurate measurements in native conditions.

CLiC with ALEX enables nanoparticle and payload characterization

By combining our proprietary CLiC microscopy with alternating laser excitation (ALEX, achieved at high-speed with NIS-Elements triggered acquisition), we can study LNP heterogeneity with high-throughput single-particle measurements of size, payload, and molecular arrangements made under suspended-solution conditions⁸. CLiC-ALEX combines physical confinement of freely diffusing nanoparticles with multiplexed fluorescence microscopy

and automated quantitative analysis to generate multidimensional particle fingerprints in solution. As shown in **Figure 2**, by combining ALEX with simultaneous excitation of distinct probes on both the nanoparticles (e.g., DiO) and their nucleic acid cargo (e.g., Cy5), we are able to separate direct excitation from dye-dye interactions, allowing for robust detection of cargo presence and molecular arrangements across various formulations.

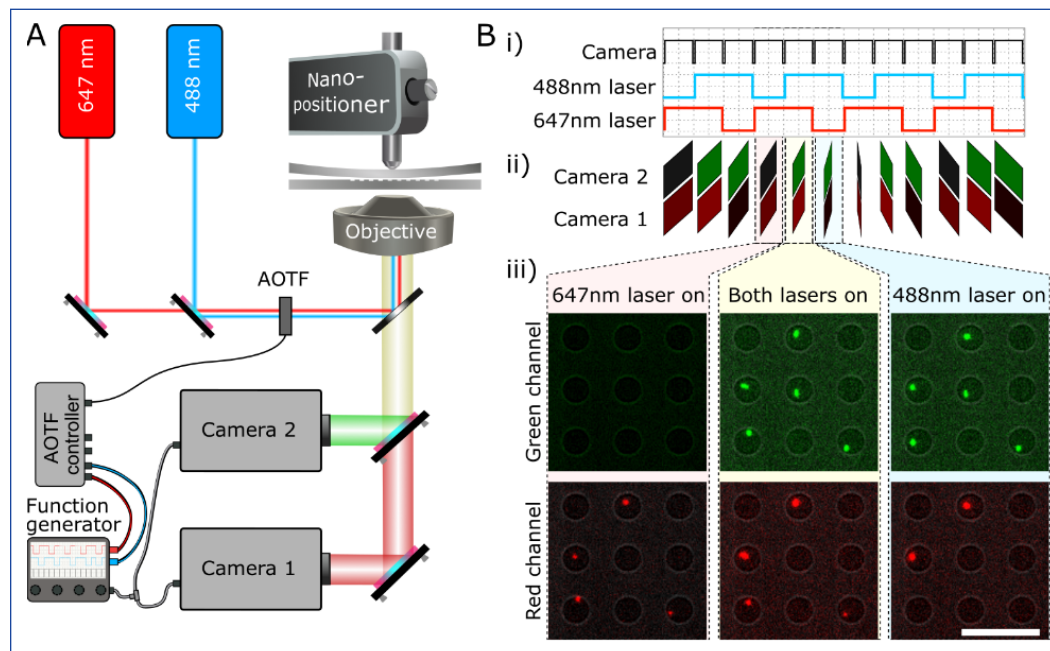


Figure 2. Panel A: Schematic of the imaging system, including: two lasers, a CLiC device on an inverted fluorescence microscope, and a dual-camera system for data collection.

Panel B: (B i.) Triggering waveforms to synchronize cameras (black line) with the excitation lasers, including a 488-nm (blue) line and a 647-nm (red) line. Using Nikon's software NIS-Elements triggered acquisition module with the lasers as shown above, we are able to combine both sequential and simultaneous excitations across our imaging channels, as shown in (B ii. & iii.). (B iii.) Example images of LNPs as observed in the two channels under various excitation conditions. Note that fluorescent images are false colored, to represent imaging channels, and are overlaid with a bright field image to indicate location of CLiC micro-wells. CLiC-ALEX enables multiparametric measurements on individual particles across heterogeneous formulations. Scale bar = 10 μm .

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Formulation-dependent sample heterogeneity for empty/full LNP sub-populations

To study the relationship between mRNA loading and nanoparticle size we analyzed the relationship between lipid associated fluorescence intensities (IDiO) and the corresponding hydrodynamic radii (r_h) for a set of clinically relevant formulations. We observed that particles containing mRNA formed subpopulations distinct from empty ones. In some cases, these differences appeared primarily in lipid dye intensities rather than particle sizes, indicating altered lipid organization even when particle diameters were similar. However, in other formulations, cargo-containing particles were both larger and brighter, pointing to coupled changes in size and lipid content. These observations demonstrated that mRNA incorporation reshapes LNP internal structure in formulation-dependent ways that are invisible to ensemble methods (**Figure 3**).

Next, we compared our results with cryogenic electron microscopy (cryo-TEM) measurements performed on the same formulation. Here we observed that the subpopulations detected by CLiC-ALEX corresponded closely to distinct structural classes observed by cryo-EM: particles exhibiting internal partitioning of aqueous and lipid volumes were more likely to contain mRNA in certain formulations, though this relationship was not universal. This partial overlap underscores two important conclusions: Particle morphology and cargo loading are correlated but not interchangeable metrics; and Single-particle CLiC measurements provide complementary information that cannot be inferred from structure alone.

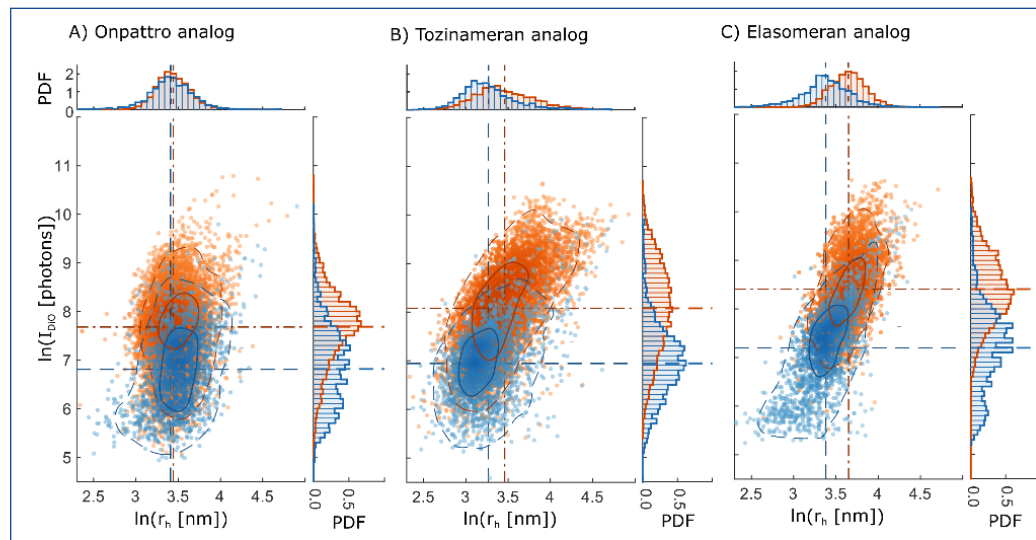


Figure 3. Lipid fluorescence (DiO) intensities are plotted as a function of corresponding particle sizes, for three clinically relevant formulations including: Onpattro analog (Panel A), Tozinameran analog (Panel B) and Elasmomeran analog (Panel C). Individual particles are classified as loaded (containing Cy5-mRNA) or empty by looking for the presence Cy5 signal co-localized with DiO (lipid dye) signal, see Kamanzi, et. al., *ACS Nano* 2026⁸ for details. Loaded particles form distinct subpopulations relative to empty ones. Depending on formulation chemistry, subpopulation separation appears either predominantly in lipid fluorescence intensity or in both particle size and fluorescence. These results demonstrate that cargo incorporation alters LNP structure in formulation specific ways that are not resolved by ensemble methods, highlighting the need for single-particle measurements.

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Quantification of mRNA copy number distributions

Next, we estimated mRNA copy numbers per particle by rescaling the fluorescence intensities of Cy5 (mRNA probes) measured per particle, with the average intensities of free Cy5-mRNA molecules. Rather than a fixed payload per particle, mRNA loading followed broad distributions

whose shapes depended strongly on formulation chemistry. Some formulations were dominated by particles carrying only one or two mRNA molecules, while others exhibited wider copy-number ranges that scaled with particle volume (**Figure 4**).

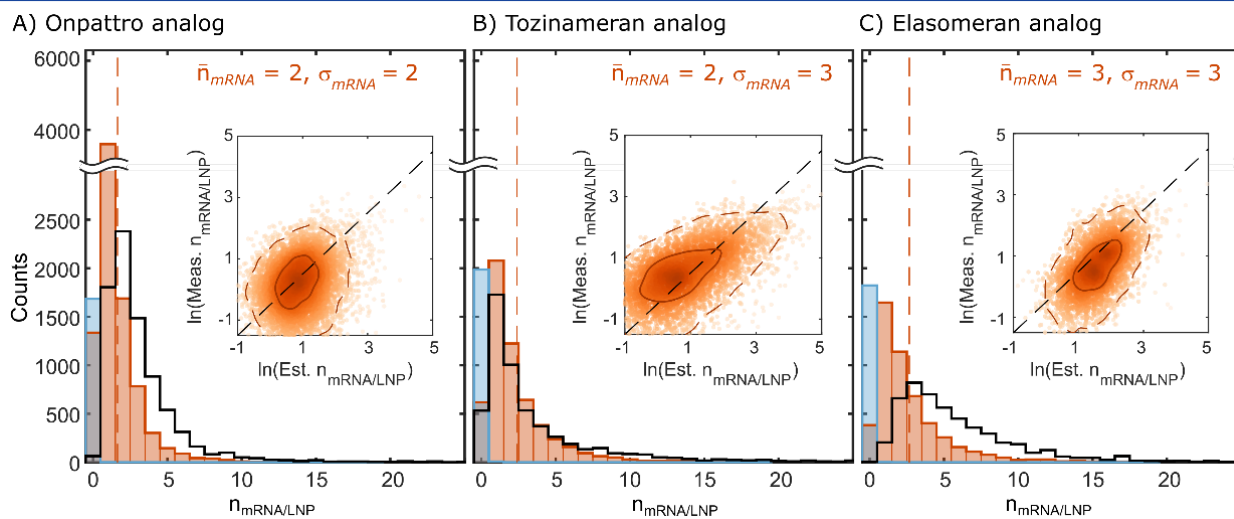


Figure 4. Fluorescence intensities of labeled mRNA cargo are rescaled using single-molecule calibration to estimate per-particle mRNA copy numbers for three clinically relevant formulations. Histograms illustrate differences between empty and loaded nanoparticle populations, while scatter plots correlate measured cargo fluorescence with predicted loading based on nanoparticle volume. The Onpattro analog formulation (A) is dominated by particles containing one to two mRNA molecules, whereas vaccine formulations (B & C) exhibit broader copy-number distributions that scale with particle size. These findings demonstrate that bulk encapsulation efficiency metrics can mask substantial particle-to-particle variability, emphasizing the importance of measuring payload distributions to predict therapeutic performance and optimize manufacturing consistency.

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Molecular organization probed by single-particle FRET

Beyond detecting whether cargo is present, we used measurements to gain insight into how lipid and mRNA molecules are arranged within individual particles. Here, FRET-stoichiometry plots showed distinct subpopulations for the various formulations measured. As shown in **Figure 5**, these subpopulations are consistent with surface-localized lipid dyes, mixed

distributions, and/or clustered lipid dye-cargo domains.

Such organizational differences are expected to influence particle stability, release kinetics, and intracellular processing, highlighting the value of measuring not just composition, but molecular arrangement with LNPs samples.

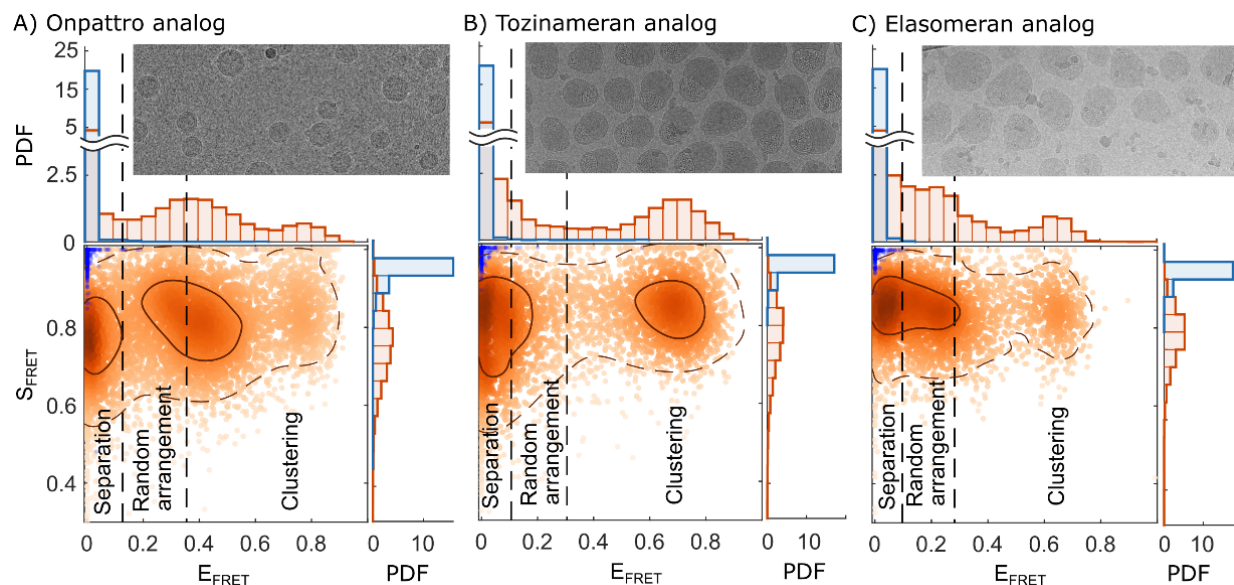


Figure 5. FRET efficiency and stoichiometry distributions are shown for multiple LNP formulations, revealing distinct clustering patterns corresponding to different lipid-cargo spatial arrangements. Contour overlays identify dominant particle subpopulations, while projected histograms illustrate distribution breadth. Representative cryo-EM images demonstrate structural classes corresponding to measured FRET profiles. Clusters correspond to distinct molecular arrangements including clustered lipid-cargo domains, mixed distributions, and spatially separated dye populations. These results demonstrate that nanoparticle composition alone does not fully describe internal structure. CLIC-ALEX provides functional insight into molecular organization, which is expected to influence nanoparticle stability, cargo release kinetics, and intracellular processing. Scale bar = 100 nm.

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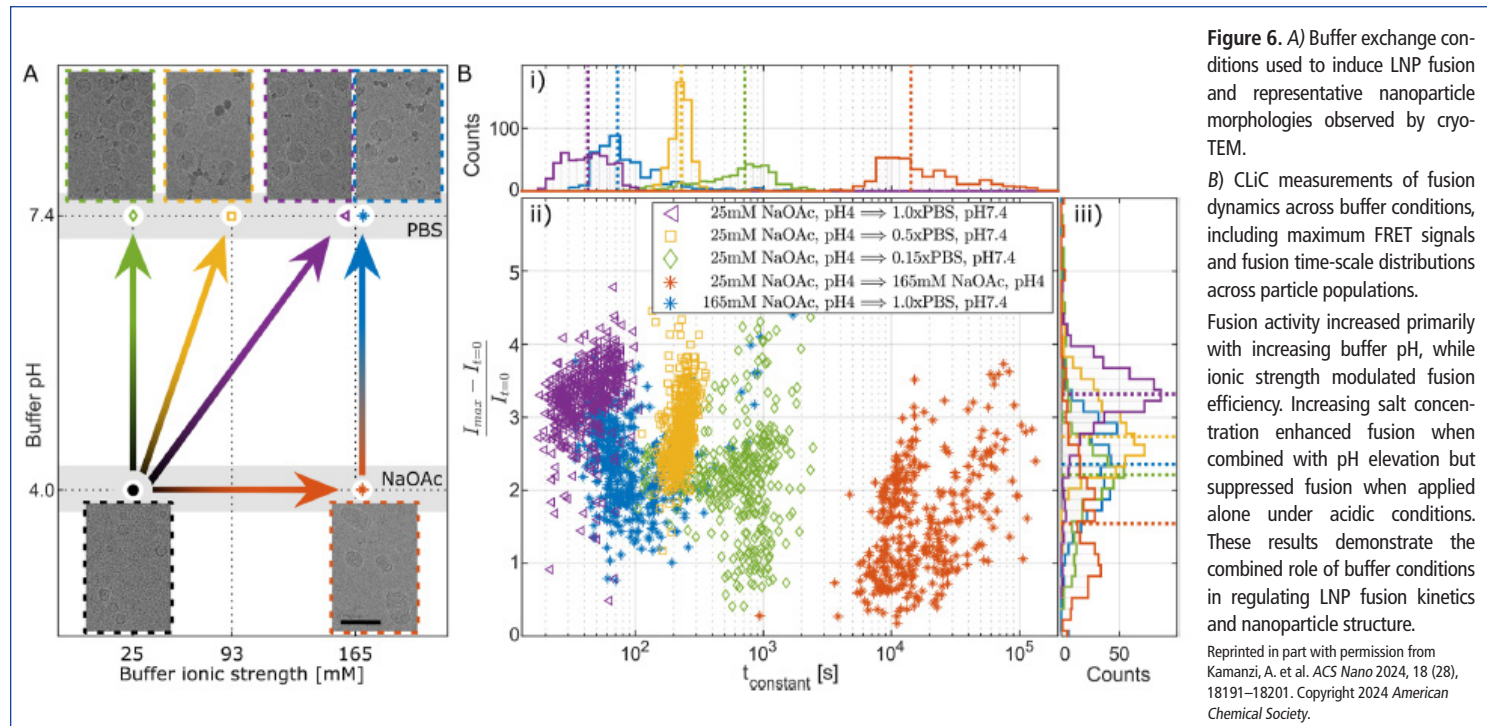
LNP fusion as a function of changing buffer conditions

We examined the impact of different buffer conditions (i.e., pH and ionic strength) on LNP fusion (Kamanzi, A.; et al. Quantitative visualization of lipid nanoparticle fusion as a function of formulation and process parameters. *ACS Nano* 2024, 18 (28), 18191-18201.). As shown in **Figure 6**, we observed that fusion activity was strongly influenced by buffer pH, with increased fusion observed when LNPs were transitioned from acidic formulation conditions to physiological pH. This behavior is consistent with deprotonation of ionizable lipids, which promotes nanoparticle restructuring and lipid mixing.

Ionic strength was found to modulate fusion efficiency. Increasing salt concentration in conjunction with pH elevation resulted in increased fusion

activity and shorter fusion time scales. In contrast, increasing ionic strength alone at constant acidic pH produced slower fusion dynamics, indicating that electrostatic screening and lipid packing effects can stabilize particle interfaces and suppress spontaneous fusion.

Structural imaging by cryo-TEM confirmed buffer-dependent nanoparticle morphologies, demonstrating structural transitions that were not fully captured by ensemble sizing measurements. Together, these results demonstrate that CLiC enables direct measurement of formulation-dependent fusion behavior and provides mechanistic insight into nanoparticle assembly, stability, and delivery performance.



Implications for development and manufacturing

The ability to resolve LNP heterogeneity at the single-particle level unlocks several capabilities critical to translational success:

Rational formulation optimization: Identify which formulation parameters shift populations toward desired structural states

Comparability assessment: Quantitatively compare batches, process changes, or scale-up conditions beyond ensemble averages

Quality control: Detect low-abundance but high-impact subpopulations that may affect efficacy or safety

Mechanistic insight: Link microscopic organization to downstream biological performance

Looking forward

As RNA therapeutics diversify toward more complex cargos and targeting strategies (e.g., co-loading of multiple drugs in the same formulation), particle-level heterogeneity will only increase in importance. Single-particle, multiparametric analytics provide a scalable path to understand and control this complexity.

Future extensions combining optical confinement with label-free detection and post-formulation labeling strategies promise even broader applicability, including native drug products without covalent modification.

Conclusion

mRNA lipid nanoparticles (mRNA-LNPs) are inherently heterogeneous systems in which subtle internal differences can significantly impact therapeutic performance. CLiC reveals this hidden diversity by confining freely diffusing nanoparticles under physiological conditions, enabling extended single-particle observation without surface tethering. The Nikon Ti2 microscope helps enable this research with large field of view enabling the collection of more data in each image, bright high-resolution optics, and PFS to keep the sample in focus for longer observation. Coupled with two-color ALEX, powered by NIS-Elements triggered acquisition, the platform simultaneously captures direct fluorescence and FRET signals from individual particles, delivering multiparametric insight into nanoparticle composition, molecular organization, and interaction dynamics. CLiC also supports reagent exchange, enabling real-time monitoring of particle behavior under changing environmental conditions. By moving beyond ensemble averages, CLiC provides the resolution needed to link formulation chemistry to nanoparticle structure, payload organization, and therapeutic function. The CLiC multiparametric nanoparticle assay described here is developed by the Leslie Team at the University of British Columbia in collaboration with ScopeSys Inc which is commercializing the technology, with the goal of enabling broader access to single-particle analytical tools for nanoparticle formulation design, process optimization, and manufacturing control.

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Product Information

AX/AX-R Confocal Microscope

The AX-R confocal system includes a resonant scanner allows for the high-speed and sensitive imaging that allows experiments such as the ones described in this application note. With the AX-R confocal, capturing high-resolution, high-speed events is easily achieved.

Find more information [here](#).



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