# Lipid-siRNA Organization Modulates the Intracellular Dynamics of Lipid Nanoparticles

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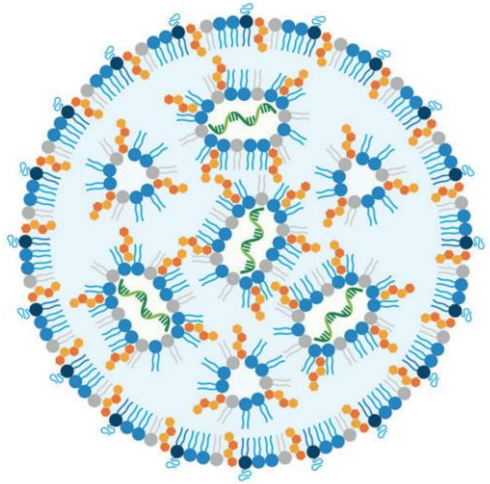
University of Toronto

CRS 2025 ANNUAL MEETING & EXPOSITION
PHILADELPHIA, PA
JULY 13-18, 2025

NEXT-GENERATION DELIVERY INNOVATIONS

# LNPs are made of simple lipid compositions and have good clinical translation profile



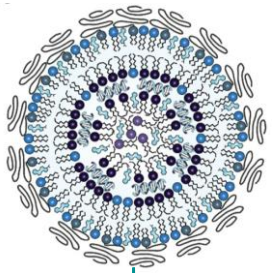
PEG-lipid

Ionizable lipid

Helper lipid

Cholesterol

Nucleic acid



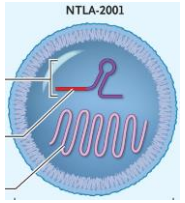
RNA interference

Protein replacement/ vaccines

Gene editing


Onpattro®, 2018
**siRNA**

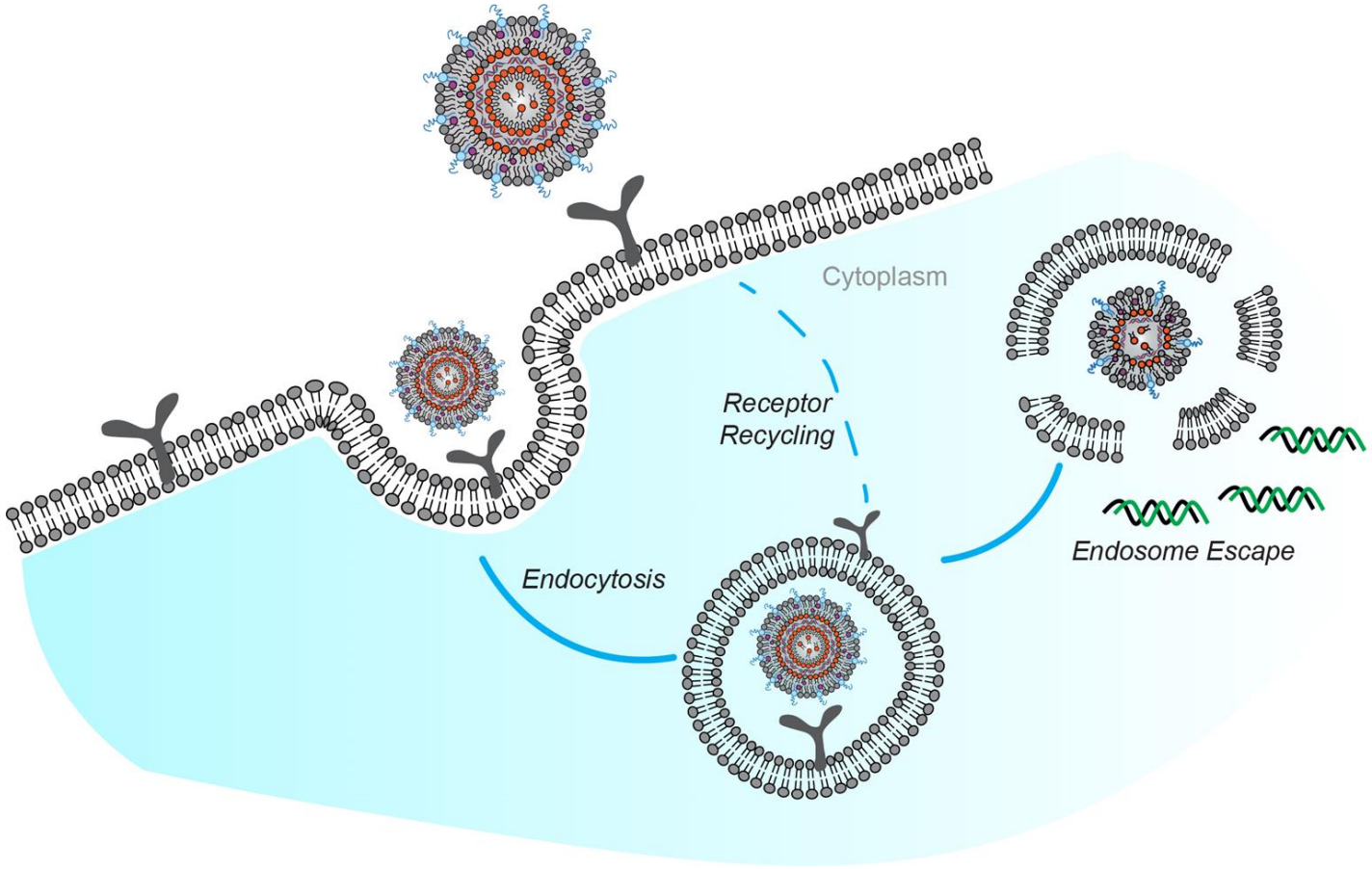

COVID vaccines, 2020
**mRNA**




CRISPR editing
**Cas9 mRNA & sgRNA**
(Phase III trial, 2024)

Kulkarni et al., Nat. Nanotechnol., 2021; Kulkarni et al., Acc Chem Res, 2019

# Unsolved questions remain for LNP intracellular delivery dynamics



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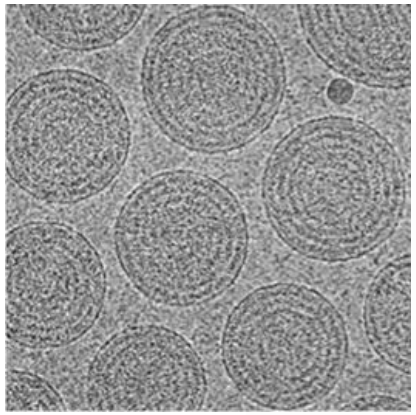
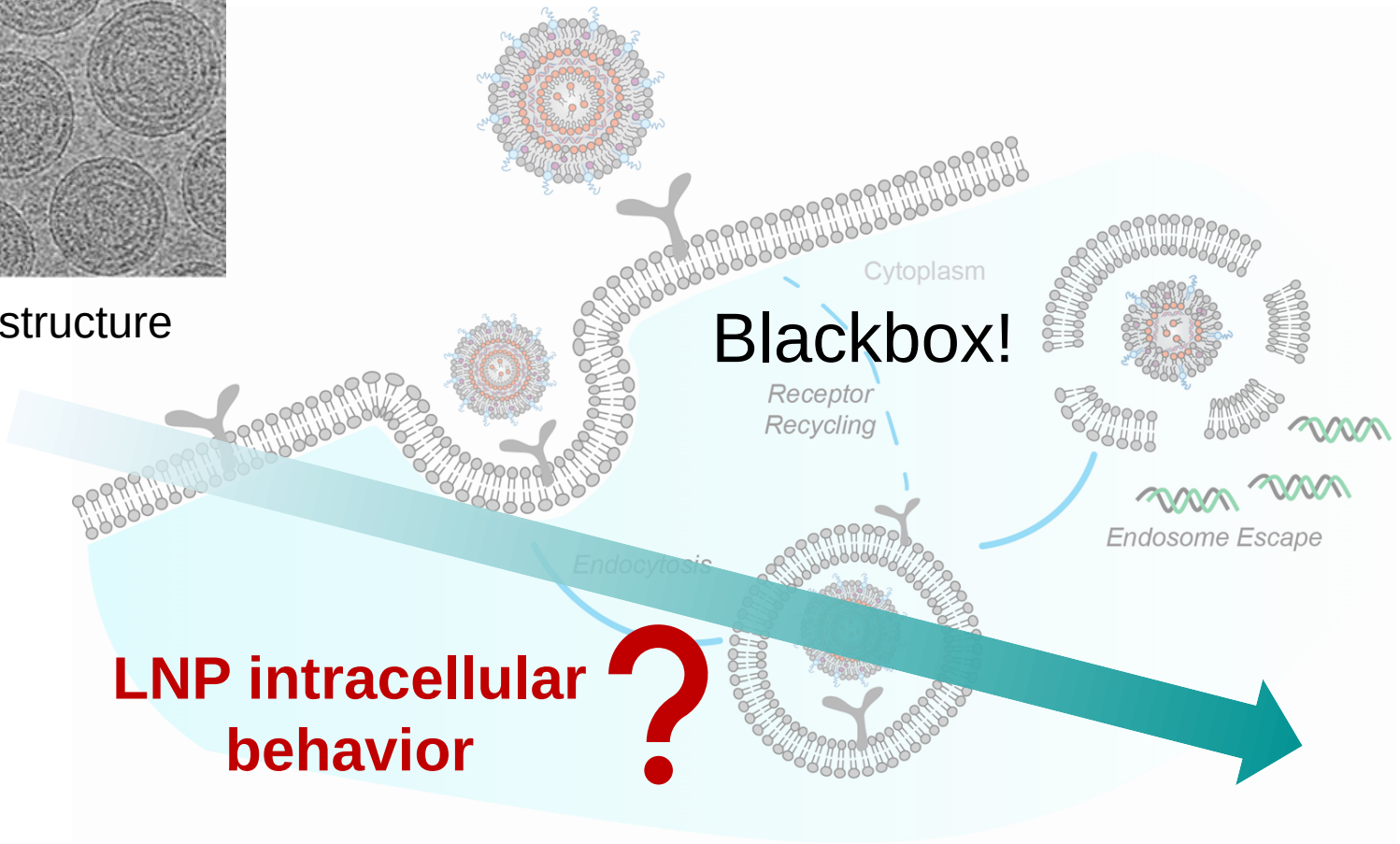
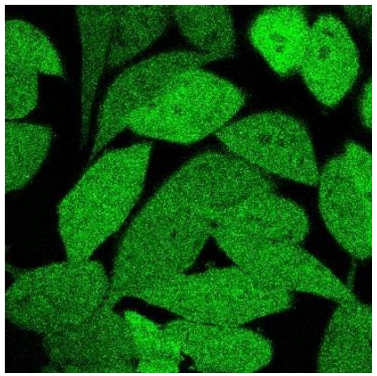


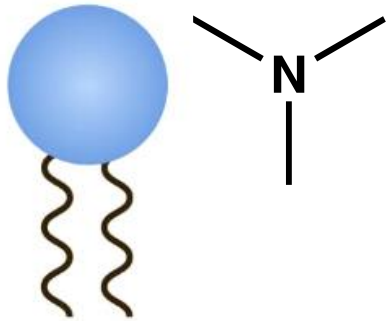
LNP structure



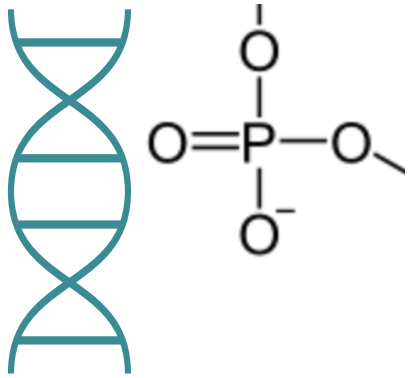

Downstream target expression (efficacy readout)



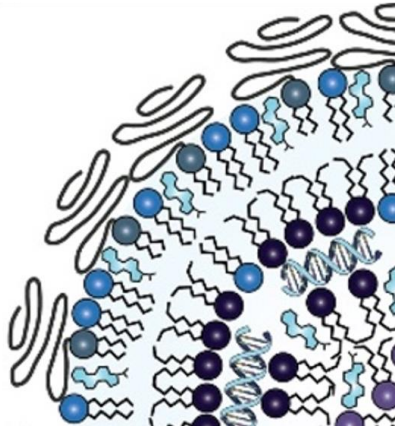
# A way to modulate LNP internal structure: nitrogen to phosphate ratio (N/P ratio)




Ionizable lipid




DNA/RNA



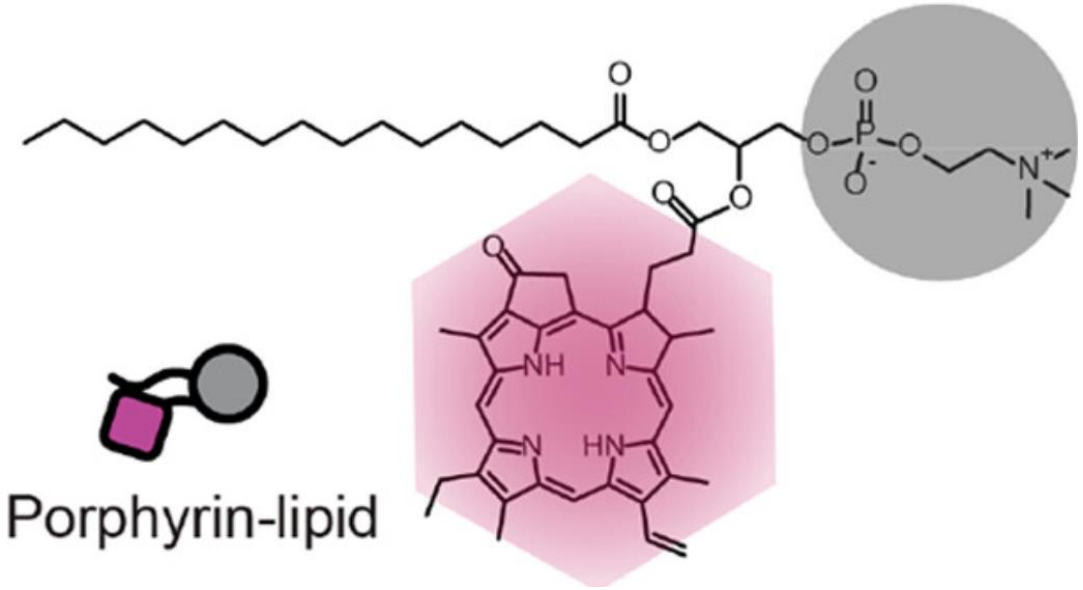
Lipid distribution

N/P ratio changes **helper lipids** distribution in LNP
(Kulkarni et al., Nanoscale, 2019)

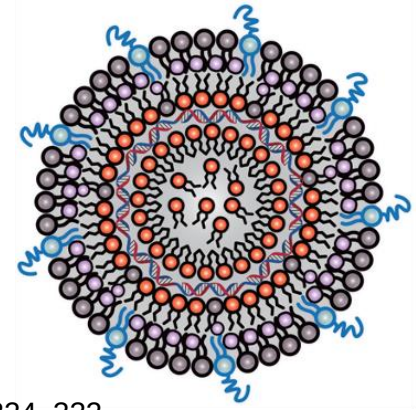
- **High N/P**: more helper lipids on LNP surface

- **Low N/P**: helper lipids partially migrate to core of LNP

# A way to monitor LNP intracellular behaviors: a functional, trackable helper lipid – porphyrin lipid




- **A fluorescent lipid**
- **Photosensitizer**: generate reactive oxygen species (ROS) upon light irradiation

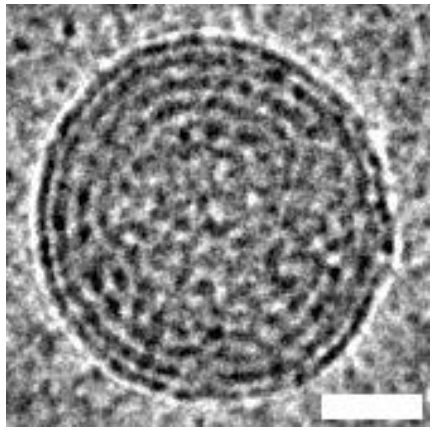


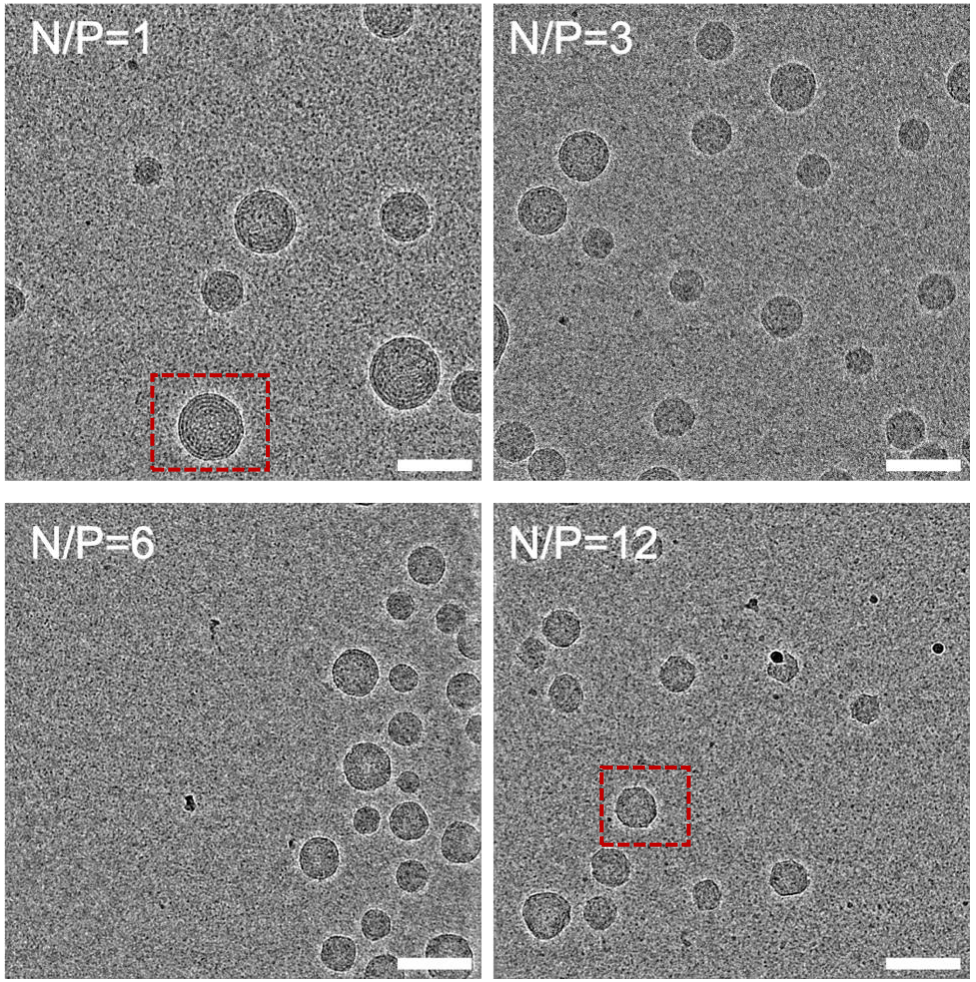
| Porphyrin-LNP Molar Ratios | | | | |
|---|---|---|---|---|
| **Dlin-MC3-DMA** | **porphyrin-lipid** | **DSPC** | **Chol** | **DMG-PEG** |
| **50** | **10** | **0** | **38.5** | **1.5** |

*Nature Mater* **2011**, *10* (4), 324–332.

# Cryo-EM is applied to visualize the structure of LNP at varying N/P ratios

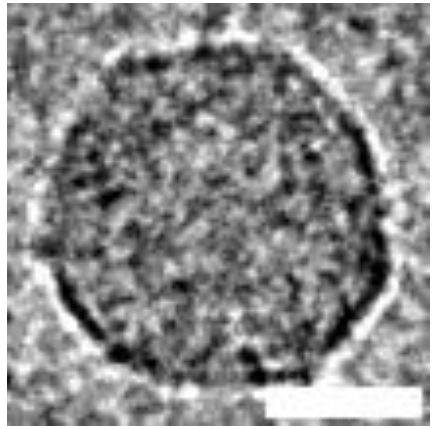
N/P=1



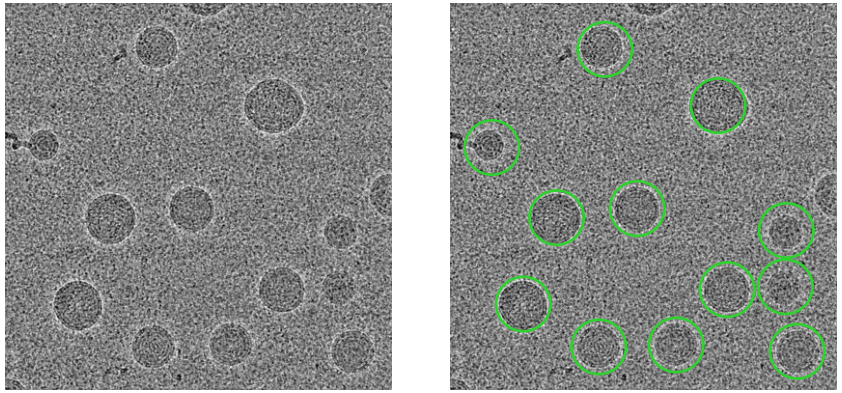



CryoEM is often used to visualize morphology of individual LNP particles but are these features representative?

N/P=12

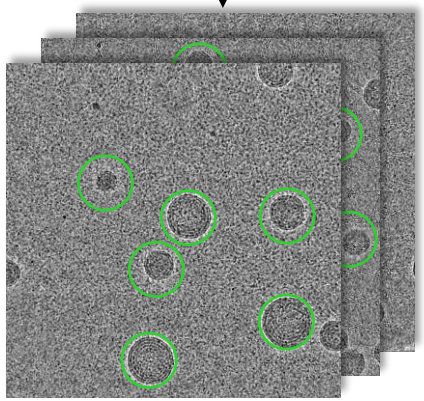


# Machine learning-enabled selecting and extracting particle images from Cryo-EM images: 2d-classification algorithm



Manual particle picking and crYOLO model training

***Thousands imges***

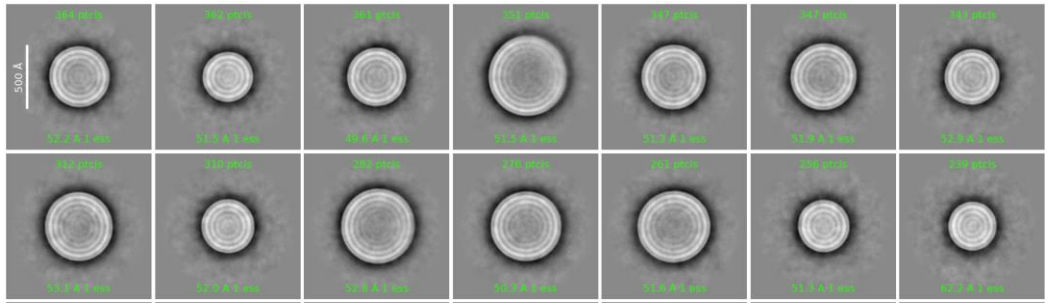


Automatic particle picking across all datasets, followed by manual curation

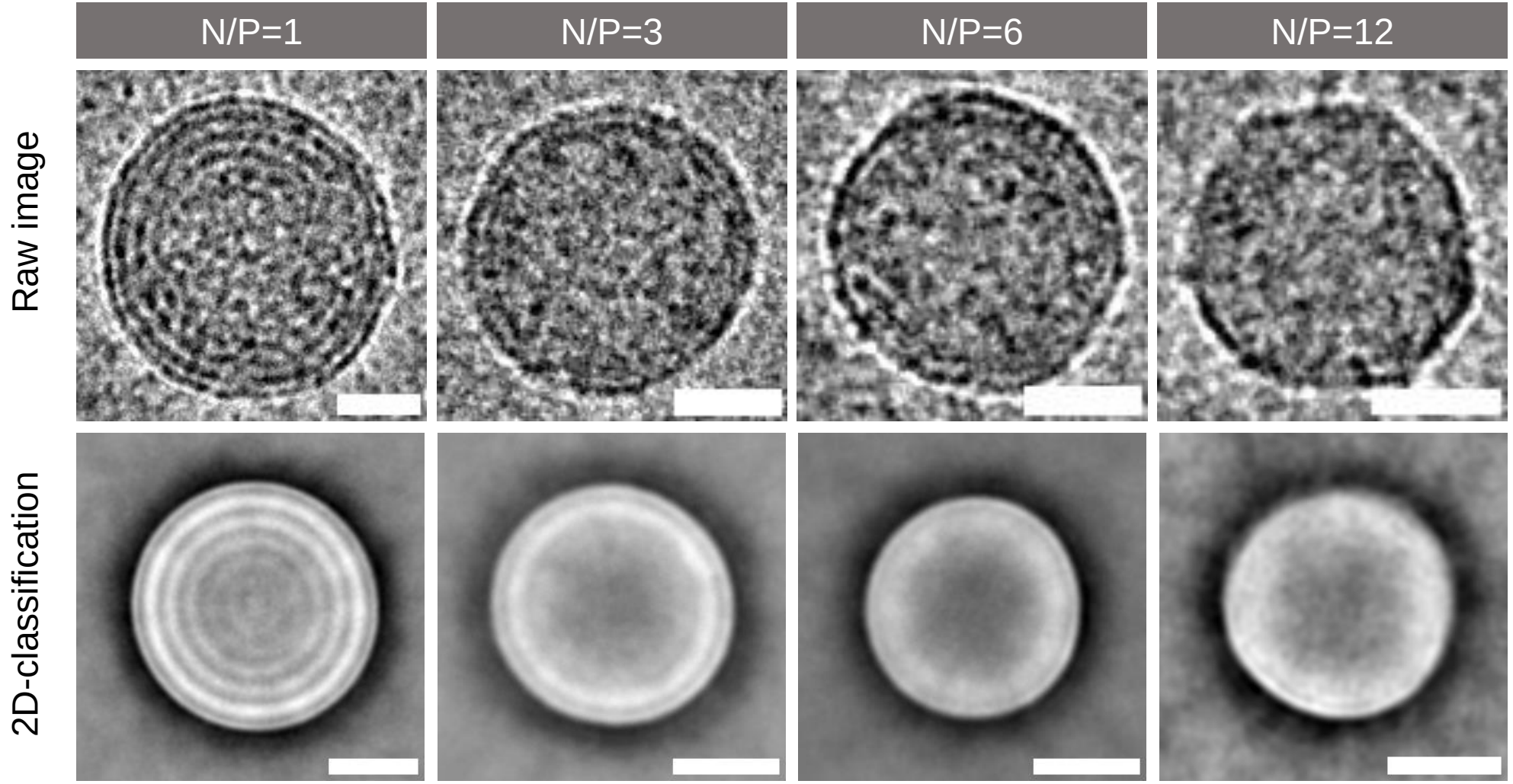
cryoSPARC import, downsampling, initial 2D-classification

Drop bad particles. Particles re-extraction, re-centering, no downsampling

Final 2D-classification



# Cryo-EM and 2d-classification revealed lipid-siRNA organization



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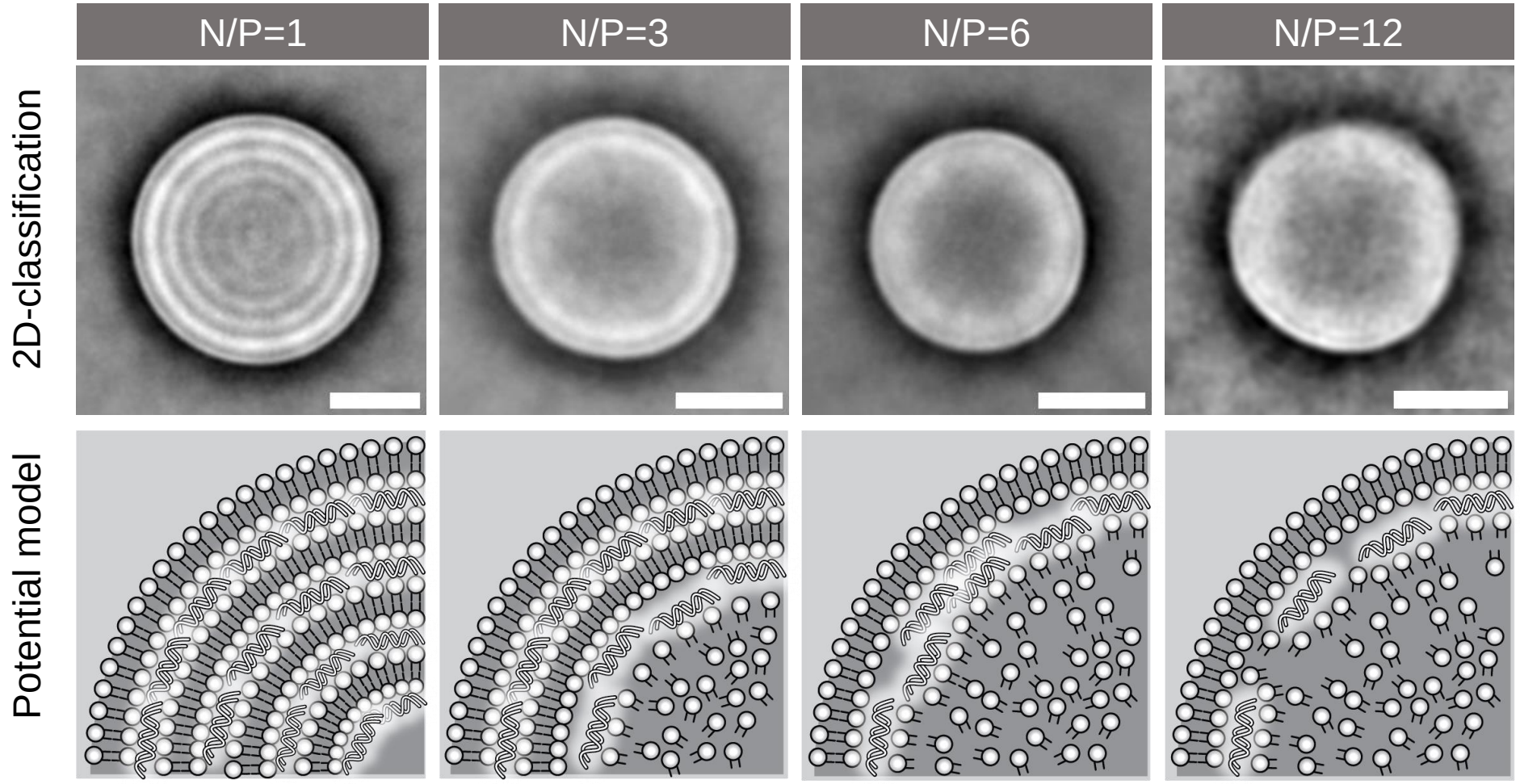
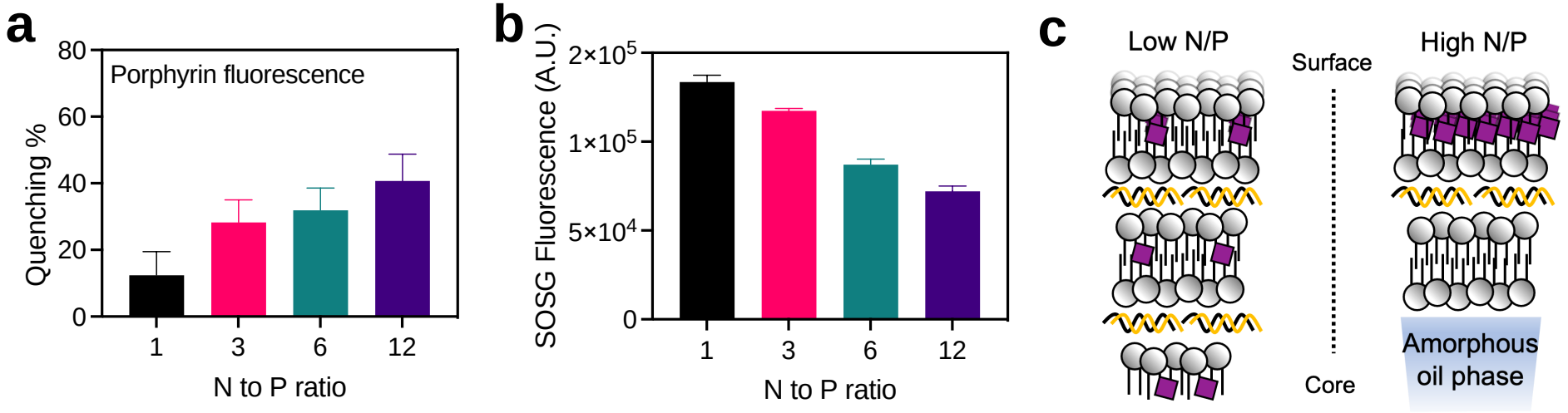



**Regardless of the siRNA payload, RNA preferentially localizes near the LNP surface**

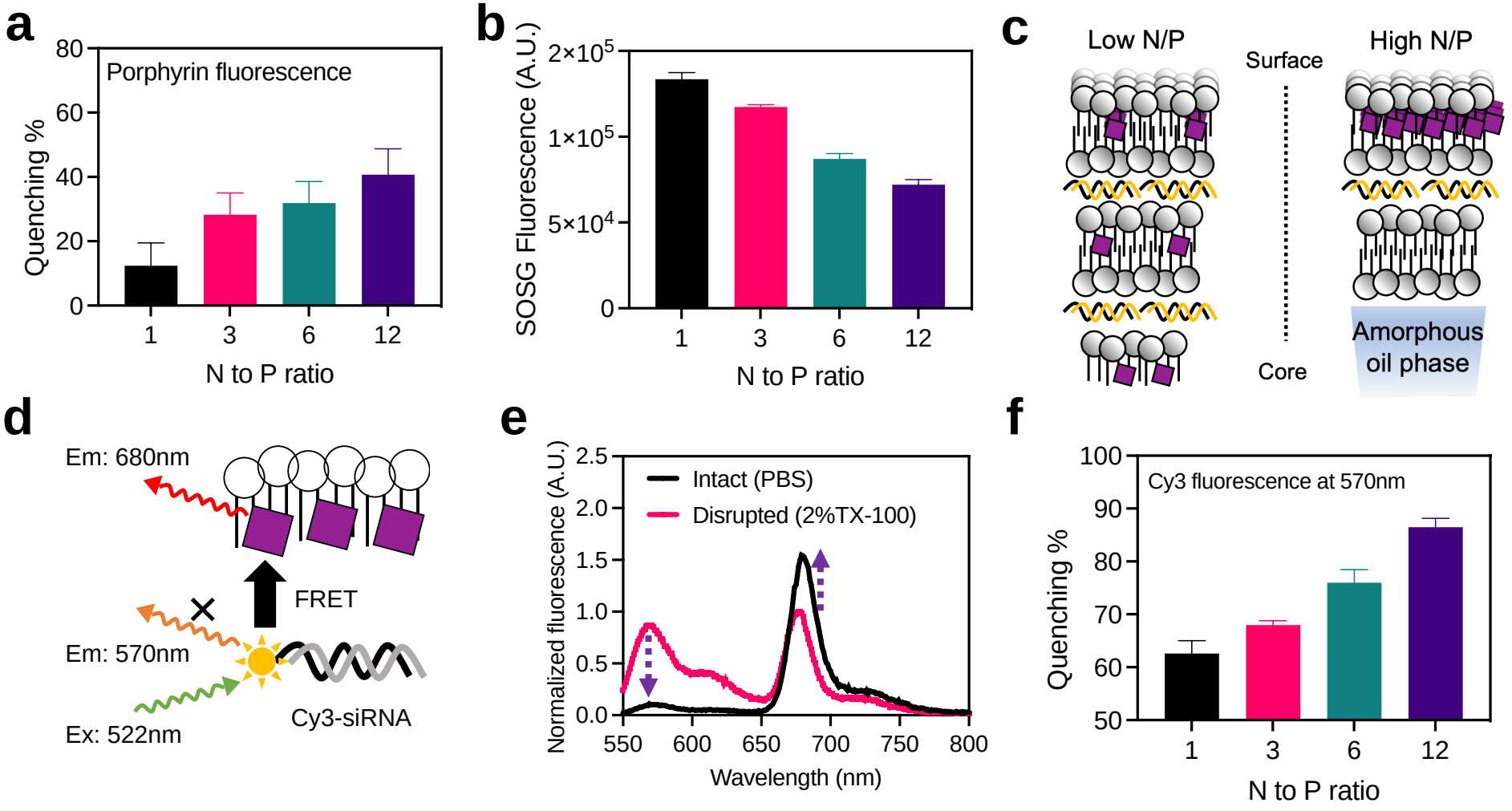
# Photochemical assays confirmed helper lipids migrating to the surface as N/P ratios increased




Higher N/P, stronger fluorescence quenching & less singlet oxygen generation

Porphyrin-lipid enrichment on LNP surface

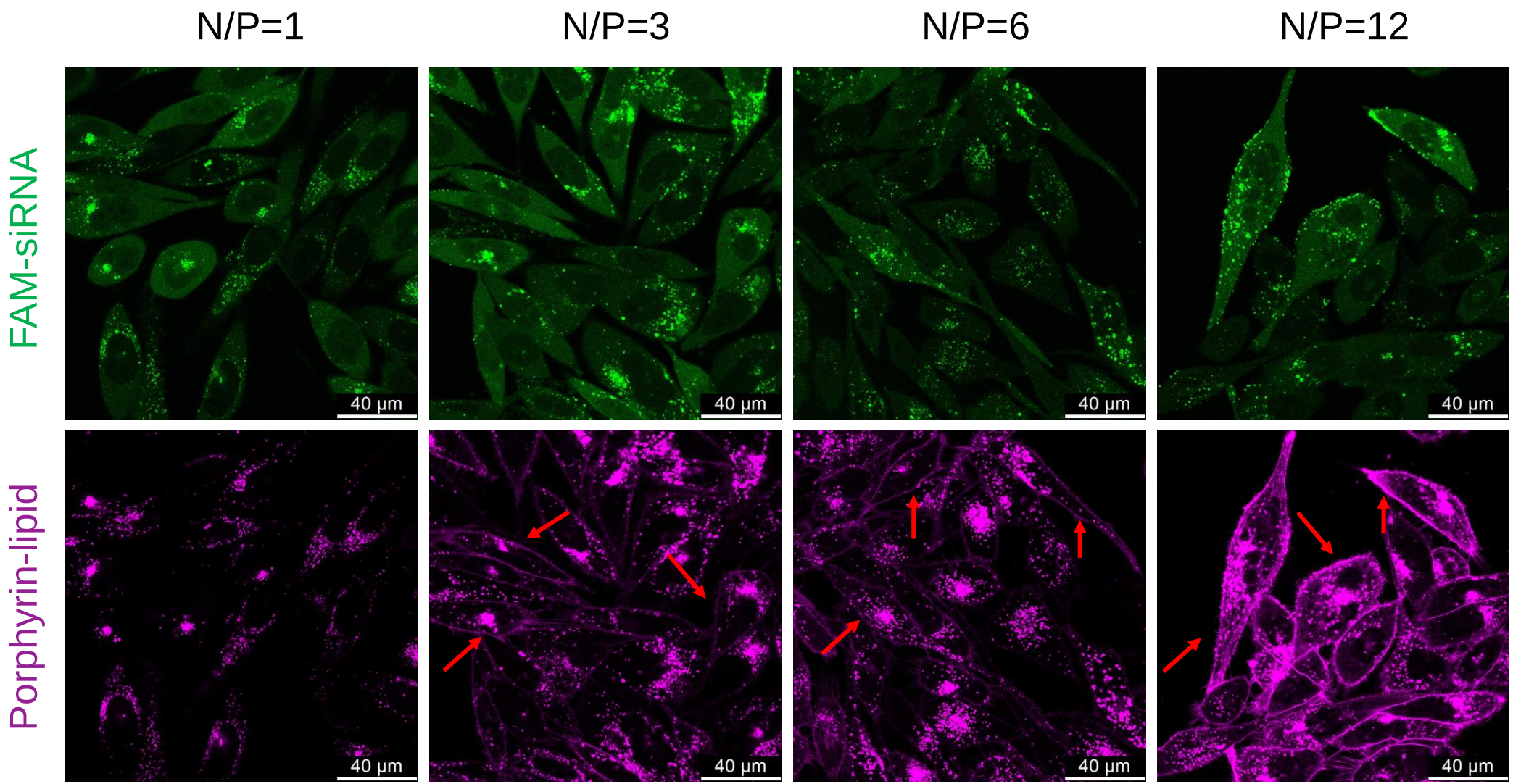
# Photochemical assays confirmed helper lipids migrating to the surface as N/P ratios increased




Higher N/P, stronger cy3 Fluo. quenching

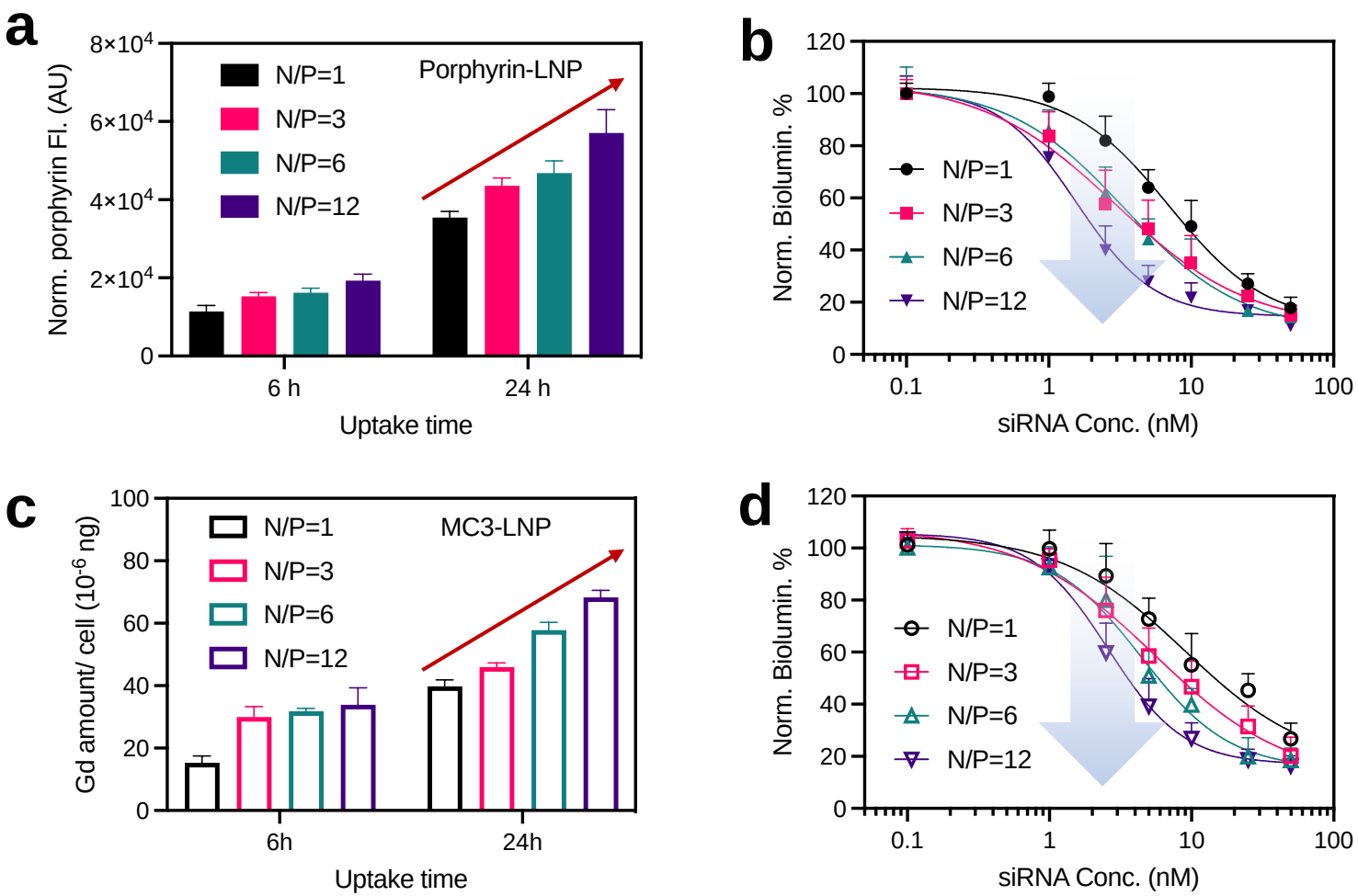
Closer siRNA-porphyrin distribution

# Lipid-siRNA organization impacts LNP interaction with cell membrane




All formulation Conc. porphyrin = 2uM, incubated with PC3-Luc6 for 6h

# Lipid-siRNA organization impacts LNP intracellular uptake amount and siRNA knockdown efficacy

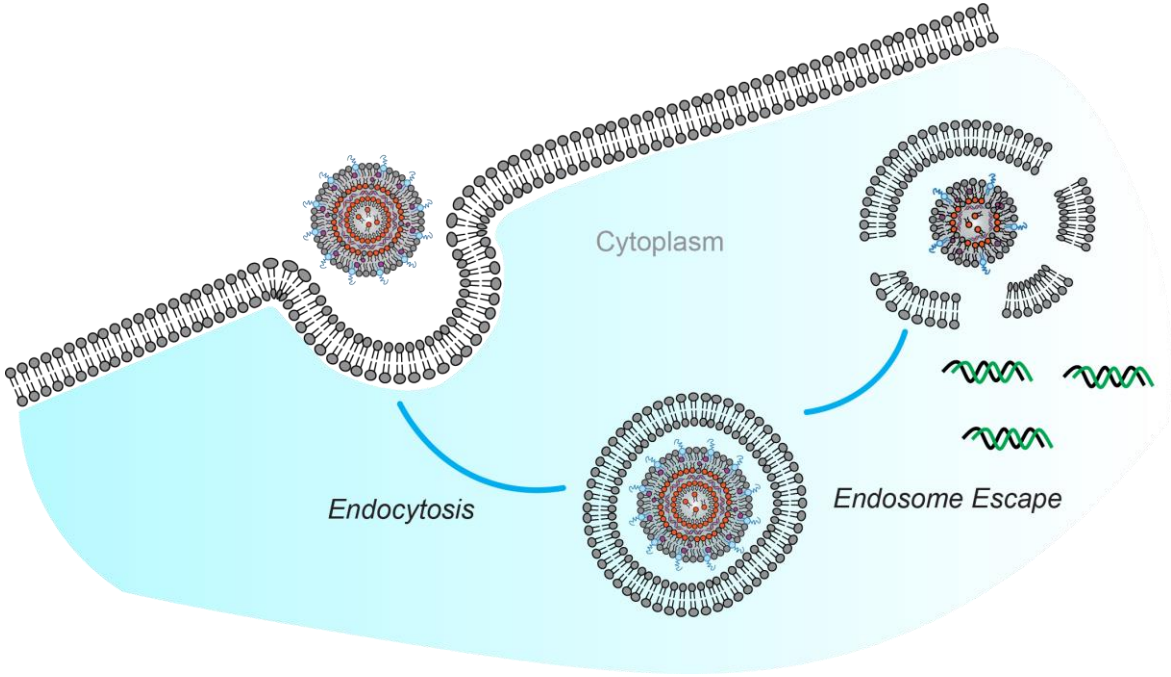



Porphyrin-LNP

MC3-LNP

✓ Higher N/P ratio --- higher intracellular uptake and better siRNA knockdown efficacy

# Does lipid-siRNA organization also impact LNP subcellular behavior?




- Porphyrin-lipid translocate to endosomal membrane
- ROS disrupted endosomal membrane, allowing siRNA endosomal release

ACS NANO
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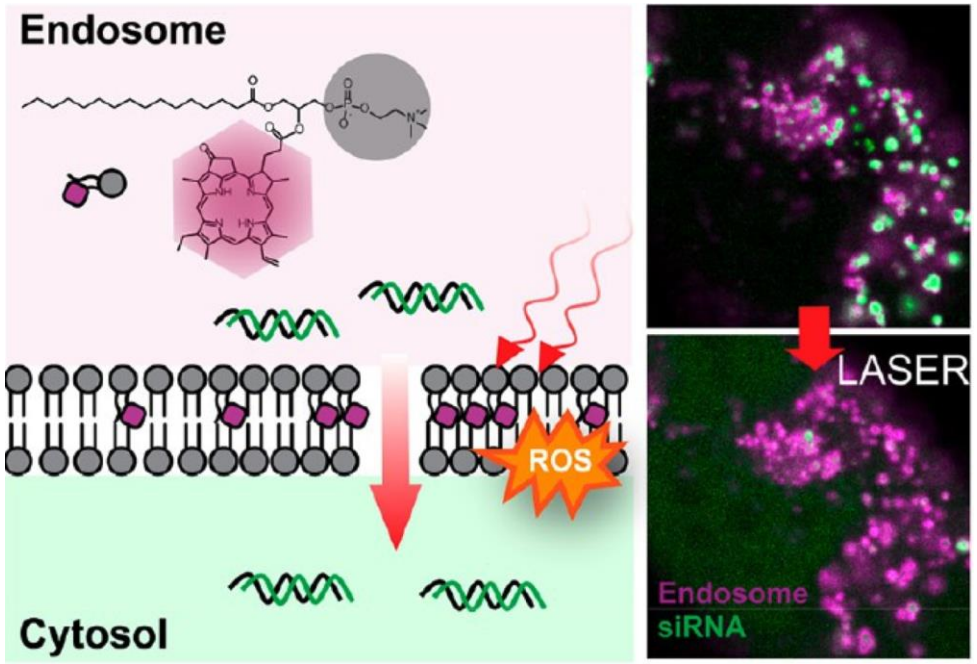
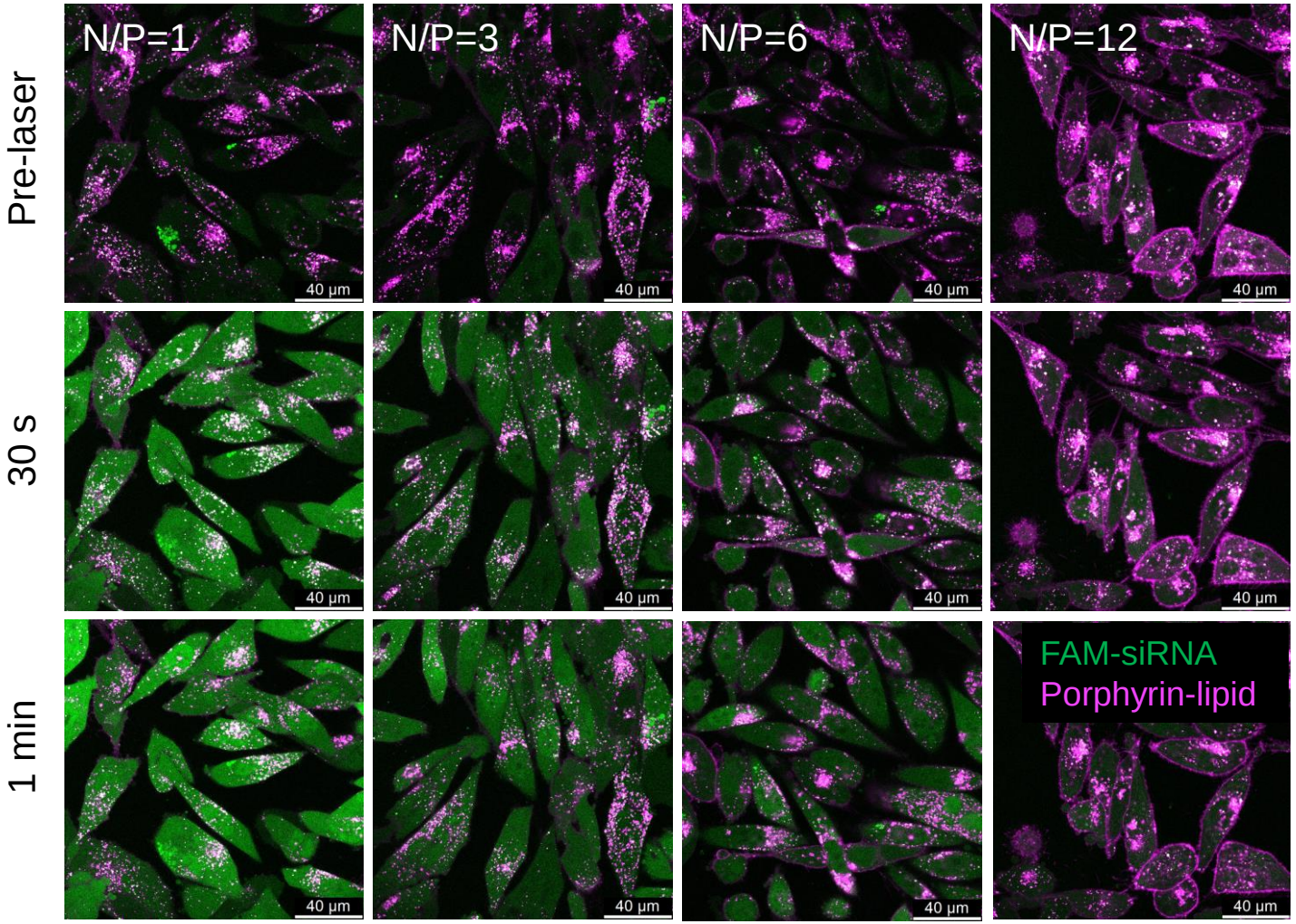
## Light-Activated siRNA Endosomal Release (LASER) by Porphyrin Lipid Nanoparticles

Yulin Mo, Miffy H. Y. Cheng, Andrew D'Elia, Katie Doran, Lili Ding, Juan Chen,* Pieter R. Cullis, and Gang Zheng*

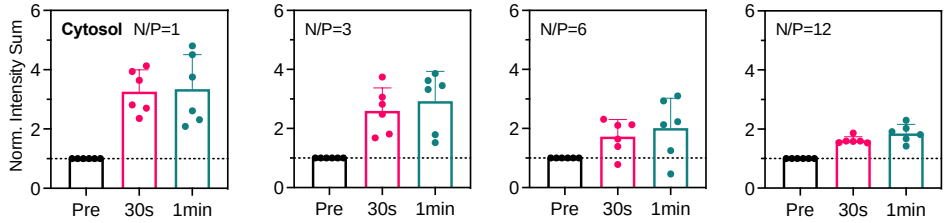
Cite This: *ACS Nano* 2023, 17, 4688–4703 Read Online



# Lipid-siRNA Organization significantly affects light-activated siRNA endosomal release




**Cytosolic** FAM-siRNA intensity change




**3.26-fold** **2.60-fold** **1.73-fold** **1.62-fold**

✓ NP1 exhibited strongest FAM-siRNA endosomal escape upon light

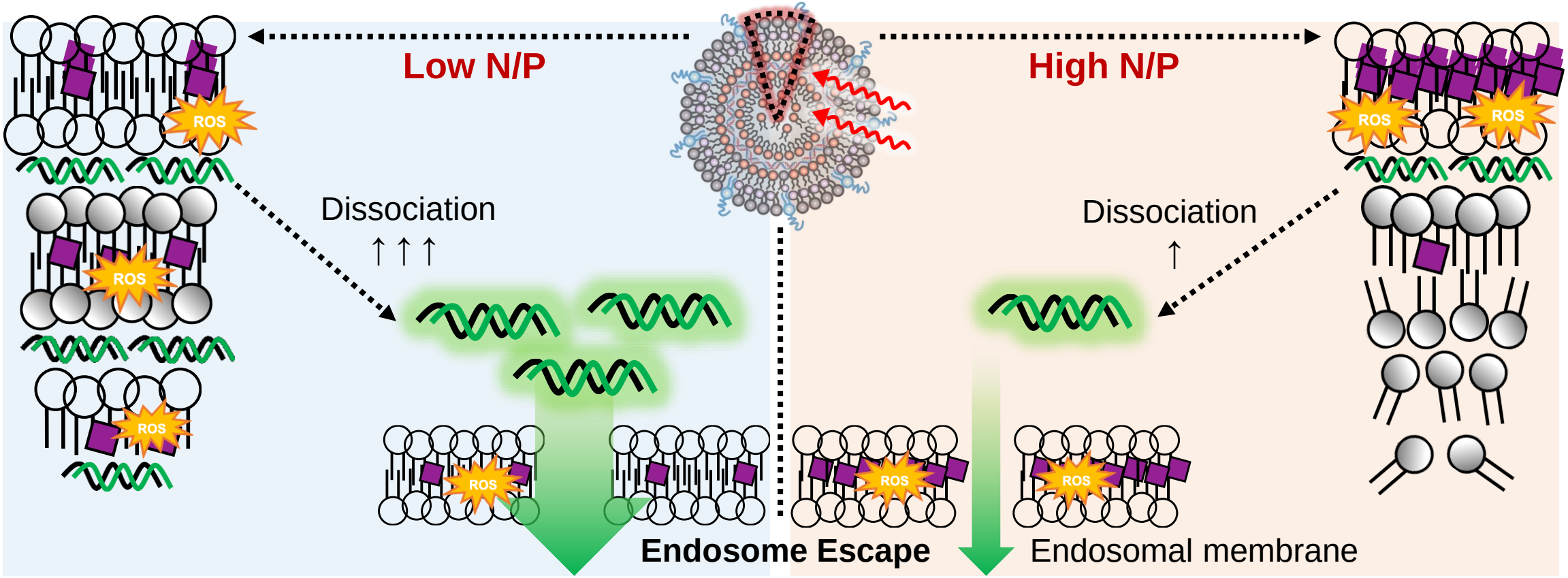
**Organelle** FAM-siRNA intensity change

| | 1 | 3 | 6 | 12 |
|---|---|---|---|---|
| 30s ↑ | **4.04** | **3.74** | **2.34** | **1.93** |
| 1min ↓ | 2.02 | 1.05 | 1.43 | 1.35 |

✓ FAM signal in ***organelles*** showed a ***"light-up, then decay"*** pattern

All formulation **Conc. porphyrin = 2uM.** LNPs have same amount of FAM-siRNA

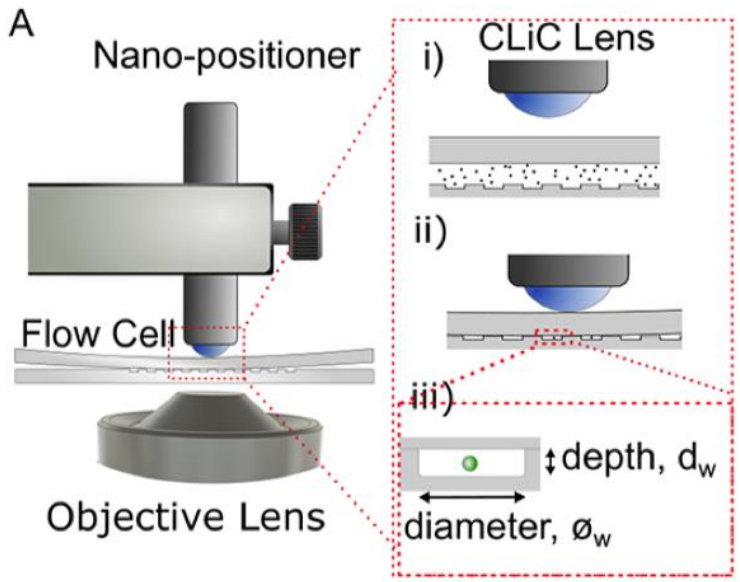
# Potential mechanism on lipid-siRNA organization affecting LASER and LNP dissociation



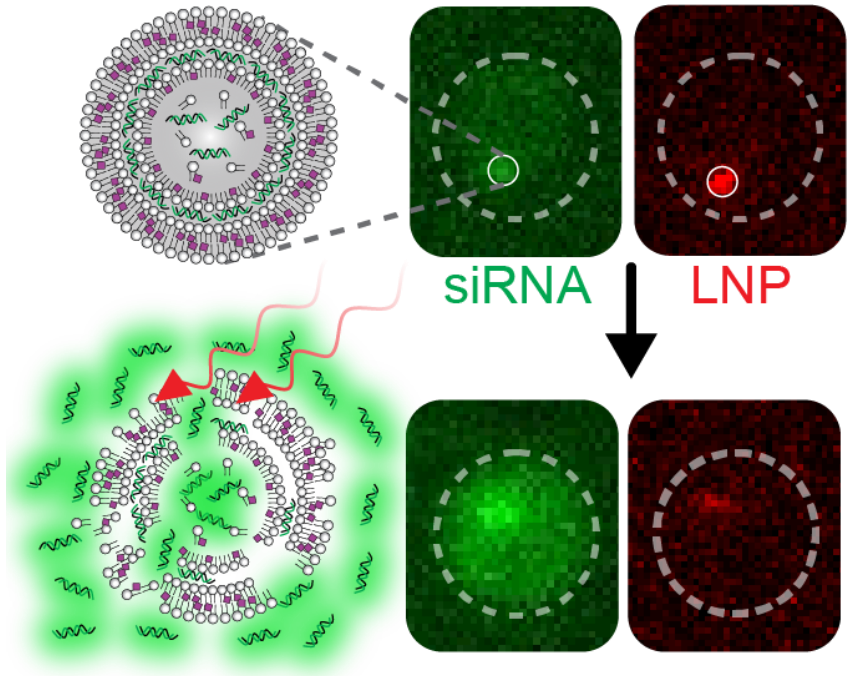

**Current knowledge:** **Endosomal escape step** is the most critical in LNP efficacy

**Our results:** **LNP dissociation** and **siRNA availability** are also limiting factors

# Can we dissect the intracellular observations and evaluate LNP dissociation directly at *single particle level?*




Single LNP imaging by Convex Lens-induced Confinement (CLiC) microscopy



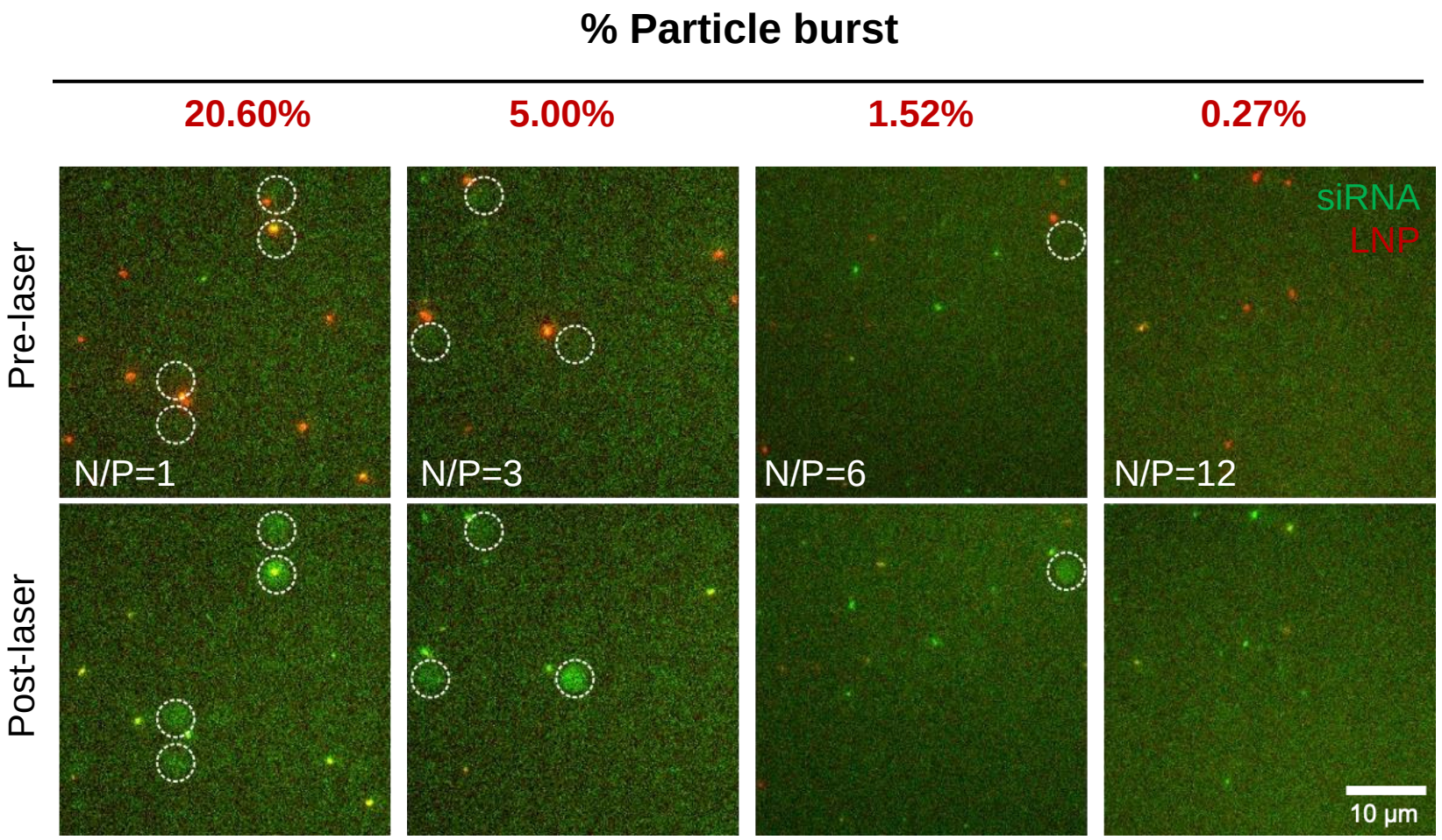

Dual-colour imaging of siRNA and LNP

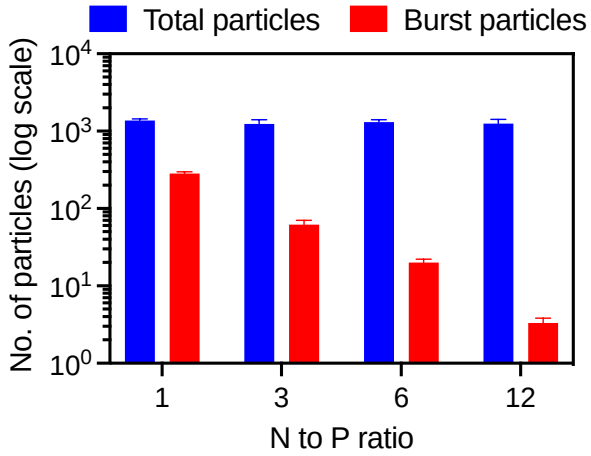
LNP dissociation siRNA release to well

**Real-time tracking** of **single LNP** dissociation upon light

In collaboration with **Dr. Sabrina Leslie** at University of British Columbia

# Lipid-siRNA organization directly impacts light-activated LNP dissociation



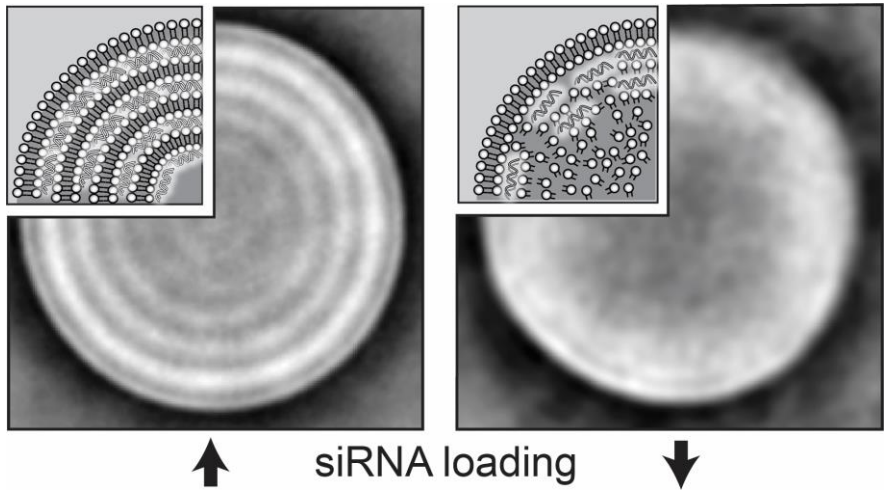

- ✓ **NP1** exhibited ***76.9*** times more burst events compared to **NP12**

Porphyrin-LNP at low N/P **dissociate more** upon light

LNP burst setting:
647nm laser, 4.2mW, 40s

# Summary

**Lipid-siRNA organization (Structure)**




- Interaction with cell membrane
- Intracellular uptake amount
- siRNA endosomal escape
- Responsiveness to external stimuli

**LNP intracellular dynamics (Function)**

**Impact:**

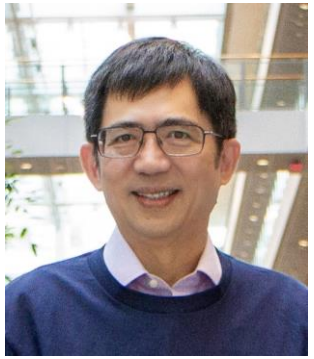
1) Develops novel imaging pipelines for LNP internal structures using Cryo-EM and 2d-classfication.

2) Identifies LNP dissociation as an overlooked factor in endosomal escape.

3) Provides rational design principles for optimizing LNP-based RNA delivery systems.

# Acknowledgement

## Zheng lab members



**Dr. Gang Zheng**

## Collaborators

Jafari lab

- Dr. Alexander Keszei
- Dr. David Dai
- Dr. Mohammad T. Mazhab-Jafari

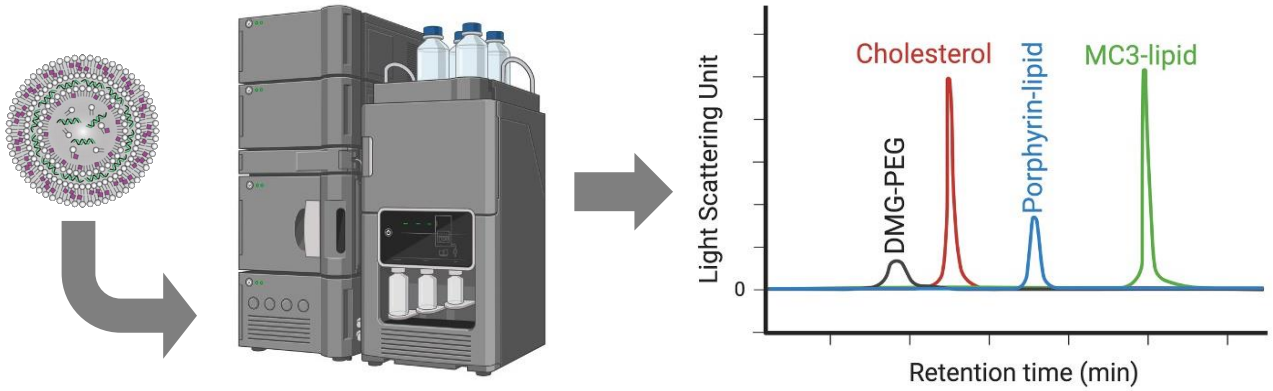
Leslie lab

- Shagun Kothari
- Dr. Albert Kamanzi
- Dr. Sabrina Leslie

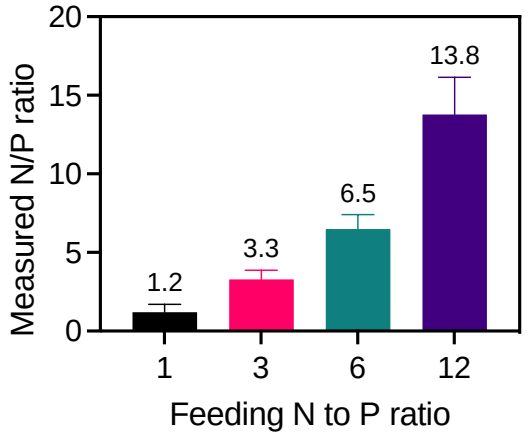
## Funding support

# Porphyrin-LNPs at N/P Ratios 1, 3, 6, and 12 were synthesized and Validated

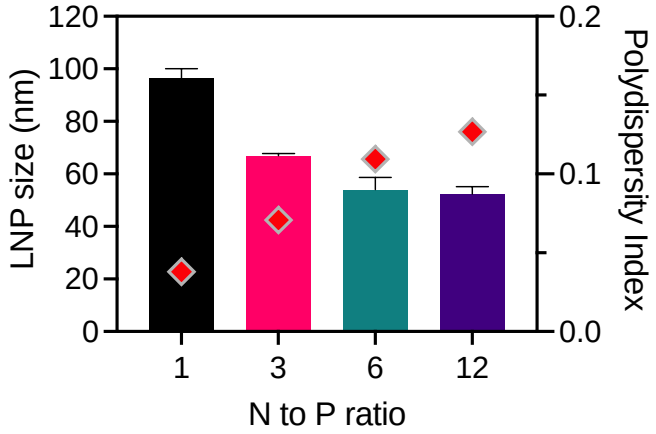
## Lipid quantification by UPLC-ELS



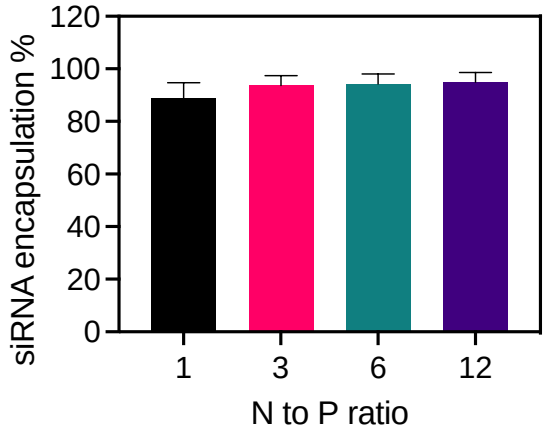

## Measured N/P ratio




## Size



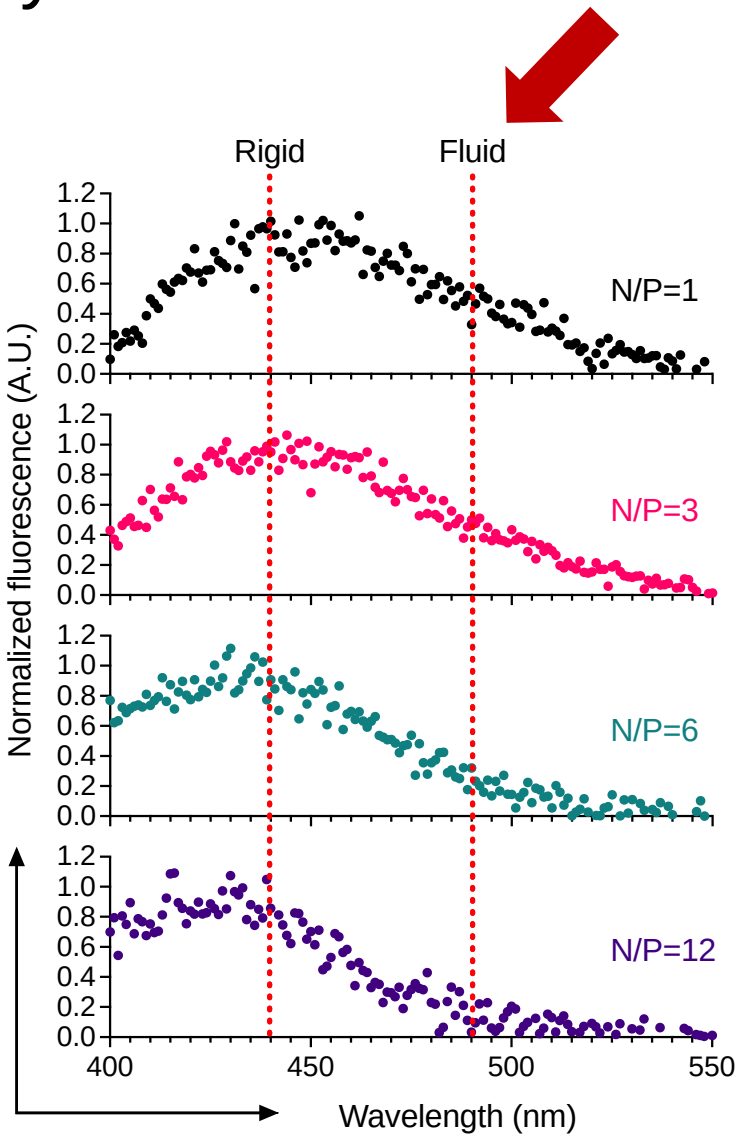

## siRNA loading




- ✓ Actual N/P ratio is closed to designed ratio
- ✓ N/P ratio increase, LNP exhibited smaller size; no impact on siRNA encapsulation efficiency

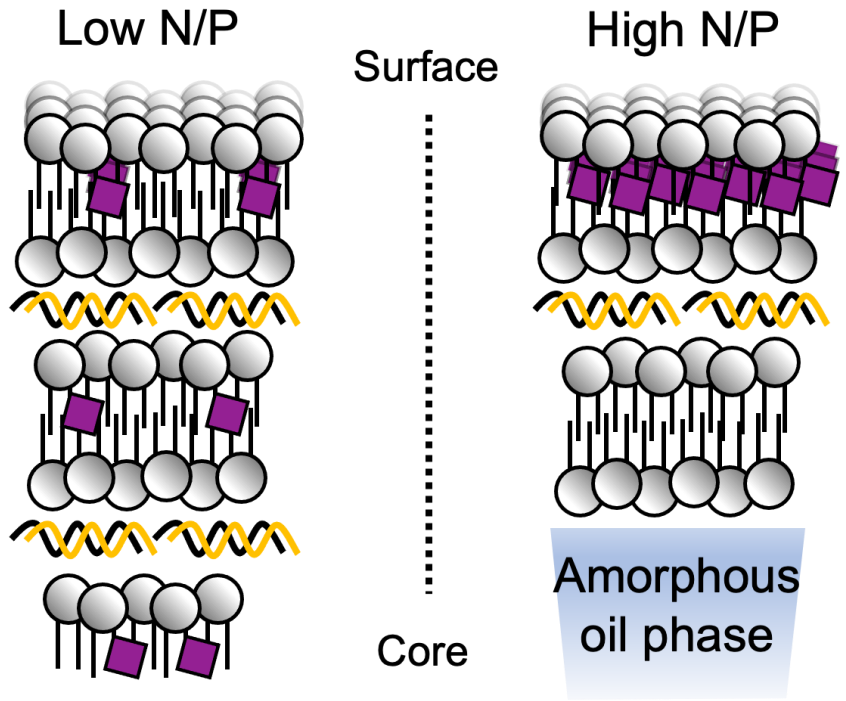
Mo et al., J. Am. Chem. Soc., 2025

# At higher N/P (less siRNA per LNP), porphyrin-LNP membrane fluidity is lower as measured by C-laurdan assay

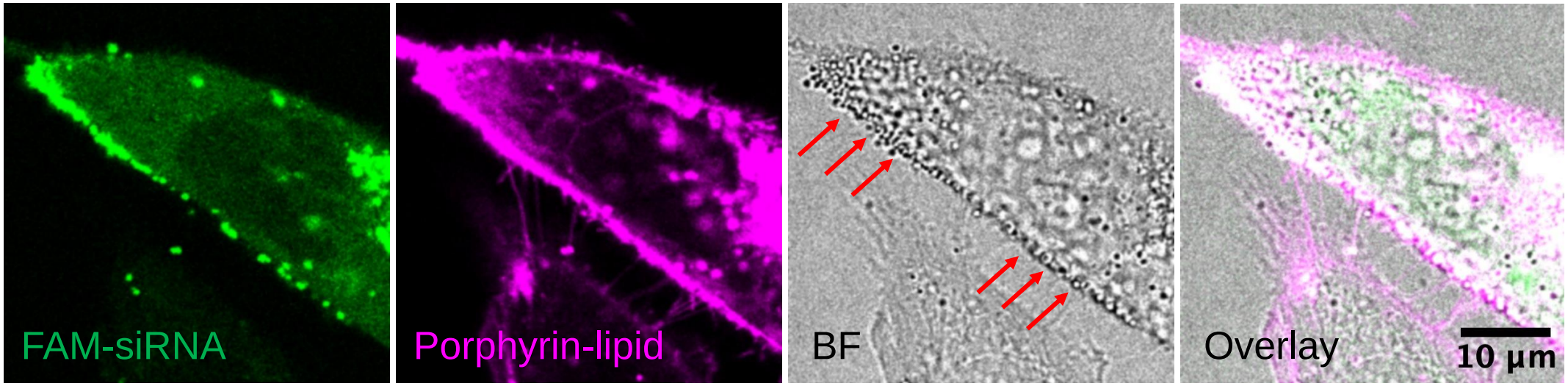


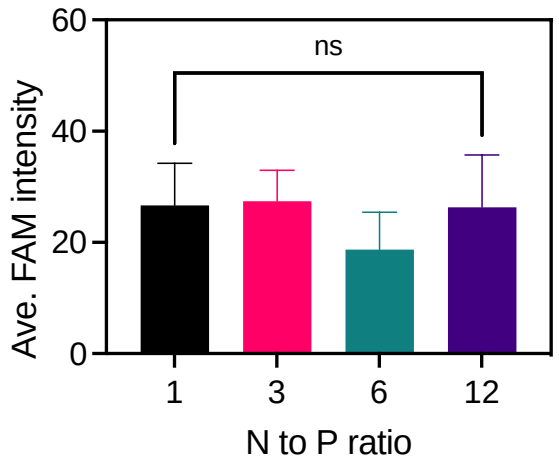

**High** membrane fluidity

**Low** membrane fluidity



# Vesicular structures forming on the cell membrane at N/P=12, supporting LNP binding and potentially membrane fusion



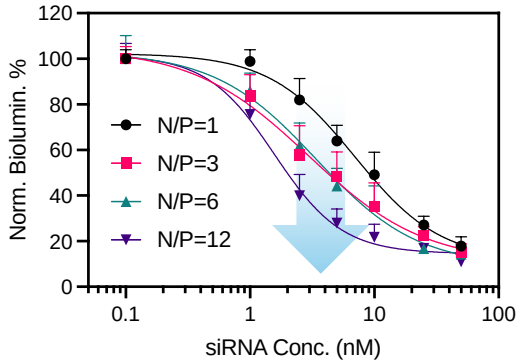




✓ Stronger cytosolic siRNA delivery at higher N/P

# Reconsider what factors contributing to LNP-RNA efficacy

**Current knowledge**

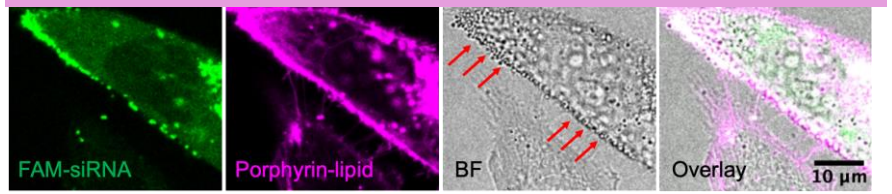
Higher N/P ratio, LNP has better efficacy



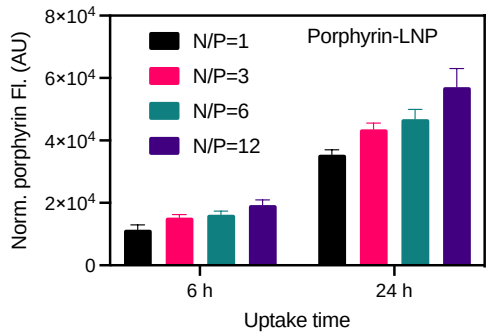

***Excessive ionizable lipids***
Stronger interaction with endosomal membrane
Stronger endosomal escape

**Novel ionizable lipids**

Cell membrane fusion; cytosolic siRNA delivery




Enhance uptake




***Lipid-RNA organization***