SUPPLEMENTARY INFORMATION

PINPOINT enables non-destructive nanoscopic quality control of individual lipid nanoparticles and their mRNA cargo

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TABLE OF CONTENTS

Table of Contents ……1

Supplementary Figures……4

Supplementary Fig. 1 | Representative RiboGreen imaging frames for mRNA detection in LNP samples ……4

Supplementary Fig. 2 | Local sequence context for probe and DNA-tiling design at the middle-region target site in mRNA1 and mRNA2……5

Supplementary Fig. 3 | Workflow for surface capture and imaging of bare mRNA and LNPs…….6

Supplementary Fig. 4 | Analysis pipeline for PINPOINT single-molecule detection using TIRF microscopy……..….7

Supplementary Fig. 5 | Impact of pre-capture versus co-capture strategies on accepted counts of bare mRNA1……9

Supplementary Fig. 6 | Cumulative dwell time analysis of bare mRNA2 and encapsulated mRNA2 using PINPOINT……...…….……..….10

Supplementary Fig. 7 | Multistep intensity transitions in kinetic fingerprinting traces indicate multiple encapsulated mRNAs per LNP……11

Supplementary Tables…….12

Supplementary Table 1 | Sequences of capture probes, fluorescent probes, and tiling probes…….12

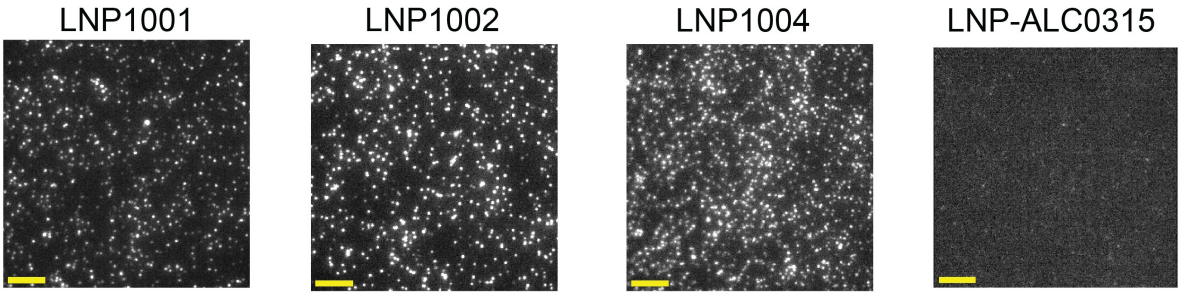
Supplementary Table 2 | mRNA and lipid nanoparticle samples and their concentrations……13

Supplementary Table 3 | Size metrics for lipid nanoparticles (LNPs) measured by DLS and PINPOINT……..…...13

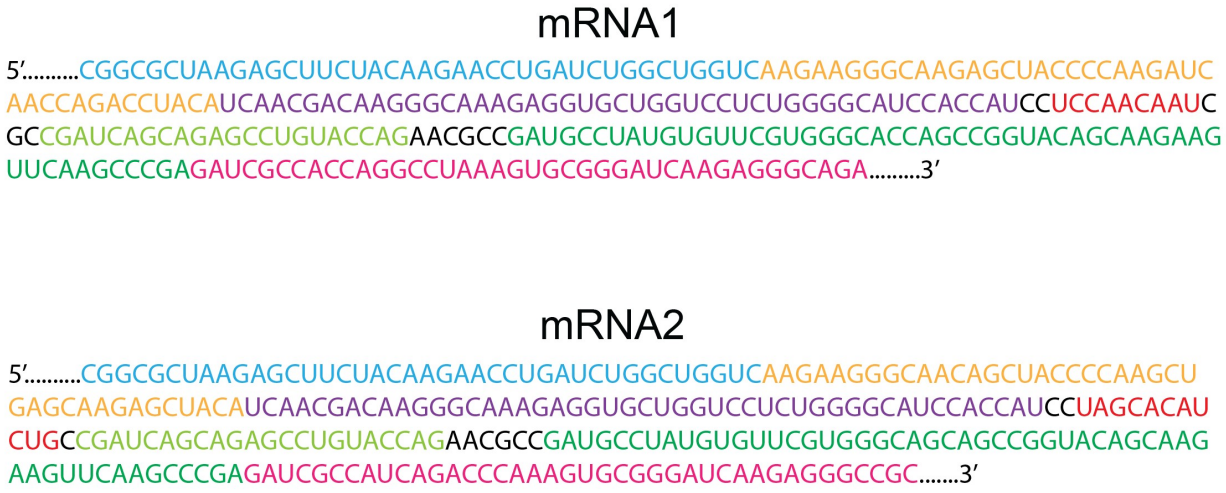
Supplementary Table 4 | General parameter sets for trace generation and analysis……..….14

Supplementary Table 5 | mRNA copy number per lipid nanoparticle determined by PINPOINT……..…15

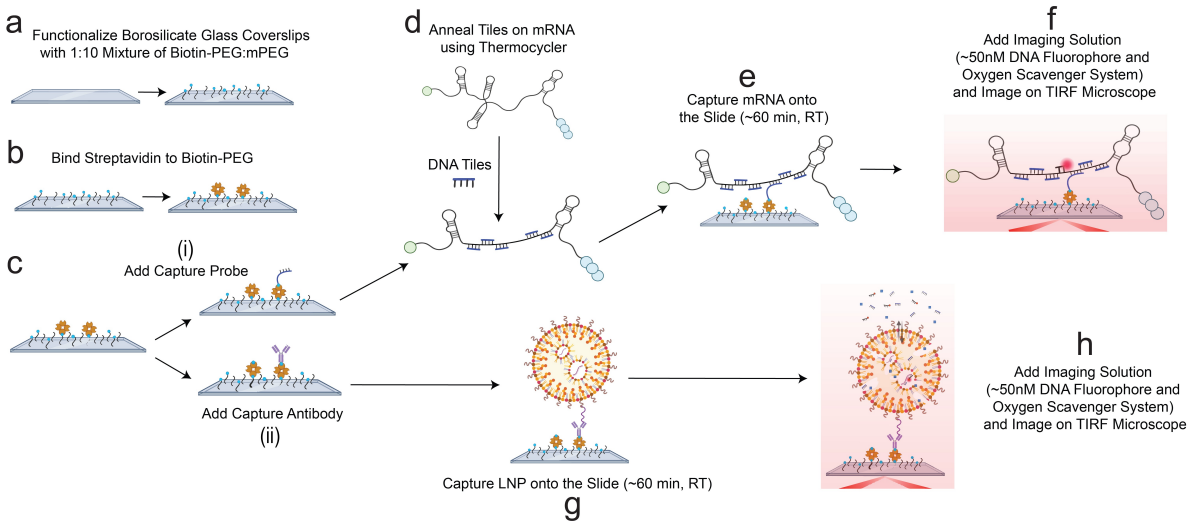
Supplementary Figures



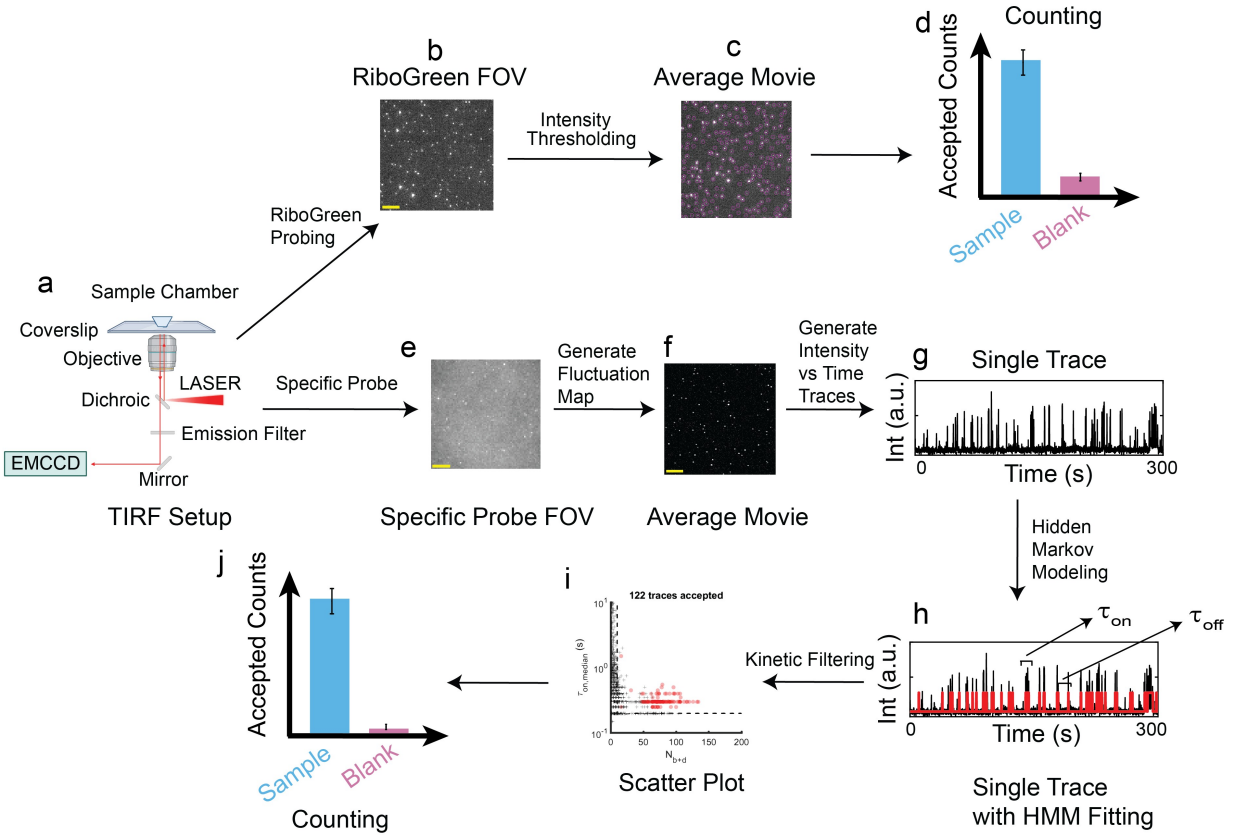
Supplementary Fig. 1 | Representative RiboGreen imaging frames for mRNA detection in LNP samples. Representative TIRF microscopy frames from the single-molecule RiboGreen assay used to assess mRNA-associated fluorescence in LNP1001, LNP1002, LNP1004, and LNP-ALC0315. RiboGreen-positive puncta were observed for LNP1001, LNP1002, and LNP1004, while LNP-ALC0315 showed minimal detectable signal in the representative field of view. Scale bars, 5 μm.



Supplementary Fig. 2 | Local sequence context for probe and DNA-tiling design at the middle-region target site in mRNA1 and mRNA2. mRNA1 and mRNA2 sequences spanning nt 520–782 (5′→3′) are shown, with colour-coded segments marking the RNA regions targeted by the DNA-tiling and capture architecture used for kinetic fingerprinting. Cyan, tile 1 binding region; yellow, tile 2 binding region; purple, tile 3 binding region; red, fluorophore-labelled probe binding region; light green, biotinylated capture probe/tile 4 binding region; deep green, tile 5 binding region; magenta, tile 6 binding region. Colours denote RNA segments complementary to the corresponding DNA tiles/probes. Sequence differences between mRNA1 and mRNA2 within the probed window are apparent within the red probe-target region. Black segments indicate deliberate unprobed gaps (no complementary DNA tile/probe) incorporated between the probe and capture-probe binding sites.

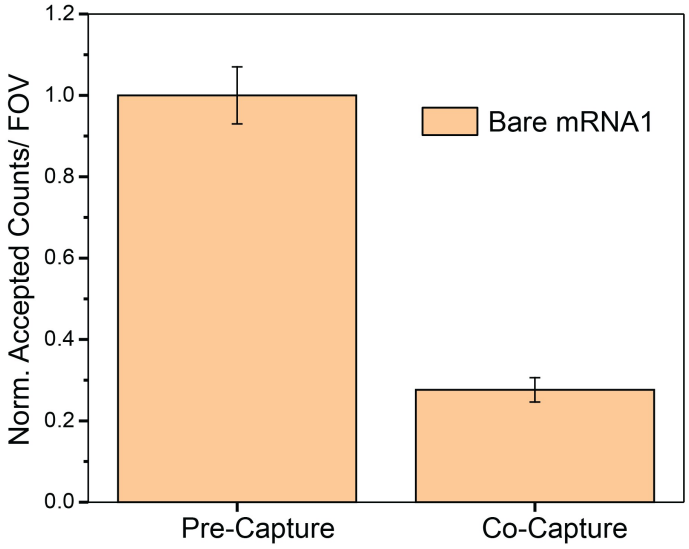


Supplementary Fig. 3 | Workflow for surface capture and imaging of bare mRNA and LNPs. **a,** Functionalization of borosilicate glass coverslips with a 1:10 mixture of biotin-PEG:mPEG. **b,** Binding of streptavidin to biotin-PEG surface. **c,** Addition capture probe: (i) DNA capture probe for bare mRNA, (ii) capture antibody for LNPs. **d,** Annealing DNA tiles onto bare mRNA using a thermocycler. **e,** Capture of bare mRNA onto the slide. **f,** Single-molecule imaging of surface captured bare mRNAs with DNA fluorophores in TIRF microscopy. **g,** Capture of LNP onto the slide. **h,** Single-molecule imaging of surface captured LNPs with DNA fluorophores in TIRF microscopy after achieving controlled permeability by optimized concentration of TX-100.

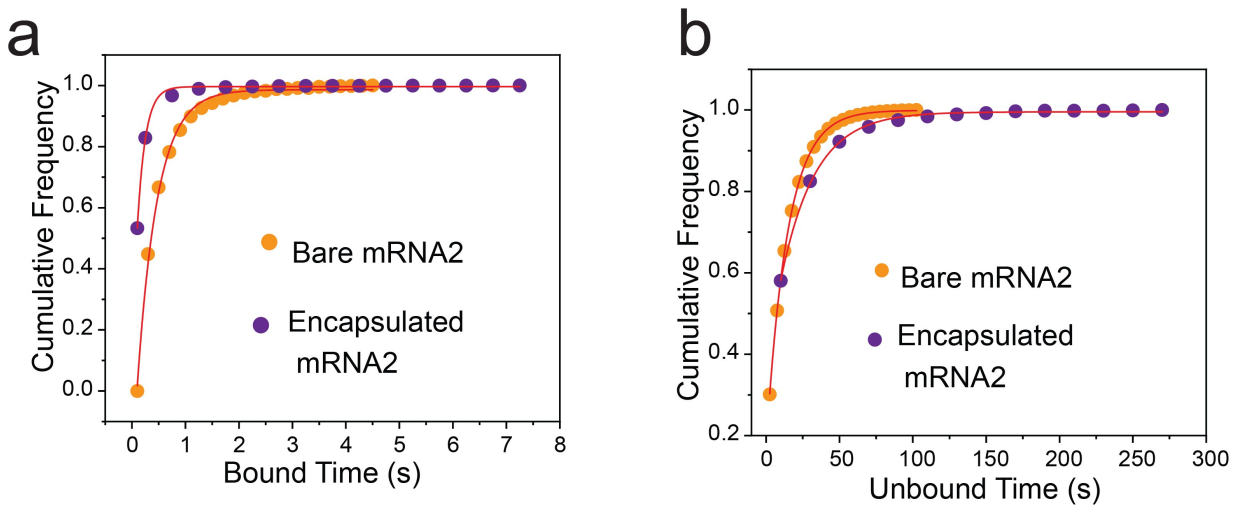


Supplementary Fig. 4 | Analysis pipeline for PINPOINT single-molecule detection using TIRF microscopy. a, Schematic of single-molecule TIRF setup. **b–d,** Analysis of RiboGreen movies: **b,** Single-frame images of representative field of view (FOV) from TIRF microscopy (scale bar: 5 µm). **c,** Average movie after intensity thresholding. **d,** Accepted counts in sample and blank chambers after intensity thresholding. Black error bars represent the standard errors of the mean from three independent replicates. **e–j,** Analysis pipeline for imaging with mRNA-specific probes: **e,** Single-frame images of representative FOV from TIRF microscopy (scale bar: 5 µm). **f,** Intensity fluctuation maps of the fields of view shown in (e), with candidate ROIs identified for further analysis. **g,** Representative intensity versus time traces generated from ROIs in (f). **h,** Hidden Markov modelling (HMM) idealization (red lines) for each intensity-versus-time trace in (g), with bound and unbound dwell times ($\tau_{on,med}$ and $\tau_{off,med}$) indicated. **i,** Scatterplots of dwell

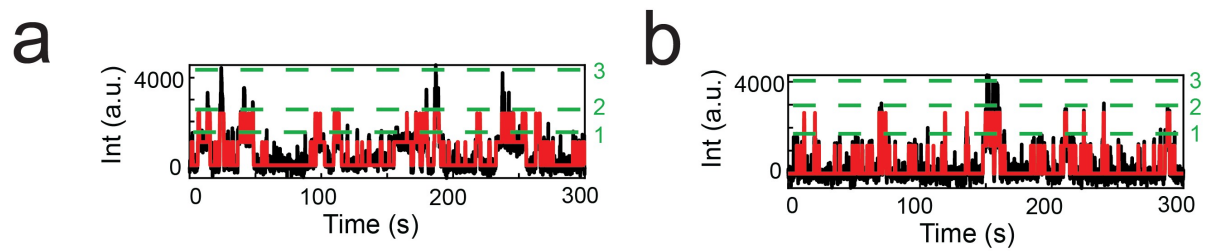
time analysis ($\tau_{on,med}$ versus $\tau_{off,med}$) for all intensity-versus-time trajectories in the field of view. **j,** Accepted counts in sample and blank chambers after kinetic filtering, with black error bars representing standard errors of the mean from three independent replicates.



Supplementary Fig. 5 | Impact of pre-capture versus co-capture strategies on accepted counts of bare mRNA1. Normalized accepted counts per FOV for pre-capture and co-capture conditions, with tiles present in both. Black error bars represent the standard errors of the mean from three independent replicates. Sample: Bare mRNA1; Temperature: 25°C; Acquisition time: 5 min/FOV; Probe: mRNA1 FP (650-658) _Middle Region_9 nt_Cy5 at 50 nM.



Supplementary Fig. 6 | Cumulative dwell time analysis of bare mRNA2 and encapsulated mRNA2 (LNP1004) using PINPOINT. Cumulative **a,** bound and **b,** unbound dwell time histograms comparing bare mRNA2 and encapsulated mRNA2, with $\tau_{on,med}$ and $\tau_{off,med}$ values determined from single-exponential fitting of cumulative dwell time distributions of accepted single-molecule traces. Temperature: 25°C; Acquisition time: 5 min/FOV; Probe: mRNA2 FP (650–660) _Middle Region_11 nt_Cy5 at 50 nM.



Supplementary Fig. 7 | Multistep intensity transitions in kinetic fingerprinting traces indicate multiple encapsulated mRNAs per LNP. a, b, Representative intensity-versus-time trajectories for the interaction of encapsulated mRNA1 (LNP1001, with tiles) with the sequence-specific probe. Red segments indicate HMM-idealized bound states. Green dashed lines mark discrete intensity levels consistent with multiple probe-binding contributions within a single particle, supporting the presence of more than one mRNA molecule per LNP. Temperature: 25 °C; acquisition time: 5 min/FOV; target: LNP1001, 100 pM; probe: mRNA1 FP (nt 650–658, middle region, 9 nt, Cy5), 50 nM; DNA tiles at 6 nM.

Supplementary Tables

Supplementary Table 1 | Sequences of capture probes, fluorescent probes, and tiling probes.

Sample Type	Name	Sequence
DNA Capture Probes (CPs)	Capture probe-DNA (662-683) _both mRNA	3'-bioTEG/ GCTAGTCGTCTCGGACATGGTC/ 5'
Fluorescent Probes (FPs) (with Cy5/ Cy3)	mRNA1 FP (650-658) _Middle Region_9 nt_Cy5	5'/Cy5/ATTGTTGGA/ 3'
	mRNA1 FP (650-658) _Middle Region_9 nt_Cy3	5'/Cy3/ATTGTTGGA/ 3'
	mRNA1 FP (2033-2040) _End Region_8 nt_Cy5	5'/Cy5/GTGTGGCT/ 3'
	mRNA1 FP (2033-2040) _End Region_8 nt_Cy3	5'/Cy3/GTGTGGCT/ 3'
	mRNA2 FP (650-660) _Middle Region_11 nt_Cy5	5'/Cy5/CAGATGTGCTA/ 3'
	mRNA2 FP (650-660) _Middle Region_11 nt_Cy3	5'/Cy3/CAGATGTGCTA/ 3'
Tiles	Tile 1 (520-560) _both mRNA	5'/ GAC CAG CCA GAT CAG GTT CTT GTA GAA GCT CTT AGC GCC G/3'
	Tile 2 (561-600)-mRNA 1	5'/ TGT AGG TCT GGT TGA TCT TGG GGT AGC TCT TGC CCT TCT T/3'
	Tile 2 (561-600)-mRNA 2	5'/ TGT AGC TCT TGC TCA GCT TGG GGT AGC TGT TGC CCT TCT T/3'
	Tile 3 (601-647) _both mRNA	5'/ ATG GTG GAT GCC CCA GAG GAC CAG CAC CTC TTT GCC CTT GTC GTT GA/3'
	Tile 5 (701-742) _both mRNA	5'/ TCG GGC TTG AAC TTC TTG CTG TAC CGG CTG GTG CCC ACG A/3'
	Tile 6 (743-782) _both mRNA	5'/ TCT GCC CTC TTG ATC CCG CAC TTT AGG CCT GGT GGC GAT C/3'

Supplementary Table 2 | mRNA and lipid nanoparticle samples and their concentrations.

Sample	mRNA concentration (mg/mL)
Unformulated- Strain 1 (mRNA1)	2.17
Unformulated-Strain 2 (mRNA2)	2.83
LNP1001	0.1 (100 μg/mL)
LNP1002	0.1 (100 μg/mL)
LNP1004	0.1 (100 μg/mL)

Supplementary Table 3 | Size and shape metrics for lipid nanoparticles (LNPs) measured by DLS and PINPOINT.

Sample	DLS Average Diameter (nm)	PINPOINT Average Diameter (nm)	PINPOINT Average Area (nm^2)
LNP1001	79.16 ± 0.70	79.52 ± 1.12	4740.12 ± 213.94
LNP1002	78.74 ± 0.93	80.98 ± 1.07	4958.26 ± 159.31
LNP1004	73.53 ± 0.91	73.61 ± 1.03	4081.03 ± 141.40
LNP-ALC0315	78.32 ± 0.72	76.93 ± 1.20	4509.44 ± 140.32
LNP1001_After five freeze thaw cycles	66.33 ± 1.47 (1^{st} peak), 114.76 ± 7.70 (2^{nd} peak)	60.79 ± 1.61 (1^{st} peak), 94.66 ± 2.10 (2^{nd} peak)	2549.80 ± 162.89 (1^{st} peak), 6428.60 ± 608.81 (2^{nd} peak)

Supplementary Table 4 | General parameter sets for trace generation and analysis.

General Trace Generation Parameters	
Spot fixing method	Fluctuation map
Spot fixing threshold	4
Edge Pixel	20
Start frame	1
End frame	3000
Percentilecut	0.95
ROI size (pixels)	3

General Trace Analysis Parameters	
Start frame	1
End frame	3000
Exposure time (s)	0.1
Remove_single_frame_events	True
Intensity Threshold	100
S/N Threshold (Event)	2
S/N Threshold (Trace)	2
Minimum N_{b+d}	10
Minimum N_{b+d}	Inf
Minimum $\tau_{on,med}$	0.2
Maximum $\tau_{on,med}$	Inf
Minimum $\tau_{off,med}$	0.2
Maximum $\tau_{off,med}$	Inf
Maximum individual τ_{on}	Inf
Maximum individual τ_{off}	Inf
Maximum τ_{on} (C.V.)	Inf
Maximum τ_{off} (C.V.)	Inf

Supplementary Table 5 | mRNA copy number per lipid nanoparticle determined by PINPOINT.

Sample	mRNA Copy Number
LNP1001	2.26 ± 0.12
LNP1002	2.04 ± 0.32
LNP1004	2.19 ± 0.04