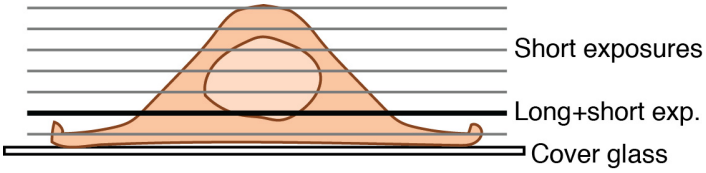
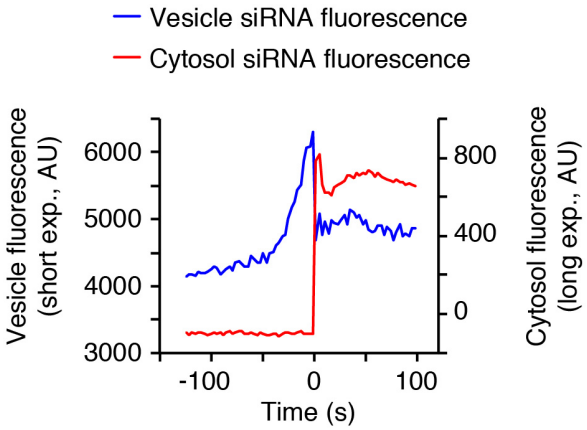


Supplementary Figure 1

High dynamic range (HDR) imaging of live cells with spinning disk confocal microscopy.

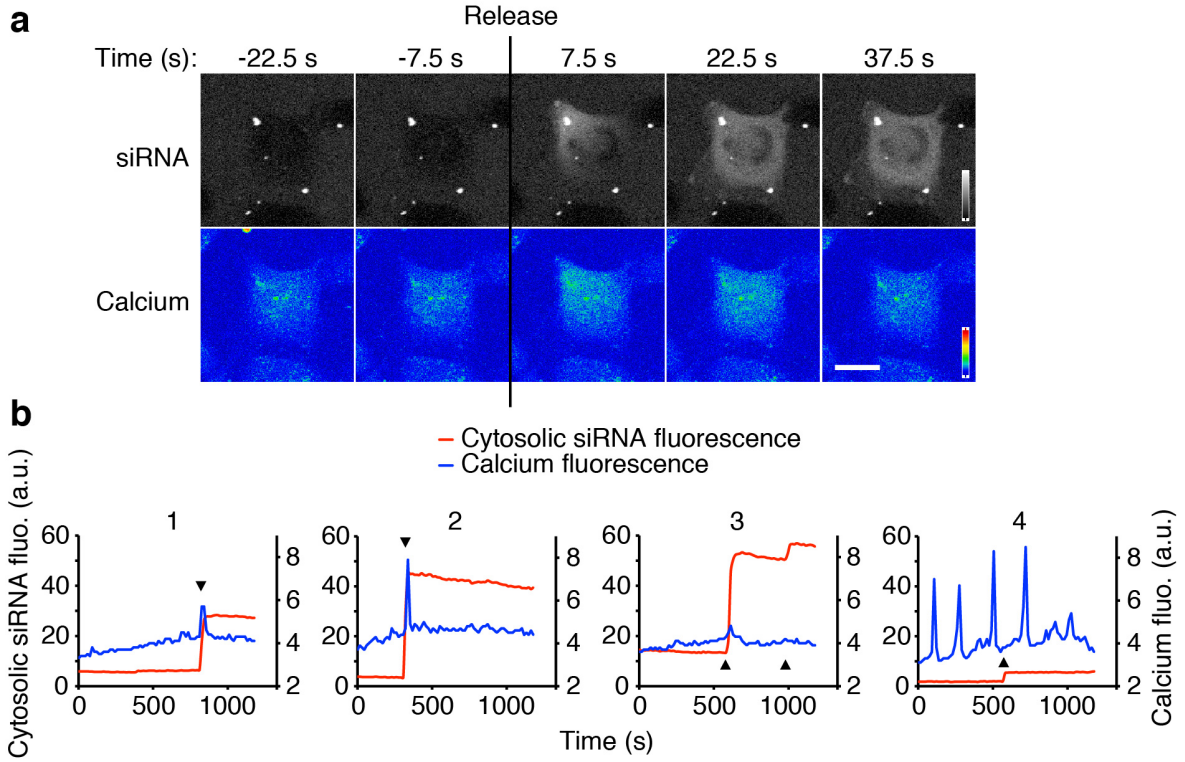
During image acquisition, z-sections, encompassing most of the cellular volume, are captured with short exposure times adjusted to the brightest structures in the cell. After all short exposures are captured, a single plane with approximately 10 times longer exposure time, is captured from the lower third of the cell. Only a single plane with long exposure time is cap- tured in order to limit phototoxicity.



Supplementary Figure 2

Cytosolic siRNA release coincides with decrease in vesicular siRNA fluorescence.

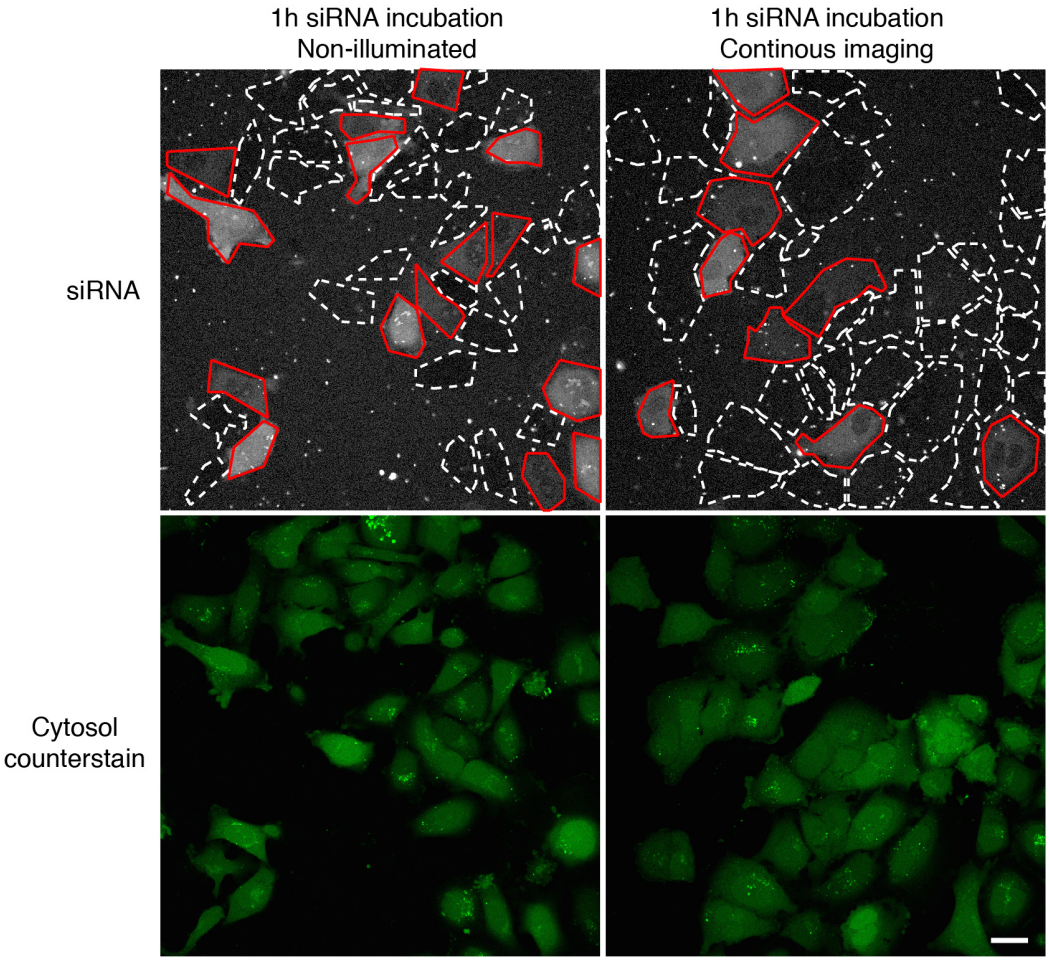
HeLa cells were incubated with siRNA-AF647 lipoplexes and imaged every 3 s. Blue line: fluorescence intensity traces of releasing vesicle (from maximum projection of short exposure images). Red line: fluorescence intensity traces from cytosolic Region of Interest (ROI) adjacent to the releasing vesicle (from single plane long exposure images). This representative tracing suggests release occurs as sudden discrete events without prior leakage.



Supplementary Figure 3

Cytosolic release of siRNA is associated with cytosolic calcium transients.

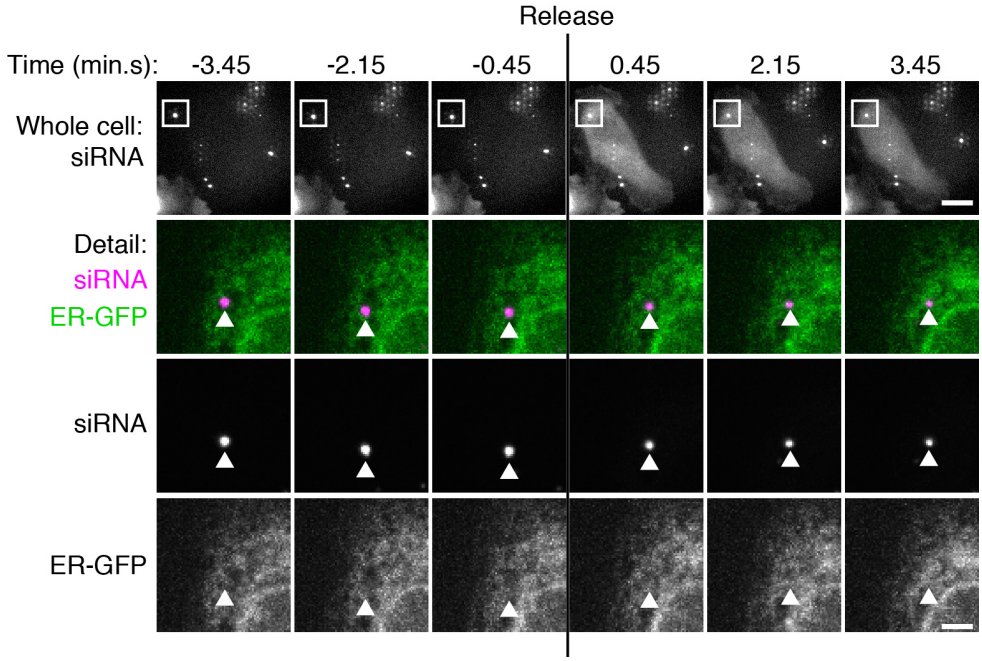
HeLa cells pre-loaded with the fluores- cent Ca++ indicator Fluo-4 were incubated with siRNA-AF647 lipoplexes and imaged every 15 s. a, Representative cell exhibiting a small localized calcium release coincident with siRNA release. Scale bar, 10 µm. b, Quantitation of whole-cell cytosolic siRNA fluorescence (red curve, left axis) and Ca++/Fluo-4 fluorescence (blue curve, right axis) in four typical cells (1-4). siRNA release events are indicated with black triangles. Spontaneous Ca++ spikes are seen in cell 4, for comparison. Ca++ transients during release are of similar or smaller magnitude than spontaneous Ca++ fluctuations.



Supplementary Figure 4

Cytosolic release of siRNA in both non-illuminated and continously imaged cells.

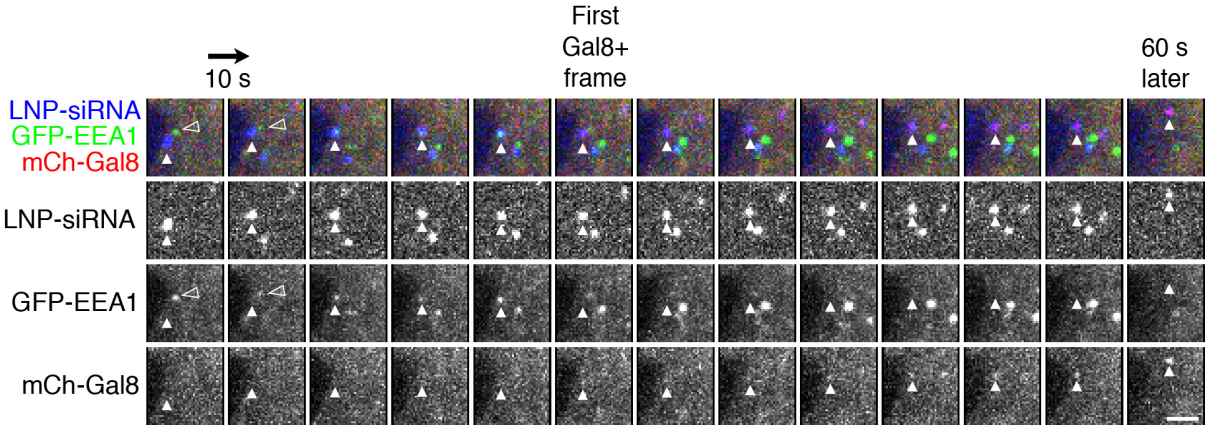
HeLa cells were incubated with 100 nM siRNA-AF647 lipoplexes for 1 h and either continously imaged every 10 s (right panels) or left non-illuminated until the end of the incuba- tion (left panels). Red outlines indicate cells that have experienced a release event and white dashed outlines indicate cells without release events. Cells are counterstained to facilitate localization (Fluo-4). Scale bar, 20 µm. Shown is representative fields of >10 imaged. The same observation, i.e., no difference in release probability between illuminated and adjacent fields has been observed throughout the imaging experiments reported herein. In addition, the extent of knockdown measured with flow cytometry in end-point analyses recapitulates the extent of knockdown seen during continous imaging (not shown).



Supplementary Figure 5

Endosomes releasing lipoplex siRNA do not associate with the ER.

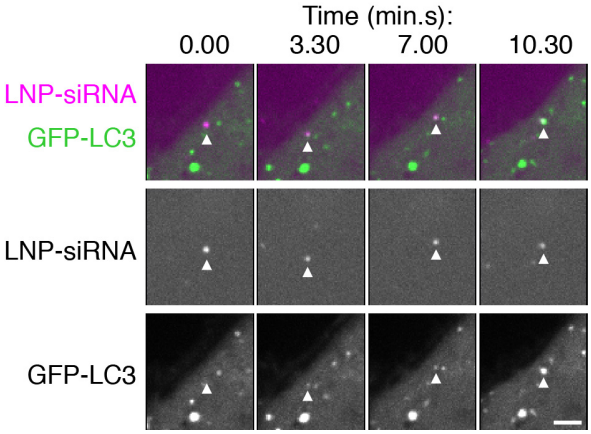
HeLa cells expressing an ER-GFP marker were incubated with siRNA-AF647 lipoplexes and imaged every 1.5 min. Whole cell (upper row, scale bar 10 μm) and detail time-lapse images of cell exhibiting a release event. Scale bar, 2 μm. White triangles indicate a representative releasing endosome.



Supplementary Figure 6

LNPs merge with pre-existing EEA1+ struc- tures and rapidly lose EEA1 association around the time of release.

HeLa cells, expressing mCherry-galectin-8 and GFP-EEA1, were incubat- ed with LNP-siRNA-AF647 and imaged every 10 s. Detail time-lapse images of an LNP containing vesicle (indicated with filled white triangles) associating with mCherry-galectin-8, are shown. Open triangles indicate pre-existing EEA1+ vesicle. Scale bar, 2 μm.



Supplementary Figure 7

LC3 is recruited to LNP containing vesicles.