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Supporting Information

Quantitative Mapping of Endosomal DNA Processing by Single Molecule Counting

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Author Contributions

V.P. Conceptualization: Equal; Data curation: Lead; Formal analysis: Lead; Investigation: Equal; Methodology: Lead; Project administration: Supporting; Visualization: Lead; Writing – original draft: Equal
K.T. Formal analysis: Supporting; Software: Supporting
M.V. Writing – original draft: Supporting; Writing – review & editing: Supporting
L.H. Methodology: Supporting
S.P. Funding acquisition: Supporting; Supervision: Supporting
Y.K. Conceptualization: Lead; Data Curation: Lead; Funding acquisition: Lead; Investigation: Lead; Supervision: Lead; Writing - original draft: Lead; Writing - review & editing: Lead
N.W. Conceptualization: Equal; Data curation: Supporting; Funding acquisition: Supporting; Investigation: Supporting; Supervision: Supporting; Writing – review & editing: Supporting.

METHODS

Materials

All the oligonucleotides used were obtained from Integrated DNA Technologies (IDT). Labeled oligonucleotides were subjected to ethanol precipitation to remove any contaminating fluorophores. Peptide inhibitor for DNase II, ID2-3 was procured from Selleckchem.[1] Oligonucleotides and peptide were dissolved in Milli Q water and was stored at –20°C.

Oligonucleotide sequences used in this study

Devices	Sequence (5'-3')
dsDNA-488	**A488-**ATA ACA CAT AAC ACA TAA CAA AAT ATA TAT CCT AGA ACG ACA GAC AAA CAG TGA GTC–3′ TAT TGT GTA TTG TGT ATT GTT TTA TAT ATA GGA TCT TGC TGT CTG TTT GTC ACT CAG–5′
dsDNA-Cy5	**Cy5-**ATA ACA CAT AAC ACA TAA CAA AAT ATA TAT CCT AGA ACG ACA GAC AAA CAG TGA GTC–3′ TAT TGT GTA TTG TGT ATT GTT TTA TAT ATA GGA TCT TGC TGT CTG TTT GTC ACT CAG–5′
I4Cy5	**Cy5-**CCC CTA ACC CCT AAC CCC TAA CCC CAT ATA TAT CCT AGA ACG ACA GAC AAA CAG TGA GTC GAC TCA CTG TTT GTC TGT CGT TCT AGG ATA TAT ATG GGG TTA GGG GTT AGG GGT TAG GGG

Cargo DNA sample preparation

Constituent complementary strands of $I4_{Cy5}$ (I4 and I4') were mixed in 20 mM sodium buffer pH 5.5 containing 100 mM KCl at 5 µM concentration. For dsDNA DNA, constituent strands dsDNA-Cy5 and I4' or dsDNA-A488 and I4') were mixed at 5 µM concentration in 50 mM sodium phosphate buffer pH 7. For both cases, the resultant solution was heated from 25°C to 90°C in 15 min and was then cooled to room temperature at 1°C/2 min and equilibrated at 4°C overnight.

Protein conjugation

Ovalbumin was obtained from Sigma and labeled with FITC using a standard protein labeling protocol.[2] Briefly, 200 µl of 1.25 mg/ml FITC solution in 0.1 M sodium phosphate buffer at pH 8 was added to 500 µl of 10 mg/ml protein solution. The reaction mixture was adjusted to pH 9.0 with 0.1 M trisodium phosphate. The reaction mixture was maintained at 25°C for 3 hours. Labeled

ovalbumin was purified from reaction mixture using 10 kDa cutoff Amicon filter using PBS and was then stored in PBS at –20^{o}C.

Mouse Apo-transferrin was obtained from Sigma and was converted to holo-transferrin by loading with Fe(III) as described previously.[3] Briefly, 4.49 mg of $FeCl_3$ was dissolved in 2 ml of water and was neutralized with sodium hydroxide. 106 mg of nitrilotriacetic acid was added to it and the solution was neutralized again. 0.5 mg of apo-transferrin was dissolved in 100 µl of buffer 1 (0.1 M $NaClO_4$/20 mM $NaHCO_3$/10 mM Tris-HCl, pH 7.6) (5 mg/ml protein concentration). To this protein solution, 1 µl of above Fe^{3+} solution was added, incubated for 1-hr at room temperature and was subjected to 30 kDa cutoff Amicon. Buffer was exchanged with buffer2 (100 mM sodium bicarbonate buffer pH 9) and volume was concentrated to 5 mg/ml.

In order to label holo-transferrin with Rhodamine B, 0.2 ml of 5 mg/ml holo-transferrin solution in pH 9, and 0.1 M sodium bicarbonate buffer was mixed with 6.7 µl of 20 mg/ml Rhodamine B isothiocyanate solution. Solution was allowed to stir at RT for 1 hour and was then subjected to 30 kDa cutoff Amicon purification using perchlorate buffer (0.1 M $NaClO_4$/20 mM $NaHCO_3$/10 mM Tris-HCl, pH 7.6).

Cell culture and labelling with endocytic markers

J774A.1 macrophages (ATCC No. TIB-67) were a kind gift from Prof. Deborah Nelson, Department of Pharmacological and Physiological Sciences, the University of Chicago. They were cultured in Dulbecco's Modified Eagle's Medium/F-12 (1:1) (DMEM-F12) (Invitrogen Corporation, USA) containing 10% heat inactivated Fetal Bovine Serum (FBS) (Invitrogen Corporation, USA), 100 U/ml penicillin and 100 g/ml streptomycin at 37^{o}C in 5% CO2, and were used at 60% confluence.

In order to label early endosomes, J774A.1cells were co-pulsed with a cocktail of 1 µM Rhodamine labeled holo-transferrin and 500 nM Cy5 labeled dsDNA for 10 min at 37^{o}C. Cells were immediately washed with PBS and placed on ice to prevent endocytosis progression. Cells were surface stripped by incubating them in surface stripping buffer (160 mM sodium ascorbate, 40 mM ascorbic acid, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 4.5.) for 10 min on ice. Cells were then washed with PBS and fixed using 2.5 % paraformaldehyde (PFA) at room temperature for 20 min. For labeling late endosomes, cells were pulsed with Cy5 labeled DNA dsDNA in complete medium for 5 min at 37^{o}C followed by addition of FITC labeled ovalbumin such that its final concentration in pulsing medium was 1 µM. After 5 min pulse at 37^{o}C, cells were washed with PBS and were chased at 37^{o}C for 30 min in complete medium. Cells were then washed, surface stripped and fixed as it were done for early endosome sample. For labeling lysosomes, cells were pulsed with 0.5 mg/ml TMR labeled 10 kDa dextran for 16 hours in complete medium at 37^{o}C followed by 2 hours chase in complete medium. Cells were then pulsed with 500 nM Cy5 labeled dsDNA for 10 min followed by 2 hours chase in complete medium. Cells were then washed, surface stripped, fixed and imaged on confocal microscope.

Labeling endosomes for molecule counting

In a typical molecule counting experiment, for labeling early or late endosomes J774A.1 cells were pulsed with a cocktail of 25 nM of dsDNA-Cy5 (reporter) + 475 nM dsDNA-A488 (endocytic tracer) for 10 min and chased for indicated time in DMEM with 0.1% BSA (without serum) at 37^{o}C.

In the same way, lysosomes were labeled with 100 nM of dsDNA-Cy5 (reporter) + 400 nM dsDNA-A488 (endocytic tracer) for no inhibitor sample and with 50 nM of dsDNA-Cy5 (reporter)

+ 450 nM dsDNA-A488 (endocytic tracer) for 10 µM inhibitor sample. Cells were then washed with PBS, surface stripped, incubated at room temperature for 3 hours and imaged in imaging buffer (Tris-base 50mM, NaCl 10mM, Glucose 10%, oxygen-scavenging system (0.1 mg/ml glucose oxidase, 0.02 mg/mL catalase, pH=8) on Total Internal Reflection Fluorescence (TIRF) microscope.

DNase II inhibitor treatment

In order to block DNase II activity, J774A.1cells were pretreated with 10 µM DNase II inhibitor peptide ID2-3 in DMEM with 0.1% BSA (without serum) for 1 hour at 37^{o}C.[1] Cells were then pulsed with cargo DNA dissolved in DMEM with 0.1% BSA and 10 µM DNase II inhibitor peptide (without serum) at 37^{o}C for 10 min and were chased in DMEM with 0.1% BSA (without serum) and 10 µM DNase II inhibitor peptide at 37^{o}C for indicated time.

Image acquisition

Confocal images were acquired Olympus FV1000 confocal laser scanning microscope set up equipped with IX81 body, 60x / NA 1.42 oil (PlanApoN) objective, multi alkali PMTs and laser lines for 488, 543 and 633 nm excitation.

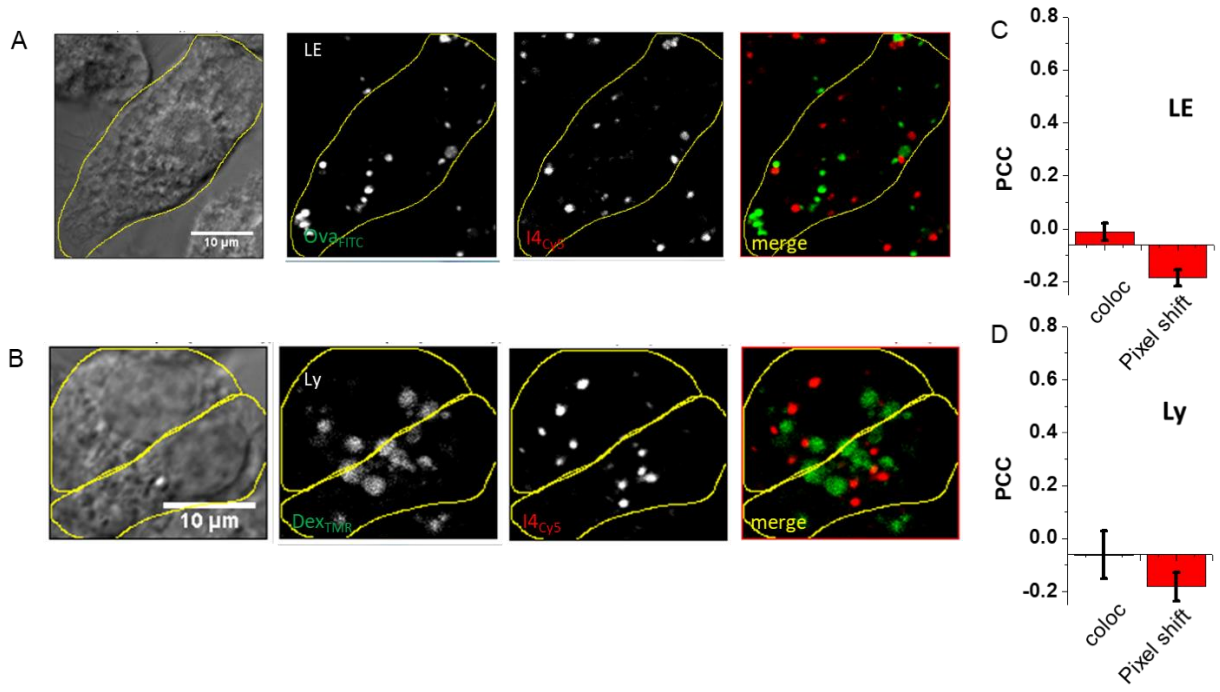
TIRF images for molecule counting were acquired on Leica four-color (405nm, 488nm, 532nm and 642nm) Total Internal Reflection fluorescence (TIRF) microscope equipped with automated critical angle positioning, 160x NA 1.43 state of the art, adhesive-free objective, Suppressed Motion (SuMo) stage which locks in the 160x objective to minimize sample drift and iXon Ultra EMCCD camera. Before image acquisition, samples were allowed to sit on microscope

undisturbed for thermal equilibration. This prevented z-drift during image series acquisition. Image series of 4,000 to 12,000 frames was acquired with 100 ms exposure.

Image analysis

J774A.1 cells treated with Cy5 labeled DNA devices labeled various cellular compartments (EE, LE & Ly). Using Fiji, acquired TIRF microscopy slices were used to measure the ratio of number of endosomes in the first plane (closest to coverslip) to the number of endosomes in the entire cell (Converting the image stack into Maximum Z-Projection). In three independent experiments, we detected 51.66% of early endosomes (n=6 cells), 37.34% of late endosomes (n=5 cells) and 23.47% of lysosomes (n=5 cells) illuminated in the first slice of the microscopy image.

Acquired images were opened in Fiji and were exported into image sets for small areas using custom written ImageJ macro. This was a vital step as too large image sets can't be opened in LabView program due to memory limits. Images were manually analyzed and spots where significant colocalization in endocytic tracer and reporter channels was observed were marked and fluorescence photobleaching trace for each such spot was exported. More than 200 such traces for each sample were then analyzed using the previously reported Python program "Photobleach".[4] Results were then exported into excel and were plotted in OriginPro software.



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118 **Supplementary Figure S1 | DNA cargo does not co-localize with LE or Ly at 10 min pulse.**

119 *Representative single-plane confocal images showing co-localization of cargo DNA with various*

120 *compartment markers at 10 min pulse. (A) J774A.1cells were pulsed with 500 nM of LE marker*

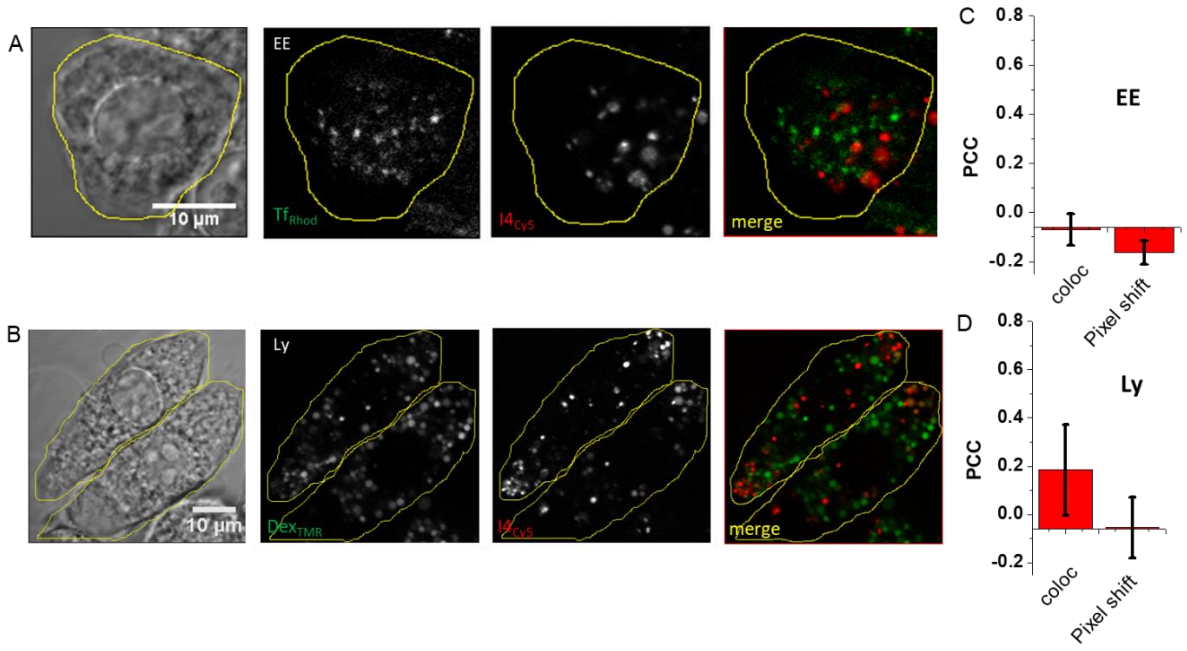
121 *Ovalbumin-FITC (Ova$_{FITC}$) for 5 min followed by a chase of 20 min. These cells were then pulsed*

122 *with 500 nM of I4$_{Cy5}$ for 10 min. (B) Lysosomes were labeled by 16 hours pulse of 0.5 mg/ml*

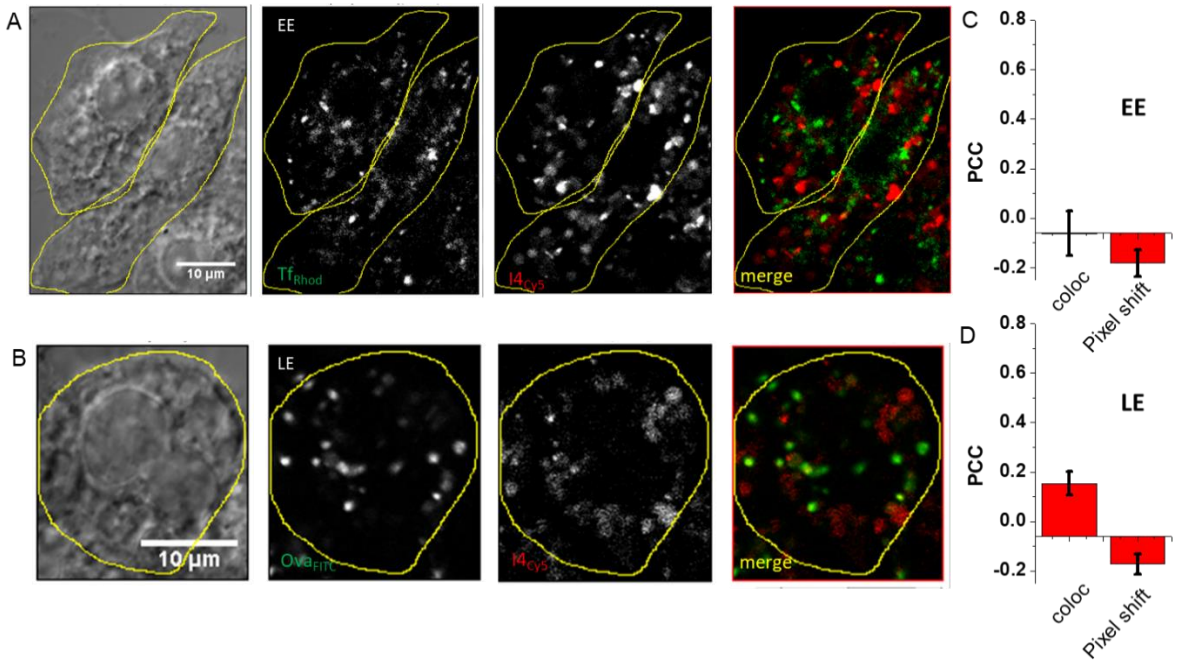
123 *Dextran-TMR (Dex$_{TMR}$) followed by 4 hours chase. These cells were then labeled with I4$_{Cy5}$ for 10*

124 *min. Cell boundaries are demarcated by yellow outlines. (C & D) Quantification of co-localization*

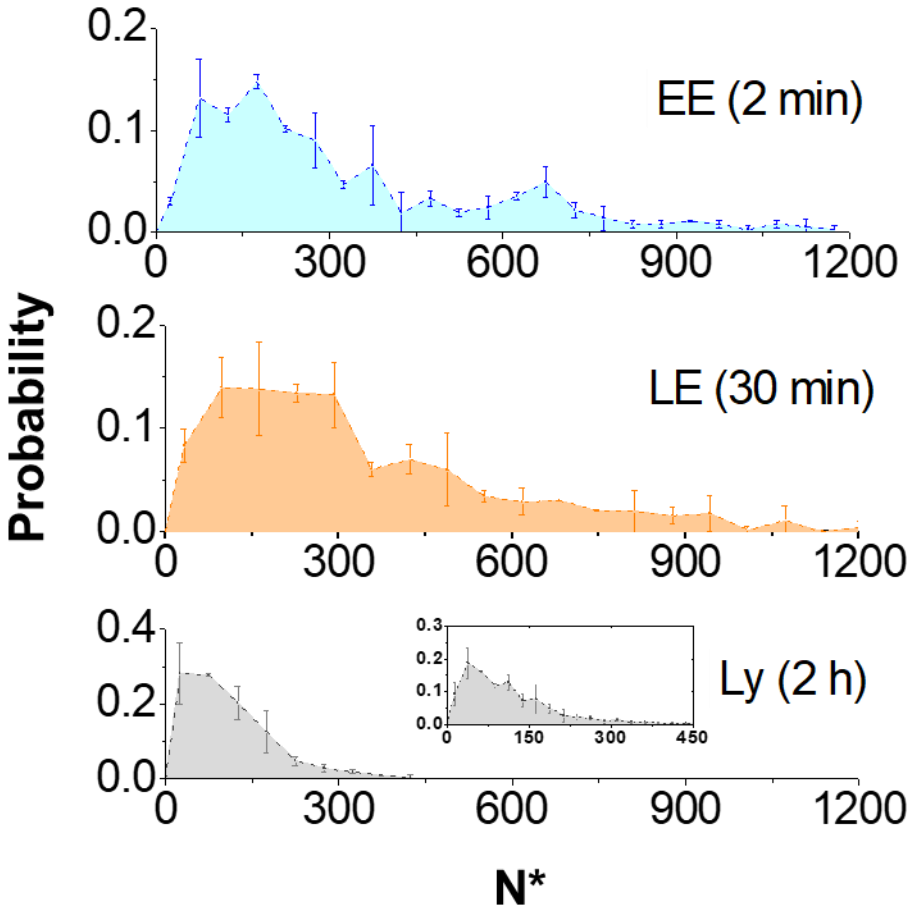
125 *between cargo DNA and endosomal markers used in a & b. Values indicate mean of n~20 cells.*



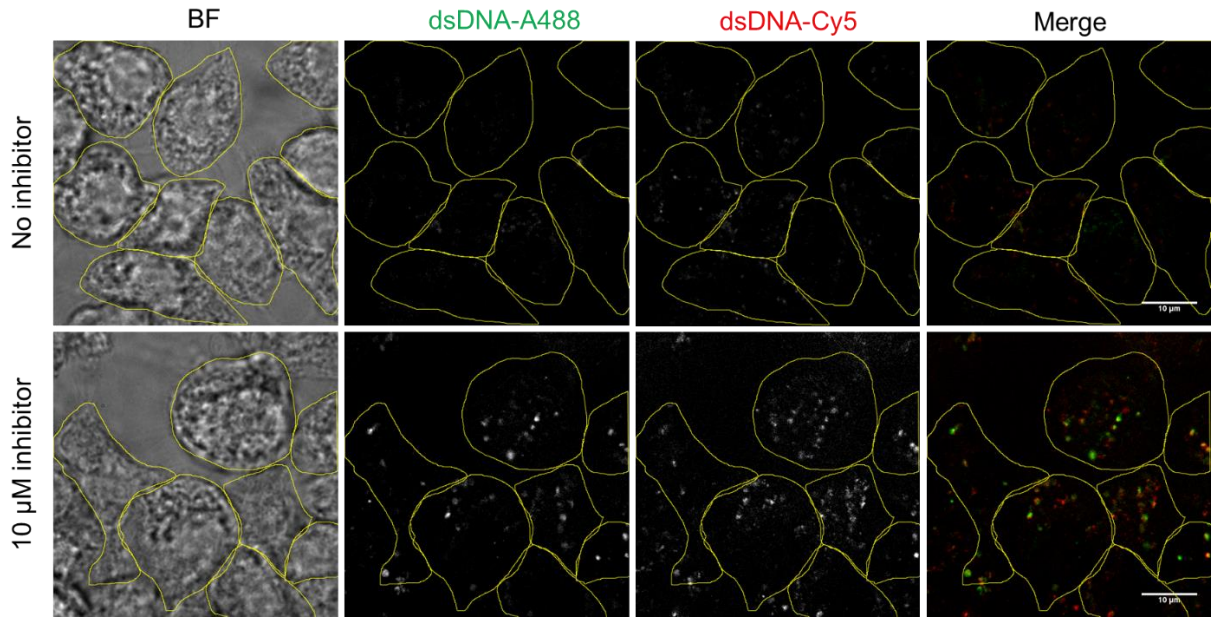
Supplementary Figure S2 | DNA cargo does not co-localize with EE or Ly at 10 min pulse and 30 min chase. *Representative single-plane confocal images showing co-localization of cargo DNA with various compartment markers at 10 min pulse and 30 min chase. (A) J774A.1cells were pulsed with 500 nM I4$_{Cy5}$ for 10 min and then chased for 20 min. These cells were labeled with 1 µM EE/SE marker transferrin-Rhodamine B (Tf$_{Rhod}$) for 10 min. (B) Lysosomes were labeled by 16 hours pulse of 0.5 mg/ml Dextran-TMR (Dex$_{TMR}$) followed by 3.5 hours chase. These cells were then labeled with I4$_{Cy5}$ for 10 min followed by a chase for 30 min. Cell boundaries are demarcated by yellow outlines. (C & D) Quantification of co-localization between cargo DNA and endosomal markers used in a & b. Values indicate mean of n~20 cells.*



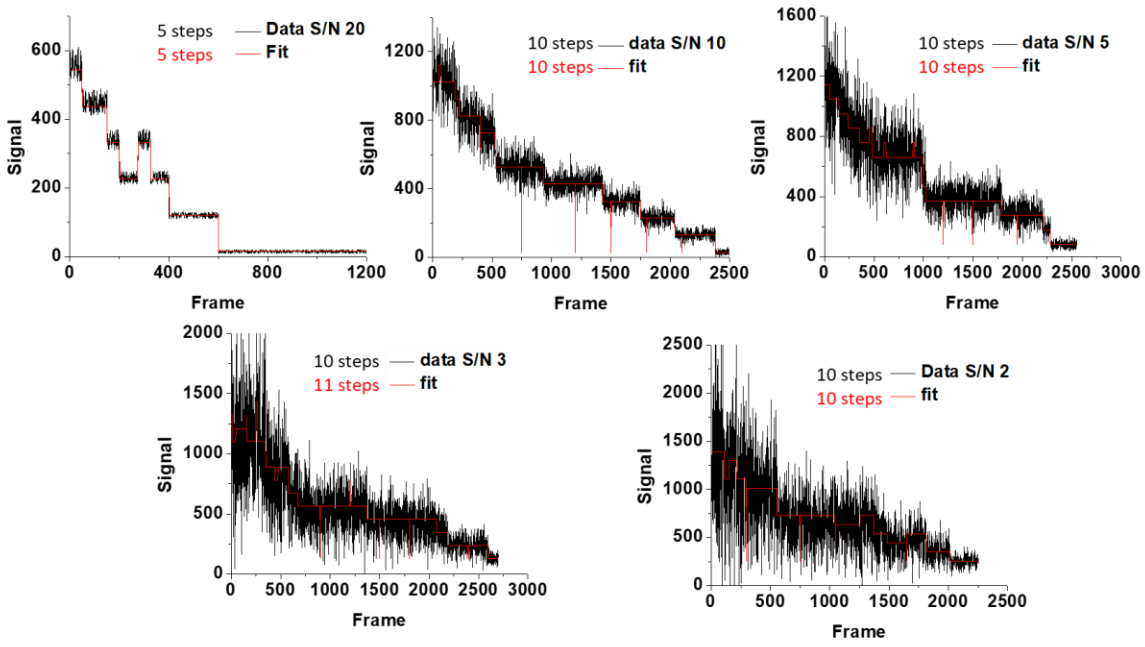
Supplementary Figure S3 | DNA cargo does not co-localize with EE or LE at 10 min pulse and 2 h chase. *Representative single-plane confocal images showing co-localization of cargo DNA with with various compartment markers at 10 min pulse and 2 h chase. (A) J774A.1cells were pulsed with 500 nM* $I4_{Cy5}$ *for 10 min and then chased for 2 h. These cells were labeled with 1 µM EE/SE marker transferrin-Rhodamine B (*Tf_{Rhod}*) for 10 min. (B) J774A.1cells were pulsed with 500 nM of* $I4_{Cy5}$ *for 10 min followed by a chase for 85 min. These cells were then labeled with 500 nM of Ovalbumin-FITC (*Ova_{FITC}*) for 5 min followed by a chase of 30 min to mark LE. Cell boundaries are demarcated by yellow outlines. (C & D) Quantification of co-localization between cargo DNA and endosomal markers used in a & b. Values indicate mean of n~20 cells.*



Supplementary Figure S4 | Molecule counting in endosomes. Histograms of the number of devices observed per compartment in early endosomes (EE), late endosomes (LE) and lysosomes (Ly) of J774A.1 cells. Early and late endosomes were labeled with 25 nM of dsDNA-Cy5 (reporter) + 475 nM dsDNA-A488 (endocytic tracer). Lysosomes were labeled with 100 nM dsDNA-Cy5 (reporter) + 400 nM dsDNA-A488 (endocytic tracer). Error bars indicate the mean of two independent experiments ± standard deviation. $N^* = n_p \times d$ where N^* = total number of devices per compartment, n_p = number of photobleaching steps observed and d = dilution factor. n = 200 endosomes (duplicate). Inset shows a zoom of histogram for lysosome sample with smaller bin size showing distribution at lower N^*.



Supplementary Figure S5 | Effect of DNase II inhibitor on lysosomal cargo DNA processing. Lysosomes of J774 cells were labeled with 100 nM of dsDNA-Cy5 (reporter) + 400 nM dsDNA-A488 (endocytic tracer) for no inhibitor sample and with 50 nM of dsDNA-Cy5 (reporter) + 450 nM dsDNA-A488 (endocytic tracer) for DNase II 10 µM inhibitor sample. Brightness of red channel image for no inhibitor sample has been scaled to half intensity to compensate for double concentration of dsDNA-Cy5. DNase II inhibitor sample shows bright and large lysosomes.



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168 ***Supplementary Figure S6 | Photobleaching software benchmarking.*** To test the robustness of

169 the algorithm, synthetic photobleaching traces were generated using a MATLAB program for

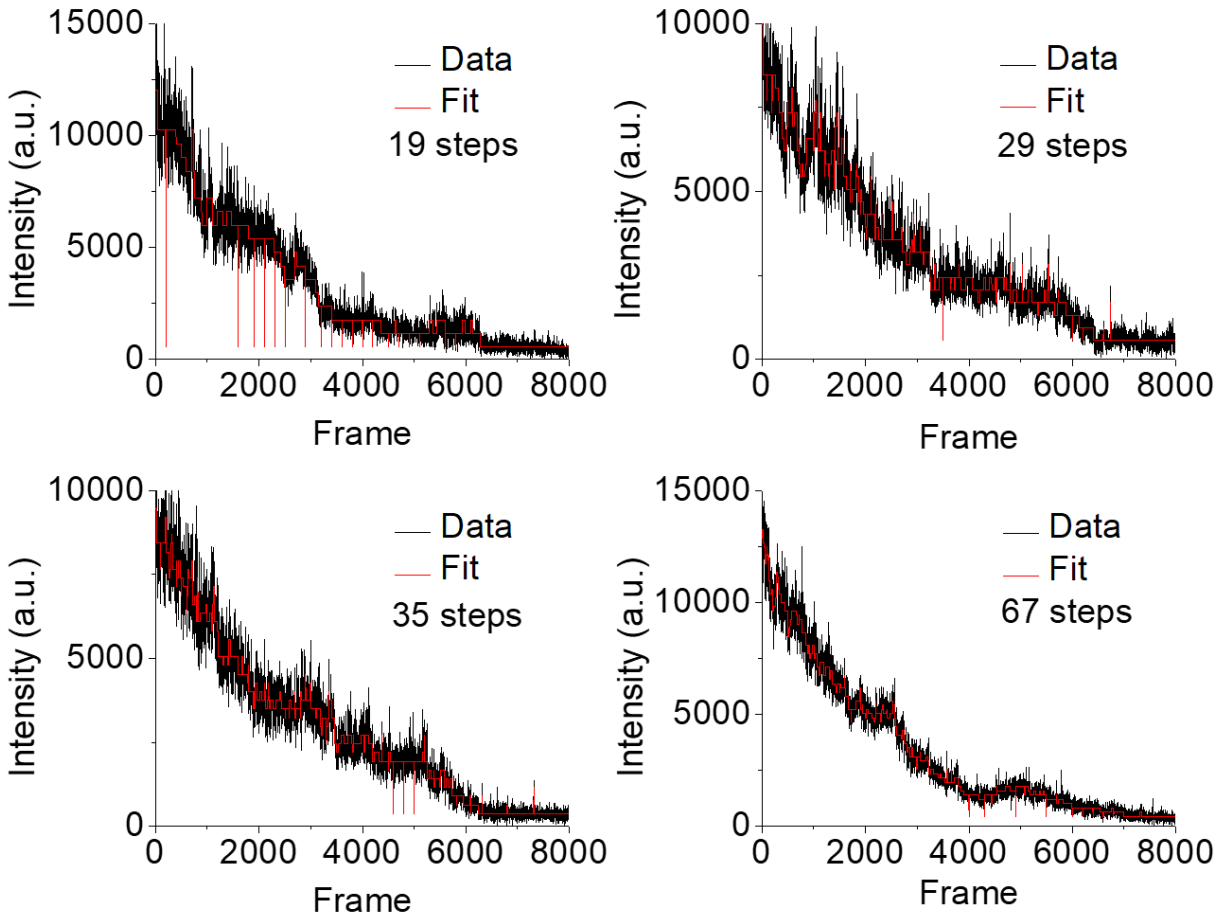
170 various S/N whose steps were detected using the algorithm written by Tsekouras *et al*. Note that

171 sharp downward spikes in the fit (see also Fig. S6) are an artifact of the fitting software and can

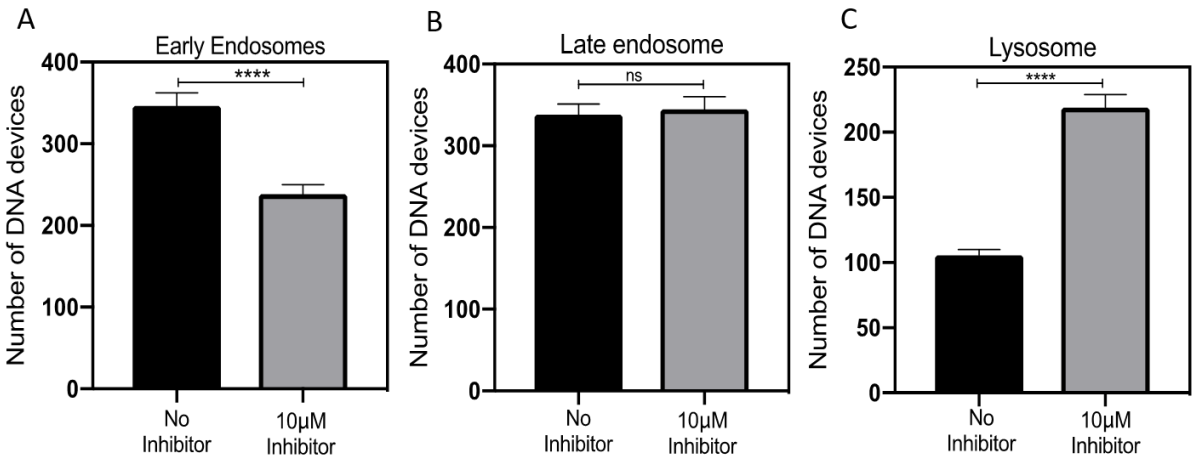
172 be eliminated via a simple post-processing step detailed in the software user guide.

173 Synthetic data = Signal + Poisson noise (fluorophore) + Gaussian noise (detector) + background

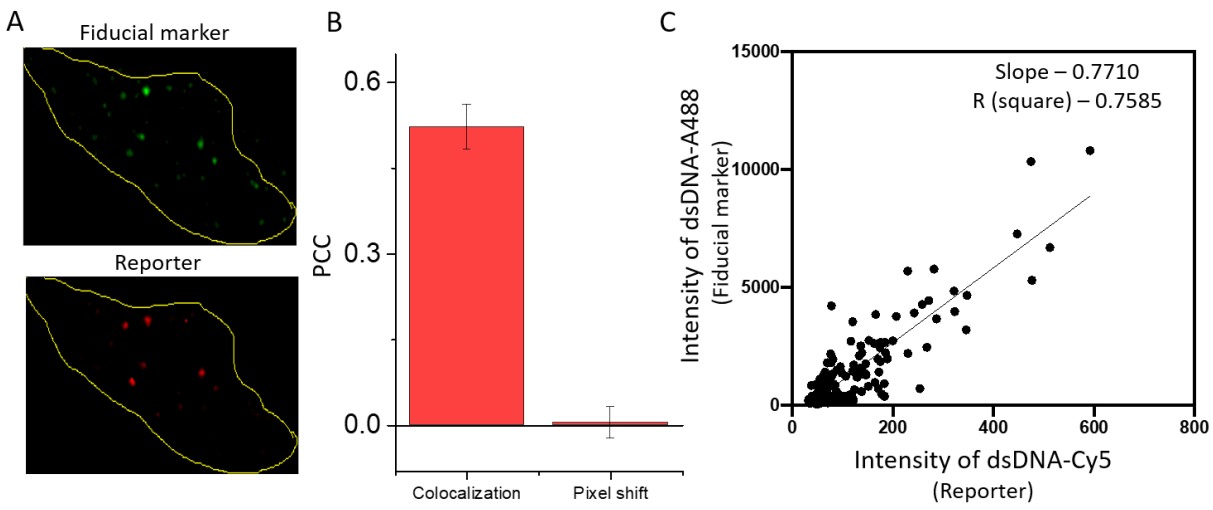
174



Supplementary Figure S7| Photobleaching step detection. Representative photobleaching decay traces (black) and their detected steps (red) for early endosomes.



Supplementary Figure* S8**: ***Quantification of DNA nanodevices inside endosomal compartments during pharmacological treatment. Bar graphs of the number of DNA devices in (A) early endosomes (B) late endosomes and (C) lysosomes in the presence (grey) and absence (black) of DNase II inhibitor. ****p<0.0001, ns = non-significant, where p<0.6139.



Supplementary Figure 9: Quantitative analysis of the DNA devices inside endosomes. A. Endosomes of J774A.1 cells co labeled with 500nM of dsDNA-488 (Fiducial marker) and dsDNA-Cy5 (Reporter) in 4:1 stoichiometry. B. Pearson Correlation Coefficients (PCC) of colocalized and pixel shifted images of A488 and Cy5 puncta (n-12 cells). C.A plot of intensity in A488 channel versus that in the Cy5 channel per endosome. Black line is a linear fit of the data and the slope and R2 values are shown.

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(5) *Salkind Neil J & Kristin Rasmussen, Encyclopedia of Measurement and Statistics (2007), pages 566-567*