Supporting Information

Quantitative tracking of protein trafficking to the nucleus enabled by cytosolic protein delivery

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Methods

Construction of plasmids and *Escherichia coli* strains. 6xHis-eGFP expression vector (pET21d-eGFP) was obtained from Novagen. Briefly, using 6xHis-eGFP as the template, PCR was performed with primers listed in the **Table S1**. Subsequently, PCR products were digested using BamHI and HindIII restriction enzymes and inserted into pQE80 vector, downstream of nucleotides for six histidine tag to construct pQE80-6xHis-NLS-eGFP or pQE80-6xHis-eGFP-NLS expression vectors. Successful cloning was confirmed by DNA sequencing.

Protein Expression. To produce recombinant proteins, plasmids carrying 6xHis-NLS-eGFP or 6xHis-eGFP-NLS were transformed into *Escherichia coli* BL21 (DE3) strain. A transformed colony was picked up to grow small cultures in 50 mL 2XYT media at 37°C overnight. The following day, 15 mL of grown culture was inoculated into 1 L 2XYT media and allowed to grow at 37°C until OD reaches 0.6. At this point, the protein expression was induced by adding isopropyl-*β*-D-thiogalactopyranoside (IPTG; 1 mM final concentration) at 25°C. After 16 hours of induction, the cells were harvested and the pellets were lysed using a microfluidizer. His-tagged fluorescent proteins were purified from the lysed supernatant using His-Pur cobalt columns. The integrity and the purity of native protein were determined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). **Figure S1** shows the SDS-PAGE gel of the purified proteins.

Protein-NPSC Complex Formation. HKRK gold nanoparticles (HKRK AuNPs) were synthesized according to a previous report.[1] To make the NLS-eGFP-NPSC complex, 2.5 μM HKRK AuNPs were incubated with 1 μM eGFP in 30 μL of phosphate buffer (5 mM, pH = 7.4) for 10 min. Then, 1 μL of linoleic acid was mixed with 500 μL of phosphate buffer (5 mM, pH = 7.4) containing 1 μM HKRK AuNPs and agitated by an amalgamator (Yinya New Materials Co. Ltd, Hangzhou, China) at 5000 rpm for 100 s to form emulsions. Finally, the mixture of the protein and HKRK AuNPs was diluted to 45 μL with phosphate buffer (5 mM, pH = 7.4) followed by the addition of 5 μL of the emulsion. The NLS-eGFP-NPSC complexes were ready to use after 10 min incubation at room temperature. The final concentrations of HKRK AuNPs and eGFP were 1.5 μM and 600 nM, respectively.

Cell viability assay (Alamar Blue). 15,000 HeLa cells were cultured in a 96-well plate for 24 h prior to the experiment. The cells were washed by cold phosphate buffer saline (PBS) thrice before the delivery, then different amounts of the $NLS^{c\text{-}Myc}$-eGFP-NPSC complex (prepared as mentioned above) were diluted by DMEM and incubated with the cells for 1 h followed by the incubation with DMEM containing 10% FBS and 1% antibiotics for 23 h. After washing with PBS 3 times, the cells were then incubated with 200 μL DMEM containing 10% Alamar Blue for 3 h. Cell viability was calculated by measuring the fluorescence intensity of Alamar Blue at 590 nm, with an excitation of 535 nm.

Cell culture. HeLa cells were cultured in a humidified atmosphere (5% CO_2) at 37°C and grown in Dulbecco's modified eagle's medium (DMEM, low glucose) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

NLS-eGFP Delivery. A total of 60,000 or 240,000 HeLa cells were cultured in a 24-well plate or confocal dish, respectively, for 24 h prior to delivery. The cells were washed by cold phosphate buffer saline (PBS) three times right before delivery. After the preparation of cells, NLS-eGFP-NPSC complex solution (50 or 150 μL of the NLS-eGFP-NPSC complex diluted by 450 μL or 1.35 mL of the DMEM without FBS, respectively) was incubated with the cells for 1 h in a 24-well plate or confocal dish, followed by incubation with fresh DMEM with 10% FBS for 1 h unless otherwise mentioned.

Cell Imaging. 240,000 HeLa cells were cultured in the confocal dish for 24 h prior to the experiment. Before imaging, cells were washed by PBS for three times followed by the incubation with NLS-eGFP-NPSC in cell culture media. The cells were then observed by LSCM (LSM 510, Zeiss, Germany) microscope.

Live Cell Imaging. 240,000 HeLa cells were cultured in the confocal dish for 24 h prior to delivery. Before imaging, cell culture media were replaced PBS with 10% FBS to eliminate the fluorescence of media. The confocal dish was then placed in the live cell

imaging chamber with 5% CO_2 and at 37°C on the fluorescent (IX51, Olympus, Japan) or LSCM (LSM 510, Zeiss, Germany) microscope. A series of images were taken at 4 s interval on fluorescent microscope or 1 min interval on LSCM microscope.

ATP Depletion. Cells were treated with cell culture media containing 3 mg/mL NaN_3/50 mM 2-deoxyglucose 1 h prior to delivery. NLS-eGFP was then delivered using the same method mentioned above. However, during the delivery, 3 mg/mL NaN_3 and 50 mM 2-deoxyglucose were supplemented in the media to maintain the ATP depleted status of cells.

Image Analysis. Images obtained from LSCM were in 8-bit grayscale format containing both fluorescent and bright field channels. The fluorescent channel was extracted by ImageJ. The pixel intensity profile along line segment was also performed by ImageJ. The cytosol and nucleus of each cell were separated by Photoshop and saved as 8-bit grayscale Tiff files with black backgound without any intensity adjustment. The resulted images were processed and plotted by R (**Code S1**).

Table S1. Primers for PCR cloning

SV40	Forward	5’- ACGATGGATCCATGGTGAGCAAGGGCGAGGA -3’
	Reverse	5’-GTGTAAGCTTTTACAGTTCGCGTTTTTCTTTGG CCTTGTACAGCTCG -3’
NLP	Forward	5’-ACGATGGATCCATGGTGAGCAAGGGCGAGGA -3’
	Reverse first run	5’-GCTTTCTTAGTTGCGGCAGGGCGCTTAACAGC CTTGTACAGCTCGTCC-3’
	Reverse second run	5’-GTGTAAGCTTTTAATCTAGCTTTTTCTTTTTAGCC TGACCTGCTTTCTTAGTT-3’
c-Myc	Forward	5’- ACGATGGATCCATGGTGAGCAAGGGCGAGGA -3’
	Reverse	5’-GTGTAAGCTTTTAGTCTAGTTTAACGCGTTTGGC AGCAGGCTTGTACAGCTCGTCC-3’
Tus	Forward	5’- ACGATGGATCCATGGTGAGCAAGGGCGAGGA -3’
	Reverse	5’-GTGTAAGCTTTTACTTTACAGGCCGTTTTATCTTG AGTTTCTTGTACAGCTCGTCC-3’
EGL-13	Forward first run	5’- GAAAACGCGAAGAAGCTTGCCAAGGAAGTTGAA AATATGGTGAGCAAGGGCGAGGA-3’
	Forward second run	5’-ATATGGATCCATGAGCCGTAGACGAAAAGCGAAT CCGACAAAACTGAGTGAAAACGCGAAGAAGCTTG-3’
	Reverse	5’-TATAAAGCTTTTACTTGTACAGCTC-3’

Table S2. Sequences of NLS-eGFPs (NLSs have been labeled with color)

eGFP-SV40: MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLP VPWPTLVTTFXYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYK TRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGI KVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRD HMVLLEFVTAAGITHGMDELYKPKKKRKV
eGFP-NLP: MRGSHHHHHHGSMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDAXYG KLTLKFICTTGKLPVPWPTLVTTFXYGVQCFSRYPDHMKQHDFFKSAMPEGYVQ ERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHN VYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLST QSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYKAVKRPAATKKAGQAKKK KLD
eGFP-c-Myc: MRGSHHHHHHGSMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYG KLTLKFICTTGKLPVPWPTLVTTFTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQ ERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHN VYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLST QSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYKPAAKRVKLD
eGFP-TUS: MRGSHHHHHHGSMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDAXYG KLTLKFICTTGKLPVPWPTLVTTFTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQ ERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHN VYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLST QSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYKKLKIKRPVK
EGL-13-eGFP: MRGSHHHHHHGSMSRRRKANPTKLSENAKKLAKEVENMVSKGEELFTGVVPIL VELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTFXYGVQ CFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRI ELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQL ADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHG MDELYK

Code S1. R code for the analysis of single cell fluorescence intensity.

```
#Begin, using SV40 as an example. Cytosol and nucleus have been splitted and
#saved separately as Tiff files.
library("tiff")
#Read pixels of tiff files for nucleus and cytosol separately as matrices and
#convert them to one dimension vectors.
nucleus<-as.vector(readTIFF("sv40_nu.tif"))
cytosol<-as.vector(readTIFF("sv40_cyto.tif"))
#Remove background and saturated pixels
nucleus<-nucleus[nucleus<1 & nucleus>0]
cytosol<-cytosol[cytosol<1 & cytosol>0]
#The value of each pixel is between 0 and 1. It should multiply 255 to obtain the
#value in 8-bit image.
compare<-list(nucleus=(nucleus)*255,cytosol=(cytosol)*255)
#Data analysis and plotting
mean_nu<-mean(nucleus)*255
mean_cyto<-mean(cytosol)*255
print(mean_nu/mean_cyto*100-100) #Normalization for percentage increase
pdf(file="sv40.pdf")
boxplot(compare,pch=20,col=c("red","blue"),ylab="Absolute Pixel Intensity",
      ylim=c(10,255),boxwex=0.5,outline=FALSE)
#Use the following plotting if necessary
#boxplot(compare,pch=20,col=c("red","blue"),ylab="Absolute Pixel Intensity",
#       boxwex=0.5,outline=FALSE)
dev.off()
#End. Generated pdf file can be further processed for illustration.
```

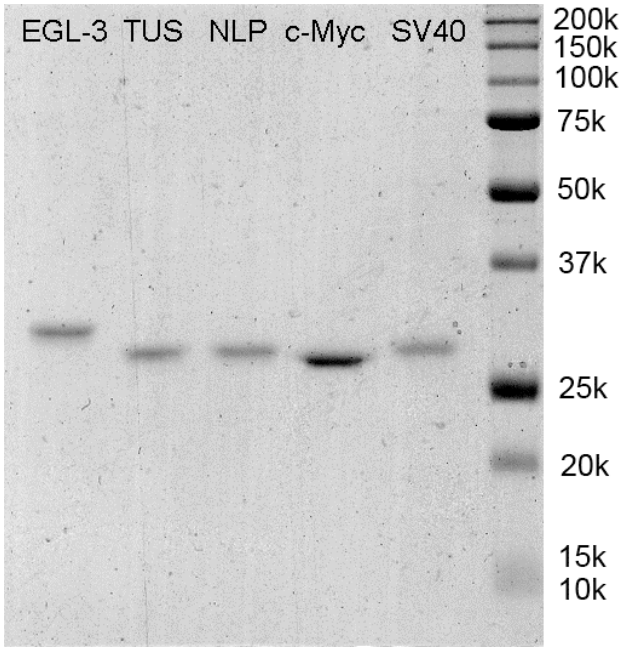
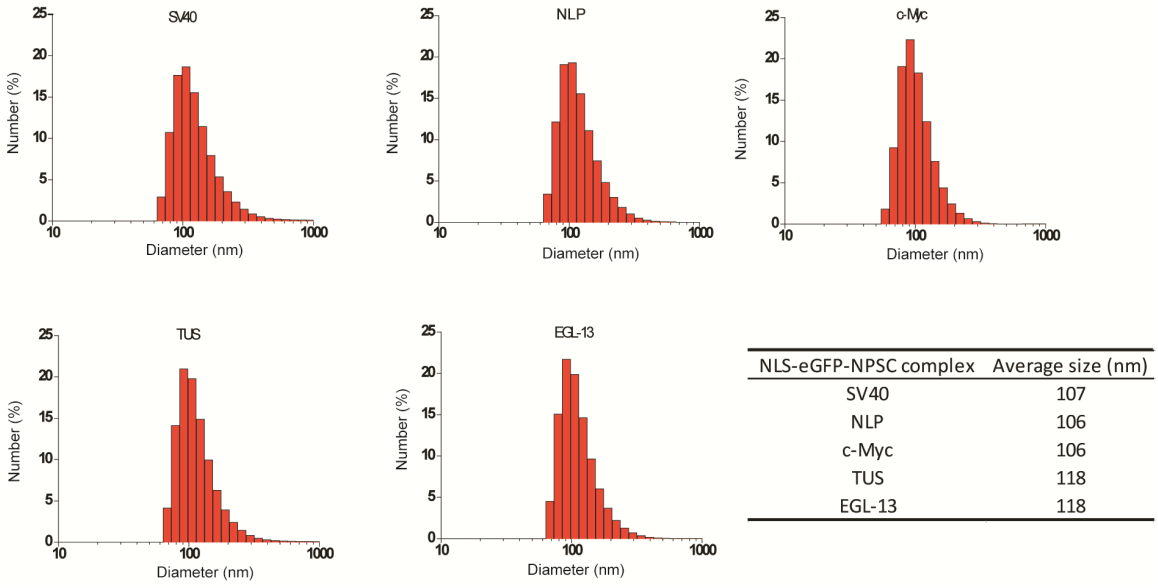


Figure S1. SDS-PAGE analysis of five NLS-eGFPs.



NLS-eGFP-NPSC complex	Average size (nm)
SV40	107
NLP	106
c-Myc	106
TUS	118
EGL-13	118

Figure S2. Dynamic Light Scattering measurements for NLS-eGFP-NPSC complexes

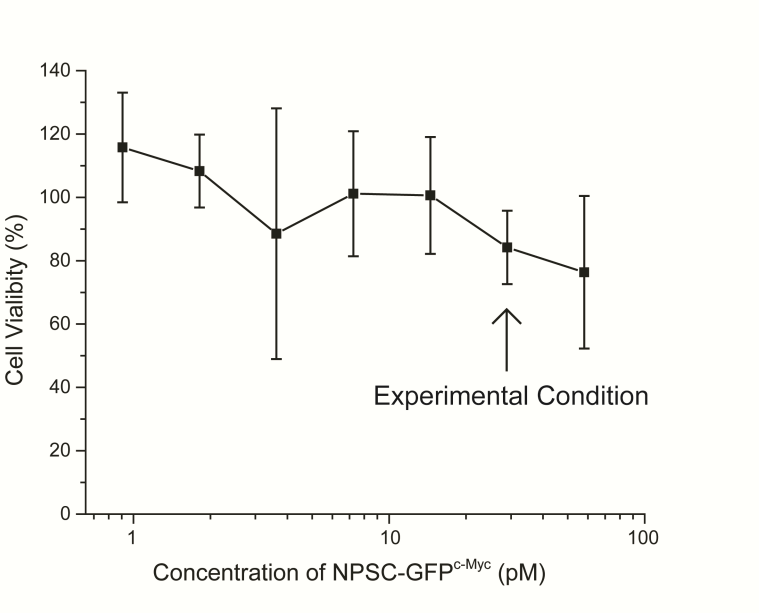


Figure S3. Viability of HeLa cells at different concentrations of NPSC-NLS$^{c\text{-}Myc}$ -eGFP complexes measured by Alamar Blue assay.

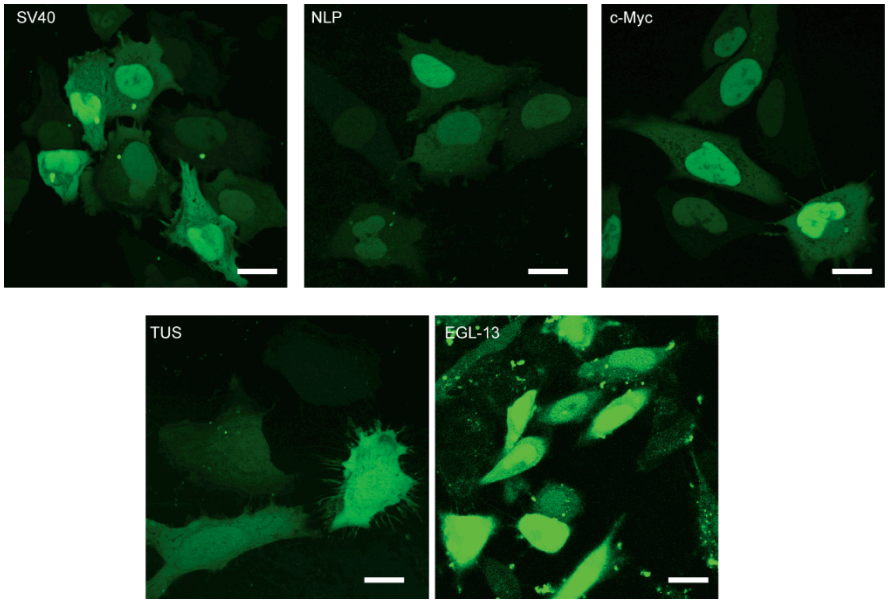


Figure S4. Large scale images of NLS-eGFPs delivered into HeLa cells. Bars: 20 μm.

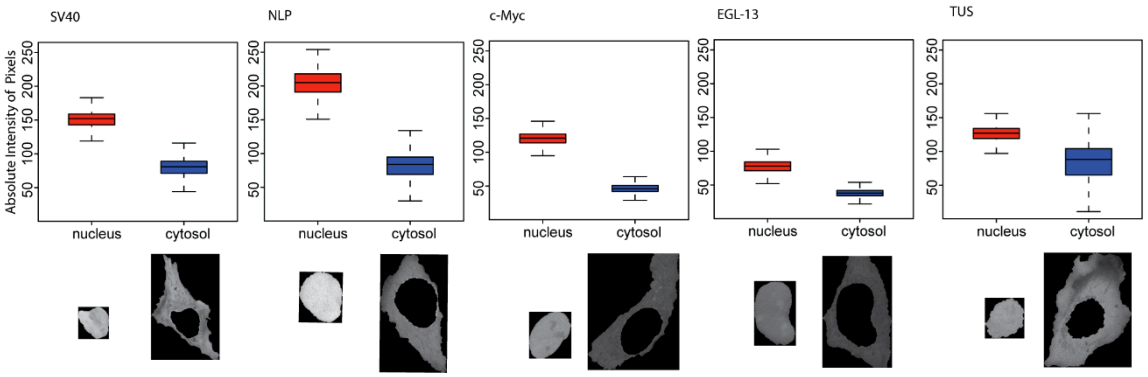


Figure S5. Typical results of quantitative fluorescence intensity analyses of NLS-eGFPs in single cells. Quartiles of pixel intensities were illustrated as box plots.

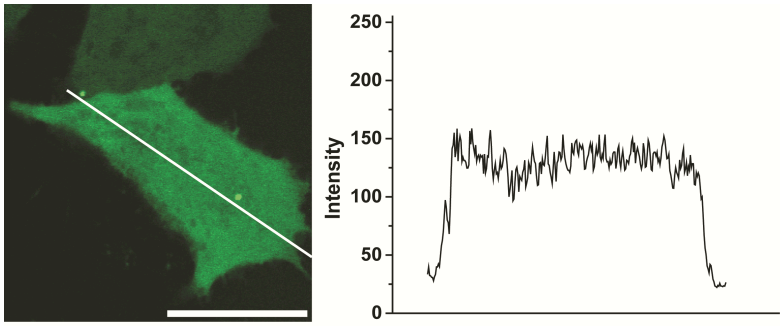


Figure S6. LSCM image showing cellular distribution pattern of eGFP without NLS. Bar: 20 μm.

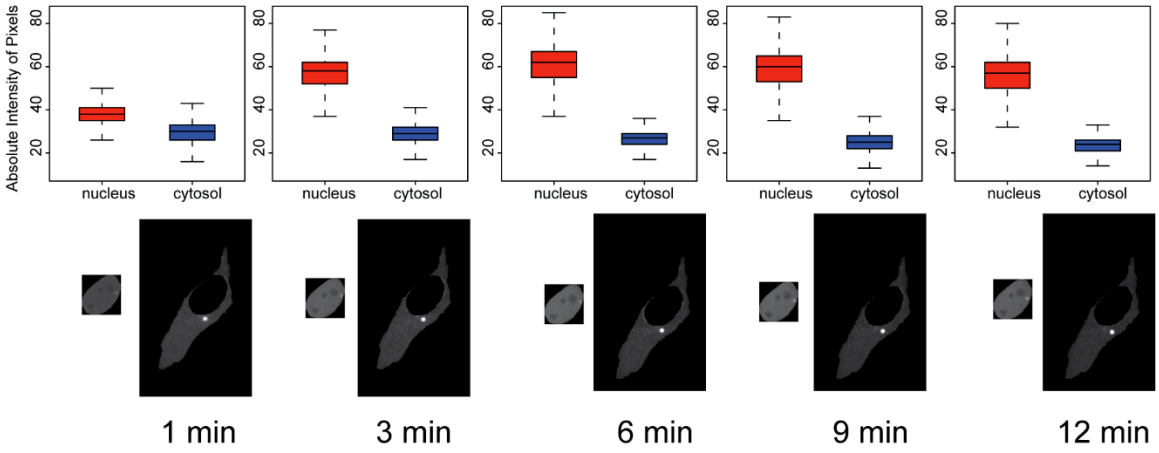


Figure S7. Typical results of quantitative fluorescence intensity analyses of NLS$^{c\text{-}Myc}$-eGFPs in single cell at different time scales. Quartiles of pixel intensities were illustrated as box plots.

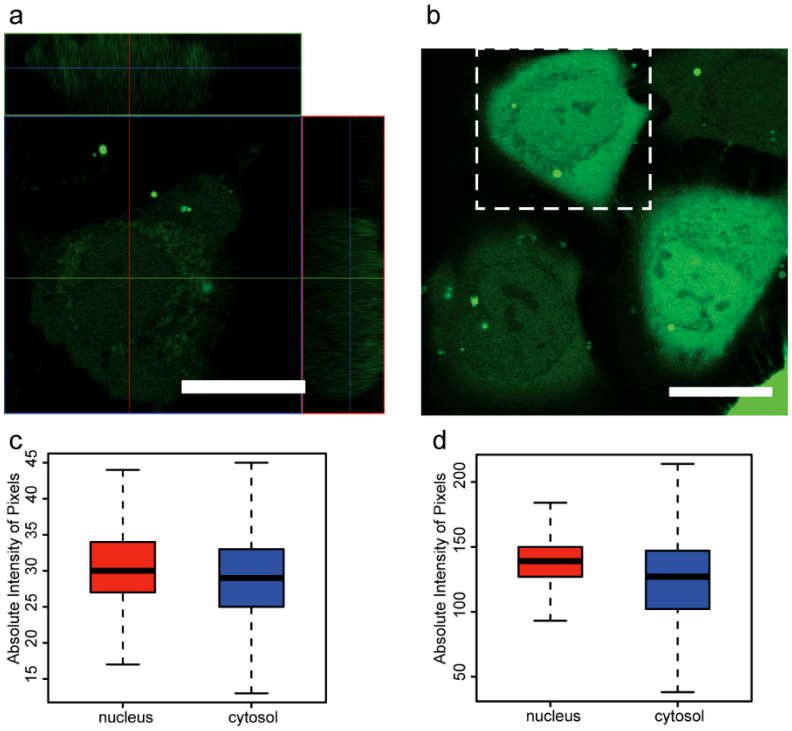


Figure S8. A comparison between the cytosolic delivery of (**a**) NLS$^{c\text{-}Myc}$-eGFP at ATP depletion condition and (**b**) eGFP without NLS into HeLa cells. Bars: 20 μm. (**c,d**) Quartiles of pixel intensities were illustrated as box plots. For (**d**) Analysis of the boxed cell in **b**.

References for Supporting Information

1. Tang, R., Kim, C. S., Solfiell, D. J., Rana, S., Mout, R., Velázquez-Delgado, E. M., Chompoosor, A., Jeong, Y., Yan, B., Zhu, Z. J., Kim, C., Hardy, J. A., and Rotello, V. M. (2013) Direct delivery of functional proteins and enzymes to the cytosol using nanoparticle-stabilized nanocapsules. *ACS Nano 7,* 6667–6673.