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VIPER^{nano}: Improved Live Cell Intracellular Protein Tracking

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Abstract

Tracking intracellular proteins in live cells has many challenges. The most widely used method, fluorescent protein fusions, can track proteins in their native cellular environment and has led to significant discoveries in cell biology. Fusion proteins add steric bulk to the target protein and can negatively affect native protein function. The use of exogenous probes such as antibodies or protein labels is problematic because these cannot cross the plasma membrane on their own and thus cannot label intracellular targets in cells. We developed a labeling platform, VIPER^{nano}, for live cell imaging of intracellular proteins using a peptide fusion tag (CoilE) to the protein of interest and delivery of a fluorescently labeled probe peptide (CoilR). CoilR and CoilE form an α -helical heterodimer with the protein of interest, rendering a labeled protein. Delivery of CoilR into the cell uses hollow gold nanoshells (HGNs) as the primary delivery vehicle. The technology relies on the conjugation and light-activated release of the CoilR peptide on the surface of the HGNs. We demonstrate light-activated VIPER^{nano} delivery and labeling with two intracellular proteins, localized either in the mitochondria or the nucleus. This technology has the ability to study intracellular protein dynamics and spatial tracking while lessening the steric bulk of tags associated with the protein of interest.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsami.9b12679](https://doi.org/10.1021/acsami.9b12679).

Time-lapse video demonstrates the dynamic movement of tagged mitochondria with mEmerald and CoilR. Tagged mitochondria movement (MP4)

The authors declare no competing financial interest.

Keywords

live cell imaging; protein tagging; coiled coil; hollow gold nanoshells; peptide delivery

INTRODUCTION

Chemical biology tools can interrogate protein function within live cells and provide high resolution spatial and temporal information. In particular, visualizing and tracking proteins through fluorescence microscopy has transformed cell biology.¹ Antibody-based methods form the basis of many such studies, yet these methods require specific reagents, and many proteins are not amenable to this approach. The reliance on cell fixation forms another barrier.^{2,3}

Fluorescent protein (FP) fusion tags provide an enormous improvement over immunolabeling by allowing the tracking of proteins in live cells.^{4–7} However, FP fusion tags rely on proteins that in many cases are larger than the protein of interest (see Figure 1), with concomitant alterations in protein function, trafficking, stability, and localization.^{8,9} More recently, smaller peptide epitopes consisting of ~10 amino acids (e.g., FLAG) have been explored as alternatives.¹⁰ However, these methods still require membrane permeabilization, fixation, and incubation with anti-FLAG antibodies which limits their capabilities for live cell imaging. Smaller molecule-based probes like tetracysteine or His6tag have been used to label intracellular proteins; however, they are limited by the number of proteins that can be observed at the same time^{11,12}

An alternative labeling strategy uses coiled-coil interactions as a basis for protein tagging. In this method, an endogenous protein–peptide fusion is labeled by incubation and dimerization with a second peptide conjugated to a fluorophore as shown in Figure 2a.^{13–16} We recently developed VIP (versatile interacting peptide) tags, which use a heterodimeric coiled-coil between two peptides as seen in Figure 2a.^{13,14} In the latest variation, VIPER, a fusion peptide tag (CoilE) binds with reporter-conjugated peptide (CoilR) and is used to label both extracellular and intracellular proteins. While the VIP tags are small (~5 kDa) and highly specific, the delivery of the probe peptide to cytosolic targets requires

permeabilization and fixation of the cells.^{13,14} Here we sought to improve the VIP tags by introducing a technology for accessing intracellular targets in living cells.^{13,15,16}

VIPER^{nano} (Versatile Interacting Peptides E and R with hollow gold nanoshells) demonstrates the capability of live cell imaging with small fluorescent tags using the VIPER coils attached to hollow gold nanoshells (HGNs) for light-activated protein labeling.^{17–19} This delivery strategy relies on orthogonal linkers attached to the HGN surface, one for internalization using a cell penetrating peptide and the other for CoilR attachment. Particle internalization via endocytosis results in endosomal entrapment until irradiation with NIR light as demonstrated in Figure 3a. For many nanoparticle delivery strategies, endosomal entrapment is a major obstacle.^{20,21} VIPER^{nano} allows for controlled release of the HGNs from the endosome²² as well as release of the CoilR from the HGN surface with near-infrared light.

Herein we describe a proof of concept colocalization study of intracellular targets on cellular components (mitochondria and histone H2B). Target proteins are expressed as a genetic fusion with the CoilE peptide and the complementary, CoilR, conjugated to a Sulfo-Cy5 dye (CoilR-Cy5), is delivered using the HGNs. Expansion of this technology will allow for the study of dynamic protein tracking in live cells with less impact on the protein function.

RESULTS AND DISCUSSION

Particle Design, Characterization, and Internalization.

VIP tags were previously developed to enable cellular proteins to be labeled and imaged without the need to add a large protein fusion, such as green fluorescent protein. VIPER was used to distinctly label the histone protein H2B and an inner membrane mitochondrial protein (using a COX8 fragment encoding a localization sequence: “Mito”) with a significantly smaller tag.¹⁴ The CoilE tag lessened the steric bulk around the protein of interest, which allows for the use of fluorescent labels with a more accurate representation of protein dynamics.

For example, a nucleosome core particle consists of a histone octamer wrapped with DNA²³ (Figure 1a). The GFP fusion to H2B creates steric bulk at the site where the DNA wraps around the histone proteins. Our model of this interaction suggests that the size of the GFP barrel would impact the structural role of histones in regulating transcription and in assembling specialized chromatin domains.²³ Prior studies of fluorescent proteins such as GFP have shown unanticipated issues with protein dynamics.^{8,9} A comparison of H2B fused to GFP (Figure 1b) and H2B fused to CoilE (Figure 1c) shows the size variation between the two tags. Notably, GFP is double the size of H2B whereas CoilE is just one-third the size of H2B. Our study relies on the use of protein FP fusions involving both coiled coil tags as well as FPs to enable a direct comparison of the localization as revealed by our technology; future applications of VIPER^{nano} will track proteins without fluorescent protein fusions.

Our HGN functionalization strategy relies on the robust affinity of the hexahistidine-tagged CoilR to nitrilotriacetic acid (NTA) in the presence of copper, which is attached to a thiolated PEG (poly(ethylene glycol)) linker assembled on the surface of the HGN.¹⁸ Cellular internalization of the HGNs is guided by the transactivating transcriptional activator

(TAT) peptide attached via streptavidin to a biotinylated PEG linker orthogonal to our CoilR component (Figure 2b). The two different thiolated PEG strands were loaded onto the HGN in equimolar amounts to allow for proper loading of the internalization peptide, TAT, as well as the CoilR-Cy5. Characterization of our nanoparticles showed that the final HGN particle size was ~150 nm (Figure 2c). The Cy5 fluorophore is a red-fluorescent probe that is bright and pH insensitive, which makes it compatible with observing proteins in lysosomes and other acidic organelles.²⁶

We previously demonstrated that the CoilR peptide alone, as well as gold particles without the TAT cell penetrating peptide, do not enter cells through passive diffusion.^{14,27} To confirm the internalization of PEGylated HGN-CoilR-Cy5 particles, HeLa cells (100 000 cells) were incubated with 3.2 pM HGN-CoilR-Cy5 for 2 h and visualized using both fluorescent and darkfield microscopy. The Cy5 fluorescent puncta within the HeLa cells indicated internalization of HGN-CoilR-Cy5 while the scattered light from the HGN surface in the darkfield microscopy image (orange puncta) demonstrated internalization of the HGN (Figure 3b).

We controlled the internalization of the HGN construct into human cells by using the TAT peptide.^{28,29} The modular design of the HGN construct allows for the use of other targeting peptides depending on the target cell application (Figure 2).

Load and Release Characterization.

Quantification of peptide load and release was determined in a cell free environment through analysis of the fluorescent Cy5 label on the CoilR peptide. HGNs coated with a 1:1 layer of thiolated PEG-biotin and PEG-NTA (32 pM) were incubated with 1 μ M CoilR and then washed to remove the excess CoilR-Cy5 via centrifugation. The particles were then exposed to a pulsed 800 nm laser at 300 mW for 15 s to release the loaded peptide from the surface of the particle. The particles were concentrated via centrifugation, and the supernatant was analyzed using a Tecan M200 fluorescent plate reader and compared to a titration curve of known Cy5 fluorescence and CoilR concentration. The HGN-CoilR-Cy5 samples that were not irradiated with NIR light were treated with a buffer containing KCN to dissolve the gold particles and therefore chemically released the bound peptide for total loading quantification of the CoilR-Cy5 peptide. We determined that 10 000 CoilR-Cy5 peptides were loaded per particle, which is comparable to the HGN-DNA-NTA construct previously reported.²⁷ Comparison of the supernatants in Figure 4a demonstrates the release of 63% of the loaded CoilR peptide only upon NIR irradiation which agrees with previous PEG release studies using HGNs.¹⁷

Further release studies were conducted to demonstrate the release capabilities of CoilR-Cy5 from HGN once internalized into HeLa cells. Samples were irradiated with a commercial two-photon confocal microscope equipped with a scanning, tunable NIR laser set to 800 nm and 240 mW. Comparison of images taken before and after irradiation show release of CoilR-Cy5 from puncta upon irradiation with NIR light (Figure 4b,c). Diffusion of the Cy5 dye upon irradiation is within seconds and visualized in a low magnification image in Figure 4b. Individual cells were isolated and compared for their overall intensity and distribution of Cy5 dye using the ImageJ Plot Profile analysis tool (Figure 4b,c). Pixel plot profiles of four

selected cells (in blue boxes shown in Figure 4b) were analyzed before and after laser irradiation to demonstrate the overall increase in both intensity and distribution of Cy5 throughout the cells before (black trace) and after (magenta trace) laser irradiation (Figure 4d). The gray value demonstrates the overall intensity of pixels at a given location in the box (distance, pixels).

Colocalization of CoilR with Subcellular Targets Is Laser Dependent and Allows for Dynamic Protein Tracking in Live Cells.

Many labeling strategies that use endogenous tags lack the ability to control how much of the labeled target is released.³⁰ The delivery of CoilR with HGN provides a means to tune the amount of CoilR delivered through changing the NIR laser power used to release the CoilR. To demonstrate this feature, we plated HeLa cells on a gridded ibidi culture dish (60 μ -Dish, High Grid 500) to allow us to relocate the irradiated areas using a two-photon confocal microscope. HeLa cells transfected with Mito-CoilE-mEmerald were incubated with HGN-CoilR-Cy5 and subjected to NIR irradiation at different laser powers (0, 168, 240 mW). The samples were later visualized on a Leica SP8 resonant scanning confocal microscope to assess colocalization between the green fluorescent mitochondria target and the delivered red fluorescent Coil-Cy5 peptide (Figure 5a). An increase in protein labeling was observed with higher laser powers and no mitochondrial protein label was observed in samples that were not irradiated with NIR light. Cells that do not express the CoilE fusion protein still internalized the CoilR-Cy5; however, the mitochondria in these cells were not labeled due to the lack of CoilE fusion peptide (Figure 5a).

Super-resolution microscopy can track single molecules in live cells and reveal details of cellular structures.³¹ We used the Bruker/Vutara 352 super-resolution microscope to observe HeLa cells expressing H2B-CoilE-mEmerald and treated with HGN-CoilR-Cy5 after laser irradiation and tracked the migration of CoilR-Cy5 over 10 s using the Vutara 2D particle tracking module.³² The software was able to track the movement of CoilR peptide through analysis of Cy5 dye movement in the selected cell, demonstrating release of the CoilR after laser irradiation. The Cy5 dye was stationary in nonirradiated samples (Figure 5b). From this tracking experiment, we conclude that VIPER^{nano} colocalization can be used for super resolution tracking of target protein movement in live cells in a laser power-dependent manner.

We tracked protein labeling of the mitochondria and H2B targets over the course of hours and days to visualize protein movement in specific cells that were relocated using the gridded tissue culture plates. Time-lapse images were collected over a period of 2.5 h for the mitochondria CoilR Cy5 labels to demonstrate the dynamic movement of the mitochondria overtime validated by simultaneously tracking mEmerald movement (Figure 6a,c). A super resolution microscopy (SRM) technique, Super-Resolution Radial Fluctuations (SRRF), was used to extract subdiffraction information from a quick, short burst of 100 images taken in time lapse on a confocal fluorescent microscope.³³ The SRRF analysis allows for long-term SRM time-lapse acquisition using lower intensity illumination to prevent phototoxicity during the live cell microscopy analysis.³⁴ A SRRF time lapse was acquired over the period of 2.5 h, showing the movement of mitochondria over a short period of time (Figure 6c). A

time-lapse video demonstrates the dynamic movement of tagged mitochondria with mEmerald and CoilR in the Supporting Information. The video tracking of CoilR movement shows how VIPER^{nano} can be applied to study protein dynamics in live cells.

For H2B protein labeling we imaged at two time points 24 h apart to observe the longevity of the VIP tags in a system undergoing cellular division. Similar to previous histone-mEmerald fusion protein analysis of histone dynamics,³⁵ chromatin bridges can be visualized during certain stages of mitosis.³⁶ The same cells were located ~24 h later using the gridded culture dish and imaged to show proper division into two individual cells (Figure 6b). The CoilR and mEmerald colocalization demonstrates the capability of VIPER^{nano} for longer term protein dynamics analysis.

CONCLUSIONS

Our results show the light-activated delivery of CoilR using HGN unleashes a way to study intracellular protein dynamics and tracking while lessening the steric bulk of tags associated with the protein of interest. This development provides a new strategy to track proteins without the introduction of large fusion proteins. We demonstrate VIPER^{nano}, which utilizes HGN for protein tagging of intracellular targets with the addition of a small tag, a peptide less than 6 kDa to the protein of interest. The HGN construct design for delivery is advantageous, as it can be modulated to allow for particle internalization into diverse cell lines using an alternative cell-penetrating peptide motif on the particle. For example, substitution of the TAT peptide for a biotinylated RPARPAR peptide lends to specific cell targeting to PPC-1 cells that overexpress the neuropilin-1 receptor.^{17,37} We demonstrated the light-controlled capability of the technology that allows for control over when the labeling of intracellular targets occurs, how much of the CoilR peptide is released, and cellular specification of peptide release.

MATERIALS AND METHODS

PEG Adsorption onto HGN.

HGNs were synthesized using sacrificial silver template in a previously described protocol.^{27,38} The particles were then dialyzed overnight in sodium citrate buffer (500 mM) with 0.03% diethyl pyrocarbonate (DEPC) (Biochemica) in dialysis cassettes (MWCO 20 kDa).

To coat the particles with the 1:1 PEG layer, HS-PEG-biotin (5K) and HS-PEG-NTA (3.4K) 1 mM stocks were incubated with 12.5 mM TCEP for 10 min to reduce dithiols in solution. A final concentration of 10 μ M PEG (5 μ M SH-PEG-NTA and 5 μ M SH-PEG-biotin) was added to 64 pM HGNs, incubated on a rocker overnight at room temperature, and washed 3 $\times$ in PBS with 0.01% Tween 20 (PBST) at 20 000g for 20 min to remove excess PEG.

Streptavidin and Biotin-TAT Functionalization.

For particle internalization, streptavidin–biotin–TAT was added to the SH-PEG-biotin strands on the 1:1 PEG particles. A 2 mg/mL streptavidin stock in PBST was added in equal volume to 64 pM pegylated particles and quickly sonicated and vortexed. After a 1 h incubation period, excess streptavidin was removed with three washes in PBST at 10 000g

for 10 min. A final concentration of 20 μM biotin-TAT (Anaspec) was achieved through two equal volume additions with a 30 min incubation at room temperature after each addition. The particles were then washed another three times with PBST to remove any excess biotin-TAT and stored at 4 °C.

Quantification Cell-Free CoilR-Cy5 Load and Release.

In brief, the CoilR peptide was previously expressed in *E. coli* BL21 (DE3) cells (thermofisher) using a pET28b(+)-CoilR plasmid and purified under denaturing conditions.¹⁴ The purified CoilR peptide was labeled with Cy5 dye for peptide tracking with a maleimide dye which reacted with the single reactive cysteine residue on the CoilR peptide.¹⁴

CoilR-Cy5 (1 μM initial) was loaded onto 32 pM HGNs incubated with 500 μM CuCl_2 for 20 min on ice. Excess CoilR-Cy5 was removed with three washes in PBST via centrifugation at 10 000g for 10 min. Total peptide loading per particle was determined using a previously described KCN etch procedure where 32 pM particles were incubated in a KCN solution [0.1 M KCN, 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$] to dissolve the gold particles and release the labeled peptide.²⁷ The concentration of the released peptide was quantified using a standard linear calibration curve between the concentration of CoilR-Cy5 and the corresponding fluorescence intensity detected using the Tecan M200 plate reader. HGN loaded with CoilR-Cy5 were irradiated with NIR light using a pulsed laser generated from a femtosecond Ti:sapphire regenerative amplified (Spectraphysics Spitfire) running at 1 kHz repetition rate for 15 s at 500 mW laser power (power determined using a thermopile power meter). Quantification of peptide released was determined after centrifugation of the samples and removal of the supernatant. The supernatant was plated on a 96-well plate for Cy5 fluorescence analysis and the pellets were then etched to determine the amount of peptide retained on the particle.

Cell Culture and Transfection.

VIPER^{nano} Imaging experiments were conducted in transiently transfected HeLa cells cultured at 37°C in 5% CO_2 . To create the plasmid constructs for transfection, the CoilE gene was inserted into fusion protein mEmerald constructs obtained from Addgene (Michael Davidson's Collection): Mito-7-mEmerald (Addgene #54160) and H2B-mEmerald (Addgene #54111).¹⁴ To generate the cell lines expressing the CoilE peptide, HeLa cells were plated in a six-well tissue culture plate seeded at 3×10^5 cells per well 48 h prior to transfection. Each plasmid transfection was performed using lipofectamine 2000 (ThermoFisher Scientific) according to the manufacturer's protocol. Each transfection mixture included 1.4 μg of plasmid DNA and 7 μL of lipofectamine reagent in 400 μL of Opti-MEM. The cells were washed after 2 h, and the transfection media was replaced with DMEM + 10% FBS.

Darkfield and Fluorescence Microscopy for Internalization Analysis.

HeLa cells were plated in an eight-well chambered glass slide (Millipore) at 4×10^5 cells per well in 400 μL of DMEM + 10% FBS approximately 24 h prior to particle internalization. HGNs loaded with CoilR-Cy5 were suspended in DMEM + 10% FBS (3.2

pM) and incubated with plated HeLa cells for 2 h at 37 °C at 5% CO₂. Cells were washed with PBS twice, and then one drop of PBS was added to each well before a cover glass was applied to the glass slide containing the HeLa cells. Samples were observed on an Olympus BX51 upright compound microscope with a dark field condenser and a 20× objective lens.

Cellular Release Using Two-Photon Microscope and Analysis with ImageJ.

Transfected cells were plated on Ibidi μ -Dish (35 mm, high Grid-50) at 100 000 cells 2–3 days post transfection and 24 h prior to laser excitation. HGNs were loaded with CoilR-Cy5 as in the previously described method and filtered using a 0.22 μ m filter to remove any large particle contaminants prior to incubation with HeLa cells. The 4 pM particles were incubated in 400 μ L of DMEM with 10% FBS at 37 °C at 5% CO₂ in an ibidi culture dish. After 2 h the samples were washed with PBS three times to remove excess particles and then suspended in 2 mL of DMEM, high glucose, HEPES, no phenol red supplemented with 10% FBS. Samples were focused using a Olympus Fluoview 1000MPE two-photon and confocal microscope equipped with a 25× water immersion objective lens and irradiated using a mode locked Ti:sapphire tunable femtosecond pulsed laser (100 fs pulse duration, 80 mHz repetition rate, Mai Tai HP, Newport Spectra Physics). The cells were kept in a humidified chamber at 37 °C and 5% CO₂ through the duration of the two-photon microscopy experiment. The NIR laser irradiation was set to irradiate at 800 nm in 0.69 nm slices through the cell volume at varying NIR power percentages to get to 168 and 240 mW. Images capturing mEmerald and Cy5 fluorescence were collected before and after laser irradiation to confirm release of the CoilR peptide, and these images were later analyzed using (Fiji Is Just) ImageJ for diffusion of the Cy5 dye. Single cells were selected and analyzed using the pixel plot profile analysis tool. The data was later visualized in GraphPad Prism using second order smoothing with 80 neighbors for qualitative analysis of pixel distribution with and without laser. The cell samples were later analyzed for colocalization using a Leica SP8 resonant scanning confocal microscope or the Vutara 352 Superresolution microscope for particle tracking analysis.

Super Resolution Particle Tracking.

Post NIR irradiation, samples were imaged on the Vutara 352 super resolution microscope for analysis of Cy5 localization with and without laser irradiation. HeLa cells transfected with H2B-CoilE-mEmerald previously incubated with HGN-CoilR-Cy5 were initially visualized by focusing on the green fluorescence in the nucleus. Once a single cell was located, a 10 000 ms time lapse was obtained on both the mEmerald and Cy5 fluorescence channels. The peptide location was later analyzed using the SRX software capable of high resolution single molecule tracking for the Cy5 movement through the duration of the time lapse. Cy5 movement in cells irradiated with NIR light was compared to the movement in those that were not irradiated with NIR light.

Confocal Imaging for Colocalization Analysis.

Confocal microscopy was conducted on a Leica SP8 resonant scanning confocal microscope with a 65× oil objective lens in a live cell chamber which kept the cells at 37 °C and 5% CO₂ throughout the data collection. Excitation source for mEmerald and Cy5 fluorescence was a white light laser at 488 and 633 nm, respectively. Time-lapse images (100 frames) for

Mito colocalization for later SRRF analysis were collected using the xyt function for 10 min per final SRRF image. SRRF analysis was conducted using the NanoJ-SRRF plug-in for (Fiji Is Just) ImageJ. All images were false-colored using standard lookup tables: mEmerald (green) and Cy5 (magenta). These two colors when merged in ImageJ to create white for colocalization analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

HGN	hollow gold nanoshell
VIPER	versatile interacting peptides E and R
FP	fluorescent protein
FM	fluorescence microscopy
NTA	nitrilotriaceticacid
PEG	poly(ethylene glycol)
TAT	trans activator
NIR	near-infrared
ROI	region of interest
POI	protein of interest
SRM	super-resolution microscopy
SRRF	super-resolution radial fluctuations
PBS	phosphate-buffered saline solution

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(A) Nucleosome with H2B-GFP (B) H2B-GFP fusion (C) H2B-CoilE fusion

Figure 1.

VIPER^{nano} relies on much smaller fluorescent protein tags. (A) Nucleosome (PDB ID: 1kx5) with H2B-GFP (GFP PDB ID: 2yog) shows the DNA (tan) wrapped around the octamer of histone proteins (light blue) including the two H2B proteins (dark blue) fused to GFP (green). (B) H2B-GFP fusion monomer with size markers for GFP (27 kDa) and H2B (15 kDa) (C) H2B-CoilE fusion monomer with size markers for CoilE (5.2 kDa) and H2B (15 kDa). Structure prediction for CoilE peptide model with PEPFOLD3.^{24,25}

Figure 2.

Particle assembly strategy and size analysis. (a) VIPER technology strategy. A target protein is genetically tagged with the CoilE peptide. The tagged protein can then be labeled by dimerization with a CoilR peptide conjugated to a fluorescent dye delivered with a HGN. (b) Illustration of HGN construct with 1:1 loading of thiol-PEG-NTA (CoilR-Cy5 strand): Thiol-PEG-biotin (internalization strand). The biotin-terminated PEG was labeled with streptavidin TAT for internalization while the orthogonal NTA-terminated strand allowed for CoilR-Cy5 loading. (c) Dynamic light scattering size distribution of nanoparticles at different stages of synthesis and conjugation steps.

Figure 3.

(a) Schematic of HGN-CoilR-Cy5 (magenta) internalization and release from the endosome with NIR light colocalizes with cellular targets (e.g., H2B-CoilE-mEmerald and Mito-CoilE-mEmerald). (b) Streptavidin–TAT coating on nanoparticles allows for internalization of HGN-CoilR-Cy5 into HeLa cells observed by Cy5 fluorescence and light scattered from the gold nanoparticles with dark field microscopy (orange puncta).

Figure 4. Release of CoilR-Cy5 from HGN is NIR light activated.

(a) Release of CoilR-Cy5 from 32 pM HGNs quantified by fluorescence intensity observed in the supernatant solution after NIR irradiation and centrifugation. Samples were centrifuged, and their pellets were analyzed for total peptide loading with KCN etch and fluorescence detection. (b) HeLa cells were treated with HGN-CoilR-Cy5 and irradiated using a two-photon microscope at 800 nm. Release of CoilR-Cy5 upon NIR irradiation is demonstrated by the diffusion of CoilR-Cy5 across HeLa cells as the peptide was released from the endosomal membrane into the cytosol. The blue boxes indicate region of interests (ROIs) selected to demonstrate CoilR-Cy5 diffusion through pixel distribution plots. (c) Magnification of selected ROIs demonstrating CoilR-Cy5 peptide diffusion from fluorescent puncta after NIR laser irradiation. (d) Smoothed pixel distribution plot (2nd order, 80 neighbors) where an increase in gray value demonstrates the increase in Cy5 fluorescence observed in an averaged plot from $N=4$ before laser irradiation (black trace) and $N=4$ after laser irradiation (magenta trace).

Figure 5.

(a) Confocal imaging of HeLa cells transfected with Mito-CoilE-mEmerald 24 h post laser irradiation of a region of interest (ROI) with internalized HGN-CoilR shows specific labeling (white merge) of cellular mitochondria (Mito-CoilE-mEmerald in green) with CoilR-Cy5 (magenta) increases with higher laser irradiation power. Two-photon NIR excitation at full power was 2.4 W. (b) Overlay of mEmerald and Cy5 in high resolution fluorescence microscopy with Bruker Vutara super resolution microscope of CoilR-Cy5 in HeLa cells with H2B-CoilE-mEmerald. Peptide tracking over 10 s shows peptide movement only in irradiated samples (top image) and little to no movement in nonirradiated samples (bottom image) $N = 3$ for both laser conditions.

Figure 6.

Live tracking of cellular dynamics using HGN-CoilR (a) HeLa cells transfected with Mito-CoilE-mEmerald were treated with 3.2 pM HGN-CoilR and irradiated using a two-photon microscope at 800 nm. Twenty-four hour post-laser-irradiated images were collected using a Leica SP8 resonant scanning confocal microscope over a period of 2.5 h to demonstrate the ability to track mitochondria movement over time. Each photo is a combination of 100 images taken as a time lapse to generate one NanoJ-SRRF high resolution image. (b) HeLa cells transfected with H2B-CoilE-mEmerald were treated with 3.2 pM HGN-CoilR and irradiated. The cells were later relocalised using gridded culture dishes 24 and 48 h post laser irradiation to observe the separate of two nuclei using the CoilR peptide. (c) Time lapse imaging of (a) demonstrates dynamic movement of mitochondria with both CoilR and mEmerald. White scale bar in images corresponds to 8 μm .