

Simultaneous intracellular delivery of targeting antibodies and functional nanoparticles with engineered protein G system

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By,
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Work done

1. Synthesis of gold-coated iron oxide nanoparticles and modification of their surfaces
2. Surface modification of quantum dots with a NTA–Ni(II)-phospholipids micelle
3. Construction of expression vectors
4. Expression and purification of recombinant fusion proteins
5. Intracellular delivery of engineered proteins and nanoparticle/engineered protein hybrids

Synthesis of gold-coated iron oxide nanoparticles

Iron(III) acetylacetonate (5 mmol)
+
oleic acid (10 mmol)
+
Oleylamine (10 mmol)
+
hydrazine monohydrate (20 mmol)
+
Trioctylamine (TOA, 50 mL)

(250 mL, vigorous
stirring at 180°C for
18 hrs)



cooling to RT



addition of 50mL Toluene



Filteration and washing ppt with acetone/methanol 1:1
And stored in methanol/oleylamine/oleic acid [5/1/1]

Synthesis of gold-coated iron oxide nanoparticles

iron oxide nanoparticles
(0.1 g in 40 mL of benzyl ether)
+
gold(III) acetate (2.2 mmol)
+
1,2-hexadecanediol (12 mmol)
+
oleic acid (1.5 mmol)
+
oleylamine (6 mmol)

(vigorous stirring at
190°C for 2 h)



cooling to RT



washing with ethanol



NP collection by magnet

Surface modification of gold-coated iron oxide nanoparticles

A = gold-coated iron oxide NPs reacted with mercaptoundecanoic acid (500 μL , 2mM in ethanol) for 12 hrs

B = amine–nitrilotriacetic acid (200 μL , 1 mg mL⁻¹) + sodium hydroxide (800 μL , 1 mM) + EDC (40 μL , 0.4 M)

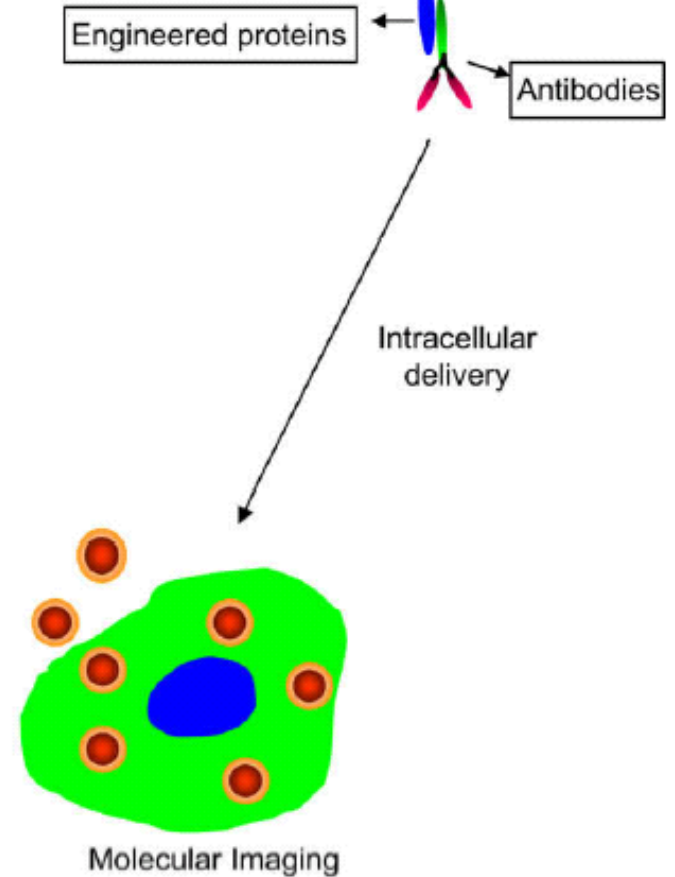
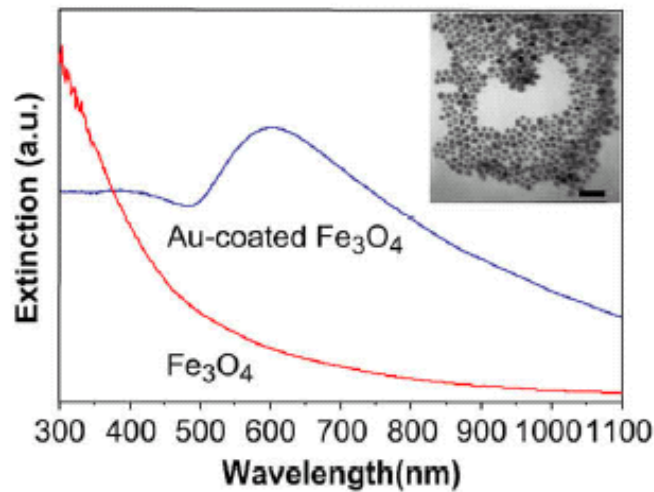
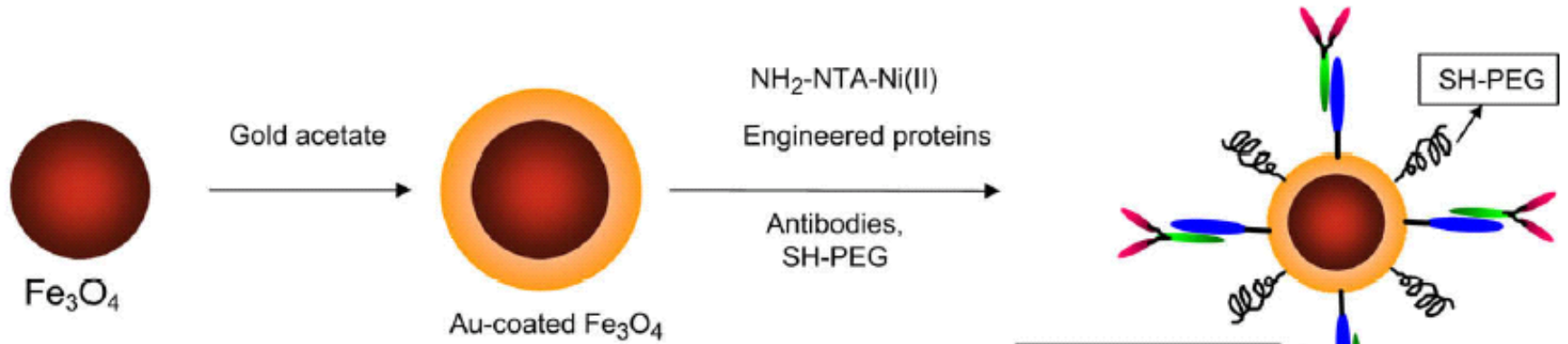
A + B $\xrightarrow{\hspace{1.5cm}}$ product + nickel(II) sulfate (1 mL, 50 mM)
(30 min incubation) ↓ (15 min)

centrifugation (10,000 rpm, 10 min)



Gold-coated iron oxide NPs modified with (NH₂–NTA–Ni(II))

gold-coated iron oxide nanoparticles, general strategy



Cell lysis

Target separation

Surface modification of quantum dots with a NTA–Ni(II)-phospholipids micelle

A = QDs in toluene (525 nm emission) washed three times with methanol and re-dispersed in chloroform (3.7 mg/mL)

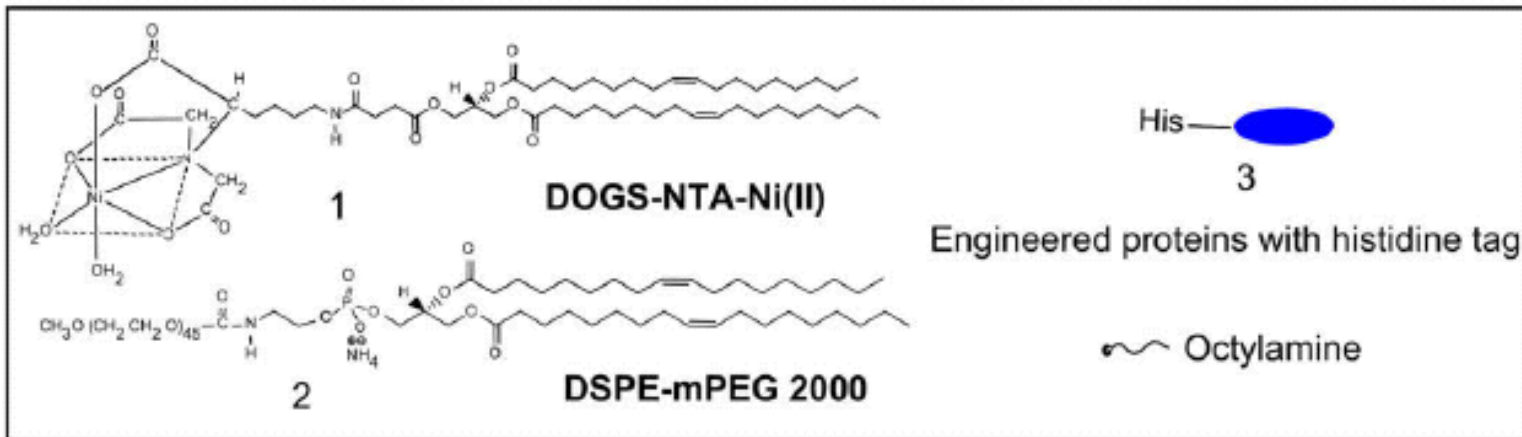
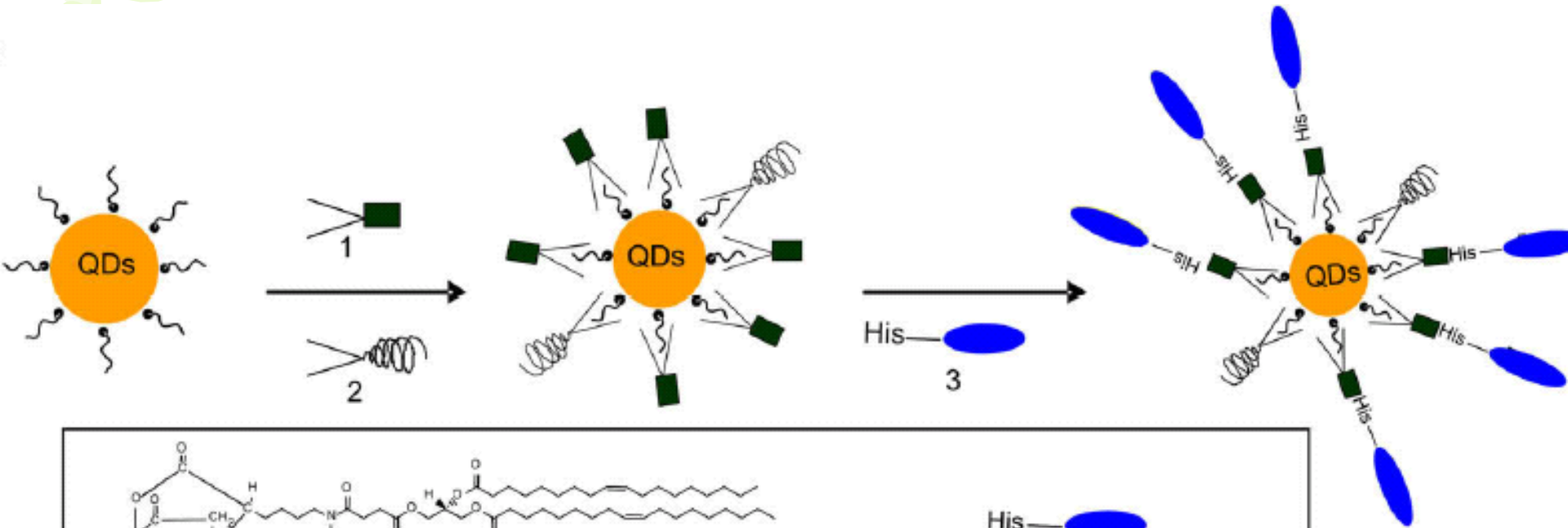
B = DSPE-mPEG2000 : DOGS-NTA-Ni(II) (3:7) in 1.6 mL of chloroform + 400 μ L of A

B was dried and re-suspended in DI water or PBS and filtered using a 0.2 μ m syringe filter.

DSPE-mPEG 2000 - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (PEG) 2000

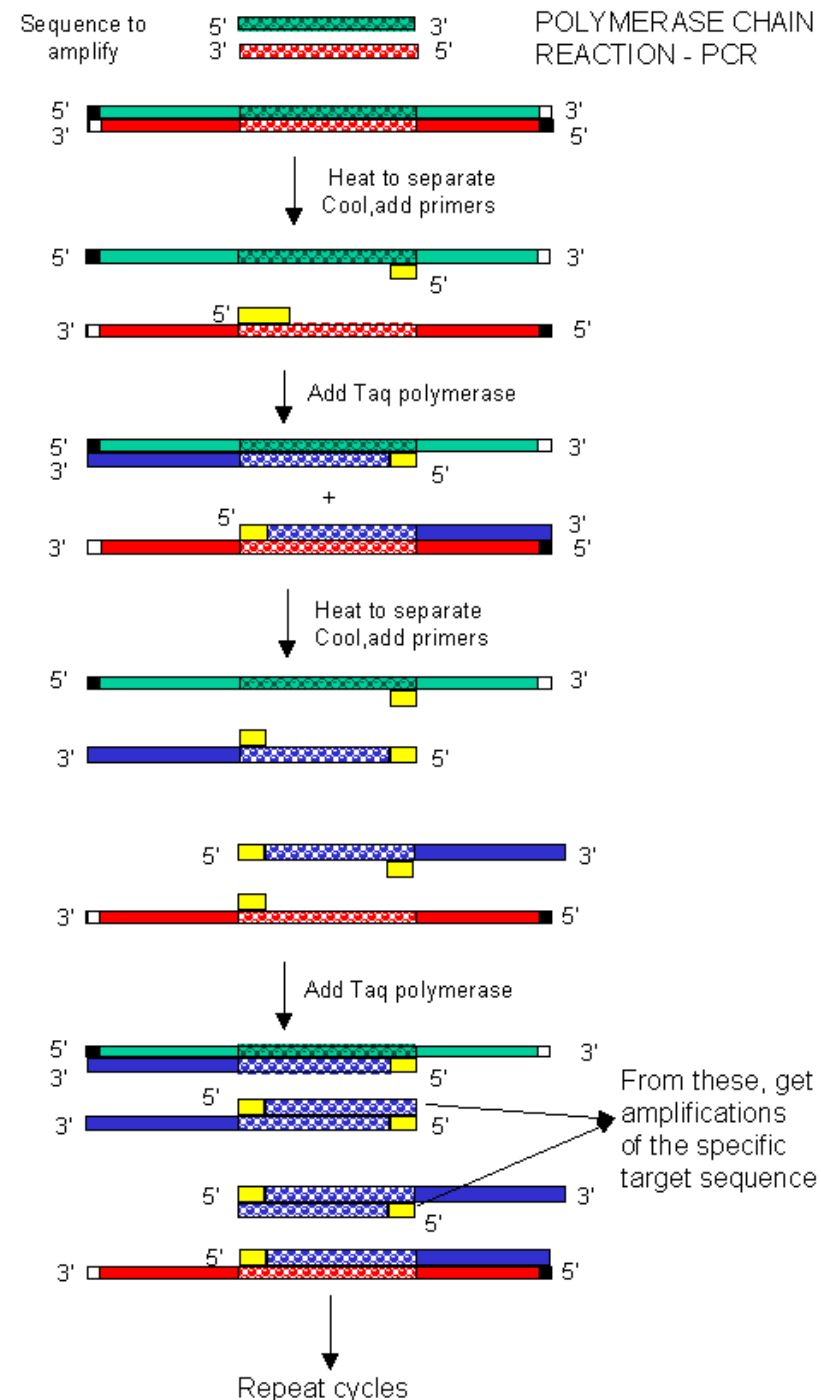
DOGS-NTA-Ni(II) - 1,2-dioleoyl-sn-glycero-3-[[N(5-amino-1-carboxypentyl)iminodiacetic acid]-succinyl](nickel salt)

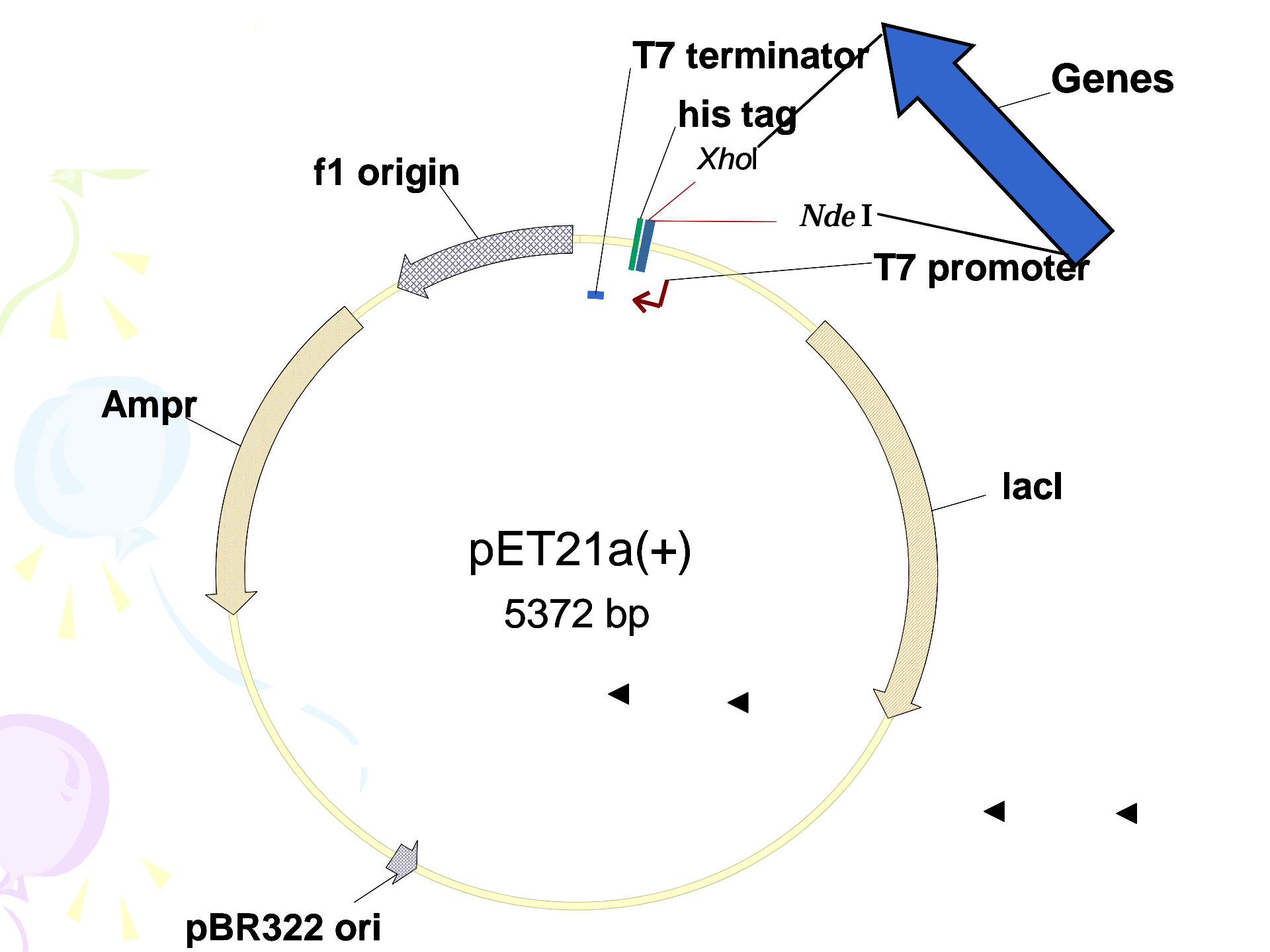
Surface modification of quantum dots with a NTA-Ni(II)-phospholipids micelle



Construction of expression Vectors

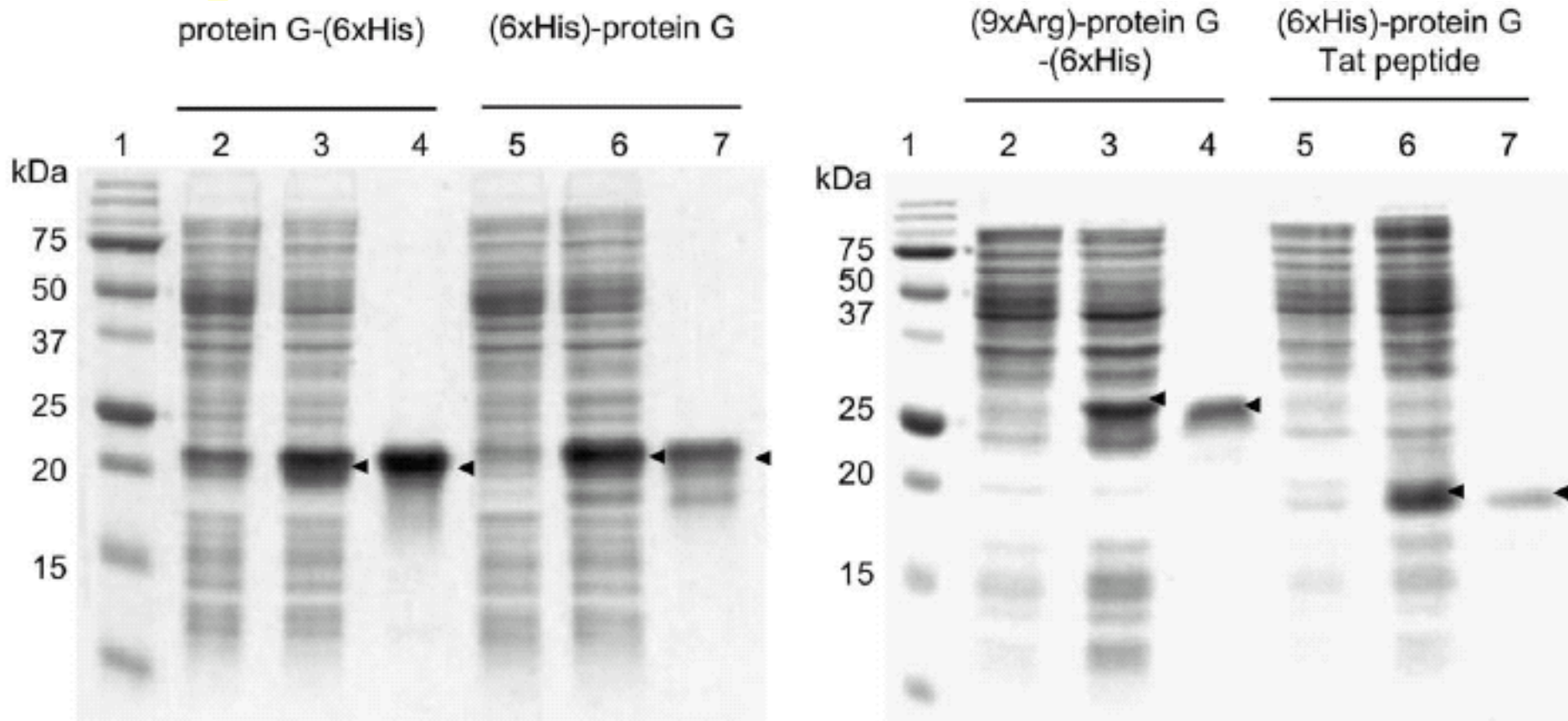
- Vectors produced,
 - pET-(6 His)-protein G-Tat peptide
 - pET-(6His)-protein G
 - pET-(9 Arg)-protein G-(6His)
 - pET-protein G-(6 His)
- Restriction enzymes used,
 - NdeI & XhoI
- Vectors used - pET21a vector





Expression and purification of recombinant fusion Proteins

1. Vectors transformed into Ecoli BL21(DE3)
2. Ecoli grown in LBB
3. Expression was induced by adding IPTG and cells were grown for 12 h at 25 C
4. Cells centrifuged at 6,000g for 10 min and lysed by sonication.
5. The soluble and insoluble fractions were separated by centrifugation at 12,000g for 20 min at 4 C.
6. Recombinant proteins were purified from supernatants by IMAC (Immobilized Metal Affinity Chromatography), eluted with 50 mM Tris-HCl/0.5 M NaCl (pH = 8.0) containing 0.5 M imimidazole
7. Product was dialyzed against PBS (pH = 7.4)



Lane 1, protein molecular weight marker;

Lanes 2 and 5, total cellular fraction, no IPTG induction;

Lanes 3 and 6, total cellular fraction, IPTG induction;

Lanes 4 and 7, elutes after IMDA affinity chromatography.

Intracellular delivery of engineered proteins

HeLa cells were grown in DMEM [including 10% (v/v) FBS and antibiotics]



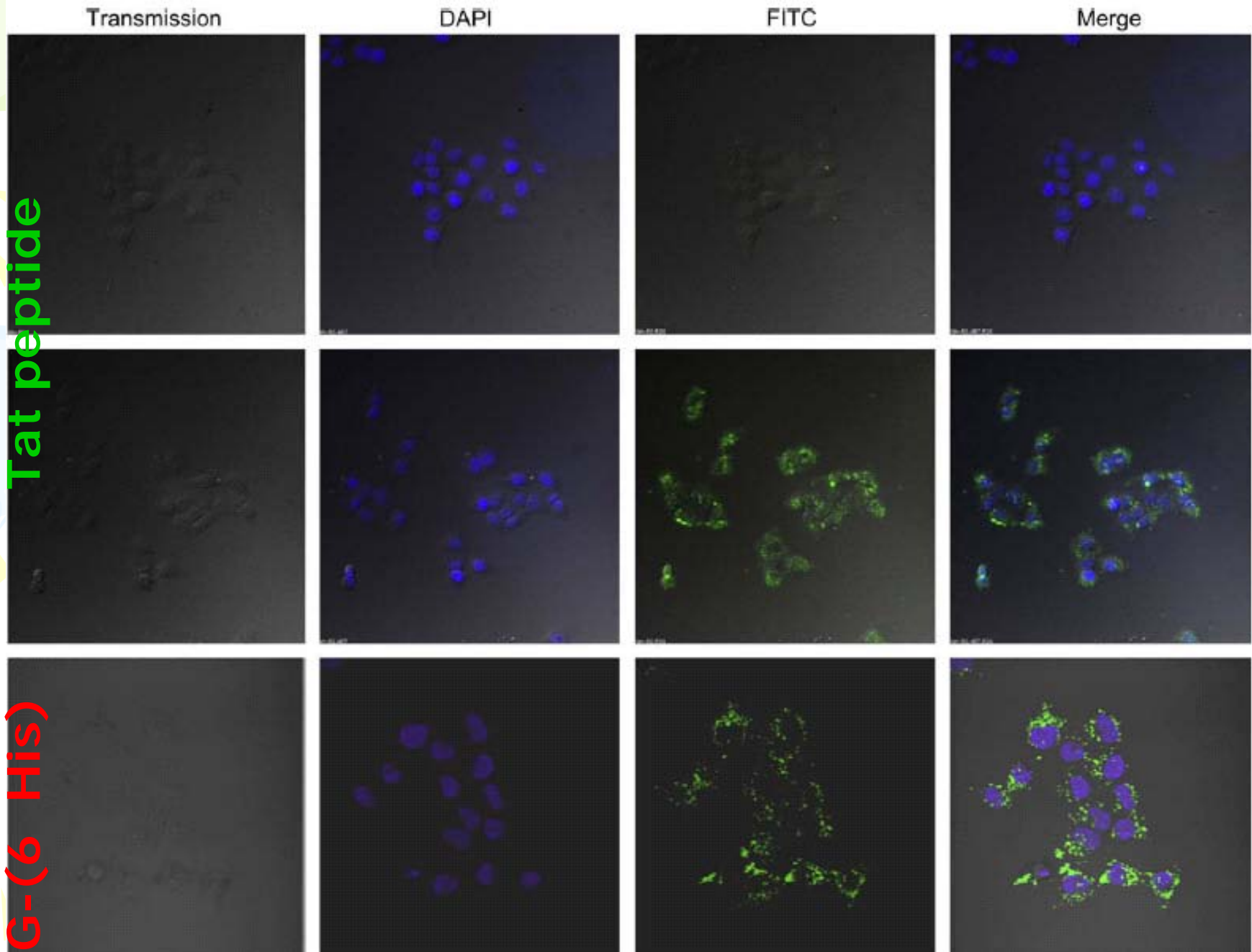
(6 His)-protein G and (6His)-protein G-Tat peptide were reacted with Alexa Fluor 488 goat anti-mouse IgG antibody at RT for 30 min.



DMEM was exchanged for fresh DMEM and the 30 μ L of engineered protein solution conjugated with antibodies was added; the culture continued at 37 C for 1 h.

Intracellular delivery of engineered proteins

(9 Arg)-protein (6 His)-protein G- (6 His)-proteing
G-(6 His)
Tat peptide



Mitochondria targeting by proteins

Engineered proteins were reacted with mouse monoclonal antibody against mitochondria (MTC02) at RT for 30 min.



Above solution was reacted with QDs coated with a NTA–Ni(II)-phospholipid micelle for 10 min and separated by ultracentrifugation



HeLa cells were loaded with MitoTracker Deep Red 633 (25 nM) and then QDs coated with engineered proteins containing mitochondria-targeting antibodies were added. Followed by incubation at 37°C for 15 min.



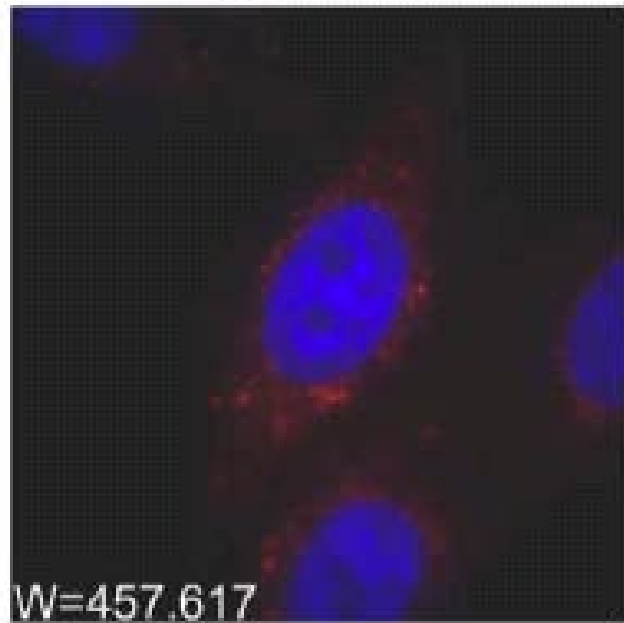
Cells were washed with DPBS and fixed with 4% paraformaldehyde for 20 min at RT. After further washing with DPBS, blocked using 1% (w/v) BSA in DPBS for 30 min at RT



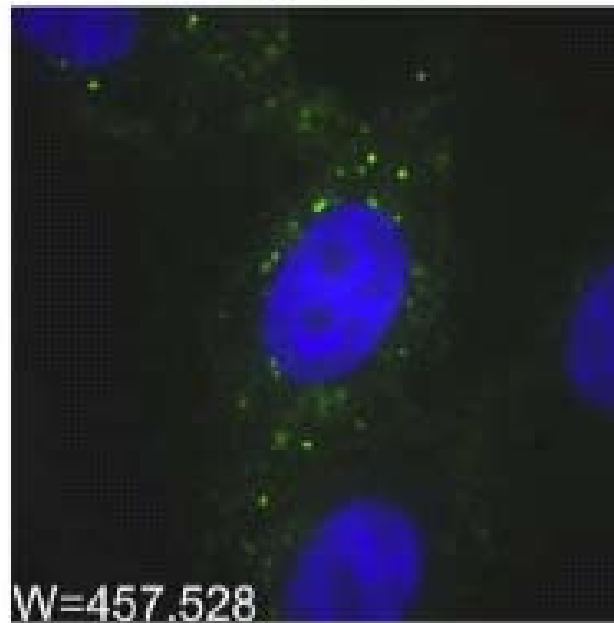
The cells were then treated with DAPI in DPBS for 10 min, and washed with DPBS.

Mitochondria targeting by proteins/QD system

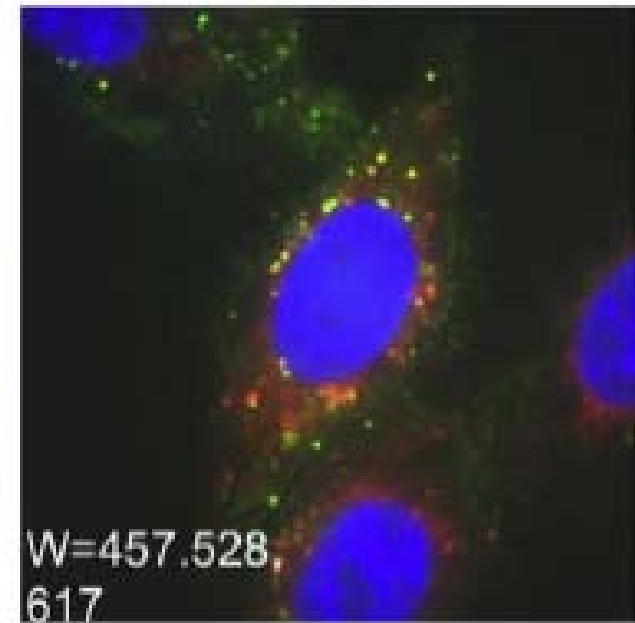
Mitotracker



QD525



Merge



Mitochondria targeting by protein/NP system

Engineered proteins/mitochondria-targeting antibodies /gold-coated magnetic nanoparticle conjugates were prepared



Then nanoparticles were washed and re-dispersed in serum-free DMEM



The medium of HeLa cells was exchanged with fresh DMEM and suspension of nanoparticles conjugated with anti-mitochondrial antibodies, and the culture continued at 37°C for 3 hr.

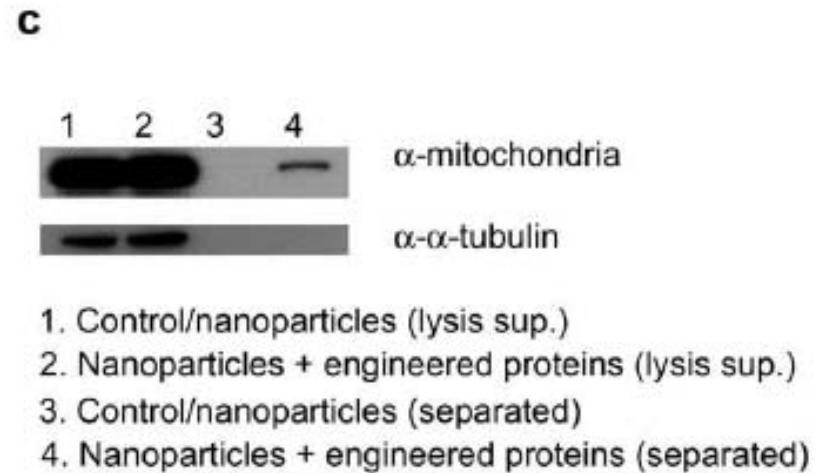
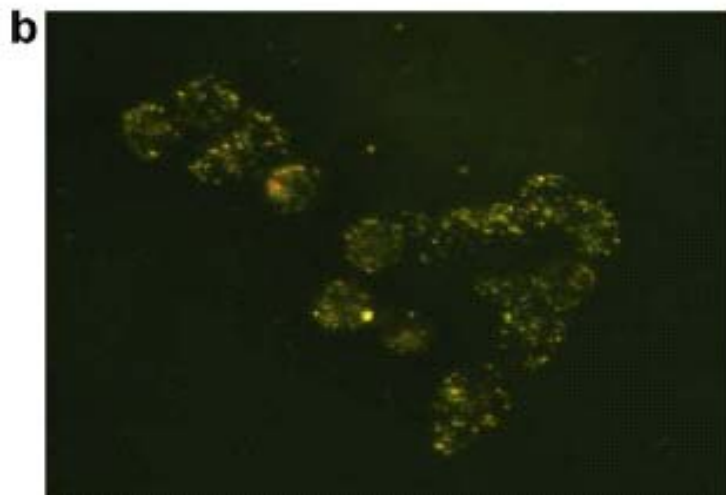
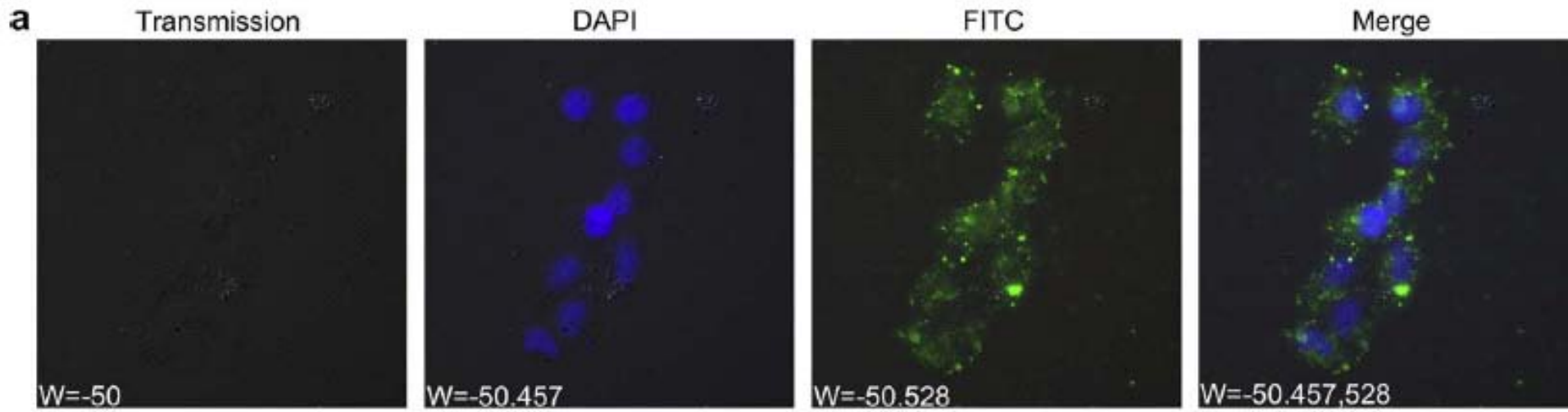


Cells were washed three times with DPBS and fixed with 4% (v/v) paraformaldehyde at room temperature for 20 min, then washed three times with DPBS, and permeabilized with 0.3% (v/v) Triton X-100 for 5 min.



Cells were then blocked with 1% (w/v) BSA , incubated with Alexa Fluor 488 goat anti-mouse IgG antibody, stained with DAPI, washed three times with DPBS, and mounted with Fluorescence Mounting Medium (DAKO Cytomation).

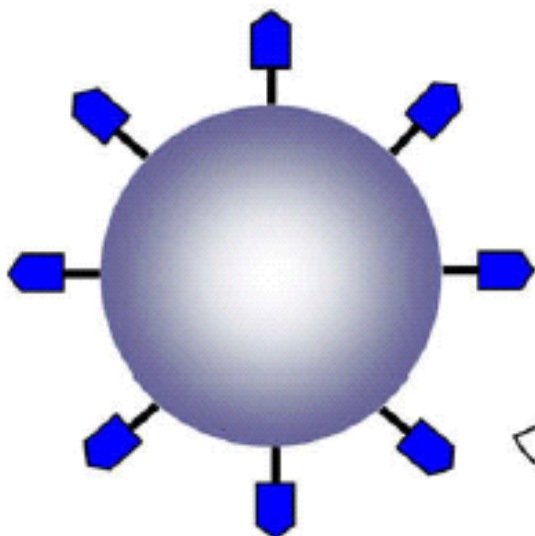
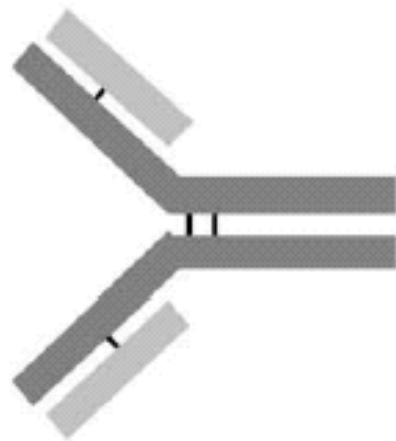
Mitochondria targeting by protein/NP system



- a) Fluorescence microscopy and,
- b) Dark-field scattering microscopy
- c) Western blot analysis

Antibody

(targeting, therapeutic, etc)

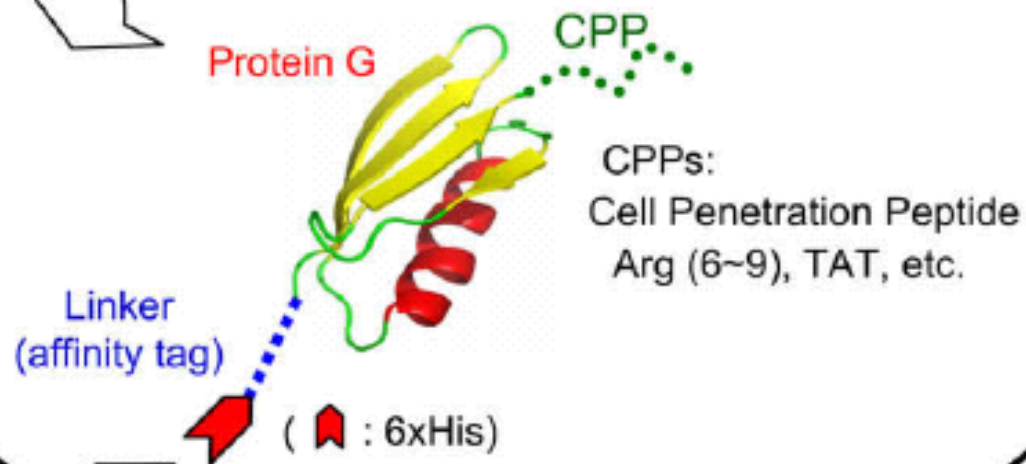


■: NTA-Ni(II)

Surface modified Nanoparticles

(quantum dots, magnetic nanoparticles, etc)

Engineered proteins



Future plans

A decorative graphic on the left side of the slide features three balloons in shades of green, blue, and purple, with yellow streamers and triangular flags trailing from them.

1. Synthesis of clusters with engineered proteins
2. Conjugation of clusters with CPPs and then drug/organelle targeting antibody
3. Separation of organelles using FACS



Thank you

For Western blotting experiments,

1. HeLa cells were washed with DPBS harvested using a scraper and lysed with lysis buffer [1 M Tris-HCl, 1 M NaCl, 100 mM EDTA, 0.1% (v/v) NP-40] and magnetic nanoparticles were separated using a conventional magnet.
2. The NPs were washed three times with DPBS, and boiled after addition of 70 μ L sample buffer and separated by SDS-PAGE, and transferred from the gel onto a PVDF membrane
3. Nonspecific antibody binding sites were blocked by incubation in PBS with 0.1% (v/v) Tween 20 and 4% (w/v) skim milk for 1 h at room temperature.
4. The membrane was washed in PBS containing 0.1% (v/v) Tween 20 and incubated overnight at 4 C with anti-mitochondria monoclonal antibody.
5. After washing for 1 h, the membrane was incubated with HP-conjugated secondary antibody for 1 h at room temperature.
6. The resulting blot was visualized by ECL-Plus Western detection reagents