Molecular beacon-quantum dot-Au nanoparticle hybrid nanoprobes for visualizing virus replication in living cells

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Experimental Procedures

BGMK Cell Culture: BGMK cells obtained from American Type Culture Collection (passages 50–60) were grown in 400 mL of 1× autoclavable minimum essential medium (AMEM; Irvine Scientific) containing 1% of 7.5% $NaHCO_3$, 2% of 1 M Hepes, 1% of nonessential amino acids (NEAA; HyClone, Thermo Scientific), 100 μg/mL of penicillin and 100 U/mL of streptomycin (HyClone, Thermo Scientific), 1% of 200 mM L-glutamine in 0.85% NaCl (HyClone, Thermo Scientific), and 10% of FBS (Sigma–Aldrich) at 37°C in a 5% CO_2 atmosphere. PBS [1× PBS = 0.01 M phosphate (pH 7.4), 0.138 M NaCl, and 2.7 mM KCl] and Tris-buffered saline solution [1× TBSS = 0.05 M Tris (pH 7.4), 0.28 M NaCl, 10 mM KCl, and 0.82 mM Na_2HPO_4] were used for washing steps in the plaque assay and MB analysis, respectively.

Virus Preparation: Virus stocks of Coxsackie B6 (CVB6) Schmitt strain (ATCC VR-155) were allowed to proliferate on BGMK cells for 2 days at 37°C in a 5% CO_2 atmosphere and collected by freeze–thawing (3 times) infected flasks demonstrating >80% lysis and extracting the cell lysate with chloroform. The CVB6 virus stock was stored at −80°C.

Plaque Assay: The CVB6 virus stock was thawed, and then a series of 10-fold serial dilutions in 1× PBS were prepared. Confluent, 1-day-old BGMK cell monolayers in 12-well, 22.1-mm dishes (Costar; Corning) were infected with 1 mL of virus dilution. After 90 min of adsorption at room temperature, the solutions were aspirated, and 2% carboxymethylcellulose (CMC) sodium salt (Sigma–Aldrich) containing 1 volume of 2× AMEM (Irvine Scientific) with 2% of 7.5% $NaHCO_3$, 4% of 1 M Hepes, 2% of NEAA, 200 μg/mL of penicillin and 200 U/mL of streptomycin, 2% of 200 mM L-glutamine in 0.85% NaCl (HyClone, Thermo Scientific), and 4% of FBS (Sigma–Aldrich) was added into each well. After 3 days of incubation at room temperature, the CMC overlay was removed, and the cells were treated with 0.8% crystal violet/3.7% formaldehyde solution overnight. Excess stain was removed by washing with de-ionized water and the virus plaques were counted.

Design of Nuclease-Resistant MB CVB6: MB CVB6 was designed on the basis of an alignment of the sequences of enterovirus strains obtained from GenBank database. The DNA folding program *mfold* (www.bioinfo.rpi.edu/) and *IDT SciTools* (www.idtdna.com/SciTools/SciTools.aspx) were used to predict the thermodynamic properties and the secondary structures of MBs. MB CVB6 5′-SH-CGCACC***GTAGTC***CGCATTCAGGGGCCGGAG***GACTAC***CAATTA-NH-3′ (probe sequence is underlined and stem sequence is bold italic) possessing a 2′-*O*-methylribonucleotide backbone with phosphorothioate internucleotide linkages was synthesized (TIB Molbiol) to be specifically hybridized to an 18-bp region of the 5′ untranslated region of the enterovirus genome. The thiol group at the 5’ end is for the reaction with a maleimide group attached to the N terminus of the $(His)_6$ peptide (Pi Proteomics, LLC) to form a thiol–maleimide bridge. The 3’ amino-labeled end

forms covalent amide linkage with the mono-sulfo-NHS ester on the surface of Au nanoparticles (NPs). MB CVB6 was suspended in 100 mM Tris·HCl (pH 8.0) buffer containing 1 mM $MgCl_2$ to make the concentration 100 μM for the subsequent studies.

Peptide: One hundred and fifty micromolar N-terminal maleimide-modified H-$(His)_6$-NH-CH_2-CH_2-N-maleimide (Pi Proteomics, LLC) suspended in 10 mM Hepes buffer containing 1 mM $MgCl_2$ was mixed with 100 μM thiolated MB CVB6 in the dark for 2 h to form a stable thiol–maleimide linkage. The peptide-linked MB complex was dialyzed overnight in Slide-A-Lyzer Mini Dialysis Units 10,000 molecular weight cutoff (MWCO) to remove the unconjugated peptide/MBs (Pierce).

QD-MB CVB6-Au NP Conjugation: Several steps were taken to attach QD and Au NP to MB CVB6 in 10 mM Hepes buffer containing 1 mM $MgCl_2$ at pH 8.5 in the ambient environment. To make colloidal QDs compatible with biological environments, the TOP/TOPO capped CdSe-ZnS QDs (Evident Technologies, Inc., NY) were cap-exchanged with dihydrolipoic acid (DHLA) ligands to replace the hydrophobic shell. The basic procedure followed for DHLA-capping is described by Clapp et al. After the QD surface replacement, the DHLA-capped QDs were resuspended in 10 mM Hepes buffer. To form a thiol–maleimide bridge, the thiol group at the 5' end of MB sequence reacted with a maleimide group attached to the N terminus of the $(His)_6$ peptide (Pi Proteomics, LLC) as described above. The Mono-Sulfo-NHS-NANOGOLD (Nanoprobes, NY) reagent was dissolved and mixed with MB CVB6 at molar ratio of 2:1 in 200-μl 10 mM Hepes buffer. The 3' amino-labeled end on the MB sequence formed a covalent amide linkage with the mono-sulfo-NHS ester on the surface of Au NPs. The mixture was incubated for

1 h and then quenched with 10 μl of 10 mM glycine to deactivate any remaining NHS on the NP surface. Unreacted MB sequences and NPs were removed by spin dialysis in Millipore Microcon 10,000 MWCO at 7000×g for 5 min and the NP-labeled MB solution was resuspended in 200-μl 10 mM Hepes buffer. DHLA-capped QD solution was mixed with an increasing molar ratio of NP-labeled MB solution for 1 h in 200-μl 10 mM Hepes buffer. The $(His)_6$ peptide linker at the 5' end of MB sequence facilitated the self-assembly of MB onto the QD surface via metallic affinity. Unreacted NP-labeled MB sequences were removed by spin dialysis in Millipore Microcon 50,000 MWCO at 7000×g for 5 min. The final QD-MB-Au NP conjugate was resuspended in 200-μl 10 mM Hepes buffer. After conjugation, the QD emission spectra of a 100-μl QD-MB-Au NP solution (QD concentration 0.1 μM) were measured on an RF-551 spectrofluorometric detector (Shimadzu, MD) from 450 to 650 nm with a fixed excitation wavelength of 400 nm. For AFM imaging and *in vivo* experiments the desirable concentrations are described below. All QD-based MBs were stored in the dark at 4 °C and were used within 1 day.

Cellular Delivery of QD-based MBs: For intracellular delivery, the hexahistidine-appended Tat peptide H-$(His)_6$-Trp-Gly-Leu-Ala-Aib-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-$CONH_2$ was synthesized (Pi Proteomics, LLC), where Aib is α-aminoisobutyric acid. The peptide solution was mixed with QD-MB-Au NP solution for 1 h at room temperature in 200-μl 10 mM Hepes buffer at molar ratio of 10:1. $(His)_6$-expressing peptides can be directly attached to the QD-surface based on the strong interaction between Zn^{2+} and histidine. BGMK cells were seeded into the 8-well Lab-Tek Chambered Coverglass (Fisher Scientific) at 37°C in 5% CO_2 in air and cultured to >90% confluence. After removing the incubation medium, the cell monolayer

was washed twice with 1× TBSS. To facilitate determining the efficiency of Tat peptide-mediated intracellular delivery, nonconjugated QD-based MB CVB6 or QD-based MB CVB6-Tat was mixed with complementary oligonucleotides (5′-CTCCGGCCCCTGAATGCG-3′) to the loop region at an MB/oligonucleotide molar ratio of 1:1. BGMK cells were incubated at 37°C in the dark with 1× Leibovitz L-15 medium (Invitrogen) containing either preformed nonconjugated QD-based MB CVB6 hybrids or QD-based MB CVB6-Tat hybrids at QD concentrations of 50 nM. To record the image, the chamber well was placed on the Zeiss Axiovert 40 CFL inverted fluorescence microscope stage and was marked to permit the repeated observation of the chosen region in the cell monolayer. As soon as the positive fluorescent signals were observed inside the cells, the chamber well was kept on the microscope stage instead of returning it to the 37°C incubator. All assays were carried out over a period of 12 h, and the fluorescence images were taken at intervals of 15 min.

Progression of Viral Infection in Living Cells: BGMK cells were cultured to >90% confluence in the 8-well Lab-Tek Chambered Coverglass (Fisher Scientific) at 37°C in 5% CO_2 atmosphere. After incubation for predetermined time periods, the slides were removed from the 37°C incubator, and the growth medium was aspirated. Following 2 washes with 1× TBSS, the cells were incubated with 50 nM QD-based MB CVB6-Tat in 1× Leibovitz L-15 medium (Invitrogen) at 37°C in the dark for 3 h. After 2 washes with 1× TBSS, the chamber wells were oriented on the microscope stage; the cells were infected with 10-fold virus dilutions in 1× Leibovitz L-15 medium and were observed under the Zeiss Axiovert 40 CFL inverted fluorescence microscope at room temperature for 12 h. A movie showing the real-time spreading of viruses is included. Snapshots were taken from 30 min p.i. to 12.5 h p.i.. From 30 min p.i. to 2.5 hours p.i., they were

taken every 15 min; thereafter they were taken every 30 min. Cd-rich fraction of IEC was dialyzed for 3-7 days against water to remove potassium chloride before taking images. One drop of the fractionated sample was placed on a carbon-coated 400 mesh copper grid and dried under an incandescent lamp for about 15 min. Transmission electron microscopy (TEM) was performed on a FEI CM300 transmission electron microscope, operated at an accelerating voltage of 300 kV.

Fluorescence Microscopy and Image Processing: Living cell imaging was performed on a Zeiss Axiovert 40 CFL inverted microscope equipped with a 12-V, 35-W halogen lamp (for the phase-contrast images) and an HBO 50 W/AC mercury lamp (for the fluorescence images). The objectives used were a 5×/0.12 A-Plan, a 10×/0.25 A-Plan, a 20×/0.50 EC Plan-NEOFLUAR, and a 40×/0.50 LD A-Plan (Zeiss). Fluorescent hybrids were detected by using a filter set consisting of a D436-nm exciter, a D535/50-nm emitter, and a 475 nm-dichroic long pass beam splitter (Chroma Technology). Images were acquired by using a ProgRes MF^{scan} Monochrome CCD camera (Jenoptik). Both phase-contrast and fluorescence images were analyzed by using Image-Pro PLUS analysis software (Media Cybernetics). All settings for image processing were kept constant, and the exposure time for image capture was adjusted, if necessary, to maintain output levels similar to those observed under the fluorescence microscope.

Enumeration of Fluorescent Cells: To calculate the infected cells (fluorescent cells) in each chamber well, 30 fields within the well were randomly chosen, and the fluorescence images were collected at 10× magnification. The number of fluorescent cells within the area was counted by Image-Pro PLUS analysis software.

Fig. S1. Intracellular delivery of QD-MB-Au NP probes without TAT modification. BGMK cells were incubated with QD-MB-Au NP conjugates for 12 h without Tat modification. Rapid aggregation of the probes was observed outside the cells. After washing away the medium, there was no significant fluorescence detected inside the cells, confirming that the conjugates were not taken up by the cells without the help of Tat peptides.

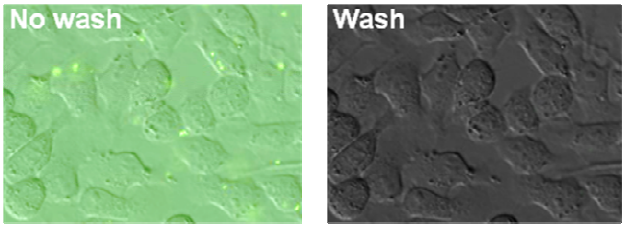

Fig. S2. The correlation between the number of fluorescent cells and PFU at 4 h p.i.

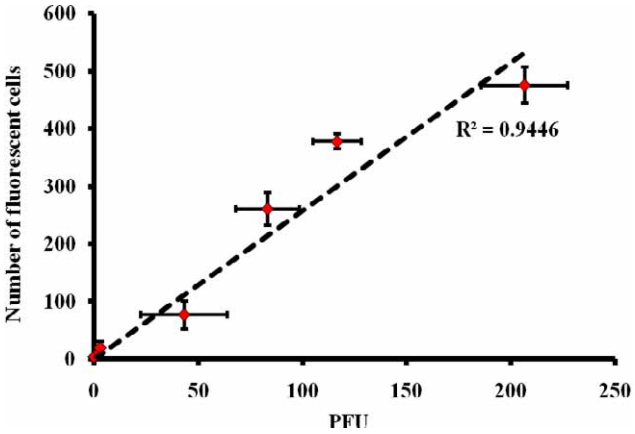

Fig. S3. Intracellular delivery of Tat-QD-MB-Au NP probes without hybridization. BGMK cells were incubated with probes for 12 h and no significant fluorescence was detected inside the cells, confirming their resistant to intracellular degradation.