A new stable and reliable method for labeling nucleic acids of fully replicative viruses

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Materials and methods:

Synthesis of Ru complexes. $[Ru(phen)_2(dppz)]^{2+}$ were synthesized as described previously (1-2).

Uptake of $[Ru(phen)_2(dppz)]^{2+}$ by Vero cell and cell viability assay. Vero cells were placed in glass-bottom dishes (NEST, http://www.cell-nest.com) at $1x10^4$ cells per well, cultured with $[Ru(phen)_2(dppz)]^{2+}$ at different concentrations in the culture medium for 24 hours, and washed with PBS, The cells were imaged on a confocal microscope (Leica SP5, Germany) excited with 454 nm (for $[Ru(phen)_2(dppz)]^{2+}$) Argon laser, and the fluorescence was collected in the wavelength range of 590nm-620nm. Cell viability assays were performed using Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). Vero cells were cultured with $[Ru(phen)_2(dppz)]^{2+}$ at different concentrations in the culture medium for 48 hours. Then, the cells were washed with PBS, added with the CCK-8 solution and incubated for 4 h. Finally, the absorbance (450 nm) for each well was measured with a Microplate System (354 model). The absorbance at 450nm corresponds to the concentration of reduced 2-(2-methoxy-4- nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt, (product name: WST-8), which reflects the viability of the cells.

Labeling vaccinia virus with $[Ru(phen)_2(dppz)]^{2+}$. Vaccinia virus was inoculated with Vero cells in a monolayer for 2 h. Then the medium was changed into Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 2% fetal bovine serum (FBS) (Gibco) and $[Ru(phen)_2(dppz)]^{2+}$ (at a final concentration of 15μM). At 48 h post-infection, the infected cells were centrifuged at 1,500 X g for 10min and the cell pellet was suspended in 20ml of PBS. After three rounds of freeze-thaw, the cell debris was removed by centrifugation at 1,500 X g for 10min. The virus in the supernatant was layered on to a 36% sucrose cushion and centrifuged at 80,000 X g with a Beckman rotor SW40 for 90min at 4˚C. The resulting virus pellet was suspended in 1ml of PBS and layered onto a continuous 40% to 60% sucrose gradient and centrifuged at 58,000 X g for 65min at 4˚C. The clearly visible virus band was collected and washed with PBS by repeated centrifugation. The final virus pellet was suspended in 1ml of PBS and stored at -80˚C (3). The size and the morphology of the virions were characterized by TEM (JEOL 1400), for which samples were dropped onto a copper grid and negatively stained with uranyl acetate.

Virus titer assays. The purified virus was quantitated by 50% Tissue Culture Infective Dose ($TCID_{50}$).Vero cells were maintained as stock cultures in DMEM and replated for 2 days before infection. The virus samples and their paired controls were immediately serially diluted with Hank's balanced salt solution (HBSS; Freshney, 1983) supplemented with 25mM HEPES Buffer and 4 mM sodium bicarbonate, pH 7.2. They were subsequently added

to the cells and infected for 1 h. Post infection (pi) TCID50 cultures were washed and fed with DMEM containing 2%FBS. Leave TCID50 cultures in incubator at 37˚C with CO_2 for about 5 days. Count the number of Cytopathic Effect (CPE), the TCID50 was calculated by Formula of Reed-Muench: TCID50=the dilution above 50% CPE + [(% next above 50%)-50%] / (% next above 50% - (% next below 50%) X log10. pfu/ml=0.69XTCID50/ml, where pfu is plaque forming unit.

Immunofluorescence analysis of $[Ru(phen)_2dppz]^{2+}$-labeled vaccinia virus. Cells, cultured in glass-bottom dishes, were grown to 50% confluence. Then the cells were incubated with $[Ru(phen)_2(dppz)]^{2+}$-labelled vaccinia virus for 30 min at 4°C to allow virus binding. Subsequently, Vero cells were fixed with 4% paraformaldehyde for 20min and permeabilized with 0.1% Triton X-100 in PBS for 5-10min at room temperature. After being blocked in PBST containing 1% bovine serum albumin (BSA) for 1 h, the samples were incubated with anti-H_3 protein (vaccinia virus) mouse monoclonal antibody (Immune Technology, USA) for 1 h at room temperature, followed by goat anti-mouse dylight488 conjugate (Earthox LLC, USA) for 1 h in dark. The samples were rinsed three to four times with PBS between two successive steps (4). The fluorescence images were acquired using the UltraView VOX spinning disc confocal microscope (Improvision, Perkin Elmer) with Volocity image analysis software. Dylight488 were excited with 488nm laser and a 525nm±25nm band-pass filter was used for emission. $[Ru(phen)_2dppz]^{2+}$ was excited with 488 nm laser, and the fluorescence was collected through a 615 nm±35nm long-pass filter.

Plaque of the vaccinia virus. The concentration of virus was determined by absorbance measurement at 595nm (5). A U-3900UV-vis spectrophotometer (U-3900, Hitach, Japan) was used to acquire the UV-vis absorption spectra. The binding of the dye to virus protein causes a shift in the absorption maximum of the dye from 365 to 595 nm. ($[Ru(phen)_2(dppz)]^{2+}$ has little absorption at 595 nm). 100μL of $[Ru(phen)_2(dppz)]^{2+}$-labelled vaccinia virus or unlabelled vaccinia virus at the same concentration were respectively placed on Vero cells in a monolayer. The mixture was incubated at 37˚ for 30min to allow virus binding the cells. Then unbound virions were washed with PBS. The monolayer Vero cells were covered with a DMEM containing 0.5% agar and 2%FBS. After 2 days, plaques were stained with crystal violet. Enlarge one plaque of each monolayer in order to show the fluorescing cell more clearly in fluorescence microscope with a 4 **X** objective.

The preparation and imaging of the dual-labeled vaccinia virus. $[Ru(phen)_2(dppz)]^{2+}$-labelled vaccinia virus ($0.69X10^8$ pfu/ml) were suspended in 0.5 ml PBS, pH 7.4, to which 0.1 ml of 1 mg/ml biotinylation reagent (sulfo-NHS-LC-biotin, Pierce) was added in PBS pH 7.4. Biotinylation was done at room temperature for 1 h while the sample was rotated (6). Excess biotin was removed by via buffer exchange into 50 mM HEPES buffer (pH 7.4, 145 mM NaCl) using gel filtration columns (Nap5; GE Healthcare).

$[Ru(phen)_2(dppz)]^{2+}$-labelled and biotinylated virions were plated in polylysine-coated slides 37°C for 1h, unbound virions were removed by washing with PBS .The slide was then incubated with QDs525-Streptavidin Conjugate (Wuhan Jiayuan Quantum Dots Co., Ltd.) for 10 min at 37°C. After three additional washes with PBS, the dual-labeled virions were imaged with the laser confocal fluorescence microscope (TCS SP5). QDs525 were excited with 454 nm Argon laser and the fluorescence was collected in the wavelength range of 515-540nm. The $[Ru(phen)_2(dppz)]^{2+}$ was excited with 454 nm Argon laser, and the fluorescence was collected in the wavelength range of 590nm-620nm.

Fluorescence microscopic imaging of live cells. Cells, cultured in glass-bottom dishes, were grown to 70% confluence. Then the cells were incubated with $[Ru(phen)_2(dppz)]^{2+}$-labelled and biotinylated vaccinia virus for 30 min at 4°C to allow virus binding, the unbound virions were removed by washing with PBS containing 1% glucose. Then QDs525-streptavidin (1nM) was added to cells at 4°C. After incubated for 15 min, the unbound QDs525-streptavidin was removed. Fresh phenol red–free DMEM (Invitrogen) containing 1% glucose and 50 mM HEPES was added to the cells (7).

Unless otherwise noted, cells were infected at a multiplicity of infection (5 pfu per cell) of vaccinia virus.

The fluorescence images were acquired using the Leica laser confocal fluorescence microscope (TCS SP5).

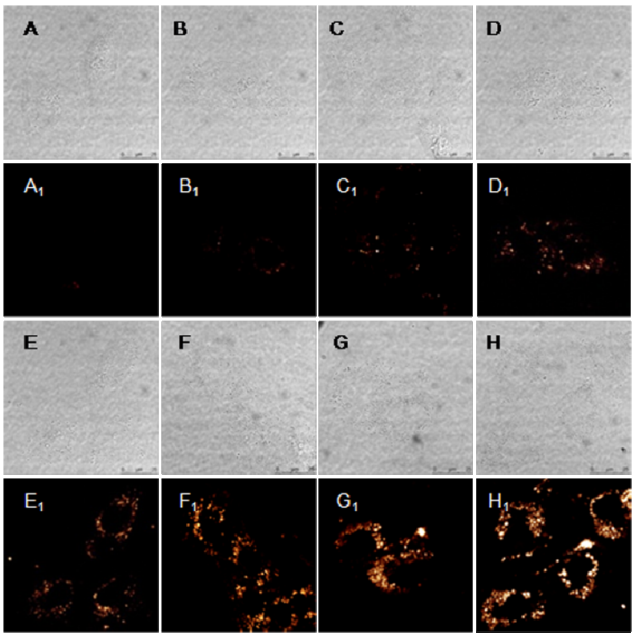

Fig. S1 The uptake of the $[Ru(phen)_2(dppz)]^{2+}$ by Vero cells (from A-H, the concentration of $[Ru(phen)_2(dppz)]^{2+}$ was 1μM, 5μM, 10μM, 15μM, 20μM, 40μM, 60μM and 80μM respectively. Scale bar: 25μm)

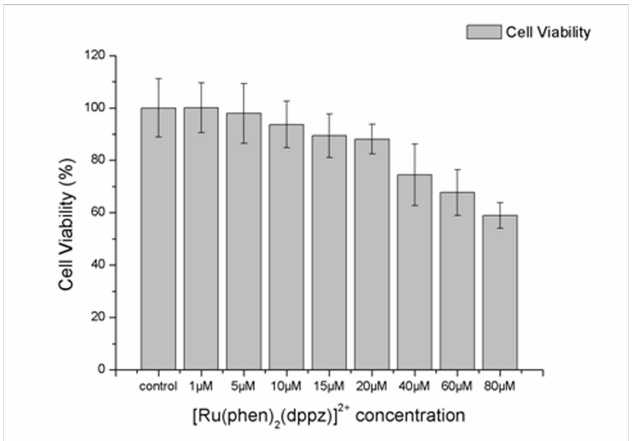

Fig. S2 CCK-8 detection of the viability of Vero cells incubated with $[Ru(phen)_2(dppz)]^{2+}$ at different concentrations.

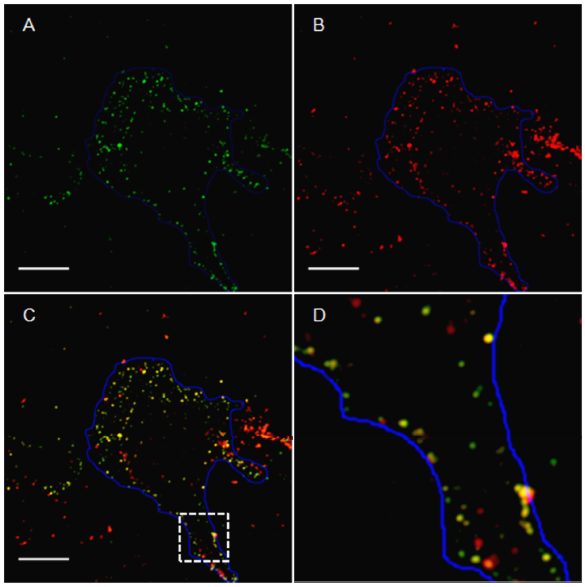

Fig. S3 Colocalization of H_3 protein with nucleic acid of vaccinia virus (The sequence of pictures shows the green fluorescence channel of the anti-vaccinia virus H_3 protein mAb (A), the red fluorescence channel of $[Ru(phen)_2(dppz)]^{2+}$ labeled vaccinia virus nucleic acid (B), the merging of the green and red fluorescence channels (yellow indicates colocalization of the two markers) (C) and a partial enlarged image of C, blue line is the Vero cell contour; Scale bar: 20μm)

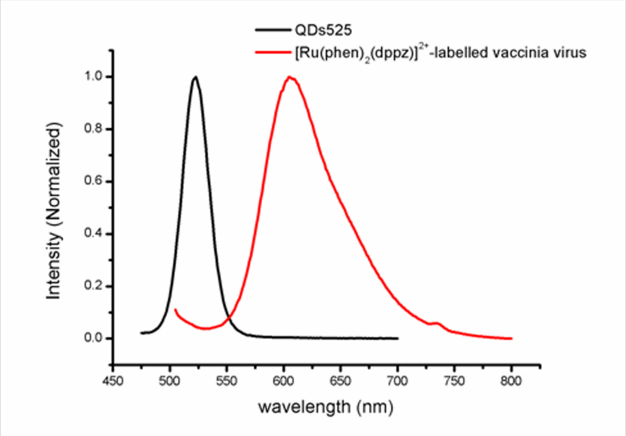

Fig. S4 Fluorescence spectra of the $[Ru(phen)_2(dppz)]^{2+}$ (EX: 488nm, EM: 605nm) and QDs525 (EX:400nm, EM:525nm).

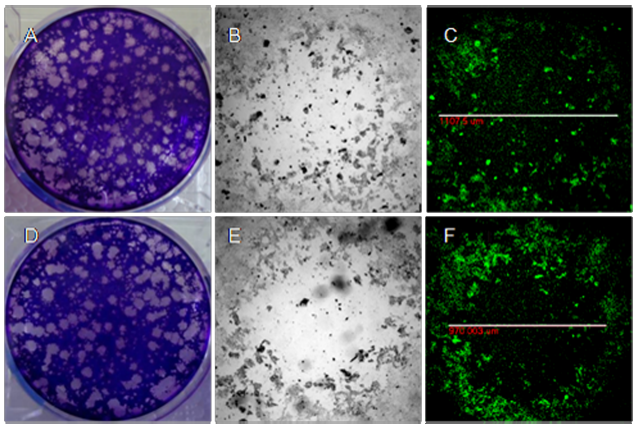

Fig. S5 Plaque phenotype of vaccinia virus. (A-C: Vaccinia virus strain WR; D-F: $[Ru(phen)_2(dppz)]^{2+}$ -labeled vaccinia virus).

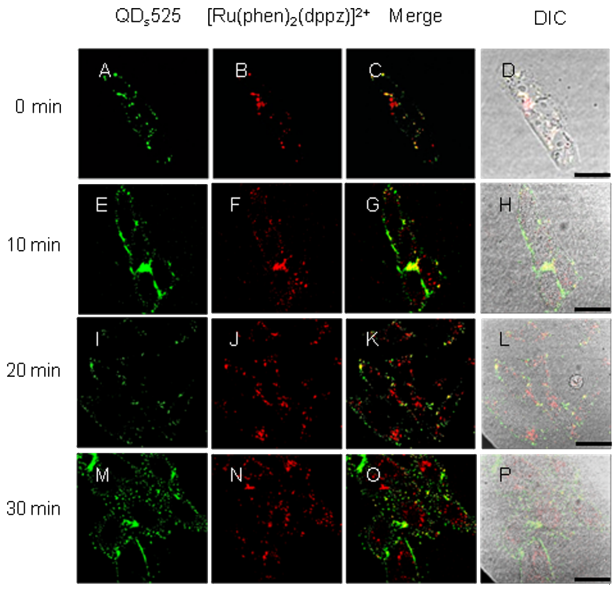

Fig. S6 Dynamic fluorescence imaging of the infection of the dual-labeled vaccinia virus (Scale bar: 25μm).

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