Electronic Supplementary Information

Stepwise Fluorescence Changes of Quantum Dots: Single-Molecule Spectroscopic Studies on the Properties of Turn-On Quantum Dots

Yea Seul Kim, Min Young Kim, Jae Kyu Song, Tae Jung Kim, Young Dong Kim, and Sang Soo Hah

Materials. Reagents were obtained from commercial suppliers and were used without further purification. Double-distilled deionized water was used throughout the experiments. Quantum dots (QDs) modified with PEG and amino groups (emission maxima at 605 nm) were obtained from Invitrogen. QD concentrations were measured by optical absorbance, using extinction coefficients provided by the supplier.

Preparation of biotinylated QDs conjugated with Nile Blue (NB). As shown in Scheme S2, biotinylated CdSe/ZnS QDs functionalized with NB were prepared according to the literature with slight modification (Refs. 5 and 6 in the text). Briefly, QDs were mixed with a l000-fold excess of bovine serum albumin (BSA) in 10 mM HEPES buffer (pH 7.4) containing 10 mM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC). The mixture was then shaken at 4 ^{o}C for 1 h. Next, the QDs were purified by precipitation and the particles were subsequently dissolved in 10 mM HEPES buffer (pH 7.4). In the third step, as a homobifunctional crosslinking agent, bis(sulfosuccinimidyl)suberate (BS^3) stock solution (50 μL, 1 mg/ml in 10 mM HEPES buffer, pH 8) was added, and the mixture was shaken for 15 min. The resulting QDs were purified by precipitation, and the particles subsequently dissolved in 10 mM HEPES buffer (pH 7.4). This was followed by mixing with a 20-fold excess of amino-PEG-biotin (EZ-Link Amino-PEG-Biotin, Thermo) and a 50-fold excess of NB in stock solution (3:2 ethanol/water), and shaking at 4 ^{o}C for 16 h. Excess biotin and NB was removed by a precipitation step of the QDs using NaCl and methanol followed by centrifugation at 1500 rpm for 1 min. The resulting QDs were dissolved in 1 ml of 10 mM phosphate buffer (pH 8.8) for further experiments.

Total internal reflection (TIR)-based single-molecule fluorescence resonance energy transfer (smFRET). Single-molecule fluorescence detection was performed by a TIR-based smFRET instrument (Figure S2) which had been installed according to the literature (R. Roy, S. Hohng and T. Ha, *Nature Methods* 2008, **5**, 507-516). Our system is constructed around a commercial inverted microscope (Zeiss), equipped with 532-nm DPSS laser system (Nd:YAG

continuous crystal laser, 50 mW) and electron-multiplying charged-couple device (CCD) camera (iXon, Andor Technology). The fluorescence signals from QDs that were collected by a water-immersion (prism-type) objective lens went through a long-pass filter to block out laser scattering, and the signals lower and higher than 611 nm were separated by a dichroic mirror and detected by a CCD camera with up to 0.2 sec time resolution. The observation area was 25 μm × 50 μm (Figure 1 in the text).

Single-molecule fluorescence detection. Single-molecule fluorescence detection was carried out according to the literature. For a chamber slide preparation, 100 μL of biotinylated BSA (1 mg/mL) was deposited on the naked glass surface for 5 min. After washing the surface with T50 buffer (10 mM Tris-HCl, 50 mM NaCl), 100 μL of streptavidin solution (0.2 mg/mL) was introduced to the glass surface via biotin-streptavidin binding and incubated for 5 min. The glass surface was washed with T50 buffer, followed by incubation with biotinylated QDs conjugated with NB for 10 min and by washing with T50. For the single-molecule fluorescence detection using the TIR-based smFRET instrument, the chamber slide assembled with a coverslip was mounted on the inverted microscope as shown in Figure S3. The resulting fluorescence signals were recorded in real time with a resolution of 0.2 sec using home-written Visual C++ software (Microsoft) which had been generously donated by Prof. Seok-Cheol Hong (Korea University). The software was used to obtain each frame of the movie from the camera, and the single-molecule traces were extracted from the recorded movie file using scripts written in IDL (Research Systems)

Figure Legends:

Scheme S1 Scheme to turn-on quantum dots (QDs) induced by NADPH-dependent biocatalyzed transformations, which is slightly modified from the literature (Refs. 5 and 6 in the text). Nile Blue (NB) can nonradiatively quench QD fluorescence and act as a redox dye in the presence of NADPH, resulting in fluorescence emission at 605 nm when NB is reduced.

Scheme S2 Procedure for conjugating NB and amino-PEG-biotin on QDs, resulting in biotinylated QDs functionalized with NB.

Figure S1 (a) Fluorescence spectra of 8.00 nM unmodified quantum dots and 8.00 nM NB-functionalized quantum dots. (b) UV spectra of biotinylated quantum dots functionalized with NB (0.8 pmol in 1 ml of 10 mM phosphate buffer, pH 8.8).

Figure S2 (a) Optical configuration for two-color emission measurements. The collimated beam goes through a dichroic mirror where the donor signal is reflected. After passing through a lens, the donor and acceptor images are each projected onto one-half of the CCD screen. (b) Excitation part of prism-type TIR microscopy. (c) The photo is a bird's eye view of the excitation optics including the inverted microscope and the intensified CCD camera.

Figure S3 Single-molecule image of the biotinylated QDs functionalized with NB in the absence and presence of 4 mM NADPH. The image is split into a QD emission channel (below 611 nm) and a NB emission channel (above 611 nm), each 25 μm × 50 μm. Single QDs immobilized on the same chamber, which are shown in the left window, are distinctly brighter than the molecules in the right window. This demonstrates that the fluorescence of QDs conjugated with NB turned on upon addition of NADPH.

Figure S4 Non-equilibrium fluorescence dynamics of single QDs immobilized on the glass chamber. (a) QD emission window for single-molecule image of the biotinylated QDs functionalized with NB right after addition of NADPH. Letters with arrows point the single QDs for non-equilibrium fluorescence dynamics, data of which are shown in (b): Time trace data for the fluorescence emission of each QD was recorded under continuous 532-nm laser excitation for 20 sec with a resolution of 0.2 sec/scan (every 5 min over the time course of 50 min).

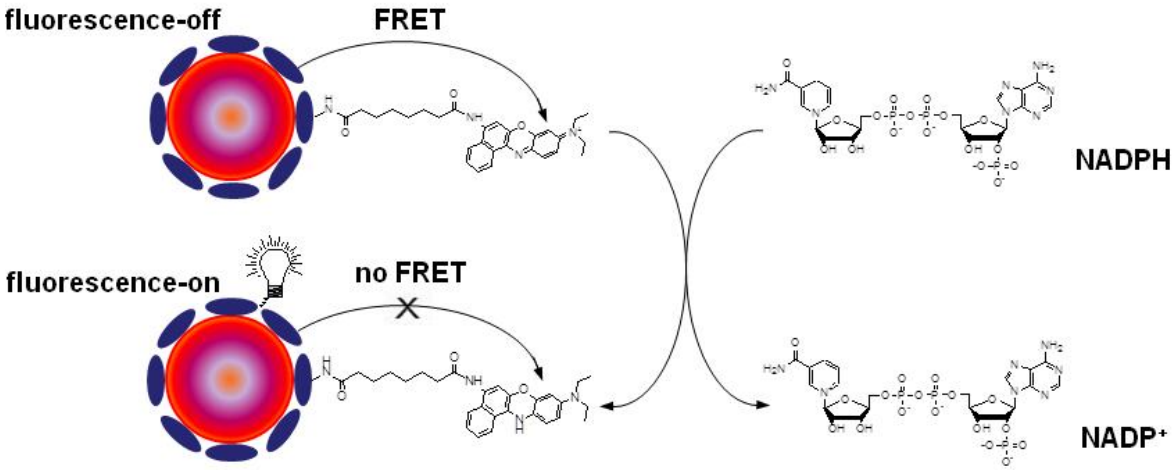
Figure S5 Hypothesis for the stepwise fluorescence emission of NB-functionalized QDs. It can be assumed that the fluorescence intensity of each NB-functionalized QD are subdivided into small but measurable increments, probably because of the differentiated number of the oxidized and reduced dyes attached to the individual QDs. In the present study, for example, four redox dyes are hypothesized to be actively involved in the stepwise fluorescence emission, although approximately seven units of NB are determined on average to be conjugated with each QD. It is hypothesized that the NB-functionalized QDs used cannot fluoresce in the absence of NADPH or reducing agents (F_{off} state) and the NB units can be reduced one by one, resulting in stepwise fluorescence changes of QDs ($F_{on(4\text{-}3)}$, $F_{on(4\text{-}2)}$, $F_{on(4\text{-}1)}$, and $F_{on(4\text{-}0)}$, respectively), where $F_{on(4\text{-}0)}$ represents that the NB units of the particle are fully reduced so that the particle can fluoresce as unmodified QDs do.

Figure S6 (a) Single-molecule image of the biotinylated QDs functionalized with NB in the absence and presence of 40 mM NADPH. The image is split, as described above. This demonstrates that the fluorescence of most of QDs conjugated with NB fully turned on when NADPH was added. (b) Non-equilibrium fluorescence dynamics of single QDs immobilized on the glass chamber. QD emission window for single-molecule image of the biotinylated QDs functionalized with NB right after addition of 40 mM NADPH. Time trace data for the

fluorescence emission of each QD was recorded under continuous 532-nm laser excitation for 20 sec with a resolution of 0.2 sec/scan (before, right after and 30 min after the addition of 40 mM NADPH, respectively).

Figure S7 Biotinylated QDs conjugated with NB were similarly prepared as controls, except that a 50-fold excess of amino-PEG-biotin and a 20-fold excess of NB in stock solution (3:2 ethanol/water) were mixed with BS^3-activated QDs. Approximately three units of NB are determined on average to be conjugated with each QD by UV absorbance measurement, as described. (a) Single-molecule image of the biotinylated QDs functionalized with NB in the absence and presence of 4 mM NADPH. The image is split, as described above. The number of the fluorescent QDs on the imaging area was approximately 800, presumably because of the higher unit number of biotin conjugated with each QD particle. This result demonstrates that the fluorescence of QDs conjugated with NB turned on even in the absence of NADPH. (b) Non-equilibrium fluorescence dynamics of single QDs immobilized on the glass chamber. QD emission window for single-molecule image of the biotinylated QDs functionalized with NB right after addition of 4 mM NADPH. Time trace data for the fluorescence emission of each QD was recorded under continuous 532-nm laser excitation for 20 sec with a resolution of 0.2 sec/scan (before, right after and 30 min after the addition of 4 mM NADPH, respectively).

Scheme S1



Scheme S2

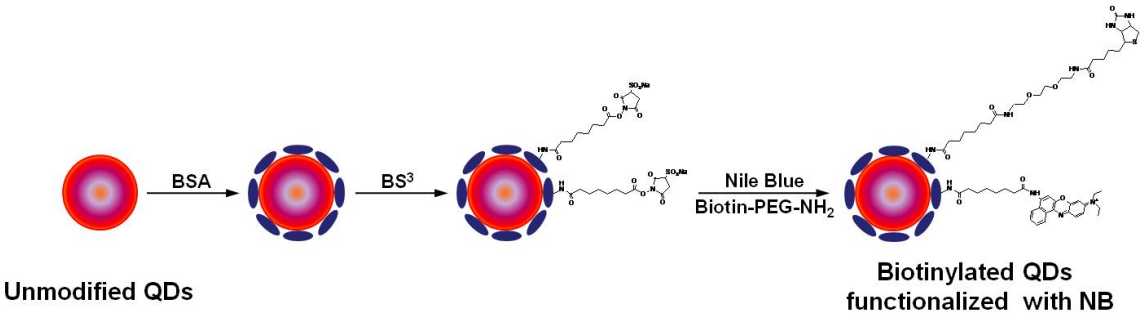

BSA
BS3
Nile Blue
Biotin-PEG-NH2
Unmodified QDs
Biotinylated QDs
functionalized with NB

Figure S1

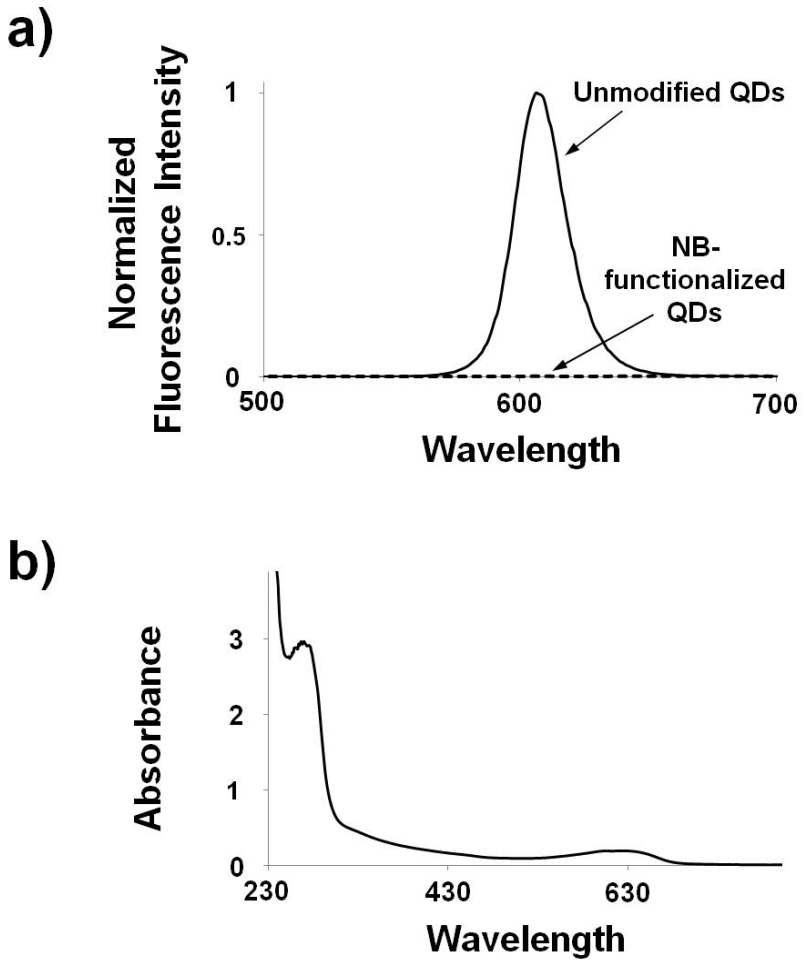
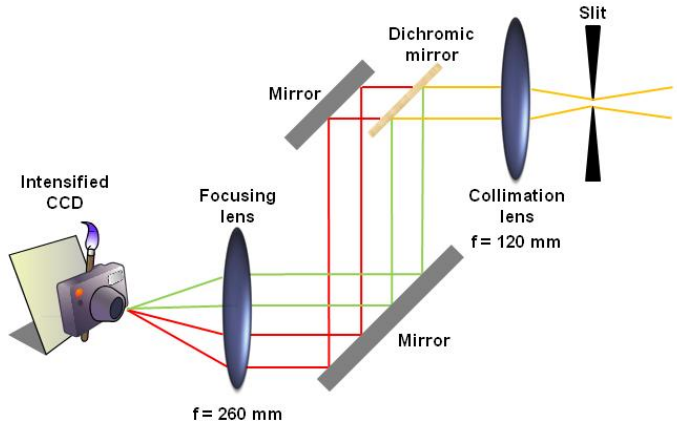
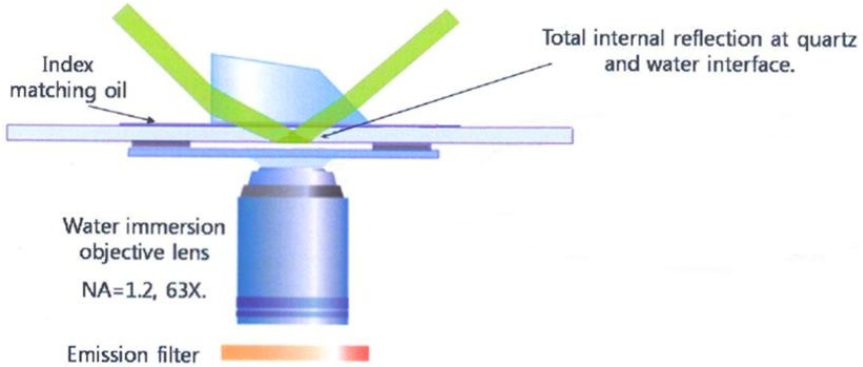

a)
Normalized Fluorescence Intensity
1
0.5
0
500
600
700
Wavelength
Unmodified QDs
NB-functionalized QDs
b)
Absorbance
3
2
1
0
230
430
630
Wavelength

Figure S2

a)



b)



c)

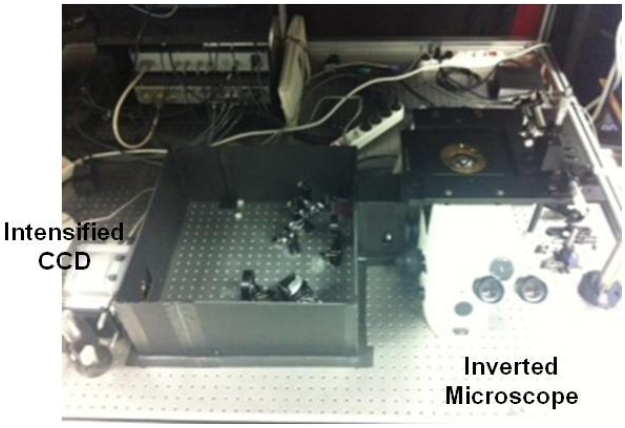


Figure S3

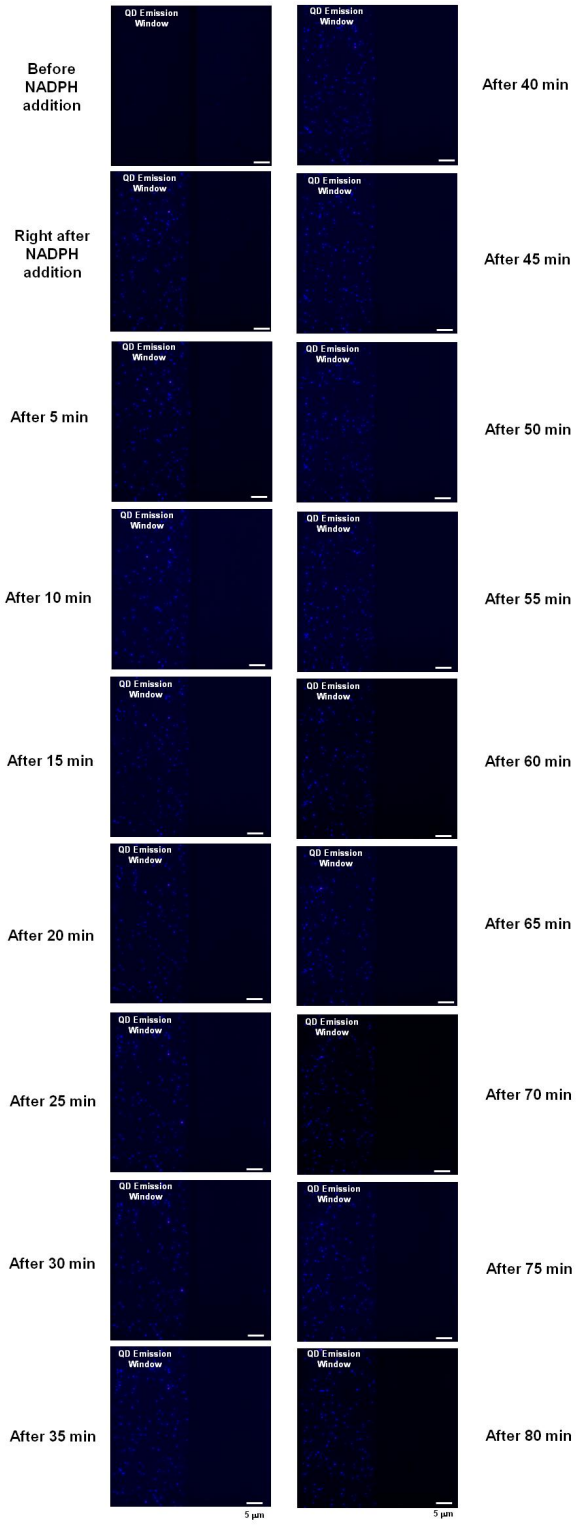

QD Emission Window
Before NADPH addition
After 40 min
Right after NADPH addition
After 45 min
After 5 min
After 50 min
After 10 min
After 55 min
After 15 min
After 60 min
After 20 min
After 65 min
After 25 min
After 70 min
After 30 min
After 75 min
After 35 min
After 80 min
5 μm

Figure S4

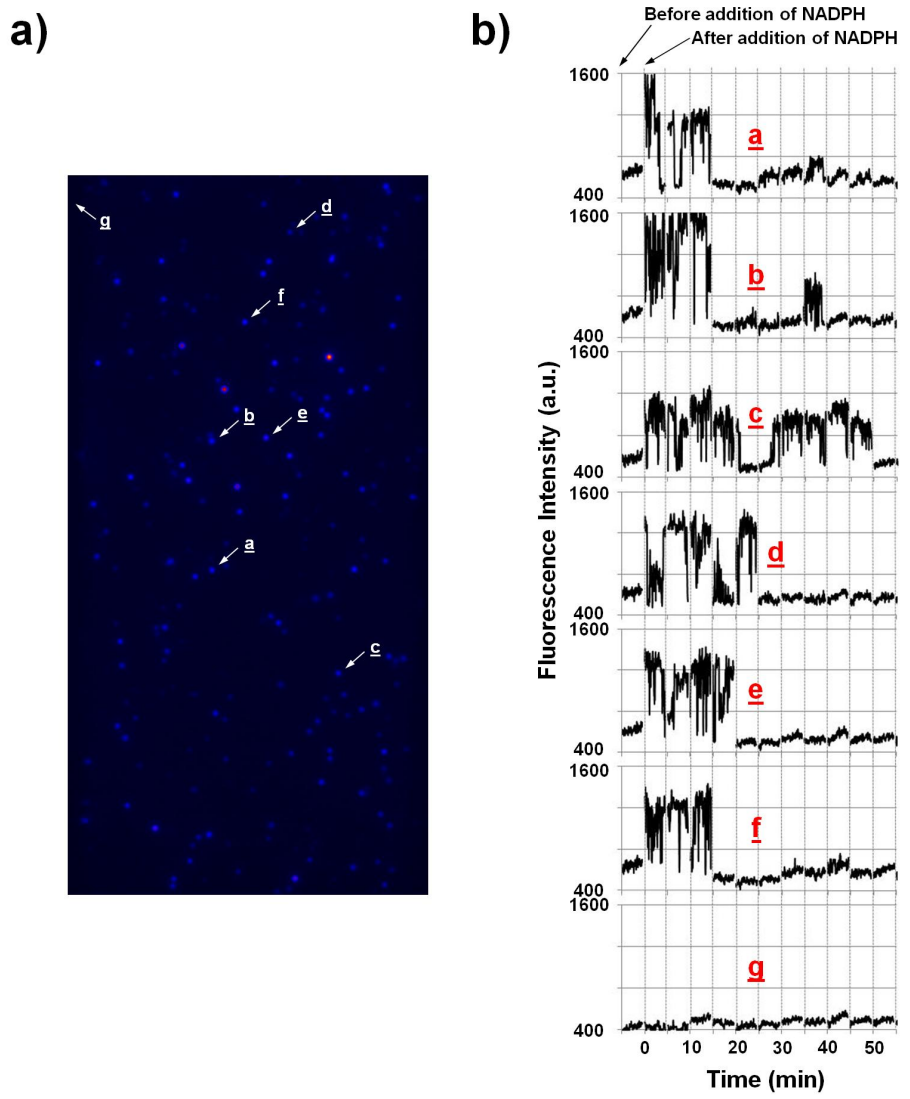

a)
b)
g
d
f
b
e
a
c
Before addition of NADPH
After addition of NADPH
1600
400
a
b
c
d
e
f
g
Fluorescence Intensity (a.u.)
0
10
20
30
40
50
Time (min)

Figure S5

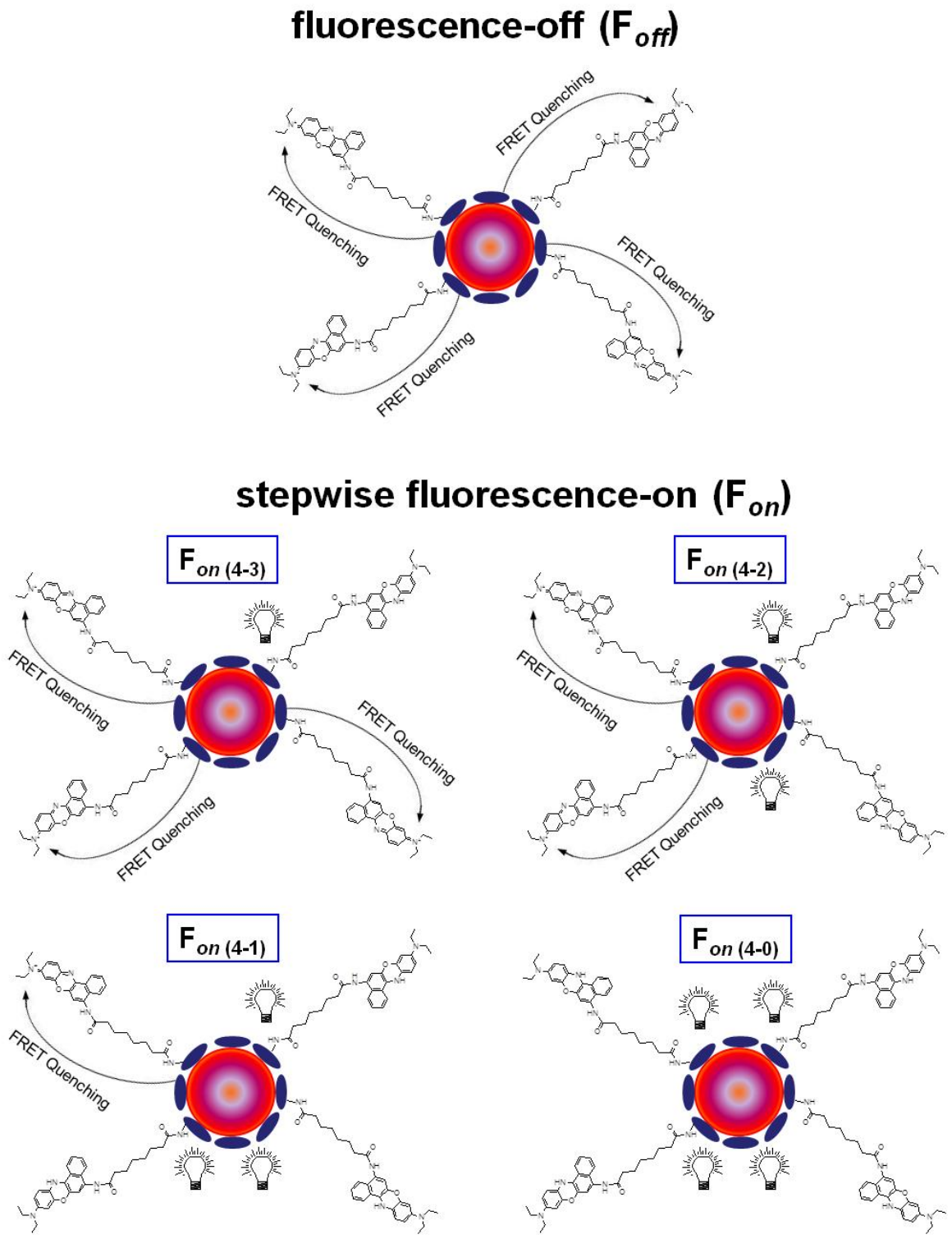

fluorescence-off (F_{off})
FRET Quenching
FRET Quenching
FRET Quenching
FRET Quenching
stepwise fluorescence-on (F_{on})
F_{on} (4-3)
FRET Quenching
FRET Quenching
FRET Quenching
F_{on} (4-2)
FRET Quenching
FRET Quenching
F_{on} (4-1)
FRET Quenching
F_{on} (4-0)

Figure S6

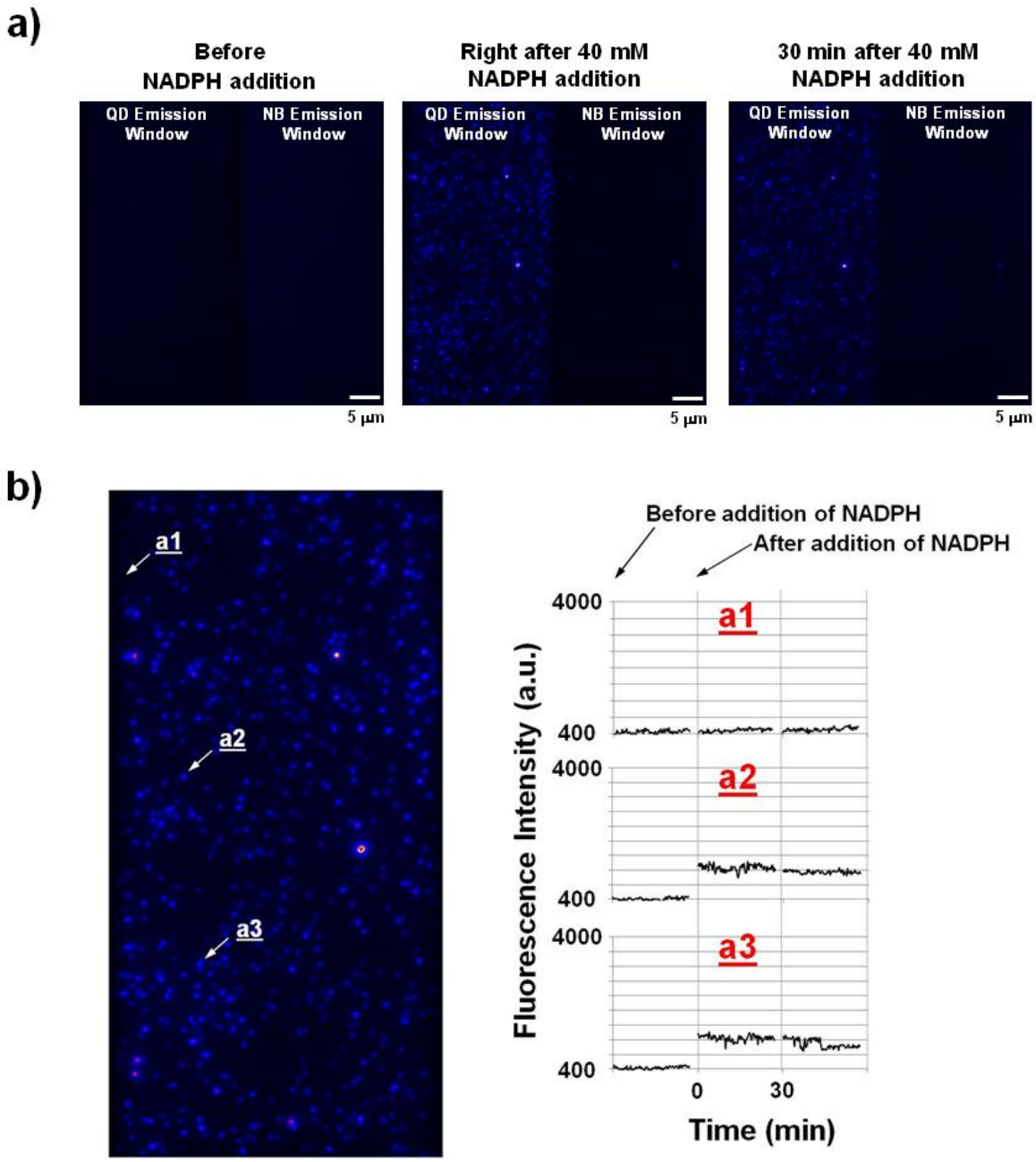
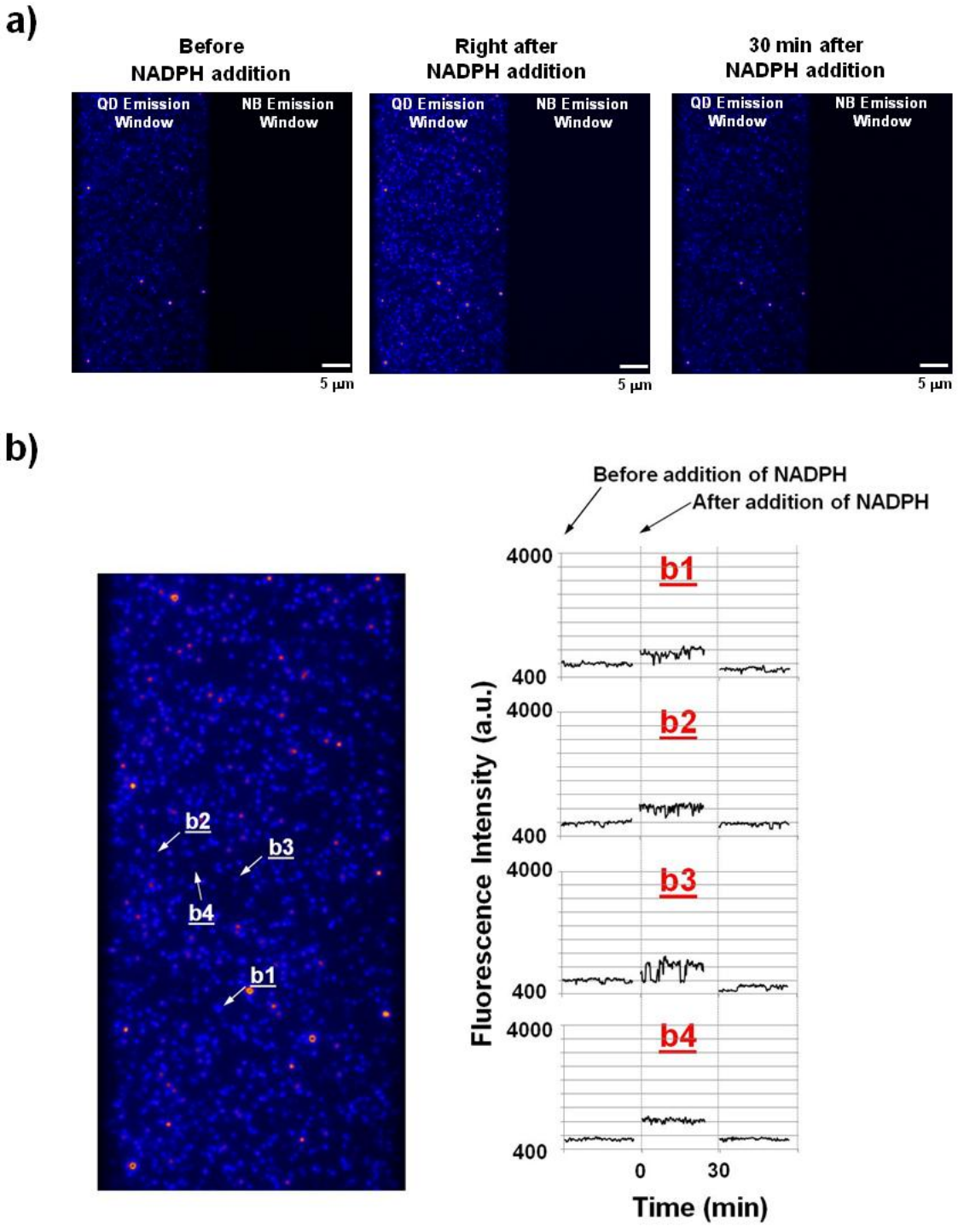

a)
Before NADPH addition
Right after 40 mM NADPH addition
30 min after 40 mM NADPH addition
QD Emission Window
NB Emission Window
5 μm
b)
a1
a2
a3
Before addition of NADPH
After addition of NADPH
Fluorescence Intensity (a.u.)
4000
400
Time (min)
0
30

Figure S7


a)
Before NADPH addition
Right after NADPH addition
30 min after NADPH addition
QD Emission Window
NB Emission Window
5 μm
b)
b1
b2
b3
b4
Before addition of NADPH
After addition of NADPH
Fluorescence Intensity (a.u.)
4000
400
0
30
Time (min)