Electronic Supplementary Information (ESI) for ChemComm.

Electronic Supplementary Information for

A Graphene Oxide-based tool-kit capable of characterizing and classifying exonuclease activities

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Supplementary Information

1. General experimental sections

Materials and apparatus

The DNA was synthesized and HPLC-purified by IDT (Integrated DNA Technologies). Graphene oxide (GO), water dispersion was purchased (Graphenea, Cambridge, MA 02142, U.S.A). The 96-well assay black plates were purchased from Corning (CORNING, NY 14831 USA). Fluorescence spectra were recorded on a Fluorescence spectrometry (Model: FS-2, SCINCO Inc., Nonhyeon-dong, Gangnam-gu, Seoul, Korea). Imaging and intensity measurements were performed on a Flexstation3 Multi-Mode Microplate Reader.

Protein (Exonuclease III) purification

Exonuclease III[1], Lambda exonuclease[2], and Phi29 polymerase[3] were purified as previously described. T7 exonuclease was purchased from New English BioLabs (NEB, catalog# M0263S). The genes of three enzymes were amplified by PCR from genomic DNA using primers designed for ligation-independent cloning (LIC). The PCR product was cloned into the pB3 ((His)6-) vector via the LIC method(1). The cloned vector was confirmed by DNA sequencing, and ExoIII was expressed in E. coli cells. The fusion plasmid construct was transformed into BL21-Star E. coli (General experimental section) and expressed in 1 L LB. Bacterial cultures were grown to an OD600 of 0.5. IPTG was added at to final concentration of 1 mM. After shaking for 3.0 h at 37 °C, the bacteria were harvested in a rotor at 5000×g and lysed by sonication in a buffer (20 mM Tris–HCl, pH 7.5, and 500 mM NaCl). The cell lysate was centrifuged for 30 min at 35,000×g and re-suspended in 20 mM Tris–HCl (pH 7.5), 500 mM NaCl. ExoIII was purified by HisTrap FF (GE Healthcare) using a buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl and 500 mM Imidazole) in a gradient method. The purified protein was stored in a buffer (50 mM Tris–HCl, pH 7.5, 1 mM DTT, and 50% glycerol).

Cy3 and Cy5 labeling and annealing

Two kinds of DNA (3'-dsDNA and 5'-dsDNA) were prepared. Both non-hydrolyzed strands of 3'-dsDNA and 5'-dsDNA (sequences shown in Table 1) have amine-modified oligonucleotides that are labeled Cy5 (Ex/Em=650/670 nm) and Cy3 (Ex/Em=550/570 nm), respectively. The fluorescently labeled DNA was annealed with the complementary strand in Tris buffer (pH 8.0) with 100 mM NaCl by heating for 2 min at 85 °C and cooling slowly to room temperature. Following fluorescence labeling, the fluorescence efficiencies were determined by Nanodrop™.

Neutravidin attachment assay

Following fluorescent and biotin labeling, neutravidin was attached to the DNA, following the method[4]. The neutravidin (5 mg/ml) was diluted 10x. 10 µl of DNA (10 µM), and 10 µl of neutravidin (500 µg/ml) were mixed in the total volume of 30 µl, making 1x binding buffer (12% Glycerol, 12 mM HEPES-NaOH [pH 7.9], 4 mM Tris-HCl [pH 7.9], 60 mM KCL, 1 mM EDTA, 1 mM DTT). After mixing, the sample was placed in a 37 °C incubator with shaking for 30 min.

Fluorescence spectrometer measurement

Fluorescence images were collected using a fluorescence spectrometer (Model: FS-2, SCINCO Inc., Nonhyeon-dong, Gangnam-gu, Seoul, Korea) at room temperature. The basic conditions were as follows. Firmware Version is 110120. Accessory Type is Single-Cell type. PMT Voltage(V) is 600, PMT Integration Time is 20 ms. Scan Speed was 600 nm/min. Scan Mode was Emission. Data Mode was Fluorescence. Excitation Slit Width and Emission Slit Width were set to 5 nm. Excitation filter and Emission filter was Air. The excitation wavelength and emission range were set depending on the fluorescent dye used. To detect the Cy3 fluorescence signal, the excitation wavelength was 532 nm and the emission range was 550 to 850 nm. For the Cy5 fluorescence signal, the excitation wavelength was 633 nm and the emission range was 650 to 850 nm. The graphene oxide solution (4.5 µl, 90µg/ml), DNA, and exonuclease in reaction buffer were added to a cuvette for measurement.

Gel based degradation assay

Two DNA probes were prepared. The 3'–dsDNA fluorescent dye-conjugated bottom-strand DNA was fluorescently labeled via 3' amino-modification with a Cy5 NHS ester (GE Healthcare LifeSciences), and biotin was attached to the 5'-end. Next, the DNA substrates are prepared such that only one end is accessible to enzyme. Our initial plan was to use biotinylated DNA. However, biotinylation at the 5'-

end of DNA does not prevent cleavage by exonucleases, as observed by the previous study[4] and by ourselves. ExoIII can digest biotinylated and fluorescently labeled DNA (Fig. S2). In the gel assay, ExoIII degradation left only the dyes. However, Lambda exonuclease cannot digest both the biotin and fluorescent labels part and fluorescence dye (Fig. S1). To prevent digestion of the biotinylated ends, we further blocked these ends with neutravidin, taking advantage of the power neutravidin-biotin interaction. With neutravidin in place, the exonuclease reaction by ExoIII was successfully prevented (Fig. S3). Previous results had also shown that when streptavidin was allowed to bind to the biotinylated substrates prior to addition of the nuclease, no cleavage was observed[4].

Fluorometric analysis of exonuclease activity in a 96-well plate.

Exonuclease (1 μM) was used for degradation in vitro as stated above, and buffer used as a negative control. Intensity measurements were taken every few seconds. The volume of each well was 100 μL. The reaction product was added to a solution containing 10 μl 1 mg/mL GO (a final concentration of 100 μg/mL), 10 μL 10X reaction buffer, 63 μl distilled water, 10 μl exonuclease (a final concentration of 1 μM), 7 μl 1 μM DNA (a final concentration of 70 nM) in a 96-well plate. After incubation for 30 min at room temperature, Cy3/Cy5 fluorescence was measured using a Multi-Mode Microplate Reader (FlexStation 3). Fluorescence images of the ExoIII reaction in a black 96-well plate (CORNING, NY 14831 USA) were obtained using a fluorescent imaging system. The fluorescence intensities were measured using a Multi-Mode Microplate Reader (FlexStation 3) with an excitation wavelength of 548 nm and an emission wavelength of 561 nm in the case of 5'dsDNA•Cy3. For 3'dsDNA•Cy5, an excitation wavelength of 649 nm and an emission wavelength of 661 nm were used. Using the Well-scan function, images related to the intensity were obtained above the table.

Optimizing the graphene oxide concentrations

Keeping DNA concentration constant at 70 nM, we needed to know what concentration of GO results in ssDNA quenching. The ssDNA and dsDNA intensity was measured as GO concentration was increased from 0 μg/ml to 150 μg/ml. Under the experimental conditions, we used 4.5 μl 1 mg/ml GO (a final concentration of 90 μg/ml) with 70 nM DNA. The ssDNA is fully quenched at 90 μg/ml, but at that loading of GO, quenching of dsDNA is also observed. For example, the intensity of 3'–dsDNA becomes 47% lower than initial intensity when GO loading is 90 μg/ml. Likewise, the 5'–dsDNA intensity is quenched by 42% of the initial intensity. However, in the case of dsDNA, this signal remains flat and stabilized.

Assay of Protein concentration and cofactor

Enzyme activity is dependent on the concentration and the presence of important cofactors, and we wanted to calculate the values of the Km value and Vmax for Michaelis-Menten fitting. GO interacts weakly with dsDNA and strongly with ssDNA. The presence of all components complicates the data due to the presence of dsDNA-GO and ssDNA–Enzyme complexes.

To simplify the measurements, the process was divided into two steps. During the first 20 min, we only measure dsDNA and GO. That signal is taken as the basal interaction signal. After 20 min, we add the exonuclease and measure the intensity for 10 min. The exonuclease concentration ranged from 10 nM to 3 mM in 50 μl samples containing 3.5 μl 1 μM DNA, 10X reaction buffer, and distilled water.

To test the cofactor effect, we titrated the cofactor into the sample. Both ExoIII and λexo require the presence of the cofactor Mg2+. This cofactor was titrated into 50 μl samples containing 3.5 μl 1 μM DNA, 10X reaction buffer, 1 μM exonuclease, and distilled water.

2. DNA Sequence table

DNA	Sequence
3’-dsDNA: hydrolyzed ssDNA	/5Biosg/GGT GGC GGC GGG ATG ATG AAG AGA ACA GTG CGT CGG AGA GGA GAG AGA GAA GGA AGT GTG GCG GAA GG
3’-dsDNA: Cy5-labled non-hydrolyzed ssDNA	/5Biosg/CCT TCC GCC ACA CTT CCT TCT CTC TCT CCT CTC CGA CGC ACT GTT CTC TTC ATC ATC CCG CCG CCA CC/3AmMO/

5’-dsDNA: hydrolyzed ssDNA	/5Phos/ CTG CCT AAA TTA CAT GTT GGC GTG AGA ATC GCC ATA TTT AAC AAA TTA AGC CTC GCT GCC GTC GCA A/3Bio/
5’-dsDNA: Cy3-labeled non-hydrolyzed ssDNA	/5AmMC6/ TGG CGA CGG CAG CGA TTA ATT TGT TAA ATA TGG CGA TTC TCA CGC CAA CAT GTA ATT TAG GCA G/3Bio/

Table 1.

Fluorescent cyanine dyes (Cy3 and Cy5) were labeled at amine-modified sites denoted by /AmMO/ or /AmMC6/. 3’-dsDNA and 5’-dsDNA are 68bp and 67bp long, respectively.

3. Supplementary Figures

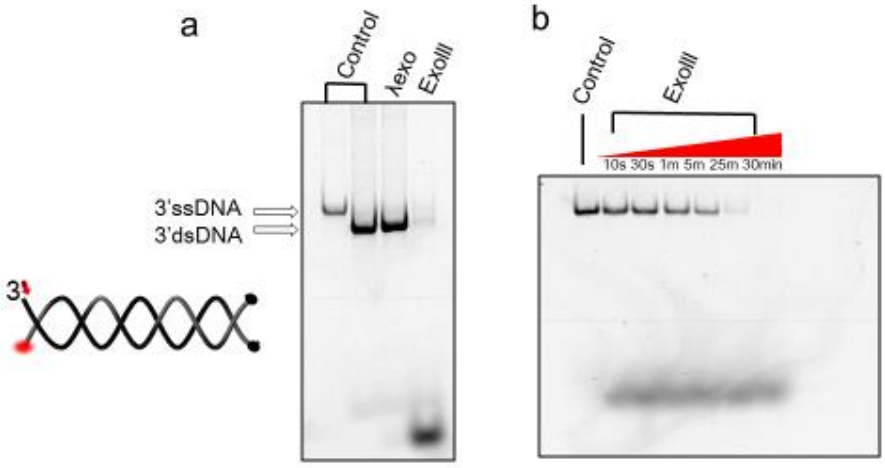

Fig. S1 Degradation assay by gel. The 3'-dsDNA was degraded by ExoIII, but not by λ-Exo, suggesting blocking the 5' end of DNA via a biotin efficiently prevents the degradation by λ-Exo. (a) Lane 1: ssDNA only; lane 2: 3'-dsDNA only; lane 3: 500nM λ-Exo + 3'-dsDNA in the reaction buffer; lane 4: 500nM ExoIII + 3'-dsDNA in the reaction buffer. (b) Time course of degradation by ExoIII

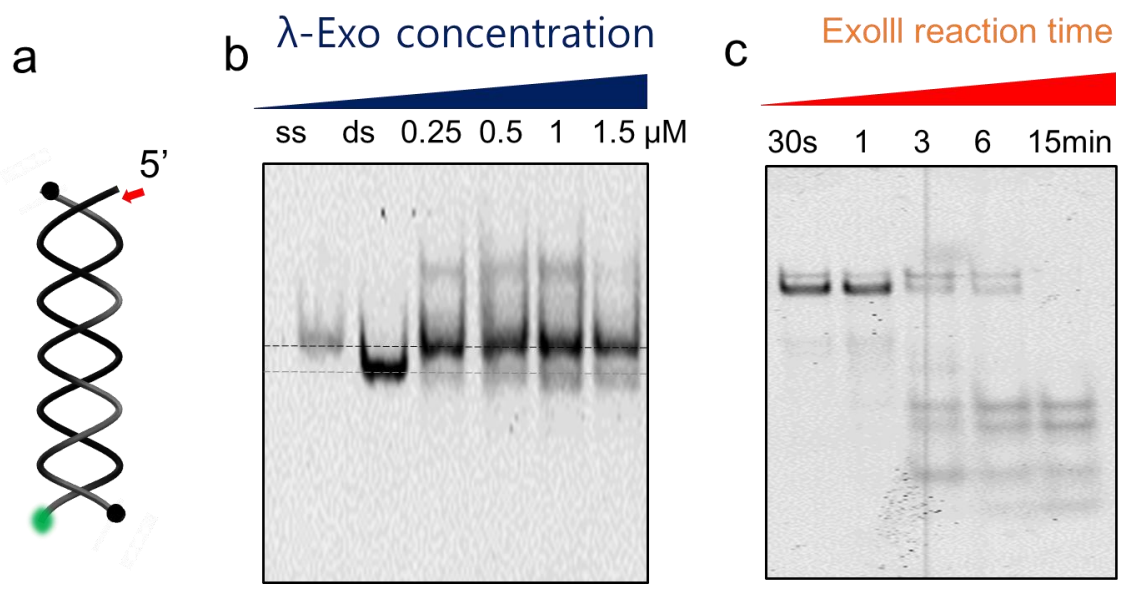

Fig. S2 The 5’-dsDNA was degraded by both λ-Exo and ExoIII, suggesting blocking the 3’ end of DNA via a biotin could not prevent the degradation by ExoIII. (a) 5’-dsDNA. (b) Lanes 1-2: ssDNA emerged slightly higher than 5’-dsDNA presumably due to its secondary structure formation in a native gel, but the final product after degradation appeared the exactly same position as ssDNA; lanes 3-6: 0.25, 0.5, 1, 1.5 μM λ-Exo in the reaction buffer containing 5’-dsDNA. (c) Time course of degradation showing that ExoIII degraded the 5’-dsDNA, even with biotin capping at the 3’ ends of dsDNA.

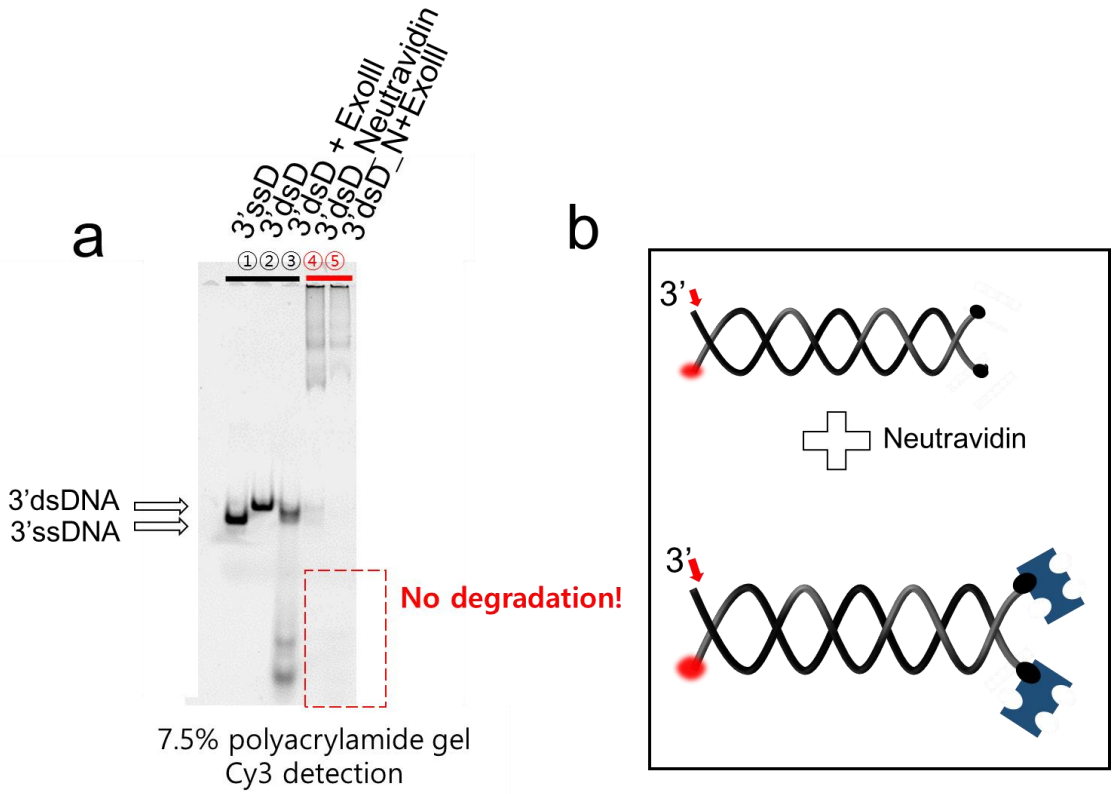

Fig. S3 The 3' end-capping via biotin and neutravidin (a deglycosylated version of avidin) sufficiently prevents the degradation from the 3'ends via ExoIII. (a) Lane 1: ssDNA; lane 2: 3'-dsDNA; lane 3: ExoIII + 3'ds-DNA in the reaction buffer; lane 4: 3'-dsDNA + neutravidin; lane 5: 3'-dsDNA + neutravidin + Exo III in the reaction buffer. If the 3'-dsDNA-netravidin complex was degraded by ExoIII, then the degradation product would appear in the dotted red box in the lanes 4-5. This result was very consistent with the previous report[4] and our GO-based fluorescence-quenching assay (main Figs.1- 3). (b) top: 3'-dsDNA alone; bottom: 3' dsDNA conjugated with neutravidin.

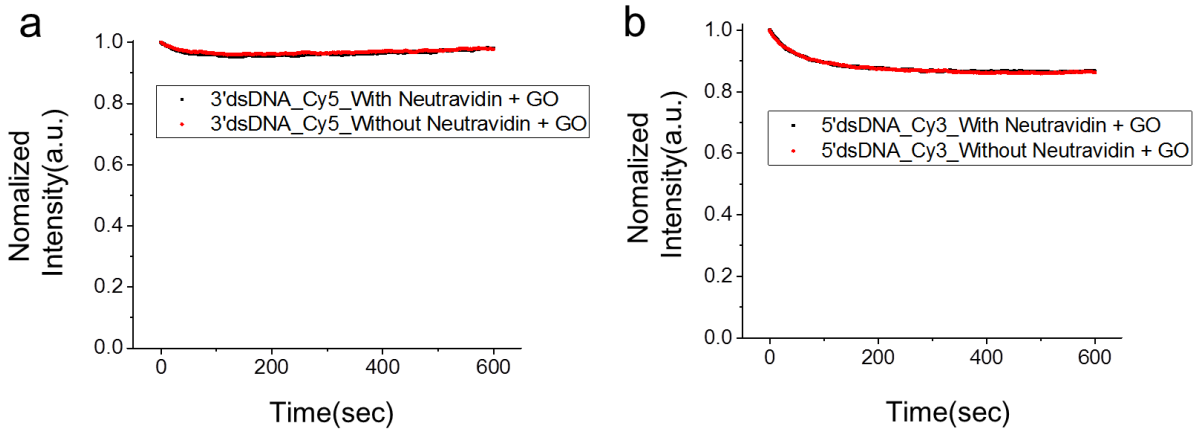

Fig. S4 The effect of neutravidin conjugation at the ends of dsDNA on fluorescence-quenching between GO and dsDNA. (a) Cy5-labeled 3'-dsDNA with (black) or without (red) neutravidin. (b) the same as in a, but for Cy3-labeled 5'-dsDNA.

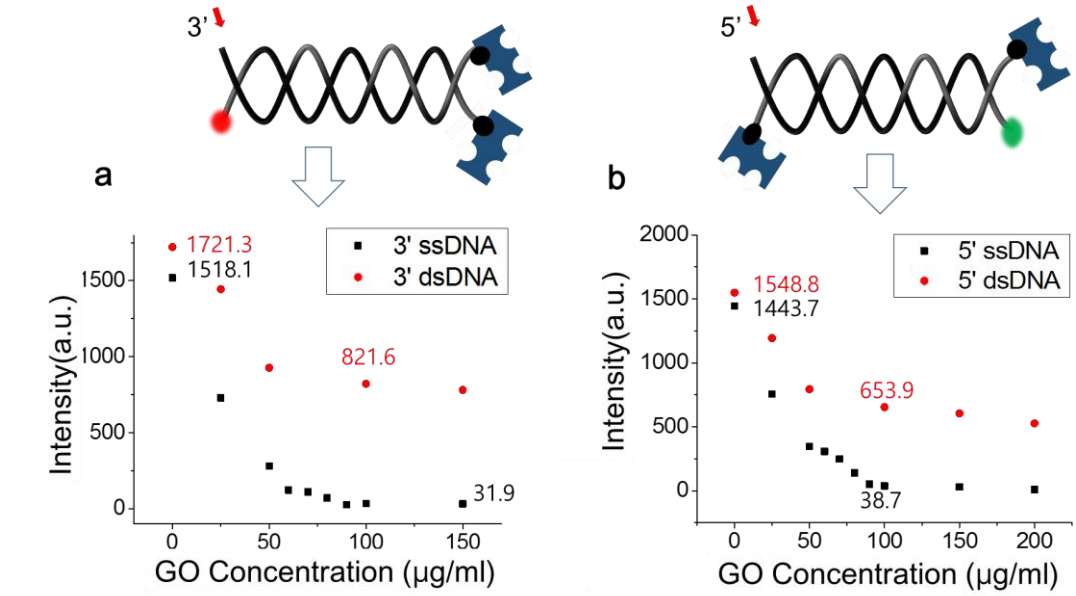

Fig. S5 Determination of the minimum amount of GO for GO-based fluorescence-quenching assay (Optimization of GO). (a, b) Fluorescence intensity vs GO concentration plots. To achieve efficient quenching, 90 μg/ml of GO was required for both 3'-dsDNA and 5'-dsDNA at the fixed concentration of dsDNA (70 nM).

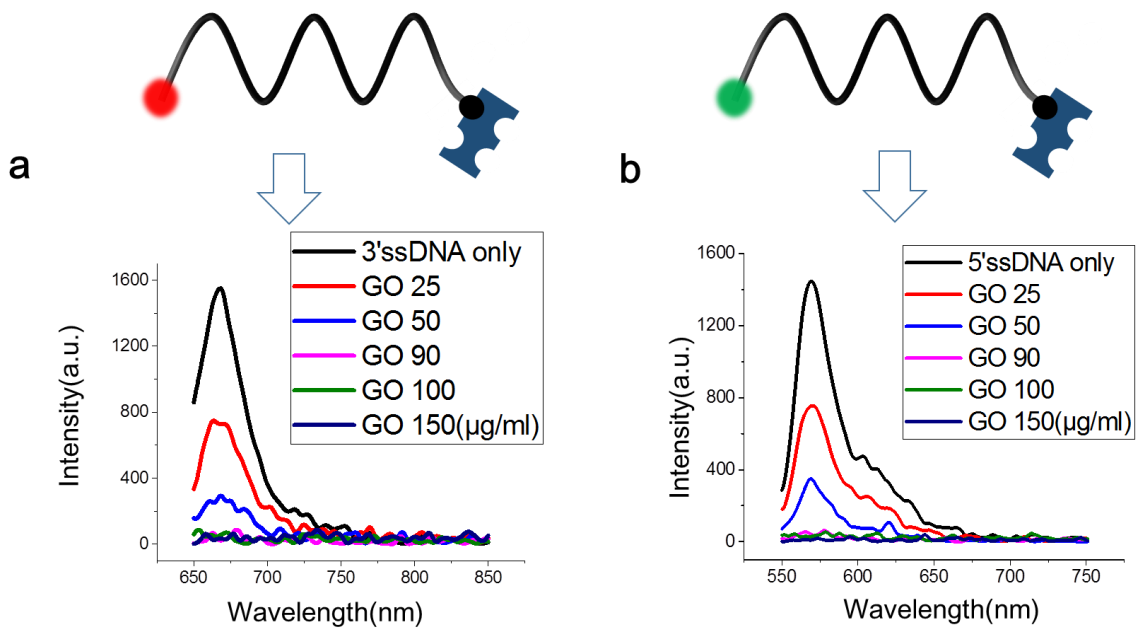

Fig. S6 Quenching effects by ssDNA as a function of GO concentration. (a, b) To efficiently quench fluorescently labeled-ssDNA resulting from exonuclease degradation, 90μg/ml of GO was required for both 3'-dsDNA and 5'-dsDNA at the fixed concentration of dsDNA (70 nM).

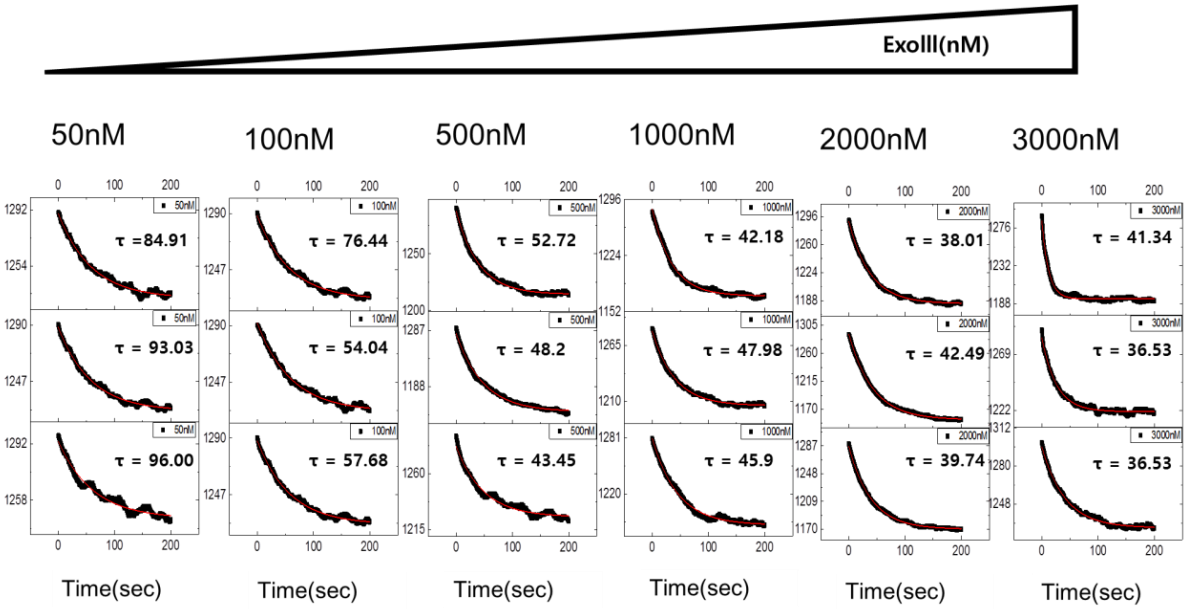

Fig. S7 Kinetic measurements to determine quenching rates as a function of ExoIII concentration, using fluorescence time-scan. The quenching rates were determined by fitting fluorescence intensity vs time curves to a single exponential decay. Three independent measurements were repeated for each experimental condition to obtain standard errors of the mean.

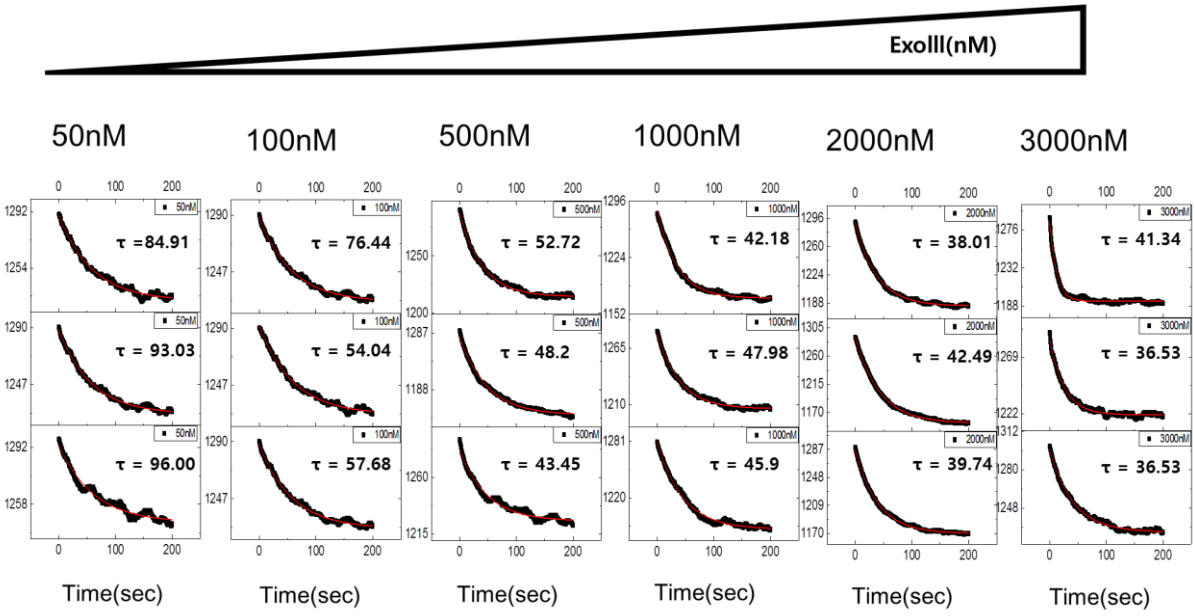

Fig. S8 Determination of quenching rates as a function of λ-Exo concentration. The quenching rates were determined the same as in Fig. S7

References

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(4) Murante, R. S.; Rust, L.; Bambara, R. A. *J. BIOL. CHEM.* **1995**, *270*, 30377.