Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information for "Direct characterization of protein oligomers and their quaternary structures by single-molecule FRET"

Cheolhee Kim,[a] Jae Yeol Kim,[a] Seung Hyeon Kim,[a] Byung Il Lee,[b] and Nam Ki Lee*[a]

[a] *Department of Physics and School of Interdisciplinary Bioscience & Bioengineering, Pohang University of Science and Technology, Pohang 790-784, South Korea.*

[b] *National Cancer Center, Goyang 410-769, South Korea.*

CORRESPONDING AUTHOR Email: nklee@postech.ac.kr

1. Expression and purification of drRecR and drRecO

The *recR* gene from *D. radiodurans* was amplified and cloned into the pET21a vector in the C-terminal His-tagged form (pNK-RecR1). drRecR was expressed in the *E. coli* strain BL21(DE3) (invitrogen) using Luria-Bertani media (BD) supplemented with 50 μg/mL ampicillin at 37 degree. Cells were harvested by centrifugation and lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 400 mM NaCl, 20 mM Imidazole, 5 mM DTT, and 2 mM AEBSF (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride), by applying sonication on ice. After the insoluble cellular material was removed by centrifugation, the expressed drRecR was purified using Ni-NTA super flow (Qiagen). His-tagged proteins were eluted with a buffer supplemented with 500 mM Imidazole. Then the proteins were dialyzed overnight at 4 degree with a storage buffer (15 % Glycerol (v/v), 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM DTT). drRecO was expressed and purified using the same method described above. The *recO* gene was cloned into pET21a vector from pDRO5 (the kind gift of Dr. Sergey Korolev) in the N-terminal Hig-tagged form (pNK-RecO). Protein stocks were stored at -80 degree.

2. Construction of drRecR mutants (Q133C, R37C and Q133C$_{1\text{-}173}$) and fluorescent labeling

Two mutants (R37C, Q133C) for dye labeling were prepared by point mutations using the PrimeSTAR DNA polymerase (TAKARA, Japan). In order to test stable dimer form, we deleted 50 residues from C-terminal end (Q133C$_{1\text{-}173}$). All mutations were confirmed by sequencing plasmids (SOLGENT, Korea). For dye labeling, we used the Maleimide-reactive Cy3 and Cy5 (GE healthcare) according to the manufacturer's direction. Free dyes were removed using a size-exclusion chromatography column (PD minitrap G-25, GE healthcare).

3. ALEX measurement for stable oligomers

To measure the oligomeric form of drRecR, we prepared Cy3 and Cy5-labeled drRecR. Typical

labeling efficiency was 50 %, but to reduce the probability of having three (or four) dyes in a tetramer we added unlabeled drRecR, so that the final labeling efficiency of drRecR was ~ 20 %. We mixed Cy3-labeled drRecR together with Cy5-labeled drRecR (2 μM final concentration) in buffer A (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 % Glycerol (v/v), 1 mM DTT, 5 mM $MgCl_2$, 100 μg/mL BSA) and incubated it 0.5−3 hours at 37 degree. For single-molecule measurement, we diluted the mixture in the single-molecule buffer (buffer A + 1 mM MEA) to be 0.4 nM drRecR concentration, *i.e.*, 80 pM dye-labeled drRecR concentration. In the case of the mixture with drRecO, we added 10 μM drRecO to both buffer A and the single-molecule buffer (Fig. 3B). All data acquisition time was 10 min.

4. Single-molecule Alternating-laser excitation (ALEX) setup

The microscope setup, data acquisition, and data analysis of alternating laser excitation method (ALEX) have been described in other papers.[1] Two lasers of 532-nm solid state green laser (TECGL-20, World Star Tech) and 633-nm HeNe laser (25-LHP-925, Melles-Griot) were alternated using Acoustic-optic modulators (AOM, 23080-1, Neos technologies) with a period of 100 μs. The excitation intensities were 80 μW and 24 μW for the 532-nm and 633-nm lasers, respectively, in the alternating mode. Two laser lights were coupled by a dichroic mirror (z532bcm, Chroma), and then directed to an inverted microscope (IX51, Olympus). After reflected on a dichroic mirror (Z532/633RPC, Chroma), the lights were focused at 20 μm from the surface of a coverslip by an objective (a water-immersion objective, 60×, 1.2 NA, UPLAPO, Olympus). Fluorescence emissions were collected through the objective and passed through a 100 μm pinhole, and then refocused onto silicon avalanche photodiode detectors (APD) (SPCM AQR-13, EG&G Perkin Elmer). The fluorescence was separated into two streams by a beamsplitter (625DCLP,Chroma) and filtered by HQ580/60m for Cy3 and HQ660LP for Cy5. All data were analyzed by LABVIEW software (National Instruments). From the 1 ms binned time traces (Figure S1), we selected fluorescent bursts (single molecules passing through the excitation volume) using a threshold ~30 photons and each burst was registered with three photon counts. We calculated stoichiometric ratio (***S***) and FRET efficiency (***E***) for each molecule from the three photon counts of a burst. The data were typically collected for 10 minutes.

5. Calculation of *E* and *S* of a single molecule.

The method to obtain ***E*** and ***S*** of a single molecule from ALEX measurement has been well described before.[1a] Briefly, from ALEX measurement the fluorescence time traces of molecules

were obtained as raw data (Figure S1). Because the lasers are focused tightly in femto-liter volume by an objective, in sufficiently diluted solution only one molecule passes through the excitation area. This diffusing-in-and-out event of a molecule results in fluorescent burst in the time trace (Figure S1). The unique feature of ALEX is to alternate two lasers (532 nm and 633 nm) faster (100 μs) than the transit time (~ ms) of a molecule through the excitation area. As a result, three different types of photons for each burst are obtained: I_D^D is a fluorescent emission of donor dyes excited by donor-excitation laser, I_D^A a fluorescent emission of acceptor dyes excited by donor-excitation laser, which is a FRET signal, and I_A^A a fluorescent emission of acceptor dyes excited by acceptor-excitation laser. These three intensities are used to calculate ***E*** and ***S***.

$$\mathrm{S} = \frac{I_D^D + I_D^A}{I_D^D + I_D^A + I_A^A}, \qquad \mathrm{E} = \frac{I_D^A}{I_D^D + I_D^A}.$$

Here, ***S*** is used as a sorting parameter. Theoretically, ***S*** of donor-only is 1, because $I_A^A = 0$ (no acceptor dyes), while ***S*** of acceptor-only is 0, because $I_D^D + I_D^A = 0$ (no donor dyes). In the cases of FRET species, containing both donor and acceptor dyes, ***S*** becomes 0.5 by adjusting the ratio of laser powers to be $I_D^D + I_D^A \approx I_A^A$.

6. Estimating distance from *E* value

Detailed procedures to calculate distance from measured ***E*** have been well described in ref (1). The ***E*** presented in Figures 2 and 3 are corrected only for the backgrounds. To obtain "accurate ***E***", the leakage, direct excitation of acceptor by donor excitation laser, and the ratio of detection channel efficiency (γ) have to be corrected. Since the dyes are attached to protein with a considerable length of linker, the dye-to-dye distance calculated from the "accurate ***E***" is still approximated one for the distance between the labeled positions on a protein. In this work we used $\boldsymbol{E}^c$ [$\mathrm{E^c} = (I_D^A - \mathrm{lk} * I_D^D)/(\gamma * I_D^D + I_D^A - \mathrm{lk} * I_D^D)$] for the calculation of the approximated distances, where $\boldsymbol{E}^c$ is the corrected ***E*** for the leakage (the fluorescence of Cy3 bleeds through the detection channel of Cy5) and gamma factor (γ), and *lk* is leakage ratio. We measured γ = 1.44, using the method described in ref (1). Then we applied equation of $\mathrm{E^c} = 1/(1 + \left(\frac{\mathrm{R}}{\mathrm{R_0}}\right)^6)$ to estimate distance, where ***R*** and $\boldsymbol{R_0}$ denote the distance (dye-to-dye) and the Förster radius, respectively. For $\boldsymbol{R_0}$ of Cy3-Cy5 pair, we used literature value of 6 nm.[2] As a result, the dye-to-dye distances were estimated to be 5.6 nm and 8.1 nm for distance ***a*** and ***b*** of Q133C (Figure 2a), respectively, which are in line with the distances (between labeled positions) of 4.5 nm and 8.9 nm, estimated from the crystal structure. In the same way, the dye-to-dye distances of **a** and ***b*** of R37C (Figure 2c) were estimated

to be 8.3 nm and 4.7 nm, respectively, which are in line with the distances of 7.3 nm and 2.5 nm, derived from the crystal structure. Again, it is to be noted that due to the considerable length of the dye linker and the free-rotation assumption of the dyes the measured distances from "accurate E" is not exactly matched with the values obtained from the crystal structure.

7. Dissociation constant measurement of drRecR tetramer by ALEX

The dissociation constant of drRecR tetramer in the presence of drRecO was studied by measuring the amount of high ***E*** tetramer as a fraction (***F***) of the total FRET species at the various drRecR concentrations (Figure 3c). The number of the total FRET species was calculated by selecting the molecules in the range of $0.30 < \boldsymbol{S} < 0.75$ (Figure 3b). This number includes dimer (if it exists) and tetramers that have both Cy3 and Cy5. The high ***E*** tetramer was selected in the range of $0.6 < \boldsymbol{E} < 1.0$ among the FRET species. As we have demonstrated in Figure 3b, this population corresponds only to the tetramer. As the concentration of drRecR is increased, the population of the tetramer is increased. At the saturation concentration, *i.e.*, all drRecR exist as the tetramers, a third of the tetramers would have high ***E*** value (distance ***a*** of Figure 2a), while two third of the tetramer have low ***E*** value (distance ***b*** and ***c*** of Figure 2a). Thereby, ***F*** reaches approximately 0.33 at the saturation condition. Indeed, Figure 3c shows that ***F*** reached 0.38 as we increased the concentration of drRecR. The dissociation constant of drRecR was determined at the concentration that ***F*** gained half of the increment, which was 6 ± 2 nM. For this measurement, we incubated various concentrations of drRecR (equal amount of Cy3- and Cy5-labeled Q133C) in the buffer A + 10 μM drRecO for 1 hour at 37 degree. Then the mixture is diluted to be 0.4 nM of drRecR in the single-molecule buffer that contains 10 μM drRecO, too. It is to be noted that the tetramers in the presence of drRecO are very stable that less than 10 % of the tetramers were dissociated during the 10 min ALEX measurement.

8. Dissociation constant measurement of drRecR tetramer by bulk FRET

As for bare drRecR (*i.e.*, without drRecO), the tetramer was immediately dissociated into dimer when drRecR was diluted to be 0.4 nM concentration for the ALEX measurement (Figure 2b). Thereby, we used the bulk FRET to estimate the dissociation constant of bare drRecR tetramer.[3] Because the ***E*** value increased from 0.2 of the dimer to 0.7 of the tetramer, the increment of FRET signal is a good indicator for the tetramer formation of drRecR (Q133C, Figure 3b). To measure the bulk FRET, we used ALEX setup with only 532-nm laser excitation. We prepared each concentration of drRecR by mixing the equimolar amounts of Cy3-labeled and Cy5-labeled drRecR. After incubating the mixture for 20 minutes at 37 degree, we obtained the fluorescent signals of

Cy3 and Cy5 simultaneously using two detection channels for 1 minute. The FRET value of Figure 3d was obtained by,

$\frac{(\text{total Cy5 fluorescence} - \text{leakage} * \text{total Cy3 fluorescence})}{(\text{total Cy3 fluorescence} + \text{total Cy5 fluorescence} - \text{leakage} * \text{total Cy3 fluorescence})}$, where *leakage* is the ratio that the fluorescence of Cy3 bleeds through the detection channel of Cy5. The laser intensity was varied to get the similar number of photons for each measurement. From the result of Figure 3d we estimated the dissociation constant of drRecR without drRecO when the FRET signal gained half of the total FRET increment, which was 12 ± 2 μM. We note that this result is in line with that the size-exclusion chromatography which showed the tetramer size of drRecR at the tens of μM drRecR concentration.[4]

Reference

[1] a) N. K. Lee, A. N. Kapanidis, Y. Wang, X. Michalet, J. Mukhopadhyay, R. H. Ebright, S. Weiss, *Biophys. J.* 2005, *88*, 2939; b) N. K. Lee, A. N. Kapanidis, H. R. Koh, Y. Korlann, S. O. Ho, Y. Kim, N. Gassman, S. K. Kim, S. Weiss, *Biophys. J.* 2007, *92*, 303; c) A. N. Kapanidis, N. K. Lee, T. A. Laurence, S. Doose, E. Margeat, S. Weiss, *Proc. Natl. Acad. Sci. USA* 2004, *101*, 8936.

[2] R. Roy, S. Hohng, T. Ha, *Nat. Methods* 2008, *5*, 507.

[3] N. Makharashvili, T. Mi, O. Koroleva, S. Korolev, *J. Biol. Chem.* 2009, *284*, 1425.

[4] I. Leiros, J. Timmins, D. R. Hall, S. McSweeney, *EMBO J.* 2005, *24*, 906.

Supporting Figures

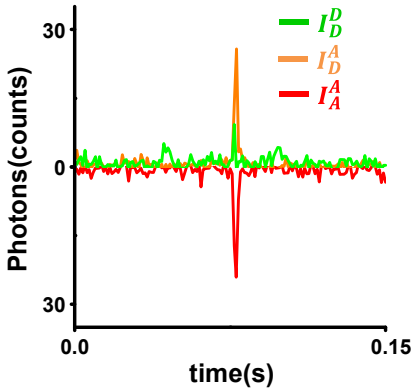

Figure S1. The fluorescence time trace obtained by ALEX measurement. A fluorescent burst is generated by a single molecule passing through the excitation area. I_D^D is a fluorescent emission of donor dyes excited by donor-excitation laser, I_D^A a fluorescent emission of acceptor dyes excited by donor-excitation laser, which is a FRET signal, and I_A^A a fluorescent emission of acceptor dyes excited by acceptor-excitation laser. The time trace is binned by 1 ms.

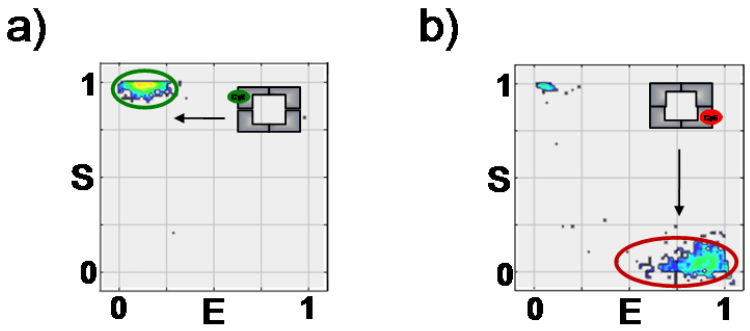

Figure S2. 2D ***E-S*** graphs for Cy3-only and Cy5-only species. After labeling drRecR with Cy3 or Cy5, each labeled protein was measured by ALEX. (a) Cy3-labeled drRecR. (b) Cy5-labeled drRecR.

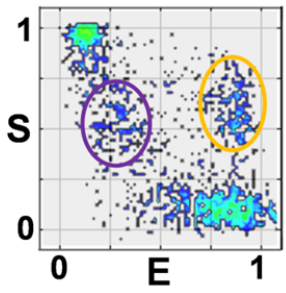

Figure S3. 2D ***E-S*** graph of the mixture of the labeled drRecR (R37C) in the presence of 10 μM drRecO. The yellow ellipse denotes the dimer (Figure 2d), while the purple ellipse at the low ***E*** denotes the tetramer for the distance ***a*** in Figure 2c.

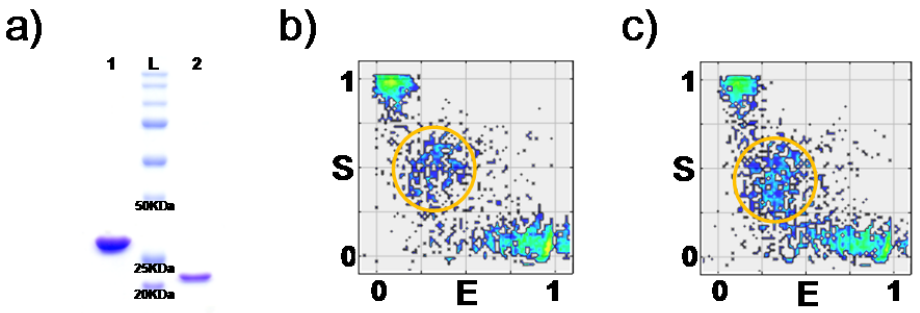

Figure S4. To confirm that drRecR formed tetramer by its specific interactions with drRecO, we prepared another drRecR mutant by deleting the 50 residues at the C-terminal end ($Q133C_{1\text{-}173}$). This mutant does not contain the binding sites for drRecO (J. Timmins, I. Leiros and S. McSweeney, EMBO J., 2007, 26, 3260). (a) SDS-PAGE gel image of drRecR. Lane 1, Q133C; lane 2, $Q133C_{1\text{-}173}$. (b) The mixture of $Q133C_{1\text{-}173}$ in the absence of drRecO. The yellow ellipse denotes the dimer (the same as Figure 2b). Although the C-terminal domain interaction has been abolished in this mutant by deleting 50 residues from C-terminal end, this mutant showed the exactly same feature as Q133C (Figure 2b). This result supports again that drRecR forms dimer by the N-terminal domain interaction. (c) The mixture of $Q133C_{1\text{-}173}$ in presence of 10 μM drRecO. The tetramer of drRecR (high FRET species, purple ellipse in Figure 3b) was not generated in this mutant. This result confirmed that the tetramer formation in Figure 3b is due to the specific interactions between drRecR and drRecO.

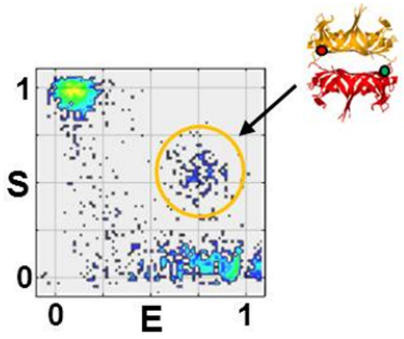

Figure S5. 2D ***E-S*** graph of drSSB. drSSB has been well known to form dimer (Bernstein *et al*., *PNAS* **101**, 8575, 2004). We prepared drSSB mutant (E114C) for dye labeling using the same procedures we used for drRecR: the positions of dye labeling are denoted by the green and red dots in the dimer form. We incubated Cy3-labeled drSSB together with Cy5-labeled drSSB (2 μM final concentration) in a buffer (20 mM Tris-HCl pH 8.3, 500 mM NaCl, 10 % Glycerol (v/v), 1 mM DTT) for 2 hours at 37 degree. For ALEX measurement, we diluted the mixture in single-molecule buffer (incubation buffer + 1 mM MEA and 0.01 % BSA) to be 30 pM dye-labeled drSSB concentration. We observed one cluster of FRET species (***E*** ~ 0.75), denoted by the yellow ellipse, which is in line with the dimer form of drSSB: the distance between the dyes in dimer form is estimated to be ~ 4 nm from the structure of drSSB.