

## Supplementary Information

### **A Co-expression Strategy to Achieve Labeling of Individual Subunits within a Dimeric Protein for Single Molecule Analysis**

**Fei Lou<sup>a b</sup>, Jie Yang<sup>a b</sup>, Si Wu<sup>a b\*</sup>, and Sarah Perrett<sup>a b\*</sup>**

<sup>a</sup>National Laboratory of Biomacromolecules, CAS Center for Excellence in Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, Beijing 100101, China.

<sup>b</sup>University of the Chinese Academy of Sciences, 19A Yuquan Road, Shijingshan District, Beijing 100049, China.

The Supplementary Information includes:

1. Experimental Procedures
2. Supplementary Figures (Figure S1-S4)
3. Supplementary Tables (Table S1-S2)

## Experimental Procedures

### *Cloning and construction of the hetero-dimer of Ure2*

The pQLinkN expression plasmid (Addgene, Plasmid #13670) was used for the construction of the hetero-dimer of Ure2<sup>1</sup>. For the cloning of the Ure2 mutants with both Cys mutations in one monomer, we first constructed the pQLinkN plasmids including the WT Ure2 gene with N-terminal His-tag and the double-Cys mutant Ure2 gene without His-tag. Similarly, for the cloning of the Ure2 mutants with Cys mutations on each monomer within a dimer, we constructed the pQLink plasmids carrying one Cys mutant Ure2 gene with an N-terminal His-tag and the other Cys mutant gene with a Strep-tag (sequence-WSHPQFEK) at the C-terminal with SA linker.

To construct the co-expression plasmid for the Ure2 hetero-dimer from the above two pQLink plasmids, about 0.5 mg of the plasmid with WT-Ure2 gene was digested overnight with 5 units PacI at 37 °C, while the plasmid carrying the double-Cys mutant gene of Ure2 was cleaved with 5 units SmaI at 25 °C. The enzymes were inactivated at 65 °C for 20 min and the DNA was treated with LIC-qualified T4 DNA polymerase in 40 µL reaction buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 5 mg/ml BSA, 5 mM DTT, 2.5 mM dCTP for the product digested by PacI and 2.5 mM dGTP for the product digested by SmaI). Upon incubation for 30 min at 25 °C and then heat inactivation at 65 °C for 20 min, the digested product of the two plasmids were mixed and heated to 65 °C and cooled to room temperature for annealing. Then 10 µL of the product was transformed into *E. coli* Top10 competent cells. Transformants were tested for the expected inserts by colony PCR with primers pQTEV3U, 5'-TATAAAAATAGGCGTATCACGAGG-3' and pQTEV3L, 5'-CCAGTGATTTTTTCTCCATTTT-3'. These primers are upstream of the LINK1 sequence and downstream of the LINK2 sequence of the pQLink vector, respectively.

### *Preparation and labeling of proteins*

All Ure2 mutants were expressed in *E. coli* BL21(DE3) cells and purified by nickel chromatography as described previously<sup>2</sup>. The purity of each protein was above 98% judged by SDS-PAGE and was stored at -80 °C. All proteins were thawed and centrifuged at 18000 g for 15 min prior to use. For the mutants including a His-tag and a Strep-tag in each monomer within the dimer, the eluent after the nickel column was further subjected to a Strep-tag affinity column (IBA Lifesciences, Germany) purification step. A solution containing 2.5 mM desthiobiotin was used to elute the final protein. The concentration of the protein was determined from the absorbance at 280 nm using a molar extinction coefficient of 48,220 M<sup>-1</sup> cm<sup>-1</sup><sup>2</sup>.

Fluorescence labeling of the protein was carried out by incubating the Ure2 Cys mutant with a mixture of Alexa Fluor 555 (AF555) maleimide and Alexa Fluor 647 (AF647) maleimide (Invitrogen) at a molar ratio of 1:3. The mixture was incubated at 25 °C for 30 minutes. The unreacted free dye was removed using a PD-10 Desalting Column (GE Healthcare). The labeled protein was flash frozen and stored at -80 °C before use.

### *Q-TOF-MS experiment*

HPLC-Q-TOF-MS was used to measure the MW of the hetero-dimer of Ure2. Chromatogram separation was carried out on an HPLC system (Agilent) equipped with a shield RP C8 column (4.6 × 250 mm, 5 µm, Zorbax 300SB Corp.). The mobile phase was composed of 0.1% formic acid water solution (v/v, A) and acetonitrile (B), and was pumped at a flow rate of 1 mL/min. The injection volume of each sample was 1.5 µL. The chromatograms were monitored at 280 nm and

obtained through the following gradient elution program: A started at 95% and decreased linearly from 95% to 10% (0 to 15 min). The column temperature was maintained at 40 °C.

MS analysis was performed on an HPLC-Q-TOF-MS system (Agilent) equipped with an electrospray ionization source in both positive and negative ion modes. The atomization pressure was 45 psi and the capillary voltage was 3.5 kV. The temperature of sheath gas was set at 325 °C with the sheath gas flow rate at 9 L/min. The mass spectra were recorded within the scan range of 30000 to 100000 Da.

### ***Single molecule FRET experiments and data analysis***

The single molecule FRET instrument was built on an inverted fluorescence microscope (Ti-U, Nikon) equipped with a 100× objective (N.A. = 1.4, Nikon), which is similar to that described previously<sup>3</sup>. The beam of a 532 nm laser (Pavilion) was directed to the back port of the microscope for excitation with a laser power of 40 μW. The fluorescence emission was collected via the same objective, filtered with a 50 μm pinhole and split into donor and acceptor channels with dichroic mirrors (T660LPXR, Chroma) before being focused on to two avalanche photodiode detectors (SPCM-AQRH-14, Excelitas) with fluorescence filters (Chroma, ET595/50 for AF555 and ET690/50 for AF647). The fluorescence signal was recorded using a two-channel photon counting card (PMS-400A, Becker & Hickl).

AF555/AF647 labeled Ure2 was centrifuged at 18000 g for 15 min to remove any aggregates, diluted to 10 pM and loaded onto the coverslip. The detection buffer (50 mM Tris, 200 mM NaCl, pH 8.4) contained 0.1 μM unlabeled Ure2 protein and 0.005% Tween 20 (Thermo) in order to avoid possible dimer dissociation and surface adsorption of labeled proteins to the coverslip. For each sample, smFRET data collection lasted for 1 h, and at least three repetitions were performed in order to ensure the result was reproducible. The fluorescent bursts, which have the sum of donor and acceptor signal that are above the 30 photons per 1 ms time bin, were selected as effective FRET events. The FRET efficiency was calculated and calibrated considering the donor fluorescence leakage to the acceptor channel, the direct excitation of acceptor, the difference in quantum yield and detection efficiency of donor and acceptor, according to established methods<sup>4</sup>. The histogram of the FRET distribution was plotted and fitted to a Gaussian function to obtain the central position and width of the FRET peaks.

The FRET data were further analyzed to obtain the distance distribution and plot the probability density profile of the position of N-terminal labeling dyes using FRETnpsTools<sup>5</sup> run by MATLAB (Math Works). We used the coordinates of the gamma-carbon of the residues in the C-terminal domain (Glu108, Lys240, Phe295, L351) as the locations of the sulfur atoms when they were mutated to cysteines. In the following calculation, we used  $D_{\text{dye}}=7 \text{ \AA}$  and  $L_{\text{linker}}=7 \text{ \AA}$  and  $D_{\text{linker}}=4.5 \text{ \AA}$  for both AF555 and AF647 dyes, the same as that used in Ref [5]. Other steps were performed according to the instruction of FRETnpsTools. The resulting spatial density distribution of N-terminal residues Val9, Ser26, and Ser52 were plotted using PyMOL as shown in Fig. 5.

### ***Fluorescence correlation spectroscopy (FCS)***

FCS experiments were performed on the same instrument as used in smFRET measurements but with a slightly different fluorescence detection pathway. The fluorescence emission was filtered by an ET595/50 filter (Chroma) and a 50 μm pinhole (Thorlabs), split by a 50/50 splitter mirror (Chroma), focused onto two avalanche photodiode detectors. The pseudo cross-correlation of the

donor fluorescence (Cy3B) signal from two APD detectors was collected using a Flex02-01D correlation card (Correlator.com). The collection of each FCS curve lasted 1 hour. The FCS curve of Cy3B-only labeled sample was also measured as a control and used to divide the values of the Cy3B/AF647 labeled sample to obtain the relative FRET fluctuation.

#### ***Fluorescence anisotropy assay***

Steady-state fluorescence anisotropy experiments were carried out on a spectrofluorophotometer (RF-5301PC, Shimadzu) equipped with excitation and emission polarizers. The donor AF555 and acceptor AF647 were excited at 532 nm and 640 nm respectively. The peak fluorescence intensities of four different combinations of polarization orientations were recorded, where both the excitation and emission can be vertical or horizontal. The detection efficiency of the spectrometer in different orientations was corrected by the factor  $G = I_{HV}/I_{HH}$ , and the anisotropy was calculated according to  $r = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH})$ . All of the anisotropy experiments were performed at 25 °C.

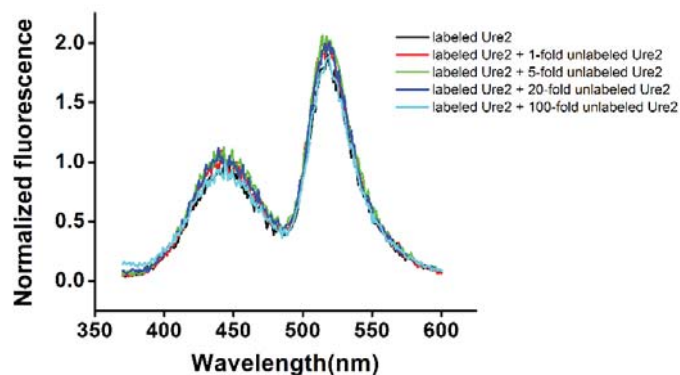
#### ***Photocrosslinking and mass spectrometry***

Ure2 K240C protein was labeled using a ten-fold excess of benzophenone-4-maleimide (BPM, Invitrogen) and incubated overnight at 4 °C. The reaction was then stopped with 1 mM DTT. Labeled protein was passed through a PD-10 desalting column (GE Healthcare) to remove the excess BPM. Then the BPM-labeled Ure2 was irradiated under UV light (365 nm) for up to 30 min to trigger the crosslinking reaction.

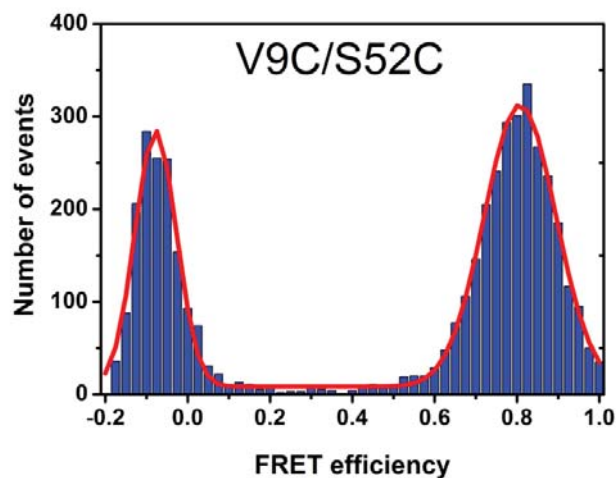
The photocrosslinking product and unlabeled Ure2 K240C (as a control) was reduced with DTT and alkylated with iodoacetamide, and digested with trypsin overnight. The peptides obtained after digestion were desalted using C18ZipTip (Millipore) according to the protocol provided by the manufacturer. The samples were loaded into the MALDI-TOF/TOF Ultraflex<sup>TM</sup> (Brucker, Germany) mass spectrometer in a crystalline matrix of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA, 5 mg/ml). The experimental peptide mass fingerprint was compared with the theoretical peptides calculated using the FindPept tool (<http://web.expasy.org/findpept>).

#### **References:**

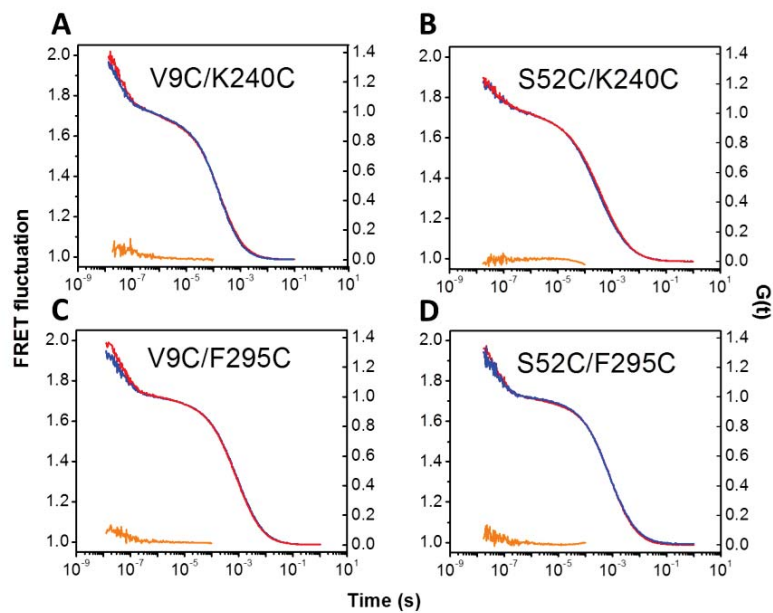
1. C. Scheich, D. Kummel, D. Soumailakakis, U. Heinemann and K. Bussow, *Nucleic Acids Res*, 2007, **35**, e43.
2. L. Fei and S. Perrett, *J Biol Chem*, 2009, **284**, 11134-11141.
3. S. Wu, X. Ge, Z. Lv, Z. Zhi, Z. Chang and X. S. Zhao, *Biochem J*, 2011, **438**, 505-511.
4. A. Hoffmann, A. Kane, D. Nettels, D. E. Hertzog, P. Baumgartel, J. Lengefeld, G. Reichardt, D. A. Horsley, R. Seckler, O. Bakajin and B. Schuler, *Proc Natl Acad Sci U S A*, 2007, **104**, 105-110.
5. A. Muschielok, J. Andrecka, A. Jawhari, F. Bruckner, P. Cramer and J. Michaelis, *Nat Methods*, 2008, **5**, 965-971.



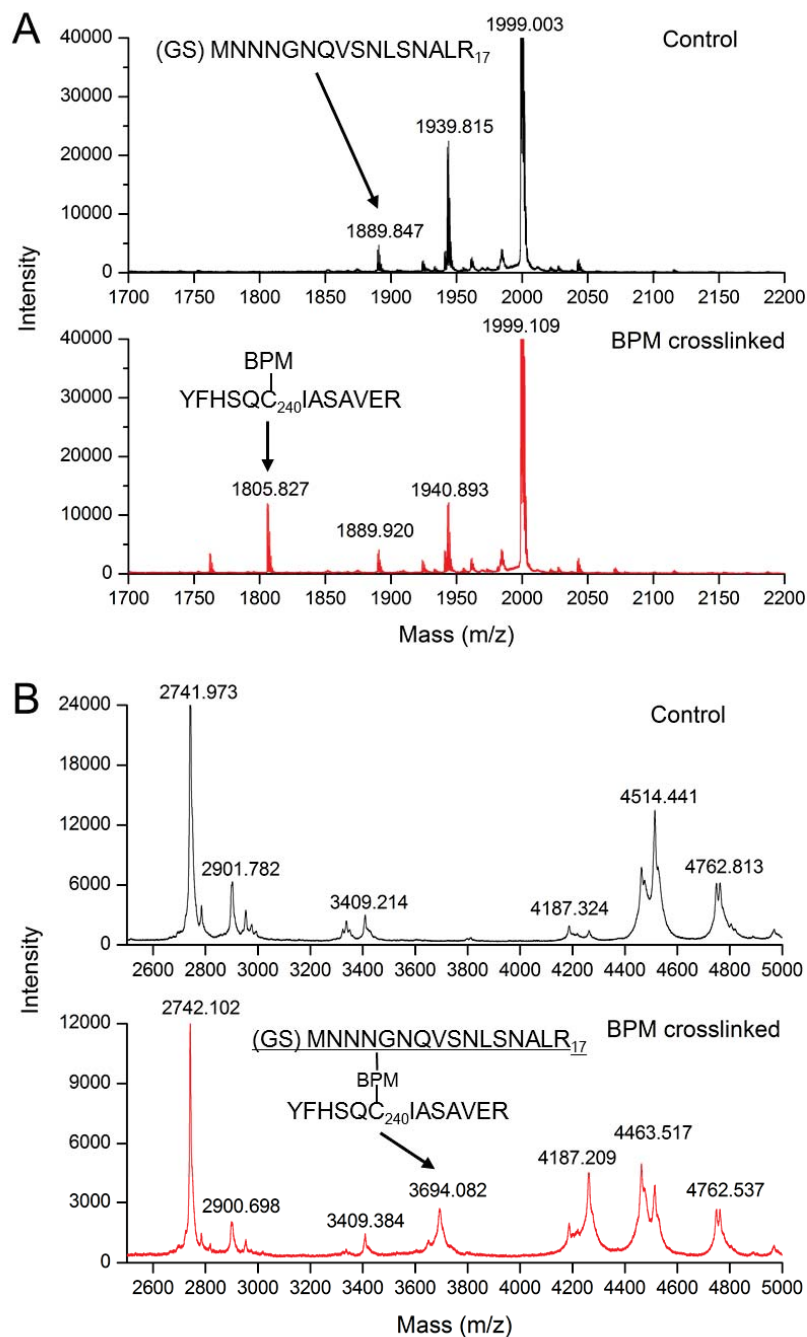
**Figure S1. Ure2 D204C subunit exchange reflected by inter-monomer FRET.** AF350/AF488 labeled Ure2 D204C dimer was incubated with different amounts of unlabeled D204C dimer, as indicated, overnight. No significant decrease of the FRET between monomers could be observed, indicating no significant degree of subunit exchange occurs between Ure2 dimers on an experimentally accessible timescale.



**Figure S2. Single-molecule FRET histogram of Ure2 WT/V9C-S52C.** The V9C/S52C heterodimer of Ure2 gives a high FRET peak at 0.8, which indicates that the Val9 and Ser52 residues of the prion domain are in close proximity to each other.



**Figure S3. FCS results for Cy3B/AF647 labeled and Cy3-only labeled heterodimers of Ure2.** (A) WT/V9C-K240C, (B) WT/V9C-F295C, (C) WT/S52C-K240C and (D) WT/S52C-F295C. Red and blue curves are the pseudo cross-correlation of Cy3B of the Cy3B/AF647 double-labeled protein and the Cy3B-only labeled protein respectively. Orange curves are the ratio between red and blue correlation curves that reflect the FRET fluctuation caused by fast dynamics of the chain.



**Figure S4. MALDI-TOF/TOF analysis of Ure2 K240C upon photocrosslinking.** (A) The peptide mass fingerprint of unlabeled Ure2-K240C (upper panel) and Ure2-K240C-BPM after photocrosslinking (bottom panel) in the molecular weight range of 1700-2200 Da. The peak at 1805.827 is the BPM labeled fragment (YFHSQC<sub>240</sub>IASAVER) and 1889.847 is the fragment of the N-terminal prion domain (residues 2-17 with two residues from the GS linker: GS/MNNNGNQVSNLSNALR). (B) The peptide mass fingerprint of unlabeled Ure2-K240C (upper panel) and Ure2-K240C-BPM after photocrosslinking (bottom panel) in the molecular weight range of 2500-5000 Da. A peak at 3694.082 was detected after photocrosslinking, which is the product of the K240C fragment crosslinked with the N-terminal fragment (residues 2-17).

**Table S1.** Mean FRET efficiency and width obtained from smFRET measurements

| Ure2 constructs | FRET efficiency | Half width |
|-----------------|-----------------|------------|
| V9C/E108C       | 0.60            | 0.16       |
| V9C/K240C       | 0.71            | 0.18       |
| V9C/F295C       | 0.58            | 0.16       |
| V9C/L351C (OP)  | 0.66            | 0.18       |
| S26C/E108C      | 0.65            | 0.16       |
| S26C/K240C      | 0.70            | 0.14       |
| S26C/F295C      | 0.58            | 0.17       |
| S26C/L351C (OP) | 0.65            | 0.18       |
| S52C/E108C      | 0.71            | 0.13       |
| S52C/K240C      | 0.80            | 0.14       |
| S52C/F295C      | 0.61            | 0.15       |
| S52C/F295C (OP) | 0.56            | 0.13       |

OP: The N-terminal and C-terminal mutations are in opposite chains.

**Table S2.** Fluorescence anisotropy data for AF555/AF647 dual labeled Ure2

| Ure2 Mutants    | AF647 | AF555 |
|-----------------|-------|-------|
| V9C/E108C       | 0.17  | 0.23  |
| V9C/K240C       | 0.22  | 0.30  |
| V9C/F295C       | 0.23  | 0.30  |
| V9C/L351C (OP)  | 0.22  | 0.33  |
| S26C/E108C      | 0.19  | 0.28  |
| S26C/K240C      | 0.19  | 0.30  |
| S26C/F295C      | 0.16  | 0.26  |
| S26C/L351C (OP) | 0.16  | 0.22  |
| S52C/E108C      | 0.20  | 0.28  |
| S52C/K240C      | 0.17  | 0.26  |
| S52C/F295C      | 0.16  | 0.26  |
| S52C/F295C (OP) | 0.22  | 0.33  |
| V9C/S52C        | 0.17  | 0.26  |

OP: The N-terminal and C-terminal mutations are in opposite chains.