Direct sensing and discrimination among Ubiquitin and Ubiquitin chains using solid-state nanopores

Supporting Information

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1. NANOPORE FABRICATION

Nanopore chips were fabricated on a double side polished 4" silicon wafer (350 µm thick) with (100) crystal orientation (Virginia Semiconductors). The wafer was coated with 0.5 µm of thermal oxide and 60 nm of low-stress chemical vapor deposition silicon nitride (LPCVD, SiN_x). Standard UV photolithography was used to pattern circular wells (1.5-2 µm) on one side of the wafer, through which the SiN_x was locally thinned to 10 nm using a controlled reactive ion etch (RIE) process. Freestanding membranes of SiN_x (20x20 μm^2) were created by defining pattern square openings on the reverse side of the wafer using photolithography and RIE processes, followed by anisotropic KOH etch through the silicon wafer, with the locally etched wells aligned to the etched freestanding SiN_x membranes.

Nanopores were fabricated in the thinned SiN_x regions using a high resolution aberration-corrected TEM (Titan 80-300 FEG-S/TEM, FEI), as previously reported (1). In the current studies we used nanopore with diameters between 3 to 4 nm. The membrane effective thickness was in the range of 7 nm – 10 nm.

2. NANOPORE SETUP, DATA ACQIZITION AND ANALYSIS

Before the experiments, pores were treated with heated piranha (3:1 H_2SO_4/H_2O_2), followed by extensive was with deionized, filtered Milli-Q water. Nanopore chips were then dried under vacuum, and quickly assembled in a PTFE cell sealed by painting with a quick-curing PDMS to reduce the excess capacitance. Electrolyte solution was then flowed using a syringe to hydrate both chambers (1.5 M KCl, 10 mM Tris, buffered to pH as indicated). The PTFE cell was inserted in a matching part (also PTFE), which created *cis* and *trans* small liquid chambers (~ 100 µl) and contact to two Ag/AgCl pellet electrodes. Temperature of the cell was actively regulated using a Thermoelectric device as explained in Wannun et al (2) and included a double Faraday shield electrical insulation.

Proteins were added to the *cis* chamber using a pipette tip and thoroughly mixed to the indicated final concentrations. Ub monomers, Lys48 dimers, Lys63 dimers, Lys48 pentamers and USP8 samples were purchased from BostonBiochem (Cambridge, MA). All experiments were carried out at darkness with T = 22.0 ± 0.2 °C. For calibration experiments we used 2.5 kbp double-stranded DNA (Fermentas NoLimits, Thermo Scientific), as explained below.

The nanopore current signal was acquired continuously and digitally at a rate of 4.17 MS/s using a Chimera Instruments (New York, NY) amplifier (3). Before the introduction of a protein sample, several seconds of current data were collected to verify the pores' stability by checking that no spikes are observed.

A custom LabVIEW translocation analysis software (2) was used to perform low-pass filtering and to record each translocation event that crosses a fixed threshold with its a pre-set padding time before and after each event (typically 1 ms). Three main parameters were

extracted from the data: the dwell time, t_D, which is the spike duration at a median level between the open pore level and the lowest point, the average ion current blockage level, i_b during its dwell-time, and the intervened waiting time between events, δ_t, from which protein capture rates can be extracted. The fractional blocking current, I_B, was calculated using: $I_B=i_b/i_o$, where i_o is the event's open pore current calculated by averaging the mean current in the padding data of each event.

Using I_B and t_D, a heatmap is created by a custom Matlab code: The I_B versus t_D space was divided into 2D bins (hence creating effective pixels) and the number of events in each pixel was evaluated and presented using the color scale (hotter color reflects higher number of events – see Figure S1). The heatmap is then smoothed for better visualization as described in (4). The same t_D and I_B data used to produced the heatmaps were further histogrammed using Igor Pro. For I_B we typically used bins of 0.01 and for t_D bins of 5 µs. Except otherwise noted Gaussian and exponential tail fits to the data showed in this study have P values < 0.00001 which means that the results are significant at significance level of 0.05.

3. CALIBRATION MESURMENTS USING DNA

To calibrate our system and data analysis routine we used 2.5 Kbp dsDNA molecules (Fisher Scientific, NoLimits), which provide an excellent reference. Nanopore measurements where performed using a ~4 nm pore in thinned down membrane and V=300 mV. Figure S1 (top) shows the resulting heatmap (N = 835) where a single cluster of events is clearly visible. Further histogram analysis is provided in Figure SI (bottom). From the fits we obtain: I_p = 0.745±0.050 and characteristic decay dwell time: 1.81±0.02 ms, with a excellent agreement with previous experiments under similar conditions (2) (5).

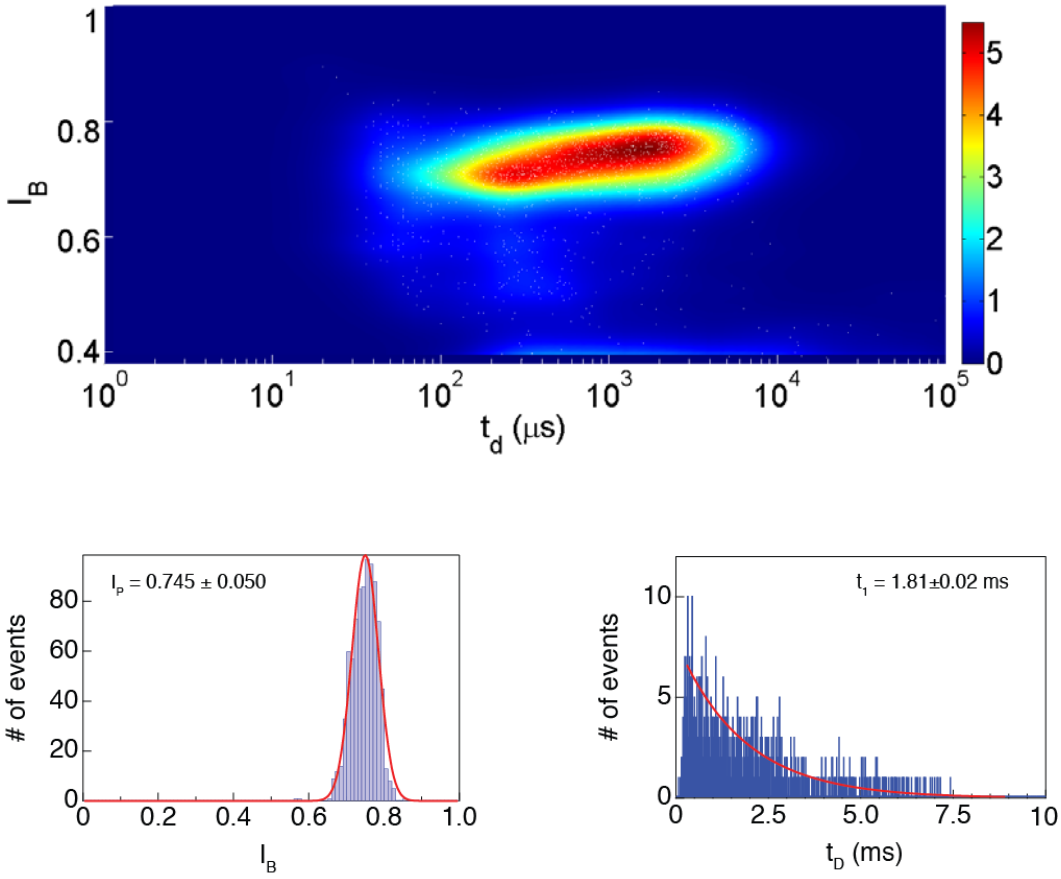

Figure S1. Top: A 2D heatmap of 2.5 kbp dsDNA translocations (N = 835 events) measured using a 4 nm nanopore in thin membrane (~ 8 nm) and V = 300 mV. Bottom: the corresponding fractional blockade current and dwell time histograms of the data shown in the heatmap, fitted by a Gaussian and exponential functions, respectively.

4. GEL CHARACTERIZATION OF PROTEINS USED IN THIS STUDY AND CATALYSIS QUANTIFICATION

Ubiquitin, diubiquitin (K48), diubiquitin (K63) and penta ubiquitin (0.75 microgram of each) were separated on 15% SDS-PAGE and stained with Coomassie.

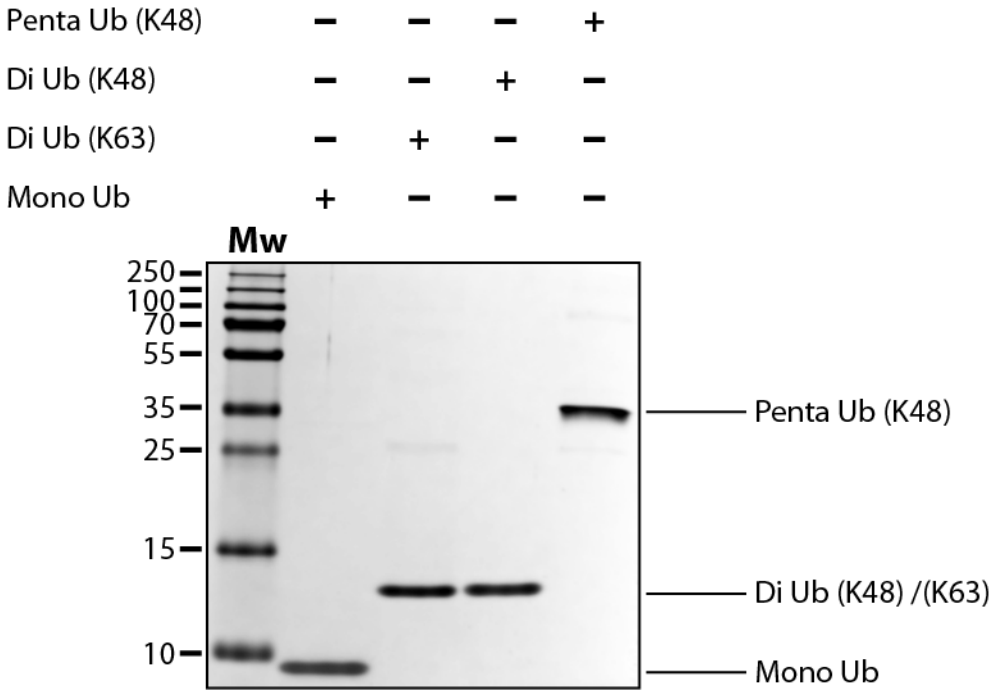

Figure S2. 15% SDS-PAGE stained with Coomassie of the proteins used in this study.

Explanation on the gel-based quantification for the catalysis experiment (additional details for Figure 6 in the main text): The samples from the deubiquitination assay were separated on 15% SDS-PAGE, stained with Coomassie and imaged with Gel Doc EZ (BioRad). The gel images were analyzed using the public domain software imageJ (NIH). The intensity of the bands corresponding to the substrate (Di Ub K48) and the product (Ub) was measured, and the ratio product/substrate was calculated. The values presented in the graph are an average ratio of three independent gels loaded with the same samples. The error bars represent the standard deviation from the mean for each time point.

5. ADDITIONAL DWELL TIME DISTRIBUTIONS (LINKAGE TYPE EXPERIMENT)

As mentioned in the article, the experiment constituted of translocating either one of the two di-Ubs (Lys63- and Lys48-linked di-Ub), or a mixed population (molar ratio 1:1) of the two proteins using the same nanopore. The shift in dwell times for K63 linked di Ub chains, vs. K48 linked di Ub chains is easily seen and quantified in the detailed dwell-time histograms (figure),

which give characteristic time constants of 95.9±9.3 µs and 34.2±1.2 µs for the K63 and K48 di-Ub, respectively.

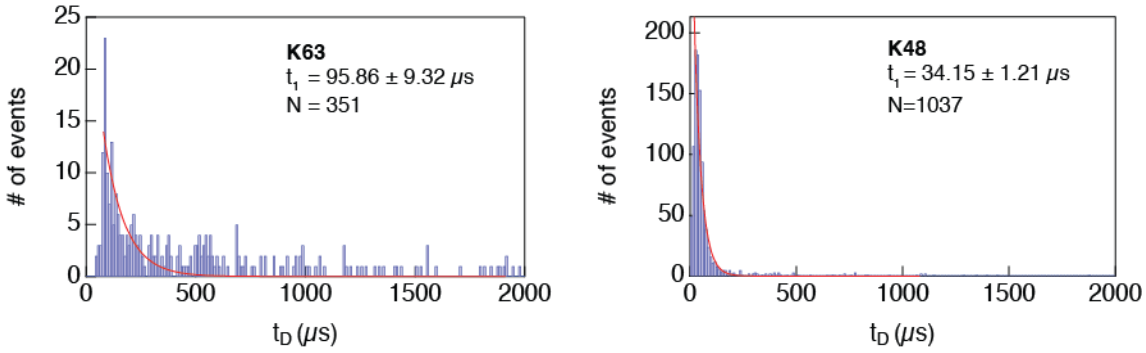

Figure S3. Dwell time histograms of the two kinds of di Ub proteins (linked via either K63 or K48), used in Figure 4 of the main paper. Red lines are exponential tail fits used to characterize the dwell times.

References

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