Supporting information

Quantifying nanomolar protein concentrations using designed DNA carriers and solid-state nanopores

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S1. Nanopore pulling programs and size distribution

Detailed nanopore fabrication methods are described previously. Two slightly different programs are used for biotin–streptavidin system and the digoxigenin-antidigoxigenin system with the pulling programs listed in Table S1. With lower heat, program 2 produces nanocapillaries of larger size as determined by the distribution of ionic current drop caused by double stranded DNA translocation shown in Figure S1. It is clear that the dsDNA current drops from program 2 are smaller indicating the nanopore size is larger.

| Program No. | Heat | Velocity | Delay | Pull |
|-------------|------|----------|-------|------|
| 1           | 480  | 25       | 170   | 200  |
| 2           | 460  | 25       | 170   | 200  |

Table S1. Parameters of capillary pulling programs.

Figure S1. Distributions of dsDNA translocation ionic current drop from nanopores produce by pulling program 1 (a) and 2 (b).

S2. Occupied translocation event threshold determination.

To differentiate the blank and occupied DNA carrier translocation event, a threshold factor \( \frac{I}{I_{DNA}} \) based on the dsDNA current amplitude was set to determine the protein binding peak, where \( I \) is the peak current drop amplitude in the searching window and \( I_{DNA} \) is the
current drop amplitude caused by dsDNA translocation. The fraction of occupied events decreases with the increasing threshold factor as shown in Figure S2.

For the blank carrier events (black), the fraction drops from the factor of 1 and reaches 0 at the factor of about 1.5. Since this control measurement should ideally show no occupied fraction, all events detected between the factor 1 and 1.5 are background noise or caused by DNA with complex folds or knots. As for the fully occupied events (red and green), the occupied fraction, which ideally should be around 1, starts to drop for factors of about 1.4 and larger, meaning some of the occupied events start to be lost. The optimal threshold factor varies among different nanopores: with smaller diameter nanopores and lower noise (green), higher threshold factor can be set without losing occupied detection while in the in red, smaller factor is needed. We choose the factor of 1.4 as a compromise to keep the occupied events for most of the nanopores and filter out most of background noise.

![Figure S2. Occupied fraction of DNA translocation events verses different threshold factors. All data are acquired with biotin – streptavidin system. The blank carrier control is shown in black and the fully occupied data sets from two nanopores are shown in red and green, in which the dsDNA current drop amplitude is -0.18 nA for the green and -0.13 nA for the red.](image)

**S3. Electrophoresis gel shift assay**

A 38 bp duplex with one oligonucleotide modified with 5’ three thymine and biotin or digoxigenin was titrated against the respective binding protein. The DNA duplexes were incubated with the target proteins at different concentration for 30 minutes at room temperature before gel loading. The incubations were carried out in 100 mM NaCl, 2 mM MgCl$_2$ buffered with 10 mM Tris-HCl in the same condition as for the nanopore measurements. 3% agarose gel and 1× TAE as running buffer was used at the voltage of 100 V for 1 H. The gel is then stained with Gel Red (Biotium) in all the assays.
Figure S3. EMSA images of the three anti-dig samples. Lane L is a DNA ladder reference (low molecular weight from 25 bp to 766 bp). Lanes 1-4 correspond to the DNA duplex incubated with antidiagoxigenin of different concentration ratio \( \left( \frac{c_{\text{Binding site}}}{c_{\text{Oligo}}} \right) \) of 0, 0.5, 1, and 2, respectively. The concentration of digoxigenin labeled DNA duplex was 80 nM for each lane.

As a reference to the nanopore measurements, EMSA is used to measure the differences among the three samples. The concentration ratio \( \left( \frac{c_{\text{Binding site}}}{c_{\text{Oligo}}} \right) \) is used here given the divalent nature of the antibody. Unlike the short DNA duplex which could bind to both sites of one antibody molecule, we did not observe a significant number of DNA carrier dimers at lower protein concentrations. This shows that for the 7.2 kbp DNA carrier, it is not possible for two carriers to be connected by one antibody. The weak band seen above the dominant oligo-antibody complexes band is probably an antibody with only one oligo attached.

**S4. Fluorescent polarization measurement**

A 16mer (16 thymine) DNA oligo modified with 6FAM (5') and digoxigenin (3') was used. 8nM DNA modified oligo was titrated against anti-dig of the concentrations from 1 nM to 120 nM. The incubations were carried out in 100 mM NaCl, 2 mM MgCl\(_2\), 10 mM Tris-HCl with and without 4 M LiCl. Setup runs of the FP assay were performed in 96-well plates and read on a CLARIOstar plate reader (BMG Labtech) with a 488/520 FP filter. The binding curve in high salt (4 M LiCl) buffer shows that the binding curve is not significantly affected by this high salt and that the binding fraction is still saturated at a few nM. This can be expected given that shape complementarity (and not electrostatic interaction) was explained as the primary reason for the anti-digoxigenin-digoxigenin affinity based on an analysis of crystal structures.\(^2\)

![Figure S4. Binding curve based on FP results. The data set measured in 100 mM NaCl, 2 mM MgCl\(_2\), 10 mM Tris-HCl, 4M LiCl buffer is in red and the one in 100 mM NaCl, 2 mM MgCl\(_2\), 10 mM Tris-HC LiCl is in black. The fitting of the black data set is shown as the grey dash curve with the fitted K\(_d\) of 3.5 nM.](image)

All fitting in this work are based on an equilibrium calculation of the simple binding model \(^3\):

\[
K_d = \frac{[P][L]}{[PL]}
\]
Where \([P], [L], [PL]\) are the concentration of target protein, ligand (carrier) and protein-carrier complex at the equilibrium state. \(K_d\) is the dissociation constant. Taking into account the concentration change towards reaching the equilibrium state, we can rewrite this equation as:

\[
K_d = \frac{([P_0] - [PL])([L_0] - [PL])}{[PL]}
\]

where \(P_0\) and \(L_0\) are the initial concentration of protein and ligand added. The binding fraction can be calculated as:

\[
\text{Binding fraction} = \frac{[PL]}{[P_0]} = \frac{[P] + [L] + K_d - \sqrt{([P] + [L] + K_d)^2 - 4[P][L]}}{2}
\]

All the fittings in this work were done with the model described here using OriginPro9.

**S5. Minimum sampling size discussion**

The translocation event recording is considered as a Binomial distribution. The confidence interval \(E\) is obtained

\[
E = p - z^* \frac{\sqrt{p(1-p)}}{\sqrt{N}}, p + z^* \frac{\sqrt{p(1-p)}}{\sqrt{N}}
\]

where \(p\) is the occupied fraction, \(z^*\) is the z value of the chosen confident level and \(N\) is the sample size. The minimum \(N\) can be estimated with

\[
N = \frac{z^*^2 p(1-p)}{\text{MOE}^2}
\]

where \(\text{MOE}\) is the margin of error or the accuracy we aim to maintain.\(^4\) When 1.96 is used as the \(z\) value for 95% confident level, the equation produces the minimum \(N\) for 10% and 1% accuracy shown in Figure S5. The event numbers needed for different occupied fraction to achieve certain margin of error are listed in table S2.

![Figure S5. Estimated minimum sample size for defined margin of error expected.](image)
| Occupied fraction | 1% accuracy | 5% accuracy | 10% accuracy |
|-------------------|-------------|-------------|--------------|
| 0                 | 0           | 0           | 0            |
| 0.1               | 3457.4      | 138.3       | 34.6         |
| 0.2               | 6146.6      | 245.9       | 61.5         |
| 0.3               | 8067.4      | 322.7       | 80.7         |
| 0.4               | 9219.8      | 368.8       | 92.2         |
| 0.5               | 9604        | 384.2       | 96           |
| 0.6               | 9219.8      | 368.8       | 92.2         |
| 0.7               | 8067.4      | 322.7       | 80.7         |
| 0.8               | 6146.6      | 245.9       | 61.5         |
| 0.9               | 3457.4      | 138.3       | 34.6         |
| 1                 | 0           | 0           | 0            |

Table S2. Estimated minimum sample size for defined margin of error expected.

S6. Translocation event rate comparison

The translocation rates of the proteins with and without DNA carrier under the same experimental conditions were compared. It should be noted that for protein only translocations that it is likely that some translocations are missed due to bandwidth restrictions. As shown in Figure S4(a), the normalized event rate of both streptavidin and anti-dig translocation alone increases as a function of the bias voltage applied which is consistent with the previous literature.

The event detection threshold was set as 50 pA for streptavidin and 70 pA for Anti-Dig. The event rate of unfolded DNA carrier is slightly higher: 0.08 ± 0.004 Hz /nM (Biotin carrier) and 0.10 ± 0.013 Hz /nM (Digoxigenin carrier) at 600 mV voltage compared to the protein translocation event rate of 0.052 ± 0.008 Hz /nM (streptavidin) and 0.057 ± 0.007 Hz /nM (anti-dig) under the same voltage of 600mV. In addition, the event rates of both biotin carrier and digoxigenin carrier are independent from the incubated protein concentration (Figure S4b).
Figure S6. (a) Normalized event rate of DNA carrier and proteins as a function of the voltage applied. The event rate of protein translocation (streptavidin in green and Anti-dig in red) increases with the bias voltages applied. (b) Normalized event rate of DNA carrier as a function of protein concentrations in the incubation. The event rates are independent from protein concentrations in both of the systems. Error bars are the standard error of mean among different nanopores under each experimental condition.

S7. Raw statistics of DNA carrier translocation events after incubation with target proteins

Detailed statistics of translocation event numbers and nanopore information is listed in Table S2 (biotin-streptavidin system) and Table S3 (digoxyigenin system).

| Concentration ratio (protein: carrier) | Nanopores No. | dsDNA current drop amplitude (nA) | All carrier No. | Unfolded carrier No. | Occupied carrier No. | Occupied fraction |
|----------------------------------------|---------------|-----------------------------------|----------------|----------------------|---------------------|------------------|
| 0 N=434                                | 01            | -0.144                            | 352            | 67                   | 4                   | 0.060            |
|                                        | 02            | -0.152                            | 536            | 76                   | 6                   | 0.079            |
|                                        | 03            | -0.145                            | 297            | 69                   | 3                   | 0.043            |
|                                        | 04            | -0.144                            | 170            | 25                   | 1                   | 0.040            |
|                                        | 05            | -0.146                            | 242            | 41                   | 6                   | 0.14             |
|                                        | 06            | -0.138                            | 186            | 29                   | 2                   | 0.069            |
|                                        | 07            | -0.159                            | 75             | 22                   | 1                   | 0.045            |
|                                        | 08            | -0.169                            | 577            | 11                   | 11                  | 0.14             |
|                                        | 09            | -0.159                            | 204            | 24                   | 0                   | 0                |
| 0.38 N=479                             | 10            | -0.161                            | 681            | 68                   | 31                  | 0.46             |
|                                        | 11            | -0.171                            | 1147           | 153                  | 38                  | 0.25             |
|                                        | 12            | -0.153                            | 820            | 161                  | 27                  | 0.17             |
|                                        | 13            | -0.115                            | 244            | 28                   | 5                   | 0.18             |
|                                        | 14            | -0.159                            | 351            | 37                   | 7                   | 0.19             |
|                                        | 15            | -0.139                            | 201            | 32                   | 10                  | 0.31             |
| 0.94 N=2044                             | 16            | -0.179                            | 793            | 90                   | 74                  | 0.82             |
|                                        | 17            | -0.145                            | 387            | 40                   | 29                  | 0.72             |
|                                        | 18            | -0.133                            | 236            | 21                   | 18                  | 0.86             |
|                                        | 19            | -0.137                            | 187            | 21                   | 17                  | 0.81             |
|                                        | 20            | -0.131                            | 782            | 125                  | 107                 | 0.86             |
|                                        | 21            | -0.133                            | 400            | 63                   | 49                  | 0.78             |
|                                        | 22            | -0.162                            | 605            | 79                   | 60                  | 0.76             |
|                                        | 23            | -0.187                            | 732            | 112                  | 43                  | 0.38             |
|                                        | 24            | -0.172                            | 492            | 99                   | 40                  | 0.40             |
|                                        | 25            | -0.160                            | 313            | 35                   | 12                  | 0.34             |
|                                        | 26            | -0.164                            | 1442           | 197                  | 90                  | 0.46             |
|                                        | 27            | -0.170                            | 249            | 24                   | 19                  | 0.79             |
|                                        | 28            | -0.155                            | 277            | 50                   | 20                  | 0.40             |
|                                        | 29            | -0.142                            | 536            | 72                   | 34                  | 0.47             |
|                                        | 30            | -0.153                            | 1470           | 181                  | 63                  | 0.34             |
Table S3. Raw statistics of biotin carrier translocation event and the nanocapillaries used.

| Concentration ratio (protein/carrier) | Nanopore No. | dsDNA current drop amplitude (nA) | All carrier No. | Unfolded carrier No. | Occupied carrier No. | Occupied fraction |
|--------------------------------------|--------------|-----------------------------------|----------------|---------------------|---------------------|------------------|
| 0 N=237                              |              |                                   |                |                     |                     |                  |
| 1                                    | -0.127       | 124                               | 18             | 1                   | 0.056               |                  |
| 2                                    | -0.172       | 142                               | 19             | 2                   | 0.11                |                  |
| 3                                    | -0.122       | 87                                | 21             | 5                   | 0.24                |                  |
| 4                                    | -0.124       | 263                               | 36             | 4                   | 0.11                |                  |
| 5                                    | -0.138       | 78                                | 24             | 3                   | 0.12                |                  |
| 6                                    | -0.168       | 114                               | 31             | 3                   | 0.10                |                  |
| 7                                    | -0.144       | 410                               | 88             | 12                  | 0.14                |                  |
| Sample A                             |              |                                   |                |                     |                     |                  |
| 0.42 N=141                           |              |                                   |                |                     |                     |                  |
| 8                                    | -0.159       | 104                               | 13             | 2                   | 0.15                |                  |
| 9                                    | -0.126       | 98                                | 26             | 6                   | 0.23                |                  |
| 10                                   | -0.134       | 316                               | 59             | 12                  | 0.20                |                  |
| 11                                   | -0.152       | 289                               | 43             | 6                   | 0.14                |                  |
| 1.26 N=159                           |              |                                   |                |                     |                     |                  |
| 12                                   | -0.124       | 596                               | 40             | 14                  | 0.28                |                  |
| 13                                   | -0.112       | 326                               | 31             | 12                  | 0.39                |                  |
| 14                                   | -0.111       | 128                               | 22             | 6                   | 0.27                |                  |
| 15                                   | -0.118       | 292                               | 38             | 8                   | 0.27                |                  |
| 16                                   | -0.119       | 183                               | 26             | 10                  | 0.38                |                  |
| 2.09 N=145                           |              |                                   |                |                     |                     |                  |
| 17                                   | -0.125       | 289                               | 31             | 19                  | 0.61                |                  |
| 18                                   | -0.146       | 538                               | 35             | 21                  | 0.60                |                  |
Table S4. Raw statistics of digoxigenin carrier translocation event and the nanocapillaries used.

Reference:
1. (a) Steinbock, L. J.; Otto, O.; Chimerel, C.; Gornall, J.; Keyser, U. F., Detecting DNA folding with nanocapillaries. Nano letters 2010, 10 (7), 2493-2497; (b) Bell, N. A.; Thacker, V. V.; Hernández-Ainsa, S.; Fuentes-Perez, M. E.; Moreno-Herrero, F.; Liedl, T.; Keyser, U. F., Multiplexed ionic current sensing with glass nanopores. Lab Chip 2013, 13 (10), 1859-1862.
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