# Supporting Information

## Label-Free, Single Protein Detection on a Near Infrared Fluorescent Single-Walled Carbon Nanotube/Protein Microarray Fabricated by Cell-Free Synthesis

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## Materials

ATP, GTP, UTP, CTP, creatine phosphate, creatine kinase and the *E. coli* total tRNA mixture were purchased from Roche Applied Science (Indianapolis, IN). All the other reagents for cell-free system were purchased from Sigma (St. Louis, MO). The *E. coli* strain BL21-Star$^{TM}$(DE3) was purchased from Invitrogen (Carlsbad, CA). Acetic acid, chitosan, succinic anhydride, *N*,*N*-diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbo diimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS), *Nα*,*Nα*-bis(carboxymethyl)-L-lysine (NTA), nickel (II) sulfate and glutaraldehyde solution (50 vol%) were purchased from Sigma-Aldrich. The patterned glass functionalized with poly-L-lysine was purchased from Tekdon Inc. Single-walled carbon nanotubes were purchased from Nano-C Inc.

## Preparation of cell extract

The S30 cell extracts were prepared from *E. coli* strain BL21 Star$^{TM}$ (DE3) (Novagen, Madison, WI) according to the method reported elsewhere[1-3]. The cells were grown at 37 °C in 4 L of 2xYT medium with agitation and aeration. When the cell density ($OD_{600}$) reached 0.5, isopropyl-thiogalactopyranoside (IPTG, 0.5 mM) was added to the culture media to induce T7 RNA polymerase expression. The cells were harvested when the $OD_{600}$ reached 4.0 and cells were washed three times by suspending them in 20 mL of S30 buffer per gram of wet cells and then centrifuged. S30 buffer contained 10 mM Tris–acetate buffer (pH 8.2), 14 mM magnesium acetate, 60 mM potassium glutamate, and 1 mM dithiothreitol (DTT) containing 0.05% (v/v) 2-mercaptoethanol (2-ME). The resulting cell pellets were weighed and then suspended with 12.7 mL of S30 buffer without 2-ME and disrupted in a French press cell (Thermo Scientific) at a constant pressure of 20,000 psi. The crude lysate was then centrifuged at 12,000 RCF for 10 min,

and the recovered supernatant was briefly incubated at 37 °C. The resulting extract was divided into small aliquots and stored at −80 °C before use for cell-free expression.

## Gene preparation

Target ORFs were amplified using primers P1s and P2s. The first PCR products were purified by gel extraction and used for the second-round PCR, in which the full expression templates were synthesized using the P3 and P4 (**Supporting Fig. 3**). Primer sequences are listed in **Supporting Table 1**. After amplification, the final PCR products were used in cell-free protein synthesis reaction without purification[3].

## Spectroscopy and microscopy

Near-infrared photoluminescence spectra were acquired using 785 nm excitation and an Acton SP-500 spectrograph coupled to a Princeton instruments OMA V InGaAs detector. Absorption measurements were taken with a Shimadzu UV-3101 PC UV-VIS-NIR scanning spectrophotometer.

## Microscopy and Data Analysis for Single Molecule Detection of Protein

After SWNT/CHI film was functionalized with Ni-NTA, the nIR fluorescence response of SWNT to His-tag EGFP as a capture protein and anti-His-tag antibody as a analyte was imaged and monitored in real-time for 25 min through a 100x TIRF objective using an inverted microscope (Carl Zeiss, Axiovert 200) attached with a 2D InGaAs array (Princeton Instruments OMA 2D) with a 658 nm laser excitation (LDM-OPT-A6-13, Newport Corp., 35 mW). The nIR fluorescence response movies were acquired at 1.0 sec/frame using the WinSpec data acquisition

program (Princeton Instruments). Before the experiment, a control movie was taken for 25 min to ensure a stable baseline. A 20 μL portion of His-tag EGFP (final concentration: 100 μg/ml) was added into the SWNT/CHI film bearing Ni-NTA 5 min after taking the movie in PBS (pH 7.4, 10 mM) without addition of proteins, and the fluorescence response was further imaged and monitored for 10 min. Then, a 10 μL portion of anti-His-tag antibody (final concentration: 23 μg/ml) was added into EGFP-immobilized SWNT/CHI film, and the fluorescence response was monitored for 10 min. The fluorescence response within a 2 × 2 pixel spatial binning region in the movie images was examined, and the analysis algorithm is similar to that reported before[4]. The four-pixel area in the image corresponds to a 600 × 600 $nm^2$ region in the real sample, representing the PL from a single SWNT, which is determined by the diffraction limit in the nIR range[5]. Hidden Markov Modeling (HMM) is employed to correlate the rate constants of immobilization of His-tag EGFP to Ni-NTA and binding of anti-His-tag antibody to EGFP on the SWNT/CHI film.

## Sensitivity of SWNT/CHI microarray to protein detection

A 40 μL of His-tag EGFP (initial concentration; 5 mg/mL) was added to SWNT/CHI microarray bearing $Ni^{2+}$ in 1 mL of PBS (pH 7.4, 10 mM), and then incubated for 30 min at room temperature. After washing out the unbound His-tag EGFP with PBS, the SWNT/CHI bearing His-tag EGFP in 1 mL of PBS was placed on the nIR fluorescence microscope (100x objective, 658 nm laser) to focus on the array of single SWNT. The 15 μL of the serially-diluted analyte protein from 100 nM to 10 pM (anti-His-tag antibody, initial concentration; 1 mg/mL) was added to the SWNT/CHI microarray in 1 mL of PBS 100 sec after taking the fluorescence image of

SWNT without addition of the analyte protein. The fluorescence response was further measured for 20 min at room temperature, and the collected movie was then analyzed as described above.

## Control experiments

Various control experiments including negative and positive controls were performed (**Supporting Fig. 4**). Each reagent as positive or negative controls was added to each array wells in a humidified chamber at 37ºC and incubated for 2 h. The fluorescence response of each SWNT/CHI well was measured. The arrays were washed three times for 10 min each with PBS buffer (pH 7.4, 10 mM) at RT and then PL spectra were taken. As shown in Supporting Figure 4, all reagents and buffers that are used as components in cell-free reaction mixture were tested and DNA expression templates including no ribosome binding site and no His-tag were also tested as negative control. On the other hand, His-tag coding expression templates, Fos, Jun, EGFP were tested as positive controls. Only His-tag containing proteins cause fluorescence decrease. Hence, the capture proteins are effectively and selectively expressed by cell-free synthesis on each spot of the array and no fluorescence decrease induced from other reagents in a cell-free protein expression system was detected.

## References for Supporting Information

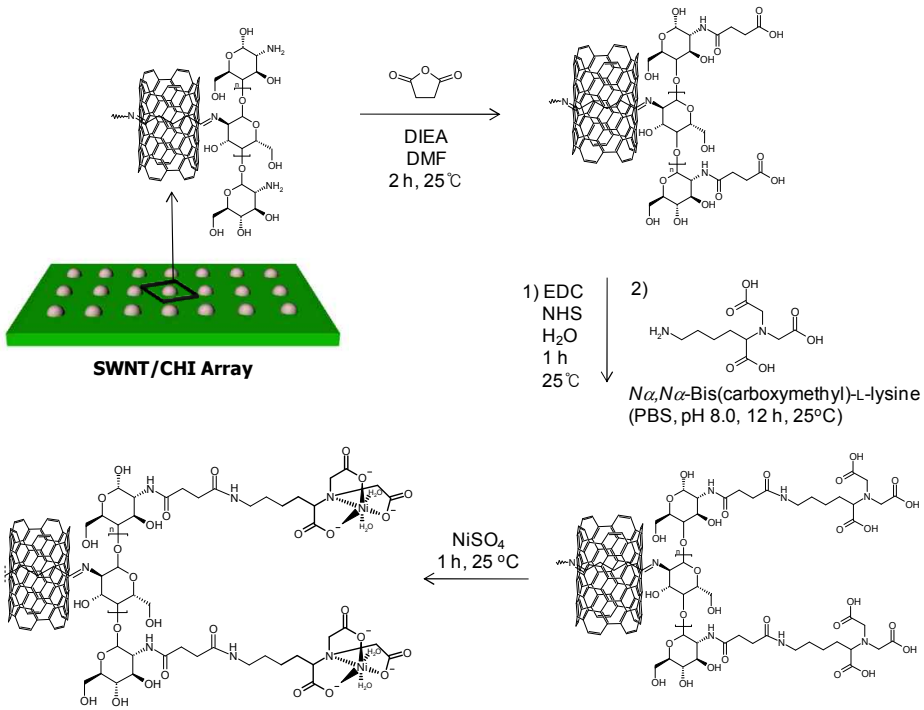

1. Kim, T.W., Oh, I.S., Ahn, J.H., Choi, C.Y. & Kim, D.M. Cell-free synthesis and in situ isolation of recombinant proteins. *Protein Expres Purif* **45**, 249-254 (2006).
2. Ahn, J.H. et al. Cell-free synthesis of recombinant proteins from PCR-amplified genes at a comparable productivity to that of plasmid-based reactions. *Biochem Bioph Res Co* **338**, 1346-1352 (2005).
3. Ahn, J.H., Keum, J.W. & Kim, D.M. High-throughput, combinatorial engineering of initial codons for tunable expression of recombinant proteins. *J Proteome Res* **7**, 2107-2113 (2008).
4. Jin, H., Heller, D.A., Kim, J.H. & Strano, M.S. Stochastic Analysis of Stepwise Fluorescence Quenching Reactions on Single-Walled Carbon Nanotubes: Single Molecule Sensors. *Nano Letters* **8**, 4299-4304 (2008).

5. Cognet, L. et al. Stepwise quenching of exciton fluorescence in carbon nanotubes by single-molecule reactions. *Science* **316**, 1465-1468 (2007).

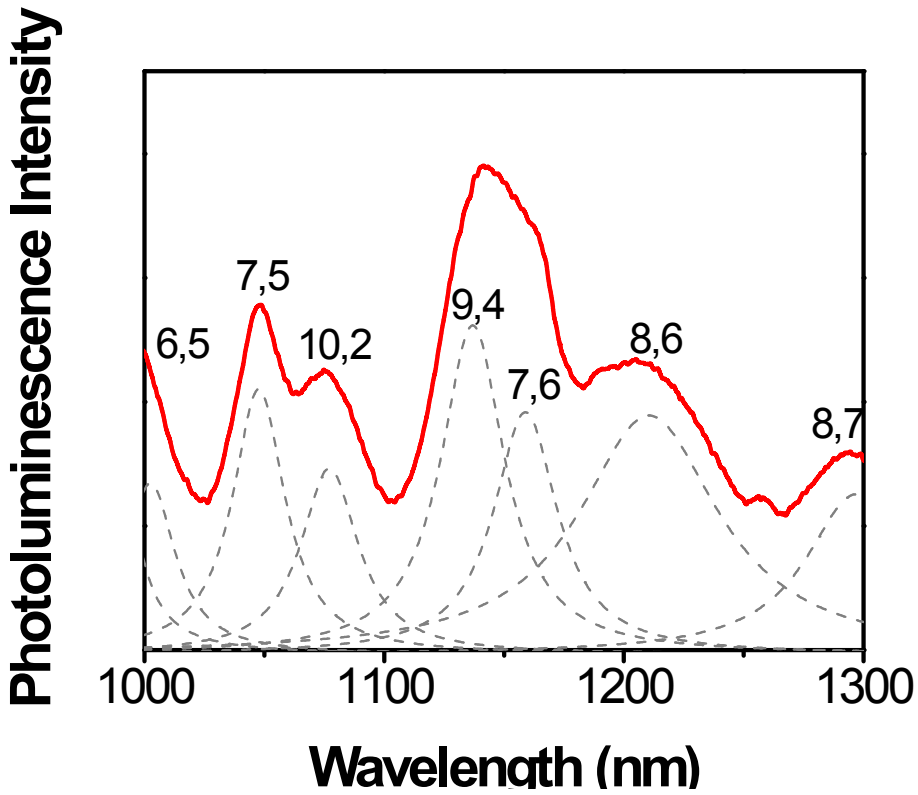
**Supporting Table 1**. Oligonucleotide primers used in this study.

| Protein[a] | P1 | P2 |
|---|---|---|
| Ack (P0A6A3) | aagaaggagatatacatatgtcgagtaagttagtactggt | ttaatgatgatgatgatgggcagtcaggcggctcgcgt |
| Dnak (P0A6Y8) | aagaaggagatatacatatgggtaaaataattggtatcga | ttaatgatgatgatgatgtttttgtctttgacttctt |
| FbaA (P0AB71) | aagaaggagatatacatatgtctaagatttttgatttcgt | ttaatgatgatgatgatgcagaacgtcgatcgcgttca |
| GlyA (P0A825) | aagaaggagatatacatatgttaaagcgtgaaatgaacat | ttaatgatgatgatgatgtgcgtaaaccgggtaacgtg |
| LpdA (P0A9P0) | aagaaggagatatacatatgagtactgaaatcaaaactca | ttaatgatgatgatgatgcttcttcttcgctttcgggt |
| RpoA (P0A7Z4) | aagaaggagatatacatatgcagggttctgtgacagagtt | ttaatgatgatgatgatgctcgtcagcgatgcttgccg |
| RplB (P60422) | aagaaggagatatacatatggcagttgttaaatgtaaacc | ttaatgatgatgatgatgtttgctacggcgacgtacga |
| RpsB (P0A7V0) | aagaaggagatatacatatggcaactgtttccatgcgcga | ttaatgatgatgatgatgctcagcttctacgaagcttt |
| Tsf (P0A6P1) | aagaaggagatatacatatggctgaaattaccgcatccct | ttaatgatgatgatgatgagactgcttggacatcgcag |
| Ada (P06134) | aagaaggagatatacatatgaaaaaagccacatgcttaac | ttaatgatgatgatgatgcctctcctcattttcagctt |
| Cdd (P0ABF6) | aagaaggagatatacatatgcatccacgttttcaaaccgc | ttaatgatgatgatgatgagcgagaagcactcggtcga |
| CDK4 (P11802) | aagaaggagatatacatatggctacctctcgatatga | ttaatgatgatgatgatgcaactccggattaccttcat<br>atgatgatgatgatgttacaactccggattaccttcat (noHis) |
| p16 (P42771) | aagaaggagatatacatatggtgcgcaggttcttggt | ttaatgatgatgatgatgcaagccaggtccacgggcag<br>atgatgatgatgatgttacaagccaggtccacgggcag (noHis) |
| Jun (P05412) | aagaaggagatatacatatgactgcaaagatggaaac | ttaatgatgatgatgatggtcaaatgtttgcaactgct<br>atgatgatgatgatgttagtcaaatgtttgcaactgct (noHis) |
| Fos (P01100) | aagaaggagatatacatatgatgttctcgggcttcaa | ttaatgatgatgatgatgcaacagggccagcagcgtgg<br>atgatgatgatgatgttacaacagggccagcagcgtgg (noHis) |
| P3: Mega-F | TCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAAT<br>AATTTTGTTTAACTTTAAGAAGGAGATATACATATG | |
| P4: Mega-R | CAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGCTCGAGAAGCTTG<br>TCGACGAATTCGGATCCTTAATGATGATGATGATGATG | |

[a]ExPASy accession ID in parenthesis

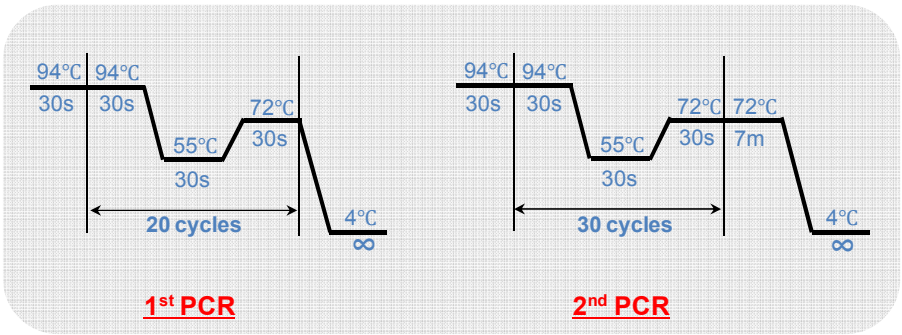



**Supporting Figure S1**. Functionalization of SWNT/CHI array with Ni-NTA.

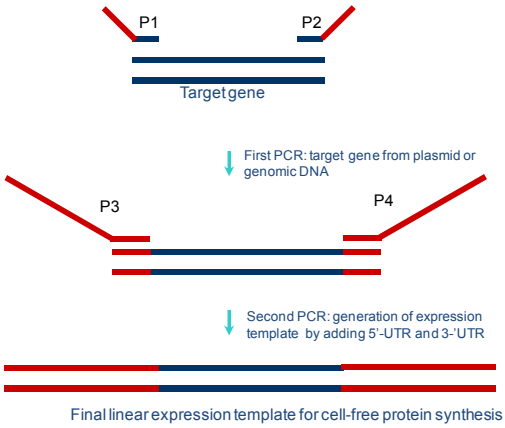



**Supporting Figure S2**. Deconvolution of nIR fluorescence of SWNTs in response to His-tag protein (Ack) in SWNT/CHI array.

a




b




P1: 5'-AAGAAGGAGATATACATATG + (20 nt sequence for target gene)-3'
P2: 5'-TTAATGATGATGATGATGATG + (complementary 20 nt sequence for target gene)-3'
P3: 5'-
TCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGT
TTAACTTTAAGAAGGAGATATACATATG-3'
P4: 5'-
CAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGCTCGAGAAGCTTGTCGACGA
ATTCGGATCCTTAATGATGATGATGATGATG-3'

c

T7 promoter
TCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACA

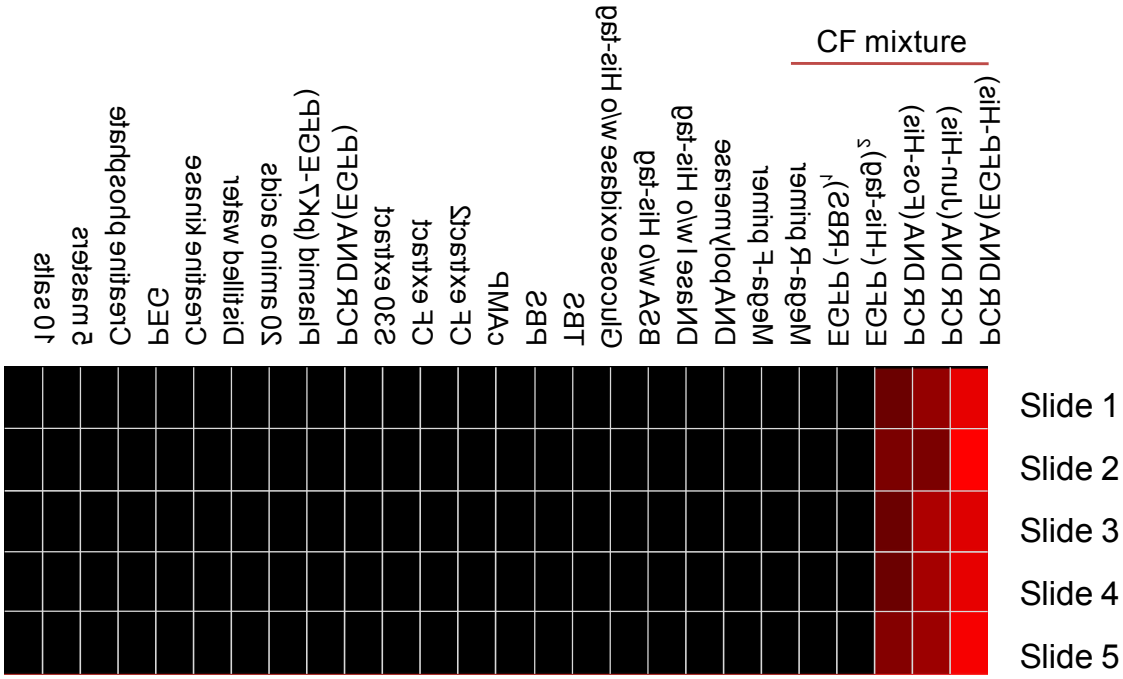
RBS
ACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAG

Start codon
ATATACAT**ATG** + **gene of interest** + CATCATCATCATCATCAT (6x Histag)

Stop codon
**TAA**GGATCCGAATTCGTCGACAAGCTTCTCGAGCTAGCATAAC

T7 terminator
CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG

**Supporting Figure S3**. Generation of expression template by PCR. (a) Conditions for two-step PCR. (b) Two-step PCR, the gene of interest is amplified in the primary PCR via specific primers that are introducing an overlap region (primers P1 and P2). In a second PCR, the outer primers P3 and P4 bind to the overlapping region and add all regulatory elements necessary for transcription and translation. (c) Nucleotide sequence of amplified linear expression template for cell-free protein expression.

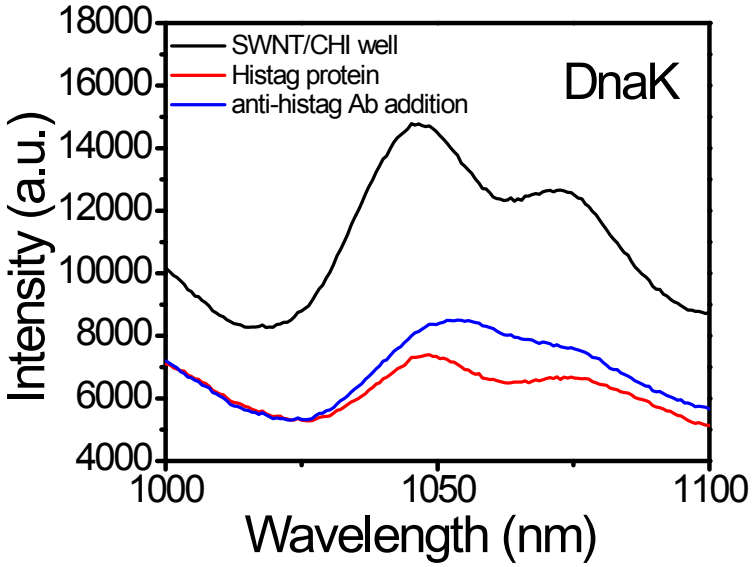



[1] EGFP(-RBS): only transcription
TCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTA
AGUUCCUCATATACCATG-

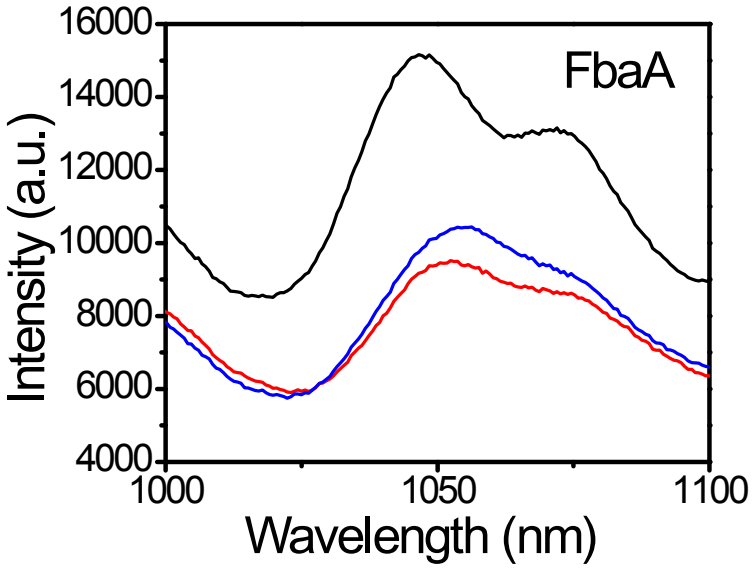
[2] EGFP(-stop): no His-tag
TAACATCATCATCATCATTAAGGATCCGAATTCGTCGACAAGCTTCTCGAGCTAGCATAACCCCTTGGGGCCTC
TAAACGGGTCTTGAGGGGTTTTTTG

**Supporting Figure S4**. Investigation of reproducibility on SWNT/CHI protein array with various control experiments. All reagents and buffers used in cell-free reaction mixture were tested and DNA expression templates including no ribosome binding site and no His-tag were also tested as negative control. Each reagent indicated above as positive or negative controls was added to each array wells in a humidified chamber at 37ºC and incubated for 2 h. The fluorescence response of each SWNT/CHI well was measured. The arrays were washed three times for 10 min each with PBS buffer (pH 7.4, 100 mM) at RT and then PL spectra were taken. 10 salts (90 mM of potassium glutamate, 80 mM of ammonium acetate, 12 mM of magnesium acetate); 5 masters (57 mM of Hepes-KOH (pH 8.2), 1.2 mM of ATP, 0.85 mM each of CTP, GTP and UTP, 0.64 mM of cAMP, 34 μg/ml of L-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), 1 mM each of 20 amino acids, 0.17 mg/ml of *E. coli* total tRNA mixture (from strain MRE600)); CF extract (cell-free reaction mixture w/o expressible DNAs).

a


SWNT/CHI well
Histag protein
anti-histag Ab addition
DnaK
Intensity (a.u.)
Wavelength (nm)
18000
16000
14000
12000
10000
8000
6000
4000
1000
1050
1100

b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Intensity (a.u.)
Wavelength (nm)
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14000
12000
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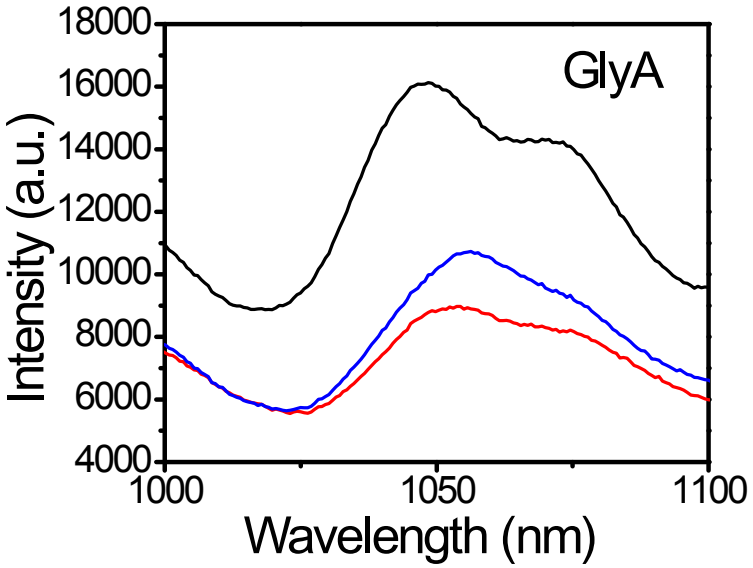
8000
6000
4000
1000
1050
1100

c


GlyA
Intensity (a.u.)
Wavelength (nm)
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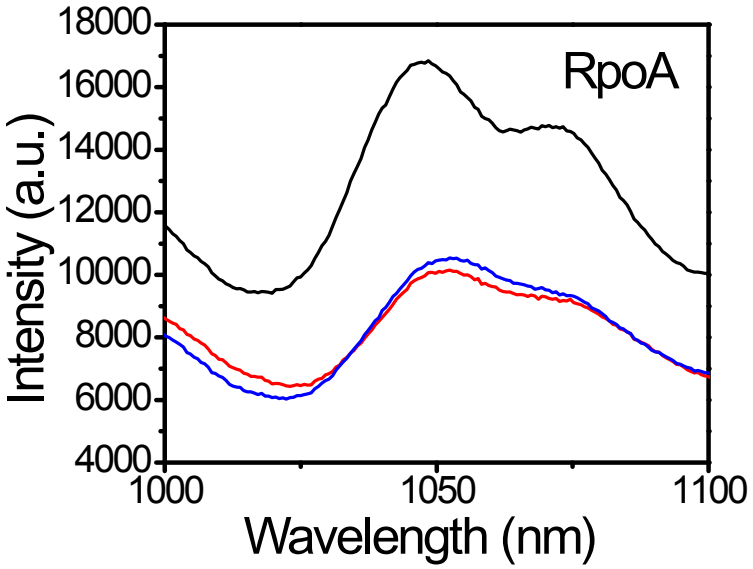
16000
14000
12000
10000
8000
6000
4000
1000
1050
1100

d


RpoA
Intensity (a.u.)
Wavelength (nm)
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14000
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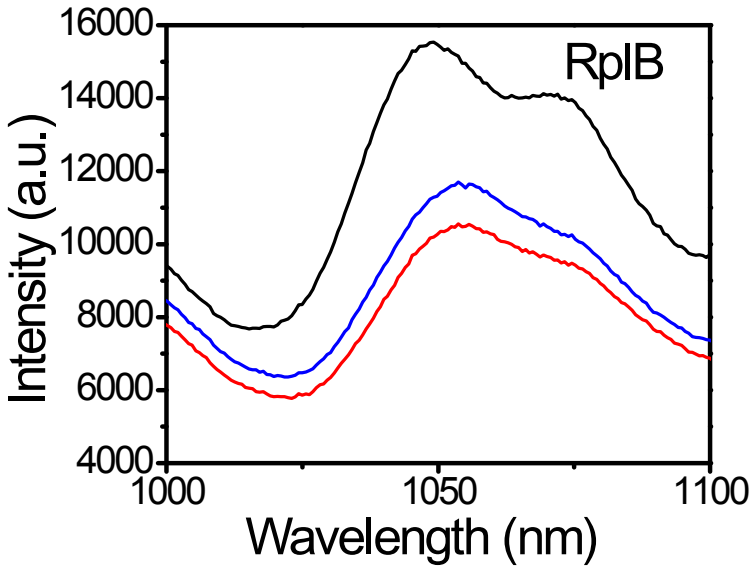
10000
8000
6000
4000
1000
1050
1100

e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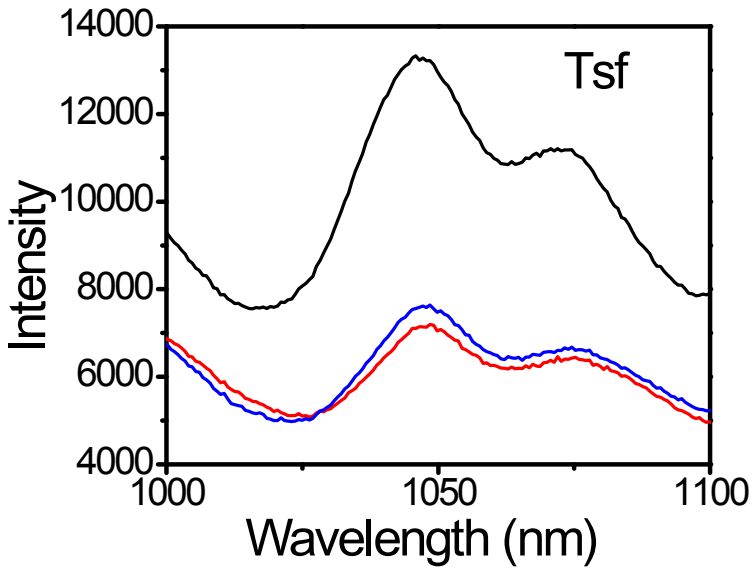
1100
Wavelength (nm)

f


Tsf
Intensity
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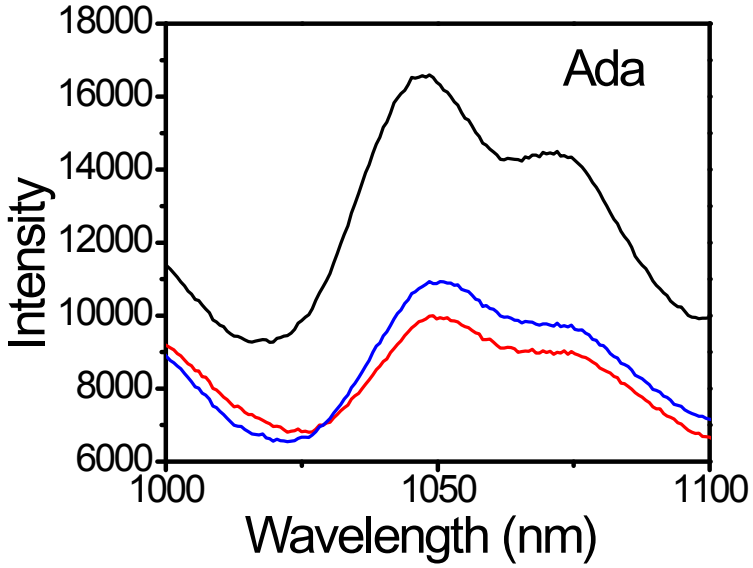
Wavelength (nm)

g


Ada
Intensity
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12000
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1000
1050
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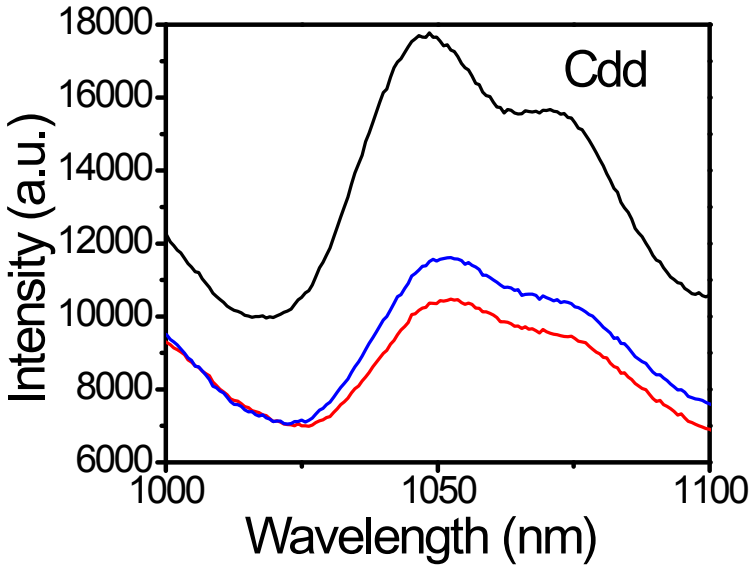
Wavelength (nm)

h


Cdd
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1000
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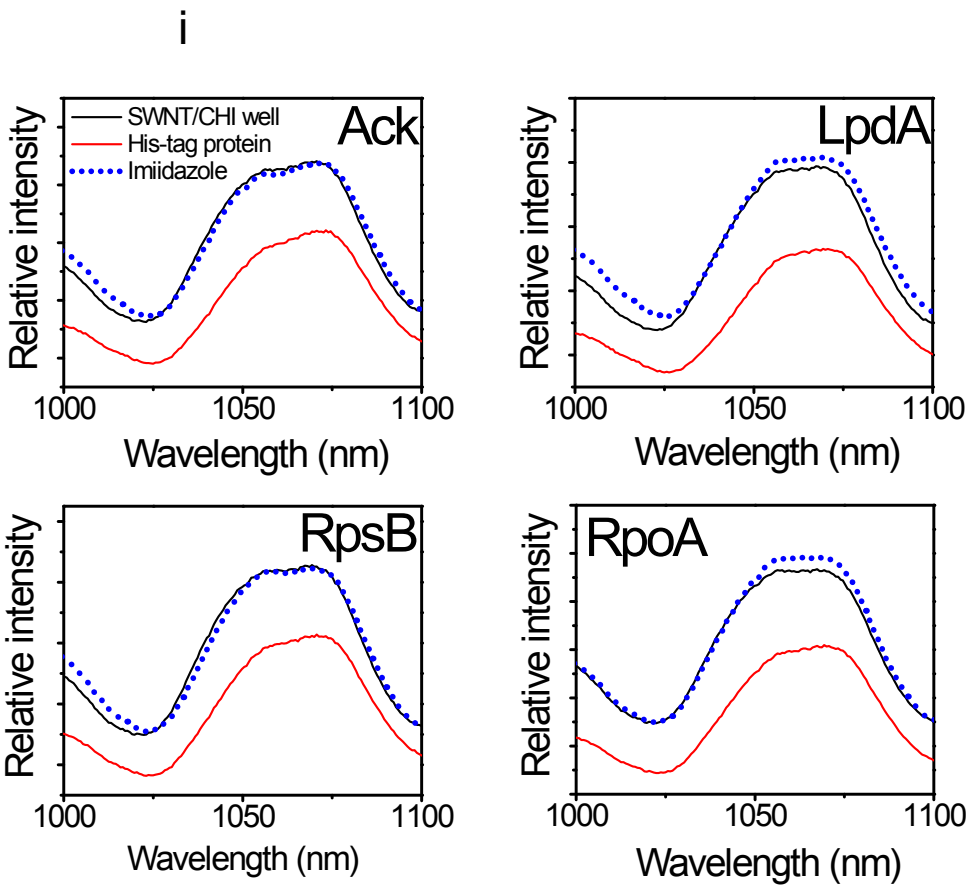
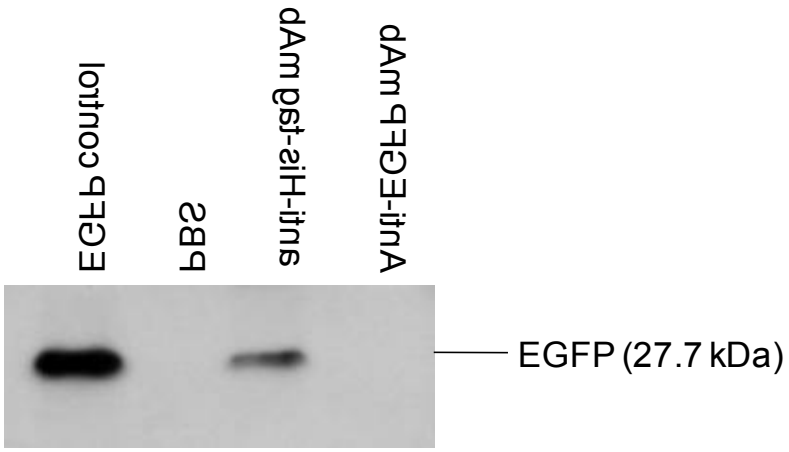
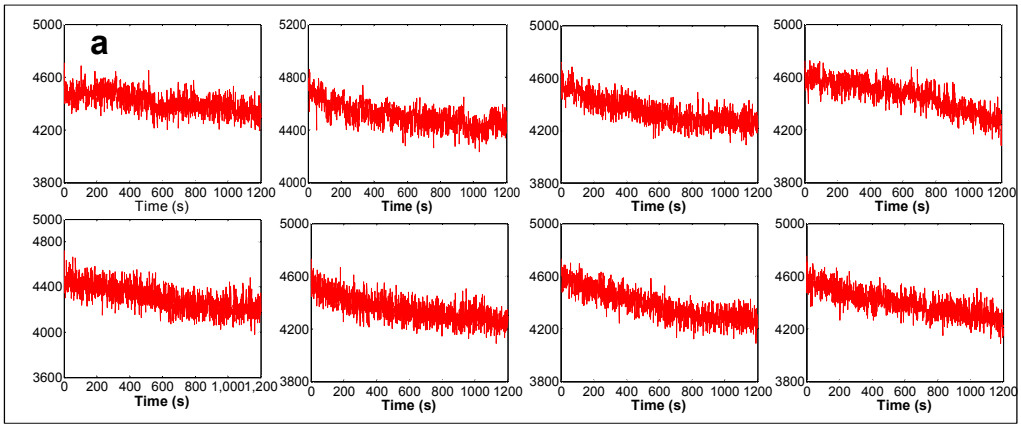
Wavelength (nm)

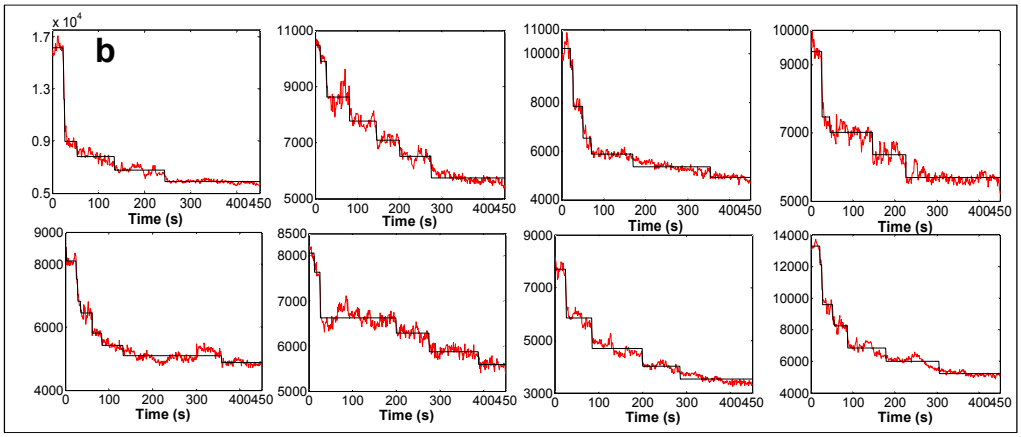


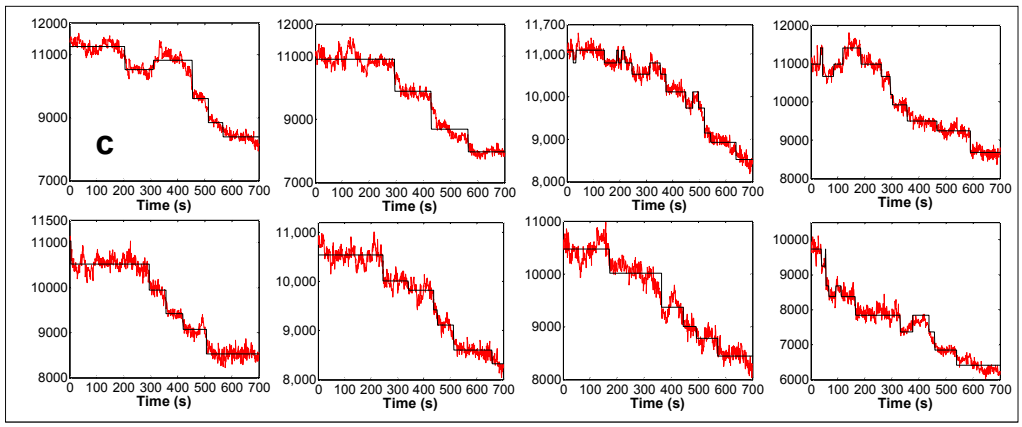

**Supporting Figure S5**. Selective recognition of protein-protein interactions on SWNT/CHI array. nIR fluorescence response of SWNT to protein immobilization and interaction with anti-His-tag antibody, showing fluorescence diminution for immobilization of His-tag proteins and increase for binding anti-His-tag antibody (a-h). (i) Imidazole addition: demonstration of protein binding reversibility on the SWNT/CHI array; quenched fluorescence is completely restored after treatment with an imidazole elution buffer (250 mM).

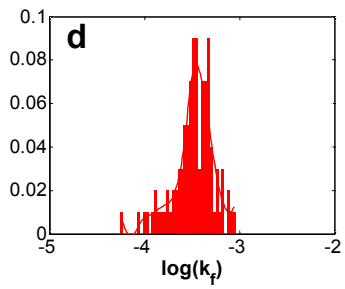



**Supporting Figure S6**. Western blot analysis. After incubation of His-tagged EGFP, each well was treated with monoclonal anti-His-tag antibody and anti-EGFP antibody for 30 min, and the eluted fractions were analyzed by 15% SDS-PAGE/Western blot using the monoclonal anti-His-tag antibody to detect EGFP protein.

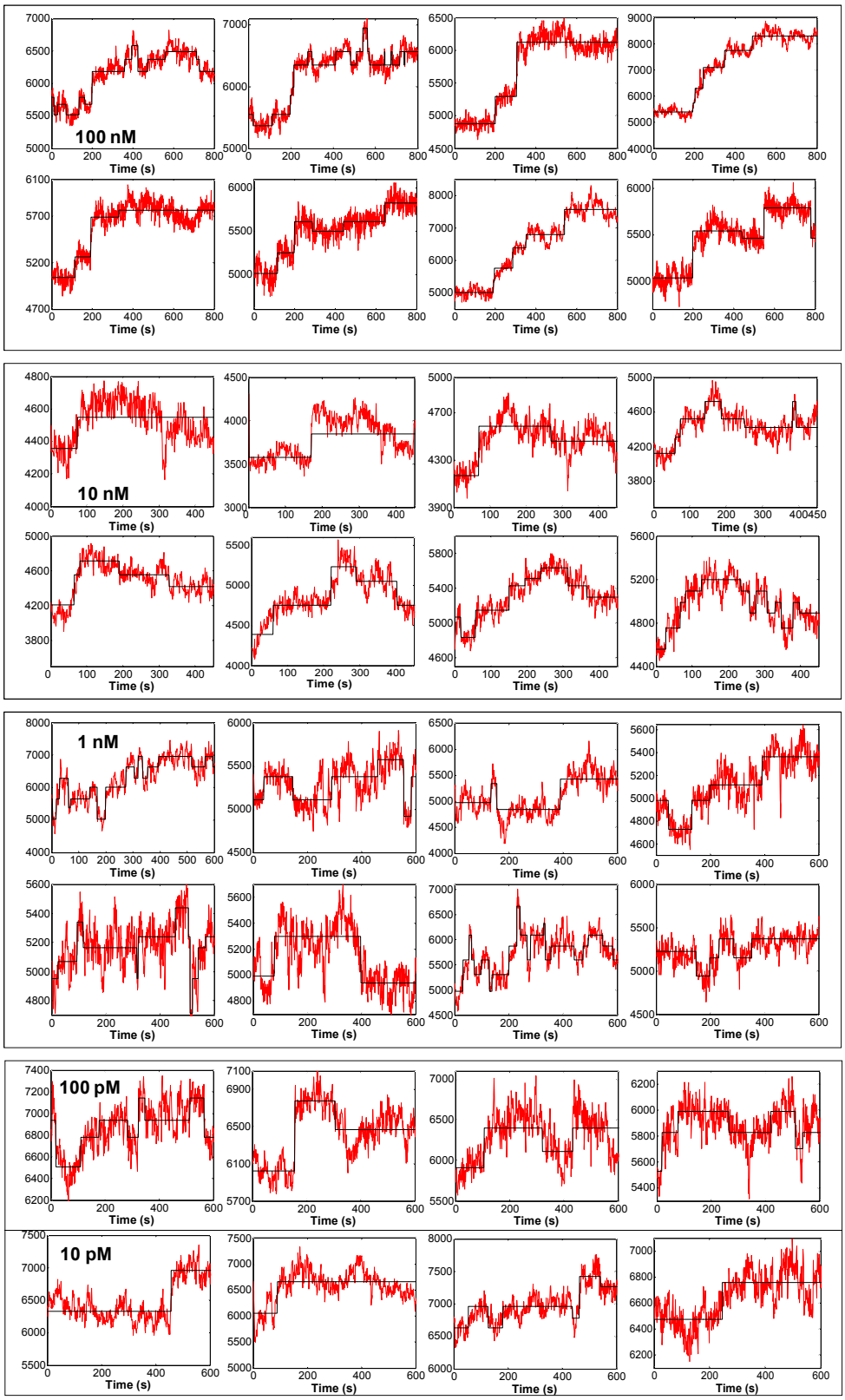

a
Time (s)


b
x 10^4
Time (s)


c
Time (s)

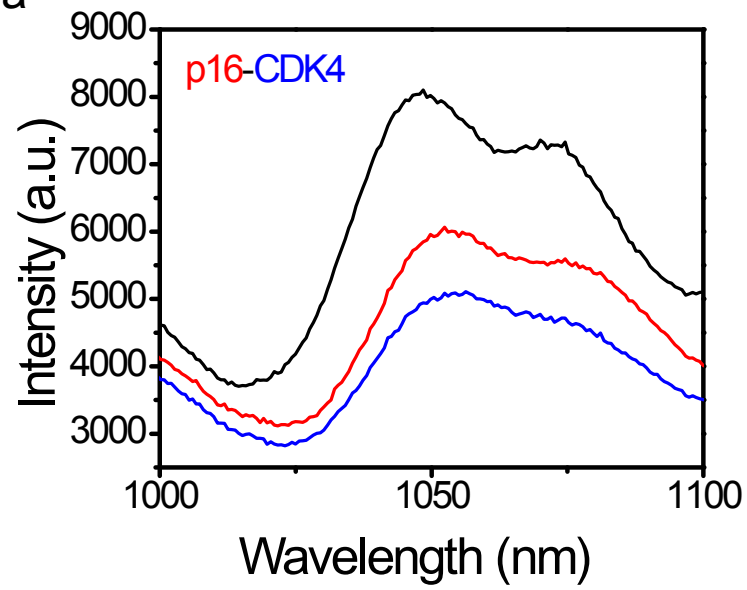

d
log(k_f)

**e**



**Supporting Figure S7.** Single molecule detection of protein-protein interaction on SWNT/CHI microarray. (a) Representative fluorescence time-traces (red) without protein addition as a control, showing that SWNT emission is stable with zero mean deflection. (b) Representative fluorescence time-traces (red) for addition of $Ni^{2+}$ to the NTA-bearing SWNT/CHI spot, showing stepwise quenching response. (c) Representative fluorescence time-traces (red) for the addition of His-tag EGFP (3.61 μM) to the SWNT/CHI microarray bearing Ni-NTA, demonstrating additional stepwise fluorescence quenching. (d) Histogram of the rate constants for binding His-tag EGFP to Ni-NTA on SWNT/CHI microarray. (e) Representative fluorescence time-traces (red) for the addition of anti-His-tag antibody (100 nM to 10 pM), showing the clear stepwise fluorescence increase. This stepwise increase response indicates single protein-protein interaction on SWNT. Black line in all traces denotes the fitted trace from Chisquared error-minimizing step-finding algorithm.

a


p16-CDK4
Intensity (a.u.)
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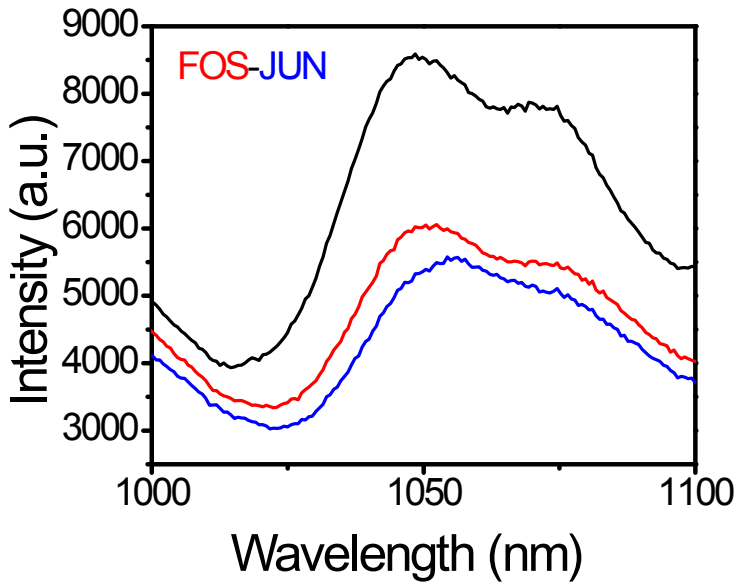
8000
7000
6000
5000
4000
3000
1000
1050
1100
Wavelength (nm)

b


FOS-JUN
Intensity (a.u.)
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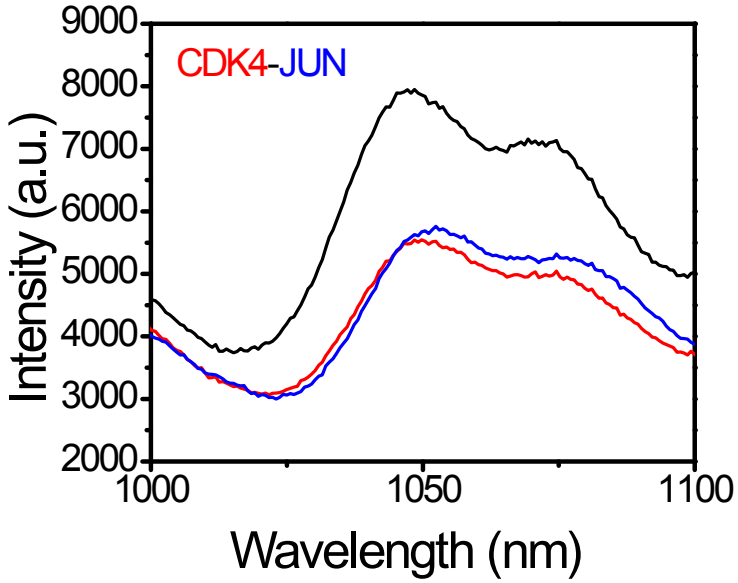
7000
6000
5000
4000
3000
1000
1050
1100
Wavelength (nm)

c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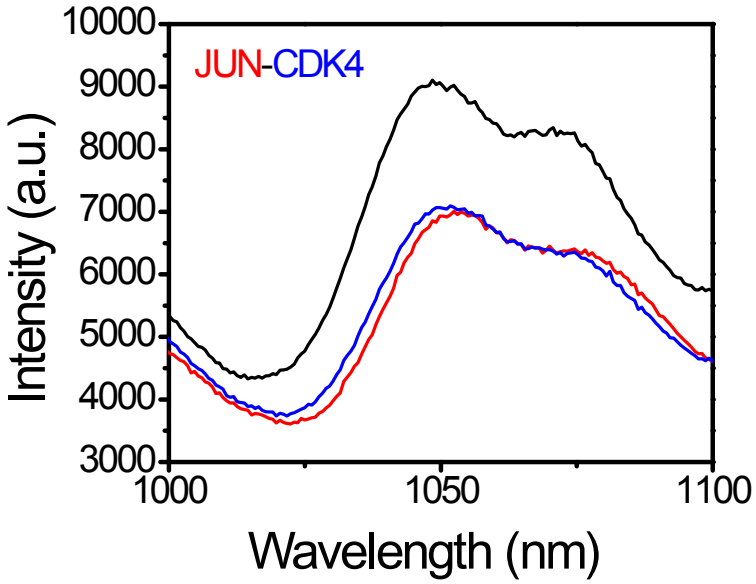
Wavelength (nm)

d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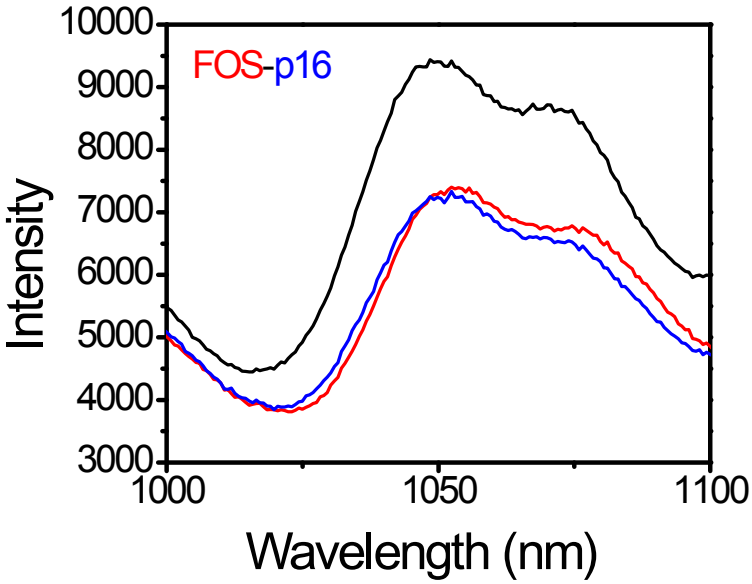
Wavelength (nm)

e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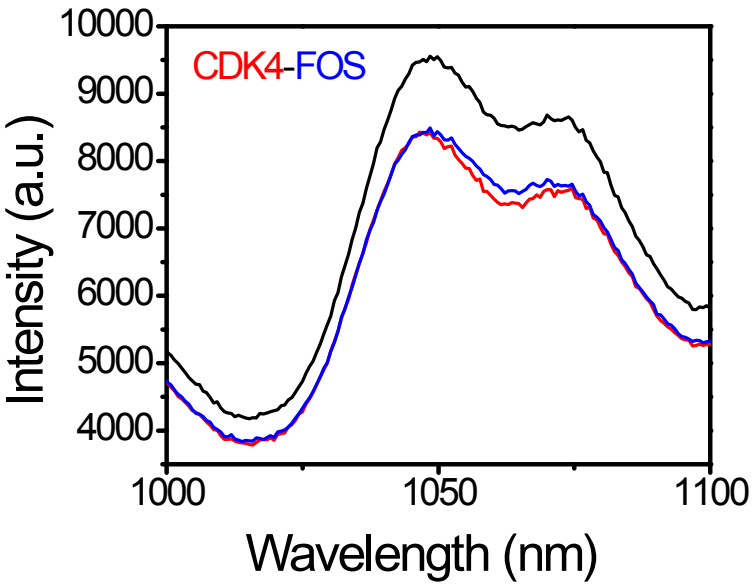
Wavelength (nm)

f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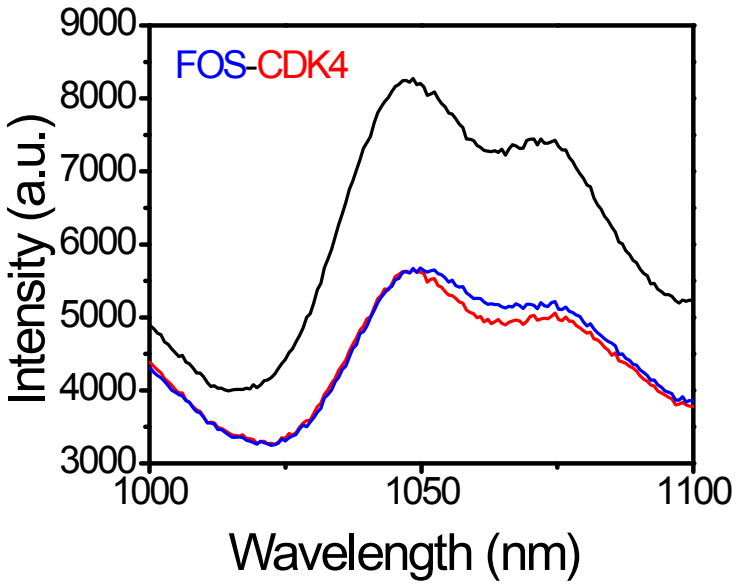
Wavelength (nm)

g


FOS-CDK4
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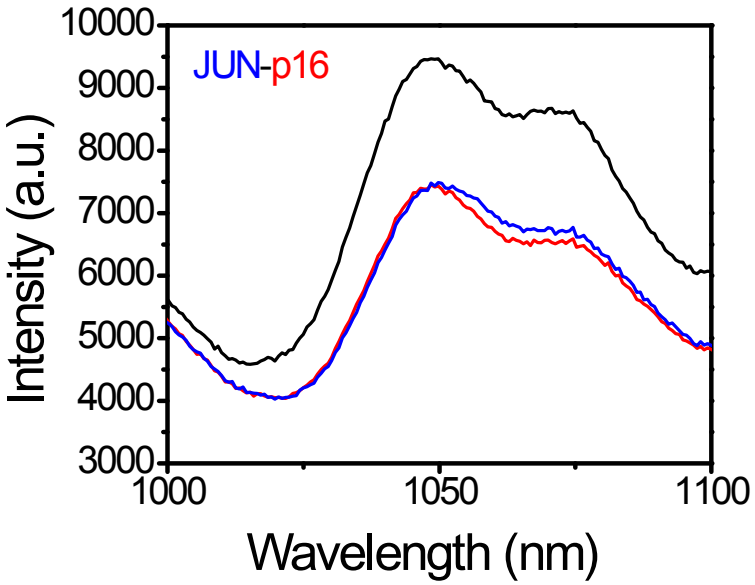
Wavelength (nm)

h


JUN-p16
Intensity (a.u.)
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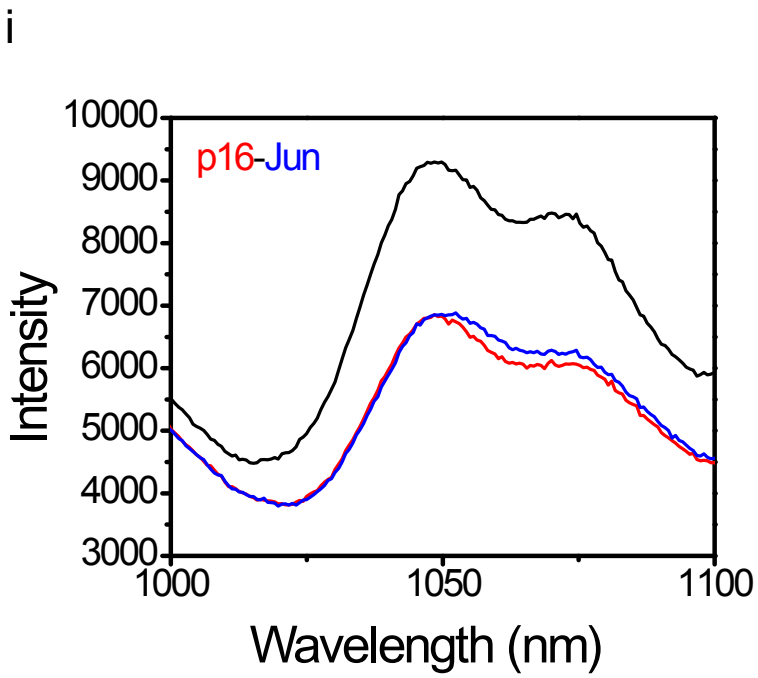
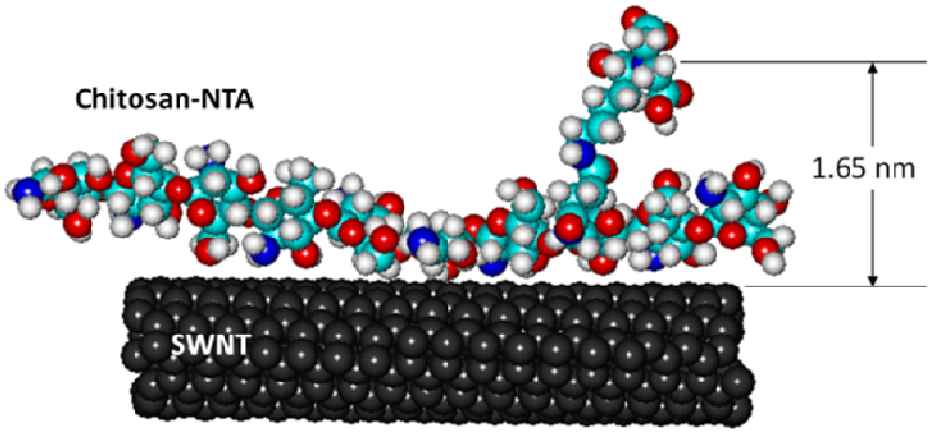
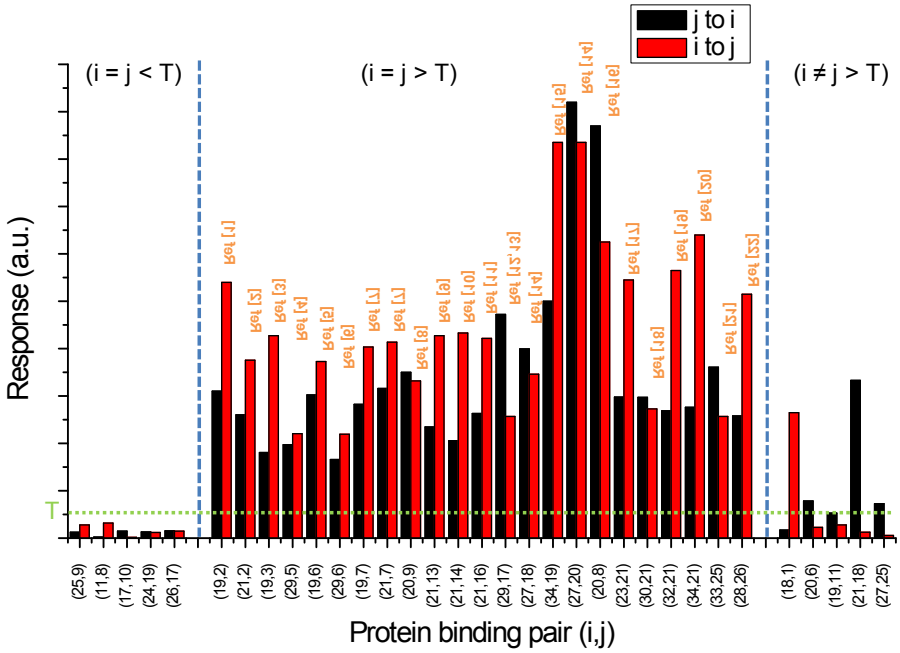
1050
1100
Wavelength (nm)




**Supporting Figure S8**. Protein-protein interactions on SWNT/CHI array. nIR fluorescence spectra of SWNT before and after protein addition. Using Jun, Fos, CDK4, and p16 as queries, protein-protein interaction was analyzed by detecting the fluorescence changes. (a) p16-CDK4, (b) FOS-JUN, (c) CDK4-JUN, (d) Jun-CDK4, (e) FOS-p16, (f) CDK4-FOS, (g) FOS-CDK4, (h) JUN-p16, (i) p16-JUN. The first His-tag protein indicated in red was expressed on SWNT/CHI array, and then the second protein without His-tag indicated in blue was added to each well, respectively. Nanotubes were excited at 85 mW with a 785 nm laser.




**Supporting Figure S9**. Distance calculation between the SWNT and Ni-NTA moiety. The distances corresponding to 10 monomer chitosan units and the Ni-NTA moiety were estimated from a Hyperchem molecular model. Geometry optimization was performed in the presence of water at 300K for 1ps providing the shortest possible distance between the SWNT and NTA is 1.65 nm.




**Supporting Figure S10**. Published literature of protein-protein interaction shown in Figure 5b.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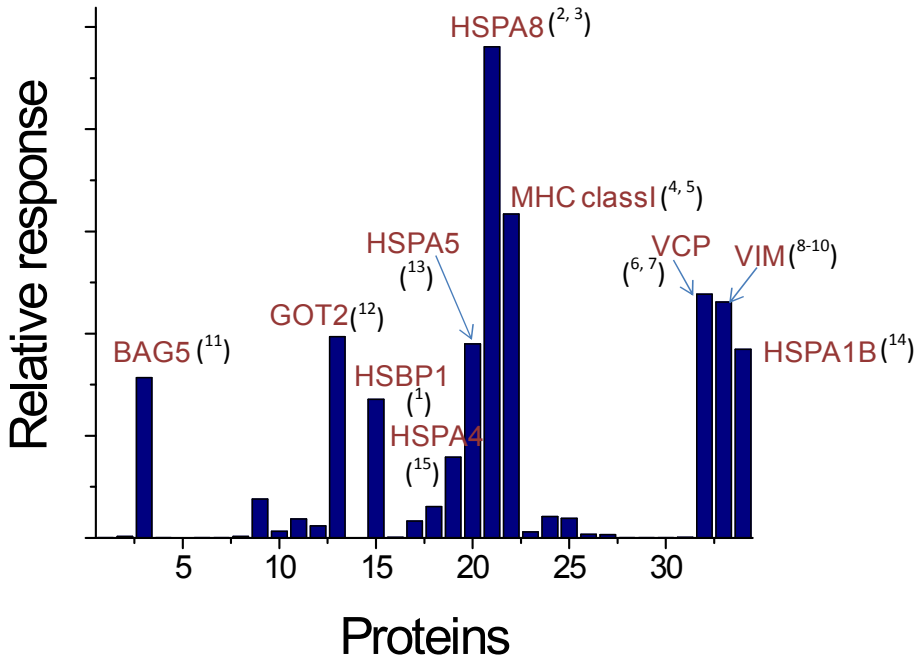


**Supporting Figure S11**. Homo-multimer interaction analysis based on literature survey.


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