Supporting Information

The force of transporting amino-acid into the living cell measured by atomic force microscopy

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Illustration of transporting cysteine into the cell

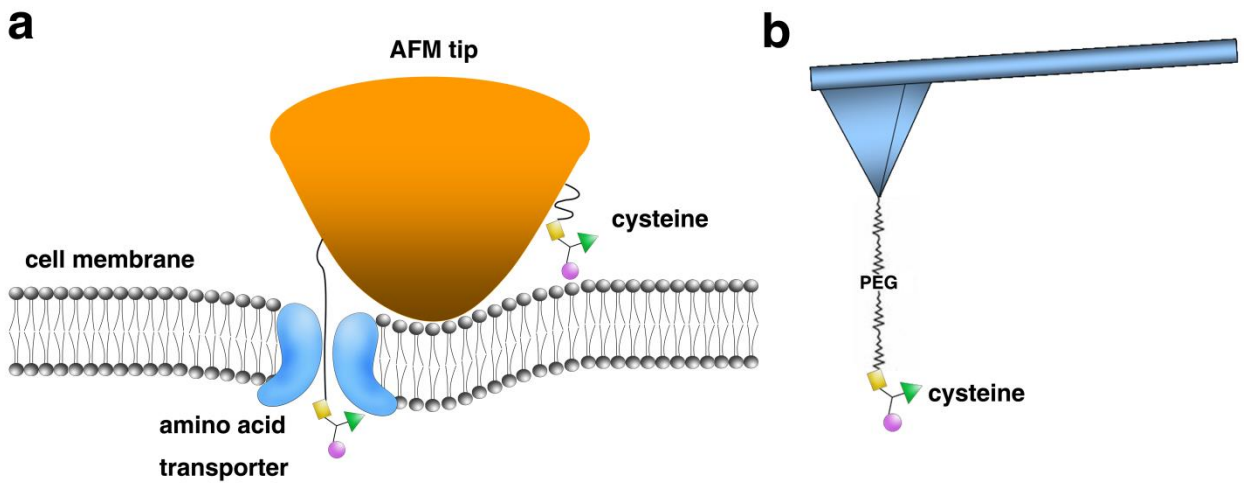

Figure S1. (a) Scheme of transporting the cysteine on AFM tip into the cell via the amino acid transporter. (b) The surface chemistry functionalization of AFM tip with cysteine. PEG linker was used to attach the cysteine on the AFM tip.

Morphology of HeLa cells in HBSS buffer solution

HeLa cells, the well-known cancer cells that proliferated rapidly，were used to perform AFM force measurements. The living HeLa cells in Hanks's BSS buffer were imaged by AFM. Figure S2a and Figure S2b show the topography and amplitude images of living HeLa cells growing on glass slides for 24 h. The height of cells measured by AFM is about 2 μm, which is consistent with the height of the cellular monolayer reported in references from AFM imaging[1]. The actual height of cells obtained from fluorescence microscopy is about 8-10 μm. Clearly, the AFM tip deformed the cells during imaging but not destroyed them. The region around the nuclei appeared to be the cell membranes and cytoplasm, which was chosen for AFM force measurements.

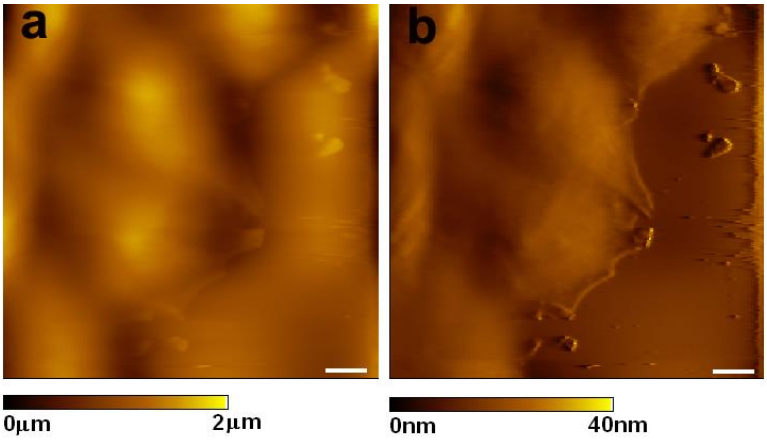

Figure S2. Morphology of living HeLa cells. (a) Topographical image of living cells. The bright regions of the image are the nuclei. (b) The corresponding amplitude image of living cells. The scale bar is 10 μm.

In-situ trypan blue dye test of HeLa cells

To verify the survivability and plasma membrane integrity of HeLa cells during the AFM force measurements, we did the in-situ trypan blue dye test. The AFM force experiment was performed on adherent HeLa cells in the definite regions. After up to 50-100 force-distance cycles were recorded for each cell, the trypan blue dye test were in situ done on the same cells. Figure S3a and Figure S3b show the optical image of HeLa cells before AFM force experiment and after AFM force experiment staining with trypan blue. After the AFM force experiments, there was no significantly difference in morphology and survivability compared with the control experiment (Figure S3c, Figure S3d and Figure S3f). The results indicate that the cells lived in good conditions during the AFM experiments.

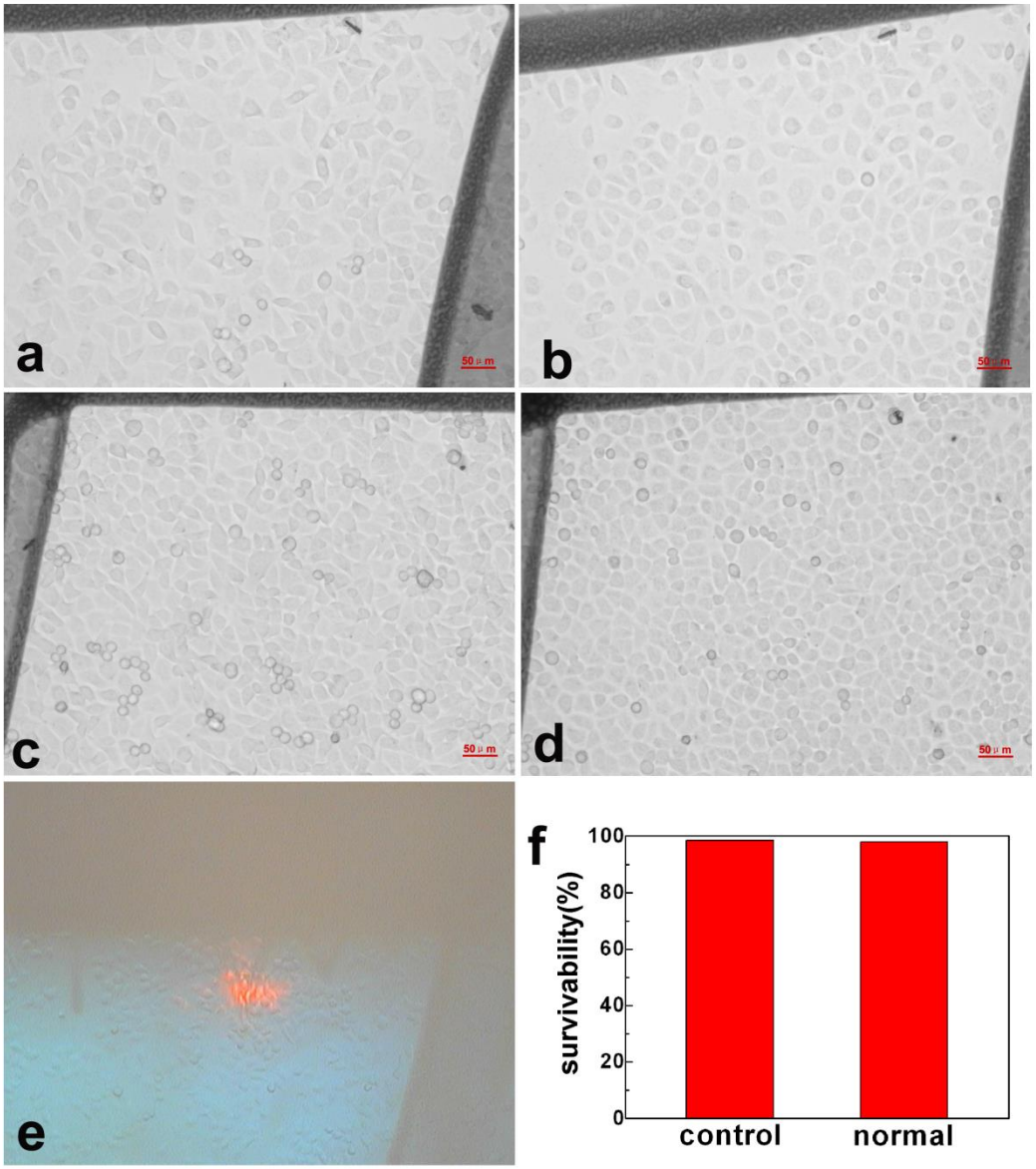

Figure S3. Trypan blue dye test of HeLa cells. (a) Optical images of HeLa cells before force measurement. The black lines on the top and right of the image were the markers to locate the position of cells. (b) Optical images of HeLa cells after force measurement and staining with trypan blue. (c) Optical images of control HeLa cells before AFM experiment. The black lines on the top and left of the image were the markers. (d) Optical images of control HeLa cells staining with trypan blue. (e) Optical images of positioning the AFM cantilever on the cell monolayers with the assistance of a CCD camera. (f) The percentage of survivability from the control and force measurement. 700 cells were counted for both force experiment and control experiment. The scale bar is 50 μm.

Electronic Supplementary Material (ESI) for Chemical Communications

Control experiments for the specificity of transporting events

To test the specificity of blocking experiments, we tried to block the transporting events by non-related amino acids. We performed force measurements with cysteine-coated tip via NHS-PEG-MAL cross-linker on HeLa cells in HBSS buffer, and then 0.1 mM phenylalanine or tryptophane were injected into the solution. The results reveal that binding probability don't change significantly by the addition of free non-related amino acid (Figure S4a and Figure S4b). To determine whether the linker's end groups (MAL or Aldehyde) contribute to the binding events observed in Figure 2e and Figure 2f, we performed control experiments by linker-modified AFM tips without amino acids. There was no force signal either with NHS-PEG-MAL-modified tip or NHS-PEG-aldehyde-modified tip (Figure S4c and Figure S4d). The results indicate that the peaks observed in Figure 2e and Figure 2f were not from the contribution of the linkers.

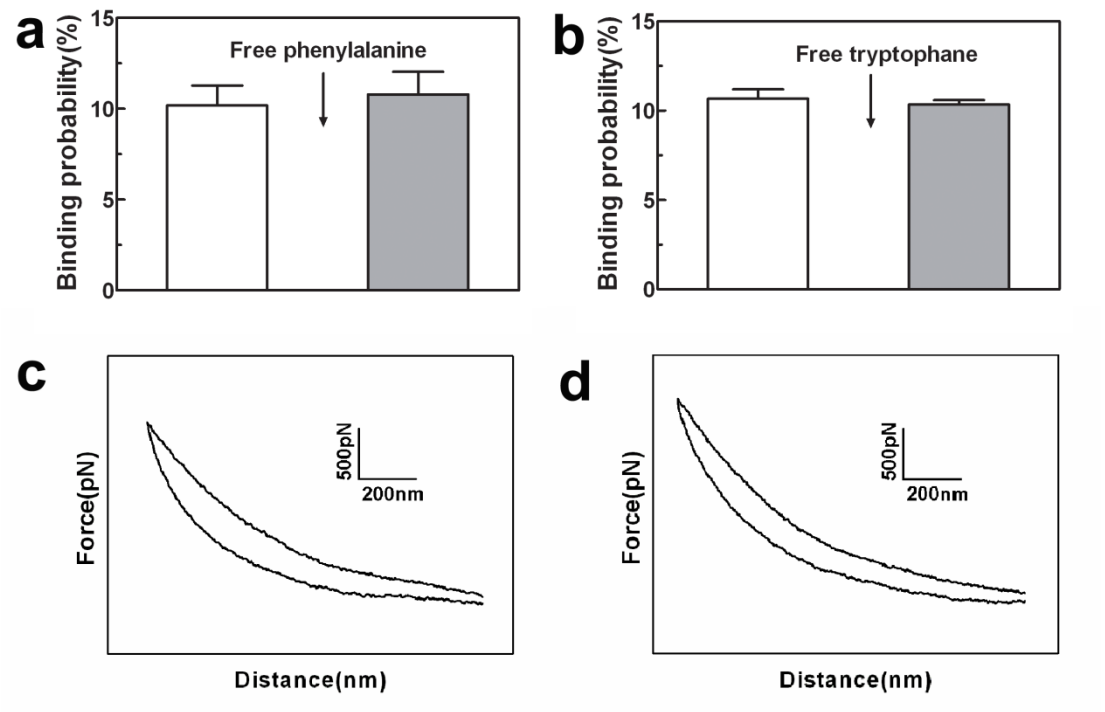

Figure S4. Control experiments to verify the specificity of transporting event. Binding probabilities of cysteine-coated AFM tips on HeLa cells without (left) and with free phenylalanine ((a), right bar) or tryptophane ((b), right bar) in solution. Values are mean ± s.e.m., n=1000-2000. (c) The force curve with a NHS-PEG-MAL-modified tip on living HeLa cells in the HBSS buffer. (d) The force curve with a NHS-PEG-aldehyde-modified tip on living HeLa cells in the HBSS buffer.

The hypothetical process of transporting cysteines into living cell

The hypothetical process of transporting cysteines into living cells via the amino acid transporter is shown in Figure S5. Firstly, the AFM tip with a cysteine coupled to cross-linker NHS-PEG-MAL via a thiol group moves towards the outer surface of cell membranes (Figure S5a). Amino acid transporter specifically recognizes and binds the cysteine (Figure S5b), and subsequently the transporter changes their conformations to transport the amino acid from the extracellular medium into the cell (Figure S5c). With the tip retraction from the cell, the cysteine is pulled out of cell membranes (Figure S5d). In contrast, while the AFM tip carrying a cysteine coupled to the cross-linker aldehyde-PEG-NHS via α-amino group moves towards the outer surface of cell membranes (Figure S5a'), the cysteine binds to the amino acid transporter without the following translocation process happening (Figure S5b'). Finally, the AFM tip is drawn away from the cell (Figure S5c'). Our results support the "two-step" hypothesis about amino acid translocation[2], and suggest that the conformation of cysteine in the presence of free α-amino group is important for discriminating the substrate for translocation. The scheme (Figure S5) just shows a simple concept about the process of transporting amino acid on the tip into the cell, which can be easily understood about the concept of "fishing" emphasized in this work. For the real process that could be more complicated, the amino acid is very small compared to the AFM tip, so that the amino acids may be also attached to the AFM tip sidewall. The cell membranes are very soft, the AFM tip sidewall is not absolutely smooth, and there are also water molecules between the cell membranes and the tip, therefore there should be many contacting points between the tip sidewall and cell membranes for amino acid transporting. While the apex of AFM tip touched the cell membranes, the amino acid on the side wall of AFM tip could interact with the soft cell membranes and be transported. In our experiments, we could also observe the

force curves that the unbinding force "fu" appears during the contact between the AFM tip and cell membranes. This result indicates that the amino acid on the AFM tip could be transported after the apex of AFM tip contacting with the cell membranes, and the amino acid may be detached from the cell membranes before the end of AFM tip departing from the cell, which totally supports our thought that the amino acid may be on the sidewall of AFM tip.

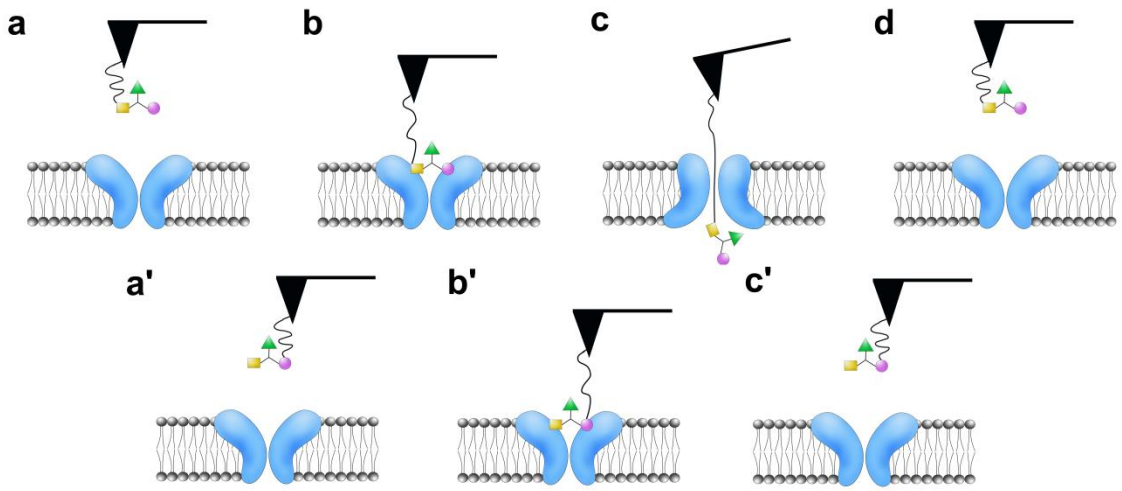

Figure S5. Scheme of the interactions between the cysteine functionalized onto AFM tip via two types of linkers and the amino acid transporter in the living cell membranes. The blue parts represent the amino acid transporter. The yellow tetragon represents the thiol group of cysteine. The purple ball represents the α-amino group of cysteine. The green triangle represents the α-carboxyl group of cysteine. (a) The AFM tip modified with cysteine which is coupled to cross-linker NHS-PEG-MAL via a thiol group, moves towards the cell membrane surface. (b) The cysteine binds to amino acid transporter. (c) The cysteine is transported into the cell. (d) The cysteine is pulled out of the cell. (a') The AFM tip modified with cysteine which is coupled to cross-linker aldehyde-PEG-NHS via α-amino group, moves towards the cell membrane surface. (b') The cysteine binds to amino acid transporter. (c') The cysteine is drawn away from the cell.

The criterion to select the force events

We chose S/N (signal/noise) ＞3 as the criterion to select the force peaks. For example, we considered the force events in Figure S6a and S6c as the obvious peaks; however, the force events in Figure S6b and S6d were considered as no peak.

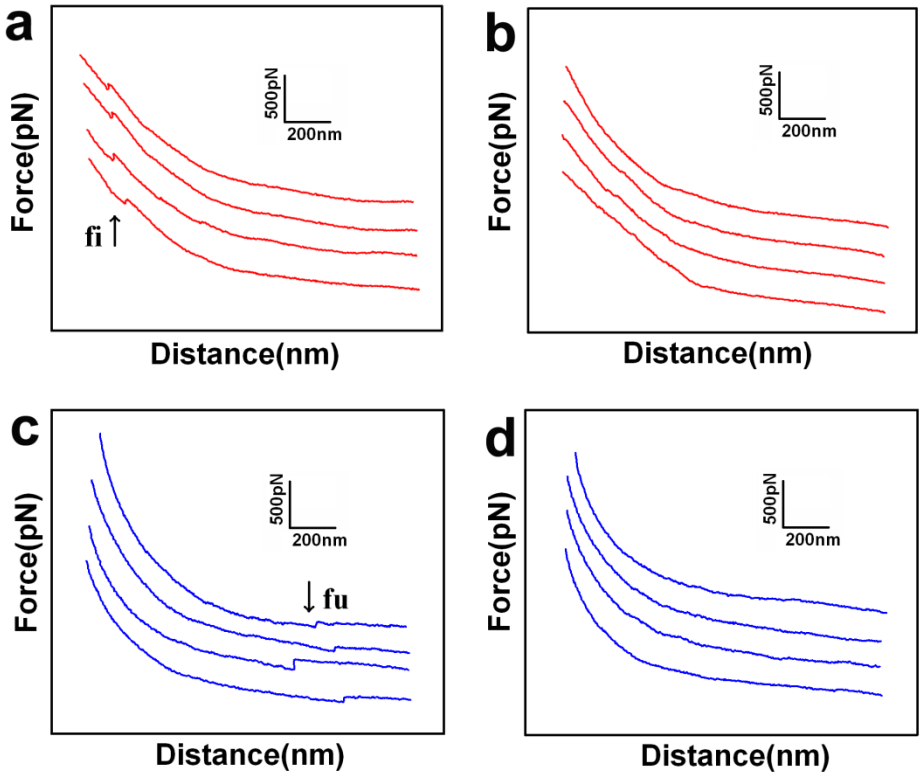

Figure S6. Raw data of the clear and not so clear cases of fi (a, b) and fu (c, d) for all experiments.

Experimental Section

Cell culture: HeLa cells were cultivated on glass slides in Dulbecco Modified Eagle's Mmedium (DMEM) supplemented with 10% heat-inactivated bovine serum and 1% of penicillin/streptomycin under 5% CO_2 at 37°C. For AFM experiments, the cells were subcultured at 80% confluence and incubated for 1h at 37°C in Hanks's BSS buffer solution to remove the extracellular amino acid and then to reduce the intracellular amino acid concentration before performing force spectroscopy.

Trypan blue dye test: The trypan blue dye test was a simple and common assay for examining the survivability of cultured cells. Trypan blue dye was added to cell culture medium in a ratio of 1:1 for 10 min. The number of survival cells (not stained) and dead cells (stained) was counted under a microscope. The absolute survivability was calculated as a percentage of survival cells against total cells. The total number of cells counted is 700 for both AFM experiment and control experiment.

Conjugation of cysteine to AFM tips via different PEG-cross-linkers: The length of the PEG (polyethylene glycol) cross-linker is about 20 nm, which is longer than the dimension of transporter (less than 15 nm) so that the amino acid on AFM tip could be transported into the membrane transporter. The conjugation of cysteine to AFM tips (Microlevers, Veeco, Santa Barbara, CA) was done using a flexible PEG-cross-linker (NHS-PEG-MAL) as described previously[3]. AFM tips were functionalized with APTES. NHS-PEG-MAL cross-linkers were attached to AFM tips by incubating the tip for 2 h with 1 mg/mL of the PEG linker in chloroform containing 0.5% of triethylamine. Cysteine was conjugated to MAL -PEG tips via its intrinsic thiol group in buffer A (100 mM NaCl, 50 mM NaH_2PO_4, 1 mM $EDTANa_2$, adjusted to pH 7.5 with NaOH) at a concentration of 10 μg/mL for 1 h. Functionalized AFM tips were extensively washed using PBS for three times and stored at 4°C until use.

An alternative method for conjugating the cysteines to AFM tips was to use the aldehyde-PEG-NHS cross-linker. Aldehyde cross-linkers were attached to APTES-functionalized AFM tips by incubating the tips with 1 mg PEG linkers in 0.1 mL chloroform containing 6% of triethylamine for 2 h. Cysteine was conjugated to aldehyde-PEG tips via its α-amino-aldehyde coupling in PBS at a concentration of 10 μg/mL. In addition, 2 μL of 1 M $NaCNBH_3$ were added to the PBS and mixed carefully with the pipette. One hour later, 5 μL of 1M aqueous ethanolamine were added to the PBS in order to passivate unreacted aldehyde groups. Finally, functionalized AFM tips were extensively washed using PBS for three times and stored at 4°C until use.

Atomic force microscopy and force spectroscopy: All AFM experiments were performed using AFM 5500 (Agilent Technologies, Chandler, AZ). Topographical and amplitude images of cells were acquired with AAC mode AFM in HBSS buffer at room temperature with non-functionalized AFM cantilevers. Force spectroscopy measurements were performed at 37°C in HBSS buffer with functionalized AFM cantilevers. In order to assess sodium-dependent cysteine transport, the AFM force experiments on cells were performed at 37°C in Na^+-containing KRH (Krebs-Ringer-HEPES) buffer solution or Na^+-free KRH-NMG buffer solution. The KRH-NaCl buffer solution contained 120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4 with Tris base). In the KRH-NMG buffer, NaCl was replaced by N-methylglucamine (NMG). Force-distance cycles were recorded on cell surfaces with the assistance of a CCD camera for positioning the AFM cantilever on isolated cells or cell monolayers (living cells). Up to 500-1000 force-distance cycles were performed for each location on the surface of cells and up to four locations for each condition, i.e., initial condition, cysteine addition, sodium substitution, and washout condition. For the concentration-dependent

blocking experiment, 200 μL 0.1 mg/mL cysteine, 1 mg/mL cysteine and 5 mg/mL cyesteine were separately added to the solution in-situ. Force-distance cycles were recorded after incubation for 30 min. The washing periods were about 30 min to 1 h for each condition. The binding probability for each condition was derived and expressed as the mean value ± the standard error of the mean (n =1500–2500). The statistical significance was tested using a Student's t test. Several experiments were performed, and one typical experiment for each condition is shown.

For the quantification of the forces, the AFM cantilevers were calibrated by a reference cantilever (CLFC, Veeco, Santa Barbara, CA) as described. The deflection sensitivity of the photo-detector was determined by the slope of the force–distance curves taken on the bare surface of mica[4]. The loading rate was calculated from $r = k_{eff}$*scan size (nm)*2/sweep time, and the Gaussian fit of the highest for k_{eff} (0.95 pN nm^{-1}) was calculated using Matlab software with the true spring constant. Analysis of retrace of force-distance cycles was performed using the Matlab Version 7 as previously described[5]. During the process of analyzing the force curves with the Matlab, the parameter "probability" in the software could detect the validity of force peak, as the function of the parameter, if the letters of "probability" were not red color then the force signal was effective. Analysis of trace of force-distance cycles was performed by PicoScan 5.3.3 software. The force signal with the S/N $\geqslant$ 3 was selected as the transporting force peak. The transporting force was calculated from $f = k * \Delta z$ (k is the spring constant of the cantilever; Δz is the deflection up (+) or down (-) of the cantilever).

Reference:

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