Supplementary Information

1. Comparison of the typical force-distance curves of chicken and fish erythrocyte membranes

The similarity of protein distribution in both sides of chicken and fish erythrocyte membranes can be further demonstrated by the comparison of the force-distance curves on the inner leaflets. As shown in Fig.S1, the typical force-distance curves in the inner leaflets of chicken (Fig.S1a) and fish (Fig.S1b) erythrocyte membranes similarly display multiple force peaks with the binding probability 86.4% and 98.5%, respectively, both of which are remarkably larger than the corresponding value from the outer leaflet (Table 1). This consistency in the percentage of exposed amino groups in both sides of chicken and fish erythrocyte membranes provides additional evidence for their similar membrane structure.

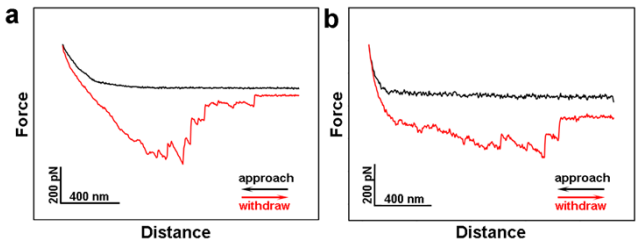

Fig.S1 The typical force-distance curves acquired on the inner leaflet of chicken (a) and fish (b) erythrocyte membranes by glutaraldehyde-functionalized AFM tips. In (a) and (b), the force curves show multiple force peaks when withdrawing the AFM tip from the membrane surface.

Experimental section

Isolation of the chicken erythrocytes

This study was performed with the approval of the Regional Ethics Committee for Animal Experiments and carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Jilin University. Isolation of chicken erythrocytes was carried out at room temperature. Fresh blood was drawn from wing vein into a syringe (with the needle diameter of 0.6 mm) containing a few drops of heparin (1000 U/mL). Then the erythrocytes were washed five times in 1 mL PBS buffer (136.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) by a low speed centrifuge (1300 rpm) and resuspended in PBS buffer. APTES-mica substrate was prepared as described [1]. 200 μL erythrocyte suspension were deposited onto APTES-mica for 20 min. Thereafter, the unadsorbed erythrocytes were washed away by PBS buffer. The prepared sample was mounted into the AFM liquid flow cell containing PBS buffer and imaged immediately. Alternatively, the adsorbed erythrocytes were fixed with 4% paraformaldehyde for 60 min before imaged by AFM.

Preparation of the outer and inner leaflet of erythrocyte membranes

High resolution image of the outer leaflet of the chicken erythrocyte membrane was obtained on the smooth edge of the intact unfixed erythrocyte. And the inner membrane leaflet was prepared by shear-open method as described [2].

Digestion of the inner leaflet of erythrocyte membranes by Proteinase K and MβCD

The inner leaflet of erythrocyte membranes was prepared as described above and digested by 0.5 mg/mL Proteinase K (Sigma) for 30 min at 37℃. Then the membranes were washed in PBS buffer for three times and imaged by AFM. After this, 10 mM MβCD was injected into the AFM fluid cell and the changes occurred to the membranes were followed by in situ AFM.

AFM imaging and force spectroscopy

The AFM imaging and force spectroscopy of isolated erythrocytes and erythrocyte membranes were performed using AFM 5500 (Agilent Technologies, Chandler, AZ) [1]. All the images were obtained at room temperature in PBS buffer and recorded with 512 × 512 pixels. Erythrocyte size and membrane heights were measured using PicoScan 5.3.3 software (Agilent Technologies, Chandler, AZ). The AFM tips were functionalized with glutaraldehyde as described [2]. For statistical analysis, thousands of force curves were recorded in different positions of the inner and outer leaflet of erythrocyte membranes. The force curves were processed with MathLab 7.9 (Math Works Inc.).

References

1. Y. Tian, J. Li, M. Cai, W. Zhao, H. Xu, Y. Liu and H. Wang, *RSC Adv.*, 2013, **3**, 708-712.
2. H. Wang, X. Hao, Y. Shan, J. Jiang, M. Cai and X. Shang, *Ultramicroscopy*, 2010, **110**, 305-312.