Electronic Supplementary Information (ESI) to the manuscript:

"Single Molecule distribution of RhD binding epitopes on ultraflat erythrocyte ghosts"

by Sarah Stainer et al.

Alternative TREC Specificity Proof: One way to verify specificity of molecular recognition within a TREC image is performing an amplitude block.[1] Obtaining recognition signals is highly dependent on the oscillation amplitude in relation to the linker length. If the oscillation amplitude of the cantilever is higher than the stretched linker length, the receptor ligand bond ruptures at each oscillation cycle, thus no reduction in the upper amplitude can be recorded. Measuring at an amplitude too low also results in a lack of recognition spots as the linker does not get stretched sufficiently. Hence, if the amplitude where recognition was gained is doubled, the recognition spots should disappear proving that the interaction is dependent on the oscillation amplitude. Fig. S1 shows two TREC images recorded on an erythrocyte ghost membrane. The two topographical images (A, B) were recorded on the very same spot, however with different working amplitudes. The optimized TREC amplitude shows recognition events (C) whereas the doubled TREC amplitude does not (D). (E) and (F) show an overlay of the regions recognized as recognition after applying the previously described criteria for recognition. It is well visible that the doubled amplitude resulted in a significant reduction of recognition events and led to a decrease of recognition area of 89 %.

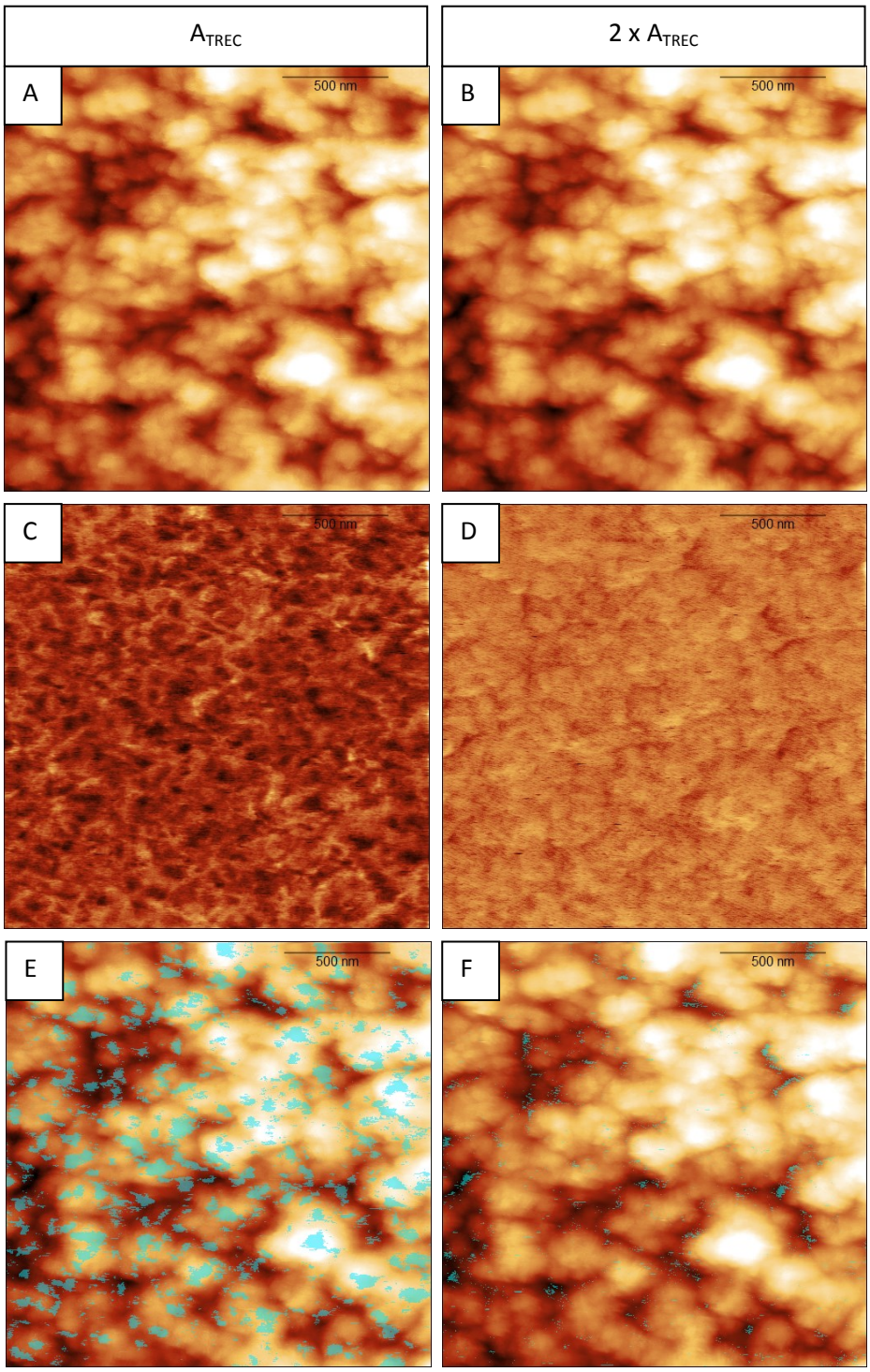


Figure S1: Specificity proof via Amplitude Block. (A) topography image obtained with the optimized TREC cantilever oscillation amplitude. (B) topography imaged with the doubled TREC amplitude. (C) recognition image recorded with the optimized TREC amplitude. Dark recognition spots are visible. (D) recognition image recorded with the doubled amplitude. No dark recognition spots are visible. (E) overlay of the recognition spots over the topographical image (optimized amplitude). (F) overlay of the recognition spots over the topographical image (doubled amplitude). Scale bar is 500 nm. Z scale is 100 nm.

Detailed scheme of the vlsb washing step: The washing step (Fig. S2) with vlsb is crucial for the formation of ultra-flat erythrocyte ghosts. First of all the remaining erythrocyte solution should be withdrawn before the washing step is conducted to prevent dilution of the vlsb with the PBS buffer that contains a high salt concentration (Fig S2 step 3). The pressure applied while spraying vlsb on the immobilized erythrocytes plays an important role. If the stream is to firm, the upper plasma membrane layer of the cell will rupture. If the stream is to gentle, the ghosts formed will not collapse in an ultra-flat manner. It is important that the vlsb flows over the cells, creating a shear force and forcing the emptying of the cell content in the direction of the flow. Immersing the cells completely in vlsb will result in wrinkled ghosts.

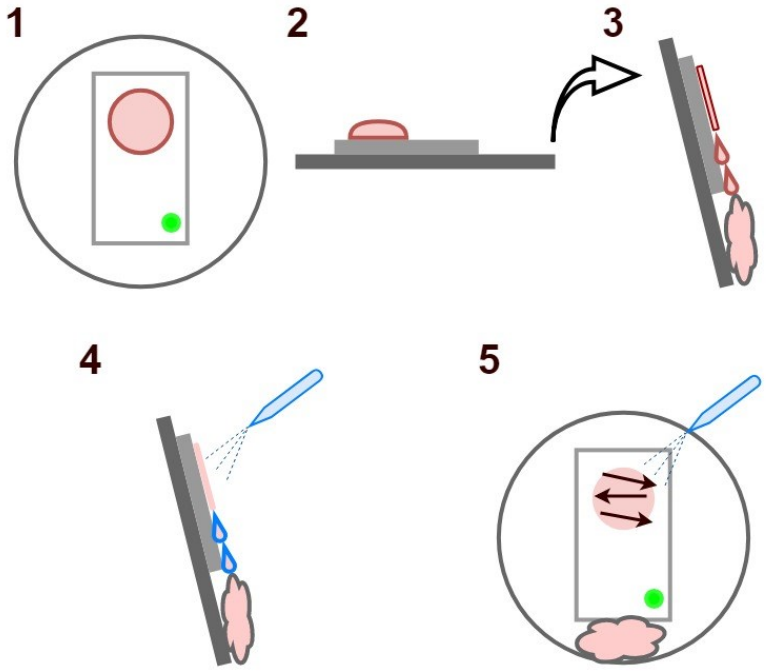


Figure 2: Detailed scheme of the vlsb washing step. (1) Glass plate (rectangle) with the erythrocyte solution (red) in a petri dish (circle). (2) horizontal view. (3) before applying the vlsb, make sure the erythrocyte solution is removed by tilting (a tissue helps (cloud)). (4) apply a gentle but firm stream of vlsb in a zick.zack motion to the glass slide. Make sure that the erythrocyte region is not immersed in vlsb (a tissue helps to prevent flooding). (5) zick zack movement while applying the vlsb.

Raw data of force spectroscopy experiments on RhD positive red blood cells: Single rupture forces are plotted versus their loading rate. The binning intervals for the binning fit are shown in figure 3 for BRAD5 mAb and in figure 4 for ESD-1 mAb functionalized tips.

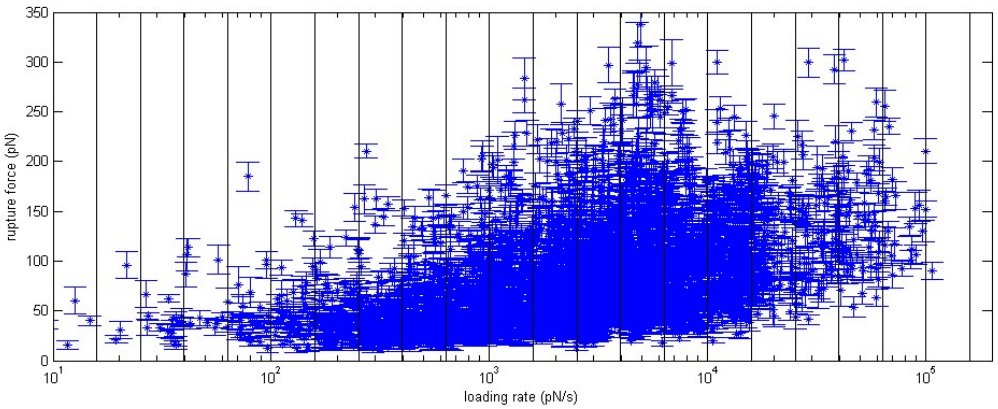


Figure 3: Plot of individual rupture forces versus force loading rate of force spectroscopy experiments on ultraflat erythrocyte ghosts, using a BRAD5 functionalized tip.

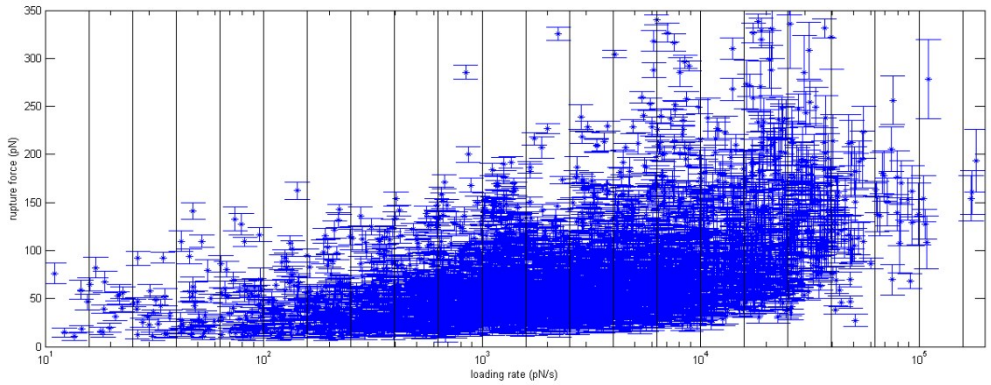


Figure 4: Plot of individual rupture forces versus force loading rate of force spectroscopy experiments on ultraflat erythrocyte ghosts, using an ESD-1 functionalized tip.

Fluorescence measurements on RBCs: To get a quick overview of the distribution of RhD receptors on intact RBC, we labelled BRAD5 mAbs with Cy5 and incubated the labelled antibody solution. Figure 5 shows the fluorescence signal on unopened (freshly adhered) RBC. A rather homogeneous distribution was observed.

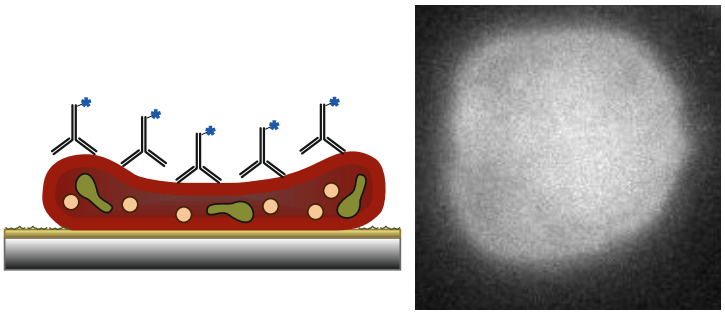


Figure 5: Cy5 labelled BRAD5 mAbs were incubated on PLL adhered red blood cells resulting in a rather homogeneous distribution.

Probability density functions (PDF) for block and specificity proof: For both monoclonal antibodies, the error-weighted histograms (i.e. the PDF) are shown in figure 6. The pdfs for block and specificity proof were set relative to the measurement on RhD positive sample without block (the values for the block were divided by the binding probability of the RhD positive sample without block multiplied with the binding probability of the block.

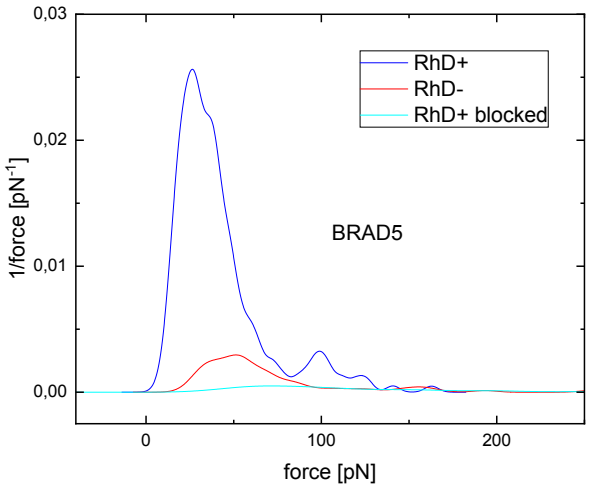
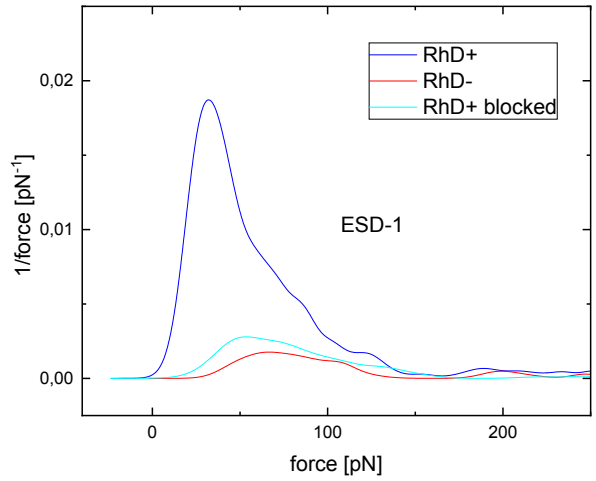


Figure 6: PDFs (error-weighted histograms) of the rupture forces of BRAD5 (left, blue line) and ESD-1 (right, blue line) with RhD epitopes on ultraflat RhD positive erythrocytes ghosts. For the specificity proof using the same tip - but on RhD negative samples (red line) - the y-scale was set relative to the ratio of the binding probabilities. The same was done for the blocking experiment (cyan line). The binding probabilities were for BRAD5: RhD+: 18.9, RhD- 1.0 and 3.1 for the block and for ESD-1: RhD+ 25.1, RhD- 3.5 and for the block 5.5. All measurements were done at similar force loading rates.



Citations

1. Preiner, J., et al., *Simultaneous topography and recognition imaging: physical aspects and optimal imaging conditions.* Nanotechnology, 2009. **20**(21): p. 215103.