Supplementary information

High-efficiency localization of Na^+-K^+ ATPases on the cytoplasmic side by direct stochastic optical reconstruction microscopy

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Experimental Section

Sample Preparation.

To characterize the Na^+-K^+ ATPases distribution in the inner membrane of hRBC, Cy5-NHS ester (GE Life Sciences, Buckinghamshire, U.K.) was firstly added in DMSO at approximately 10 mg/ml and then diluted in 0.01 M phosphate buffer at different working concentrations. The immunoglobins (IgG, IgA, IgM) and Na^+-K^+ ATPase antibody were stained by Cy5 at 30 nM in 0.1% (w/v) BSA in PBS. In order to maintain an appropriate pH condition (pH 8.0) and protect the NHS ester from degradation, 10% (v/v) 1M sodium bicarbonate was added into the reaction system, and then the solution were vortexed in dark for 1h at room temperature. Unreacted Cy5 molecules were filtered out by gel filtration using PD spintrap G-25 column (GE Life Sciences, Buckinghamshire, U.K.). The degree-of-labeling between Cy5 and Na^+-K^+ ATPase monoclonal antibody (Abcam mab2871) was determined to be about 1.0 dye/protein by absorption spectroscopy assay.

In order to eliminate surface pollution, glass slides and cover slips were cleaned by sonication in 1 M potassium hydroxide for 20 min at room temperature. Both of them were rinsed with Milli-Q water and then stored in absolute ethyl alcohol. Prior to use, the glass sides and cover slips were washed for three times with sterile distilled water and dried with pure Argon.

About 50 μl human blood from the same donor were washed 5 times in 150 mM PBS buffer (pH 7.4). The diluted resuspension solution was deposited on prepared cover slips for 30 min. To obtain the inner membrane, the attached hRBCs were sheared open by a quick squirt of 7.5 mM PBS as described. In that case, the transmembrane proteins were still resident in the lipid bilayers intactly and the membrane skeletons were washed away by the hypotonic salt buffer. To block the nonspecific binding site, 4% (w/v) bovine serum albumin (BSA) was added to the surface of cover slips for 30 min at room temperature. After washing out the blocking buffer by PBS buffer (150 mM, pH 7.4) for three times, ten microliters of purified anti-ATPase-Cy5 were added onto the inside-out hRBC membrane and incubated for 1h.

Before imaging, the cover slips were washed up with PBS buffer for more than three times. Buffer solutions were replaced with an imaging buffer containing 50 mM Tris (pH 7.5), 10 mM NaCl, 10% (w/v) glucose, 0.5 mg/mL glucose oxidase (Sigma- Aldrich), 40 μg/mL catalase (Sigma-Aldrich) and 1% (v/v) β-mercaptoethanol.

Instrumentation

The imaging experiment was performed on a Nikon Ti-E microscope. An objective-type total internal reflection fluorescence (TIRF) configuration using an oil-immersion objective (100×, NA 1.49, Nikon, Japan) was applied in the experiment. Fluorescence images were recorded at rates of 20 - 50Hz in laser-excitation widefield. The sample was excited with a 640 nm laser (≈ 40 mW). Meanwhile, a 405 nm laser (≈ 25 μW) was added to reactivate the bleached Cy5 molecules in a pulse mode and maintain an optimal number of emitting molecules in the field of view. The switch between the activation wavelength and the excitation wavelength, and the intensity of each laser were controlled via an acoustooptic tunable filter (AOTFnC-400.650, A-A Opto-Electronic, Orsay Cedex, France). The laser beams were coupled by dichroic mirrors and widened by a telescope consisting of the achromatic doublet lenses before injection into the microscope. The fluorescence was filtered with emission filters and imaged on an EMCCD camera (Photometrics, Cascade II). This condition is approximately satisfied by choosing a tube lens of 400 mm focal length, yielding a 167 nm effective pixel size and a maximum field of view of 85 × 85 μm^2. The z-drift was eliminated by the means of a focus lock, and 150 nm diameter fluorescent polystyrene beads were embeded to correct the *x*-*y* drift of the sample independently.

***d*STORM Image Analysis**

The localization accuracy that defines the intrinsic resolution of *d*STORM was calculated using a previously established statistical equation (Thompson et al., 2002). Based on the total number of collected photons (*N*) from a single dye molecule, the localization accuracy is given in terms of the error in localization, represent as $\langle(\Delta x)^2\rangle = 4\pi^{1/2}s^3b^2/aN^2$, where *s* represents the standard deviation of the center of the point-spread function (PSF), *a* is the pixel size of the EMCCD, *b* is the background noise. Detailed spatial information is usually lost when the distance between fluoresecnt molecules is shorter than the diffraction limit so that multiple PSFs overlap

with each other. Therefore, the labeling density of dye molecules should be sufficiently low and Gaussian fitting of segregate PSFs can pinpoint the localization of individual fluorophores with nanometer precision. The theoretical localization accuracy of a single Cy5 molecule is calculated as about 22 nm. In this work, we adopt FWHM for a Gaussian fitting function to describe imaging resolution of ATPases molecules. Using the TIRF imaging by a 640 nm laser, a typical *d*STORM image was generated from a sequence of 5000 - 40000 frames recorded at 30 Hz. For each frame, fluorescent spots were isolated as far as possible and then the center of positions were identified and fitted to an elliptical Gaussian function by Image J according to a threshold to the peak height, the coordinates in the two lateral dimensions, and the peak widths in the two lateral dimensions. The centroid positions of peaks occurring in consecutive frames with an offset less than one pixel were considered to the origin of the same fluorescent molecule. The final localization of the single anti-ATPase-Cy5 molecule was determined by the weighted average of these centroid positions of peaks and insured to be located in an limited area that was wide enough to populate several subjected localizations.

Analysis of Cluster Characteristics

The distribution types of Na^+-K^+ ATPases were complex; nevertheless, most of the protein molecules were localized in several restricted domains. To determine the level of Na^+-K^+ ATPases clustering, we utilize the Ripley's *K*-function analysis on the localization data established as described above. Ripley's *K*-function measures the degree of clustering of a spatial point pattern relative to a random distribution by counting the number of encircled localizations within the circles. The Ripley's *K*-function is calculated as:

$$K(r) = A\sum_{i=1}^{n}\sum_{j=1}^{n}\left(\frac{\delta_{ij}}{N^2}\right)$$

where $K(r)$ is the *K*-function, A is the image area, N is the total number of localized points, δ_{ij} is the distance between the i-th and j-th points, and r is the spatial scale. Here, if δ_{ij} is less than r, the value will be zero. In principle, the number of localized points encircled in different circles of r can be counted. Nevertheless, the *K*-value scales linearly with area and radius, given by the *L*-function:

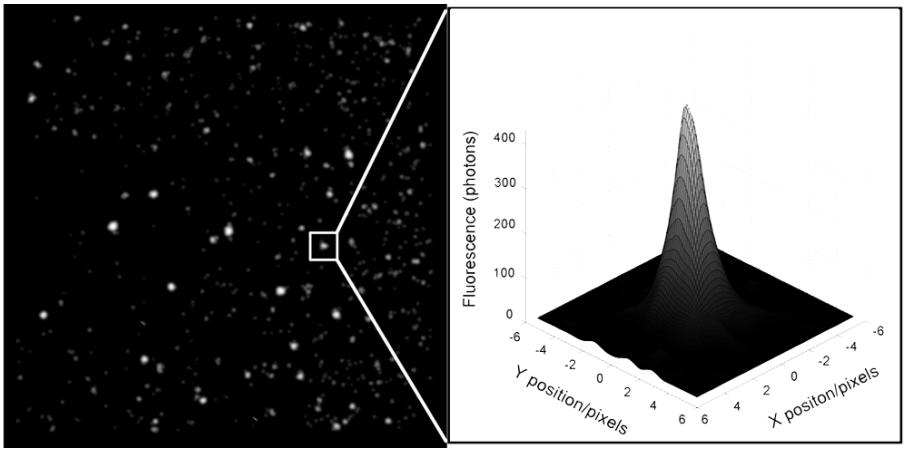
$$L(r) = \sqrt{\frac{K(r)}{\pi}}$$

Therefore, the higher value of $L(r)$ increases at any given r, the more localized points are circled. The K-function can be further transformed to the so-called H-function, represented as:

$$H(r) = L(r) - r$$

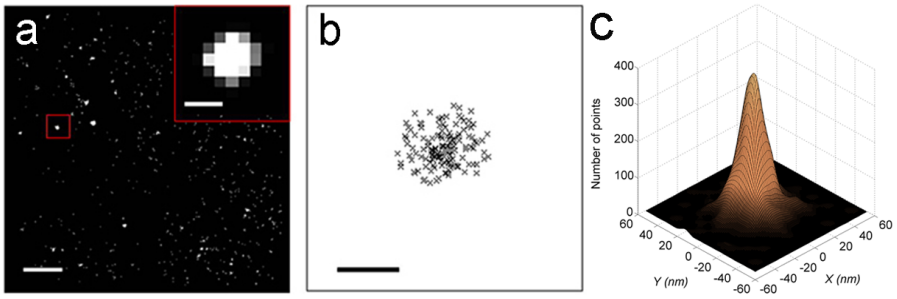
For a completely spatially randomness (CSR), the value of $H(r)$ will be zero for all r. Positive values of $H(r)$ indicate that the localized points can be classified as clusters. If the value of $H(r)$ is negative, fewer localized points have been encompassed if they were randomly distributed. Thus, $H(r)$ can be defined as a measurement of the $K(r)$'s deviation from CSR at each radius. The maximum value of r, corresponding to the inflection points in $H(r)$ curve, indicates the radius of a circle area in which a centered point on average includes the most points. Edge effects must be correctly weighted to remove the bias and confidence intervals should be calculated to confirm the value is not due to the randomness.

Supplementary Figure 1



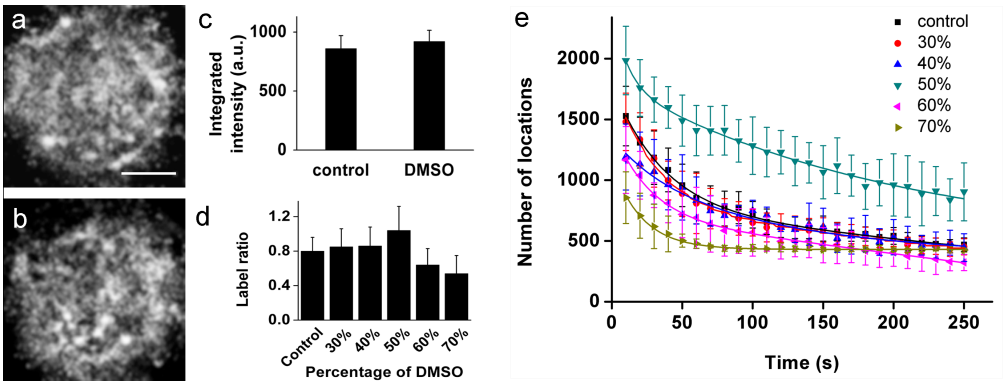
Supplementary Figure 1. The localization principle of single fluorescent molecules during *d*STORM imaging. The resolution of *d*STORM is limited by the localization accuracy of individual switches of dye molecules during each switching cycle. In our experiment, the antiATPase-Cy5 molecules were sufficiently dispersed in reaction solution and then immobilized on the cleaned coverslips before imaging. Each acquired fluorescence image frame (left) from a single switch give a PSF. The PSFs are commonly assumed as 2D Gaussian distributions and Gaussian mask approach is used to calculate the center of mass of the PSFs, weighted by the number of photons detected in each frame (right).

Supplementary Figure 2



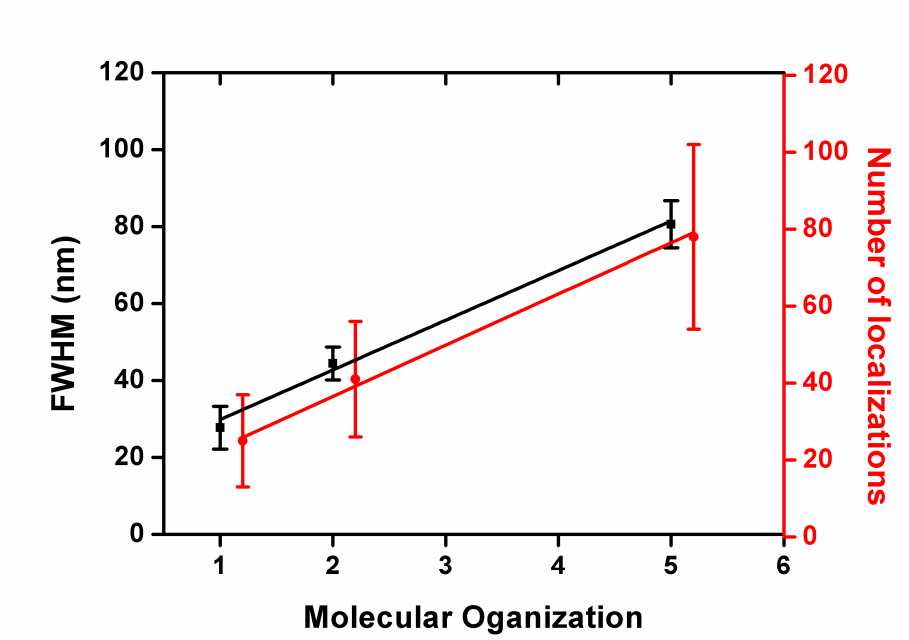
Supplementary Figure 2. Determination and analysis of the localization precision of single Cy5-Ab. (**a**) Adequately diluted anti-ATPase-Cy5 molecules (≈ 6.25 nM) were deposited on the exposed inner membrane of hRBC fixed on APTES-coverslips and conventional fluorescence image shows the typical emission pattern of a single antiATPase-Cy5 molecule. (**b**) Repetitive localizations from single antiATPase-Cy5 molecule between its fluorescent and dark states were represented and accurate positions were plotted after sample drift correction. (**c**) Aligned two-dimensional distribution of localizations from 300 anti-ATPase-Cy5 molecules demonstrates a spatial resolution of 30 ± 14 nm. Scale bars: 2 µm for **a** and 500 nm for red box in **a**, 50 nm for **b**.

Supplementary Figure 3



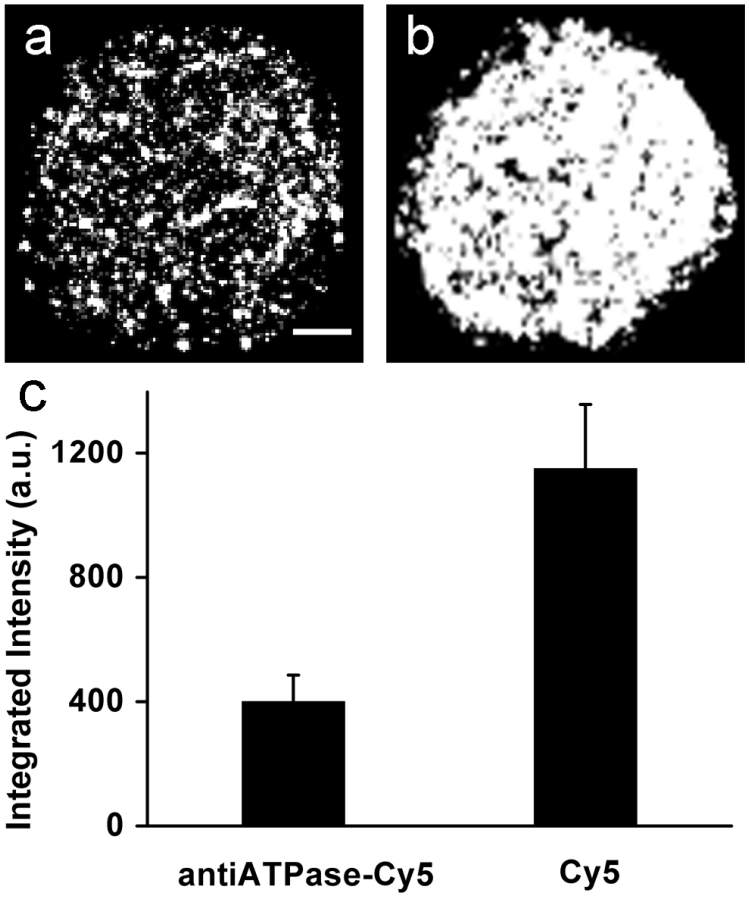
Supplementary Figure 3. Optimization of functionalization of ATPase antibody with Cy5-NHS. In order to protect the NHS group of Cy5 dye from hydrolysis, a different dose of DMSO was added into the reaction solution and the statistics were derived from 20 different samples. The result of staining experiment performing in the intact inner membrane of hRBC showed that the presence (**a**) or the absence of DMSO (**b**) didn't significantly affect the combination between antibodies and membrane proteins. (**c**) The comparison of integrated fluorescence intensity in the intact inner membrane of normal (control) or DMSO treated hRBC. (**d**) The labeling ratio of Cy5-NHS and ATPases antibody in different dose of DMSO. It had been determined that the presence of 50% dose of DMSO in the reaction buffer was optimal for the combination of dyes and antibodies. (**e**) The influence of *d*STORM imaging was indicated by the number of localizations for different DMSO conditions at various time points after staining. It shows the significant effect on antiATPase-Cy5 reaction in the percentage of 50% DMSO. Scale bars, 2 μm for **a**, **b**.

Supplementary Figure 4.



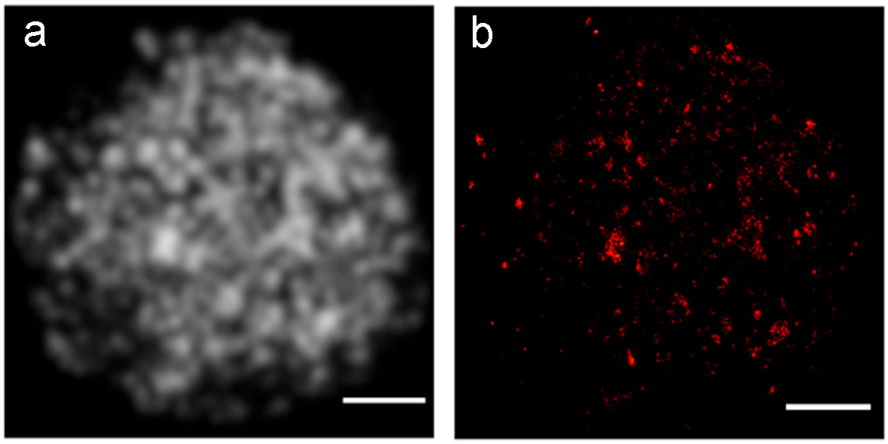
Supplementary Figure 4. The mean FWHM values of three types of immunoglobulins which were determined by *d*STORM imaging in two dimensions show a good linear relation as well as the localization numbers for each dye-labeled immunoglobulin.The mean FWHM values and the localization numbers for each sample obviously increased corresponding to the molecular organization so we could roughly estimate the number of dye-labeled antibody molecules within a complex cluster based on this statistical data.

Supplementary Figure 5



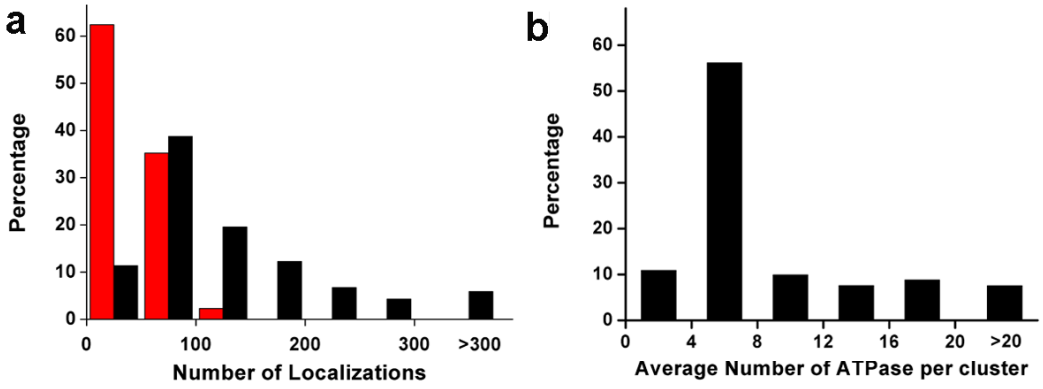
Supplementary Figure 5. Fluorescence imaging of inside-out hRBC membranes stained with anti-ATPase-Cy5 (**a**) or Cy5 (**b**). (**a**) It can be seen that only the ATPase domains were labeled with anti-ATPase-Cy5. (**b**) In contrast, almost all the proteins on the inner membrane were stained by Cy5. Therefore, the ATPases antibody labeled with Cy5 can specifically recognize the epitopes. (**c**) Quantitative results show that the integrated fluorescence intensity in anti-ATPase-Cy5-labeled cells is much lower than the Cy5-labeled cells. Data were collected from 18 cells. Scale bars in **a,b**: 2 μm.

Supplementary Figure 6



Supplementary Figure 6. Comparison between conventional TIRF (a) and *d*STORM (b) images of Na^+-K^+ ATPases distributions in the inner membrane of hRBC. It can be distinctly seen that the application of the *d*STORM imaging approach significantly improve the effective resolution as compared to conventional imaging (~30 nm vs 250 nm, respectively).

Supplementary Figure 7



Supplementary Figure 7. Estimated probability of residency of Na^+-K^+ ATPases in single Na^+-K^+ ATPases cluster domains using *d*STORM. **(a)** Histogram of the number of localizations for single antiATPase-Cy5 molecule on coverslips (*red bars*) and single Na^+-K^+ ATPases cluster domains on hRBC labeled with antiATPase-Cy5 (*balck bars*). **(b)** Histogram of the average number of Na^+-K^+ ATPases per cluster domain on hRBC, which were calculated via dividing the number of localizations per cluster by the average number of localizations from a single anti-ATPase-Cy5.