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Atomic Force Microscopy Demonstration of Cytoskeleton Instability in Mouse Erythrocytes with Dematin-Headpiece and β -Adducin Deficiency

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Abstract

The pattern of disassembly of the cytoskeletal network of murine erythrocytes with deficiency of either dematin-headpiece or β -adducin or both proteins were investigated using atomic force microscopy. A heterogeneous complex structure with fine filament features and coarse features was observed in the cytoskeleton of wild type mouse erythrocytes, whereas a significant amount of rearrangement and aggregation occurred in the mutants, particularly in the cells carrying double gene mutations. These results are consistent with the cellular and biochemical phenotype of the mutant cell membranes as being more fragile due to weakened vertical connections with the plasma membrane.

Keywords

Atomic force microscopy; Erythrocyte; Cytoskeleton; Dematin; Adducin

INTRODUCTION

Atomic force microscope (AFM) imaging continues to provide unique insights about the structure and properties of biological membranes at sub-micron spatial resolution (Frederix et al., 2009; Muller, 2008). A goal is to bridge the gap between structural understanding from high resolution electron microscopy studies of highly processed specimens (Byers and Branton, 1985; Liu et al., 1987) and functional understanding obtained from biochemical and biological tests performed under physiological conditions (Bennett and Gilligan, 1993; Hassoun et al., 1997; Peters et al., 1996; Shi et al., 1999; Wandersee et al., 2003).

In samples where the molecules are arranged in a repetitive, regular or crystalline pattern, AFM has provided resolutions that are comparable to TEM (Muller et al., 1998; Muller et al., 2002). However, for specimens with irregular and complicated features, such as the

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erythrocyte cytoskeleton, it is challenging to obtain precisely repeatable high-resolution images and common image analysis techniques like Fast Fourier Transform filtering are generally difficult to apply (Frederix et al., 2009; Swihart et al., 2001; Takeuchi et al., 1998). Newly developed AFM techniques such as immuno-recognition imaging show much promise (Jiang et al., 2009; Shan et al., 2010), but specimen preparation and image analysis methods still require significant scrutiny and advancement.

In previous work we developed a gentle method for the specific attachment of human erythrocyte membranes onto glass coverslips via the exposed carbohydrate moiety on glycophorin C transmembrane protein (Liu et al., 2003) allowing the attached cytoskeleton network to be exposed and directly interrogated with AFM either in liquid or in glutaraldehyde-reinforced air-dried form. We showed that it was possible to use detailed image analysis methods to quantitatively estimate ligand binding constants of certain transmembrane proteins in the supported specimen (Liu et al., 2005).

In this work we show the first report of AFM imaging of the morphology and structure of the intact, glutaraldehyde-reinforced murine erythrocyte cytoskeletons still attached to their plasma membrane. Applying a sequence of image analyses, we also quantitatively compare the imaged cytoskeleton network structures of murine erythrocytes altered by site-specific genetic modifications. The results demonstrate that the AFM can provide useful information about differences between genetically modified erythrocyte cytoskeletons, and could be developed into a technique that reflects the relative severity and nature of structural consequences of specific mutations.

Murine erythrocytes contain similar major cytoskeleton components as human erythrocytes, including spectrin, actin, protein 4.1, dematin, and adducin (Gilligan et al., 1999; Khanna et al., 2002; Yi et al., 1997) and the cytoskeletal architecture is assumed to be similar, comprising of an approximate hexagonal network of spectrin tetramers, connected to one another at junctional complexes (Byers and Branton, 1985; Liu et al., 1987; Shen et al., 1986; Ursitti et al., 1991; Ursitti and Wade, 1993). Both dematin and adducin are actin-binding and bundling proteins that are located at the spectrin-actin junctional complex in human cytoskeletons (Bennett, 1990; Bennett and Gilligan, 1993). Though individually dematin and adducin exert modest effects on the structural resilience of the erythrocyte membrane, as revealed by their gene knock-out phenotypes in mice (Gilligan et al., 1999; Khanna et al., 2002), mutant mice lacking both dematin-headpiece domain and β -adducin display severe defects in erythrocyte shape, membrane instability, and hemolysis (Chen et al., 2007). Architectural changes in the cytoskeleton structure accompanying these observations could help understand the mechanism by which these features arise.

MATERIALS AND METHODS

Generation of Knock-out Mice

Dematin headpiece null mice (DKO) and β -adducin null mice (AKO) were generated through gene target deletion as described previously (Gilligan et al., 1999; Khanna et al., 2002). Double knockout mice (DAKO) were generated by crossing of DKO and AKO mice (Chen et al., 2007). All mice used were of C57BL/6J and 129/SVJ mixed genetic background. Generation of knock-out mice have been verified with PCR and Western blots analysis and reported earlier (Chen et al., 2007). The RBC samples used in the current study were obtained from the same sets of mice

Erythrocyte Attachment and Cytoskeleton Exposure

Peripheral blood was collected from wild type (WT), DKO, AKO and DAKO mice in sodium-heparin tubes. All erythrocyte samples were immediately washed with PBS (145

mM NaCl, 5 mM KH₂PO₄/K₂HPO₄, pH 7.4), stored on ice until used within 5 hours. Cell attachment on surface-modified glass coverslips was performed as previously described (Liu et al., 2003). Briefly, erythrocytes were attached to chemically modified glass coverslips coated with erythroagglutinating phytohemagglutinin (E-PHA). Excess cells were washed away and those erythrocytes attached to the lectin-coated coverslips were sheared open by subjecting the attached cells to a stream of a hypotonic solution of 5 mM KH₂PO₄/K₂HPO₄ and 70 mM NaCl, pH 7.4 (5P7-70) at a rate of ~ 0.75 mL/s for 20 s through a 23-gauge needle. The coverslips with mounted membranes were rinsed gently with 5P7-70 to remove loose fragments and other cell contents. All specimens were fixed with 0.05% glutaraldehyde (freshly prepared in 5P7-70) for at least 30 min on ice, and rinsed with 5P7-70 and deionized water to remove excess reagents before air drying in a covered Petri dish. To prepare unopened erythrocytes ghosts, the shear step and the washing step were replaced by incubating coverslips with attached cells in 5P7-70 for 5 min.

Atomic Force Microscopy

AFM topographic images of the exposed cytoplasmic surface of the glutaraldehyde-fixed erythrocyte membranes were recorded with a Nanoscope III Multimode SPM™ system (Veeco, Santa Barbara, CA). An E-scanning tube with total scan size of 15 μm was used. All AFM imaging was performed in tapping-mode at room temperature in PBS solution or in air. Standard Silicon Nitride Probes NP-20 (Veeco, Santa Barbara, CA) with a tip radius of 20–60 nm and a spring constant $k = 0.12$ N/m were used for imaging in PBS solution. NanoSensors TappingMode™ Etched Silicon Probes OTESPA (Veeco, Santa Barbara, CA) with a tip radius of 5–10 nm, a spring constant $k = 20$ –100 N/m and a resonant frequency $f_0 = 200$ –400 kHz were used for imaging in air. The AFM system was calibrated following the manufacture's recommended procedure. Briefly, the sensitivity and linearity of the scanner was calibrated with a NanoGauge™ NGR-1 calibration reference (NanoDevices, Santa Barbara, CA); The X-Y calibration was performed using Capture Calibration. Fine-tuning was performed to correct both X-Y and Z measuring accuracy. The z-piezo sensitivity was calibrated to be ~7.56 nm/V. In liquid, the working frequency of the cantilever was selected manually at the first major resonant peak, typically around 6–30 kHz. The working frequency in air was selected automatically by the system and the typical values were around 300 kHz. In one typical experiment, the free amplitude of the cantilever was 1.5 V and amplitude set point was adjusted to 1.08 V for imaging in air. Topographic images with a resolution of 512 × 512 pixels were obtained at scan rate of about 1–2 Hz for scan sizes ranging from 15 μm to 0.5 μm.

Image Analysis

AFM topographic images (1 × 1 μm² image dimension) were processed and analyzed for height profile, histogram and bearing (grain size, volume) analysis using Nanoscope III 5.12r3 software (Veeco, Santa Barbara, CA). Pixel height histogram (H) was defined as follows:

$$H(\%) = n_p(z) / N_p \times 100\% \approx \text{Probability density of the pixel height} \quad (1)$$

where $n_p(z)$ is the number of pixels in a topographic image with a corresponding height of z nm, and N_p is the total number of pixels in the topographic images. The histograms generated by Nanoscope III were fitted with Gaussian peaks in Origin Pro 7.0 (OriginLab, Northampton, MA) to calculate peak positions. Topographic images were thresholded at the pixel height corresponding to the central position of the first Gaussian peak of the height distribution histogram, which was assigned to be the average height level of the plasma membrane above the coverslip glass surface. Grain size framed by cytoskeleton filaments

and cytoskeleton volume was then calculated with bearing analysis in Nanoscope III (Liu et al., 2005; McMaster et al., 1996).

Skeletonization analysis of the images was performed with Fovea Pro v.3 software (Reindeer Graphics, NC) to generate cytoskeleton connectivity maps. A Euclidean Distance Map (EDM)-constrained function using a relatively isotropic set of erosion criteria was applied on topographic images:

$$Skeleton(Z) \iff \exists p_1, p_2 \in \partial Z | p_1 \neq p_2 \text{ and } d_z(z, \partial Z) = d_z(z, p_1) = d_z(z, p_2) \quad (2)$$

where Z is the boundary of the image feature Z , and d_z is the Euclidean distance:

$$d_z[(x_1, y_1), (x_2, y_2)] = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2} \quad (3)$$

in which (x_i, y_i) are the coordinates of a pixel p_i in the image. An EDM Distance function defined as:

$$[D(f)](x) = \min \{d_z(x, y) | f(y) = 0\} \quad (4)$$

was used to give each pixel within the feature a brightness value proportional to its distance from the nearest edge (Russ, 1989; Soille, 1999). In general terms, the algorithm for skeletonization was as follows: EDM was first constructed for the topographic image after thresholding at the first Gaussian peak; then, a “wave front” propagation approach was applied to permit the skeletal erosion to take place in the new areas covered by the front as the wave propagated. This process was continued on each brightness level until no additional erosion took place. Based on the number of neighbors the skeleton pixels had in the final connectivity map, jointing nodes of the skeleton lines could be identified. Individual skeleton branches corresponding to cytoskeleton filaments were separated at the jointing nodes and the number of filaments within a specific area calculated.

RESULTS

Overview of AFM images of WT membranes

Fig. 1A shows a typical AFM image of the cytoplasmic surface of WT membranes attached to a lectin-functionalized glass coverslip exposed by shear washing using hypotonic buffer imaged in PBS solution. The brighter semicircle at the edge of the central feature is the remaining edge of the upper membrane that was not removed, while the darker grey central area is the exposed cytoplasmic surface of the cytoskeleton network resting on top of the plasma membrane underneath. The attached WT (both in PBS and in air) specimen show a similar footprint size of $\sim 5.5 \mu\text{m}$ in diameter (Table 1), close to the reported value of the diameter of mouse erythrocytes (Gregory, 2000), indicating little lateral shrinkage or expansion of the membrane occurred during attachment to the coverslip. A similar result, summarized in Table 1, was obtained with the AKO and DKO mutants with the footprint of the DAKO mutant being slightly smaller. The attached pieces of WT membranes imaged in PBS had an average thickness of $\sim 25 \text{ nm}$ as indicated by the section profile (Fig. 1A, Table 1), which is close to the thickness of the human erythrocyte plasma membrane plus the cytoskeletal network as previously reported (Shohet and Bicknese, 1993; Takeuchi et al., 1998). On the other hand, the thickness at the sample edge with un-removed upper membrane is $\sim 50 \text{ nm}$ (square marked in the height profile below the AFM image in Fig. 1A), twice the thickness of the single membrane.

High-resolution images of erythrocyte cytoskeleton in aqueous medium (i.e., Fig. 1C) are challenging to obtain because of the inherent compliance of the biological specimen

compared to the much stiffer AFM probe (Berdyeva et al., 2005; Takeuchi et al., 1998). Therefore we used a glutaraldehyde treatment procedure that allows us to reinforce the cytoskeleton specimen prior to air-drying and imaging to capture the configuration of complex structures with higher resolution (Fig. 1B and 1D). The increase in image resolution of air-dried samples is accompanied by a reduction in the specimen thickness due to dehydration (Fig. 1B and Table 1).

In contrast, images of the outer surface of the cell membrane (inset Fig. 1D) show a very smooth surface with little fine details. These differences are supported by the cross sectional height profile traces shown in Fig. 2D.

Overview of AFM images of mutant membranes

Fig 2 displays the AFM morphology of AKO (Fig 2A), DKO (Fig 2B) and DAKO (Fig 2C) mutant membranes prepared identically and imaged, as were the WT membranes shown in Fig. 1D. Visually, the network organizations appear less uniform than WT with the greatest disruption observed in samples of the DAKO cytoskeleton. From an analysis of the cross sectional lines it is found that the membrane thickness of WT, AKO and DKO samples are similar at ~8.5 nm, while the membrane thickness of DAKO erythrocytes is slightly thicker ~10.5 nm (Table 1). Fine structure was observed in the images of WT and AKO specimens but absent in others.

Comparison of WT and mutant higher resolution images

At a higher magnification ($1 \times 1 \mu\text{m}^2$ scan) (Fig. 3), it is very clear that the murine WT cytoskeleton consists of both coarse features (arrowheads) and fine network filaments (arrows). The fine “meshes” are readily seen spanning the “window” regions of the coarse features. Here, “mesh” refers to small single dark area framed by thin filaments (dark holes near arrows in WT and AKO), while “window” refers to large gray area (vertical open arrows) at the intermediate height level framed by coarse white features at the highest height level. For the AKO mutant, a network structure similar to the WT was observed, but the fine filaments are more obscured. No fine filament structure was observed in DKO or DAKO specimens. Few deep holes were observed indicating the underlying plasma membrane remained intact in all cases.

An image of WT murine cytoskeleton prepared similarly was obtained with a ($500 \times 500 \text{ nm}^2$) scan (Fig. 4A). Individual cytoskeleton filaments were clearly seen. Arrowheads in the upper left quadrant of the image point to a typical filament and another identified in the height profile below is ~6.8 nm. Their apparent length is ~20–40 nm. These values are similar to the dimensions of spectrin filaments reported from intact, unspread cytoskeletons of human erythrocytes imaged with TEM (Ursitti et al., 1991). The size of the mesh framed by the thin filaments (horizontal arrows in the lower left and right quadrant of the image and in the height profile below) is about 20 nm. Only a few filaments are seen at similar magnification in images of AKO cytoskeletons, and none at all in DKO or DAKO cytoskeletons (Fig. 4B), but the coarse features remain.

Histogram analysis of WT and mutant high resolution images

Histogram analysis was performed on ($1 \times 1 \mu\text{m}^2$) images from each of the four specimen types to quantify the extent and statistical relevance of the structural differences among the specimens (Fig. 5, reproduced from (Chen, et al. 2007)). The pixel height histograms obtained from these specimens contain three distinct peaks (Fig. 5A). Histogram-topography correlations using the Nanoscope III software indicate that the peak 1 correspond to pixels from the deepest parts (arrows) in the cytoskeleton topography of Fig. 3, and are at height close to that expected for the cytoplasmic surface of the plasma membrane. The remaining

peaks (2 and 3) represent components of the cytoskeleton that occupy, on average, two different height zones. Peak 2 represents the height zone largely occupied by the visible fine filaments, and the Peak 3 to the tallest coarse features of the imaged network.

In Fig. 5B is a detailed view of the Peak 1 region of Fig. 5A showing the relative contribution of these features to the overall histogram. In wild type and AKO specimens a greater percentage of the height features correspond to the height of the cytoplasmic surface of the plasma membrane. In DKO and DAKO mutants the cytoplasmic surface of the plasma membrane is obscured and so those height features comprise a smaller percentage of the overall topography. As seen from Fig. 4B, these are also the two mutants in which the fine intermediate height filament mesh is absent.

Gaussian fitting of a typical multi-peak histogram obtained from WT images is shown in Fig. 5C. Peak separations, D(2-1) and D(3-1), represent the average elevation of the fine filament features and the coarse aggregates from the cytoplasmic surface of the plasma membrane, respectively. These values are listed in Table 2 (adapted from Chen et al., 2007) along with the same measurements for the three mutant specimens. No significant difference for D(2-1) or D(3-1) elevations exists among WT, AKO and DKO specimens. The height (D3-1) coarse features in the DAKO specimens are significantly greater.

Grain analysis was used to measure the area expanse of the exposed cytoplasmic surface of the plasma membrane in the images. These features were isolated by a two-step thresholding method. In the first step, the highest parts of the image were removed by thresholding the image at the height level corresponding to the center position of the middle peak in the histogram corresponding to the intermediate height filaments (Fig. 5). A second thresholding to remove features at and below the heights of the cytoplasmic surface of the plasma membrane was performed at the center position of peak 1. The images in Fig. 6B were then reconstructed from the thresholded data set. The black and grey areas represent open expanses with height features between the height of the cytoplasmic surface of the plasma membrane and the intermediate height filament or aggregates. The area occupied by these grains was smallest for WT specimens and became progressively larger for AKO->DKO->DAKO mutants (Table 2).

Skeletonization analysis of WT and mutant images

Skeletonization analysis was performed to evaluate the network architecture in typical images of the various specimens. As indicated by the name, skeletonization traces the “crest of the ridge” of topographic features in the image, and therefore includes both fine filaments and coarser features. Conventionally, lines obtained by skeletonization processing are assumed to be the spectrin tetramers or their aggregates. The crossing points represent the center of junctional complexes of the network filaments (Swihart et al., 2001; Takeuchi et al., 1998). In Fig. 7A, overlaid with its original image, is a representative result of skeletonization of the WT cytoskeleton. From the skeletonization map it is possible to obtain the filament density of ~ 800 per μm^2 , higher than the value of $\sim 300\text{--}350$ per μm^2 reported for the WT human erythrocyte cytoskeleton (Liu et al., 1987; McGough and Josephs, 1990; Vertessy and Steck, 1989). A significant decrease in the filament density from WT to AKO->DKO->DAKO mutant specimens was noted (Table 2), suggesting a marked breakdown of network connectivity.

Volume analysis of images

One of the unique advantages of the AFM technique is that the specimen volume information can be obtained from voxel analysis of the topographic image (Quist et al., 2000). This provides a means to compare specimen images containing roughly the same

protein content or area density. If, in a given set of images of the same type of specimen, the voxel value is similar, then other topographic differences are likely to be the result of rearrangement of components rather than problems associated with stretching of the membranes or loss of components to damages taking place during processing.

The cytoskeleton volume over a given scan area ($1 \times 1 \mu\text{m}^2$) was calculated by adding the total voxels lying above the plasma membrane surface (McMaster et al., 1996). These voxels were defined by thresholding everything below the elevation of the plasma membrane as described previously. As shown in Table 2, the total cytoskeleton volume in WT, AKO, DKO and DAKO specimens was roughly equivalent over identical image areas. This is expected since adducin and dematin proteins are a relatively small part of the entire cytoskeleton as compared to the dominant spectrin molecules.

A relatively constant cytoskeleton volume is observed among these specimens suggesting that the observed structural differences, i.e., change in height, expansion in coarse features, and decrease in filament density are primarily due to the network reorganization and not variance in the protein content among specimens. In previous biochemical studies no significant quantitative difference in protein content except for the deletion was noted (Chen et al., 2007).

DISCUSSION

Factors affecting the cytoskeleton morphology

The agreement between the footprint size of attached WT and mutant specimens and the known diameters of intact cells of each type suggests that there is no significant stretching of the membranes at the time of cell attachment to the coverslips. If this is true, then there are three possible explanations for the observed topological differences between WT and mutant murine cytoskeletons: 1) the morphological differences are intrinsic to the membranes at the time of attachment, 2) they are created or worsened as a consequence of shear-opening process, and that state is later preserved by glutaraldehyde fixation, or 3) they are a consequence of air drying of the glutaraldehyde-treated specimen after it is attached to the coverslip.

Let us address the last item first. In our previous work in human WT erythrocytes (Liu et al., 2005), we suggested that glutaraldehyde fixation prior to air drying is a way to preserve the liquid-phase network architecture during the air-drying process. This assumed there was no damage to the surface attached cytoskeletons during shear opening, since the images that were obtained were consistent with many reported high-resolution images of unstretched human WT cytoskeletons obtained by AFM and TEM. Assuming this is correct, we can compare the independently measured strength of unfixed WT membranes to the shear forces experienced by the cytoskeletons during air-drying. If the shear forces existing during specimen drying are not strong enough to rupture cells, then it is unlikely that a glutaraldehyde fixed membrane (even with cytoplasmic surface of the cytoskeleton exposed) would rupture either.

The critical shear modulus of normal unfixed WT membranes has been measured to be $\mu \sim 10 \mu\text{N/m}$ (Boal, 1994; Li et al., 2005; Mills et al., 2004). In contrast, the drag stress experienced by a microparticle being propelled by the receding edge of an evaporating water droplet on a hydrophilic surface was estimated to be only $\sim 0.2 \mu\text{N/m}$ (Jung et al., 2010). This rough comparison suggests that the forces experienced during liquid drop evaporation at the plane of the specimen surface are much weaker than the forces needed to rupture the membranes of intact cells. Since our mounted specimens are further reinforced with

glutaraldehyde fixation, membrane disruption of the specimens via mechanism 3) appears to be even more unlikely.

Mechanisms 1) and 2) are more difficult to distinguish. If shear forces applied to remove the upper membranes are too strong they might permit horizontal displacement of a cytoskeleton network whose connection to junctional complexes tethered to the coverslip are weakened and lead to major topology change of the structure (Hategan et al., 2003). The glutaraldehyde treatment step applied immediately after shear opening would preserve the rearranged configuration. Consistent with this idea is the fact that the mutants intended to have increasingly weaker vertical connections (Khan et al., 2008) and greater susceptibility to shear lysis (Chen et al., 2007; Gilligan et al., 1999; Khanna et al., 2002) also exhibit increasing topological differences compared with WT.

Comparison of WT murine and human cytoskeletons

The current study is one of the first reports about the morphology and structure of the intact glutaraldehyde-reinforced mouse erythrocyte cytoskeletons still attached to its plasma membrane. Generally the cytoskeletal structure of murine erythrocytes is assumed to be similar to that found in human cells (Byers and Branton, 1985; Liu et al., 1987; Shen et al., 1986; Ursitti et al., 1991; Ursitti and Wade, 1993) and so it is a reasonable model for genetic manipulation to study structural defects in pathological states found in humans (Evans, 1989; Mohandas and Chasis, 1993). However details of the AFM-derived topography of exposed WT human cytoskeletons are distinct from that of the WT murine specimens interrogated here (Liu et al., 2005). For example, in the WT murine erythrocytes a complex structure comprised of both fine filaments and higher coarse features was observed, but in WT human specimens fine filament architecture was not observed. The significance of these differences is not fully understood at this stage.

Advantages of a combined AFM image analysis approach

The combined application of pixel histogram, grain size, volume and connectivity analysis, while taking advantage of AFM extra resolution in the Z direction to evaluate the volume of the imaged specimens, makes it possible to systematically compare the subtle structural changes accompanying genetic mutation, which previously has not been fully explored. For a given type of specimen prepared using the same procedures similarity of voxel values is a way to distinguish between naturally occurring architectural variations in the samples and more significant damage caused by processing. Once a comparable set of specimen images is identified, then more advanced image analysis procedures can be applied to distinguish architectural differences. Among these are pixel height histogram, grain size and skeletonization analyses. Together these methods can quantitatively describe the degree of architectural change in an imaged specimen without having to identify the chemical nature of each component in the specimen. Their application provides an objective method to screen for significant differences between specimens, and to evaluate the statistical relevance of any differences.

While the results obtained are consistent with biochemical studies reported previously, this study suggests a need for further high-resolution study of WT murine erythrocytes and perhaps those of other species used as a surrogate for human erythrocytes in genetic modification studies.

CONCLUSIONS

Cytoskeletal networks of mouse erythrocytes lacking either dematin headpiece or β -adducin or both proteins were imaged using AFM. A heterogeneous complex structure was observed

in the cytoskeletal network of WT mouse erythrocyte. A large amount of network reorganization was observed in the cytoskeleton of mutant cells, consistent with the biochemical and mechanical evidence showing the fragility of mutant erythrocyte membranes. A comprehensive image analysis indicates that the pattern of disassembly observed in mutant erythrocyte cytoskeleton could be related to the extent of disruptions of vertical interactions between the cytoskeleton and plasma membrane. This work demonstrates that AFM imaging with a logical application of image analysis methods applied to appropriately processed specimens can provide useful information about complex cellular structures that can be compared with other data, but care must be taken to scrutinize all steps of specimen preparation before firm conclusions are drawn.

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Figure 1. AFM topographic images of WT mouse erythrocyte membrane after shear-opening (A) WT membrane imaged in PBS solution. (B) WT membrane imaged in air. Air-dried membranes are compressed in height, and finer details are observed. Images from AKO and DKO samples show a similar morphology trend. Corresponding height profiles below each image indicate variations in membrane thickness and size across the white line marked in the images. Scale bars: 2 μm . (C, D) $2.5 \times 2.5 \mu\text{m}^2$ scans of WT membrane in PBS (C) and in air (D). (D, inset) Image of the extracellular surface of the cell membrane show a very smooth surface with little fine details. Scale bars: 500 nm.

Figure 2. AFM topographic images of mutant mouse erythrocyte cytoskeleton
 (A–C) Cytoskeleton of AKO, DKO and DAKO imaged in air. Images from WT (Fig. 1D), AKO and DKO show similar morphology with relatively flat surface and two distinct height levels, while more dissembled and irregular features were observed on the DAKO sample. (D) Representative height profiles show higher density of cytoskeleton filaments in WT, AKO and DKO compare to that in DAKO, while that from WT in PBS and WT extracellular surface is more smooth and featureless. Scale bars: 500 nm.

Figure 3. AFM images ($1.0 \times 1.0 \mu\text{m}^2$ scans) of mouse erythrocyte cytoskeleton obtained in air Fine filament features are observed in WT erythrocyte cytoskeleton. AKO cytoskeleton has similar morphology as WT but fewer fine details and more coarse features. More aggregation with no fine filament features appears in DKO cytoskeleton. DAKO cytoskeleton shows more aggregation and network damage. The shaded bar in the middle of the image shows the vertical heights depicted by various grayscale levels in the image. Arrowheads, coarse features; arrows, fine filaments; vertical open arrows, intermediate height aggregates. Scale bars: 200 nm.

Figure 4. AFM images ($500 \times 500 \text{ nm}^2$ scans) of mouse erythrocyte cytoskeleton obtained in air (A) WT cytoskeleton with details of fine filaments marked by arrows. Height profile below panel A shows the relative height of the coarse feature ($\sim 7.5 \text{ nm}$), the thickness of the fine filaments ($\sim 6.8 \text{ nm}$) and the mesh size framed by thin filaments ($\sim 20 \text{ nm}$ in diameter). (B) Cytoskeleton in AKO, DKO, DAKO and corresponding height profiles below each image. Only a few fine filaments are seen in AKO cytoskeleton, but none at all in DKO or DAKO cytoskeleton. Scale bars: 100 nm.

Figure 5. Histogram of AFM images ($1.0 \times 1.0 \mu\text{m}^2$ scans) (reproduced from (Chen, et al. 2007)). (A) WT and AKO samples have similar peak distributions revealing more structural details as indicated by the extra small peaks at lower pixel height. The DKO histogram demonstrates a larger peak at higher pixel height, suggesting increased aggregation of the cytoskeleton filaments, although the average apparent cytoskeleton height (distance between peak 2 and peak 3) is not significantly changed. The average apparent cytoskeleton height in DAKO is much larger than that of WT, indicating an increase in filaments aggregation. (B) Detailed view of the peak 1 region of (A) showing the alignment of peaks of the cytoplasmic surfaces. (C) Representative Gaussian fittings of a typical histogram from WT topographic image. Peak separations, D(2-1) and D(3-1), represent the average elevation of the fine filament features and the coarse aggregates from the cytoplasmic surface, respectively.

Figure 6. Grain size analysis of mouse cytoskeleton mesh size and window region
 (A) Thresholding at the height level (white lines) corresponding to the center position of histogram peak 2. (B) Isolated window regions (gray) and cytoskeleton meshes (black).
 Scale bars: 200 nm.

Figure 7. Skeletonization analysis of mouse erythrocyte cytoskeleton connectivity

(A) Cytoskeleton connectivity map overlaid with the original topographic image from WT sample. (B) The skeletonized lines in the connectivity map correspond to cytoskeleton filaments, while the junctional nodes represent places in the image where the filaments intersect. Scale bars: 200 nm.

TABLE 1

Dimensions of erythrocyte membranes as measured with AFM

Sample	Membrane diameter, μm (n)	Membrane height, nm (n)
WT (in PBS)	$5.95 \pm 0.32^*$ (5)	$24.87 \pm 2.85^{\wedge}$ (5) $53.47 \pm 2.31^{\&}$ (5)
WT	$5.54 \pm 0.46^*$ (15)	$8.93 \pm 1.20^*$ (23)
AKO	$5.59 \pm 0.43^*$ (12)	$8.24 \pm 0.89^*$ (22)
DKO	$5.43 \pm 0.25^*$ (13)	$8.45 \pm 1.71^*$ (19)
DAKO	$4.65 \pm 0.27^{\#}$ (13)	$10.54 \pm 1.42^{\#}$ (13)

(n) = number of cells measured; each value represents the mean $\pm$ S.D.* indicates no significant difference between the data sets according to one-way ANOVA test at 0.05 significant level ($p > 1$);#, &, $\wedge$ indicates significant difference of data set from the others ($p < 0.01$).

TABLE 2

&. Characteristic parameters of mutant cytoskeleton

Sample	WT	AKO	DKO	DAKO
D(2-1)	$3.5 \pm 1.2^*$	$3.7 \pm 1.4^*$	$3.8 \pm 1.5^*$	$4.8 \pm 1.8^*$
D(3-1)	$7.4 \pm 1.8^*$	$7.1 \pm 2.0^*$	$7.9 \pm 2.4^*$	$10.5 \pm 2.7^\#$
Volume (10^6 nm^3)	$4.78 \pm 0.43^*$	$4.13 \pm 0.57^*$	$5.07 \pm 0.72^*$	$4.53 \pm 0.67^*$
Grain size (10^4 nm^2)	$0.26 \pm 0.18^*$	$0.32 \pm 0.18^*$	$0.44 \pm 0.27^*$	$0.93 \pm 0.39^\#$
Filament no. (per μm^2)	$807 \pm 93^*$	$674 \pm 50^*$	$109 \pm 25^\#$	$74 \pm 20^\#$

& adapted from (Chen, H., et al., 2007)

* indicates no significant difference between the data sets according to one-way ANOVA test at 0.05 significant level ($p > 0.4$);

indicates significant difference of data set from the others ($p < 0.01$).