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研究課題名（和文）Imaging myosin-driven stress fiber contraction with molecular resolution by high-speed AFM

研究課題名（英文）Imaging myosin-driven stress fiber contraction with molecular resolution by high-speed AFM

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研究成果の概要（和文）：このプロジェクトでは、細胞のデルーフィングとHS-AFMイメージングを組み合わせることで、収縮したアクチンストレスファイバー（SF）を、本来の細胞環境内でほぼ分子レベルの分解能でイメージングする方法を確立した。開発したHS-AFM用超広域スキャナーにより初めてネイティブSF全体の分子分解能マップが作成され、SFの超微細構造に関する新たな知見が得られた。さらに、SF収縮中の個々の非筋ミオシンIIモーターの配列と構造変化を初めて可視化することができた。これと並行して、SICMイメージングを脱ルーフ細胞の細胞内イメージングにも初めて応用し、SF収縮に関するさらなる構造的・力学的知見を提供した。

研究成果の学術的意義や社会的意義
アクチンストレスファイバー（SF）の収縮は、マトリックス接着、遊走、メカノセンシングなど、多くの細胞プロセスに影響を与える一方、SF機能の誤制御は、アテローム性動脈硬化症、骨粗鬆症、がんなどの疾患の原因となりうる。このプロジェクトで得られたSFの超微細構造とSF収縮を駆動する分子メカニズムに関する新たな知見は、SFの正常および異常機能についての理解を広げるものである。

研究成果の概要（英文）：By combining cell de-roofing and HS-AFM imaging, this project established a method to image contracting actin stress fibers (SFs) to near molecular resolution within native cellular environments. Achieving these goals required (1) the development of suitable de-roofing methods, (2) establishment of an ultrawide scanner system for large resolution HS-AFM imaging in combination with fluorescence microscopy, and (3) developing an optimized AFM tip design for imaging highly corrugated intracellular structures. Ultrawide HS-AFM scanning generated the first molecular-resolution maps of entire native SFs, providing novel insight into SF ultrastructure. Furthermore, arrangement and conformational changes of individual non-muscle myosin II motors during SF contraction could be visualized for the first time. In parallel, SICM imaging was also adapted for intracellular imaging in de-roofed cells for the first time, providing additional structural and mechanical insight into SF contraction.

研究分野：Bioimaging

キーワード：AFM　SICM　myosin　actin　stress fiber　contractility

１．研究開始当初の背景

Stress fibers (SFs) are large intracellular bundles of actin filaments found in a variety of non-muscle cells. SF contraction driven by embedded non-muscle myosin II motor proteins enables cells to exert forces against the extracellular surrounding, which is crucial for maintaining intracellular tension and resisting external deformation. SF-based contractility underlies numerous cellular processes, such as cell-matrix adhesion, migration, and mechanosensing, and changes in the direction or magnitude of SF-dependent forces can contribute to diseases such as atherosclerosis, osteoporosis and cancer. Like striated myofibrils, the contractile elements in muscle cells, SF display a striking sarcomeric organization featuring alternating bands of myosin II and α-actinin, and SFs have been called “intracellular muscles” as a result. However, despite these obvious similarities, there are important structural and functional differences and overall, the molecular architecture of the SF sarcomere is much less understood than its muscle cell counterpart. Likewise, the molecular mechanism driving SF contraction and sarcomere shortening are still incompletely understood. Better understanding the molecular processes driving SF contraction thus requires innovative bioimaging tools able to resolve dynamic conformational changes of individual biomolecules within native cellular environments in real-time.

２．研究の目的

The aim of the project was to establish an experimental system in which cytoskeletal changes driven by dynamic myosin motor action can be visualized and analyzed on the molecular scale directly within native cellular environments. High-speed atomic force microscopy (HS-AFM) is unique because it allows the observation of dynamic biological samples in liquid with subnanometer resolution in real-time. HS-AFM has previously visualized dynamic myosin action using recombinant proteins *in vitro*, but so far not been applied to study dynamic intracellular processes. Extending its application to studying dynamic intracellular molecular processes requires methods for introducing the AFM nanotip into the cell. For this, a gentle cell “de-roofing” technique must be developed to remove part of the outer cell membrane, thereby exposing intracellular structures, including SFs, for subsequent HS-AFM imaging. Several key questions related to SF structure, dynamics, and function, are then be addressed, including visualizing the sarcomere ultrastructure, the nature of actin/myosin filament packing, or the arrangement of different non-muscle myosin II isoform (A,B,C). Furthermore, conformational changes occurring within the SF sarcomere during non-muscle myosin II-driven SF contraction after Mg2+/ATP stimulation will be imaged by HS-AFM. Besides providing novel insight into SF structure and function, the developed experimental approach developed in this study should　open future opportunities to perform sub-molecular level HS-AFM studies on the structure and dynamics of additional functional intracellular components.

３．　研究の方法

(1) First, different cell de-roofing methods were tested for their suitability in exposing functional (contractile) SFs, including detergent extraction- and sonication based protocols.

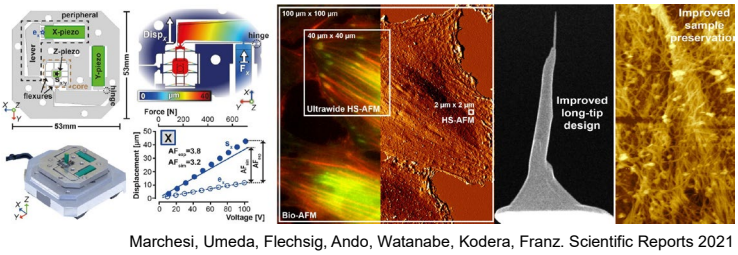
(2) The suitability of different HS-AFM and scanning ion conductance microscopy (SICM) platforms for intracellular SF imaging was then tested. A dedicated ultrawide HS-AFM scanner was developed for large scale HS-AFM scanning. Moreover, AFM tip design was optimized for intracellular HS-AFM imaging.

(3) HS-AFM and SICM measurements were complemented by quantitative AFM imaging methods to obtain complementary structural and mechanical SF information.

４．研究成果

(1) Optimizing cell de-roofing, AFM tip design, and applying an ultrawide HS-AFM scanner system

A cell de-roofing method was developed that combines microsonication with pre-stabilization of actin filaments with jasplakinolide. Mechanical damage during de-roofing was further minimized by adding high molecular weight polymers to enhance the viscosity of the de-roofing solution. Furthermore, conventional short (~300 nm) EBD tips were replaced with optimized long AFM tips consisting of a stable ~2µm support beam carrying a sharp ~150 nm long tip. In a collaborative effort between NanoLSI scientist from different sections, we also developed an ultrawide HS-AFM scanner with coupled fluorescence and a data acquisition system able to record large topographic images (≤40µm^2) with high pixel densities (≤16 megapixels), providing near-molecular resolution throughout the large image frame (Marchesi et al.



Marchesi, Umeda, Flechsig, Ando, Watanabe, Kodera, Franz. Scientific Reports 2021

2021). In combination, these innovations yielded high quality images of extremely well-preserved SFs, revealing surprisingly complex arrays of additional associated cytoskeletal structures.

(2) Generating the first molecular-resolution maps of stress fibers by wide scanning HS-AFM

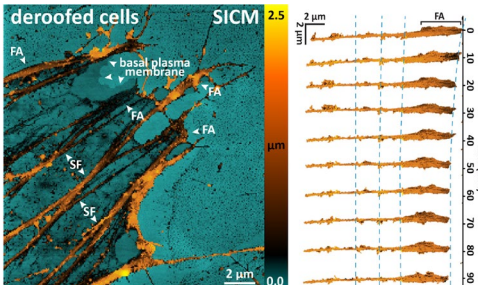
The wide-scanning capabilities of the new scanner system enabled us for the first time to generate the first molecular-scale topographic images of entire exposed actin stress fibers in de-roofed cells. Stitching several overlapping scans together further increases the achievable pixel counts. After reducing the scan area, subregions of the overview scans can then be imaged at enhanced frame rates. The composite AFM images demonstrated that actin filaments form contiguous structures spanning from focal adhesion into central SF area. Moreover, after switching from slow overview scanning to conventional high-speed scanning with increased frame rates, reversible conformational changes in individual motor proteins within their native cellular environment could be visualized for the first time. Being able to identify such ATP-driven conformational changes in individual motor protein molecules was an important milestone of the project.

(3) Revealing the arrangement of myosin II motor proteins within gelsolin digested SFs

From fluorescence microscopy studies it is assumed that myosin II motors are arranged in a sarcomere-like manner within SFs. The initial expectation was therefore that HS-AFM imaging could visualize the re-arrangement of these motors during SF contraction. However, high-resolution HS-AFM images showed no discernable myosin bundles or suborganization into sarcomere boundaries on the SF surface. This suggested that myosin motors may be embedded deep with SFs. To further analyze myosin arrangement, the F-actin in exposed SF was depolymerized with gelsolin. Surprisingly, actin-depleted SFs retained the regular, sarcomere-like arrangement of myosins, indicating that their arrangement is organized independently of F-actin. Also, myosins localized directly on the cell membrane, suggesting that myosin II could form an elastic, modulable linker between the plasma membrane and SFs.

(4) First application of SICM for intracellular imaging of contracting stress fibers

While not achieving the high resolution of AFM, SICM is a contactless SPM technique and therefore especially suitable for imaging fragile biological samples, such as membrane protrusions on the surface of living cells. However, so far it has not been used for intracellular imaging. In collaboration with Prof. Korchev and Prof. Takahashi, we employed SICM imaging to visualize the dynamic contraction of SF in de-roofed cells. Even individual actin filaments within SFs could be easily visualized by SICM, as well as ultrastructural changes during SF contraction. Tracing of individual SFs demonstrated that contraction occurs predominantly at the SF ends. Due to the gentler imaging mode of SICM, the experimental success rate was significantly higher compared to HS-AFM. These experiments provided complementary insight into SF contraction to the HS-AFM data and expanded the application of SICM to the intracellular space.



(5) Comprehensive nanomechanical mapping of contracting stress fibers by HS-, QI-AFM and SICM

AFM and SICM are unique SPM tools, as they permit collecting topographic images and performing nanomechanical characterization of fragile biological samples simultaneously. To complement the nanostructural analysis, nanomechanical changes of exposed SFs during contraction were investigated both by SICM and different AFM techniques: HS-AFM indentation force spectroscopy, QI-mode imaging (JPK NanoWizard), and peak-force-tapping mode (Bruker Resolve). While all tested SPM techniques demonstrated significant SF stiffening during contraction, absolute elasticity values obtained by the different methods varied by several orders of magnitude. The different stiffness ranges likely reflect the different ways by which SICM (low indentation forces, small deformations) and AFM (larger forces and indentation depths) probe the biological sample. These experiments provided the first direct nanomechanical analysis of contracting SFs and important comparative insight between different SPM techniques.

(6) Applying cell de-roofing and AFM to study S100A11 localization to SFs

In a separate project we recently identified localization of the small Ca^{2+} binding protein S100A11 to SFs and focal adhesions (Mohammed et al., 2024). The cell de-roofing/AFM protocol developed in this project was instrumental in demonstrating the Ca^{2+}-dependency of the S100A11/SF interaction. Moreover, cell de-roofing and exposure of the intracellular side of the plasma membrane was used in another study investigating ion channel structure and function (Ye et al. 2024).

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〔図書〕　計0件

〔産業財産権〕

〔その他〕

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８．本研究に関連して実施した国際共同研究の実施状況

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