Seminars in Cell & Developmental Biology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Seminars in Cell & Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

Probe microscopy methods and applications in imaging of biological materials

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ARTICLE INFO

Article history:
Received 23 May 2017
Received in revised form 4 August 2017
Accepted 4 August 2017
Available online xxx

Keywords:
Atomic force microscopy
Probe modification
Biological specimens

ABSTRACT

Atomic force microscopy is an emerging tool for investigating the biomolecular aspects of cellular interactions; however, cell and tissue analyses must frequently be performed in aqueous environment, over rough surfaces, and on complex adhesive samples that complicate the imaging process and readily facilitate the blunting or fouling of the AFM probe. In addition, the shape and surface chemistry of the probe determine the quality and types of data that can be acquired from biological materials, with certain information becoming available only within a specific range of tip lengths or diameters, or through the assistance of specific chemical or biological functionalization procedures. Consequently, a broad range of probe modification techniques has been developed to extend the capabilities and overcome the limitations of biological AFM measurements, including the fabrication of AFM tips with specialized morphologies, surface coating with biologically affine molecules, and the attachment of proteins, nucleic acids and cells to AFM probes. In this review, we underline the importance of probe choice and modification for the AFM analysis of biomaterials, discuss the recent literature on the use of non-standard AFM tips in life sciences research, and consider the future utility of tip functionalization methods for the investigation of fundamental cell and tissue interactions.

Contents

1. Introduction

Although mechanotransductive elements such as focal adhesion points, calcium-gated channels, matrix metalloproteinases, extracellular matrix proteoglycans and YAP/TAZ signaling components have long been appreciated to play an important role in the biology of tissues that are regularly exposed to significant external forces [1–4], recent discoveries have made it abundantly clear that mechanical signaling is much broader in scope and contributes significantly to the biological activity of every cell and tissue. Disparate and far-reaching processes such as nuclear organization [5], cellular differentiation [6] and embryonic development [7] have been demonstrated to depend on mechanical stimuli in addition to biochemical signals, suggesting that mechanical aspects of cell-cell and cell-extracellular matrix (ECM) signaling are as important for the regulation of cellular behavior as well-established receptor/ligand interactions. Methods for determining the elastic moduli of cells and tissues have consequently joined the arsenal of existing molecular biology tools for the investigation of cellular signaling mechanisms, and atomic force microscopy (AFM) in particular has attracted considerable attention for its compatibility with liquid

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http://dx.doi.org/10.1016/j.semcdb.2017.08.018
1084-9521/

environments and live cells in adherent condition. However, it must be noted that AFM results in the literature are highly variable due to differences in sample preparation, measurement and analysis, all of which can significantly impact the topographies and elastic moduli of biological materials. In addition, small differences in these conditions may be further magnified by the natural heterogeneity that is observed even in well-characterized tissues and cell lines, resulting in the creation of major discrepancies between numerical values reported for identical cell or tissue types [8].

Characterization of biological materials by AFM is further complicated by the fact that cells and tissues are far from ideal substrates for this technique. AFM was originally developed for the measurement of nano-to-microscale samples, but eukaryotic cells on flat surfaces may measure in excess of 10 μm in height and 100 μm in diameter, which are beyond the vertical and horizontal ranges of many commercial systems. In addition, mammalian cells do not survive in the absence of certain environmental conditions, and while the necessary temperature and CO_2 ranges can be maintained by specialized AFM attachments for biological imaging, measurements must still be performed in a growth or differentiation medium that contains a wide range of proteins and growth factors. These biomolecules readily attach to available surfaces and create a corona [9] that may interfere with measurement, while physical contact with the sample may also foul or abrade the probe during contact-mode imaging or force-displacement measurements. Furthermore, biological research often entails the measurement of interactions between specific biomolecules, and it may be desirable to determine the adhesive forces between a sample molecule on the probe and another on the surface, or to utilize the probe itself as a biosensor for the detection of a specific moiety in a complex sample such as blood or saliva. However, these functionalities are outside the capabilities of conventional AFM probes and necessitate either the modification of standard cantilevers with new surface molecules, or the wholesale fabrication of AFM probes with alternate designs and materials.

Modification of AFM probe morphology and its surface chemistry is therefore a convenient means of improving the technique's functionality with respect to biological measurements, as well as eliminating the problems associated with its use for this purpose. Consequently, probe modifications have been developed through a broad spectrum of material fabrication and biochemical functionalization methods, such as thin film coating, two-photon polymerization, focused ion beam (FIB) etching and whole cell attachment through biotin-avidin interactions, for an equally broad range of reasons, including the investigation of protein-protein interactions [10,11] and unfolding kinetics [12,13], real-time imaging of cytoskeletal movements [14] and early diagnosis of cancer [15]. As such, the present topical paper provides a broad overview of the physical, chemical and biological aspects of probe modification and the use of "non-standard" probe designs in the imaging and quantification of cell and tissue interactions, with emphasis on the potential expansion of AFM-based basic research in biology through novel probe designs.

2. Effect of probe morphology, material properties and surface chemistry

Biological AFM measurements are typically performed using silicon nitride probes with nominal spring constants in the range of 0.006–0.10 N/m; however, changes in probe size, material and morphology are commonly made to meet the demands of specific samples and experiments. It should be noted that these choices contribute to the variance in results reported in the literature, as it is now widely appreciated that tip morphology may have a significant impact on AFM measurements. Chiou et al., for example, found that effective Young's moduli of NIH3T3 and 7-4 cells were two-folds higher when measured by sharp tips compared to flat or bead-attached cantilevers [16], while Carl and Schillers similarly demonstrated that spherical probes produce significantly lower Young's modulus values compared to conventional, sharp-tipped AFM probes for the elasticity analysis of Chinese hamster ovary cells, despite exhibiting consistent results over a wide range of probe radii (0.5-26 μm) [17]. The dimensions of the sample should also be considered when choosing a suitable probe for AFM measurements: Whole cells and tissues are frequently imaged using colloidal probes, as conventional AFM probes would produce data from individual cell and ECM components that would not necessarily represent the mechanical characteristics of the sample as a whole. But while microindentation results are useful for the measurement of large, heterogenous AFM samples, the ability to analyze tissue components at nanoscale is also potentially valuable. Stolz et al. for example found that age-dependent differences in the elastic moduli of arthritic cartilage could be monitored with sharp AFM tips but not microindenters, as the former is able to measure the elasticity of individual collagen fibers that constitute cartilage tissue [18].

While tip modifications are sometimes performed for sampling reasons, they are more often used to develop specialized tip morphologies that extend the capabilities of AFM imaging, or combine the technique with other imaging or analysis methods for synchronized measurement (a list of such applications is provided in Table 1). Difficulties associated with the real-time AFM imaging of live cells have been circumvented in this manner by Shibata et al., who used ~3 μm-long, ~5 nm-thin, stilt-like probes to minimize tip abrasion and sample damage during high-speed AFM of cytoskeletal dynamics in COS-7, HeLa and neural cells [14]. Likewise, Liu et al. used focused ion beam etching to produce 3–6 μm-long, 150–250 nm-thick needles to directly probe cellular nuclei after entering through the cell membrane [19], while Meister et al. integrated a microfluidics channel inside the AFM tip for the precise delivery of fluids into cells [20]. Sahin et al. also fabricated an unusual probe design, in which a sharp tip was fabricated on one side of the AFM cantilever, and the torsional forces acting upon the tip during tapping-mode imaging were used to determine the sub-microsecond changes that occur in adhesive and repulsive forces during approach and retraction [21]. Modified probes can also facilitate the integration of molecular biology methods into AFM, as a conductive layer at the apex of the AFM tip was used by Kim et al. to deliver currents for site-specific electroporation and transfection on individual cells [22], and Li et al. described a modified AFM probe to extract mRNA molecules within live HeLa cells through dielectrophoretic forces generated by an AC current [23]. In addition, other imaging modalities can be combined with AFM to provide more comprehensive information about cellular processes: Gold- and silver-coated AFM tips are known to strongly enhance Raman signals, and nucleic acids [24], proteins [25], bacterial [26,27] and eukaryotic cell surfaces [28], and sectioned erythrocytes [29] have been investigated using a combination of AFM and Raman spectroscopy using TERS-compatible tips, even allowing the nucleotide-level detection in DNA strands [30]. Similarly, AFM can be performed alongside other scanning probe techniques such as scanning near-field optical microscopy (SNOM) [31], and AFM tip-based nanoneedles and nanoscalpels have also been fabricated for performing highly precise measurements in living cells [32–34].

The diversity in probe types is reflected by the diversity of material fabrication tools used in their production, although most modifications can be performed even by non-specialist laboratories: Attachment of colloidal probes only requires glass beads, glue and a steady hand under the light microscope, while relatively common cleanroom processes such as focused ion beam milling

Please cite this article in press as: A.D. Ozkan, et al., Probe microscopy methods and applications in imaging of biological materials, Semin Cell Dev Biol (2017), http://dx.doi.org/10.1016/j.semcdb.2017.08.018