Review

Probe microscopy methods and applications in imaging of biological materials

Alper D. Ozkan^a, Ahmet E. Topal^a, Fatma B. Dikecoglu^a, Mustafa O. Guler^{a,b},
Aykutlu Dana^{a,*}, Ayse B. Tekinay^{a,*}

^a Bilkent University, Institute of Materials Science and Nanotechnology, Ankara, 06800, Turkey

^b Institute for Molecular Engineering, University of Chicago, Chicago, IL 60637, USA

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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.

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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

* Corresponding authors.

E-mail addresses: aykutlu@unam.bilkent.edu.tr (A. Dana),
atekinay@bilkent.edu.tr (A.B. Tekinay).

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 $\sim 3 \mu\text{m}$ -long, $\sim 5 \text{ nm}$ -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

Table 1
Examples of morphological probe modifications used in AFM imaging and measurements.

Probe type	Fabrication method	Material(s) used	Advantages and application areas	Reference
Photopolymerizable hydrogel nano-probes	Bottom-up fabrication with compressible replica moulding	Poly(ethylene glycol)diacrylate (PEG-DA), polymerized by ultraviolet light	Can be utilized as soft matter-based nanomechanical sensors with strong control over molecular characteristics, compatible with biological measurements	[35]
Soft acrylic polymer-based cantilever and tips	Micromachining techniques	PMMA	Allows fabrication of acrylic polymer cantilevers with spring constants and tip radii comparable to standard Si-based probes	[37]
Tipless SU-8 AFM cantilevers	Photolithography	SU-8 photoresist	Suitable for cell adhesion studies, allows physical and chemical surface modification steps, easy to mass-produce	[38]
Hollow tips	Micromachining techniques	Polycrystalline silicon	Injects soluble molecules into live cells with high precision, proof-of-concept demonstrated through dye injection into NG108 and C2C12 cells	[20]
Hydrophobic perfluoropolyether-based AFM tips	Two-photon polymerization (2PP) technology	PFPE-PETA oligomer	Allows the measurement of mechanical properties with minimal tip-sample interaction in biological environments	[39]
Needle-like tips	Focus ion beam (FIB) etching of sharp tips into needle morphology	Silicon	Penetrates the cell membrane to directly measure the elastic moduli of intracellular elements	[19]
Wedged cantilevers (<i>i.e.</i> parallel plate)	Epoxy wedging by using a glass block mounted on a micrometer gauge	Epoxy	Allows the uniaxial confinement of cells	[40–42]
Ultra-compliant polyimide-based AFM tips	Wet etching followed by deposition of a sacrificial layer, probe, and two gold regions that flip the probe from surface through thermocompression	Two layers of polyimide between a resistive metallic thin film	Minimizes sample damage during biological imaging due to soft material composition	[43]
Conductive colloidal AFM-SECM probes	Mask deposition of gold on a tipless cantilever, followed by attachment of a gold probe and deposition of polymer material	Poly(3,4-ethylenedioxythiophene) doped with polystyrene sulfonate (PEDOT:PSS)	Can be prepared with different material characteristics by switching polymers, compatible with high-throughput biological screening	[44]
μ -TERS tips	Electrochemical etching for deposition of gold layer on commercial silicon cantilevers	Gold	Performs tandem topographical imaging and Raman characterization of biological materials	[45]
Electrolyte-filled, glass micropipette probes	Glass microcapillaries formed using commercial micropipette puller	Borosilicate glass	Offers precise control over tip-sample distance in biological environments for SICM/SNOM applications	[46]
Flat-ended, boron-doped Si AFM tips	SiO ₂ deposition for tip insulation, followed by FIB etching of the tip apex to allow conductance	SiO ₂	Directly delivers currents to single cells for electrophoresis applications	[47]
Nanofountain probes	Etching of SiO ₂ precursors to produce microchannel-bearing probe array and microfluidics system on tip	SiO ₂	Injects the contained fluid into live cells following local electroporation	[48]
Engineered nanoparticle (ENP)-functionalized AFM tips	Attachment of multiple ENMs on sharp AFM tip, resulting in an even coating with a single nanoparticle at the apex	CeO ₂ and Fe ₂ O ₃	Permits AFM measurement of interactions between ENPs and cells, especially with respect to protein corona formation	[49]
Magnetic cantilevers	Gluing a magnet to cantilever by epoxy glue	Fragments of samarium cobalt magnet, c. 20 μ m diameter	Allows single-cell rheology measurements of the creep response	[50]
Insulator-coated, bent optical fiber probes	Chemical etching, gold sputtering and voltage-mediated deposition of insulating paint, followed by sectioning by FIB at a 30° angle	Gold and Elecoat AE-X electrophoretic paint	Can perform tandem AFM/scanning electrochemical microscopy/near-field optical microscopy on live PC12 neuronal cells	[51]
Graphene oxide (GO)-functionalized AFM probes	Polydopamine-assisted adsorption of graphene oxide to AFM probe	Graphene oxide (GO)	Facilitates measurement of cell membrane–GO interactions and the toxicity of GO to bacteria	[52]
Single-walled carbon nanotube probes	CVD deposition on commercial sharp-tip cantilevers	SWNTs	Used successfully for high-resolution imaging of the chaperonin GroES under two distinct morphologies (representing either end of the protein)	[53]
Gold-coated AFM/tip-enhanced near-field lifetime microscopy tips	Sputter coating	Gold	Can be used for AFM/FLIM imaging of soft biological samples, such as stained DNA molecules	[54]
Micromachined cantilevers	Removal of the central region of the cantilever by focused ion beam (FIB) lithography	Si ₃ N ₄ (Biolever mini)	Reduction of cantilever's stiffness and hydrodynamic drag surfaces, high force precision and stability in protein stretching measurements	[55]

and inductively-coupled plasma deposition can be used to etch tip surfaces or coat them with metal and polymer layers, and wet chemistry methods can be employed for further surface modification. In addition to surface coating, AFM probes have also been

constructed wholly from alternative materials, such as photoreactive polymers: Kim et al. reported the fabrication of soft, epoxy resin-based AFM tips through two-photon polymerization [36], while Lee et al. produced both cantilevers and soft tips using PEG-

Fig. 1. Hydrogel AFM probes with different tip geometries, fabricated using a bottom-up fabrication strategy.

This figure is reprinted with permission from Lee et al. [35].

DA as the pre-polymer solution (Fig. 1) [35]. It is also worth noting that a wide variety of AFM probe types can now be supplied from commercial sources without the need for additional functionalization – bead-attached probes, tipless cantilevers, SNOM and TERS tips and functionalized surfaces for protein and cell attachment can be purchased directly, and the availability of suitable equipment (such as a SNOM-Raman-AFM system for combined imaging or liquid cell, temperature, CO₂ and fluorescence microscope attachments for live cell studies) is a bigger barrier of entry for biological AFM research.

3. Surface attachment of proteins, antibodies and other biomolecules

Many fundamental processes in molecular biology depend on highly specific interactions between two biomolecules, and the coating of AFM probes with proteins, nucleic acids, glycosaminoglycans and other bioactive molecules allows the determination of the forces associated with these interactions. However, the strong affinity between matching pairs of biomolecules is difficult to overcome, and require both materials to be bound to their respective substrates (AFM probe and sample surface) to allow the force-mediated detachment of non-covalent bonds without pulling the interacting partners off the AFM probe or the surface [56]. In addition, while tethering can be performed by using anti-

bodies, avidin-biotin linking, or directly attaching the molecule of interest to the probe through covalent bonds, these materials also show little inherent affinity to probe surfaces and necessitate additional functionalization steps prior to their binding. Both the probe and the material can be modified for this purpose: Nucleic acids, for example, can be synthesized with thiol extensions to facilitate their absorption on gold probes [57], while aminopropyltriethoxysilane (APTES), aminophenyl-trimethoxysilane (APhS) or ethanolamine treatment can be used to produce a layer of amine groups directly on silicon nitride probe surfaces [58]. Polyethylene glycol (PEG) and similar molecules can also be incorporated as linker residues during surface functionalization and provide the attached molecules with a degree of flexibility that better facilitates binding interactions [59,56,60]. Such an incorporation of a PEG linker between an AFM tip and an antibody was used in a recent study to show that the impairment of LRP-1 function results in stronger rupture forces between surface integrins and anti-integrin- β 1 coated tips, suggesting that integrin clustering is enhanced in the absence of this protein (Fig. 2). In addition, a bifunctional linker that binds to aminated AFM probe surfaces at one end and lysine residues in proteins on the other has been developed to simplify the binding of antibodies to probes [60], and a cyclooctyne/silatrane “anchor” terminating in a PEG linker has likewise been used to allow biomolecule attachment through click chemistry, reducing the number of steps required for the surface functionalization of AFM probes [61].

Fig. 2. A PEG linker was used in this study for the surface functionalization of AFM tips with monoclonal anti-β1 antibodies to investigate rupture events between the antibody and integrin under different conditions. The silicon nitride AFM probe was amino-terminated in an ethanolamine hydrochloride bath (DMSO) and successively incubated in PEG linker, citric acid and monoclonal antibody solutions. Surface clustering (as indicated by the distribution of binding interaction forces) is weak in the presence of LRP-1, decreases further with free antibody treatment (which blocks available integrins), and is enhanced following exposure to the LRP-1 antagonist RAP.

The figure is reprinted with permission from Le Cigne et al. [62].

A summary of biomolecules used in the surface functionalization of AFM probes is provided in Table 2. Adhesion profiles of a wide range of biomolecules have been characterized by AFM studies: Fernandez et al. have performed a series of experiments on the importance of domain order, amino acid identity and intermediate states during unfolding using the I27 and I28 domains of human cardiac titin [63], while comprehensive reviews of AFM investigations of DNA repair, replication, recombination and protein complexation are provided by Lyubchenko et al. [64,65]. Antibody-functionalized probes are also used in the imaging of live cells: Quisenberry et al. for example have used anti-β1-integrin functionalized AFM probes to determine the distribution of this molecule on human adipose-derived stem cells surfaces, and found that β1-integrin density depends on the cellular environment during the chondrogenesis in these cells [66]. Likewise, Askarova et al. functionalized silicon nitrate cantilevers with sialyl-Lewis^x to investigate whether amyloid-β aggregations alter the interaction of this sugar moiety with p-selectin on cerebral endothelial cell membranes, and reported that amyloid deposition weakens sLe^x/p-selectin binding despite increased p-selectin expression on the cell surface [67]. Hinterdorfer group also developed a method,

called simultaneous topography and recognition imaging (TREC), that allows the tandem imaging and affinity mapping of cells and tissues. This technique was used to identify binding sites of vascular endothelial cadherin on endothelial cells of the murine myocardium [68], hERG channels on human embryonic kidney (HEK 293) cells [69] and CD1d-glycolipid interactions on THP natural killer cells [70]. Other molecular recognition experiments were performed for the detection of angiotensin II type I receptor (AT₁R) distribution on H295R adrenocortical cells, as well as to identify the differences in heat shock protein 60 (HSP60) expression in stressed and non-stressed human umbilical cord endothelial cells (HUVECs) [71] (see also the review by Senapati and Lindsay [72]).

Probes capable of detecting small quantities of specific molecules in complex samples also show potential as diagnostic tools in cancer and other disorders. Blood microparticles (MPs), cellular fragments that have been implicated in processes such as thrombosis and cancer, have been analyzed in this manner using AFM probes functionalized with an antibody for CD41 (which is indicative of platelet origin for MPs). CD41-positive MPs in three cancer patients were found to be smaller compared to healthy donors (51.4 ± 14.9 nm vs. 67.5 ± 26.5 nm average diameters), and

Table 2
Biomaterial functionalization methods used in AFM studies.

Probe type	Functionalization material	Functionalization method	Sample	Reference
Si ₃ N ₄	Human spleen ferritin protein	Surface silanization and glutaraldehyde activation followed by protein binding	Anti-ferritin mouse monoclonal IgG2a antibody-coated substrates	[80]
Si ₃ N ₄	Hemagglutinin (HA) antibodies	Functionalization by 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) linkage in MES buffer	HA-GluR2 receptors on hippocampal neuron surfaces	[81]
Si ₃ N ₄	Fibrinogen	Silanization with 3-aminopropyl-triethoxysilane (APTES), followed by activation with glutaraldehyde and protein functionalization	Erythrocytes on poly-L-lysine-coated glass slide	[82]
Si ₃ N ₄	Disuccinimidyl suberate (DSS)	Surface silanization by APTES followed by DSS functionalization	3T3 fibroblast cells	[83]
Gold-coated Si ₃ N ₄	Fibrinogen	Self-assembled hexadecanethiol monolayer formation by vapor-phase deposition, followed by protein absorption (non-functionalized probes were also exposed to fibrinogen as control)	Freshly cleaved mica, hexadecanethiol monolayer on gold, oligo(ethylene glycol)-terminated monolayer on gold	[84]
Gold-coated Si ₃ N ₄	CH ₃ -terminated alkanethiol molecules	Gold-coating followed by incubation in HS(CH ₂) ₁₁ CH ₃	<i>Aspergillus fumigatus</i> and <i>Mycobacterium bovis</i> cells	[85]
Carbon nanotube-attached silicon tip	Biotin	Amine-coupling at the hanging end of carbon nanotubes	Streptavidin on biotinylated surface	[86]
Gold-coated Si	BRAF-specific oligonucleotide sequences	Direct attachment of oligonucleotides with thiol groups at the 5' end	Total RNA from melanoma cells (for wild-type and mutated BRAF analysis)	[75]
T-shaped Si ₃ N ₄ cantilevers with Si tips	DNA	Surface silanization by APDES, followed by SM(PEG) ₂ linker attachment and functionalization by thiolated DNA molecules	Protein domains	[87]
Gold-coated Si ₃ N ₄	Nitrilotriacetic acid-Ni ²⁺	Adsorption of NTA-Ni ²⁺ -terminated alkanethiols on gold surfaces	<i>Aspergillus fumigatus</i> and Mid2-expressing <i>Saccharomyces cerevisiae</i> cells	[88]
Si ₃ N ₄	Anti-myoglobin antibody or myoglobin-specific aptamer	Silanization by MPTMS, followed by functionalization with NHS-PEG-maleimide and antibody/aptamer attachment	Myoglobin-immobilized gold film on glass	[89]
Si	Cadherin	Attachment of PEG spacer through N-hydroxysuccinimide groups to APTES-functionalized surface, followed by streptavidin and biotinylated cadherin binding to the biotin-containing end of PEG spacer	Cadherin-functionalized surface	[90]
Magnetically coated silicon cantilevers (MAC levers, Agilent)	ATP or anti-UCP1 antibody	APTES silanization, followed by NHS-PEG-aldehyde linker attachment and binding of ethylenediamine derivative of ATP (EDA-ATP) or anti-UCP1 antibody	Proteins reconstituted in lipid bilayer (i.e. uncoupling protein family member UCP1-reconstituted lipid bilayer)	[91]
Si ₃ N ₄ and tip pen lithography probe DPN Type B (Nanolnk)	Therminator γ DNA polymerase, with and without DNA primer template	Surface silanization, followed by functionalization with a 27-acid dendron, deprotection of dendron end groups with TFA, generation of N-hydroxysuccinimide groups from exposed amines by disuccinimidyl carbonate, and DNA polymerase attachment	Biotin-labeled dNTPs immobilized on glass slide	[92]
Si ₃ N ₄	Rituximab	Amino-silanization followed by maleimide-PEG-NHS linker chemistry	Natural killer (NK) cells and tumor cells	[93]
Si ₃ N ₄ tips or magnetically coated tips (MACLevers, Type VII, Agilent)	Human gonadotropin-releasing hormone receptor (GnRH-R; or type I GnRH-R)	Functionalization by NHS-PEG-aldehyde cross linker following amino-silanization	Human bladder cancer cells (T24)	[94]
Si ₃ N ₄	Sialyl-Lewis ^x	Conjugation of biotinylated sialyl-Lewis ^x to streptavidin-functionalized probe surface	p-Selectin receptors expressed on endothelial cell surfaces	[67]
Gold-coated Si ₃ N ₄	Heparin	Heparin immobilization through thiol-PEG-NH ₂ conjugation	PF4 tetramers immobilized on substrate by thiol-PEG-COOH linker	[95,96]
Si ₃ N ₄	acetolactate synthase (ALS)	APTES functionalization followed by self-assembled glutaraldehyde monolayer (SAMs) formation	imazaquin and metsulfuron-methyl	[97]
Gold-coated Si ₃ N ₄	Ni ²⁺ -N-nitrilotriacetate (Ni ²⁺ -NTA)	Coating with Al and Au and immersion in a mixture of NTA- and triethylene glycol-terminated alkanethiols, followed by exposure to NiSO ₄ solution	SAS-6 protein	[98]
Si ₃ N ₄ (BioLever mini)	Cohesin E (CohE) and carbohydrate-binding module (CBM) domain of dockerin	APDMES silanization and PEGylation with α -maleimide-hexanoic- ω -NHS PEG	ybbR-tagged peptides and proteins	[99]

AFM was capable of detecting 1000-fold greater numbers of platelet-derived MPs compared to flow cytometry [73]. AFM probes can also be functionalized with bacterial biofilms for the investigation of cellular invasion in pathogens and the surface-adhesion process biofilm-forming strains, the elimination of which is a high

priority in medical and food processing industries. Lau et al., for example, have shown that *Pseudomonas aeruginosa* biofilms are significantly less adhesive in *wapR* mutants compared to the wild-type strain [74]. Although they cannot be classified as atomic force microscopes in the strict sense, cantilever array sensors also warrant

Fig. 3. A commonly applied approach for producing a cell probe, in which a biotinylated BSA is first immobilized on the AFM probe, followed by the attachment of a streptavidin layer and biotinylated Concanavalin A (ConA) protein molecules. ConA binds to glycoproteins such as selectins, allowing its use for the functionalization of almost any cell type.

This figure is reprinted with permission from Friedrichs et al. [102].

mention as a related design: These systems involve a parallel series of cantilevers that are functionalized with a biomolecule of interest, such as an antibody or complementary oligonucleotide sequence, to detect and/or isolate a specific biomarker, and have been developed for the detection of DNA and RNA sequences [75,76], tumor proteins such as prostate-specific antigen and carcinoembryonic antigen [77], cardiac markers such as creatin kinase and myoglobin [78], and other interactions such as protein A/immunoglobulin binding [79].

4. Investigation of cell-substrate interactions by cell-functionalized probes

Cell attachment can in many ways be considered as a subset of surface coating, as it is typically preceded by chemical functionalization with a cell-binding material to facilitate the adhesion process. Concanavalin A, a lectin with strong affinity to cell membrane carbohydrates, is commonly used for this purpose (and attached to probe surfaces through biotin-avidin interactions) (Fig. 3), although yeast and other eukaryotic cells have been immobilized on AFM cantilevers by gelatin, polylysine, cyanoacrylate and the commercial cell adhesive Cell-Tak [56], and bacterial cells have been attached to probe surfaces through fixative agents such as glutaraldehyde and cationic polymers such as polydopamine and polyethyleneimine [100]. Cell survival can generally be attained in methods involving non-fixative agents, many of which are commonly used in tissue culture for attachment to microplate surfaces; however, stresses associated with measurement may eventually disrupt the morphology and behavior of the attached cell. Consequently, cellular reusability depends on the identity of the cell and the nature of the interaction in question. Krieg et al., for example, have successfully used individual germ layer cells for up to 40 force-displacement measurements (with regular observation to ensure that cell integrity is not compromised, and discarding the cells that showed aberrant morphology) [7], while Hosseini et al. used fresh cells for each measurement in the analysis of T-cell attachment to antigen-presenting cells (APCs), due to the tendency of this interaction to permanently transfer membrane fragments between the participants (it should also be noted that the T-cell/APC interaction required ~30 min for optimal binding, while other cellular adhesion processes are seldom observed over time periods exceeding 1 min) [101].

Following the attachment process, tip-bound cells can be brought over a sample surface to directly study cell-substrate inter-

actions on a single-cell basis. Interaction studies of this type are called *single-cell force spectroscopy* (SCFS) experiments, a selection of which are listed in Table 3. Zhang, Wojcikiewicz and Moy, for example, reported that Jurkat cells (T-lymphocytes) formed stronger adhesions to HUVECs with longer interaction times, and used antibody blocking to demonstrate that, following 0.25 s of binding, the adhesion molecules E-cadherin, ICAM-1 and VCAM-1 were responsible for 18%, 39% and 41% of the interaction between the two cell types [103]. In another study, this group also demonstrated that ICAM-1 and VCAM-1 partially mediated the interactions between monocytic human promyelocytic leukemia cells (HL-60 cells) and HUVECs, while β_1 -integrins played a comparatively stronger role in attachment and $\alpha V\beta_3$ -integrins had no significant role in this process [104]. In addition, the leukocyte-endothelial cell association was inhibited through the disruption of VLA-4/VCAM-1 binding by the cRGD sequence, which is a well-known motif for cell attachment and presumably competes with adhesive cell membrane proteins for binding sites [105]. Forced cellular detachment was associated in both studies with multiple rupture events that potentially correspond to the stretching and/or breaking of different types of receptor-ligand interactions between the cells.

Adhesive interactions are also of fundamental importance for tumorigenesis and cancer metastasis, and Yu et al. have found that surface-coated ephrin-A1 (but not soluble ephrin-A1) stimulates collagen I binding in PC3 prostate cancer cells by enhancing the adhesive capacity of β_1 -integrins, which may be relevant to the metastasis of these cells into collagen I-rich bone tissue [106]. In addition to mammalian cell lines, the adhesion of bacterial and single-celled eukaryotic cells can also be quantified through cell-functionalized probes; for instance, a single *Staphylococcus aureus* bacterium can be used to obtain adhesion images by probing the interaction forces between the bacterium and skin corneocytes (Fig. 4). Like cancer cells, cellular recognition and binding mechanisms are crucial for the initial invasion, immune detection and phagocytic destruction of bacterial and fungal pathogens, and Mostowy et al. have demonstrated the importance of septins for the cellular entry of *Listeria monocytogenes* by measuring the force of interaction between invasion proteins on *Listeria* and Met receptors on HeLa cells in the presence and absence of these proteins [107]. In a similar vein, El-Kirat-Chatel and Dufrêne have shown that the yeast *Candida albicans* adheres strongly to the J774A.1 murine macrophage cell line through mannan/mannose receptor interactions in a time-dependent manner [108]. It is also known that *C. albicans* can form biofilms alongside the bacterial pathogen *Staphylococcus aureus*, and this group also used mutant *Candida* strains to show that bacteria adhere preferentially to fungal hyphae but not the cells themselves, and that the Als protein family and O-mannosyl groups are crucial for the establishment of *Candida-Staphylococcus* interactions [109].

5. Conclusions and future directions

AFM is now commonly used for the investigation of biological phenomena, and the flexibility of the technique makes it a strong candidate for the development of new methods for this purpose. While surface functionalization and single-cell force spectroscopy have become relatively well-established as fields of study, the development of new AFM methods for tissue characterization is currently limited, and given the importance of cell-cell and cell-ECM junctions for tissue integrity, future efforts in this direction are likely to be fruitful. In addition, despite the great diversity of AFM-derived techniques for biomaterial characterization, high-throughput methods for potential diagnostic applications are rare, and specialized cantilevers are promising for converting AFM from

Table 3
Cell attachment techniques used in single-cell force spectroscopy studies. Surface modification methods are summarized for brevity. The reader is advised to refer to the original literature citations for full experimental details.

Probe type	Attached cell type	Surface modification(s)	Sample	Reference
Colloidal silica beads (6.1 μm diameter), attached on tipless cantilevers by glue	<i>Staphylococcus aureus</i> (Newman and Newman <i>srtA</i> strains)	Coating in 4 mg/mL dopamine hydrochloride for 1 h, followed by washing and bacterial attachment	Primary human corneocytes, collected by tape from forearm of healthy donor	[111]
Pyramidal sharp-tipped silicon nitride probes (OTR4)	<i>Lactococcus lactis</i> ssp. <i>cremoris</i> strain MG1820	Coating in 0.1% (w/v) polyethylenimine for 1 h, followed by washing and bacterial attachment	Pig gastric mucin	[112]
FluidFM cantilever	MCF-7 cells	Adhesive-free cell attachment through the application of negative pressure from the FluidFM probe cavity	Adherent MCF7, MCF10A, or HS5 cells	[113]
Tipless silicon nitride cantilevers	MCF-7 cells	Coating in 1 mg/mL poly-L-lysine for 1 h, followed by fibronectin coating (20 $\mu\text{g}/\text{mL}$, 1 h) and MCF-7 cell attachment	SbpA protein (isolated from <i>Lysinibacillus sphaericus</i> CCM2177) and other MCF-7 cells on surface	[114]
Triangular, tipless cantilevers	<i>Candida albicans</i> CA14 yeast cells	Coating in 4 mg/mL dopamine hydrochloride for 1 h, followed by drying by N_2 gas and yeast cell attachment	J774A.1 murine macrophages	[108]
Triangular cantilevers with tips chipped off	<i>Dictyostelium discoideum</i> AX2-214 wildtype and derived mutants	Surface silanization, followed by functionalization by activated carboxyamylase, coating with 50 $\mu\text{g}/\text{mL}$ wheatgerm agglutinin for 1 h, and cell attachment	Other <i>D. discoideum</i> cells on surface	[115]
300- μm long, tipless, gold-coated silicon nitride cantilevers	RT112, T24 and J82 bladder cancer cell lines	Surface activation with acetone and UV treatment, coating with biotinamidocaproyl-labeled BSA, binding of streptavidin to the biotin layer, secondary functionalization with 0.5 mg/mL biotinylated ConA, and cell attachment	BSA, ICAM-1 and HUVECs on surface	[116]
Tipless silicon nitride cantilevers	A549 lung cancer cells	Coating in 10% APTES for 20 min, followed by exposure to 2.5% glutaraldehyde for 15 min and 50 $\mu\text{g}/\text{mL}$ fibronectin for 20 min, and cell attachment	Pulmonary human aorta endothelial cells	[117]
200- μm long, V-shaped, tipless silicon nitride cantilevers	HeLa cells	Surface activation by plasma cleaning, incubation in 2 mg/mL ConA overnight or 50 $\mu\text{g}/\text{mL}$ human plasma fibronectin for 2 h, and cell attachment	Other HeLa cells on surface	[118]
200- μm long, V-shaped, tipless silicon nitride cantilevers	PC3 prostate cancer cells	Surface activation by plasma cleaning, incubation in 2 mg/mL ConA, 50 $\mu\text{g}/\text{mL}$ ephrin-A1-fc, 50 $\mu\text{g}/\text{mL}$ fc region or 63 $\mu\text{g}/\text{mL}$ Cell-Tak cell adhesive, and cell attachment	Ephrin-A1-, collagen- and fibronectin-functionalized surfaces	[106]
Triangular cantilevers	<i>Pseudomonas putida</i> KT2440 and <i>Bacillus subtilis</i> JH642 strains	Tip cleaning in piranha solution and UV/ozone treatment, followed by coating in 4 mg/mL decarboxylated dopamine for 1 h, washing, drying and bacterial attachment	SW3+, NF90, NF90PVA and SWHR membranes	[119]
Colloidal silica beads (6.1 μm diameter), attached on tipless cantilevers by glue	SdrF-expressing and non-expressing <i>Lactobacillus lactis</i> MG1363 strains	Coating in 4 mg/mL dopamine hydrochloride for 1 h, followed by washing and bacterial attachment	Collagen-coated surfaces	[120]
200- μm long, V-shaped, tipless silicon nitride cantilevers	HeLa cells and mouse embryonic kidney fibroblasts	Tip cleaning using plasma, followed by overnight incubation in 2 mg/mL ConA, and cell attachment	Surfaces functionalized with collagen I, fibronectin fragment FN _{III} 7–10 and fibronectin fragment FN _{III} 7–10 lacking RGD	[121]
Tipless silicon cantilevers	CCL-61T Chinese hamster ovary cells	Surface coating with biotinamidocaproyl-labeled BSA, binding of streptavidin to the biotin layer, secondary functionalization with 0.25 mg/mL biotinylated ConA, and cell attachment	PDL- and PEI-coated substrates	[122]
SCS12 tipless silicon cantilevers	Human blood platelet cells, taken from healthy donors	UV cleaning, followed by incubation in 50 $\mu\text{g}/\text{mL}$ collagen G, and cell attachment	Collagen, fibronectin, and poly-L-lysine surfaces	[123]
Tipless V-shaped silicon nitride cantilevers	Patient-derived glioma-initiating cells from high-grade and low-grade tumors	O_2 plasma cleaning, followed by incubation in 10 μM ConA and cell attachment	Patient-derived glioma-associated cells from high-grade and low-grade tumors	[124]
Sephacryl S-1000 beads (80 $\pm$ 20 μm diameter), attached on tipless cantilevers by glue	HTB 112 human trophoblasts	Coating in 0.01% poly-D-lysine and cell attachment	RL95-2 human uterine epithelial cells	[125]
Veeco MLCT soft cantilevers	Zebrafish endoderm, mesoderm and ectodermal cells	Plasma cleaning, followed by overnight incubation in 2.5 mg/mL ConA and cell attachment	Other zebrafish germ layer cells on surface	[7]
Arrow TL-1 tipless cantilevers	Red blood cells	Immersion in 1 mg/mL wheat germ agglutinin solution	EA.hy926 endothelial cells	[126]
Bead attached to V-shaped tipless cantilever (PNP-TR-TL-Au)	Bead loaded with multiple or single <i>E. coli</i>	Loading of <i>E. coli</i> in suspension to aminated silica beads coated further with polyethyleneimine (PEI)	Planar and structured aluminum oxide surfaces	[127]
Ethanolamine-coated cantilevers (PFQNM-LC)	Modified rabies virus (RABV) expressing the envelope glycoprotein of avian sarcoma leukosis virus subgroup A (EnvA)	NHS chemistry and PEG ₂₇ linker coupling to a free amino group of the EnvA-RABV(Δ G) glycoprotein	MDCK cells with and without avian tumor virus receptor A (TVA) expression	[40,128]

Fig. 4. Height and adhesion images of corneocytes as measured by a *Staphylococcus aureus* probe, produced by the attachment of a single bacterium on a polydopamine-coated colloidal probe. Adhesion is very weak when probes are functionalized by the *S. aureus* Newman srtA strain, which is deficient in adhesive proteins. Reprinted from with permission from Alsteens et al. [110].

a strictly research-based method to a platform for the development of new diagnostic tools. Nevertheless, AFM's current role as the go-to method for cell-cell and cell-surface interaction studies is not to be underestimated, and future advances in tip functionalization will no doubt open new avenues for testing the importance of biological tether molecules and their relevance on the mechanical environment that is created and experienced by cells.

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