Published in final edited form as:

Methods. 2013 April 1; 60(2): 111–112. doi:10.1016/j.ymeth.2013.05.009.

Nanoimaging methods for biomedicine

Yuri L. Lyubchenko

Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, 986025 Nebraska Medical Center, Omaha, NE 68198-6025, USA

lyubchenko@unmc.edu

Numerous biomedical discoveries have been made on the ability to image biological objects. Cellular processes involve the actions and interactions of biomolecules and biomolecular nanosystems, and these processes are the targets for drug design and delivery. Understanding how these dynamic molecular systems operate requires methods that can analyze dynamics at the nanoscale level. Nanoimaging techniques, such as Atomic Force Microscopy (AFM), offer these capabilities. Traditional biophysical techniques available to researchers and clinicians provide an obscured picture of a living system. The problem lies in the ensemble techniques, which average the states of a large population of molecules and/or freeze complex systems in time; therefore, information on the subtle, intermediate, or short-lived conformations is lost. In actuality, mechanisms of physiological systems and diseases are conducted on a very transient level through highly dynamic molecular-scale interactions. Similarly, a process that appears simple by existing techniques may actually have many steps and a variety of important intermediate states, each with its own unique dynamics. Importantly, intermediate states are stabilized by weak interactions that are typically transient and difficult to measure; however, they are often the key control points of disease development. The different modalities of AFM provide single-molecule nanoimaging capabilities to image and probe these intermediate states.

The prototype scanning tunneling microscope (STM) was conceived by Binnig and Rohrer [1,2], an invention for which the authors were awarded the 1986 Nobel Prize in Physics. The atomic force microscope (AFM) was invented in 1986 [3], and its brilliant development by the Hansma group [4] resulted in the commercial production of the AFM, allowing the instrument to become available to the biological community. The implementation of the AFM operation mode in which the tip oscillates and touches the sample gently dramatically facilitated imaging of biological samples, specially the nanoscale visualization of samples in a fully hydrated state, enabling time-lapse imaging of sample dynamics [5]. Initially, the tip oscillation mode was termed Tapping mode [6], but the name has been trademarked by the AFM manufacturer Veeco. Therefore, other manufacturers utilize this methodology under different names, such as intermittent contact (IC) and alternating contact (AC). Recent advances in high-speed AFM (HS-AFM) instrumentation enable time-lapse observations of biological nanosystem dynamics in real time [7].

The progress made in the utilization of AFM and the improvements made in instrumentation required the development of reliable and routine sample preparation techniques that are fundamental for any imaging method, particularly for AFM. The scanning tip can move or even sweep samples that are weakly bound to the surface. Ignoring the sweeping effect has

led to a number of scanning artifacts in early attempts to image DNA with STM [8]. Therefore, the sample preparation procedure received extensive attention in AFM studies of biological samples, resulting in a slow start in the development of AFM biological applications. Breakthroughs made in the early 1990s in DNA preparation techniques for AFM [9-15] enabled the analysis of DNA structure and dynamics, and various protein-DNA complexes at the nanoscale resolution level (reviewed in [5]).

Unlike other types of microscopes, AFM is capable of topographic imaging and measuring intermolecular interactions. Therefore, during the past decade, AFM has evolved from primarily a topographic technique into a multifaceted methodology that combines the visualization and probing modalities of the instrument. Furthermore, the force spectroscopy mode of AFM has achieved a piconewton level of sensitivity that is capable of measuring weak intermolecular interactions. Likewise, the development of a force spectroscopy methodology for imaging at the single-molecule level made it possible to probe transient molecular complexes. The current Methods issue summarizes a number of technological advances of AFM in both application areas.

AFM is widely used for imaging DNA-protein complexes, and the characterization of the protein stoichiometry in these complexes is one of the goals of AFM analyses. Fuentes-Perez and colleagues review various approaches to measure protein volumes in different types of protein-DNA complexes and describe their methodology for these measurements [16]. The contribution from the tip geometry is a complicating factor; therefore they used DNA fragments as fiducial markers to normalize the volume measurements. The quantitative analysis of single-stranded DNA is complicated by the low contrast of these molecules that appear as globular features on the AFM images. The application of the DNA fiducial method enabled the authors to estimate the DNA size from the volume measurements.

AFM is also widely used to map specific positions of proteins on a DNA template. One of the problems complicating unambiguous protein mapping is being able to distinguish the two DNA ends. Chammas and colleagues developed the DNA end-labeling procedure that circumvents the problem [17] by labeling the ends with relatively small loops formed by single-stranded DNA. The approach was utilized in the analysis of RNA polymerase's transcription direction.

The stability of the instrument during operation is a critical issue in all modes of AFM. Sullan and colleagues describe an approach enabling them to dramatically reduce instrument drift [18]. They showed that gold coating of the cantilever could be a major factor that increases instrument drift, and that by stripping the gold layer it was possible to achieve sub-pN force precision.

It is critical to be able to reliably detect and measure individual rupture events in single-molecule AFM force spectroscopy (SMFS). Three papers address this issue. Noy and Friddle provide a practical guide for the quantitative analysis of SMFS experiments [19]. They review the basic physics of the measurements, the model used for data interpretation, and outline a number of practical aspects to the analysis of single-molecule force

spectroscopy experiments. Bujalowski and Oberhauser describe the use of single-molecule AFM to track protein unfolding and refolding pathways, enzymatic catalysis, and the effects of osmolytes and chaperones on protein stability and folding [20]. They also outline the operating principles for two different AFM pulling techniques, length clamp and force-clamp, and discuss prominent applications. SMFS practitioners and novices would benefit from protocols for the: construction of poly-proteins, the preparation of AFM substrates, calibration of AFM cantilevers, protein sample preparations, and the analysis of the obtained data.

In AFM force spectroscopy, polymer linkers are necessary for the covalent attachment of molecules of interest to the AFM tip and the surface. Additionally, the polymer linkers provide the proper orientation of interacting molecules in probing experiments, separate specific interactions from nonspecific short-range adhesion, and serve as a reference point for the quantitative analysis of single-molecule probing events. A paper by Tong et al. describes a novel flexible linker for tethering molecules on the surface and the tip based on the well-developed phosphoramidate (PA) chemistry [21]. PA linkers with different functional groups have been synthesized and tested in experimental systems utilizing different immobilization chemistries. One of the most attractive features of PA linkers is their homogeneity in length. Furthermore, the properties of the tether (length, functional groups) can be adjusted to meet the specific requirements for different force spectroscopy experiments and system characteristics, allowing PA linkers to be used for a variety of single molecule probing applications.

The use of AFM to probe cells is an important area for various AFM applications. Friedrichs et al. provide a practical guide to quantify the adhesive strength of living animal cells to various substrates at the single-cell level [22]. The paper describes a number of useful protocols for the control cell state, including: methods to attach the probe to the AFM cantilever, substrate functionalization, performing cell adhesion measurements, and the analysis and interpretation of the force spectroscopy data. The report by Posch et al. describes approaches for force spectroscopy measurements of living platelets, enabling the exploration of morphological changes and receptor activities [26]. SMFS measurements of living platelets enabled the determination of the activation state of the most prominent membrane receptor, integrin $\alpha\text{IIb}\beta_3$, at different phases of activation. Celik et al. describe the application of AFM to probe microtubule networks under biochemical and mechanical stimulations, revealing the depolymerization and displacement of the microtubules out of the contact zone [23]. They additionally identified a slower response characterized by tubulin polymerization at the periphery of the indented area. Notably, the approach allowed the authors to detect cytoskeletal changes in response to the mechano-chemical stimuli in real-time, which could be applicable to other living systems.

Sokolov et al. [24] developed an approach for the quantitative analysis of elastic modulus of cells, enabling the measurement of mechanical properties of cells in indentation experiments. Importantly, the contribution from the inelastic effect of cellular brushes was incorporated. The proposed method is applicable to eukaryotic cells and to the mechanical probing of bacteria and non-biological soft materials. Stewart et al. describe a method for manufacturing tip-less wedged cantilevers for probing cell mechanics [27]. This

modification of the cantilever prevents cell sliding caused by the tilted cantilever when it is mounted in the AFM tip holder and improves the assessment of cell mechanical properties.

Kuznetsov and Evans describe a non-AFM approach for probing cell surfaces in which a levitated spherical particle held by an optical trap interrogates the surface of the cell [25]. The probe's precise positioning (nanometer range) enabled the authors to probe the topography of a soft lamella cell with a nanometer resolution. The glass probe was functionalized with a ligand and was capable of probing individual integrin-ligand interaction events and measuring complex stability.

References

- [1]. Binnig G, Rohrer H, Gerber C, Weibel E. Phys. Rev. Lett. 1982; 49:57–61.
- [2]. Binnig G, Rohrer H. Helvetica Phys. Acta. 1982; 55:726–735.
- [3]. Binnig G, Quate CF, Gerber C. Phys. Rev. Lett. 1986; 56:930–933. [PubMed: 10033323]
- [4]. Hansma PK, Elings VB, Marti O, Bracker CE. Science. 1988; 242:209–216. [PubMed: 3051380]
- [5]. Lyubchenko YL, Shlyakhtenko LS, Ando T. Methods. 2011; 54:274–283. [PubMed: 21310240]
- [6]. Zhong Q, Inniss D, Kjoller K, Elings VB. Surf. Sci. Lett. 1993; 290:L688–L92.
- [7]. Ando T, Uchihashi T, Kodera N. Annu. Rev. Biophys. 2013
- [8]. Clemmer CR, Beebe TP Jr. Science. 1991; 251:640–642. [PubMed: 1992517]
- [9]. Bustamante C, Vesenka J, Tang CL, Rees W, Guthold M, Keller R. Biochemistry. 1992; 31:22–26. [PubMed: 1310032]
- [10]. Thundat T, Allison DP, Warmack RJ, Brown GM, Jacobson KB, Schrick JJ, Ferrell TL. Scanning Microsc. 1992; 6:911–918. [PubMed: 1295085]
- [11]. Vesenka J, Guthold M, Tang CL, Keller D, Delaine E, Bustamante C. Ultramicroscopy. 1992; 42-44(Pt B):1243–1249. [PubMed: 1413262]
- [12]. Weisenhorn AL, Römer DU, Lorenzi GP. Langmuir. 1992; 8:3145–3149.
- [13]. Bezania M, Drake B, Nudler E, Kashlev M, Hansma PK, Hansma HG. Biophys. J. 1994; 67:2454–2459. [PubMed: 7696484]
- [14]. Hansma HG, Hoh JH. Annu. Rev. Biophys. Biomol. Struct. 1994; 23:115–139. [PubMed: 7919779]
- [15]. Bustamante C, Rivetti C. Annu. Rev. Biophys. Biomol. Struct. 1996; 25:395–429. [PubMed: 8800476]
- [16]. Fuentes-Perez ME, Dillingham MS, Moreno-Herrero F. Methods. 2013; 60:113–121. [PubMed: 23454289]
- [17]. Chammas O, Billingsley DJ, Bonass WA, Thomson NH. Methods. 2013; 60:122–130. [PubMed: 23500656]
- [18]. Sullan RM, Churnside AB, Nguyen DM, Bull MS, Perkins TT. Methods. 2013; 60:131–141. [PubMed: 23562681]
- [19]. Noy A, Friddle RW. Methods. 2013; 60:142–150. [PubMed: 23531626]
- [20]. Bujalowski PJ, Oberhauser AF. Methods. 2013; 60:151–160. [PubMed: 23523554]
- [21]. Tong Z, Mikheikin A, Krasnoslobodtsev A, Lv Z, Lyubchenko YL. Methods. 2013; 60:161–168. [PubMed: 23624104]
- [22]. Friedrichs J, Legate KR, Schubert R, Bharadwaj M, Werner C, Müller DJ, Benoit M. Methods. 2013; 60:169–178. [PubMed: 23396062]
- [23]. Celik E, Abdulreda MH, Maignel D, Li J, Moy VT. Methods. 2013; 60:195–201. [PubMed: 23466787]
- [24]. Sokolov I, Dokukin ME, Guz NV. Methods. 2013; 60:202–213. [PubMed: 23639869]
- [25]. Kuznetsov IR, Evans EA. Methods. 2013; 60:214–224. [PubMed: 23643864]

- [26]. Posch S, Neundlinger I, Leitner M, Siostrzonek P, Panzer S, Hinterdorfer P, Ebner A. *Methods*. 2013; 60:179–185. [PubMed: 23571313]
- [27]. Stewart MP, Hodel AW, Spielhofer A, Cattin CJ, Müller DJ, Helenius J. *Methods*. 2013; 60:186–194. [PubMed: 23473778]