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Three Powerful Research Tools from Single Cells into Single Molecules: AFM, Laser Tweezers, and Raman Spectroscopy

Yongkuan Wu · Kun Liu · Kedong Song · Shi Pan

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Abstract By using three physical techniques (atomic force microscopy (AFM), laser tweezers, and Raman spectroscopy), many excellent works in single-cell/molecule research have been accomplished. In this review, we present a brief introduction to the principles of these three techniques, and their capabilities toward single-cell/molecule research are highlighted. Afterward, the advances in single-cell/molecule research that have been facilitated by these three techniques are described. Following this, their complementary assets for single-cell/molecule research are analyzed, and the necessity of integrating the functions of these three techniques into one instrument is proposed.

Keywords Single cells · Single molecules · Atomic force microscopy · Laser tweezers · Raman spectroscopy

Introduction

Cell cloning, stem cell/cell transplantation, and cell/gene therapy have drawn great attention for their potential human health applications, but there are many challenges ahead. Cells are the structural and functional units of all living things, whereas the details of various interactions within cells remain elusive. The investigations of the microstructure and physicochemical properties of single cells can provide clues to those unsolved issues. To decode the working mechanisms of single cells, the further exploration of the biomolecules

Y. Wu · K. Liu (✉) · S. Pan
Institute of Near-field Optics and Nano Technology, School of Physics and Optoelectronic Technology, Dalian University of Technology, Dalian 116024, People's Republic of China
e-mail: liukun@dlut.edu.cn

K. Song
Dalian R&D Center for Stem Cell and Tissue Engineering, Dalian University of Technology, Dalian 116024, People's Republic of China

within cells (DNA, RNA, proteins, etc.) are indispensable. The micro-/nanostructure of cells/biomolecules, microsecond processes of biological reactions, piconewton level of biological interactions, and the complexity and diversity of biomolecules suggest that conventional biotechniques would be inadequate.

Thereby, the selection of methodology and techniques for single-cell/molecule research is crucial. Most traditional experiments measure the ensemble average of the target cells/molecules and cannot directly reveal the unsynchronized biological behavior, such as the heterogeneity in the population, transient intermediate states, and parallel reaction pathways. Single-cell/molecule experiments can provide access to the fluctuations of individual cells/biomolecules. Atomic force microscopy (AFM), laser tweezers, and Raman spectroscopy are appropriate tools to probe the structures and properties of single cells/molecules [1–5].

Atomic Force Microscopy

Figure 1a displays the schematic diagram of AFM. The AFM tip at the terminal of a flexible micro-cantilever scans the sample surface through controlling the tip–sample interactions. A laser beam, reflected from the cantilever into the quadrant detector (a position detector), is used to stabilize the deformation of the cantilever through extending/retracting the piezoelectric ceramics. The three-dimensional morphology of the sample can thus be depicted at nanometer by recording the variation of the piezoelectric ceramics. In the pulling mode, the tip–sample interactions can be measured as a function of the tip–sample distance, named the force–extension curves, which reflect the mechanical properties of the sample surface.

Single-Cell Applications of AFM

With high-resolution (0.5 nm lateral resolution and 0.1 nm axial resolution) and simple sample preparation (without fixation and staining (compared to electron microscopes and fluorescence microscopes, respectively)), AFM is used to image single cells, even the biomolecular assemblies, special structures, and native membrane proteins on cell surfaces

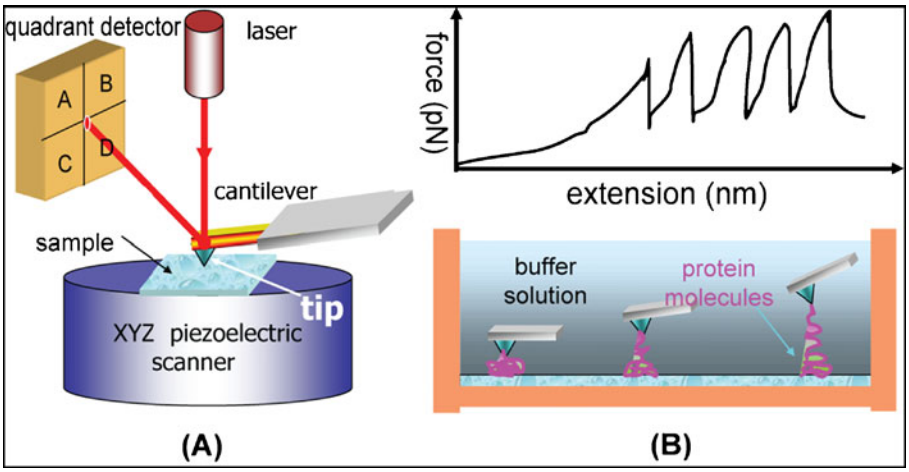

Fig. 1 **a** Schematic diagram of AFM; **b** SMFS experiment of protein molecules in buffer solution

under physiological conditions [2, 6–8]. Time-lapse AFM also allows monitoring the dynamic bioprocesses in situ, such as the pH-induced conformational changes in connexin26 hemichannels [9] and the cell surface dynamics of germinating *Aspergillus fumigatus* conidia [10]. All of these features offer insights into the microstructures and dynamic processes of single cells.

Cell surface mechanical properties (adhesion, elasticity, and hydrophobicity), which are essential for many biological functions (cell migration, tissue formation, wound healing, etc.), can be detected via the force–extension curves with an accuracy of 10^{-13} N [2, 6, 7, 11, 12]. By utilizing chemical force microscopy (the AFM tips are modified with specific functional groups), the hydrophobicity of single living cells was mapped at nanometer resolution [13, 14]. Importantly, cylindrical AFM tips were fabricated to achieve a constant tip–cell contact area during the mechanical measurement, which is impossible with the common pyramidal or spherical AFM tips, and were used to characterize the adhesion and elasticity of single cells [15]. Besides, one novel application of AFM is that single-cell transfection was achieved by using plasmid decorated AFM tips that were forcibly incorporated into the cell to transfer the genetic material through cell membrane without cell damage. Thus, single-cell surgery can be performed by AFM with minimal disturbance [16].

To date, cancer diagnosis relies on biopsy, which is sometimes inconvenient or unavailable. Recently, the mechanical properties of cancer cells were probed by AFM, and it was found that they were far softer than normal cells [17–19]. These studies indicate that this mechanical method is a new candidate for cancer diagnostics.

Single-Molecule Advances of AFM

By immobilizing the target biomolecules onto flat substrates, AFM can further image single biomolecules, such as plasmid DNA and viral RNA [20], single-stranded DNA-binding protein–DNA complexes [21], and GDP-tubulin protofilaments [22]. Time-lapse AFM also permits the monitoring of the dynamic processes of biomolecules in real time, such as protein movements [23], the disassembly of a single RecA–DNA–ATPγS complex [24], and DNA unwinding and transport processes [25]. Moreover, fast-scanning AFM (several images per second) can capture the short-lived bioprocesses. Yokokawa et al. visualized the GroES binding to and dissociation from individual GroEL with a lifetime of 6 s, as well as the ATP/ADP-induced open/closed conformational changes of individual GroEL [26]. Besides, based on special interactions between ligands and receptors, the biomolecules could be recognized and localized at nanometer resolution by bonding the corresponding molecules onto AFM tips [27–29].

Molecular interactions trigger many bioprocesses, such as DNA replication, protein synthesis, and information transfer between/within cells. By utilizing single-molecule force spectroscopy (SMFS) as shown in Fig. 1b, the intermolecular interactions [30–33] and the intramolecular forces during the unfolding and refolding [31, 34–38] were quantified at piconewton level. Liu et al. probed the RNA–coat protein interactions directly in intact tobacco mosaic virus by extracting the RNA from the helical groove [33]. Besides, Kufer et al. used AFM to cut and paste single DNA molecules under the monitoring of SMFS [39], which shed new light on gene surgery.

Limitations and Developments of AFM in Single-Cell/Molecule Research

Although fast-scanning AFM has increased the imaging speed to several frames per second [26], AFM is still not capable of visualizing some short-lived bioprocesses. Therefore,

improving time sensitivity is undoubtedly one of the key challenges for AFM in the future applications [7]. Moreover, to depict the fine biostructures at nanometer resolution or below and to detect the weak molecular interactions at femtonewton level via AFM, sharper tips and more sensitive cantilevers are on demand.

Laser Tweezers

Laser tweezers or optical tweezers are a stable three-dimensional light trap formed through tightly focusing a laser beam with a high numerical aperture objective lens and enable the manipulation of small particles and precise force measurement. A dielectric particle with higher refractive index than the surroundings will withstand a resultant force pointing at the focus due to momentum transfer; thus, small particles near the focus can be captured (Fig. 2a, b). Due to the reason that the deflection of the dielectric particle relative to the laser focus is a function of the applied force, the force can be quantified by measuring the deflection distance.

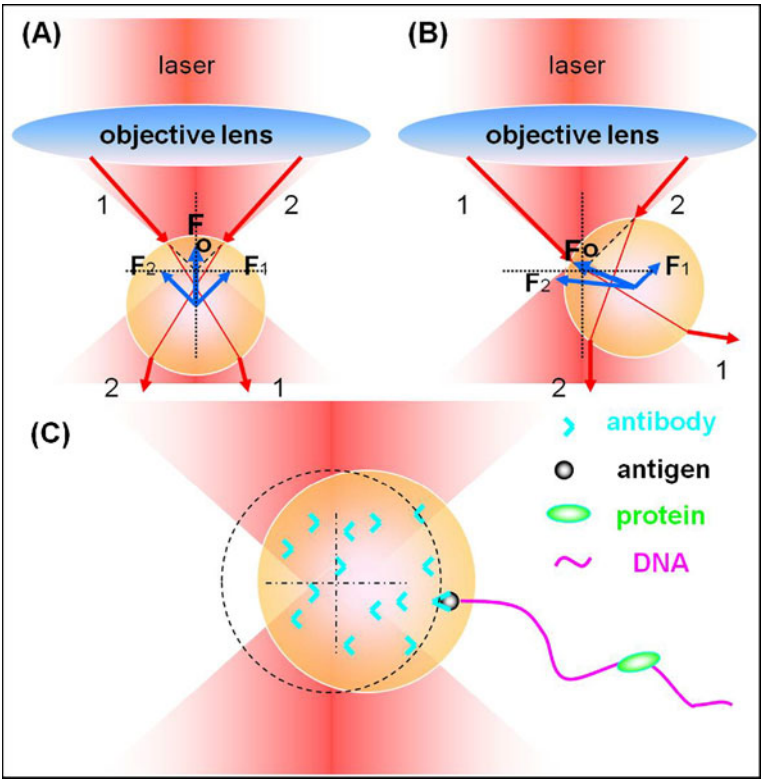

Fig. 2 Schematic of optical trapping: F_1 and F_2 arise from two conjugated laser beams 1 and 2; F is the resultant force of F_1 and F_2 and O is the focus of the laser beam. **a–c** shows the axial stability, the gradient force, and how laser tweezers manipulate a protein molecule indirectly, respectively [3]

Single-Cell Applications of Laser Tweezers

Except for the functions of trapping and sorting single cells, laser tweezers were successfully used to detect the mechanical properties of single cells without mechanical damage due to its non-contact control. For instance, the mechanical properties of human mesenchymal stem cells and differentiated fibroblasts were measured through extracting tethers from cell membranes [40]. By exerting a point load, it was found that the protrusion stiffness of neutrophils depended on the force-loading rate and the cytoskeletal integrity, not on the force location, medium osmolality, or temperature [41]. Moreover, the frequency-dependent viscoelasticity of alveolar epithelial cells could be measured through the forced oscillation by laser tweezers, and the results suggested that the oscillation of endogenous organelles could be useful for investigating intracellular heterogeneity and temporal fluctuations [42].

Dynamic processes of single cells as well as the organelles and macromolecules within cells can be monitored via laser tweezers. Typically, laser tweezers were used to analyze sperm motility (swimming force in terms of escape laser power), which might be useful to quantitatively assess sperm quality and viability [43]. Moreover, the dynamic response of cells to external stimulations can be detected, such as the dynamic processes of a growing yeast cell in an optical trap [44] and the dynamic deformation of a red blood cell in dual-trap laser tweezers [45]. By using multiple laser tweezers, it was found that the extracellular matrix-cytoskeleton linkages along a rigidity gradient were regulated by controlling the adhesion area and the actomyosin recruitment to maintain a constant deformation of the extracellular matrix [46]. Furthermore, by exploring infrared laser to heat single molecules of *Escherichia coli* RNA polymerase and monitoring the transcription activity simultaneously, a significant change in the rates of transcript elongation with temperature was found [47]. Besides, the initial interactions of HIV infected cells with uninfected cells were also investigated with laser tweezers [48].

Single-Molecule Advances of Laser Tweezers

With high force sensitivity (10^{-13} N), laser tweezers are particularly suitable for quantifying the mechanical properties of single biomolecules. For most biomolecules, direct trapping is impossible, but it is accessible by tethering the interested biomolecules to a mechanical handle (dielectric bead) as shown in Fig. 2c. By using this strategy, non-specific, protein-mediated DNA–DNA interactions were investigated systematically with a laser tweezers instrumentation that could assess two DNA molecules independently [49]. The intramolecular forces of DNA, RNA, and proteins during folding and unfolding could also be probed and located [50–52]. Cecconi et al. directly observed the three states (folding, unfolding, and intermediate) of a single protein molecule by using laser tweezers to induce the mechanical unfolding and refolding of individual *E. coli* ribonuclease H molecule and found that the intermediate state displayed unusual force compliance [50]. Similarly, laser tweezers were used to detect the sequence-dependent energy landscapes of DNA hairpins under controllable loads [51, 52]. Additionally, the folding-energy landscape could be manipulated to control the fate of RNA (native or misfolding pathways) by modulating the relaxation rate of the applied force [53].

The piconewton/nanometer resolving power and high time sensitivity makes laser tweezers an irreplaceable tool to investigate the kinetic properties of single biomolecules. Typically, the study of molecular motors, which provides the original motivity for majority

of the bioprocesses, yields a large number of outstanding achievements. By tethering the target molecular motors to the dielectric beads, the strength of the motivity, step length, and pausing times were quantified, and their relationships with loads and ATP hydrolysis were detected [54–58]. The kinetic details of DNA transport in *Bacillus subtilis* and transcription termination by bacterial RNA polymerase were also investigated via laser tweezers [59, 60].

Disadvantages and Developments of Laser Tweezers in Single-Cell/Molecule Research

Using near-infrared laser can relieve the radiation damage of laser tweezers to biological sample, but the corresponding heating effect will increase the temperature and induce convection currents around the laser focus. By placing copper jackets on both sides of the microchamber, a stable and homogeneous temperature control (4.5–68 °C) inducing little convection current was achieved [61]. Normally, the efficiency of laser tweezer experiments is low (one particle per time). Recently, Tam et al. presented a method to selectively capture and release 2×2 microparticles by using an imaging fiber-bundle-based optical tweezer array that enabled individual control of the size, shape, and position of each trap [62].

Raman Spectroscopy

Figure 3 shows the schematic of light scattering. When photons collide with the scattering center, most photons do not change their frequency (defined as elastic scattering or Rayleigh scattering), but a small number of photons change their frequencies due to energy exchange (defined as inelastic scattering). Raman scattering belongs to inelastic scattering. The frequency difference between Raman scattering and incident photons (called Raman shift) is related to the vibration–rotation bands of the target molecules. Raman scattering includes Stokes scattering (the lower frequency) and anti-Stokes scattering (the higher frequency); ordinarily Stokes scattering is collected. Raman spectroscopy is widely applied to detect the composition and structure of target molecules.

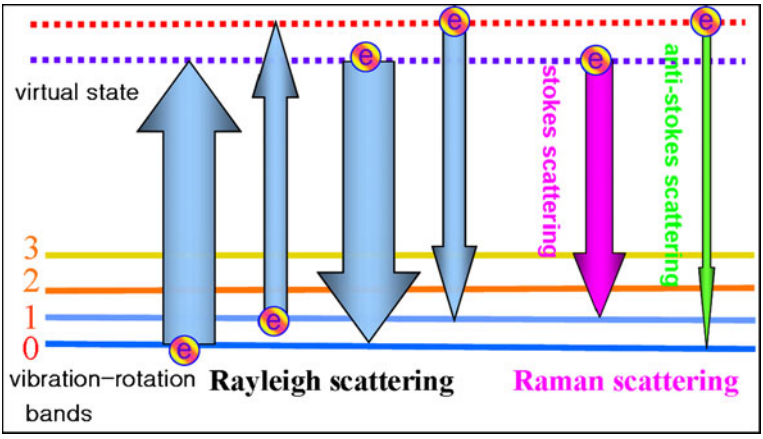

Fig. 3 Schematic of light scattering

Single-Cell Applications of Raman Spectroscopy

Immunostaining is regularly used to identify special cells, but the processed cells are unviable and nonrecoverable, whereas the nondestructive and label-free identification via Raman spectroscopy is an ideal candidate [63, 64]. For instance, mouse embryonic stem cells were imaged using coherent anti-Stokes Raman spectroscopy (CARS). Based on the selective observation of specific molecular vibrations, clear differences between differentiated and undifferentiated cells were revealed [63]. With the help of multivariate statistical methods, embryonic stem cells were separated from their cardiac derivatives through their intrinsic biochemical characteristics [64]. These experiments are meaningful for stem cell transplantation [63, 64]. Similarly, by virtue of computer-based data processing methods (principal component analysis (PCA) and linear discrimination analysis (LDA)), Raman spectroscopy was successfully used to discriminate lung cancer cells from normal cells [65]. Moreover, the distribution of astaxanthin in a single microalgal cell was revealed via Raman imaging [66]. Besides, by using noble metal (gold or silver) nanoparticles as Raman signal amplifier (surface enhanced Raman spectroscopy, SERS), the membrane proteins of living cells [67] and the intracellular chemical composition could be detected [68]. Lately, gold nanoparticles with ultra-thin silica/alumina shells were proved to be favorable Raman signal amplifiers due to its inert and biocompatible surfaces [69].

The composition and structure of biomolecules vary with their physiological activities, and the understanding of these bioprocesses is essential for further cell investigations. According to the "fingerprint spectroscopy" of specific biomolecules, it is possible to monitor the cell activities in situ using Raman spectroscopy [70, 71], such as the dynamic changes during electrical stimulation and cell remodeling in a cardiac myocyte [70] and the metabolic history of pseudomonas fluorescence [71].

Single-Molecule Advances of Raman Spectroscopy

The high information content and the weak Raman signal of water molecules makes Raman spectroscopy a wonderful tool to detect the structure information of biomolecules in aqueous solution. Conventional Raman experiments reveal the ensemble averages of the target molecules and cannot reveal the fluctuations of single molecules. However, owing to the small scattering cross section ($\sim 10^{-30}$ cm^2 per molecules) of Raman scattering, Raman spectra of single molecules is hard to acquire. The development of SERS with an enhancement factor up to 10^{14}–10^{15} [72] makes single-molecule detection possible. Lately, several types of biomolecules have been detected at the single-molecule level via SERS [4, 72, 73]. Koo et al. confirmed the applicability of combining SERS with CARS for high-sensitivity detection of single biomolecules (deoxyguanosine monophosphate and deoxyadenosine monophosphate) and found that this combination of the two techniques provided more than three orders of signal enhancement compared with SERS [74].

Obstacles and Developments of Raman Spectroscopy in Single-Cell/Molecule Research

Raman spectroscopy has not been used extensively in single-cell/molecule research. On one hand, Raman scattering of biological samples is weak, which results in the lengthy acquisition time and high excitation power. The development of SERS can enhance the Raman scattering by 14–15 orders of magnitude but introduces a new issue—the preparation of reproducible, biocompatible, and high enhancement activity SERS substrates. On the other hand, the analysis of Raman spectra of cells/biomolecules is

difficult due to their heterogeneous contents, whereas computer-based data processing methods, such as PCA and LDA, can offer assistance [64, 65].

Complementary Assets of These Three Techniques

The roles of these three techniques in single-cell/molecule research are specified in general terms: AFM is a nanoscale microscope and a piconewton detector; laser tweezers are nanometer and piconewton manipulator; Raman spectroscopy is a molecular structure detector. Obviously, there are complementary assets between these three techniques in single-cell/molecule research (Fig. 4).

It is feasible that biological researchers acquire the required information by using these three techniques one by one. However, the samples/environments will change (normally in undesirable ways) if the experimental equipments are replaced. Unifying the functions of these three techniques into one instrument is a feasible method to avoid this problem, but it is difficult for technical reasons. Luckily, such instrumentations have been developed. A prominent example is laser tweezers Raman spectroscopy (LTRS), which can successfully trap single cells and acquire its Raman spectrum in aqueous solution. Several significant works have been accomplished with the assistance of LTRS, such as the detection of recombinant proteins in single transgenic microbial cells [75], the monitoring of single cell bacterial lysis [76], single-cell identification of leukemia [77], and single-cell lipid profiling of oil-producing microalgae [78]. The real-time spectral changes of single pancreatic β-cell under high glucose stimulation were also monitored by LTRS [79]. Recently, the capability of LTRS was further strengthened. Ye and Zhang designed a LTRS system equipped with two lasers (an infrared laser and a visible laser) for trapping and Raman excitation, respectively [80]. In addition, a multiple-trap LTRS array for simultaneously acquiring the Raman spectra of several cells was developed, which markedly improved the work efficiency of LTRS [81, 82]. Besides, tip-enhanced Raman spectroscopy, the combination of AFM with Raman spectroscopy, was successfully used to study the membrane surface chemistry of a single living cell with ultra-high morphology/spectroscopy spatial resolution (less than 10 nm) [83, 84]. In a word, a more powerful tool for single-cell/molecule

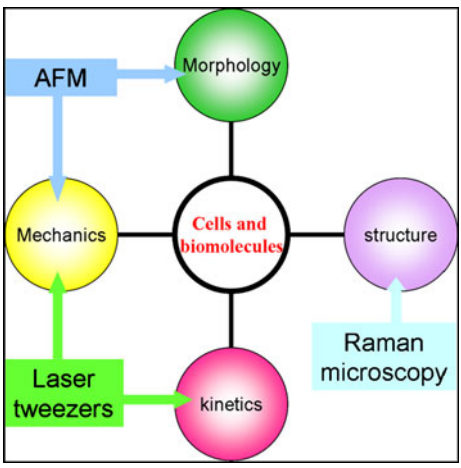

Fig. 4 The functions of AFM, laser tweezers, and Raman spectroscopy in single-cell/molecule research

research will emerge if the functions of these three techniques are successfully integrated into one instrument.

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