1
A Road Map to Single Molecule Dynamics

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1.1
Visualization of Single Molecules

Signals from single fluorescent dye molecules were first measured in non-biological environments. Some of the earliest studies acquired signals from single fluorescence dyes embedded in a solid matrix at the temperature of liquid helium [1] and from excitation fluorescence signals [2]. In early studies carried out at room temperature, single fluorescence dye molecules spread over a film were observed using near-field scanning fluorescence microscopy [3].

In a biological environment where fluorescence is largely quenched by the surrounding water molecules, single molecule imaging was first accomplished in 1995 using total internal reflected fluorescence (TIRF) and epi-fluorescence microscopy [4] (see Chapter 2). Single fluorophores attached to myosin molecules were visualized in aqueous solution in the vicinity of a glass surface while single ATP turnover from the same myosin molecule was measured using fluorescently-labeled ATP. TIRF microscopy has been widely used for single molecule imaging. Other studies have measured single fluorophores bound to biomolecules in solution while passing through a fixed small volume using confocal microscopy [5].

1.2
Single Molecule Position Tracking

The motion of biomolecules has been tracked by following the visualization of immobilized single biomolecules. The motion of processive motors has been visualized by using large particles or by attaching if fluorescent dyes to the motor. In 1996, the smooth and processive motion of a molecular motor, kinesin attached to a single fluorescence dye was detected along microtubules immobilized on a glass surface using TIRF microscopy [6]. Epi- and TIRF microscopy were used to monitor the 2D diffusion of fluorescently-labeled phospholipids on phospholipid membranes

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[7] while 3D diffusion of single fluorescence molecules was observed in gel structures [8]. One-dimensional diffusion, which was discovered by single molecule measurements, is a biomolecular mechanism used in the search for specific reaction sites on DNA. This mechanism was first observed directly during studies to elucidate the promoter binding site of RNA polymerase on DNA molecules which had been extended with laser traps [9].

It has been demonstrated that the high-resolution analysis of individual fluorescence spots enables the position of a single molecule to be tracked with nanometer accuracy, thus overcoming the diffraction limit of light [10]. In 2003, this nanometer accuracy proved crucial in the detection of the step movement of myosin V, a processive motor, along actin filaments at no load [11]. Taken together with the laser trap data, the step movement of molecular motors containing two motor domains (heads) can then be defined by a hand-over-hand mechanism (see Chapter 3). Thus single molecule detection has been instrumental in establishing the basic operations of molecular motors. This technique was also used to detect the step movement of microtubule-based motors in live cells by tracking cargoes fluorescently labeled with GFP [12] or carrying QDs (quantum dots) introduced via endocytosis [13].

In addition to fluorescence imaging, dark-field imaging of gold nanoparticle-labeled molecular motors provides another method for high sensitivity tracking. Tracking labeled myosin V heads during single step movements to the adjacent landing position with sub-milliseconds accuracy, enabled investigators to directly observe the diffusive search pattern in the step movement [14].

1.3 Single Molecules in Live Cells

Single fluorescently labeled molecules have been visualized in artificial lipid bilayers. The model bilayer was formed at the tip of a pipette near the surface of the glass in a TIRF microscope, which enabled simultaneous imaging and electric measurements of single ion channels [15]. Given that the ion current through a single ion channel has already been reported [16], the visualization of single molecules in the lipid bilayer has opened up the possibility of studying the structure and function of ion channels directly (see Chapter 4).

In 2000 single molecule imaging was successfully applied to living cells using TIRF and epi-fluorescence microscopy [17]. The binding of fluorescently-labeled signaling molecule epidermal growth factor (EGF) to its receptor on the surface of live cells was monitored at the single molecule level, which provided further information relating to the kinetics of binding and the behavior of the EGF–receptor complex (see Chapter 5). Single molecule tracking in live cells has also been achieved using fluorescent labels. In particular quantum dots (QDs) have been frequently used for quantitative and prolonged tracking (see Chapter 6). The trajectory of QDs bearing EGF revealed a variety of transport paths used by the ligand–receptor complex [18]. There have been a large number of papers on both single molecule tracking in live cells and the dynamic properties of the corresponding biomolecules, including

diffusion. The motion of single molecules on the cell surface and inside the cell has also been measured using fluorescence correlation spectroscopy (FCS) [19, 20].

The interaction between ligands and receptors has been studied using single molecule imaging and mechanical measurements (see Chapter 7). The binding of ligands to receptors triggers signal transduction processes leading to the activation of whole cells (see Chapter 5). The activation of a cell can be directly related to the binding events of the ligands inside the cell. Such an experiment was first carried out by increasing the cytoplasmic Ca^{2+} concentration to determine the number of peptide-MHC ligands bound to a T-cell [21]. In general, only a small number of binding ligands have been reported in various systems. In fact, results have emphasized that small input signals within large noisy signals can generate consistent output. For example, in dictyosterium cells, in which motion is biased in one direction in response to a small cAMP concentration gradient, the kinetics of cAMP binding was observed to be dependent on location, leading to the polarity of the moving cell [22].

The expression of RNA and protein has also been detected in live cells at the single molecule level. RNA plays a pivotal role in gene expression in which RNA molecules work dynamically in the nucleus and cytoplasm (see Chapter 8). The trafficking of messenger RNA was monitored in real time after being expressed inside the nucleus of live cells [23]. It was demonstrated that diffusion is the basis for mRNA displacement. Regarding proteins, individual proteins expressed in single cells have been counted in real time. YFP fluorescence showed [24] that protein expression occurs in bursts. The stochastic nature of the gene expression has also been studied.

1.4
Fluorescence Spectroscopy and Biomolecular Dynamics

Combined with single molecule imaging, fluorescence spectroscopy can be used to measure structural dynamics and biomolecule interactions in real time (see Chapter 9). In particular, fluorescence resonance energy transfer (FRET) between single fluorophores has become a popular tool of choice following its application in dried DNA using scanning near-field microscopy [25]. Conformational dynamics were observed using chymotrypsin inhibitor 2 [26] and *Tetrahymena thermophila* ribozyme [27]. These conformational studies have been followed by a large number of studies on multiple conformations and rugged energy landscapes. Single molecule FRET has also been used for protein folding studies. Subpopulations of folded and denatured conformations of proteins freely diffusing in solution were directly determined by confocal microscopy [28, 29]. These measurements have provided useful tests for a new view on dynamic structures of biomolecules in which they behave according to the energy landscape. In addition to FRET, spectral shift and fluorescence lifetime polarizations have been used to study protein dynamics. Single molecule fluorescence polarization imaging was first carried out to monitor the axial rotation of actin filaments sliding over myosin immobilized on a glass surface [30]. It was recently demonstrated using polarization imaging from fluorescence dyes attached bifunctionally to the neck domain of myosin V that the orientation of the

neck domain is associated with the step movement, in agreement with a walking model of myosin V [31] (see Chapter 3).

By monitoring fluorescence changes associated with enzymatic reactions, the operation of enzymes can be studied. Taking advantage of the cycle between the active fluorescent oxidized form (FAD) and non-fluorescent reduced form (FADH) of flavin adenine dinucleotide, the turnover of cholesterol oxidase was monitored [32]. The reaction was stochastic, consistent with Michaelis–Menten mechanics while a memory effect was observed between several turnover intervals as a result of slow conformational isomerizations. Similar memory effects were observed for RNA enzymes (ribozymes) [27].

Proteins, DNA and RNA fulfill their function by assembling and disassembling dynamically. Single molecule spectroscopy is capable of determining the dynamic structure of particular molecules and molecular interactions in such biomolecular complexes. A more physiological environment has been observed during protein folding in more complex systems such as Gro EL and ES complexes [33]. The dynamic structures of certain molecular complexes such as helicase, DNA and holiday junctions, which appear in gene recombination and repair, have been extensively studied (see Chapter 11).

1.5
Single Molecule Manipulation and Molecular Motors

Laser and magnetic trapping and glass microneedles are common tools used to manipulate single biomolecules. After Ashkin applied optical trapping techniques to trap viruses [34], single kinesin was trapped using an attached dielectric bead to monitor its movement along microtubules immobilized on a glass surface [35], followed by the finding of an 8-nm unit step associated with the hydrolysis of single ATP molecules [36]. Microneedles were first used to manipulate actin filaments in 1988 [37]. This led to the measurement of the mechanical properties of actin filaments and the force generated by both myosin filaments and single muscle myosin heads [38]. Actin filaments have also been manipulated using the laser trap. Actin filaments, in which each end was attached to a trapped bead, were manipulated to interact with single non-processive muscle myosin molecules. The step movement of muscle myosin was measured by monitoring the displacement of the actin filaments [39, 40]. Alternatively, instead of manipulating actin filaments, single myosin molecules have been captured on the tip of a microneedle and manipulated to interact with actin filaments [41]. The movement of myosin was monitored directly by measuring the displacement of myosin. Given that these measurements have sensitivity to detect thermal motion, the effect of thermal motion on the mechanism of molecular motor step movement is discussed in Chapter 2.

Unlike muscle myosin, it is relatively easy to measure step movement in processive motors. Manipulation by laser trap has been used for mechanical studies on several processive myosin motors including myosin V [42], myosin VI [43] and dynein [44, 45] demonstrating that ATP hydrolysis reactions are strain-dependent, suggesting that

cooperation between the two heads is necessary for processive movement [46]. When excess load (greater than stall force) was applied, motors such as kinesin showed stepwise movement in the backward direction [47].

1.6
Mechano-Chemical Coupling of Molecular Motors

In order to understand the mechanism of molecular motors, it is critical to describe how mechanical steps are related to ATP hydrolysis (see Chapter 2). Simultaneous imaging of ATP turnover and mechanical measurements using a single molecule optical trap was used to determine the mechano-chemical coupling in myosin [48].

In contrast to ATP-driven but irreversible actomyosin motors, the ATP synthase F_0F_1 is a reversible mechano-chemical machine. Using single molecule detection, ATP synthase F_0F_1 was found to be a rotary motor (see Chapter 10). The rotation of F_1 was first observed by monitoring the rotation of fluorescent actin filaments attached to the rotor [49]. The 120° rotation unit steps generated by a single ATP molecule were detected at low ATP concentrations [50]. It was also demonstrated that ATP is synthesized when F_1 is forced to rotate in the backward direction [51]. This mechanism has been extensively studied.

1.7
DNA-Based Motors

DNA-based motors translocate along a DNA molecule powered by nucleotide hydrolysis while they transcribe gene information. As compared with actin- and microtubules-based motors, the properties of the DNA-based motors are not yet known. Individual DNA molecules were first visualized by staining with fluorescent dyes and using video-enhanced optical microscopy [52]. This led to single DNA molecules being manipulated by magnetic traps [53], laser traps [54], and by pipette [55]. These manipulation techniques have been used to measure various mechanical properties of DNA such as the force–extension relationship and its interaction with motor molecules.

Transcription by a single RNA polymerase molecule has also been observed by laser trap. The displacement and force exerted on a trapped bead attached to the end of a DNA molecule being pulled by an RNA polymerase was measured while the polymerase was immobilized on a slide glass during transcription [56, 57]. In 2005, RNA polymerase was shown to have a step-size of 3.7 Å, equivalent to a DNA base pair [58]. This technique also proved that RNA polymerase moved along a DNA helix [59] as confirmed by monitoring the rotation of a magnetic bead attached to the end of a DNA molecule during transcription while the RNA polymerase was immobilized on the slide glass. Unlike actin filament- and microtubule-based molecular motors, some DNA-based motors change the topology of DNA while they translocate. This change has been used to monitor the movement of DNA-based

motors. For example, Topoisomerase II removed supercoils that had been created by twisting DNA through magnetic beads [60]. The removal of the supercoil was measured by monitoring the expansion between the two DNA ends. Another example is DNA polymerase, which converts single-stranded DNA into double-stranded DNA. This DNA conformation change was detected by measuring the extension of DNA at constant load [61]. The unwinding of DNA and RNA as catalyzed by helicase has also been measured using mechanical and optical methods (see Chapter 11)

1.8 Imaging with AFM and Force Measurements

AFM [62] is a unique tool for force measurements and imaging, offering single molecule sensitivity. It has been applied to both single protein [63] and DNA molecules [64]. In contrast to fluorescence imaging, which is based on local information around the fluorophores, AFM provides topological imaging and specific electrostatic interaction mapping without modifying the biomolecules. Recently, both temporal and spatial resolutions have been dramatically improved [65] allowing the dynamic operations of biomolecules and the molecular machines responsible for their assembly to be visualized in real time (see Chapter 12).

The interaction force between single molecules has been measured for streptavidin-biotin using AFM [66, 67] and the forces involved in ligand–receptor complexes, antibody–antigen complexes, adhesion of complementary strands of DNA, carbohydrate–carbohydrate complexes, lectin–carbohydrate complexes, and cell adhesion proteins have all been studied (see Chapter 6). AFM and other manipulation techniques have also been used to study the folding and unfolding of proteins. In 1997, the unfolding and folding of titin which is an extremely large protein, was measured when its two ends were pulled and shortened [68–70]. Force clamp methods of AFM allow the detailed processes of individual protein molecule folding to be scrutinized rather than depending on the stepwise transition observed in ensemble measurements [71] (see Chapter 13).

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