REVIEW ARTICLES

Single-molecule studies of DNA by molecular combing*

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Abstract Molecular combing is a powerful method for aligning a large array of DNA molecules onto a surface. It is a process whereby DNA molecules are stretched and aligned on a glass surface by the force via fluid flow. The ability to comb up to several hundred DNAs on a single cover slip allows for a statistically significant number of measurements to be made. These features make molecular combing an attractive tool for genomic studies, such as DNA replication, DNA transcription, DNA-protein interaction and so on. In this review article, we discuss the molecular combing principle, method and its applications.

Keywords: DNA molecule, molecular combing, DNA-protein interaction.

Life, at its most fundamental level, is the subtle interplay of large numbers of proteins, DNAs and RNAs that interact through enzymatic reactions and complex signaling pathways. Current high-resolution methods, such as X-ray crystallography and NMR spectroscopy, have provided a vast array of structural details on biological molecules. Yet, a quantitative understanding of complex, multistep biochemical processes involving multiple species requires a new set of tools that goes beyond both the static molecular view and ensemble averaging. Detecting and controlling macromolecules at the single molecule level has attracted much attention in biological studies[1]. DNA, the carrier of genetic information, is the central molecule in life. When free in solution, a DNA helix behaves as if it were a random elastic coil. To observe this linear molecule, recently, DNA stretching has become the important method to control DNA. Several physical methods have been employed to stretch single DNA molecules, such as magnetic tweezer[2—4], laser tweezer[5—8], micropipette[9] and molecular combing method[10—12]. These biomechanical techniques and optical imaging have demonstrated that it is possible to make observations on the dynamic behavior of single molecules, to determine their mechanisms of action.

In the following, we describe molecular combing method, a novel approach of stretching DNA, and focus on its biological applications.

1 Molecular combing

Extension and manipulation of individual biopolymers (such as DNA) is generally performed by anchoring one end of the molecule at a solid matrix; viscous drag, electrophoresis, or optical forces may then achieve stretching. Molecular combing method extends a DNA molecule with a receding air-water interface and fixes the molecule in this state on the dry substrate. This process leads to a complete, controlled and reproducible alignment of all DNA fragments, thus allowing accurate position of determinations along the molecule[10]. Recently, DNA stretching has attracted much attention in the field of manipulation and detection of individual molecules. Especially DNA straightening method and its application in biology have been reported. The fast and accurate building of genome physical maps, positioning gene and sequencing whole genome are important issues, which will be resolved in the Human Genome Project and post-genome plan. To reach this target, straightening and manipulating DNA by molecular combing technique has made an effective methodology exploration. Single molecule detection methods include fluorescent microscopy and atomic force microscopy. Since the 1980s, fluorescent microscopy has become

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an imaging method of single molecule. Nevertheless, the high quality image of single DNA molecule depends on the new method of using highly efficient fluorescent probe and charge coupled device (CCD).

1.1 The principle of molecular combing

Molecular combing was discovered during attempts to specifically anchor individual DNA molecules to a solid surface. The simple principle behind this method involves the physical-chemical binding of the molecules by one or both of their extremities to a hydrophobic surface, and the uniform alignment and homogenous extension of all attached molecules.

The purity of the DNA is a critical feature of the combing process. The key feature of this method is attaching either end of the molecule to the surface. In solution, the molecule assumes the form of a random coil which fluctuates in size and shape due to Brownian motion. Once in contact with the surface, the molecule binds spontaneously[13], and is then stretched and aligned by a receding meniscus. That is, the configuration of DNA changes to a linear shape from a random coil by molecular combing. During the stretching process, the force making DNA bind to surface might be the hydrophobic or electrostatic interaction.

1.2 Methods of achieving molecular combing

Since molecular combing method was discovered firstly by Bensimon in 1994[10], up to now, it has been developed significantly and a variety of combing approaches have been devised.

Molecular combing is a simple way to bind unmodified double-stranded DNA by its extremities on hydrophobic surfaces in a given range of pH[14]. So in recent years, many combing methods have been developed for stretching DNA. Such as dynamic molecular combing (meniscus moving on coverslip[12]), spin-stretching[15,16], evaporation of small droplets of DNA solution[17,18], stretching DNA with a cover slip[10,19], mechanical movement of a meniscus[20], nitrogen gas flow driven droplet motion[21], precisely controlled meniscus motion[22], ordered stretching of DNA between micro fabricated polystyrene lines[23], and so on. Though a variety of combing approaches have been developed they obey the same principle, that is hydrophobic surface preparation, DNA molecule labeled with a fluorescent dye, and DNA stretching. The difference between these combing methods lies in stretching DNA with a different procedure. In the following, we describe three combing methods: dynamic molecular combing, spin-stretching, evaporation of small droplets of DNA solution.

1.2.1 Dynamic molecular combing

Dynamic molecular combing (DMC) approach is an important, effective and independent method which is different from polymerase chain reaction (PCR) based approaches and cytogenetics techniques. It allows precise and quantitative measurements of the sizes of hybridized DNA clones and distances between them, at a resolution ranging from a few kilo bases up to a few hundred-kilo bases for both, without requiring normalization by other methods. DMC has been applied to the refinement of a sequence-ready cosmid map, measurement of gaps between contigs on total human genomic DNA, and the measurement of micro deletions on patients' DNA. This approach is of interest not only in genomics, for instance in genome sequencing projects that require validation of sequence-ready maps, but also in diagnostics, where new tools for well-characterized diseases are needed. DMC can be used to check the integrity and stability of large DNA clones.

The procedure of dynamic molecular combing is as follows: (1) Prepare silica or PMMA hydrophobic film coated on glass; (2) prepare DNA solution at given concentrations, then label DNA with fluorescent dye (YOYO-1); (3) dip the hydrophobic surface in the solution, and leave it for 5 min, attaching either end of DNA molecules to the surface; (4) pull the cover slip at a constant speed, at the interface of cover slip and meniscus, where DNA is stretched by the surface tension of solution. The hydrophobic nature of silica surface make it dry quickly, and then the stretched DNA fibres adhere to surface reversibly. These DNA fibres are aligned to the surface parallelly, just like comb. All DNA stretching is uniform, without the influence of DNA length.

1.2.2 Spin stretching

DNA droplets labeled with fluorescent dye (5—10 μL) were deposited on the hydrophobic surface and incubated for 5 min. During this period, either end of DNA molecules was anchored to the hydrophobic surface. Then the coverslip was accelerated up to its final rotation speed ranging from 2000 to 7000 r/min, spinning 1 min. After spinning, the straightened DNA molecules were aligned and combed well on the hydrophobic surface (Fig. 1(a)). DNA molecules were stretched during spinning. If the linear velocity of DNA was too high, the adhered DNA would be thrown from the surface. So it is very important to control an appropriate velocity and time for single DNA combing.

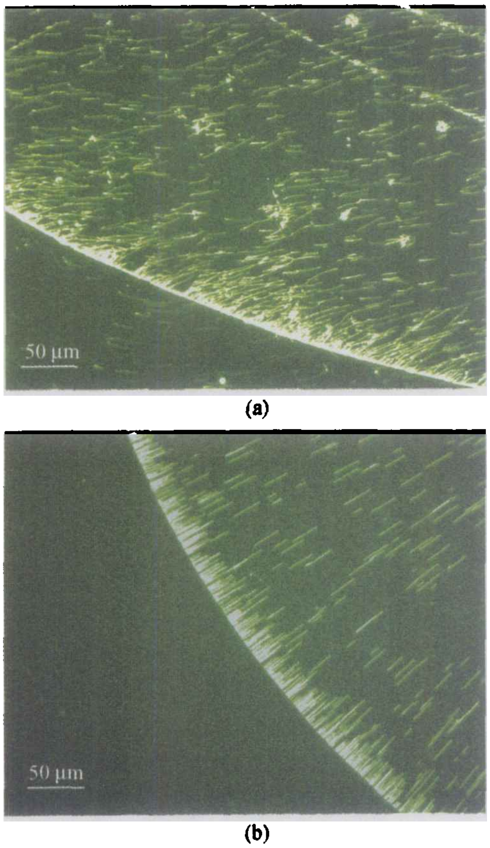

Fig. 1. The combing of single λDNA. (a) Spin-stretching; (b) evaporation of DNA droplet.

1.2.3 Evaporation of small droplets of DNA solution

Evaporation of DNA droplet solution is a very efficient and simple method to stretch DNA. A droplet (1—2 μL) of the stained DNA solution was deposited onto a PMMA surface. At first, the interface of droplet does not move at all. After about 5—10 minutes, the air-water interface starts to recede due to significant drying of the droplet, and DNA molecules originally bound to the surface with one extremity are extended and immobilized on the dried surface, whereas unbound molecules are swept away by the moving interface. As a result, a significant number of fixed DNA molecules are fully elongated and aligned radially. As most of them are concentrated near the droplet's round periphery, usually a "sunburst" pattern is formed (Fig. 1 (b)). Single DNA molecule can be observed directly using molecular combing and fluorescent microscopy.

1.3 The application of molecular combing

Molecular combing has been developed significantly and a variety of combing approaches have been devised to investigate DNA. Molecular combing method has the virtues that it can stretch a large array of DNA molecules simultaneously and reproducibly. Combined with other technique, the application of molecular combing method can much be widened. For example, when combined with fluorescent *in situ* hybridization (FISH), an unprecedented view of DNA analysis at the single-molecule level can be obtained, thus applying to the detection of cancer[24] and the detection of drug effect. Combed DNA has a great potential in molecular nano electronic devices as template for two-dimensional nano device[25].

1.3.1 Gene positioning on chromosomes

DNA molecule carries the gene. It is important to resolve the gene position on chromosome and the sequence not including gene(s). Because chromosome is highly condensed and compacted, its structure cannot be observed easily. Using molecular combing method, then fluorescent hybridization of DNA probes on combed DNA allows direct mapping of their respective positions along the fibers, such as measuring the deletions involving the *TSC2* gene region in affected patients[12]. The procedure is as follows: probe labeling, combing, FISH analysis on combed DNA and detection. Using molecular combing, the high-resolution physical mapping can be obtained (1—4 kb). It has also been applied to the genomic studies, and to the positional cloning (intragenic rearrangements, map translocation breakpoints) in disease genes loci[12] (Fig. 2).

1.3.2 Genomic studies of DNA replication

DNA, the carrier of gene, its replication is highly organized timely and spatially, whereas the dynamics of genome replication has just been elucidated primarily. But using FISH analysis and molecular combing method allows for the genome study of replication at a single molecular level.

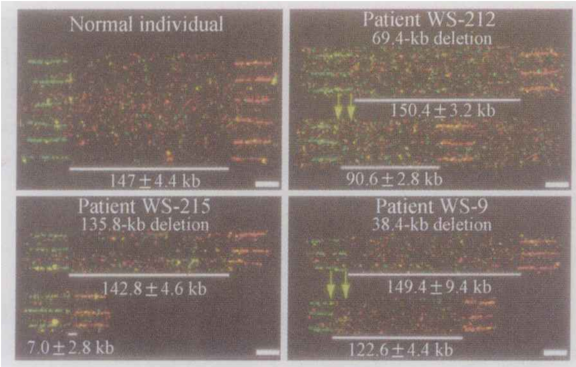

Fig. 2. Measurement of deletions involving the *TSC2* gene region in affected patients[12]. Cosmids CBFS1 (at the distal end of *TSC2*) and GGG4A (at the proximal end) were hybridized onto combed total genomic DNA slides and detected with two different fluorochromes. For each experiment, a montage of six representative pairs of signals is shown. The top three pairs of signals from each patients' slide (WS-212, WS-9, WS-215) represent the normal allele, whereas the lower three show the allele with the deletion. In two patients (WS-212 and WS-9), one cosmid signal was partially deleted (Bars: 10 μm).

Early replicating sequences labeled with biotin-dUTP are visualized using red fluorescing antibodies (Texas Red). Biotin-dUTP is added before the cell enters S-phase in order to label the entire genome. Later replicating sequences (genome after entering S-phase at successive time points) are labeled with dig-dUTP, and visualized using green (FITC) fluorescing antibodies. DNA taken from each time point is combed. Thus the fluorescent micrograph of labeled and combed DNA molecule is made (Fig. 3). Dynamics of replication can be analyzed, such as replication origins, bidirectional replication, replicon sizes, replication fork densities and so on. Thus the spatial and temporal organization of DNA replication can be directly deduced on a genome wide basis[26].

In addition, the molecular combing platform combined with FISH improves the analysis of the structure and function of whole genome, such as the measurements of the distance between gene sites, the absence of gene on patients' DNA and the cloning of the gene related to cancer. Molecular combing method has been shown to be the only tool that currently permits physical identification and kinetic analysis of all replication origins. It is well known that the replication of cancer cell is endless and uncontrollable. Molecular combing method can provide DNA physical maps at different replication phases. For this reason, molecular combing method is especially powerful for studying the transformation of a normal cell to a cancer cell. The combing platform has already demonstrated its remarkable capacities in several applications involving breast cancer and uterine cancer[24].

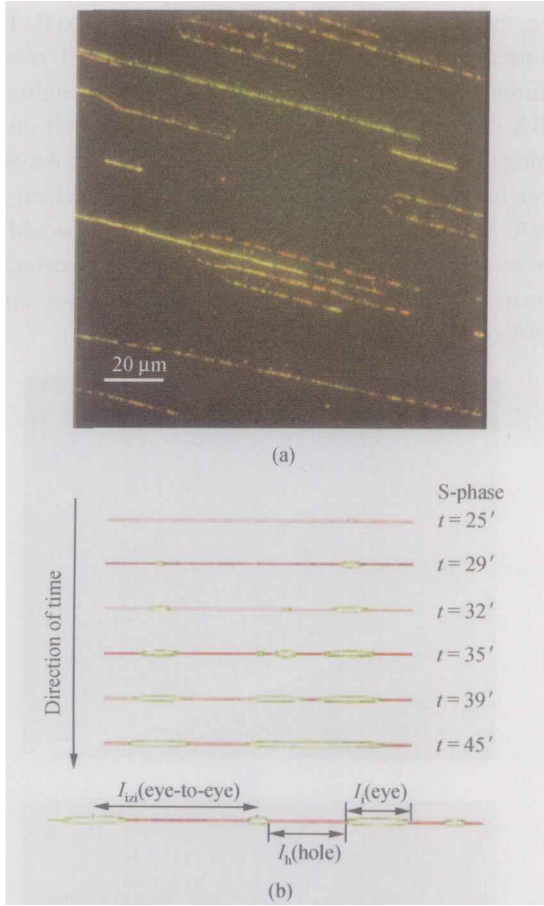

Fig. 3. Fluorescent micrograph and schematic representation of labeled and combed DNA molecule. (a) Fluorescent micrograph. Early replicating sequences labeled with biotin-dUTP are visualized using red fluorescing antibodies (Texas Red). Later replicating sequences are labeled with digdUTP and visualized using green (FITC) fluorescing antibodies[26]. (b) The hole corresponds to sequences synthesized in the presence of a single dye (red). The green segment (eye) corresponds to those sequences synthesized after the second dye (green) is added.

1.3.3 Observation of transcription on single combed DNA

Using molecular combing and fluorescent microscopy, the transcription activity on DNA can be detected by labeling and observing newly synthesized RNA with fluorescent nucleotides. The procedure is as follows: stretching DNA on hydrophobic surface by molecular combing, rehydrate combed DNA with transcription buffer, which contains T7DNA, NTPS, T7RNAP, RNase inhibitor, UTP-Alexa etc. After being incubated in transcription buffer, transcription on combed DNA is performed. In the presence of NTPs and UTP-Alexa, RNA synchronized by RNAP will emit fluorescence. Fluorescent UTP-Alexa is added for RNA labeling. The transcription efficiency is detected by fluorescence as a sensitive probe[27]. No transcription activity occurs on overstretched DNA molecules, whereas a transcription activity occurs for nonoverstretched DNA molecules[27].

1.3.4 Study of interaction between DNA, enzyme and histone

DNA is labeled with fluorescent dye. *Eco*RI-conjugated nanometer-sized fluorescent particles are incubated with DNA, combed, and observed by fluorescence microscopy. Site-specific binding is observed when *Eco*RI-conjugated particles are incubated with λ-DNA[28]. By using scanning tunneling microscopy (STM) and molecular combing, DNA infinite structure and binding site of enzyme can be imaged. Such a new approach might be very powerful in quick gene positioning and gene physical mapping[29].

Eukaryotic genes do not exist naturally as naked DNA molecules. Instead, they combine with some proteins, especially the basic proteins called histones, to form a substance known as chromatin. We have stretched and aligned DNA-histone complexes on the hydrophobic surface, then observed single DNA and DNA-histone complex by fluorescent microscopy[30] (Fig. 4). The effect of influencing factors on the interaction between DNA and histone can be analyzed by observing the length and the density of the stretched DNA-histone complex. This method is helpful to investigation into the interaction between DNA and histone at the single molecular level[31]. We have studied the effects of metallic ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}) on the combination of DNA and histones with molecular combing technique[32].

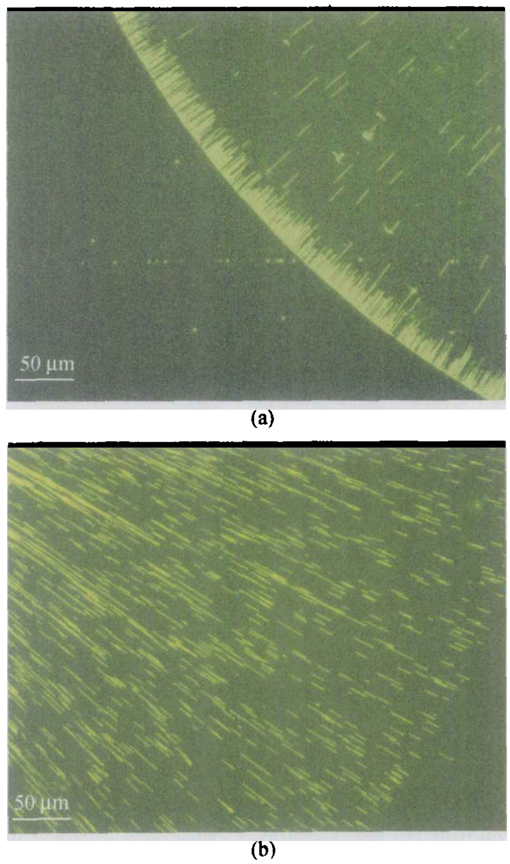

Fig. 4. CCD images of single combed DNA, and DNA-histone complexes[30]. (a) DNA (6.5 pmol, no adding histone); (b) DNA-histone complexes ([Histone]/[DNA] = 50).

We also investigated the interaction of DNA and *E. coli* RecQ helicase by molecular combing and fluorescent microscopy. DNA helicases use the chemical energy derived from nucleoside 5′-triphosphate binding or hydrolysis to mechanically disrupt the hydrogen bonds between the two strands in dsDNA and to translocate along DNA for processive unwinding[33]. DNA helicases are motor proteins responsible for separating the individual strands of double-stranded DNA to provide single-stranded DNA (ssDNA) for key cellular processes such as DNA repair, DNA recombination, and replication[34, 35]. *E. coli* RecQ helicase with 610 amino acids is a member of the RecQ helicase family, and it is widespread in diverse organisms. In our experiment, DNA-RecQ complexes were combed on hydrophobic surface by molecular combing. RecQ helicase binding to and unwinding of lambda DNA were observed directly with fluorescence microscopy. By atomic force microscopy (AFM), we observed that RecQ helicase is monomeric in solution and also functions as a monomer in DNA binding[36]. These methods were simple and effective. Most importantly, they can observe binding and unwinding directly.

1.3.5 The conductivity of DNA

DNA molecules are aligned between two electrodes by molecular combing technique, thus the conductivity of a large array of DNA molecules can be measured. It has been reported that the conductance of DNA molecules is relative to stretching shape, helix structure, dye molecule and character of the hydrophobic surface[37].

However, the conductance behavior of DNA remains poorly understood. It appears that drying DNA can lead to DNA conformations with localized electronic π states, although hole doping of the backbone by counterions might be possible. On the other hand, wet DNA may support electrical current, partly due to solvent impurity states sitting in the large π-π^* energy gap[38]. DNA molecule's conducting property makes it have potential use, since it can be used as molecular wire in nanoelectronics[39]. Deng et al. stretched and aligned linear DNA molecules into parallel or crossed patterns by molecular combing method. Subsequent metallization of DNA through electroless palladium deposition yielded 1D parallel or 2D crossed metallic nanowire arrays[25]. This research is very important to nanodevice construction.

2 Conclusion and future prospects

In summary, molecular combing method has the advantages as follows:

(1) The stretching process is simple. Every stretching method obeys the same principle: the surface of the substrate is firstly processed to be hydrophobic and then one end of DNA molecular in the solution attaches to the hydrophobic surface. Then the receding of the air-water interface extends DNA molecule in the flowing direction of the solution. Finally, DNA molecules are fixed and spread on the hydrophobic surface. Molecular combing method, which is simple and reproducible, can stretch a large array of DNA molecules.

(2) The preparation of sample solution is simple. The preparation of the experiment is much simplified only by staining DNA molecule with fluorescent dyes, without special chemical modification of DNA ends or coating micron beads.

(3) Combination with other techniques much widens the application field of molecular combing technique. Till now, molecular combing technique has become very mature, since combing DNA can be made on various surfaces such as some kinds of hydrophobic surface, mica and highly oriented pyrolitic graphite (HOPG) surfaces of bulk crystals and nanosheets[40].

In the following work, we will stretch DNA molecule or DNA-protein complex on surfaces of mica, silica or other flat hydrophobic surface by using molecular combing technique. Then through observing DNA-protein complex by an atomic force microscope we will try to solve some problems on the interaction between DNA and protein, such as in what form histone combines with DNA and how their combining mode will change with buffer condition. Molecular combing technique combined with fluorescence microscopy and atomic force microscopy must enrich our knowledge about the interaction between DNA and protein. Moreover, molecular combing technique integrating with fiber-FISH methods can analyze DNA quickly, thus it can be used in the detection of cancer and the drug effect. Therefore, this research field will be very important in medical applications.

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