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Micro- and nanofluidic technologies for epigenetic profiling

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This short review provides an overview of the impact micro- and nanotechnologies can make in studying epigenetic structures. The importance of mapping histone modifications on chromatin prompts us to highlight the complexities and challenges associated with histone mapping, as compared to DNA sequencing. First, the histone code comprised over 30 variations, compared to 4 nucleotides for DNA. Second, whereas DNA can be amplified using polymerase chain reaction, chromatin cannot be amplified, creating challenges in obtaining sufficient material for analysis. Third, while every person has only a single genome, there exist multiple epigenomes in cells of different types and origins. Finally, we summarize existing technologies for performing these types of analyses. Although there are still relatively few examples of micro- and nanofluidic technologies for chromatin analysis, the unique advantages of using such technologies to address inherent challenges in epigenetic studies, such as limited sample material, complex readouts, and the need for high-content screens, make this an area of significant growth and opportunity. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4816835]

I. INTRODUCTION

The biological "instruction manual" for structure, function, and growth has long been considered to be encoded in the DNA of organisms. Interactions between DNA and external factors drive differential transcription of these genes into proteins, producing transmittable signals that regulate function. However, cells can present distinct heritable phenotypes, without changes in the underlying DNA. Alterations to the instruction manual via these "epigenetic" changes, such as DNA methylation or histone modifications, have recently generated tremendous scientific interest.[1] As these changes are transmitted from parent to child, the existence of epigenetic mechanisms suggest that lifestyle choices may impact future generations.[2–7] Conversely, epigenetic mechanisms may enable us to manipulate or avoid the conditions that drive phenotypic expression of a genetic disorder. Hence, with implications in cancer,[8–10] autism,[11,12] and Alzheimer's disease,[13,14] the epigenetic paradigm provides a powerful set of tools with which to understand the transmission and etiology of diseases, and ultimately design treatments for genetic disorders that need not rely on potentially hazardous genetic modification.[15]

Epigenetics also have significant implications in the fields of tissue engineering,[16] regenerative medicine,[17,18] and creating artificial *in vitro* cell culture models;[19] particularly in sourcing cells for these technologies. A particularly promising approach is the use of embryonic, adult, and induced pluripotent stem cells,[20] which can be differentiated towards a specified phenotype.[21]

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This powerful technique enables basic scientists and clinicians to create autologous artificial tissues for transplantation,[22] or to better study various aspects of disease using genetically identical source materials; but the epigenetic paradigm raises a significant concerns about this process.[23] Differentiated cells may contain significant and previously unsuspected epigenetic variations (Fig. 1), arising from the use of chemical differentiation reagents[24] and from requirements to prepare undifferentiated stem cells for long periods in culture.[25] These epigenetic differences may lead to unexpected and potentially detrimental cell functionality, such as uncontrolled cancerous proliferation.[23–25] Hence, techniques to rapidly assess the epigenetic profiles of differentiated and diseased cells are of critical importance in a broad variety of applications.

The most commonly used method for chromatin analysis is the chromatin immunoprecipitation (ChIP) assay.[26] In this technique, chromatin (the contents of the cell nucleus, including DNA and associated proteins) protein, and DNA are temporarily bonded together and the chromatin is chopped up by mechanical shear into multiple small DNA-protein fragments. Select fragments containing proteins of interest are then precipitated from the solution using bead-attached antibodies. Proteins are then removed from the collected fragments and the DNA is purified and sequenced. The result is a list of short DNA fragments that had been bound by a specific protein of interest. While ChIP is very useful, the technique can analyze only one histone modification at a time. With over 30 different types of histone modifications known,[28] the amount of time, money, and cellular material required to do a thorough mapping is prohibitive (Table I). Alternative technologies to current ChIP techniques are required.

In this review, we provide an overview of the importance and need for analysis of epigenetic markers on chromatin in eukaryotes. We then review technologies aimed at improving chromatin mapping capabilities particularly in terms of using fewer cells and less starting material. For the technology part of this review, we first focus on microfluidic methods that aim to improve upon ChIP (2nd generation epigenetic analysis tools), then describe nanofluidic, non-ChIP technologies (3rd generation epigenetic analysis tools) to perform chromatin mapping.

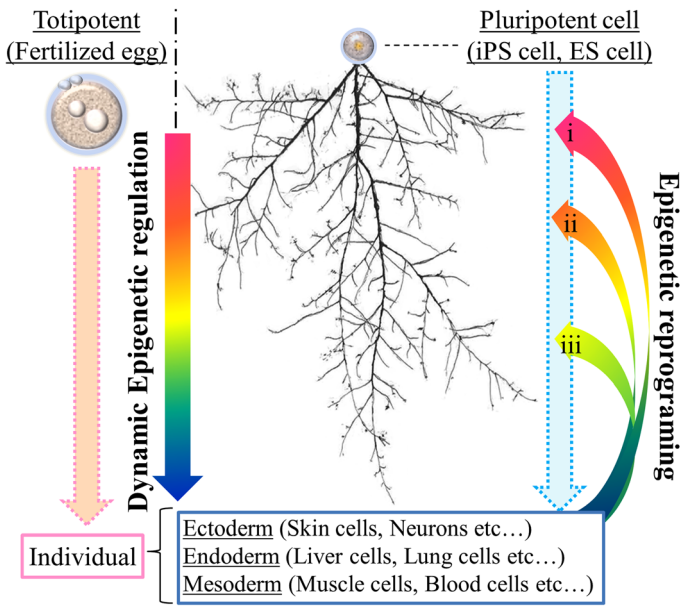

FIG. 1. The concept of epigenetic regulation and reprogramming. Fertilized egg represents totipotency or iPS and ES represents pluripotency, they have the potential to differentiate down all pathways through dynamic epigenetic regulation. Bottom roots represent differentiated cells such as skin, neurons, liver, lung, muscle, blood cells, etc. Alternatively, differentiated cell types in an individual can reverse their fate through epigenetic reprogramming. These induced pluripotent cells can be re-differentiated to various cell types through dynamic epigenetic regulation, but cannot develop into an individual.

TABLE I. Comparison for methods of conventional ChIP, improved ChIP, and ChIP in micro/nanofluidics.

	Operation time	Starting number of cells	No. of target antibodies (reaction spots)	No. of genomic sites	Ref.
Conventional ChIP	Several days	10^6–10^7 cells	One	Many	27
Improved ChIP methods					
Carrier ChIP	2–3 days	100 cells	One	One	67
Fast ChIP	5 h[a]	10^6–10^7 cells	24	Several	68
Quick and quantitative ChIP	A day	10^5 cells	100–1000	One	69
Matrix ChIP	4–5 h[a]	10^6 cells/well	96 (ideal)	One/well	70
Micro ChIP	A day	100–1000 cells	1–16	Few	71
	8 h[a]	Biopsy ($\sim$1 mm^3)	16	One	72
ChIP in micro/nanofluidics					
DNA-enrichment microfluid ChIP	2–3 h[b]	2.5×10^6 cells	One	Several	73
Automated microfluidic ChIP	A day	8000 cells	4	Several	74
High-throughput automated ChIP	A day	10^4 cells	16	Several	75
Microfluidic ChIP	8.5 h[b]	50 cells	One	Several	76

[a]Except PCR time.
[b]Except DNA purification and PCR time.

II. CHALLENGES IN CHROMATIN ANALYSIS

A. Chromatin structure influences cell function

DNA in a living cell must be accessible so as to readily perform vital biological functions such as gene transcription, DNA replication, DNA recombination, and DNA repair. At the same time, however, the meter-long eukaryotic DNA must be highly condensed so as to fit into the nucleus, which affords space of only a few microns in size. The two seemingly incompatible requirements of extremely compact storage and rapid accessibility have prompted a decade of intense studies of chromatin organization. The hierarchical structure of chromatin necessary to achieve this degree of packing and accessibility of DNA has subtle and diverse features at all levels of organization, some of which have only recently been understood.[27] Since the structure of chromatin itself likely regulates aspects of gene transcription,[28] understanding these factors is of critical importance in probing epigenetics. Intensive research has been conducted to characterize the structural and functional properties of chromatin using diverse experimental and modeling techniques including atomic force microscopy (AFM),[29–31] optical/magnetic tweezers,[32,33] electron microscopy,[34,35] and X-ray scattering methods.[36,37]

DNA is coiled around a nucleosome core comprising four pairs of histone proteins, and nucleosomes are connected to each other by linker DNA (often represented by a "beads-on-a-string" model) (Fig. 2(a)).[38] However, the higher-order structure that the primary chromatin strand coils into is still actively debated.[35] A regularly arranged secondary structure known as the "30 nm fiber" is believed to exist.[34,35,39] The organization of this fiber is achieved through two major structures: the "one-start" or solenoid model, in which two successive nucleosomes follow a helical path and are connected by bent linker DNA; or the "two-start" or zig-zag model, in which nucleosomes directly interact with each other with minimal bending of linker DNA between them (Fig. 2(b)).[40] *In vitro* and modeling studies provide considerable evidence that this secondary structure is strongly dependent on various properties such as the length of linker DNA, the existence and properties of linker histones, and experimental conditions such as ion valence and concentration (see references reviewed in Ref. 40). At physiological ion concentrations, the secondary chromatin contains uneven proportions of both type of structures ("heteromorphic fibers").[35,38] Furthermore, more recent evidence suggests that even the regular 30-nm fiber structure does not exist consistently in living mammalian cells.[37,41,42] Hence, our

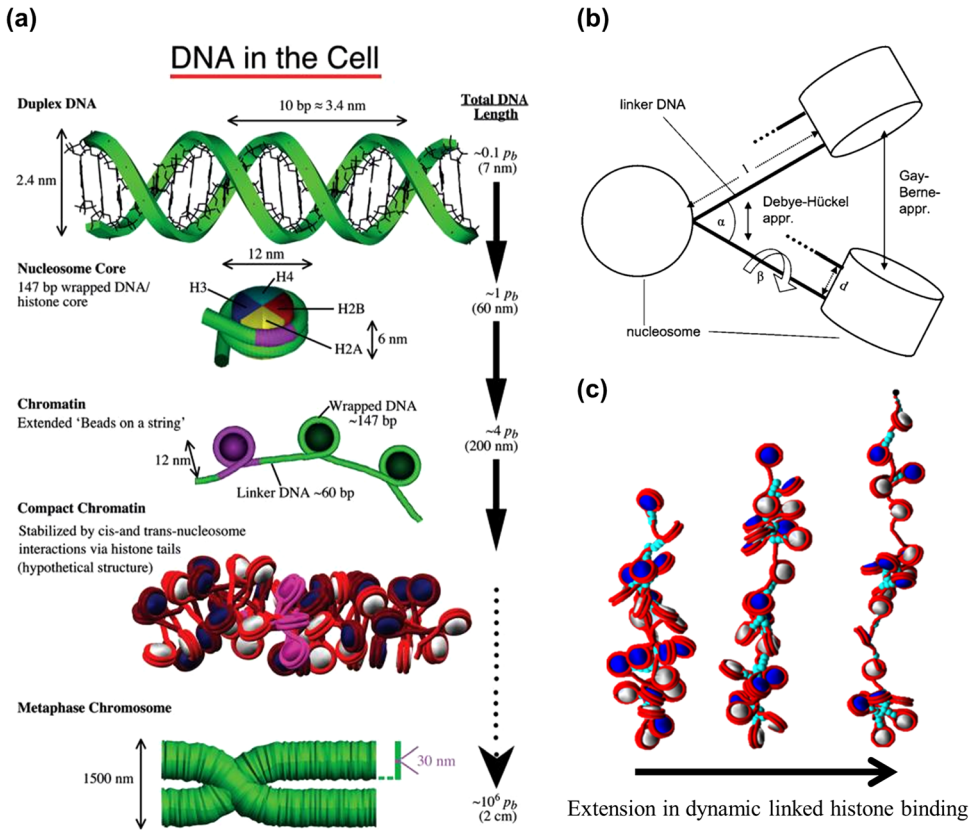

FIG. 2. Schematic of DNA folding in the cell and higher coarse-grained models of chromatin. (a) Bare DNA is folded to form highly condensed chromatin structures such as a chromosome. The structure of compact chromatin is still uncertain but a compact chromatin fiber is a likely candidate. Reprinted with permission from Schlick *et al*., J. Biol. Chem. **287**, 5183 (2012). Copyright 2012 American Society for Biochemistry and Molecular Biology. (b) A chromatin fiber model can be defined by the opening angle between the incoming and outgoing linker DNA of length l, torsional angle of the linker DNA, and the interactions between nucleosomes and linker DNA. Reprinted with permission from Aumann *et al*., Phys. Rev. E **73**, 041927 (2006). Copyright 2006 American Physical Society. (c) A heteromorphic-chromatin stretching simulation where the components such as linker histone and the nucleosome surface charge distribution are explicitly modeled. Reprinted with permission from Schlick *et al*., J. Biol. Chem. **287**, 5183 (2012). Copyright 2012 American Society for Biochemistry and Molecular Biology.

current understanding of chromatin structure suggests that the chromatin in a cell exists in a melt-like dynamic state, providing greater accessibility for biological purposes than the conventional static model.[41] Also on a primary structural level, there are small variations in amino-acid sequences even though core histones are highly conserved proteins across species. The variations can influence the stability and dynamic state of the primary structure, thereby influencing the rate and duration of gene activation. Taken together, the variability, irregularity, and dynamics in the hierarchy of chromatin structures represent an epigenetic mechanism, as they form based on specific needs and conditions in a given biological environment. Simply mapping end-point protein-DNA interactions using ChIP assays are unable to recreate and capture these dynamic complexities.

Computational models have greatly enhanced our understanding of the structure of chromatin. In most studies of chromatin structure, only the essential elements of the system are retained to reduce the degrees of freedom in the model.[40] In the most basic course-grain (CG) models, chromatin is modeled as a linear string of nucleosomes connected by linker DNA ("beads-on-a-string") (Fig. 2(c)).[43] The nucleosomes can then be modeled as cylindrical objects to which two torsional springs are attached. The angle between the two (entry/exit) springs at a nucleosome and the torsional angle in each spring are the variables in the dynamics of the CG model. The interactions between nucleosomes can be modeled by using an "effective potential"

term that accounts for all the interactions among the simplified neighboring elements such as surface charges, ions, and solvent interactions. Even this simple model provides insights into how the global bending and stretching stiffness depend on the chromatin primary structure.[40] Recently, the Schlick group[43] has developed a more refined CG model that explicitly includes realistic geometric shapes of the nucleosome, histone tails, linker histone, and ion valence/concentration dependence, using a Debye-Hückel model. The model can quantitatively reproduce the force vs. extension curve in various regimes and predict the structural role of the chromatin components in higher-order compaction and organization of chromatin.[43] Furthermore, as demonstrated by Yang *et al.*,[44] explicit modeling of the ion and charge distribution in multivalence/high concentration polyelectrolytes (such as in the case of salt-induced chromatin aggregation; Fig 2(c)) is necessary to adequately capture model dynamics.[43] The CG model of higher-order chromatin structures in solution has matured enough to complement experimental results.[27]

B. Broad variety of histone modifications

A key structural unit of chromatin is the nucleosome. Nucleosomes in eukaryotes consist of DNA coiled around an octamer core comprised four types of histone proteins: H2A, H2B, H3, and H4, each of which has a high affinity for DNA. Nucleosomes are bound at the entry and exit sites of the DNA by the linker histone H1.[45–47] About 147 base-pairs of DNA are tightly wound around a histone octamer. The terminal regions of these histones are exposed to modifications, such as acetylation and methylation that can dynamically change chromatin structure, regulating accessibility of proteins to the DNA.[48] For example, activity of transcription factors can be blocked or enhanced based on modification of the histone. This mechanism makes it physically possible to regulate gene expression independently from the DNA sequence, creating an epigenetic effect (Fig. 3(a)).[49]

The variety of known histone modifications is large and growing. The most extensively documented modifications include acetylation, methylation, phosphorylation, ubiquitination, and biotinylation.[50] In particular, acetylation on histone H4 and methylation of lysine 9 on histone H3 can significantly influence chromatin structure of euchromatin (decondensed chromatin) and heterochromatin (highly condensed chromatin), respectively.[51–54] Hence, patterns of these histone modifications, collectively referred to as the "histone code" can physically regulate gene expression and transcription.[55] Currently, researchers commonly consider 24 histone modifications of acetylation and methylation on 22 modification sites (Fig. 3). Modifications are quickly being added to this list to include ubiquitination,[56,57] adenosine diphosphate (ADP)-ribosylation,[58,59] citrullination,[60] and sumoylation.[61] Analysis of each of these modifications requires the development and validation of uniquely specific antibodies. As ChIP analysis is typically limited to precipitation of protein-DNA fragments using a single antibody at a time, histone

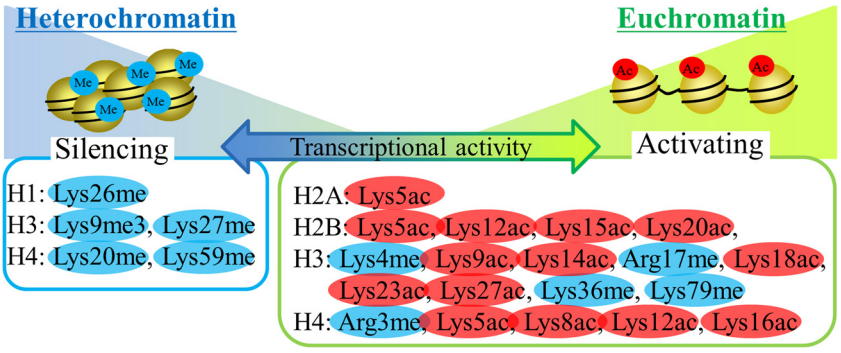

FIG. 3. Transcriptional relationship between chromatin structure and epigenetic modifications on histones. Gene transcriptional activity can be controlled by chromatin structure such as heterochromatin and euchromatin. These chromatin structures can also be controlled by histone modifications, as demonstrated in the diagram which shows post-translational histone modifications on each histone (Ac, acetylation; Me, Methylation).

mapping of the epigenetic state grows more complex and time consuming with the discovery of each new modification. Given the quantity of potential modifications, and the rate of discovery of new modifications, new methods to study these modifications and their effects on chromatin structure are important to develop.

C. One genome but many epigenomes per person

Generally, the genomic DNA sequence of cells within an individual person is all the same. The human genome project, successfully achieved over 10 years ago and progressively improved upon, enables an individual person's DNA to be mapped for less than $1000, from amplification of genetic contents of a single cell.[62] While knowledge of the complete genomic sequence is useful to detect genomic variations such as single-nucleotide polymorphism analysis, this information is not always sufficient to understand individual cell type-based function and phenotype. To understand cell type-dependent functional differences, not only the DNA sequence but also knowledge of how that DNA is packaged within the cell's nucleus is required. In other words, epigenomic information of that specific cell that includes patterns of histone modifications across the whole genome is needed.[63] This is because significant epigenomic diversity exists not only between individuals in a population but also from tissue to tissue, and between individual cells within the same tissue.[9,64]

Unfortunately, no technology currently exists with which to study the epigenetic profile of chromatin from a single cell. DNA amplification cannot be utilized, as it does not replicate the modified histones and structure of the chromatin necessary for epigenetic analysis. Recently, the Encyclopedia of DNA Elements (ENCODE) project undertook a massive research program involving the compilation of many researchers' efforts in mapping the epigenome.[65] However, even this well-funded consortium chose to study cell lines, at least in part, due to the need to produce sufficient numbers of cells for the epigenetic analyses such as ChIP. The ability to map histone states using chromatin from a single cell would greatly reduce the time and cost involved in such endeavors. Additionally, the ability to perform histone mapping of single cells would open new opportunities in understanding cell-to-cell variability of the epigenome as well as in identifying and understanding rare cells, such as cancer progenitors or pluripotent stem cells, which play critical roles in disease and repair but are currently very difficult to analyze due to lack of starting cellular materials.

III. MICRO- AND NANOFLUIDIC APPROACHES

Conventional methods to study chromatin are limited in their ability to provide sufficient information at a single cell level—information critical for rare cell or cell differentiation research.[26,66–71] These issues are compounded by the complexity and insufficient-throughput of such technologies given the challenges associated with having over 30 histone modification possibilities and multiple epigenomes per person. Recent advancements in micro/nanotechnology offer opportunities for the study of chromatin at the single cell level using mechanisms that allow for precise control, improved handling, and higher throughput studies. In this section, current studies related to chromatin in micro/nanofluidics platforms as well as potential future applications will be discussed. We divide this section into two parts: part one consists of methods that aim to improve upon ChIP and are mainly microfluidic, and the second part focuses on non-ChIP methods comprised 3rd generation nanofluidic systems.

A. ChIP in micro/nanofluidics

ChIP has been exploited to study DNA-protein interactions and histone modifications for many years. Despite the capabilities and history of this technology, ChIP assays can be challenging to perform. It requires several sequential steps: (1) cell lysis, (2) binding of an antibody to a target DNA-binding protein, (3) immunoprecipitation of the antibody and antigen complex using a secondary antibody and beads, (4) purification, and (5) further analysis such as polymerase chain reaction (PCR) and sequencing. Multiple processing steps in any assay typically

results in larger sample requirements and assay time. In contrast to conventional technologies, miniaturized micro/nanofluidics platforms require only small amount of sample volume, can simplify and accelerate the assay, and enables the study of single cells in a high-throughput manner.

Microfluidic systems can greatly improve antibody reaction and immunoprecipitation times in ChIP assays. Lee *et al*. utilize microfluidics to demonstrate an efficient approach for ChIP assays (Fig. 4(a)).[72] The microfluidic polydimethylsiloxane (PDMS) chip is separated into four parts; an inlet, a bead reservoir, three dispersion channels, and an outlet. A cell suspension, containing fragmented chromatin sheared by sonication, is introduced by a peristaltic pump. The solution is dragged into the microbeads reservoir where target protein/DNA is captured by antibody-coated microbeads. The rest of the solution flows to an outlet through a small dam interface between the bead reservoir and dispersion channels. After a washing step to remove non-specific binding molecules, the histone/DNA molecules bound to the microbeads are extracted and the DNA molecules are isolated. Further DNA analysis is performed by PCR. Optimized dispersion and DNA shearing conditions for efficient immunoprecipitation are identified based on computational fluid dynamic (CFD) modeling. This filtration design allows simultaneous concentration and precipitation of target chromatin, resulting in small sample requirements, shorter operation time, and skill-independent work. They utilized the technology to rapidly purify a target gene, *GAPDH*, in a human cell line. As a result, experimental efficiency was verified in comparison to a commercial ChIP assay kit (Table I).

Microfluidics can also be used to improve assay throughput and automation. Quake and coworkers designed an automated microfluidic platform for ChIP that requires small cell populations (Fig. 4(b)).[73] Fluid flow within the microfluidic chip was controlled using integrated pneumatic valves and pumps, enabling rapid and automated processing of immunoprecipitation procedures. More specifically, mixtures of fixed cells and microbeads are loaded in ring 1, and then sequential buffer solutions are applied to create a cell lysate; NP40 buffer increases cell permeability, micrococcal nuclease (MNase) breaks chromatin into several DNA fragments, and

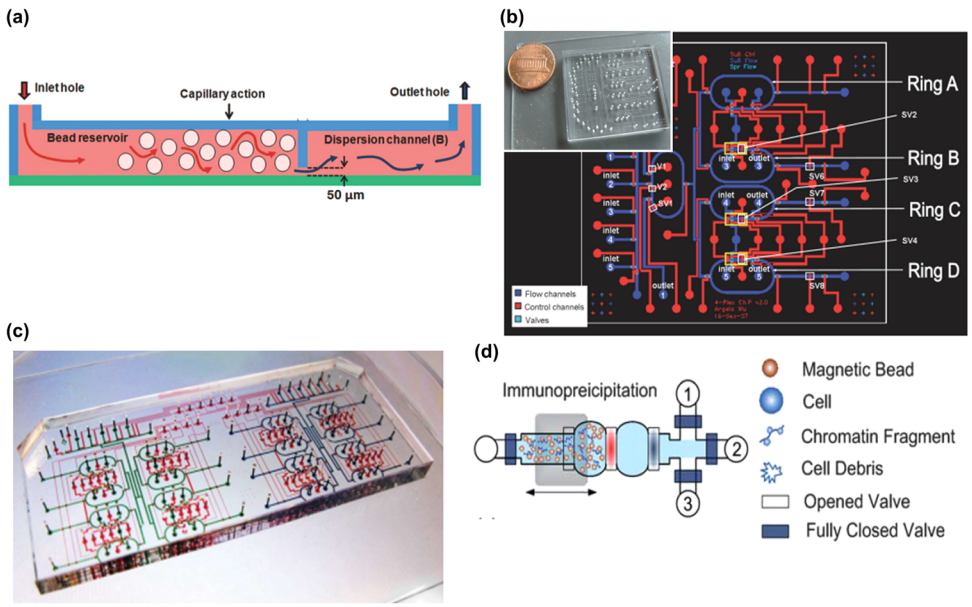

FIG. 4. Micro/nanofluidic technologies for ChIP assays. (a) Schematic of a DNA-enrichment microfluidic chip. Reprinted with permission from Oh *et al*., Anal. Chem. **81**, 2832 (2009). Copyright 2009 American Chemical Society. (b) Automated microfluidic ChIP assay device. Reprinted with permission from Wu *et al*., Lab Chip **9**, 1365 (2009). Copyright 2009 Royal Society of Chemistry. (c) High throughput automated ChIP assay platform. Reprinted with permission from Wu *et al*., Lab Chip **12**, 2190 (2012). Copyright 2012 Royal Society of Chemistry. (d) ChIP assay platform for histone modification analysis of a low number of cells. Reprinted with permission from Geng *et al*., Lab Chip **11**, 2842 (2011). Copyright 2011 Royal Society of Chemistry.

sodium dodecyl sulfate (SDS)/ethylenediaminetetraacetic acid (EDTA) solution is used not only to neutralize the MNase but also to break the remaining cell membrane completely. The beads utilized in ring 1 function as a matrix to keep the cells in the gap between each beads as solutions are exchanged. When the cell lysate meets antibody tagged microbeads, precipitation occurs in ring A to D regions in which different antibody tagged microbeads are deposited using sieve valves. During this immunoprecipitation procedure, the cell lysate and antibody tagged microbeads are well-mixed by active pumping, resulting in reduced incubation times. With careful washing that does not strip target proteins bound to the antibody beads, the cross-linked DNA on the target proteins is isolated and amplified with real time qPCR. The study precipitated two target proteins, H3K4me3 and H3Ac in parallel. Compared to the conventional ChIP assay protocol, the ratio of acquired target molecules over total input molecules from cells was improved. The key to improvements include increased interaction between concentrated cell lysate and antibody in the small channel as well as the active mixing during immunoprecipitation. This method reduces the total operation time to less than a day, and the required cell population to approximately 2000 cells. In contrast, conventional ChIP assays = require large numbers of cells (over 1×10^6 cells) and longer times (up to several days; Table I). Building on this work, the group has scaled up their platform to establish a high throughput automated chromatin immunoprecipitation (HTChIP) platform for drug screening and antibody validation (Fig. 4(c)).[74] The HTChIP is designed to use conventional sample preparation protocols of cell lysis and chromatin fragmentation in bulk to utilize existing infrastructures and to make the microfluidics chip more focused on the immunoprecipitation step. The HTChIP has been used to successfully observe changes in transcription factor binding in murine embryonic fibroblast (MEF) under TNF-α stimulation.

Another approach for improved ChIP based histone modification analysis in a PDMS microfluidic device was reported by Lu *et al*. In conjunction with magnetic beads, rapid immunoprecipitation and purification of target DNA are achieved. (Fig. 4(d)).[75] Similar to the microfluidics devices described above, fluids are controlled in the PDMS microfluidic device by pumps and valves. Antibody conjugated magnetic beads are initially introduced by pumps and an external magnetic force. Cells are then introduced among the magnetic beads by partially opening a valve to allow fluid flow, creating a bead/cell capture area. The cells are ruptured with a cell lysis buffer, and chromatin is fragmented by MNase. After neutralizing the enzyme activity, immunoprecipitation is conducted under enhanced mixing by both periodic actuation of valves at a high frequency, and by movement of an external magnetic field. The magnetic beads capturing the target proteins are sorted after thorough washing to flush non-specifically bound materials. Increased interaction between the target protein in chromatin and the antibody on the magnetic beads in the microscale chamber results in needing fewer cells and reduces operation time. Coupled with real-time PCR, they characterized histone acetylation in immature 6C2 cells, using as few as 50 cells to provide biologically meaningful results within a few hours.

Despite advances made in using microfluidics to improve ChIP methodologies, there is still a need to enable detection of multiple types of histone modifications simultaneously across the whole genome, on a single-cell level, in order to study epigenetic influences of rare cells. Achieving this goal requires technologies that can bypass two limitations associated with the conventional ChIP process: (1) The need for large number of cells to provide the required amount of nuclear material; and (2) The inability to amplify chromatin. These challenges have led to development of alternatives to ChIP to identify histone modifications directly on chromatin. Some micro-nanofluidic methods to achieve this are highlighted below.

B. Chromatin linearization

Since chromatin exists in a highly compact state, specific markers in chromatin are quite difficult to discriminate when tangled. ChIP assays include a shearing step to generate chromatin fragments to overcome the entanglement problem. The harsh mechanical forces, however, may damage epigenetic information. More importantly, extensive fragmentation of the chromatin strand destroys positional information for epigenetic markers on the genomic structure. An

alternative approach is to directly observe the position of epigenetic markers along a long strand of chromatin. This approach requires chromatin to be linearized, a task suited to micro and nano-fluidics. Nanochannels are well known to provide confinement conditions that allow for reliable linearization of DNA.[76–80] More recently, chromatin has also been linearized by nanoconfinement. With fluorescent labeling using multiple antibodies against specific histone modification, positions of epigenetic markers can then be directly imaged.[81–83]

To date, efforts to study DNA linearization in micro/nanofluidics have been oriented toward the visualization of single DNA molecules and the detection of target markers on DNA.[84–86] In this short review, we focus on fluidic approaches that linearize chromatin where DNA with its associated proteins is stretched intact. The first demonstration of linearizing chromatin in nanochannels was performed by Streng *et al.*[87] This group fabricated nanochannels with approximately 80×80 nm^2 cross-sectional area on silica wafers. Due to the difficulty of estimation of DNA lengths in a chromatin molecule extracted from a single cell, they reconstituted chromatin by combining unfractionated whole histones with the well characterized λ-DNA. The reconstituted chromatin was dragged into the nanochannels using an electrical field (Fig. 5(a)). Frictional force involved with forcing the globular chromatin to stretch into the nanochannel and entropic effects of nanoconfinement linearize the chromatin and maintain it in that state. Stretched chromatin and DNA were visualized by staining with the intercalating dye, YOYO-1, and lengths with and without histones were compared (Fig. 5(b)). Consequently, they demonstrated that elongated reconstituted chromatin contained 2.5 times the number of compact structures as compared to bare DNA in its stretched state. Considering normal diffraction-limited optical microscopy limits, the measured length of elongated chromatin corresponds to a chromatin mapping resolution of 6 kbp. A high throughput approach to analyze epigenetic markers in single DNA or chromatin molecules in nanofluidics was reported by the Craighead group.[81] A few hundred nanometer sized channels, fabricated on a fused silica wafer by projection photolithography and reactive ion etching, combined with multicolor fluorescence confocal microscopy enable detection of multiple target markers on single molecules simultaneously at a high rate of approximately 10 Mbp/min. Here, the main purpose of the nanochannels is to space out chromatin fragments from each other so that each fragment is analyzed one by one as they are electrokinetically driven through nanochannels. Two lasers focused on the nanochannel allow coincident multi-fluorescence color detection (Fig. 5(c)). For example, the group used TOTO-3 (red color to stain DNA) and histone H2B-GFP (green) to simultaneously measure DNA and histone content of each chromatin fragment as it passed the detection region (Fig. 5(d)). They also successfully detected methylation of DNA and presence of histones by use of a fluorescently tagged methyl binding domain protein-1 (MBD1). Recently, they analyzed the coincidence of three gene silencing/activating marks—H3K9me3 and H3K27me3 on histone and cytosine methylation (mC) on DNA—in normal and cancer cells to reveal higher incidents of "contradicting" marks (activating histone marks together with silencing DNA methylation marks) in cancer cells.[88] Multi-color analysis enabled direct confirmation of the presence or absence of multiple modifications on each chromatin fragment enabling direct confirmation of these markings which could only be indirectly inferred previously.

Recently, Matsuoka *et al.* presented multi-color histone mapping in nanochannels. In this method, an array of nanochannels are fabricated based on cracking of a brittle PDMS surface layer.[83] The key to achieving chromatin linearization in these nanochannels is the ability to modulate the channel cross-sectional dimensions. This channel tuneability is useful not only for easily loading of chromatin into the channels using the channel-widened state but also for actively linearizing the chromatin structure by the application of elongational squeezing shear flows as the channels are narrowed (Fig. 5(e)). This chromatin linearization is gentle and quick enough to allow anti-histone antibody-labeled chromatin to be elongated with their markings maintained but vigorous enough to linearize to near estimated contour lengths. The elongated chromatin was visualized by combinations of fluorescent DNA intercalating dyes, fluorescently labeled antibodies for specific target histone modifications, or histone-GFP. Examples of multi-color chromatin analysis demonstrated include simultaneous mapping of histone-H3K9me3, histone-H4Ac, and DNA on the linearized chromatin (Fig. 5(f)).

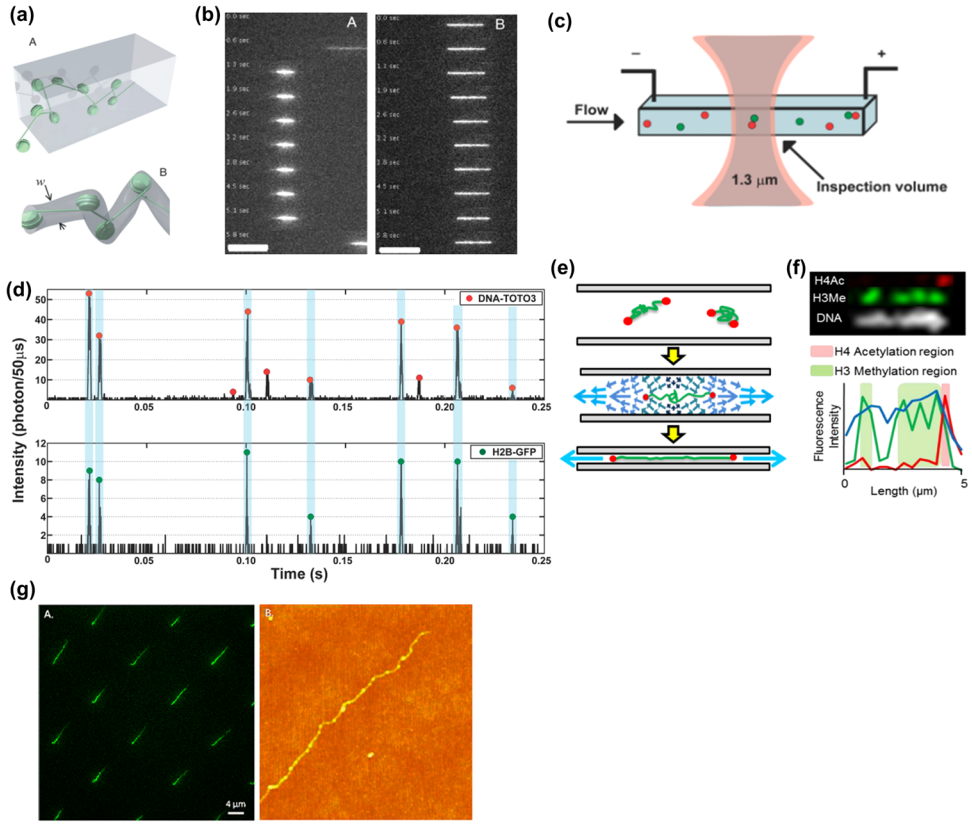

FIG. 5. Chromatin linearization in micro/nanofluidic systems. (a) Schematics of reconstituted chromatin linearization in nanochannels. (b) Linearization of chromatin assembled from lambda-DNA and bare lambda DNA in nanochannels. Reprinted with permission from Streng *et al.*, Lab Chip **9**, 2772 (2009). Copyright 2009 Royal Society of Chemistry. (c) Experimental platform for single chromatin analysis at the nanoscale. (d) Coincidence two color measurements of stained DNA and GFP expressed histone on a single chromatin. Reprinted with permission from Cipriany *et al.*, Anal. Chem. **82**, 2480 (2010). Copyright 2010 American Chemical Society. (e) Chromatin elongation induced by hydrodynamic squeezing flow as well as confinement effect as channel narrows. (f) Multi-color epigenetic marker mapping for histone-H3K9me3 or histone-H4Ac on linearized chromatin. Reprinted with permission from Matsuoka *et al.*, Nano Lett. **12**, 6480 (2012). Copyright 2012 American Chemical Society. (g) Individual chromatin molecules contain valuable genetic and epigenetic information. Reprinted with permission from Cerf *et al.*, ACS Nano **6**, 7928 (2012). Copyright 2012 American Chemical Society.

As an alternative to fluidic channels, chromatin molecules can also be linearized and arrayed on a substrate via soft lithography and fragment immobilization. Cerf *et al.* recently demonstrated this approach (Fig. 5(g)).[89] Chromatin molecules are elongated on a PDMS stamp having microfeatures and the array of linearized chromatin transferred to a functionalized cover slip for high resolution imaging. Stretched chromatin extracted from cancerous cell lines such as M091 and HeLa were utilized for epigenetic analysis. Target histone in the arrayed chromatin was observed with fluorescence microscope and conformations of the elongated chromatin were characterized by AFM.

To enhance our understanding of chromatin behaviour at the molecular scale in these nanofluidic environments, it will be critical to utilize computational simulations, since direct observation is difficult. Computational modeling of chromatin states and dynamics in confined geometries has only just begun.[83,87] There are unique challenges and opportunities for performing such analyses. In addition to the interaction with boundaries, the hydrodynamic effects and polymer drift (such as in electrophoresis), must be properly addressed in the model. For analysis of hydrodynamic effects, the CG model may also need to explicitly include a solvent which would increase computational loads. Simple models such as the semi-flexible chain model based on the persistence length of the polymer may not be generally reliable. The reported

persistence length spans a broad range from 30 nm to 300 nm,[41,42] reflecting the sensitivity of the system to the environmental variables previously discussed.

IV. SUMMARY AND FUTURE PERSPECTIVES

The ability to map the whole human genome in combination with more recent advances in chromatin analysis based on histone modifications by the ENCODE project have provided the basis for scientists to identify the dynamic processes involved in epigenetics. The limitations imposed by available techniques have prompted the development of novel technologies to conduct a genome-wide epigenetic analysis, using very few cells while minimizing time, cost and assay complexity. Furthermore, the ability to analyze positional relationships between multiple histone markers simultaneously from a single chromatin molecule will enhance our mechanistic understanding of the roles of the various markings and how it contributes to normal physiology as well as disease. There are still relatively few micro- and nanofluidic methods that have been developed for chromatin analysis. Micro- and nano-engineering approaches described in this review provide a glimpse of the promising approaches developed to towards this goal. Combined with developments in super resolution imaging techniques,[90,91] the nanofluidic single molecule-level direct chromatin analysis looks poised for further advances. We hope this review will encourage increased participation in this important field.

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