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Assaying the epigenome in limited numbers of cells

William J. Greenleaf

Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, United States

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ABSTRACT

Spectacular advances in the throughput of DNA sequencing have allowed genome-wide analysis of epigenetic features such as methylation, nucleosome position and post-translational modification, chromatin accessibility and connectivity, and transcription factor binding. However, for rare or precious biological samples, input requirements of many of these methods limit their application. In this review we discuss recent advances for low-input genome-wide analysis of chromatin immunoprecipitation, methylation, DNA accessibility, and chromatin conformation.

1. Introduction

The advent of cost effective massively-parallel short-read sequencing has led to the sequencing of thousands of human genomes [1,2] providing a glimmer of the much vaunted "personalized genomics revolution." But perhaps more importantly, high-throughput sequencing has brought about a sea change in the types of mechanistic biological questions that can be addressed at genome-wide scale. Any biological question that might be transformed into DNA fragments may now be investigated with hundreds of millions to billions of individual measurements, providing a powerful window into genome-wide molecular functions. Arguably, nowhere has this fundamental methodological change been more apparent than in the field of epigenomics [3,4].

For the purposes of this review, we will define the epigenome as the set of chemical and physical modifications of the genome that do not comprise changes in the primary sequence of the DNA. These changes encompass a diverse set of transformations, from DNA methylation [5,6], to changes in positions and chemical composition of nucleosomes [1,7], to the binding of transcription factors [3,8], to higher-order changes in the manner in which the genome is folded and or made accessible within the nucleus [5,6,9,10].

Substantial insight into the epigenetic information coded within the nucleoprotein structure of chromatin have come from molecular biological methods that then couple into high-throughput sequencing [5,11]. Chromatin immunoprecipitation and sequencing (ChIP-seq) protocols have enabled investigation of nucleosome modifications and their correlation with functional elements genome-wide, as well as a more comprehensive understanding of binding sites of transcription factors [11,12]. Methods to sequence fragments generated from the digestion of chromatin with MNase have allowed comprehensive cataloging of nucleosome position in human cells [13,14]. A number of strategies for assessing the methylation state of bases within the genome, either in a defined subset of genomic loci or genome wide, have also transformed our understanding of the dynamics of methylation changes in early development, during differentiation, and in cancer [1,15–21]. Finally, chromatin "openness" – the accessibility of DNA to transcription factors, RNA polymerases, and other components involved in gene expression – has been explored by coupling DNase I hypersensitivity assays and high throughput sequencing. The assay of DNA accessibility in particular has proven an information-rich, genome-wide analysis tool, allowing identification of areas of active transcription factor binding, active transcription start sites, enhancers, microRNA expression, and insulators in a wide variety of cell lines and tissue samples [22–26]. To determine how these regulatory regions, or even all regions in the genome, fold and interact with other regions, a number of methods capable of recording the conformation of chromatin have been employed to link regions that have a high probability of interaction [27–32]. In sum, these methods, when amplified by their application by scientific consortia such as the ENCODE project [33] and the Roadmap Epigenomics Project [34], have clearly demonstrated that the physical position, compaction, and chemical modifications of histone particles encode a complex and dynamic molecular "state machine" of the cell, defining cell type specific functional regions of the genome.

E-mail address: wjg@stanford.edu

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Despite these substantial insights, current methods for assaying chromatin structure and composition are often substantially limited by cell input requirements on the order of tens to hundreds of millions of cells as starting material [3,12,35–38], and such requirements are limiting in a number of ways. First, these methods average out or drown out both dynamic heterogeneity and standing variation in cellular populations. In this way, fine-scale, subpopulation-level variability in epigenomic structure that may be crucial to understanding the drivers of phenotype in complex samples is lost to ensemble averaging over cellular populations. Second, cells must often be grown and expanded in culture to obtain sufficient starting material. These *ex vivo* methods of cellular propagation may well modulate the epigenetic state in unknown ways. Indeed, for many dynamic and transient cellular populations, the time required to expand cultures *in vitro* to the degree necessary for genome-wide investigations will allow the dynamic state of the sorted population to interconvert, frustrating ensemble genome-wide assays. Finally, the requirements of large amounts of input material, especially when coupled with relatively complex workflows that often accompany methods for generating genome-wide epigenomic information, make application of these powerful methods more difficult in primary tissues, complicating possible clinical applications. Indeed, as our mechanistic understanding of epigenetic drivers of phenotypic change grows, the application of these methods to a broad diversity of human samples, both normal and diseased, promises to provide valuable, and potentially clinically actionable insights.

Thus while powerful genome-wide epigenome analysis methods would provide a picture of epigenomic composition within phenotypically isolated, homogenous, and/or rare primary cells, these ensemble methods still wash out the single-cell variability that may be present within the population. As an ultimate goal, we might hope these genome-wide techniques to be adapted for the fundamental limit of input material – the single cell [39,40]. Indeed single cell methods might be applied to two important classes of problem. The first where individual cells might be selected from a group of relatively abundant cells to unravel fine-scale epigenomic variation, and the second wherein a small group of rare cells might be profiled to increase the sensitivity of assessing epigenomic state of the selected population. Such capabilities will provide a never before seen window into cellular epigenomic and gene-regulatory variation, and adding this crucial dimension of analysis to the lists of single cell genome-scale investigation, which currently include genome sequencing and gene expression analysis. Recent work has begun to apply a variety of methodologies, from microfluidics to novel enzymes to clever molecular biological manipulations, to drive down the input requirements of these methods while, ideally, maintaining data quality. This review will focus on four methodological areas that have seen recent developments on this front: ChIP-seq, methyl-seq, DNA accessibility, and chromosome conformation capture.

2. ChIP-seq

While a number of reports have detailed protocols for low-input (i.e. <100,000 cells) ChIP-qPCR and ChIP-chip assays [41–44], a smaller subset have been demonstrated compatible with a high-throughput sequencing output. Two notable papers from the laboratory of Bradley Bernstein have described ChIP-seq data generated from as few as 10,000 cells [45,46]. This protocol, referred to as nano-ChIP-seq, relies on a workflow fairly similar to a standard ChIP-seq workflow, coupled with well-calibrated sonication dosage and antibody concentrations, as well as two separate PCR amplification steps, to extend the sensitivity of the ChIP-seq assay for post-translationally modified nucleosomes to the level of 10,000 input cells. This methodology was initially applied to hematopoietic progenitors, providing evidence that developmental regulators in these HSC cell types are enriched for bivalent domains. While the reduction in cell number from $\sim 2 \times 10^7$ to $\sim 10^4$ reduces overall signal intensity, peak calls of H3K4me3 modifications from nano-ChIP were highly concordant with calls from a standard ChIP-seq protocol [45].

A distinct approach from the same laboratory involves the direct single molecule sequencing of ChIP fragments using the Helicos single molecule sequencing methodology [47]. In this approach a standard protocol for ChIP of three types of modified histones, as well as CTCF, was carried out, and the DNA fragments obtained were then poly(A)-tailed and annealed onto a HeliScope instrument for single molecule sequencing. This technique allows for sequencing with no PCR amplification, thereby allowing a better sampling of fragments independent of GC content. Overall the method is compatible with as little as ~50 pg (or approximately 25–50,000 cell equivalents) of input DNA, and while using this limiting input material reduced the number of overall reads, the correlation with larger input data sets was extremely high [47]. While Helicos sequencing has become harder to come by in recent years, it is likely that this direct-sequencing approach may be translatable to the single-molecule sequencing platforms of other providers such as Pacific Biosciences, or Oxford Nanopore.

Another pair of papers describing an approach from the Gronemeyer lab rely on distinct molecular biological mechanisms to generate ChIP-seq libraries compatible with small input requirements [48,49]. Amplification of ChIP-seq fragments in this protocol was carried out with linear amplification of DNA (linDA), which relies on the T7 RNA polymerase to linearly amplify DNA fragments. In short, fragments generated from standard cross-linking based ChIP-seq protocol are amplified by (1) poly-A tailing, (2) addition of bidirectional T7-initiation promoter, and (3) the transcription of RNA from ChIPed DNA fragments (Fig. 1). All of these initial steps can occur in a single tube. After RNA generation, cDNA is then generated from the RNA, and this DNA can then be used in the standard sequencing library preparation protocol for high-throughput sequencing. While this linear amplification methodology is suitable for T7-RNAP-based linear pre-amplification of any fragment library, it has been successfully applied to produce ChIP-seq maps of estrogen receptor alpha (ERα) from as few as 5000 cells [49]. In principle, this protocol might be applied to the linear amplification and subsequent sequencing library generation for any set of nucleic acid fragments, making it potentially broadly applicable to genome-wide assays of limited input materials. The concept of linear pre-amplification leading to a more faithful representation of the initial fragment distribution has also been used in whole-genome amplification methods [50].

Finally, another recent method relies on an indexing-first approach for profiling both post-translational modifications of nucleosomes, as well as transcription factors in scarce cell populations [5]. In this iChIP methodology, cells are fixed, sorted and then sheared, then immobilized on beads loaded with antibody against histone H3. Fixed on this surface, the fragments that are still associated to the nucleosomes are indexed via ligation of indexing oligos unique to the specific cell type of interest, then the indexed chromatin is released and pooled with other samples. This multiplexed pool of indexed chromatin is then divided among a variety of ChIP experiments (i.e. to assess H3K4me1, H3K4me2, H3K4me3, H3K27Ac, and PU.1 binding). After sequencing, the ChIP-seq data is demultiplexed using the cell-specific barcode. When applied to a variety of cell types within the hematopoietic lineage, this methodology provided excellent signal-to-noise ratio and reproducibility when starting with as few as 500 cells per individual sorted population. This technique is especially powerful when assessing a large cohort of potentially related cell populations, as the pooling of

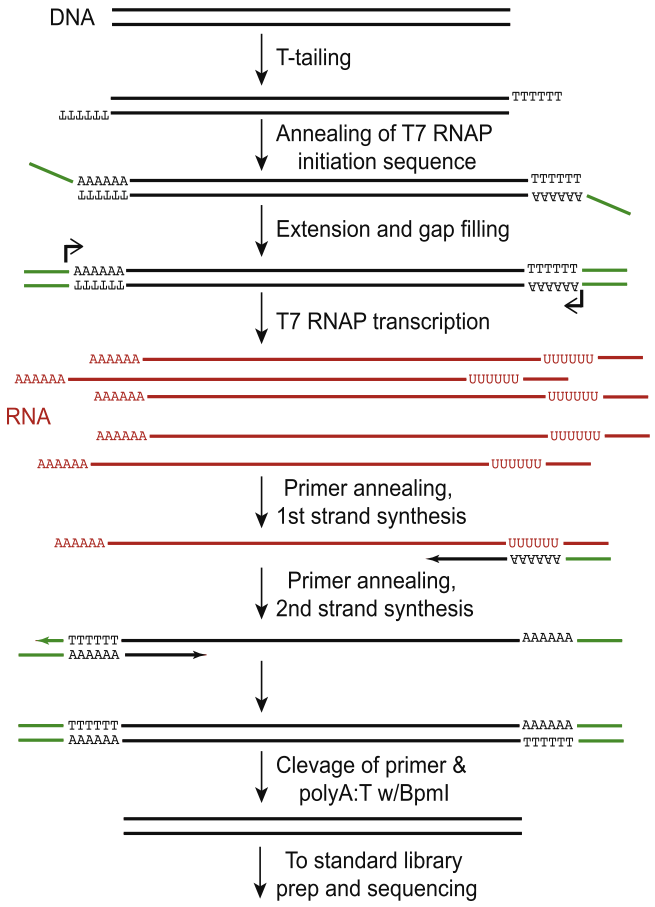

Fig. 1. Linear amplification of DNA fragments with T7 RNA polymerase: DNA fragments to be amplified are first poly-T tailed, then a primer (including a BmpI recognition sequence and T7 RNA polymerase (RNAP) transcription initiation site (green)) are annealed, and added via primer annealing, extension, and gap filling. Then *in vitro* transcription generates linearly amplified copies of the DNA (red). These strands are then re-converted to DNA via primer annealing, 1st strand synthesis, 2nd primer (identical to primer 1) annealing and 2nd strand synthesis. Finally, the sequences added are cleaved off via BpmI digestion. Adapted from [48].

indexed samples allows for increased total amount of material for each distinct ChIP-seq experiment.

3. Methyl-seq

5-Methylcytosine is one of the most extensively studied epigenetic features, and differential, or variable, methylation has been detected in a variety of disease states including cancer [1]. A number of recent directions for decreasing the requirements for input material for these methods have led to improvement in sensitivity of sequencing-based methylation detection [51], but here we focus on genome-wide methods. The laboratory of Jay Shendure and Dieter Weichenhan has described a tagmentation-based approach for ultra-low-input whole-genome bisulfite sequencing [52,53]. This method relies on the Tn5 transposase to simultaneously tag and fragment the genome of interest (Fig. 2A). This Tn5 transposase uses a "cut and paste" mechanism to deliver its DNA payload into the genome, generating PCR-amplifiable fragments rapidly from genomic DNA. To generate a bisulfite sequencing library, the transposase is loaded with specially protected adapter segments (with methylated CpGs) such that these adapters are unreactive to bisulfite treatment. After an adapter-replacement and gap repair step, which allows PCR amplifiable fragments to be generated after the transposition reaction, the fragments are bisulfite-converted, and then PCR amplified, and sequenced using Illumina chemistry. In these steps, harsh bisulfite treatment might cleave the DNA backbone of some members of the library, leaving the fragments incapable of amplification in subsequent PCR steps, whereas full-length fragments that have been chemically converted will be competent for amplification. Thus instead of ligating adapters to DNA fragments and bisulfite converting the libraries, this protocol eliminates a potentially inefficient and time consuming ligation step, improving the number of sequenced fragments per input cell. This strategy allows for a reported ~100-fold improvement in the input requirements for bisulfite whole-genome sequencing, allowing for high-quality bisulfite maps to be generated from ~2000 cells.

The laboratory of Takashi Ito has also reported a genome-wide sequencing based assay of 5-methylcytosine by reordering crucial steps in the sequencing library construction procedure. Instead of ligating adapters prior to bisulfite conversion, bisulfite conversion is instead carried out as the first step (thus the technique is named Post-Bisulfite Adaptor Tagging or PBAT). Unlike standard bisulfite library preparation methods that add adapters to dsDNA prior to conversion, any fragmentation occurring during the bisulfite conversion does not render these fragments non-amplifiable because these fragments may still serve as substrates for subsequent steps that sequentially add adapters to the now single stranded DNA fragments (Fig. 2B). To add adapters to these post-bisulfite treated ssDNA fragments, two rounds of sequential primer annealing and extension are employed. In addition, biotinylated primers were used to simplify purification steps. This purification strategy can allow DNA libraries to be directly sequenced with no PCR steps, improving the representation of regions of the genome. Alternately, libraries can be made from even smaller amounts of input material with a smaller number of global PCR steps prior to sequencing. Overall, a whole genome bisulfite map of methyl-C positions was generated without subsequent amplification at an average depth of 21-fold on the mouse genome from 100 ng of astrocyte DNA [54]. Methylation maps generated with the PBAT process are highly concordant with standard methylC-seq methods.

Other work has described a reduced representation bisulfite sequencing (RRBS) approach for assaying methylation state genome-wide at the level of individual cells [55]. While RRBS provides a picture of a subset of genomic methylation [37], this technique has proven useful in providing a deep and targeted picture of methylation in genomic subregions [17]. Guo et al. [55] shows a one-pot RRBS approach that involves isolation and lysis of an individual cell, followed by MSPI digestion, end repair and terminal-A addition, then standard Y-adapter ligation followed by bisulfite conversion and PCR. The approach can assay the methylation state of up to 1.5 M CpG sites in the genome. They also applied this technique to haploid cells (sperm and pronuclei).

Finally, very recent work from the labs of Wolf Feik and Gavin Kelsey has described true single cell genome-wide bisulfite sequencing, applying the method to study embryonic stem cell heterogeneity [56]. This single-cell bisulfite sequencing method (scBS-seq) is able to measure DNA methylation of up to 48% (10.1 M) CpG sites within a single cell. This procedure also relies on efficiency gains obtained by carrying out the bisulfite conversion prior to construction of the sequencing library by using a modified PBAT procedure (Fig. 2B), allowing DNA fragments that are cleaved by bisulfite treatment to be competent for generation of sequencing library in later steps. With these data, the variance in methylation state of within specific genomic regions (as distinct from the mean methylation state, as observed in the bulk) could be assessed. The variance of regions associated with enhancer marks were higher than the genome average, consistent with the presumption that these distal regulatory elements may be showing

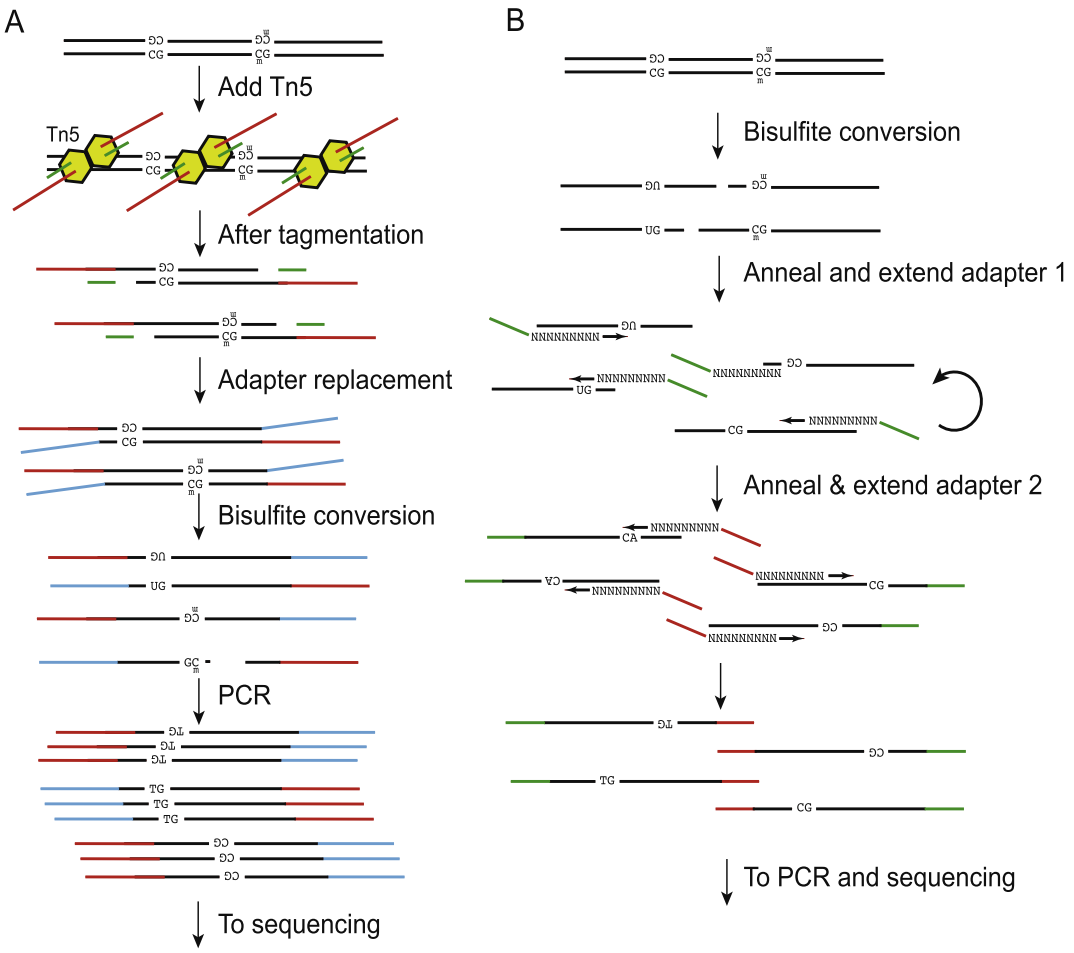

Fig. 2. Strategies for small-sample methylation analysis: (A) Genomic DNA is exposed to Tn5 transposase (yellow), generating fragments. Then adapter fragments are replaced and added to the strand, generating independently amplifiable fragments from each strand of the DNA. Bisulfite conversion then transforms un-methylated Cs to Us, which are read as As during PCR. Bisulfite treatment breaks some fragments, which are not amplified in subsequent steps. (B) Genomic DNA is sheared, then bisulfite converted, transforming un-methylated Cs to Us. Primers are added by sequential extensions of random primers. In Ref. [56] this first step is repeated multiple times, whereas in ref [55] these steps are carried out on beads attached through biotinylated primers. After addition of the second primer, optional pre-sequencing PCR converts Us to Ts and generates a final sequencing library.

the first signs of development-specific methylation. Overall, these synergistic works have opened the door to single-cell analysis of methylation variation, blazing a trail that will be further reinforced by further improvements in recovery efficiency of fragments from individual cells, and methods for highly multiplexed single-cell methylation analysis.

4. DNA accessibility

DNAse hypersensitivity has been used for more than 30 years to identify regions of the genome that are capable of interacting with DNA binding proteins [57,58]. Recent work coupling this method to high-throughput sequencing [59] has produced genome-wide maps of chromatin accessibility that have transformed our understanding of gene regulation [22,23,26]. Such assays can identify portions of the genome accessible to the machinery of transcription and to transcription factor binding within different cell types, thereby highlighting phenotype-specific regulatory regions [22,23]. However, protocols for DNAse-seq generally require tens of millions of reads to generate deep data sets [3,35], making exploration of rare or precious samples difficult.

To address these input requirements, recent work has described a transposase-based method for probing the accessibility of chromatin [60]. Instead of relying upon DNase I to create nicks in accessible regions of DNA, this assay of transposase accessible chromatin, or ATAC-seq, relies on the Tn5 transposase to simultaneously fragment and insert sequencing adapters into the genome (Fig. 3A–C). In this manner, the complex, multi-day protocol for generating DNAse-seq libraries [3] is reduced to a workflow comprising the steps of (1) isolating native chromatin, (2) exposing this chromatin to purified transposase loaded with sequencing adapters, and (3) amplifying and quantitating library for sequencing. The procedure generates complex (~50 M fragment) libraries from approximately 50,000 cells, and allows the identification of a subset of DNAse-seq peaks from as few as 500 cells [60]. Furthermore, by sequencing both ends of the DNA fragments generated from the ATAC-seq assay, the fragment size distribution of ATAC-seq fragments can be bioinformatically separated into fragments originating from nucleosome free regions, and reads likely originating from nucleosomes (Fig. 3D). These differently sized fragments can then be used to call regions that appear nucleosome free, as well as nucleosomal regions, generating data akin to MNase-seq data [13,61,62] within regulatory regions. In a similar manner to DNAse-seq, the insertion pattern of the Tn5 transposase can be used to infer the presence of proteins that interact with DNA [60]. The workflow simplification coupled with reduction in sample requirements enables the possibility of functional investigations of gene regulation from clinical tissues, or from fluorescently-sorted sub-populations of cells [60]. While the field has

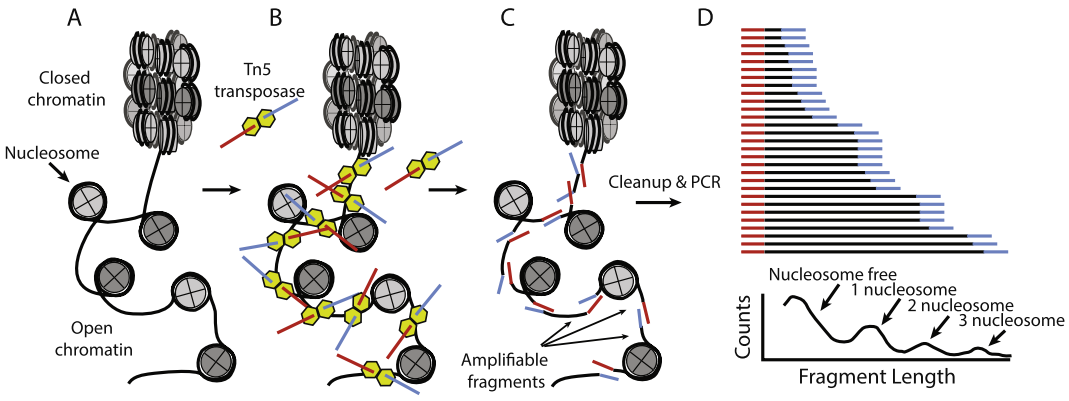

Fig. 3. Assaying accessible chromatin with Tn5 transposase: To map accessible DNA within chromatin, (A) native chromatin is isolated, then (B) exposed to Tn5 transposase loaded with sequencing adapters. Tn5 can only insert its adapter payload into regions of the genome that are accessible. Fragments generated (C) can be amplified after primer extension steps. (D) The distribution of fragments comprises short fragments generated from nucleosome free regions of the genome, as well as fragments generated from protection of integer multiples of individual nucleosomes. Adapted from [60].

already begun to implement ATAC-seq in the context of limited samples, for example in the hematopoietic niche [5], ATAC-seq remains to be applied broadly to a wide variety of different cell types, and the specific sensitivity of ATAC-seq data as compared to DNAse-seq data for determining regulatory elements or inferring TF binding remains to be broadly assessed.

5. Chromatin connectivity

A full two meters of DNA is folded into a ~5 μm cell nucleus within every human cell. The topology of the packaging of chromatin is expected to play a role in gene regulation – from setting the background abilities of different enhancers to interact with promoters to drive expression, to partitioning highly expressed regions and less expressed genomic regions [28]. A variety of methods for understanding these interactions, including 3-C, 4-C, 5-C, Hi-C, and ChIA-pet have been developed to probe these types of interactions at varying levels of generality [29]. The most general, Hi-C [28], uses a library preparation whereby two distal regions of the genome that were in close proximity are fragmented and ligated together to produce one chimeric read. When each end of this read is aligned to the genome, the distant alignments provide a pairwise, genome-wide contact map. Initial implementations of the Hi-C protocol required millions of input cells [28], thereby producing a ensemble average picture of the sorts of interactions that occurred within the population of cells of interest.

A recent study described methods to generate Hi-C data sets from individual cells to asses the variability of this higher order genome organization from cell to cell [63]. To achieve this substantial improvement, many aspects of the bulk Hi-C protocol, including the crosslinking of chromatin, restriction enzyme digestion, biotin fill-in and ligation to generate chimeric reads, were all done within the nuclei of the cells of interest. These nuclei were then hand selected and placed into individual tubes, where the rest of the standard Hi-C protocol (i.e. reversion of crosslinks, purification of ligation junctions, and the rest of library preparation) was carried out. The resulting libraries generated more than 1000 distinct Hi-C read pairs in 37 of the 74 cells that were investigated. While relatively modest number of reads per cell were obtained, extensive analysis of these data demonstrated substantial cell-to-cell stochasticity in the structure of condensed DNA – however some global organizational aspects, including the localization of active gene domains to the boundaries of chromosome territories, remained constant [63]. Future improvements in the efficiency of this single-cell investigation promise an even higher-resolution view of the intricacies of DNA folding, and how this folding varies in larger numbers of cells.

6. Conclusion

The development of methods capable of interrogating epigenomic components of small numbers of cells – even to the level of individual cells – are enabling the epigenomic profiling of precious or rare tissue samples. However, further thought must also be given to making these methods highly quantitative when input material reaches such very low levels. Unlike RNAseq methodologies, where transcript abundance can reach into the thousands per cell, the dynamic range associated with assessment of epigenomic features often ranges between 0 and ~2 reads per genomic locus per cell. Fundamentally, limiting input materials substantially blunts the dynamic range of assessment, a problem further magnified at the level of single cell analysis. This problem also manifests itself as a fundamental limit to the complexity of the sequencing library, i.e. the number of unique fragments that can be sequenced. The library complexity per cell unit is often the most relevant figure of merit, along with sensitivity of signal-to-noise ratio to input amount, for small-sample epigenomic methods. Indeed, the complexity limitations inherent in small-sample assays make identification of reads originating from PCR duplicates (using methods such as molecular barcoding strategies [64–67]) all the more important.

Because of these issues of dynamic range and library complexity, substantial work remains for algorithm development that might take these relatively sparse data sets and extract maximally biologically meaningful insights from these promising methodologies. Alternately, this problem of dynamic range might be addressed by highly parallel methods for the probing of many individual cells, allowing per-cell technical noise to be combated by large numbers of observations. In short, the future of small-sample epigenomic analysis is bright, with a number of recent innovative methodological solutions poised to diffuse to the epigenomics community at large, unlocking exciting new directions of investigation.

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