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Review

Discovering enhancers by mapping chromatin features in primary tissue

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ABSTRACT

Enhancers work with promoters to refine the timing, location, and level of gene expression. As they perform these functions, active enhancers generate a chromatin environment that is distinct from other areas of the genome. Therefore, profiling enhancer-associated chromatin features can produce genome-wide maps of potential regulatory elements. This review focuses on current technologies used to produce maps of potential tissue-specific enhancers by profiling chromatin from primary tissue. First, cells are separated from whole organisms either by affinity purification, automated cell sorting, or microdissection. Isolating the tissue prior to analysis ensures that the molecular signature of active enhancers will not become lost in an averaged signal from unrelated cell types. After cell isolation, the molecular feature that is profiled will depend on the abundance and quality of the harvested material. The combination of tissue isolation plus genome-wide chromatin profiling has successfully identified enhancers in several pioneering studies. In the future, the regulatory apparatus of healthy and diseased tissues will be explored in this manner, as researchers use the combined techniques to gain insight into how active enhancers may influence disease progression.

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1. Introduction

Enhancers are regulatory elements that direct their target genes to express in the right place, at the right level, and at the right time. These segments of DNA recruit sequence-specific transcription factors and transcriptional co-activators to regulate gene expression. They are distinct from promoters in that they generally are not directly upstream of the transcription unit. One gene may have several different enhancers that regulate its expression in specific cells at precise developmental times. Since cell identity is defined by a stable transcriptional program, identifying which enhancers are active in a given cell type will advance the understanding of how cell identity is established and maintained.

Historically, enhancers were identified by a time-consuming process of cloning candidate DNA elements into reporter constructs with minimal promoters, transforming a genetic model, and screening for reporter expression in vivo. More recently, several different research trajectories have combined to assist the unbiased discovery of tissue- and cell-specific enhancers across an entire genome. Researchers investigating chromatin and transcription have revealed that active enhancers exhibit a distinguishing molecular signature. At the same time, cell

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and developmental biologists have refined techniques for isolating tissues and cells of interest from whole organisms. This enables molecular studies of the regulatory apparatus of single tissues instead of an average of many tissue types. The combination of these approaches enables the molecular definition of active enhancers in purified cells and tissues on a genome-wide scale.

This review will highlight recent advances in techniques that produce genome-wide maps of candidate enhancers. In particular, it will focus on the discovery of active enhancers via the molecular analysis of cells isolated from whole organisms. There are two major steps in producing such a genome-wide map. First, the cell or tissue of interest is purified. Second, a molecular assay that identifies enhancers is performed on the purified material, yielding sufficient DNA for high-throughput sequencing. The choice of methodology for the first step depends on the targeted cell or tissue, and whether the organism studied is amenable to transgenesis. The assay applied in second step depends on the quality and quantity of isolated cells. Since researchers have been improving the efficiency of production of high throughput sequencing libraries, many molecular assays can succeed even when applied to very small, very pure amounts of primary tissue.

2. Purifying cells and tissues from whole organisms

Active enhancers have a distinctive chromatin landscape, and they are also associated with characteristic RNAs. One way to identify candidate enhancers is to map the location of their chromatin or RNA features back to the genome. Assays like chromatin immunoprecipitation followed by sequencing (ChIP-seq) or microarray (ChIP-chip), DNaseI digestion followed by sequencing (DNase-seq), and sequencing of total RNA (RNA-seq), are routinely used to interrogate chromatin structure and transcriptional activity genome-wide. The discriminatory power of these experiments requires homogenous populations of cells. If a chromatin signature is present only in a certain cell or tissue type, the cell-specific signal can be completely obscured when the molecular assay is performed on a mixed cell population. Therefore, to define tissue and cell-specific enhancers using molecular techniques, the first step is to segregate the cells of interest away from the rest of the organism.

This section will outline current methods used to isolate cells or tissues prior to performing the molecular assays. An excellent recent review covers many of these technologies from the perspective of defining cell-specific transcriptomes [31]. Here, the focus will be on what cellular or molecular features are preserved by each isolation method, and the ease of acquiring sufficient material for downstream molecular analysis.

2.1. Bench-top affinity purification

Of the three different categories of cell isolation, purifying cells or nuclei on the bench-top requires limited technology beyond transgenesis. The protocol typically involves expressing a transgenic tag in the cells of interest, disrupting the organism or tissue to make a cell suspension, and exposing the suspension to magnetic beads that capture the desired cells. Because sorting depends on a physical interaction between the tag and the magnetic bead, the tag must be on the cell surface or the nuclear envelope. In *Drosophila*, one early example of this type of sorting involved expressing the mouse cell surface molecule CD8 with the GAL4/UAS system. Exposing the native cell suspension to beads coupled to anti-CD8 antibody resulted in a 30- to 100-fold enrichment of the tag-expressing cells. The purified population was used to determine tissue-specific gene expression via microarray [42]. GFP is another tag used for cell isolation purposes. Fusion of the GFP molecule to an outer or inner nuclear membrane protein allows nuclear isolation when expressed in target cells [20,25]. Isolating nuclei can be preferable for downstream methods that interrogate chromatin, because the contaminating cytoplasm is left behind. Nuclear isolation is also feasible after formaldehyde crosslinking, which can destroy the ability to make suspensions of whole cells. Beads-based separation of crosslinked nuclei has also been successful with the INTACT tag. This tag consists of three parts: an organism-specific protein domain that localizes to the nuclear membrane, a fluorescent protein for visualization, and the biotin ligase recognition peptide [14,15]. Expression of this tag together with *Escherichia coli* biotin ligase (endogenous biotinylation is also possible) generates biotinylated nuclei that can be harvested with streptavidin beads. This method has been put to use in native cells from *Xenopus* [1], *Arabidopsis* [14,15], and *Drosophila* [37]. Fixed *Caenorhabditis elegans* nuclei have also been purified successfully [37].

2.2. Automated cell sorting

Separating cells or nuclei using a cell sorter is more technology-intensive than separation on beads, it may require the assistance of a FACS operator, and it takes more time to yield the same number of cells. Otherwise it has many of the same experimental requirements, and what it lacks in yield it may make up for in purity. Unlike with beads, automated sorting can sort single cells and exclude clumps. This ensures that contaminating negative cells do not end up in the positive sample simply because they are stuck to a positively marked cell. An additional difference is that a cell sorter depends on detectable fluorescence in the cells of interest and not on a physical interaction between a tag and a magnetic bead. This means transgenic tags internal to the cell or nucleus may be used. Alternatively, cells such as pancreatic islet progenitor cells or ventral foregut endoderm can be isolated using antibodies directed against cell surface markers [38,44].

One example of using transgenic tags internal to the nucleus is batch isolation of tissue specific chromatin for immunoprecipitation (BiTS-ChIP). In this method, GFP-tagged Histone H2B is expressed in the cell type of interest using the GAL4/UAS system in *Drosophila*. Following fixation, fluorescently labeled nuclei are sorted using FACS and prepared for ChIP [5,6]. Other GFP-tagged native nuclei have been sorted successfully in *Drosophila* [32], *C. elegans* [16,18,41], and *Arabidopsis* [45]. The fluorescent protein portion of the INTACT tag has also been used for automated sorting of crosslinked *Drosophila* nuclei [7]. That approach was combined with a ChIP protocol optimized for hundreds of thousands of cells. This reduced the amount of time spent sorting, which is one of the major limitations of this cell separation technique. The time spent sorting will depend upon the percentage of the fluorescent cells within the suspension, and the desired purity of the sort: isolating a small fraction at high purity takes longer. Many downstream molecular analyses require millions of cells (see next section), and in order to achieve that yield some protocols require up to 8 h of sorting for relatively prevalent cell types like mesoderm [5]. By contrast, when the cell number requirement is reduced by an order of magnitude, only 1 or 2 h of sorting is necessary, even for tagged nuclei that are around 5% of the total population [7].

2.3. Microdissection

In some systems the tissue of interest cannot be isolated using cell-specific molecular tags. In this case, microdissection may be required. This is the least automated and most skill-intensive cell separation approach. Typically, the tissue is crosslinked with formaldehyde before dissection. This approach has enabled the discovery of enhancers via ChIP-seq in mouse embryonic forebrain, midbrain, limb, and heart [4,40]. Microdissected mouse tail buds provided enough material to describe the chromatin landscape of the HoxD cluster via ChIP-chip [35]. As the molecular assays become optimized for small amounts of material (see below), fewer and fewer dissections will be required for the production of robust and reproducible datasets.

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