

DNA microarray technologies for measuring protein–DNA interactions

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DNA-binding proteins have key roles in many cellular processes, including transcriptional regulation and replication. Microarray-based technologies permit the high-throughput identification of binding sites and enable the functional roles of these binding proteins to be elucidated. In particular, microarray readout either of chromatin immunoprecipitated DNA-bound proteins (ChIP-chip) or of DNA adenine methyltransferase fusion proteins (DamID) enables the identification of *in vivo* genomic target sites of proteins. A complementary approach to analyse the *in vitro* binding of proteins directly to double-stranded DNA microarrays (protein binding microarrays; PBMs), permits rapid characterization of their DNA binding site sequence specificities. Recent advances in DNA microarray synthesis technologies have facilitated the definition of DNA-binding sites at much higher resolution and coverage, and advances in these and emerging technologies will further increase the efficiencies of these exciting new approaches.

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Introduction

DNA-binding proteins perform a variety of important functions in cells, including transcriptional regulation, chromosome maintenance, replication and DNA repair. The interactions between transcription factors (TFs) and their DNA-binding sites are of particular interest, as these interactions control crucial steps in development and responses to environmental stresses. Moreover, in humans the dysfunction of TFs can contribute to the progression of various diseases. Also of significant interest is the location of histones and their post-translational modifications, as they too contribute significantly to gene regulation. The labor involved in traditional techniques

for examining the DNA-binding sites of proteins limits analysis to a fairly small number of DNA sequences.

DNA microarrays [1,2] together with the availability of whole-genome sequences have revolutionized mRNA expression analysis and, more recently, have facilitated biochemical and functional genomics studies of DNA-binding proteins. Chromatin immunoprecipitation of a protein of interest followed by microarray-based detection of enriched DNA fragments, referred to as 'ChIP-chip' or 'genome-wide location analysis', is currently the most widely used method for identifying *in vivo* TF-binding sites in a high-throughput manner [3–6]. An alternative microarray-based approach for the genome-scale identification of *in vivo* binding sites utilizes a DNA-binding protein fused to DNA adenine methyltransferase (Dam), which marks DNA near the protein's target sites [7]. The protein-binding microarray (PBM) technology permits rapid, high-throughput characterization of the *in vitro* DNA-binding specificities of DNA-binding proteins by assaying their binding to double-stranded DNA microarrays [8,9,10**].

Advances in DNA microarray synthesis technology have increased feature (spot) density, which has allowed higher resolution definition of *in vivo* target sites and greater coverage of genomic sequence space. Such advances have also permitted a larger fraction of sequence space to be assayed *in vitro* by PBMs. Because the ChIP-chip and DamID technologies and early studies using these techniques have been described in depth in several recent reviews [11–13], this review will focus primarily on recent studies that included technological improvements. This review will also focus on PBM technology, which previously has not been reviewed. Table 1 provides a comparison of the three technologies: ChIP-chip, DamID and PBM.

ChIP-chip

In ChIP-chip, cells are treated with a reagent, typically formaldehyde, which creates covalent crosslinks between protein and DNA. An antibody specific for a protein of interest is then used to immunoprecipitate protein-bound DNA fragments, which are subsequently labeled in an amplification reaction and hybridized to DNA microarrays to identify the protein-bound fragments (Figure 1). Initial ChIP-chip studies were performed on *Saccharomyces cerevisiae* regulatory TFs [3–6] and on replication origin recognition proteins [14], using microarrays spotted with PCR amplicons covering essentially all intergenic regions in yeast. Studies of yeast TFs in multiple cellular

Table 1

Comparison of ChIP-chip, DamID and PBM technologies

Technology	Advantages	Disadvantages
ChIP-chip	<p><i>In vivo</i></p> <p>Provides a snapshot of DNA interactions in a given cell type under the examined cellular conditions</p> <p>Use of modification-specific antibodies permits the identification of genomic sites associated with a protein containing a particular post-translational modification</p> <p>Identifies genomic sites associated either directly or indirectly (via protein–protein interactions) with the protein</p>	<p>Typically requires a protein-specific antibody, which may be a challenge to obtain</p> <p>To obtain enrichment of bound fragments, experiments must be performed in cellular conditions under which the protein of interest is expressed, nuclear, and regulating its target genes</p> <p>Might require a potentially limiting tissue source</p> <p>Even with the use of densely tiled oligonucleotide arrays, difficulties in reducing the size of immunoprecipitated DNA fragments limit resolution of DNA-binding site identification</p>
DamID	<p><i>In vivo</i></p> <p>Provides a snapshot of DNA interactions in a given cell type under the examined cellular conditions</p> <p>Does not require a protein-specific antibody</p> <p>Could identify genomic sites associated either directly or indirectly (via protein–protein interactions) with the protein</p>	<p>Requires slight overexpression of the protein because of expression of the fusion protein</p> <p>The fusion protein might not exhibit the same binding properties as the endogenous protein</p> <p>To obtain enrichment of bound fragments, experiments must be performed in cellular conditions under which the protein of interest is expressed, nuclear, and regulating its target genes</p> <p>Currently does not permit direct identification of genomic sites associated with a protein containing a particular post-translational modification</p> <p>Might require a potentially limiting tissue source</p> <p>Resolution has been limited so far to ~1 kb owing to the spread of methylation</p>
PBM	<p>Does not require a protein-specific antibody</p> <p>Highly rapid</p> <p>Provides a comprehensive survey of DNA-binding site sequence variants, including data on non-binding sequences</p> <p>Can have very high binding-site resolution, down to the exact binding site sequence variant</p>	<p><i>In vitro</i></p> <p>Experiments are performed typically at arbitrary protein concentrations, in arbitrary buffer conditions</p> <p>Requires analysis of additional data types, such as phylogenetic sequence conservation and gene expression data, in order to identify which genomic sites are likely to be utilized <i>in vivo</i></p> <p>Observed binding might not correspond to endogenous binding because of missing cofactors or chromatin context</p> <p>Fusion protein might not exhibit same binding properties as the endogenous protein</p>

states [15] or under different environmental conditions [16•] highlighted that binding of TFs can be condition-dependent. ChIP-chip has also been used in yeast to examine TATA-binding protein (TBP) [17], RNA polymerase (Pol) II transcription initiation and elongation apparatuses [18], the RNA Pol III transcription apparatus [19], and a centromere-related factor [20].

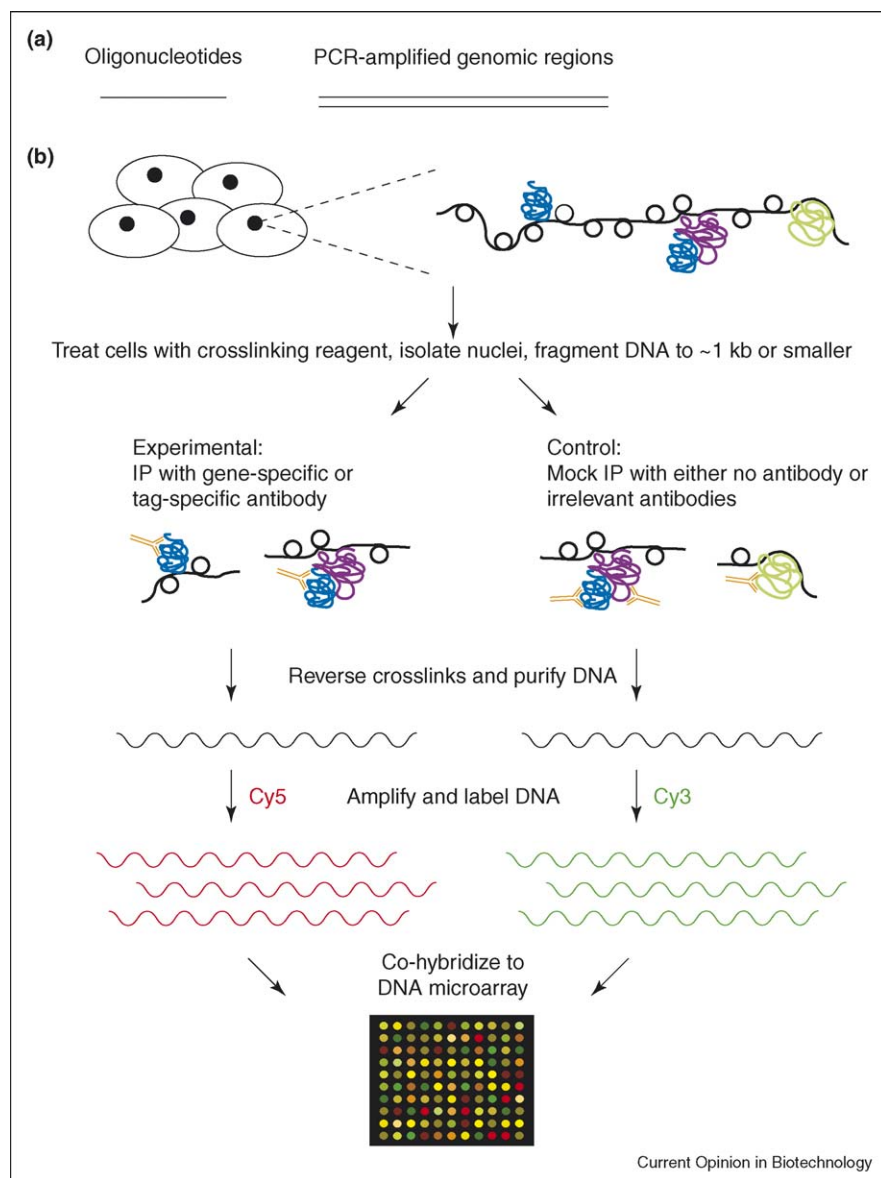
Recent ChIP-chip studies have focused on two main areas: histone modifications, histone-modifying proteins and chromatin remodeling; and combinatorial and condition-specific regulation by TFs. Many recent studies have utilized arrays of oligonucleotides designed to tile a portion of the genome ('tiling arrays'), permitting high-resolution definition of the genomic binding sites of a given protein.

ChIP-chip studies to analyse histone modifications and histone-modifying proteins

A recent study provided high-resolution mapping of the positions of nucleosomes (i.e. the chromosomal packing unit of DNA wrapped around a histone core) in yeast. This

was accomplished by the purification of mononucleosomal DNA (i.e. DNA from a single nucleosome) resulting from a digestion of genomic DNA with micrococcal nuclease, and the use of microarrays printed with 50-mer oligonucleotides tiled every 20 bp across almost all of yeast chromosome III and ~1 kb of 230 additional promoters. Interestingly, Rando and colleagues [21••] observed nucleosome-free regions of ~150 bp located ~200 bp upstream of many annotated coding regions, possibly to permit accessibility of the intergenic DNA to regulatory TFs; this finding is consistent with a previous study by Lieb and colleagues [22] who found decreased nucleosome occupancy at active promoters. In contrast to other studies in which data from PCR amplicon arrays suggested the existence of a discrete, complex, combinatorial 'histone code', Rando and colleagues observed a continuous pattern of modifications across nucleosomes, suggesting a simpler, redundant 'code' [23••]. Interestingly, the histone variant H2A.Z was found to occur at the transcription start sites of most genes, including those at undetectable transcription levels, suggesting a mechanism for marking the 5'

Figure 1



ChIP-chip. (a) Types of DNAs used in ChIP-chip. Oligonucleotides tiling genomic regions (left); PCR-amplified genomic regions (right).

(b) ChIP-chip experimental design. A large variety of protein–DNA and protein–protein crosslinks are created nonspecifically, owing to the nonspecific nature of formaldehyde crosslinking. An antibody (orange) either specific for the protein of interest (blue) or specific for an epitope tag fused to the protein of interest is used in immunoprecipitation (IP) in the experimental sample. This IP will enrich for the target protein, including protein directly bound to genomic DNA-binding sites, and also for protein indirectly associated with DNA via protein–protein interactions. A control (‘mock’) IP is performed using either no antibody, an irrelevant antibody or pre-immune IgG antibodies. This mock IP is not expected to enrich for the target protein of interest.

ends of both active and inactive genes [24]. A separate study used an Agilent DNA microarray containing ~44 000 60-mer oligonucleotides covering most of the yeast genome at an average probe density of 266 bp to map histone acetylation and methylation at high resolution [25]. In another study, histone methylations in mouse embryonic stem cells were mapped using Affymetrix arrays custom-designed to tile noncoding regions that are highly conserved over mammalian genomes and

may be important for gene regulation during development [26]. ChIP-chip studies of chromatin and its epigenetic modifications are described in a recent review [12], so are not discussed in detail here.

ChIP-chip studies to analyse transcription factor binding

The construction of microarrays for genome-scale ChIP-chip studies in metazoans has been a significant challenge

owing to the increased size of these genomes. Early ChIP-chip studies in mammalian genomes utilized various types of PCR amplicon arrays, including arrays tiling a specific genomic region of interest [27], CpG island arrays [28], and promoter arrays [29]. Promoter arrays covering roughly –750 bp to +250 bp relative to transcription start sites have been used to analyze binding of human nuclear factor 1 α (HNF1 α), HNF4 α , and HNF6 in post-mortem human liver and pancreas [30]. These arrays were also used to study the muscle regulatory TFs MyoD, myogenin and MEF2 (myocyte enhancer factor-2) in differentiating murine C2C12 skeletal muscle cells [31].

The definition of *in vivo* binding sites at higher resolution has resulted largely from the switch to oligonucleotide tiling arrays. In a study using the oligonucleotide tiling arrays of Rando and colleagues [21^{••}], the nuclear pore associated protein Mlp1 was found to associate with α -factor-induced genes in an RNA-dependent manner, suggesting a mechanism for chromosome conformational changes in response to the α -factor mating pheromone [32[•]]. In another study, a 10-slide set of Agilent 60-mer oligonucleotide arrays covering –8 kb to +2 kb relative to the transcript start sites for nearly 18 000 human genes, was used to identify binding sites in human embryonic stem (ES) cells for the TFs OCT4, SOX2 and NANOG, which are important in ES cell renewal and maintenance of pluripotency [33^{••}]. Even though important regulatory interactions can occur in promoters, a study using Affymetrix arrays representing essentially all nonrepetitive sequences on human chromosomes 21 and 22 found that most binding sites for the TFs Sp1, cMyc and p53 were located far from the transcription start sites of known protein-coding genes [34^{••}].

ChIP-chip using anti-RNA Pol II and anti-TBP-associated factor 1 antibodies to isolate pre-initiation complexes from four human cell lines has enabled improved promoter mapping [35]. In a follow-up study, Ren and colleagues [36^{••}] mapped promoters in the entire human genome using a set of 38 NimbleGen arrays containing roughly 14.5 million 50-mer oligonucleotides, designed to represent all non-repetitive DNA throughout the human genome at 100 bp resolution. Interestingly, the authors found that a large number of genes contained two or more active promoters, and also defined a set of 1239 putative promoters that correspond to previously unannotated transcription units.

Limitations of ChIP-chip technology

A major limitation in applying ChIP-chip to other model organisms has been the availability of suitable microarrays. Using a PCR amplicon array tiling *Drosophila* chromosome arm 2L, Bell and colleagues [37[•]] found that sites of active transcription correlated with binding by the origin recognition complex and early replicating origins. Such arrays have also been used to examine heat shock

factor binding in *Drosophila* embryos [38]. NimbleGen 60-mer arrays tiling over 36 Mb of the *Drosophila* genome at 100 bp resolution have also been used [39]. Binding by *Escherichia coli* TFs has been examined using off-the-shelf Affymetrix *E. coli* antisense arrays [40], whereas NimbleGen tiling arrays have been used to map *E. coli* RNA Pol binding sites [41].

Changes in the crosslinking procedure have also led to improvements in ChIP-chip. To improve the efficiency of crosslinking when formaldehyde treatment alone did not result in significant enrichment in ChIP of the histone deacetylase and repressor Rpd3, cells were first treated with dimethyl adipimidate, a protein–protein crosslinking reagent, and then with formaldehyde to create protein–DNA crosslinks [42].

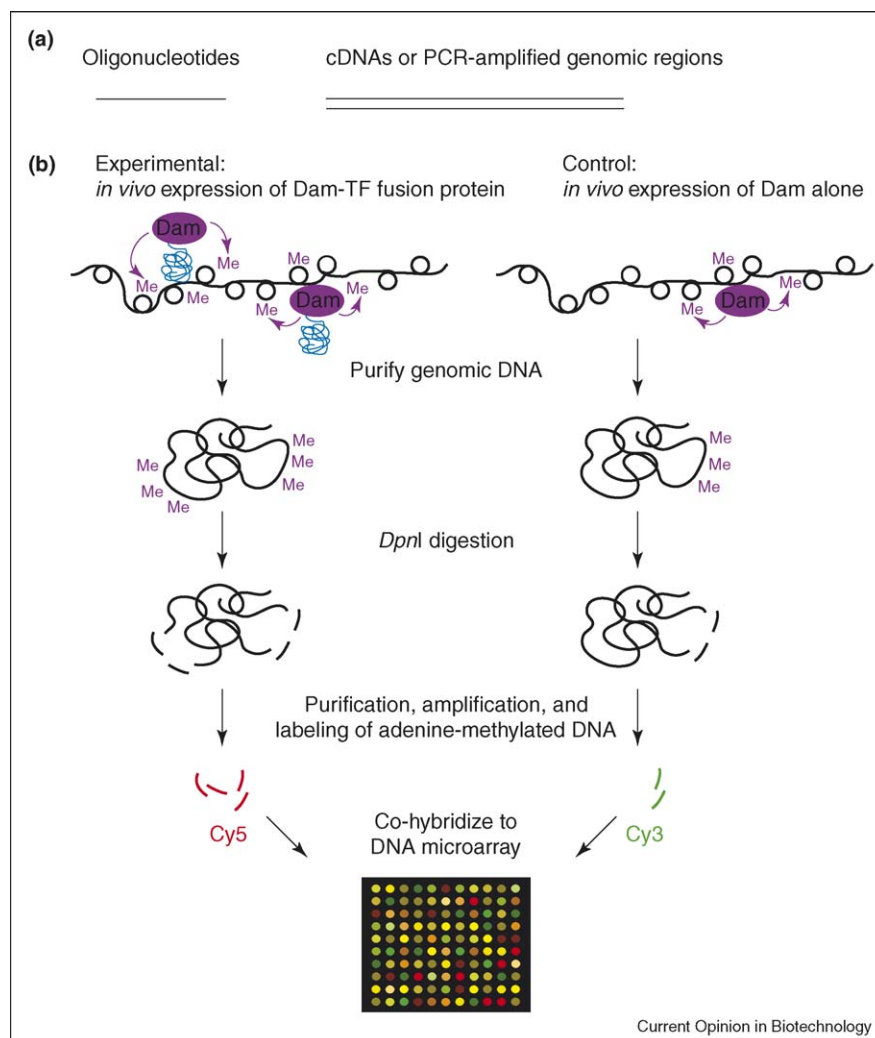
Despite all the benefits of identifying *in vivo* binding locations, ChIP has some inherent caveats that can make the identification of DNA-binding sites difficult [43]. In particular, both antibody limitations and condition-specific protein binding can result in ChIP experiments that do not provide significant enrichment of bound fragments in the immunoprecipitated sample [16[•],43].

DamID

In the DamID approach, the protein of interest is over-expressed *in vivo* from a plasmid as a fusion to Dam. Wherever the protein binds DNA, Dam will methylate adenines within GATC sites in the vicinity of the binding sites. The methylated sites in the experimental versus control (Dam alone) samples are detected by digestion with a methyl-specific restriction enzyme, amplification, labeling and hybridization to a microarray (Figure 2). DamID has been used to identify *in vivo* binding sites in *Drosophila* [7] and *Arabidopsis* [44]. Following this approach, binding sites for sequence-specific TFs [45], DNA methyltransferase [44], chromatin [7] and chromatin-associated proteins have been identified using cDNA or PCR amplicon arrays and, more recently, NimbleGen 60-mer tiling arrays [46[•]].

Comparison of ChIP-chip and DamID indicates that these two methods for mapping *in vivo* TF-binding sites can yield very similar results [47]. DamID has the advantage that it does not require a TF-specific antibody; however, DamID is performed using slight overexpression of a tagged TF from a plasmid, raising concerns that even this slight increase in TF concentration might result in artifactual binding at non-native binding sites. In addition, DamID is not suitable for the detection of post-translational modifications. Finally, DamID does not permit high-resolution mapping of binding sites, because methylation by the tethered Dam can extend over a few kilobases from the TF-binding site [7]. The advantages and disadvantages of DamID and papers using DamID are discussed in-depth in a recent review [13].

Figure 2



DamID. (a) Types of DNAs used in DamID microarray hybridizations. Oligonucleotides tiling genomic regions (left); cDNAs or PCR-amplified genomic regions (right). (b) DamID experimental design. The protein of interest (blue) is overexpressed *in vivo* from a plasmid as a fusion to Dam (purple). Wherever the protein binds DNA, Dam will methylate adenines within GATC sites in the vicinity of the binding sites. The methylated sites are digested with the methyl-specific restriction enzyme *DpnI*, which cuts only at methylated GATC sites. The smaller *DpnI* digestion fragments, corresponding to the methylated regions, are either purified by sucrose gradient centrifugation or specifically amplified using a methylation-specific PCR protocol. (Panel (b) was adapted from [7] and [51] with permission by Macmillan Publishers Ltd.).

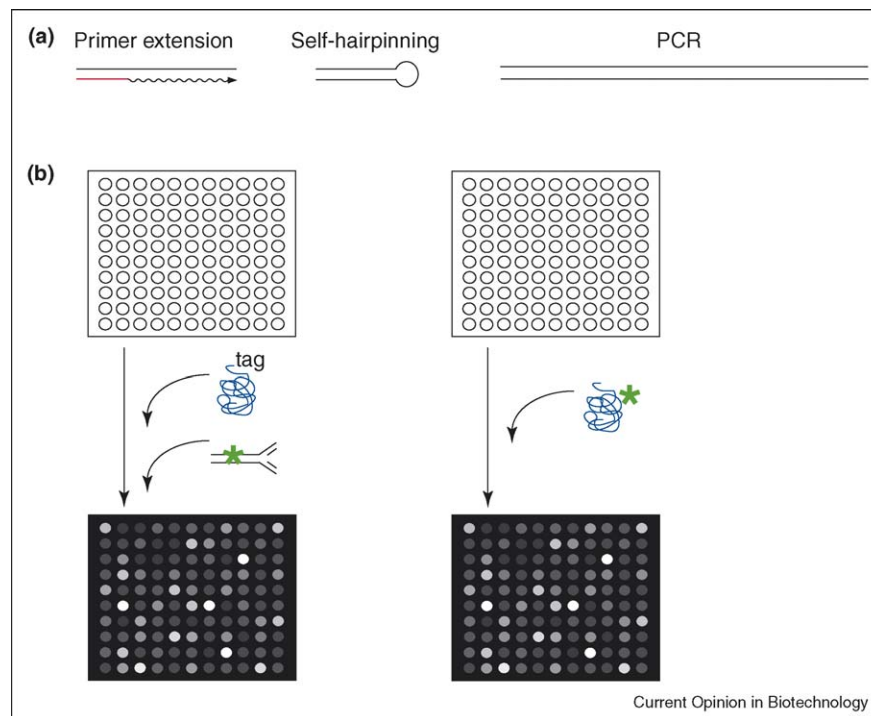
Protein-binding microarrays

The PBM technology permits high-throughput characterization of the sequence specificities of DNA–protein interactions *in vitro* [8,9,10**] (see Figure 3). Briefly, a purified, epitope-tagged protein is allowed to bind directly to a double-stranded DNA (dsDNA) microarray. The protein-bound microarray is then stained with a fluorophore-conjugated antibody (alternatively, a directly labeled protein can be used). The protein's DNA-binding specificity is determined from the significantly bound spots. Three types of DNA molecules can be used to construct the dsDNA array: short double-stranded oligonucleotides created by primer extension; short double-stranded DNAs created using self-hairpinning oligonucleotides; and longer

double-stranded DNAs resulting from the PCR amplification of genomic regions (Figure 3a). Although they do not assay binding *in vivo*, PBMs offer several advantages: an *in vitro* approach does not require prior knowledge of the conditions in which a TF binds its genomic sites; PBMs can provide extensive binding preference data for each DNA sequence variant; and PBM technology is rapid, allowing the determination of the DNA-binding specificities of a purified protein in a single day.

In a proof-of-principle study, Bulyk and colleagues [8] biochemically converted Affymetrix oligonucleotide arrays to dsDNA arrays by extension of a universal primer that was complementary to a universal primer sequence

Figure 3



Protein binding microarrays (PBMs). **(a)** Types of DNAs used in PBMs. Short double-stranded oligonucleotides created by primer extension using a universal primer (red), complementary to a sequence present on spots either on an oligonucleotide array [8] or in solution [9,10**] (left); regions of short double-stranded DNA created by self-hairpinning oligonucleotides on an oligonucleotide array [50**] (center); longer double-stranded DNAs resulting from PCR amplification of genomic regions [10**] (right). **(b)** PBM experimental design. Double-stranded DNA microarrays can be either bound by an epitope-tagged TF (blue) and labeled by a fluorophore-conjugated (green) antibody specific for the tag [10**] (left), or bound by a directly fluorophore-labeled TF [50**] (right). (Panel (b) was adapted from [10**] with permission from Nature Publishing Group.).

present in each oligonucleotide on the array. The authors observed that the methylation-sensitivity of restriction enzymes could be detected by the use of dsDNA arrays treated with *Dam* methylase.

In the first study to assay TF binding to dsDNA arrays, Bulyk *et al.* [9] used a DNA microarray spotted with short synthetic dsDNAs designed to examine a phage display library of wild-type and mutant Cys₂His₂ zinc finger DNA-binding domains of Zif268 (Egr1). Importantly, spots with higher signal intensities were found to contain higher affinity binding sites. In a similar study, Udalova and colleagues [48] examined the DNA-binding specificities of Oct-1 and the NF- κ B p52 homodimer. As in the earlier study [9], the correlation of the PBM signal intensities with binding affinity data allowed the authors to approximate the relative binding affinities for other binding-site variants.

More recently, Mukherjee *et al.* [10**] examined glutathione *S*-transferase (GST)-tagged yeast TFs using whole-genome yeast intergenic microarrays spotted with DNAs resulting from the PCR amplification of genomic regions. The PBM-derived binding-site motifs for the

TFs Abf1 and Rap1 were highly similar to motifs derived from ChIP-chip data [43]. Moreover, analysis of PBM data obtained for Mig1 resulted in the identification of the Mig1 binding site motif [10**], whereas analysis of the ChIP-chip data [43] did not. Many of the newly identified binding sites of these TFs are highly conserved across five sequenced *sensu stricto* yeast species and, thus, are potentially regulatory [10**].

The use of microarrays constructed using longer DNAs obtained from the PCR amplification of genomic regions has the advantages of covering much sequence space with relatively few spots and representing TF-binding sites in the context of their native genomic flanking sequences, including potential cofactor-binding sites. In a proof-of-principle study, Doi *et al.* [49] showed that the TF Jun with its protein partner Fos bound to microarrays upon formation of the Jun/Fos heterodimer. However, inherent in the use of intergenic arrays are two key limitations. First, the relative binding preferences for each of potentially multiple sites in a given intergenic region are not readily distinguished. Second, it can be difficult to separate the specific versus nonspecific contributions to the signal intensities for spotted DNAs of variable lengths.

In contrast, microarrays created with short synthetic dsDNAs representing all possible sequence variants of a given length (all ' k -mers', where k is the length of the DNA-binding sites to be examined) [8,9,50**] permit the relative preferences for variant binding site sequences to be extracted more readily. In one recent study, Warren *et al.* created microarrays of 34-mer oligonucleotides, each containing a 14 bp double-stranded hairpin region. On these arrays, each possible 8-mer binding site sequence variant was synthesized at a distinct feature on the array. The authors used these hairpinned dsDNA arrays to examine binding by fluorophore-conjugated engineered polyamides and the *Drosophila* TF Extradenticle, Cy3-labeled at a unique cysteine residue [50**]. However, as the DNA-binding site length (k) to be examined increases, the number of possible variant binding site sequences can far exceed the number of spots that can currently be manufactured on a single microarray. To overcome this limitation, one could sample binding-site space instead of exhaustively examining all possible binding site sequence variants. Alternatively, instead of devoting a unique spot to each binding-site variant, one can instead employ a compact universal DNA microarray design, whereby distinct k -mers are allowed to overlap within a given DNA probe; for example, there are 31 overlapping 10-mers within 40 bp of dsDNA. Such universal arrays permit comprehensive examination of all possible sequence variants of a given length in a space- and cost-efficient manner (MF Berger, AA Philippakis, *et al.*, unpublished).

Conclusions

Most TFs in human and various model organisms have undetermined DNA-binding specificities and their regulatory functions are not well understood on a genomic scale. Significant challenges include characterization of their DNA-binding specificities and determination of the differential usage of their binding sites in various cell types — through development and response to cellular and environmental conditions — and in normal and disease states. Thus, the target genes of TFs and potential combinatorial modes of transcriptional regulatory control will need to be discovered and recent advances will help us to reach this goal. Continued improvements in the synthesis of high-density DNA microarrays will allow even greater coverage of DNA-binding site space. Furthermore, as more genomes are sequenced and comprehensive data on TF-DNA binding are generated, it will become feasible to examine the co-evolution of TF protein sequence and their corresponding DNA-binding sites.

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