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Development of a Novel Oligonucleotide Array-Based Transcription Factor Assay Platform for Genome-Wide Active Transcription Factor Profiling in *Saccharomyces cerevisiae*

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Abstract: Transcription factors (TFs) play a central role in regulating gene expression and in providing interconnecting regulatory networks between related pathway elements. Although single TF assays provide some insights into pathway regulation, a method that allows the parallel investigation of all active TFs is highly desired to elucidate the complex inter-regulated cellular mechanisms. We have developed a novel oligonucleotide array-based transcription factor assay platform for genome-wide active TF profiling of *Saccharomyces cerevisiae*, which can simultaneously analyze the activities of 93 different TFs. The platform has been validated using 28 purified TFs produced in *Escherichia coli*, cell extracts from yeast strains overexpressing particular TFs, and by detailed control experiments. We then used the platform to examine the activity changes of all yeast TFs during diauxic shift, and results showed, in good agreement with previous studies, that the Sip4 was induced specifically. Other individual TFs required for growth in synthetic complete medium were also identified. Genome-wide analysis of TF activity is extremely useful in investigating complex gene regulatory networks and for the development of systematic understanding of the complexity of genomic functions. These results obtained in this report demonstrate the validity, and for the first time the utility, of this technology for genome-wide investigation of TF activities.

Keywords: oligonucleotide array-based transcription factor assay • transcription factor profiling • DNA-binding activity • nitrocellulose-filter binding assay • *Saccharomyces cerevisiae*

Introduction

Transcription factors (TFs) play a central role during the responses of cells to environmental change. They link external environmental variations to downstream changes in the genomic expression program. The identification of active TFs is required to understand cellular responses to environmental changes at the level of target gene activities. Although mRNA profiling[1,2] can provide expression changes of all genes, including TF-coding genes, gene expression at the mRNA level does not always correlate well with expression at the protein level.[3–5] Several direct TF activity assay strategies have been developed including electromobility shift assays (EMSA),[6] microwell colorimetric TF assays,[7] nitrocellulose-filter binding assays,[8] and reporter gene assays.[9] Although all of these methods can detect the activity of TFs of interest, they were designed to analyze the activity of one specific TF per assay, providing a clear statement of biological interest, but do not allow for the simultaneous high-throughput analysis of multiple TF activities.

We initially demonstrated the feasibility of an oligonucleotide array-based transcription factor assay (OATFA) system[10] employing a limited experimental design that enriched some 4 mammalian TF-capture targets in a multiplex manner by EMSA, then detected the signals by microarray and subsequently confirmed the correct identity of the bound TFs by antibody-based microwell colorimetric TF assays. The TF-capture targets containing different TF binding sequences were first mixed with either purified TFs or cell extracts, then the TF−DNA complexes were separated from the unbound DNAs by running on a high-concentration agarose slab-gel. The bound DNAs in the TF−DNA complexes were extracted and allowed to hybridize with the immobilized probes on the array. Here, we report the further development of OATFA technology in yeast by systematically expanding the scale of analyses as well as introducing improvements such as single primer amplification (SPA)[11] to make the technology more sensitive. We have adopted Wong and Lohman's[8] nitrocellulose-filter binding method to isolate protein−DNA complexes away from the free DNAs in a single filter handling step, enhancing both its simplicity and reproducibility.[12] The new OATFA method is faster and easier to perform than gel-based EMSA for protein−DNA complex isolation by combining several well understood techniques in a multiplex manner. These steps

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include TF protein−DNA binding, nitrocellulose capture of TF protein−DNA complexes, recovery of TF-bound DNAs, single primer amplification, microarray hybridization, and signal quantification.

Our yeast OATFA platform has some 109 different yeast TF-capture targets, which can analyze the activities of 93 different TFs. Using these improved assay conditions, for the first time we can simultaneously monitor many known transcription factors of the yeast *Saccharomyces cerevisiae*. The paper describes the detailed analysis of each step of the OATFA platform, detailing the specificity and accuracy of the platform measurements. These individual steps were independently validated and confirmed by extensive control experiments using both TF-capture targets alone and binding assays using sub-libraries of TF-capture targets, by both *Escherichia coli* expressed yeast TFs and cell extracts from yeast strains overexpressing particular TFs, as well as by antibody-based microwell colorimetric TF assays for some individual TFs.

In addition, we analyzed the activity profiles of all yeast TFs during the metabolic changes induced when the carbon sources for growth were shifted from glucose to glycerol and ethanol (diauxic shift). Our results clearly showed that the yeast TF Sip4 was specifically induced, which correlated well with previous studies by DeRisi et al.[1] who used gene expression analysis alone. The spectrum of yeast TFs activated for growth in synthetic complete (SC) medium were also identified for the first time using the OATFA platform, and the data were independently confirmed by antibody-based microwell colorimetric TF assays. We believe that this series of independent assays shows that the yeast OATFA platform has a useful role for indicating the changes in activities of the numerous nuclear TFs during defined cell states and represents the most comprehensive analysis to date of the complement of DNA-binding TFs that regulate the yeast transcriptome.

Materials and Methods

DNA Oligonucleotides. The probe design scheme is shown in Supplementary Figure S1, and the detailed procedures can also be found in the Supporting Information. Yeast TF binding core sequences were collected from published literature and are listed in Supplementary Table S1. DNA oligonucleotides used in this study were synthesized and PAGE purified by Sangon Inc. (Shanghai, China), and only the "TF-1" sequences from each TF group are listed in Supplementary Table S2. The long oligonucleotide "TF-3" from each TF group was allowed to anneal with its cognate unmodified short oligonucleotide "TF-2" from the same group to form each respective TF-capture target. A mixture of all 109 different yeast TF-capture targets and five different negative controls, called a "TF-capture DNA library", was used in OATFA experiments as indicated in the text. Each TF-capture target was present at a final concentration of 10 nM.

Fabrication of OATFA Arrays. The 5′ amino-linked complementary TF-1 oligonucleotides for each TF-capture target were used as immobilized probes for microarray fabrication. These probes were first dissolved in water and then diluted with 50% DMSO aqueous solution to make the final concentration 10 μM prior to spotting. One 5′ amino-linked and 3′ HEX-linked oligonucleotide was used as the spotting control to align the spots in the array, and 50% DMSO aqueous solution was used as the negative control to monitor possible contaminations during array fabrication. There were triplicate spots of each probe on the array. The array format is shown in Supplementary Figure S2. A commercial arrayer SmartArrayer-48 (CapitalBio, Beijing, China) was utilized to spot the samples onto the aldehyde-modified glass slides (CapitalBio). The center-to-center space between two adjacent spots was 200 μm. After spotting, the arrayed probes were immobilized as described (http://www.arrayit.com/Products/Substrates/SMA/sma.html).

Expression of Yeast TFs. Yeast TF genes *ABF1*$_{1–530}$, *ACE2*$_{490–770}$, *AFT1*$_{1–313}$, *AFT2*$_{2–214}$, *BAS1*$_{1–343}$, *CBF1*, *ECM22*$_{1–497}$, *GAL4*$_{1–147}$, *GLN3*, *HAP1*$_{1–247}$, *HSF1*$_{166–285}$, *MET31*, *MET32*, *MIG1*, *MOT3*, *NRG1*, *PHO4*, *PPR1*$_{1–143}$, *RFX1*, *RIM101*$_{1–300}$, *SIP4*$_{1–130}$, *SKO1*$_{333–581}$, *SWI5*$_{384–709}$, *STP2*, *TBF1*, *TEA1*$_{3–232}$, and *YAP1* were cloned into the plasmids pET30a or pET32a (Novagen, Madison, WI). The sequences of PCR primers were shown in the Supplementary Table S3 and contained the enzyme recognition site to enable the PCR products to be ligated into plasmids. The recombinant proteins expressed from *E. coli* BL21 (DE3) were purified using His SpinTrap (GE Healthcare Bio-Sciences, Piscataway, NJ).

Yeast Strains and Genetic Methods. *Saccharomyces cerevisiae* strains used in this study were BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), Y01334 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sip4Δ::G418*), DBY9653 (*MATa ade2-1 trp1-1 leu2-3,112 his3-11, 15 ura3 can1-100*), and S288c FY2 (*MATa ura3-52*). Standard genetic methods were followed, and yeast cultures were grown in SC medium lacking appropriate amino acids to maintain selection for plasmids.[13] The Myc-6 × His epitope tag coding sequences were introduced into the genomic sequences encoding the C-terminus of TFs, as described previously.[14] The appropriate insertion of the Myc-6 × His tag was confirmed by PCR and sequence analysis.

Diauxic Shift. Yeast was grown to exponential growth phase in SC + 2% glucose, and derepressed cultures were prepared by shifting to SC + 2% glycerol + 2% ethanol for 5.5 h.

Preparation of Yeast Whole Cell Extracts. Preparation of the protein extracts was carried out using a procedure described previously.[15] The protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL).

Nitrocellulose Filter Binding-Based OATFA Method. (1) Binding reaction. The binding reaction occurred at 4 °C for 45 min. *E. coli*-expressed purified TF assays were performed in 20 μL of binding mixture containing 1 × HDB buffer (25 mM HEPES, pH 7.6, 60 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 5 mM $MgCl_2$),[16] 20 μg of BSA, 7.5% glycerol, 1 μg of poly(dIdC) (GE Healthcare Bio-Sciences) competitor DNA, TF-capture DNA library, and *E. coli*-expressed purified TF. The whole cell extract assay was performed in a 20 μL of binding mixture containing 1 × HDB buffer, 7.5% glycerol, 2 μg of poly(dIdC) competitor DNA, the TF-capture DNA library, and 20 μg of the cell extract. The TF-capture DNA library was added to the binding mixture which was preincubated at 4 °C for 5 min to reduce possible nonspecific binding. The final concentration of each of the TF-capture targets was 1 nM. The 20 μL of reaction products was used in the following nitrocellulose filter binding separation steps.

(2) Nitrocellulose filter binding-based separation and collection. A binding mixture was filtered through a BA85 nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) in a spin-column apparatus. The filter was presoaked for 10 min in 0.4 M KOH followed by three 5-min washings in distilled water to bring the pH to neutral and equilibrated in 1 × HDB buffer at 4 °C for 1 h. Then the spin-column was centrifuged at 3500*g* for 30 s at 4 °C followed by three washings with 600

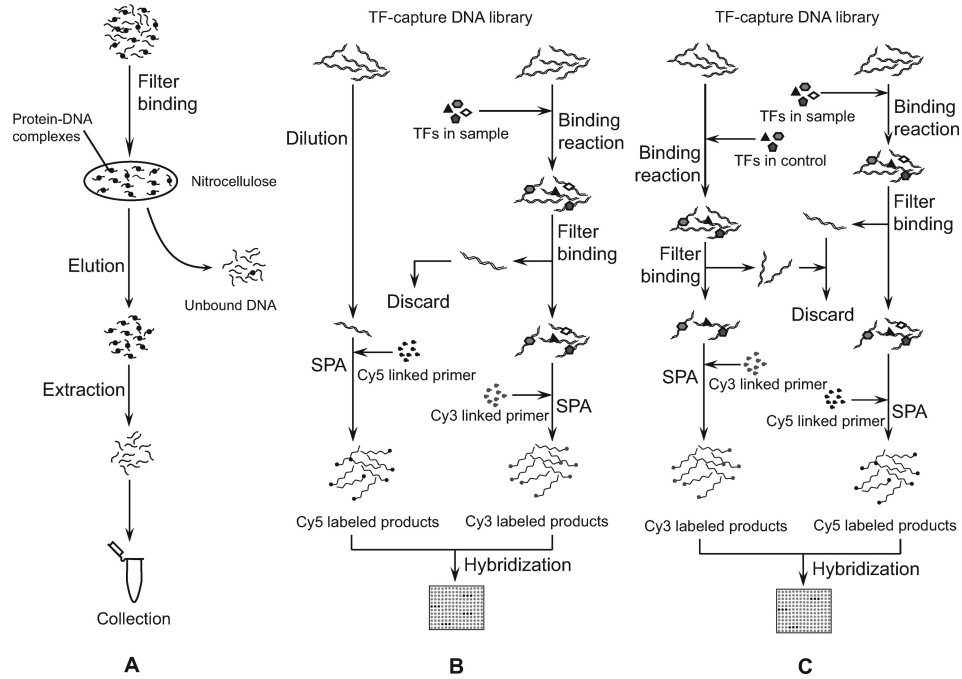


Figure 1. Scheme depicting the OATFA platform. (A) The principle of nitrocellulose filter binding-based separation and collection. The TF−DNA complexes were trapped on nitrocellulose filters after filtration, while the unbound dsDNAs passed straight through the nitrocellulose filter and were discarded. The TF−DNA complexes were then eluted from the filters, and the bound dsDNAs were extracted for single primer amplification and microarray hybridization. (B) The OATFA single sample assay procedure. The TF-capture DNA library was mixed with proteins and then isolated by nitrocellulose filter binding. Bound dsDNAs were extracted, then considered as the SAMPLE and amplified using Cy3-labeled T7 promoter primer. The TF-capture DNA library, without added proteins, was diluted and used as the CONTROL and amplified using Cy5-labeled T7 promoter primer. The products with different labels were then hybridized to the same array. The data were analyzed using the single-array error model. (C) The OATFA dual sample assay procedure. Equal volumes of the TF-capture DNA library were mixed with "sample" and "control" protein extracts, and the resulting protein−DNA complexes were then isolated by nitrocellulose filter binding. The respective bound dsDNAs were extracted and amplified using either Cy5 or Cy3-labeled T7 promoter primers. The differently labeled products were then hybridized to the same array, and the resulting data were analyzed using the single-array error model.

μL of 1 × HDB buffer. The retained protein−DNA complexes were eluted by incubating the filter in the elution buffer (300 mM NaCl, 1% SDS, 10 mM Tris-HCl, pH 8, and 1 mM EDTA) at 50 °C for 10 min. The recovered DNA molecules were phenol-extracted and precipitated by ethanol.[17]

(3) Single primer amplification. The recovered DNA, 200 nM Cy5 or Cy3-labeled T7 promoter primer (5′-GGG GTA ATA CGA CTC ACT ATA GGG-3′), 200 μM dNTPs (TaKaRa, Dalian, China), and 2.5 units of Taq polymerase (TaKaRa) were mixed into a 20 μL mixture and amplified for 40 cycles. The SPA products were heated to 45 °C and vacuumed dry.

(4) Hybridization and scanning. The dried SPA products were resuspended with 2.9 μL of distilled water, respectively. The 5.8 μL of resuspension solution containing Cy5 and Cy3 labeled molecules, with 0.24 μL of 10% SDS, 1.8 μL of 20 × SSC, 3 μL of formamide, and 1.2 μL of 50 × Denhardts was added into one array and hybridized at 42 °C overnight. The slides were then washed once in 2 × SSC/0.1% SDS at room temperature for 4 min followed by a 0.2 × SSC wash at room temperature for 4 min. Finally, the slides were centrifuged dry at 800*g* for 1 min. The slides were then scanned by a laser confocal scanner LuxScan 10KA (CapitalBio). The signal intensities of the spots were quantified by GenePix 4.0 software (Molecular Devices, Union City, CA).

(5) Data analysis. Data were analyzed using the single-array error model.[18] The detailed procedure can be found in the Supporting Information. The normalization factor was calculated based on the intensities of negative control. The median of the intensity ratios, Cy5/Cy3, was then used to adjust the Cy3 channel intensity to the same level as that of the Cy5 channel. Data from independent replicate experiments were combined. An average enrichment ratio was calculated for each DNA probe on the array from at least three replicate data sets. The DNA-binding activity of a TF was deemed significant if the median ratio was >1.8 and the *P*-value was $<10^{-3}$.

Results

Design of the OATFA Method. The original OATFA method[10] was designed to simultaneously analyze multiple TF activities by combining gel-based EMSA to fractionate TF−DNA capture target complexes with DNA microarray hybridization for multiparallel semiquantitative assay of the TF-capture targets. Here, the method has been improved by replacing gel-based EMSA, by a nitrocellulose filter-binding technique which required only a single filter handling procedure to isolate protein−DNA complexes away from the free, unbound TF-capture targets (Figure 1). In our design, each TF being assayed has one "specific" TF-capture target which is recovered in amounts representative of the cellular concentration of its bound TF and which is then semiquantitatively assayed. This new OATFA method involves four steps: (1) the interaction of the yeast cell extract with a specific TF-capture target library, (2) the selection of TF−DNA complexes on nitrocellulose filters (Figure 1A), (3) the recovery of the bound TF-capture targets and their single

primer amplification (Figure 1B and 1C), and (4) the microarray-based discrimination and estimation of each of the TF-capture targets (Figure 1).

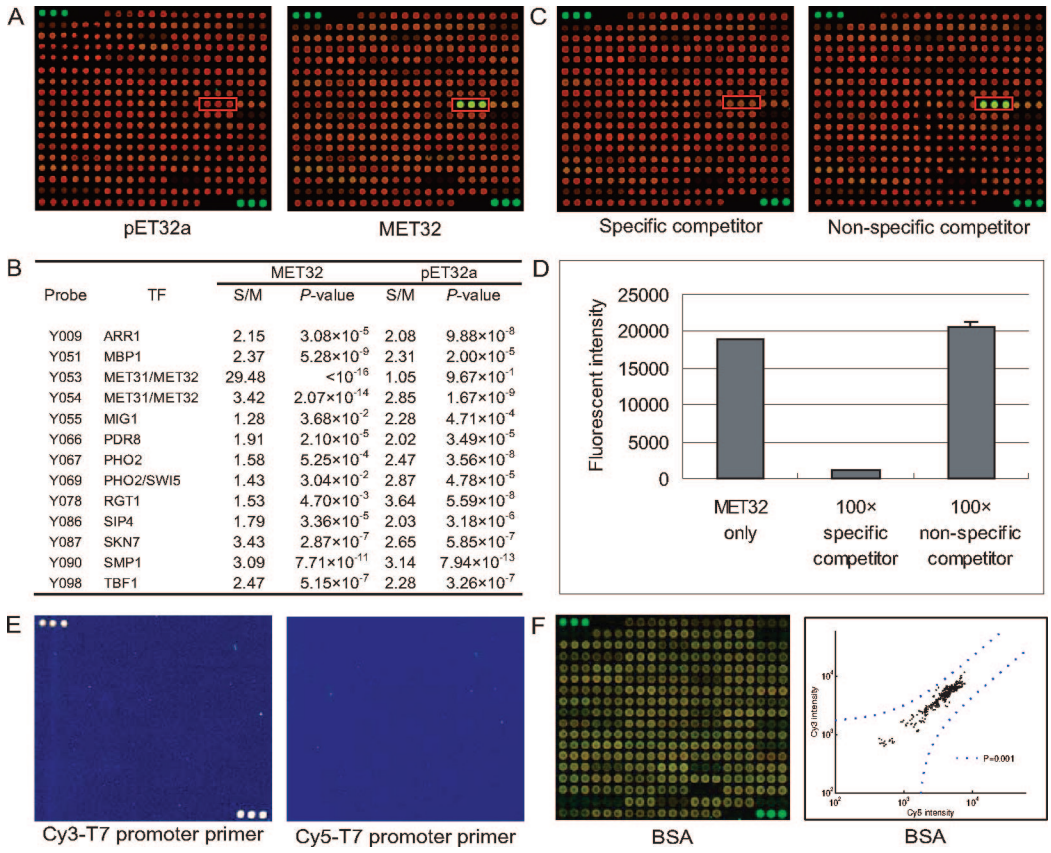
Care was taken to ensure that each of these selected "specific" TF-capture targets have (1) a high specificity of unique TF-binding such that it binds exclusively to the target TF with high affinity and (2) a high specificity of hybridization to array-bound cDNA sequence which do not cross hybridize with other TF-capture complements. The design of the TF-capture targets was based on the core TF-binding sequences/matrix identity collected from the literature (Supplementary Table S1). The principles of our TF-capture target designs are complex but involve one or multiple copies of core TF-binding sequences plus additional nonspecific sequences to produce molecules with roughly equivalent hybridization characteristics for optimal hybridization to the detection array (Supplementary Table S2). Five sequences for negative controls were also designed, which did not contain homologies to any known yeast core TF-binding sequences. An overhanging T7-promoter complementary sequence (5′-CCC TAT AGT GAG TCG TAT TAC CCC-3′) was added at the 3′ ends of all the designed sequences to facilitate further amplification and for DNA preparation during the validation experiments.

Two different TF assay models were used concurrently in this study, "sample comparison assay" (SCA) and "single sample assay" (SSA) (Figure 1). In the SCA model, two different yeast cell extract samples are each incubated with aliquots from the same TF-capture DNA library. The resulting protein−DNA complexes are captured by binding to the nitrocellulose membrane, while most of the free DNA molecules are not retained and pass directly through the membrane. The DNA molecules from the bound protein−DNA complexes are then extracted and amplified by the SPA method[11] using either Cy5 or Cy3 labeled T7 promoter primers, respectively, for each of the two samples. The Cy5 and Cy3 labeled products are then mixed together and hybridized to the assay microarray on which the complementary sequences to each of the 109 TF-capture targets and the 5 negative control sequences are immobilized. Experimental data are analyzed statistically using the single-array error model[18] to identify the positive signals (Figure 1C) after performing at least three parallel replicates. The positive signals are identified if the median of ratios is >1.8 and P-value $< 10^{-3}$. In the second single sample assay (SSA) model, for each single aliquot of cell extract to be assayed, one aliquot from the TF-capture DNA library without the added protein sample was also directly amplified using the SPA method for competitive microarray hybridization assay as described for SCA, following differential Cy3 and Cy5 labeling of the probes (Figure 1B).

Initially, "self-to-self" experiments were undertaken with the TF-capture DNA library to evaluate any possible systematic errors. Almost all of the yeast TF probes worked well in our yeast "total TF" OATFA assay system, except for four probes with distinct fluorescent channel preferences (Supplementary Figure S3). These findings suggest that parallel fluorescent swap experiments should always be performed to filter-out possible false-positive signals.

Assay of Selected *E. coli* Produced TFs. We then tested the performance of the OATFA assay with 28 purified yeast TFs (more than 30% of total) produced in *E. coli* to explore the TF-capture target specificities. This can be illustrated with the full length yeast TF gene *MET32* (YDR253C) which was cloned into the prokaryotic expression vector pET32a. The DNA-binding activity of the recombinant Met32 protein was first validated by microwell colorimetric TF assays (Supplementary Figure S4). The proteins purified from *E. coli* transformed with the pET32a-MET32 and the control proteins purified from *E. coli* transformed with the blank vector pET32a were analyzed by OATFA according to the single sample assay model (Figure 1B), and the results are shown in Figure 2A. There were 13 positive probe signals in either the Met32 or the pET32a assays (Figure 2B). Eight probes (Y009, Y051, Y054, Y066, Y086, Y087, Y090, and Y098) were positive in both assays, which suggested that the signals were likely caused by exogenous interactions with residual *E. coli* proteins. A further four probes (Y055, Y067, Y069, and Y078) were only positive in the pET32a vector (control) assay. The signal of the Y053 probe was much stronger than all other probes in the Met32 assay, and this discrepancy introduced relatively large P-values using the single-array error model. The Y053 probe was designed specifically to capture the yeast TF Met31/32, and the signal was strongly positive only in the Met32 positive assay (Ratio = 29.48 and P-value $< 10^{-16}$) and not in the pET32a assay (Ratio = 1.05), indicating that the signal was most likely caused by the specific binding of the expressed recombinant Met32 protein to its capture target. Another TF-capture target of Met32 is Y054 (5′-AAA CTG TG-3′) which only differed one nucleotide from Y053 (5′-AAA CTG TGG-3′). However, the signals for the two probes differed greatly, which strongly suggests the G at the end of the core binding sequence is very important in DNA-Met32 protein binding.

To further investigate the specificity of the Met32 signal, competition experiments were performed. The signal remained steady when nonspecific competitor dsDNA molecules were added; however, the signal reduced greatly when specific competitor dsDNA molecules were added (Figure 2C and 2D), indicating the specificity of Met32 binding. The Cy5/Cy3 labeled T7 promoter primers used in the SPA were applied to the OATFA array to evaluate possible nonspecific hybridization with the probes immobilized on the arrays, but no cross-hybridization was detected (Figure 2E). When BSA was assayed as a control non-TF protein on the OATFA array, no positive signals (ratio > 1.8 and P-value $< 10^{-3}$) were detected (Figure 2F). Overall these experiments provide a clear indication of the high specificity of the OATFA assay of Met32 by the Y053 target and the absence of interactions with other capture targets. Twenty-six other *E. coli*-expressed yeast TFs (Abf1, Ace2, Aft1, Aft2, Bas1, Cbf1, Ecm22, Gal4, Gln3, Hap1, Hsf1, Met31, Mig1, Mot3, Nrg1, Pho4, Ppr1, Rfx1, Rim101, Sip4, Sko1, Swi5, Stp2, Tbf1, Tea1, and Yap1) and human TF AP1 (the core binding sequence of AP1 is 5′-TGA CTC A-3′, identical to Gcn4 of yeast) were similarly analyzed, and their expected specific signals were all detected successfully (Supplementary Figure S5). These experiments each independently suggested that the yeast OATFA platform specifically detected their target yeast TFs with a low nonspecific cross-reactivity to exogenous proteins and nucleic acids and was suitable for the global assay of the activity of yeast TFs. It was notable that for these 27 purified TFs eight probes (Y009, Y051, Y054, Y066, Y086, Y087, Y090, and Y098) were always slightly positive, as was the case for TF Met32 and its plasmid control as described above. The commonality of these weak signals regardless of the identity of the purified TF confirms they were caused by exogenous interactions with residual *E. coli* proteins present in common in the purified TF preparations. We also examined the detection limitations of the OATFA assay with different concentrations of purified TFs,



Probe	TF	MET32 S/M	MET32 *P*-value	pET32a S/M	pET32a *P*-value
Y009	ARR1	2.15	3.08×10^{-5}	2.08	9.88×10^{-8}
Y051	MBP1	2.37	5.28×10^{-9}	2.31	2.00×10^{-5}
Y053	MET31/MET32	29.48	$<10^{-16}$	1.05	9.67×10^{-1}
Y054	MET31/MET32	3.42	2.07×10^{-14}	2.85	1.67×10^{-9}
Y055	MIG1	1.28	3.68×10^{-2}	2.28	4.71×10^{-4}
Y066	PDR8	1.91	2.10×10^{-5}	2.02	3.49×10^{-5}
Y067	PHO2	1.58	5.25×10^{-4}	2.47	3.56×10^{-8}
Y069	PHO2/SWI5	1.43	3.04×10^{-2}	2.87	4.78×10^{-5}
Y078	RGT1	1.53	4.70×10^{-3}	3.64	5.59×10^{-8}
Y086	SIP4	1.79	3.36×10^{-5}	2.03	3.18×10^{-6}
Y087	SKN7	3.43	2.87×10^{-7}	2.65	5.85×10^{-7}
Y090	SMP1	3.09	7.71×10^{-11}	3.14	7.94×10^{-13}
Y098	TBF1	2.47	5.15×10^{-7}	2.28	3.26×10^{-7}

Figure 2. Assays with purified TF Met32. (A) The DNA-binding activity of the purified proteins from *E. coli* transformed with both pET32a-MET32 and blank expression vector pET32a were analyzed using OATFA according to the single sample assay model. The TF-capture targets enriched by exposure to the Met32 or pET32a were amplified and labeled with Cy3, and the diluted TF-capture DNA library was amplified and labeled with Cy5. (B) Analysis of positive signals (ratio > 1.8 and *P*-value < 10^{-3}) seen in either the Met32 or pET32a assays. (C) A binding mixture containing 100 × specific competitor dsDNA or 100 × nonspecific competitor dsDNA (TF-capture target for Abf1) was added. The TF-capture targets enriched by Met32 binding were amplified and labeled with Cy3, while an aliquot of diluted TF-capture DNA library was amplified and labeled with Cy5 as a control. (D) The change of fluorescent intensity of the Met32 signal with or without competitor. (E) Cy5- or Cy3-labeled T7 promoter primers were hybridized directly to the array. The setting used for the scanner was laser power 85 and PMT 85. (F) BSA was used as a non-TF protein control to bind the TF-capture DNA library, and the products were analyzed on the OATFA array. The TF-capture targets enriched by BSA binding were amplified and labeled with Cy3, while the diluted TF-capture DNA library was amplified and labeled with Cy5 as a control.

which were 0.25 nM (Abf1), 2.5 nM (Met32), and 1.25 nM (Gal4), respectively (data not shown).

Overexpressed Yeast TF Assays. The amount of recombinant protein produced by the yeast expression system is lower than that by the *E. coli* system, but it is believed to be comparatively closer to the physiological expression levels. TFs overexpressed in yeast were analyzed using OATFA according to the sample comparison assay model (Figure 1C). The plasmid pGBKT7 was transformed into the yeast strain DBY9653 to express the yeast TF Gal4 DNA-binding domain. Only two probes (Figure 3A), both designed for Gal4, met the cutoff setting: Y026 (Ratio = 3.89 and *P*-value = 3.33×10^{-14}) and Y027 (Ratio = 11.46 and *P*-value < 10^{-16}). A third probe Y025 was also designed for Gal4, but its signal was not positive (Ratio = 0.84). Comparing the information listed in Supplementary Table S1, the core binding sequences of Y026 and Y027 obviously included more specific information than that of Y025, which suggested that the core binding sequences of Y026 and Y027 were more precise. The core binding sequence of Y027 was the most detailed and was derived from the matrix information of the TRANSFAC database, and it is perhaps not surprising that the signal of Y027 was the strongest. The array results were further validated by microwell colorimetric TF assays in which dsDNA molecules containing the Y027 sequence were immobilized (Figure 3B). Whole cell extracts of AH109 transformed with pGBKT7 were also analyzed (Supplementary Figure S6A), and the expected specific signals were detected. Yeast TF coding gene *SIP4* (YJL089W) was cloned into the vector pGADT7 and transformed into the yeast strain BY4741 to express Sip4. Only the probe Y086 (Ratio = 3.37 and *P*-value = 3.07×10^{-8}) designed for Sip4 was positive (Figure 3C). The array results were further examined by microwell colorimetric TF assays in which dsDNA molecules containing the Y086 sequence were immobilized (Figure 3D). The whole cell extracts of Y01334 transformed with pGADT7-SIP4 were also analyzed (Supplementary Figure S6B), and the expected specific signals were detected. Potential nonspecific signals from unexpected amplification products during single primer amplification (templated by contaminating yeast genomic DNA from the yeast whole cell extracts) were examined in purified yeast genomic DNA, amplified using Cy5/Cy3 labeled T7 promoter primers, but no signals were detected (Figure 3E).

We also examined whether our TF−DNA binding buffer allowed equivalent binding of the whole range of TFs potentially found in a yeast cell (data not shown). We chose to use HDB buffer[16] with our OATFA platform experiments because

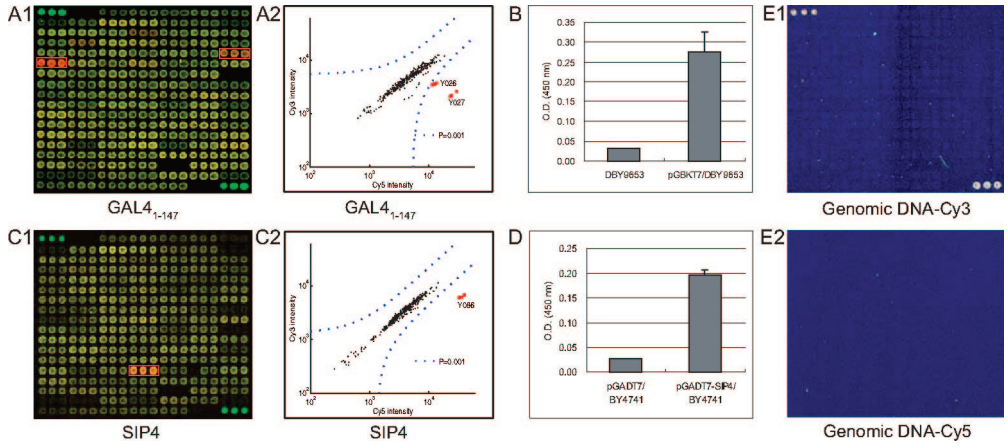


Figure 3. Assays with selected TFs overexpressed in yeast cells. (A) Yeast strain DBY9653 was transformed with pGBKT7 expressing the TF Gal4 DNA-binding domain. Whole cell extracts of yeast DBY9653 were then exposed to the TF-capture DNA library. The recovered DNAs were amplified and labeled with Cy3, while the TF-capture targets recovered after binding to extracts from DBY9653 cells transformed with pGBKT7 were amplified and labeled with Cy5. (B) The array results were further validated using microwell colorimetric TF assays in which dsDNA molecules containing the Y027 sequence were immobilized on microtitre plates. (C) The whole cell extract assays of BY4741 transformed with pGADT7-SIP4 expressing the TF Sip4 or with blank expression vector pGADT7. The TF-capture targets enriched by binding to extracts of BY4741 transformed with pGADT7 were amplified and labeled with Cy3, while the TF-capture targets enriched by binding to extracts of BY4741 transformed with pGADT7-SIP4 were amplified and labeled with Cy5. (D) The array results were further validated by microwell colorimetric TF assays in which dsDNA molecules containing the Y086 sequence were immobilized. (E) Yeast genomic DNA was used as a template and amplified using Cy5 or Cy3 labeled T7 promoter primers. The setting used for the scanner was laser power 85 and PMT 85.

it had shown good EMSA results with a number of different yeast TFs. Comparison with three other binding TF-binding buffers with different compositions[19–21] showed relatively similar active-TF profiles for each buffer (data not shown), and in particular, the HDB buffer[16] showed little evidence of interference between nonspecific TF-binding sequences and their nontarget TFs.

Carbon Source Shift TF Assays. Inoculation of yeast into a medium rich in sugar such as glucose is followed by rapid growth fueled by fermentation, with the production of ethanol. When the fermentable sugar is exhausted, the yeast cells turn to ethanol as a carbon source for aerobic growth. This switch from anaerobic growth to aerobic respiration upon depletion of glucose, referred to as the diauxic shift, correlates with widespread changes in the expression of genes involved in fundamental cellular processes such as carbon metabolism, protein synthesis, and carbohydrate storage.

We used the OATFA platform to characterize the changes in yeast TF activities that take place during this process on a total TF scale, according to the sample comparison assay model (Figure 1C). It is notable that probe Y086 (designed to detect yeast TF Sip4) was the only positive signal generated (Ratio = 4.06 and *P*-value = 8.53×10^{-8}) of all 109 potentially detectible probes after the metabolic treatment (Figure 4A). To confirm the OATFA result, several independent tests were performed. First reporter gene assays were undertaken, and the result correlated well with OATFA array data in which the signal from the Sip4 reporter plasmid increased 23-fold after treatment (Figure 4B). Second, a Myc-6 × His tag was fused to the C-terminus of the yeast TF gene *SIP4*. Microwell colorimetric TF assays were performed to analyze the change of the recombinant Sip4 using the anti-Myc antibody, and again the DNA-binding activity of Sip4 was greatly increased (Figure 4C). Finally, a *sip4*Δ mutant strain Y01334 was also analyzed by OATFA, and the results were negative after metabolic treatment (Figure 4D). Together, these results showed that the DNA-binding activity of Sip4 was greatly induced during the diauxic shift and suggests that Sip4 plays an important role in the altered metabolic activities during this process and that the OATFA assay faithfully reflects this activation. Both the mRNA expression[1] and protein expression[22] of the *SIP4* gene have been previously shown to be induced during diauxic shift, which suggests that increased expression of the *SIP4* gene also contributes to our observed change in Sip4−DNA-binding activity. DeRisi et al.[1] found that only two TF coding genes, including *SIP4*, were induced by a factor of more than 3-fold at the diauxic shift. The other induced TF coding gene is *HAP4* which codes for a TF with transcriptional activity but not with any known DNA-binding activity.[23] It forms a complex with Hap2/3/5 and activates downstream gene expression in which Hap2/3/5 binds to a specific CCAAT regulatory sequence. Although the expression of *HAP4* was induced, the signal of probe Y039 (designed for Hap2/3/5) was negative by OATFA assay (Ratio = 1.04) suggesting that the diauxic metabolic shift may regulate Hap2/3/4/5-dependent target genes via the activation of the transcriptional activity of Hap4, without significant changes in the DNA-binding activity of Hap2/3/5.

Using data from promoter sequence analysis, four typical TF regulatory motifs including the carbon source response element (CSRE), stress response element (STRE), CCAAT box, and Rap1-binding were identified from the other non-TF coding genes whose mRNA expression obviously changed.[1] Sip4, Msn2/4, and Hap2/3/4/5 are the regulators for CSRE, STRE, and CCAAT box motifs, respectively. In response to stress or nutritional limitation, the protein synthesis of Msn2 and Msn4 is nearly unchanged; however, these factors migrate from the cytoplasm to the nucleus to effect metabolic regulation,[24] and Rap1 levels do not change as it remains bound to nearly all its targets.[25] Here, the yeast OATFA platform was used to analyze DNA-binding activities of multiple TFs from yeast whole cell extracts, but not from nuclear extracts, thus garnering TF proteins from all cell compartments. This is a likely explanation of why changes in the levels of Msn2/4 and Rap1 were not detected by the OATFA assays.

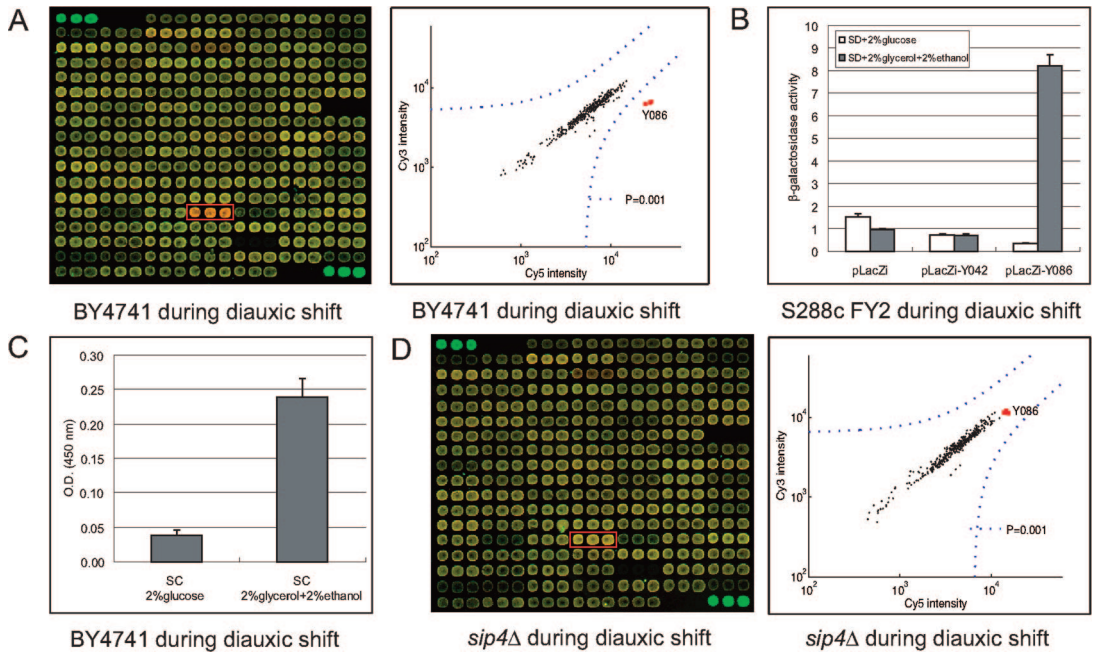


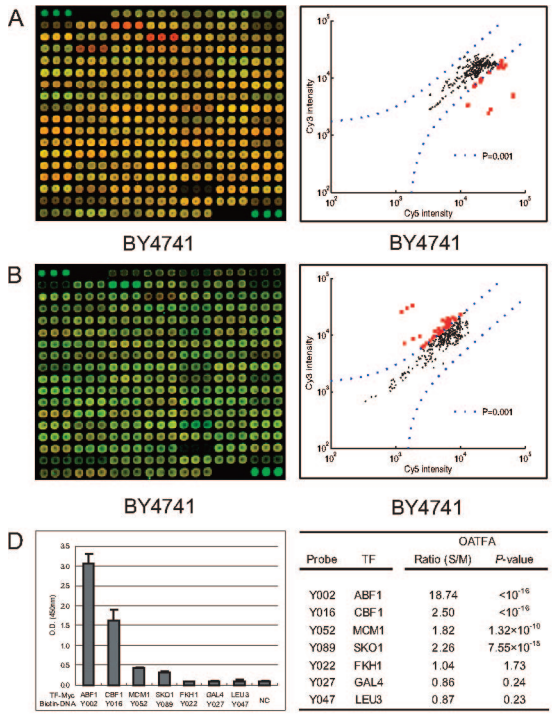
Figure 4. Whole yeast active TFs during the carbon source shift. (A) Yeast strain BY4741 was grown to exponential growth phase in SC + 2% glucose and was then shifted to SC + 2% glycerol + 2% ethanol for 5.5 h. The TF-capture targets enriched by exposure to extracts of BY4741 grown in 2% glucose were amplified and labeled with Cy3, while the TF-capture targets enriched in extracts of BY4741 grown in 2% glycerol + 2% ethanol were amplified and labeled with Cy5. (B) The array results were validated by reporter gene assays. (C) The array results were further validated by microwell colorimetric TF assays in which dsDNA molecules containing the Y086 sequence were immobilized in microtitre plates. (D) The *sip4*Δ mutant strain Y01334 was treated as described above in (A). The TF-capture targets enriched by exposure to extracts of Y01334 grown in 2% glucose were amplified and labeled with Cy3, while the TF-capture targets enriched in Y01334 grown in 2% glycerol + 2% ethanol were amplified and labeled with Cy5.

TFs Required for Yeast Cell Growth in SC Medium. Considering the large number of yeast TFs (>100) and the large number of potential metabolic states, a high-throughput genome-wide assay such as the OATFA can potentially provide a flexible platform for detailed cellular metabolic analysis. Information is largely unknown concerning the activity of individual TFs during particular culture conditions, and here we used growth in SC medium to investigate transcription factor requirements during active cell growth. Whole cell extracts (20 μg) from the yeast cells cultured in SC medium were analyzed according to the single sample assay model (Figure 5A and 5B). Some 25 positive probe signals (6 replicate experiments) were within this range, corresponding to the 25 different yeast TFs listed in Figure 5C.

We also explored the specificity of the OATFA platform with additional sublibrary experiments (Supplementary Figure S7). The sublibrary experiments were designed to present TF-capture targets in groups with minimal potential for cross-interaction with alternative TF capture targets for the same TFs and also with minimal competition with other potential alternative binding TFs. This design was also intended to examine whether a TF displayed strong interactions with several different capture targets and thus to assess the potential for cross interaction within the complex whole capture library. The activation ratios seen for TFs for both the whole TF-capture DNA library and the TF-capture sublibrary experiments are similar for a large majority of the factors (34 probes), except for five probes indicated in purple in which an additional four probes satisfy the 1.8 ratio in the sublibraries that are not satisfied in the whole library experiments, and one probe satisfying the 1.8 ratio in the whole library was below this value in the sublibrary tests.

Four positive probes seen during growth in SC medium whose ratios ranged from high to low (Y002–18.74, Y016–2.50, Y089–2.26, and Y052–1.82) and three randomly selected negative probes (Y022, Y027, and Y047) were chosen for microwell colorimetric TF assays (Figure 5D) to independently assay the OATFA data. The lack of commercially available antiyeast TF antibodies necessitated the insertion of the Myc-6 × His tag at the C-terminus of corresponding genomic TF coding genes to facilitate the microwell colorimetric TF assays. The extracts (150 μg) were analyzed using the microwell colorimetric TF assay, and the activities of each of the seven TF probes correlated well with their corresponding OATFA data (Figure 5D), further substantiating the OATFA array as a robust and reliable platform to detect metabolically activated TFs from among the total complement of cellular TFs. The signal of probe Y002 was the strongest (Ratio = 18.74 and *P*-value < 10^{-16}) suggesting the largest activation of its binding protein, yeast TF Abf1. Lee et al.[26] have shown in the ChIP-chip assays of all the yeast TFs that Abf1 bound the largest number (181) of promoter regions and that around 24% of yeast intergenic regions possessed the Abf1 consensus binding sequence (5′-TCR-n_6-ACG-3′). Out of the 93 TFs represented on our OATFA platform, Giaever et al.[27] identified only eight TFs (Abf1, Met4, Gcr1, Hsf1, Mcm1, Rap1, Reb1, and Tbf1) as critical to normal yeast physiological functions and whose null mutants are totally nonviable. Six of these eight TFs (except Hsf1 and Reb1) were activated positively in our OATFA analysis of growth in SC medium and were also activated in our OATFA analysis of growth in SC + 2% glycerol + 2% ethanol (Supplementary Table S4).

Twenty-five TFs identified as activated during growth in SC medium by our OATFA assays could be classified into five



C

Probe	TF	Ratio (S/M)	*P*-value
Y002	**ABF1**	18.74	$<10^{-16}$
Y008	ARG81	1.80	7.87×10^{-9}
Y009	ARR1	2.12	3.41×10^{-6}
Y014	CAD1/CIN5	1.86	1.34×10^{-8}
Y016	**CBF1**/MET28/MET4	2.50	$<10^{-16}$
Y031	GCN4	1.85	1.32×10^{-7}
Y034	GCR1/HAP2	1.99	4.39×10^{-12}
Y050	MCM1	1.90	3.59×10^{-12}
Y052	MCM1	1.82	1.32×10^{-10}
Y055	**MIG1**	2.10	1.24×10^{-12}
Y062	**NRG1**	2.81	$<10^{-16}$
Y067	PHO2	1.82	4.10×10^{-9}
Y068	PHO2	1.83	2.23×10^{-9}
Y070	**PHO4**	1.91	1.21×10^{-12}
Y071	**PHO4**	2.06	1.95×10^{-11}
Y073	RAP1	2.57	$<10^{-16}$
Y074	RAP1	1.96	1.91×10^{-12}
Y077	**RFX1**	2.52	$<10^{-16}$
Y086	**SIP4**	1.97	3.93×10^{-14}
Y089	**SKO1**	2.26	7.55×10^{-15}
Y090	SMP1	1.98	1.33×10^{-14}
Y095	SWI5	2.02	1.78×10^{-14}
Y098	**TBF1**	2.08	1.72×10^{-12}
Y100	TYE7	1.94	1.37×10^{-10}
Y102	UME6	2.03	1.85×10^{-13}

E

Metabolism	Environmental response	DNA/RNA/protein biosynthesis	Cell cycle	Developmental processes
ARG81	ARR1	ABF1	MCM1	UME6
CBF1	CAD1	RAP1	SWI5	
MET28	CIN5	TBF1		
MET4	RFX1			
GCN4	SKO1			
GCR1	SMP1			
HAP2				
MIG1				
NRG1				
PHO2				
PHO4				
SIP4				
TYE7				

Figure 5. Active TFs identified in yeast grown in SC medium. (A) The whole cell extract of BY4741 grown in SC was analyzed using OATFA according to the single sample assay model. The TF-capture targets enriched by exposure to extracts of BY4741 were amplified and labeled with Cy5, while the diluted whole TF-capture DNA library was amplified and labeled with Cy3 as a control. (B) Parallel fluorescent dye-swap experiment. (C) List of the statistically significant positive signals resulting from 6 replicate experiments. The TFs (bold) have been validated using purified *E. coli*-produced yeast TFs (Supplementary Figure S5). (D) Four positive TF signals and three negative TF signals were evaluated by independent microwell colorimetric TF assays. (E) List of active TFs classified into five categories, according to the primary functions of their target genes.

categories (Figure 5E) according to the functions of their target genes.[26] About half of the positive TFs (13/25) belonged to the "metabolism" category; six TFs (6/25) were related to "environmental response" activities; three TFs (3/25) were related to "DNA/RNA/protein biosynthesis" functions; two TFs (2/25) took part in "cell cycle" regulation; and the function of one TF related to regulation of "developmental processes". This illustrates the wide range of metabolic and regulatory pathways that may be activated during any particular cellular state that the OATFA platform can detect using the single sample analysis model.

Discussion

Presently, about 141 different TFs are reported to have DNA-binding activity or transcriptional regulatory activity in the yeast *Saccharomyces cerevisiae*.[28] Some 93 of those TFs with DNA-binding activity were selected for inclusion in the OATFA assay, but all TFs that lack DNA-binding were not assayed. Detailed information about 23 and 24 yeast TF binding matrices, respectively, from the TRANSFAC 7.0[29] and SCPD[30] databases provided a starting point, but most matrices in the two databases correspond to identical factors. Through additional literature searches, we collected an additional 74 TF binding sequences/matrices, giving a total of 109 TF binding sequences/matrices, which correspond to a total of some 93 different yeast TFs (Supplementary Table S1).

The quality of the consensus TF binding sequences/matrices is critical for the accuracy and efficiency of detection of each TF by the yeast OATFA assay platform. For example, the probes Y025, Y026, and Y027 were designed as targets for Gal4, while probes Y053 and Y054 were designed as targets for Met31 and Met32 according to the binding sequences identified by dif-

ferent authors (Supplementary Table S1). In the Gal4 assays (Figure 3A), Y025 was not effective which suggested that the motif 5′-CGG n_{11} CCG-3′ was not sufficiently specific for Gal4 binding. Both Y026 and Y027 probes had more sequence definition than Y025, and the binding of Gal4 to these two probes was positive. The signal of Y027 was better than that of Y026 which further suggests that the Gal4 matrix provided in this case by TRANSFAC was the best.

Blaiseau et al.[31,32] noted two similar core binding sequences for Met31 and Met32 (Supplementary Table S1) allowing design of probes Y053 and Y054, respectively. Although differing by only one nucleotide, their binding with the two TFs differed greatly. In the pET32a-MET32 assay, the enriched ratio of Y053 is 29.48, but Y054 is 3.42 (Figure 2B). Blaiseau et al.[31] used a longer 5′-AAA CTG TGG-3′ element of the MET28 promoter for EMSA assays, though they argued the core binding sequence was 5′-AAA CTG TG-3′. The core binding sequences for yeast Met31/32 given recently by Gonze et al.[33] strongly suggested the G at the 3′-end of the core binding sequence was very important in DNA-Met32 protein binding, which is confirmatory of our data and indicates the critical need for accurate matrix information for the design of the OATFA platform.

Ideally, a convincing evaluation of all the binding sequences and their individual consensus matrix is necessary. Yet, that information is not always available in databases or from the scientific literature, and it is not initially possible to ensure that every probe possesses the most specific core binding sequences. We have initiated the testing of the specificity of individual TF-capture targets by two methods. First, we introduced Myc-6 × His tags coding sequences into the genomic sequences encoding the C-terminus of TFs. Specific binding of the tagged-TF to any DNA-capture sequence could then be determined by microwell colorimetric TF assays against the Myc-6 × His tag. Second, we overexpressed selected TFs in either *E. coli* or yeast cells and demonstrated the high specificity of overexpressed-TF−DNA capture target binding. We plan to clone and express all the yeast TFs in the future to test the potential cross-reactivity between each of the yeast TFs with each of the TF-capture target designs. A further possibility would be to assay TF-capture targets using either the yeast proteome array[34] or the yeast TF array,[20] both of which can test the potential for cross-reactivity between each designed capture-target with all of the yeast TF proteins. We also intend to regularly update our OATFA platform by trialing any newly reported yeast TF-binding sequence information and maintain a database of current best consensus matrices for all yeast TFs.

The OATFA microarray assay described here is the first platform capable of simultaneously monitoring many known active yeast *Saccharomyces cerevisiae* (DNA-binding) TFs in a single multiplex experiment. It involves the use of nitrocellulose-filter binding to isolate DNA-binding proteins away from the free unbound TF-capture targets and subsequently to assay the presence of bound TF-capture targets by the DNA microarray. Compared with traditional TF assay techniques such as EMSA, standard nitrocellulose-filter binding, microwell colorimetric TF assays, and others, OATFA is the first high-throughput TF assay technology that is potentially capable of estimating changes in the levels of all active DNA-binding TFs in a cell. Yet, the assay could potentially be limited in several key ways, including (1) the availability of exclusive and specificity of TF-binding sequence information, (2) the potential for cross-reactivity of the multiple protein−DNA binding reactions, and (3) the generality of the binding buffer for all TFs. We have systematically attempted to address each of these questions. We used individual hybridization of TF-capture targets to microarrayed complement sequences to demonstrate unique hybridization, as well as individual melting analysis to demonstrate the close similarity of the hybridization parameters of all of our selected probes (data not shown). We confirmed the specificity of TF-capture targets using 28 purified TFs, and to examine any potential for cross-reactivity between TFs and several (unrelated) TF-capture targets, we created sublibraries of 10 TF-capture targets and repeated the single sample assays of activated TFs found during growth in SC medium for all 109 of the targets (Supplementary Figure S7). The overall findings of these experiments is that the TF-capture DNA library has reproducible and specific interactions with individual TFs, and there is little cross-reaction with nontarget TFs. Additional experiments with a sublibrary containing three binding sequences (plus seven other unrelated binding sequences) that showed interference during overexpression of TF Sip4 in *E. coli* (Supplementary Figure S5) did not occur at normal cellular levels of the TF (Supplementary Figure S8) and indeed showed close correlation to the TF activities observed with the other sublibrary experiments described above, as well as with the entire TF-capture DNA library. We also examined whether our TF−DNA binding buffer allowed equivalent binding of the whole range of TFs potentially found in a yeast cell. We chose to use HDB buffer[16] with our OATFA platform experiments because Kuras and colleagues had shown good EMSA results with a number of different yeast TFs. We also tested three other binding TF-binding buffers with different compositions[19–21] to reanalyze the active TFs found during growth in SC medium. Each of these binding buffers produced similar, relative activity-TF profiles (data not shown). Taken together, these experiments suggest that different binding buffers have little effect on the relative equivalent binding between yeast TFs and their capture DNAs, further suggesting that the TF activities observed with the OATFA platform using the entire TF-capture DNA library accurately reflect the TF activities of cells.

High-throughput mRNA profiling assays[1,2] are widely used for profiling the expression of new TFs in response to physiological change. The mRNA profiling arrays provide both direct and indirect TF information. The direct TF information concerns changes in the expression of TF coding genes; however, the major disadvantage of mRNA profiling technology is the molecular gap between mRNA expression and the functional protein. First, TF expression at the mRNA level does not always correlate well with TF expression at the protein level.[3–5] Second, since the functionality of a TF is usually directed by post-translational modifications,[35,36] such as phosphorylation, glycosylation, or acetylation, knowledge of the quantity of mRNA of the TF coding gene solely does not necessarily provide information on TF function, and it is questionable how far transcriptional assays are truly able to provide information on the activities of TFs that finally contribute to the transcription of target genes.

The indirect information is acquired by prediction of the potential regulatory TFs based on genome-wide mRNA profiling data. Using promoter analysis tools, Brown and colleagues identified the yeast TFs Msn2 and Msn4 as potential regulators during response to certain environmental changes.[37] There are around 100 TFs in the yeast *Saccharomyces cerevisiae*, and Young and colleagues[26] identified nearly all of their target genes using ChIP-chip technology.[18,38] It is possible to deduce the

potential regulatory TFs by combining the ChIP-chip data and mRNA expression data together.[39] These methods however are both indirect, and other independent direct validations are needed. Changes in genomic expression programs are complex, and different combinations of TFs can elicit different patterns of gene expression from a particular gene. Similarly, almost identical genomic expression profiles might be elicited by very different combinations of TFs under different circumstances, making prediction of the role or combination of TFs difficult to infer from indirect assay information. Although genomic expression program prediction is sometimes possible, the consequences may not be exclusive. Antibody array technology[40] can profile the expression of all TFs at the protein level. However, this technology is still in its infancy, and antiyeast TF antibodies, and more particularly antiactive yeast TF antibodies, are largely unavailable. Unlike some surface receptors and metabolism-related enzymes, most TFs are present at levels that are not readily detectable by current proteomic technologies.[41] OATFA technology involves a semiquantitative PCR-amplification of captured DNA-binding sequences, allowing the changes in the amount of these low abundance TFs to be measured, and thus provides a direct and high-throughput approach for examining active TFs at the protein level (via protein−DNA capture). In addition, OATFA is an inexpensive method for profiling TFs compared with other current technologies, as: (1) the TF-capture targets are label-free, and a dye-labeled universal primer is introduced during SPA; (2) the detection does not require specific antibodies for each TF; and (3) all TFs with a well characterized DNA binding consensus sequence can be simultaneously monitored in a single multiplex experiment.

Further development and improvements can be envisioned for application of the yeast OATFA platform. We used yeast whole cell extracts in this study but not the nuclear extracts. The robust yeast cell wall requires aggressive rupture methods to isolate nuclei and active nuclear extracts.[42] The unavailability of high quality yeast nuclear extracts restricted our exploration to some physiological phenomenon, such as the activation of some TFs by cytoplasm to nucleus localization.[24,43,44] It is probably advisable to also examine yeast nuclear extracts in light of the failure to detect activity changes in several known activated TFs from total cell extracts.

Like all technologies, the yeast OATFA system has limitations. First, similar to EMSA or nitrocellulose-filter binding assays, OATFA is an in vitro method to analyze the DNA-binding activity of proteins. The activity of a TF at a minimum includes DNA-binding activity and transcriptional activity. Active TFs detected by EMSA, nitrocellulose-filter binding, or our OATFA method do not measure the actual "active" TFs but are rather a measure of active TFs with DNA-binding activity. The detection of those TFs only with transcriptional activity changes are outside the scope of OATFA. Additional in vivo methods such as reporter gene assays are needed to examine transcriptionally active TFs that do not bind DNA targets.

Second, a small number of TFs may share the same or very similar consensus DNA-binding sequences. The OATFA assay, similar to both EMSA and nitrocellulose-filter binding assays, does not use antibodies, thus a TF "identified" by its TF-capture target binding may potentially represent a composite of several such factors. Similarly, the individual activated TF cannot be identified with certainty if a capture sequence has binding redundancy. We have shown that the overexpression of individual TFs and their assay by OATFA can help confirm the accuracy of the cognate TF-capture target binding of that TF and equally eliminate the contribution of that TF to the signal at any other TF-capture targets. Yet, it is often difficult to decide which TF is bound, and ideally further antibody-based tests should be performed to confirm the identity of each TF and validate the OATFA data. We suggest that researchers may combine several technologies together to gain the advantages of each: using OATFA as the first stage screening tool to rapidly and economically identify changes in the abundance of potential TF candidates, and later using antibody-based methods to identify and confirm the real TFs. Finally, in vivo reporter gene assays and more cell-based assays can be applied to explain the transcriptional activities of the TFs thought to be involved in the process.

There are two questions related to transcriptional regulation. The first is which genes are regulated by each specific TF. The second is which TFs are activated in the cell. Any methods able to fully answer both of these questions will clarify how genes are regulated at the transcription level. A more complete understanding of the activation of TFs, their gene binding sites, and their protein–protein interactions will lead to more comprehensive and quantitative mapping of the regulatory pathways within cells. The microarray-based assay of chromatin immunoprecipitation ("ChIP-chip"), also referred to as genome-wide location analysis,[18,38] is currently the most widely used method for identifying genomic TF-binding sites in vivo in a high-throughput manner. As the target genes regulated by many known yeast TFs are thought to have been identified,[26,45] the role of a multiple TF profiling technology such as OATFA that may help to provide the answers for the second question will become obvious. Once both ChIP-chip data and OATFA data are coanalyzed, a great amount of information will be available to elucidate the gene regulation network. A database of TF-related information to correlate gene expression data, ChIP-chip data, and OATFA data will be essential for the analysis of the gene regulatory network.

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Supporting Information Available: Detailed descriptions of the design of TF-capture sequences, β-galactosidase assays, microwell colorimetric TF assays, and single-array error model. TF binding core sequences for probe design (Table S1). TF-capture target sequences (Table S2). List of the primers used in the cloning of TFs (Table S3). Active TFs identified in yeast grown in SC + 2% glucose and SC + 2% glycerol + 2% ethanol (Table S4). Oligonucleotide design scheme (Figure S1). The array format (Figure S2). Self-to-self-assays (Figure S3). DNA-binding activity of recombinant Met32 protein by microwell colorimetric TF assays (Figure S4). OATFA assay of single purified TFs produced in *E. coli* (Figure S5). Assays of specific TFs overexpressed in yeast (Figure S6). Comparison between whole OATFA TF-capture DNA library and sublibraries of 10 TF-capture targets (Figure S7). The assays of sublibraries of 10 TF-capture targets during diauxic shift (Figure S8). This material is available free of charge via the Internet at http://pubs.acs.org.

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