Finding regulatory modules through large-scale gene-expression data analysis

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The use of gene microchips has enabled a rapid accumulation of gene-expression data. One of the major challenges of analyzing this data is the diversity, in both size and signal strength, of the various modules in the gene regulatory networks of organisms. Based on the Iterative Signature Algorithm [Bergmann, S., Ihmels, J. & Barkai, N. (2002) Phys. Rev. E 67, 031902], we present an algorithm—the Progressive Iterative Signature Algorithm (PISA)—that, by sequentially eliminating modules, allows unsupervised identification of both large and small regulatory modules. We applied PISA to a large set of yeast gene-expression data, and, using the Gene Ontology database as a reference, found that the algorithm is much better able to identify regulatory modules than methods based on high-throughput transcription-factor binding experiments or on comparative genomics.

I. INTRODUCTION

The introduction of DNA microarray technology has made it possible to acquire vast amounts of gene expression data, raising the issue of how best to extract information from this data. While basic clustering algorithms have been successful at finding genes that are coregulated for a small, specific set of experimental conditions, these algorithms are less effective when applied to large data sets due to well-recognized limitations. First, standard clustering algorithms assign each gene to a single cluster, while many genes in fact belong to multiple transcriptional regulons. Second, each transcriptional regulon may only be active in a few experiments, and the remaining experiments will only contribute to the noise.

A number of approaches have been proposed to overcome one or both of these problems. A particularly promising approach, the Signature Algorithm (SA) was introduced in 2002 by Ihmels et al. [11]. Based on input sets of related genes, SA identifies “transcription modules” (TMs), i.e. sets of coregulated genes along with the sets of conditions for which the genes are strongly coregulated. SA is well grounded in the biology of gene regulation. Typically, a single transcription factor regulates multiple genes; a TM naturally corresponds to a set of such genes and the conditions under which the transcription factor is active. The authors tested the algorithm on a large data set for the yeast Saccharomyces cerevisiae. By applying SA to various sets of genes that were known or believed to be related, they identified a large number of TMs.

Soon after, Bergmann et al. [12] published the Iterative Signature Algorithm (ISA), which uses the output of SA as the input for additional runs of SA until a fixed point is reached. By applying ISA to random input sets and varying the threshold coefficient \( t_G \) (see below), the authors found almost all the TMs that had been identified using SA, as well as a number of new modules. Many of these modules proved to be in excellent agreement with existing knowledge of yeast gene regulation.

While ISA can identify many transcriptional regulons from gene-expression data, the algorithm has significant limitations. The modules found depend strongly on the value of a threshold coefficient \( t_G \) used in the algorithm. To find all the relevant modules, a large range of threshold values must be considered, and for each threshold the algorithm may find thousands of fixed points, many of which are spurious. While the largest, strongest modules are easily identified, among the smaller, weaker modules it is a major challenge to identify the real transcriptional regulons. Weak modules can even be completely “absorbed” by stronger modules.

The performance limitations of ISA are related to a number of algorithmic limitations. The need for a large range of thresholds is partially due to the threshold definition, and the large number of fixed points is due to the large positive feedback in the algorithm. The main conceptual limitation of ISA, however, is that it only considers one transcription module at a time. The algorithm does not use knowledge of already identified modules to help it find new modules, and it may find a strong module hundreds of times before it finds a given weak module. An even worse case is shown in Fig. 1. When a strong and a weak module are coexpressed for a significant fraction of conditions, it may be impossible to find the weak module by itself—ISA will find only a single stable fixed point, dominated by the strong module.

A simple way to ensure that the same module is not found repeatedly is to directly subtract the module from the expression data. (this approach is used in [10]). A more robust approach is to require the condition vector, i.e. the weighted condition set, of each new transcription module to be orthogonal to the condition vectors of all previously found modules. In essence, this procedure corresponds to successively removing transcription factors to reveal smaller and weaker transcription mod-
modules. The successive removal of condition vectors is the central new feature in our approach, and it is illustrated schematically in Fig. 2. We call the modified algorithm the Progressive Iterative Signature Algorithm (PISA). Returning to the example in Figs. 1 and 2 one finds that PISA can easily identify both TMs: it first finds the strong module, removes its condition vector, and then the new fixed point found by PISA. Genes of module 1 (solid line) have been separated from genes of module 2 (dotted fill) and the background (solid fill).

Progressively eliminating transcription modules à la PISA can also improve the prospects for finding unrelated modules. The gene regulation from one module will contribute to the background noise for all unrelated modules. Therefore, eliminating large, strong modules can significantly improve the signal to noise ratio of the remaining modules.

II. METHODS

A. The Algorithms SA/ISA

We briefly review the algorithms SA and ISA. A transcription module $M$ can be specified by a condition vector (“experiment signature”) $m^C$ and a gene vector (“gene signature”) $m^G$, where nonzero entries in the vectors indicate conditions/genes that belong to the transcription module (TM).

Given an appropriately normalized$^1$ matrix $E$ of log-ratio gene expression data and an input set $G_t$ of genes, SA scores all the conditions in the data set according to how much each condition upregulates the genes in the input set (downregulation gives a negative score). The result is a condition-score vector $s^C$:

$$s^C = \frac{E^T m^G}{|m^G|},$$  (1)

where $E^T$ is the transpose of $E$ and

$$(m^G)_{g} = \begin{cases} 1 & g \in G_t \\ 0 & g \notin G_t \end{cases}$$  (2)

is the gene vector corresponding to the input set. The entries of $s^C$ that are above/below a threshold $\pm t_C$ constitute the condition vector $m^C$:

$$(m^C)_{c} \equiv (s^C)_{c} \cdot \Theta(|(s^C)_{c}| - t_C),$$  (3)

where $\Theta(x) = 1$ for $x \geq 0$ and $\Theta(x) = 0$ for $x < 0$.

Similarly, the gene-score vector $s^G$ measures how much each gene is upregulated by the conditions in $m^C$, using the entries of $m^G$ as weights:

$$s^G \equiv \frac{E \cdot m^C}{|m^C|}.$$  (4)

The entries of the gene-score vector $s^G$ that are more than $t_G$ standard deviations $\sigma_{sg}$ above the mean gene score in the vector $s^G$ constitute the gene vector $m^G$:

$$(m^G)_{g} \equiv (s^G)_{g} \cdot \Theta((s^G)_{g} - (s^G)_{g} - t_G \sigma_{sg}),$$  (5)

ISA uses $m^G$ as the input $m^G_0$ for the next iteration, i.e. the genes are now weighted according to their gene scores, until a fixed point is reached.

B. The Algorithm PISA

Orthogonalization. Within PISA, each condition-score vector $s^C$ is required to be orthogonal to the condition-score vectors of all previously found transcription

$^1$ SA actually uses two matrices with different normalizations [11].
modules (TMs). Therefore, whenever PISA finds a TM and its associated condition-score vector $s^C$, the component along $s^C$ of each gene is removed from the gene expression matrix (see Implementation of PISA below). This requirement of orthogonality in PISA conflicts with the condition-score threshold as used in ISA. If we make the condition-score vector orthogonal first and then apply the threshold, the vector will no longer be orthogonal, whereas if we apply the threshold first, orthogonalization will give nonzero weight to all conditions, eliminating the noise-filtering benefit of thresholding. We have chosen to eliminate the condition-score vector completely. In any event, condition that in ISA would fall below the threshold will have low weight and will give only a small contribution to the noise.

The gene-score threshold. In ISA, to find all modules, it is necessary to run the algorithm with many different threshold coefficients $t_G$. For low thresholds one finds a few very large modules (many genes), while for high thresholds one finds many small modules (few genes). Without prior knowledge of the module one is searching for, it is difficult to know what $t_G$ to use. Within PISA, we wish to find all the modules using a single threshold. This requires modifying the threshold definition. In ISA, the gene-score threshold is $t_G = \sigma^\text{ISA}$ where the standard deviation $\sigma^\text{ISA}$ is computed using the full distribution of gene scores, and includes contributions both from the background and from the module of interest (Fig. 3). For large, strong modules, the module contribution may be larger than the background contribution. As a result, $\sigma^\text{ISA}$ is module dependent and $t_G$ must be adjusted to prevent false-positives from the background.

We eliminate this problem in PISA by specifying the threshold relative to the background, which we estimate using the mean, $\langle x \rangle_{70\%}$, and the standard deviation, $\sigma_{70\%}$, of the gene scores within the shortest interval that contains at least 70% of all the gene scores. By excluding extreme gene scores in this way, we minimize the influence of the module on the means and standard deviations of gene scores (Fig. 3). As a test, we used $\sigma_{70\%}$ in place of $\sigma^\text{ISA}$ in ISA and found both very large and very small modules with a single value of $t_G$.

We need to be conservative when selecting the gene-score threshold because, if PISA misidentifies a module, elimination of its condition vector can lead to errors in other modules. Therefore, the number of genes included in modules due to noise should be very low. We have used a threshold of 7.0$\sigma_{70\%}$, which for a Gaussian distribution corresponds to about 3.9$\sigma$. The chance of including a gene due to noise is about $10^{-4}$ per gene, e.g. with the 6206 genes in the yeast data set, the average number of genes included by mistake in each module would be about 0.62. Using a high threshold means that we may miss genes that should belong to a module, however this is less risky than including genes by mistake. As PISA proceeds by eliminating condition-score vectors, it does not matter whether we identify all the genes in a module, as long as the condition-score vector is accurate. Once, PISA has finished, we can easily see which genes would be included when using various gene-score thresholds for the same condition-score vector.

ISA only considers sets of genes that have high gene scores, i.e. positive signs. As discussed in [11], this can lead to two modules that are regulated by the same conditions but with opposite sign. In contrast, PISA includes all genes with sufficiently extreme scores in a single module, and the relative signs of gene scores specify whether the genes are coregulated or counter-regulated.

Implementation of PISA. To begin, PISA requires a matrix $E$ of log-ratio gene-expression data, with zero average for each condition. Two matrices are obtained from $E$: The first $E_C$ is normalized for each gene

$$
\langle (E_{Cg})_c \rangle_g = 0, \quad \langle (E_{Cg})^2 \rangle_g = 1 \quad \forall g \in G.
$$

Normalizing of $E_C$ is essential so that the gene-score threshold can be applied to all genes on an equal footing. The second matrix $E_C$ is obtained from $E_C$ by normalizing for each condition, $\langle (E_{Cg,0})_c \rangle_g = 1$, where $E_{Cg,0}$ denotes the initial $E_C$. (Note that this is essentially the opposite of the notation used in [11].) We then apply a modified version of ISA, mISA (see below), a large number of times (typically 10,000), and whenever mISA finds a module, we remove from $E_C$ the components along the module’s condition score vector $s^C$:

$$
E_C^{\text{new}} = E_C - E_C s^C (s^C)^T / |s^C|^2
$$

(6)
As mISA is repeatedly applied, new modules are found less and less frequently. For example, one run of 10,000 applications of mISA found 496 modules, and 287 of them were found in the first 1,000 applications. As the later modules are also generally smaller and less reliable, the exact number of times mISA is applied is not very important.

mISA. As input, the modified Iterative Signature Algorithm (mISA) requires the two matrices $E_G$ and $E_C$. We start each application of mISA by generating a random set of genes $G_0$ and a corresponding gene vector $m_i^G$:

$$(m_i^G)_g = \begin{cases} 1 & g \in G_0 \\ 0 & g \notin G_0 \end{cases}$$

Each iteration $i$ within mISA consists of multiplying the transpose of $E_C$ by the gene vector $m_i^G$ to produce the condition-score vector $s_i^C$:

$$s_i^C \equiv E_C^T m_i^G,$$

and then multiplying $E_G$ by the normalized condition-score vector to produce the gene-score vector $s_i^G$:

$$s_i^G \equiv \frac{E_G s_i^C}{|s_i^C|}.$$

From $s_i^G$, one calculates the gene vector $m_{i+1}^G$ for the next iteration:

$$(m_{i+1}^G)_g = (s_i^G)_g \theta(|(s_i^G)_g - \langle (s_i^G)_g \rangle_{70\%}| - t_G s_{i+1}^G(70\%)).$$

We iterate until: (a) $(m_i^G)_g$ and $(m_{i+1}^G)_g$ have the same sign (0, + or -) for all $g$, (b) the iteration number is $i = 20$, or (c) fewer than two genes have nonzero weight. If fewer than five genes have nonzero weight (for (a) or (b)), the result is discarded, otherwise we have found a module with condition-score vector $s_i^C = s_i^G$, gene-score vector $s_i^G = s_i^G$, and gene vector $m_{i+1}^G = m_i^G$.

We chose a threshold coefficient $t_G = 7.0$ so that the expected number of genes included in each module due to background noise would be less than one. However, with this high threshold, starting from a random set of genes there was only a very low chance that two or more genes would score above the threshold in the first iteration. To increase the chance of finding a module, we used a different formula for $m_i^G$. Instead of selecting only genes with scores above the threshold, we kept a random number $2 \leq n \leq 51$ of the genes with the most extreme scores. This procedure was generally adequate to produce a correlated set of genes for the next iteration.

Consistent modules. ISA typically finds many different fixed points corresponding to the same module, each differing by a few genes. PISA only finds each module once during a run, but the precise genes in the module depend on the random input set of genes and also on which modules were already found and eliminated. Furthermore, PISA sometimes finds a module by itself, while other times it may find the module joined with another module, or PISA may find only part of a module, or not find the module at all. To get a reliable set of modules, it was necessary to perform a number of runs of PISA and identify the modules that were consistent from run to run. To identify consistent modules, we first tabulated preliminary modules – transcription modules found by individual runs of PISA. A preliminary module contributes to a consistent module if the preliminary module contains more than half the genes in the full module, regardless of gene-score sign, and these genes constitute at least 20% of the genes in the preliminary module. A gene is included in the consistent module if the gene occurs in more than 50% of the contributing preliminary modules, always with the same gene-score sign.

Correlations between condition-score vectors. Once we identified a consistent module, $m_i^C$, we calculated the raw condition-score vector $r = E_C^T m_i^G$, using the initial value of the gene-expression-data matrix $E_C$. From the $r$’s we evaluated the condition correlations $r \cdot r'/(|r| |r'|)$ between different modules.

Additional details are discussed in the supporting material.

C. p-Values

Given a set containing $m$ genes out of the total of $N_G$, the $p$-value for having at least $n$ genes in common with a Gene Ontology (GO) category containing $c$ of the $N_G$ genes is

$$p = \sum_{i=n}^{\min\{c,m\}} \frac{\binom{N_G-c}{m-i} \binom{c}{i}}{\binom{N_G}{m}},$$

We ignore any genes that are not present in our expression data when counting $c$.

III. RESULTS

We applied PISA to the yeast data set used in [12], which consists of log-ratio gene-expression data for $N_G = 6206$ genes and $N_C = 1011$ experimental conditions (approximately 10% of the values are missing or invalid). Normalization gives the matrices $E_G$ and $E_C$ (see Methods for details).
As a preliminary test, we repeatedly applied PISA to one fully scrambled version of the matrix $E_G$ (and the corresponding $E_C$). From run to run, the algorithm identified many large modules derived almost entirely from a single condition, as expected in light of the broad distribution of the raw gene-expression data (Fig. S1 in supporting material). PISA also found many small modules, but these differed from one run to the next. We were able to eliminate both these classes of false positives using filters for consistency, recurrence, and number of contributing conditions (Fig. S2 in supporting material).

We performed 30 runs of PISA on the yeast data set and identified the modules that appeared consistently, using the filters derived above. At the start of each run, only a few modules could be found with our single choice of gene threshold $t_G$. Nevertheless, PISA did consistently find new modules after eliminating others, demonstrating that removing the condition vectors of found modules improves the signal to noise for the remaining ones.

For most of the modules we found, the genes were coregulated, i.e. all the gene scores had the same sign. (In contrast, the modules that were eliminated by the filters often had about equal numbers of genes of either sign.) There were, however, a significant number of modules with a few gene scores differing in sign from the rest, and a few modules with many gene scores of both signs, e.g. the $\alpha/\alpha$ pheromone production/detection module. Furthermore, many of the modules found by PISA agreed closely with modules identified by ISA at various thresholds, while other PISA modules were subsets of ISA modules. Some PISA modules, for example the de novo purine synthesis module (Fig. 4), were significantly more complete than the ones found by ISA (at any threshold).

PISA found several small modules that agree very well with known gene regulation in yeast. For example, the arginine-biosynthesis module consists of ARG1, ARG3, ARG5,6, ARG8, CPA1, CTF13, and CAR2; out of these CAR2 has a negative gene score, i.e. it is counter-regulated relative to the others. The first five genes are precisely the arginine-synthesis genes known to be repressed by arginine, while CAR2 and CAR1 (which is the 2nd highest scoring gene that failed to make the threshold) are catabolic genes known to be induced by arginine \cite{18}.

PISA also found a zinc (zap1 regulated) module consisting of ZRT1, ZRT2, ZRT3, ZAP1, YOL154, INO1, ADH4, and YNL254C. These are almost exactly the highest scoring genes in a microarray experiment comparing expression under zinc starvation of a zap1 mutant vs. wild type \cite{15}; however, our data set does not include this or any other zinc starvation (or zap1 mutant) experiment—indeed, there are no experimental conditions that have a remarkably high score for this module, although conditions from the Rosetta compendium \cite{16}, most of which are deletion mutant experiments, tend to have much higher scores than the other conditions (see supporting material). This module, as well as the star-

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**Module:** De novo purine biosynthesis

**Number of genes:** 32

**Average number of contributing conditions:** 14.6

**Consistency:** 0.83

**Best ISA overlap:** 0.59 at threshold 5.0, frequency 16

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**FIG. 4:** The de novo purine synthesis module found with PISA.

**FIG. 5:** Correlations between modules identified by PISA. The modules are ordered to form clusters: In the lower left corner are the main amino acid biosynthesis module and several smaller, more specific biosynthesis modules; the other main clusters are, roughly, stress response, mating, and ribosomal proteins/rRNA processing.
Database B have a clear disadvantage: their binding sites are assigned to intergenic regions, and if the two genes bordering an intergenic region are divergently transcribed, then the databases do not identify which of the genes is regulated. In many cases, we found that by comparing sets of genes in database A to PISA modules, we could decide which of divergently transcribed genes were actually regulated. For example, Database A lists 6 intergenic regions as binding site for zap1 at an internal p-value threshold of $10^{-5}$, and 4 of these lie between divergently transcribed genes. However, 5 of the 6 intergenic regions border the genes ZRT1, ZRT2, ZRT3, ZAP1, and YNL254C which PISA identifies as part of the zinc module.

Database A appears to have an additional source of false positives. Intergenic regions that are close to intergenic regions with very low p-values often have low p-values themselves, even when there is no apparent connection between the genes and no evidence of a binding site in the DNA sequence. For example, for the de novo purine-biosynthesis module, which is primarily regulated by the bas1 transcription factor, the intergenic region controlling GCV2 has the lowest p-value within Database A, $1.1 \cdot 10^{-16}$, and all the four closest intergenic regions have p-values below $10^{-5}$. Comparison to PISA modules can help eliminate these potential false positives: out of the 29 genes assigned a p-value below $10^{-4}$ for bas1 binding in database A, 13 belong to a single PISA module, 4 others are divergently transcribed adjacent genes, and 6 others are genes transcribed from nearby intergenic regions.

### IV. DISCUSSION

The Progressive Iterative Signature Algorithm (PISA) embodies a new approach to analysis of large gene-expression data sets. The central new feature in PISA is the robust elimination of transcription modules as they are found, by removing their condition-score vectors. Also new to PISA, compared to its precursors SA \cite{11} and ISA \cite{12}, is the inclusion of both coregulated and counter-regulated genes in a single module, and the use of a single gene-score threshold.

Altogether, these new features result in an algorithm that can reliably identify both large and small regulatory modules, without supervision. We confirmed the performance of PISA by comparison to the Gene Ontology (GO) database – PISA performed considerably better against GO than either high-throughput binding experiments or comparative genomics. PISA therefore provides a practical means to identify new regulatory modules and to add new genes to known modules.

Can PISA shed any light on the organization of gene expression beyond the level of individual transcription modules? In \cite{12}, the authors argued that they could trace the relationship between modules from the effects of changing the threshold $t_G$. For instance, a large
module might split into two smaller ones as $t_c$ was increased. With PISA, we were able to use a more direct approach. Once we identified the modules, we computed the “raw” (i.e., pre-eliminations) condition-score vector $r$ for each module, and from these raw condition-score vectors, we evaluated the condition correlations between modules (see Methods). Figure 4 shows the condition correlations between 40 of the modules that we can put a name to. A large, positive correlation between two modules can either indicate that the modules have many genes in common, e.g., the genes of the arginine-biosynthesis module are essentially a subset of the genes of the amino-acid-biosynthesis module, or, as in the toy model in Figs. 1 and 2, the modules have few/no genes in common, but the two sets of genes are similarly regulated under many conditions. In the toy model, the raw condition-score vectors $r_1$ and $r_2$ correspond to the vectors in Fig. 1(a) and their correlation, $r_1 \cdot r_2 / (|r_1||r_2|)$, is simply the cosine of the angle between them. A real example of this second type of correlation is provided by the ribosomal-protein module (104 genes) and the rRNA-processing module (144 genes). They have no genes in common, but the correlation between them is very high, 0.76.

Out of the 6206 genes included in the expression data, 2626 genes appeared in at least one module, and 923 genes appeared in more than one module. No genes appeared in more than 4 different modules.

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. SUPPORTING MATERIAL

A. Normalization

Here we review in detail the normalization procedure employed in PISA. The most obvious requirement for the normalization is that scores for different genes must be comparable. The procedure itself is as follows: Given a matrix $E$ of log-ratio gene-expression data, we first set the average to zero for each condition, $$(E')_{gc} = (E)_{gc} - \langle (E)_{g'c'} \rangle_{g'} ,$$ and then normalize to zero mean and unit variance for each gene, giving $E_G$, which is used in PISA to calculate gene scores:

$$(E'')_{gc} = (E')_{gc} - \langle (E')_{g'c'} \rangle_{c'} ,$$

$$(E)_{gc} = (E'')_{gc} / \sqrt{\langle (E'')^2_{g'c'} \rangle_{c'}} .$$

For this normalization to be consistent through the iterations in mISA, the different condition scores must also be comparable. To get the initial value $E_{G,0}$ of the matrix used to calculate condition scores, we divide $E_G$ by the rms value for each condition:

$$(E_{G,0})_{gc} = (E_G)_{gc} / \sqrt{\langle (E_G)^2_{g'c'} \rangle_{g'}} .$$

Note that a simple approach would be to normalize for both genes and conditions simultaneously and thus use only a single set of data—this could be easily accomplished by alternately normalizing over conditions and genes a few times; the data converge quickly. There is, however, a risk of losing significant features of the data through excessive normalization. For some conditions, the typical change in expression levels may be very large, while for others it may be negligible, and it would be misleading to always normalize these to the same level; at the very least, this would give a lower signal to noise ratio. Therefore, we have chosen to normalize $E_G$ over genes but not conditions, allowing conditions with large changes in expression level to make a proportionately larger contribution to gene scores. For genes, however, it is reasonable to always normalize to the same level. If two genes are in the same module, then there is little reason to consider the gene with the larger dynamical range to be more reliable than the other. That is why we use $E_G$ to calculate $E_{C,0}$.

Also note a the difference between genes and conditions: The variance for a gene often depends on a small number of outlying values, and normalizing over genes prevents these from dominating. In contrast, the variance for a condition typically depends on many genes, and as such is a far more reliable quantity.

If $E_G = E_{C,0}$ initially, then it is equivalent to keep $E_G$ constant or use $E_G = E_C$, which is updated every time PISA finds a module.

B. Avoiding Positive Feedback

The basic principle of SA, or an iteration of ISA/mISA, is to find the set of genes whose expression profiles most resemble those of the genes in the input set, either for all conditions (mISA) or for a selected subset of conditions (SA/ISA). Of course, the gene whose expression profile most resembles that of a given gene is the gene itself, thus there is a potential for significant positive feedback. Adding one gene to the input set would typically increase the score of that gene far more than the score of any other gene. As a consequence of positive feedback, adding one gene to the gene vector of a fixed point would have a considerable chance of yielding another fixed point, and a small set of genes could be a fixed point even if the genes were completely uncorrelated.

In PISA, we only find each module (or combination) once for each run, and it is important to be as certain as possible that we have the correct genes. We avoid positive feedback by using leave-one-out scoring for genes that had nonzero weight at the start of the iteration, i.e. we remove the contribution from gene $g$ from the condition scores $s^C_i$ before we use these scores to calculate the new score for gene $g$:

$$(s^C_i)_g = \frac{(E_G)_{g,1} (s^C_i) - (E_G)_{g,0} (m^G_i)_g}{| s^C_i - (E_G)^T (m^G_i)_g |} ,$$

where $(A)_j$ is row $j$ of matrix $A$, and $(A)_{i,j}$ is column $j$ of matrix $A$. With a Gaussian distribution of the background noise, this approach is very close to neutral, i.e. adding a gene will neither affect that gene’s score, nor will it significantly change $\sigma^{70\%}$ of the gene-score distribution.

Without positive feedback, fixed points may be marginally stable (or even unstable, i.e. a limit cycle), thus we do not require a true fixed point; we accept any gene vector reached after 20 iterations in mISA, as long as it contains at least 5 genes.

In SA/ISA, the authors do not eliminate positive feedback. Indeed it would be difficult to do so, as adding/removing a gene can change which conditions have scores exceeding the condition threshold. Apart from this complication, the feedback in SA/ISA is proportional to the number of conditions that make the threshold. For small modules, typically only a small fraction of the conditions have scores above the threshold, thus the feedback is lower than it would have been for PISA, which includes all conditions. For large modules, the feedback is only a minor effect in the first place. Nevertheless, the total number of fixed points for ISA is huge due to positive feedback—at a gene threshold coefficient $\tau_G = 4.0$, there are, at a minimum, more than a million fixed points.
C. Filters

We chose the gene-score threshold as $7.0\sigma^{70\%}$ so that, on average, less than one gene would be included in a module purely due to background noise. This estimate assumed that the background noise had a Gaussian distribution. For most modules, the gene scores are the sums of contributions from many different conditions, and if these contributions are independent, as they should be for background noise, then the total background noise will have approximately a Gaussian distribution, regardless of the distribution for a single condition (central limit theorem). For modules that derive almost entirely from one or very few conditions, however, the distribution of gene scores may not be Gaussian.

While we do not know the true distribution of the background noise, it is reasonable to use the full distribution of the data as a worst case scenario. As shown in Fig. S1 this distribution is far from Gaussian: it has a fairly sharp cusp at zero and long tails, both before and after normalization (Eqs. S1–S3).

We applied PISA to a matrix $E_{G}$ that had been fully scrambled after normalization. As shown in Fig. S2, PISA found many large modules that were based almost entirely on a single condition (however, as the modules were not based on only one condition, they were not as large as our estimate of 200, above), whereas modules based on many conditions were much smaller. We also applied PISA to a random matrix generated from a Gaussian distribution, and in that case PISA did not find any large modules (in 30 runs, PISA found 8 modules with 20 or more genes; the largest contained 26 genes). In both cases, the small modules found by PISA varied from run to run.

In order to eliminate these false modules we introduced a set of filters. For each preliminary module $M$ we calculate the “number of contributing conditions”, given as $n_{C}^{M} = \sum_{c} (s_{C})^{2} / (\max\{ (s_{C})^{2} \})^{2}$. We ignored any module for which the median of the numbers of contributing conditions for its preliminary modules was below 6 (this threshold worked well; it is somewhat above the threshold required to remove the false positives for the scrambled matrix). We also ignored all modules that had fewer than 5 genes or fewer than 5 contributing preliminary modules, and for modules with fewer than 10 genes we required that the “consistency”, defined as the average fraction of the genes in the preliminary modules that are in the full module, was above 0.55 (during post processing, we required that this fraction was above 0.2 for each preliminary module). These filters removed all but one of the modules found by PISA when applied to the scrambled matrix.

FIG. S1: Distributions of the yeast microarray data used (6206 genes/ORFs, 1011 conditions). Roughly 10% of the data was invalid/missing (not included in the distributions). The distribution is sharply cusped and has long tails, both before and after normalization (Eqs. S1, S3).

We applied PISA to a scrambled expression matrix (black) only yielded modules close to the axes (small $n_{G}^{M}$ or small $n_{C}^{M}$), while PISA run on the real data (green) yielded modules with both large $n_{G}^{M}$ and large $n_{C}^{M}$.

FIG. S2: The number of genes $n_{G}^{M}$ in a module $M$ and the number of contributing conditions $n_{C}^{M}$ (see text) were two of the properties we used in our filters to eliminate false modules. PISA applied to a scrambled expression matrix (black) only yielded modules close to the axes (small $n_{G}^{M}$ or small $n_{C}^{M}$), while PISA run on the real data (green) yielded modules with both large $n_{G}^{M}$ and large $n_{C}^{M}$. 
| Function                                      | # genes | # cond. | Cons. | Over. w/ISA | Best $t_G$ | Freq. |
|-----------------------------------------------|---------|---------|-------|-------------|------------|-------|
| Amino acid biosynthesis                       | 96      | 31.2    | 0.83  | 0.89        | 3.7        | 10000 |
| Arginine biosynthesis                         | 6       | 5.7     | 0.72  | 0.83        | 6.0        | 60    |
| Biotin synthesis & transport                  | 6       | 6.5     | 0.80  | 0.67        | 5.5        | 7     |
| Lysine biosynthesis                           | 11      | 9.0     | 0.82  | 0.82        | 4.6        | 10    |
| De novo purine biosynthesis                   | 32      | 13.1    | 0.83  | 0.59        | 5.0        | 16    |
| Oxidative stress response                    | 69      | 23.8    | 0.91  | 0.32        | 3.4        | (1)   |
| Aryl alcohol dehydrogenases                   | 6       | 15.4    | 0.62  | 0.83        | 4.9        | 8     |
| Proteolysis                                   | 27      | 82.1    | 0.80  | 0.86        | 3.6        | 1661  |
| Trichalose & hexose metabolism/conversion    | 21      | 34.9    | 0.55  | 0.67        | 3.2        | 910   |
| COS genes                                     | 11      | 9.2     | 0.49  | 1.00        | 3.3        | 756   |
| Heat shock                                    | 52      | 42.8    | 0.78  | 0.38        | 3.2        | (1)   |
| Repair of disulphide bonds                    | 26      | 41.6    | 0.73  | 0.58        | 3.5        | 15    |
| Calcium-calmodulin related                   | 41      | 32.5    | 0.78  | 0.73        | 3.0        | 2198  |
| Oxidative phosphorylation                     | 42      | 48.3    | 0.89  | 0.95        | 3.7        | 2600  |
| Gluconeogenesis, fatty acid beta-oxidation    | 38      | 18.2    | 0.81  | 0.63        | 2.9        | 264   |
| Mitochondrial ribosomal genes                 | 52      | 57.6    | 0.79  | 0.89        | 3.3        | 2291  |
| Transcription (RNA polymerase etc.)++         | 22      | 70.4    | 0.59  | 0.52        | 3.2        | 1     |
| SubtelomERICally-encoded proteins             | 36      | 48.2    | 0.94  | 1.00        | 3.9        | 6174  |
| Iron/copper uptake                            | 38      | 10.8    | 0.82  | 0.79        | 3.7        | 1704  |
| Coated vesicles/secretion                     | 25      | 47.6    | 0.61  | 0.64        | 3.4        | 4     |
| Phosphoglycerides biosynthesis                | 33      | 36.1    | 0.86  | 0.61        | 2.9        | 27    |
| Hexose transporters                           | 10      | 33.9    | 0.74  | 0.60        | 3.8        | 41    |
| Galactose utilization                         | 23      | 17.4    | 0.84  | 0.74        | 3.2        | 686   |
| Mid sporulation                               | 97      | 11.7    | 0.90  | 0.70        | 2.7        | 6556  |
| Mating factors/receptors: a/α difference       | 26      | 15.8    | 0.57  | 0.58        | 3.8        | 6     |
| Mating                                        | 110     | 31.1    | 0.89  | 0.75        | 2.7        | 24622 |
| Mating type a signaling genes                 | 6       | 18.6    | 0.26  | 0.83        | 5.5        | 22    |
| Mating genes for mating type a                | 15      | 13.6    | 0.41  | 0.53        | 8.0        | 16    |
| Phosphate utilization                         | 27      | 24.4    | 0.89  | 0.81        | 3.3        | 5796  |
| Glycolysis                                    | 19      | 26.9    | 0.54  | 0.89        | 3.7        | 91    |
| Ergosterol biosynthesis                        | 36      | 28.3    | 0.89  | 0.69        | 3.1        | 57    |
| Cell cycle G1/S                               | 66      | 39.1    | 0.80  | 0.81        | 3.7        | 4382  |
| Cell wall (bud emergence)                     | 17      | 42.7    | 0.76  | 0.94        | 4.0        | 63    |
| Cell cycle M/G1                               | 35      | 31.4    | 0.82  | 0.89        | 3.9        | 952   |
| Cell cycle G2/M                               | 31      | 25.0    | 0.82  | 0.90        | 3.7        | 1258  |
| Uracil synthesis/permeases                    | 8       | 11.4    | 0.75  | 0.88        | 3.5        | 19    |
| Fatty acid synthesis++                        | 22      | 49.4    | 0.86  | 0.50        | 3.1        | 2     |
| Histones                                      | 19      | 34.6    | 0.67  | 0.53        | 3.4        | 2972  |
| Ribosomal proteins                            | 126     | 49.2    | 0.91  | 0.87        | 3.0        | 18661 |
| rRNA processing                               | 117     | 46.0    | 0.85  | 0.64        | 2.7        | 13355 |

**TABLE I**: 40 of the modules found by PISA that we could assign a name to. For each module we list the number of genes in the module, the number of conditions that had a significant contribution to the module, how consistent the module was from each run to the next, the maximal overlap with a module found by ISA (using 200,000 seeds at each threshold from 1.8 to 15.0), the threshold value $t_G$ at which that overlap was found, and how many times such an ISA module was found.
Module: Galactose induced genes

Number of genes: 23
Average number of contributing conditions: 18.1
Consistency: 0.84
Best ISA overlap: 0.74 at threshold 3.2, frequency 686

- **GAL10**
- **GAL7**
- **GAL1**
- **GAL3**
- **GAL2**
- **YPL066W**
- **YOR121C**
- **GAL80**
- **PCL10**
- **GCY1**
- **MLF3**
- **YDR010C**
- **YLR201C**
- **FUR4**
- **MUP3**
- **MRPL24**
- **OPT2**
- **YEL057C**
- **HXT1**
- **HXT2**
- **HXT4**
- **HSL1**
- **HXT5**

0  Unknown
1  Galactose induced genes
2  Hexose transporters (downregulated)
3  Other, downregulated
4  Other

**Raw condition scores**

FIG. S3: The galactose induced module found with PISA.  
This module turns on GAL genes and also, as a weaker effect, represses a number of hexose transporters.
Module: Hexose transporters

Number of genes: 10  
Average number of contributing conditions: 33.7  
Consistency: 0.74  
Best ISA overlap: 0.6 at threshold 3.8, frequency 41

Glucose transporter
Galactose/glucose transporter
Glucose suppression regulator
Similar to glucose suppression regulator

FIG. S4: The hexose transporter module found with PISA. In this module (which is consistently found after the galactose induced module), the hexose transporter genes are co-regulated with GAL2, the galactose permease, whereas they were counter-regulated in the galactose induced module.
Module: Peroxide shock

Number of genes: 69
Average number of contributing conditions: 23.9
Consistency: 0.91
Best ISA overlap: 0.34 at threshold 3.4, frequency (1)

FIG. S5: The oxidative stress response module found with PISA. This module is significantly more complete than the modules of comparable size found by ISA.
Module: Zinc regulated genes

Number of genes: 8
Average number of contributing conditions: 29.0683
Consistency: 0.638515
Best ISA overlap: 0.88 at threshold 4.6, frequency 2

FIG. S6: The zinc module found with PISA. This module has a high overlap with the group of genes bound by ZAP1 in database A (at p-value 0.001): The ZRT1, ZRT2, ZRT3, ZAP1 and YNL254C genes make up 5 of the 6 lowest p-values (counting each pair of divergently transcribed genes only once), and the remaining hits from database A (most with p-values above $10^{-4}$) are likely to be mostly false positives. Based on this, it seems very likely than YNL254C, if functional, is regulated by and related to zinc. (ADH4 has also been shown to be zinc-regulated elsewhere.)
Module: Arginine regulation

Number of genes: 7
Average number of contributing conditions: 14.0667
Consistency: 0.548283
Best ISA overlap: 0.71 at threshold 6.0, frequency 60

ARG8 ARG3 ARG1 CPA1 CTF13 ARG5.6
CAR2

Arginine biosynthesis
Arginine degradation, downregulated
Other

Raw condition scores

FIG. S7: The arginine regulated module found with PISA. The module agrees very well with what is known about regulation of arginine metabolism [F. Mesenguy and E. Dubois (2000) Food tech. bio. 38, 277-285]: ARG1, ARG3, ARG5.6 and ARG8 are repressed by arginine through the Arg80/Arg81/Mcm1 complex, while CAR2 (and CAR1, which is the 2nd highest scoring gene that failed to make the module) is activated by the same complex. We also find CPA1, which is claimed to be regulated by arginine at the translational level—the mRNA is destabilized by a small peptide in the presence of arginine. However, database A indicates that ARG1, ARG3, ARG5.6, ARG8 and CPA1 are all bound by the Arg80/Arg81/Mcm1 complex.