Combining optimization and machine learning techniques for genome-wide prediction of human cell cycle-regulated genes

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\begin{abstract}
\textbf{Motivation:} The identification of cell cycle-regulated genes through the cyclicity of messenger RNAs in genome-wide studies is a difficult task due to the presence of internal and external noise in microarray data. Moreover, the analysis is also complicated by the loss of synchrony occurring in cell cycle experiments, which often results in additional background noise.

\textbf{Results:} To overcome these problems, here we propose the LEON (L\textit{earning} and Optimizatio\textit{N}) algorithm, able to characterize the ‘cyclicity degree’ of a gene expression time profile using a two-step cascade procedure. The first step identifies a potentially cyclic behavior by means of a Support Vector Machine trained with a reliable set of positive and negative examples. The second step selects those genes having peak timing consistency along two cell cycles by means of a non-linear optimization technique using radial basis functions. To prove the effectiveness of our combined approach, we use recently published human fibroblasts cell cycle data and, performing \textit{in vivo} experiments, we demonstrate that our computational strategy is able not only to confirm well-known cell cycle-regulated genes, but also to predict not yet identified ones.

\textbf{Availability and implementation:} All scripts for implementation can be obtained on request.

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\end{abstract}

1 \textbf{INTRODUCTION}

Cell cycle progression is achieved by a highly regulated temporal program of gene expression where transcription is coordinated in a series of consecutive interdependent waves. Such ‘just-in-time’ strategy is suggested by the experimental observation that genes are expressed at peak levels at the time they are needed (Breeden \textit{et al.}, 2003). To study genes regulated in a cell cycle-dependent manner, researchers have deeply used messenger RNA expression profiling microarrays of synchronously growing cells progressing through the cell cycle. Nevertheless, the genome-wide identification of cycling genes is a difficult task for a number of reasons, including cell synchronization loss and intrinsic microarray noise. The most critical experimental issue is the degree of synchronization as well as its loss during a time course sampling. This is the reason why various computational methods have been proposed to correct such experimental artifact. The key problem is that the loss of synchronization results in a ‘flattening’ of the timing due to the presence of cells in different stages of the cell cycle. To overcome this problem, Bar-Joseph \textit{et al}. (2008) have recently developed a combined experimental and computational approach to recover ‘true’ cell cycle expression profiles. A number of different techniques have been proposed in the literature for the identification of cycling genes in many organisms, all of them relying on a cyclicity scores based on the degree of regulation (magnitude) and/or on a shape parameter (Fourier analysis). Unfortunately, there is a remarkably poor overlap between the gene sets identified as cyclic in different experiments even for the same organism (Zhao \textit{et al.}, 2001). Therefore, none of them can be considered as the ‘best method’ and this field is still an open area of research.

Here, we provide a new tool to identify cycling transcripts by integrating different features of gene expression time profiles. In fact, the LEON (L\textit{earning} and Optimizatio\textit{N}) algorithm combines, in two separate steps, information contained in the amplitude of the response (learning step) and information contained in the shape of the response by considering the consistency of peak timing between minima and maxima along two cell cycles (optimization step). In particular, this last feature is suitable to overcome the loss of synchronization problem, as peak timing of upregulation or downregulation is not significantly affected by such artifact. In other words, the shape of the expression profile of the population of cells changes as a consequence of different cell progress rate, but the up or down expression peak positions in time remain approximately the same. This property is illustrated in Figure 1 through the example used by Bar-Joseph \textit{et al}. (2008) where raw and deconvolved data display the same peak timing. The authors hypothesize that cell progress rates are distributed as a Gaussian with a mean of 1 (average time) and that observed expression values result from a convolution with such Gaussian kernel. Because the Gaussian kernel function is symmetric around the mean, if a certain group of cells has a cell progress rate, say \(1 - d\), there is also a ‘symmetric’ group of cells having a progress rate \(1 + d\). The total effect of these contributions leaves the
maxima points (and the minima points) in the same time positions.

To prove the effectiveness of our approach, we used recently published human fibroblasts cell cycle data (Bar-Joseph et al., 2008) and performed in vivo experiments to validate our computational predictions.

2 METHODS

The LEON algorithm identifies cycling genes by a two-step procedure. The first step computes a suitably trained Support Vector Machine (SVM) classifier to decide whether a given profile is cycling or not and, accordingly, a binary cyclicity score of \( c = 1 \) or \( c = 0 \) is assigned. The second step consists in an optimization-based approach, which identifies consistency of peak timing along two cell cycles and accordingly assigns to each gene a cyclicity score \( p \) between 0 and 1. The SVM prediction and the optimization procedure are then combined into a single overall score \( p_{\text{comb}} \) characterizing the degree of cyclicity for each transcript.

2.1 First step: SVM classification

The SVMs, first described by Vapnik in the 90’s (Vapnik, 1998), have been successfully applied to a large number of pattern recognition problems, including classification of microarray data (Brown et al., 2000). Each expression profile vector with \( n \) time samples can be seen as a point in an \( n \)-dimensional space. To separate in this space cycling genes from non-cycling genes, the simplest approach is to consider a linear decision boundary region (i.e. a hyperplane) that separates the two given classes (positive and negative examples) in the original \( n \)-dimensional space. In our case such simple rule is not satisfactory, as the ‘best’ hyperplane separating the two classes still misclassifies a large number of genes. Therefore, we adopted the common approach of mapping the dataset into a higher dimensional space (the so-called feature space).

Fig. 1. Experimental gene expression time profile (triangles) and deconvolved data (squares) using BIRC5 (data taken from Bar-Joseph et al., 2008). It is worth noting that peaks remain located approximately at the same position in time both in raw and deconvolved data.

This point is illustrated in Figure 2. Then, a reliable subset of cycling and non-cycling genes was used as positive and negative examples for the training set. On this basis, the first step of LEON algorithm, classifies all genes as ‘cyclic’ or ‘not cyclic’ (see Fig. 3) using an SVM (see Section 3).

2.2 Second step: non-linear optimization-based gene expression profile analysis

Cyclicity of a time series can be characterized in many ways. A common choice is the use of the first Fourier coefficient (Spellman et al., 1998), but often the shape of a time series is different from a pure sinusoid. Additionally, owing to the already mentioned synchronization loss, the shape of the curve is also different between two subsequent cycles of the same transcript.

Among the many possible alternatives, we selected a feature that is independent from the overall shape and robust with respect to the loss of synchronization problem (see Fig. 1), as previously described. Such feature is the distance in time \( d_{\text{min}} \) between two subsequent minima and the distance \( d_{\text{max}} \) between two subsequent maxima (illustrated in Fig. 4). Therefore, cell cycle time series having \( d_{\text{min}} \) and \( d_{\text{max}} \) near to the duplication time \( \Delta \), namely, the time needed by a cell to complete a cell division cycle obtained by flow cytometry [Fluorescence-Activated Cell Sorting (FACS)] analysis, are considered good candidates for being associated to cycling genes.

Unfortunately, microarray time series are noisy and provide expression values only at sampling times. To robustly evaluate peak times, data were preprocessed to reduce noise and then values outside the range of sampling times were extrapolated. By doing so, we could obtain a precise estimation of times at which maxima or minima are attained.

Therefore, we needed an appropriate mathematical tool to get a smooth continuous representation of the experimental data that enables us to estimate unobserved time points. Radial basis functions (RBFs) are tools to approximate functions by linear combinations of terms based on a single univariate function (the RBF).

The use of RBFs is motivated by the fact that they have excellent approximation properties (as shown, e.g. in Girosi and Poggio, 1990) and are widely used also in the bioinformatics field (Chen et al., 2011; Chiang and Ho, 2008; Takasaki et al., 2006). They are usually applied to approximate data that are only known at a limited number of points—as for the case of microarray experiments—so that the approximating function can be evaluated often and efficiently. In fact, once the RBFs are generated, one can resample the curve to estimate expression values at any time points.

The second step of LEON algorithm consists in the approximation and resampling of (noisy) expression time profiles using a linear combination of RBF. When estimating RBFs from expression data, we did not fit each time course individually. Owing to noise and missing values, such an approach could lead to overfitting. Instead, we constrained the RBF coefficients to have good smoothness (or regularity) properties (see Section 3.2) based on the same training set (golden standard genes) used for SVM. Then, a cyclicity score \( p \) can be computed for each gene using a non-linear optimization-based method to find optimal parameter values of the RBFs. That is, a set of optimal RBF parameters (the same for all
genes) is calculated by means of an optimization-based method. The rationale behind this choice is to determine the set of parameters that best represent the biological process under analysis. Then, for a given gene, we calculated the RBF approximation and executed a cyclicity analysis to get a score \( p \). Precisely, once the smoothed expression profile is computed, the cyclicity score \( p \) is defined as follows:

\[
p = \frac{m_{\text{max}} + m_{\text{min}}}{2}
\]

where \( d_{\text{max}} \), \( d_{\text{min}} \) are, respectively, the distances in time between the two maximum peaks and the two minimum peaks of the approximation curve and \( \Delta \) is the duplication period. A score value close to 1 indicates a high consistency of peak timing and, therefore, a good indication of the presence of a cyclic behavior. To find optimal parameters values, we evaluated the performance of the smoothing algorithm by generating a Receiver-Operating Characteristic (ROC) curve according to the \( p \) score (Fig. 5). Then, a global optimization algorithm (namely, the PRICE algorithm see e.g. Brachetti et al., 1997; Luzzi et al., 2003) found the parameters giving the best ROC curve (larger area under curve) obtaining \( A.U.C. = 0.86 \). The final RBF coefficients were \( \frac{1}{C_0} \frac{4}{10^4} \), \( \frac{1}{C_1} 2 \), and the number of RBFs used was 8. The overall algorithm scheme is illustrated in Figure 6.

### 2.3 Combined cyclicity score

In sum, the proposed methodology is the following: first, we decided whether a given gene expression is sufficiently ‘fluctuating’ (cycling genes usually have such a kind of expression). Then, we identify among those fluctuating genes, the ones that are cycling by means of a peak consistency analysis. In particular, the SVM is used to extract the information contained in the amplitude of fluctuations and to get rid of bad shaped expression profiles (e.g. flat profiles) that usually identify not cycling genes. Then, the \( p \) score, which is related to the consistency of peak timing along two cell cycles, identifies those genes that are cycling.

Once each gene in our test set was classified (\( c \) score) by the learning machine (first step) and the \( p \) score for that gene was calculated (second step), we built a combined score as follows:

\[
p_{\text{comb}} = c + p
\]

where \( c \) is the SVM output related to the gene under analysis (\( c = 1 \) cyclic, \( c = 0 \) not cyclic). Then we ranked genes according to their \( p_{\text{comb}} \) value. Ideally, the first gene in the list should be the one classified as cycling (\( c = 1 \)) and with a \( p_{\text{comb}} \) score equal to 1, whereas the last one should be the one classified as non-cycling (\( c = 0 \)) and with a \( p_{\text{comb}} \) score equal to 0.

As a preliminary step, we generated synthetic data to evaluate the differentiating ability of the proposed method. To produce realistic synthetic data, we followed the approach of Zhao et al. (2001). The authors propose an algorithm to generate synthetic time series by assuming that cell cycle-regulated genes are transcribed at one invariant time and that synchrony deteriorates with time, leading to the attenuation of simple pulses into smooth peaks that dampen out with time. Using this algorithm, we generate 1000 synthetic time courses covering two cell cycles, and 1000 randomly fluctuating profiles, obtained by random time shuffling of cyclic data. Then, we generated, using the same algorithm, 50 positive and 50 negative synthetic examples for the learning step. We also added to all synthetic data a multiplicative white Gaussian noise having 10 and 20% of noise standard deviation. We applied the proposed method to such data and computed ratios of genes scored \( >0.5 \) and \( <0.5 \) for cyclic (Re) and non-cyclic (Rnc), respectively, and obtained a ratio of \( \frac{\text{Re}}{\text{Rnc}} = 1/0.997 = 1.003 \) with 10% of noise and a ratio of \( \frac{\text{Re}}{\text{Rnc}} = 1/0.993 = 1.007 \).
Supplementary Table S3 reports, for each gene of the low Cyclebase ranking is not available, we considered PRKD1 for agreement with our analysis. Moreover, among the genes for which a the genes in this group classified by Cyclebase as non-cyclic, in good genes, of which 18 are classified as cyclic, 44 non-cyclic and 29 not clas-
group, the annotation and the Cyclebase classification. We found 91

Figure 7. The picture shows a clear bimodal distribution composed of a

Figure 7. $p_{comb}$ score distribution for the 480 genes considered as cyclic by


Fig. 7. $p_{comb}$ score distribution for the 480 genes considered as cyclic by


3 IMPLEMENTATION

3.1 SVM classification

We selected 50 gene expression profiles as positive examples, compiled from the literature that had been shown to be cell cycle-regulated in cells synchronized experiments (Supplementary Table S1). Known cell cycle-regulated genes were limited to those regulated at the messenger RNA level during a continuous human cell cycle, as determined by traditional experimental methods. We selected also 50 profiles generated by a random time shuffling procedure, considered as negative ones. Eventually, we obtained a sample of 100 genes each one having dimension 17 (time points).

Then, we trained the SVM using the LIBSVM software, a library for SVMs developed by the Machine Learning Group at National Taiwan University (Chang et al., 2008) and selected a radial basis kernel. The parameters $C$ and $\gamma$ were determined by a standard $k$-fold cross-validation procedure (with $k=5$) combined with a grid search. This procedure is done to prevent overfitting problems (Bishop et al., 1996; Hsa et al., 2010). In particular, we first divided the training set into five subsets of equal size. Sequentially one subset was tested using the classifier trained on the remaining four subsets. Thus, each instance of the whole training set was predicted once, so the $k$-fold cross-validation accuracy is the percentage of data that are correctly classified. Various pairs of $C$ and values (selected from a suitably chosen grid) were tried and the one with the best $k$-fold cross-validation accuracy was picked. The final values were

$$C = 1 \text{ and } \gamma = 0.06.$$ The final $k$-fold cross-validation accuracy and standard error were, respectively, 93.1%, and 0.02.

3.2 Radial basis approximation

The approximating function $\hat{y} : R \rightarrow R$ used was as follows:

$$\hat{y}(t) = \sum_{j=1}^{m} a_j \phi(|t - c_j|)$$
where $\phi$ is a suitably chosen RBF, $a_j$ are the coefficients of the linear expansion and $c_i$ are the centers of the RBFs. We used the inverse multiquadratic RBF in our experiments:

$$\phi(t) = (\sigma^2 + \tau^2)^{-1/2}$$

where $\sigma$ is a positive scalar. This choice was mainly motivated by the fact that the multiquadratic function showed the best performance in expression profile approximation. To find the approximation, we solved the following regularized problem:

$$\min_{\text{a.c.}} \sum_{i=1}^{p} (y(t_i) - \tilde{y}(t_i))^2 + \tau_1\|a\|^2 + \tau_2\|c\|^2$$

where $y(t_i)$ is the expression of the gene at the sampling instant $t_i$.

The number of RBFs, the RBF parameter $\sigma$ and the regularization parameters $\tau_1$ and $\tau_2$ were the same for all the approximations and were obtained by means of a global optimization algorithm. The idea behind this approach is that of determining the fact that the multiquadric function showed the best performance in expression profile approximation. To find the approximations and were obtained by means of a global optimization algorithm. For a given choice of $m$, $\sigma$, $\tau_1$ and $\tau_2$, we computed the expression profile approximations and the corresponding scores $p$ of the genes in the dataset used for the training of the SVM.

3.3 Cell culture, synchronization and FACS analysis

Early passage of human foreskin fibroblasts was grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% Fetal Calf Serum (FCS). For G0/G1 synchronization, cells were arrested with 0.5% FCS (48 h) and then released in 10% FCS. Cells were harvested at specific time points following serum stimulation and were processed. The progression of cells through the cell cycle was monitored by flow cytometric (FACS) analysis of replicate samples of propidium iodide-stained cells (Gurtner et al., 2008).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted using the Trizol reagent (Gibco BRL) and following the manufacturer’s instructions. The first strand of complementary DNA was synthesized according to manufacturer instructions of the M-MLV RT kit (Invitrogen). PCR was performed with HOT-MASTER Taq (Eppendorf) using 2 ml of complementary DNA reaction. The primer sequences of the human genes are as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCycA2f</td>
<td>5'-AGCAGGCCTGCAACTGCAAATGTTG</td>
<td>3'-GCCATAGGGAAGCCAGAGA</td>
</tr>
<tr>
<td>hCycA2r</td>
<td>5'-TGGTGGGTTAGGAGAGAACAACC</td>
<td>3'-CTCCTACCTTTGCACTTCCTTCCG</td>
</tr>
<tr>
<td>hCycB1f</td>
<td>5'-CCTCTACCTTTGCACCTTCTTCCG</td>
<td>3'-GGCAGTAGTGGATTGCTCTG</td>
</tr>
<tr>
<td>hCycB1r</td>
<td>5'-GAGTGTGCTGCTTTAGCATGCTTCCG</td>
<td>3'-CTCAGTCACCACGTACACG</td>
</tr>
<tr>
<td>hMTX2f</td>
<td>5'-CTGCAGAAGACCTTGGGCTGAA</td>
<td>3'-GTAGCCGTCATCGGCTTCT</td>
</tr>
<tr>
<td>hMTX2r</td>
<td>5'-CTGCACTGCAAGAGAAGCTG</td>
<td>3'-GGTTGCTGTTGATGAAGTCAGGAGAC</td>
</tr>
<tr>
<td>hEDF1f</td>
<td>5'-GCACGAGACAGGAGGAGAAGA</td>
<td>3'-CCAAGTGCAACAAGGAGGAG</td>
</tr>
<tr>
<td>hEDF1r</td>
<td>5'-ACCTTGTGGCATACCTTCTTGC</td>
<td>3'-GTACCAGCTGGGCTGATGAC</td>
</tr>
<tr>
<td>hDNAJf</td>
<td>5'-TCAAGGAGATCGCTGAGGCC</td>
<td>3'-GGTACCTGGTGGGCTGATGAC</td>
</tr>
<tr>
<td>hDNAJR</td>
<td>5'-GCCATAGGGAAGCCAGAGA</td>
<td>3'-GGCAGTAGTGGATTGCTCTG</td>
</tr>
<tr>
<td>hLMO4f</td>
<td>5'-GGAAATTACGCGGTCTGCAG</td>
<td>3'-GGCAGTAGTGGATTGCTCTG</td>
</tr>
<tr>
<td>hLMO4r</td>
<td>5'-GGCAGTAGTGGATTGCTCTG</td>
<td>3'-GGCAGTAGTGGATTGCTCTG</td>
</tr>
</tbody>
</table>

4.4 In vivo validation of LEON predicted cell cycle-regulated genes

Using LEON algorithm we selected 50 genes having $P_{\text{comb}}$ score and excluded 5 genes that were identified as cycling also by Bar-Joseph et al. (2008). Among the remaining 45 new putatively cycling genes, we experimentally validated the cell cycle-dependent expression of 9 of them. To this purpose, primary human fibroblasts prepared from human foreskin were grown to ~50% confluence and synchronized in G0 by serum deprivation. Cultures were then released from arrest, and cells were collected at different time points covering one complete cell cycle (Fig. 8A).

We used RT-PCR to measure the expression level of the nine genes with higher combined score (see Supplementary Table S2 and Fig. 7) along synchronized cell populations. None of these genes result to be cell cycle-regulated in the study by Bar-Joseph et al. (2008), demonstrating the strength of the LEON algorithm in identifying cell cycle-expressed genes. As shown in Figure 8B (RT-PCR) and Supplementary Figure S1 (densitometric analysis), the expression of these nine genes is cell cycle-regulated being maximum on S phase for six and on G1 phase for four of them, respectively.

Interestingly, the expression of two of these genes, NCOR1 and EDF-1, is already known to be cell cycle-regulated further.
confirming the predictive power of the LEON algorithm (Altintas et al., 2011; Bolognese et al., 2006). Next we measured the expression level of four genes with a low combined score. Of note, the expression of these genes is not regulated during the cell cycle further demonstrating that the LEON algorithm specifically identifies the cell cycle gene expression. We measured cyclin A and cyclin B1 as well as GAPDH and aldolase genes as positive and negative controls, respectively. It is well known that cyclin A and B1 expression is cell cycle dependent being high in S phase and in G2, respectively (Pines and Hunter, 1989, 1990). On the contrary, the expression of GAPDH and aldolase genes is constant during the cell cycle (Eisenberg and Levanon, 2003). As expected, cyclin expression is cell cycle dependent, whereas GAPDH and aldolase expression is constant. These results demonstrate that our approach is successful in identifying cell cycle-regulated genes.

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**Conflict of Interest:** none declared.

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