Improving Clustering of Gene Expression Patterns

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I certify that all material in this dissertation which is not my own work has been identified and that no material is included for which a degree has previously been conferred upon me.

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Signed:

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Abstract

The central question investigated in this project was whether clustering of gene expression patterns could be done more biologically accurate by providing the clustering technique with additional information about the genes as input besides the expression levels. With the term biologically accurate we mean that the genes should not only be clustered together according to their similarities in expression profiles, but also according to their functional similarity in terms of functional annotation and metabolic pathway. The data was collected at AstraZeneca R&D Mölndal Sweden and the applied computational technique was self-organizing maps. In our experiments we used the combination of expression profiles together with enzyme classification annotation as input for the self-organising maps instead of just the expression profiles. The results were evaluated both statistically and biologically. The statistical evaluation showed that our method resulted in a small decrease in terms of compactness and isolation. The biological evaluation showed that our method resulted in clusters with greater functional homogeneity with respect to enzyme classification, functional hierarchy and metabolic pathway annotation.

Keywords: gene expression analysis | functional annotation | clustering techniques | self-organizing maps

Contents

1 Introduction

Bioinformatics is a rather new scientific discipline that has not been around for more than a decade or so, and it encompasses all aspects of biological information acquisition, processing, analysis and storage (Attwood *et al.* 1999). It is a science that combines mathematics, computer science and biology with the aim of understanding life from the biological point of view. The science of bioinformatics has emerged because of the rapidly growing amount of biological data produced (Bassett *et al.* 1999).

In the past, investigations of the genome, i.e. the entire set of genes for a species, were focused on one gene at a time. In the past decade, however, new and sophisticated techniques have made it possible to map out entire genomes, e.g. yeast and several bacteria (Lipshutz *et al.* 1999). In 1990, the Human Genome Project (HUGO) was started with the goal of mapping the entire human genome[1], which is estimated by the HUGO foundation to contain somewhere between 70,000 – 100,000 genes.

There are four different kinds of building blocks, called nucleotides, in a gene and the number of nucleotides needed to code for a gene varies from a few hundred to several hundred thousands. If we look upon the genes as “blueprints” (Brown and Botstein, 1999), the proteins are the finished building structure. Proteins are large

[1] Although the goal is set to the year of 2003, a “working draft” was already accomplished in June this year. Human Genome Project Information Site: http://www.ornl.gov/hgmis/home.html

molecules that we are dependent on to perform all tasks necessary for life, such as for example the regulation of blood sugar levels, *Insulin*, and *enzymes* that are organic catalysts that speed up chemical reactions. An overview of the Human Genome project and other related information can be found at http://www.ornl.gov/hgmis/home.html.

Some of the computational contributions to bioinformatics include the development of database methods suitable for storing and maintaining genomic information. This is information such as gene sequences, three dimensional protein structures and additional information, *annotation*, about the genes. Annotation can be anything that is known about a gene, e.g. name, length of sequence and what is known about its function. Other important contributions are algorithms for searching and analysing large numbers of sequences (Durbin *et al.* 1998). For instance, we may want to compare several sequences from different species for equality and see if they belong to the same gene family. Furthermore, prediction algorithms (Sterberg, 1996) that are applied to try to predict what three-dimensional structure the protein of a certain gene sequence will fold into and how to visualise and modell all kinds of biological information are other important issues.

It is a fact that not all genes in each cell are expressed, or put more simply – not all kinds of protein are produced in all cells all the time, but only a small part (Waterman 1995). The Insulin protein for example, is only produced in the cells of the pancreas. Furthermore, the same amount of protein is not produced all the time, e.g. it can vary with the cell’s lifecycle or with the organism’s state of health. To help understand the activity of a certain gene in a certain cell, the expression level of that gene is measured, i.e. is it expressed very often the expression level is high.

By measuring expression levels very useful information can be collected. Suppose for example, that we have two organisms of the same species, one treated with some substance and the other untreated. Then the expression levels of all the genes in both organisms are measured. The expression level data from the two organisms can then be compared and the, by the substance, affected genes can be determined (Gwynne *et al.* 1999). If an expression level is increased it is said to be *up-regulated* and if it is decreased it is *down-regulated*.

With traditional methods the work was carried out on one gene in one experiment at a time, but recently a new technology for measuring thousands of genes at a time was developed, the so called microarrays (Shi, 1998). Nowadays, these microarrays are used in laboratories around the world for measuring thousands of genes at several timesteps creating large datasets that have to be analysed and understood, (Brown and Botstein, 1999).

1.1 The project and its perspective

The computational algorithms applied to this domain of data are essentially different kinds of clustering techniques, such as for example hierarchical clustering (Jobson, 1992; Hartigan, 1975; Gordon, 1981) or self-organising maps (Kohonen, 1997). In experiments carried out with clustering techniques, the expected outcome is clusters of genes with similar expression *profiles*, i.e. genes that are regulated in a similar manner, and with similar biological function (Michaels *et al.*, 1998; Eisen *et al.*, 1998; Wen *et al.*, 1998; Tamayo *et al*., 1999). In reality, the resulting clusters only contain genes with similar expression profiles. The reason for this is that the input data only consists of gene expression profiles and genes with different functions can have the same expression profile. Biologically, the clusters are not accurate since they lack functional coherence among the genes.

In other experiments as in Tamames *et al.* (1998), it has been tried to automatically categorise the proteins in functional classes by considering the annotation stored about them in the databases. With this method the outcome is “clusters” that reflect genes with similar function, but they do not reflect which genes with a certain function are up- or down-regulated. The reason for this is obvious since expression profiles are not stored in databases as annotation and therefore not included in the categorisation. Although these clusters are in some sense biologically accurate, they do not identify the affected genes we would be looking for in experiments such as in the example above.

In this project the aim was to find a technique that combines previous techniques of clustering expression profiles on one hand, and annotation on the other. The hypothesis was that a combination of these techniques gives more biologically accurate results. The experiments were concentrated on investigating if the use of both gene expression patterns and annotation as input for the clustering technique could provide the improvements in biological accuracy we were looking for.

Gene expression data was collected at AstraZeneca R&D Mölndal Sweden and we were also provided with additional data containing gene annotation from them. Because of time constraints, we delimited the project and worked only with self-organising maps (Kohonen, 1997). We chose to work with self-organising maps because it has been used in previous works such as, for instance, the work by Tamayo *et al*., 1999 and since we have been using the technique before and are familiar with it. The results are validated in collaboration with biologists at AstraZeneca R&D Mölndal Sweden.

The annotation used in the experiments as input besides the expression profiles is called *enzyme classification*, which is an annotation that divides the genes into categories according to their enzymatic function. The results are evaluated both statistically and biologically and both evaluation methods indicate that expression profiles should not be clustered alone, but together with annotation.

1.2 The structure of the thesis

Chapter two contains necessary background information - such as the biological concepts, measuring techniques for gene expressions, techniques for clustering and computational analysis such as self-organising maps - and aims at giving the reader an introduction to the problem area of the thesis. Furthermore, related work is discussed in chapter 2.4. In chapter three the thesis statement is described. First the hypothesis is presented and discussed and in chapter 3.1 the reader will find a summary of the objectives needed for the fulfilment of the aim of the project. Further, methods and experiments are described in chapter 4 with chapters dealing with each objective separately and a chapter describing the experimental design. All results are presented and analysed in chapter 5. Drawn conclusions are presented in chapter 6 and finally, an overall discussion along with future work is found in chapter 7.

2 Background

This chapter describes the background of the problem area and elaborates on both the biological and the computational foundation of this project. First, in chapter 2.1, the basic biological concepts underlying gene expression analysis are presented. These are concepts such as *DNA transcription*, *protein synthesis* and *annotation*. In chapter 2.2 the concept of gene expression and how it can be measured is discussed. Here, the reader will be introduced to techniques such as *microarrays*, *genechips* and *RT-PCR*. Chapter 2.3 comprises a description of the computational techniques used in the problem area, self-organising maps in particular, but other techniques such as hierarchical clustering are also mentioned. The last chapter (2.4), lists related work and thus also puts this project in its context.

2.1 Biological concepts

All higher lifeforms depend on cells to produce proteins and enzymes in order to become a living organism and stay alive (Waterman, 1995). This is true for all organisms except for certain viruses. Cells in the liver, for instance, produce enzymes that detoxify poisons, and pancreas cells produce insulin, which regulate the blood sugar levels. Responsible for the production of these specific proteins and enzymes, which is a highly complex process, are the genes transcribed in each cell. The way the cells produce protein and enzymes, called protein synthesis, is commonly referred to as the central dogma of biology (Waterman, 1995), the dogma is illustrated in figure 1. In this chapter, we first explain the steps in this process and start with DNA transcription.

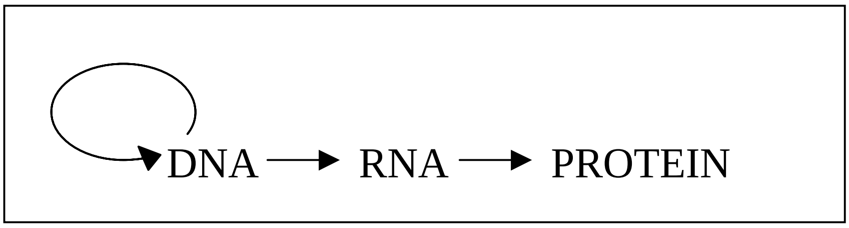


Figure 1. Schematic picture of the central dogma of biology.

2.1.1 DNA transcription

There is a loop from DNA to DNA, which means that DNA molecules can be copied, which is called replication. This makes it possible for a cell to be divided and become two new cells with their own copy of the entire DNA. DNA is transcribed into RNA and the RNA in turn is then translated into protein. Deoxyribonucleic acid (DNA) sequences store the genetic information, called the genome, for any given organism. The genome can be looked upon as the “blueprint” of that organism (Brown and Botstein, 1999). The only exceptions are some viruses, which instead of DNA transcribe RNA and retroviruses that can transcribe RNA to DNA, which is called reverse transcription (Waterman, 1995).

The DNA sequences are made up of molecules, called nucleotides, which contain one of the four bases: adenine (A), cytosine (C), guanine (G), and thymine (T). All DNA sequences can therefore be considered as strings comprised of only these four letters, e.g. “ATGGCA”. However, not all DNA codes for protein. For example, it is believed that only about 5% of the human genome is used, but in some bacteria over

90% of the genome is used (Attwood *et al.* 1999). The coding parts that code for certain proteins have themselves intermediate regions that appear to be non-coding DNA. These non-coding regions are called introns and the actual coding parts are called exons. See Figure 2 for further explanation. What function the introns have is not yet fully understood (Waterman, 1995).

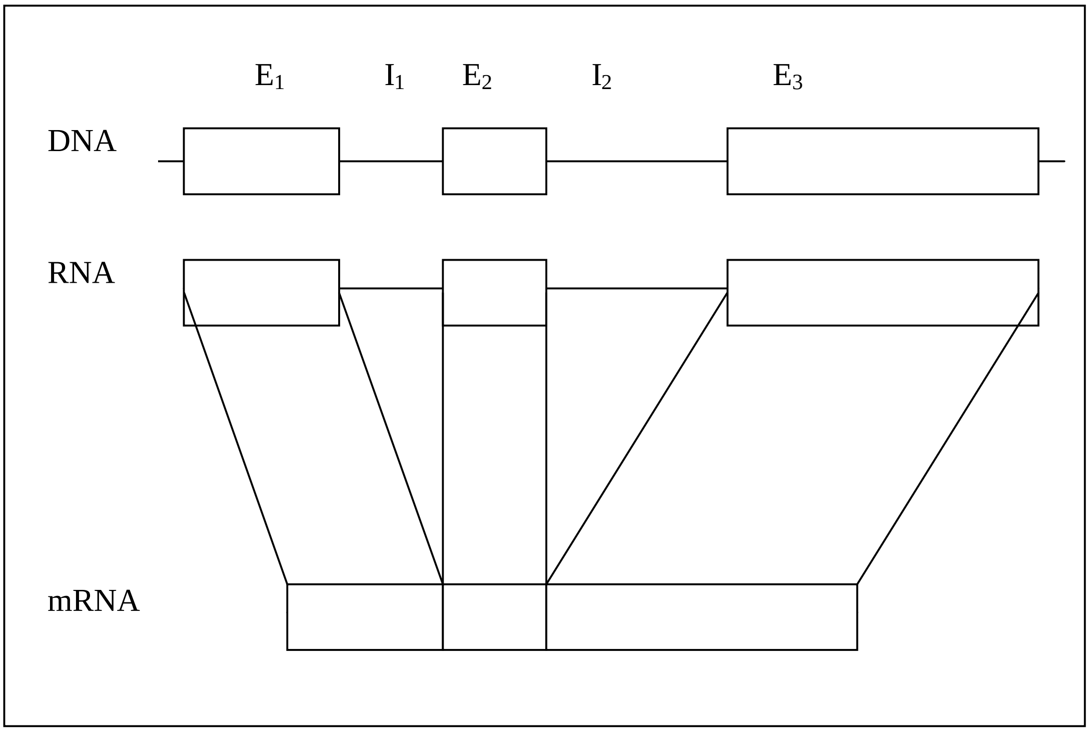


Figure 2. DNA is transcribed into RNA with both the exons $E_{1\text{-}3}$ and the introns $I_{1\text{-}2}$. The introns are spliced out and discarded from the RNA and the finished, uninterrupted exon-sequence is called messenger-RNA, or mRNA. The mRNA, which now only contains the gene, is then translated into some protein or enzyme.

2.1.2 Protein synthesis

DNA is transcribed into messenger ribonucleic acid (mRNA), which after the filtering process, where the introns are spliced out and discarded, only contains the exons of the DNA. The information in the mRNA is interpreted in the form of *codons*, which basically are triplets of nucleotides. In the ribosome, the codons of the mRNA are translated into polypeptide chains. The transfer RNA (tRNA) provides *anticodons* for the codons of the mRNA and in the interaction between these molecules the codons are translated into amino acids. Each codon corresponds to a single amino acid (Brown, 1998). In the last step of the protein synthesis these chains, sometimes with the help of other enzymes called chaperons (Attwood *et al*. 1999), fold into various proteins (see Figure 3).

It is not a fact that without the chaperons the polypeptide chain could fold in a different way and become a different protein, but with the chaperons many dead ends in the folding process are avoided and thus the efficiency of the folding process is increased (Attwood *et al.* 1999).

The polypeptide chains are assembled from twenty amino acids. Similar to the DNA sequences, these polypeptide chains can be viewed as words or strings over this amino acid “alphabet”, where every amino acid is tokened by a certain letter, e.g. “ANCWMP”. One specific polypeptide chain can only fold into one specific protein (Attwood *et al.* 1999). The protein can have several different conformations depending on, for instance, its environment and energy state.

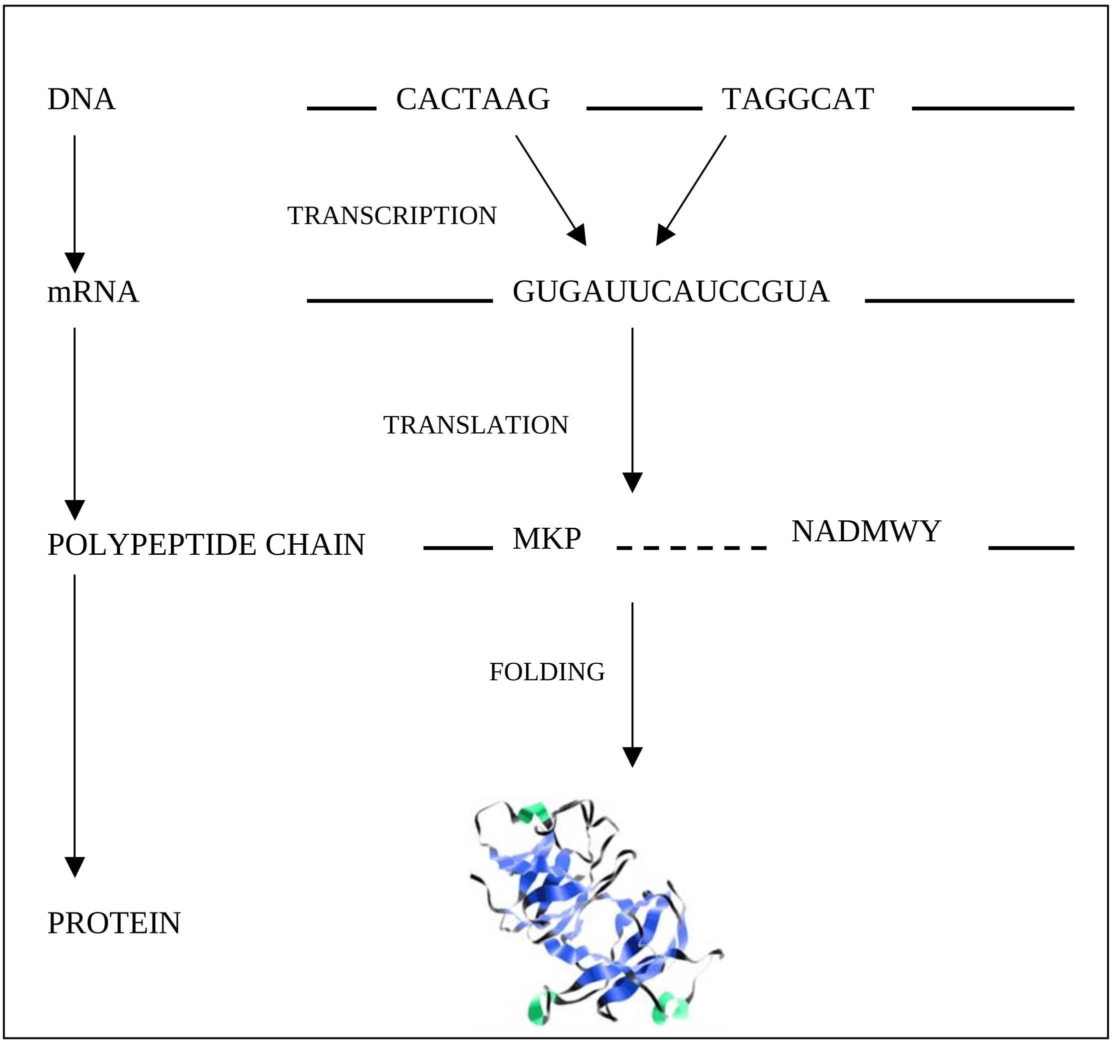


Figure 3. The process of protein synthesis. From the DNA the introns are spliced out and the resulting mRNA contains only the exons, i.e. the gene. This mRNA sequence is then translated three nucleotides at a time, a codon, with the help of transfer RNA (tRNA) into a polypeptide chain. This chain in turn, folds into a protein.

2.1.3 Gene annotation

Gene annotation can be anything that says something about a gene. For example, the length of the DNA sequence corresponding to the gene, on what chromosome it is located, in which tissue it is expressed and something as simple as its name. Furthermore, genes are divided into functional categories. The enzymes, that constitute a subset of all the genes, are classified according to their enzymatic function. Examples of other classifications are which metabolic pathway or process the genes are involved in, or where in the functional hierarchy they belong. Below follows a couple of examples of different types of annotation that are stored in databases. Most of the databases are publically available on the Internet. The data in table 1 exemplifies the type of annotation that can be found in a database[2]. Figure 4 illustrates one type of information, metabolic pathways, that can be found in the KEGG database. Examples of other information that can be found in KEGG are regulatory pathways and gene- and disease catalogs.

[2] This set of annotation should only be viewed as an example and comprises annotation from several different databases.

PROBESET	Msa.1022.0_at
EMBL_ID	L09104
EMBL_DESC	Mus musculus glucose phosphate isomerase mRNA, 3' end.
LSG_DE	INCY:Human neuroleukin mRNA, complete cds.
LSG_AI	A.5.3.1.0
LSG_AD	Enzyme hierarchy>Isomerases> Intramolecular oxidoreductases> Interconverting aldoses and ketoses>
KEGG_MAPNO	MAP00030
KEGG_DESC	Metabolism; Carbohydrate Metabolism; Pentose phosphate cycle
UNIGENE_ACCNO	Mm.589
UNIGENE_GENE	Gpi1
UNIGENE_CHR	7
UNIGENE_BAND	7 11.0 cM

Table 1. In this table we see annotation about the gene **Gpi1**. From this information we can see, for example, that it is an enzyme and that it is involved in carbohydrate metabolism.

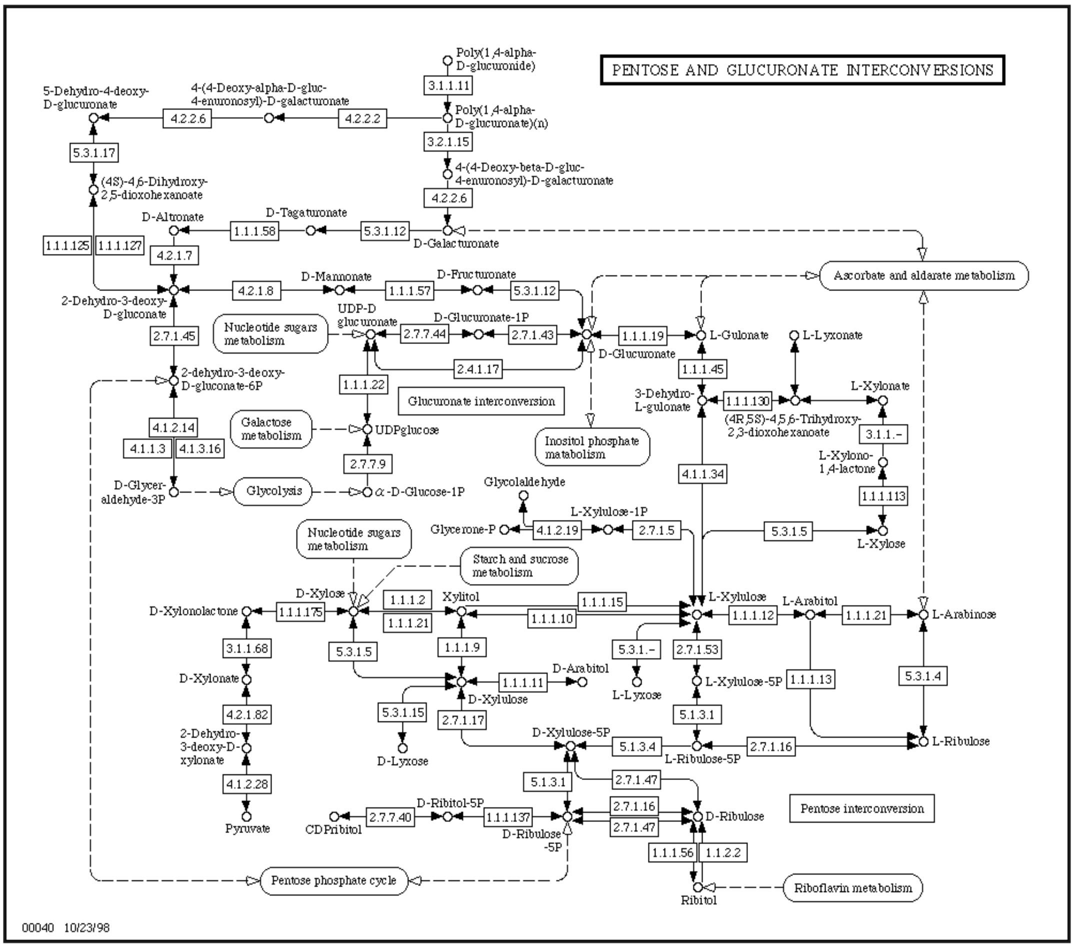


Figure 4. This figure shows an example of a metabolic pathway and is taken from the KEGG database[3]. This map is also an example of an annotation. The numbers in the squares are enzyme classification numbers and they represent the genes that are in this particular pathway.

[3] The KEGG database can be accessed at http://www.genome.ad.jp/kegg/kegg2.html.

A final example of different kinds of annotation is the GeneCards database [4] where several different databases have been linked together for easy access. The listed annotation for each gene is in fact a set of hyperlinks and each link leads to information in other databases such as PubMed for example, which contains publications on the subject, such as research articles about the specified gene.

A problem with the annotation stored in databases is that it is very often incomplete and can even sometimes be wrong, e.g. misclassification of enzymes. The following citation from Bassett *et al.* (1999) presses on this problem:

> "It is important to remember, however, that for even the best characterized organisms, functional information is usually incomplete and exists for only a fraction of the genes." (p. 54).

2.2 Gene expression analysis

Through some process, still unknown to scientists, different cells activate only some of their genes, producing different proteins so that each cell specializes on specific tasks in an organism (Waterman, 1995). To come to an understanding on how cells specialize, scientists need to somehow identify which genes each type of cell activates, also known as a cell's gene expression profile. Furthermore, by studying gene expressions, we can find out how the organism reacts to external stimulus. It is also believed that the expression pattern of a gene provides indirect information about its function (Debouck and Goodfellow, 1999). In chapter 2.2.2 and 2.2.3 different techniques for extracting the gene expression profile of a gene are

[4] The GeneCards database can be accessed at http://bioinformatics.weizmann.ac.il/cards/.

discussed. The expression levels can be measured both on the mRNA level and the protein level. It is important to recognise that not all mRNA is translated into protein and thus, the mRNA levels may not reflect the protein levels. Taking it further, it is not always true that the expression of a protein has a physiological consequence (Debouck and Goodfellow, 1999). In chapter 2.2.1 we first elaborate on the concept and use of gene expression.

2.2.1 Gene expression

By preparing some test tissue with certain substances, it is possible to measure what effect these substances have on the tissue (Debouck and Goodfellow, 1999). This is done by studying which genes are expressed and how much, giving a measure of the amount of protein produced in the cell. By comparing the test tissue with normal tissue, the deviations from the normal protein production can be calculated and possible drug targets can be identified (Gwynne *et al.* 1999). These assumptions are based on the concept that a protein’s function is strictly determined by the structure and activity of the gene that encodes it (Wen *et al.*, 1998).

Traditional methods in molecular biology generally work with one gene in one experiment at a time, which led to only limited amounts of data on a small group of genes (Shi, 1998), and these methods are time consuming (Debouck and Goodfellow, 1999). In the past several years a new technology called microarrays has emerged which helps researchers analyse more of the genome and under several different conditions, e.g. using different stimulus (D’haeseleer *et al.* 1999). This technique is a potentially powerful tool for investigating the mechanism of drug action (Debouck and Goodfellow, 1999). A microarray makes it possible to monitor a massive number of genes simultaneously and thus gives the researcher a better picture of the effect of the interactions between genes in a specific gene group (Shi, 1998). Another advantage is that, as the microbiologists used to spend years studying bacteria one gene at a time *in vitro*, in test tubes, they now can study and identify genes that are turned on *in vivo*, in living tissue. Not all genes that are turned on in vitro are turned on in vivo - and the other way around, not all genes are turned on in vitro when in vivo (Debouck and Goodfellow, 1999).

Different terminologies have been used for these microarrays in literature, such as: bio chip, DNA chip and gene chip. Affymetrix' chips are called gene chips and this is the terminology that will be used from now on in this report when referring to a single chip. With the traditional methods, the researcher produced only small amounts of data which could be analysed manually, e.g. compare a couple of genes and rank them according to their relative expression level regulation. With this microarray technology, a new challenge has arrived, namely: How to make sense of the massive amounts of data? Each dataset can contain from hundreds to several thousands of gene samples and each gene sample can be comprised of the complete developmental time series for that specific gene (Bassett *et al.* 1999; Tamayo *et al*. 1999; Eisen *et al.*, 1998). As mentioned, gene expression data can be extracted on the mRNA and the protein level. In the next chapter we discuss the mRNA techniques.

2.2.2 Measuring techniques for mRNA levels

There are several different techniques for measuring gene expression levels as reported in D'haeseleer *et* al. (1999), e.g. cDNA microarrays, DNA chips, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), Serial Analysis of Gene Expression (SAGE).

The technology of cDNA microarrays was first developed at Stanford University and consist basically of glass slides upon which cDNA has been attached. The measurements are carried out by flushing the microarray simultaneously with mRNA from two different sources. One sample contains control mRNA and the second sample contains drug treated mRNA. The two samples are labelled with differently coloured fluorescent dyes. The flourescence levels of the two samples are measured independently and the ratio between them can be calculated (D'haeseleer *et al.*, 1999). At Stanford University, these microarrays have been used to measure gene expression levels for the entire yeast genome and there are also arrays for parts of human, mouse and plant genomes available from Incyte Pharmaceuticals, Inc.

DNA chips are produced by Affymetrix, Inc., called GeneChip®, among others and are glass slides with millions of short oligonucleotides, i.e. short DNA sequences complementary to the target DNA sequences, each of which identifies a given gene (Southern *et al.*, 1999). This array can be flooded with a solution containing fluorescently tagged cDNA samples from test cells, so that sequences complementary to the probed samples will hybridise. Hybridisation is the process in which the nucleotides of two DNA strands pair up and results in a double strand. After hybridisation, the amount of fluorescence on any given gene can be measured, so that the abundance of the sequence of each gene can be measured (Brown and Botstein, 1999). Among the different GeneChips Affymetrix is manufacturing, there is one that can measure 42,000 human genes simultaneously, one with 11,000 mouse genes and another one that contains the entire yeast genome[5].

[5] See http://www.affymetrix.com/ for more information.

When measuring gene expression levels with reverse transcriptase polymerase chain reaction (RT-PCR)[6], the probed mRNA is reverse transcribed into cDNA. The cDNA is amplified many times through the polymerase chain reaction (PCR) and coloured with fluorescent dyes. In the process of amplification, the flourescence levels of the target genes, i.e. the genes that are studied, will increase and these genes can then be easily detected against the background noise because of the intensity differences in the fluorescence (D’haeseleer *et al.*, 1999). This method requires that PCR-primers, which are short oligonucleotides that match the beginning of the genes they are to prime in the RT-PCR, are available for all the genes that we want to measure. The RT-PCR method is very accurate, but the amplification is very time consuming. It is accurate because if the genes of interest are present the primers will amplify them. The process is time consuming because the steps of RT-PCR include heating of the DNA strands, cooling, and reaction with the primers over and over again (Diaz and Sabino, 1998).

With the serial analysis of gene expression method (SAGE), double stranded cDNA sequences are created from the mRNA. Then, from each cDNA a short sequence is cut. This short sequence should be long enough to uniquely identify the gene it comes from (D’haeseleer *et al.*, 1999). The short sequences are concatenated into a long double stranded DNA, which can be amplified and then sequenced. Because the mRNA sequence does not have to be known a priori, new genes can be discovered in the analysis. This technique has, for example, been used to monitor the expression levels of over 45,000 human genes (D’haeseleer *et al.*, 1999).

[6] On http://hepatitis-c.de/pcr.htm a step-by-step description on how to perform RT-PCR is given.

2.2.3 Measuring techniques for protein levels

It is a much harder task to quantify protein levels than mRNA levels (D'haeseleer *et al.*, 1999). Proteins can be separated on a two-dimensional sheet of gel. First, the proteins are applied on the sheet in one direction separating the different proteins according to their isoelectric point[7] and then in the opposite direction, which separates the proteins in accordance with their molecular weight. The result is an image with a number of protein spots. The more intense the spot is, the larger amount of the specific protein is present, and thus the higher the expression level is (D'haeseleer *et al.*, 1999).

Some of the problems with this technique are that it is not known beforehand which protein each spot represents, this has to be investigated afterwards, but the position of known proteins can be estimated. Another problem is that results are hard to reproduce due to the sensitivity to operating parameters (D'haeseleer *et al.*, 1999).

2.3 Statistical and computational analysis techniques

Statistical techniques can be used to detect and extract the internal structure of a dataset (Bassett *et al.*, 1999). Many gene expression studies make the assumption that important information about a gene's function is carried in expression profiles. The first step is thus to organise the genes based on similarities in their profiles.

[7] The pH at which a protein or peptide has zero charge.

The idea of clustering genes together according to their expression levels is not new, but it is only recent that data have become available so that the techniques can be tested on a genomic scale (Bassett *et al.*, 1999). The computational techniques applied to this domain of data are essentially different kinds of clustering techniques, which cluster points in multi-dimensional space. This is good because these techniques can be directly applied to gene expression data by considering the quantitative expression levels of *k* genes in *n* time-steps as *k* points in *n*-dimensional space, i.e. the input data consist of several variables for each datapoint. For example, if we have expression levels measured at three timepoints for each gene the input vectors would be on the form:

$$x_i(a_1,a_2,\ldots,a_n) \quad i = 1\ldots k,\ n = 3$$

As gene expression time series almost always consist of several time-steps for each gene, the data is usually multi-dimensional (Tamayo *et al*. 1999), e.g. in our example above the data is multi-dimensional since $n = 3$.

A simple method to do clustering would be to group together genes by visually comparing their expression patterns. Cho *et al.* (1998) used this technique to cluster gene expressions that correlated with phases of the cell cycle. This method does not scale well when handling larger datasets such as data from a whole genomic, which could mean hundreds of datapoints for tens of thousands of genes (Eisen *et al.*, 1998). Furthermore, new and unexpected patterns cannot easily be discovered in this manner, (Tamayo *et al.* 1999).

Some of the clustering techniques that have been applied on this domain of data are techniques such as hierarchical clustering (Jobson, 1992; Hartigan, 1975; Gordon, 1981) and self-organizing maps (Kohonen, 1997). Hierarchical clustering has been used more frequently (Michaels *et al.*, 1998; Eisen *et al.*, 1998; Wen *et al.*, 1998) than self-organising maps (Tamayo *et al*., 1999). Further clustering techniques that have been applied are k-means clustering (Soukas *et al.*, 2000), decision trees, bayesian networks and affinity grouping (Friedman *et al.*, 2000; D’haeseleer *et al.*, 1999; Bassett *et al.*, 1999).

There are basically two types of hierarchical clustering, the splitting and the merging method. The splitting method first splits the dataset into two subsets trying to maximise the distance between these two. Then each subset is further divided into subsets and a binary tree structure is built (Kohonen, 1997). The more commonly used merging method starts with the two datapoints that are the most similar and at each iteration step more datapoints are added according to how similar they are, i.e. what distance they have to the first datapoints (Kohonen, 1997).

In this work, self-organising maps will be used and this technique is therefore further elaborated in the next chapter.

2.3.1 Self-organizing maps

Self-organizing maps (SOMs) have several features that make them well suited to clustering and analysis of gene expression patterns: they are scalable to large datasets and the technique is fast (Kohonen, 1997). Furthermore, SOMs have been studied in a large variety of problem domains (Kohonen *et al.*, 1998). Next, a general view of the SOM algorithm is given and thereafter a more thorough explanation of the underlying functions in the method.

Construction and training of SOMs

First, the number of nodes must be decided. Depending on the size of the data set, i.e. the number of input vectors $x_i(a_1,a_2,\ldots,a_n)$, the number of nodes should be chosen so that clearly separable clusters can be formed, i.e. the more datapoints and less nodes we have, the less compact clusters we get. This is obvious, because the more datapoints that are mapped to a certain node, the more generalised the node gets. The geometry itself, or topology, can vary from a single line to a multidimensional grid of nodes. Through an iterative process, the nodes are mapped into the *n*-dimensional space of the dataset, see figure 5. Each node consists of a *codebook* vector that is of the same dimensionality as the input vectors. The nodes can be randomly initialised or with values taken directly from the dataset.

In each iteration, the dataset is scanned one data point at a time and the map nodes are moved towards this point. The closest node N_j, according to some distance measure, is moved the most and the surrounding nodes, the neighbours of N_j, are moved depending on their distance from N_j. The larger the distance, $\|N_j – N_c\|$, the lesser they move toward the data point, i.e. this is regulated with a smoothing kernel called *neighbourhood* function. The smoothing means that we get a smooth transition between two neighbouring nodes regarding what datapoints they are mapping, i.e. the closest neighbour to N_j will map very similar datapoints, and the further away in the map, the less similar datapoints it will map. In this way, neighbouring nodes on the map are mapped to close data points in the *n*-dimensional space, where the closest datapoints are mapped to the same node N_j. When the process is finished, the final map can be said to define clusters of the data points (Kohonen, 1997).

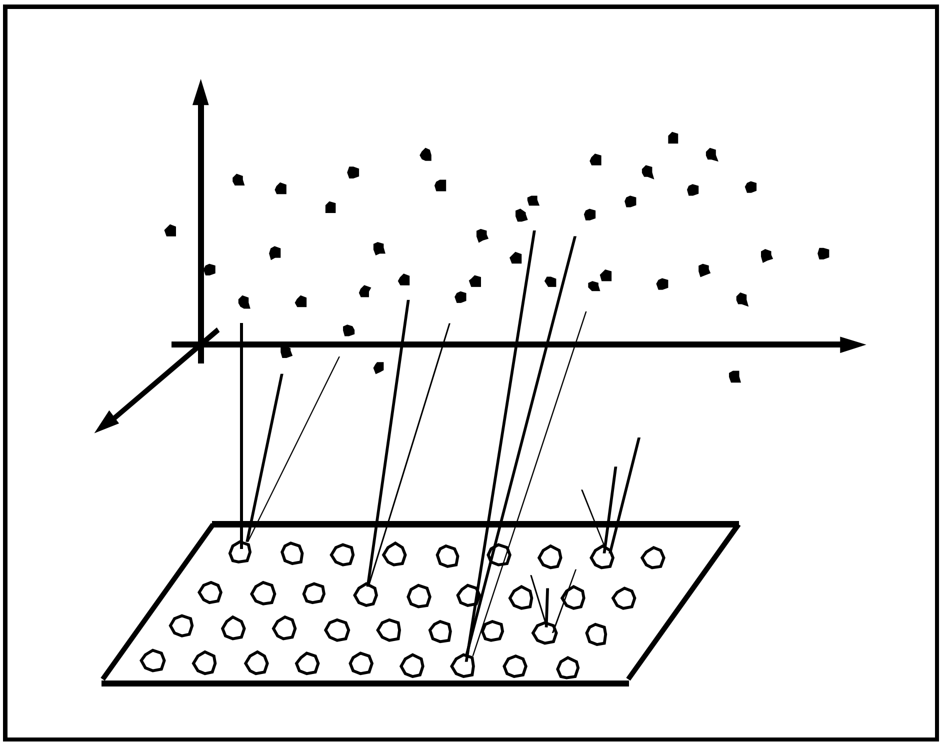


Figure 5. Illustration of self-organising maps. The nodes are arranged in a two dimensional lattice at the bottom of the picture. During training each node is mapped to a corresponding reference vector in the dataspace, above in the picture. One single node can map several datapoints and thus we get clusters.

Underlying function and algorithm of SOMs

For every input vector $x_i(t)$, at iteration step t, the distance to each node N_j is measured to find the closest node, called “the winner”. The distance measure that is used mostly in self-organising maps is the Euclidean distance (Kohonen, 1997) and is defined as:

$$D_E(x,y) = \|x - y\| = \sqrt{\sum_{i=1}^{n} (\xi_i - \eta_i)^2} \qquad (2.1)$$

Another measure that has been widely used is the Minkowski metric (Kohonen, 1997), or Minkowski norm (Mirkin, 1996), which is a generalisation of (2.1). The distance in the Minkowski metric is defined as:

$$D_M(x,y) = \left(\sum_{i=1}^{n} \left| \xi_i - \eta_i \right|^{\lambda} \right)^{1/\lambda}, \ \lambda \in \Re \qquad (2.2)$$

If $\lambda = 1$, we get yet another popular distance measure, namely the City-block distance (Mirkin, 1996; Kohonen, 1997).

When the winner is located, its codebook vector is updated according to the following algorithm[8] (Kohonen, 1997):

$$N(t+1) = N_j(t) + \alpha(t) h_{ci}(t)[x_i(t) - N_j(t)] \qquad (2.3)$$

Where $N_j(t)$ is the codebook vector at iteration t, $x_i(t)$ is the input vector and $\alpha(t)$ is a learning rate factor that usually decreases monotonically for each iteration, e.g. $\alpha(t) = A/(B+t)$ where A and B are constants. The neighbourhood function, denoted by $h_{ci}(t)$ can vary between application, but a widely used kernel can be written in terms of the Gaussian function (Kohonen, 1997):

$$h_{ci} = \alpha(t) * \exp\left(-\frac{\left\| r_c - r_i \right\|^2}{2\sigma^2(t)} \right) \qquad (2.4)$$

Where $\alpha(t)$ is the learning rate factor and the parameter $\sigma(t)$ defines the width of the kernel, which also decreases through time, i.e. in every iteration step. This means that the farther the ordering process has come, the smaller the neighbourhood will be

[8] This is the original SOM algorithm (Kohonen, 1997).

that is updated and the smaller the learning rate will be. A simpler neighbourhood kernel that is called “bubble” (Kohonen, 1997) is defined as:

$$h_{ci} = \alpha(t) \text{ if } i \in N_c(t) \text{ and } h_{ci} = 0 \text{ if } i \notin N_c(t) \tag{2.5}$$

This kernel updates every neighbour in the specified neighbourhood N_c with the same learning rate factor and results in a less smooth transition than the Gaussian based kernel.

Because the neighbouring nodes are learning from the same input vector as the winning node, we get a relaxation effect or smoothing effect on these nodes that in the continuation leads to a global ordering of the nodes (Kohonen, 1997). As Kohonen (1997) points out, if the network is not very large[9] the choice of process parameters is not very crucial when it comes to learning rate factor or neighbourhood function. The choice of neighbourhood size must be treated with a little caution though. If the size is too small the map will not be globally ordered.

[9] The author speaks of “a few hundred nodes at most” as being small (Kohonen, 1997).

2.4 Related Work

In this chapter, the reader will get acquainted with other work that is somehow related to our problem statement. Foremost, the work by Tamayo *et al.* (1999) will be discussed, because they used self-organising maps in their experiments when clustering expression profiles, and the work by Tamames *et al.* (1998) where they categorised proteins in functional classes on the basis of the annotation stored about them in databases.

2.4.1 Gene expression clustering

Tamayo *et al.* (1999) were the first to use self-organising maps to cluster gene expression profiles. They note a couple of shortcomings with other clustering techniques. Hierarchical clustering, for example, they mean is best suited for datasets that have true hierarchical order and expression profiles are not included in that list. They developed their own computer package which implements the SOM algorithm, called "Genecluster", which is publically available at the website of the Whitehead/Massachusetts Institute of Technology Center for Genome Research. The datasets they used involved the yeast cell cycle and hematopoietic differentiation and was preprocessed by excluding genes that did not change significantly across samples or genes that did not show a significant relative change, i.e. up- or downregulation.

In their results, they report clusters that contain genes that are known to be regulated at different phases of the cell cycle, e.g. G1, S, G2 and M. When comparing with results from the visual inspection of the same dataset by Cho *et al.* (1998), Tamayo *et al.* (1999) reports to identify the same clusters.

For a dataset containing 828 genes they recognised a SOM of 6X5 nodes to be suitable for their needs, as cited from (Tamayo *et al.*, 1999):

> "Although there is no strict rule governing such exploratory data analysis, straightforward inspection quickly identified an appropriate SOM geometry in each of the examples below." (p. 2909).

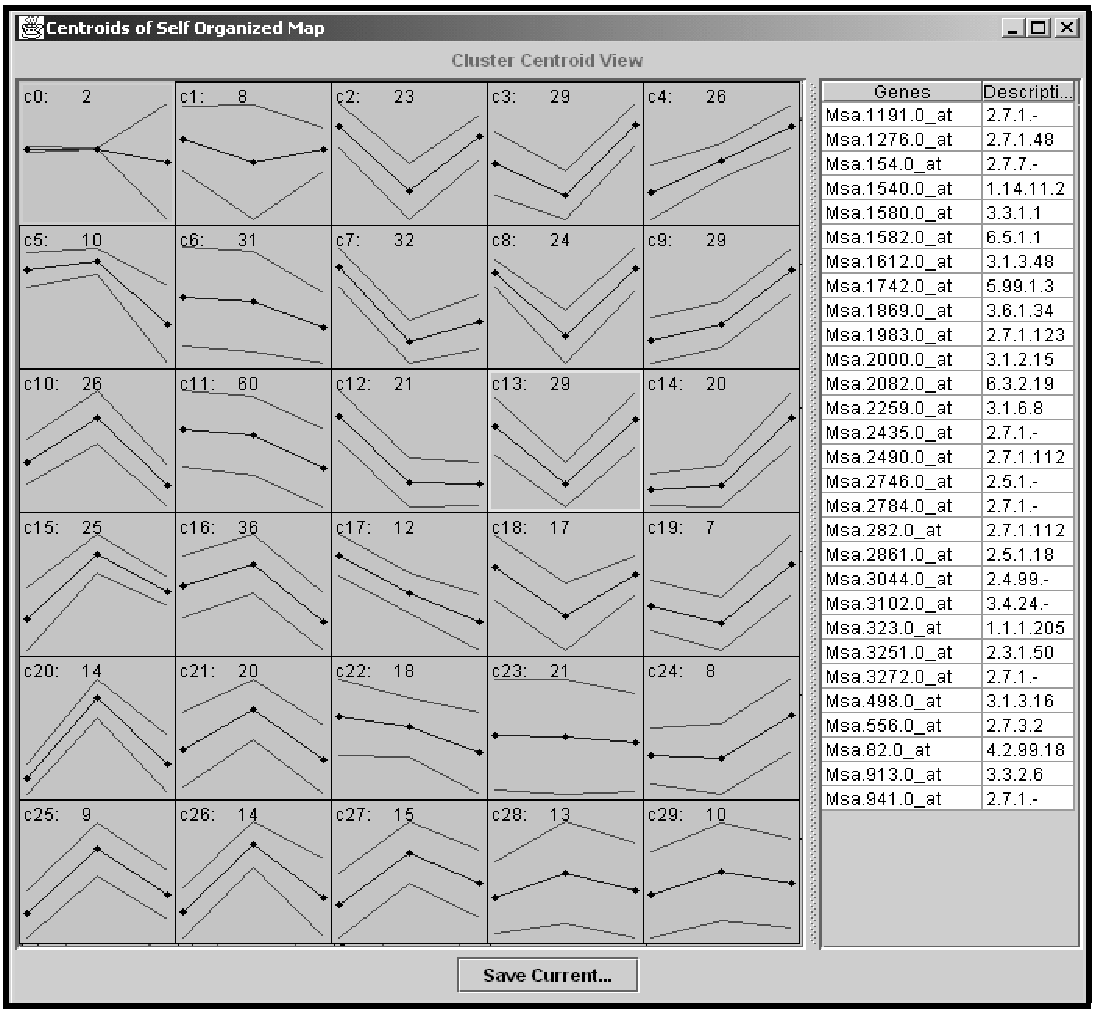
It is very hard, if not impossible, to gain information from their report about how the other process parameters of the SOM were set. Figure 6 illustrates the interface of the Genecluster program.

Eisen *et al.* (1998) used hierarchical clustering, according to the merging method, to cluster data from budding yeast *Saccharomyces cerevisiae* and human fibroblasts. The gene similarity metric they used is a form of correlation coefficient:

$$c_{ij} = \frac{\sum (x_{ki} - \overline{x}_i)(x_{kj} - \overline{x}_j)}{\sqrt{\sum (x_{ki} - \overline{x}_i)^2 (x_{kj} - \overline{x}_j)^2}} \tag{2.6}$$

The coefficient is a variant of the Pearson correlation coefficient (Eisen *et al.*, 1998). Their clustering approach resulted in clusters with genes that share similar functionality, e.g. genes that are known to be regulated and transcribed at a particular point of the cell cycle. Thus, the results show to some extent that expression data in some cases has tendency to organise genes into functional classes. Figure 7 illustrates the output from the software implementation by Eisen *et al.* (1998) of the hierarchical clustering method.

In a study by Wen *et al.* (1998) they examined the profiles of gene expression of 112 genes during development of the rat spinal cord. They used the FITCH software from the PHYLIP package (Felsenstein, 1993), which implements the hierarchical clustering method, in order to cluster gene expression time series. This study resulted in the five “waves” of gene expression that correspond to the different phases in spinal cord development of rats.



Genes	Descripti...
Msa.1191.0_at	2.7.1.-
Msa.1276.0_at	2.7.1.48
Msa.154.0_at	2.7.7.-
Msa.1540.0_at	1.14.11.2
Msa.1580.0_at	3.3.1.1
Msa.1582.0_at	6.5.1.1
Msa.1612.0_at	3.1.3.48
Msa.1742.0_at	5.99.1.3
Msa.1869.0_at	3.6.1.34
Msa.1983.0_at	2.7.1.123
Msa.2000.0_at	3.1.2.15
Msa.2082.0_at	6.3.2.19
Msa.2259.0_at	3.1.6.8
Msa.2435.0_at	2.7.1.-
Msa.2490.0_at	2.7.1.112
Msa.2746.0_at	2.5.1.-
Msa.2784.0_at	2.7.1.-
Msa.282.0_at	2.7.1.112
Msa.2861.0_at	2.5.1.18
Msa.3044.0_at	2.4.99.-
Msa.3102.0_at	3.4.24.-
Msa.323.0_at	1.1.1.205
Msa.3251.0_at	2.3.1.50
Msa.3272.0_at	2.7.1.-
Msa.498.0_at	3.1.3.16
Msa.556.0_at	2.7.3.2
Msa.82.0_at	4.2.99.18
Msa.913.0_at	3.3.2.6
Msa.941.0_at	2.7.1.-

Figure 6. A 6X5 SOM has been trained. On the left, the clusters are visualised with the codebook vectors of the SOM illustrated as the lines in the middle and the standard deviation is also marked with lines. On the right the genes of some selected cluster are presented. When saving the result, two files are generated. One that contains the codebook vectors for the nodes and the other contains the datapoints together with their respective cluster number. The picture is a snapshot taken from a fictive training with the Genecluster program[10].

[10] The GeneCluster program can be found at http://www.genome.wi.mit.edu/MRP/software.html.

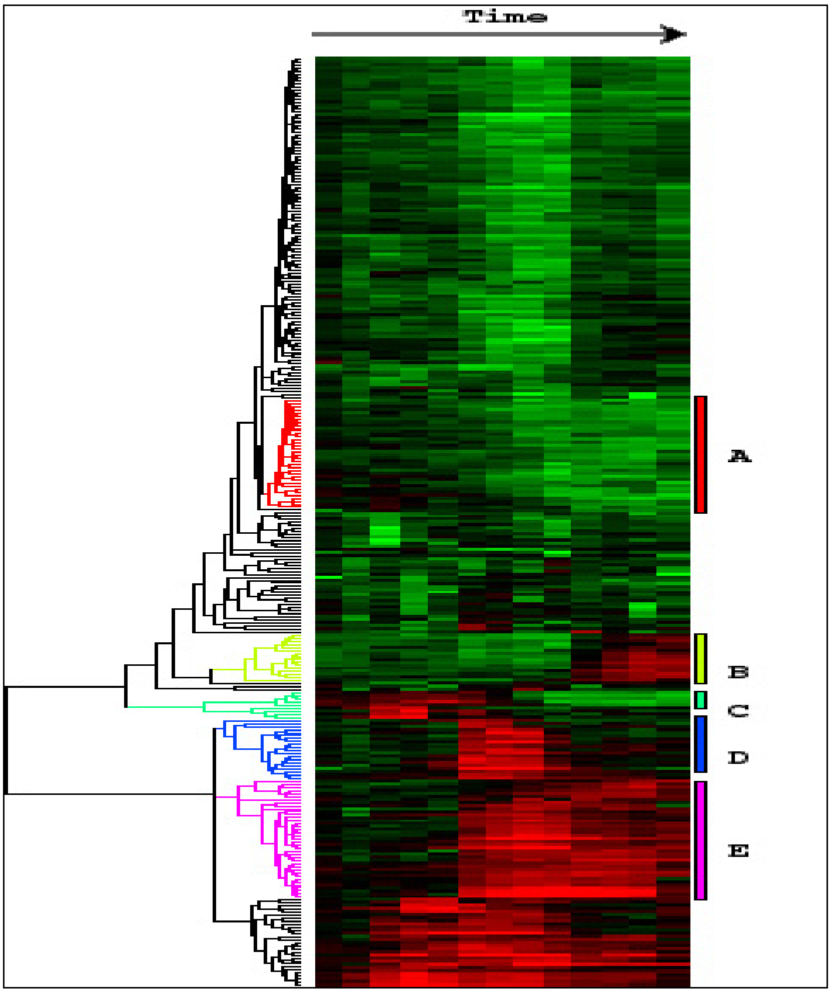


Figure 7. Results from a hierarchical clustering obtained from the Cluster and TreeView implementation[11]. The tree is taken from Eisen *et al.* (1998) by courtesy of Eisen.

2.4.2 Classification of annotation

Tamames *et al.* (1998) presents the construction of the EUCLID system, which uses the functional information provided by databases to classify proteins according to their major functionality such as transcription or intra-cellular communication. A problem with this method that they have come across is that the annotation in the

[11] The software can be found at http://rana.stanford.edu/clustering.

databases often is very specialised and not on the major functional level. They write for example:

> “…annotated as a cdc2 kinase, but not as being involved in intra-cellular communication.” (p. 542).

So, instead of using the annotation directly, they extracted characteristic keywords for each functional group from a set of proteins that had been classified by a human expert. These keywords, constituting a dictionary, were then used to extract from the database other proteins that matched with the keywords. These new proteins were analysed and additional keywords were added to the dictionary. This is an iterative process that finishes when there is no increase in classification quality. In their experiments they created a dictionary for three functional classes, i.e. ENERGY, INFORMATION and COMMUNICATION. With this method, they managed to correctly classify over 90% of the proteins.

Biologically, the gene expression clusters that are produced with some clustering technique are not completely accurate since they lack functional coherence among the genes. It is, for example, possible for a housekeeping gene and a target gene to have the same expression profile. Housekeeping genes perform the functions that are required for the viability for the cells, such as making cell membranes and controlling cell division. That they have the same expression profiles means that they are clustered together, even though this is something we do not want. We want to discard the housekeeping genes because they are of no interest. Although the clusters obtained through some annotation classification method are in some sense biologically accurate, they do not identify the affected genes we would be looking for in experiments where we compare sick to healthy tissue for instance.

In Ben-Dor *et al.* (2000), they note that clustering methods such as hierarchical clustering or self-organising maps do not use any tissue annotation as input to the learning algorithm. Further, they point out that the annotation is only used to assess the success of the clustering technique.

The central question investigated in this project is whether gene expression profiles can be clustered together with annotation as input and produce the more biologically correct clusters we want as a result. We will confine our studies to one clustering technique and it will be the self-organizing maps (SOMs). Next chapter holds the thesis statement where the aim is defined along with the objectives that have to be met in order to fulfil the aim.

3 Thesis statement

The aim of this project is to investigate whether clustering of gene expression patterns can be done more biologically accurate by providing the clustering technique with additional information, annotation, about the genes besides the expression profiles. This aim lets us formulate the following hypothesis, which will be tested in this thesis:

Hypothesis

By providing the clustering technique with not only data on the gene expression levels, but also annotation about the genes, the clusters formed will show a higher biological accuracy, than when just the expression levels are used as in the approach of Tamayo et al.,(1999).

The hypothesis will be falsified if no clear improved correlation to the biological reality can be shown regarding the clusters.

3.1 Motivating the aim

In chapter 2.4 two different approaches to clustering, or classification of genes were presented. The gene expression clustering on one hand and the annotation classification on the other. Clearly, both approaches contribute with something important to the area of gene function analysis. Clustering techniques on expression data help us identify clusters of genes that have changed their expression levels in a similar way over a certain time interval due to, for instance, some drug treatment. The classifying method that worked on functional annotation stored in databases helps us classify genes in functional categories by comparing keywords in its annotation with what is stored for the different classes.

A problem with the first method is that there are always ”unwanted” genes in the good clusters found, e.g. with housekeeping genes, or with genes where the function is unknown. Nothing can be said about these genes of unknown function. Maybe they really should belong to the cluster and have something to do with the functionality of the other, interesting genes. Or maybe, by coincidence, they just have the same expression profile as the other genes, but not as an effect of the drug treatment. A problem with the latter method is that it cannot identify genes that have changed their expression profile due to some treatment, or health condition.

Therefore, we believe that a combination of both these methods, where we cluster genes on the basis of both expression profiles and annotation, can produce clusters that are more informative. Informative in such a way that when an interesting cluster is found, it can be assumed that all the genes that are in it really should be there and have some functional coherence with each other.

3.2 The objectives of the project

For the fulfilment of the aim, six objectives have been identified and they can be summarised in the following six questions:

- **What datasets should be used?**
- **What annotation should be used?**
- **How can the annotation be used?**
- **How to set the parameters for the SOM?**
- **How to measure "biological correctness"?**
- **How to evaluate the results?**

Datasets must be decided upon. There are numerous datasets publically available to use. Most of them contain gene expression profiles collected from yeast or mouse cells. The more the dataset has been studied, the more data we can use in the evaluation process of our method. A problem with the publically available datasets on gene expression profiles is that they do not contain annotation. This is information that has to be extracted separately from databases.

When it comes to the choice of annotation, it must say something about the gene's specific function to help in the clustering. It must be an annotation that helps divide the genes in natural categories. Gene name, or sequence length are surely too individual annotations that cannot help group genes together according to their functionality. Some description field that elaborates on a gene's function could be used, but the problem is how to encode it to an acceptable form that SOMs can take as input.

This means that it has to be encoded into real numbers, or vectors of real numbers. These vectors should not vary in size and should not impose an internal order on the annotation. For example, if the annotation identifies four totally different categories, we should not come up with a coding scheme that turns the category names into one, two, three and four. That would mean that category one and two are more similar than one and four since Euclidean distance is used in the training algorithm. The similarities of the function of the genes in the categories might be the other way around.

As explained in chapter 2.3.1, when training SOMs, there are many parameters that have to be taken into consideration. Some parameters, for example the topology of the map, can be empirically tested and decided upon early in the project, while other parameters can vary from experiment to experiment throughout the project. Although desirable, there will be no need to find an optimal map, defining which would probably be an entire project by itself, since we only want to show relative improvements regarding the clustering with more detailed data on a map with the same parameter values.

The results can be evaluated both statistically and biologically. Statistical measures such as standard deviation, compactness and isolation of the clusters can be applied. Furthermore, correlations between clusters and annotation can be computed. While the former measures belong strictly to the statistical evaluation process and nothing can be said about biological validity, the latter measure does and can perhaps be used to measure the biological correctness of a clustering. An important component in the biological evaluation is to use current knowledge of metabolic pathways, functional categories and known gene clusters where it is known which genes are clustered together and should be clustered that way. This evaluation has to mostly be done manually. The annotation of the genes that are clustered together, (other annotation than the one used in the clustering of course), can be compared and scanned for similarities. The similarities, in turn, could indicate that they are biologically alike and thus that, the clusters are biologically accurate to some extent. In addition, interesting clusters can, in this project, be sent to AstraZeneca R&D Mölndal Sweden for evaluation in order to see if the results make biological sense.

4 Method and experiments

This chapter discusses the methods used and how the objectives are met. Furthermore, it deals with the experiments carried out in this project. Each objective is discussed in a separate chapter in the same order that they were presented in chapter 3.2. First, in chapter 4.1, the choice of what dataset to use is discussed and what implementation of the SOM algorithm to use. In chapter 4.2 the choice of annotation is made clear along with a thorough explanation of the chosen annotation. Chapter 4.3 elaborates on how the annotation was used and presents the form of the input vectors. In chapter 4.4 decisions are made regarding how to set the parameters for SOMs, chapter 4.5 presents the experimental design and finally chapter 4.6 describes the measures that were used in the statistical evaluation and how the qualitative, or biological, evaluation was done.

4.1 What dataset should be used?

There are numerous datasets publically available to use. Most of them contain gene expression profiles collected from yeast or mouse cells and contain data collected at several time points, e.g. often nine or even fifteen. They have been widely studied and used in previous experiments on gene expression clustering, e.g. (Tamayo *et al.*, 1999; Eisen *et al.*, 1998). Since this project aimed to *improve* clustering of gene expression data, it would have been ideal to use a well-studied dataset. The more it has been studied, the more data could we use in the evaluation of our method.

There are, however, some disadvantages with using previous findings in the evaluation process that takes the advantages away from using the well-studied datasets. In previous experiments (Tamayo *et al.*, 1999; Eisen *et al.*, 1998), they report only positive findings in their results and not bad, such as *misplaced* genes, i.e. genes that are clustered together with other genes that have totally different functionality. When evaluating against such results, we cannot use the results themselves as the right answers. We would have to compare their results with ours, with some kind of method that takes advantage of the annotation about the genes. Only then could we show if our method has improved the clustering.

A big problem with the method above is how to collect the annotation for the genes in these datasets. There are no finished and preparated datasets that contain both expression profiles *and* every annotation that is known about the genes in it. We would have to compile our own dataset by extracting the information we need from public databases for each gene. Depending on what annotation we use and since the annotation might be spread between different databases, we could end up needing something from every database. This could turn out to be inefficient and timeconsuming.

Through AstraZeneca R&D Mölndal Sweden we have gained access to a dataset that is not public and thus less well studied, but it has another, big advantage instead. The dataset already contains plenty of annotation, e.g. enzyme classification, functional hierarchy and metabolic hierarchy. With this dataset it could be a problem to evaluate the results, because it has not been studied before, but as it turns out we still have enough foundation for the evaluation process in the study of the annotation. Furthermore, as mentioned above, results from other experiments could not be used as right answers. The evaluation process is further discussed in chapter 4.6. Another advantage is that since this project is in collaboration with AstraZeneca R&D Mölndal Sweden we have help from their experts to evaluate our results. This dataset will be used in this project.

The dataset consists of data from several different mouse tissues: liver, brown fat, epididymus, mesenterial fat and white quadriceps. Each contains expression profiles for ~6500 genes[12] collected with GeneChips from Affymetrix. Since genes have diverse behaviour in different tissues we have made the assumption that genes would be clustered differently in each of the above datasets. To evaluate all these different clusters and keep track of when and where the genes are up- or down-regulated would be very time consuming and beyond the scope of this dissertation. Thus, we have decided to delimit ourselves and only conduct our experiments on the data from the mesenterial fat. The tissue has been treated with a substance called *Rosiglitazone* (Barman Balfour *et al.* 1999), which is a thiazolidinedione. This is a new class of antidiabetic agents, which enhances sensitivity to insulin in the liver, adipose tissue and muscle. The result is improved glucose disposal, i.e. reduction of bloodsugar. Insulin resistance often underlies type 2 diabetes mellitus (non-insulin-dependent diabetes) (Barman Balfour *et al.* 1999).

Expression levels have been collected at three timepoints: day zero, three and seven. The expression levels from day zero indicate the expression levels under normal conditions, i.e. the tissue is yet untreated. Day three and seven indicates the expression levels after the mice have been treated for three and seven days

[12] A mouse has somewhere between 50,000 and 100,000 genes in total.

respectively with the substance. The target genes, i.e. the genes we are expecting to be affected by the substance, are genes that are related to leptin and obesity and also food-intake.

A noteworthy disadvantage regarding the amount of datapoints compared to other datasets is that we only have three timepoints to build the profiles on. Since they are spread over seven days, we pretty much catch the trends of up- and down-regulations, but the more fine-grained the time-series, the more fine-grained clusters we would get with the SOMs. This disadvantage is not so serious, since in the resulting clustering we are looking for clusters of genes that are up- or down regulated at least two-fold in their expression levels. Only these genes are considered to be affected by the substance according to personal communication with AstraZeneca R&D Mölndal Sweden. Thus minor changes, or variations, in between the time-points can be disregarded (Tamayo *et al.* 1999).

4.1.1 Choice of clustering technique and implementation thereof

As earlier mentioned, we have delimited ourselves to use SOMs in our experiments. We chose to work with the implementation by Tamayo *et al.* (1999) that is publically available and can be found at their website[13]. This choice was made mainly because it saved us time on not having to implementing the algorithm by ourselves and that it had a built-in visualisation tool.

[13] The Genecluster program can be found at http://www.genome.wi.mit.edu/MRP/software.htm.

4.2 What annotation should be used?

From chapter 2.1.3 we know that there are all kinds of annotation. To help in the clustering the annotation must say something about the gene’s function. It must be an annotation that helps divide the genes into natural categories. Furthermore, the annotation should be known for all, or sufficiently many of the genes in the dataset. It would be no point in using an annotation that is known for only 1% of the genes, this would not help the clustering in large. If it is too little it could be either that the annotation is known for only one category of genes and thus only help making one cluster with these genes. The other possibility would be that only a few, one or two, genes are known to belong to each category and it seems it would be difficult for those to direct the other genes into meaningful clusters.

The enzyme classification (EC) annotation is known for about 10% of the genes in the mesenterial fat dataset. Now, 10% may seem to be low, but it is as good as it gets, because the reality is that most functional annotation is known for only a fraction of the genes as explained in Bassett *et al.* (1999). So, these 10% is a set of 609 genes, which we considered to be large enough for our experiments. Furthermore, because it is a functional annotation it helps divide the genes into natural categories. Next chapter contains a detailed description of the enzyme classification annotation.

4.2.1 Enzyme classification

The enzymes are classified according to the catalytic reaction they are involved in. There are six main classes of enzymes[14] and these are:

1. Oxidoreductases
2. Transferases
3. Hydrolases
4. Lyases
5. Isomerases
6. Ligases

Each classified enzyme has an EC number, but this number is not unique. This is because several different ezymes can have the same catalytic function. The number is on the form EC 1.2.3.4, where the first number stands for which of the main classes above the enzyme belongs to: the 1 in 1.2.3.4 denotes that this enzyme is an oxidoreductase. The next two numbers are subclasses and the last number is the serial number of the enzyme in its sub-subclass. The subclasses stand for different things depending on which main class it is under. To complete the example above, the first subclass (2 in the example) stands for which molecule is oxidated, and the sub-subclass (3 in the example) indicates which acceptor that is involved in the process. The last figure in the code number is the serial number (4) of the enzyme in its class.

[14] The information on EC is taken from Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) website, www.chem.qmw.ac.uk/iubmb/enzyme/.

For the second class – the transferases – the first subclass stands for which group that is transferred and the sub-sublevel gives further information on the transferred group. Common for the six classes is that it is mainly the main class belonging that decides the function of the enzyme.

In our mesenterial fat dataset the distribution of the enzymes in the classes is shown in table 2.

Enzyme main class	Number of enzymes	percentage of whole set
EC 1	120	20%
EC 2	217	36%
EC 3	200	33%
EC 4	29	5%
EC 5	21	3%
EC 6	22	4%
SUM	**609**	**100%**

Table 2. The distribution of the enzymes.

To recapitulate, this is a functional annotation that we had for about 10% of the genes, which gave us a dataset of 609 genes for experiments testing the hypothesis. It divides genes in six functional classes, which seems to be sufficient, i.e. not too many and not too few. If there were too many classes it could be hard to keep track of all of them and if there were too few, e.g. two classes, they would be too general for the purpose of functional categorisation. Moreover, the annotation is based on numbers, which seems to be easier to encode into SOM input than a description field would have been. Next chapter elaborates on how this was done.

4.3 How can the annotation be used?

The EC numbers must be coded in such a way that any order between the classes is lost. This is because SOM uses the Euclidean distance as similarity measure and thus class 1 and 2 would be considered more similar to each other than class 1 and 5. Since EC 1.1.1.1 is not a numeric value, it first has to be converted to a value. Otherwise we cannot use it as input for the SOMs, since we cannot measure the Euclidean distance directly between symbols. The easiest way would be to just cut the last two sublevels of the EC number and encode the example into 1.1 and we would still have the overall function of the enzyme included in the encoded annotation. By doing so, we would get potentially about 60 categories that we could divide the genes into. As explained in chapter 4.2.1 the first number stands for the overall function of the enzyme and the second number, the first sublevel, stands for on what kind of molecule it functions. This indicates that it could be enough to just use the first number, i.e. we would get six categories instead of sixty.

Since the enzyme classes are not intentionally ordered, but defined in an order, we have to encode each of the classes 1-6 into some other representation that does not reflect order among them. This can be done by encoding each class into a vector of three random real numbers between 0-1, e.g. <0,24521; 0,98332; 0,44304>. If each attribute in the vector is considered as a dimension, we get a three dimensional space and the classes can be represented in such way that they have near to equal distance between each other in the three dimensional space and we would lose the order between them. Table 3 shows the Euclidean distances between the coding vectors used in the experiments of this project.

EC-number	2	3	4	5	6
1	0.72266	0.39744	0.62093	0.57456	0.58787
2	-	0.70934	0.39561	0.58364	0.44393
3	-	-	0.51519	0.4498	0.81371
4	-	-	-	0.66715	0.6371
5	-	-	-	-	0.71997

Table 3. The distances between the vectors coding for the annotation. The largest distance is ~0.81 and the smallest distance is ~0.40 between the vectors.

4.3.1 Construction of input vectors for the SOM

We prepared three different sets of input vectors for the SOM.

Set 1. Only the expression profiles

Set 2. Expression profiles and their slopes

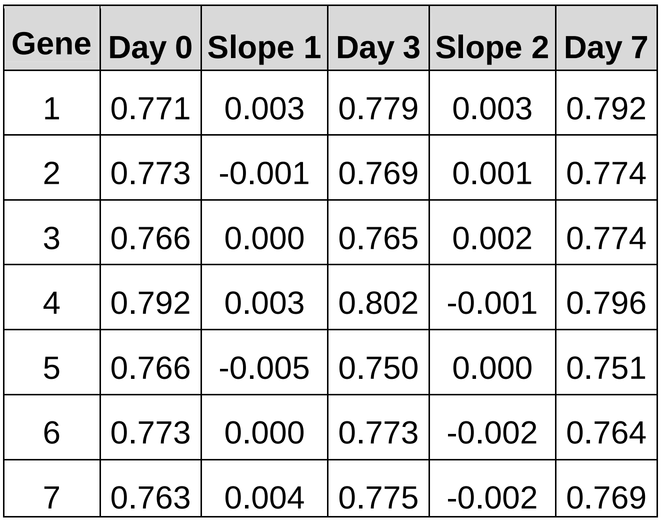
Set 3. Expression profiles, their slopes and the encoded EC numbers

Set 1 consisted of only expression profiles. In order to minimise the effects of the large variations in the expression levels, we did not use the raw data directly. We used the 2-logarithm of the expression levels and normalised them to be in the interval 0 < x <1, for an example see table 4. The relative variation between each gene was, of course, still preserved in the data, but the interval is smaller and the concentration is more on similarities between the actual profiles and not between the actual levels.

Gene	Day 0	Day 3	Day 7
1	0.771346017	0.779162098	0.792259907
2	0.772803864	0.76874314	0.774173496
3	0.766410688	0.765115896	0.774163847
4	0.791746196	0.801860519	0.796274477
5	0.766040194	0.750149866	0.751099564
6	0.773106583	0.772519855	0.76380967
7	0.762513435	0.775424828	0.768936377

Table 4. Example of input vectors for set 1.

Set 2 consisted of the same set as above except for that the slopes between the timepoints were added, see figure 8. This set was prepared mainly for the reason of comparing it with set 1. When the slopes are added more attention is put on the profile itself, than the expression levels at each timepoint. Basically, we added information about whether the gene was down- or up-regulated. Table 5 shows how the input vectors in set 2 looked like.



Gene	Day 0	Slope 1	Day 3	Slope 2	Day 7
1	0.771	0.003	0.779	0.003	0.792
2	0.773	-0.001	0.769	0.001	0.774
3	0.766	0.000	0.765	0.002	0.774
4	0.792	0.003	0.802	-0.001	0.796
5	0.766	-0.005	0.750	0.000	0.751
6	0.773	0.000	0.773	-0.002	0.764
7	0.763	0.004	0.775	-0.002	0.769

Table 5. Example of input vectors for set 2.

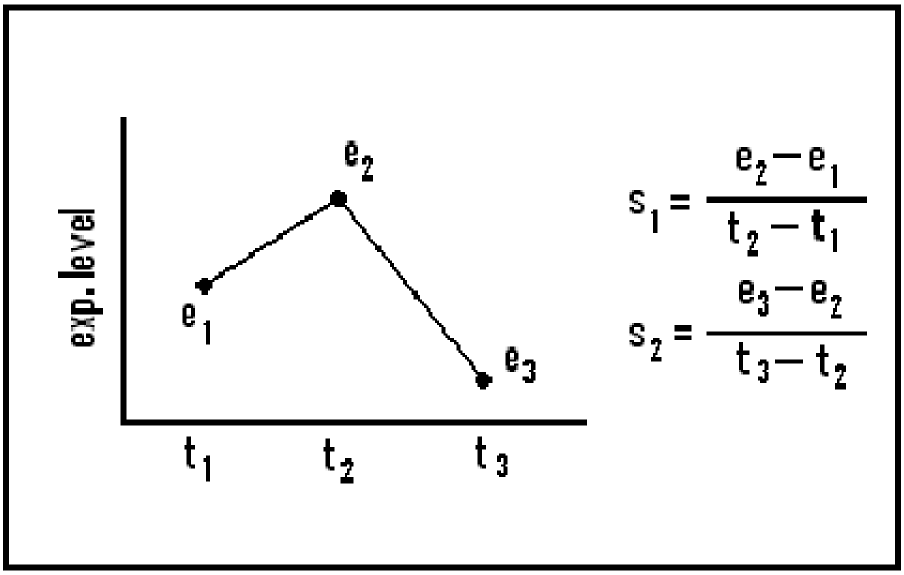

exp. level
e_2
e_1
e_3
t_1
t_2
t_3
$s_1 = \frac{e_2 - e_1}{t_2 - t_1}$
$s_2 = \frac{e_3 - e_2}{t_3 - t_2}$

Figure 8. Calculation of the slopes.

Set 3 was the same as set 2 with the annotation added to it. Table 6 shows the input vectors for set 3. The annotation was inserted in between the timepoints and the slopes so that all features, i.e. timepoints, slopes and annotation, were mixed and not treated separately.

Gene	Day 0	EC# 1	Slope 1	Day 3	EC# 2	Slope 2	Day 7	EC# 3
1	0.771	0.499	0.003	0.779	0.051	0.003	0.792	0.594
2	0.773	0.448	-0.001	0.769	0.438	0.001	0.774	0.517
3	0.766	0.058	0.000	0.765	0.062	0.002	0.774	0.039
4	0.792	0.627	0.003	0.802	0.021	-0.001	0.796	0.164
5	0.766	0.448	-0.005	0.750	0.438	0.000	0.751	0.517
6	0.773	0.014	0.000	0.773	0.003	-0.002	0.764	0.427
7	0.763	0.058	0.004	0.775	0.062	-0.002	0.769	0.039

Table 6. Example of input vectors for set 3.

4.4 How to set the parameters for the self-organising maps?

As discussed in chapter 2.3.1 there are a few parameters that have to be set when training SOMs, such as learning rate and the map's topology. Since there is no analytic solution on how to set them to give an optimal clustering, this had to be empirically tested. We trained a series of SOMs[15] with different parameter settings, and to evaluate how much they differed depending on the settings we calculated the number of genes that had been clustered differently between two clusterings. That is, if two genes are clustered together in one clustering and not in another – 1 point is added to the score. This means that the higher the score, the less similar, or dissimilar, the two compared clusterings are. We refer to this score as the similarity

[15] These experiments were conducted on set 1, see chapter 4.3.1.

score between two clusterings. The effects of the following process parameters were tested, see Appendix A for extensive results:

1. Learning rate
2. Learning radius (size of neighbourhood)
3. Neighbourhood function (gaussian and bubble)
4. Topology of map (number of nodes)

The baseline for how many genes that changed clusters between clusterings was approximately 2,000-3,000. Since we in fact count how many pairs of genes that are clustered together in both clusterings, the maximum score would be (609*608)/2=185,136. This gives a baseline of approximately 1% genes that 'jump' clusters in the different clusterings.

The results from these experiments showed that changing learning rate and neighbourhood function only produced changes in the clusterings that were around the baseline, see figure 9. As mentioned in chapter 2.3.1, learning radius has a little more effect on the result. The mean score when comparing clusterings with high learning radius, half the map for instance, was well below baseline. These results can be found in figure 10 .

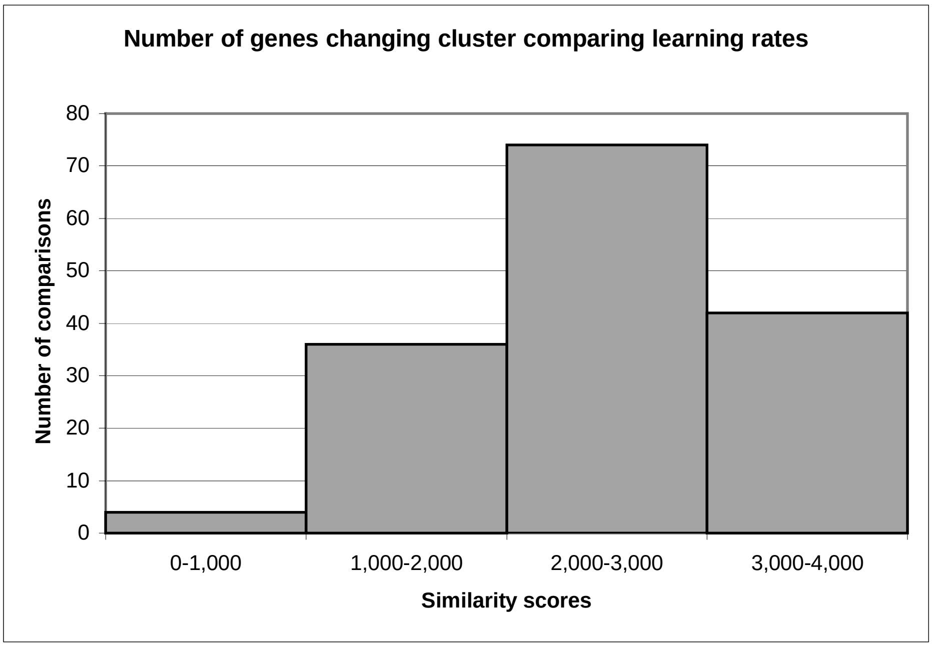


Figure 9. The similarity scores when comparing different learning rate settings. The x-axis shows the similarity scores and the y-axis shows the number of comparisons in a certain interval. 13 clusterings were compared, generating 12*13=156 similarity scores. For example, 74 of the comparisons generated scores in the interval 2,000-3,000 and 4 of the comparisons generated scores under 1,000. The mean score between the compared clusterings was 2,461 genes changing cluster, which is around the baseline.

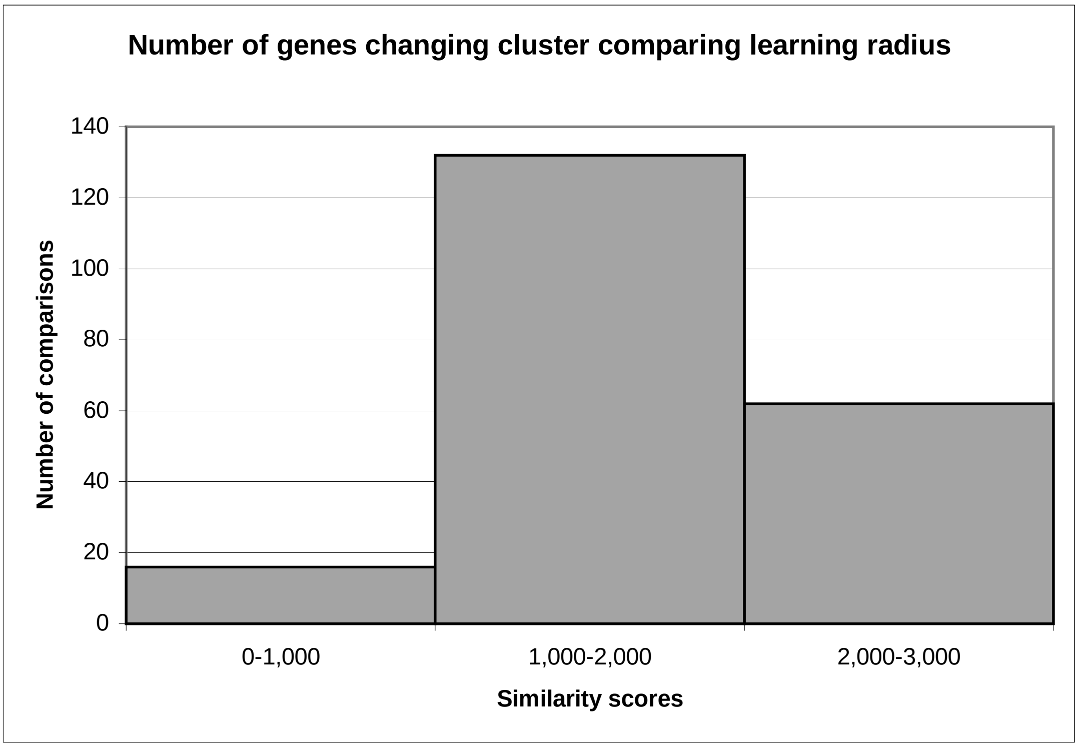


Figure 10. This figure illustrates the scores when comparing different learning radius settings. 15 clusterings were compared. In most of the clusterings the genes are clustered in the same way with scores of the comparisons below the baseline The mean score was 1,692 genes changing cluster, which is below the approximative baseline.

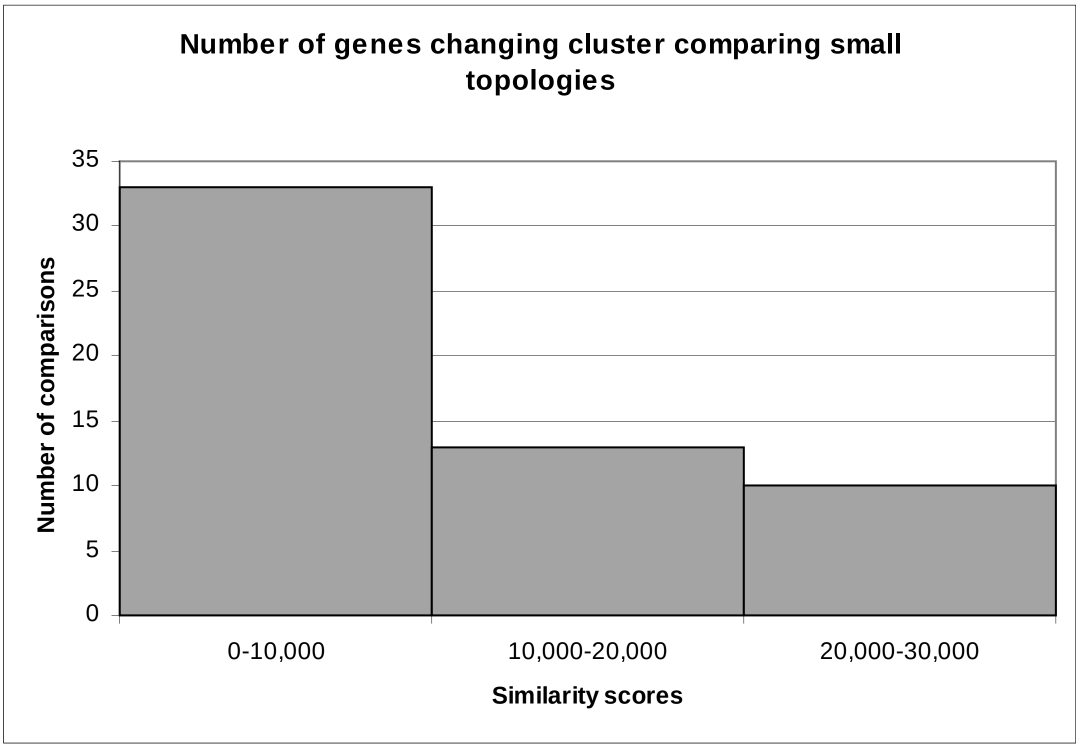


Figure 11. This diagram shows that when using small topologies, 9-25 nodes, the clusters are very generalised and genes often jump to different clusters between different clusterings. Eight clusterings were compared and the bigger the topology got, the smaller the score. Thus the clusters get more stable and fewer genes jump clusters as the topology grows. The mean score of 11,140 gives that on average 6% of the genes are clustered differently between the clusterings.

When testing different topologies, the results varied drastically between small and large topologies. On small topologies, approximately 6% of all the genes were clustered differently between clusterings. On large topologies, approximately 2% of the genes were clustered differently, see figures 11 and 12.

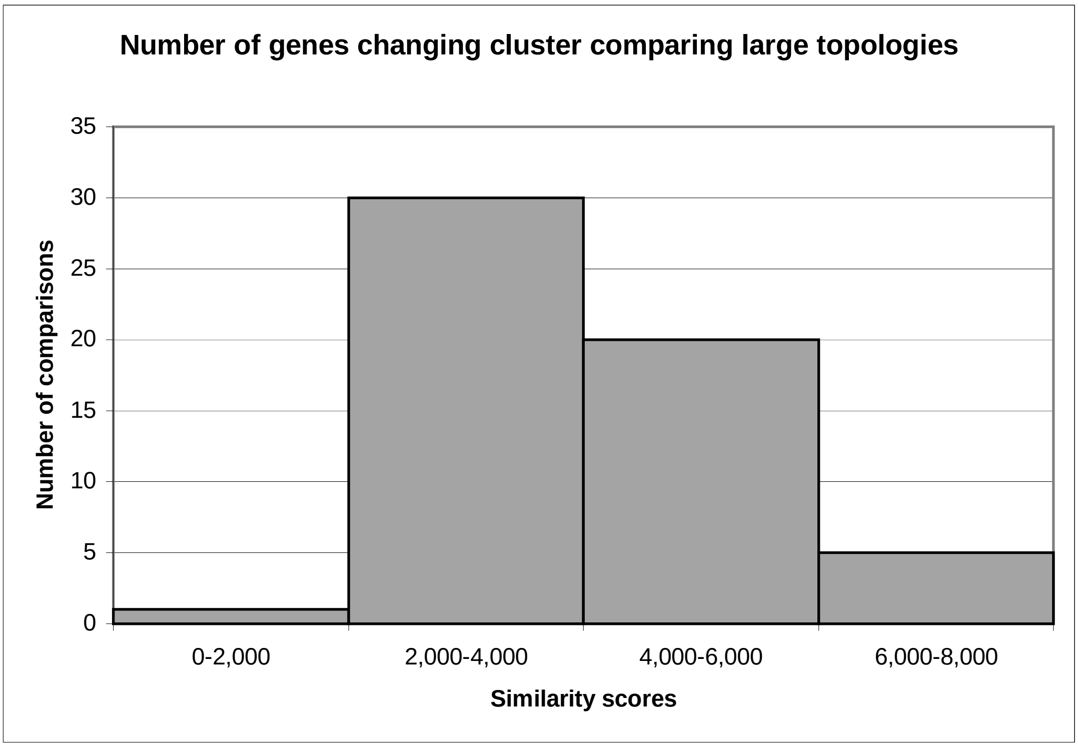


Figure 12. In this diagram, eight clusterings with large topologies, 30-60 nodes, are compared. The mean score of 4,018 is more or less a third of the mean score for the small topology comparison. It means that 98% of the genes are clustered the same way in all clusterings on large topologies, see Appendix A for extensive testing results.

Thus, after having tested the different parameter settings it was clear that, under the premises of this project, neighbourhood function and learning rate did not affect the resulting clustering significantly and the default settings of the software were used. The learning radius parameter was set high, which was approximately half the topology size, in order to help the global ordering as discussed in chapter 2.3.1. The only parameter that really affected the outcome was the topology of the SOMs. Tamayo *et al.* (1999), for example, states nothing about the impact of other parameters than the amount of nodes. With very small topologies, we get large and messy clusters and the larger we make the map, the smaller and tighter the clusters get. After enough nodes have been added, adding more nodes do not contribute to other results, i.e. 99% of the genes are clustered in the same way. There is a limit at which point nodes are starting to be left empty and the more nodes that are added, the more empty nodes we get. The drawn conclusion is that the map topology must be adjusted according to the size of the dataset and the rest of the parameters are of relatively small importance and therefore the default settings in the Genecluster program were used except for the adjustment of the learning radius.

4.4.1 Experimental design

When clustering on sets 1-3 (see chapter 4.3.1), 30-60 nodes were found to be sufficient because with this ~98.5% of the genes were clustered in the same way in all our experiments. Twelve clusterings were performed on each of the three sets 1-3 with varied topology, e.g. 36 nodes. The evaluation was done on all of these clusterings.

4.5 How to measure “biological correctness”?

The approach to evaluate the biological correctness of a clustering that often is used in gene expression studies is visual inspection of clusters in order to find clusters of interest. This is often performed in an ad hoc manner and the validation is performed by investigating the functional annotations of the genes. If genes that are clustered together also share the same functionality we can say that the cluster is biologically correct.

4.6 How to evaluate the results?

In this chapter we present the evaluation methods that were used. The first subchapter explains the statistical measures and the second subchapter deals with how the biological evaluation was performed.

4.6.1 Statistical evaluation

We have used the following statistical measures in the evaluation:

1. Standard deviation within the clusters as a measure of compactness
2. Similarity score (as explained in chapter 4.4)
3. Compactness and Isolation
4. Conditional entropy
5. Mutual information

The standard deviation is defined as:

$$\mathrm{SD} = \sqrt{\frac{\sum (\mathrm{X} - \mu)^2}{N - 1}} \tag{4.1}$$

Where X is the sample set, μ is the mean value of the sample set and N is the number of samples. The standard deviation was calculated for each of the time points of the genes in a cluster, giving three standard deviations per cluster. The standard deviation gave a measure of the variation of the expression profiles within a cluster, i.e. how compact the cluster was. The standard deviation measure let us compare the clusterings done on sets 1 and 2 with the clusterings done on set 3 to see how the use of annotation affected the compactness of the clusters. The standard deviation was calculated for all clusterings and the results are presented in chapter 5.

The similarity score measure that was introduced in chapter 4.4 was calculated for the different clusterings. Clusterings with datasets 1 and 2 were compared to see if it was basically the same genes that were clustered together, despite the use of slopes as extra information in set 2. The assumption was that adding the slope information would result in more compact clusters. Clusterings with datasets 2 and 3 were compared for the same reason. We wanted to see if the use of annotation in set 3 would affect which genes that were clustered together.

As explained in Jain and Dubes (1988), the two main properties of a cluster are compactness and isolation. Compactness measures the cohesion of the data points in a cluster and isolation measures the separation between the cluster and the other patterns in the dataspace. The measures are illustrated in figure 13.

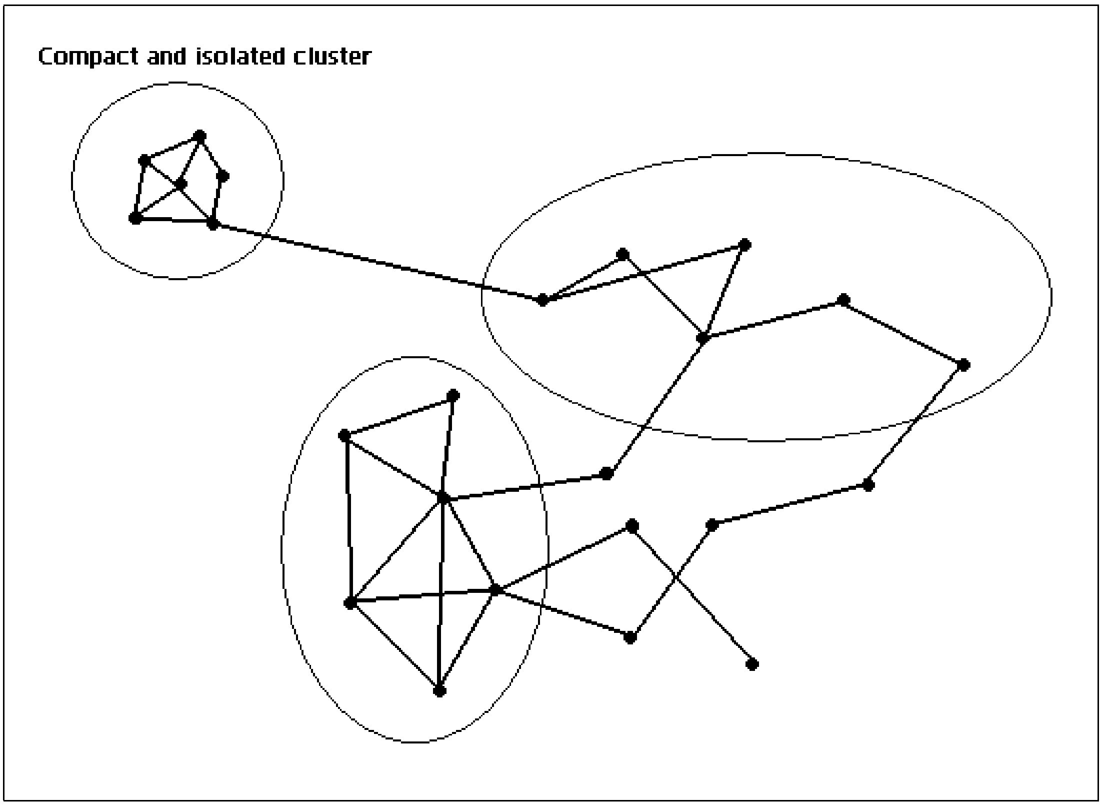


Figure 13. Illustration of compactness and isolation. Not all edges between the datapoints are drawn. The cluster in the top left corner exhibits both high compactness and isolation. The inner edges of the cluster are short and the edges linking the cluster to the other points in the dataspace are longer. The other two depicted clusters are less compact and isolated. This is because their inner edges are longer, relatively speaking, and the linking edges to other datapoints are shorter.

The compactness index is defined as the mean of the number of edges between the datapoints internal to a cluster, as a proportion of all inner edges of the cluster, at each level of proximity. Each level of proximity means that all edges between all the genes in the dataset are ordered in a vector of proximities. These edges, or distances, are taken one by one, starting with the shortest, to see if it corresponds to a pair of genes in the cluster we are calculating the index for. At the first level of proximity we look to see if the shortest edge is internal to the cluster. If it is, we increase the compactness index. At the second level of proximity we look to see if the second from shortest edge is internal to the cluster and so on and so forth.

$$\text{Compactness Index} = \frac{\sum_{i=1}^{N} \frac{e_i}{k}}{N} \qquad (4.2)$$

Here, e_i is the number of inner edges of a certain cluster at proximity i and k is the total number of inner edges of the cluster. N is the total number of edges, or distances, between the genes, i.e. 609*608/2=185,136 edges in our dataset. The higher the compactness index, the more compact the cluster is.

The isolation index is defined as:

$$\text{Isolation Index} = \frac{\sum_{i=1}^{N} \left(1 - \frac{b_i}{l}\right)}{N} \qquad (4.3)$$

Where b_i is the number of linking edges from the cluster to other patterns in the dataspace at proximity i, and l is the total number of linking edges from the cluster. N is the total number of edges. The higher the isolation index, the more isolated the cluster is.

As the question investigated was to find out if we could obtain more biologically correct clusters when clustering with both expression profiles and annotation, we used conditional entropy and mutual information measures. This to find out how well our different datasets, sets 1-3, were correlated to the biological annotation we used in the biological evaluation, see chapter 4.6.2.

The conditional entropy is defined as (Pierce, 1980):

$$H_x(y) = \sum_{x=1}^{m}\sum_{y=1}^{m} - p(x)p_x(y)\log p_x(y) \tag{4.4}$$

Where *x* is the datapoint in the set 1, 2 or 3 and *y* is the annotation that corresponds to the certain gene and *m* is the total number of samples. The conditional entropy gives us a measure of how good the input to the SOM for a gene is on predicting the annotation, i.e. is there a correlation between the expression profiles of a gene and its annotation.

The mutual information is a measure of the reduction of the entropy of the annotation if we know the expression profile for the gene, i.e. how much information do we acquire about the annotation from the expression profile of a gene (Durbin *et al.*, 1998). It is defined as (Durbin *et al.*, 1998):

$$MI(x,y) = \sum_{x=1}^{m}\sum_{y=1}^{m} p(x,y)\log\frac{p(x,y)}{p(x)p(y)} \tag{4.5}$$

Where *x* is the datapoint in the set 1, 2 or 3 and *y* is the annotation of a certain gene and *m* is the total number of samples. The annotation we tried to 'predict' was on the same form as the EC-numbers, i.e. B1.2.3.4 and C4.3.2.1. The first is a functional annotation and the second is a metabolic pathway classifying annotation. Experiments were performed on trying to predict the two annotations separately and together.

The conditional entropy and mutual information were calculated for the following combinations:

1. Expression profiles, i.e. set 1, against the functional annotation.
2. Expression profiles against the metabolic pathway annotation.
3. Expression profiles against a combination of the functional and the pathway annotation.
4. Expression profiles in combination with the EC annotation, i.e. set 3, against the functional annotation and the pathway annotation separately and in combination.
5. EC annotation against the functional- and the pathway annotation separately and combined.

If the expression profile in combination with the EC annotation gives better results than the other two inputs, it supports our hypothesis that the expression profiles really should be clustered together with annotation and not by themselves.

4.6.2 Biological evaluation

We used Hubert's Γ statistics in normalised form (see equation 4.6) to measure the correlation between how the genes were clustered and what annotation they had (Jain and Dubes, 1988). The more the annotation agrees among the genes of a cluster, the higher the correlation.

The correlation coefficient was computed for the following combinations:

1. Clustering of expression profiles – EC annotation.

2. Clustering of expression profiles – functional annotation.

3. Clustering of expression profiles – metabolic pathway annotation.

As with the statistical measures, a higher correlation between set 3 and the annotation, than between set 1-2 and the annotation would support our hypothesis. As explained in Jain and Dubes (1988), the Hubert's Γ is applicable if we have two *n* by *n* matrices, *X*=[x(i, j)] and *Y*=[y(i, j)], of the same *n* objects. There should be no implied relationship between the matrices. In our case, *X* denotes the observed similarities in the clusterings and *Y* denotes the annotations:

$$x(i,j) = \begin{cases} 1 & \text{if objects i and j are in the same cluster} \\ 0.5 & \text{if objects i and j are in neighbouring clusters} \\ 0 & \text{otherwise} \end{cases}$$

$$y(i,j) = \begin{cases} 1 & \text{if objects i and j have the same annotation} \\ 0 & \text{otherwise} \end{cases}$$

Hubert's Γ in normalised form is defined as (Jain and Dubes, 1988):

$$\Gamma = \left\{ (1/M) \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} [x(i,j) - m_x][y(i,j) - m_y] \right\} / s_x s_y \tag{4.6}$$

M is the number of entries in the matrices, m_x and m_y are the mean values of the entries, s_x and s_y are the standard deviations of the entries. The coefficient is between –1 and 1. A perfect correlation would give a coefficient of 1 and a perfect negative correlation would give a coefficient of –1. If there is no correlation the coefficient is zero (0).

5 Results and analysis

All results from the experiments are presented in this chapter. Twelve clusterings were performed on datasets 1, 2 and 3 each. Table 7 shows the results from the standard deviation calculations (see equation 4.1). As explained in chapter 4.6.1, the standard deviation was calculated for each timepoint separately, but the figures presented in table 7 are averages over all clusters over all timepoints in a certain clustering.

Topology	Set 1	Set 2	Set 3
5 x 6	0.00829	0.00810	0.01939
5 x 6	0.0085	0.00757	0.01765
5 x 6	0.0082	0.00797	0.01985
6 x 6	0.00738	0.007	0.01573
6 x 6	0.00749	0.00679	0.01548
6 x 6	0.00756	0.0073	0.01728
5 x 8	0.007	0.00643	0.01408
5 x 8	0.00698	0.0065	0.01098
5 x 8	0.0074	0.00696	0.01661
6 x 8	0.00605	0.0065	0.0102
6 x 8	0.00599	0.00593	0.01134
6 x 8	0.00653	0.0059	0.01534

Table 7. Standard deviations for topologies between 30 and 50 nodes in size.

In figure 14 the averages of the standard deviations for each topology are presented. The standard deviations for the clusterings made on set 3, the one with annotation, is twice as high as for the other two sets. This indicates that we lose compactness when clustering with annotation.

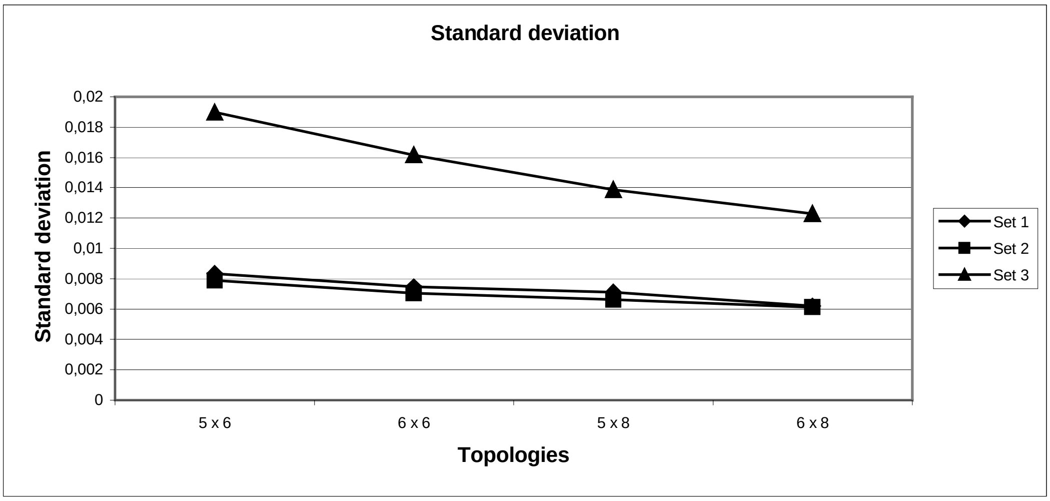


Figure 14. The average standard deviation for each topology. The deviation decreases as the size of the map grows. This is because the more nodes that are added the more specialised they get and map fewer datapoints. The higher standard deviations for the clusterings made on set 3 indicates that clusters of genes with larger variations in expression profiles are generated.

The mean values of the similarity score measure are presented in table 8. The similarity scores are decreasing as more nodes are added to the topology. As discussed in chapter 4.4 this was expected and the scores are still around or just above the baseline (~2,500 genes changing cluster) when comparing clusterings on set 1 and 2. The much higher scores when comparing set 2 and 3 indicates that the genes are clustered differently when using annotation as extra information in the clustering than just the profiles. Nothing can be said about whether these clusterings are more biologically correct or not just comparing similarity scores. The histograms in figure 15-18 show the results from the similarity calculations for each clustering.

Number of nodes	Comparing set 1 and 2	Comparing set 2 and 3
30	4,113	10,488
40	3,191	7,792
36	3,034	8,243
48	2,573	6,113

Table 8. The mean similarity scores for the different comparisons. It shows that clusterings on set 1 and 2 are similar and the added slope information in set 2 does not change the way the genes are clustered together. The last column shows that the genes are clustered differently when using annotation in set 3 than when just using the expression profiles.

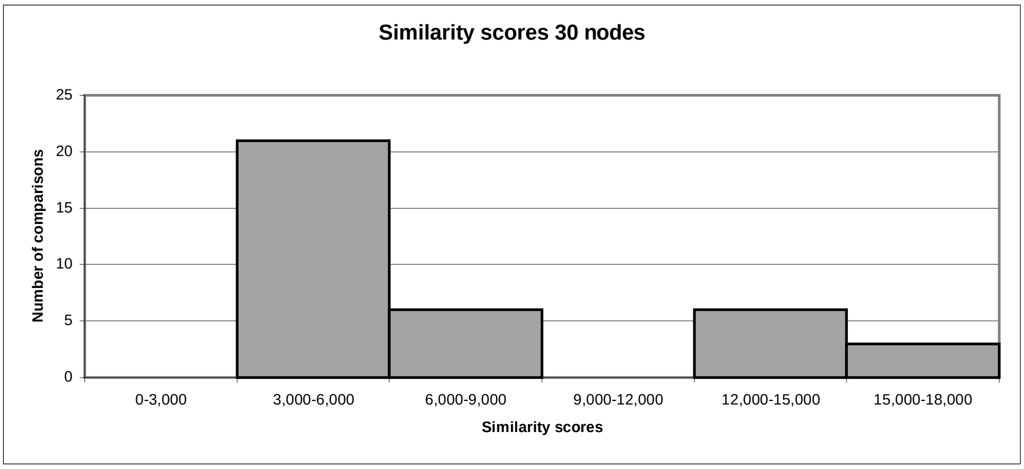


Figure 15. The comparisons of clusterings made on set 1 and 2, the leftmost piles, generates similarity scores that lay just above the baseline (~2,500). The two rightmost piles show the higher scores when comparing clusterings made on set 2 and 3.

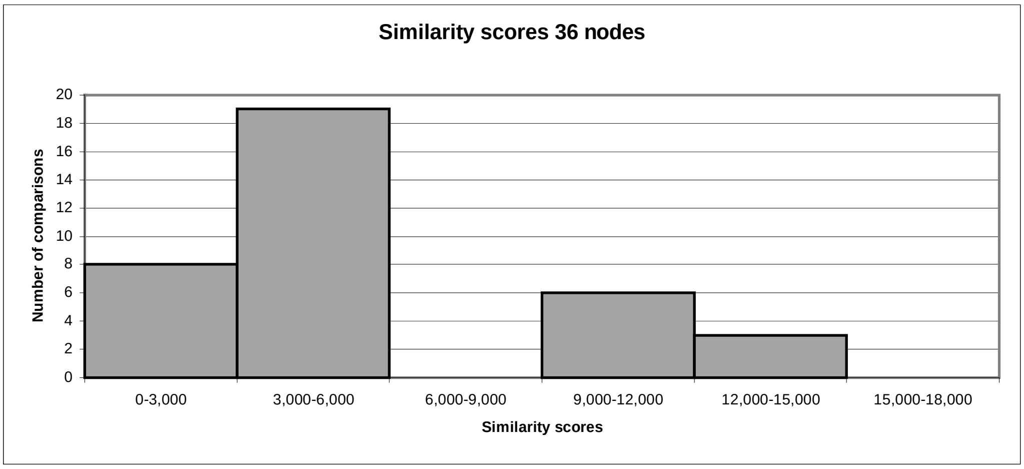


Figure 16. Histogram showing the similarity scores when comparing clusterings made on topologies with 36 nodes.

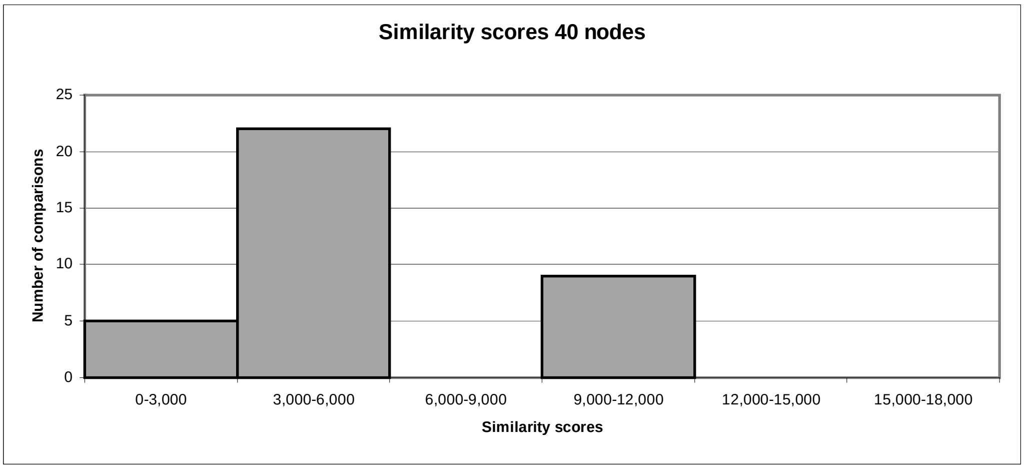


Figure 17. Histograms showing the similarity scores when comparing clusterings made on topologies with 40 nodes. The scores from the comparisons of set 2 and 3 are decreasing (all scores are in the interval 9,000-12,000) and this indicates that when more nodes are added, the effect of using annotation in the input is decreasing.

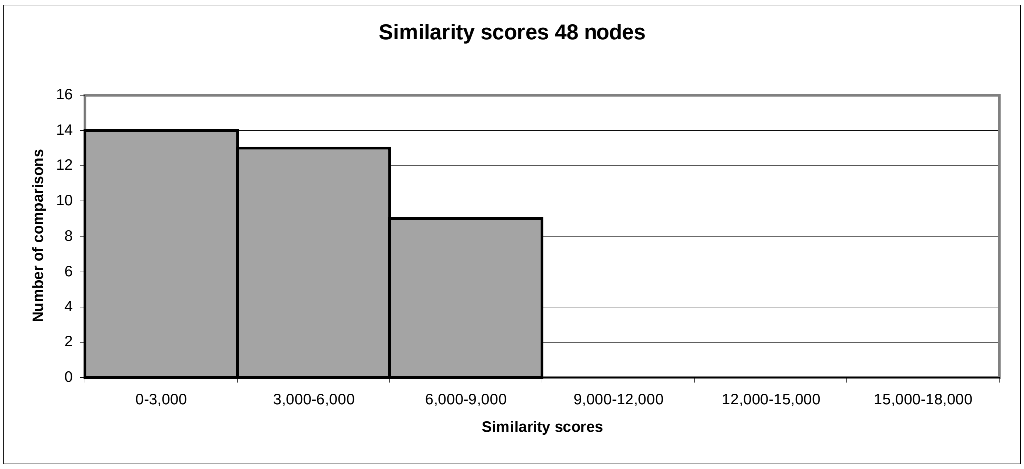


Figure 18. The histogram for the similarity scores when comparing clusterings on topologies with 48 nodes. The trend is similar as with the other topologies, the nodes are getting more specialised.

The compactness and isolation indices for the clusterings were all in the same range. We cannot say that the clusterings on set 3 resulted in more compact, or more isolated clusters in general. The indices also turned out to have an almost perfect negative correlation. In figures 19-21 the indices are shown for the clusterings on 36 nodes.

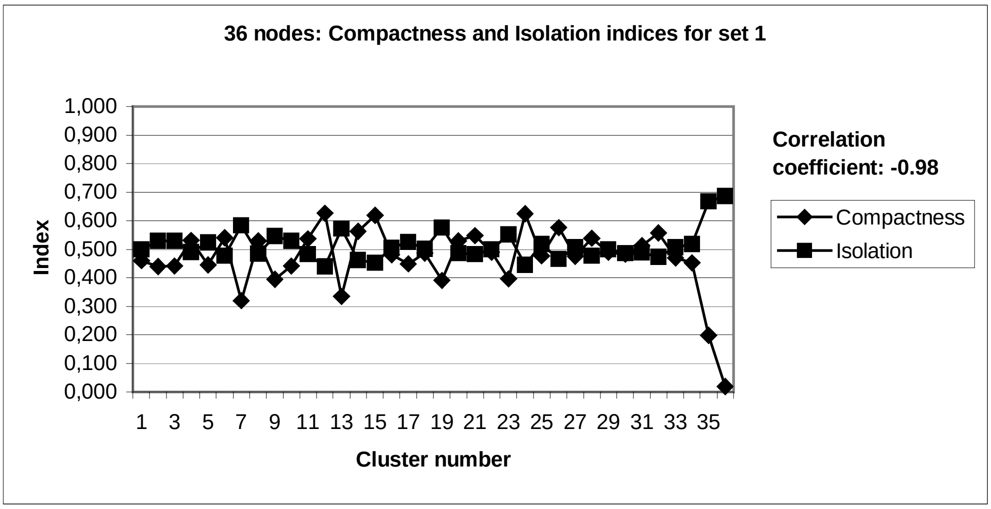


Figure 19. Compactness and isolation indices for one of the clusterings on set 1. Both the compactness and isolation indices are around 0.5 for every cluster, which is neither good nor bad since the interval is 0-1. The correlation coefficient between the two indices is –0.98. Cluster number 36 contained only two genes, which is the biggest reason for the low compactness index (0.018) for that cluster.

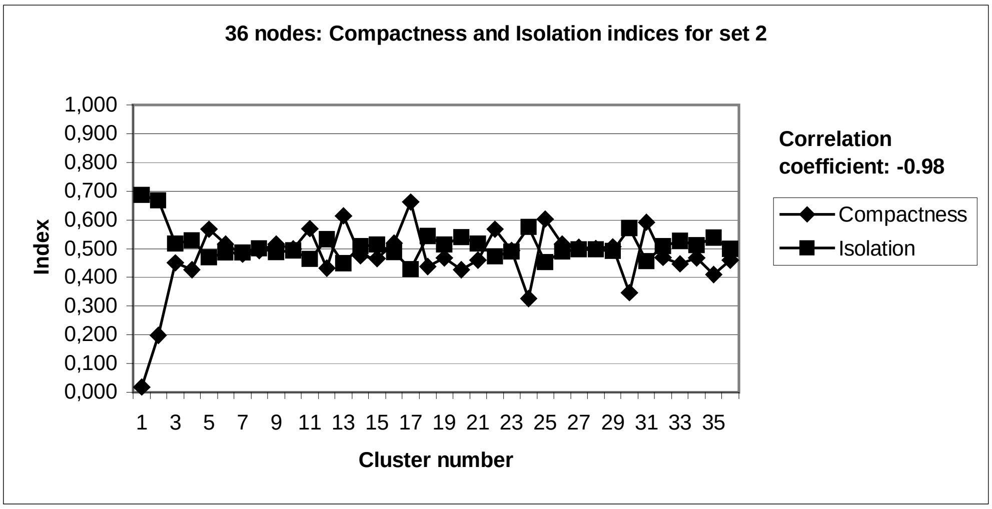


Figure 20. Compactness and isolation indices for one of the clusterings on set 2. The results are much the same as in the previous example. The difference was that it was cluster 1 that contained only two genes and not cluster 36. A closer inspection revealed that the genes in cluster 1 in this figure, were the same two genes as in cluster 36 in figure 19.

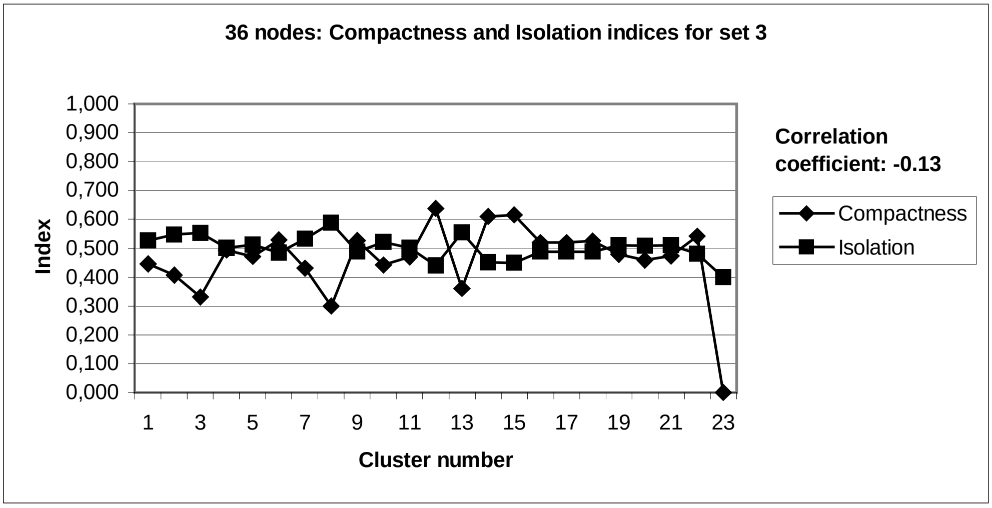


Figure 21. Compactness and isolation indices for clustering on set 3. In this clustering, only 24 clusters were formed. The last cluster contains only one gene, one of the same two genes that were clustered separately in the previous examples. The low correlation coefficient is a result of this single gene cluster and without cluster 24 the coefficient would be 0.99.

In tables 9-11 the results from the biological evaluation are presented. Hubert's Γ was measured for each clustering with respect to the EC annotation, functional annotation and metabolic pathway annotation. Clusterings with set 3 are of course expected to have higher correlation with the EC annotation than set 1 and 2, since this annotation was already used in the clustering process.

Topology	Set 1	Set 2	Set 3
5x6	0.010	0.007	0.470
5x6	0.009	0.008	0.328
5x6	0.013	0.007	0.399
6x6	0.013	0.012	0.422
6x6	0.013	0.011	0.428
6x6	0.010	0.014	0.440
5x8	0.007	0.000	0.171
5x8	-0.001	0.004	0.202
5x8	-0.004	-0.003	0.212
6x8	0.004	0.005	0.200
6x8	0.000	0.002	0.251
6x8	0.003	0.002	0.257

Table 9. Hubert's Γ with respect to the EC annotation. As expected, the clusterings with set 3 have higher correlation with the annotation.

Topology	Set 1	Set 2	Set 3
5x6	0.011	0.005	0.111
5x6	0.006	0.017	0.070
5x6	0.004	0.016	0.089
6x6	0.018	0.013	0.096
6x6	0.022	0.021	0.098
6x6	0.023	0.024	0.104
5x8	0.000	-0.010	0.059
5x8	0.008	0.005	0.049
5x8	0.008	0.006	0.048
6x8	0.003	0.009	0.063
6x8	0.004	0.002	0.060
6x8	0.006	0.009	0.063

Table 10. Hubert's Γ with respect to the functional annotation. The higher values in the last column, the correlations between the clusterings on dataset 3 and the functional annotation, indicates that the clusters formed when using the EC annotation as extra input for the SOM are more biologically correct than when just using the expression levels for the clustering.

Topology	Set 1	Set 2	Set 3
5x6	0.007	0.014	0.109
5x6	0.006	0.021	0.042
5x6	0.005	0.023	0.081
6x6	0.014	0.015	0.107
6x6	0.012	0.011	0.104
6x6	0.012	0.017	0.088
5x8	0.018	0.002	0.081
5x8	0.005	0.008	0.067
5x8	0.002	0.001	0.054
6x8	0.015	0.015	0.090
6x8	0.007	0.013	0.050
6x8	0.008	0.011	0.062

Table 11. Hubert's Γ with respect to the metabolic pathway annotation. The correlation between the clustering with dataset 3 and this annotation is consequently higher than the clusterings on dataset 1 and 2.

In figure 22-24 we have plotted the correlations against each other for each annotation. It can clearly be seen that the clusterings made with dataset 3 are consistently more correlated to the annotations than the clusterings made on the other two datasets (that only contain the expression profiles).

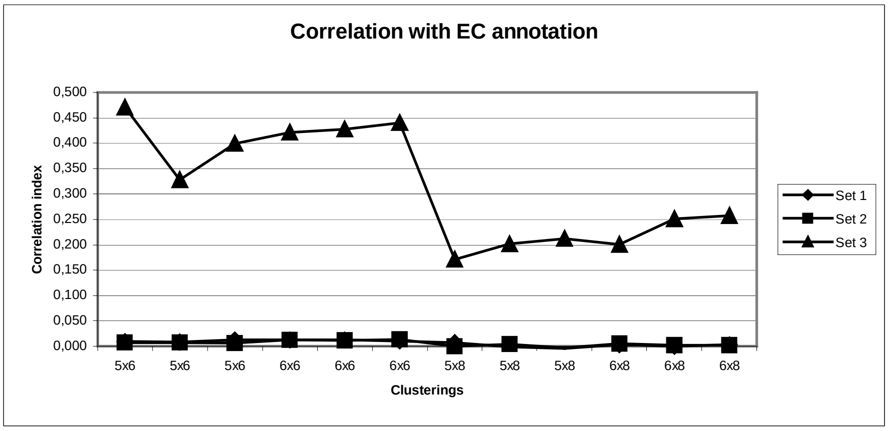


Figure 22. The relative high correlation between the clusterings with dataset 3 and the EC annotation is no surprise since the same annotation was used as part of the dataset. It should be noticed that the correlation between the clusterings with dataset 1 and 2 and the annotation is very low. This suggests that these clusterings does not produce biologically accurate clusters.

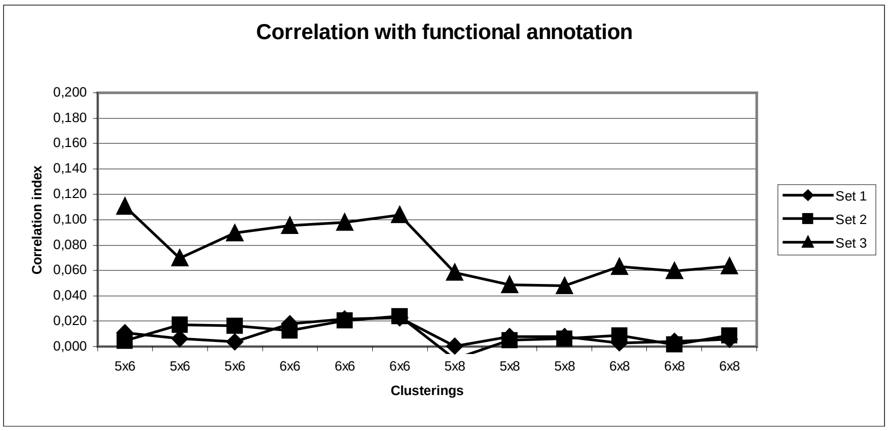


Figure 23. The correlation is very low, but clusterings with set 3 always produces a higher correlation with respect to the functional annotation.

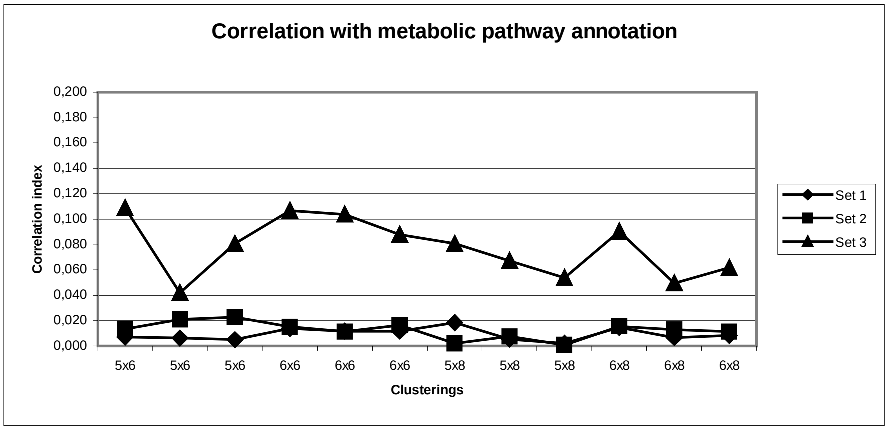


Figure 24. As with the other two annotations, the most correlated clusterings are the ones done on dataset 3.

The results from the conditional entropy and mutual information calculations are presented in tables 12 and 13, respectively.

Conditional entropy	**Functional annotation**	**Metabolic pathway annotation**	**Combination of both annotations**
EC annotation	1.1549	1.3742	1.8867
Expression profiles	1.8537	2.1281	2.6459
EC annotation and expression profiles	0.7393	0.8299	1.1115

Table 12. The conditional entropy. The smaller the value the more correlated the input is with the annotation. In each case, the EC annotation in combination with the expression profiles are best correlated with the annotation.

Mutual Information	**Functional annotation**	**Metabolic pathway annotation**	**Combination of both annotations**
EC annotation	1.0137	1.1263	1.6757
Expression profiles	0.3148	0.3725	0.9164
EC annotation and expression profiles	1.4292	1.6706	2.4509
Maximum entropy	**6.0113**	**6.1821**	**5.8406**

Table 13 The mutual information indices. The higher the index the more correlated the input is with the annotation. In each case, the EC annotation in combination with the expression profiles yields the best correlation with the annotation. The maximum entropy is calculated as the logarithm of the number of possible outcomes.

6 Conclusion

The aim of this project was to investigate whether clustering of gene expression patterns could be done more biologically accurate by providing the clustering technique with additional information, annotation, about the genes besides the expression profiles. Our hypothesis was:

> *By providing the clustering technique with not only data on the gene expression levels, but also annotation about the genes, the clusters formed will show a higher biological accuracy, than when just the expression levels are used.*

We also stated that the hypothesis would be falsified if no clear improved correlation to the biological reality could be shown regarding the clusterings. Our statistical evaluation indicated that when using annotation the compactness and isolation of the clusters were negatively affected. The standard deviation measure, for instance, showed that the compactness was decreased, see figure 14 for an example. The decrease in compactness and isolation was relatively small, see figures 19-21, and these are pure statistical measures that cannot say anything about the biological relevance of the clusters. Thus, more weight should be put on the results from the biological evaluation.

The biological evaluation showed that the clusterings made with the dataset that contained the annotation were more biologically accurate clusterings. This conclusion is drawn from the fact that the correlation between the clusterings and the three different annotations was consistently higher than the correlation obtained when just clustering with the expression profiles, see figures 22-24.

Furthermore, the conditional entropy and the mutual information indices, see tables 12 and 13, also showed that the expression profiles together with the EC annotation were more correlated with the functional and metabolic pathway annotation than by themselves.

Thus, we consider our aim fulfilled and our conclusion is that expression profiles should be clustered together with annotation and not alone.

7 Discussion

The intention of this work was to find a method to improve clustering of gene expression profiles. In our experiments we used the enzyme classification annotation as additional input for the clustering technique. When using the EC annotation we came to the conclusion that the clusterings were improved. Further investigations will show if different kinds of annotation, such as functional hierarchy or metabolic pathway information, can be used instead and what effect it would have on the clustering. We used self-organising maps and cannot say that the result would be the same with a different clustering technique and the drawn conclusion holds, of course, only for the tested dataset and it could be that we would get different results using another dataset.

The evaluation showed that when using statistical measures, we have to be careful about how we interpret the results. Negative results coming from a statistical measure does not necessarily mean that the clustering is bad in terms of biological accuracy.

7.1 Future work

Other clustering techniques, such as hierarchical clustering and k-means, must be applied to see if we would get the same result with them. It would be interesting to see if the method of using annotation as extra input would improve the results of the clusterings with these clustering techniques too. We have to test our method on other datasets. For instance, datasets that have been treated with other substances.

Furthermore, it would be interesting to see what effect the additional annotation have when clustering datasets that contain more genes than there is annotation for. We used enzyme classification annotation and a possible continuation would be to use other annotations such as functional annotation or metabolic pathway information, or a combination of these.

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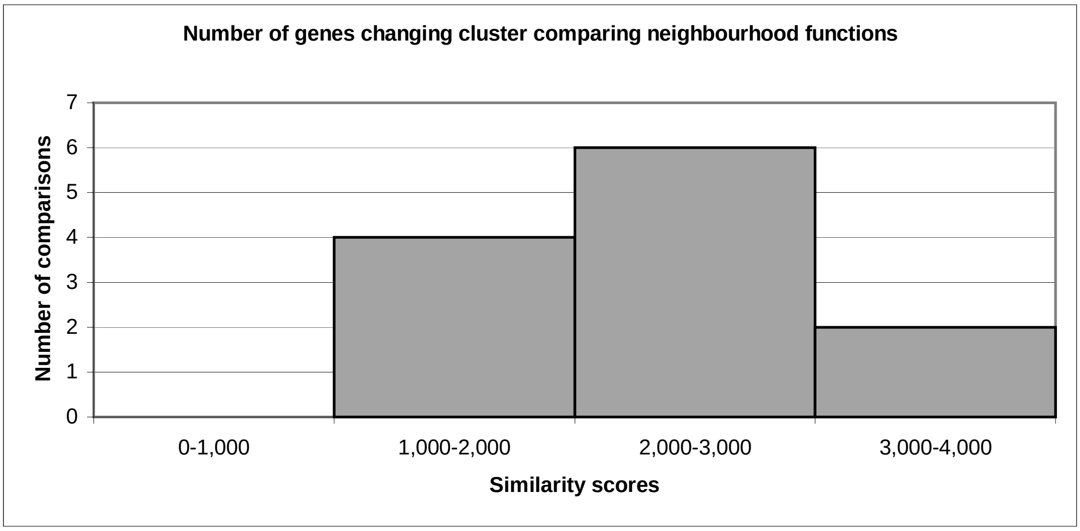
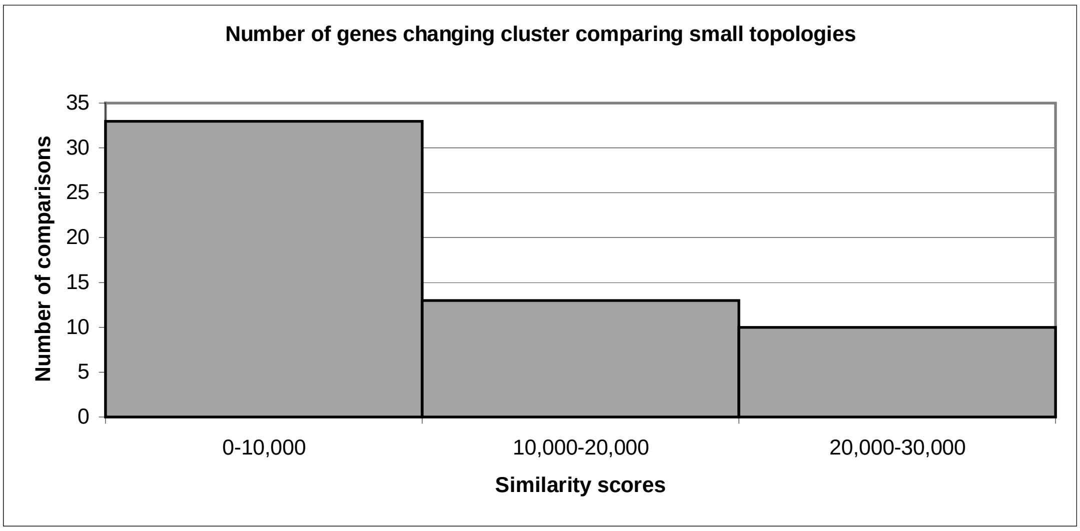
Appendix A

Here are the results from the comparisons that were made between different parameter settings for the SOMs. To compare how similar two different clusterings are, we calculated the number of gene pairs that were clustered differently between the two clusterings. The experiments were performed on dataset 1, see chapter 4.3.1 for further information on this set. The total number of gene pairs is 185,136. Learning- rate and radius and neighbourhood function did not affect the outcome significantly, although learning radius can be chosen with a little caution to improve the global ordering of the SOMs. Each graph below shows the distribution of how many genes that changed cluster between clusterings.

For most of the process parameters over 98% of the genes were clustered in the same way despite of the variation of the parameter setting. Variation of topology size did, as expected, have greater effect on the outcome. The x-axis shows similarity score intervals and the y-axis how many of the comparisons that had similarity scores in the given intervals.


Number of genes changing cluster comparing neighbourhood functions
Number of comparisons
0
1
2
3
4
5
6
7
0-1,000
1,000-2,000
2,000-3,000
3,000-4,000
Similarity scores


Number of genes changing cluster comparing small topologies
Number of comparisons
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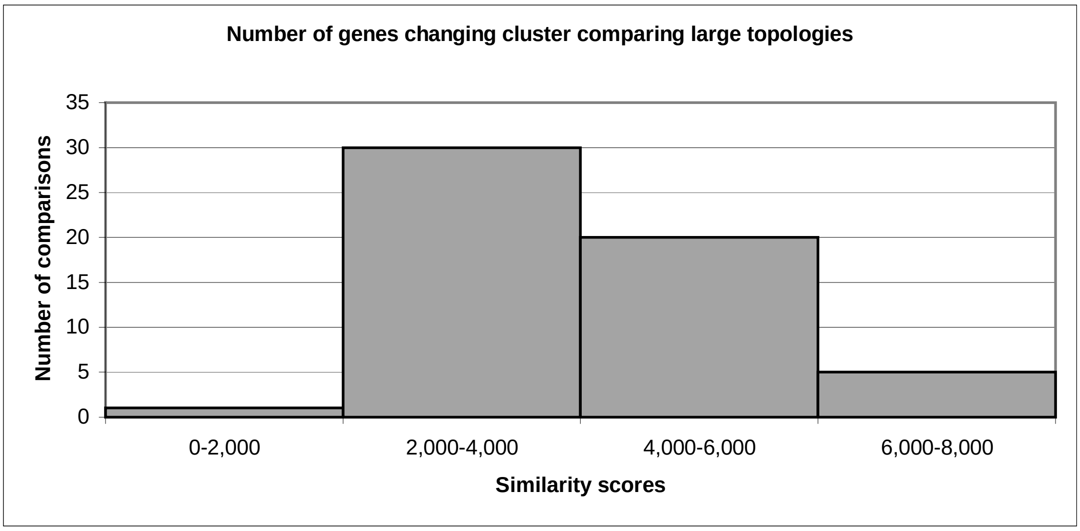
5
10
15
20
25
30
35
0-10,000
10,000-20,000
20,000-30,000
Similarity scores


Number of genes changing cluster comparing large topologies
Number of comparisons
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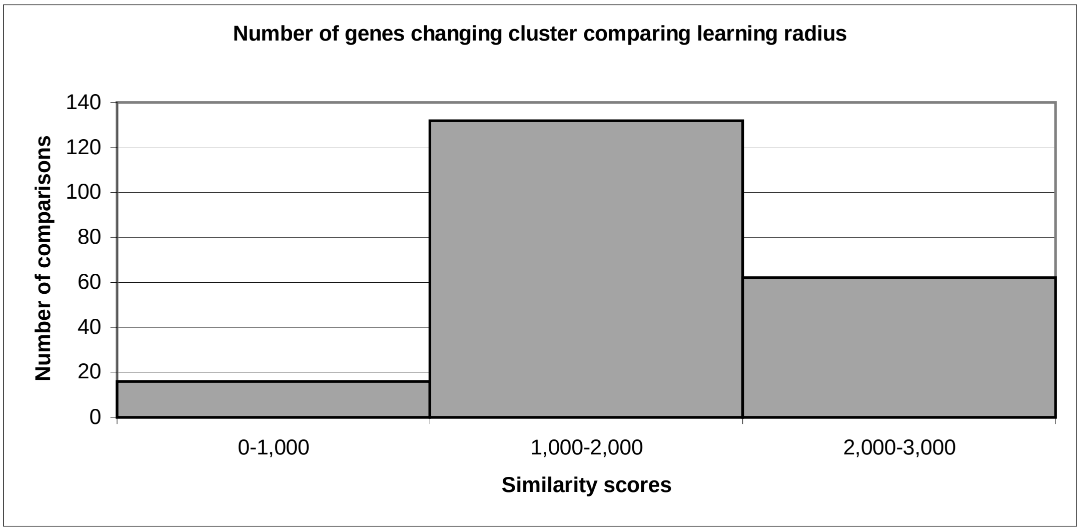
10
15
20
25
30
35
0-2,000
2,000-4,000
4,000-6,000
6,000-8,000
Similarity scores


Number of genes changing cluster comparing learning radius
Number of comparisons
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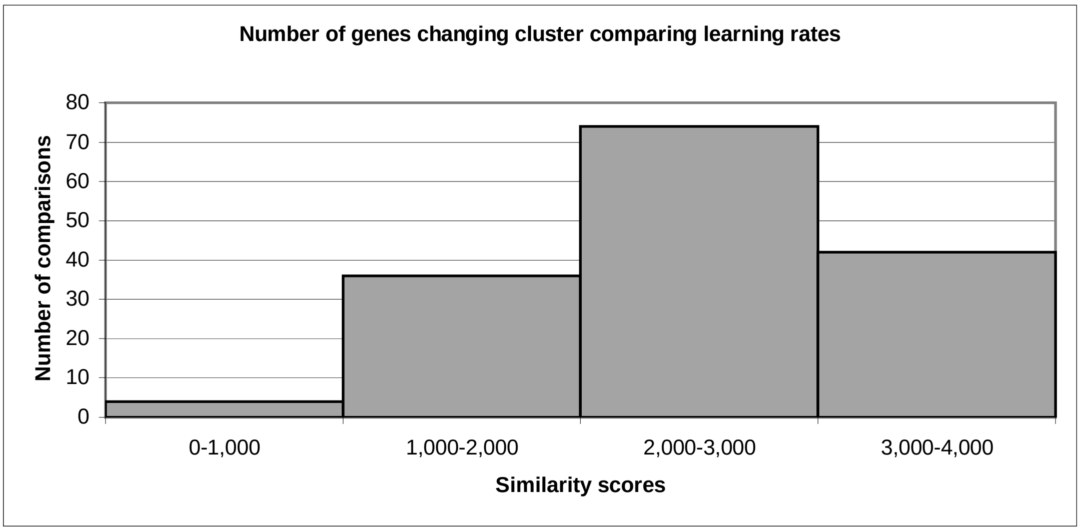
40
60
80
100
120
140
0-1,000
1,000-2,000
2,000-3,000
Similarity scores


Number of genes changing cluster comparing learning rates
Number of comparisons
0
10
20
30
40
50
60
70
80
0-1,000
1,000-2,000
2,000-3,000
3,000-4,000
Similarity scores