

## Pathway-based visualization of cross-platform microarray datasets

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Received on XXXXX; revised on XXXXX; accepted on XXXXX

Associate Editor: XXXXXXXX

## ABSTRACT

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**Availability:** The described method is implemented as part of the InCroMAP application that is freely available at [www.ra.cs.uni-tuebingen.de/software/InCroMAP](http://www.ra.cs.uni-tuebingen.de/software/InCroMAP).

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## 1 INTRODUCTION

The first generation of microarray platforms was developed as a high-throughput technique for profiling the transcriptome of diverse biological systems (i.e., cells, organs or organisms) under various experimental conditions (Golub *et al.*, 1999; Schena *et al.*, 1995). As these traditional gene-centered arrays were mostly limited to mRNA transcripts, the vast majority of visualization tools is focused on mRNA datasets (e.g., Expression Profiler – Kapushesky *et al.*, 2004 or KegArray – Kanehisa *et al.*, 2006). To date, a plethora of different microarray platforms are readily available. These include gene-centered platforms which rely on current genome annotations as well as unbiased tiling arrays which interrogate large non-repetitive regions of the genome. Diverse types of platforms have been specifically designed for the interrogation of different genomic features, ranging from mRNA or miRNA transcripts, through proteins or protein modifications, to relevant functional elements such as exons, SNPs or promoters (Hoheisel, 2006). In addition to arrays serving for the quantification of global gene expression on the RNA or protein level, also epigenetic modifications such as DNA methylation can be monitored on a genome-wide level using microarray technology (Schumacher *et al.*, 2006).

Several tools exist for the visual inspection of datasets from individual platforms (see Gehlenborg *et al.*, 2010, for some examples). However, the current inventory of publicly available tools that are capable of integrating and jointly visualizing data from multiple microarray platforms, is still very limited. Here, we

introduce a method for integrated pathway-centered visualization of datasets, generated from the same biological samples using different microarray platforms, which monitor complementary genomic and epigenomic features.

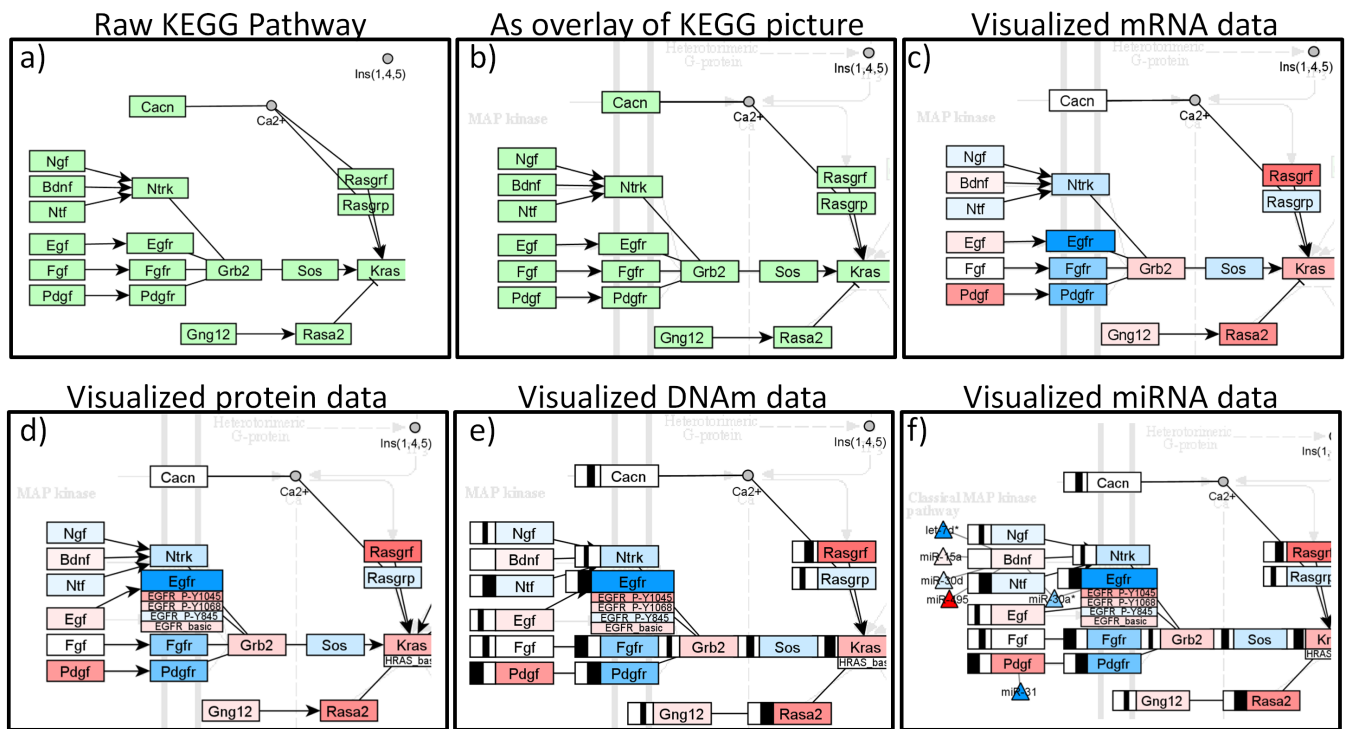
In contrast to commonly used region-based visualization methods (e.g., the UCSC genome browser – Kent *et al.*, 2002), we propose to visualize the microarray data in the context of specific signaling or metabolic pathways, which can in many cases be more easily related to the biological problem under study, than individual genes or genomic regions. In recent years, diverse tools were developed, which are specialized in pathway analysis (e.g., Ingenuity) or pathway visualization (e.g., Cytoscape – Cline *et al.*, 2007 or KEGG Atlas – Okuda *et al.*, 2008). Some of these tools offer visualizing experimental data in a pathway (e.g., KegArray – Kanehisa *et al.*, 2006, GenMAPP – Salomonis *et al.*, 2007 or MGv – Symons and Nieselt, 2011). For this purpose, the experimental data is typically mapped to a color gradient and displayed in the background color of the pathway nodes. GenMAPP or MGv even have capabilities to display multiple colors in a single node (e.g., for the visualization of time-series experiments). MGv goes one step further and offers additional features to put profile plots or heatmaps inside nodes. However, all of these tools are not able to handle data from genomic features which have no direct reference to the genes in a pathway (e.g., microRNAs or genomic regions). Furthermore, none of these tools offers viable solutions that are tailored for the integration of multiple datasets obtained from heterogeneous microarray platforms.

## 2 METHODS

Before visualization, the microarray datasets of interest have to be preprocessed and annotated using platform-specific workflows (Smyth, 2004; López-Romero, 2011). These workflows usually involve (1) the quality control of the raw data (Kauffmann *et al.*, 2009), (2) the data normalization to correct for background noise and experimental variation (Lim *et al.*, 2007), and (3) the mapping of probes to genes or genomic regions. After these preprocessing steps the microarray data has to be exported in tabular format. These tables have to contain two types of columns: (1) annotation columns, containing probe or probeset IDs (e.g., Affymetrix IDs) and database IDs of the corresponding genes (e.g., Ensembl or Entrez IDs), and (2) data columns containing either fold-changes

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**Fig. 1.** These pictures show an excerpt of the KEGG “MAPK signaling” pathway. It is demonstrated how the pathway and each of the four supported platforms are visualized in the pathway. Picture a) shows the visualized KGML document as nodes and edges. b) demonstrates that the overall quality of KEGG pathway visualizations can be improved by underlaying the original KEGG pathway picture. c) presents the excerpt with visualized mRNA data, whereas red means upregulation, blue downregulation and brighter colors represent smaller fold-changes. d) shows how protein modification datasets are visualized in the pathway. Each of the boxes below “Egfr” represent different modifications of this protein and the color of the box reflects the corresponding fold-change. e) illustrates DNA methylation peaks in promoter regions. A bar from the middle of the black box to the left represents hypomethylation and hypermethylation is expressed by a bar to the right. The size of the bar determines the size of the maximum DNA methylation peak. f) demonstrates the visualization of microRNA data by adding small triangles, representing microRNAs, and connecting them with grey edges to their mRNA targets. The color of the microRNA nodes determines the corresponding fold-change.

and/or  $p$ -values resulting from basic statistical analysis of the microarray data.

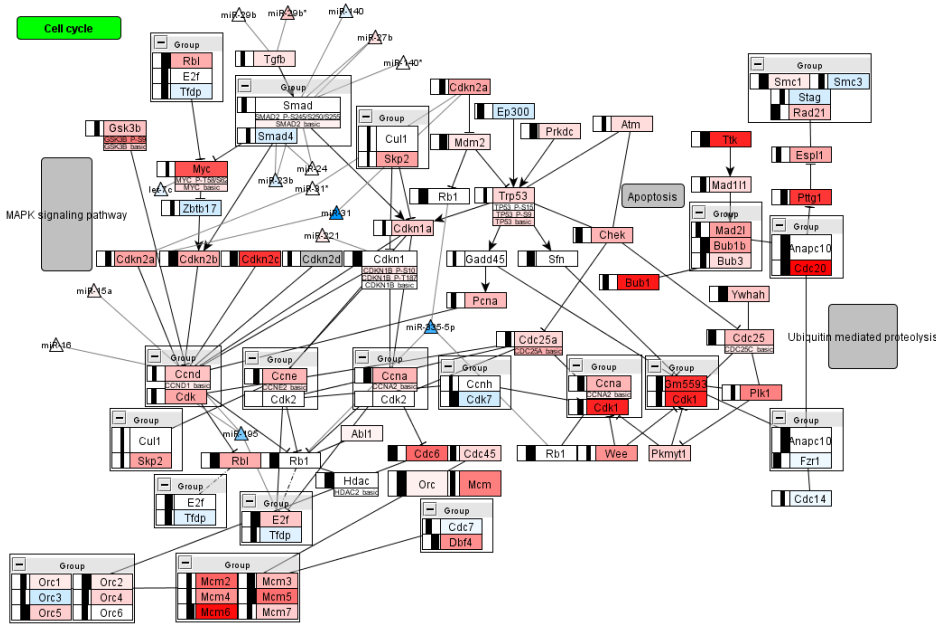
The pathway data is automatically imported from KEGG. In the KEGG PATHWAY database each pathway map is available for download as a KGML document which is internally converted into a GraphML document by InCroMAP. These GraphML files can be interactively visualized by InCroMAP using the yWorks graph viewer (TODO: Ref.). In order to overcome limitations of the KGML format, one can create an overlay graph that shows the original KEGG pathway image in the background, which may provide the user with additional information about cellular structure and compartmentalization.

After uploading the data, the KEGG pathway nodes, which correspond to genes or gene families, can be overlaid with fold-changes measured on mRNA or protein expression level. Furthermore, DNA methylation changes observed in the proximal promoter regions can be visualized. In the final step, additional nodes corresponding to miRNAs can be added to the pathway and colored according to the expression changes measured in the underlying experiment. See Figure 1 for an illustration of all the above-mentioned visualization steps.

## 2.1 Pathway visualization

The basic prerequisite for generating a pathway-based visualization is visualizing the pathway itself. For this purpose, we are using KEGGtranslator (see Wrzodek *et al.*, 2011), which performs a basic conversion of the KEGG KGML documents to GraphML. In short, KEGGtranslator converts all KGML entries to nodes and all relations to edges. Some basic errors are corrected automatically and appropriate shapes, colors and labels are inferred. Then, all nodes are annotated with diverse identifiers (e.g., Entrez Gene identifiers – Maglott *et al.*, 2005), descriptions, and further information. The resulting document provides the basis for the subsequently generated visualizations.

At least for some pathways, the KGML document available at KEGG does not contain all information that is depicted in the corresponding pathway map. Thus, an overlay graph can be generated which contains the original pathway map as a static transparent image in the background of the interactive graph plot (see Figure 1b). Owing to this feature, additional information on cellular structure (e.g., schematic drawings of receptors involved in cell signaling) can be maintained.



**Fig. 2.** Integrated visualization of datasets from four different platforms in the KEGG "Cell Cycle" pathway. The colors reflect the fold-changes, whereas red means upregulated and blue means downregulated. White corresponds to a fold-change of zero and darker colors represent stronger differential expression. The color of each node itself reflects the mRNA fold-change, e.g., "Ttk" shows a strong upregulation and Smc3 is downregulated. Smaller boxes below nodes show the protein and protein modification expression. For example, we visualized three different forms of "Trp53": a phosphorylation at the Serine 15 (S15) site, which shows almost no expression change, a modification of the S9 site, which is upregulated and the basic protein itself, which is also upregulated. The black boxes left of the nodes represent the maximum DNA methylation fold-change peak. A bar to the left represents hypomethylation and a bar to the right hypermethylation. In our example, "Ttk" shows a strong hypermethylation. MicroRNAs are added as small triangles to the pathway and connected with a grey edge to their mRNA targets. The color of the microRNA nodes reflects the fold-change, as described for the mRNA nodes.

## 2.2 Visualization of messenger RNA expression data

As mRNA expression data is typically available for the whole genome and thus, also for the majority of nodes in a particular KEGG pathway, these data are displayed in the background color of the nodes. As input, our method requires preprocessed mRNA datasets with annotation columns (e.g., gene identifiers) and data columns, which are referred to as *observations*. In this context, observations can be any statistical significance (e.g., *p*-values) or comparative measure (e.g., fold-changes or log-ratios).

Next, these data have to be broken down to a single value for each pathway node, which then determines the color of the node. As single nodes can represent multiple genes in KEGG, the intensities measured by probes, corresponding to the same node, have to be summarized. To this end, the mean or median is calculated across probes, which were mapped to the same node, or the probe with the strongest or most significant signal (i.e.,  $\min p - \text{value}$  or  $\max |\text{fold} - \text{change}|$ ) is decisive for coloring the node.

To visualize fold-changes or log-ratios in the context of pathways, a color gradient ranging from blue through white to red is used to illustrate down- and upregulation, respectively. Non-differentially expressed genes are shown in white, and pathway nodes for which no mRNA data is available are displayed in grey. If desired, *p*-values can be shown instead of fold-changes. For this purpose, we propose to map the negative logarithm of the *p*-values to a color gradient, which leads to a more intuitive illustration of the observed significances. See Figure 1c) for an example of visualized mRNA data.

## 2.3 Visualization of protein expression data

Visualization of protein datasets is performed by adding small boxes below pathway nodes and changing the color of the boxes

according to the corresponding protein expression data. As state-of-the-art experimental techniques (e.g., reverse-phase protein arrays, quantitative mass spectrometry) facilitate the distinction between different protein modifications (e.g., phosphorylated or acetylated forms of proteins) (TODO: Refs), multiple measurements may correspond to the same gene. In this particular case, the expression change observed for each individual protein form is represented as a separate box below the corresponding node. Each of these boxes is then labeled according to the respective protein form and colored based on the underlying expression data, as described previously for mRNA datasets. We require protein datasets to be annotated with database identifiers referring to proteins or genes (e.g. Entrez Gene IDs), which allows us to perform a straightforward mapping to pathway nodes. For protein modification dataset, we further require a column, determining the actual modification for every protein.

## 2.4 Visualization of DNA methylation data

The DNA methylation (DNAm) status, observed in the proximal promoter of a gene, is graphically represented by adding boxes to the left side of the pathway nodes. These boxes are drawn on a white background and contain a black bar which stretches from the middle to the left, in the case of hypomethylation and from the middle to the right, to indicate hypermethylation. Instead of discriminating between hyper- and hypomethylation, one can simply visualize the amount of differential methylation. In this case, the black bar would stretch from the left border of the box to the right border.

The length of the black bar is proportional to a summary value reflecting the DNAm status of the promoter of a certain gene. This value is by default computed from the probes in a region ranging from -2000 bps upstream of the transcription start site to 500 bps downstream. Normally, the value of the

maximum peak found in the promoter of a gene is visualized. For peak detection, Nimblegen proposed two algorithms which are called "Windowed Threshold Detection" and "Second Derivative Peak Detection". A detailed description of these methods can be found in the user's guide of Nimblegen's SignalMap software (see NimbleGen Systems Inc., 2006). In addition to those sophisticated peak detection methods, one can also approximate peaks by using simple methods like picking the probe with maximum differential methylation (i.e.,  $\max|fold - change|$ ). As an alternative to visualizing peaks derived from the probe-level fold-changes one can also use a statistical approach to estimate the differential methylation in a promoter region. For this purpose, we propose to divide the promoter region  $r$  into segments  $b_1, \dots, b_n$ , each containing a fixed number of probes. For each segment a statistical test (e.g., ordinary or moderated t-test) can be employed to detect differential methylation between two experimental groups. An overall significance value  $s$  can then be computed from the  $p$ -values  $p(b_1), \dots, p(b_n)$  using the formula  $s = \frac{1}{n} \sum_{i=1}^n -\log(p(b_i))$ .

This binning approach has the advantage of being sensitive to local changes in DNA methylation, as promoter segments with significant changes in DNA methylation are highly weighted. Conversely, segments for which slight changes were observed only contribute marginally to the overall significance score.

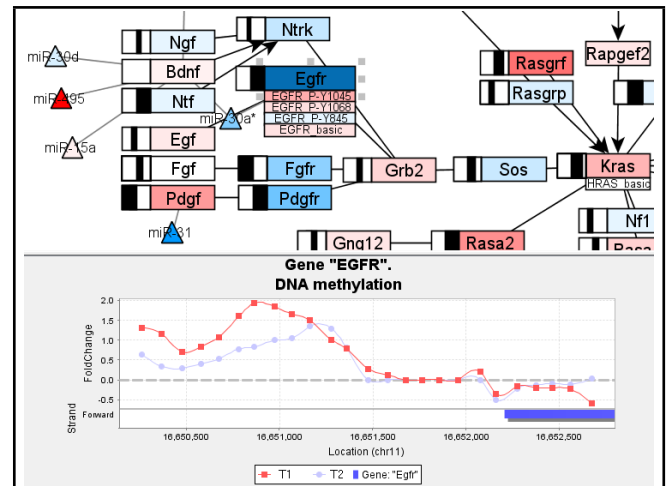
The purpose of this visualization technique is giving researchers a hint if, and how many methylation changes are present in a gene promoter. For more details, the genomic location of individual peaks can be visualized in a DNAm profile plot (see Figure 3). To this end, the fold-changes observed for the probes covering the promoter region of a certain gene are plotted along their genomic coordinates. This depiction is particularly useful for comparing the DNAm profiles and peaks found for different observations (i.e., sample groups).

In order to relate the DNAm data to data from gene-centered microarrays (e.g., mRNA expression arrays), each probe needs to be mapped to a gene. Thus, we require annotation columns containing the chromosome and the genomic position of each probe to facilitate the mapping from probes to genes.

## 2.5 Visualization of microRNA expression data

Visualizing microRNA (miRNA) datasets in the context of KEGG pathways is not straightforward, as these pathways do not contain miRNAs *a priori*. Therefore, in order to incorporate the data into a pathway, a connection must be established between the miRNAs and the protein-coding genes in the pathway. As the common mechanism of miRNAs involves the binding to complementary mRNA transcripts (Bartel, 2004), we propose to link miRNAs to their known target mRNAs. These target mRNAs can be obtained from diverse databases, which either contain experimentally verified targets (e.g., miRecords – Xiao *et al.*, 2009, miRTarBase – Hsu *et al.*, 2011, TarBase – Papadopoulos *et al.*, 2009) or predicted miRNA targets (reviewed in Alexiou *et al.*, 2009). We used a union of the three mentioned experimentally verified miRNA target databases for the figures in this publication.

Based on a map containing all connections between miRNAs and their target mRNAs, the miRNAs monitored in a specific experiment can be added to a pathway of interest as small triangular nodes, which have outgoing edges to the pathway nodes corresponding



**Fig. 3.** Integrated visualization of the "MAPK signaling" pathway with a detailed view of DNA methylation in the proximal promoter of "Egfr". The DNA methylation bar next to the "Egfr" gene in the pathway plot indicates hypermethylation. A profile plot of this genes genomic location reveals an increase in DNA methylation in this genes promoter.

to their target mRNAs. The triangular miRNA nodes are colored according to their expression, as described previously for mRNA datasets. This leads to an integrated visualization of a pathway, overlaid with miRNA and mRNA expression data, and extended with putative miRNA-mRNA interactions. Figure 1f) shows an example result of the described procedure.

## 3 RESULTS AND DISCUSSION

In this work, we present a novel methodology for the combined visualization of DNA methylation data, as well as mRNA, (phospho-) protein, and miRNA expression data in the context of canonical pathways. This methodology involves strategies for mapping the data from heterogeneous platform types to a common functional element, namely a gene, embedded into a pathway which regulates higher-order cellular functions or processes. Furthermore, we propose visualization techniques which are particularly suited for displaying quantitative data from diverse genomic features in an integrated graph plot, which represents a metabolic or signaling pathway of interest. However, generating a joint visualization of epigenomics, transcriptomics and proteomics data is challenging, as each data type has its own characteristics.

For proteomics data we propose to map basic and modified proteins to their common source gene and draw each protein form as a separate box below the pathway node representing the source gene. Using this kind of visualization one can, for instance, determine the activation state of a protein by comparing the color (i.e., expression state) of the box corresponding to the basic form with the one which corresponds to the phosphorylated form.

In brief, for most mRNA expression datasets it is sufficient to annotate the probesets with appropriate gene identifiers, in order to link the data with the pathways of interest. For miRNA datasets this linkage is complicated by the fact that miRNAs are normally not contained in pathways. Thus, we make use of known interactions

between miRNAs and their target mRNAs which can be extracted from public databases. By identifying protein coding genes, whose mRNAs are targets of miRNAs, one can add the miRNAs to the pathways and connect them to the protein coding genes by inserting edges to the corresponding targets.

The probe design of DNA methylation microarrays is typically not gene-centered, but region-based. Thus, in contrast to gene-centered arrays the probes are not organised in probesets which from the outset correspond to the same gene. Instead these arrays cover well-defined genomic regions of interest, as for instance promoters. The connection to protein coding genes is usually established by defining a region around the transcription start site (TSS) of a gene and assigning all probes in this region to the gene.

As visualizing the signal levels of all probes assigned to a certain gene in one pathway node would clutter the picture and reduce the clarity of it, a method for the computation of a single summary value is needed. For mRNA expression arrays the summarization of probes corresponding to the same transcript is usually achieved by the median polishing algorithm (TODO: cite RMA/GCRMA paper) which effectively alleviates the influence of outlier probes. However, this algorithm is only applicable under the assumption that ideally the probe intensities are expected to run evenly along transcripts. As a matter of fact, this assumption does not hold for the DNA-methylation of promoter regions, which is for one not expected to be evenly distributed along the region. Secondly, outlier probes corresponding to DNA-methylation peaks are quite likely not due to probe-specific effects, but rather reflect a local change in the DNA-methylation pattern, which may be biologically meaningful. Thus, one of the summarization methods, we consider advisable is the detection and visualization of maximum peaks (TODO: cite Nimblegen's user's guide), as it is sensitive to local changes in DNA-methylation. Alternatively, we suggest to use the mean probe-level fold-changes as a summary value, if global changes of DNA-methylation in the proximal promoter region are of peculiar interest. Nevertheless, both methods have obvious limitations. The maximum peaks method is definitely less robust against probe-specific effects and the results may be inconclusive as a single positive peak may suggest hypermethylation of a region, while the major part of the promoter is clearly hypomethylated. On the other hand the mean fold-change summarization clearly suffers from the fact that localized differential methylation is averaged out. Despite these limitations, in principle, we deem both methods suited for indicating differential methylation in the promoters of most genes. However, due to the heterogeneity and complexity of DNAm patterns, plotting the probe-level DNAm data along genomic coordinates often leads to more reliable conclusions about the DNAm state of promoters.

An example for a KEGG pathway with visualized mRNA, miRNA, protein and DNA methylation data can be seen in Figure 2. The visualization of multiple heterogenous datatypes can not be performed with either a loss of information or a loss of clarity. Since these integrated pathway visualizations should provide an overview, rather than a detailed listing of all possible information, we tried to keep the clarity and summarize information wherever possible.

## 4 CONCLUSION

Pathway enrichment analysis is a common microarray data analysis tool to discover pathways, whose genes show significant expression changes. In most applications, these enrichments are endpoints in analysis workflows. There are some methods or applications that can show pictures of significantly altered pathways and a few applications can even change node shapes or colors according to a single expression dataset. However, not only the amount of available microarray datasets is growing rapidly, but also the number of available platforms. Thus, today we have datasets that not only consist of mRNA data, but also DNA methylation, miRNA, protein or even more different levels. Analysis and especially visualization methods for such cross-platform datasets are very rare.

Here, we present a pathway-based cross-platform microarray visualization method that is perfectly suitable, e.g., as visualization method after pathway enrichment analyses. Especially the visualization of DNA methylation and miRNA datasets is a feature that can not be found in other visualization approaches. By integratively visualizing all datatypes, it helps researchers to discover potential relationships across multiple layers. For example, a miRNA can be upregulated and the corresponding mRNA target downregulated, whereupon a connected pathway protein could also be downregulated in protein expression. This effect, which involves information about pathway relations and miRNA targets, as well as miRNA, mRNA and protein expression, would be directly visible from the here presented visualization method.

## ACKNOWLEDGEMENT

We gratefully acknowledge contributions from Andreas Dräger and Finja Büchel, as well as the whole MACRCAR consortium.

**Funding:** The research leading to these results has received funding from the Innovative Medicine Initiative Joint Undertaking (IMI JU) under grant agreement nr. 115001 (MACRCAR project).

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