Supplementary Material To

Deconvolution of complex DNA methylation data - a detailed protocol

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Supplementary Text

Gene expression data processing

1. Download matched RNA-seq data from the TCGA legacy archive using the *TCGAbiolinks* [1] R package as normalized results.

```
library(TCGAbiolinks)
query <- GDCquery(project = "TCGA-LUAD",
                          data.category = "Gene expression",
                          data.type = "Gene expression quantification",
                          platform = "Illumina HiSeq",
                          experimental.strategy = "RNA-Seq",
              file.type  = "normalized_results",
                          legacy = TRUE)
GDCdownload(query, method = "api", files.per.chunk = 10)
data <- GDCprepare(query,summarizedExperiment=F)
```

2. Use *edgeR* [2] to further process the data to obtain counts per million (CPM) values per gene and sample and then use the marker genes *EPCAM*, *CLDN5*, *COL1A2*, and *PTPRC* to correlate sample-specific marker gene expression values to LMC proportions across the samples.

```
obj <- DGEList(data)
row.names(obj$samples) <- unlist(lapply(strsplit(row.names(obj$samples),"_"),
  function(x)x[3]))
colnames(obj$counts) <- unlist(lapply(strsplit(colnames(obj$counts),"_"),
  function(x)x[3]))
row.names(obj$samples) <- substr(row.names(obj$samples),1,16)
colnames(obj$counts) <- substr(colnames(obj$counts),1,16)
cpm.obj <- cpm(obj)
```

3. Plot each marker gene expression values per gene versus the LMC proportions.

```
load("FactorViz_outputs/medecom_set.RData")
props <- getProportions(medecom.set,K=7,lambda=0.001)
load("FactorViz_outputs/ann_S.RData")
colnames(props) <- substr(ann.S$Comment..TCGA.Barcode.,1,16)
marker.genes <- c("EPCAM","CLDN5","COL1A2","PTPRC")
in.exp <- colnames(cpm.obj) %in% colnames(props)
in.props <- colnames(props) %in% colnames(cpm.obj)
props <- props[,in.props]
cpm.obj <- cpm.obj[,in.exp]
cpm.obj <- cpm.obj[,colnames(props)]
row.names(cpm.obj) <- unlist(lapply(strsplit(row.names(cpm.obj),"[[:punct:]]"),
  function(x)x[1]))
cors.all <- sapply(marker.genes,function(marker){
  if(!marker %in% row.names(cpm.obj)){
    cors.gene <- NA
```

```
  }else{
    sel.exp <- cpm.obj[marker,]
    cors.gene <- apply(props,1,function(prop){
      cor(unlist(sel.exp),unlist(prop))
    })
  }
  cors.gene
})

cors.p.vals <- sapply(marker.genes,function(marker){
  if(!marker %in% row.names(cpm.obj)){
    cors.gene <- NA
  }else{
    sel.exp <- cpm.obj[marker,]
    cors.gene <- apply(props,1,function(prop){
      cor.test(unlist(sel.exp),unlist(prop))$p.value
    })
  }
  cors.gene
})
library(corrplot)
corrplot(cors.all,"ellipse")

plot.path <- "analysis/gene_expression/"
cors.all <- sapply(marker.genes,function(marker){
  if(!marker %in% row.names(cpm.obj)){
    cors.gene <- NA
  }else{
    sel.exp <- cpm.obj[marker,]
    for(j in 1:nrow(props)){
      prop <- props[j,]
      lmc <- paste0("LMC",j)
      to.plot <- data.frame(CPM=sel.exp,Proportion=prop)
      plot <- ggplot(to.plot,aes(x=Proportion,y=CPM))+geom_point(size=.1)+
        geom_smooth(method="lm",size=.5)+theme_bw()+
        theme(panel.grid=element_blank(),text=element_text(color="black",size=20),
          axis.ticks=element_line(size=0.5,color="black"),axis.ticks.length=unit(2,"mm"),
          axis.title=element_blank(),axis.text=element_blank())
      ggsave(file.path(plot.path,paste0(lmc,"_",marker,"_new.pdf")),
        plot,width=35,height=35,unit="mm")
    }
  }
})
```

Supplementary Tables

Supplementary Table 1: Overview of published DNA methylation based deconvolution tools. The methods are stratified according to the type and then ordered chronologically according to their date of publication.

Tool	Type	Short description	Reference
Houseman	reference-based	The method employs constrained projection to infer proportions of reference profiles and was particularly developed for deconvolution of whole blood samples.	Houseman *et al.* [3], 2012
EpiDISH	reference-based	*EpiDISH* is a reference-based method using robust partial correlations to compute proportions of reference profiles. The authors propose a method based on DNase hypersensitive sites to determine appropriate reference profiles.	Teschendorff *et al.* [4], 2017
hEpiDISH	reference-base	*hEpiDISH* is an extension of *EpiDISH* that hierarchically performs deconvolution, and along with a new reference database, improves devonvolution results	Zheng *et al.* [5], 2018
Methyl-CIBERSORT	reference-based	An extension of the *CIBERSORT* (Newman *et al.* [6], 2015) algorithm created for RNA-seq data that employs support vector regression (SVR) to estimate the proportions of given reference profiles across the samples.	Chakravarthy *et al.* [7], 2018
methylCC	reference-based	*methylCC* uses latent components and a region-based, rather than an individual CpG-based, model to compute the proportions of given reference profiles independent of the technology (RRBS, WGBS, or BeadArray) used.	Hicks & Irizarry [8], 2019
IDOL	selection of cell type markers	*IDOL* presents an improved strategy to determine cell-type specific marker CpGs, which improves deconvolution results	Salas *et al.* [9], 2018
FaST-LMM-EWASher	confounding factor in EWAS	The *EWASher* approach is based on factored spectrally transformed linear mixed models to account for differences in cellular compositions in EWAS.	Zou *et al.* [10], 2014
ReFACTor	confounding factor in EWAS	*ReFACTor* is based on Principal Component Analysis based on sites that are differentially methylated between cell types. The first few principal components are then used to adjust for cell type composition differences in EWAS.	Rahmani *et al.* [11], 2016
RefFreeCellMix	reference-free	*RefFreeCellMix* from the *RefFreeEWAS* R-package uses non-negative matrix factorization (NMF) of the input DNA methylation matrix to compute a matrix of proportions and estimated reference profiles.	Houseman *et al.* [12], 2014
EDec	reference-free	*EDec* is a two-step approach that combines reference-based and reference-free estimations using constrained matrix factorization.	Onuchic *et al.* [13], 2016
MeDeCom	reference-free	*MeDeCom* uses regularized non-negative matrix factorization (NMF) of the input DNA methylation data matrix to create a matrix of proportions and of latent methylation components (LMCs).	Lutsik *et al.* [14], 2017
TCA	reference-free	*TCA* uses tensor composition analysis to obtain sample-specific cell type profile estimates. In contrast to classical NMF, the method does not produce a single LMC matrix, but sample-specific LMCs using the same proportions matrix.	Rahmani *et al.* [15], 2019
CONFINED	reference-free	*CONFINED* uses two matrices as input and employs canonical correlation analysis (CCA) to obtain purely biological sources of variations.	Thompson *et al.* [16], 2019
BayesCCE	semi-reference-free	*BayesCCE* is a semi-supervised method to estimate proportions of different cell types that requires some prior knowledge on the cell-type composition of the studied tissue.	Rahmani *et al.* [17], 2018

Supplementary Table 2: Computational configurations in which software installation and the protocol have been tested. In case of an unexpected installation error, use the docker image available from https://hub.docker.com/r/mscherer/medecom.

Type	Distribution	Version	R-version	Installation successful	Protocol tested	Comments
Linux	Debian	Wheezy (7)	R-3.5.2	Yes	Yes	
			R-3.6.0	Yes	Yes	
		Jessie (8)	R-3.5.3	Yes	Yes (reduced[1])	
			R-3.6.1	Yes	No	
			R-4.0	Yes	No	
		Buster (10)	R-3.5.2	Yes	Yes (reduced)	
	Fedora	28	R-3.5.3	Yes	No	
		31	R-3.6.1	No	Yes (reduced)	'igraph' dependency fails to install
	CentOS	8.0	R-3.5.2	Yes	Yes (reduced)	
			R-3.6.1	Yes	Yes (reduced)	
	Ubuntu	19	R-3.6.1	Yes	Yes (reduced)	
MacOS		Mojave	R-3.5.1	Yes	Yes (reduced)	binary release used
		Catalina	R-3.6.0	Yes	Yes (reduced)	
Windows	10	Pro	R-3.6.1	No	Yes (reduced)	Use docker image
	7	Pro	R-3.6.1	No	No	Docker is not available for Windows 7

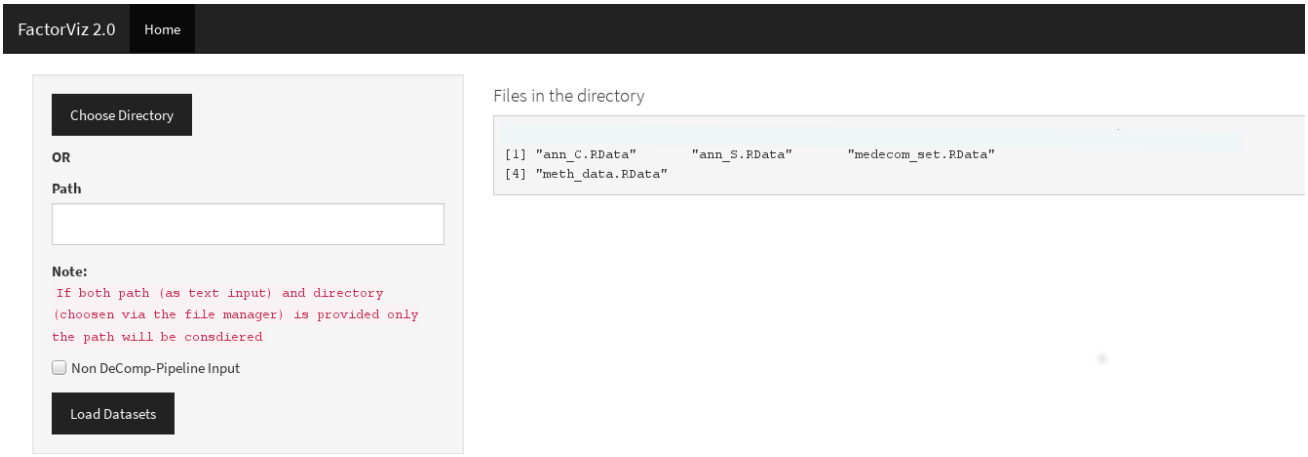
[1] *In the reduced protocol, we executed preprocessing and a single MeDeCom run on a reduced dataset.*

Supplementary Table 3: Genomic annotations of the sites that had an absolute difference between LMC4 and the median of the other LMCs larger than 0.75. The distance corresponds to the distance of the CpG to the gene body of the closest gene (0 distance refers to sites located within the gene). CGI=CpG island, CTCF=CTCF binding site, ENSEMBL annotation=annotation according to the ENSEMBL regulatory build, proximal=proximal enhancer, TFBS=transcription factor binding site

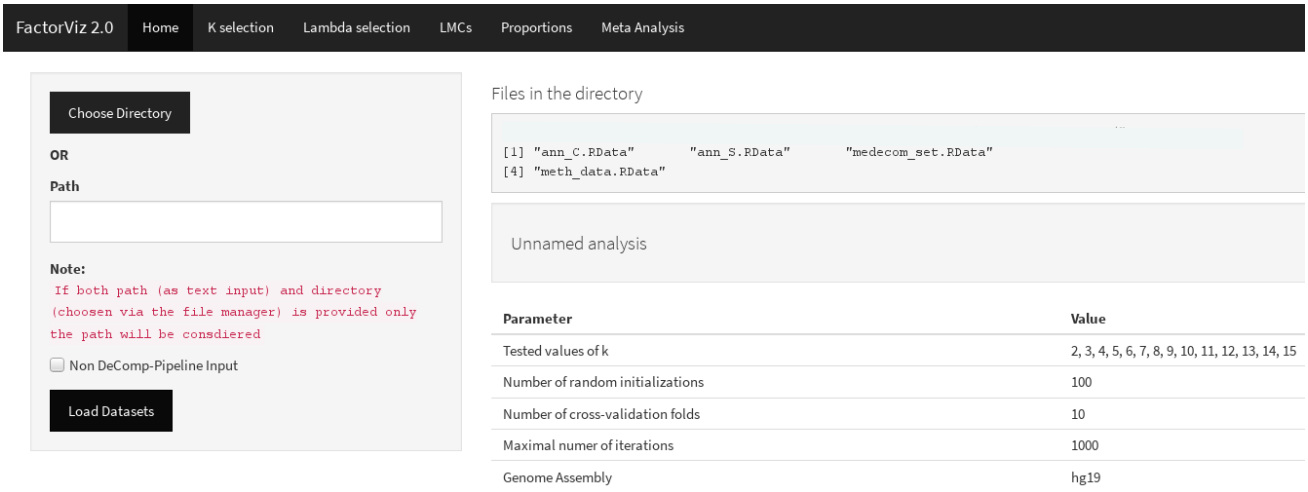
CpG ID	Chr	Start	End	Strand	CGI Relation	Difference	Closest gene	Closest gene (ENSEMBL)	Nearest gene distance	ENSEMBL annotation
cg00319661	chr5	2632178	2632179	+	Open Sea	-0.93	C5orf38	ENSG00000186493	120065	
cg03415617	chr16	34726856	34726857	+	Open Sea	-0.922		ENSG00000260341	0	CTCF
cg05789595	chr6	56555274	56555275	+	Open Sea	-0.864		ENSG00000231441	153524	TSS
cg11006453	chr8	141599185	141599186	-	Open Sea	-0.851	AGO2	ENSG00000123908	0	
cg08440178	chr2	2737278	2737279	+	Open Sea	-0.841	MYT1L-AS1	ENSG00000225619	406395	
cg26992600	chr14	37054509	37054510	-	South Shore	0.84	NKX2-8	ENSG00000136327	2696	TFBS
cg25153741	chr5	177913468	177913469	+	Open Sea	-0.833	RN7SL646P	ENSG00000242341	108248	
cg13157980	chr8	141599141	141599142	-	Open Sea	-0.826	AGO2	ENSG00000123908	0	
cg24066980	chr2	1864151	1864152	+	Open Sea	-0.821		ENSG00000232057	31711	
cg23731089	chr8	141599208	141599209	-	Open Sea	-0.82	AGO2	ENSG00000123908	0	
cg15616496	chr17	73860607	73860608	-	Open Sea	0.816	WBP2	ENSG00000132471	8018	
cg22986569	chr5	2659008	2659009	+	Open Sea	-0.811	C5orf38	ENSG00000186493	93235	
cg26845946	chr5	2137653	2137654	-	South Shore	-0.803		ENSG00000248597	170384	
cg03003434	chr6	159141722	159141723	-	Open Sea	-0.797	AMZ2P2	ENSG00000219249	5055	
cg02896768	chr1	154179996	154179997	-	Open Sea	0.793	C1orf43	ENSG00000143612	0	
cg06255006	chr16	86653215	86653216	-	Open Sea	-0.786		ENSG00000260387	16717	
cg11573608	chr2	503193	503194	-	Island	-0.779		ENSG00000223985	10537	
cg16783478	chr16	9943657	9943658	-	Open Sea	-0.779	GRIN2A	ENSG00000183454	0	
cg06334134	chr7	142986693	142986694	+	South Shore	-0.779	CASP2	ENSG00000106144	0	TSS
cg25453625	chr7	4347751	4347752	+	Island	-0.774	SDK1	ENSG00000146555	39118	
cg03877767	chr2	11680057	11680058	+	Open Sea	0.774	GREB1	ENSG00000196208	0	TSS
cg14584961	chr7	157533065	157533066	+	Open Sea	-0.773		ENSG00000233038	114154	TFBS
cg11761483	chr17	70723386	70723387	-	Open Sea	0.772	SLC39A11	ENSG00000133195	0	CTCF
cg02756683	chr10	99449502	99449503	-	South Shelf	-0.772	AVPI1	ENSG00000119986	2421	TSS
cg17167920	chr1	154127537	154127538	+	Open Sea	0.771	UBAP2L	ENSG00000143569	65116	
cg19075377	chr13	112770169	112770170	+	Open Sea	-0.77	LINC00403	ENSG00000224243	7839	
cg26165146	chr12	27484656	27484657	+	North Shore	-0.765	ARNTL2	ENSG00000029153	1129	TSS
cg05721751	chr10	1707576	1707577	+	Open Sea	-0.763	ADARB2-AS1	ENSG00000205696	108396	
cg03945777	chr7	157514049	157514050	+	Open Sea	-0.759		ENSG00000222012	102591	
cg20696049	chr7	157551890	157551891	+	South Shore	-0.759		ENSG00000233038	95329	proximal
cg05726239	chr6	107816677	107816678	-	South Shelf	0.757		ENSG00000234206	14328	TSS
cg03262885	chr7	157710179	157710180	-	Open Sea	-0.757	PTPRN2	ENSG00000155093	0	
cg14462553	chr7	157444239	157444240	-	South Shore	-0.757	PTPRN2	ENSG00000155093	0	
cg06809074	chr8	976415	976416	-	Island	-0.756		ENSG00000254160	220372	
cg26109981	chr5	2175329	2175330	+	Open Sea	-0.755		ENSG00000201026	9493	
cg03540794	chr5	2112109	2112110	-	Island	-0.752		ENSG00000248597	144840	
cg00327669	chr5	1950782	1950783	+	Island	-0.752		ENSG00000248994	0	
cg26577252	chr15	99212332	99212333	+	Open Sea	-0.75	IGF1R	ENSG00000140443	0	

Supplementary Figures

a **Step I:** Start *FactorViz*

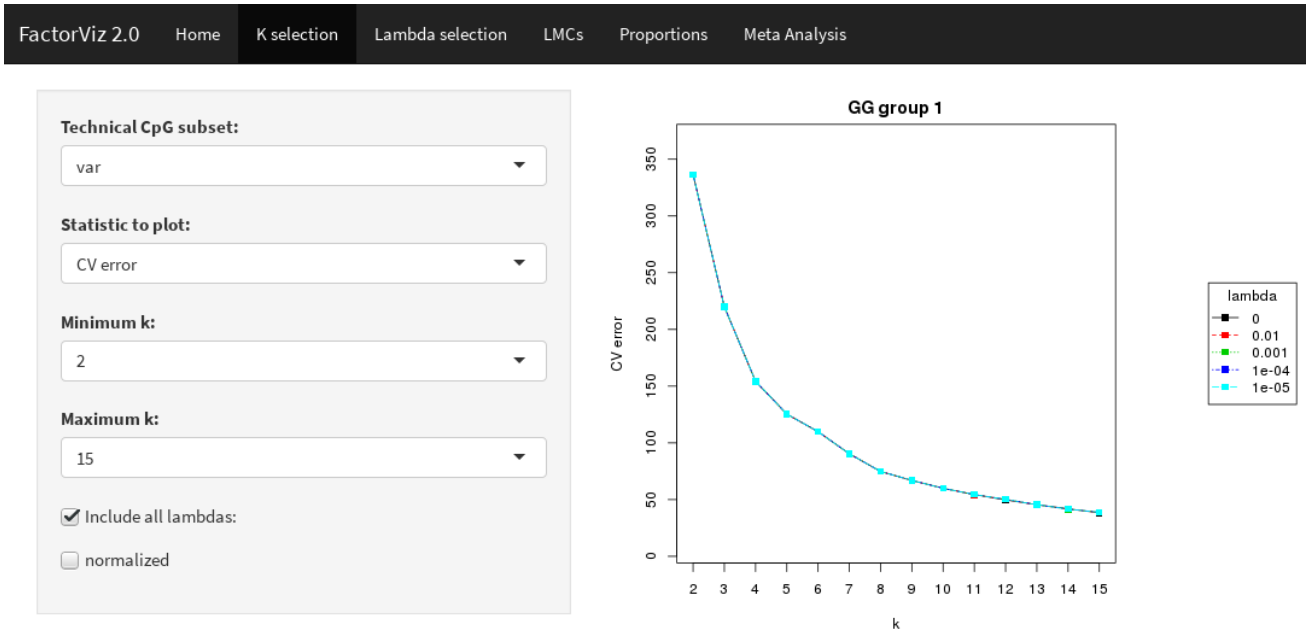


b **Step II:** Load *MeDeCom/DecompPipeline* output

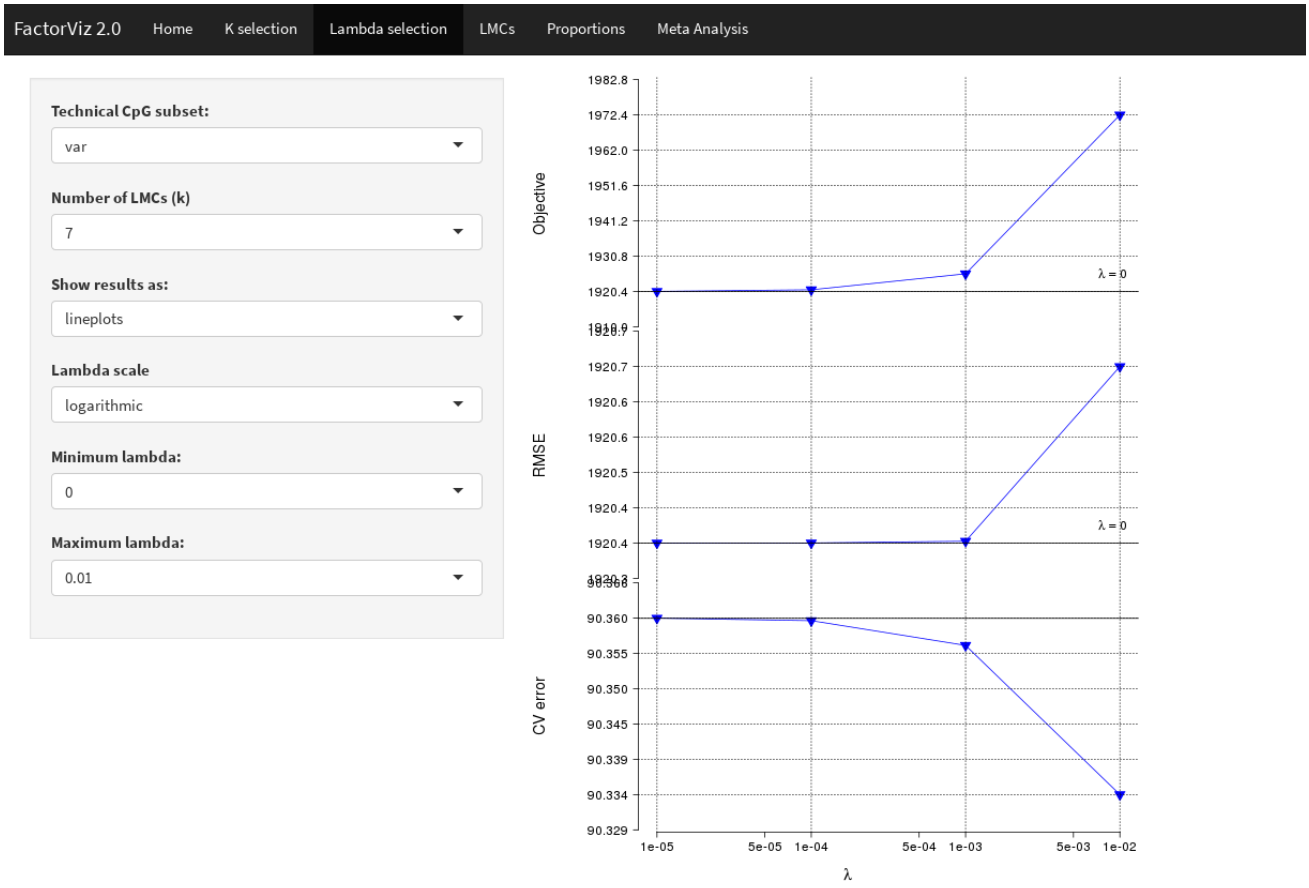


Parameter	Value
Tested values of k	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15
Number of random initializations	100
Number of cross-validation folds	10
Maximal numer of iterations	1000
Genome Assembly	hg19

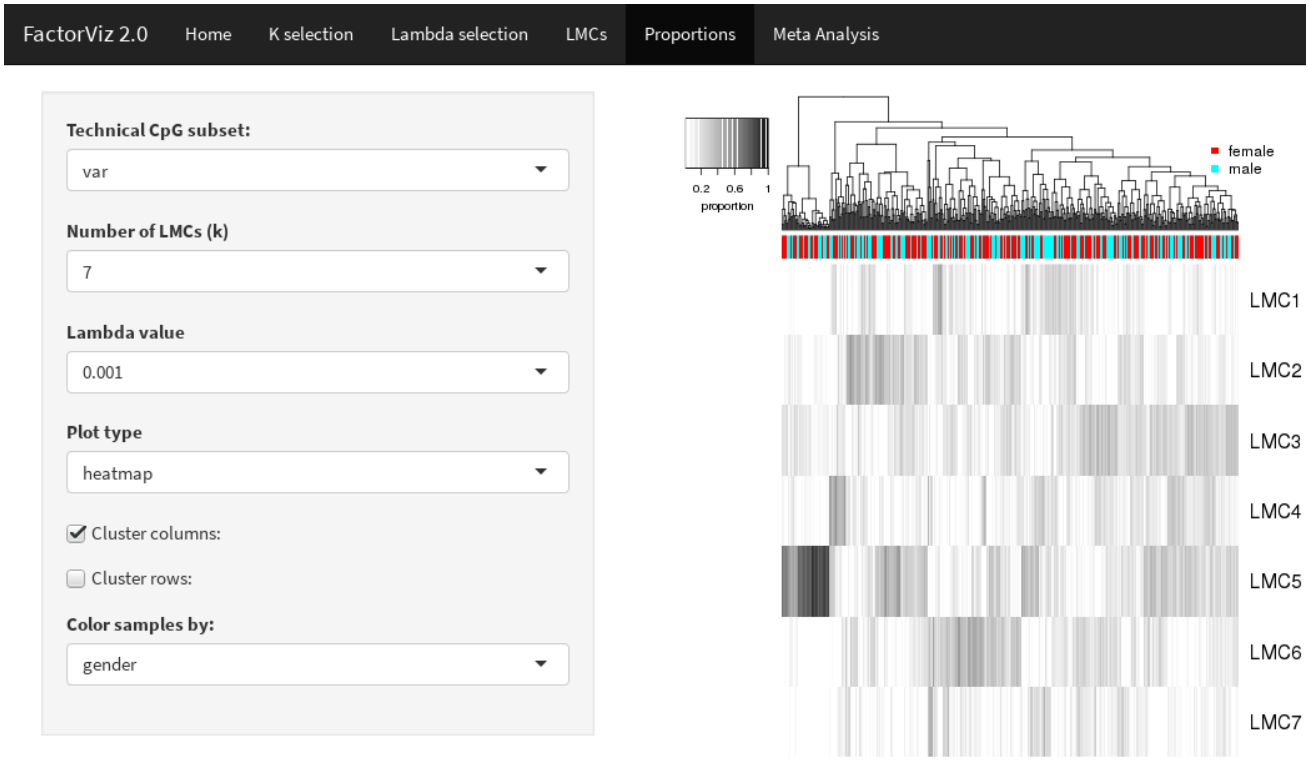
c **Step III:** Select number of components (K)



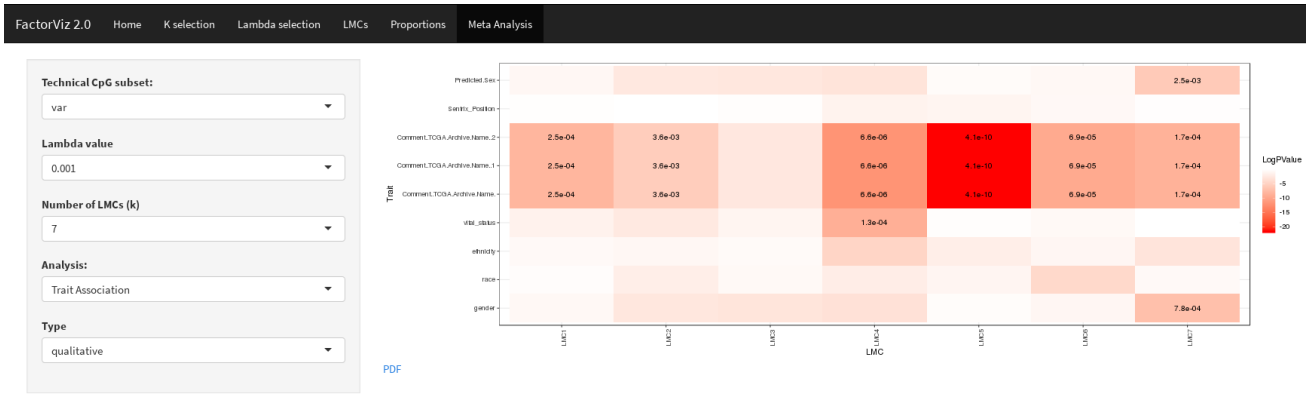
d **Step IV:** Select regularizer (λ)



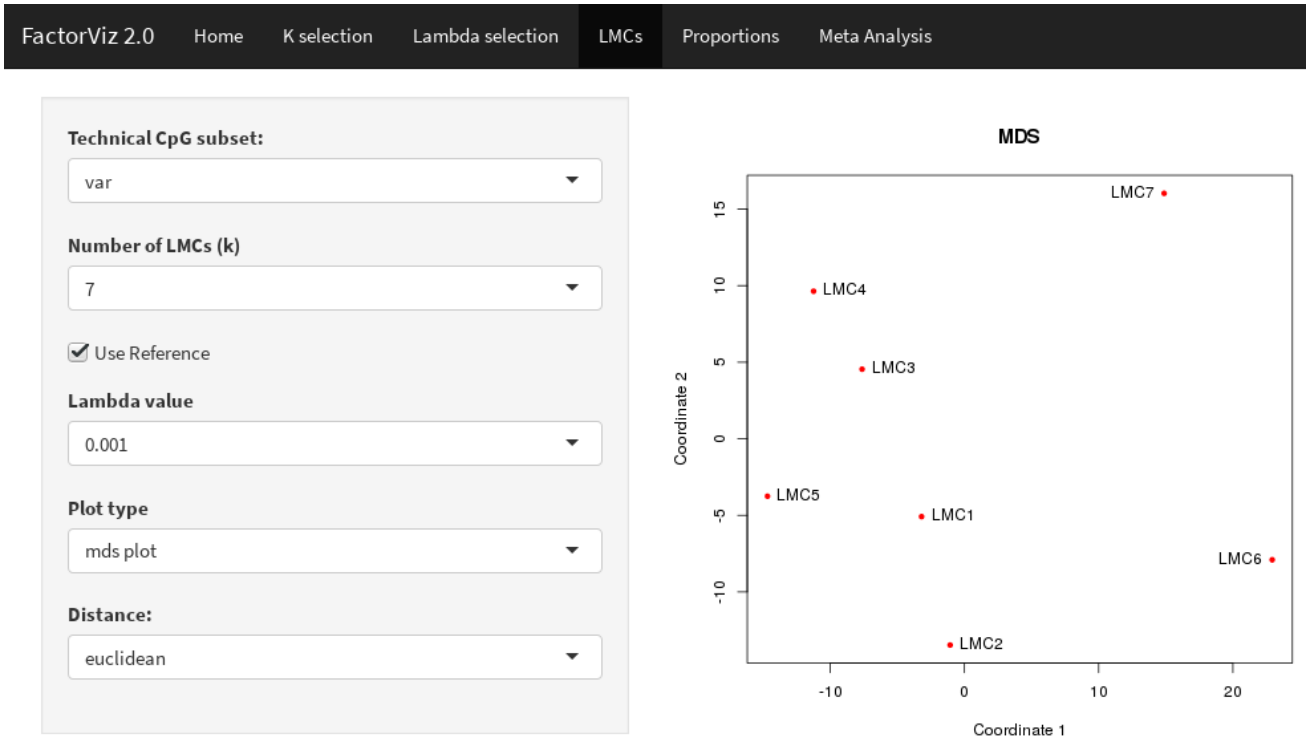
e **Step V:** Visualize proportions matrix (A)



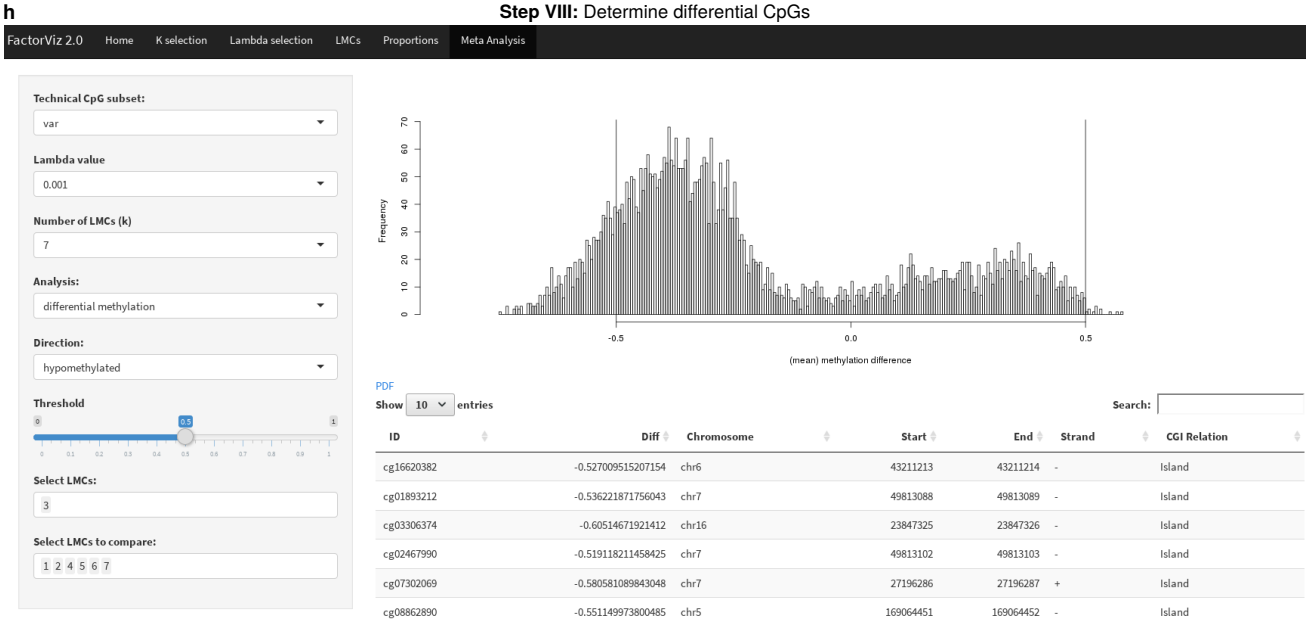
f **Step VI:** Associate proportions with phenotypes



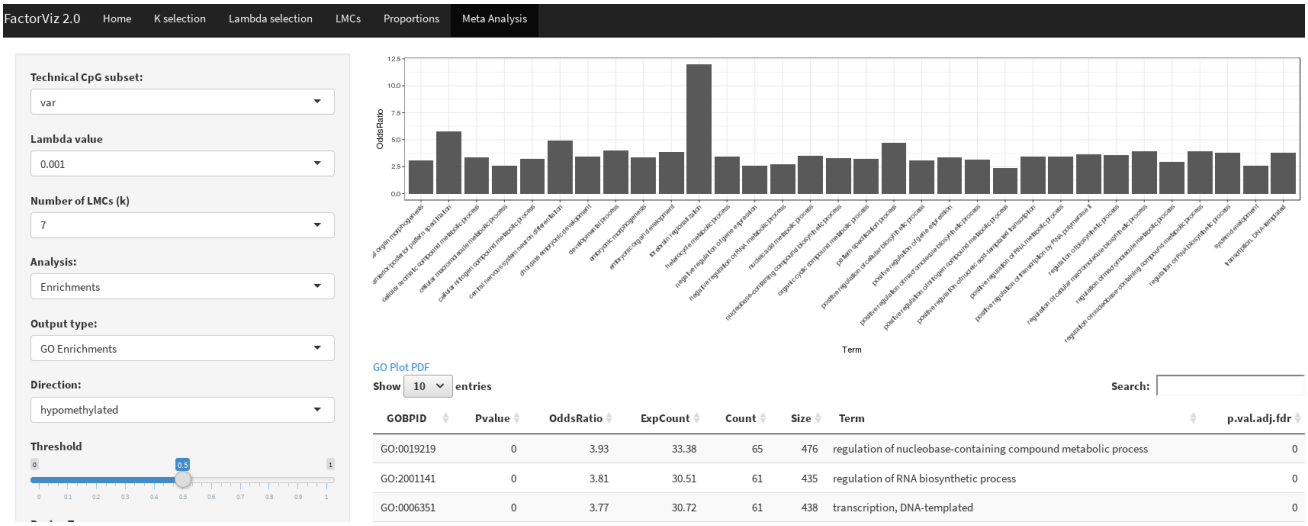
g **Step VII:** Visualize LMC matrix (T)



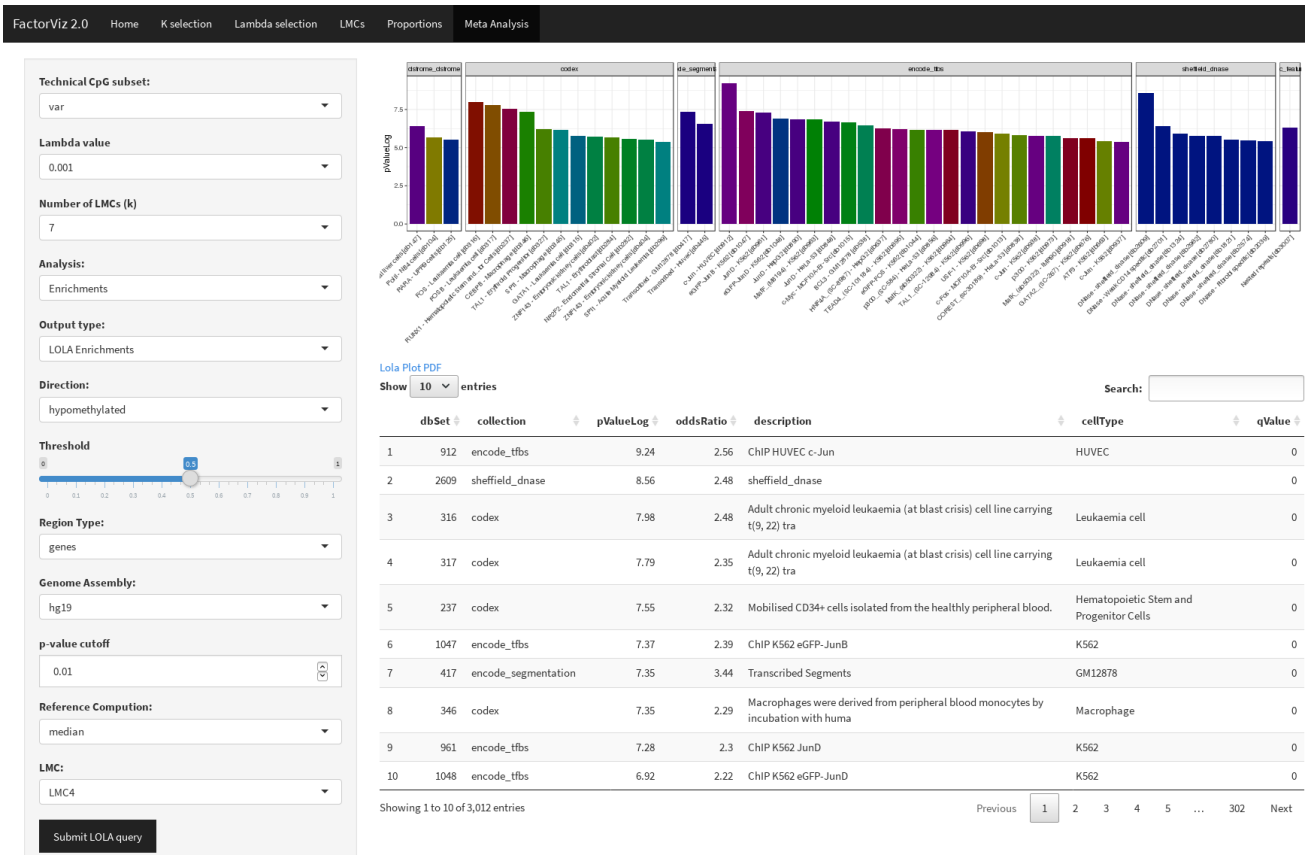
h **Step VIII:** Determine differential CpGs



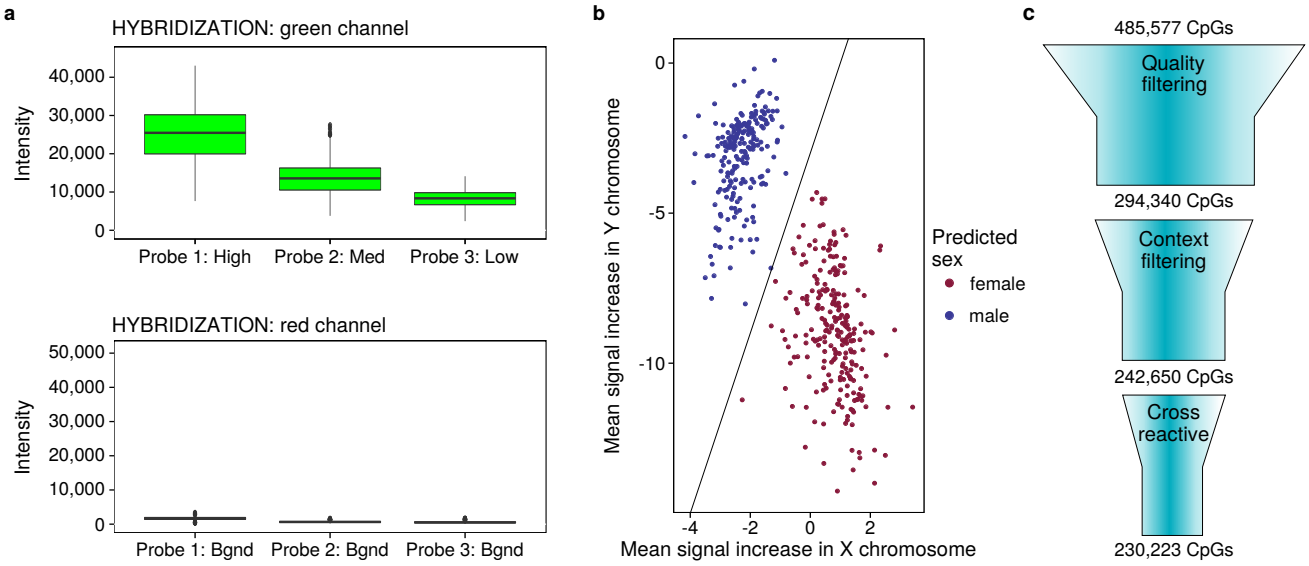
i **Step IX:** LMC GO enrichment analysis



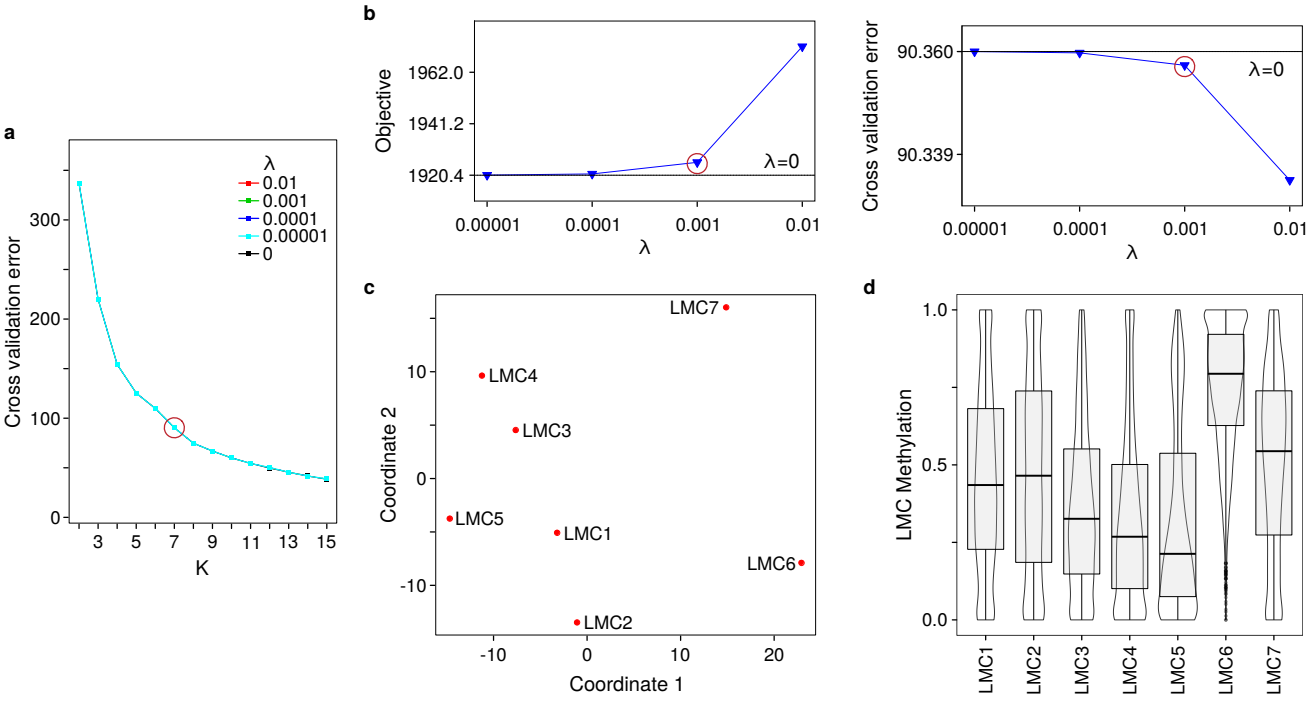
j **Step X:** LMC LOLA enrichment analysis



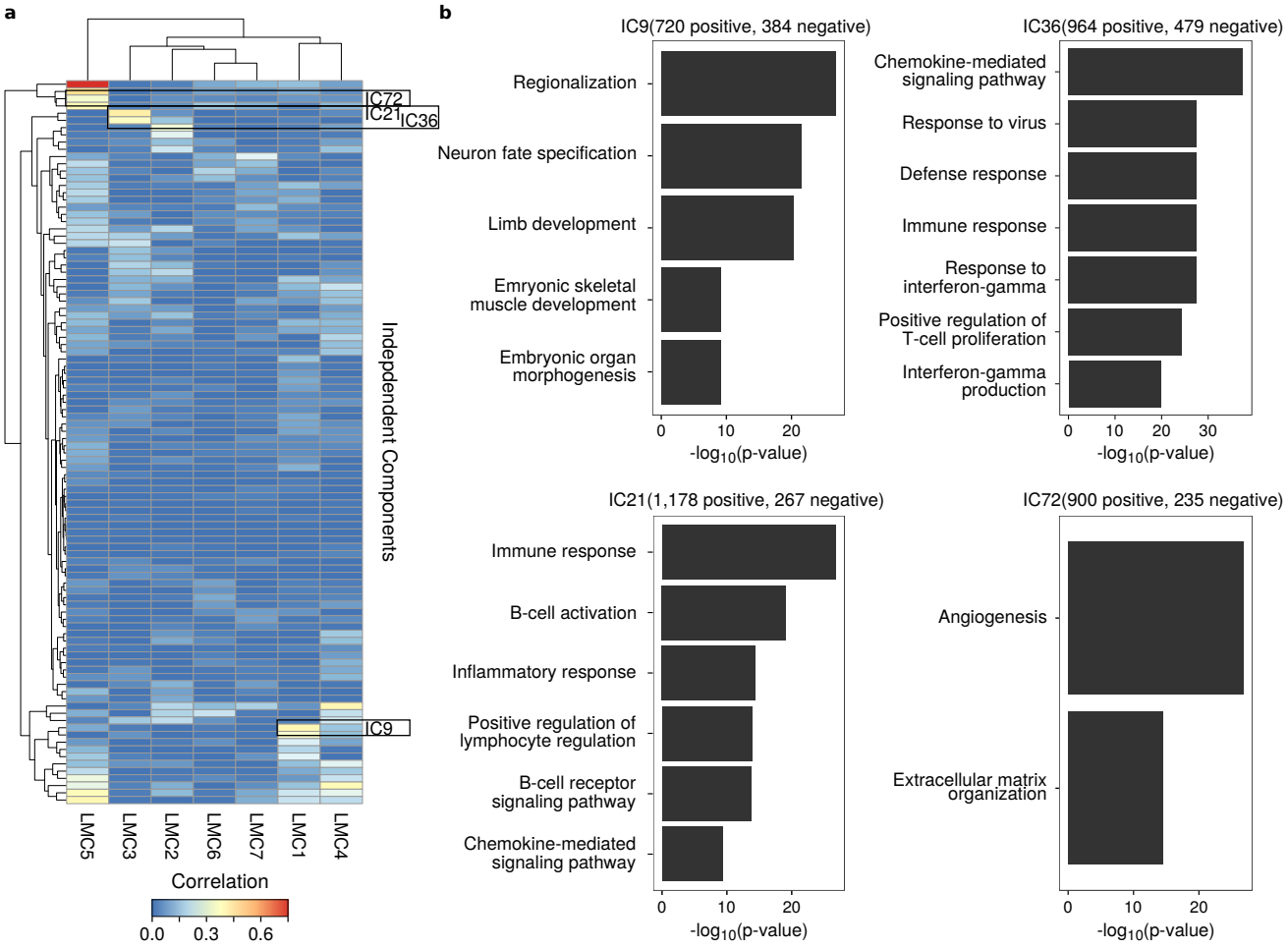
Supplementary Fig. 1: Interpreting *MeDeCom*'s results with *FactorViz*. For each of the steps, a screenshot of the *FactorViz* User Interface is shown for the TCGA LUAD dataset, and the ten performed steps are briefly described. **a, b** Specify the input, **c, d** Select the best parameters for the deconvolution, **e, f** Visualize proportion matrix and associate it with phenotypic traits, **g, h** Visualize LMCs matrix and determine differential CpGs, and **i, j** GO and LOLA enrichment analysis of differential CpGs.



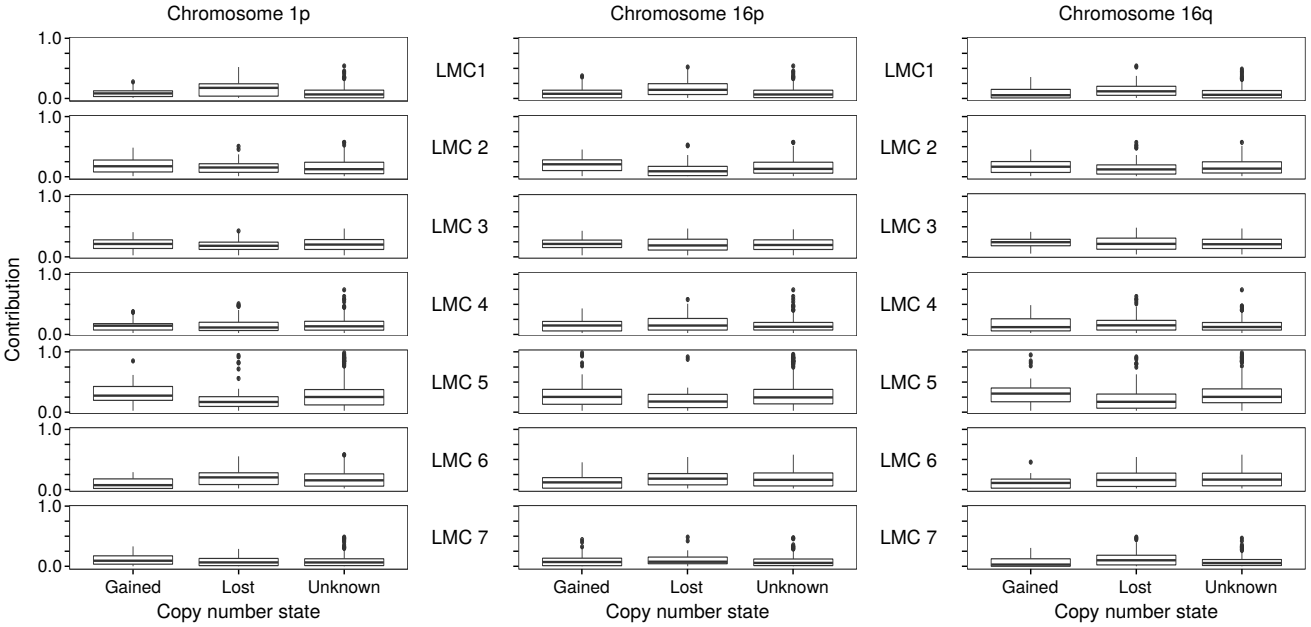
Supplementary Fig. 2: Quality control of TCGA data. **a** Boxplot for hybridization control probes for the green and the red channel, respectively. **b** Sex prediction based on the intensities of the probes on the sex chromosomes. A logistic regression classifier was employed to differentiate between female and male samples. **c** Outline of the CpG filtering procedure. The sites on the 450k array are filtered according to quality scores (coverage, overall intensity), genomic sequence context (SNPs, sex chromosomes), and cross-reactive sites are discarded.



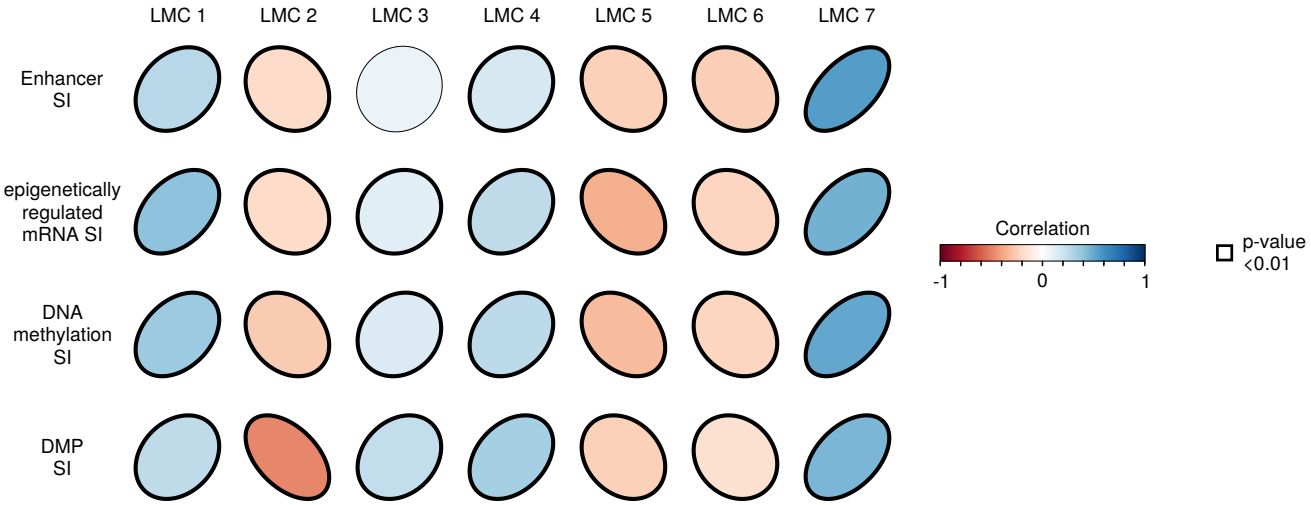
Supplementary Fig. 3: Selecting the number of components and the regularization parameter for *MeDeCom*. **a** Cross-validation error plotted against the number of latent components K for different values of the regularization parameter λ. **b** Objective value and cross-validation error for different values of λ after fixing the number of components to 7. **c** Multidimensional scaling of the LMC data matrix after fixing the number of components to 7 and the regularization parameter to 0.001. Shown are the first two multidimensional components. **d** Violin plots of the LMC methylation matrix for the selected parameters.



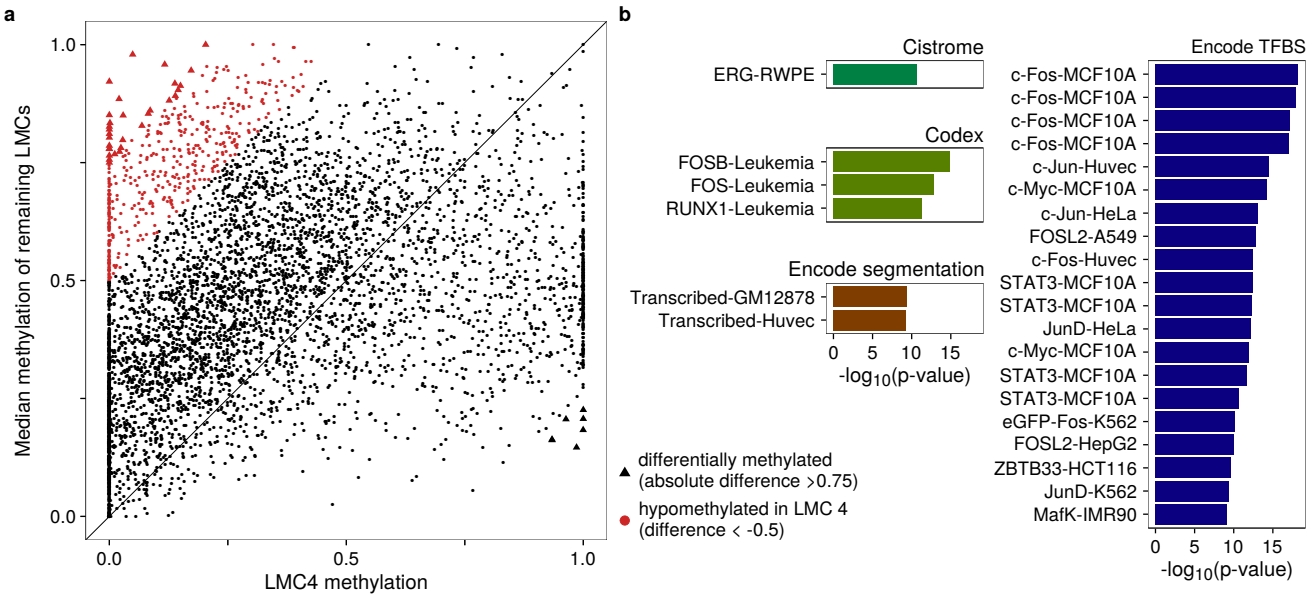
Supplementary Fig. 4: Comparing LMCs with independent components (ICs). **a** Correlation heatmap between the detected LMCs and the 100 detected independent components using ICA. Higher correlation is indicated by red and lower by blue colors. **b** GO enrichment analysis of the CpGs that contributed either positively or negatively (depicted in parentheses) to a particular independent component for IC9, IC21, IC36 and IC72.



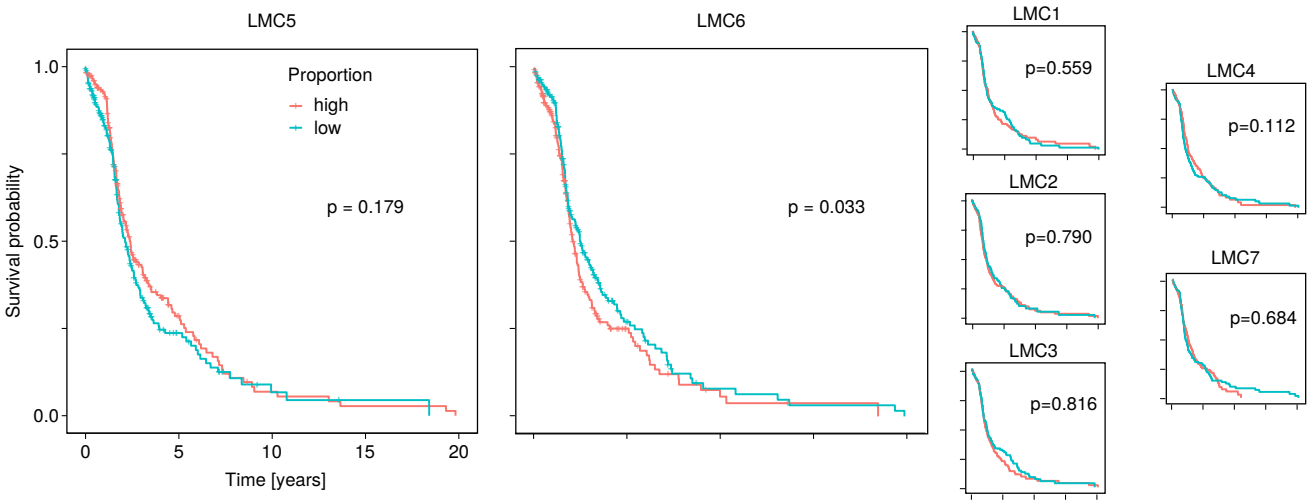
Supplementary Fig. 5: LMC (K=7, λ=0.001) contributions for different copy number states of different chromosomal parts in the TCGA LUAD dataset. The contributions have been stratified for each sample according to overall gain or loss of chromosomal parts. The copy number states were obtained from https://www.cbioportal.org/study/summary?id=luad_tcga_pan_can_atlas_2018 [18, 19].



Supplementary Fig. 6: Pearson correlation between the different cancer stemness indices (SI) computed in Malta *et al.* [20] and the LMC proportions. The ellipses are directed towards the upper right for positive and to the lower right for negative correlations, respectively, while statistical significance is indicated by bold borders. DMP=differentially methylated probes



Supplementary Fig. 7: Differential analysis for LMC4. **a** Scatterplot between the methylation values of LMC4 (x-axis) and the median methylation values of the remaining six LMCs. Each point represents a CpG and points in red indicate the LMC-specific hypomethylated sites (difference less than 0.5), while the bold points represent those with an absolute difference larger than 0.75 (listed in **Supplementary Table 3**). **b** LOLA enrichment analysis of the LMC4-specific hypomethylated sites (the red points). Shown is the negative logarithm of the enrichment p-value.



Supplementary Fig. 8: Survival analysis comparing different levels of LMC proportions. Shown are Kaplan-Meier curves, while samples were stratified according to the LMC proportions into two groups according to the median (high vs. low proportions). P-values were computed using the Cox proportional hazards model with the LMC proportions as input, and age, sex, and tumor stage as covariates [21].

References

1. Colaprico, A. *et al.* TCGAbiolinks: An R/Bioconductor package for integrative analysis of TCGA data. *Nucleic Acids Res.* http://doi.org/10.1093/nar/gkv1507 (2015).
2. McCarthy *et al.* Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res.* **40,** 4288–4297 (2012).
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