ITMO UNIVERSITY

CT Lab
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Exploring gene expression datasets

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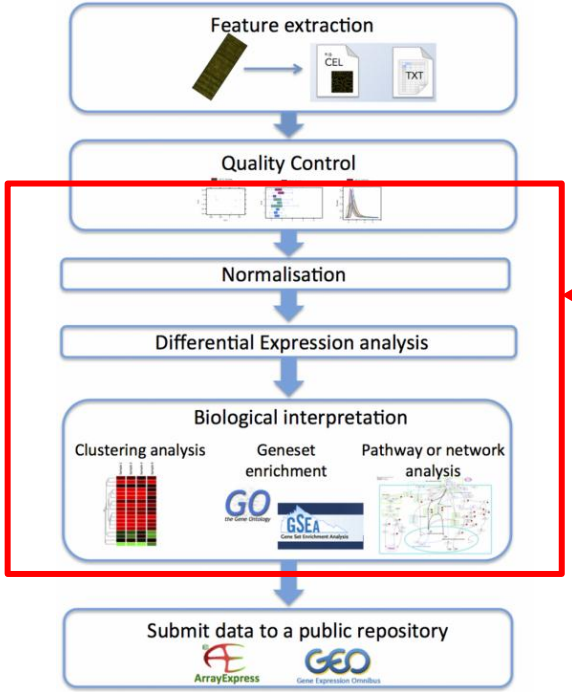
About the module

- We will cover the basic analysis of gene expression matrices
- The focus is on being able to do a quick analysis, not the perfect one

Outline

- **Exploring gene expression datasets**
- Simple analysis methods
- Working with public datasets

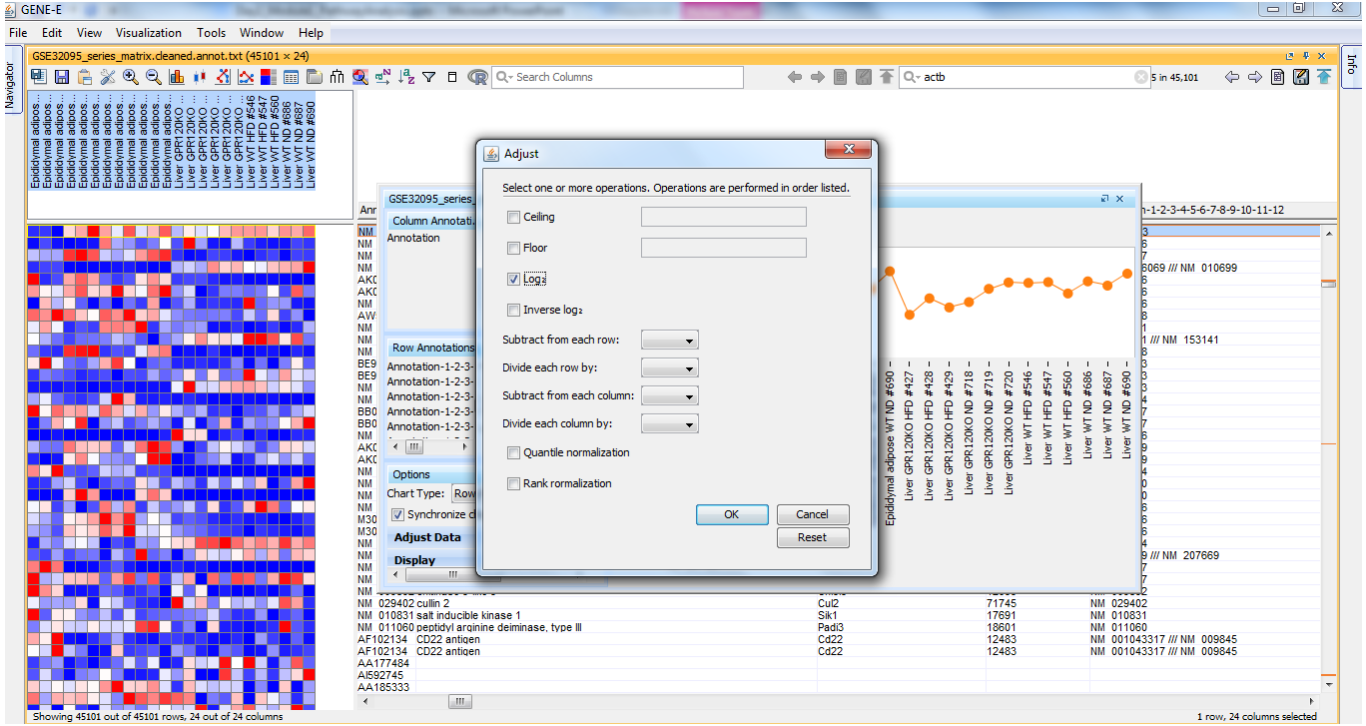
Overall gene expression pipeline



This module

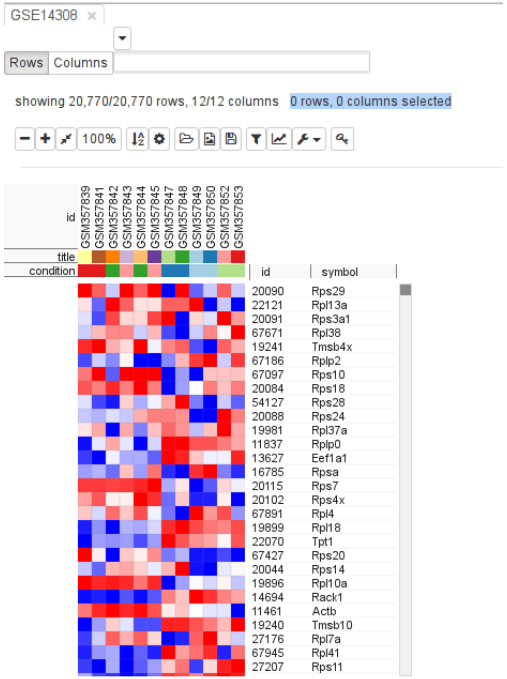
https://www.ebi.ac.uk/training/online/course/functional-genomics-ii-common-technologies-and-data-analysis-methods/analysis-microarray-data

GENE-E: software for working with gene expression data (obsolete)



Morpheus – heatmap visualization software replacing GENE-E

- Developed at Broad Institute (by Joshua Gould)
- Works in browser
- Fully client-side application
  - data is not sent to server!
- Open source
- Limited functionality



https://software.broadinstitute.org/morpheus/

Phantasus – Morpheus integrated with R environment

- An extension developed by Daria Zenkova & Vlad Kamenev at ITMO University
- Server-side application -> requires internet access
  - unless installed locally
- Can be easily extended to support different R/Bioconductor packages
- Free and open-source

- Feedback is welcome!

https://github.com/ctlab/phantasus

Phantasus can be accessed in multiple ways

Online:

- https://ctlab.itmo.ru/phantasus/
- https://artyomovlab.wustl.edu/phantasus/

It can be installed locally from Bioconductor

- http://bioconductor.org/packages/phantasus

As a docker image:

- https://hub.docker.com/r/dzenkova/phantasus

Where datasets are coming from?

- From papers!

LETTER

doi:10.1038/nature13152

NRROS negatively regulates reactive oxygen species during host defence and autoimmunity

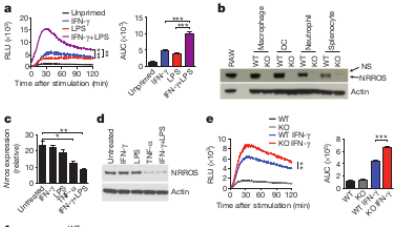
Rajkumar Noubade[1]†, Kit Wong[1], Naruhisa Ota[1], Sascha Rutz[1], Celine Eidenschenk[1], Patricia A. Valdez[1]†, Jiabing Ding[1], Ivan Peng[1], Andrew Sebrell[2], Patrick Caplazi[3], Jason DeVoss[1], Robert H. Soriano[4], Tao Sai[2], Rongze Lu[1], Zora Modrusan[4], Jason Hackney[5] & Wenjun Ouyang[1]

Reactive oxygen species (ROS) produced by phagocytes are essential for host defence against bacterial and fungal infections. Individuals with defective ROS production machinery develop chronic granulomatous disease[1,2]. Conversely, excessive ROS can cause collateral tissue damage during inflammatory processes and therefore needs to be tightly regulated. Here we describe a protein, we termed negative regulator of ROS (NRROS), which limits ROS generation by phagocytes during inflammatory responses. NRROS expression in phagocytes can be repressed by inflammatory signals. NRROS-deficient phagocytes produce increased ROS upon inflammatory challenges, and mice lacking NRROS in their phagocytes show enhanced bactericidal activity against *Escherichia coli* and *Listeria monocytogenes*. Conversely, these mice develop severe experimental autoimmune encephalomyelitis owing to oxidative tissue damage in the central nervous system. Mechanistically, NRROS is localized to the endoplasmic reticulum, where it directly interacts with nascent NOX2 (also known as gp91^{phox} and encoded by *Cybb*) monomer, one of the membrane-bound subunits of the NADPH oxidase complex, and facilitates the degradation of NOX2 through the endoplasmic-reticulum-associated degradation pathway. Thus, NRROS provides a hitherto undefined mechanism for regulating ROS prodution—one that enables phagocytes to produce higher amounts of ROS, if required to control invading pathogens, while minimizing unwanted collateral tissue damage.

In response to microorganisms and inflammatory stimuli, professional phagocytes can generate ROS either within mitochondria or through a process named oxidative burst mediated by the NADPH oxidase 2 (NOX2) complex[1–3]. Although many regulatory factors for

(Fig. 1b and Extended Data Fig. 1d, e). Interestingly, priming with a combination of IFN-γ and LPS or tumour necrosis factor (TNF)-α alone markedly repressed *Nrros* messenger RNA and protein expression in wild-type BMDMs (Fig. 1c, d).

To reveal the biological functions of NRROS, we generated NRROS-specific antibody and NRROS-deficient mice (Extended Data Fig. 1f–j). At 6 weeks of age, all mice were viable and immune organs and leukocyte subsets were indistinguishable from those of wild-type mice (Extended Data Table 1 and data not shown). However, significantly augmented ROS production was observed from NRROS-deficient primary BMDMs upon zymosan stimulation after priming for 24 h with either IFN-γ (Fig. 1e) or LPS (Fig. 1f). These observations were confirmed in a variety of phagocytes, under several priming and activation



http://www.nature.com/nature/journal/v509/n7499/full/nature13152.html

There is a mention of microarray

tion in phagocytes. Gene expression analysis by microarray under these conditions identified a previously uncharacterized gene, EMSMUSG 00000052384, which we named *Nrros* (negative regulator of ROS, previously known as *Lrrc33*) that was markedly downregulated upon priming with a combination of IFN-γ and LPS (Extended Data Fig. 1a). The

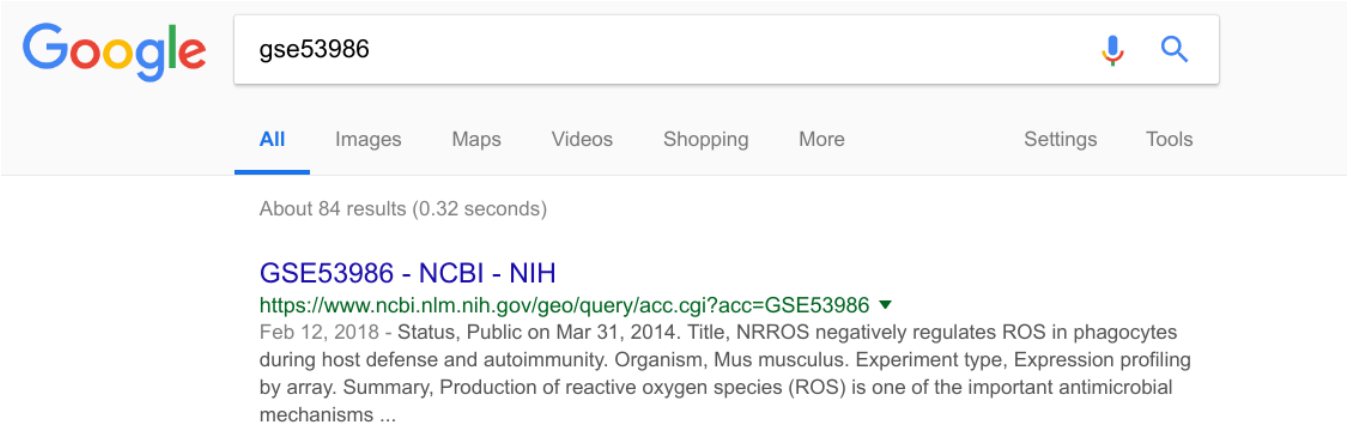
The data should be available from somewhere!

Check the methods section

“accession number GSE53986”

Microarray analysis. Statistical analyses of microarray data were performed using the R programming language (http://r-project.org). Microarray data were normalized using the RMA method[27]. Data were prefiltered to remove probes that were not mapped to an annotated Entrez gene. We also filtered our data to retain only a single probe per gene, selecting the probe with the highest variance, if multiple probes were found for the gene[28]. For differential expression analysis, the limma R package was used[29]. We modelled the synergistic regulation of gene expression by the combined IFN-γ and LPS treatment as an interaction term in our linear model. This model will identify changes that are significantly different from the sum of the individual treatments. Multiple test correction was done using the method of Benjamini and Hochberg[30]. Genes were considered significantly different if they changed more than 1.4-fold at a false discovery rate of 0.05. Genes were further filtered for immune-cell-specific expression using the gene sets defined by the Immune Response In Silico (IRIS) project[31]. As the IRIS-defined gene sets were derived from human immune cells, we mapped the human genes to mouse orthologues using the HomoloGene database[32]. Genes from all IRIS-defined categories were included in the analysis. Data were submitted to the NCBI (accession number GSE53986).

Let’s google that



Let’s look at GSE53986

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53986

Series GSE53986	Query DataSets for GSE53986
Status	Public on Mar 31, 2014
Title	NRROS negatively regulates ROS in phagocytes during host defense and autoimmunity
Organism	Mus musculus
Experiment type	Expression profiling by array
Summary	Production of reactive oxygen species (ROS) is one of the important antimicrobial mechanisms of phagocytic cells. Enhanced oxidative burst requires these cells to be primed with agents such as IFNg and LPS with a synergistic effect of these agents on the level of the burst. However, excessive ROS generation will lead to tissue damage and has been implicated in a variety of inflammatory and autoimmune disease. Therefore, this process needs to be tightly regulated. In order to understand the genes regulating this process, we will treat bone marrow derived macrophages with above mentioned priming agents and study the gene expression. We used microarrays to determine the changes in gene expression that occur in bone marrow derived macrophages after treatment with IFNg, LPS, or a combination of IFNg and LPS
Overall design	Four condition experiment; Biological replicates: four replicates per condition
Contributor(s)	Noubade R, Wong K, Ota N, Rutz S, Eidenschenk C, Ding J, Valdez PA, Peng I, Sebrell A, Caplazi P, DeVoss J, Soriano RH, Modrusan Z, Hackney JA, Sai T, Ouyang W
Citation(s)	Noubade R, Wong K, Ota N, Rutz S et al. NRROS negatively regulates reactive oxygen species during host defence and autoimmunity. *Nature* 2014 May 8;509(7499):235-9. PMID: 24739962

Samples from GSE53986

Samples (16)
Less...

GSM1304836	BMDM, untreated, 1
GSM1304837	BMDM, untreated, 2
GSM1304838	BMDM, untreated, 3
GSM1304839	BMDM, untreated, 4
GSM1304840	BMDM, IFNg, 1
GSM1304841	BMDM, IFNg, 2
GSM1304842	BMDM, IFNg, 3
GSM1304843	BMDM, IFNg, 4
GSM1304844	BMDM, LPS, 1
GSM1304845	BMDM, LPS, 2
GSM1304846	BMDM, LPS, 3
GSM1304847	BMDM, LPS, 4
GSM1304848	BMDM, IFNg+LPS, 1
GSM1304849	BMDM, IFNg+LPS, 2
GSM1304850	BMDM, IFNg+LPS, 3
GSM1304851	BMDM, IFNg+LPS, 4

A lot of datasets can be found at GEO

(will come back to this later)

NCBI Resources How To Sign in to NCBI

GEO Home | Documentation | Query & Browse | Email GEO

Gene Expression Omnibus

GEO is a public functional genomics data repository supporting MIAME-compliant data submissions. Array- and sequence-based data are accepted. Tools are provided to help users query and download experiments and curated gene expression profiles.

Gene Expression Omnibus

Keyword or GEO Accession | Search

Getting Started

- Overview
- FAQ
- About GEO DataSets
- About GEO Profiles
- About GEO2R Analysis
- How to Construct a Query
- How to Download Data

Tools

- Search for Studies at GEO DataSets
- Search for Gene Expression at GEO Profiles
- Search GEO Documentation
- Analyze a Study with GEO2R
- GEO BLAST
- Programmatic Access
- FTP Site

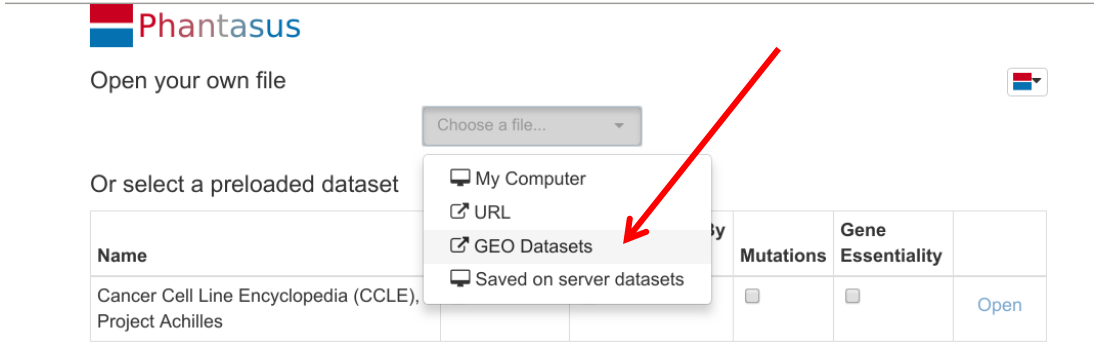
Browse Content

Repository Browser

DataSets:	4348
Series:	83420
Platforms:	17091
Samples:	2042680

Let’s explore this dataset

- Open https://ctlab.itmo.ru/phantasus/ or
- Open https://artyomovlab.wustl.edu/phantasus/
- Load dataset into phantasus:
  - Choose a file/GEO Datasets/GSE53986

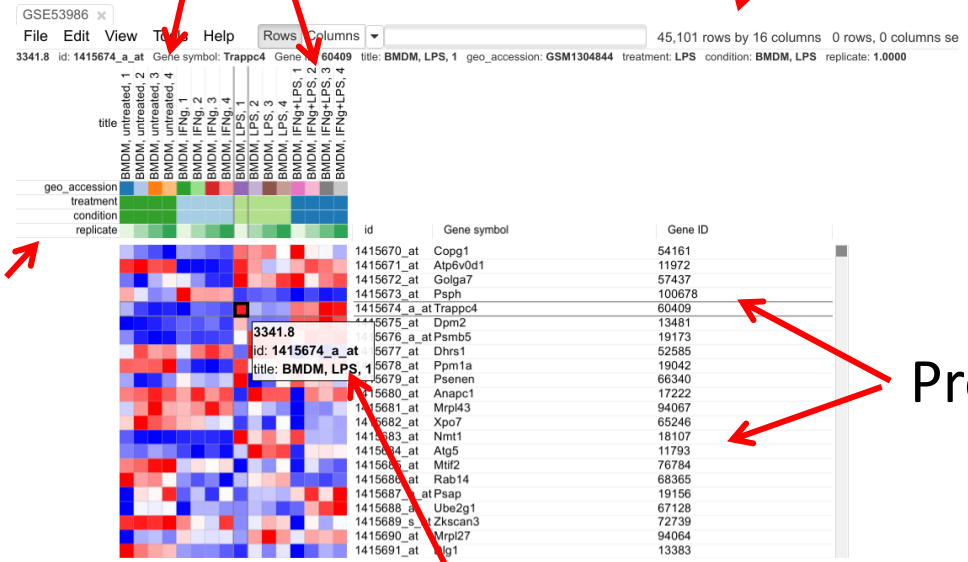


Interface overview

Samples

Dataset dimension

Sample annotations (right click for context menu)



Probes/genes

Expression value (color scheme is relative)

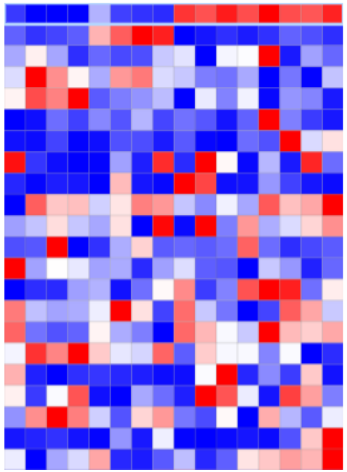
Exploring individual genes

GSE53986

File Edit View Tools Help | Rows Columns | Acod1 | 1 match | 45,101 rows by 16 columns | 1

title: BMDM, untreated, 1; BMDM, untreated, 2; BMDM, untreated, 3; BMDM, untreated, 4; BMDM, IFNg, 1; BMDM, IFNg, 2; BMDM, IFNg, 3; BMDM, IFNg, 4; BMDM, LPS, 1; BMDM, LPS, 2; BMDM, LPS, 3; BMDM, LPS, 4; BMDM, IFNg+LPS, 1; BMDM, IFNg+LPS, 2; BMDM, IFNg+LPS, 3; BMDM, IFNg+LPS, 4

geo_accession
treatment
condition
replicate



id	Gene symbol	Gene ID
1427381_at	Acod1	16365
1427382_a_at	Suv39h1	20937
1427383_at	Irx6	64379
1427384_at	Chd6	71389
1427385_s_at	Actn1	109711
1427386_at	Arhgef16	230972
1427387_a_at	Itgb4	192897
1427388_at	Lrrc2	74249
1427389_at	Mfsd4b5	215928
1427390_at	Bloc1s3	232946
1427391_a_at	Col12a1	12816
1427392_at	Dscaml1	114873
1427393_at	F9	14071
1427394_at	Igf2os	111975
1427395_a_at	Aldh1a3	56847
1427396_a_at	Csde1	229663
1427397_at	Proser1	212127
1427398_at	Muc4	140474
1427399_a_at	Nxf7	170722
1427400_at	Lbx1	16814
1427401_at	Chrna5	110835
1427402_at	Polr2f	69833

1. Enter “Acod1”

2. Click to scroll to the next hit

Row profile chart

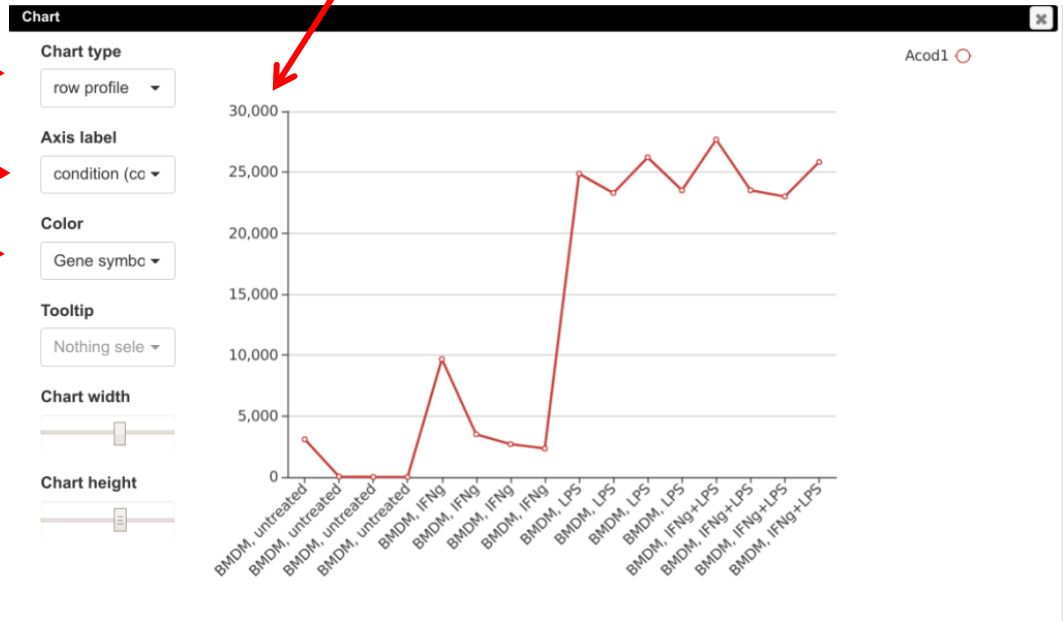
- Select all columns and Acod1 row
- Tools/Plots/Chart

Data is in linear scale!

row profile →

condition →

gene symbol →

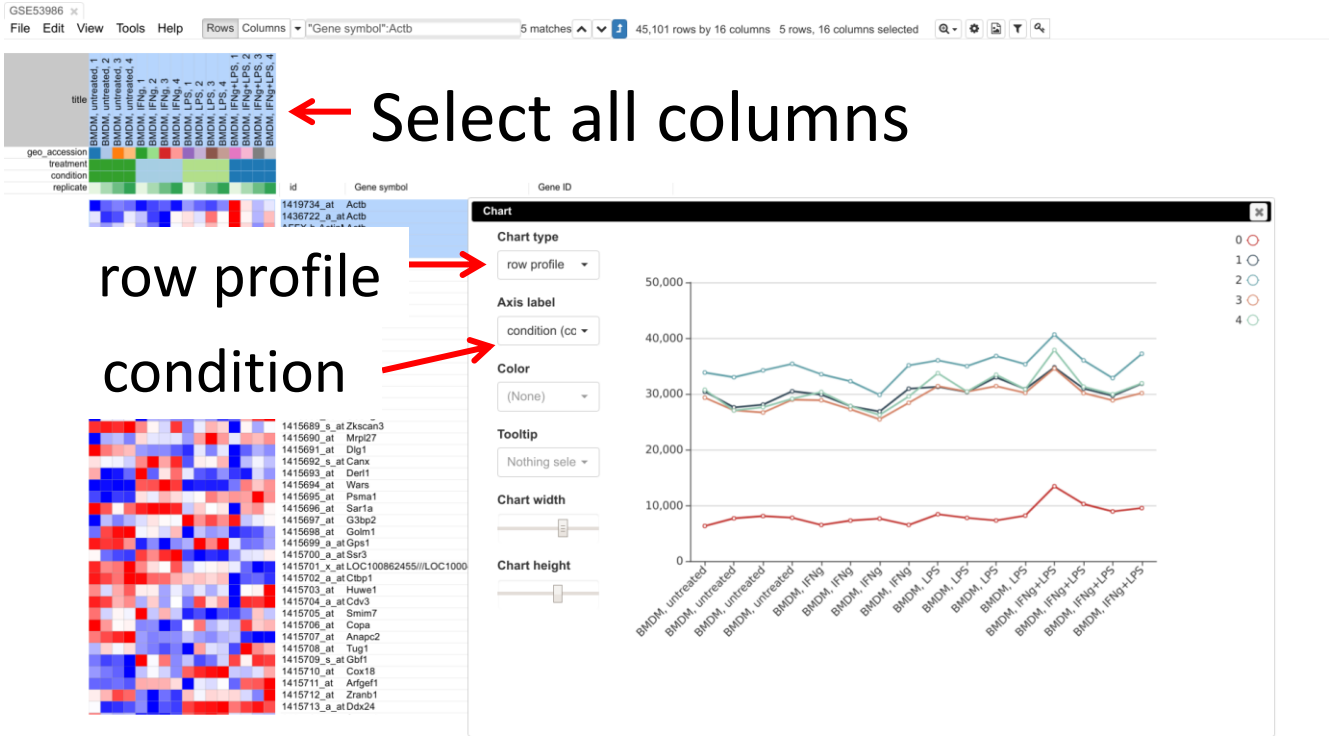


Let’s look at Actb as a control

1. Enter “Actb”

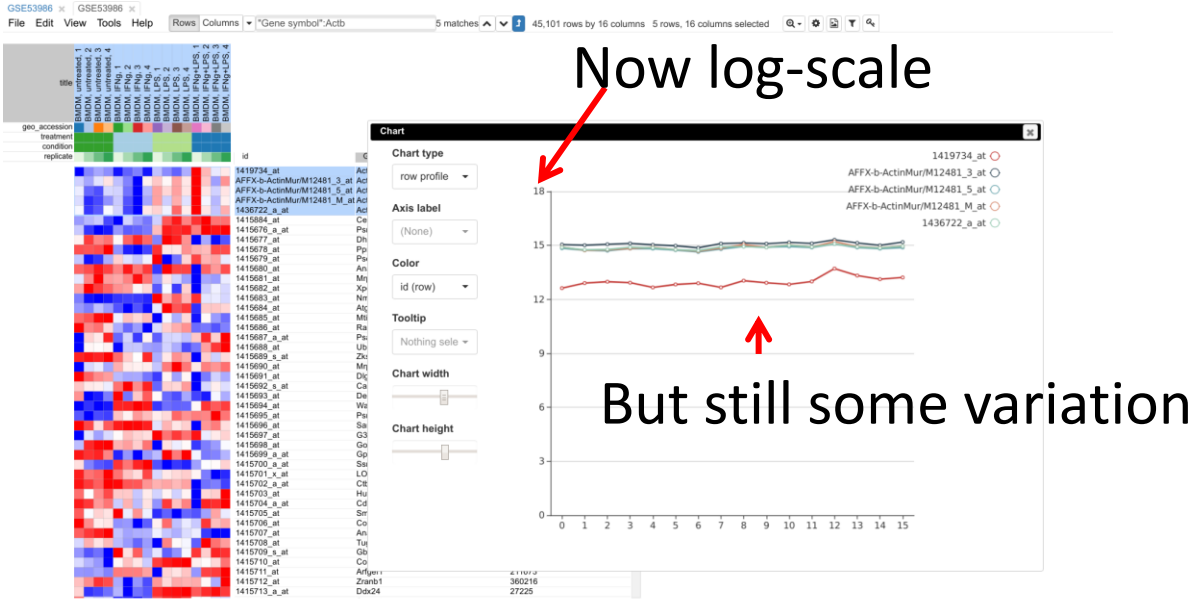
2. Click “Matches to top”

Actb expression chart: high variation (but in a linear scale)



Log 2 normalization

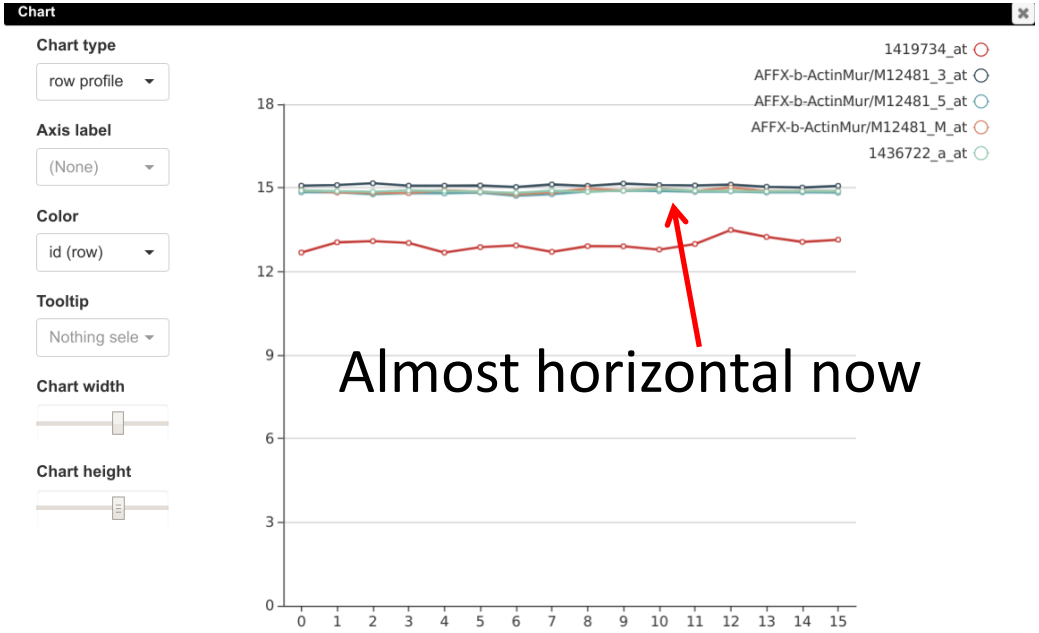
- Close the chart window
- Tools/Adjust, check “Log 2”
- Redo the plot



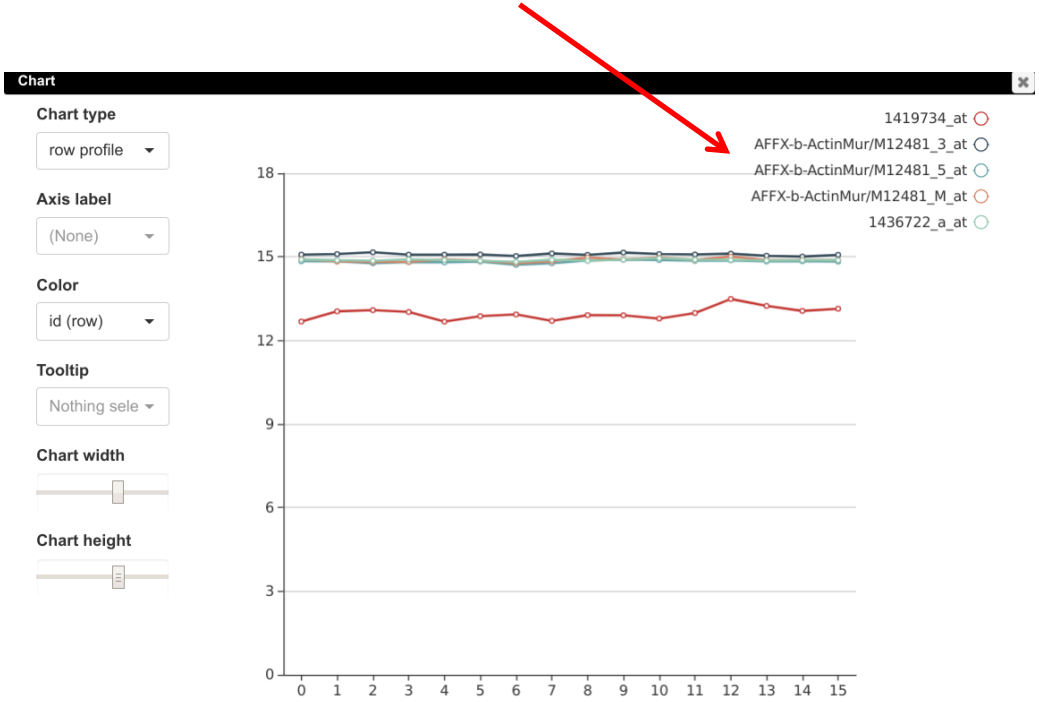
Quantile normalization

- Close the chart window
- Tools/Adjust, check “quantile”
- Redo the plot

- Log2 and quantile can be done in one step
- Don’t do Log2 twice, twice quantile is OK

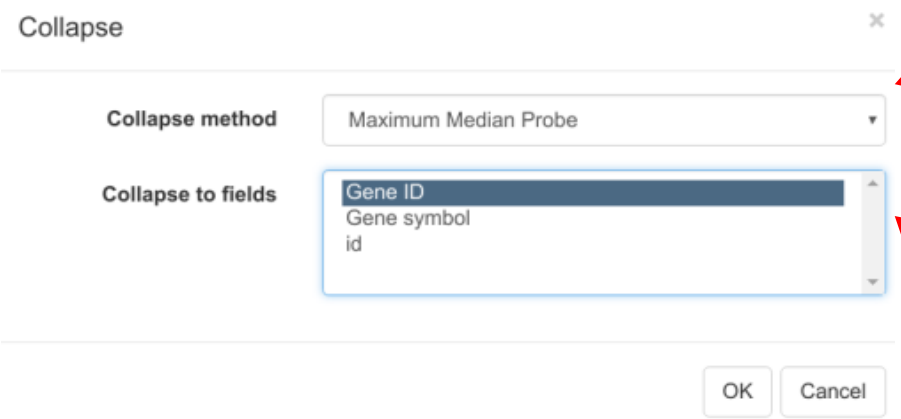


There are multiple probes per gene in microarrays



Collapsing duplicated probes to genes: keeping only one probe per gene

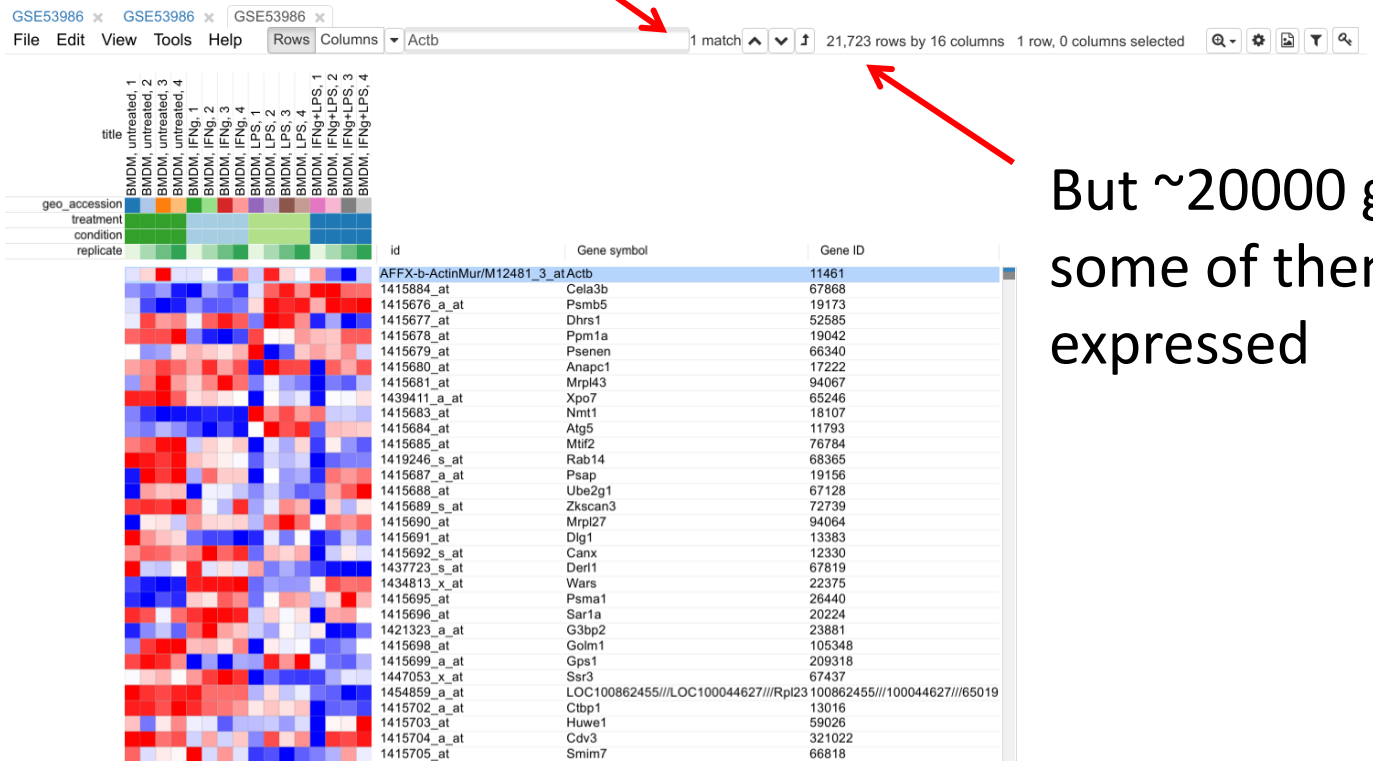
- Tools/Collapse



method = maximum median probe

Grouping by Gene ID

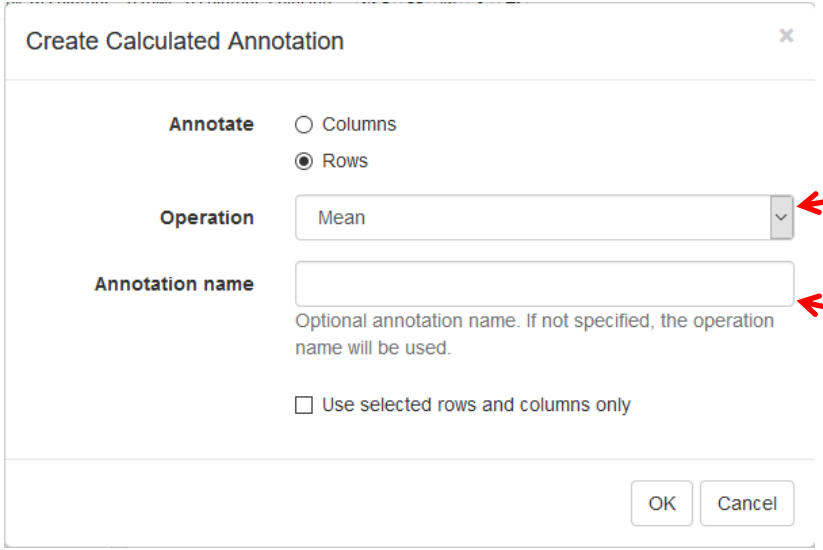
No more duplicates



But ~20000 genes, some of them are not expressed

Filtering lowly expressed genes: calculating mean expression

- Tools/Create Calculated Annotation



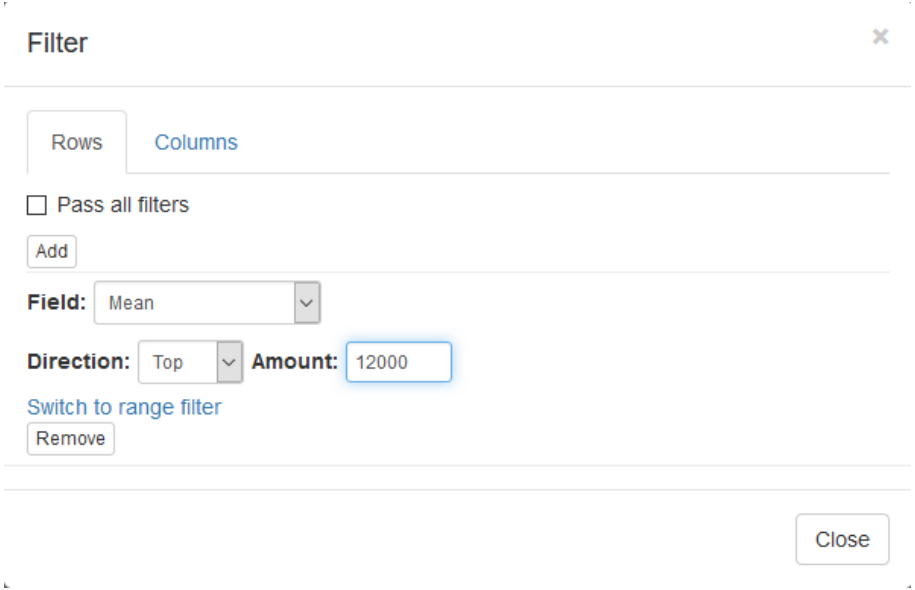
Operation: Mean

Optional name
(e.g. “mean_expression”)

Filtering lowly expressed genes: calculating mean expression result

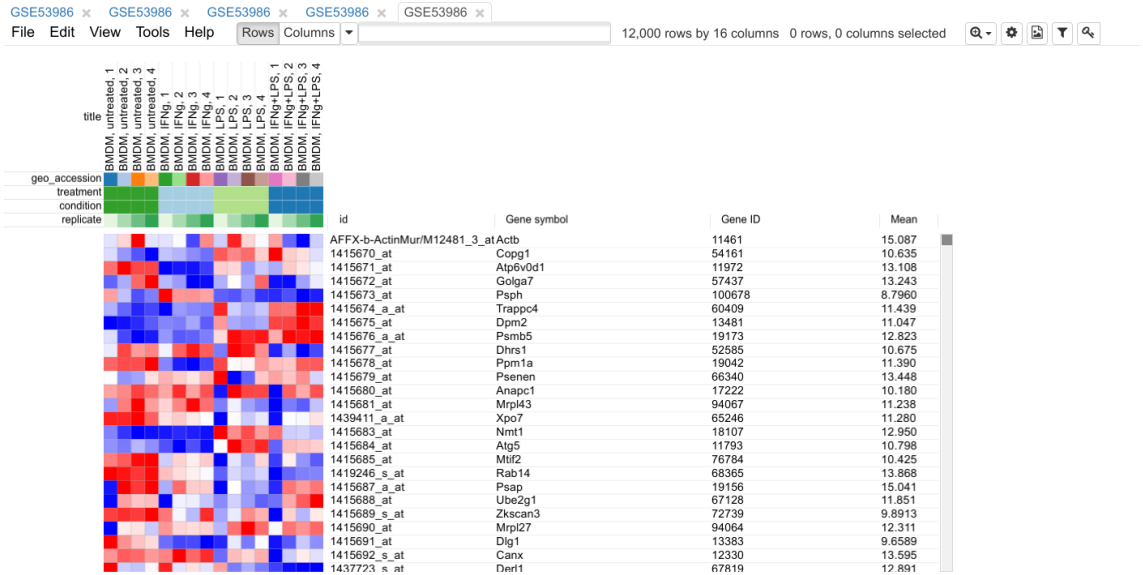
Filtering lowly expressed genes: keeping only top 12000 genes

- Tools/Filter
- Add
- Field <- Mean
- Switch to top filter
- N <- 12000



Filtering lowly expressed genes: creating new dataset

- Select all genes (click on any gene and Ctrl+A)
- Hit Ctrl-X to create new dataset (or Tools/New Heat Map)



Saving dataset

- File/Save Dataset

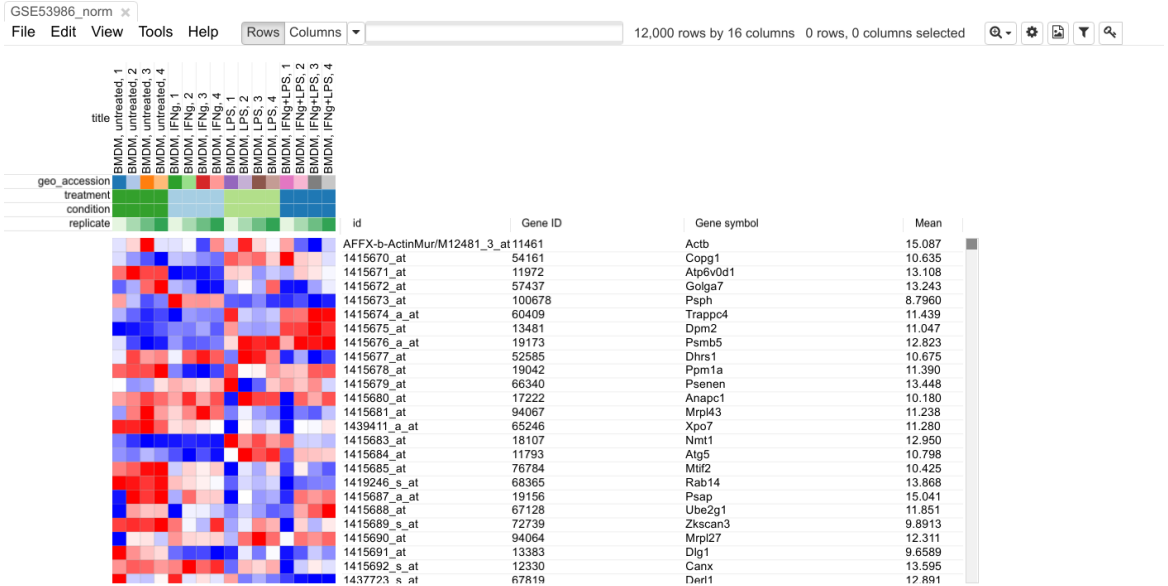
Let’s look at what we got

- Open gct file in Excel/Calc/Notepad

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
1	#1.3																				
2	12001	16	3	7																	
3	id/title	Gene ID	Gene sym	mean_exp	BMDM, ur	BMDM, ur	BMDM, ur	BMDM, ur	BMDM, IF	BMDM, IF	BMDM, IF	BMDM, IF	BMDM, LP	BMDM, LP	BMDM, LP	BMDM, LP	BMDM, IF	BMDM, IF	BMDM, IF	BMDM, IFNg+LPS, 4	
4	geo_acces	na	na	na	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304	GSM1304851	
5	strain	na	na	na	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	C57BL/6	
6	tissue	na	na	na	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone mari	bone marrow	
7	cell type	na	na	na	macropha	macropha	macropha	macropha	macropha	macropha	macropha	macropha	macropha	macropha	macropha	macropha	macropha	macropha	macropha	macrophages	
8	treatment	na	na	na	Untreated	Untreated	Untreated	Untreated	IFNg	IFNg	IFNg	IFNg	LPS	LPS	LPS	LPS	IFNg+LPS	IFNg+LPS	IFNg+LPS	IFNg+LPS	
9	condition	na	na	na	BMDM, ur	BMDM, ur	BMDM, ur	BMDM, ur	BMDM, IF	BMDM, IF	BMDM, IF	BMDM, IF	BMDM, LP	BMDM, LP	BMDM, LP	BMDM, LP	BMDM, IF	BMDM, IF	BMDM, IF	BMDM, IFNg+LPS	
10	replicate	na	na	na	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	
11	AFFX-b-A	11461	Actb	15.087	15.078	15.1	15.165	15.078	15.078	15.085	15.03	15.122	15.072	15.155	15.1	15.085	15.115	15.04	15.009	15.072	
12	1415884_a	67868	Cela3b	6.5234	5.998	5.8081	6.0273	5.391	5.2858	6.0805	5.8875	5.5574	6.3775	7.2931	7.6495	7.0469	7.7264	7.7597	7.2534	7.2321	
13	1415676_a	19173	Psmb5	12.823	12.663	12.208	11.995	12.073	12.451	12.264	12.29	12.374	12.879	13.51	13.418	13.476	13.02	13.527	13.48	13.54	
14	1415677_a	52585	Dhrs1	10.675	10.594	10.873	10.756	10.785	10.602	10.836	10.933	10.839	10.443	10.965	10.929	10.774	10.322	10.502	10.277	10.372	
15	1415678_a	19042	Ppm1a	11.39	11.555	11.594	11.592	11.708	11.135	10.976	10.933	11.04	11.602	11.326	11.339	11.474	11.422	11.407	11.568	11.573	
16	1415679_a	66340	Psenen	13.448	13.431	13.291	13.312	13.475	13.536	13.502	13.468	13.535	13.771	13.094	13.225	13.506	13.556	13.51	13.583	13.377	
17	1415680_a	17222	Anapc1	10.18	10.162	10.205	10.322	10.252	10.155	10.354	10.161	10.293	9.6225	10.427	10.315	10.31	9.5902	10.267	10.15	10.293	
18	1415681_a	94067	Mrpl43	11.238	11.154	11.352	11.459	11.324	11.278	11.344	11.455	11.363	11.128	11.227	11.159	11.13	11.021	11.127	11.1	11.188	
19	1439411_a	65246	Xpo7	11.28	11.654	11.647	11.712	11.533	11.291	11.33	11.274	11.237	10.749	11.238	11.132	11.188	10.77	11.227	11.217	11.284	
20	1415683_a	18107	Nmt1	12.95	12.86	12.73	12.646	12.669	12.68	12.713	12.694	12.673	13.48	13.25	13.358	13.225	13.294	12.982	12.989	12.954	
21	1415684_a	11793	Atg5	10.798	10.699	10.667	10.751	10.695	10.608	10.497	10.607	10.514	10.849	11.205	11.092	11.152	10.633	10.944	10.933	10.919	
22	1415685_a	76784	Mtif2	10.425	10.596	10.642	10.751	10.766	10.35	10.48	10.441	10.49	10.07	10.382	10.303	10.474	10.153	10.441	10.272	10.193	
23	1419246_s	68365	Rab14	13.868	14.43	14.388	14.309	14.409	14.001	13.938	13.892	13.843	13.5	13.77	13.704	13.764	13.285	13.514	13.571	13.58	
24	1415687_a	19156	Psap	15.041	14.874	15.191	15.155	15.191	14.973	15.122	15.059	15.059	14.861	15.026	14.96	14.973	14.886	15.115	15.092	15.115	
25	1415688_a	67128	Ube2g1	11.851	11.647	11.963	11.93	11.936	11.642	11.863	11.86	11.824	11.768	11.833	11.785	11.73	11.744	11.955	12.026	12.109	
26	1415689_s	72739	Zkscan3	9.8913	10.083	10.11	10.09	10.173	10.003	9.7937	9.7065	10.111	9.6763	9.7773	9.9733	9.9161	9.439	9.8711	9.7016	9.8362	
27	1415690_a	94064	Mrpl27	12.311	11.718	12.249	12.265	12.107	12.414	12.291	12.278	12.299	12.072	12.487	12.703	12.495	12.2	12.479	12.421	12.505	
28	1415691_a	13383	Dlg1	9.6589	10.055	9.8835	9.825	9.796	9.5786	9.5297	9.5348	9.4491	9.4932	9.7261	9.5939	9.7468	9.4707	9.5531	9.6885	9.6174	

Loading a gct file in Phantasus

- File/Open, choose GSE53986_norm.gct



https://artyomovlab.wustl.edu/publications/supp_materials/SBW2018-StL/GSE53986_norm.gct

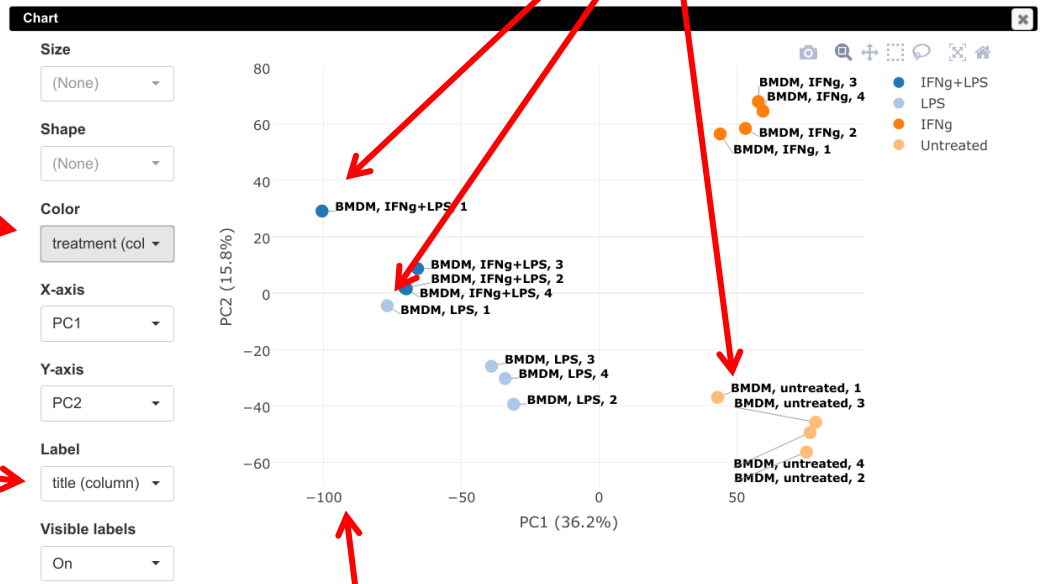
Exploring dataset: principal component analysis (PCA) plot

- Tools/Plots/PCA plot

Outliers!

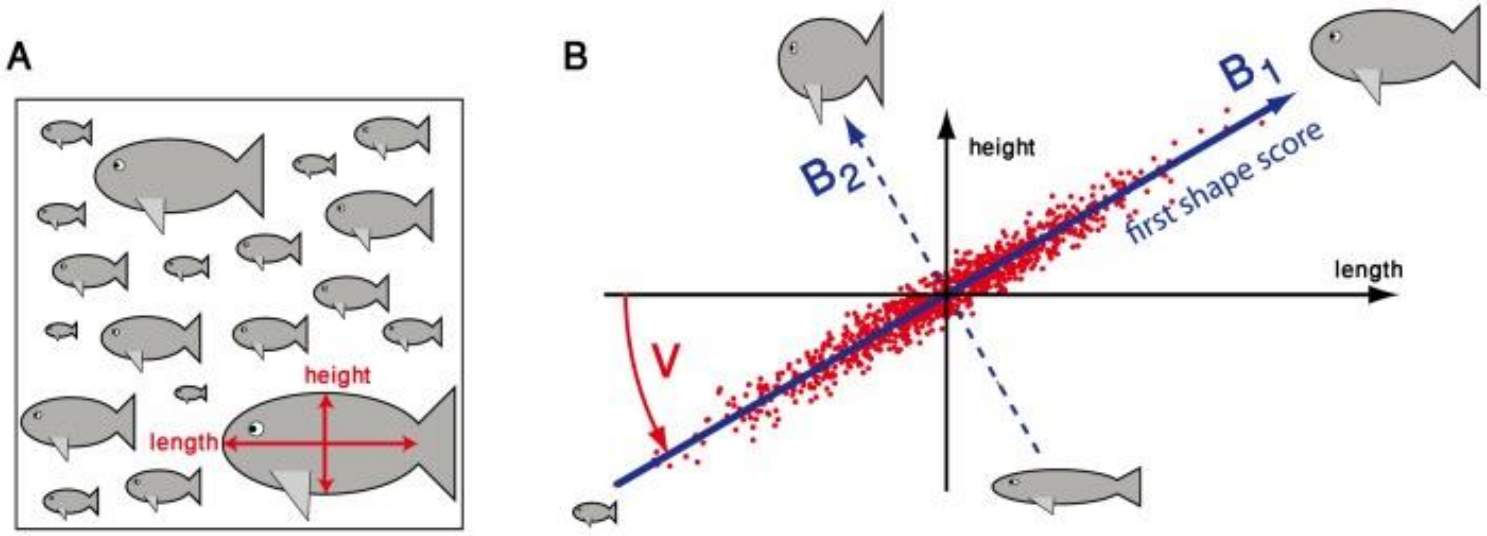
color <- treatment

label <- title



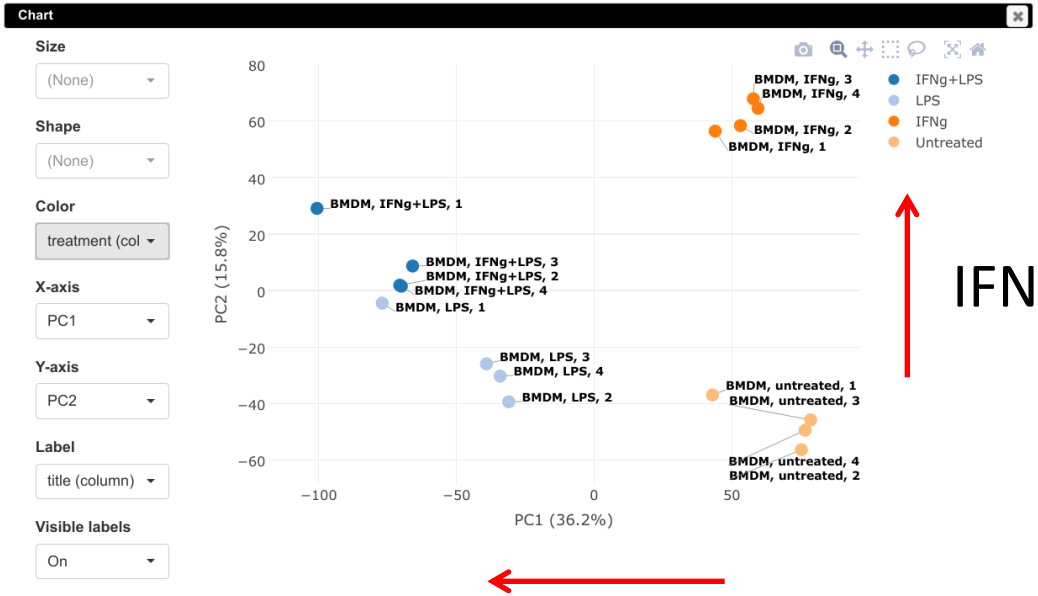
Scale should be ~10-100, not 1000000

What is PCA?



https://www.ncbi.nlm.nih.gov/pubmed/25426857

Exploring dataset: principal components can be meaningful

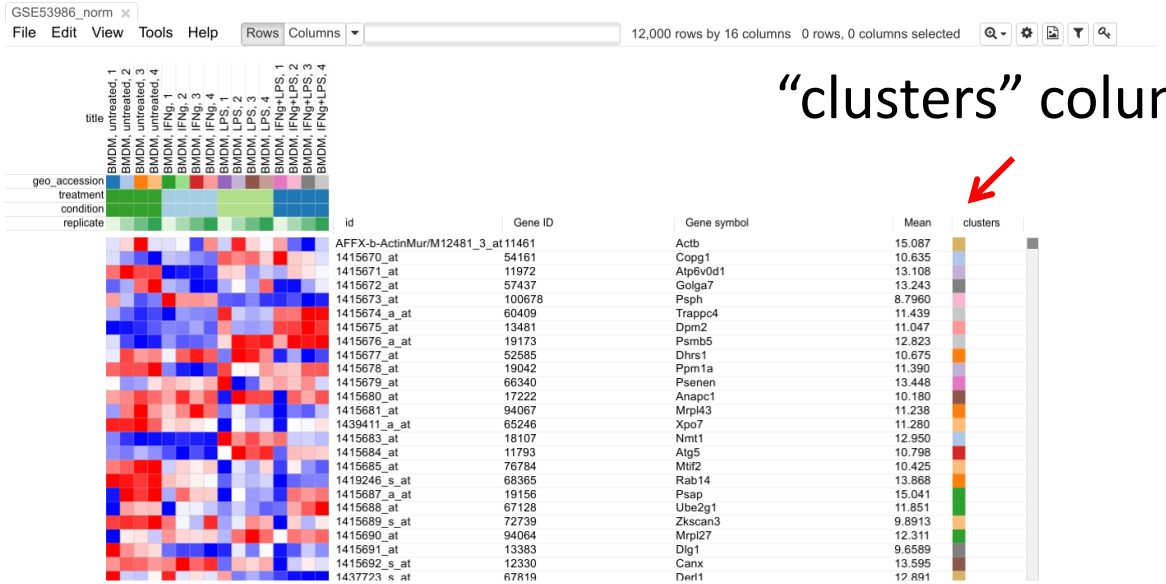


IFNg response

LPS response

Exploring dataset: k-means

- Tools/Clustering/k-means
- Number of cluster = 16

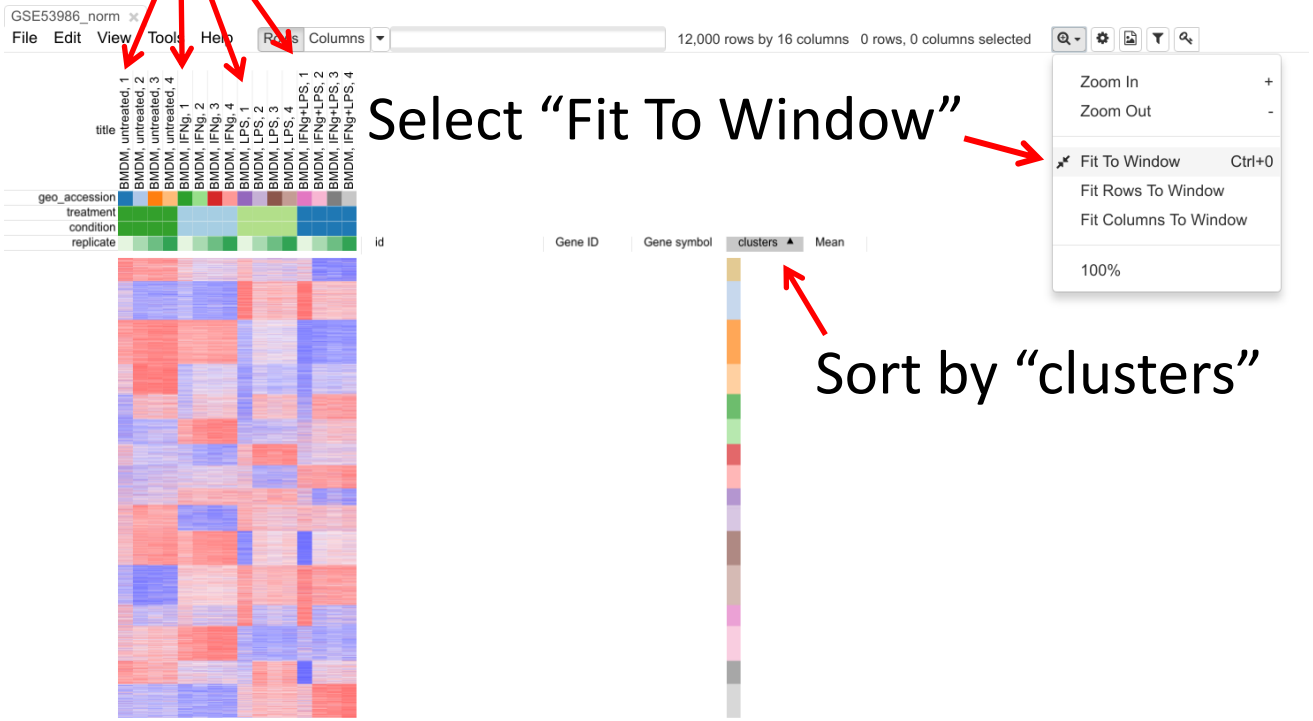


id	Gene ID	Gene symbol	Mean	clusters
AFFX-b-ActinMur/M12481_3_at	11461	Actb	15.087	
1415670_at	54161	Copg1	10.635	
1415671_at	11972	Atp6v0d1	13.108	
1415672_at	57437	Golga7	13.243	
1415673_at	100678	Psph	8.7960	
1415674_a_at	60409	Trappc4	11.439	
1415675_at	13481	Dpm2	11.047	
1415676_a_at	19173	Psmb5	12.823	
1415677_at	52585	Dhrs1	10.675	
1415678_at	19042	Ppm1a	11.390	
1415679_at	66340	Psenen	13.448	
1415680_at	17222	Anapc1	10.180	
1415681_at	94067	Mrpl43	11.238	
1439411_a_at	65246	Xpo7	11.280	
1415683_at	18107	Nmt1	12.950	
1415684_at	11793	Atg5	10.798	
1415685_at	76784	Mtif2	10.425	
1419246_s_at	68365	Rab14	13.868	
1415687_a_at	19156	Psap	15.041	
1415688_at	67128	Ube2g1	11.851	
1415689_s_at	72739	Zkscan3	9.8913	
1415690_at	94064	Mrpl27	12.311	
1415691_at	13383	Dlg1	9.6589	
1415692_s_at	12330	Canx	13.595	
1437723_s_at	67819	Derl1	12.891	

“clusters” column appeared

Exploring dataset: k-means, bird's eye view

Our outliers

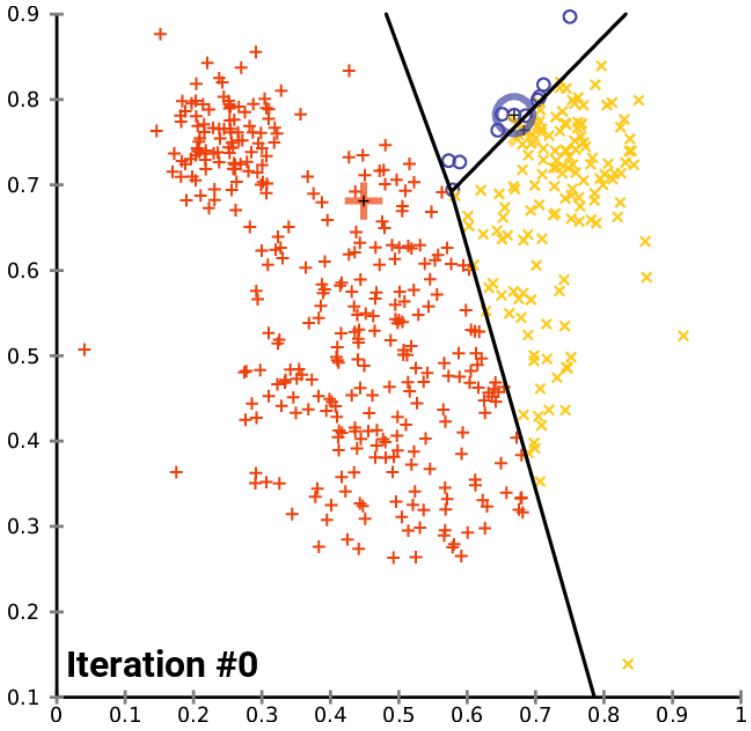


Select "Fit To Window"

Sort by "clusters"

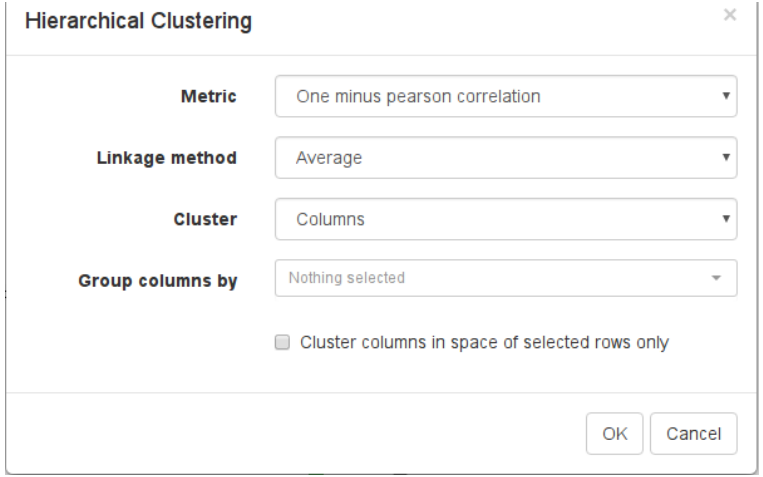
How k-means clustering works

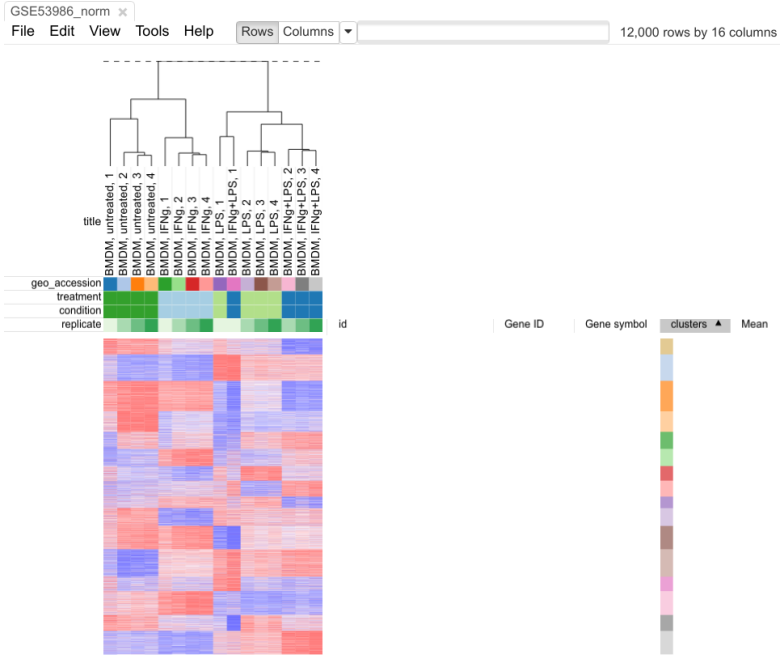
- Select k *random* centers
- Assign each gene to the closes cluster center
- Refine center
- Repeat until convergence



Exploring dataset: hierarchical clustering

- Tools/Hierarchical clustering
- Metric <- 1 - pearson correlation

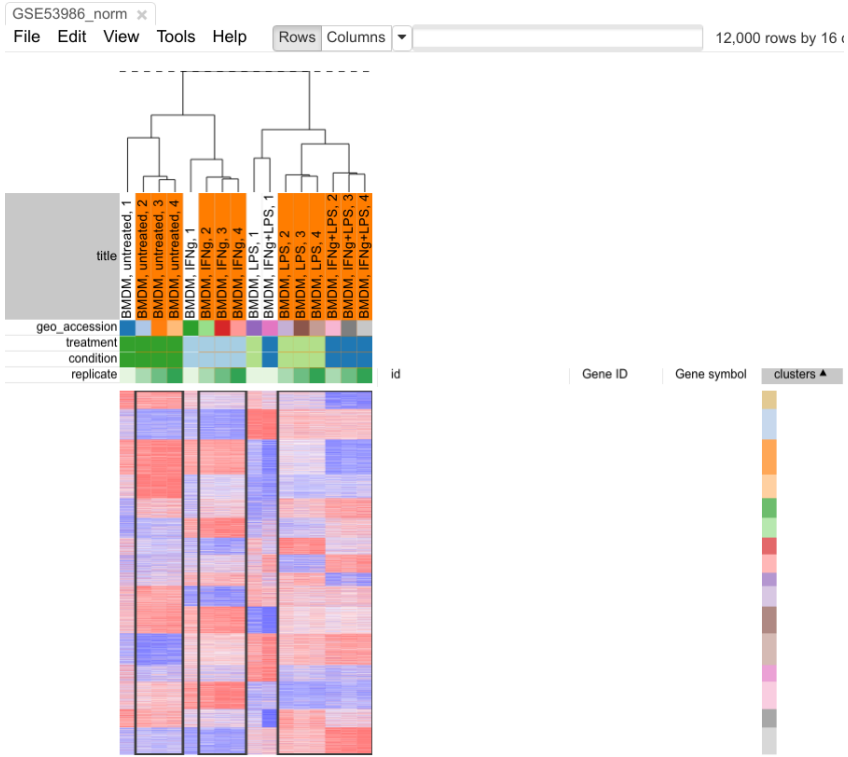




Filtering outliers

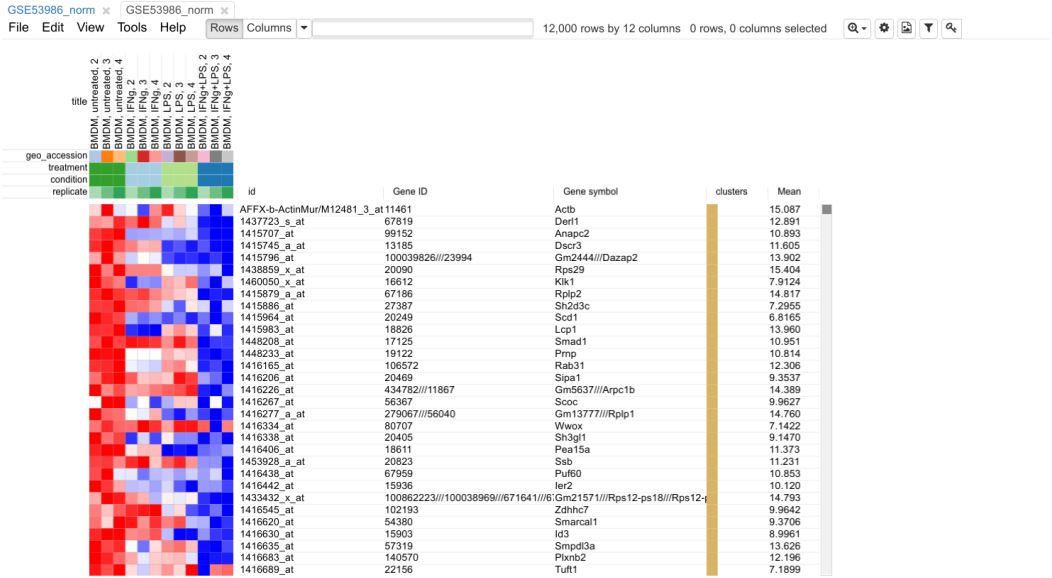
- Select good samples
- Tools/New heatmap (Ctrl-X)

- Very bad outlier should be removed at the start of the analysis, before normalization



Saving filtered dataset

- File/Save dataset
- Name like GSE53986_filtered.gct



id	Gene ID	Gene symbol	clusters	Mean
AFFX-b-ActinMur/M12481_3_at	11461	Actb		15.087
1437723_s_at	67819	Derl1		12.891
1415707_at	99152	Anapc2		10.893
1415745_a_at	13185	Dscr3		11.605
1415796_at	100039826///23994	Gm2444///Dazap2		13.902
1438859_x_at	20090	Rps29		15.404
1460050_x_at	16612	Klk1		7.9124
1415879_a_at	67186	Rplp2		14.817
1415886_at	27387	Sh2d3c		7.2955
1415964_at	20249	Scd1		6.8165
1415983_at	18826	Lcp1		13.960
1448208_at	17125	Smad1		10.951
1448233_at	19122	Prnp		10.814
1416165_at	106572	Rab31		12.306
1416206_at	20469	Sipa1		9.3537
1416226_at	434782///11867	Gm5637///Arpc1b		14.389
1416267_at	56367	Scoc		9.9627
1416277_a_at	279067///56040	Gm13777///Rplp1		14.760
1416334_at	80707	Wwox		7.1422
1416338_at	20405	Sh3gl1		9.1470
1416406_at	18611	Pea15a		11.373
1453928_a_at	20623	Ssb		11.231
1416438_at	67959	Puf60		10.853
1416442_at	15936	Ier2		10.120
1433432_x_at	100862223///100038969///671641///6	Gm21571///Rps12-ps18///Rps12-		14.793
1416545_at	102193	Zdhhc7		9.9642
1416620_at	54380	Smarcal1		9.3706
1416630_at	15903	Id3		8.9961
1416635_at	57319	Smpdl3a		13.626
1416683_at	140570	Plxnb2		12.196
1416689_at	22156	Tuft1		7.1899

Summary

- Check expression scale (should be log2)
- Data should be normalized
- Do a quality check by looking at markers, PCA, k-means and hierarchical clustering
- Save processed datasets

- Next: doing a differential expression analysis