Measuring Gene Expression Part 3

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Bioinformatics 301

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Objectives*

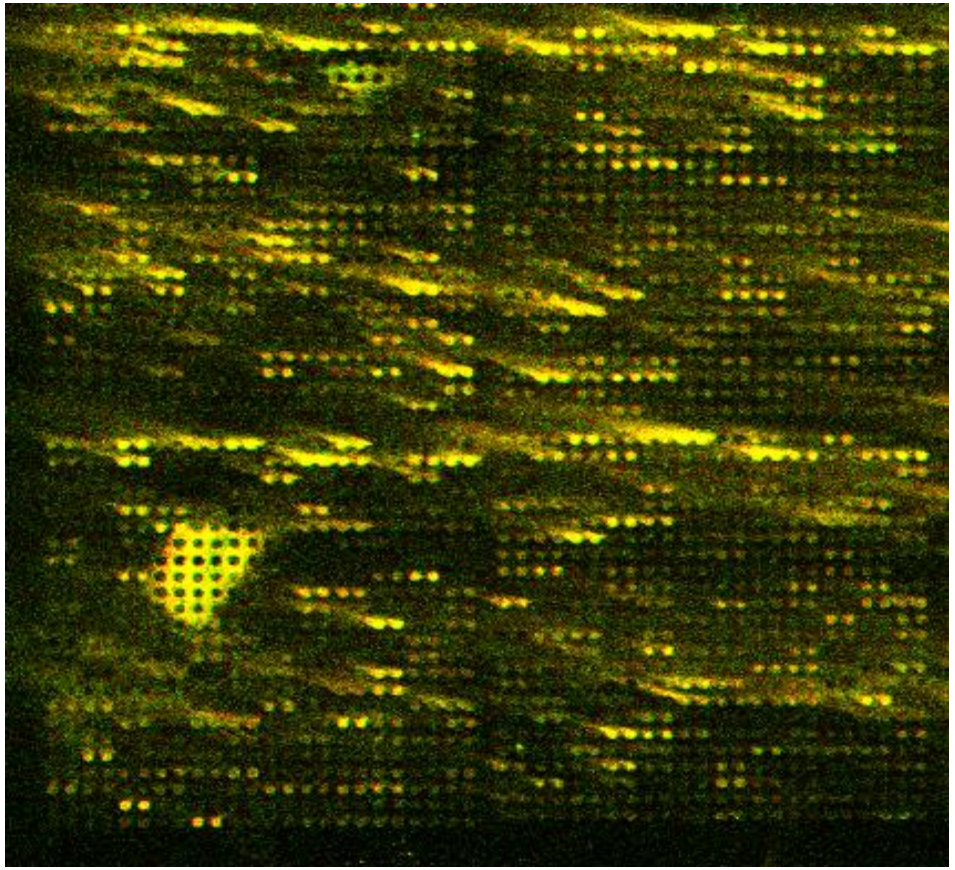
- Become aware of some of the causes of low quality microarray data
- Become familiar with gridding, spot picking, intensity determination & quality control issues
- Become familiar with normalization, curve fitting and correlation
- Understand how microarray data is analyzed

Key Steps in Microarray Analysis*

- Quality Control (checking microarrays for errors or problems)
- Image Processing
  - Gridding
  - Segmentation (peak picking)
  - Data Extraction (intensity, QC)
- Data Analysis and Data Mining

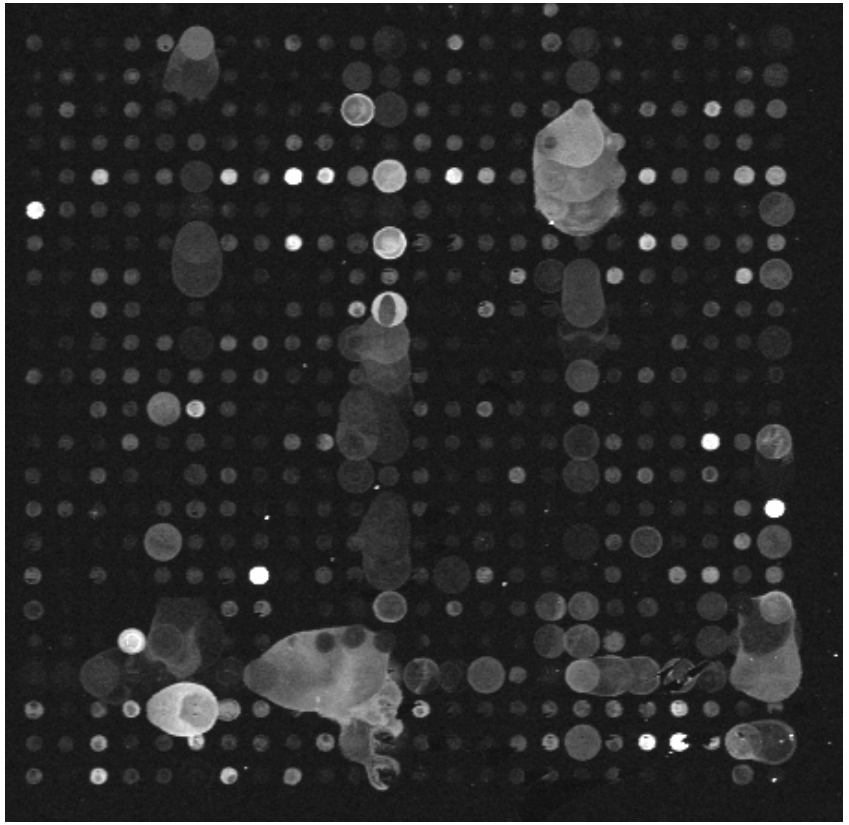
Comet Tailing*

- **Often caused by insufficiently rapid immersion of the slides in the succinic anhydride blocking solution.**

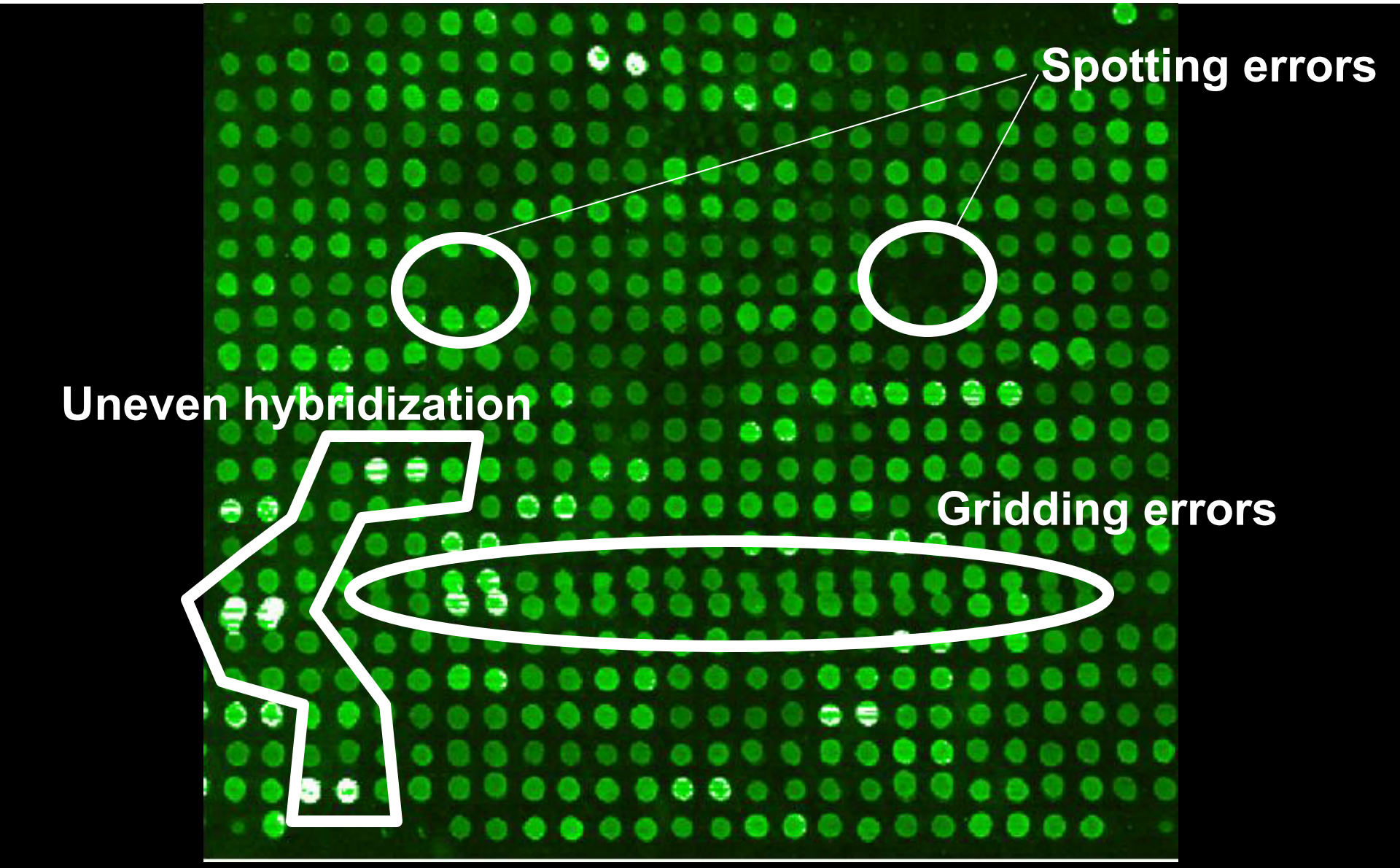


Uneven Spotting/Blotting

- Problems with print tips or with overly viscous solution
- Problems with humidity in spottiing chamber



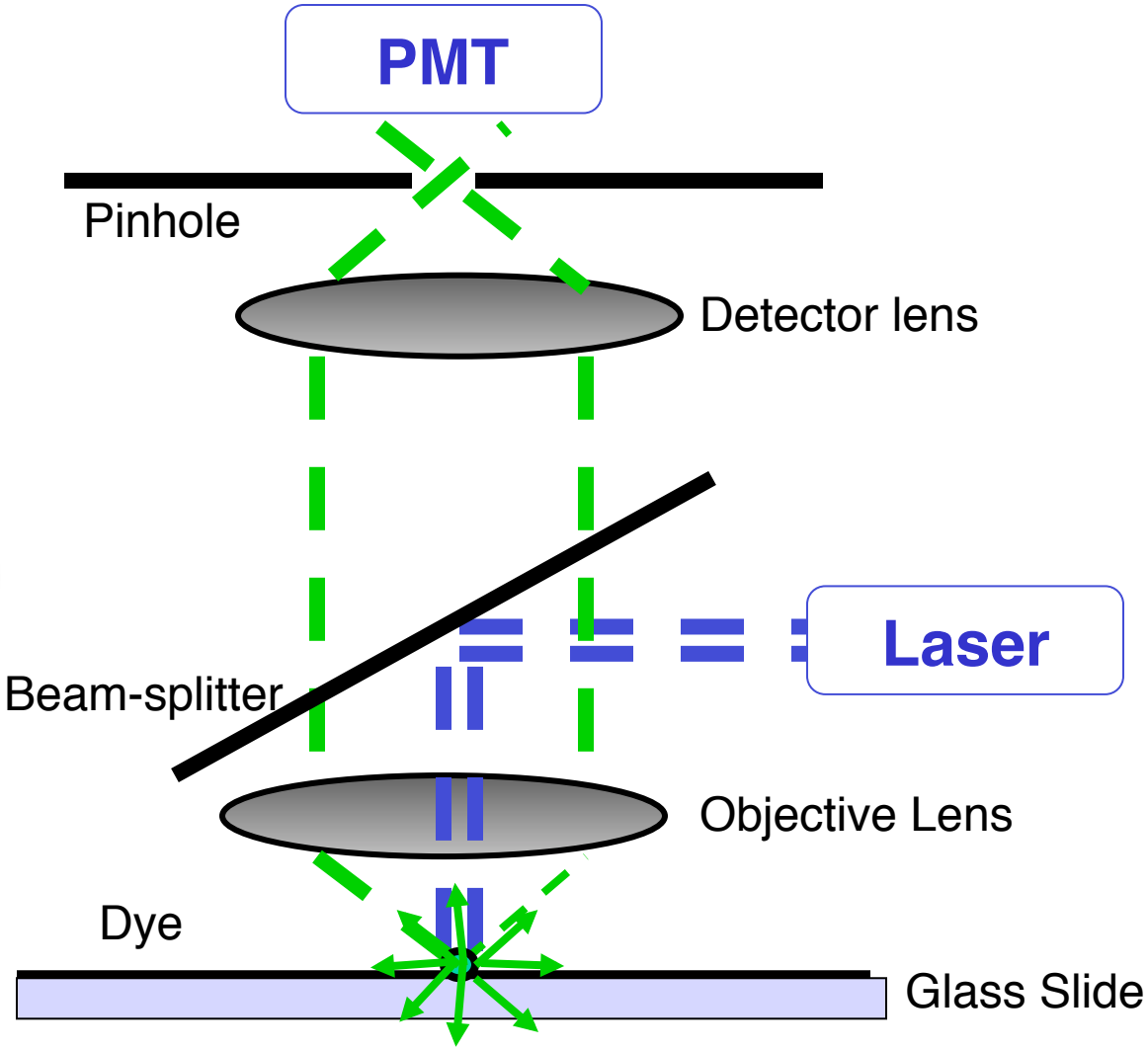
Gridding Errors



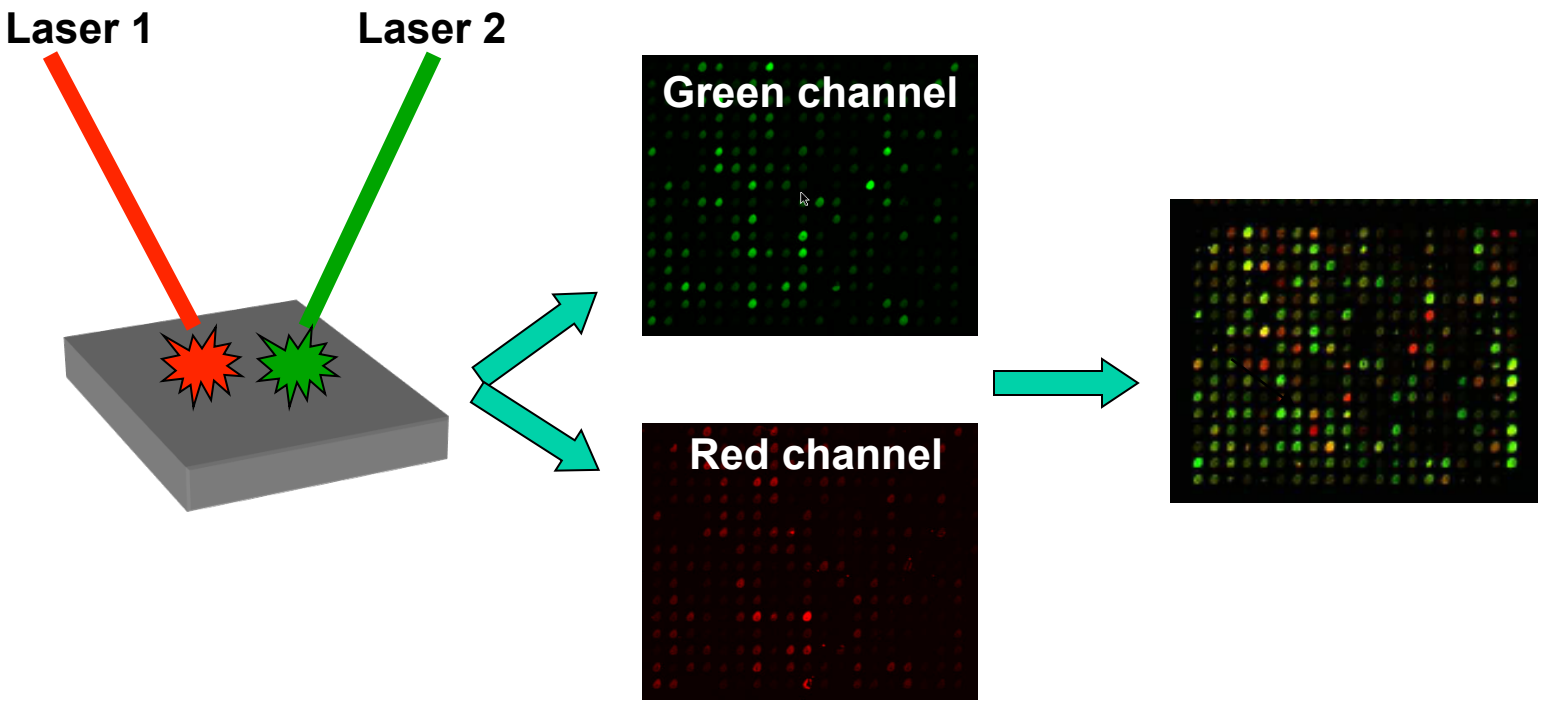
Key Steps in Microarray Analysis

- Quality Control (checking microarrays for errors or problems)
- Image Processing
  - Gridding
  - Segmentation (spot picking)
  - Data Extraction (intensity, QC)
- Data Analysis and Data Mining

Microarray Scanning*



Microarray Principles*



Scan and detect with confocal laser system

overlay images and normalize

Image process and analyze

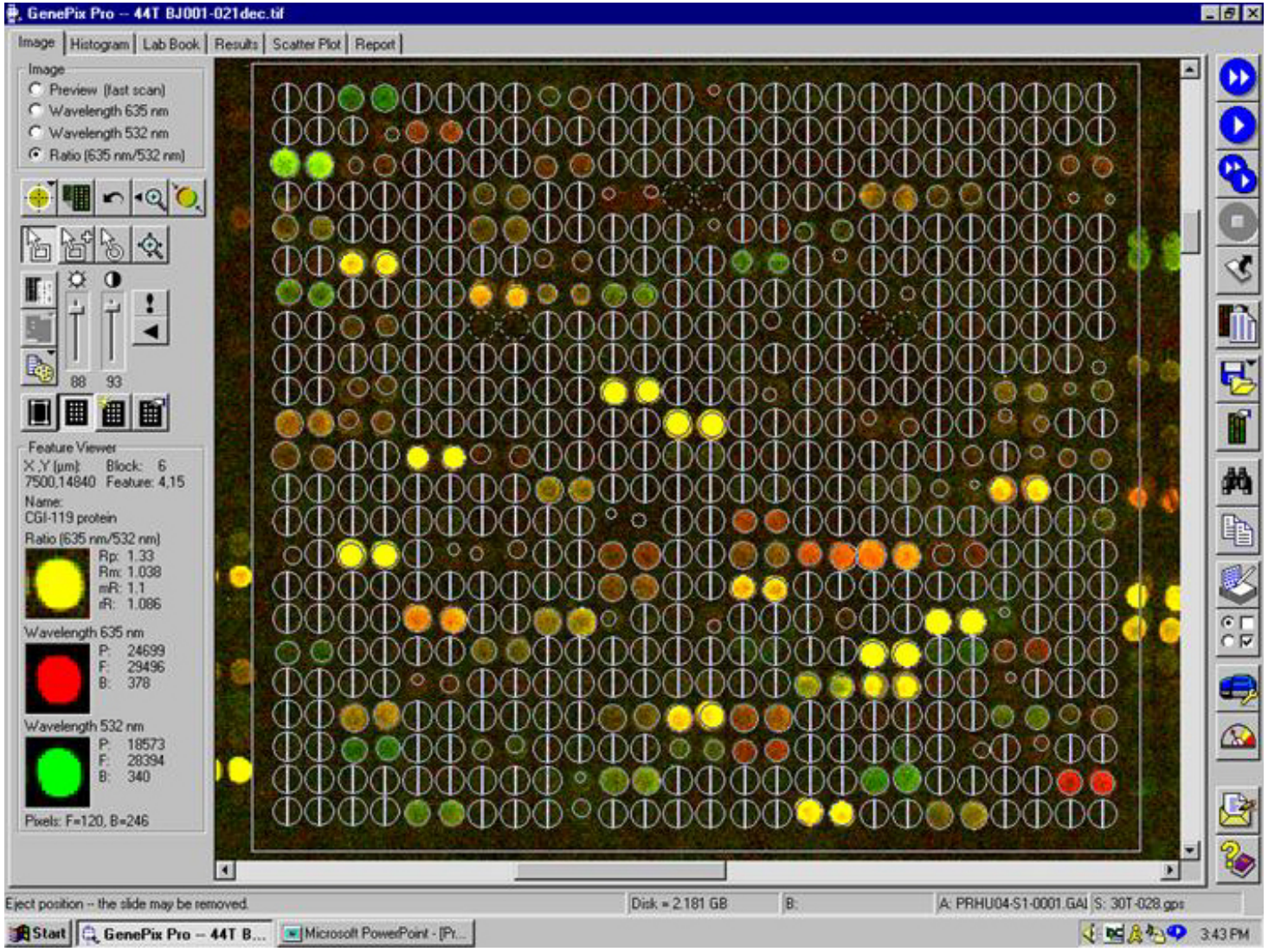
Microarray Images

- **Resolution**
  - **standard 10μm [currently, max 5μm]**
  - **100μm spot on chip = 10 pixels in diameter**
- **Image format**
  - **TIFF (tagged image file format) 16 bit (64K grey levels)**
  - **1cm x 1cm image at 16 bit = 2Mb (uncompressed)**
  - **other formats exist i.e. SCN (Stanford University)**
- **Separate image for each fluorescent sample**
  - **channel 1, channel 2, etc.**

Image Processing*

- **Addressing or gridding**
  - **Assigning coordinates to each of the spots**
- **Segmentation or spot picking**
  - **Classifying pixels either as foreground or as background**
- **Intensity extraction (for each spot)**
  - **Foreground fluorescence intensity pairs (R, G)**
  - **Background intensities**
  - **Quality measures**

Gridding

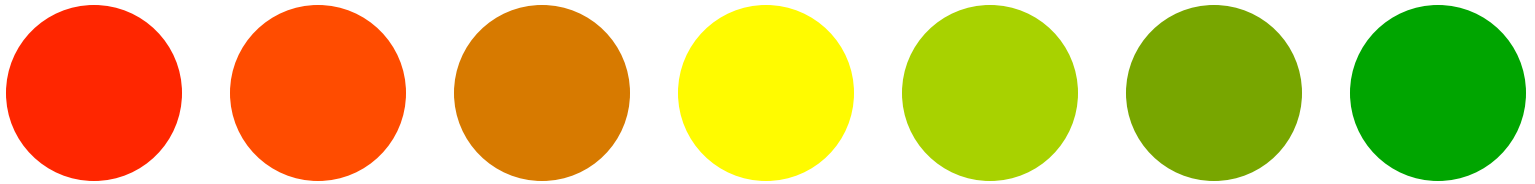

GenePix Pro -- 44T BJ001-021dec.tif
Image
Histogram
Lab Book
Results
Scatter Plot
Report
Image
Preview (fast scan)
Wavelength 635 nm
Wavelength 532 nm
Ratio (635 nm/532 nm)
88
93
Feature Viewer
X,Y (µm): 7500,14840
Block: 6
Feature: 4,15
Name:
CGI-119 protein
Ratio (635 nm/532 nm)
Rp: 1.33
Rm: 1.038
mR: 1.1
sR: 1.086
Wavelength 635 nm
P: 24699
F: 29496
B: 378
Wavelength 532 nm
P: 18573
F: 28394
B: 340
Pixels: F=120, B=246
Eject position -- the slide may be removed.
Disk = 2.181 GB
B:
A: PRHU04-S1-0001.GAI
S: 30T-028.gps
Start
GenePix Pro -- 44T B...
Microsoft PowerPoint - [Pr...
3:43 PM

Gridding Considerations*

- Separation between rows and columns of grids
- Individual translation of grids
- Separation between rows and columns of spots within each grid
- Small individual translation of spots
- Overall position of the array in the image
- *Automated & manual methods available*

Spot Picking

- **Classification of pixels as foreground or background (fluorescence intensities determined for each spot are a measure of transcript abundance)**
- **Large selection of methods available, each has strengths & weaknesses**

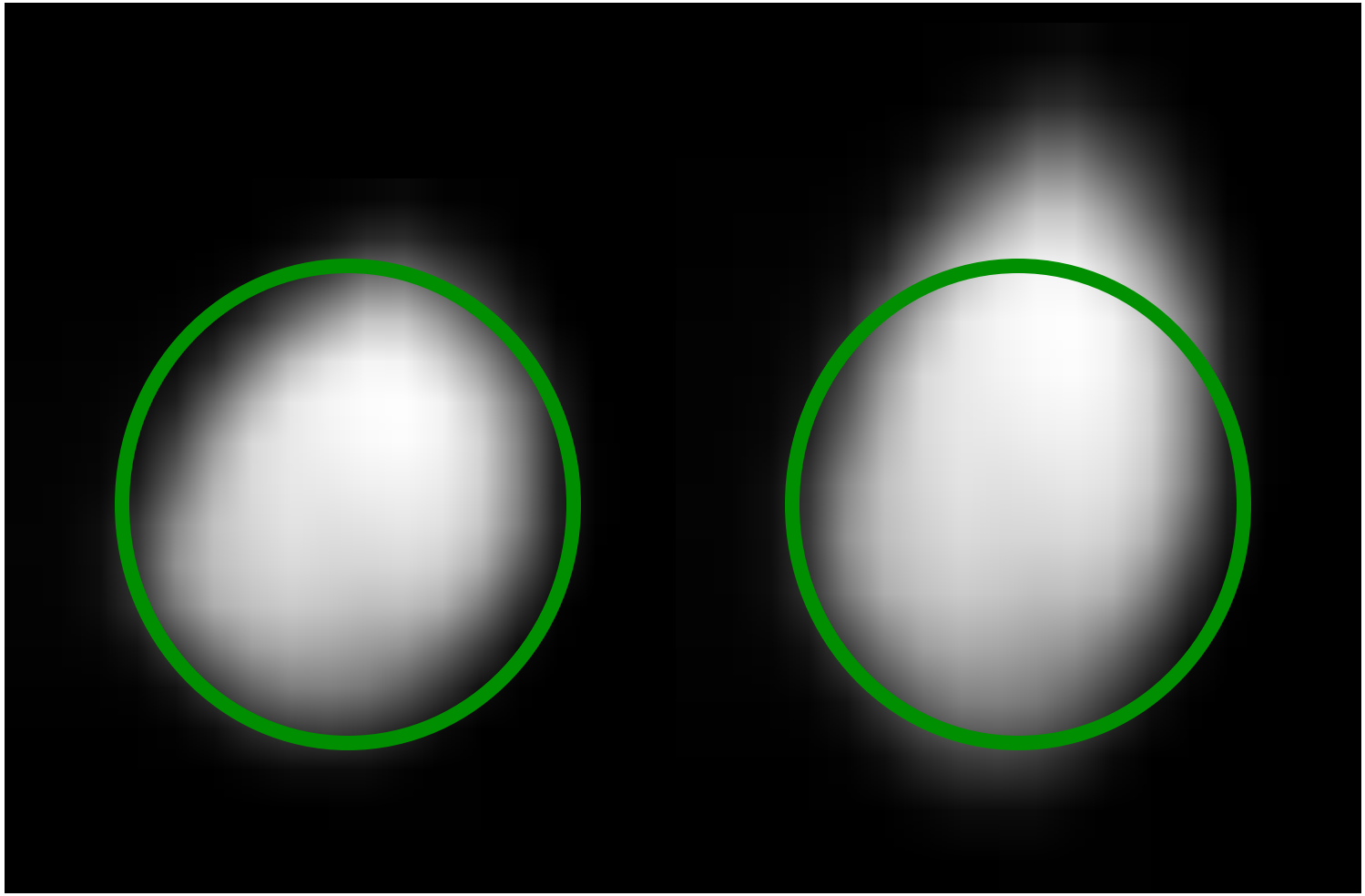


Spot Picking*

- **Segmentation/spot picking methods:**
  - **Fixed circle segmentation**
  - **Adaptive circle segmentation**
  - **Adaptive shape segmentation**
  - **Histogram segmentation**

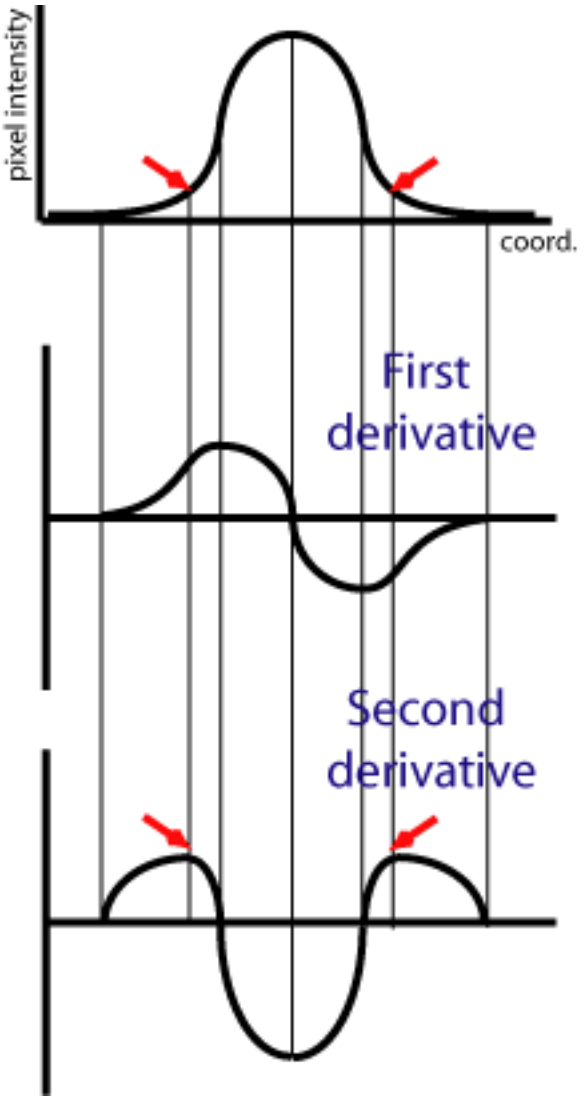
Fixed circle	**ScanAlyze, GenePix, QuantArray**
Adaptive circle	**GenePix, Dapple**
Adaptive shape	**Spot, region growing and watershed**
Histogram method	**ImaGene, QuantArraym DeArray and adaptive thresholding**

Fixed Circle Segmentation*

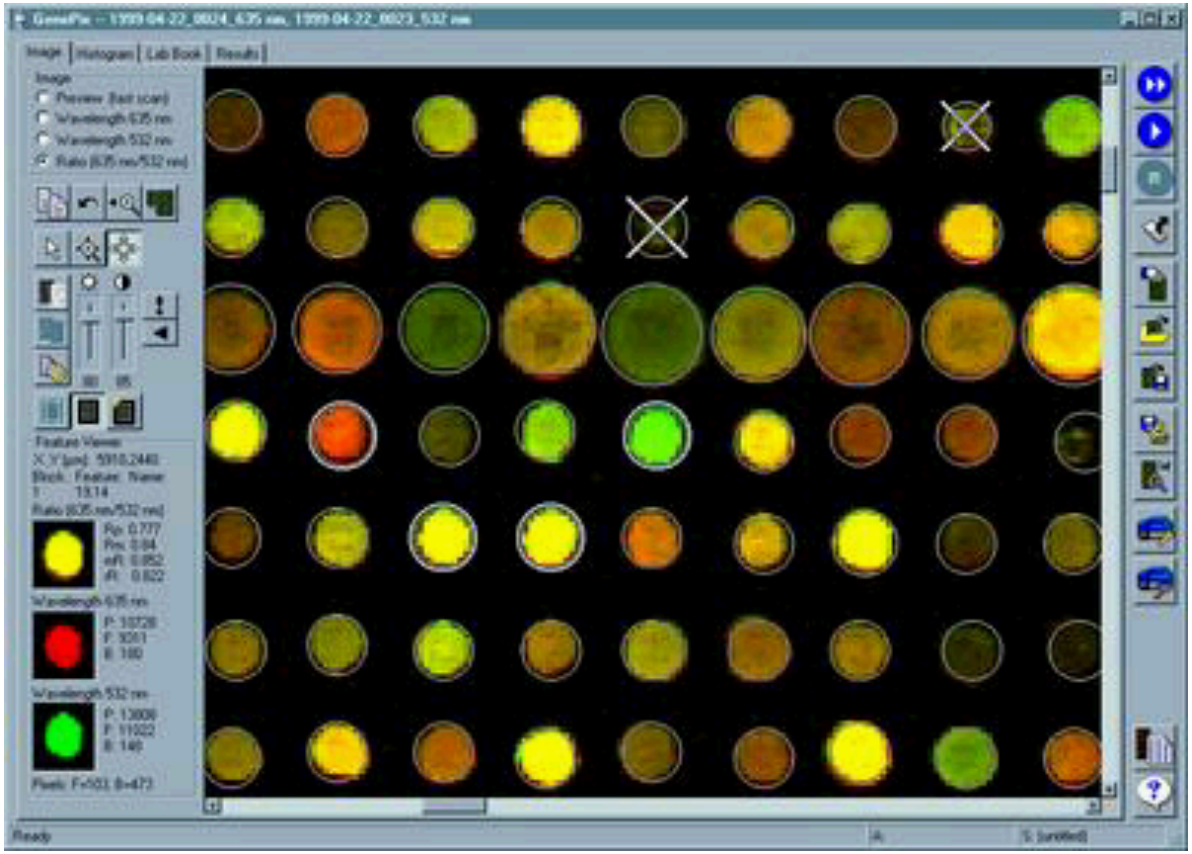


Adaptive Circle Segmentation*

- **The circle diameter is estimated separately for each spot**
- ***GenePix* finds spots by detecting edges of spots (second derivative)**
- **Problematic if spot exhibits oval shapes**

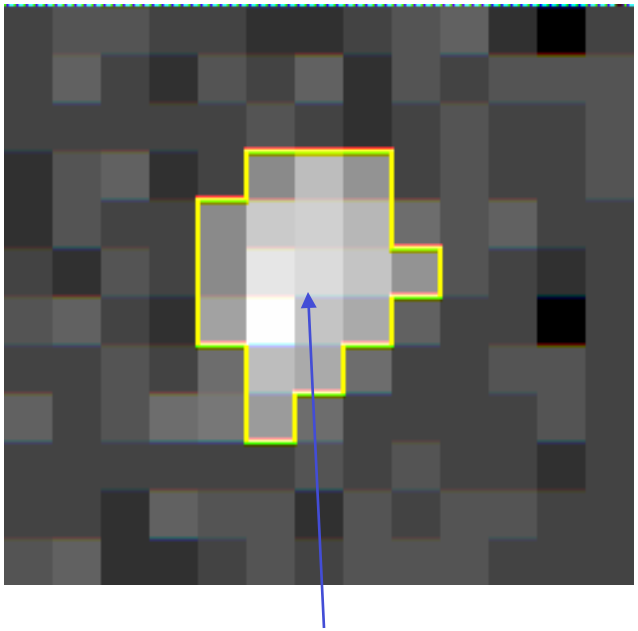


Adaptive Circle Segmentation



Information Extraction

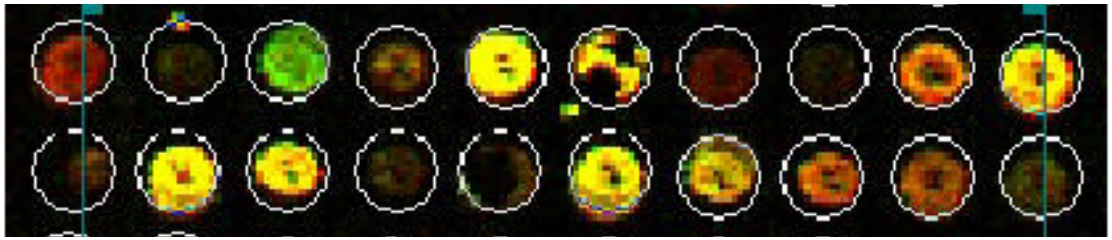
- **Spot Intensities**
  - mean (pixel intensities)
  - median (pixel intensities)
- **Background values**
  - Local Background
  - Morphological opening
  - Constant (global)
- **Quality Information**



Spot Intensity*

- **The total amount of hybridization for a spot is proportional to the *total fluorescence* at the spot**
- **Spot intensity = sum of pixel intensities within the spot mask**
- **Since later calculations are based on ratios between cy5 and cy3, we compute the average* pixel value over the spot mask**
- **Can use ratios of medians instead of means**

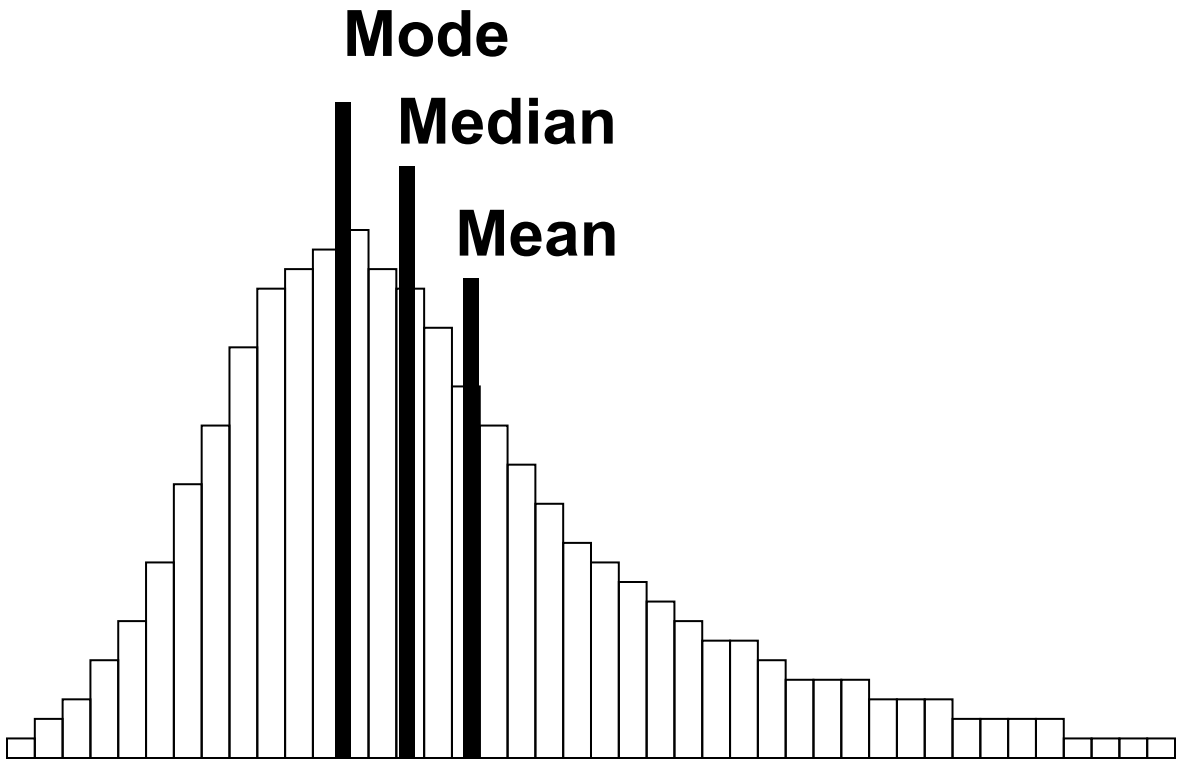
Means vs. Medians*



row	col	ch1_sig_mea	ch2_sig_mea	ch1_sig_med	ch2_sig_med
1	1	56000	2000	58000	1900
1	2	1000	600	600	800
1	3	2000	60000	3000	59000

etc.

Mean, Median & Mode



Mean, Median, Mode*

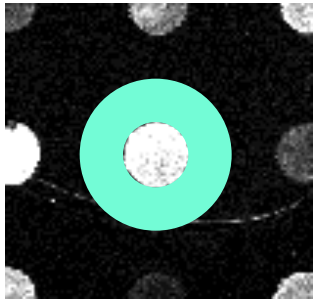
- In a Normal Distribution the mean, mode and median are all equal
- In skewed distributions they are unequal
- **Mean** - average value, affected by extreme values in the distribution
- **Median** - the "middlemost" value, usually half way between the mode and the mean
- **Mode** - most common value

Background Intensity

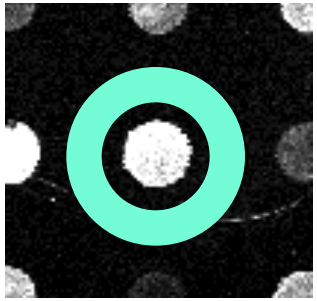
- A spot's measured intensity includes a contribution of non-specific hybridization and other chemicals on the glass
- Fluorescence intensity from regions not occupied by DNA can be different from regions occupied by DNA

Local Background Methods*

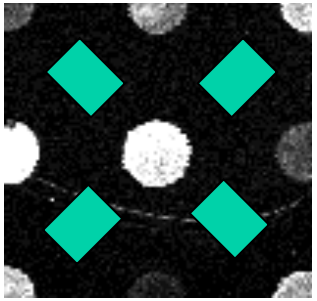
- Focuses on small regions around spot mask
- Determine median pixel values in this region
- Most common approach



ScanAlyze



ImaGene



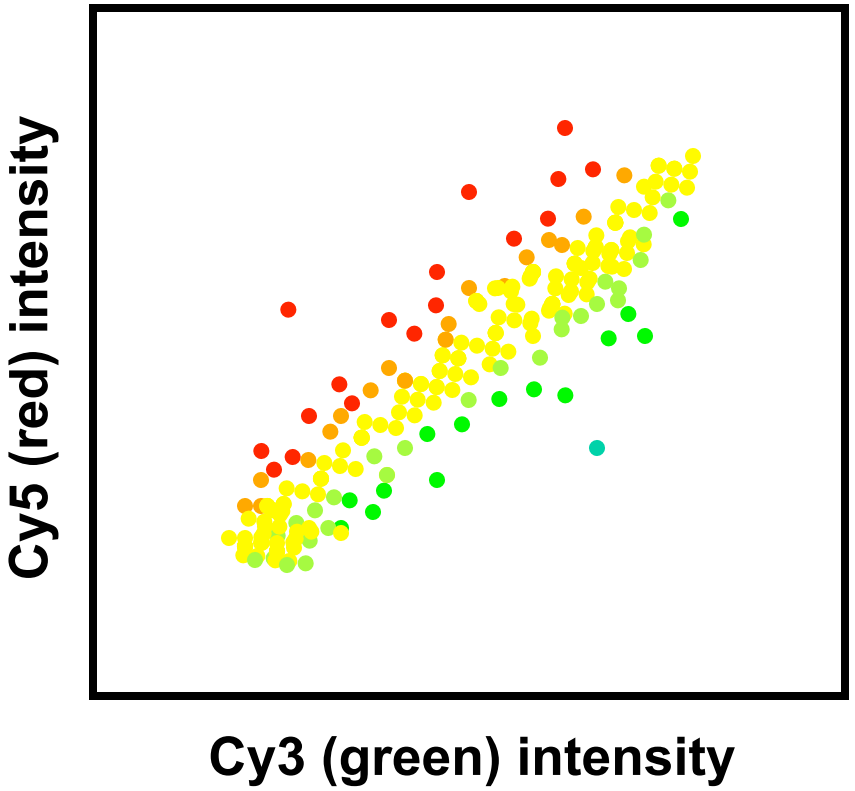
Spot, GenePix

- ***By not considering the pixels immediately surrounding the spots, the background estimate is less sensitive to the performance of the segmentation procedure***

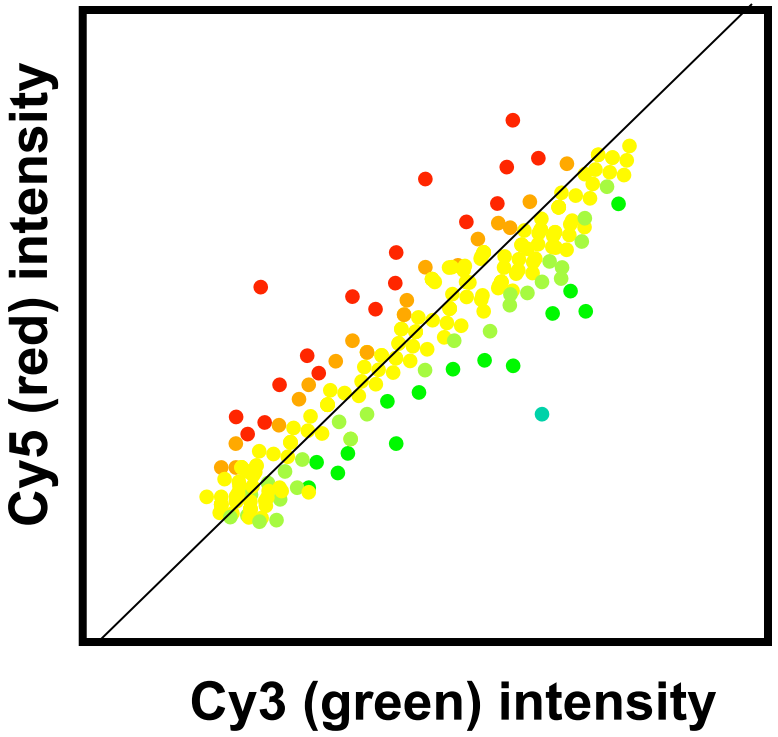
Quality Measurements*

- **Array**
  - ***Correlation between spot intensities***
  - **Percentage of spots with no signals**
  - **Distribution of spot signal area**
  - **Inter-array consistency**
- **Spot**
  - **Signal / Noise ratio**
  - **Variation in pixel intensities**
  - **ID of "bad spots" (spots with no signal)**

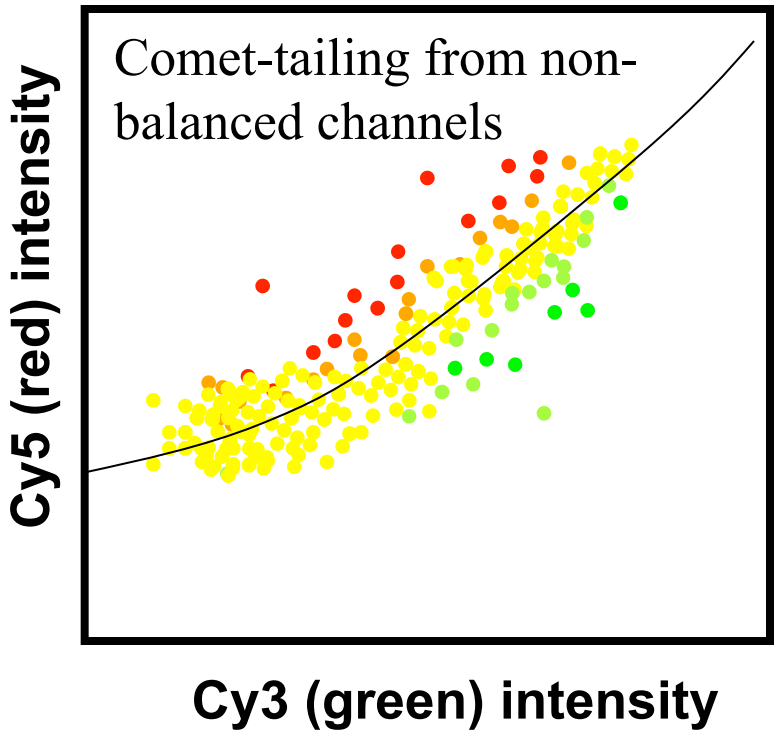
A Microarray Scatter Plot



Correlation*

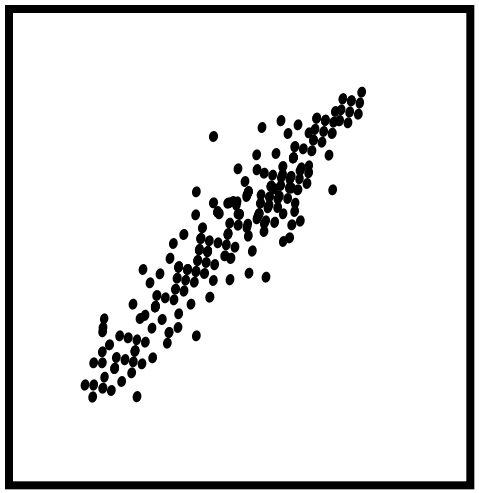


Linear

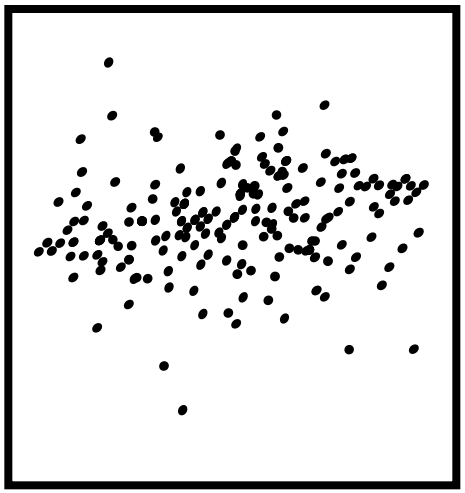


Non-linear

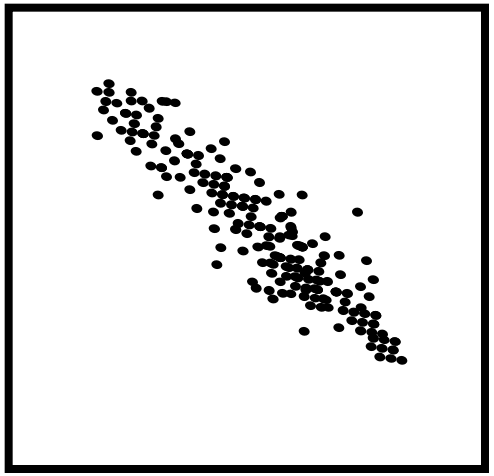
Correlation



“+” correlation

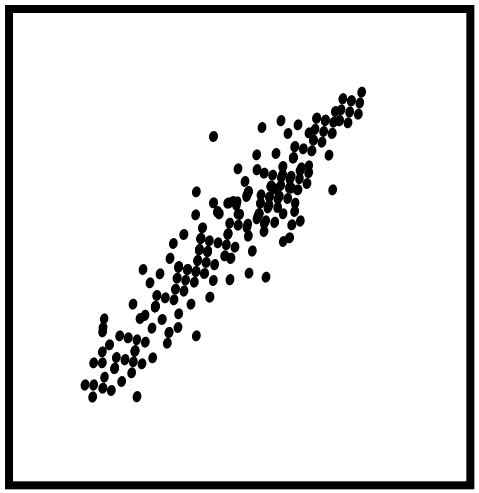


Uncorrelated



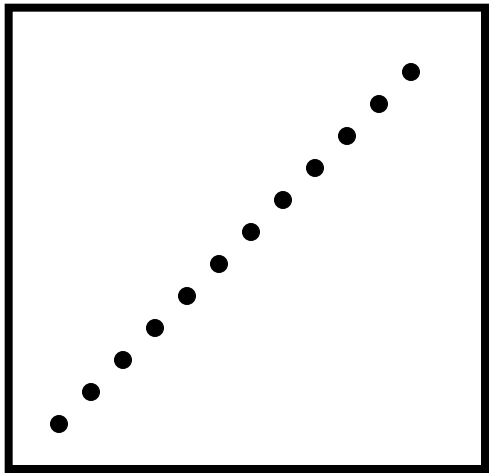
“-” correlation

Correlation



High correlation

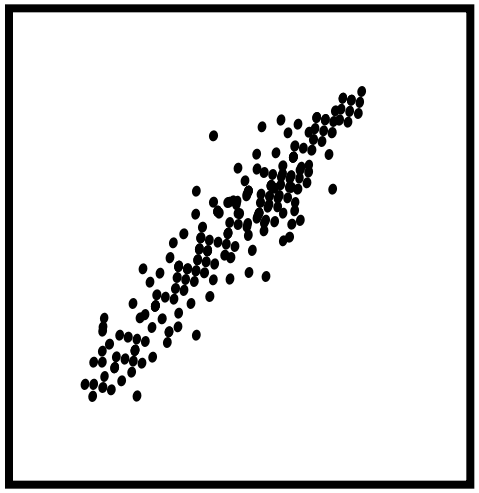
Low correlation



Perfect correlation

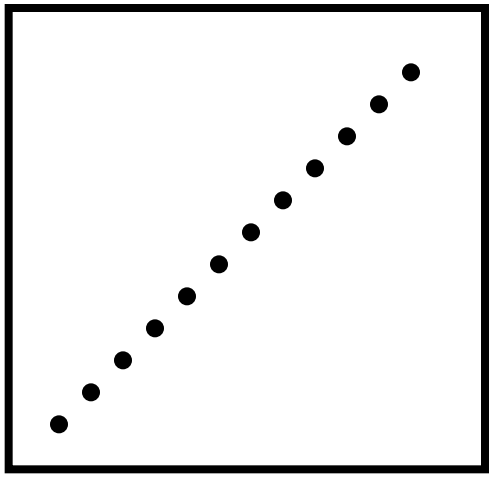
Correlation Coefficient*

$$r = \frac{\Sigma(x_i - \mu_x)(y_i - \mu_y)}{\sqrt{\Sigma(x_i - \mu_x)^2(y_i - \mu_y)^2}}$$



r = 0.85

r = 0.4

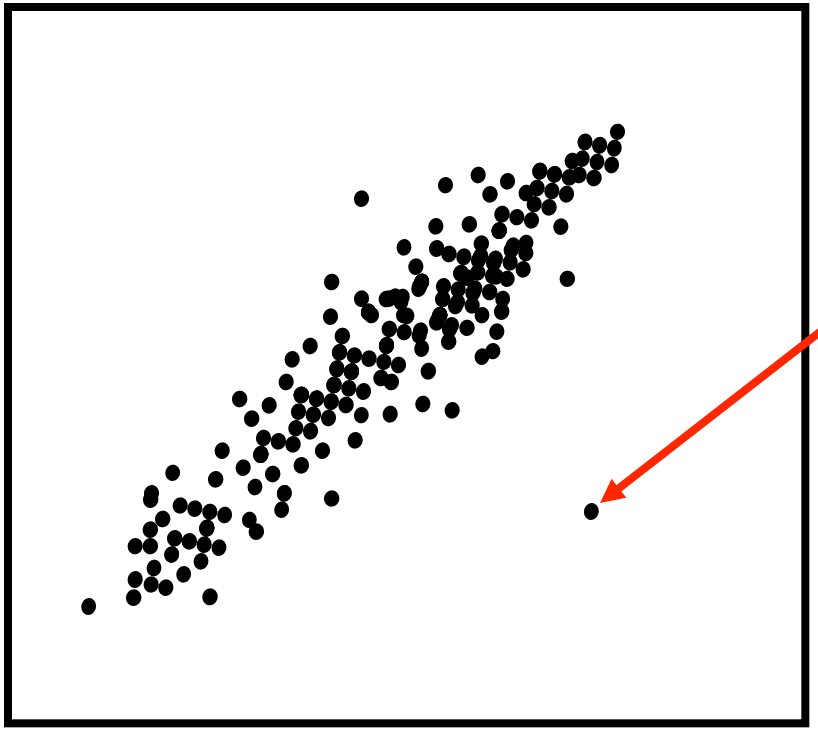


r = 1.0

Correlation Coefficient

- Sometimes called coefficient of linear correlation or Pearson product-moment correlation coefficient
- A quantitative way of determining what model (or equation or type of line) best fits a set of data
- Commonly used to assess most kinds of predictions or simulations

Correlation and Outliers

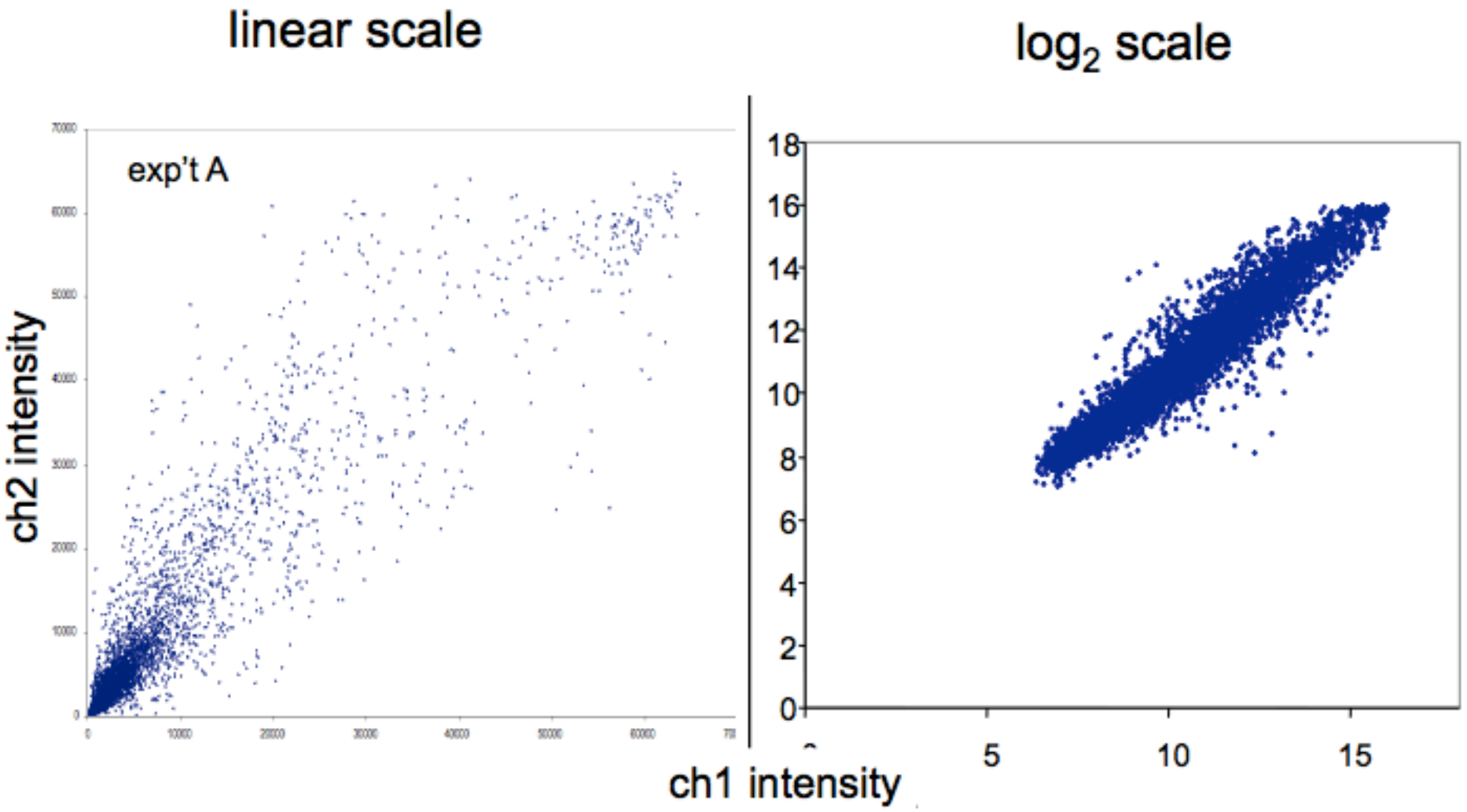


A single “bad” point can destroy a good correlation

Outliers*

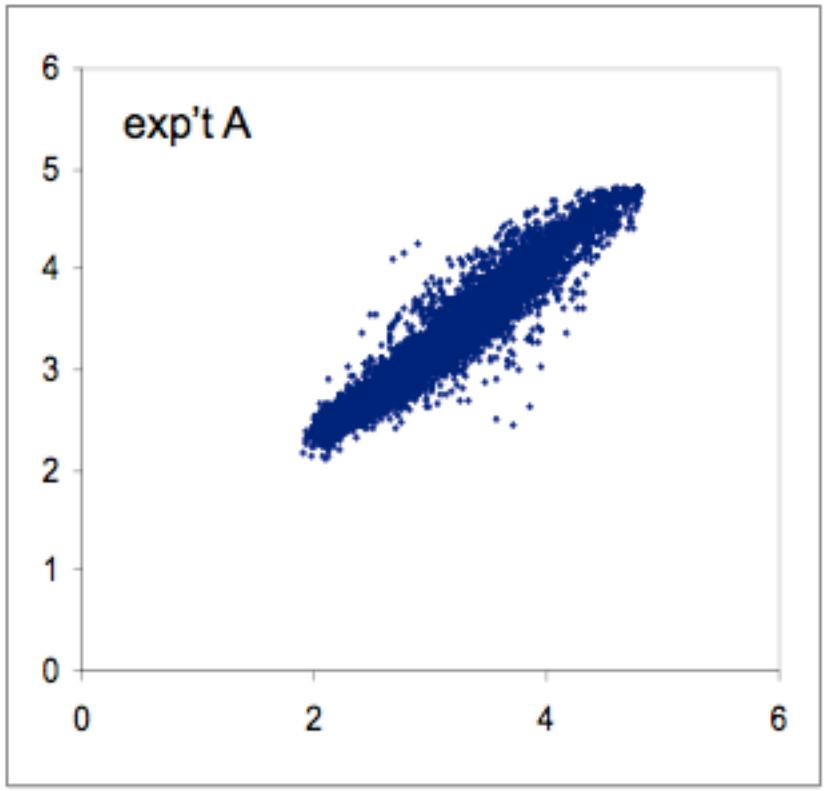
- Can be both “good” and “bad”
- When modeling data -- you don’t like to see outliers (suggests the model is bad)
- Often a good indicator of experimental or measurement errors -- only you can know!
- When plotting gel or microarray expression data you do like to see outliers
- A good indicator of something significant

Log Transformation*

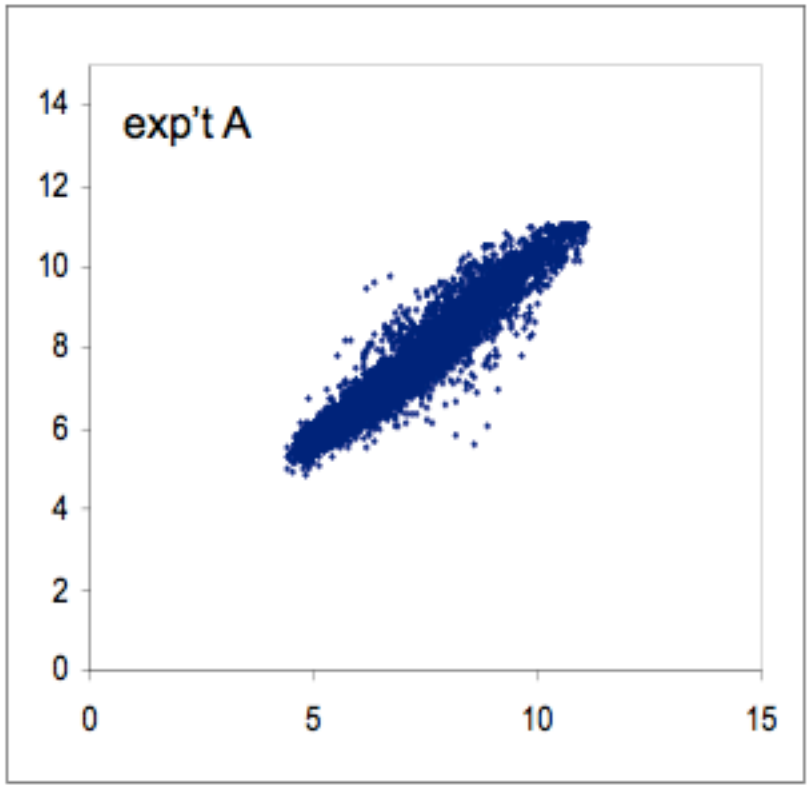


Choice of Base is Not Important

$\log_{10}$



ln



Why Log_2 Transformation?*

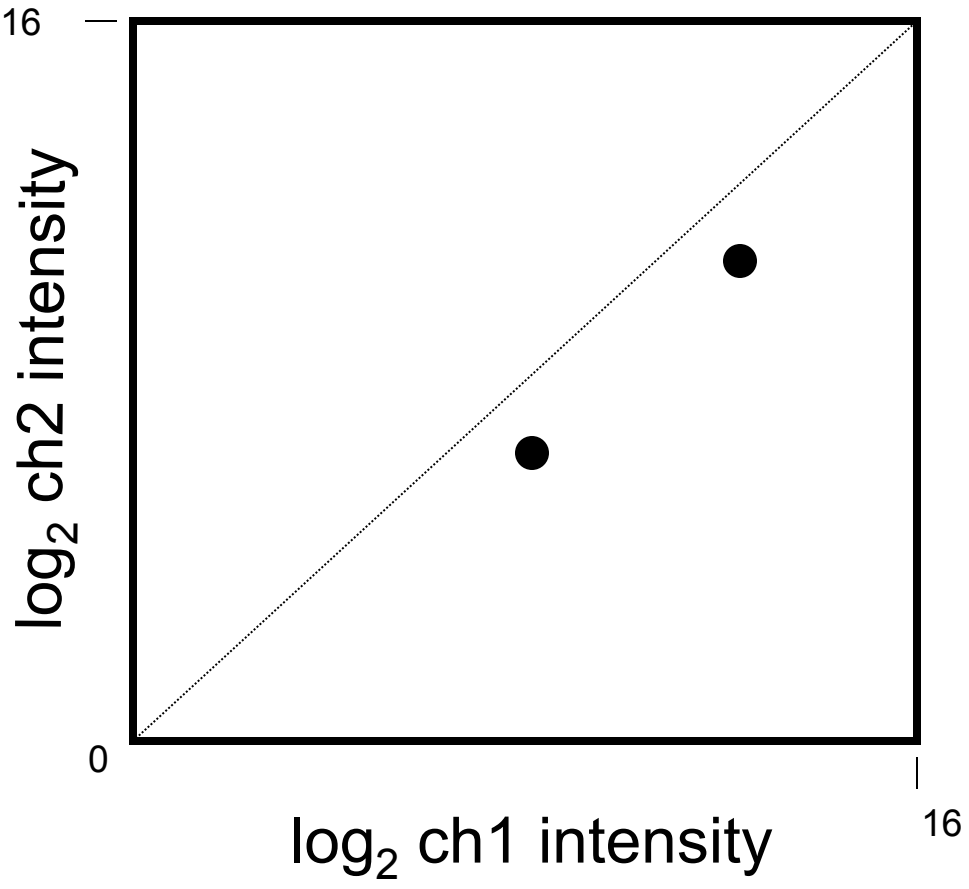
- Makes variation of intensities and ratios of intensities more independent of absolute magnitude
- Makes normalization additive
- Evens out highly skewed distributions
- Gives more realistic sense of variation
- Approximates normal distribution
- Treats up- and down- regulated genes symmetrically

Log Transformations

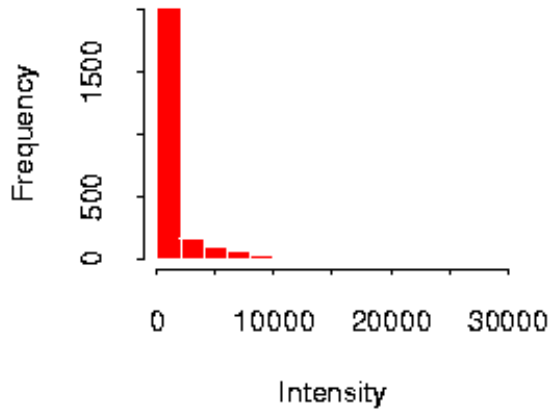
Applying a log transformation makes the variance and offset more proportionate along the entire graph

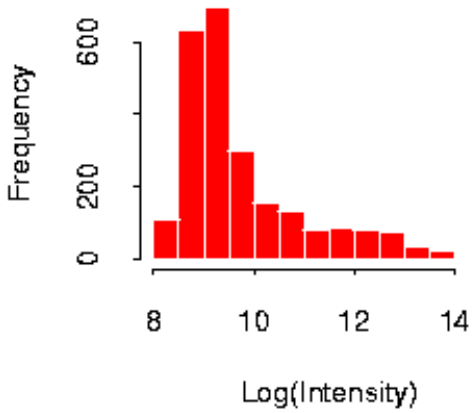
ch1	ch2	ch1/ch2
60 000	40 000	1.5
3000	2000	1.5

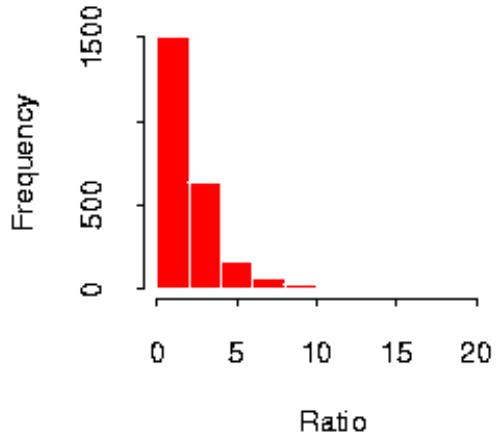
$\log_2$ ch1	$\log_2$ ch2	$\log_2$ ratio
15.87	15.29	0.58
11.55	10.97	0.58

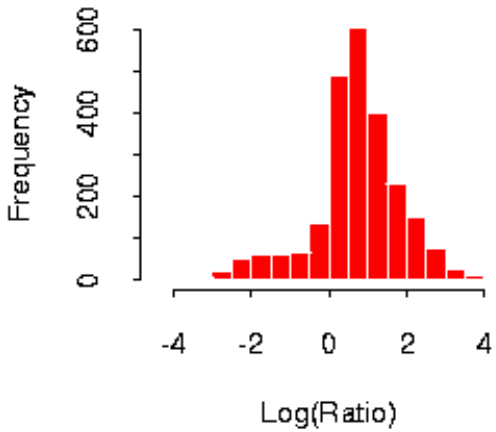


Log Transformations*

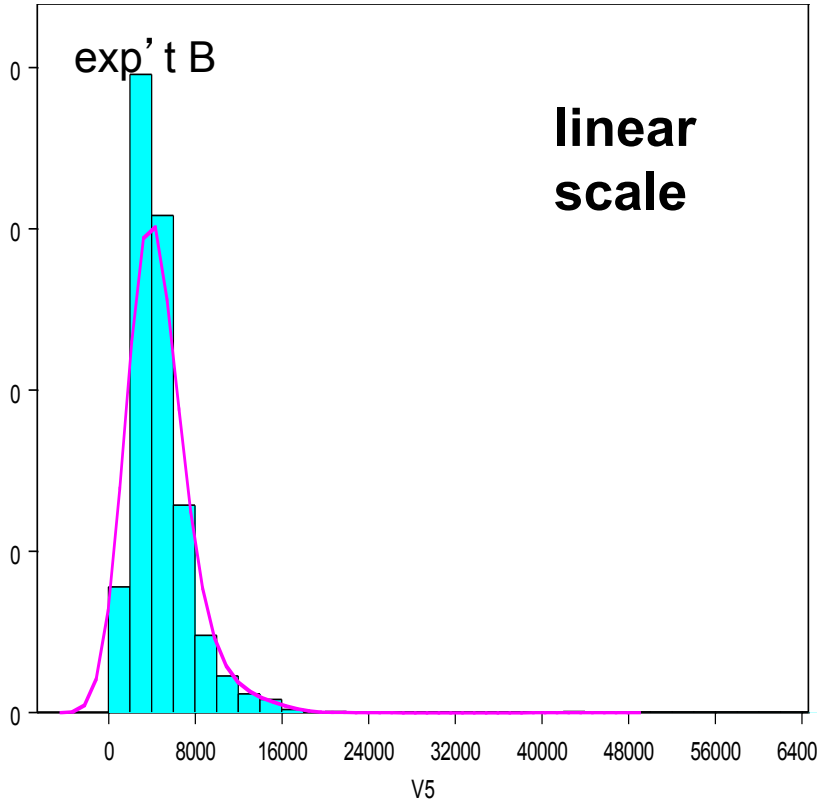


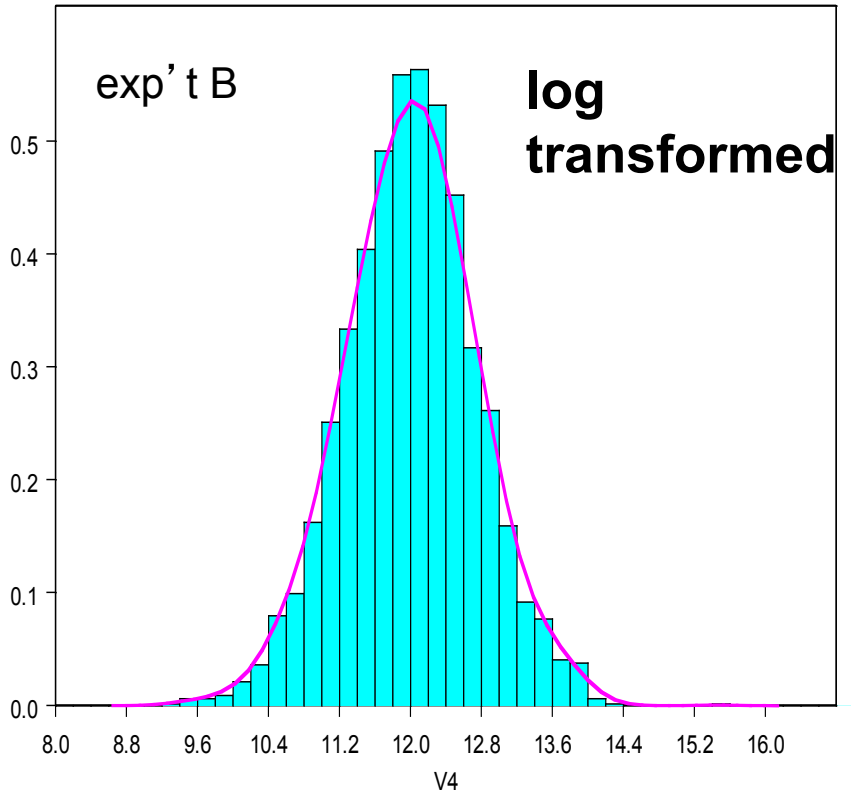






Log Transformation



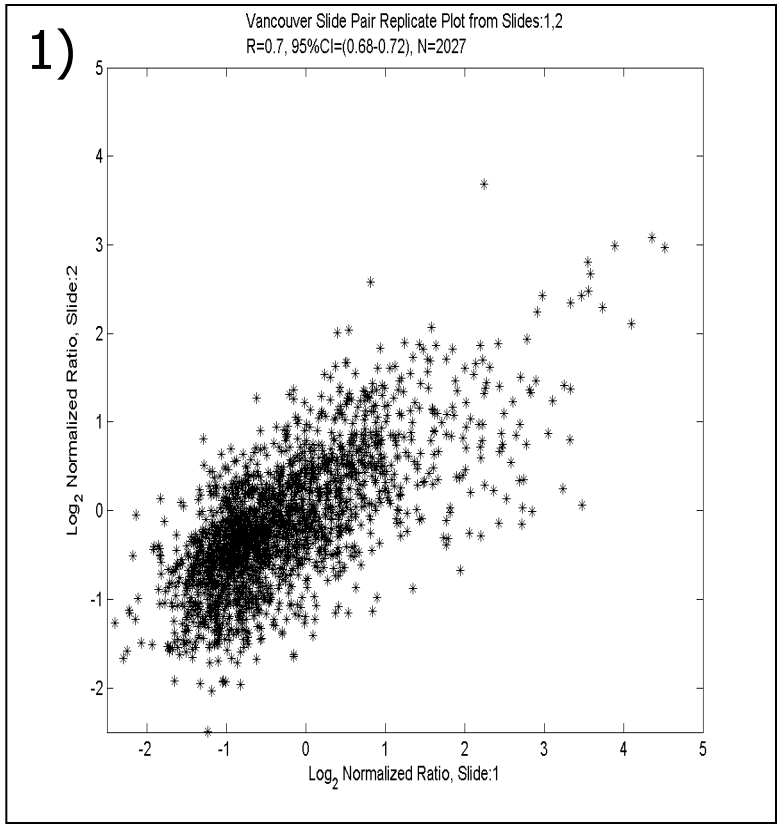


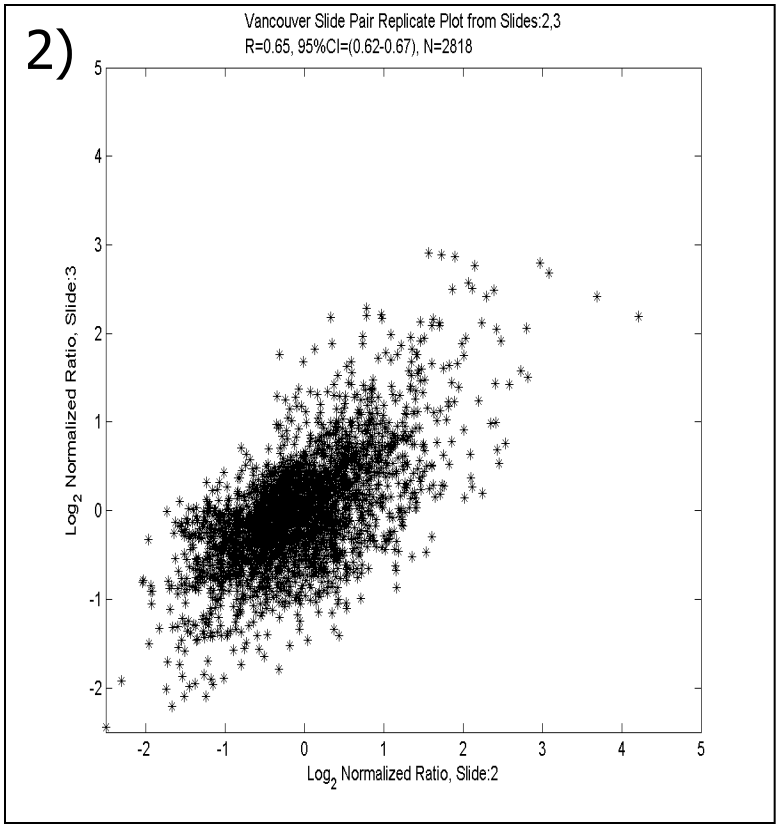
Normalization*

- **Reduces systematic (multiplicative) differences between two channels of a single hybridization or differences between hybridizations**
- **Several Methods:**
  - **Global mean method**
  - **(Iterative) linear regression method**
  - **Curvilinear methods (e.g. loess)**
  - **Variance model methods**

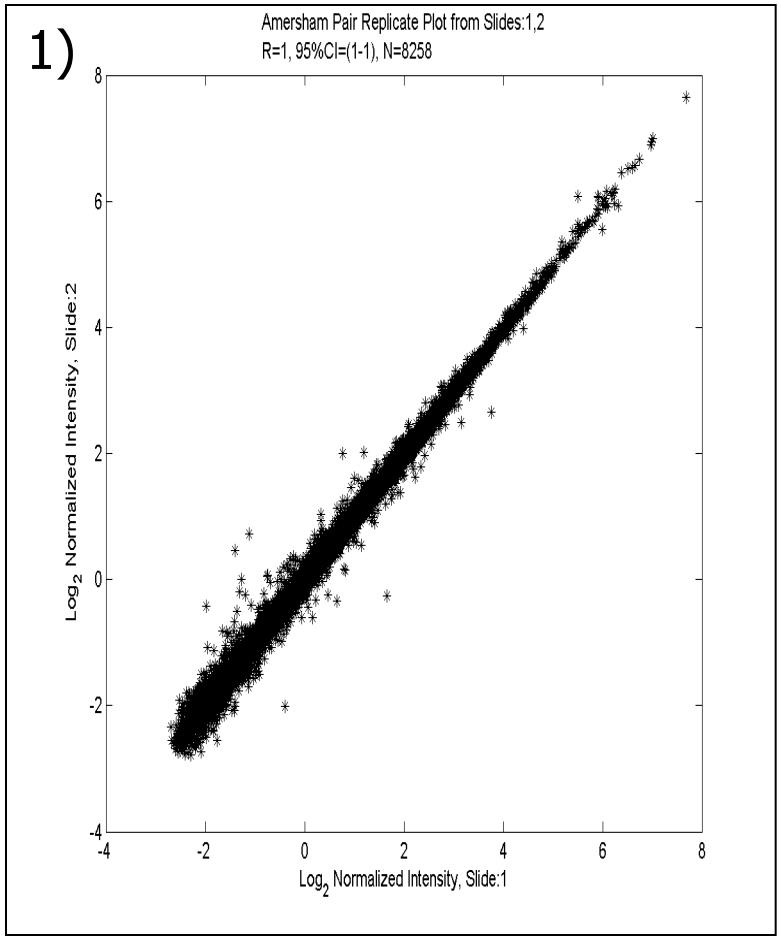
Try to get a slope ~1 and a correlation of ~1

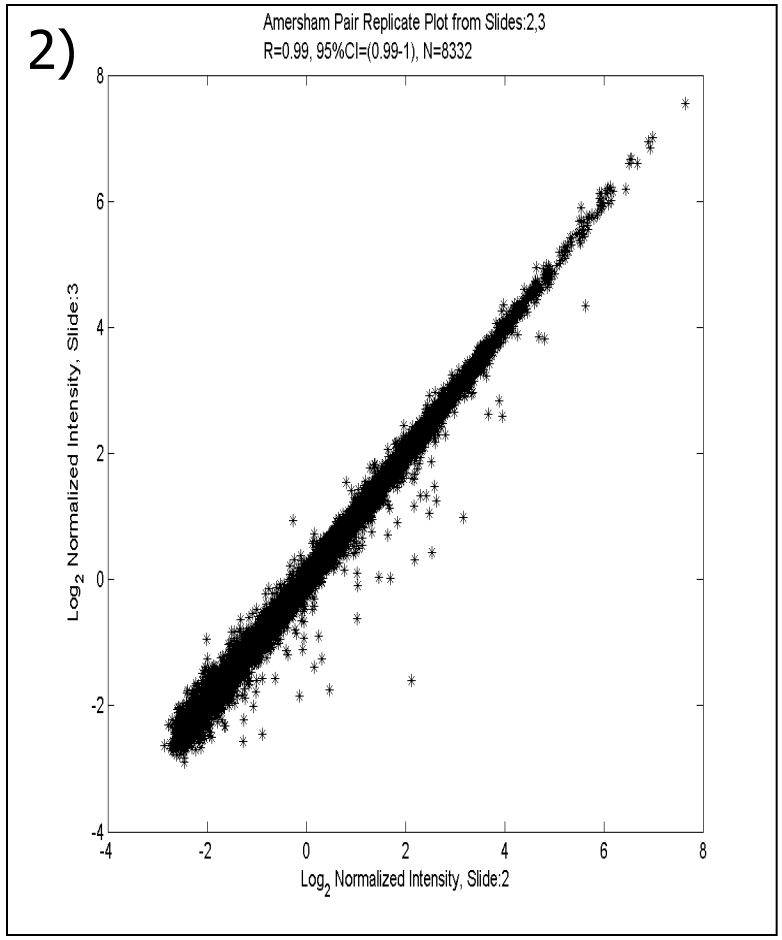
Example Where Normalization is Needed





Example Where Normalization is Not Needed

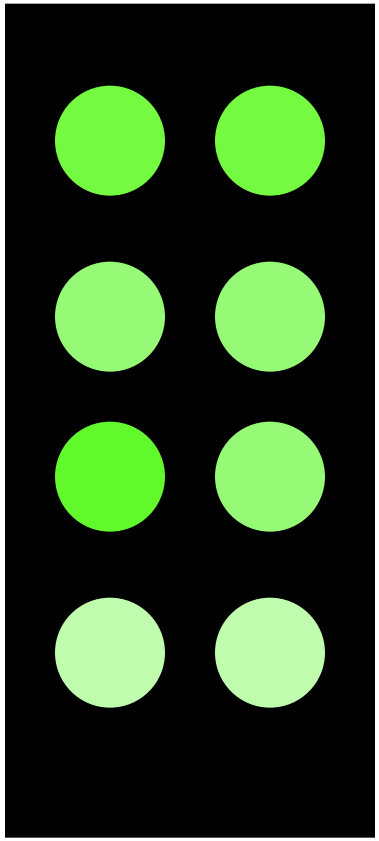




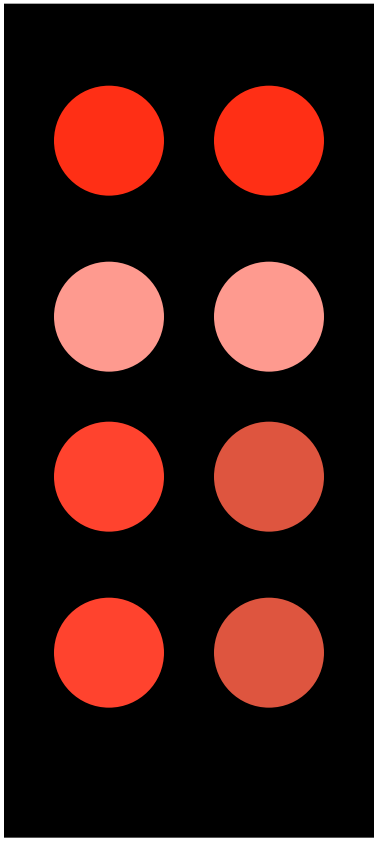
Normalization to a Global Mean*

- **Calculate mean intensity of all spots in ch1 and ch2**
  - e.g. μch2 = 25 000 μch2/μch1 = 1.25
  - μch1 = 20 000
- **On average, spots in ch2 are 1.25X brighter than spots in ch1**
- **To normalize, multiply spots in ch1 by 1.25**

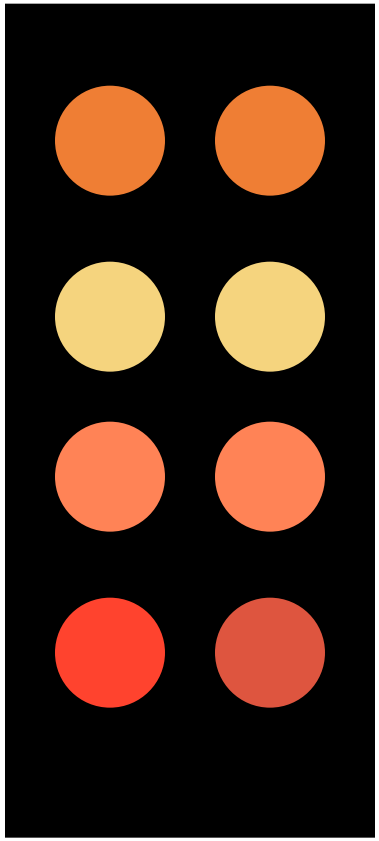
Visual Example: Ch2 is too Strong



Ch 1

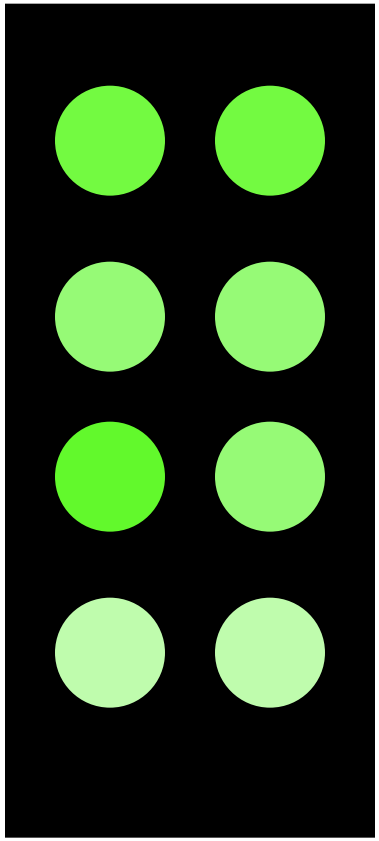


Ch 2

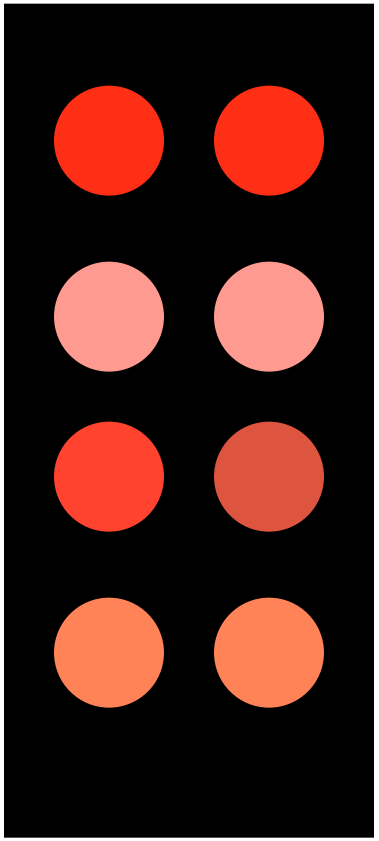


Ch1 + Ch2

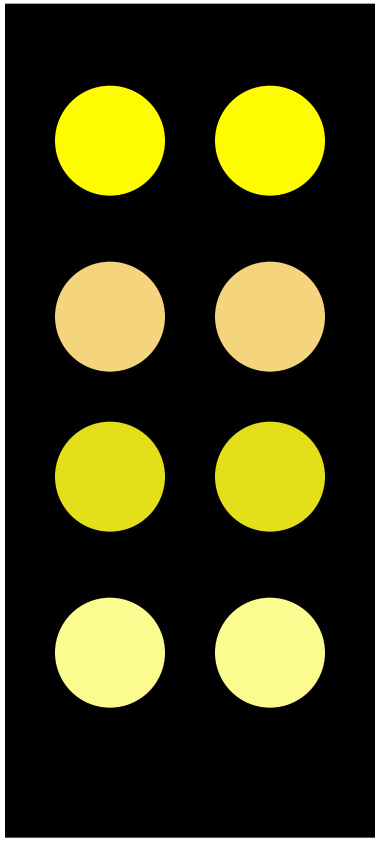
Visual Example: Ch2 and Ch1 are Balanced



Ch 1

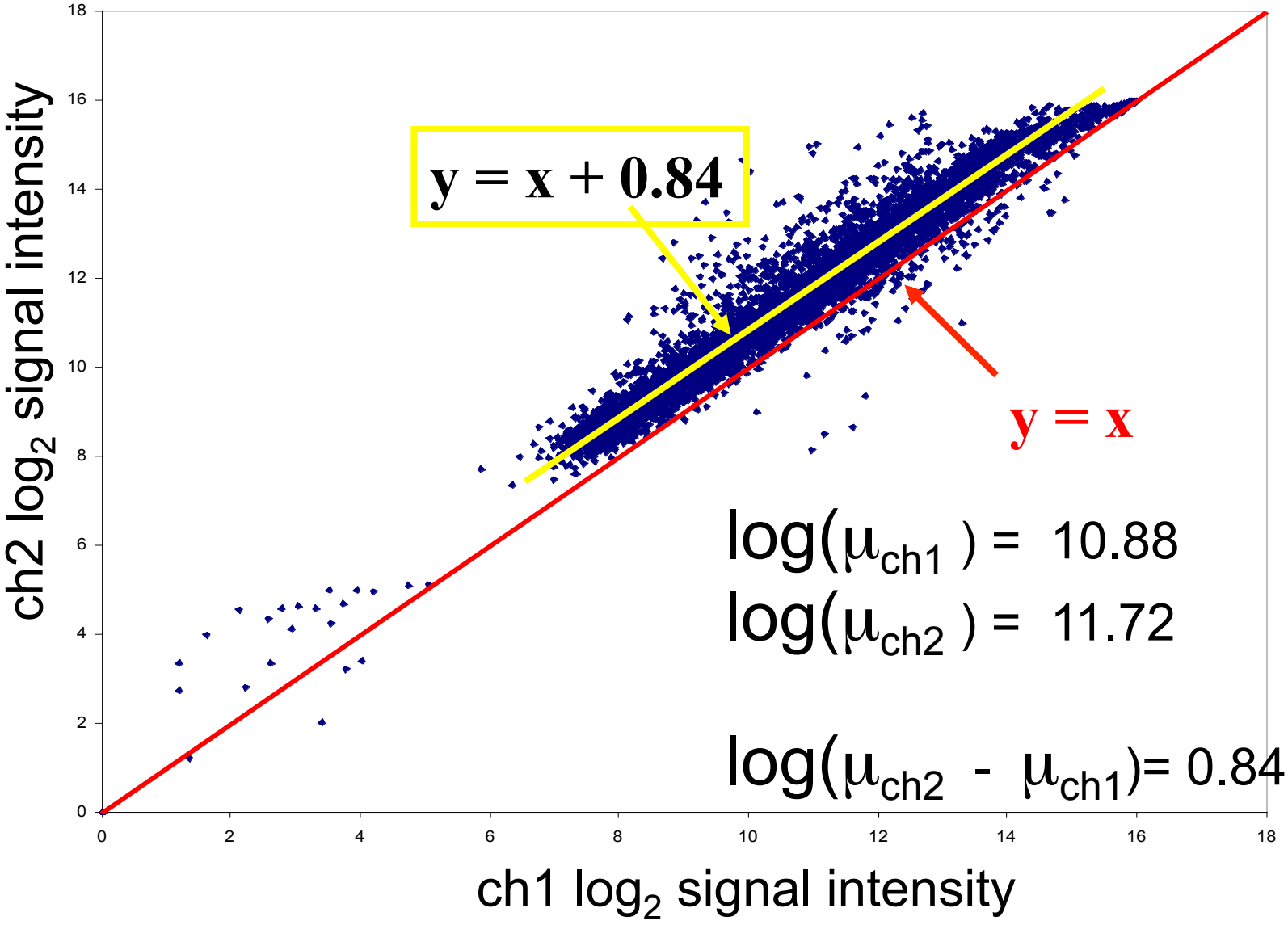


Ch 2

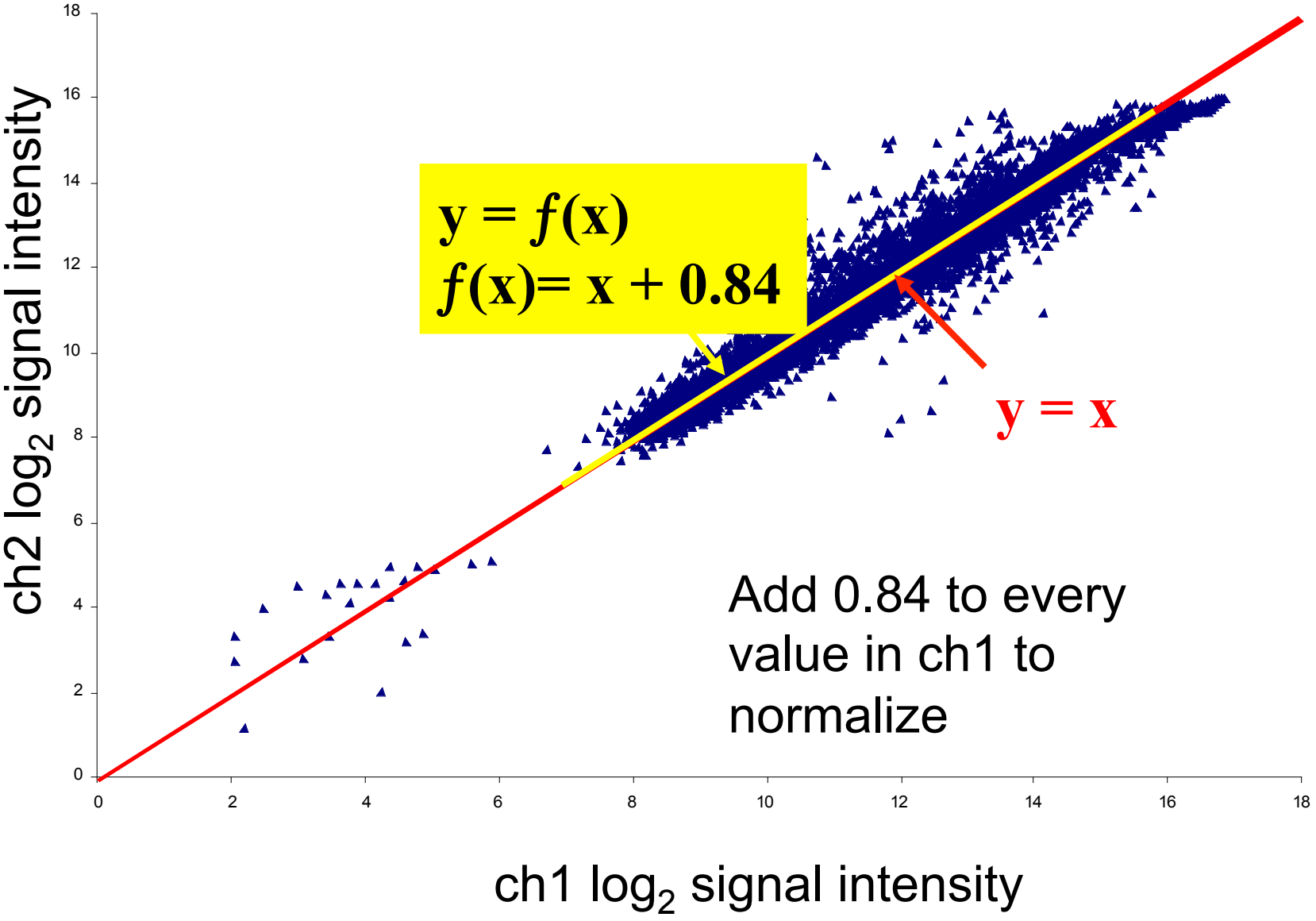


Ch1 + Ch2

Pre-normalized Data



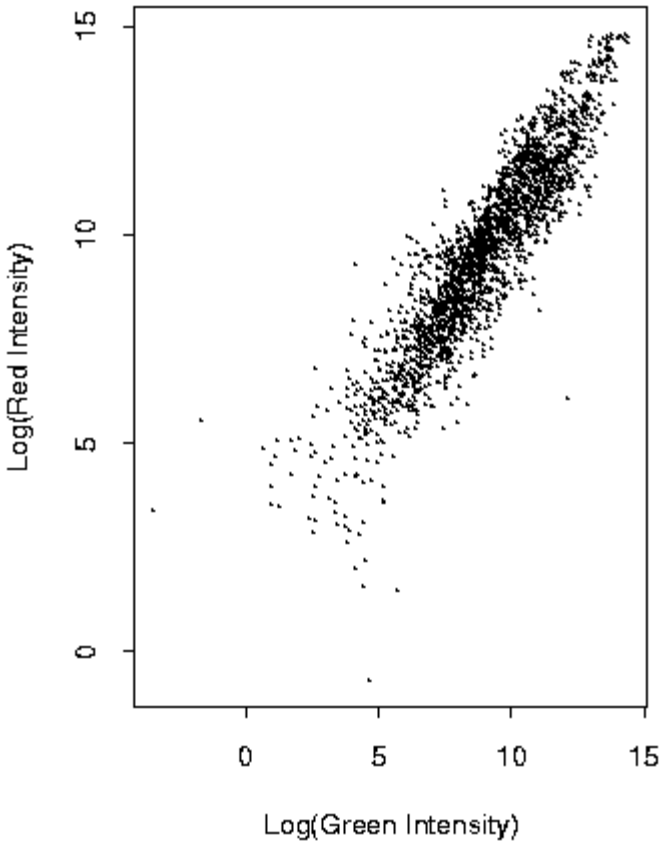
Normalized Microarray Data



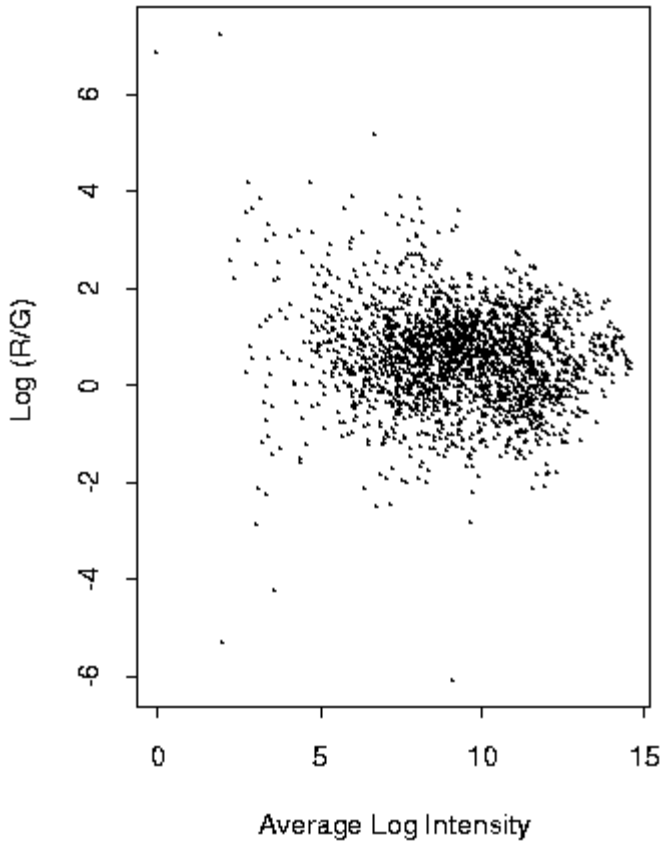
Normalization to Loess Curve*

- A curvilinear form of normalization
- For each spot, plot ratio vs. mean (ch1,ch2) signal in log scale (A vs. M)
- Use statistical programs (e.g. S-plus, SAS, or R) to fit a loess curve (local regression) through the data
- Offset from this curve is the normalized expression ratio

The A versus M Plot*



More Informative Graph

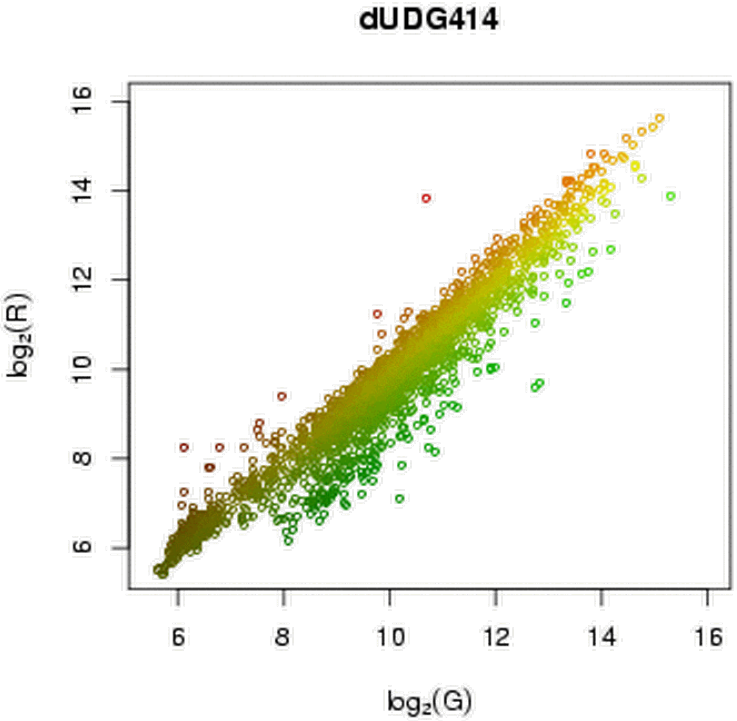


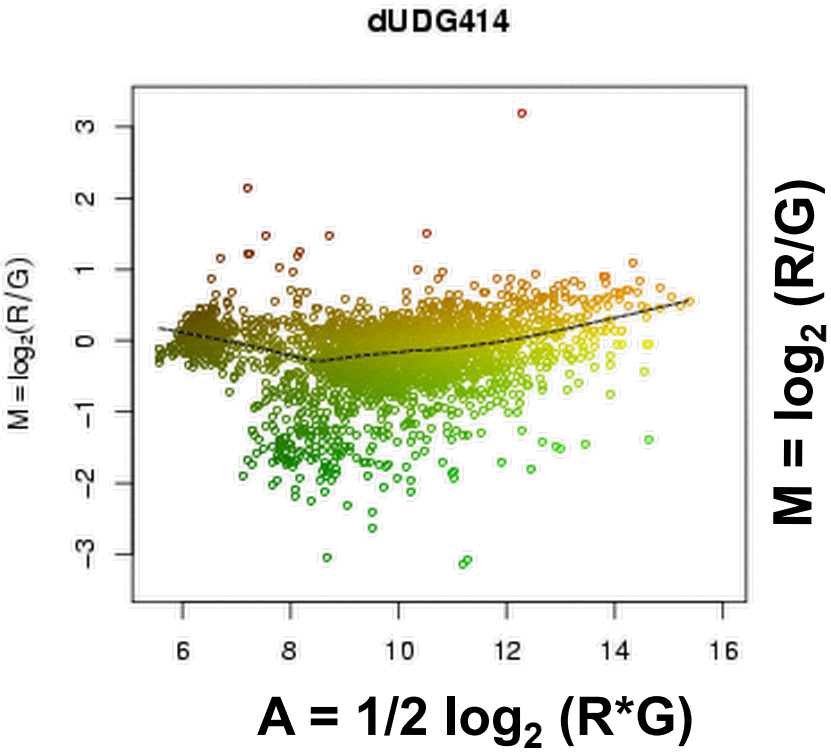
M = $\log_2$ (R/G)

A = 1/2 $\log_2$ (R*G)

A vs. M Plot

More Informative Graph



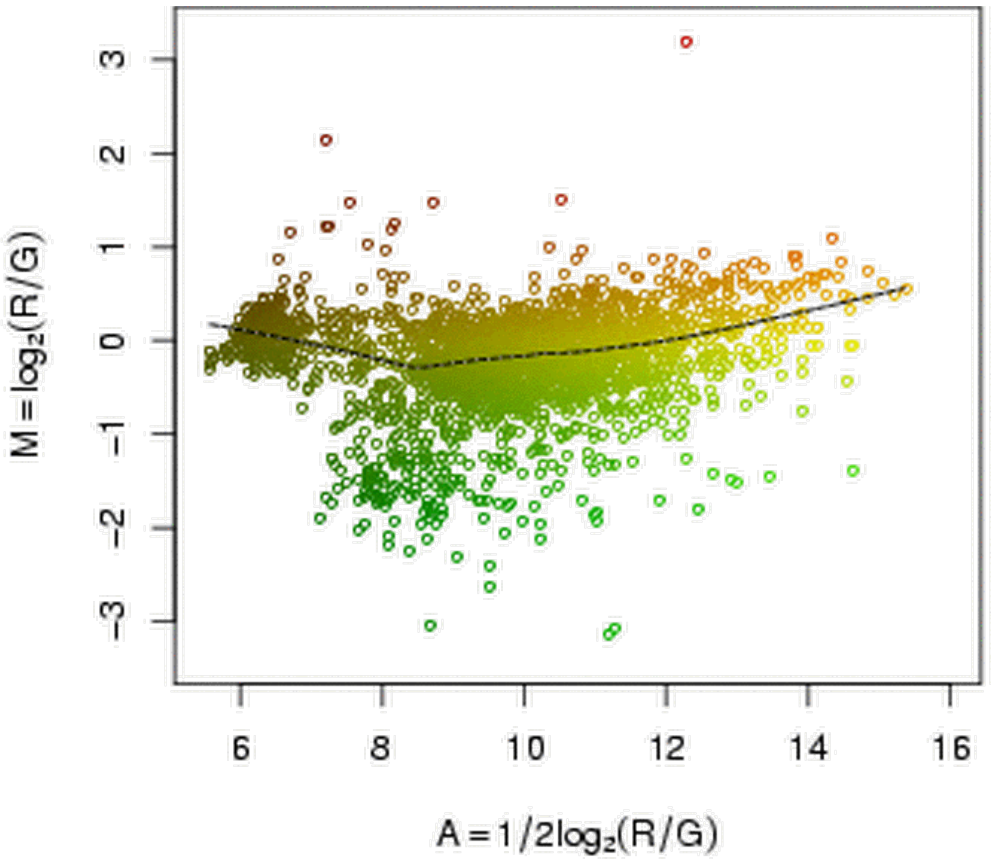


A = 1/2 $\log_2$ (R*G)

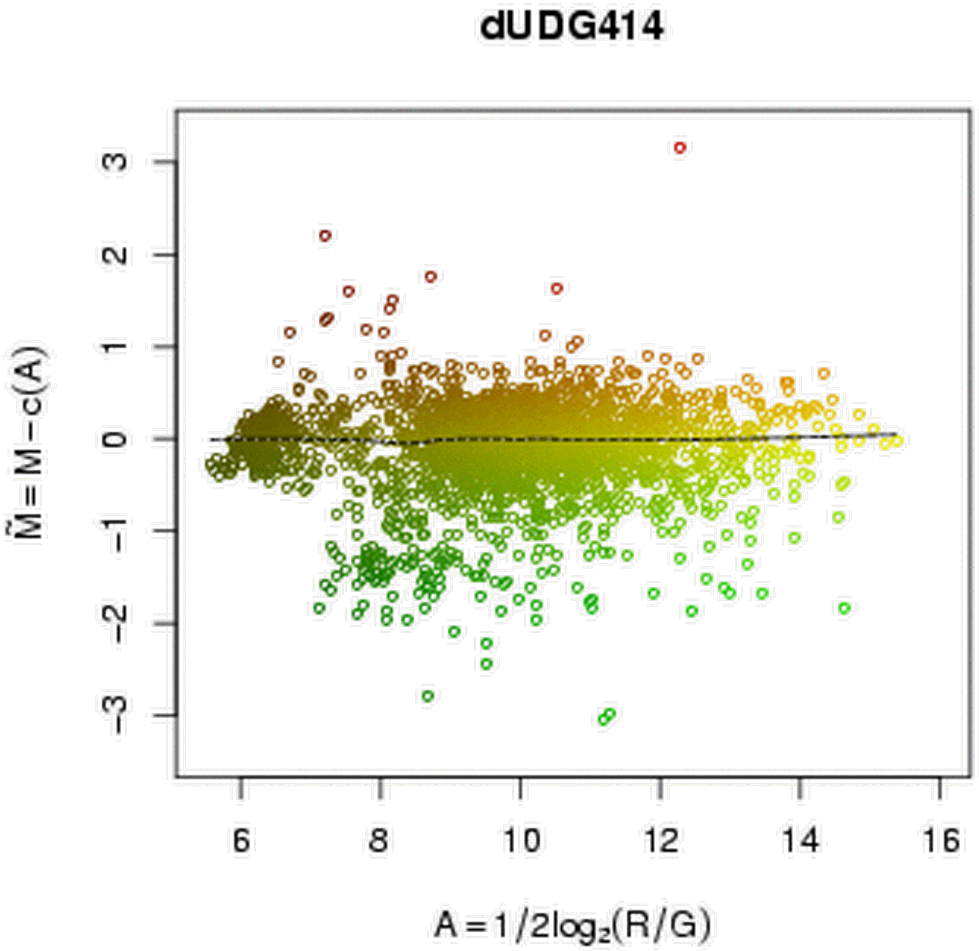
M = $\log_2$ (R/G)

Prior To Normalization

Non-normalized data $\{(M,A)\}_{n=1..5184}$:
$M = \log_2(R/G)$



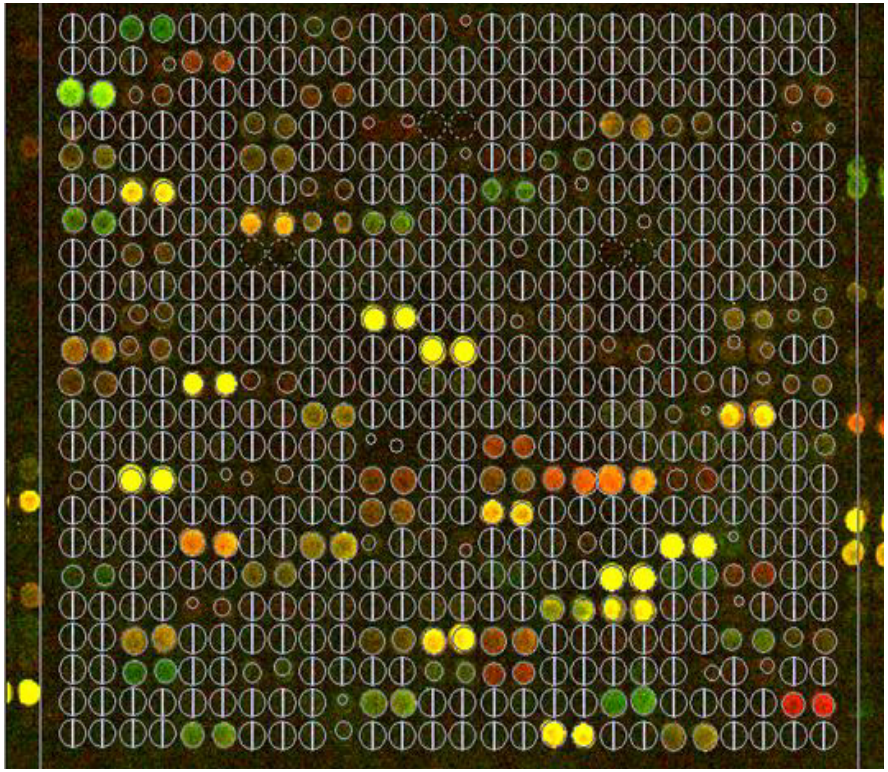
Global (Loess) Normalization



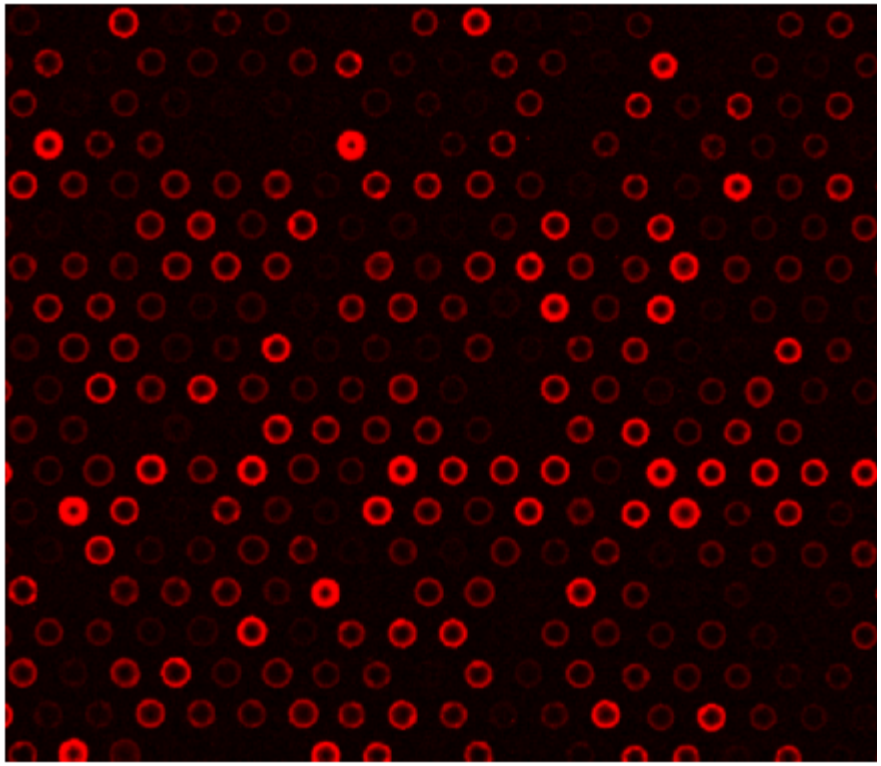
Quality Measurements

- **Array**
  - ***Correlation between spot intensities***
  - **Percentage of spots with no signals**
  - **Distribution of spot signal area**
  - **Inter-array consistency**
- **Spot**
  - **Signal / Noise ratio**
  - **Variation in pixel intensities**
  - **ID of “bad spots” (spots with no signal)**

Quality Assessment

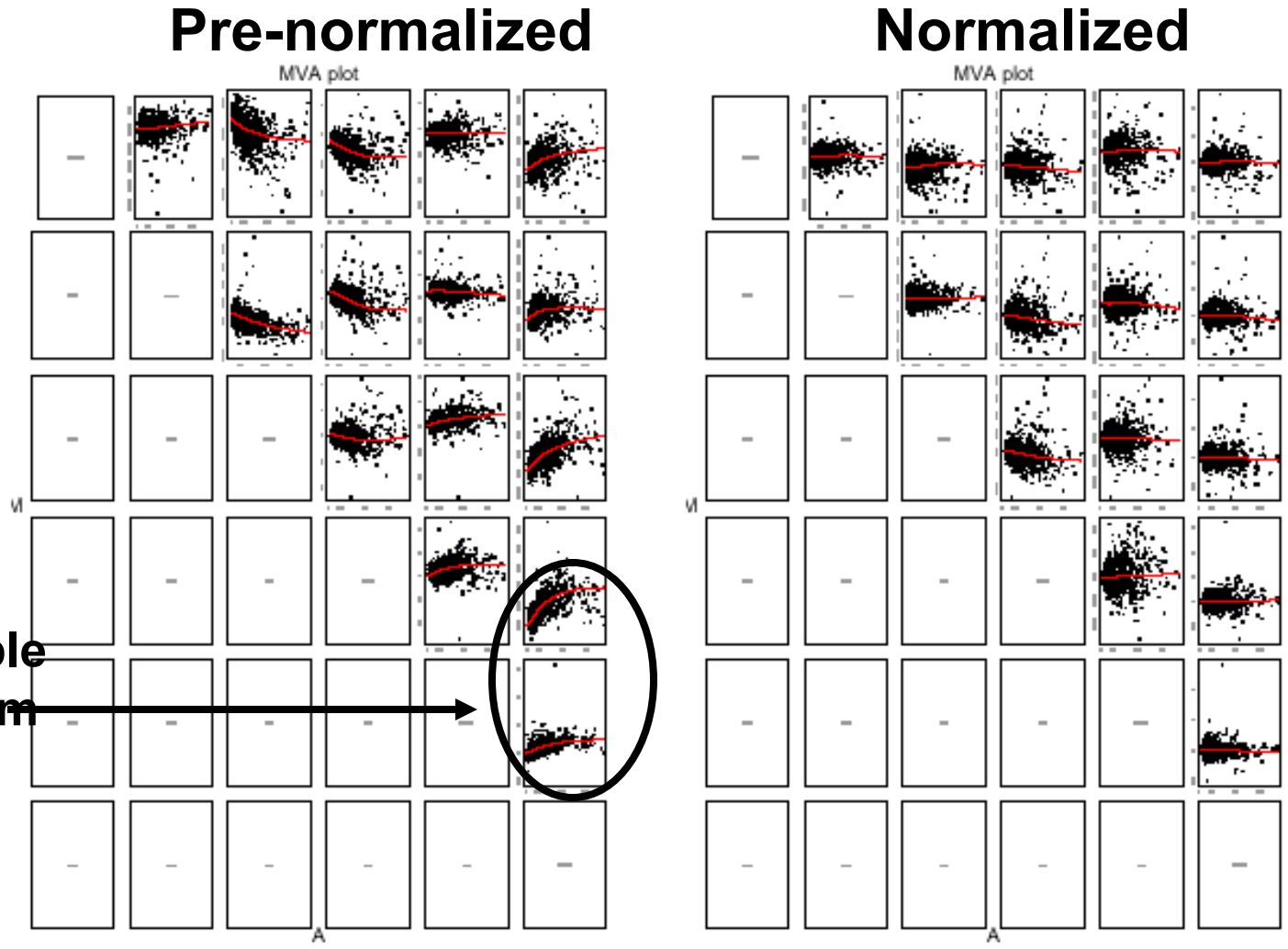


OK quality



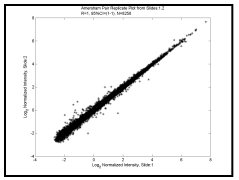
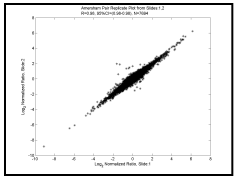
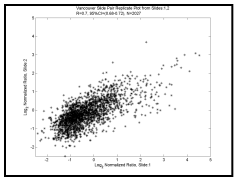
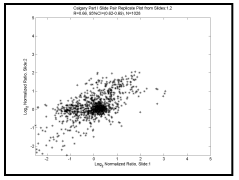
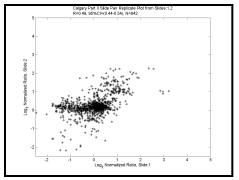
High quality

Inter-Array Consistency*

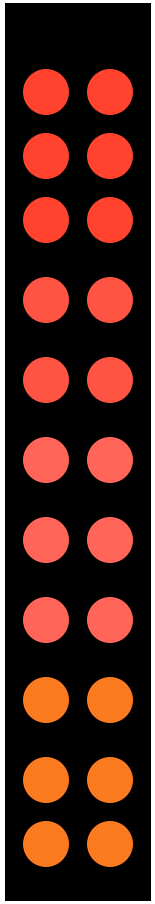


Quality Assessment

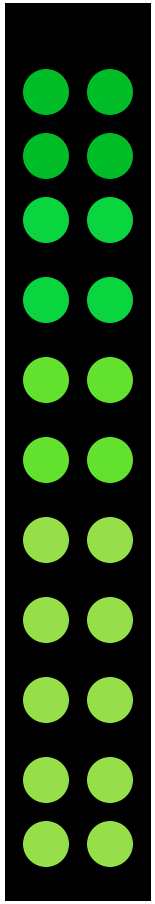
High Quality Array	1)	R=1	95%CI=(1-1)	N=8258
	2)	R=0.99	95%CI=(0.99-1)	N=8332
	3)	R=0.99	95%CI=(0.99-0.99)	N=8290
High Quality Array	1)	R=0.98	95%CI=(0.98-0.98)	N=7694
	2)	R=0.97	95%CI=(0.97-0.98)	N=7873
	3)	R=0.97	95%CI=(0.97-0.97)	N=7694
Good Quality Array	1)	R=0.7	95%CI=(0.68-0.72)	N=2027
	2)	R=0.65	95%CI=(0.62-0.67)	N=2818
	3)	R=0.61	95%CI=(0.59-0.64)	N=2001
Poor Quality Array	1)	R=0.66	95%CI=(0.62-0.69)	N=1028
	2)	R=0.86	95%CI=(0.85-0.87)	N=1925
	3)	R=0.64	95%CI=(0.61-0.68)	N=1040
Poor Quality Array	1)	R=0.49	95%CI=(0.44-0.54)	N=942
	2)	R=0.81	95%CI=(0.8-0.83)	N=1700
	3)	R=0.57	95%CI=(0.52-0.61)	N=973

Final Result



Highly Exp		Reduced Exp	
Trx	16.8	GPD	0.11
Enh1	13.2	Shn2	0.13
Hin2	11.8	Alp4	0.22
P53	8.4	OncB	0.23
Calm	7.3	Nrd1	0.25
Ned3	5.6	LamR	0.26
P21	5.5	SetH	0.30
Antp	5.4	LinK	0.32
Gad2	5.2	Mrd2	0.32
Gad3	5.1	Mrd3	0.33
Erp3	5.0	TshR	0.34



Fold change

Key Steps in Microarray Analysis

- **Quality Control (checking microarrays for errors or problems)**
- **Image Processing**
  - **Gridding**
  - **Segmentation (peak picking)**
  - **Data Extraction (intensity, QC)**
- **Data Analysis and Data Mining (Differential gene expression)**

Identifying Patterns of Gene Expression*

- **Key Goal: identify differentially & co-regulated groups of genes via clustering**
- **This leads to:**
  - **inferences about physiological responses**
  - **generalizations about large data sets**
  - **identification of regulatory cascades**
  - **assignment of possible function to uncharacterized genes**
  - **identification of shared regulatory motifs**

Clustering Applications in Bioinformatics*

- Microarray or GeneChip Analysis
- 2D Gel or ProteinChip Analysis
- Protein Interaction Analysis
- Phylogenetic and Evolutionary Analysis
- Structural Classification of Proteins
- Protein Sequence Families

Clustering*

- **Definition - a process by which objects that are logically similar in characteristics are grouped together.**
- **Clustering is different than Classification**
- **In classification the objects are assigned to pre-defined classes, in clustering the classes are yet to be defined**
- **Clustering helps in classification**

Clustering Requires...

- A method to measure similarity (a similarity matrix) or dissimilarity (a dissimilarity coefficient) between objects
- A threshold value with which to decide whether an object belongs with a cluster
- A way of measuring the “distance” between two clusters
- A cluster seed (an object to begin the clustering process)

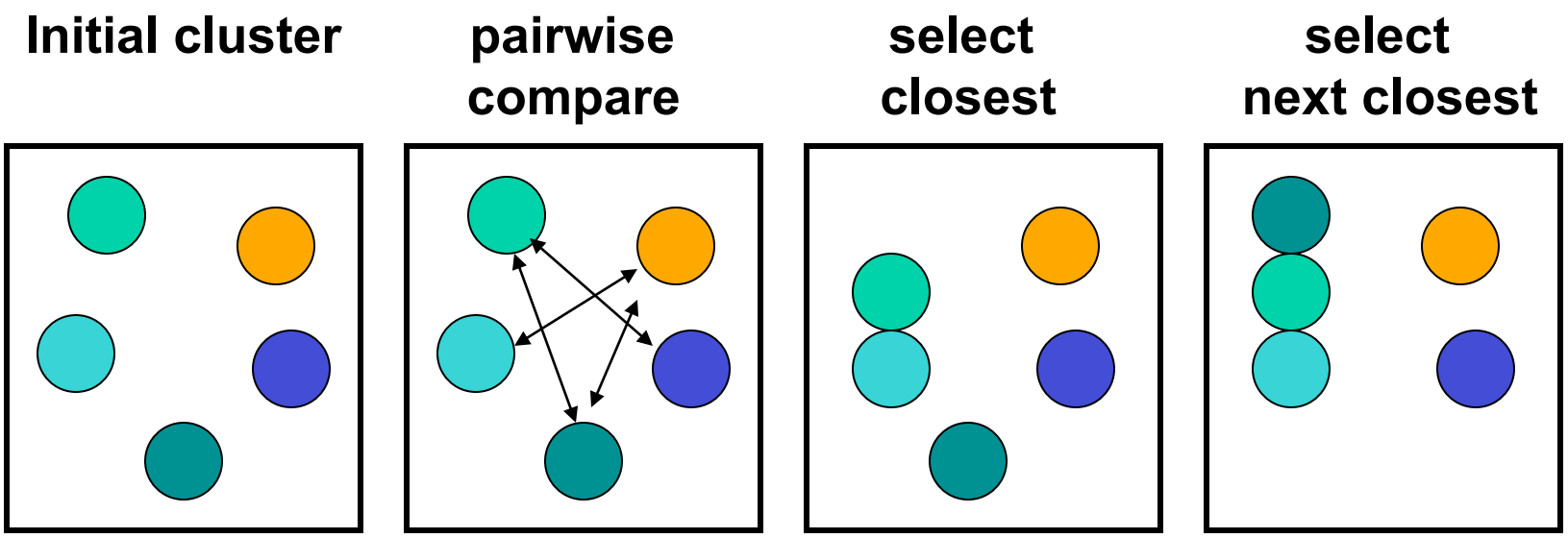
Clustering Algorithms*

- **K-means or Partitioning Methods - divides a set of N objects into M clusters -- with or without overlap**
- **Hierarchical Methods - produces a set of nested clusters in which each pair of objects is progressively nested into a larger cluster until only one cluster remains**
- **Self-Organizing Feature Maps - produces a cluster set through iterative "training"**

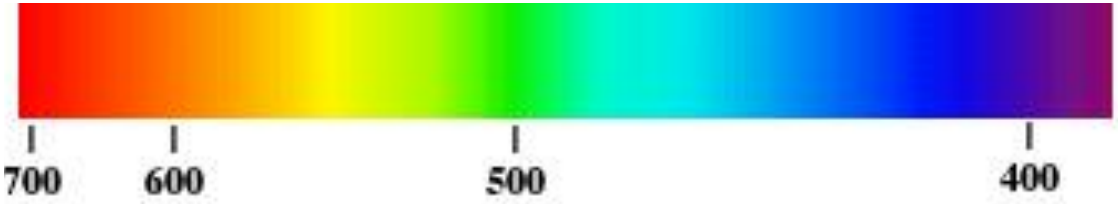
Hierarchical Clustering*

- ***Find the two closest objects and merge them into a cluster***
- ***Find and merge the next two closest objects (or an object and a cluster, or two clusters) using some similarity measure and a predefined threshold***
- ***If more than one cluster remains return to step 2 until finished***

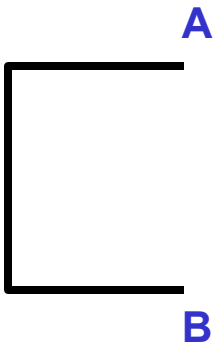
Hierarchical Clustering*



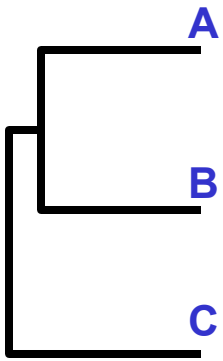
Rule: $\lambda_T = \lambda_{obs} \pm 50$ nm



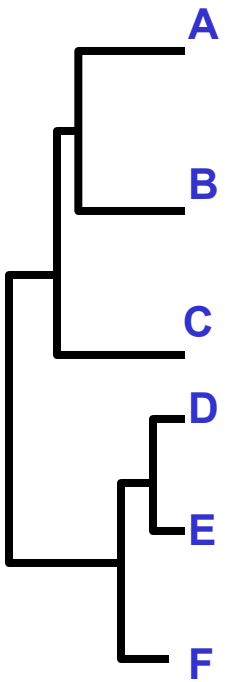
Hierarchical Clustering*



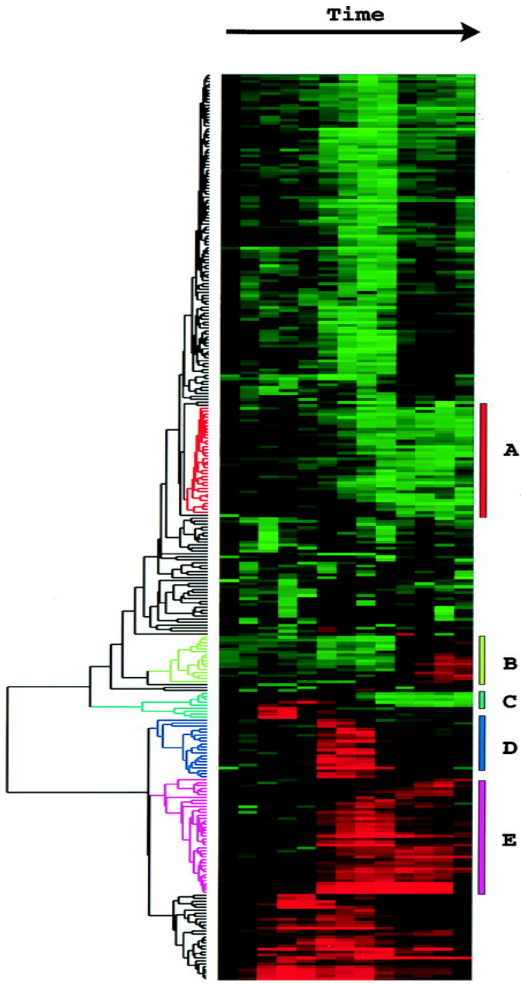
Find 2 most similar gene express levels or curves



Find the next closest pair of levels or curves

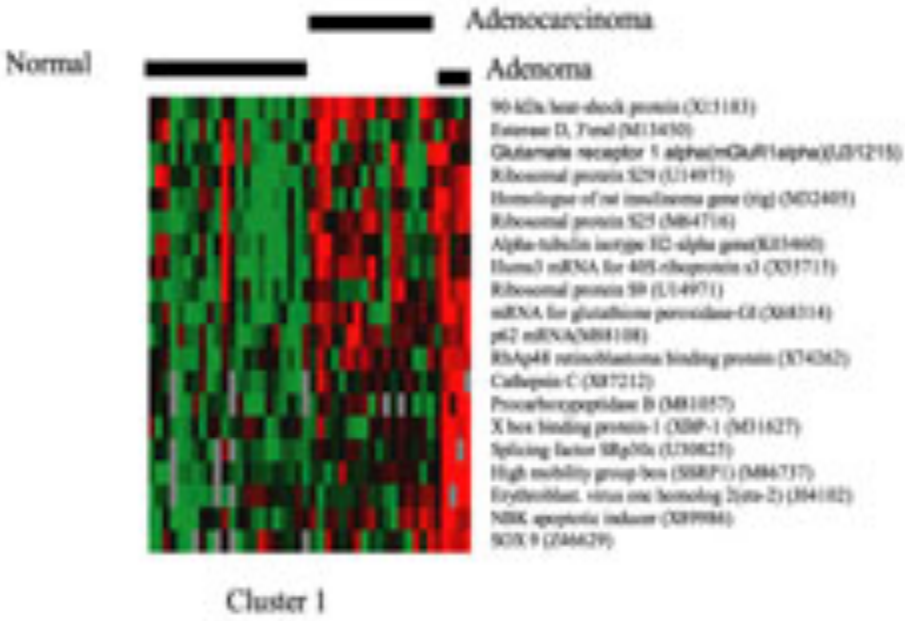


Iterate

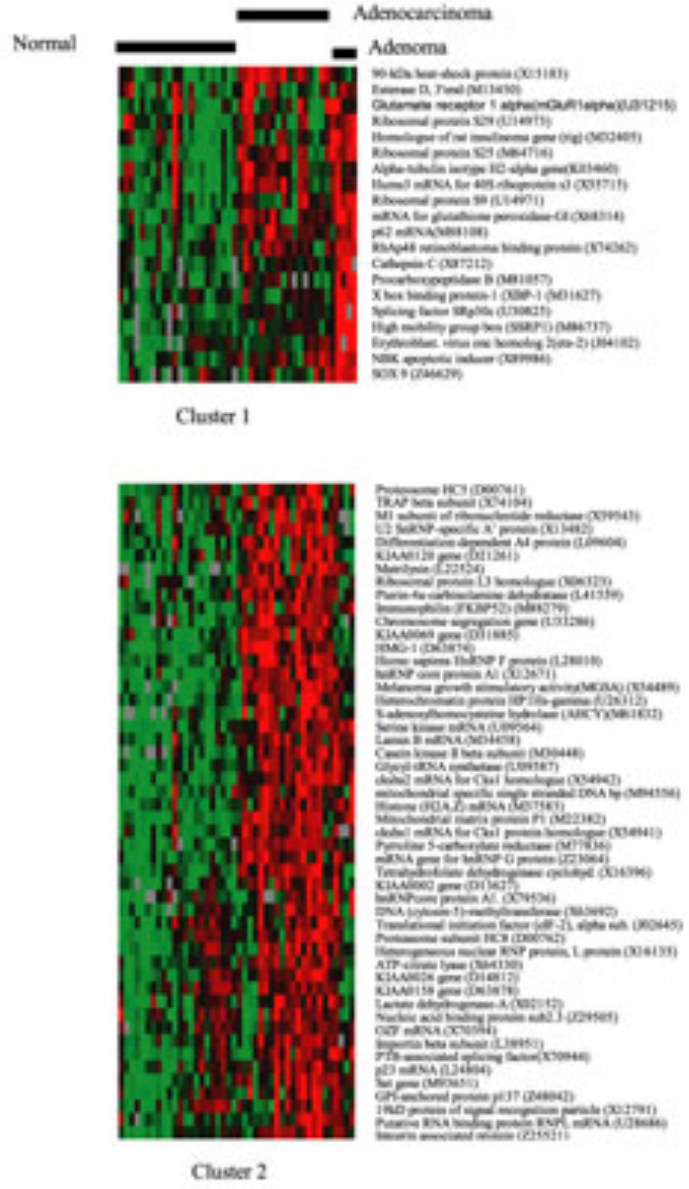


Heat map

Results



Heat Map

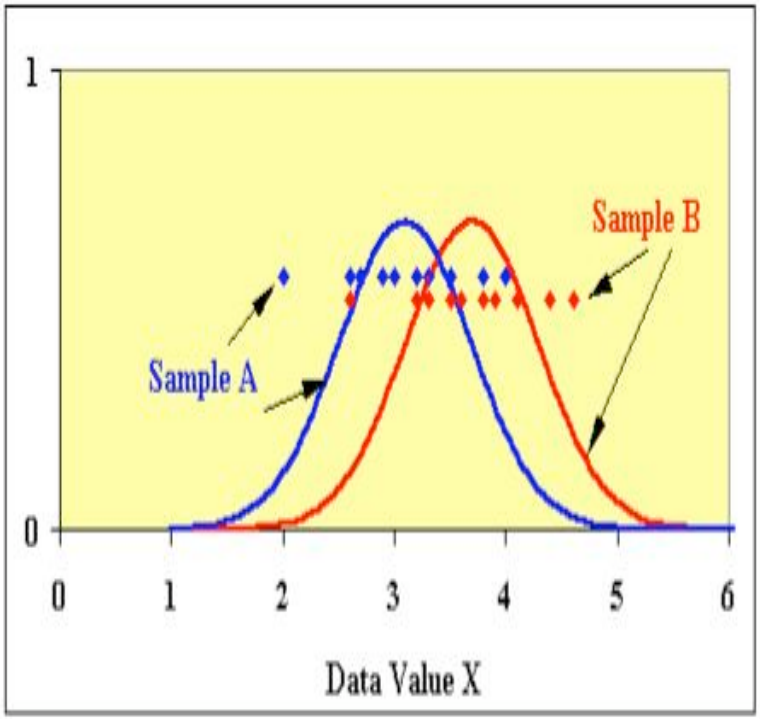


Putting it All Together*

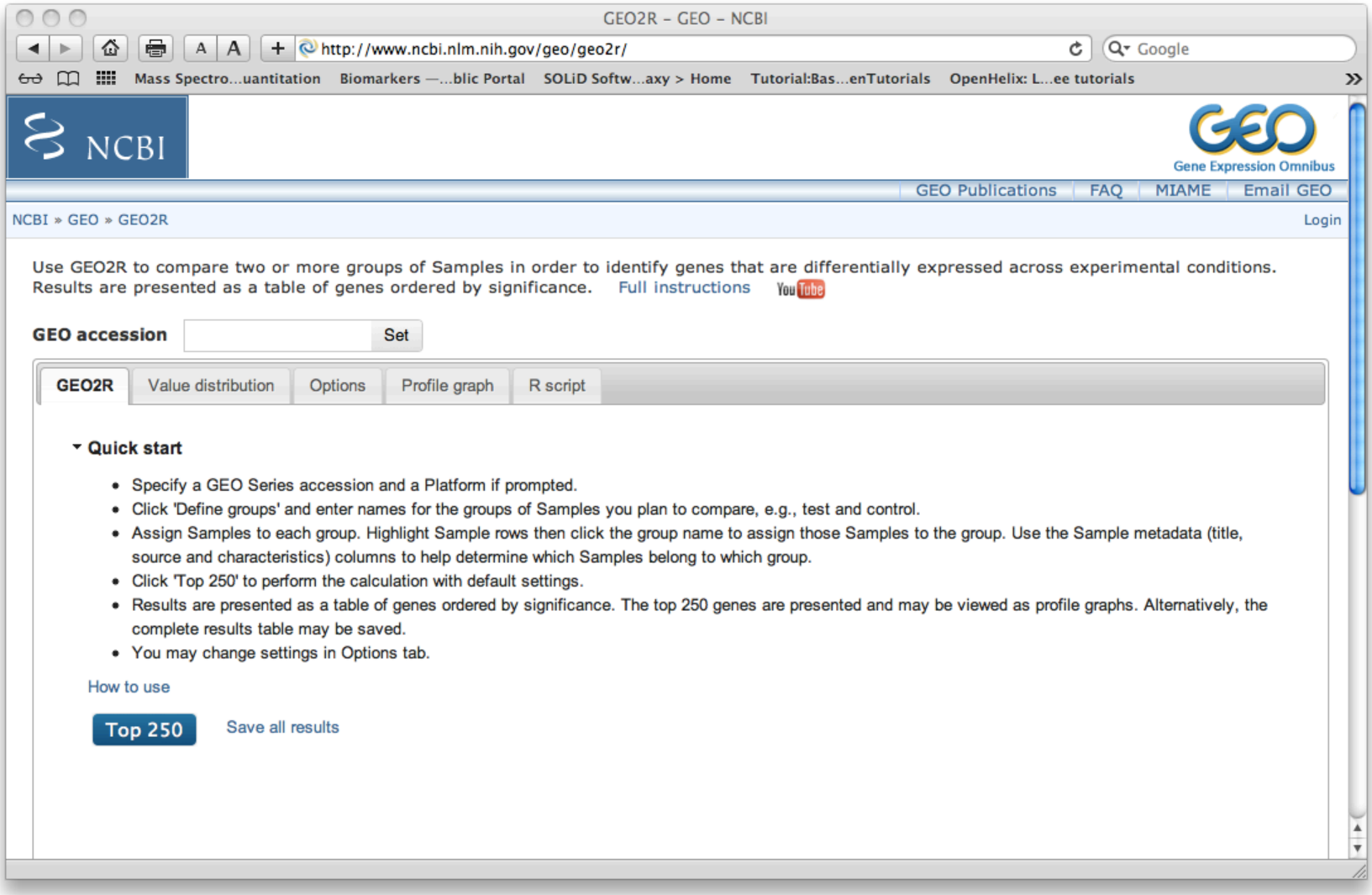
- Perform normalization
- Determine if experiment is a time series, a two condition or a multi-condition experiment
- Calculate level of differential expression and identify which genes are significantly ($p<0.05$ using a t-test) over-expressed or under expressed (a 2 fold change or more)
- Use clustering methods and heat maps to identify unusual patterns or groups that associate with a disease state or conditions
- Interpret the results in terms of existing biological or physiological knowledge
- Produce a report describing the results of the analysis

The Student's t-test*

- The Student's t-distribution was first introduced by W.S. Gossett in 1908 under the pen name Student
- Used to establish confidence limits (error bars) for the mean estimated from smaller sample sizes
- Used to test the statistical significance of a non-zero mean
- Used to test the statistical significance of the difference between means from two independent samples
- A p value or t-stat of <0.05 is significant



GEO2R



http://www.ncbi.nlm.nih.gov/geo/geo2r/

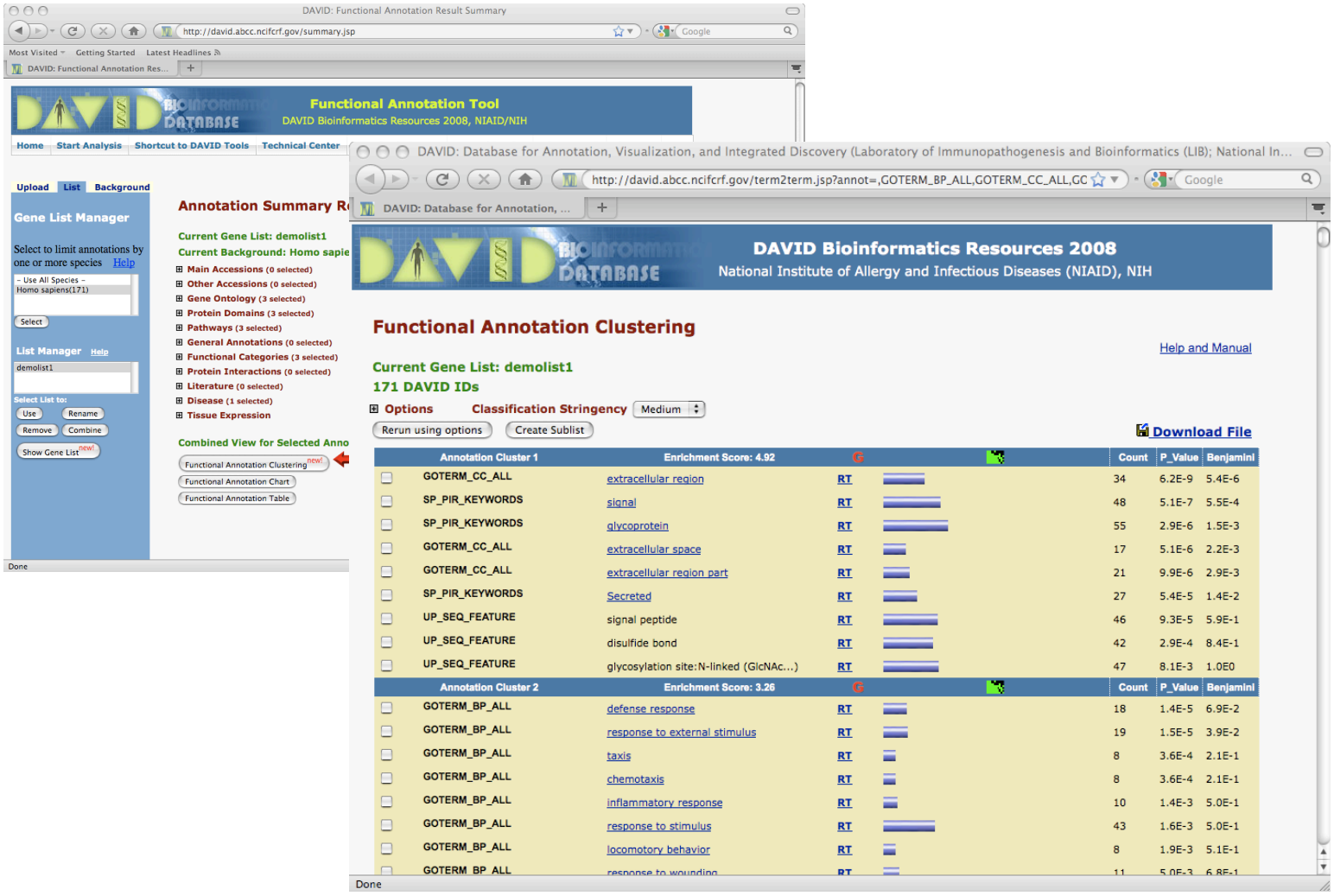
GEO2R

- Web-based GeneChip/Microarray analysis pipeline written in R
- Designed to handle microarray data deposited in the GEO (Gene Expression Omnibus) database
- Performs relatively simple analysis of microarray data
- Generates lots of tables and plots
- Supports many different microarray platforms
- User-friendly, with several tutorials

DAVID*

http://david.abcc.ncifcrf.gov/

DAVID - Output



DAVID-Annotation

- Takes “significant” gene lists (from microrarray expts or proteomic experiments) and allows users to plot heatmaps, generate graphs, identify possible pathways, common or shared functions, clusters of similar genes as well as shared gene ontologies (GO terms)
- Facilitates biological interpretation

How To Do Your Assignment

- **Read the assignment instructions carefully**
- **Follow the instructions listed on the GEO2R website. If you are not clear on how to use the site, look at the YouTube video. Part of the assignment grade depends on you being able to follow instructions on your own**
- **The assignment has several tasks. Make sure to complete all tasks. Use graphs and tables to make your point or answer the questions**
- **Do not plagiarize text from the web or from papers when putting your answers together**
- **You can cut and paste tables and images from tasks you perform on webservers**

How To Do Your Assignment

- **The assignment should be assembled using your computer (cut, paste, format and edit the output or data so it is compact, meaningful and readable)**
- **No handwritten materials unless your computer/printer failed**
- **A good assignment should be 5-6 pages long and will take 4-5 hours to complete**
- **Hand-in hard copy of assignment on due date. Electronic versions are accepted only if you are on your death bed**