3000788 Intro to Comp Molec Biol

Lecture 8: Transcriptomics technology

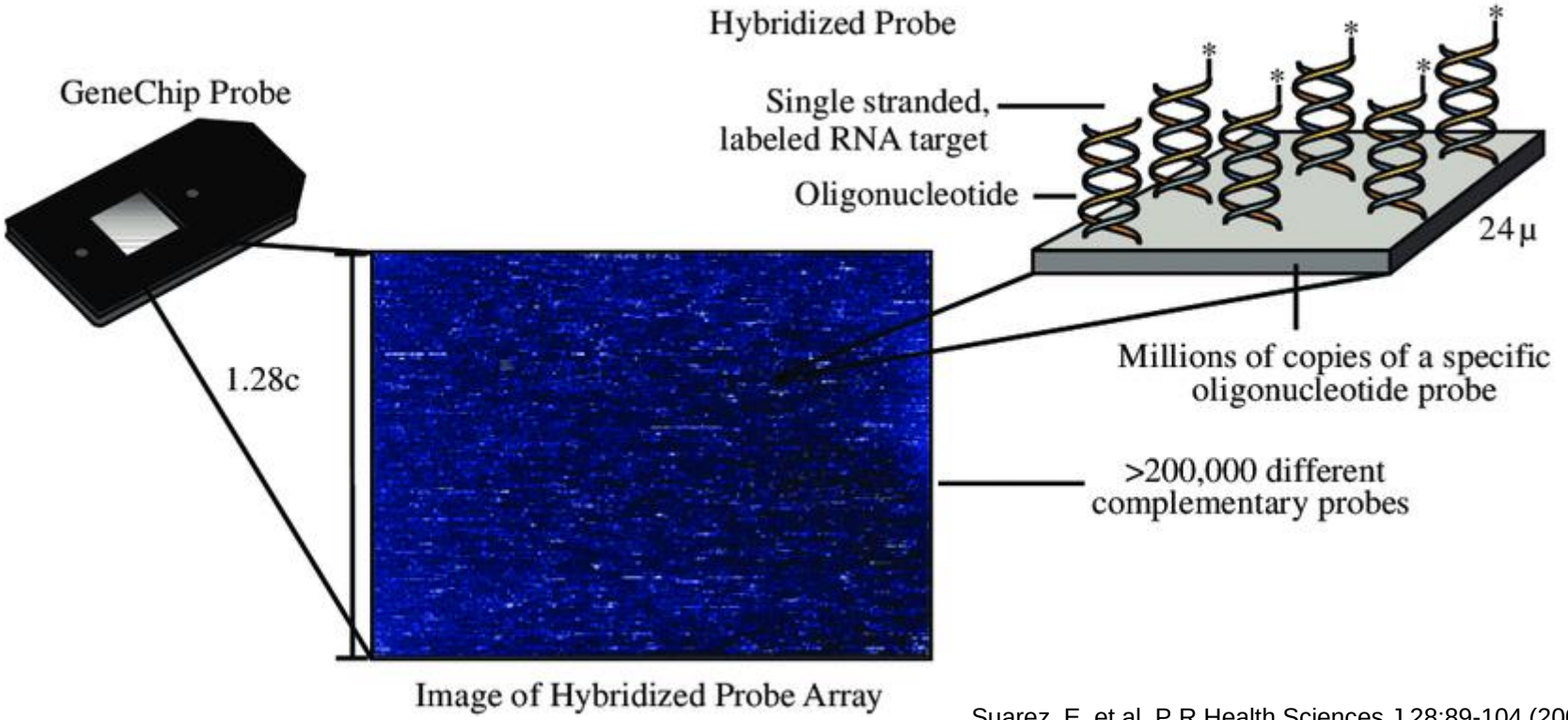
September 8, 2022

Sira Sriswasdi, PhD

- Research Affairs
- Center of Excellence in Computational Molecular Biology (CMB)
- Center for Artificial Intelligence in Medicine (CU-AIM)

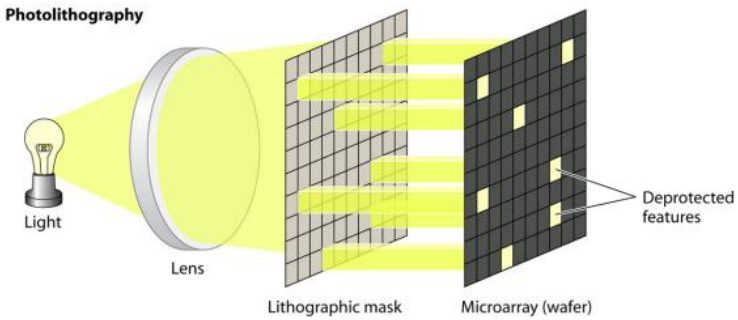
Oligonucleotide microarray

Microarray technology overview

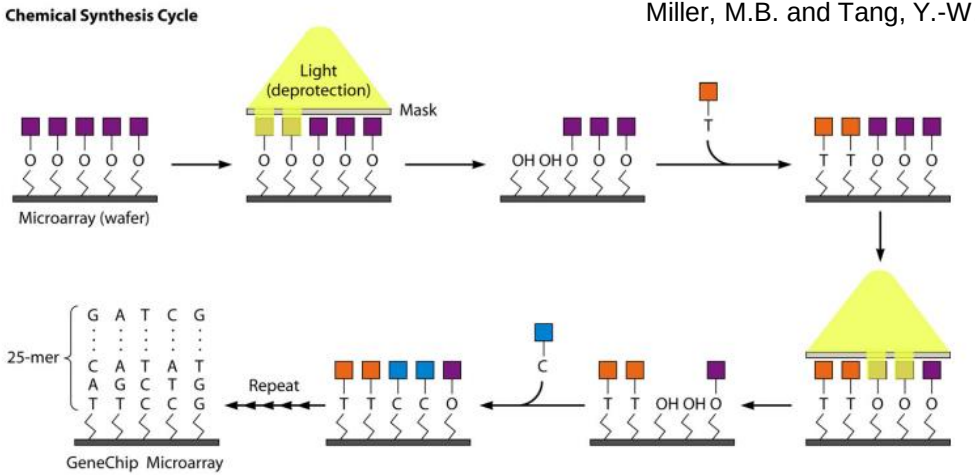


Suarez, E. et al. P R Health Sciences J 28:89-104 (2009)

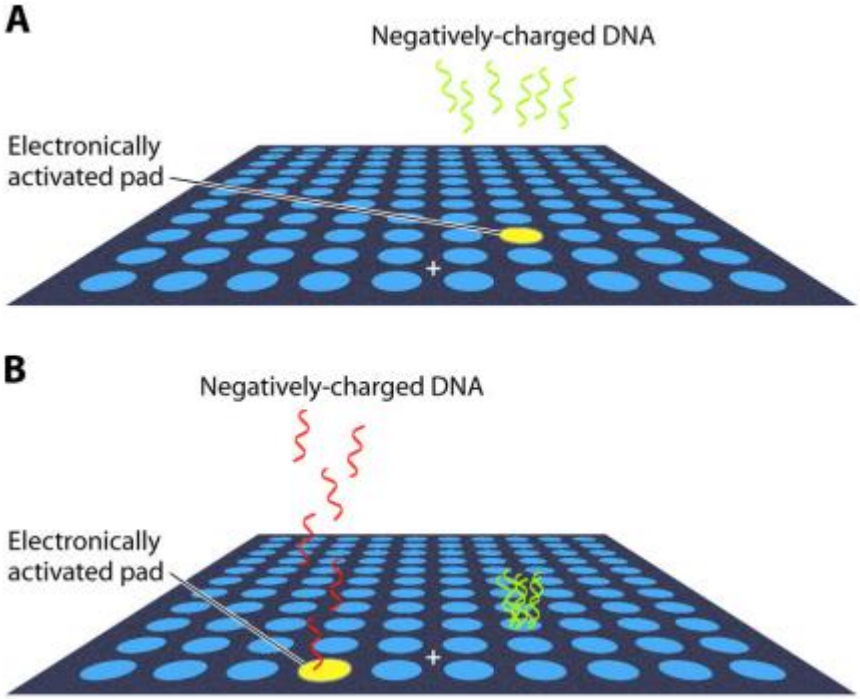
Microarray fabrication



Miller, M.B. and Tang, Y.-W. Clin Microbiol Rev 611-633 (2009)



Microarray fabrication



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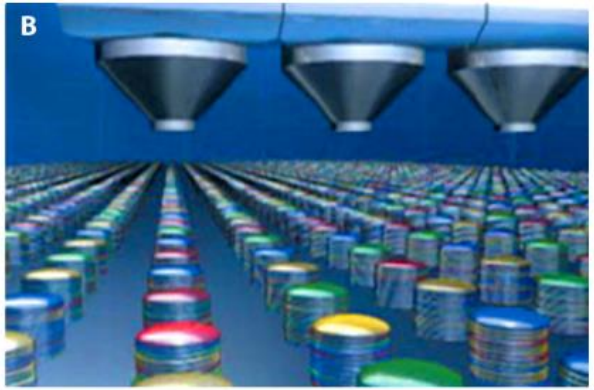
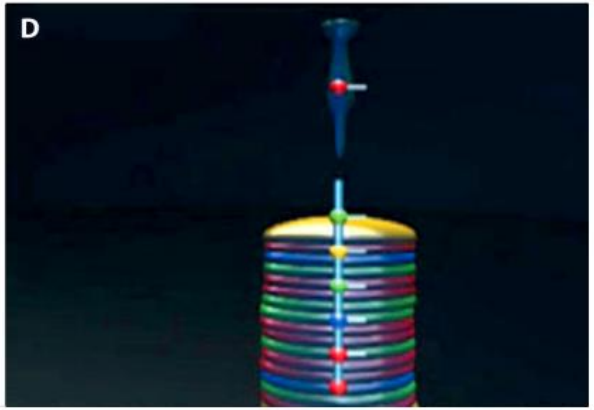
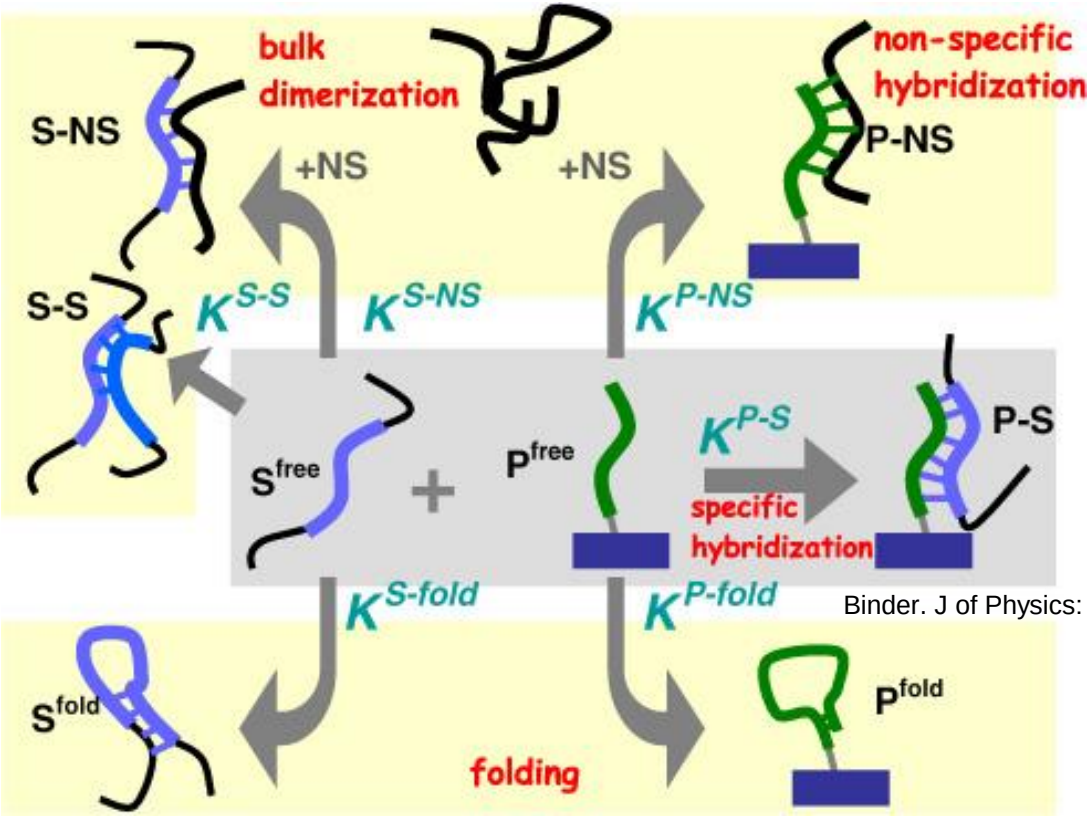




Probe design for microarray

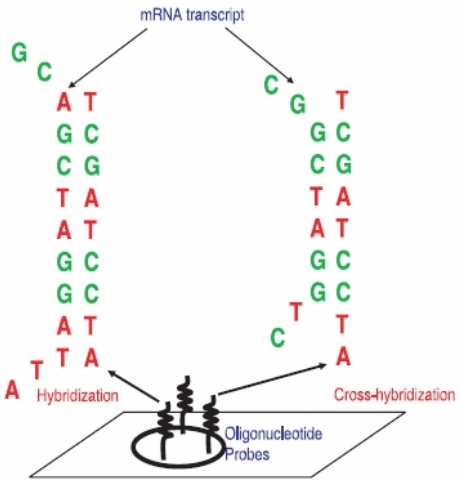
Unwanted probe interactions



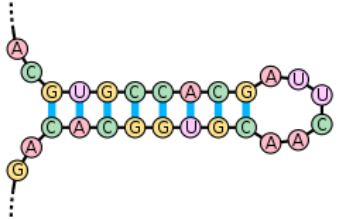
Binder. J of Physics: Condensed Matter 18. (2006)

Probe design principles

- **Sensitivity**
  - Complementary to each target genomic region
  - Multiple probes for each target
- **Specificity**
  - Cross-hybridization: similarity to other targets
  - BLAST
  - Negative control probes
- **Technical issues**
  - Secondary structure: self-complementary
  - Hybridization energy
  - Position-specific bias

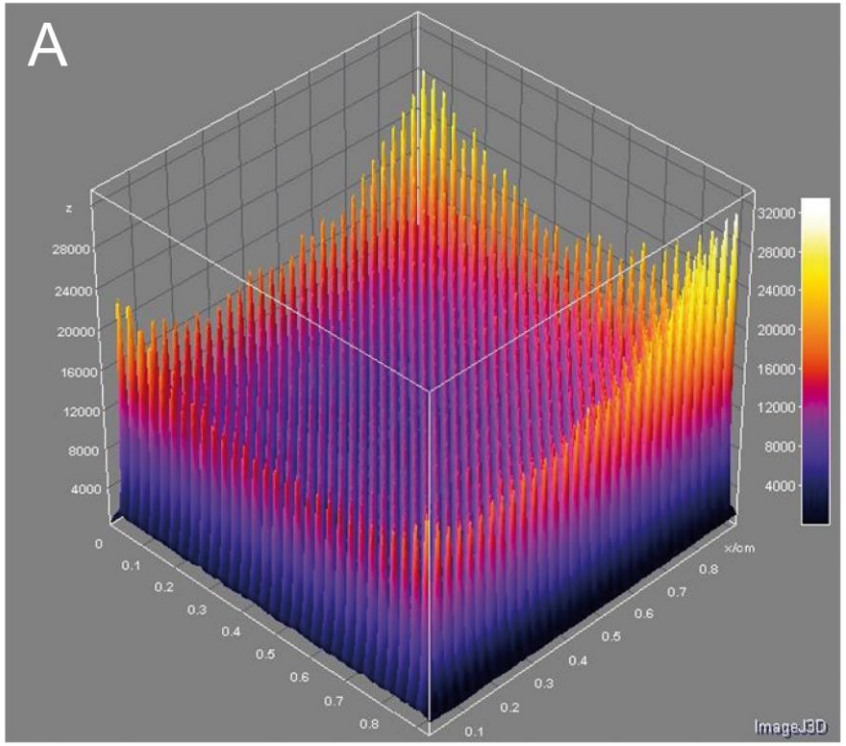


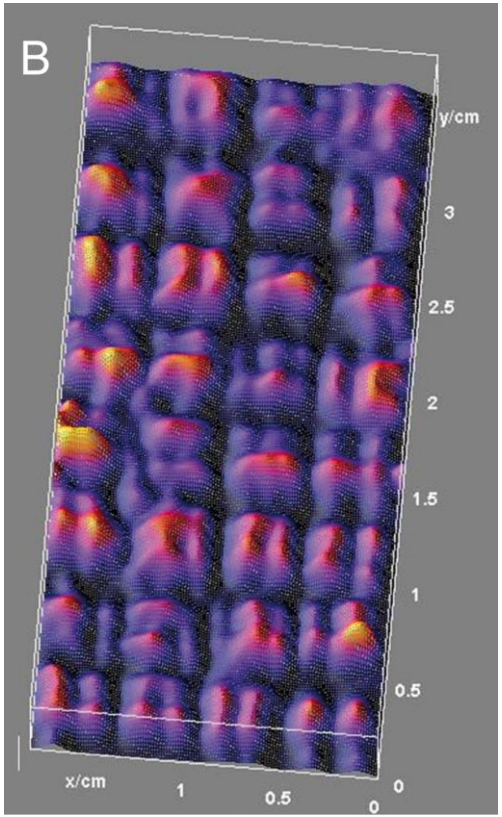
Malutan, R. and Vilda, P.G. Thermodynamics of Microarray Hybridization (2011)



https://en.wikipedia.org/wiki/Complementarity_%28molecular_biology%29

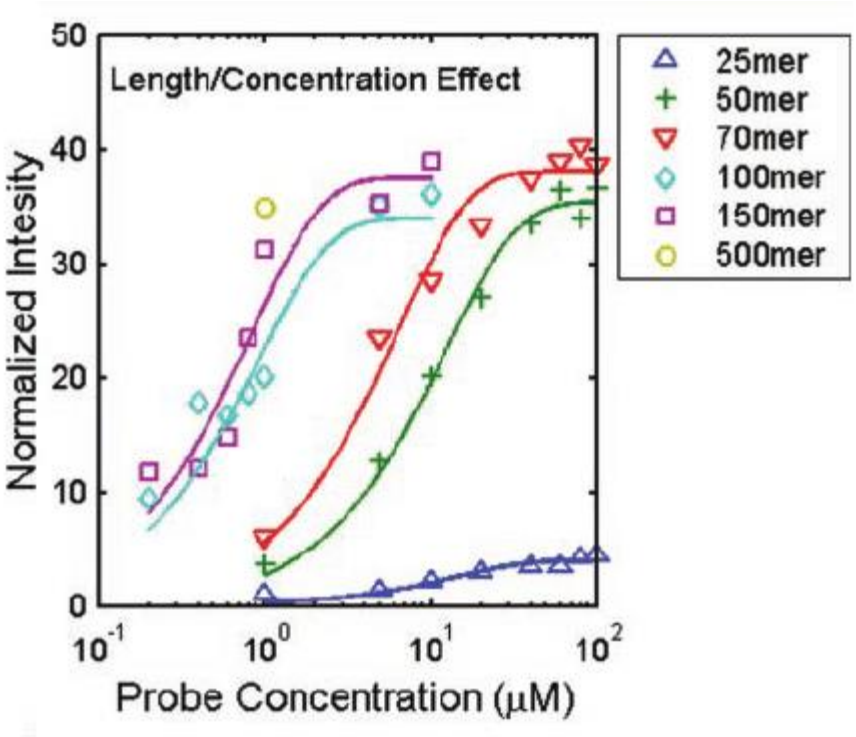
Position-specific intensity bias

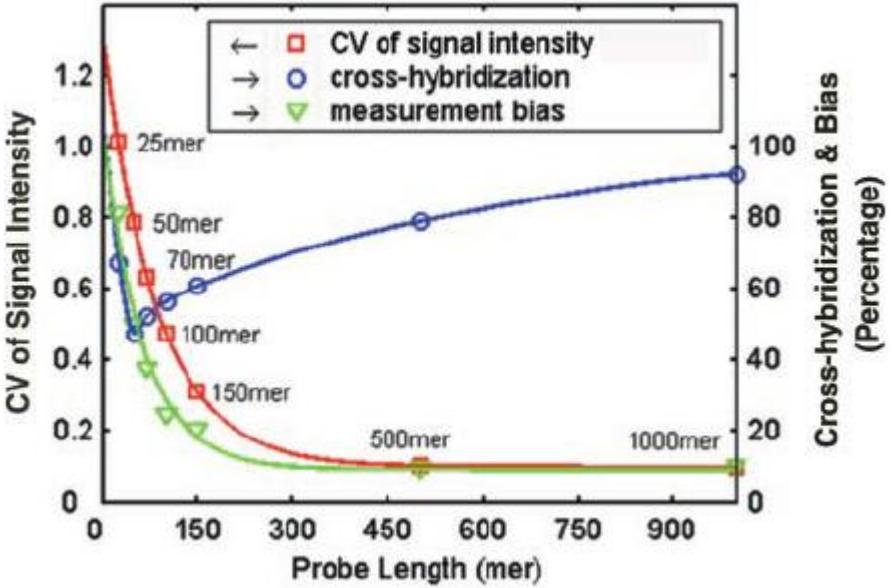




Steger, D. et al. PLoS ONE 6:e23727 (2011)

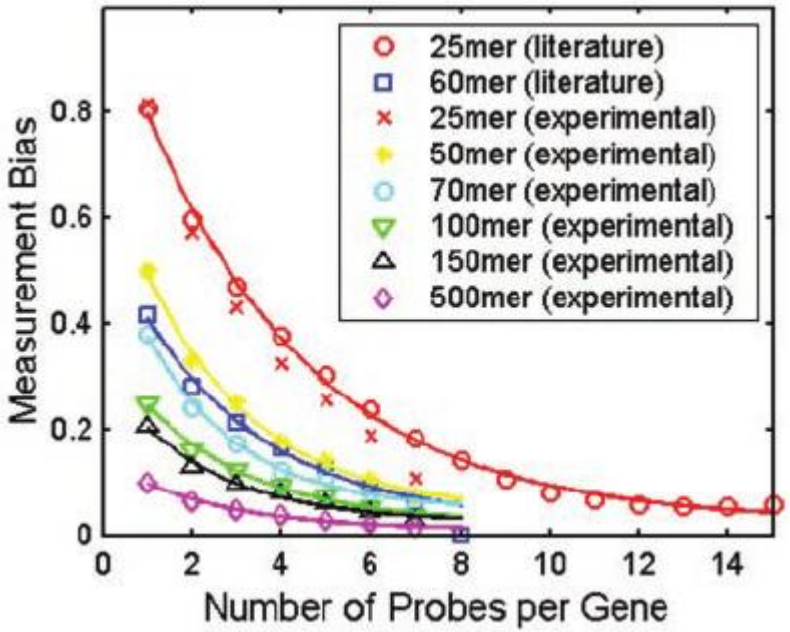
Impact of probe length

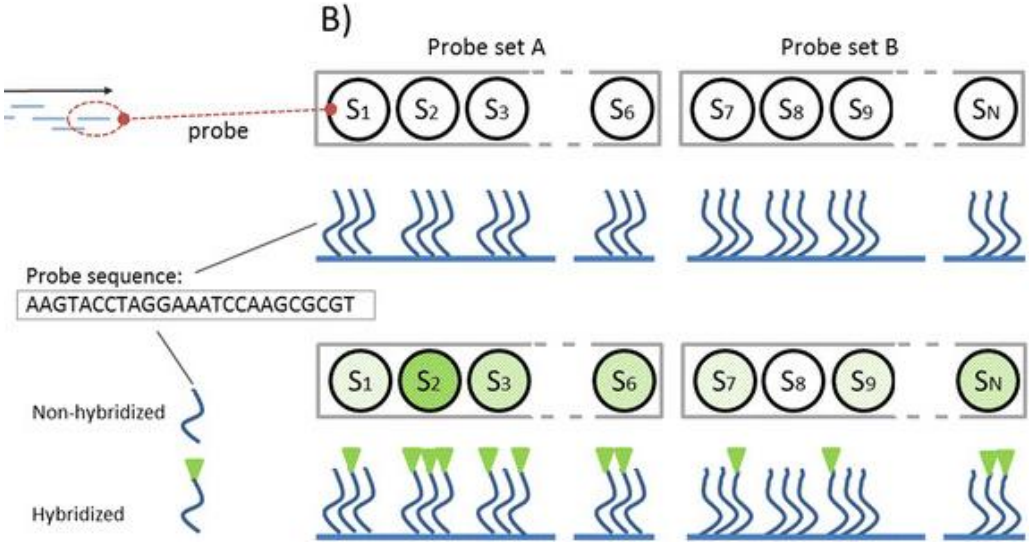




Chou, C.-C. NAR 32:e99 (2004)

Probe set = multiple probes per gene

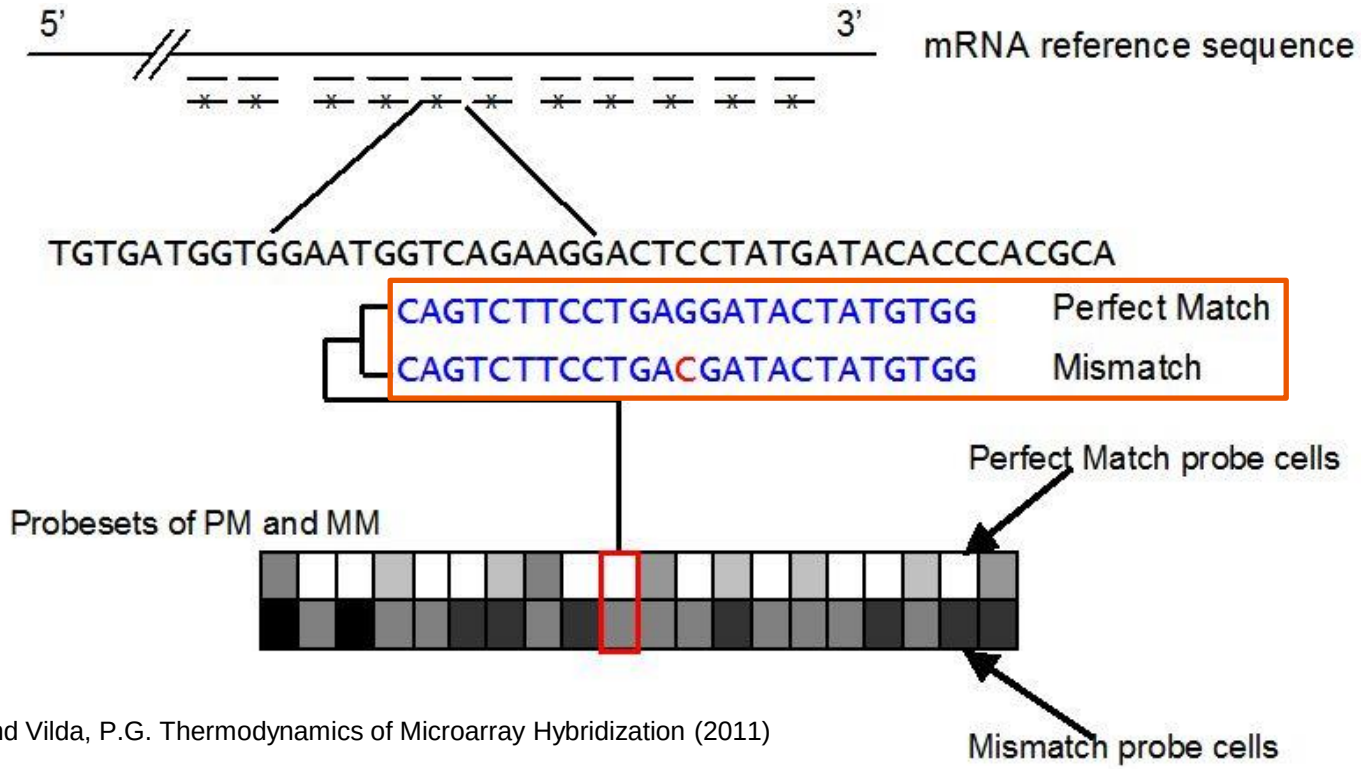




Chou, C.-C. NAR 32:e99 (2004)

Jaksik, R. et al. Biology Direct 10:46 (2015)

Perfect match (PM) and mismatch (MM)



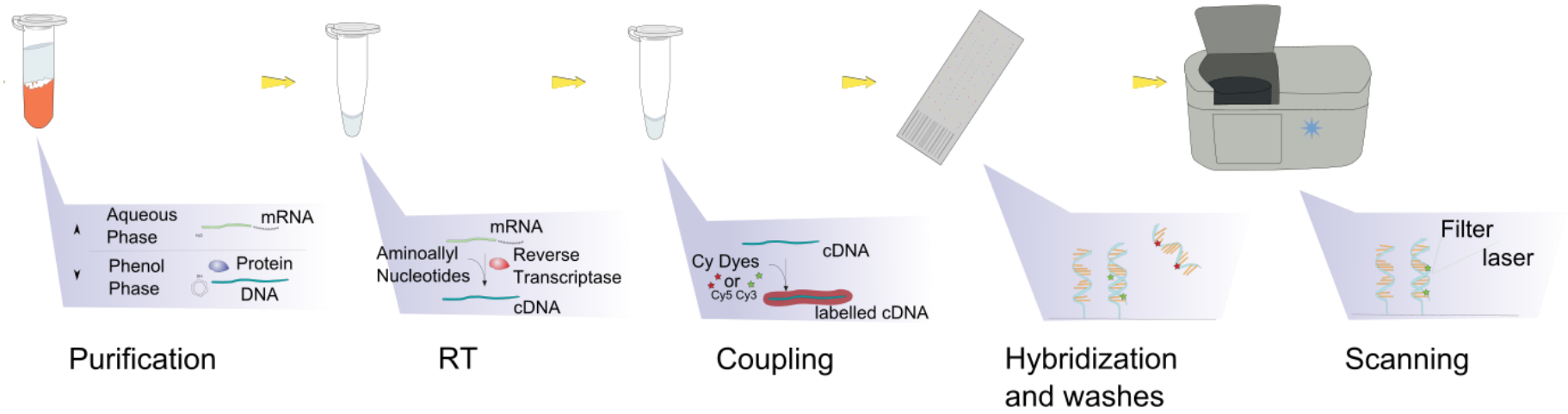
Malutan, R. and Vilda, P.G. Thermodynamics of Microarray Hybridization (2011)

Example microarray metadata

ControlType	ProbeName	SystematicName	PositionX	PositionY
1	GE_BrightCorner	GE_BrightCorner	584.922	4464.27
1	DarkCorner	DarkCorner	606.433	4464.3
1	DarkCorner	DarkCorner	626.841	4464.18
0	A_23_P326296	NM_144987	648.069	4464.19
0	A_24_P287941	NM_013290	669.667	4464.39
0	A_24_P325046	BC022434	691	4464.5
0	A_23_P200404	NM_001625	712	4464.5
0	A_19_P00800513	lincRNA:chr7:226042-232442_R	733.224	4464.48
0	A_23_P15619	NM_032391	754.4	4464.41
0	A_33_P3402354	L40403	775.5	4464.32
0	A_33_P3338798	NM_001145251	798.041	4464.16
0	A_32_P98683	NM_005937	817.068	4464.27
0	A_23_P137543	NM_152493	838.533	4464.4
0	A_19_P00803040	lincRNA:chr8:104254399-104295074_F	859.965	4464.37
0	A_23_P117852	NM_014736	881	4464.3
0	A_33_P3285585	AK127191	902.5	4464.5
0	A_24_P328231	NM_017871	923.214	4464.57
0	A_33_P3415668	NR_028328	944.776	4464.52
0	A_23_P73609	NM_000266	966	4464.5
0	A_24_P186124	NM_182501	986.871	4464.53

Microarray data pre-processing

Microarray data collection steps



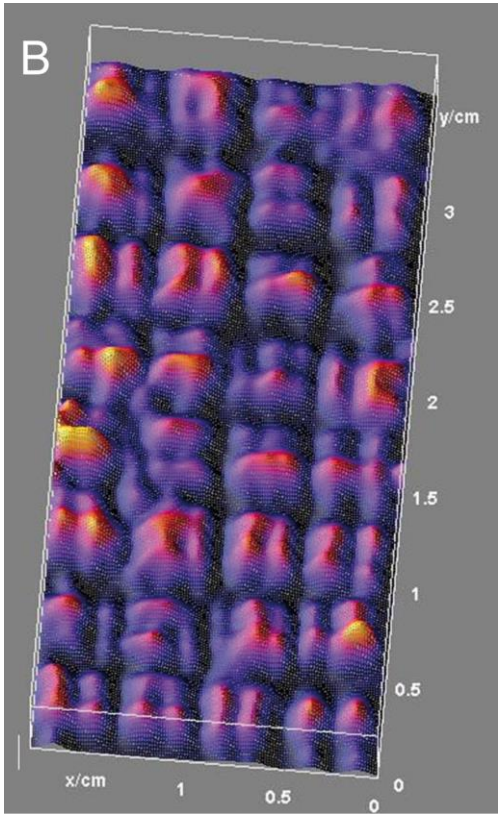
https://en.wikipedia.org/wiki/Microarray_analysis_techniques

Key processing steps

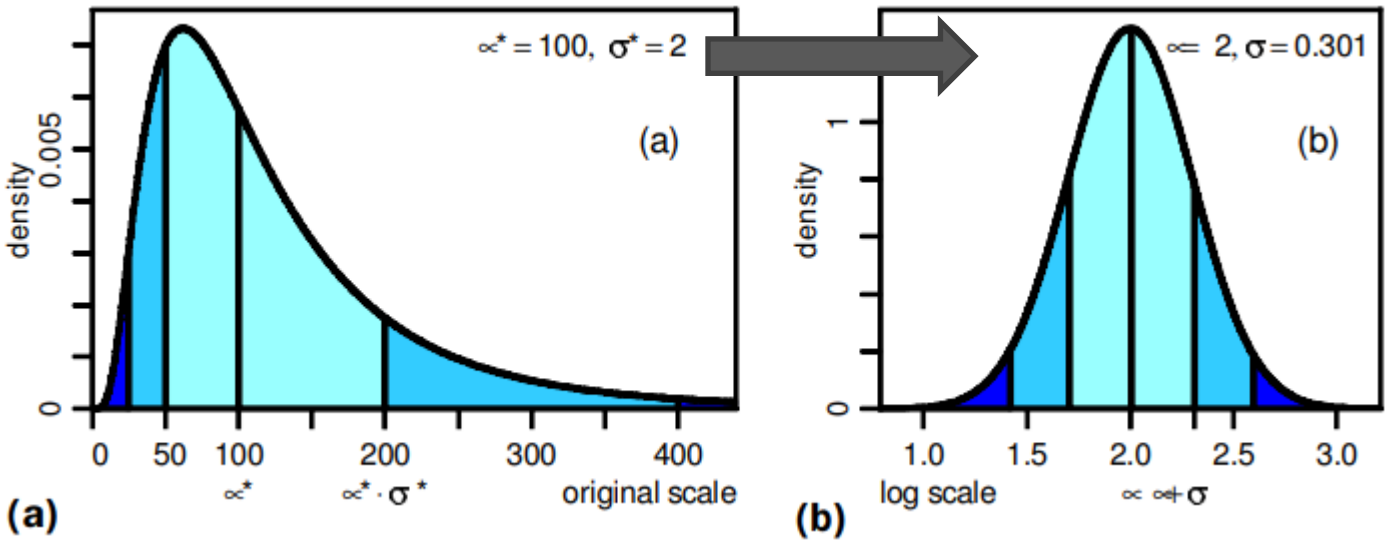
- Redefining probe set
  - BLAST to latest genome annotation

- Intensity correction
  - Model background using probe location & sequence
  - Perfect match (PM) vs mismatch (MM)
  - Global & local correction
- Outlier removal

- Probe set aggregation
- Log transform

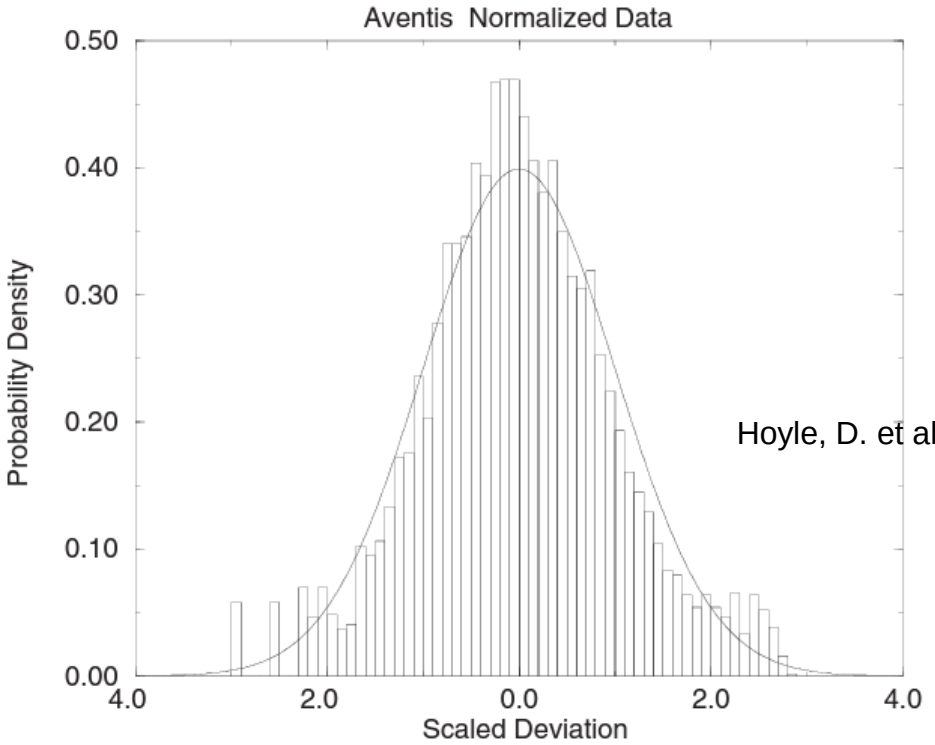


Log-normal distribution



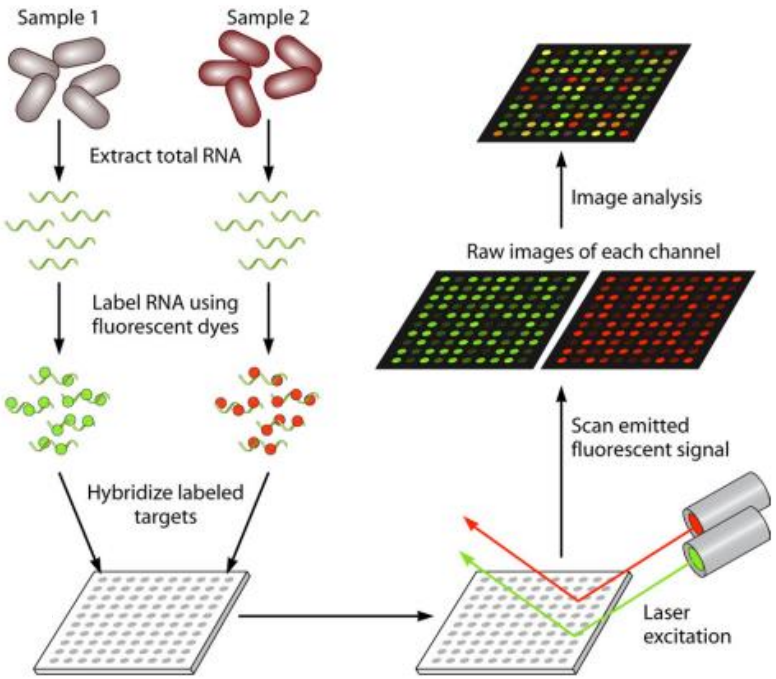
Limpert, Stahel, and Abbt. BioScience 2001.

Microarray data are log-normal distributed



Hoyle, D. et al. Bioinformatics 18:576-584 (2001)

Two-channel microarray

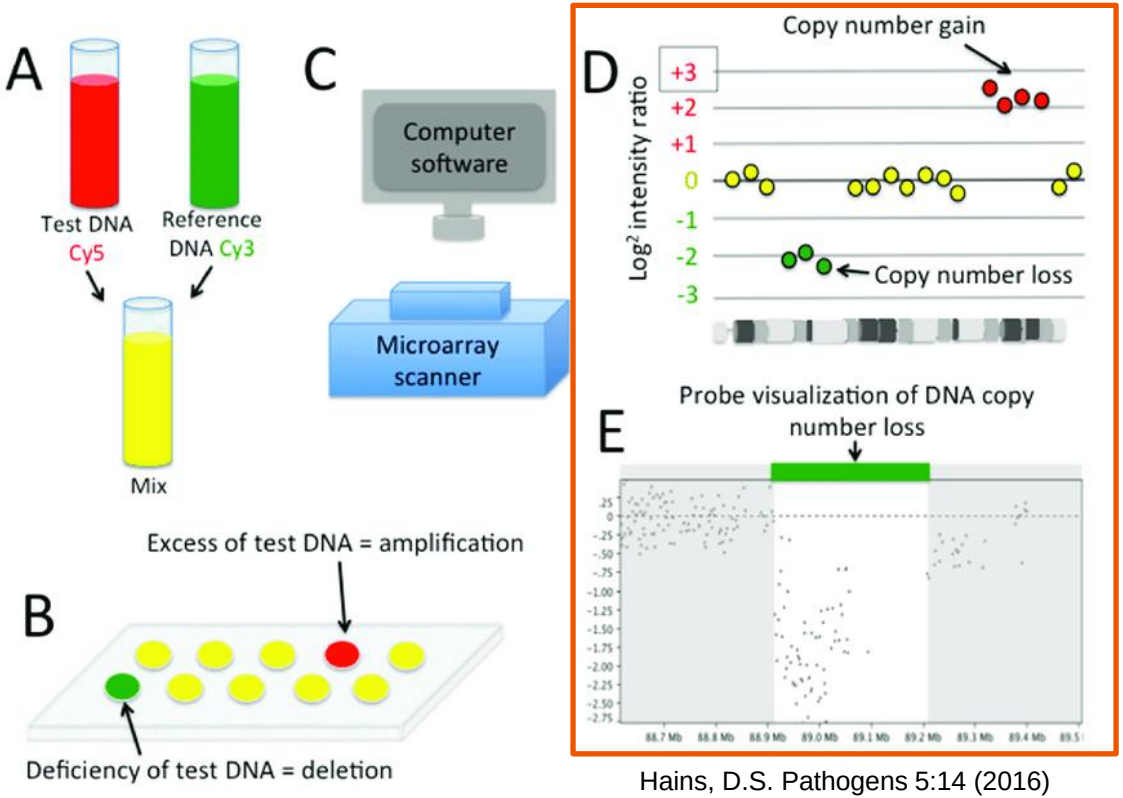


Miller, M.B. and Tang, Y.-W. Clin Microbiol Rev 611-633 (2009)

- Two samples are labeled with different dyes
- Mix and hybridize to microarray
- Relative fluorescence signal (ratio) directly indicates fold difference in gene expression
- Minimize technical variance

Beyond transcriptomics

Comparative genome hybridization (CGH)

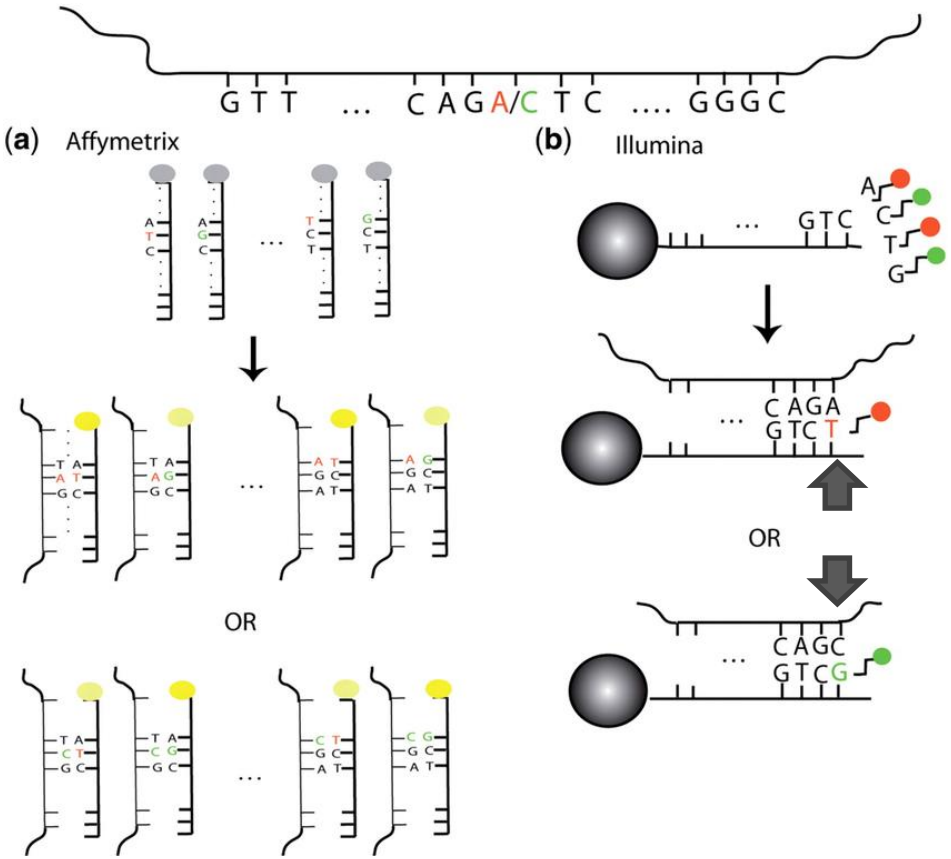


Hains, D.S. Pathogens 5:14 (2016)

SNP genotyping array

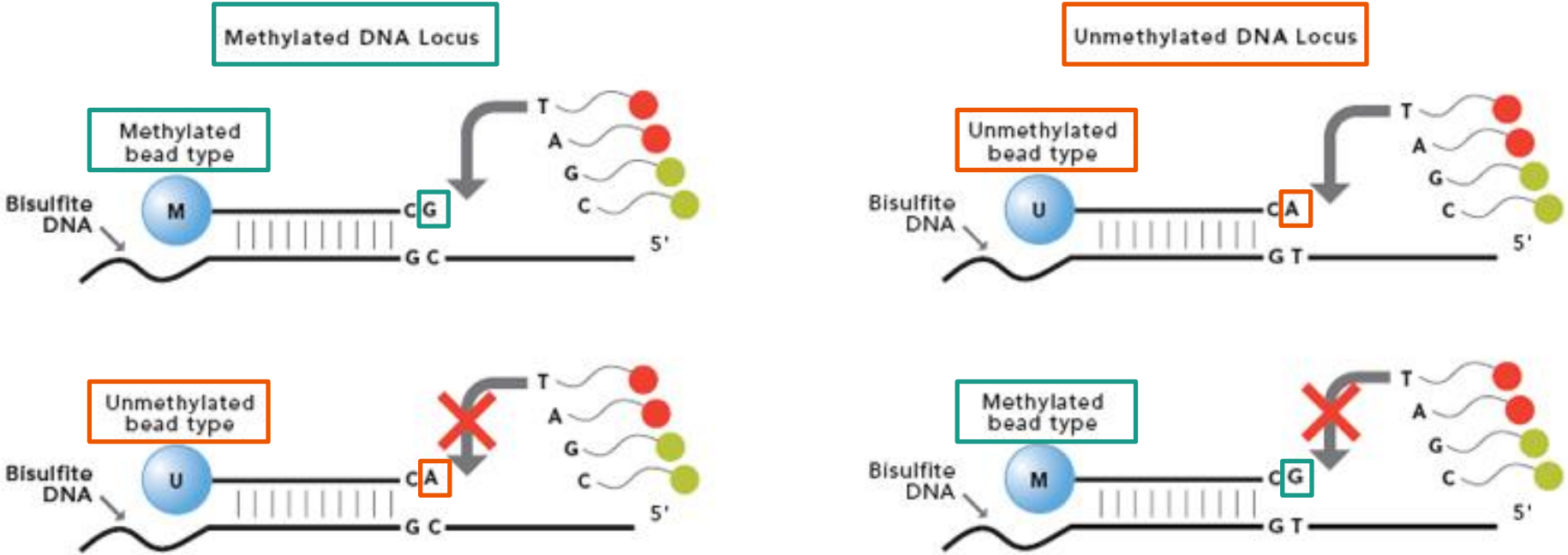
- Design probes for alternative SNPs at each position
  - Relative hybridization

- Single-nucleotide sequencing
  - Probe acts as primer
  - Match to the position up until right before the SNP
  - Incorporation of the next nucleotide determine the genotype



LaFramboise, T. NAR 37:4181-93 (2009)

Methylation array



https://www.illumina.com/science/technology/microarray/infinium-methylation-assay.html

Microarray vs DNA sequencing

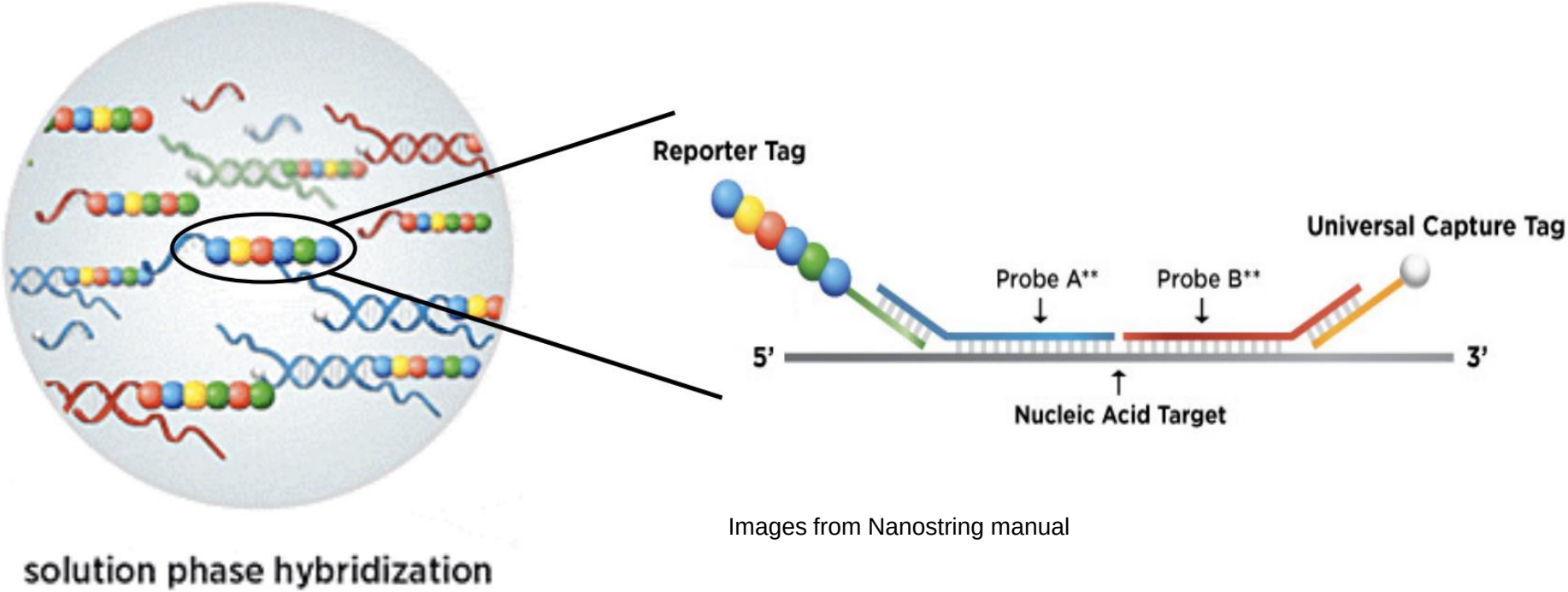
- Microarray and DNA sequencing are interchangeable in many applications
  - Genome tiling array
  - Fusion gene
  - ChIP-chip
  - Pathogen-specific probes

- Designed once for each task and can be reused

- Cheaper than DNA sequencing
  - But lack the ability to detect novel molecules

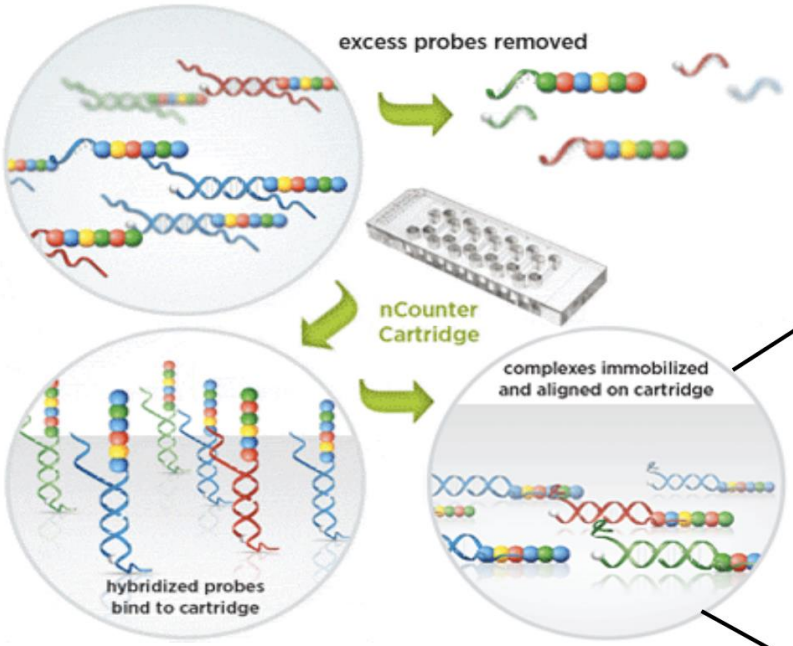
Nanostring

Transcript-specific probes & fluorescence barcodes

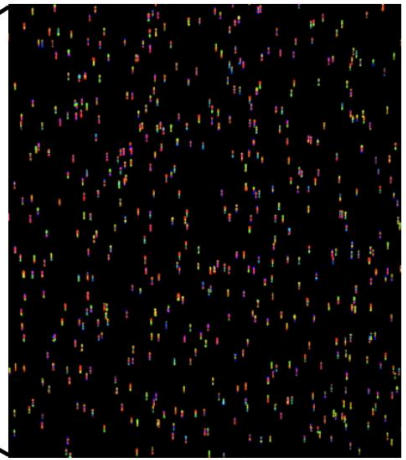


Images from Nanostring manual

Counting number of molecules



Barcode	Counts	Identity
	3	XLSA
	2	FOX5
	1	INSULIN



Images from Nanostring manual

Prebuilt barcode set (up to 800 targets)

PanCancer IO 360

Human Mouse

750 cancer-related genes involved in the complex interplay between the tumor, microenvironment and immune response including 20 internal reference controls.

Application:	Oncology
Species:	Human, Mouse
Genes in panel:	770, 770
% Match:	100%, 100%
Panel type:	Inventoried
Platform:	nCounter Analysis System

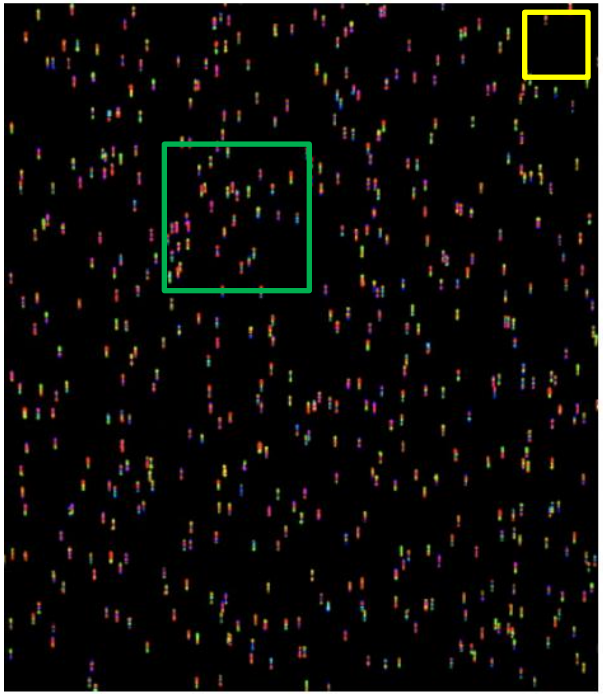
Canine IO

Canine

The nCounter® Canine IO Panel includes 780 genes covering 47 annotated pathways involved in canine immune response to IO treatments, and 20 internal reference genes for .... show more

Application:	Oncology
Species:	Canine
Genes in panel:	800
% Match:	100%
Panel type:	Inventoried
Platform:	nCounter Analysis System

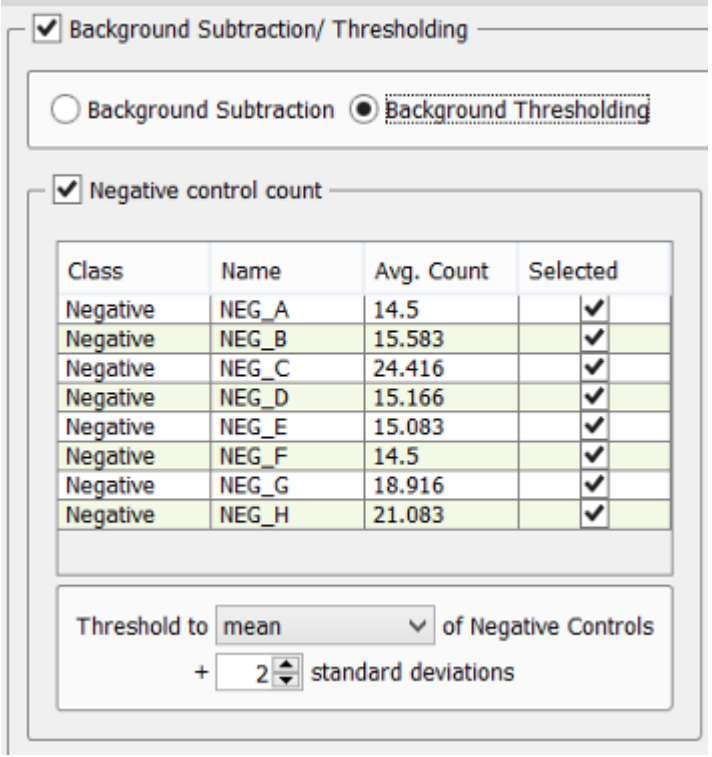
Nanostring quality control



- Imaging QC
  - % of successful imaging field of view > 75%
- Binding QC
  - 0.1-2 molecules per square micron
- Positive control
  - Six synthetic DNA ranging from 0.125-128 fM
- Negative control
  - Eight synthetic DNA that do not bind to probe

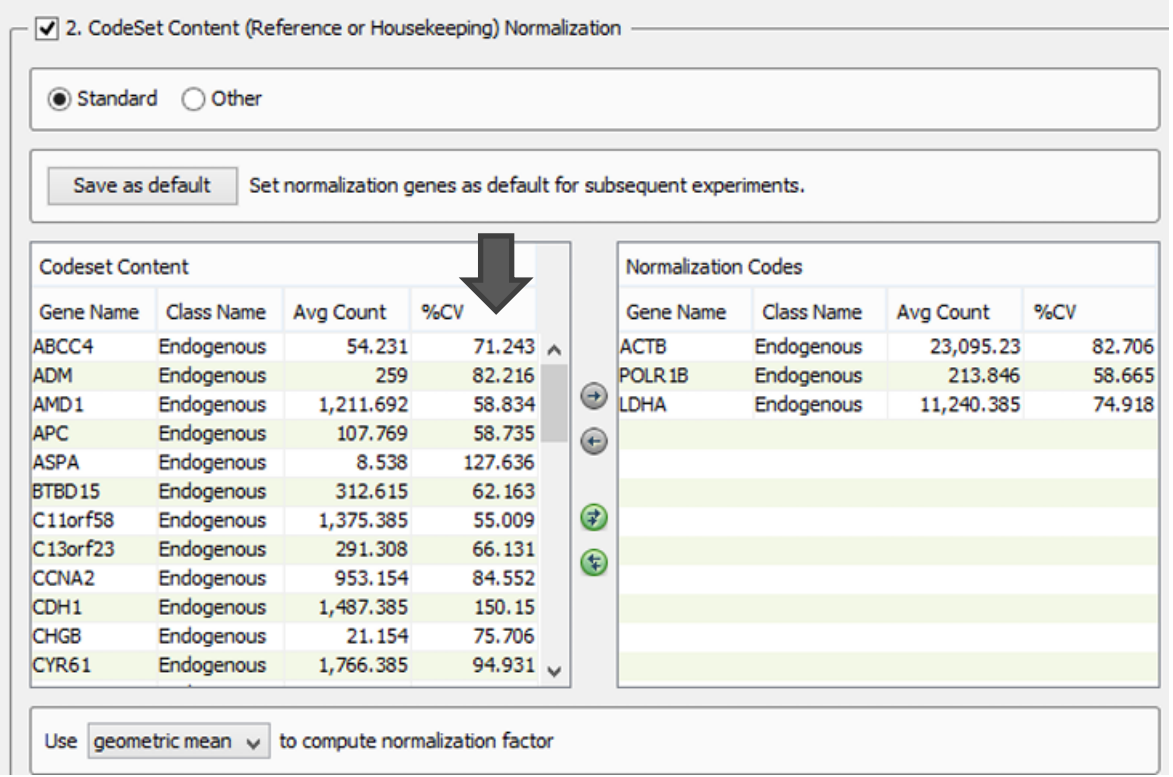
Nanostring data preprocessing

Negative and positive control

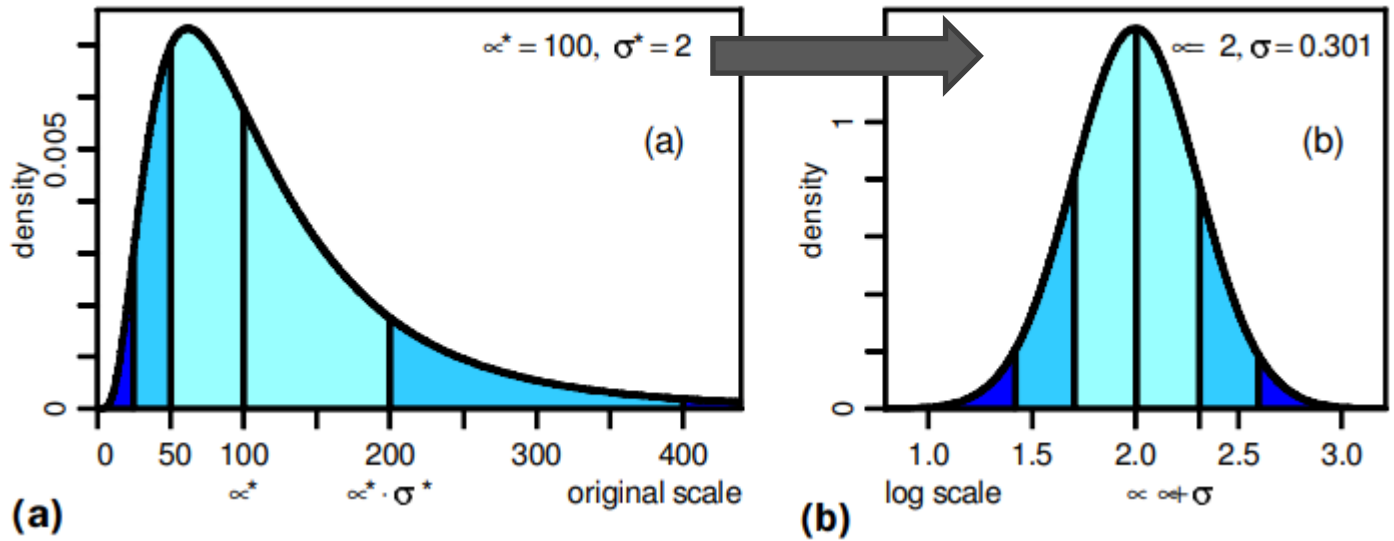


Raw Data					
			Sample 1	Sample 2	Sample 3
Positive	POS_A	ERCC_00117.1	24573	21007	21856
Positive	POS_B	ERCC_00112.1	6948	6414	6589
Positive	POS_C	ERCC_00002.1	2123	1826	1932
Positive	POS_D	ERCC_00092.1	432	363	425
Positive	POS_E	ERCC_00035.1	52	68	53
Positive	POS_F	ERCC_00034.1	49	38	52
		Geomean of POS:	858.01	783.19	829.55
		Arithmetic mean of geomeans:	823.58		
		POS control normalization factors:	0.96	1.05	0.99

Housekeeping control



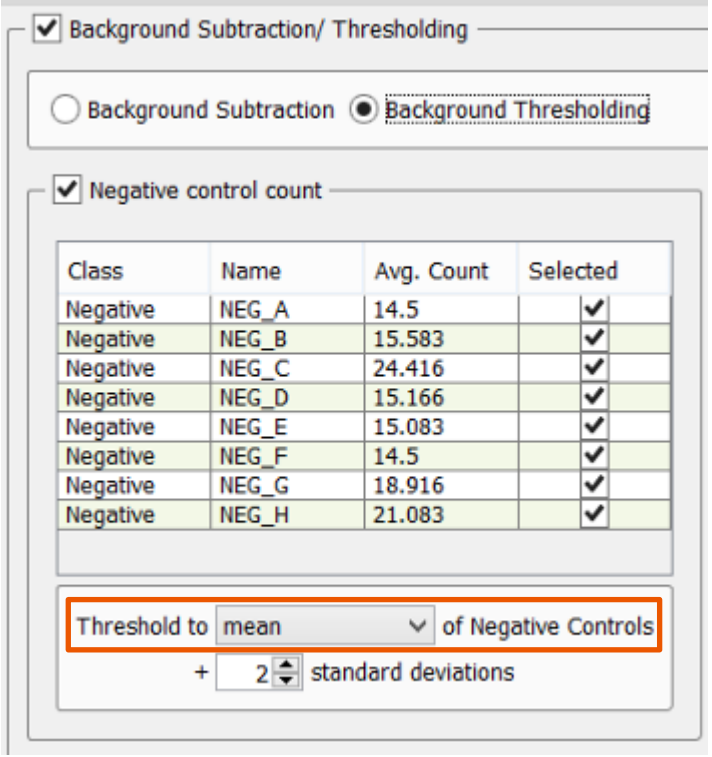
Arithmetic Mean vs Geometric Mean



Limpert, Stahel, and Abbt. BioScience 2001.

- $\frac{\log(x)+\log(y)}{2} = \log\left(\sqrt{xy}\right)$ → AM of log-transformed = GM of original data

Arithmetic mean of background noises



- Background noises are Normal
- Arithmetic Mean is ok

Geometric mean of molecule counts

Raw Data					
			Sample 1	Sample 2	Sample 3
Positive	POS_A	ERCC_00117.1	24573	21007	21856
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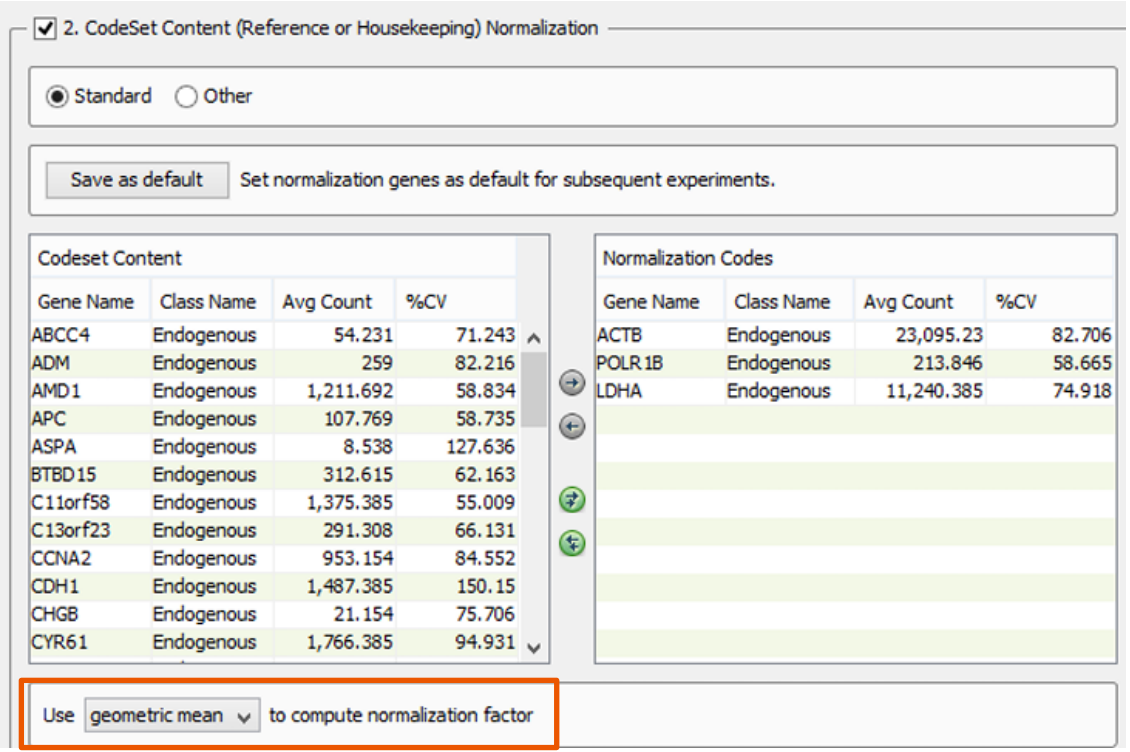
- Real expression data are closer to Log-Normal
- Use GM to represent AM of log-transformed data

Geometric mean of molecule counts



- Real expression data are closer to Log-Normal
- Use GM to represent AM of log-transformed data

Simple transcriptomics analysis

Transformed data can be analyzed with *t*-test

Control Treatment

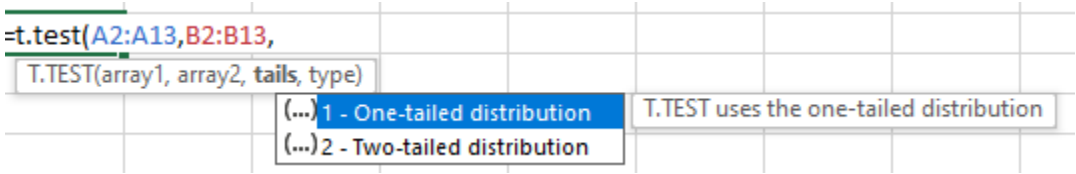
J15

	A	B	C	D	E	F	G	H
1	Acc ID	Exp1	Exp2	Exp3	Exp4	Exp5	Exp6	
2	NM_007818	67540.89	70924.09	80243.76	3501.2	5697.47	2426.72	
3	NM_001105160	811.93	801.36	740.71	128.67	104.42	101.33	
4	NM_028089	190.41	211.06	236.19	9.05	23.33	8.44	
5	NM_016696	66.77	57.56	101.09	750.9	659.84	491.89	
6	NM_013459	3.3	11.29	1.89	735.82	816.46	118.22	
7	NM_007809	45.34	36.12	51.02	245.27	372.13	335.67	
8	NM_009999	103.04	370.21	200.29	17.09	13.33	8.44	
9	NM_133960	7708.78	6976.38	6569.04	1731	1641.81	1853.55	
10	NM_027881	31.32	10.16	24.56	268.39	186.62	135.11	
11	NM_054053	31.32	24.83	19.84	323.68	428.78	116.11	
12	NM_007377	47.81	89.17	70.86	370.93	378.79	279.72	
13	NM_028064	703.95	689.62	662.29	214.11	168.85	144.61	
14	NM_008182	222.56	339.73	226.75	30.16	63.32	26.39	
15	NM_013661	12.36	11.29	8.5	97.51	77.76	71.78	
16	NM_007815	20613.09	25218.13	31540.46	5209.07	7680.3	6312.2	

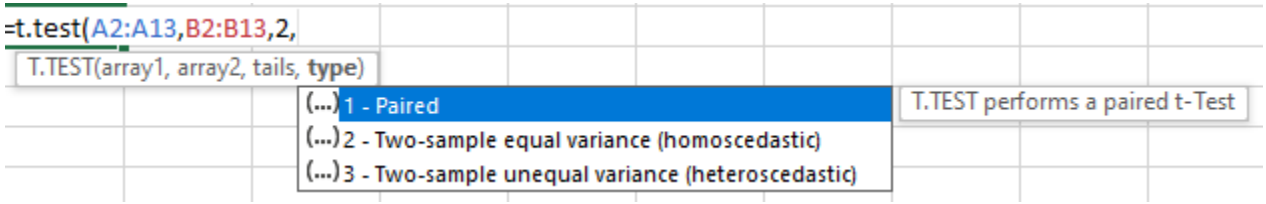
- Log transform
- Perform *t*-test on each gene
- Correct the p-values for multiple testing

http://homer.ucsd.edu/homer/basicTutorial/clustering.html

Choosing the right *t*-test



- Two-tailed tests whether the expression is higher or lower



- Use paired only for before & after treatment data of the same sample
- Otherwise, assuming unequal variance (Welch) is safer

Correction for multiple testings

- P-value cutoff of 0.05 means that under the null hypothesis, there is only 5% chance of observing the same or more extreme result

- Applying similar test 1,000 times will result in 50 tests on average with smaller p-value than 0.05 just by chance
  - Differential expression analysis tests thousands of gene at once

- This in unacceptable if a conclusion relies on multiple tests
  - Biological interpretation assumes that selected genes are truly differentially expressed

Bonferroni method

- Divide the p-value cutoff by the number of test
- Adjusted p-value cutoff = 0.05 / 1000 = 0.00005

- Applying similar test 1,000 times will result in 0.05 tests on average with smaller p-value than 0.00005 just by chance

- Easy to calculate but lose power

False discovery rate (FDR)

- P-value operates under the null hypothesis
- But in practice, we want to control the number of errors in the output
  - The number of DEGs that were incorrectly proposed

- False Discovery Rate (FDR) = Probability of getting a false positive
  - Probability that a DEG is not truly differentially expressed
- But FDR involves alternative hypothesis

- There are ways to control FDR through p-value!

Benjamini-Hochberg procedure

- Valid under broad assumptions (independent tests, positively correlated tests, etc.)

- Given a series of tests with p-values, $p_1, p_2, \ldots, p_n$

- To control FDR to be within 0.05
  - Sort p-values from low to high, $p'_1, p'_2, \ldots, p'_n$
  - Find largest k such that $p'_k \leq 0.05 \times k / n$
    - For the smallest p-value, this is equivalent to Bonferroni
    - For other p-values, this technique gradually loosens the cutoff
  - Reject null hypothesis for tests corresponding to $p'_1, p'_2, \ldots, p'_k$

Benjamini-Yekutieli procedure

- Valid under broader assumption (some dependence between tests)

- Given a series of tests with p-values, $p_1, p_2, \ldots, p_n$

- To control FDR to be within 0.05
  - Sort p-values from low to high, $p'_1, p'_2, \ldots, p'_n$
  - Find largest k such that $p'_k \leq (0.05 \times k) / (n \times c(k))$
    - $c(k) = \sum_{i=1}^{k} \frac{1}{i}$
    - For the smallest p-value, this is equivalent to Benjamini-Hochberg & Bonferroni
    - For other p-values, this technique gradually loosens the cutoff – but not as much as Benjamini-Hochberg
  - Reject null hypothesis for tests corresponding to $p'_1, p'_2, \ldots, p'_k$

Correction method comparison

P-value	Bonferroni	B-H	B-Y
Smallest	0.0005	0.0005	0.0005
2nd smallest	0.0005	0.001	0.000667
3rd smallest	0.0005	0.0015	0.000818
4th smallest	0.0005	0.002	0.00096
5th smallest	0.0005	0.0025	0.001095

- There are 100 tests
- Target p-value or FDR cutoff = 0.05

Effect of correction

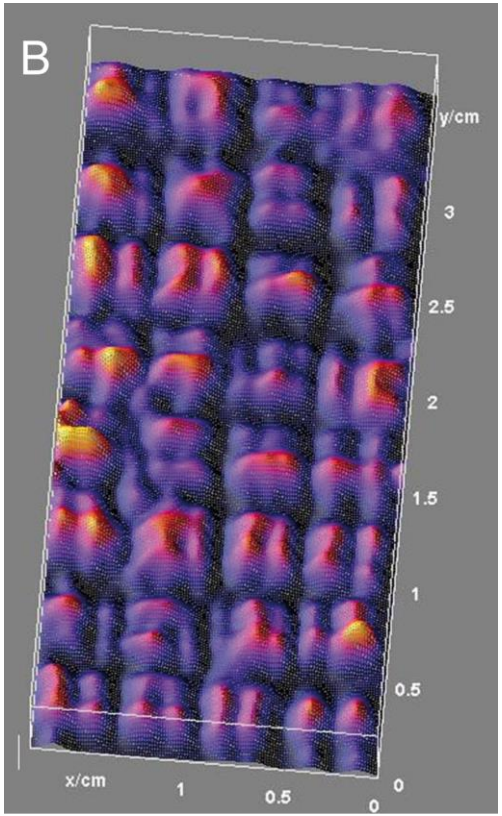
Gene	Sorted p-value	Rank	Benjamini-Hochberg	Result	c(rank)	Benjamini-Yekutieli	Result
Gene M	0.0000001	1	0.0005	Pass	1	0.0005	Pass
Gene S	0.0000035	2	0.001	Pass	1.5	0.00067	Pass
Gene A	0.00028	3	0.0015	Pass	1.83	0.00082	Pass
Gene C	0.0011	4	0.002	Pass	2.08	0.00096	Fail
Gene P	0.06	5	0.0025	Fail	2.28	0.0011	

Linear effect model

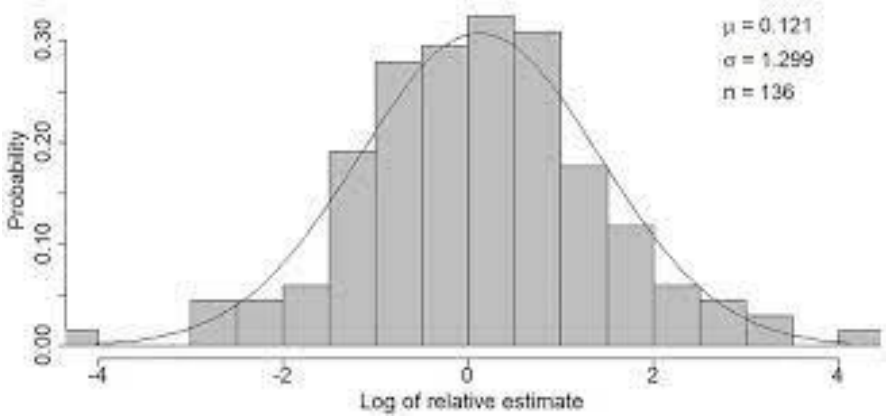
Background noise correction models

- Null hypothesis
  - Background noise is normally distributed and is the same over the entire array

- Linear effect model
  - Background noise is normally distributed with mean depending on (x, y) positions and a fixed variance



Fitting normally distributed data



- Negative control probe intensities: $n_1, n_2, \ldots, n_k$
  - Fitted mean and variance: $\mu = \frac{\sum_i n_i}{k}$ and $\sigma^2 = \frac{1}{k-1}\sum_i (n_i - \mu)^2$
  - Likelihood: $\prod_i P(n_i | \mu, \sigma^2) = \left(\frac{1}{\sigma\sqrt{2\pi}}\right)^k e^{-\frac{1}{2}\sum_i \left(\frac{n_i - \mu}{\sigma}\right)^2}$

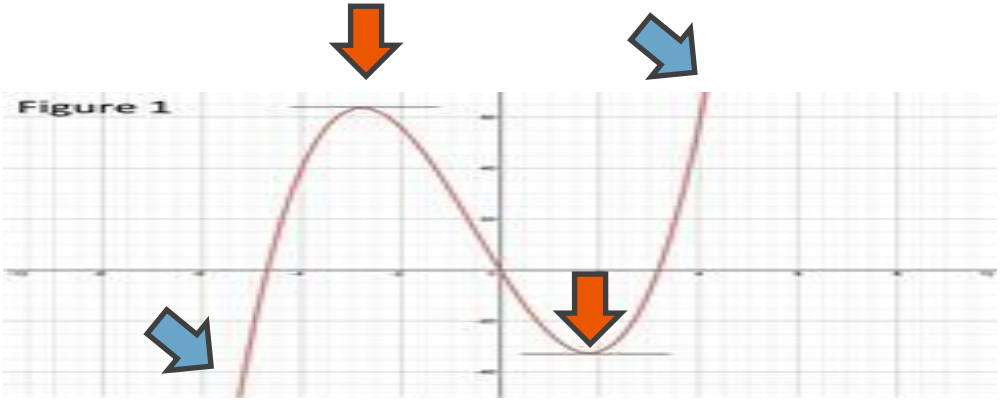
Linear effect model

- Position of probe *i* with intensity n_i is (x_i, y_i)

- Fitted mean: $\mu(x_i, y_i) = ax_i + by_i + c$
  - Solve for a, b, c that minimize MSE: $\sum_i \left(n_i - (ax_i + by_i + c)\right)^2$

- Recall calculus:
  - $\frac{\delta MSE}{\delta a} = \sum_i 2\left(n_i - (ax_i + by_i + c)\right)(-x_i)$
  - $\frac{\delta MSE}{\delta b} = \sum_i 2\left(n_i - (ax_i + by_i + c)\right)(-y_i)$
  - $\frac{\delta MSE}{\delta c} = \sum_i 2\left(n_i - (ax_i + by_i + c)\right)(-1)$

Finding optimal solution by setting derivative to zero



- Boundary: a, b, or c approaches infinity
- Local optima: setting partial derivatives to zero
  - $0 = \sum_i 2\big(n_i - (ax_i + by_i + c)\big)(-x_i)$
  - $0 = \sum_i 2\big(n_i - (ax_i + by_i + c)\big)(-y_i)$
  - $0 = \sum_i 2\big(n_i - (ax_i + by_i + c)\big)(-1)$

Some algebra exercises

- Setting partial derivatives to zero
  - $0 = \sum_i \big(n_i - (ax_i + by_i + c)\big)(-x_i)$
  - $0 = \sum_i \big(n_i - (ax_i + by_i + c)\big)(-y_i)$
  - $0 = \sum_i \big(n_i - (ax_i + by_i + c)\big)$

- Or equivalently
  - $a \sum_i x_i^2 + b \sum_i x_i y_i + c \sum_i x_i = \sum_i n_i x_i$
  - $a \sum_i x_i y_i + b \sum_i y_i^2 + c \sum_i y_i = \sum_i n_i y_i$
  - $a \sum_i x_i + b \sum_i y_i + ck = \sum_i n_i$

- Three linear equations with three variables ☺

Hypothesis testing

- Fitted mean: $\mu(x_i, y_i) = ax_i + by_i + c$
  - Solve for a, b, c that minimize MSE: $\sum_i \left(n_i - (ax_i + by_i + c)\right)^2$
- Fitted variance: $\sigma^2 = \frac{1}{k-1} \sum_i (n_i - \mu(x_i, y_i))^2$
- Likelihood: $\prod_i P(n_i | \mu(x_i, y_i), \sigma^2) = \left(\frac{1}{\sigma\sqrt{2\pi}}\right)^k e^{-\frac{1}{2}\sum_i \left(\frac{n_i - \mu(x_i, y_i)}{\sigma}\right)^2}$
  - Compare to likelihood from Null hypothesis
  - Likelihood ratio test or nested model testing (degree of freedom = 2)

Incorporating confounding variable

- Design matrix

Sample	Condition	Batch	Patient's Age
S1	Control	1	35
S2	Control	2	21
S3	Control	3	45
S4	Treatment	1	18
S5	Treatment	2	37
S6	Treatment	3	52

Any question?

- See you next week on September 13rd 9-10:30am

- Preparation for upcoming classes:
  - Install R and RStudio
  - Install Python
  - Specific version info and instructions will be posted on Teams