Measuring Gene Expression Part 2

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Bioinformatics 301

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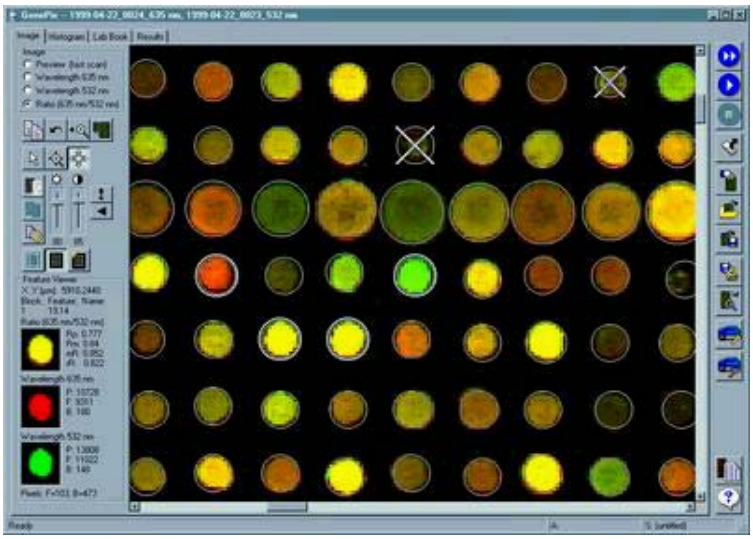
Measuring Gene Expression

- **Differential Display**
- **Serial Analysis of Gene Expression (SAGE)**
- **RT-PCR (real-time PCR)**
- **Northern/Southern Blotting**
- **DNA Microarrays or Gene Chips**

Microarrays



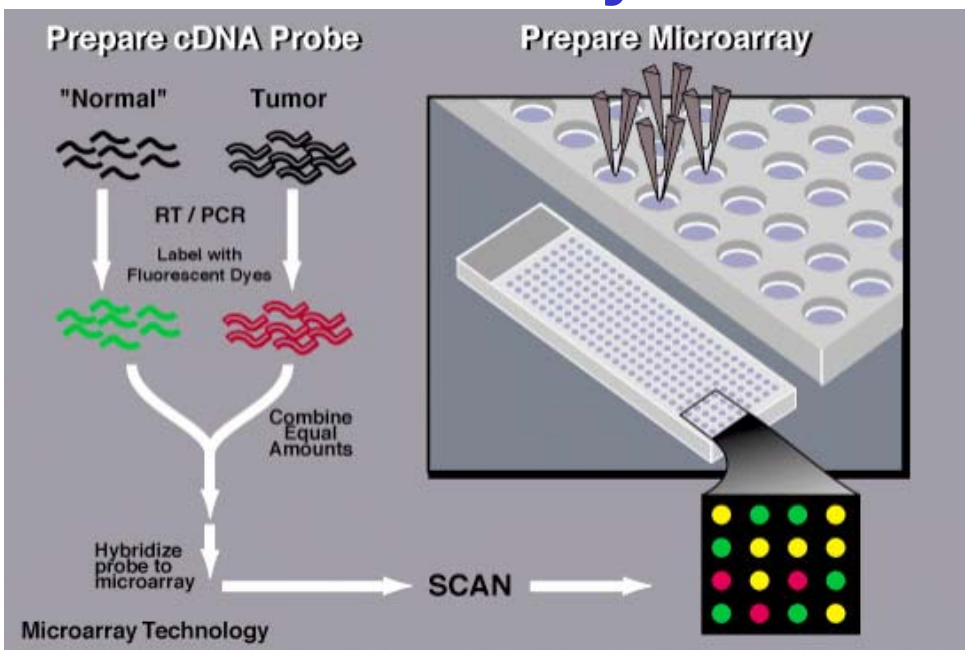
DNA Microarrays

- Principle is to analyze gene (mRNA) or protein expression through large scale non-radioactive Northern (RNA) or Southern (DNA) hybridization analysis
- Essentially high throughput Northern Blotting method that uses Cy3 and Cy5 fluorescence for detection
- Allows expressional analysis of up to 20,000 genes simultaneously

Four Types of Microarrays

- **Photolithographically prepared short oligo (20-25 bp) arrays (1 colour)**
- **Spotted glass slide cDNA (500-1000 bp) arrays (2 colour)**
- **Spotted nylon cDNA (500-1000 bp) arrays (1 colour/radioactive)**
- **Spotted glass slide oligo (30-70 bp) arrays (1 or 2 colour)**

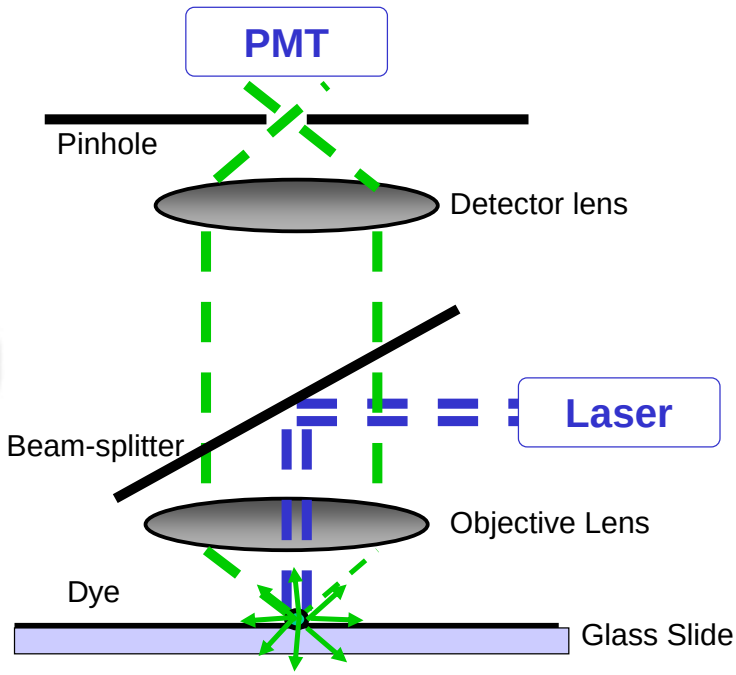
Principles of 2 Colour Microarrays



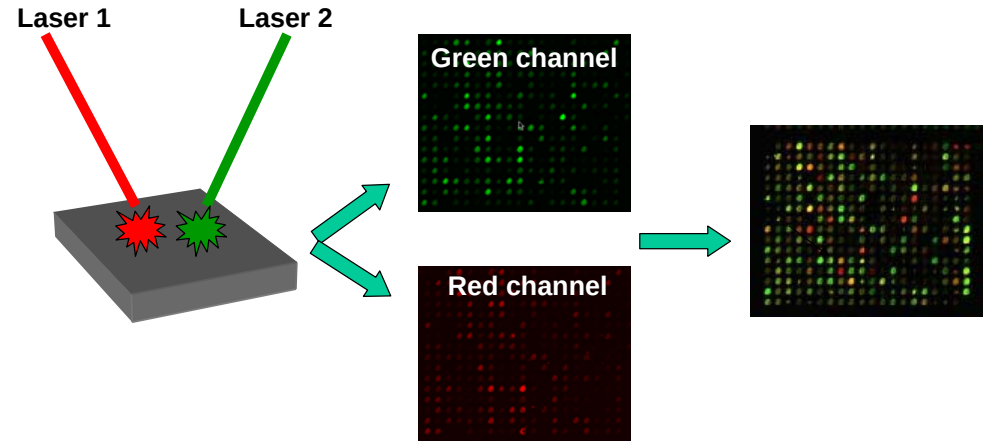
Microarray Definition of Probe and Target

- There are two acceptable and completely opposite definitions. We will use:
- Target = the DNA that is spotted on the array
- Probe = the DNA that is labeled with the fluorescent probe

Microarray Scanning



2-Colour Microarray Principles

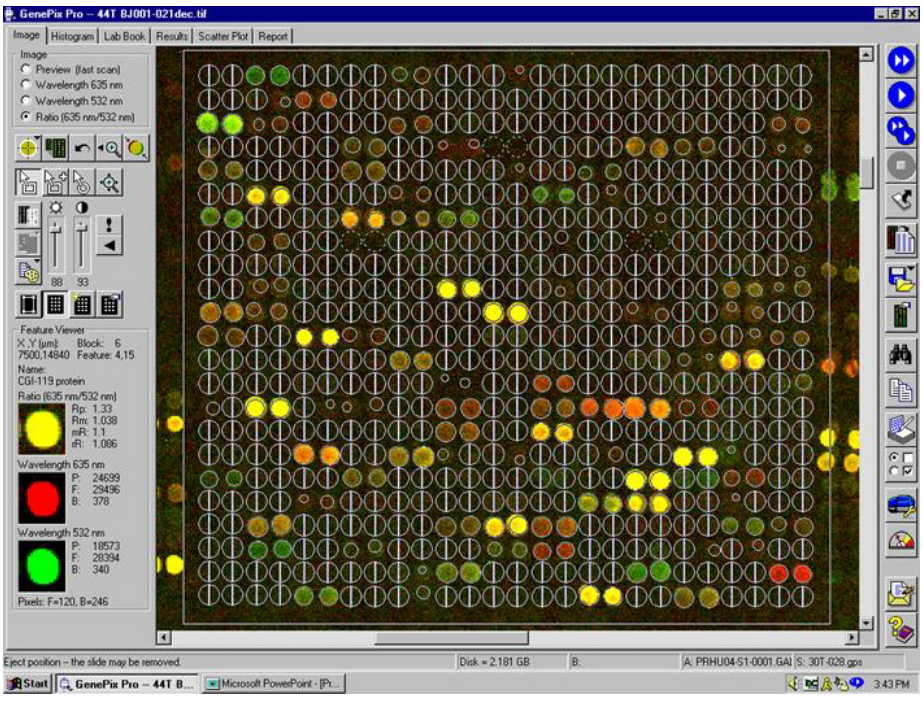


Scan and detect with confocal laser system

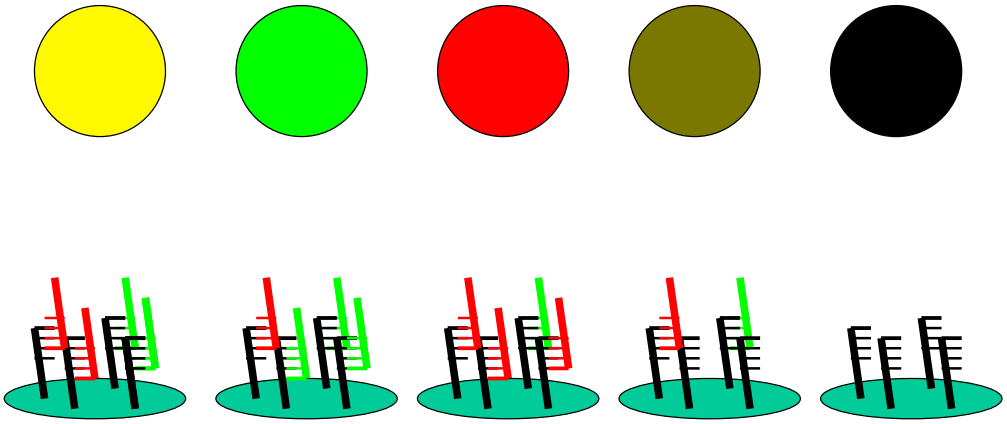
overlay images and normalize

Image process and analyze

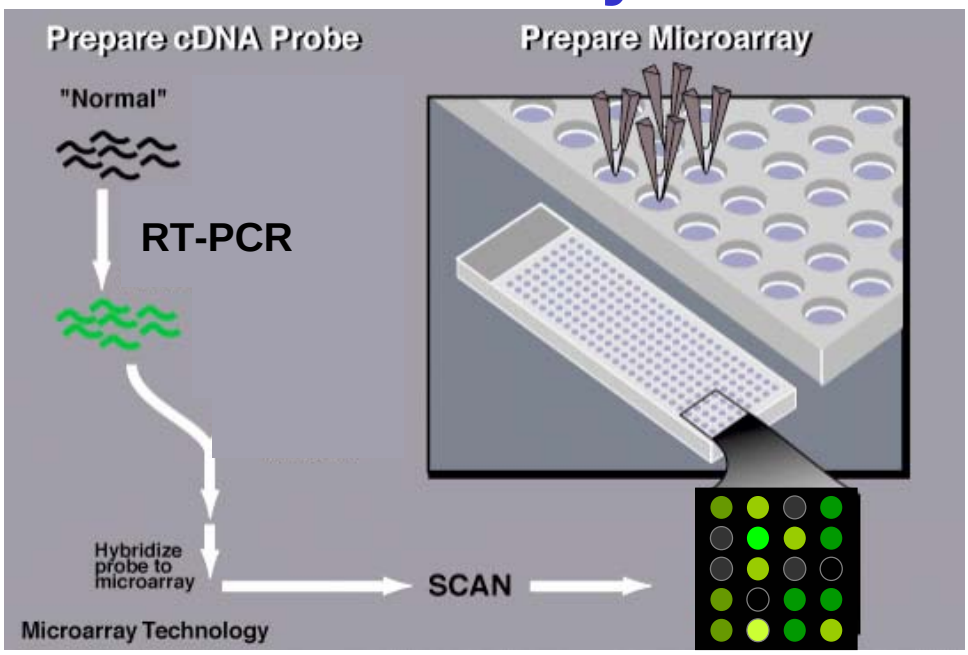
Typical 2-Colour Data



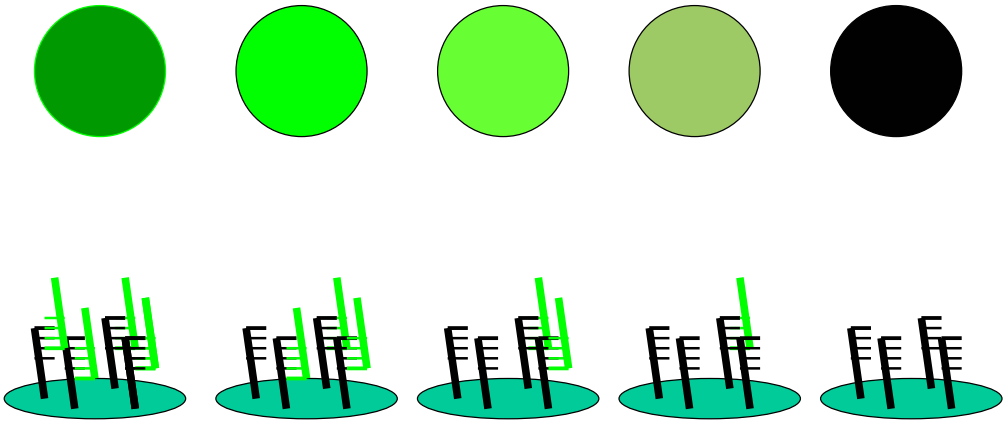
Microarrays & Spot Colour



Principles of 1 Colour Microarrays



Microarrays & Spot Colour



Two Colour vs. One Colour

- **Two-colour hybridization eliminates artifacts due to variation in:**
  - **quantity of DNA spotted**
  - **stringency of hybridization**
  - **local concentration of label**
- **However,**
  - **both samples *must* label with equivalent efficiency**
  - **Information is lost for genes not expressed in the reference or control sample**

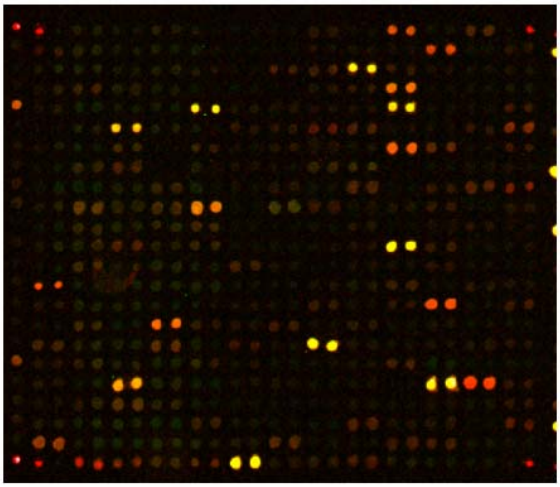
Two Colour vs. One Colour

- One-colour hybridization may have artifacts due to variation in:
  - quantity of DNA spotted
  - stringency of hybridization
  - local concentration of label
- However good quality control (QC) means,
  - fewer artifacts
  - less manipulation, lower cost
  - reduced loss of information (due to reference sample transcript content)

Specific Arrays of Interest

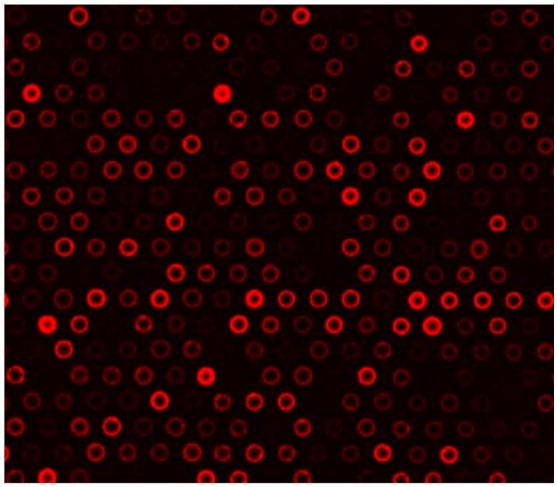
- Home-made Spotted Oligo Arrays
  - Made using glass slides, Operon oligos and robotic spotting equipment
- Amersham CodeLink Microarrays
  - Made using specially treated slides, QC'd oligos and robotic spotting equipment
- Affymetrix Gene Chips
  - Made using photolithographically produced systems with multi-copy oligos

Array Images



Oligo Microarray

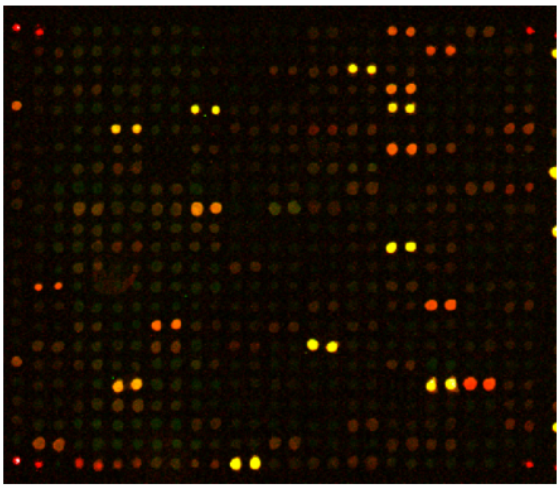
2 colour



Amersham

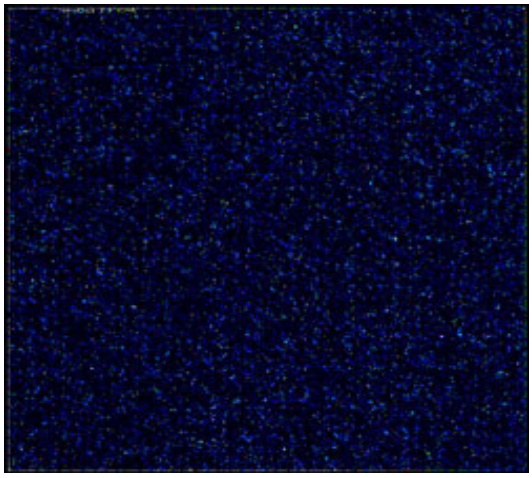
1 colour

Array Images



Oligo Microarray

2 colour



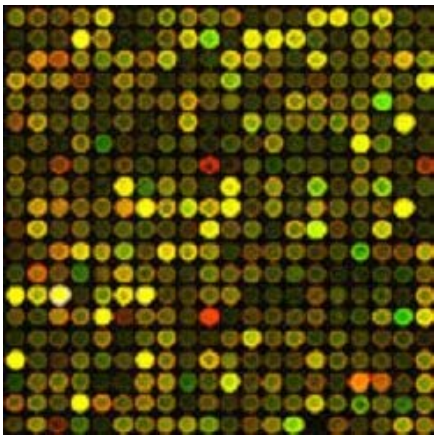
Affymetrix Gene Chip

1 colour

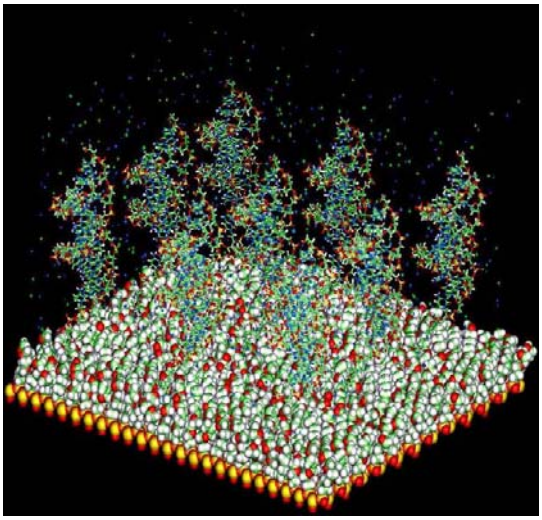
Home-made Spotted Arrays

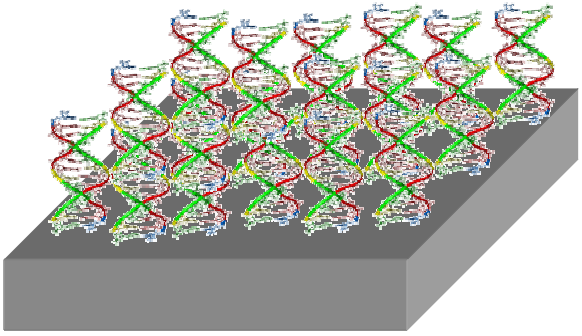
Spotted Microarrays

- **Probes are >100μm and are usually deposited on glass**
- **Probes can be:**
  - **oligos (usually >40mers)**
  - **PCR fragments from cDNA/EST or genomic templates**
- **Not reused; 2-colour hybridizations**

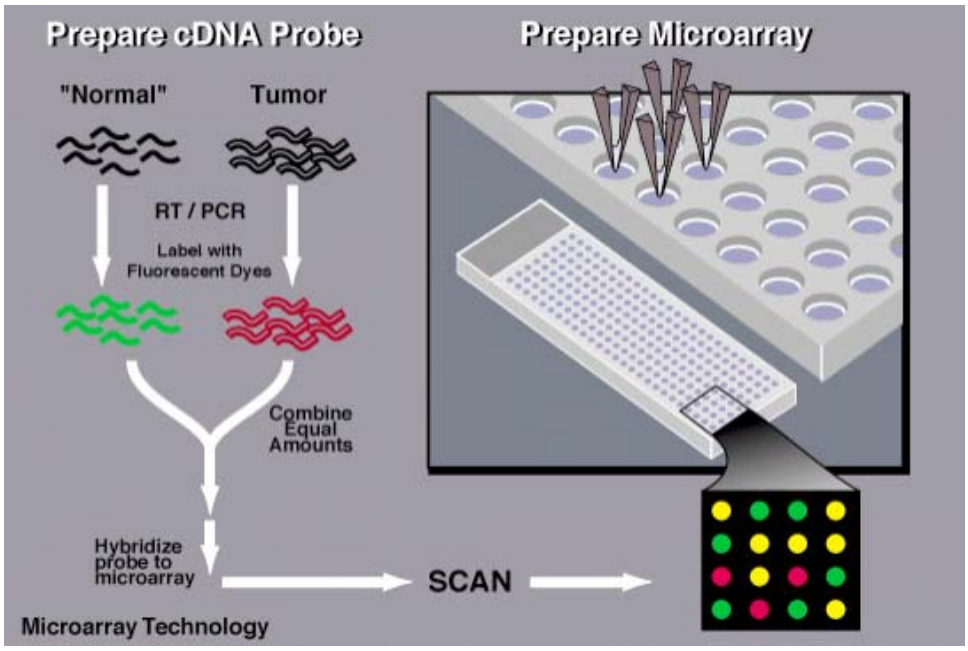


Standard Spotted Array

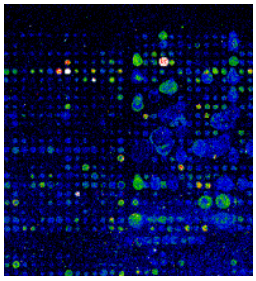




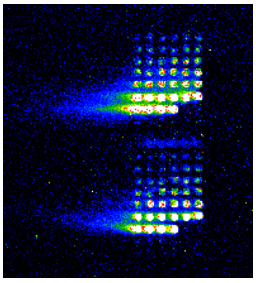
Home-made Microarrays



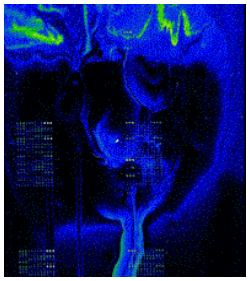
Common Home-made Microarray Errors



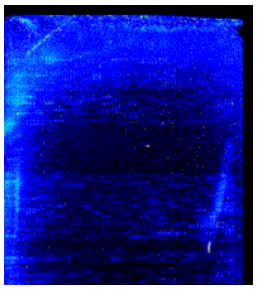
Irregular Spot



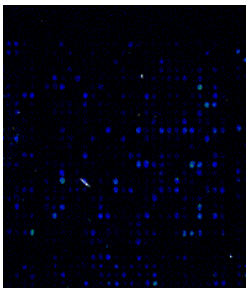
Comet Tail



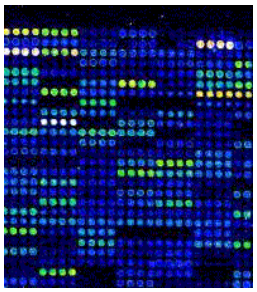
Streaking



Hi Background



Low Intensity

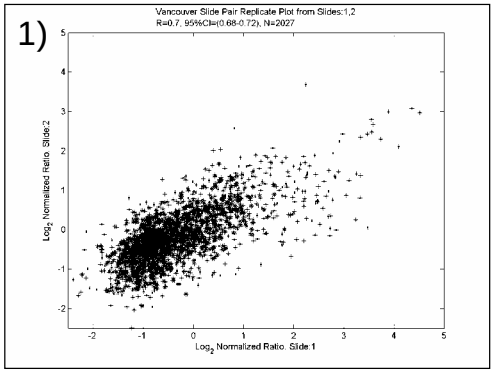


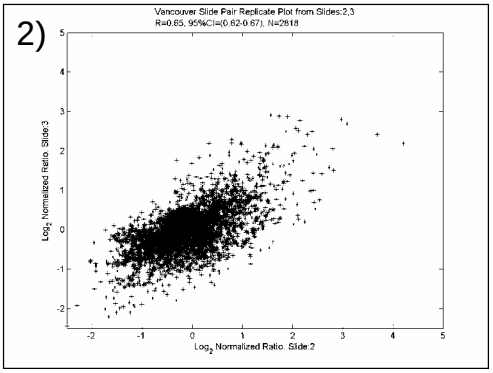
A Good Array

Testing Reproducibility

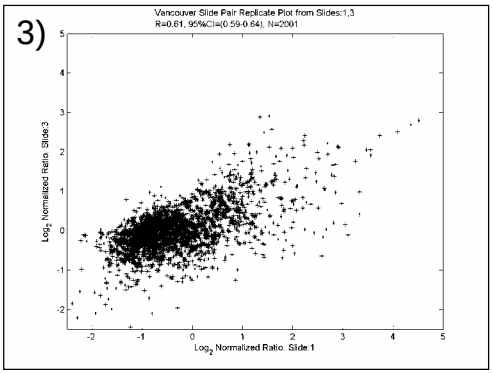
- Breast tumor tissue biopsy
- mRNA prepared using standard methods
- Control sample made from pooled mRNA from several cell types
- 3 RNA samples prepared from 1 tissue source – arrayed onto two sets of home-made chips from different suppliers
- Conducted pairwise comparison of intensity correlations & no. of spots

Home-made Arrays



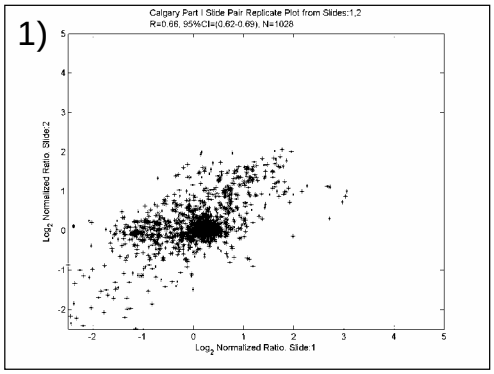


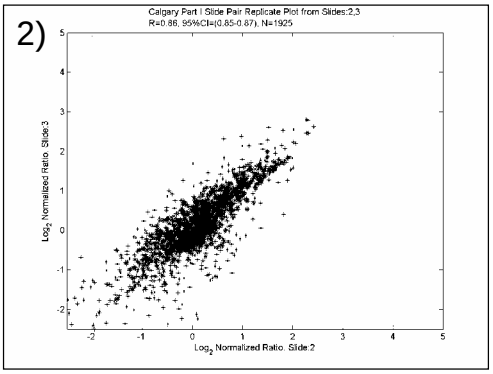
Oligo Microarray 1



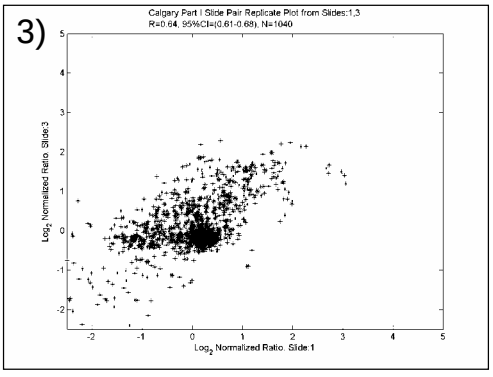
1)	R=0.7	95%CI=(0.68-0.72)	N=2027
2)	R=0.65	95%CI=(0.62-0.67)	N=2818
3)	R=0.61	95%CI=(0.59-0.64)	N=2001

Home-made Arrays





Oligo Microarray 2



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

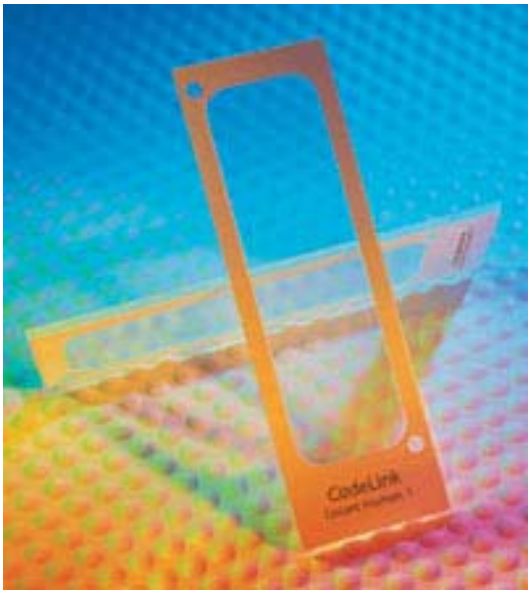
Advantages to Home-made Systems

- Cheapest method to produce arrays ($100 to $300/slide)
- Allows lab full control over design and printing of arrays (customizable)
- Allows quick adaptation to new technologies, new probe sets
- Allows more control over analysis

Disadvantages to Home-made Systems

- Quality and quality-control of oligo probe set is highly variable
- Quality of spotting and spot geometry is highly variable
- Technology is very advanced, difficult and expensive to maintain (robotics)
- Reproducibility is poor

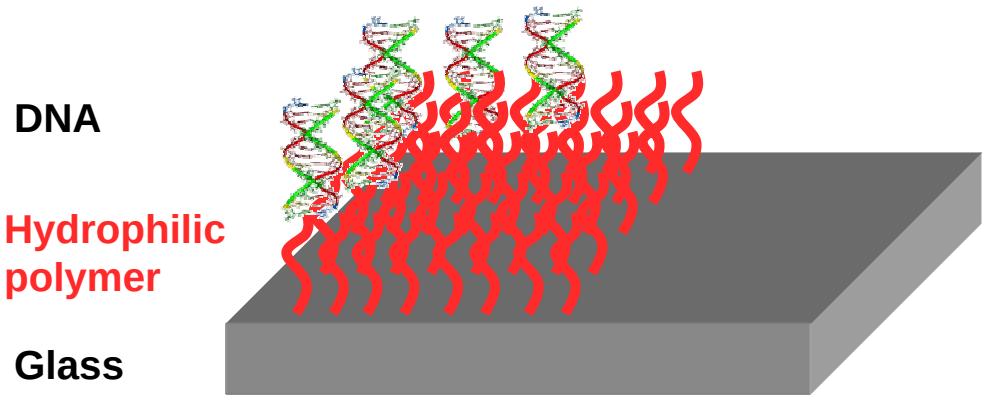
Amersham CodeLink Arrays



Amersham CodeLink Arrays

- **Amersham synthesizes its 30-nucleotide oligos offline, tests them by mass spectrometry, deposits them on specially coated array, and then assays them for quality control**
- **Uses a special Flex Chamber™—a disposable hybridization chamber already attached to the slide to improve hybridization consistency**

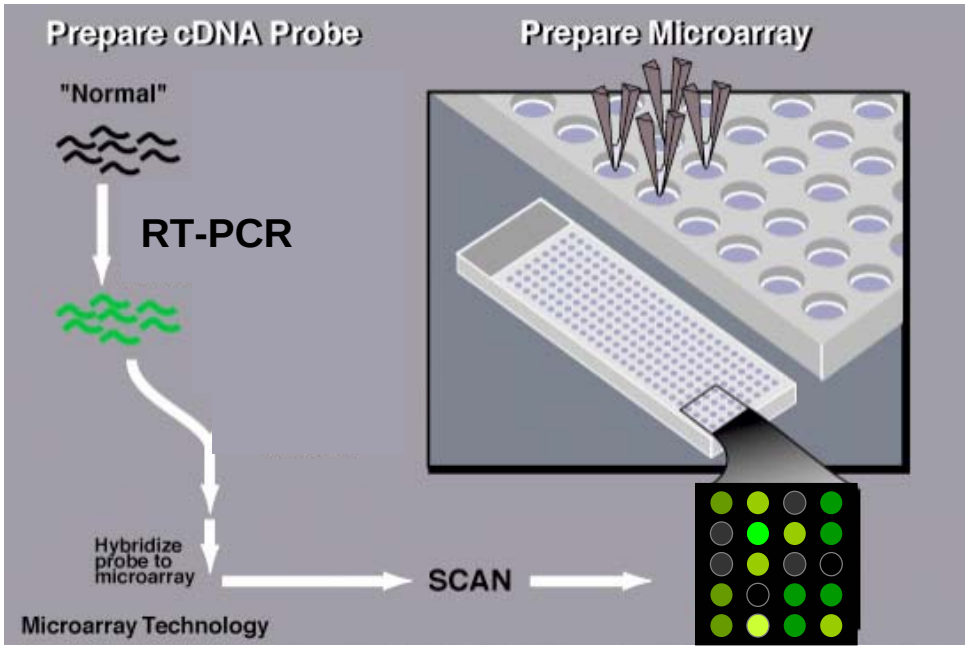
Amersham CodeLink Oligo Chip



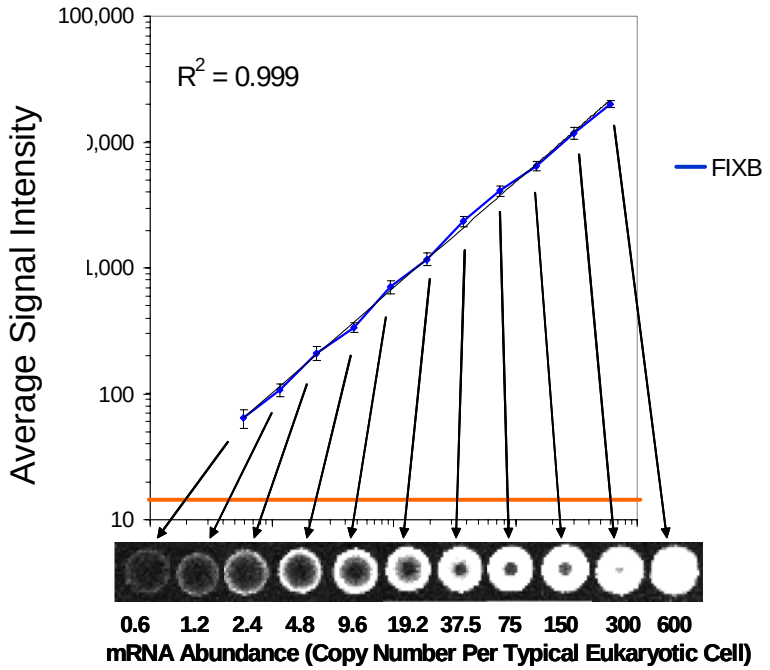
CodeLink Special Coating

- Most glass substrates are quite hydrophobic
- This hydrophobicity affects the local binding and surface chemistry of most glass-slide chips making most of the attached DNA oligo inaccessible
- Coating the slide with a hydrophilic polymer allows the cDNA to pair up with the substrate oligos much better

Amersham Microarrays



Morphology Does Not Affect Dynamic Range
CodeLink Bioarrays Can Achieve Linearity Across 3 Logs*



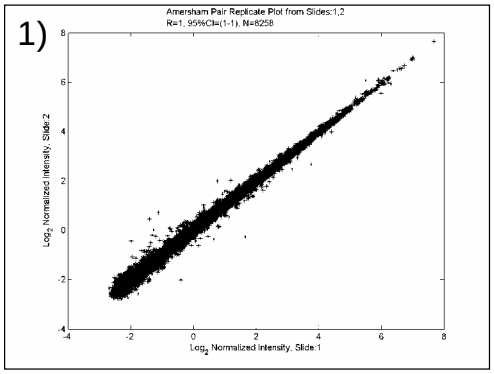
- The red line indicates the signal level for non-spiked target.
- Error bars represent one standard deviation for each mean (n=18) signal

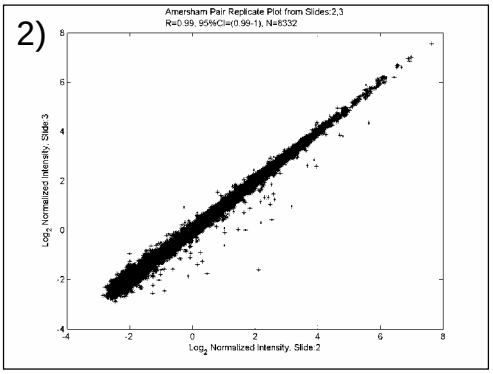
*Data obtained from cRNA dilution series.

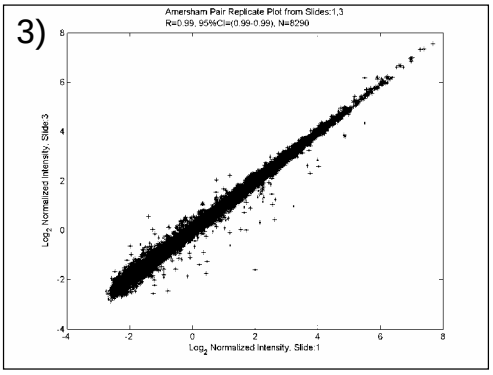
Testing Reproducibility

- **Breast tumor tissue biopsy**
- **mRNA prepared using standard methods**
- **3 RNA samples prepared from 1 tissue source – arrayed onto 3 different sets of CodeLink chips**
- **Conducted pairwise comparison of intensity correlations, intensity ratio correlations & number of "passed" spots**

Intensity, Pairwise Comparisons



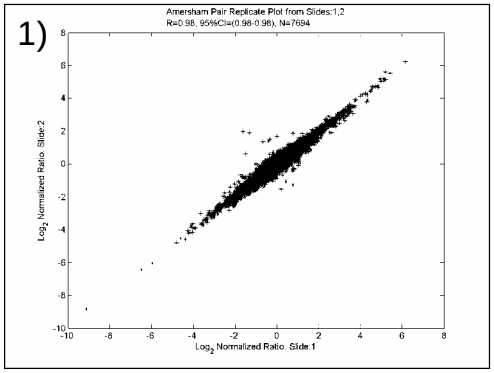


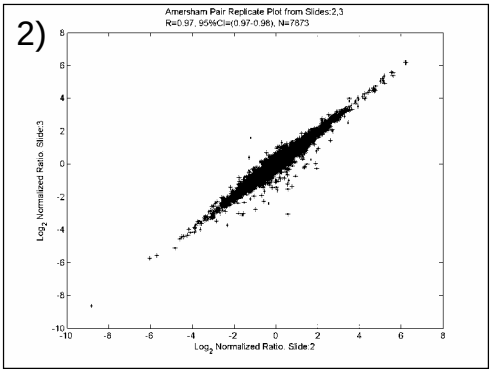


Amersham Slides

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

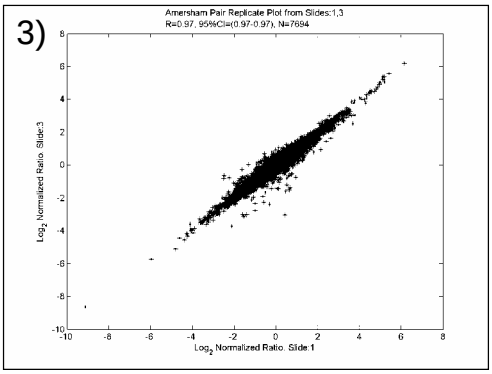
Ratio, Pairwise Comparisons



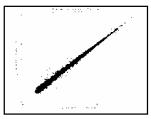
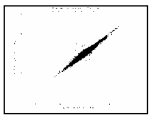
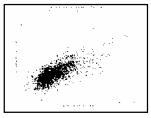
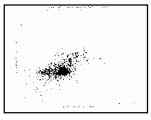
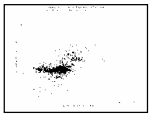


Amersham Slides

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



General Comparison

Amersham Intensity	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	
Amersham Ratio	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	
Vancouver	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	
Calgary I	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	
Calgary II	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	

Comparative Accuracy

	RT-PCR	Spotted Array	CodeLink
GENES	Expression Pattern TaqMan	Expression Pattern Operon	Expression Pattern Amersham
hENT1	+	-	+
hENT2	+	-	+
hCNT1	-	-	-
hCNT2	-	+	-
dck	+	-	+
ER	+	-	+

CodeLink Advantages

- **Exceptional reproducibility because of:**
  - **careful probe design**
  - **QC of oligo preparations and spotting**
  - **high proportion of oligo binding to cDNA substrate due to hydrophilic coating**
  - **well controlled/uniform hybridization**
- **Allows users to continue using same scanners/software as in spotted arrays**

CodeLink Disadvantages

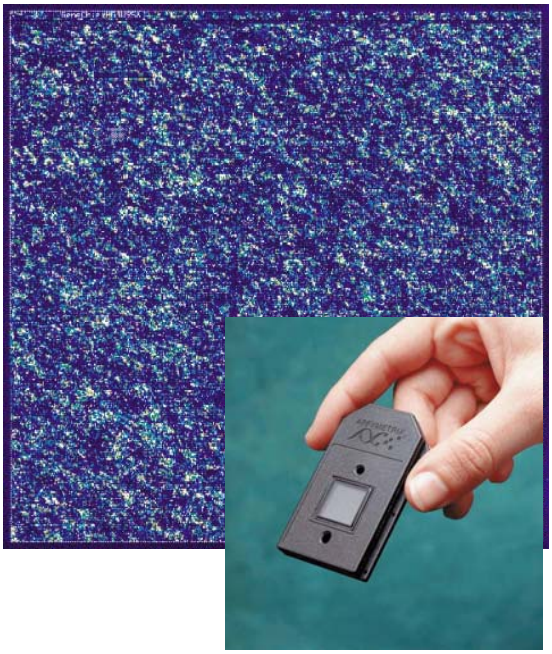
- Lack of flexibility or customizability (users depend on Amersham to provide & design chips)
- Dependent on proprietary kits and reagents
- More expensive than spotted arrays ($700/chip)

Cost per Sample in Triplicate

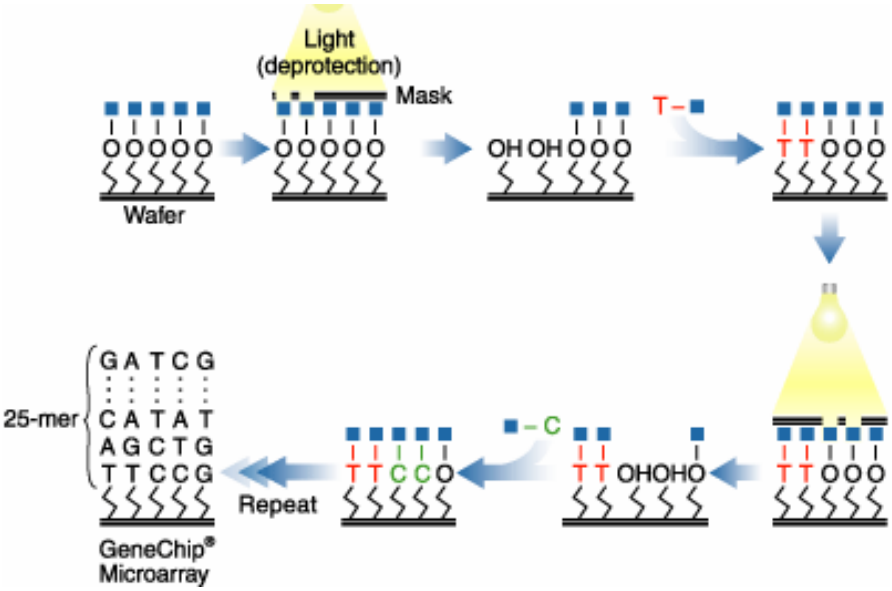
- Amersham Slides (single channel)
  - $2000
- Vancouver Spotted Arrays (two colour)
  - $800
- Calgary Spotted Arrays (two colour)
  - $1100

Affymetrix Gene Chips

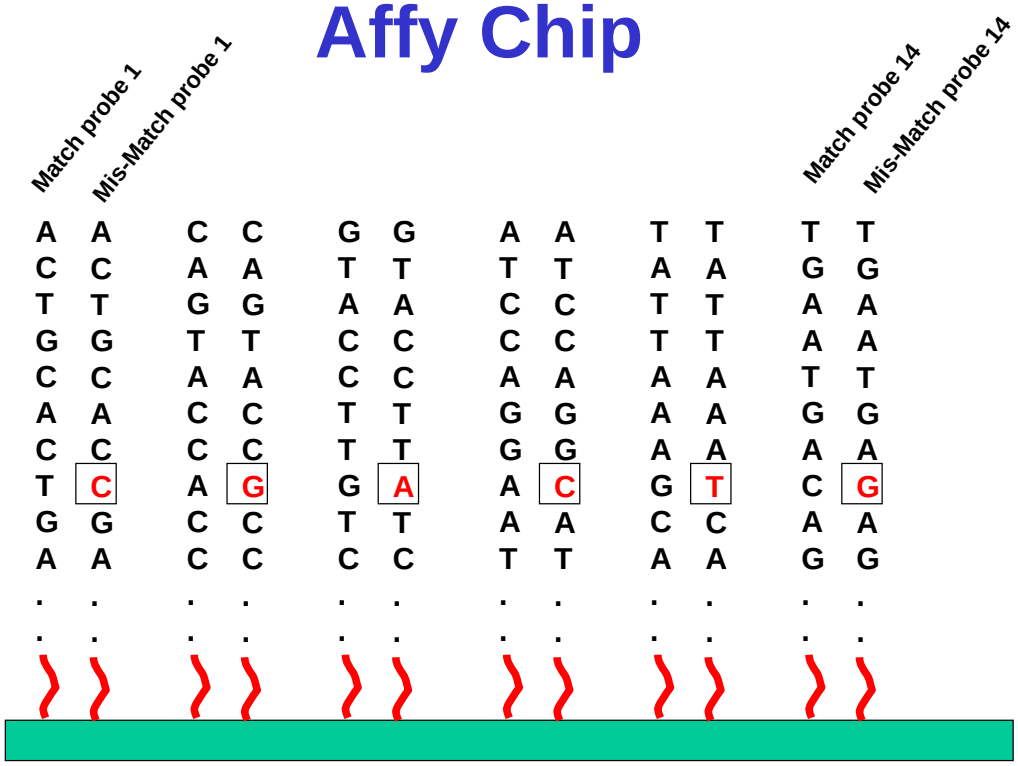
- Chips are 1.7 cm^2
- 400,000 oligo probe pairs
- Probe “spots” are 20μ x 20μ
- Each probe is 25 bases long
- 11-20 “match” probes and 11-20 “mismatch” probes per gene



Affymetrix Gene Chip



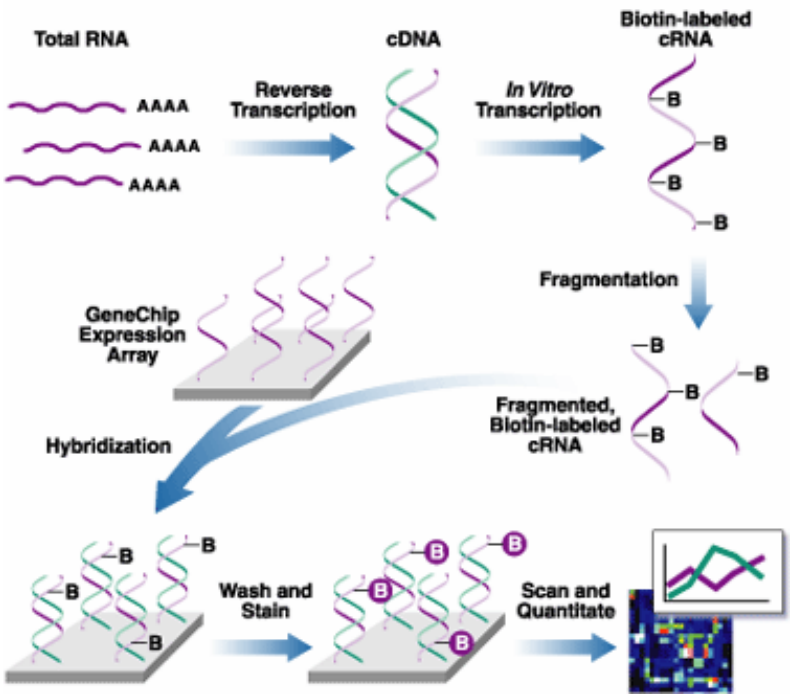
Affy Chip



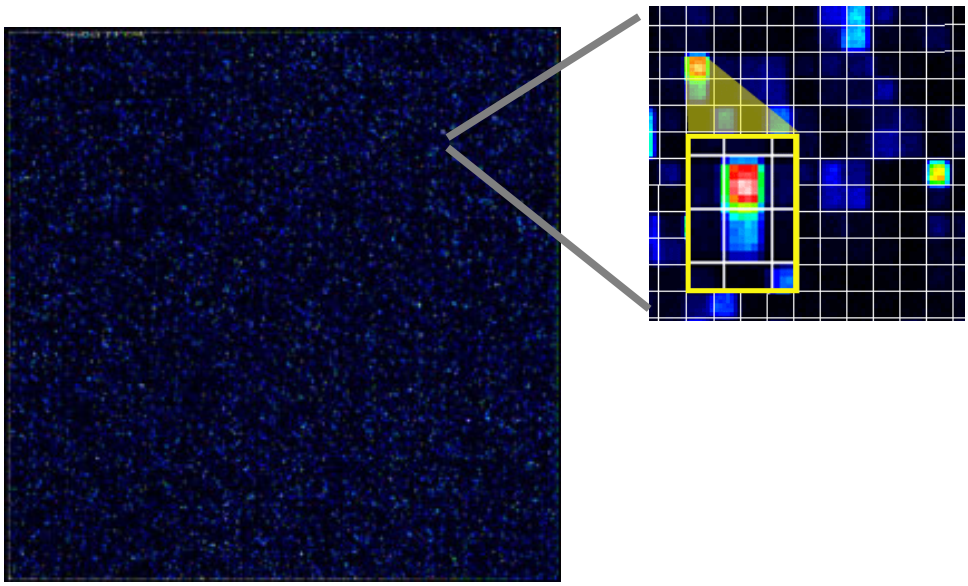
Affy Chip

- 11-20 probes for each gene/EST
- Each probe is 25 bases long
- 1 has exact match, the other is mismatched in the middle base
- Match (M) and mismatch (MM) pairs are placed next to each other
- Expression levels calculated using intensity difference between M & MM for all probe pairs

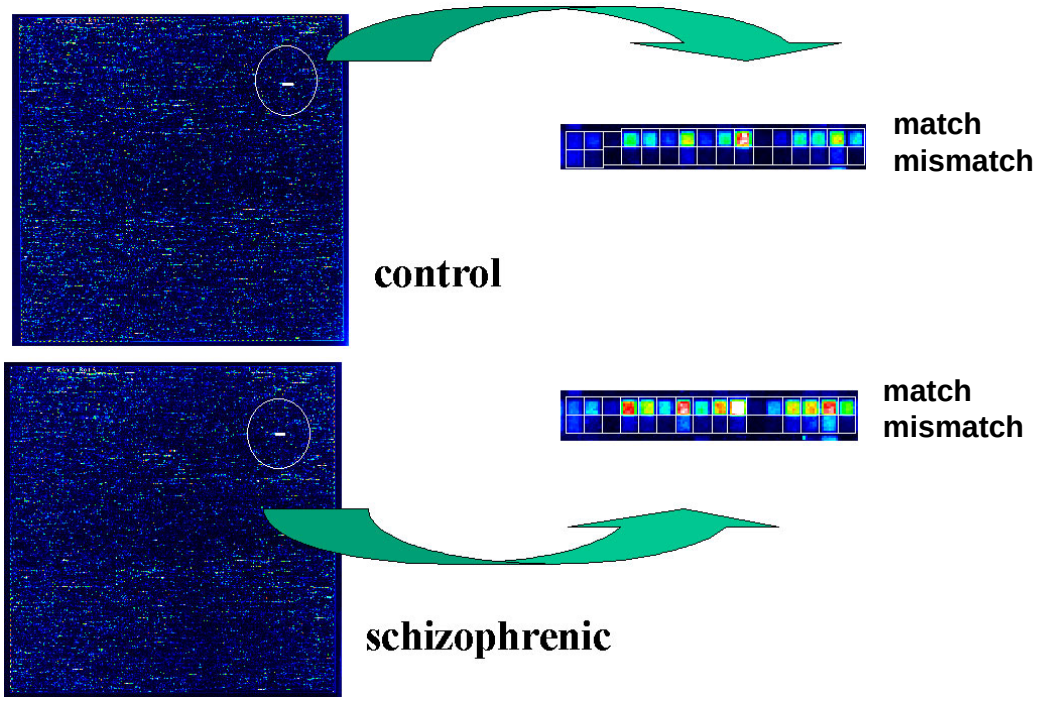
Affymetrix Hybridization



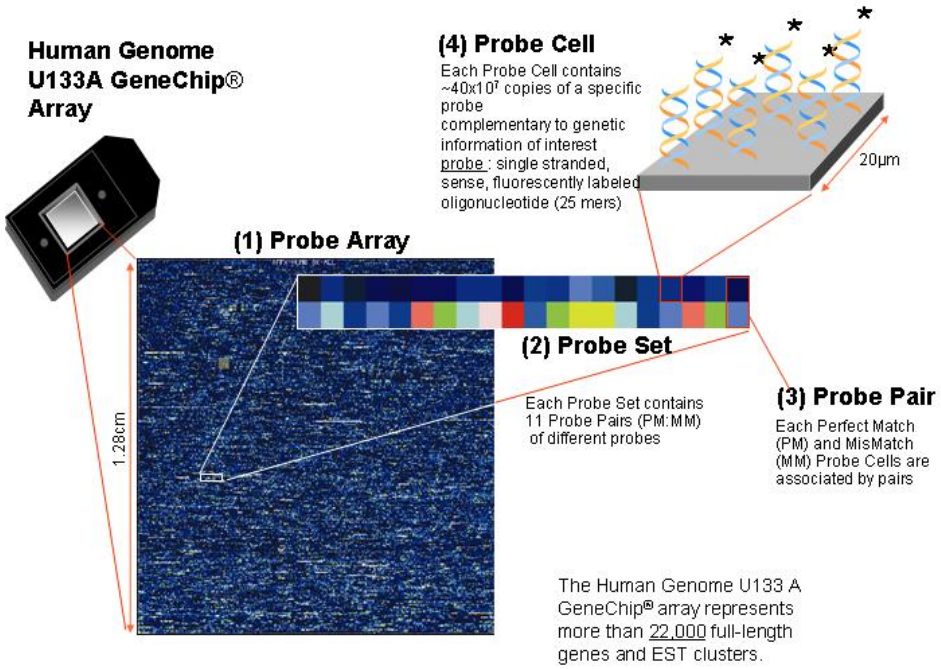
Affy Chips



Affy Chips



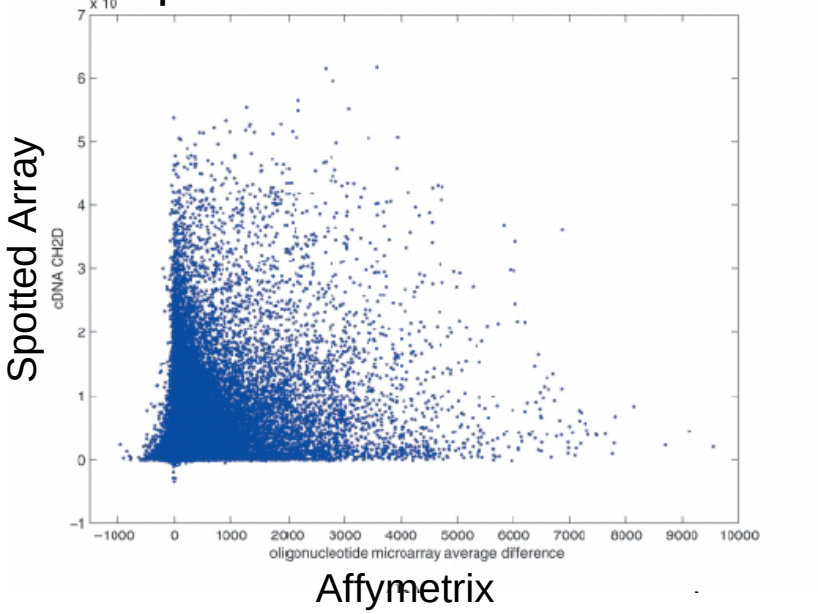
Affy Chips



Kuo et al. (2002) Bioinformatics

Comparison of Affymetrix and Spotted cDNA Arrays

161 620 matched pairs of measurements from 56 cell lines



Affymetrix GeneChip Advantages

- **High precision because of:**
  - **careful probe design**
  - **up to 20 probes per gene**
  - **up to 20 mismatch probes**
- **Very precise measurements**
- **Very high density (500,000 elements/array)**

Affymetrix GeneChips Disadvantages

- Inflexible: each array requires custom photolithographic masks
- More expensive than spotted arrays ($1000-$1200 per chip)
- Proprietary technology
  - not all algorithms, information public
  - only one manufacturer of readers, etc.

General Comments

- Spotted arrays are still wildly popular and widely used – a great learning tool for expression analysis
- Spotted arrays are generally unreliable and provide only gross indications of RNA expression
- Commercial systems (CodeLink and Affy) offer much greater reliability but are expensive & inflexible

Microarray Production

- Probe design and selection
- Printing
- RNA extraction
- Labeling
- Hybridization and washing
- Scanning
- Data analysis

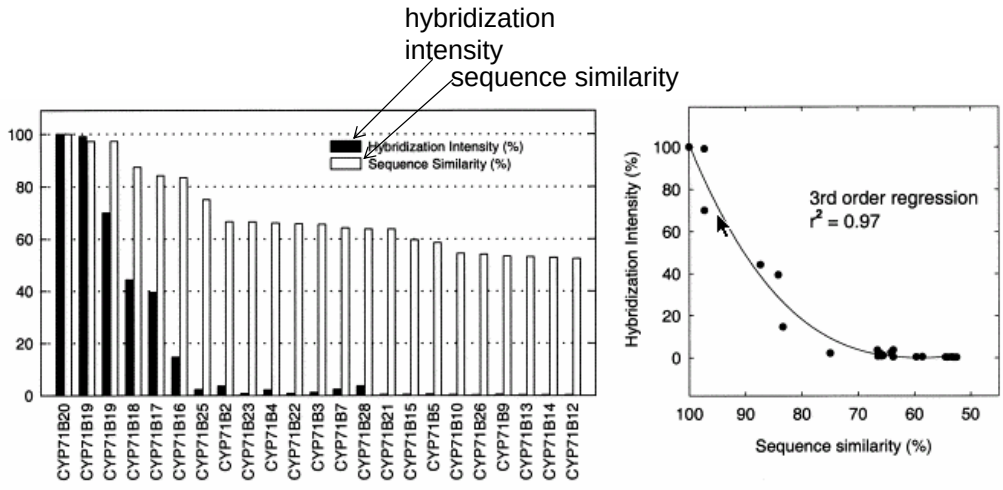
Probe Design & Selection

- Synthetic oligos 25-70 bases in length
- Choose sequences complementary to mRNA of interest
- Random base distribution and average GC content for organism
- Avoid long A+T or G+C rich regions
- Minimize internal secondary structure (hairpins or other loops)
- 1 M salt + 65 ^{o}C thermostability

Probe Design & Selection

- **Design and select oligo sequences that are less than 75% identical to existing genes elsewhere in the genome (i.e. do a BLAST search)**
- **Sequences with >75% sequence identity to other sequences will cross-hybridize – leading to confounding results**

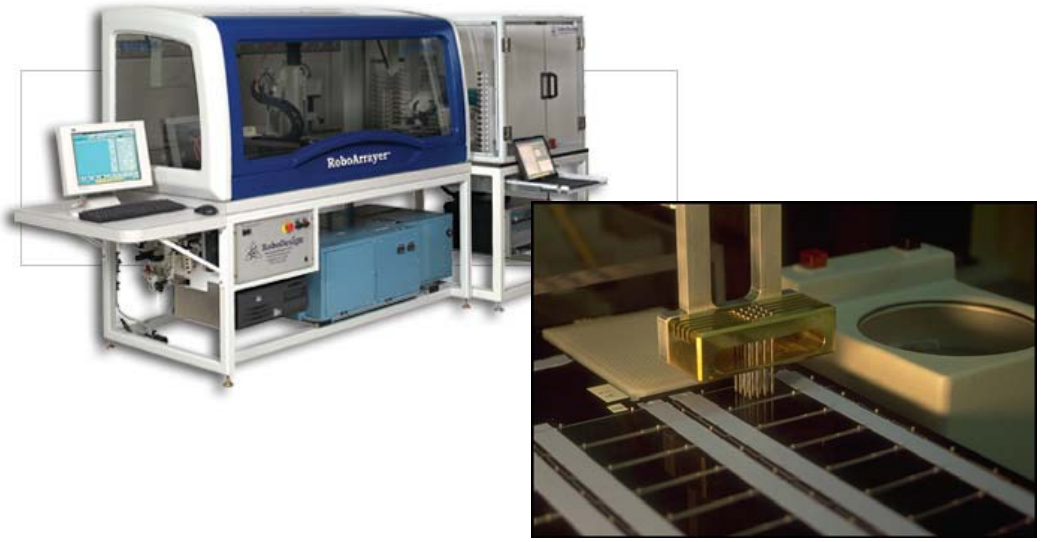
Cross-hybridization



Analysis of a cross-hybridization within the CYP450 superfamily

Xu et al. (2001) Gene

Microarray Printing



Microarray Printing

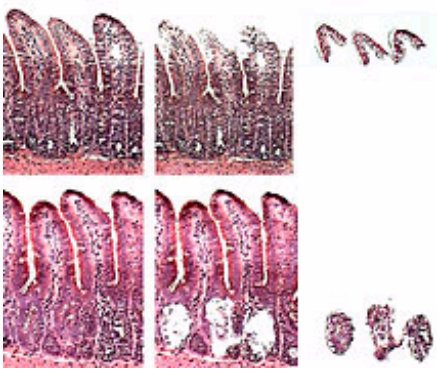
- **Probes are deposited by robots using:**
  - **piezo-electric jets**
  - **microcapillaries**
  - **split or solid pins**
- **Coated glass is the most common substrate**
  - **aminosilane, poly-lysine, etc. give non-covalent linkages**
  - **covalent linkage is possible with modified oligos + aldehyde (etc.) coatings**

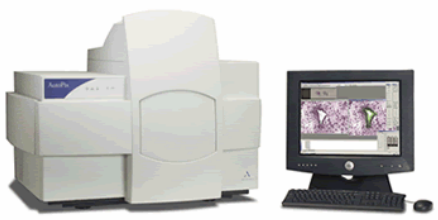
RNA Extraction

- RNA is extremely unstable
- Probably the most problematic step in all microarray analysis
- RNA is extracted as “total RNA”
  - only 1-2% is mRNA
  - remainder is rRNA, tRNA, etc.
- RNA extracted from tissue is often very heterogeneous (many cells and cell types) – watch selectivity

Laser Capture Microdissection

- Cells of interest are visually selected and exposed to an IR laser, which adheres them to a transfer film



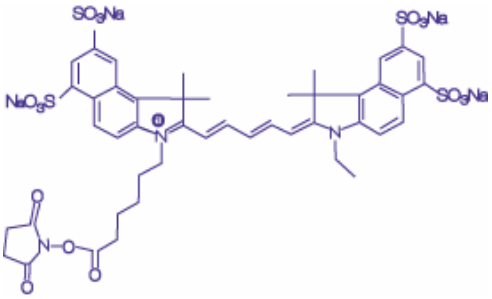


arcturus.com

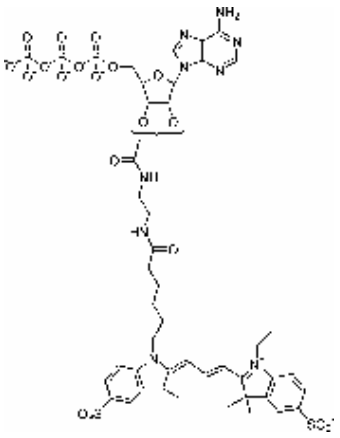
RNA Labeling

- Common source of systematic error (freshness, contaminants)
- Direct labeling
  - fluorescent nucleotides are incorporated during reverse transcription (“first strand”)
- Indirect labeling
  - reactive nucleotides (aminoallyl-dUTP) are incorporated during RT; first strand product is mixed with reactive fluorescent dyes that bind to amino group

Direct Labeling

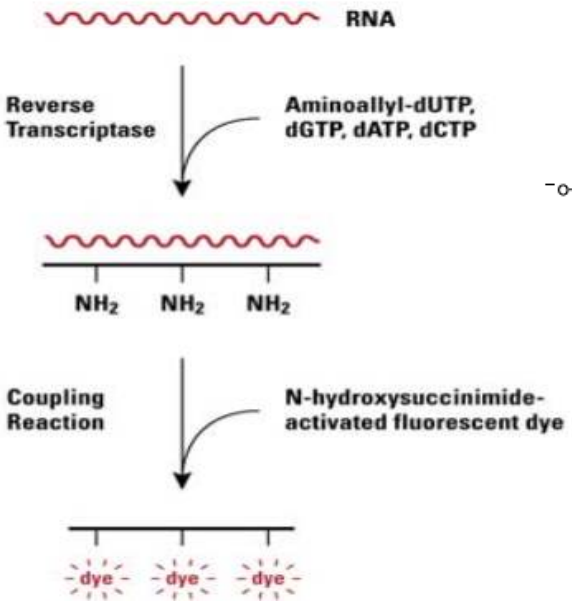


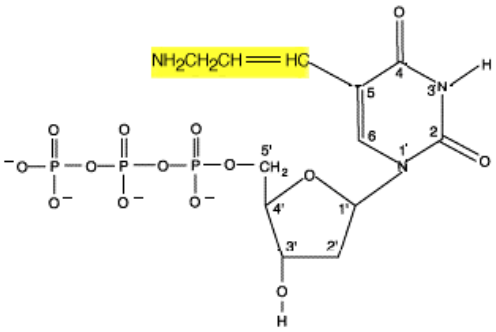
Cy5



Cy3-ATP

Indirect Labeling





aminoallyl-dUTP

Hybridization

- **Stringency of hybridization is affected by ions, detergents, formamide, temperature, time...**
- **Hybridization may be an important source of systematic error**
- **Automated hybridization systems exist; value is debatable**

Lee et al. (2000) *PNAS*

How Many Replicates?

Table 5. Misclassification percentages for different combinations of replicates

Classification Outcome	Combination of Replicates (1)	(2)	(3)	(1, 2)	(1, 3)	(2, 3)	(1, 2, 3)
False positive, %	8.3	1.4	9.0	1.0	2.1	0.7	0.7
False negative, %	0.3	0.0	0.0	0.3	0.3	0.0	0.0
Misclassified, %	8.7	1.4	9.0	1.4	2.4	0.7	0.7

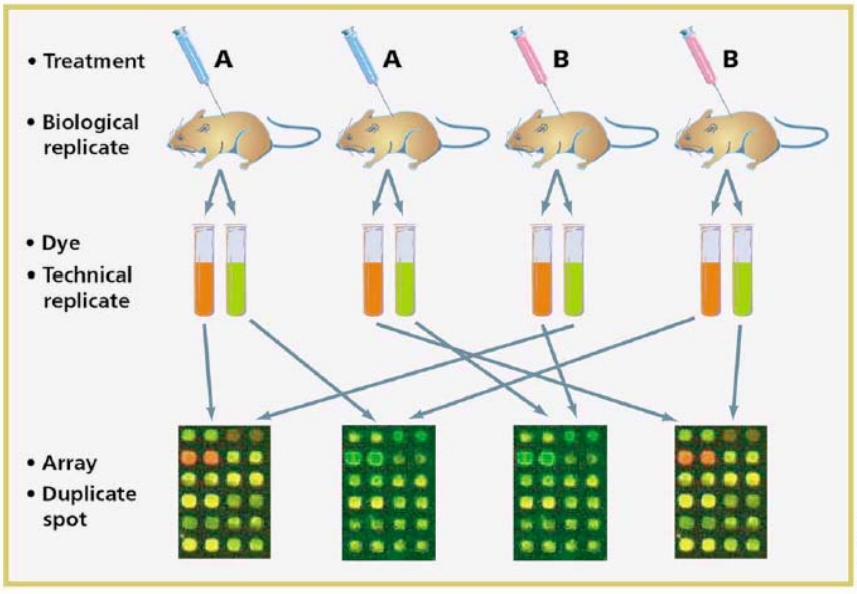
Singletons **Duplicates** **3X**

- **Substantial error when only one array analyzed, standard is to use 3 replicates**

What Types of Replicates?

Biological replicates

Technical replicates



Bob Crimi

Biological replication is most important because it includes all of the potential sources for error

Microarray Production

- Probe design and selection
- Printing
- RNA extraction
- Labeling
- Hybridization and washing
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- Data analysis