20.109

LABORATORY FUNDAMENTALS IN BIOLOGICAL ENGINEERING

MODULE 2

EXPRESSION ENGINEERING

Lecture # 4

Leona Samson

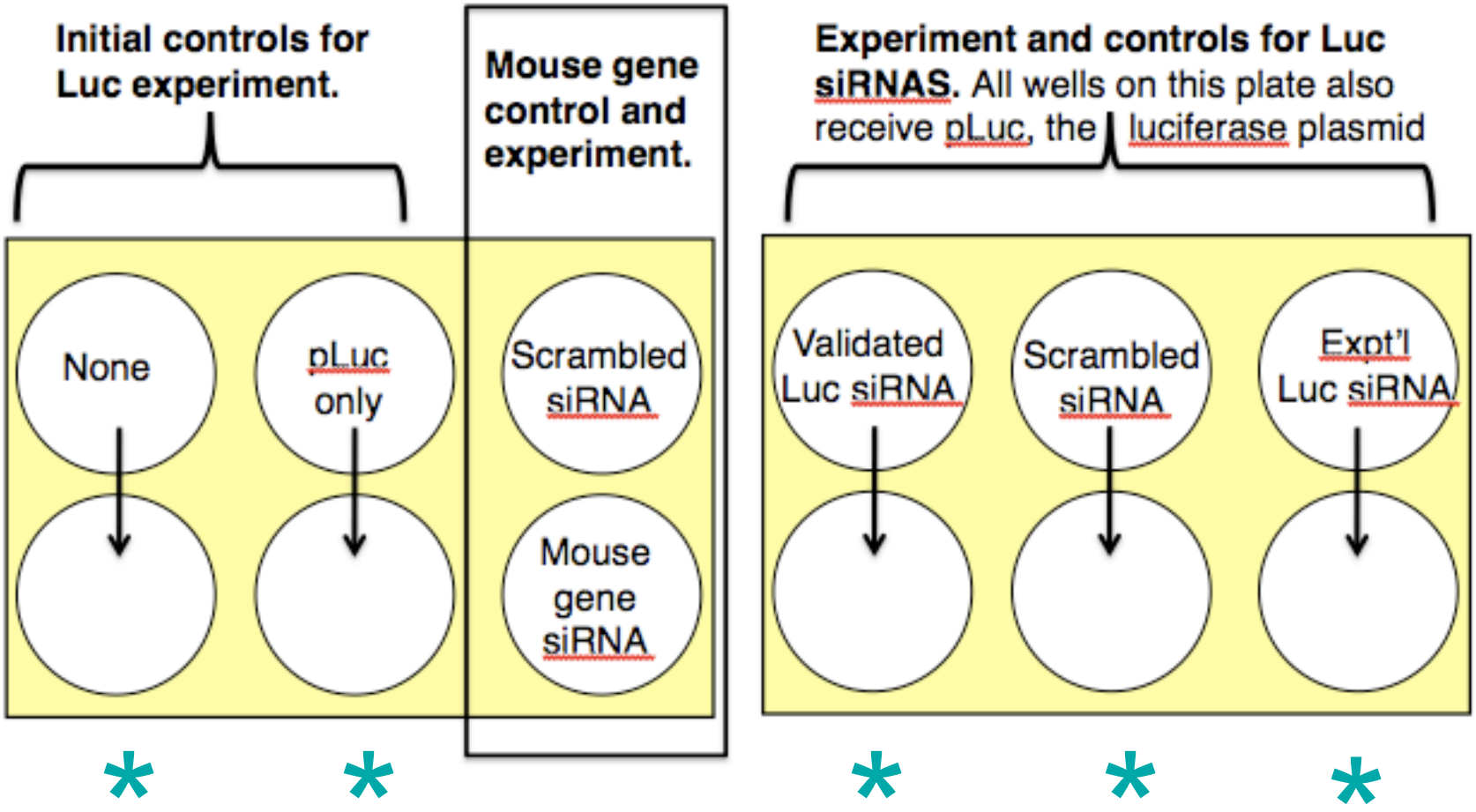
April 2nd 2009

Snapshot of the next four weeks

We will eliminate the expression of various genes using

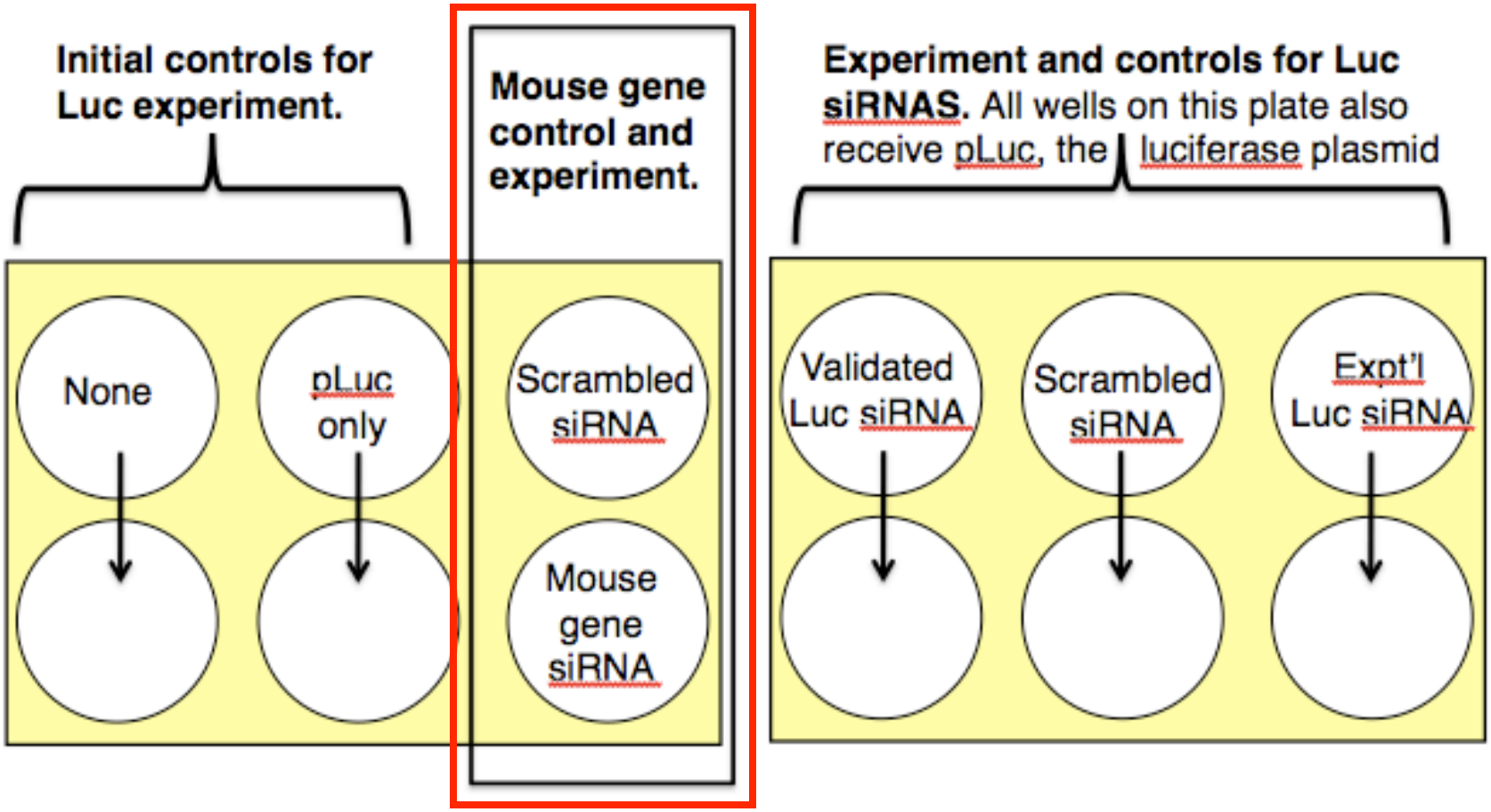
(i) RNA interference technology
(ii) Cultured mouse ES cells
(iii) Chemiluminescent proteins
(iv) DNA microarrays

siRNA knockdown of expression of Renilla Luciferase plus various mouse gene



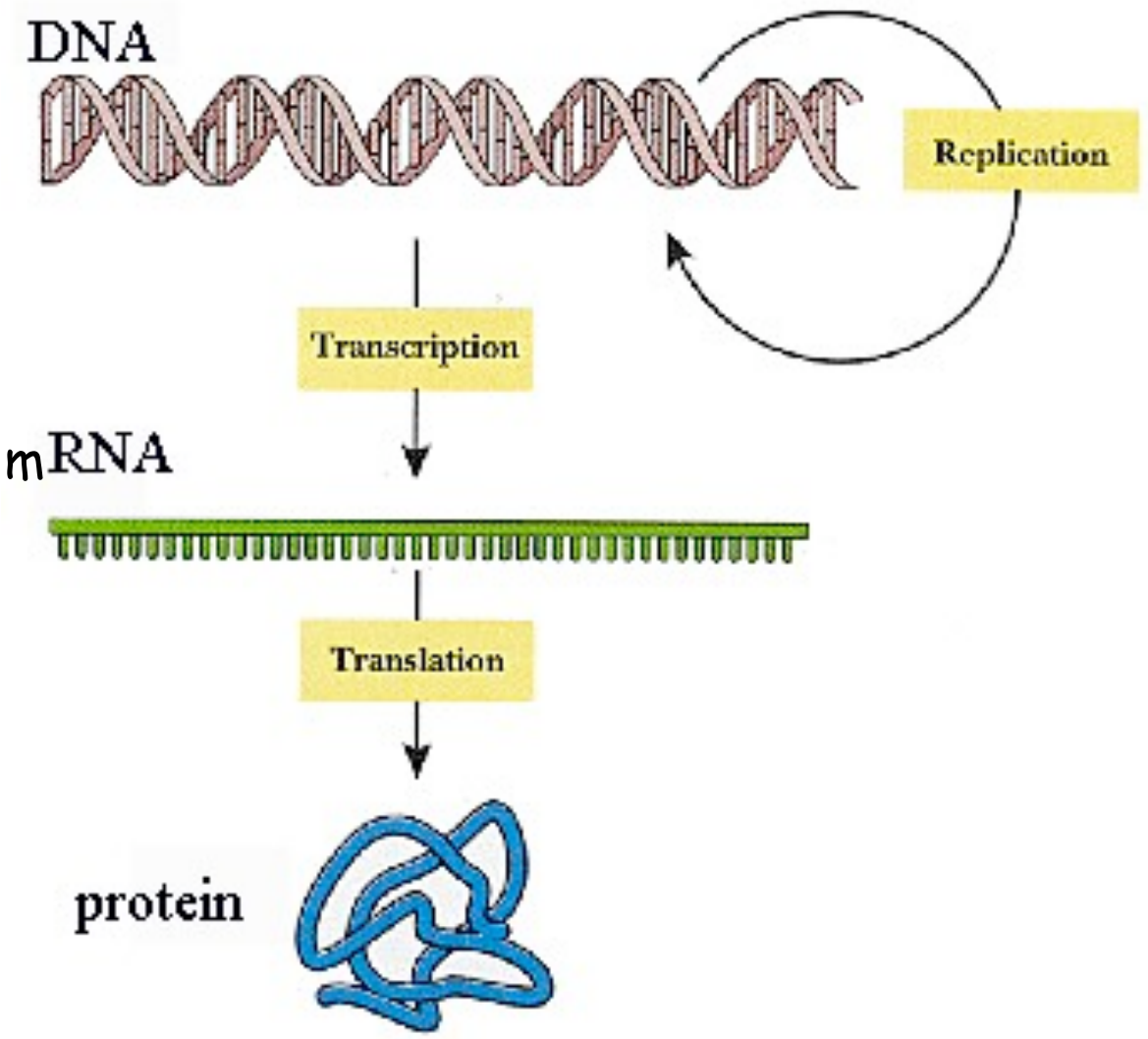
* Prepare cell extracts and measure Luciferase activities

siRNA knockdown of expression of Renilla Luciferase plus various mouse gene



Isolate total RNA in order to measure relative levels of all mRNAs – with special attention to YGI

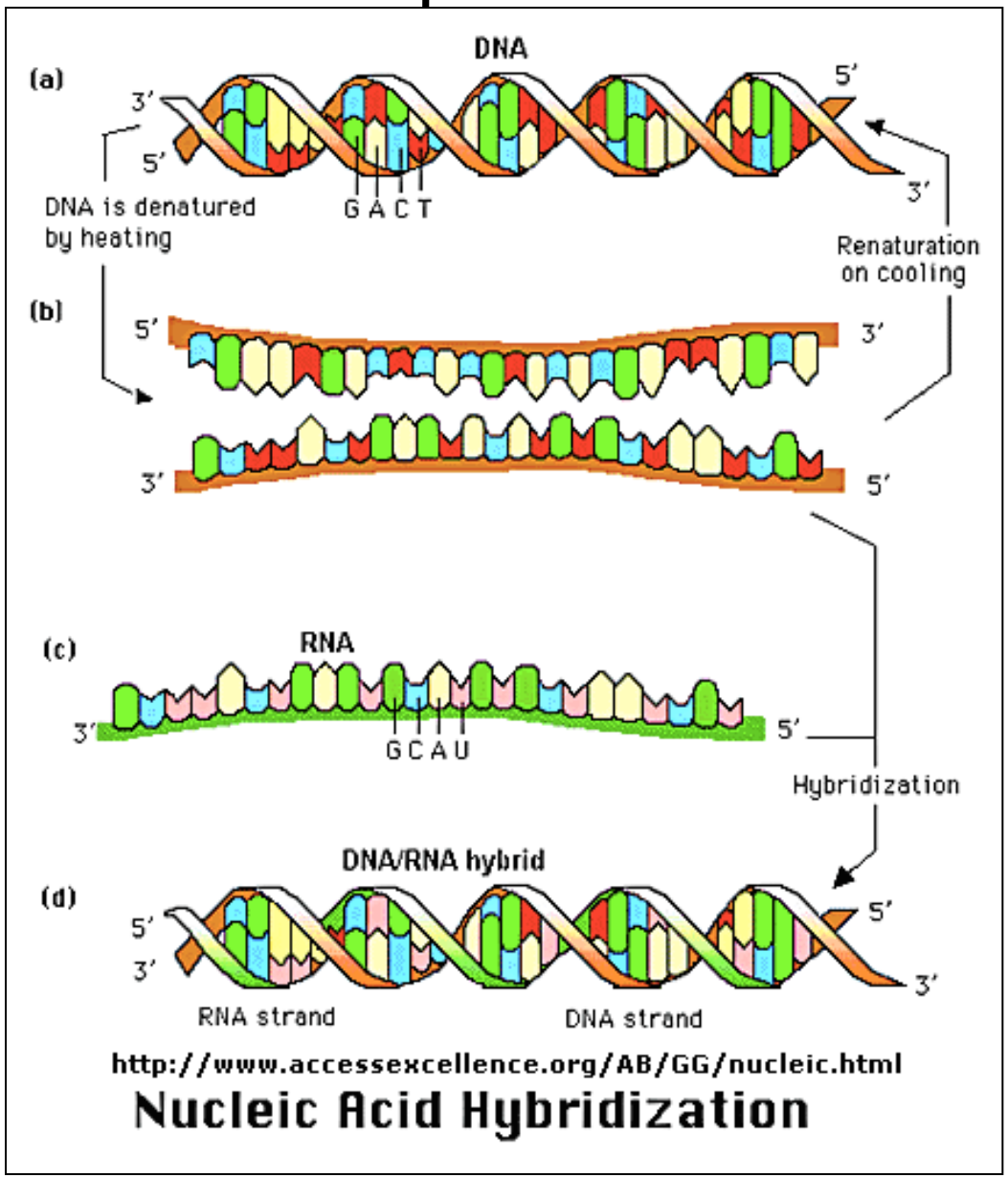
Monitor mRNA expression level for every mouse gene in one single experiment.



How can we measure the level of **thousands** of mRNA species present in a particular cell type?

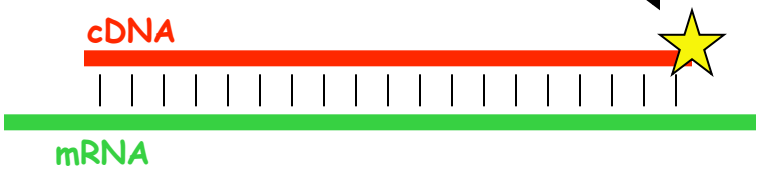
Now that we know the DNA sequence for every gene, this is possible!

How did we measure mRNA levels one at a time?
This depends on Nucleic Acid Hybridization

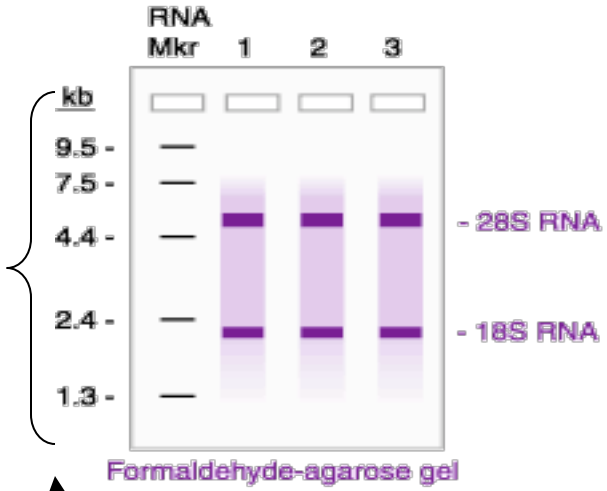


The specificity of G pairing with C and A pairing with T (or U) drives hybridization and provides a mechanism for quantitatively assessing the amount of a specific mRNA species in cells.

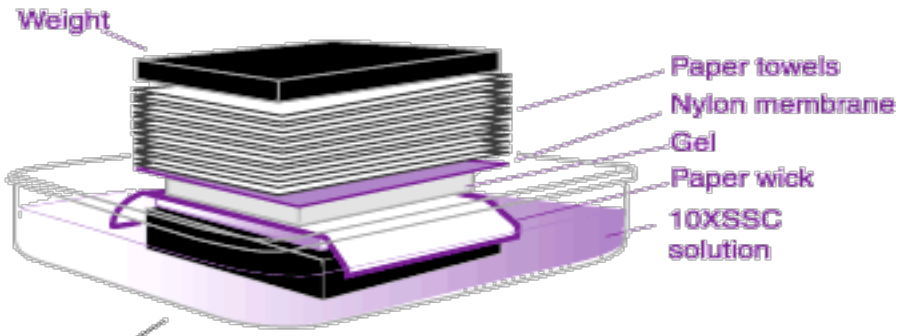
^{32}P- label



Lets first back-up. How did we measure mRNA levels one or two at a time? Northern Blots



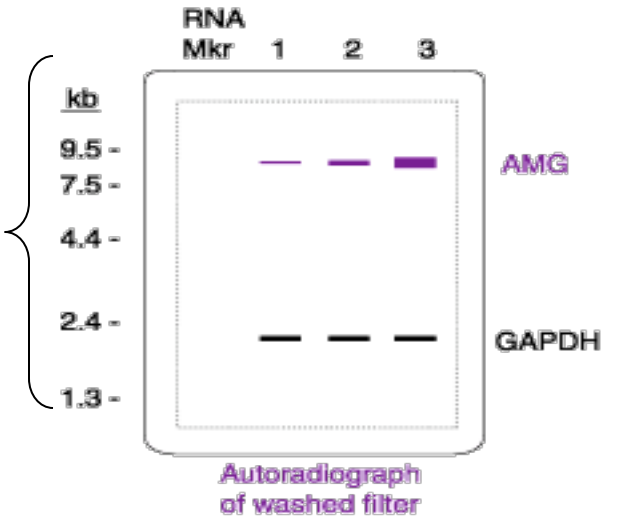
Transfer RNA by membrane blotting

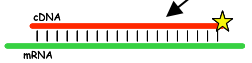


Cross-link RNA to the membrane using UV light

Hybridize membrane with denatured ^{32}P-cDNA probe

All mRNAs separated by size





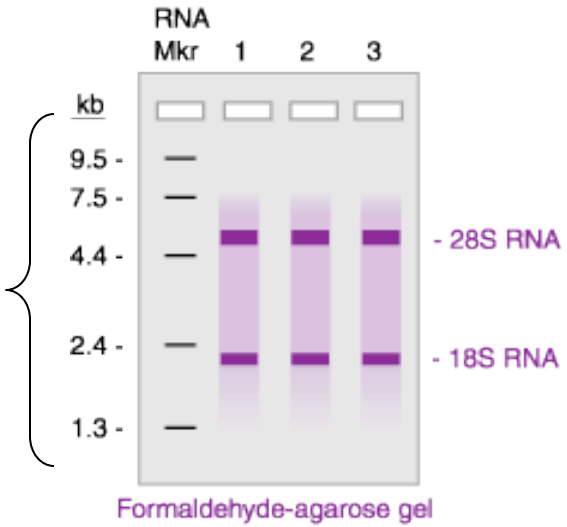
AMG probe

GAPDH probe

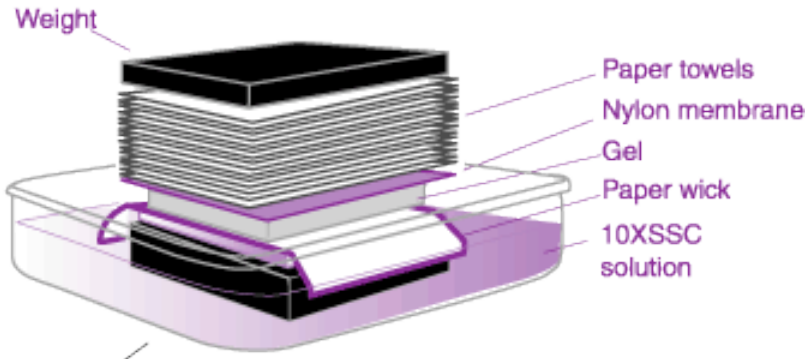
Specific mRNAs Lit-up by radioactive cDNA probes

How to monitor mRNA expression level for every gene: Global transcriptional profiling

- Carry out thousands Northern Blots?
- Instead – DNA microarrays were developed
- DNA microarrays for global transcriptional profiling were not feasible before the sequencing of whole genomes.



Transfer RNA by membrane blotting



Cross-link RNA to the membrane using UV light

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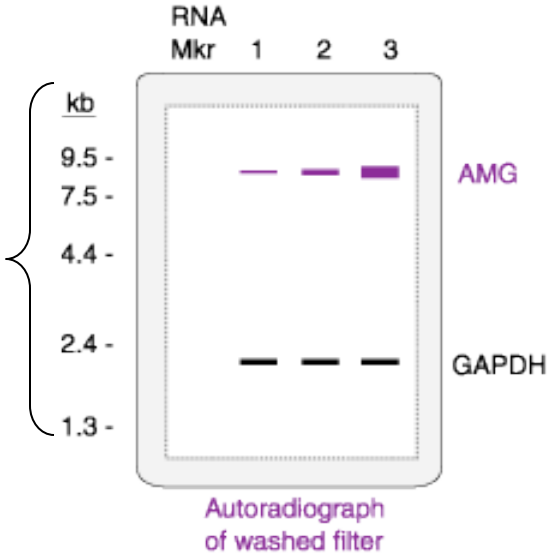
All mRNAs separated by size



The immobilized mRNA population is probed (hybridized) with ^{32}P-labeled DNA sequences specific for one or two genes

Northern Blots

Immobilized mRNA population hybridized with labeled DNA probe representing one or two genes

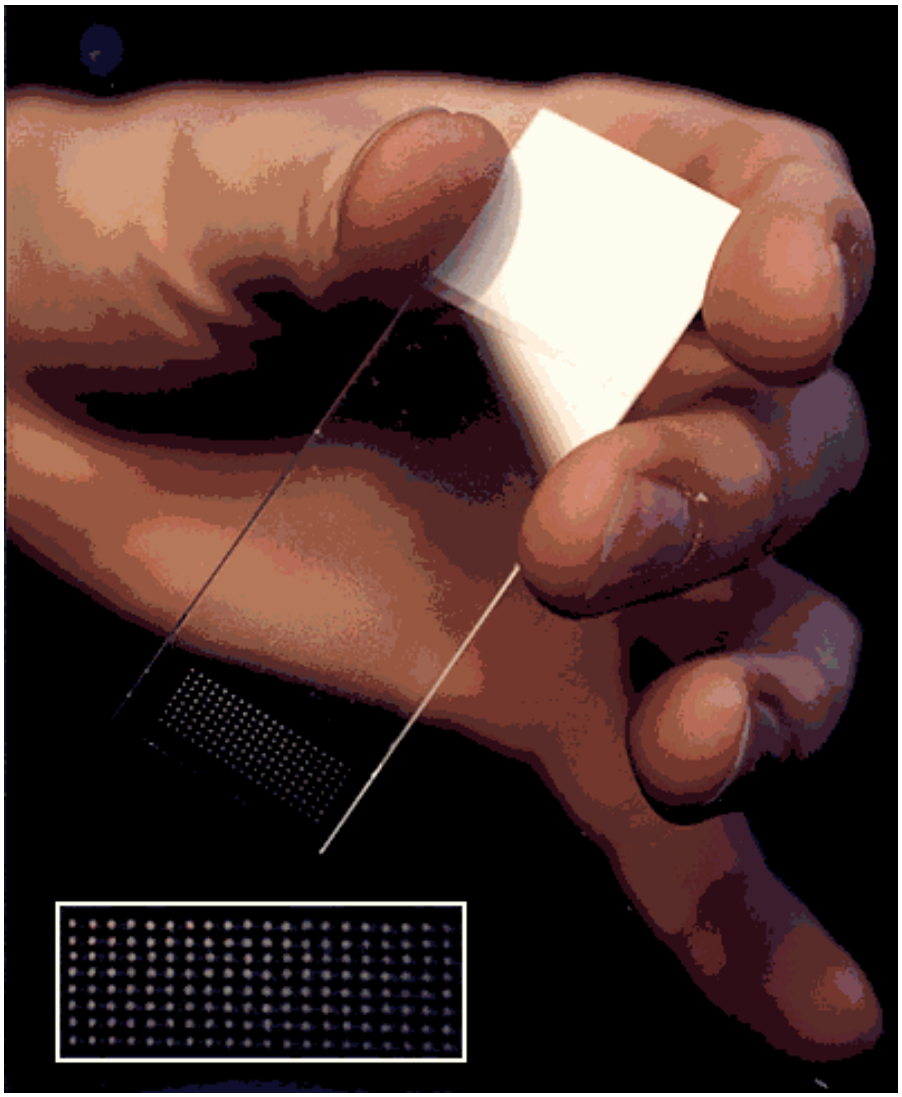
DNA Microarrays

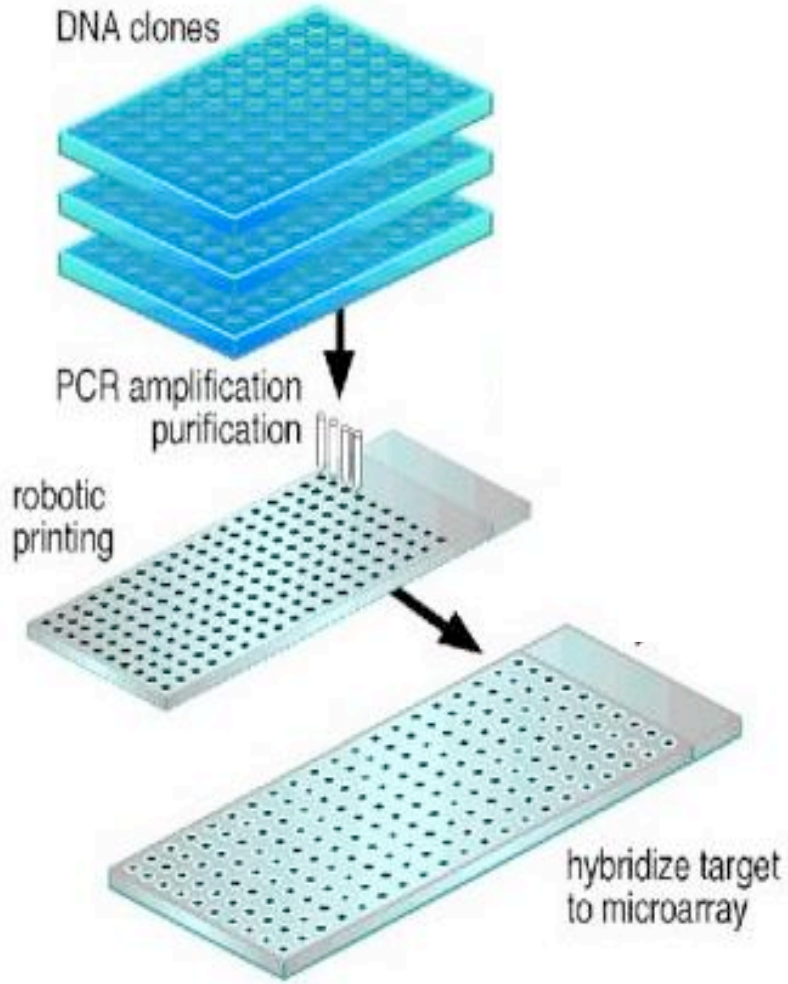
Immobilized DNA probes representing **all** possible genes hybridized with labeled mRNA population

Need to achieve two things:

(i) Immobilize (array) thousands of DNA probes specific for each individual mRNA gene product

(ii) Label mRNA populations





Up to 20,000 probes per slide

The probes can be cDNAs (~ 1Kb) or oligonucleotides (20-70 mers)

Robots designed to spot up to 20,000 DNA probes per slide

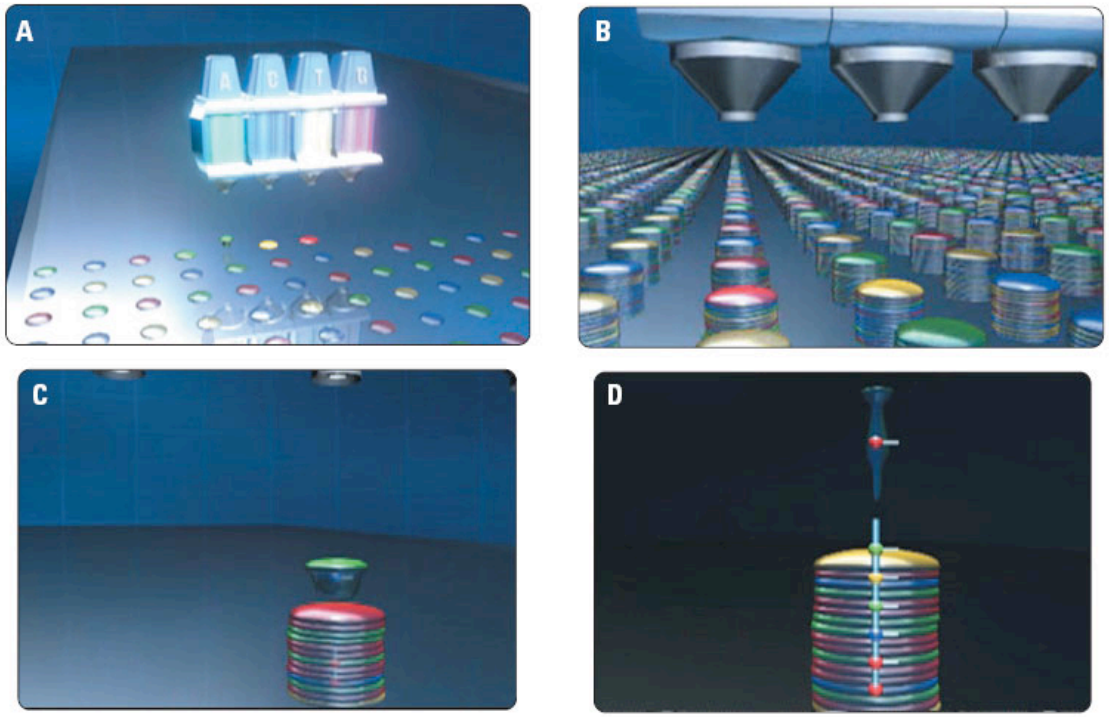
The probes can be cDNAs (~ 1Kb) or oligonucleotides (20-70 mers)

Catalog Oligo Microarrays

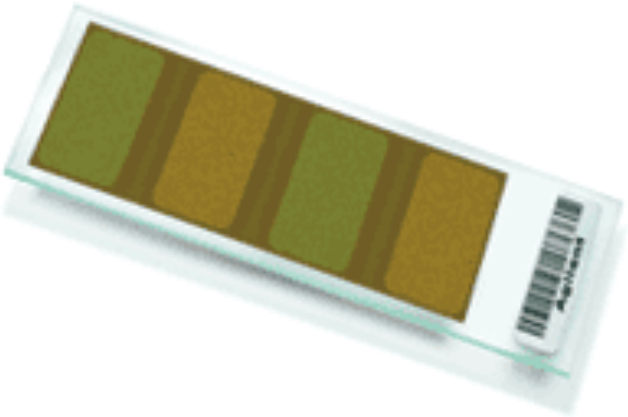
The arrays we'll be using…….

Agilent's non-contact industrial inkjet printing process uniformly deposits oligo monomers onto specially-prepared glass slides. Both the catalog and custom microarrays are manufactured using Agilent's non-contact in situ synthesis process of printing 60-mer length oligonucleotide probes, base-by-base, from digital sequence files. This is achieved with an inkjet process which delivers extremely small, accurate volumes (picoliters) of the chemicals to be spotted. Standard phosphoramidite chemistry used in the reactions allows for very high coupling efficiencies to be maintained at each step in the synthesis of the full-length oligonucleotide. Precise quantities are reproducibly deposited "on the fly." This engineering feat is achieved without stopping to make contact with the slide surface and without introducing surface-contact feature anomalies, resulting in consistent spot uniformity and traceability.

Ink-jet Technology



4x44K spots "features"



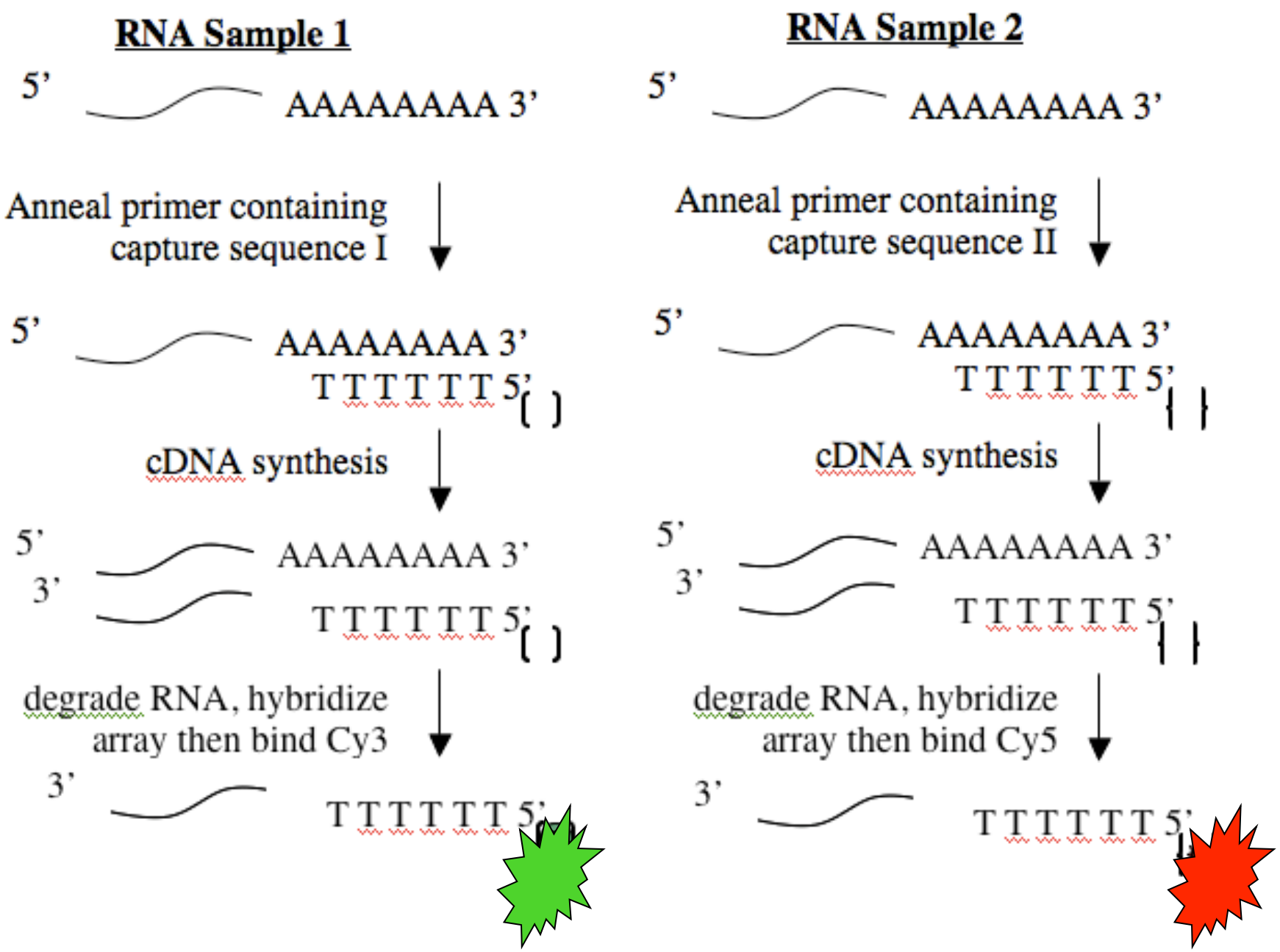
http://www.chem.agilent.com/en-US/products/instruments/dnamicroarrays/pages/gp557.aspx

Need to achieve two things:

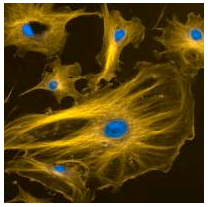
(i) Immobilize (array) thousands of probes specific for each individual gene

(ii) Label mRNA populations

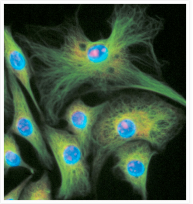
Copy the population of purified mRNA species such that they are fluorescently labeled – hybridize to the array


RNA Sample 1
5'
AAAAAAAA 3'
Anneal primer containing capture sequence I
5'
AAAAAAAA 3'
T T T T T T 5'
cDNA synthesis
5'
AAAAAAAA 3'
3'
T T T T T T 5'
degrade RNA, hybridize array then bind Cy3
3'
T T T T T T 5'
RNA Sample 2
5'
AAAAAAAA 3'
Anneal primer containing capture sequence II
5'
AAAAAAAA 3'
T T T T T T 5'
cDNA synthesis
5'
AAAAAAAA 3'
3'
T T T T T T 5'
degrade RNA, hybridize array then bind Cy5
3'
T T T T T T 5'

Cells in state A



Cells in state B



Isolate mRNA populations

AAAAA
AAAAA
AAAAA
AAAAA

AAAAA
AAAAA
AAAAA
AAAAA

Label copies of mRNA species with RED or GREEN

TTTTT
TTTTT
TTTTT
TTTTT

TTTTT
TTTTT
TTTTT
TTTTT

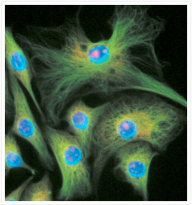
MIX

TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT

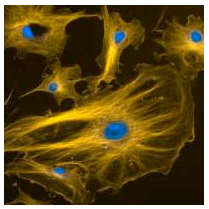
Hybridize to the microarray

DYE SWAP

Cells in state B



Cells in state A



Isolate mRNA populations

AAAAA
AAAAA
AAAAA
AAAAA

AAAAA
AAAAA
AAAAA
AAAAA

Label copies of mRNA species with RED or GREEN

TTTTT
TTTTT
TTTTT
TTTTT

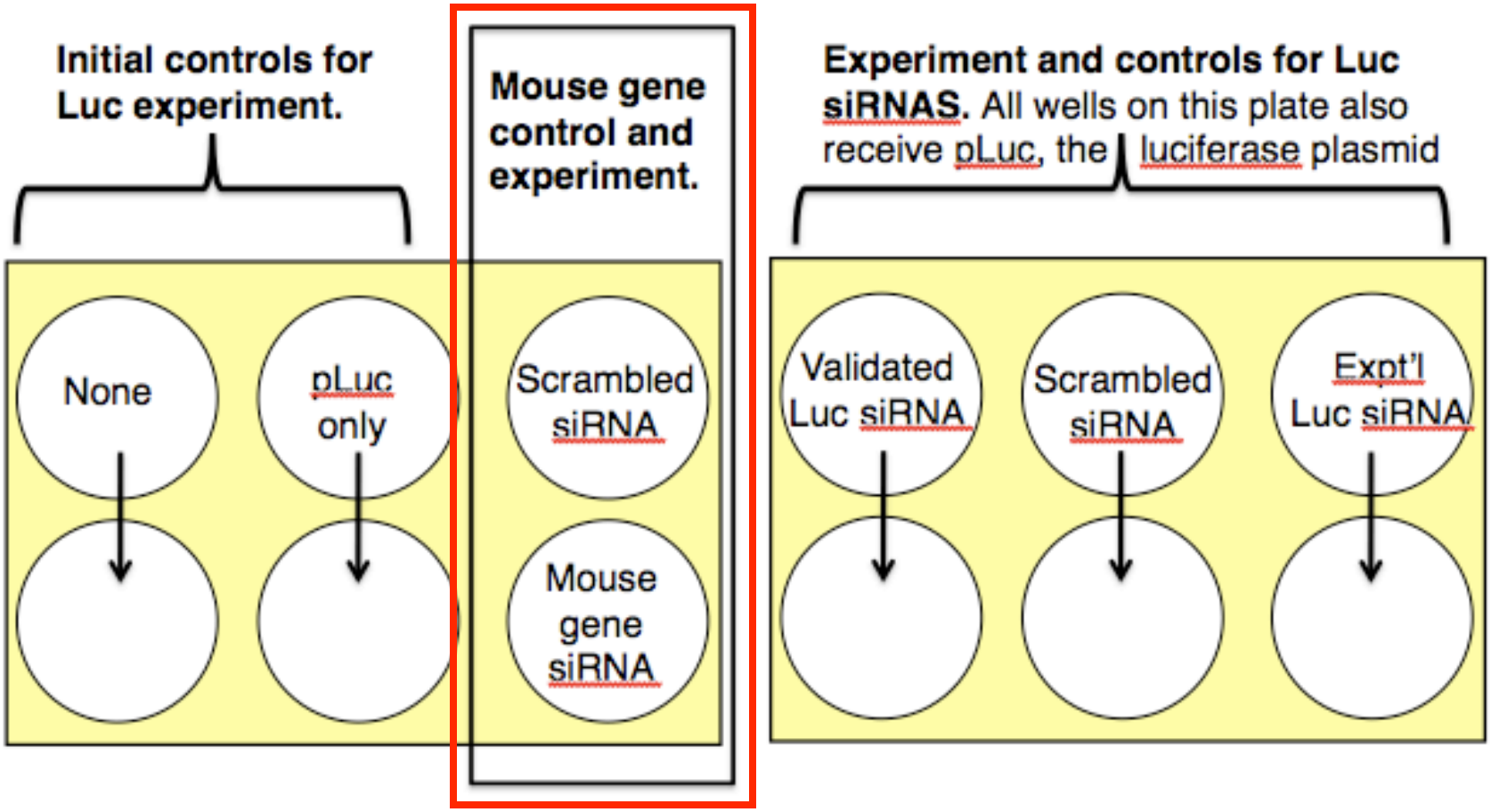
TTTTT
TTTTT
TTTTT
TTTTT

MIX

TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT

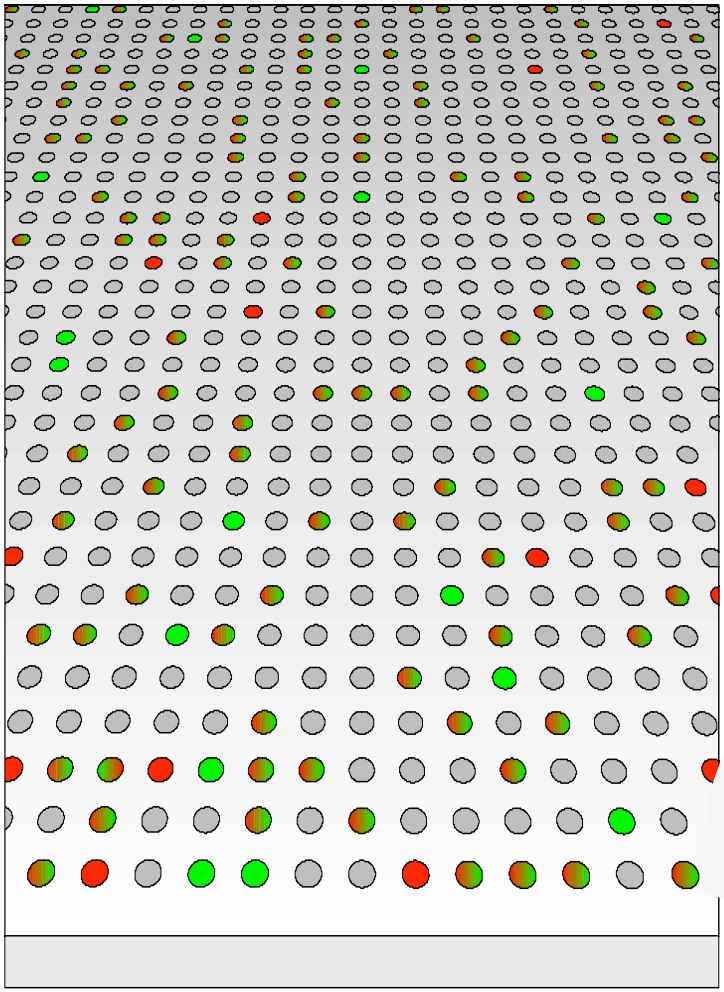
Hybridize to the microarray

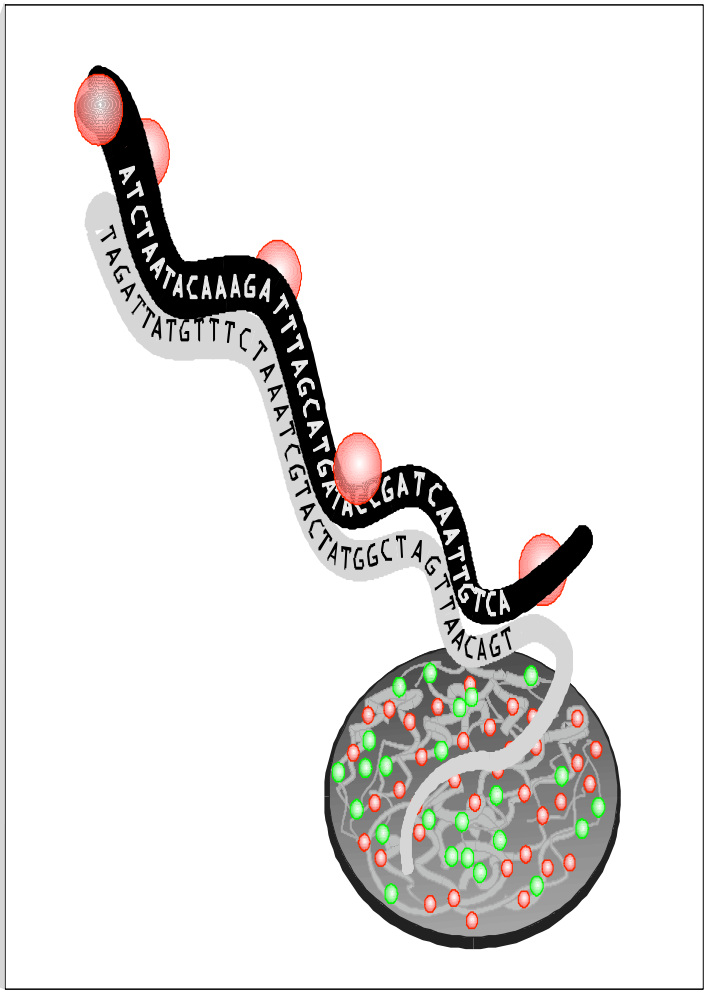
siRNA knockdown of expression of Renilla Luciferase plus various mouse gene



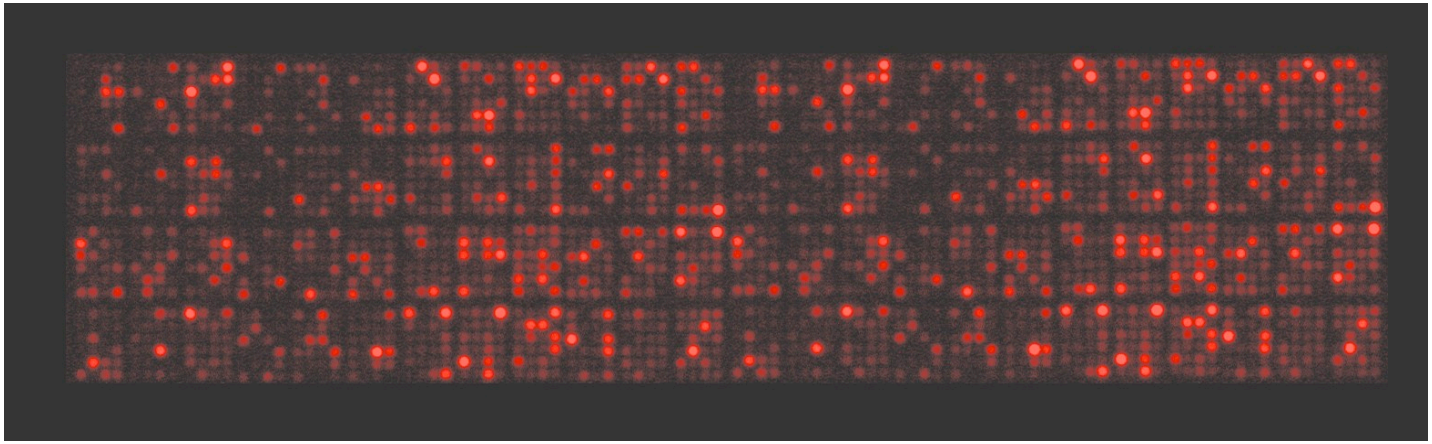
Isolate total RNA in order to measure relative levels of all mRNAs – with special attention to YGI

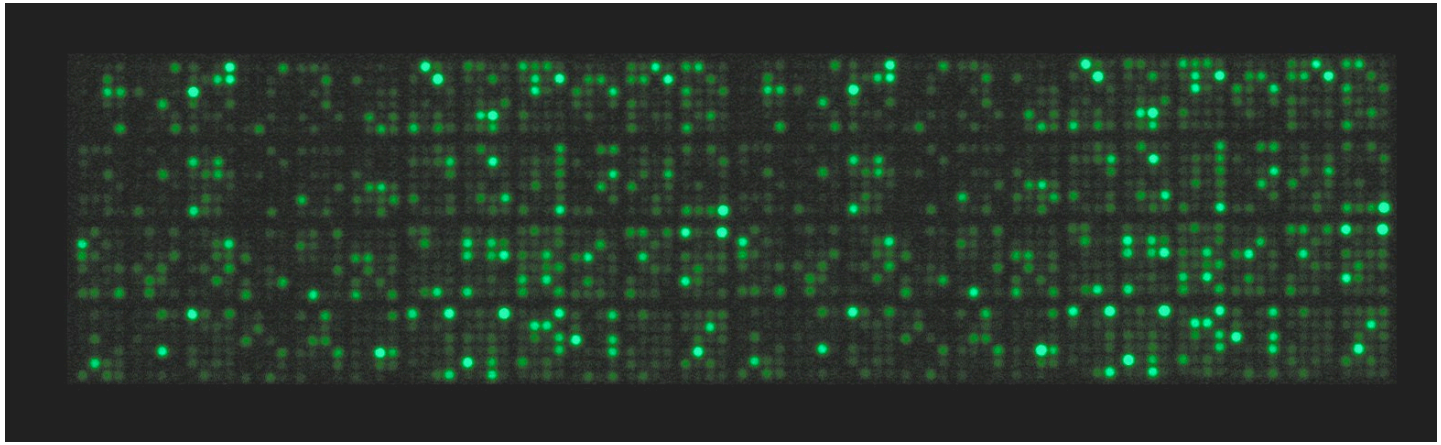
What's happening at each spot?

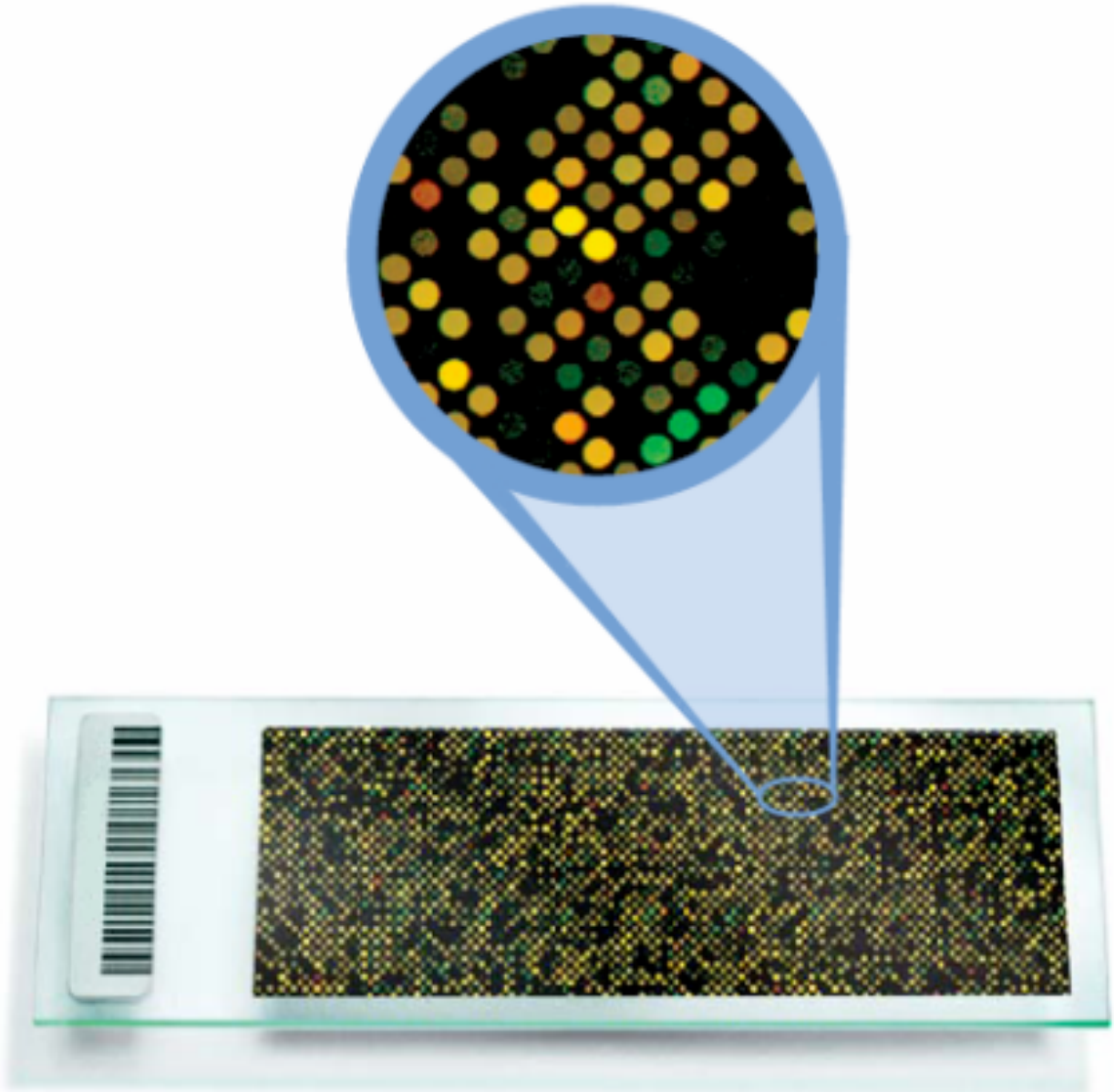


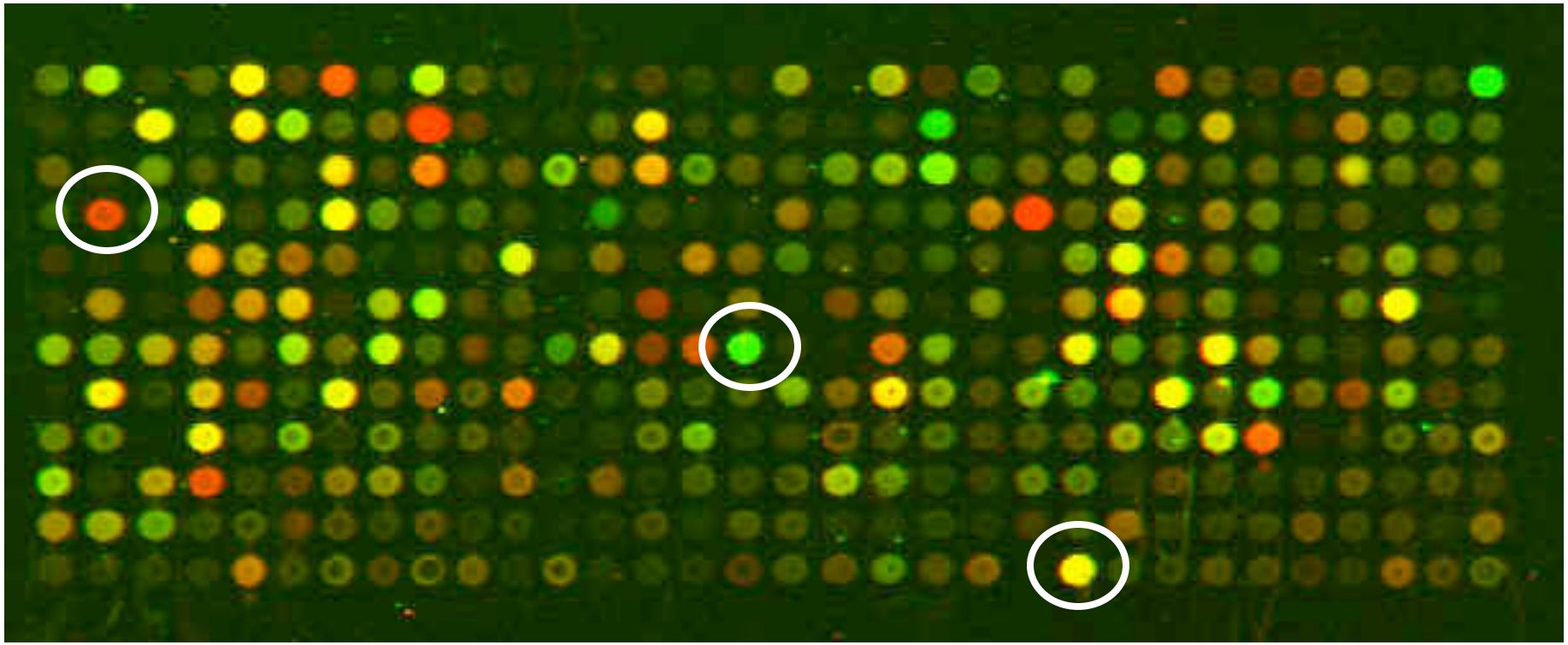


Hybridization





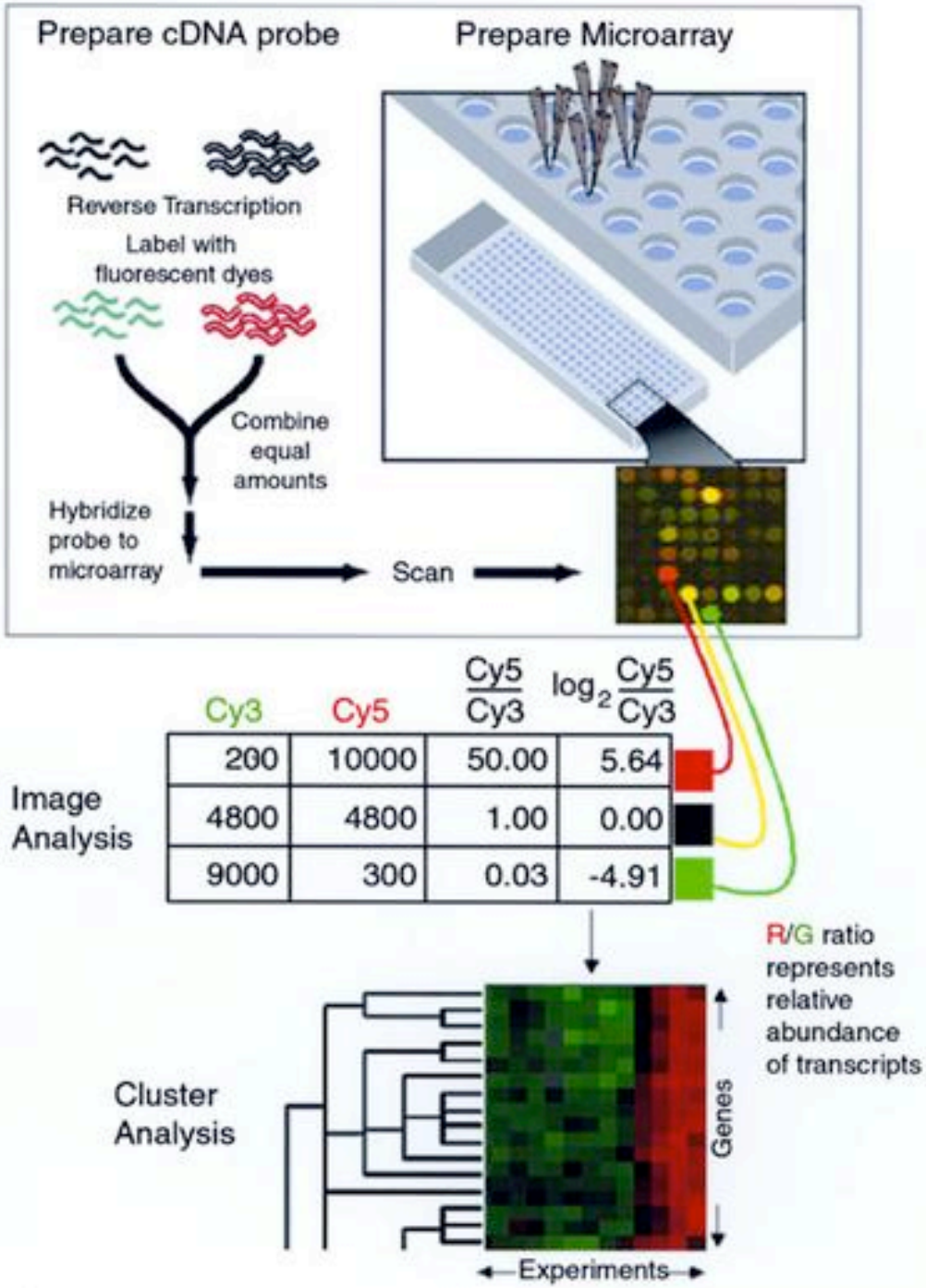




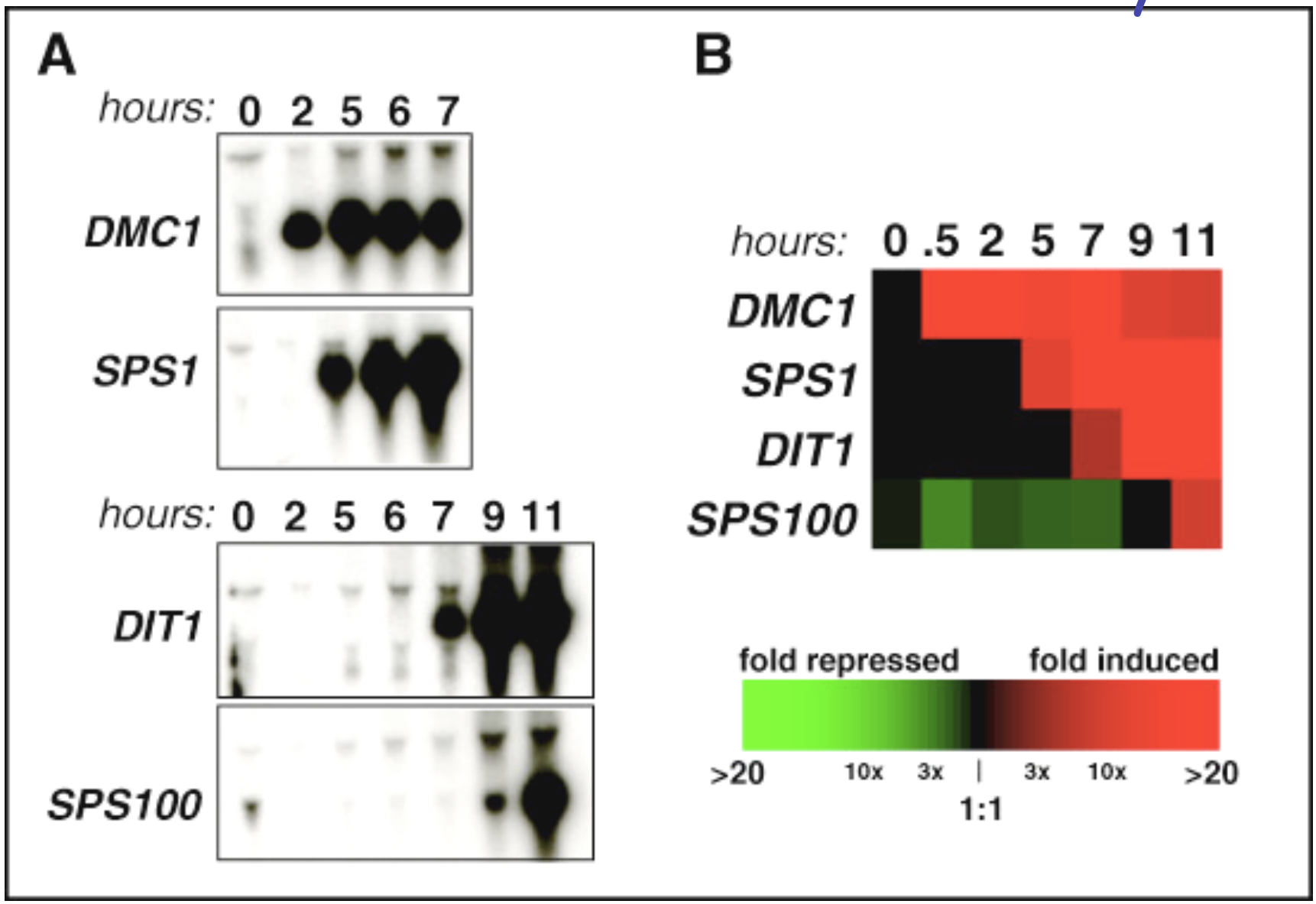
mRNA present much higher in State A than State B

mRNA present much higher in State B than State A

mRNA present at equal levels in States A and B

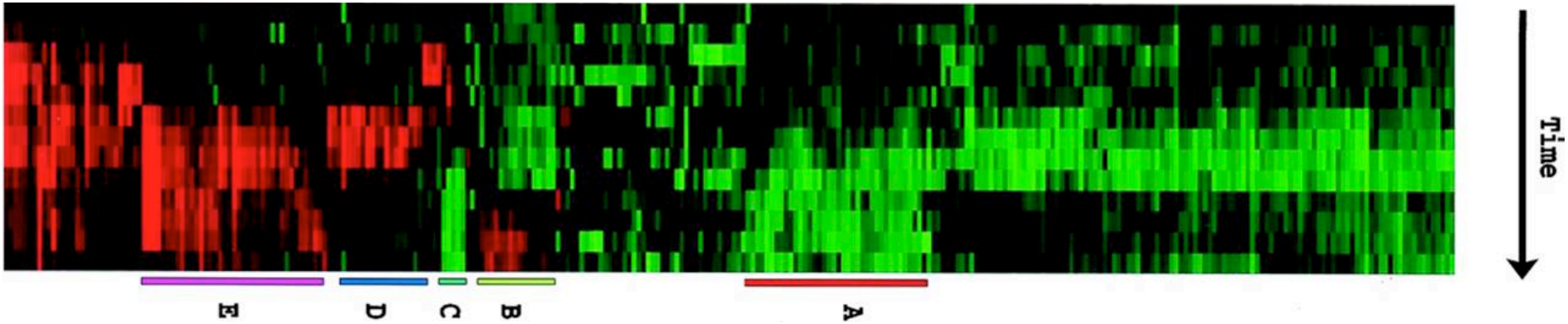

Prepare cDNA probe
Prepare Microarray
Reverse Transcription
Label with fluorescent dyes
Combine equal amounts
Hybridize probe to microarray
Scan
Cy3
Cy5
Cy5/Cy3
log2 Cy5/Cy3
200
10000
50.00
5.64
4800
4800
1.00
0.00
9000
300
0.03
-4.91
Image Analysis
R/G ratio represents relative abundance of transcripts
Cluster Analysis
Genes
Experiments

Northern Blot vs. Microarray

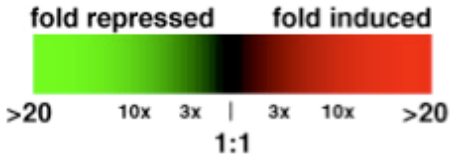


Hierarchical clustering to group together similarly regulated genes

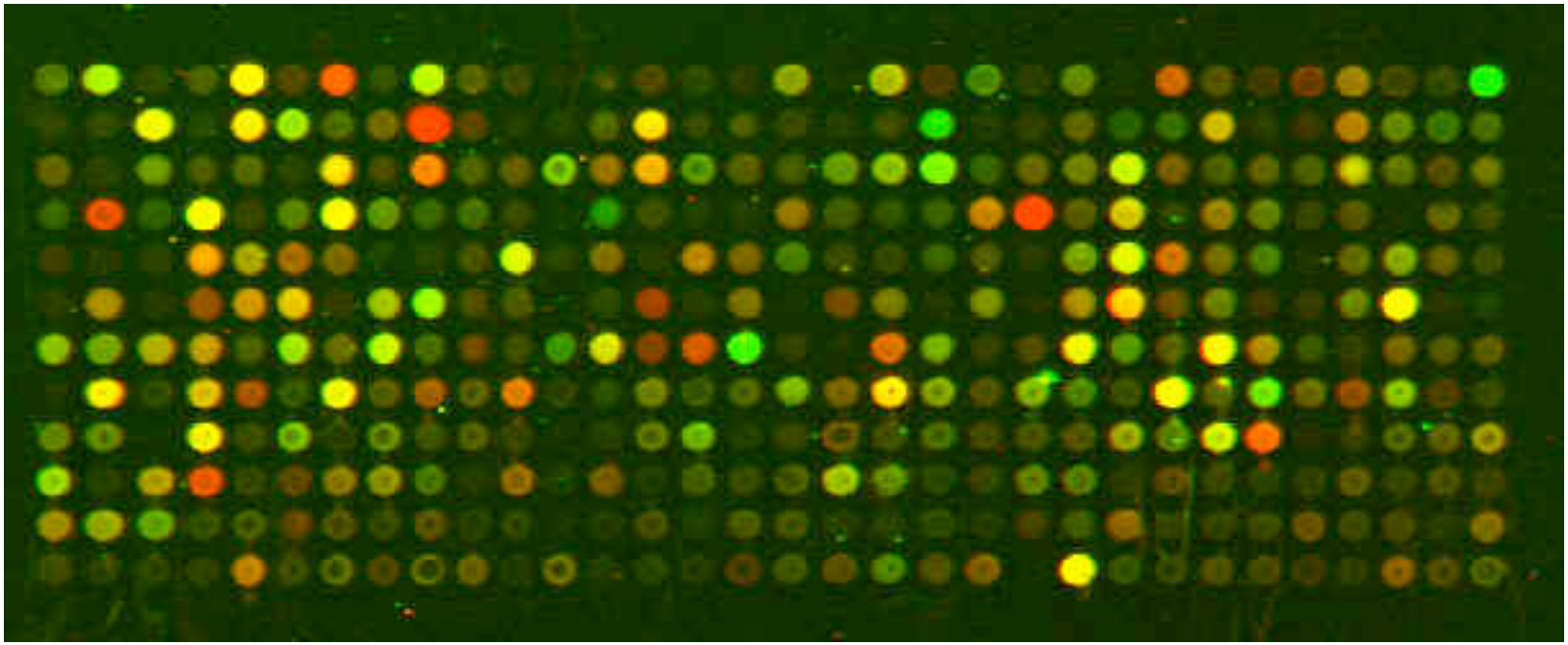
Each colored vertical line in the horizontal lane displays the relative expression level of a single mRNA



13 time points, and several thousand genes

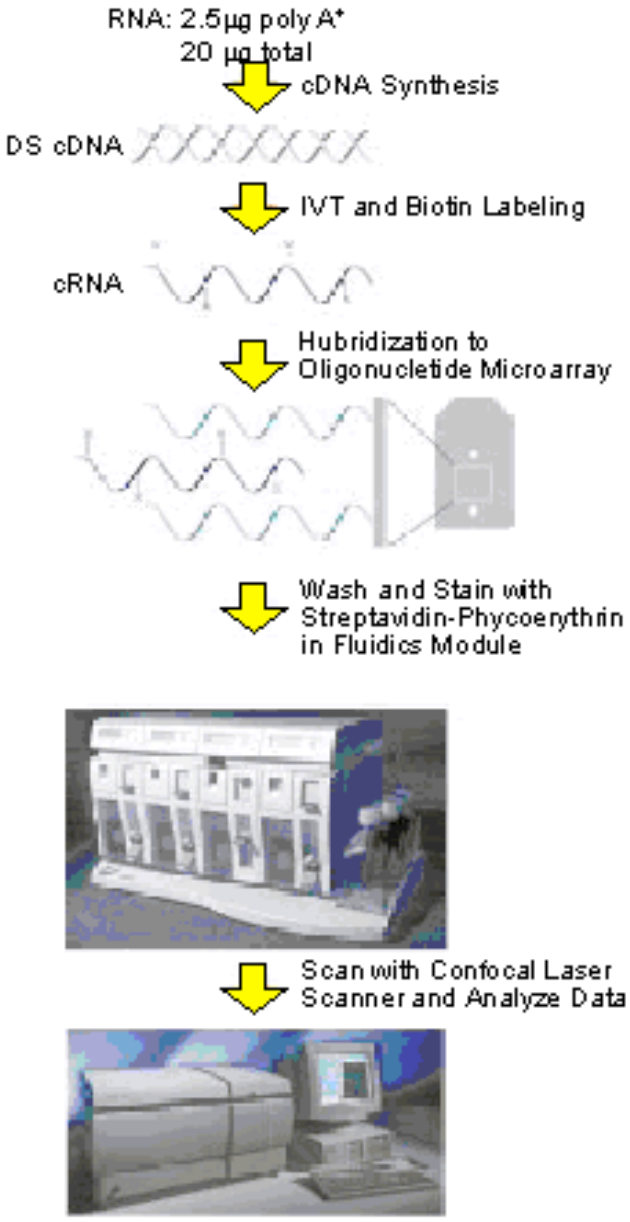


Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 14863–14868, December 1998
Cluster analysis and display of genome-wide expression patterns



This is the "two color" technology (you will use something similar), but there is another common technology that uses one color….

Affymetrix Technology



Affymetrix has focused on light-directed synthesis for the construction of high-density DNA probe arrays using two techniques: photolithography a solid-phase DNA synthesis. Synthetic linkers are attached modified with photochemically removable protecting groups to a glass substrate and direct light through a photolithographic mask to specific areas on the surface to produce localized photodeprotection.

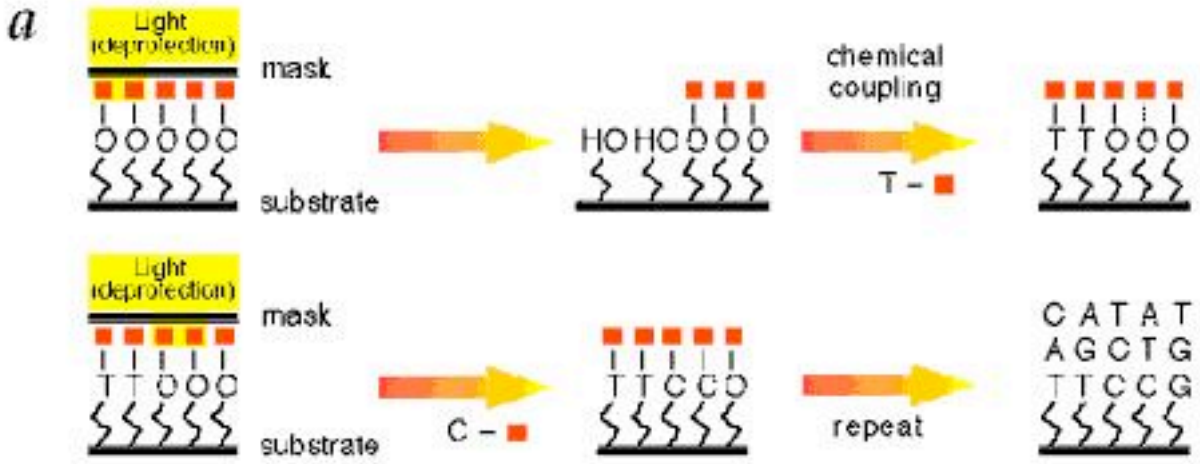
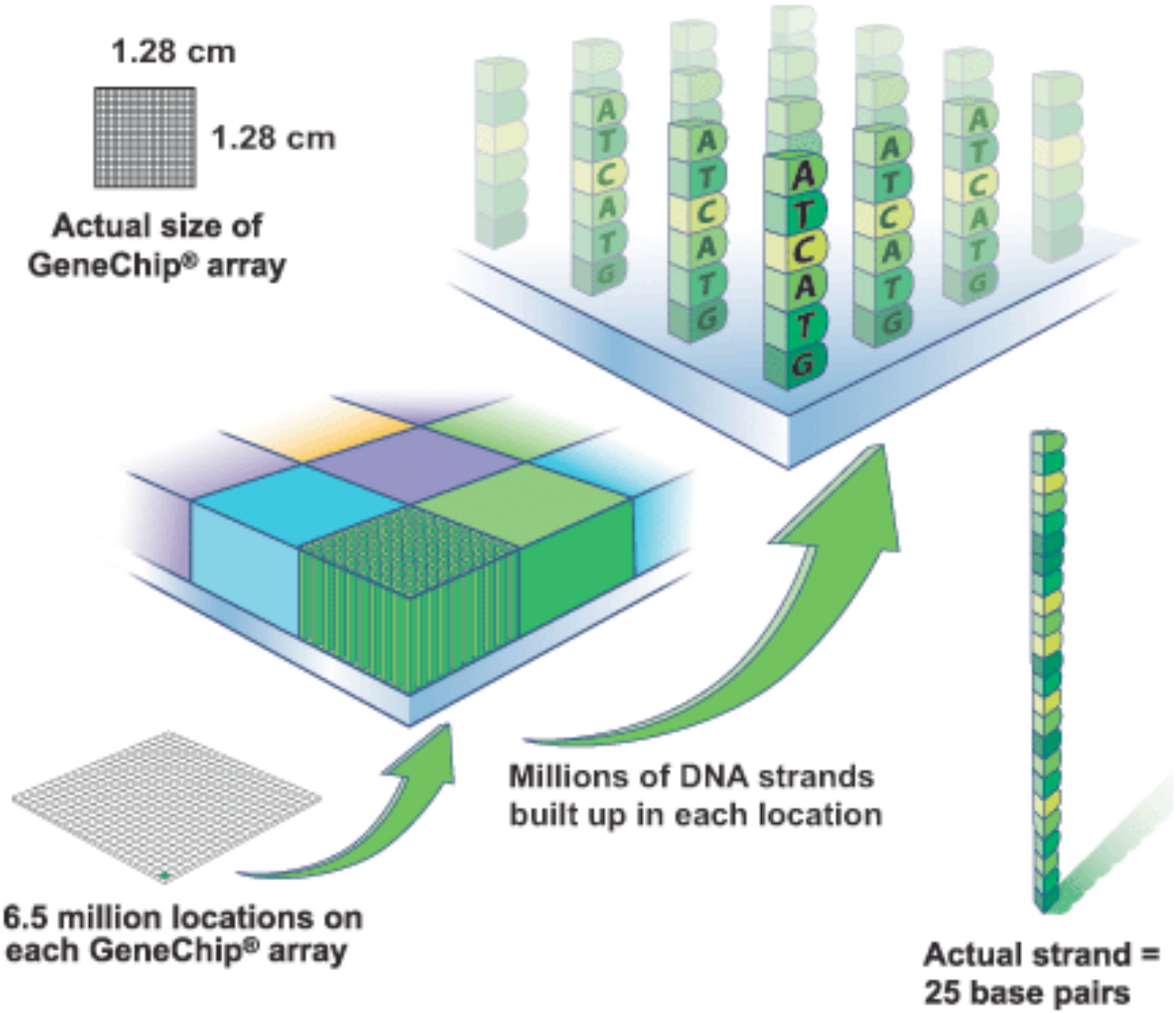
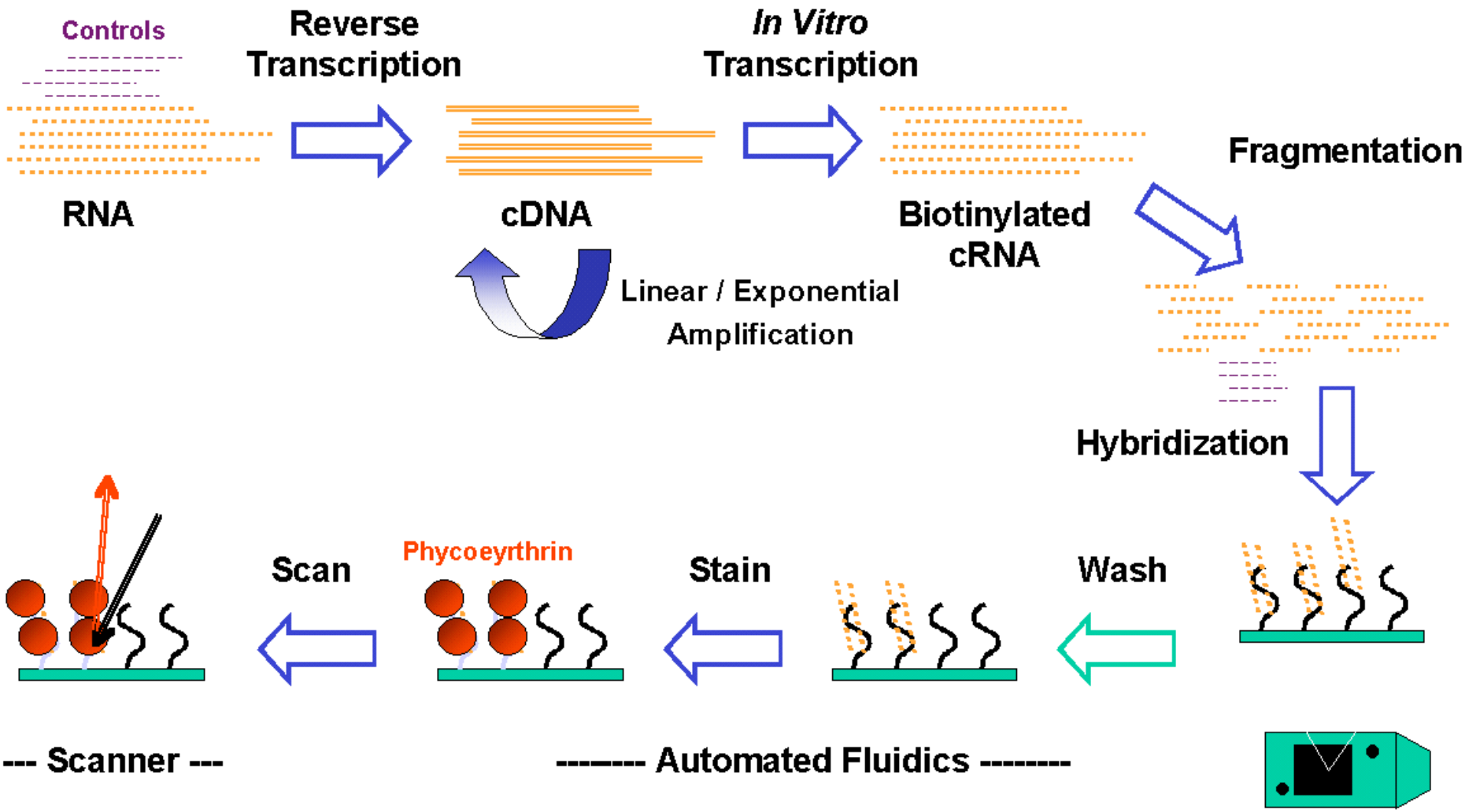


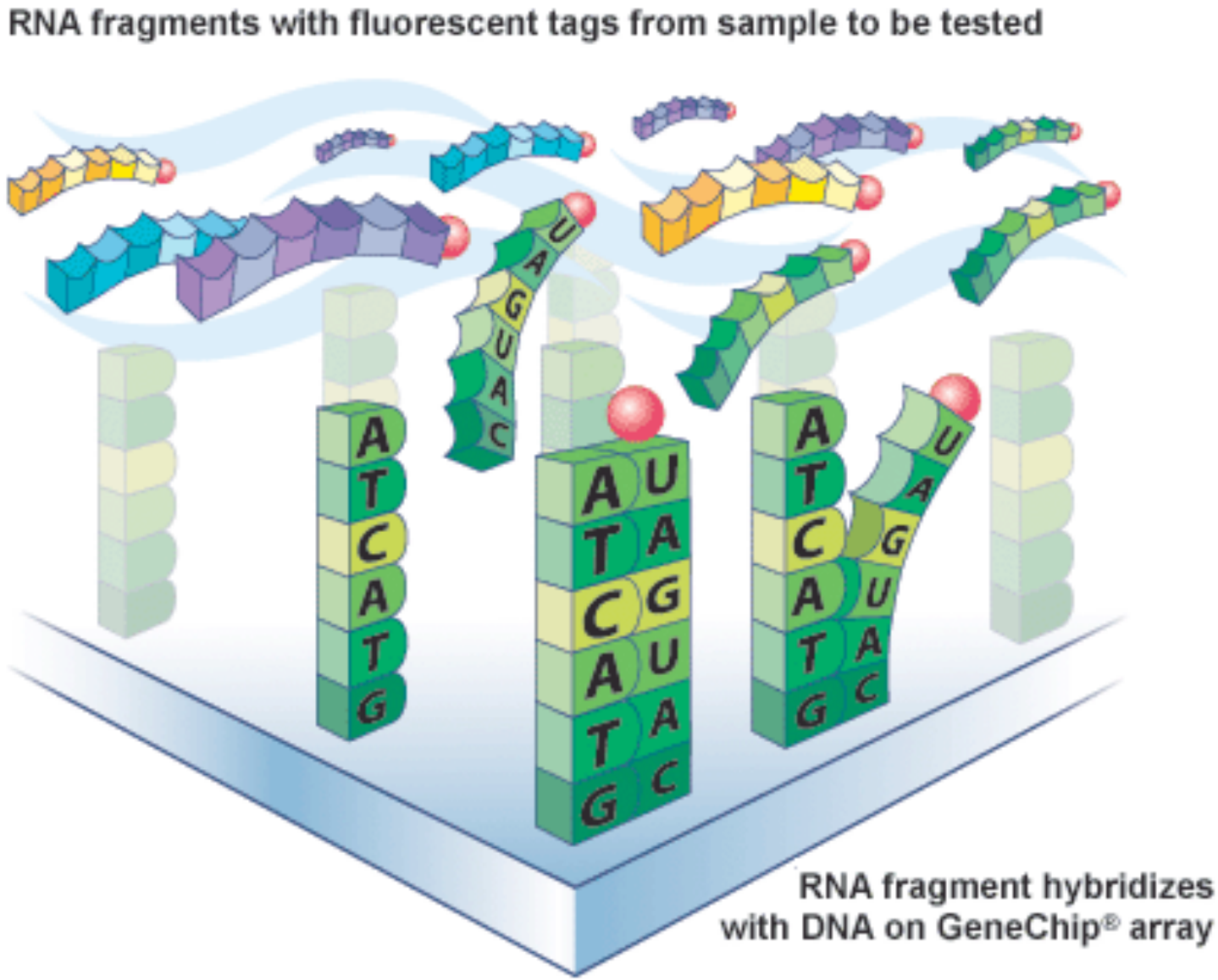
Figure 1. Synthesis Confirmation.

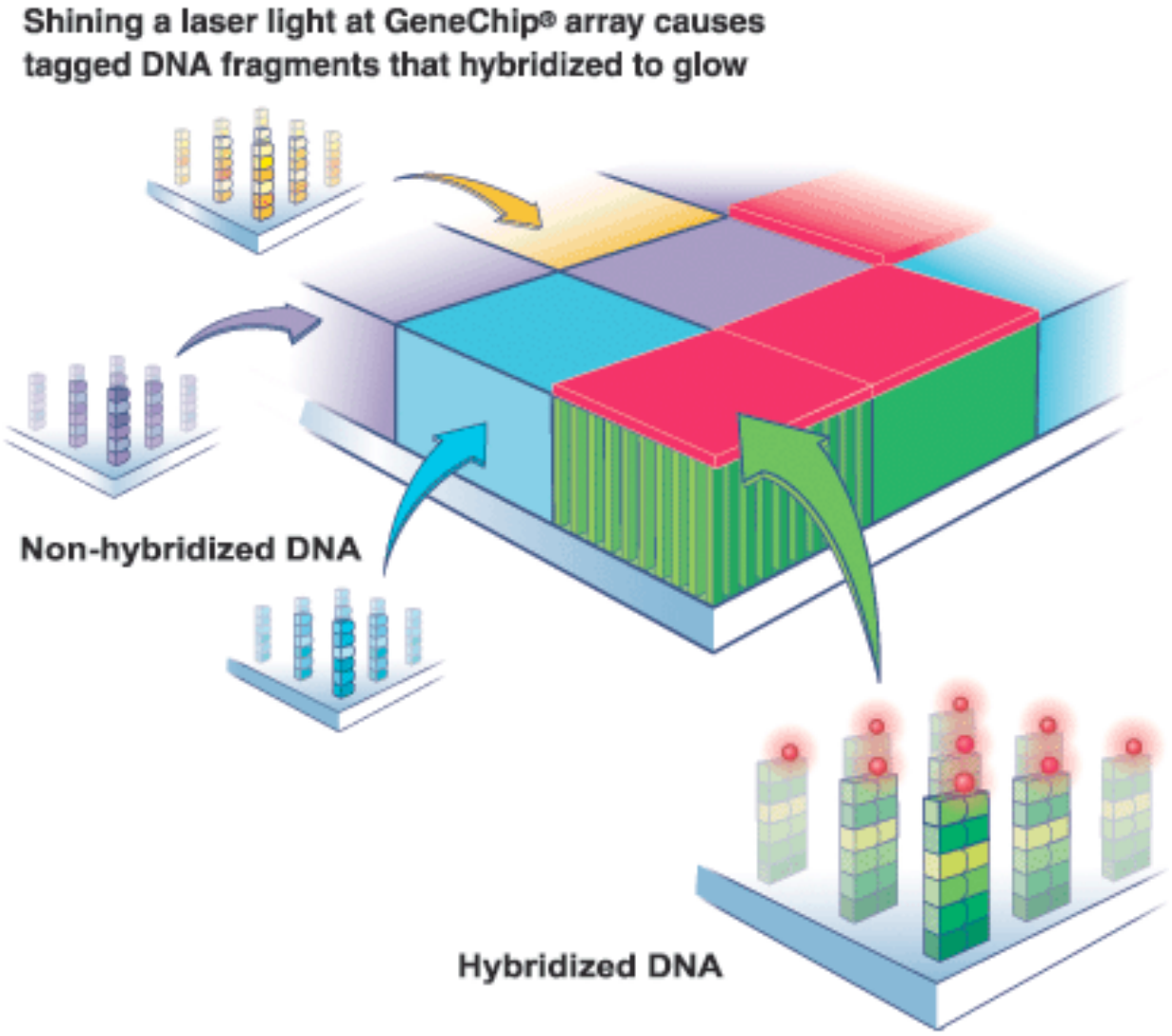
Mask Cycles	1	2	3	4	5	6	7	8	Probes Synthesized
	A	C	G	T	A	C	G	T	
Probe 1	A	C	-	T	-	C			A C T C
Probe 2			G	-	A	-	G	T	G A G T
Probe 3	A	-	G	-	A	-	G		A G A G
Probe 4		C	-	T	-	C	-	T	C T C T

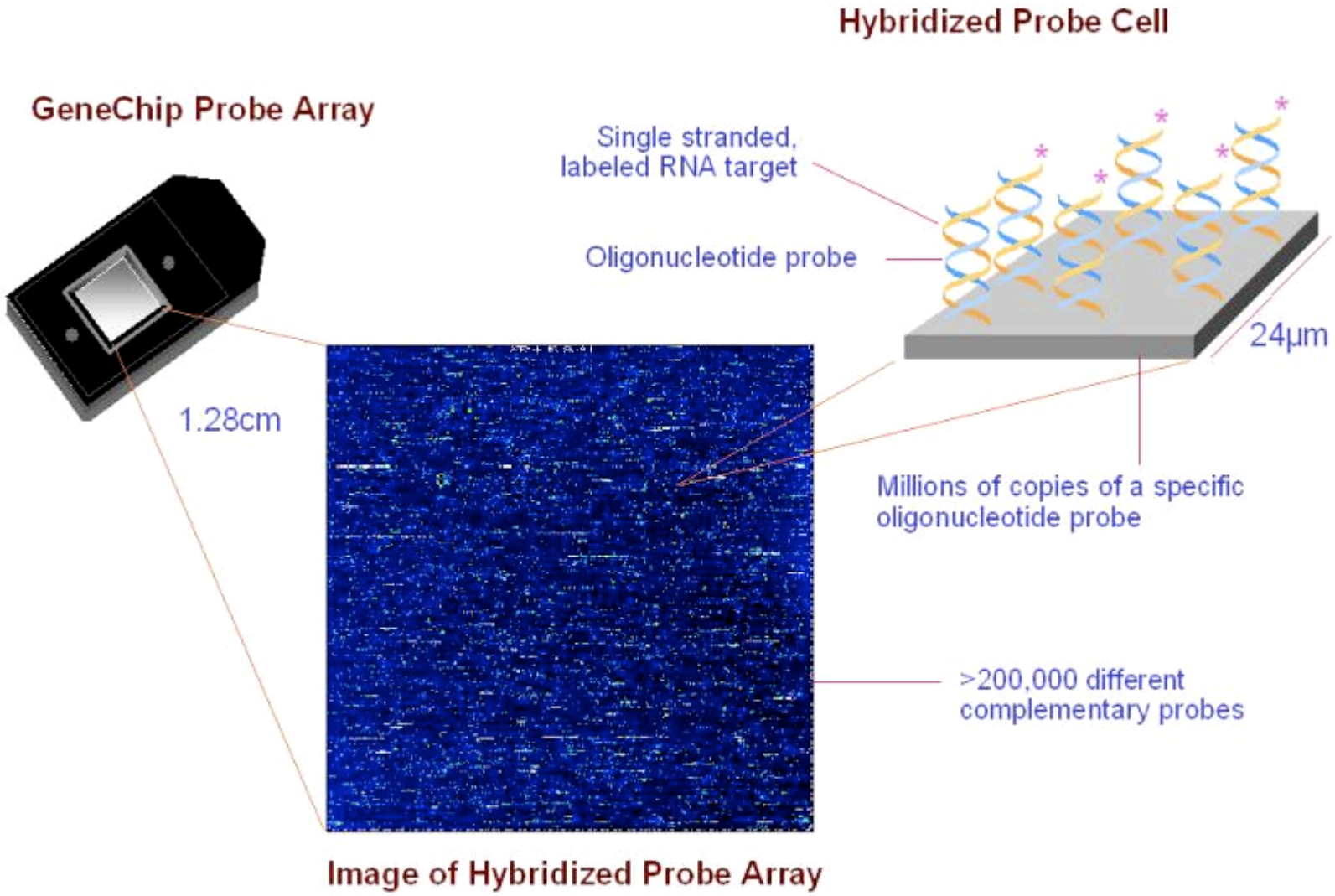


Affymetrix Technology

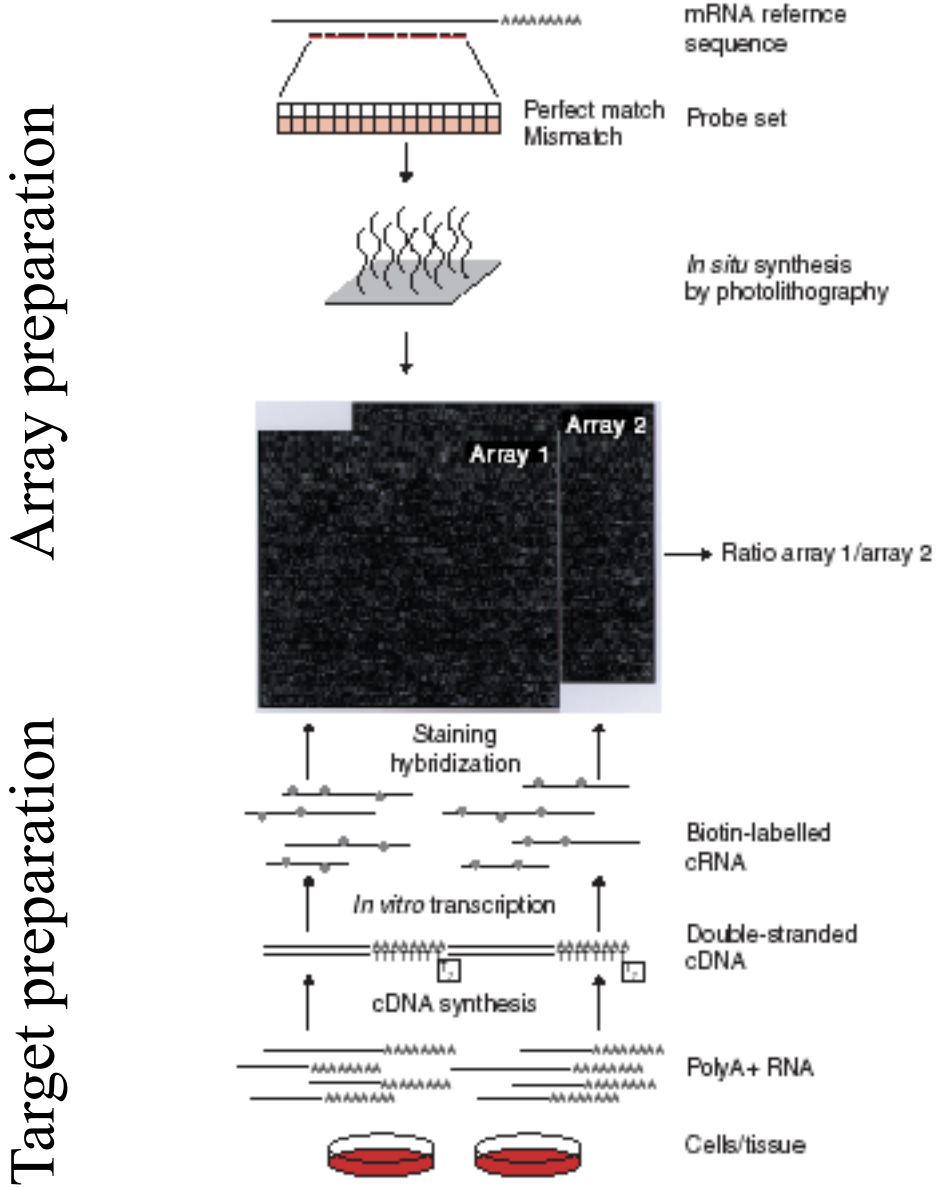


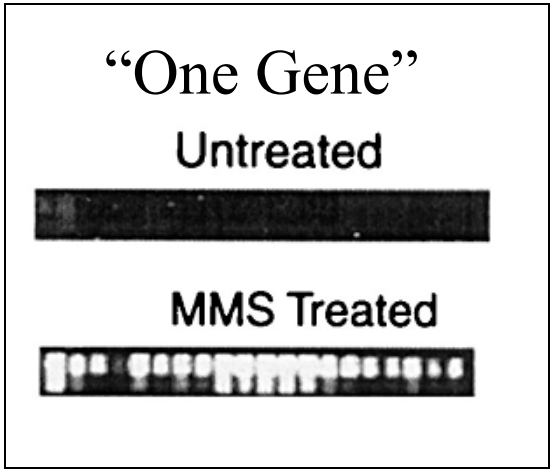


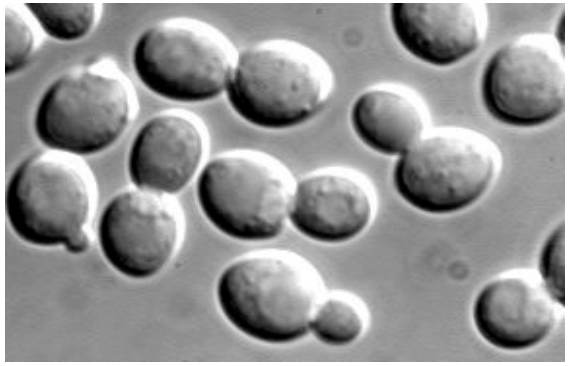



Hybridized Probe Cell
GeneChip Probe Array
Single stranded,
labeled RNA target
Oligonucleotide probe
24µm
1.28cm
Millions of copies of a specific
oligonucleotide probe
>200,000 different
complementary probes
Image of Hybridized Probe Array

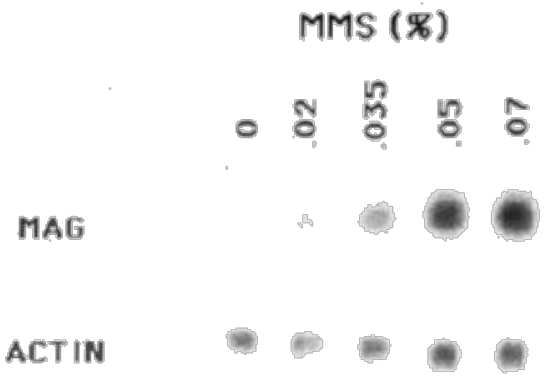
Oligonucleotide "One Color Chips"

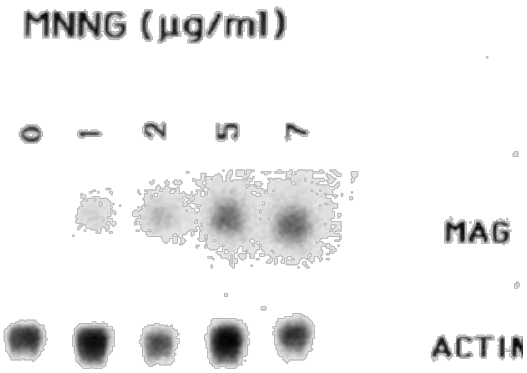


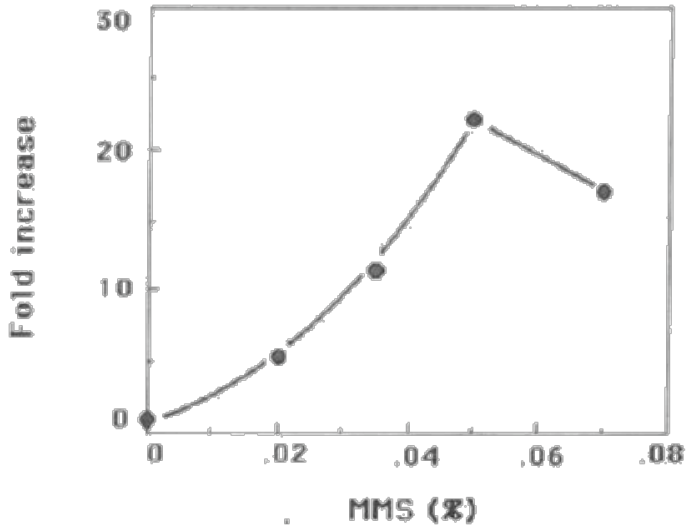


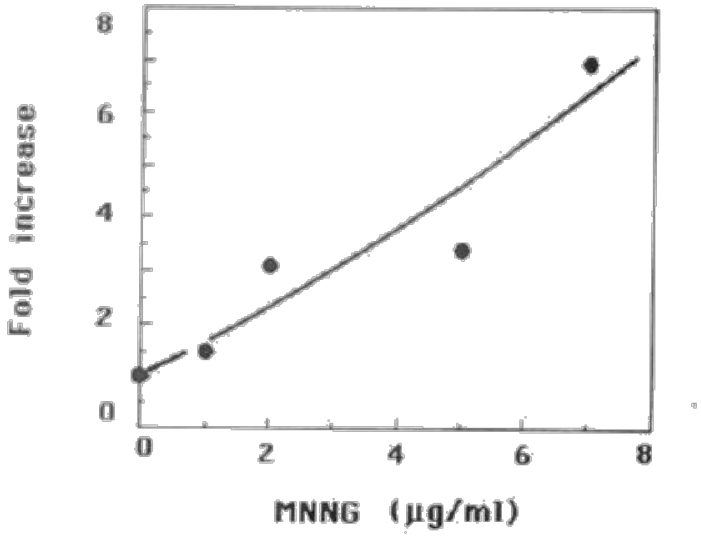


Yeast cells treated with DNA damaging agents like those in tobacco smoke

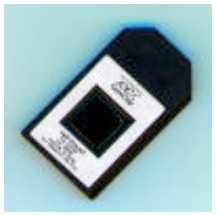




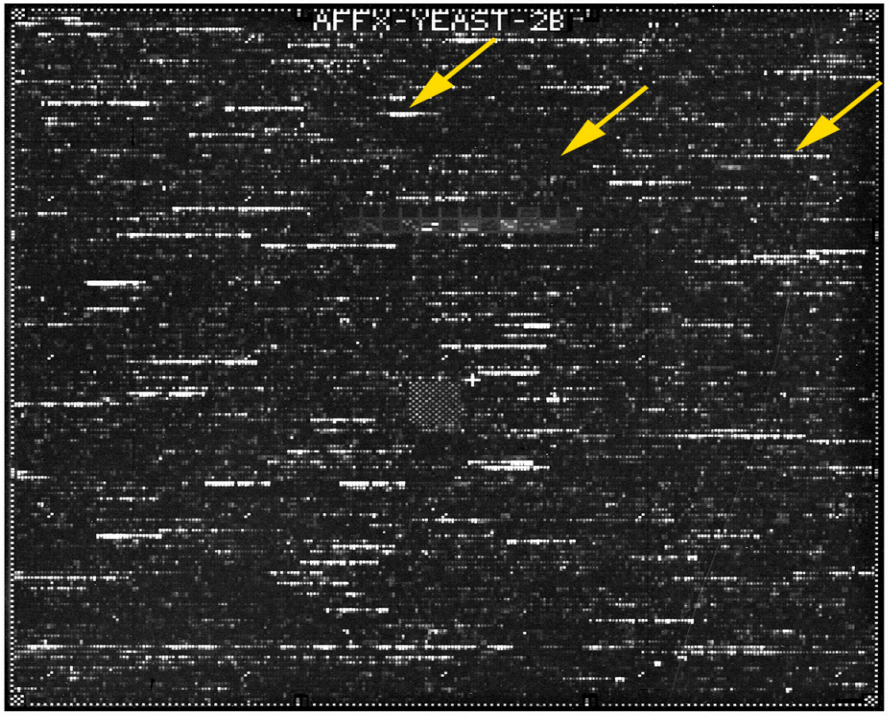


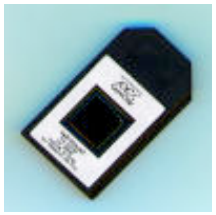


Affymetrix Oligonucleotide Based Expression Arrays

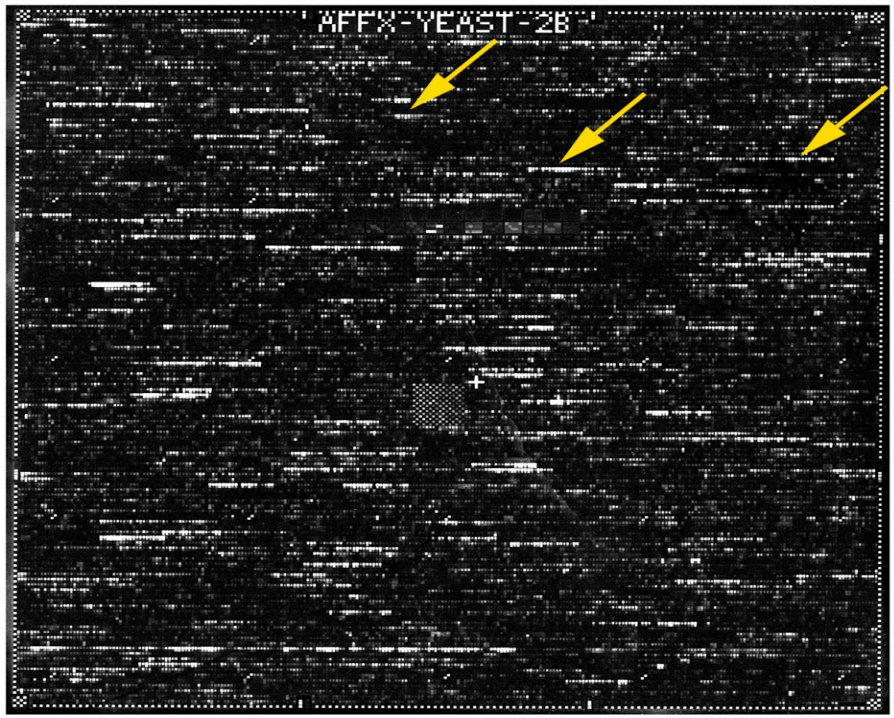


Untreated



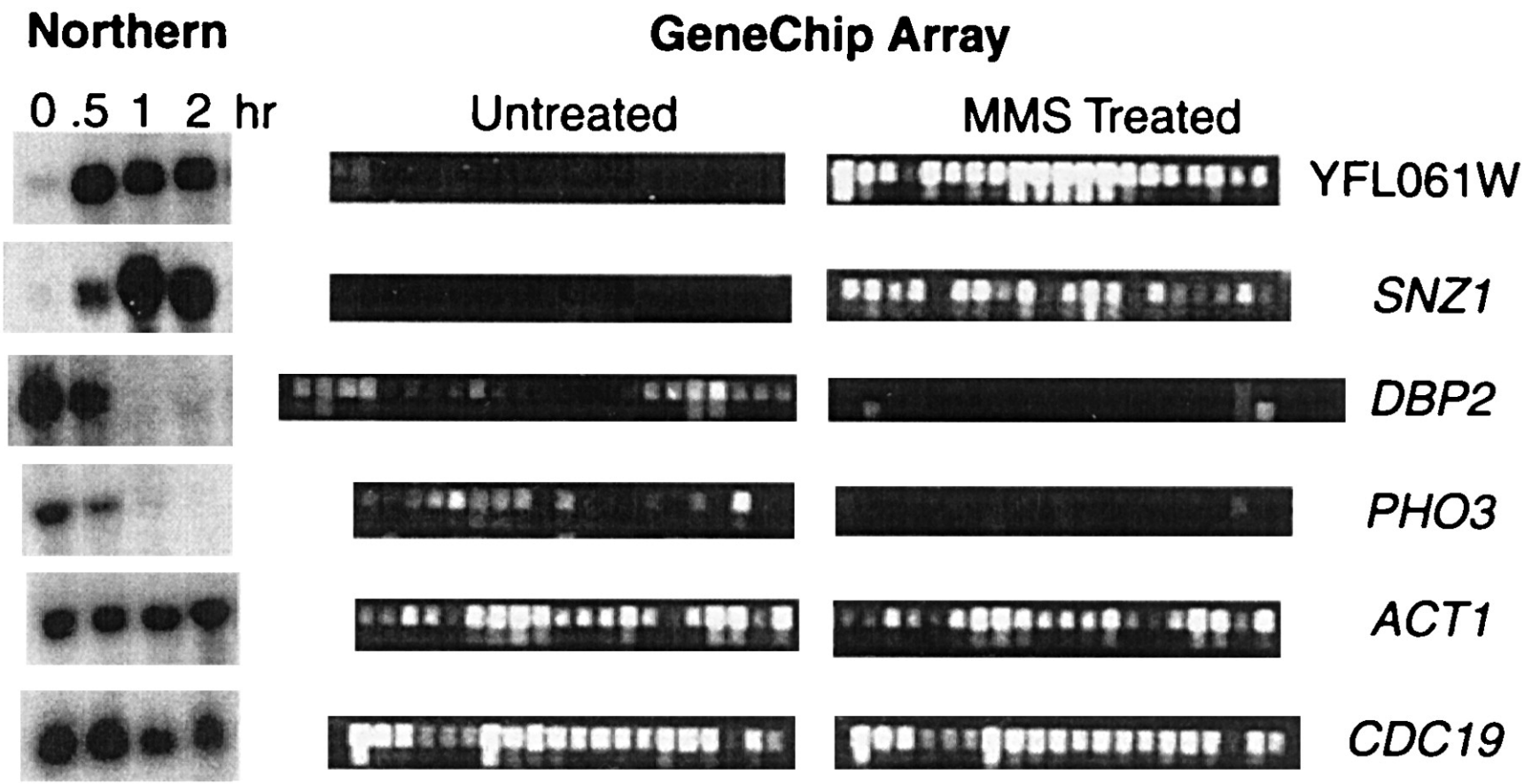


MMS treated

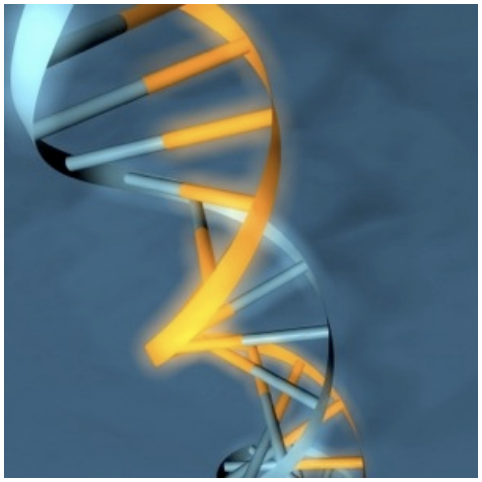


Jelinsky and Samson,1999

Northern Blot vs. Affymetrix Chip



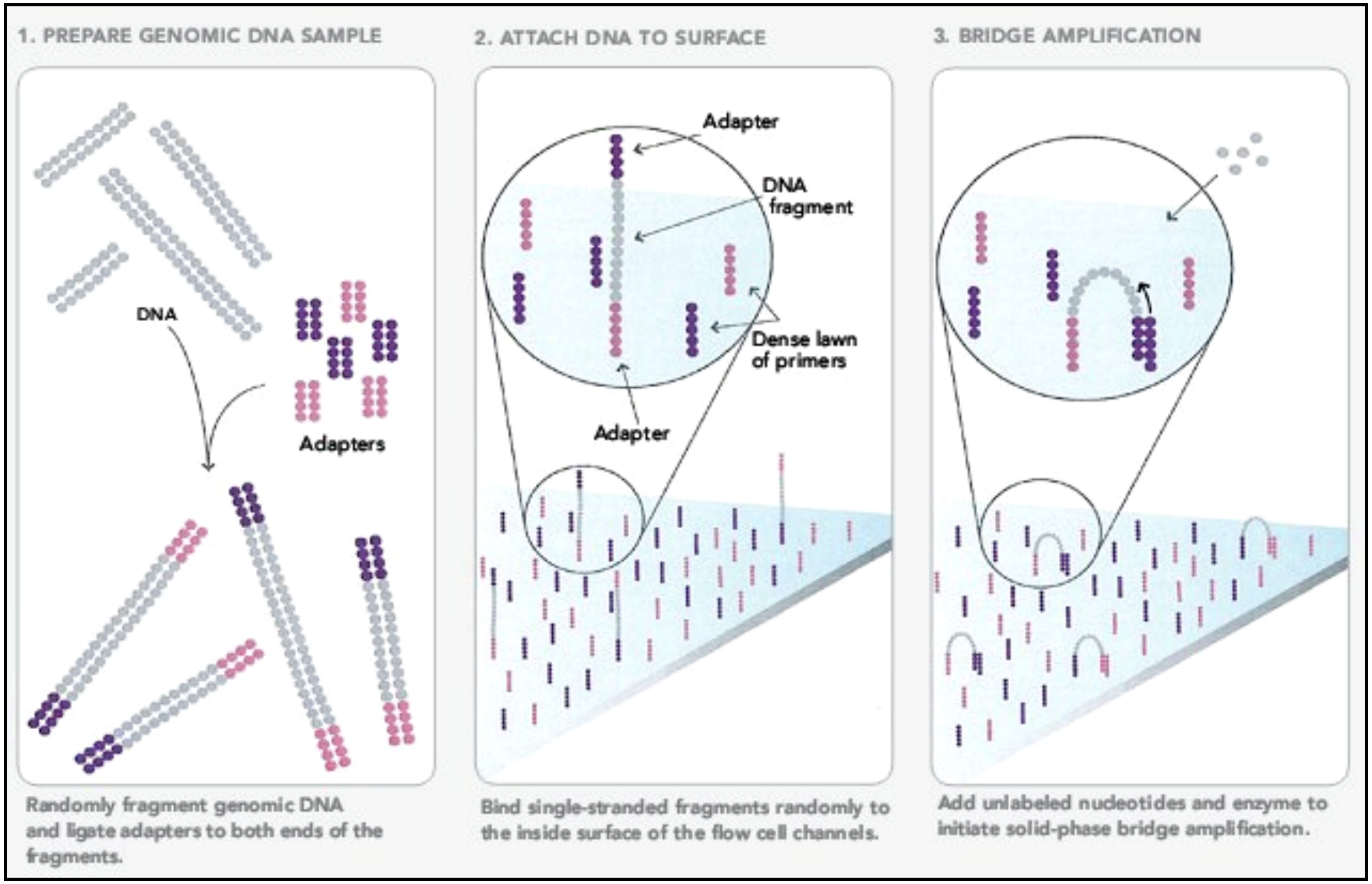
'Next Generation' Sequencing Technologies



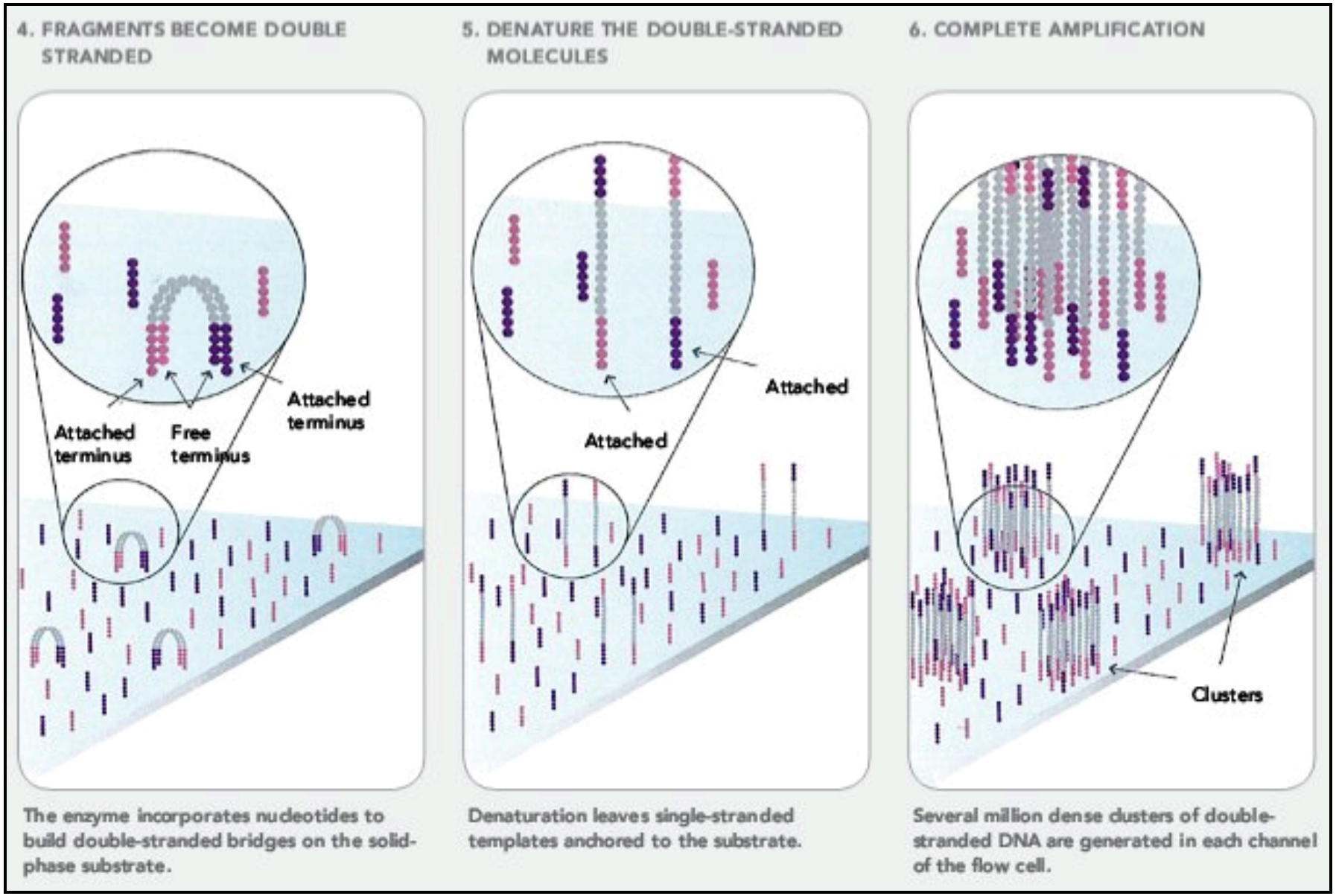
Platform	Amplification	Sequencing
Harvard/Danaher/Agencourt/ABI	Emulsion PCR	Ligase
454 Life Sciences / Roche	Emulsion PCR	Polymerase - pyrosequencing
Solexa/Illumina	Bridge PCR	Polymerase - reversible terminator
Helicos	None	Polymerase - single base extension
Pacific Biosciences	None	Polymerase - active site occupancy

Shendure et al. Curr. Protocols Mol. Biol. 2008

Solexa/Illumina DNA Sequencing Platform



Solexa/Illumina DNA Sequencing Platform



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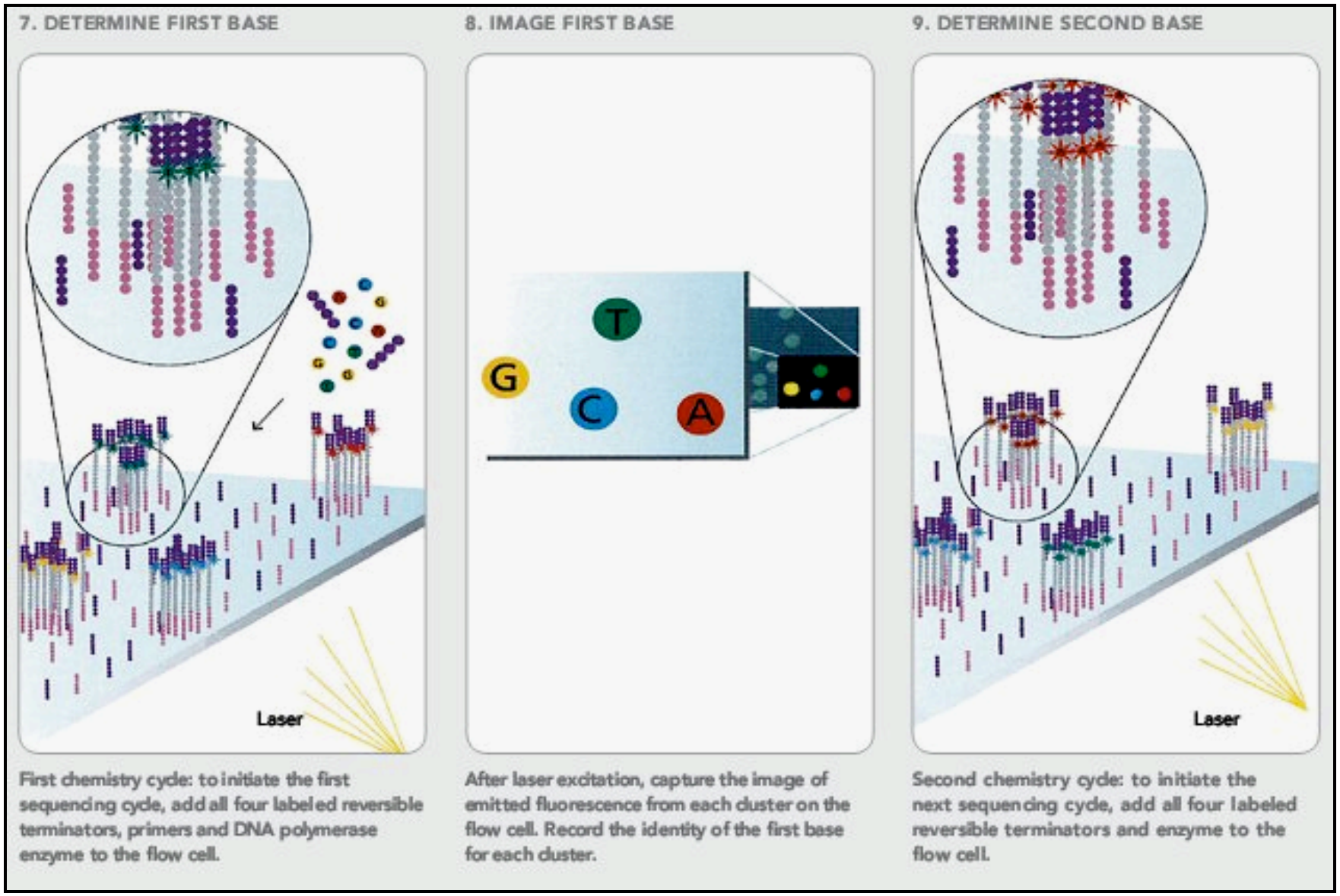
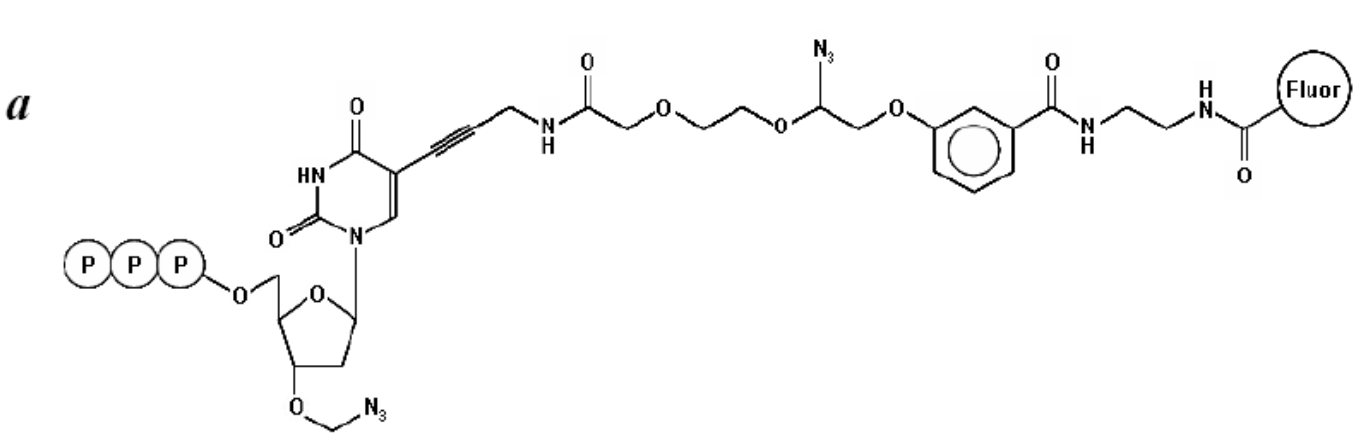
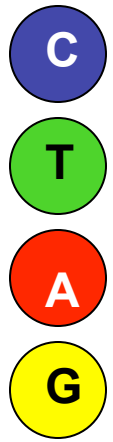
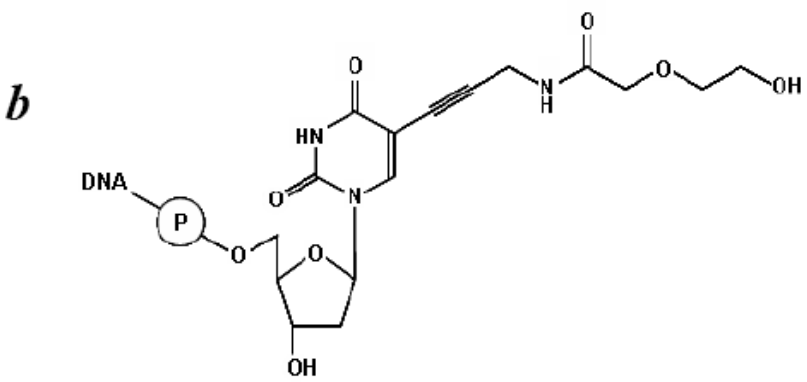
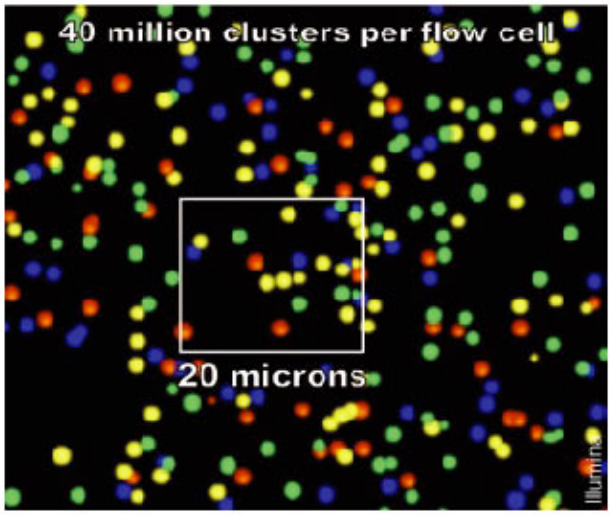


Reversible Terminator Chemistry



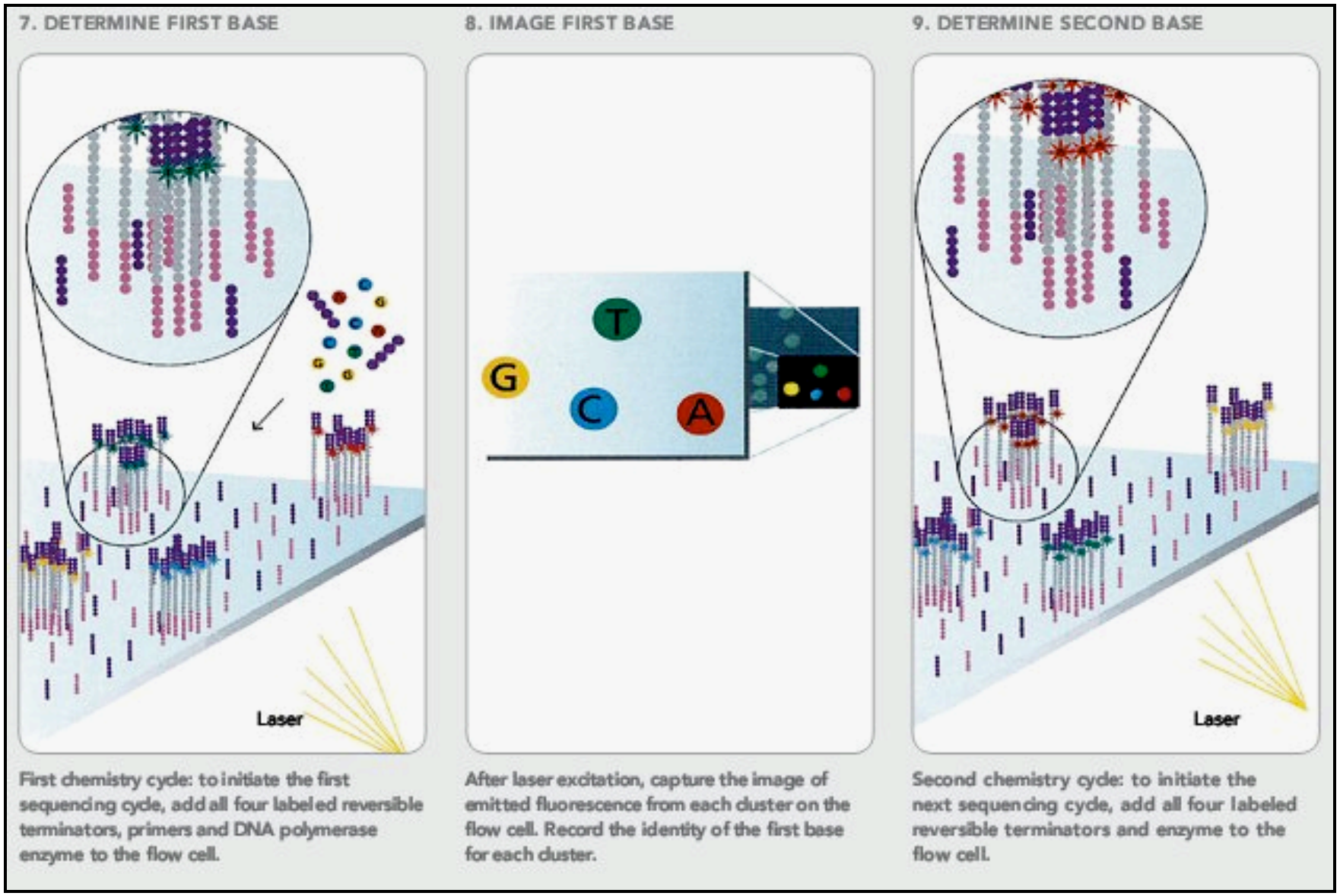






Bentley et al. Nature 456, 53-, 2008.

Solexa/Illumina DNA Sequencing Platform



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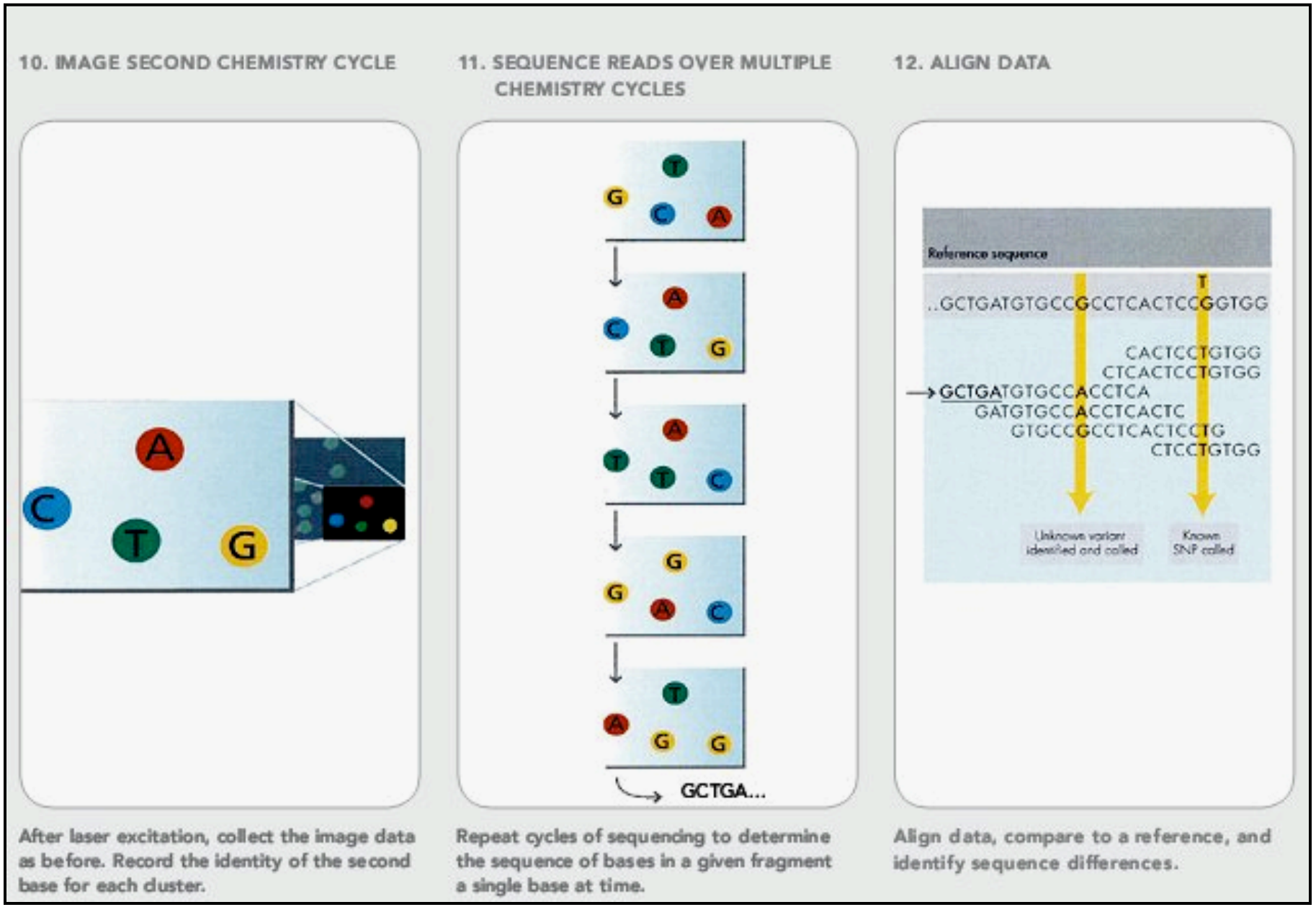
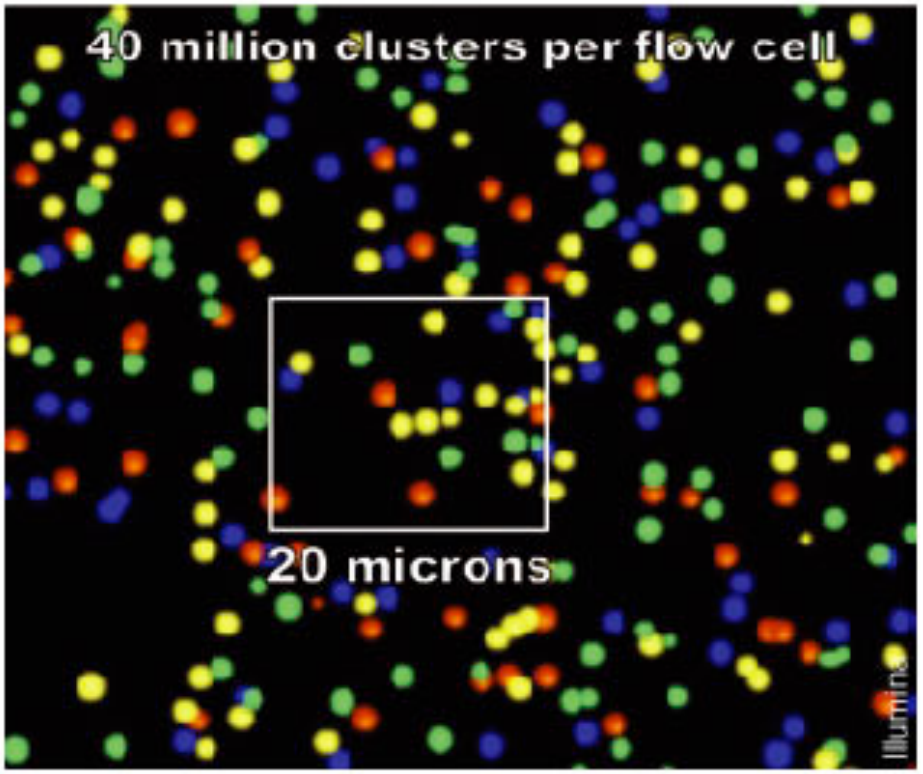




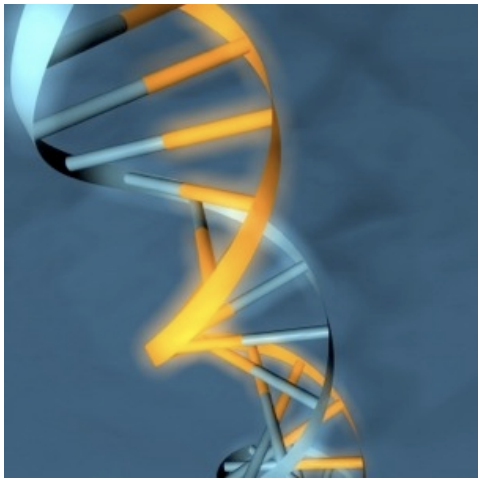
In the MIT BioMicro Center

~70bp "reads"

7 million reads per channel (flow cell)!!!

From 7 million sequences can count/ calculate the relative abundance of each original mRNA species – i.e. the transcriptional profile

'Next Generation' Sequencing Technologies



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http://www.pacificbiosciences.com/video_lg.html

Shendure et al. Curr. Protocols Mol. Biol. 2008