20.109

LABORATORY FUNDAMENTALS IN BIOLOGICAL ENGINEERING

MODULE 2

EXPRESSION ENGINEERING

Lecture # 3

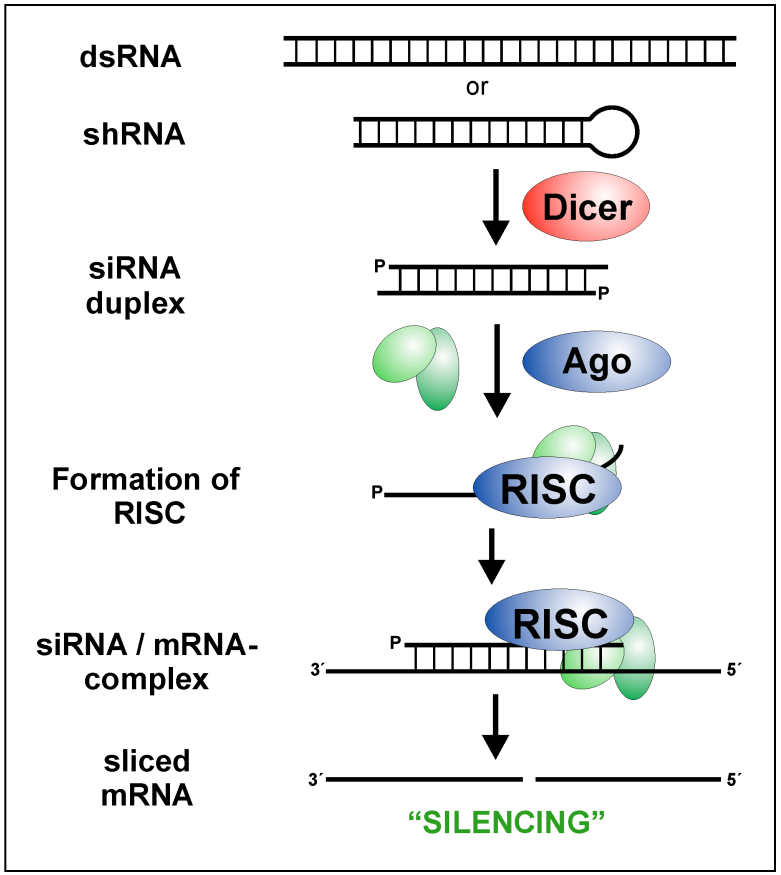
Leona Samson

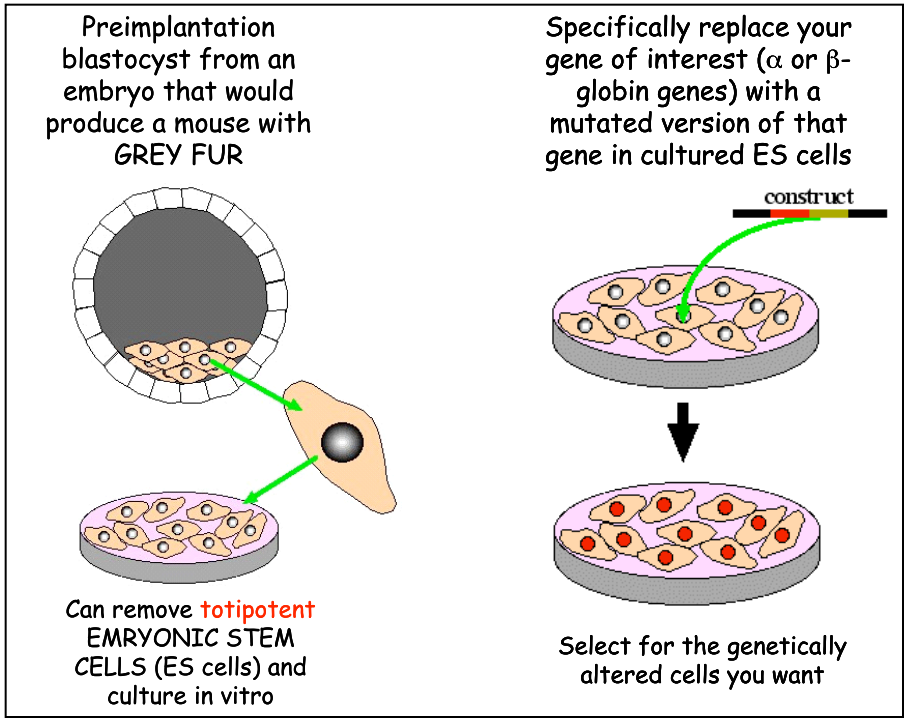
March 31st 2009

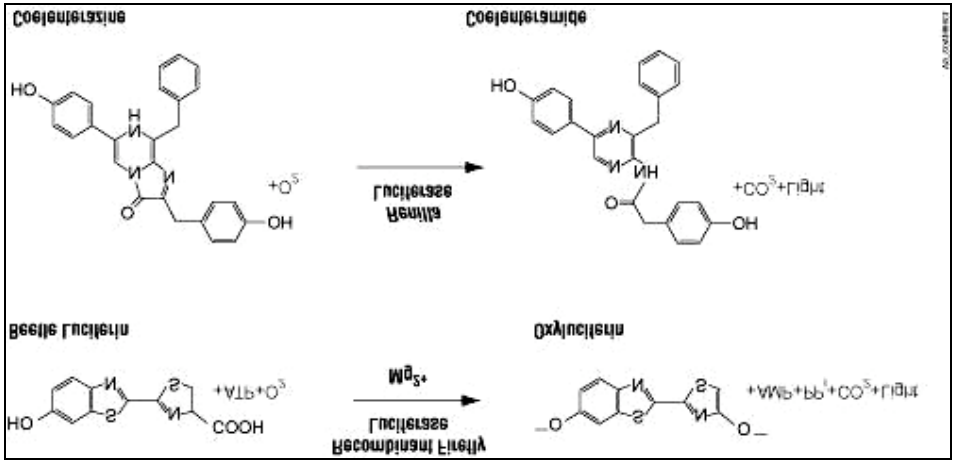
Snapshot of the next four weeks

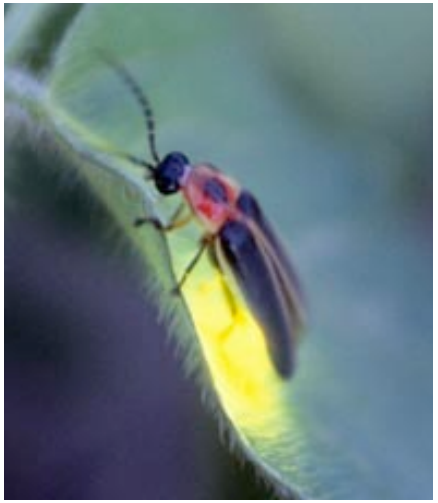
We will eliminate the expression of various genes using

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


dsRNA
or
shRNA
Dicer
siRNA duplex
P
P
Ago
Formation of RISC
P
RISC
siRNA / mRNA-complex
RISC
P
3´
5´
sliced mRNA
3´
5´
"SILENCING"


Preimplantation blastocyst from an embryo that would produce a mouse with GREY FUR
Can remove totipotent EMRYONIC STEM CELLS (ES cells) and culture in vitro
Specifically replace your gene of interest (α or β-globin genes) with a mutated version of that gene in cultured ES cells
construct
Select for the genetically altered cells you want





Snapshot of the next four weeks

We will eliminate the expression of various genes using

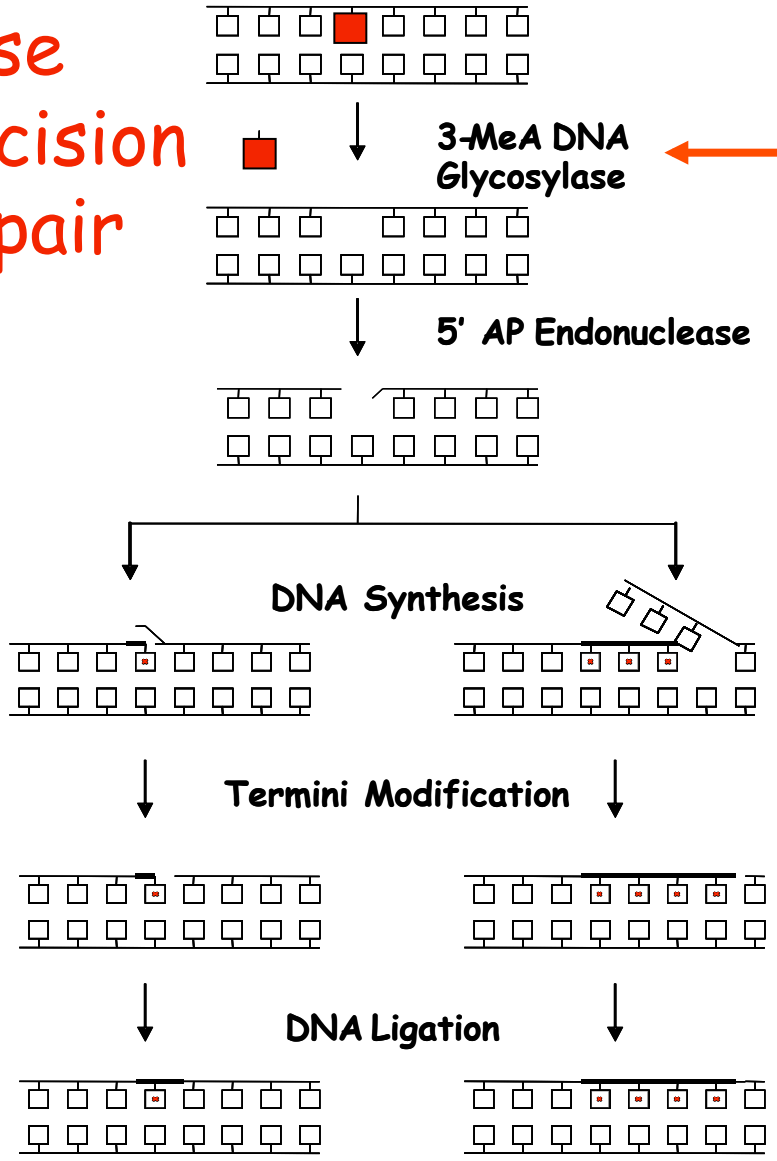
(i) RNA interference technology
(ii) Cultured mouse ES cells
(iii) Chemiluminescent proteins
(iv) DNA microarrays

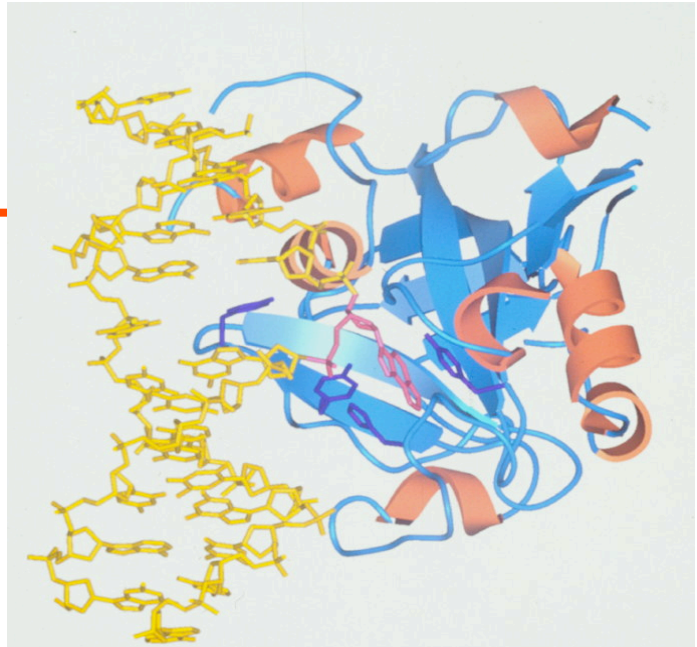
Various Genes

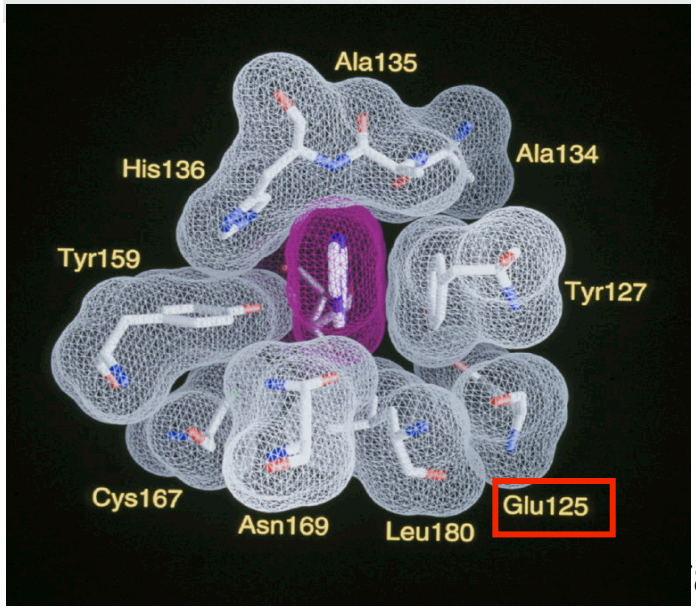
Mpg – Methyl Purine Glycosylase	DNA Repair gene
Trp 53 - transformation related protein	Transcription factor and tumor suppressor
Polr2d – RNA polymerase II polypeptide D	Non-essential subunit of RNA polymerase II
Tada2L – transcriptional adaptor 2	Transcriptional activator by chromatin remodeling
Nanog – transcription factor	ES cell-specific transcription factor

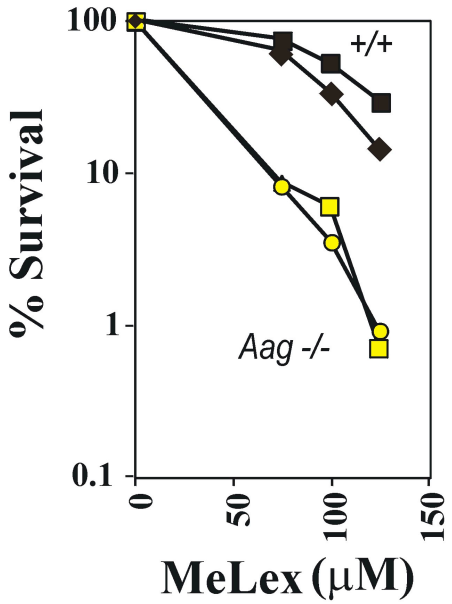
Mpg/Aag: Mammalian 3MeA DNA Glycosylase

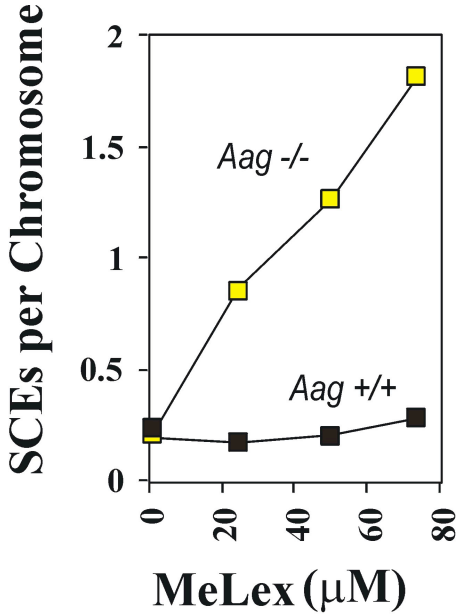
Base Excision Repair

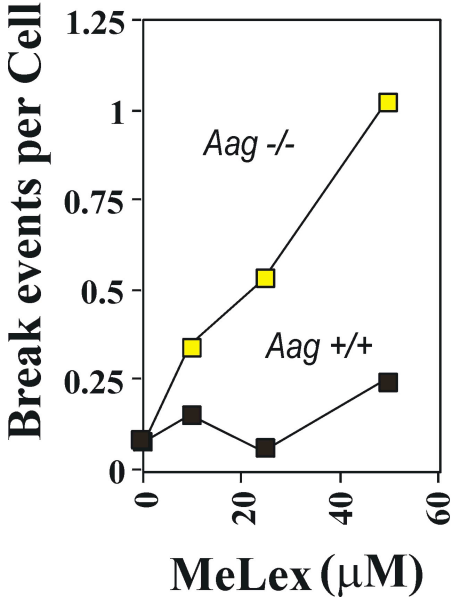


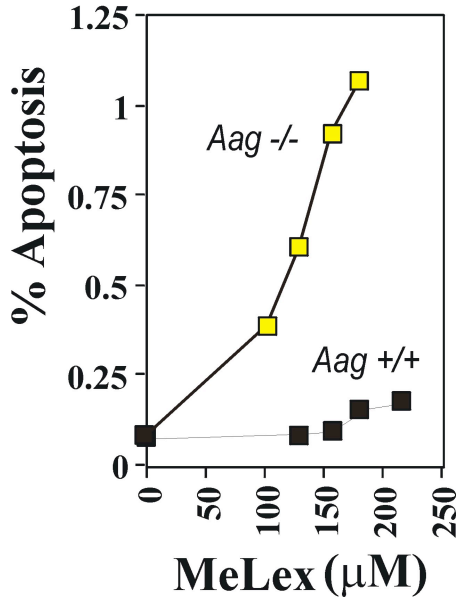








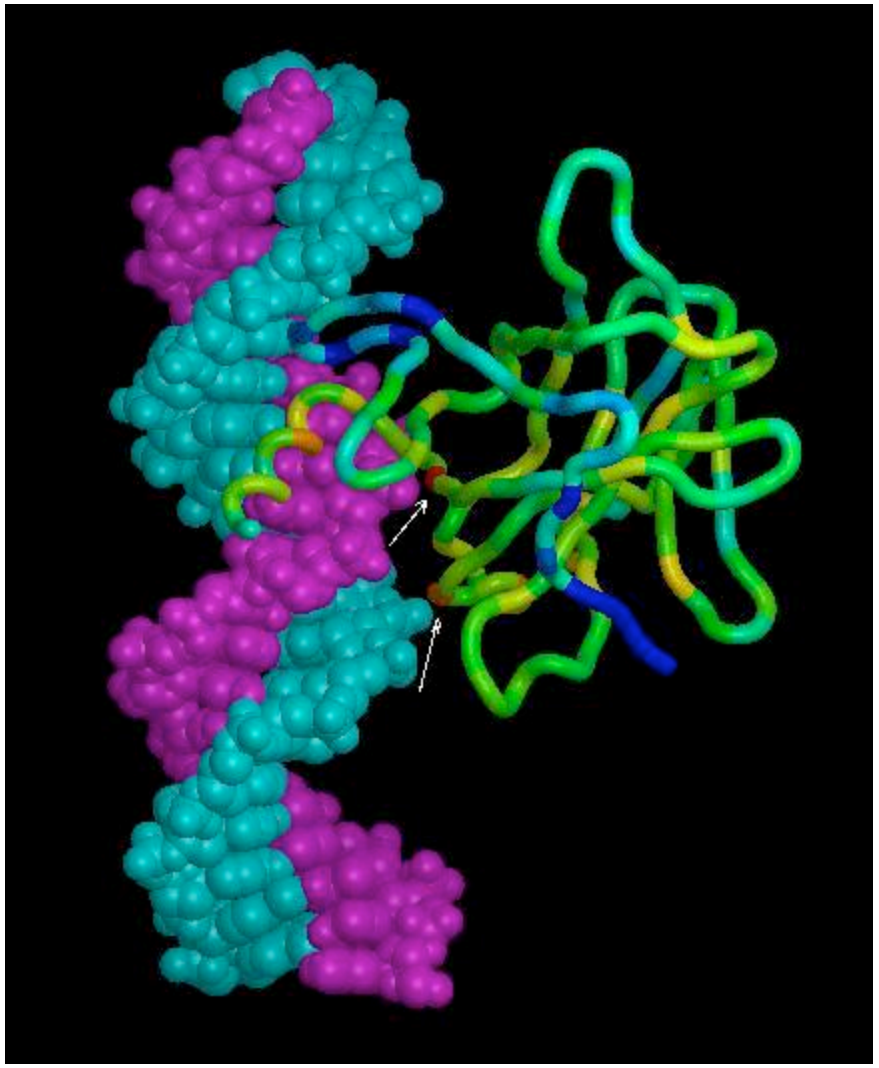


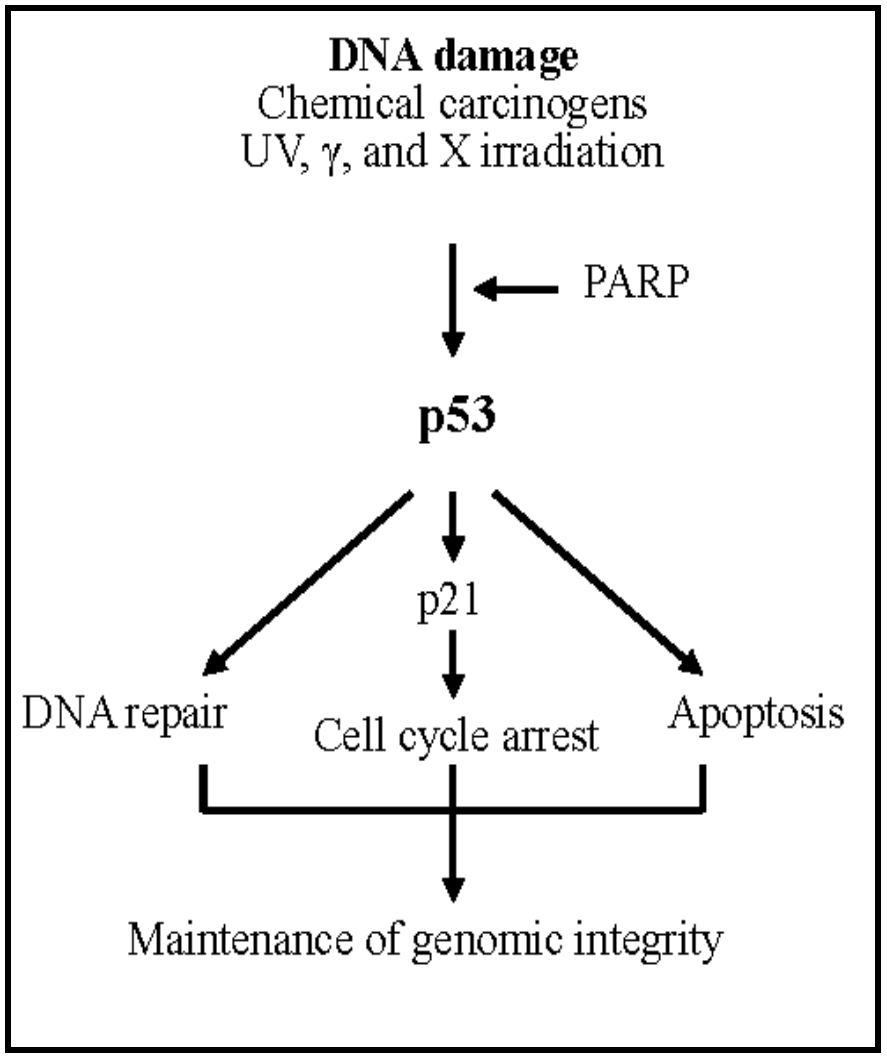


Aag -/- Mouse ES cells are sensitive to methylating agents MMS and MeLex that produce 3MeA DNA lesions

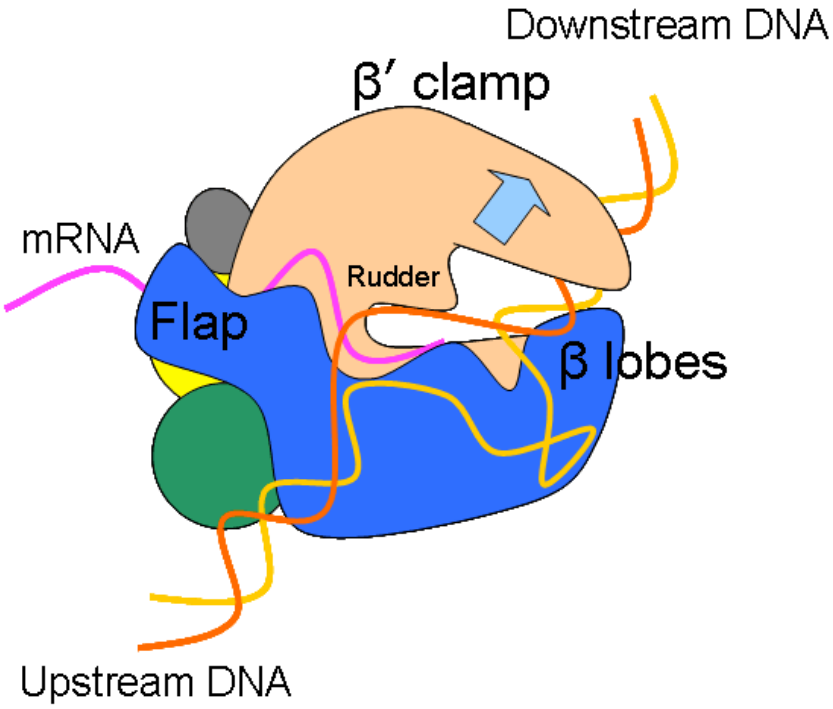
Bevin Engelward

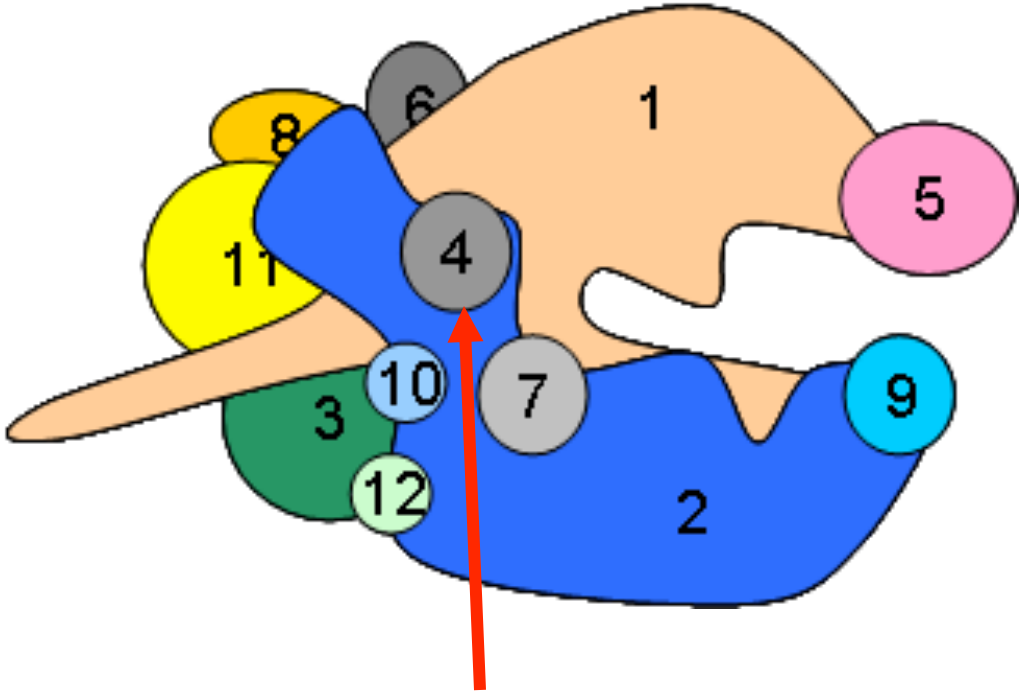
Trp53 – p53 – Transcription factor and tumor suppressor





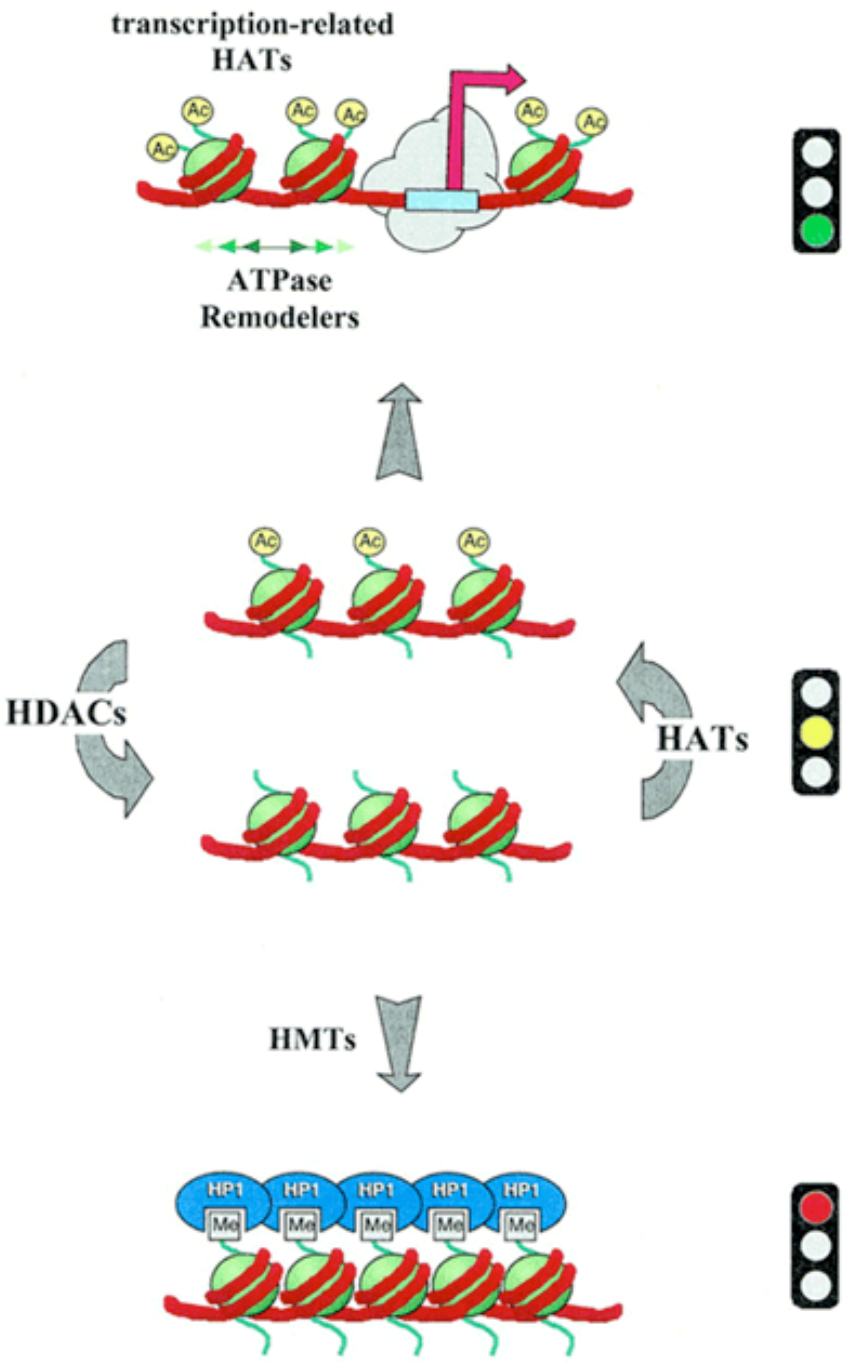
Polr2d – RNA polymerase II polypeptide D





Non-essential subunit of RNA polymerase II

http://images.google.com/imgres?imgurl=http://www.steve.gb.com/images/science/rna_polymerase_ii.png&imgrefurl=http://www.steve.gb.com/science/transcription.html&usg=__yYwIZurQR0P7pwFMUJzpTlyZfM8=&h=199&w=331&sz=8&hl=en&start=6&um=1&tbnid=qJKtKx0oraa0NM:&tbnh=72&tbnw=119&prev=/images%3Fq%3DRNA%2Bpol%2BII%2Bsubunits%26hl%3Den%26rls%3Dcom.microsoft:en-us:IE-SearchBox%26rlz%3D1I7SUNA_en%26sa%3DN%26um%3D1



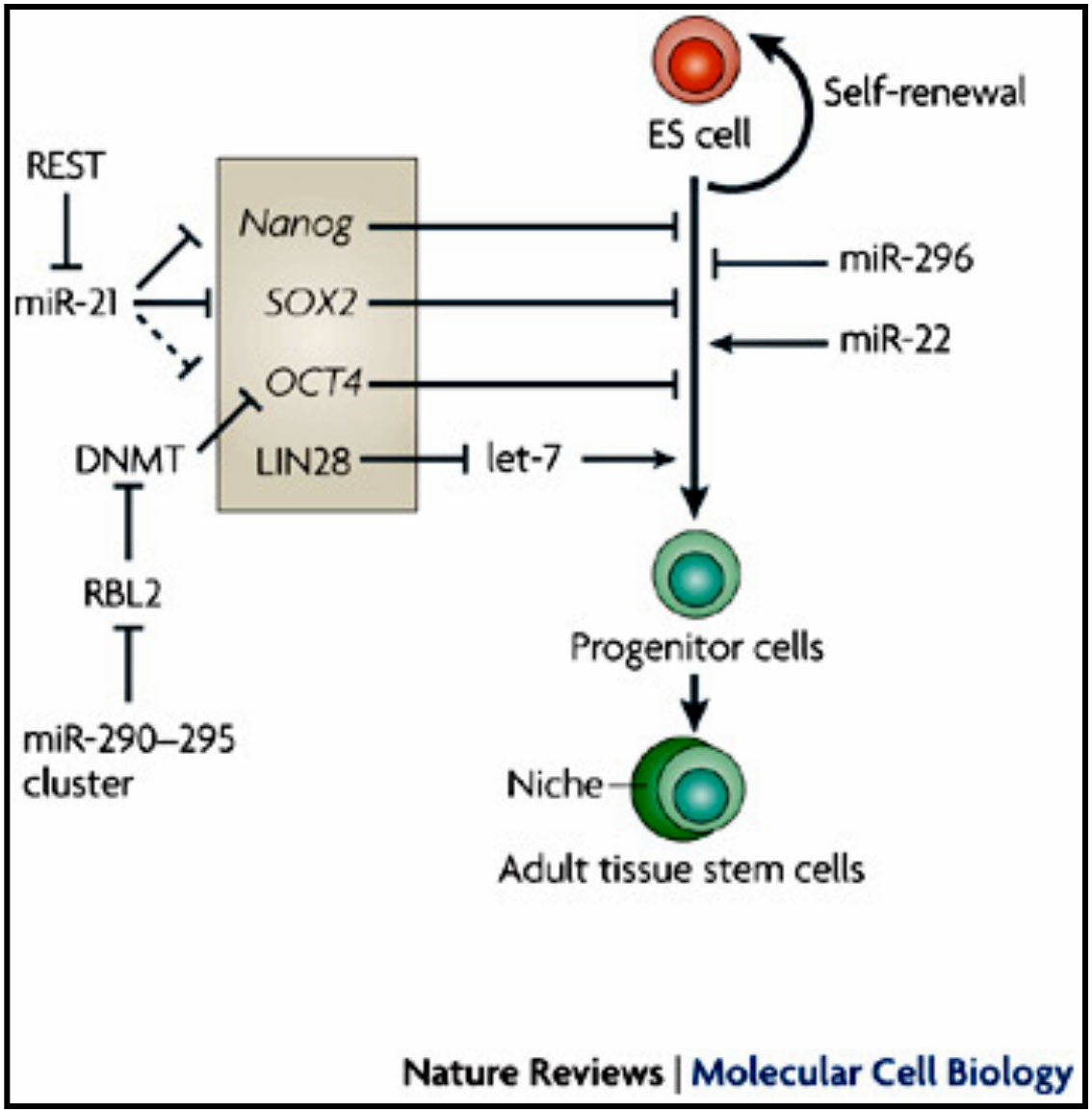
Tada2L – transcriptional adaptor 2, activates by chromatin remodeling

Tada2L is part of a Histone Acetylase (HAT) complex

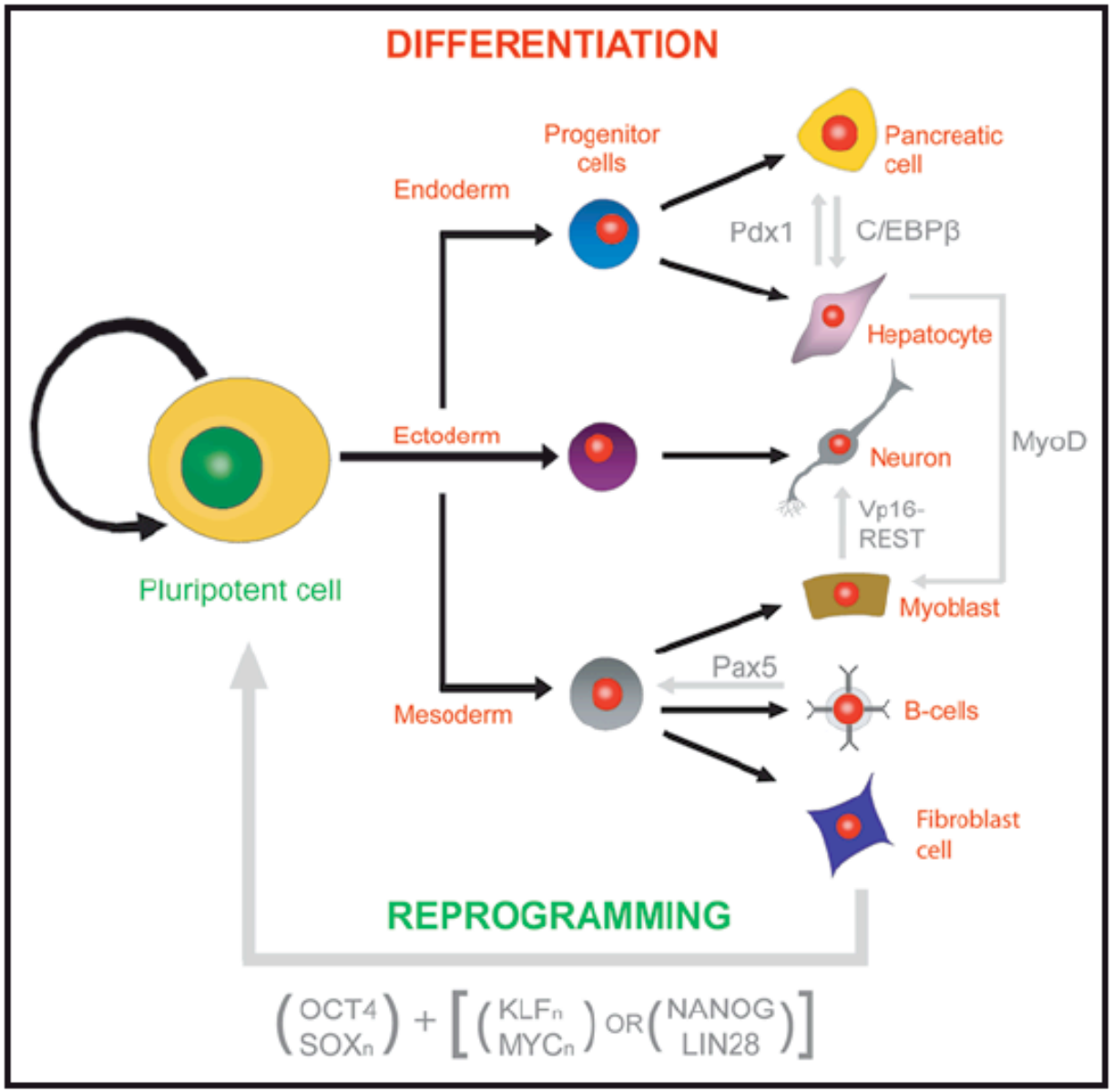
HDAC – Histone Deacetylase

HMT – Histone methyltranferase

Nanog – transcription factor



Nanog – transcription factor



Tír na nÓg - Land of the Ever-Young

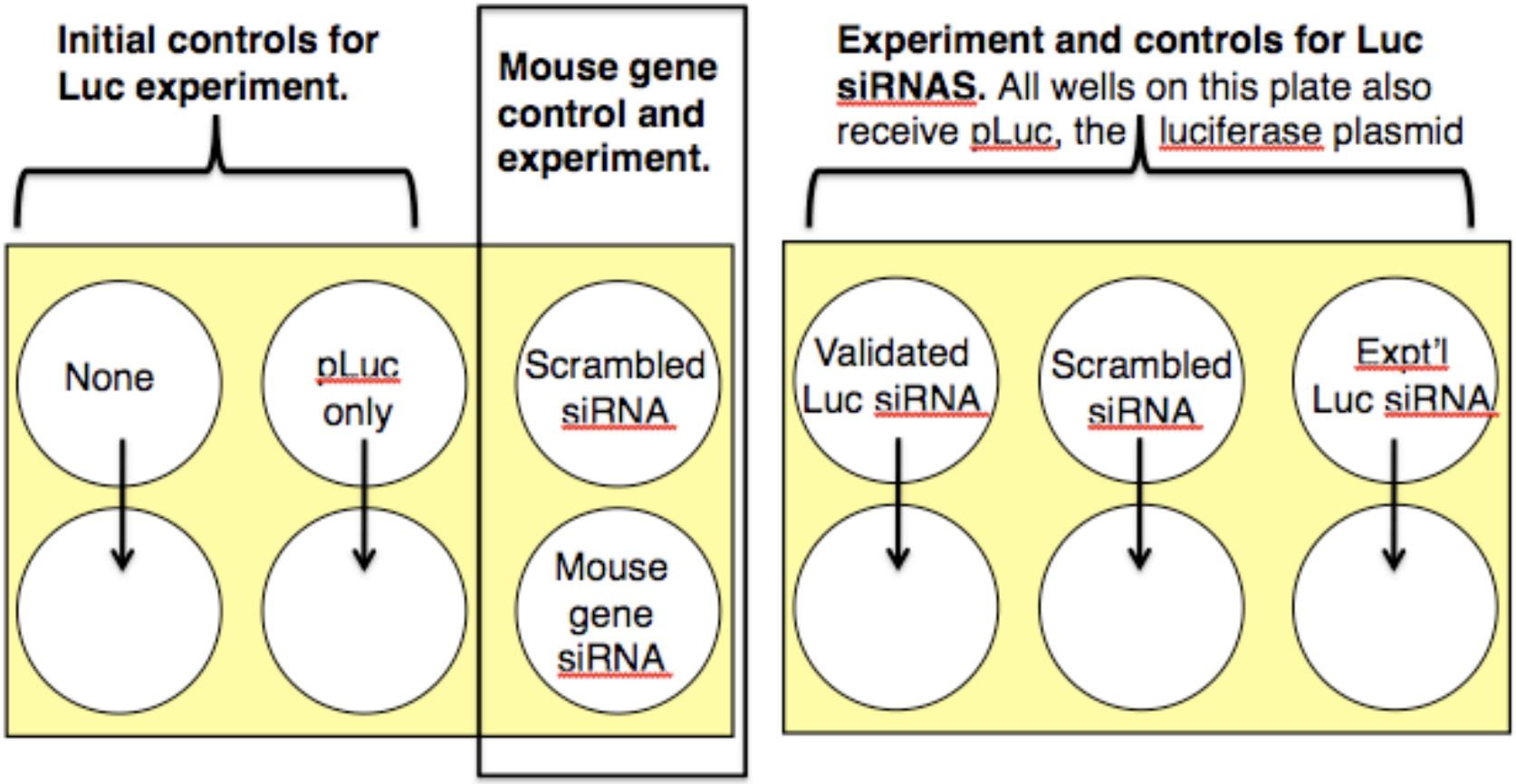
A Mythological Celtic Land where fairies live

Snapshot of the next four weeks

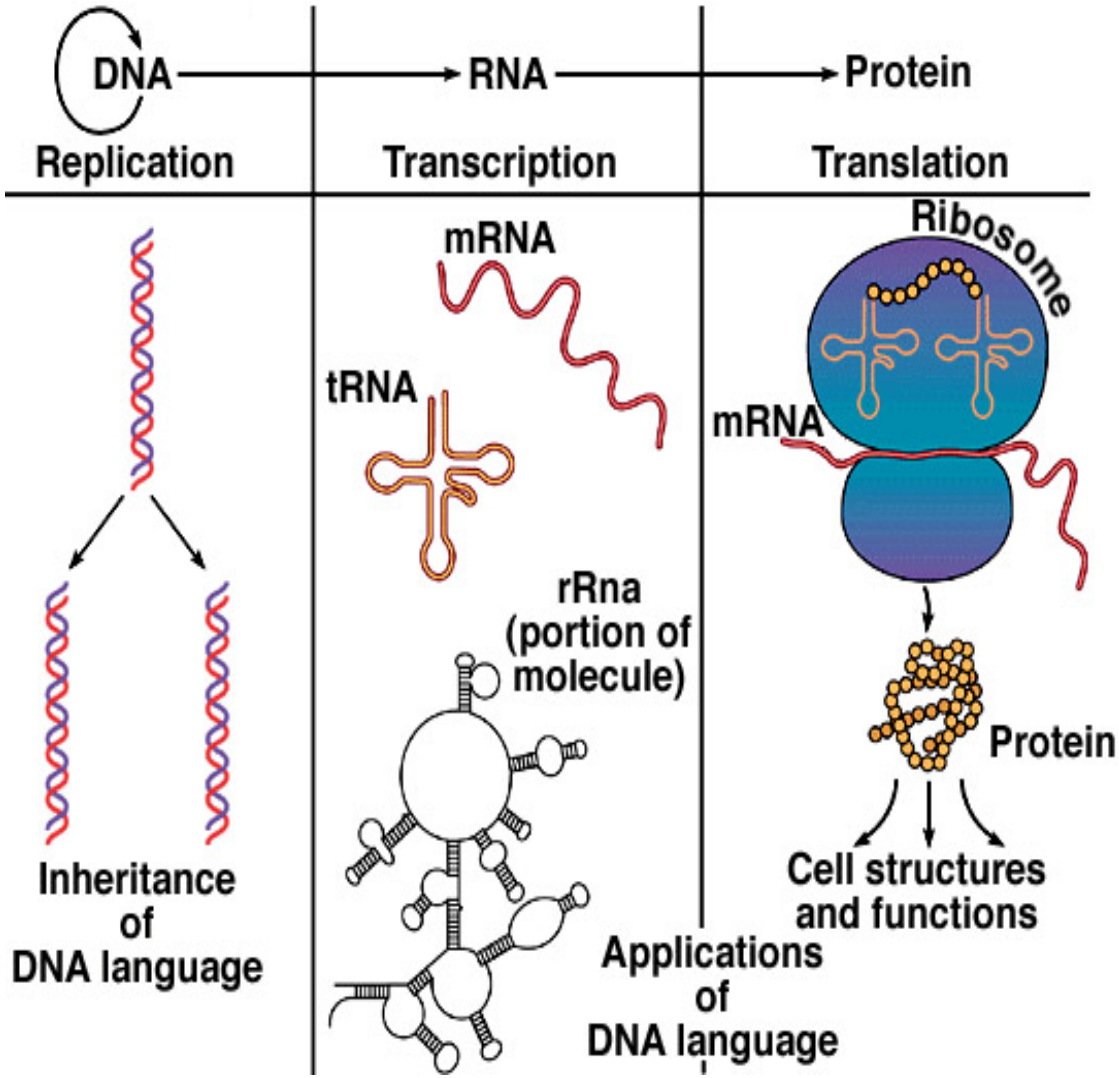
We will eliminate the expression of various genes using

(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



Isolate total RNA in order to measure levels of specific mRNAs

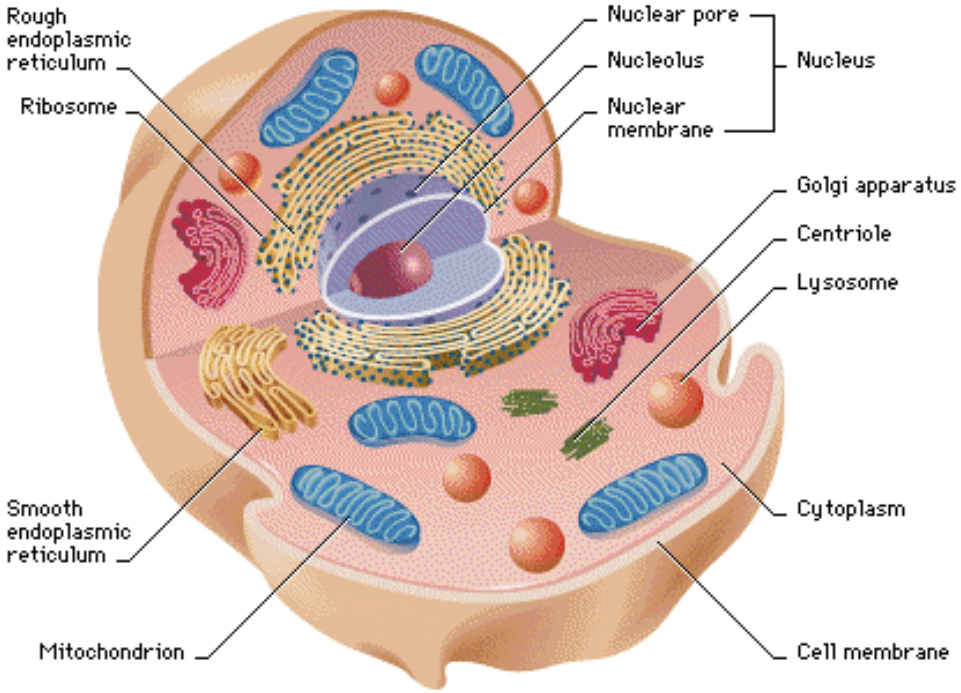


Let's think about isolating RNA from mouse ES cells....what will we get?

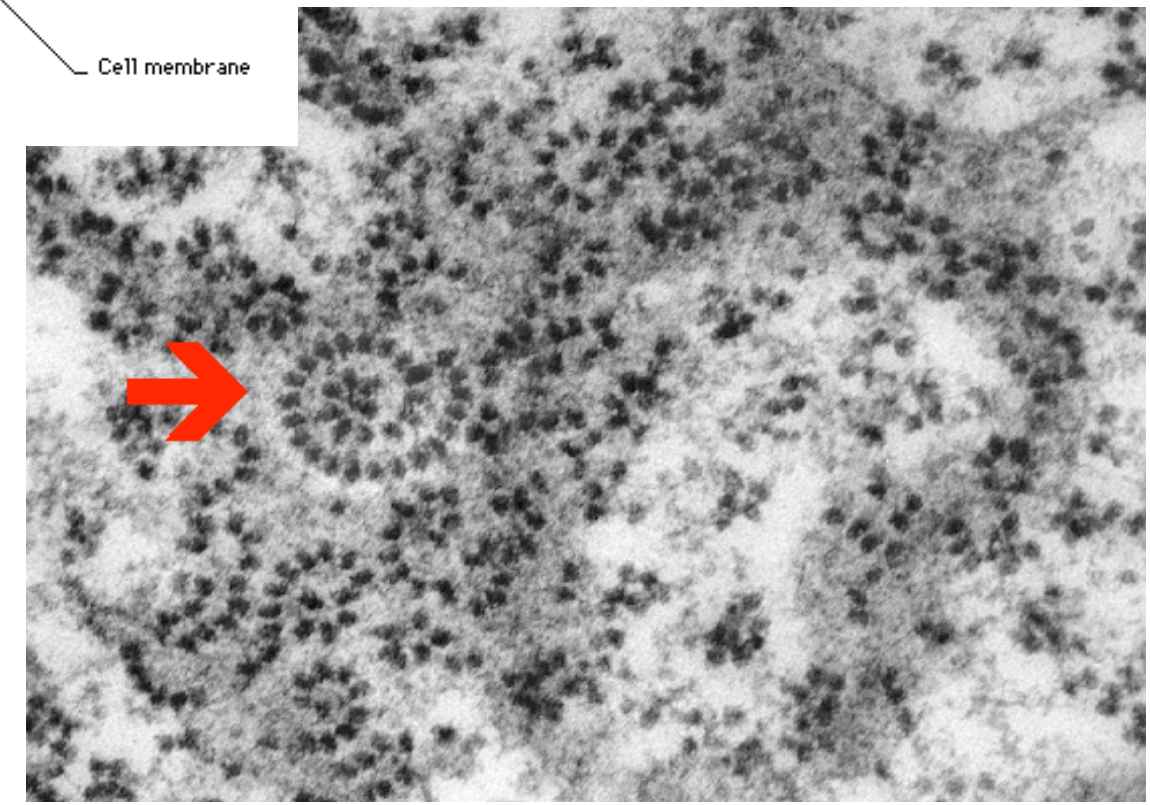
mRNA

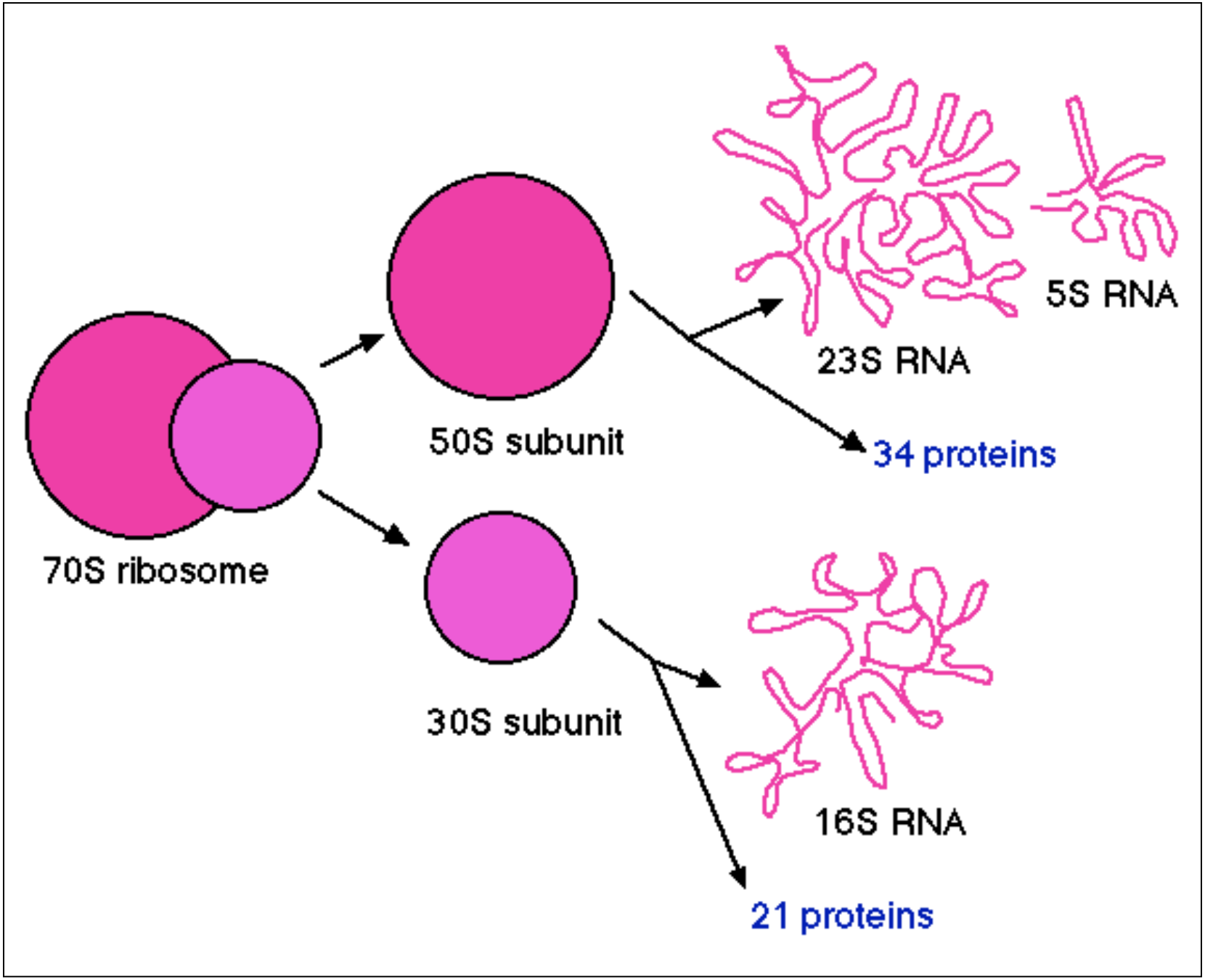
tRNA

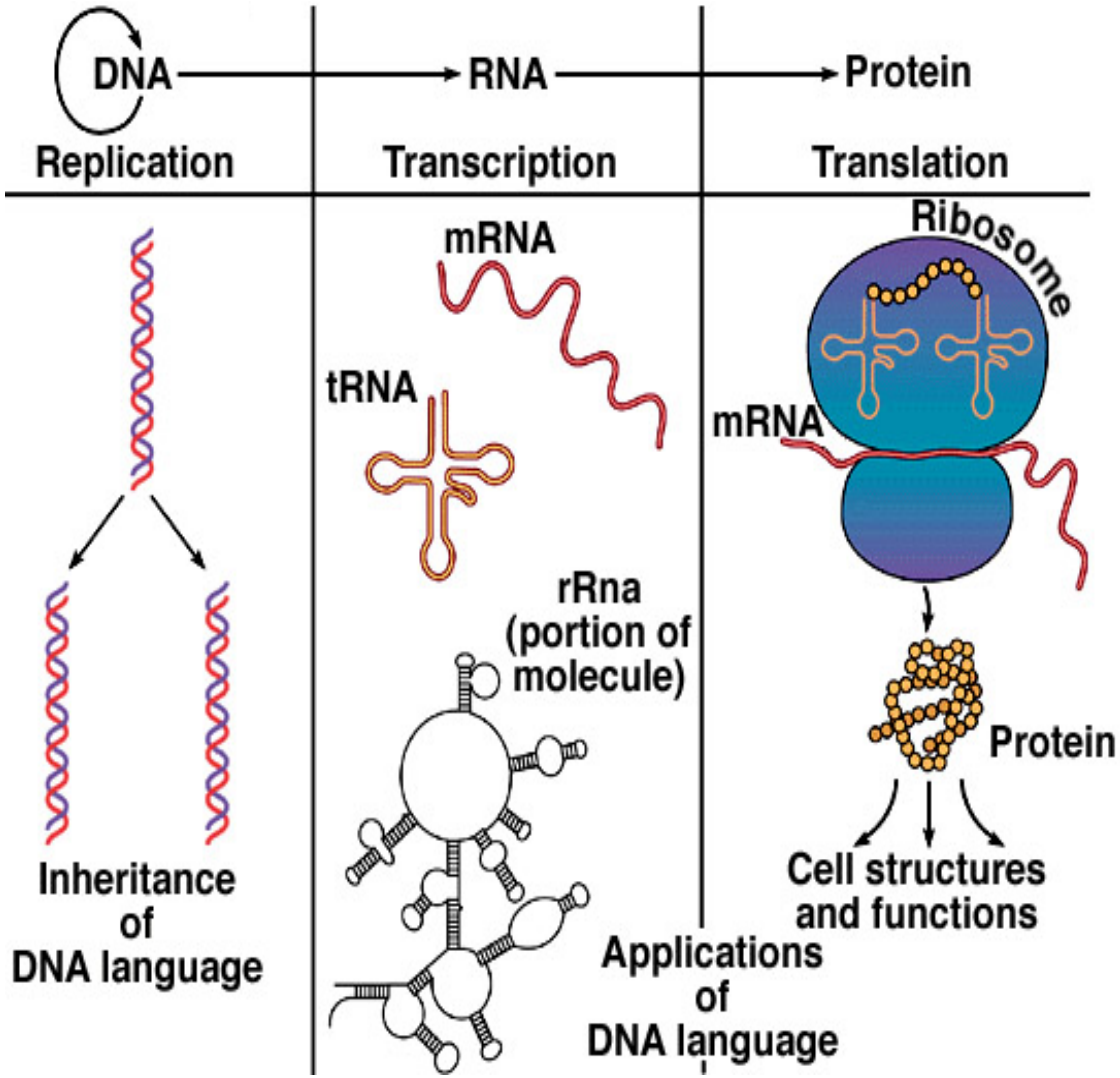
rRNA



Cells have lots and lots of ribosomes, most are lined up as poly-ribosomes in the Rough Endoplasmic Reticulum




70S ribosome
50S subunit
30S subunit
23S RNA
5S RNA
34 proteins
16S RNA
21 proteins



Let's think about isolating RNA from mouse ES cells....what will we get?

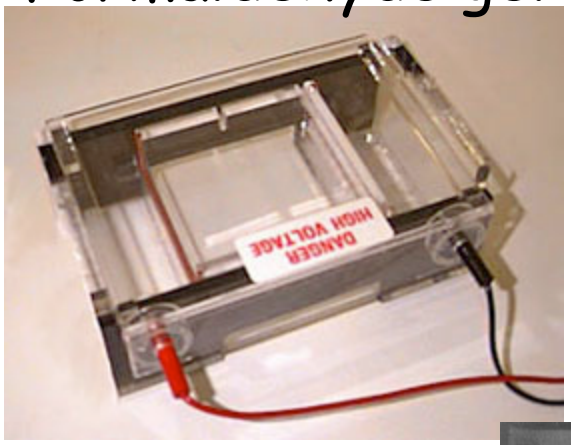
mRNA

tRNA

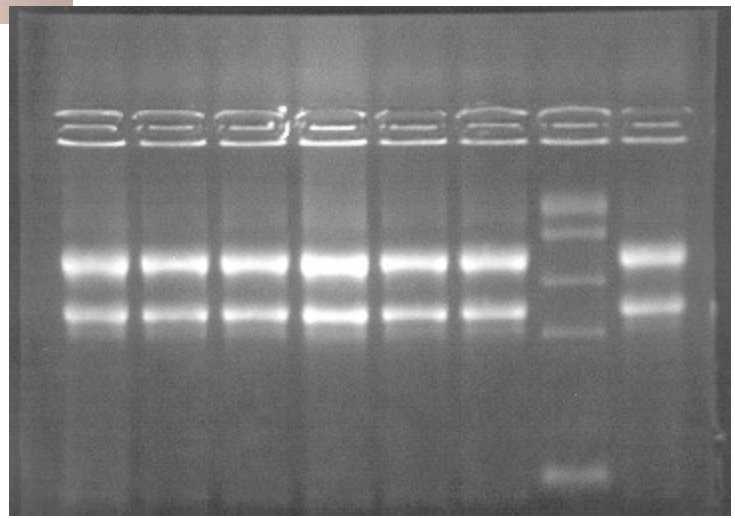
rRNA

RNA Analysis

Formaldehyde gel

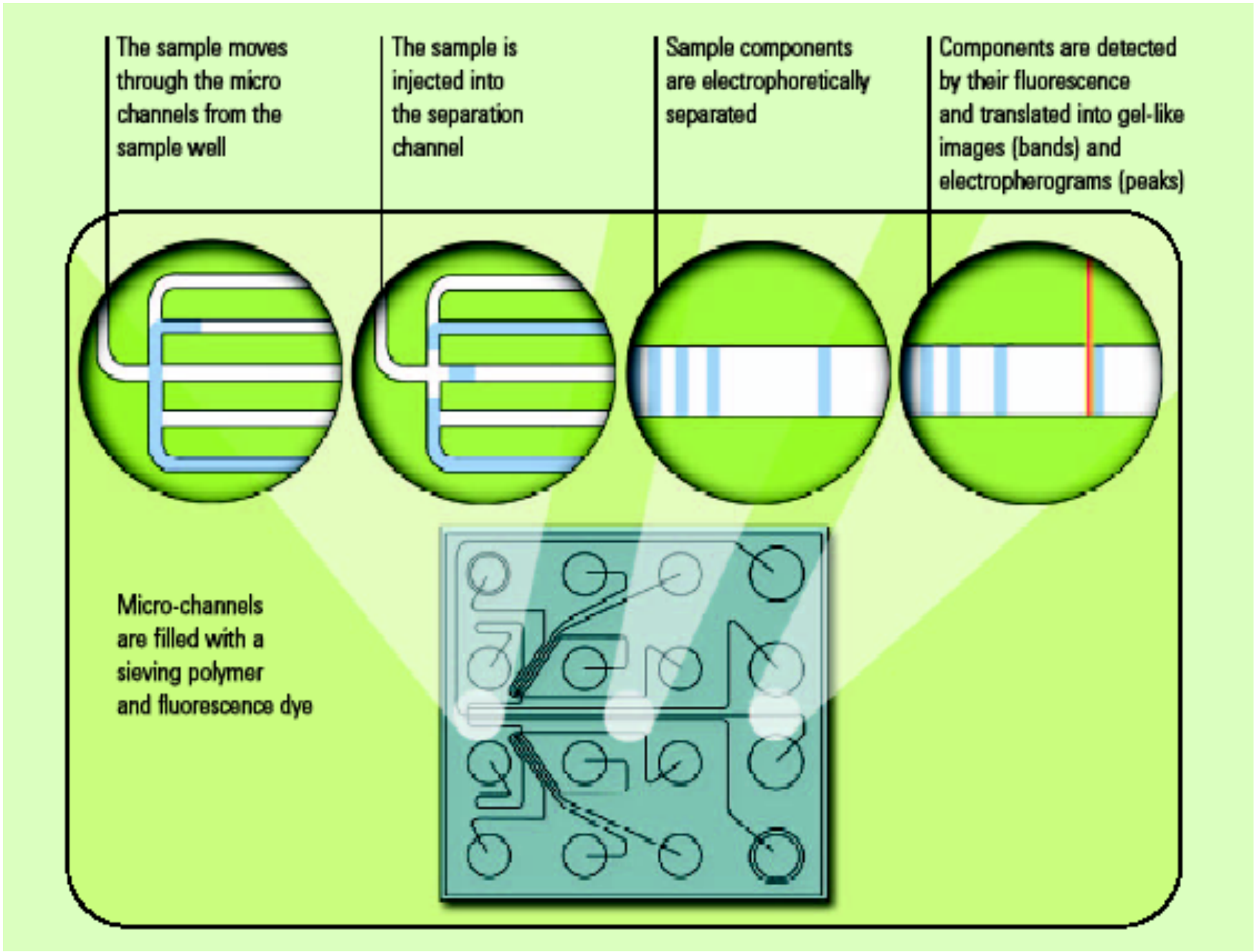


Hours of preparation and
Run time



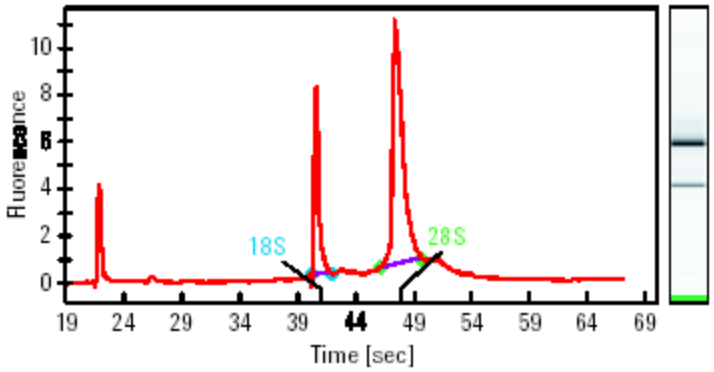
Ribosomal RNA bands – mRNA smeared in background

Agilent 2100 Bioanalyzer
Automated Analysis System

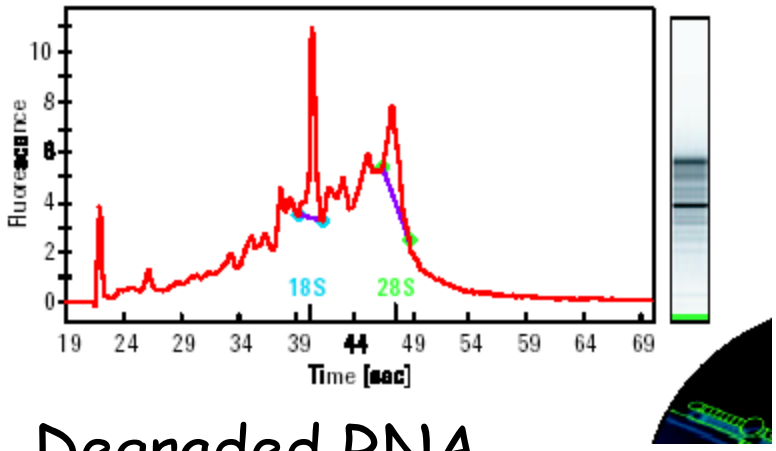

The sample moves through the micro channels from the sample well
The sample is injected into the separation channel
Sample components are electrophoretically separated
Components are detected by their fluorescence and translated into gel-like images (bands) and electropherograms (peaks)
Micro-channels are filled with a sieving polymer and fluorescence dye

RNA solutions

Guarantee high quality RNA
for microarray sample preparation

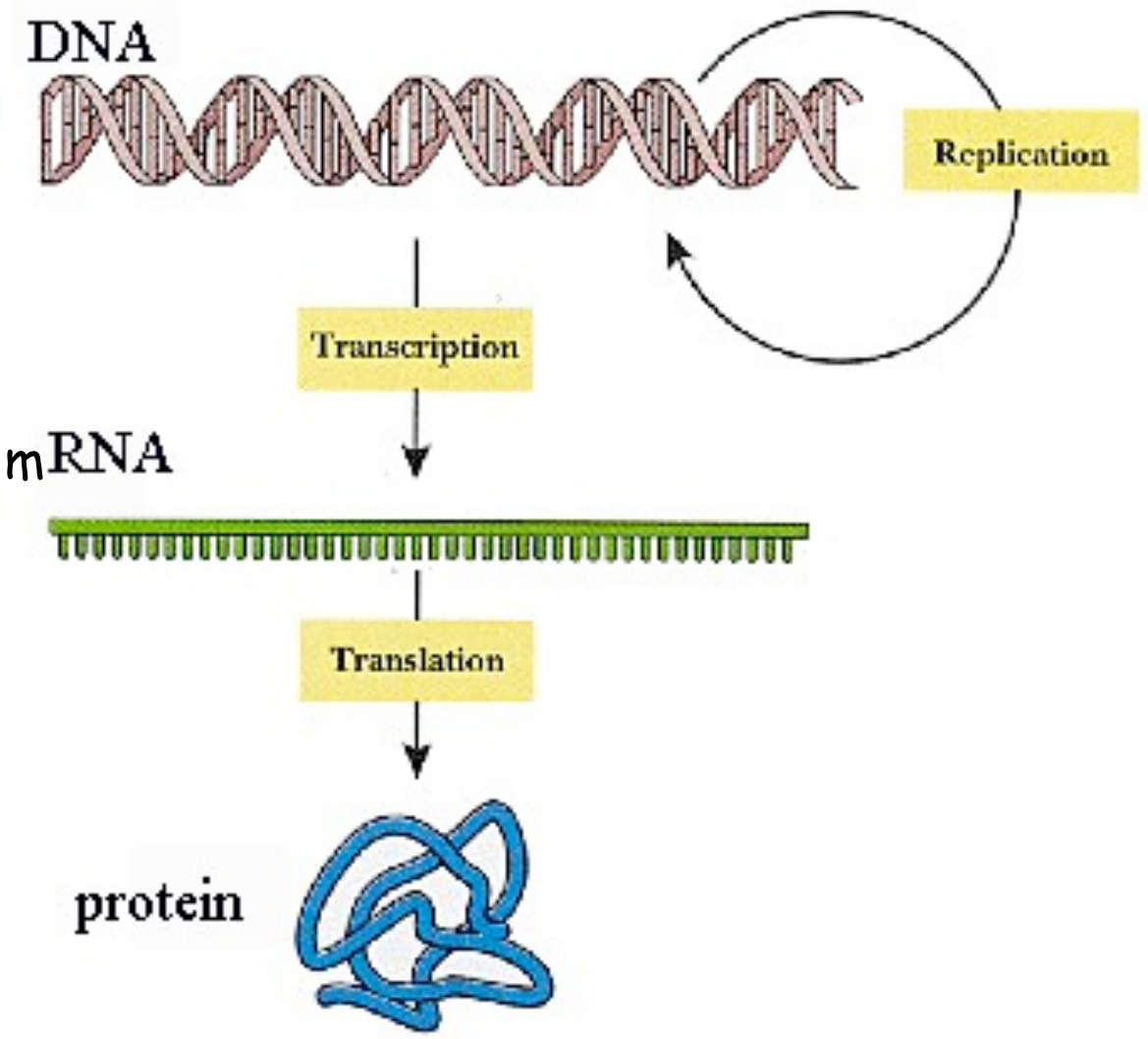


Intact RNA



Degraded RNA

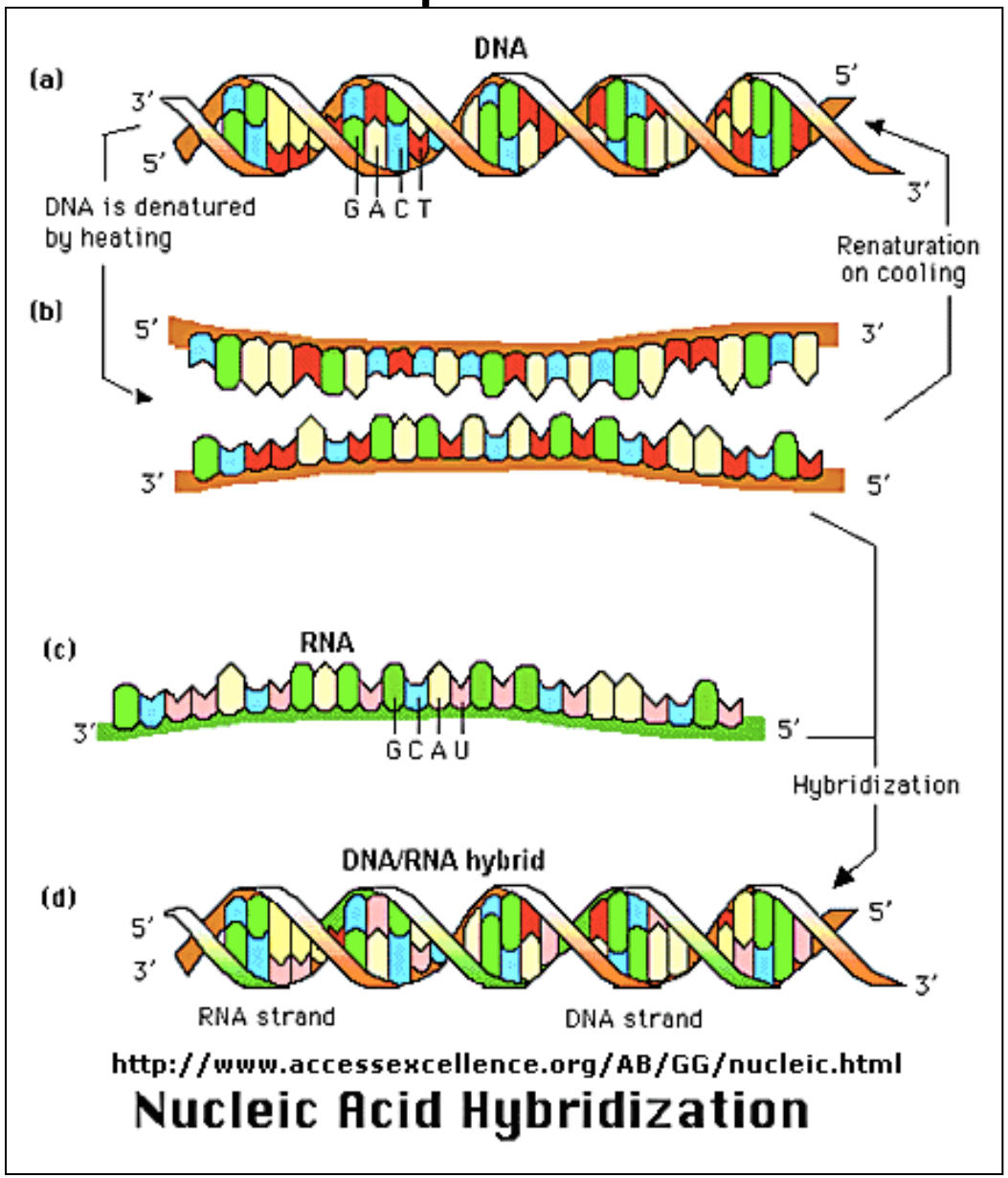
Monitor mRNA expression level for every mouse gene in one single experiment.



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

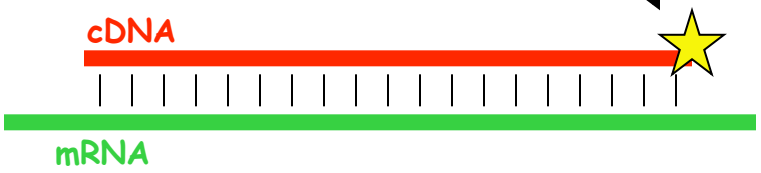
Now that we know the DNA sequence for every gene, this is possible!

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

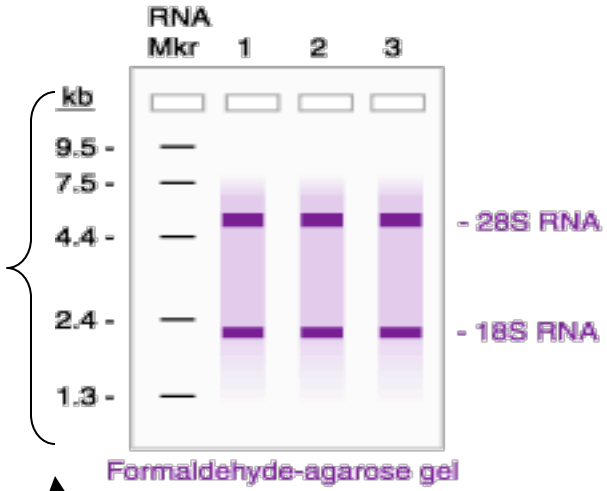


The specificity of G pairing with C and A pairing with T (or U) drives hybridization and provides a mechanism for quantitatively assessing the amount of a specific mRNA species in cells.

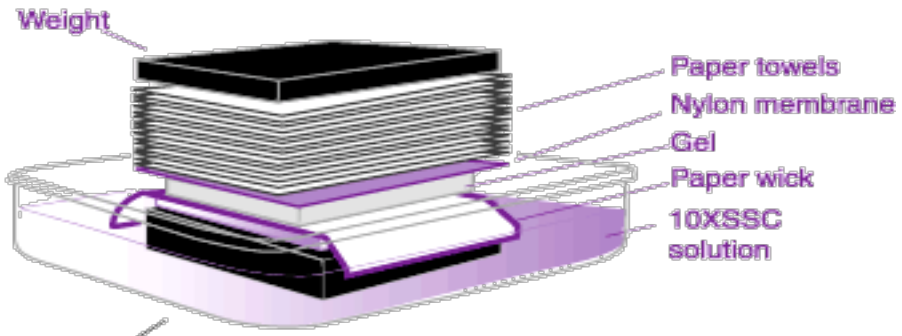
^{32}P- label



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



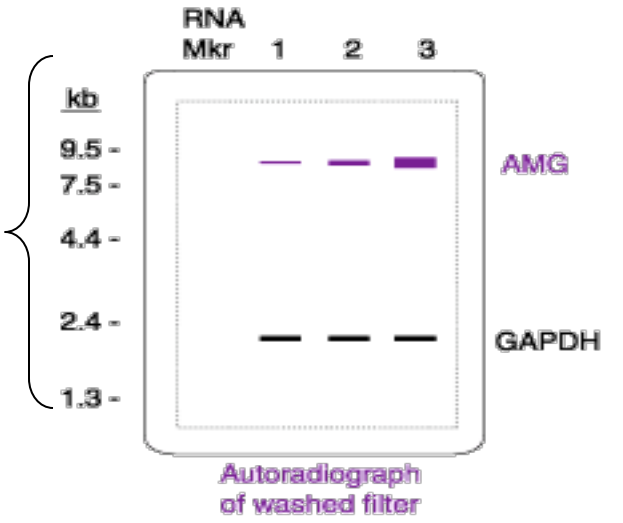
Transfer RNA by membrane blotting

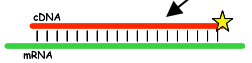


Cross-link RNA to the membrane using UV light

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

All mRNAs separated by size





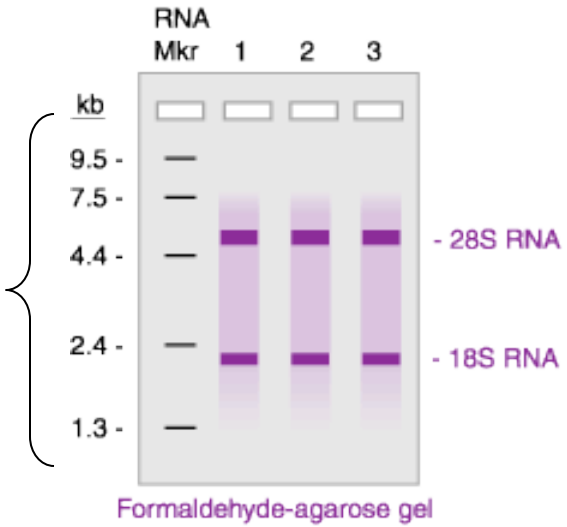
AMG probe

GAPDH probe

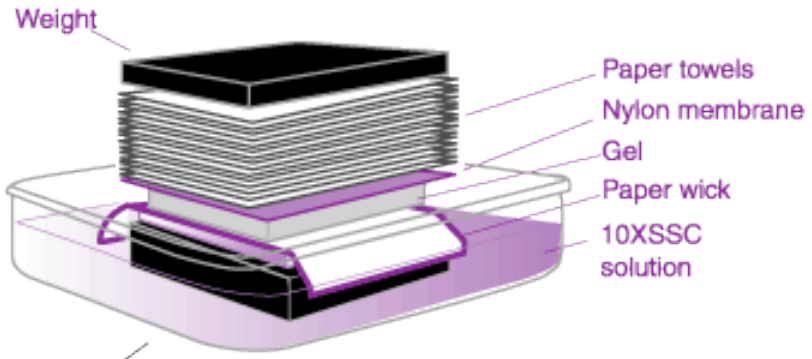
Specific mRNAs Lit-up by radioactive cDNA probes

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.



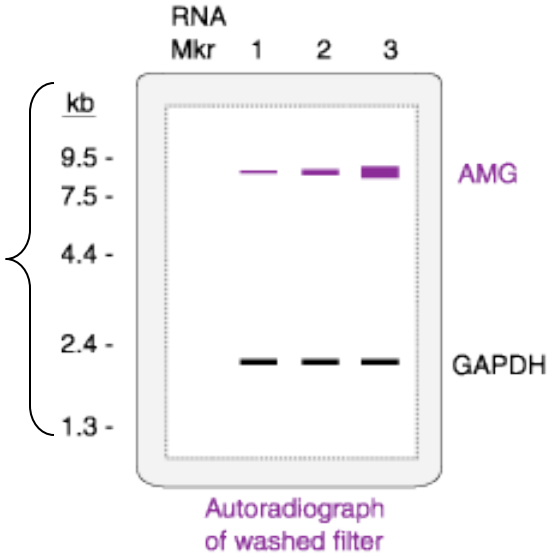
Transfer RNA by membrane blotting



Cross-link RNA to the membrane using UV light

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

All mRNAs separated by size



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

Northern Blots

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

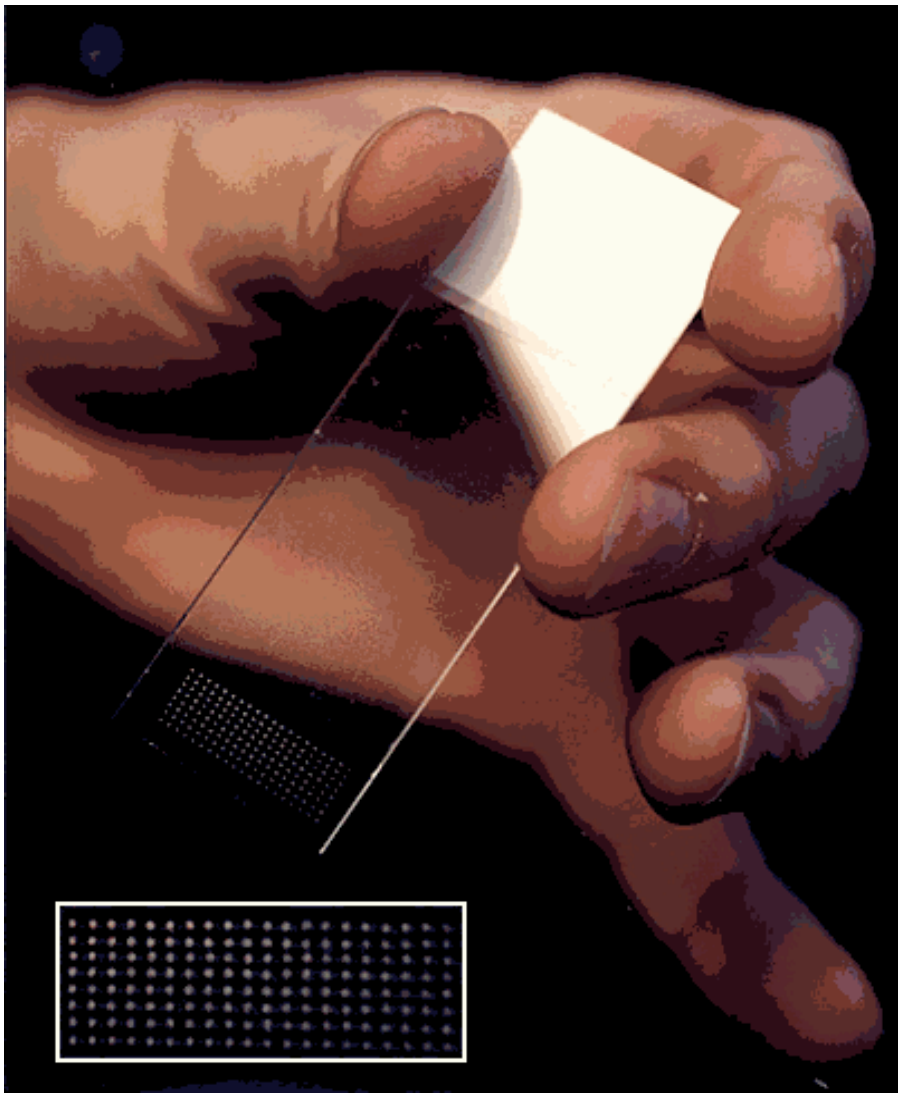
DNA Microarrays

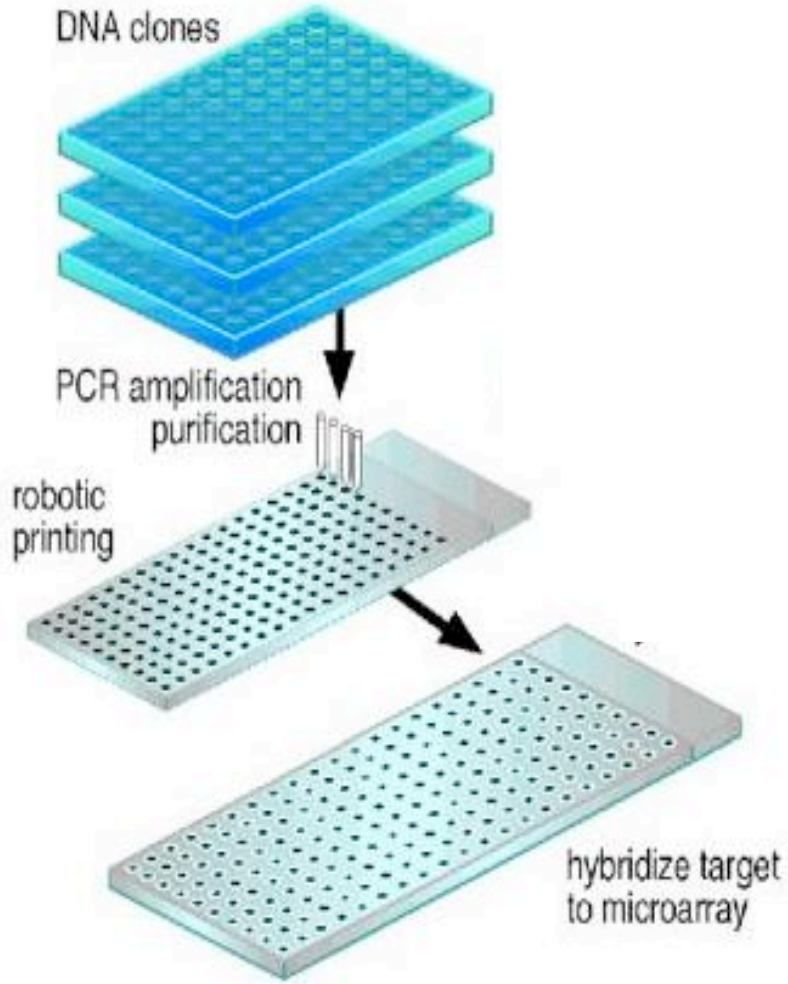
Immobilized DNA probes representing **all** possible genes hybridized with labeled mRNA population

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 probes per slide

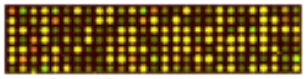
The probes can be cDNAs (~ 1Kb) or oligonucleotides (20-70 mers)

Microarray

the arrays we'll be using

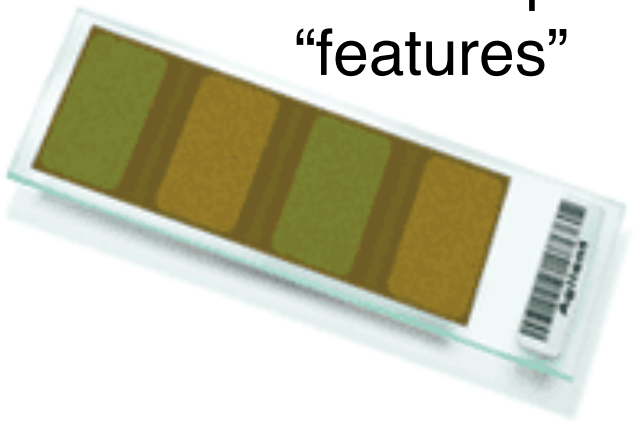
Catalog Oligo Microarrays

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.



Agilent's *in situ*
Oligonucleotide Microarray

4x44K spots
"features"

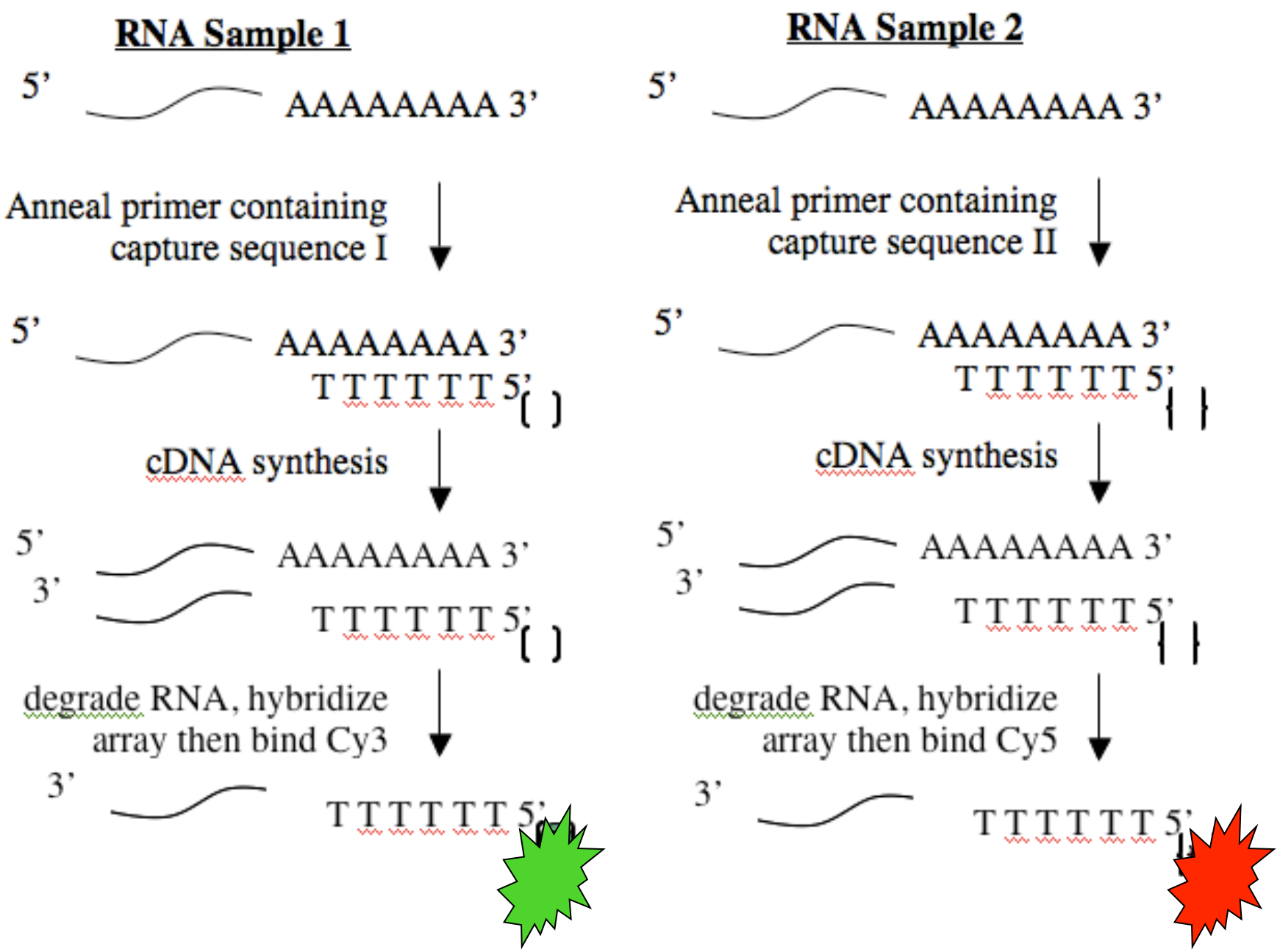


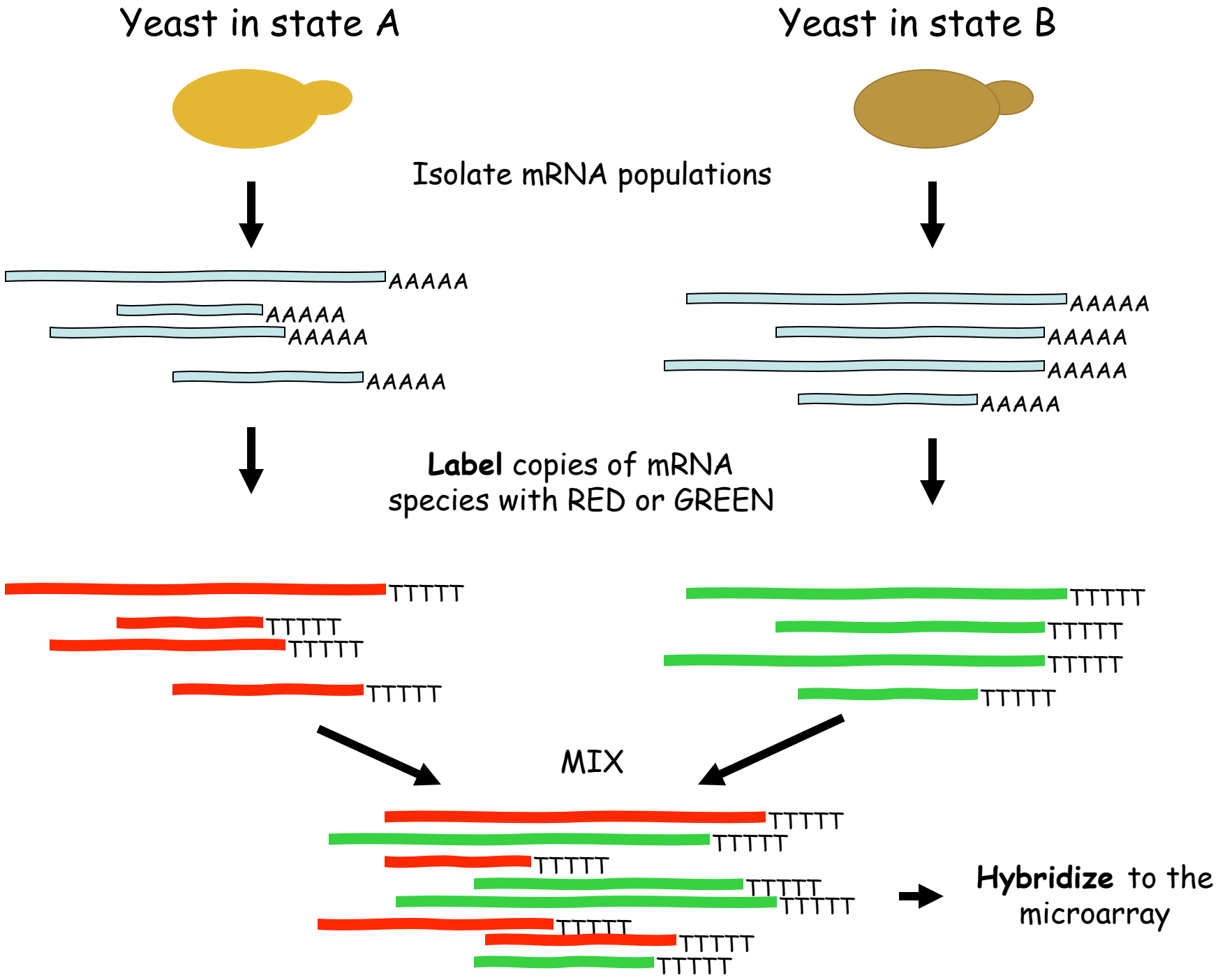
Need to achieve two things:

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

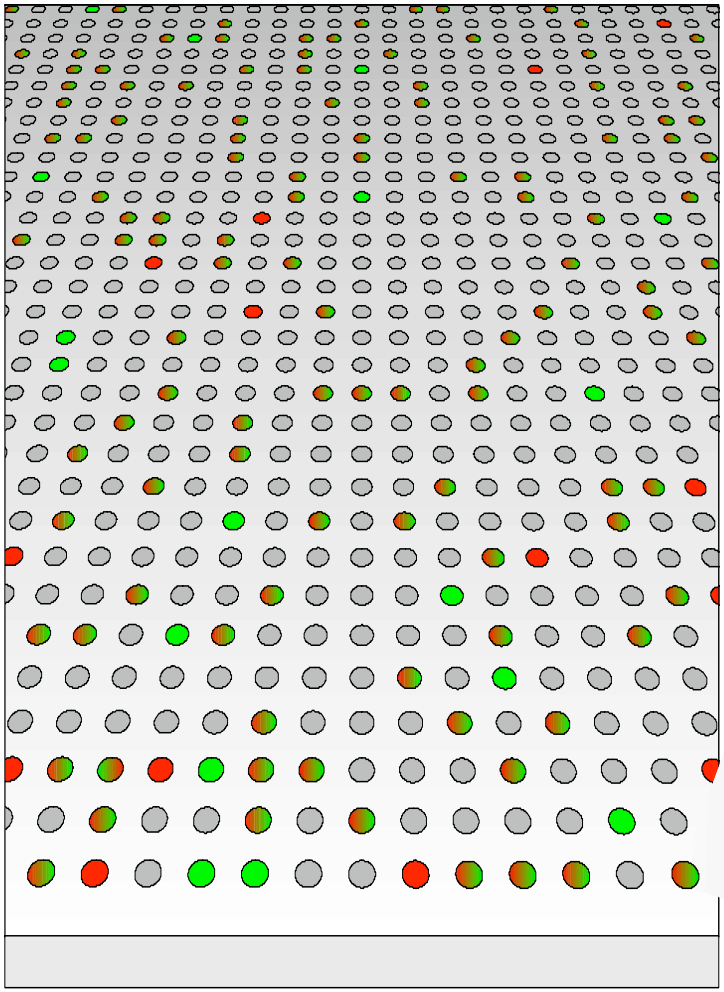
(ii) Label mRNA populations

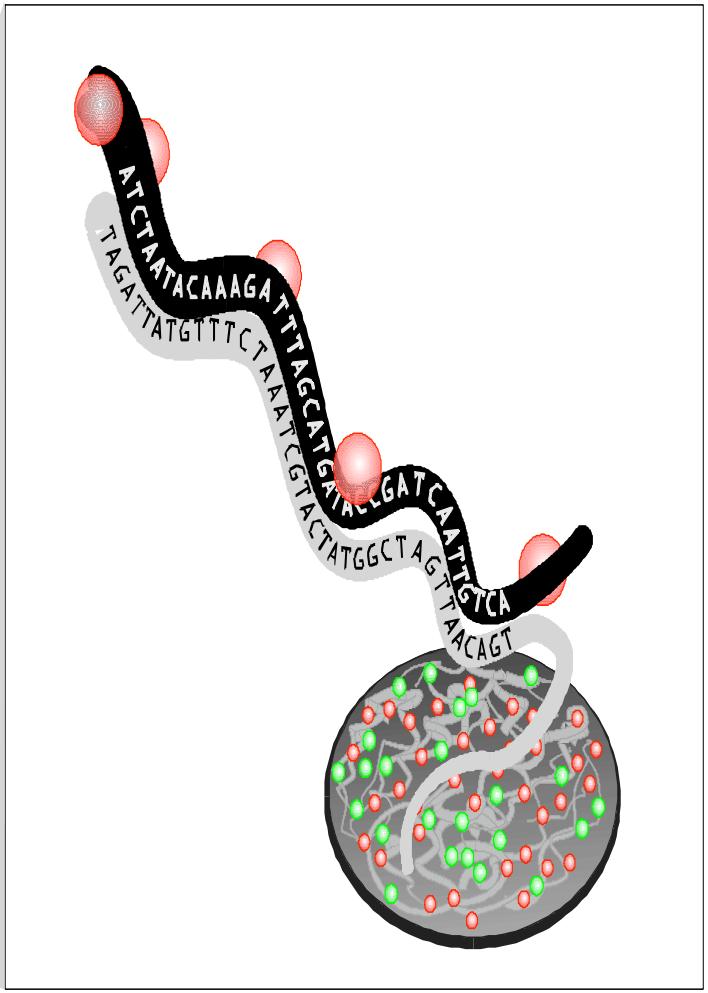
Copy the population of purified mRNA species such that they are fluorescently labeled – hybridize to the array


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'

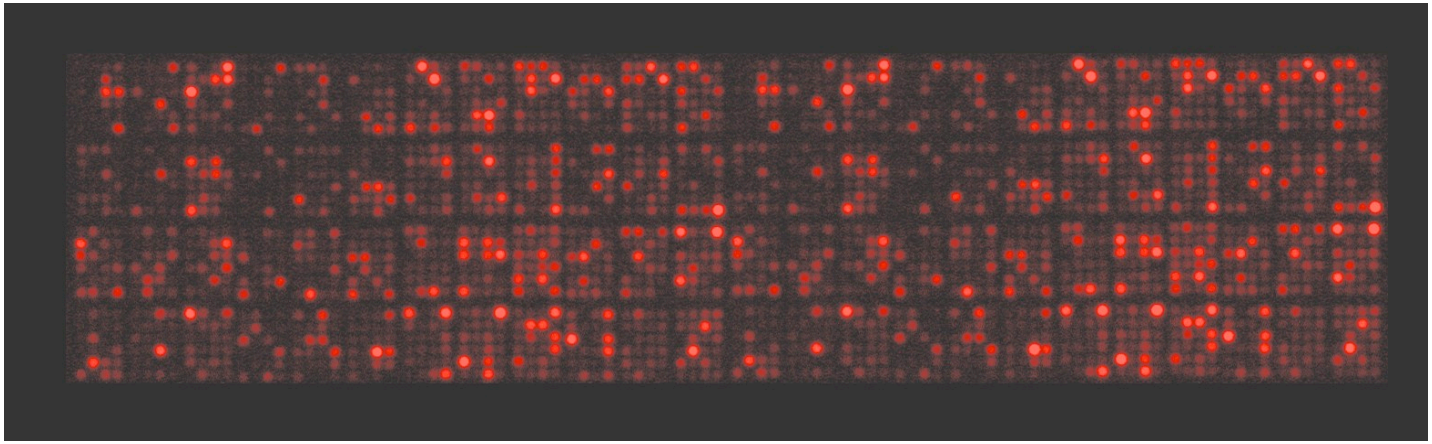

Yeast in state A
Yeast in state B
Isolate mRNA populations
AAAAA
AAAAA
AAAAA
AAAAA
AAAAA
AAAAA
AAAAA
AAAAA
Label copies of mRNA species with RED or GREEN
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
MIX
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
TTTTT
Hybridize to the microarray

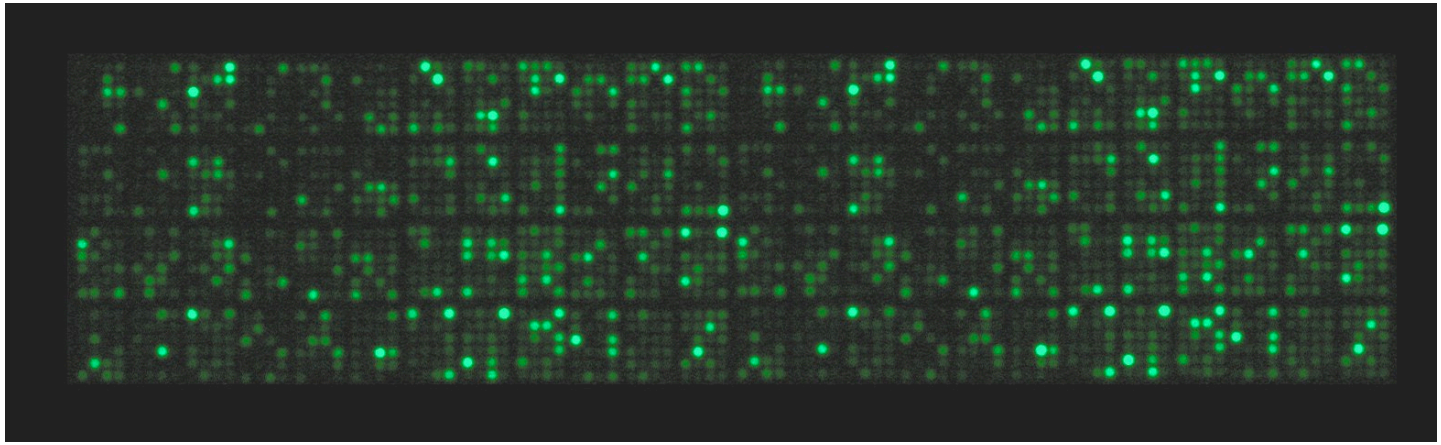
What's happening at each spot?

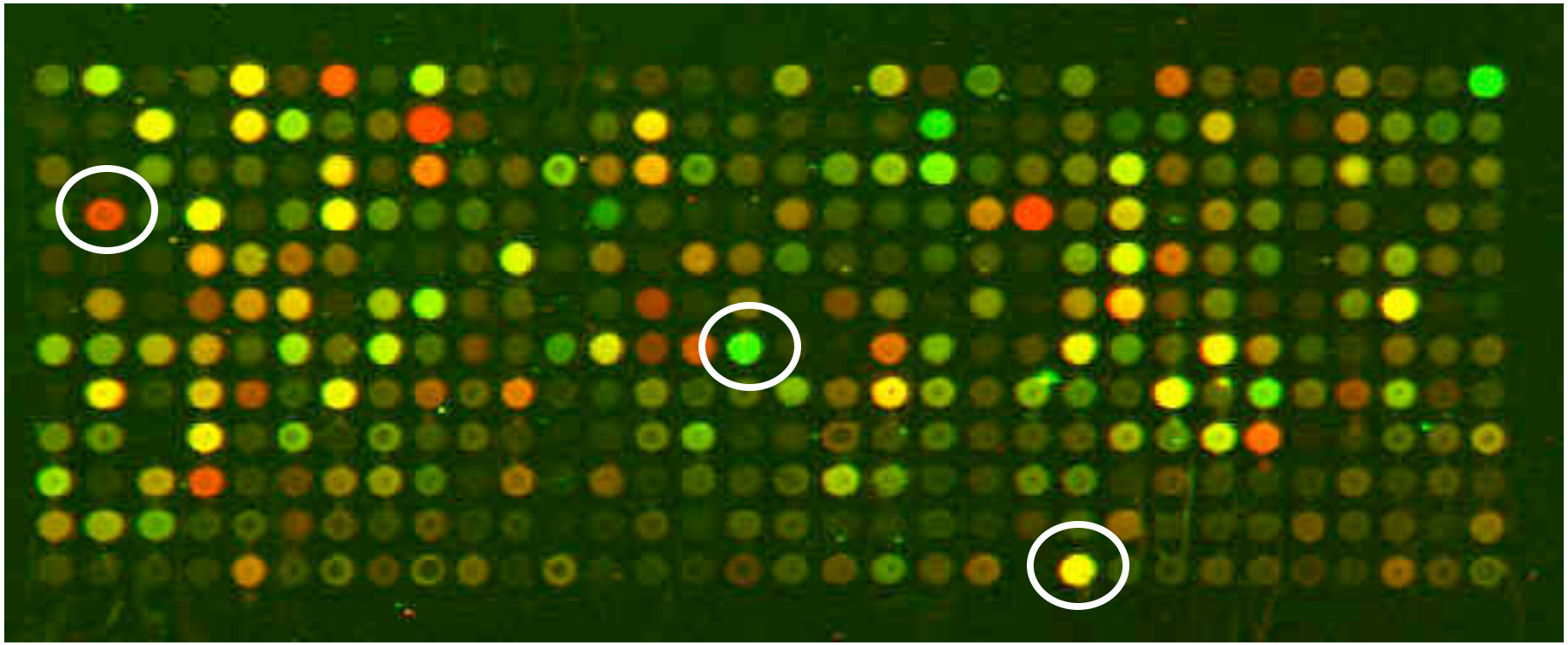




Hybridization



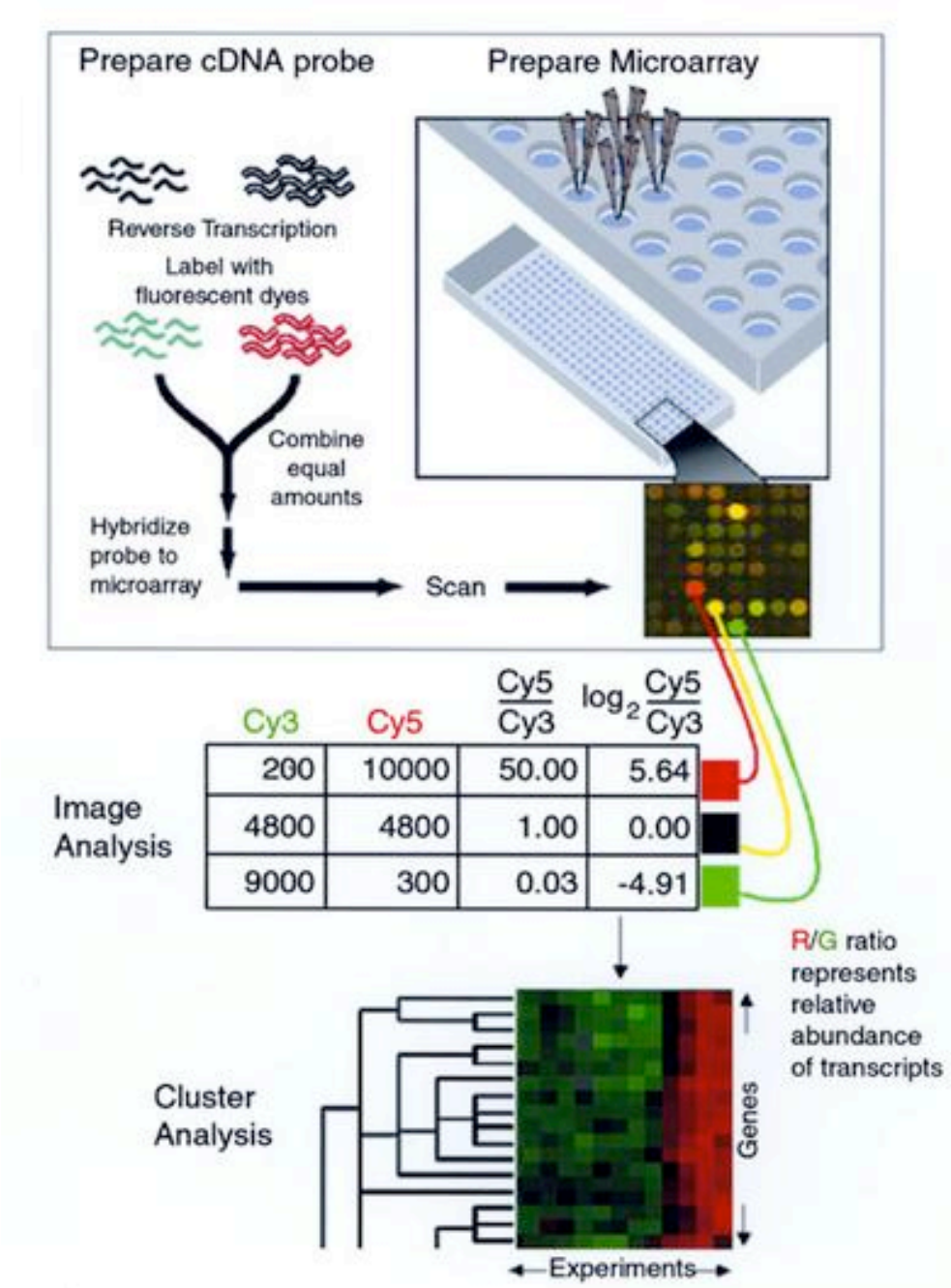




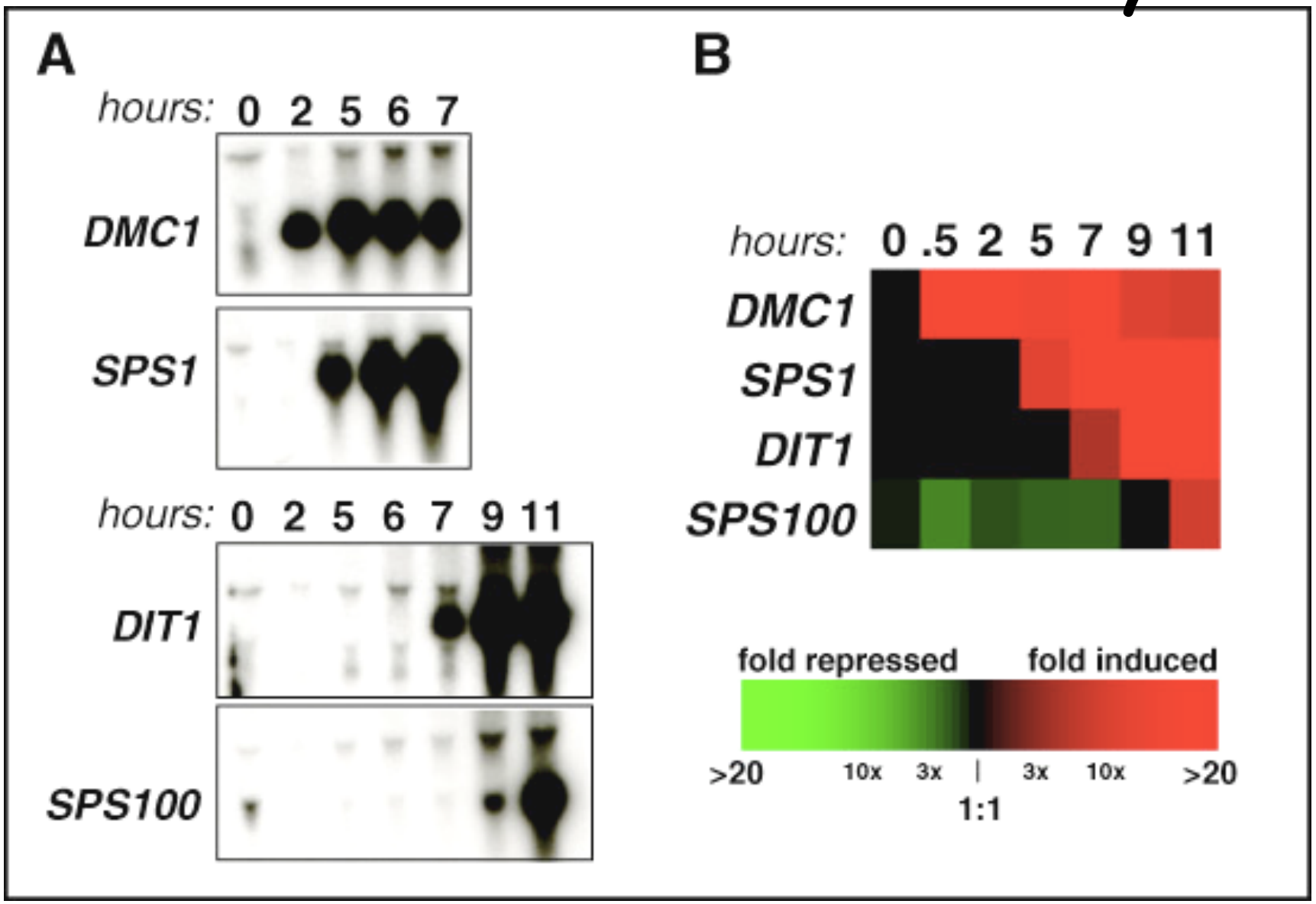
mRNA present much higher in State A than State B

mRNA present much higher in State B than State A

mRNA present at equal levels in States A and B

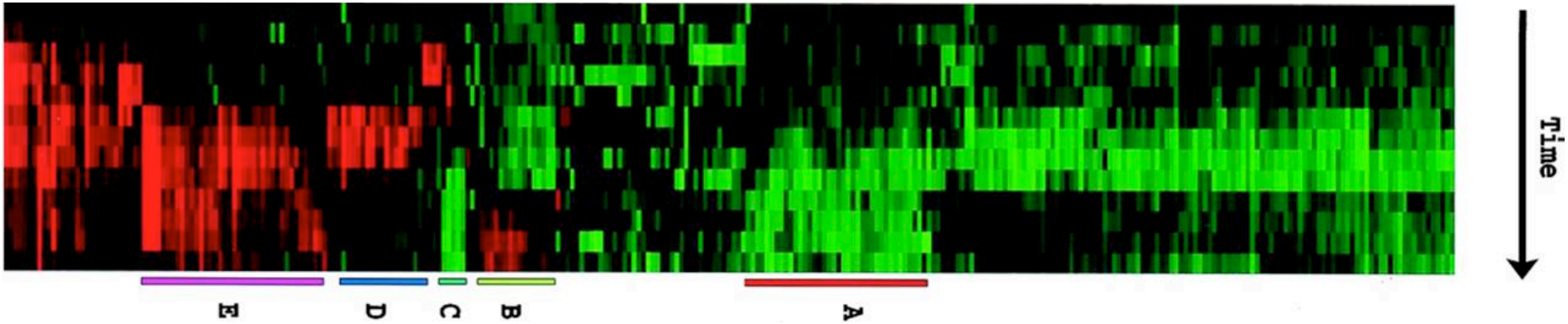

Prepare cDNA probe
Prepare Microarray
Reverse Transcription
Label with fluorescent dyes
Combine equal amounts
Hybridize probe to microarray
Scan
Cy3
Cy5
Cy5/Cy3
log2 Cy5/Cy3
200
10000
50.00
5.64
4800
4800
1.00
0.00
9000
300
0.03
-4.91
Image Analysis
Cluster Analysis
R/G ratio represents relative abundance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

Northern Blot vs. Microarray

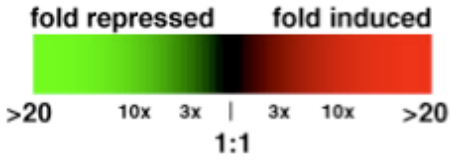


Hierarchical clustering to group together similarly regulated genes

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