MOLECULAR BIOLOGY

بسم الله الرحمن الرحيم

FINAL – Lecture 15

Analysis of Gene Expression and RNA Levels

﴿ وَإِن تَتَوَلَّوْا يَسْتَبْدِلْ قَوْمًا غَيْرَكُمْ ثُمَّ لَا يَكُونُوا أَمْثَالَكُم ﴾

اللهم استعملنا ولا تستبدلنا

Written by :

- Mahmoud Aljunaidi
- Muthanna Khalil

APPROVED
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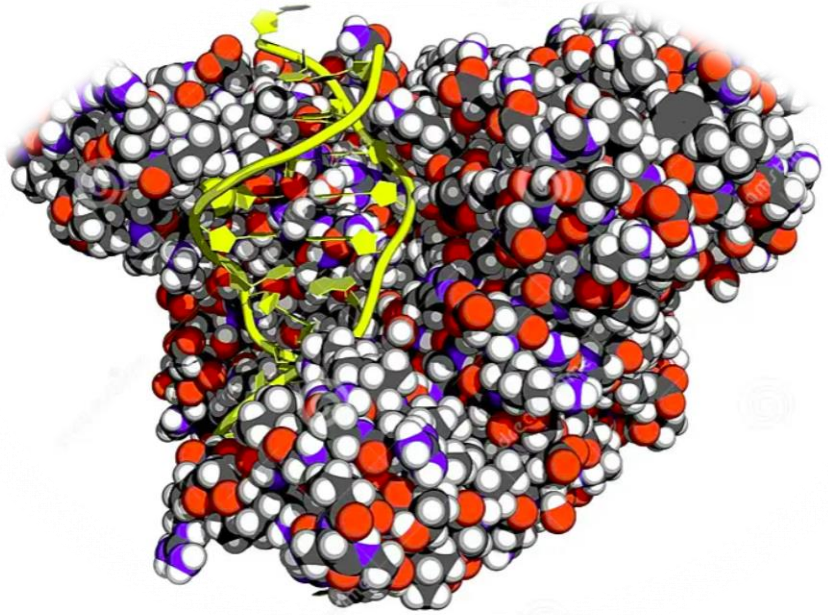
Quiz on the previous lecture



Analysis of gene expression

RNA level

This lecture discusses techniques for gene expression analysis, categorized into three levels of complexity:

1. *Basic methods:*
   - *Northern blotting.*
   - *in situ hybridization.*
2. Advanced methods:
   - real-time PCR.
   - DNA microarray.
3. Very advanced methods:
   - RNA-seq (sequencing)

Northern blotting

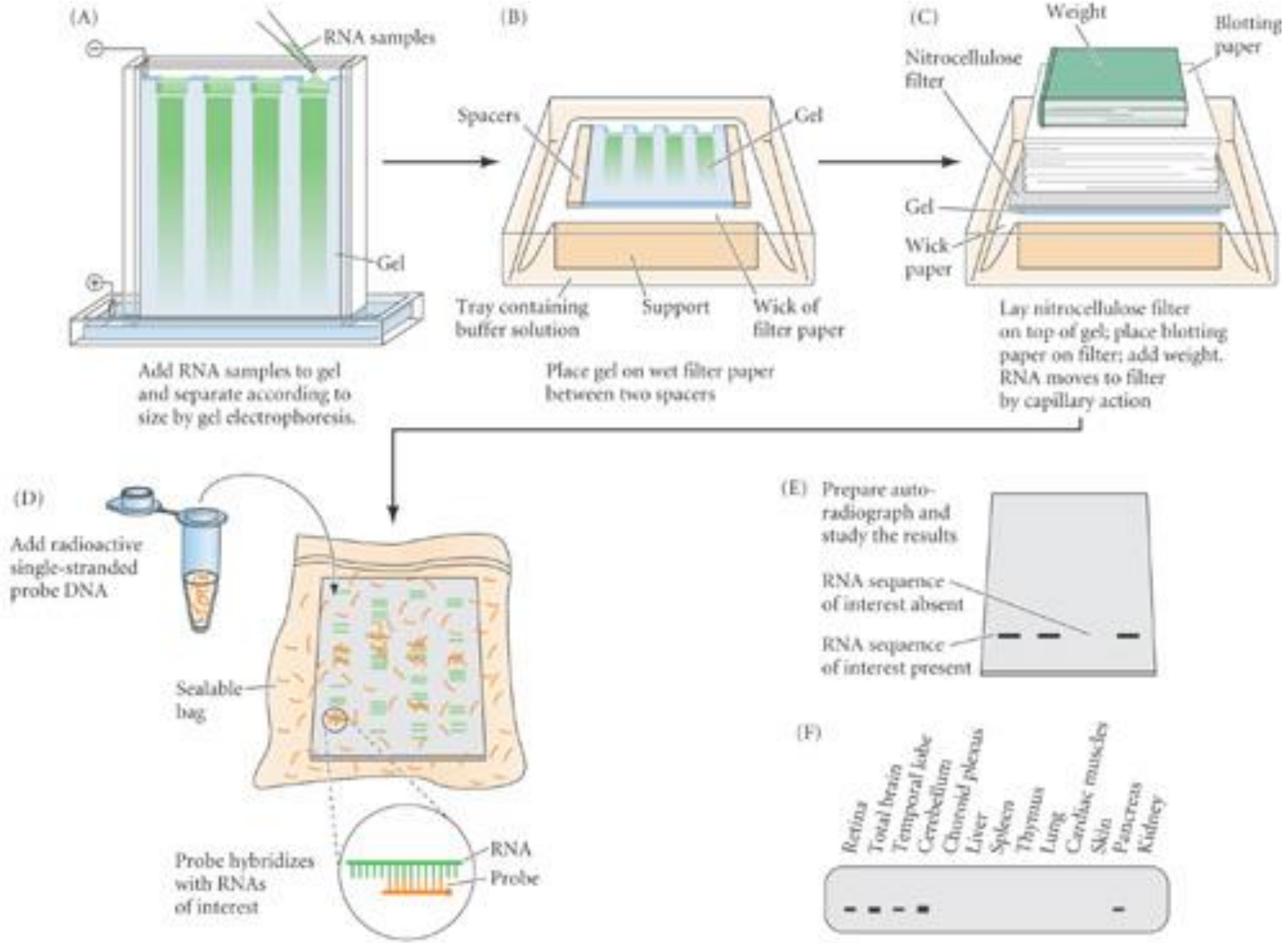
- This is done exactly like Southern blotting except that:
  - RNA from cells is isolated instead of DNA.
  - RNA molecules are fractionated based on size by gel electrophoresis.
  - The fractionated RNA molecules are transferred onto a membrane.
  - RNA molecules are targeted by a labeled DNA probe with a sequence that is complementary to a specific RNA molecule.

    (usually, DNA probes are used because they're more stable than RNA probes)

- What information can you deduce from it?

1. If the gene expressed or not. (if expressed, mRNA (transcript) would be detected)
2. Size of the RNA of interest.
3. How active the gene is/ to what extent is the gene expressed. (More band intensity, more expression)

Examine the figure carefully before proceeding to the explanation in the following slides.



Northern Blotting Technique

Here, the term "RNA" specifically refers to mature mRNA.

- It is similar to Southern blotting, but specifically used for detecting RNA.

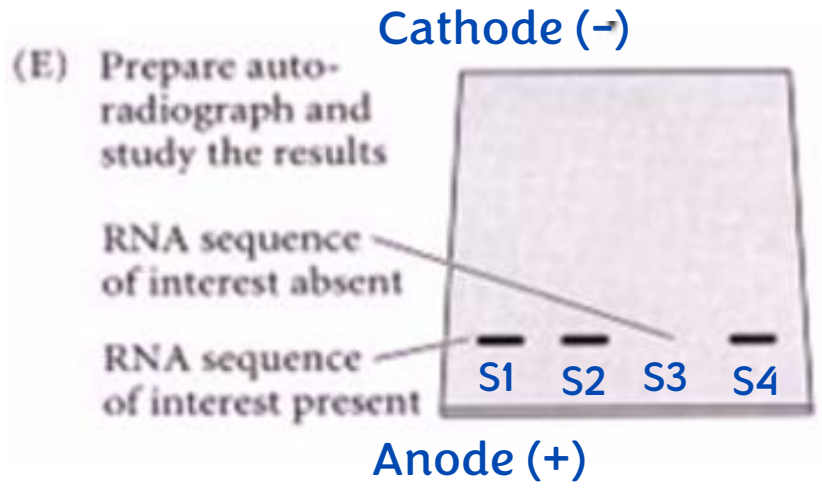
1. First, **RNA** is obtained from cells, NOT DNA.
   - RNA molecules obtained are variable in size, depending on the size of the gene.
2. RNA molecules are then fractionated by gel electrophoresis.
   - RNA molecules are separated based on size, with smaller fragments migrating further and faster than larger ones.
3. After that, RNA molecules are transferred onto a membrane.
   - RNA molecules are already separated by size, serving as an exact replica of the gel.
4. Next, radioactively labeled complementary DNA probes bind to the target RNAs of interest (hybridizing to it).
5. A signal is emitted where the probe binds, identifying the target RNA.

Northern Blotting- Results interpretation(1)

- In this example there are 4 samples, put in different wells.

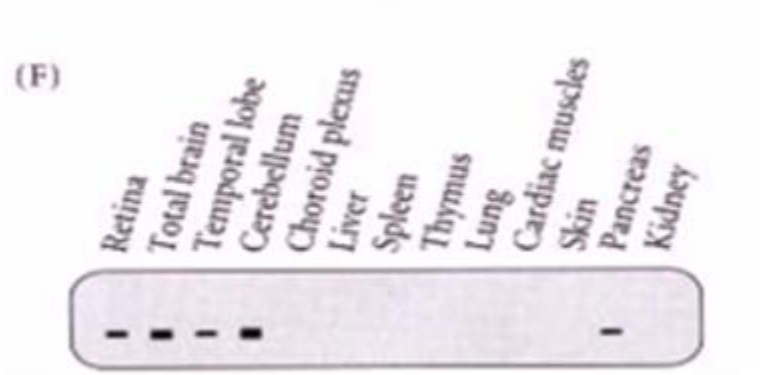
❖From the results, the following can be determined:

1. Samples 1, 2, and 4 **express** the gene, as bands are observed, whereas sample 3 does not express the gene, as no band is present.
2. RNA molecules are relatively small, as they are positioned closer to the anode than the cathode.



Northern Blotting- Results interpretation(2)

Same gene, different tissues.



- Here, the expression of the **same gene** but in **different tissues** is examined.

❖ For this specific gene:

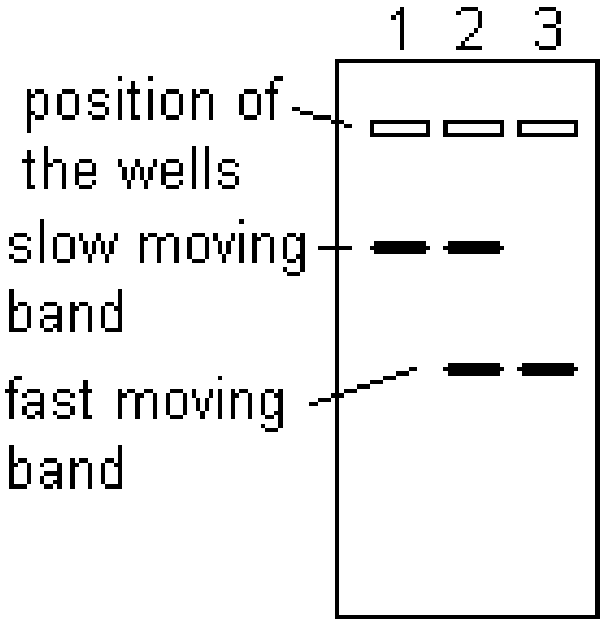
- The cerebellum (sample 4) expresses the gene at a much higher level than the temporal lobe (sample 3), as indicated by the greater intensity of band 4 compared to band 3.
- In tissues such as the liver and spleen, where no RNA band is observed, there is no expression of this gene.

- So, northern blotting can determine if a specific gene is expressed in a particular tissue and to what extent.

What are your interpretations?

- Here, we have three samples, and after performing northern blotting, the following results were observed:
  1. Sample 1 expresses the gene and produces a relatively large mRNA.
  2. Sample 2 expresses the gene but produces 2 mRNA molecules; a large mRNA and a smaller mRNA.
  3. Sample 3 expresses the gene but produces a small mRNA. But why?
- The generation of different RNA molecules of varying lengths from the same gene can be attributed to:
  1. RNA molecules can be produced from **different promoters**.
  2. RNA can be **alternatively spliced**.
  3. Variation in the **termination** sites of transcription.

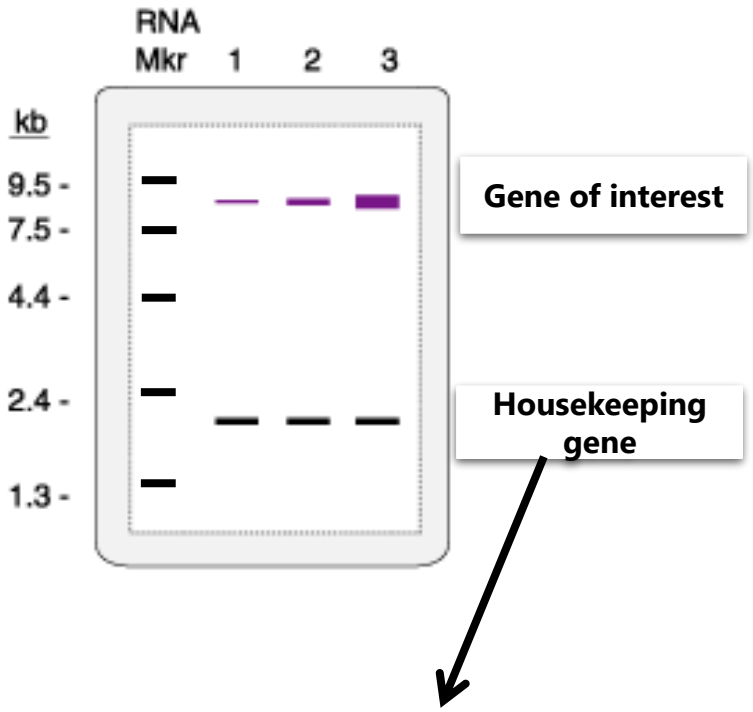
(Recall lecture 11)



What are your interpretations?

REMEMBER, RNA is single stranded so no base pairs → kb not kbp.

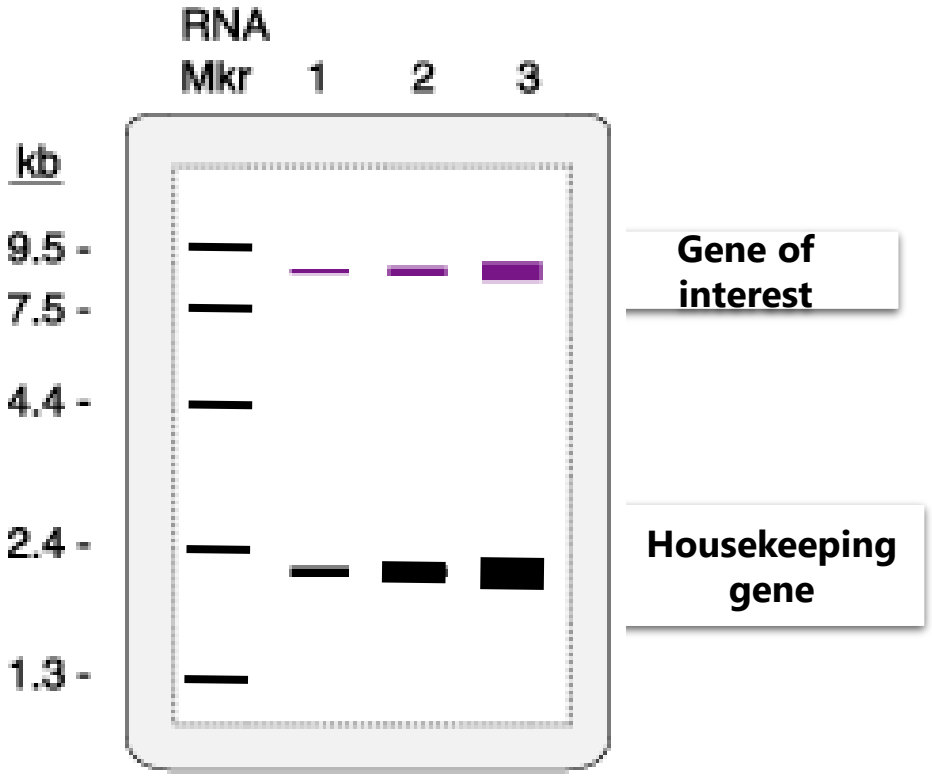
- Here comparative expression is done among 3 samples.
  - **Comparative expression**: comparing the level of a certain gene expression among different samples, meaning that samples can be placed under varying conditions to observe changes in gene expression levels.
- The gene of interest produces RNA molecules about 9 kb long.
- Sometimes different conditions (ex. volumes) lead to false results (see next slide), so, to ensure that the interpretations are error-free, the expression of house keeping genes is observed.
  - **House keeping genes**: genes whose expression isn't changed under different conditions (constant), for example: histone genes are always expressed and doesn't change, also actin, tubulin and certain metabolic enzymes; all cells need them, so their expression isn't changed.
- Here real changes in intensity can be observed (3 most intense), because the expression of the housekeeping gene is equal among all 3 samples.



A gene with constant expression (examples: actin, tubulin)

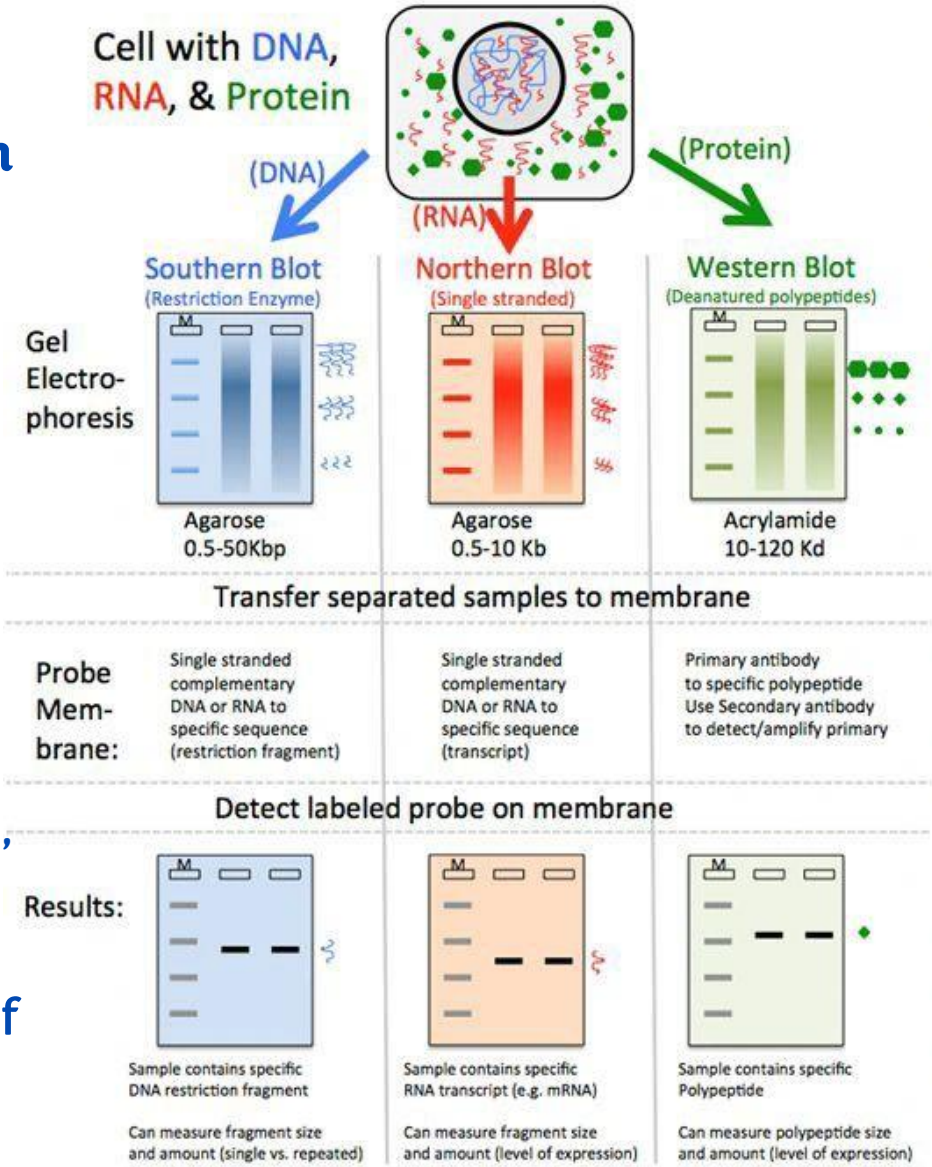
What are your interpretations?

- ❖ Here, we have three samples, but the expression of the housekeeping gene varies among them, indicating that the initial amounts of the samples are **not equal**.
- ✓ As a result, the observed increases in intensity are not real.



Summarizing Techniques – Macromolecules Analysis

1. Proteins.
   - Proteins are separated based on size, then targeted with antibodies is process known as **immunoblotting** or **western blotting.**
   - ❖ Mechanism.
     - SDS-PAGE is done, separating proteins based on size.
     - Proteins are transferred into a membrane.
     - Addition of specific antibodies.
     - Detection of proteins and their sizes (MW).
2. RNA.
   - **Northern blotting** can be used to analyze gene expression.
   - Examining the presence of RNA bands, specifically RNA fragments that are complementary to a particular probe.
   - ✓ We assess not only their **presence** but also their **size** and **intensity**, with the latter reflecting the level of gene expression.
3. DNA.
   - **Southern blotting** is a technique used to analyze the sizes of DNA fragments, typically generated by restriction endonucleases, for example.



SDS: sodium diacyl sulfate.
PAGE: polyacrylamide gel electrophoresis.

In place

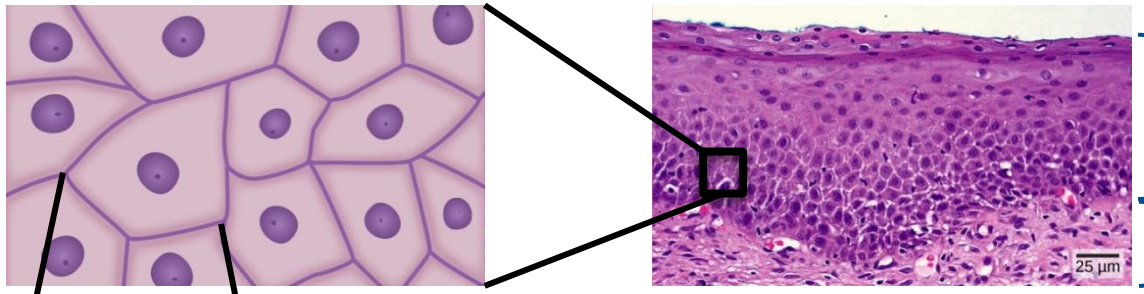
In situ hybridization

- In situ hybridization methods reveals the distribution of specific RNA molecules in cells in tissues.
- RNA molecules can hybridize when the tissue is incubated with a complementary DNA or RNA probe.
- In this way the patterns of differential gene expression can be observed in tissues, and the location of specific RNAs can be determined in cells.

- Looking for exactly where the gene is expressed in a tissue section.
- A tissue section has multiple cell types (differentiated, less differentiated and stem cells, ex: epithelial cells, endothelial cells, fibroblasts, etc.), and this allows the identification of which specific cell is expressing a particular gene.

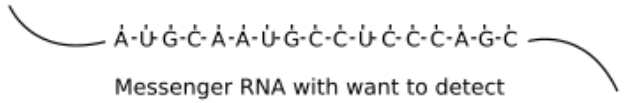
Procedure of in situ hybridization

Tissue section with multiple cell types.



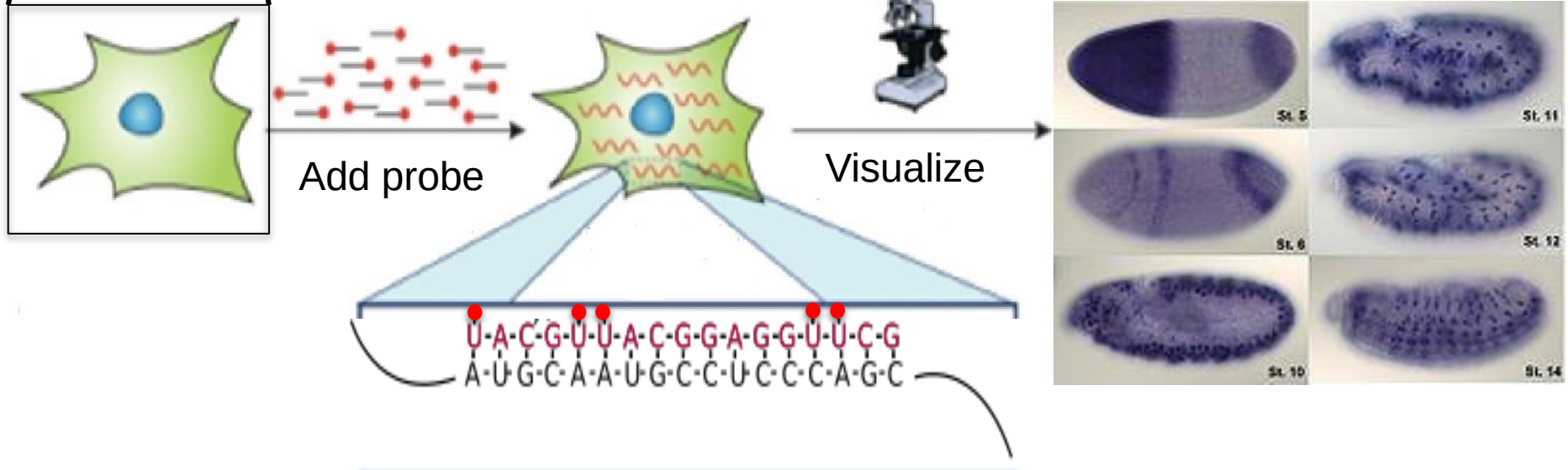
Well differentiated cells, immature cells, ex: epithelial cells (keratinocytes for example).

Probe with labeled nucleotides



Messenger RNA with want to detect

Stromal cells; fibroblasts, inflammatory cells, endothelial cells, muscle cells, etc.



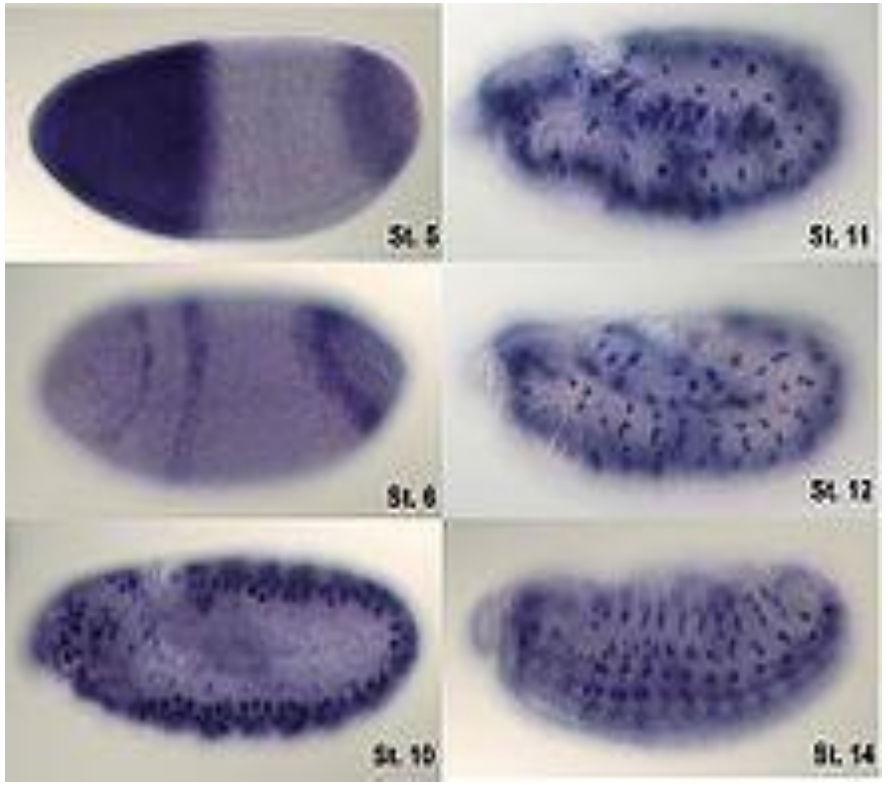
In Situ Hybridization – Procedure

1. A tissue section is obtained.
2. A probe (DNA or RNA) is added to the tissue section.
3. Upon hybridization with an mRNA, the probe emits a signal which allowing the identification of the specific cell where the signal originates.
   - Zooming in on a particular cell, the probe binds to messenger RNA (mRNA) molecules in that cell.
4. The signal is visualized, confirming that a specific cell expresses the gene of interest.

In Situ Hybridization – Visualization

- This is taken from a Drosophila embryo, the embryo of fruit flies.
- Various in situ hybridization techniques were performed using different probes targeting specific genes.

➢ These techniques reveal the distinct patterns of expression for certain proteins involved in processes like development and embryogenesis in fruit flies.



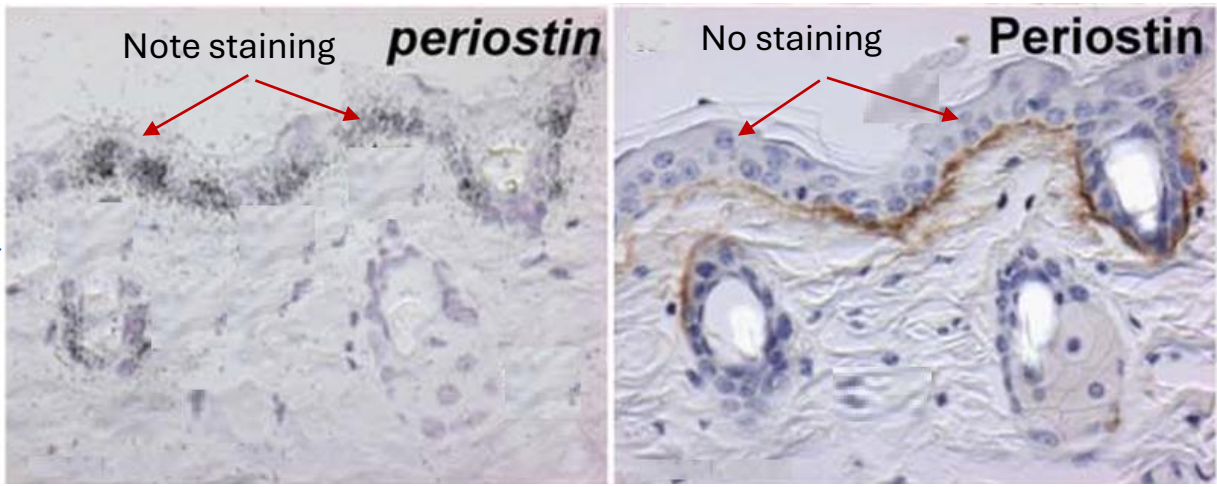
Why northern blotting and not immunohistochemistry?

- Sometimes, a gene may be expressed in certain cells, but the protein is located elsewhere.
- For example,
  - In in situ hybridization (ISH), the expression of a gene is observed at the RNA level in cells lining the basement membrane (this example ➔).
  - However, when using immunohistochemistry (IHC), the protein was found in the basement membrane.

✓ This suggests that the cells express the gene, and the protein is immediately exported to the basement membrane.

➢ **In situ hybridization helps identify the source of gene expression and provides insight into protein localization within tissue sections.**

ISH (RNA) **IHC (protein)**



RNA and protein molecules do not coexist and are present in different places.

mRNA: inside cells along the basement membrane

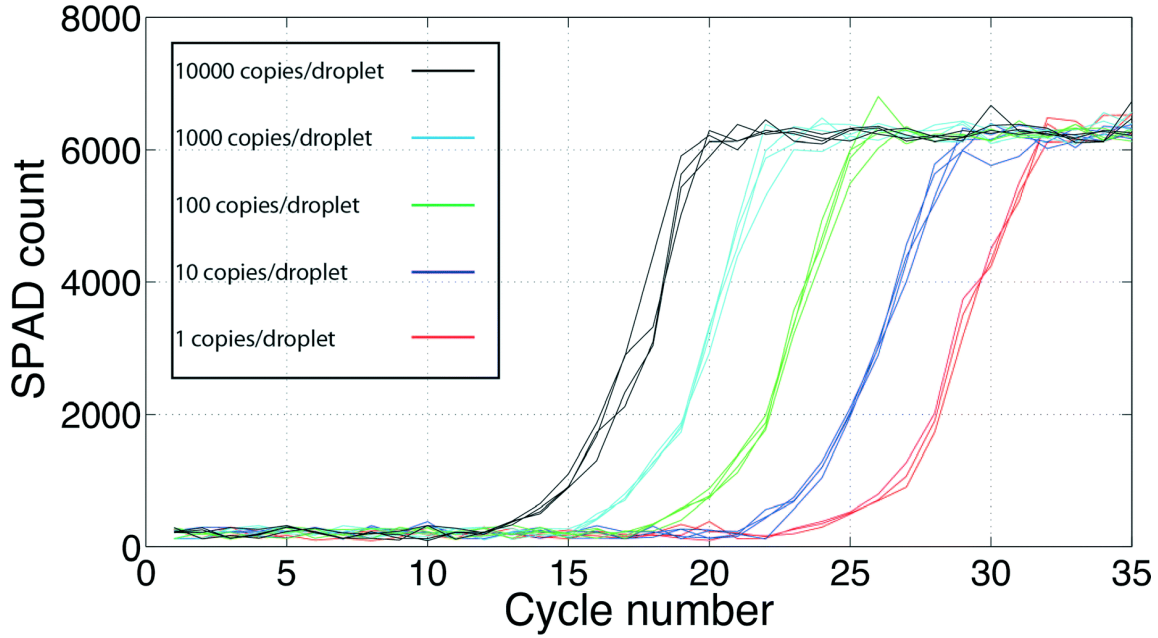
Protein: outside cells in the basement membrane

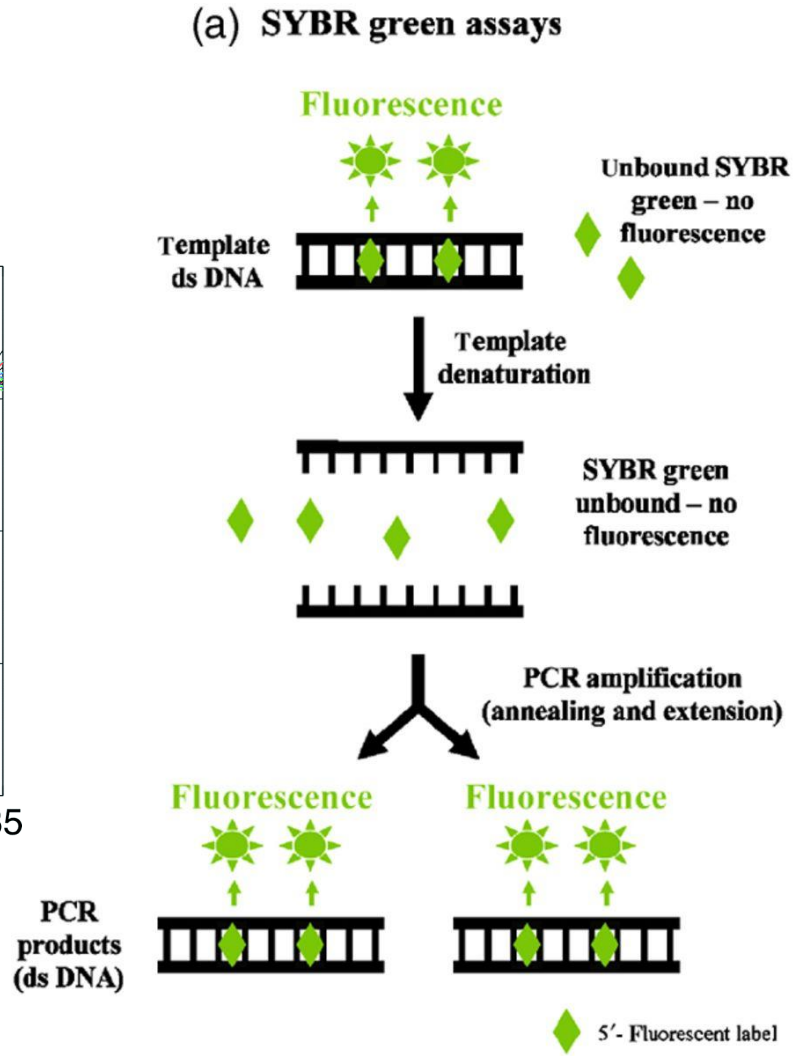
So, you can know that stained cells are the source of the protein, but not any other cell.

RT ⇔ Reverse Transcriptase (RNA → cDNA)

Quantitative reverse transcriptase real-time RT-qPCR of mRNA

- Another way of relative quantitation of RNA expression is by converting RNA into cDNA followed by PCR in the presence of SYBR green.
- The higher the amount of RNA (cDNA), the sooner it is detected.



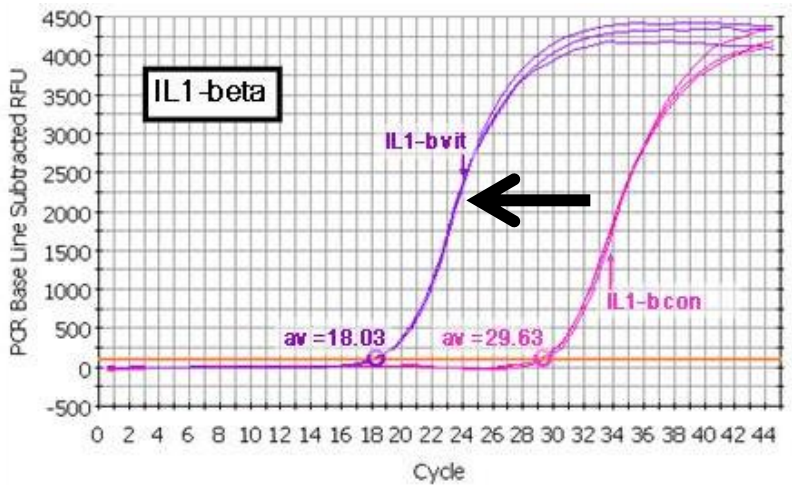


Apart from the reverse transcription here, this is completely equivalent to the technique discussed in *Lecture 6*.

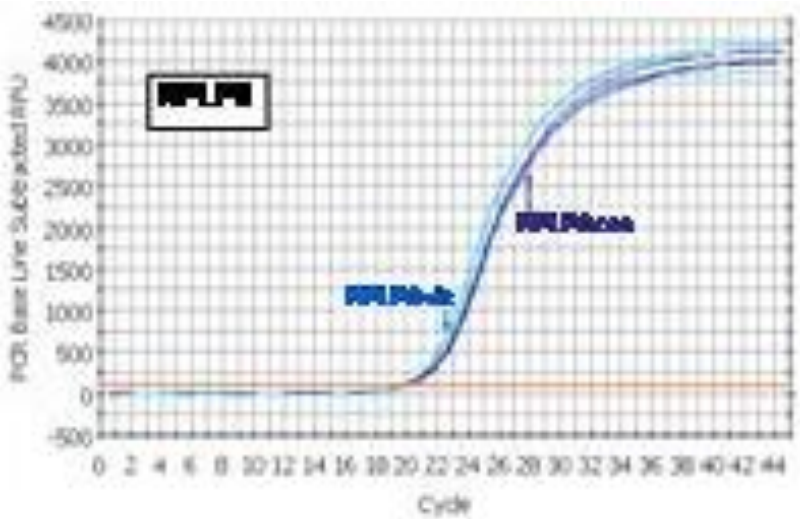
Recall that the **higher** the initial load (RNA load here), the **lower** the threshold cycle (see x-axis in the graph above).

Example

A gene of interest



Housekeeping gene



Notice the 2 curves in the graph above:

The right has higher threshold cycle → less RNA (cDNA)
The left has lower threshold cycle → more RNA (cDNA)

This indicates that this particular gene is transcribed to a greater extent in the sample shown on the left.

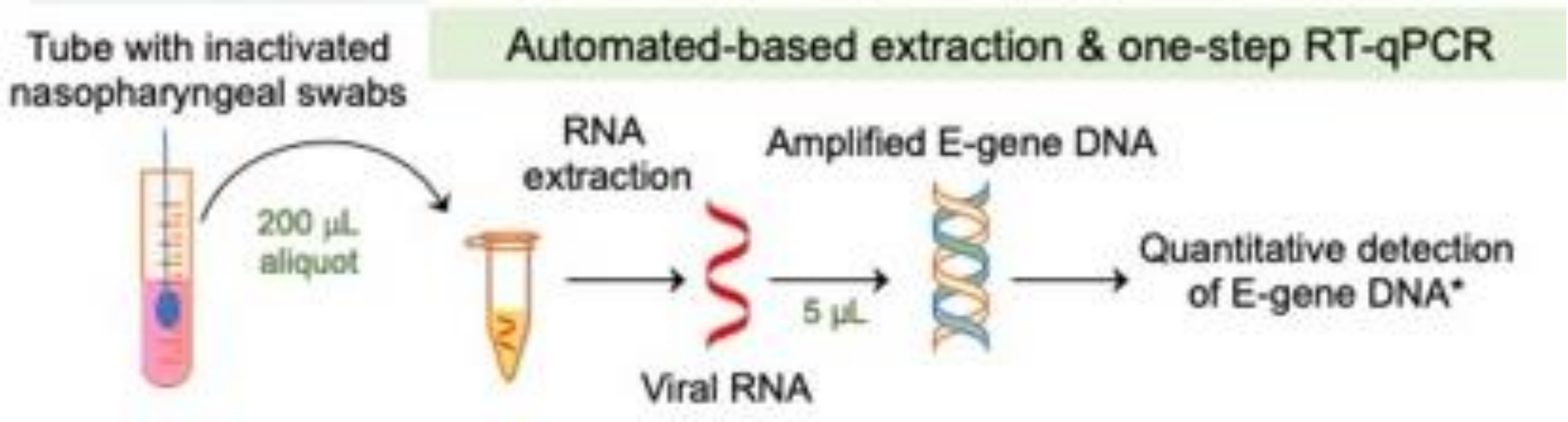
Unaltered expression

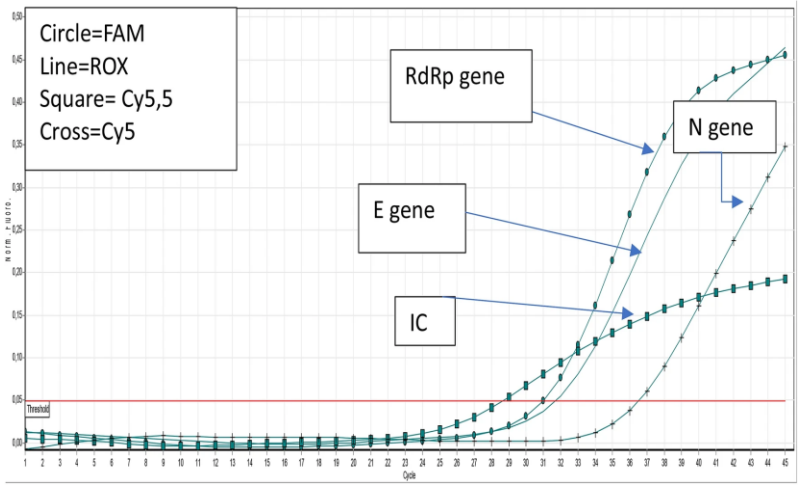
As discussed earlier in slide 10, we use a housekeeping gene to ensure that the experiment was well-conducted, and that both samples are initially equivalent.

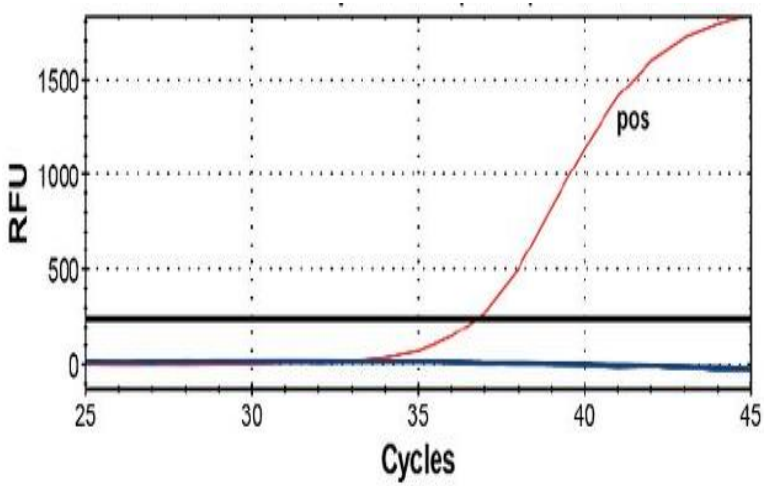
Detection of SARS-Co-2

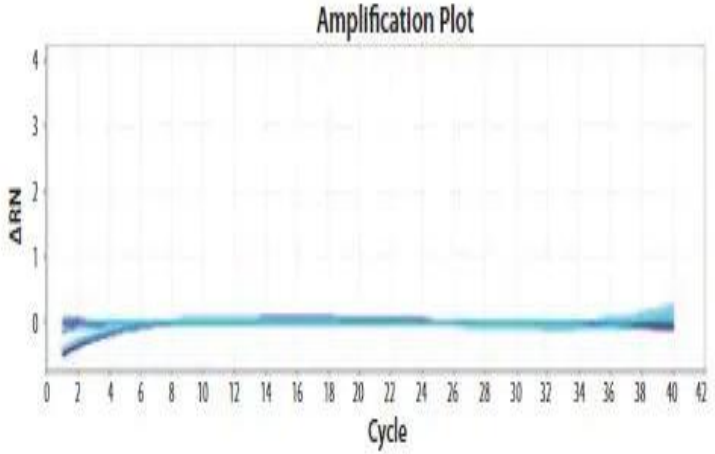
This RT-qPCR was famously used during the COVID-19 pandemic to assess the presence of coronavirus.

3 genes were monitored simultaneously (multiplexing).



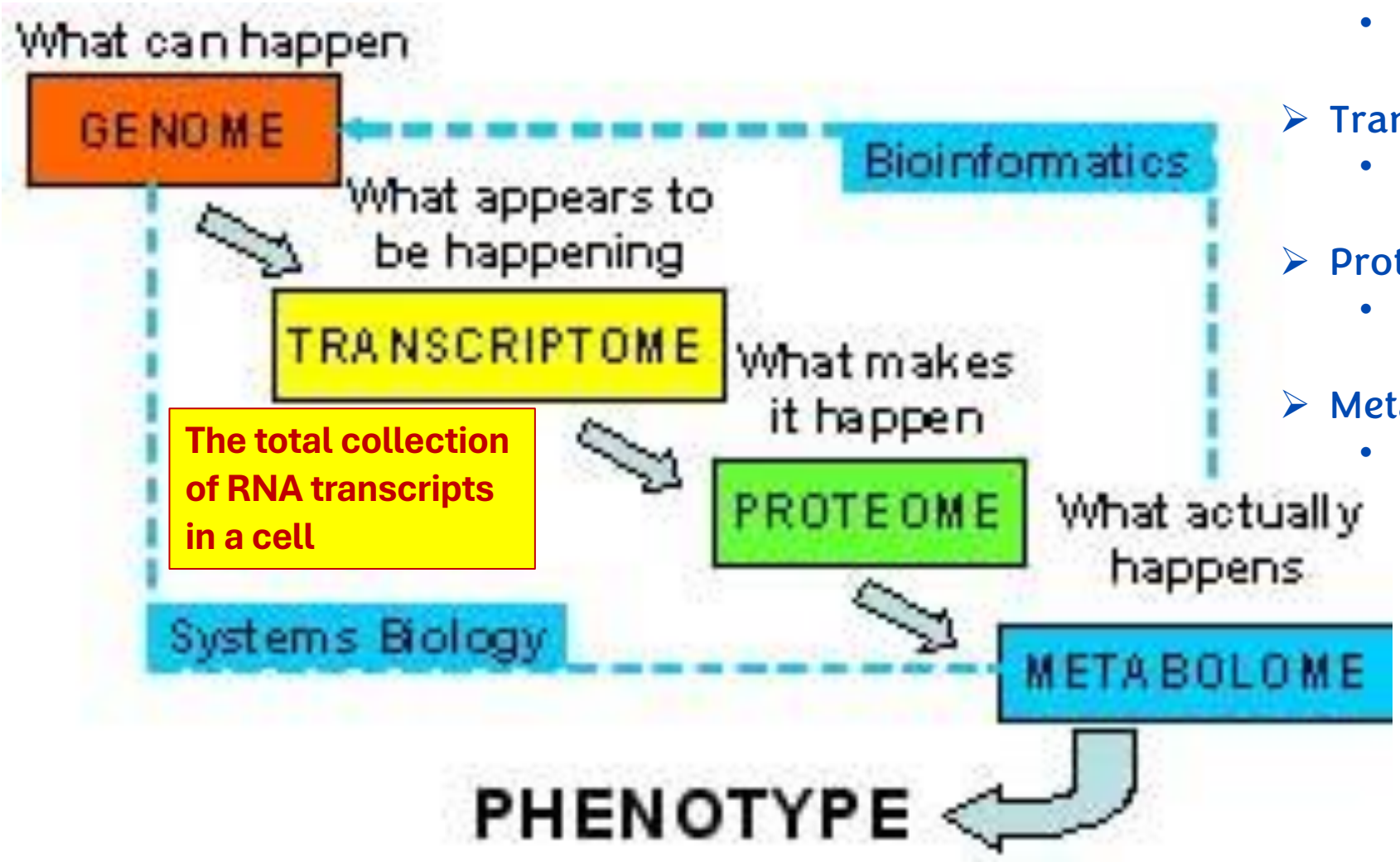






The 3 genes are expressed → positive.

The science of -omics



The total collection of RNA transcripts in a cell

On a larger scale, similar to the genome which accounts for all the DNA in a sample, there exist other concepts:

- Genomics (the study of genomes)
  - DNA sequence
- Transcriptomic (// of transcriptomes)
  - RNA produced by transcription
- Proteomics (// of proteomes)
  - Proteins translated from mRNA
- Metabolomics (// of metabolomes)
  - Metabolites altered by enzymes

Studying the transcriptome

- One such method in studying transcriptomes is DNA microarrays, which allow the analysis of the RNA products of thousands of genes all at once.

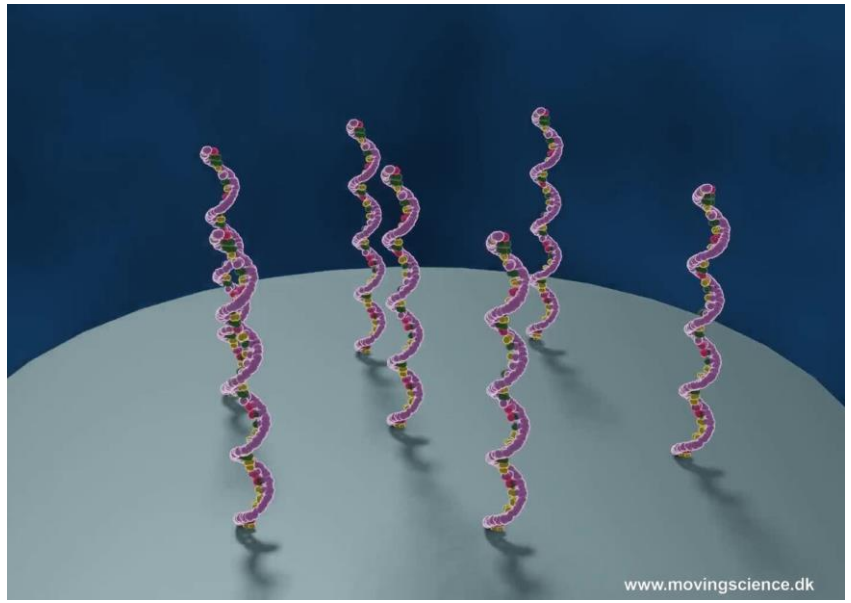
- By examining the expression of so many genes simultaneously, we can understand gene expression patterns in physiological and pathological states.

DNA microarrays

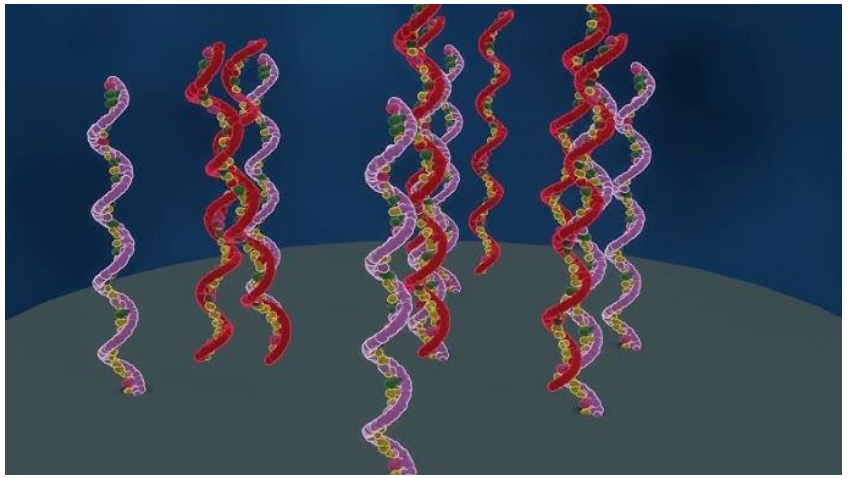
- DNA microarrays are solid surfaces (glass microscope slides or chips) spotted with up to tens of thousands of DNA fragments in an area the size of a fingernail.
- The exact sequence and position of every DNA fragment on the array is known.

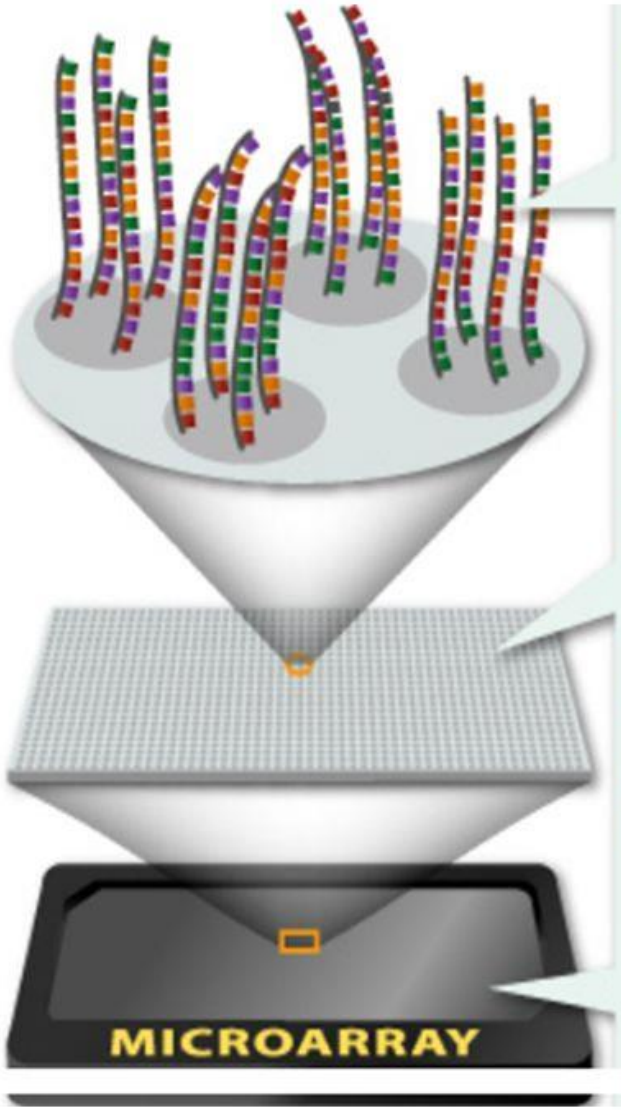
- http://learn.genetics.utah.edu/content/labs/microarray/
- http://www.sumanasinc.com/webcontent/animations/content/dnachips.html

Nonhybridized spot



Hybridized spot (partially)





A DNA micorarray allows scientists to perform an experiment on thousands of genes at the same time.

Each spot on a microarray contains multiple identical strands of DNA.

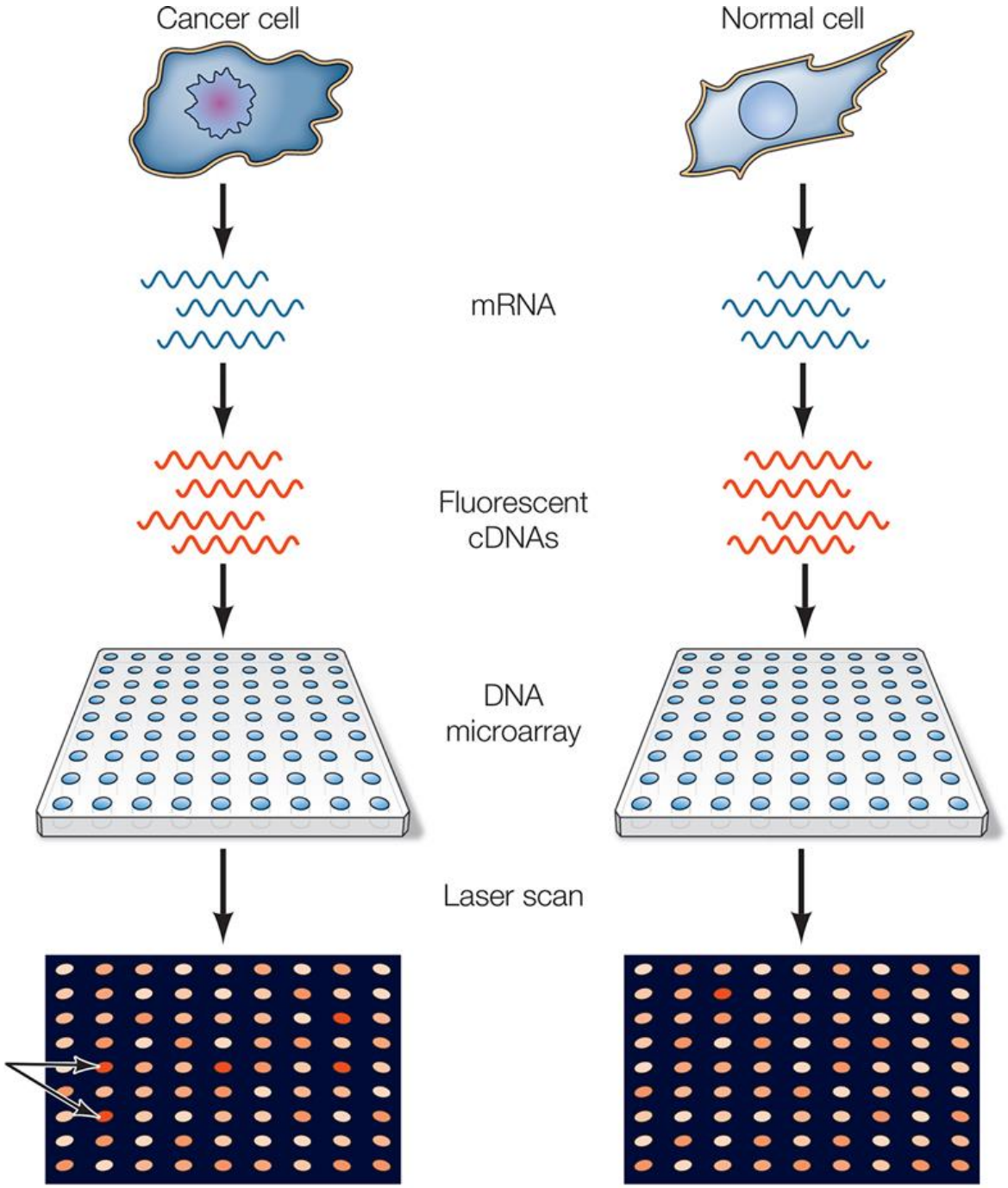
The DNA sequence on each spot is unique.

Each spot represents one gene.

Thousands of spots are arrayed in orderly rows and columns on a solid surface (usually glass).

The precise location and sequence of each spot is recorded in a computer database.

Microarrays can be the size of a microscope slide, or even smaller.



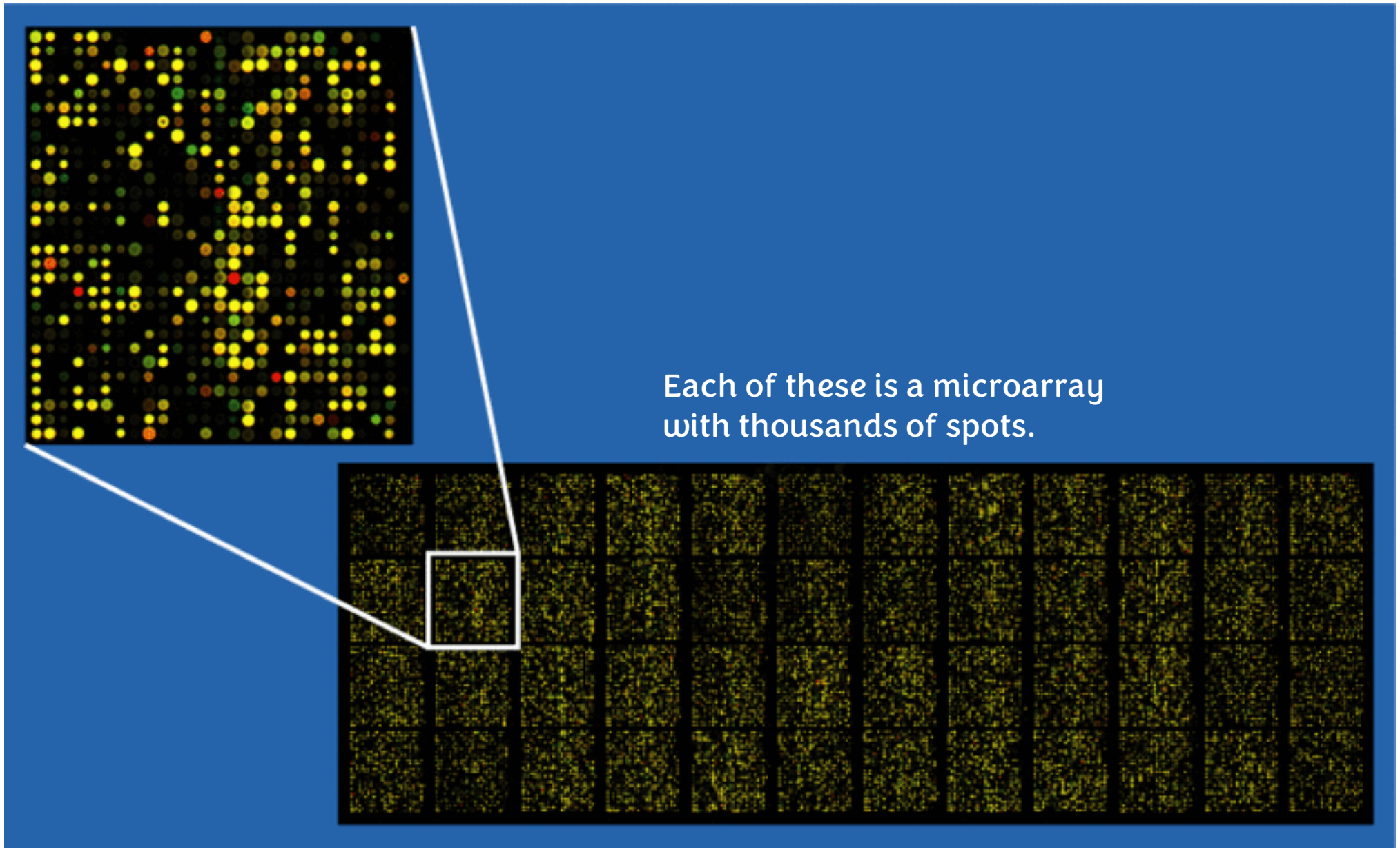
The procedure

- mRNAs are extracted from cancer cells and normal cells and converted to cDNAs, which are labeled with a fluorescent dye.
- The cDNAs are then hybridized to a DNA microarray containing spots of oligonucleotides corresponding to 20,000 or more distinct human genes.
- The relative level of expression of each gene is indicated by the intensity of fluorescence at each position on the microarray, and the levels of expression in cancer cells and normal cells can be compared.

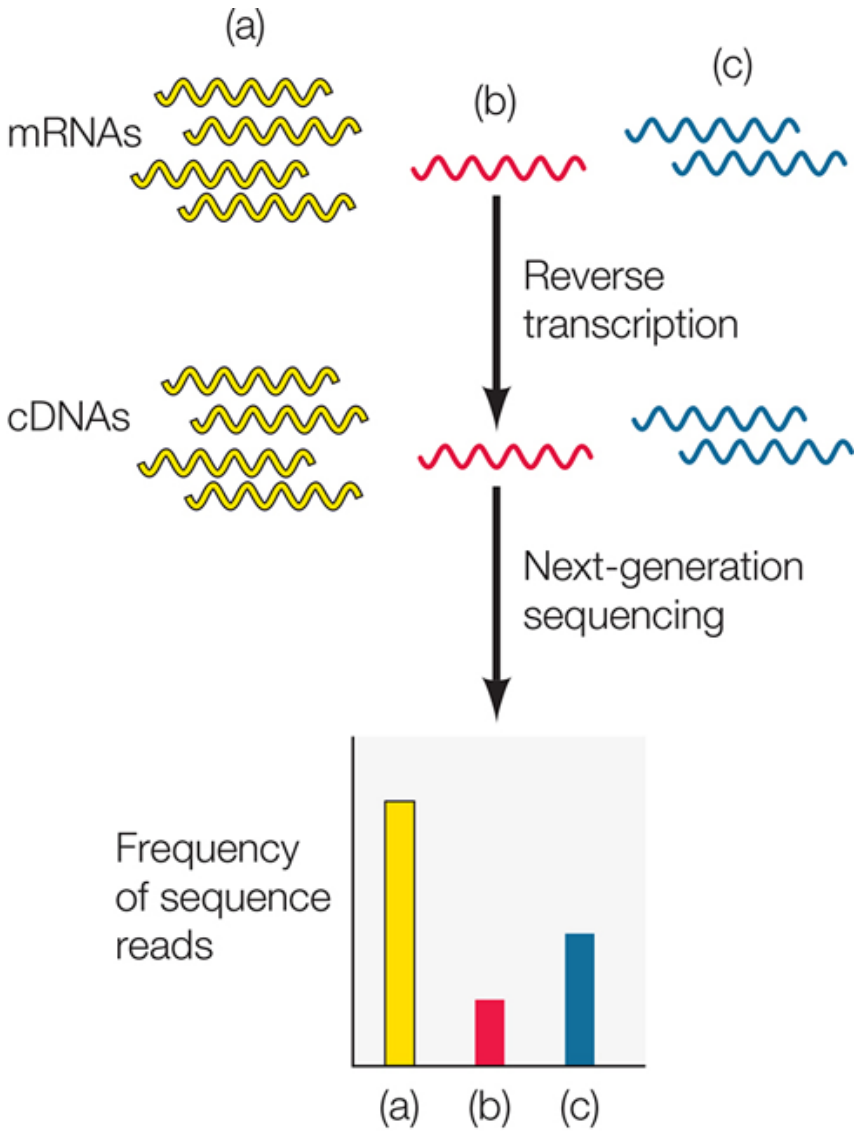
DNA microarrays – More explanation

- The microarray is divided into thousands of spots.
- 20000 for protein-coding RNA + others for other RNA types.
- Each spot is composed of many copies of DNA probes attached to the underlying surface.
- DNA fragments at each spot are identical and are complementary to the cDNA of exactly one gene.
- After exposition to the cDNA sample, the microarray obtains a unique configuration related to the intensity of the signal from the fluorescent cDNA from the sample; the amount of cDNA of a gene is proportional to the extent of expression of that gene in the sample, and thus the intensity of the signal at each spot reports the expression of the gene whose cDNA is complementary to the DNA fragments at the spot.
- E.g., RED may indicate strong signal, PINK is weaker, and WHITE with no signal.

Notice that each spot reflects the expression of one gene.



RNA sequencing (RNA-seq)



- Cellular RNA is reverse transcribed to cDNAs, which are subjected to next-generation sequencing.
- The relative amount of each cDNA (mRNA) is indicated by the frequency at which its sequence is represented in the total number of sequences read.

Recall Lecture 7 where next-generation DNA sequencing was discussed.

The idea here is that reverse-transcripted RNA molecules → cDNA are sequenced just like any DNA.

The more frequent a **cDNA** is sequenced, the more its **RNA** was initially made, and the more active the gene is.

RNA-seq vs. microarray

- RNA-seq can be used to
  - characterize novel transcripts
  - Identify splicing variants
  - profile the expression levels of all transcripts

This technique, unlike microarrays, doesn't need a known complementary sequence to capture the DNA sought for.

Instead, it "sequences" the cDNA for all genes present; after that we can refer to databases to identify the sequences.

This is how it was known that about 75% of the genome is transcribable (not only protein-coding genes).

- Microarrays are limited to detect transcripts corresponding to known genomic sequences. RNA-seq can overcome these limitations.

For any feedback, scan the code or click on

Corrections from previous versions:

Versions	Slide # and Place of Error	Before Correction	After Correction
V0 → V1			
V1 → V2			

Additional Resources:

Extra sources:

1. Northern blotting

رسالة من الفريق العلمي:

اللهم لا ترفع ليهود راية
ولا تحقق لهم غاية
واجعلهم لمن خلفهم عبرة وآية
اللهم عليك بمن خذل غزة وأهلها
اللهم أعنا على نصرتهم ولا تؤاخذنا بما فعل السفهاء منا

اللهم أغثنا
اللهم اسقنا الغيث ولا تجعلنا من القانطين