Cellular and Molecular Biology I

BIO-205-12

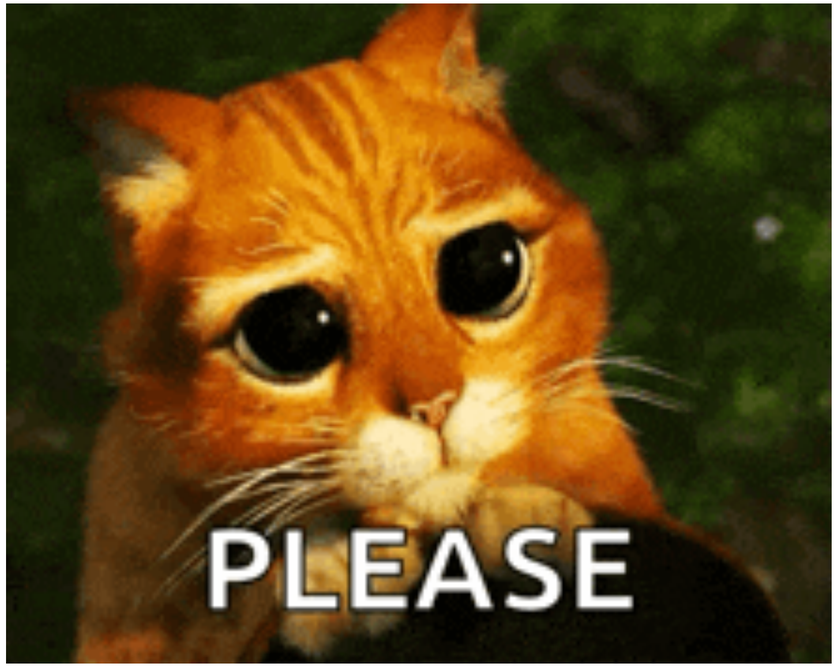
Camille Goemans - 2024

End-of-year organization

Week	Exercises	Lecture
12	10	12
13	11+12	**questions only**
14	no	no

My evaluation

- Teacher’s evaluation
    - improve next year class
    - improve my teaching in general
    - adapt the exam



Your evaluation

- 30 multiple choice questions
- 30 True/False (negative points)
- French translation
- Automatic correction —> bring a black pencil and eraser
- Additional info on Moodle (room, place to sit, etc.)
- Open book
    - make sure to understand and know the basic concepts/terminology
    - make sure to know how the course is organised, where to find the information

Summary

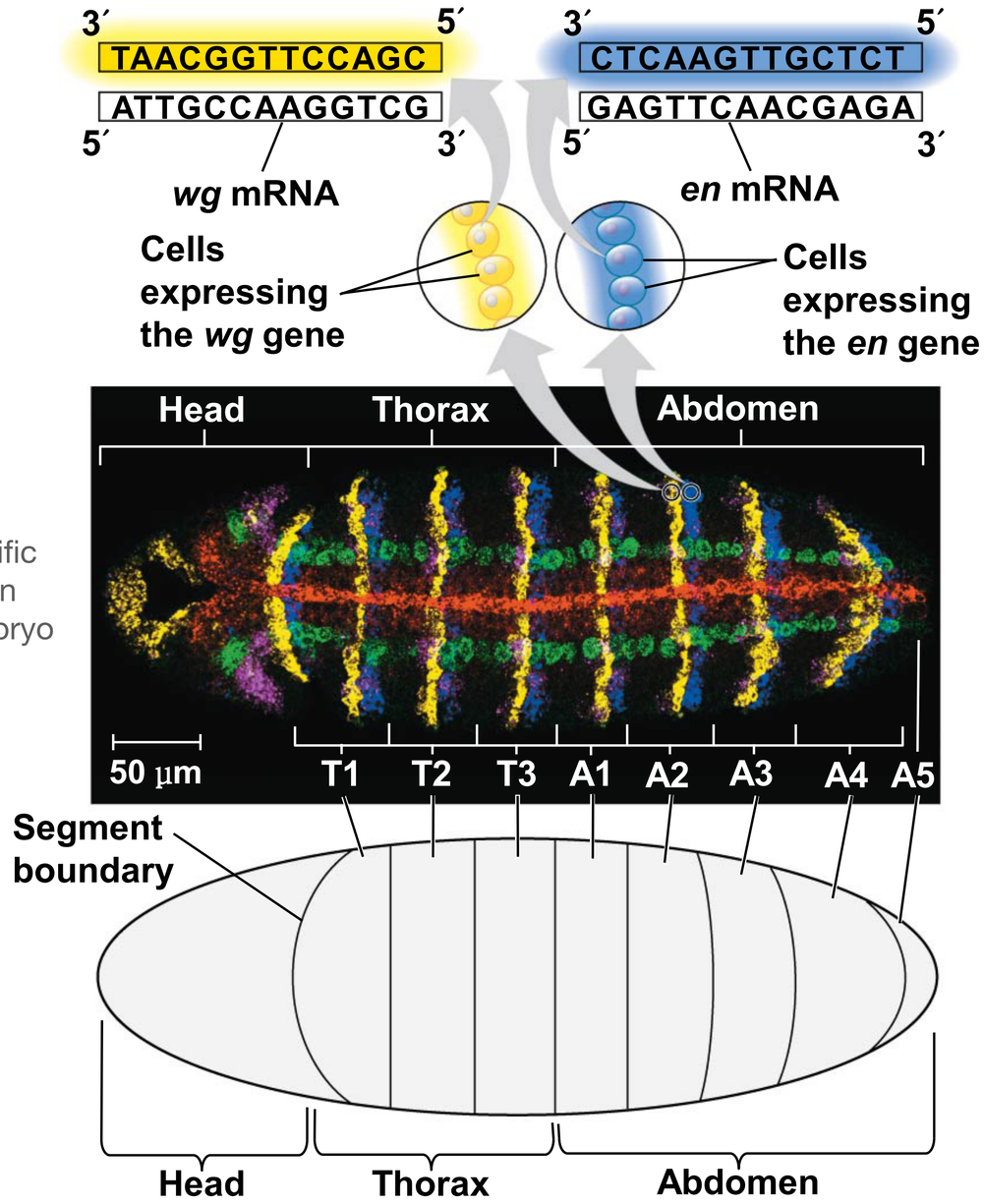
- Studying DNA
    - DNA sequencing
    - DNA extraction
    - DNA amplification
    - DNA cloning
    - Cloned organisms and stem cells
    - Practical applications of DNA-based technology

Plan

- **Studying gene expression**
- Determining gene function
- Functional genomics
- Visualizing cells and molecules

How to study gene expression ?

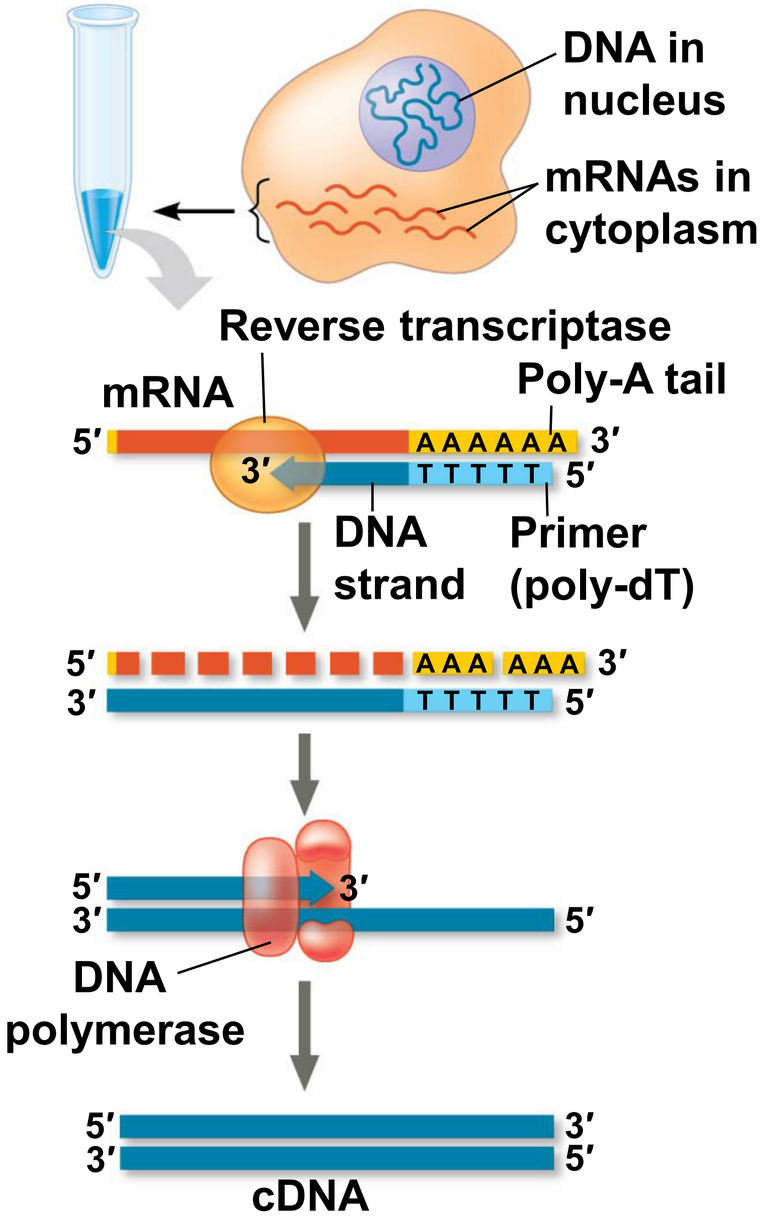
- by looking at the **mRNAs: *in situ* hybridization**
  - Where and when is the **RNA** produced?
  - Based on **nucleic acid hybridization**
  - Tissues are fixed and probes added
  - **No genetic engineering** required
  - Not good for **quantification**



Expression of specific regulatory genes in Drosophila early embryo

Studying gene expression

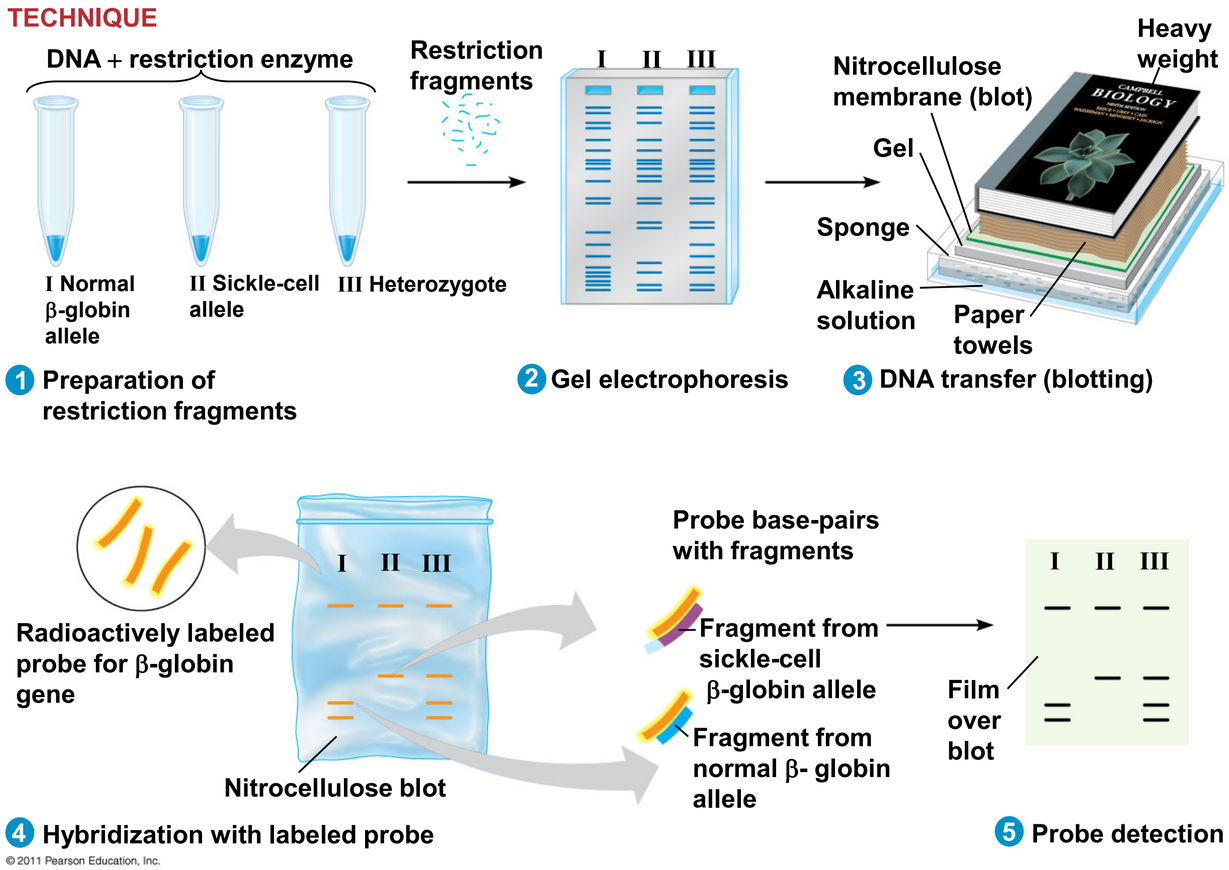
- using **Northern Blotting**: gel electrophoresis of mRNA followed by hybridization with a probe on a membrane
- using ***in situ* hybridization:** fluorescent dyes attached to probes to identify the location of specific mRNAs in the intact organism
- using Reverse transcriptase-PCR or **RT-PCR**



Studying gene expression

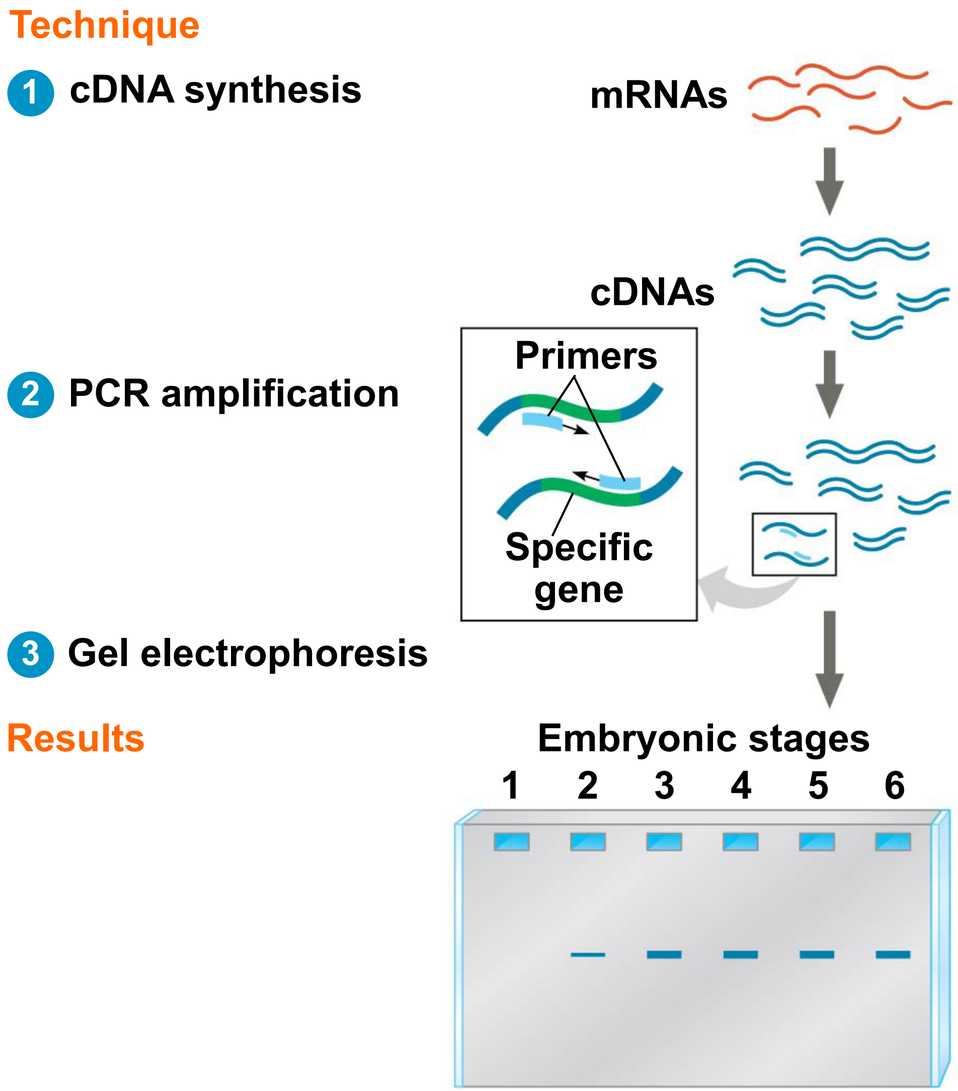
- using **Northern Blotting**: gel electrophoresis of mRNA followed by hybridization with a probe on a membrane

Here, southern blotting but same principle



Studying gene expression

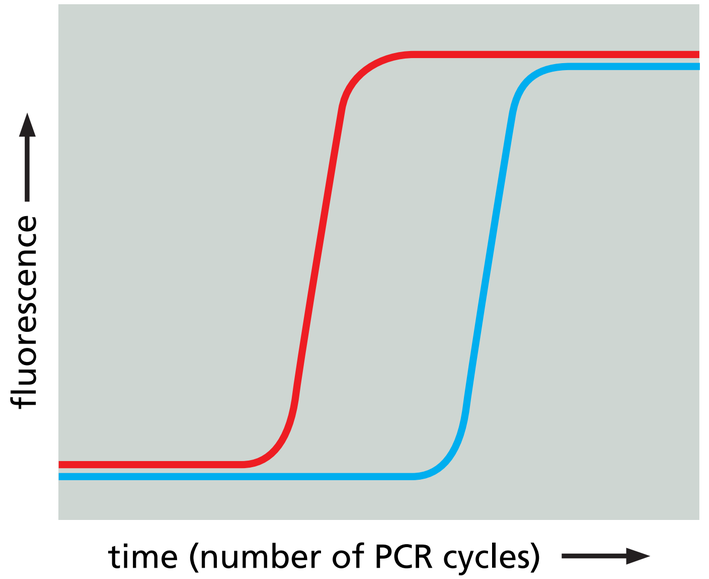
- using Reverse transcriptase-PCR or **RT-PCR**



Studying gene expression

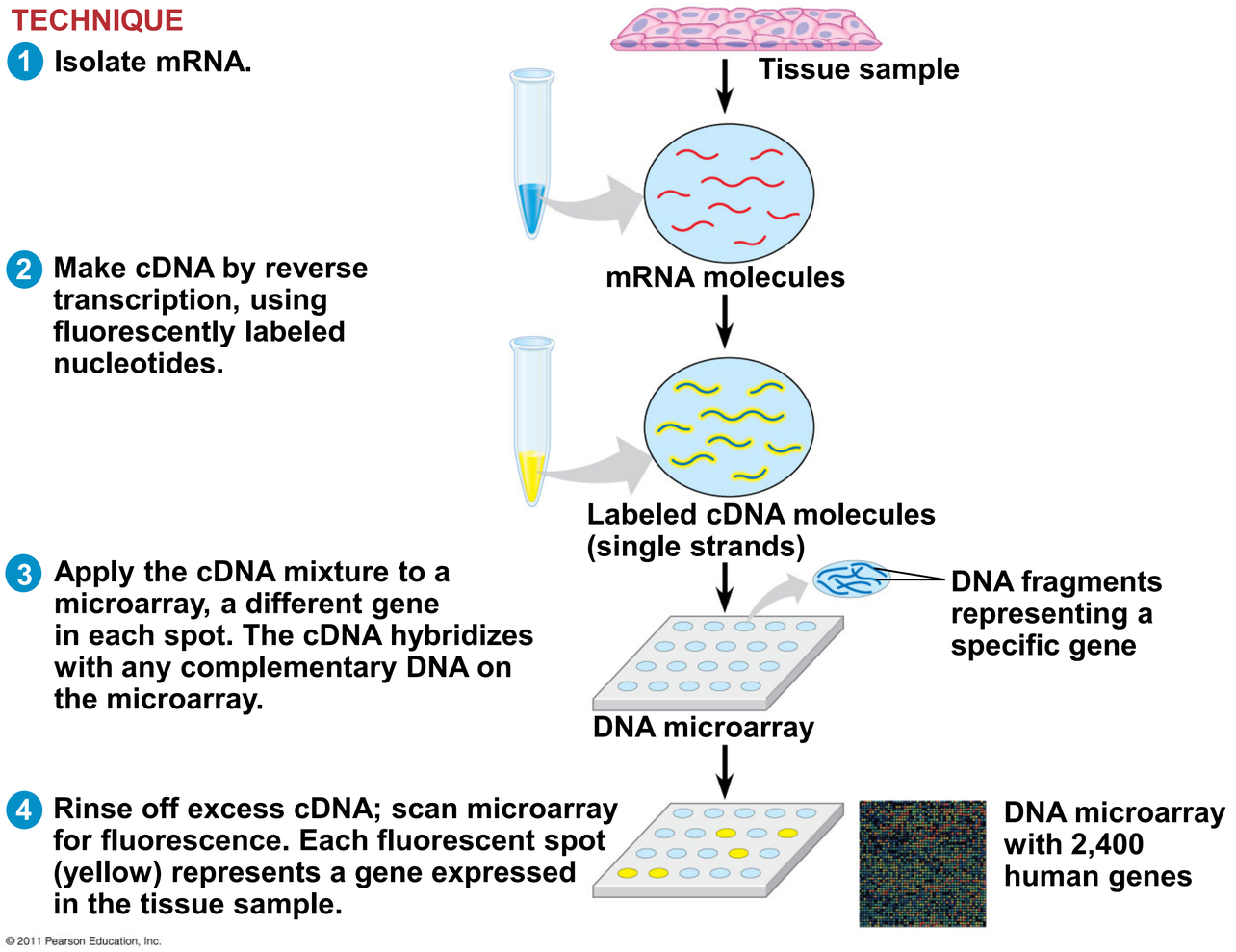
- or quantitative **RT-PCR (qRT-PCR)**

  - **Quantifying** RNAs using **quantitative reverse-transcription polymerase chain reaction**
  - Isolation of the **whole pool of RNAs** from a sample (no DNA!)
  - Addition of **primers specific to the RNA** of interest + reverse transcriptase+ DNA polymerase+ nucleotides
  - Addition of **chemical dyes** that are fluorescent when bound to dsDNA
  - Direct relationship between the **number of PCR cycles needed to detect the product** and **initial amount of RNA** in the sample



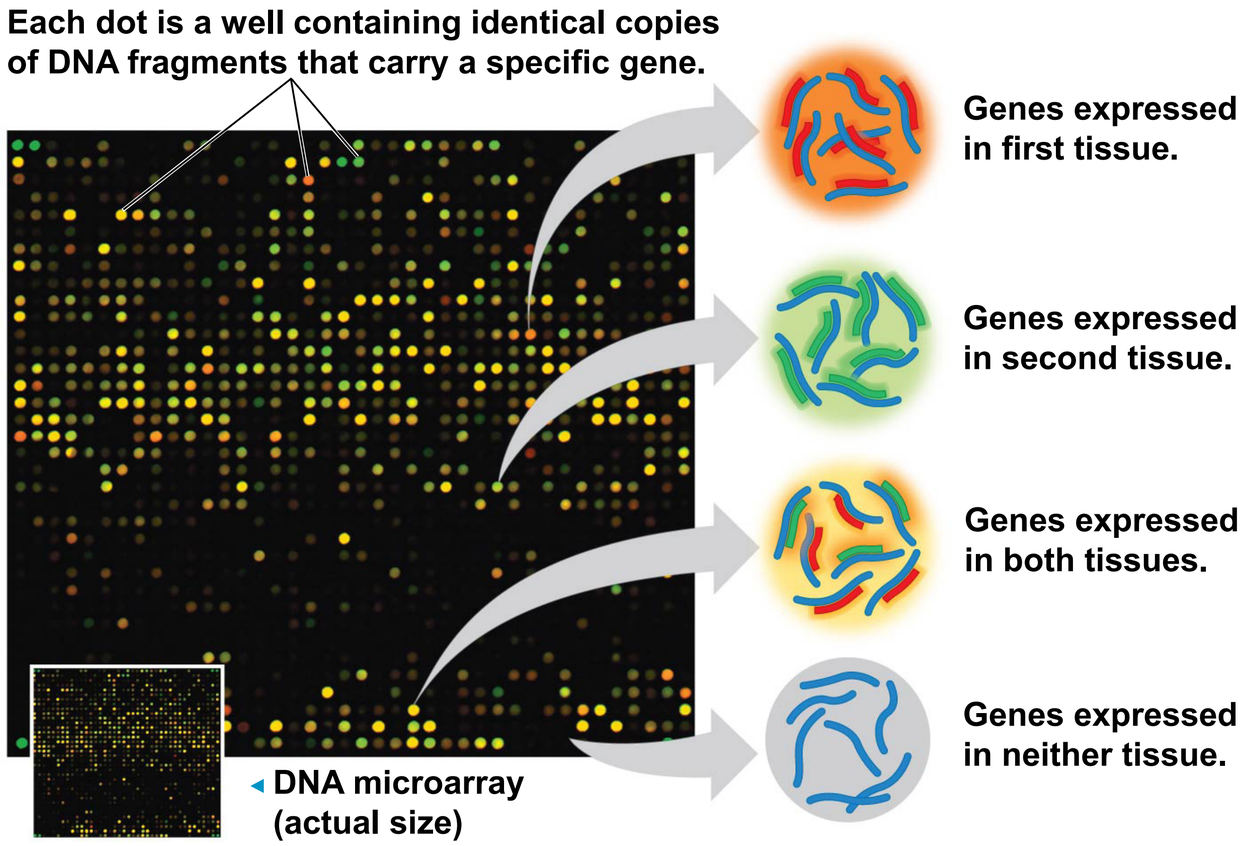
Studying gene expression

- using **microarrays**



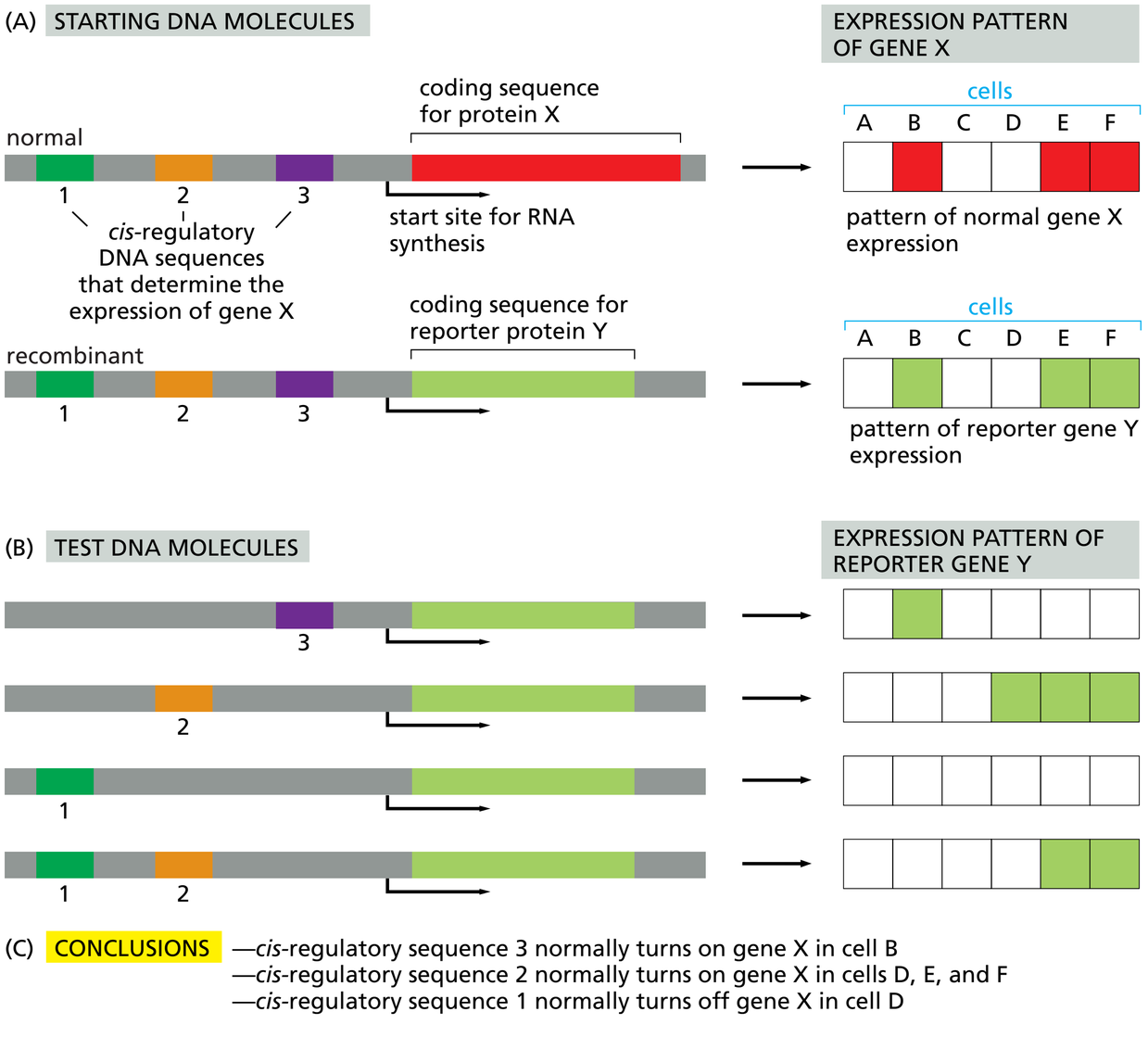
Studying gene expression

- using **microarrays**



Studying gene expression

- using **reporter genes**



Studying gene expression

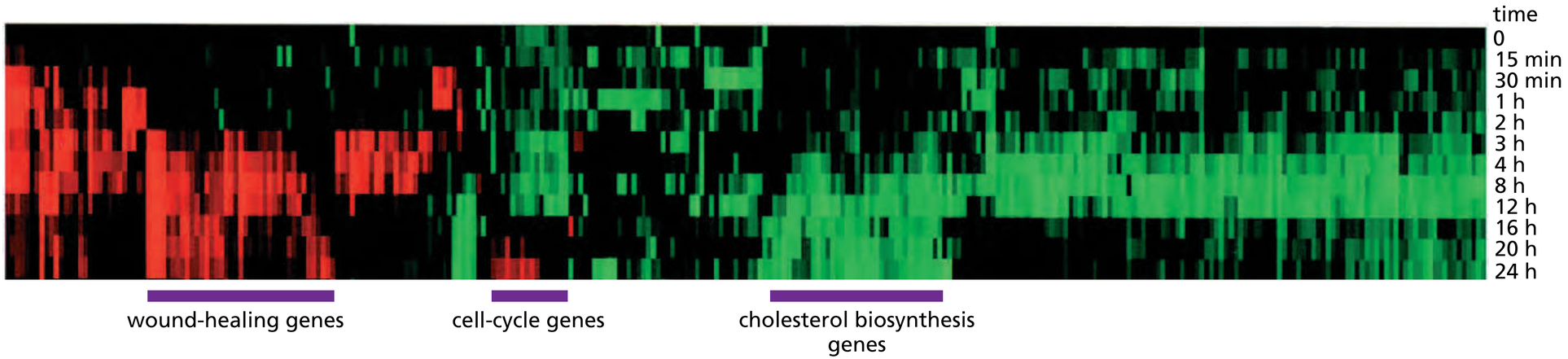
- using **RNA sequencing (RNA-seq)**
  - Measures **which genes are being transcribed** at a given time under given conditions
  - Uses **reverse transcriptase** that to copy all RNAs into cDNAs
  - cDNAs are then **fragmented** and **sequenced** by **next-generation sequencing**
  - Abundant **RNAs** have more **cDNA** copies and therefore more sequencing **reads**

Studying gene expression

- using **RNA sequencing (RNA-seq)**

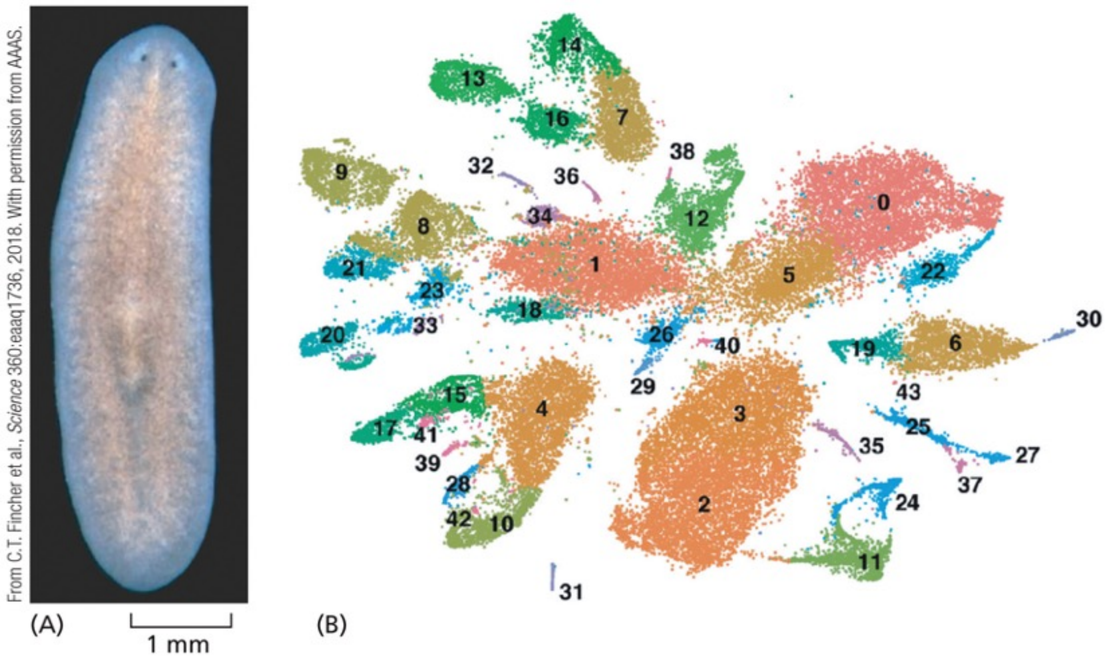
  ➡ **Identification** of RNAs present and **quantification**

  ➡ Using **cluster analysis** (computational approach), one can identify genes that are regulated together



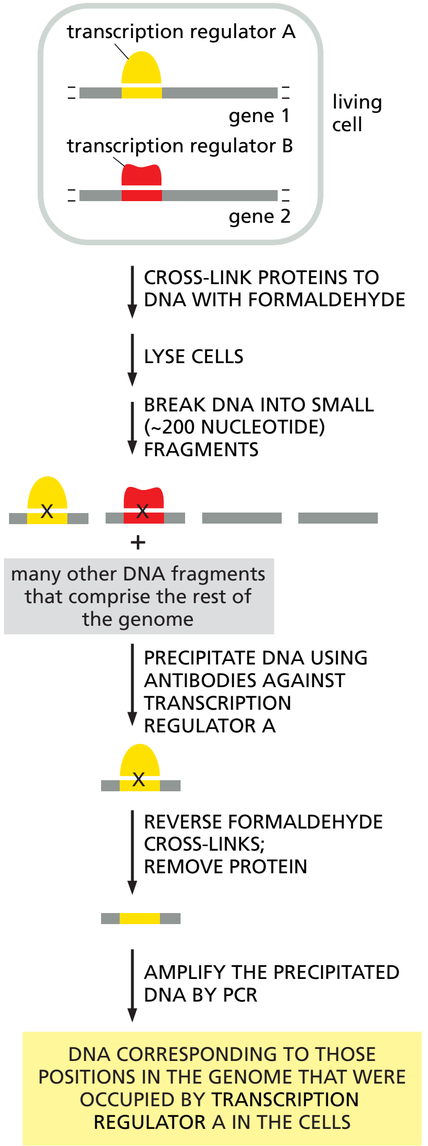
Studying gene expression

- using single cell **RNA sequencing (RNA-seq)**
  - Tissue dissociated into **single cells**
  - **Microfluidics** to separate single cells
  - Each cell is processed for **RNA-seq**
  - **Cluster analysis** algorithm that group cells with similar gene expression patterns



Studying gene expression

- Using **genome-wide chromatin immunoprecipitation** (ChiP) to identify sites on the **genome occupied** by transcription factors
  - **transcription regulators** are responsible for changing **transcription patterns**
  - Proteins are **cross-linked** to DNA
  - Cells are **open**
  - DNA is **fragmented**
  - **Antibodies** that recognise a specific transcription regulator are used to precipitate it with its bound DNA
  - DNA is **sequenced**
  - Can also be used to identify positions bound to specific **modified histones** (using antibodies that recognise those modifications)



Studying gene expression

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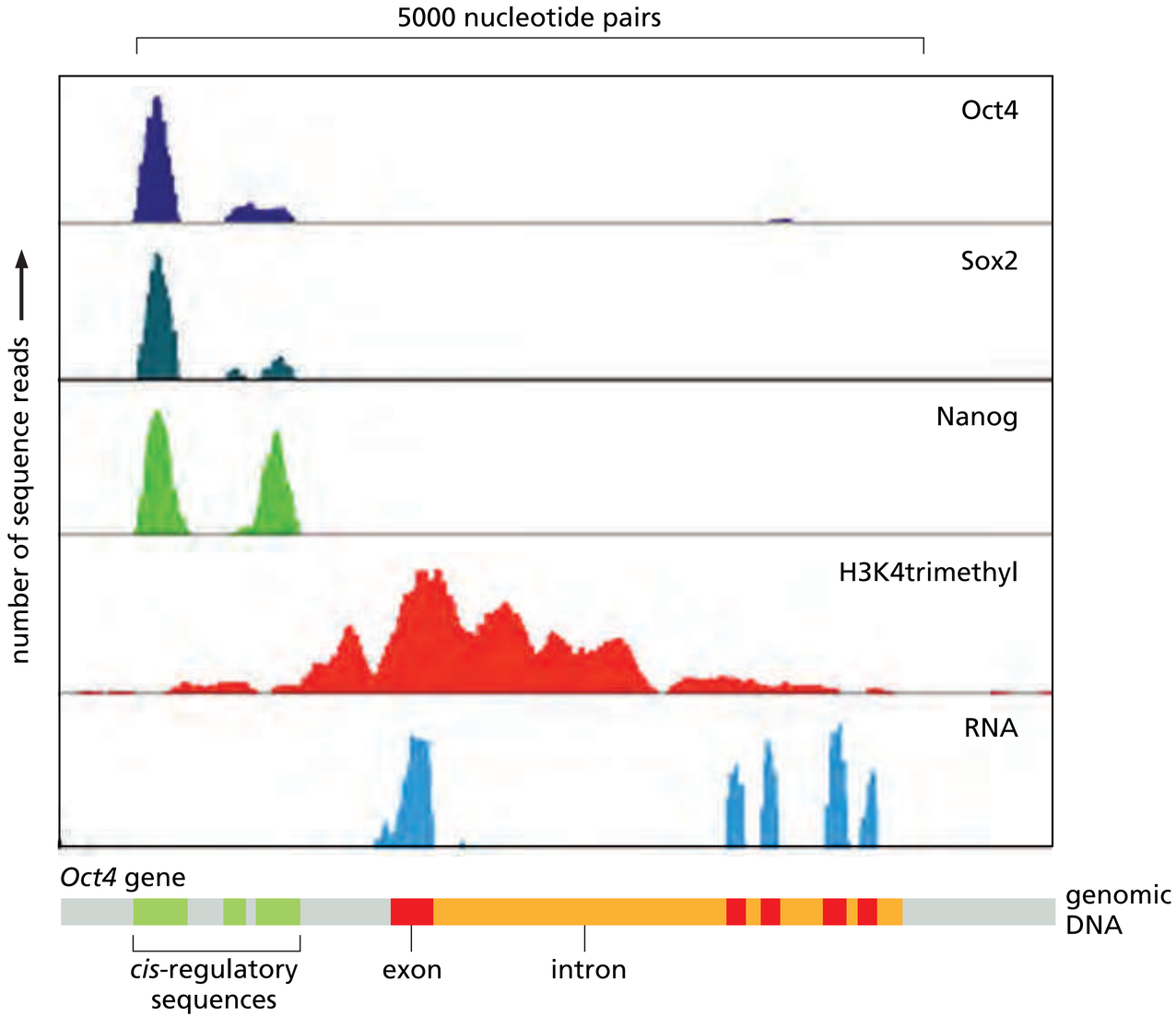
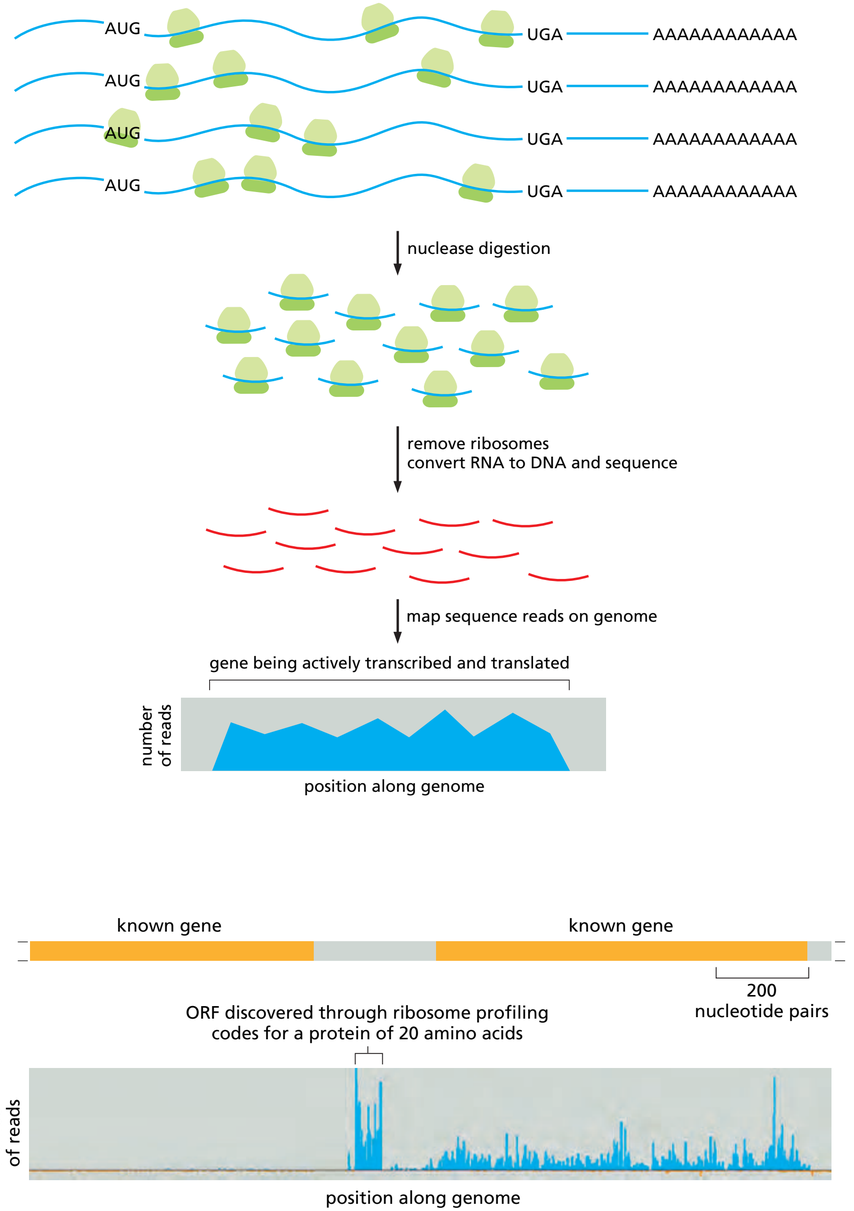


Studying gene expression

- Using **ribosome profiling** to identify **RNAs being transcribed** at a given moment in the cell
  - Gives a map of the **instantaneous position** of ribosomes on each mRNA in the cells
  - Total **RNA** is exposed to a **ribonuclease**
  - RNA sequences **covered by ribosomes** are spared
  - Protected RNAs are converted to **DNA** and **sequenced**
  - Allowed the discovery of **new (small) ORFs**



Plan

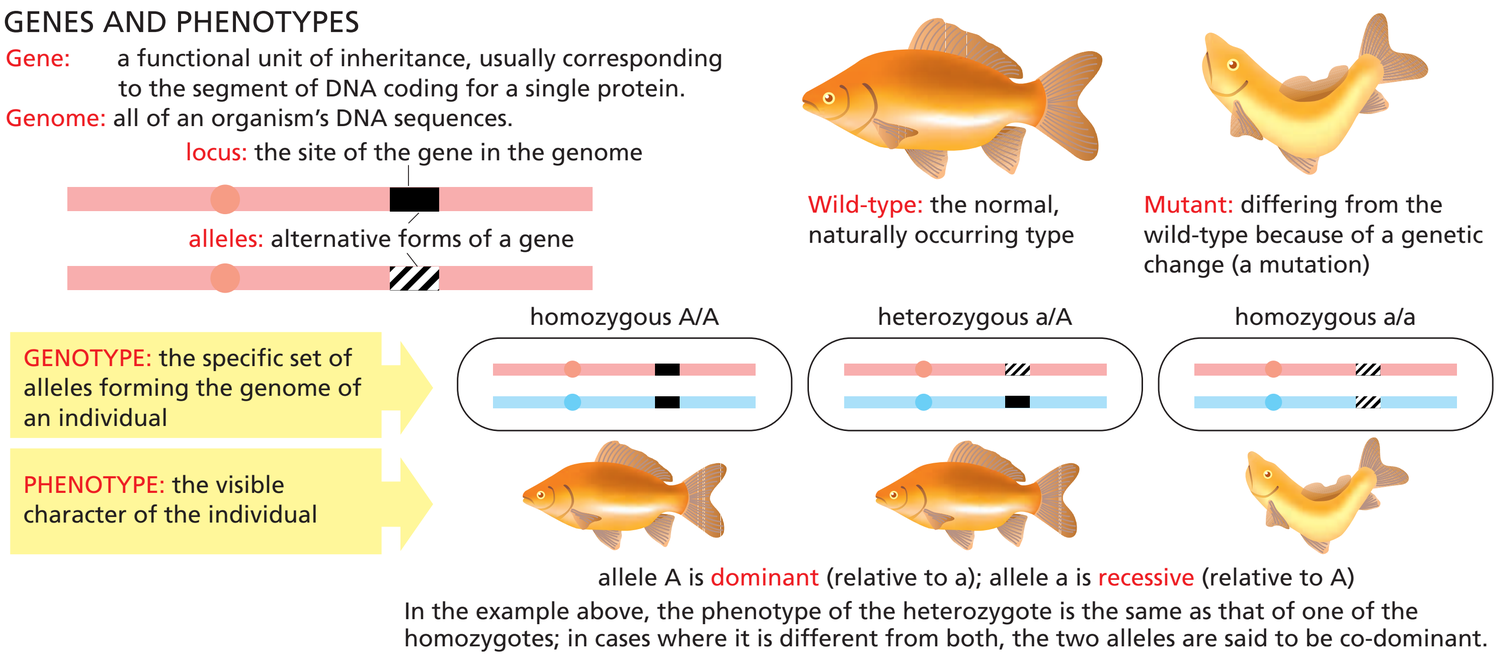
- Studying gene expression
- **Determining gene function**
- Functional genomics
- Visualizing cells and molecules

Determining gene function

- The classical approach is to **mutate or delete** a gene and look for associated **phenotype**
- **Mutations/deletions** are introduced by the **cloning methods** described before
- Gene expression can be silenced by **RNA interference**

Determining gene function: deletion mutants

- We want to understand how genes (and the proteins they encode) **function**
- One of the most direct way is to **remove the gene (=deletion mutant)** and see what happens
- Basis of **genetics**



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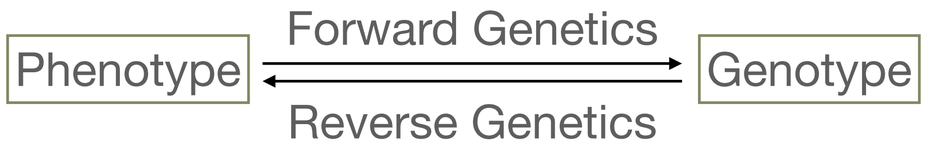

Determining gene function: creating mutations

- With **chemicals** or **radiations** that mutate DNA
- By **insertional mutagenesis** (with external DNA such as transposons)
- Using gene **cloning** (knock-out, knock-down, CRISPRi, …)

Building mutant libraries

Molecular Systems Biology (2006) doi:10.1038/msb4100050

www.molecularsystemsbiology.com
Article number: 2006.0008

molecular systems biology

Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection

Tomoya Baba[1,2], Takeshi Ara[1], Miki Hasegawa[1,3], Yuki Takai[1,3], Yoshiko Okumura[1], Miki Baba[1], Kirill A Datsenko[4], Masaru Tomita[1], Barry L Wanner[4,*] and Hirotada Mori[1,2,*]

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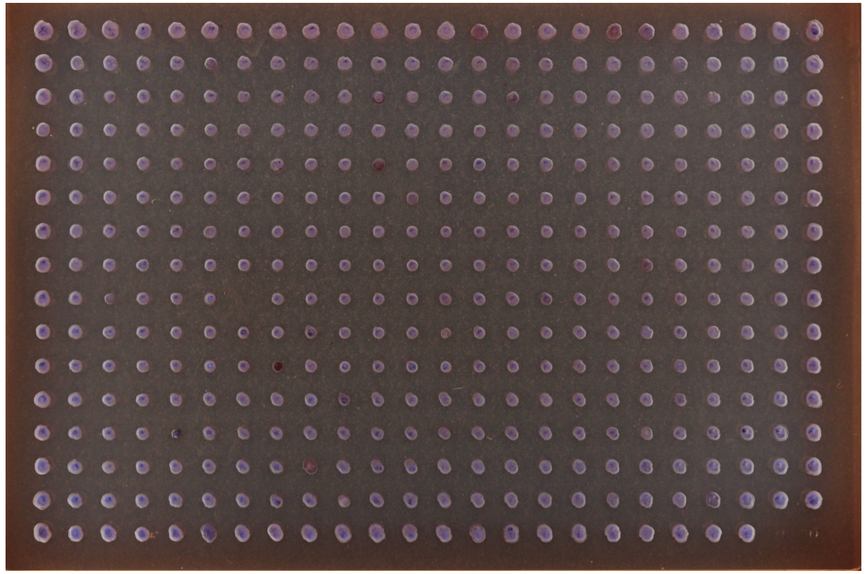
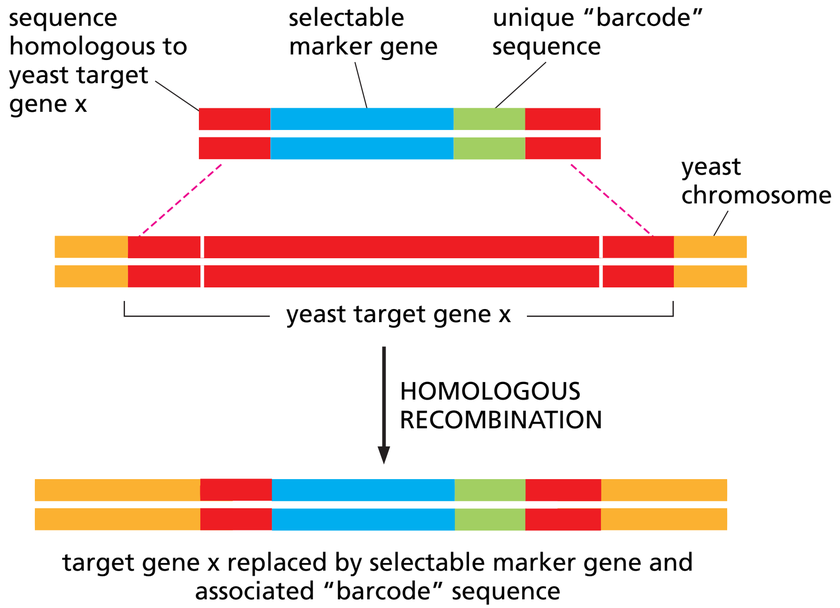


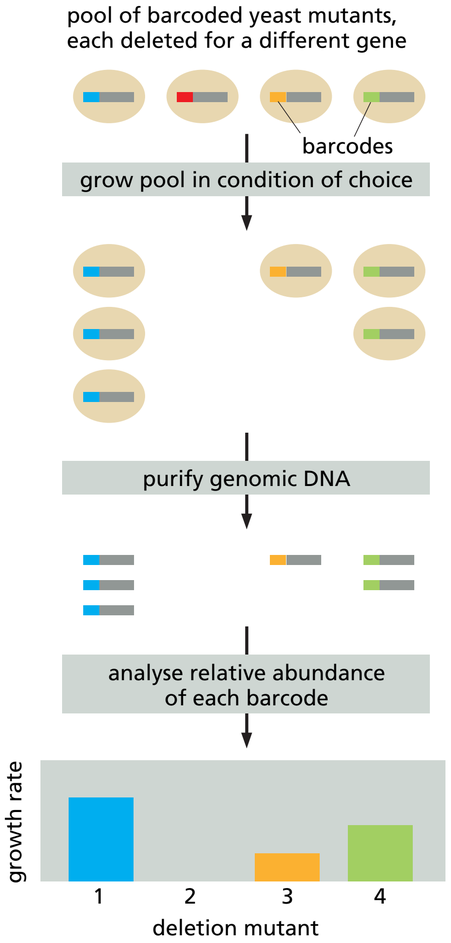
Building mutant libraries

- Efforts to produce this in more **complex organisms**
- Invaluable resource to investigate **gene function on a genomic scale**
- Use of **DNA barcodes** to facilitate mutant identification



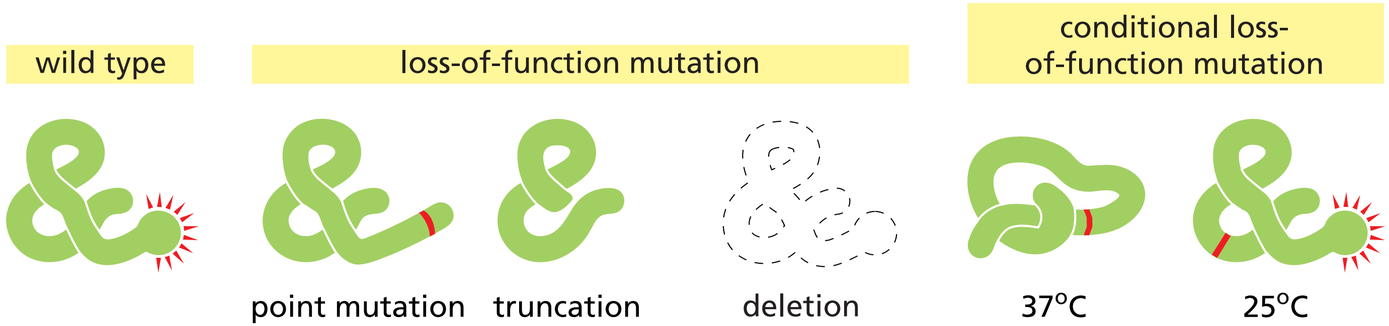
Building mutant libraries

- **Pooled library** versus arrayed library

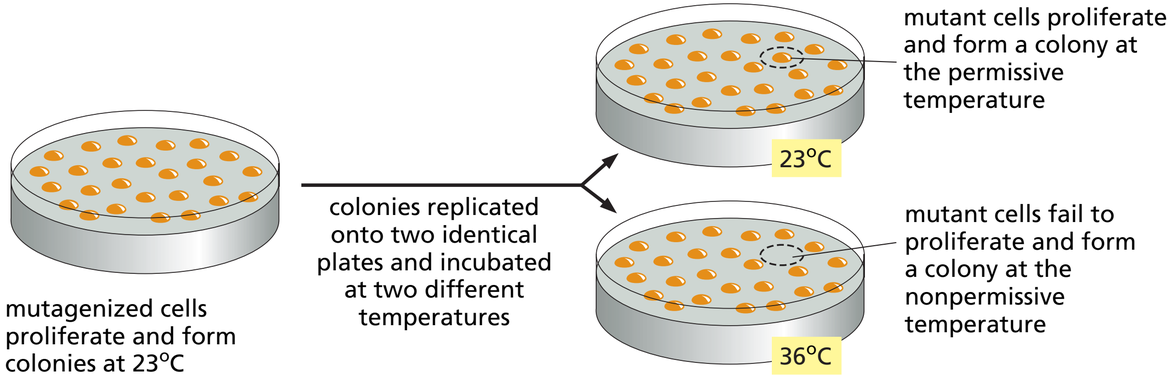


Gene mutations

- Can lead to gene **loss-of-function** = gene product does not work
- Can lead to gene **gain-of-function** = works too much or in a different way

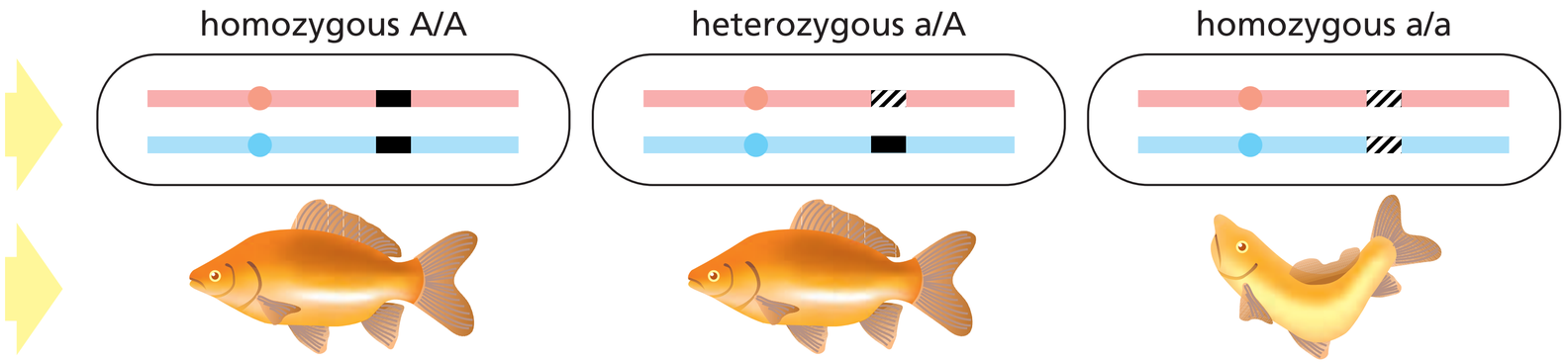


- **Conditional mutant** only shows a phenotype in a given condition



Gene mutations

- A mutation can be **dominant** or **recessive**
- **Dominant mutations** cause the mutant phenotype when only present in one copy
- **Recessive mutations** cannot cause the mutant phenotype when a wild-type copy is also present



allele A is dominant (relative to a); allele a is recessive (relative to A)

In the example above, the phenotype of the heterozygote is the same as that of one of the homozygotes; in cases where it is different from both, the two alleles are said to be co-dominant.

Complementation

- **Different mutants** can exhibit the **same phenotype**
- A **complementation test** can be used to test whether different mutations appear in the same gene or not
- In diploid organisms: mating of an individual that is homozygous for one mutations with an individual that is homozygous for the other mutation
  - if the mutations occur in the same gene, the offspring will show the phenotype
  - if the mutations occur in different genes, the offspring will not show the phenotype as it will have one copy of each WT gene

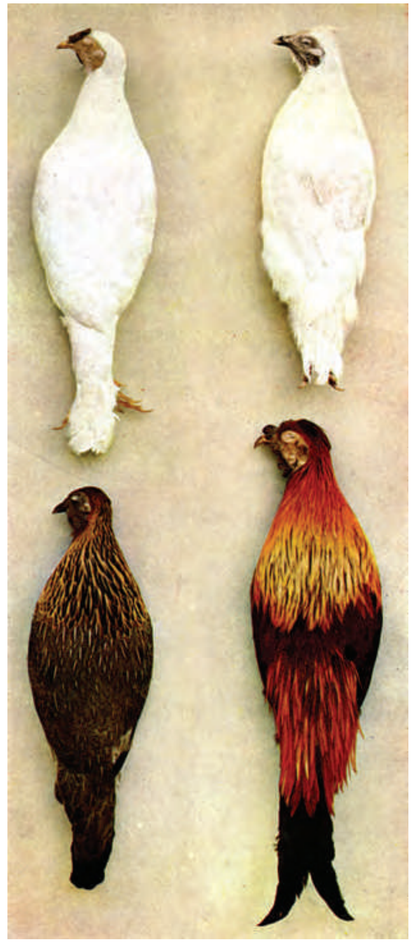


Figure 8–48 **A complementation test can reveal that mutations in two different genes are responsible for the same abnormal phenotype.** When an albino (white) bird from one strain is bred with an albino from a different strain, the resulting offspring (bottom) have normal coloration. This restoration of the wild-type plumage indicates that the two white breeds lack color because of recessive mutations in different genes. (From W. Bateson, Mendel's Principles of Heredity, 1st ed. Cambridge, UK: Cambridge University Press, 1913.)

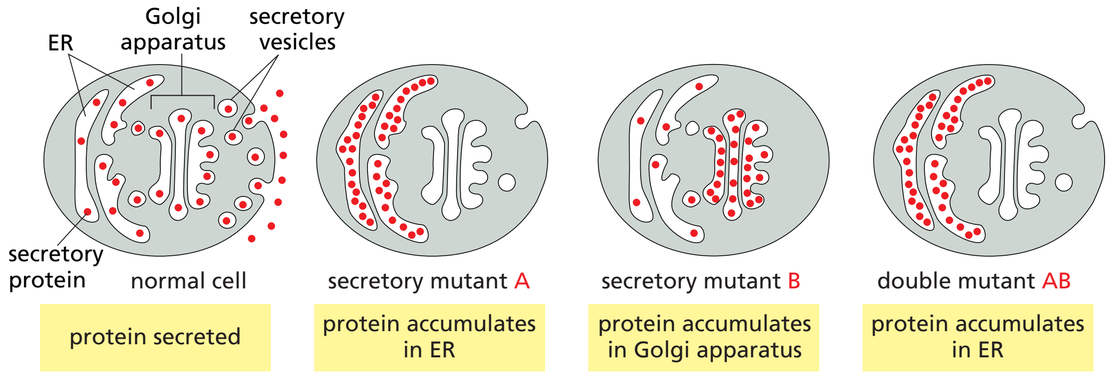
Epistasis analysis

- A **set of genes** participates in a given biological pathway - **in which order?**

Step A gene A	→	Step B gene B	gene A acts upstream of gene B; gene B acts downstream of gene A
Step A gene A*	→	Step B gene B	gene A null mutation — the process is **stopped** at step A even if gene B is functional
Step A gene A	→	Step B gene B*	gene B null mutation — the process is **stopped** at step B

- The term **epistasis** describes a certain relationship between genes, where one gene hides or masks the visible output, or phenotype, of another gene

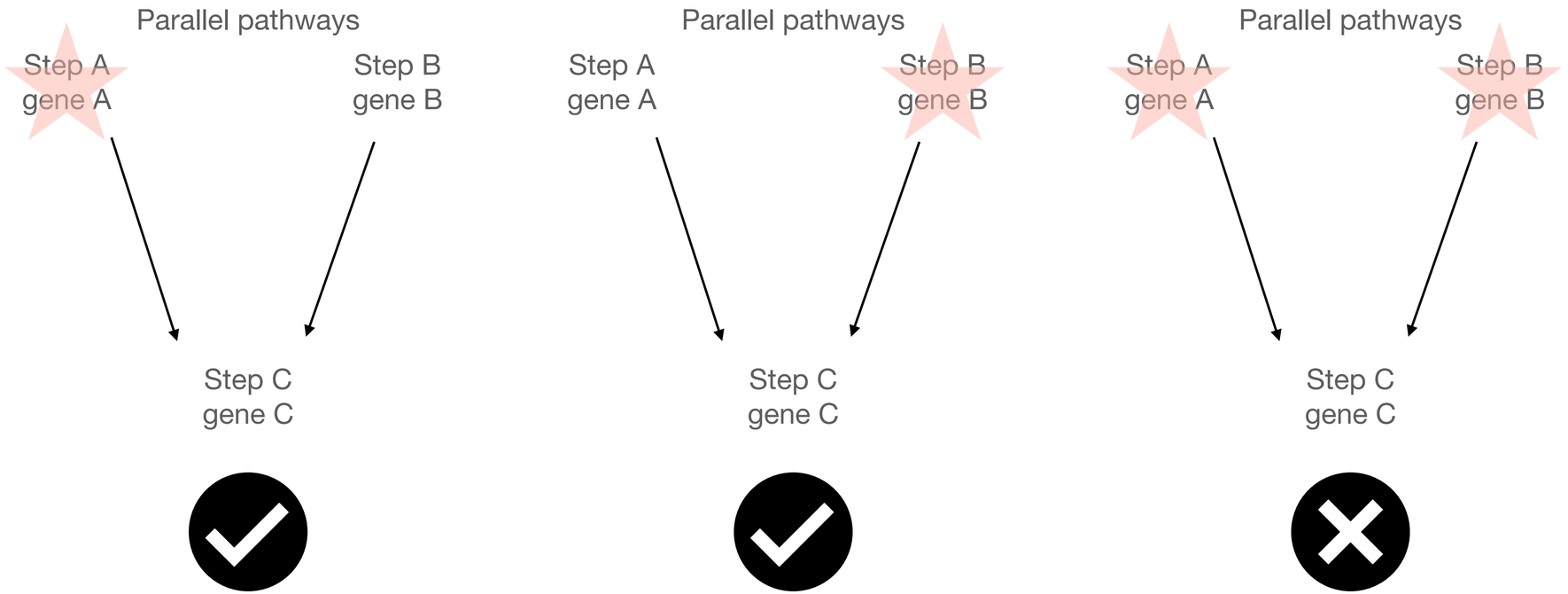
Do secreted proteins first go into the ER or the Golgi?



Epistasis analysis

- **Synthetic phenotype**: the phenotype of a double mutant is more severe than each of the single mutants

- **Synthetic lethality**: the phenotype of a double mutant is death, whereas each of the single mutants survives



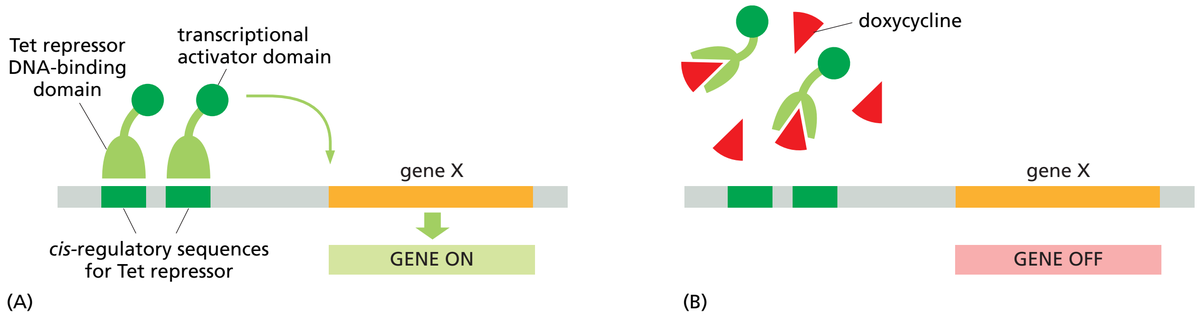
Identification of the mutated gene

- **Insertional mutagenesis:** by PCR
- **For random mutations:** whole genome sequencing

➡ re-introduction of the identified mutation in a clean WT background to prove causality

Starting from a known gene - reverse genetics

- Starting with a gene —> **making mutations** —> observing the phenotype
  - gene **deletions** (or gene knock-out) are only possible is the gene is not essential
  - **inducible** gene expression



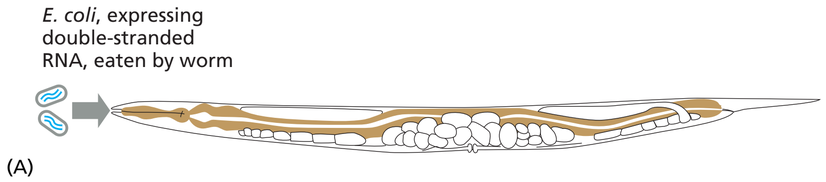
  - **cell-type dependent** gene expression
  - **overexpression**
  - any other more **“subtle” mutation** (catalytic site, structure, fusing a marker,…)

Determining gene function: RNA interference

- Introduction of a **dsRNA molecule** with a sequence that matches the target sequence
- RNA **processing**
- RNA **binding** to complementary sequence (mRNA or non-coding RNA)
- Expression is **reduced**
- Frequently used in ***Drosophila*, mammalian cells or *C. elegans***

Determining gene function: RNA interference

- In *C. elegans*, the dsRNA can be injected directly in the intestine or it can be fed with *E. coli* producing the RNA



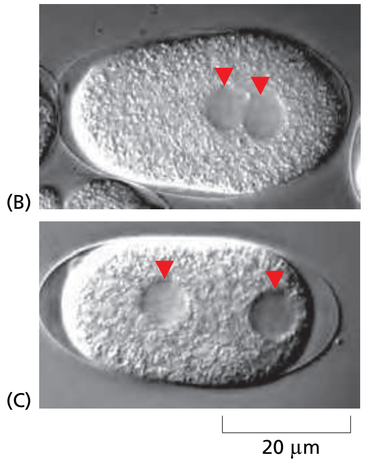
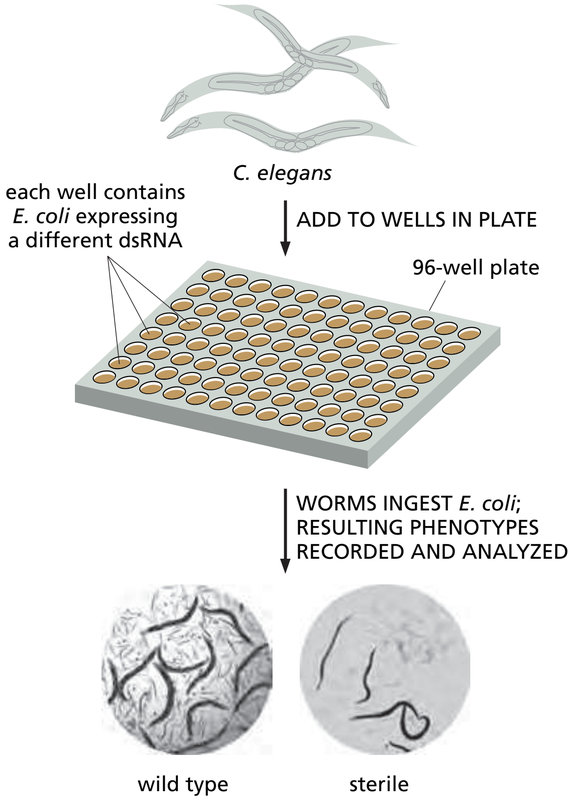


Figure 8–58 Gene function can be tested by RNA interference. (A) Double-stranded RNA (dsRNA) can be introduced into *C. elegans* by (1) feeding the worms *E. coli* that express the dsRNA or (2) injecting the dsRNA directly into the animal's gut. (B) In a wild-type worm embryo, the egg and sperm pronuclei (*red* arrowheads) come together in the posterior half of the embryo shortly after fertilization. (C) In an embryo in which a particular gene has been inactivated by RNAi, the pronuclei fail to migrate. This experiment revealed an important but previously unknown function of this gene in embryonic development. (B and C, from P. Gönczy et al., *Nature* 408:331–336, 2000. With permission from Macmillan Publishers Ltd.)

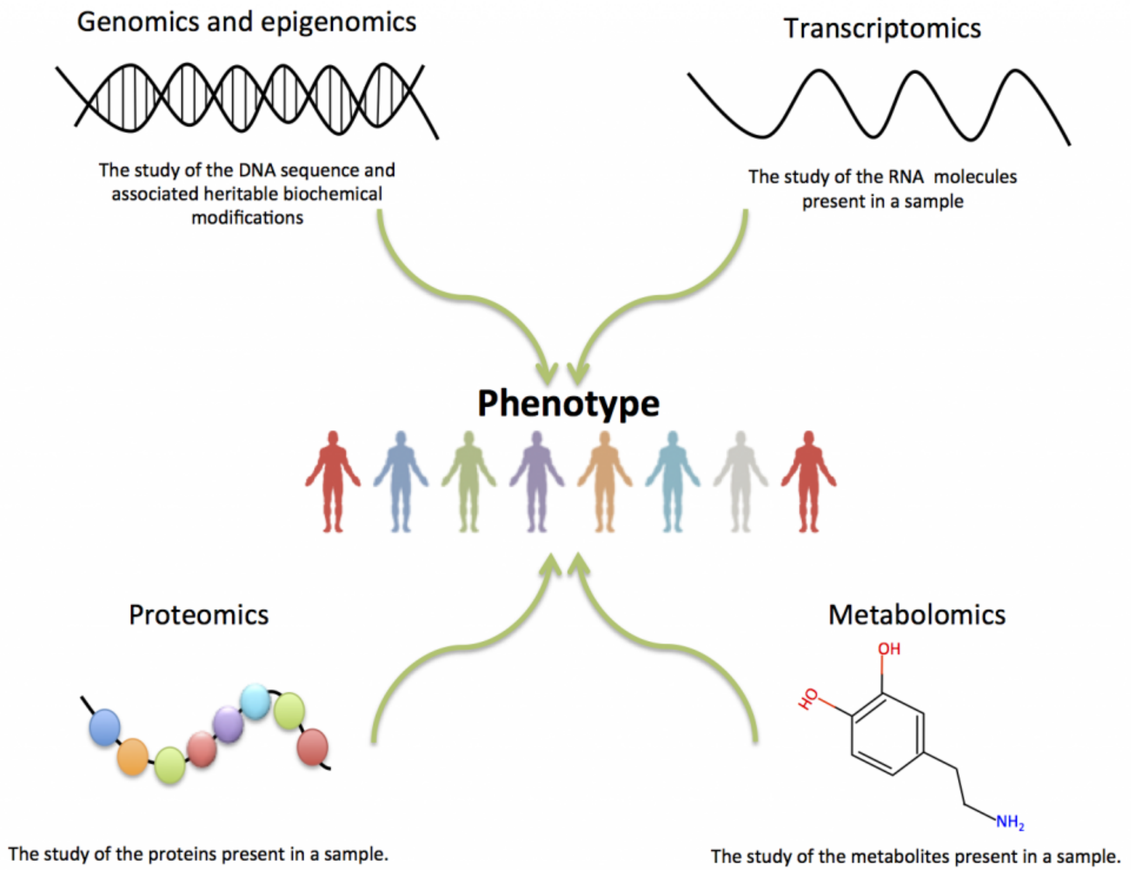


Plan

- Studying gene expression
- Determining gene function
- **Functional genomics**
- Visualizing cells and molecules

Functional genomics

- **Functional genomics** is the study of how the genome, transcripts (genes), proteins and metabolites work together to to produce a particular phenotype.



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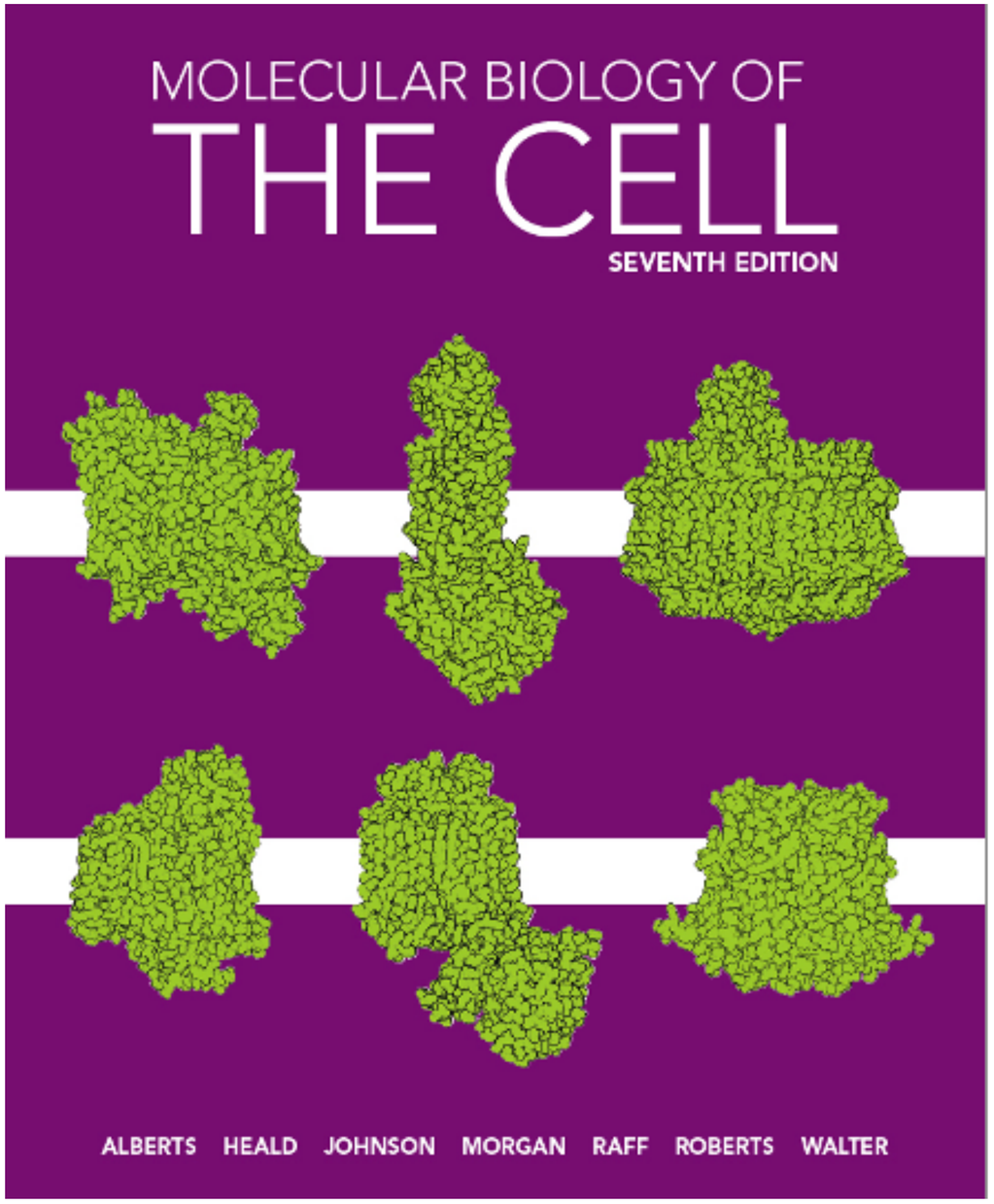
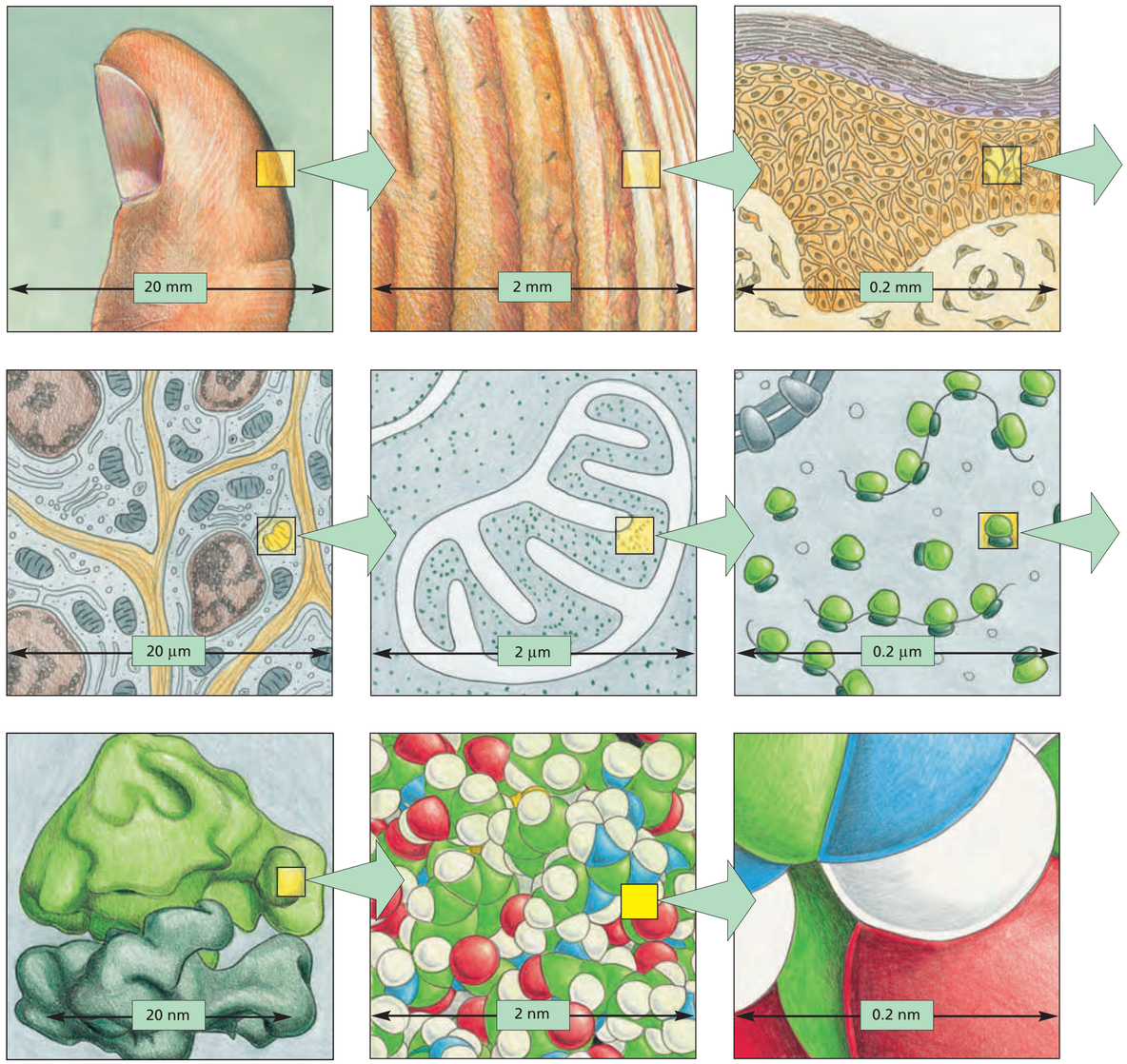


Chapter 9

Visualizing Cells and Their Molecules

Cells sizes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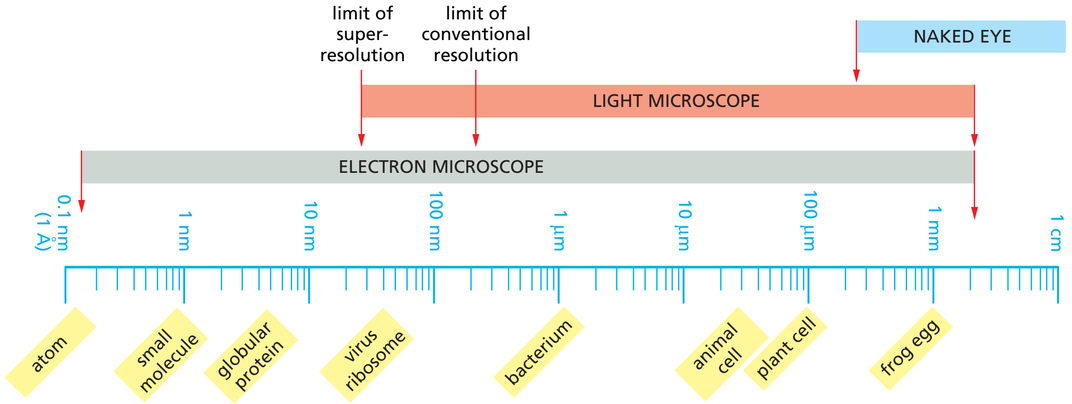
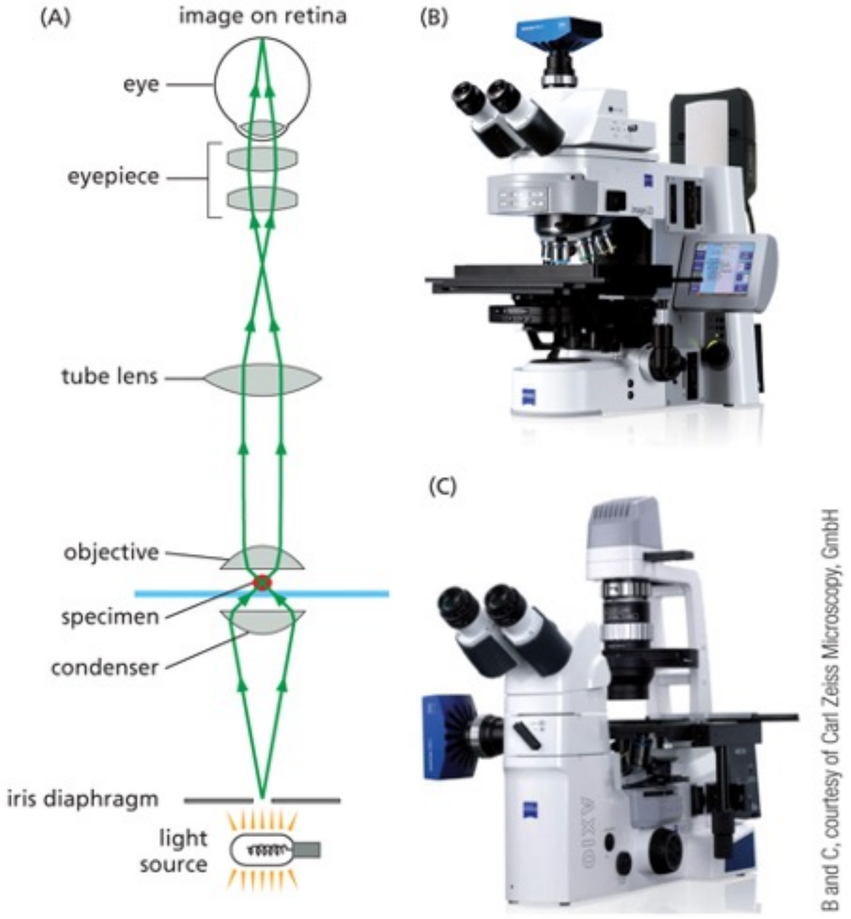

limit of super-resolution
limit of conventional resolution
NAKED EYE
LIGHT MICROSCOPE
ELECTRON MICROSCOPE
0.1 nm (1 Å)
1 nm
10 nm
100 nm
1 µm
10 µm
100 µm
1 mm
1 cm
atom
small molecule
globular protein
virus ribosome
bacterium
animal cell
plant cell
frog egg

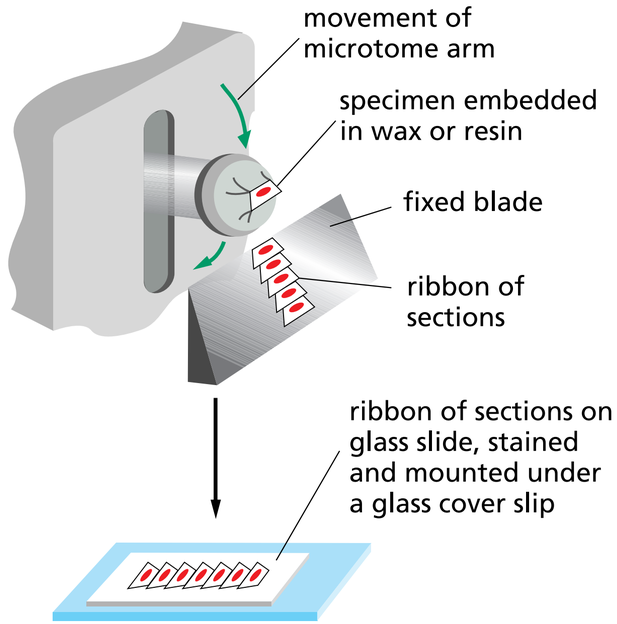
Light microscope

- Details **0.2 um apart**
- In practical terms, **bacteria or mitochondria** are the smallest objects that can be seen
- **Phase-contrast** microscopy increases the contrast in a sample
- **Digital imaging systems** improve light microscopy (camera detects dim lights and small intensity changes in light better than the eye)



Preparing tissues

- Tissues are too **thick**, they are cut in **slices or sections**
- Tissues are **fixed**, then **frozen or embedded in wax or resin** (as they are fragile)
- Tissues are **sliced** with a **microtome**
  - Cells are typically **transparent**
    - Can be **stained** with organic dyes that have affinity for specific compartments
    - Use of **fluorescent probes and markers**



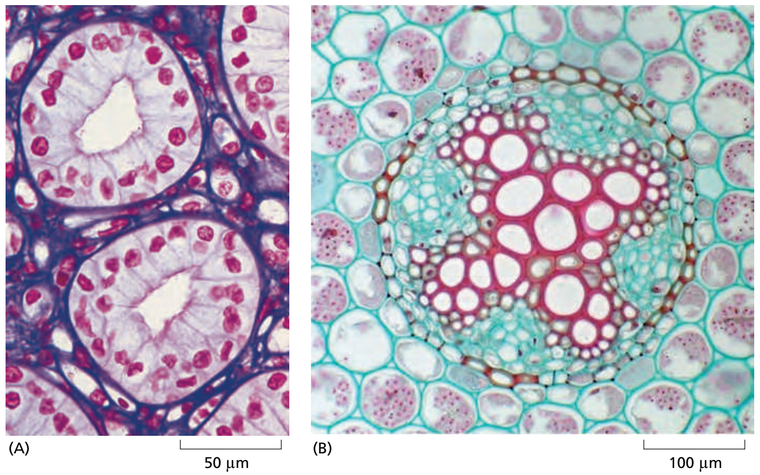
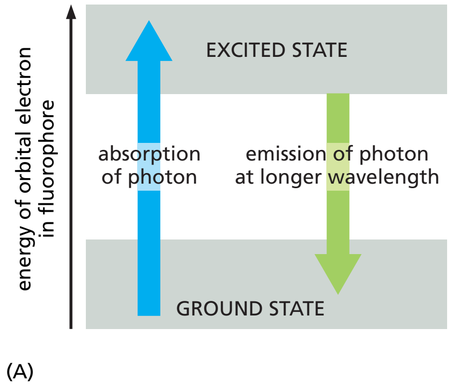
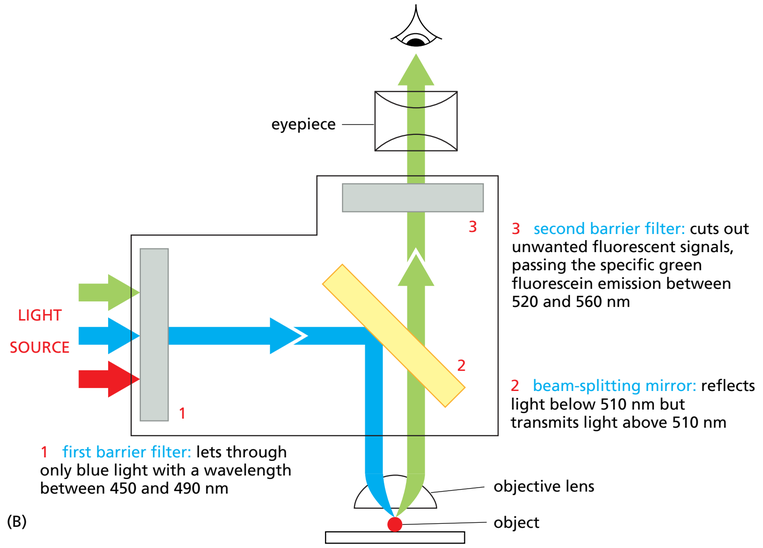


Figure 9–10 Staining of cell components. (A) This section of cells in the urine-collecting ducts of the kidney was stained with hematoxylin and eosin, two dyes commonly used in histology. Each duct is made of closely packed cells (with nuclei stained *red*) that form a ring. The ring is surrounded by extracellular matrix, stained *purple*. (B) This section of a young plant root is stained with two dyes, safranin and fast green. The fast green stains the cellulosic cell walls while the safranin stains the lignified xylem cell walls bright red. (A, from P.R. Wheater et al., Functional Histology, 2nd ed. London: Churchill Livingstone, 1987; B, courtesy of Stephen Grace.)

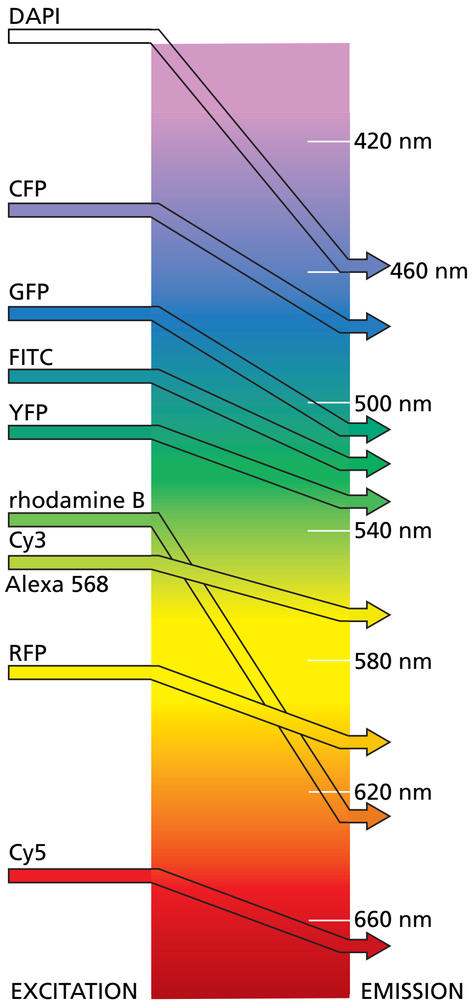
Fluorescence microscopy



(A)

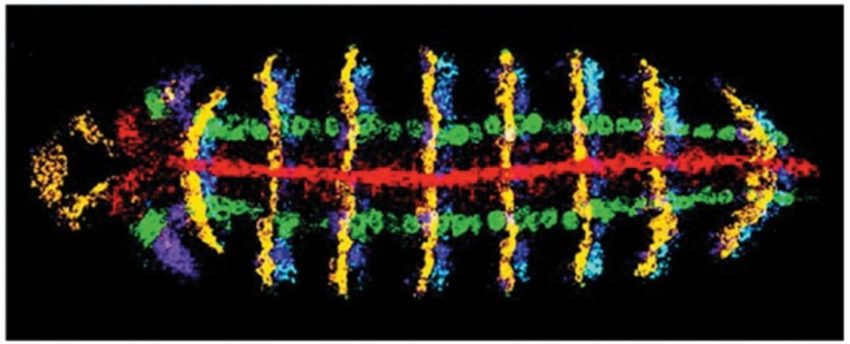


(B)



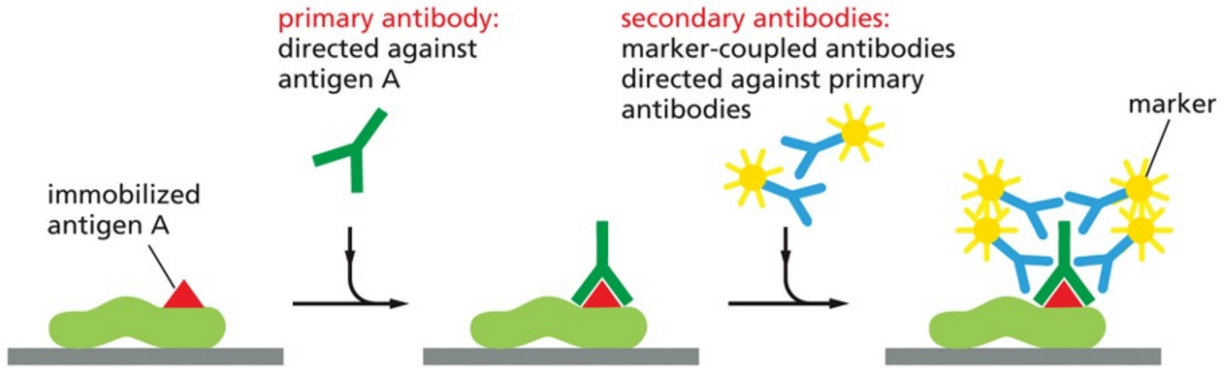
- Typically used to detect **specific molecules or proteins**

In situ hybridization with fluorescent probe:
RNAs in the fruit fly embryo

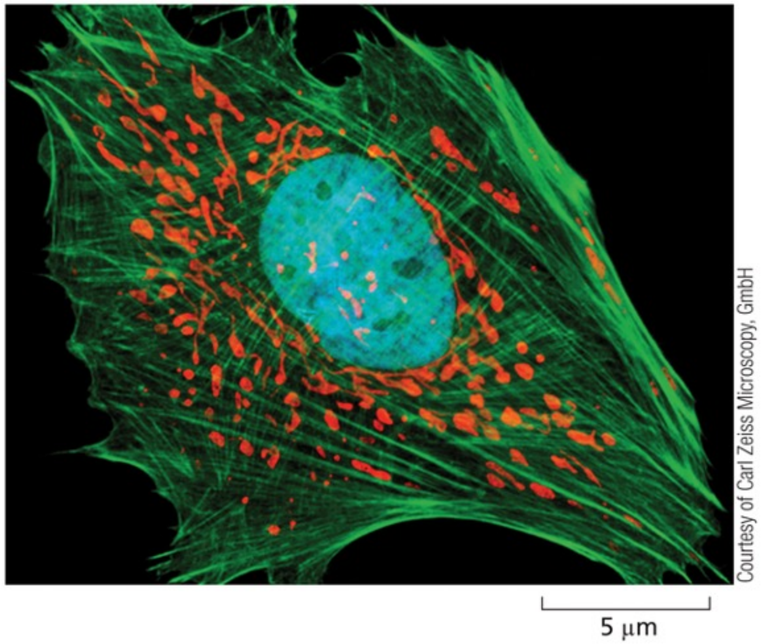


Fluorescence microscopy

- **Antibodies** can be coupled to fluorescent probes



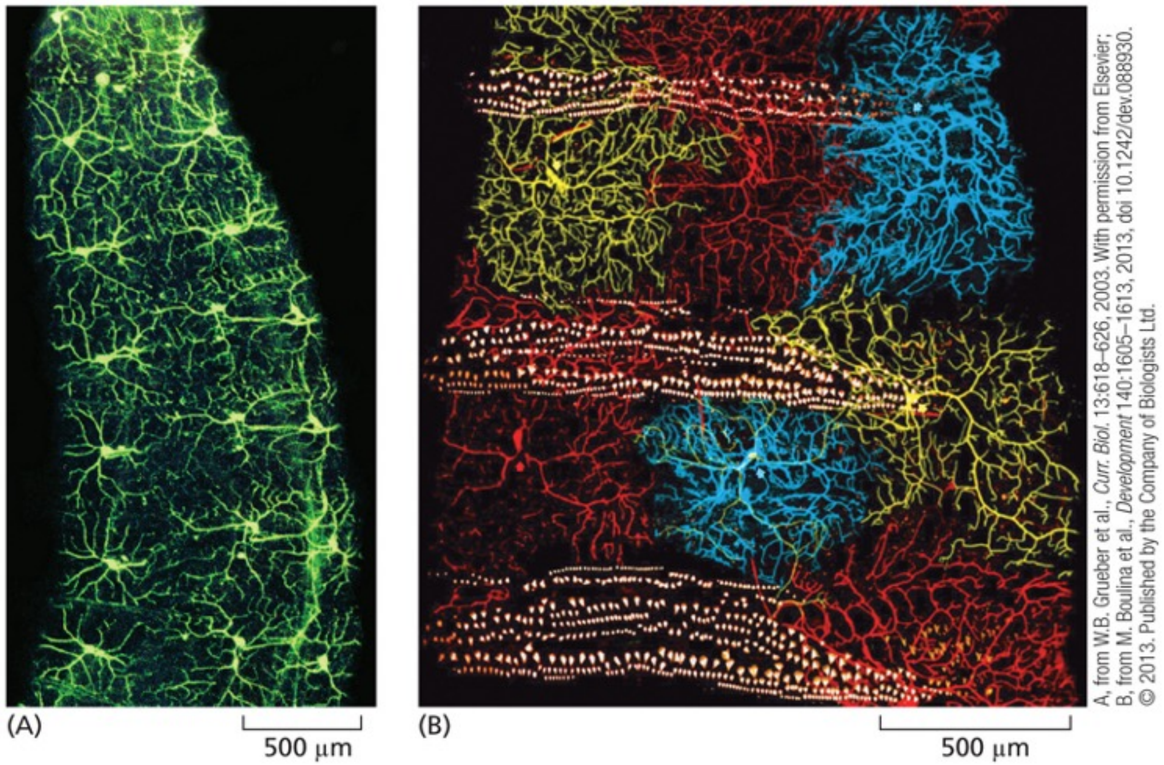
- Multiple **dyes** specifically bind to different types of molecules in the cell



Epithelial cell
- Actin (cytoskeleton) is labelled in green
- Mitochondria in ref
- Nucleus in blue

Fluorescence microscopy

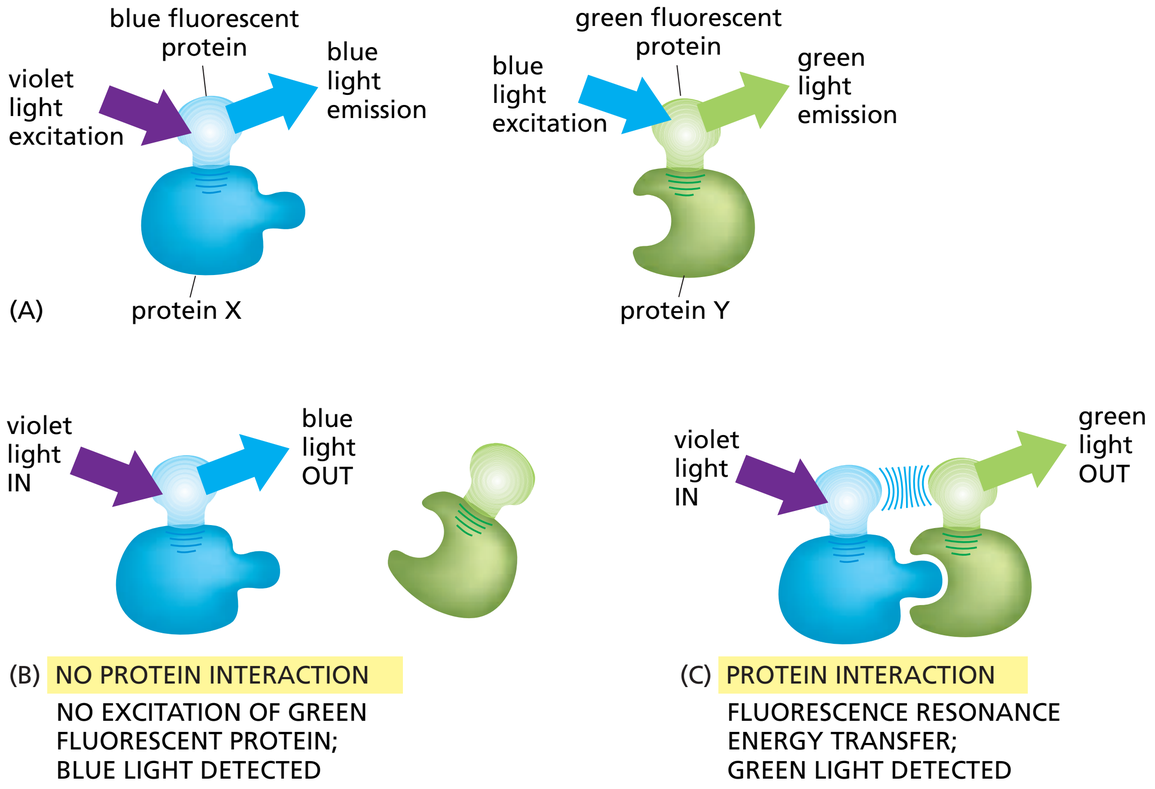
- Individual **proteins** can be **fluorescently tagged**



Fluorescent proteins as reporter molecules. (A) For this experiment, carried out in the fruit fly, the GFP gene was joined (using recombinant DNA techniques) to a fly promoter that is active only in a specialized set of neurons. This image of a live fly embryo was captured by a fluorescence microscope and shows approximately 20 neurons, each with long projections (axons and dendrites) that communicate with other (nonfluorescent) cells. These neurons are located just under the surface of the animal and allow it to sense its immediate environment. (B) In a variation of this method, three different fluorescent proteins, red, yellow, and cyan, can be expressed at random in neurons of the live fly embryo. The genetic constructs have been arranged such that a strong pulse of blue light will activate the expression of one or other of the three fluorescent proteins at random in neuronal cells, where they are then targeted to the plasma membrane. This noninvasive control of the timing of cell labeling allows the behavior of individual cells to be followed subsequently over time. The fine detail of all the dendrites of individual sensory neurons can be clearly seen. The lines of pale dots arise from the autofluorescence of the bands of denticles in the cuticle that define the segments of the embryo

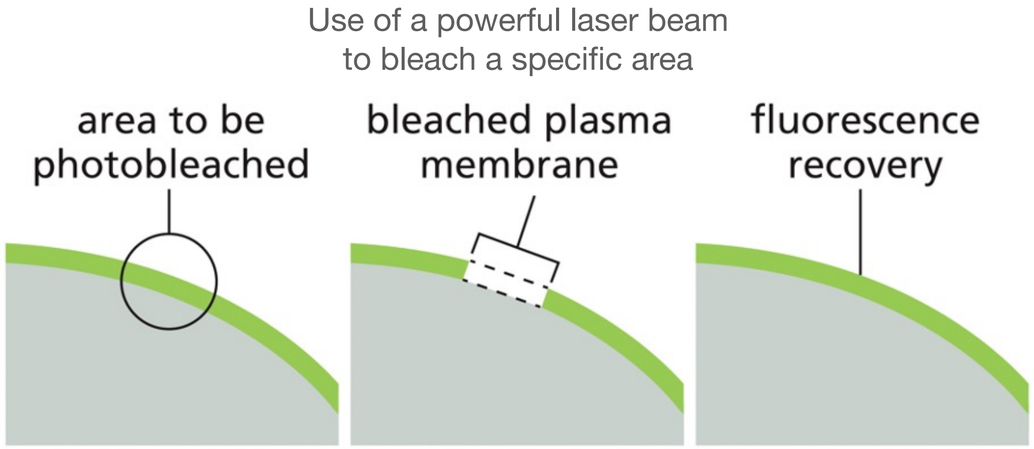
FRET

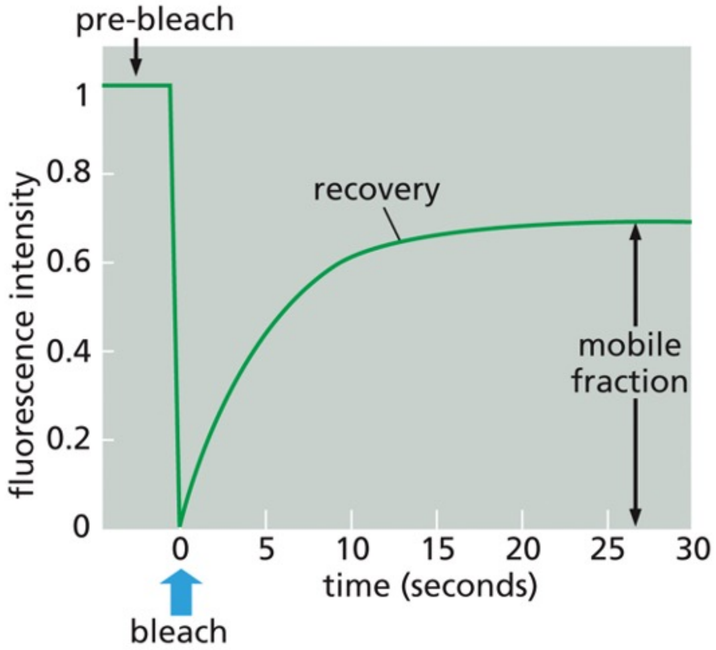
- FRET = **fluorescence resonance energy transfer**
- Monitors **interactions** between proteins



FRAP

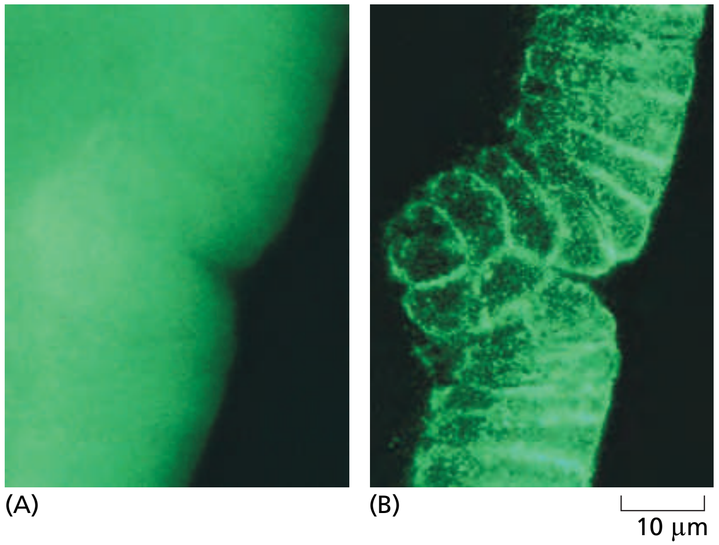
- FRAP = **fluorescence recovery after photobleaching**
- Provides quantitative data about a **protein's kinetics**

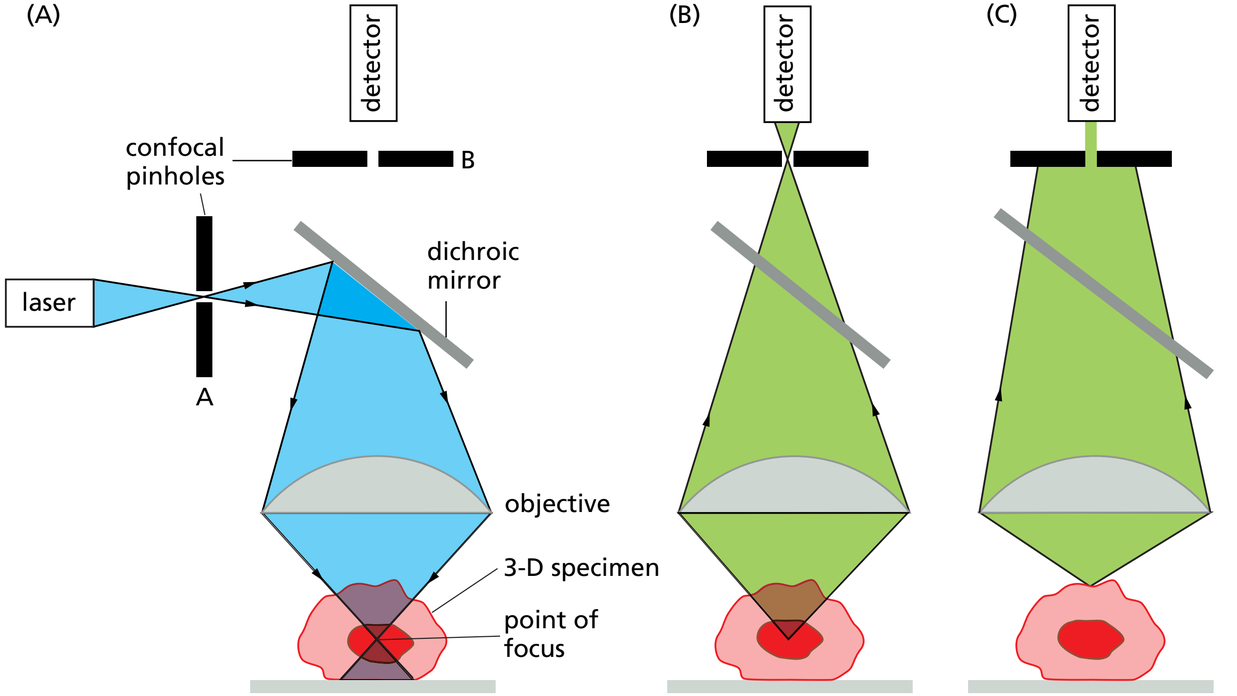




Confocal microscope

- Instead of illuminating the **whole sample**, it focuses a point of light onto a **single point** at a **specific depth** of the specimen





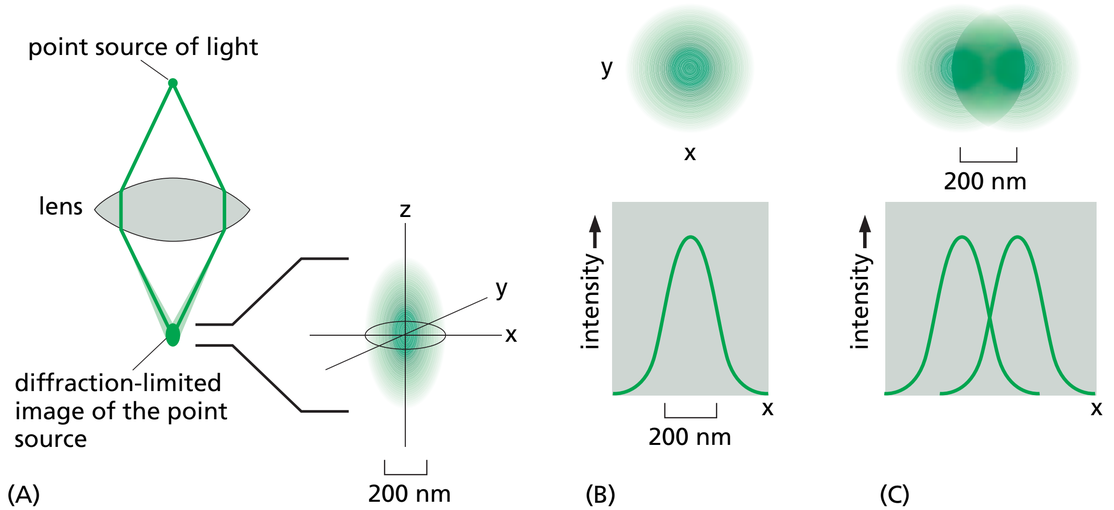
fluorescent specimen is illuminated with a focused point of light from a pinhole

emitted fluorescent light from in-focus point is focused at pinhole and reaches detector

emitted light from out-of-focus point is out of focus at pinhole and is largely excluded from detector

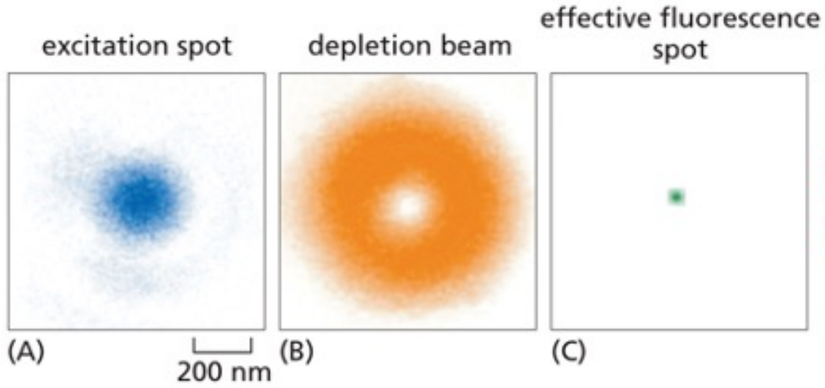
Superresolution fluorescence techniques

- Resolution of light microscopy is **limited** (0.2 um)
- **Superresolution** approaches:
    - The concept of the point spread function

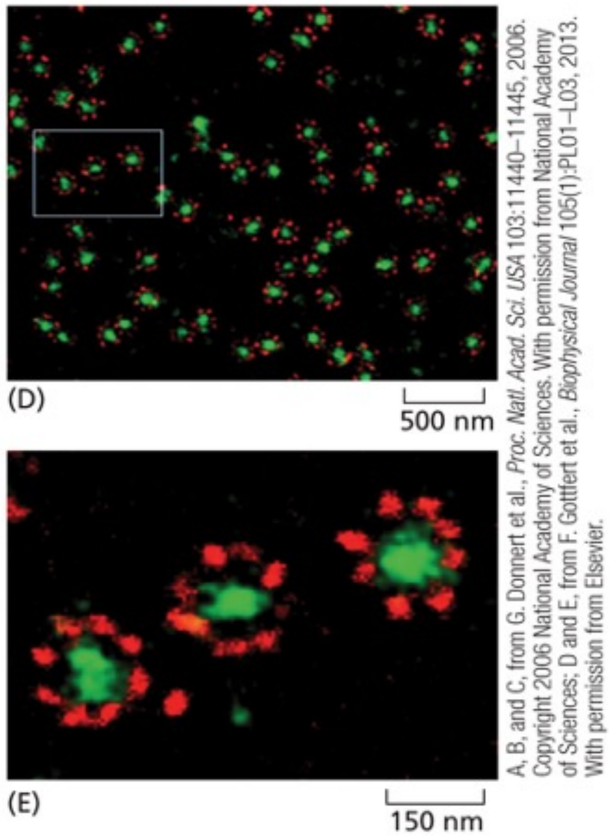


Superresolution fluorescence techniques

- Resolution of light microscopy is **limited** (0.2 um)
- **Superresolution** approaches:



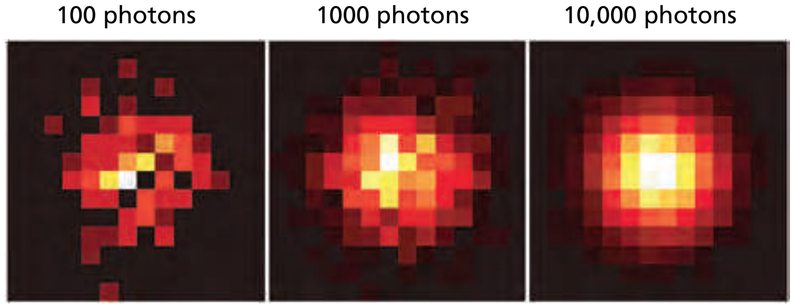
- use of a **depletion beam** (very bright laser beam around the excitation beam)
- **switch off the fluorescent molecules** around the excitation spot
- Increases **resolution**



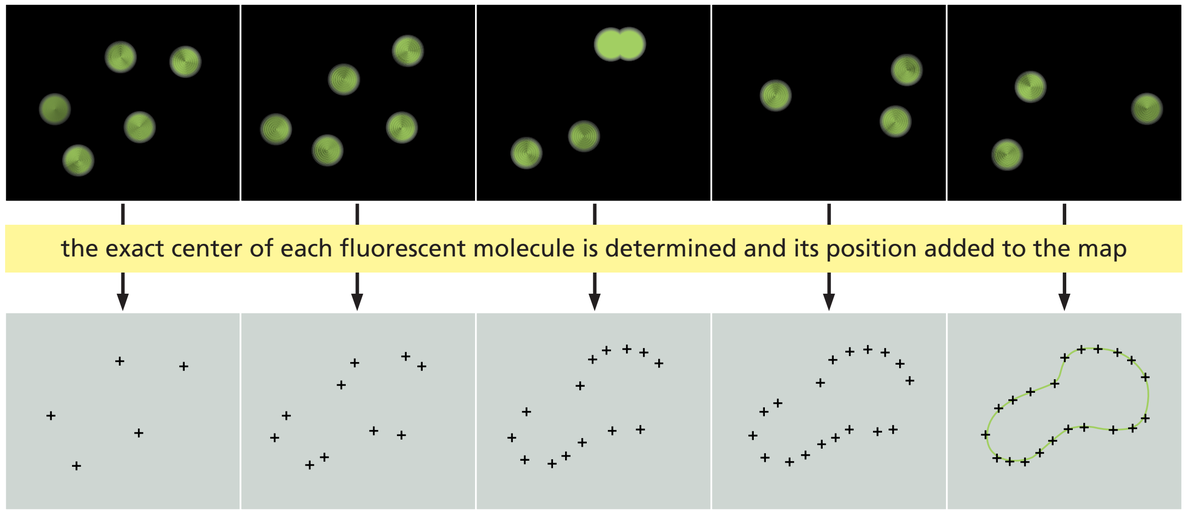
An enlargement of the boxed region shows the clear eightfold symmetry of the membrane ring proteins and the central fibrillar region with a resolution of about 20 nm.

Superresolution fluorescence techniques

- **Single molecule** localization

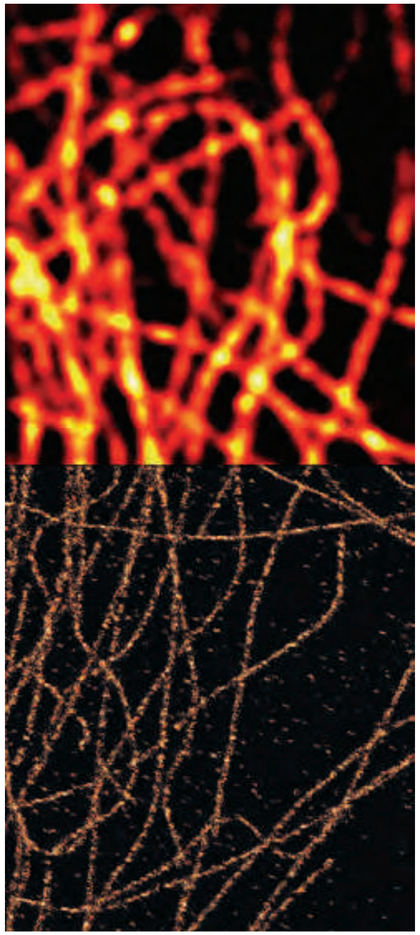


successive cycles of activation and bleaching allow well-separated single fluorescent molecules to be detected



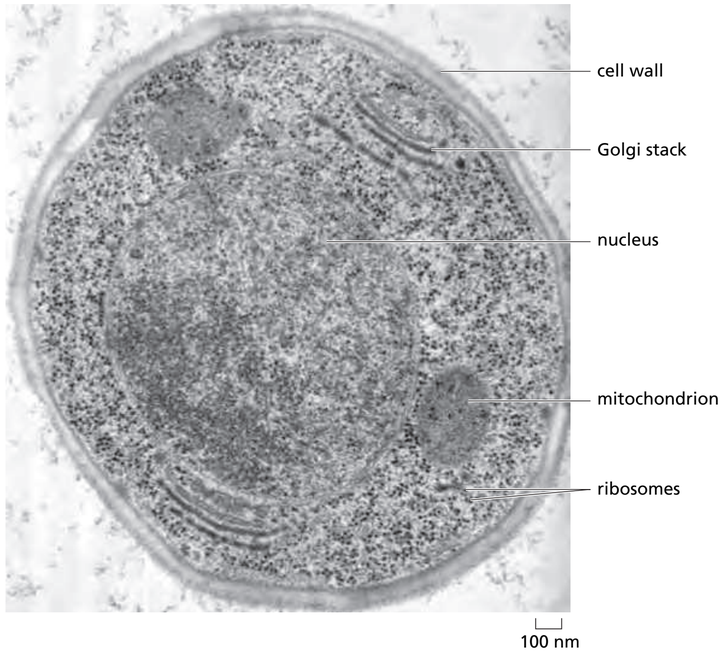
a super-resolution image of the fluorescent structure is built up as the positions of successive small groups of molecules are added to the map

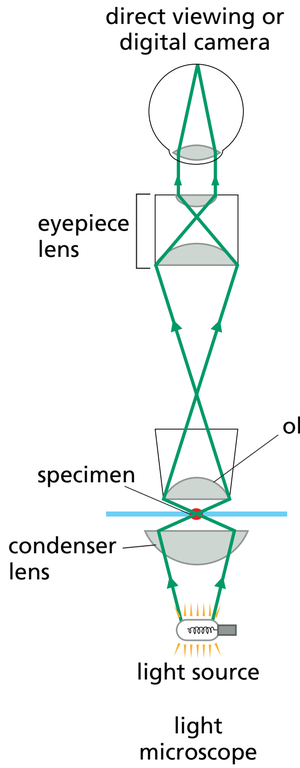
(B)

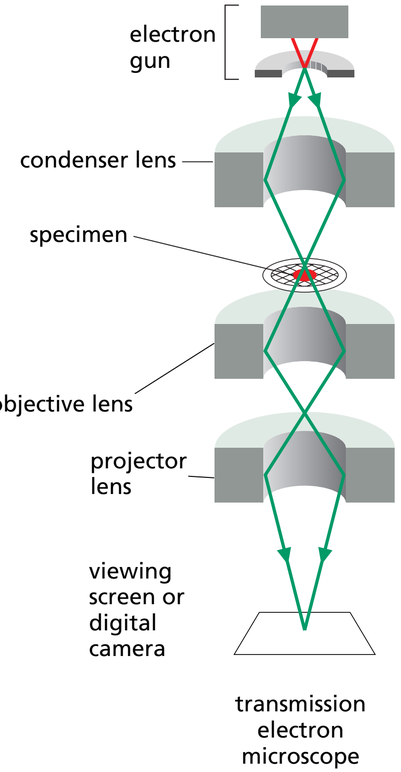


Electron Microscopy

- Resolution **0.05 nm**
- Based on an **electron beam**
- Staining with **electron-dense** material

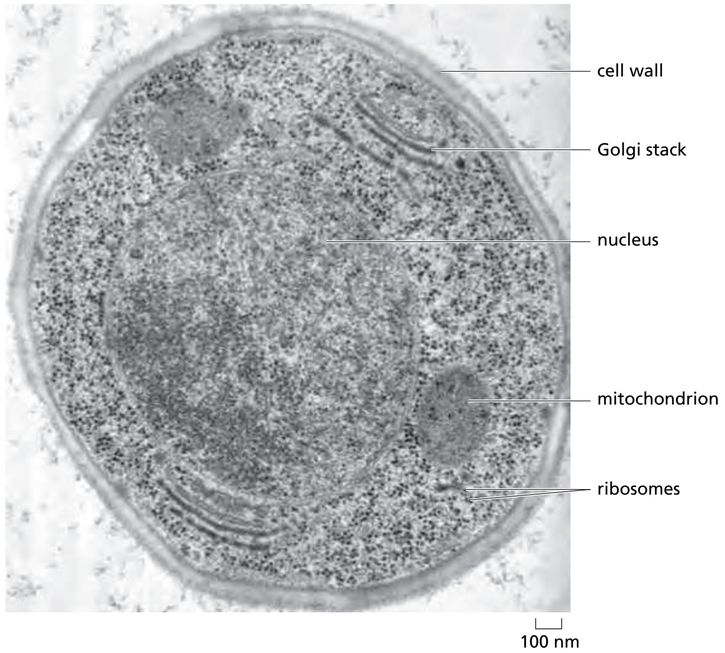


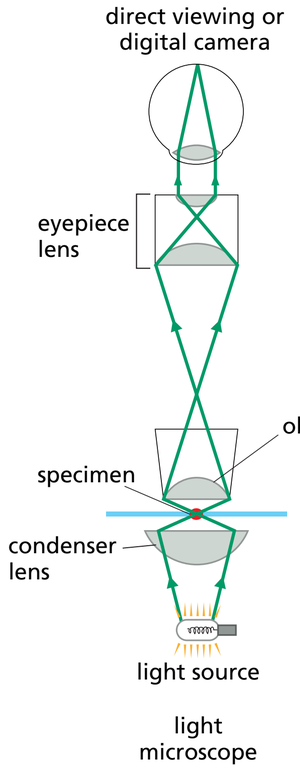


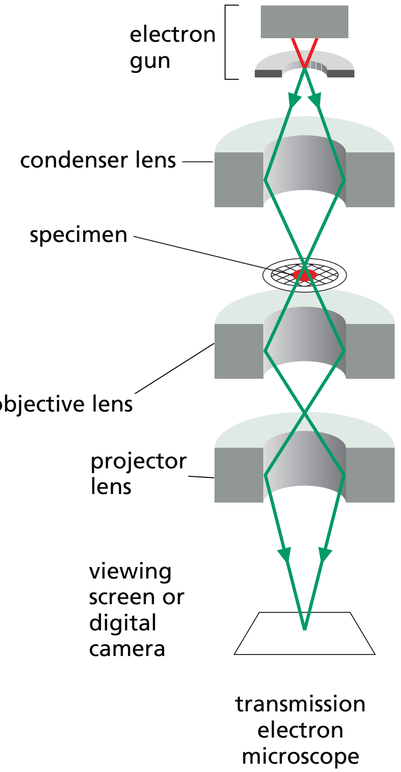


Electron Microscopy

- Resolution **0.05 nm**
- Based on an **electron beam**
- Staining with **electron-dense** material (heavy metals)







Summary

- Studying gene expression
- Determining gene function
- Functional genomics
- Visualizing cells and molecules

Have a nice day!