

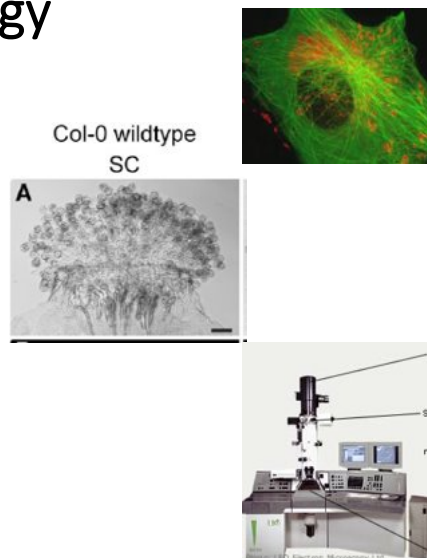
# BIOL 377 – cell biology

Week 2 & 3 - methods

1

## Microscopy is a key technique used in cell biology

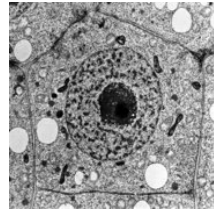
- Transmitted light
  - (Bright field & DIC)
- Fluorescence and confocal light microscopy
- Electron Microscopy (TEM and SEM)



2

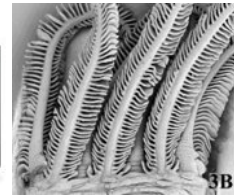
## Microscopes used to observe cells

Transmission Electron Microscope



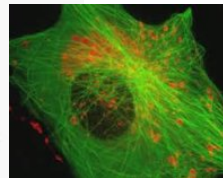
TEM of root cell

Scanning Electron Microscope



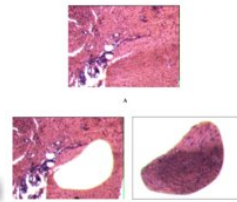
SEM of fish gill

Confocal Scanning Laser Microscope



CLSM of human cell

Laser Capture Microdissection Scope



LCM of tumor cells

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## Microscopy: the cell biologist's key tool but also drawback

- Entire field was founded on the observations of the early microscopists
- Leeuwenhoek's "wee beasties"
- Were able to observe cellular processes long before we understood what they were looking at
- Made predictions that proved correct 100 years later
  - others were dead wrong

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## Two issues when using microscopy:

- How do you add contrast when biological specimens usually lack intrinsic contrast?
  - How do you “see” specific structures or molecules w/in cells
- To what extent can you resolve two closely proximal objects?
  - How low can you go?
  - How close can things be to distinguish them apart?

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## The resolution of each microscope has a clear distance limit

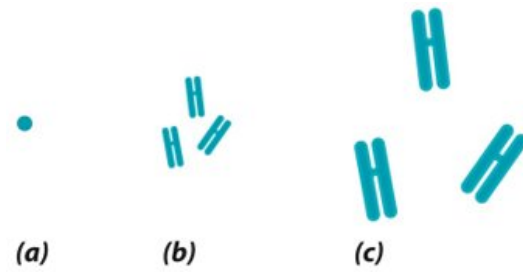
- |                                              |   |         |
|----------------------------------------------|---|---------|
| • Transmitted light                          | } | ~200 nm |
| • Fluorescence and confocal light microscopy |   |         |
| • Super resolution microscopy                | } | ~50 nm  |
| • Electron Microscopy                        | } | ~0.1 nm |



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## Resolution

Fig 18.3



- Each objective has a magnification
- 10x, 20x, 100x, etc
- However, resolution of an objective is not solely a function of magnification
  - NA: numerical aperture
  - True measure of resolution
  - NA 1.4 is the highest

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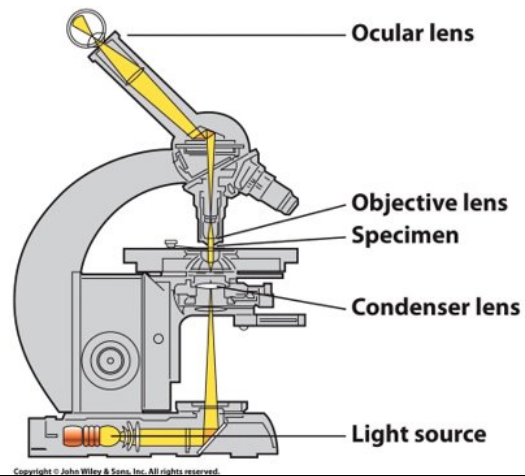
## To visualize most samples you must create contrast

- Most specimens have no contrast
- Most lack pigments, or any coloration or contrast
- Standard brightfield microscope won't show much
- Stain fixed specimens
- For live specimens, can use optical "tricks" to add contrast

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## The basic transmitted light microscope

Fig 18.1



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## Brightfield view of an onion root tip stained with Feulgen

Fig 18.5



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### *Paramecium* viewed by 3 flavors of transmitted light microscopy

- Top: Brightfield
  - Can see some organelles
- Middle: phase contrast
  - refractive properties of structures yields bright/darkness
- Bottom: Nomarski/DIC
  - Refractive properties of structures result in illusion of contour



(a)

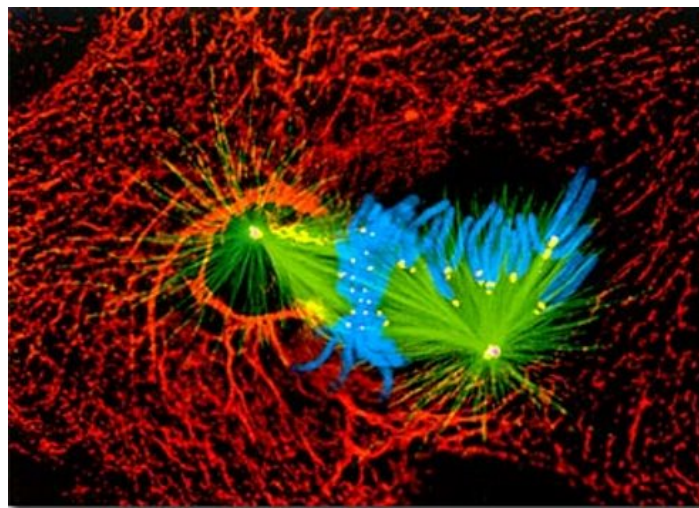


(b)



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## Fluorescence Microscopy



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## Fluorescence has multiple applications in cell biology

Fluorescence can be used to:

- Localize molecules in live or preserved specimens
- Track protein-protein interactions
- Monitor second messengers, enzyme activities

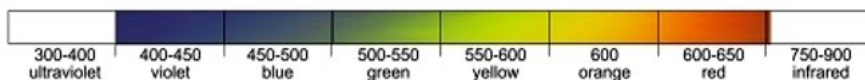
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## Fluorescence has multiple applications in cell biology

- Indirect fluorescence
  - Fixed cells or tissues are incubated with specific antibodies, and those antibodies are detected with fluorophore (dye)-labeled secondary antibodies
- Physiological indicators- pH,  $\text{Ca}^{++}$
- Direct probes: small molecules that directly bind a structure
- Fluorescently-labeled proteins
  - tubulin, actin
- Proteins with intrinsic fluorescence
  - aequorin, green fluorescent protein, red fluorescent protein

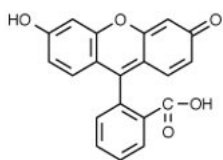
14

## How is fluorescence generated?

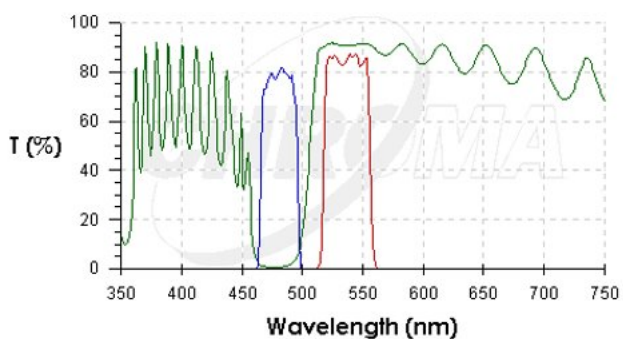


- Property of a substance that when excited by a photon of one  $\lambda$  of light (excitation), will emit a new wavelength of light of lower energy and thus longer  $\lambda$  (emission)
  - Some E lost to vibration or heat, as fluorophore sinks back to ground state, energy given off as light

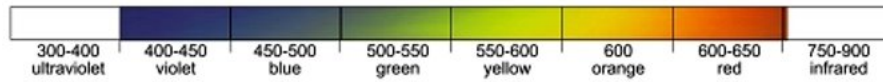
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Fluorescein



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You have a fluorophore that is excited with 546 nm light. What is the most likely emission wavelength?

- A. 400 nm
- B. 488 nm
- C. 510 nm
- D. 546 nm
- E. 610 nm

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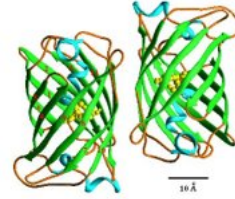
## Green fluorescent protein: a revolution in cell biology

- Isolated from the jellyfish *Aequoria victoria*
- When excited with blue light, emits in the green
- Can be expressed as a chimeric protein, with GFP fused to any protein of interest
- Allows scientists to look at protein dynamics in living cells!!
- 2008: three scientists were awarded the Nobel prize for the discovery and applications of GFP

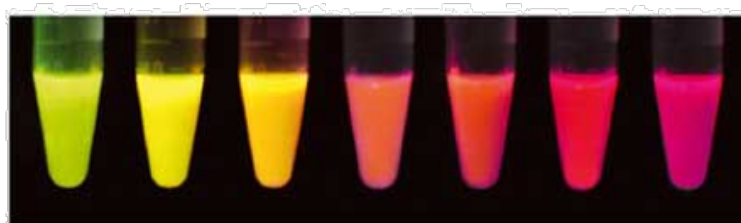


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## Green fluorescent protein: a revolution in cell biology

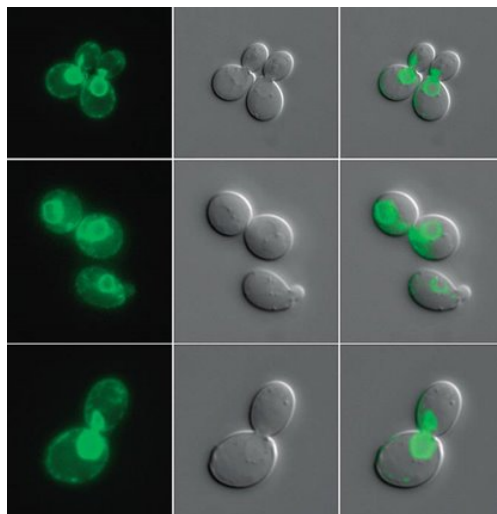


- Barrel-shaped protein w/Chromophore consists of modified amino acids within polypeptide
- Mutations were created to generate blue, cyan, yellow, forms of GFP
- New generation of fluorescent proteins span most of the visible range

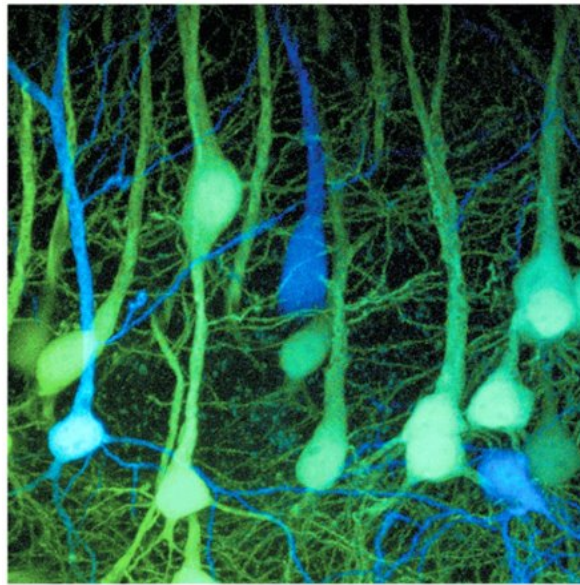


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## GFP images

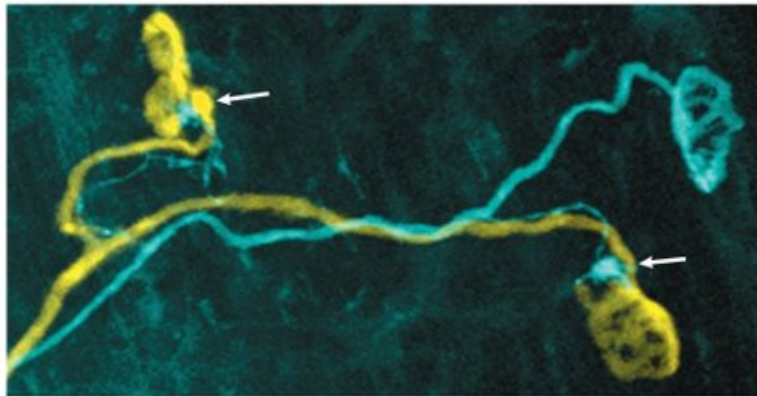


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Courtesy N. Kasthuri & J.W. Lichtman,  
Washington University School of Medicine.

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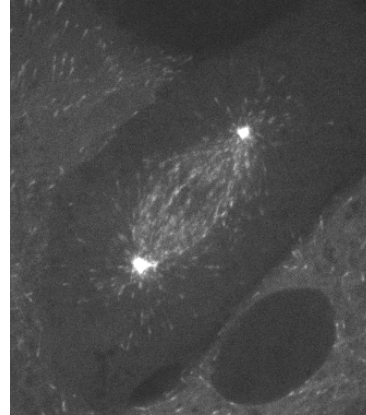
From N. Kasthuri and Jeff W. Lichtman, *Nature* 424: 429, 2003, Fig. 4a. Reprinted by permission from Macmillan Publishers Ltd.

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## GFP movies



mCherry-protein bound to DNA  
GFP-tubulin



GFP-EB1

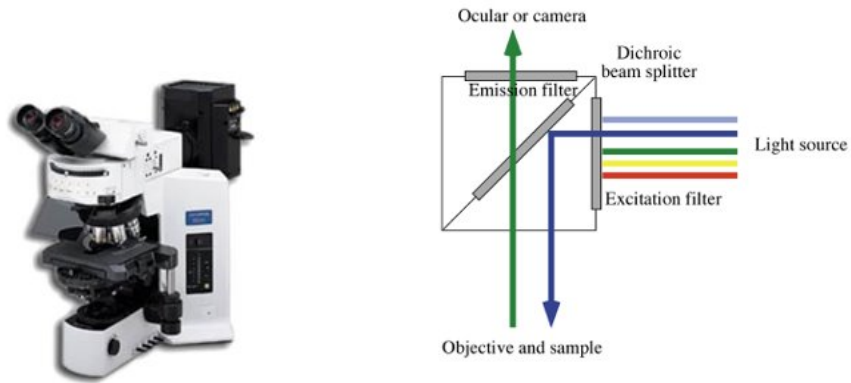
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## Imaging fluorescence: epifluorescence

- A light source emits multiple wavelengths
- 1st filter allows one wavelength (488nm, blue) which is reflected by a mirror *through the objective* and onto the sample
- Excited fluorophore emits green light, which is collected through the objective and back through the mirror
- The second filter allows only the emission (515nm) wavelength to pass through the filter to the eyepieces or camera

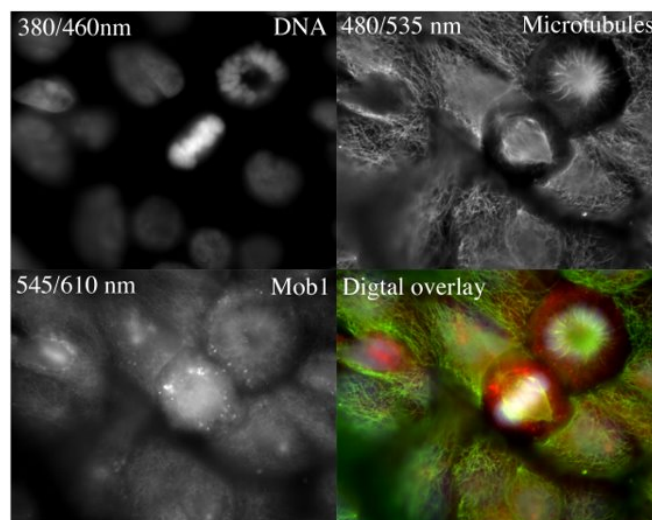
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## Imaging fluorescence: epifluorescence



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## How do we generate these images?

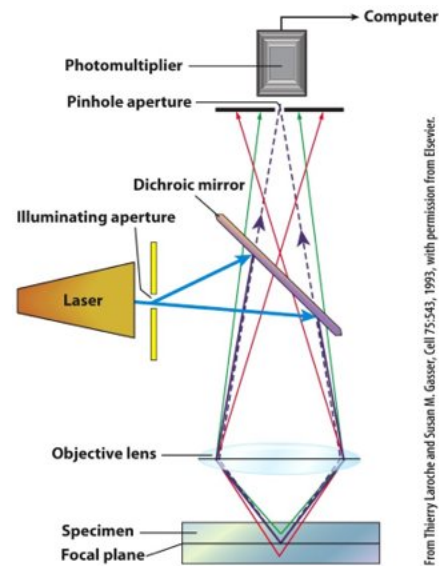


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## Confocal Microscopy.

Fig 18.11

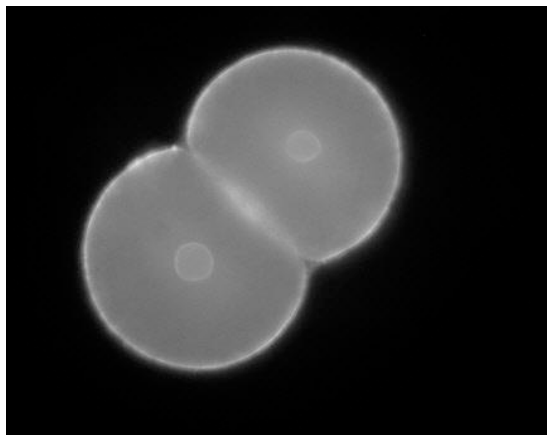
- Conventional microscopes capture light from both the focal plane as well as above/below
  - Results in a blurry image
- Confocal microscopes combine lasers, motorized control of focal plane, and pin holes to eliminate out of focal plane
- Allow for 3D reconstructions, 4D (xyzt) imaging



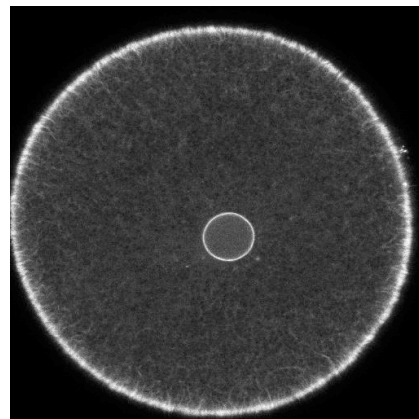
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## Wide-field vs confocal microscopy

Wide-field



Confocal

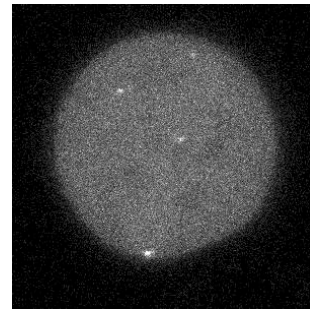
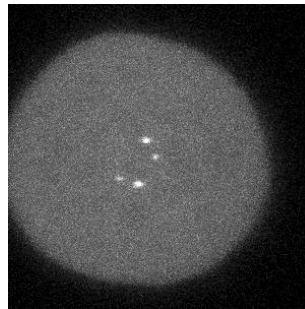
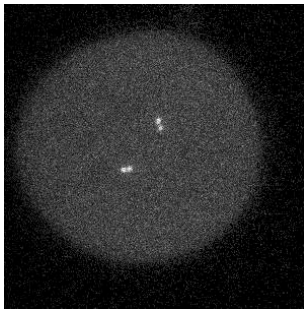


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## Example of 4D imaging (3D imaging over time): Keeping track of centrioles during cell division

WT

8hr mitotic arrest



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## What are the limits of resolution?

- Transmitted light
  - Fluorescence and confocal light microscopy
  - Super resolution microscopy
  - Electron Microscopy
- }

}

}

~200 nm

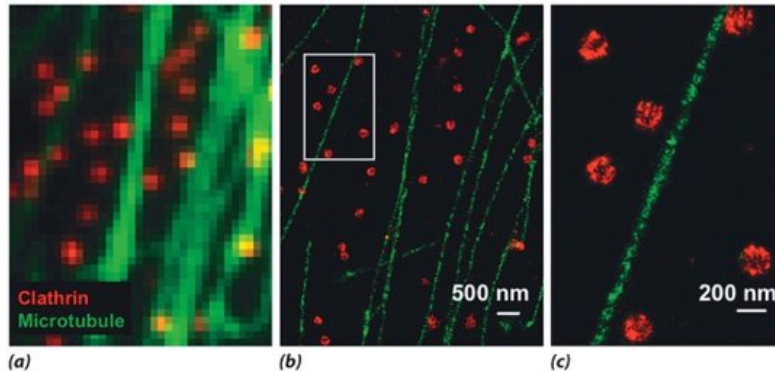
~50 nm

~0.1 nm

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## Super resolution microscopy

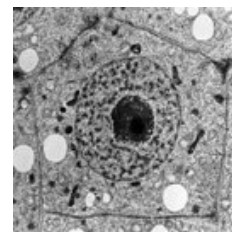
- Breaks the diffraction limit of the light microscope (200 nm) using a variety of techniques
  - Can resolve objects as close as 30nm!
  - Inventors shared the 2014 Nobel prize in Chemistry



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## Transmission Electron Microscopy: resolution below the level of the light microscope

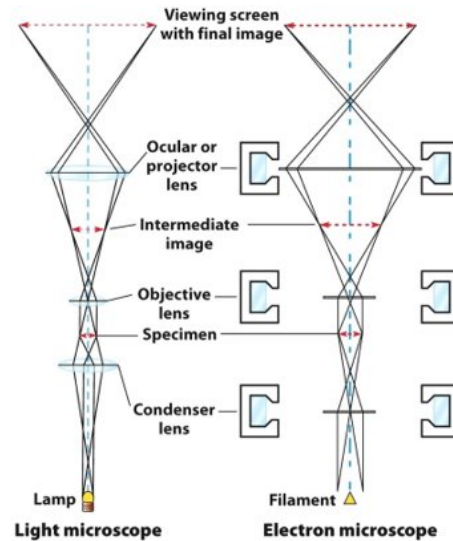
- High energy electron beam focused by electromagnetic lenses in a vacuum
- 0.1nm resolution
- Samples fixed, stained with heavy metal stains to generate contrast
- dehydrated, embedded and thin sectioned
- High voltage TEM
  - Allows for examination of thick sections at high resolution



TEM of root cell

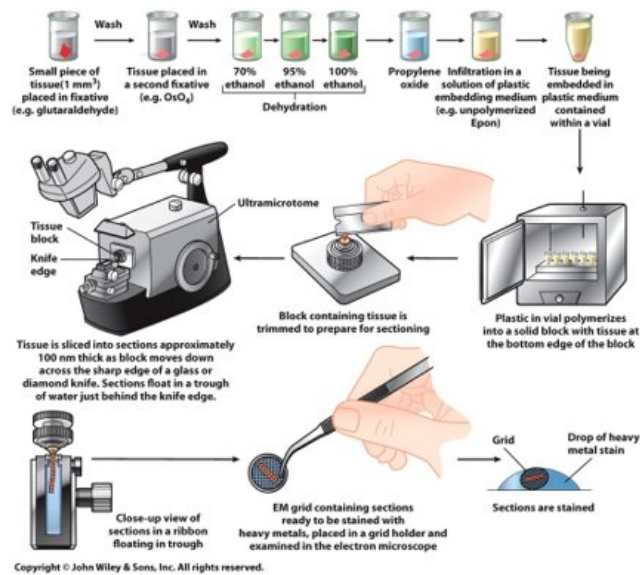
32

## Electron microscope use magnets instead of lenses. Figure 18.15



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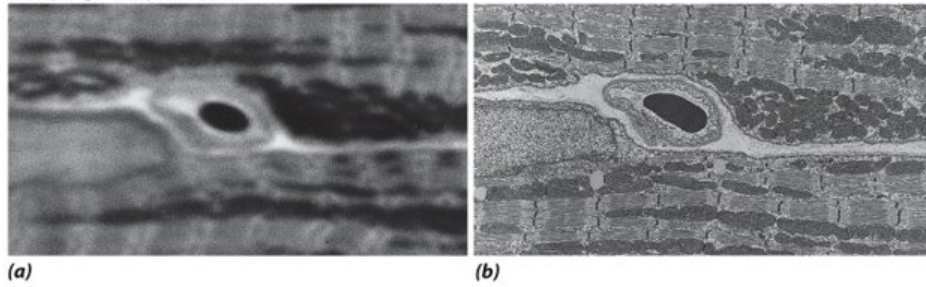
## Sample preparation for transmission electron microscopy. Figure 18.16



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## Comparing light vs electron microscope. Figure 18.14

Courtesy Douglas E. Kelly and M.A. Cahill



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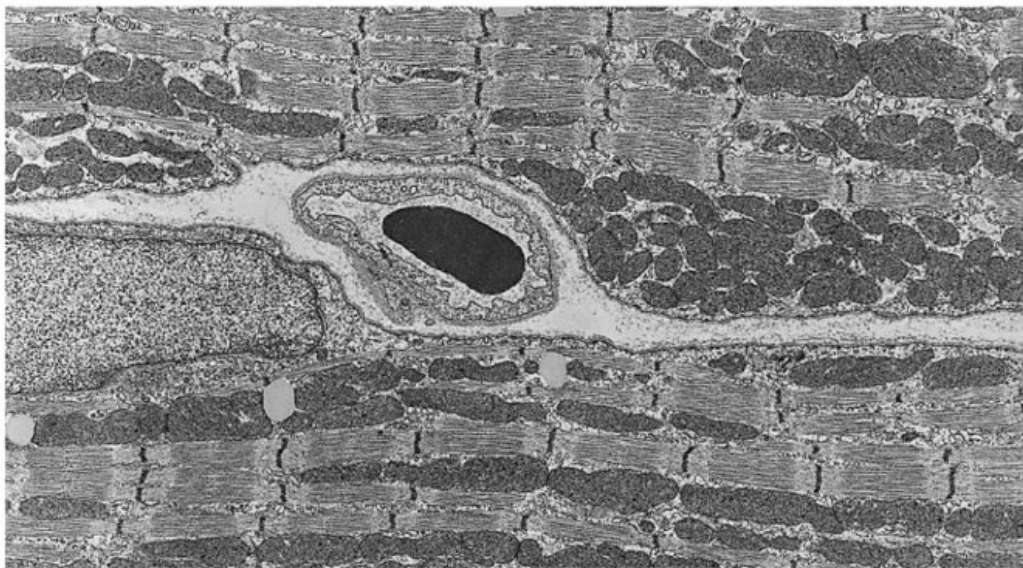


Figure 18-11b Cell and Molecular Biology, 5/e (© 2008 John Wiley & Sons)

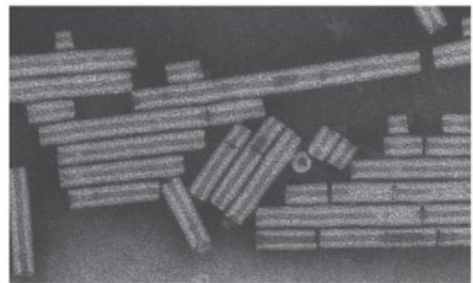
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## Variations of TEM

- Rotary shadowing: metal replica made of a sample
- Freeze-fracture: sample is frozen split open, and a metal replica is made
- Immunoelectron microscopy
  - Prior to staining and drying, samples are incubated with antibodies, and gold labeled secondary antibodies are seen by EM as black dots in the thin sections

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- **Figure 18.17**
- **Negatively stained vs rotary shadowed Tobacco Mosaic Viral particles**



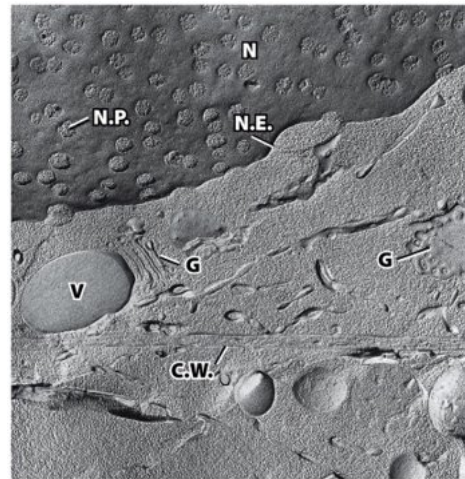
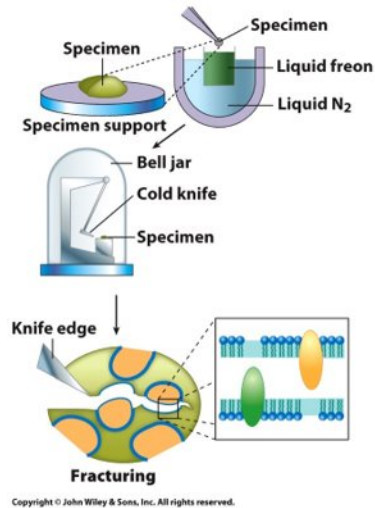
(a)



(b)

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## Freeze-fracturing. Fig. 18.16 and 18.17



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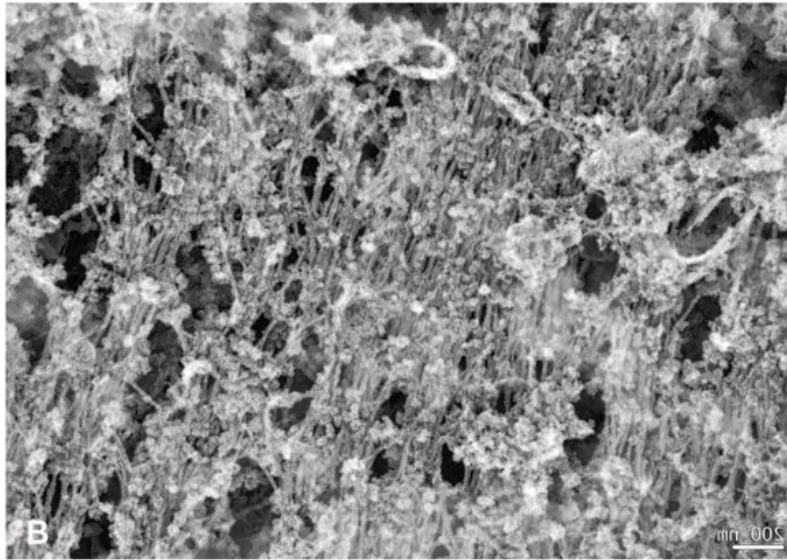
## Scanning electron microscopy. Fig 18.22

- Used for examination of surface structures
  - Samples are fixed, dehydrated, critical point dried, and sputter coated with platinum
- Electron microscope visualizes metal replica



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## Super resolution vs Electron Microscopy



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If you wanted to localize a protein in a living cell, what would be the preferred method?

- Antibodies and indirect immunofluorescence
- Express a fusion protein of your protein of interest and GFP
- Express GFP alone

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You are interested in localizing two proteins that are localized on opposite sides of a neuromuscular junction (~40 nm), what technique would you use?

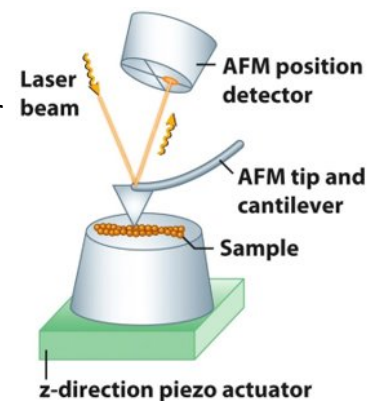
- A. phase contrast microscopy
- B. Nomarski DIC
- C. Fluorescence microscopy
- D. Super resolution microscopy
- E. Electron microscopy

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## Atomic Force Microscopy

### Fig 18.23

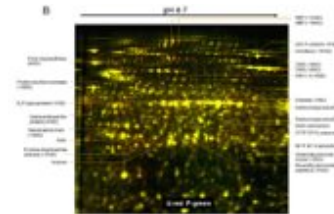
- Drags a calibrated tip over the surface of cells or isolated molecules
  - Reconstructs a surface profile of the sample
- Sensitivity is a function of the tip and cantilever
- Can get EM-level resolution
- Can even be used on live cells!



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## Biochemical methods for cell biology

- understand the activities of their component parts (reductionist)
  - Requires the use of quantitative biochemical approaches
  - Many cellular proteins do not have an enzymatic activity *per se*
  - Most proteins and lipids are not found as soluble monomers but as large aggregates

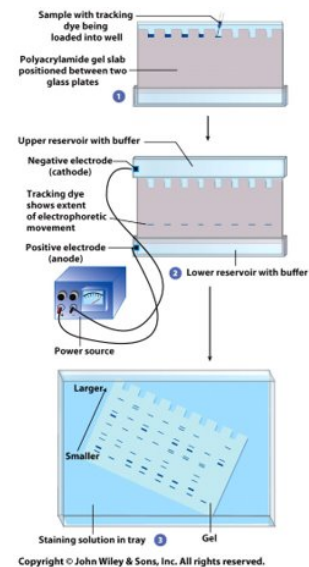


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## SDS-PAGE for protein separation and analysis

### Figure 18.30

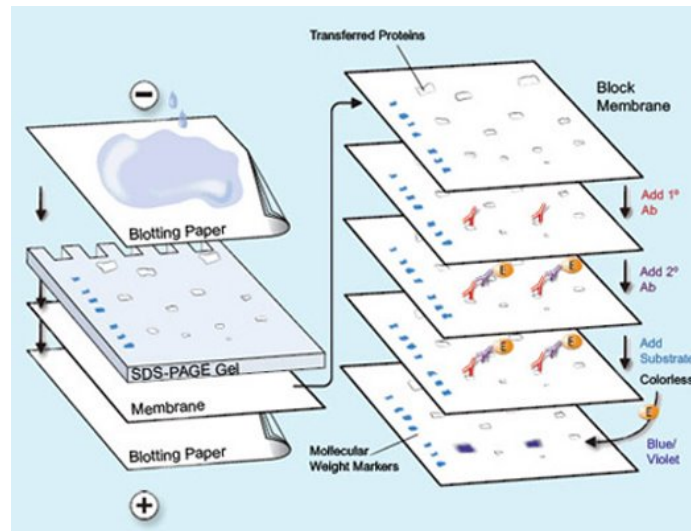
- Native proteins migrate in an electric field based on overall net charge
- To separate proteins by molecular weight they need a net negative charge
  - Sodium dodecyl sulfate (SDS)
  - uniform negative charge
  - proteins migrate inversely proportional to molecular weight



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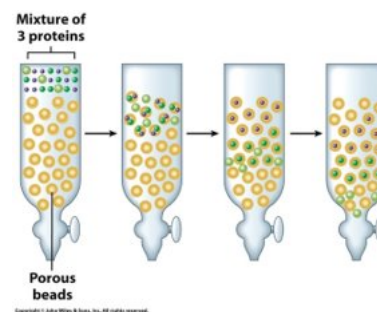
## Western Blotting uses antibodies to detect a protein of interest



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## Protein purification by column chromatography Fig 18.26-18.28

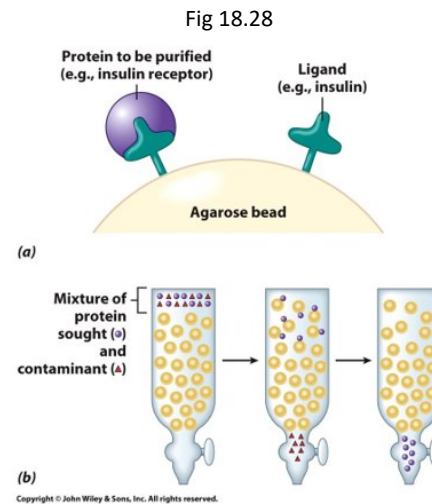
- Proteins can be separated by:
  - Molecular weight
    - (gel filtration)
  - Charge
    - (ion-exchange)
  - Binding to ligands
    - (affinity)



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## Affinity chromatography allows for the purification of a single protein from a mixture

- Specific interaction of a protein is used to pull it out of a mixed solution
- This could be through several different interactions
  - substrate
  - ligand
  - antibody
- When would this method not work?



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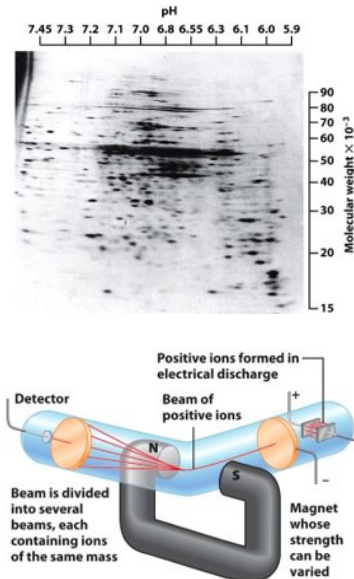
## Immunoprecipitation uses antibodies to isolate proteins or protein complexes

- Draw this on the “board”
- <https://www.youtube.com/watch?v=OrVVZ8X3n6k>

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## Proteomics: identification of all of the proteins in a cell or tissue

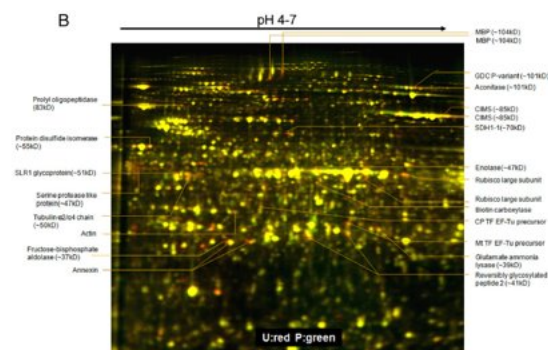
- Fig. 18.31 and 18.32
- Can identify single proteins or mixtures of proteins using mass spectroscopy
- Mass spectroscopy determines the precise molecular mass of peptide
- Mass spec can also identify post-translational modifications
  - Phosphorylation
  - Ubiquitination



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## 2D- DIGE gel electrophoresis

- Comparison of two different protein samples e.g. control vs. treatment
- Shows a change in protein levels
- Cut out shifted proteins from gel
- Verify shifted proteins using Mass Spec

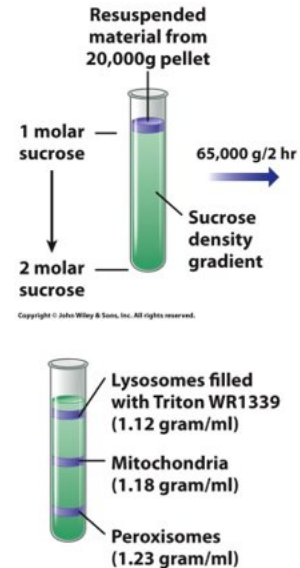


*Samuel et al 2011*

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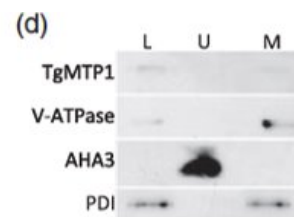
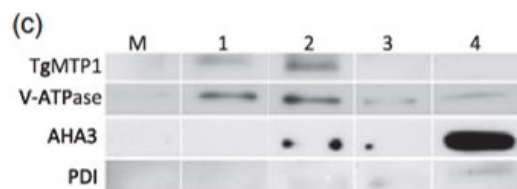
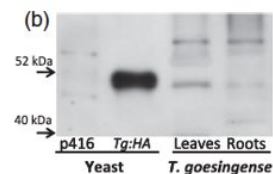
## Sucrose gradients allow for the separation of membrane proteins

- Membrane proteins are found in membranes and are not soluble in the aqueous phase
- Different membranes have varying density
- Use a density gradient to separate them
- Then examine each membrane fraction to identify proteins
- What do you need to know for this to work?



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## Sucrose gradients allow for the separation of membrane proteins



Gustin et al 2009

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Hark, a vagrant by Kate Beaton

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## Radioisotopes can be used to trace synthesis of biological macromolecules *in vivo*

**Table 18.1** Properties of a Variety of Radioisotopes Used in Biological Research

| Symbol and atomic weight | Half-life | Type of particle(s) emitted |
|--------------------------|-----------|-----------------------------|
| $^3\text{H}$             | 12.3 yr   | Beta                        |
| $^{11}\text{C}$          | 20 min    | Beta                        |
| $^{14}\text{C}$          | 5700 yr   | Beta                        |
| $^{24}\text{Na}$         | 15.1 hr   | Beta, Gamma                 |
| $^{32}\text{P}$          | 14.3 d    | Beta                        |
| $^{35}\text{S}$          | 87.1 d    | Beta                        |
| $^{42}\text{K}$          | 12.4 hr   | Beta, Gamma                 |
| $^{45}\text{Ca}$         | 152 d     | Beta                        |
| $^{59}\text{Fe}$         | 45 d      | Beta, Gamma                 |
| $^{60}\text{Co}$         | 5.3 yr    | Beta, Gamma                 |
| $^{64}\text{Cu}$         | 12.8 hr   | Beta, Gamma                 |
| $^{65}\text{Zn}$         | 250 d     | Beta, Gamma                 |
| $^{131}\text{I}$         | 8.0 d     | Beta, Gamma                 |

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