

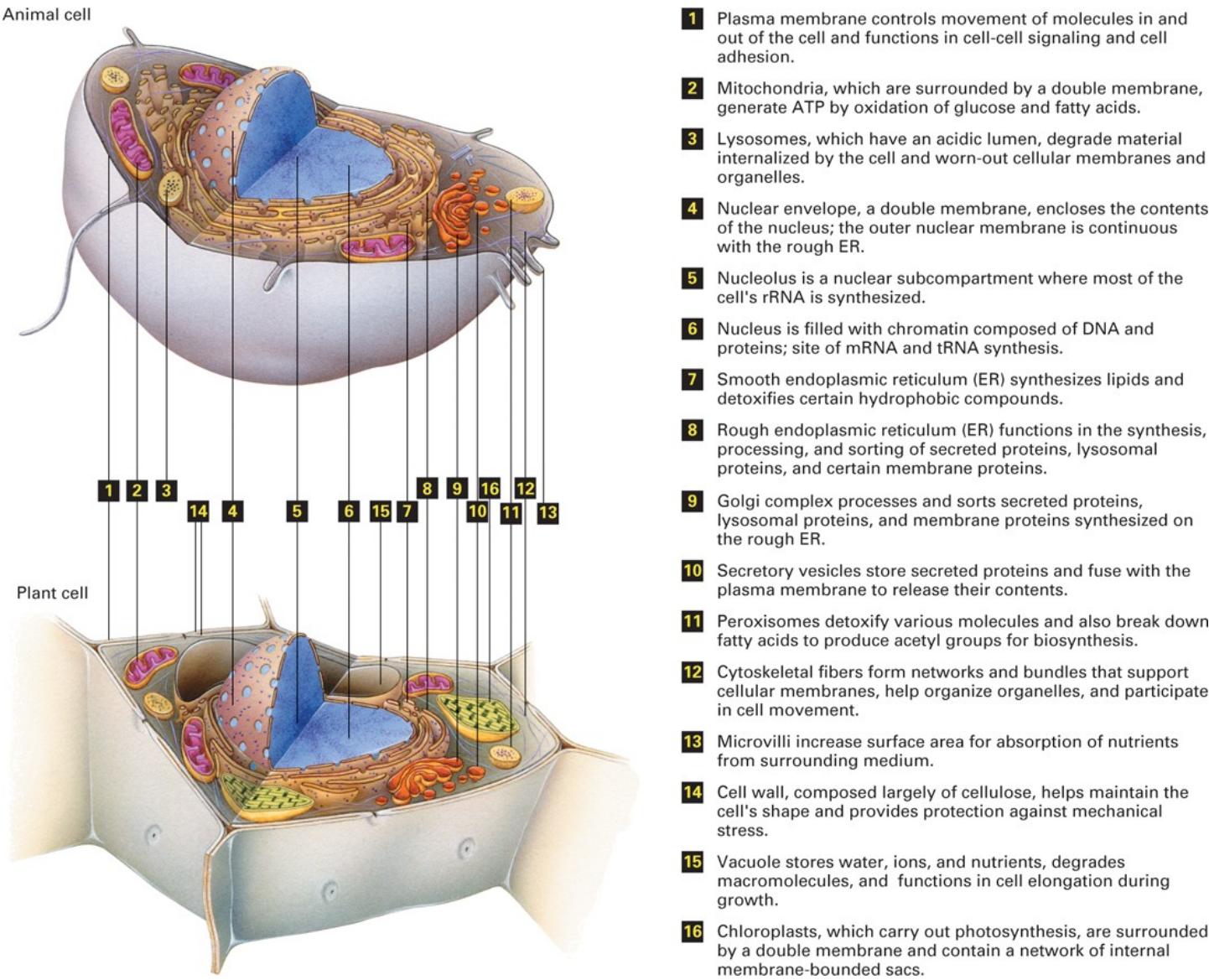
Perturbing, and visualizing cells

Chapter 9

Visualizing cells

Chapter 9

Figure 9.32 Schematic overview of a “typical” animal cell (top) and plant cell (bottom) and their major substructures.



FLUORESCENCE MICROSCOPY

What is fluorescence?

**Absorb light
at one
wavelength
and emit light
at a specific
and longer
wavelength**

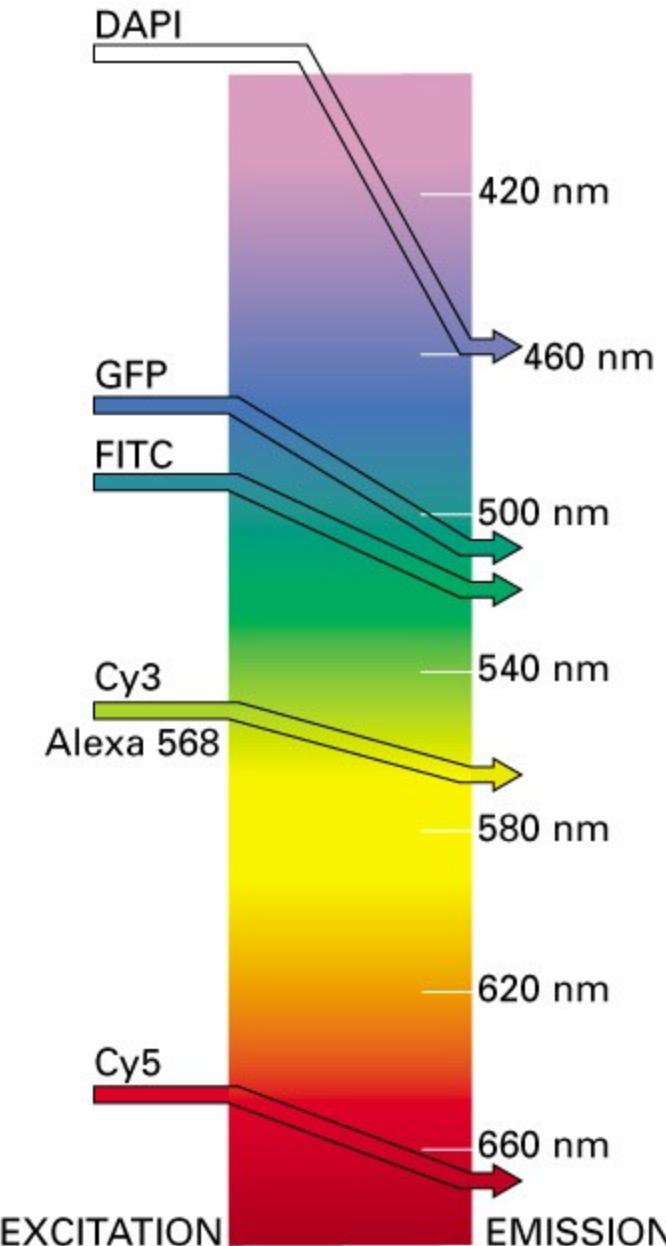


Figure 9–13. Molecular Biology of the Cell, 4th Edition.

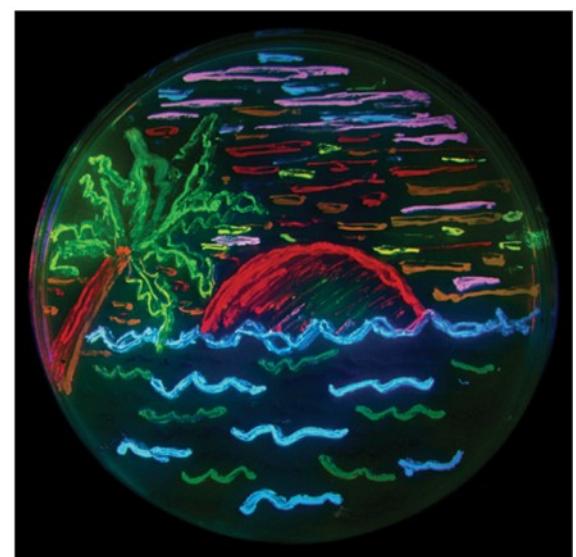
Fluorescent colors that are available

(a)



mPlum
mGrape2
mRaspberry
mGrape1
mCherry
mStrawberry
1dTomato
mTangerine
mBanana
mHoneydew
Citrine
EGFP
ECFP
EBFP

(b)



Fluorescence Microscopy

- Major Function: Localization of specific cellular molecules – example proteins
- Expression of fluorescent proteins – recombinant DNA technology.
- Major Advantages:
 - Sensitivity: “glow” against dark background
 - Specificity: immunofluorescence
 - **CELLS MAY BE FIXED OR LIVING**
- Fluorescent dyes or proteins (Flurochromes)
 - flurochromes may be indirectly or directly associated with the cellular molecule
 - Multiple flurochromes may be used simultaneously

HYDRA EXPRESSING GFP

**Fluorescent protein
in live cells**
**Plasmid containing
the GFP gene is
driven by the
H.vulgaris beta-actin
promoter.**
**GFP - green
fluorescent protein
(jelly fish)**



Figure 9-12
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RECOMBINANT DNA TECHNIQUE TO TAG PROTEINS WITH GFP FOR VISUALIZATION IN LIVING CELLS

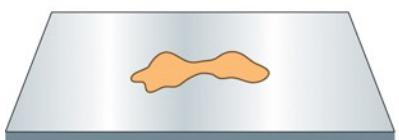
- Gene of interest X is fused with GFP gene
- This recombinant DNA is transfected (introducing foreign DNA into eukaryotic cells) into living cells.
- Wait for protein expression
- Chimeric fusion protein (Protein X-GFP) can be visualized in living cells
- Very useful in studying protein localization.

Immunofluorescence Microscopy of Specific Proteins in **FIXED CELLS**

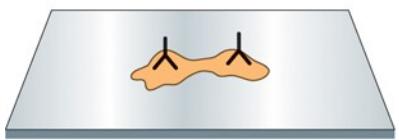
- Fluorescently tagged primary anti body
 - Commonly used fluorochromes include Texas red, and rhodamine (emit red light), Cy3 (emits orange light), fluorescein (emits green light).
- Fluorescently tagged secondary antibody- Indirect Immunofluorescence microscopy
- Fluorescently labelled antibody to tagged proteins such as myc or FLAG
- Fluorescent dye fura-2 used to monitor Ca²⁺ concentrations within the cell
- SPED (stimulated emission depletion) fluorescence microscopy has a

Purpose- to visualize the localization of Glucose Transporter

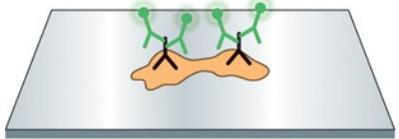
- 1 Prepare sample and place on microscope slide



- 2 Incubate with primary antibody; wash away unbound antibody

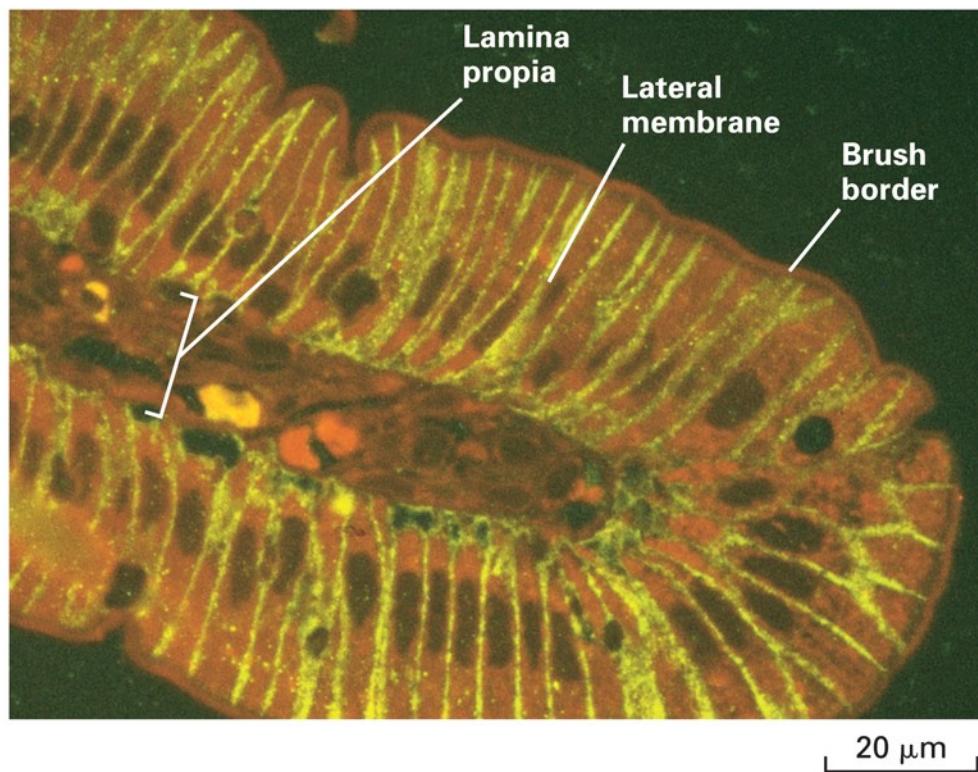


- 3 Incubate with fluorochrome-conjugated secondary antibody; wash away unbound antibody



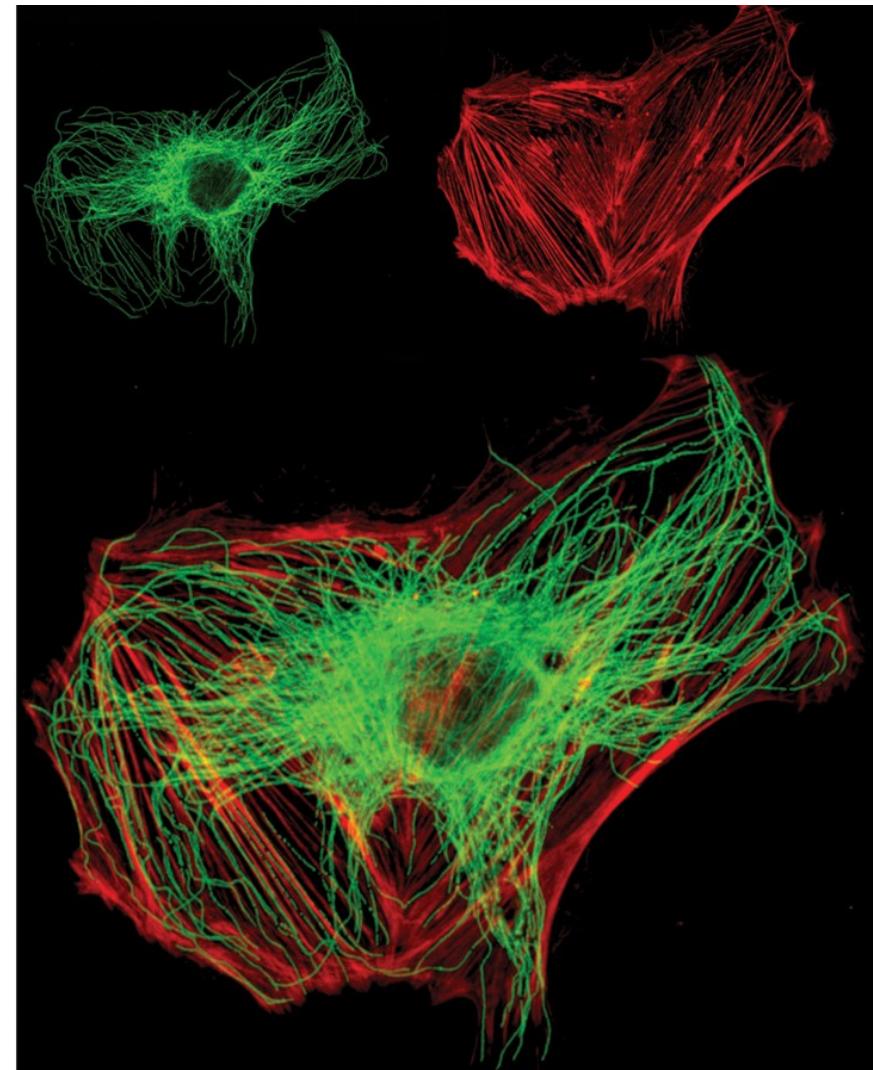
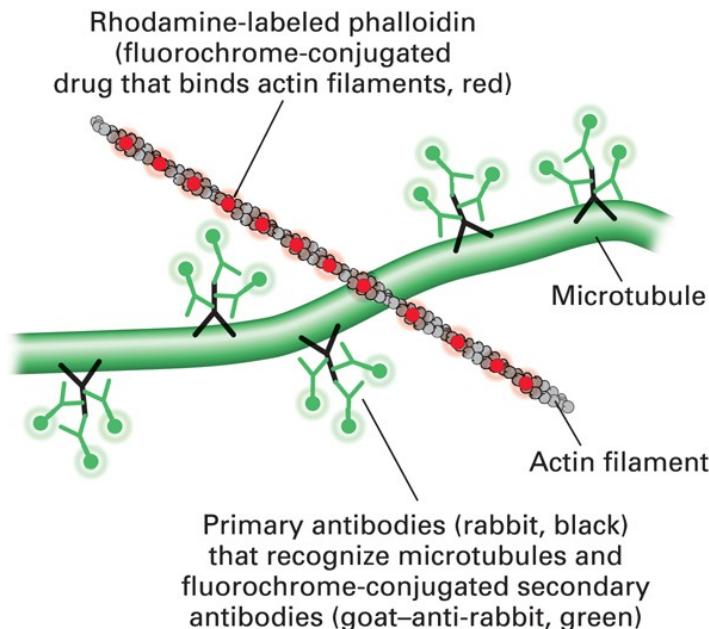
→
4

- Mount specimen and observe in fluorescence microscope



Tagging with fluorescent tags/proteins allows visualization of specific proteins

Double-label fluorescence microscopy can visualize the relative distributions of two proteins.



CONFOCAL AND DECONVOLUTION MICROSCOPY

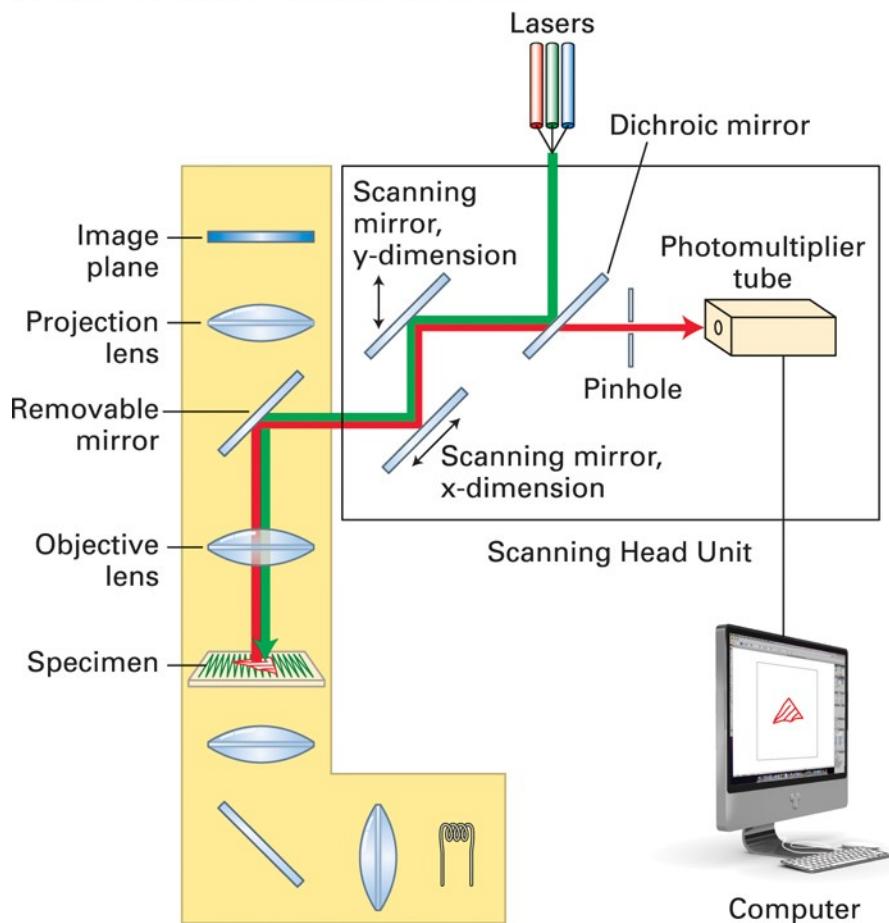
- This overcomes the limitations of Fluorescence microscopy
 - Blurred images
 - Thick specimens

CONFOCAL MICROSCOPY

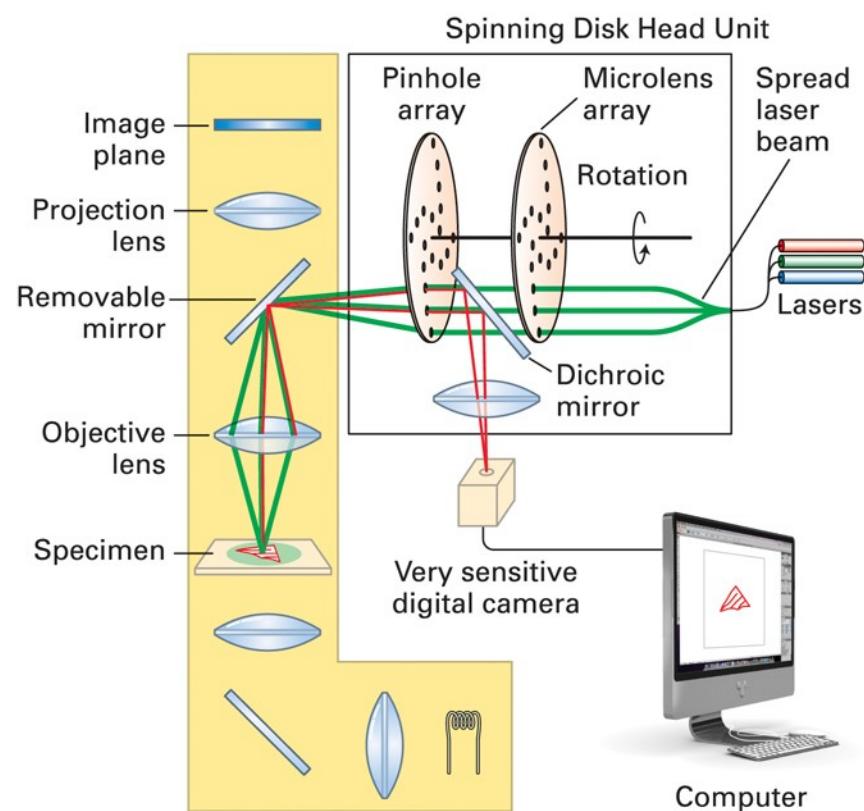
- Laser as the energy source
- The laser scans the specimen across and down to build the image
- Uses a pinhole in front of the detector so light from other focal planes are blocked

Figure 9.17 Light paths for two types of confocal microscopy.

(a) Laser-Scanning Confocal Microscope



(b) Spinning Disk Confocal Microscope

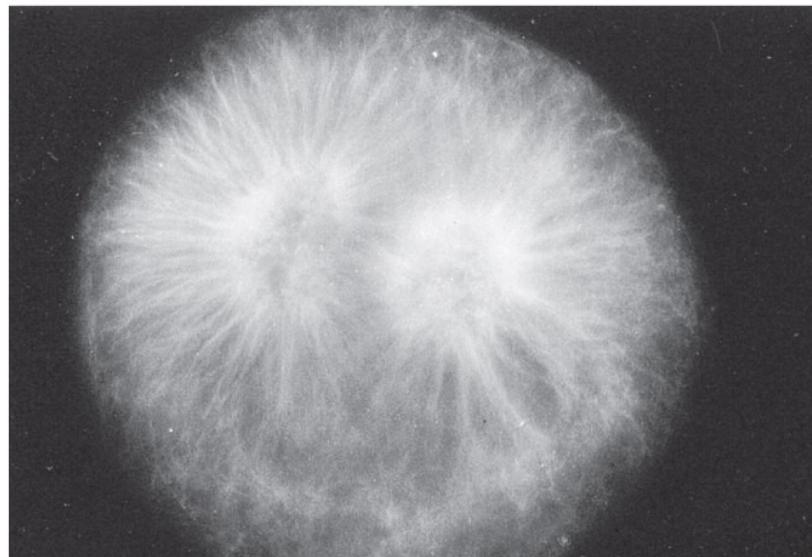


REMOTES OUT OF FOCUS

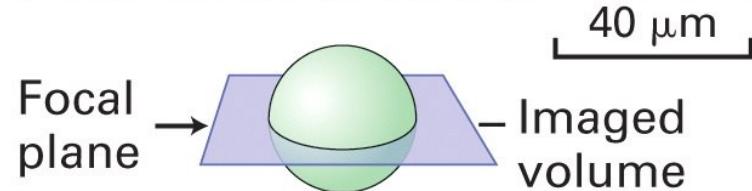
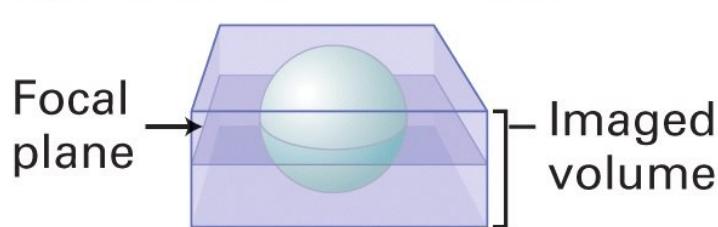
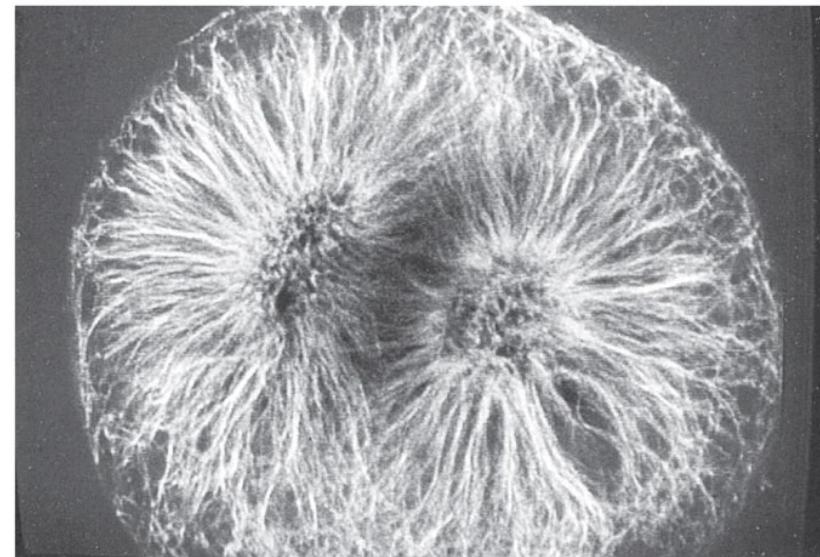
IMAGES

Sea Urchin egg undergoing mitosis using fluorescein-tagged secondary antibody that binds to primary anti-tubulin antibody

(a) Conventional fluorescence microscopy



(b) Confocal fluorescence microscopy



EXAMPLE OF IMAGE RECONSTRUCTED AFTER DECONVOLUTION MICROSCOPY

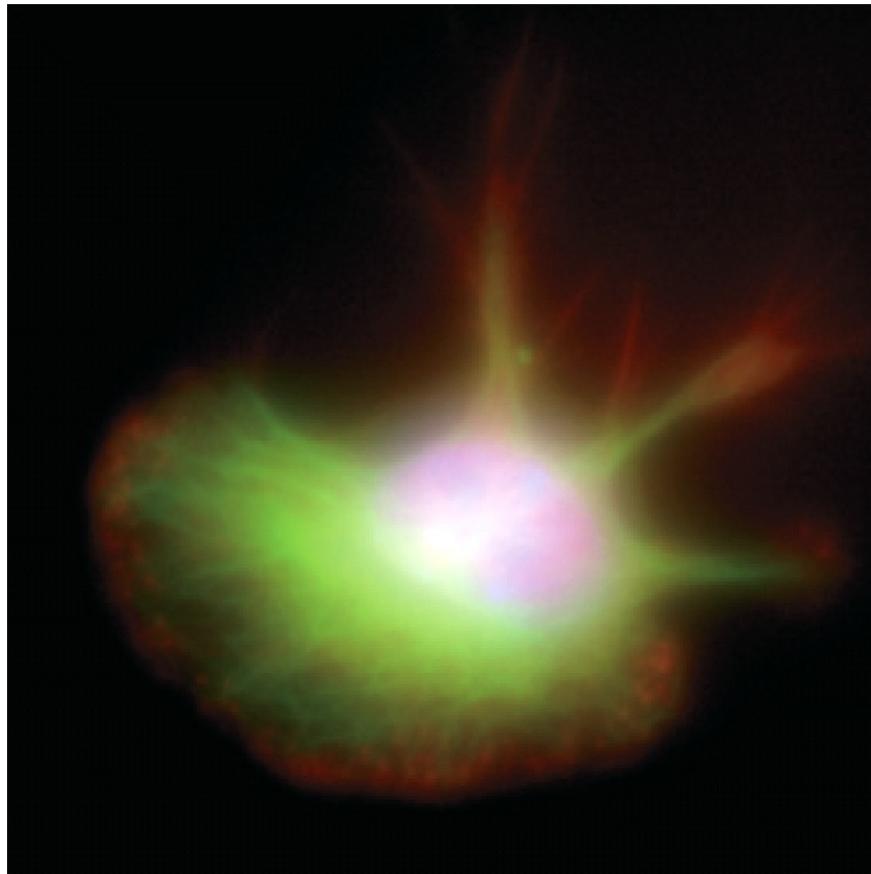


Figure 9-19a
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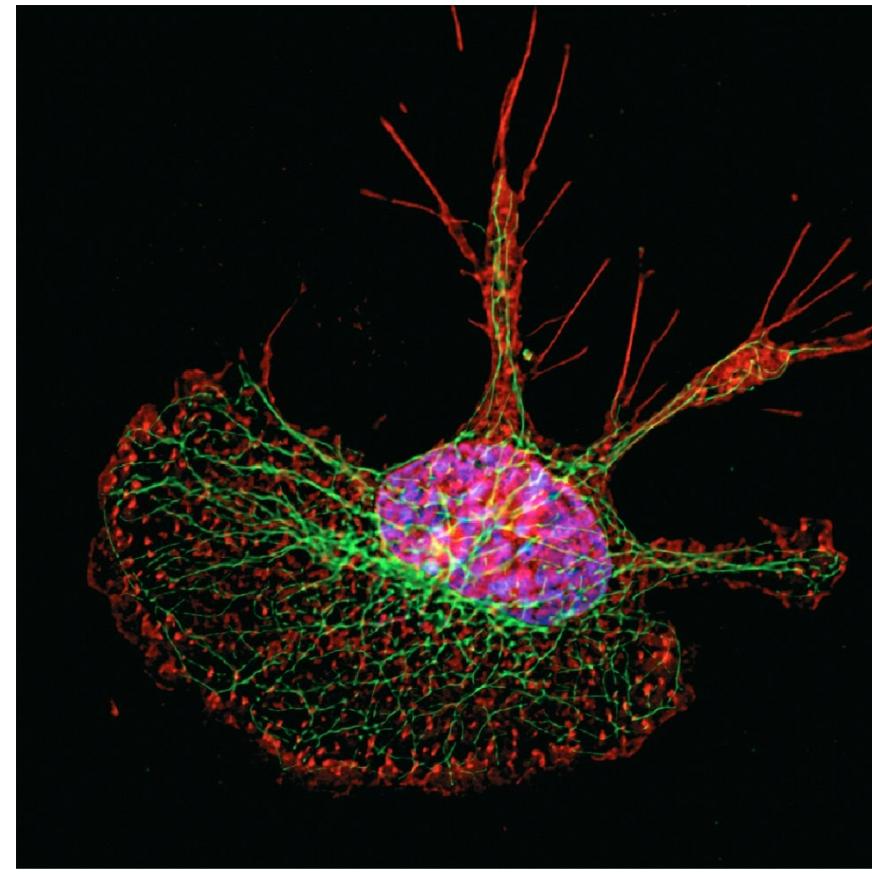
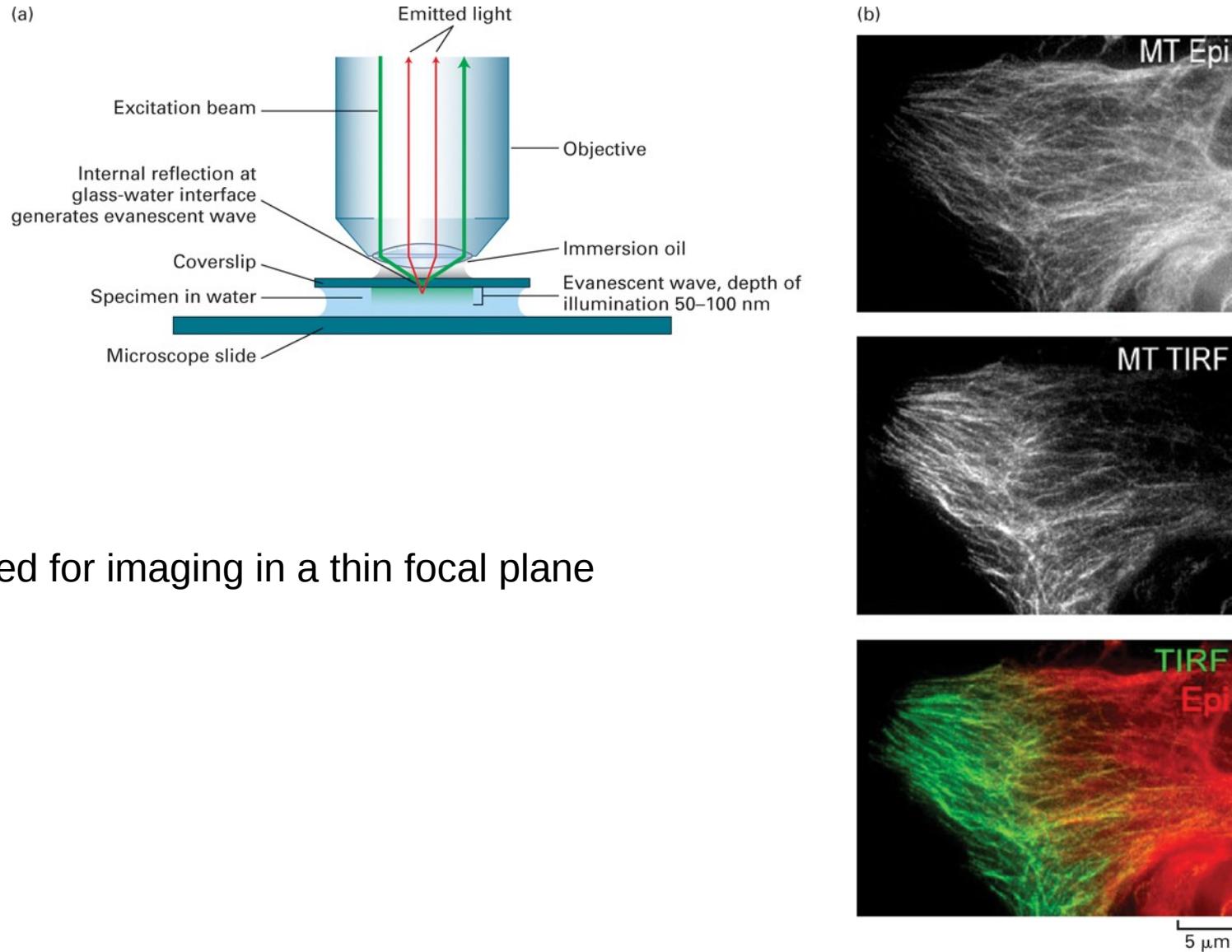


Figure 9-19b
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DNA - blue, Microtubules - green and Actin microfilaments - red

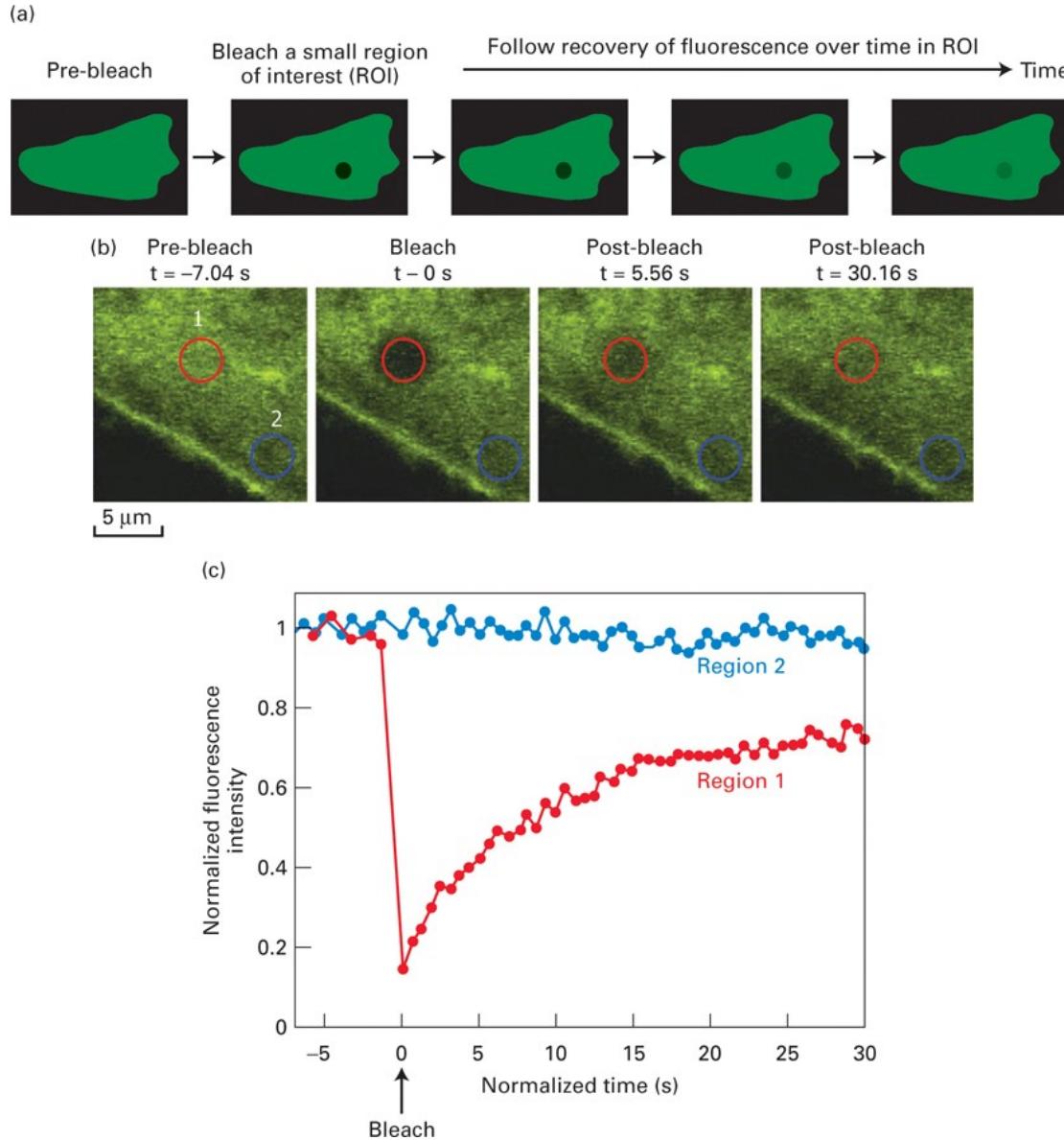
Fluorescent samples in a restricted focal plane can be imaged by total internal reflection (TIRF) microscopy.



Used for imaging in a thin focal plane

FRAP shows dynamics of cellular components

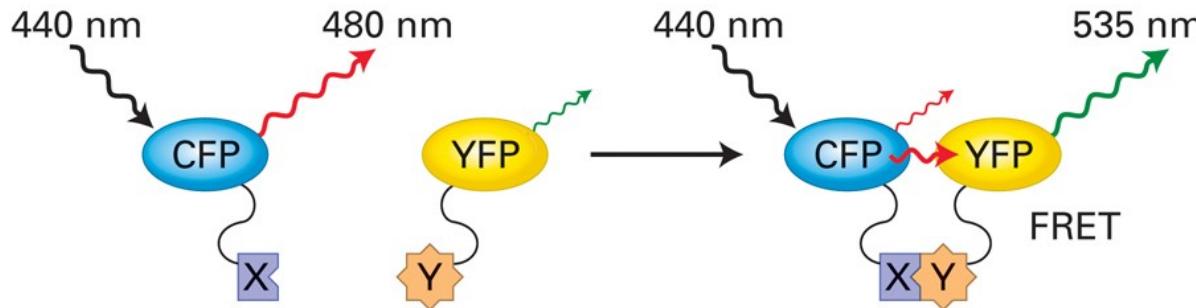
Fluorescence recovery after photobleaching (FRAP) reveals the dynamics of molecules.



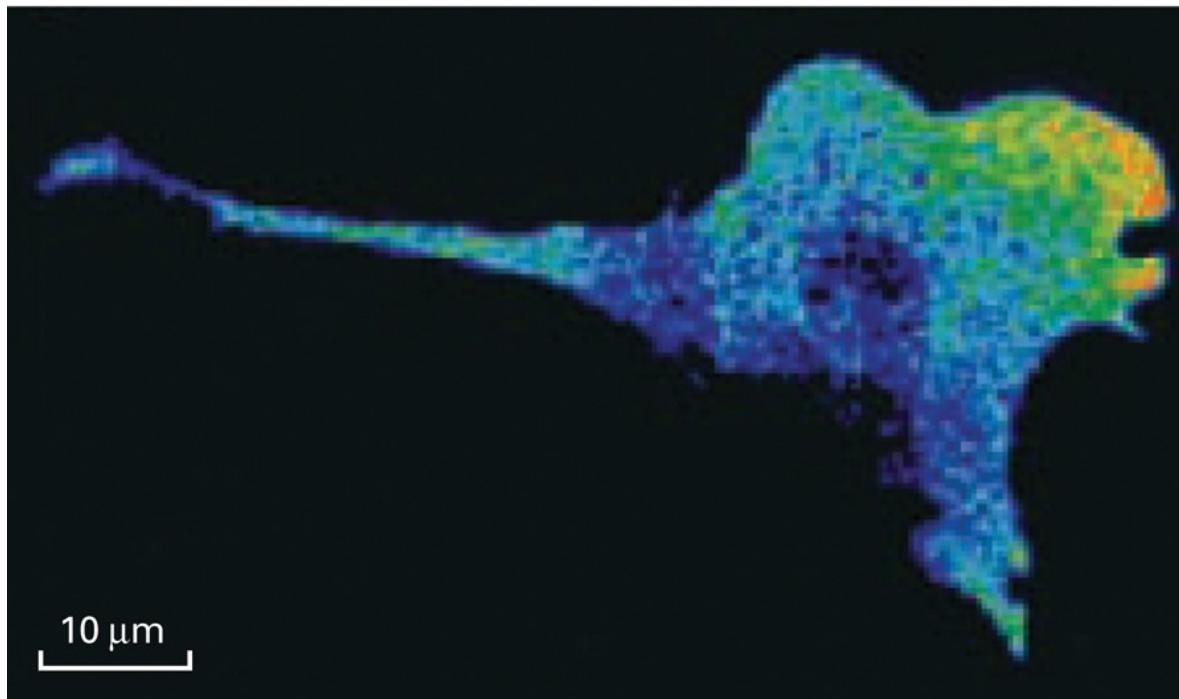
FRET measures distance between proteins using chromophores

Protein-protein interactions can be visualized by FRET.

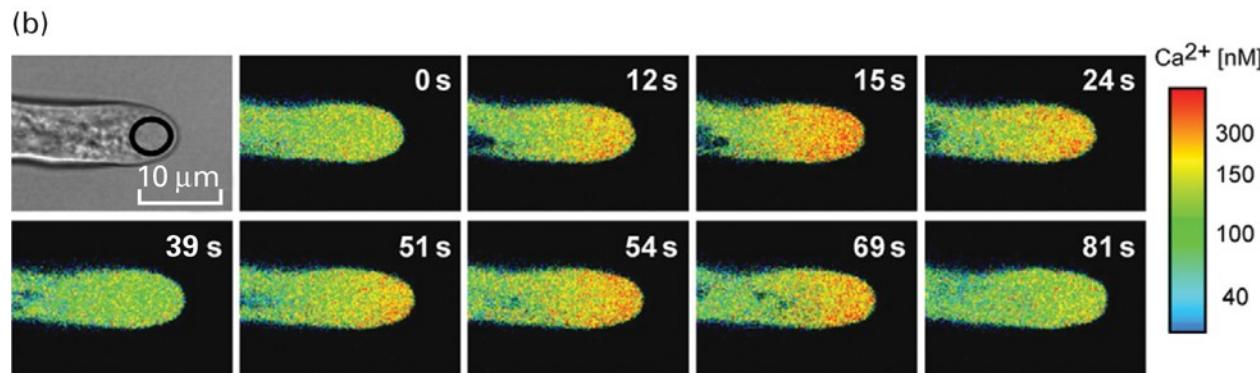
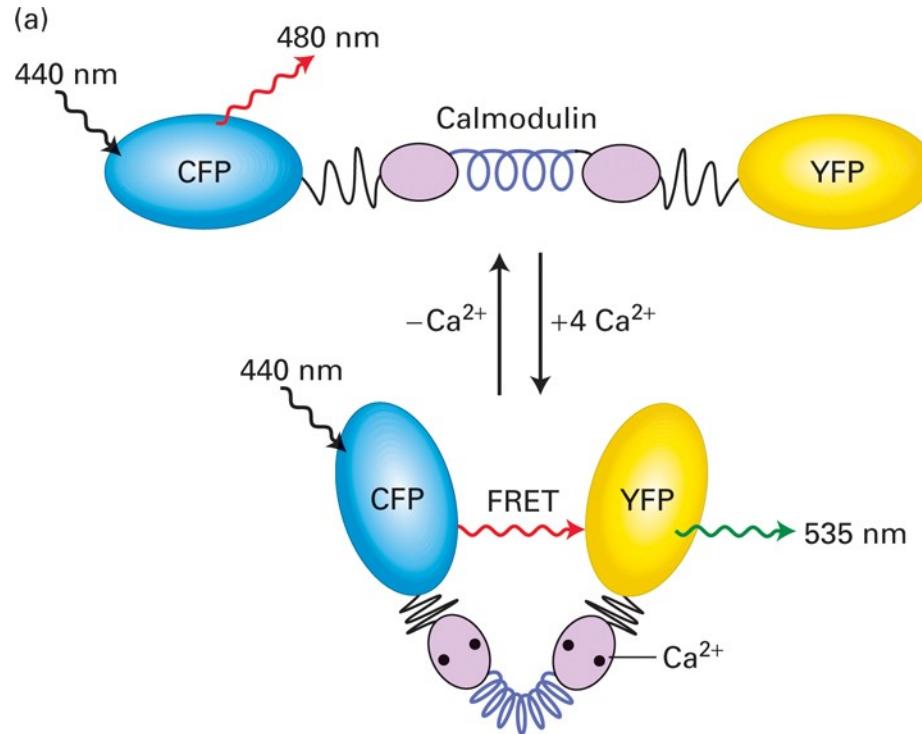
(a)



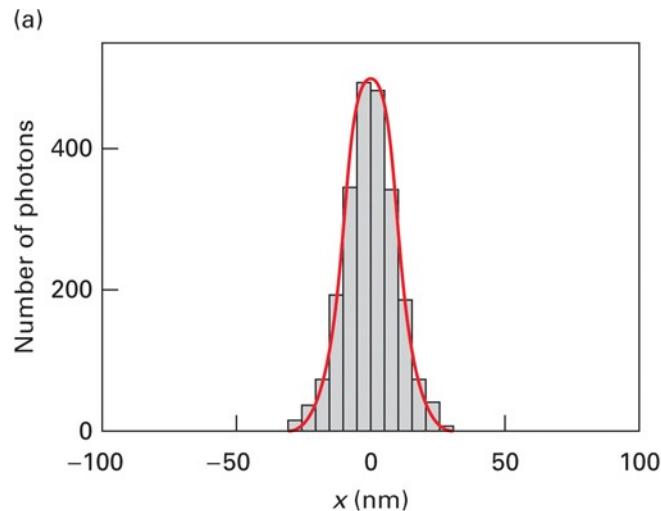
(b)



FRET biosensors can detect local biochemical environments.

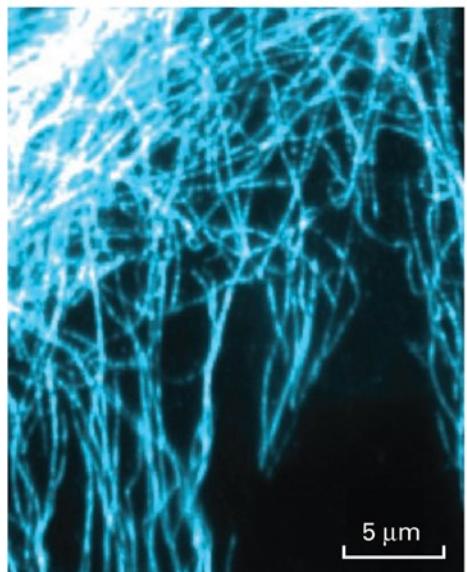


Super-resolution microscopy can generate light-microscope images with nanometer resolution.



Overcomes resolution limitations of Fluorescence microscopy .

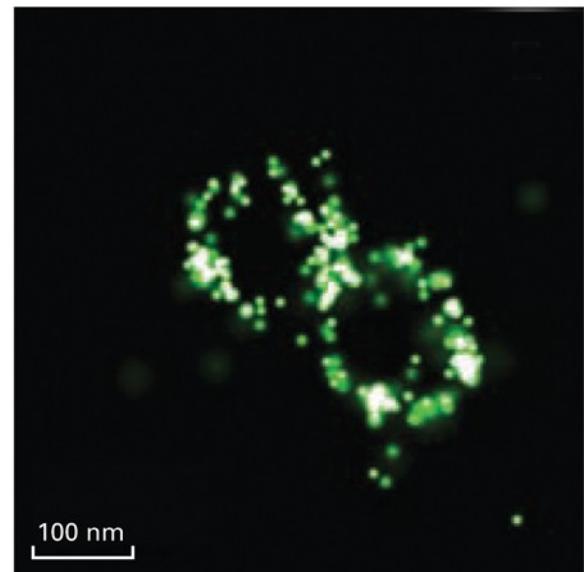
(b) Confocal



Super-resolution



Clathrin-coated pit by super-resolution



3-D structure of microtubues

PREPARATION FOR BOTH LIGHT AND ELECTRON MICROSCOPY

FIX –Formaldehyde

cross links amino groups on adjacent molecules

EMBED – For light microscopy in Paraffin; For electron microscopy in liquid plastic

SECTION – 0.5-50 micrometers for Light microscopy; 50 -100 nm for electron microscopy

STAIN –

Hematoxylin binds to basic amino acids and

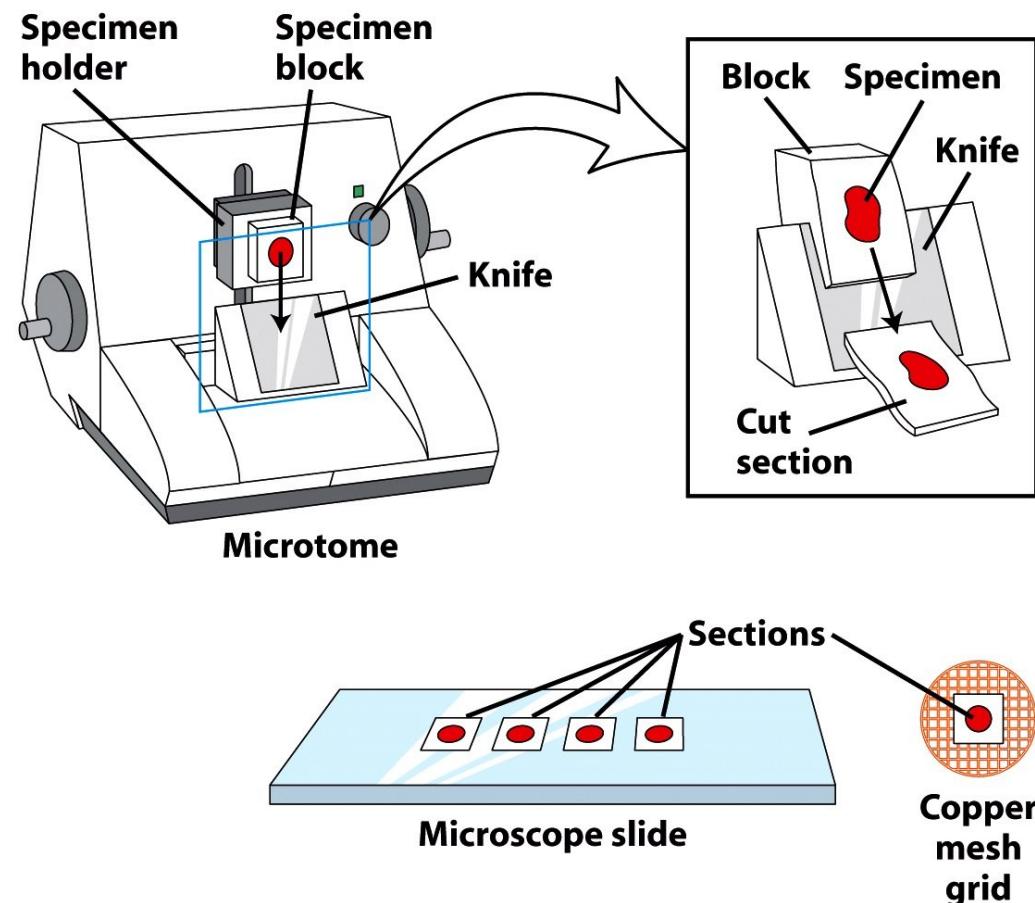


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ELECTRON MICROSC OPY

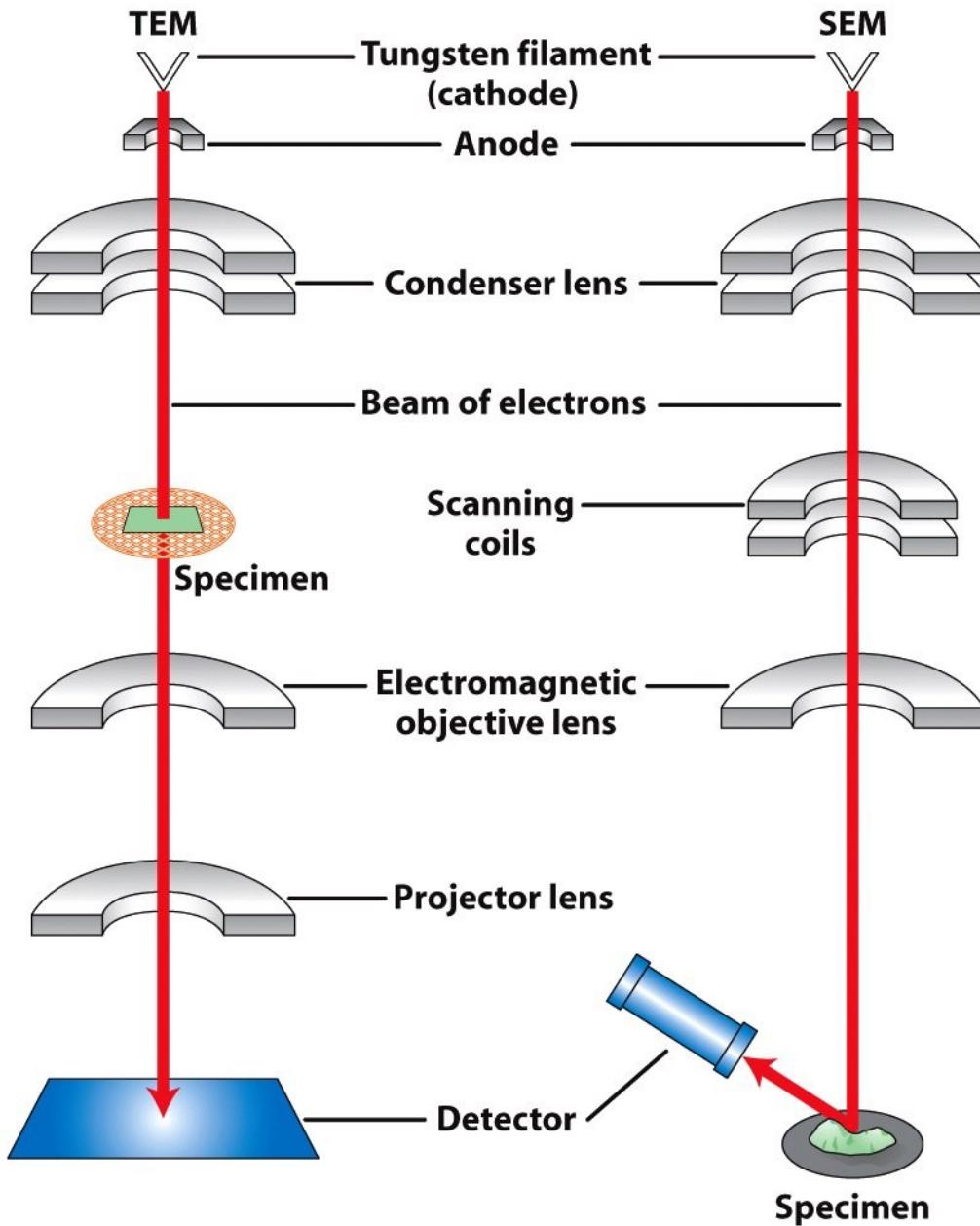


Figure 9-20
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- Transmission EM
 - theoretically 0.005 nm; practically 0.1 nm -1 nm (2000x better than LM)
 - High – velocity electron beam passes through the sample
 - 50-100 nm thick sections
 - 2-D sectional image – surface details are revealed
 - Subcellular organelles
 - Samples need to be fixed and stained and so cannot image living cells
 - Stained with heavy metals such as uranium, lead, Osmium tetroxide (stains membranes)
- Scanning EM
 - Resolution about 10 nm
 - Used to view unsectioned, metal coated specimens
 - 3-D surface image

SURFACE DETAILS BY METAL SHADOWING in TEM

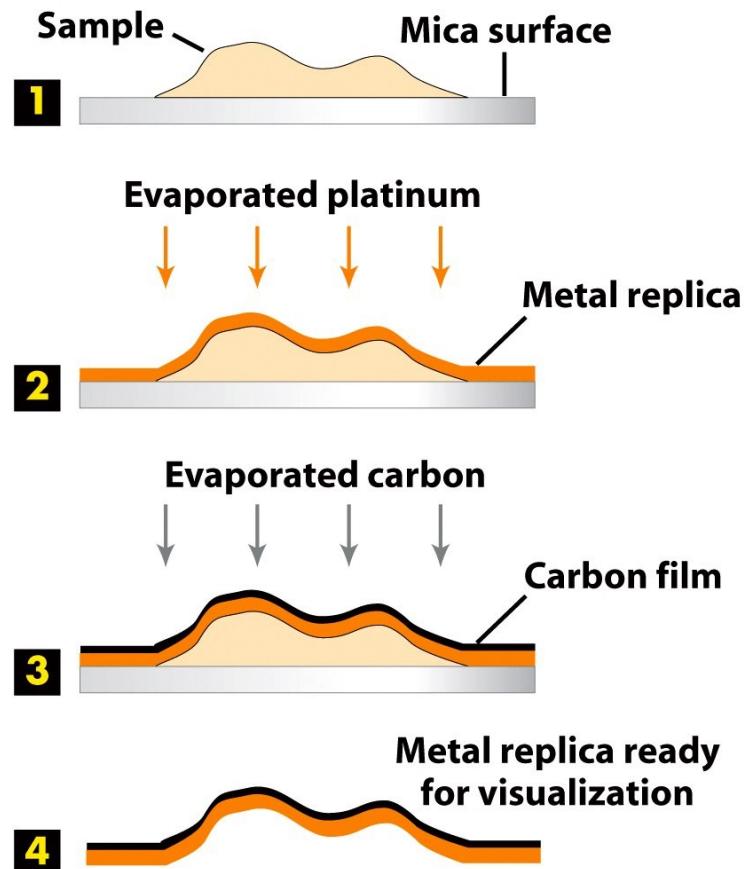


Figure 9-23a
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PLATINUM SHADOWED REPLICA OF THE SUBSTRUCTURAL FIBERS OF CALFSKIN COLLAGEN

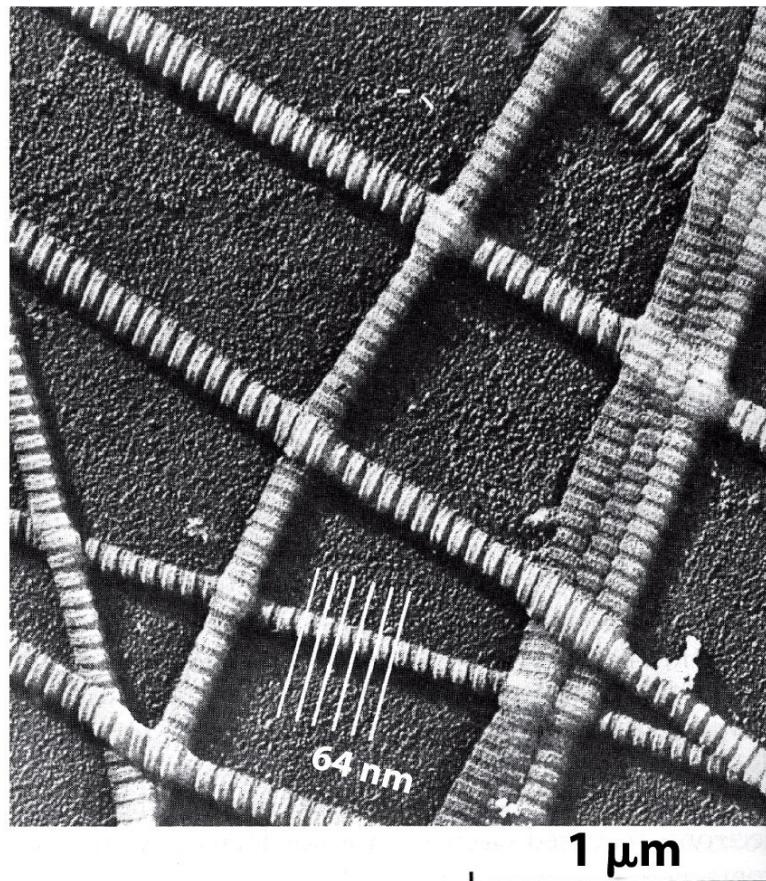


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SEM OF EPITHELIUM LINING THE INTESTINAL LUMEN

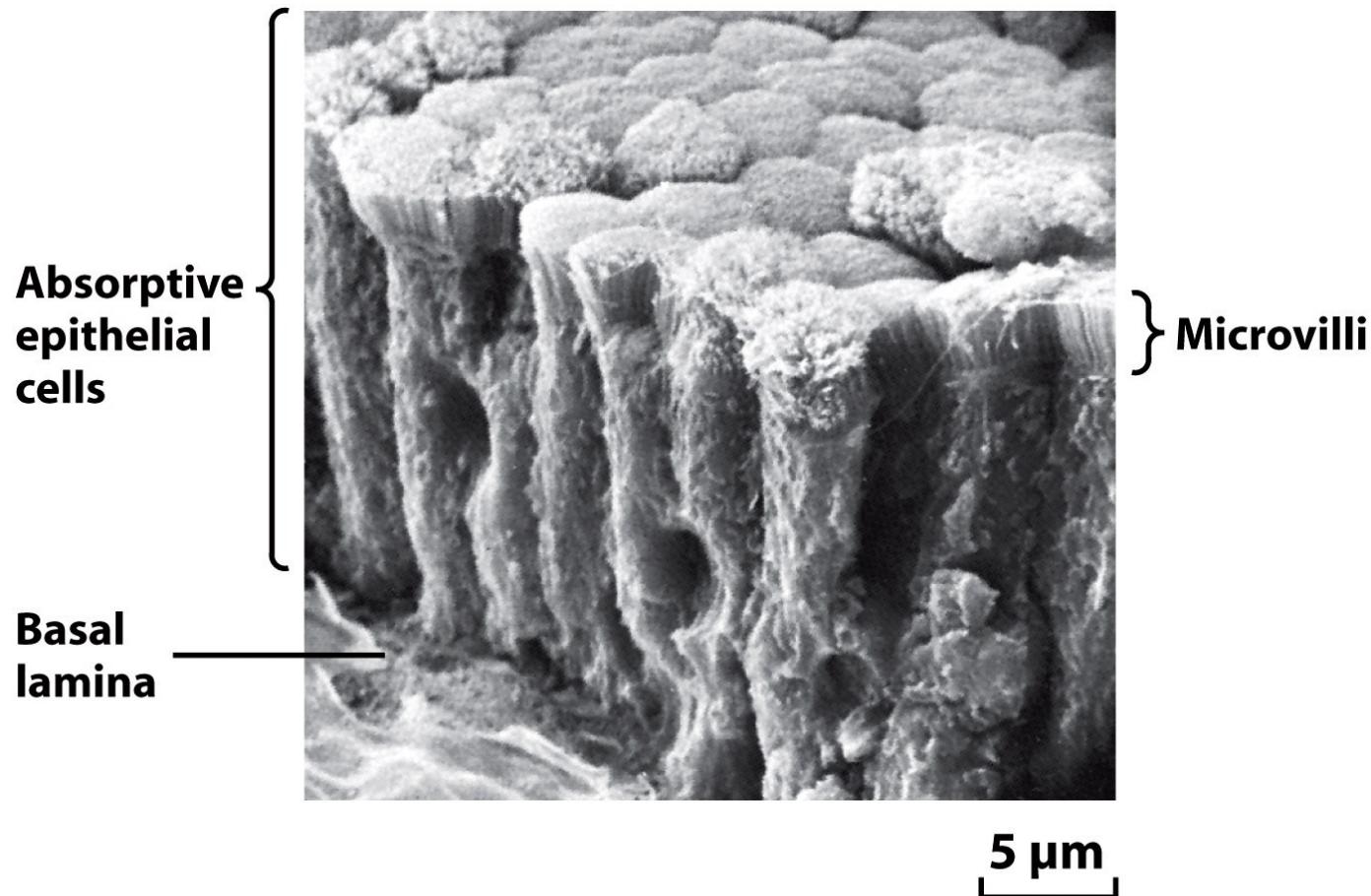


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GOLD PARTICLES COATED WITH PROTEIN A ARE USED TO DETECT ANTIBODY BOUND TO PROTEIN

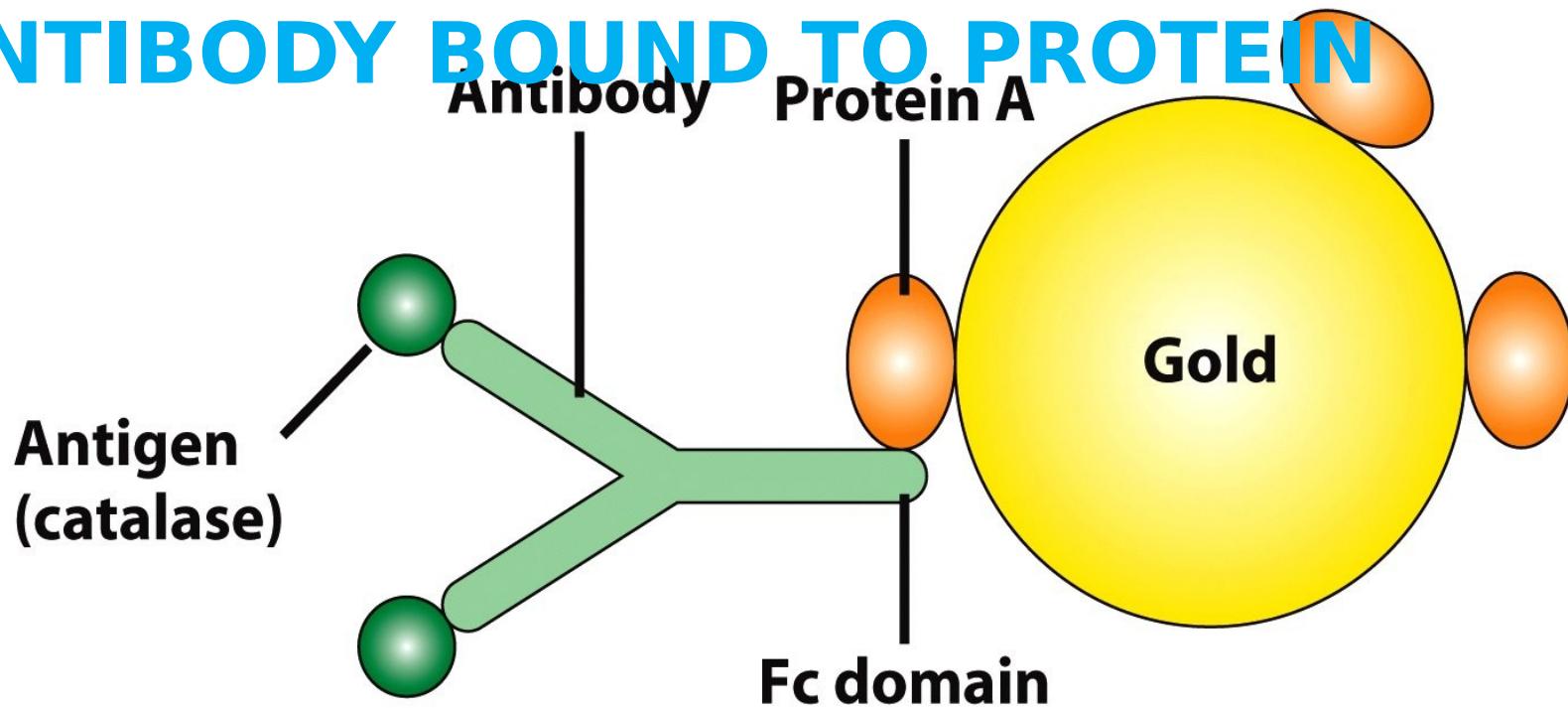


Figure 9-21a
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Protein A binds to the Fc segment of all antibody molecules

Purpose: To visualize catalase

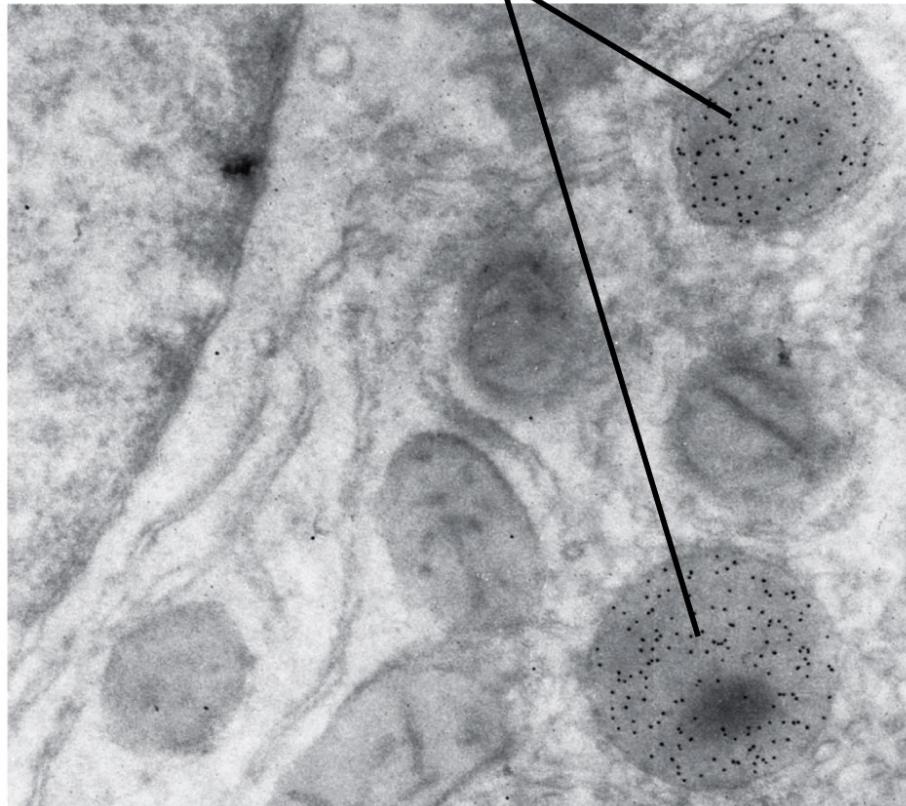
Method:

A slice of rat liver fixed with glutaraldehyde, sectioned and then treated to visualize catalase molecules.

Conclusion:
Black dots indicate

TEM IMAGE

Peroxisomes



0.5 μm

Figure 9-21b
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CRYOELECTRON MICROSCOPY

- HYDRATED, UNFIXED AND UNSTAINED SAMPLES
- SAMPLES ARE OBSERVED IN ITS NATIVE HYDRATED STATE
- METHOD - AN AQUEOUS SUSPENSION OF SAMPLE IS APPLIED ON A GRID (frozen in liquid nitrogen) AND HELD BY A SPECIAL MOUNT
- 5 nm RESOLUTION
- Variation of this is CRYOELECTRON TOMOGRAPHY. This allows determination of 3D structure .

Perturbing cells to study organelles

PURIFICATION OF CELL ORGANELLES

- CELL DISRUPTION -in isotonic solution
- SEPARATION OF DIFFERENT ORGANELLES USING CENTRIFUGATION
- PREPARATION OF PURIFIED ORGANELLES USING SPECIFIC ANTIBODIES

BREAKING OPEN PLASMA MEMBRANES IN CELLS

CELLS ARE SUSPENDED IN ISOTONIC
SUCROSE AFTER

- SONICATION
- HOMOGENIZATION
- CELLS IN HYPOTONIC SOLUTION –
RUPTURE OF CELL MEMBRANES

SEPERATING ORGANELLES

- DIFFERENTIAL CENTRIFUGATION
- DENSITY GRADIENT CENTRIFUGATION

DIFFERENTIAL CENTRIFUGATION

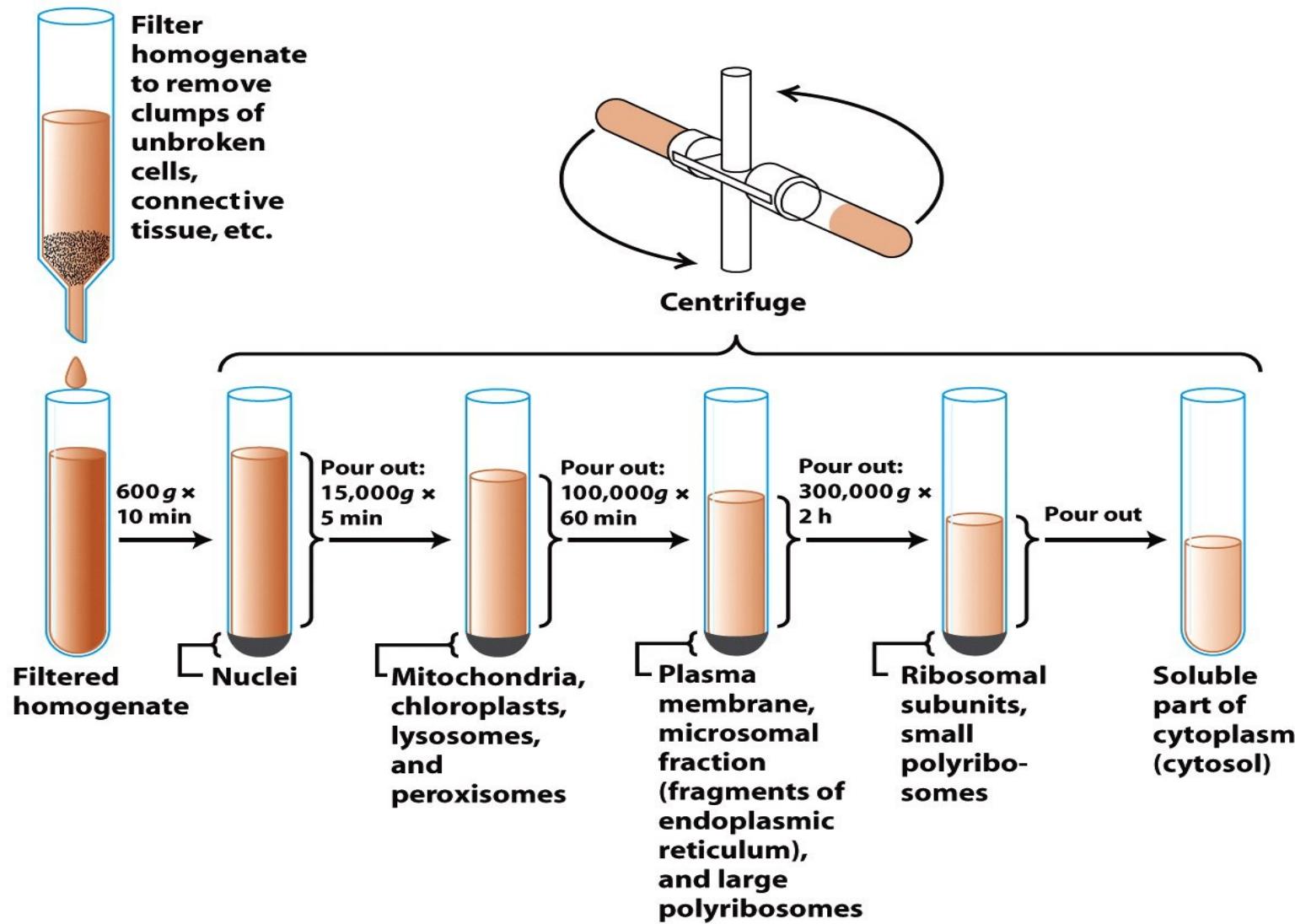


Figure 9-25

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DENSITY GRADIENT CENTRIFUGATION

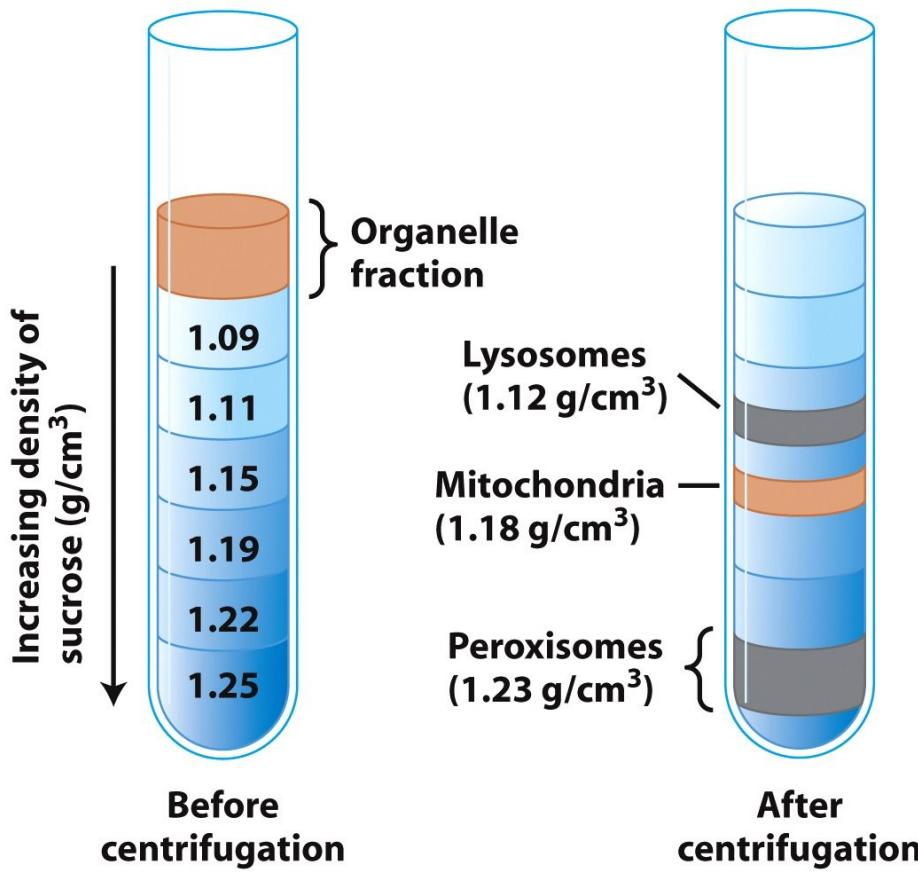


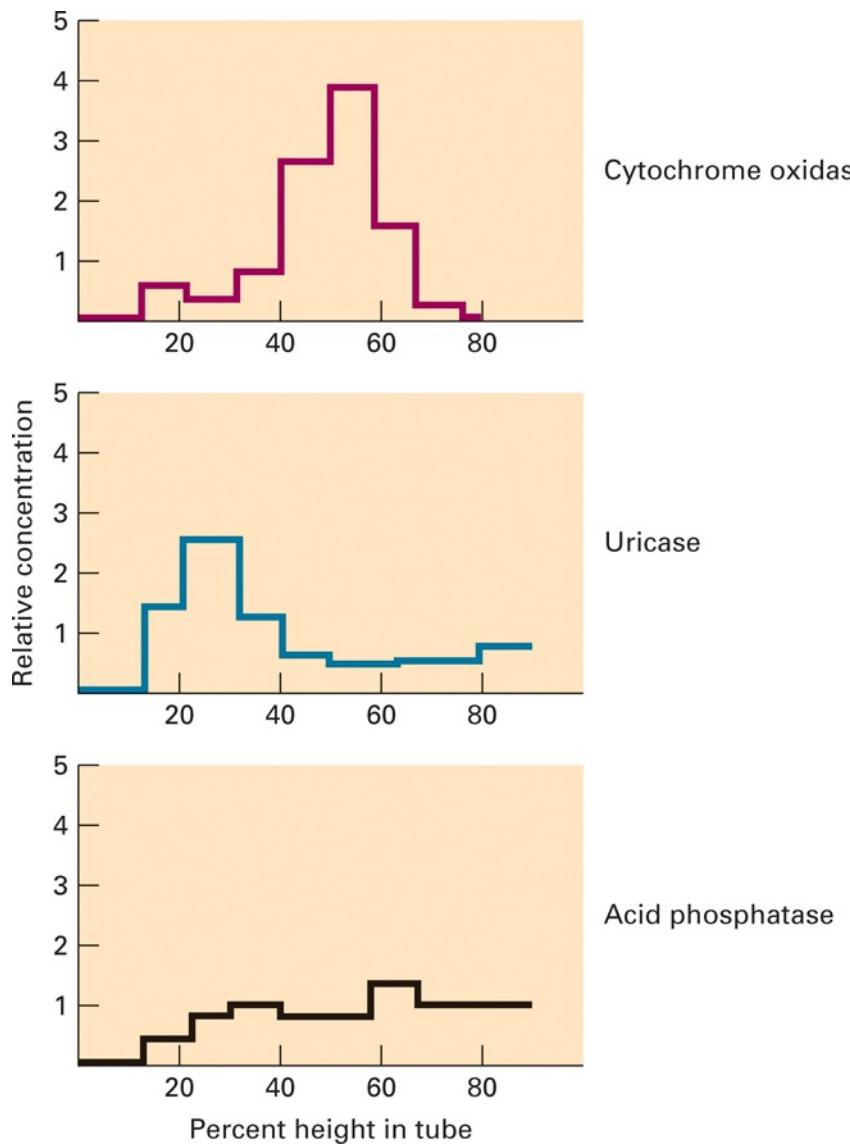
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PURIFICATION OF ORGANELLE FRACTION USING SPECIFIC ANTIBODIES

- CATALASE – PROXISOMES
- CYTOCHROME C – MITOCHONDRIA
- ACID PHOSPHATASE – LYSOSOMES
- The proteins listed above are specific for each organelle. Western blots of protein fractions are probed with antibodies specific to the proteins of interest. Cross contamination of organelle fractions is tested as mentioned in class, by analyzing the western blot results.

CLASSIC EXPERIMENT 9.1

Figure 1 Graphical representation of the enzyme analysis of products from a sucrose gradient.



ANTIBODIES ARE USED TO MAKE PURIFIED VESICLES

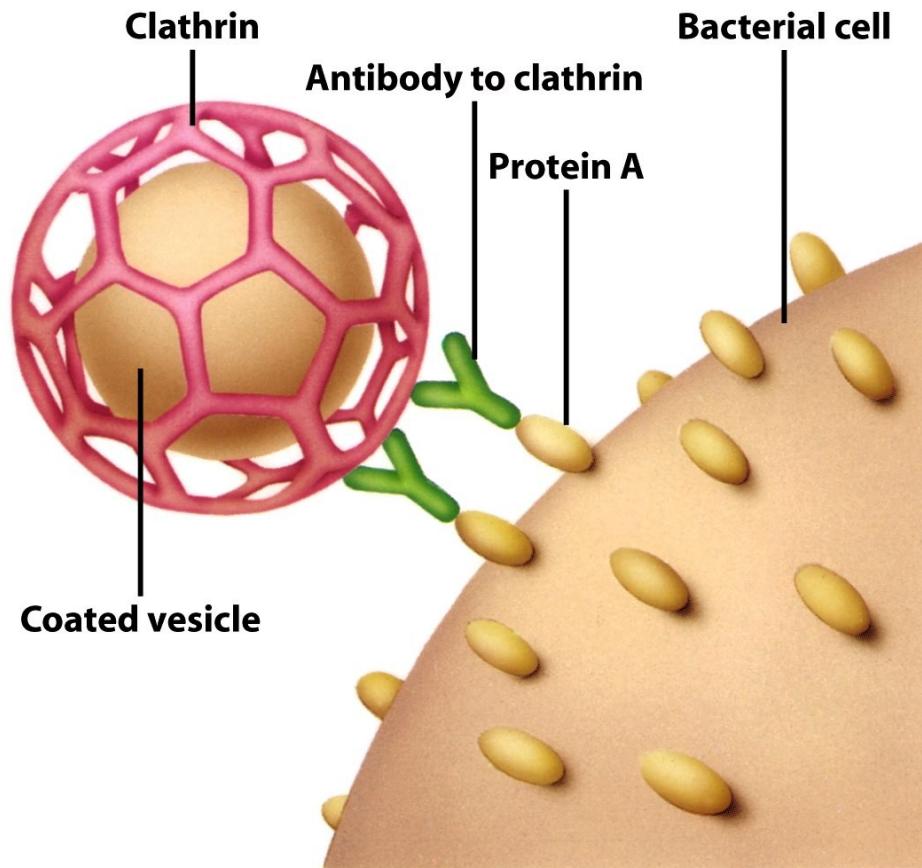


Figure 9-27a
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Antibodies against organelle-specific proteins are used.

Membrane suspension from Rat liver is incubated with antibody to clathrin. Staphylococcus Aureus bacterial suspension is added to the mix. S.Aureus membrane contains Protein A

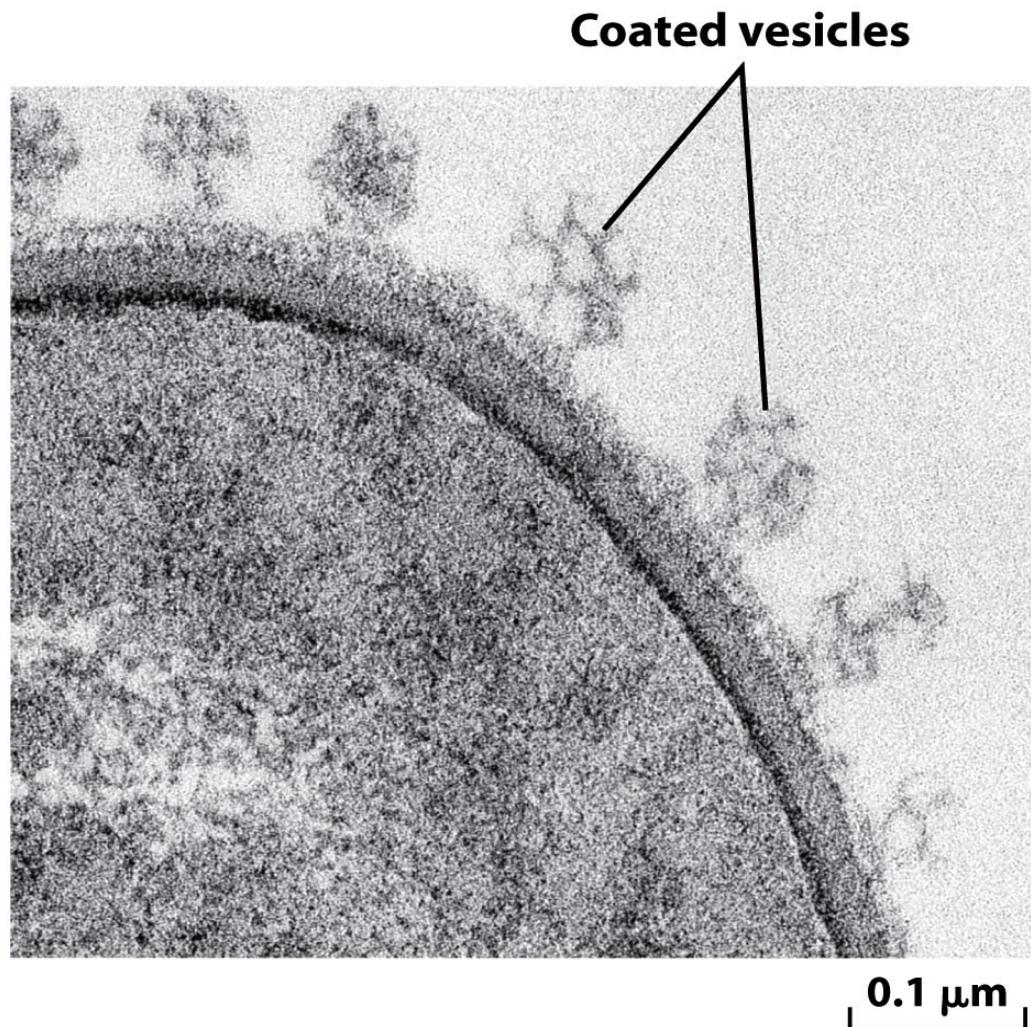


Figure 9-27b
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TABLE 9-1

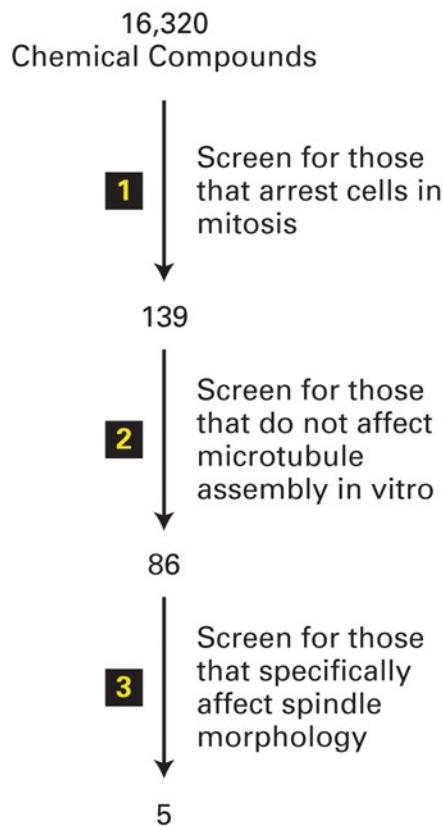
Selected Set of Small Molecules Used in Cell Biological Research

Some of the following molecules have broad specificity, whereas others are highly specific. More information about many of these compounds can be found in the relevant chapters in this text.

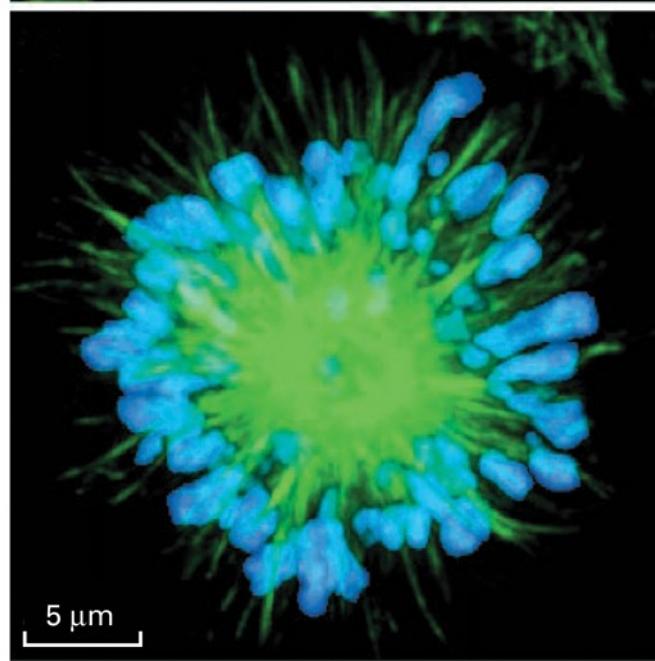
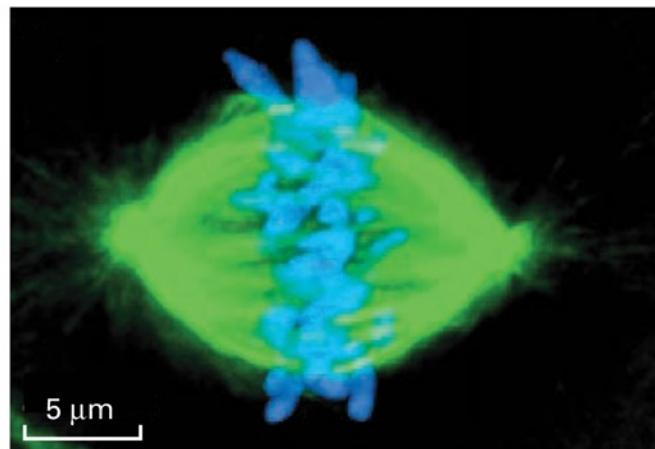
DNA replication inhibitors	Aphidicolin (eukaryotic DNA polymerase inhibitor); camptothecin, etoposide (eukaryotic topoisomerase inhibitors)
Transcription inhibitors	α -Amanitin (eukaryotic RNA polymerase II inhibitor); actinomycin D (eukaryotic transcription elongation inhibitor); rifampicin (bacterial RNA polymerase inhibitor); thiolutin (bacterial and yeast RNA polymerase inhibitor)
Protein synthesis inhibitors—block general protein production; toxic after extended exposure	Cycloheximide (translational inhibitor in eukaryotes); geneticin/G418, hygromycin, puromycin (translation inhibitors in bacteria and eukaryotes); chloramphenicol (translation inhibitor in bacteria and mitochondria); tetracycline (translation inhibitor in bacteria)
Protease inhibitors—block protein degradation	MG-132, lactacystin (proteasome inhibitors); E-64, leupeptin (serine and/or cysteine protease inhibitors); phenylmethanesulfonyl fluoride (PMSF) (serine proteases inhibitor); tosyl-L-lysine chloromethyl ketone (TLCK) (trypsin-like serine protease inhibitor)
Compounds affecting the cytoskeleton	Phalloidin, jasplakinolide (F-actin stabilizer); latrunculin, cytochalasin (F-actin polymerization inhibitors); taxol (microtubule stabilizer); colchicine, nocodazole, vinblastine, podophyllotoxin (microtubule polymerization inhibitors); monastrol (kinesin-5 inhibitor)
Compounds affecting membrane traffic, intracellular movement and the secretory pathway, protein glycosylation	Brefeldin A (secretion inhibitor); leptomycin B (nuclear protein export inhibitor); dynasore (dynamin inhibitor); tunicamycin (N-linked glycosylation inhibitor)
Kinase inhibitors	Genistein, rapamycin, gleevec (tyrosine kinase inhibitors with various specificities); wortmannin, LY294002 (PI3 kinase inhibitors); staurosporine (protein kinase inhibitor); roscovitine (cell cycle CDK1 and CDK2 inhibitors)
Phosphatase inhibitors	Cyclosporine A, FK506, calyculin (protein phosphatase inhibitors with various specificities); okadaic acid (general inhibitor of serine/threonine phosphatases); phenylarsine oxide, sodium orthovanadate (tyrosine phosphatase inhibitors)
Compounds affecting intracellular cAMP levels	Forskolin (adenylate cyclase activator)
Compounds affecting ions (e.g., K⁺ Ca²⁺)	A23187 (Ca ²⁺ ionophore); valinomycin (K ⁺ ionophore); BAPTA (divalent cation (e.g., Ca ²⁺) binding/sequestering agent); thapsigargin (endoplasmic reticulum Ca ²⁺ ATPase inhibitor); ouabain (Na ⁺ /K ⁺ ATPase inhibitor)
Some drugs used in medicine	Propranolol (β -adrenergic receptor antagonist), statins (HMG-CoA reductase inhibitors, block cholesterol synthesis)

Screening for drugs that affect specific biological processes.

(a)



(b)

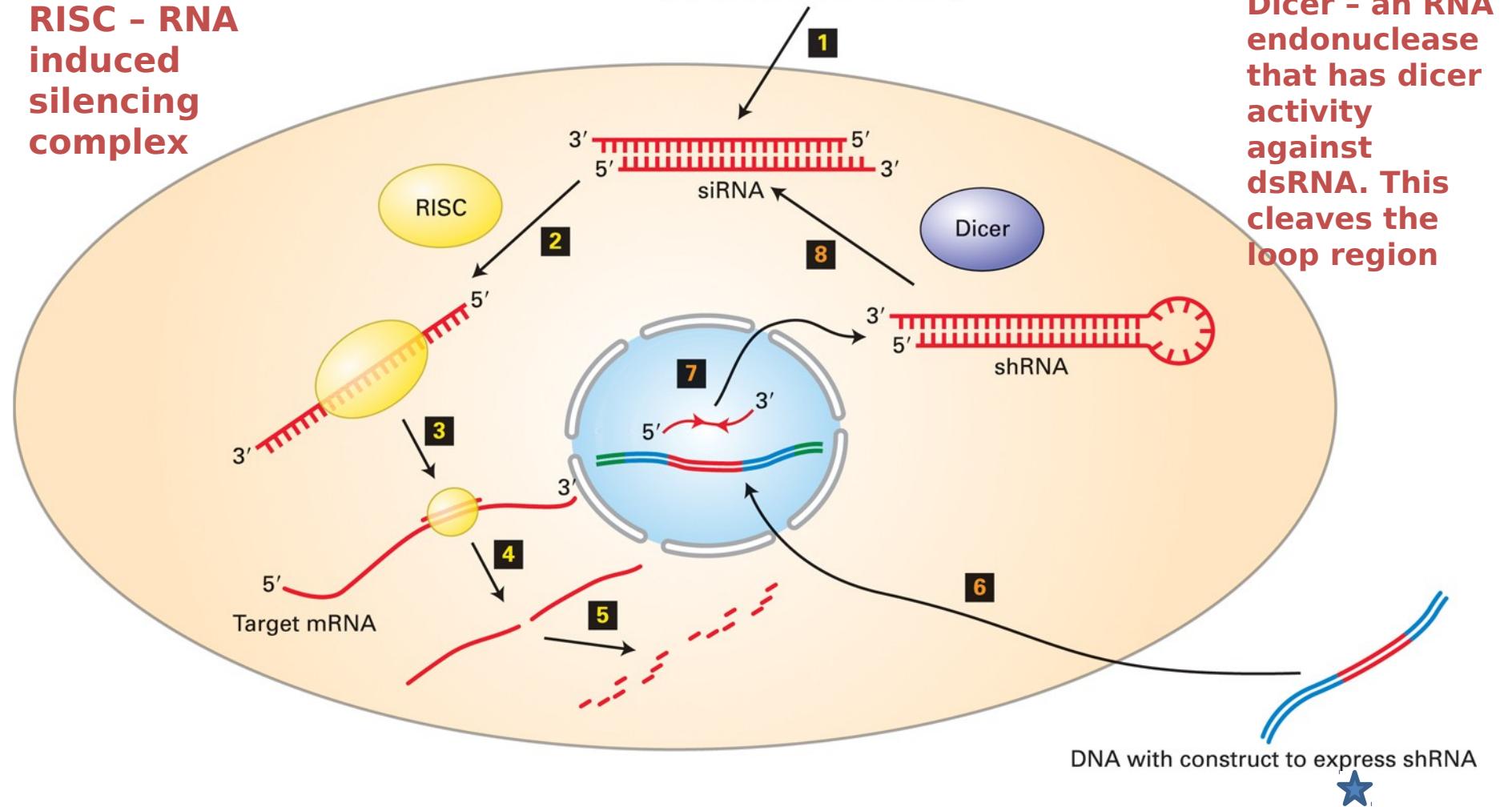


siRNA – methods and analysis

SMALL INHIBITORY RNA (siRNA) can knock down specific protein expression

★ siRNA or DNA expressing shRNA can target the degradation of

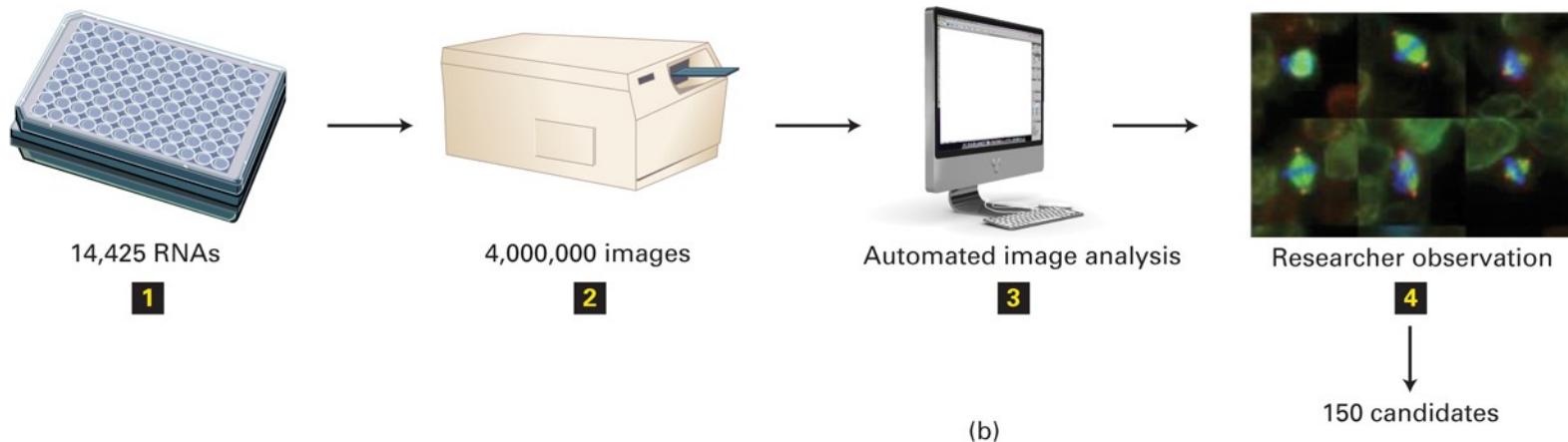
(a) RISC - RNA induced silencing complex



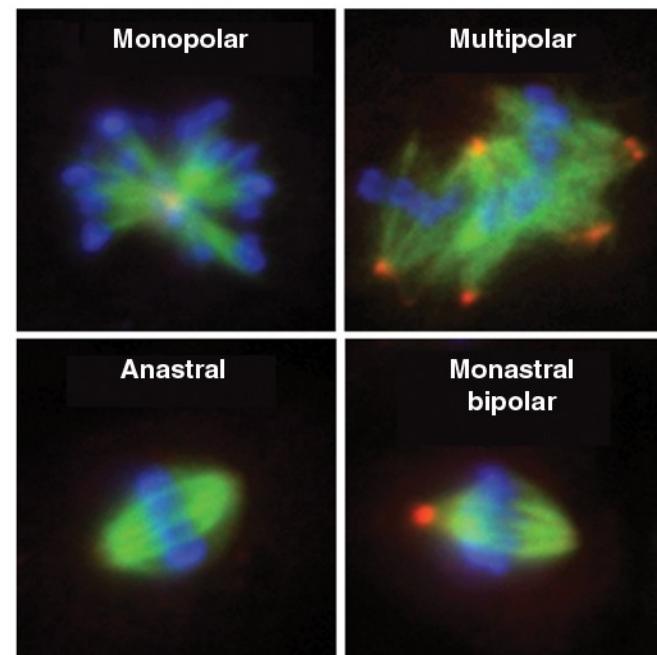
Genomic screens using siRNA

RNAi screens can explore the function of all the genes in cultured cells.

(a)



(b)



RNAi can be used to suppress genes in a tissue-specific manner in the fruit fly.

