Cell Biology

Unit 6 · Lesson 3 · Seeing and spinning cells

The notes for Lesson 3 of Unit 6: chapters 6.5 to 6.6 of the unit book, with their figures, worked examples, exam traps and quizzes, in one file. The full unit book, with every chapter, the exam question bank and the glossary, is linked at the end.

Lesson 3 of 4 | 2 chapters | Chapters 6.5 to 6.6

About these notes

These are the notes for **Lesson 3 · Seeing and spinning cells** of the University free n easy video lessons on Cell Biology Unit 6, Specialized Cells and Cell Study Methods. They are chapters 6.5 to 6.6 of the unit book, unchanged, so a page here is the same page in the full book.

6.5 Electron and confocal microscopy

When light cannot resolve a structure, use electrons; when a fluorescent sample is too thick and blurred, use a pinhole. Electron microscopes (TEM and SEM) replace light with an electron beam whose wavelength is about 100,000 times shorter, and show membranes, ribosomes and viruses. The confocal microscope keeps fluorescence but rejects out-of-focus light, giving sharp optical slices and 3D images of cells.

You need: resolution and fluorescence (Chapter 6.4).

Transmission electron microscope (TEM)

A heated filament or field-emission gun produces electrons, which are accelerated by a high voltage (typically 80 to 300 kV) down a column kept under **vacuum** (air would scatter them). Electromagnetic coils act as lenses. Electrons pass *through* a very thin sample; dense regions scatter electrons away and look dark, giving a 2D projection on a fluorescent screen or camera. For biological sections the practical resolution is a few nanometres, set by the sample, not the beam.

Sample preparation for thin-section TEM:

1. **Fixation** with glutaraldehyde (cross-links proteins), then osmium tetroxide (fixes and stains lipids).
2. **Dehydration** through rising concentrations of ethanol or acetone, since water boils in vacuum.
3. **Embedding** in epoxy resin, which hardens into a block.
4. **Ultrathin sectioning** (about 50–100 nm) on an ultramicrotome with a diamond or glass knife.
5. **Heavy-metal staining** with uranyl acetate and lead citrate, which bind membranes and nucleic acids and scatter electrons.

Other routes: **negative staining** surrounds viruses or protein complexes with heavy-metal salt so they appear light on dark; **cryo-EM** freezes samples so fast that water forms glass-like (vitreous) ice and averages thousands of images to solve protein structures (2017 Nobel Prize in Chemistry).

Scanning electron microscope (SEM)

An SEM scans a focused beam across the *surface* of the sample. Secondary electrons knocked out of the surface are collected and turned into an image with a striking 3D look, ideal for cell shape, microvilli, cilia and bacteria on a surface. Samples are fixed, dehydrated, dried by **critical-point drying** (to avoid collapse from surface tension) and coated with a thin layer of gold or another metal by sputter coating so they conduct. Resolution is usually lower than TEM, a few nanometres or more.

Confocal microscope

In a widefield fluorescence microscope the whole depth of the sample glows, so a thick specimen gives a blur. A confocal microscope (the idea patented by Marvin Minsky) focuses a **laser** to a point in the sample and places a **pinhole** in front of the detector at the plane conjugate to that focal point. Light from the focal point passes the pinhole; light from planes above and below is out of focus at the pinhole and is blocked. Scanning the point across the field builds one sharp **optical section**; moving the focus in steps gives a **z-stack** that software turns into a 3D image. The price: slower imaging and more photobleaching than widefield.

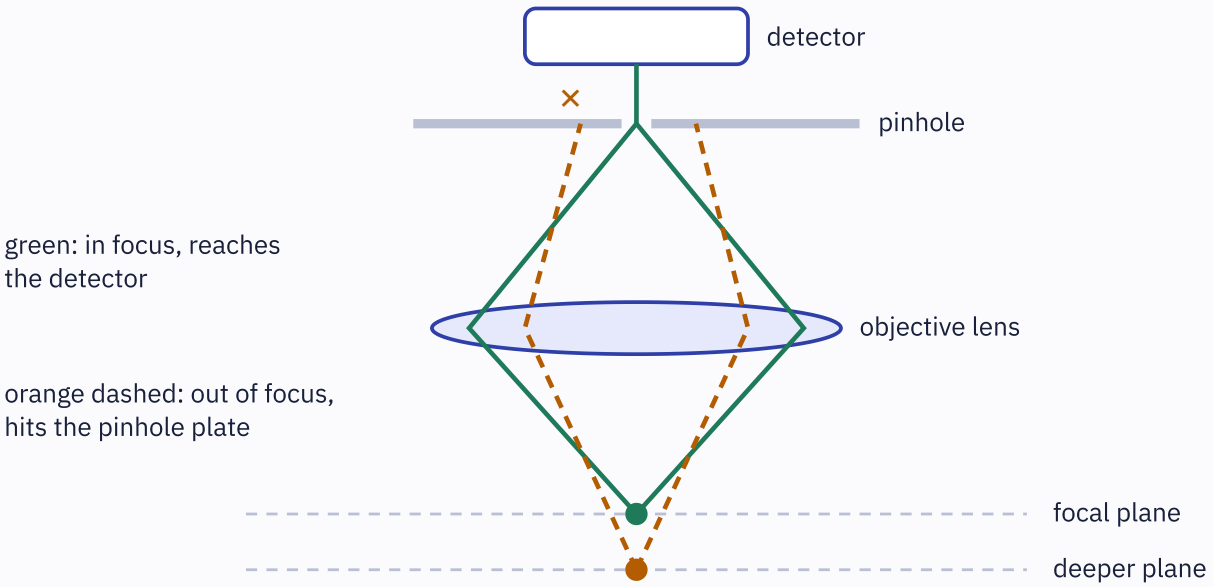


Figure 6.5.1 Why a confocal image is sharp (laser path omitted). Light from the focal plane (green) converges exactly on the pinhole and reaches the detector; light from a deeper plane (orange) converges in the wrong place and is mostly stopped by the plate.

WORKED EXAMPLE

Question (5 marks). The de Broglie wavelength of an electron accelerated through V volts is about $\lambda = 1.226 / \sqrt{V}$ nm (ignoring relativity). Find λ at 100 kV, compare it with green light (550 nm), and explain why biological TEM images do not reach this limit.

1. $\sqrt{V} = \sqrt{100{,}000} = 316.2$.
2. $\lambda = 1.226 / 316.2 =$ **0.0039 nm** (the relativistic value is a little shorter, about 0.0037 nm).
3. Ratio: $550 / 0.0039 \approx 1.4 \times 10^5$, so the electron wavelength is over 100,000 times shorter.
4. In practice, electron lenses have a small aperture and large aberrations, and biological samples are damaged by the beam, have low contrast and are limited by fixation, sectioning and stain grain. So stained sections resolve a few nanometres, still about 100 times better than the light microscope (about 200 nm).

EXAM TRAPS

- TEM: electrons pass through a thin section, 2D internal detail. SEM: surface scanning, 3D-looking surface. Do not swap them.
- Living cells cannot be seen in a conventional EM: vacuum, fixation and dehydration are required.
- The confocal pinhole sits in front of the detector, not in front of the lamp alone; it is the detector pinhole that rejects out-of-focus light.
- Electron lenses are electromagnetic coils, not glass.

CHECK YOURSELF

– REMEMBER **Name the two heavy-metal stains used for TEM sections.** 2 marks

Uranyl acetate and lead citrate (osmium tetroxide also fixes and stains lipids).

– UNDERSTAND **Why must an electron microscope column be under vacuum?** 2 marks

Gas molecules would scatter the electrons and blur or stop the beam (and the filament would oxidise).

– UNDERSTAND **Why are SEM samples coated with gold?** 2 marks

The metal makes the surface conductive, so charge from the beam drains away, and it gives a strong secondary-electron signal.

– APPLY **Choose a method: (a) shape of pollen grain surfaces; (b) cristae inside mitochondria; (c) 3D shape of a GFP-tagged Golgi in a living cell.** 3 marks

(a) SEM; (b) TEM of thin sections; (c) confocal microscopy with a z-stack.

– ANALYZE **What happens to a confocal image if the pinhole is opened very wide, and why?** 3 marks

More light reaches the detector (brighter), but out-of-focus light also passes, so the optical section gets thicker and the image blurs towards a widefield picture.

BEYOND THE SYLLABUS

Not for the exam. Cryo-electron tomography images frozen cells from many angles to see molecular machines in their native place. Volume EM (serial block-face SEM) maps the wiring of whole brain regions. Two-photon microscopes excite with infrared pulses to image deep inside living tissue. Search next: "cryo-electron tomography" and "two-photon microscopy".

6.6 Cell fractionation and isolation of organelles

Cell fractionation breaks cells open gently and separates their parts, so that nuclei, mitochondria or ER membranes can be studied in a test tube. It is how biologists proved what each organelle does: Albert Claude, Christian de Duve and George Palade shared the 1974 Nobel Prize in Physiology or Medicine for this work, which found lysosomes and peroxisomes. The method has two steps, breaking the cells (homogenisation) and sorting the pieces by centrifugation.

You need: organelle sizes and functions (Unit 3: Mitochondria; Endoplasmic reticulum; Lysosomes, peroxisomes and vacuoles).

Step 1: homogenisation

The aim is to break the plasma membrane (and the cell wall in plants or fungi) but keep organelles whole. Common methods:

- **Potter–Elvehjem or Dounce homogeniser**: a pestle turning or moving in a close-fitting glass tube; shear forces tear soft tissue such as liver.
- **Blender** for tough tissue; **sonication** (ultrasound) and the **French press** (forcing cells through a narrow valve under high pressure) for bacteria and yeast.
- **Osmotic lysis**: cells without a wall swell and burst in a hypotonic solution.
- **Enzymes**: cellulase and pectinase for plant walls, lysozyme for bacteria.

The homogenisation buffer matters as much as the force. It is **isotonic** (commonly 0.25 M sucrose; mannitol is also used), so organelles neither swell nor shrink; **buffered** near pH 7.4; **ice-cold** (0–4 °C) to slow enzymes; and often contains protease inhibitors and EDTA (which binds metal ions that activate degrading enzymes).

Step 2: differential centrifugation

In a centrifuge, a particle feels a force many times gravity. This **relative centrifugal force (RCF)**, quoted as "× g", depends on the radius r and the speed:

$$RCF = 1.118 \times 10^{-5} \times r \times (\text{rpm})^2 \qquad (r \text{ in cm})$$

Larger and denser particles sediment faster. A particle's sedimentation rate per unit field is its **sedimentation coefficient**, $s = v / (\omega^2 r)$, where v is its velocity, ω the angular speed in rad/s and r the distance from the axis; s is measured in svedbergs ($1\ S = 10^{-13}$ s). That is why ribosomes are called 70S and 80S.

Differential centrifugation spins the homogenate at a series of rising speeds. After each spin the pellet is kept and the supernatant moves to the next, faster spin. Typical values (protocols differ):

1. About 1,000 × g for 10 min: nuclei, unbroken cells and large debris pellet.
2. About 10,000–20,000 × g for 20 min: mitochondria, lysosomes and peroxisomes (and chloroplasts from plant tissue).
3. About 80,000–100,000 × g for 1 h (ultracentrifuge): **microsomes**, small vesicles formed from ER and plasma membrane during homogenisation.
4. About 150,000 × g for several hours: ribosomes and large macromolecular complexes. The final supernatant is the **cytosol**.

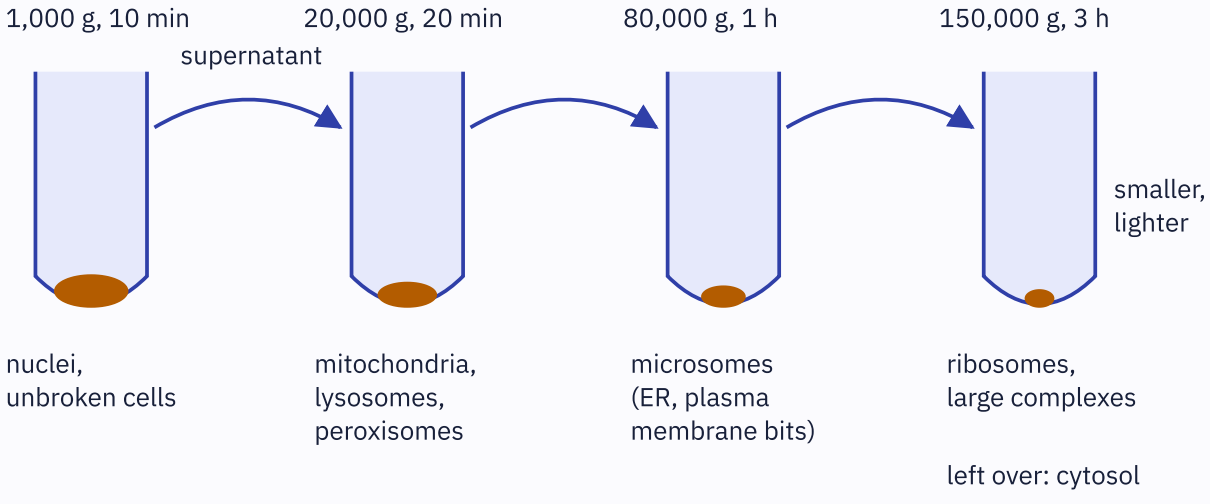


Figure 6.6.1 Differential centrifugation of a liver homogenate. Each spin is faster and longer than the one before; the pellet (orange) is kept and the supernatant moves right. Notice that pellets get smaller as the particles do, and that the 20,000 g pellet mixes three organelles of similar size.

Density-gradient centrifugation: cleaning up a fraction

Differential pellets are enriched, not pure: mitochondria, lysosomes and peroxisomes overlap in size. A **density gradient** separates them further.

- **Rate-zonal** (velocity) centrifugation: the sample is layered on a shallow gradient (for example 5–20% sucrose) and spun for a set time. Particles move as bands by size and shape (their s value); the run is stopped before they reach the bottom. Used for ribosomal subunits and polysomes.
- **Isopycnic** (equilibrium) centrifugation: a steep gradient (sucrose, Percoll or Nycodenz for organelles; caesium chloride for DNA) is spun until each particle stops at the layer whose density equals its own. Separation is by density only, not size. Peroxisomes are denser than mitochondria and band lower.

Checking the fractions: marker enzymes

Each organelle has an enzyme or molecule found almost nowhere else. Measuring it in every fraction tells you where that organelle went and how pure each fraction is.

ORGANELLE	MARKER
Nucleus	DNA
Mitochondria	Succinate dehydrogenase, cytochrome c oxidase
Lysosomes	Acid phosphatase
Peroxisomes	Catalase
Endoplasmic reticulum (microsomes)	Glucose-6-phosphatase
Golgi	Galactosyltransferase
Plasma membrane	Na^+/K^+-ATPase, 5′-nucleotidase
Cytosol	Lactate dehydrogenase

Purity is reported as **specific activity** (enzyme units per mg protein). **Enrichment** (fold purification) = specific activity in the fraction ÷ specific activity in the homogenate, and **yield** = total units in the fraction ÷ total units in the homogenate × 100%. Today, Western blots for organelle marker proteins (Chapter 6.8) are also used.

WORKED EXAMPLE 1: SETTING THE CENTRIFUGE

Question (4 marks). A rotor has a radius of 8.0 cm. What speed in rpm gives (a) 10,000 × g and (b) 100,000 × g?

1. Rearrange: rpm = √[RCF / (1.118 × 10^{-5} × r)].
2. 1.118 × 10^{-5} × 8.0 = 8.944 × 10^{-5}.
3. (a) rpm = √(10,000 / 8.944 × 10^{-5}) = √(1.118 × 10^{8}) ≈ **10,600 rpm**.
4. (b) rpm = √(100,000 / 8.944 × 10^{-5}) = √(1.118 × 10^{9}) ≈ **33,400 rpm**. Ten times the force needs only √10 ≈ 3.2 times the speed.

WORKED EXAMPLE 2: ENRICHMENT AND YIELD

Question (5 marks). A liver homogenate has 500 mg protein and 25 units of succinate dehydrogenase (SDH). The 20,000 × g pellet has 20 mg protein and 12 units of SDH. Find the specific activities, the enrichment and the yield of mitochondria. The pellet also shows high catalase activity: what does that mean?

1. Homogenate specific activity = 25 / 500 = 0.05 U/mg.
2. Pellet specific activity = 12 / 20 = 0.60 U/mg.
3. Enrichment = 0.60 / 0.05 = **12-fold**.
4. Yield = 12 / 25 × 100 = **48%**; the rest of the mitochondria were lost in other fractions or broken.
5. Catalase marks peroxisomes, which co-sediment with mitochondria. The pellet is contaminated; an isopycnic gradient would separate them.

EXAM TRAPS

- Quote centrifuge settings in × g, not rpm: the same rpm gives different forces in rotors of different radius.
- Microsomes are not an organelle in the living cell; they are vesicles made by breaking the ER and other membranes.
- Rate-zonal separates by size (stopped before equilibrium); isopycnic separates by density (run to equilibrium). This is the classic 8-mark comparison.
- In differential centrifugation the pellet is kept and the supernatant spun again; students reverse this.
- High enrichment with low yield is common and is not an error.

CHECK YOURSELF

– REMEMBER **Give the marker enzyme for lysosomes, peroxisomes and ER.** 3 marks

Acid phosphatase; catalase; glucose-6-phosphatase.

– REMEMBER **What is one svedberg?** 1 mark

10^{-13} seconds, the unit of sedimentation coefficient.

– UNDERSTAND **Why is the homogenisation buffer isotonic and ice-cold?** 3 marks

Isotonic sucrose stops organelles swelling and bursting by osmosis; the cold slows proteases, lipases and nucleases released from broken lysosomes and cytosol.

– UNDERSTAND **Why do mitochondria and peroxisomes end up in the same differential pellet, and how are they separated?** 3 marks

They are similar in size, so they sediment at similar speeds. They differ in density, so isopycnic gradient centrifugation bands them at different heights.

– APPLY **A rotor of radius 10 cm runs at 15,000 rpm. What is the RCF?** 2 marks

RCF = $1.118 \times 10^{-5} \times 10 \times (15{,}000)^2 = 1.118 \times 10^{-4} \times 2.25 \times 10^8 \approx$ **25,200 × g**.

– APPLY **A homogenate has catalase specific activity 0.2 U/mg; a gradient fraction has 3.0 U/mg. What is the enrichment?** 2 marks

3.0 / 0.2 = **15-fold**.

– ANALYZE **After homogenising liver too vigorously, acid phosphatase appears mostly in the final supernatant. Explain and suggest a fix.** 4 marks

Harsh homogenisation broke lysosome membranes, so the soluble enzyme leaked into the cytosol and stays in the supernatant instead of pelleting with lysosomes. Use fewer or gentler pestle strokes, keep the buffer isotonic and cold, and check intactness with a latency assay.

BEYOND THE SYLLABUS

Not for the exam. Organelle proteomics combines gradient fractionation with mass spectrometry: by matching each protein's profile across fractions to known markers, thousands of proteins can be assigned to organelles in one experiment ("spatial proteomics"). Immuno-isolation pulls out one organelle with antibody-coated magnetic beads for very pure samples. Search next: "spatial proteomics LOPIT" and "organelle immunoprecipitation".

Key terms of this lesson

TERM	MEANING OR FORMULA	CHAPTER
Electron wavelength	$\lambda \approx 1.226 / \sqrt{V}$ nm (non-relativistic)	6.5
RCF	$RCF = 1.118 \times 10^{-5} \times r(cm) \times rpm^2$	6.6
Enrichment and yield	Enrichment = SA fraction ÷ SA homogenate; yield = units fraction ÷ units homogenate × 100%	6.6

Glossary (terms used in this lesson)

Confocal microscope A point-scanning fluorescence microscope whose detector pinhole rejects out-of-focus light.

Differential centrifugation Spinning at rising speeds to pellet particles in order of size.

Marker enzyme An enzyme found in one organelle only, used to trace it in fractions.

Microsomes Small vesicles formed from ER and other membranes during homogenisation.

Photobleaching	Loss of fluorescence as light destroys the fluorophore.
Resolution	The smallest distance at which two points can be seen as separate.
Specific activity	Enzyme units per mg of protein; a measure of purity.

The full unit book

Every chapter of Unit 6, the unit summary, the exam question bank with model answers and the full glossary: Cell Biology · Unit 6 · Specialized Cells and Cell Study Methods (one file, works offline).

Sources and further reading

- Alberts B. et al., *Molecular Biology of the Cell*, 7th edition (2022), W. W. Norton: chapters on visualising cells, analysing cells and molecules, and the cytoskeleton (from memory; check).
- Lodish H. et al., *Molecular Cell Biology*, W. H. Freeman: chapters on culturing, visualising and fractionating cells, and on nerve cells (from memory; check).
- Karp G., *Cell and Molecular Biology: Concepts and Experiments*, Wiley: chapter on techniques in cell and molecular biology (from memory; check).
- Cooper G. M., *The Cell: A Molecular Approach*, Sinauer/Oxford University Press: chapter on tools of cell biology (from memory; check).
- Hall J. E., *Guyton and Hall Textbook of Medical Physiology*, Elsevier: chapters on membrane potentials, skeletal muscle contraction, the retina and insulin (from memory; check).
- Wikipedia, "Cell fractionation": en.wikipedia.org/wiki/Cell_fractionation (overview of homogenisation and isotonic, cold buffers).
- Abcam, "Cell fractionation: a complete overview": abcam.com knowledge center (differential and density-gradient centrifugation, purity checks by Western blot).

Syllabus: the University free n easy universal syllabus, built from top universities' syllabi.

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