Cell Biology

Unit 6 · Lesson 4 · Flow cytometry and immunostaining

The notes for Lesson 4 of Unit 6: chapters 6.7 to 6.8 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 4 of 4 | 2 chapters | Chapters 6.7 to 6.8

About these notes

These are the notes for **Lesson 4 · Flow cytometry and immunostaining** of the University free n easy video lessons on Cell Biology Unit 6, Specialized Cells and Cell Study Methods. They are chapters 6.7 to 6.8 of the unit book, unchanged, so a page here is the same page in the full book.

6.7 Flow cytometry

A flow cytometer pushes cells one at a time through a laser beam and measures, for each cell, how it scatters light and how brightly it glows in several colours. It reads thousands of cells per second, so instead of looking at a few cells under a microscope you get numbers for a whole population: what percentage are CD4 T cells, how many are dividing, how many are dying. A cell sorter (FACS) can then separate the cells you want.

You need: fluorescence and fluorophores (Chapter 6.4); apoptosis and the cell cycle (Unit 4).

The three systems

1. **Fluidics.** The cell suspension is injected into a faster stream of sheath fluid. **Hydrodynamic focusing** squeezes the sample into a narrow core, so cells pass the laser in single file.
2. **Optics.** One or more lasers (488 nm blue is the classic) hit each cell. Light scattered at small angles along the beam is **forward scatter (FSC)**, which rises with cell size. Light scattered at about 90° is **side scatter (SSC)**, which rises with internal complexity or granularity. Fluorescence from dyes or labelled antibodies is split by dichroic mirrors and filters into colour channels.

3. **Electronics.** Photodetectors (photomultiplier tubes or photodiodes) turn each flash into a voltage pulse. The pulse height, area and width are digitised for every channel of every cell (each cell is an "event").

Reading the data: gates and plots

Data are shown as **histograms** (one parameter: number of cells against intensity) or **dot plots** (two parameters, one dot per cell). A **gate** is a region you draw to keep only some events: first FSC against SSC to keep intact cells and drop debris, then a pulse area against width plot to drop doublets (two cells stuck together), then the fluorescence of interest. Because dye emission spectra overlap, part of one dye's signal leaks into the next channel; **compensation** subtracts that leak using single-stained controls. Blood is the classic example: on FSC against SSC, lymphocytes (small, smooth), monocytes (larger) and granulocytes (high SSC from granules) form three separate clouds.

Common uses

- **Immunophenotyping**: fluorescent antibodies against surface CD markers count cell types; CD4 T-cell counts guide HIV care, and marker panels classify leukaemias.
- **Cell cycle**: a DNA dye such as propidium iodide (PI) on fixed cells gives a histogram with a G1 peak (2N DNA), a G2/M peak at twice the intensity (4N) and S phase in between.
- **Apoptosis**: annexin V binds phosphatidylserine, which flips to the outer leaflet early in apoptosis; PI enters only cells whose membrane has broken.
- **Sorting (FACS)**: the stream is vibrated into droplets; a droplet holding a wanted cell is given a charge and deflected by charged plates into a collection tube.

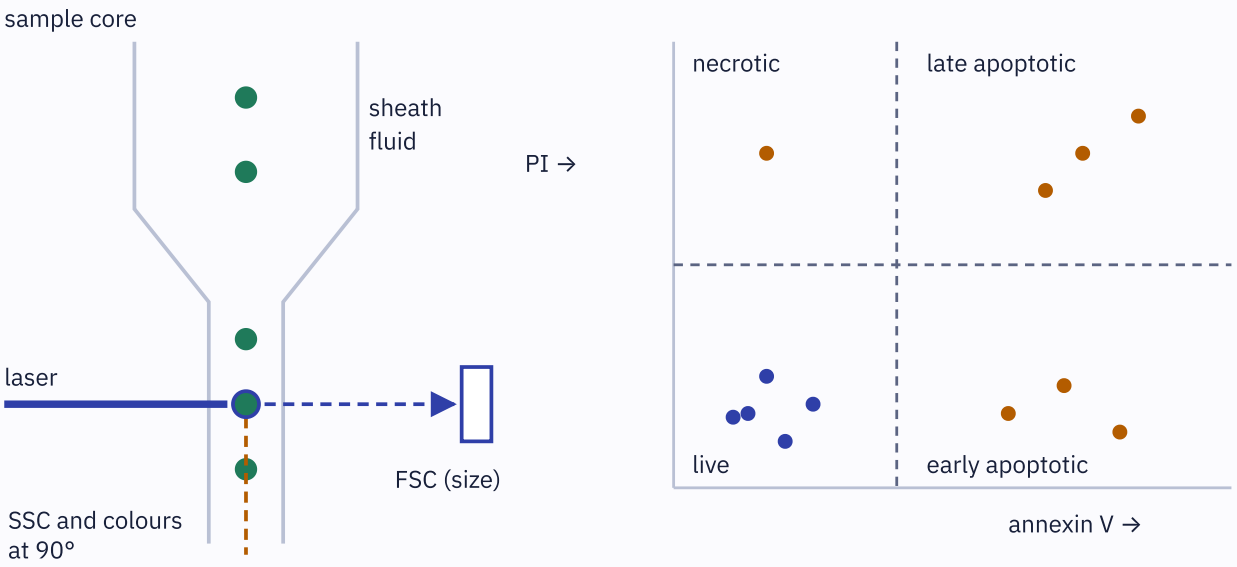


Figure 6.7.1 Left: hydrodynamic focusing lines cells up in single file; each cell crossing the laser sends scattered light forward (FSC) and sideways (SSC, plus fluorescence). Right: an annexin V against PI dot plot; each dot is one cell, and the quadrant it falls in tells you its state.

WORKED EXAMPLE

Question (5 marks). Cancer cells are treated with a drug and stained with annexin V–FITC and PI. Of 20,000 gated events: 14,000 are annexin$^-$ PI$^-$, 3,000 annexin$^+$ PI$^-$, 2,400 annexin$^+$ PI$^+$ and 600 annexin$^-$ PI$^+$. Give the percentage in each state and the total apoptotic fraction.

1. Live (annexin$^-$ PI$^-$): 14,000 / 20,000 = **70%**.
2. Early apoptotic (annexin$^+$ PI$^-$: PS exposed, membrane intact): 3,000 / 20,000 = **15%**.
3. Late apoptotic or secondary necrotic (annexin$^+$ PI$^+$): 2,400 / 20,000 = **12%**.
4. Necrotic or damaged (annexin$^-$ PI$^+$): 600 / 20,000 = **3%**.
5. Total apoptotic = 15 + 12 = **27%**. Compare with an untreated control before saying the drug caused it.

EXAM TRAPS

- FSC relates to size, SSC to granularity or internal complexity; not the other way round.
- Flow cytometry gives no image of the cell (imaging flow cytometers aside); it gives numbers per cell.
- FACS means fluorescence-activated cell sorting; analysis alone is flow cytometry.
- Annexin V binds phosphatidylserine on the outside of the cell; PI marks broken membranes, not apoptosis itself.

CHECK YOURSELF

– REMEMBER **What do FSC and SSC measure?** 2 marks

FSC: roughly cell size. SSC: internal complexity or granularity.

– UNDERSTAND **What is hydrodynamic focusing and why is it needed?** 3 marks

The sample stream is injected into faster sheath fluid, which narrows it to a thin core so cells pass the laser one at a time; otherwise two cells would be read as one event.

– UNDERSTAND **Why is compensation needed in multicolour experiments?** 2 marks

Emission spectra overlap, so part of one dye's light is detected in another dye's channel; compensation subtracts that spillover.

– APPLY **In a PI cell-cycle histogram the G1 peak is at intensity 200. Where is the G2/M peak, and where are S-phase cells?** 2 marks

G2/M at about **400** (twice the DNA); S-phase cells lie between 200 and 400.

– ANALYZE **Why do you gate on FSC against SSC before looking at fluorescence?** 3 marks

Debris, dead fragments and clumps also give fluorescence events. Gating on intact single cells by size and complexity first removes them, so percentages refer to real cells.

BEYOND THE SYLLABUS

Not for the exam. Spectral flow cytometers record each cell's whole emission spectrum and unmix 30 or more colours. Mass cytometry (CyTOF) labels antibodies with metal isotopes and reads them by mass spectrometry. In CAR-T cell therapy, flow cytometry checks every manufactured batch. Search next: "spectral flow cytometry" and "mass cytometry CyTOF".

6.8 Immunostaining and protein localization

Immunostaining uses antibodies to find one protein among thousands in a fixed cell or tissue and make it visible, by a fluorescent dye (immunofluorescence) or a coloured enzyme product (immunohistochemistry). It answers the question "where in the cell is my protein?": in the nucleus, on mitochondria, at the plasma membrane. Protein localisation also uses GFP fusions in live cells and fractionation with Western blots.

You need: fluorescence microscopy (Chapter 6.4), confocal microscopy (Chapter 6.5) and cell fractionation (Chapter 6.6).

Antibodies as probes

An IgG antibody is a Y-shaped protein of two heavy and two light chains. The two tips (the **Fab** arms) bind one small region of the target, the **epitope**; the stem (the **Fc** region) is the same for all antibodies of one species and class. A **monoclonal** antibody comes from one B-cell clone and binds one epitope; a **polyclonal** serum is a mixture binding several epitopes, which gives a stronger but less defined signal.

- **Direct** staining: the primary antibody itself carries the label. Fewer steps, weaker signal.
- **Indirect** staining: an unlabelled primary antibody (say, raised in rabbit) binds the target; a labelled **secondary** antibody (goat anti-rabbit IgG) binds the primary's Fc. Several secondaries bind each primary, so the signal is amplified, and one labelled secondary works with any rabbit primary.

The immunofluorescence protocol

1. **Fix**: 4% paraformaldehyde cross-links proteins and holds them in place; cold methanol instead precipitates proteins and also permeabilises.
2. **Permeabilise** (after aldehyde fixation): a detergent such as Triton X-100 or saponin opens membranes so antibodies (about 150 kDa) reach inside.
3. **Block** with BSA or serum from the secondary's host species, which coats sticky sites and cuts background.
4. **Primary antibody**, diluted (often 1:100 to 1:1000), usually 1 h at room temperature or overnight at 4 °C; wash.
5. **Secondary antibody** with a fluorophore, in the dark; wash.

6. **Counterstain** nuclei with DAPI, mount in antifade medium and image by fluorescence or confocal microscopy.

Controls make the result believable: a **secondary-only** control (no primary) shows background; an **isotype** control for monoclonals; and the best test of specificity, cells where the gene is knocked out or knocked down, which should lose the signal.

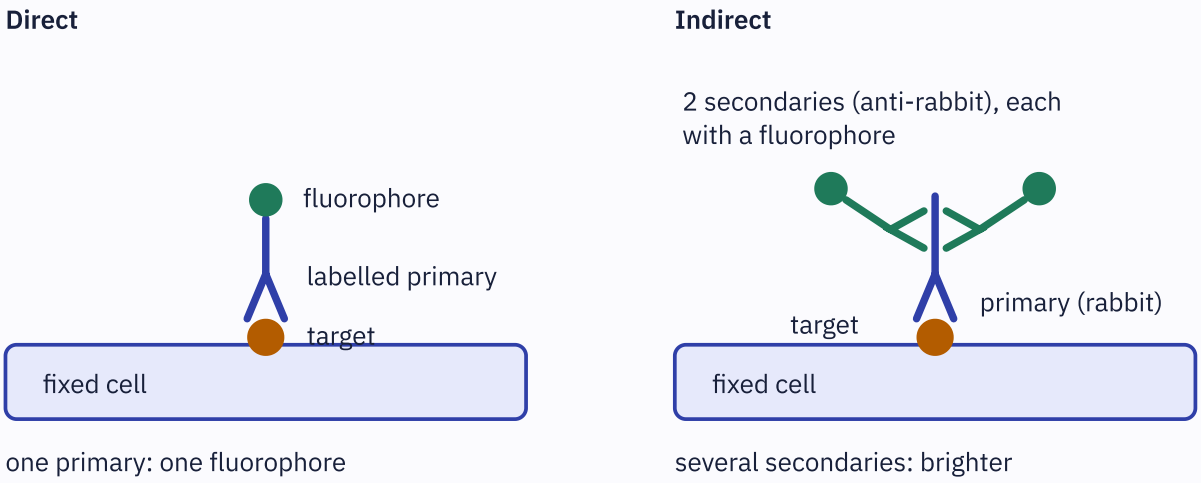


Figure 6.8.1 Direct against indirect immunostaining (antibodies drawn as Y shapes, not to scale). Each antibody grips its target with the tips of its arms. In the indirect method labelled secondary antibodies grip the stem (Fc) of the primary, which amplifies the signal.

Immunohistochemistry and histochemical staining

In **immunohistochemistry (IHC)**, used in pathology on tissue sections, the secondary antibody carries an enzyme, usually horseradish peroxidase (HRP). HRP with its substrate DAB (diaminobenzidine) and hydrogen peroxide gives a brown precipitate where the target is; the section is counterstained with haematoxylin (nuclei blue) and viewed in bright-field. Breast cancer biopsies are routinely stained this way for oestrogen receptor and HER2. Plain **histochemical stains** work without antibodies: haematoxylin and eosin (H&E) shows nuclei purple-blue and cytoplasm pink; periodic acid–Schiff (PAS) stains glycogen and mucins magenta.

Finding where a protein lives

Three methods support one another:

- **Co-localisation** with organelle markers: stain your protein green and a known marker red, for example TOM20 (mitochondria), calnexin (ER), GM130 (Golgi) or LAMP1 (lysosomes). Overlap shows yellow in a merged image, and a correlation value such as Pearson's coefficient (from −1 to +1) puts a number on it. Use confocal images, since widefield blur creates false overlap.

- **Fluorescent protein fusions**: fuse GFP to your protein's gene and watch it in living cells, including how it moves. Check that the tag does not hide a targeting signal, such as an N-terminal mitochondrial presequence.
- **Fractionation and Western blot**: separate nuclear, mitochondrial, membrane and cytosolic fractions (Chapter 6.6), run each on a gel and probe with your antibody plus marker antibodies.

WORKED EXAMPLE 1: DESIGN AN EXPERIMENT

Question (8 marks). A new human protein X is suspected to sit in mitochondria. You have a rabbit anti-X antibody. Design an experiment to test this and say what result would support it.

1. Grow cells on coverslips; fix in 4% paraformaldehyde for 15 min; permeabilise with 0.1% Triton X-100; block with 5% goat serum.
2. Primary antibodies together: rabbit anti-X and mouse anti-TOM20 (mitochondrial outer membrane marker).
3. Secondaries: goat anti-rabbit with a green dye; goat anti-mouse with a red dye. Counterstain DAPI.
4. Controls: secondaries only; cells with X knocked down by siRNA.
5. Image by confocal microscopy in separate channels to avoid bleed-through.
6. Support: green and red overlap in thread-like networks (yellow in the merge), Pearson's coefficient near +1, and the signal falls after knockdown. Confirm by fractionation: X should appear in the mitochondrial fraction with TOM20 in a Western blot, not with the cytosolic marker LDH.

WORKED EXAMPLE 2: DILUTING AN ANTIBODY

Question (2 marks). You need 5 mL of primary antibody at 1:500. How much stock and how much blocking buffer?

1. Stock volume = 5 mL ÷ 500 = 0.010 mL = **10 µL**.
2. Buffer = 5,000 µL − 10 µL = **4,990 µL**.

EXAM TRAPS

- The secondary antibody binds the primary antibody's Fc region, not the target protein.
- Aldehyde-fixed cells need a separate permeabilisation step; methanol fixation already permeabilises.
- The secondary must be raised against the primary's host species: a rabbit primary needs an anti-rabbit secondary.
- Overlap in a widefield image is weak evidence; confocal sections plus a number (Pearson's) and a second method are expected.
- Immunofluorescence uses fluorophores; IHC usually uses HRP–DAB and a bright-field microscope.

CHECK YOURSELF

– REMEMBER **Name the substrate that HRP turns into a brown precipitate in IHC.** 1 mark

DAB (3,3′-diaminobenzidine), with hydrogen peroxide.

– REMEMBER **Give one marker protein each for mitochondria, ER and Golgi.** 3 marks

TOM20 (mitochondria), calnexin (ER), GM130 (Golgi).

– UNDERSTAND **Why does indirect staining give a brighter signal than direct staining?** 2 marks

Several labelled secondary antibodies bind each primary antibody, so each target carries more fluorophores.

– UNDERSTAND **What is the purpose of the blocking step?** 2 marks

Proteins such as BSA or serum coat non-specific binding sites, so antibodies stick only to their epitope and background falls.

– APPLY **A secondary antibody is used at 1:1000 in 20 mL. How much stock?** 1 mark

20 mL ÷ 1000 = **20 µL** in 19.98 mL buffer.

– APPLY **You see bright staining in the secondary-only control. What does it mean and what will you change?** 3 marks

The secondary binds non-specifically, so the real staining cannot be trusted. Block longer or with serum of the secondary's host, dilute the secondary more, wash more, or change the secondary.

– ANALYZE **A GFP fusion of protein Y shows cytosol, but antibody staining shows mitochondria. Give two explanations.** 4 marks

(1) GFP on the N-terminus masked the mitochondrial targeting presequence, so the fusion stays in the cytosol; try a C-terminal tag. (2) The antibody cross-reacts with a mitochondrial protein; test it in knockout cells and by Western blot of fractions.

BEYOND THE SYLLABUS

Not for the exam. The Human Protein Atlas has mapped the subcellular location of most human proteins by immunofluorescence and makes the images free online. Proximity labelling (BioID, APEX) tags neighbouring proteins with biotin in living cells to map compartments by mass spectrometry. Multiplexed imaging stains 40 or more proteins on one tissue section, now used in cancer research. Search next: "Human Protein Atlas subcellular" and "proximity labelling APEX".

Key terms of this lesson

TERM	MEANING OR FORMULA	CHAPTER
FSC and SSC	Forward scatter ≈ size; side scatter ≈ granularity	6.7
Indirect immunostaining	Unlabelled primary + labelled secondary against the primary's Fc	6.8

Glossary (terms used in this lesson)

Annexin V A protein that binds phosphatidylserine exposed on the outer surface of apoptotic cells.

Compensation Correction for one dye's light spilling into another dye's detector in flow cytometry.

Dichroic mirror A mirror that reflects short wavelengths and transmits longer ones.

Epitope The small part of an antigen that an antibody binds.

FACS Fluorescence-activated cell sorting: a flow cytometer that also separates cells.

Immunohistochemistry	Antibody staining of tissue sections, usually with an enzyme such as HRP that makes a coloured product.
Secondary antibody	A labelled antibody that binds the Fc region of a primary antibody.
Side scatter (SSC)	Light scattered at about 90°, reflecting a cell's internal complexity.

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.

Built for the University free n easy YouTube channel from the universal syllabus of Cell Biology (Oct 2026). Written with top universities' syllabi and standard textbooks; check a formula against your prescribed book before an exam, and report mistakes on the channel.