

Unit 5: Cell Culture Techniques and Biosimilars

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5.1 The cell culture laboratory: basic equipment

Cell culture keeps living cells growing outside the body, which demands sterility, stable warmth and gas, and gentle handling. The basic equipment follows from those demands:

Equipment	Purpose
Class II biosafety cabinet (laminar airflow)	Sterile working space; filtered air protects both the culture and the worker
CO2 incubator	Holds cultures at 37 degrees C with 5 percent CO2 and high humidity; the CO2 sets the pH of bicarbonate buffered media
Inverted microscope	Examines cells growing on the base of flasks and plates from below, without opening them
Centrifuge	Pellets cells for media changes, isolation and subculture
Autoclave	Sterilises media components, glassware and waste with pressurised steam
Water bath	Warms media and thaws frozen cells gently and quickly
Refrigerators, minus 80 freezer and liquid nitrogen storage	Staged cold storage, ending at minus 196 degrees C for the cell bank
Haemocytometer and cell counter	Counts cells and checks viability for seeding and experiments
pH meter, balance, sterile filtration units	Media preparation and quality control

5.2 Cell culture media

A culture medium must replace blood: salts for osmotic balance, glucose and amino acids as fuel and building blocks, vitamins and trace elements, a buffering system, and usually a growth supplement. The standard basal media are named recipes, **MEM**, **DMEM** and **RPMI-1640** among them, differing in richness and intended cell types (RPMI favours blood and suspension cells, DMEM adherent lines). Buffering is by bicarbonate in equilibrium with the incubator's CO2, and the pH indicator **phenol red** turns the medium into a visible gauge: pink at the working pH, yellow as growing cells acidify it, purple if it turns alkaline. The decisive supplement is **serum**, usually fetal bovine serum at 5 to 10 percent, supplying growth factors, hormones, attachment factors and carrier proteins; its undefined composition and batch variation are also the medium's weakest point, driving the move to serum free and chemically defined media for production work. Glutamine is added fresh or in stabilised form because it degrades in storage. Antibiotics such as penicillin and streptomycin are available but good laboratories use them sparingly: they mask low grade contamination instead of preventing it.

5.3 Types of cell culture

Cultures divide first by origin. A **primary culture** is taken directly from tissue (section 5.5): the cells keep much of their specialised character but divide a limited number of times before senescing. An **established or continuous cell line** has escaped that limit, spontaneously or by transformation, and divides indefinitely: HeLa, from a human cervical carcinoma in 1951, was the first and remains the most used; the pharmacologist's working set includes CHO cells (production and toxicology), HEK293 (transfection and

channel expression, host of the hERG safety assay), HepG2 (liver metabolism models) and Caco-2 (intestinal absorption models). Cultures divide again by habit: **adherent** cells must attach to a treated surface and grow as a monolayer, while **suspension** cells, mostly of blood origin, grow floating. **Organ culture** keeps a tissue fragment's architecture for short studies, and the modern extensions, three dimensional spheroids and organoids, restore some structure that flat monolayers lose.

5.4 The general procedure for cell culture

The general procedure is a discipline more than a recipe, built on **aseptic technique**: work in the cabinet with surfaces and gloved hands disinfected with 70 percent alcohol, flame or filter sterilise what enters, minimise the time vessels stand open, and never let a pipette pass over an open sterile vessel. The working rhythm runs:

A WORKING DAY IN THE CELL CULTURE LAB

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observe cultures under the inverted microscope
(confluence, morphology, contamination check)
|
warm fresh medium and reagents to 37 degrees C
|
feed: replace spent medium, or subculture if confluent (5.6)
|
return vessels to the incubator, record passage and observations
|
experiments seeded from counted, healthy, log phase cells
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Only healthy cells in the logarithmic growth phase give reproducible assays; an overconfluent, starved or quietly mycoplasma contaminated culture will fail an experiment weeks before the failure is traced back to it.

5.5 Isolation of cells

Isolation converts a tissue into a suspension of its cells. The tissue is washed free of blood in sterile balanced salt solution, minced finely to increase surface area, and then disaggregated. **Enzymatic digestion** is the standard route: trypsin breaks the protein links between cells, collagenase digests the collagen scaffold of solid tissues, and dispase gives a gentler release; enzymes are stopped with serum containing medium once the tissue softens. A mechanical route (sieving, gentle pipetting) suits soft tissues such as spleen and liver. The suspension is filtered to remove debris, centrifuged, washed and resuspended in complete medium, and counted (section 5.6) before seeding. Cells then sort themselves in part: in a mixed primary culture the adherent cells attach within hours while blood cells float, and differential attachment is itself a first purification.

5.6 Subculture: passaging the culture

Subculture, or passaging, is the routine transfer of cells into fresh vessels that keeps a culture alive and expanding. Adherent cells are passaged when they reach about 70 to 80 percent confluence, before crowding stops their growth: the medium is removed, the layer is washed with phosphate buffered saline, and **trypsin EDTA** is added briefly; the protease cuts the attachment proteins and the EDTA chelates the calcium adhesion depends on, so the cells round up and float free. Serum containing medium stops the enzyme, the suspension is centrifuged and counted, typically on a haemocytometer with trypan blue to exclude dead cells, and a defined fraction is seeded into new vessels (the split ratio, or an exact seeding density where the assay demands it). Suspension cultures are simpler: dilute or centrifuge and reseed. Every passage is recorded with its number, because cells drift in growth rate, karyotype and drug sensitivity with passage count, and an experiment done at passage 40 is not the same experiment done at passage 8.

5.7 Cryopreservation

Cryopreservation banks cells against loss, contamination and drift. Healthy log phase cells are suspended in freezing medium, standardly complete medium with about 10 percent **dimethyl sulfoxide (DMSO)**, a cryoprotectant that limits the ice crystal formation which would otherwise shred membranes. The vials are cooled slowly, about one degree per minute in an insulated freezing container, to minus 80 degrees, then transferred to liquid nitrogen at minus 196 degrees, where biological time effectively stops. Banks are organised as a small master stock and a larger working stock, so the master vials are rarely disturbed. Revival inverts the logic: thaw fast in a 37 degree water bath, because slow warming lets crystals regrow, dilute the DMSO away at once (it is toxic at room temperature), wash, seed into fresh medium, and confirm viability and identity before the cells are trusted.

5.8 Characterisation of cells and their application

A culture is only useful when its identity and health are proven, so **characterisation** runs on a checklist: **morphology** under the microscope (shape, growth pattern, the look of a healthy culture of that line); **growth characterisation**, the growth curve and doubling time; **viability** (section 5.9); **karyotyping** to confirm the chromosome complement has not drifted; identity testing, in human lines by short tandem repeat profiling, which exists because cross contamination between lines is a historically common and embarrassing error; marker expression, confirming the cell still displays the proteins of its tissue of origin, by immunostaining or flow cytometry (section 5.11); and sterility testing, including the PCR based hunt for **mycoplasma**, the invisible contaminant that alters cell behaviour without clouding the medium. The **applications** in pharmacology justify the whole apparatus: screening compounds for cytotoxicity and activity, expressing a single receptor or channel for clean drug testing (the hERG assay on which cardiac safety screening depends), modelling absorption and metabolism with Caco-2 and liver cells before animal studies, producing recombinant medicines (Unit 3.10), and replacing animal experiments at the earliest stages of drug evaluation.

5.9 Cell viability assays: principles and applications

A **viability assay** reports how many cells in a culture are alive, or behaving as if alive, after treatment, and it is the readout behind most cell based drug screening. The assays measure different definitions of alive, and the syllabus expects the principles distinguished:

Assay	Principle	What it really measures
Trypan blue exclusion	The dye cannot cross an intact membrane; dead cells stain blue	Membrane integrity, counted manually on a haemocytometer
MTT assay	Living cells' dehydrogenases reduce the yellow tetrazolium salt MTT to purple formazan crystals, dissolved and read as absorbance	Metabolic activity as a proxy for viable cell number
Resazurin (Alamar Blue)	Living cells reduce blue resazurin to pink, fluorescent resorufin	Metabolic activity, read without killing the cells, so it can be repeated
ATP luminiscence	Luciferase light output is proportional to ATP present	The energy charge of the culture; the most sensitive format
LDH release	Lactate dehydrogenase leaking from damaged cells is measured in the medium	Membrane damage (a death readout, complementary to the others)

The **applications** are the daily currency of screening: cytotoxicity ranking of anticancer candidates and IC50 determination, safety testing of new compounds on normal cells, and quality control of cultures themselves. The critical skill is caveat recognition: the MTT assay reports metabolism, so a drug that

sedates mitochondria without killing cells falsifies it, coloured test compounds can absorb at the reading wavelength, and the honest experiment confirms a hit with a second assay built on a different principle.

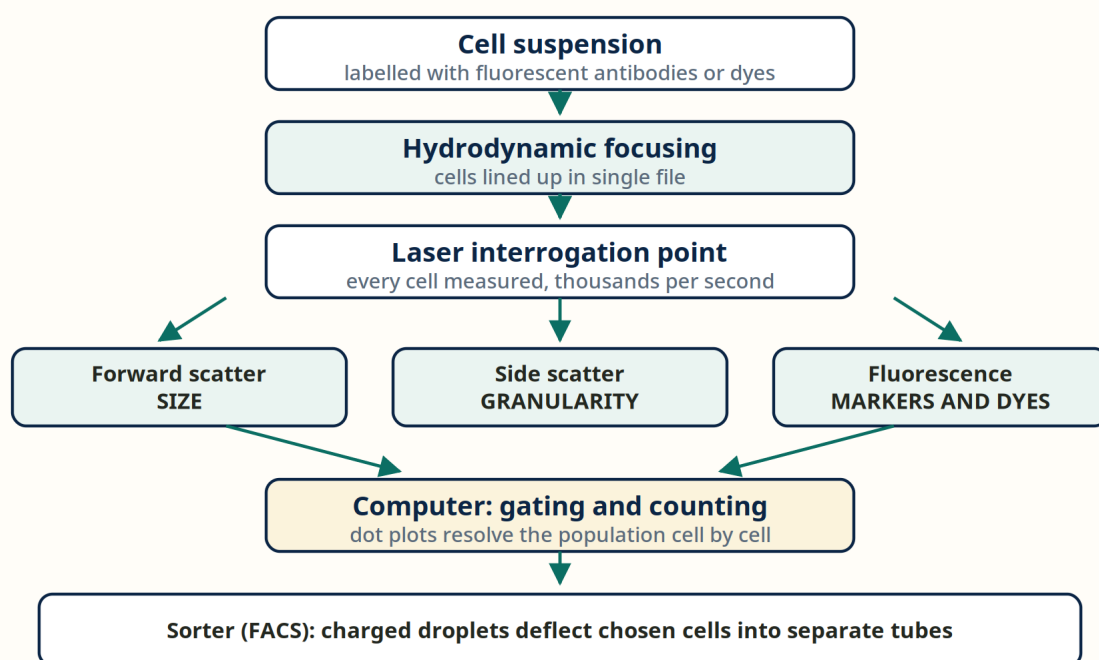
5.10 Glucose uptake and calcium influx assays

Two functional assays extend the laboratory from counting cells to watching pathways work. The **glucose uptake assay** measures transport of glucose into cells, classically in insulin responsive models, the adipocyte line 3T3-L1 and the muscle line L6. The tracer is either radiolabelled 2-deoxyglucose, which enters through the glucose transporters and is trapped inside after phosphorylation, or the fluorescent analogue 2-NBDG, read on a plate reader. Insulin sharply raises uptake by moving GLUT4 transporters to the surface (Unit 2.5's pathway, measured end to end), and the assay, with cytochalasin B as the transporter blocking control, screens for insulin sensitising and antidiabetic compounds and probes the mechanism of insulin resistance in cells made resistant by chronic insulin or fatty acid exposure. The **calcium influx assay** watches the messenger of Unit 2.9 in real time: cells are loaded with a calcium sensitive fluorescent dye (Fura-2, whose signal is ratiometric and self calibrating, or the brighter single wavelength Fluo-4, both entering as membrane permeant ester forms) and fluorescence reports cytosolic calcium second by second. Run on a fluorescence plate reader of the FLIPR type, it turns Gq coupled receptor activation and ion channel opening into a high throughput signal, and it is one of the standard primary screens of industrial drug discovery for GPCR and channel targets.

5.11 Flow cytometry: principles and applications

Flow cytometry measures cells one at a time, thousands per second, as they stream past lasers. The **fluidics** system uses hydrodynamic focusing to line the cells up in single file. The **optics** then record, for every cell, **forward scatter** (roughly, size), **side scatter** (roughly, internal granularity) and the fluorescence of any labels the cell carries: antibodies tagged with fluorochromes against surface markers, DNA dyes, or functional probes. Software plots the population, and the analyst **gates**, selecting subpopulations by their scatter and fluorescence to count and characterise them. In the sorting form (FACS), droplets containing single chosen cells are charged and deflected into separate tubes, physically purifying the selected population.

Flow Cytometry: Principle and Layout



Not an average but a census: every cell counted and classified on its own evidence.

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Figure: Flow cytometry in one pass. Single file cells, laser interrogation, scatter for physical character, fluorescence for identity, and, in the sorter, physical separation of the cells the gates select.

The **applications** in pharmacology and medicine are wide: immunophenotyping, counting CD4 T cells in HIV care and classifying leukaemias and lymphomas by their marker patterns; apoptosis measurement with Annexin V (which binds the phosphatidylserine that apoptotic cells expose, Unit 1.9) paired with a viability dye; cell cycle analysis from DNA content staining, reading out the phase distribution of Unit 1.7 and the effect of cycle active drugs; and the characterisation work of section 5.8. Its strength is statistical: it does not average a culture, it censuses it.

5.12 Biosimilars

Unit 5 closes with the products that the technologies of Units 3 and 4 created. A **biologic** medicine, an antibody, insulin, growth factor or vaccine, is made by living cells, is large and structurally complex, and carries the manufacturer's fingerprints in its glycosylation and folding: the process defines the product in a way that never applies to a small molecule. A **biosimilar** is a biologic shown to be **highly similar** to an already licensed reference product, with no clinically meaningful differences in quality, safety or efficacy. It is therefore not a generic: a generic small molecule is chemically identical to its reference and proves itself by bioequivalence, while a biosimilar can only be shown similar, through a stepwise **comparability exercise** that starts with extensive analytical and functional comparison (the tools of Unit 3) and confirms with comparative pharmacokinetic, pharmacodynamic and immunogenicity studies in patients. Regulation reflects this: the European agency built the first dedicated pathway, India regulates these products as **similar biologics** under guidelines issued by the CDSCO and the Department of Biotechnology (2012, revised 2016), and the United States reserves the additional label of interchangeable for products shown to support switching. Biosimilars of filgrastim, insulin glargine, trastuzumab, rituximab and adalimumab are now routine, several of them developed and manufactured in India. Their value is the value of competition: lower prices and wider access to the immunotherapeutics of Unit 4, guarded by pharmacovigilance and

traceability, since a similar product is a distinct product and must be tracked as one.

Frequently Asked Questions

Q: What separates the intrinsic from the extrinsic pathway of apoptosis, and where do they meet?

The intrinsic pathway is started from inside the cell, by damage, stress or withdrawal of growth signals, and runs through the mitochondria: Bax and Bak open the outer mitochondrial membrane, cytochrome c forms the apoptosome with Apaf-1, and caspase 9 is activated. The extrinsic pathway is started from outside, by death ligands such as Fas ligand and TNF binding their surface receptors, and activates caspase 8 at the death inducing signaling complex. They meet at the executioner caspase 3, and they are cross connected, because caspase 8 also cuts Bid to tBid, which engages the mitochondrial pathway and amplifies the signal.

Q: How do siRNA and microRNA differ, given that both silence genes? Both are short RNAs loaded into the RISC complex, but siRNA comes from long double stranded RNA (or is supplied synthetically), matches its single target messenger RNA perfectly, and directs Argonaute 2 to slice and destroy that message. microRNA is encoded by the cell's own genes, is processed from a hairpin precursor by Drosha and Dicer, matches its targets only partially, chiefly in the 3 prime untranslated region, and mainly represses translation and promotes decay; one microRNA therefore tunes many messages at once. siRNA is the precision tool and the drug (patisiran, inclisiran), microRNA is the cell's own fine tuning system.

Q: Why does the JAK/STAT pathway need no second messenger, and why does that matter for drugs? Cytokine receptors carry no enzyme of their own; the JAK kinases attached to their cytoplasmic tails do the work. Ligand binding activates the JAKs, which phosphorylate the receptor and then the STAT transcription factors, and the phosphorylated STATs dimerise and go straight to the nucleus. Receptor and gene are therefore only three proteins apart, the shortest signaling route in the subject. It matters because the borrowed kinase is a single druggable bottleneck for many cytokines at once: JAK inhibitors such as ruxolitinib and tofacitinib block a whole family of signals with one small molecule, which explains both their broad efficacy in immune disease and their broad adverse effect burden.

Q: RT-PCR and real time PCR sound like the same thing. Are they? No, and the similarity of the names is a standard examination trap. RT-PCR means reverse transcription PCR: a first step in which reverse transcriptase converts an RNA template into complementary DNA, without which RNA targets (RNA viruses, gene transcripts) could not be amplified at all. Real time PCR means the amplification is monitored as it happens by fluorescence, giving a Ct value that quantifies the starting template. The two combine freely: viral load tests for HIV and SARS-CoV-2 are reverse transcription real time PCR, doing both jobs in one run.

Q: Why can the same dose of codeine be harmless in one patient, useless in a second and fatal in a third? Codeine itself is nearly inactive; it must be converted to morphine by CYP2D6, a highly polymorphic enzyme. Poor metabolisers make little morphine, so the drug simply fails. Normal metabolisers make the expected amount. Ultrarapid metabolisers, who carry extra copies of the CYP2D6 gene, convert so fast that morphine levels spike into the toxic range, causing respiratory depression that has killed adults and breastfed infants of ultrarapid mothers. Identical prescription, three different drugs in practice: this is the founding clinical example of pharmacogenomics.

Q: What does it mean to call an antibody humanised, and why is it worth the engineering? A mouse monoclonal antibody is read as foreign protein by the human immune system, which raises anti mouse antibodies that neutralise the drug and cause reactions on repeat dosing. A chimeric antibody replaces the constant regions with human ones; a humanised antibody keeps only the mouse binding loops (the

complementarity determining regions) grafted into a human framework, leaving about 5 to 10 percent mouse sequence; a fully human antibody has none. Each step cuts immunogenicity and extends usable life in the patient, which is why the stems in antibody names (-xi-, -zu-, -u-) record how far the engineering went.

Q: ELISA and western blotting both use antibodies. When is each the right tool? ELISA is the bulk measurement tool: fast, quantitative against standards, run in 96 well plates on hundreds of samples, but it reports only that something bound, in aggregate. Western blotting is the verification tool: the sample is first separated by size on SDS-PAGE, so the antibody signal comes with a molecular weight attached, confirming that the band is the right protein and revealing cut or modified forms. In practice ELISA screens and quantifies, western blotting confirms identity, and strong work uses each to police the other.

Q: How does the cell cycle explain the scheduling and toxicity of cancer chemotherapy? Most cytotoxic drugs kill cells in a particular phase: antimetabolites in S phase, vinca alkaloids and taxanes in M phase. A tumour's cells are not synchronised, so a single dose catches only the fraction currently in the sensitive phase, which is why chemotherapy is given in repeated cycles as more cells cycle through. The same logic predicts the toxicity pattern: the normal tissues that cycle fastest, bone marrow, gut lining and hair follicles, are hit hardest, producing myelosuppression, mucositis and alopecia. Checkpoint aware drugs such as the CDK4/6 inhibitors attack the control system itself, arresting cells in G1.

Q: The MTT assay is called a viability assay, but it does not count dead cells. What does it actually report? It reports metabolic activity. Living cells' dehydrogenases reduce the yellow MTT salt to purple formazan, so the colour intensity tracks active metabolism in the well, which under controlled conditions is proportional to viable cell number. The distinction matters: a drug that suppresses mitochondrial activity without killing cells reads as cytotoxic when it is not, and coloured or reducing compounds interfere directly. A viability claim from MTT alone is a proxy claim, and screening practice confirms hits with an assay on a different principle, such as ATP luminescence or membrane integrity.

Q: Why is a biosimilar not simply called a generic biologic? Because identity cannot be shown. A generic small molecule is the same chemical as its reference and needs only bioequivalence. A biologic is made by living cells, is far larger and more complex, and differs in fine details such as glycosylation between manufacturers and even between processes, so a follow on product can be demonstrated highly similar, through analytical, functional and clinical comparability studies, but never identical. The different name carries a different evidence standard, different regulation (similar biologics in India), and different clinical handling, including traceability of which product a patient received.

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