Methods and Techniques Course 2020

Cell Culture

Louise Johnson

MRC Human Immunology Unit

Thursday, 5th November, 2020

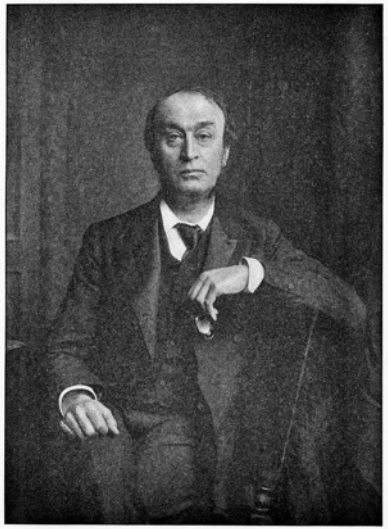
What is Cell Culture?

- Process by which cells are grown under controlled conditions, usually outside their natural environment
- Cells are removed from living tissue (animal or plant) and grown in an artificial environment
- Purposes:
  - *In vitro* assays and basic cell biology
  - Produce biological reagents (e.g. recombinant proteins, antibodies, vaccines)

What is Cell Culture?

- **Primary culture**. Cells are isolated from tissue and proliferate under appropriate conditions until they occupy all of the available substrate (i.e. attain confluency). At this stage, they must be subcultured (passaged) by transferring them to a new flask with fresh growth medium, to permit further growth. Now there are known as a cell line
- **Cell line** (or subclone)
  - → Senescence → Death. Finite cell line
  - → Transformation (chemical or viral) → Continuous cell line, dividing indefinitely

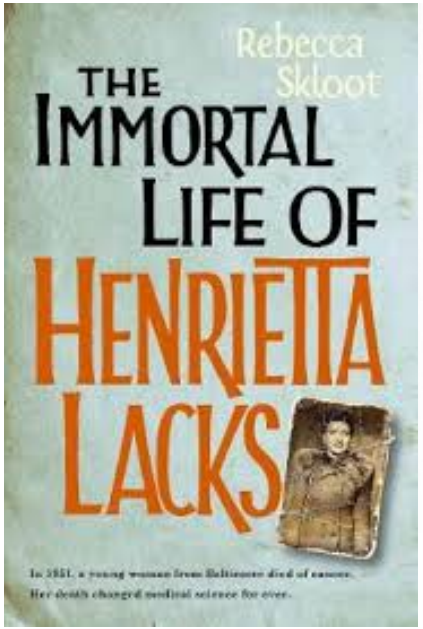
History of Cell Culture



Sydney Ringer
1882

George Gey
(Margaret Gey)
(Mary Kubicek)
1951

Henrietta Lacks
(1st August, 1920 –
4th October, 1951)



What is required for cell culture conditions?

- Essential nutrients (amino acids, carbohydrates, vitamins, minerals)
- Growth factors
- Hormones
- Gases (O_2, CO_2)
- Regulated physico-chemical environment
  - pH
  - Osmotic pressure
  - temperature

Adherent/monolayer culture (anchorage-dependent)
Or
suspension culture

Artificial basal media

- DMEM (Dulbecco's Modified Eagle Medium)
- RPMI 1640 (Roswell Park Memorial Institute 1640)
- MEM (Minimum Essential Medium)
- F-12
- + many more

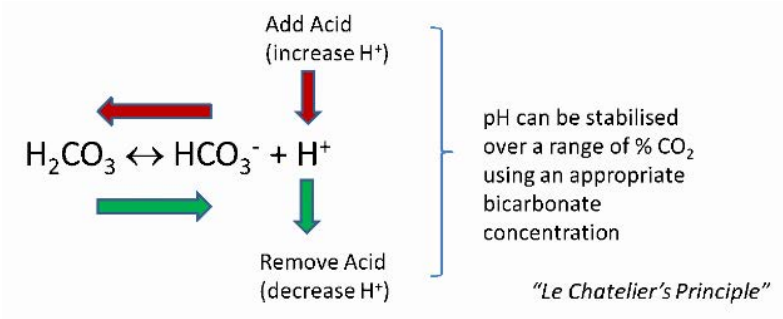
- Composition:
  - Inorganic salts (Ca, Mg, K, Na, PO4, Cl, CO4)
  - Amino acids
  - Vitamins

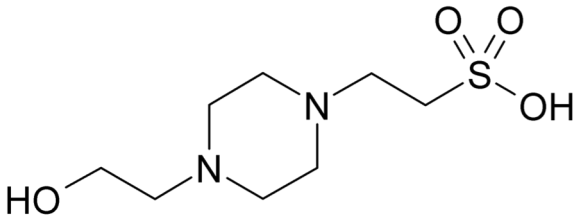
Regulated physico-chemical environment – humidified CO2 incubator

- CO_2 → into solution →+H_2O → carbonic acid → + bicarbonate ions → maintains stable physiological pH (7.2-7.4)
- Humidified to reduce evaporation of media
- May be a hypoxia incubator, for cells/stimulation requiring low O_2

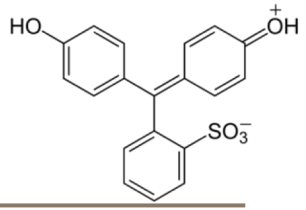
pH control and buffers

- Bicarbonate buffering system
  - Occurs naturally in the human body
  - Minimal side-effects
  - Not the most efficient chemical system for controlling pH
- Synthetic buffers e.g. HEPES
  - More effective buffering agent
  - When exposed to ambient light, undergoes photochemical oxidation → H_2O_2

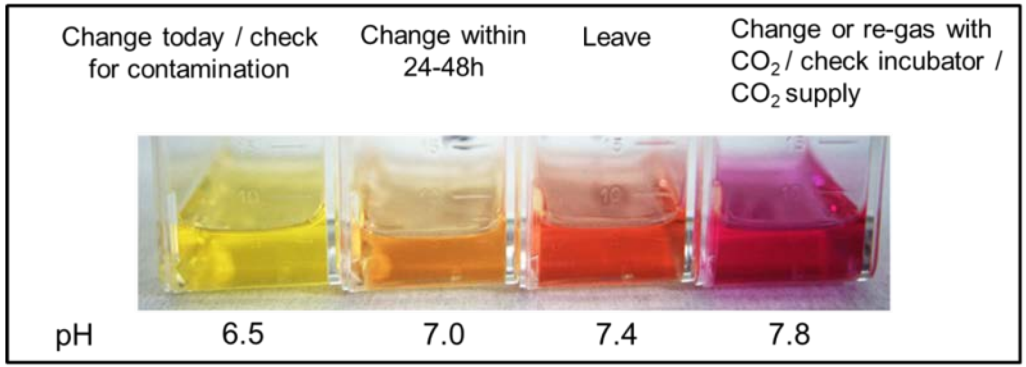




Phenol red



- pH indicator, included in cell culture media
- Metabolic products from growing cells lead to acidification of the media
- Weak oestrogen mimic
- Can obtain phenol red-free media

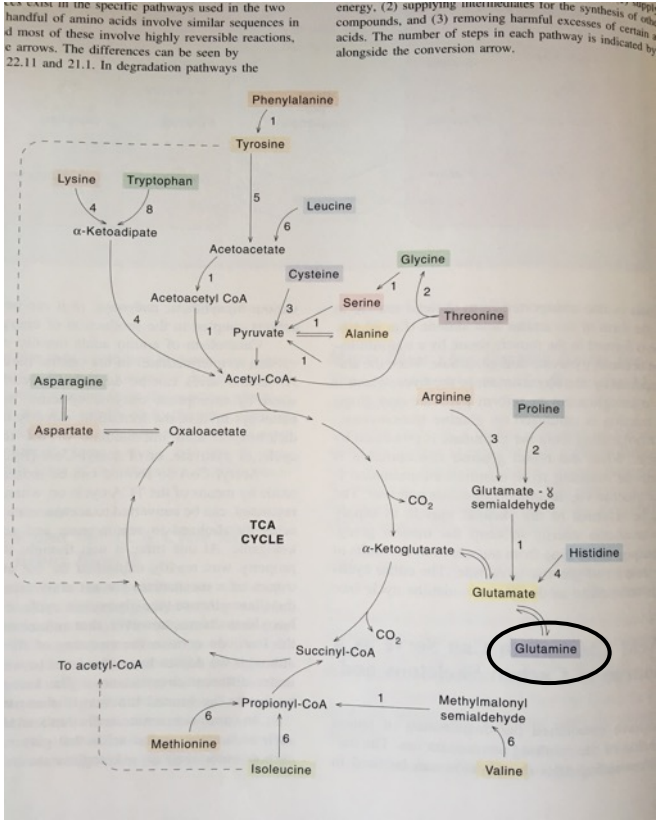


https://www.phe-culturecollections.org.uk/news/ecacc-news/co2-concentration-and-ph-control-in-the-cell-culture-laboratory.aspx

Foetal calf serum (Foetal bovine serum)

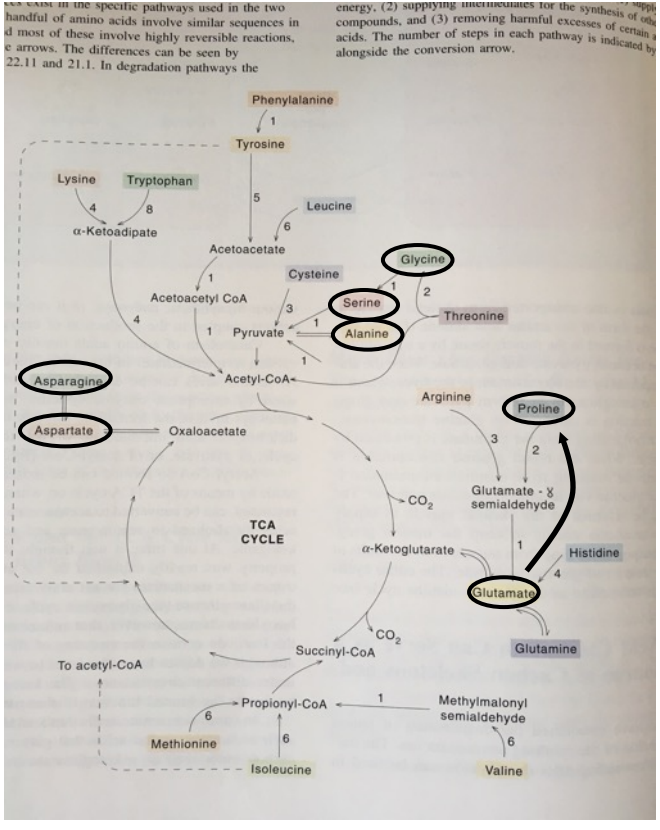
- Liquid fraction of clotted blood from foetal calves
- Main component is bovine serum albumin (BSA)
- High levels of growth factors
- Low levels of antibodies
- Composition cannot be fully defined and may vary between batches
  - batch-testing needed
  - undefined proteins can cause unwanted stimulation of cells so serum starvation is used, e.g. to detect subtle changes in cytokine production

L-Glutamine



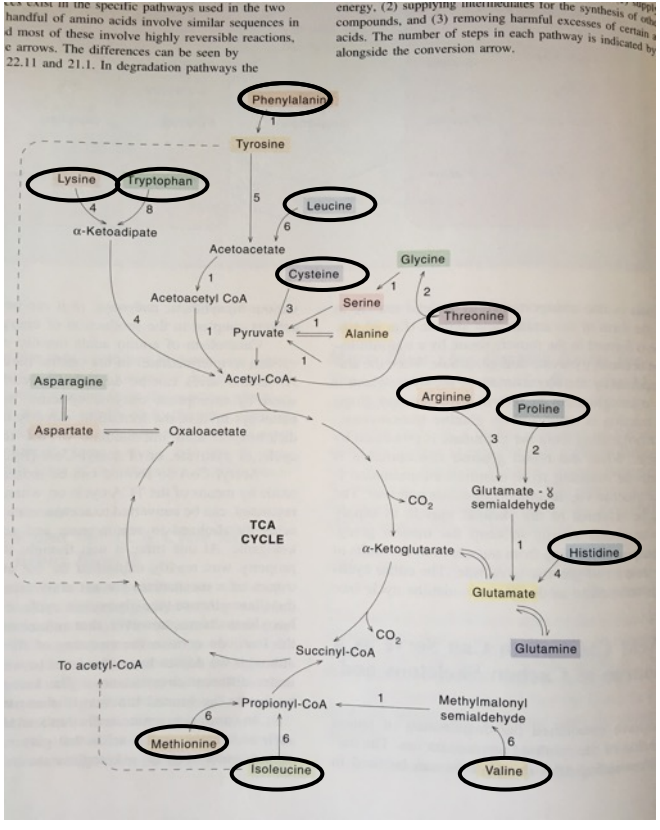
- Supports growth of cells that have high energy demands and synthesize large amounts of proteins and nucleic acids
- Alternative energy source for rapidly dividing cells and those that use glucose inefficiently
- Unstable at physiological pH (breaks down into ammonium and pyroglutamate

MEM Non-Essential Amino Acid Solution



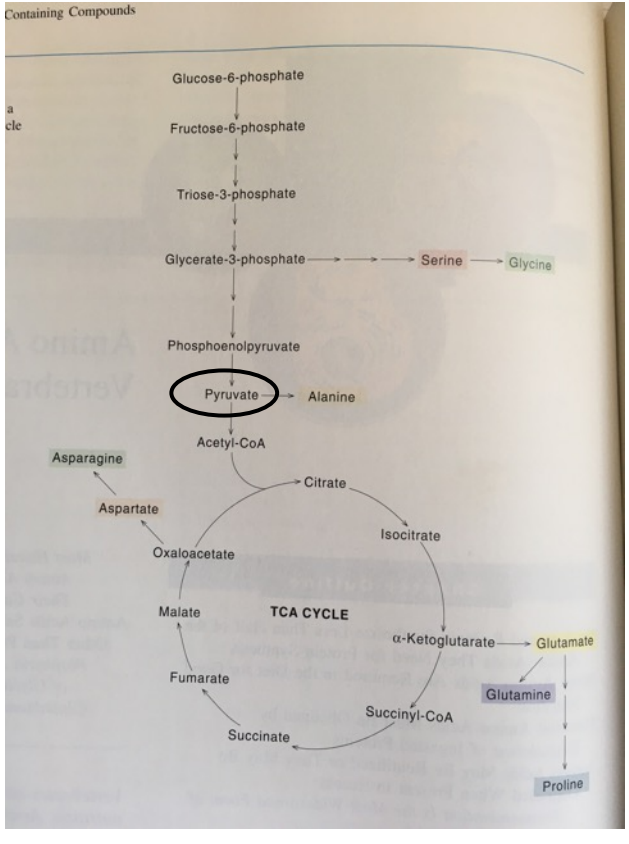
- Glycine, Alanine, Asparagine, Aspartic acid, Glutamic acid, Proline, Serine
- Contains the same non-essential amino acids as in standard Minimum Essential Medium (MEM) but more concentrated
- Prolong cell viability and stimulate growth

MEM Amino Acid Solution



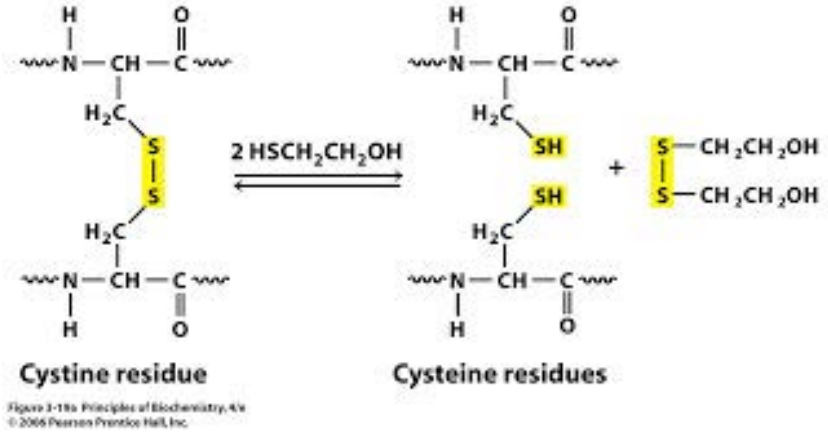
- Increases cell growth and viability
- Contains all essential amino acids except L-glutamine found in Minimum Essential Medium (MEM)

Sodium Pyruvate



- Additional carbon source to glucose
- Not an essential supplement for all cell cultures but cell growth may lag if it is withdrawn once used

2-Mercaptoethanol



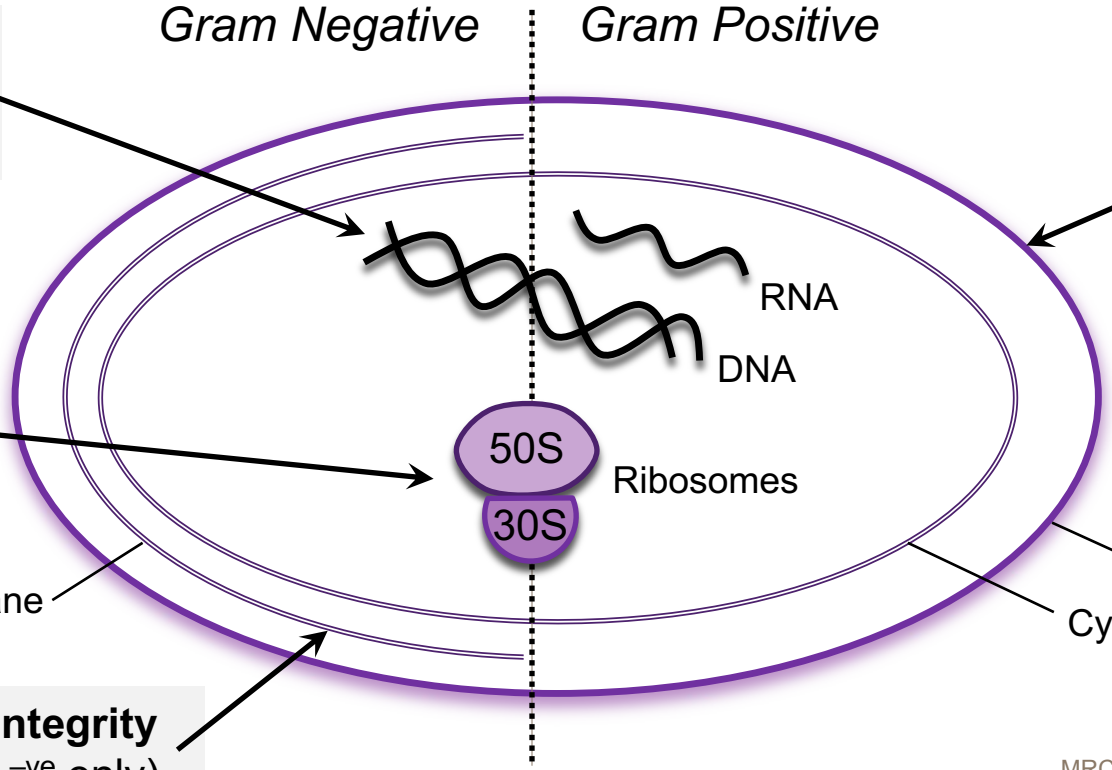
- Scavenges oxygen radicals
- Reduces disulphide bonds
- Biological antioxidant
- Not stable in solution so may require daily supplementation

Antibiotics

Transcription Inhibitors
Actinomycin D

Protein synthesis Inhibitors
Streptomycin
Gentamicin
Kanamycin
Neomycin

Outer membrane integrity
Polymyxin B (Gram $^{-ve}$ only)

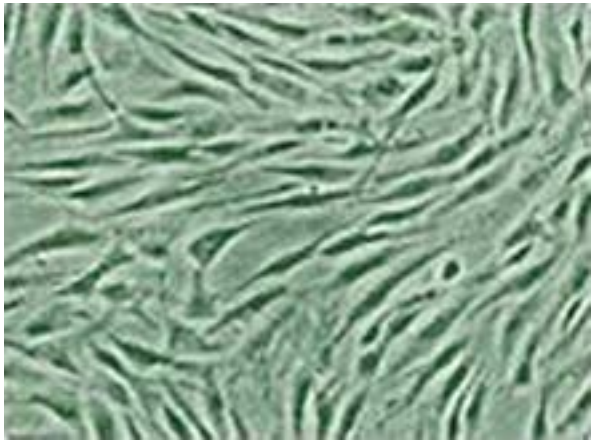


Cell Wall Synthesis Inhibitors
Penicillins
Ampicillin
(Gram $^{+ve}$ and only some Gram $^{-ve}$)

Antimycotics

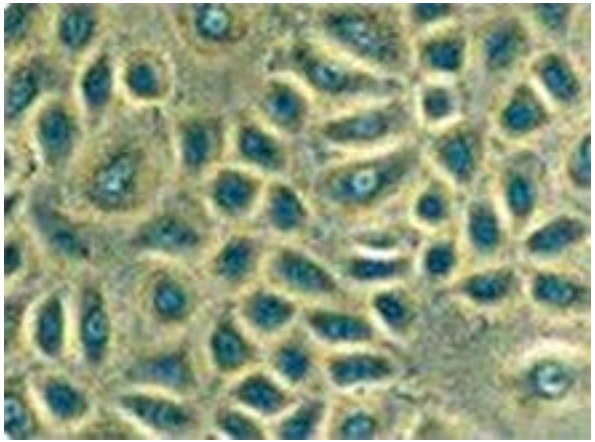
- Prevents contamination by yeast and multicellular fungi
- E.g. Amphotericin B (Fungizone®) and Nystatin (mycostatin)
- Disrupts permeability of cell membranes by forming a complex with cholesterol
- Toxic to some cell lines

Morphology of cells in culture



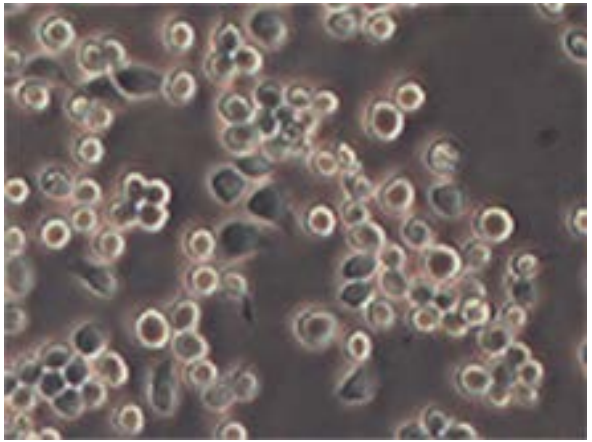
Fibroblast-like

- Bipolar or multipolar
- Elongated
- Adherent



Epithelial-like

- Polygonal with more regular dimensions
- Adherent



Lymphoblast-like

- Spherical
- Non adherent
- Grown in suspension

Subculturing/Passaging

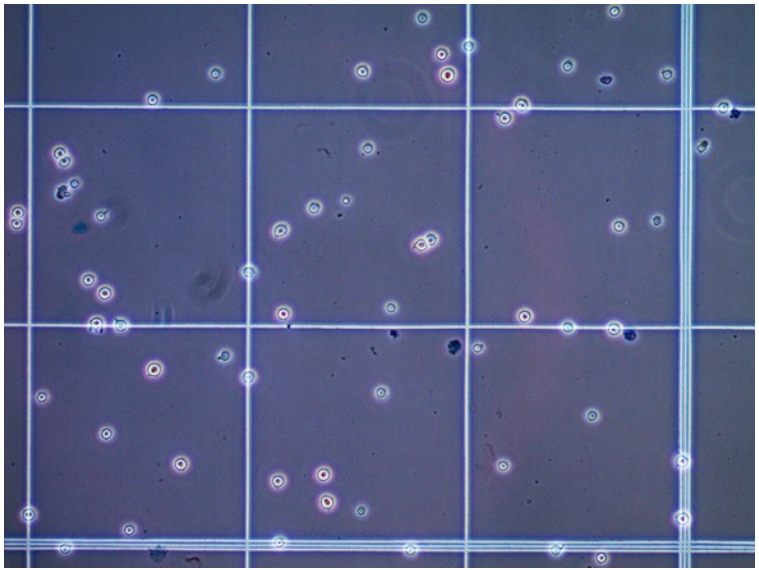
- Some cells require culture-ware to be pre-coated with extracellular matrix proteins e.g. collagen, fibronectin, laminin)
- Wash cells with balanced salt solution e.g. PBS (no Ca or Mg)
- Apply pre-warmed dissociation reagent to separate adherent cells from the substrate and from each other
  - EDTA
  - Trypsin +/- EDTA
  - Accutase®
- Cell scraping
- Incubate at 37°C and check every few minutes by microscope
- Centrifuge with pre-warmed complete growth medium
- Count cells and replate
- Keep everything warm and avoid bubbles

Transfection

- Purposes:
  - To study the function of genes/proteins, by enhancing (or inhibiting) specific gene expression in cells
  - To produce recombinant proteins
- Transient pores are opened within the cell's lipid bilayer to allow nucleic acids (DNA, RNA, siRNA RNAi), proteins, nanoparticles, Abs into the cellular milieu
- Calcium phosphate
- Dendrimers (branched organic polymers)
- Liposomes (lipid bilayer analogs)
- Electroporation (electricity to create pores)
- Speciality reagents

Cell viability

- 0.4% trypan blue in buffered isotonic salt solution, pH 7.2 – 7.3
- Add 1:1 to a sample of cells
- Count cells by haemocytometer or Countessa etc
- Live cells exclude trypan blue, dead cells don’t
- Cell viability should be > 95 % for healthy log-phase cultures



How to become immortal

- Hayflick limit
- Proliferation → telomeres shorten → DNA damage → cellular senescence
- How to overcome the Hayflick limit and achieve immortality:
  1. Spontaneously immortalized cells e.g. HeLa
  2. Introduce a viral gene that over-rides cell cycle e.g. HEK293T cells and SV40
  3. Expression of genes conferring immortality e.g. hTert
  4. Combining tumour suppressor inactivation and telomerase expression
  5. Fuse with an immortalized cell e.g. B-cell secreting mAb + myeloma cell line
  5. Viral infection

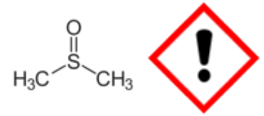
Virus	Integration into genome	Expression	Cells infected	Packaging capacity (kb)	Insertional mutagenesis
Adeno-	No	Transient	Dividing only	7-8	No
Lenti-	Yes	Long-term	Non-dividing and dividing	10-11	Maybe
Retro-	Yes	Long-term	Dividing only	~8	Maybe
Adeno-assoc	Yes	Long-term	Require co-infection with helper virus	<4.9	No. Integrates into specific region

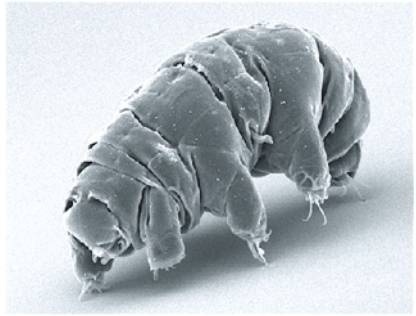
BUT immortality may not be the best route

Cell populations, cellular mechanisms and responses, activation status will be altered

Cryopreserving

- Unprotected freezing is (normally) lethal… except for tardigrades with their trehalose
- Cryoprotectants
  - Increase the total concentration of solutes present and reduce the amount of ice formed
  - Allow a slower cooling rate
  - Reduce the freezing point of the medium
  - Must be able to penetrate into cells
  - Low toxicity
  - E.g. Glycerol, DMSO
  - 10 % DMSO in FCS

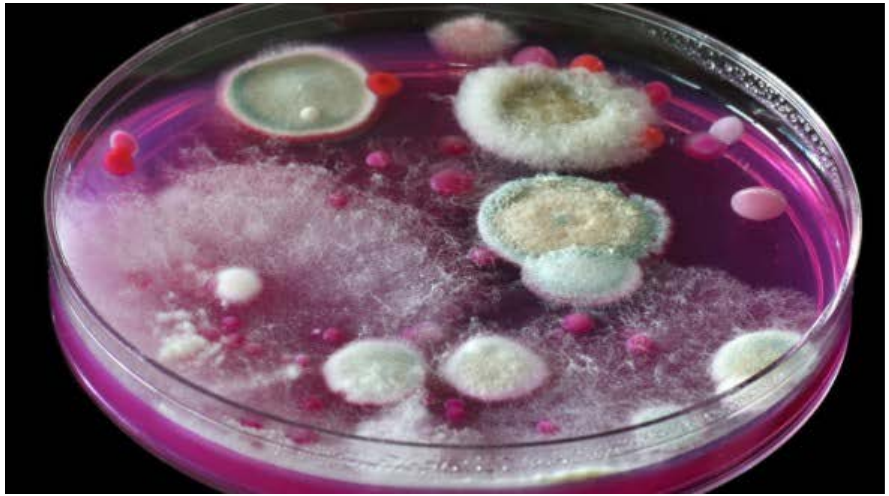




- Cells should be in log phase of growth
- Cool slowly (-1°C/min) e.g. in isopropanol chamber
- Store in -80°C for short-term (< 1 year) or liquid nitrogen (-135°C)
- When recovering the cells, thaw rapidly in 37°C water bath, use pre-warmed media

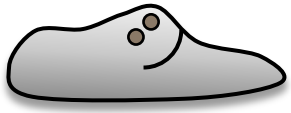
What can go wrong?

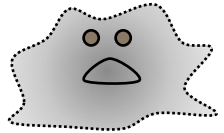
- Poor attachment of cells to substrate
- Infections
- Mycoplasma contamination
- Endotoxin contamination
- Contamination with other chemicals
- Contamination with other cell lines



Poor attachment of adherent cells

- Check for signs of contamination/infection
- Coat cultureware with ECM proteins
- Check incubator (temperature, CO_2)

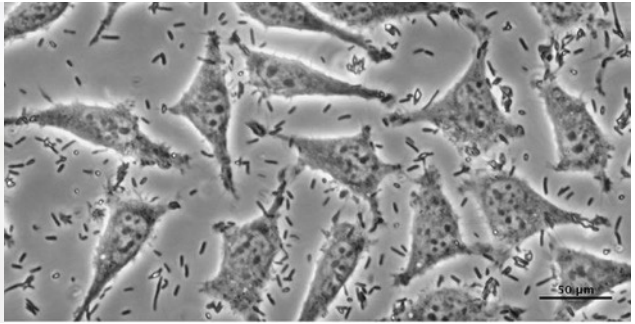




- Lipid peroxidation can degrade cell membranes and cause cells to detach. Ascorbate reduces this and glutathione regenerates ascorbate in solution
- Calcium is required for cell attachment and signaling; check there is no EDTA (calcium chelator) present
- Oxidative stress. Add glutathione/cysteine, or 2-mercaptoethanol
- H_2O_2. Keep all reagents in the dark; add pyruvate to bind H_2O_2

Infections - Bacterial

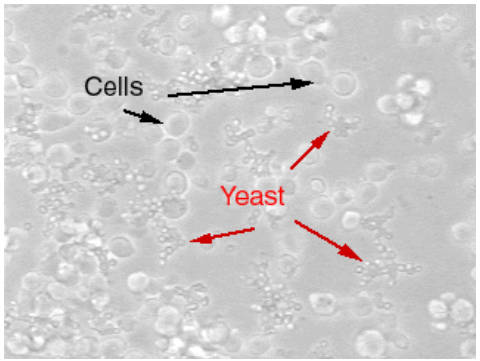
- Smelly
- “Milky” media
- High magnification → round, rod-shaped or spiral-shaped
- 0.5-1 µm (or up to 20 µm for some spiral forms)
- Cells in culture look unhealthy



https://handling-solutions.eppendorf.com/cell-handling/contamination/scientific-background/bacterial-contamination/

Infections - Yeast

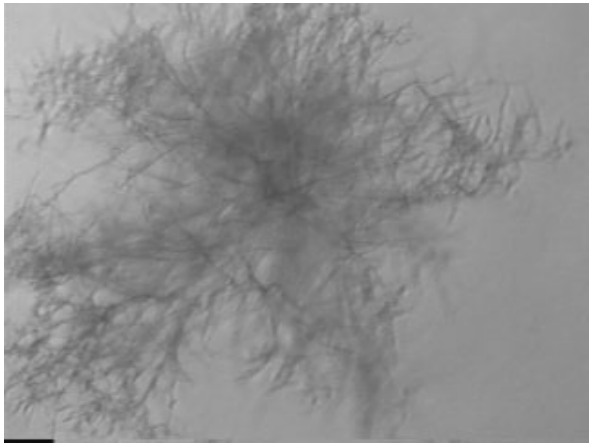
- Smelly
- Coloured media
- High magnification → separate round or ovoid particules, or in chains
- Larger than bacteria and smaller than mammalian cells



https://unclineberger.org/tissueculture/contaminant/yeastcontam/

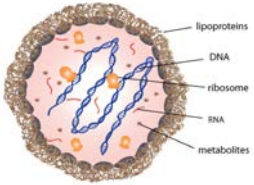
Infections - Fungus

- Usually visible under a low power microscope (or without magnification)
- Whiteish, yellowish or black
- Large furry patches when in advanced mycelial growth
- Hard to detect the spores
- Seasonal
- Can be “cured” at early stages with anti-mycotics and usually no toxic effects on mammalian cells
- BUT
  - Proteases secreted
  - PRRs activated

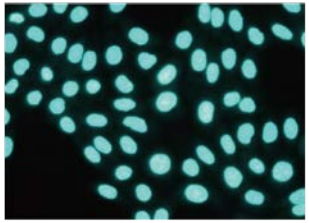


https://unclineberger.org/tissueculture/contaminant/funguscontam/

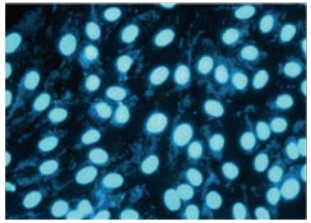
Mycoplasma



- Small (0.2-0.3 µm) wall-less bacteria that can grow to high concentrations in cell culture (10^7 – 10^8/ml).
- Typically fuse with mammalian cell membranes and invade the host cell
- Very hard to detect!
  - Not smelly
  - Unobserved by regular light microscopy
  - View by high magnification fluorescence microscopy with DNA detection agents e.g. DAPI, Hoechst, or use PCR-based detection methods



Mycoplasma-free cells

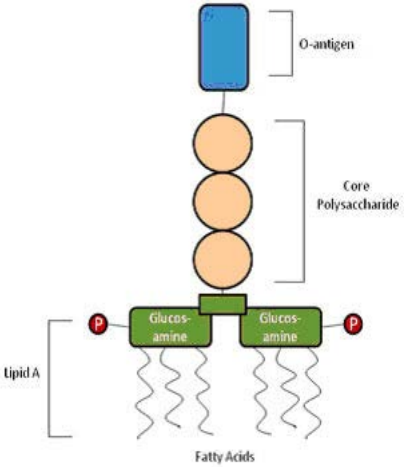


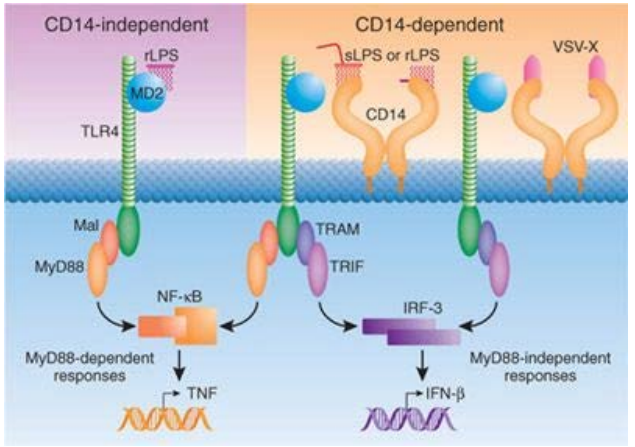
Mycoplasma-contaminated cells

- Alter cell growth characteristics, inhibition of cell metabolism, altered cell attachment, disrupted nucleic acid synthesis, chromosomal abberation, altered transfection rates and virus susceptibility, experimental variation, lipoproteins are recognized by PRRs (e.g. TLR2) → NFκB-mediated activation
- Typical routes of infection:
  - Cross-contamination from untested cells to other cell lines, e.g. Airborne microscopic aerosolization when multiple cell lines/media are handled in the same hood
  - From human skin/hair/mouth

Endotoxins

- Hydrophobic lipopolysaccharides found in the outer membrane of gram negative bacteria
- Commonly found in water, sera, some culture additives (especially those manufactured using microbial fermentation) and plasticware/glassware
- Consequences:
  - Source of experimental variation
  - Elicit an inflammatory response
  - Will compromise the use of cell culture products e.g. antibodies and vaccines) in therapeutics and experiments
- To avoid this:
  - Use endotoxin-tested reagents/supplements; practice good aseptic technique
  - Measure endotoxin concentration in reagents using the Limulus Amebocyte Lysate (LAL) assay, or a luciferase-based NFκB reporter assay using highly LPS-sensitive cells (overexpressing CD14, TLR4, MD-2)





From Godowski, Nat. Immunol. 2005

Other chemical contaminants

- Toxic metal ions from glassware or metal pipes
- Plasticizers from plastic storage vessels or tubing
- Detergents used in washing
- Fluorescent lights. Photoactivation of HEPES buffer, riboflavin and tryptophan → H_2O_2
- Incubators
  - Contamination of CO2 input (v rare)
  - Disinfectants

Contamination with other cell lines

- Robust, immortal cell lines such as HeLa grow really well
- By 1967, HeLa cells had contaminated 19 other human cell lines (shown by electrophoretic polymorphisms of isoenzymes e.g. G6PD)
- In 1974, 5 cell lines (human cells infected with animal viruses) were revealed to be HeLa in origin (karyotyping)
- In 2000s, 45 of 252 human cell lines (18%) supplied by 27 of 93 originators (29%) were contaminated with HeLa cells
- Inter- and intra-species contaminations, virus infections, somatic cell hybridization between the original cell line and a contaminating cell line… all contribute to a mix-ups
- HeLa contamination has been reported from air droplets

Lucey *et al* (2009) Archives of Pathology and Laboratory Medicine

How to avoid problems

- **Good aseptic technique** (uncluttered hood, avoid turbulence, avoid talking), disinfect and clean surfaces, incubators and pipette aids regularly
- **PPE** specifically for tissue culture use
- **Clean and disinfect** incubators and water baths regularly
- Use **antimicrobial treatment** in water baths and incubator reservoirs
- **Regularly test** cells for mycoplasma and reagents for endotoxin
- **Deal with problems** promptly
  - Virkon-treat and dispose of infected cells
  - Mycoplasma removal using Normocin™ (or i.p. into mice)
- **Do not share** media/reagents etc
- Only have **one** cell line/media at once in the hood
- **Avoid "gifts"** of cells unless you check them thoroughly
- Only use **tissue culture grade** plasticware and **ultrapure** water
- Do not create **bubbles** in media or in pipette, to avoid **aerosols**
- **Be observant**