BRITISH JOURNAL OF HOSPITAL MEDICINE

CORE TRAINING FOR DOCTORS

Edited by Dr Daniel JB Marks, Academic Clinical Fellow in Translational Medicine, University College London and **Dr Jacob de Wolff**, Consultant Acute Physician, North West London Hospitals NHS Trust, Middlesex

Cell culture

Introduction

In 1885, Wilhelm Roux isolated cells from a chick embryo and sustained them in vitro for several days (Alberts et al, 2002). Since then, cell culture has played an integral role in scientific development with applications including exogenous protein expression, vaccine production, pseudotyped virus production and gene mapping. Technological advances mean that it is now possible to grow cells on specially developed scaffolds, demonstrated by the successful transplantation of a bioartificial tracheobronchial airway (Jungebluth et al, 2011).

A primary culture will grow and replicate, given the correct environmental conditions. However, after a finite number of cell divisions, referred to as the Hayflick limit, the cells will enter replicative senescence and cease to divide (Hayflick and Moorhead, 1961). In 1951, George Gey isolated human cervical carcinoma cells that could continue to propagate in vitro without entering cellular senescence (Scherer et al, 1953). The progeny of this immortalized cell line, named HeLa, are still widely used in laboratories worldwide and have even been sent to space (Ohnishi et al, 2002). Cells from other multicellular organisms, such as fungi and plants, can also be cultured.

Choosing the right cells

It can often be difficult to decide which cell line to choose for a particular experiment. Points to consider include:

- The species and tissue type of the cell line, dependent on the desired phenotype. As an example, human liver cells are particular useful for studying toxicity (Soldatow et al, 2013)
- Growth characteristics, e.g. are they fast or slow dividing cells?
- Are they adherent or suspension cells? Suspension cells are particularly useful for efficient recombinant protein production (Plasson et al, 2009)
- Do the cells require any specific chemicals or environmental conditions?
- Are they permissive to the chemical agent or pathogen of interest? This is exemplified by HIV, which displays CD4+ cell tropism (Clapham and McKnight, 2001)
- Were the cells obtained from an approved and accredited source? Have they been authenticated and tested for contamination?

New cell lines can also be established. For human cells, this can be particularly time-consuming as there are many ethical and legal requirements to address including patient consent, ethics and the Human Tissue Act 2004.

Working with cells

This section details some important points to consider when working with live cells.

Safety

The material being handled will determine the laboratory category level and the type of biosafety cabinet required. Safety information should be adhered to and certification provided by the employer. Individuals should have a separate set of personal protective equipment specifically for cell culture. Ideally, there should also be a separate set of equipment with transfer between other laboratory environments involving rigorous cleaning with an appropriate disinfectant or 70% ethanol.

Medium

The cell line governs the choice of medium. Its basic constituents include amino acids, vitamins and inorganic salts. Fetal bovine serum is frequently added to mammalian cells to provide growth factors and other macromolecules. It also contains low concentrations of immune molecules, including immunoglobulin and complement. Owing to variation, serum should be bought in batches and tested before use.

Antibiotics are often added to media to help prevent contamination. This will change the culture environment and is inappropriate when studying pathogens susceptible to the antibiotics. It may also

Mr CD Donaldson *is PhD Student and* **Dr KN Bishop** *is Programme Leader in the Division of Virology, MRC National Institute for Medical Research, London NW7 1AA*

Correspondence to: Mr CD Donaldson (cdonald@nimr.mrc.ac.uk)

encourage poor aseptic technique and lead to mycoplasma infection (Nikfarjam and Farzaneh, 2012).

Media should be stored at 4°C and warmed to 37°C before use. The addition of particular molecules to the medium can alter cellular behaviour, demonstrated by the influence of interferons on the induction of an antiviral state (Samuel, 2001).

Subculturing

Table 1 includes a number of signs of poor cell culture health. Regular subculturing prevents contact inhibition and cell death through overcrowding. The confluency of adherent cells can be estimated by eye with a light microscope. Cell counting machines are used to measure suspension cell density and for more accurate measurement of adherent cell numbers. Suspension cells can be simply subcultured by taking a proportion of the cell/media solution and placing in a new culture vessel with fresh medium. However, adherent cells must first be detached from the growing surface. Trypsin, an enzyme that disrupts the extracellular matrix between cells and their attachment to the culture vessel, is often used. The cells can also be physically removed with a sterile scraper, although this can cause cell damage. Consequently, enzymatic detachment is usually preferred when subculturing, while cell scrapers are used for harvesting cells that are to be lysed anyway (e.g. for a Western blot).

The speed of enzymatic detachment depends on cell type; for example, HeLa cells will take longer to detach than 293T cells. Putting the enzyme at room temperature 30 minutes before use and incubating the culture vessel transiently at 37°C following addition of the enzyme can speed up the process. Once the cells have detached, addition of serum-containing media will prevent cellular damage by diluting and inactivating the enzyme. Causes of inappropriate adherent cell detachment are listed in *Table 2*.

There are four main stages of cell population growth (*Figure 1*). Many experiments require the cells to be in the logarithmic (log) growth phase. Subculturing just before confluence prevents contact inhibition and maintains the cells in this phase. The proportion of cells taken from a culture vessel to reseed a new vessel is often referred to as the split ratio. This is dependent on the cell type but 1:3 and 1:4 are commonly used daily split ratios to maintain cells in the log phase. When leaving the laboratory for a few days a higher split ratio can be used (e.g. 1:12 or 1:24). However, these cells should be subcultured a number of times at a higher split ratio (e.g. 1:3 or 1:4) before use.

Subculturing can result in genetic and phenotypic drift: U-251 glioblastoma cells have been shown to lose their original profile with high passage number (Torsvik et al, 2014). Low passage number cells should be regularly thawed to replace working stocks.

Incubation

Different cell lines should be separated to prevent cross-contamination. Many of the commonly used mammalian cell lines are incubated at 37°C, 5% CO_2 in a humid environment. The CO_2 helps maintain a constant pH by interacting with the bicarbonate buffering systems in many commonly used medias. A pH of 7.4 is desirable for most mammalian cells and changes in pH as a result of fluctuations in CO_2 concentration may affect cell growth.

Some cell lines may need different incubation conditions for optimal growth and in this instance a bioreactor may be helpful.

Storage

Storing cell lines prevents overcrowding in the workspace, reduces the risk of cross-contamination and provides reserves if working stocks become con-

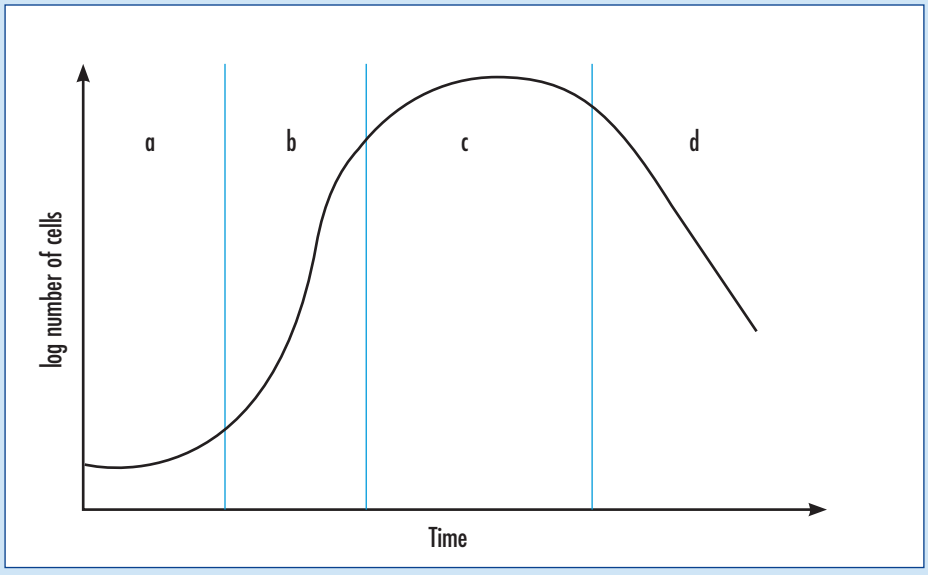
Table 1. Are my cells healthy?

Cell morphology is a good indicator of culture health. Although dependent on cell type, general poor prognostic signs include:	Detachment of adherent cells
	Cytoplasmic vacuolation
	Perinuclear granularity (Freshney, 2010)
Some media contain pH indicators. For example, media containing phenol red will change from red to yellow as the concentration of acidic toxic metabolites increases. Subculturing and changing the medium regularly prevents the build up of toxins that can inhibit cell growth	

Table 2. Why do my cells detach from the plate?

Some cells, such as 293T cells, are naturally less adherent. This can be improved by lining the plate or flask with gelatin
Cells can be disrupted if culture vessels are not handled gently
If cells are left for insufficient time in the incubator between manipulations, the cells may detach

Figure 1. Schematic showing the characteristic growth of a population of cells in a culture vessel. The graph can be split into four main phases: a = lag; b = logarithmic (log)/exponential; c = plateau/ stationary and d = death.



taminated. For finite cells, storage is essential if they are to be used beyond their life span. Commonly, cells are cryopreserved in liquid or vapour nitrogen containers with cryoprotectants added to the medium to protect them from ice crystal formation. Dimethyl sulfoxide (DMSO) is commonly used but can cause cell differentiation as seen in mesenchymal stem cells, where it causes hepatic differentiation (Alizadeh et al, 2014); in this instance, glycerol is a suitable alternative. As a general rule, cells should be cooled slowly to help prevent ice crystal formation, and thawed quickly so that the toxic cryoprotectants can be removed through dilution in media and/or centrifugation. Initially, cells should be plated at a high density to allow recovery.

Contamination

This is a surprisingly widespread problem (*Figure 2*). Some common causes of contamination are shown in *Table 3*.

Figure 2. Contaminated culture vessel. This flask contained 293T cells in media with insufficient antibiotics. Areas of contaminant growth can be seen within the red boxes.

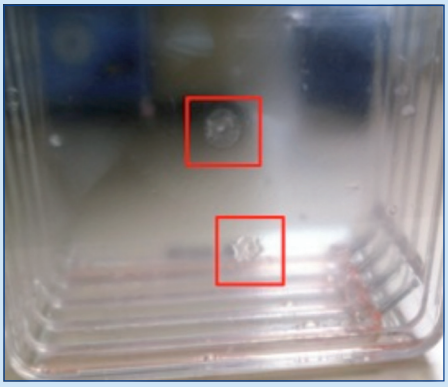


Table 3. Why do my cell cultures keep on getting infected?

Causes of infection include:	Contaminated media and reagents
	Contaminated equipment
	Poor aseptic technique
	Receipt of a contaminated cell line

Repeated contaminations should trigger an investigation to identify the source and a review of cell culture techniques. An aseptic technique not only helps prevent contamination but also protects the operator from pathogenic organisms that may be present in the cell culture

Mycoplasma and fungal infections are particularly problematic as they can be hard to identify and eliminate. Contaminations should be dealt with quickly and efficiently by disposal of contaminated material, and sterilization or replacement of equipment and reagents.

Aseptic technique

Meticulous hygiene and cleaning practices are essential in preventing contamination of sterile cultures. Cells that are to be used clinically, such as stem cells, will need to adhere to Good Manufacturing Practice requirements. Some practical tips for reducing the risk of contamination are given below.

Cabinet (*Figure 3*)

- Use of a laminar flow machine
- Thorough cleaning with disinfectant or 70% ethanol before and after use
- Regular deep cleaning and servicing
- Prevention of airflow disruption by keeping items in the cabinet to a minimum, avoiding quick and forceful movements, keeping windows shut and ensuring the cabinet is in an area of low footfall.

Medium

- Pouring directly from a bottle should be avoided: single use serological pipettes from sterile packaging should be used to transfer media
- Bottle lids should be removed for the minimum time possible and if put down, the opening should face the work surface.

Equipment

- Disinfectant or 70% ethanol should be used to clean any items being placed in the cabinet
- Autoclaving can be used to sterilize reagents and equipment as necessary.

Cell cultures

- Ideally, cell lines should be managed in separate cabinets and incubators. If this is not possible, only one cell line should be in the cabinet at a time, with thorough cleaning practices between changes
- Any spillages should be dealt with immediately with an appropriate disinfectant.

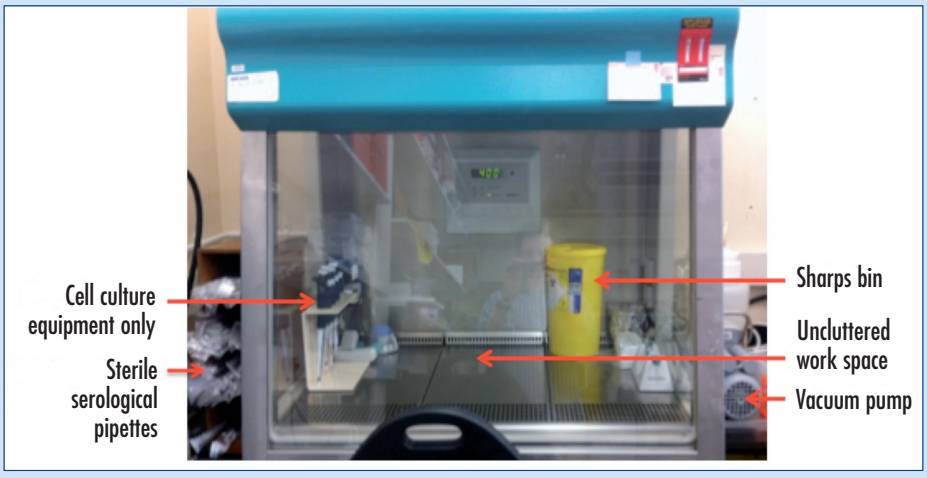
Operator

- Gloves should be changed frequently (this is imperative if they become contaminated). In between glove changes, they can be wiped regularly with 70% ethanol
- Cell culture personal protective equipment should be worn, and hands washed thoroughly before and after.

Conclusions

The uses of cell culture are wide and varied, and it is an invaluable tool for scientists and clinicians. However, to be used effectively, it is a skill that requires practice and patience. Advancements in fields such as tissue engineering and stem cell therapy mean cell culture has an exciting role to play in medical developments, as well as continuing to underpin basic scientific research. BJHM

Figure 3. Biosafety cabinet. Different types of cabinet provide varying degrees of protection from contamination to both the scientist and cell culture. They work by filtering the air and controlling airflow.



Conflict of interest: none.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) Isolating cells and growing them in culture. In: Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P, eds. *Molecular Biology of the Cell.* 4th edn. Garland Science, New York: 469–78

Alizadeh E, Zarghami N, Eslaminejad MB, Akbarzadeh A, Barzegar A, Mohammadi SA (2014) The effect of dimethyl sulfoxide on hepatic differentiation of mesenchymal stem cells. Artificial Cells, Nanomedicine, and Biotechnology Early Online: 1–8 (doi: 10.3109/21691401.2014.928778)

Clapham PR, McKnight A (2001) HIV-1 receptors and cell tropism. *Br Med Bull* **58**: 43–59 (doi: 10.1093/bmb/58.1.43)

Freshney RI (2010) Subculture and Cell Lines. In: Freshney RI, ed. *Culture of Animal Cells: A Manual of Basic Techniques and Specialised Applications.* 6th edn. John Wiley and Sons, Hoboken: 187–206

Hayflick L, Moorhead PS (1961) The serial cultivation of human diploid cell strains. *Exp Cell Res* **25**: 585–621 (doi: 10.1016/0014-4827(61)90192-6)

Jungebluth P, Alici E, Baiguera S et al (2011) Tracheobronchial transplantation with a stem-cell-seeded bioartificial nanocomposite: a proof-of-concept study. *Lancet* **378**(9808): 1997–2004 (doi: 10.1016/S0140-6736(11)61715-7)

Nikfarjam L, Farzaneh P (2012) Prevention and detection of Mycoplasma contamination in cell culture. *Cell J* **13**(4): 203–12

Ohnishi T, Ohnishi K, Takahashi A et al (2002) Detection of DNA damage induced by space radiation in Mir and space shuttle. *J Radiat Res* **43** (Suppl): S133–6 (doi: 10.1269/jrr.43.S133)

Plasson C, Michel R, Lienard D et al (2009) Production of recombinant proteins in suspension-cultured plant cells. *Methods Mol Biol* **483**: 145–61 (doi: 10.1007/978-1-59745-407-0_9)

Samuel CE (2001) Antiviral actions of interferons. *Clin Microbiol Rev* **14**(4): 778–809 (doi: 10.1128/CMR.14.4.778-809.2001)

Scherer WF, Syverton JT, Gey GO (1953) Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *J Exp Med* **97**(5): 695–710 (doi: 10.1084/jem.97.5.695)

Soldatow VY, Lecluyse EL, Griffith LG, Rusyn I (2013) In vitro models for liver toxicity testing. *Toxicol Res* **2**(1): 23–39 (doi: 10.1039/C2TX20051A)

Torsvik A, Stieber D, Enger PØ et al (2014) U-251 revisited: genetic drift and phenotypic consequences of long-term cultures of glioblastoma cells. *Cancer Med* **3**(4): 812–24 (doi: 10.1002/cam4.219)

KEY POINTS

- Proper aseptic technique is important to protect both the scientist and cell culture from contamination. The temptation to skip steps should be avoided.
- When producing a new cell line, guidance should be sought on the ethical and legal considerations.
- Forward planning is essential, as cells require regular subculturing and fresh media to prevent toxicity and death. Many experiments require cell populations to be in the log growth phase.
- Subculturing cells regularly before experimentation will allow an understanding of the characteristics, such as adherence and replicative speed, of a cell line to be developed thus optimizing performance.
- A detailed record of cell lines should be kept, including information on the isolation of the primary culture, and subsequent manipulations and passage numbers.
- Cell culture should be performed in an appropriate containment laboratory and approved cabinet. Equipment should be cleaned frequently with 70% ethanol or an appropriate disinfectant.
- Working with different cell lines simultaneously should be avoided. Meticulous cleaning habits and good organization help prevent cross-contamination.

Glossary

Adherent cells – cells that require attachment to a surface in order to grow and replicate. Also known as anchorage dependent cells

Aseptic technique – the various methods used to ensure that a cell culture remains sterile

Cabinet – a specially designed container in which cell culture can be performed. Different biosafety levels determine the degree of protection. Commonly referred to as the 'hood'

Cell culture – the isolation of cells from a host organism and their subsequent propagation in vitro

Cell line – the cells produced following subculture of a primary culture. Cells that are immortalized are known as continuous cell lines, while those that are unable to divide indefinitely are known are finite cell lines

Confluency – the density of cells in their culture vessel. For adherent cells, this is often expressed as a percentage of the growing surface of the culture vessel

Contact inhibition – in high density cell cultures, the close proximity of cells prevents further growth

Immortalization – cells that are able to replicate indefinitely beyond their Hayflick limit without entering cellular senescence

Primary culture – the initial cells that are isolated from the host organism and placed in a culture vessel

Subculturing – the propagation of cells in vitro by removal of a proportion of cells from a culture vessel and their placement in a new culture vessel with fresh medium. Also referred to as 'passaging' or 'splitting'

Suspension cells – cells that are able to grow and replicate while in solution with the medium. Also called anchorage independent cells