CHAPTER 1

Introduction

1.1 TERMINOLOGY

1.1.1 Tissue Culture and Cell Culture

Tissue culture, in its various guises, has been in use for more than a century (Harrison 1907; Burrows 1910). Tissue culture methodology was initially devised to study the behavior of animal cells, free of systemic variations that might arise *in vivo* during normal homeostasis and under the stress of an experiment. As the term implies, fragments of tissue were originally used to provide a source of cells; this form of culture is commonly known as "explant" culture. Culture of cells within and from such tissue fragments came to dominate the field for more than 50 years (Fischer 1925; Parker 1961). Because culture conditions were initially suboptimal, growth was restricted to the radial migration of cells from the explant, with occasional mitoses in the outgrowth.

Throughout the first three decades of tissue culture, efforts were made to induce an "immortal" line of cells that would continue to replicate outside the tissue fragment. Success was initially reported by Alexis Carrel, who maintained a culture for more than 30 years that was originally derived from embryonic chick heart (Carrel 1912). Carrel's achievement could not be reproduced by other laboratories; it was subsequently shown that this cell type does not survive in culture for more than a year (Hayflick 1965). In retrospect, Carrel's "immortal" cells were probably an artifact due to cross-contamination (see Chapter 17) (Witkowski 1980). The first continuous cell lines were established by Wilton Earle and his colleagues at the National Cancer Institute (NCI). Earle's "L" strain was initiated in 1941 and cloned to give rise to the L-929 cell line (see Figure 1.1) (Earle et al. 1943; Sanford et al. 1948). After a further decade of effort, George Gey and colleagues succeeded in establishing the HeLa cell line from a young woman with cervical carcinoma (Gey et al. 1952; Skloot 2010). L-929 and HeLa were used to develop many of the culture conditions and techniques that are still in use today (Ham 1974).

"Tissue culture" is used throughout this book as a generic term, referring to the culture of cells that were initially derived from a tissue sample. The word "culture" implies that cells can undergo replication outside the organism. In some senses, "tissue culture" is an historical term. Most of the explosive expansion in the field in the second half of the twentieth century was made possible by the use of dispersed cell cultures and, in particular, by the availability of L-929, HeLa, and other cell lines (see Figure 1.2). It is not surprising that the term "cell culture" has become increasingly popular within the scientific literature. However, dispersed cell cultures make up only one type of culture. "Tissue culture" has taken on a deeper meaning in the last few years, thanks to the development of three-dimensional (3D) cultures. These 3D models are truly tissue culture in all senses of the term; for example, organoid cultures develop complex structures that reflect the behavior of tissues within the original organism (see Figure 1.3).

1.1.2 Sources of Terminology

Although we have tried to use consistent terminology throughout this book to reduce confusion, the meanings of some terms may be controversial. The Tissue Culture Association (now the Society for In Vitro Biology) developed

Freshney's Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, Eighth Edition. Amanda Capes-Davis and R. Ian Freshney.

Companion website: www.wiley.com/go/freshney/cellculture8

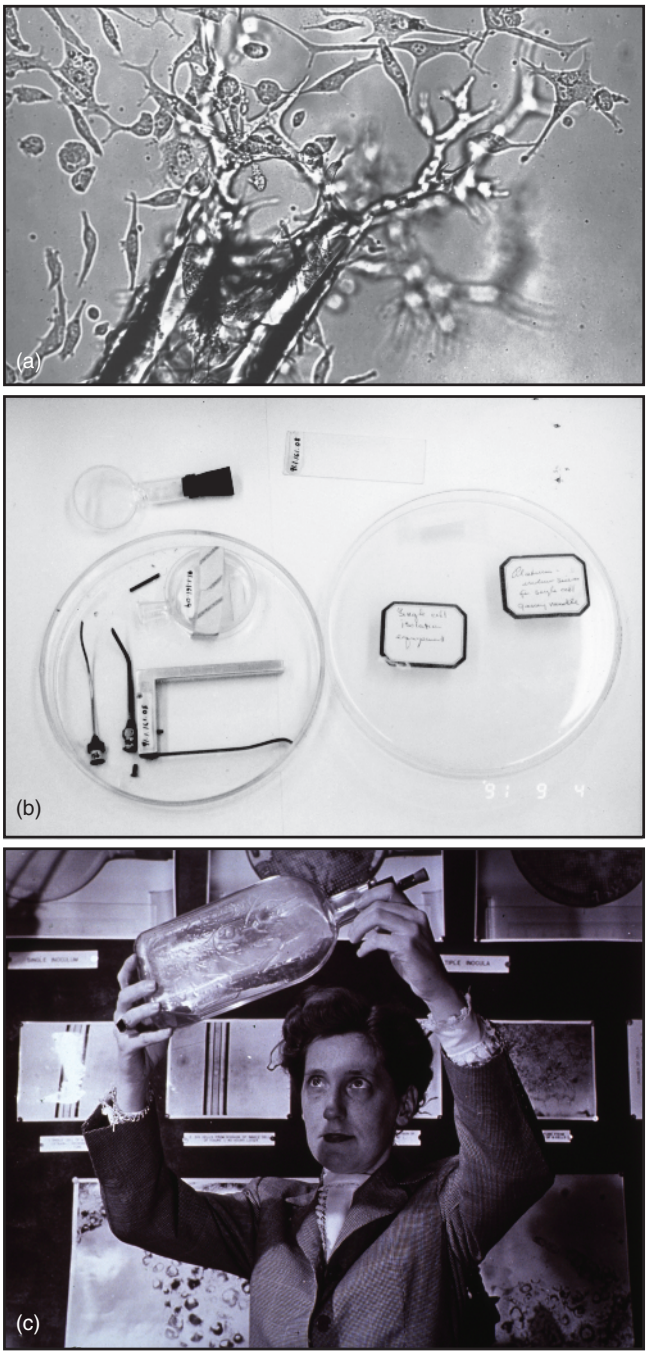


Fig. 1.1. Establishment of the L-929 cell line. (a) Cloning of the L strain to give L-929 (also known as NCTC clone 929), as photographed by Wilton Earle. Single cells were isolated and cultured in sealed glass capillary tubes. If proliferation was observed, the tube was broken and the cells allowed to spill out. (b) Equipment used to perform single cell isolation. The Carrel flask, shown top left, was later modified in Earle's laboratory for large-scale tissue culture. (c) Virginia Evans, who worked with Earle to develop the first defined substrates for tissue culture, examining growth within a modified tissue culture flask ("T-flask"). *Source*: (a) National Cancer Institute, image AV-4300-4382; (b) Office of NIH History and Stetten Museum, item 91.0001.161; (c) US National Library of Medicine, image 101393939. Public domain.

consensus terminology in an effort to reduce confusion and improve communication (Schaeffer 1990). This book primarily follows the consensus terminology, as do several guidelines on Good Cell Culture Practice (GCCP) (Coecke et al. 2005; Geraghty et al. 2014). Terms that are particularly controversial or confusing are discussed at the beginning of the relevant chapter; for example, "cell line" and "cell strain" are discussed at the beginning of Chapter 14. In some cases, a term is not included in the consensus terminology and the meaning may vary across the field. These terms are clarified and source references provided in a relevant chapter; for example, "organoid culture" is discussed in Chapter 27. Tissue culture-related terms are listed at the end of this book (see Appendix A). Other terms (e.g. from molecular biology) may be found elsewhere (Cammack et al. 2008).

1.2 HISTORICAL DEVELOPMENT

1.2.1 Substrates and Media

L-cells were initially grown in clotted plasma that was bathed in a mixture of horse serum, chick embryonic extract, and Earle's balanced salt solution (EBSS) (Earle et al. 1943). These conditions were almost completely undefined (apart from the EBSS), leading to great difficulty in generating reproducible results. The first defined substrate was not introduced until 40 years after the first experiments, when Virginia Evans and Wilton Earle used perforated cellophane to culture L-cells (see Table 1.1) (Evans et al. 1947). Cells grew exceptionally well on the cellophane, which provided a 3D matrix, since the cells grew through the perforations in the sheet. The cellophane matrix could also be extensively folded and used for scale-up in 3D culture (Sanford and Evans 1982). Once the culture adapted to growing on the cellophane, the cells would usually grow on the glass floor of the flask, leading to increasing use of glass (and later plastic) for cell culture (Evans and Sanford 1978). The further development of culture vessels and substrates is described in Chapter 8.

The increasing number of cells grown *in vitro* (which translates to "in the glass") meant that a standardized medium formulation was necessary to provide consistent nutritional requirements. The first medium formulation, Medium 199, was developed for the culture of chick embryonic fibroblasts (Morgan et al. 1950). Although it was effective for some cell types and applications, Medium 199 did not support the extensive proliferation that was seen with "natural" media containing serum and chick embryo extract (Morgan et al. 1955). A variety of other formulations were developed to address this concern, particularly for use with L-929 and HeLa (Ham 1974). The most popular were Eagle's Basal Medium (BME) and Minimum Essential Medium (MEM), which supported growth of a wide variety of cell lines (Eagle 1955, 1959). The development and use of defined media are described in Chapter 9.

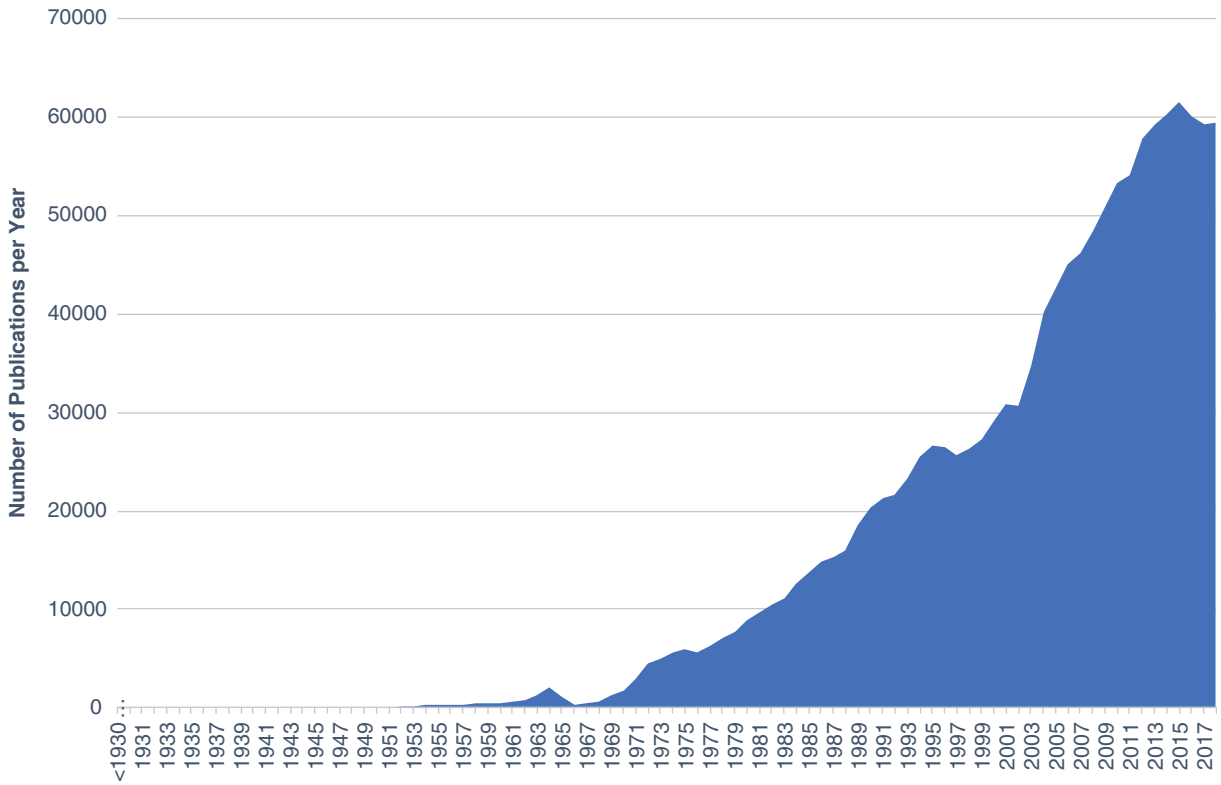


Fig. 1.2. Growth of cell and tissue culture. A search was performed in the PubMed database using the text string ("tissue culture" OR "cell culture" OR "cultured cells" OR "cell line"). A total of 1 395 112 items were found and are displayed by year of publication. Results shown here date from before 1930 to 2018; the years 2019–2020 (n = 32 279) were incomplete and were thus omitted. More than half of these results (n = 809 041) could be found using a single term "cell line."

Although many of the classic media formulations were intended for use without embryo extract or serum, it has been difficult to remove serum entirely, in what Honor Fell once described as a "tiresome fact" of tissue culture (Fell 1972). Extensive progress has since been made in developing serum-free culture media, thanks to the efforts of Richard Ham, Gordon Sato, and colleagues in the 1970s and 1980s (Ham and McKeehan 1978; Barnes and Sato 1980). More recently, we are indebted to James Thomson and other modern pioneers who have developed defined media for stem cell culture (Ludwig et al. 2006; Chen et al. 2011). The development and use of serum-free media are described in Chapter 10.

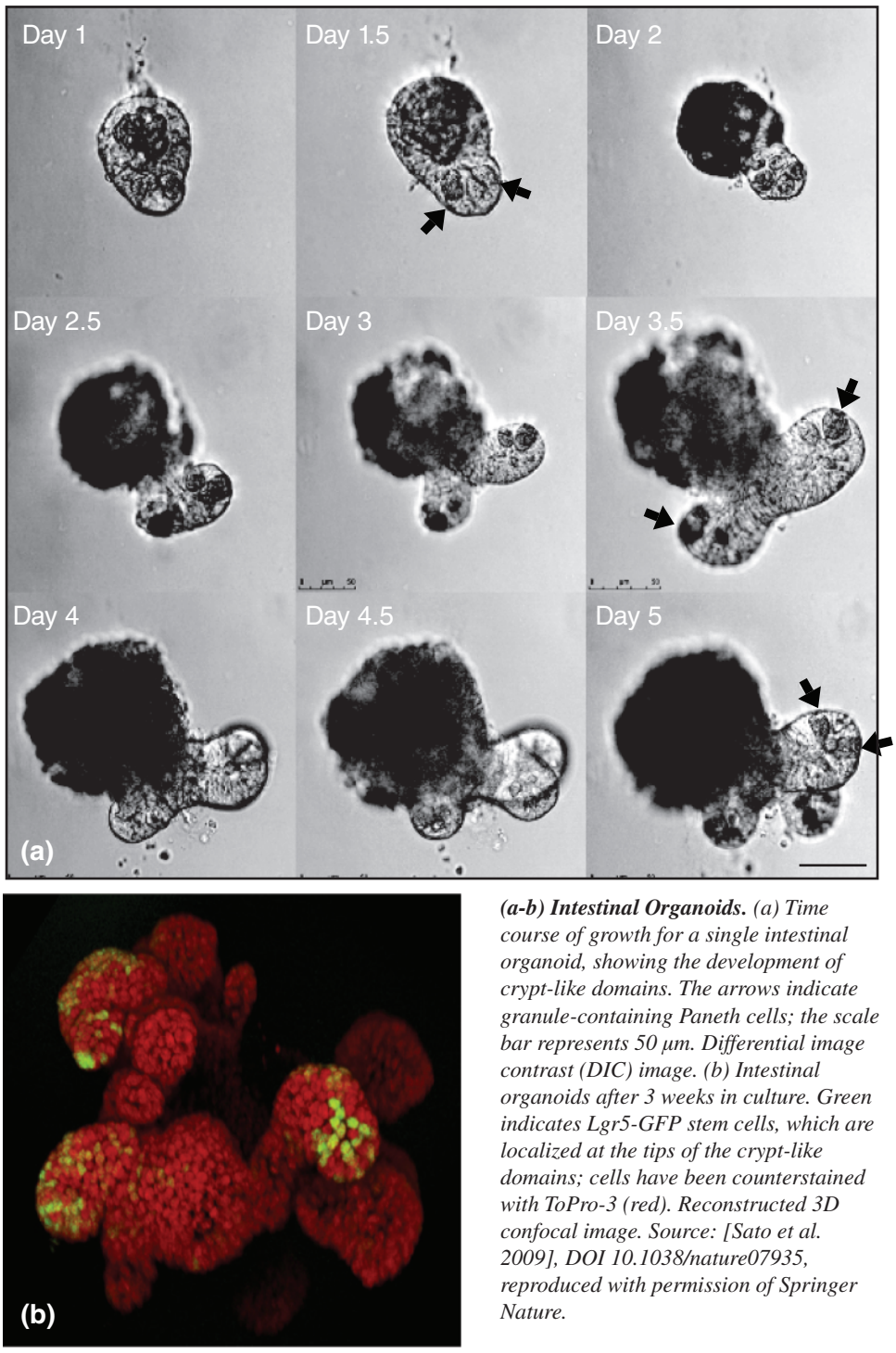
1.2.2 Primary Cultures and Cell Lines

The development of suitable substrates and media meant that cells could be grown at a larger scale and for longer time periods, requiring transfer from one culture vessel to another. This process is known as subculture or passage. Before the first subculture is performed, the sample is known as a primary culture. Cells in a primary culture may have migrated from an explant or may be released from it through disaggregation (see Figure 1.4). Disaggregation of tissue fragments and plating out of the dispersed cells was initially performed in 1916 using trypsin, although this enzyme was not adopted for subculture until some years later (Rous and Jones 1916; Dulbecco 1952). Once a primary culture has been passaged, it becomes a cell line, at least according to the consensus terminology (see Section 1.1.2). More information on the changes that occur throughout the lifespan of a culture can be found in Chapter 3. Procedures for generating primary cultures and passaging cell lines are described in Chapters 13 and 14.

Subculture (particularly the first subculture to form a cell line) implies (i) an increase in the total number of cells over several generations (population doublings) and (ii) the ultimate predominance of cells or cell lineages with a high proliferative capacity, resulting in (iii) increased uniformity in the cell population. These are all advantages, but there are also disadvantages that must be considered (see Table 1.2). Subculture results in an increased risk of contamination by microorganisms or by other cultures. Prevention of contamination relies on good aseptic technique, which is discussed in Chapter 12.

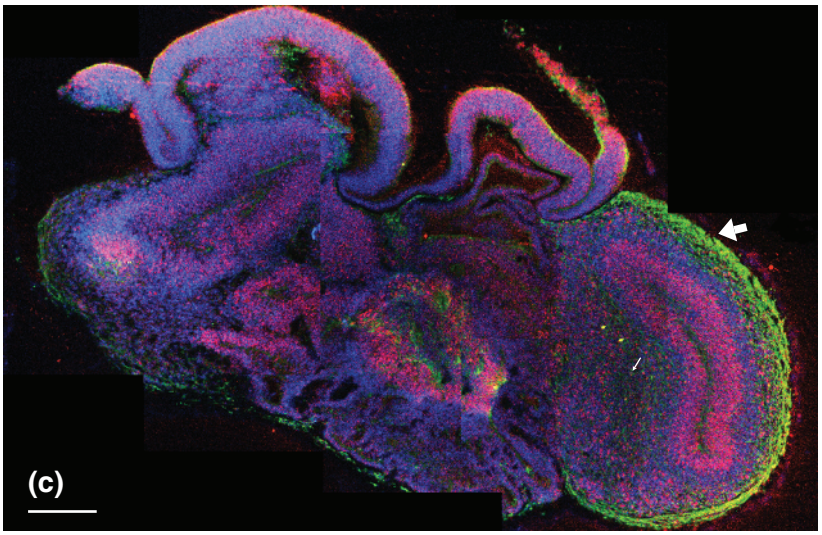
With continued subculture, cells may display changes in their genetic makeup (genotype) or their observable physical characteristics (phenotype). Cell lines must be preserved at early passage if these problems are to be avoided. Procedures for cryopreservation and cell banking are described in Chapter 15; validation and characterization of cell lines are discussed in Chapters 16–19.

Many cell lines cease to grow after repeated subculture. This was originally thought to be due to poor culture conditions, until Leonard Hayflick and Paul Moorhead



(a-b) Intestinal Organoids. *(a) Time course of growth for a single intestinal organoid, showing the development of crypt-like domains. The arrows indicate granule-containing Paneth cells; the scale bar represents 50 μm. Differential image contrast (DIC) image. (b) Intestinal organoids after 3 weeks in culture. Green indicates Lgr5-GFP stem cells, which are localized at the tips of the crypt-like domains; cells have been counterstained with ToPro-3 (red). Reconstructed 3D confocal image. Source: [Sato et al. 2009], DOI 10.1038/nature07935, reproduced with permission of Springer Nature.*

Fig. 1.3. Examples of organoid culture.



(c) Cerebral Organoid. *Cerebral organoids can be generated using a multistep procedure (see Fig. 27.12). Sectioning and immunohistochemistry reveal complex morphology, with heterogeneous regions containing neural progenitors (Sox2, red) and neurons (Tuj1, green; see arrow). DNA is indicated by Hoechst staining (blue). The scale bar represents 200 μm. Source: [Lancaster et al. 2013], DOI 10.1038/nature12517, reproduced with permission of Springer Nature.*

Fig. 1.3. (*Continued*)

proved that normal fibroblasts consistently ceased to divide after a certain number of generations (now known as the Hayflick limit) (Hayflick and Moorhead 1961). A subset of cell lines continue to divide even after the Hayflick limit has been reached and are known as continuous or immortalized cell lines. Hilary Koprowski and colleagues discovered that simian virus 40 (SV40) could be used to induce "transformation" of the culture, which was associated with an immortal lifespan (Koprowski et al. 1962; Girardi et al. 1965). Study of oncogenic viruses led to a better understanding of the genes that were required for immortalization and how to deliver them to the cell with greater specificity. Gene delivery, editing, and immortalization are discussed in Chapter 22.

Some cell types are more difficult to grow than others, leading to the need for optimized culture conditions. The feeder layer, which was developed by Theodore Puck and Philip Marcus for cell cloning, was a particularly important innovation in this area (Puck and Marcus 1955; Marcus et al. 2006). Increasing familiarity with the feeder layer and its interaction with other cell types led to the first cellular therapies, with Howard Green and colleagues using feeder layers to culture human keratinocytes for the treatment of burns (Green et al. 1979; Green 2008). In retrospect, Green's work represented the first use of stem cell therapy, since keratinocyte expansion depends on the maintenance of epidermal stem cells (Hynds et al. 2018). Feeder layers were then used for the culture of embryonic stem cells (ESCs) and for induction of pluripotency in adult cells (Evans and Kaufman 1981; Martin 1981; Thomson et al. 1998; Takahashi and Yamanaka 2006; Takahashi et al. 2007). Although stem cells can now be grown feeder-free, feeder layers continue to play an important role in the culture of specific cell types. Procedures for the culture of stem cells (including induction of pluripotency), specific cell types, and tumor cells are described in Chapters 23–25. Induction of differentiation is a challenge for many cell types and is discussed in Chapter 26.

1.2.3 Organ, Organotypic, and Organoid Culture

Although cell lines are a mainstay of most tissue culture laboratories, concerns have been expressed for many years regarding their suitability to model *in vivo* behavior. Perhaps the earliest concern was expressed by David Thomson, who observed that dispersed cells displayed uncontrolled growth compared to cells that are maintained *in situ* (Thomson 1914). This observation was the first step in what came to be known as "organ culture" (see Figure 1.4). Rather than trying to establish a cell line, the proponents of organ culture aimed to study cells in their original environment with minimal changes to the tissue architecture. Organ culture techniques were pioneered by Thomas Strangeways, Honor Fell, and colleagues at the Strangeways Research Laboratory (Strangeways and Fell 1925; Fell and Robison 1929; Fell 1972). Organ culture was technically challenging but was believed to be the best way to study physiological behavior,

TABLE 1.1. Technical innovations in tissue culture development.

Year of publication	Technical innovation	Book section where innovation discussed	References
1907	Primary explant of frog embryo nerve fiber in a hanging drop, leading to outgrowth *in vitro*	1.1.1, 27.4.1	Harrison (1907)
1910	Use of plasma clot to culture tissue fragments; culture of chick embryonic tissue fragments (explants)	1.1.1	Burrows (1910)
1916	Trypsinization of explants	1.2.1	Rous and Jones (1916)
1923	Development of Carrel flasks	8.5.3	Carrel (1923)
1925	Differentiation of embryonic tissue in organ culture	1.2.3, 27.8	Strangeways and Fell (1925)
1928	Time-lapse recording of live cell behavior	18.4.3	Canti (1928)
1929	Use of watch-glass method for organ culture	1.2.3	Fell and Robison (1929)
1933	Development of the roller tube	28.3.1	Gey (1933)
1936	Culture of poliovirus in primary embryonic nervous tissue	1.3	Sabin and Olitsky (1936)
1943	Establishment of the first continuous cell line (L)	1.1.1	Earle et al. (1943)
1947	Culture of L-cells on the first defined substrate	1.2.1	Evans et al. (1947)
1948	Capillary cloning of L-cells to give L-929	1.1.1	Sanford et al. (1948)
	Use of antibiotics in culture	9.6.2	Keilova (1948)
1949	Use of glycerol as a cryoprotective agent	15.1.1	Polge et al. (1949)
	Use of the Coulter principle for automated cell counting	19.1.2	Coulter (1949)
1950	Development of Medium 199 for chick embryo fibroblasts and organ culture	1.2.1, 9.4	Morgan et al. (1950)
1951	Culture of cells in three dimensions using cellulose sponge, folded cellophane, or glass rings	1.2.1, 27.5	Earle et al. (1951); Leighton (1951)
	Use of a hemocytometer for counting cultured cells	19.1.1	Sanford et al. (1951)
1952	Establishment of the first human cell line (HeLa)	1.1.1, 1.3	Gey et al. (1952)
	Use of trypsin for subculture	1.2.2	Dulbecco (1952)
	Organotypic culture performed by inducing dissociated cells to reaggregate in 3D culture	1.2.3	Moscona and Moscona (1952)
1953–1955	Mass production of HeLa cells for polio vaccination program	1.3	Syverton and Scherer (1953)
1953	Demonstration of contact inhibition by time-lapse cinematography	3.6.2	Abercrombie and Heaysman (1953)
	Culture of cells on either side of a membrane filter (leading to the development of filter well inserts)	27.5.2	Grobstein (1953)
1954	Culture of cells in suspension; development of shaker culture	8.6.2, 14.7	Earle et al. (1954); Owens et al. (1954)
	Development of the modified Carrel flask (the "T-flask")	8.5.3	Earle and Highhouse (1954)

(*continued*)

TABLE 1.1. (*continued*)

Year of publication	Technical innovation	Book section where innovation discussed	References
1955	Production of irradiated feeder layers for use in cloning	8.4, 20.6.2	Puck and Marcus (1955)
	Development of BME for culture of L-929 and HeLa cells	9.4	Eagle (1955)
1956	Culture of cells on rat tail collagen	8.3.3	Ehrmann and Gey (1956)
	Demonstration of mycoplasma contamination	16.4	Robinson et al. (1956)
1957	Use of collagenase for disaggregation of mammary glands	13.5.3	Lasfargues (1957)
	Development of spinner culture	28.2.1	McLimans et al. (1957)
	Development of "Friend cells" as a model for erythroid differentiation	24.5.3, 26.3.1	Friend (1957)
1958	Use of stirred-tank systems for scale-up of mammalian culture	28.2.2	Ziegler et al. (1958)
1959	Use of dimethyl sulfoxide (DMSO) as a cryoprotective agent	15.1.1	Lovelock and Bishop (1959)
	Demonstration of interspecies contamination	17.3.4	Rothfels et al. (1959)
1961	Definition of finite lifespan of normal human fibroblasts	1.2.2, 3.2.1	Hayflick and Moorhead (1961)
1962	Transformation of cells obtained from tissue explants using SV40	22.3.1	Koprowski et al. (1962)
	Development of Grace's medium for insect culture	24.6.2	Grace (1962)
1963	Establishment of the WI-38 cell line for viral vaccine production	1.3	Hayflick (1963)
	Development of protocols to establish 3T3 mouse embryonic fibroblast (MEF) cell lines	3.2.1	Todaro and Green (1963)
1964	Cloning of cells in suspension using soft agar	20.3.1	Macpherson and Montagnier (1964)
	Formation of embryoid bodies; cloning of embryonal carcinoma cells to assess pluripotency	23.2	Kleinsmith and Pierce (1964)
1965	Development of serum-free media and defined growth factors for cloning and for normal and specialized cell types	10.2, 10.3	Ham (1965); Barnes and Sato (1980)
1966	Colony formation by hematopoietic cells	20.3	Bradley and Metcalf (1966); Ichikawa et al. (1966)
1967	Demonstration of intraspecies cross-contamination with HeLa	17.3.1	Gartler (1967)
	Isolation of lymphoblastoid cell lines (LCLs)	22.3.1	Moore et al. (1967)
	Culture of cells on microcarriers	27.5.4, 28.3.3	van Wezel (1967)
1968	Use of a "biohazard hood" to prevent infection during microbiological procedures	6.3.4	Coriell and McGarrity (1968)
1969	Transfer of human tumor cells to "nude" mice	25.4.5	Rygaard and Povlsen (1969)

(*continued*

TABLE 1.1. (*continued*)

Year of publication	Technical innovation	Book section where innovation discussed	References
1970	High density culture using perfusion	28.2.3	Kruse et al. (1970)
1971	Culture of mouse epidermal keratinocytes	24.2.1	Fusenig (1971)
	Production of feeder layers using mitomycin C	20.6.2	Macpherson and Bryden (1971)
	Culture of tumor cells as spheroids in suspension	25.4.4, 27.4.1	Sutherland et al. (1971)
1972	Primary culture of hepatocytes disaggregated in collagenase	24.2.6	Leffert and Paul (1972)
	Perfused culture in hollow fibers	27.5.3	Knazek et al. (1972)
1973	Transfection using calcium phosphate for gene delivery	22.1.1	Graham and van der Eb (1973)
1974	Use of Giemsa banding for detection of misidentified cell lines	17.3.4	Nelson-Rees et al. (1974)
1975	Culture of human epidermal keratinocytes using feeder layers of 3T3 mouse fibroblasts	1.2.2, 24.2.1	Rheinwald and Green (1975)
	Culture of hepatocytes on floating collagen membranes	8.3.3	Michalopoulos and Pitot (1975)
	Production of monoclonal antibodies by hybridomas (mouse myelocyte–splenocyte hybrids)	24.5.4	Köhler and Milstein (1975)
1977	Culture of normal human fibroblasts under hypoxic conditions	5.3.1	Packer and Fuehr (1977)
	Characterization of matrix from Engelbreth-Holm-Swarm (EHS) mouse sarcoma (resulting in Matrigel® and other products)	8.3.2	Orkin et al. (1977)
	Detection of mycoplasma by DNA fluorescence	16.4.1	Chen (1977)
	Suspension cloning using Methocel	20.3.2	Dao et al. (1977)
1979	Growth of human epidermal keratinocytes for use as skin grafts	1.2.2, 24.2.1	Green et al. (1979)
1981	Culture of mouse embryonic stem cells (mESCs)	23.2.1	Evans and Kaufman (1981); Martin (1981)
1982	Use of electroporation for gene delivery	22.1.3	Neumann et al. (1982)
1983	Immortalization of human diploid fibroblasts by SV40	22.3.1	Huschtscha and Holliday (1983)
	Development of baculovirus expression vectors (BEVs)	22.1.4	Smith et al. (1983)
1984	Induction of differentiation in pluripotent embryonal carcinoma cells	23.2	Andrews et al. (1984)
	Development of stereolithography (later used for 3D bioprinting)	28.7.3	Hull (1984)
1986	Development of confocal microscopy	18.4.4	Petran et al. (1986)
1987	Use of lipofection for gene delivery	22.1.2	Felgner et al. (1987)
1990	Use of retroviral vectors for gene delivery	22.1.4	Miller et al. (1990)
1991	Culture of human adult mesenchymal stromal cells (MSCs)	23.5, 23.6	Caplan (1991)

(*continued*

TABLE 1.1. (*continued*)

Year of publication	Technical innovation	Book section where innovation discussed	References
1992	Culture of primary breast epithelial cells within an EHS matrix, resulting in 3D structures	27.5.1	Petersen et al. (1992)
1993	Generation of chimeric antigen receptor (CAR) T-cells	24.5.5	Eshhar et al. (1993)
1996	Development of zinc finger nucleases (ZFNs)	22.2.1	Kim et al. (1996)
1998	Culture of human embryonic stem cells (hESCs)	23.2.2	Thomson et al. (1998)
1999	Use of short tandem repeat (STR) profiling for authentication of cell lines	17.3.2	Tanabe et al. (1999); Masters et al. (2001)
	Large-scale culture of suspension cells in disposable bioreactor using wave-like agitation	28.2.2	Singh (1999)
2006	Induction of pluripotency in mouse embryonic and adult fibroblasts using defined factors	23.3	Takahashi and Yamanaka (2006)
	Establishment of hESC cultures using defined conditions	23.4.2	Ludwig et al. (2006)
2007	Induction of pluripotency in human adult fibroblasts	23.3	Takahashi et al. (2007)
	Use of DNA barcoding for species identification in cell lines	17.3.3	Cooper et al. (2007)
2008	Development of cortical structures in spheroid microplates	27.6	Eiraku et al. (2008)
2009	Establishment of intestinal organoids from adult stem cells in the intestinal crypt	27.6	Sato et al. (2009)
2010	Use of CAR T-cells for treatment of lymphoma	24.5.5	Kochenderfer et al. (2010)
2011	Development of chemically defined conditions for culture of human induced pluripotent stem cells (iPSCs)	23.4.2	Chen et al. (2011)
2012	Use of clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) proteins for gene editing	22.2.3	Jinek et al. (2012)
	Development of conditional reprogramming using Rho kinase (ROCK) inhibitor and 3T3 feeder layers	22.3.4	Liu et al. (2012)
2013	Establishment of cerebral organoids that model human brain development	27.6	Lancaster et al. (2013)

particularly in complex structures such as the nervous system and the developing embryo.

In 1952, Aron Moscona performed a classic experiment that would eventually lead to today's 3D culture models (Moscona and Moscona 1952). Using techniques that he learned at the Strangeways Research Laboratories, Moscona used trypsin to dissociate cells from several chick embryonic organs, and then placed the cells in close association in the hollow of a ground slide – a technique known as the "watch-glass" method (Fell 1972). The dissociated cells reaggregated to form structures that reflected their tissues of origin. This type of culture, where cells from multiple lineages are brought together to recapitulate the original tissue, is referred to here as organotypic culture (see Figure 1.4). Organotypic culture has provided new prospects for the study of cell interaction among discrete, defined populations of homogeneous and potentially genetically and phenotypically defined cells. It has also provided exciting opportunities to develop "tissue equivalent" models for toxicity testing, such as keratinocyte/fibroblast co-culture systems, and to build constructs for tissue engineering (Stark et al. 1999; Vunjak-Novakovic and Freshney 2006).

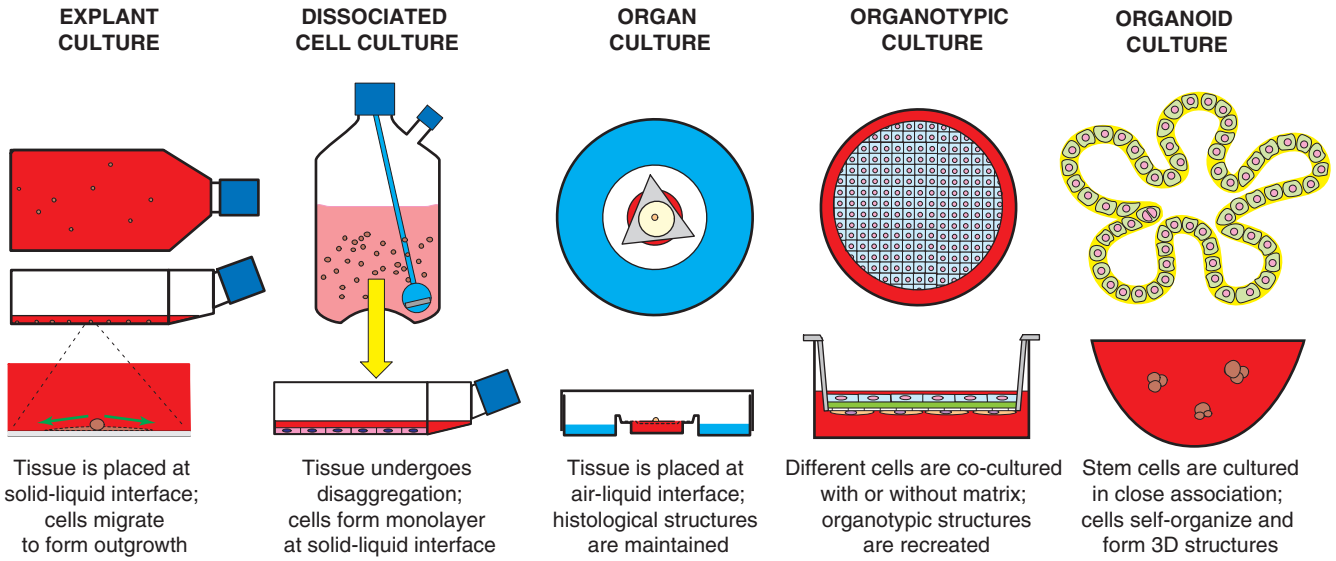


Fig. 1.4. Types of tissue culture. Additional terms may be used to describe 3D culture; see Figure 27.1.

TABLE 1.2. Subculture.

Advantages	Disadvantages
Propagation	Trauma of enzymatic or mechanical dissociation
More cells	Overgrowth of unspecialized or stromal cells
Possibility of cloning	Selection of cells adapted to culture
Expanded stocks	Loss of differentiated properties
Increased homogeneity	Genetic instability
Characterization, authentication	Change in relative abundance of clonal populations
Cryopreservation	Increased risk of microbial contamination
Distribution to multiple laboratories	Increased risk of cross-contamination

In 2008, Yoshiki Sasai and colleagues discovered that ESCs could spontaneously self-organize to form polarized cortical tissues if they were grown in close association in a spheroid microplate – an environment that is strikingly similar to the "watch-glass" method (Eiraku et al. 2008). Such "organoid cultures" can be grown from pluripotent stem cells (PSCs) or adult stem cells. The potential of the latter approach has been shown by Hans Clevers and colleagues, in a series of elegant studies on LGR5-positive stem cells in the intestinal crypt and in other epithelial tissues (see Figure 1.3a, b) (Clevers 2016). This type of culture has tremendous possibilities for personalized therapy. For example, it is now possible to develop a biobank of organoids from patients with colorectal carcinoma, and use these cultures for genomic characterization and personalized drug screening (van de Wetering et al. 2015).

1.3 APPLICATIONS

Initially, the development of cell culture owed much to the needs of two major branches of medical research: the cultivation of viruses and the understanding of neoplasia. The standardization of conditions and cell lines for the cultivation of viruses undoubtedly provided much impetus to the development of modern tissue culture technology, particularly the production of large numbers of cells that were suitable for biochemical and molecular analysis. This and other technical improvements, which were made possible by the commercial supply of reliable media and sera and by the greater control of contamination with antibiotics and laminar flow equipment, made tissue culture more accessible and resulted in a broad range of applications (see Figure 1.5). The field has moved from being exploratory research, conducted by a few individuals, to becoming a major tool in cell and molecular biology,

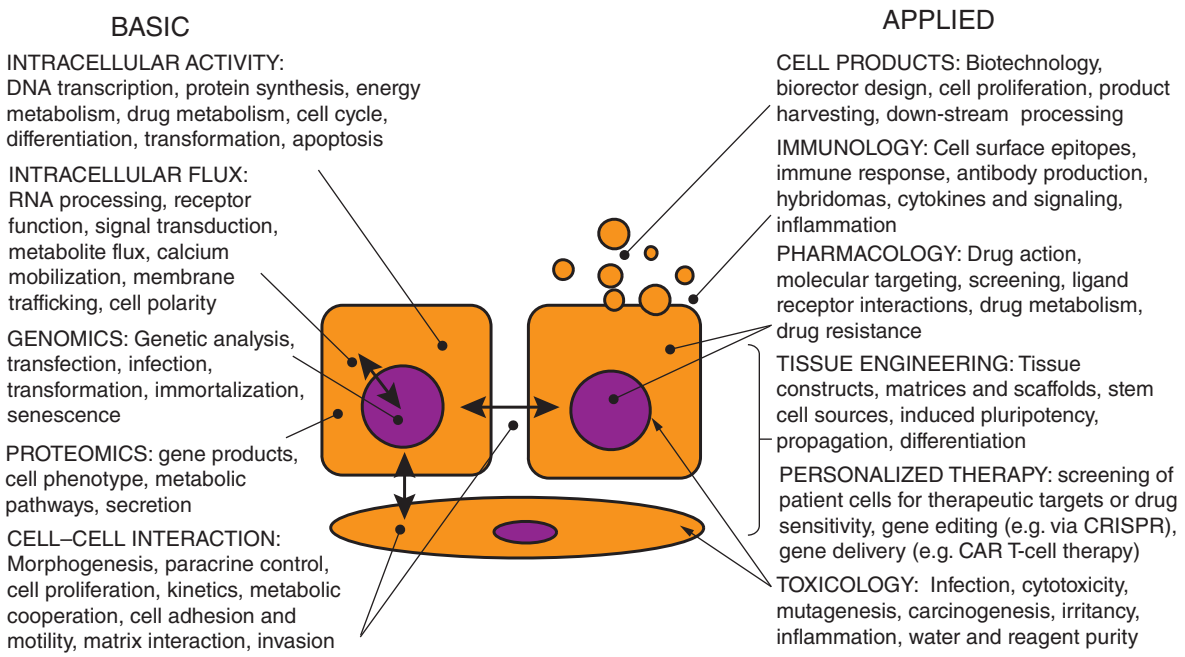


Fig. 1.5. Tissue culture applications. Applications are broadly divided into "basic" and "applied," but in many cases, tissue culture is applied in both areas.

virology, bioengineering, and industrial pharmaceutics on a scale that would have astonished the early workers.

Vaccine development is perhaps the oldest tissue culture application and can be used to illustrate its effect on society. Tissue culture was initially used to grow poliovirus in the 1930s (Sabin and Olitsky 1936). In 1952, the newly established HeLa cell line was found to act as a good host for poliovirus and was mass produced for evaluation of the Salk polio vaccine (see Figure 1.6) (Brown and Henderson 1983). Primary cultures were initially used to prepare the vaccine itself, but it was quickly recognized that this resulted in unacceptable safety concerns (Koprowski et al. 1962). The WI-38 cell line was established in an effort to provide a safer alternative and selected, after extensive testing, for the production of polio vaccine and, subsequently, for rubella and other vaccines (Hayflick et al. 1962; Plotkin et al. 1969). Recently, it was estimated that 450 000 deaths have been averted in the United States (and roughly 10.3 million globally) due to vaccines developed using WI-38 cells (Olshansky and Hayflick 2017). More than 50 human viral vaccines have now been manufactured using WI-38 and other cell lines (Gallo-Ramirez et al. 2015). The Vero cell line, which was derived from a female monkey (*Chlorocebus sabaeus*), is highly susceptible to many viruses and can now be used for rapid vaccine development against emerging pathogens. Vero cells have been used to cultivate H5N1 influenza virus, severe acute respiratory system (SARS) coronaviruses, and other global threats to public health (Barrett et al. 2017).

Other applications that have arisen from tissue culture and are now widespread include (i) *in vitro* fertilization (IVF), which developed from early advances in embryo culture (Edwards 1996); (ii) manufacture of tissue grafts and other cellular therapies, starting with Howard Green's skin transplants and extending to today's 3D constructs (Hynds et al. 2018); (iii) generation of hormones, growth factors, antibodies, and other biological products (Sato et al. 2010); (iv) *in vitro* diagnostic techniques such as amniocentesis; and (v) *in vitro* toxicity testing for pharmaceuticals, medical devices, and numerous other products (Shukla et al. 2010). Although the benefits of these applications are clear, tissue culture has had a complex impact on society in a broader sense. Concerns regarding lack of consent for the establishment of cell lines in the early years of tissue culture have led to clear ethical requirements regarding the use of human tissue for research (Beskow 2016). Ethical issues will continue to arise in relation to gene editing, organoid culture, and other emerging applications (Munsie and Gyngell 2018). The basic principles that underlie safety and ethics in tissue culture are discussed in Chapter 6.

1.4 ADVANTAGES OF TISSUE CULTURE

1.4.1 Environmental Control

Two major advantages of tissue culture (see Table 1.3) are the ability to control the physiochemical environment (including

Fig. 1.6. Mass production of HeLa cells for polio vaccine testing. James C. Harris of the Tuskegee Institute, preparing culture tubes for use in field trials of the polio vaccine. The stainless-steel racks shown were designed by William F. Scherer to minimize handling of individual tubes. By July 1955, the Tuskegee team had shipped approximately 600 000 cultures to laboratories across the United States. *Source*: Brown and Henderson (1983), reproduced with permission of Oxford University Press.

pH, temperature, osmotic pressure, and O_2 and CO_2 tension) and the physiological conditions. The former can be controlled very precisely, whereas the latter must be kept relatively constant but cannot always be defined where cell lines still require supplementation of the medium with serum or other poorly defined constituents. These supplements are prone to batch variation and contain undefined elements such as hormones and other stimulants and inhibitors. The identification of some of the essential components of serum, together with a better understanding of factors regulating cell proliferation, has made the replacement of serum with defined constituents feasible, as explained in Chapters 9 and 10.

The role of the extracellular matrix (ECM) is important but subject to similar limitations as the use of serum – that is, the matrix is often necessary, but not always precisely defined. Prospects for defined ECM have improved, however, as its individual constituents have been identified and manufactured. It is now possible to develop "designer matrices" that provide an optimized microenvironment for specific cell types. Although matrix design requires specialized expertise, most laboratories now have access to a range of ECM coatings and mimetic treatments, as described in Chapter 8.

1.4.2 Homogeneity and Characterization

Tissue samples are invariably heterogeneous. Replicates, even from one tissue, vary in their constituent cell types. By contrast, cultured cell lines assume a homogeneous (or at least uniform) constitution after one or two passages. The cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. Hence, subdivided cultures will act as biological replicates, allowing further expansion, cryopreservation, characterization, or use in experiments. Homogeneity can be increased by performing cell cloning or by selecting or separating cells within the culture based on specific characteristics. These techniques allow the characteristics of the culture to be better understood and preserved for as long as suitable frozen stocks are retained in cryostorage. Cryopreservation and cell line characterization are discussed in Chapters 15 and 18. Techniques for cell cloning, selection, and separation are discussed in Chapters 20 and 21.

1.4.3 Economy, Scale, and Automation

Exposure of a culture to a drug or other reagent can occur at a lower, and more precisely defined, concentration and with direct access to the cell. Consequently, less reagent is required than for injection *in vivo*, where > 90% may be lost by distribution to tissues other than those under study and by metabolism and excretion. Cell lines can be scaled up to increase the number of cells that are produced (e.g. for manufacture of biological products at industrial scale) or the number of replicates (e.g. for high-throughput screening of therapeutic products). Large-scale procedures can also be automated for increased speed, reproducibility, or convenience. Scale-up of cell culture processes, including high-throughput screening and automation, is described in Chapter 28.

1.4.4 Replacement of *In Vivo* Models

As the various types of tissue culture have become more extensively characterized, they have gained acceptance as models for toxicology testing alongside *in vivo* animal models. Cell lines can be used to detect environmental pollutants and to test various commercial products for hazardous properties, including pharmaceuticals and personal care products such as shampoos and cosmetics. For example, the rainbow trout cell line RTgill-W1 is sensitive to many environmental toxins, making it a suitable model for toxicity testing of environmental samples (Lee et al. 2009). Panels of tumor cell

TABLE 1.3. Advantages of tissue culture.

Category	Advantages
Physicochemical environment	Control of pH, temperature, osmolality, and dissolved gases
Physiological conditions	Control of hormone, growth factor, and nutrient concentrations
Microenvironment	Regulation of matrix, cell–cell interaction, and gaseous diffusion
Cell line homogeneity	Replicates can be prepared; cell cloning or other techniques can be used to increase homogeneity
Preservation	Cells can undergo cryopreservation and be stored in cryofreezers
Characterization	Genomic analysis, immunostaining, and other forms of characterization are easily performed
Validation	Purity and authenticity can be specifically tested to demonstrate lack of contamination or misidentification
Certification	Safety and efficacy testing can be performed to confirm that a cell line is fit for purpose, e.g. for use in vaccine production
Replicates and variability	Quantitation is easy and statistical analysis is usually straightforward; multiple replicates are easily generated
Reagent saving	Reduced volumes, direct access to cells, lower costs
Control of $C \times T$	Ability to define dose, concentration, and time (duration of exposure)
Automation and mechanization	Scale-up and automation can be performed using bioreactors, high-throughput liquid handling systems, or robotic platforms
Scale	Culture volumes from a few microliters to 20 000 l
Time saving	Assay time can be reduced at least by an order of magnitude
Reduction of animal use	Toxicity testing of pharmaceutics, cosmetics, etc. can be performed using validated culture systems

lines have been assembled for high-throughput screening of anticancer drugs, resulting in the discovery of new therapeutic substances. For example, ovarian cell lines played an important role in the development of cisplatin and related drugs for ovarian carcinoma (Kelland et al. 1992).

The development of 3D culture models, with a more accurate replication of the *in vivo* cell phenotype, has increased the accuracy of *in vitro* modeling and the relevance of targeted metabolic pathways. This field has been particularly driven by expressions of concern by many community members and advocacy groups over the use of animals for scientific purposes. The need for replacement, reduction, and refinement (the 3Rs) was proposed by William Russell and Rex Birch more than 50 years ago (Russell and Birch 1959). Although Russell and Birch foresaw that tissue culture could replace animal models, cell lines and the other models available in their era could not be used to study complex physiological processes such as inflammation. Over time, the continued push for alternative testing has led to the development of organotypic "tissue equivalent" models that have undergone validation for use in toxicity testing (Sheasgreen et al. 2009). These systems can be used to replace animals in some assays, such as the Draize test for acute skin and eye irritation. Alternative systems have become more widely used and promoted following the adoption of regulations to limit animal-based testing in the European Union and elsewhere. The role of tissue culture in toxicity testing is discussed in Chapter 29.

1.5 LIMITATIONS OF TISSUE CULTURE

1.5.1 Quality and Expertise

Cell lines are the commonest cultures to be found in today's research laboratories. Although the advantages of cell lines are obvious, their development has brought with it a number of unforeseen problems (see Table 1.4). The observation that differentiated properties were lost in culture alerted early workers to the problems of dedifferentiation and selection and the fact that cell lines may be genetically, as well as phenotypically, unstable. Unfortunately, the early workers could not predict that cell lines would (i) provide an ideal substrate for the growth of mycoplasma, and (ii) extend the risks of cross-contamination and misidentification that were already familiar to microbiologists. Both problems continue to be frequently overlooked by today's workers, despite access to modern detection methods. This book and a number of expert guidelines on GCCP reinforce the need for validation to minimize the risks of microbial contamination, misidentification, and cross-contamination (Coecke et al. 2005; Geraghty et al. 2014; OECD 2018). More information on these topics and

TABLE 1.4. Limitations of tissue culture.

Category	Disadvantaged or limitations
Quality	Microbial contamination or cross-contamination may occur, e.g. due to contact with aerosols from other cultures
	Chemical contamination may arise, e.g. due to use of shared glassware from experimental work
Genotype	Continuous cell lines display genomic instability
	Passaging leads to clonal evolution and changes in genotype
Phenotype	Selective overgrowth may occur with unwanted cell types
	Passaging leads to clonal evolution and changes in phenotype
	Conditions favor uncontrolled proliferation over differentiation
Expertise	Aseptic technique must be used to minimize contamination risks
	Training is necessary to teach GCCP
	Validation testing is necessary to detect some forms of contamination
Laboratory	A separate sterile handling area is required
	Biological containment may require laboratory certification
	Waste may require decontamination as part of safe disposal
Equipment	A biological safety cabinet (BSC) is typically used, based on risk assessment and requirements for laminar airflow
	Basic equipment is required for tissue culture
Reagents	Reagents, substrates, etc. must be sterile and of suitable purity
	Procedures for preparation and sterilization must be adhered to
Quantity	Scale-up or high-throughput applications require capital investment
Cost	Serum, growth factors, and other reagents can be expensive
	Disposable plasticware is expensive and results in increased waste
Application	Geometry and microenvironment changes cell function
	Difficult to grow some species and cell types
	Difficult to model ingestion, absorption, distribution, metabolism, and excretion in pharmacokinetic studies

problems with reproducibility when using cell culture models can be found in Chapters 7, 16, and 17.

Tissue culture requires training and expertise for such problems to be avoided. Handling must be performed under strict aseptic conditions, because animal cells grow much less rapidly than many of the common contaminants, such as bacteria, molds, and yeasts. Contaminants may include pathogens, so containment is also necessary for safe handling of living cultures. Unlike microorganisms, cells from multicellular animals do not normally exist in isolation and, consequently, are not able to sustain an independent existence without the provision of a complex environment simulating blood plasma or interstitial fluid. These conditions imply a level of skill and understanding on the part of the operator in order to appreciate the requirements of the system and to diagnose problems as they arise. Hence, tissue culture should not be undertaken casually to run one or two experiments, but requires proper training, strict control of procedures, and a controlled environment. The design and layout of a tissue culture laboratory is discussed in Chapter 4, and the necessary equipment and safety requirements are described in Chapters 5 and 6. Training and problem solving are explored in Chapters 30 and 31.

1.5.2 Quantity and Cost

A major limitation of cell culture is the expenditure of effort and materials that goes into the production of relatively little tissue. A realistic maximum per batch for most small laboratories (with two or three people doing tissue culture) might be 1–10 g (wet weight) of cells. With a little more effort and the facilities of a larger laboratory, 10–100 g is possible; > 100 g implies industrial pilot-plant scale, a level that is beyond the reach of most laboratories but is not impossible if special facilities are provided. If industrial manufacture is performed, it is possible to generate kilogram quantities of cells.

The cost of producing cells in culture, excluding capital and labor costs, is about 10 times that of animal tissue. When the world's first tissue culture grown hamburger was eaten in London in 2013, the creator, Mark Post of Maastricht, reckoned it cost about €250 000! If large amounts of tissue (> 10 g) are required, the reasons for providing them by culture must be very compelling. For smaller amounts of tissue (~ 10 g), the costs are more readily absorbed into routine expenditure, but it is always worth considering whether assays or preparative procedures can be scaled down or automated. Automation is likely to come with a significant up-front cost but is worthwhile if costs are balanced by long-term savings

due to reduced manual labor. Microscale and nanoscale assays can often be quicker (because of reduced manipulation times, volumes, etc.) and may be more readily automated. Such assays typically involve microfluidic systems, which are discussed in Chapter 21. Scale-up and automation are explored in Chapter 28.

1.5.3 Limited Species and Cell Types

Ross Harrison chose the frog as his source of tissue for initial experiments in tissue culture (Harrison 1907), presumably because it was a cold-blooded animal and, consequently, incubation was not required. Because tissue regeneration is more common in lower vertebrates, Harrison may also have felt that growth was more likely when compared to mammalian tissue. Although his technique initiated a new wave of interest in the cultivation of tissue *in vitro*, few later workers were to follow his example in the selection of species. The accessibility of different tissues, many of which grew well in culture, originally made the embryonated hen's egg a favorite choice. However, the well-established genetic background of the mouse, the success of experimental animal husbandry (particularly with genetically pure strains of rodents), and the development of transgenic mouse technology led to the selection of this animal as a favorite species.

Once the first human cell line had been established, human tissue became a favorite source of cells, helped later by the classic studies of Leonard Hayflick on the finite lifespan of cells in culture (Hayflick and Moorhead 1961) and the preference of virologists and molecular geneticists to work with human material. However, it has been difficult to establish cell lines from some human tissues. Over time, serum-free selective media have been optimized for epidermal keratinocytes, bronchial epithelium, vascular endothelium, and many other cell types. Some of these selective media are available commercially, although the cost remains high relative to the cost of regular media. Techniques have also been developed to extend the lifespan of certain cell types, such as the use of feeder layers for keratinocyte cultures and for conditional reprogramming of some epithelial cells (Rheinwald and Green 1975; Liu et al. 2012).

Analysis of data from the Cellosaurus knowledge resource shows that although cell lines have been established from more than 660 species, most are derived from a small subset of species (Bairoch 2018). As of January 2020, the most common species were (i) human (*Homo sapiens*, 87 495 cell lines); (ii) mouse (*Mus musculus*, 20 817 cell lines); (iii) rat (*Rattus norvegicus*, 2131 cell lines); (iv) Chinese hamster (*Cricetulus griseus*, 1736 cell lines); and (v) domestic dog (*Canis lupus familiaris*, 680 cell lines). More than 370 species are represented by only one or two cell lines (all data: personal communication, Amos Bairoch). This distribution is perhaps not surprising; each species may require an investment in time and effort before culture conditions are optimized for its growth. However, the effort is worthwhile when future applications are considered. For example, culture conditions have been optimized for the establishment of cell lines from the black flying fox (*Pteropus alecto*) (Crameri et al. 2009). Bats are important reservoirs of infection for viruses that may cross the species barrier, such as SARS coronaviruses (Ge et al. 2013). Recently, snake cells were successfully grown in organoid culture, resulting in the development of 3D glandular structures that produce venom *in vitro* (Post et al. 2020). These snake venom gland organoids will allow research to be performed on species where venom has been difficult to obtain *in vivo*.

1.5.4 Limited Understanding of the Cell and its Microenvironment

When the first major advances in cell line propagation were achieved in the 1950s, many workers observed loss of the phenotypic characteristics typical of the tissue from which the cells had been isolated. This effect was blamed on dedifferentiation, a process that was assumed to be the reversal of differentiation. Gordon Sato demonstrated that this initial finding was largely due to selective overgrowth by fibroblasts, and would go on to develop enrichment techniques and serum-free selective media to avoid this problem (Sato et al. 2010). However, it remains true that a culture's differentiated properties are often lost under normal conditions of serial propagation. It is not clear how this happens. Either the differentiated cells dedifferentiate when they start to proliferate or, more likely, the culture becomes dominated by undifferentiated precursor cells with greater proliferative capacity. Continuous cell lines may also be affected by genotypic instability, resulting from their unstable aneuploid chromosomal constitution. All tissue culture practitioners should understand that their cultures undergo evolution with continued handling, resulting in unforeseen consequences that may threaten the reproducibility of experimental work. These consequences can be almost entirely avoided if the worker freezes the culture early in its lifespan using cell banking procedures. The changes that are likely to occur throughout the lifespan of a culture are described in Chapter 3. Cryopreservation and cell banking are explained in Chapter 15. In some cases, it is possible to induce differentiation within a culture even when dedifferentiation is believed to have occurred; this topic is discussed in Chapter 26.

Most of the differences in cell behavior between cultured cells and their counterparts *in vivo* come from the dissociation of cells from their 3D geometry *in situ* and their propagation on a two-dimensional (2D) substrate. Specific cell interactions that are characteristic of the histology of the tissue are lost and the matrix that surrounds the cell is replaced by a foreign substrate that fails to mimic its chemical composition or its physical properties. As Mina Bissell once observed, "half the secret of the cell is outside the cell" (Bissell 2016). Many of the technical innovations described in this book come from tissue culture pioneers who have increased our understanding

of how cells behave *in vivo* and what they require for a more physiological environment. The discoveries made by these scientists – from Ross Harrison onwards – are the bedrock on which today's exciting discoveries are built.

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