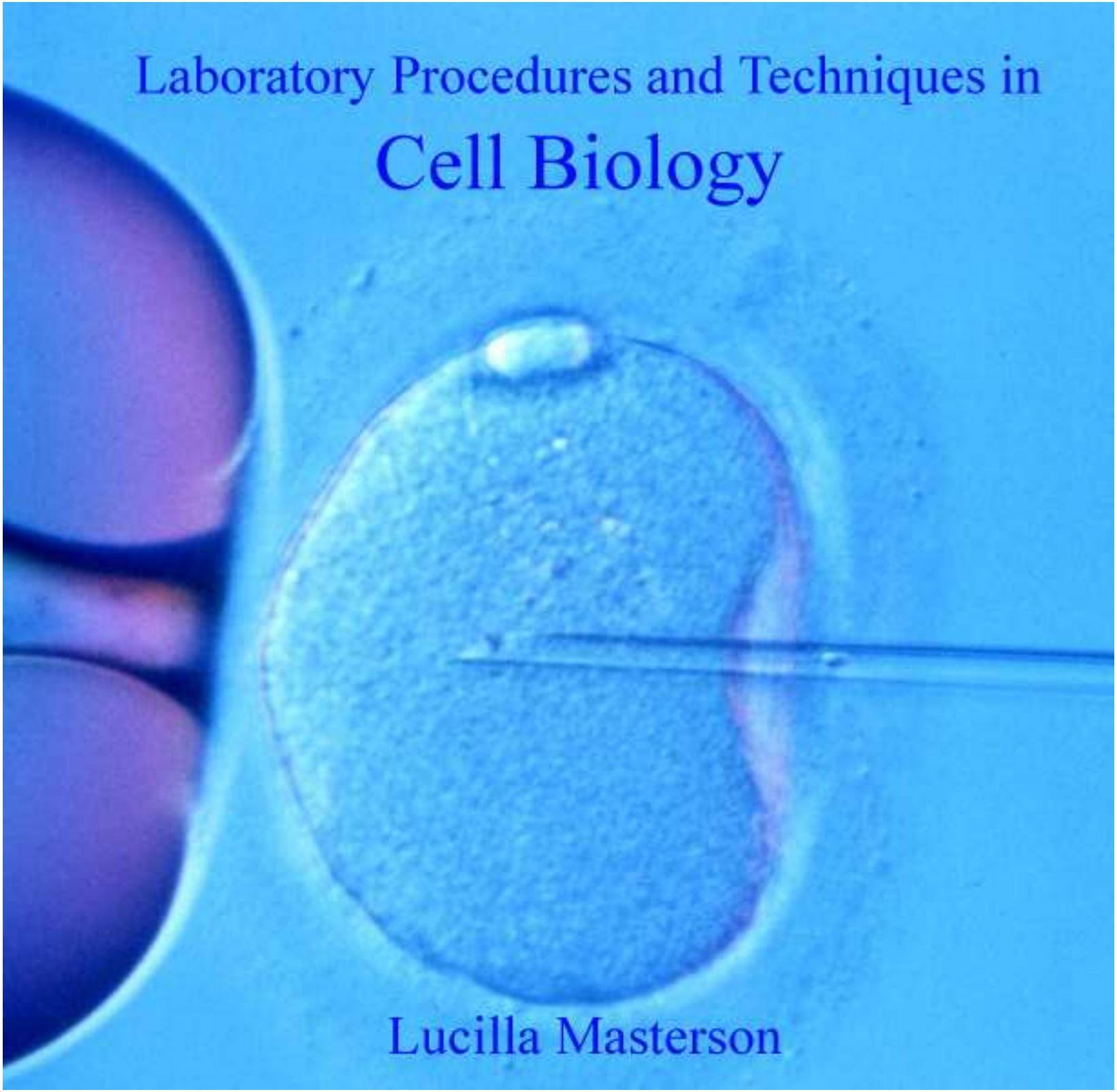
Laboratory Procedures and Techniques in
Cell Biology
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First Edition, 2012

ISBN 978-81-323-3153-7

Published by:
Research World
4735/22 Prakashdeep Bldg,
Ansari Road, Darya Ganj,
Delhi - 110002
Email: info@wtbooks.com

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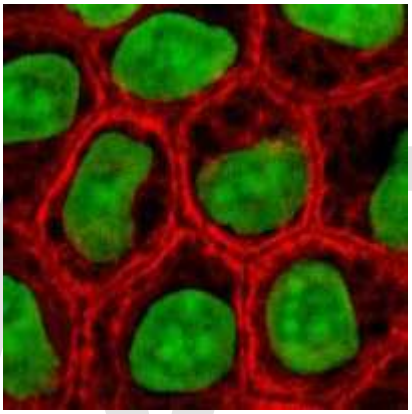
Chapter- 1

Cell Culture



Epithelial cells in culture, stained for keratin (red) and DNA (green)

Cell culture is the complex process by which cells are grown under controlled conditions. In practice, the term "cell culture" has come to refer to the culturing of cells derived from multicellular eukaryotes, especially animal cells. However, there are also cultures of plants, fungi and microbes, including viruses, bacteria and protists. The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture.

Animal cell culture became a common laboratory technique in the mid-1900s, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century.

History

The 19th-century English physiologist Sydney Ringer developed salt solutions containing the chlorides of sodium, potassium, calcium and magnesium suitable for maintaining the beating of an isolated animal heart outside of the body. In 1885 Wilhelm Roux removed a portion of the medullary plate of an embryonic chicken and maintained it in a warm saline solution for several days, establishing the principle of tissue culture. Ross Granville Harrison, working at Johns Hopkins Medical School and then at Yale

University, published results of his experiments from 1907–1910, establishing the methodology of tissue culture.

Cell culture techniques were advanced significantly in the 1940s and 1950s to support research in virology. Growing viruses in cell cultures allowed preparation of purified viruses for the manufacture of vaccines. The injectable polio vaccine developed by Jonas Salk was one of the first products mass-produced using cell culture techniques. This vaccine was made possible by the cell culture research of John Franklin Enders, Thomas Huckle Weller, and Frederick Chapman Robbins, who were awarded a Nobel Prize for their discovery of a method of growing the virus in monkey kidney cell cultures.

Concepts in mammalian cell culture

Isolation of cells

Cells can be isolated from tissues for *ex vivo* culture in several ways. Cells can be easily purified from blood, however only the white cells are capable of growth in culture. Mononuclear cells can be released from soft tissues by *enzymatic digestion* with enzymes such as collagenase, trypsin, or pronase, which break down the extracellular matrix. Alternatively, pieces of tissue can be placed in growth media, and the cells that grow out are available for culture. This method is known as *explant culture*.

Cells that are cultured directly from a subject are known as ***primary cells***. With the exception of some derived from tumors, most primary cell cultures have limited lifespan. After a certain number of population doublings (called the Hayflick limit) cells undergo the process of senescence and stop dividing, while generally retaining viability.

An established or **immortalised** *cell line* has acquired the ability to proliferate indefinitely either through random mutation or deliberate modification, such as artificial expression of the telomerase gene. There are numerous well established cell lines representative of particular cell types.

Maintaining cells in culture

Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37°C, 5% CO_2 for mammalian cells) in a cell incubator. Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed.

Aside from temperature and gas mixture, the most commonly varied factor in culture systems is the growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. The growth factors used to supplement media are often derived from animal blood, such as calf serum. One complication of these blood-derived ingredients is the potential for contamination of the culture with viruses or prions, particularly in biotechnology medical applications. Current practice is to minimize or eliminate the use of these ingredients wherever possible, but

this cannot always be accomplished. Alternative strategies involve sourcing the animal blood from countries with minimum BSE/TSE risk such as Australia and New Zealand, and using purified nutrient concentrates derived from serum in place of whole animal serum for cell culture.

Plating density (number of cells per volume of culture medium) plays a critical role for some cell types. For example, a lower plating density makes granulosa cells exhibit estrogen production, while a higher plating density makes them appear as progesterone producing theca lutein cells.

Cells can be grown in *suspension* or *adherent* cultures. Some cells naturally live in suspension, without being attached to a surface, such as cells that exist in the bloodstream. There are also cell lines that have been modified to be able to survive in suspension cultures so that they can be grown to a higher density than adherent conditions would allow. Adherent cells require a surface, such as tissue culture plastic or microcarrier, which may be coated with extracellular matrix components to increase adhesion properties and provide other signals needed for growth and differentiation. Most cells derived from solid tissues are adherent. Another type of adherent culture is *organotypic culture* which involves growing cells in a three-dimensional environment as opposed to two-dimensional culture dishes. This 3D culture system is biochemically and physiologically more similar to *in vivo* tissue, but is technically challenging to maintain because of many factors (e.g. diffusion).

Cell line cross-contamination

Cell line cross-contamination can be a problem for scientists working with cultured cells. Studies suggest that anywhere from 15–20% of the time, cells used in experiments have been misidentified or contaminated with another cell line. Problems with cell line cross contamination have even been detected in lines from the NCI-60 panel, which are used routinely for drug-screening studies. Major cell line repositories including the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ) have received cell line submissions from researchers that were misidentified by the researcher. Such contamination poses a problem for the quality of research produced using cell culture lines, and the major repositories are now authenticating all cell line submissions. ATCC uses short tandem repeat (STR) DNA fingerprinting to authenticate its cell lines.

To address this problem of cell line cross-contamination, researchers are encouraged to authenticate their cell lines at an early passage to establish the identity of the cell line. Authentication should be repeated before freezing cell line stocks, every two months during active culturing and before any publication of research data generated using the cell lines. There are many methods for identifying cell lines including isoenzyme analysis, human lymphocyte antigen (HLA) typing, Chromosomal analysis, Karyotyping, Morphology and STR analysis.

One significant cell-line cross contaminant is the immortal HeLa cell line.

Manipulation of cultured cells

As cells generally continue to divide in culture, they generally grow to fill the available area or volume. This can generate several issues:

- Nutrient depletion in the growth media
- Accumulation of apoptotic/necrotic (dead) cells.
- Cell-to-cell contact can stimulate cell cycle arrest, causing cells to stop dividing known as contact inhibition or senescence.
- Cell-to-cell contact can stimulate cellular differentiation.

Among the common manipulations carried out on culture cells are media changes, passaging cells, and transfecting cells. These are generally performed using tissue culture methods that rely on sterile technique. Sterile technique aims to avoid contamination with bacteria, yeast, or other cell lines. Manipulations are typically carried out in a biosafety hood or laminar flow cabinet to exclude contaminating micro-organisms. Antibiotics (e.g. penicillin and streptomycin) and antifungals (e.g. Amphotericin B) can also be added to the growth media.

As cells undergo metabolic processes, acid is produced and the pH decreases. Often, a pH indicator is added to the medium in order to measure nutrient depletion.

Media changes

In the case of adherent cultures, the media can be removed directly by aspiration and replaced.

Passaging cells

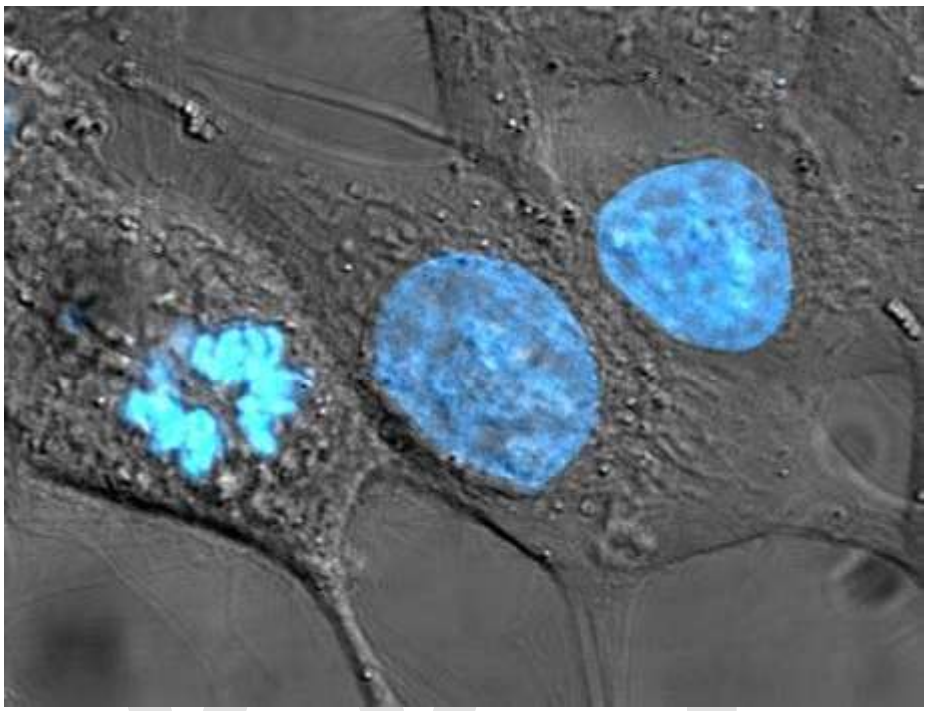
Passaging (also known as subculture or splitting cells) involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence associated with prolonged high cell density. Suspension cultures are easily passaged with a small amount of culture containing a few cells diluted in a larger volume of fresh media. For adherent cultures, cells first need to be detached; this is commonly done with a mixture of trypsin-EDTA, however other enzyme mixes are now available for this purpose. A small number of detached cells can then be used to seed a new culture.

Transfection and transduction

Another common method for manipulating cells involves the introduction of foreign DNA by transfection. This is often performed to cause cells to express a protein of interest. More recently, the transfection of RNAi constructs have been realized as a convenient mechanism for suppressing the expression of a particular gene/protein. DNA can also be inserted into cells using viruses, in methods referred to as transduction,

infection or transformation. Viruses, as parasitic agents, are well suited to introducing DNA into cells, as this is a part of their normal course of reproduction.

Established human cell lines



One of the earliest human cell lines, descended from Henrietta Lacks, who died of the cancer that those cells originated from, the cultured HeLa cells shown here have been stained with Hoechst turning their nuclei blue.

Cell lines that originate with humans have been somewhat controversial in bioethics, as they may outlive their parent organism and later be used in the discovery of lucrative medical treatments. In the pioneering decision in this area, the Supreme Court of California held in *Moore v. Regents of the University of California* that human patients have no property rights in cell lines derived from organs removed with their consent.

Generation of hybridomas

It is possible to fuse normal cells with an immortalised cell line. This method is used to produce monoclonal antibodies. In brief, lymphocytes isolated from the spleen (or possibly blood) of an immunised animal are combined with an immortal myeloma cell line (B cell lineage) to produce a hybridoma which has the antibody specificity of the primary lymphoctye and the immortality of the myeloma. Selective growth medium (HA or HAT) is used to select against unfused myeloma cells; primary lymphoctyes die quickly in culture and only the fused cells survive. These are screened for production of the required antibody, generally in pools to start with and then after single cloning.

Applications of cell culture

Mass culture of animal cell lines is fundamental to the manufacture of viral vaccines and other products of biotechnology

Biological products produced by recombinant DNA (rDNA) technology in animal cell cultures include enzymes, synthetic hormones, immunobiologicals (monoclonal antibodies, interleukins, lymphokines), and anticancer agents. Although many simpler proteins can be produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified) currently must be made in animal cells. An important example of such a complex protein is the hormone erythropoietin. The cost of growing mammalian cell cultures is high, so research is underway to produce such complex proteins in insect cells or in higher plants, use of single embryonic cell and somatic embryos as a source for direct gene transfer via practicle bombardment, transit gene expression and confocal microscopy observation is one of its applications. It also offers to confirm single cell origin of somatic embryos and the asymmetry of the first cell division, which starts the process. -

Tissue culture and engineering

Cell culture is a fundamental component of tissue culture and tissue engineering, as it establishes the basics of growing and maintaining cells *ex vivo*. The major application of human cell culture is in stem cell industry where mesenchymal stem cells can be cultured and cryopreserved for future use.

Vaccines

Vaccines for polio, measles, mumps, rubella, and chickenpox are currently made in cell cultures. Due to the H5N1 pandemic threat, research into using cell culture for influenza vaccines is being funded by the United States government. Novel ideas in the field include recombinant DNA-based vaccines, such as one made using human adenovirus (a common cold virus) as a vector, , such as adjuvants.

Culture of non-mammalian cells

Plant cell culture methods

Plant cell cultures are typically grown as cell suspension cultures in liquid medium or as callus cultures on solid medium. The culturing of undifferentiated plant cells and calli requires the proper balance of the plant growth hormones auxin and cytokinin.

Bacterial and yeast culture methods

For bacteria and yeast, small quantities of cells are usually grown on a solid support that contains nutrients embedded in it, usually a gel such as agar, while large-scale cultures are grown with the cells suspended in a nutrient broth.

Viral culture methods

The culture of viruses requires the culture of cells of mammalian, plant, fungal or bacterial origin as hosts for the growth and replication of the virus. Whole wild type viruses, recombinant viruses or viral products may be generated in cell types other than their natural hosts under the right conditions. Depending on the species of the virus, infection and viral replication may result in host cell lysis and formation of a viral plaque.

Common cell lines

Human cell lines

- National Cancer Institute's 60 cancer cell lines

- DU145 (Prostate cancer)
- Lncap (Prostate cancer)
- MCF-7 (breast cancer)
- MDA-MB-438 (breast cancer)
- PC3 (Prostate cancer)
- T47D (breast cancer)
- THP-1 (acute myeloid leukemia)
- U87 (glioblastoma)
- SHSY5Y Human neuroblastoma cells, cloned from a myeloma
- Saos-2 cells (bone cancer)

Primate cell lines

- Vero (African green monkey *Chlorocebus* kidney epithelial cell line initiated 1962)

Rat tumor cell lines

- GH3 (pituitary tumor)
- PC12 (pheochromocytoma)

Mouse cell lines

- MC3T3 (embryonic calvarial)

Plant cell lines

- Tobacco BY-2 cells (kept as cell suspension culture, they are model system of plant cell)

Other species cell lines

- zebrafish ZF4 and AB9 cells.
- *Madin-Darby Canine Kidney (MDCK)* epithelial cell line
- Xenopus A6 kidney epithelial cells.

List of cell lines

Cell line	Meaning	Organism	Origin tissue	Morphology	Link
293-T		Human	Kidney (embryonic)		Derivative of HEK 293ECACC
3T3 cells	"3-day transfer, inoculum 3 x 10^5 cells"	Mouse	Embryonic fibroblast		Also known as NIH 3T3 ECACC
721		Human	Melanoma		
9L		Rat	Glioblastoma		
A2780		Human	Ovary	Ovarian Cancer	ECACC
A2780ADR		Human	Ovary	Adriamycin-resistant derivative	ECACC
A2780cis		Human	Ovary	Cisplatin-resistant derivative	ECACC
A172		Human	glioblastoma	malignant glioma	ECACC
A20		Murine	B lymphoma	B lymphocyte	
A253		Human	Head and neck carcinoma	submandibular duct	
A431		Human	Skin epithelium	squamous carcinoma	ECACCCell Line Data Base
A-549		Human	Lungcarcinoma	Epithelium	DSMZECACC
ALC		Murine	bone marrow	Stroma	PubMed
B16		Murine	Melanoma		ECCAC
B35		Rat	Neuroblastoma		ATCC
BCP-1 cells		Human	PBMC	HIV+ Lymphoma	ATCC

BEAS-2B	Bronchial epithelium + Adenovirus 12-SV40 virus hybrid (Ad12SV40)	Human	Lung	Epithelial	ATCC
bEnd.3	*Brain endothelial*	Mouse	Brain / Cerebral cortex	Endothelium	ATCC
BHK-21	"Baby Hamster Kidney Fibroblast cells"	Hamster	Kidney	fibroblast	ECACCOlympus
BR 293		Human	Breast	Breast cancer	
BxPC3	Biopsy xenograph of pancreatic carcinoma line 3	Human	pancreatic adenocarcinoma	Epithelial	ATCC
C3H-10T1/2		Mouse	Embryonic mesenchymal cell line		ECACC
C6/36		Asian tiger mosquito	larval tissue		ECACC
Cal-27		Human	Tongue	squamous cell carcinoma	
CHO	*Chinese hamster ovary*	hamster	Ovary	Epithelium	ECACCICLC
COR-L23		Human	Lung		ECACC
COR-L23/CPR		Human	Lung		ECACC
COR-L23/5010		Human	Lung		ECACC
COR-L23/R23		Human	Lung	Epithelial	ECACC
COS-7	*Cercopithecus aethiops, origin-defective SV-40*	Ape - *Cercopithecus aethiops* (Chlorocebus)	Kidney	fibroblast	ECACCATCC
COV-434		Human	Ovary	Metastatic granulosa cell carcinoma	ECACC
CML T1	*Chronic Myelod Leukaemia T-lymphocyte 1*	Human	CML acute phase	T cell leukaemia	Blood
CMT	*canine*	Dog	Mammary	Epithelium	

	mammary tumor		gland		
CT26		Murine	Colorectal Carcinoma	Colon	
D17		canine	osteosarcoma		ECACC
DH82		canine	histiocytosis	monocyte/macrophage	ECACC J Vir Meth
DU145		Human	Androgen insensitive carcinoma	Prostate	PubMed
DuCaP	Dura mater Cancer of the Prostate	Human	Metastatic Prostate Cancer	Epithelial	EAC { Ehrlich Ascites Carcinoma } mice
EL4		Mouse		T cell leukaemia	ECACC
EM2		Human	CML blast crisis	Ph+ CML line	Cell Line Data Base
EM3		Human	CML blast crisis	Ph+ CML line	Cell Line Data Base
EMT6/AR1		Mouse	Breast	Epithelial-like	ECACC
EMT6/AR10.0		Mouse	Breast	Epithelial-like	ECACC
FM3		Human	Metastatic lymph node	melanoma	
H1299		Human	Lung	Lung cancer	
H69		Human	Lung		ECACC
HB54		hybridoma	hybridoma	secretes L243 mAb (against HLA-DR)	Human Immunology
HB55		hybridoma	hybridoma	secretes MA2.1 mAb (against HLA-A2 and HLA-B17)	Journal of Immunology
HCA2		Human	fibroblast		Journal of General Virology
HEK-293	*Human*	Human	Kidney	Epithelium	ATCC

	embryonic kidney		(embryonic)		
HeLa	*Henrietta Lacks*	Human	Cervical cancer	Epithelium	DSMZECACC
Hepa1c1c7	clone 7 of clone 1 hepatoma line 1	Mouse	Hepatoma	Epithelial	ECACC ATCC
HL-60	*Human leukemia*	Human	Myeloblast	bloodcells	ECACCDSMZ
HMEC	*Human mammary epithelial cell*	Human		Epithelium	ECACC
HT-29		Human	Colon epithelium	Adenocarcinoma	ECACC Cell Line Data Base
Jurkat		Human	T-Cell-Leukemia	white blood cells	ECACC DSMZ
JY cells		Human	Lymphoblastoid	EBV immortalised B cell	
K562 cells		Human	Lymphoblastoid	CML blast crisis	ECACC
Ku812		Human	Lymphoblastoid	erythroleukemia	ECACC LGCstandards
KCL22		Human	Lymphoblastoid	CML	
KG1		Human	Lymphoblastoid	AML	
KYO1	Kyoto 1	Human	Lymphoblastoid	CML	DSMZ
LNCap	Lymph node Cancer of the Prostate	Human	prostatic adenocarcinoma	Epithelial	ECACCATCC
Ma-Mel 1, 2, 3....48		Human		a range of melanoma cell lines	
MC-38		Mouse		Adenocarcinoma	
MCF-7	*Michigan Cancer Foundation-7*	Human	Mammary gland	Invasive breast ductal carcinoma	ER+, PR+
MCF-10A	*Michigan*	Human	mammary	Epithelium	ATCC

	Cancer Foundation		gland		
MDA-MB-231	M.D. Anderson - Metastatic Breast	Human	Breast	Cancer	ECACC
MDA-MB-468	M.D. Anderson - Metastatic Breast	Human	Breast	Cancer	ECACC
MDA-MB-435	M.D. Anderson - Metastatic Breast	Human	Breast	melanoma or carcinoma (disputed)	Cambridge Pathology ECACC
MDCK II	*Madin Darby canine kidney*	Dog	Kidney	Epithelium	ECACC ATCC
MDCK II	*Madin Darby canine kidney*	Dog	Kidney	Epithelium	ATCC
MOR/0.2R		Human	Lung		ECACC
MONO-MAC 6		Human	WBC	myeloid metaplasic AML	Cell Line Data Base
MTD-1A		Mouse		Epithelium	
MyEnd	*Myocardial endothelial*	Mouse		Endothelium	
NCI-H69/CPR		Human	Lung		ECACC
NCI-H69/LX10		Human	Lung		ECACC
NCI-H69/LX20		Human	Lung		ECACC
NCI-H69/LX4		Human	Lung		ECACC
NIH-3T3	*NIH, 3-day transfer, inoculum 3 x 10^5 cells*	Mouse	embryo	fibroblast	ECACCAT CC
NALM-1			peripheral blood	blast-crisis CML	Cancer Genetics and Cytogenetic s
NW-145				Melanoma	ESTDAB
OPCN / OPCT cell lines	Onyvax Prostate Cancer....			Range of prostate tumour lines	Asterand
Peer		Human	T cell leukemia		DSMZ
PNT-1A / PNT 2				Prostate tumour lines	ECACC
RenCa	Renal Carcinoma	Mouse		renal carcinoma	
RIN-5F		Mouse	Pancreas		

RMA/RMAS		Mouse		T cell tumour	
Saos-2 cells		Human		Osteosarcoma	ECACC
Sf-9	*Spodoptera frugiperda*	insect - *Spodoptera frugiperda* (moth)	Ovary		DSMZECACC
SkBr3		Human		Breast carcinoma	
T2		Human		T cell leukemia/B cell line hybridoma	DSMZ
T-47D		Human	Mammary gland	ductal carcinoma	
T84		Human	colorectal Carcinoma / Lungmetastasis	Epithelium	ECACCATCC
THP1 cell line		Human	Monocyte	AML	ECACC
U373		Human	Glioblastoma-astrocytoma	Epithelium	
U87		Human	glioblastoma-astrocytoma	Epithelial-like	Abcam
U937		Human	Leukaemic monocytic lymphoma		ECACC
VCaP	Vertebra Prostate Cancer	Human	Metastatic prostate cancer	Epithelial	ECACC ATCC
Vero cells	'Vera Reno' ('Green kidney') / 'Vero' ('truth')	African Green Monkey	Kidney epithelium		ECACC
WM39		Human	skin	Primary melanoma	
WT-49		Human	Lymphoblastoid		
X63		Mouse	Melanoma		
YAC-1		Mouse	Lymphoma		Cell Line Data Base
YAR		Human	B-cell	EBV transofrmed	ECACC Human Immunology

Chapter- 2

Cell Disruption

Cell disruption is a method or process for releasing biological molecules from inside a cell.

Choice of disruption method

The production of biologically-interesting molecules using cloning and culturing methods allows the study and manufacture of relevant molecules.Except for excreted molecules, cells producing molecules of interest must be disrupted. This page discusses various methods.

Major factors

Several factors must be considered.

Volume or sample size of cells to be disrupted

If only a few microliters of sample are available, care must be taken to minimize loss and to avoid cross-contamination.

Disruption of cells, when hundreds or even thousands of liters of material are being processed in a production environment, presents a different challenge. Throughput, efficiency, and reproducibility are key factors.

How many different samples need to be disrupted at one time?

Frequently when sample sizes are small, there are many samples. As sample sizes increase, fewer samples are usually processed. Issues are sample cross contamination, speed of processing, and equipment cleaning.

How easily are the cells disrupted?

As the difficulty of disruption increases (e.g. E. coli), more force is required to efficiently disrupt the cells. For even more difficult samples (e.g. yeast), there is a parallel increase in the processor power and cost. The most difficult samples (e.g. spores) require mechanical forces combined with chemical or enzymatic efforts, often with limited disruption efficiency.

What efficiency of disruption is required?

Over-disruption may impact the desired product. For example, if subcellular fractionation studies are undertaken, it is often more important to have intact subcellular components, while sacrificing disruption efficiency.

For production scale processes, the time to disrupt the cells and the reproducibility of the method become more important factors.

How stable is the molecule(s) or component that needs to be isolated?

In general, the cell disruption method is closely matched with the material that is desired from the cell studies. It is usually necessary to establish the minimum force of the disruption method that will yield the best product. Additionally, once the cells are disrupted, it is often essential to protect the desired product from normal biological processes (e.g. proteases) and from oxidation or other chemical events.

What purification methods will be used following cell disruption?

It is rare that a cell disruption process produces a directly usable material; in almost all cases, subsequent purification events are necessary. Thus, when the cells are disrupted, it is important to consider what components are present in the disruption media so that efficient purification is not impeded.

Is the sample being subjected to the method biohazardous?

Preparation of cell-free extracts of pathogens presents unique difficulties. Mechanical disruption techniques are not always applicable owing to potential biohazard problems associated with contamination of equipment and generation of aerosols.

Lysis

For easily disrupted cells such as insect and mammalian cells grown in culture media, a mild osmosis-based method for cell disruption (lysis) is commonly used. Quite frequently, simply lowering the ionic strength of the media will cause the cells to swell and burst. In some cases it is also desirable to add a mild surfactant and some mild mechanical agitation or sonication to completely disassociate the cellular components.

Due to the cost and relative effort to grow these cells, there is often only a small quantity of cells to be processed, and preferred methods for cell disruption tend to be a manual mechanical homogenizer, nitrogen burst methods, or ultrasound with a small probe. Because these methods are performed under very mild conditions, they are often used for subcellular fractionation studies.

For cells that are more difficult to disrupt, such as bacteria, yeast, and algae, hypotonic shock alone generally is insufficient to open the cell and stronger methods must be used, due to the presence of cell walls that must be broken to allow access to intracellular components. These stronger methods are discussed below.

Laboratory-scale methods

Enzymatic method

The use of enzymatic methods to remove cell walls is well-established for preparing cells for disruption, or for preparation of protoplasts (cells without cell walls) for other uses such as introducing cloned DNA or subcellular organelle isolation. The enzymes are generally commercially available and, in most cases, were originally isolated from biological sources (e.g. snail gut for yeast or lysozyme from hen egg white). The enzymes commonly used include lysozyme, lysostaphin, zymolase, cellulase, mutanolysin, glycanases, proteases, mannase etc.

Disadvantages include:

- Not always reproducible.

In addition to potential problems with the enzyme stability, the susceptibility of the cells to the enzyme can be dependent on the state of the cells. For example, yeast cells grown to maximum density (stationary phase) possess cell walls that are notoriously difficult to remove whereas midlog growth phase cells are much more susceptible to enzymatic removal of the cell wall.

- Not usually applicable to large scale.

Large scale applications of enzymatic methods tend to be costly and irreproducible.

The enzyme must be removed (or inactivated) to allow cell growth or permit isolation of the desired material.

Bead method

Another common laboratory-scale mechanical method for cell disruption uses small glass, ceramic, zirconium, or steel beads and a high level of agitation by stirring or shaking of the mix. The method, often referred to as "beadbeating", works well for all types of cellular material - from spores to animal and plant tissues.

At the lowest levels of the technology, beads are added to the cell or tissue suspension in a test tube and the sample is mixed on a common laboratory vortex mixer. While processing time is 3-10 times longer than that in specially machines, it works for easily disrupted cells and is inexpensive.

At the more sophisticated level, beadbeating is done in closed vials, centrifuge tubes, or sealed titer plates. The sample and the beads are vigorously agitated at about 2000 oscillations per minute in a specially designed clamp driven by a high energy electric motor. In some machines hundreds of samples can be processed simultaneously. To prevent degradation of RNA and proteins, some form of cooling is required because samples heat due to collisions of the beads. This can be accomplished by placing titer plates or vials in chilled aluminum blocks. Another configuration suitable for larger sample volumes uses a rotor inside a sealed 15, 50 or 200 ml chamber to agitate the beads. The chamber can be surrounded by a cooling jacket. Using this same configuration, commercial machines capable of processing many liters of cell suspension are available.

Disadvantages include:

- Occasional problems with foaming and sample heating, especially for larger samples.
- Tough tissue samples such as skin or seeds are difficult to disrupt unless the sample is very small or has been pre-chopped into small pieces.

Sonication

Another common laboratory-scale method for cell disruption applies ultrasound (typically 20–50 kHz) to the sample (*sonication*). In principle, the high-frequency is generated electronically and the mechanical energy is transmitted to the sample via a metal probe that oscillates with high frequency. The probe is placed into the cell-containing sample and the high-frequency oscillation causes a localized low pressure region resulting in cavitation and impaction, ultimately breaking open the cells. Although the basic technology was developed over 50 years ago, newer systems permit cell disruption in smaller samples (including multiple samples under 200 µL in microplate wells) and with an increased ability to control ultrasonication parameters.

Disadvantages include:

- Heat generated by the ultrasound process must be dissipated.
- High noise levels (most systems require hearing protection and sonic enclosures)
- Yield variability
- Free radicals are generated that can react with other molecules.

Detergent methods

Detergent-based cell lysis is an alternative to physical disruption of cell membranes, although it is sometimes used in conjunction with homogenization and mechanical grinding. Detergents disrupt the lipid barrier surrounding cells by disrupting lipid:lipid, lipid:protein and protein:protein interactions. The ideal detergent for cell lysis depends on cell type and source and on the downstream applications following cell lysis. Animal cells, bacteria and yeast all have differing requirements for optimal lysis due to the presence or absence of a cell wall. Because of the dense and complex nature of animal tissues, they require both detergent and mechanical lysis to effectively lyse cells.

In general, nonionic and zwitterionic detergents are milder, resulting in less protein denaturation upon cell lysis, than ionic detergents and are used to disrupt cells when it is critical to maintain protein function or interactions. CHAPS, a zwitterionic detergent, and the Triton X series of nonionic detergents are commonly used for these purposes. In contrast, ionic detergents are strong solubilizing agents and tend to denature proteins, thereby destroying protein activity and function. SDS, an ionic detergent that binds to and denatures proteins, is used extensively for studies assessing protein levels by gel electrophoresis and western blotting.

In addition to the choice of detergent, other important considerations for optimal cell lysis include the buffer, pH, ionic strength and temperature.

Solvent Use

A method was developed for the extraction of proteins from both pathogenic and nonpathogenic bacteria. The method involves the treatment of cells with sodium dodecyl sulfate followed by extraction of cellular proteins with acetone. This method is simple, rapid and particularly well suited when the material is biohazardous.

Simple and rapid method for disruption of bacteria for protein studies. S Bhaduri and P H Demchick Disadvantages include:

- Proteins are denatured

The 'cell bomb'

Another laboratory-scale system for cell disruption is rapid decompression or the "cell bomb" method. In this process, cells in question are placed under high pressure (usually nitrogen or other inert gas up to about 25,000 psi) and the pressure is rapidly released. The rapid pressure drop causes the dissolved gas to be released as bubbles that ultimately lyse the cell.

Disadvantages include:

- Only easily disrupted cells can be effectively disrupted (stationary phase E. coli, yeast, fungi, and spores do not disrupt well by this method).
- Large scale processing is not practical.
- High gas pressures have a high risk of personal hazard if not handled carefully.

High-shear mechanical methods.

High-shear mechanical methods for cell disruption fall into four major classes: rotor-stator disruptors, valve-type processors, fixed-geometry processors and fixed orifice and constant pressure processors. (These fluid processing systems also are used extensively for homogenization and deaggregation of a wide range of materials – uses that will not be discussed here.) These processors all work by placing the bulk aqueous media under shear forces that literally pull the cells apart. These systems are especially useful for larger scale laboratory experiments (over 20 mL) and offer the option for large-scale production.

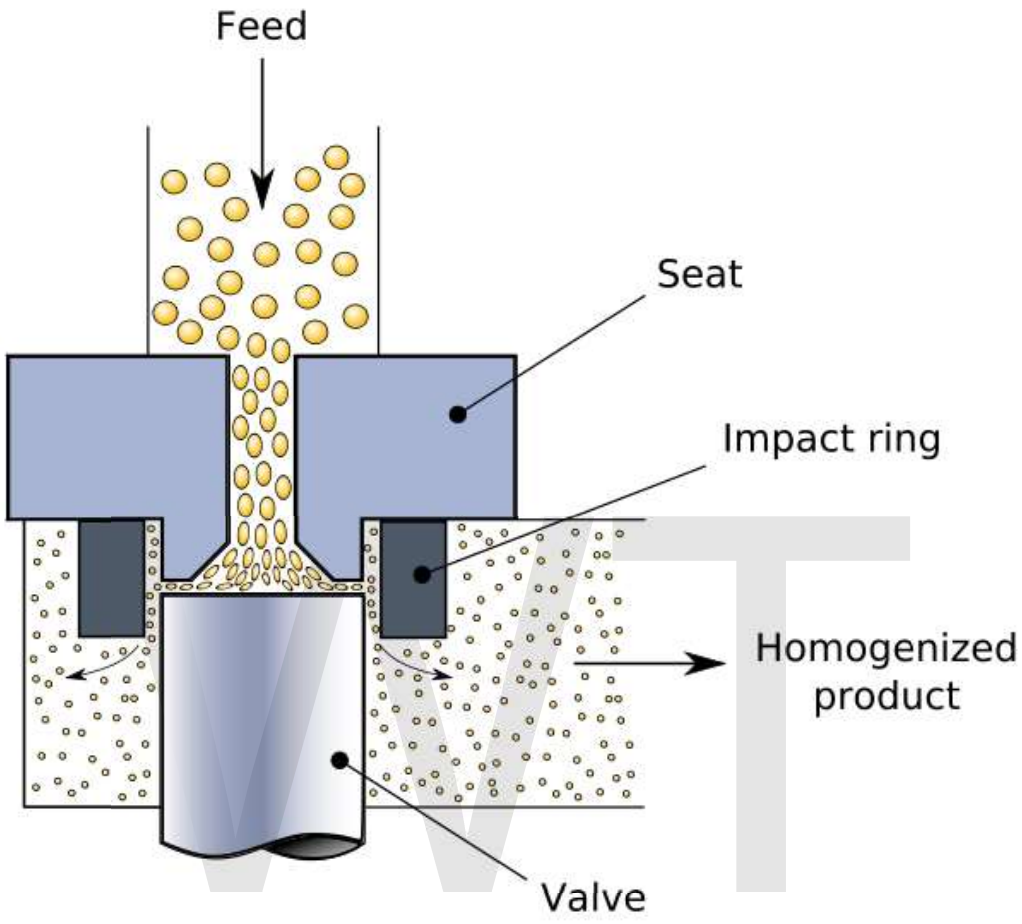
Rotor-stator Processors

Most commonly used as tissue disruptors.

Disadvantages include:

- Do not work well with difficult-to-lyse cells like yeast and fungi
- Often variable in product yield.
- Poorly suited for culture use.

Valve-type processors



Homogenizing valve, a method to homogenize at high pressure.

Valve-type processors disrupt cells by forcing the media with the cells through a narrow valve under high pressure (20,000–30,000 psi or 140–210 MPa). As the fluid flows past the valve, high shear forces in the fluid pull the cells apart. By controlling the pressure and valve tension, the shear force can be regulated to optimize cell disruption. Due to the high energies involved, sample cooling is generally required, especially for samples requiring multiple passes through the system. Three major implementations of the technology exist: the French pressure cell press, *Constant Cell Disruption Systems* and pumped-fluid processors.

French press technology uses an external hydraulic pump to drive a piston within a larger cylinder that contains the sample. The pressurized solution is then squeezed past a needle valve. Once past the valve, the pressure drops to atmospheric pressure and generates shear forces that disrupt the cells. Disadvantages include:

- Not well suited to larger volume processing.
- Awkward to manipulate and clean due to the weight of the assembly (about 30 lb or 14 kg).

Mechanically pumped-fluid processors function by forcing the sample at a constant volume flow past a spring-loaded valve.

Disadvantages include:

- Requires 10 mL or more of media.
- Prone to valve-clogging events.
- Due to variations in the valve setting and seating, less reproducible than fixed-geometry fluid processors.

Fixed-geometry fluid processors

Fixed-geometry fluid processors are marketed under the name of Microfluidizer processors. The processors disrupt cells by forcing the media with the cells at high pressure (typically 20,000–30,000 psi or 140–210 MPa) through an interaction chamber containing a narrow channel. The ultra-high shear rates allow for:

- Processing of more difficult samples
- Fewer repeat passes to ensure optimum sample processing

The systems permit controlled cell breakage without the need to add detergent or to alter the ionic strength of the media. The fixed geometry of the interaction chamber ensures reproducibility. Especially when samples are processed multiple times, the processors require sample cooling.

Fixed Orifice and Constant Pressure

Constant Cell Disruption Systems by Constant Systems part of Score Group plc - these systems are fully contained and operate using a finely controlled hydraulic system powered by electricity only. The sample is taken in and instantly pressurised up to a maximum of 40,000 PSI before being passed through a very small and fixed orifice and then returned back to atmospheric pressure. As the sample is being processed this type of cell disruptor ensures that the pressure is maintained throughout the process, ensuring repeatability throughout the sample run.

Both fluid and non fluid samples can be processed through this type of cell disruptor, plant leaves and skin samples being a good example of non fluid samples. Having a maximum process pressure achievable of 40,000 PSI enables this type of unit to process more difficult sample types with fewer repeat passes. A built-in cooling jacket ensures temperature control of the sample (Water Bath or Chiller Unit is required)

Cell disruption by nitrogen decompression

Cell disruption by rapid decompression is one of several methods of cell disruption and is also called explosive decompression or cell bomb.

Applications

The technique is used to:

- Homogenize cells and tissues
- Release intact organelles
- Prepare cell membranes
- Release labile biochemicals
- Produce uniform and repeatable homogenates without subjecting the sample to extreme chemical or physical stress.

According to manufacturers of nitrogen decompression devices, the method is particularly well suited for treating mammalian and other membrane bound cells. It has also been used successfully for treating plant cells, for releasing virus from fertilized eggs and for treating fragile bacteria. It is not recommended for untreated bacterial cells. Yeast, fungus, spores and other materials with tough cell walls do not respond well to this method.

How it works

Large quantities of nitrogen are first dissolved in the cell under high pressure within a suitable pressure vessel. Then, when the gas pressure is suddenly released, the nitrogen comes out of the solution as expanding bubbles that stretch the membranes of each cell until they rupture and release the contents of the cell.

Nitrogen decompression is claimed to be more protective of enzymes and organelles than ultrasonic and mechanical homogenizing methods and to compare favorably to the controlled disruptive action obtained in a PTFE and glass mortar and pestle homogenizer. While other disruptive methods depend upon friction or a mechanical shearing action that generate heat, the nitrogen decompression procedure is accompanied by an adiabatic expansion that cools the sample instead of heating it.

The blanket of inert nitrogen gas that saturates the cell suspension and the homogenate offers protection against oxidation of cell components. Although other gases: carbon dioxide, nitrous oxide, carbon monoxide and compressed air have been used in this technique, nitrogen is preferred because of its non-reactive nature and because it does not alter the pH of the suspending medium. In addition, nitrogen is preferred because it is generally available at low cost and at pressures suitable for this procedure. Once released, subcellular substances are not exposed to continued attrition that might denature the sample or produce unwanted damage. There is no need to watch for a peak between

enzyme activity and percent disruption. Since nitrogen bubbles are generated within each cell, the same disruptive force is applied uniformly throughout the sample, thus ensuring unusual uniformity in the product. Cell-free homogenates can be produced.

Chapter- 3

Cell Fractionation and Incubator (Laboratory Device)

Cell fractionation

Cell fractionation is the separation of homogeneous sets, usually organelles, from a heterogeneous population of cells.

Steps

There are three principal steps involved:

1. Disruption (homogenization) of cells and liberation of organelles.
2. Macro Filtration
3. Purification of cell components.

Homogenization

Tissue is typically homogenized in an isotonic buffer solution using a variety of mechanisms. A 'Potter-Elvehjem homogeniser' is often used as it is relatively gentle. Other procedures include grinding, mincing, chopping, pressure changes, osmotic shock, freeze-thawing, and ultra-sound homogenization.

The solution is homogenized in an isotonic solution to stop osmotic damage, with a pH buffer to regulate pH, and at an ice-cold temperature to prevent enzyme damage. The organelles are kept either cold, isotonic or buffered.

Filtration

This step may not be necessary depending on the source of the cells. Animal tissue however is likely to yield connective tissue which must be removed. Commonly, filtration is achieved either by pouring through gauze or with a suction filter and the relevant grade ceramic filter.

Purification

Invariably achieved by Differential centrifugation - the sequential increase in gravitational force resulting in the sequential separation of organelles according to their density.

Incubator (laboratory device)

A Bacteriological incubator

Interior of a CO_2 incubator used in cell culture

In biology, an **incubator** is a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains optimal temperature, humidity and other conditions such as the carbon dioxide(CO_2) and oxygen content of the atmosphere inside. Incubators are essential for a lot of experimental work in cell biology, microbiology and molecular biology and are used to culture both bacterial as well as eukaryotic cells.

Incubators are also used in the poultry industry to act as a substitute for hens. This often results in higher hatch rates due to the ability to control both temperature and humidity. Various brands of incubators are commercially available to breeders.

The simplest incubators are insulated boxes with an adjustable heater, typically going up to 60 to 65 °C (140 to 150 °F), though some can go slightly higher (generally to no more than 100 °C). The most commonly used temperature both for bacteria such as the frequently used E. Coli as well as for mammalian cells is approximately 37 °C, as these organisms grow well under such conditions. For other organisms used in biological experiments, such as the budding yeast Saccharomyces cerevisiae, a growth temperature of 30 °C is optimal.

More elaborate incubators can also include the ability to lower the temperature (via refrigeration), or the ability to control humidity or CO_2 levels. This is important in the cultivation of mammalian cells, where the relative humidity is typically >95% and a slightly acidic pH is achieved by maintaining a CO_2 level of 5%.

Most incubators include a timer; some can also be programmed to cycle through different temperatures, humidity levels, etc. Incubators can vary in size from tabletop to units the size of small rooms.

There are many other ways to create an incubator. The renowned scientist Louis Pasteur used the small opening underneath his staircase as an incubator.

The maximum temperature for sterilization is 200^{o}C.

Incubator

If a hot room is not available, it may be necessary to buy an equivalent dry incubator. Even with a hot room, it is sometimes convenient to have another incubator close to the hood for trypsinization. The incubator should be large enough, ~50–200 L (1.5–6 ft^3) per person, and should have forced-air circulation, temperature control to within ±0.2 °C, and a safety thermostat that cuts off if the incubator overheats or, better, that regulates the incubator if the first thermostat fails. The incubator should be resistant to corrosion (e.g., stainless steel, although anodized aluminum is acceptable for a dry incubator) and easily cleaned. A double chamber, or two incubators stacked, one above the other, independently regulated, is preferable to one large incubator because it can accommodate more cultures with better temperature control, and if one half fails or needs to be cleaned, the other can still be used. Many incubators have a heated water jacket to distribute heat evenly around the cabinet, thus avoiding the formation of cold spots. These incubators also hold their temperature longer in the event of a heater failure or cut in power. However, new high-efficiency insulation and diffuse surface heater elements have all but eliminated the need for a water jacket and make moving the incubator much simpler. (A water jacket generally needs to be emptied if the incubator is to be moved.) Incubator shelving is usually perforated to facilitate the circulation of air. However, the perforations can lead to irregularities in cell distribution in monolayer cultures, with variations in cell density following the pattern of spacing on the shelves. The variations may be due to convection currents generated over points of contact relative to holes in the shelf, or they may be related to areas that cool down more quickly when the door is opened. Although

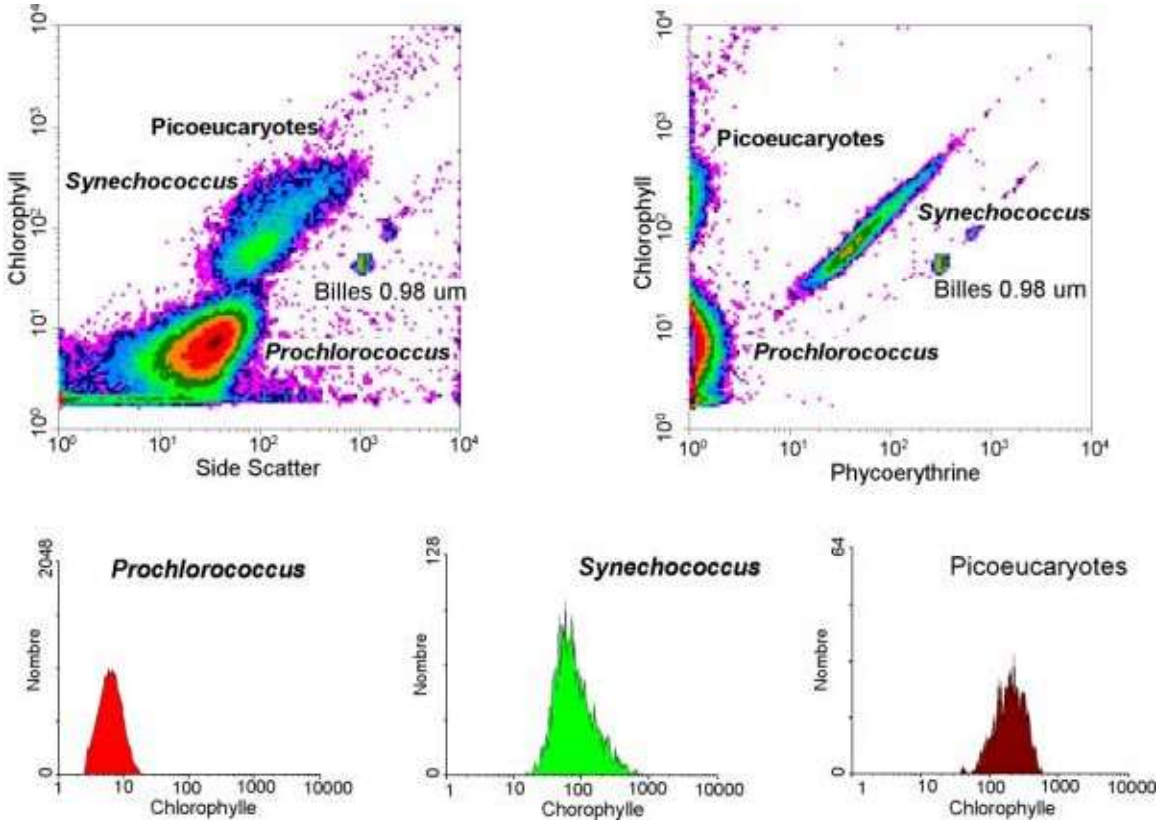
no problem may arise in routine maintenance, flasks and dishes should be placed on an insulated tile or metal tray in experiments in which uniform density is important.

Humid CO_2 Incubator

Although cultures can be incubated in sealed flasks in a regular dry incubator or a hot room, some vessels, e.g., Petri dishes or multiwell plates, require a controlled atmosphere with high humidity and elevated CO2 tension. The cheapest way of controlling the gas phase is to place the cultures in a plastic box, or chamber (Bellco, MP Biomedicals): Gas the container with the correct CO2 mixture and then seal it. If the container is not completely filled with dishes, include an open dish of water to increase the humidity inside the chamber. CO2 incubators are more expensive, but their ease of use and superior control of CO2 tension and temperature (anaerobic jars and desiccators take longer to warm up) justify the expenditure. A controlled atmosphere is achieved by using a humidifying tray (Fig 5.16) and controlling the CO2 tension with a CO2-monitoring device, which draws air from the incubator into a sample chamber, determines the concentration of CO2, and injects pure CO2 into the incubator to make up any deficiency. Air is circulated around the incubator by natural convection or by using a fan to keep both the CO2 level and the temperature uniform. It is claimed that fan-circulated incubators recover faster after opening, although natural convection incubators can still have a quick recovery and greatly reduce the risks of contamination. Dry, heated wall incubators also encourage less fungal contamination on the walls, as the walls tend to remain dry, even at high relative humidity. Some CO2 controllers need to be calibrated every few months, but the use of gold wire or infrared detectors minimizes drift and many models reset the zero of the CO2 detector automatically. The size of incubator required will depend on usage, both the numbers of people using it and the types of cultures. Five people using only microtitration plates could have 1000 plates (~100,000 individual cultures) or 10 experiments each in a modest-sized incubator, while one person doing cell cloning could fill one shelf with one or two experiments. Flask cultures, especially large flasks, are not an economical use of CO2 incubators. They are better incubated in a regular incubator or hot room. If CO2 is required, flasks can be gassed from a cylinder or CO2 supply.

Chapter- 4

Flow Cytometry



Analysis of a marine sample of photosynthetic picoplankton by flow cytometry showing three different populations (*Prochlorococcus*, *Synechococcus*, and picoeukaryotes)

Flow cytometry (abbreviated: **FCM**) is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers, but has many other applications in both research and clinical practice. A common variation is to physically sort particles based on their properties, so as to purify populations of interest.

History

The first impedance-based flow cytometry device, using the Coulter principle, was disclosed in U.S. Patent 2,656,508, issued in 1953, to Wallace H. Coulter. The first fluorescence-based flow cytometry device (ICP 11) was developed in 1968 by Wolfgang Göhde from the University of Münster and first commercialized in 1968/69 by German developer and manufacturer Partec through Phywe AG in Göttingen. At that time, absorption methods were still widely favored by other scientists over fluorescence methods . Soon after, flow cytometry instruments were developed, including the Cytofluorograph (1971) from Bio/Physics Systems Inc. (later: Ortho Diagnostics), the PAS 8000 (1973) from Partec, the first FACS instrument from Becton Dickinson (1974), the ICP 22 (1975) from Partec/Phywe and the Epics from Coulter (1977/78).

Name of the technology

The original name of the flow cytometry technology was "pulse cytophotometry" (German: *Impulszytophotometrie*). Only 20 years later in 1988, at the Conference of the American Engineering Foundation in Pensacola, Florida, the name was changed to "flow cytometry", a term that quickly became popular.

Principle of flow cytometry

A beam of light (usually laser light) of a **single wavelength** is directed onto a hydrodynamically-focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and, by analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Some flow cytometers on the market have eliminated the need for fluorescence and use only light scatter for measurement. Other flow cytometers form images of each cell's fluorescence, scattered light, and transmitted light.

Front of desktop flow cytometer - the Becton-Dickinson FACSCalibur.

Flow cytometers

Modern flow cytometers are able to analyze several thousand particles every second, in "real time," and can actively separate and isolate particles having specified properties. A flow cytometer is similar to a microscope, except that, instead of producing an image of the cell, flow cytometry offers "high-throughput" (for a large number of cells) automated quantification of set parameters. To analyze solid tissues, a single-cell suspension must first be prepared.

A flow cytometer has 5 main components:

- a flow cell - liquid stream (sheath fluid), which carries and aligns the cells so that they pass single file through the light beam for sensing
- a measuring system - commonly used are measurement of impedance (or conductivity) and optical systems - lamps (mercury, xenon); high-power water-cooled lasers (argon, krypton, dye laser); low-power air-cooled lasers (argon (488 nm), red-HeNe (633 nm), green-HeNe, HeCd (UV)); diode lasers (blue, green, red, violet) resulting in light signals

- a detector and Analogue-to-Digital Conversion (ADC) system - which generates FSC and SSC as well as fluorescence signals from light into electrical signals that can be processed by a computer
- an amplification system - linear or logarithmic
- a computer for analysis of the signals.

The process of collecting data from samples using the flow cytometer is termed 'Acquisition'. Acquisition is mediated by a computer physically connected to the flow cytometer, and the software which handles the digital interface with the cytometer. The software is capable of adjusting parameters (i.e. voltage, compensation, etc.) for the sample being tested, and also assists in displaying initial sample information while acquiring sample data to insure that parameters are set correctly. Early flow cytometers were, in general, experimental devices, but technological advances have enabled widespread applications for use in a variety of both clinical and research purposes. Due to these developments, a considerable market for instrumentation, analysis software, as well as the reagents used in acquisition such as fluorescently-labeled antibodies has developed.

Modern instruments usually have multiple lasers and fluorescence detectors (the current record for a commercial instrument is **4 lasers and 18 fluorescence detectors**). Increasing the number of lasers and detectors allows for multiple antibody labeling, and can more precisely identify a target population by their phenotypic markers. Certain instruments can even take digital images of individual cells, allowing for the analysis of fluorescent signal location within or on the surface of cell.

Data analysis

Gating

The data generated by flow-cytometers can be plotted in a single dimension, to produce a histogram, or in two-dimensional dot plots or even in three dimensions. The regions on these plots can be sequentially separated, based on fluorescence intensity, by creating a series of subset extractions, termed "gates." Specific gating protocols exist for diagnostic and clinical purposes especially in relation to hematology.

The plots are often made on logarithmic scales. Because different fluorescent dyes' emission spectra overlap, signals at the detectors have to be compensated electronically as well as computationally. Data accumulated using the flow cytometer can be analyzed using software, e.g., WinMDI(deprecated), Flowjo, FCS Express, VenturiOne or CellQuest Pro. Once the data is collected, there is no need to stay connected to the flow cytometer. For this reason, analysis is most often done on a separate computer. This is especially necessary in core facilities where usage of these machines is in high demand.

Computational analysis

Recent progress on automated population identification using computational methods has offered an alternative to traditional gating strategies. Automated identification systems could potentially help findings of rare and hidden populations. Representative automated methods include FLOCK in Immunology Database and Analysis Portal (ImmPort) , FLAME in GenePattern and flowClust ,, in Bioconductor. Collaborative efforts have resulted in an open project called FlowCAP (Flow Cytometry: Critical Assessment of Population Identification Methods,) to provide an objective way to compare and evaluate the flow cytometry data clustering methods, and also to establish guidance about appropriate use and application of these methods.

Fluorescence-activated cell sorting

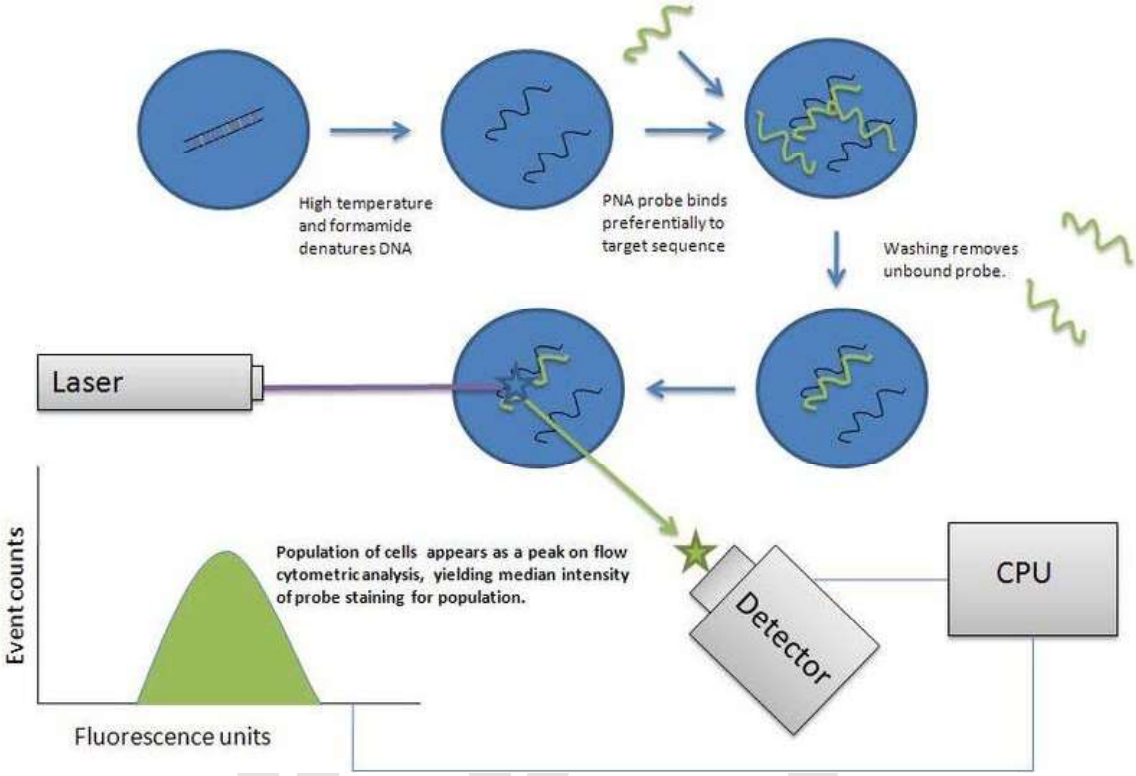
Fluorescence-activated cell sorting is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. The acronym FACS is trademarked and owned by Becton, Dickinson and Company. While many immunologists use this term frequently for all types of sorting and non-sorting applications, it is not a generic term for flow cytometry. The first cell sorter was invented by Mack Fulwyler in 1965, using the Coulter principle, a relatively difficult technique and one no longer used in modern instruments. The technique was expanded by Len Herzenberg who was responsible for coining the term FACS. Herzenberg won the Kyoto Prize in 2006 for his work in flow cytometry.

The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately-prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off.

Fluorescent labels

A wide range of fluorophores can be used as labels in flow cytometry. These each have a characteristic peak excitation and emission wavelength. Also, the emission spectra of the

labels often overlap. Consequently, the combination of labels which can be used depends on the wavelength of the lamp(s) or laser(s) used to excite the fluorochromes and on the detectors available



Use of flow cytometry to measure copy number variation of a specific DNA sequence (Flow-FISH)

Measurable parameters

This list is very long and constantly expanding.

- volume and morphological complexity of cells
- cell pigments such as chlorophyll or phycoerythrin
- total DNA content (cell cycle analysis, cell kinetics, proliferation, etc.)
- total RNA content
- DNA copy number variation (by Flow-FISH)
- chromosome analysis and sorting (library construction, chromosome paint)
- protein expression and localization
- Protein modifications, phospho-proteins
- transgenic products *in vivo*, particularly the Green fluorescent protein or related fluorescent * cell surface antigens (Cluster of differentiation (CD) markers)
- intracellular antigens (various cytokines, secondary mediators, etc.)
- nuclear antigens
- enzymatic activity

- pH, intracellular ionized calcium, magnesium, membrane potential
- membrane fluidity
- apoptosis (quantification, measurement of DNA degradation, mitochondrial membrane potential, permeability changes, caspase activity)
- cell viability
- monitoring electropermeabilization of cells
- oxidative burst
- characterising multidrug resistance (MDR) in cancer cells
- glutathione
- various combinations (DNA/surface antigens, etc.)
- cell adherence (for instance pathogen-host cell adherence)

Applications

The technology has applications in a number of fields, including molecular biology, pathology, immunology, plant biology and marine biology. It has broad application in medicine (especially in transplantation, hematology, tumor immunology and chemotherapy, genetics and sperm sorting for sex preselection). In marine biology, the auto-fluorescent properties of photosynthetic plankton can be exploited by flow cytometry in order to characterise abundance and community structure. In protein engineering, flow cytometry is used in conjunction with yeast display and bacterial display to identify cell surface-displayed protein variants with desired properties. It is also used to determine ploidy of grass carp fry.

Chapter- 5

Animal Testing

Animal testing	
 A white Wistar lab rat	
Description	Around 50–100 million vertebrate animals are used in experiments annually.
Subjects	Animal testing, science, medicine, animal welfare, animal rights, ethics.

Animal testing, also known as **animal experimentation**, **animal research**, and **in vivo testing**, is the use of non-human animals in experiments. Worldwide it is estimated that the number of vertebrate animals—from zebrafish to non-human primates—ranges from the tens of millions to more than 100 million used annually. Invertebrates, mice, rats, birds, fish, frogs, and animals not yet weaned are not included in the figures; one estimate of mice and rats used in the United States alone in 2001 was 80 million. Most animals are euthanized after being used in an experiment. Sources of laboratory animals vary between countries and species; most animals are purpose-bred, while others are caught in the wild or supplied by dealers who obtain them from auctions and pounds.

The research is conducted inside universities, medical schools, pharmaceutical companies, farms, defense establishments, and commercial facilities that provide animal-testing services to industry. It includes pure research such as genetics, developmental biology, behavioral studies, as well as applied research such as biomedical research, xenotransplantation, drug testing and toxicology tests, including cosmetics testing. Animals are also used for education, breeding, and defense research. The practice is regulated to various degrees in different countries.

Supporters of the use of animals in experiments, such as the British Royal Society, argue that virtually every medical achievement in the 20th century relied on the use of animals in some way, with the Institute for Laboratory Animal Research of the U.S. National Academy of Sciences arguing that even sophisticated computers are unable to model interactions between molecules, cells, tissues, organs, organisms, and the environment, making animal research necessary in many areas. A number of scientists, animal welfare, and animal rights organizations—such as PETA and BUAV—question the legitimacy of

it, arguing that it is cruel, poor scientific practice, poorly regulated, that medical progress is being held back by misleading animal models, that some of the tests are outdated, that it cannot reliably predict effects in humans, that the costs outweigh the benefits, or that animals have an intrinsic right not to be used for experimentation.

Definitions

The terms animal testing, **animal experimentation**, animal research, ***in vivo* testing**, and **vivisection** have similar denotations but different connotations. Literally, "vivisection" means the "cutting up" of a living animal, and historically referred only to experiments that involved the dissection of live animals. The term is occasionally used to refer pejoratively to any experiment using living animals; for example, the *Encyclopædia Britannica* defines "vivisection" as: "Operation on a living animal for experimental rather than healing purposes; more broadly, all experimentation on live animals", although dictionaries point out that the broader definition is "used only by people who are opposed to such work". The word has a negative connotation, implying torture, suffering, and death. The word "vivisection" is preferred by those opposed to this research, whereas scientists typically use the term "animal experimentation".

History

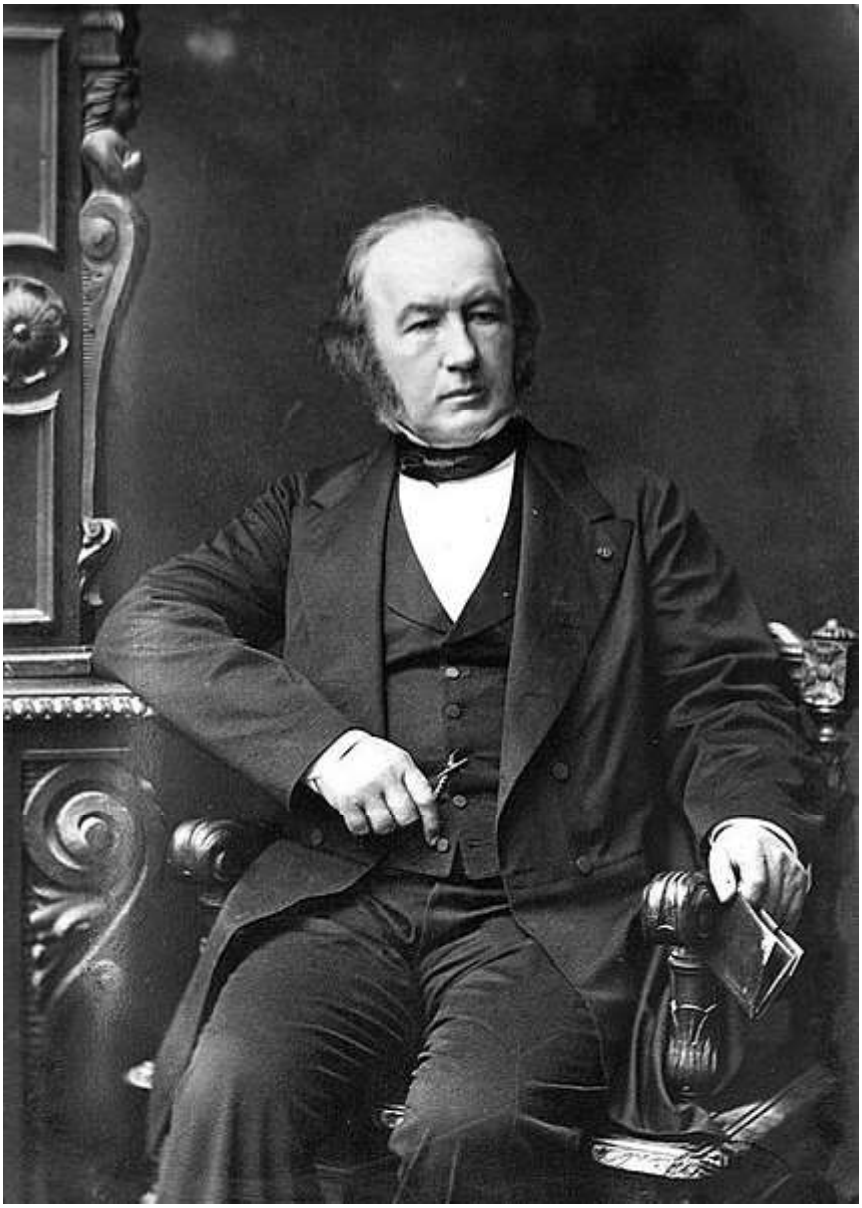
An Experiment on a Bird in an Air Pump, from 1768, by Joseph Wright

The earliest references to animal testing are found in the writings of the Greeks in the 2nd and 4th centuries BCE. Aristotle (Αριστοτέλης) (384–322 BCE) and Erasistratus (304–258 BCE) were among the first to perform experiments on living animals. Galen, a physician in 2nd-century Rome, dissected pigs and goats, and is known as the "father of vivisection." Avenzoar, an Arabic physician in 12th-century Moorish Spain who also practiced dissection, introduced animal testing as an experimental method of testing surgical procedures before applying them to human patients.

Animals have been used repeatedly through the history of biomedical research. In the 1880s, Louis Pasteur convincingly demonstrated the germ theory of medicine by inducing anthrax in sheep. In the 1890s, Ivan Pavlov famously used dogs to describe classical conditioning. Insulin was first isolated from dogs in 1922, and revolutionized the treatment of diabetes. On November 3, 1957, a Russian dog, Laika, became the first of many animals to orbit the earth. In the 1970s, antibiotic treatments and vaccines for leprosy were developed using armadillos, then given to humans. The ability of humans to change the genetics of animals took a large step forwards in 1974 when Rudolf Jaenisch was able to produce the first transgenic mammal, by integrating DNA from the SV40 virus into the genome of mice. This genetic research progressed rapidly and, in 1996, Dolly the sheep was born, the first mammal to be cloned from an adult cell.

Toxicology testing became important in the 20th century. In the 19th century, laws regulating drugs were more relaxed. For example, in the U.S., the government could only ban a drug after a company had been prosecuted for selling products that harmed customers. However, in response to the Elixir Sulfanilamide disaster of 1937 in which the eponymous drug killed more than 100 users, the U.S. congress passed laws that required safety testing of drugs on animals before they could be marketed. Other countries enacted similar legislation. In the 1960s, in reaction to the Thalidomide tragedy, further laws were passed requiring safety testing on pregnant animals before a drug can be sold.

Historical debate



Claude Bernard, regarded as the "prince of vivisectors" argued that experiments on animals are "entirely conclusive for the toxicology and hygiene of man".

As the experimentation on animals increased, especially the practice of vivisection, so did criticism and controversy. In 1655, the advocate of Galenic physiology Edmund O'Meara said that "the miserable torture of vivisection places the body in an unnatural state." O'Meara and others argued that animal physiology could be affected by pain during vivisection, rendering results unreliable. There were also objections on an ethical basis, contending that the benefit to humans did not justify the harm to animals. Early objections to animal testing also came from another angle — many people believed that

animals were inferior to humans and so different that results from animals could not be applied to humans.

On the other side of the debate, those in favor of animal testing held that experiments on animals were necessary to advance medical and biological knowledge. Claude Bernard, known as the "prince of vivisectors" and the father of physiology—whose wife, Marie Françoise Martin, founded the first anti-vivisection society in France in 1883—famously wrote in 1865 that "the science of life is a superb and dazzlingly lighted hall which may be reached only by passing through a long and ghastly kitchen". Arguing that "experiments on animals ... are entirely conclusive for the toxicology and hygiene of man...the effects of these substances are the same on man as on animals, save for differences in degree," Bernard established animal experimentation as part of the standard scientific method.

In 1896, the physiologist and physician Dr. Walter B. Cannon said “The antivivisectionists are the second of the two types Theodore Roosevelt described when he said, ‘Common sense without conscience may lead to crime, but conscience without common sense may lead to folly, which is the handmaiden of crime.’ ” These divisions between pro- and anti- animal testing groups first came to public attention during the brown dog affair in the early 1900s, when hundreds of medical students clashed with anti-vivisectionists and police over a memorial to a vivisected dog.

One of Pavlov’s dogs with a saliva-catch container and tube surgically implanted in his muzzle, Pavlov Museum, 2005

In 1822, the first animal protection law was enacted in the British parliament, followed by the Cruelty to Animals Act (1876), the first law specifically aimed at regulating animal testing. The legislation was promoted by Charles Darwin, who wrote to Ray Lankester in March 1871: "You ask about my opinion on vivisection. I quite agree that it is justifiable for real investigations on physiology; but not for mere damnable and detestable curiosity. It is a subject which makes me sick with horror, so I will not say another word about it, else I shall not sleep to-night." Opposition to the use of animals in medical research first arose in the United States during the 1860s, when Henry Bergh founded the American Society for the Prevention of Cruelty to Animals (ASPCA), with America's first specifically anti-vivisection organization being the American AntiVivisection Society (AAVS), founded in 1883. Antivivisectionists of the era generally believed the spread of mercy was the great cause of civilization, and vivisection was cruel. However, in the USA the antivivisectionists' efforts were defeated in every legislature, overwhelmed by the superior organization and influence of the medical community. Overall, this movement had little legislative success until the passing of the Laboratory Animal Welfare Act, in 1966.

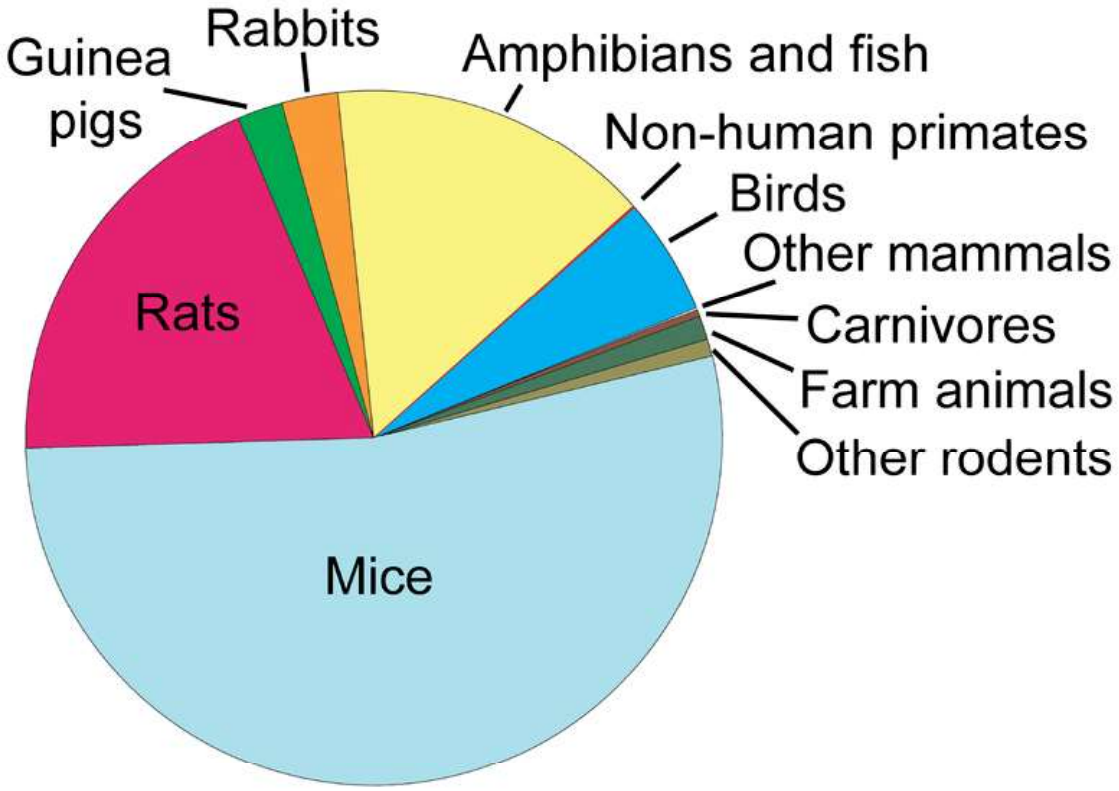
Care and use of animals

Regulations

The regulations that apply to animals in laboratories vary across species. In the U.S., under the provisions of the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals* (the *Guide*), published by the National Academy of Sciences, any procedure can be performed on an animal if it can be successfully argued that it is scientifically justified. In general, researchers are required to consult with the institution's veterinarian and its Institutional Animal Care and Use Committee (IACUC), which every research facility is obliged to maintain. The IACUC must ensure that alternatives, including non-animal alternatives, have been considered, that the experiments are not unnecessarily duplicative, and that pain relief is given unless it would interfere with the study. Larry Carbone, a laboratory animal veterinarian, writes that, in his experience, IACUCs take their work very seriously regardless of the species involved, though the use of non-human primates always raises what he calls a "red flag of special concern." A study published in *Science* magazine in July 2001 confirmed the low reliability of IACUC reviews of animal experiments. Funded by the National Science Foundation, the three-year study found that animal-use committees that do not know the specifics of the university and personnel do not make the same approval decisions as those made by animal-use committees that do know the university and personnel. Specifically, blinded committees more often ask for more information rather than approving studies.

The IACUCs regulate all vertebrates in testing at institutions receiving federal funds in the USA. Although the provisions of the Animal Welfare Act do not include purpose-bred rodents and birds, these species are equally regulated under Public Health Service policies that govern the IACUCs. Animal Welfare Act regulations are enforced by the USDA, whereas Public Health Service regulations are enforced by OLAW and in many cases by AAALAC.

Numbers



Types of vertebrates used in animal testing in Europe in 2005: a total of 12.1 million animals were used.

Accurate global figures for animal testing are difficult to obtain. The British Union for the Abolition of Vivisection (BUAV) estimates that 100 million vertebrates are experimented on around the world every year, 10–11 million of them in the European Union. The Nuffield Council on Bioethics reports that global annual estimates range from 50 to 100 million animals. None of the figures include invertebrates such as shrimp and fruit flies. Animals bred for research then killed as surplus, animals used for breeding purposes, and animals not yet weaned are also not included in the figures.

According to the U.S. Department of Agriculture (USDA), the total number of animals used in that country in 2005 was almost 1.2 million, but this does not include rats and mice, which make up about 90% of research animals. In 1995, researchers at Tufts University Center for Animals and Public Policy estimated that 14–21 million animals were used in American laboratories in 1992, a reduction from a high of 50 million used in 1970. In 1986, the U.S. Congress Office of Technology Assessment reported that estimates of the animals used in the U.S. range from 10 million to upwards of 100 million each year, and that their own best estimate was at least 17 million to 22 million.

In the UK, Home Office figures show that 3.2 million procedures were carried out in 2007, a rise of 189,500 since the previous year. Four thousand procedures used non-human primates, down 240 from 2006. A "procedure" refers to an experiment that might last minutes, several months, or years. Most animals are used in only one procedure: animals either die because of the experiment or are euthanized afterwards.

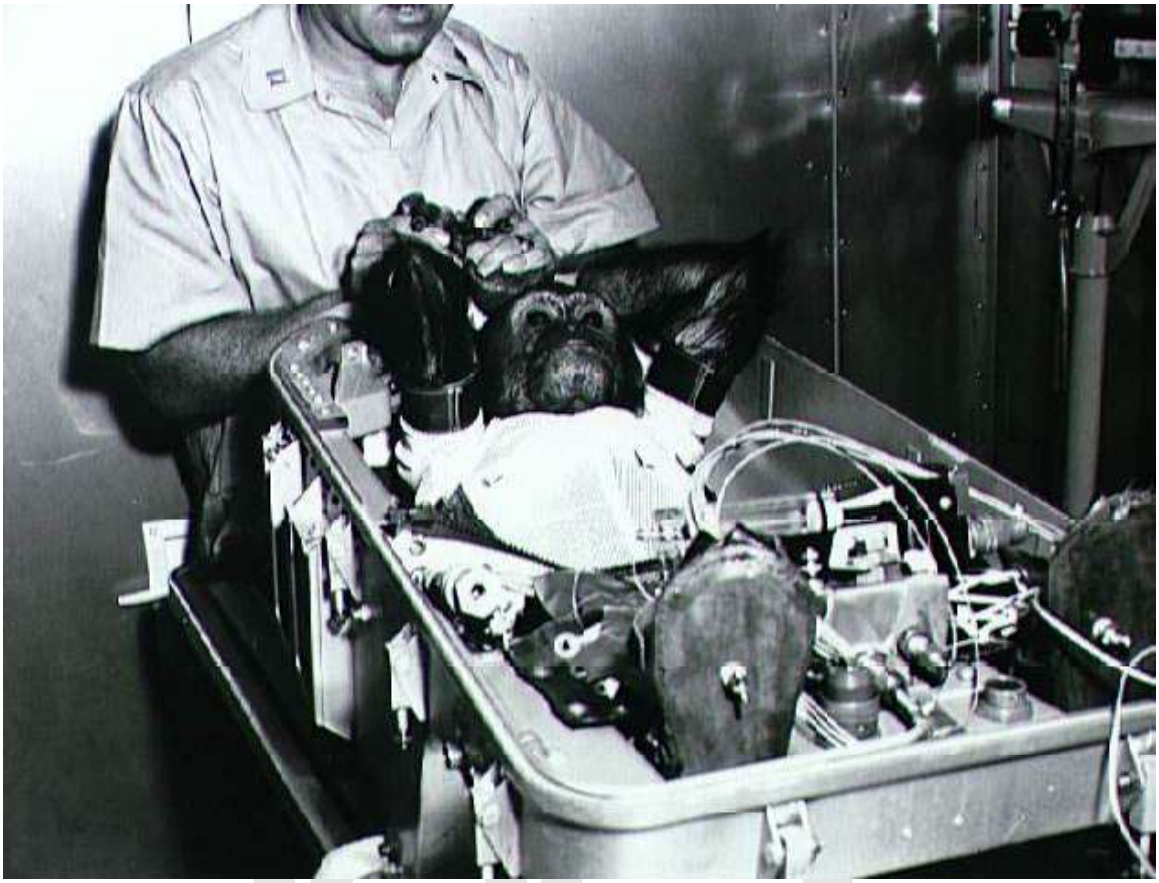
Species

Invertebrates

Fruit flies are commonly used.

Although many more invertebrates than vertebrates are used, these experiments are largely unregulated by law. The most used invertebrate species are *Drosophila melanogaster*, a fruit fly, and *Caenorhabditis elegans*, a nematode worm. In the case of *C. elegans*, the worm's body is completely transparent and the precise lineage of all the organism's cells is known, while studies in the fly *D. melanogaster* can use an amazing array of genetic tools. These animals offer great advantages over vertebrates, including their short life cycle and the ease with which large numbers may be studied, with thousands of flies or nematodes fitting into a single room. However, the lack of an adaptive immune system and their simple organs prevent worms from being used in medical research such as vaccine development. Similarly, flies are not widely used in applied medical research, as their immune system differs greatly from that of humans, and diseases in insects can be very different from diseases in vertebrates.

Vertebrates



Enos the space chimp before insertion into the Mercury-Atlas 5 capsule in 1961

This rat is being deprived of restful REM sleep by a researcher using a single platform ("flower pot") technique. The water is within 1 cm of the small flower pot bottom platform where the rat sits. At the onset of REM sleep, the rat would either fall into the water only to clamber back to its pot to avoid drowning, or its nose would become submerged into the water shocking it back to an awakened state.

In the U.S., the numbers of rats and mice used is estimated at 20 million a year. Other rodents commonly used are guinea pigs, hamsters, and gerbils. Mice are the most commonly used vertebrate species because of their size, low cost, ease of handling, and fast reproduction rate. Mice are widely considered to be the best model of inherited human disease and share 99% of their genes with humans. With the advent of genetic engineering technology, genetically modified mice can be generated to order and can provide models for a range of human diseases. Rats are also widely used for physiology, toxicology and cancer research, but genetic manipulation is much harder in rats than in mice, which limits the use of these rodents in basic science.

Nearly 200,000 fish and 20,000 amphibians were used in the UK in 2004. The main species used is the zebrafish, *Danio rerio*, which are translucent during their embryonic stage, and the African clawed frog, *Xenopus laevis*. Over 20,000 rabbits were used for animal testing in the UK in 2004. Albino rabbits are used in eye irritancy tests because rabbits have less tear flow than other animals, and the lack of eye pigment in albinos

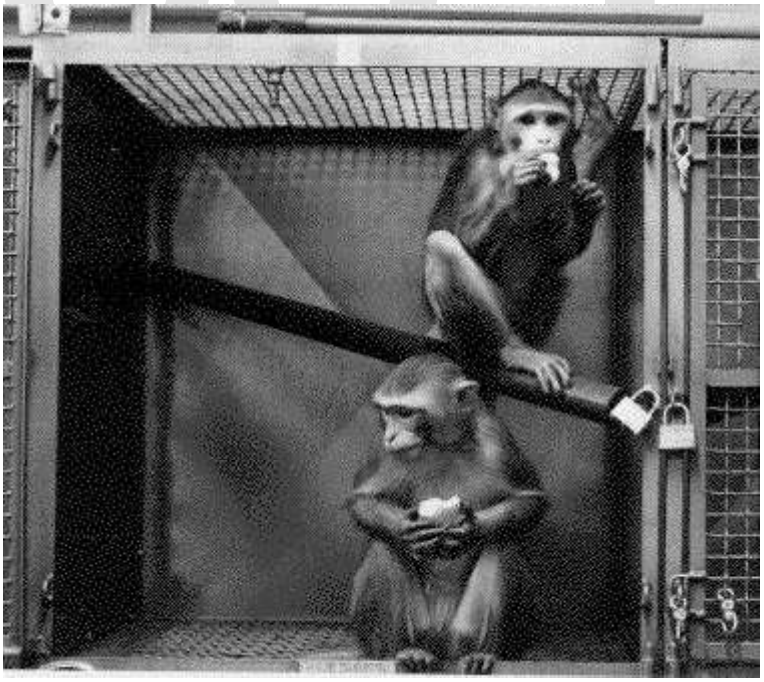
make the effects easier to visualize. Rabbits are also frequently used for the production of polyclonal antibodies.

Cats and dogs

Cats are most commonly used in neurological research. Over 25,500 cats were used in the U.S. in 2000, around half of whom were used in experiments which, according to the American Anti-Vivisection Society, had the potential to cause "pain and/or distress".

Dogs are widely used in biomedical research, testing, and education — particularly beagles, because they are gentle and easy to handle. They are commonly used as models for human diseases in cardiology, endocrinology, and bone and joint studies, research that tends to be highly invasive, according to the Humane Society of the United States. The U.S. Department of Agriculture's Animal Welfare Report for 2005 shows that 66,000 dogs were used in USDA-registered facilities in that year. In the U.S., some of the dogs are purpose-bred, while most are supplied by so-called Class B dealers licensed by the USDA to buy animals from auctions, shelters, newspaper ads, and who are sometimes accused of stealing pets.

Non-human primates



Around 65,000 primates are used each year in the U.S. and Europe.

Non-human primates (NHPs) are used in toxicology tests, studies of AIDS and hepatitis, studies of neurology, behavior and cognition, reproduction, genetics, and xenotransplantation. They are caught in the wild or purpose-bred. In the U.S. and China, most primates are domestically purpose-bred, whereas in Europe the majority are imported purpose-bred. Rhesus monkeys, cynomolgus monkeys, squirrel monkeys, and

owl monkeys are imported; around 12,000 to 15,000 monkeys are imported into the U.S. annually. In total, around 70,000 NHPs are used each year in the United States and European Union. Most of the NHPs used are macaques; but marmosets, spider monkeys, and squirrel monkeys are also used, and baboons and chimpanzees are used in the U.S; in 2006 there were 1133 chimpanzees in U.S. primate centers. The first transgenic primate was produced in 2001, with the development of a method that could introduce new genes into a rhesus macaque. This transgenic technology is now being applied in the search for a treatment for the genetic disorder Huntington's disease. Notable studies on non-human primates have been part of the polio vaccine development, and development of Deep Brain Stimulation, and their current heaviest non-toxicological use occurs in the monkey AIDS model, SIV. In 2008 a proposal to ban all primates experiments in the EU has sparked a vigorous debate.

Sources

Animals used by laboratories are largely supplied by specialist dealers. Sources differ for vertebrate and invertebrate animals. Most laboratories breed and raise flies and worms themselves, using strains and mutants supplied from a few main stock centers. For vertebrates, sources include breeders who supply purpose-bred animals; businesses that trade in wild animals; and dealers who supply animals sourced from pounds, auctions, and newspaper ads. Animal shelters also supply the laboratories directly. Large centers also exist to distribute strains of genetically-modified animals; the National Institutes of Health *Knockout Mouse Project*, for example, aims to provide knockout mice for every gene in the mouse genome.

A laboratory mouse cage. Mice are either bred commercially, or raised in the laboratory.

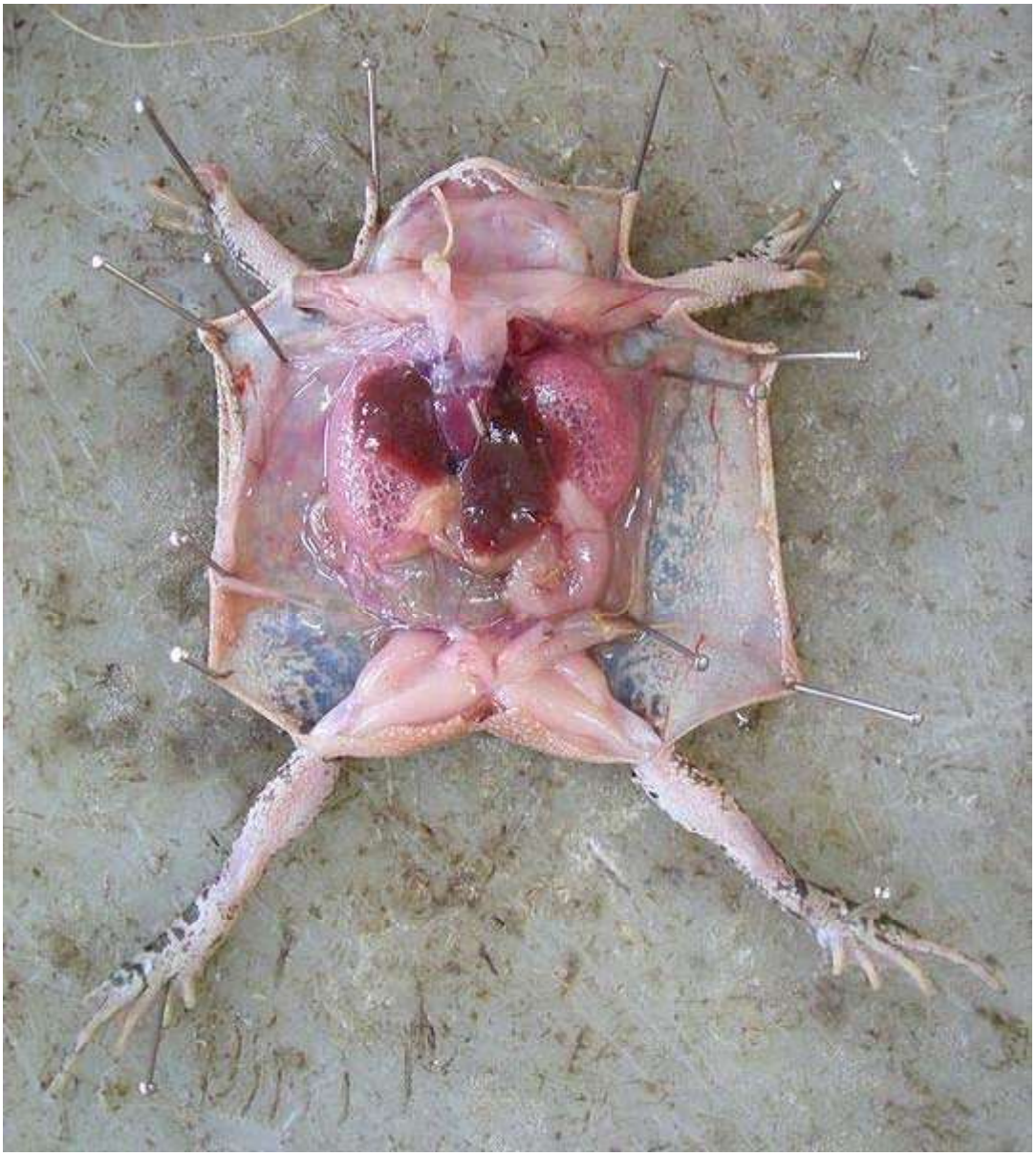
In the U.S., Class A breeders are licensed by the U.S. Department of Agriculture (USDA) to sell animals for research purposes, while Class B dealers are licensed to buy animals from "random sources" such as auctions, pound seizure, and newspaper ads. Some Class B dealers have been accused of kidnapping pets and illegally trapping strays, a practice known as *bunching*. It was in part out of public concern over the sale of pets to research facilities that the 1966 Laboratory Animal Welfare Act was ushered in — the Senate Committee on Commerce reported in 1966 that stolen pets had been retrieved from Veterans Administration facilities, the Mayo Institute, the University of Pennsylvania, Stanford University, and Harvard and Yale Medical Schools. The USDA recovered at least a dozen stolen pets during a raid on a Class B dealer in Arkansas in 2003.

Four states in the U.S. — Minnesota, Utah, Oklahoma, and Iowa — require their shelters to provide animals to research facilities. Fourteen states explicitly prohibit the practice, while the remainder either allow it or have no relevant legislation.

In the European Union, animal sources are governed by *Council Directive 86/609/EEC*, which requires lab animals to be specially bred, unless the animal has been lawfully imported and is not a wild animal or a stray. The latter requirement may also be exempted by special arrangement. In the UK, most animals used in experiments are bred for the purpose under the 1988 Animal Protection Act, but wild-caught primates may be

used if exceptional and specific justification can be established. The United States also allows the use of wild-caught primates; between 1995 and 1999, 1,580 wild baboons were imported into the U.S. Over half the primates imported between 1995 and 2000 were handled by Charles River Laboratories, Inc., or by Covance, which is the single largest importer of primates into the U.S.

Pain and suffering



Prior to vivisection for educational purposes, chloroform was administered to this common sand frog to induce terminal anesthesia.

The extent to which animal testing causes pain and suffering, and the capacity of animals to experience and comprehend them, is the subject of much debate.

According to the U.S. Department of Agriculture, in 2006 about 670,000 animals (57%) (not including rats, mice, birds, or invertebrates) were used in procedures that did not include more than momentary pain or distress. About 420,000 (36%) were used in procedures in which pain or distress was relieved by anesthesia, while 84,000 (7%) were used in studies that would cause pain or distress that would not be relieved.

In the UK, research projects are classified as mild, moderate, and substantial in terms of the suffering the researchers conducting the study say they may cause; a fourth category of "unclassified" means the animal was anesthetized and killed without recovering consciousness, according to the researchers. In December 2001, 1,296 (39%) of project licenses in force were classified as mild, 1,811 (55%) as moderate, 63 (2%) as substantial, and 139 (4%) as unclassified. There have, however, been suggestions of systemic underestimation of procedure severity.

The idea that animals might not feel pain as human beings feel it traces back to the 17th-century French philosopher, René Descartes, who argued that animals do not experience pain and suffering because they lack consciousness. Bernard Rollin of Colorado State University, the principal author of two U.S. federal laws regulating pain relief for animals, writes that researchers remained unsure into the 1980s as to whether animals experience pain, and that veterinarians trained in the U.S. before 1989 were simply taught to ignore animal pain. In his interactions with scientists and other veterinarians, he was regularly asked to "prove" that animals are conscious, and to provide "scientifically acceptable" grounds for claiming that they feel pain. Carbone writes that the view that animals feel pain differently is now a minority view. Academic reviews of the topic are more equivocal, noting that although the argument that animals have at least simple conscious thoughts and feelings has strong support, some critics continue to question how reliably animal mental states can be determined. The ability of invertebrate species of animals, such as insects, to feel pain and suffering is also unclear.

The defining text on animal welfare regulation, "Guide for the Care and Use of Laboratory Animals" defines the parameters that govern animal testing in the USA. It states "The ability to experience and respond to pain is widespread in the animal kingdom...Pain is a stressor and, if not relieved, can lead to unacceptable levels of stress and distress in animals." The Guide states that the ability to recognize the symptoms of pain in different species is vital in efficiently applying pain relief and that it is essential for the people caring for and using animals to be entirely familiar with these symptoms. On the subject of analgesics used to relieve pain, the Guide states "The selection of the most appropriate analgesic or anesthetic should reflect professional judgment as to which best meets clinical and humane requirements without compromising the scientific aspects of the research protocol". Accordingly, all issues of animal pain and distress, and their potential treatment with analgesia and anesthesia, are required regulatory issues in receiving animal protocol approval.

Euthanasia

There is general agreement that animal life should not be taken wantonly, and regulations require that scientists use as few animals as possible. However, while policy makers consider suffering to be the central issue and see animal euthanasia as a way to reduce suffering, others, such as the RSPCA, argue that the lives of laboratory animals have intrinsic value. Regulations focus on whether particular methods cause pain and suffering, not whether their death is undesirable in itself. The animals are euthanized at the end of studies for sample collection or post-mortem examination; during studies if their pain or suffering falls into certain categories regarded as unacceptable, such as depression, infection that is unresponsive to treatment, or the failure of large animals to eat for five days; or when they are unsuitable for breeding or unwanted for some other reason.

Methods of euthanizing laboratory animals are chosen to induce rapid unconsciousness and death without pain or distress. The methods that are preferred are those published by councils of veterinarians. The animal can be made to inhale a gas, such as carbon monoxide and carbon dioxide, by being placed in a chamber, or by use of a face mask, with or without prior sedation or anesthesia. Sedatives or anesthetics such as barbiturates can be given intravenously, or inhalant anesthetics may be used. Amphibians and fish may be immersed in water containing an anesthetic such as tricaine. Physical methods are also used, with or without sedation or anesthesia depending on the method. Recommended methods include decapitation (beheading) for small rodents or rabbits. Cervical dislocation (breaking the neck or spine) may be used for birds, mice, and immature rats and rabbits. Maceration (grinding into small pieces) is used on 1 day old chicks. High-intensity microwave irradiation of the brain can preserve brain tissue and induce death in less than 1 second, but this is currently only used on rodents. Captive bolts may be used, typically on dogs, ruminants, horses, pigs and rabbits. It causes death by a concussion to the brain. Gunshot may be used, but only in cases where a penetrating captive bolt may not be used. Some physical methods are only acceptable after the animal is unconscious. Electrocution may be used for cattle, sheep, swine, foxes, and mink after the animals are unconscious, often by a prior electrical stun. Pithing (inserting a tool into the base of the brain) is usable on animals already unconscious. Slow or rapid freezing, or inducing air embolism are acceptable only with prior anesthesia to induce unconsciousness.

Research classification

Pure research

Basic or pure research investigates how organisms behave, develop, and function. Those opposed to animal testing object that pure research may have little or no practical purpose, but researchers argue that it may produce unforeseen benefits, rendering the distinction between pure and applied research—research that has a specific practical aim—unclear. Pure research uses larger numbers and a greater variety of animals than applied research. Fruit flies, nematode worms, mice and rats together account for the vast

majority, though small numbers of other species are used, ranging from sea slugs through to armadillos. Examples of the types of animals and experiments used in basic research include:

- Studies on *embryogenesis* and *developmental biology*. Mutants are created by adding transposons into their genomes, or specific genes are deleted by gene targeting. By studying the changes in development these changes produce, scientists aim to understand both how organisms normally develop, and what can go wrong in this process. These studies are particularly powerful since the basic controls of development, such as the homeobox genes, have similar functions in organisms as diverse as fruit flies and man.

- Experiments into *behavior*, to understand how organisms detect and interact with each other and their environment, in which fruit flies, worms, mice, and rats are all widely used. Studies of brain function, such as memory and social behavior, often use rats and birds. For some species, behavioral research is combined with enrichment strategies for animals in captivity because it allows them to engage in a wider range of activities.

- Breeding experiments to study *evolution* and *genetics*. Laboratory mice, flies, fish, and worms are inbred through many generations to create strains with defined characteristics. These provide animals of a known genetic background, an important tool for genetic analyses. Larger mammals are rarely bred specifically for such studies due to their slow rate of reproduction, though some scientists take advantage of inbred domesticated animals, such as dog or cattle breeds, for comparative purposes. Scientists studying how animals evolve use many animal species to see how variations in where and how an organism lives (their niche) produce adaptations in their physiology and morphology. As an example, sticklebacks are now being used to study how many and which types of mutations are selected to produce adaptations in animals' morphology during the evolution of new species.

Applied research

Applied research aims to solve specific and practical problems. Compared to pure research, which is largely academic in origin, applied research is usually carried out in the pharmaceutical industry, or by universities in commercial partnerships. These may involve the use of animal models of diseases or conditions, which are often discovered or generated by pure research programmes. In turn, such applied studies may be an early stage in the drug discovery process. Examples include:

- Genetic modification of animals to study disease. Transgenic animals have specific genes inserted, modified or removed, to mimic specific conditions such as single gene disorders, such as Huntington's disease. Other models mimic complex, multifactorial diseases with genetic components, such as diabetes, or even transgenic mice that carry the same mutations that occur during the

development of cancer. These models allow investigations on how and why the disease develops, as well as providing ways to develop and test new treatments. The vast majority of these transgenic models of human disease are lines of mice, the mammalian species in which genetic modification is most efficient. Smaller numbers of other animals are also used, including rats, pigs, sheep, fish, birds, and amphibians.

- Studies on models of naturally occurring disease and condition. Certain domestic and wild animals have a natural propensity or predisposition for certain conditions that are also found in humans. Cats are used as a model to develop immunodeficiency virus vaccines and to study leukemia because their natural predisposition to FIV and Feline leukemia virus. Certain breeds of dog suffer from narcolepsy making them the major model used to study the human condition. Armadillos and humans are among only a few animal species that naturally suffer from leprosy; as the bacteria responsible for this disease cannot yet be grown in culture, armadillos are the primary source of bacilli used in leprosy vaccines.

- Studies on induced animal models of human diseases. Here, an animal is treated so that it develops pathology and symptoms that resemble a human disease. Examples include restricting blood flow to the brain to induce stroke, or giving neurotoxins that cause damage similar to that seen in Parkinson's disease. Such studies can be difficult to interpret, and it is argued that they are not always comparable to human diseases. For example, although such models are now widely used to study Parkinson's disease, the British anti-vivisection interest group BUAV argues that these models only superficially resemble the disease symptoms, without the same time course or cellular pathology. In contrast, scientists assessing the usefulness of animal models of Parkinson's disease, as well as the medical research charity *The Parkinson's Appeal*, state that these models were invaluable and that they led to improved surgical treatments such as pallidotomy, new drug treatments such as levodopa, and later deep brain stimulation.

Xenotransplantation

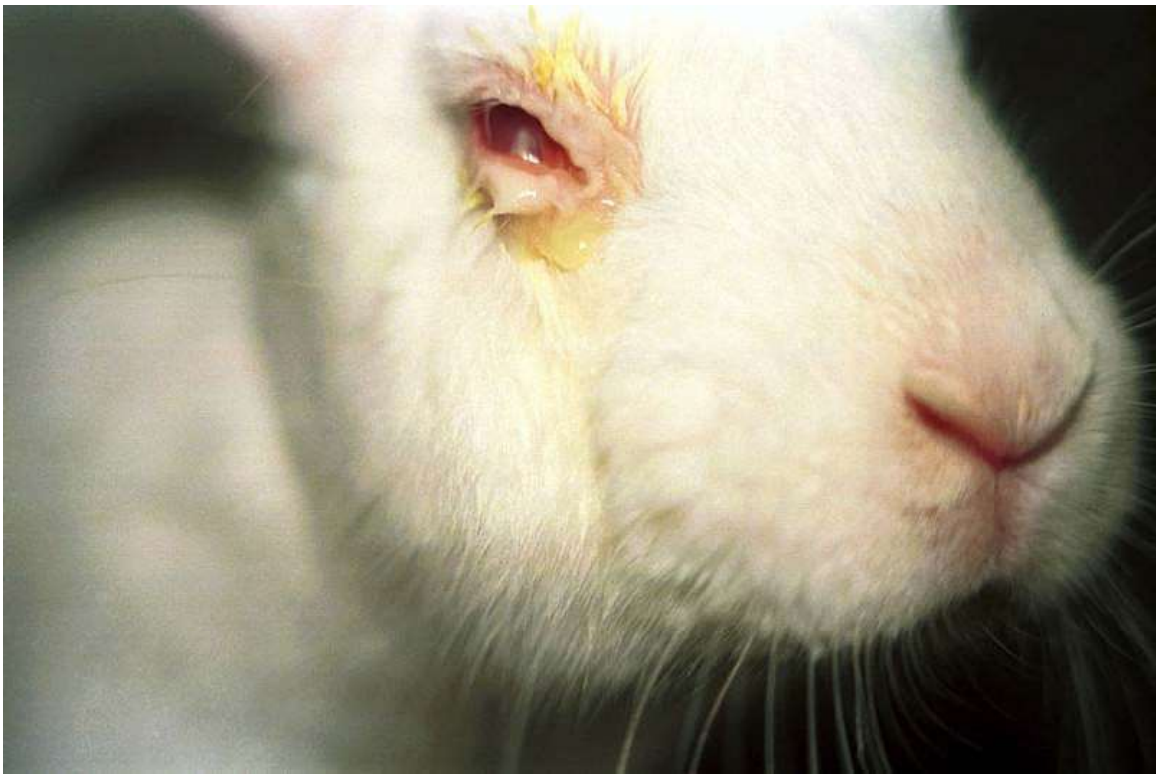
Xenotransplantation research involves transplanting tissues or organs from one species to another, as a way to overcome the shortage of human organs for use in organ transplants. Current research involves using primates as the recipients of organs from pigs that have been genetically-modified to reduce the primates' immune response against the pig tissue. Although transplant rejection remains a problem, recent clinical trials that involved implanting pig insulin-secreting cells into diabetics did reduce these people's need for insulin.

Documents released to the news media by the animal rights organization Uncaged Campaigns showed that, between 1994 and 2000, wild baboons imported to the UK from Africa by Imutran Ltd, a subsidiary of Novartis Pharma AG, in conjunction with

Cambridge University and Huntingdon Life Sciences, to be used in experiments that involved grafting pig tissues, suffered serious and sometimes fatal injuries. A scandal occurred when it was revealed that the company had communicated with the British government in an attempt to avoid regulation.

Toxicology testing

Toxicology testing, also known as safety testing, is conducted by pharmaceutical companies testing drugs, or by contract animal testing facilities, such as Huntingdon Life Sciences, on behalf of a wide variety of customers. According to 2005 EU figures, around one million animals are used every year in Europe in toxicology tests; which are about 10% of all procedures. According to *Nature*, 5,000 animals are used for each chemical being tested, with 12,000 needed to test pesticides. The tests are conducted without anesthesia, because interactions between drugs can affect how animals detoxify chemicals, and may interfere with the results.



A rabbit during a Draize test

Toxicology tests are used to examine finished products such as pesticides, medications, food additives, packing materials, and air freshener, or their chemical ingredients. Most tests involve testing ingredients rather than finished products, but according to BUAV, manufacturers believe these tests overestimate the toxic effects of substances; they therefore repeat the tests using their finished products to obtain a less toxic label.

The substances are applied to the skin or dripped into the eyes; injected intravenously, intramuscularly, or subcutaneously; inhaled either by placing a mask over the animals and restraining them, or by placing them in an inhalation chamber; or administered orally, through a tube into the stomach, or simply in the animal's food. Doses may be given once, repeated regularly for many months, or for the lifespan of the animal.

There are several different types of acute toxicity tests. The LD_{50} ("Lethal Dose 50%") test is used to evaluate the toxicity of a substance by determining the dose required to kill 50% of the test animal population. This test was removed from OECD international guidelines in 2002, replaced by methods such as the fixed dose procedure, which use fewer animals and cause less suffering. *Nature* writes that, as of 2005, "the LD50 acute toxicity test ... still accounts for one-third of all animal [toxicity] tests worldwide." Irritancy can be measured using the Draize test, where a test substance is applied to an animal's eyes or skin, usually an albino rabbit. For Draize eye testing, the test involves observing the effects of the substance at intervals and grading any damage or irritation, but the test should be halted and the animal killed if it shows "continuing signs of severe pain or distress". The Humane Society of the United States writes that the procedure can cause redness, ulceration, hemorrhaging, cloudiness, or even blindness. This test has also been criticized by scientists for being cruel and inaccurate, subjective, over-sensitive, and failing to reflect human exposures in the real world. Although no accepted *in vitro* alternatives exist, a modified form of the Draize test called the *low volume eye test* may reduce suffering and provide more realistic results and this was adopted as the new standard in September 2009. However, the Draize test will still be used for substances that are not severe irritants.

The most stringent tests are reserved for drugs and foodstuffs. For these, a number of tests are performed, lasting less than a month (acute), one to three months (subchronic), and more than three months (chronic) to test general toxicity (damage to organs), eye and skin irritancy, mutagenicity, carcinogenicity, teratogenicity, and reproductive problems. The cost of the full complement of tests is several million dollars per substance and it may take three or four years to complete.

These toxicity tests provide, in the words of a 2006 United States National Academy of Sciences report, "critical information for assessing hazard and risk potential". *Nature* reported that most animal tests either over- or underestimate risk, or do not reflect toxicity in humans particularly well, with false positive results being a particular problem. This variability stems from using the effects of high doses of chemicals in small numbers of laboratory animals to try to predict the effects of low doses in large numbers of humans. Although relationships do exist, opinion is divided on how to use data on one species to predict the exact level of risk in another.

Cosmetics testing

Products in Europe not tested on animals carry this symbol.

Cosmetics testing on animals is particularly controversial. Such tests, which are still conducted in the U.S., involve general toxicity, eye and skin irritancy, phototoxicity (toxicity triggered by ultraviolet light) and mutagenicity.

Cosmetics testing is banned in the Netherlands, Belgium, and the UK, and in 2002, after 13 years of discussion, the European Union (EU) agreed to phase in a near-total ban on the sale of animal-tested cosmetics throughout the EU from 2009, and to ban all cosmetics-related animal testing. France, which is home to the world's largest cosmetics company, L'Oreal, has protested the proposed ban by lodging a case at the European Court of Justice in Luxembourg, asking that the ban be quashed. The ban is also opposed by the European Federation for Cosmetics Ingredients, which represents 70 companies in Switzerland, Belgium, France, Germany and Italy.

Drug testing

Beagles used for safety testing of pharmaceuticals in a British facility

Before the early 20th century, laws regulating drugs were lax. Currently, all new pharmaceuticals undergo rigorous animal testing before being licensed for human use. Tests on pharmaceutical products involve:

- *metabolic tests*, investigating pharmacokinetics – how drugs are absorbed, metabolized and excreted by the body when introduced orally, intravenously, intraperitoneally, intramuscularly, or transdermally.

- *toxicology tests*, which gauge acute, sub-acute, and chronic toxicity. Acute toxicity is studied by using a rising dose until signs of toxicity become apparent. Current European legislation demands that "acute toxicity tests must be carried out in two or more mammalian species" covering "at least two different routes of administration". Sub-acute toxicity is where the drug is given to the animals for four to six weeks in doses below the level at which it causes rapid poisoning, in order to discover if any toxic drug metabolites build up over time. Testing for

chronic toxicity can last up to two years and, in the European Union, is required to involve two species of mammals, one of which must be non-rodent.
- *efficacy studies*, which test whether experimental drugs work by inducing the appropriate illness in animals. The drug is then administered in a double-blind controlled trial, which allows researchers to determine the effect of the drug and the dose-response curve.
- Specific tests on *reproductive function*, *embryonic toxicity*, or *carcinogenic potential* can all be required by law, depending on the result of other studies and the type of drug being tested.

Education, breeding, and defense

Animals are also used for education and training; are bred for use in laboratories; and are used by the military to develop weapons, vaccines, battlefield surgical techniques, and defensive clothing. For example, in 2008 the United States Defense Advanced Research Projects Agency used live pigs to study the effects of improvised explosive device explosions on internal organs, especially the brain.

There are efforts in many countries to find alternatives to using animals in education. Horst Spielmann, German director of the Central Office for Collecting and Assessing Alternatives to Animal Experimentation, while describing Germany's progress in this area, told German broadcaster ARD in 2005: "Using animals in teaching curricula is already superfluous. In many countries, one can become a doctor, vet or biologist without ever having performed an experiment on an animal."

Ethics

Background

Monument for animals used in testing at Keio University

The ethical questions raised by performing experiments on animals are subject to much debate, and viewpoints have shifted significantly over the 20th century. There remain disagreements about which procedures are useful for which purposes, as well as disagreements over which ethical principles apply to which species. The dominant ethical position worldwide is that achievement of scientific and medical goals using animal testing is desirable, so long as animal suffering and use is minimized. The British government has additionally required that the cost to animals in an experiment be

weighed against the gain in knowledge. Some medical schools and agencies in China, Japan, and South Korea have built cenotaphs for killed animals. In Japan there are also annual memorial services (*Ireisai* 慰霊祭) for animals sacrificed at medical school.

A wide range of minority viewpoints exist. The view that animals have moral rights (animal rights) is a philosophical position proposed by Tom Regan, among others, who argues that animals are beings with beliefs and desires, and as such are the "subjects of a life" with moral value and therefore moral rights. Regan still sees ethical differences between killing human and non-human animals, and argues that to save the former it is permissible to kill the latter. Others, such as Bernard Rollin, argue that benefits to human beings cannot outweigh animal suffering, and that human beings have no moral right to use an animal in ways that do not benefit that individual. Another prominent position is that of philosopher Peter Singer, who argues that there are no grounds to include a being's species in considerations of whether their suffering is important in utilitarian moral considerations.

Although these arguments have not been widely accepted, governments such as the Netherlands and New Zealand have responded to the concerns by outlawing invasive experiments on certain classes of non-human primates, particularly the great apes.

Prominent cases

Various specific cases of animal testing have drawn attention, including both instances of beneficial scientific research, and instances of alleged ethical violations by those performing the tests.

Muscle physiology

The fundamental properties of muscle physiology were determined with on work done using frog muscles (including the force generating mechanism of all muscle, the length-tension relationship, and the force-velocity curve), and frogs are still the preferred model organism due to the long survival of muscles in vitro and the possibility of isolating intact single-fiber preparations (not possible in other organisms). Modern physical therapy and the understanding and treatment of muscular disorders is based on this work and subsequent work in mice (often engineered to express disease states such as muscular dystrophy).

University of California, Riverside

1985 was a pivotal year in the debate about animal research in the United States, with the enactment of amendments to the Animal Welfare Act. Britches, a macaque monkey, was born that year inside the University of California, Riverside, removed from his mother at birth, and left alone with his eyelids sewn shut, and a sonar sensor on his head, as part of an experiment to test sensory substitution devices for blind people. The Animal Liberation Front raided the laboratory on April 20, 1985, removing Britches and 466 other animals, and reportedly inflicting $700,000-worth of damage to equipment. A

spokesman for the university said the allegations of mistreatment were false, and that the raid caused long-term damage to its research projects. The National Institutes of Health conducted an eight-month investigation and concluded that no corrective action was necessary.

Huntingdon Life Sciences

Huntingdon Life Sciences showed staff mistreating beagles.

In 1997, People for the Ethical Treatment of Animals filmed staff inside Huntingdon Life Sciences (HLS) in the UK, Europe's largest animal-testing facility, hitting puppies, shouting at them, and simulating sex acts while taking blood samples. The company said the employees were dismissed. Two pleaded guilty to "cruelly terrifying dogs," and were given community service orders and ordered to pay £250 costs, the first lab technicians to have been prosecuted for animal cruelty in the UK. The broadcast of the video on Britain's Channel 4 Television in March 1997 triggered the formation of Stop Huntingdon Animal Cruelty (SHAC), an international leaderless resistance campaign to close HLS, which has been criticized for its sometimes violent tactics. In January 2009, several British SHAC activists were jailed for blackmailing companies linked to HLS.

Roslin Institute

Dolly the sheep: the first clone produced from an adult animal

In February 1997 a team at the Roslin Institute in Scotland announced the birth of Dolly the sheep, a ewe that had been cloned from tissue taken from another adult sheep. Dolly was produced through nuclear transfer to an unfertilised oocyte, and was the only lamb that survived from 277 attempts at this technique. Dolly appeared to be a normal sheep, living for six years and giving birth to several lambs, but was euthanized in 2003 after contracting a progressive lung disease. Although the production of Dolly was a scientific breakthrough, it was controversial, since it showed that not only could cloned animals be produced for use in farming, but also that it would now be, in principle, possible to clone a human being.

University of Cambridge

A marmoset after being brain damaged

The British Union for the Abolition of Vivisection (BUAV) raised concerns about primate experiments at the University of Cambridge in 2002. In a series of court cases, the BUAV alleged that monkeys had undergone surgery to induce a stroke, and were left alone after the procedure for 15 hours overnight. Researchers had trained the monkeys to perform certain tasks before inflicting brain damage and re-testing them. The monkeys were only given food and water for two hours a day, to encourage them to perform the tasks. The judge hearing BUAV's application for a judicial review rejected the allegation that the Home Secretary had been negligent in granting the university a license. The British government's chief inspector of animals conducted a review of the facilities and experiments. It concluded the veterinary input at Cambridge was "exemplary"; the facility "seems adequately staffed"; and the animals afforded "appropriate standards of accommodation and care."

Columbia University

CNN reported in October 2003 that Catherine Dell'Orto, a veterinarian at Columbia University, had approached the university's Institute of Comparative Medicine about the treatment of baboons who were undergoing surgery as part of an experiment into stroke treatment. She said the baboons, who were in some cases having an eyeball removed, were left to suffer in their cages after the surgery. She alleged there was systemic maltreatment, poor record-keeping, and other violations of regulations, according to CNN. She presented her evidence in October 2002 and, dissatisfied with the response, contacted People for the Ethical Treatment of Animals two months later.

In March 2003, a lab technician shot video inside the lab, which according to *The New York Daily News* showed primates in cages without pain medication; the video included one baboon with a metal cylinder screwed into its head, according to the newspaper. Dell'Orto told the newspaper that primates were often not euthanized or given painkillers after surgery; she said other primates had torn their fingers off out of fear. The U.S. Department of Agriculture upheld Dell'Orto's complaint that there was shoddy record-keeping, and that 11 animals had been provided with "inadequate or questionable care." They found no evidence that the experiments violated federal guidelines or that there had been retaliation against Dell'Orto. CNN reported that Columbia responded by ordering better record-keeping, a review of the veterinary care program, and tighter criteria for euthanasia of laboratory animals.

Covance

In 2004, German journalist Friedrich Mülln shot undercover footage of staff in Covance, Münster, Europe's largest primate-testing center, making monkeys dance in time to blaring pop music, handling them roughly, and screaming at them. The monkeys were kept isolated in small wire cages with little or no natural light, no environmental enrichment, and high noise levels from staff shouting and playing the radio (video). Primatologist Jane Goodall described the living conditions of the monkeys as horrendous. Another primatologist, Stephen Brend, told BUAV that using monkeys in such a stressed state is bad science, and trying to extrapolate useful data in such circumstances is what he

called an untenable proposition. In 2004 and 2005, PETA shot footage inside the company in the United States. According to *The Washington Post*, PETA said an employee of the group filmed primates being choked, hit, and denied medical attention when badly injured. The U.S. Department of Agriculture fined Covance $8,720 for 16 citations, three of which involved lab monkeys; the other citations involved administrative issues and equipment.

Threats to researchers

In 2006, a primate researcher at the University of California, Los Angeles (UCLA) shut down the experiments in his lab after threats from animal rights activists. The researcher had received a grant to use 30 macaque monkeys for vision experiments; each monkey was anesthetized for a single physiological experiment lasting up to 120 hours, and then euthanized. The researcher's name, phone number, and address were posted on the website of the Primate Freedom Project. Demonstrations were held in front of his home. A Molotov cocktail was placed on the porch of what was believed to be the home of another UCLA primate researcher; instead, it was accidentally left on the porch of an elderly woman unrelated to the university. The Animal Liberation Front claimed responsibility for the attack. As a result of the campaign, the researcher sent an email to the Primate Freedom Project stating "you win," and "please don't bother my family anymore." In another incident at UCLA in June 2007, the Animal Liberation Brigade placed a bomb under the car of a UCLA children's ophthalmologist who experiments on cats and rhesus monkeys; the bomb had a faulty fuse and did not detonate. UCLA is now refusing Freedom of Information Act requests for animal medical records.

These attacks, as well as similar incidents that caused the Southern Poverty Law Center to declare in 2002 that the animal rights movement had "clearly taken a turn toward the more extreme," this prompted the US government to pass the Animal Enterprise Terrorism Act and the UK government to add the offense of "Intimidation of persons connected with animal research organisation" to the Serious Organised Crime and Police Act 2005. Such legislation, and the arrest and imprisonment of extremists may have decreased the incidence of attacks.

Alternatives to animal testing

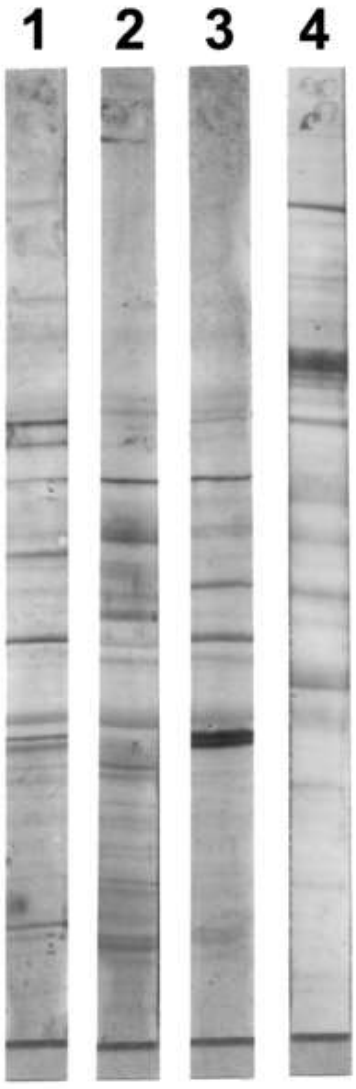
Scientists and governments state that animal testing should cause as little suffering to animals as possible, and that animal tests should only be performed where necessary. The "three Rs" are guiding principles for the use of animals in research in most countries:

1. **Replacement** refers to the preferred use of non-animal methods over animal methods whenever it is possible to achieve the same scientific aim.
2. **Reduction** refers to methods that enable researchers to obtain comparable levels of information from fewer animals, or to obtain more information from the same number of animals.
3. **Refinement** refers to methods that alleviate or minimize potential pain, suffering or distress, and enhance animal welfare for the animals still used.

Although such principles have been welcomed as a step forwards by some animal welfare groups, they have also been criticized as both outdated by current research, and of little practical effect in improving animal welfare.

Chapter- 6

Western Blot



Western blot analysis of proteins separated by SDS-PAGE.

The **Western blot** (alternatively, **protein immunoblot**) is an extremely useful analytical technique used to detect specific proteins in the given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein.

There are now many reagent companies that specialize in providing antibodies (both monoclonal and polyclonal antibodies) against tens of thousands of different proteins. Commercial antibodies can be expensive, although the unbound antibody can be reused between experiments. This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines.

Other related techniques include using antibodies to detect proteins in tissues and cells by immunostaining and enzyme-linked immunosorbent assay (ELISA).

The method originated from the laboratory of George Stark at Stanford. The name *Western blot* was given to the technique by W. Neal Burnette and is a play on the name Southern blot, a technique for DNA detection developed earlier by Edwin Southern. Detection of RNA is termed northern blotting and the detection of post-translational modification of protein is termed eastern blotting.

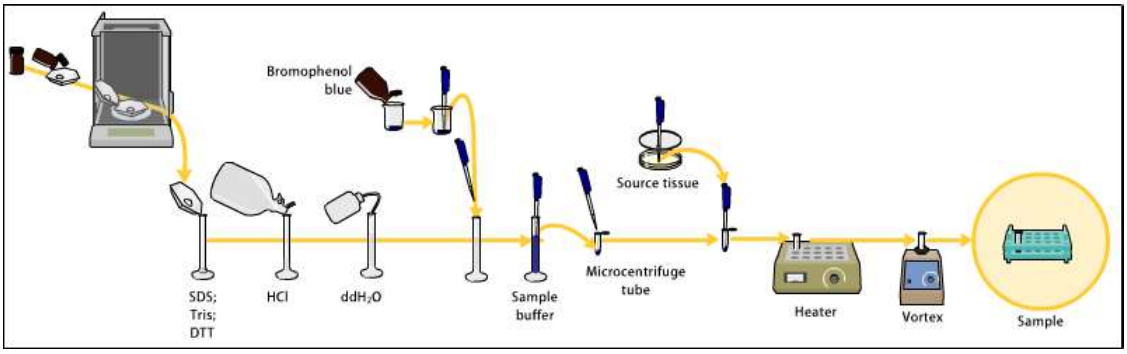
Steps in a Western blot

Tissue preparation

Samples may be taken from whole tissue or from cell culture. In most cases, solid tissues are first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication. Cells may also be broken open by one of the above mechanical methods. However, bacteria, virus or environmental samples can be the source of protein and thus Western blotting is not restricted to cellular studies only.

Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes. Tissue preparation is often done at cold temperatures to avoid protein denaturing and degradation.

A combination of biochemical and mechanical techniques – including various types of filtration and centrifugation – can be used to separate different cell compartments and organelles.

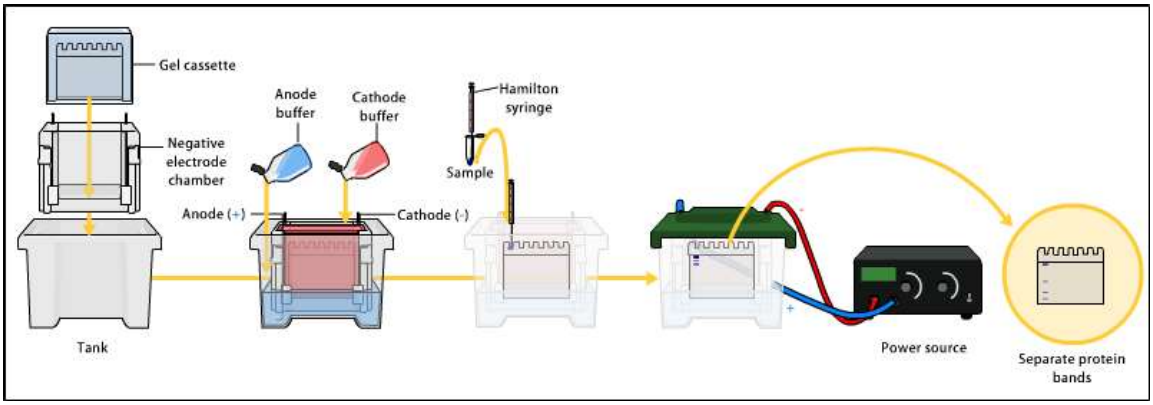


Gel electrophoresis

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge, or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel. This is a very useful way to determine a protein.

By far the most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilodaltons, kDa). The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots.

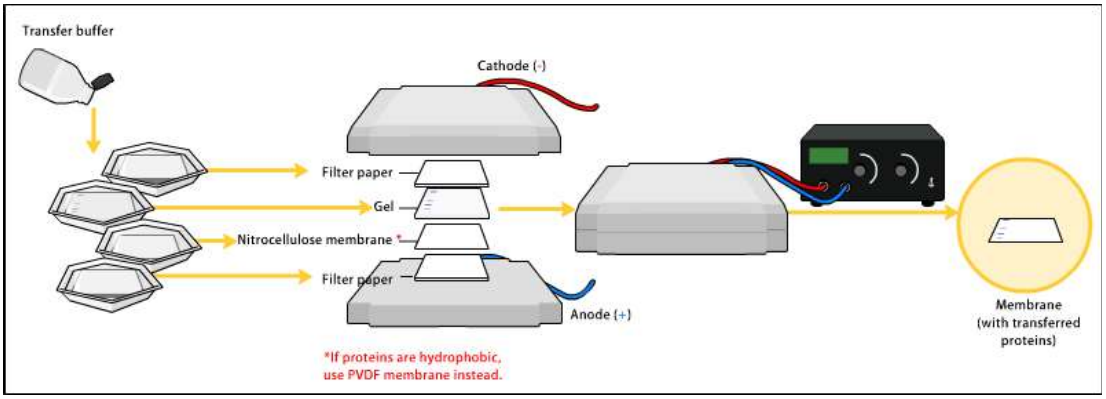
Samples are loaded into *wells* in the gel. One lane is usually reserved for a *marker* or *ladder*, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, coloured bands. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different *electrophoretic mobilities*) separate into *bands* within each *lane*.



It is also possible to use a two-dimensional (2-D) gel which spreads the proteins from a single sample out in two dimensions. Proteins are separated according to isoelectric point (pH at which they have neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

Transfer

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of *nitrocellulose or polyvinylidene difluoride (PVDF*). The membrane is placed on top of the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. Another method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The protein move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. Both varieties of membrane are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated probings.



The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie Brilliant Blue or Ponceau S dyes. Ponceau S is the more common of the two, due to Ponceau S's higher sensitivity and its water solubility makes it easier to subsequently destain and probe the membrane as described below.

Blocking

Since the membrane has been chosen for its ability to bind protein and as both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive) in Tris-Buffered Saline (TBS), with a minute percentage of detergent such as Tween 20 or Triton X-100. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

Detection

During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colourimetric reaction and produces a colour. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

Two steps

- Primary antibody

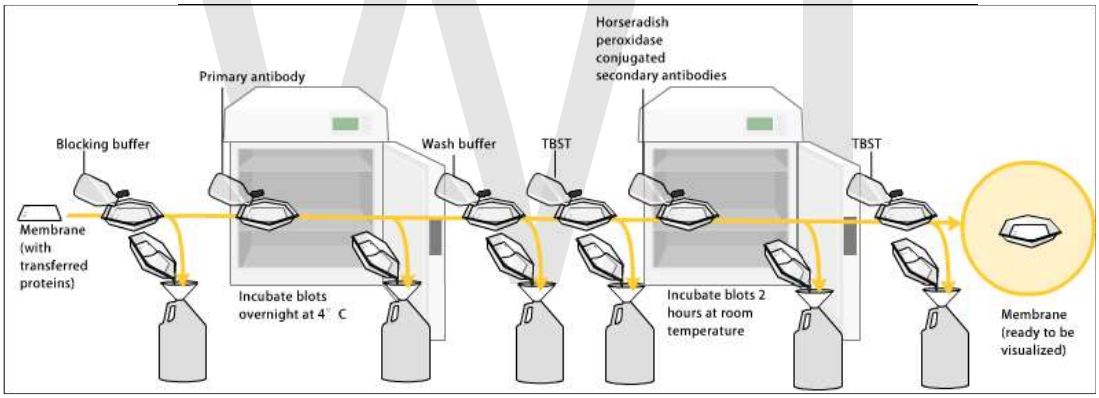
Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest (or a part thereof). Normally, this is part of the immune response, whereas here they are harvested and used as sensitive and specific detection tools that bind the protein directly.

After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/mL) is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise").

- Secondary antibody

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary will bind to almost any mouse-sourced primary antibody, which allows some cost savings by allowing an entire lab to share a single source of mass-produced antibody, and provides far more consistent results. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

Most commonly, a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. A cheaper but less sensitive approach utilizes a 4-chloronaphthol stain with 1% hydrogen peroxide; reaction of peroxide radicals with 4-chloronaphthol produces a dark brown stain that can be photographed without using specialized photographic film.



As with the ELISPOT and ELISA procedures, the enzyme can be provided with a substrate molecule that will be converted by the enzyme to a colored reaction product that will be visible on the membrane.

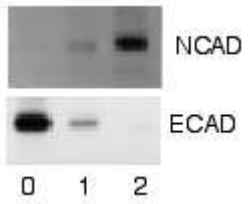
Another method of secondary antibody detection utilizes a near-infrared (NIR) fluorophore-linked antibody. Light produced from the excitation of a fluorescent dye is static, making fluorescent detection a more precise and accurate measure of the difference in signal produced by labeled antibodies bound to proteins on a Western blot. Proteins can be accurately quantified because the signal generated by the different amounts of proteins on the membranes is measured in a static state, as compared to chemiluminescence, in which light is measured in a dynamic state.

A third alternative is to use a radioactive label rather than an enzyme coupled to the secondary antibody, such as labeling an antibody-binding protein like *Staphylococcus* Protein A or Streptavidin with a radioactive isotope of iodine. Since other methods are

safer, quicker, and cheaper, this method is now rarely used; however, an advantage of this approach is the sensitivity of auto-radiography based imaging, which enables highly accurate protein quantification when combined with optical software (e.g. Optiquant).

One step

Historically, the probing process was performed in two steps because of the relative ease of producing primary and secondary antibodies in separate processes. This gives researchers and corporations huge advantages in terms of flexibility, and adds an amplification step to the detection process. Given the advent of high-throughput protein analysis and lower limits of detection, however, there has been interest in developing one-step probing systems that would allow the process to occur faster and with less consumables. This requires a probe antibody which both recognizes the protein of interest and contains a detectable label, probes which are often available for known protein tags. The primary probe is incubated with the membrane in a manner similar to that for the primary antibody in a two-step process, and then is ready for direct detection after a series of wash steps.



Western blot using radioactive detection system

Analysis

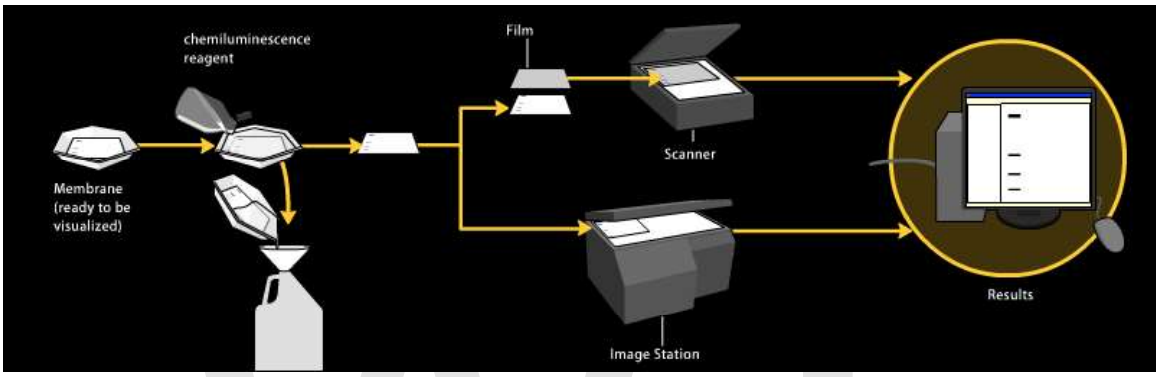
After the unbound probes are washed away, the Western blot is ready for detection of the probes that are labeled and bound to the protein of interest. In practical terms, not all Westerns reveal protein only at one band in a membrane. Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis. The process is repeated for a structural protein, such as actin or tubulin, that should not change between samples. The amount of target protein is indexed to the structural protein to control between groups. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

Colorimetric detection

The colorimetric detection method depends on incubation of the Western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry (how intense the stain is) or spectrophotometry.

Chemiluminescent detection

Chemiluminescent detection methods depend on incubation of the Western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film, and more recently by CCD cameras which capture a digital image of the Western blot. The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used.



Radioactive detection

Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the Western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest. The importance of radioactive detections methods is declining, because it is very expensive, health and safety risks are high, and ECL (enhanced chemiluminescence) provides a useful alternative.

Fluorescent detection

The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as CCD camera equipped with appropriate emission filters which captures a digital image of the Western blot and allows further data analysis such as molecular weight analysis and a quantitative Western blot analysis. Fluorescence is considered to be among the most sensitive detection methods for blotting analysis.

Secondary probing

One major difference between nitrocellulose and PVDF membranes relates to the ability of each to support "stripping" antibodies off and reusing the membrane for subsequent antibody probes. While there are well-established protocols available for stripping nitrocellulose membranes, the sturdier PVDF allows for easier stripping, and for more reuse before background noise limits experiments. Another difference is that, unlike

nitrocellulose, PVDF must be soaked in 95% ethanol, isopropanol or methanol before use. PVDF membranes also tend to be thicker and more resistant to damage during use.

2-D gel electrophoresis

2-dimensional SDS-PAGE uses the principles and techniques outlined above. 2-D SDS-PAGE, as the name suggests, involves the migration of polypeptides in 2 dimensions. For example, in the first dimension polypeptides are separated according to isoelectric point, while in the second dimension polypeptides are separated according to their molecular weight. The isoelectric point of a given protein is determined by the relative number of positively (e.g. lysine and arginine) and negatively (e.g. glutamate and aspartate) charged amino acids, with negatively charged amino acids contributing to a high isoelectric point and positively charged amino acids contributing to a low isoelectric point. Samples could also be separated first under nonreducing conditions using SDS-PAGE and under reducing conditions in the second dimension, which breaks apart disulfide bonds that hold subunits together. SDS-PAGE might also be coupled with urea-PAGE for a 2-dimensional gel.

In principle, this method allows for the separation of all cellular proteins on a single large gel. A major advantage of this method is that it often distinguishes between different isoforms of a particular protein - e.g. a protein that has been phosphorylated (by addition of a negatively charged group). Proteins that have been separated can be cut out of the gel and then analysed by mass spectrometry, which identifies the protein.

Medical diagnostic applications

- The confirmatory HIV test employs a Western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody.
- A Western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').
- Some forms of Lyme disease testing employ Western blotting.
- Western blot can also be used as a confirmatory test for Hepatitis B infection.
- In veterinary medicine, Western blot is sometimes used to confirm FIV+ status in cats.

Chapter- 7

Chemotaxis Assay

Chemotaxis assays are experimental tools for evaluation of chemotactic ability of prokaryotic or eukaryotic cells. A wide variety of techniques are known and applied for such reason. Some of them qualitative and investigator can determine whether the cells prefer or not the tested chemical, others are quantitative and we can get information about the intensity of the responses in a more detailed way.

Quality control

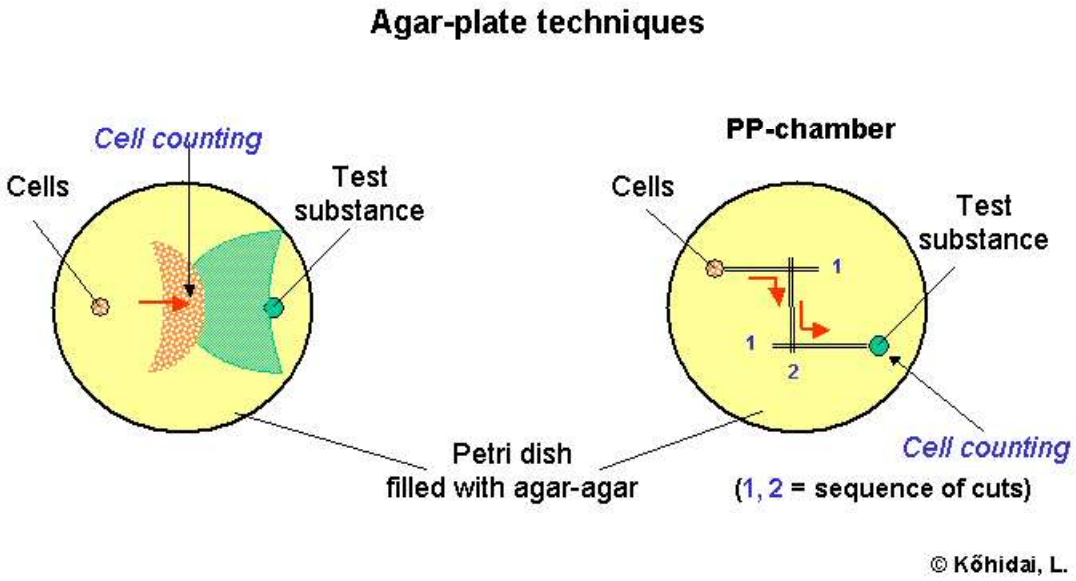
In general, the most important requisite to calibrate the incubation time of the assay both to the model cell and the ligand to be evaluated. Too short incubation time results no cells in the sample, while too long time perturbs the concentration gradients and measures more chemokinetic than chemotactic responses.

The most commonly used techniques are grouped into two main groups:

Agar-plate techniques

This way of evaluation deals with agar-agar or gelatine containing semi-solid layers made prior to the experiment. Small wells are cut into the layer and filled with cells and the test substance. Cells can migrate towards the chemical gradient in the semi solid layer or under the layer as well. Some variations of the technique deal also with wells and parallel channels connected by a cut at the start of the experiment (PP-technique). Radial arrangement of PP-technique (3 or more channels) provides the possibility to compare chemotactic activity of different cell populations or study preference between ligands.

Counting of cells: positive responder cells could be counted from the front of migrating cells, after staining or in native conditions in light microscope.



Two-chamber techniques

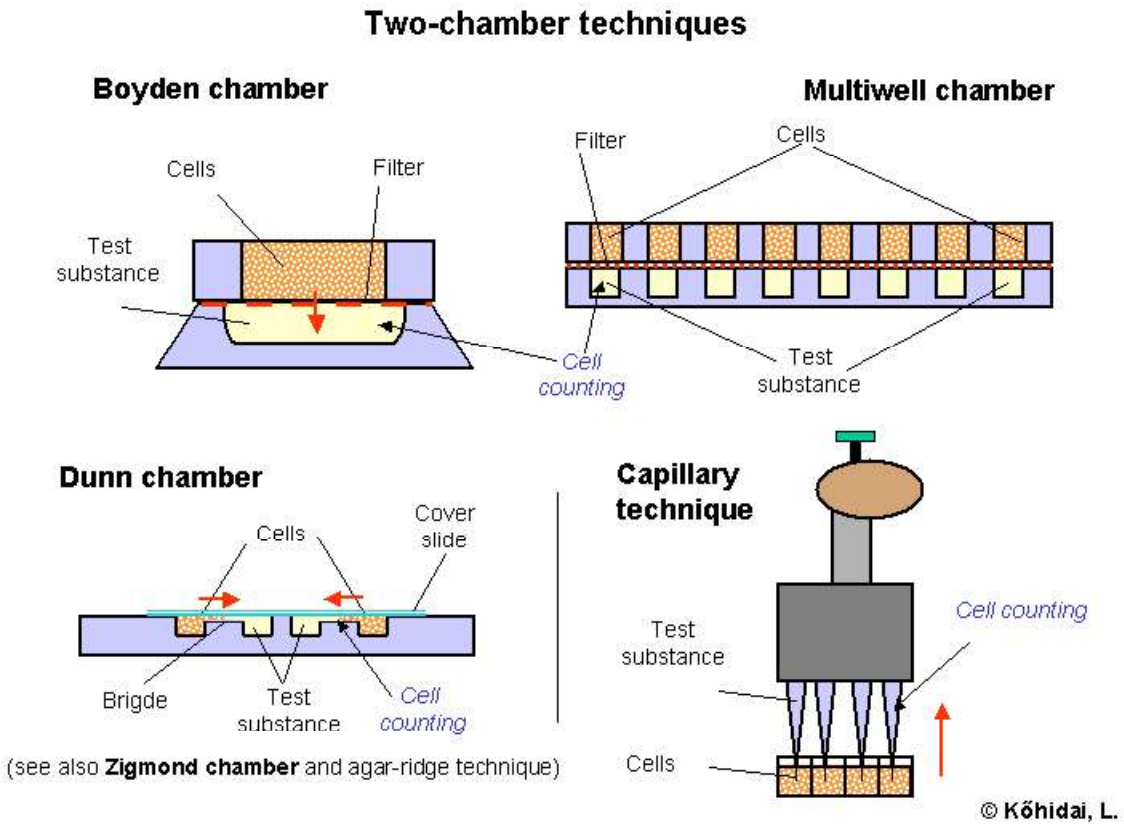
Chambers isolated by filters are proper tools for accurate determination of chemotactic behaviour. The pioneer type of these chambers was constructed by Boyden. The motile cells are placed into the upper chamber, while the test substance containing fluid is filled into the lower one. The size of the motile cells to be investigated determines the pore size of the filter; it is essential to choose a diameter which allows active transmigration. For modelling *in vivo* conditions, several protocols prefer coverage of filter with molecules of extracellular matrix (collagen, elastin etc.) Efficiency of the measurements was increased by development of multiwell chambers (e.g. NeuroProbe), where 24, 96, 384 samples are evaluated in parallel. Advantage of this variant is that several parallels are assayed in identical conditions.

In another setting the chambers are connected side by side horizontally (e.g. Zigmond chamber) or as concentric rings on a slide (e.g. Dunn chamber) Concentration gradient develops on a narrow connecting bridge between the chambers and the number of migrating cells is also counted on the surface of the bridge by light microscope. In some cases the bridge between the two chambers is filled with agar and cells have to "glide" in this semisolid layer.

Some capillary techniques provide also a chamber like arrangement, however, there is no filter between the cells and the test substance. Quantitative results are gained by the

multiwell type of this probe using 4-8-12-channel pipettes. Accuracy of the pipette and increased number of the parallel running samples is the great advantage of this test.

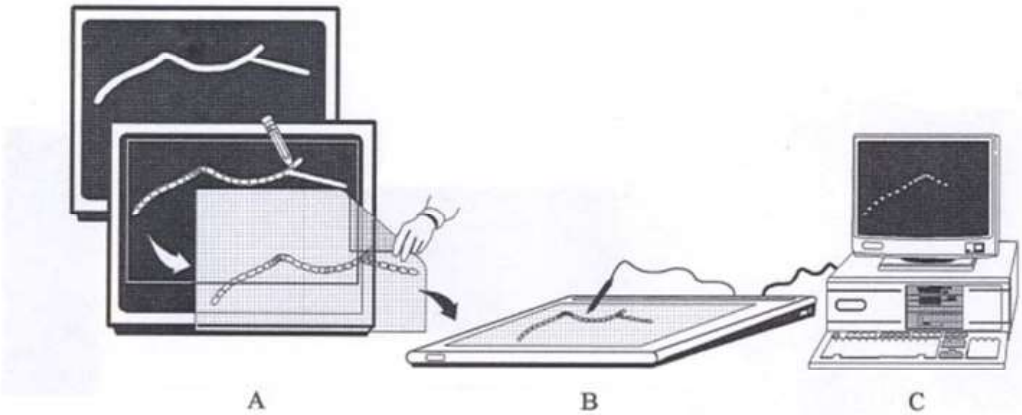
Counting of cells: positive responder cells are count from the lower chamber (long incubation time) or from the filter (short incubation time). For detection of cells general staining techniques (e.g. trypan blue) or special probes (e.g. mt-dehydrogenase detection with MTT assay) are used. Labelled (e.g. fluorochromes) cells are also used, in some assays cells get labelled during transmigration the filter.



Micro-video-recording technology

The ethograms of *Tetrahymena*, *Euplotes*, *Oxytricha* and other ciliates contain rightward and leftward arcs, which are combined with straight segments and several types of directional change to locomotory patterns that lead walking specimens in a random manner over an area. Within the diversity of all behavioural traits the arcs, considered as segments of circles, constitute the basic elements. The reason is that each limitation of behavioural capacity leads to circular walking. Behaviour registration is performed by real-time digitized dark-field tracks of walking cells, with a time resolution of around 80 µm. Registration is complemented by light-field videotracking (at different magnifications) of freely running specimens where movements of the whole cell and (part of) their cirri could be analyzed. All tracks are representative for at least 20, mostly 50 specimens.

Micro-video-recording technology



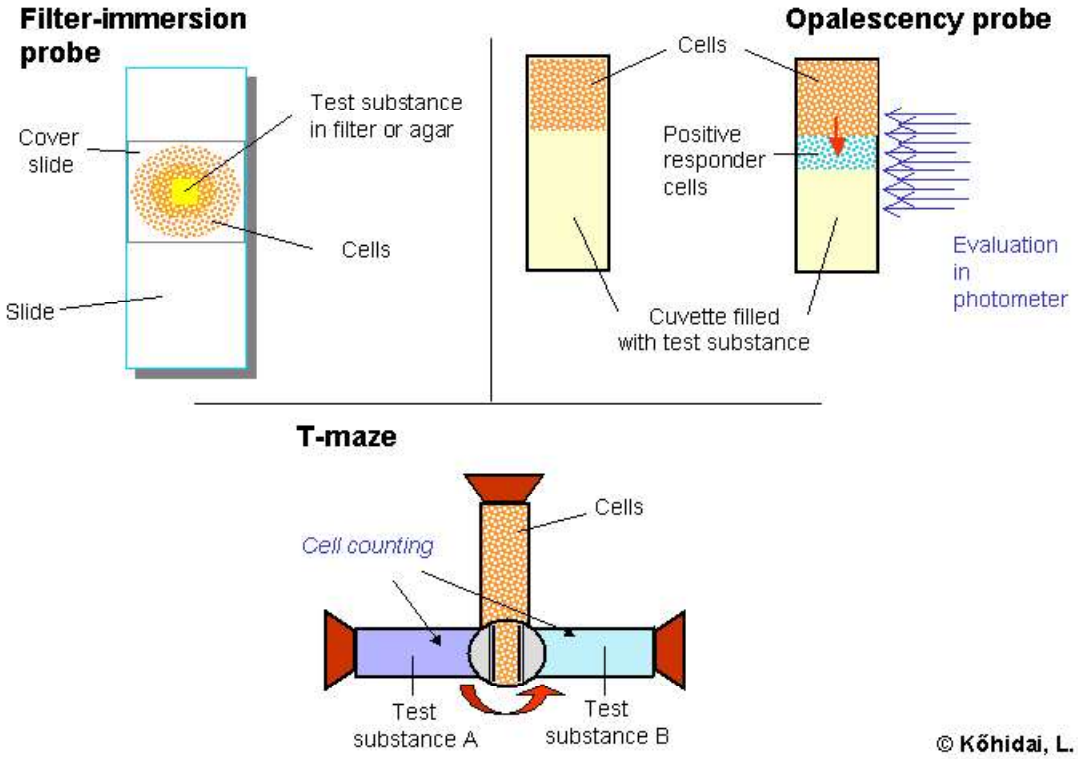
Other techniques

Besides the above mentioned two, most commonly used family of techniques a wide range of protocols were developed to measure chemotactic activity. Some of them are only qualitative, like aggregation tests, where small pieces of agar or filters are placed onto a slide and accumulation of cells around is measured.

In another semiquantitative technique cells are overlayed the test substance and changes in opalescence of the originally cell-free compartment is recorded during the incubation time.

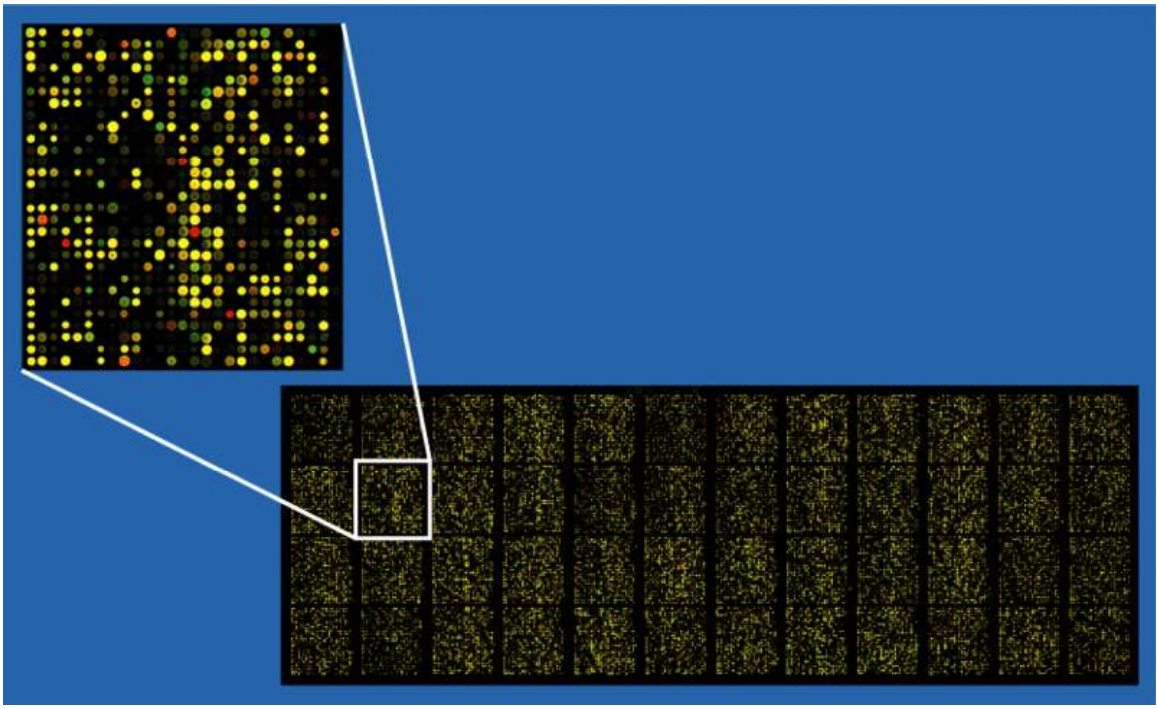
The third very frequently used, however, qualitative technique is the T-maze and its adaptations for microplates. In the original version a container drilled in a peg is filled with cells. Then the peg is twisted and the cells get contact with two other containers filled with different substances. The incubation is stopped with resetting the peg, the cell number is counted from the containers.

Other chemotaxis techniques



Chapter- 8

DNA Microarray



Example of an approximately 40,000 probe spotted oligo microarray with enlarged inset to show detail.

A **DNA microarray** is a multiplex technology used in molecular biology. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles (10^{-12} moles) of a specific DNA sequence, known as *probes* (or *reporters*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many genetic tests in parallel. Therefore arrays have dramatically accelerated many types of investigation.

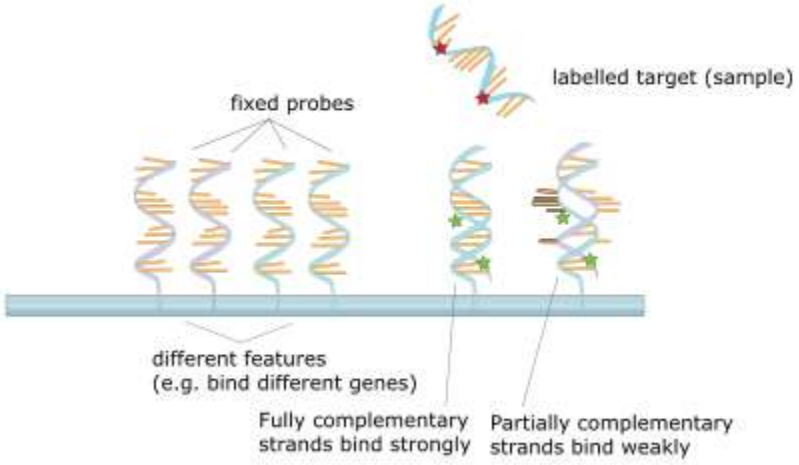
In standard microarrays, the probes are attached via surface engineering to a solid surface by a covalent bond to a chemical matrix (via epoxy-silane, amino-silane, lysine, polyacrylamide or others). The solid surface can be glass or a silicon chip, in which case they are colloquially known as an *Affy chip* when an Affymetrix chip is used. Other microarray platforms, such as Illumina, use microscopic beads, instead of the large solid support. DNA arrays are different from other types of microarray only in that they either measure DNA or use DNA as part of its detection system.

DNA microarrays can be used to measure changes in expression levels, to detect single nucleotide polymorphisms (SNPs), or to genotype or resequence mutant genomes. Microarrays also differ in fabrication, workings, accuracy, efficiency, and cost. Additional factors for microarray experiments are the experimental design and the methods of analyzing the data.

History

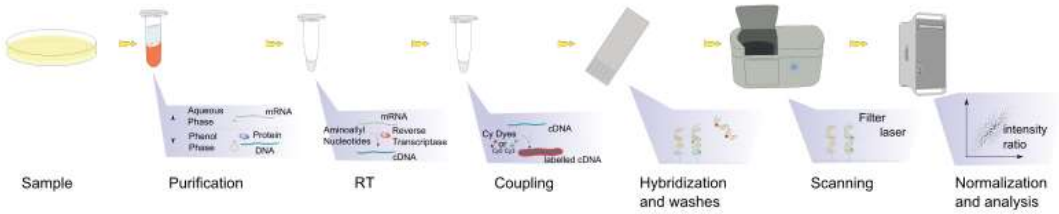
Microarray technology evolved from Southern blotting, where fragmented DNA is attached to a substrate and then probed with a known gene or fragment. Nucleic Acids Res. 1992 Apr 11;20(7):1679-84. Oligonucleotide hybridizations on glass supports: a novel linker for oligonucleotide synthesis and hybridization properties of oligonucleotides synthesised in situ. Maskos U, Southern EM. The first reported use of this approach was the analysis of 378 arrayed lysed bacterial colonies each harboring a different sequence which were assayed in multiple replicas for expression of the genes in multiple normal and tumor tissue (Augenlicht and Kobrin, Cancer Research, 42, 1088–1093, 1982). This was expanded to analysis of more than 4000 human sequences with computer driven scanning and image processing for quantitative analysis of the sequences in human colonic tumors and normal tissue (Augenlicht *et al.*, Cancer Research, 47, 6017-6021, 1987) and then to comparison of colonic tissues at different genetic risk (Augenlicht *et al.*, Proceedings National Academy of Sciences, USA, 88, 3286-3289, 1991). The use of a collection of distinct DNAs in arrays for expression profiling was also described in 1987, and the arrayed DNAs were used to identify genes whose expression is modulated by interferon. These early gene arrays were made by spotting cDNAs onto filter paper with a pin-spotting device. The use of miniaturized microarrays for gene expression profiling was first reported in 1995, and a complete eukaryotic genome (*Saccharomyces cerevisiae*) on a microarray was published in 1997.

Principle



Hybridization of the target to the probe

The core principle behind microarrays is hybridization between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. A high number of complementary base pairs in a nucleotide sequence means tighter non-covalent bonding between the two strands. After washing off of non-specific bonding sequences, only strongly paired strands will remain hybridized. So fluorescently labeled target sequences that bind to a probe sequence generate a signal that depends on the strength of the hybridization determined by the number of paired bases, the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot. Microarrays use relative quantization in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position. An alternative to microarrays is serial analysis of gene expression, where the transcriptome is sequenced allowing an absolute measurement.



The step required in a microarray experiment

Uses and types

Two Affymetrix chips

Many types of array exist and the broadest distinction is whether they are spatially arranged on a surface or on coded beads:

- The traditional solid-phase array is a collection of orderly microscopic "spots", called features, each with a specific probe attached to a solid surface, such as glass, plastic or silicon biochip (commonly known as a *genome chip*, *DNA chip* or *gene array*). Thousands of them can be placed in known locations on a single DNA microarray.
- The alternative bead array is a collection of microscopic polystyrene beads, each with a specific probe and a ratio of two or more dyes, which do not interfere with the fluorescent dyes used on the target sequence.

DNA microarrays can be used to detect DNA (as in comparative genomic hybridization), or detect RNA (most commonly as cDNA after reverse transcription) that may or may not

be translated into proteins. The process of measuring gene expression via cDNA is called expression analysis or expression profiling.
Applications include:

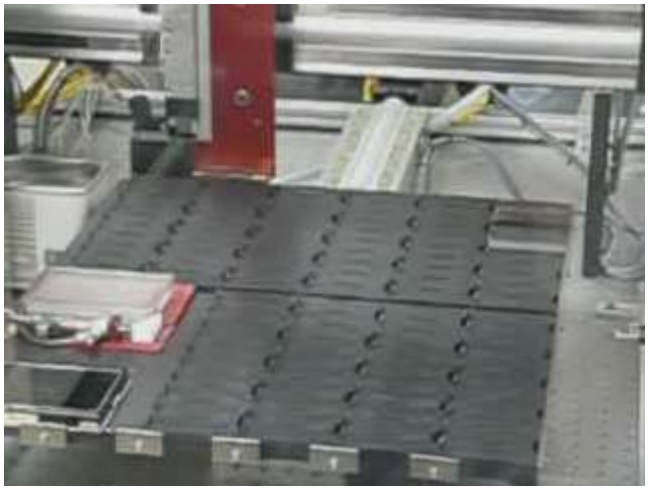
Application or technology	Synopsis
Gene expression profiling	In an mRNA or gene expression profiling experiment the expression levels of thousands of genes are simultaneously monitored to study the effects of certain treatments, diseases, and developmental stages on gene expression. For example, microarray-based gene expression profiling can be used to identify genes whose expression is changed in response to pathogens or other organisms by comparing gene expression in infected to that in uninfected cells or tissues.
Comparative genomic hybridization	Assessing genome content in different cells or closely related organisms.
GeneID	Small microarrays to check IDs of organisms in food and feed (like GMO), mycoplasms in cell culture, or pathogens for disease detection, mostly combining PCR and microarray technology.
Chromatin immunoprecipitation on Chip	DNA sequences bound to a particular protein can be isolated by immunoprecipitating that protein (ChIP), these fragments can be then hybridized to a microarray (such as a tiling array) allowing the determination of protein binding site occupancy throughout the genome. Example protein to immunoprecipitate are histone modifications (H3K27me3, H3K4me2, H3K9me3, etc.), Polycomb-group protein (PRC2:Suz12, PRC1:YY1) and trithorax-group protein (Ash1) to study the epigenetic landscape or RNA Polymerase II to study the transcription landscape.
DamID	Analogously to ChIP, genomic regions bound by a protein of interest can be isolated and used to probe a microarray to determine binding site occupancy. Unlike ChIP, DamID does not require antibodies but makes use of adenine methylation near the protein's binding sites to selectively amplify those regions, introduced by expressing minute amounts of protein of interest fused to bacterial DNA adenine methyltransferase.
SNP detection	Identifying single nucleotide polymorphism among alleles within or between populations. Several applications of microarrays make use of SNP detection, including Genotyping, forensic analysis, measuring predisposition to disease, identifying drug-candidates, evaluating germline mutations in individuals or somatic mutations in cancers, assessing loss of heterozygosity, or genetic linkage analysis.
Alternative splicing detection	An '*exon junction array* design uses probes specific to the expected or potential splice sites of predicted exons for a gene. It

	is of intermediate density, or coverage, to a typical gene expression array (with 1-3 probes per gene) and a genomic tiling array (with hundreds or thousands of probes per gene). It is used to assay the expression of alternative splice forms of a gene. Exon arrays have a different design, employing probes designed to detect each individual exon for known or predicted genes, and can be used for detecting different splicing isoforms.
Fusion genes microarray	A Fusion gene microarray can detect fusion transcripts, *e.g.* from cancer specimens. The principle behind this is building on the alternative splicing microarrays. The oligo design strategy enables combined measurements of chimeric transcript junctions with exon-wise measurements of individual fusion partners.
Tiling array	Genome tiling arrays consist of overlapping probes designed to densely represent a genomic region of interest, sometimes as large as an entire human chromosome. The purpose is to empirically detect expression of transcripts or alternatively splice forms which may not have been previously known or predicted.

Fabrication

Microarrays can be manufactured in different ways, depending on the number of probes under examination, costs, customization requirements, and the type of scientific question being asked. Arrays may have as few as 10 probes or up to 2.1 million micrometre-scale probes from commercial vendors.

Spotted vs. in situ synthesised arrays



A DNA microarray being printed by a robot at the University of Delaware

Microarrays can be fabricated using a variety of technologies, including printing with fine-pointed pins onto glass slides, photolithography using pre-made masks, photolithography using dynamic micromirror devices, ink-jet printing, or electrochemistry on microelectrode arrays.

In *spotted microarrays*, the probes are oligonucleotides, cDNA or small fragments of PCR products that correspond to mRNAs. The probes are synthesized prior to deposition on the array surface and are then "spotted" onto glass. A common approach utilizes an array of fine pins or needles controlled by a robotic arm that is dipped into wells containing DNA probes and then depositing each probe at designated locations on the array surface. The resulting "grid" of probes represents the nucleic acid profiles of the prepared probes and is ready to receive complementary cDNA or cRNA "targets" derived from experimental or clinical samples. This technique is used by research scientists around the world to produce "in-house" printed microarrays from their own labs. These arrays may be easily customized for each experiment, because researchers can choose the probes and printing locations on the arrays, synthesize the probes in their own lab (or collaborating facility), and spot the arrays. They can then generate their own labeled samples for hybridization, hybridize the samples to the array, and finally scan the arrays with their own equipment. This provides a relatively low-cost microarray that may be customized for each study, and avoids the costs of purchasing often more expensive commercial arrays that may represent vast numbers of genes that are not of interest to the investigator. Publications exist which indicate in-house spotted microarrays may not provide the same level of sensitivity compared to commercial oligonucleotide arrays, possibly owing to the small batch sizes and reduced printing efficiencies when compared to industrial manufactures of oligo arrays.

In *oligonucleotide microarrays*, the probes are short sequences designed to match parts of the sequence of known or predicted open reading frames. Although oligonucleotide probes are often used in "spotted" microarrays, the term "oligonucleotide array" most often refers to a specific technique of manufacturing. Oligonucleotide arrays are produced by printing short oligonucleotide sequences designed to represent a single gene or family of gene splice-variants by synthesizing this sequence directly onto the array surface instead of depositing intact sequences. Sequences may be longer (60-mer probes such as the Agilent design) or shorter (25-mer probes produced by Affymetrix) depending on the desired purpose; longer probes are more specific to individual target genes, shorter probes may be spotted in higher density across the array and are cheaper to manufacture. One technique used to produce oligonucleotide arrays include photolithographic synthesis (Agilent and Affymetrix) on a silica substrate where light and light-sensitive masking agents are used to "build" a sequence one nucleotide at a time across the entire array. Each applicable probe is selectively "unmasked" prior to bathing the array in a solution of a single nucleotide, then a masking reaction takes place and the next set of probes are unmasked in preparation for a different nucleotide exposure. After many repetitions, the sequences of every probe become fully constructed. More recently, Maskless Array Synthesis from NimbleGen Systems has combined flexibility with large numbers of probes.

Two-channel vs. one-channel detection

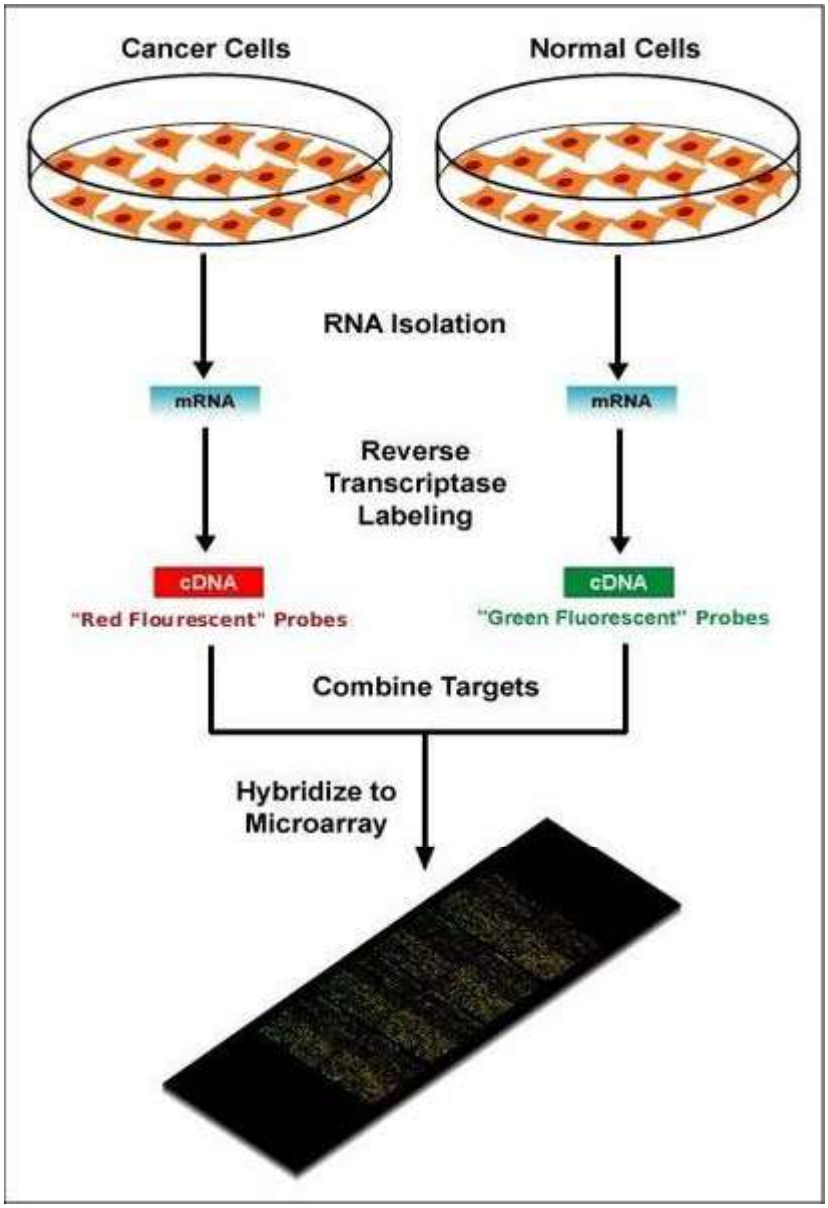


Diagram of typical dual-colour microarray experiment.

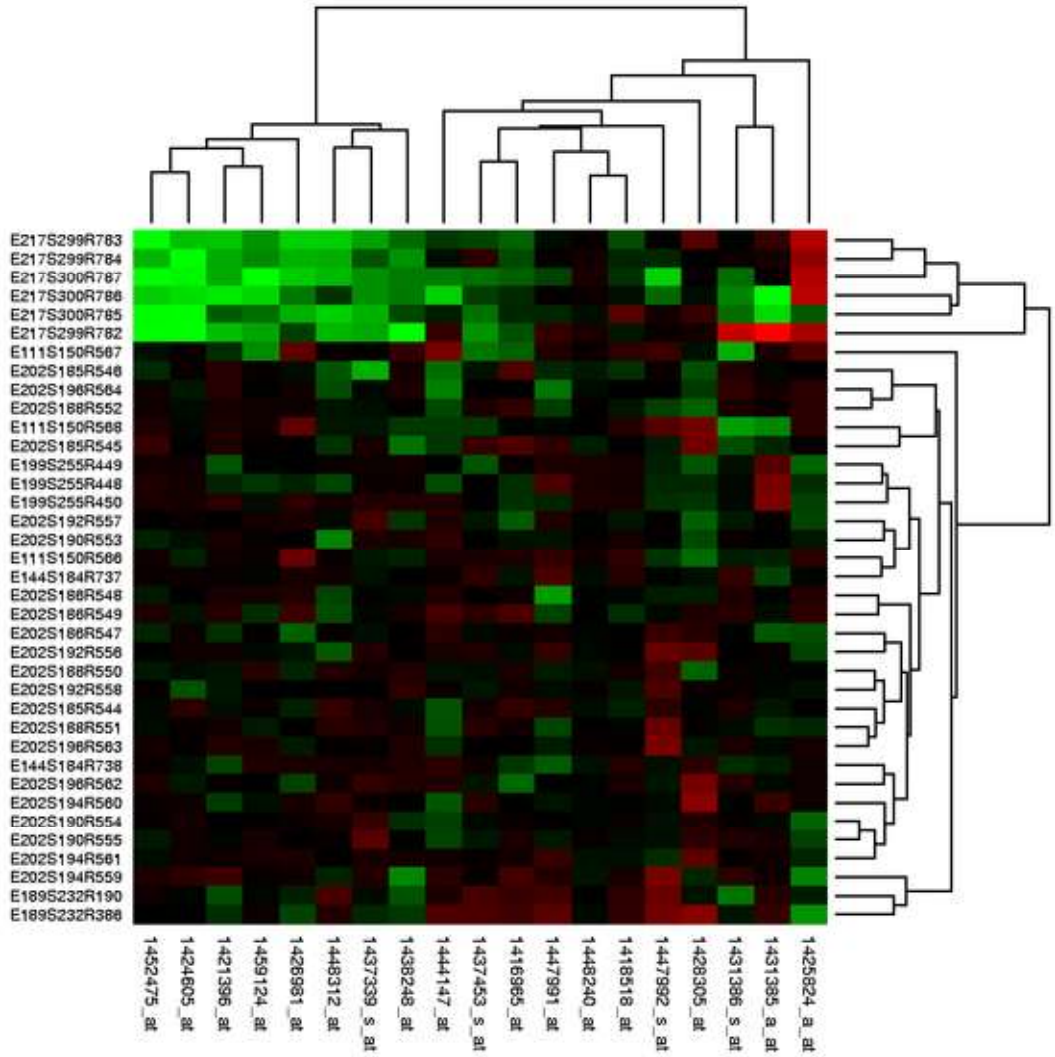
Two-color microarrays or *two-channel microarrays* are typically hybridized with cDNA prepared from two samples to be compared (e.g. diseased tissue versus healthy tissue) and that are labeled with two different fluorophores. Fluorescent dyes commonly used for cDNA labeling include Cy3, which has a fluorescence emission wavelength of 570 nm (corresponding to the green part of the light spectrum), and Cy5 with a fluorescence emission wavelength of 670 nm (corresponding to the red part of the light spectrum). The two Cy-labeled cDNA samples are mixed and hybridized to a single microarray that is then scanned in a microarray scanner to visualize fluorescence of the two fluorophores after excitation with a laser beam of a defined wavelength. Relative intensities of each

fluorophore may then be used in ratio-based analysis to identify up-regulated and down-regulated genes.

Oligonucleotide microarrays often carry control probes designed to hybridize with RNA spike-ins. The degree of hybridization between the spike-ins and the control probes is used to normalize the hybridization measurements for the target probes. Although absolute levels of gene expression may be determined in the two-color array in rare instances, the relative differences in expression among different spots within a sample and between samples is the preferred method of data analysis for the two-color system. Examples of providers for such microarrays includes Agilent with their Dual-Mode platform, Eppendorf with their DualChip platform for colorimetric Silverquant labeling, and TeleChem International with Arrayit.

In *single-channel microarrays* or *one-color microarrays*, the arrays provide intensity data for each probe or probe set indicating a relative level of hybridization with the labeled target. However, they do not truly indicate abundance levels of a gene but rather relative abundance when compared to other samples or conditions when processed in the same experiment. Each RNA molecule encounters protocol and batch-specific bias during amplification, labeling, and hybridization phases of the experiment making comparisons between genes for the same microarray uninformative. The comparison of two conditions for the same gene requires two separate single-dye hybridizations. Several popular single-channel systems are the Affymetrix "Gene Chip", Illumina "Bead Chip", Agilent single-channel arrays, the Applied Microarrays "CodeLink" arrays, and the Eppendorf "DualChip & Silverquant". One strength of the single-dye system lies in the fact that an aberrant sample cannot affect the raw data derived from other samples, because each array chip is exposed to only one sample (as opposed to a two-color system in which a single low-quality sample may drastically impinge on overall data precision even if the other sample was of high quality). Another benefit is that data are more easily compared to arrays from different experiments so long as batch effects have been accounted for. A drawback to the one-color system is that, when compared to the two-color system, twice as many microarrays are needed to compare samples within an experiment.

Microarrays and bioinformatics



Gene expression values from microarray experiments can be represented as heat maps to visualize the result of data analysis.

The advent of inexpensive microarray experiments created several specific bioinformatics challenges:

- the multiple levels of replication in experimental design (Experimental design)
- the number of platforms and independent groups and data format (Standardization)
- the treatment of the data (Statistical analysis)
- accuracy and precision (Relation between probe and gene)
- the sheer volume of data and the ability to share it (Data warehousing)

Experimental design

Due to the biological complexity of gene expression, the considerations of experimental design that are discussed in the expression profiling article are of critical importance if statistically and biologically valid conclusions are to be drawn from the data.

There are three main elements to consider when designing a microarray experiment. First, replication of the biological samples is essential for drawing conclusions from the experiment. Second, technical replicates (two RNA samples obtained from each experimental unit) help to ensure precision and allow for testing differences within treatment groups. The technical replicates may be two independent RNA extractions or two aliquots of the same extraction. Third, spots of each cDNA clone or oligonucleotide are present as replicates (at least duplicates) on the microarray slide, to provide a measure of technical precision in each hybridization. It is critical that information about the sample preparation and handling is discussed, in order to help identify the independent units in the experiment and to avoid inflated estimates of statistical significance.

Standardization

Microarray data is difficult to exchange due to the lack of standardization in platform fabrication, assay protocols, and analysis methods. This presents an interoperability problem in bioinformatics. Various grass-roots open-source projects are trying to ease the exchange and analysis of data produced with non-proprietary chips:

- For example, the "Minimum Information About a Microarray Experiment" (MIAME) checklist helps define the level of detail that should exist and is being adopted by many journals as a requirement for the submission of papers incorporating microarray results. But MIAME does not describe the format for the information, so while many formats can support the MIAME requirements, as of 2007 no format permits verification of complete semantic compliance.
- The "MicroArray Quality Control (MAQC) Project" is being conducted by the US Food and Drug Administration (FDA) to develop standards and quality control metrics which will eventually allow the use of MicroArray data in drug discovery, clinical practice and regulatory decision-making.
- The MGED Society has developed standards for the representation of gene expression experiment results and relevant annotations.

Statistical analysis

Microarray data sets are commonly very large, and analytical precision is influenced by a number of variables. Statistical challenges include taking into account effects of background noise and appropriate normalization of the data. Normalization methods may be suited to specific platforms and, in the case of commercial platforms, the analysis may be proprietary. Algorithms that affect statistical analysis include:

- Image analysis: gridding, spot recognition of the scanned image (segmentation algorithm), removal or marking of poor-quality and low-intensity features (called *flagging*).
- Data processing: background subtraction (based on global or local background), determination of spot intensities and intensity ratios, visualisation of data, and log-transformation of ratios, global or local normalization of intensity ratios.
- Identification of statistically significant changes: t-test, ANOVA, Bayesian method Mann–Whitney test methods tailored to microarray data sets, which take into account multiple comparisons or cluster analysis. These methods assess statistical power based on the variation present in the data and the number of experimental replicates, and can help minimize Type I and type II errors in the analyses.
- Network-based methods: Statistical methods that take the underlying structure of gene networks into account, representing either associative or causative interactions or dependencies among gene products.

Microarray data may require further processing aimed at reducing the dimensionality of the data to aid comprehension and more focused analysis. Other methods permit analysis of data consisting of a low number of biological or technical replicates; for example, the Local Pooled Error (LPE) test pools standard deviations of genes with similar expression levels in an effort to compensate for insufficient replication.

Relation between probe and gene

The relation between a probe and the mRNA that it is expected to detect is not trivial. Some mRNAs may cross-hybridize probes in the array that are supposed to detect another mRNA. In addition, mRNAs may experience amplification bias that is sequence or molecule-specific. Thirdly, probes that are designed to detect the mRNA of a particular gene may be relying on genomic EST information that is incorrectly associated with that gene.

Data warehousing

Microarray data was found to be more useful when compared to other similar datasets. The sheer volume (in bytes), specialized formats (such as MIAME), and curation efforts associated with the datasets require specialized databases to store the data.