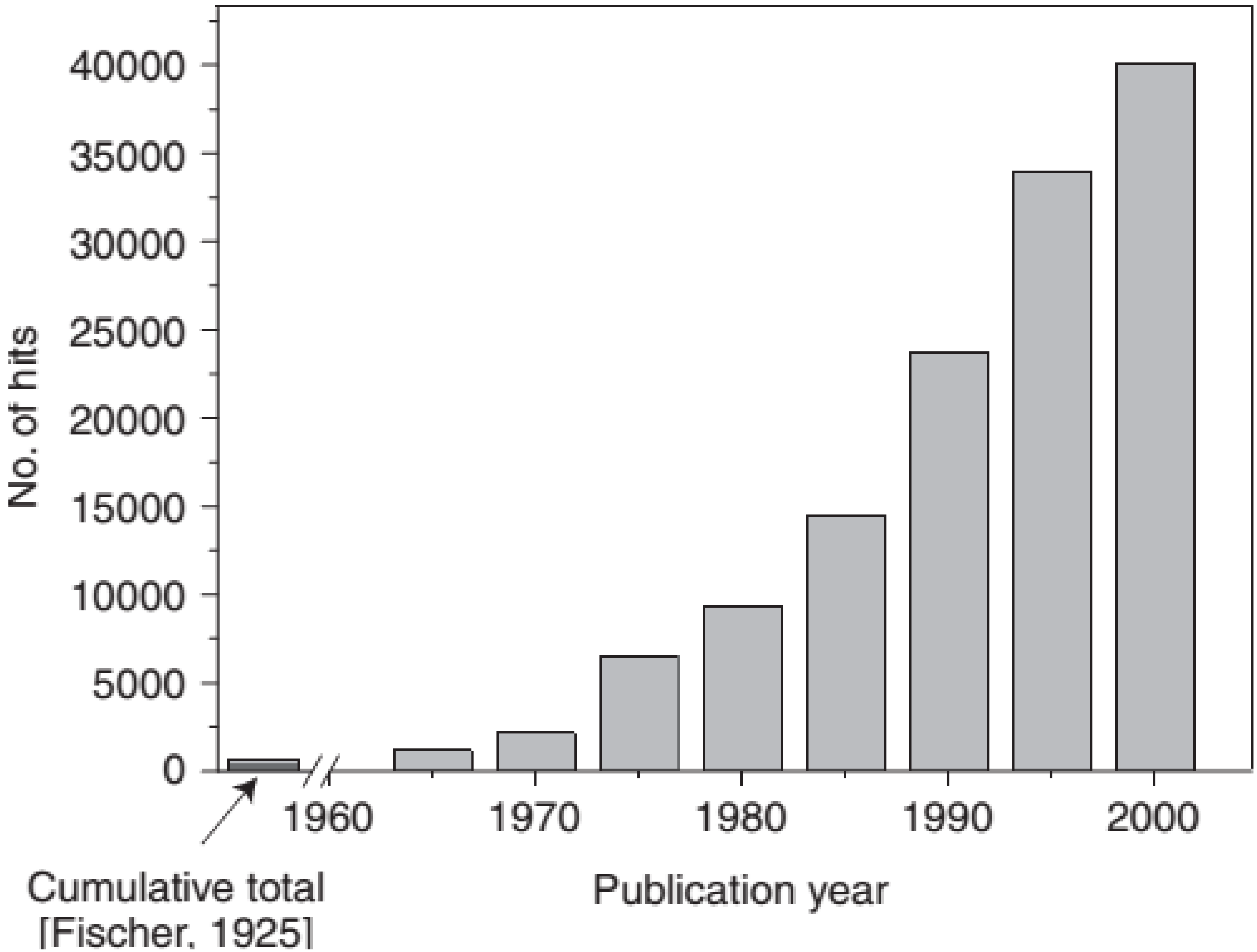
Eukaryote Cell Culture Workshop

Dr E. Eftekhar (PhD)
Associate professor of Clinical Biochemistry



Growth of Tissue Culture. Number of hits in PubMed for "cell culture" from 1965. (2015 =350000 article)

Applications of Cell Culture

1- Model System:

Cell culture are used as model system to study basic cell biology and biochemistry, to study the interaction between cell and disease causing agents like bacteria, virus, to study the effect of drugs.

2- Drug Screening and Development:

- ACC are used to study the cytotoxicity of new drug. This is also used to find out the effective and safe dosage of new drugs.

3- Cancer Research

- The basic difference between normal cell and cancer cell can be studied using animal cell culture technique, as both cells can be cultured in laboratory.

- Normal cells can be converted into cancer cells by using radiation, chemicals and viruses. Thus, the mechanism and cause of cancer can be studied.

- Cell culture can be used to determine the effective drugs for selectively destroy only cancer cells

4-Vaccine Production

- ACC are used in the production of viruses and these viruses are used to produce vaccines.

- For example vaccines for deadly diseases like polio, rabies, chicken pox, measles and hepatitis B are produced using animal cell culture.

5- Virology:

- ACC are used to replicate the viruses instead of animals for the production of vaccine.
- Cell culture can also be used to detect and isolate viruses.

6- Genetic Counseling:

- Fetal cell culture extracted from pregnant women can be used to examine the abnormalities of chromosomes, genes using karyotyping, and these findings can be used in early detection of fetal disorders.

7-Genetic Engineering:

- ACC can be used to introduce new genetic material like DNA or RNA into the cell.

- These can be used to study the expression of new genes and its effect on the health of the cell.

- Animal cell cultures (ACC) are used to produce commercially important genetically engineered proteins such as monoclonal antibodies, insulin, hormones, and much more.

TABLE 1.2. Advantages of Tissue Culture

Category	Advantages
Physico-chemical environment	Control of pH, temperature, osmolality, dissolved gases
Physiological conditions	Control of hormone and nutrient concentrations
Microenvironment	Regulation of matrix, cell–cell interaction, gaseous diffusion
Cell line homogeneity	Availability of selective media, cloning
Characterization	Cytology and immunostaining are easily performed
Preservation	Can be stored in liquid nitrogen
Validation & accreditation	Origin, history, purity can be authenticated and recorded
Replicates and variability	Quantitation is easy
Reagent saving	Reduced volumes, direct access to cells, lower cost
Control of C × T	Ability to define dose, concentration (C), and time (T)
Mechanization	Available with microtitration and robotics
Reduction of animal use	Cytotoxicity and screening of pharmaceutics, cosmetics, etc.

TABLE 1.3. Limitations of Tissue Culture

Category	Examples
Necessary expertise	Sterile handling Chemical contamination Microbial contamination Cross-contamination
Environmental control	Workplace Incubation, pH control Containment and disposal of biohazards
Quantity and cost	Capital equipment for scale-up Medium, serum Disposable plastics
Genetic instability	Heterogeneity, variability
Phenotypic instability	Dedifferentiation Adaptation Selective overgrowth
Identification of cell type	Markers not always expressed Histology difficult to recreate and atypical Geometry and microenvironment change cytology

Tissue Culture Terminology

- **Tissue culture:** is used as a generic term to include organ culture and cell culture.

- **Organ culture:** will always imply a three-dimensional culture of undisaggregated tissue retaining some or all of the histological features of the tissue in vivo.

- **Cell culture:** Term used to donate the maintenance or cultivation of cells in vitro, including the culture of single cells. In cell culture , the cells are no longer organized into tissue.

- **Histotypic culture:** implies that cells have been reaggregated or grown to re-create a three-dimensional structure with tissue like cell density.

- **Organotypic culture:** implies the same procedures but recombining cells of different lineages, e.g., epidermal keratinocytes in combined culture with dermal fibroblasts, in an attempt to generate a tissue equivalent.

Main Types of Cell Culture

1- Primary culture: are derived directly from excised, normal animal tissue and cultured either as an explant culture or following dissociation into a single cell suspension by enzyme digestion (primary cell).

- The preparation of primary cultures is labor intensive and they can be maintained *in vitro* only for a limited period of time.

- **Cell line:** After the first subculture, or passage, the primary culture becomes known as a cell line and may be propagated and subcultured several times.

- With each successive subculture, the component of the population with the ability to proliferate most rapidly will gradually predominate, and nonproliferating or slowly proliferating cells will be diluted out.

- This type of cell line has a finite lifespan, during which cells with the highest growth capacity will predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.

2- Continuous Cultures:

Continuous cultures are comprised of a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefinitely.

Cell lines of a finite life are usually diploid and maintain some degree of differentiation.

- **Cell strain:** is derived either from a primary culture or a cell line by the selection or cloning of cells having specific properties or characteristics which must be defined.

- **Passaging:** (also known as subculture or splitting cells) involves transferring a small number of cells into a new vessel.

- **Passage number**: refers to the number of times that a cell population has been removed from the culture vessel and undergone a subculture (passage) process, in order to keep the cells at a sufficiently low density to stimulate further growth.

- **Population doubling**: is a two-fold increase in the total number of cells in a culture, and is most commonly referred to during the exponential, or “log”, phase of growth.

- **Explant:** Tissue taken from its original site and transferred to artificial medium for growth.

- **Feeder layer**: Feeder cell layers usually consist of adherent growth-arrested but viable and bioactive cells (primary cells or continuous cell lines) that have been incapacitated, for example by irradiation.

- These cells are used as a substratum on which other cells are grown in a co-culture system. Sometimes, the term filler cells is used.

- **There are two basic systems for growing cells in culture:**
- **1-As monolayers on an artificial substrate (i.e., adherent culture)**
- **2- free-floating in the culture medium (suspension culture).**
- The majority of the cells derived from vertebrates, with the exception of hematopoietic cell lines, are anchorage-dependent and have to be cultured on a suitable substrate that is specifically treated to allow cell adhesion and spreading (i.e., **tissue-culture treated**).

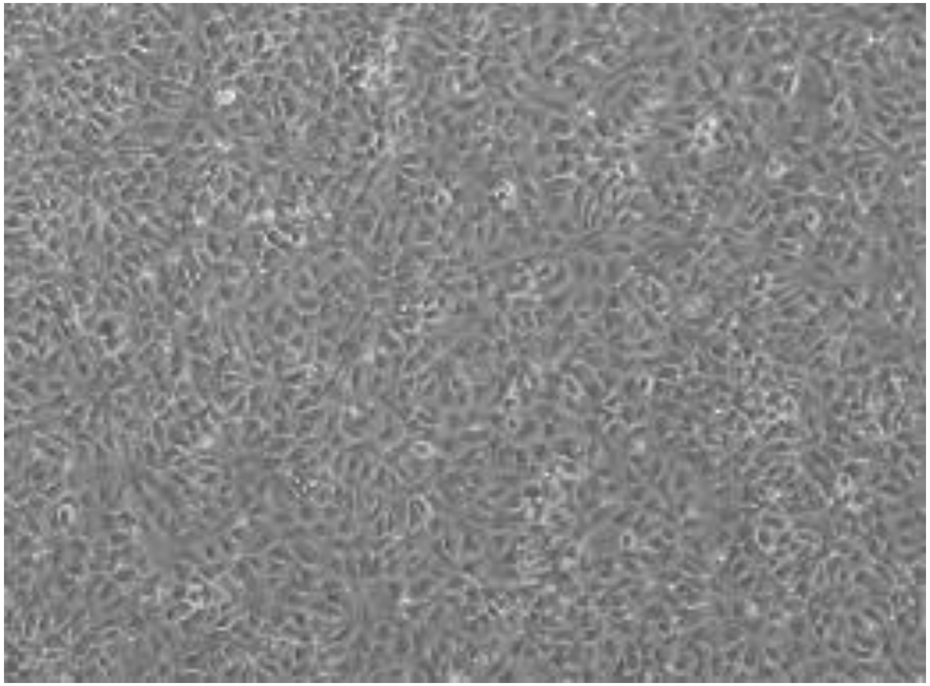
Adherent Cell Culture	Suspension Cell Culture
Appropriate for most cell types, including primary cultures	Appropriate for cells adapted to suspension culture and hematopoieti cell
Requires periodic passaging, but allows easy visual inspection under inverted microscope	Easier to passage, but requires daily cell counts and viability determination to follow growth patterns; culture can be diluted to stimulate growth
Cells are dissociated enzymatically or mechanically	Does not require enzymatic or mechanical dissociation
Growth is limited by surface area, which may limit product yields	Growth is limited by concentration of cells in the medium, which allows easy scale-up
Requires tissue-culture treated vessel	Can be maintained in culture vessels that are not tissue-culture treated

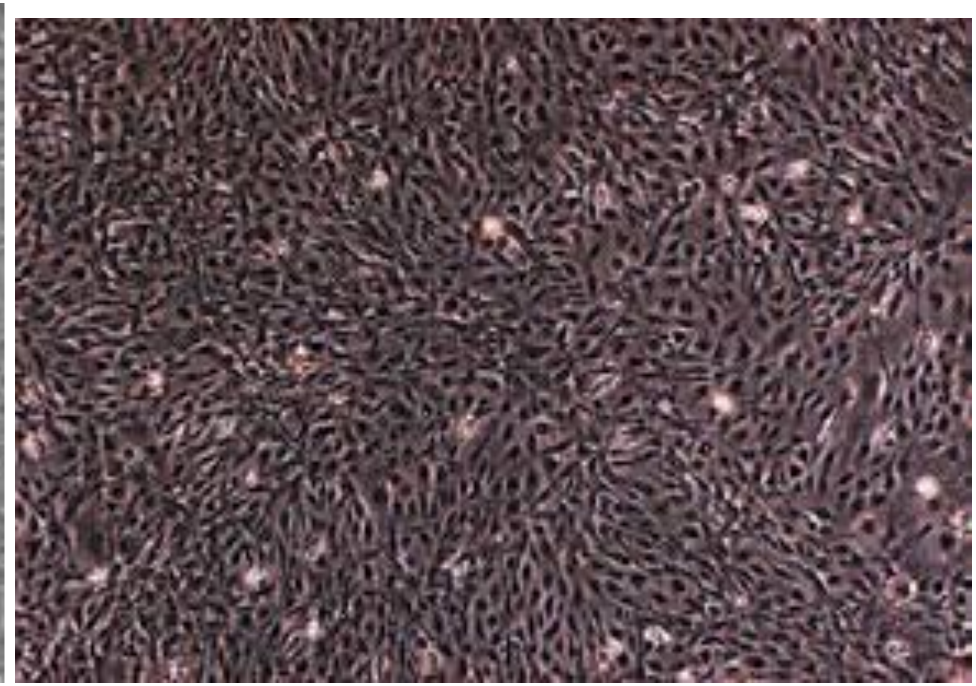
- **Confluency:** an estimate of the number of adherent cells in a culture dish or a flask, referring to the proportion of the surface which is covered by cells.(e.g 50% confluence).

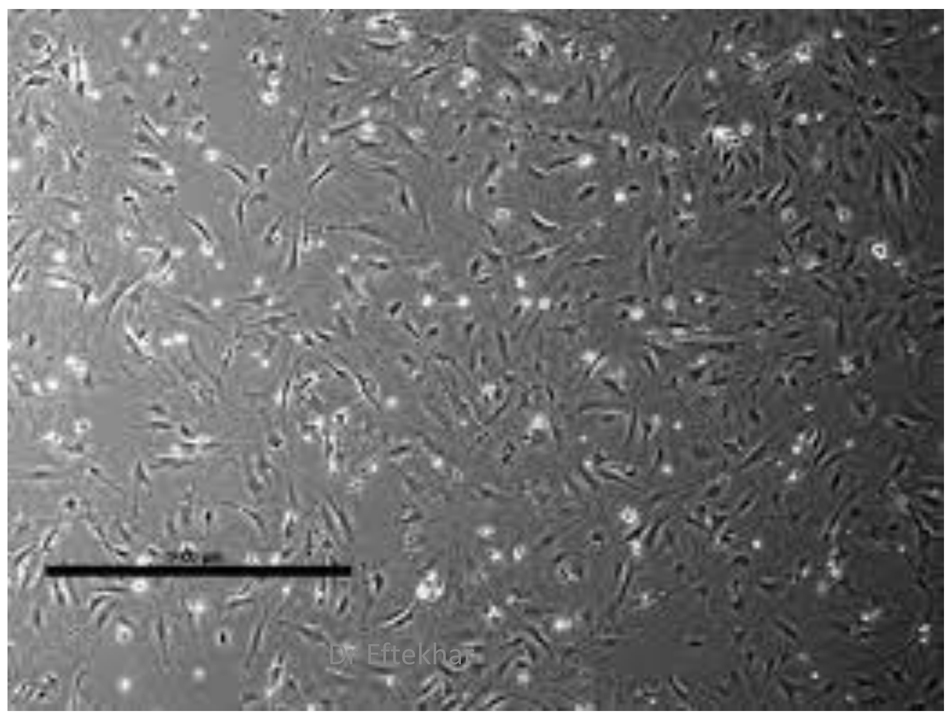
- Many cell lines exhibit differences in growth rate or gene expression depending on the degree of confluence.

- Cells are typically passaged before becoming fully confluent in order to maintain their proliferative phenotype.

- To achieve optimal and consistent results, experiments are usually performed using cells at a particular confluence, depending on the cell type.



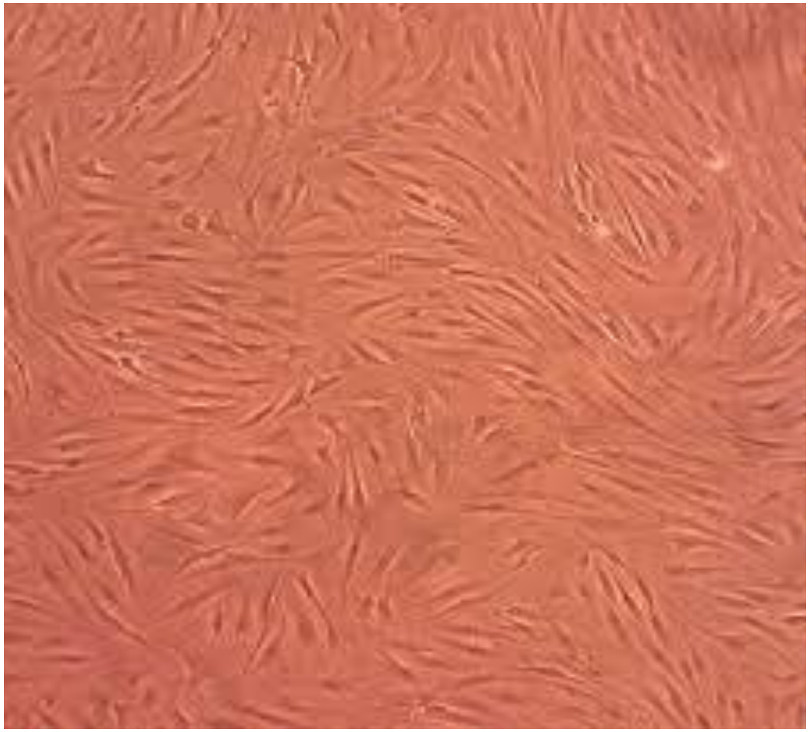




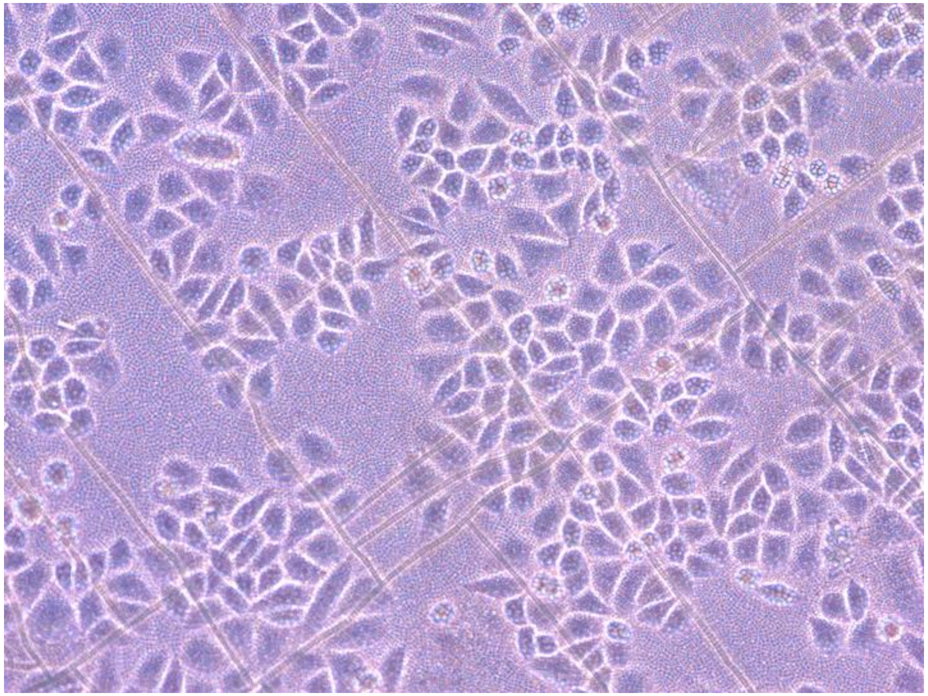
Morphology of Cells in Culture

- Cells in culture can be divided in to three basic categories based on their shape and appearance (i.e., morphology).

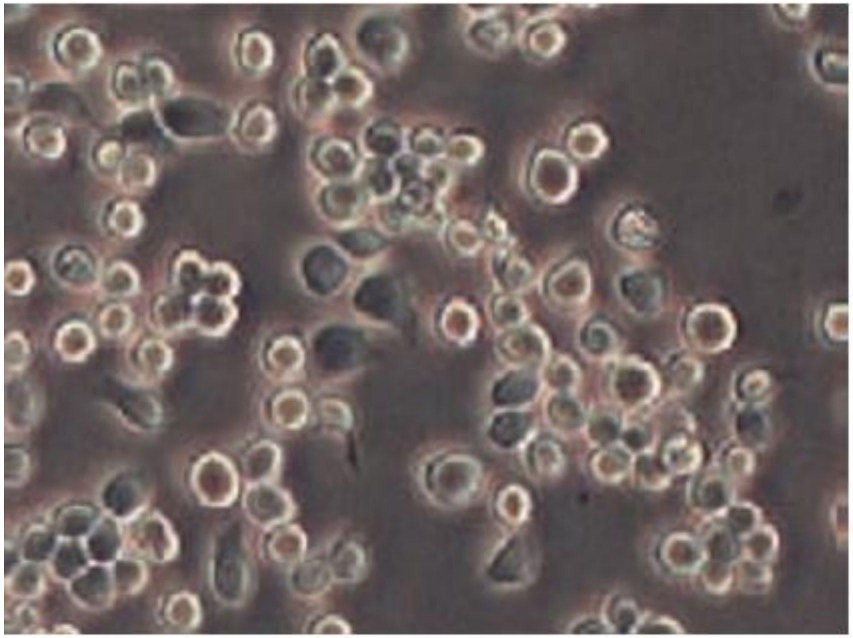
- 1-Fibroblastic (or fibroblast-like) cells are bipolar or multipolar, have elongated shapes, and grow attached to a substrate.

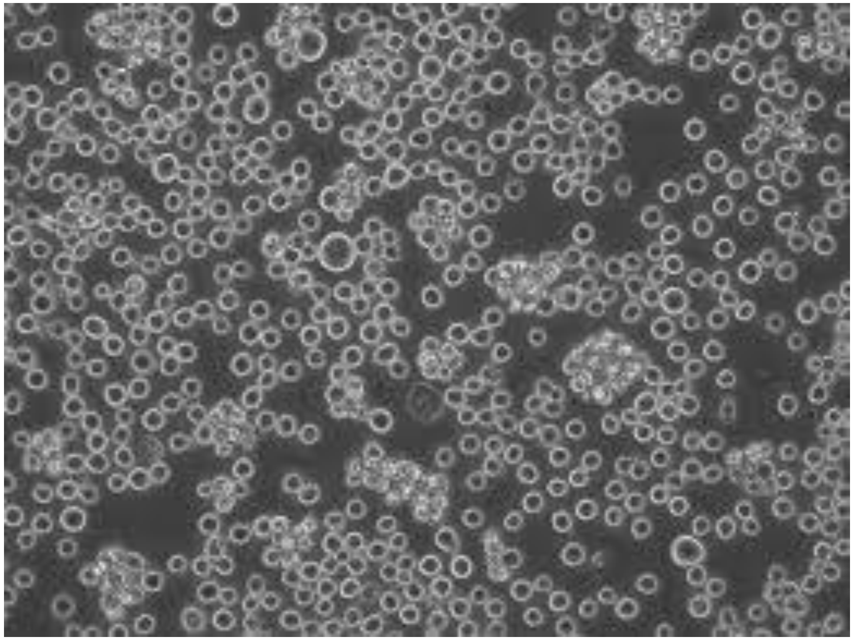


2- Epithelial-like cells are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches.



3-Lymphoblast-like cells are spherical in shape and usually grown in suspension without attaching to a surface.





Safety

- A cell culture laboratory has a number of specific hazards associated with handling and manipulating human or animal cells and tissues, as well as toxic materials.

- The fundamental objective of any biosafety program is to reduce or eliminate exposure of laboratory workers and the outside environment to potentially harmful biological agents.

- **Biosafety Levels**:

- Biosafety Level 1 (BSL-1): is the basic level of protection common to most research and clinical laboratories, and is appropriate for agents that are not known to cause disease in humans.

- Biosafety Level 2 (BSL-2): is appropriate for moderate-risk agents known to cause human disease of varying severity by exposure. Most cell culture labs should be at least BSL-2.

- BSL3:

- BSL4:

- BSL2 requirement:

1-laboratory personnel have specific training in handling pathogenic agents and are supervised by scientists competent in handling infectious agents and associated procedures.

2) access to the laboratory is restricted when work is being conducted

3) all procedures in which infectious aerosols or splashes may be created are conducted in BSCs or other physical containment equipment.

Disinfection

- Important means for minimizing the risk of harm.
- The major disinfectants:

1-hypochlorites : good general purpose disinfectant, Active against viruses.

2-Alcohol (e.g. ethanol, isopropanol)

- Effective concentrations 70% for ethanol, 60-70% for isopropanol
- Effective against bacteria. Ethanol is effective against most viruses, isopropanol is not effective against viruses

Waste Disposal

Different forms of waste require different treatment.

1-Tissue culture waste (culture medium): Inactivate overnight in a solution of hypochlorite (10,000ppm) prior to disposal to drain with an excess of water.

2- Contaminated pipettes should be placed in hypochlorite solution (2500ppm) overnight before disposal by autoclaving and incineration.

3- Solid waste such as flasks, centrifuge tubes, contaminated gloves, tissues etc. should be placed inside heavy duty sacks for contaminated waste and autoclaved prior to incineration.

Material Safety Data Sheet (MSDS) or SDS

- MSDS, is a form containing information regarding the properties of a particular substance.

- The SDS includes physical data such as melting point, boiling point, and flash point, information on the substance's toxicity, reactivity, health effects, storage, and disposal, as well as recommended protective equipment and procedures for handling spills.

Safety Equipment

- The biosafety cabinet(i.e., cell culture hood) is the most important equipment to provide containment of infectious splashes or aerosols generated by many microbiological procedures as well as to prevent contamination of your own cell culture.

- personal protective equipment (PPE): gloves, laboratory coats and gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles.

Safe Laboratory Practices

1-Always wear appropriate personal protective equipment. Change gloves when contaminated.

2- Wash your hands after working with potentially hazardous materials and before leaving the laboratory.

3-Decontaminate all work surfaces before and after your experiments, and immediately after any spill or splash of potentially infectious material with an appropriate disinfectant.

4-Decontaminate all potentially infectious materials before disposal (sodium hypochlorite,or Savlon or bleach 10% for 24 h).

TABLE 11.1. Methods of Sterilization

Method	Conditions	Materials	Limitations
Dry heat	160°C, 1 h	Heat stable: metals, glass, PTFE.	Some charring may occur, e.g., of indicating tape and cotton plugs.
Moist heat	121°C, 15–20 min	Heat-stable liquids: water, salt solutions, autoclavable media. Moderately heat-stable plastics: silicones, polycarbonate, nylon, polypropylene.	Steam penetration requires steam-permeable packaging. Large fluid loads need time to heat up.
Irradiation:			
γ-Irradiation	25 kGy	Plastics, organic scaffolds, heat-sensitive reagents and pharmaceuticals.	Chemical alteration of plastics can occur. Macromolecular degradation.
Electron beam	25 kGy	Plastics, organic scaffolds, heat-sensitive reagents and pharmaceuticals.	Needs high-energy source. Not suitable for average laboratory installation.
Microwave	5 min full power	Aqueous solutions and gels such as agar.	Only useful for small volumes; usually just for melting agar
Short-wave UV	254 nM, 50–100 W, 30 min	Flat surfaces, circulating air.	Will not reach shadow areas. Spores resistant.
Chemical:			
Ethylene oxide	1 h	Heat-labile plastics.	Items must be ventilated for 24–48 h; leaves toxic residue.
Hypochlorite	300–2500 ppm 30 min	Contaminated solutions. Plastics.	Needs extensive washing. May leave residue.
70% Alcohol	Soak for 1 h	Dissecting instruments (combined with flaming). Some plastics.	Does not kill spores. Fire risk with flaming. Precast Perspex or Lucite may shatter if immersed in alcohol.
Filtration	0.1- to 0.2-µm porosity	All aqueous solutions; particularly suitable for heat-labile reagents and media. Specify low protein binding for growth factors, etc.	Not suitable for some solvents, e.g., DMSO. Slow with viscous solutions.

TABLE 11.2. Sterilization of Equipment and Apparatus

Item	Sterilization
Ampoules for freezer, glass	Dry heat[1]
Ampoules for freezer, plastic	Autoclave[2] (usually bought sterile)
Apparatus containing glass and silicone tubing	Autoclave
Disposable tips for micropipettes	Autoclave in autoclavable trays or nylon bags
Filters, reusable	Autoclave; do not use prevacuum or postvacuum
Glassware	Dry heat
Glass bottles with screw caps	Autoclave with cap slack
Glass coverslips	Dry heat
Glass slides	Dry heat
Glass syringes	Autoclave (separate piston if PTFE)
Instruments	Dry heat
Magnetic stirrer bars	Autoclave
Pasteur pipettes, glass	Dry heat
Pipettes, glass	Dry heat
Plexiglas, Perspex, Lucite	70% EtOH (*see* text)
Polycarbonate	Autoclave
Repeating pipettes or syringes	Autoclave (separate PTFE pistons from glass barrels)
Screw caps	Autoclave
Silicone grease (for isolating clones)	Autoclave in glass Petri dish
Silicone tubing	Autoclave
Stoppers, rubber and silicone	Autoclave
Test tubes	Dry heat

[1] Dry heat, 160°C/L h.

[2] Autoclave, 100 kPa (1 bar, 15 lb/in.^{2}), 121°C for 20 min.

TABLE 11.3. Sterilization of Liquids

Solution	Sterilization	Storage
Agar	Autoclave[1] or boil	Room temperature
Amino acids	Filter[2]	4°C
Antibiotics	Filter	−20°C
Bacto-peptone	Autoclave	Room temperature
Bovine serum albumin	Filter (use stacked filters)	4°C
Carboxylmethyl cellulose	Steam, 30 min[3]	4°C
Collagenase	Filter	−20°C
DMSO	Self-sterilizing; dispense into aliquots in sterile tubes	Room temperature; keep dark, avoid contact with rubber or plastics (except polypropylene)
Drugs	Filter (check for binding; use low- binding filter, e.g., Millex-GV, if necessary)	−20°C
EDTA	Autoclave	Room temperature
Glucose, 20%	Autoclave	Room temperature
Glucose, 1–2%	Filter (low concentrations; caramelizes if autoclaved)	Room temperature
Glutamine	Filter	−20°C
Glycerol	Autoclave	Room temperature
Growth factors	Filter (low protein binding)	−20°C
HEPES	Autoclave	Room temperature
HCl, 1 M	Filter	Room temperature
Lactalbumin hydrolysate	Autoclave	Room temperature
Methocel	Autoclave	4°C
$NaHCO_3$	Filter	Room temperature
NaOH, 1 M	Filter	Room temperature
Phenol red	Autoclave	Room temperature
Salt solutions (without glucose)	Autoclave	Room temperature
Serum	Filter; use stacked filters	−20°C
Sodium pyruvate, 100 mM	Filter	−20°C
Transferrin	Filter	−20°C
Tryptose	Autoclave	Room temperature
Trypsin	Filter	−20°C
Vitamins	Filter	−20°C
Water	Autoclave	Room temperature

[1] Autoclave, 100 kPa (15 lb/in.^{2}), 121°C for 20 min.

[2] Filter, 0.2-μm pore size.

[3] Steam, 100°C for 30 min.

Cell Culture Equipments

- The specific requirements of a cell culture laboratory depend mainly on the type of research conducted.

- For example the needs of mammalian cell culture laboratory specializing in cancer research is quite different from that of an insect cell culture laboratory that focuses on protein expression.

- However, all cell culture laboratories have the common requirement of being free from pathogenic microorganisms (i.e., asepsis), and share some of the same basic equipment that is essential for culturing cells.

Basic Equipment

1- Cell culture hood (i.e., laminar-flow hood or biosafety cabinet)

2-Incubator (humid CO_2 Incubator recommended)

3-Water bath

4-Centrifuge

5-Refrigerator and freezer (–20°C)

6-Cell counter or hemacytometer

7-Inverted microscope

8-Liquid nitrogen (N_2) freezer or cryostorage container

9-Sterilizer (i.e., autoclave)

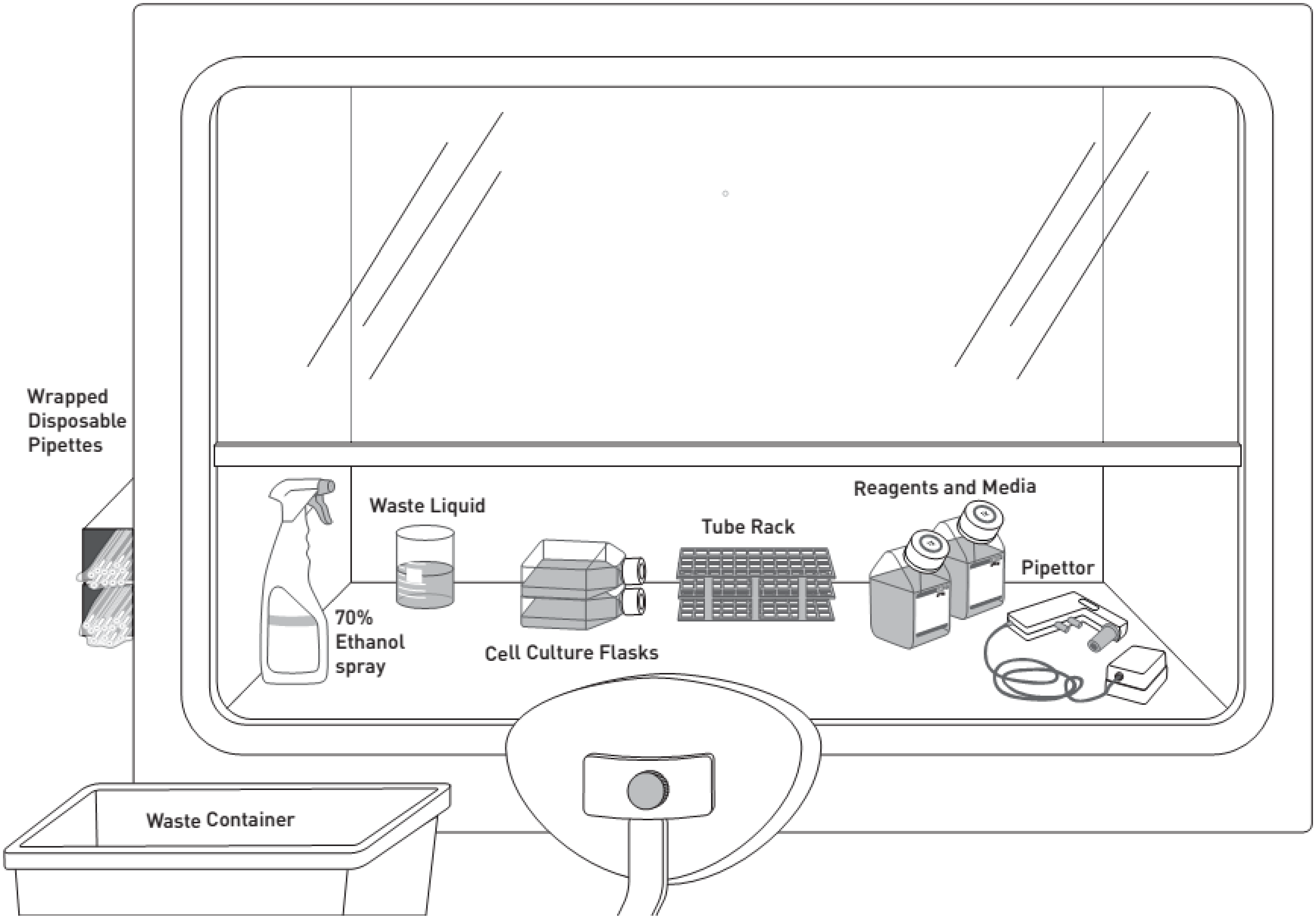
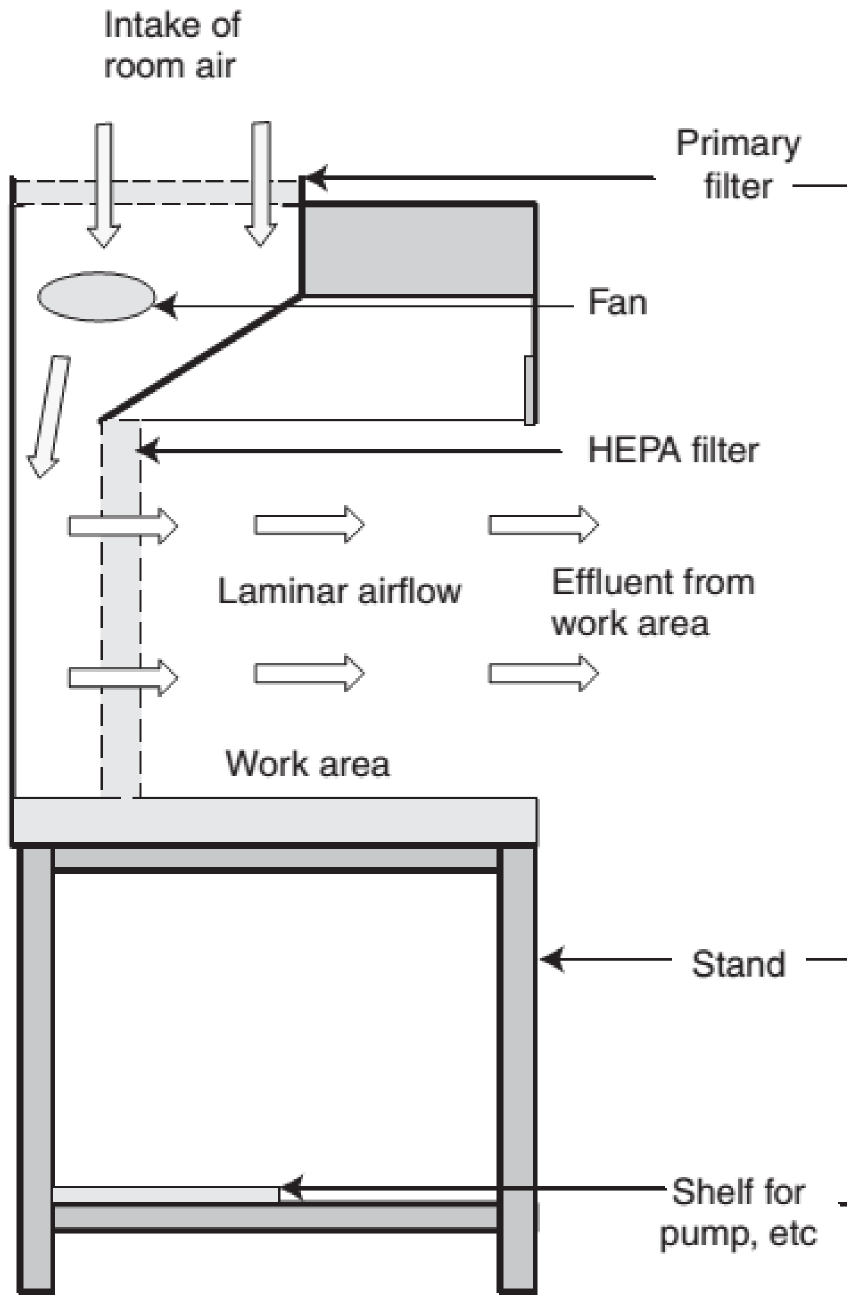
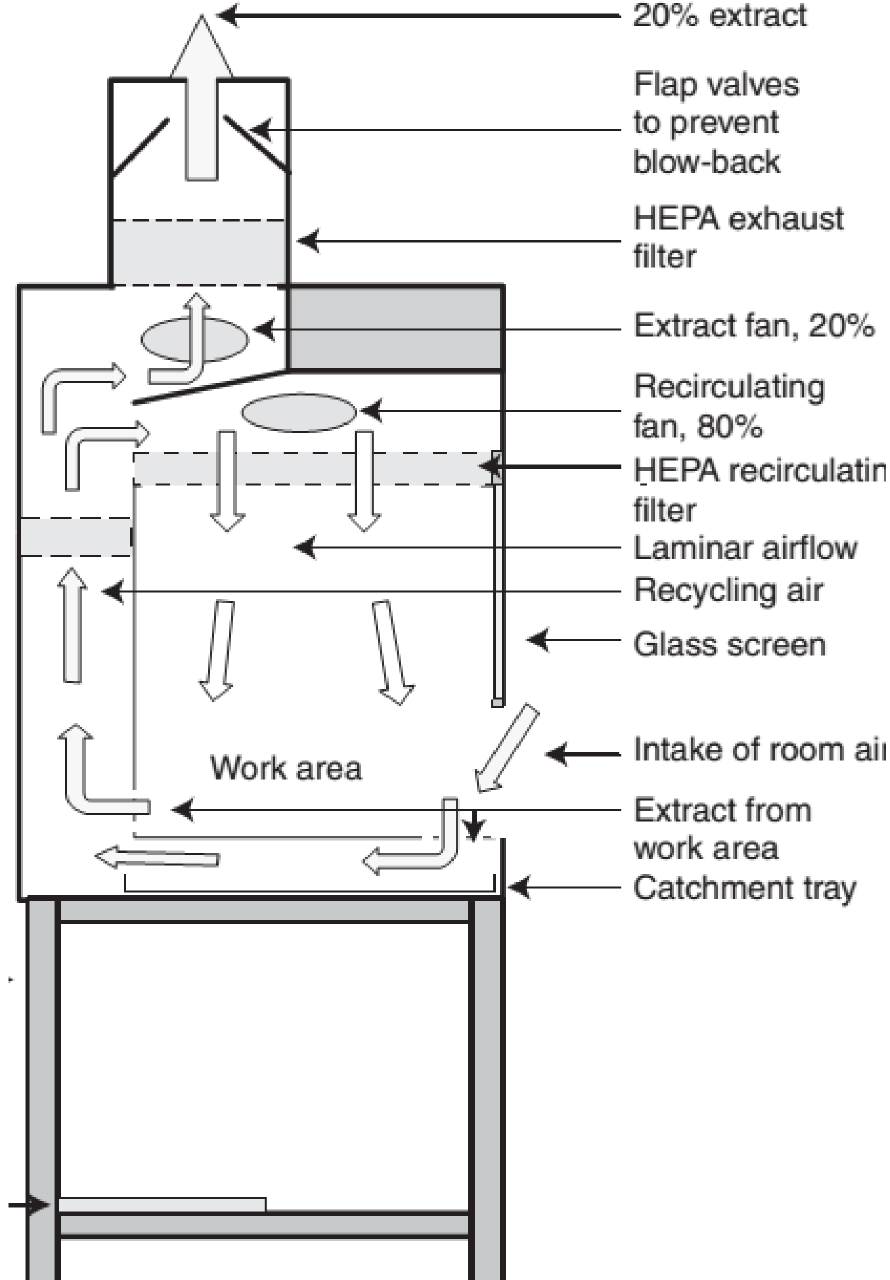


Figure 2.1. The basic layout of a cell culture hood for right-handed workers. Left-handed workers may switch the positions of the items lai out on the work surface.



(a) HORIZONTAL LAMINAR FLOW



(b) VERTICAL LAMINAR FLOW

2-Incubator

- Provide the appropriate environment for cell growth.

- There are two basic types of incubators:

1-Dry incubators

2-Humid CO_2 incubators

- Humidified incubators are a major source of contamination.


Cole-Parmer

Culture Vessels and Surfaces

- Culture vessels provide a contamination barrier to protect the cultures from the external environment while maintaining the proper internal environment.

- For anchorage-dependent cells, the vessels provide a suitable and consistent substrate for cell attachment.

1)Flasks

Description	Growth area (cm^2)	Recommended working volume (mL)	Cell yield*
T-25	25	5 to 10	2.5×10^6
T-75	75	15 to 25	7.5×10^6
T-150	150	30 to 50	15.0×10^6
T-175	175	35 to 60	17.5×10^6
T-225	225	45 to 75	22.5×10^6

*Cell line dependent. Based upon a density of 1×10^5 cells/cm^2.

2) Cell culture dishes

- Cell culture dishes offer the best economy and access to the growth surface.
- This makes them the vessels of choice for cloning or other manipulations such as scraping that require direct access to the cell monolayer.

- Cell culture dishes are available with either specially treated surfaces for growing anchorage-dependent cells, or untreated (native) surfaces for growing suspension cultures where attachment is not desired.

- Most manufacturers offer dishes in four diameters.

Description	Growth area (cm^2)	Working volume (mL)	Cell yield*
35	8	1 to 2	0.8×10^6
60	21	4 to 5	2.1×10^6
100	55	10 to 12	5.5×10^6
150	148	28 to 32	14.8×10^6

*Cell line dependent. Based upon a density of 1×10^5 cells/cm^2.

3)Multiwell plates

- Multiwell plates offer significant savings in space, media, and reagents when compared to an equal number of dish.

Description	Growth well (cm^2)	Working volume/ well (mL)	Cell yield*
96-well	0.32	0.1 to 0.2	0.32×10^5
48-well	1.00	0.3 to 0.6	0.8×10^5
24-well	1.88	0.5 to 1.2	1.9×10^5
12-well	3.83	1.0 to 2.4	3.8×10^5
6-well	9.40	2.0 to 3.0	9.5×10^5

*Cell line dependent. Based upon a density of 1×10^5 cells/cm^2.


PAA
GLASGOW MEM
DMEM

- The three basic classes of media are:
- (they differ in their requirement for supplementation with serum).

1- **Basal media** : The majority of cell lines grow well in basal media, which contain amino acids, vitamins, inorganic salts, and a carbon source such as glucose, but these basal media formulations must be further supplemented with serum.

Serum is vitally important as a source of growth and adhesion factors, hormones, lipids and minerals for the culture of cells in basal media.

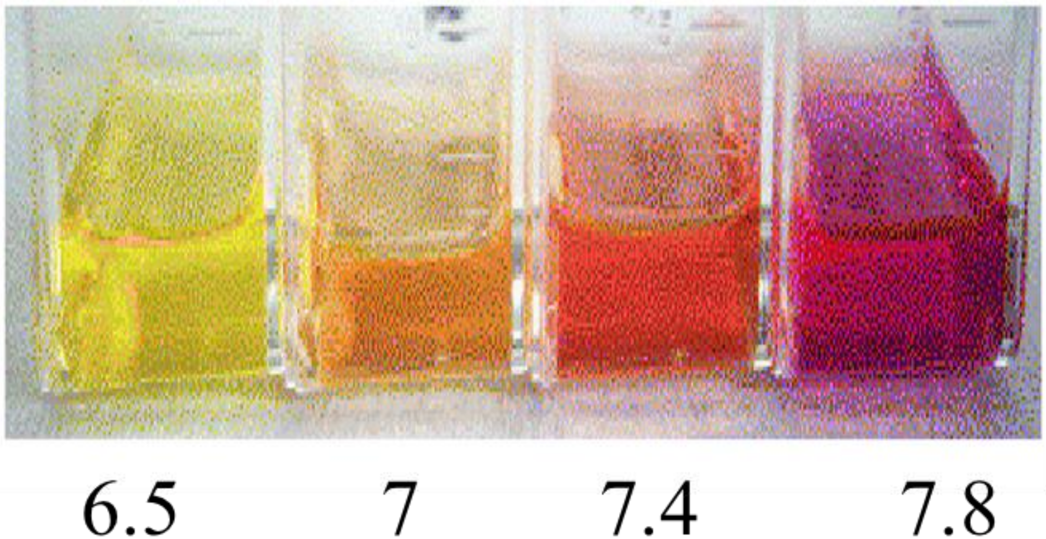
Media Ingredients

1-Sodium bicarbonate and buffering:

- In culture media, dissolved CO_2 is in equilibrium with bicarbonate ions and many medium formulations take advantage of this CO_2/bicarbonate reaction to buffer the pH of the medium.

- The optimal pH range of 7.2 to 7.4 can be maintained by supplementing the medium with sodium bicarbonate and regulating the level of CO_2 in the atmosphere.

- Phenol red is frequently omitted from studies with flow cytometry as its color interferes with detection



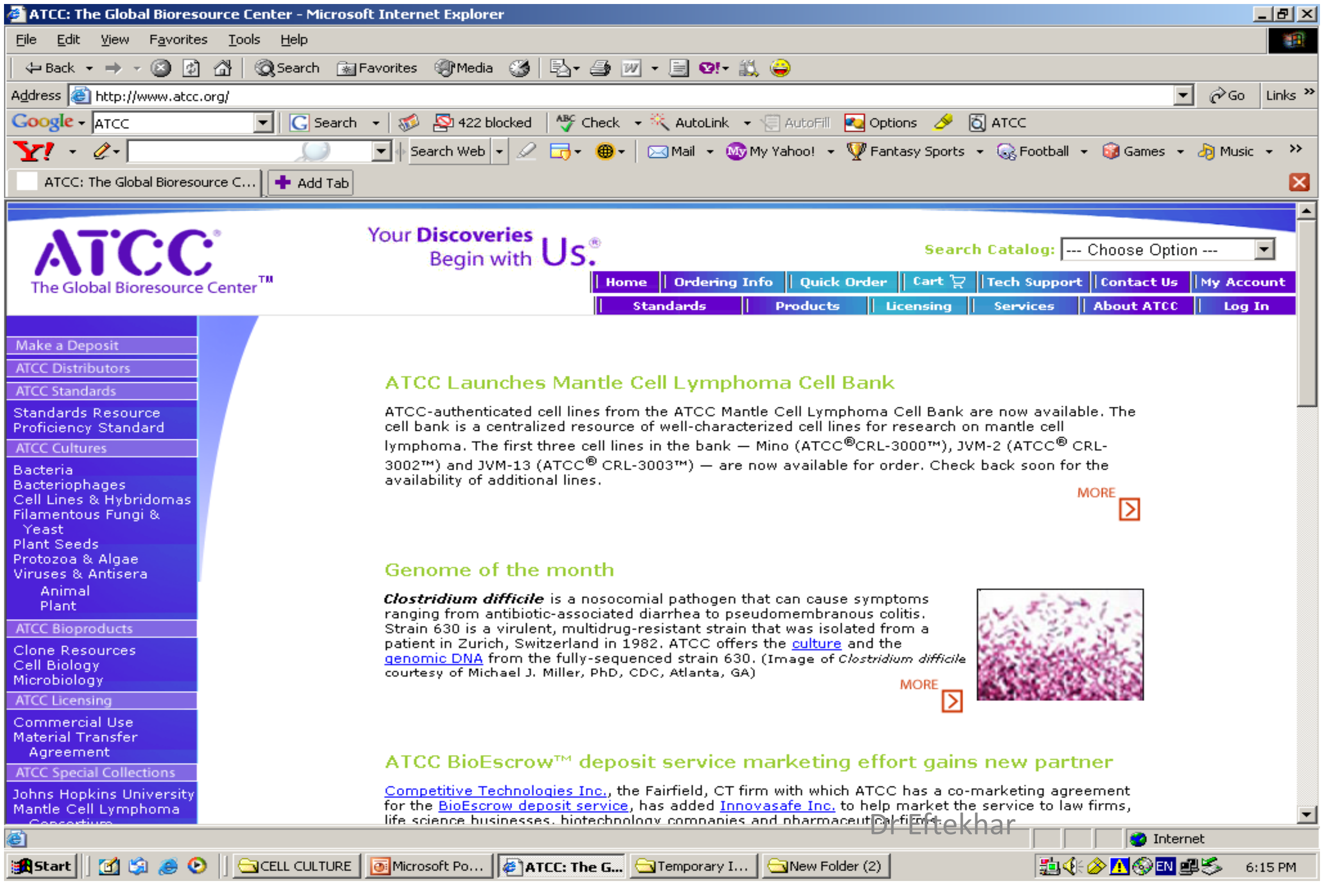
5-Antibiotics and Antimycotics

- Routine use of antibiotics is not recommended, it can mask contamination by mycoplasma and resistant bacteria.
- While cell lines can be cured of microbial contamination with antibiotics, this is not recommend unless the cell line is irreplaceable; the process is lengthy and there is no guarantee contamination will be eliminated. Even if the contamination is eliminated, there is no way of ensuring that the resulting cell line will have the same characteristics as the initial one due to the stress of the treatment.
- It is best to discard the cell line and start over with new stocks.

- Penicillin: final concentration of 50 to 100 IU/mL
- streptomycin: final 50 to 100 μg/mL
- Gentamicin sulfate: final 50 to 100 μg/mL.
- The antimycotic amphotericin B: final 2.5 μg/mL.
- These concentrations apply to media that contain serum. For serum-free media, reduce the concentrations by at least 50%.

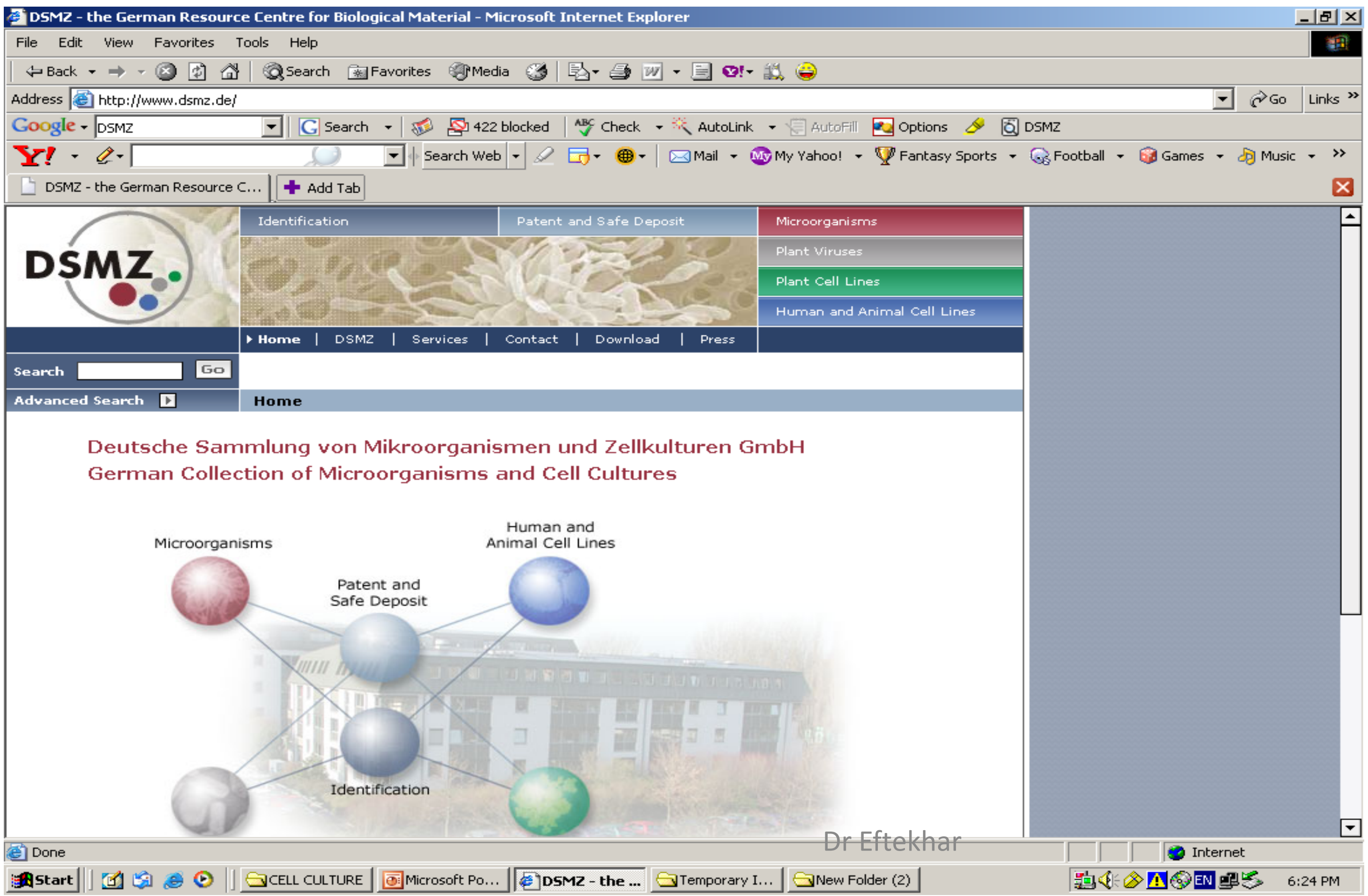
Useful links

- The American Type Culture Collection
  http://www.atcc.org



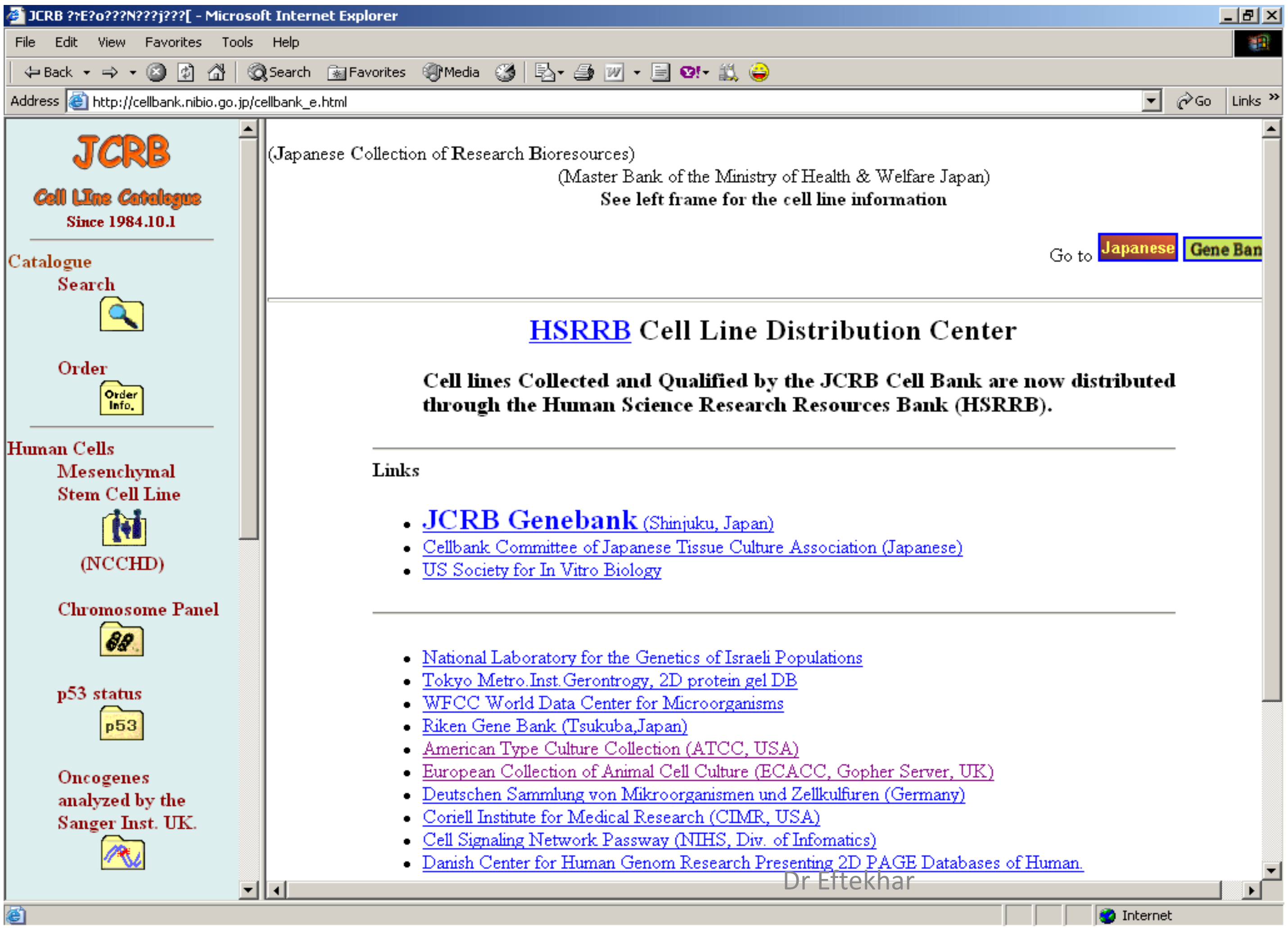
- The German Resource Centre for Biological Material and cell cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

  http://www.dsmz.de



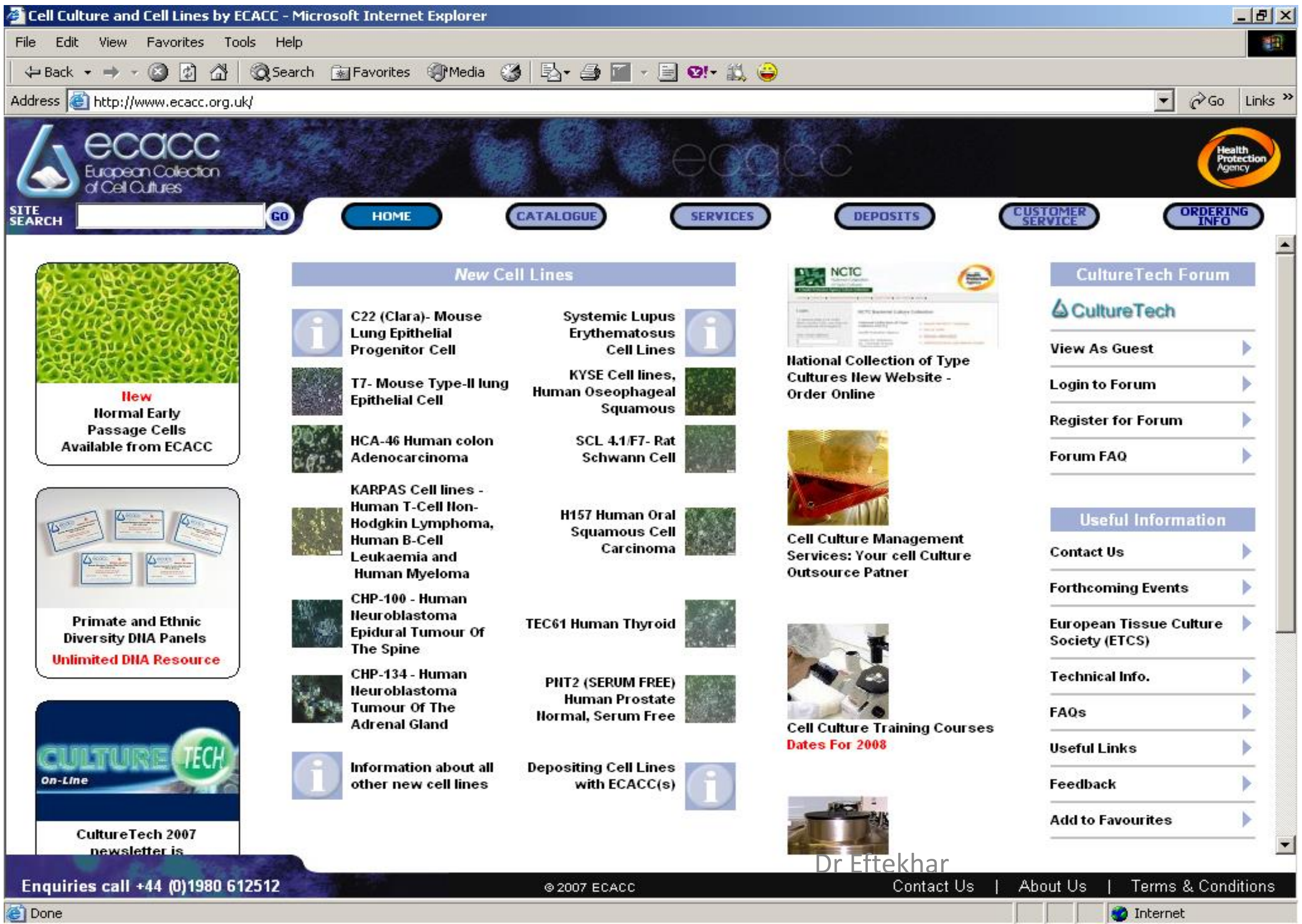
- Japanese Collection of Research Bioresources

  http://cellbank.nibio.go.jp



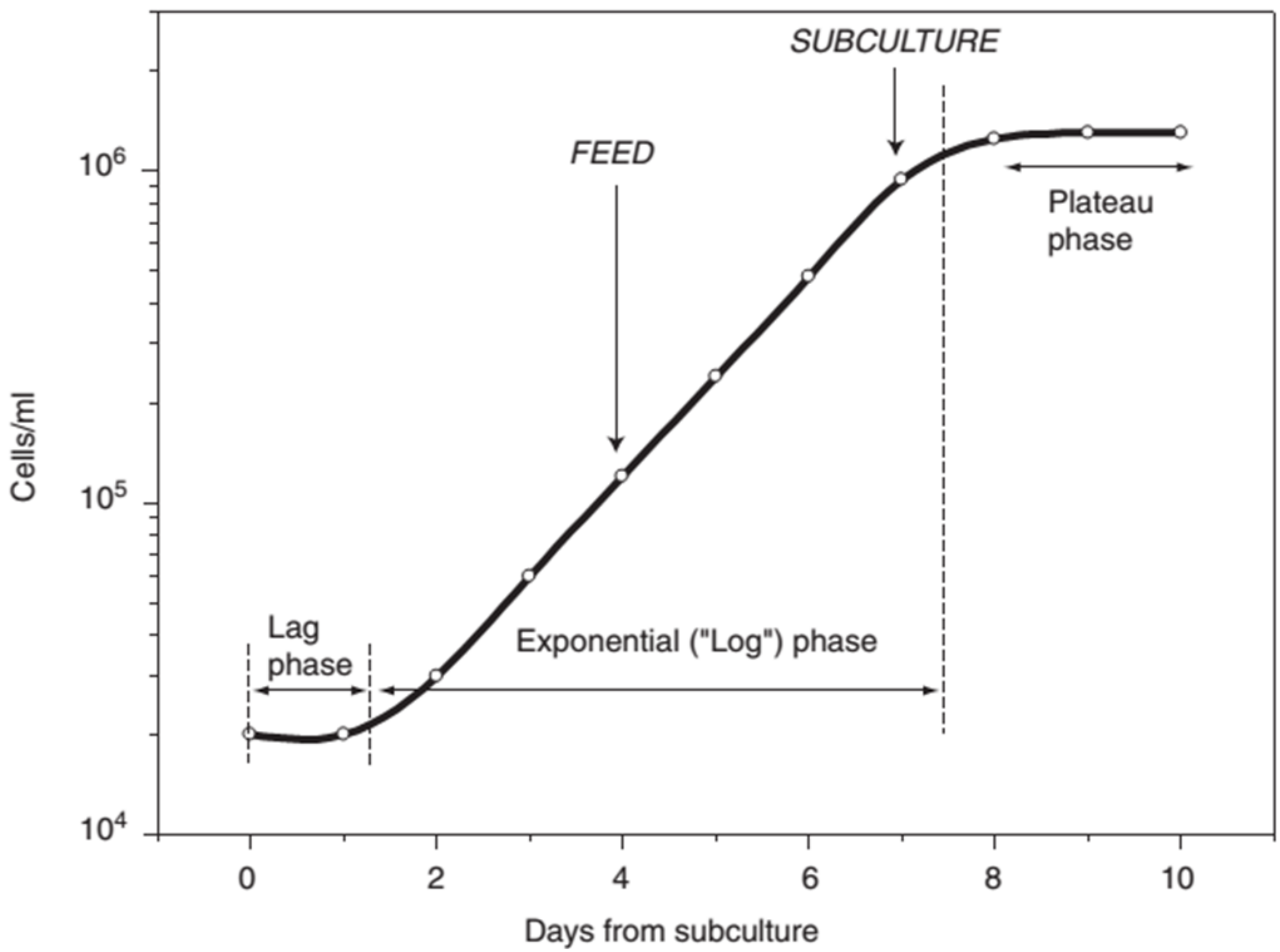
- European Collection of Cell Cultures

  http://www.ecacc.org.uk



- NCBI -National Cell Bank of Iran- Pasteur Institute of Iran

http://ncbi.pasteur.ac.ir



Growth Curve and Maintenance

- To ensure viability, genetic stability, and phenotypic stability, cell lines need to be maintained in the exponential phase.

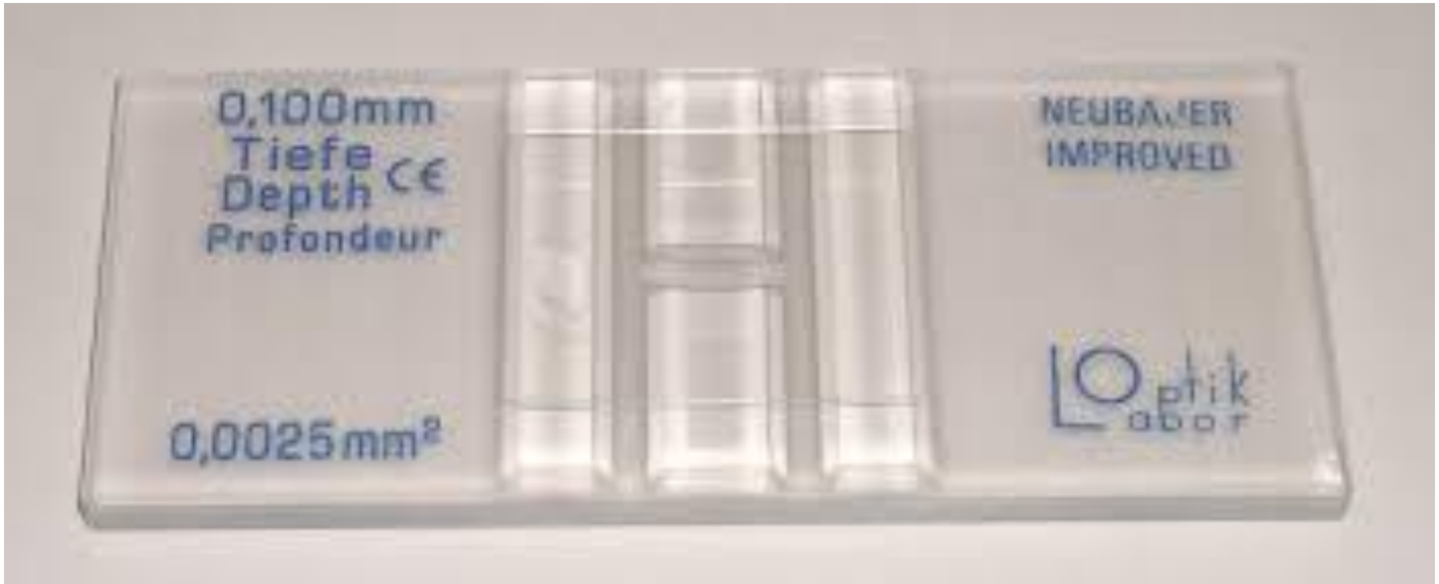
- This means that they need to be subcultured on a regular basis before they enter the stationary growth phase, before a monolayer becomes 100% confluent or before a suspension reaches its maximum recommended cell density.

When the monolayer cells are near the end of exponential growth (roughly 70% to 90% confluent), they are ready to be subcultured.

Cell Counting

- Cell counts are necessary in order to establish or monitor growth rates as well as to set up new cultures with known cell numbers.

- Hemocytometers (also spelled hemacytometers) are commonly used to estimate cell number and determine cell viability.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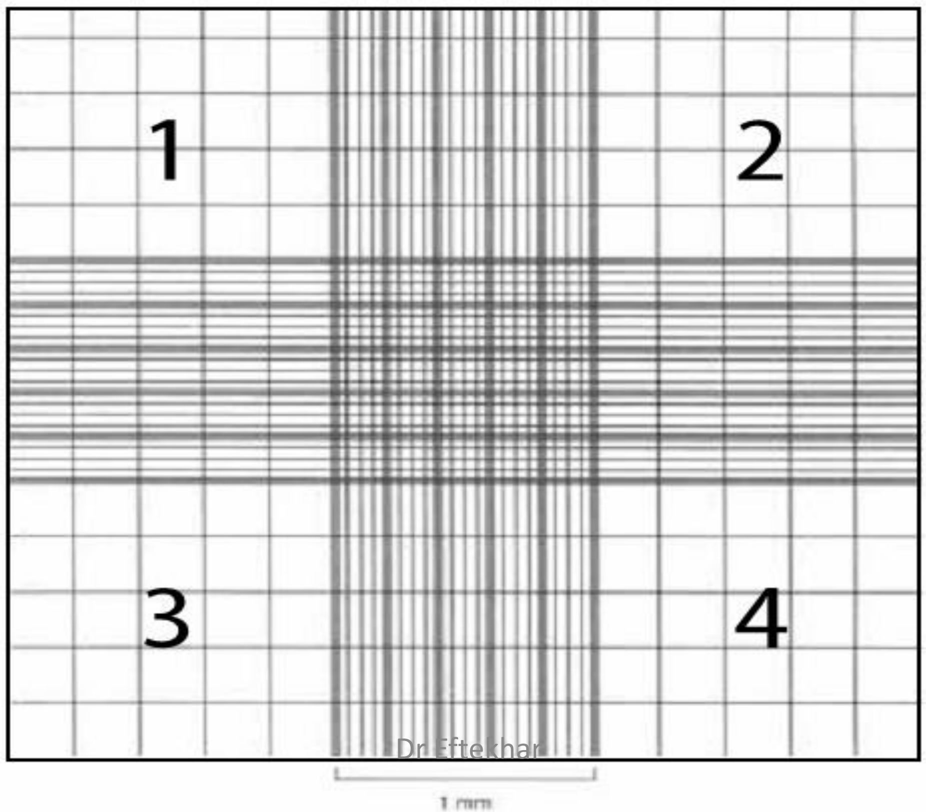
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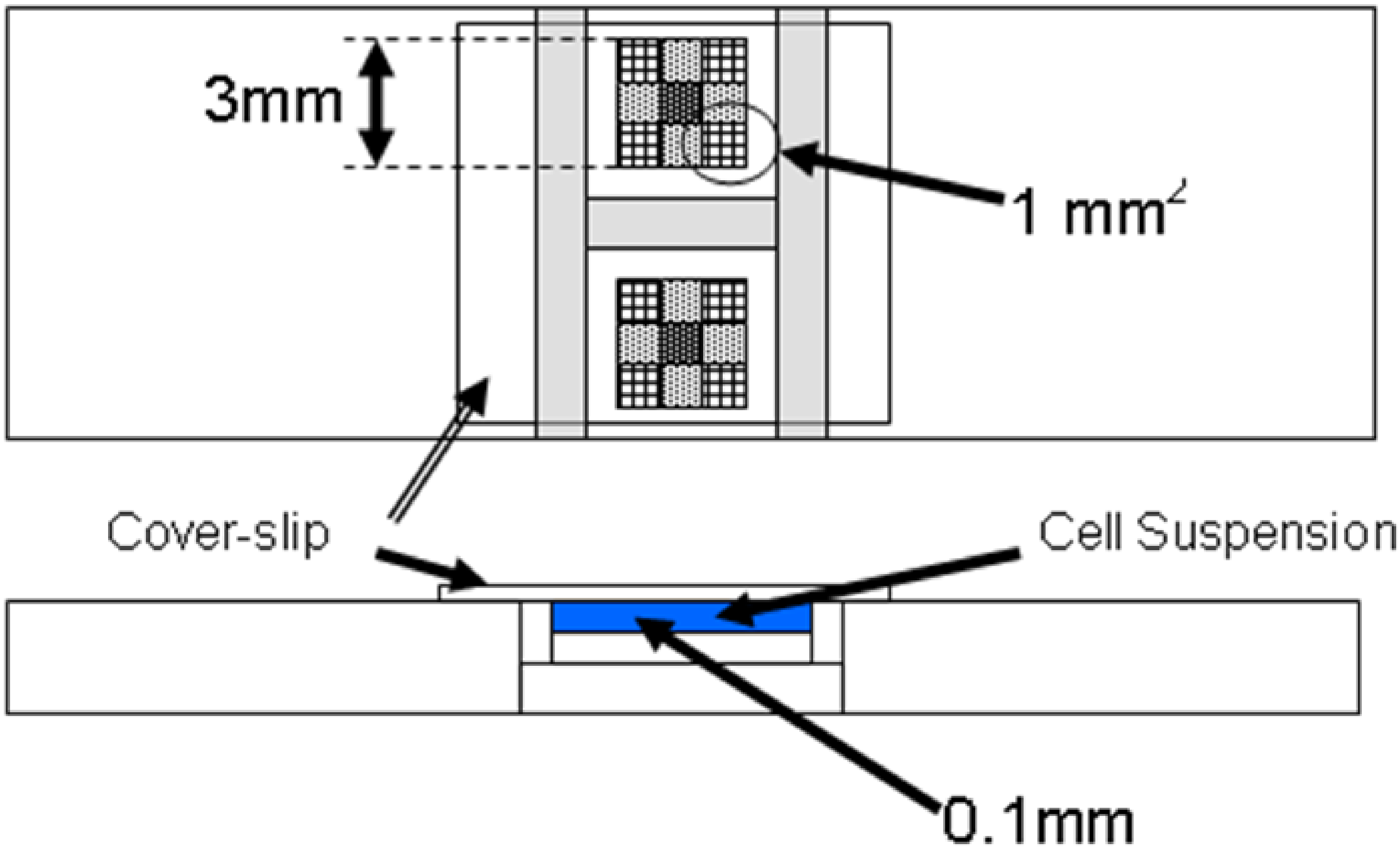
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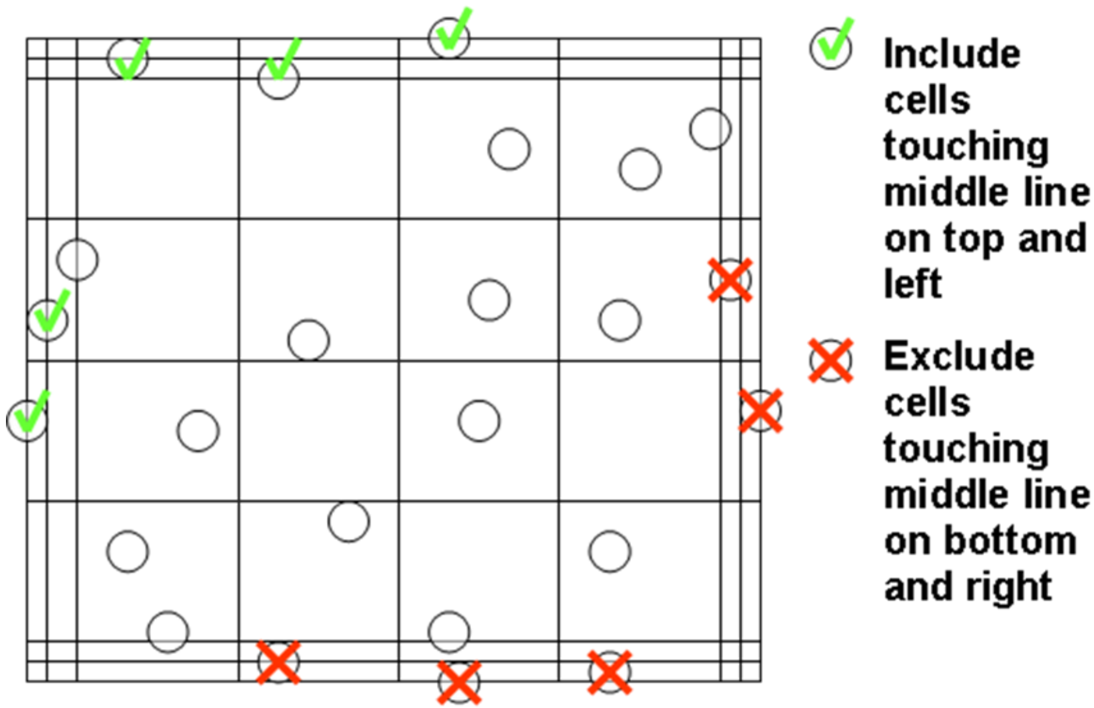
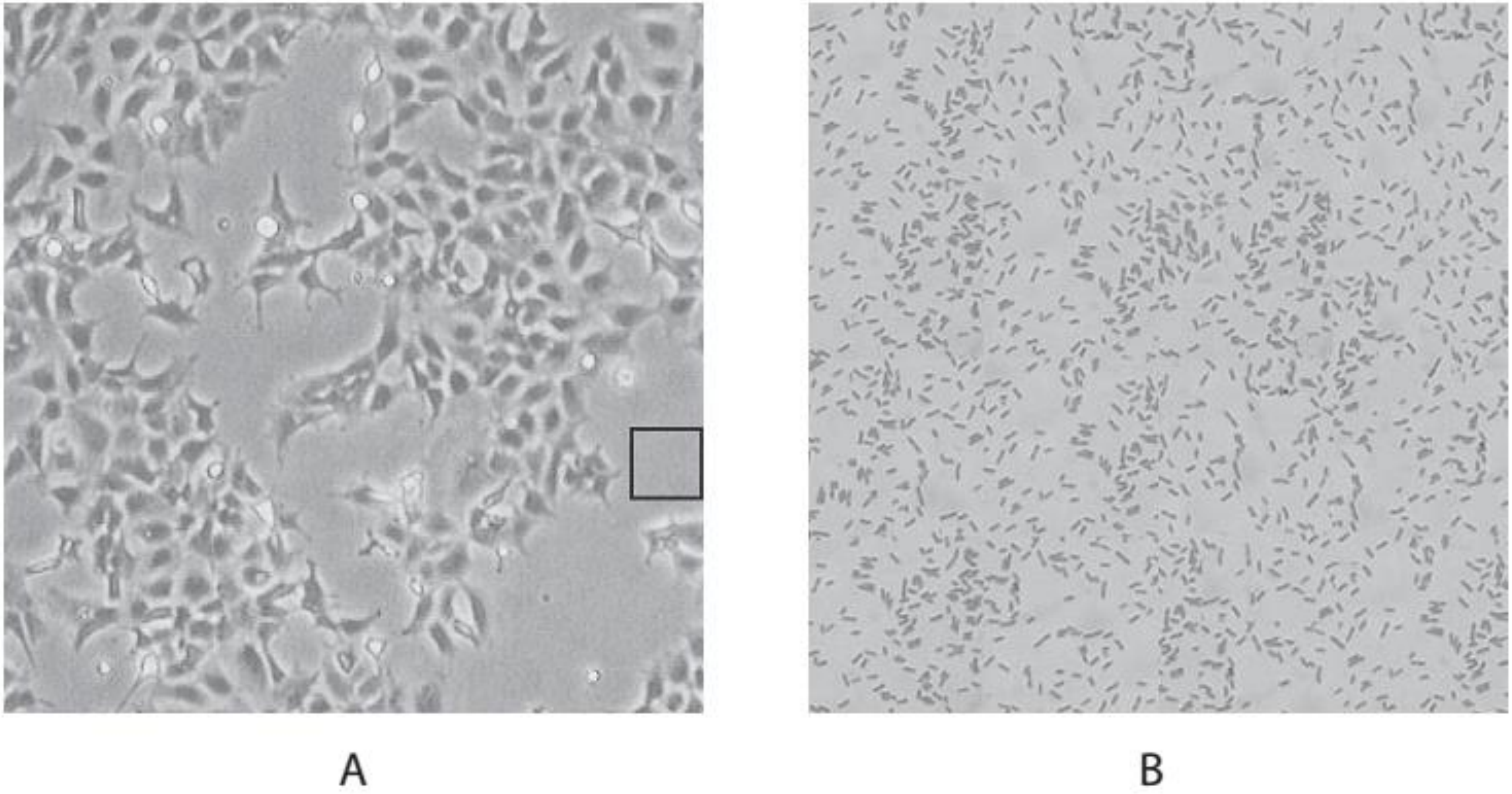
Cell Suspension
0.1mm


Include cells touching middle line on top and left
Exclude cells touching middle line on bottom and right

Biological Contamination

- Contamination of cell cultures is easily the most common problem encountered in cell culture laboratories.
- Biological contaminants such as bacteria, molds, yeasts, viruses, mycoplasma, as well as cross contamination by other cell lines.
- While it is impossible to eliminate contamination entirely, it is possible to reduce its frequency and seriousness by gaining a thorough good aseptic technique.

- **Bacteria**:
- Bacterial contamination is easily detected by visual inspection of the culture within a few days of it becoming infected; infected cultures usually appear cloudy (i.e., turbid).
- Sudden drops in the pH of the culture medium is also frequently encountered (yellow color).
- Under a low power microscope, the bacteria appear as tiny, moving granules between the cells.



Simulated phase contrast images of adherent 293 cells contaminated with E. coli .

- **Yeasts:**

- Yeast cells are larger than bacteria, but may not appreciably change the pH of the medium, and will appear as separate round or ovoid particles that may bud off smaller particles.

- Molds

- Under microscopy, the mycelia usually appear as thin, wisp-like filaments.

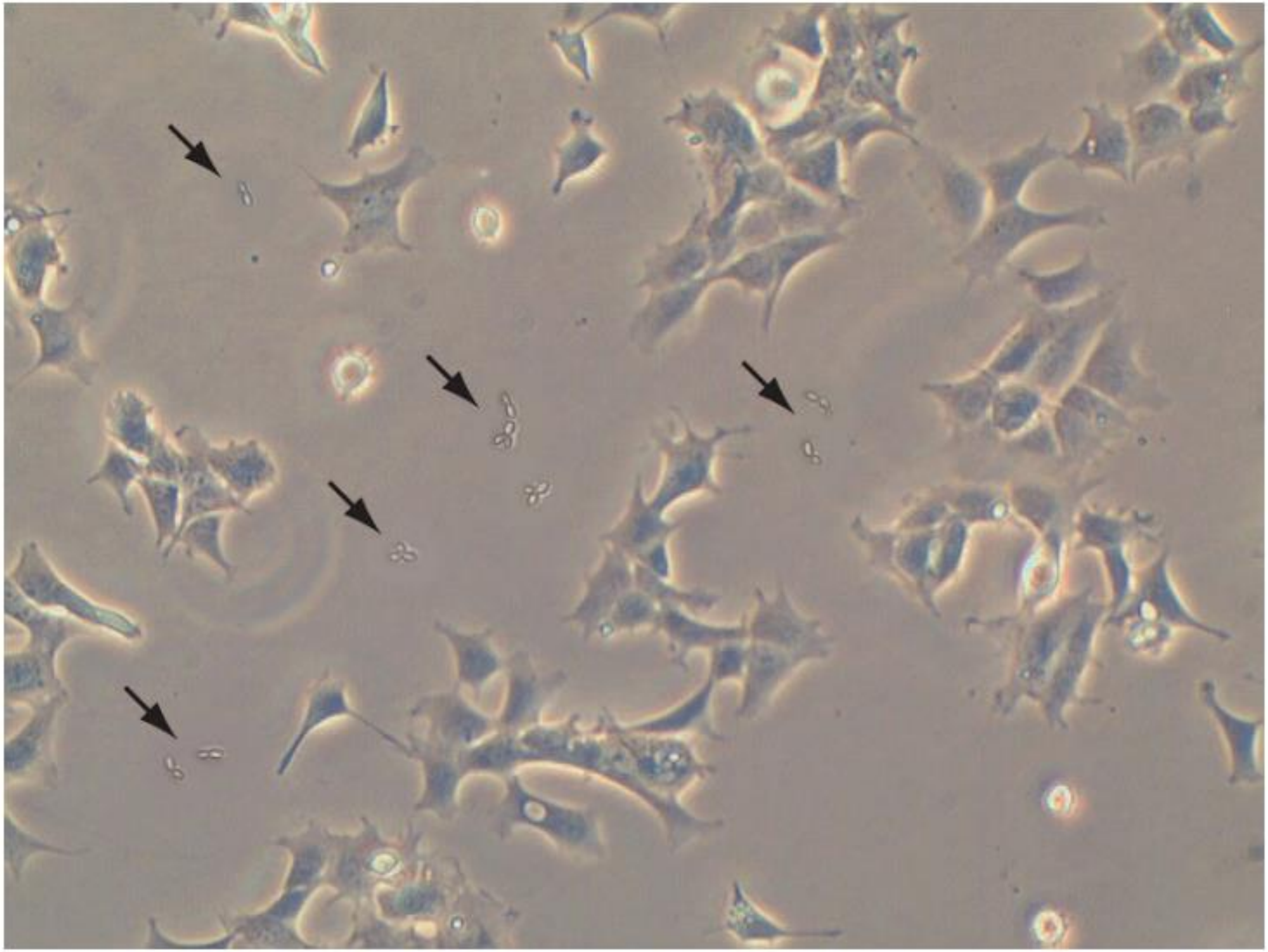


Figure 2.3. Simulated phase contrast images of 293 cells in adherent culture that is contaminated with yeast. The contaminating yeast cells appear as ovoid particles, budding off smaller particles as they replicate.

- **Mycoplasma**:
- Because of their extremely small size, mycoplasma are very difficult to detect until they achieve extremely high densities and cause the cell culture to deteriorate.

- Chronic mycoplasma infections might manifest themselves with decreased rate of cell proliferation, reduced saturation density, and agglutination in suspension cultures.
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- The only assured way of detecting mycoplasma contamination is by testing the cultures periodically using fluorescent staining (e.g., Hoechst 33258), ELISA, PCR assays.