Cell Culture

Basics of Cell Culture
Introduction

- Cell culture is the process by which prokaryotic, eukaryotic or plant cells are grown under controlled conditions. But in practice it refers to the culturing of cells derived from animal cells.
- Cell culture was first successfully undertaken by Ross Harrison in 1907.
- Roux in 1885 for the first time maintained embryonic chick cells in a cell culture.

Major development's in cell culture technology

- First development was the use of antibiotics which inhibits the growth of contaminants.
- Second was the use of trypsin to remove adherent cells to subculture further from the culture vessel.
- Third was the use of chemically defined culture medium.
Why is cell culture used for?

Areas where cell culture technology is currently playing a major role.

- Model systems for
  Studying basic cell biology, interactions between disease causing agents and cells, effects of drugs on cells, process and triggering of aging & nutritional studies
- Toxicity testing
  Study the effects of new drugs
- Cancer research
  Study the function of various chemicals, virus & radiation to convert normal cultured cells to cancerous cells

Contd....

- Virology
  Cultivation of virus for vaccine production, also used to study there infectious cycle.

- Genetic Engineering
  Production of commercial proteins, large scale production of viruses for use in vaccine production e.g. polio, rabies, chicken pox, hepatitis B & measles

- Gene therapy
  Cells having a functional gene can be replaced to cells which are having non-functional gene
Tissue culture

- In vitro cultivation of organs, tissues & cells at defined temperature using an incubator & supplemented with a medium containing cell nutrients & growth factors is collectively known as tissue culture.

- Different types of cells grown in culture include connective tissue elements such as fibroblasts, skeletal tissue, cardiac, epithelial tissue (liver, breast, skin, kidney) and many different types of tumor cells.

Primary culture

- Cells when surgically or enzymatically removed from an organism and placed in suitable culture environment will attach and grow are called as primary culture.

- Primary cells have a finite life span.

- Primary culture contains a very heterogeneous population of cells.

- Sub culturing of primary cells leads to the generation of cell lines.

- Cell lines have limited life span, they passage several times before they become senescent.

- Cells such as macrophages and neurons do not divide in vitro so can be used as primary cultures.

- Lineage of cells originating from the primary culture is called a cell strain.
Continous cell lines

- Most cell lines grow for a limited number of generations after which they cease.
- Cell lines which either occur spontaneously or induced virally or chemically transformed into Continous cell lines.
- Characteristics of continous cell lines:
  - Smaller, more rounded, less adherent with a higher nucleus/cytoplasm ratio.
  - Fast growth and have aneuploid chromosome number.
  - Reduced serum and anchorage dependence and grow more in suspension conditions.
  - Ability to grow up to higher cell density.
  - Different in phenotypes from donor tissue.
  - Stop expressing tissue specific genes.

Types of cells

On the basis of morphology (shape & appearance) or on their functional characteristics. They are divided into three.

- Epithelial like–attached to a substrate and appears flattened and polygonal in shape.
- Lymphoblast like–cells do not attach remain in suspension with a spherical shape.
- Fibroblast like–cells attached to an substrate appear elongated and bipolar.
Culture media

- Choice of media depends on the type of cell being cultured
- Commonly used Medium are GMEM, EMEM, DMEM etc.
- Media is supplemented with antibiotics viz. penicillin, streptomycin etc.
- Prepared media is filtered and incubated at 4°C

Why sub culturing?

- Once the available substrate surface is covered by cells (a confluent culture) growth slows & ceases.
- Cells to be kept in healthy & in growing state have to be sub-cultured or passaged
- It’s the passage of cells when they reach to 80–90% confluency in flask/dishes/plates
- Enzyme such as trypsin, dipase, collagenase in combination with EDTA breaks the cellular glue that attached the cells to the surface
Culturing of cells

- Cells are cultured as anchorage dependent or independent
- Cell lines derived from normal tissues are considered as anchorage-dependent and grow only on a suitable substrate e.g. tissue cells
- Suspension cells are anchorage-independent e.g. blood cells
- Transformed cell lines either grow as monolayer or as suspension

Adherent cells

- Cells which are anchorage dependent
- Cells are washed with PBS (free of ca & mg) solution.
- Add enough trypsin/EDTA to cover the monolayer
- Incubate the plate at 37°C for 1-2 mts
- Tap the vessel from the sides to dislodge the cells
- Add complete medium to dissociate and dislodge the cells
- With the help of pipette which are remained to be adherent
- Add complete medium depends on the subculture requirement either to 75 cm or 175 cm flask
**Suspension cells**

- Easier to passage as no need to detach them
- As the suspension cells reach to confluency
- Asceptically remove 1/3rd of medium
- Replaced with the same amount of pre-warmed medium

**Transfection methods**

- Calcium phosphate precipitation
- DEAE-dextran (dimethylaminoethyl-dextran)
- Lipid mediated lipofection
- Electroporation
- Retroviral Infection
- Microinjection
Cell toxicity

- Cytotoxicity causes inhibition of cell growth
- Observed effect on the morphological alteration in the cell layer or cell shape
- Characteristics of abnormal morphology is the giant cells, multinucleated cells, a granular bumpy appearance, vacuoles in the cytoplasm or nucleus
- Cytotoxicity is determined by substituting materials such as medium, serum, supplements flasks etc.

Working with cryopreserved cells

- Vial from liquid nitrogen is placed into 37°C water bath, agitation of the vial continuously until medium is thawed
- Centrifuge the vial for 10 mins at 1000 rpm at RT, wipe top of vial with 70% ethanol and discard the supernatant
- Resuspend the cell pellet in 1 ml of complete medium with 20% FBS and transfer to properly labeled culture plate containing the appropriate amount of medium
- Check the cultures after 24 hrs to ensure that they are attached to the plate
- Change medium as the colour changes, use 20% FBS until the cells are established
Freezing cells for storage

- Remove the growth medium, wash the cells by PBS and remove the PBS by aspiration
- Dislodge the cells by trypsin-verse
- Dilute the cells with growth medium
- Transfer the cell suspension to a 15 ml conical tube, centrifuge at 200g for 5 mts at RT and remove the growth medium by aspiration
- Resuspend the cells in 1–2ml of freezing medium
- Transfer the cells to cryovials, incubate the cryovials at −80 C overnight
- Next day transfer the cryovials to Liquid nitrogen

Cell viability

- Cell viability is determined by staining the cells with trypan blue
- As trypan blue dye is permeable to non-viable cells or death cells whereas it is impermeable to this dye
- Stain the cells with trypan dye and load to hemocytometer and calculate % of viable cells
  \[ \% \text{ of viable cells} = \frac{\text{Nu. of unstained cells}}{\text{total nu. of cells}} \times 100 \]
Common cell lines

- Human cell lines
- MCF-7  breast cancer
- HL 60  Leukemia
- HEK-293  Human embryonic kidney
- HeLa  Henrietta lacks
- Primate cell lines
- Vero  African green monkey kidney epithelial cells
- Cos-7  African green monkey kidney cells
- And others such as CHO from hamster, sf9 & sf21 from insect cells

Contaminant’s of cell culture

Cell culture contaminants of two types
- Chemical—difficult to detect caused by endotoxins, plasticizers, metal ions or traces of disinfectants that are invisible
- Biological—cause visible effects on the culture they are mycoplasma, yeast, bacteria or fungus or also from cross-contamination of cells from other cell lines
Effects of Biological Contamination's

- They compete for nutrients with host cells
- Secreted acidic or alkaline by-products cease the growth of the host cells
- Degraded arginine & purine inhibits the synthesis of histone and nucleic acid
- They also produce H₂O₂ which is directly toxic to cells

Detection of contaminants

- In general indicators of contamination are turbid culture media, change in growth rates, abnormally high pH, poor attachment, multi-nucleated cells, graining cellular appearance, vacuolization, inclusion bodies and cell lysis
- Yeast, bacteria & fungi usually shows visible effect on the culture (changes in medium turbidity or pH)
- Mycoplasma detected by direct DNA staining with intercalating fluorescent substances e.g. Hoechst 33258
- Mycoplasma also detected by enzyme immunoassay by specific antiserum or monoclonal abs or by PCR amplification of mycoplasmal RNA
- The best and the oldest way to eliminate contamination is to discard the infected cell lines directly
Basic equipments used in cell culture

- Laminar cabinet–Vertical are preferable
- Incubation facilities– Temperature of 25–30 C for insect & 37 C for mammalian cells, co2 2–5% & 95% air at 99% relative humidity. To prevent cell death incubators set to cut out at approx. 38.5 C
- Refrigerators– Liquid media kept at 4 C, enzymes (e.g. trypsin) & media components (e.g. glutamine & serum) at −20 C
- Microscope– An inverted microscope with 10x to 100x magnification
- Tissue culture ware– Culture plastic ware treated by polystyrene

Rules for working with cell culture

Never use contaminated material within a sterile area
Use the correct sequence when working with more than one cell lines.
- Diploid cells (Primary cultures, lines for the production of vaccines etc.)
- Diploid cells (Laboratory lines)
- Continuous, slow growing line
- Continuous, rapidly growing lines
- Lines which may be contaminated
- Virus producing lines
Basic aseptic conditions

- If working on the bench use a Bunsen flame to heat the air surrounding the Bunsen
- Swab all bottle tops & necks with 70% ethanol
- Flame all bottle necks & pipette by passing very quickly through the hottest part of the flame
- Avoiding placing caps & pipettes down on the bench: practice holding bottle tops with the little finger
- Work either left to right or vice versa, so that all material goes to one side, once finished
- Clean up spills immediately & always leave the work place neat & tidy

Safety aspect in cell culture

- Possibly keep cultures free of antibiotics in order to be able to recognize the contamination
- Never use the same media bottle for different cell lines. If caps are dropped or bottles touched unconditionally touched, replace them with new ones
- Necks of glass bottles prefer heat at least for 60 secs at a temperature of 200 C
- Switch on the laminar flow cabinet 20 mts prior to start working
- Cell cultures which are frequently used should be subcultered & stored as duplicate strains
**Other key facts**

- Use actively growing cells that are in their log phase of growth, which are 80–90% viable
- Keep exposure to trypsin at a minimum
- Handle the cells gently. Do not centrifuge cells at high speed or roughly re-suspend the cells
- Feeding & sub culturing the cells at more frequent intervals than used with serum containing conditions may be necessary
- A lower concentration of 10^4 cells/ml to initiate subculture of rapidly growing cells & a higher concentration of 10^5 cells/ml for slowing growing cells

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**Experimental Animals**
Model systems

*E. coli*
- easy and inexpensive to maintain
- 장내 서식, 배설물의 주성분
- 해로운 박테리아의 생장 저해
- Pathogenic은 sickness, death 유발
- 돌연변이 잘 유발 → Metabolism 연구에 이용
- Eukaryote에서의 과정 일어나지 않음

*Yeast*
- Eukaryote와 prokaryote 사이의 차이점
- Genetic study easy
- haploid, single-celled
- Grow very rapidly, inexpensive
- 2가지 종류 사용
  - Saccaromyces cerevisiae; budding
  - Schizosaccaromyces pombe; fission
Cloning the yeast origin of replication
- yeast genome cut restriction enzyme
- fragments clone into plasmid vector
- recombinant plasmids growth and select
- yeast survive in media lacking leucine
- selected yeast cells contain leu2+ gene

Nematoda worm
- Small and easy to keep
- Three days to develop
- Multicellular organism
  Caenorhabditis elegans

Fruit fly
Identification of mutant
→ mutation 특징 확인이 쉬움
- 짧은 시간에 많은 자손 생산
- 알에서 성체가 되기까지 12일 소요
- Drosophila melanogaster
Zebrafish
- Reproduction rapidly and high number
- vertebrate
  - 발생 과정이 사람과 유사
  - 알이 뚜껑하여 발달 확인 가능
  *Danio rerio*

Amphibians
- 알의 크기가 큼 → 발생학 연구에 이용
  - 1920년 슈페만 형성체 발견
  - *Xenopus leavis*

Chicken
- 배 발생 연구에서 알 사용됨
  ; 진화상 인간과 유사
  - 알이 크고, 분화 관찰에 좋음
  - *Gallus gallus*

Mouse
- vertebrate, mammals
  - Human과 더욱 가까움
  ; 생리학, 발생학적으로 유사
  - Expensive to maintain, Reproduce slowly
  - Knock out mouse 이용
    → 특정 *gene*의 기능 확인
Human cell culture
- Human blood 나 tissue에서 분리
- Primary culture
  - derived directly from living tissue
  - Grow for a short period time and stop
  - 세포 분열 횟수 제한

Plants
- Grow slowly, long generation time
- 세포벽 때문에 형질전환이 어려움
- Large genome
- Transposon 연구에 이용; corn
- Arabidopsis thaliana

유전자 cloning
Cloning & Plasmid
두 종류의 다른 유전자를 가진 대장균 사이에 유전자의 재조합이 일어나 새로운 대장균이 만들어 지는 것을 발견했다. 이 원리를 바탕으로 1970년대에 DNA재조합 기술이 발전하게 되었다.

원핵생물에서 DNA의 이동방법

1. 형질전환 (Transformation)
세포 주변에 있는 유전물질을 받아 들어가는 방법 (Frederick Griffith 1920)

2. 형질도입 (Transduction)
세균의 유전자를 박테리오파지가 전달

3. 접합 (Conjugation)
두 개의 세포사이에 접합을 통한 DNA 이동
플라스미드를 이용한 cloning

1. 미생물로부터 플라스미드를 분리한다.
2. 동물, 식물로부터 특정한 유전자가 포함된 DNA를 분리한다.
3. 분리한 유전자를 포함하고 있는 DNA조각을 플라스미드에 삽입하여 재조합 DNA를 만든다.
4. 박테리아 세포에 재조합 플라스미드를 넣어 형질전환을 한다.
5. 재조합 박테리아 클론이 확보 사용된다.

Plasmid selection

selectable markers
- plasmid 삽입된 cell과 삽입되지 않는 cell 구별을 위해 사용
- antibiotic resistance 이용
  ; plasmid 삽입된 cell만 항생제 함유 배지에서 성존
- antibiotic resistance
  → chemical modification 통해서 target antibiotics inactivation
  → 세포막을 통한 antibiotics transport 방해


**DNA Recombination**

inserting new genes into plasmids
- gene cloning technology
  ; cut and past DNA fragment
- plasmid vector는 cloning site(제한효소 인식 서열) 포함
  ; 원형의 plasmid 절단하여 open, DNA 삽입 가능
- 외래의 제한효소 → sticky end 형성
  ; 보완적인 다른 fragment와 수소 결합 형성하므로 DNA ligase를
  위해 충분한 시간 동안 DNA fragment 점착
- DNA ligase ; 인접한 nucleotide 사이에 phosphodiester 결합 재형성
  → stable double helix

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**The host cell : Escherichia Coli**

DNA 증식을 위해 transformation 중요
Transformation을 위해 요구되는 요소
- gene 도입을 위한 적당한 숙주
- 숙주 안으로 gene을 도입할 운반체
- gene을 받아들인 숙주 선별할 수단

Bacterium E. coli
- 가장 널리 사용 : simple, genetic environment
- genome 완전히 분석됨
- genetic code 분명하기 때문에 다른 생명의 외래 DNA 받아들임
  → DNA의 구성, 구조, 기본 mechanism 동일하므로 복제 가능
- 배르게 분열하고, cell 분열할 때마다 도입된 DNA도 복제
- culture medium에서 37℃ 일 때 최고 성장
- bacterial growth
Transformation

*E. Coli*

; CaCl2 + heat shock(42℃) 조건에서 형질전환 일어남

다른 이온(Mg2+, Mn2+, Ba2+ 등)도 사용
; mixture of positive ion 사용시 → 효율 증가

DNA size 와 conformation이 형질전환 효율에 영향
A subset of cell에 제한 발음
→ plasmid 수 증가해도 형질 전환된 cell 수 변화 없음
*E. coli*가 DNA 받아들이는 정확한 메커니즘 밝혀지지 않음
→ adhesion zone 가설
; 세포막의 adhesion zone 에서 channel 형성
단점; Large DNA는 성공적으로 형질전환 되기 힘들

Proposed molecular mechanism of DNA transformation of *E. coli*

0℃ 처리하여 세포막 고형화,
charged phosphate stabilizing
- Transformation solution 속의 양이온들이
phosphate group과 complex 이룹
→ (-) charge 가리므로 DNA 분자 이동 가능
- Heat shock
→ 세포막의 열적 불균형 형성하여
    adhesion zone 를 통한 DNA pumping 도움
**Directional cloning**

1. Source DNA containing gene of interest (GOT) and insert downstream into vector
2. Separate fragments on agarose gel
3. Make fragments from gel and mix together with DNA vector

**Genomic library** (유전자 도서관)

무차별 유전자 클로닝 방법

1. 제한효소를 이용하여 DNA를 수천 조각으로 절단
2. 각 DNA조각은 서로 다른 박테리아 분자에 실려 박테리아 세포에 형질 전환
3. 수많은 종류의 박테리아 클론을 genomic library라 함

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Plasmids

DNA fragments

Construct recombinant DNA molecules

Each carries a different fragment
특정 유전자를 갖고 있는 클론을 찾는 방법

**Probe**
찾고자 하는 유전자가 TAGGCT라는 염기서열을 가지고 있다면, 방사성 동위원소 표지 후 특정 유전자 발견하는데 사용

**Hybridization**
탐지자가 준비되면, 클론으로부터 단일가닥의 DNA를 준비하며, 탐지자와 DNA 표본을 혼합하면 방사능을 가진 RNA probe가 상 보적인 서열을 가진 DNA에 수소결합을 함으로써 클론을 표지한 다.

*Single stranded (SS) DNA can pair with a complementary strand to regenerate DS DNA*
**Southern Blotting**

DNA fragments separated in a gel can be transferred to a membrane for hybridization to a SS DNA Prob. The extent of hybridization can be quantitated by using a radioactive DNA probe and auto-radiography.

![Southern Blotting Diagram](image)

**The plasmid vector**

propagation of plasmids
- bacterial cell의 빠른 증식 능을 이용하여 특정 gene를 증폭 시킬 때 plasmid 이용 (host cell division시에 plasmid duplication)
- origin of replication(ori) sequence 필요
  - host cell 안에서 복제 가능하게 함
- 복제 조절에 따라 2 group으로 구분
  - stringent control : bacterial cell 분열에 조절 받음(1개씩 replication)
  - relaxed : bacterial cell 과 자율적(cell당 수 백개의 copy 축적)
**Laboratory of Molecular Genetics, KNU**

- **pUC19**
  - 2686 bp
  - Polylinker region

- **GST-Vector (pGEX 6p, T7)**

*Image of diagrams and sequences*
**Isolation of recombinant plasmids**

1. **E.coli**을 EDTA, Glucose 섞인 buffer로 현탁

2. SDS, NaOH Mixture 첨가 → cell lysis, DNA denature

3. potassium acetate, acetic acid 첨가 → neutralization

4. 상층액에 ethanol 이나 isopropanol 첨가 → plasmid DNA 침전

5. pellet = clean plasmid DNA

6. 전기 영동 하여 재조합 확인

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**The Boyer-Cohen- Chang experiment. 1973**

Proof that The Boyer-Cohen- Chang experiment created a recombinant DNA molecule
유전자 cloning
Useful Enz.

DNA polymerases

**DNA template**

**RNA template**

DNA copies

Figure 2.4a: Genes 3 (© Garland Science 2009)
DNA synthesis is done by an enzyme (DNA polymerase) adding nucleotides to the 3'-end of a primer DNA chain.

Polymerase Chain Reaction (PCR)–1

A pre-defined DNA sequence in the genome can be greatly amplified by repeated Polymerization cycles using 2 primers which hybridize to the ends of the target DNA. In each cycle, the amount of target DNA is doubled. After 10, 20 and 30 cycles, there is a 1000-, million- and billion-fold amplification respectively.
Polymerase Chain Reaction (PCR) – 2

Each PCR cycle has 3 steps:

a. Melting of DNA

b. Hybridization of primer

c. DNA synthesis

Nucleases

- Endonuclease
  - Internal cuts

- Exonuclease
  - Nucleotides removed from the ends

Figure 2-4b: Oligonucleotides (© Garland Science 2007)
1. 진핵세포로부터 mRNA를 분리 후 DNA 함성을 위한 주형으로 사용
2. RNA 주형으로부터 DNA를 합성하는 것을 역전사라 한다.
3. DNA 단일 가닥이 만들어진 후, RNA 가닥은 분해되고, 첫 번째 합성된 DNA 가닥을 주형으로 두 번째 가닥의 DNA가 합성된다.
4. 합성된 DNA에는 인트론이 존재하지 않는 DNA (cDNA)로 단백질 합성에 대한 유전정보를 포함

Reverse trasncriptase (역전사효소)
Restriction endonucleases

- cut DNA
  - DNA의 특정 sequence 인지, 절단(break phosphodiester bond)

발견과정

-1950s; bacteria에서 원시적인 immune system 발견
-1960s; enzyme system (E. coli 추출물) 발견
  → self DNA 보호, 외래 DNA 인지- 절단
  modification activity (methylolation)
-1970s; New restriction endonuclease 발견
  → Hind III (haemophilus influenzae 에서 발견)
  → modification activity 없음, 인식자리 안의 정확한 지점 절단

Three major class
- type I, III; restriction and modification activity ,
  인식자리 밖 절단, ATP를 에너지원으로 사용
  → 예측 불가능, ATP 요구성 때문에 사용 안 함
- type II; restriction activity만 있음, ATP필요 없음, Mg2+ 필요,
  인식자리 안이나 인접부위 예측 가능하게 절단
  → DNA 조작에 이상적

절단방법

- middle of the site → blunt end
- 3’ of center, 5’ of center → sticky end

Frequency of cutting
; 제한효소가 인지하는 sequence의 길이에 의존
Restriction map
; 제한효소에 의해 절단한 DNA fragment의 크기 비교
  → genetic map과 연관
Restriction enzymes cleave DNA at a specific sequence.

Molecular detail of EcoR1 restriction-modification
Cleavage map of the SV40 genome

![SV40 genome diagram]

Properties of restriction enzymes–2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Species/Strain</th>
<th>Cut Site</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaeIII</td>
<td><em>Haemophilus aegiptius</em></td>
<td>GG/CC</td>
<td>Blunt cut</td>
</tr>
<tr>
<td>Sau3A</td>
<td><em>Staphylococcus aureus</em></td>
<td>/GATC</td>
<td>5’-overhang</td>
</tr>
<tr>
<td>Hhal</td>
<td><em>Haemophilus haemolyticus</em></td>
<td>GCG/C</td>
<td>3’-overhang</td>
</tr>
<tr>
<td>Smal</td>
<td><em>Serratia marcescens</em></td>
<td>CCC / GGG</td>
<td>Blunt cut</td>
</tr>
<tr>
<td>EcoRI</td>
<td><em>Escherichia coli RY13</em></td>
<td>G / AATTCC</td>
<td>5’-overhang</td>
</tr>
<tr>
<td>PstI</td>
<td><em>Providencia Stuartii</em></td>
<td>CTGCA / G</td>
<td>3’-overhang</td>
</tr>
<tr>
<td>NotI</td>
<td><em>Nocardia otitidis</em></td>
<td>GC / GGCCGC</td>
<td>8 nt sequence</td>
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