Cell culture and passaging (Practice in English/Japanese)

朝比奈 欣治・山元 武文・森 康博・寺戸 勅雄・岡本 久美・福永 祥子（実験実習支援センター）
Kinji Asahina, Takefumi Yamamoto, Yasuhiro Mori, Tokio Terado, Kumi Okamoto, Sachiko Fukunaga (Central Research Laboratory)

始めに細胞培養の原理について 30 分講義したのち、細胞培養室に移動し、実際の細胞培養の方法について実習を行う。実習では、培養皿上で培養した細胞を観察後、トリプシン-EDTA を使って細胞を剥離する方法を学び、剥離した細胞の数を血球計算盤でカウントし、新しい培養皿に播種する方法を習得する。

At the beginning of this lecture/practice, we will explain how to culture a cell line on a plastic dish in a sterile condition. In the following hands-on training, we will show you how to collect cells growing on the dish using a trypsin-EDTA solution, count the cell number with a cell counter, and passage them to a new dish.

Program of 2024 Intensive Course in Basic Science Fundamentals &
Multidisciplinary Seminars / Central Research Laboratory Special Seminars

Sep 13, 2024
17:00-18:20

Introduction of cell culture and passaging

細胞培養の基礎と細胞の継代方法

Kinji Asahina, PhD, FAASLD
Associate Professor
Central Research Laboratory

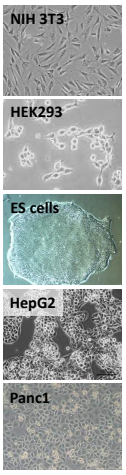
朝比奈 欣治
実験実習支援センター

Cell culture

Adherent cells
Plastic dish (plate)

Cell lines
NIH 3T3 (mouse fibroblasts)
HEK 293 (human embryonic kidney cells)
COS-7 (monkey fibroblasts)
Embryonic stem cells, etc...

Cancer cell lines
HepG2 (human hepatoma)
Panc1 (human pancreatic cancer)
etc...



Culture medium

Dulbecco's Modified Eagle Medium (DMEM)
Buy either powder or liquid (filtered)

Components
D-Glucose (1,000 mg/ml low or 4,500 mg/ml high)
L-Glutamine ← Unstable, need to be added
Sodium pyruvate
$NaHCO_3$ ← Makes pH stable
HEPES ← Makes pH stable
Phenol red ← pH indicator

Inorganic components
$CaCl_2$, KCl, $MgSO_4$, NaCl, NaH_2PO_4, $Fe(NO_3)_3$

Amino acids
L-Arginine, L-Cystine, Glycine, etc...

Vitamins
Folic acid, Inositol, etc...

There are different media available (MEM, RPMI 1640, IMDM, etc...).
Select the best medium for your cell type

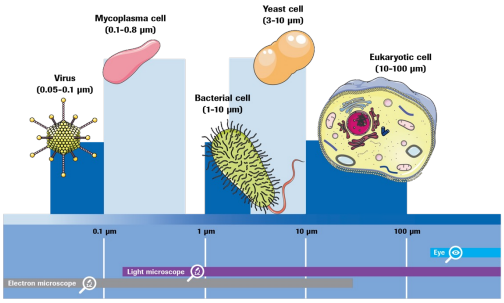
Sterilization of liquid by filtration

Culture media or bio reagents

0.2 μm filter
(cannot exclude mycoplasma)

Large units for making culture medium (100-1,000 ml)
Small unit for making reagents (1-10 ml)

Mycoplasma



Mycoplasma cannot be removed by filtration using a 0.2 μm filter.

Antibiotics

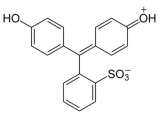
Anti-bacteria

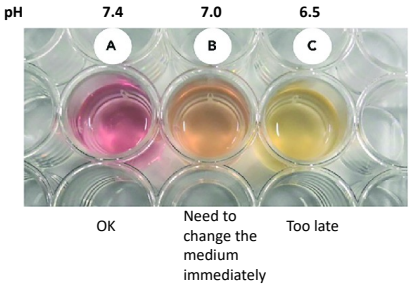
Penicillin G: Inhibition of the bacterial cell wall synthesis
Streptomycin: Inhibition of protein synthesis
Gentamycin: Inhibition of protein synthesis

Anti-fungal
Amphotericin B: Suppression of the growth of fungi

<u>These antibiotics cannot prevent the contamination of viruses, mycoplasma, and yeast.</u>

Phenol red

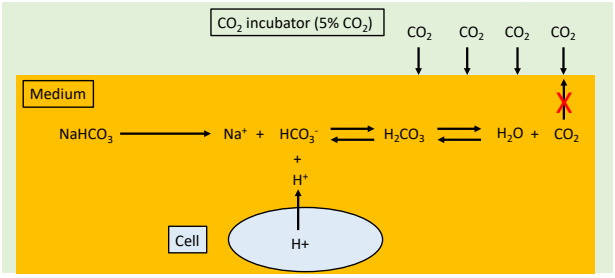




CO_2 incubator

1. 37 °C temperature
2. 90-95% moisture (use water chamber)
3. 5% CO_2 for pH stabilization

Available on the 4th floor of the Central Research Laboratory



Serum for cell growth

Sera contain many factors that help cell growth (proteins, lipids, other components)

Fetal bovine serum (2-10%)
Horse serum (5%)

Heat-inactivation of fetal bovine serum (FBS)

Inactivate the component system containing in FBS by incubation at 56 °C for 30 min

Cell culture dish/plate/flask

Adherent cells

Plastic dish, plate, flask
Glass plate (cover glass)

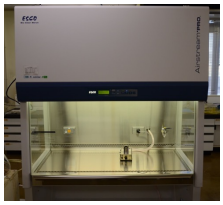
Dish coating

Poly-D-Lysine
Extracellular matrices (collagen, fibronectin, etc...)

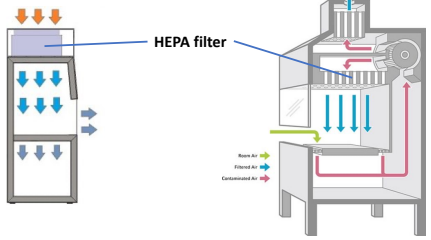
Dish **Plate** **Flask**

Cell culture hood

Available on the 4th floor of the Central Research Laboratory



Laminar flow hood **Biosafety cabinet**

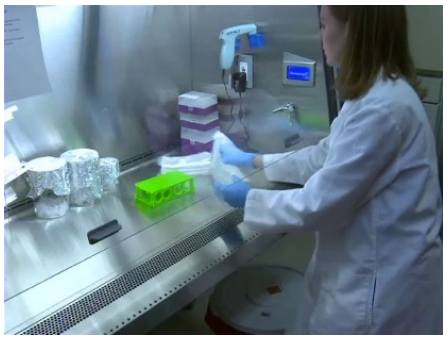


Aseptic techniques in cell culture

Cell culture hood

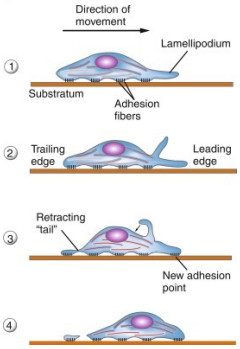
Gloves

70% Alcohol

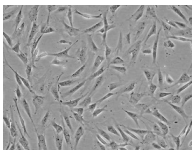


Cell adhesion on a culture dish

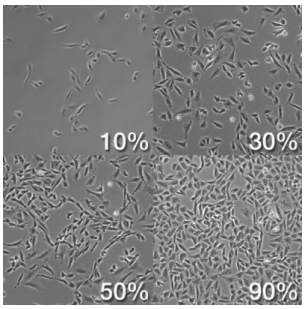
Epithelial cells and fibroblasts
Adhere the extracellular matrix on the dish via proteins, such as integrins.



NIH 3T3



Confluency

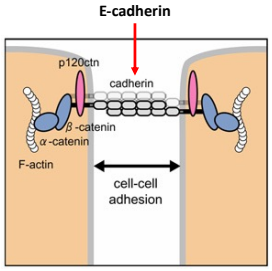


In general, cells grow well at a low confluency

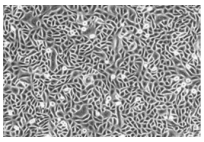
Cells at a high confluency do not grow and respond to cytokines by their cell-cell contact inhibition.

E-cadherin expressed on epithelial cells

Epithelial cells
Form epithelial colonies by E-cadherin, a Ca^{2+}-dependent cell-cell adhesion molecule

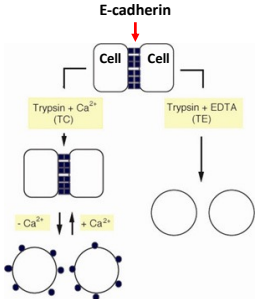


Keratinocytes



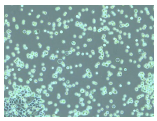
Passaging cells

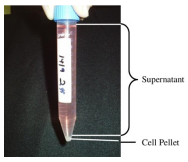
Use of Trypsin-EDTA for dissociation of cells from culture dishes
Trypsin (0.05-0.5%)
Digest proteins involving the cell attachment
EDTA
Chelate Ca^{2+} for dissociation of E-cadherin bindings



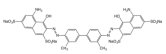
Passaging cells

General procedure
Check cells cultured on a dish under microscope
Aspirate the culture medium
Add PBS for washing
Aspirate the PBS
Add 2 ml of a Trypsin-EDTA solution to a 10 cm dish
Incubate at 37°C for 1-5 min (until cells detach from the dish)
Add 3 ml culture medium containing FBS
Dissociate cells by repeating pipetting 3-10 times gently
Transfer cell suspension to a 15 ml tube
Centrifuge at 3,000 rpm for 3 min
Aspirate the culture medium
Suspend the cell pellet in 1 ml medium
Take 10 ul of cell suspension
Add 10 ul of trypan blue to detect dead cells
Count the cell number





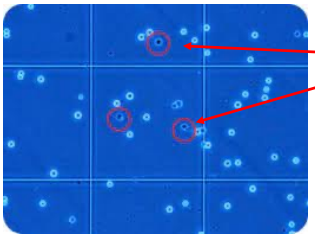
Trypan blue dye



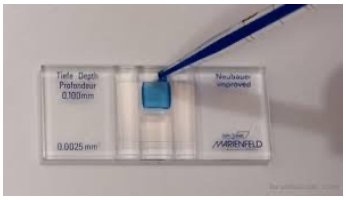
Add 10 ul of trypan blue solution to 10 ul of cell suspension
Apply 10 ul of the solution to a hemocytometer

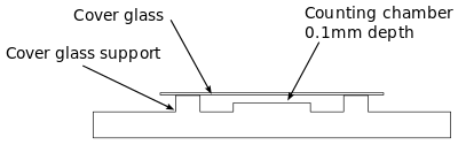
Live cells: translucent, no blue staining

Dead cells: blue because trypan blue goes to the cytoplasm

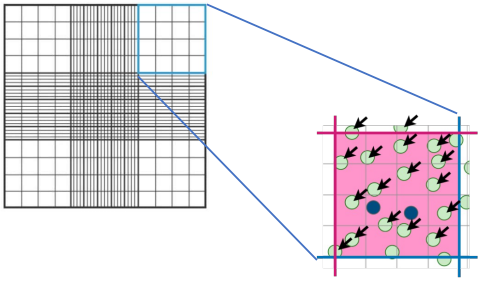


How to count the cell number using a hemocytometer?



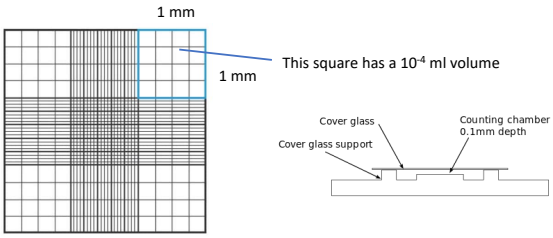


Under the microscope, you will see these squares



Arrows indicate live cells.
You need to count cells on the 2 border lines (red lines in this figure).
Do not count cells on the all 4 border lines (red and blue lines).
In this picture, there are 17 live cells (arrows) and 2 dead cells (blue).
The viability is 17/(17+2) x 100= 89.5%.

One square has a 10^{-4} ml volume



One square of this plate: **1 mm x 1 mm x 0.1 mm**

1 ml volume: **10 mm x 10 mm x 10 mm**

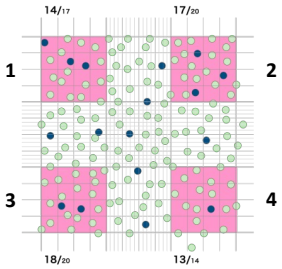
10-fold x 10-fold x 100-fold = 10,000-fold = $1x10^4$ cells/ml

Cell concentration = average number x 10^4 cells/ml
(keep this number in mind)

Count cell number in each of 4 squares

Example
Cell suspension in 5 ml
Take 10 ul of cells
Add 10 ul of trypan blue

Use 10 ul for cell counting



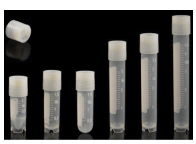
Total cell number
(17+20+20+14) / 4 squares x 2 (trypan blue dilution) =35.5 cells
35.5 x 10^4 = 3.55 x 10^5 cells/ml (cell concentration)
3.55 x 10^5 cells/ml x 5 ml = 1.78 x 10^6 cells (total number)

Viability
Live cells: (14+17+18+13) / 4 x 2 = 31 cells x 10^4 x 5 ml = 1.55 x 10^6 cells
1.55 x 10^6 cells / 1.78 x 10^6 cells x 100 = 87.1%

How to preserve cultured cells?

Collect cells from the dish with a trypsin-EDTA solution
Count cell numbers
Suspend 10^6-10^7 cells in 1-2 ml of the stock solution
(Stock solution: 10% dimethyl sulfoxide, 20% FBS in DMEM)

Aliquot cell suspension in a cryovial
Put the vial in a freezing container
Freeze cells slowly in -80°C freezer overnight



Add isopropanol

Storage of cells

Liquid Nitrogen Dewar

Liquid nitrogen (-196°C)

Liquid nitrogen increases its volume 800-fold when it becomes gas.

Never store liquid nitrogen in closed containers or tubes.

If liquid nitrogen is present in the closed tube, the tube will explode during thawing.

Liquid nitrogen Dewar
Store cell vials in liquid nitrogen

Need to add liquid nitrogen periodically

Mycoplasma might be contaminated from tubes to tubes via liquid nitrogen

Storage of cells

Air liquid nitrogen tank

Store cell vials in the very cool air generated by liquid nitrogen
Available on the 4th floor of the Central research laboratory
Need to add liquid nitrogen periodically
Free from mycoplasma contamination

Next

Observe cultured cells under the microscope

Remove cells using trypsin-EDTA

Count cell numbers

Passage to a new dish

Make a freezing stock

Take a frozen cell vial from a liquid nitrogen tank
Plate cells to a new dish

September 13, 2024, 17:00-18:20

Introduction of cell culture and passaging

Kinji Asahina, Futoshi Toyoda, Takefumi Yamamoto, Yasuhiro Mori, Tokio Terado, Sachiko Fukunaga, Kumi Okamoto (Central Research Laboratory)

Lecture (17:00-17:20)
Room 416, Central Research Laboratory

Practice (17:20-18:20)
Room 416, Central Research Laboratory

Split the attendees into two groups for the practice
Group A (English): Asahina
Group B (Japanese): Okamoto

Materials
Cells
COS-7 fibroblast-like cell line (obtained from Dr. Akio Shimizu)
(transformed by polyoma virus SV40 derived from African green monkey kidney)

Culture medium
DMEM high glucose
10% fetal bovine serum
Glutamine
Sodium pyruvate
Sodium bicarbonate ($NaHCO_3$)
Antibiotics

Freezing medium (x2 concentration)
DMEM high glucose
20% fetal bovine serum (10% final concentration)
20% dimethyl sulfoxide (DMSO) (10% final concentration)

Trypsin-EDTA solution
2.5 g/L trypsin
1 mM EDTA

Trypan blue solution
0.05% trypan blue

Note. General plate area, cell number, and medium volume in different plates/dishes

	Area	Medium volume	Plate number	Confluent number
96-well plate	0.3 cm^2	0.1 ml	$6.0x10^2$ - $3.0x10^3$	$5.0x10^4$
48-well plate	0.7 cm^2	0.2 ml	$1.4x10^3$ - $7.0x10^3$	$1.3x10^5$
24-well plate	2 cm^2	0.5 ml	$4.0x10^3$ - $2.0x10^4$	$2.5x10^5$
12-well plate	4 cm^2	1 ml	$8.0x10^3$ - $4.0x10^4$	$5.0x10^5$
6-well plate	10 cm^2	2 ml	$2.0x10^4$ - $1.0x10^5$	$1.2x10^6$
35 mm dish	10 cm^2	2 ml	$2.0x10^4$ - $1.0x10^5$	$1.2x10^6$
60 mm dish	20 cm^2	5 ml	$4.0x10^4$ - $2.0x10^5$	$2.5x10^6$
100 mm dish	60 cm^2	10 ml	$1.2x10^5$ - $6.0x10^5$	$7.5x10^6$

Procedures

1. Passaging

1. Take the 60 mm culture dish from the CO_2 incubator
2. Observe cells under the microscope
3. Move the dish into the culture hood
4. Aspirate the culture medium using a Pasteur pipette
5. Add 2 ml of PBS to the dish
6. Aspirate PBS
7. Add 1 ml of Trypsin-EDTA solution to the dish
8. Incubate the dish in the CO_2 incubator for 3 min
9. Check whether cells detach from the dish
10. Add 2 ml of the culture medium
11. Dissociate cells by gentle pipetting around 10 times
12. Aliquot 10 μl from the cell suspension
13. Add 10 μl of Trypan blue solution to cells and mix well
14. Apply 10 μl of cell suspension to a hemocytometer
15. Count the cell number
    The average cell number in a square x 10^4 cells / ml
16. Meanwhile, transfer cells to a 15 ml tube
17. Centrifuge the tube at 3,000 rpm for 3 min
18. Suspend the cell pellet in 1 ml of the medium
19. Plate 1 x 10^5 cells in 5 ml medium into a new 6 cm dish

2. Cryo-preservation

1. Transfer 500 μl of the cell suspension prepared in (1-18) into a cryotube
2. Add 500 μl of a freezing medium
3. Mix the cells gently
4. Put the tube into a freezing container
5. Keep the freezing container in -80°C freezer for 24 hrs
6. Transfer the freezing tube into the liquid nitrogen tank next day

3. Plating cells from freezing stock

1. Aliquot 10 ml of culture medium in a new 15 ml tube
2. Incubate the tube in the water bath at 37°C
3. Take a cryotube from the liquid nitrogen tank
4. Thaw the cryotube in the water bath at 37°C
5. Transfer cells from the cryotube to the pre-incubated medium
6. Centrifuge the 15 ml tube at 3,000 rpm for 3 min
7. Suspend the cell pellet in 1 ml of the culture medium
8. Count the cell number
9. Plate 1x10^5 cells in a new 6 cm dish