Introduction of Cell Culture (Lecture in English)
Cell culture and passaging (Practice in English/Japanese)

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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 2023 Intensive Course in Basic Science Fundamentals &
Multidisciplinary Seminars / Central Research Laboratory Special Seminars

Sep 15, 2023
15:30-16:00

Introduction of cell culture and passaging

細胞培養の基礎と細胞の継代方法

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Learning objectives

1. To learn how to culture cells.

2. To understand how to avoid contamination.

3. To understand how to passage cells in culture.

4. To learn how to store cells in liquid nitrogen.

Cell culture

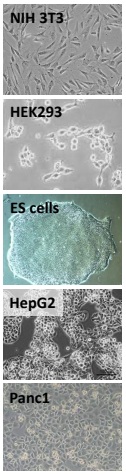
Adherent cells

Plastic dish (plate)

Cell lines

NIH 3T3 (mouse fibroblasts)
HEK293 (human embryonic kidney cells)
Embryonic stem cells, etc...

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



Cell lines

Pros

grow indefinitely
have a stable phenotype

Cons

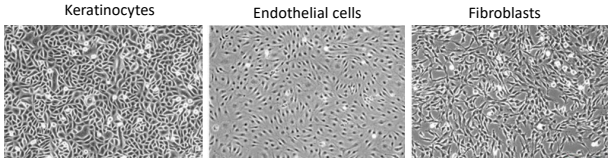
may not represent their original phenotype or function
may not be used to understand *in vivo* mechanism
may change their phenotypes by mutations

Buy cell lines

JCRB Cell Bank (Japan)
RIKEN BRC Cell Bank (Japan)
ATCC (USA)
Companies (Sigma, etc...)

Primary cells

Skin (keratinocytes, fibroblasts)
Brain (neuron, astrocytes)
Bone marrow (mesenchymal stem cells)
Liver (hepatocytes, hepatic stellate cells), etc...



Primary cells

Pros

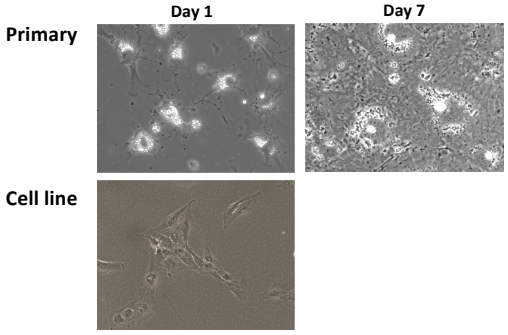
may keep the original phenotype in culture
may be used to understand the *in vivo* mechanism

Cons

are not easy to obtain from organs
may not survive in culture in a long-term
may decline their function rapidly in culture
may not be passaged many times

Primary cells vs cell lines

Hepatic stellate cells



Primary cells lose their morphology in culture.
Cell lines keep their morphology, but they look different from the primary one.

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...)

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...

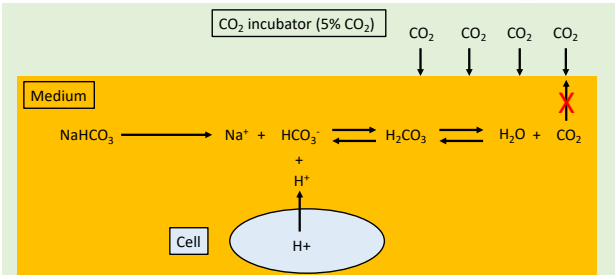
Vitamins
- Folic acid, Inositol, etc...

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

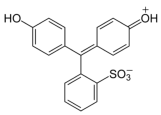
CO_2 incubator

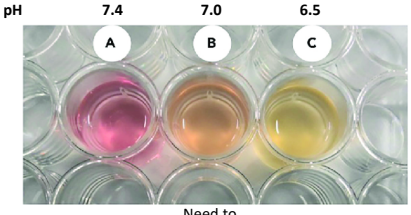
1. 37 °C temperature
2. 90-95% moisture (use water chamber)
3. 5% CO_2 for pH stabilization

Available on the 4th floor of the Central Research Laboratory



Phenol red





OK | Need to change the medium immediately | Too late

Serum for cell growth

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

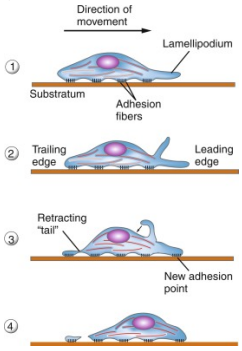
Fetal bovine serum (2-10%)
Horse serum (5%)

Heat-inactivation of fetal bovine serum (FBS)

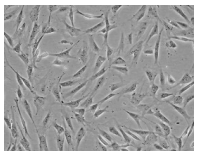
Inactivate the component system containing in FBS by incubation at 56 °C for 30 min

Cell adhesion on a culture dish

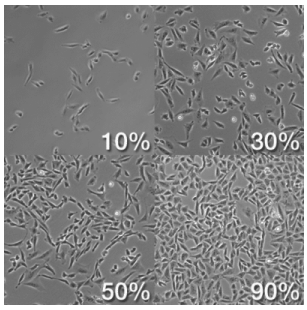
Epithelial cells and fibroblasts
Adhere the extracellular matrix on the dish via proteins, such as integrins.



NIH 3T3



Confluency

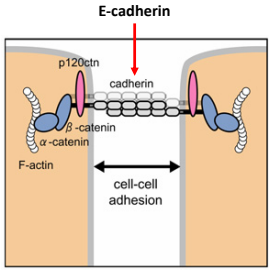


In general, cells grow well at a low confluency

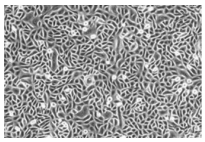
Cells at a high confluency do not grow and respond to cytokines by their cell-cell contact inhibition.

E-cadherin expressed on epithelial cells

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

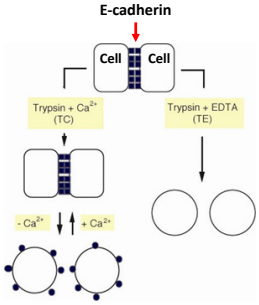


Keratinocytes



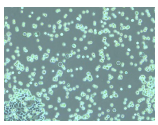
Passaging cells

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

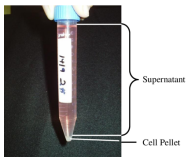


Passaging cells

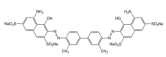
General procedure



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



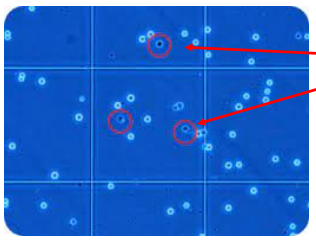
Trypan blue dye



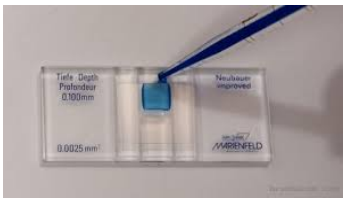
Add 10 ul of trypan blue solution to 10 ul of cell suspension
Apply 10 ul of the solution to a hemocytometer

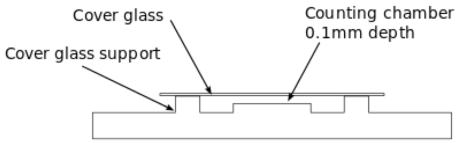
Live cells: translucent, no blue staining

Dead cells: blue because trypan blue goes to the cytoplasm

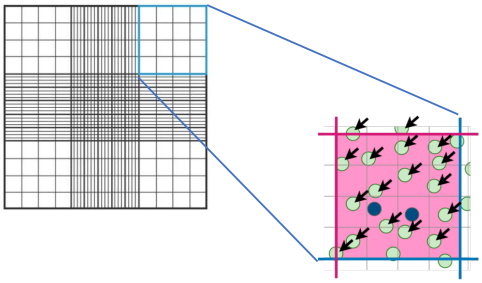


How to count the cell number using a hemocytometer?



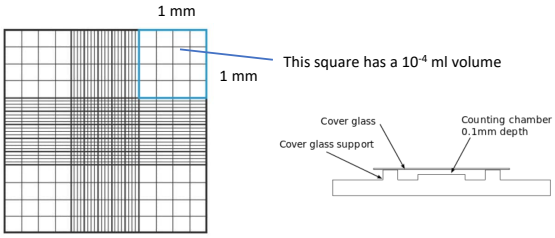


Under the microscope, you will see these squares



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**

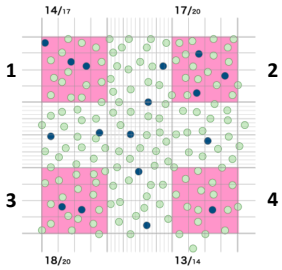
10-fold x 10-fold x 100-fold = 10,000-fold = $1x10^4$ cells/ml

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)

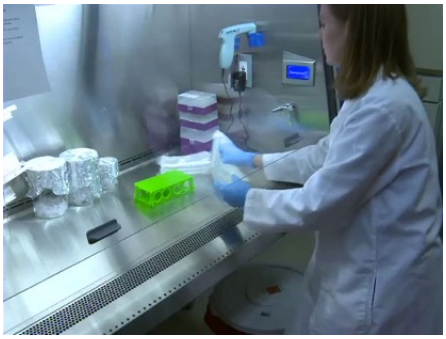
Viability
Live cells: 14+17+18+13 / 4 x 2 = 31 cells x 10^4 x 5 ml = 1.55 x 10^6 cells
1.55 x 10^6 cells / 1.78 x 10^6 cells x 100 = 87.1%

Aseptic techniques in cell culture

Cell culture hood

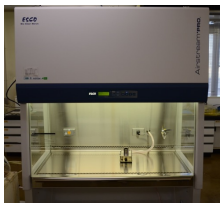
Gloves

70% Alcohol

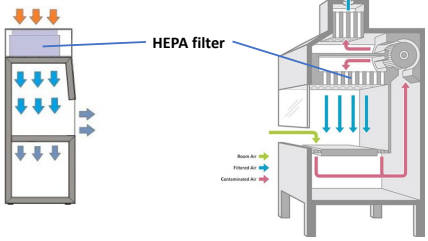


Cell culture hood

Available on the 4th floor of the Central Research Laboratory



Laminar flow hood **Biosafety cabinet**



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)

Antibiotics

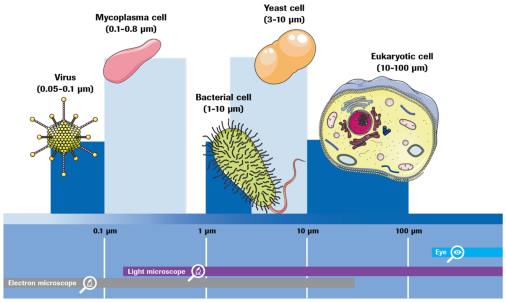
Anti-bacteria

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>

Mycoplasma



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

Buy disposable items that were already sterilized by γ-irradiation

Cobalt-60 (γ-ray, half life 5.27 years)

Sterilization techniques in cell culture

Autoclave
121 °C, 20 min

Buffer (PBS, etc...)

Pipet tips
Tubes (1.5 ml, 15 ml, 50 ml, etc...)

Pasteur pipettes
Glass plates
Cover glass
Surgery tools (forceps, etc...)

Dry heat sterilization

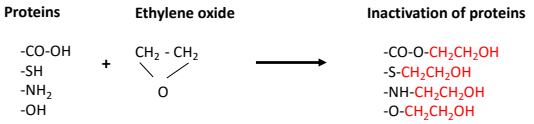
160 °C, 2hr
Pasteur pipet
Surgical tools (forceps, etc...)

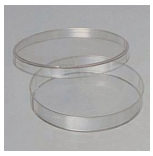
260 °C, 2hr
Surgical tools (forceps, etc...) for inactivation of bacterial endotoxin (LPS)

Available on the 4th floor of the Central Research Laboratory

Buy items that were sterilized with ethylene oxide gas

Ethylene oxide gas inactivates proteins by alkylation





Sterilization with ethylene oxide gas by yourself

Ethylene oxide gas is available on the 4th floor of the Central Research Laboratory

Ethylene oxide gas is toxic

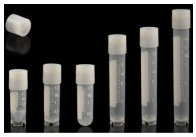
You need to ask the center staff for sterilization by ethylene oxide gas of heat-sensitive surgical tools, catheter, plastic equipment, lubber, etc..

After treatment, make sure the gas was gone

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

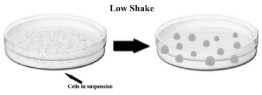
Free from mycoplasma contamination

Other cell culture techniques

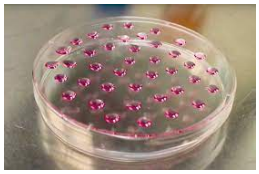
Floating cells

Suspension culture of floating cells

Anti-adhesive culture dish



Hanging drop culture

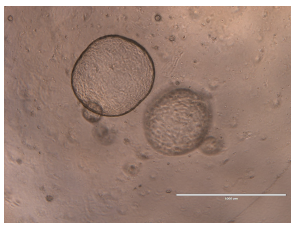


Asahina *et al*. **Genes Cells** 2004;9:1297-1308
Asahina *et al*. **Curr Stem Cell Res Ther** 2006;1:139-156

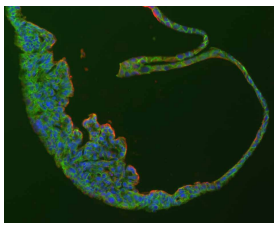
Other cell culture techniques

Organoid culture

EPCAM+ pancreatic cancer



KRT19 CDH1



Skrypek *et al*. **Pancreas** 2021;50:219-226
Asahina. **Biol Trace Elem Res** 2022;200:1667-1676

Next
Move to the 4th floor of the Cell Culture room (416)

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