Virus Cultivation Systems

In-vivo Systems

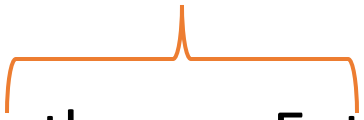
Animals

- Inoculation: The introduction of a virus or infective material into an organism by any of several routes.
- Infection: the multiplication of a virus or infectious material after being introduced naturally or artificially into a susceptible individual.
- Disease: It is a collection of abnormal findings in vital functions and behavior as a result of pathologies that occur after viral infection.
- Challenge: It is the re-inoculation of previously vaccinated individuals with virulent virus.

Postinoculation Infection Follow-up

Antemortem (before death)

- Fever
- Isolation of agents from secretions and excretions
- Antibody determination
- Monitoring clinical disease



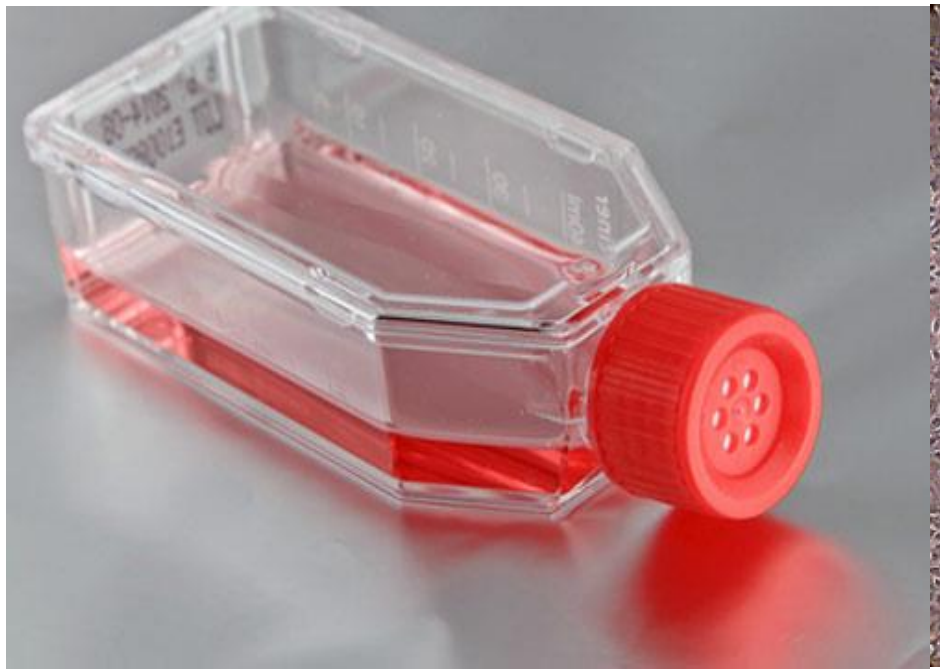
Natural death Euthanasie=Sacrifice

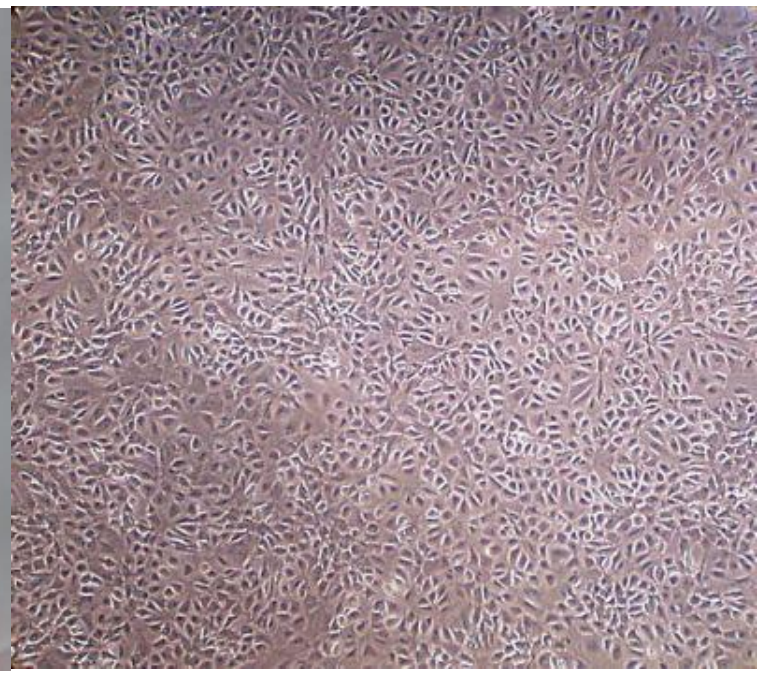
Postmortem (After death)

- Macro (necropsy) and micropathology

Virus Cultivation Systems

- In-vivo
  - Experimental animals
  - Embryonated eggs
- In-vitro
  - Cell and tissue cultures

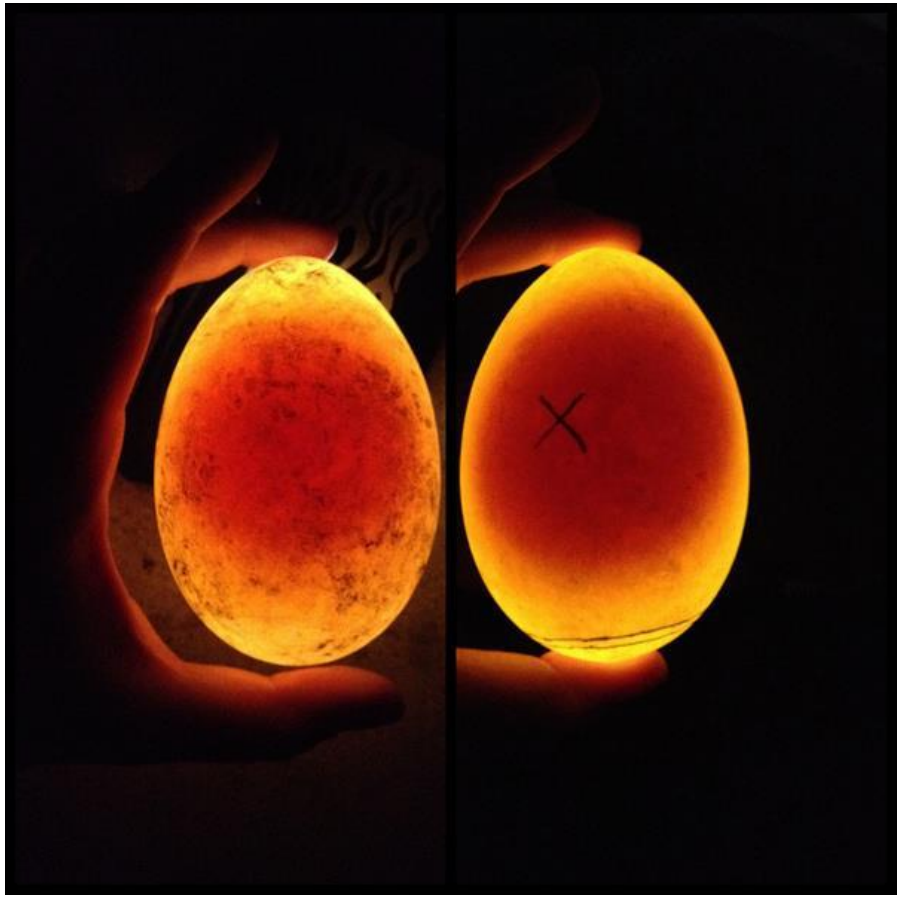




http://www.backyardchickens.com/t/662123/help-clueless-about-incubating-a-rescued-goose-egg-photos

Embryonated Eggs

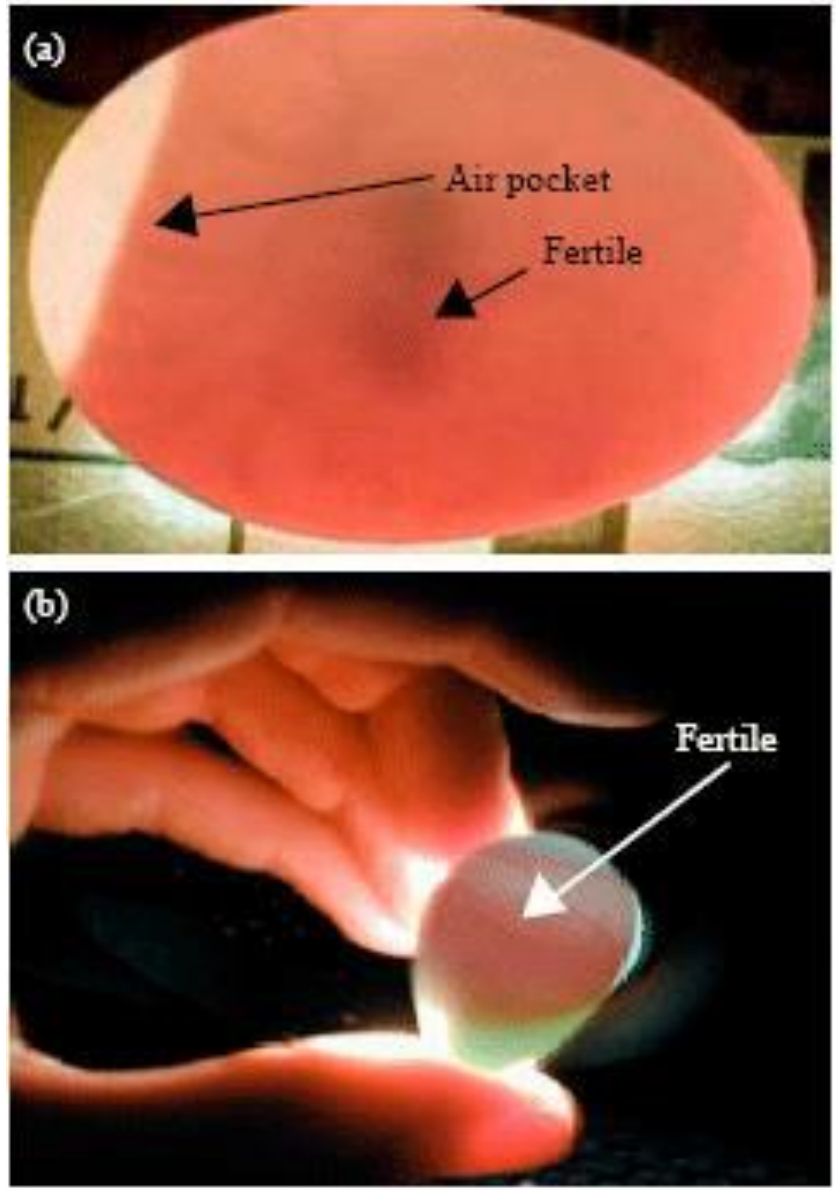
- Embryonated chicken eggs (ECE) are most commonly used.
- Duck eggs are also preferred from time to time.
- Although ECEs have remained in the background due to the widespread use of cell cultures today, they are still the ideal cultivation environment for some viruses. Such as
  - many avian viruses,
  - Bluetongue virus
  - Influenza A



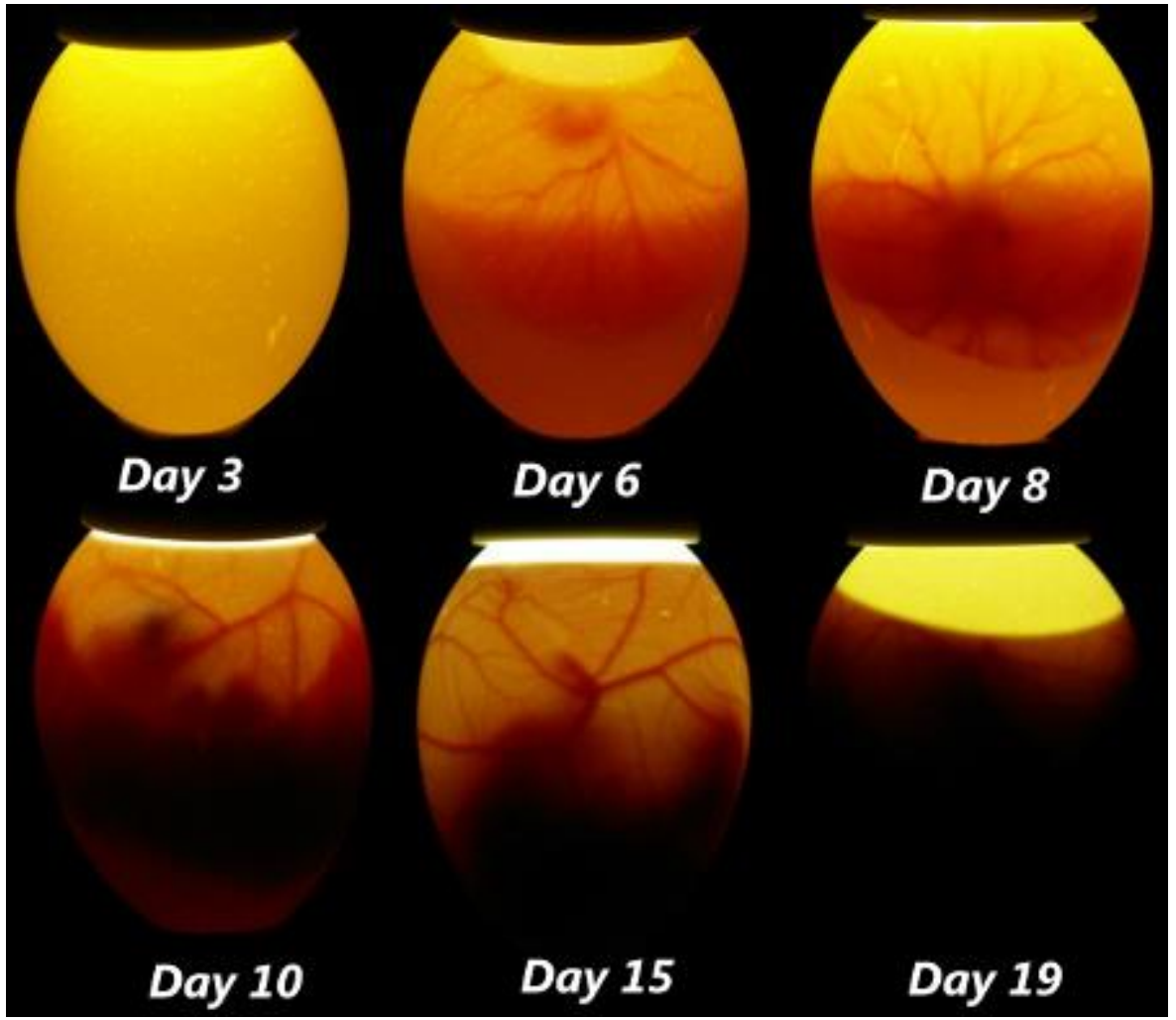
http://www.backyardchickens.com/t/662123/help-clueless-about-incubating-a-rescued-goose-egg-photos

- Egg Selection: Fertile eggs are taken from disease-free flocks. It is desired that they be white in color and weigh approximately 5-6 g.
- In order to be successful in ECE applications, hatcheries, where eggs are obtained;
  - –should be constantly monitored,
  - –should be free of important diseases,
  - –should have a high fertility rate.

- **Incubation:** Fertilized eggs taken from the coops are placed into an incubator at 35-37ºC, and 40-70% humidity for pre-development.
- Eggs are turned by hand or mechanically every day
- About a week later, they are taken to the dark room and checked for vitality. Viability checks are made every day during incubation.
- In this examination, the movement and veins of the embryo are observed under a strong light source.
  - Initially, the veins are only detected in a narrow area on the yolk. However, they increase in size every day, and the eyeball and limbs become more prominent as the embryo becomes more mobile.
  - Dead embryos remain still, and their veins appear pale gray and of a constant size. If opened, they emit a foul odour.



A. Farhangi, A. Akbarzadeh, M.R. Mehrabi, M. Chiani, Z. Saffari, S. Ghassemi, M. KheiriandR. Bashar, 2010. Safetyof Human TherapeuticMorphineVaccineEmployingLohmannSpecificPathogenFreeEggs.Pakistan Journalof BiologicalSciences, 13: 1047-1051.



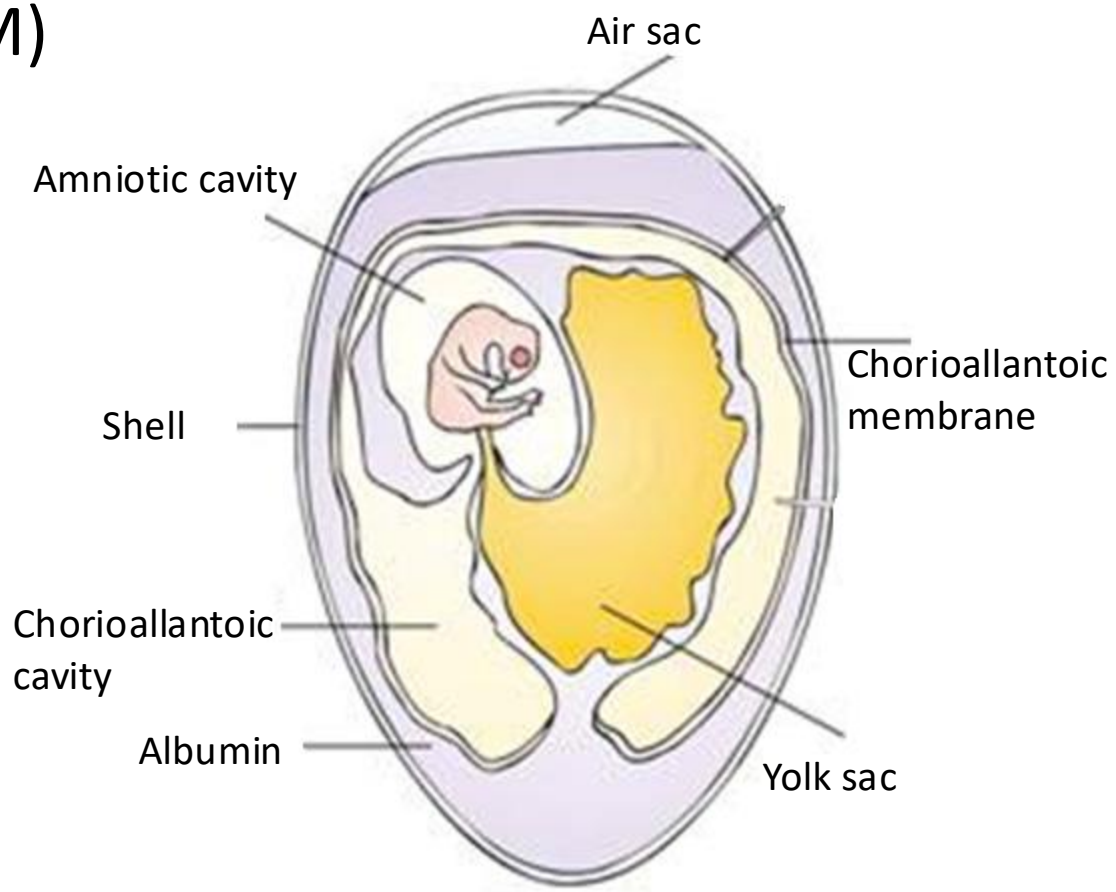
http://incubatorwarehouse.com/egg-candling

- Under light, the immobile and dark mass of the embryo indicates its dead.
- Live embryos are put back in the incubator for proper use.
- The inoculation process should be done immediately after the markings, before changing the location of the embryo.

- Inoculation: It can be done directly to the embryo (iv, sc, etc.) or to accessory egg components (CAM, CAC, AC, YS).
- Incubation and Daily Monitoring: Follow the previously stated protocol. Embryo mortality is monitored as an indicator of viral replication. Mortality occurring within the first 48 hours is attributed to procedural errors.
- Follow-up of Infection: To check for virus growth, fluid and tissue samples from the deceased or euthanized embryo are examined.
  - **Chorio-allantoic membrane (CAM)** Local vascularization, opacity, pox formation
  - **Chorio-allantoic cavity (CAC)** Hemagglutination from fluid
  - **Amniotic cavity (AC)** Hemagglutination from fluid
  - **Yolk sac (YS)** The yolk sac membrane is stained, and inclusion bodies are scanned.

Areas Frequently Used for Virus Inoculation to ECE

1. Chorio-allantoic membrane (CAM)
2. Chorio-allantoic cavity (CAC)
3. Amniotic cavity (AC)
4. Yolk sac (YS)



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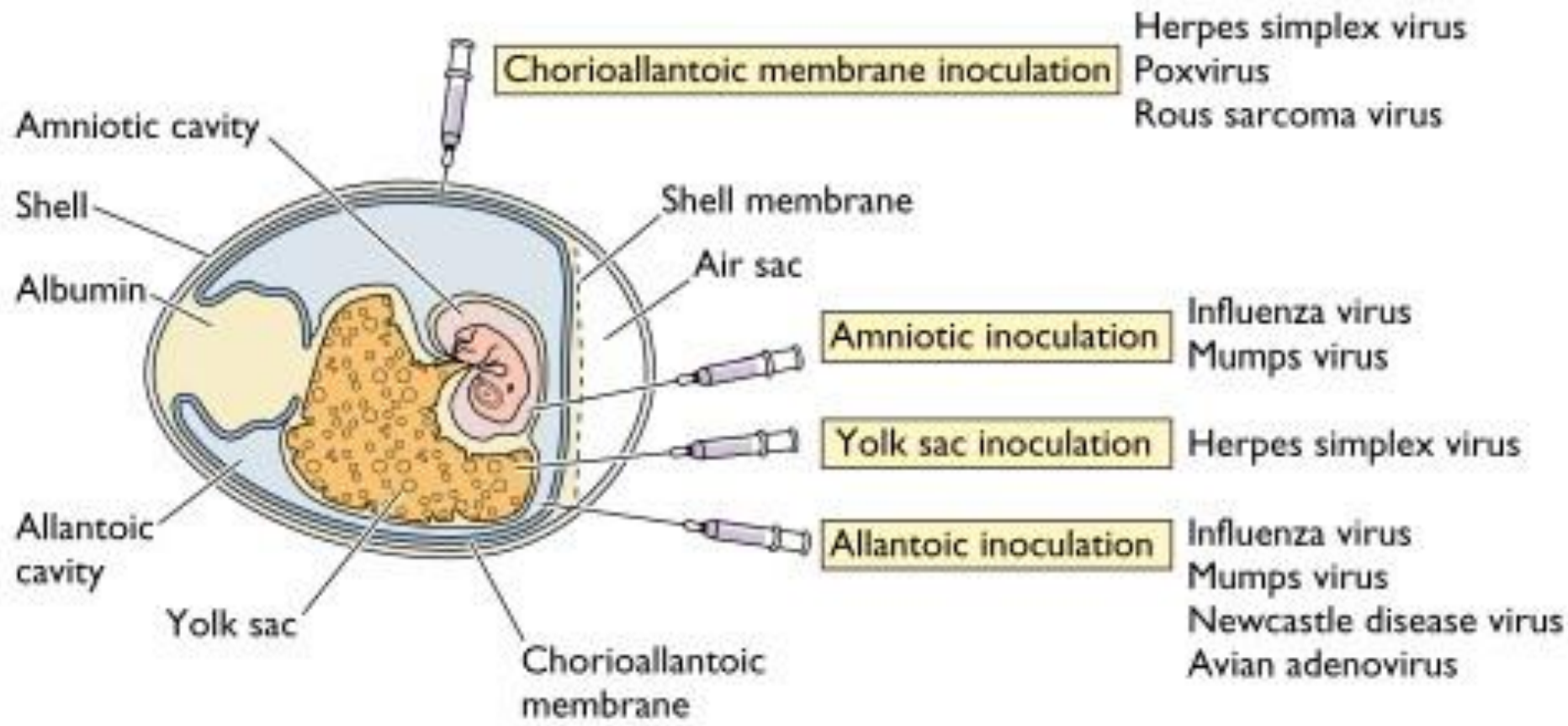
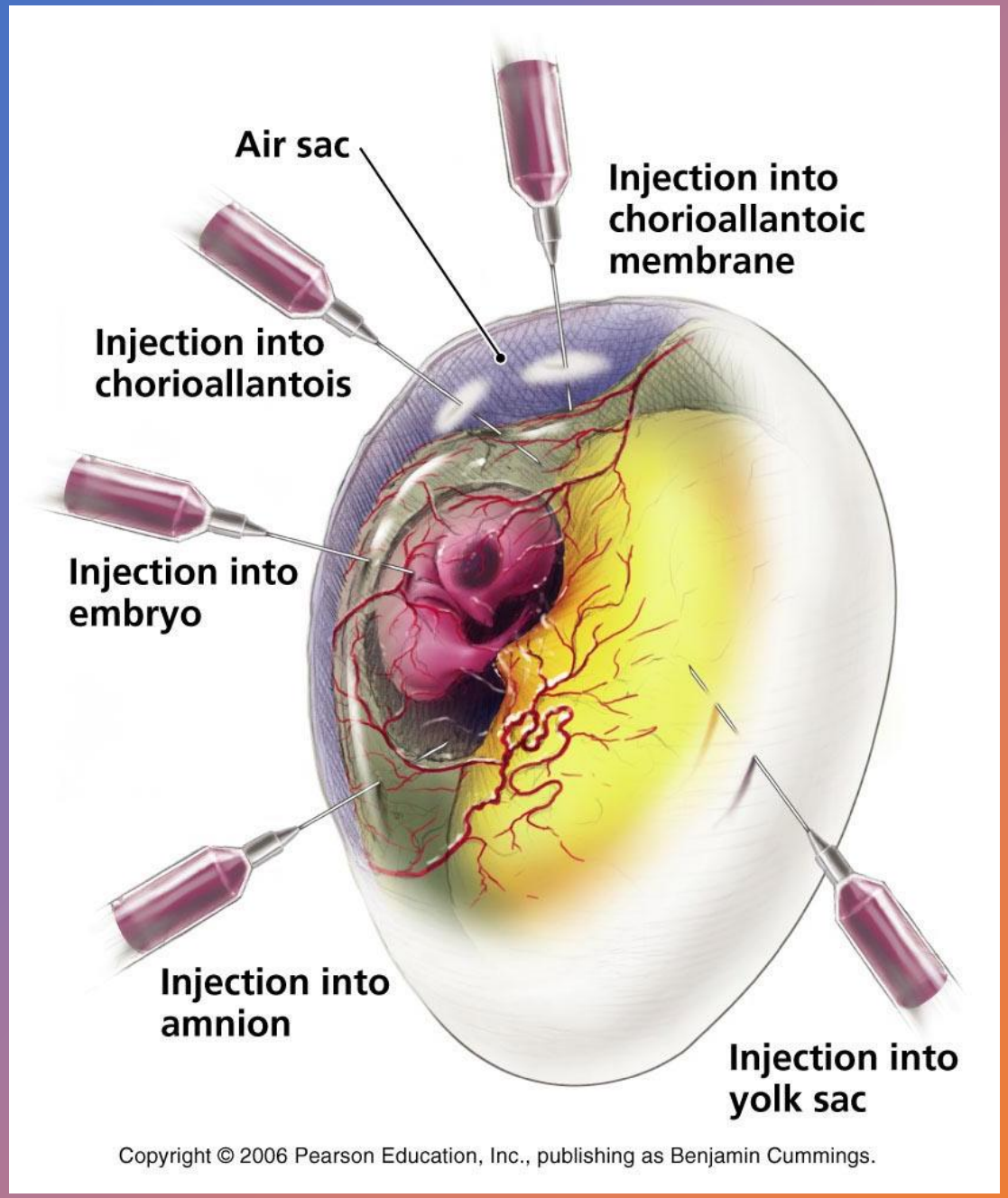


http://www.virology.ws/2009/12/10/influenza-virus-growth-in-eggs/



Apart from these regions, implantation can also be made to embryonal vessels on the chorio-allantoic membrane and directly to the embryo. The virus replicates in the membrane cells that form these sacs, and mature virions accumulate in the sacs.

INFERTILE
- No development.

DAY 1
- Appearance of tissue development.

DAY 2
- Tissue development very visible.
- Appearance of blood vessels.

DAY 3
- Heart beats.
- Blood vessels very visible.

DAY 4
- Eye pigmented.

DAY 5
- Appearance of elbows and knees.

DAY 6
- Appearance of beak.
- Voluntary movements begin.

DAY 7
- Comb growth begins.
- Egg tooth begins to appear.

DAY 8
- Feather tracts seen.
- Upper and lower beak equal in length.

DAY 9
- Embryo starts to look bird-like.
- Mouth opening appears.

DAY 10
- Egg tooth prominent.
- Toe nails.

DAY 11
- Comb serrated.
- Tail feathers apparent.

DAY 12
- Toes fully formed.
- First few visible feathers.

DAY 13
- Appearance of scales.
- Body covered lightly with feathers.

DAY 14
- Embryo turns head towards large end of egg.

DAY 15
- Gut is drawn into abdominal cavity.

DAY 16
- Feathers cover complete body.
- Albumen nearly gone.

DAY 17
- Amniotic fluid decreases.
- Head is between legs.

DAY 18
- Growth of embryo nearly complete.
- Yolk sac is still on outside of embryo.
- Head is under the right wing

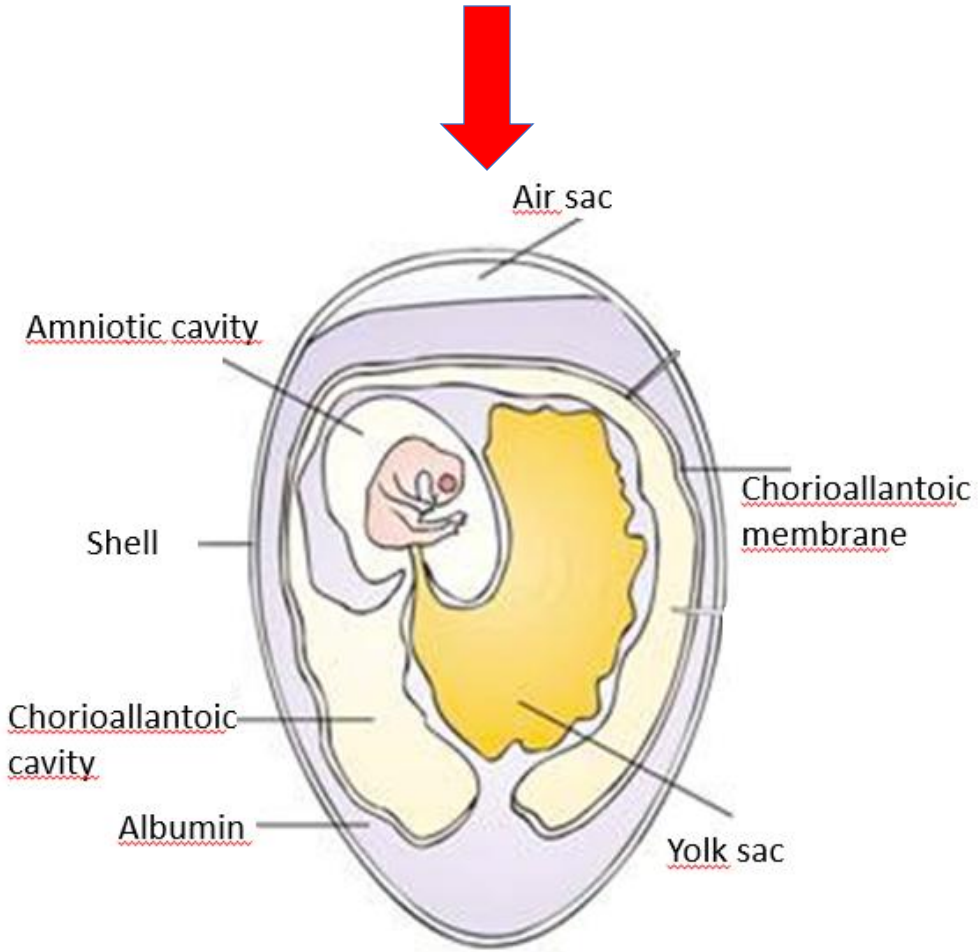
DAY 19
- Yolk sac draws into body cavity.
- Amniotic fluid gone.
- Embryo occupies most of space within egg (not in the air cell).

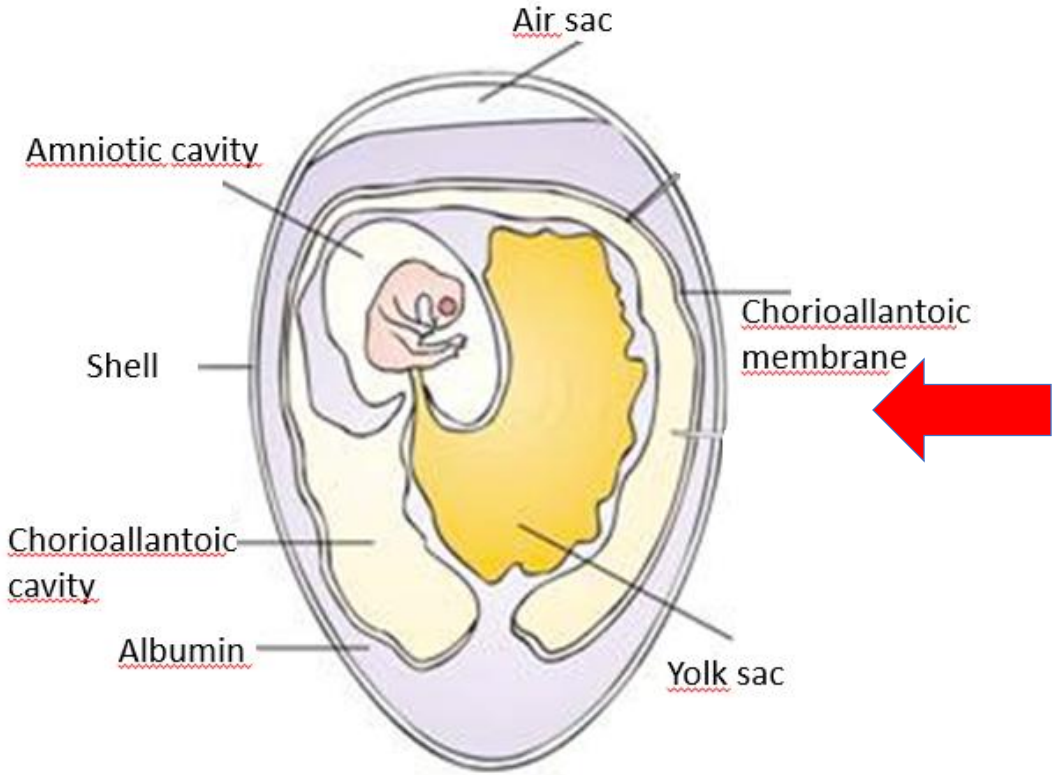
DAY 20
- Yolk sac drawn completely into body.
- Embryo becomes a chick (breathing in air cell).
- Internal and external pip.

http://www.riverbendfarmtasmania.com/incubation-info

Routes of inoculation:

1. from the side of the air sac





2. from the side

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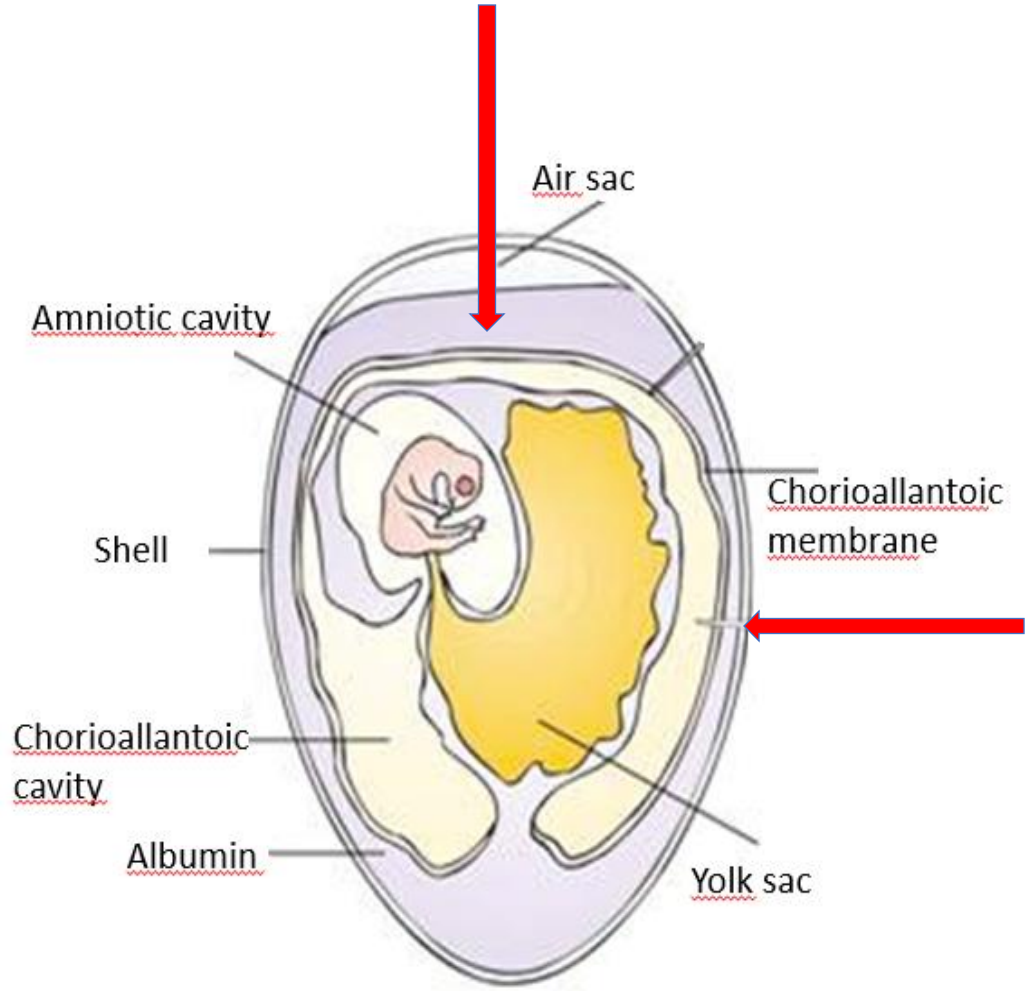
1. Inoculation to Chorioallantoic membrane (CAM)

- 10-12 days old embryo
- Poxvirus and some epitheliotropic virus cultivation



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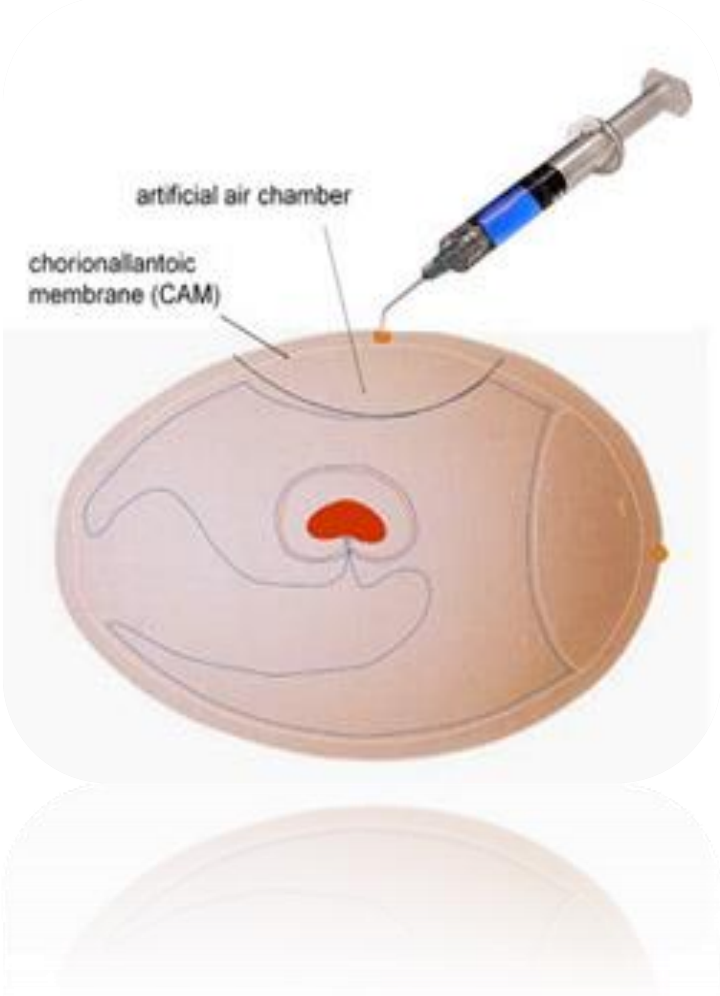
a. from the side of the air sac

- mark the position of air cell.
- Drill a small hole through the eggshell just above the air cell.
- Open a bigger hole by scissors and forceps
- Through the shell, which opens in the form of a window, and a drop of virus suspension is dropped on the membrane.
- The areas under the drop are scratched with the syringe tip (scarification).
- Seal the holes and keep eggs positioned horizontally for a few hours. Eggs are usually incubated for 5-7 days.

Amie J Eisfeld, Gabriele Neumann , Yoshihiro Kawaoka, (2014) Nature Protocols 9, 2663–2681

b. From the side

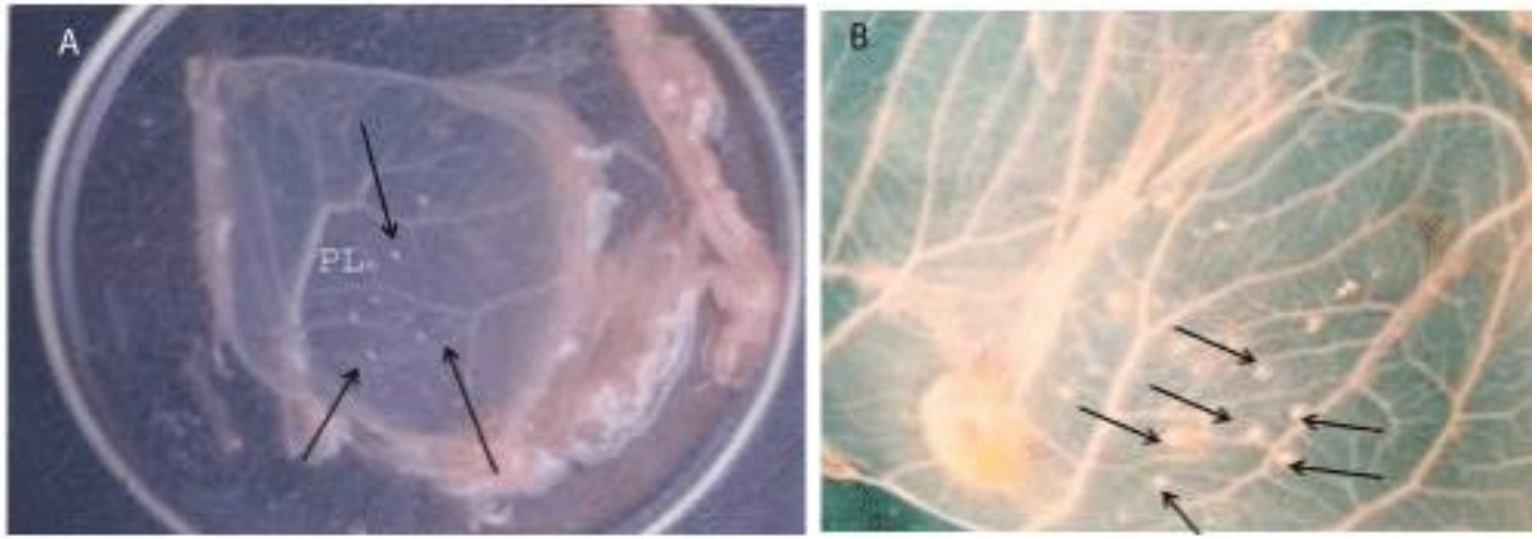
- mark the position of air cell.
- Drill a small hole through the eggshell just above the air cell.
- Place eggs horizontally on the egg flat and disinfect the eggshell.
- Drill a second hole on the side of egg in area devoid from blood vessels or between large blood vessels.
- Apply gentle vacuum to the hole in the air cell, this will cause the CAM to drop, thus forming a new false air cell directly over CAM.
- Open a bigger hole by scissors and forceps
- Through the shell, which opens in the form of a window, and a drop of virus suspension is dropped on the membrane.
- The areas under the drop are scratched with the syringe tip (scarification).
- Seal the holes and keep eggs positioned horizontally for a few hours. Eggs are usually incubated for 5-7 days.



https://slideplayer.com/slide/14474145/

Evaluation

- The deaths in the first 48 hours should be discarded as nonspecific or traumatic death due to inoculation.
- Death and pathological changes occurring in the later period are because of the virus.
- The shell on the air sac is removed and the membrane is taken into the petri dish.
- After washing with PBS, thickening and pox nodules are sought on the membrane.
- It is evaluated by looking at the control and making a comparison.



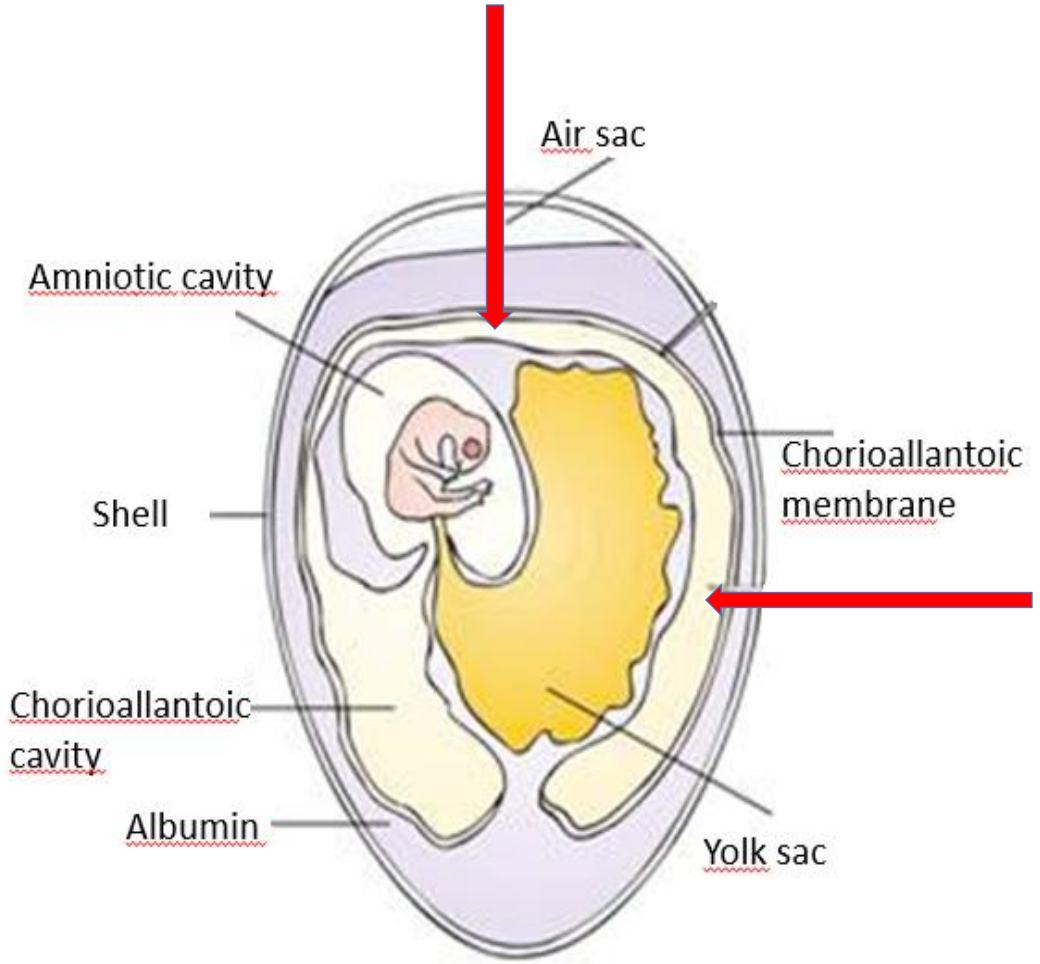
Chorio-allantoic membranes of the ECE inoculated with ORF virus produced the characteristic pock lesions (black arrows). **(A)** very small pock lesion, **(B)** well developed pock lesion

https://www.omicsonline.org/articles-images/JAA-06-154-g004.html

Nagasse-Sugahara TK, Kisielius JJ, Ueda-Ito M, Curti SP, Figueiredo CA, Cruz AS, Silva MM, Ramos CH, Silva MC, Sakurai T, Salles-Gomes LF (2004). Human vaccinia-like virus outbreaks in São Paulo and Goiás States, Brazil: virus detection, isolation and identification. Rev Inst Med Trop Sao Paulo. 46(6):315-22.

2. Inoculation to Chorioallantoic cavity

- It is routinely used in the production of Newcastle virus.
- 9-11 days old eggs are preferred.



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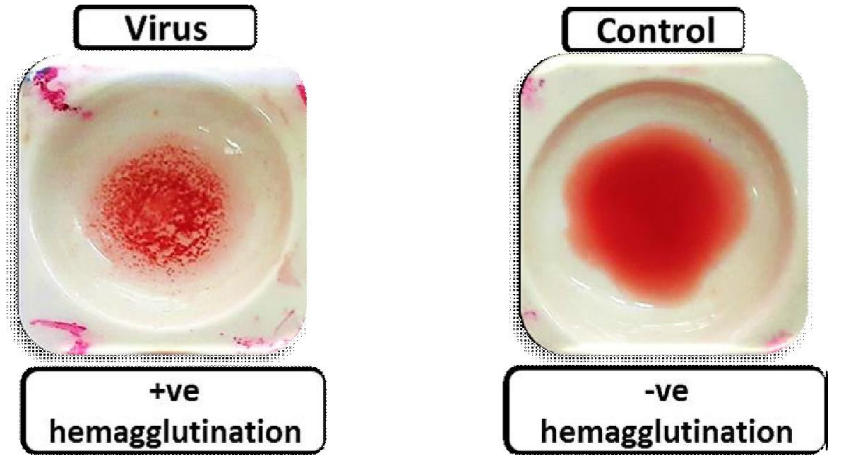
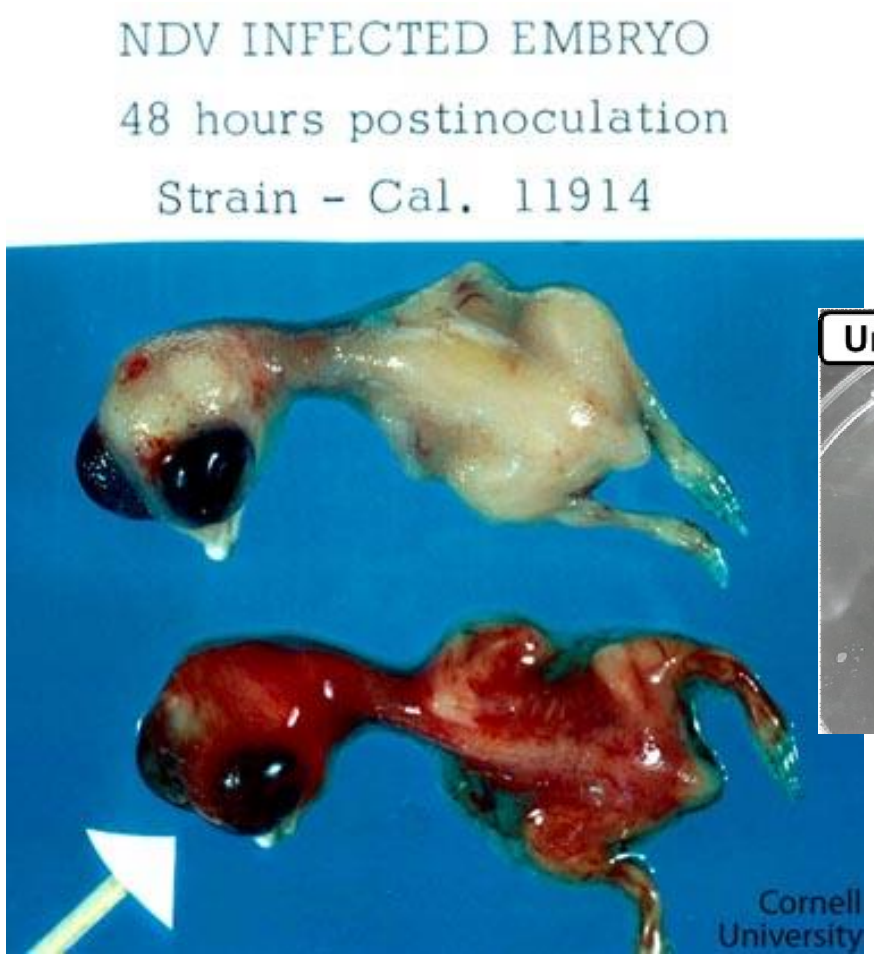
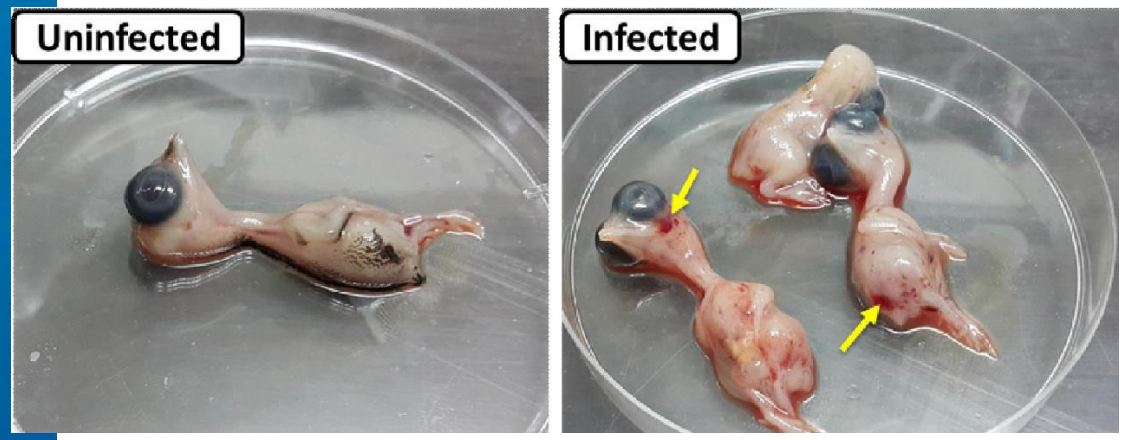
Evaluation

- The deaths in the first 48 hours should be discarded as nonspecific or traumatic death due to inoculation.
- Death and pathological changes occurring in the later period are because of the virus.
- If the embryo has not died during this period, it is kept for 2-4 hours at 4 ° C to die.
- Hemagglutination test is performed after the chorio-allatoic fluid is taken into a sterile tube.
- A positive result indicates that the virus has replicates.



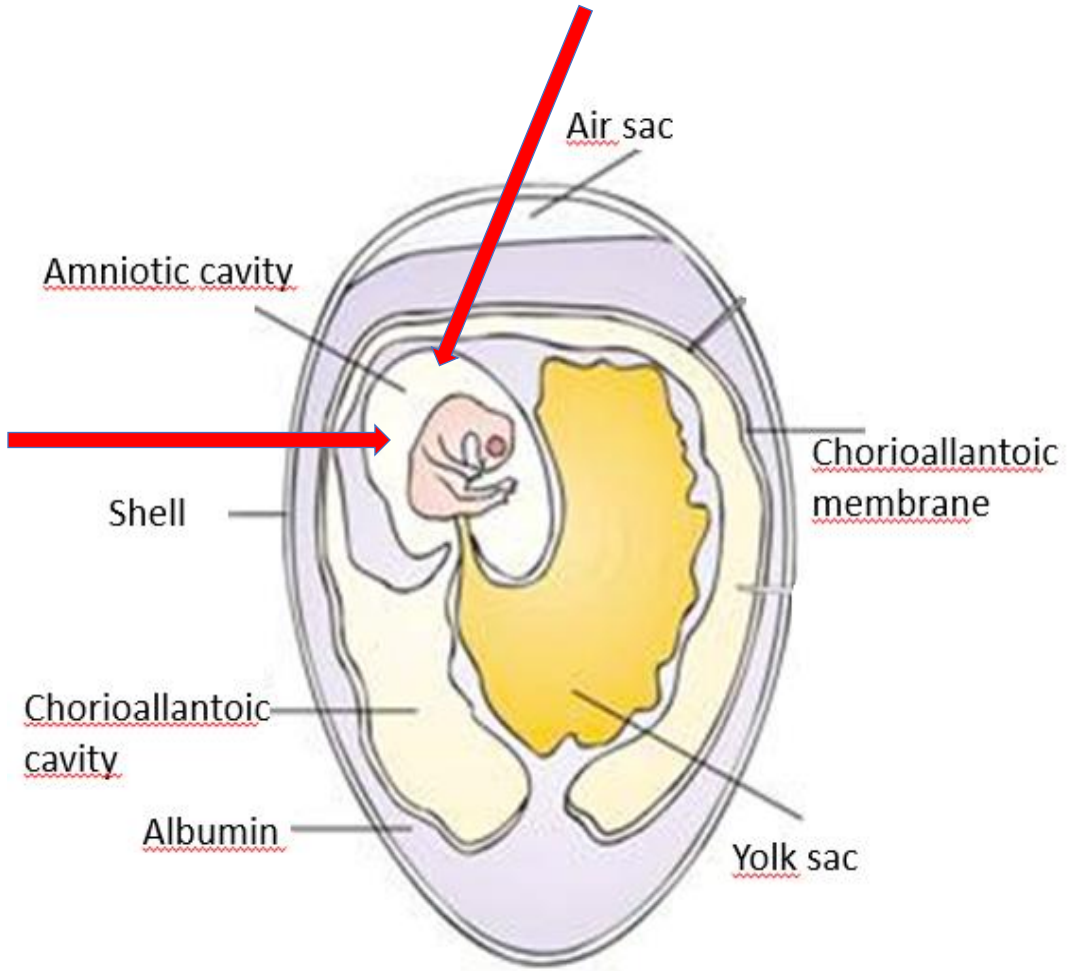




http://www.poultrydisease.ir/Atlases/avian-atlas/search/lesion/661.html

3. Inoculation to Amniotic cavity (AC)

- Measles, Mumps and Influenza viruses
- The embryo is large;
- 12-14 days old eggs



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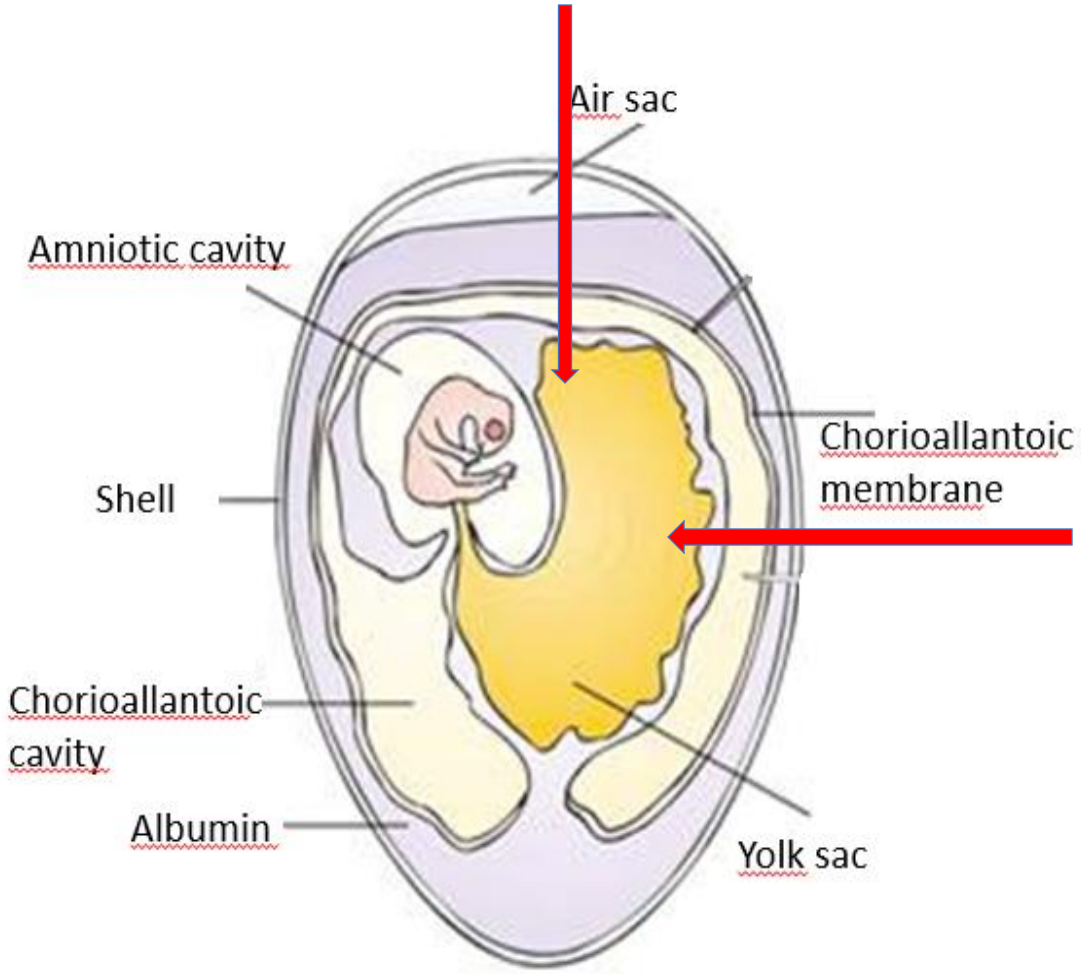
- Hemagglutination test is performed after the amniotic fluid is taken into a sterile tube.
- A positive result indicates that the virus has replicates.

4. Inoculation to Yolk Sac

- Bluetongue virus also Equine Herpesvirus and rabies virus
- When the yellow sac is largest, 6-8 days old eggs



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Evaluation

- The deaths in the first 48 hours should be discarded as nonspecific or traumatic death due to inoculation.
- Death and pathological changes occurring in the later period are because of the virus.
- The egg is opened and the yolk sac is removed.
- After the membrane is washed with PBS, they are placed on a slide and stained.
- Then, Inclusion bodies are searched under the microscope.

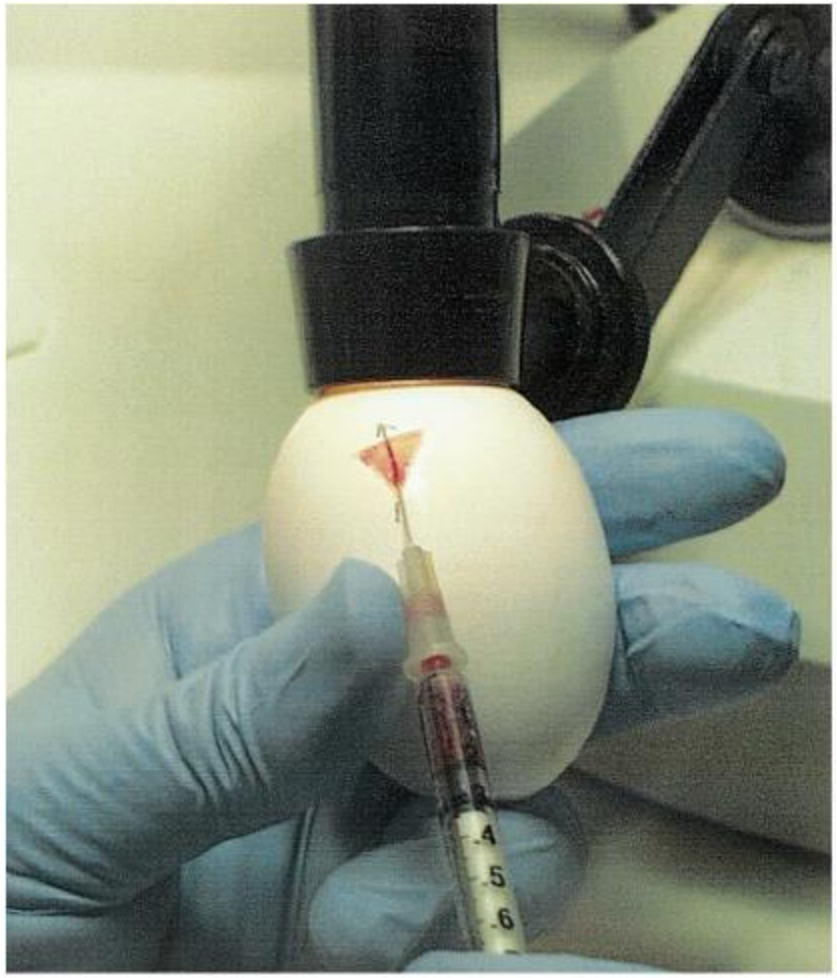


Fig. 1

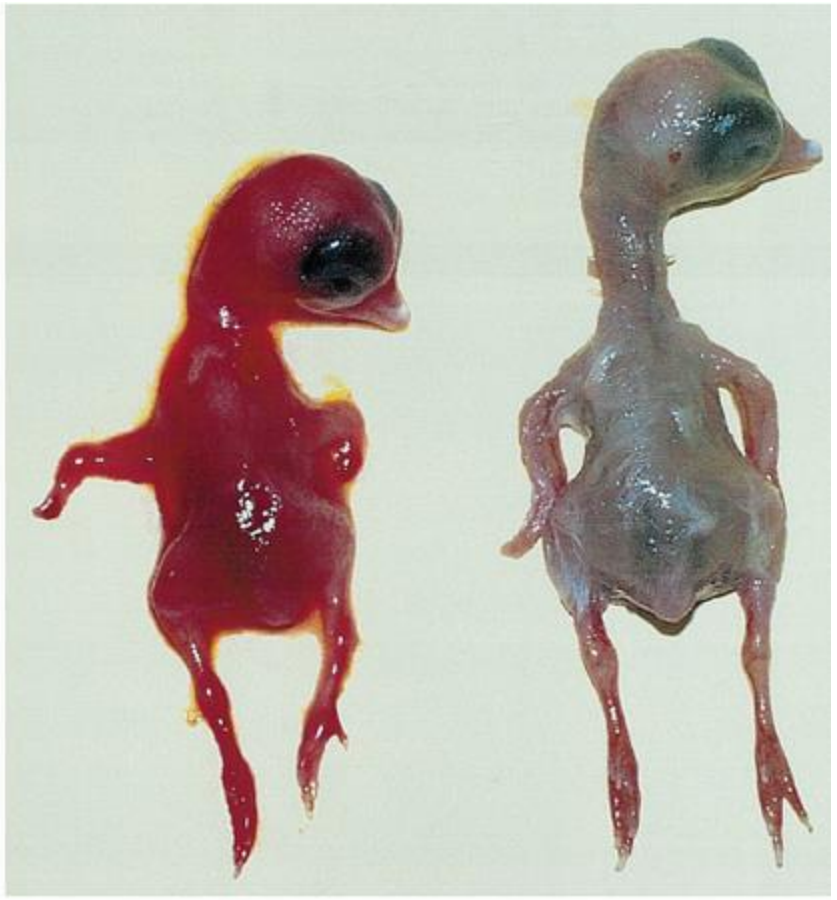


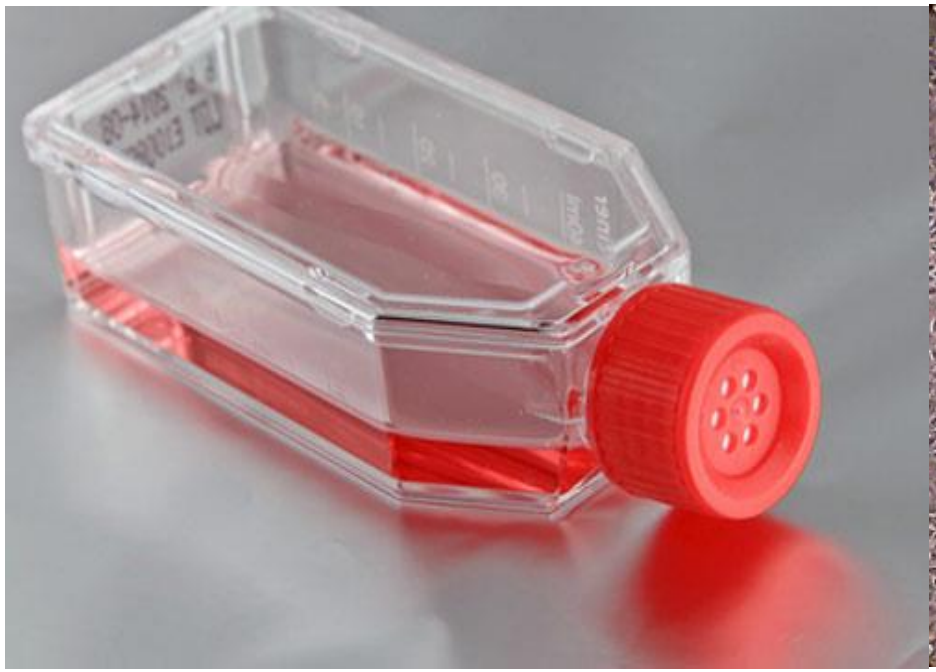
Fig. 2

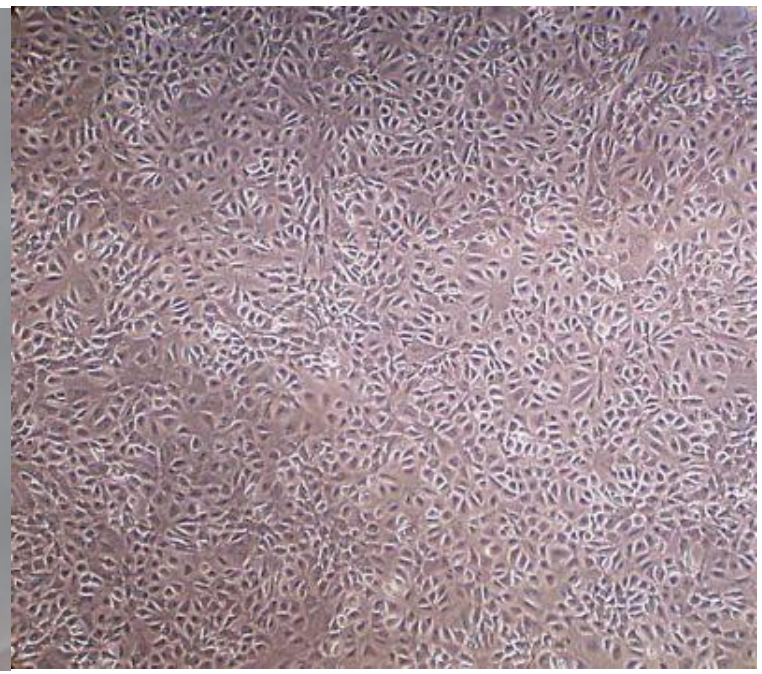
Clavijo, A., Heckert, R. A., Dulac, G. C., & Afshar, A. (2000). Isolation and identification of bluetongue virus. *Journal of virological methods*, *87*(1), 13-23.

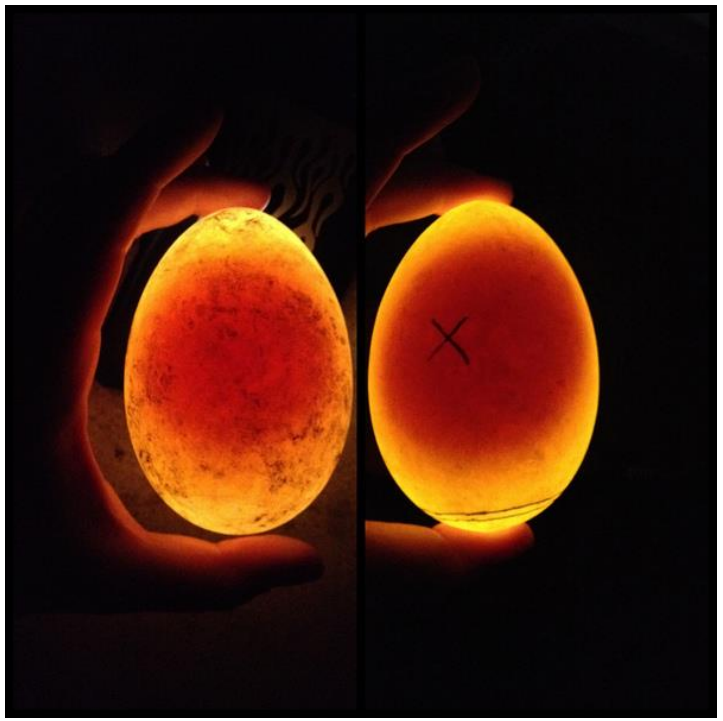
- Fig 2. Bluetongue infected embryo (left). Following embryo inoculation, the embryo usually dies within 3-6 days and multiple hemorrhage and edema are observed.

Virus Cultivation Systems

- In-vivo
  - Experimental animals
  - Embryonated eggs
- In-vitro
  - Cell and tissue cultures







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Organ culture: It is the preservation of a certain part or all of the organs in-vitro, provided that their function and structure are maintaned.

Tissue Culture: It is the storage and production of tissues in-vitro without disrupting their function and structure.

Cell Culture: The production of cells under in-vitro conditions by multiplication from a single cell. Cells never exhibit an organization leading to tissue.

In-Vitro Systems

Cell cultures

Definitions

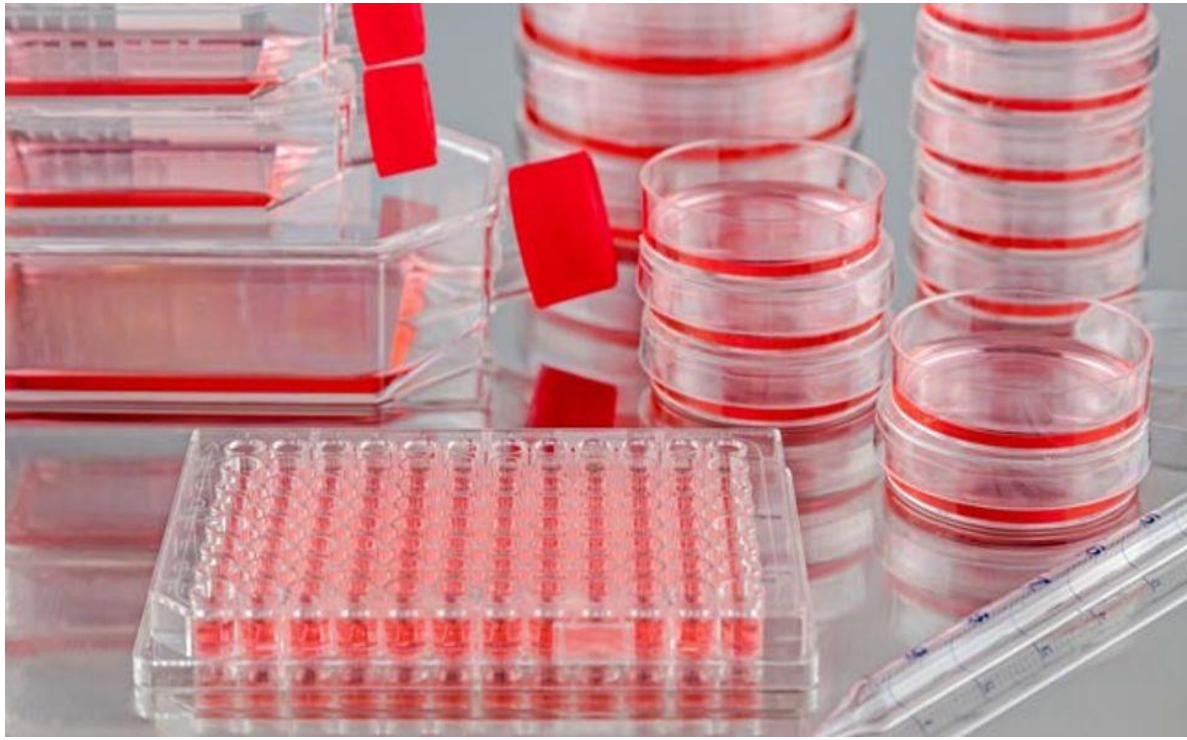
- Primary Cell Culture: It is the form in which cells are first adapted to in vitro conditions from the tissue or organ from which they originate. This definition is valid until the first subculture is made. They are generally mortal (finite) cells.
- Permanent cell culture: It refers to a type of cell culture that has the ability to multiply indefinitely and can be subcultured. These cells are karyotypically differentiated from the tissue or cells from which they originate.
- Diploid cell cultures: They are obtained from subcultures of primary cultures. It is important to note that karyotypic features may differ to a certain extent (20-30%) from the tissue of origin.
- Fibroblast Cell Culture: These cells originate from embryonic tissues and exhibit spindle-like morphology.
- Subculture: It refers to the transfer or transplantation of cells that have completed their reproduction in a culture medium to another culture medium, with or without dilution.

Cell Culture Environment

- Cell culture flasks
  - Glass (recycling)
  - Plastic (disposable)

Sterile
Neutral

- Cell production media
  - Sterile
  - Nutritious
  - Isotonic
  - Neutral pH
  - Body temperature (depends on cell type)



Medium (MEM, DMEM, etc.)

+

Serum (fetal calf/bovine serum, horse serum etc.)

=

The cell culture medium

- The cell culture medium is added to as 10% of the flask volume and contains inactive calf serum at a rate varying between 5-20%.

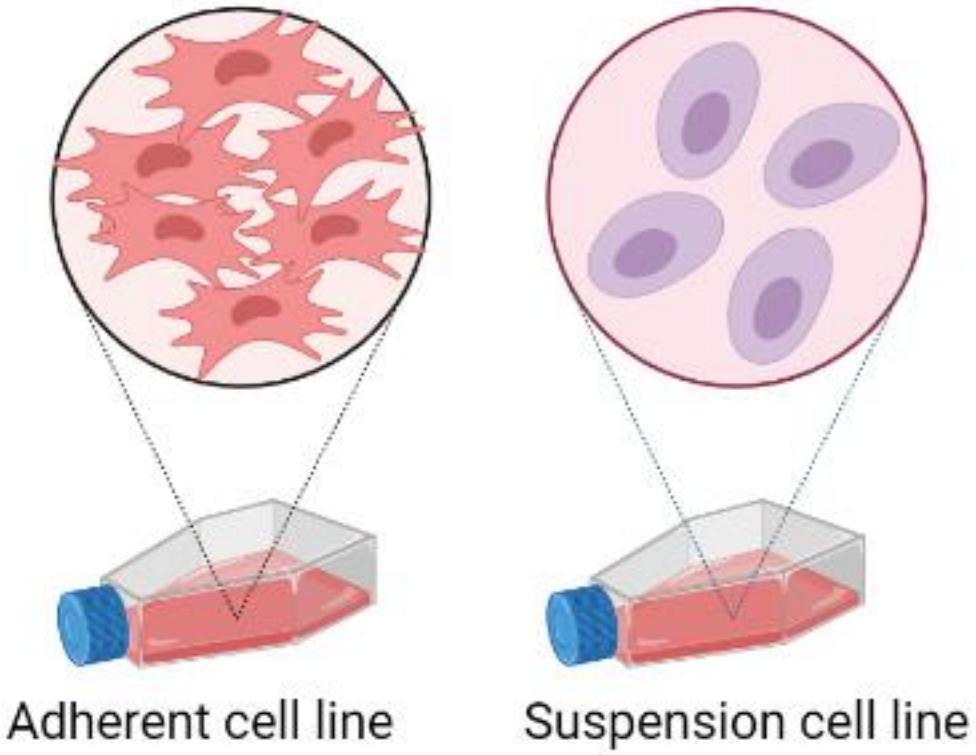
The functon of the cell medium,

- Nutrition source for the cell it contains,
- Stimulant and mineral source for cell functions,
- Providing the homeostatic environment like in vivo conditions
- Waste site for metabolites,

The function of the serum,

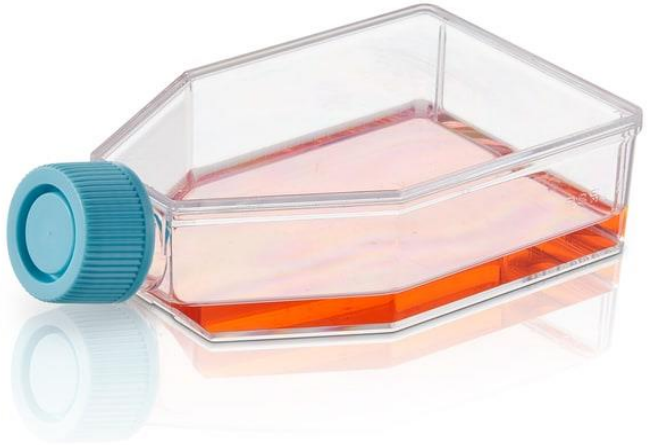
- regulates pH,
- Includes adhesion and bonding factors (fetin protein),
- Contains hormones, lipids, and minerals for the culture of cells

- Cell cultures are divided into two groups: Adherent and Suspension cultures, depending on the environment in which they are cultured (relationship with the surface of the flasks).

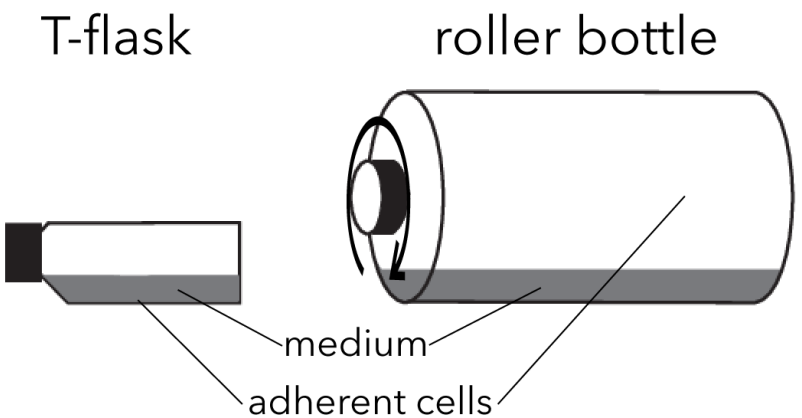


https://lab-label.com/blogs/main/cellculture-essential-methods

- 1. Adherent cells: types of cell lines that grow in the monolayer attached to the surface.
  - ➢Stationary Cultures: They can be incubated on incubator shelves
  - ➢Roller Cultures: in a rotating system with a special mechanism.



https://www.fishersci.ca/shop/products/biolite-cell-culture-treated-flasks/p-4524967



https://pharsol.com/knowledge-hub/blog/scaling-up-or-out

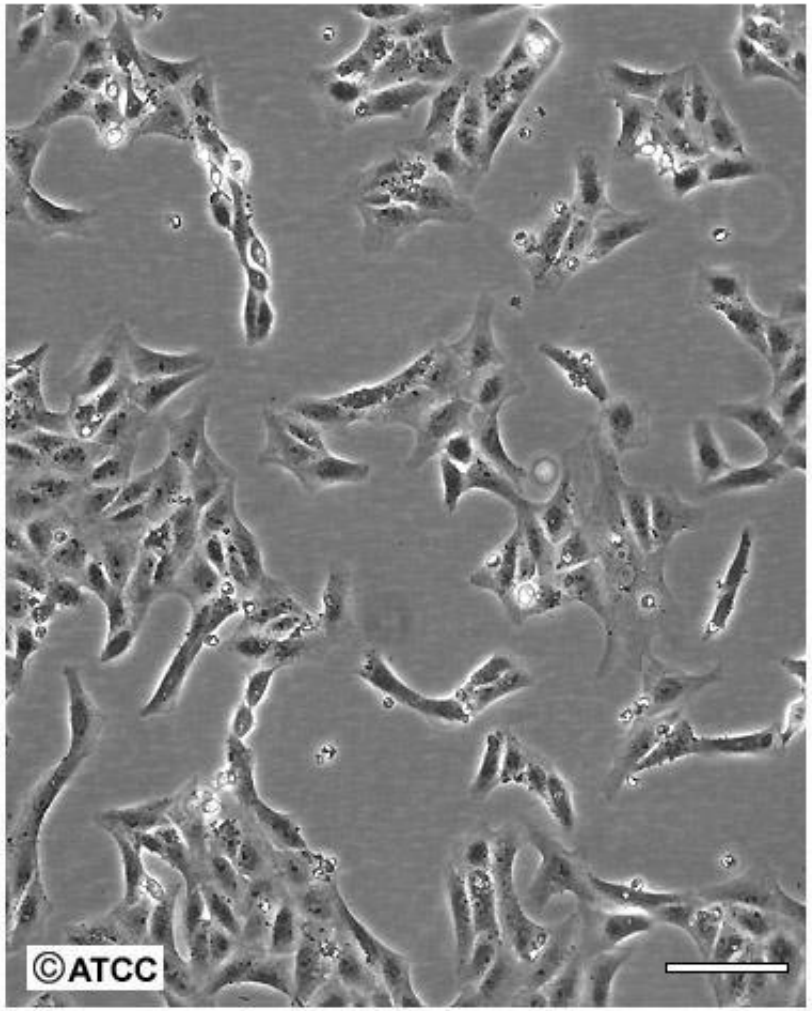
https://pfee.de/cellroll/?lang=en

- These kinds of cells, will only grow and survive in culture when attached to a surface such as glass or plastic.
- The flasks in which such cells grow are specifically treated to allow cell adhesion.
- The cells grow in a monolayer until they reach a 100% confluence (completely covering the flask surface), after which they usually stop proliferating.
- While passaged a detaching agent (e.g., trypsin) needs to be used to detach them from the surface.
- They re-attach to the surface within a few hours upon passaging.

https://lab-label.com/blogs/main/cellculture-essential-methods

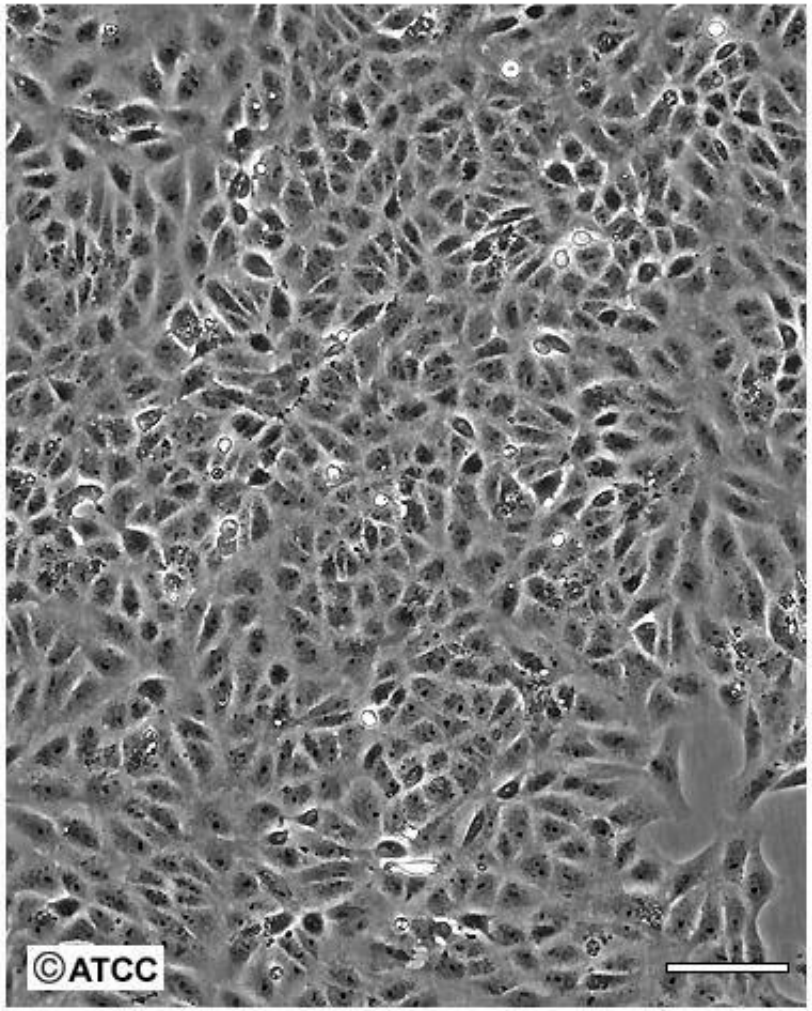
Vero- African green monkey kidney

ATCC Number: **CCL-81**
Designation: **Vero**



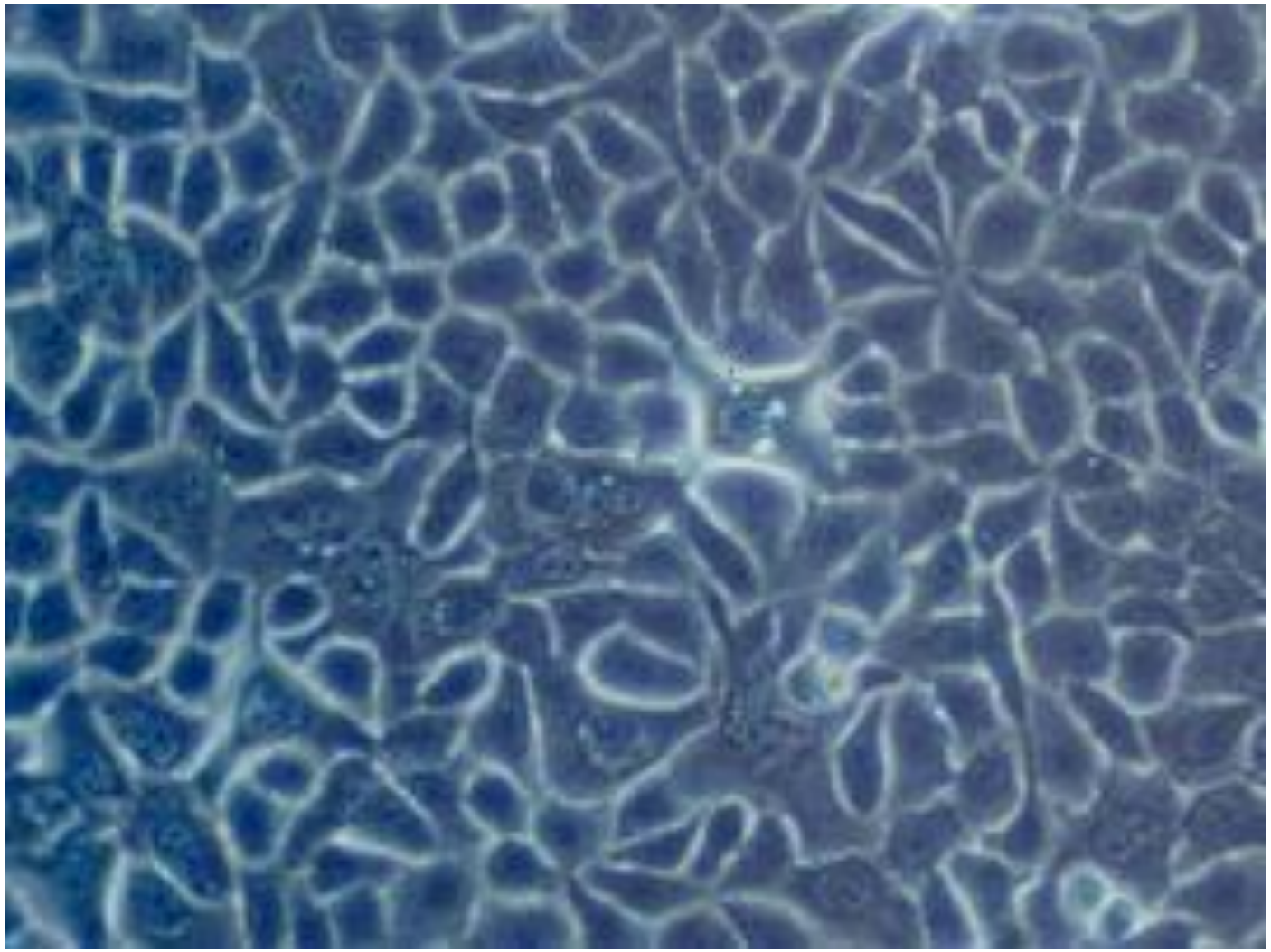
Low Density

Scale Bar = 100µm



High Density

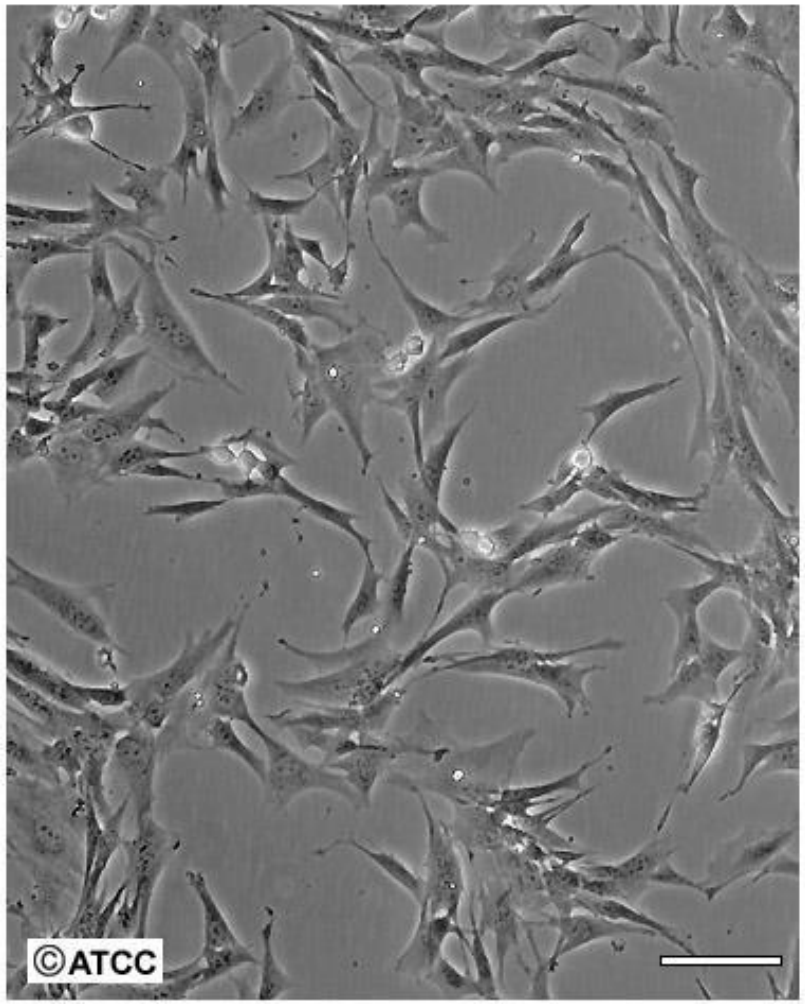
Scale Bar = 100µm



MDBK
Madin-Darby bovine kidney

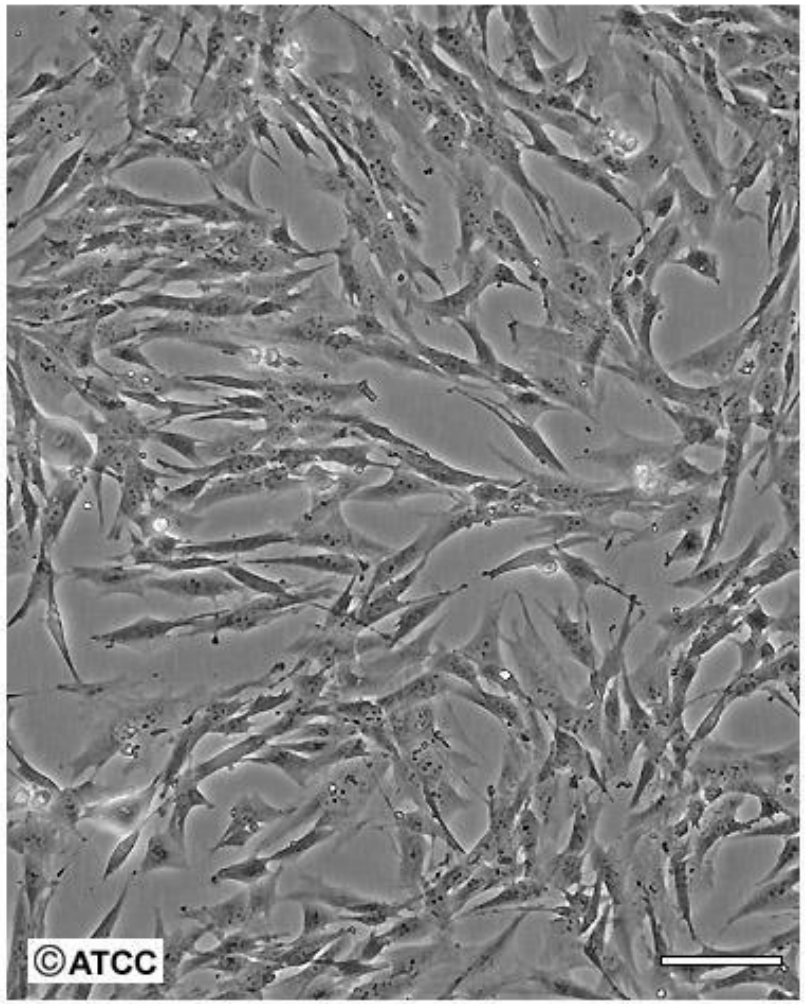
- Fibroblastic Cell

ATCC Number: **CCL-10**
Designation: **BHK-21**



Low Density

Scale Bar = 100µm



High Density

Scale Bar = 100µm

2. Suspension cells: type of cell lines that grow in suspension and do not form monolayers on the surface. Cells form clumps, especially at high density.

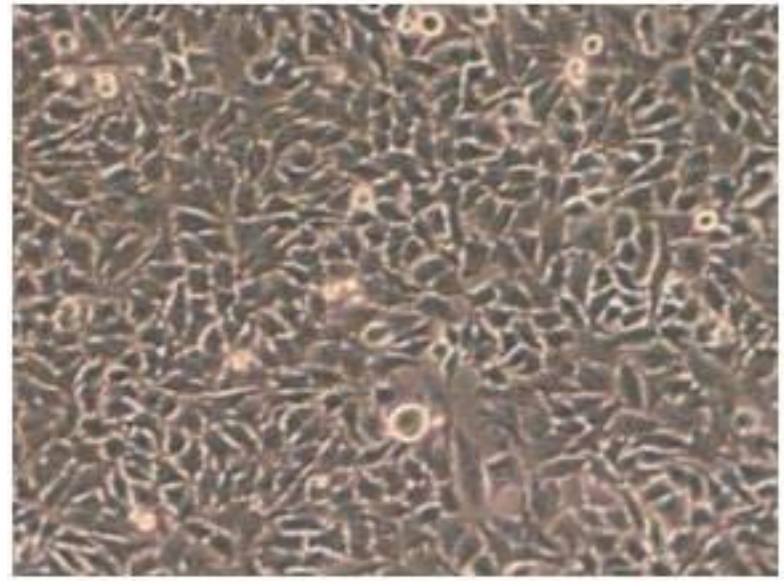
- The culture medium is constantly rotated with a magnetic or mechanical system to prevent the cells from sinking to the bottom and to remain in suspension.
- Suspension cultures are extremely useful in vaccine production activities where large amounts of cells are required.

Cell lines may have different microscopic appearances:

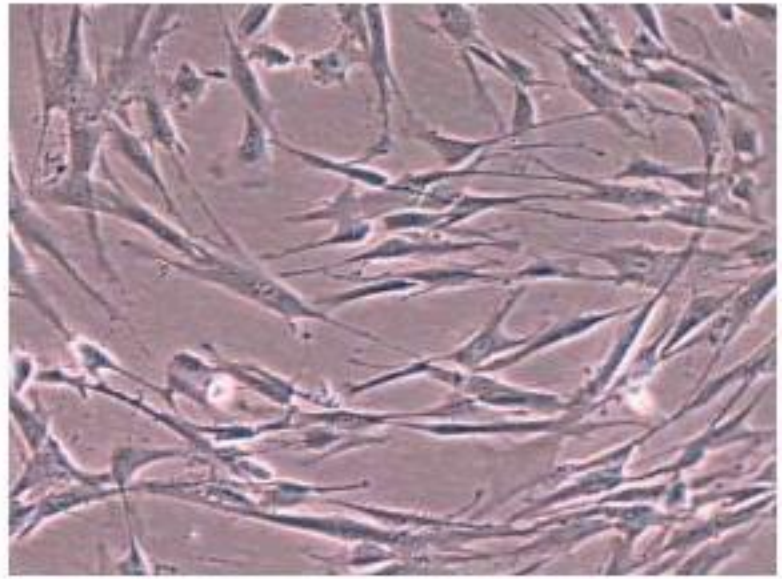
1. Morphologically, cells with a polygonal structure and harmonious dimensions is defined as epithelial morphology.
2. Cells that have a bipolar structure and a spindle-shaped appearance create a fibroblastic cell morphology.
3. Some cell lines cannot be included in any of these groups and exhibit an intermediate morphology. The morphology of these types of cells is described as epithelioid or fibroblastoid.

- Most continuous cell lines are epithelial in character.
- Fibroblast morphology is mostly observed in primary or diploid cell lines.

Epithelial Cell Type



Fibroblast Cell Type



https://www.sigmaaldrich.com/TR/en/technical-documents/technical-article/cell-culture-and-cell-culture-analysis/mammalian-cell-culture/cell-types-culture

Primary Cell Culture Preparation

1. Organ or tissue selection:

A. Donors should be young, fetuses if possible.

B. Donors should be healthy

C. There should be no pathology on the tissue/organ taken.

D. The obtained tissue/organ should not be frozen

E. The obtained tissue/organ should not come into contact with any chemicals.,

F. It should be transported to the laboratory in a cold chain.

Primary Cell Culture Preparation

2. Laboratory equipment

A. UV sterilized work surface

B. Glass material (beaker with cheesecloth, petri dish, erlenmeyer, pipette, etc.)

C. Operation set (scissors, forceps, scalpel, spatula)

D. Magnetic stirrer and rod

E. Mask, gloves, iodine, cotton, gauze

F. Cooling centrifuge

G. 37^{o}C adjustable water bath and incubator

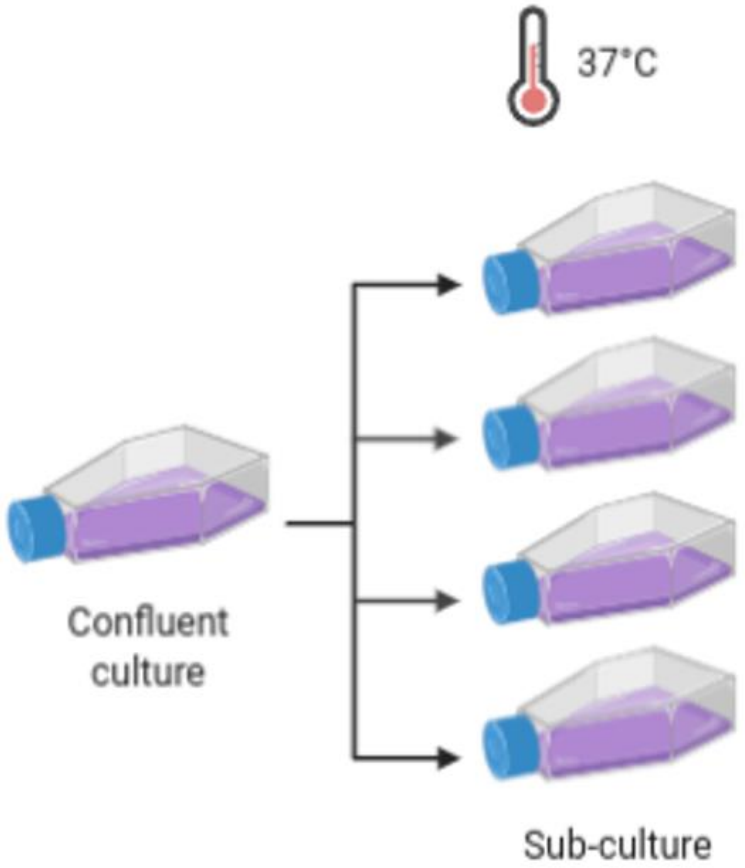
H. 0.25% trypsin, PBS, medium, inactive calf serum

Primary Cell Culture Preparation

1. The membrane or capsule on the tissue is peeled off.
2. Tissue pieces are taken into a petri dish with scissors.
3. The tissues were thoroughly minced using scissors and two scalpels. T
4. The minced tissue fragments were transferred to an Erlenmeyer and washed repeatedly with sterile phosphate-buffered saline (PBS).
5. Intercellular connective tissue is dissociated using pre-warmed trypsin (37°C).
6. The cell trypsin mixture is taken into the beaker every 20 minutes, and fresh trypsin was added to the remaining tissue fragments.
7. The trypsinization continues until the tissues are completely lysed.
8. The resulting cell - trypsin mixture is separated by centrifugation.
9. The cell pellet was resuspended in culture medium supplemented with calf serum, and viable cells were counted before being seeded into culture flasks.
10. The cultures were incubated at 37°C and monitored daily under an inverted tissue culture microscope.

Cell Maintenance

1. Subculture (passage): It refers to the transfer of cells that have completed their reproduction in a culture medium to another culture medium, with or without dilution.



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https://www.thermofisher.com/tr/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/freezing-cells.html?gclid=CjwKCAiA_5WvBhBAEiwAZtCU72aMH_AN0mNW2LvMnt48g9TuUBoijHWOHENe_qtXvXVgtOz87nWj6BoCQxIQAvD_BwE&ef_id=CjwKCAiA_5WvBhBAEiwAZtCU72aMH_AN0mNW2LvMnt48g9TuUBoijHWOHENe_qtXvXVgtOz87nWj6BoCQxIQAvD_BwE:G:s&s_kwcid=AL!3652!3!530416915615!!!g!!!382790548!12548700878&cid=bid_clb_cce_r01_co_cp0000_pjt0000_bid00000_0se_gaw_dy_pur_con&s_kwcid=AL!3652!3!530416915615!!!g!!&gad_source=1

- 2. Freezing: The method for freezing adherent and suspension cells is the same, except that adherent cells must be removed from the culture plates prior to freezing.
- The optimal approach is to store the cells in complete medium with a cryoprotective agent, such as dimethyl sulfoxide (DMSO), at -80°C or in liquid nitrogen.
- Cryoprotectants reduce the freezing point of the medium and allow a slower cooling rate. This greatly decreases the risk of ice crystal formation, which can damage cells and cause cell death.

https://www.thermofisher.com/tr/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/freezing-cells.html?gclid=CjwKCAiA_5WvBhBAEiwAZtCU72aMH_AN0mNW2LvMnt48g9TuUBoijHWOHENe_qtXvXVgtOz87nWj6BoCQxIQAvD_BwE&ef_id=CjwKCAiA_5WvBhBAEiwAZtCU72aMH_AN0mNW2LvMnt48g9TuUBoijHWOHENe_qtXvXVgtOz87nWj6BoCQxIQAvD_BwE:G:s&s_kwcid=AL!3652!3!530416915615!!!g!!!382790548!12548700878&cid=bid_clb_cce_r01_co_cp0000_pjt0000_bid00000_0se_gaw_dy_pur_con&s_kwcid=AL!3652!3!530416915615!!!g!!&gad_source=1