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Microbiology

Doctor 2017 | Medicine | JU

◉ Sheet ○ Slides

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Concepts to be covered in this lecture:

1. The Infectious Cycle
2. Virus cultivation
3. Viruses cytopathic effects
4. Viruses Quantification, Plaque assay, End-point dilution assay

The Infectious Cycle

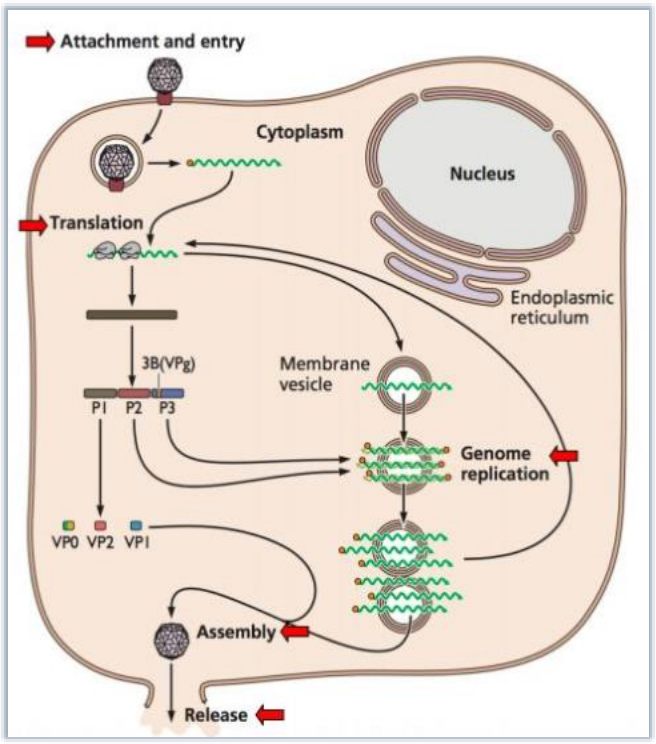
"Reproductive Cycle"

-Inclusive of everything that happens from beginning (when a virus gets into the cell) to end in an infected cell (the cycle for a virus to reproduce).

-Virologists divide the infectious cycle into stages to facilitate their study, but there are no artificial boundaries.

Stages of Infectious Cycle:

1. **Attachment and Entry**:
   the virus attaches to receptors on the cell via its own receptors and then internalizes.
2. **Translation**:
   the virus hijacks the translational machinery of the cell i.e. it will use the cell's RNA polymerase to make its own RNA and the cell's ribosomes to make its own proteins.
3. **Genome Replication**:
   the virus hijacks the cell's DNA replicational machinery (the cell's DNA Polymerase) in order to amplify its own genetic material.



4. **Assembly and Release**:
   the different viral components which are synthesized eventually assemble together and the virus is released from the cell. This is the final stage.

NOTE: In an infected cell everything happens in continuum, there are no labeled segments of the infectious cycle.

⇒Important Terms:

♦**A susceptible cell**: has a functional receptor for a given virus (virus will enter the cell), but it may or may not be able to support viral replication.

♦**A resistant cell**: has no receptor – it may or may not be competent to support viral replication.

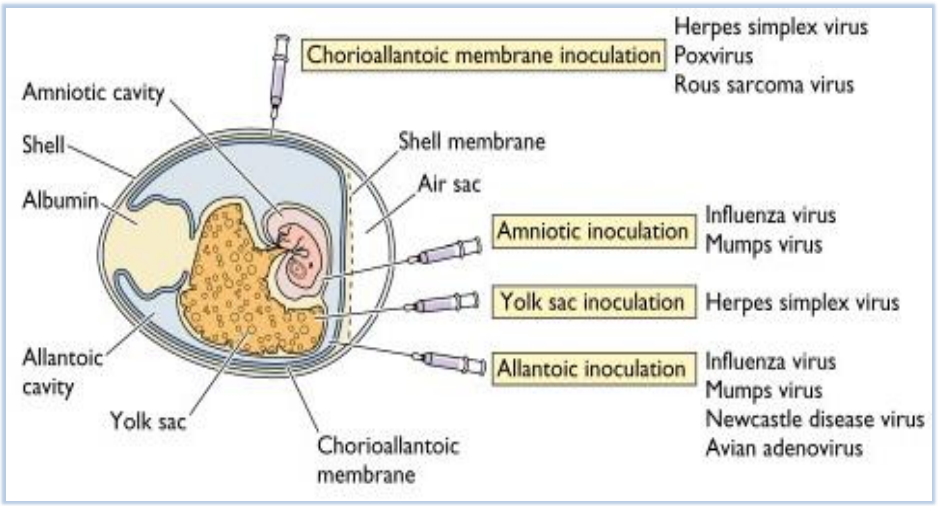
♦**A permissive cell**: has the capacity for viral replication, regardless if it has the receptor or not (it may or may not be susceptible).

♦**A susceptible AND permissive cell**: has a receptor for the virus and it permits viral replication (it's the only cell that can take up a virus and replicate it).

□ Before the development of cell culture, many viruses were propagated in embryonated chicken eggs.

The illustration below shows a cross section of an embryonated chicken egg. Different routes of inoculation (injection) into the egg are displayed (Amniotic inoculation, Yolk sac inoculation, Allantoic inoculation, Chorioallantoic membrane inoculation). Different viruses replicate in different compartments and thus require different routes of inoculation. E.g. Poxvirus replicates via the chorioallantoic membrane inoculation route.

→ You don't have to memorize these viruses just have a general idea about how it was done.



Later on, three scientists (John Enders, Thomas Weller and Frederick Robbins) were able to grow viruses (Poliovirus) **in vitro** (in cells but outside a biological system).

The idea behind their experiment was the following:

 Take cells and place them in a petri dish or a flask containing a nutrient enriched medium (nutrients such as sterile water, sugar, amino acids). This development led to the isolation of many new viruses in tissue culture → they won Nobel prize.

Different types of Cell Cultures used In Viral Cultivation

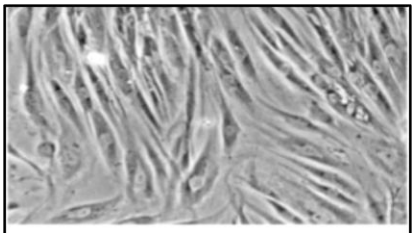
-> A cell line is a cell culture originating from a single type of cell.

1. Primary Cell Lines:

- Take a tissue (human or animal)
- Chop the tissue up and digest it with trypsin to partially dissolve the ECM and get individual/ separated cells (*Advantage: these cells mimic the actual cells in our body/ mimic cells **in vivo**)
- Plate the cells in cell culture dishes (flask, petri dish or multi-well plate) + nutrients
- Add the viruses
- Place the flask in an incubator to obtain physiological conditions (37°C, 5% CO2).

*Disadvantage: These cells will grow up to 20-30 cell divisions since cells don't live forever. The reason behind this is that the cell itself ages, the telomeres get shorter and shorter, and eventually the cell dies. Thus, these cells can't grow forever, when needed: they are taken from donors, babies or companies providing primary cell lines.

* An example of primary cell lines is the cell culture provided by the **primary human foreskin fibroblasts**. The cells from the foreskin are obtained from the retractable skin covering the end of the penis in babies and are cultured to form fibroblasts.



Primary human foreskin fibroblasts

- Extraction of collagen from these fibroblasts occurs. This collagen is later incorporated in women skincare products. A new trend in cosmetics has been surfacing and it involves the injection of Fibroblasts' Growth Factors (FGFs) into the skin of the face.

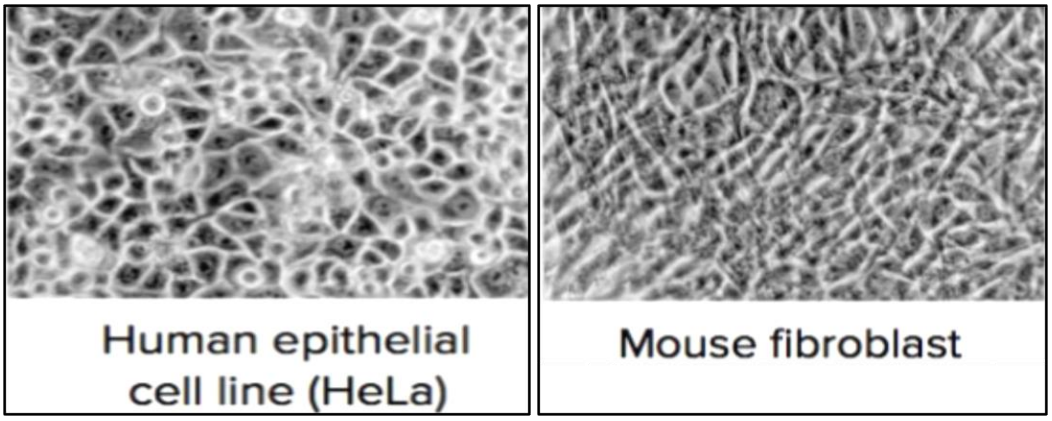
2. Immortal Cell Lines

Cancerous cells that last forever as long as they are provided with nutrients.

- The most common cells used in immortal cell lines are the **human epithelial cell lines (HeLa cells),** they are the first immortal cell lines ever made. (HeLa cells come from a sample taken and cultured from a woman called Henrietta Lacks & were named using the two initials of her first (He) and last (La) names. She had a cervical tumor, which had sequentially caused her death some months later, so she never knew that her cells became cell lines that would be widely used in science.)

HeLa cells can be frozen at (-80°C) or in liquid nitrogen at (-150°C), & can be brought back to life.

- The second type of immortal cell lines is the **mouse fibroblast cell line (3T3).**

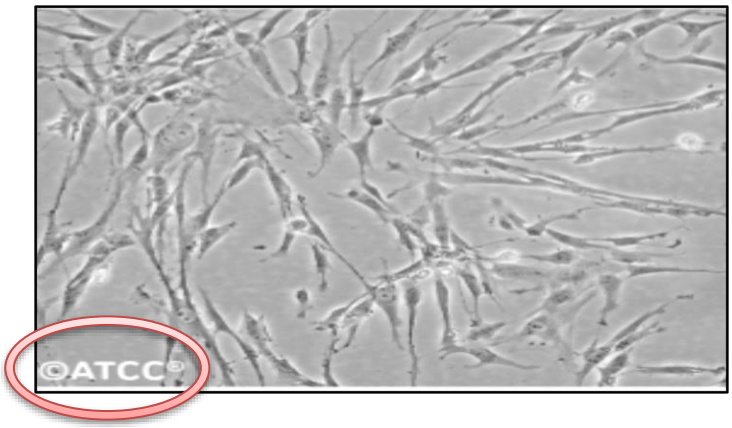


Pros and Cons of using Immortal Cell Lines:

- (+) HeLa and 3T3 cell lines are great because they grow forever. They can outlive us if they were treated well.
- These cancerous cells are immortal, grow fast and can be used to replicate viruses. (-) However, they lack many genes, have too many copies of some other genes, have DNA mutations + other mutations, and may contain an abnormal number of chromosomes (Their genetic material is a mess!!!) → So, they are suitable to grow and replicate viruses but can't be used to study viral behavior inside our bodies because they don't mimic our normal cells (in this case primary cell lines will be used instead).
- The cells cultured in the immortal cell line won't be consistent (due to the genetic abnormalities) from batch to batch. If they were consistent, the cells will die & one must make them repeatedly.

3. Diploid Cell Strains

- They have the right number of chromosomes (48).
- A very famous example is called **(WI 38)**, and these don't last forever, but do last longer than primary cell lines.
- The most common source of these cells is **ATCC** (an organization/bank which collects, stores, and distributes standard reference microorganisms).



Viruses Cytopathic Effects (CPE)

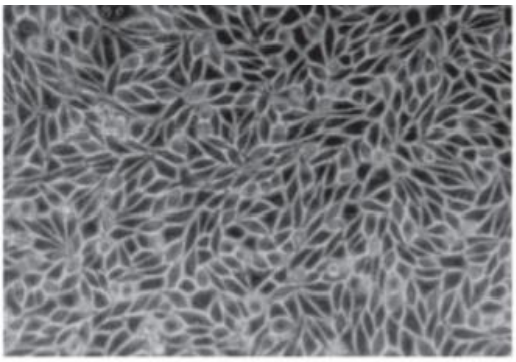
"changes in host cells that are caused by viral infection"

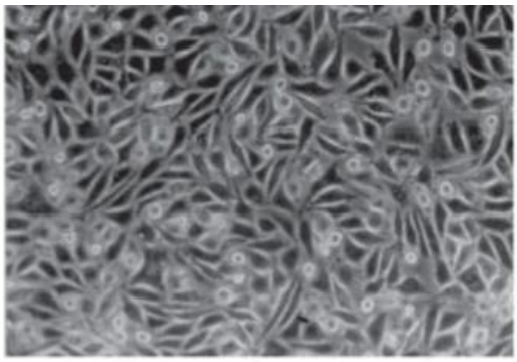
There are many CPE depending on the cell and the virus.

1. Rounding up, Detachment, and Dying

"especially in case of Herpes simplex virus 1 infected cells"

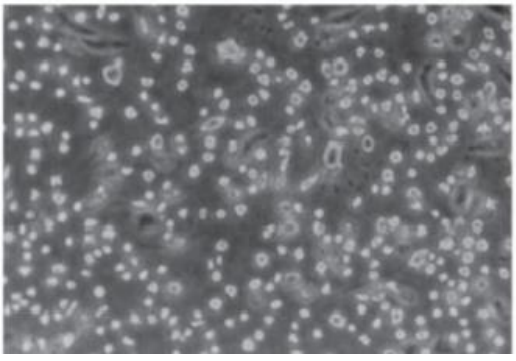
Pic 1

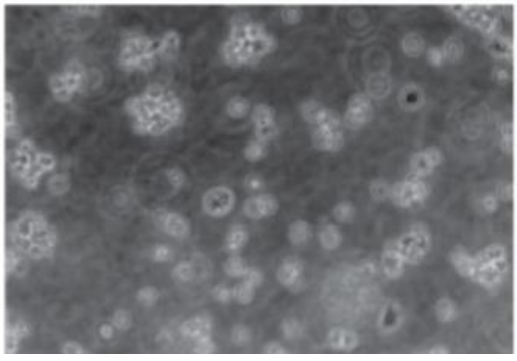




Pic 2

Pic 3





Pic 4

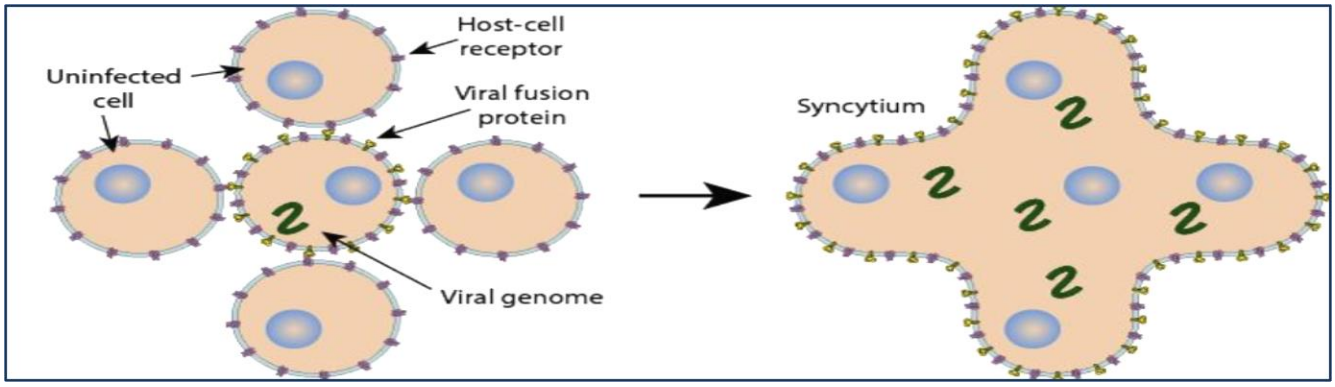
- ✓ In the 1st picture: Normal monolayer cells appear as **confluent** (no space between cells, they are adjacent to one another, and appear elongated).
- ✓ In the 2nd picture: After 2 hours of the infection. Few cells getting **rounded** (rounding up) and they are detaching. This means that the virus is infecting the cells and that the cells are dying.
- ✓ In the 3rd picture: Most of the cells are floating.
- ✓ In the 4th picture: Many of the cells have broken open.

"These CPEs can be seen under the microscope and can be used as a diagnostic sign for the virus".

2. Formation of Syncytia:

"cells are fusing together"

- Initially, you can see single cells, then these cells get infected with a virus and this causes them to fuse together forming one big individual cell with multiple nuclei called a syncytium.
- This event is induced by the surface expression of a viral fusion proteins. (Characteristic of viruses that encode for fusion proteins)
- The viral protein used by the virus to get into the cell will appear on the surface of the cell that the virus is replicating in.
- Syncytia are highly unstable/ abnormal and die quickly.
- Many viruses can generate this effect, most commonly the HIV virus infecting the brain cells.



- The yellow surface protein, shown in the diagram above, is the viral protein that catalyzes the fusion.

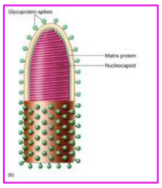
Mechanism:

1. HIV is a single stranded RNA virus (group 6-> single stranded RNA+ reverse transcriptase) and has glycoproteins on its surface (gp120)

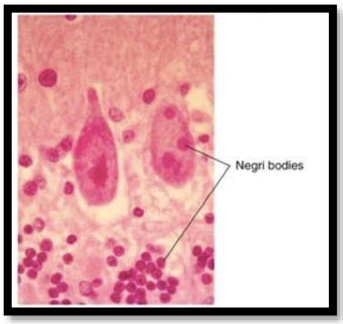
2. Gp120 is used to attach the virus to the host cell
3. Once the virus internalizes, the cell will express these viral glycoproteins on its surface
4. Infected cells will attach to other receptors of neighboring cell inducing the fusion of the cells and forming a syncytium (Syncytia is plural)

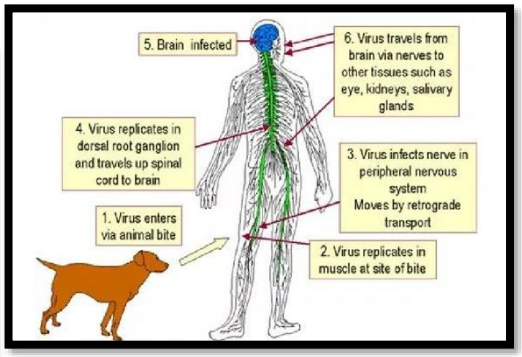
3. Inclusion Bodies

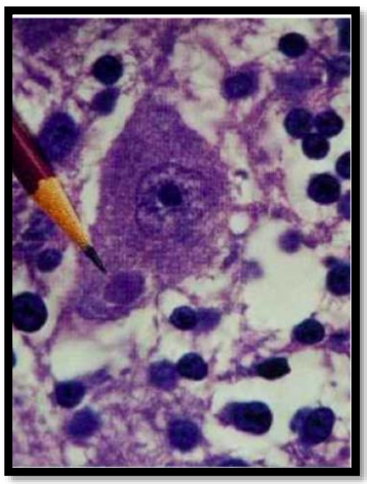
- Caused by **Rabies Virus**:
  - A lyssavirus (extra info: is a genus of RNA viruses in the family Rhabdoviridae, order Mononegavirales.)
  - Enveloped (with protein spikes that bind to cells)
  - Contains single stranded RNA
  - Bullet-shaped virus

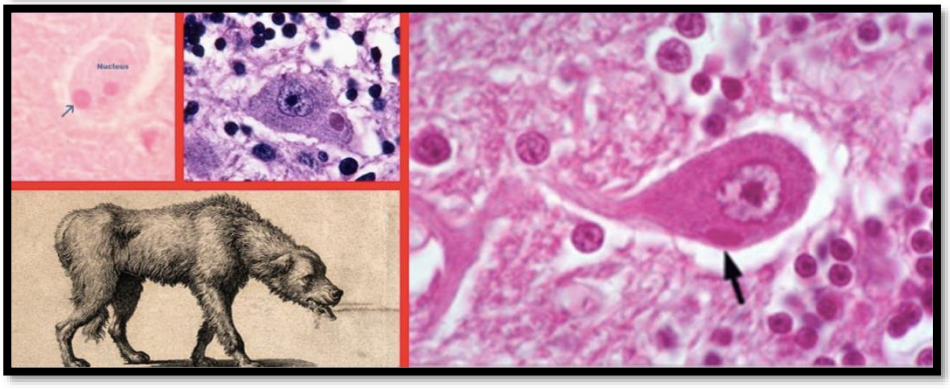


- Pets (dogs or cats) are the main agent for the transmission of the virus, the virus is later passed onto humans via a bite by an infected pet, leading to brain damage and other serious problems. The pet itself gets the rabies virus via a bite by a racoon or a wild animal → That's the reason why pets should be vaccinated and even tested for the virus.
- Negri bodies are eosinophilic, sharply outlined, pathognomonic inclusion bodies (2–10 µm in diameter) found in the cytoplasm of certain nerve cells containing the virus of rabies.
- Negri bodies are looked for in diagnosing Rabies virus infection.









Virus Quantification

"How many viruses in a sample?"

It's essential to know how many viruses the infected cell is producing (quantification of viruses).

We can't see viruses with the naked eye.

Virus quantification can be divided into 2 broad categories:

1. measure the physical particles of the viruses and their parts and components
   ex: 2*10^6 virus particles (VP)/mL

   but this doesn't mean that all of these virus particles measured have the capacity to become infectious.

2. measure infectivity (how many infectious viruses are in a sample): this method tells the infectivity/mL not virus particles/mL
   ★ **Virion**: a virus that has the capacity to be infectious.

The most commonly used methods to quantify viruses can be subdivided into:

I. Techniques measuring the viral infectivity "virions"
   a) **Viral Plaque Assay** → the gold standard one
   b) TCID50

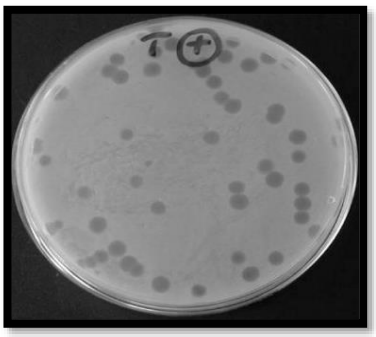
II. Techniques that examine viral nucleic acid and protein
   a) qPCR (quantitative Polymerase Chain Reaction) or real time PCR: amplifying the DNA→ introduction of viral DNA/ RNA + primers unique for viruses, amplifying that region of DNA/RNA, and predicting the initial quantity from the amplified one. (since the amplification is exponential)
   b) Western Blotting: examine and analyze the proteins of the virus.

III. Immunoassays (ELISA): using antibodies to detect the virus.

IV. Techniques that rely on direct counting of physical viral particles
   a) Transmission Electron Microscope.
   b) Viral Flow Cytometry: cells, bacteria or viruses pass through very narrow needle determining how many particles are there.

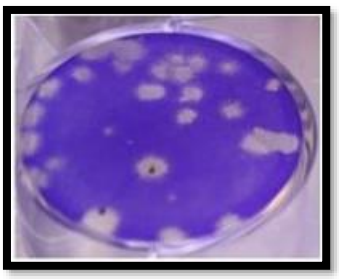
Viral Tittering (Quantification)

- Researchers perform viral titers to measure the number of infectious particles in a sample.
- The gold standard method is the Plaque Assay.
- It was first developed in 1930s for people who were studying bacteriophages.
- In 1952, Renato Dulbecco, developed plaque assay for animal viruses. He won Nobel prize in 1975 for several discoveries, including this one.



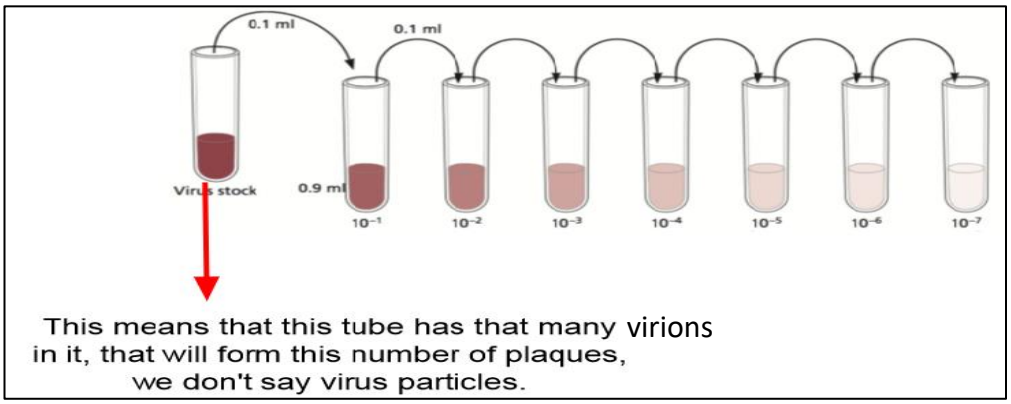
Overall Concept of Viral Tittering:

- The area that appears cloudy is the area where the cells are growing.
- Clear zone is the area where the virus is killing the cells.
- Each clear zone arises from one viral particle; the number of plaques gives an estimation of the number of the viruses in the sample.



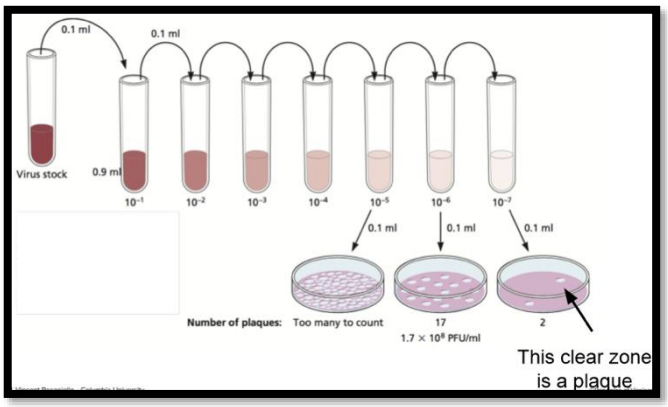
1. Cells are grown (in petri dish) in a confluent monolayer covered by agar and are infected with the virus at varying concentrations → the purpose of agar is restricting the diffusion of the virus; the overall result of this is the formation of discrete plaques.
2. Virus infects cells, cells die, grown viruses infect neighboring cells and so on.
3. Clear plaques appear on the monolayer where cells were killed by the virus.

★Quantifying Virions using Plaque Assay:



1. A sample of virus (virus stock) will undergo ten-fold serial dilutions.

2. 0.1ml of the original sample is mixed with 0.9ml of water, another 0.1ml from the second sample is taken and is mixed with another 0.9ml of water and so on.
3. Dilutions will be 10^{-1}, 10^{-2}….. until 10^{-7} (for example).
4. Plate samples from 10^{-5}, 10^{-6}, 10^{-7} diluted tubes (from the tubes -> so susceptible and permissive to the virus).
5. We count the plaques appearing on each plate:
    - 10^{-5} plate is rich in overlapping plaques that we can't count (not convenient).
    - 10^{-7} plate has only two plaques which may not be statistically significant (not convenient).
    - So, we have to find a plate that has <u>enough</u> number of plaques we can count → 10^{-6} plate in this example (17 plaques).



6. The concentration of the original sample =
   (number of plaques counted *dilution factor)/volume of applied virus solution (ml)
   = $17*10^6$ / 0.1 ml = $1.7*10^8$ PFU/ml

Typically, for the same dilution we duplicate the plating from the same tube to find the <u>average</u> between them, since it will not be the exact number of plaques when repeated.

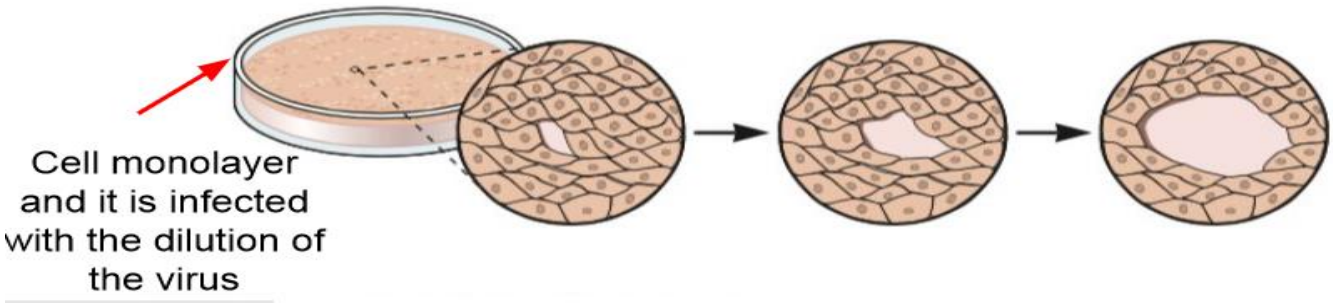
Formula for calculating virus concentration (Titre)

- $Virus\ titre = \frac{(Number\ of\ virus\ plaques) * (Dilution\ factor)}{Volume\ of\ apllied\ virus\ solution\ (mL)}$
- E.g. After applying 200 µL of a virus 10^4 dilution, 40 plaques in well A, 45 plaques in well B. What is the pfu/ml?

$$Virus\ titre\ in\ well\ A = \frac{40 * 10^4}{0.2\ (mL)} = 2 * 10^6 \frac{pfu}{mL}$$

$$Virus\ titre\ in\ well\ B = \frac{45 * 10^4}{0.2\ (mL)} = 2.25 * 10^6 \frac{pfu}{mL}$$

$$Average\ virus\ titre = \frac{(2 + 2.25) * 10^6}{2} = 2.125 * 10^6 \frac{pfu}{mL} \approx 2 * 10^6$$

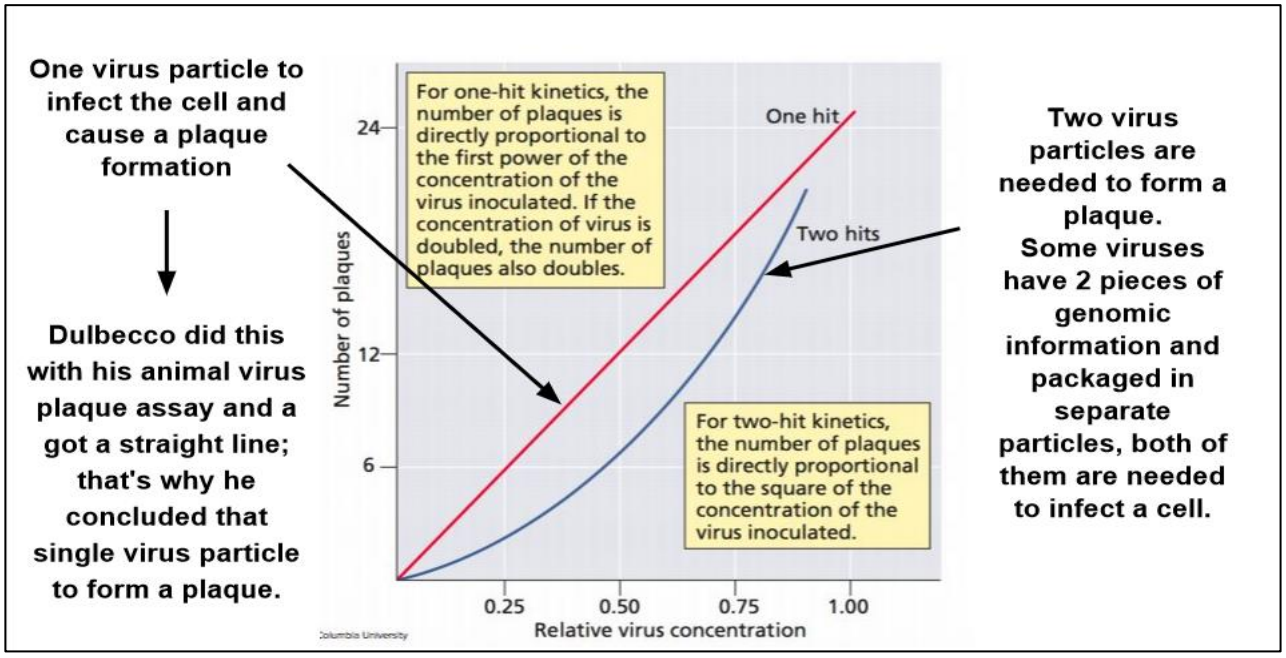


"Cells in the center are dying upon viral infection forming plaques"

- ✓ By this method, we only detect infectious viruses (virions) not viral particles.
- ✓ One virus is going to infect a single cell, that virus may kill that cell, so it is shown missing from that monolayer.
- ✓ This cell will release new viruses and those will infect neighboring cells.
- ✓ Then these cells will die and release more viruses which in turn will infect more cells.
- ✓ This makes a bigger and bigger hole in the monolayer, because cells are dying.
- ✓ **Remember** there is an agar overlay on top of the cell monolayer which is restricting the diffusion of the virus; that's why what we get is discrete plaques.
- ✓ If the overlay is liquid, the virus will swim into the liquid and spread throughout the cell monolayer infecting various cells. The cells will be dead in a day or two, and there will be no discrete plaques to count.

Dose-Response Curve

- ➢ Dose response curve means: dilutions of a sample are carried out, followed by numerous observations. This can be done with drugs or with viruses.
- ➢ It is a XY plot.
- ➢ The X-axis is the relative virus concentration and it is increasing from left to right; these values are dilutions plotted in the opposite way. (no dilution means 1.00 is relative [] of virus/ 100% of virus found)
- ➢ The Y-axis is the number of plaques.
- ➢ What is done is a series of dilutions for the wanted virus, plaquing samples of these dilutions out and counting the number of plaques.
- ➢ The various lines that result, whether lines or curves, indicate if one or more viruses are needed to form a plaque. (correlates the **relative** [virus] with the number of plaques formed.)



I. **Two hits curve (the blue one)** indicates that two viral particles together are needed to form a plaque, each alone won't be able to do so → this happens in plant viruses.

II. **One hit curve (the red linear one)** indicates that one virus is needed to infect the cell and form a plaque. Based on the One hit curve: assume we have an absolute sample of virus (stock/no dilution), then the relative virus concentration will be 1. Consequently, we can know how many plaques will be formed. We can also predict the number of plaques when the sample is diluted to a relative [] of 0.50.

Significance of Infectious Quantification (PFU/Plaque-forming Units)

Scientists are studying gene therapy nowadays using viruses especially the AAV (Adeno-associated virus). Quantification of the virus is essential and is achieved using PCR (Polymerase Chain Reaction), so from this amplified genetic material one can determine the number of viral particles/ml. However, the important quantification needed is the infectious quantification (no. of PFUs/ml) i.e. How many infectious particles enter the cells, infect them, and deliver the therapeutic gene and express it.

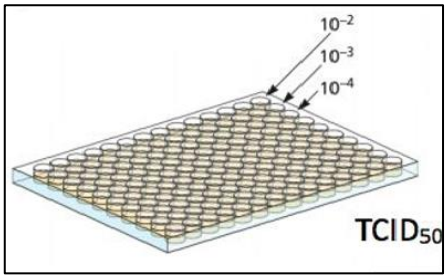
End-Point Dilution Assay

Sometimes we can't use the PFU method because some viruses simply don't form plaques and others are hard to work with when it comes to the agar overlay, so the solution to these complications would be to culture the viruses in liquid media.

Thus, there should be another way to quantify infectivity (virions): End-Point dilution assay.

- End-point dilution assay is typically done in 96-well plates.
- Host cells are placed in each well with an appropriate medium.
- Serial dilutions (10^{-2} - 10^{-7} for example) of a virus stock are inoculated into replicate test units (wells).
- Inoculate each 10 wells, for example, with the same dilution of virus sample.
- This can be done for multiple samples and multiple replicates.
- Then incubate the cultures and observe which wells show CPEs.

Virus dilution	Cytopathic effect									
10^{-2}	+	+	+	+	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	+	+	+	+	+
► 10^{-4}	+	+	–	+	+	+	+	+	+	+
► 10^{-5}	–	+	+	–	+	–	–	+	–	+
10^{-6}	–	–	–	–	–	–	+	–	–	–
10^{-7}	–	–	–	–	–	–	–	–	–	–



*According to the table:

- The number of the wells that become infected (show CPE) is counted for each dilution. (+ means showing a CPE).
- At low dilutions ($10^{-2}$and 10^{-3}) every culture is infected (10 wells showed CPEs out of the 10 inoculated).
- At 10^{-4} dilution: 9 wells are infected.
- At 10^{-5} dilution: 5 wells are infected, 5 wells aren't.
- At 10^{-6} dilution: 1 well is infected, 9 wells aren't.
- At high dilution (10^{-7}), none of the cell cultures are infected; because no infectious particles are delivered to the cells.
- Some wells aren't showing the CPEs, while others are showing them, even though samples are taken from the same tube of the same dilution (the distribution is randomized).
- The **end-point** is the dilution of virus that affects 50% of the test units (50% CPEs).
- the end-point here is at the dilution (10^{-5}), where half of the wells displayed CPEs and half of them did not.
- This number is called **infectious dose 50% (ID50).**
- Most of the time **(ID50)** is between two dilutions and is calculated using math.
  -*Question*: If the ID50 of a certain virus for a patient is 10^{-5} and the ID50 of the same virus but for a different patient is 10^{-7} which patient has more viruses? The second one, since he/she has an ID50 that is 100 times more diluted than the 1st patient.