Lecture 2

- The Infectious Cycle
- Virus cultivation
- Viruses cytopathic effects
- Viruses Quantitation Plaque assay, End-point dilution assay

The infectious cycle

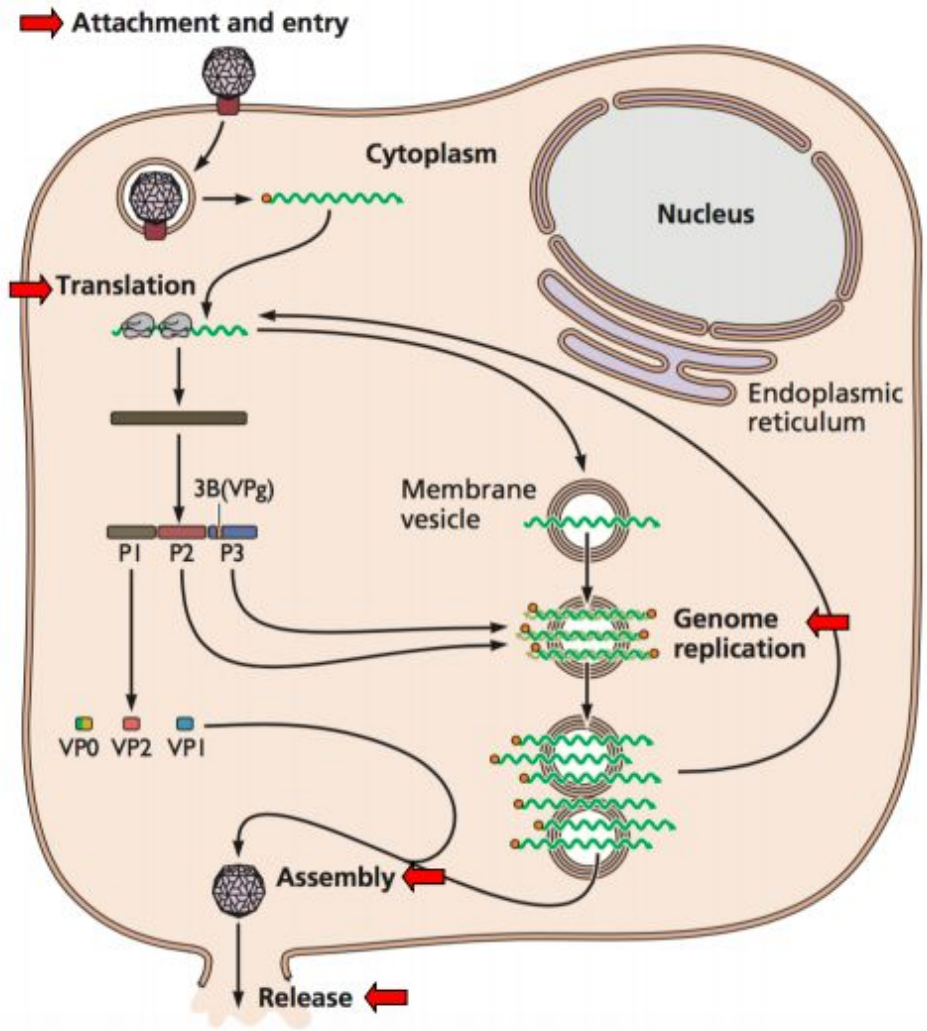
- Infectious cycle:Everything that happens from the start (when a virus gets in the cell) to the finish in the infected cell.

- Sometimes it is called reproductive cycle.

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

- They are divided into stages:
  - Attachment and entry,
  - Translation,
  - Genome replication,
  - Assembly and release

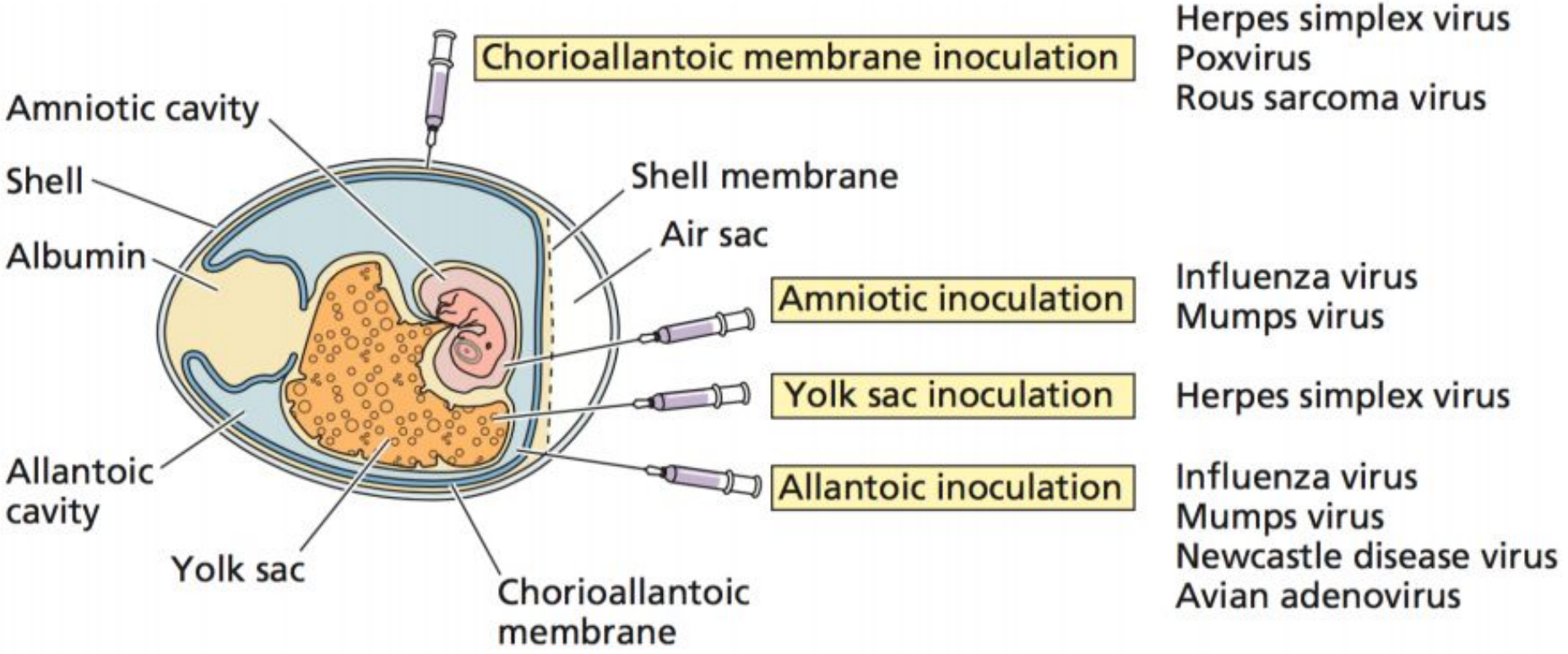
- In an infected cell everything happen in continuum, there are no labeled segments of the infectious cycle.



Some important definitions

- A **susceptible** cell has a functional receptor for a given virus - *the cell 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 to replicate virus - *it may or may not be susceptible*
- A **susceptible** AND **permissive** cell is the only cell that can take up a virus particle and replicate it

The places where viruses can be injected in and the different viruses that were grown.



The Year 1949

John Enders (1897-1985)

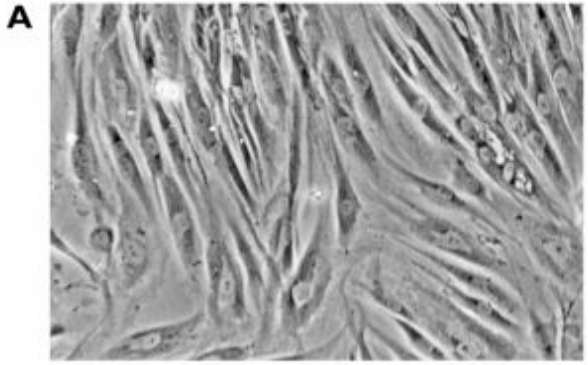
Thomas Weller (1915–)

Frederick Robbins (1916–)

John Enders, Thomas Weller (1915–) and Frederick Robbins (1916–) were able to grow **poliovirus** in vitro using human tissue culture. (Nobel Prize, 1954) This development led to the isolation of many new viruses in tissue culture.

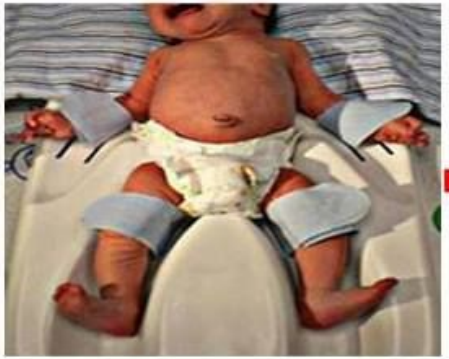
Virus cultivation: Primary cell lines

- Today we have all sort of different kinds of cell cultures that we use to grow viruses in.
- You take a tissue (human or animal), chop it up and digest it with trypsin to get individual cells, plate them in cell culture dishes
- They will grow to 20-30 cell divisions, but because cells don't live forever, because the telomeres gets shorter and shorter and eventually they will die.

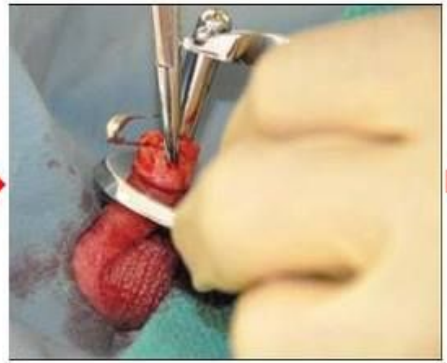


Primary human foreskin fibroblasts

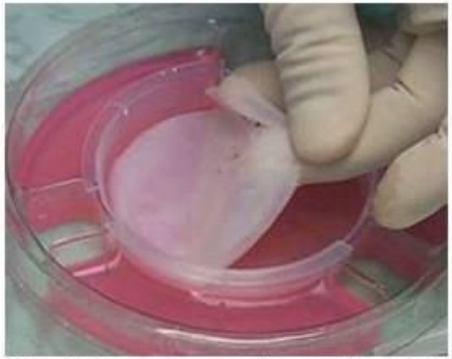
https://youtu.be/tJz7Ipve8OU



Child Strapped down



Foreskin surgically removed from penis, no anesthetic used.



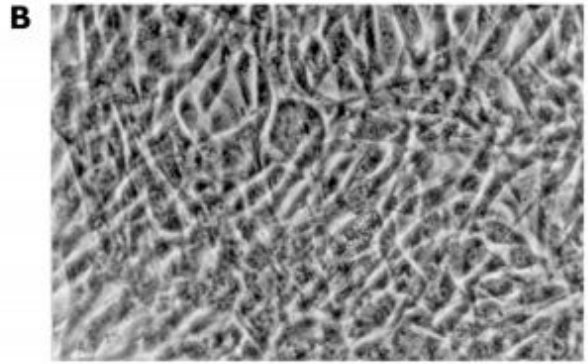
Fibroblasts from the cells of the foreskin is used to make collagen

Foreskin collagen inserted into womens skin care products

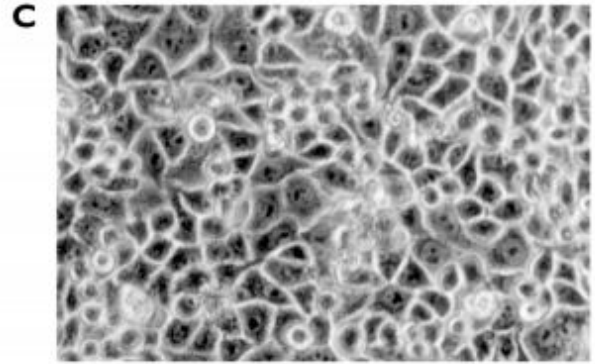
Virus cultivation: immortal cell lines

- They won't be consistent from batch to batch; because the cells will die and you have to make them over and over again.
- We found out we that we can make cell lines, and sometimes these are immortal (they live forever).
- We have a mouse fibroblast cell line called 3T3 and -the famous- human epithelial cell line HeLa cell.
- HeLa cells are the first immortal cell line ever made.
- HeLa and 3T3 cell lines are great because they grow forever. They can outlive us if they were treated well.

continuous cell lines



Mouse fibroblast cell line (3T3)



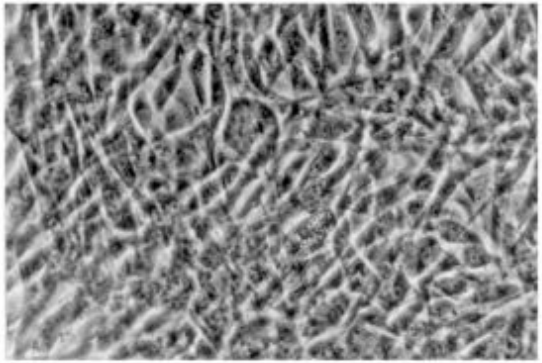
Human epithelial cell line (HeLa)

Virus cultivation: immortal cell lines

- The problem is they are weird, they have abnormal chromosomes, they have all sort of mutations; so depending on what the purpose is thay may not be perfect.
- To grow a stocks of a virus it is okay but to understand what this virus doing in the cell probably not.
- These cells lack many genes, they have too many copies of some other genes and they may not give the right answer.

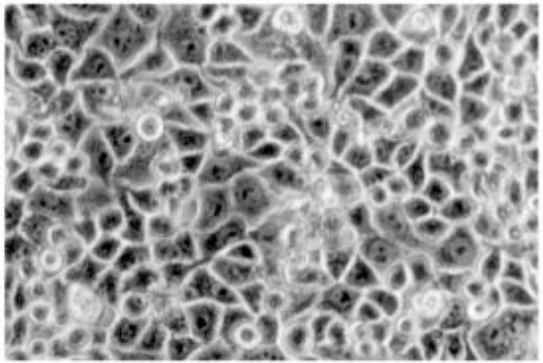
continuous cell lines

B



Mouse fibroblast cell line (3T3)

C

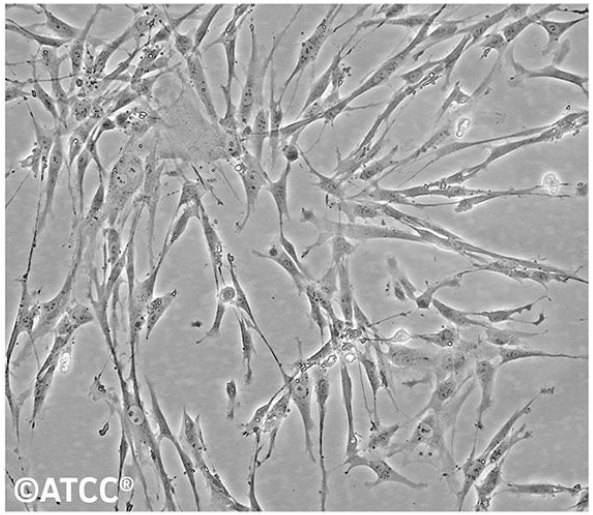


Human epithelial cell line (HeLa)

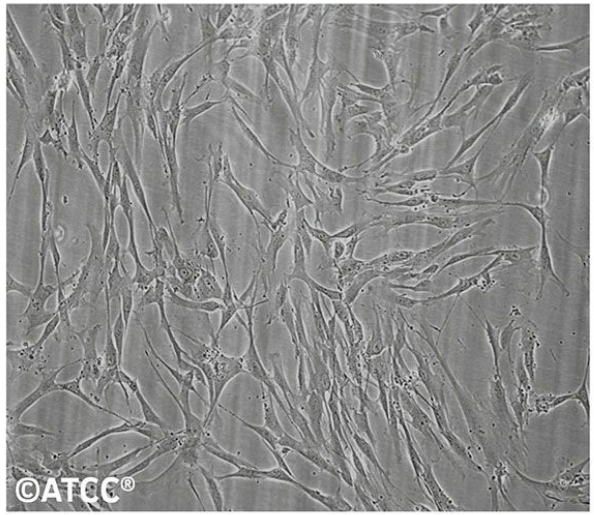
Virus cultivation: diploid cell strains

- They have the right number of chromosomes.
- A very famous one is called WI 38, and these don't last forever, but do last longer than primary cell line.

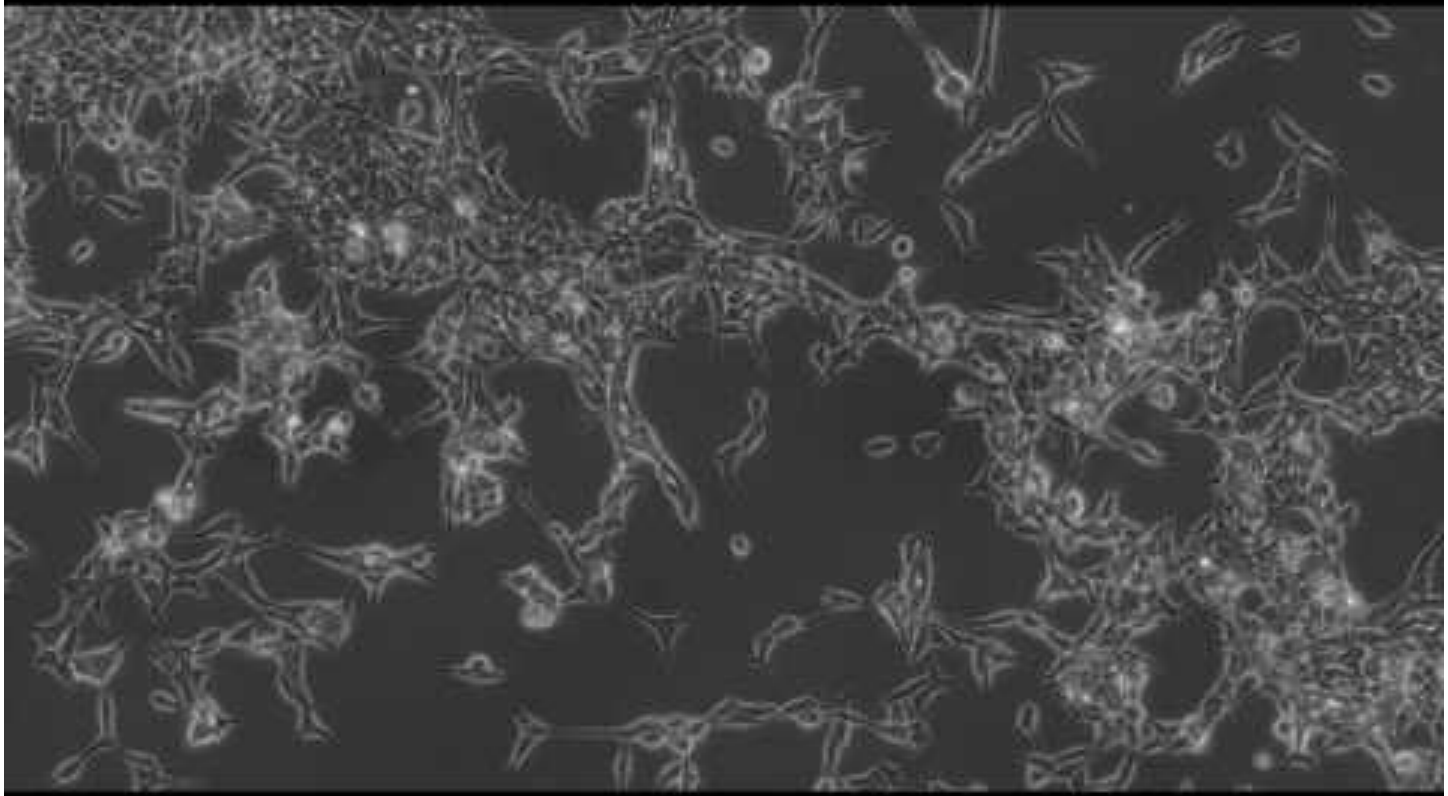
ATCC Number: **CCL-75**
Designation: **WI-38**



Low Density



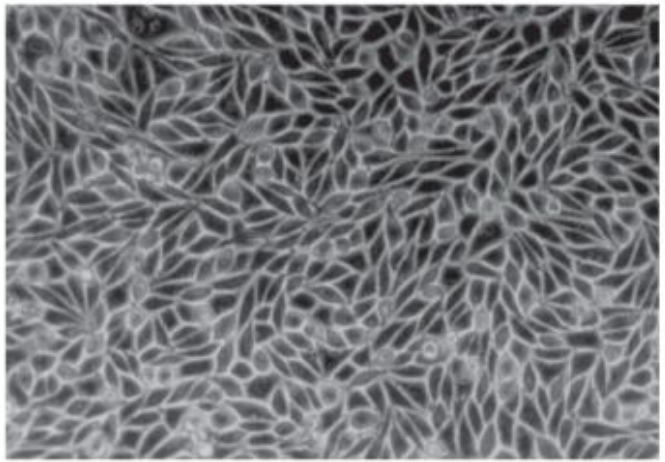
High Density

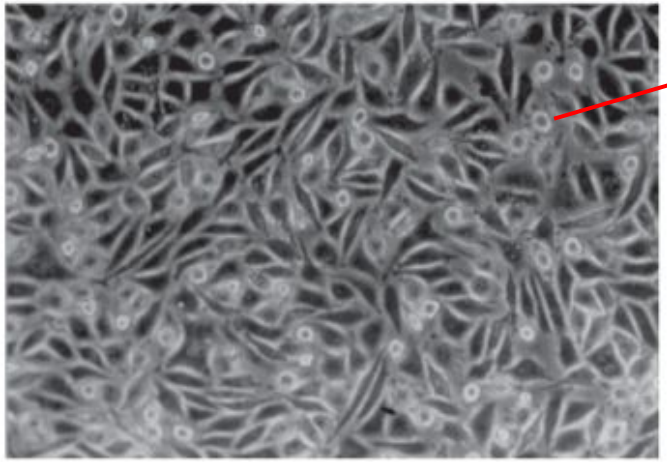


Viruses cytopathic effects (CPE)

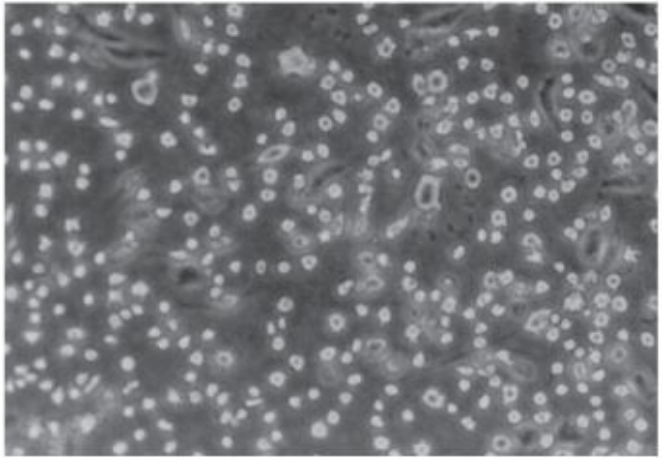
- When having a cell line to grow virus and infect it, how to know that the virus is growing in the cultured cells?
- The following figure in the next slide will show a series of panels of cells infected with poliovirus.
- They are shown after different time of the infection.

Monolayer of cells →

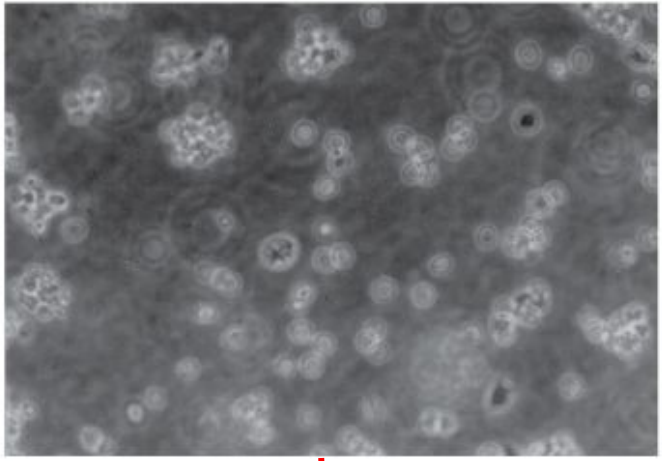




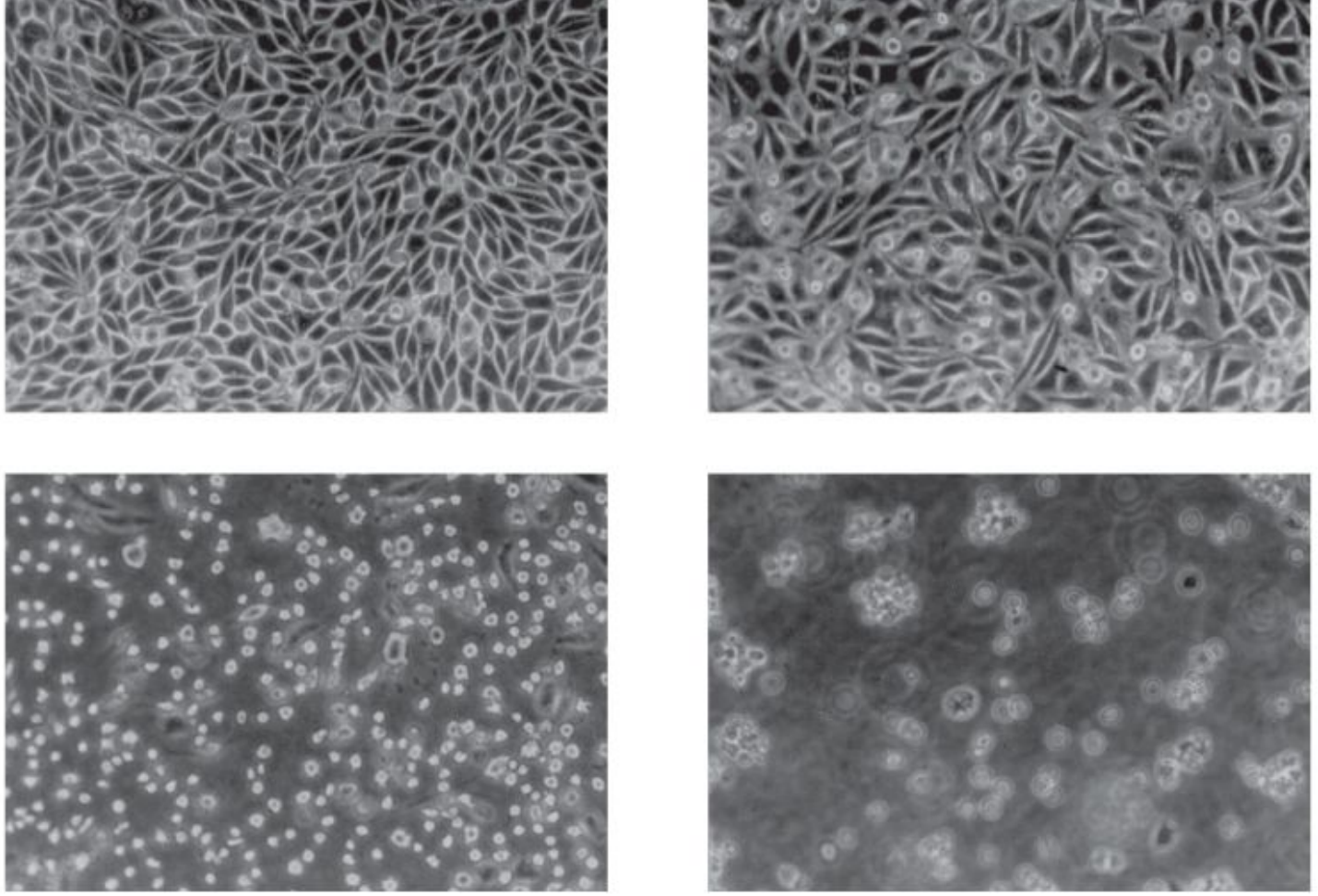
After 2 hours of the infection.
Few cells getting round and they are detaching.,



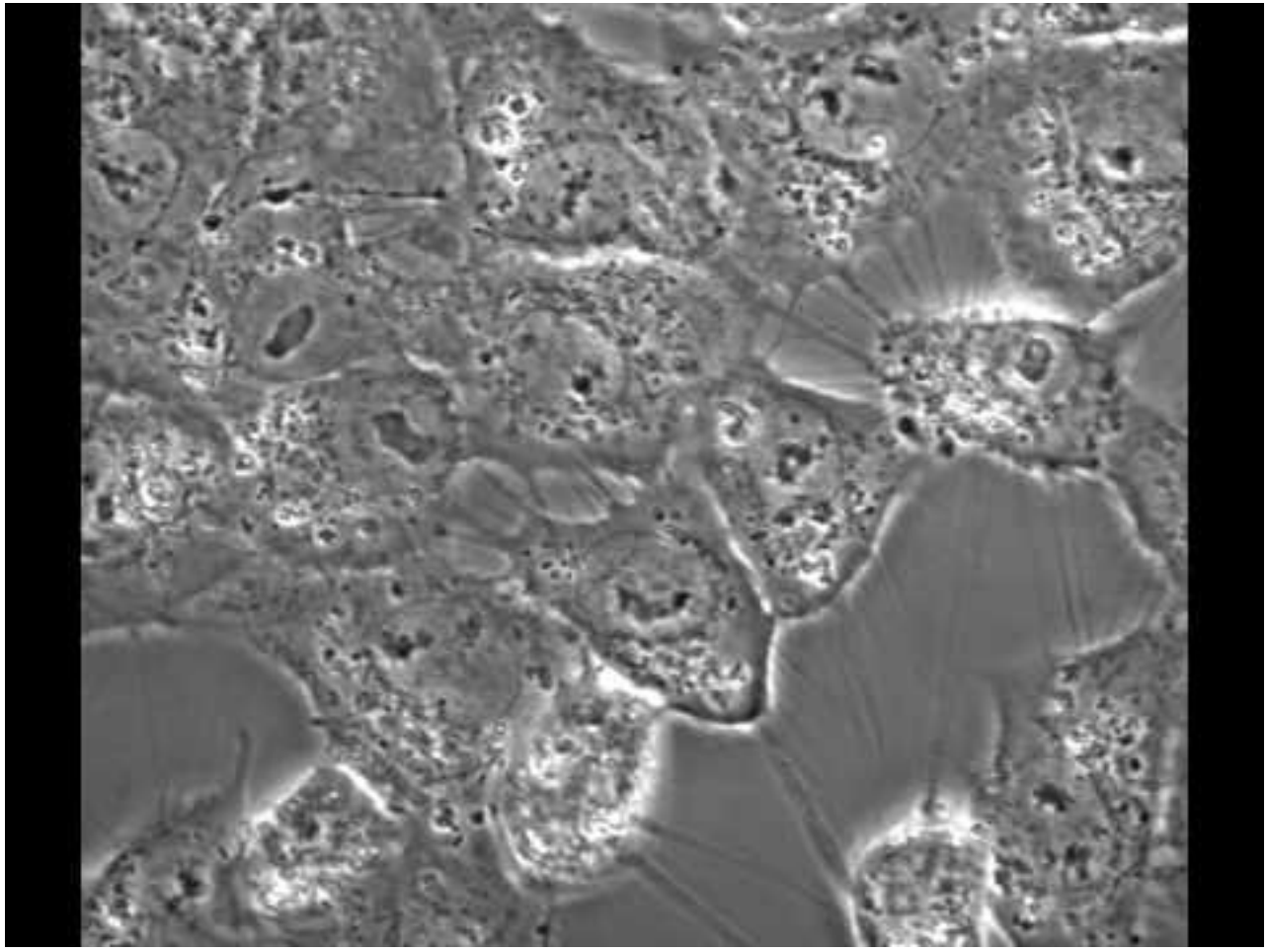
At this point, most of the cells are floating.



At the end many of the cells have actually broken open



cytopathic effect (CPE)



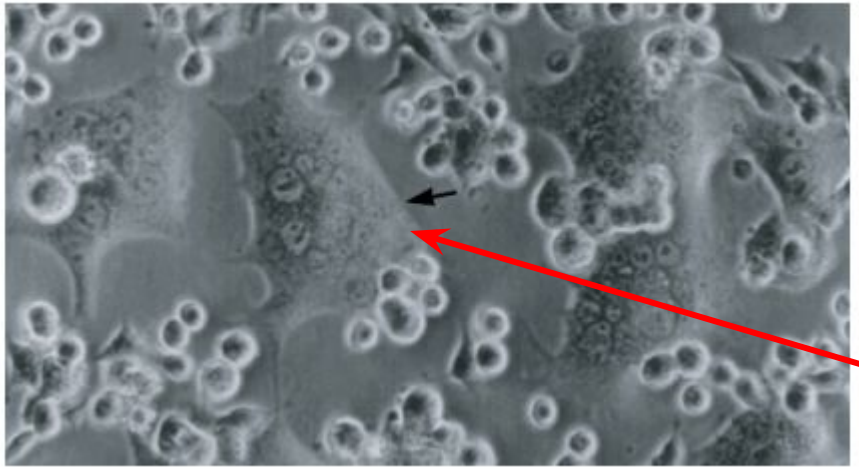
Cytopathic Effect: Herpes simplex virus 1 infected cells

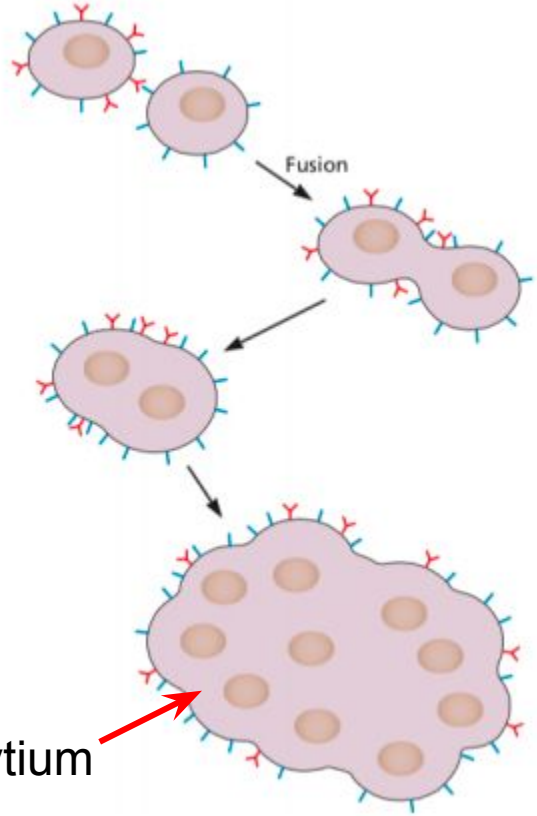
Viruses cytopathic effects (CPE)

- The changes that was shown before is called cytopathic effect CPE.
- It is the changes that happen to the cell after infecting them with a virus, these changes can be seen under the microscope.
- There are many CPE depending on the cell and the virus.
- This is one kind of change; rounding up, detachment and dying.

Formation of syncytia

- Another kind of CPE is when cells **fuse**.
- So here we have cells in culture.
- You can see single cells and these have been infected with a virus that causes the cells to fuse together.

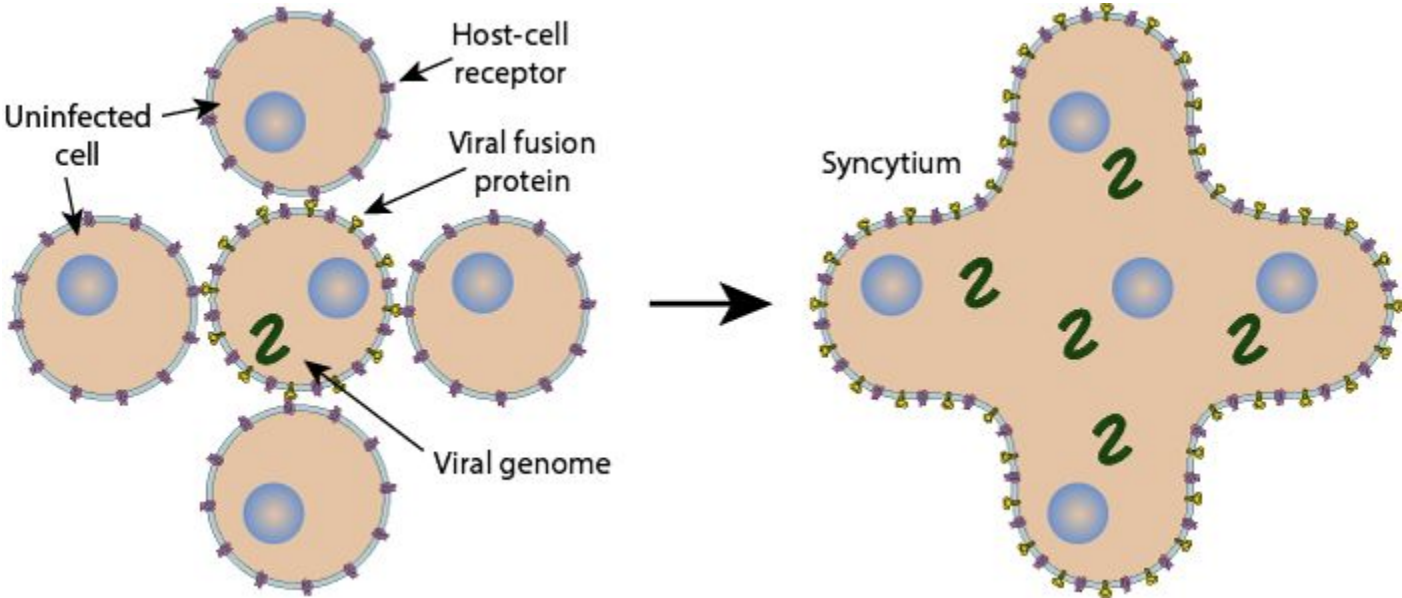




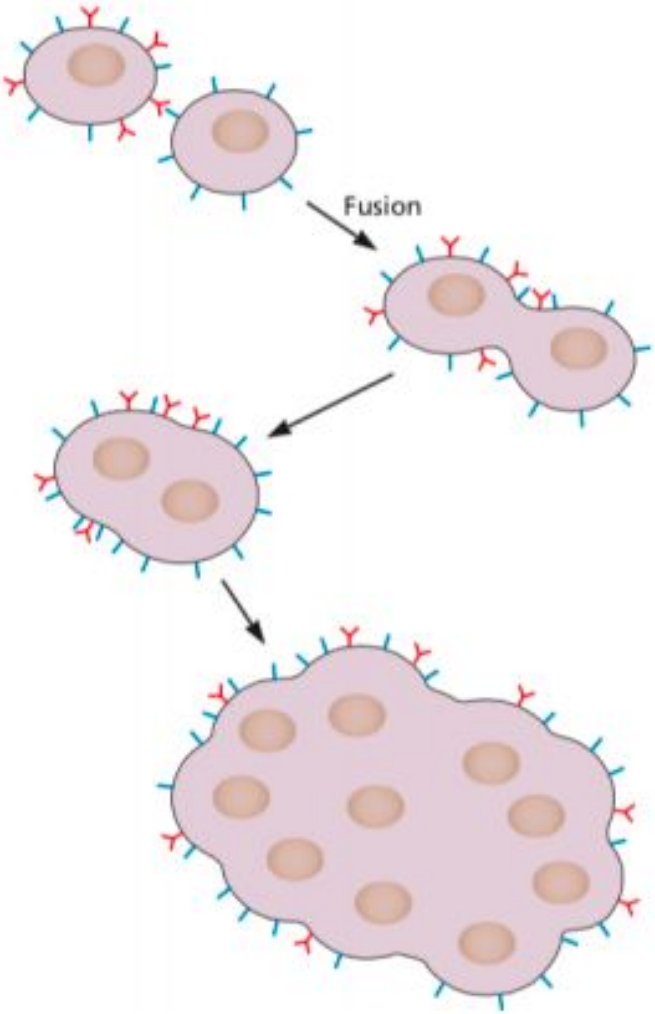
Formation of syncytia

- You can see a cell with many nuclei and that is called syncytium. (Syncytia is plural)
- Many viruses can make this effect.
- A viral protein that the virus use to get into the cell will appear on the surface of the cell that the virus is replicating in.

This protein help with the fusion of the virus and the cell, but also if the protein is on the surface of two neighboring cells , they will fuse together.

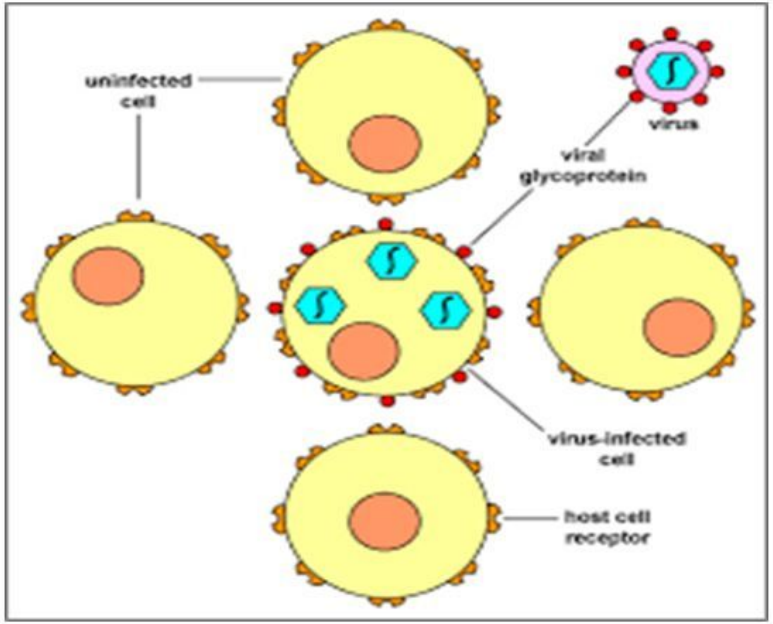


- The red surface protein is the viral protein that catalyzing fusion
- It attaches to a receptor on the neighboring cell, those two cell are going to fuse and on and on and on and so forth until a big syncytium is formed
- This a characteristic of viruses that encode fusion proteins.



Syncytium Formation

- Observed in HIV infection, most commonly in the brain
- Uninfected cells may then bind to infected cells due to viral gp120
- This results in fusion of the cell membranes and subsequent syncytium formation.
- These syncytia are highly unstable and die quickly



Large diversity of the cytopathic effect you viruses

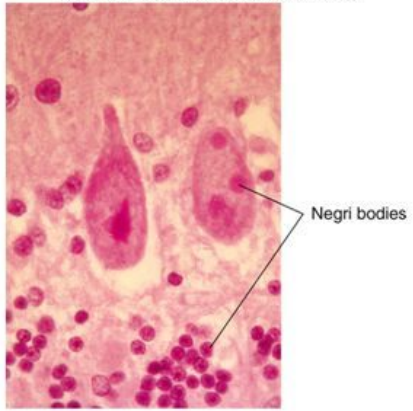
- Some of these CPE can be a diagnostic sign for the virus.
- For example; Negri bodies in the inclusion bodies are looked for in diagnosing Rabies virus infection.

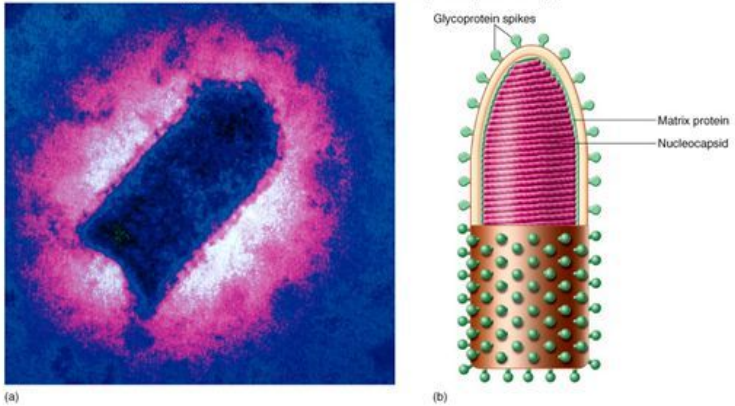
Examples of cytopathic effects

Cytopathic effect(s)	Virus(es)
Morphological alterations	
Nuclear shrinking (pyknosis), proliferation of membrane	Picornaviruses
Proliferation of nuclear membrane	Alphaviruses, herpesviruses
Vacuoles in cytoplasm	Polyomaviruses, papillomaviruses
Syncytium formation (cell fusion)	Paramyxoviruses, coronaviruses
Margination and breaking of chromosomes	Herpesviruses
Rounding up and detachment of cultured cells	Herpesviruses, rhabdoviruses, adenoviruses, picornaviruses
Inclusion bodies	
Virions in nucleus	Adenoviruses
Virions in cytoplasm (Negri bodies)	Rabies virus
"Factories" in cytoplasm (Guarnieri bodies)	Poxviruses
Clumps of ribosomes in virions	Arenaviruses
Clumps of chromatin in nucleus	Herpesviruses

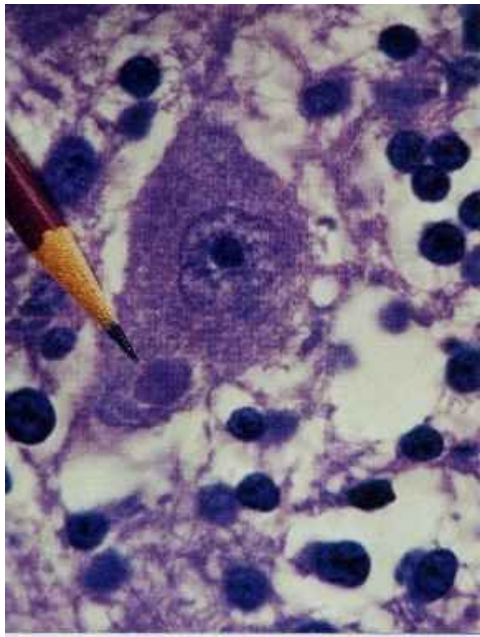
Rabies

- Causative agent:
  - Rabies virus
    - Lyssavirus
    - Enveloped ssRNA
    - Spiked bullet shaped virus
  - Virus multiplies in brain forming Negri bodies

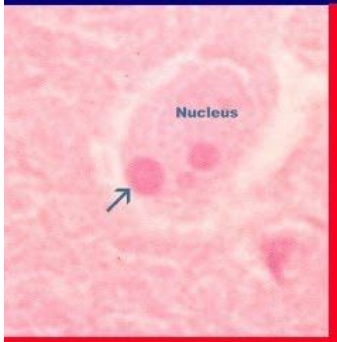


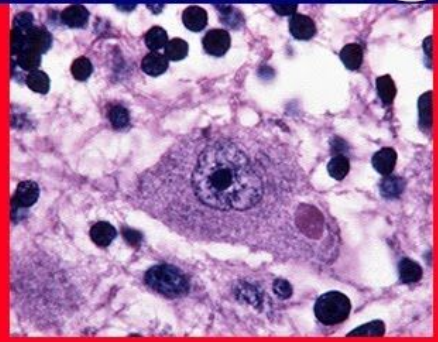


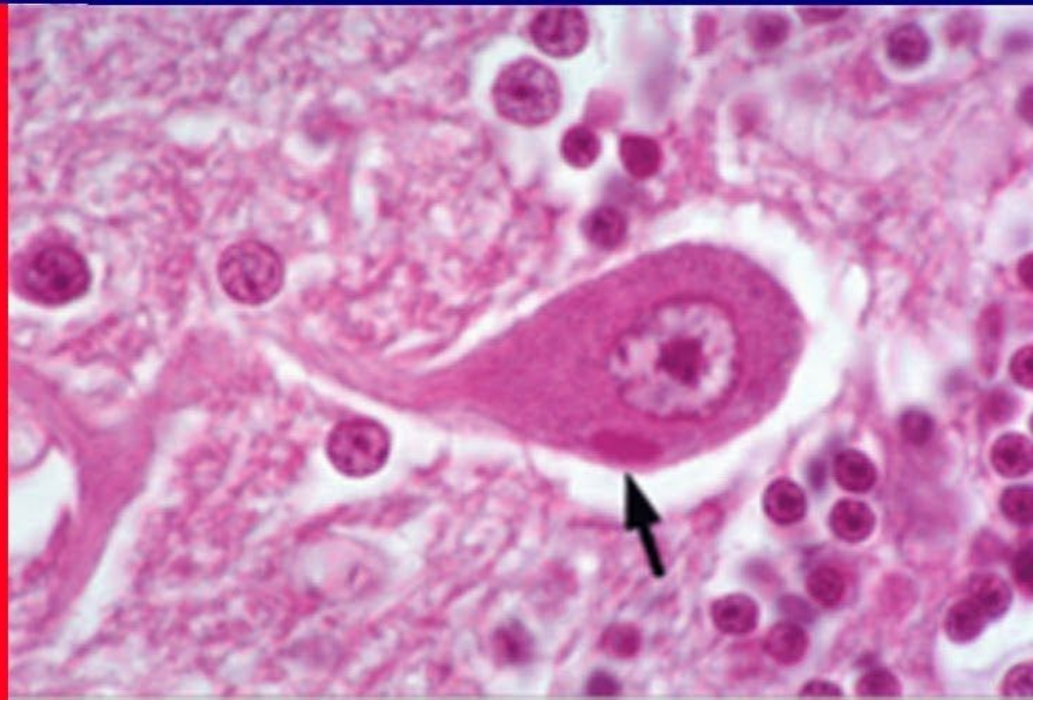
Negri bodies are eosinophilic, sharply outlined, pathognomonic inclusion bodies (2–10 μm in diameter) found in the cytoplasm of certain nerve cells containing the virus of rabies

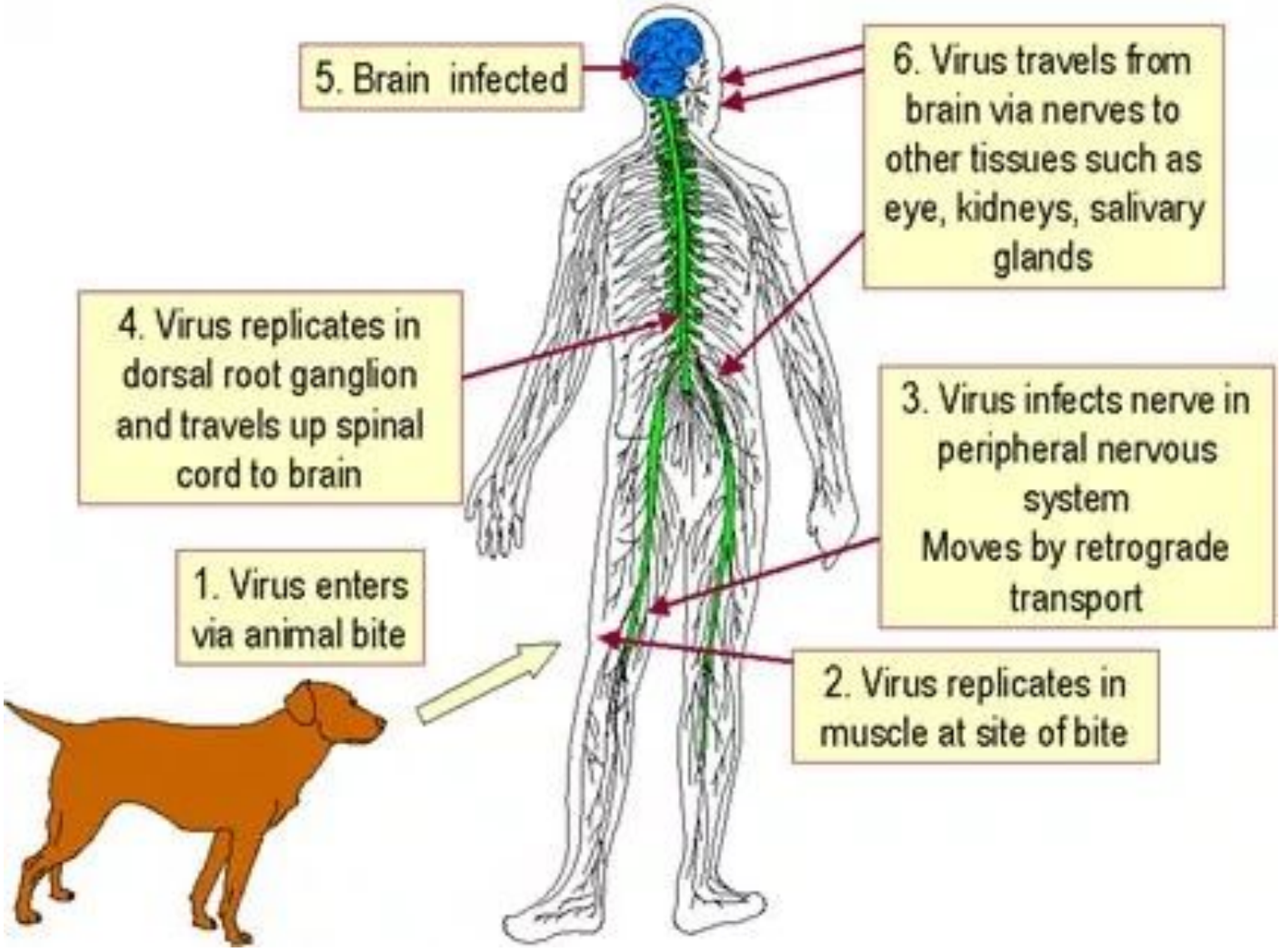


Negri Bodies ?








5. Brain infected
6. Virus travels from brain via nerves to other tissues such as eye, kidneys, salivary glands
4. Virus replicates in dorsal root ganglion and travels up spinal cord to brain
3. Virus infects nerve in peripheral nervous system
Moves by retrograde transport
1. Virus enters via animal bite
2. Virus replicates in muscle at site of bite

How many virus in a sample? (Viruses Quantitation)

- After observing the changes virus, we have to know how many viruses the infected cell is producing. (quantification of viruses)

- Virus quantification can be divided into 2 broad categories:

  First: we can measure infectivity (how many infectious viruses are in a sample)

  Second: measure the physical particles of the viruses and their parts and components.

Quantification of Viruses

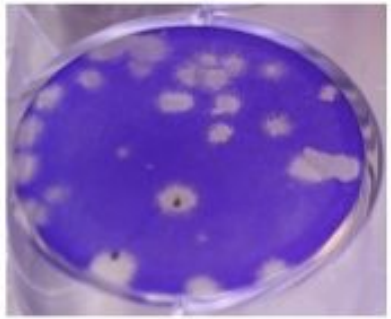
- The most commonly used methods to quantify viruses can be subdivided into three broader categories:
  - Techniques measuring viral infectivity
    - Viral plaque assay
    - $TCID_{50}$
  - Those that examine viral nucleic acid and protein
    - qPCR (real time PCR)
    - Western blotting
  - Immunoassyas
    - ELISA
  - Those that rely on direct counting of physical viral particles
    - viral flow cytometry
    - and transmission electron microscopy

Viral Titering

Researchers perform viral titers to measure the number of infectious particles in a sample

The gold standard method is the plaque assay:

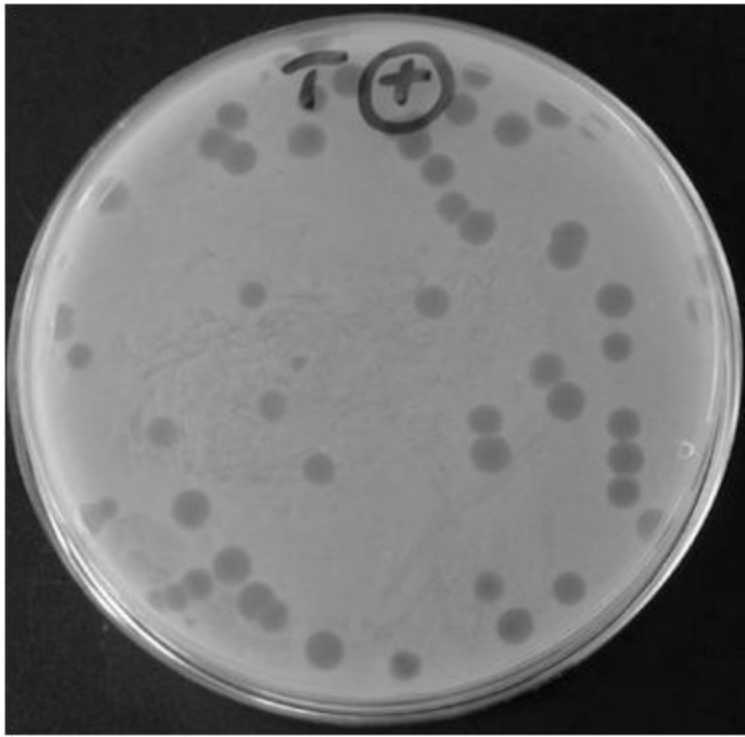
- Cells are grown in a monolayer and infected with virus at varying concentrations
- Clear plaques appear on the monolayer where cells were killed by the virus

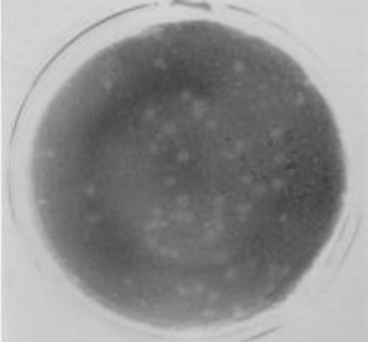


LumaCyte

Plaque assay

- It was first developed in 1930s for people who were studying bacteriophages.
- Where it is cloudy is where the bacteria is growing.
- Clear zone is where the virus is killing the bacteria.
- Each clear zone arise from one viral particle; the number of plaques gives an estimation about the number of the viruses in the sample.
- It is called plaques forming units per milliliter. PFU/ml





Plaque assay

- In 1952, Renato Dulbecco, developed plaque assay for animal viruses.
- This is his paper, published on April 29th 1952.
- He won Nobel prize in 1975 for a number of discoveries, including this one.

PRODUCTION OF PLAQUES IN MONOLAYER TISSUE CULTURES BY SINGLE PARTICLES OF AN ANIMAL VIRUS

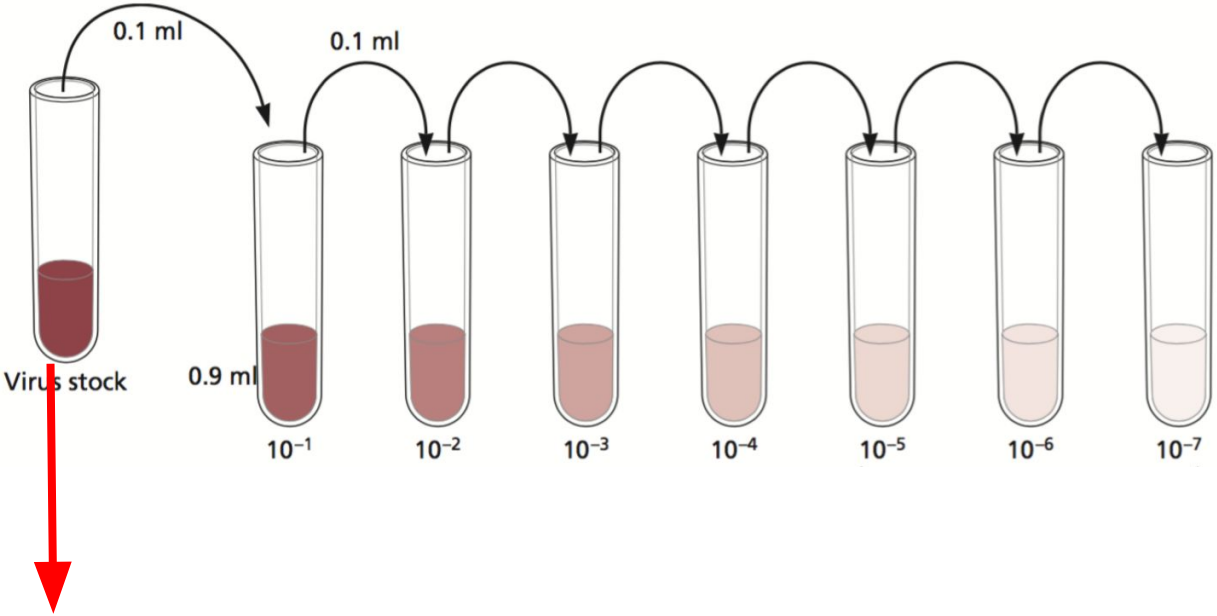
By Renato Dulbecco

California Institute of Technology, Pasadena, California

Read before the Academy, April 29, 1952

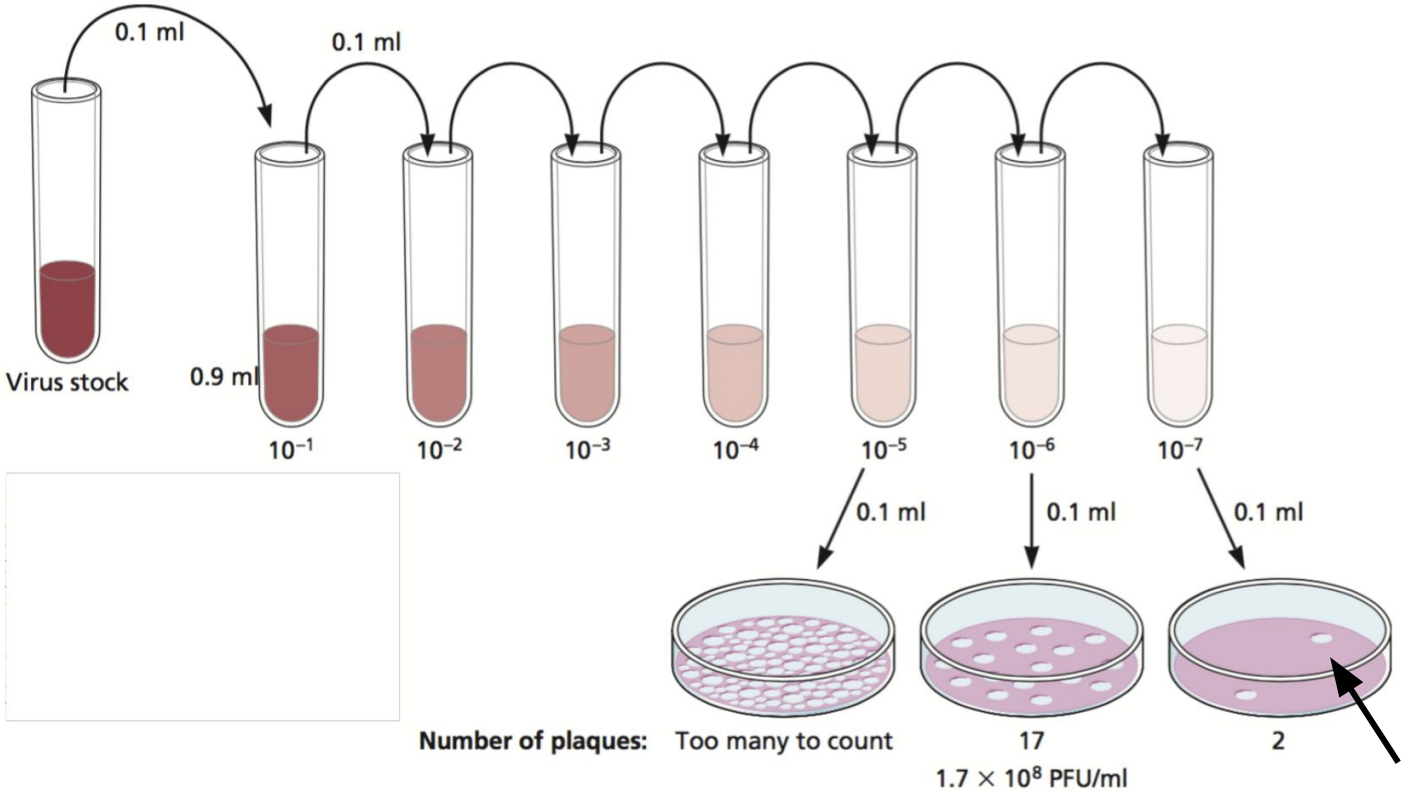
Research on the growth characteristics and genetic properties of animal viruses has stood greatly in need of improved quantitative techniques, such as those used in the related field of bacteriophage studies.

The requirements for a quantitative virus technique are as follows: (1) The use of a uniform type of host cell; (2) an accurate assay technique; (3) the isolation of the progeny of a single virus particle; and (4) the separate isolation of each of the virus particles produced by a single infected



This means that this tube has that many in it, that will form this number of plaques, we don't say virus particles.
And you will see why in a moment.

Plaque assay



This clear zone is a plaque

Plaque assay

- The way plaque assay is done:
- A solution of virus then do a ten fold serial dilutions (0.1 mL in 0.9 mL)
- Then, you have to have an idea of what your titer is going to be, if not yo can use all of the dilutions.
- One point 1 ml into three different cell culture plates, which we know is susceptible and permissive to the virus, and then we cover these plates with agar overlay and incubate them overnight
- Plaques will be formed and then the monolayer will be stained to see the plaques very clearly.

Plaque assay

- The plaques are counted, in the middle there are a good number.
- There are 17 plaques on the dilution of 10^{-6} but remember there is another tenfold dilution.
- 0.1 is another tenfold so that is 10^{7} dilution.
- So 17 X 10^{7} PFU/ml OR 1.7 X 10^{8} and that is the titer in PFU/ml.

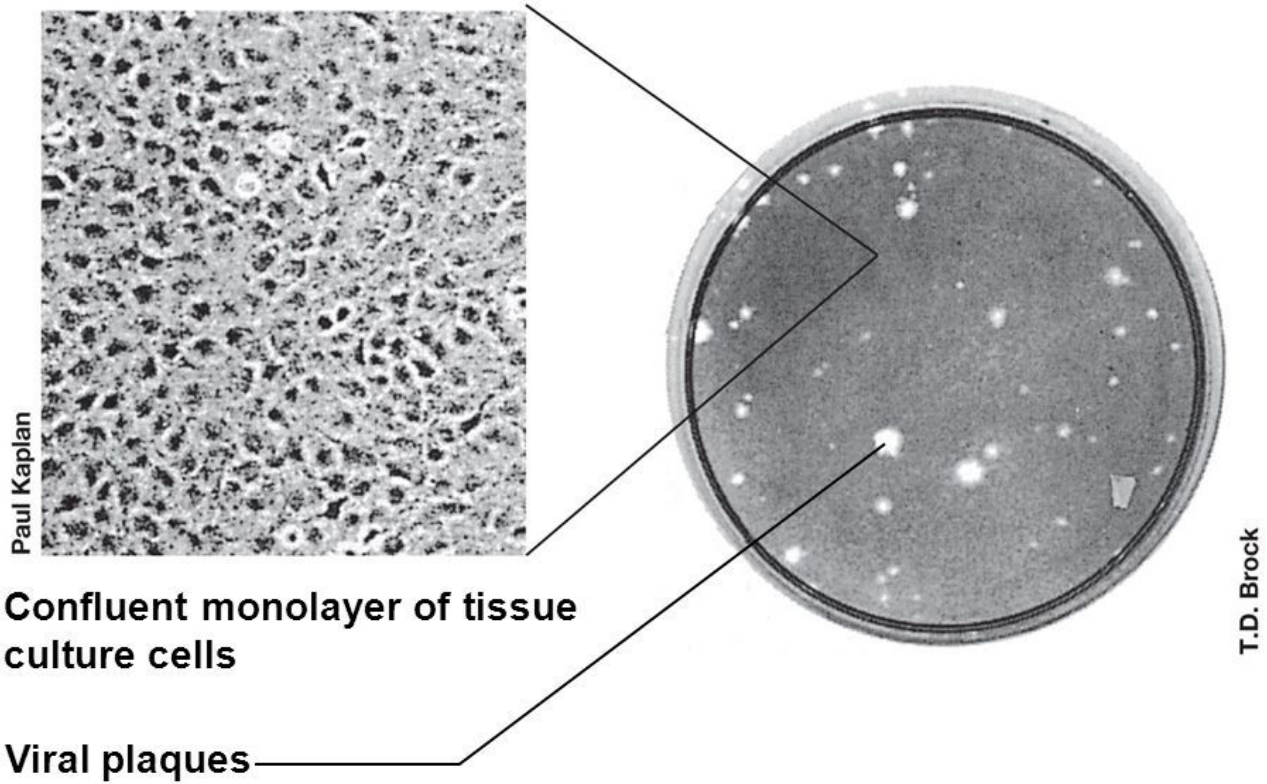
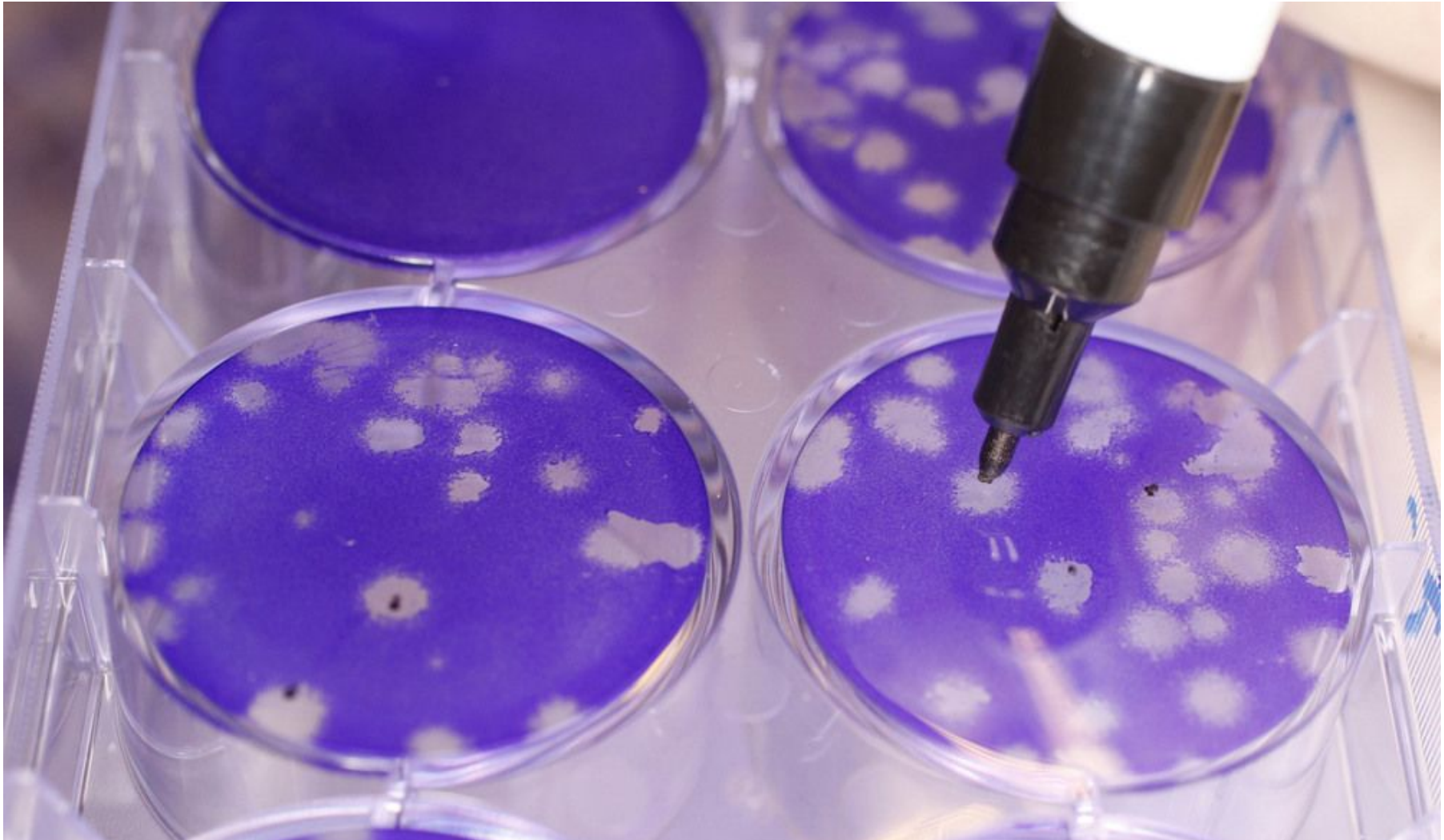


Figure 8.9



Formula for calculating virus concentration (Titre)

- $Virus\ titre = \frac{(Number\ of\ virus\ plaques) * (Dilution\ factor)}{Volume\ of\ apllied\ virus\ solution\ (mL)}$

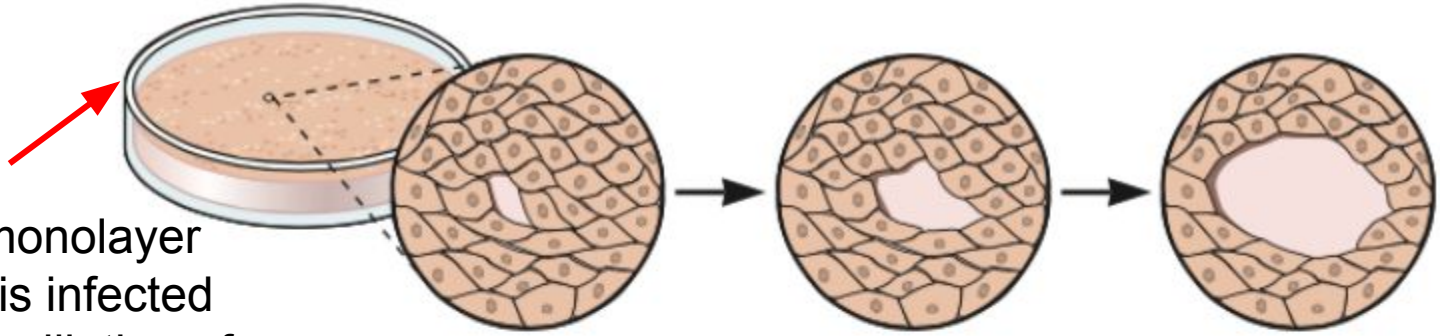
- E.g. After applying 200 µL of a virus 10^4 dilution, 40 plaques in well A, 45 plaques in well B. What is the pfu/ml?

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

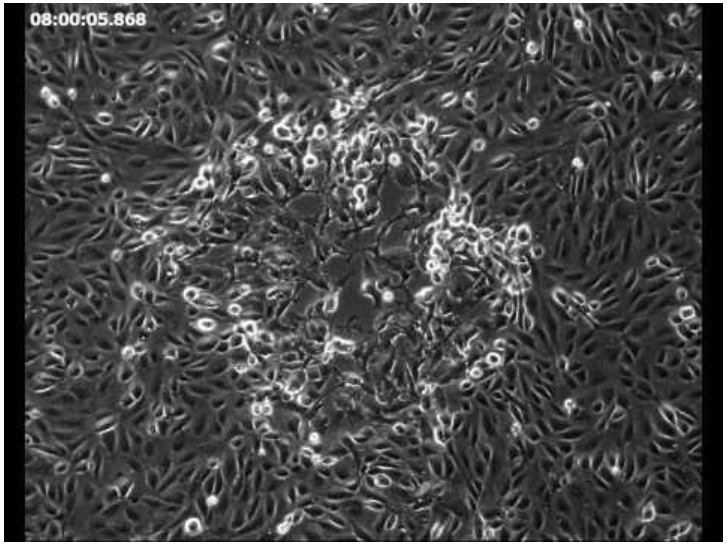
$$Virus\ titre\ in\ well\ B = \frac{45 * 10^4}{0.2\ (mL)} = 2.25 * 10^6 \frac{pfu}{mL}$$

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

NYP



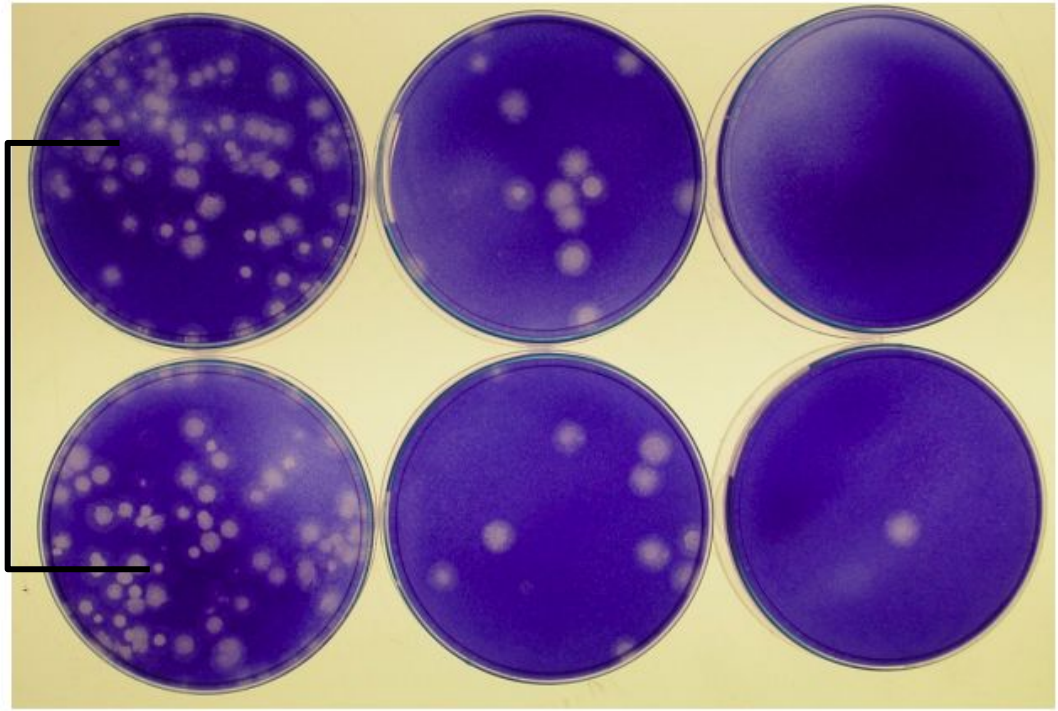
Cell monolayer and it is infected with the dilution of the virus



Virus plaque formation

- One virus is going to infect a single cell, that virus maybe 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 new viruses who in turn will infect more cells.
- That make a bigger and bigger hole in the monolayer, because the clle are dying.
- Remember there is an agar overlay on top of the 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 go to the liquid and spread throughout the cells, and the cells will be dead in a day or two, and there will be no plaques to count.

Pairs of dilutions, this is influenza virus.



How many viruses are needed to form a plaque?

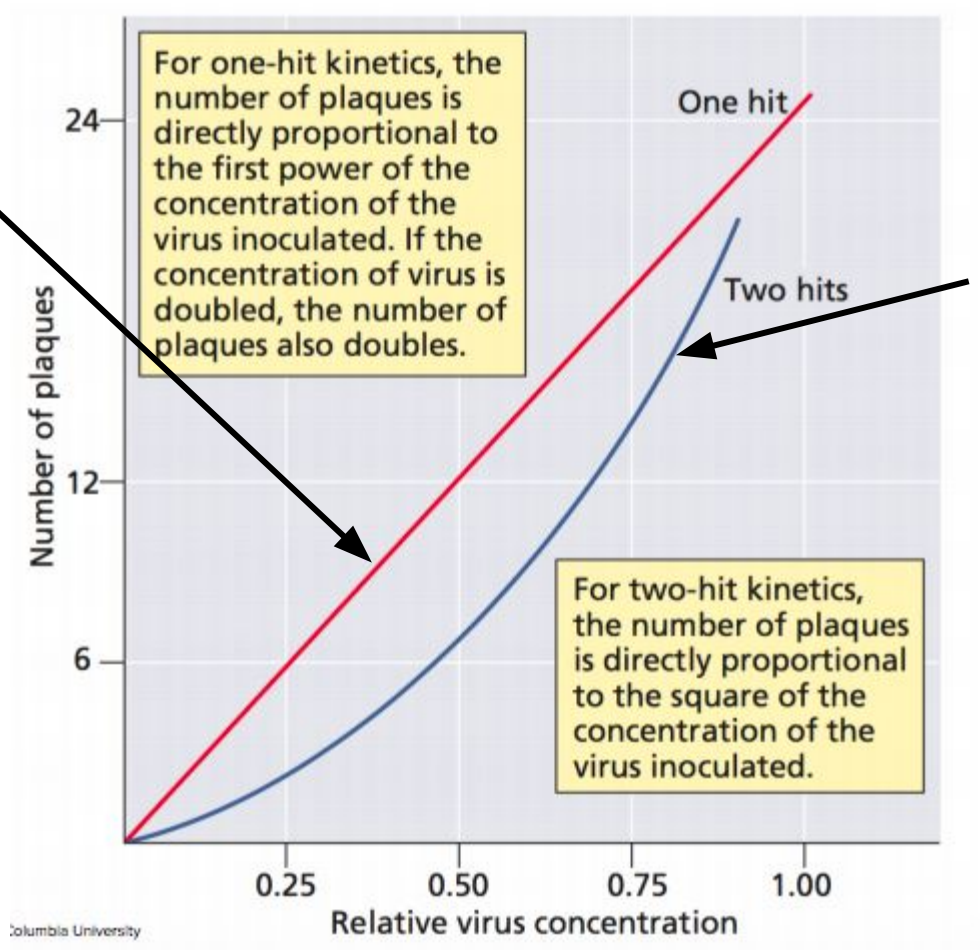
Why we use PFU/ml instead of virus particles/ml?
To figure this out, we use dose response curve.

Dose response curve

- Dose response curve means; you make dilutions of something and you, and then observe what happens, it can be done with drugs or with viruses.
- It is a XY plot.
- The X-axis is the relative concentration of the virus and it is increasing from left to right; these values are dilutions plotted in the opposite way.
- The Y-axis is the number of plaques.
- What is done is a series of dilutions of the wanted virus, plaque it out and count the number of plaques.
- The kind of line that results, whether it is a line or a curve indicate if one or more viruses are needed to form a plaque.

One virus particle to infect the cell and cause a plaque formation

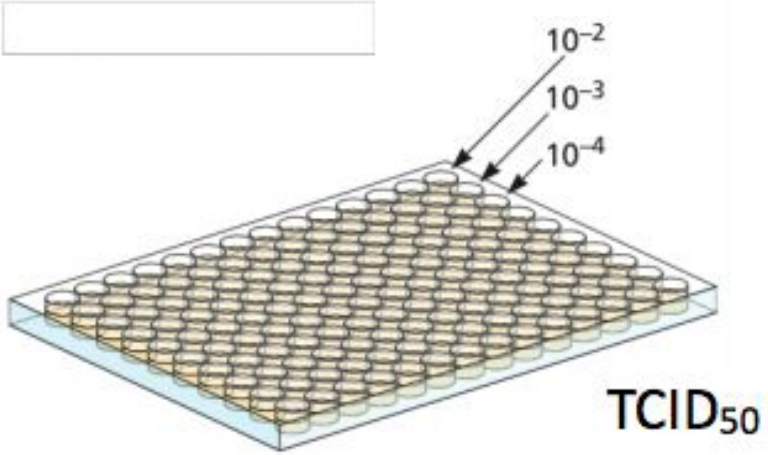
Dulbecco did this with his animal virus plaque assay and a got a straight line; that's why he concluded that single virus particle to form a plaque.

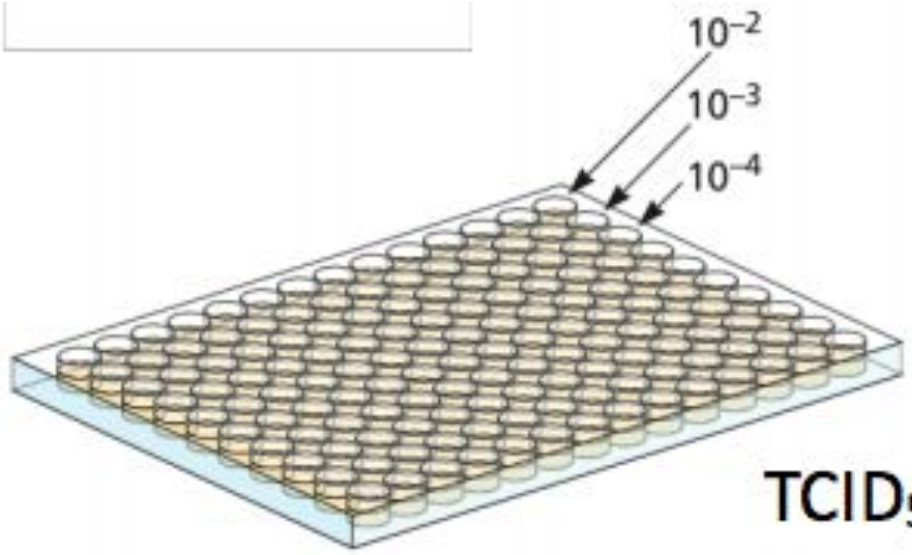


Two virus particles are needed to form a plaque.
Some viruses have 2 pieces of genomic information and packaged in separate particles, both of them are needed to infect a cell.

End-point dilution assay

- Sometimes viruses don’t form a plaque, how hard you work and try different agars. Some viruses just cannot infect when agar is present
- So there should be another way to quantify infectivity:
- End-point dilution assay: these are done typically in 96-well plates
- Cells are put in each well and a dilution is made of the virus
- Infect each well with a dilution
- This can be done for multiple samples and multiple replicates.
- Then incubate the cultures and observe which wells shows CPE





$TCID_{50}$

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

End-point dilution assay

- If the virus is showing CPE why it is not forming a plaque?
- It happens that whatever the agar you chose will be inhibitory to the virus forming a plaque, but in liquid the virus can kill cells.
- Serial dilutions of a virus stock inoculated into a replicate test unites (wells).
- The number of the wells that become infected (shows CPE) is counted for each dilution as shown in the previous table. (+ means showing a CPE).
- At high dilution (10^{-7}) none of the cell cultures are infected; because no infectious particles are delivered to the cells
- At low dilution (10^{-2} and 10^{-3}) every culture is infected.

End-point dilution assay

- The end-point, is the dilution of virus that affects 50% of the tests units

- From the tabel, the end-point here is at dilution 10^{-5}, where half of the wells displayed CPEs and half of them did not

- This number is called infectious doses 50% (ID_{50})
- Most of the time ID_{50} will get between two dilutions and is calculated using math.

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