Cell Counting using the Trypan Blue Exclusion Method

Learning outcomes

- Understand the basic structure of hemocytometer.
- Learn to load sample on hemocytometer.
- Defining Neubauer factor?
- Understand formulae for calculating cell viability and cell number in a sample.
- Calculate cell numbers using hemocytometer.
- Calculate cell viability in a sample after staining the cells with trypan blue.
- Practising focusing an inverted microscope to visualize mammalian cells.
- Identify the morphology of cells (sample given during practical)
- Estimating the confluency of cells in three different samples.

Trypan Blue

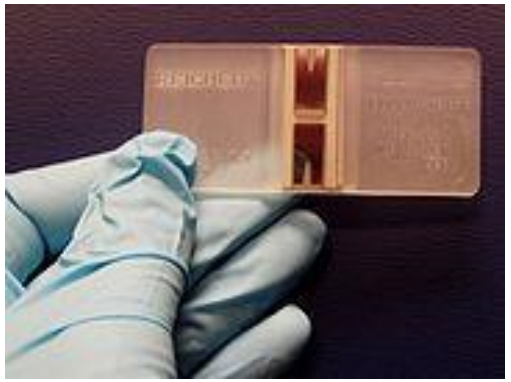
- Trypan Blue Solution, 0.4%, is routinely used as a cell stain to assess cell viability using the dye exclusion test.
- This test is often performed while counting cells with the hemocytometer during routine sub-culturing, but can be performed any time cell viability needs to be determined quickly and accurately.
- The dye exclusion test is based upon the concept that viable cells do not take up impermeable dyes (like Trypan Blue), but dead cells are permeable and take up the dye.

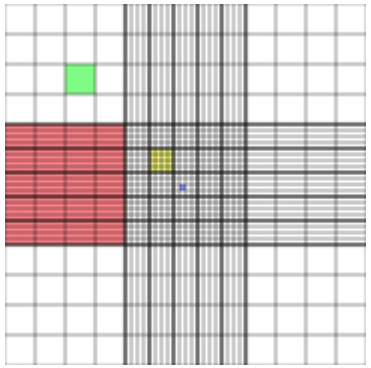
https://www.thermofisher.com/order/catalog/product/15250061

Hemocytometer

- The **hemocytometer** or haemocytometer is a device originally designed for the counting of blood cells. It is now also used to count other types of cells as well as other microscopic particles.

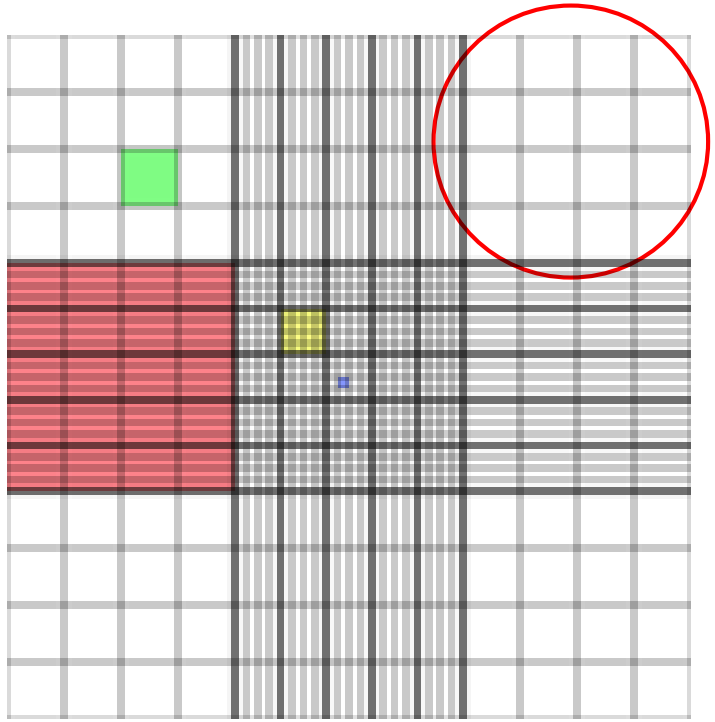
- The hemocytometer was invented by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. It is therefore possible to count the number of cells or particles in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall.





Neubauer – Hemocytometer

- In an improved Neubauer hemocytometer, the total number of cells per ml can be discovered by simply multiplying the total number of cells found in the hemocytometer grid (16 Squares) by 10^4.

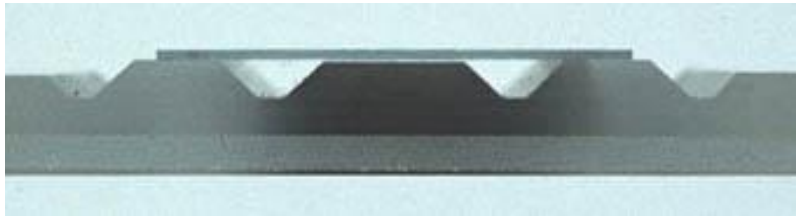


Hemocytometer grid:
Red square = 1 mm^2
Green square = 0.0625 mm^2
Yellow square = 0.04 mm^2
Blue square = 0.0025 mm^2

Count cells in 4 corner squares, on top and left border, take average

Neubauer Factor (10^4)

- With the coverslip on the chamber, the coverslip sits 0.1mm over the chamber. Therefore, the volume of each square, contained under the coverslip is:
- 1mm x 1mm x 0.1mm = $0.1mm^3$ / $0.1\mu l$ / 10^{-4} ml **(10mm = 1cm; 1mm = 0.1cm; $1mm^3 = 10^{-3}$ ml; $0.1mm^3 = 10^{-4}$ ml (the vol. of 1 square))**



http://e-learning.studmed.unibe.ch/hemosurf_demo/Demo_E/Lab/count_manual.htm

Neubauer Factor (10^4)

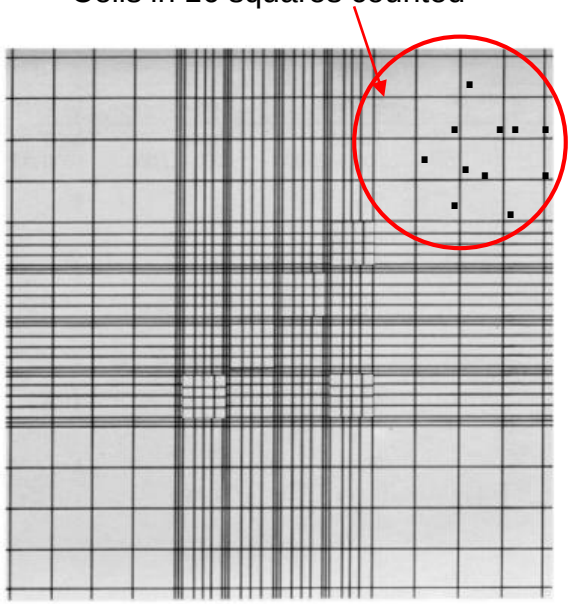
- If 0.1 µl has = 1 cell
- Then 1000 µl would have = $\frac{1x\ 1000}{0.1}$ = 10000 = **10^4 cells**

So, for calculating cell number per mL, multiply the counted number of cells with **Neubauer Factor (10^4)**

Cell Count Determination - Hemocytometer

Neubauer Hemocytometer

Cells in 16 squares counted



Light Microscope: Magnification 100 x

You got your cell suspension (10 ml) and want to know how many cells you might have in your cell suspension.

You add 1 Vol of cell suspension to 1 Vol of Trypan Blue solution.

Trypan Blue stains dead cells only.

You count cells in **16 squares**: e.g. **80 white** (unstained/white cells)
And **20 blue cells** (stained cells). Makes a total of **100 cells**.

Total Cell count determination (total cell concentration):

100 x 2 (due to 1:1 dilution) = 200
200 x 10^4 (**Neubauer Factor**) = **2 x 10^6/ml** (total cell concentration)

Total Cell Number:

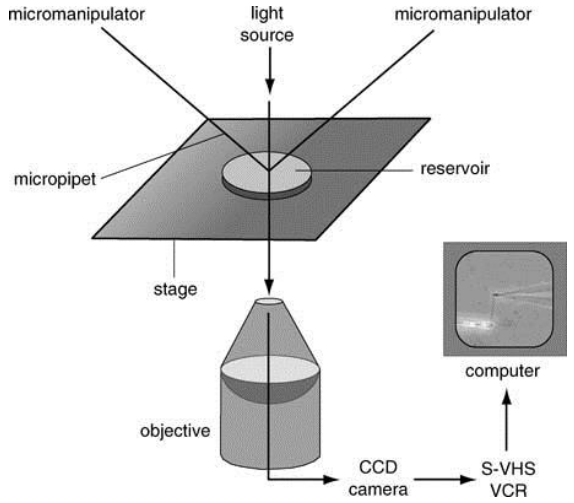
10 ml of cell solution: 10 x 2 x 10^6 = 2 x 10^7 cells (total cell number)

Cell Viability (%): $\frac{\text{number of white cells x 100}}{\text{total cell number}}$

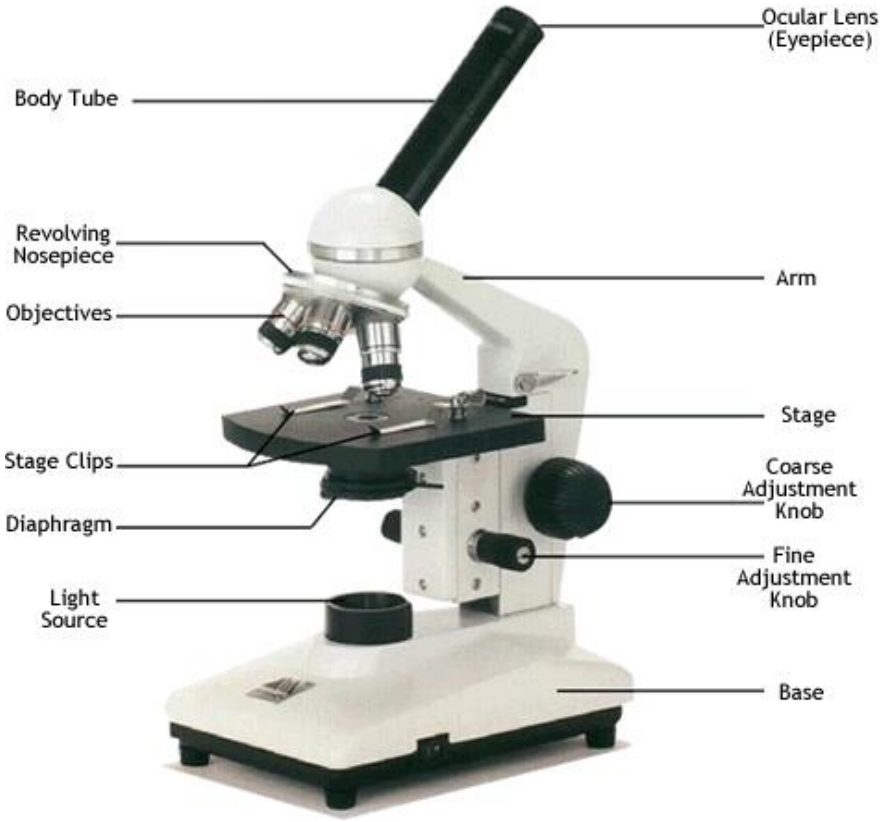
80 x 100 / 100 = 80% Cell Viability

Inverted Microscope

- An **inverted microscope** is a microscope with its light source and condenser on top, whereas the objectives are below the stage pointing up. It was invented in 1850 by J. Lawrence Smith.
- Inverted microscopes are useful for observing living cells (or organisms) at the bottom of a tissue culture flask under more natural conditions than on a glass slide, as is the case with a conventional light microscope.



Light Microscope



Inverted Microscope

Laboratory Practical

The purpose of this laboratory exercise is to acquaint you with some of the fundamentals of culturing cells *in vitro*.

(1) What do cell cultures look like?

(2) Where do the cells grow?

(3) What do the cells in the culture look like?

In this exercise you will:

(1) Observe the cell culture provided;

(2) Count cells to determine how many cells were in the original plate;

(3) Determine the viability of cells.

MATERIALS

- Hemocytometer plus a supply of cover slips.
- 0.4% Trypan Blue stain (fresh & filtered) in phosphate buffered saline.
- Cell Suspension.
- Micropipettes and tips.
- Inverted microscope.

Lab Coats
Gloves

Workflow to be followed

Visualize cells under microscope (record confluency, morphology, cell line name)

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Prepare the hemocytometer

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Prepare a 1:1 dilution of the cell suspension in trypan blue

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Load the hemocytometer

↓

Determine the number of cells (total, live and dead)

↓

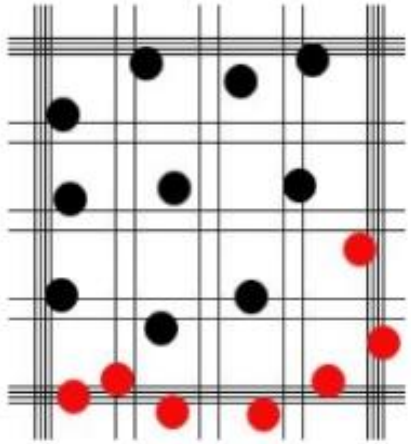
Calculations

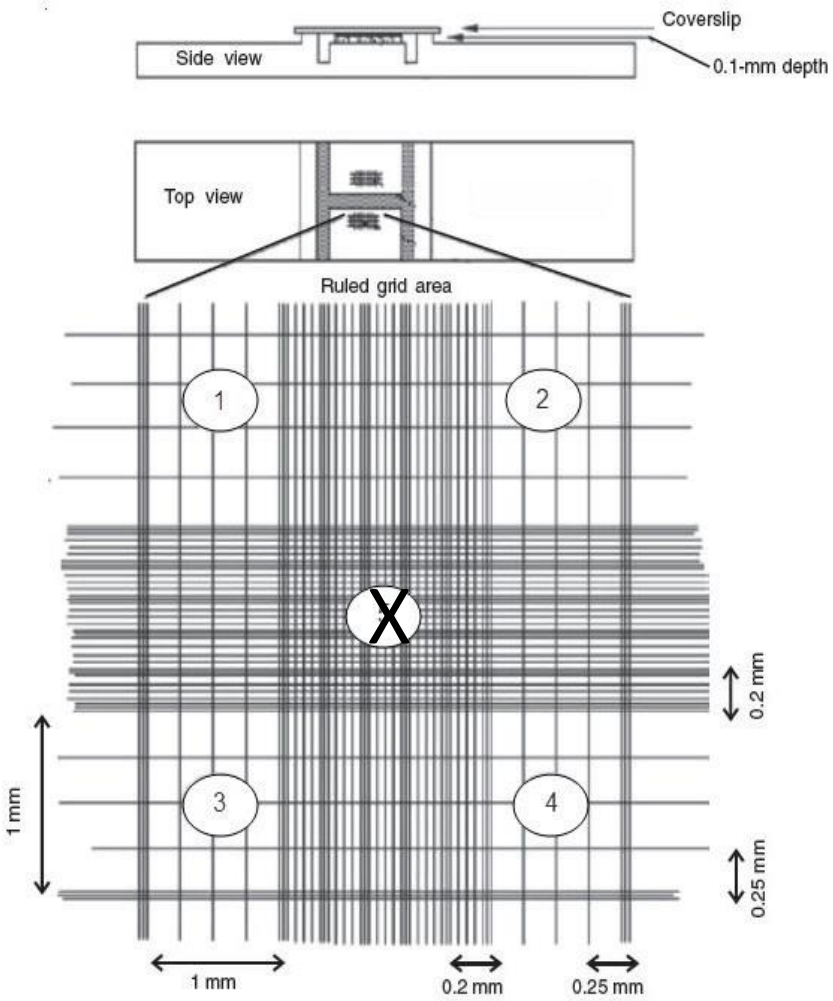
↓

Cleaning the hemocytometer

Cell Counting

- For purposes of this practical, you will only count cells touching the top and left border (count ‘black’ and not ‘red’ as shown in figure). You will count the cells in four large corner squares (i.e. those divided further into sixteen small squares).





Calculations

Calculate the average #cells from chamber

Average # Live Cells _____**150**_____

Average # Dead Cells___**20**_____

Average # Total Cells_____**170**_____

Calculate the cell density or Total cell concentration in the original suspension (in cells/ml) is:

 # Cells/ml = (Average # total cells) X dilution factor X 10^4

Dilution Factor = Total Volume (Volume of sample + Volume of diluting liquid) / Volume of sample. The dilution factor for this example is 2 because 20 µl of cell suspension was diluted with 20 µl Trypan Blue.

 # Cells/ml = _____**170**_____ **X 2 x 10^4**

 # Cells/ml = _____**340 x 10^4** _____**= 3.4 x 10^6/ml**

Calculate cell viability

Average # live cells / Average # total cells x 100

____**150**___ / ___**170**_____ **x 100**

Cell viability = _____**88.23**_____**%**