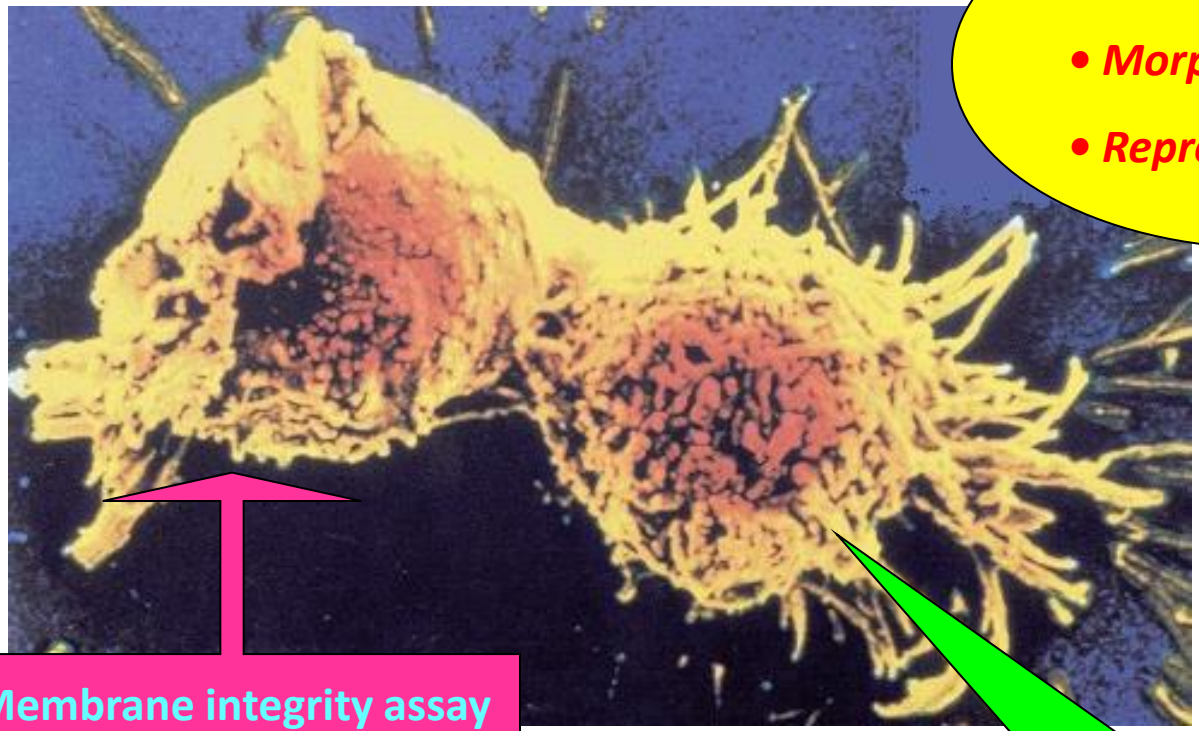
Cell Viability I



- *DNA labeling assay*
- *Morphological assay*
- *Reproductive assay*

Membrane integrity assay

Functional assay

Prof. Arttatrana Pal
Department of Zoology
School of Life Sciences
Mahatma Gandhi Central University, Bihar

The Goal

To distinguish live cells from dead and apoptotic cells in order to calculate the percentage of viable cells for each experiment

Major criteria employed in viability assay

Category of viability assay	Assays	Principles
Membrane integrity assay	-Exclusion dyes -Fluorescent dyes -LDH leakage	The determination of membrane integrity via dye exclusion from live cells
Functional assay	-MTT, XTT assay -Crystal violet/ Acid phosphatase(AP) assay -Alamar Blue oxidation-reduction assay - Neutral red assay -[3H]-thymidin/ BrdU incorporation	Examining metabolic components that are necessary for cell growth
DNA labeling assay	-Fluorescent conjugates	Simultaneous cell selection and viabilityassay
Morphological assay	-Microscopic observation	Determination of morphological change
Reproductive assay	-Colony formation assay	Determination of growth rate

Cell counting

Measurement	Characteristics	Methods
Direct	- Distinguish viable and non-viable cell - Simple, quick, cheap - A small fraction of the total cells from a cell suspension	- Microscopic counting (Hemocytometer) : Trypan blue, Crystal violet - Electronic counters : Coulter counter (an apparatus for counting and sizing particles sus pended in electrolytes. It is used for cells, bacteria, Eukaryotic cells, pro karyotic cells and virus particles)
Indirect	- Monolayer - Immobilized in matrix	- Lactate dehydrogenase (LDH) activity - Functional assay - DNA labeling assay

Category of viability assay

- **Membrane integrity assay**
  - Hemocytometer
  - Trypan blue assay / Automatic trypan blue method/ LDH leakage / Fluorescent dyes
- **Functional Assay**
  - MTT / XTT assay
  - Crystal violet / Acid phosphatase(AP) assay
  - Alamar Blue oxidation-reduction assay / Neutral red assay ([3H]-thymidin and BrdU incorporation)
- **DNA Assay**
  - Enzymatic DNA labeling / DNA-binding dye labeling
- **Morphological assay**
- **Reproductive Assay**
  - Colony-forming efficiency
- **Laser scanning confocal microscopy**
- **Nuclear magnetic resonance methods**

Membrane integrity assays

Exclusion dyes

- Features distinguishing live from dead cells include the loss of transport function across plasma membrane which results from loss of membrane integrity
- Cells must be counted within 3-5 min because the number of blue-staining cells increases with time after addition of the dye
- Large numbers of samples have to be counted, it may be inconvenient to perform all the tests on the same day by counting one cell suspension at a time before staining the next sample
- In engineered tissues, because of the affinity of the dye for protein, trypan blue exclusion cannot be used to assess cell viability and proliferation in matrices

Materials and Equipment

- Trypan blue (0.4 g trypan blue in 100 ml physiological saline) → pass through a 0.22 μm filter
- Hemocytometer with coverslip
- Hand-held counter
- Microscope

Trypan blue

- A stain which will only enter across the membranes of dead/non-viable cells
  - Cause cancer in lab. animals
  - Appropriate precaution should be taken when handling trypan blue (use of extraction hood and gloves)

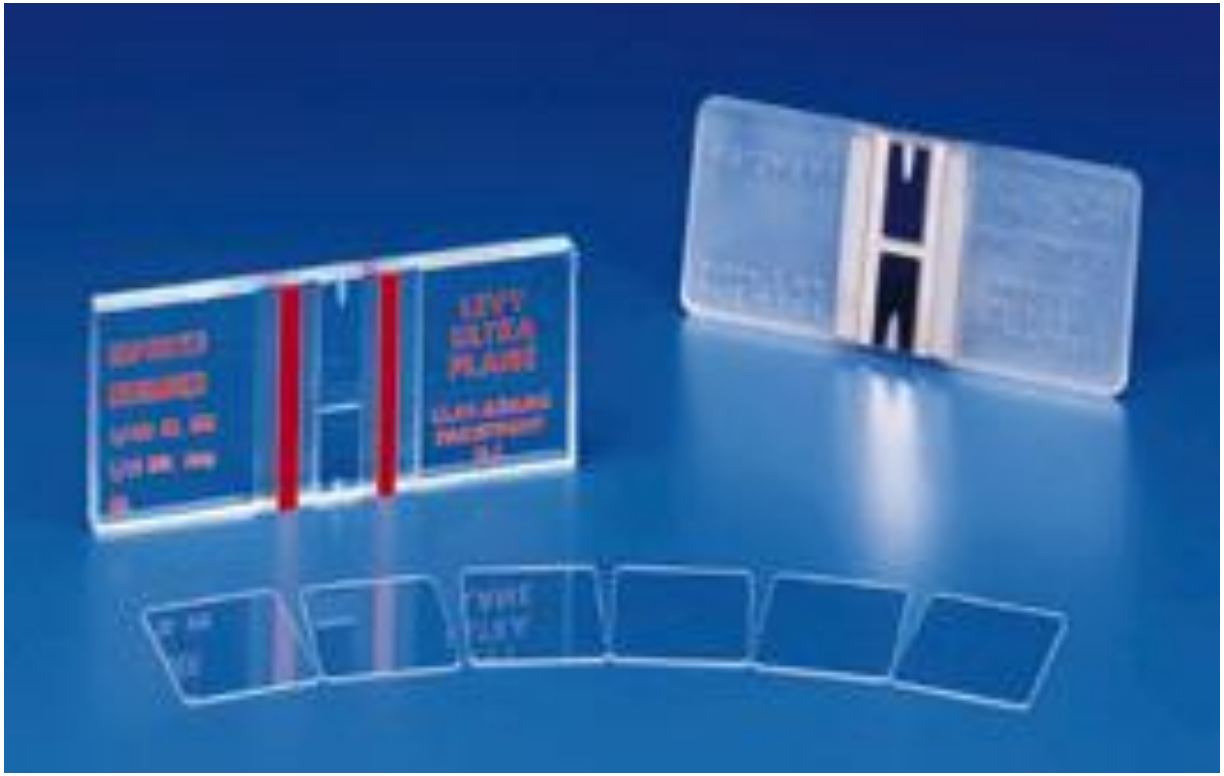
- Dilution by trypan blue

- Viable cells : small, round and refractive
- Non-viable cells : swollen, larger, dark blue

Hemocytometer Cell Counts

Hemocytometer

The most common routine method for cell counting which is efficient and accurate is with the use of a hemocytometer



Methods

- Clean hemocytometer & coverslip and wipe with 70% alcohol before use
- Place coverslip on hemocytometer
- Mix the cell suspension gently
- Aliquot 0.1 ml cell suspensions
- Add 0.1 ml (2-fold dilution), 0.3 ml (4-fold dilution) or 0.9 ml (10-fold dilution) trypan blue : appropriate range of cells to be counted
- Draw a sample into a Pasteur pipette after mixing
- Draw the cell suspension in to fill the chamber
- Using a light microscope at low power, count the number of cells
- Count the viable & non-viable cells in both halves of the chamber

Calculations

A = Vol. Of cell solution (ml)

B = Dilution factor in trypan blue

C = Mean number of unstained cells

D = Mean number of dead/stained cells

10^4 = Conversion of 0.1 mm^3 to ml

(1) Total number of viable cells

$A \times B \times C \times 10^4$

(2) Total dead cell count

$A \times B \times D \times 10^4$

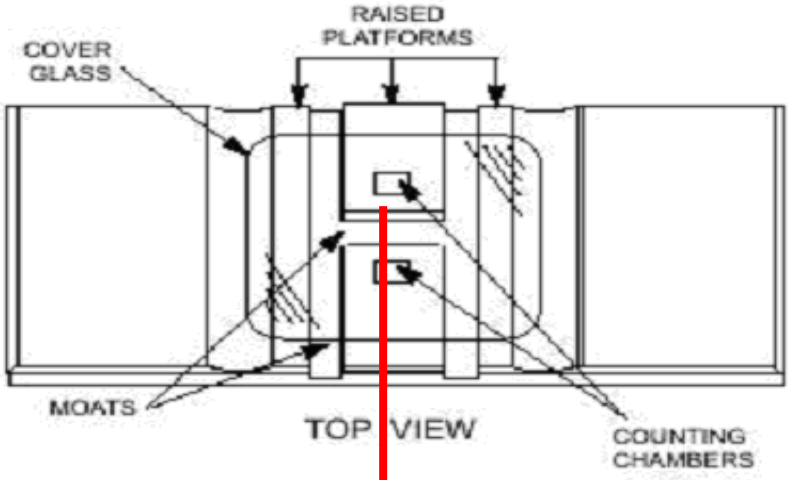
(3) To give a total cell count

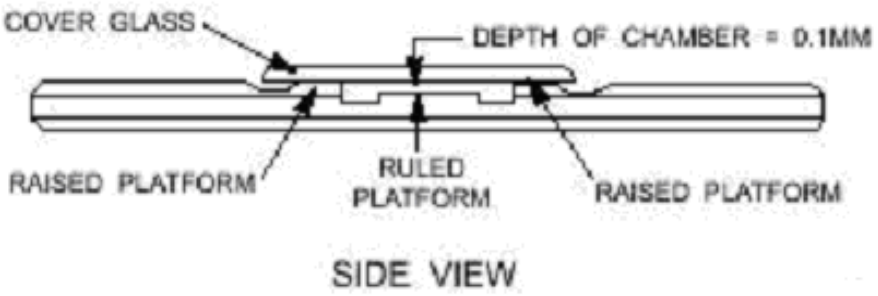
Viable cell count + dead cell count

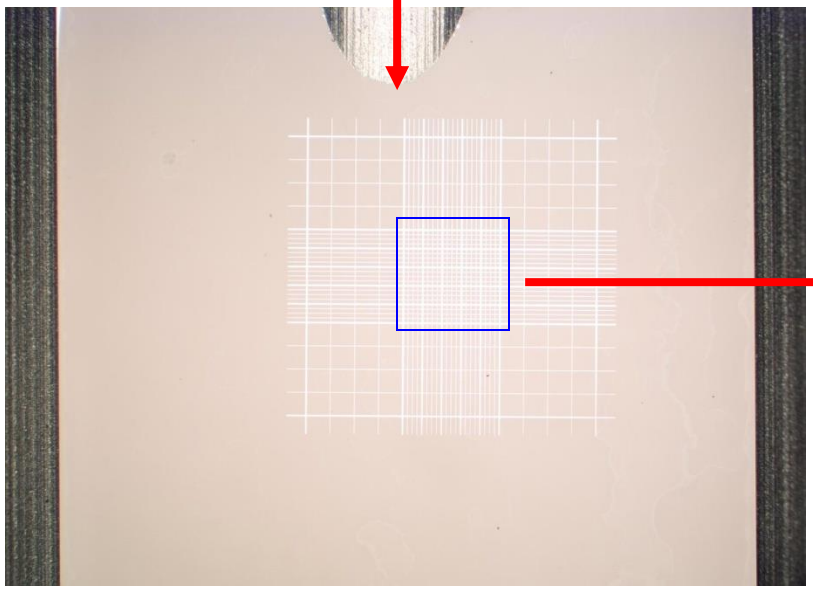
(4) % viability

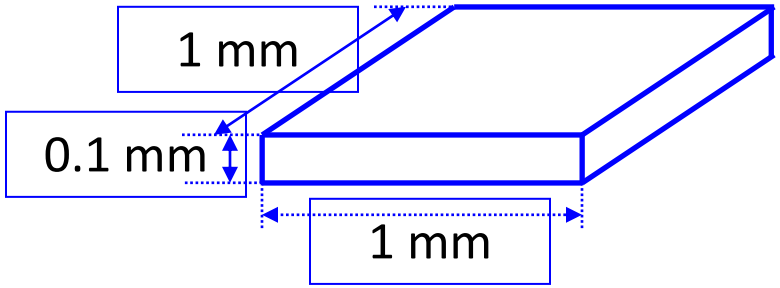
(Viable cell count/Total cell count) X 100

Hemocytometer



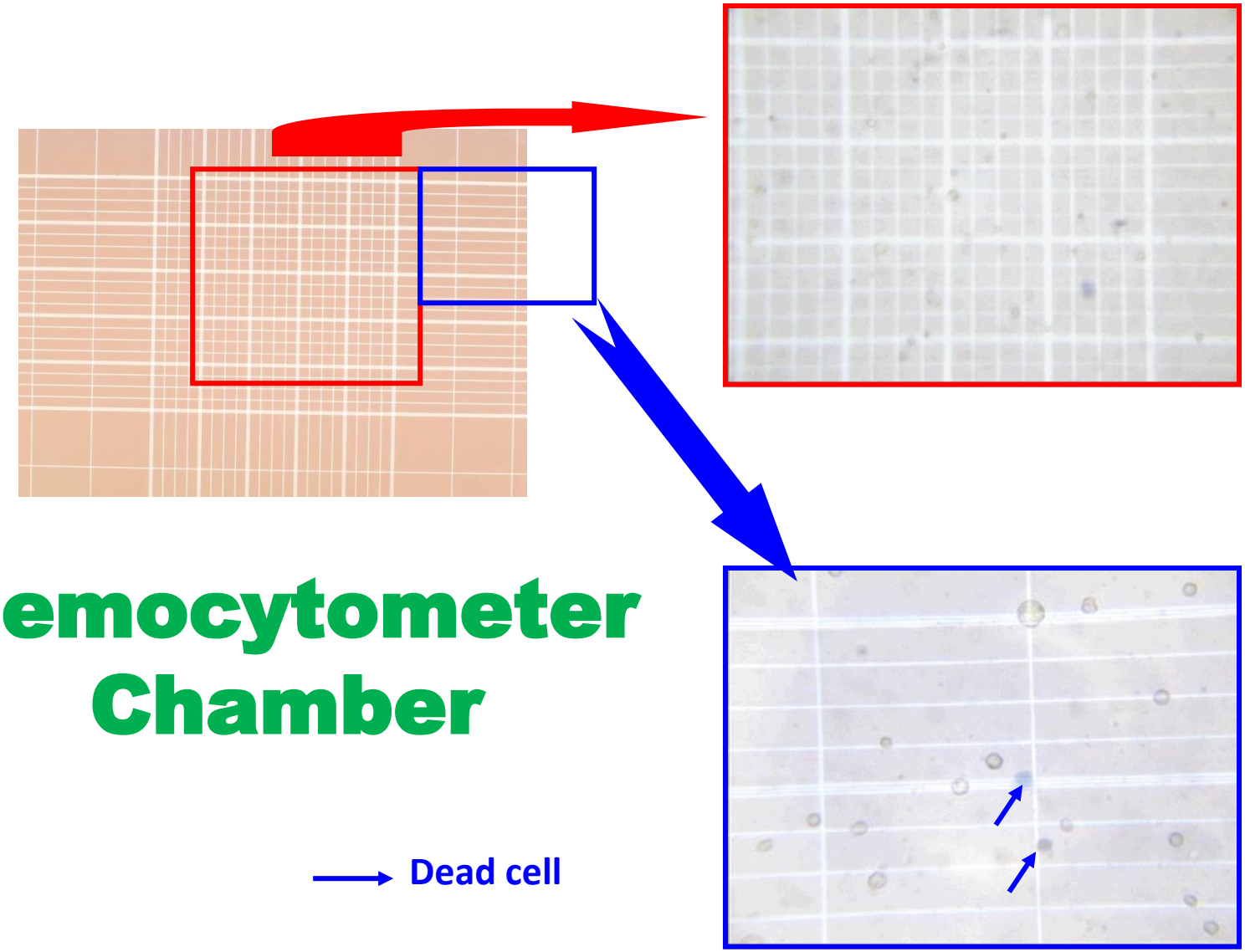






Volume : $0.1mm^3$

$1\ ml = 1\ cm^3 = 1000\ mm^3$

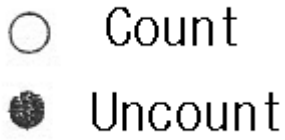


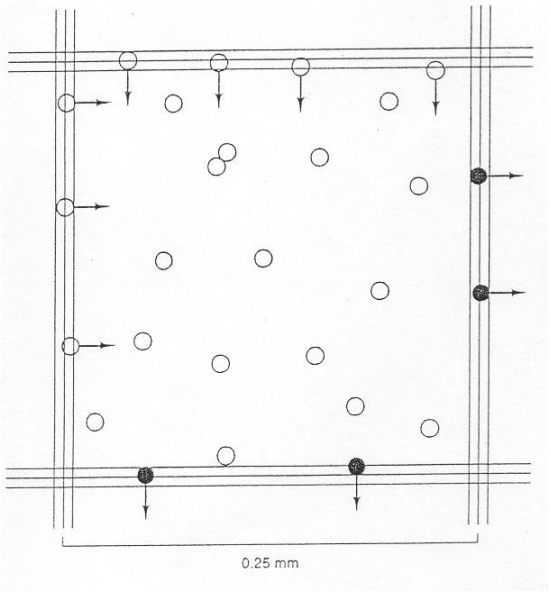
Hemocytometer Chamber

→ Dead cell

Example

1) **Vol. : Volume**
2) **CS : Cell Solution**
3) **TB : Trypan blue**





Dilution factor	Vol. of CS	Cell count	Total viable cells
0.1 ml CS + 0.1 ml TB (2)	20 ml	23	$20X2X23X10^4 = 9.2\ X10^6$ cells
0.1 ml CS + 0.3 ml TB (4)	15 ml	//	$15X4X23X10^4 = 1.38\ X10^7$ cells
0.1 ml CS + 0.9 ml TB (10)	10 ml	//	$10X10X23X10^4 = 2.3\ X10^7$ cells

Automated trypan blue method for optimal cell viability determination

www.innovatis.com

Background: Trypan blue

- The trypan blue dye exclusion assay is the most commonly used and accepted method for the measurement of cell viability

- It relies on the alteration in membrane integrity as determined by the uptake of dye by dead cells, thereby giving a direct measure of cell viability

- Based on optimal image analysis, the technology allow precise cell-viability and cell-density determination

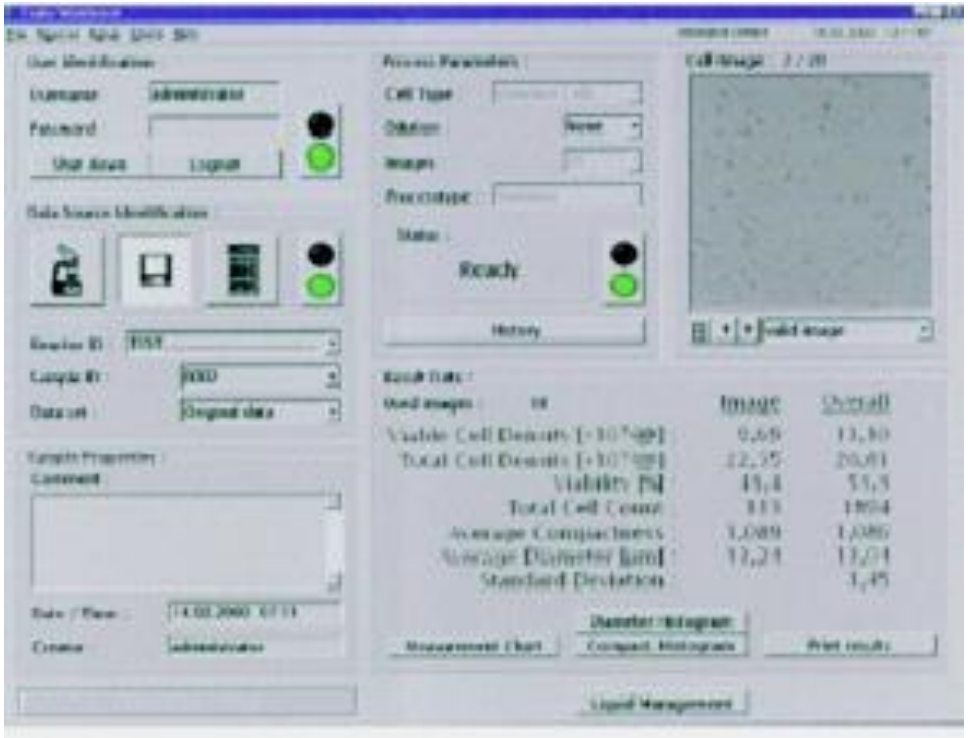
Background: Trypan blue

- The system performs **automatic and reproducible measurements** of human or animal suspension cell densities as well as standardized differentiation between viable and dead cells, **based on the trypan blue dye exclusion method**

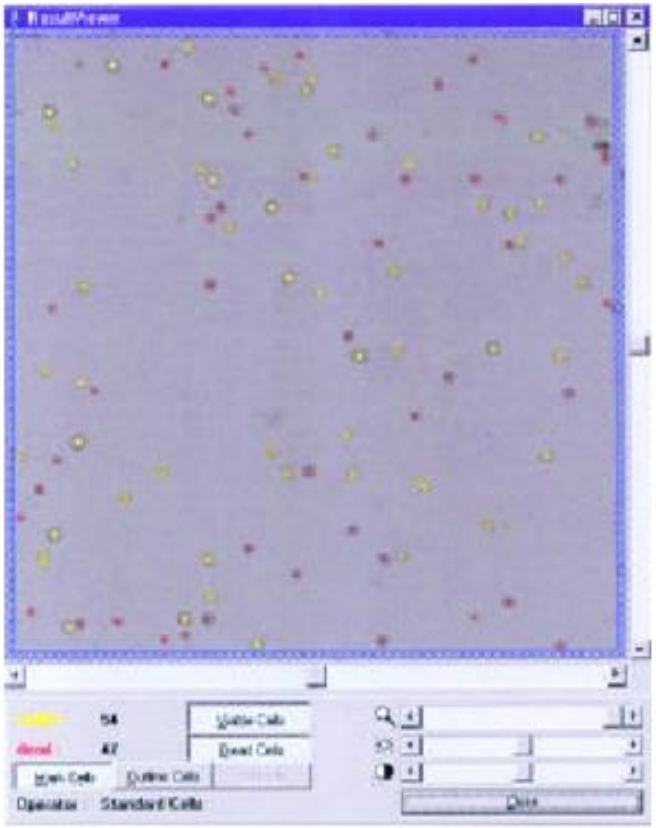
- **The direct and automated optical** analysis by means of modern pattern recognition methods allows cell identification and a **standardized differentiation between viable and dead cells** and also cell debris

The system consist of three functional part:

- the liquid handling unit
- image capture hard ware
- a data processing system, including the user interface

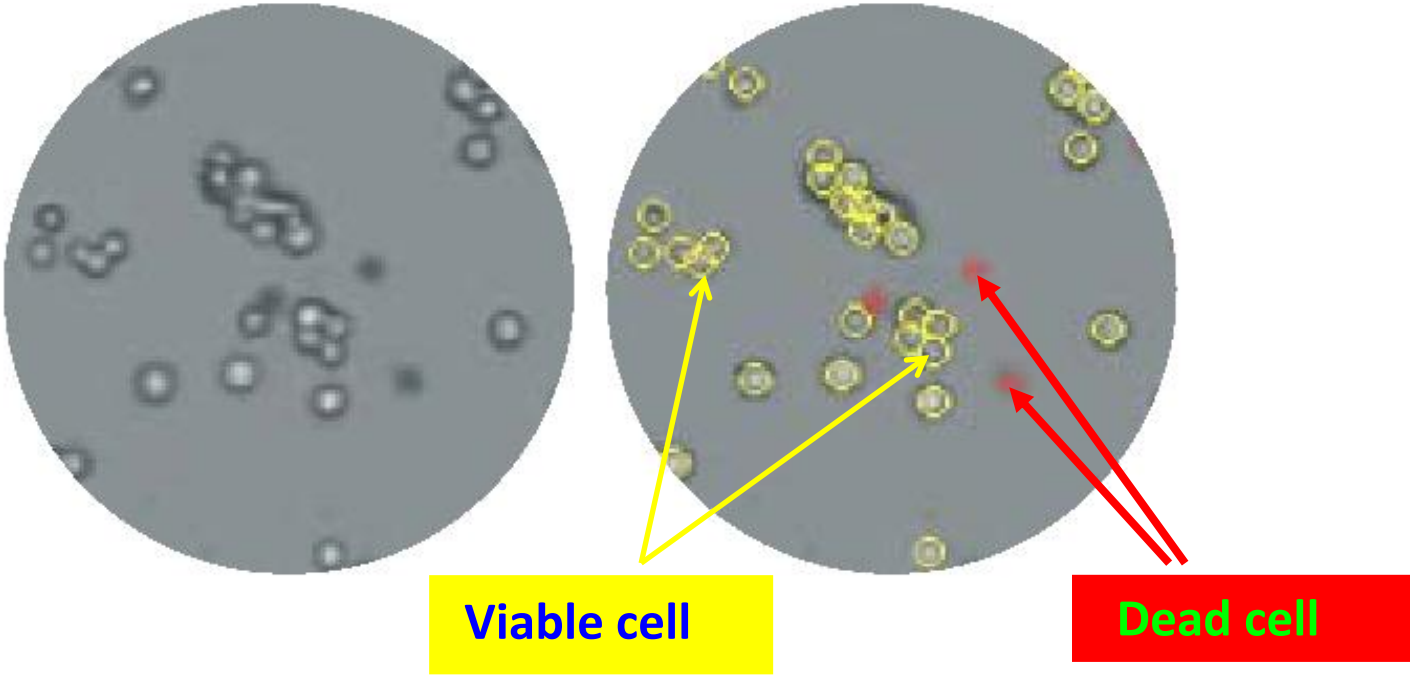


Cedex workbench



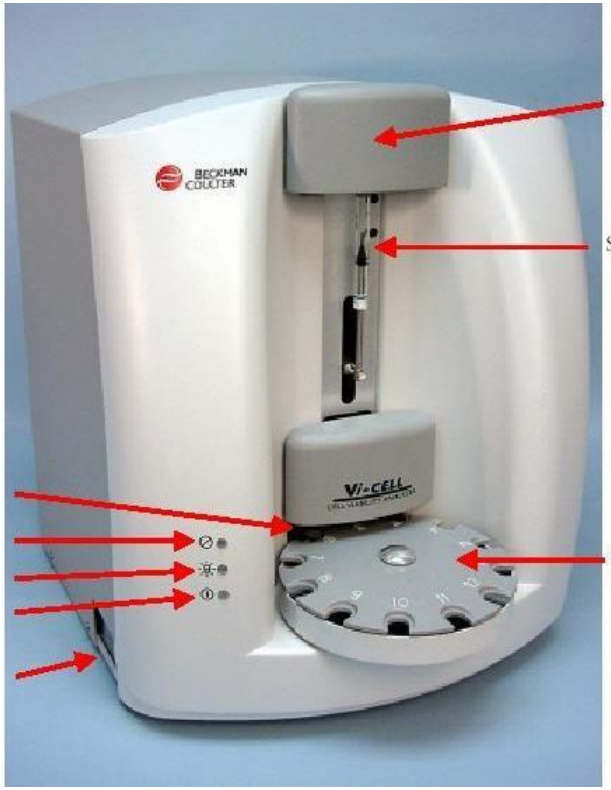
The IP Result viewer enables the user to control whether the Cedex system recognizes the cells correctly, and whether it reliably differentiates and between viable and dead cells

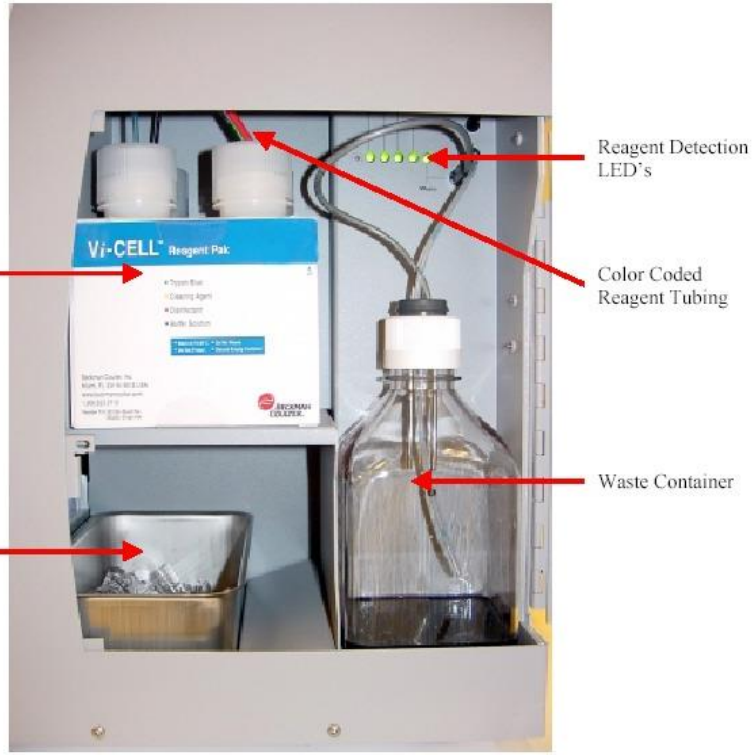
Result view of the image processing



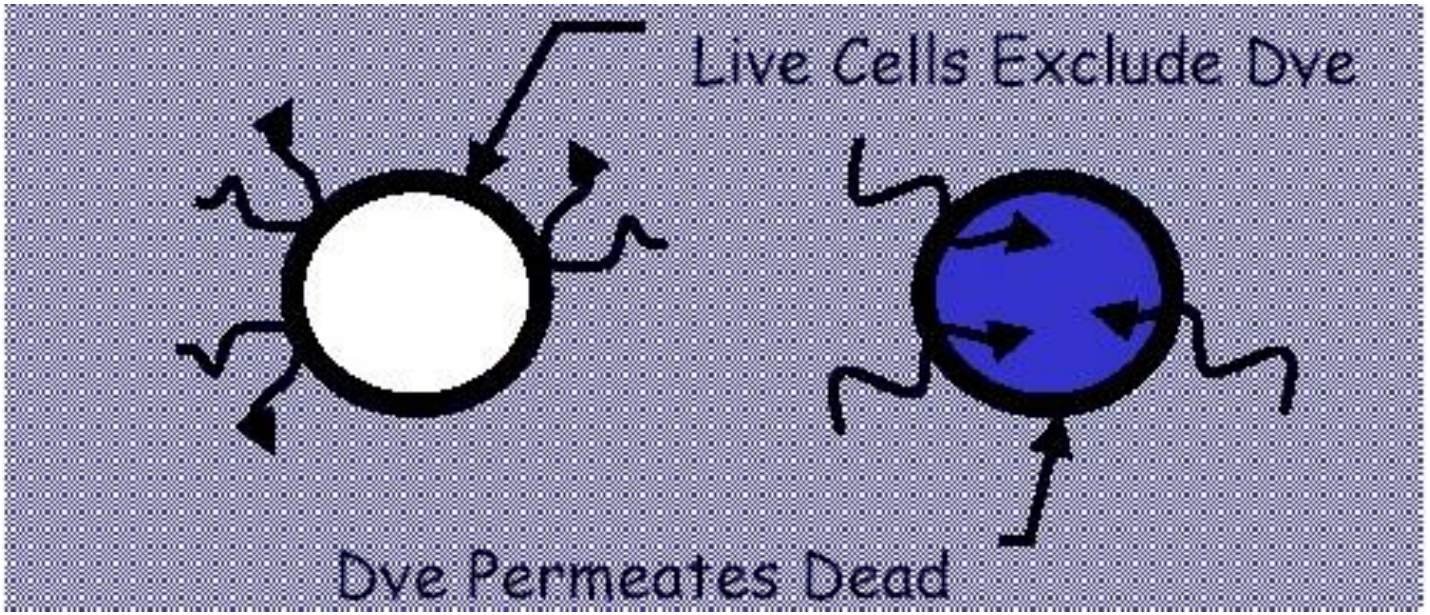
Marked viable and dead cells

Vi-CELL ™ CELL Viability Analyzer



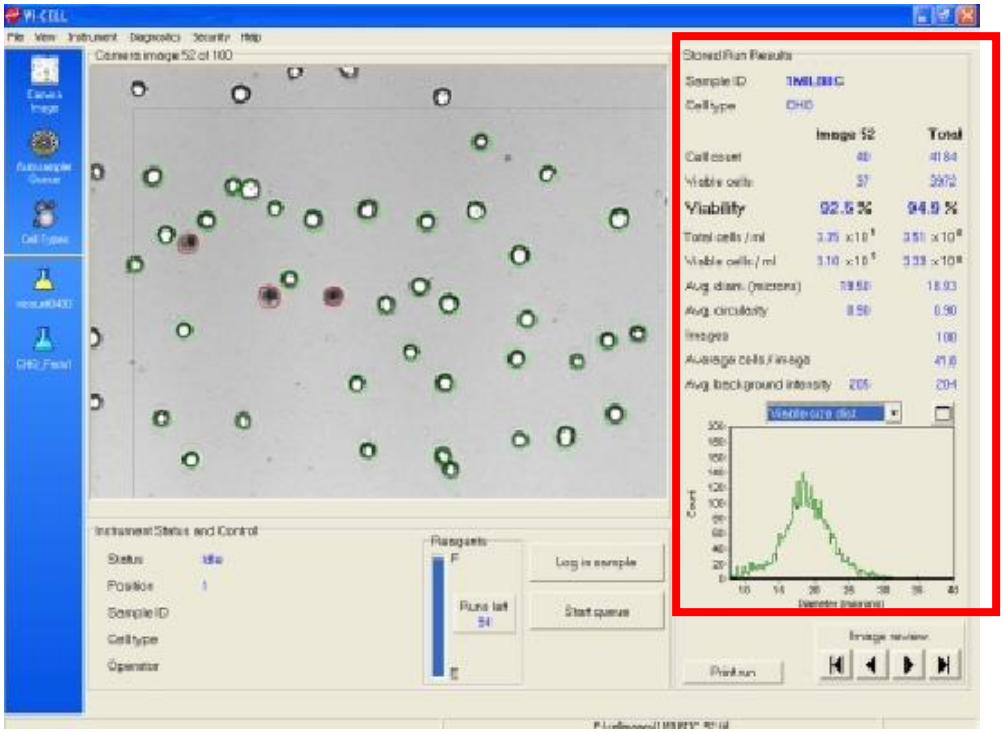


Principle



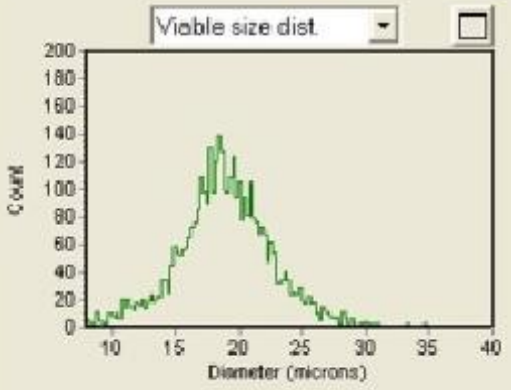
Trypan Blue dye Exclusion Methods

Run results



Stored Run Results

Sample ID	1MILBIIC	
Cell type	MILBIIC	
	Image 52	Total
Cell count	40	4181
Viable cells	37	3966
Viability	92.5 %	94.9 %
Total cells / ml	2.83 x10^6	2.96 x10^6
Viable cells / ml	2.62 x10^6	2.80 x10^6
Avg. diam. (microns)	19.50	19.00
Avg. circularity	0.94	0.93
Images		100
Average cells / image		41.8
Avg. background intensity	205	204



Electric Cell Counting using a COULTER® COUNTER

Introduction

- **Rapid, accurate, reproducible counts of cultivated cells can be obtained using electronic cell-counting techniques**
- **Used in range of cell counters and particle sizers**
- **Used extensively in the biomedical field for routine application and cell biology field for research tool**

  - blood cell counting, enumeration of immune complexes and bacteriological investigation

- Particles suspended in an electrolyte solution are drawn through a small aperture or orifice in the wall of an electrical insulator, across which a current path has been established by two immersed electrodes

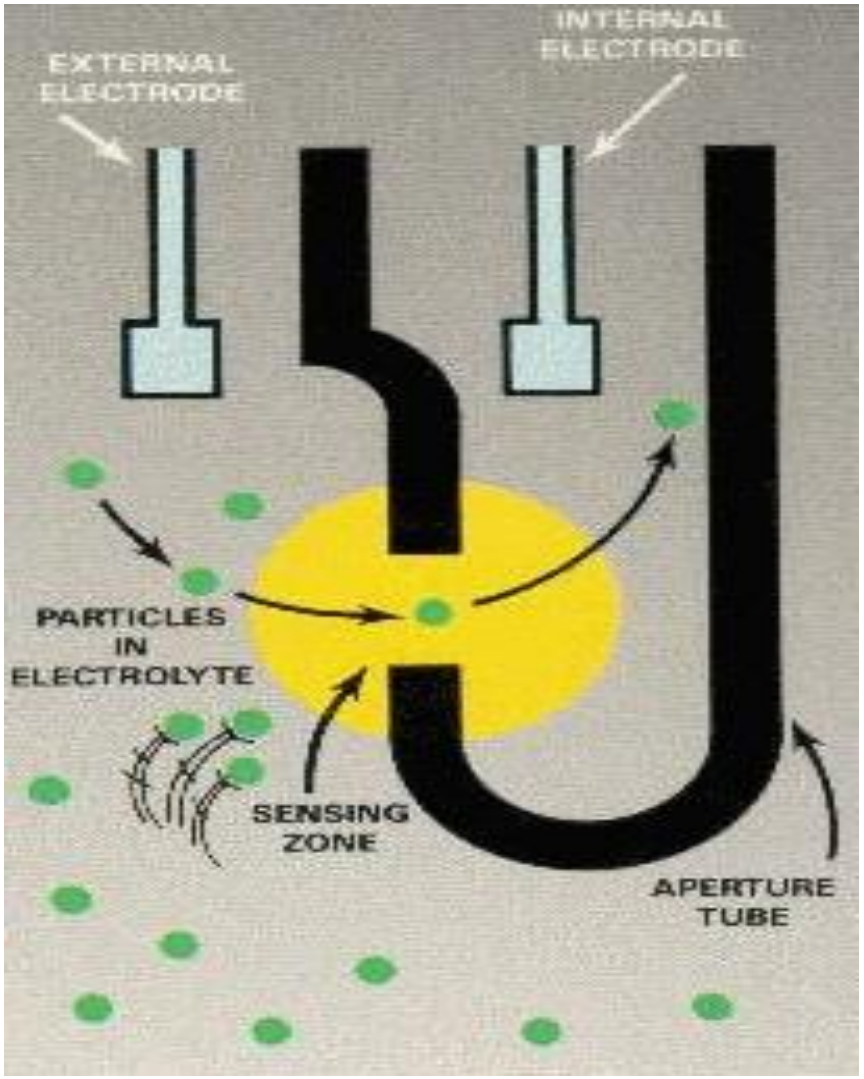
- As each particle enters the aperture, it displaces its own volume of electrolyte, thereby modulating the basic impedance of the current

- This is detected as a voltage pulse of short duration

- Proportional in height to the volume of the displaced electrolyte

- Also proportional to the magnitude of the particle size

The pulses produced by the passage of cells through the aperture can then be observed by oscilloscope and analyzed electrically to give a number versus particle volume distribution



- Almost cells are spherical form and the results are expressed conventionally as ‘spherical equivalents’ rather than by volume

- By setting threshold limits to eliminate pulses being produced by sub cellular particles, and coincidence corrections for doublets, triplets etc. of cells passing through aperture, the cell concentration within any suspension can be determined quickly and accurately

- Tens of thousands of cells are counted, providing low statistical deviations with an accuracy of measurement estimated by Coulter to be within 1% of the true count, if the coincident particle count is less than 15% of the total

Calculation

❖**The cell counts obtained at each time point may then be graphically analyzed to estimate the cell-doubling time**

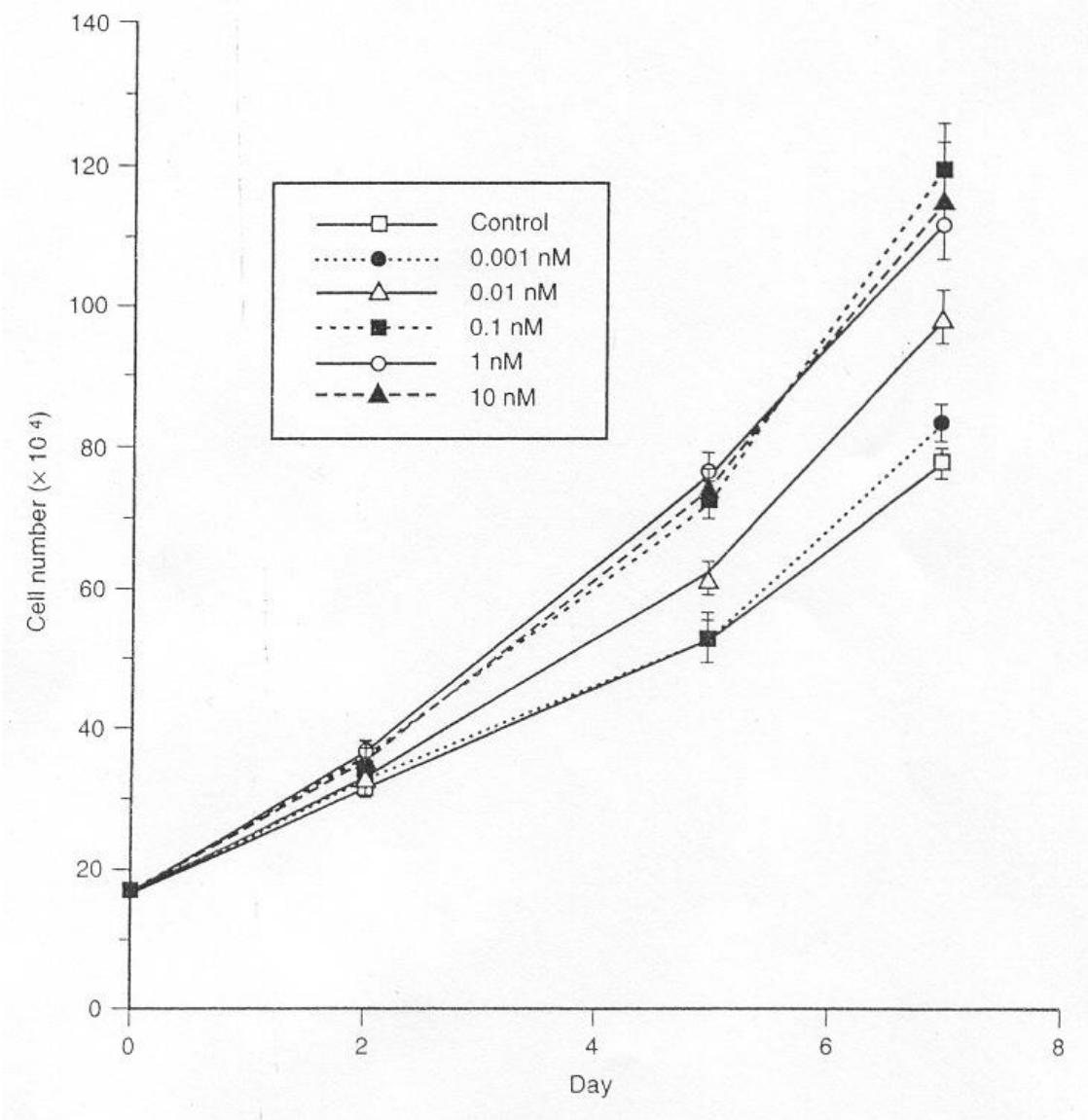


Fig. Growth curve demonstrating the effect of various concentrations of EGF (Epidermal growth factor) on the growth of human ovarian adenocarcinoma cells

LDH (lactate dehydrogenase) Leakage

Introduction

- **Quantitative value** for the loss of cell viability
- The activity of LDH can be measured **as the reduction of pyruvate to lactate**.
- The reduction is coupled to the oxidation of NADH to NAD^+, which is followed **spectrophotometrically at 340nm**

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightleftharpoons{\text{LDH}} NAD^+ + \text{lactate}$$

- As NADH has a high absorbance at 340nm compared to NAD^+, the reaction is **measured as the rate of decrease** in absorbance at 340nm.

Requirement of LDH assay

Greater process productivity (e.g. High-cell-density entrapped-culture systems) : difficulty of cell isolation

→ **Metabolic parameters(glucose uptake) : compromised because uptake/production rates can alter as a result of the cell switching carbon source**

→ **Analysis of the release of intracellular enzymes can be used enzyme in cell culture studies is LDH**

❖ **Assumptions of LDH assay**

: Intracellular enzymes are only released after damage to the cell membrane

: Rapidly released from damaged cells

Pitfalls of LDH assay

- The release of LDH activity can be related to the total No. of dead & lysed cells
- The stability of LDH can vary considerably, ranging from the loss of a few percent per day to a half-life of 12h depending upon the cell type
- Assumed that the release of LDH occurs rapidly after damage to the cell membrane. This assumption is not necessarily correct
- The release of LDH can be complete in cells which are considered dead by dye exclusion methods
- Complete release may only occur upon cell lysis
- This point is further complicated because dye exclusion methods do not measure lysed cells

Reagents and Solutions

- **Buffer** (Tris 81.3 mmol/L ; NaCl 203.3 mmol/L ; pH 7.2)

  : Dissolve 4.92 g Tris and 5.95 g NaCl in 400 ml water and adjust to pH 7.2 at 30℃ with HCl. Make up to a final volume of 500 ml with water

- **NADH solution** (β-NADH 0.17 mg/ml)

  : Dissolve 3.4 mg NADH in 20 ml buffer

- **Pyruvate solution** (9.76 mmol/L)

  : Dissolve 0.107 g monosodium pyruvate in 90 ml buffer. Make up to a final volume of 100 ml with buffer.

Stability of solutions

- **Buffer is stable at 0-4℃.**
- **The NADH solution is kept at 0-4℃ and must be prepared fresh daily**
- **The pyruvate solution should be dispensed into 1.5ml aliquots and stored at -20℃. After thawing, each aliquot should be discarded**
- **The pyruvate solution is stable for 2 months**

Materials and Equipment

- NADH solution
- Pyruvate solution
- Narrow-bandwidth spectrophotometer, fitted with a thermostatted cuvette holder capable of temperature control within ±0.1℃ and a chart-recorder

Assay Conditions

- Incubation temperature : 30.0℃
- Wavelength : 340 nm
- Final reaction volume : 1.07 ml
- Light path : 1.0 cm

Fluorescent dyes

- **Ethidium bromide (EtBr), propidium iodide (PI) and Fluorescein diacetate (FDA)**
- **PI binds to nucleic acids upon membrane damage : flow cytometric techniques depend on fluorescence, PI is ideally suitable for the rapid evaluation of the permeability properties of large numbers of cells while maintaining good statistical accuracy**
- **PI is impermeable to intact plasma membrane**
- **Intercalates with DNA or RNA $\Rightarrow$ red**

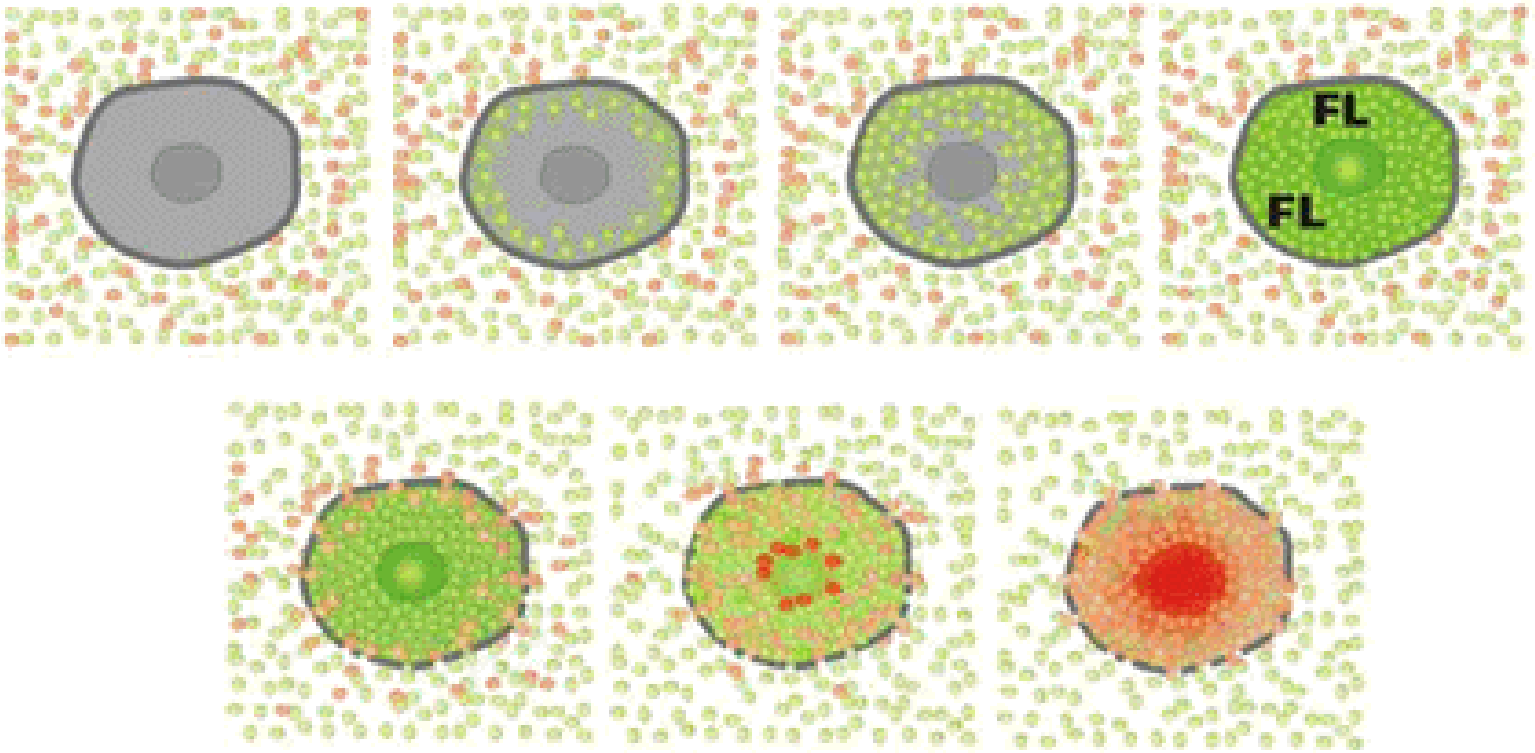
- **Fluorescein diacetate (FDA)** is a nonpolar ester which passes through plasma membranes and is hydrolyzed by intracellular esterases to produce free fluorescein, **the polar fluorescein is confined within cells which have an intact plasma membrane** and can be observed under appropriate excitation conditions
- **Undamaged cell** : **highly fluorescent** fluorescein dye
- **Damaged cell** : fluoresce only **weakly**
- greenish-yellow at 450-480 nm

Schematic illustration of the principle of PI/FDA cell viability assay

- FDA (Fluorescein diacetate)
- PI (Propidium iodide)

Intact cell –
PI and FDA is added

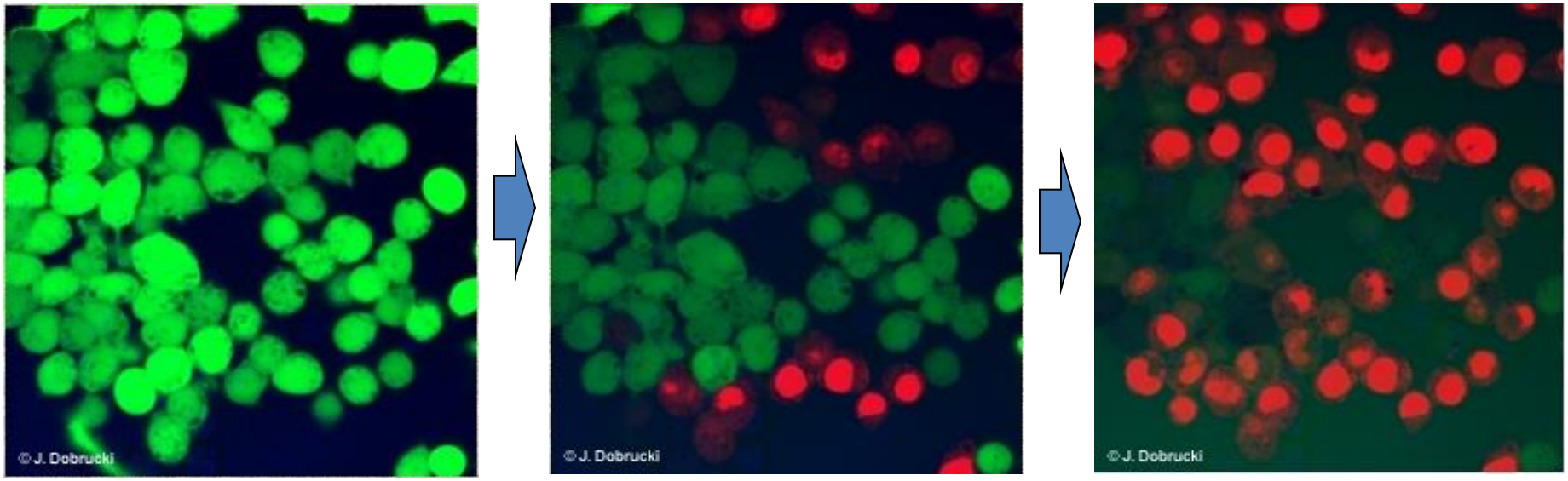
Fluorescein in intact cells



Plasma membrane is damaged; fluorescein leaks out

PI enters and strains nucleic acids

Example: Observation of cell death



A group of hepatoma cells exposed to a diffusing wave of digitonin. Intact cells (green) are damaged by digitonin, loose the green fluorescence and acquire red fluorescence of PI

Functional assays

- **Functional assays: evaluate viability by examining the metabolic components that are necessary for cell growth, on the premise that cellular damage will inevitably result in the loss of ability to maintain and provide energy for metabolic function and growth**

- **In terms of tissue remodeling in implantation, specific protein synthesis, such as collagen, by cells may be an important factor for assessing the cellular function related to cell viability**

- **The homeostasis of fibroblast collagen metabolism is regulated in a complex manner by an interplay of various different mechanisms which include hormones, cytokines, and cell-matrix interactions**

Colorimetric assay:

- Rapid and accurate assessment of viable cell number
- Miniaturized into 96-well plates
- Measure using an Microplate reader
- Permit many sample to be analyzed rapidly
- Reduce medium and plastics costs

- **These assay are read at 570 nm on a ELISA plate reader, using a 620 nm filter as reference wavelength**
- **It is important to remove any bubbles from the well before absorbance readings**

- **MTT / XTT assay**
  - **Crystal violet dye elution (CVDE)**
  - **Acid phosphatase (AP) assay**
  - **Alamar blue oxidation-reduction assay**
  - **Neutral Red (NR) assay**
  - **[^{3}H]-thymidine and BrdU incorporation**