In Vitro Cytotoxicity Assays

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Toxicity

- **Toxicity** is the potential of a chemical to induce an adverse effect in a living organism.
- **Toxicity** is the degree to which a substance can damage an organism.
- Toxicity can refer to the effect on a whole organism, such as an animal, bacterium, or plant, as well as the effect on a substructure of the organism, such as a cell (cytotoxicity) or an organ such as the liver (hepatotoxicity).

Cytotoxicity

- Cytotoxicity is a general term that **describes the detrimental effects of substances or environmental changes on cell health.**
- Exposure of cells to a cytotoxic stimulus may compromise metabolic activity, inhibit cell growth or division or ultimately produce cell death.

- Viability levels and/or proliferation rates of cells are good indicators of cell health.
- Physical and chemical agents can affect cell health and metabolism.

- These agents may cause toxicity on cells via different mechanisms such as;
  - **destruction of cell membranes,**
  - **prevention of protein synthesis,**
  - **irreversible binding to receptors,**
  - **inhibition of polydeoxynucleotide**

Cell Viability and Toxicity Tests

- In vitro cell viability and cytotoxicity assays with cultured cells are widely used for cytotoxicity tests of chemicals and for drug screening.
- Application of these assays has been of increasing interest over recent years.
- Currently, these assays are also used in oncological researches to evaluate both compound toxicity and tumor cell growth inhibition during drug development.

Cell Viability and Toxicity Tests

- Because, they are rapid, inexpensive and do not require the use of animals. Furthermore, they are useful for testing large number of samples.
- They have also advantages of **reduced cost and potential for automation, and tests using human cells may be more relevant than some in vivo animal tests**

In vitro testing of drugs

- In vitro toxicity testing is the scientific analysis of the effects of toxic chemical substances on cultured bacteria or mammalian cells.

- In vitro (literally 'in glass') testing methods are employed primarily :

  - to identify potentially hazardous chemicals
  - to confirm the lack of certain toxic properties in the early stages of the development of potentially useful new substances such as therapeutic drugs, agricultural chemicals and food additives.

- In vitro toxicity testing methods can be more useful and cost-effective than toxicology studies in living animals (which are termed in vivo or "in life" methods).

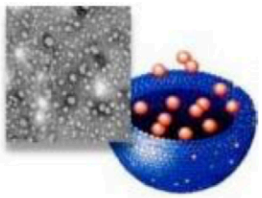
In vitro methods for the assessment of general cellular toxicity

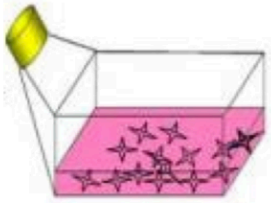
- Cell culture can be used to screen for toxicity both
  - by estimation of the basal functions of the cell (i.e. those processes common to all types of cells) or
  - by tests on specialized cell functions.
- For general toxicity studies, the more commonly used cell lines include the well characterized **diploid human fibroblast lines, WI-38 and tumour cell lines, HeLa.**
- The first and most readily observed effect following exposure of cells to toxicants **is morphological alteration in the cell layer or cell shape in monolayer culture.**

End-points for the assessment of general cellular toxicity

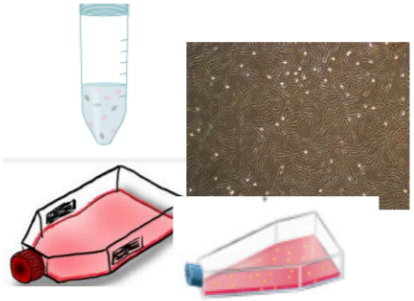
- **Cell morphology**
  - Blebbing, vacuolisation, fine ultrastructural modification
- **Cell viability**
  - Trypan blue (enters dead cells), neutral red (actively taken up by living cells), Cr51 release
- **Cell growth**
  - Cell count, plating efficiency, DNA or protein content, glucose consumption, lactate production, NR-test, MTT-test
- **Metabolic parameters**
  - O2 consumption or ATP level, pool of DNA and RNA precursors, NADH-NAD conversion.

Ekwall et al (1990)

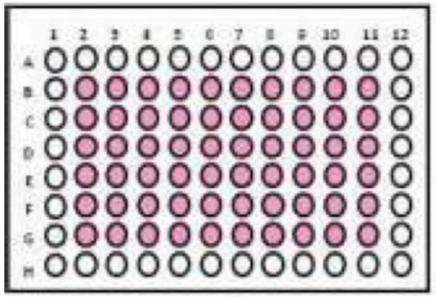


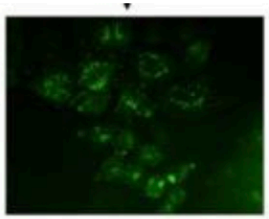


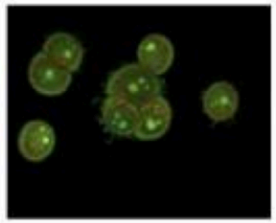
→XTT
→MTS
→SRB
→ AlamarBlue
→ [^{3}H]-thymidine inkorporasyonu
→ATP formasyonu



☑Sitotoksite analizleri
☑ Apoptoz markerlarının ölçümü
☑Hücre canlılık ölçümleri





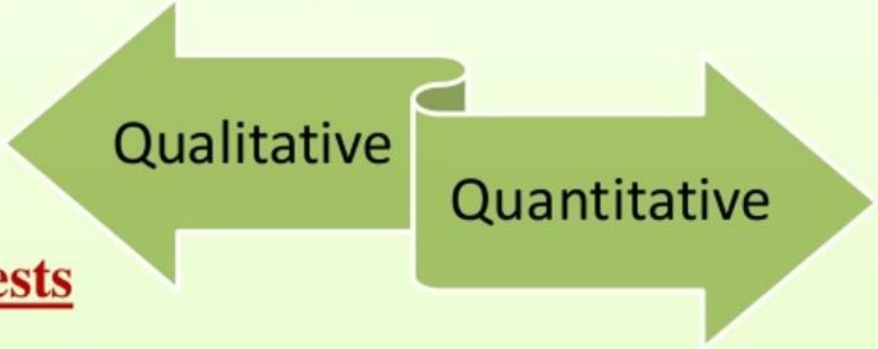


Nanomaterials **2014**, 4, 454-484.

Cell Viability Tests

1. Qualitative Tests
2. Quantitative Tests

Cytotoxicity is evaluated by qualitative and quantitative means.

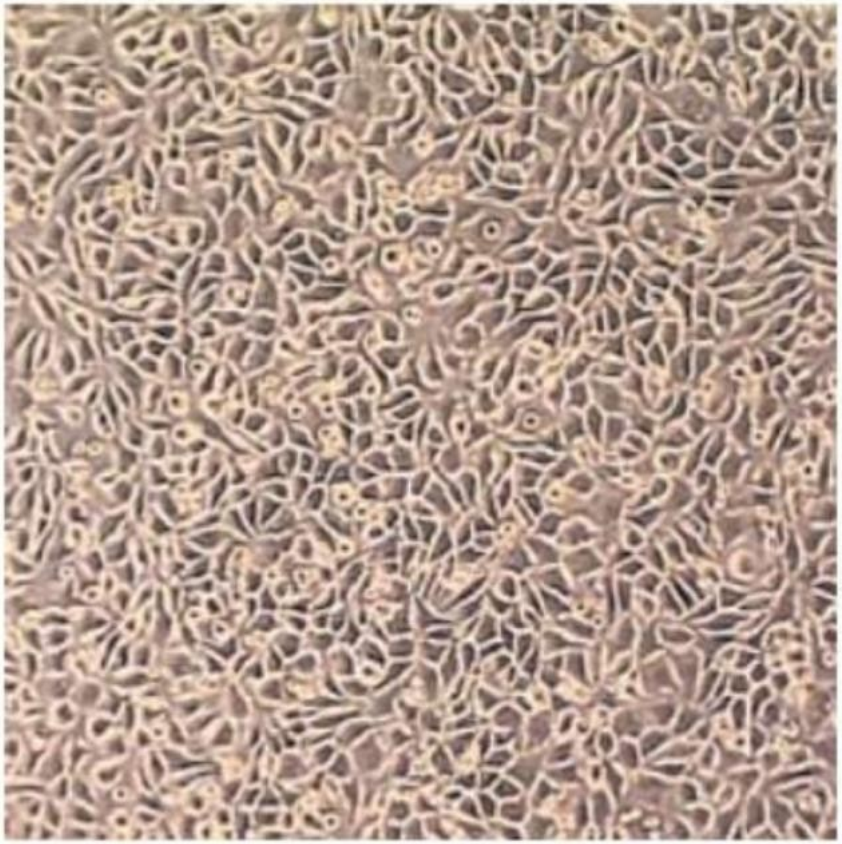


A. Qualitative Cytotoxicity Tests

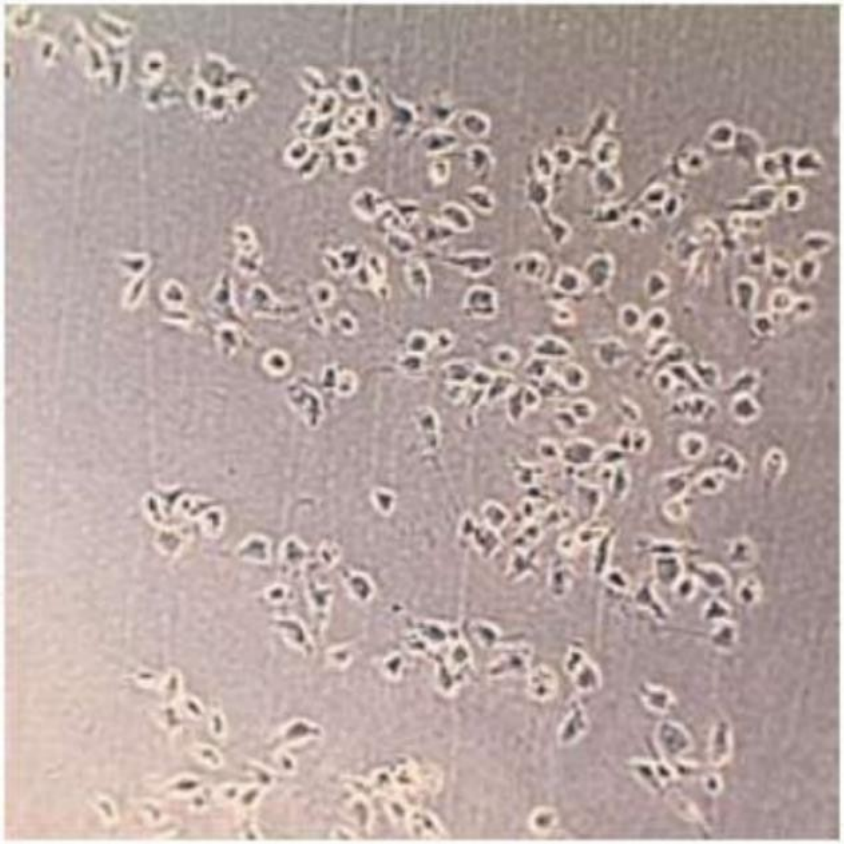
Three different qualitative cytotoxicity tests are commonly used :

1. **Extraction method/ MEM Elution**
2. **Agar Diffusion or Agarose overlay assay**
3. **Direct contact method**

In general, in these tests, toxicity is verified after a period of exposure (typically 24–72 hours) of the cells to the extract or device.



A confluent monolayer (100 x magnification) of well-defined L929 mouse fibroblast cells exhibiting cell-to-cell contact. This appearance is indicative of a non-cytotoxic (negative) response



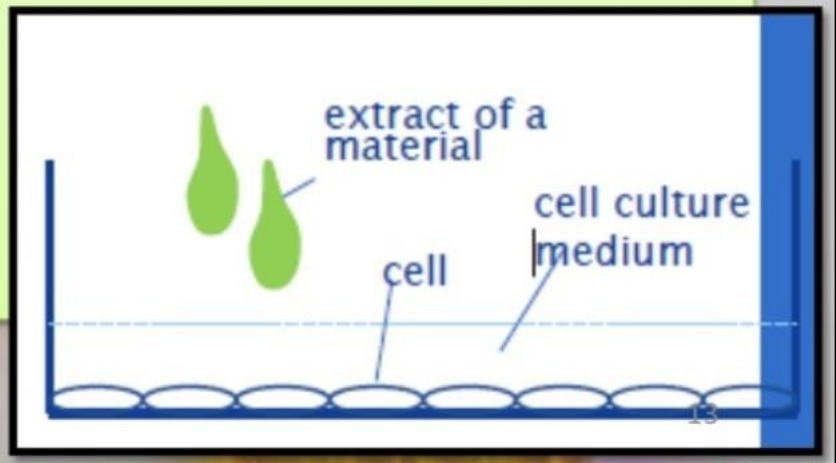
L929 mouse fibroblast cells (100 x magnification) that illustrate a positive cytotoxic reaction in the elution test method. The cells are grainy and lack normal cytoplasmic space; the considerable open areas between cells indicate that extensive cell lysis has occurred.

http://www.devicelink.com/mddi/archive/98/04/013.html

1. MEM Elution - Test on Extracts (ISO 10993-5)

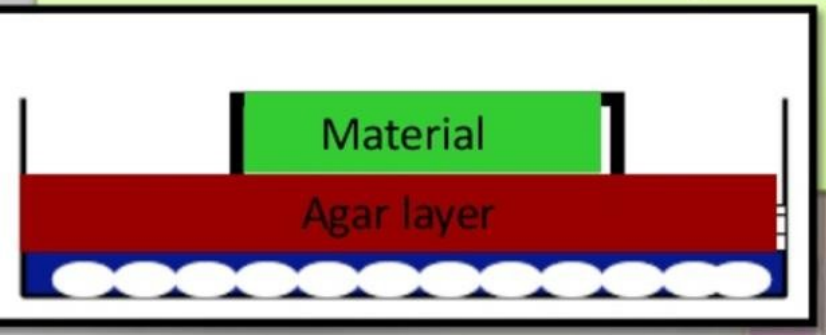
- The test material is extracted for 24 hours in Minimum Essential Medium (MEM).
- An extract is prepared from the test material which is then placed over the cultured cells. (L-929 mouse fibroblast cells)
- Following incubation, the cells are examined microscopically for morphological changes, degeneration and lysis of the cells.
- Potentially cytotoxic substances are uniformly distributed throughout the cell culture

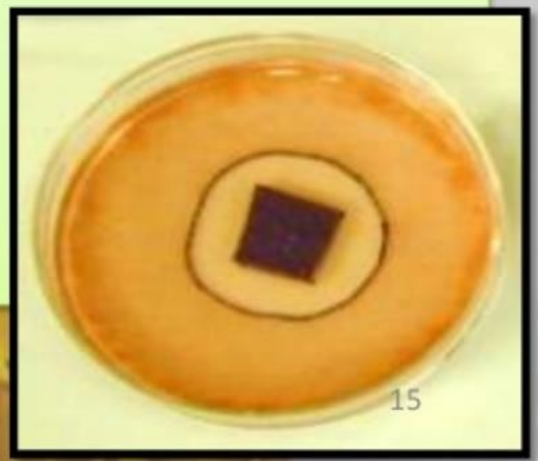
cell line L929



2. Agar Diffusion or Agarose overlay assay - (ISO 10993-5)

- In this method, a thin layer of nutrient-supplemented agar is placed over the cultured cells.(L-929 mouse fibroblast cells)
- The test material (or an extract of the test material dried on filter paper/sample) is placed on top of the agar layer, and the cells are incubated for 24 hours.
- Cytotoxic leachates diffuse into the cell layer via the agar, and a zone of malformed, degenerative or lysed cells under and around the test material indicates cytotoxicity.
- MRC-5 Human Embryonic Lung Cells

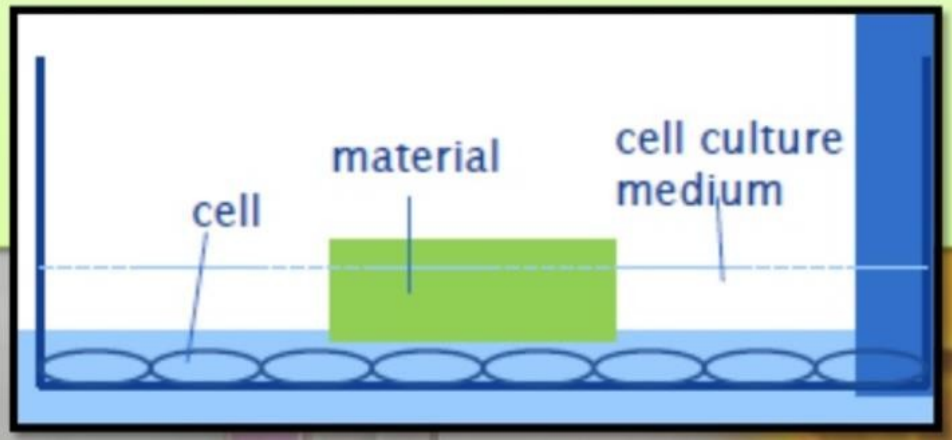




Agar overlay: positive control

3. Direct Contact (ISO 10993-5)

- In this method, a piece of test material is placed directly onto cells growing on culture medium.(without the agar layer)
- Cell cultures are grown to a standard monolayer.
- The cells are then incubated for 24 hours at 37 C.
- During incubation, leachable chemicals in the test material can diffuse into the culture medium and contact the cell layer.
- Subsequently, the monolayers are examined microscopically for the presence of morphological changes, reduction in cell density or lysis of cells around the test material.



Quantitative Tests

1.Dye exclusion: Trypan blue, eosin, Congo red, erythrosine B assays.

2.Colorimetric assays: MTT assay, MTS assay, XTT assay, WST-1 assay, WST-8 assay, LDH assay, SRB assay, NRU assay and crystal violet assay.

3. Fluorometric assays: Alamar Blue assay and CFDA-AM assay.

4. Luminometric assays: ATP assay and real-time viability assay.

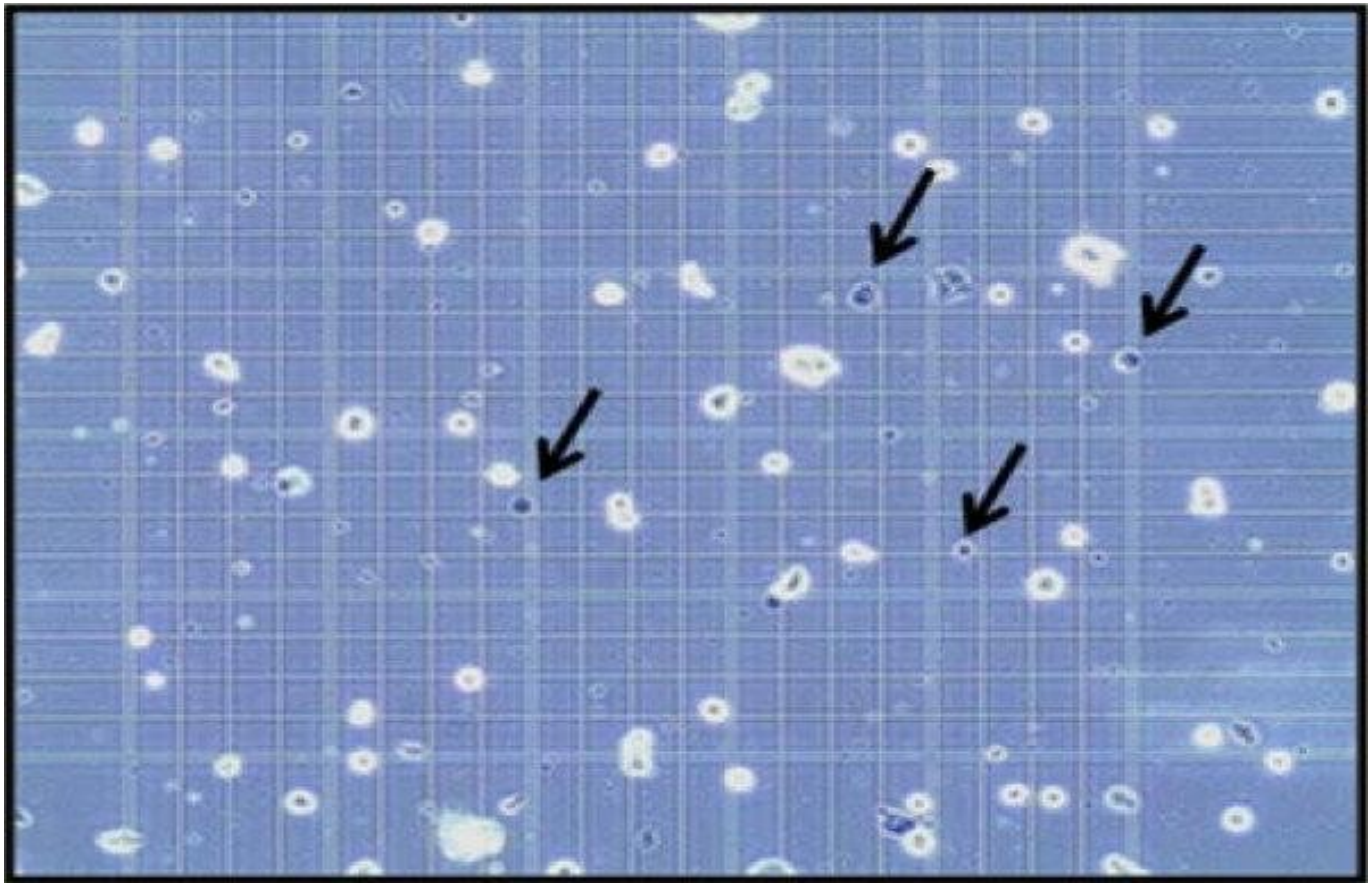
1. Dye exclusion assays

- The simplest and widely used one of the methods is dye exclusion method. In dye exclusion method, viable cells exclude dyes, but dead cells not exclude them.
- **Determination of membrane integrity** is possible via dye exclusion method.
- A variety of such dyes have been employed, including **eosin, Congo red, erythrosine B, and trypan blue.**
- Of the dyes listed, trypan blue has been used the most extensively

Trypan blue dye exclusion assay

- Trypan blue dye exclusion assay is based on the principle that live cells possess intact cell membranes that exclude this dye, whereas dead cells do not.
- **Viable cells will have a clear cytoplasm, whereas dead cells will have a blue cytoplasm**

Trypan blue dye exclusion assay

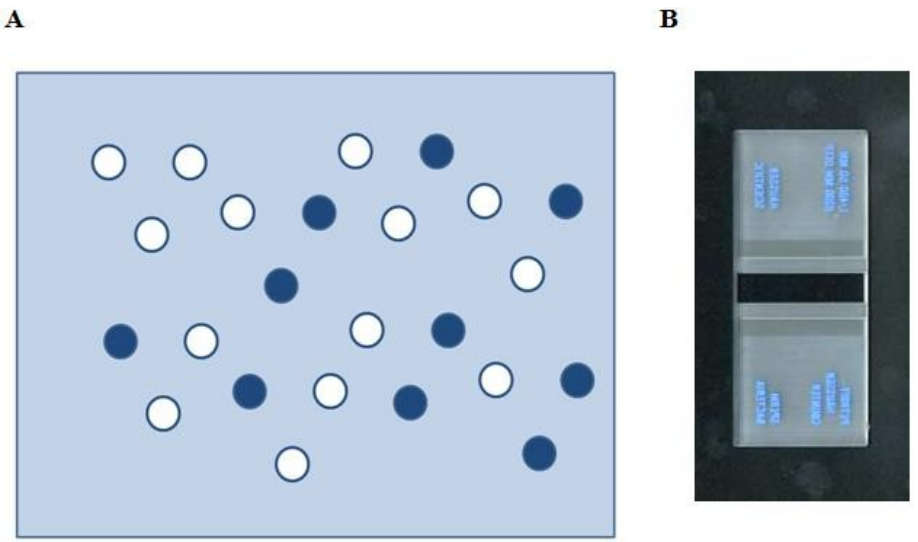


Trypan blue dye exclusion assay

- Number of viable and/or dead cells per unit volume is determined by light microscopy as a percentage of untreated control cells

Trypan blue dye exclusion assay

- **Advantages:** This method is simple, inexpensive, and a good indicator of membrane integrity and dead cells are colored blue within seconds of exposure to the dye.

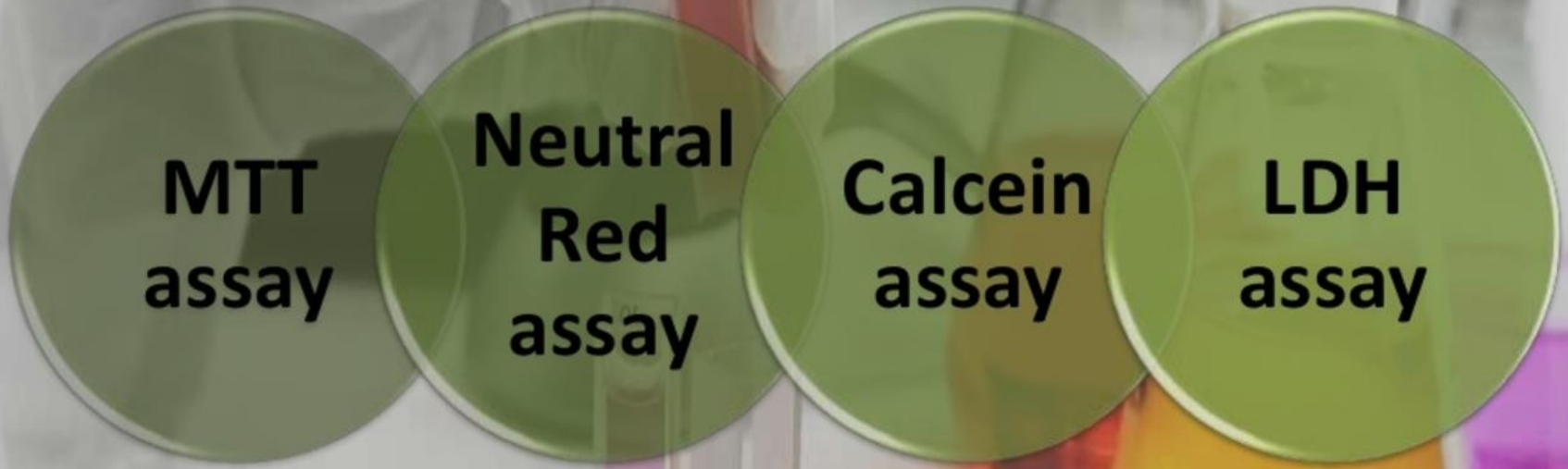


2. Colorimetric assays

- **Principle of colorimetric assays** is the measurement of a biochemical marker to evaluate metabolic activity of the cells.
- Reagents used in colorimetric assays develop a color in response to the viability of cells, allowing the colorimetric measurement of cell viability via **spectrophotometer.**
- Colorimetric assays are applicable for adherent or suspended cell lines, easy to perform, and comparably economical

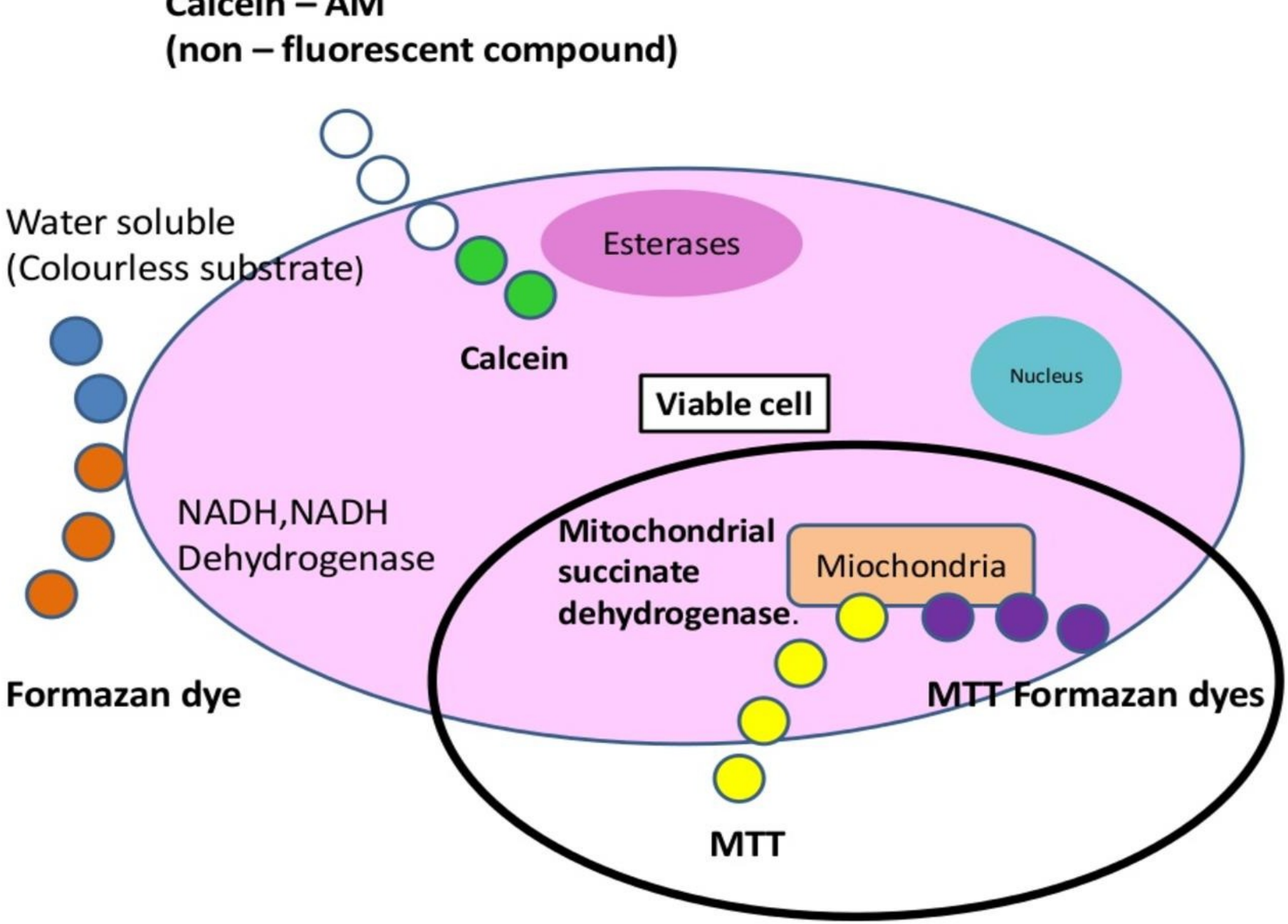
B. Quantitative cytotoxic assays:

- Evaluation of cell number and viability by measuring of metabolic activity, membrane permeability.

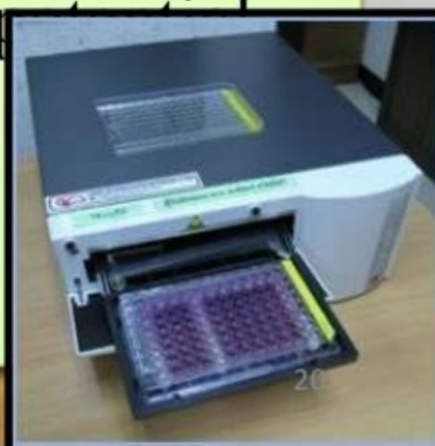


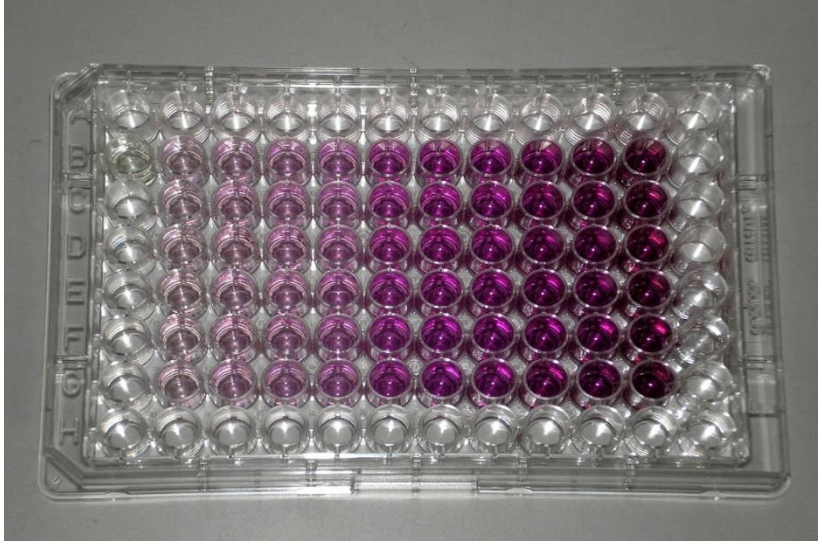
1. MTT assay:- Mitochondrial activity
[3-(4,5-dimethylthiazol-2-yl)-2,5- diphenylformazan bromide]

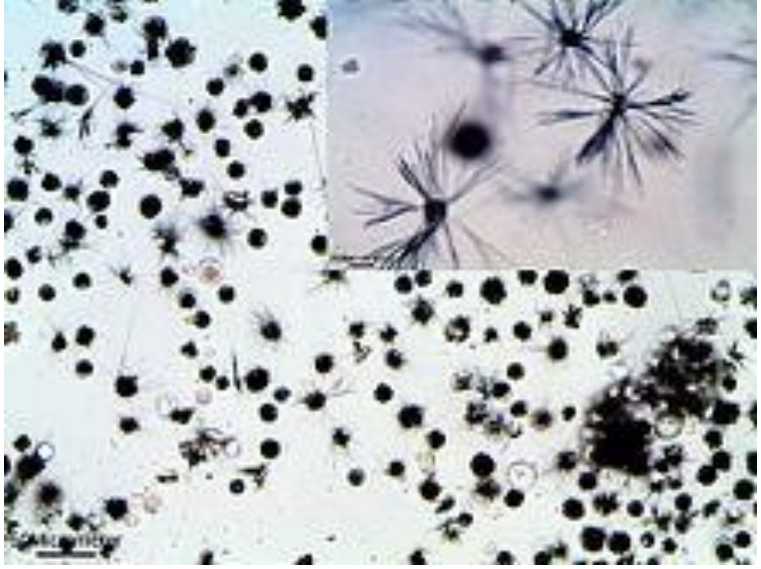
- MTT assay is used often in determining cell viability (detects viable cells).
- The MTT is a colorimetric method that measures the reduction of **yellow 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide** into an insoluble **purple formazan** product by mitochondrial succinate dehydrogenase.
- MTT being water soluble can penetrate through cell membrane, water insoluble formazan is trapped inside the cell.
- Dead cells do not have active mitochondrial reductases (as the cellular reduction is only catalyzed by living cells), MTT is not reduced and the purple formazan is not formed.

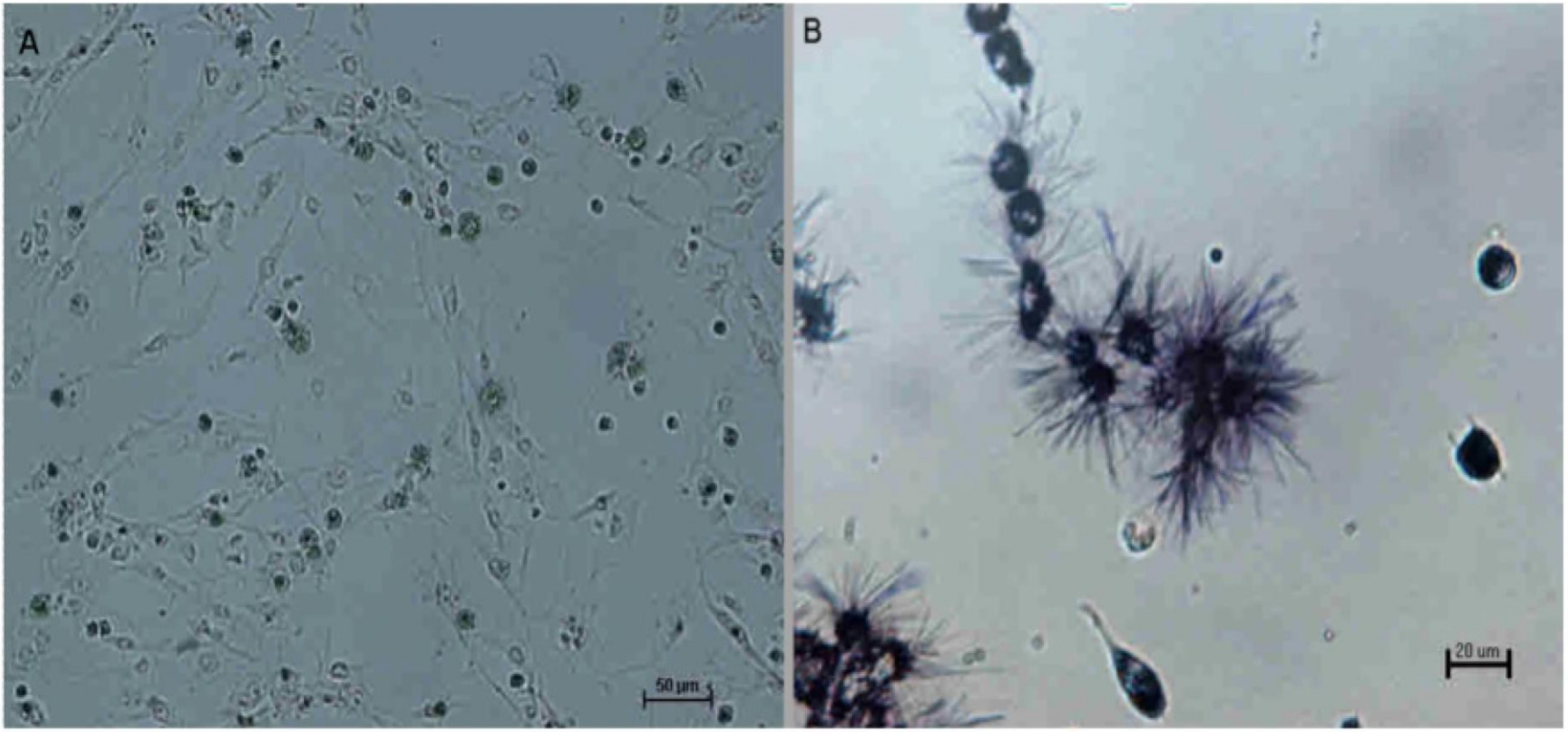

Calcein – AM
(non – fluorescent compound)
Water soluble
(Colourless substrate)
Esterases
Calcein
Nucleus
Viable cell
NADH,NADH
Dehydrogenase
Mitochondrial
succinate
dehydrogenase.
Miochondria
Formazan dye
MTT Formazan dyes
MTT

- Samples are read using an ELISA plate reader at a wavelength of 570 nm.
- The amount of color produced is directly proportional to the number of viable cells.
- The MTT can be used to evaluate the cytotoxicity of:
  - Toxic compounds
  - Toxins and environmental pollutants
  - Potential anti-cancer drugs
  - Antibodies to examine growth inhibiting p

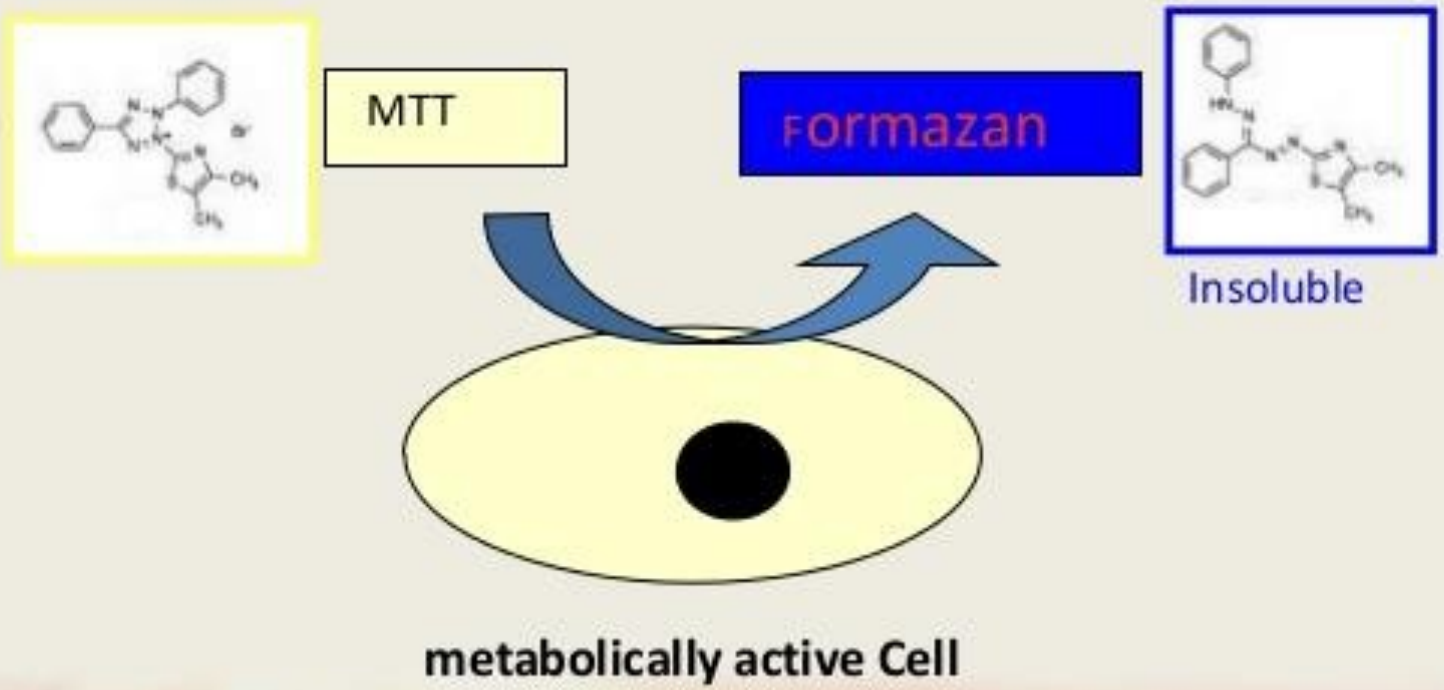







A
50 µm
B
20 um

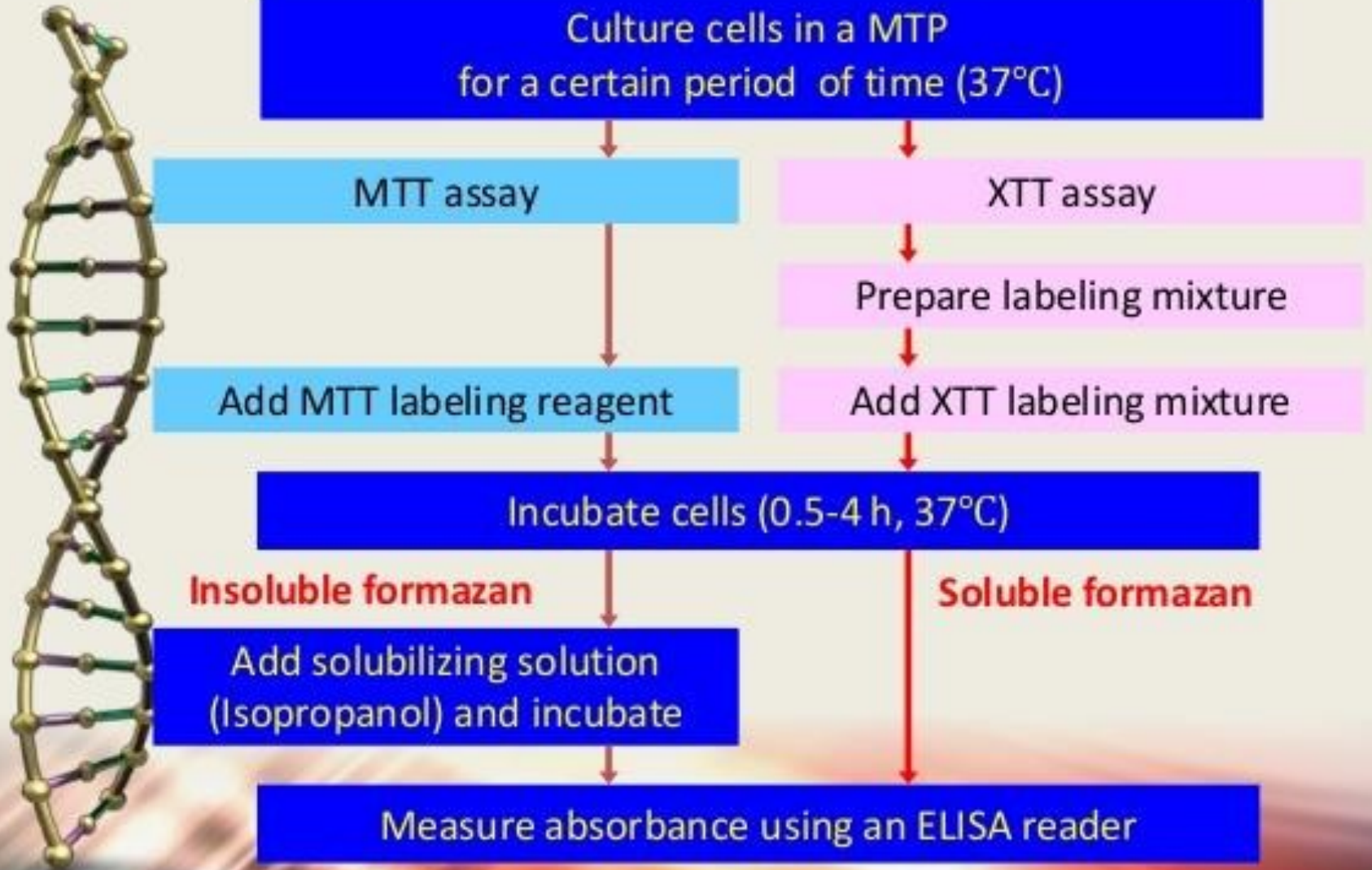
- The amount of formazan produced is directly proportional to number of viable cells present in the sample.



MTT Assay

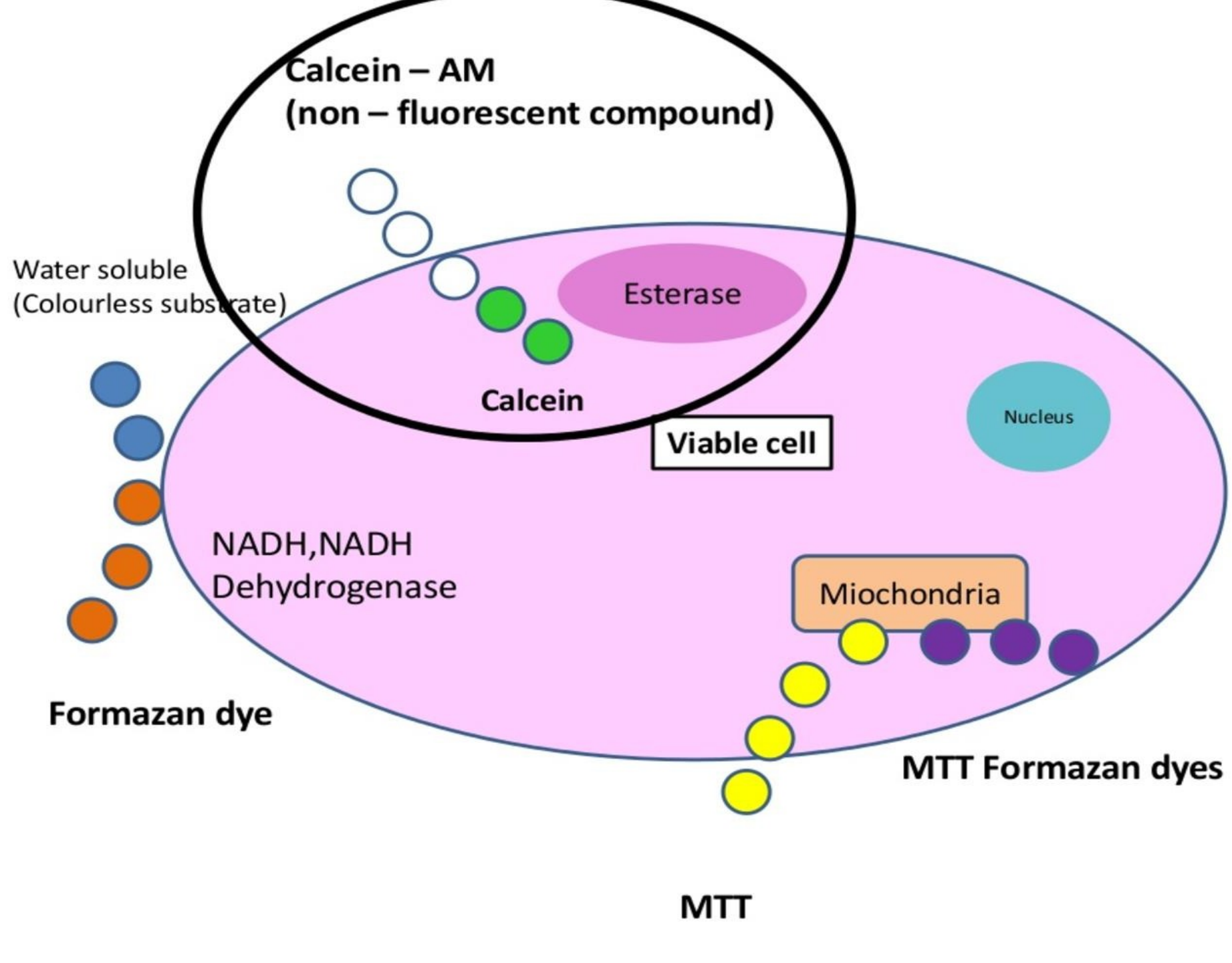
- **Advantages:** This method is far superior to the previously mentioned dye exclusion methods because it is easy to use, safe, has a high reproducibility, and is widely used to determine both cell viability and cytotoxicity tests

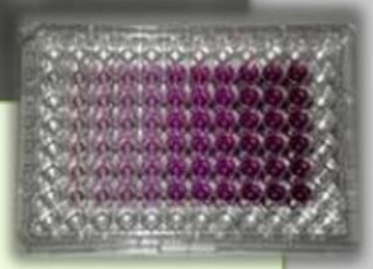
Compare with MTT assay and XTT assay



2. Calcein assay :-

- The acetomethoxy derivate of calcein (**calcein AM**) is used in testing of cell viability as it can be transported through the cellular membrane into live cells.
- After transport into the cells, intracellular esterases remove the acetomethoxy group, the molecule gets trapped inside and gives out strong **green** fluorescence.
- As dead cells lack active esterases, only live cells are labeled and counted by flow cytometry.


Calcein – AM
(non – fluorescent compound)
Water soluble
(Colourless substrate)
Esterase
Calcein
Viable cell
Nucleus
NADH,NADH
Dehydrogenase
Miochondria
Formazan dye
MTT Formazan dyes
MTT



3. Neutral Red (NR) assay:-

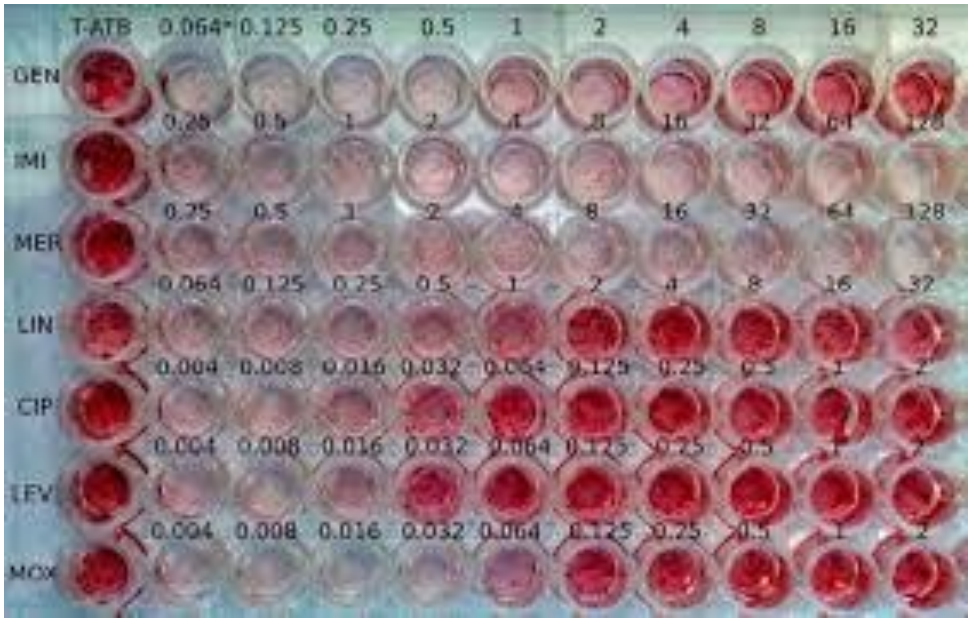
- Lysosomal activity
- Membrane permiability

- The neutral red (NR) assay is a cell survival chemo sensitivity assay.
- This assay is based on the incorporation of NR into the lysosomes of viable cells after being incubation with test agents.
- NR (3-amino-7-dimethyl-2-methylphenazine hydrochloride) is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion, accumulating intracelluarly in lysosomes, where it binds with anionic sites in the lysosmal matrix.

Neutral Red Dye

- Neutral red uptake depends on the capacity of cells to maintain **pH gradients** through the ATP production. At physiological pH, net charge of the dye is zero.
- This charge enables the dye to penetrate the cell membranes. Inside the lysosomes, there is a proton gradient to maintain a pH lower than that of the cytoplasm.

- **Thus, the dye becomes charged and is retained inside the lysosomes. When the cell dies or pH gradient is reduced, the dye cannot be retained**

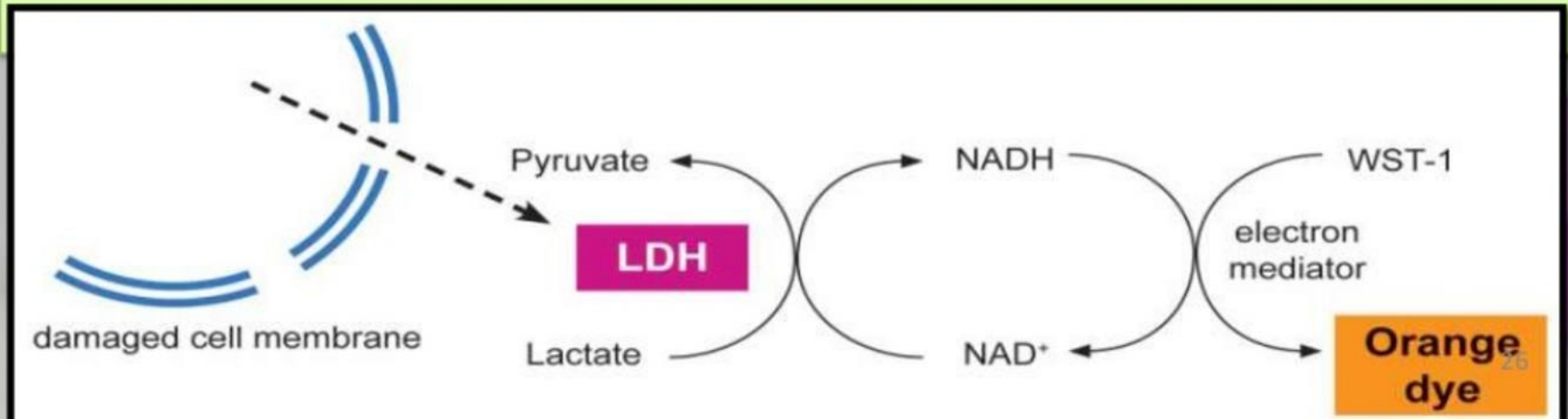


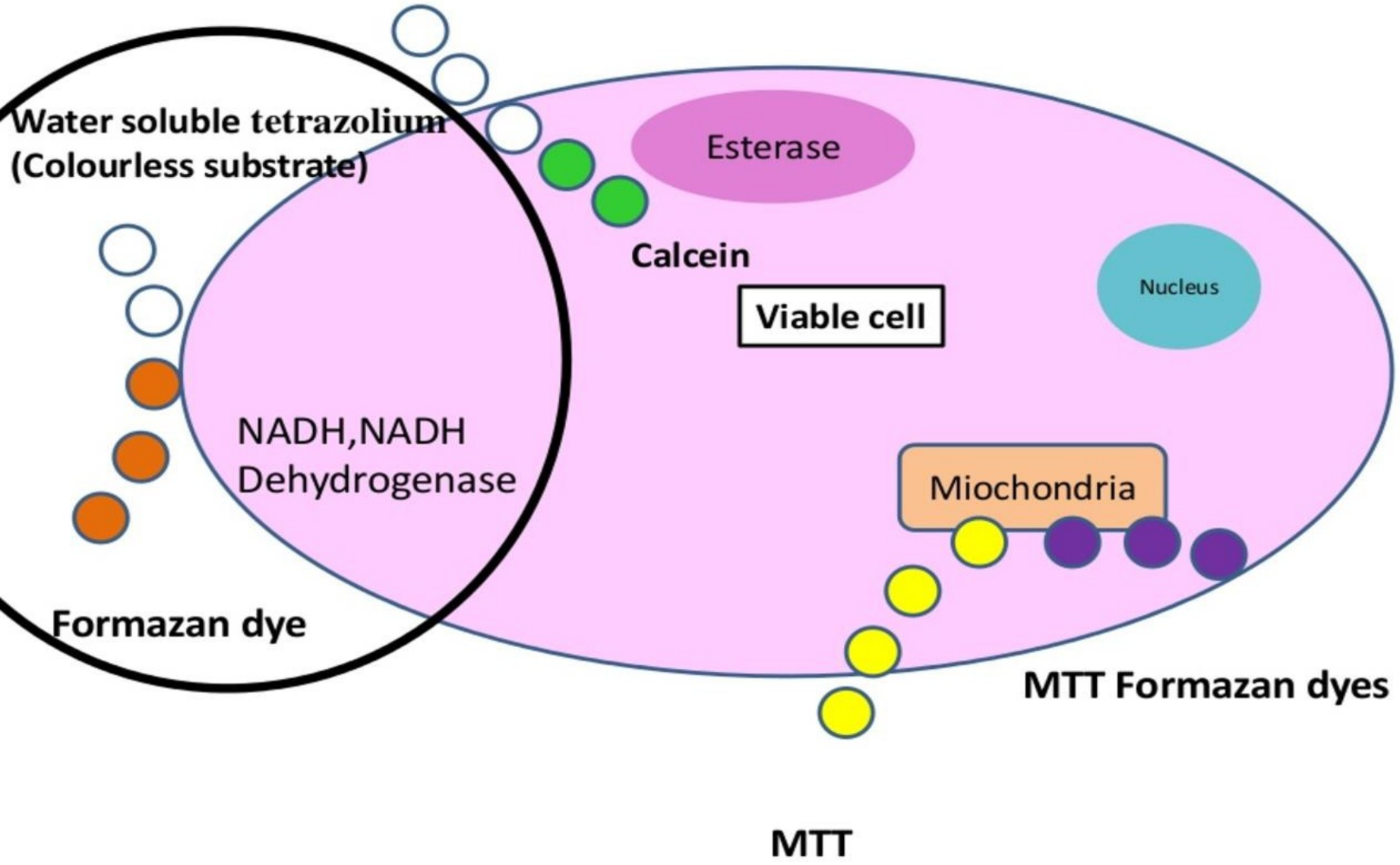
- Therefore, it is possible to distinguish between viable, damaged or dead cells as viable cells take up the NR dye, damaged or dead cells do not.
- When a reduced cytotoxic effect was determined by neutral red (NR-test), which shows the activity of lysosomal enzymes, microscopically multiplication or enlargement of lysosomes was observed.

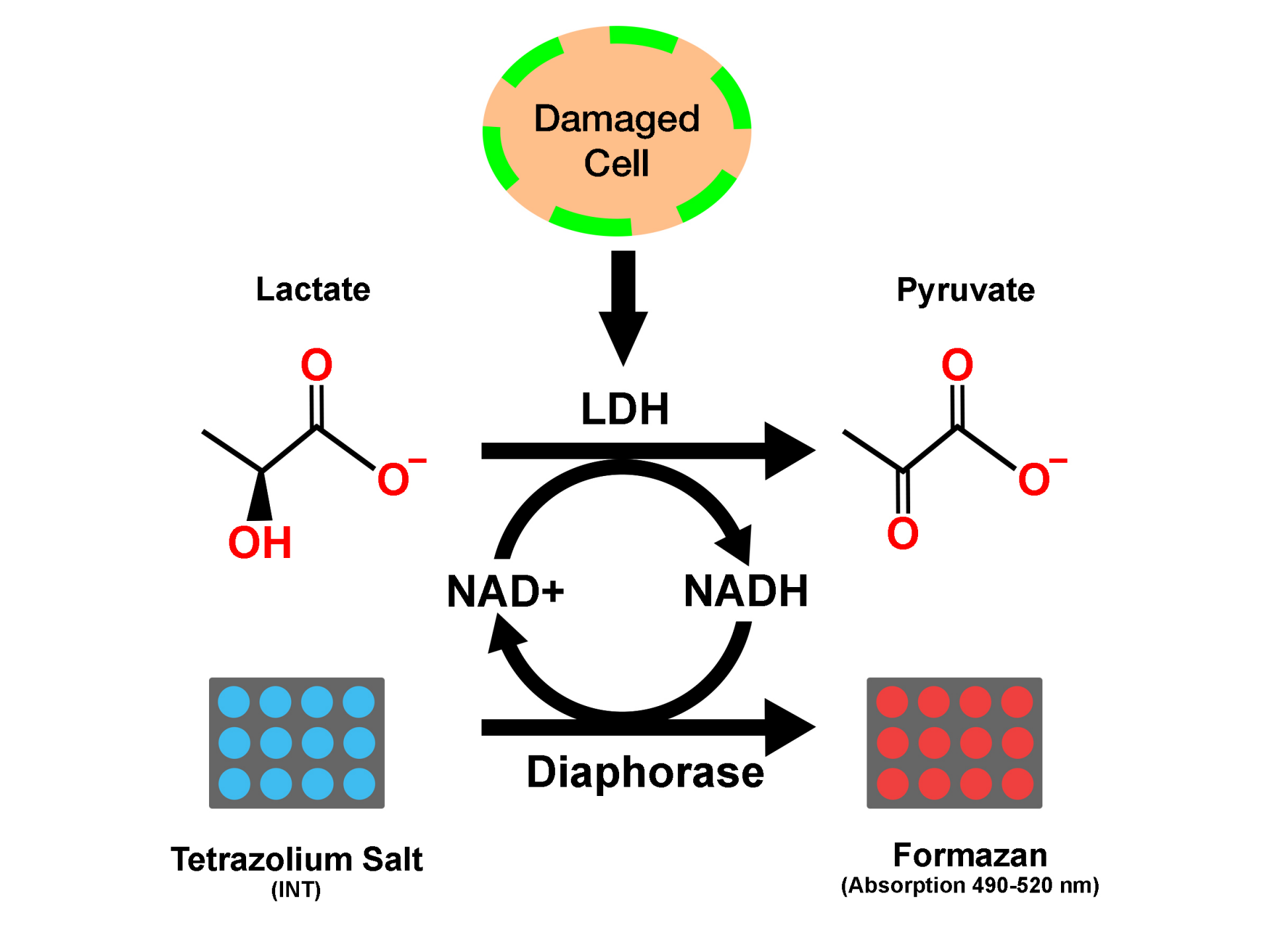
4. Lactate dehydrogenase (LDH) leakage assay : (membrane integrity)

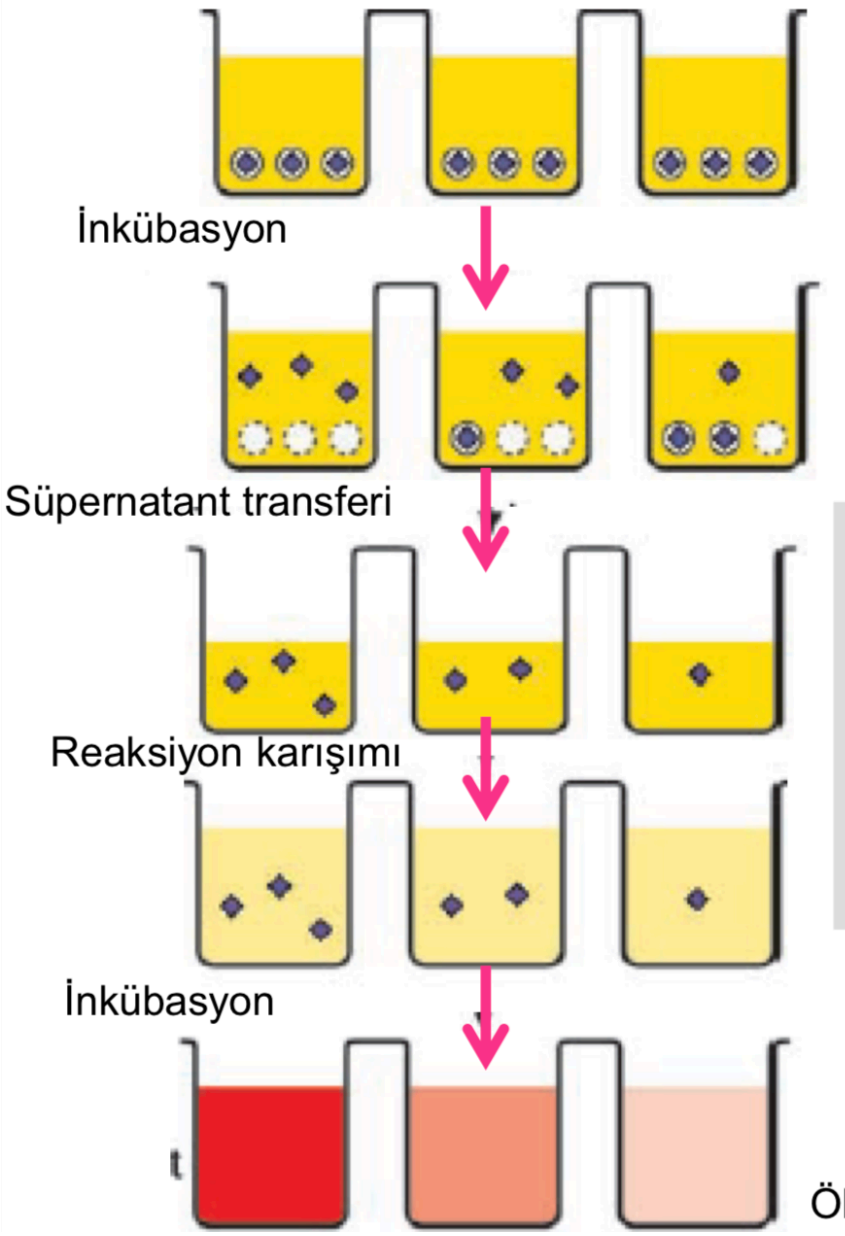
- Lactate dehydrogenase (LDH), which is a soluble cytosolic enzyme present in most eukaryotic cells, releases into culture medium upon cell death due to damage of plasma membrane.
- The increase of the LDH activity in culture supernatant is proportional to the number of lysed cells.
- LDH activity, therefore, can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from chemical compounds or environmental toxic factors.

- LDH Assay measures LDH activity present in the culture medium using a coupled two-step reaction.
- In the first step, LDH catalyzes the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate.
- In the second step of the reaction, diaphorase (NADPH dehydrogenase) uses the newly-formed NADH and H^+ to catalyze the **reduction of a tetrazolium salt** to **highly-colored formazan** which absorbs strongly at 490-520 nm.

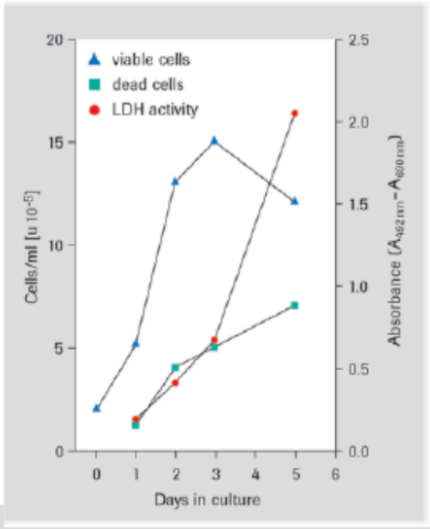


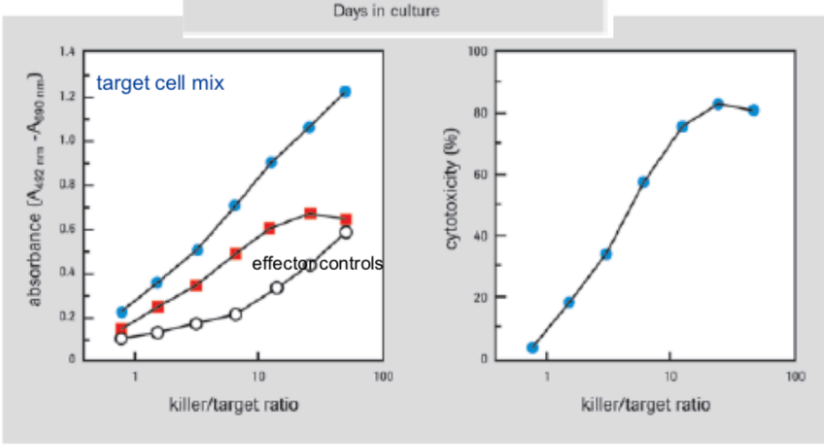

Calcein – AM
(non – fluorescent compound)
Water soluble tetrazolium
(Colourless substrate)
Esterase
Calcein
Viable cell
Nucleus
NADH,NADH
Dehydrogenase
Miochondria
Formazan dye
MTT Formazan dyes
MTT


Damaged Cell
Lactate
Pyruvate
LDH
NAD+
NADH
Diaphorase
Tetrazolium Salt
(INT)
Formazan
(Absorption 490-520 nm)


İnkübasyon
Süpernatant transferi
Reaksiyon karışımı
İnkübasyon
t

Ölçüm/ ELISA


viable cells
dead cells
LDH activity
Cells/ml [x 10⁻⁵]
Absorbance (A492nm - A690nm)
Days in culture


target cell mix
effector controls
absorbance (A492 nm - A690 nm)
killer/target ratio
cytotoxicity (%)
killer/target ratio

- **Advantages:** Reliability, speed, and simple evaluation are some of characteristics of this assay. Because, the loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage

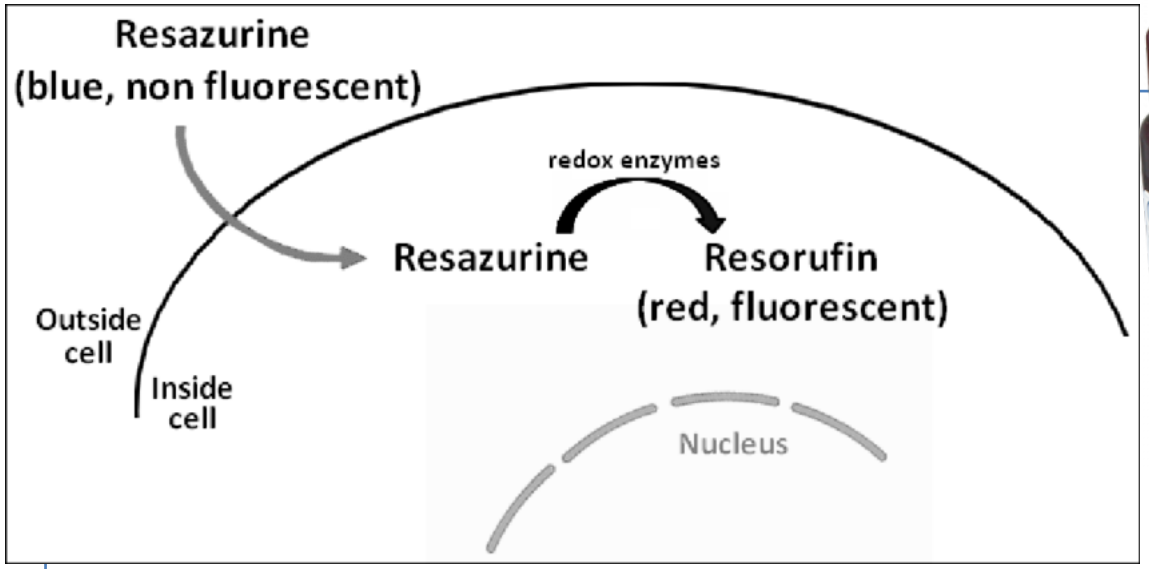
3. Fluorometric assays

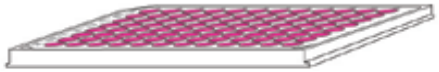
- Fluorometric assays of cell viability and cytotoxicity are easy to perform with the use of **a fluorescence microscope, fluorometer, fluorescence microplate reader or flow cytometer,** and they offer many advantages over traditional dye exclusion and colorimetric assays.
- Fluorometric assays are also applicable for adherent or suspended cell lines and easy to use. These assays are more sensitive than colorimetric assays.

Alamar Blue (AB) assay

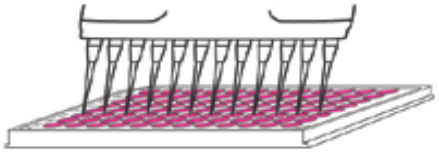
- The AlamarBlue assay is based on the conversion of the blue nonfluorescent dye resazurin, which is converted to the pink fluorescent resoruin by mitochondrial and other enzymes such as diaphorases

- Color of this compound is blue and it is nonluorescent. After entering cells, resazurin is reduced to resoruin.
- Resorufin is red in color and highly luorescent compound. Viable cells convert continuously resazurin to resofurin, increasing overall fluorescence and color of the cell culture medium.
- The quantity of produced resofurin is related to the number of viable cells.



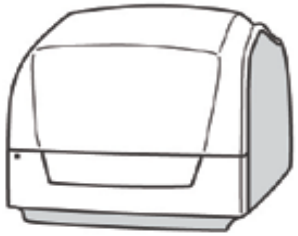


Treat cells
with compounds

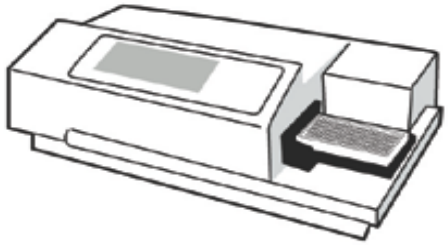


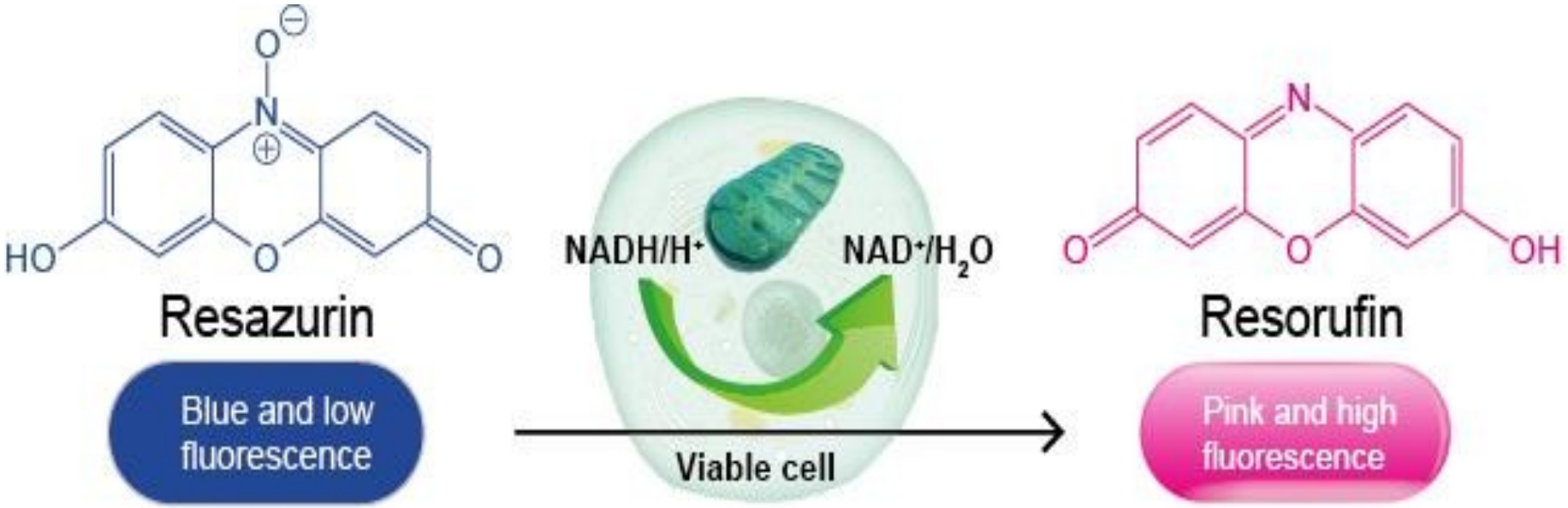
Add alamarBlue Reagent
(10% volume of culture)
directly to medium

Incubate
37°C



Measure fluorescence
(Ex 530-560nm / Em 590nm)
or
Measure absorbance
(570nm and 600nm)

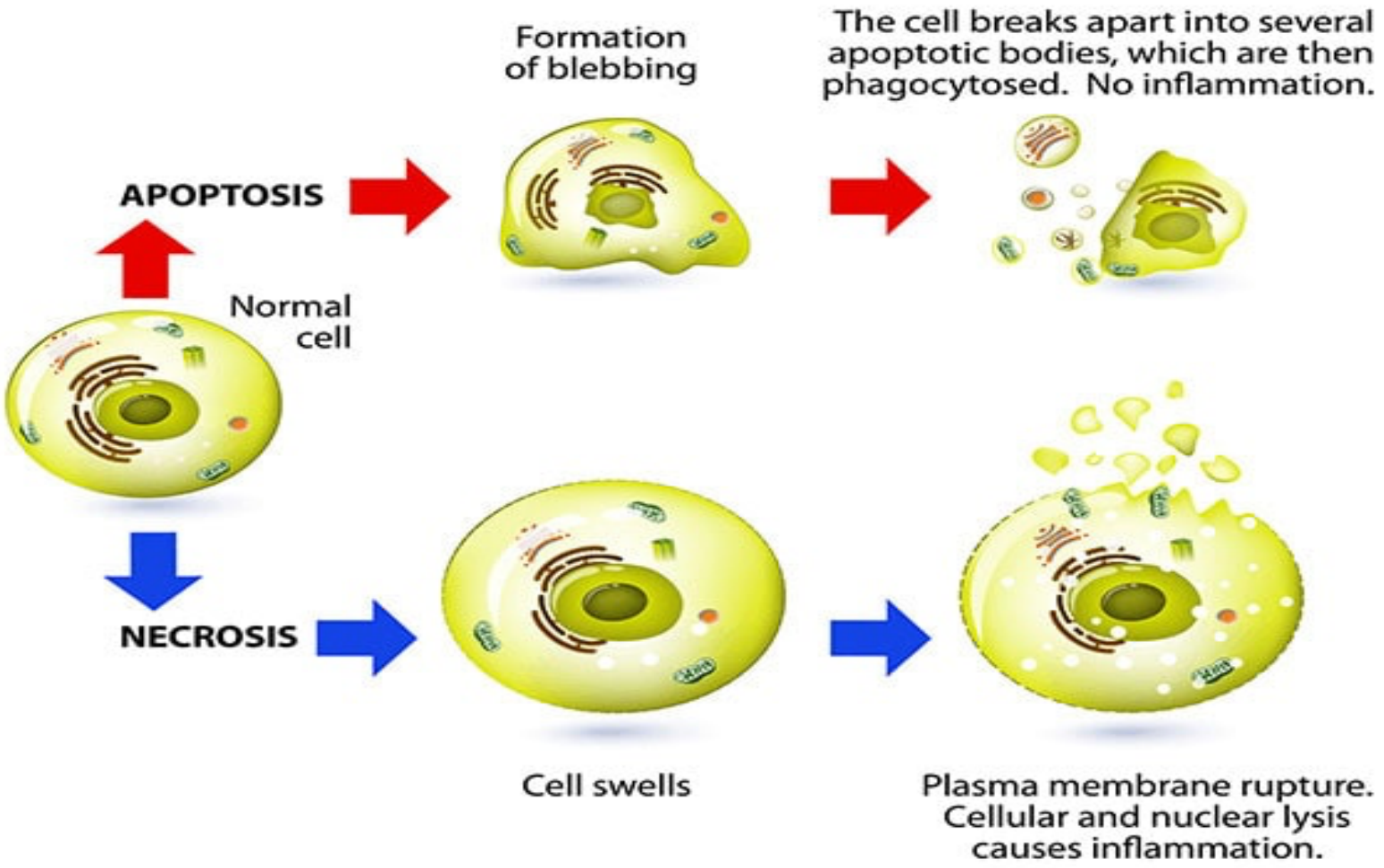



Resazurin
Blue and low fluorescence
NADH/H+
NAD+/H2O
Viable cell
Resorufin
Pink and high fluorescence

Apoptosis vs. Necrosis

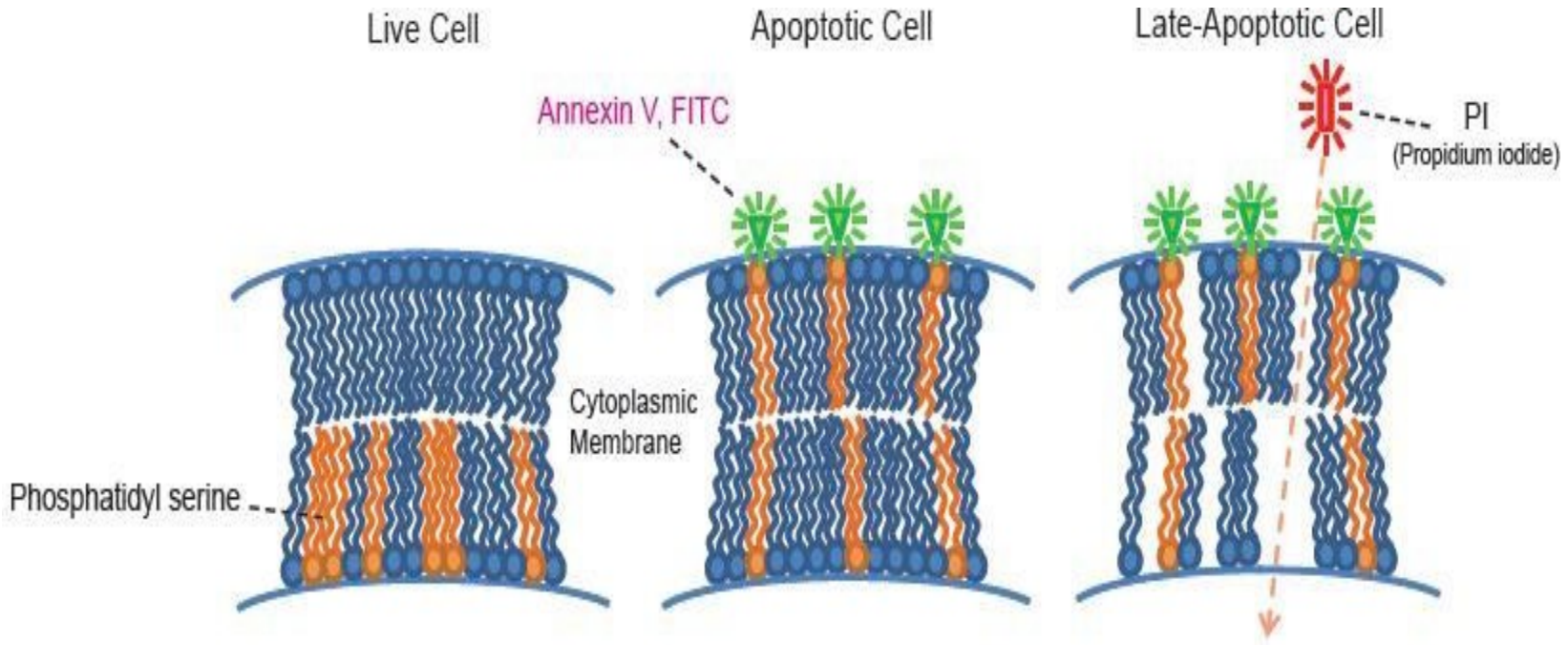
- Necrosis
  - Toxicity-induced cell death.
  - Requires no energy, passive.
  - Cells swell and then karyolysis (dissolution of the chromatin and nucleus - DNase).
  - Release of cellular contents may cause inflammation.
- Apoptosis
  - ‘Stimulation’-induced cell death.
  - Energy required.
  - Cell shrinkage, then pyknosis (chromatin condenses), followed by karyorrhexis (fragmentation of the nucleus).
  - Do not release cellular contents and are readily phagocytosed by macrophages.

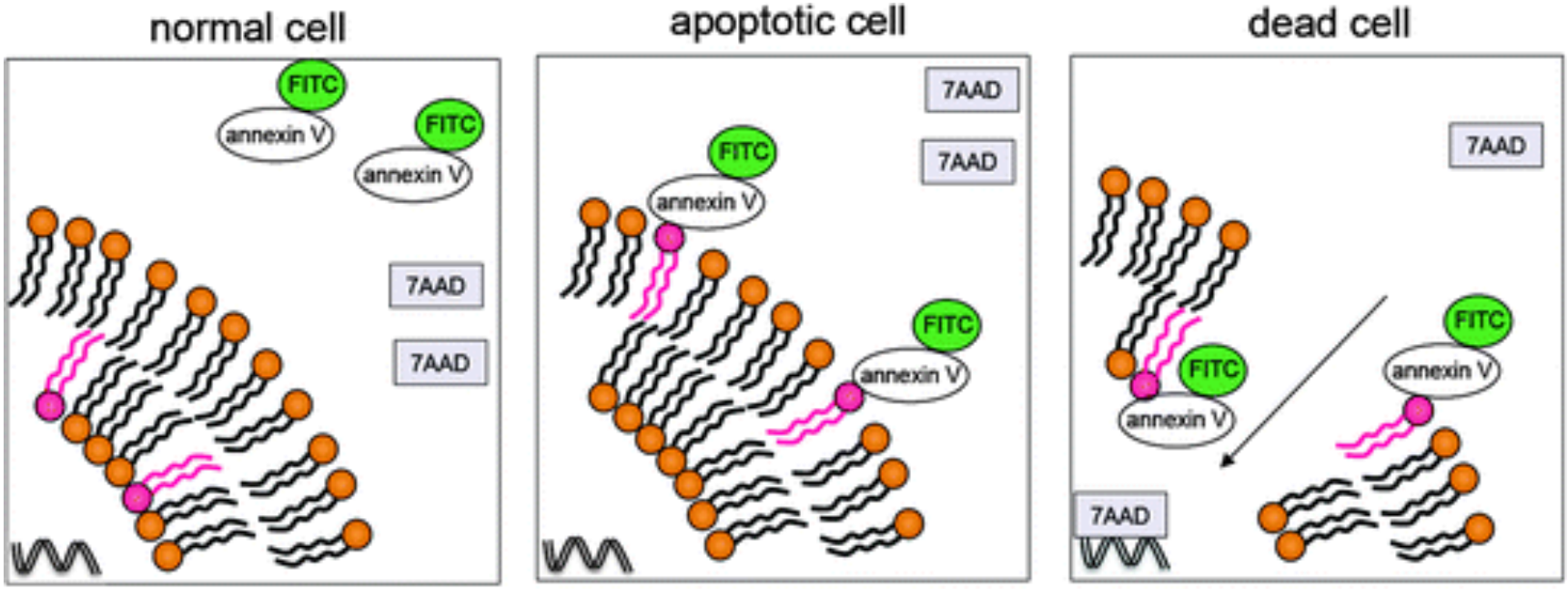
Apoptosis vs. Necrosis



Annexin V/7-amino-actinomycin (7-AAD)

Annexin V and Propidium iodide are the stains demonstrating cellular apoptosis





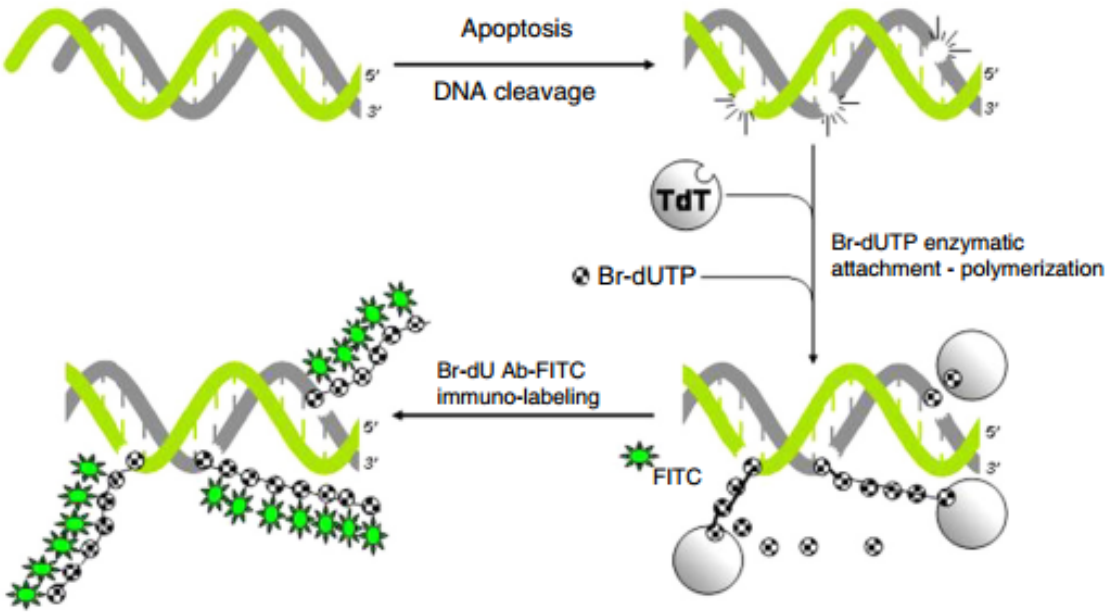
Legend:

phoshpholipids

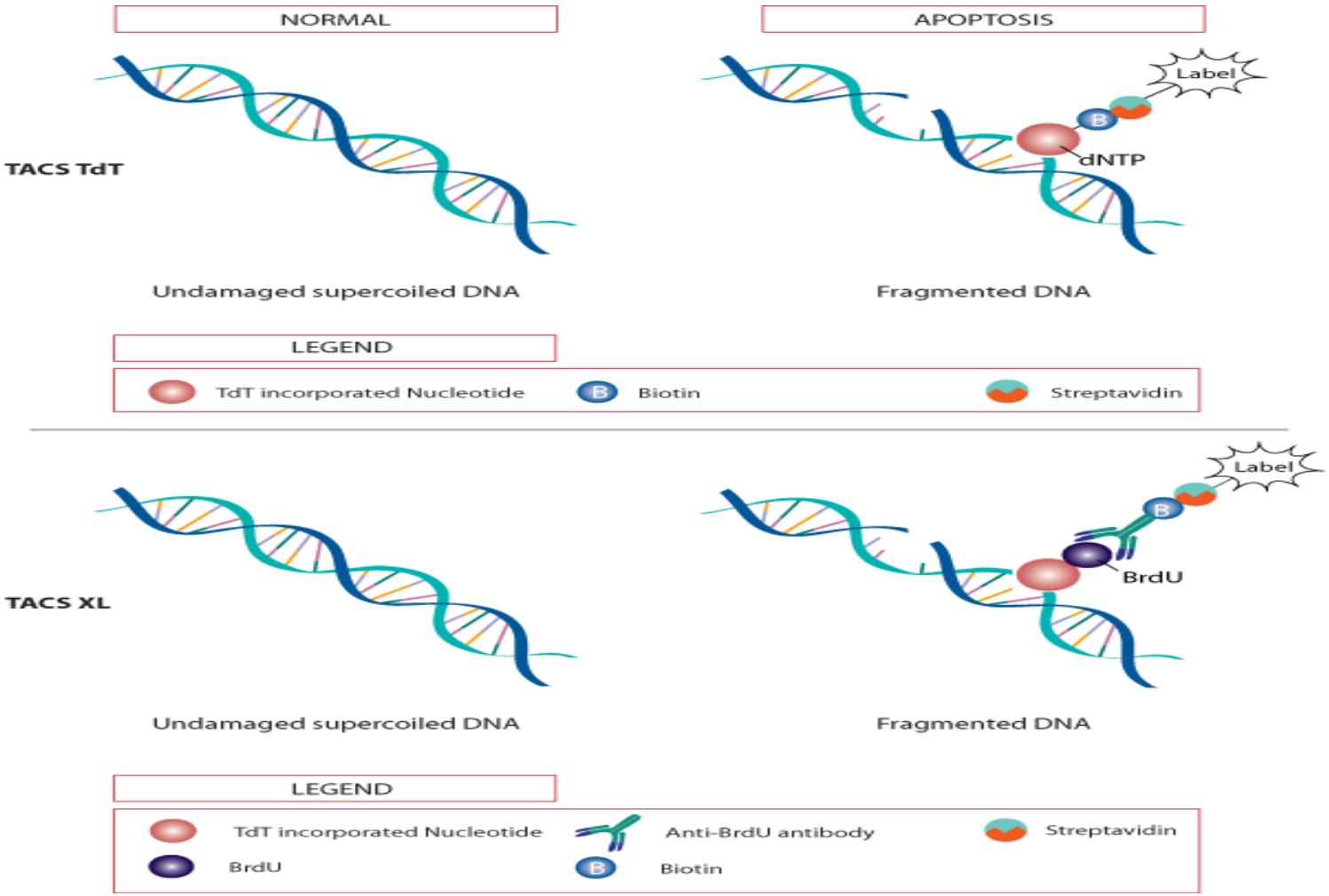
phosphatidylserine (PS)

DNA

TUNEL Test


Apoptosis
DNA cleavage
5'
3'
TdT
Br-dUTP
Br-dUTP enzymatic
attachment - polymerization
Br-dU Ab-FITC
immuno-labeling
FITC

TUNEL Test


NORMAL
APOPTOSIS
Label
B
dNTP
TACS TdT
Undamaged supercoiled DNA
Fragmented DNA
LEGEND
TdT incorporated Nucleotide
B
Biotin
Streptavidin
Label
B
BrdU
TACS XL
Undamaged supercoiled DNA
Fragmented DNA
LEGEND
TdT incorporated Nucleotide
Anti-BrdU antibody
Streptavidin
BrdU
B
Biotin

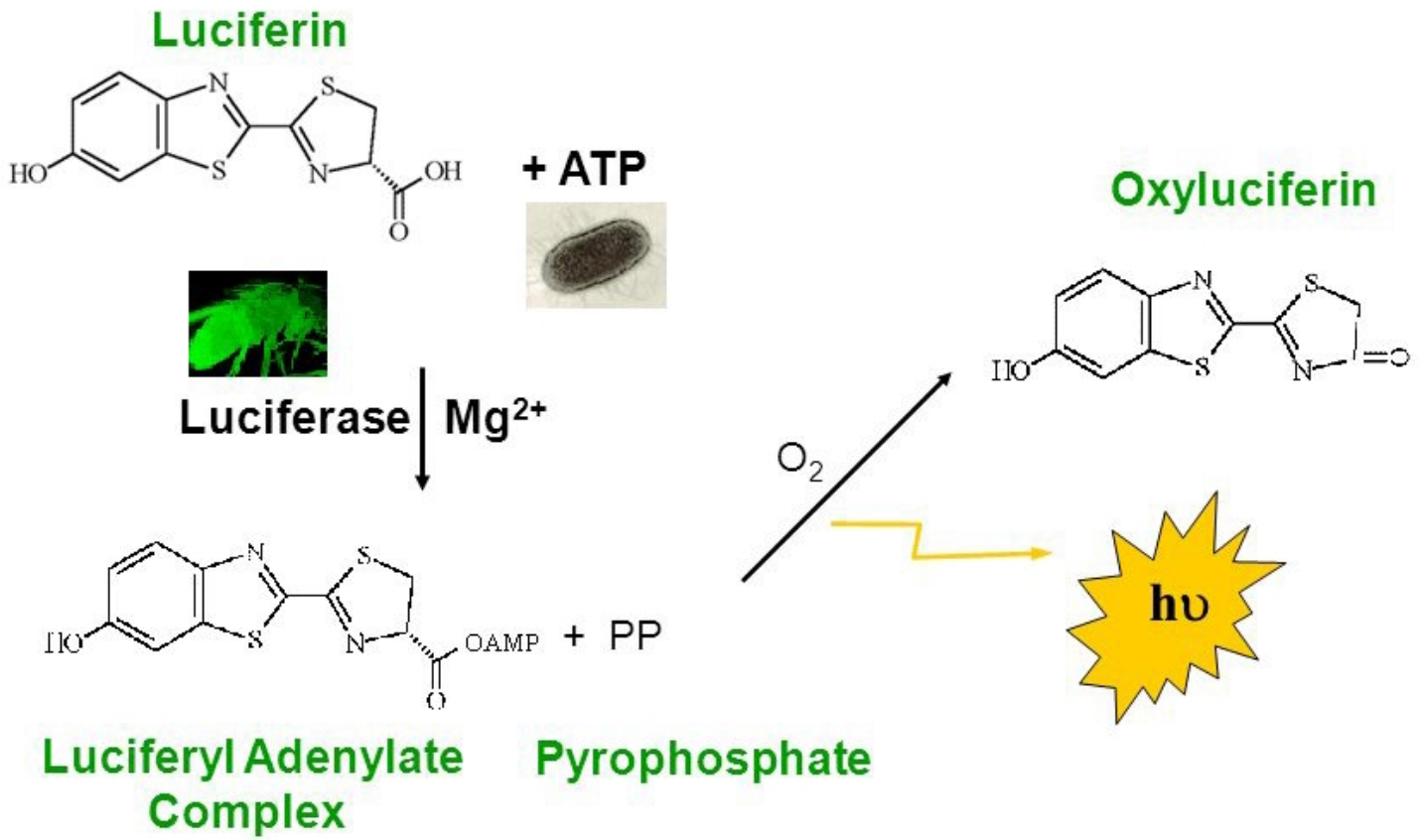
Luminometric Assays

- Luminometric assays provide fast and simple determination of cell proliferation and cytotoxicity in mammalian cells. These assays can be performed in a convenient 96-well and 384- well microtiter plate format and detection by luminometric microplate reader
- A remarkable feature of the luminometric assays is the persistent and **stable glow-type signal produced after reagent addition.** This atribute can be harnessed to produce both viability and cytotoxicity values from the same well

ATP assay

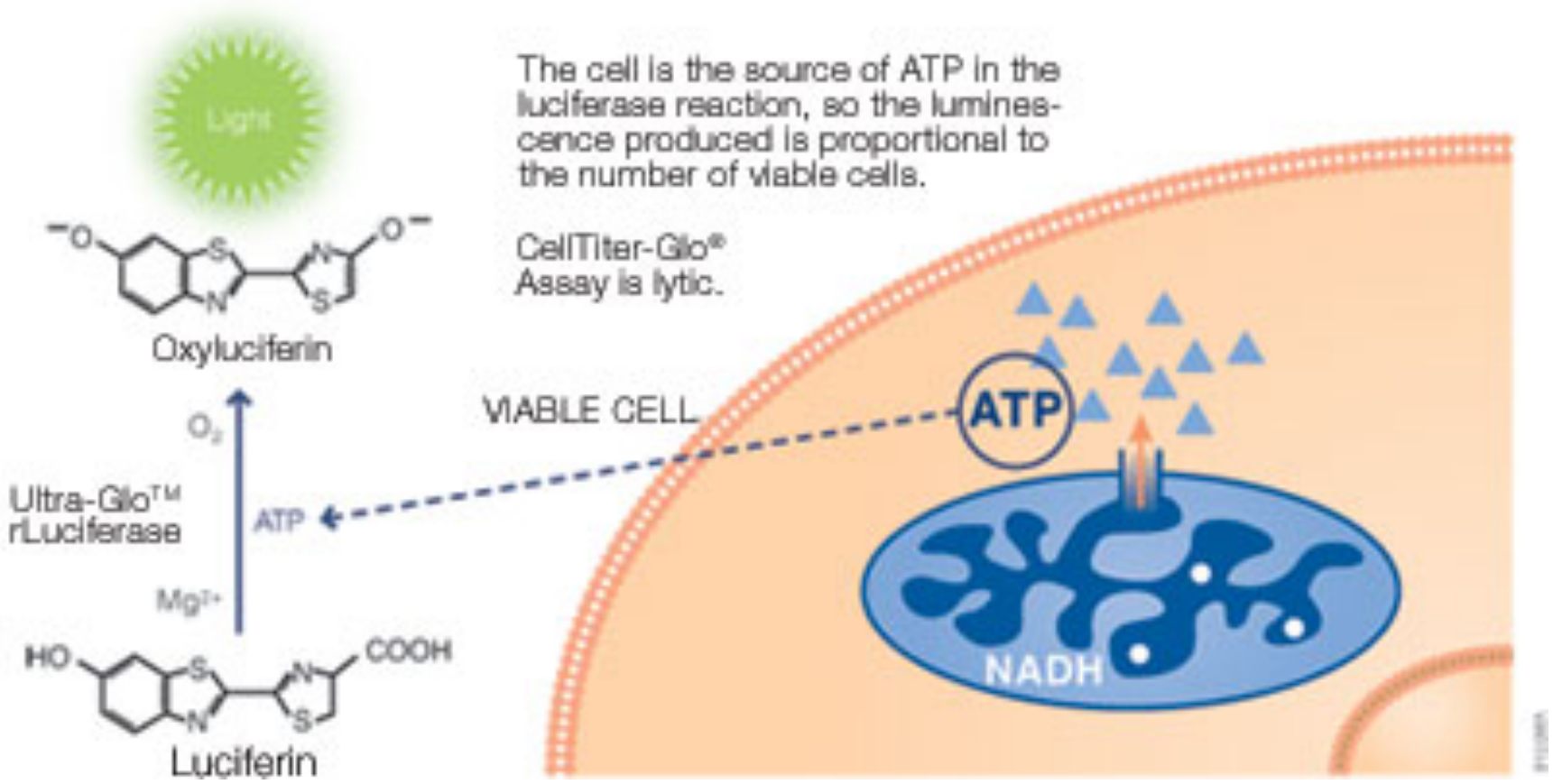
- ATP (adenosine tri-phosphate) represents the most important chemical energy reservoir in cells and is used for biological synthesis, signaling, transport, and movement processes. Therefore, **cellular ATP is one of the most sensitive end points in measuring cell viability**

ATP assay


Luciferin
HO
N
S
S
N
OH
O
+ ATP
Luciferase
Mg2+
Oxyluciferin
HO
N
S
S
N
O
O2
hυ
HO
N
S
S
N
OAMP
O
+ PP
Luciferyl Adenylate Complex
Pyrophosphate

ATP assay

- **When cells damaged lethally and lose membrane integrity, they lose the ability to synthetize ATP and the ATP level of cells decreases dramatically**
- The ATP assay is based on the reaction of luciferin to oxyluciferin. **Enzyme luciferase catalyzes this reaction in the presence of Mg2+ ions and ATP yielding a luminescent signal.**


Light
Oxyluciferin
O_2
Ultra-Glo™
rLuciferase
ATP
Mg^{2+}
HO
COOH
Luciferin
The cell is the source of ATP in the luciferase reaction, so the luminescence produced is proportional to the number of viable cells.
CellTiter-Glo®
Assay is lytic.
VIABLE CELL
ATP
NADH

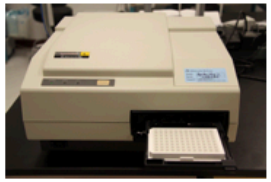
The Principle of ATP Bioluminescence Cell Proliferation Assays

Chemical energy production as ATP correlates directly with:

Viability | Proliferation | Cytotoxicity | Cell Number

Released iATP + Luciferin + Luciferase = Bioluminescence

LIGHT

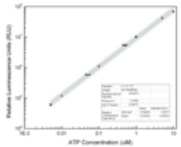


Luminescence Plate Reader

Relative Luminescence Units (RLU)

This additional step is highly recommended

Calibration and Standardization



Conversion to standardized ATP concentrations

It provides measurement assurance

- **Advantages: ATP assay is the fastest cell viability assay to use, the most sensitive, and is less prone to artifacts than other viability assays. The luminescent signal reaches steady state and stabilizes within 10 min after addition of reagent.** It does not have an incubation step for conversion of substrate into colored compound. This also eliminates a plate handling step