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# Comprehensive Overview of In Vitro Cell Viability Assays in Cancer Research

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**In cancer research, drug development, and evaluation of treatment, the most critical factor is the accurate measurement of cell viability. In the last few decades, numerous approaches that focus on specific biochemical characteristics of the cells have been created to analyse the cytotoxicity, health, and proliferation of cells. Metabolic activity-based tetrazolium assays like MTT, XTT, and MTS are able to provide accurate and quantitative colorimetric assays based on the metabolic conversion of tetrazolium to formazan. Rapid and simple assessments based on the structural integrity of cell membranes and the differential staining of viable and dead cells are provided by Trypan Blue, Eosin, and Propidium Iodide. Calcein-AM, Resazurin, and JC-1 are sensitive and reliable assays for the measurement of metabolic activity, evaluation of apoptosis, and assessment of the health of the mitochondria. This review provides a summary of the concepts, methods, advantages, and disadvantages of the described techniques.**

**Keywords:** Alamar Blue; Calcein; Cell viability; MTT; Propidium iodide; Trypan Blue.

Cell viability tests, which are essential to evaluate the percentage of living, metabolically active cells in a population, are essential aspects in biomedical research. These studies are necessary to determine how drugs(or) chemicals affects the health and proliferation of cells.[1-3]

Investigators can find out more regarding cytotoxicity, the safety of treatments, and how cells respond physiologically to different stimuli by evaluating the survival of cells.[2]

Several biological principles are essential for the development of various types of cell viability tests.[2] The ability of live cells to convert tetrazolium salts or resazurin into fluorescent products that represent mitochondrial or cytosolic metabolic activity is the basis for metabolic activity assays including the MTT, MTS, and resazurin (Alamar Blue) tests.[2,4,5]

As an indicator of active metabolic processes in cells, ATP-based assays measure intracellular ATP levels.[6] Propidium iodide staining and Trypan Blue exclusion are two assays that depend on the membrane integrity where the dead cells with the less intact membrane can take up the dye when compared to the intact cell membranes of the living cells.[2,7,8]

Clonogenic or colony formation tests, which measure single cell's capacity to withstand treatment and develop a colony over time, are commonly used to evaluate the long-term survival

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and proliferation.[2] For rapid assessment of living and dead cells, fluorescence-based LIVE/DEAD assays that combine ethidium homodimer-1 and calcein-AM are also often used. When combined together, these various methods provide a well-established foundation for understanding basic cell biology, medication development, toxicity assessment, and cellular responses.[9]

**Importance of Cell Viability Assays**

Assays for cell viability are crucial in many different biological applications. They are necessary methods in toxicology and drug development, enabling scientists to evaluate the cytotoxic effects of new chemicals on various cell types.[2,10] Viability tests are used in cancer research to assess the effectiveness of chemotherapeutic drugs and evaluate the response of tumour cells are to various treatments.[7]

Measuring the cell longevity, their proliferation, and response to environmental stresses provides an interpretation of cellular processes and signalling cascades, this is why they are important in cell biology research.[2,11] Moreover viability tests are commonly used in stem cell research and regenerative medicine to confirm that cultured cells sustain their ability to proliferate and function properly.[11]

**Tetrazolium-Based Cell Viability Assays: MTT, XTT, and MTS**

Tetrazolium-based assays are frequently used colorimetric methods for measuring cell viability, proliferation, and cytotoxicity. These assays depend on the metabolic reduction of tetrazolium salts into coloured formazan products by metabolically active cells or viable cells, predominantly through mitochondrial dehydrogenase enzyme activity.[2,4,5,7,12,13]

**Principle of Tetrazolium Assay**

Tetrazolium salts including MTT, XTT, and MTS are reduced by NAD(P)H-dependent cellular oxidoreductases into formazan products. The extent of formazan formed is directly related to the number of viable cells and the metabolically active cells.[2,14]

• MTT '! insoluble formazan

• XTT and MTS '! water-soluble formazan

**MTT Assay**

**Principle**

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to insoluble purple formazan crystals, which must be dissolved before absorbance measurement.[2,14]

**Procedure[12,15,16]**

1. Seed cells ($5\times10^3$–$1\times10$t cells/well) in a 96-well plate and incubate for 24 h.
2. Treat cells with test compounds and incubate for the desired period. Add MTT solution so that the final solution becomes 0.5mg/ml
3. Maintain under stable conditions for 2–4 h at 37°C.
4. Remove the medium carefully.
5. Add solubilization solution for solubilizing the non-soluble formazan crystals (DMSO or acidified isopropanol).
6. Mix to solubilize formazan crystals completely.
7. Analyse absorbance at 570 nm (reference: 630–690 nm).[7,16]

**Advantages**

• High sensitivity.[16]

• Widely used and standardized.[15]

**Perceived Limitations**

• Requires the solubilization step to dissolve the non -soluble crystals.[16]

• Cytotoxic to cells.

**XTT Assay**

**Principle**

XTT produces a water-soluble orange formazan dye, eliminating the need for a solubilization step. It requires an electron coupling reagent such as PMS.[2,12,14]

**Procedure[12,14]**

1. Seed and treat cells in a 96-well plate ($5\times10^3$–$1\times10$t cells/well).
2. Prepare XTT solution with electron coupling reagent.
3. Add XTT mixture directly to wells at the concentration of 0.5mg/ml.
4. Maintain under stable conditions for 2–4 h at 37°C.
5. Measure absorbance at 450–500 nm (reference ~650 nm).

**Advantages**

• No solubilization step as it forms water soluble crystals.[2]

• Less toxic to cells.

**Perceived Limitations**

• Requires additional reagent (electron mediator).

• Slightly lower sensitivity than MTT.[2]

**MTS Assay**

**Principle**

MTS is a modified Tetrazolium compound that produces a water-soluble formazan product directly in the culture medium.[2,17]

**Procedure[2,12,15,17,18]**

1. Seed and treat cells in a 96-well plate ($5\times10^3$–1×10t cells/well).
2. Add MTS reagent (0.5mg/ml) 20 µL per 100 µL medium).
3. Maintain under stable conditions for 1–4 h at 37°C.
4. Measure absorbance at 490–500 nm.

**Advantages**

• One-step, simple procedure.
• No solubilization required.[12]
• Suitable for high-throughput screening.

**Perceived Limitations[12,15]**

• More expensive
• Sensitive to media components and assay conditions.

**Dye Exclusion Assays for Cell Viability**

Dye exclusion assays are easy and rapid methods used to differentiate viable from non-viable cells based on membrane integrity.[19,20,21] These assays depend on the principle that viable cells possess intact cell membranes that don't allow the certain dyes to enter in the cell, whereas dead or damaged cells allow dye entry due to the damaged integrity of cell membrane.[2,8,19]

**Principle**

Dye exclusion assays are based on cell membrane permeability.[2,19,20]

• Viable cells '! intact membrane '! exclude dye
• Non-viable cells '! less intact membrane '! dye can be easily entered

**Common dyes used include**

• Trypan Blue
• Eosin
• Propidium Iodide

Recent developments have improved dye exclusion assays by including plate-based and high-throughput formats, decreasing subjectivity and increasing reproducibility.

**Trypan Blue Exclusion Assay**

**Principle**

Trypan Blue cannot be taken up by the viable cells due to its intact membrane but the dead cells have lost their membrane integrity and can take up the dye. Apart from this as the cell membrane of live cells is negatively charged and dye is also negatively charged which causes the repulsion and dye cannot enter the cell but different in the case of the dead cells as the damaged cell has the decreased negative charge and cells can take up the dye easily.[2,22,23]

**Procedure**

1. Prepare a single-cell suspension ($1\times10^3$).
2. Mix cells with 0.4% Trypan Blue (1:1 ratio).[24-27]
3. Maintain under stable conditions for 2–5 minutes at room temperature.
4. Place the sample onto a hemacytometer.
5. Count unstained (viable) and stained (dead) cells.
6. Calculate percentage viability.

• Automated cell counters, such as the countess™, can streamline this process by acquiring images and analysing cell count and viability automatically.[25]

**Table 1.** Classification of Cell Viability Assays

| Assay Type | Principle | Limitations |
|---|---|---|
| MTT / MTS / XTT / WST-1 | Living cells convert tetrazolium into coloured formazan | Cannot differentiate apoptosis and necrosis cells |
| Resazurin / Alamar Blue | Live cells convert resazurin fluorescent resorufin | Less accurate for low-metabolism cells ,might be influenced by external factors |
| Trypan Blue / Membrane integrity assays | Only dead cells take up the dye | No apoptosis detection |
| Propidium Iodide (PI) staining | Enters only dead cells; measured by microscopy/flow cytometry | Cannot differentiate apoptosis and necrosis cells; needs equipment |
| LIVE/DEAD fluorescence Assays | Calcein-AM stains viable cells Ethidium stains dead cells | Need confocal or fluorescence tools |

**Table 2.** Comparative summary of cell viability assays

| Parameter | Tetrazolium Assays (MTT, XTT, MTS) | Dye Exclusion Assays (Trypan Blue, Eosin, PI/SYTOX) | Fluorescence-Based Assays (Calcein-AM, Resazurin, JC-1, PI) |
|---|---|---|---|
| principle | Reduction of tetrazolium salts to formazan by metabolically active cells | Based on membrane integrity: viable cells exclude dye; dead cells take up dye. | Fluorescent probes detect viability, metabolic activity, or apoptosis. |
| Type of Detection | Colorimetric (absorbance). | Light microscopy or fluorescence (PI/SYTOX). | Fluorescence (plate reader, microscope, flow cytometry). |
| Target of Assay | Metabolic activity (mitochondrial enzymes). | Cell membrane integrity. | Metabolism, membrane integrity, or apoptotic markers |
| Solubilization Requirement | MTT: Required; XTT/MTS: Not required. | Not required (direct visualization). | Not required (direct fluorescence signal). |
| Effect on Cells | Endpoint assay; may kill cells. | Usually affects cells (Trypan Blue/Eosin); minimal for PI/SYTOX. | May affect cells (Calcein-AM, Resazurin); PI detects dead cells. |

**Advantages**

• Simple and cost-effective.

• Rapid assessment.

**Perceived Limitations**

• Need operator.

• Low throughput.

• Cannot detect early apoptosis.

**Eosin Dye Exclusion Assay**

**Principle**

Eosin dye that enters the dead cells with less membrane integrity same as the trypan blue but staining non-viable cells red or pink.[2,19,21,27,28,41]

**Procedure**

1. Mix cell suspension with eosin dye
2. Maintain under stable conditions for 2–5 minutes at room temperature.
3. Load on haemocytometer.
4. Count stained and unstained cells that represents the live and dead cells

**Advantages**

• Simple and inexpensive

**Fluorescence-Based Cell Viability Assays**

Fluorescence-based assays are highly responsive methods for measuring cell viability, cytotoxicity, and metabolic activity. They depend on fluorescent dyes or probes that either enter the viable cells ideally stain dead or apoptotic cells due to their decreased cell membrane integrity. These assays are widely used in high-throughput screening, flow cytometry, and imaging studies.[2,9,15,21,29]

**Principle of Fluorescence-Based Assays**

The major principle of fluorescent based assays depends on:

• Membrane integrity (e.g., Propidium Iodide, Calcein-AM).[31,32]

• Metabolic activity (e.g., Calcein-AM, Resazurin).[33,34,41]

• Apoptotic changes (e.g., Annexin V-FITC, JC-1)

Viable cells have the enzyme esterases that are able to convert the non-fluorescent dye to the fluorescent dyes where the dead cells are unable to stain.[19,30]

**Common Fluorescent Assays**

**Calcein-AM Assay**

**Principle**

Calcein-AM is a non-fluorescent, that has the ability to cross the viable cells that gets converted by intracellular esterases into green-fluorescent calcein in viable cells. Whereas the dead

cells don't have any effect by this calcein.[2,31-33,35]

**Procedure**

1. Seed cells ($1\times10^3$) in a microplate or chamber slide.
2. After the 24hrs of treatment remove the media wash with PBS and then Add Calcein-AM (1–5 µM) and incubate 30–60 min at 37°C.[35-38]
3. Wash cells with PBS to remove excess dye.
4. Measure fluorescence (Ex: 490 nm, Em: 520 nm) using a plate reader or fluorescence microscope.

**Advantages**

- High sensitivity
- Compatible with live-cell imaging
- Non-toxic for short-term studies

**Resazurin / Alamar Blue Assay**

**Principle**

Resazurin is also a non-fluorescent dye that has the ability to enter the viable cells and gets reduced into resorufin, a fluorescent product (Ex: 560 nm, Em: 590 nm) and it cannot enter the dead cells.[2,33]

**Procedure**[37,39,40]

1. Seed cells($1\times10^3$) in a microplate or chamber slide
2. After the 24hrs of treatment remove the media wash with PBS and then Add resazurin solution (10% v/v) and incubate 1–4 hrs at 37°C.[33,39,40]
3. Wash cells with PBS to remove excess dye.
4. Measure fluorescence using a plate reader.

**Advantages**

- Non-toxic; cells can be used for further assays.[39,40]
- High-throughput compatible.

**Propidium Iodide (PI) and SYTOX Dyes**

**Principle**

Membrane-impermeable fluorescent dyes enter only dead cells, as the membrane because of the less membrane integrity binding DNA/nucleic acids and emitting red fluorescence (Ex: 535 nm, Em: 617 nm).[2,33,41,42]

**Procedure**

1. Seed cells($1\times10^3$) in a microplate or chamber slide ($5\times10^3$).
2. After the 24hrs of treatment remove the media wash with PBS Add PI or SYTOX dye to the cell suspension.
3. Incubate 5–15 min at room temperature in the dark.
4. Analyse by fluorescence microscopy or flow cytometry.

**Advantages**

- Accurate identification of live/dead cells
- Can use along with other dyes like calcein

## CONCLUSION

Cell viability assays are important for determining cellular health, proliferation, and cytotoxicity. In this review, three major aspects tetrazolium-based assays, dye exclusion assays, and fluorescent-based assay were explained, each having various advantages and limitations.

Tetrazolium-based assays (such as MTT, XTT, and WST) facilitate a quantitative evaluation of metabolic activity, acting as an indirect measure of viable cells. These assays are sensitive, relatively simple, and suitable for high-throughput screening, but they depend on cellular metabolism, which may not always directly relates with cell number.

Dye exclusion assays, such as trypan blue staining, have straightforward and cost-effective method to differentiate live and dead cells based on membrane integrity. However, they are less sensitive, and not ideal for large-scale or automated analysis.

Fluorescent-based assays, including those using dyes like calcein-AM and propidium iodide, allow for more Accurate and multiparametric analysis of cell viability. These assays can differentiate between live, dead, and even apoptotic cells with high sensitivity and are well-established for advanced imaging and flow cytometry applications, though they need specialized equipment and can be more expensive.

Overall, no single assay is universally; the selection depends on the experimental objectives, required sensitivity, available resources, and throughput needs. Combining multiple assay types can provide a more comprehensive and reliable assessment of cell viability.

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Not Applicable.

**Author Contributions**

Korimelli Sridevi: Supervision, Designed the overall structure, Critical review, and Validation; Himaja Kommineni: Conceptualization, Design of the study, Data collection and analysis, Prepared the original manuscript draft; Gunadeep Naidu Vennela: Literature review and Data verification

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