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O.M. Sukach *, O.R. Maiorova
Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkiv, Ukraine
*an_sukach@ukr.net

USE OF METHODS FOR DETERMINING CELL VIABILITY IN CRYOBIOLOGICAL RESEARCH

Assessment of cell viability is critically important in the context of cryobiological research, as it allows determining the effectiveness of freeze-thawing procedures and low-temperature storage of isolated cells. The review systematizes the methods for determining cell viability used in cryobiological research, with an emphasis on their practical applicability, accuracy, and sensitivity. Both traditional methods based on the assessment of cell membrane integrity and metabolic activity, such as trypan blue staining, MTT assay, fluorescent dye assays, and more modern approaches, including flow cytometry, confocal microscopy, and methods for assessing cell functional activity, are reviewed. For each method, a description of the mechanism of action, a protocol, specific advantages, disadvantages, as well as equipment requirements and limitations for cell types are provided. A comparative analysis was conducted, which revealed the lack of a universal approach, but demonstrated the effectiveness of combined methods that combine fluorescent staining and metabolic assessment. Such combinations allow for high accuracy in assessing cell viability after cryopreservation. In addition, the review discusses the prospects for the development of methods for determining cell viability in cryobiology, including the introduction of the latest high-resolution imaging technologies, multiparametric analysis, as well as integration with microfluidic platforms and artificial intelligence to automate and improve the accuracy of the assessment. The review can serve as a practical tool for researchers in choosing the most optimal approach for assessing cell viability depending on the purpose of the experiment, available resources and cell type.

Key words: *cryobiology, cell viability, cryopreservation, dye exclusion method, colorimetric method, fluorescent dyes, luminometric assays, cultivation.*

Cryobiology is an interdisciplinary science that studies the behavior of biological systems under extremely low temperatures, in particular the processes of cooling, freezing, storage and recovery of cells, tissues and organs. Its practical importance is difficult to overestimate: cryopreservation methods are widely used in reproductive medicine [51], transplantology, cell therapy, biotechnology [49].

One of the main tasks of cryobiology is to preserve the viability of biological material after cryopreservation. As a result of the action of low temperatures, cells are exposed to a number of stress factors, such as dehydration, osmotic fluctuations, the formation of intracellular and extracellular ice crystals and, as a result, membrane damage and metabolic disorders [21, 41]. In this regard, there is a need to use reliable methods for determining cell viability, which allow objectively assessing the effectiveness of the chosen strategy for their cryopreservation, the level of their post-stress adaptation and suitability for further use for research or clinical purposes.

Today, researchers have in their arsenal a wide range of methods for determining cell viability —

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from classical staining techniques to highly sensitive fluorescent or metabolic analyses. Each of these methods has its own advantages, limitations, specificity and sensitivity, that necessitates their substantiated choice depending on the type of object under study and the objectives of the experiment. Choosing the optimal method allows us to objectively assess the quality of cell preservation, detect damage and optimize the conditions of their cryopreservation and storage.

Existing methods for determining cell viability can be classified by parameters, *i. e.* signs or criteria that reflect the physiological, biochemical or morphological characteristics of living and dead cells. These main parameters to be assessed are: plas-ma membrane integrity, metabolic activity, proliferation capacity, morphological characteristics, changes in membrane potential/ion balance, genetic integrity (DNA/RNA). Depending on the nature of the effect on cells, methods for determining viability are divided into destructive, which lead to cell destruction, and non-destructive, which allow cells to be preserved as viable for further use in experiments.

According to the level of required technical support, methods for determining viability can be divided into simple (light microscopy, staining methods) and high-tech (cytofluorimetry, biosensor systems based on living biochips) ones. In addition to classical approaches, in recent years the role of automated analysis systems using artificial intelligence (AI), microfluidic technologies and nanotechnologies has been increasing to increase the accuracy [4, 22], standardization and reproducibility of cell viability assessment. The introduction of these approaches into routine cryobiological practice can significantly improve the quality of cell preservation and optimize freezing protocols.

The aim of the review was to systematize existing methods for assessing cell viability used in cryobiology, identify their advantages and limitations, and analyze the prospects for implementing new technologies to increase the accuracy, informativeness, and reproducibility of the assessment.

1. PLASMA MEMBRANE INTEGRITY TESTS

1.1. Dye exclusion tests

A characteristic feature of dead or apoptotic cells is the disruption of the integrity of the plasma membrane. It is on this property that dye exclusion tests are based, which are used to determine the number of viable cells in a sample. The principle of the method is that living cells with an intact plasma membrane do not pass vital dyes, such as trypan blue (TB), eosin, Congo red, erythrosin, propidium iodide (PI), while cells with a damaged plasma membrane absorb these dyes [9, 37].

To perform such tests, the cell suspension is mixed with a dye, and then the number of viable cells (with transparent cytoplasm) and non-viable cells (with stained cytoplasm) is visually counted.

Dye exclusion tests are relatively simple, can be performed on small numbers of cells, are rapid, and allow the detection of cell death in non-proliferating populations. The main limitation is their unsuitability for the analysis of cells in monocultures, as they are designed primarily for cells in suspension [37]. One of the most common dye exclusion tests is the trypan test — a TB dye exclusion test [62]. TB is a large, negatively charged molecule. An intact cell membrane is a barrier to the passage of large charged molecules. A damaged membrane loses its barrier properties and TB passes into the cells, staining them blue. Viable cells with intact membranes are, on the one hand, impermeable to TB, and on the other hand, able to exclude the dye from their intracellular environment and remain unstained.

Typically, the TB test is performed by mixing one part of a 0.4% TB solution with one part of a diluted cell suspension. Counting of live and dead cells is performed within 3—5 minutes after mixing, as a longer incubation period with TB can cause additional cell damage and decrease viability. Mixing can be performed in a well of a plate, or in a plastic tube, using 10 to 20 μl of cell suspension and TB. Evaluation of the results is carried out microscopically using a Goryaev chamber or automated cell counting systems. TB exclusion can also be measured spectrophotometrically or using flow cytometry. It is important to note that the TB solution should be stored in a dark place, and after prolonged storage — it is subject to filtration. Since TB has a high affinity for serum proteins, which can distort the results, it is recommended to use a serum-free medium. The disadvantage of the trypan test is that it determines viability indirectly by the integrity of the cell membrane. In this case, there is a possibility of losing the functional properties of the cell (at least temporarily) while maintaining the integrity of its membrane. Conversely, the impaired

integrity of the cell membrane, which is determined by the trypan test, can be restored and the cell becomes fully viable. Another potential problem is the inaccuracy of the trypan test, since the absorption of the dye is assessed subjectively and small amounts of it, which indicate cell damage, may go unnoticed. Another disadvantage is that the trypan test is an end-point assay, since the dye for cells, as already noted earlier, is toxic. Another disadvantage of the trypan test is that it is used to analyze cell suspensions and cannot be used to assess the viability of cells in 2D- and 3D-cell structures.

As an alternative to TB, Congo red and erythrosin B dyes can be used, which are nontoxic and do not bind to serum proteins [34]. In addition, erythrosin B is able to detect early stages of membrane damage, when TB has not yet penetrated the cell, which allows for a more accurate assessment of sublethal cell damage that may occur during cryopreservation. Congo red provides a brighter color of dead cells, which facilitates counting, especially when using light microscopy. Both dyes, in contrast to TB, have higher compatibility with fluorescent assays and enzymatic tests.

1.2. Fluorescent markers of viability

More sensitive and accurate markers for determi-ning cell viability, compared with colored ones, are fluorescent dyes [36], such as ethidium bromide (EBr) and PI [5]. Like TB, they penetrate cells only in the presence of damaged membranes. Visualization of these dyes by fluorescence microscopy usually provides a more accurate assessment of cell viability compared with the use of non-fluorescent dyes.

The combined use of non-permeable (PI, EBr) and permeable (acridine orange (AO), fluorescein diacetate (FDA)) fluorescent dyes allows the simultaneous determination of the number of live and dead cells in a population [59]. Double staining is usually applied to cell suspensions, and the result is evaluated using a fluorescent microscope or flow cytometry.

1.3. Acridine orange/propidium iodide staining

AO dye is able to enter viable cells and bind to nucleic acids. When bound to double-stranded DNA (dsDNA), it fluoresces green, while binding to single-stranded DNA (ssDNA) or RNA results in red fluorescence [57]. PI only enters cells with damaged membranes, where it binds to nucleic acids [65]. This results in a 20- to 30-fold increase in its fluorescence, causing non-viable cells to fluoresce red. Through Förster resonance energy transfer (FRET), the PI signal absorbs the AO signal in non-viable cells, preventing double positives. Thus, when cells are stained with AO in combination with PI, viable cells containing a nucleus fluoresce green, and non-viable cells fluoresce red.

Determining cell viability using AO/PI staining is a rapid and informative tool, but has certain drawbacks, especially when evaluating cryopreserved cells. In particular, cells in the early stages of apoptosis or with sublethal damage to the plasma membrane may give false positive or false negative results: the membrane still retains integrity, but the cell has already lost viability in the long term. This method does not allow for a full assessment of metabolic activity, functional capacity and long-term survival potential of cells after cryopreser-vation. Morphological features of some cell types or the presence of extracellular matrix may also complicate visualization and accurate viability counting.

In cryobiological practice, these shortcomings may be particularly important, since the freeze-thaw process may cause various types of cellular damage, not always associated with direct disruption of the plasma membrane at the time of analysis. Therefore, for a comprehensive assessment of the viability of cryopreserved cells, it is recommended to use a combination of methods that assess different aspects of their functional state.

1.4. Fluorescein diacetate/propidium iodide staining

The non-fluorescent dye FDA, like AO, penetrates viable cells, where it is converted by esterases to a green fluorescent metabolite fluorescein. PI, after penetrating dead cells, binds to DNA and fluoresces red. Thus, dual FDA-PI staining allows for simultaneous assessment of live and dead cells [30]. Additional staining of cell nuclei with the blue fluorescent dye Hoechst allows for increased accuracy in assessing cell viability.

Table 1. **FDA-PI staining solution (must be freshly prepared)**

Component	Volume
Culture medium without FCS	5 ml
FDA (5 mg/ml)	8 μl
PI (2 mg/ml)	50 μl

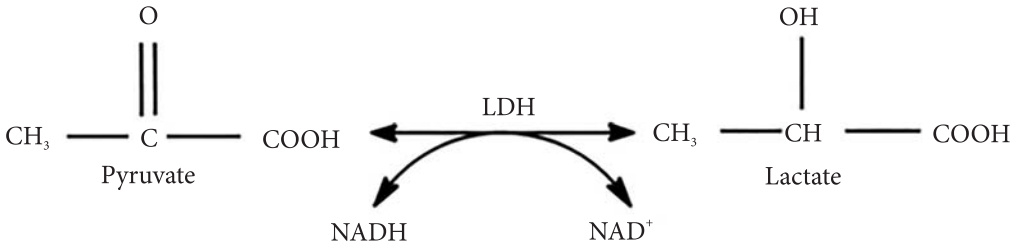

Fig. 1. Conversion of lactate to pyruvate catalyzed by LDH

FDA/PI staining can be used for cells in suspensions and monolayers, as well as cells integrated into the extracellular matrix or in formed three-dimensional clusters, in particular multicellular spheroids.

The staining protocol includes preparation of staining solutions (Table 1), addition to cells (after removal of medium), incubation at room temperature for 4—5 minutes in the dark, removal of solutions and addition of phosphate buffer or protein-free medium followed by analysis by fluorescence microscopy.

The FDA-PI staining has certain drawbacks, especially in the context of cryobiology: cells with low metabolic activity or damaged esterases may give weak fluorescent FDA staining, even if their membrane is still intact. Cells in early stages of apoptosis or with sublethal membrane damage may be impermeable to PI, leading to false positive results in the long term. FDA/PI staining does not fully reflect the functional state of the cells. Cells that appear "alive" by this test may be functionally compromised after cryopreservation.

1.5. Lactate dehydrogenase activity assay

The loss of cell membrane integrity leads to the release of cytoplasmic proteins into the extracellular space, a significant number of which are enzymes. One such enzyme is lactate dehydrogenase (LDH), which is used as a viability marker both *in vitro* and *in vivo* [16].

LDH is a stable cytoplasmic enzyme found in all cells. When the plasma membrane is damaged, it is rapidly released into the extracellular space. LDH activity can be easily quantified by using NADH, which is formed during the conversion of lactate to pyruvate (Fig. 1), to reduce the second compound in a coupled reaction to a product with properties that are easily quantified.

As a second compound, yellow tetrazolium salt or iodonitrotetrazolium, which is reduced to a red water-soluble dye of the formazan class (light absorption at 492 nm), is often used. The amount of formazan is directly proportional to the amount of LDH in dead or damaged cells.

For analysis, a substrate containing yellow tetrazolium salt, lactic acid, and NAD^+ is added to the cell supernatant, incubated at 37 °C for 15—30 min. After incubation, a stop solution is added to halt the reaction, and the absorbance is measured on a spectrophotometer at 490 nm for 1 h [31]. It should be borne in mind that serum, which is usually used in cell culture, has inherent LDH activity, which requires experiments to be carried out in its absence.

A disadvantage of the method, especially in the context of cryopreservation, is that the level of LDH in the extracellular space is not a direct indi-cator of cell viability. Cells can have damaged mem-branes and release LDH, but remain metabolically active or capable of repair. In addition, various cell types can have different levels of intracellular LDH, which affects the method sensitivity. LDH analysis is usually performed at the end point of the experiment, so it only reflects the state of the cells at the time of measurement and does not show the dynamics of cell death or repair over time. The method provides an average score across the entire cell population and does not allow the identification of subpopulations with different levels of damage or viability.

Therefore, to obtain a more complete and accurate assessment of the viability and functional status of cryopreserved cells, the LDH assay should be used in combination with other methods (e.g., live/dead cell staining, metabolic activity assay, or clonogenic assay).

2. METABOLIC TESTS

Metabolic tests are used to assess cell viability by quantifying the activity of enzymes involved in metabolic processes. At the same time, these methods can also reflect proliferative activity, since

cells that are actively dividing are characterized by an increased metabolic rate. Therefore, the results obtained can indicate not only viability, but also the level of cell proliferation.

2.1. Tetrazolium-based tests

There are a number of tests based on the metabolism of tetrazolium salts by cells [61]. These include: Methyl Thiazolyl Tetrazolium (MTT), Xenon-derived Tetrazolium (XTT), Methoxy Thiazolyl Sulfophenyl Tetrazolium (MTC), Water-Soluble Tetrazolium (WST) or its commercial implementation — Cell Counting Kit-8 (CCK-8) tests. They are highly sensitive and allow the determination of mitochondrial metabolic activity, which is present only in viable cells. These tests are based on the ability of tetrazolium salts to penetrate the outer membrane of living cells and are then reduced by NADH or oxidoreductases and dehydrogenases of metabolically active cells to various formazan derivatives, colored from red to purple [61]. Dead cells lose the ability to undergo this reduction. Some of the formazan derivatives formed are soluble, others are not. To carry out a quantitative assessment, insoluble compounds of the formazan formed in the cells are extracted with organic solvents (methanol, ethanol, acetone, dimethyl sulfoxide, dimethylformamide, sodium dodecyl sulfate and combinations of detergent with organic solvents). The choice of solvent depends on the specific test and the type of cell culture. The amount of formazan formed is determined spectrophotometrically.

2.2. MTT test

The most common tetrazolium-based test is the MTT test. It is considered to be quite simple and at the same time highly sensitive and effective for various cell types. The method is based on the ability of viable cells, unlike non-viable ones, to convert soluble yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide into insoluble purple-blue intracellular formazan crystals with an absorption maximum at about 570 nm (Fig. 2). The color change and the intensity of the reagent are determined by the activity of mitochondrial systems and reflect the number of viable cells [8, 69]. The formed formazan is dissolved by adding dimethyl sulfoxide, dimethylformamide or sodium dodecyl sulfate [17, 27, 46, 64] and the colored solution is quantitatively measured spectrophotometrically at 570 nm. The absorbance is directly proportional to the number of viable cells. However, when performing the MTT test, it should be borne in mind that the amount of formed formazan, in addition to the number of viable cells, also depends on their metabolic activity, MTT concentration and the duration of the incubation period.

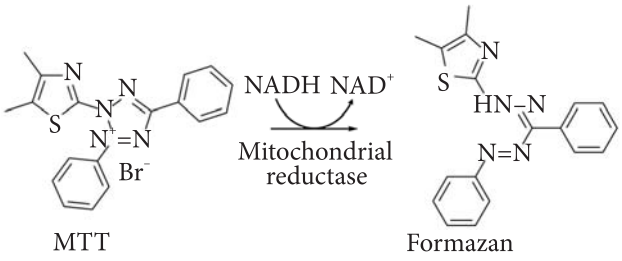

Fig. 2. Reduction of MTT to formazan crystals

Typically, to perform the MTT test, 10 μl of MTT solution is added to 100 μl of suspension or cell culture in a 96-well plate to achieve a final concentration of 0.45 mg/ml, and incubated at 37 °C for 1—4 hours. The formazan crystals formed during the incubation process are dissolved in 100 μl of solubilization solution and the absorbance at 570 nm is measured using a plate reader.

The disadvantages of MTT are its toxicity, sensitivity to the presence of serum albumin, metal ions and vitamins in the culture medium, as well as the need for a formazan dissolution step.

In addition to MTT, there are also tetrazolium reagents that are reduced by viable cells to soluble formazan products in the culture medium. These are MTS, XTT and WST tetrazolium compounds [25, 14, 48]. Due to the solubility of their formazan products, a step of dissolving the formazan precipitates is not required, making the protocols more convenient. MTS, XTT and WST tetrazolium compounds are considered to have limited cell permeability [58]. Therefore, they are used in combination with intermediate electron-withdra-wing reagents, such as phenazine methyl sulfate (PMS) or phenazine ethyl sulfate (PES), which can enter viable cells, be reduced in the cytoplasm or on the cell surface, and exit the cell, where they can convert the tetrazolium to soluble formazan (Fig. 3.) [7].

These tests are more sensitive and less toxic than MTT. The main disadvantages of tests based on the determination of the metabolic activity of cells by the reduction of tetrazolium salts are the partial instability of the solutions and the pronounced cytotoxicity, especially of the MTT reagent [54].

2.3. Alamar Blue test

Another example of a metabolic test for determining cell viability is the resazurin test, also known as the Alamar Blue or PrestoBlue test [50, 54]. The method is based on the same principle as the tetrazolium assays and uses electron transfer to convert the non-toxic water-soluble resazurin into the fluorescent substance resorufin (Fig. 4).

Non-fluorescent resazurin has a blue color, penetrates cell membranes, transforming under the influence of reductases of viable cells into resorufin, which has bright fluorescence in the red spectrum. The transformation of resazurin to resorufin in viable cells occurs continuously, without causing cell death, which allows the formation of a signal, the registration of which can be carried out both quantitatively (by spectrometric or fluorimetric methods), and qualitatively by changing the blue color to pink [2, 54]. Typically, the amount of resorufin formed, which is proportional to the number of viable cells, is determined using a microplate fluorimeter, using an excitation wave-length of 560 nm and an emission wavelength of 590 nm. The incubation time required to generate an adequate fluorescent signal ranges from 1 to 4 hours and depends on the metabolic activity of the particular cell type, cell density per well, and other assay conditions, including the type of culture medium. The incubation period is optimized to avoid reagent toxicity while still providing adequate sensitivity.

The main advantages of the resazurin reduction assay are the relative availability of the reagent, high sensitivity, and the ability to reuse the cells for other assays [75]. Disadvantages include the possibility of fluorescent interference from the test compounds.

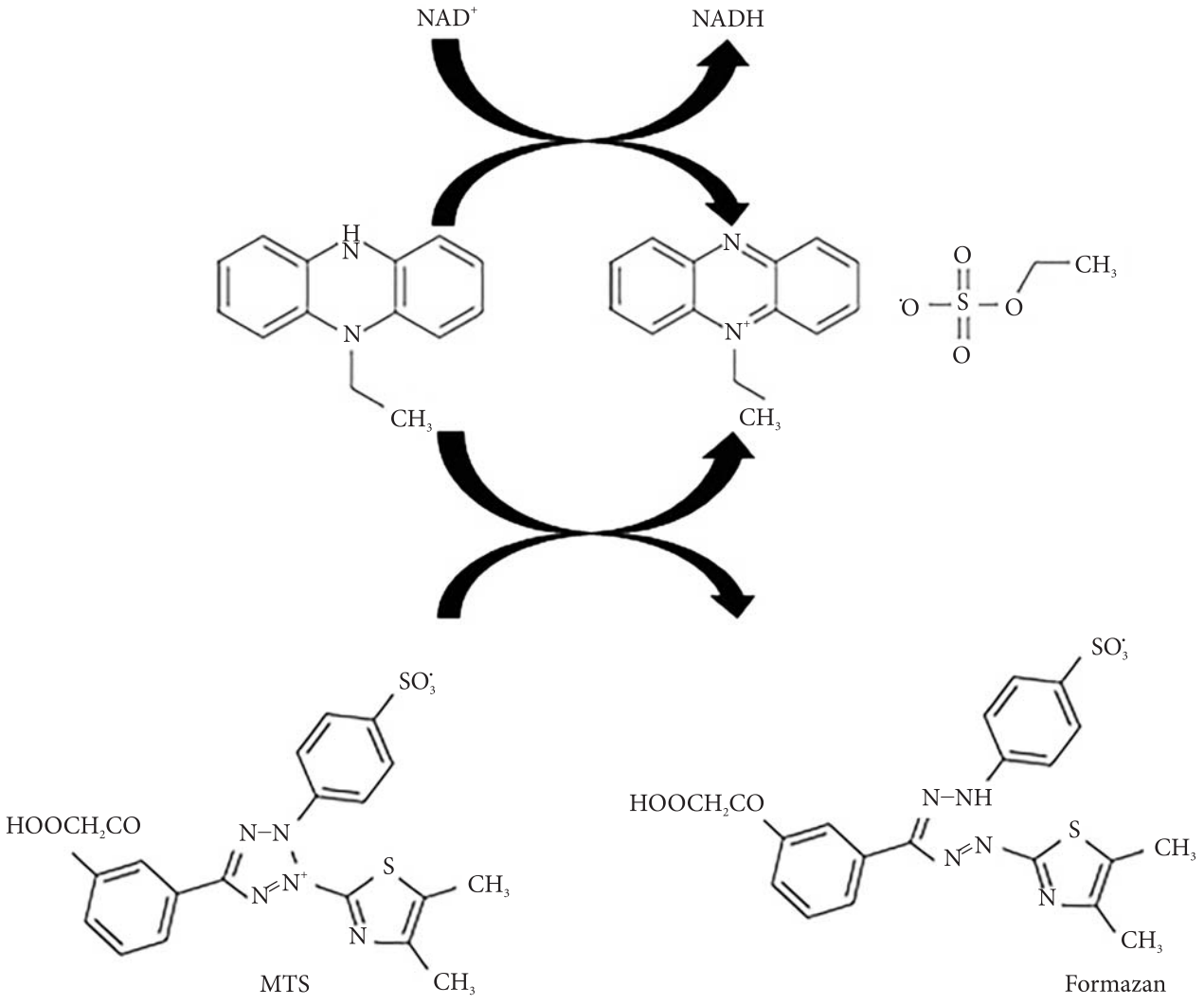

Fig. 3. Electron acceptor intermediate phenasin ethyl sulfate transfers an electron from NADH in cytoplasm to reduce MTS in culture medium to water-soluble formazan

A weakness of all tetrazolium or resazurin reduction assays is the dependence of the signal on the accumulation of colored or fluorescent products over time. The gradual increase in signal makes it difficult to determine cell viability during prolonged incubation.

It should also be noted that colorimetric assays using tetrazolium salts and resazurin are not optimal for dense three-dimensional cell structures, as the reagents have limited ability to penetrate multiple cell layers.

2.4. Cell viability assay based on ATP quantification

A widely accepted marker of cell viability is ATP, which, being a source of energy, is formed only in living cells. Thus, its quantity is directly proportional to the number of living cells [38]. Several methods can be used to determine the amount of ATP, such as spectrophotometric, fluorescent, bioluminescent and others [38]. The most common is the bioluminescent ATP assay, due to its high sensitivity, simple protocol and rapid results [39].

The bioluminescent ATP assay is based on the enzymatic reaction of firefly luciferase, which uses ATP from viable cells to generate photons of light [26]. Viable cells are pre-lysed to release ATP for detection, and reagents containing the firefly luciferase enzyme and substrate are added to catalyze the reaction of converting luciferin to oxyluciferin with the generation of luminescent light with a wavelength of 550—570 nm (from green to yellow) (Fig. 5).

The intensity of the luminescent signal is determined using a luminometer. When ATP is the limiting component in the luciferase reaction, the luminescence is proportional to the ATP concentration.

The ATP assay protocol involves adding to the cells a reagent that contains a detergent to lyse them, an ATPase inhibitor to stabilize the ATP released from the lysed cells, luciferin as a substrate, and a stable form of luciferase to catalyze the reaction that generates photons of light [56]. The luminescent signal stabilizes within 10 minutes of reagent addition and typically glows with a half-life of longer than 5 hours.

The advantage of bioluminescent ATP assays is that they do not require incubation with cells to convert a substrate (*e. g.*, tetrazolium or resazurin) to a colored compound. However, bioluminescent

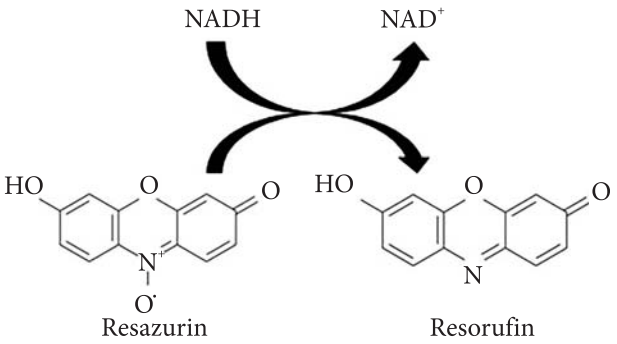

Fig. 4. Reduction of resazurin substrate to pink fluorescent resorufin

ATP assays can typically detect fewer than 10 cells per well and are widely used in 96- or 384-well formats [56]. The disadvantage is that cells must be lysed. Recently, highly sensitive, highly accurate, and flexible methods have been developed to measure ATP levels in cells using nano- or microsensors, which allow for real-time assessment of cell viability [32, 60]. However, their high cost and complexity (requiring high-precision calibration) have hindered their widespread use.

2.5. Cell viability assay using potential-dependent dyes

The mitochondrial membrane potential is closely related to the ability of cells to produce ATP and can therefore also be used as an indicator of cell viability [20, 78]. Dyes such as JC-1 and JC-10 are widely used to detect it [55, 79]. When the mitochondrial membrane potential is high, JC-1 and JC-10 aggregate in the mitochondrial matrix, emitting bright red fluorescence. At low mitochon-drial membrane potential, the aggregates in the mitochondria convert to monomers with green fluorescence. The relative ratio of red/green fluorescence can be used to determine the change in mitochondrial membrane potential, which correlates with cell viability. JC-10 differs from JC-1 in its better water solubility and higher sensitivity [79].

The staining protocols for JC-1 and JC-10 are generally rapid and do not require complex manipulations. However, the method has certain drawbacks, especially in the context of cryobiology. After cryopreservation, cells may undergo a variety of damage, which is not always manifested in changes in mitochondrial membrane potential in the early stages after thawing. Therefore, assessing viability using JC-1 and JC-10 alone may not reflect the full spectrum of damage and the long-term

ability of cells to repair and function. For a more comprehensive assessment, it is recommended to use JC-1 and JC-10 in combination with other methods that assess membrane integrity, metabolic activity, and functional capacity of cells.

2.6. Cellular autofluorescence signals as markers of viability

Most traditional methods for determining viability are inherently destructive. However, living cells express a number of intrinsic fluorophores that play a key role in cellular metabolism and can be used to non-destructively determine viability [15]. Such fluorophores include the cofactors NADH, NADPH, and FAD, whose autofluorescence imaging allows for label-free detection of cellular metabolism [24, 35]. NADH and NADPH, which cannot be distinguished by fluorescence imaging, are typically excited by light with a wavelength of approximately 370 nm for single-photon fluorescence and 750 nm for two-photon fluorescence [29]. FAD is usually excited by light with a wavelength of approximately 405 nm for single-photon fluorescence and ~890—900 nm for two-photon excitation [29]. The fluorescence peak of NAD(P)H is observed at a wavelength of 400—500 nm, and that of FAD at 500—600 nm [29]. Thus, by using ultraviolet light in the range of 370—400 nm to excite the autofluorescence of NAD(P)H and FAD, we can observe the fluorescence of living cells in the spectrum green region [63].

It should be noted that high-resolution fluorescence microscopy with lasers as the excitation light source is usually used to record the autofluorescence of NAD(P)H and FAD, which can cause phototoxicity. The use of two- or three-photon excitation allows for the reduction of the damaging effects of the excitation light, although some level of phototoxicity is inevitable.

3. FUNCTIONAL TESTS

3.1. Cultivation as a test for cell viability

A characteristic feature of viable adhesive cells is their ability to attach to the substrate, spread, migrate and proliferate in culture. Cell attachment with subsequent spreading are complex processes inherent in viable cells, which include receptor binding to ligand, activation of intracellular signaling pathways and cytoskeleton reorganization. At the same time, for freshly obtained or cryopreserved cells, the cultivation time is important, during which non-lethal damage to the plasma membrane, likely resulting from tissue disaggregation or freezing-thawing of cells, can be restored. The duration of such a lag phase may depend on the degree of cell damage, their seeding density, the type of cultivation surface, and the composition of the cultivation medium. For cryopreserved cells, the lag phase can be from 24 to 72 hours. That is, we can observe the attachment of adhesive cells to the substrate within 3 days after their extraction from the tissue or thawing. The attached cells usually spread out, acquiring a fibroblast- or epithelial-like shape. The spreading of attached cells indicates that viable cells have recovered and are ready for active division, unless they are non-dividing cells, such as neurons.

Typically, a cell attachment test is performed by seeding a known number of original or thawed cells in a culture vessel. After 24 hours of cultivation, the number of non-attached (floating) cells is counted, transferred to a clean culture vessel and continued to cultivate for another three days. The number of non-adherent cells is counted, which allows us to determine the maximum number of cells that can recover their viability after isolation or cryopreservation. [52] It should be noted that the method can only be used for adhesive cells.

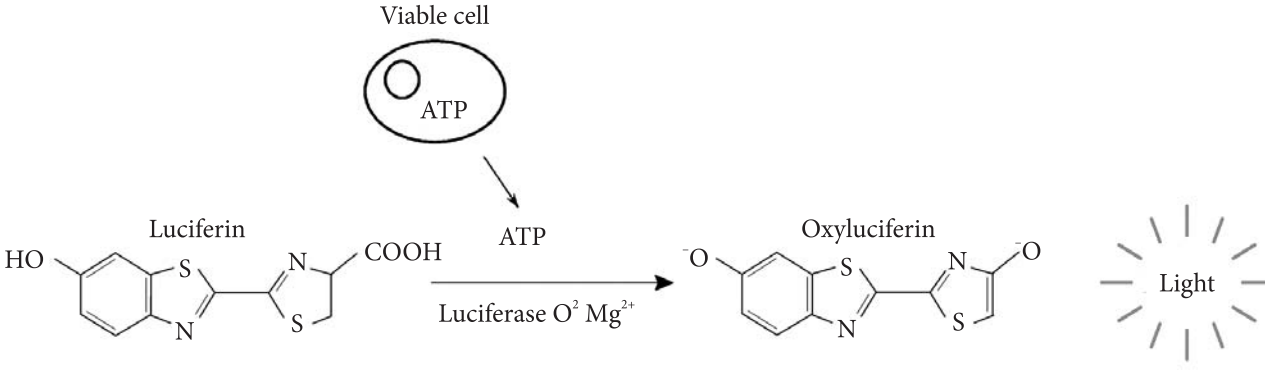

Fig. 5. Simplified reaction scheme showing ATP and luciferin as substrates for luciferase to generate light

Viable non-adherent cells should be free-floating in suspension solely or in small clusters, have a spherical or lymphoblast-like shape with a clear cytoplasm and a clearly defined plasma membrane.

Signs of cell death include their clumping (due to the release of DNA during lysis), dark color, an indistinct plasma membrane, and blebbing of the plasma membrane.

4. FEATURES OF DETERMINING CELL VIABILITY IN THREE-DIMENSIONAL STRUCTURES

Currently, pharmaceutical and biomedical research increasingly uses three-dimensional (3D) cell structures that more accurately mimic intact tissues of a living organism: organoids, spheroids, and engineered constructs created on the basis of cells and various scaffolds. These structures are characterized by high cell density, rather large sizes, and often heterogeneous composition, which complicates the assessment of their cell viability both in the native state and after cryopreservation.

Most researchers use protocols developed for two-dimensional structures with some modifications to analyze cell viability in 3D structures [71, 81]. Typically, modifications are aimed at taking into account the size and packing density of cells in 3D structures, which is expressed in a longer incubation time with reagents to ensure proper penetration.

There are several commercial protocols developed for analyzing cell viability in 3D structures, in particular, the colorimetric Cultrex® 3D assay and the luminescent CellTiter-Glo® 3D assay. In fact, as mentioned above, these assays are modified to 3D conditions by the classic MTT test and the luminescent ATP concentration determination test.

In addition to taking into account the size and packing density, determining cell viability is often accompanied by dissociation of the three-dimensional structure, or destruction of the 3D structure framework in order to obtain single cells. This leads to an increase in the experiment time and is accompanied by morphological changes in the 3D structure itself, which leads to an inaccurate assessment of cell viability and the unsuitability of using 3D structures in further experiments.

These shortcomings can be avoided by *in vivo* visualization of NADH and FAD autofluorescence of cells in 3D structures [19, 63, 81], which is non-invasive and does not use endogenous dyes. The most common methods of visualization of cell fluorescence are confocal and multiphoton microscopy. These are methods that can be used to obtain optical sections of a three-dimensional cellular structure.

By limiting the detection of the signal to one geometric plane in a three-dimensional structure, we obtain an image of cells in this plane that is similar to that obtained by standard histopathology methods. However, unlike histological methods, obtaining optical images does not require any preparation [11, 12], *i. e.* they are non-invasive. These methods are also characterized by speed and informativeness. Further, by combining images from a stack, we can obtain a three-dimensional reconstruction of 3D structures [66].

It should be noted that, compared to confocal, multiphoton microscopy provides a greater depth of light penetration, more reliable and stable optical alignment, and increased sensitivity to weak fluorophores [82]. The use of multiphoton microscopy allows for approximately five times greater imaging depth compared to confocal [74].

Thus, the use of autofluorescence is a promising method for non-invasive assessment of cell viability in complex three-dimensional structures.

5. FEATURES OF CHOOSING A METHOD FOR DETERMINING CELL VIABILITY AFTER CRYOPRESERVATION

Cryopreservation, being a method of long-term storage of cells, can at the same time cause significant damage to their structure and function (membrane, metabolic, genetic). Therefore, assessment of viability after thawing is critically important both for determining the effectiveness of the cryopreservation process itself and for further prediction of the functional activity of cells and their suitability for further use. Different methods for assessing viability reflect different aspects of the state of cells (membrane integrity, metabolic activity, proliferative potential, *etc.*). An incorrectly selected method can lead to inaccurate or incomplete results, which will complicate the data interpretation.

The choice of the optimal method for assessing cell viability after cryopreservation depends on several key factors that determine both the specificity of the method and its compliance with the set goals: available resources (light or fluorescence

microscopy, flow cytometry, spectrophotometry, *etc*.), cell type (neural, immune, stem, fibroblasts), the specific features of which must be taken into account when choosing a test.

Due to the high level of biological variability of different cell types, there is no universal approach to assessing viability. The choice of an appropriate assessment method should be justified taking into account the morphofunctional properties of cells, sensitivity to hypothermic and osmotic stress, requirements for preserving their specific activity (proliferation, differentiation, ability to implant or colony formation) and the ultimate goal of the study.

Typically, when choosing a method for assessing viability, a combined approach is used, including at least two complementary methods: assessment of membrane integrity (*e. g*., Trypan Blue or PI) and assessment of metabolic activity (MTT, XTT, Alamar Blue). In cases where viability is related to functional activity (*e. g*., in stem cells or neurons), an additional analysis of functional competence is required: the ability to differentiate, electrophysiological activity, or colony formation. This approach provides not only a qualitative but also a quantitative assessment of viability, which is especially important in the context of biobanking, regenerative medicine, and reproductive technologies.

Recommendations for the selection of viability assessment methods for different cell types after cryopreservation are presented in Table 2.

Thus, the selection of the optimal method for determining the viability of cryopreserved cells is a multifactorial process that takes into account the type of cells, the objectives of the study, the available resources, and the nature of the damage. A combination of methods (*e. g*., assessment of membrane integrity together with metabolic or functional activity tests) allows obtaining the most complete information about the state of cryopreserved cells and ensuring their effective use after thawing.

6. USE OF NEW TECHNOLOGIES FOR ASSESSMENT OF CELL VIABILITY IN CRYOBIOLOGICAL RESEARCH

In the context of the rapid development of biomedical technologies and increasing requirements for the quality of cell material, the task of objective and reproducible assessment of cell viability after cryopreservation has acquired new significance. Traditional methods, although they remain the basis of laboratory diagnostics, are increasingly giving way to high-precision, multiparametric and automated technologies that allow obtaining a comprehensive characteristic of the state of cells. At the same time, the most promising is the use of flow cytometry, microfluidic technologies, hyperspectral imaging and non-invasive spectroscopy, as well as the integration of AI algorithms into methods for determining cell viability.

Modern flow cytometers are capable of assessing dozens of parameters simultaneously at the level of individual cells [10]. In the context of cryobiology, this is especially valuable, as it allows for the differentiation of live, apoptotic and necrotic cells with high accuracy. In addition to the classic FDA/PI labeling, markers of metabolic activity (*e. g*. Calcein-AM), mitochondrial membrane potential (JC-1), oxidative stress (CellROX), etc. are being actively introduced into practice. The use of several dyes simultaneously allows not only to record the state of the cell at the time of analysis, but also to assess the possibility of its further recovery after thawing.

The advantages of flow cytometry are high accuracy, statistical reliability, and the ability to analyze large cell populations. The disadvantages are the high cost of equipment and the need for qualified personnel.

Table 2. **Recommended parameters for assessing the viability of different cell types after cryopreservation**

Cell Type	Membrane Integrity	Metabolism	Functionality	References
Stem	+	+	++	6, 72, 80
Hematopoietic	+	+	++	28, 45
Carcinogenic	+	++	++	68, 73
Neuronal	+	++	++	43, 76
Germ (Sex)	++	+	++	13, 18, 77
Embryonic	+	+	++	33, 67

Note: "+" — moderate level of manifestation of the indicator; "++" — high level of manifestation of the indicator.

Microfluidic platforms ("Lab-on-a-Chip"), which are based on the use of technology for controlling and manipulating small volumes of liquids through microchannels integrated on a chip, make it possible to simulate the microenvironment of a cell, precisely controlling the parameters of the environment during cryopreservation and thawing [23, 40, 44]. Due to the possibility of integrating sensors, these systems can continuously monitor changes in pH, osmolarity, temperature, and directly record cell responses to these changes. After thawing, cells can be analyzed *in situ* without disturbing their spatial organization. Microfluidic platforms with hydrogel microchambers allow analyzing the behavior of single cells, revealing their ability to adhesion, proliferation, and differentiation. The advantages of microfluidic technologies are high sensitivity, minimizing stress for cells, and the ability to simulate physiological conditions. The disadvantages are technological complexity, high cost, and difficulties in standardizing methods.

Hyperspectral imaging and non-invasive spectroscopy methods allow analyzing cells without using any dyes, based on the natural characteristics of the cell spectrum [1, 42, 70]. Using a hyperspectral camera or Raman spectroscopy, it is possible to determine the chemical composition, level of lipids, proteins, nucleic acids and other components in real time. In cryobiology, this is extremely promising, as it allows for multiple monitoring of the state of cells without damaging them. The advantages are non-invasiveness, high information content, the possibility of long-term monitoring, the disadvantages are high cost and the need for complex software for data processing. With the development of AI, its application to improve the analysis of the state of cells became a logical step. The introduction of AI opens up new horizons for automation, increasing accuracy and expanding the information content of the analysis of cell viability after cryopreservation. Deep learning algorithms (in particular, convolutional neural networks, CNN) are used to automatically identify both viable and non-viable cells based on microscopic or fluorescent images. Thanks to segmentation models (*e. g.*, U-Net, Mask R-CNN), AI can accurately and quickly identify individual cells in images and calculate the proportion of viable cells [47].

AI can be effectively used in the processing of complex multi-channel fluorescent images, in the analysis of large data sets, to detect trends and patterns that are impossible with manual analysis [3].

Recently, it has also become possible to use AI to create digital avatars of cells [53] that simulate their behavior during cryopreservation. This allows the simulation of the effects of freezing, predicting the cell survival under different conditions, and minimizing the number of laboratory experiments.

It should be noted that despite the successes, the widespread implementation of AI for assessing cell viability in cryobiology faces such challenges as the need for large volumes of qualitatively annotated data to develop algorithms and ensure the interpretability of AI models, as well as the validation and standardization of the developed approaches. However, with the increasing number of available bioinformatics tools, the development of open databases and the improvement of machine learning models, AI has the potential to become a standard tool in future cell viability assessments in cryobiology, transplantology, reproductive medicine and biobanking. Thus, the introduction of innovative technologies into cell viability analysis opens up a new level of accuracy, sensitivity and functional understanding of the cellular state after cryopreservation.

CONCLUSIONS

Evaluation of cell viability is a key step in cryobiological studies, which allows us to objectively determine the effectiveness of cryopreservation procedures and the suitability of cells for further use. Existing assessment methods are based on the analysis of various physiological parameters — membrane integrity, metabolic activity, functional state — and have both advantages and limitations. There is no universal method for determining cell viability, since none of the approaches is able to simultaneously cover all aspects of the structural and functional state of cells. Therefore, the most informative is considered to be a combination of at least two complementary methods, which provides a comprehensive assessment of the morphological integrity, metabolic activity and functional capacity of cells. Particular difficulties arise during the analysis of three-dimensional cellular structures (spheroids, organoids, tissue constructs), in which limited diffusion of dyes, reagents or oxygen can dis-tort the results of standard tests. In such cases, traditional methods should be adapted or combined

with modern approaches based on fluorescence microscopy, metabolic profiling, or non-invasive biophysical measurements. The integration of cutting-edge technologies — flow cytometry, microfluidic platforms, hyperspectral imaging, and AI algorithms — opens new possibilities for automated, accurate, and standardized assessment of cell viability, which is crucial for the development of cryobiology, biobanking, regenerative medicine, and cell therapy.

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О.М. Сукач, О.Р. Майорова*
Інститут проблем кріобіології і кріомедицини НАН України,
м. Харків, Україна
** an_sukach@ukr.net*

ВИКОРИСТАННЯ МЕТОДІВ ВИЗНАЧЕННЯ
ЖИТТЄЗДАТНОСТІ КЛІТИН У КРІОБІОЛОГІЧНИХ ДОСЛІДЖЕННЯХ

В огляді систематизовано методи визначення життєздатності клітин, що застосовуються у кріобіологічних дослідженнях, з акцентом на їхню практичну придатність, точність і чутливість. Розглянуто як традиційні методи, що базуються на оцінці цілісності клітинної мембрани та метаболічної активності (зокрема, фарбування трипановим синім, МТТ-тест, проби з флуоресцентними барвниками), так і сучасніші підходи, включно з протоковою цитометрією, конфокальною мікроскопією та методами оцінки функціональної активності клітин. Для кожного методу наведено опис механізму дії, протокол виконання, специфічні переваги, недоліки, а також потреби в обладнанні та обмеження щодо типів клітин. Проведено порівняльний аналіз, що виявив відсутність універсального підходу, однак засвідчив ефективність комбінованих методів, які поєднують флуоресцентне фарбування та метаболічну оцінку. Таке поєднання забезпечує високу точність оцінки життєздатності клітин після кріоконсервування. Крім того, в огляді розглянуто перспективи розвитку методів оцінювання життєздатності клітин у кріобіології, включаючи впровадження новітніх технологій візуалізації високої роздільної здатності, мультипараметричного аналізу, а також інтеграції з мікрофлюїдними платформами та штучним інтелектом для автоматизації та покращення точності оцінки. Огляд може слугувати практичним інструментом для дослідників під час вибору оптимального підходу до оцінювання життєздатності клітин залежно від мети експерименту, наявних ресурсів і типу клітин.

***Ключові слова*:** кріобіологія, життєздатність клітин, кріоконсервування, метод виключення барвника, колориметричний метод, флуоресцентні барвники, люмінометричні аналізи, культивування.