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Advances in the detection methods for assessing the viability of cryopreserved samples

Yan Hao[1], Zhicheng Liu[2], Heming Sun[3], Wang Zhai[2], Wenyu Sun[2*], Long Mu[4*]

Abstract

Since the beginning of the 21st century, modern medical technology has advanced rapidly, and the cryomedicine has also seen significant progress. Notable developments include the application of cryomedicine in assisted reproduction and the cryopreservation of sperm, eggs and embryos, as well as the preservation of skin, fingers, and other isolated tissues. However, cryopreservation of large and complex tissues or organs remains highly challenging. In addition to the damage caused by the freezing and rewarming processes and the inherent complexity of tissues and organs, there is an urgent need to address issues related to damage detection and the investigation of injury mechanisms. It provides a retrospective analysis of existing methods for assessing tissue and organ viability. Although current techniques can detect damage to some extent, they tend to be relatively simple, time-consuming, and limited in their ability to provide timely and comprehensive assessments of viability. By summarizing and evaluating these approaches, our study aims to contribute to the improvement of viability detection methods and to promote further development in this critical area.

Keywords

cryomedicine; rewarming; tissues and organs; viability; detection

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[1]*Shandong Yinfeng Life Science Research Institute, Jinan 250102, China*
[2]*Yinfeng Cryomedicine Technology Co., Ltd., Jinan 250102, China*
[3]*University of Florida, Department of Computer and Information Science and Engineering, Florida 32611, United States*
[4]*Department of Neurosurgery, The First Affiliated Hospital of Shandong First Medical University and Shandong Provincial Qianfoshan Hospital, Jinan 250014, China*

**Corresponding authors Wenyu Sun, E-mail: sunwenyumd@163.com; Long Mu, E-mail: auagcufesn@126.com*

1 Introduction

As a branch of biology, cryobiology investigates the changes, effects, and states of living organisms under low-temperature conditions. It primarily focuses on the influence of low temperatures (around 0 °C) and ultra-low temperatures (below -80 °C) on biological activity and their practical applications. The scope of research includes proteins, cells, biological tissues, and more[1]. According to the Arrhenius law, as temperature decreases, the rate of chemical reactions declines significantly, eventually approaching a complete halt. This principle highlights the profound impact of temperature on biochemical reaction rates. For instance, if a living organism can survive for 2 hours at 4 °C, it could theoretically be preserved for several days at -40 °C, several months at -80°C, and even centuries at -196 °C. At -196 °C, metabolic activities are essentially suspended, thereby minimizing biological mutations and genetic alterations[2].

With the advancement of organ transplantation technologies in modern biomedicine, the demand for effective preservation of isolated organs has grown substantially. Although a variety of preservation methods are available for fresh, viable organs, their effectiveness is limited, with optimal preservation times generally not exceeding 4 hours. To address this limitation, researchers are exploring methods for cryopreserving organs and tissues *in vitro* at ultra-low temperatures. This approach has the potential to significantly extend preservation times and reduce unnecessary loss of valuable biological materials. However, the formation of ice crystals during cryopreservation can damage tissue structures and impair organ function. The typical cryopreservation process involves multiple stages, including the addition and removal of cryoprotectants, cooling and freezing, long-term storage, and rewarming. Each of these stages poses a risk of injury, such as cryoprotectant toxicity, oxidative stress, ice crystal damage, thermal stress, and ischemia-reperfusion injury[3].

Therefore, to comprehensively understand the effects of cryopreservation and rewarming on tissues and organs, it is essential to employ a range of evaluation methods to detect damage at each stage. Optimizing these processes and improving damage

detection strategies are critical steps toward advancing cryopreservation technology. Investigating the extent of injury caused by different factors at the cellular, tissue, and organ levels is of great importance for refining preservation protocols and enhancing overall outcomes[4].

2 Detection methods

2.1 Macroscopic appearance and shape evaluation

The macroscopic appearance and morphology of tissues and organs serve as preliminary indicators for assessing viability. For certain organs, such as the liver, surgeons often rely on visual and tactile cues to make functional assessments. For example, an experienced surgeon can evaluate liver function based on its color, texture, and overall morphology, even before biopsy results are available[5]. Specific macroscopic features, such as the degree of yellowness, tissue firmness, rounded edges, and the presence of scratch marks, can provide valuable information about the extent of hepatic steatosis[6]. Skilled clinicians are particularly adept at identifying signs of severe injury, malignancy, or ischemic changes based on visual inspection alone. While such assessments are fundamental, especially in surgical practice, they are often insufficient under hypothermic or cryogenic conditions. In such cases, additional indicators and more refined methods are required to accurately evaluate organ viability[5].

2.2 Cell morphology testing assays

2.2.1 Hematoxylin and Eosin (H&E) staining assay

H&E staining is considered the golden standard for histopathological analysis. It is widely used for evaluating tissue morphology, including the assessment of primordial follicles in ovarian tissue after cryopreservation and rewarming, by distinguishing between morphologically normal and abnormal follicles[7]. Clinically, H&E staining is also employed for rapid intraoperative pathological diagnosis. In May 2023, Khan *et al.*[8] explored the application of virtual staining in H&E assays. Their study demonstrated that, with enhanced network capacity and optimized network architecture, virtual staining could replicate tissue images with high fidelity, potentially simplifying and accelerating traditional histopathological workflows.

2.2.2 Trypan blue exclusion assay

Trypan blue is one of the most used dyes for assessing cell membrane integrity. mechanism is based on the principle that viable cells with intact membranes exclude the dye, whereas non-viable cells, due to increased membrane permeability, absorb the dye and appear blue under the microscope[9]. This assay enables direct counting of stained and unstained cells either under a microscope or from captured images, allowing for relatively accurate quantitative analysis of cell viability.

2.2.3 Nissl staining assay

Nissl staining is a specialized technique used to visualize neuronal cell bodies and assess changes in neuronal structure. Nissl bodies, also known as Nissl granules, are composed of rough endoplasmic reticulum and appear as basophilic structures within the neuronal cytoplasm. Their distribution, shape, and number vary among different types of neurons. Nissl bodies are indicative of active protein synthesis in neurons. Upon neuronal injury or stimulation, these structures often decrease significantly. Thus, Nissl staining is a valuable method for evaluating neuronal integrity, cellular morphology, and the extent of neural damage and loss[10].

2.3 Apoptosis detection assay

2.3.1 Annexin V/Propidium iodide method for apoptosis detection

The annexin V is a calcium-dependent phospholipid-binding protein with a molecular weight of 35-36 kDa. It has a high affinity for phosphatidylserine, which becomes externalized on the plasma membrane of cells in early apoptosis. Thus, Annexin V serves as a sensitive marker for detecting early apoptotic cells. Propidium iodide (PI), a nucleic acid-binding dye, cannot penetrate the intact membrane of viable or early apoptotic cells. However, in late apoptotic or necrotic cells, where membrane integrity is compromised, PI enters and stains the nucleus red. Therefore, the combination of Annexin V and PI enables differentiation between early and late apoptosis, as well as between apoptotic and necrotic cells[11]. This dual-staining method allows for specific and quantitative analysis of apoptotic cells using flow cytometry[12].

2.3.2 Terminal deoxynucleotidyl transferase dUTP nick end labeling apoptosis assay

The TUNEL assay is one of the most widely used techniques for detecting apoptosis in tissue sections. Since DNA fragmentation is rare in normal or proliferating cells, TUNEL staining yields minimal signals in these cases. In apoptotic cells, however, DNA strand breaks are prominent, leading to the formation of 3'-OH termini that are specifically labeled by the TUNEL reaction. This assay is applicable across all cell types, organs, and species possessing DNA and DNases. Because of its broad applicability and high sensitivity, TUNEL is increasingly employed in the evaluation of tissue damage, such as in kidney injury. It provides accurate, quantitative results and can be combined with immunohistochemistry to identify specific cell types and elucidate the mechanisms of cell death[13].

2.3.3 Ccaspase-3 viability assay

Apoptotic signaling pathways lead to the activation of procaspases, precursors of caspases, through cleavage. Among these, caspase-3 is considered a key executioner in the apoptotic process. Present in the cytoplasm as an inactive zymogen (32 kDa), caspase-3 is activated in the early stages of apoptosis and subsequently cleaves various cytoplasmic and nuclear substrates, triggering chromatin condensation and the formation of apoptotic bodies. Due to its central role and early activation, elevated caspase-3 levels serve as a universal and reliable marker of apoptosis in a wide range of cell types[9].

2.3.4 Lactate dehydrogenase (LDH) release assay

LDH is a stable cytoplasmic enzyme that is rapidly released into the extracellular space upon cell membrane damage, particularly during the late stages of cell death. This property makes LDH release one of the most widely used markers in cytotoxicity assays[14-15]. LDH exists in five isoforms across various tissue types and catalyzes the conversion of pyruvate to lactate, coupled with the oxidation of NADH to NAD+. During cell lysis, LDH is released from the cytoplasm and can be quantitatively measured in the culture supernatant. The concentration of released LDH is directly proportional to the absorbance measured at 490 nm, allowing cell death to be quantified by plotting absorbance against cell number[16]. Recent advances in molecular biology have revealed that intracellular LDH characteristics are sensitive indicators of cellular metabolic states, including the aerobic or anaerobic direction of glycolysis, activation status, and malignant transformation[17]. Therefore, during the cryopreservation and rewarming of tissues and organs, LDH release assays are often used to assess the extent of cell death by analyzing samples taken from preserved tissues (Table 1).

2.4 Biomarkers detection for tissues

Following the cryopreservation of organs and tissues, the detection of tissue-specific biomarkers and related indicators serves as an important approach for assessing viability. For example, in the liver, various cell types contain multiple enzymes that are integral to metabolic processes. These enzymes can act as indicators of liver function and viability. Liver injury, including that caused by cryopreservation and rewarming, often leads to the leakage of intracellular enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH). During cryosurgical procedures or organ rewarming, cryoprotectant solutions are periodically collected for analysis. These samples can be examined using a blood gas analyzer to monitor dynamic changes in metabolic parameters and blood-gas composition, which reflect the physiological condition of the preserved organ[18]. This method provides real-time, quantitative information that contributes to the comprehensive evaluation of organ viability throughout the preservation and recovery processes.

2.5 Cell viability assays

2.5.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT and other related tetrazolium salts are reduced by mitochondrial succinate dehydrogenase in living cells to form insoluble purple-blue formazan crystals. In dead cells, where this enzyme is absent, MTT is not reduced. Therefore, the amount of formazan produced correlates with the number of metabolically active, viable cells. By measuring optical density (OD) at a specific wavelength, researchers can quantitatively assess cell viability and metabolic activity. Within a defined range of cell concentrations, the OD value is directly proportional to the number of viable cells[19]. Extensive cell death or mitochondrial damage results in significantly reduced or absent formazan formation.

2.5.2 Neutral red uptake assay

The neutral red uptake assay is a well-established quantitative method for assessing cell viability[20]. Neutral red penetrates intact cell membranes and accumulates within lysosomes of viable cells. In contrast, damaged or dead cells with compromised membrane integrity fail to retain the dye. This assay offers reliable, repeatable, and reproducible results across various cell types. Despite its widespread use, it has limitations, including sensitivity to metabolic state variability during different cellular life cycle stages, potential underestimation of compound potency, and

Table 1 The differences between various apoptosis detection assay methods

Detection method	Detection object	Time-consuming	Applicable scenarios
Annexin V/PI	Early apoptotic cells and late apoptotic cells	2-4 h	Flow cytometry-based apoptosis staging; drug screening & apoptosis mechanism studies
TUNEL	Apoptotic cells	6-24 h	Histopathological analysis; *in vivo*/*in vitro* apoptosis detection
Detection of caspase-3 viability	Early apoptotic cells	1-2 h	Early apoptosis event studies; apoptotic signaling pathway validation
Lactate dehydrogenase (LDH) release assay	Cell membrane damage	2-4 h	High-throughput cytotoxicity testing

non-monotonic dose-response curves due to phenotypic switching at certain concentrations[21].

2.5.3 Western blot analysis of β-actin proteins

β-actin is a structural protein encoded by the β-actin gene, forming a key component of microfilaments involved in cellular activities such as movement, secretion, and division. Owing to its typically stable expression across different cell and tissue types, β-actin is widely used as a reference or internal control protein in Western blot assays[22-24]. Its relatively constant expression can be used to normalize loading amounts and monitor changes in cell viability or structural integrity. However, some studies have shown that β-actin expression may vary under specific conditions. For example, Glare *et al.*[25] reported β-actin instability in asthmatic airway tissues and Bas *et al.*[26] observed variability in β-actin expression in human T lymphocytes when using qRT-PCR. Therefore, while β-actin is a useful reference protein, its stability must be validated under specific experimental conditions.

2.5.4 Calcein-AM cell viability assay

Calcein Acetoxymethyl Ester (Calcein-AM) is a non-fluorescent, cell-permeable dye that can enter live cells and be hydrolyzed by intracellular esterases into fluorescent Calcein (excitation: 490 nm; emission: 515 nm)[27] (excitation wavelength: 490 nm, emission wavelength: 515 nm). This fluorescence occurs only in viable cells, making Calcein-AM a reliable indicator of cell viability. Bastings *et al.*[27] applied this technique to assess both fresh and cryopreserved ovarian tissue. Calcein-AM is a sensitive, stable, and cost-effective assay for detecting live cells and for detecting early-stage apoptosis. Its ease of use and high sensitivity make it suitable for a wide range of applications in viability analysis (Table 2).

2.6 Tissue viability assay

2.6.1 Measurement of ATP content

Adenosine Triphosphate (ATP), often referred to as the "energy currency" of all viable microorganisms and cells, is a key molecule involved in cellular energy transfer. It is found only in metabolically active cells, making it an ideal indicator of tissue viability. ATP levels can be quantitatively assessed using a bioluminescent reaction in which luciferase oxidizes luciferin, producing light in proportion to the amount of ATP present[28]. Intracellular ATP can be measured by lysing cells with appropriate detergents to release the molecule[28]. This assay is highly sensitive, capable of detecting ATP from as few as 10 cells per well[29], and can also be used to evaluate cell proliferation. Since ATP levels rapidly decline after cell death, the resulting loss of bioluminescence can serve as an early indicator of reduced viability. Additionally, ATP-based detection is advantageous due to its speed, sensitivity, and ease of operation. It also enables residue detection of relevant substances, making ATP a reliable "risk" indicator for evaluating tissue health.

2.6.2 Glucose uptake assay

Glucose uptake is another useful parameter for assessing tissue viability. This assay involves measuring the amount of glucose consumed from the culture medium by tissues or cells, normalized per milligram of tissue per hour. The method, adapted and refined by Gerritse *et al.*[30], provides valuable insights into the metabolic activity and overall viability of various cell types. Glucose concentrations in the medium are measured using an analyzer, and consumption is calculated accordingly[27]. Although glucose uptake may be influenced by tissue damage, particularly during the early stages, Bastings *et al.*[27] have demonstrated its practical application in evaluating human ovarian tissue viability following cryopreservation and rewarming.

3 Conclusions and perspectives

Cryopreservation plays a vital role in maintaining the viability of biological specimens by preserving cells, tissues, and organs in a living state. Demonstrating that cryopreserved tissues or organs remain viable after thawing would mark a significant breakthrough, opening the door to a new era in biomedical science. To achieve this, it is essential to deepen our understanding of how ultra-low temperatures affect biological systems at the cellular, tissue, organ, and even whole-body levels. Furthermore, expanding the application of cryopreservation in medicine could greatly enhance

Table 2 The differences between various cell viability assays methods

Detection method	Detection object	Time-consuming	Applicable scenarios
MTT	Metabolic activity of living cells	4-6 h	High-throughput drug screening; cell proliferation & cytotoxicity studies
Neutral red uptake assay	Lysosomal activity & membrane integrity of living cells	3-4 h	Cytotoxicity assessment (chemical/environmental toxins); long-term culture viability monitoring
Western blot assay for β-actin protein	Cytoskeletal protein expression (internal reference)	12-24 h	Cell structural integrity evaluation; experimental normalization control
Calcein-AM cell viability assay	Esterase activity in viable cells	30 min-1 h	Real-time live-cell imaging; rapid viability assessment

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

the progress of modern medical technologies.

The viability assays discussed above provide multidimensional insights into the extent of cryoinjury at the cellular and tissue levels. These methods range from DNA fragmentation detection and protein expression analysis to morphological assessments and biochemical evaluations. Such techniques enable both qualitative and quantitative evaluation of cellular and tissue viability, facilitating a more comprehensive understanding of damage mechanisms and recovery potential after cryopreservation.

However, despite the availability of these diverse assays, there remains a pressing need to develop more quantitative, scalable, and integrative tools for viability assessment in cryopreservation research. Existing detection methods, although valuable, often assess viability from a single perspective. Some approaches focus on cell morphology, others on apoptosis markers or specific biomarkers, while only a limited number of techniques are applicable at the tissue or organ level. For instance, Hematoxylin and Eosin (H&E) staining remains the gold standard in histopathological analysis, yet it offers limited functional insights and lacks the ability to assess dynamic biological processes.

One major limitation is that most current methods are unable to comprehensively evaluate viability across all dimensions, morphological, functional, and molecular, especially in large, complex tissue or organ systems. Typically, only a subset of cells is sampled from a tissue, or specific cell types are isolated for analysis, leading to partial or point-specific assessments. This fragmented approach makes it difficult to accurately characterize the viability of an entire tissue or organ.

In the future, two major directions should be pursued. First, methodological innovations are needed to enhance the accuracy, depth, and integrative capacity of viability testing. Such methods should aim to assess viability in a holistic manner rather than from isolated angles. Second, the development of advanced instrumentation capable of real-time, high-resolution, and multidimensional viability assessments at the tissue and organ level will be critical. These advancements will not only improve our understanding of cryoinjury during freezing and rewarming but will also provide essential tools to support long-term preservation strategies for tissues and organs. Ultimately, these improvements will be instrumental in overcoming current limitations in cryopreservation research and advancing its clinical applications, including organ transplantation, regenerative medicine, and personalized therapy.

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Research ethics

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