CELL & GENE THERAPY INSIGHTS

CELL THERAPY CMC & QUALITY CONTROL

SPOTLIGHT

COMMENTARY/OPINION

Outcomes from a cell viability workshop: fit-for-purpose considerations for cell viability measurements for cellular therapeutic products

Laura Pierce, Sumona Sarkar, Leo Li-Ying Chan, Bo Lin & Jean Qiu

Fit-for-purpose cell viability measurement methods are urgently needed for the characterization and testing of cellular therapeutic products (CTPs) and their manufacturing processes. A cell viability workshop held at the virtual Cell Therapy Analytical Development Summit in December 2020 brought together stakeholders from academia, federal government institutes, instrument manufacturers, and cellular therapeutic product manufacturers to explore common challenges in viability measurements, as well as to address control measures and considerations for the selection of viability assays that are targeted toward the intended use of the biological sample. This report summarizes the key findings of the workshop and identifies needs and gaps for cell viability assay standards development.

Cell & Gene Therapy Insights 2021; 7(4), 551–569

DOI: 10.18609/cgti.2021.076

INTRODUCTION

Cell viability is a fundamental measurement in the manufacturing and release of cellular therapeutic products (i.e., products containing cells as the active substance) [1] such as Chimeric Antigen Receptor (CAR)-T cell therapies. It is critical to evaluate the presence of both living and dead or dying cells in cellular therapeutic products (CTPs), where dead and dying cells and cellular debris may elicit unwanted immune responses, and too few living cells may hinder the effectiveness of the products [2]. Viability assays may also serve as a method to establish strength or dose of the CTPs (i.e., viable cell concentration), and as a part of cytotoxicity assays, which have been used, for example, to evaluate CAR-T potency [3]. In addition to evaluating cell viability during product characterization and release, viability measurements can serve as a process control indicator, providing critical information for decision-making throughout the manufacturing process (Figure 1).

Viable cells are defined as cells within a sample that have an attribute of being alive (e.g., metabolically active, capable of reproduction, possessing an intact cell membrane, or with the capacity to resume these functions) defined based on the intended use [4]. When cell viability is defined as the proportion of viable to non-viable cells in a cell sample, the viability measurement necessitates a confident quantification of at least two of the three following cell numbers: the number of viable cells, the number of non-viable cells, the total number of cells. Performing this differential count means that a cell viability measurement relies on specific markers that can distinguish the viable population from the non-viable one, and an error in quantifying either the viable, non-viable, or total cell population would introduce an uncertainty into the viability measurement. In some cases, viability may also refer to assays which evaluate only the viable cell concentration, and no information is acquired regarding the dead or injured non-viable cell population. For example, impedance-based assays that monitor the growth of cells in real time [5,6], or ATP (adenosine triphosphate) assays that provide a measurement of viable cells based on their metabolic function [7], can provide valuable information on the viable cell concentration in a cell sample but may not readily provide a quantification of the relative number of live and dead cells in a sample preparation.

The definition of viable and non-viable cells is often considered on a continuum of cell health [8,9]. There are many molecular, physical, and chemical markers that can be used to monitor the health of a cell sample, where any individual marker or combination of markers can be used as a biological indicator to identify a cell as viable or non-viable in a viability assay (Figure 2) [10–13]. A wide range of assays and instrumentations are available for evaluating the biological indicators associated with cell viability. Image-based, flow cytometry-based, spectrophotometric, and electrical signal-based methods present their own measurands (i.e., the quantity or property intended to be measured) and have their own sources of measurement error and needs for control methods and materials [12,14]. Given the wide range of available cell viability measurements, different modalities for identifying viable and non-viable cells, and the many different purposes of evaluating cell viability, there is a need for more rigorous approaches to select, design, and control cell viability assays.

A cell viability workshop was held at the virtual Cell Therapy Analytical Development Summit on December 15th, 2020. The workshop, co-hosted by the National Institute for Standards and Technology (NIST) and Nexcelom Bioscience, consisted of three presentations entitled "Cell Viability Measurements: What are we Really Measuring?", "How is your Selected Method of Measurement Impacting Viability Results?" and "Use Cases and Measurement Controls", and each of these sessions explored common challenges encountered in cell viability measurements across the cellular therapy industry and discussed approaches for selecting fit-for-purpose viability assays. NIST and Nexcelom

DOI: 10.18609/cgti.2021.076

FIGURE 1

An example of the autologous cellular therapy manufacturing process, indicating viability measurements at several points in the process.

presented general concepts and considerations for cell viability measurements, studies exploring how different methods can impact viability results, and presented strategies and tools targeted at improving specific viability measurement methods. Over forty stakeholders participated in the cell viability workshop. Workshop participants came from a variety of backgrounds, with 50% of participants coming from the biotechnology industry, 42% from the pharmaceutical industry, 4% from device manufacturing companies, 3% from academic labs and 1% from governmental labs. Several key observations were made during the workshop, as described in Figure 3, and further discussed in this workshop report.

SURVEY RESULTS

Twenty-four registered workshop participants engaged in a survey inquiring about

FIGURE 2

Examples of viability measurements and the quality attribute being measured.

Membrane Integrity	Metabolism	Molecular Markers	Ability to Proliferate
• Trypan Blue • AO/DAPI • AO/PI • Calcein-AM • Impedance • Lactate dehydrogenase	• Calcein-AM • MTT • Alamar blue • ATP • Glucose consumption	• Annexin V • Caspases • TUNEL	• Count over time • Colony forming unit (CFU) assays • BrdU/EdU • CM-FDA

AM: Acetoxymethyl; AO: Acridine orange; ATP: Adenosine triphosphate; BrdU: Bromodeoxyuridine; CM-FDA: 5-chloromethylfluorescein diacetate; DAPI = 4′,6-diamidino-2-phenylindole; EdU: 5-ethynyl-2´-deoxyuridine; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PI: Propidium iodide; TUNEL: Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

common concerns and challenges encountered in conducting cell viability measurements. The most frequently cited concern with viability measurements was the lack of understanding of the accuracy of measurements, followed by the issue of relevance of measurements to product efficacy. One common challenge encountered across industry was the issue of assay selection. Seventy-nine percent of the participants indicated that their most important consideration in selecting a cell viability assay is the quality of the measurement, as measured by accuracy, precision, specificity, and reproducibility.

Workshop participants were polled as to what type of biological indicator they currently use for routine viability measurements. The most common viability measurement technique reported was fluorescent nuclear staining method of dye exclusion (83% of respondents). Interestingly, 38% reported using the Trypan blue method, indicating that some laboratories use multiple techniques for viability assessment. Other biological indicators reported included molecular markers of cell death such as apoptotic markers (4%), ability of cells to replicate (4%), and other functional abilities of cells (4%). Most respondents indicated that they consider the hemocytometer Trypan blue method to be the gold standard method for measuring viability.

A commonly discussed challenge is what action, if any, should be taken when cell viability measurements fall outside of the expected range at a specific point in the manufacturing process. More than 80% of respondents indicated that cell viability measurements are critical in monitoring the manufacturing process, however only 18% of respondents indicated that they have a high level of confidence in their cellular viability assay measurement results. Of those surveyed in the workshop poll, fewer than half (38%) indicated that a clear action is established when a viability measurement falls out of the expected range during the manufacturing process, while 50% indicated that action is taken on a case-by-case basis, and 17% indicated that the resulting out-of-range viability observation is noted, but no pre-determined action is taken. These survey results demonstrate that there remains a need to develop methods with increased measurement confidence and to develop a greater understanding of the significance of viability results in the CTP manufacturing process.

DOI: 10.18609/cgti.2021.076

FIT-FOR-PURPOSE ASSAY CONSIDERATIONS

Throughout the workshop, presenters and participants emphasized the need for a fit-for-purpose approach (fitness for the intended purpose in line with prearranged requirements for an intended use [15]) to develop cell viability assays. Based on the intended purpose of the viability measurement (e.g.in a CTP manufacturing process, characterization of the starting apheresis material, monitoring cells during expansion, or for product release), three main considerations can be taken to achieve a fit-for-purpose method:

1. Selection of an appropriate biological indicator;
2. Considerations for the properties of the cell sample; and
3. Considerations regarding the measurement method (Figure 4).

The interactions of these three areas will drive the design of the measurement system. Examples shared at the workshop for the interaction between sample properties and measurement method are given in the section titled "Selected method of measurement can impact viability results".

Figure 1 shows an example of a typical (CTP) manufacturing process, indicating several common points in the process where viability is measured. The analytical method selected for evaluating cell viability may differ for different steps of the process. For example, in the process of CAR-T production, red blood cell (RBC) residues or debris may be present in the peripheral blood mononuclear cell (PBMC) samples either

FIGURE 3

Four main concepts were discussed at the Cell Therapy Analytical Development Summit workshop on cell viability addressing challenges, considerations and future directions for improving confidence in cell viability measurements.

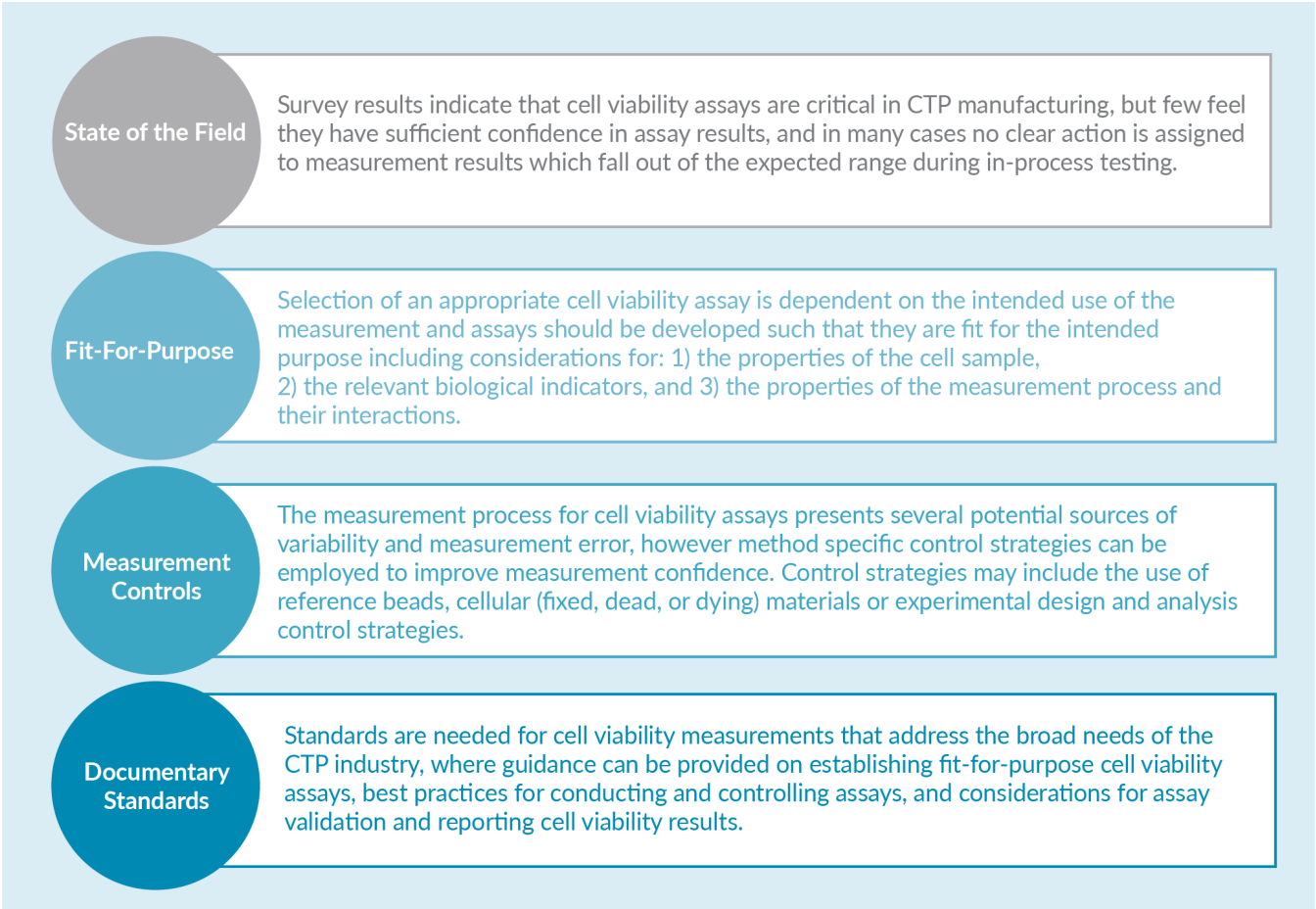
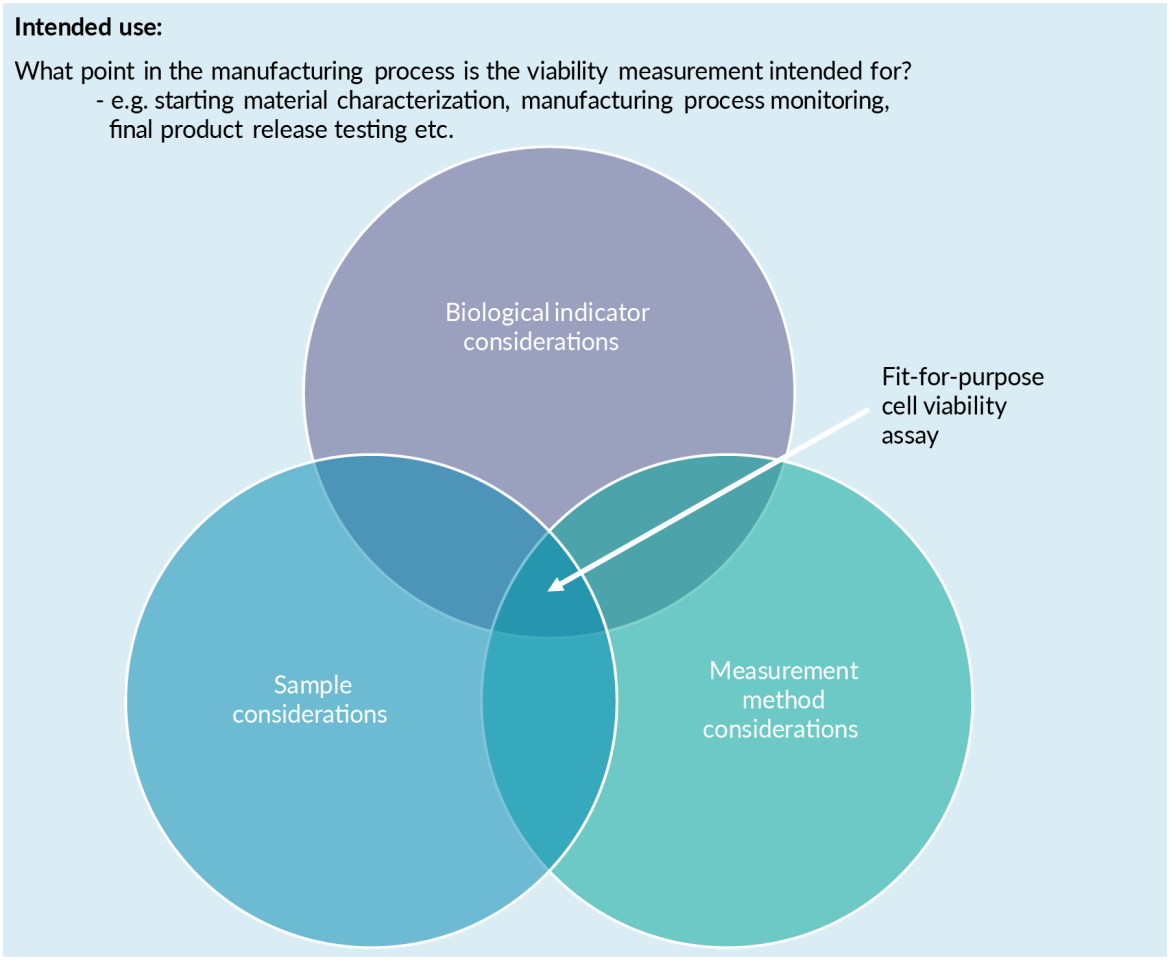


FIGURE 4

Determining the intersection of considerations for identifying a fit-for-purpose viability assay.



freshly isolated or cryopreserved. In this case, it is important to select a cell viability and counting method to consistently count nucleated cells and avoid nonspecific counting of RBCs, platelets, and debris. This selected measurement method for patient PBMC samples early in the manufacturing process will likely differ from the selected viability method in the downstream process, on more purified materials such as during cell expansion or during potency testing prior to product release.

Furthermore, virally transduced or expanded samples may have lower amounts of debris and more defined suspension medium later in the process; however, it may be more critical for viability measurements to be sensitive to potential changes in cell proliferation at the expansion stage. For example, if it is vital for cells to proliferate at a specific rate in order to have sufficient quantity of cells for the next step of the manufacturing process or in order to achieve the appropriate dosage, then a viability measurement that is sensitive to the proliferative capacity of the cells would be the most fit-for-purpose. Another intended use of cell viability measurement is *in vitro* cytotoxicity assays. Prior to performing the assay, accurate cell viability measurements of the effector and tumor target cells are required in order to properly evaluate the cytotoxic potential of the cells. Error in these cell viability measurements could incorrectly assess the specific killing function of the effector cells. In this case, the effector and target cell viability assays may be different due to the different properties (i.e. concentrations, morphologies, stabilities) of these cell materials.

DOI: 10.18609/cgti.2021.076

During workshop discussions, cell sample properties appeared to be a big driver in establishing a fit-for-purpose cell viability assay. Samples can have a wide range of properties including different levels of debris, contaminating cell lines, morphological traits, aggregation, and fragility. Basic sample properties such as concentration range, cell type, and suspension medium are commonly considered when identifying fit-for-purpose assays [10]. Further considerations include the state of the cells, contaminating cells or debris that may be present in the sample, and stability of the cell samples during the viability measurement. The state of the cells can be affected by treatments the cells may have undergone prior to viability analysis. For example, cells tested for viability directly after thawing from cryopreservation may behave differently than freshly prepared cells [16,17]. The timing of the cell viability assessment may also influence the cell viability assay results as in some cases, the properties of the cells may change given a chance to recover from activities such as cryopreservation or with continued cell culture. Furthermore, practical sample considerations can include the availability of test material for the analytical method. If sample quantity is limited, a fit-for-purpose assay would need to use as little sample volume as possible for analysis. Selection of an appropriate measurement method may also account for several practical considerations including cost per assay, throughput needs, automation needs or training requirements, and regulatory or other considerations.

Sample stability during the analytical period should also be a significant consideration when developing an appropriate viability assay. Viability measurements are often being conducted as a snapshot in time, capturing discrete information for the continual process of living and dying. The viability of a cell sample may change over time or with sample preparation processes, and the presence of contaminating debris from dead cells may increase, or cells may be lost during the measurement process as a result of centrifugation or cell adhesion to tubes or pipettes. In some cases, the cell samples may be less robust to sample handling procedures than in other cases. Workshop participants indicated, for example, that sample washing steps such as those performed during flow cytometry staining may affect the viability measurements by unintended removal of portions of the non-viable cell population. The presence of contaminating cells or debris in the cell sample is often an additional driving factor in establishing suitable viability methods, where the specificity of the viability method in isolating the cell population of interest is critical.

An additional cell sample consideration includes sample-to-sample heterogeneity. When the samples intended for analysis can vary widely, for example if samples will come from donors with varying levels of disease, a fit-for-purpose method would necessarily be robust to this variability, and should be investigated with a range of samples with the expected diversity of properties to be encountered.

In addition to developing a cell viability measurement method based on sample considerations and the selected biological indicator, an appropriate measurement method should be selected based on considerations regarding method performance criteria (e.g., accuracy, precision, sensitivity, linearity, and robustness) [18]. Control strategies for assuring measurement confidence should also be incorporated in a fit-for-purpose cell viability assay. Several control strategies were presented by NIST at the workshop and are described in the section, "Improving confidence in viability measurements through control strategies".

The workshop discussion clearly pointed to the needs to select 'fit-for-purpose' cell viability methods that can satisfy intended purposes from cell counting for passaging to performing cytotoxicity assays and establishing dose and purity of CTPs. Figure 4 suggests that cell viability assay development can best be applied at the intersection of cell sample properties, measurement methods, and biological indicators to support the development

of a viability assay which is relevant to its intended use.

SELECTED METHOD OF MEASUREMENT CAN IMPACT VIABILITY RESULTS

The method of measurement itself can affect the samples, thereby impacting viability results. There are multiple methods to determine cell viability, including methodologies that directly enumerate total, live, and dead cell counts from a sample. In this case, cell viability itself is not a measured value, but a calculated result from at least two measurand counts (total, viable, and non-viable cells). For example, the viable (bright center) and non-viable (dark blue color) cells are counted from a trypan blue-stained cell sample to calculate the viability using the equation (Figure 5).

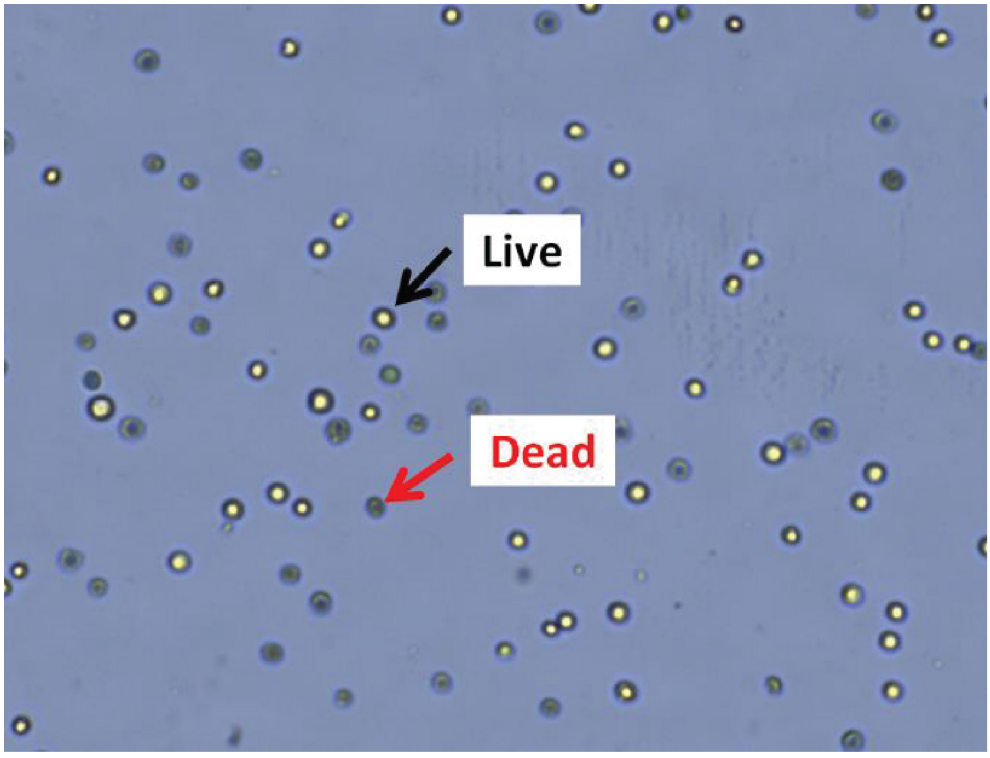
$$Viability\ \% = \frac{Viable\ Cell\ Count}{(Viable + Non\text{-}viable\ Cell\ Count)} \times 100$$

Therefore, if cell counting results are perturbed due to measurement methods, the viability results can be significantly impacted. In the workshop, we discussed three impacts of cell counting methods on cell viability results. First, primary cell sample viability can be greatly affected by the use of brightfield imaging and trypan blue staining due to residual red blood cells (RBCs) in the sample. Second, staining dye and staining time for trypan blue can affect the dead and dying immune cell populations. Finally, mechanical stresses from flow-based systems can alter the cell viability during the measurement process.

Primary cells are often the key components in CTPs. One of the main issues when utilizing primary cells such as apheresis samples or PBMCs is the existence of residual RBCs [19]. Directly counting these primary cell samples using brightfield imaging and trypan blue staining risks the possibility of erroneous counting of residual RBCs. Since the RBCs will appear as objects with bright centers in brightfield imaging, the live cell counts or

FIGURE 5

Low viability Chinese Hamster Ovary (CHO) cells stained with trypan blue showing live (viable) cells with bright center and dead (non-viable) cells with dark color.



DOI: 10.18609/cgti.2021.076

concentrations can be overestimated, thus increasing the calculation of the viability percentages (Figure 6A). In a previous publication by Chan *et al.*, it was discovered that the residual RBCs concentration is donor-dependent, thus causing high variability when calculating viabilities (Figure 6B). To improve the measurement of live cell counts for primary cells, fluorescent nuclear staining can be utilized to eliminate the counting of residual RBCs, platelets, and debris (Figure 7). It was found that fluorescent nuclear staining such as acridine orange (AO) and propidium iodide (PI) can improve identification of both viable and non-viable cells, thus improving the consistency of viability calculations [19].

Another factor that can impact viability calculation is the viability dye, specifically the traditionally used trypan blue method. In Chan *et al.*, it was demonstrated that after staining low viability Jurkat cells and mouse splenocytes with trypan blue, three morphologically distinct populations are observed: viable (bright center), non-viable (dark blue), and unknown (large diffuse object) (Figure 8A) [20]. A quantitative experiment was conducted to demonstrate the reduction of dead cell concentrations when cells are stained with trypan blue [20]. The results showed that measured viable cell concentrations are comparable between Acridine Orange/Propidium Iodide and trypan blue staining; however, the non-viable cell concentrations are significantly lower for trypan blue. Interestingly, large diffuse objects are not observed when staining a heat-shocked Jurkat cell sample (Figure 8B). After identifying the existence of these diffuse objects, an experiment was conducted to establish the origin and formation of the diffuse objects. In previously acquired videos, it was found that these diffuse objects are formed immediately from dead or dying immune cells (PBMCs) after interacting with trypan blue, demonstrating potential osmotic effects lysing the cells [21]. The consequence of this action is again an overestimation of viability by reduction of counted dead cells. Due to the disappearance of non-viable cells when stained with trypan blue, the viability percentage calculation is artificially higher. The PI viability fluorescent dye was also investigated for this phenomenon, and was found not to cause the same morphological changes as trypan blue [21].

The two previous examples demonstrate the variability of viability calculations due to the effects of viable and non-viable cell counting error. Other factors can directly affect the actual cell viability such as dye-induced cell cytotoxicity and mechanical stress-induced cell viability reduction. In Mascotti *et al.*, the authors demonstrated that trypan blue induced cell death after approximately ten minutes,

FIGURE 6

(A) Primary PBMC sample showing nucleated PBMCs with bright center (black arrows) and RBCs (red arrows) with a biconcave profile. Depending on the focus, the morphological profile may change, which can render RBCs to look like PBMCs. (B) Residual RBCs in 15 Ficoll processed PBMC samples showing different level of RBC contaminations.

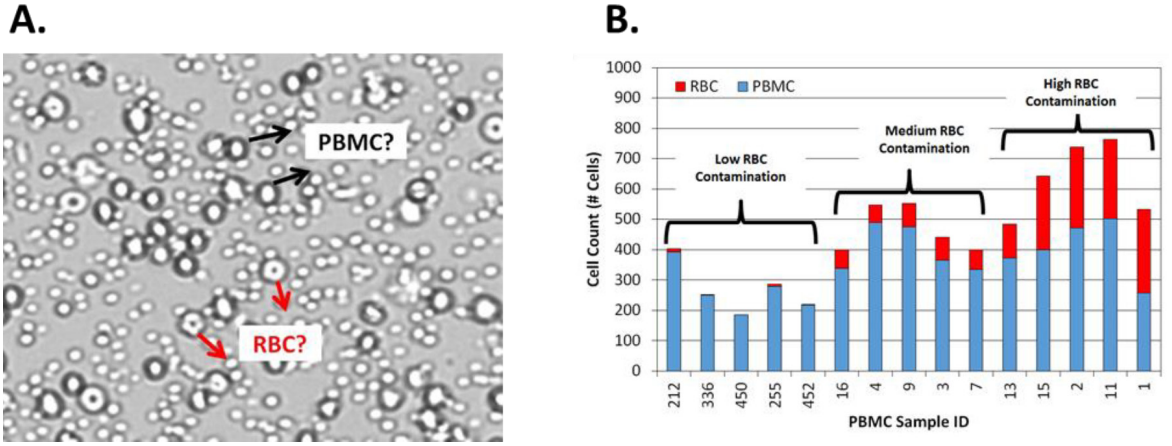
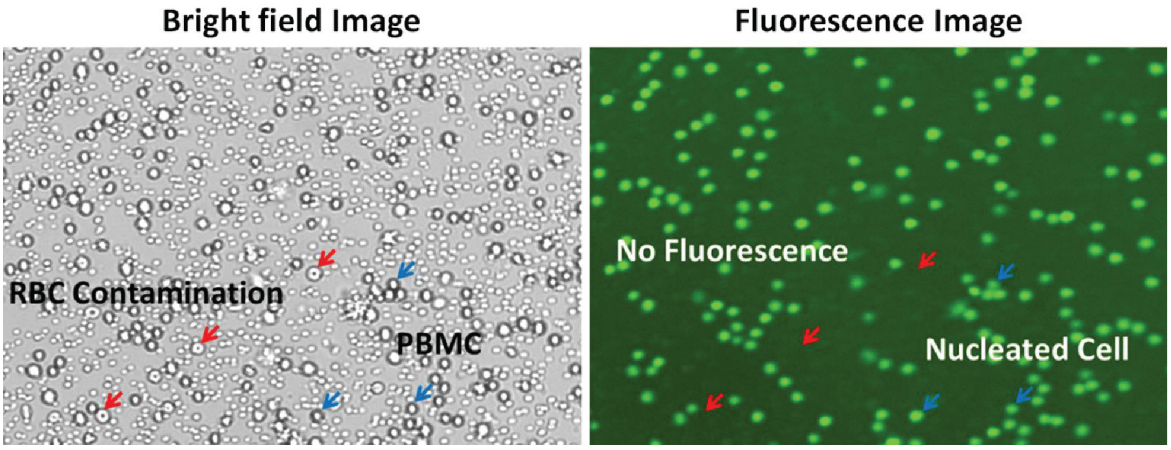


FIGURE 7

The use of AO/PI fluorescent nuclear stains to specifically stain the viable and non-viable nucleated cells, while excluding the RBCs, platelets, and other debris.



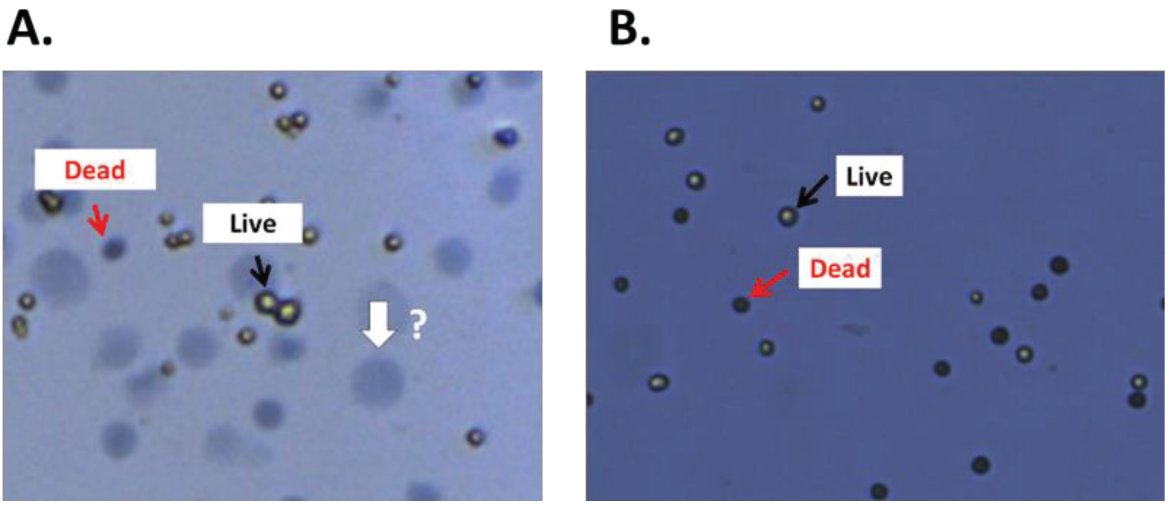
Red arrows indicate RBCs (visible in brightfield but not in fluorescence) and blue arrows indicate PBMCs (visible in both brightfield and fluorescence images). Figure adapted from [19].

causing reduction in cell viability, whereas the AO/PI staining method showed viability stability up to 120 minutes [22]. A European Pharmacopoeia standard (2.7.29) entitled, "Nucleated cell count and viability" has also noted the importance of cell incubation time in trypan blue staining solution, noting samples should not remain in trypan blue solution for more than four minutes before data acquisition [23]. Therefore, cell sample staining time can greatly affect the viability outcome and introduce variation between samples.

Finally, instrumentation-induced viability effects have also been observed in previous reports. In Chen *et al.*, the authors described their observation and quantification of mechanical stress-induced reduction in cell viability [24]. The authors discovered that phosphate buffered saline (PBS) as a diluent can present adverse effects on cell viability when

FIGURE 8

(A) Low viability Jurkat cell sample stained with trypan blue showing 3 different morphological populations for live (bright center), dead (dark color), and ruptured dead cells (large diffuse objects). (B) Heat-killed Jurkat cells stained with trypan blue showing clear distinction between live (viable) and dead (non-viable) cells, where no visible large diffuse objects are observed.



DOI: 10.18609/cgti.2021.076

used with fluidics-based automatic cell counters. The reduced cell viability was attributed to the mechanical shear stresses introduced by the system. In addition, the length of time cell samples were incubated in PBS contributed to the reduction in cell viability. Several alternative diluents were identified to maintain cell viability results over time and present more accurate representations of cell culture conditions.

In summary, cell counting methodologies that can impact viable and non-viable cell counting results can significantly alter the viability results. It is important to understand the principles of the selected cell counting methodology or process that includes the cell sample, cell sample preparation, cell counting instrument, reagents, consumables, and analysis algorithm, which can all contribute to variation in cell count and viability calculation.

IMPROVING CONFIDENCE IN VIABILITY MEASUREMENTS THROUGH CONTROL STRATEGIES

A general workflow for the cell viability measurement can be summarized into a few key steps:

1. Sampling;
2. Sample preparation;
3. Data collection; and
4. Data processing and analysis **(Figure 9)**.

Each step in this process can introduce variability to the measurement [14]. The source and level of variability will be dependent on the properties of the cell sample, as well as the type of viability assay that is being conducted. Control strategies typically target specific points in the measurement process and implement a technique which can be used to improve confidence in the measurement outcome.

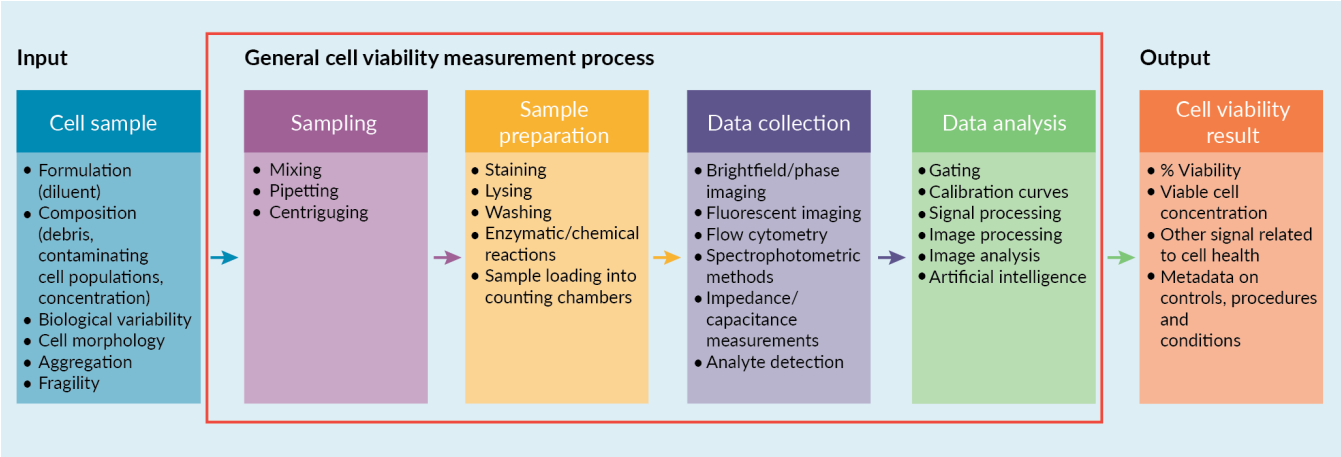
During the workshop, NIST presented several control strategies under development for improving confidence in cell viability assays, particularly for image-based viability assays. Each method establishes an approach to account for potential variability arising from specific points in the measurement process, e.g. image analysis, image acquisition, and user-determined gating analysis steps. Through these investigations, NIST illustrated the importance of closely observing each step in the measurement process, from sample preparation to final analysis, in order to target those points in the workflow which can introduce significant sources of measurement variability and error. Here we identify potential sources of variability from each general step of the cell viability measurement process as well as give examples of control strategies under development at NIST.

Sampling is an important step in the cell viability measurement process. In this step, a small aliquot of cells is typically sampled from a larger volume of cells. If mixing and pipetting are not conducted properly at this step, the test sample for the viability measurement may not be representative of the larger volume, compromising the relevance of the viability measurement. If aggregation is present in the cell sample, this may affect sampling, and require additional procedures during sample preparation and data analysis to account for aggregation. In these cases, it may be important to control for the level of aggregation in the sample, and monitoring metadata on the percent of aggregated cells may alert a user to problematic measurement conditions. Environmental factors such as temperature and humidity, as well as sample aggregation properties should be considered during sampling steps [25].

Sample preparation is another significant source of measurement variability that is often difficult to control due to the many manual steps involved. In some cases, sample preparation may be controlled by utilizing automated liquid handlers to manipulate the sample (automation can be integrated into the data acquisition device or may be an external liquid handler) or by following detailed sample preparation SOPs. In addition, reagents and consumable devices (e.g. counting chambers) can affect the quality of the measurement and

FIGURE 9

Workflow for cell viability assays, including examples of considerations for cell sample input and examples of possible data outputs from the measurement process.



A general cell viability measurement process is outlined with example considerations for each step.

may vary from lot to lot. Sample preparation may also cause changes in the properties of the sample, and any sample preparation steps that cause cell death are especially problematic for viability measurements and should be controlled for to maintain sample stability throughout the measurement process.

Data acquisition and the instrumentation used in data acquisition often present a black box scenario for cell viability measurements, especially when data acquisition is automated or semi-automated. In these cases, controls for sources of variability are sometimes built into the instrumentation and data acquisition workflow; however, as with any automated system, errors can occur that often go undetected until a measurement falls out of an expected range (at this point it would be unclear if the values were out of range due to measurement error or a true biological phenomenon). Routine instrument qualification and maintenance can help to reduce errors in data acquisition; however, it is still challenging to have confidence that individual samples are being analyzed appropriately. The incorporation of in-measurement-process controls can help to verify measurement conditions during a viability assay. For example, in image-based trypan blue cell viability measurements, image quality, particularly focus and image brightness can affect the cell viability measurement [26]. It is critical that images are of sufficient quality before automated image analysis algorithms are applied.

NIST presented an approach to incorporate a small number of beads into cell viability samples for analysis in a slide-based trypan blue imaging cell viability analyzer (Cellometer® Auto 2000, Nexcelom), where the beads serve as a sample-independent artifact for monitoring image quality. In this approach, bead features were identified that are sensitive to changes in image focus and training data sets were acquired to characterize the relationship between bead features and image focus and brightness. The beads could then be used to identify a reference focal plane for the viability measurement, and a tool was developed to help users return to the reference focal plane, based on bead features, during each measurement [27]. This approach improved the reproducibility of the automated trypan blue based viability measurement across a set of Jurkat cell samples ranging in cell viabilities from 0% viability to near 100% viability, and also improved the accuracy of the measurement relative to expert manual counting of cells in the images.

Data analysis presents another significant source of measurement error and variability. In manual data analysis, challenges include

DOI: 10.18609/cgti.2021.076

operator-to-operator variability and user bias. For many automated cell viability measurements, analysis algorithms are built into instrumentation, and are often considered proprietary. For example, in trypan-blue based cell viability analyzers, analysis software typically relies on strategic setting of parameter values that identify cells and label them as viable or non-viable based on cell features such as size, shape, brightness, sharpness, etc. These parameter settings can have a significant impact on the reported cell viabilities from the assay (Figure 10). NIST presented a study in which they systematically varied cell type parameter settings for NH-3T3 cells analyzed in a stop flow-based trypan blue imaging cell viability analyzer (Vi-Cell® XR, Beckman Coulter). NIST applied a design of experiments (DOE) approach using an orthogonal fractional factorial design to evaluate the sensitivity of the viability measurement to image analysis parameter settings, and to work towards identifying, as a proof of concept, an optimal set of parameters (i.e. image analysis protocols) for evaluating NIH3T3 cell viability on the trypan blue-based instrument. Interestingly, cell count and viability analysis for healthy cell samples was fairly robust to changes in image analysis parameter settings, but image parameter settings had a profound effect on reported viability values for samples in which cell health was compromised. Based on the DOE analysis, only a few parameter settings accurately identified viable and non-viable cells (based on comparison to expert manual identification of cells) for hydrogen peroxide treated NIH3T3 samples (i.e., cells treated with 10 mM hydrogen peroxide overnight compromising their health) (Figure 10B). This study demonstrated both the need to appropriately set image analysis parameters as well as the need to utilize health-compromised cell samples in the optimization of parameter settings.

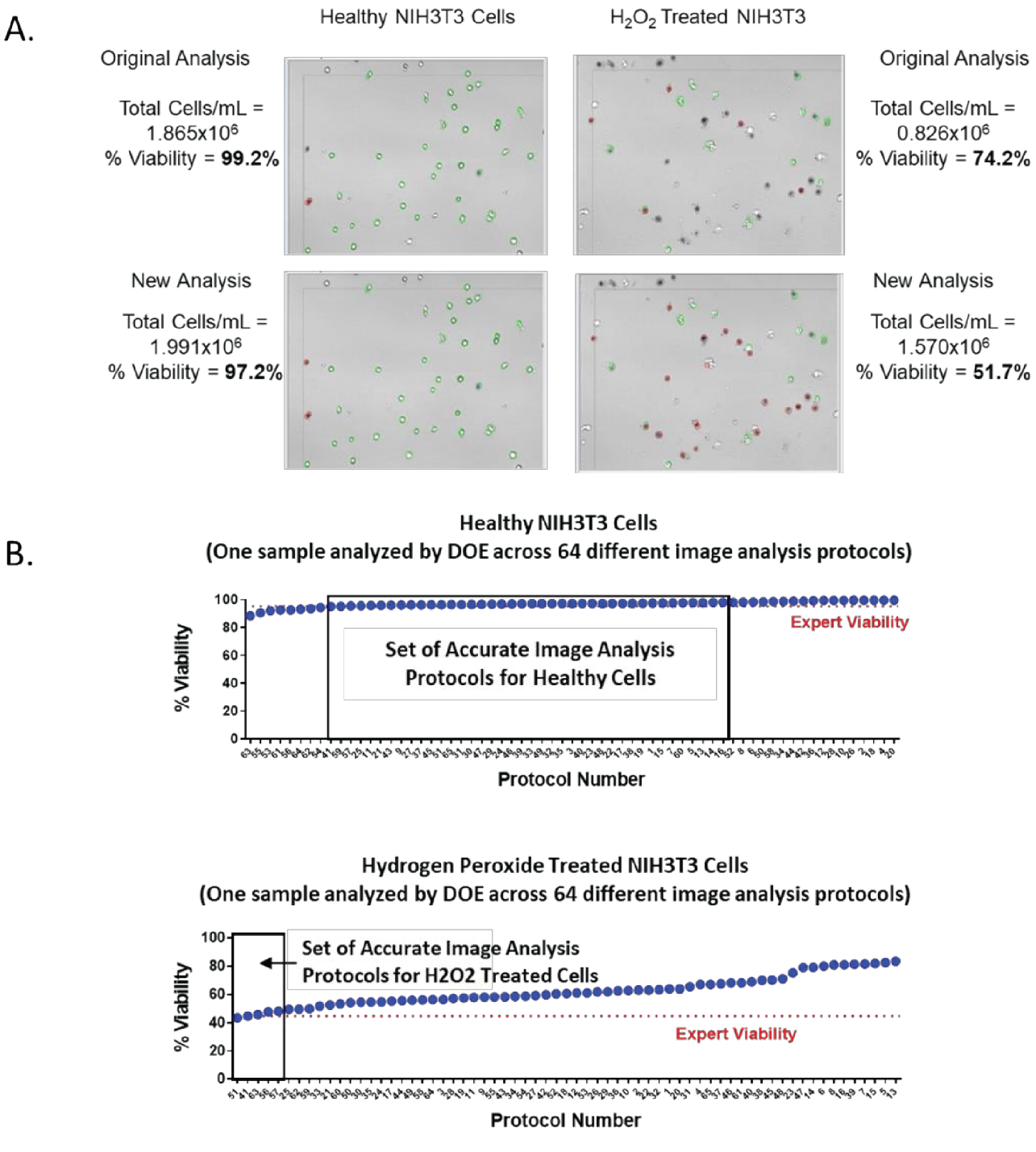
Incorporation of the appropriate control conditions and control materials during data acquisition can also be valuable in reducing error and variability that may arise during data analysis. In a plate-based MTS viability assay, for example, control wells are typically included for background correction and data calibration [28]. In addition, control experiments can be incorporated into each plate to monitor for multiple sources of variability that may arise during the measurement process including evaluation of pipetting accuracy, cell seeding density and instrument performance [29]. In some cases, variability identified from these control experiments can be incorporated into the data analysis strategy to reduce overall measurement variability. In another example presented by NIST at the workshop, reference beads can be used to benchmark the linear response of a fluorescent imaging-based cell viability analyzer to support the transfer of gating strategies from one analyzer to another. Using reference fluorescence intensity beads with NIST-assigned ERF (equivalent reference fluorophore) values, a linear function for ERF value versus mean fluorescence intensity was established on a fluorescent image-based cell viability analyzer (NC-3000™, Chemometec). The linear model was then used to assign the mean fluorescence intensity of a reference fixed Jurkat cell population to an equivalent reference value on the bead ERF scale using identical emission/excitation wavelengths (acridine orange, 495/519). This relationship was then used to establish a ratio of intensity of a test cell compared to the reference cell. The method allows for comparability of data across instruments when the ERF assigned beads, ERF assigned reference cells, and gate in the ERF scale are transferred between systems.

CONTROL MATERIALS FOR CELL VIABILITY ASSAYS

Control materials such as commercially available reference beads and fixed and dead cell control materials made within an individual lab are useful materials to consider in establishing control strategies for cell viability assays and these materials may have very specific applications depending on the chosen method of measurement. Reference beads have the advantage of being stable, commercially available, and homogeneous from lab

FIGURE 10

(A) NIH 3T3 Fibroblasts cultured under optimal growth conditions and after treatment with hydrogen peroxide (H2O2) analyzed by a stop flow-based trypan blue cell viability analyzer (Vi-Cell XR, Beckman Coulter) using two different image analysis parameter setting protocols. (B) Cell viability results reported by the analyzer using different image analysis protocols designed using a DOE strategy. Instrument reported results are compared for relative accuracy against manual identification of viable and non-viable cells in the same image. Boxed area represents the set of image analysis protocols that produce cell viability results that fall within 5% of the expert identified cell viability (red dotted line) for the sample.



to lab. Bead materials can be useful in establishing instrument qualification, or as described earlier, as stable artifacts for in-process benchmarking of measurement quality [30]. Novel synthetic reference materials that mimic cell properties such as scattering properties

DOI: 10.18609/cgti.2021.076

and staining properties may also serve as measurement process controls addressing sources of variability that arise from processes such as cell staining and data analysis such as cell gating.

Fixed cellular materials can also be a convenient tool for mimicking sample specific properties such as scattering properties in flow cytometry or staining properties in imaging methods and cells may retain specific cell features such as morphology and surface markers. For cell viability assays that are based on nuclear staining, fixed cells are particularly appealing as fixation can preserve the nuclear material of the cell and make it available for nuclear dyes that assess membrane permeability. Since many of the fixed cell characteristics may be highly similar to that of the test cells, they can potentially be sampled, stained, treated, and measured in an identical fashion to the cells of interest. As a practical consideration, fixed cell materials can be created in large batches, and potentially stored for long periods of time.

An important class of biological reference materials for cell viability assays are dead and dying cell materials. Dead or dying cell spike-in control studies (i.e., recovery studies) are critical for evaluating the specificity, linearity, and sensitivity of cell viability assays. Several approaches have been employed to obtain dead or dying cell materials including heat shock, fixation, compromised environmental growth conditions, nutrient deprivation, and chemical treatment [21,31,32]. These materials are often prepared on an ad hoc basis, as they may not have the stability needed for long-term storage. Importantly, the method used for cell killing or for compromising cell health can result in different features of the non-viable cells. For example, some killing methods may result in more cellular debris, while other methods may result in cells that are non-viable yet remain fairly intact and uniform in their properties. In particular, the range of control material properties should be consistent with the range of cell sample properties that may be encountered by the cell viability assay. For example, if cell viability will be analyzed at a particular step in a biomanufacturing process, control materials may be generated based on the types of perturbances the cells may experience from the previous step of the biomanufacturing process (e.g., extremes in fluid dynamics, environmental conditions, or nutrient conditions). It is unlikely that certified reference materials will be available for cell viability assays that adequately represent the many types of samples that will be analyzed, even in the manufacturing process for a specific CTP. Best practices and guidance for the generation of in-house dead or dying cell control materials, however, will help to support the implementation of control strategies for cell viability assays.

POTENTIAL FOR CELL VIABILITY STANDARDS DEVELOPMENT

As noted, many workshop participants indicated the need for standards development in the area of cell viability. Currently cell viability is touched upon in the recently published ISO 20391 Cell Counting Standards series, and several sector, instrument, or cell type specific standards in cell counting and viability exist or are under development [33,34]. For example, recent efforts in ASTM have focused on developing a test method for measuring cell viability in a scaffold (ASTM WK62115, "New Test Method for Measuring Cell Viability in a Scaffold") [35]. Within ISO TC 276, the Biotechnology Technical Committee (TC) of the International Standards Organization, the analytical methods working group is in the final stages of completing a documentary standard on the characterization and testing of cellular therapeutic products (CTPs), "ISO/DIS 23033 Biotechnology – Analytical methods – General guidelines for the characterization and testing of cellular therapeutic products" [4]. This standard identifies cell viability as a quality attribute important for the testing of CTPs and establishes requirements for the testing of cell viability in the final CTP. The standard also provides generally applicable guidelines for the

fit-for-purpose selection and development of analytical methods for CTP characterization and testing as well as considerations and requirements for method qualification and validation; however, it does not provide specific considerations for cell viability assays.

Currently, there are no existing standards that harmonize the terminology associated with cell viability and address the fit-for-purpose approach to cell viability assay development. A more general standard, addressing fit-for-purpose considerations and best practices for conducting viability assays would serve to address a wide range of stakeholder needs and would be best suited for the CTP sector where viability assays are needed for a wide range of intended uses and cell types. A key aspect of a general cell viability standard would be to address terminology associated with the definition of cell viability and the reporting of cell viability results, which should include a specific reference to the type of cell viability biological indicator that was measured. For example, instead of simply stating that a cell viability measurement resulted in 70% viability, one would report the value along with the assay used to obtain the value (i.e., 70% viability based on the image-based trypan blue dye-exclusion assay). In this way, viability results can be compared and utilized appropriately, recognizing the type of viability measurement that was conducted.

A general standard could also address common challenges associated with the cell samples including properties such as cell population heterogeneity, sample stability, and contaminating debris and cell types which can greatly affect a viability assay. Another key aspect of a general cell viability standard would be to address measurement control strategies including the appropriate use and generation of control materials as well as methodologies for validation of the selected measurement process, an important area for additional stakeholder consideration. General considerations could also be shared regarding different instrumentation and principles used for cell viability measurements (e.g., flow cytometry-based, imaging-based, impedance-based, metabolite-based assays). Furthermore, a documentary standard could include experimental frameworks and guidelines for experimental comparison of viability methods and subsequent analysis and approaches for evaluating the fitness for purpose of a viability assay.

CONCLUSIONS

With the recent success of CTPs in providing lifesaving treatments, the need for reliable and relevant cell viability measurements has become critical. This workshop brought together stakeholders across industry, academic, and government labs, who discussed a broad range of challenges in the area of viability measurements for CTPs and discussed possible approaches to improve confidence in viability measurements. Key considerations for fit-for-purpose cell viability method development such as sample considerations, measurement method considerations, and the selection of appropriate biological indicators for identifying viable and non-viable cells were identified by workshop participants. Importantly, the interaction between sample properties and the many measurement process steps was identified as a critical consideration in developing assays that are accurate and represent as closely as possible the true nature of the original cell sample. An important observation from the workshop was the need to identify fit-for-purpose assays which will offer the most relevant cell viability information for the intended use of their cell preparation. Reported viability measurements should include a description of the measurement technique and the calculation used to assess viability. Additionally, the application of strategically designed control materials and control studies were demonstrated to have the potential to improve cell viability measurement confidence. Standardized approaches that support the selection, development and validation of fit-for-purpose cell viability measurements will help to accelerate the development of CTPs.

DOI: 10.18609/cgti.2021.076

TRANSLATION INSIGHT

Current advances and successes in the field of CTPs have necessitated more accurate, reliable and robust cell viability measurements for these life-saving products. A recent cell viability workshop brought together stakeholders across the field who discussed challenges and solutions for the development of fit-for-purpose cell viability measurement methods. Solutions focused on a thorough analysis of sample considerations, measurement method considerations, and the selection of appropriate biological indicators for viable and non-viable cells. Standardized approaches that support the selection, development and validation of fit-for-purpose cell viability measurements will help to accelerate the development of CTPs.

REFERENCES

1. ISO. ISO/TS 20399-1: 2018 Biotechnology — Ancillary materials present during the production of cellular therapeutic products — Part 1: General requirements. ISO/TC276 Biotechnology; 2018.
2. Bauer SR. Stem Cell-based Products in Medicine: FDA Regulatory Considerations. In: Lanza R (Ed.). *Handbook of Stem Cells. 2*: Elsevier; 2004; 805–14.
3. Hoogendoorn KH, Crommelin DJA, Jiskoot W. Formulation of Cell-Based Medicinal Products: A Question of Life or Death? *J. Pharm. Sci.* 2021; 110(5): 1885–94.
4. ISO. DIS 23033 - Biotechnology – Analytical methods – General guidelines for the characterization and testing of cellular therapeutic products. ISO/TC 276 Biotechnology/WG32021.
5. Dowling M, Catríona, Herranz Ors C, Kiely A, Patrick. Using real-time impedance-based assays to monitor the effects of fibroblast-derived media on the adhesion, proliferation, migration and invasion of colon cancer cells. *Biosci. Rep.* 2014; 34(4): 415–27.
6. Ngoc Le HT, Kim J, Park J, Cho S. A Review of Electrical Impedance Characterization of Cells for Label-Free and Real-Time Assays. *BioChip J.* 2019; 13(4): 295–305.
7. Kanemura Y, Mori H, Kobayashi S *et al.* Evaluation of in vitro proliferative activity of human fetal neural stem/progenitor cells using indirect measurements of viable cells based on cellular metabolic activity. *J. Neurosci. Res.* 2002; 69(6): 869–79.
8. Raffray M, Gerald M. C. Apoptosis and necrosis in toxicology: A continuum or distinct modes of cell death? *Pharmacol. Ther.* 1997; 75(3): 153–77.
9. Cummings BS, Schnellmann RG. Measurement of Cell Death in Mammalian Cells. *Curr. Protocols Pharmacol.* 2004; 25(1): 1–30.
10. Lin-Gibson S, Sarkar S, Ito Y. Defining quality attributes to enable measurement assurance for cell therapy products. *Cytotherapy* 2016; 18(10): 1241–4.
11. Patterson MK. Measurement of growth and viability of cells in culture. In: William B. Jakoby IHP (Ed.). *Methods in Enzymology. Cell Culture*: Elsevier; 1979; 141–52.
12. Arora D, Babakhanova G, Simon Jr CG. Tissue Engineering Measurands. ACS Biomaterials Science & Engineering. 2020;6(10):5368-76.
13. Galluzzi L, Vitale I, Aaronson SA *et al.* Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Diff.* 2018; 25(3): 486–541.
14. Lin-Gibson S, Sarkar S, Elliott JT, Plant AL. Understanding and managing sources of variability in cell measurements. *Cell Gene Ther. Ins.* 2016; 6(2): 663–73.
15. ISO. ISO 20387:2018(en) Biotechnology – Biobanking – General requirements for biobanking. ISO: ISO/TC 276; 2018.
16. Woods E, Thirumala S, Badhe-Buchanan S, Clarke D, Mathew A. Off the Shelf Cellular Therapeutics: Factors to Consider during Cryopreservation and Storage of Human Cells for Clinical Use. *Cytotherapy* 2016; 18(6): S46–S.
17. Yang J, Gao L, Liu M *et al.* Advanced Biotechnology for Cell Cryopreservation. *Trans. Tianjin University* 2020; 26(6): 409–23.
18. TECHNICAL ICOHO, HUMAN RFROPF, USE. VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY Q2(R1). ICH.
19. Chan LLY, Laverty DJ, Smith T *et al.* Accurate measurement of peripheral blood mononuclear cell concentration using image cytometry to eliminate RBC-induced counting error. *J. Immunol. Meth.* 2013; 388(1–2): 25–32.

20. Chan LL-Y, Kuksin D, Laverty DJ, Saldi S, Qiu J. Morphological observation and analysis using automated image cytometry for the comparison of trypan blue and fluorescence-based viability detection method. *Cytotech*. 2015; 67(3): 461–73.

21. Chan LL-Y, Rice WL, Qiu J. Observation and quantification of the morphological effect of trypan blue rupturing dead or dying cells. *PLoS One* 2020; 15(1): 1–17.

22. Mascotti K, McCullough J, Burger SR. HPC viability measurement: trypan blue versus acridine orange and propidium iodide. *Transfusion* 2000; 40: 693–6.

23. Nucleated Cell Count and Viability. *European Pharmacopeia* 2008; 2.7.29(20729): 275–6.

24. Chen A, Leith M, Tu R, Tahim G, Sudra A, Bhargava S. Effects of diluents on cell culture viability measured by automated cell counter. *PLOS One* 2017; 12(3): 1–13.

25. ISO 20391-1:2018 Biotechnology – Cell counting – Part 1: General guidance on cell counting methods. ISO/TC 276 Biotechnology/WG3/2018.

26. ThermoFisher-Scientific. The four pillars of achieving accurate and precise cell counts [White Paper] https://assets.thermofisher.com/TFS-Assets/LSG/Application-Notes/Countess-II-FL-FourPillars-WhitePaper.pdf

27. Peskin SL, Pierce L, Kurbanov F, Chan L, Halter M, Elliott J, Sarkar S, Chalfoun J. Establishing a Reference Focal Plane Using Beads for Trypan-blue-based Viability Measurements. *J. Microscopy.* Accepted.

28. ISO 19007:2018(en) Nanotechnologies – In vitro MTS assay for measuring the cytotoxic effect of nanoparticles. ISO/TC 229 Nanotechnologies/2018.

29. Rösslein M, Elliott JT, Salit M *et al.* Use of cause-and-effect analysis to design a high-quality nanocytotoxicology assay. *Chem. Res. Toxicol.* 2015; 28(1): 21–30.

30. Lin-Gibson S, Sarkar S, Elliott JTJC. Summary of the National Institute of Standards and Technology and US Food And Drug Administration cell counting workshop: Sharing practices in cell counting measurements. 2018; 20(6): 785–95.

31. Huang L-C, Lin W, Yagami M *et al.* Validation of cell density and viability assays using Cedex automated cell counter. *Biologicals* 2010; 38(3): 393–400.

32. Cadena-Herrera D, Esparza-De Lara JE, Ramírez-Ibañez ND *et al*. Validation of three viable-cell counting methods: Manual, semi-automated, and automated. *Biotechnol. Rep.* 2015; 7: 9–16.

33. Sarkar S, Pierce L, Lin-Gibson S, Lund SP. Standards Landscape in Cell Counting: Implications for Cell & Gene Therapy. *Cell Gene Ther. Ins.* 2019; 5(1): 117–31.

34. ASTM. F2739 – 19 – Standard Guide for Quantifying Cell Viability and Related Attributes within Biomaterial Scaffolds. Subcommittee: F04432019.

35. ASTM. ASTM WK62115: New Test Method for Measuring Cell Viability in a Scaffold. Subcommittee: F0443, Committee F042021.

AFFILIATIONS

Laura Pierce
Author for correspondence:
Biosystems and Biomaterials Division, Materials Measurement Laboratory, National Institute of Standards and Technology, Gaithersburg, MD, USA
laura.pierce@nist.gov

Sumona Sarkar
Biosystems and Biomaterials Division, Materials Measurement Laboratory, National Institute of Standards and Technology, Gaithersburg, MD, USA

Leo Li-Ying Chan
Department of Advanced Technology R&D, Nexcelom Bioscience LLC, Lawrence, MA, USA

Bo Lin
Department of Advanced Technology R&D, Nexcelom Bioscience LLC, Lawrence, MA, USA

Jean Qiu
Department of Advanced Technology R&D, Nexcelom Bioscience LLC, Lawrence, MA, USA

DOI: 10.18609/cgti.2021.076

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *The authors acknowledge Carl Simon (NIST) for his contribution to the development of Table 2 and for helpful discussions. We would like to express our sincere gratitude to the conference organizers and all workshop presenters and participants for their contributions to pre-workshop activities and workshop discussions.*

Disclosure and potential conflicts of interest: *Certain commercial equipment, instruments or materials are identified in this article to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose. Some of the data and publication presented used products from Nexcelom Bioscience, where Leo Chan, Bo Lin and Jean Qiu are employed.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Mar 22 2021;* ***Revised manuscript received:*** *Apr 27 2021;* ***Publication date:*** *May 14 2021.*