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Standard Guide for ~~Quantitating~~Quantifying Cell Viability ~~Within~~within Biomaterial Scaffolds[1]

This standard is issued under the fixed designation F2739; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide is a resource of cell viability test methods that can be used to assess the number and distribution of viable and non-viable cells within porous and non-porous, hard or soft biomaterial scaffolds, such as those used in ~~tissue engineered~~ tissue-engineered medical products (TEMPs).

1.2 In addition to providing a compendium of available techniques, this guide describes ~~materials specific~~ materials-specific interactions with the cell assays that can interfere with accurate cell viability analysis, and includes guidance on how to avoid, and/or account for, scaffold material/cell viability assay interactions.

1.3 These methods can be used for 3-D scaffolds containing cells that have been cultured *in vitro* or for scaffold/cell constructs that are retrieved after implantation in living organisms.

1.4 This guide does not propose acceptance criteria based on the application of cell viability test methods.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

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2. Referenced Documents

2.1 *ASTM Standards:*[2]

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F2149 Test Method for Automated Analyses of Cells—the Electrical Sensing Zone Method of Enumerating and Sizing Single Cell Suspensions

ASTM F2739-16

F2315 Guide for Immobilization or Encapsulation of Living Cells or Tissue in Alginate Gels

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F2998 Guide for Using Fluorescence Microscopy to Quantify the Spread Area of Fixed Cells

3. Terminology

3.1 *Definitions:*

3.1.1 *non-viable cell, n*—a cell not meeting one or more of the criteria for ~~viability given in~~ a viable cell.~~3.1.2.~~

3.1.2 *viable cell, n*—a cell capable of metabolic activity that is structurally intact with a functioning cell membrane.

4. Summary of Guide

4.1 It is the intent of this guide to provide a compendium of the commonly used methods for ~~quantitating~~quantifying the number and distribution of viable and non-viable cells within, or on, a biomaterial scaffold, because cell viability is an important parameter of ~~tissue engineering~~ tissue-engineered products used to regenerate or repair lost or diseased tissue. The methods can be applied to cells residing within an intact 3-D scaffold or matrix (that is, non-destructive methods) or to cells that have been removed from the scaffold or matrix (that is, destructive methods). It should be noted that not all cells require a scaffold and some cell types, such as hematopoietic cells, cannot be cultured or grown on an adherent surface.

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[1] This guide is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.43 on Cells and Tissue Engineered Constructs for TEMPs.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the ~~standard's~~standard's Document Summary page on the ASTM website.

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4.2 Most of the methods originate from analysis of cell number on 2-D surfaces, but have been adapted for the analysis of cells within 3-D constructs that are typically used in regenerative medicine approaches. The mechanisms and the sensitivity of the assays are discussed. The limitations of the assays due to using standard curves generated from cells on 2-D surfaces are described in this document. In addition, the ways in which the biomaterial scaffold itself can affect the viability assays are described.

4.3 This guide describes ~~combinations of~~ test methods which, when used together, ~~will ensure the most~~ may enable accurate measure of the number and distribution of viable and non-viable cells. Different viability assays have different measurands, which means that the results from different assays may not correlate with one another. For instance, cell membrane integrity tests and cell metabolic tests measure fundamentally different cell properties. Although both tests are related to cell viability, they may not correlate with one another.

5. Significance and Use

5.1 The number and distribution of viable and non-viable cells within, or on the surface of, a biomaterial scaffold is one of several important characteristics that may determine *in vivo* product performance of cell/biomaterial constructs (see 5.7); therefore there is a need for standardized test methods to ~~quantitate~~quantify cell viability.

5.2 There are a variety of static and dynamic methods to seed cells on scaffolds, each with different cell seeding efficiencies. In general, static methods such as direct pipetting of cells ~~on to~~ onto scaffold surfaces have been shown to have lower cell seeding efficiencies than dynamic methods that push cells into the scaffold interior. Dynamic methods include: injection of cells into the scaffold, cell seeding on biomaterials contained in spinner flasks or perfusion chambers, or seeding that is enhanced by the application of centrifugal forces. The methods described in this guide can assist in establishing cell seeding efficiencies as a function of seeding method and for standardizing viable cell ~~number~~numbers within a given methodology.

5.3 As described in Guide F2315, thick scaffolds or scaffolds highly loaded with cells lead to diffusion limitations during culture or implantation that can result in cell death in the center of the construct, leaving only an outer rim of viable cells. Spatial variations of viable cells such as this may be ~~quantitated~~quantified using the tests within this guide. The effectiveness of the culturing method or bioreactor conditions on the viability of the cells throughout the scaffold can also be evaluated with the methods described in this guide.

5.4 These test methods can be used to ~~quantitate~~quantify cells on hard or soft 3-D biomaterials, such as ceramics and polymer gels. The test methods also apply to cells seeded on porous coatings.

5.5 Test methods described in this guide may also be used to distinguish between proliferating and non-proliferating viable cells. Proliferating cells proceed through the DNA synthesis (S) phase and the mitosis (M) phase to produce two daughter cells. Non-proliferating viable cells are in some phase of the cell cycle, but are not necessarily proceeding through the cell cycle culminating in proliferation.

5.6 Viable cells may be under stress or undergoing apoptosis. Assays for evaluating cell stress or apoptosis are not addressed in this guide.

5.7 While cell viability is an important characteristic of a TEMP, the biological performance of a TEMP is dependant on additional parameters. Additional tests to evaluate and confirm the cell identity, protein expression, genetic profile, lineage progression, extent of differentiation, activation status, and morphology are recommended.

5.8 Fundamental biocompatibility testing of the scaffold material itself as described in Practice F748 should be completed prior to using the biomaterial with cells.

5.9 Methods that remove the cells from a 3-D scaffold may reduce the cell number and viability due to the manipulation required.

6. Selection of Test Methods

6.1 Table 1 is a compendium of methods that can be used to ~~quantitate~~quantify cell viability on surfaces or in biomaterial scaffolds. Importantly, a combination of the methods listed in Table 1 is required to determine viable and non-viable (or live and dead) cells quantitatively, and additional tests must be completed to ~~quantitate~~quantify the subset of proliferating viable cells within the total number of viable cells. Proliferating cells are viable, but viable cells are not necessarily proliferating. Non-viable cells can be identified, even if they are not intact structurally or metabolically, by intact ~~nuclei~~ nuclei, DNA stains or dye entry into the cell through a disrupted cytoplasmic membrane.

6.2 The total number of cells, both alive and dead, within a 3-D construct ~~is typically~~may be determined by DNA analysis (7.2) after the cells are removed destructively (lysis) from the biomaterial scaffold and ~~solubilized. Since many cell types adhere very well to a scaffold, significant cell lysis can occur during cell removal and simple manual counting of intact stained or unstained cells (~~solubilized (with detergents or sonication, for example). It may not be possible to completely recover all cell material that is located deep within scaffold pores due to diffusion limitations.~~7.1) is not very reliable.~~

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TABLE 1 Methods for ~~Quantitating~~Quantifying Cell Viability

	Destructive (Requires cell removal from scaffold or matrix)	Non-destructive (Cells remain in scaffold or matrix during test)
I. Total Cell Number		
DNA assay	X	
Crystal violet	X	
II. Live Cell Number		
Metabolic assays	X	X
Tetrazolium salt uptake: MTT, MTS, WST, XTT	X	
Alamar Blue (resorufin)		X
Neutral Red	X	
Glucose Consumption	X	X
Cell proliferation (DNA synthesis)		
[3H] Thymidine or BrDu (Bromodoeoxyuridine) labeling	X	
Dye exclusion assays		
Trypan blue, erythrosin, and nigrosin	X	
III. Live/Dead Ratios		
Live/Dead assays using dual fluorescent stains for plasma membrane integrity	X	
Non-fluorescent dye exclusion assays	X	
IV. Imaging—density, morphology and spatial distributions of cells		
Histological sectioning	X	
Confocal microscopy	X	X
Scanning electron microscopy	X	

6.3 Counting cells harvested (by trypsinization or passaging, for example) from scaffolds may not be reliable if the scaffold specimens are small (from 96-well or 48-well plates, for example). The dilutions with cell harvesting medium or buffers may yield cell concentrations that are too low to be effectively counted (by hemocytometer, for example).

6.4 If cells in a suspension are to be counted, ~~Test Method~~ electrical sensing zone test method (F2149) or flow cytometry may be useful.

6.5 To determine the quantity of live cells only, the use of a fluorescent or colorimetric metabolic indicator that fluoresces or changes color in response to ~~chemical reduction of growth media resulting from~~ cell metabolic activity may be used (7.2). Metabolic assays are available in both destructive and non-destructive forms. The MTT (3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide) or MTS ~~assay~~ ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays (7.2.1) ~~is a~~ are destructive, commonly used ~~method~~methods that can be read with a spectrophotometer. The Alamar Blue (resorufin) (7.2.2) is a non-destructive method that requires a fluorimeter. Cell metabolism in a 2-D ~~environment, compared with~~ environment may differ from than in a 3-D environment, ~~is significantly different, even with~~even when the same cell ~~numbers.~~ numbers are the same. Accordingly, results for 3-D cell numbers can be erroneous when growth curves of cells cultured in 2-D are used for calibration (**1**).[3] It is important to note that metabolic assays are direct measures of intracellular enzyme activity produced by cells. Although the level of enzyme activity ~~is typically linearly~~may be directly proportional to the number of viable cells, it is possible that specific culture conditions ~~can~~may affect the production and activity of the enzyme being assayed or that ~~in certain circumstances, substrate may not be limiting.~~ the scaffold may interfere with the measurement (matrix effects). In this situation, the metabolic ~~assay would~~measurement may not ~~necessarily~~ be ~~linearly~~directly proportional to cell number. ~~Despite these limitations, these methods are still commonly used.~~

6.6 The quantity of live cells within the total cell population may be determined by a proliferation or metabolic assay (7.3). ~~However, this will not provide information on the distribution of live cells within a construct; hence,~~ It may be helpful to verify quantitative results with an imaging technique (7.4) ~~to visualize the morphology and~~ in order to provide visual evidence of live or dead cells. Visual evidence assures that the quantitative measurements can be trusted and did not arise due to experimental artifacts (such as the scaffold reacting with assay reagents and causing a false positive reading). Imaging also provides information on the spatial distribution of ~~dye-labeled~~ live ~~and dead cells is typically utilized.~~cells within a construct.

6.7 Non-destructive methods to determine cell viability of an entire cell population within a scaffold or bioreactor are included in this guide and are useful for conducting kinetic studies of cell number and distribution over time.

6.8 The scaffolding material may interfere with any of the following assays and must be included within the assay, typically as a control, to determine whether it has an effect. If the assay is affected by the presence of the scaffold, then either the interference should be subtracted out or an alternative assay should be selected. Notes on known interferences are included in each of the assay descriptions below.

[3] The boldface numbers in parentheses refer to the list of references at the end of this standard.

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6.9 Cell density could impact accuracy of quantification. Cells grown at low density are generally harder to wash off than cells grown to confluency, where a whole sheet of cells may be rather easy to displace. Many ~~of our products may be~~ scaffolds are seeded at as high a cell density as possible. High densities may also affect dye binding. Also, cell density generally impacts the "health" of the whole culture, since cell-to-cell interactions are important effectors of cell state.

6.10 In many instances a mixed population of cells may be present. Metabolic assays will not accurately quantify mixed cultures of cells because some cells are more metabolically active than others. There is a similar problem with dyes: nuclear sizes may not be identical (though they ~~are probably~~may be similar). Cell cytoplasm volumes may be very different, as could be the number of cellular processes. In a mixed population of cells, some cells may be proliferating rapidly, whereas others might be post-mitotic.

6.11 Some scaffolds will be translucent, others opaque. Some may be rigid, others very fragile. For more fragile scaffolds, cells may fall off during handling, so it would be preferable to use a method that minimizes handling. Scaffolds break down over time. Edges of scaffolds might be softer than internal portions. Scaffolds may not have uniform thickness or density, which may affect statistical sampling.

7. Specific Test Methods for Determining Cell Viability

7.1 *Dye Exclusion Technique to Distinguish Live from Dead:*

7.1.1 One of the simplest methods to approximate cell viability is the dye exclusion technique. This approach is based on the assumption that viable cells must have an intact membrane, which is required for life-associated cellular processes such as the conversion of food sources into energy, growth, and reproduction. This method utilizes an indicator dye to demonstrate cell membrane damage. Cells which absorb the dye become stained and are considered non-viable. Dyes such as trypan blue, erythrosin, and nigrosin are used commonly, with trypan blue being the most common in preliminary cell isolation procedures. Cells must be removed from the scaffold, mixed with the ~~dye for a few minutes,~~ dye, and then counted manually with a hematocytometer. Cells must be analyzed shortly ~~(3 to 5 min)~~ after the addition of 0.4 % trypan blue, since trypan blue is cytotoxic. There are large standard deviations with increasing cell densities; therefore samples should be diluted to the densities recommended in the hematocytometer instructions.

7.2 *Determination of Total Cell Number:*

7.2.1 *DNA Assay*—DNA analysis is a commonly used method for determining total cell number, including both viable and non-viable cells. There are several commercially available kits for assessing DNA ~~content, all of which can be used.~~ content. It is important to fully extract the cells from the scaffold prior to analysis, using for example, a solution of 0.125 mg/mL papain and 10 mmol/L L-cysteine dihydrochloride in phosphate buffered ~~EDTA~~ ethylenediaminetetraacetic acid (EDTA) in a 60°C water bath for 10 hours to extract cells from a polymer matrix (**2**). The process lyses the cells to yield soluble DNA for detection by the assay and the papain and EDTA inactivate nucleases to prevent DNA degradation. If the cell fluorescence will be measured, a protein digestion step using a proteinase ~~K digestion step ablates any residual~~may ablate endogenous fluorescence of the cells (**3**). A DNA standard curve should be run for calculating the amount of DNA.

7.2.2 *Crystal Violet Staining*—Another cell stain used for determining total cell number is crystal violet which binds to the DNA of viable and non-viable cells. Cells must be removed from the biomaterial scaffold prior to analysis. Cells are washed in phosphate buffered solution (PBS), stained with ~~0.1~~0.05 to 0.2 % (by mass) crystal violet in methanol for 15 min at 37°C, and then washed extensively prior to analysis. Absorbance is measured at a wavelength of 590 nm using ~~an ELISA~~ a plate reader.

7.3 *Proliferation or Metabolic Assays for Quantitating Live Cell Number:*

7.3.1 *MTT, MTS, XTT or WST Tetrazolium Salt Assays*—Metabolic activity ~~of~~ is often used as an indicator of cell viability, since metabolic activity is required for life-associated processes, such as conversion of food sources into energy, growth and reproduction. Metabolic activity of cells is commonly monitored colorimetrically by assaying the cell-based alteration of tetrazolium salts such as MTT (3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide). The cells take up the crystals and convert the tetrazolium salt to formazan crystals by the succinate-tetrazolium reductase system in the mitochondria. The amount of formazan dye formed correlates directly to the number of metabolically active cells in the culture. A ~~standard ELISA~~ plate reader is used to read the results. The seeded scaffolds should be rinsed in either a serum-free medium or PBS to remove unattached cells before beginning the assay. Since there can be a chemical interaction of the biomaterial scaffold with the assay components or an absorbance from the scaffold itself, an unseeded scaffold must be used as a control. ~~Standard curves are~~ Although a standard curve may be established from known cell ~~numbers. A large, but not excessive variability between~~ numbers, the cells used for establishing the standard curve may not be in the same metabolic state as the cells in the scaffold. Thus, the relationship between cell number and cell metabolic activity may vary with cell preparation. Variability between absorbance values of similar samples can be expected due to natural variability in cell output. The dissolution of the formazan crystals can be slow and difficult in the ~~original~~ MTT assay, and requires alcohol, which may damage the scaffold and cells; therefore, an aqueous MTS assay that does not require dissolving the formazan crystals is ~~now~~ available. ~~These~~MTS kits contain a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES). The XTT kit, which uses XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) instead of MTT, is also easier to run than the MTT because it produces a soluble dye. A non-destructive version of a tetrazolium salt-based proliferation assay is the WST. The WST method utilizes the tetrazolium salt

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