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# Standard Guide for Quantifying Cell Viability within Biomaterial Scaffolds[1]

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## 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 medical products (TEMPs).

1.2 In addition to providing a compendium of available techniques, this guide describes 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.*

## 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

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

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 a viable cell.

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 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-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.

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 test methods which, when used together, 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

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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 Document Summary page on the ASTM website.

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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 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 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 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 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 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 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)

**TABLE 1 Methods for 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 | |

cells quantitatively, and additional tests must be completed to 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, 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 may be determined by DNA analysis (7.2) after the cells are removed destructively (lysis) from the biomaterial scaffold and 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.

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, 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 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 ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays (7.2.1) are destructive, commonly used methods that can be read with a spectrophotometer. The Alamar Blue assay (resorufin) (7.2.2) is a non-destructive method that requires a fluorimeter. Cell metabolism in a 2-D environment may differ from than in a 3-D environment, even when the same cell 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 may be directly proportional to the number of viable cells, it is possible that specific culture conditions may affect the production and activity of the enzyme being assayed or that the scaffold may interfere with the measurement (matrix effects). In this situation, the metabolic measurement may not be directly proportional to cell number.

6.6 The quantity of live cells within the total cell population may be determined by a proliferation or metabolic assay (7.3). It may be helpful to verify quantitative results with an imaging technique (7.4) 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 live 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.

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 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 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, and then counted manually with a hematocytometer. Cells must be analyzed shortly after the addition of 0.4 % trypan blue, since trypan

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

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. 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 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 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 0.05 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 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 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 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. Although a standard curve may be established from known cell 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 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 available. 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 WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium). There has been discussion about whether these different reagents (MTT, MTS, XTT and WST-1) are metabolized by the same enzymes (**4**). Reactions with the soluble reagents may occur at the plasm membrane instead of at the mitochondria as may occur with insoluble reagents.

7.3.2 *ATP Content*—Adenonsine triphosphate (ATP) content and oxygen uptake have been shown to be highly predictive of pancreatic islet performance, for example, and it is possible to assess high-energy phosphoryl metabolism, particularly of ATP and creatine phosphate in living tissue, using non-destructive P-31 nuclear magnetic resonance (NMR) or, to obtain a more detailed picture of overall metabolic dynamics, stable isotope labeling with O-18 and mass spectrometry. There are also bioluminescence assays that use ATP-driven, luciferase-catalyzed reactions to detect ATP. These assays have the advantages of being directly calibratable with ATP standards and having low background (there are few examples of bioluminescence in mammalian cells). Although a standard curve may be established from known cell 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.

7.3.3 *Alamar Blue Assay*—The Alamar Blue assay uses a non-cytotoxic oxidation-reduction metabolic indicator that fluoresces and changes color in response to chemical reduction of growth media resulting from cell growth. Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is weakly fluorescent until it is reduced into the highly fluorescent resorufin. Since the assay is not cytotoxic, it is non-destructive and can be used repeatedly to assess cell viability and cell proliferation. It is applicable to non-proliferating cells as well. An advantage of this method is that cells do not have to be removed from the scaffold prior to analysis. Standard curves are established from known cell numbers exposed to the reagent (per unit time). A fluorimeter or a standard spectrophotometer is used to read the results.

7.3.4 *Neutral Red Assay*—The neutral red assay is based on the rapid uptake of the dye into the lysosomes of viable cells. After cells are incubated with the dye, the cells are lysed and the dye uptake is determined spectrophotometrically. When significant changes in cell viability occur, irreversible changes in the lysosome and dye uptake mechanism occur. The dye uptake is compared to that of a control sample and the % viability with respect to control cells is reported. This assay

is used routinely in high-throughput screening for cytotoxic compounds. This dye becomes toxic with light, so the reagents must be handled with safety precautions.

7.3.5 *$^{3}$H-Thymidine or BrdU Labeling*—Incorporation of tritiated thymidine into cellular DNA during the synthesis phase of the cell cycle is a widely used and sensitive protocol to monitor rates of DNA synthesis and hence cell proliferation. Cells are seeded and incubated with tritiated thymidine. During each cell division, the nucleotide becomes incorporated into the newly synthesized chromosomes. The more cell divisions (or the higher the proliferation rate), the more radioactivity is incorporated into cell DNA. After incubation, the cells are harvested, the cells are disrupted, and DNA is released. The cell fragments and intact DNA are collected on a filter. The higher the proliferation rate of the cells during incubation, the more cells are harvested along with their radioactive DNA. The filter membrane is dried and the amount of radioactivity (which corresponds to the number of cell divisions during incubation) is counted in a scintillation counter. Note that there is evidence demonstrating the ability of this radiochemical to induce cell cycle arrest and apoptosis, leading to erroneous results if the rate of proliferation is being determined (**5**). This assay is best when used as a terminal or endpoint assay. Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) is an analogue of thymidine often used in place of $^{3}$H-Thymidine because is also incorporated into the newly synthesized DNA of replicating cells. BrdU is commonly used in the detection of proliferating cells in living tissues. Antibodies specific for BrdU can then be used to detect the incorporated chemical by flow cytometry, immunocytochemistry, or ELISA-type (enzyme-linked immunosorbent assay) assays. Binding of the antibody is achieved by denaturation of the DNA, usually by exposing the cells to acid or heat. Caution should be used as this step may damage the biomaterial scaffold.

7.3.6 *Glucose Consumption*—The consumption of glucose by cultured cells is another measure of cell growth and metabolism. Glucose levels are measured before and after incubation with a given cell type and number to establish standard curves. Commercial spectrophotometric assays are available to quantify glucose depletion. The method of analysis must be selected so as to avoid spectral interference from the phenol red and serum present in cell culture media.

7.3.7 *Enzymatic Cleavage of Reporter Substrates*—There are several colorimetric, fluorescent, and luminescent assays for viability that take advantage of the ability of the enzymes in a culture supernatant to cleave reporter substrates. Enzymes such as glucose 6-phosphate dehydrogenase (G6PD) and lactate dehydrogenase (LDH) are released by cells with damaged membranes, and are thus an indication of cell death. These tests have the advantage that they can be performed on aliquots of existing cultures. It should be noted that membrane leakiness may be a late-stage cell death event, that may be preceded by other events, such as depletion of glucose, ATP or metabolic enzymes.

7.3.8 *Concerns About Proliferation Assays Used for Determining Viability Within Three-Dimensional Constructs:*

7.3.8.1 Variability in metabolic output for cells seeded on thick (more than 1 mm) 3-D scaffolds, and in cells within cell sheets, has been observed due to variability of nutrient access into the inner portions of the scaffold/cell layer (**1**). Therefore, measurements of DNA content may not always correlate with measurements of metabolic activity.

7.3.8.2 Scaffold thickness and dimensions can affect the diffusion of assay reagents into the scaffold. Care should be taken to assure that replicate scaffold sample dimensions are as consistent as possible.

7.3.8.3 Polylactic acid poly(lactic-co-glycolic acid) and poly(ε-caprolactone) are resorbable materials commonly used in scaffolds for TEMPs. As these scaffolds degrade they may release metabolic products that influence the pH or ionic strength of the medium, which may in turn influence the assay performance.

7.3.8.4 Certain dye reduction assays depend on mitochondrial metabolism. They may not be useful for tissues that generally function anaerobically.

7.4 *Imaging or Visual Methods to Determine Cell Viability:*

7.4.1 *General*—To determine spatial distributions of the cells within the biomaterial scaffold it may be necessary to visualize the cells using a microscope, such as a traditional light microscope, confocal microscope, or electron microscope. These approaches can be slow, are difficult to make quantitative, may require prohibitively expensive instrumentation and may not be appropriate for use as a routine viability assay.

7.4.2 *Histological Sectioning*—Traditional histological sectioning and staining followed by counting of cell numbers provides a measure of cell seeding uniformity.

7.4.3 *Fluorescent Markers for Live and Dead Cells*—Live/dead assays are commonly used to determine the ratio of live to dead cells. There are many chemistries for this use. A common live stain, calcein-AM (calcein acetoxymethyl ester), is weakly fluorescent and is cell-permeant. When taken up by cells, intracellular esterases metabolize it to a highly fluorescent derivative, which is membrane-impermeant and becomes trapped in cells with intact membranes. A common dead cell stain is ethidium homodimer, which enters cells with leaky membranes and stains DNA. There are assays available for both *in vitro* and *in vivo* use. Sections can be made, particularly through soft biomaterials, enabling quantitation by a plate reader on the thin slabs of approximately 1 mm. Cells may also be stained with a variety of commercially available fluorescent markers for proliferating viable or non-viable cells and then counted and classified using flow cytometry. Cells may be genetically manipulated to express fluorescence, in which case the net fluorescence could be quantitated through the use of a 2-D culture and a dose range of cells.

7.4.4 *Confocal Microscopy*—After labeling the live and dead cells with fluorescent markers, confocal microscopy can be used to distinguish distribution of live and dead cells within the construct. Since confocal microscopes typically only penetrate few hundred microns into a construct, multiple sections may be necessary to quantify the proportion of viable cells and their distribution throughout the construct. Multi-photon confocal microscopy may penetrate more deeply into constructs, but is expensive and may not be practical for routine analysis. Depending on the thickness of the sections, non-confocal

microscopes can also be used to quantify numbers of viable cells and to provide information on the 3-D cell population within a construct.

7.4.5 *Magnetic Resonance Imaging*—For very thick scaffolds (mm to cm), high-field MRI (magnetic resonance imaging) may be helpful, using either iron oxide or gadolinium tags. This approach would have the advantage that it is designed for 3-D analysis, but is limited by resolution and contrast.

7.4.6 *Scanning Electron Microscopy (SEM)*—The extent and uniformity of the cell seeding or cell ingrowth within a biomaterial can be analyzed by SEM or an environmental-SEM (E-SEM). For SEM, cells must be fixed and dehydrated (**6**). For E-SEM, hydrated samples can be imaged, resulting in a more natural cell morphology (**7**). With a cryo-SEM no sample fixation is needed. Samples are freeze-dried and transferred directly to the chamber for analysis. Typically, a section through the middle of the 3-D construct and the outer surface of the construct are analyzed.

7.4.7 *Some Concerns About Visual Observation of Cells Within Scaffolds:*

7.4.7.1 Uniform staining cannot be assumed and must be confirmed. Dye access to cells located deep within a scaffold will be limited by diffusion.

7.4.7.2 The scaffold may absorb the dye or have a background auto-fluorescence.

7.4.7.3 Quantifying cells within a scaffold or on thick sections of a scaffold is difficult because of the shallow depth of field of imaging devices and microscopes. Scaffolds do not always lie flat. Some cells will be in focus and others will be out of the focal plane. Confocal imaging can address this problem, but low-throughput may cause sampling issues. 3D reconstruction of serial sections using microscopy may also be helpful.

7.5 *Image Analysis:*

7.5.1 *General*—In addition to using image analysis software to quantify the amount of fluorescently labeled cells, there are more complex analyses that can be completed, such as determining cell density, cell spreading, and spatial uniformity of live and dead cells.

7.5.2 *Cell Density*—The cells must be stained prior to analysis. Typically a stain for cell nuclei, such as 4',6-diamidino-2-phenylindole (DAPI), is utilized so that the location of cells can be identified. Multiple color images at successive depths along the cross-sections of the constructs are taken (**8**). However, not all scaffolds can be sectioned. Some may be brittle and crumble during sectioning, while others are too elastic to be sectioned (they crush under a blade). Cell packing could affect accuracy of counts. If cells clump or form aggregates upon, or within the scaffold, cell number estimates may be inaccurate. This could be an issue for pancreatic islets or neurospheres, for example, where one cell clump may contain thousands of cells.

7.5.3 *Cell Spreading*—The cells must be stained prior to analysis (for example, Texas Red-$C_2$-maleimide to stain whole cells and 4',6-diamidino-2-phenylindole (DAPI) as a nuclear counter stain) (**9, 10**, Guide F2998). Cell density and cell spreading can be quantified within a given area using measures of object area, roundness, aspect ratio, and perimeter (**10**). Although these stains work well with cells on 2-D surfaces, stain retention in a 3-D scaffold may increase background and reduce cell contrast. Blocking (with serum albumin, for example) or washing may be required to reduce background staining.

7.5.4 *Spatial Uniformity Determinations*—In addition to determining cell density, a statistical measure of spatial uniformity of the cells within a particular scaffold can be calculated (**11**). The method involves calculating the coefficient of variation determined from statistical analysis of cell location (geometrical parameters) determined by an image analysis program.

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