Designation: F2998 – 14

Guide for Using Fluorescence Microscopy to Quantify the Spread Area of Fixed Cells[1]

This standard is issued under the fixed designation F2998; 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 describes several measurement and technical issues involved in quantifying the spread area of fixed cells. Cell spreading and the distribution of cell spread areas of a population of cells are the result of a biological response that is dependent on intracellular signaling mechanisms and the characteristics of cell adhesion to a surface. Cell spread area is a morphological feature that can be responsive to alteration in the metabolic state or the state of stress of the cells. Changes in cell spread area can also indicate an alteration in the adhesion substrate that may be due to differences in manufacturing of the substrate material or be in response to extracellular matrix secretions. High quality measurement of cell spread area can serve as a useful metric for benchmarking and detecting changes cell behavior under experimental conditions.

1.2 The measurement described in this document is based on the use of fluorescence microscopy imaging of fixed cells and the use of image analysis algorithms to extract relevant data from the images. To produce robust cell spread area measurements, technical details involved in sample preparation, cell staining, microscopy imaging, image analysis and statistical analysis should be considered. Several of these issues are discussed within this document.

1.3 This standard is meant to serve as a guide for developing methods to reliably measure the area to which cells spread at a surface. This surface can be conventional tissue culture polystyrene or sophisticated engineered biomaterial surfaces. An example of a detailed procedure to measure the spreading area of cells on a tissue culture polystyrene surface is provided in the appendix section.

1.4 Cell morphology features such as cell spreading area and perimeter are generally reported in units of length. For example, spreading area per cell (that is, cell spread area) is likely reported in units of μm^2. A spatial calibration standard is required to convert between numbers of pixels in a CCD camera image to μm^2 as an SI unit.

1.5 *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.*

1.5.1 *Sodium azide is used as a anti-bacterial reagent in the slide mounting media. This preserves the integrity of the mounting media. The toxicity of this reagent (for example, MSDS) should be considered before use of this reagent in large scale slide mounting procedures.*

2. Referenced Documents

2.1 *ASTM Standards:*

E1488 Guide for Statistical Procedures to Use in Developing and Applying Test Methods

F2150 Guide for Characterization and Testing of Biomaterial Scaffolds Used in Tissue-Engineered Medical Products

3. Terminology

3.1 *Definition of Terms:*

3.1.1 *cell morphology*—the physical shape properties of a cell such as cell volume, cell spread area, and cell perimeter; and the non-unit measures of roundness and circularity.

3.1.2 *cell spread area*—the area that encompasses a 2-dimensional (2-D) projection of a 3-dimensional (3-D) adhered cell.

3.1.3 *cell object*—a single cell or two or more adhered cells adjacent to each other such that they cannot unambiguously be segmented from one another by cell edge detection techniques.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *segmentation*—the act of classifying pixels in an image as cell or non-cell areas and the grouping of adjacent cell pixels into a cell object.

4. Summary of Practice

4.1 The measurement of a cell morphology feature such as cell spread area in a population of cells in culture can be a quantitative characteristic of cell population behavior and cell population state. This document provides guidance on measuring the 2-D morphological property (that is, cell spread area) for a population of cells in culture on a material.

[1] This test method is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.46 on Cell Signaling.

Current edition approved Jan. 1, 2014. Published May 2014. DOI: 10.1520/F2998-14.

4.2 This measurement is typically achieved by seeding a population of cells at low density on a test surface and chemically stabilizing the cellular structure by fixing the cells at a particular point in time. The cells are then treated with two stains: one that discriminates the cell from non-cell background, and another that associates with the nucleus of the cell. The dual-stained cells are then imaged with a fluorescence microscope using a low magnification and a high numerical aperture (NA) objective (for example, 10× magnification and NA>0.25) that maximizes the number of cells in a single image. The magnification and NA should provide sufficient resolution to enable accurate counting of the numbers of pixels associated with a cell, and allow large numbers of cells to be imaged, thus providing robust population statistics. Images of both the nuclei and corresponding cellular areas are collected. These images are then processed and analyzed by image analysis software to achieve both segmentation of the cell objects from the non-cell background to allow quantification of cell object spread areas, and segmentation of nuclei for determination of number of nuclei in each cell object. These two metrics allow a value for spread area per cell to be generated.

4.3 The spread area per cell measurement can be generated for each cell or cluster of cells in an image. These data can be parameterized as average spread area per cell or as a probability distribution. Each of these reported values are characteristics of the cell population adhered to a defined substrate. Statistical methods applicable to average values or distribution comparisons can be used to identify statistically significant changes.

4.4 It is important to note that the cell spread area measurement described here can be influenced by a large number of experimental factors that can influence cell state and the adhesion substrate. These include variations in reagents used during culturing of cells (such as serum, substrate vendor, media) and differences in how the cells are handled during maintenance and storage. Significant robustness testing and sensitivity analysis of these factors on the cell spread area measurement will be required to develop this measurement into a test method for benchmarking a particular cell culture system.

5. Significance and Use

5.1 Under well-controlled conditions, the quantitative evaluation of morphological features of a cell population can be used to identify changes in cellular behavior or state. Cell morphology changes may be expected when, for example, there is a response to changes in cellular cytoskeleton organization (**1**), a response of cells to toxic compounds, changes in differentiation state, and changes in adhesion properties of cells to a substrate by either chemical or mechanical-induced extracellular matrix-based (ECM-based) signaling pathways (**2**, **3**). Typically, populations of cells exhibit a range of morphologies even when the cells are genetically identical and are in a homogeneous environment (**4**). This biological variation in cell response is due to both cell-cycle variations and stochasticity in the cellular reactions that control adhesion and spreading in cells. By using cell-by-cell, microscopy-based measurements and appropriate statistical sampling procedures, the distribution of cell morphologies such as cell spreading area per cell can be measured. This distribution is highly characteristic of the culture and conditions being examined.

5.2 It is important to note that the use of this technique for cells on or in a 3-D scaffold materials can complicate the interpretation of the data. The topographic transforms of the cells on a 3-D material may require full volumetric imaging and not just wide-field fluorescence imaging as described here.

5.3 *the following are several examples of how this measurement can be used in a laboratory:—*

5.3.1 *Quantify Cellular Response to a Biomaterial*—The measurement of cell spread area can be used to characterize the response of cells to biomaterials. For example, spreading of most cell types is extremely sensitive to the stiffness of the culture substrate (**5**), (**6**). It is important to note that cell response to an ECM may be dependent on the preparation of the matrix. For example, the same ECM proteins prepared in a fibrillar or non-fibrillar surface coating can result in different morphology response

5.3.2 *Quality Control Metric for General Cell Culture Conditions*—Cell spread area may be a useful metric for monitoring a change in cell culture conditions (that is, due to a serum component, pH, passage number, confluence, etc.). Cell morphology is often altered when cells are stressed and proceeding through cell-death related processes (that is, apoptosis).

5.3.3 *Quality Control Metric for Biomaterial Fabrication*—Cell spread area measurements may be useful for generating specifications for a biomaterial. These specifications may stipulate how a particular cell line responds to a fabricated biomaterial.

5.3.4 *Quality Control Metric for Cell Line Integrity and Morphology Benchmarking*—The morphology characteristic of a cell line grown under specified conditions should ideally be the same over time and in different laboratories. Thus, cell spread area measurements may be useful for validating that no significant changes in the cell cultures have occurred. This measurement provides a benchmark that is useful for establishing the current state of the cell culture and a metric that can be charted to increased confidence for within and between laboratory comparisons of cellular measurements (**7**).

6. Test Considerations

6.1 *Fluorophore Reagents:*

6.1.1 For high quality cell morphology measurements, it is important to collect images of both the cell spreading area and images of the cell nuclei in corresponding frames. This allows the calculation of the normalized metric, average spread area per cell. An excellent method for collecting these images is with two-color fluorescence microscopy imaging. Fluorescent stains are used to highlight specific features of the cell. The use of high quality cell stains will optimize the contrast differences between the background and the cell features being probed.

6.1.2 It is critical that the cell feature stains exhibit high signal-to-background ratios. For the case of cell edge detection it is important to consider that the measurements are extracted from cell images with image analysis procedures. Reliable cell

edge detection algorithms function best with cells that exhibit high-contrast edge staining around cell features (**8**). Several cell stains (see Table 1) can be used to provide contrast at the cell and nuclear edge. It is important to note that empirical evaluation of samples treated with several stains may be required to evaluate the best staining strategy that optimizes cellular and nuclear edge detection (**9**). This is most important for cell edge stains because nuclear stains such as 4',6-diamidino-2-phenylindole (DAPI) and Hoechst 33342 typically perform very well.

6.1.3 It is important to consider the spectral properties of the stains that are used to identify the edge features and the nuclear features of a cell. To retain independence between the cell object area measurement and the nuclei count measurement, it can be useful to minimize bleed-through fluorescence (often termed crosstalk) between the nuclear stain and the cell object stain. Bleed-through is minimized by choosing fluorophore probes that have significantly different excitation and emission properties. For example, if a nucleus was stained with Hoechst 33342 (exciting wavelength = 350 nm, emission wavelength = 461 nm) and the cell edge was stained with a Texas Red based fluorophore (exciting wavelength = 590 nm, emission wavelength = 620 nm), it is unlikely that the emission from the Hoechst 33342 fluorophore would interfere with the respective Texas Red fluorophore-based images. The appropriate control for bleed-through is to evaluate a sample stained with only one fluorophore in each filter channel at the appropriate integration time, binning, and gain settings. Morphology measurements from cell edge and nuclear staining are often not highly sensitive to fluorescence bleed-through but the issue should be considered when identifying a pair of staining reagents.

6.1.4 In general, the staining reagents should not directly interact with the material on which the cells are adhered. If use of the staining reagent with a test material results in a high-level fluorescent background, it is possible that contrast at the cell edge will not be sufficient for edge detection with image analysis techniques. Control experiments that evaluate the level of fluorescent background on materials after staining can aid in selecting the most suitable staining reagents for these measurements.

6.1.5 It is desirable that the fluorophore labels are not sensitive to cell fixing and handling conditions, and that the stain does not exit the cell after extended periods of time. This criteria significantly improves the robustness of the morphology measurement as cell can be prepared and stained at one point in time and analyzed in another point in time.

6.1.6 It is desirable that the fluorophore labels are relatively photostable so that a sample can be reimaged with little change in the microscope settings. This property improves the reliability of the image analysis procedures that are used to extract data from the images.

6.2 *Cell Sample and Substrate Preparation*—Test cells are typically removed from a maintenance flask by trypsinization or other de-adhesion procedures and then seeded on the test surface. The cells are allowed to adhere to the test surface for a controlled number of hours before the cells are fixed, stained, and imaged.

6.2.1 Care must be taken to ensure that a suspension of single cells (and not clumps of cells) is achieved during de-adhesion/harvesting to increase the probability that cells seeded on the test substrate at low density will be isolated from other cells. This condition will provide the most accurate single cell morphology measurements. If large numbers of cell clusters are observed on the substrate, the ability to measure the distribution of cell spread areas may be compromised, but it should still be possible to measure the average spread area per cell

6.2.2 The cell density at several locations on the sample surface should be examined by phase or other microscopy modes to ensure that homogeneous cell adhesion over the substrate was achieved during seeding. If this is not the case, it is possible that the measurements described in this document

TABLE 1 Commercially Available Fluorophores that are Useful for Cell Morphology Measurements[A]

Fluorophore reagent Type / Names	Probe Features	Target	References
Fluorescent lipids and detergents (such as DiI, FITC-DMPE, Texas Red-DMPE)	Integrate into a lipophilic structure; spontaneous insertion into cellular membranes	Phospholipid bilayer; lipid structures in cells	(**9**)
Fluorescent maleimides (such as Fluorescein-C2-maleimide, Cy5-maleimide CPM, dylight-maleimide)	Very reactive to free thiols under physiological pH; labels cytoskeleton and edges of cells; several fluorescent derivatives of maleimides are available	Free thiol-containing proteins and smaller molecules	(**1**), (**4**), (**9**)
Fluorescent succimidyl esters (Fluorescein-NHS ester, ROX-NHS ester, etc.)	Can become inactive in physiological buffer at neutral pH	Free amine groups on intracellular proteins and other molecules	(**9**)
Nuclear fluorophores (such as DAPI, Hoechst 33342, DRAQ5)	For fixed and live cells; only fluorescent when complexed to DNA	Nuclei	(**10**), (**11**)
Affinity-based fluorescent reagents FITC-phalloidin, Texas Red-lectins) and other commercial staining reagents (cell tracker, cell mask)	For specific ligand targeting (for example, polymerized actin, cell membrane polysaccharides) and other cellular components	*f*-actin, cell surface glycoproteins; other proteins and cellular components	(**12**)

[A]The information in this table is a compilation of several staining systems that have been used to highlight cell edge and nuclear features. The table is not meant to specify the quality and performance of a particular stain.