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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.

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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.

may be biased by the selected area of a test substrate that is being examined during imaging.

6.2.3 It is important to consider the time-dependent morphology changes that occur after the cells are seeded on the sample surface. At early time points, the cells are likely round and will begin to spread on the surface. At later time points, the cells will be fully spread and depending on the cell type, the cells will begin growth and division processes. It is critical to establish a test incubation time that provides a stable measurement end point. For some cell lines such as NIH-3T3 and A10 cells (see Table 2), 17 to 24 h provide reproducible measurements (**3**, **3**).

6.2.4 During the test incubation times, it is possible that cells will divide. Cell clusters will form (single cell object with multiple nuclei) depending on if newly divided cells migrate away from each other. The procedures discussed here can detect cluster formation in the cell population (**7**).

6.2.5 Table 2 provides recommendations of seeding densities for several cell lines representative of primary cells and cell lines that are used in the laboratory.

6.2.6 If the test substrate has the tendency to adsorb significant quantities of fluorescent reagent (that is, a collagen gel or other hydrogels), the background staining intensity levels will be high and may reduce the sensitivity of the cell edge detection procedures. Robust edge detection with image analysis software requires high contrast staining at the cell edge (**8**). This is determined by differential staining between the cells and the underlying substrate. Solid surfaces often provide the highest contrast due to the ease of rinsing the stain from the substrate surface. Thicker gels may require extensive washing to reduce the background staining levels to values that are appropriate for image segmentation (**13**).

6.3 *Fixation and Staining:*

6.3.1 The measurements described here generally require that the cells are fixed before staining. The effect of the fixing procedure on the shape and integrity of the cells should be examined with phase or other modes of microscopy before the fixing procedure is used for a morphology measurement. For example, in some cases, rinsing cells with buffer before the addition of a fixative may dislodge or partially destroy the cells adhered to a surface. Solvent and other highly-reactive cross-linkers may affect morphology. Fixation by 1 % to 4 % formaldehyde in phosphate buffered saline or microtubule stabilizing buffer (**4**)is often acceptable. Permeabilization, which may be required for staining can often be achieved with low concentrations (~0.1 %) Triton X-100 without a significant loss of morphology properties (**1**)

6.3.2 After the cells are fixed, they are often immediately treated with nuclear and cell lipid or protein stains. It is often useful to assess staining protocols on trial cell experiments to ensure that accurate cell edge and nuclei discrimination can be achieved by image analysis methods. The staining conditions are sufficient if appropriate and repeatable edge detection with image analysis software can be achieved. If cell edge staining does not have sufficient intensity, increased incubation times or increased stain concentration in the protocol may be useful.

6.3.3 Stained samples are then often mounted on microscopy slides with the use of mounting media. It is critical to ensure the mounting media contain both anti-bacterial reagents (for example, sodium azide) that prevent sample contamination and anti-fade reagents that can reduce photobleaching of the fluorescence sample.

6.4 *Microscope Preparation and Imaging:*

6.4.1 Samples are typically imaged on a fluorescence microscope fitted at with a CCD camera and filter cubes for fluorophore visualization and a low magnification objective (for example, 10×) with an appropriate NA (for example, NA > 0.25). For best statistical results, stained cells should be imaged with an automated fluorescence microscope. These microscopes are fitted with a motorized *x-y-z* stage, automated focus software, motorized filter wheels, CCD camera, and a computer that is capable of controlling stage movement and image collection (**2**). The automated microscope facilitates the unbiased imaging of a large number cells on the substrate, which can assure the adequate sampling of the cell morphology data extracted from cell images. If manual image collection is used to assemble cell images, care should be taken to minimize bias during selection of fields to image. Resampling procedures that evaluate measurements from a large populations of cells can be used to determine the minimum number of cells that should be measured on a substrate.

6.4.2 A critical issue for collecting cell images with fluorescent microscopy is the need for flat-field correction. Most microscopes are illuminated by arc or LED lamps. Although many methods such as diffusers, liquid light guides, and defocusing are used to diffuse the excitation lamp homogeneously over the sample illumination area, it is likely that the illumination intensity over the collection field visualized by the CCD camera is not constant. A routine artifact is that the center of the collection field is brighter than the edges of the field. This results in higher cell edge contrast in the center of the field (i.e. higher cell pixel intensities) than the edge of the field. This effect can significantly affect the image analysis method that is used to extract cell spreading area measurements from cell images. Flat-field correction should be used to improve the reliability of the image analysis techniques (**14**), (**15**).

6.5 *Image Analysis:*

6.5.1 Once the images are collected, they must be analyzed with image analysis tools. Several open-source and commercial packages are available for this task. A very powerful package is the ImageJ, which is an open-source image analysis platform written in JAVA available from the NIH at http://

TABLE 2 Suggested Seeding Density for Various Sized Cells

Cell Type	Average Diameter, (µm)	Seeding Density, cells/cm^2
NIH 3T3[A]	30-50	1100
A10[B]	50-100	800-1000
hMSC[C]	40-60	500-800[E]
UMR106[D]	20	1500

[A]Mouse fibroblast.
[B]Rat aortic smooth muscle.
[C]Human marrow derived stem cells (#220R, Tulane Center for Gene Therapy).
[D]Rat osteosarcoma.
[E]Depending on passage.

rsb.info.nih.gov/ij/. The main requirement of the image analysis software is that it must be able to open the images collected with the microscope. The basic procedure to analyze the images is as follows: *(1)* use an image analysis technique that best identifies the edge of the cell in the images of the stained cells; *(2)* use an image analysis technique that best identifies the edge of the nucleus in the images of the stained cells; *(3)* use mask overlay techniques to identify the number of nuclei in each detected cell object; and *(4)* quantify features in the image such as cell object area and the number of nuclei per cell object. A sample macro that can be used with ImageJ software is described in X2.1.6.1. Cell area measurements extracted from image are highly dependent on the edge detection algorithm used during image analysis. Manual threshold-selection and histogram-based algorithms such as *K*-means (n = 4 or 5) can provide good segmentation of the cell objects (**8**).

6.5.2 To convert the pixel dimensions of the cell objects into physical spatial dimensions, a horizontal and vertical conversion factor shall be measured for the CCD camera on the microscope system. A standardized microscope calibration rule traceable to a NIST or similar length measurement standard can be purchased at several commercial optical suppliers and used for this purpose.

6.6 *Statistics and Analysis:*

6.6.1 There are two main metrics that can be reported from this type of measurement. The first is average cell-spread-area per cell, which is derived by dividing the total cell spread area measured in all the images by the number of nuclei measured. This metric is often recorded if there are large fraction of cell clusters identified in the cell population. This may occur with certain cell types. A second metric that can be reported is the distribution of cell spread areas for individual cells in the population. Cell spreading area data can be plotted as a histogram or as a cumulative distribution (**16**). The histogram can be generated using Microsoft Excel or similar spread sheet functions. An example of a histogram representing the distribution of spread areas for NIH 3T3 fibroblast cells on a tissue culture polystyrene surface is shown in Fig. X1.1.

6.6.2 It is important to consider how many images are required to ensure an adequate number of cells are being sampled for statistical robustness in the spreading area measurement. Techniques to evaluate the error due to undersampling (for example, resampling statistics) can be useful for addressing this issue.

6.6.3 Statistical testing between the summary statistics, such as the mean of the cell-spread area per cell, can be achieved with standard means testing (that is, student's *t*-test). The use of replicate data sets is required to provide information on the uncertainty in this measurement. More sophisticated statistical analysis procedures that take advantage of the shape of the distribution of individual cell spread areas can also be considered (**16**).

7. Future Considerations

7.1 Cellular morphology can be affected by a large number of signaling pathways with in a cell. If changes in cellular morphology measurements are not detected during an experiment, it does not indicate that intra-cellular signaling pathways are not changing. For example, if cell cycle is halted, it does not necessarily indicate that cellular morphology will change immediately. There are several classes of cell morphologies on a substrate (such as elongation, rounding, leading edge formation, etc.). Future studies to correlate activation of known pathways and their effect on cell morphology over time would be useful to aid in interpreting the cause of a morphology change.

7.2 To convert the measurements described in this guide to a test method, significant robustness testing is required to identify the experimental factors that must be specified for reducing variability in the cell spread area measurement. Furthermore, interlaboratory comparisons (for example, round-robin testing) using common cell lines and culture conditions would be useful for establishing other experimental parameters that should be controlled or measured before a standard test method can be developed.

8. Keywords

8.1 automated microscopy; cell morphology; cell cultures; segmentation; image analysis; fluorescence microscopy; cell staining; biomaterials; cytotoxicity; tissue engineering; cell therapy; stem cells; differentiation; quality control

APPENDIXES

X1. A DETAILED PROCEDURE FOR GENERATING A CELL SPREAD AREA MEASUREMENT

X1.1 Scope

X1.1.1 This is a detailed procedure for determining the spread area of fixed cells with fluorescence microscopy. This example provides the details of sample preparation, cell labeling, imaging, and analysis experiment with a specific combination of fluorophores for whole cell and nuclear labeling. These fluorophores have been used for measurements in several publications (**1-8**). This detailed procedure can be used to guide the development of cell spread area measurement in individual laboratories.

X1.1.2 This experiment describes a cell spread area measurement of A10 rat smooth muscle cells on a tissue culture polystyrene substrate.

X1.2 Hazards

X1.2.1 Reagent hazards are stated in the product MSDS. Sodium azide (NaN_3), which is used as an anti-bacterial reagent in the mounting media, is included in this procedure. This compound is highly toxic. No additional hazards have been identified during the procedure steps.

X1.3 Procedure

X1.3.1 *Cell Seeding*—A10 cells are removed from tissue culture polystyrene flasks by trypsinization, rinsed with medium, and plated in medium onto tissue culture polystyrene substrates at a cell density as described in Table 2.

X1.3.2 *Cell Fixation*—After incubation for 24 h, cells on the test substrates are washed with warm PBS, fixed in 1% (v/v) formaldehyde in DPBS (30 min) at room temperature, quenched in 0.25% (m/v) NH_4Cl in DPBS (15 min), and rinsed with DPBS containing 0.02% (m/v) sodium azide ($DPBS/N_3$). Cells can be stored at 4°C for at least a week under these conditions.

X1.3.3 *Cell Staining*—After cells are fixed and rinsed, the supernatant is removed and the cells are permeabilized and stained (2 h) with a fresh solution of 2 µg/mL Texas Red-C2-maleimide and 1.5 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) dissolved in 0.1% (v/v) Tx-100 in DPBS. The solution should completely cover the cells and the samples can be placed on a rocking table. Longer incubation times can be used, but the incubation time should be constant for all samples in a single experiment to minimize variations in the imaging settings for each sample. The stained cells are rinsed once with $DPBS/N_3$, 3% (w/v) bovine serum albumin in $DPBS/N_3$ for 10 min and $DPBS/N_3$ again. The stained cells can be stored for several days at 4ºC under these conditions.

X1.3.4 *Sample Mounting*—Fixed and stained cell samples can be mounted on a glass slide with traditional mounting medium or kept in a petri dish containing $DPBS/N_3$. The use of anti-microbial reagents such as $DPBS/N_3$ is critical for ensuring that sample damage does not occur during prolonged storage. Additionally, a mounting media with anti-fade properties that reduce photobleaching should be used during sample mounting.

X1.3.5 *Microscopy/Automated Microscopy*—To adequately sample the substrate for cell morphology measurements, it is important that significant numbers of cell images are collected. First, a grid of non-overlapping locations on the substrate is generated using an automated microscope. The microscope autofocuses on the more photostable TxRed whole cell stain and then collects an image of the cells and the nuclei with a low magnification objective (10× magnification, NA = 0.25). The microscope cycles through the grid of locations for the cell response image collection. Exposure time used for the CCD camera image collection should be sufficient to generate a high level of edge contrast at the cell and nuclear edge. This will need to be determined by ensuring image analysis routines can adequately identify the cell edges.

X1.3.6 *Image Analysis*—Cell segmentation routines are used to identify cell and non-cell pixels. For the TxRed and DAPI staining described in this example, simple manual thresholding or *K*-means (k = 5) can provide good segmentation of the cells and nuclei (**7**). An example of a macro written for ImageJ open source image analysis software to analyze the TxRed and DAPI stained TxRed cells is shown in X2.1.6.1. This procedure first identifies all cell objects in the TxRed stained images and all nuclei in the DAPI stained images. The macro then overlays the whole cell mask over the nuclei mask and the nuclei within each cell object are counted. The macro then reports cell spread area for each object and the number of nuclei for each cell in the image. The report table is then cut and pasted into a spread sheet program for further analysis.

X1.3.7 *Quantitative Comparisons*—Conventional statistical testing (that is, students *t*-test) can be used to evaluate changes in summary statistics such as the average cell spreading area per cell (**4**). Distribution comparison statistics should be used to compare cell area distributions (**16**). Replicate measurements are used to quantify the uncertainty in the cell spread area measurement.

X1.4 Potential Sources of Variability

X1.4.1 *Image Analysis*—Studies indicate that automated classification algorithms (such as *K*-means) can provide excellent segmentation of morphology images with high contrast

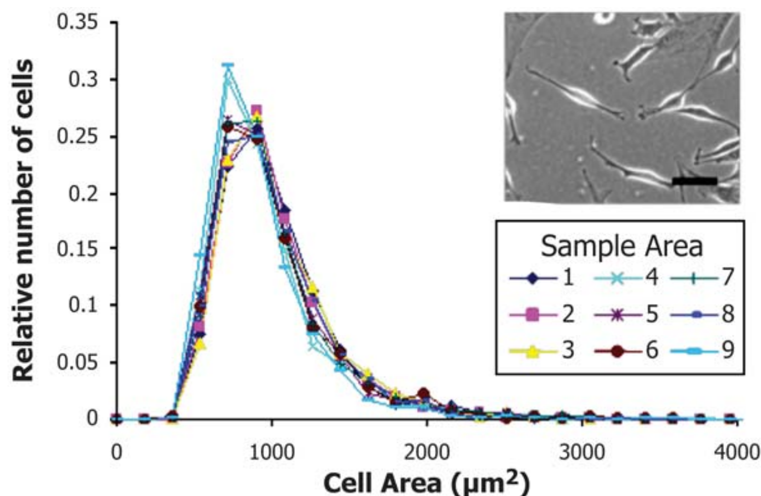

FIG. X1.1 Example of Histogram Data Generated from Morphology (Projected Cell Area) Analysis of NIH 3T3 Fibroblasts Grown on TCPS Under Normal Conditions

cell edge staining. Other pixel classification techniques, such as manual thresholding, can also provide excellent segmentation with high quality images. It is important to visually evaluate (for example, expert opinion) the segmentation procedure to ensure that adequate cell and non-cell pixels are identified (**8**).

X1.4.2 *Cell Sampling*—Due to the broad distributions of cell spread areas that are often measured, it is important that an adequate number of cells are sampled to minimize measurement error due to undersampling. In some cases, it is possible to use resampling procedures to determine a minimum number of cells to measure. For example, there is little variability in the mean and variance of the individual histograms referenced in X2.2 and Fig. X1.1 if more than 200 cells are used during the analysis.

X1.5 Report

X1.5.1 Data can be reported as average values with uncertainty measurements or histogram-based distributions with uncertainty values on each histogram bin. Alternatively, the distribution data can be presented as a cumulative distribution as described in Reference (**16**).

X2. DETAILED PROCEDURE FOR GENERATING A CELL SPREAD AREA MEASUREMENT USING IMAGE ANALYSIS SOFTWARE

X2.1 Scope

X2.1.1 The following is a procedure for analyzing the images described above with ImageJ analysis software. Similar procedures can be performed in many image analysis platforms. Therefore this procedure provides the general approach to the image analysis.

X2.1.2 The corresponding TxRed (whole cell) and the DAPI (nuclear) images should be placed in different directories. They should also be indexed so the ordering of the TxRed images correspond to the order of the DAPI images. For a small number of image files, this can be achieved by renaming the files. For example, TxR.tiff.001, TxR.tiff.002, etc. and DAPI.tiff.001, DAPI.tiff.002, etc. For a large number of files, a batch renaming program can be used. Alternatively, the images can often be stored with indices during image collection depending on the software settings.

X2.1.3 In ImageJ, open the TxRed images with *"File->Import->Image Sequence"*. Browse to the directory holding the TxRed images and double click on one of the image files. ImageJ should open a stack of image files (see Fig. X2.1). To segment the cells, use *Image->Adjust->Threshold*. Manually select a threshold value that highlights the edges of the cells. This step is critical for reproducible results. The high contrast staining of the cell edge reduces the ambiguity associated with threshold selection, but care is required to select a threshold that adequately identifies the cell edge. Scan through the images to ensure a suitable threshold value is chosen for all cells. Once the threshold value is chosen, press *Apply* in the threshold dialog box and answer*Yes* when asked to process the whole stack. The results should be black and white images of the cells (see Fig. X2.2).

X2.1.4 The black and white whole cell mask images are used to generate a list of cell objects in the ROI (region of interest) manager. In ImageJ, click *Analyze->Analyze Particles* to open the particle analyzer. Check *Add to manager* and *Exclude edges* in the dialog box. The latter selection prevents any cells on the edge of the frame from being included in the analysis. In the size box, type *100-5000*. This limits the size of the cell objects to those that are in the range of real cells. Very small object and very large objects will not be considered in the analysis. Click *OK* to start the analysis and a ROI manager should open with a region of interest for each cell object. These ROIs will be used with the DAPI images for nuclei counting in the next step. Close the Tx-Red image stack.

X2.1.5 In ImageJ, open the DAPI images with *File->Import->Image Sequence*. Browse to the directory holding the DAPI images and double click on one of the image files. ImageJ should open a stack of image files (see Fig. X2.1*(b)*). To segment the nuclei, use *Image->Adjust->Threshold*. Manually select a threshold value that highlights the edges of the nuclei. The threshold value should be set to detect each of the nuclei, but it does not need to precisely identify the edge of the nuclei. This consideration reduces the stringency of the threshold value that is used to identify the nuclei. Scan through the

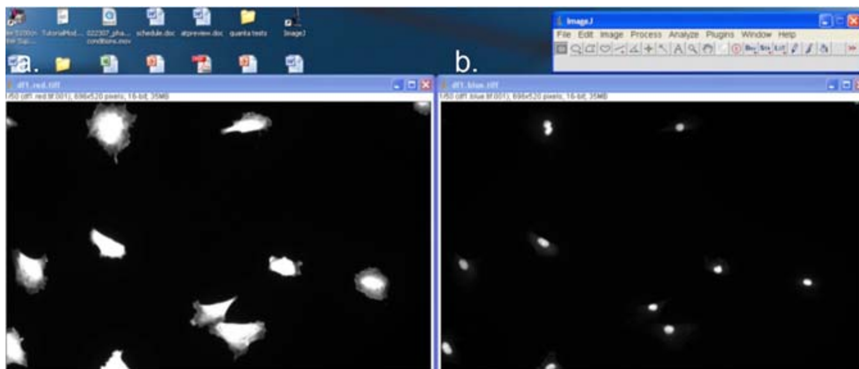

FIG. X2.1 TxRed *(a)* and DAPI *(b)* Stained A10 Smooth Muscle Cells

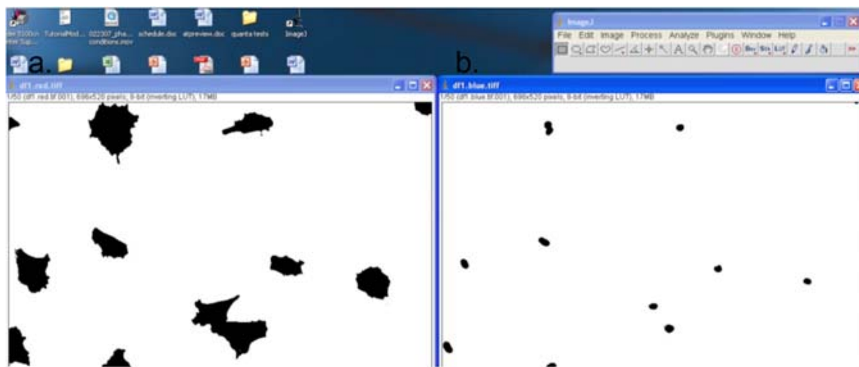

FIG. X2.2 Cell bodies *(a)* and Nuclei *(b)* After Manual Thresholding in ImageJ

images to ensure a suitable threshold value is chosen for all cells in the image stack. In some cell types, nuclear intensity varies significantly. It is important to identify a threshold value that detects most nuclei. Once the threshold value is chosen, press *Apply* in the threshold dialog box and answer *Yes* when asked to process the whole stack. The results should be black and white images of the nuclei (see Fig. X2.2*(b)*).

X2.1.6 A macro for image analysis is shown in X2.1.6.1. Copy the text into notepad.exe or similar software and save it as a text file in a known location. To run the macro in ImageJ, click *Plugins->Macro->Run*. Select the macro text file and the nuclei counting should begin. A log dialog box indicating that the macro has started should appear. Yellow outlines of cell objects should appear on the DAPI images during the analysis. On a fast computer, these yellow outlines may not be visible. When the analysis has completed, a table should appear that contains the results of the nuclei counting.

X2.1.6.1 *Image Analysis Macro for Imagej Software:*

```
//Macro was written by JTE and MH for ASTM Cell Morphology Document
//Nov 7, 2007
//A stack of TxRed labeled images are opened and thresholded to black and white mask images.
//Particle analyzer (exclude edges) is ran on the TxRed labeled images and the the regions of interest are stored in the ROI manager.
//The TxRed images are closed and the corresponding of DAPI images are opened and thresholded to black and white mask images.
//This macro is then executed.
//This macro steps through the ROI manager and counts the number of DAPI objects in each cell ROI.
//The macro then writes the area of the TxRed Object and the number of DAPI stained nuclei to the results table
var nucminSize =20; // the minimum size of a nucleus object
nold=0;
num = roiManager("count"); //number of cell objects detected
areacell = newArray(num); //arrays for measured data
frame = newArray(num);
nuc = newArray(num);
print ("Analyzing "+num+" regions of interest from TxRed stained images"); //note indicating function
for (i=0; i<num; i++) // loop through ROIs
{
roiManager("select", i);
s=getSliceNumber(); //get frame information
getStatistics(area); //get area of cell
run("Analyze Particles...", "size=20-Infinity circularity=0.00-1.00 show=Nothing exclude slice"); //count nuclei
no=nResults; //cumulating number of nuclei
frame[i]=s; //save frame area
areacell[i]=area; //save cell area
nuc[i]=no-nold; //save number of nuclei/cell
nold=no;
}
//print results table
run("Clear Results");
for (i=0; i<num; i++)
{
setResult("Frame",i,frame[i]); //print results to table
setResult("Area",i,areacell[i]);
setResult("# Nuc",i,nuc[i]);
}
updateResults(); //show results
selectWindow("Results"); //bring results to front - this data should be pasted into a spread sheet program
```

X2.1.7 The results table that is generated has three columns labeled as follows: *Frame #*, *Area*, and *# Nuc*. *Frame #* indicates which frame the cell object was located. *Area* is the area of the TxRed labeled cell object (whole cell area). *# Nuc* is the number of nuclei detected in the cell object. This table can be directly cut-and-pasted into a spreadsheet program such as Microsoft Excel. The cell spread area calculations can be performed with this data.

X2.2 Fig. X1.1 is an example of histogram data generated from morphology (projected cell area) analysis of NIH 3T3 fibroblasts grown on TCPS under normal conditions. The histograms represent nine different areas of a 10 cm polystyrene dish. One hundred fields in each area (approximately 1000 cells) were imaged using an automated microscope. The scale bar represents 30 µm.

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