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Standard Guide for Assessing the Attachment of Cells to Biomaterial Surfaces by Physical Methods[1]

This standard is issued under the fixed designation F2664; 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 protocols that can be used to measure the strength of the adhesive bond that develops between a cell and a surface as well as the force required to detach cells that have adhered to a substrate. Controlling the interactions of mammalian cells with surfaces is fundamental to the development of safe and effective medical products. This guide does not cover methods for characterizing surfaces. The information generated by these methods can be used to obtain quantitative measures of the susceptibility of surfaces to cell attachment as well as measures of the adhesion of cells to a surface. This guide also highlights the importance of cell culture history and influences of cell type.

1.2 *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]

D4410 Terminology for Fluvial Sediment

F22 Test Method for Hydrophobic Surface Films by the Water-Break Test

F2312 Terminology Relating to Tissue Engineered Medical Products

F2603 Guide for Interpreting Images of Polymeric Tissue Scaffolds

2.2 *ISO Standards:*[3]

ISO 4287 Geometrical Product Specifications (GPS)—Surface Texture: Profile Method—Terms, Definitions and Surface Texture Parameters

ISO 13565-1 Geometrical Product Specifications (GPS)—Surface Texture: Profile Method; Surfaces Having Stratified Functional Properties—Part 1: Filtering and General Measurement Conditions

3. Terminology

3.1 *Definitions:*

3.1.1 *adhesion, n*—a physiochemical state by which a cell is coupled to a non-cell surface by interfacial forces, which may consist of covalent or ionic forces.

3.1.2 *biocompatibility, n*—a material may be considered biocompatible if the materials perform with an appropriate host response in a specific application. **F2312**

3.1.3 *biomarker, n*—biochemical feature or facet that can be used to measure the progress of disease or the effects of treatment.

3.1.4 *biomaterial, n*—any substance (other than a drug), synthetic or natural, that can be used as a system or part of a system that treats, augments, or replaces any tissue, organ, or function of the body. **F2312**

3.1.5 *detachment, n*—process whereby an adhered cell or group of cells is actively detached from a surface.

3.1.6 *hydrophilic, adj*—having a strong affinity for water, wettable. **F22**

3.1.7 *implant, n—in medicine*, an object, structure, or device intended to reside within the body for diagnostic, prosthetic, or other therapeutic purposes.

3.1.8 *laminar flow, n*—well-ordered, patterned flow of fluid layers assumed to slide over one another. (See Ref (**1**).)[4]

3.1.9 *lay, n*—direction of the predominant surface pattern. **ISO 13565-1**

3.1.10 *passage, n*—the transfer or transplantation of cells, with or without dilution, from one culture vessel to another. It is understood that any time cells are transferred from one vessel to another, a certain portion of the cells may be lost and,

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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.

[3] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

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

therefore, dilution of cells, whether deliberate or not, may occur. This term is synonymous with the term *subculture*. (See Ref (2).)

3.1.11 *passage number, n*—the number of times the cells in the culture have been subcultured or passaged. In descriptions of this process, the ratio or dilution of the cells should be stated so that the relative cultural age can be ascertained. (See Ref (2).)

3.1.12 *Reynolds number, n*—a dimensionless number expressing the ratio of inertia forces to viscous forces in a moving fluid. The number is given by *VLr/m* where *V*, is the fluid's velocity, *L* is a characteristic length or distance such as pipe diameter, *r* is the fluid's mass density, and *m* is the fluid's dynamic viscosity. **D4410**

3.1.13 *scaffold, n*—a support, delivery vehicle, or matrix for facilitating the migration, binding, or transport of cells or bioactive molecules used to replace, repair, or regenerate tissues. **F2312**

3.1.14 *senescence, n—in vertebrate cell cultures*, the property attributable to finite cell cultures; namely, their inability to grow beyond a finite number of population doublings. Neither invertebrate nor plant cell cultures exhibit this property. This term is synonymous with *in vitro senescence*. (See Ref (2).)

3.1.15 *shear stress, n*—components of stress that act parallel to the plane of the surface. (See Ref (3).)

3.1.16 *surface profile, n*—the surface profile formed by the intersection of a real surface by a specified plane. It is customary to select a plane that lies perpendicular to the direction of lay unless otherwise indicated.

ISO 13565-1 and **ISO 4287**

3.1.17 *tack, n*—ability of an adhesive to form a bond to a surface after brief contact under light pressure.

4. Significance and Use

4.1 Cell attachment or, lack of it, to biomaterials is a critical factor affecting the performance of a device or implant. Cell attachment is a complicated, time-dependent, process involving significant morphological changes of the cell and deposition of a bed of extracellular matrix. Details of the adhesive bond that is formed have been reviewed by, for example, Pierres et al (2002) (4), Lukas and Dvorak (2004) (5), and Garcia and Gallant (2003) (6). The strength of this coupling can be determined either by monitoring the force of attachment between a cell and a substrate over time or by measuring the force required to detach the cell once it has adhered.

4.2 Cell adhesion to a surface depends on a range of biological and physical factors that include the culture history, the age of the cell, the cell type, and both the chemistry and morphology of the underlying surface and time. These elements that need to be considered in developing a test protocol.

4.3 Devising robust methods for measuring the propensity of cells to attach to different substrates is further complicated since either cell *adhesion* or *detachment* can be assessed. These processes that are not always similar or complementary.

4.4 Most studies of cell attachment focus on obtaining some measure of the time-dependent force required to detach, or de-adhere, cells that have already adhered to a surface (James et al, 2005) (7). More recently investigators have begun to measure the adhesive forces that develop between cells and the underlying surface during attachment (Lukas and Dvorak, 2004) (5). From a practical point of view, it is much easier to measure the force required to detach or de-adhere cells from a surface than to measure those that develop during attachment. However, in both cases, the experimental data should be interpreted with a degree of caution that depends on the intended use of the measurements. The methods of measuring cell adhesion described herein are measures of the force required to detach an adherent cell.

4.5 The purpose of this guide is to provide an overview of current generic test methods and identify the key factors that influence the assessment of cell adhesion and detachment. It is anticipated that this guide will form the basis for producing a series of standards that will describe these test methods in more detail.

5. Cell Attachment Assays

5.1 Table 1 provides examples of common cell adhesion assays, including a brief description of the forces applied. These assays are discussed in more detail in Section 6.

5.2 Cell attachment assays can be performed using single cells or a population of cells. Single cell techniques can provide quantitative measures of the adhesive force that develops with time between a cell and a substrate or that required to detach an adhered cell from a substrate. Individual ligand-surface interactions can be measured directly using, for example, a cell mounted on an atomic force microscope (AFM) tip. Single cell measurements do have their disadvantages. Variations in adhesive strength are not averaged out over a population and sophisticated equipment, such as an AFM, is required.

TABLE 1 Assays for Measuring Cell Detachment from Surfaces

Cell Requirements	Assay	Assay Description	Section
Single Cell	Micromanipulation	Measurement of the Force developed during attachment via an AFM	6.1.1-6.1.2
Single Cell	Micromanipulation	Forces applied via a micropipette, microprobe or AFM	6.1.3
Cell Population	Gravity	Detect the number of cells that remain attached after turning the culture vessel upside down	6.2.1
	Wash	Wash off adhered cells	6.2.2
	Centrifugation	Detachment of cells using centrifugal force	6.2.3
	Hydrodynamic Flow	Detachment of cells using shear forces generated by laminar flow over cells	6.2.4

5.3 Cell population based assays average out variations in cell-to-substrate adhesiveness compared with measurements performed on a single cell. This variation arises both because of variations in biomaterial surface properties, and variations in cell phenotype used as the probe (Appendix X1 and Appendix X2). Cell population techniques provide a usable measure of the biomaterial's adhesiveness for a given batch of cells and test conditions. Cell population techniques are attractive in that they provide robust measurements based on a large number of cells, which is an important consideration given the inherent variance of biological systems. Measurements that are based on large numbers of cells reduce the influences of local variations in surface chemistry and texture and in the adhesiveness of the cells themselves.

6. Measurement of Cell Detachment

NOTE 1—In principle, the strength of the adhesive bond that develops between the cell and underlying substrate will increase with time, although in practice this will depend on the cell-surface interactions. These measurements can be performed on either populations of cells or single cells. It should also be noted that it is not possible to conduct a series of measurements over time on the same cell, as these tests are destructive. Each test described below carries its own unique sources of statistical error. Users should familiarize themselves with the appropriate assay system and should consult with appropriate statistical staff to determine the necessary statistical parameters to ensure statistical significance. These parameters may include, but are not limited to: sample size, power of study, number of image fields counted (for microscope-based assays), number of cell lots tested, variability between users, what is the most appropriate statistical analysis (that is, analysis of variance, Tukeys test, *t*-test, etc.) and determination of a standard curve for analysis of detached cells.

6.1 *Micromanipulation:*

6.1.1 *Micromanipulation Methods (Single Cells)*—Single cells can be used to measure the force required to uncouple cells from the underlying substrate (measure of detachment), as a result of a time-dependent adhesion. Such measurements are made using micromanipulation or micropipettes. Cells can be seeded onto a small block of material mounted on an AFM tip, attached to a coated AFM tip or to the tip directly. The cell-coated tip can then be used to measure the tack force that develops over time.

6.1.2 There are some practical issues that need to be addressed when using this direct approach to force measurement:

6.1.2.1 Care should be taken to ensure that the measurements relate to a single cell and not to contributions from a number of cells. This is a particular issue when a block of material is mounted onto the tip.

6.1.2.2 Care should be taken to ensure that the measurement relates to the detachment force and is not a measure of cell membrane strength; this can be checked by examining the footprint left by the cell.

6.1.2.3 These measurements need to be made using a wet cell AFM. Problems have been reported with protein adsorption on the cantilever having an adverse effect on its reflectivity.

6.1.3 Micropipettes, microprobes, and AFM's have been used to measure the force required to suck or pull single cells away from the substrate to which they are attached (for example, Shao et al, 2004) (**8**). All these methods provide quantifiable sensitive and real time direct measures of the force required to detach the cell that is typically less than 10 mN (for example, Lee et al, 2004) (**9**). Control over the magnitude of the force and the rate at which it is applied can be used to explore the process of cell detachment in detail. Practical issues that need to be considered when using these methods include:

6.1.3.1 Specialized equipment, which must be calibrated to ensure that data are reproducible and repeatable, is required for such sensitive measurements.

6.1.3.2 Care should be taken to ensure that the measurement relates to detachment force and is not a measure of cell membrane strength, this can be checked by examining the footprint left by the cell.

6.1.3.3 Consideration should be given as to the direction of the applied force, that is, tensile, shear or some combination of the two and the magnitude of the applied stress. Larger area pipette tips will subject the cell to a lower stress than the tip of an AFM for a given applied force.

6.1.3.4 The period of time between exposing the cells to a surface and that at which measurements are made.

6.2 *Cell Detachment Measurements on Cell Populations:*

6.2.1 *Gravity*—Gravity can be used to differentiate between cells that are attached to a substrate and those that have not by turning the cell culture vessel upside down. Prior to using this approach, the user should consider the buoyancy of the cells with respect to medium to ensure that it is negative. Consideration should be given to the test duration to improve the consistency of repeat measurements.

6.2.2 *Wash Assays*—A simple, convenient, widely used assay that readily provides qualitative information on adhesion of cells to a substrate is to wash off non-adherent cells using culture medium. This approach may take many forms from mild shaking of the culture vessel to sluicing of the culture well. Clearly the simplicity, speed and low cost of these approaches are attractive, although lack of control of the applied force in terms of both its magnitude and the nature of the applied stress limits the sensitivity of the measurement, and hence reproducibility. For this reason comparisons between successive tests are subject to large unquantifiable uncertainties. Checks should also be made to ensure that the adherent surface is not removed or damaged during the assay.

6.2.2.1 This assay can be used to monitor cell attachment to a surface under different culture conditions, used as a measure of the biocompatibility or as a route to gauging how well cells are attached to a substrate. This approach is also a destructive method (that is, measurements should only be made using samples that have not been previously tested). This protocol will remove any contributions from residual extra-cellular matrix of fragments of cell membrane that may impact on the adhesiveness of the surface.

6.2.3 *Centrifugation*—A conventional centrifuge can be used to apply a normal or shear force to cells depending on the orientation of the cells with respect to the centrifugal force (for example, Heneweer et al, 2005) (**10**). The force that the cells are subject to can be calculated according to the following formula:

$$F = VdR\omega^2 \quad (1)$$

where:

F = centrifugal force,
V = cell volume,
d = difference in density between a cell and the surrounding medium,
R = centrifugation radius, and
ω = centrifugation speed.

6.2.3.1 Such tests are easy to conduct, do not require specialized equipment or training and the results represent a population average. Factors need to be considered when using this methodology: the test duration and the potential influence of forces applied during the period of spin up. The assay only correlates cell detachment with the maximum force applied after the centrifuge has reached its set spin speed.

6.2.4 *Hydrodynamic Flow Assays*—The basis of hydrodynamic test methods is to apply a known force to a population of cells by means of controlled movement of fluid. The assays rely on forces generated by fluid flow over adhered cells. There are several subtypes of hydrodynamic flow assays: *(1)* parallel plate flow chambers, *(2)* spinning disk chambers, and *(3)* radial flow chambers. The geometry of the flow cell and mode of operation influence both the magnitude of the applied force and its complexity, as discussed below.

6.2.4.1 The stresses that the cells are subjected to are complex and difficult to quantify. Typically cells will be subjected to a combination of shear stress and hydrodynamic drag leading to the development of torque. The geometry of the cell (that is, the amount of spreading and the presence of focal adhesions) will cause the actual stress that the cell experiences to be different from the calculated wall stress and therefore must be considered during any quantitative analysis.

6.2.4.2 *Parallel and Convergent Plate Flow Chambers*—Fig. 1 consists of parallel plates that are a known distance apart. Flow of fluid through the chamber is laminar, that is, the Reynolds number is less than 2300. In this configuration the cells are subjected to a wall shear stress, τ_w, that is, the shear stress at the wall-fluid interface according to the following equation:

$$\tau_w = \frac{6\mu Q}{wh^2} \tag{2}$$

where:

Q = flow rate of the fluid,
w = width (channel dimension),
h = height (channel dimension), and
μ = fluid viscosity.

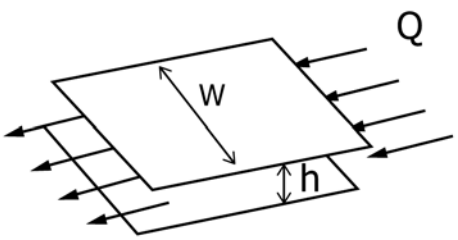

FIG. 1 A Simple Parallel-Sided Flow Cell Can Be Used to Apply a Known Shear Stress to a Bed of Adhered Cells

(1) This function applies to Newtonian fluids, of which water is an example and assumes no influences from edge effects. Care should be taken to ensure that these requirements are met for particular test geometries and culture media.

(2) The key element of this approach is to ensure that the fluid flow over the cells is laminar. The wall shear stress applied to the cells can be constant or variable, depending on the design of the flow cell. A controlled static shear stress gradient can easily be generated by converging either one or both pairs of parallel sides of the flow cell.

(3) The wall shear stress at a given point along the length of the cell is given by Eq 2.

6.2.4.3 *Spinning Disc*—The spinning disk arrangement shown in Fig. 2 can be used to subject the cells to a centripetal force and complex flow field that equates to a wall shear stress, the magnitude of which increases with increasing distance away from the pole according to:

$$\tau_w = 0.8r\sqrt{\rho\mu\omega^2} \tag{3}$$

where:

ω = rotational speed,
ρ = density of the culture medium,
r = radial position, and
μ = fluid viscosity.

6.2.4.4 *Radial Flow Cell*—The wall stress in the radial flow cell shown in Fig. 3 is given by:

$$\tau_w = \frac{3\mu Q}{\pi r h^2} \tag{4}$$

where:

Q = flow rate,
r = radial position,
h = gap between the plates, and
μ = viscosity of the fluid.

(1) This function is the same as that for the parallel plate cell shown in Eq 2. The highest wall shear stress in this configuration will be in the vicinity of the entrance port. A consequence of this will be that cells detached by the highest wall shear stress may influence detachment of cells in the lower wall stress zone. Unlike the parallel plate laminar flow chamber, cells tested in the radial flow chamber will be subject to a complex biaxial stress field.

6.3 *Additional Methods of Cell Detachment*—Tissue Engineered Medical Products (TEMPs), where, for example, cells may be attached to, or inside of, a three-dimensional scaffold, for example, may present unique requirements for cell detachment. Detachment resulting from physical forces, where a mechanical force or shear is the driving force, may result in cell injury or death. Likewise, if the investigator's interest is in studying the extracellular matrix proteins, mechanical forces may interfere with ligand-receptor studies (such as in the case

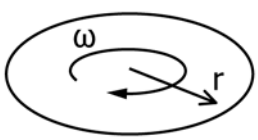

FIG. 2 A Schematic Representation of a Spinning Disk

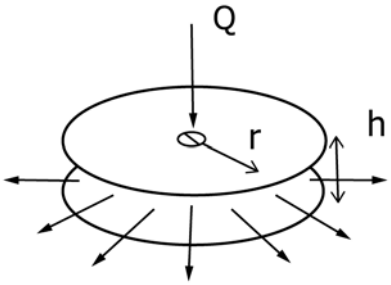

FIG. 3 A Schematic Representation of a Radial Flow Cell

where the mechanical force is applied to break or form bonds between fibronectin—integrin and tissue) and subsequently impact cell function. In such cases, the use of cell detachment methods which do not involve physical forces (that is, mechanical force is the driving force for detachment), should be applied. A non-exhaustive list of examples of these types of cell detachment assays include: *(1)* chemical chelation (that is, ethylenediaminetetraacetic acid, EDTA), *(2)* enzyme activity (that is, trypsin), and *(3)* temperature gradients (that is, use of ice to dislodge adherent cells or shift temperature to release cells from bioengineered surfaces). Users should familiarize themselves with each of these methods, including the caveats and the limitations, prior to selecting an appropriate method. The use of non-physical force to detach cells may be very important in the manufacture, testing and quality control of TEMPs.

6.4 *Calibration of Cell Detachment Assays*—In order to have an assay system that is not only reproducible in the user's laboratory, but reproducible across many laboratories, use of a reference cell line should be considered. This could be a cell line obtained from a cell repository, such as the American Type Culture Collection, or a similar type organization. This would ensure that the cell type would be available to all interested users. The user should determine the phenotype and reactivity of the cells under a known set of culture, harvest and flow conditions and for a defined range of passage numbers. The representative cell type should be consistent with the cell type to be employed in the TEMP construct, including, but not limited to, mesenchymal, epithelial, normal, transformed, human, murine, etc.). Once the user obtains data as to the reactivity of this reference cell type to a defined set of culture, harvest and assay conditions, the cells can be used to compare data on cell detachment between laboratories.

7. Additional Considerations for Cell Adhesion/ Detachment Assays

7.1 *Measurement Objective*—The major division of detachment assays is between those which measure the adhesion of a population of cells to a surface, with the measured parameter usually being the number of cells left adhering to the surface after some attempt at their removal (for example, Reyes C.D. and Garcia A. J., 2003) (**11**), and those which measure the adhesion of a single cell to a surface, with the measurement usually being of the force required to remove the cell from the surface (for example, Huang W et al 2003) (**12**). The approach selected depends upon the requirements of the investigator and methodologies seem to arise with individual laboratories. All of these assays require a measure of the number of cells that remain after the procedure has been applied. Physical, chemical, enzymatic or temperature-dependent mechanisms may be applied to remove cells remaining attached following a cell detachment assay. These cells can then be subject to further histomorphological, biochemical, mechanical, phenotypic, genetic, etc. testing to verify how closely the detached cells correspond to the original seeded cell type.

7.1.1 *Quantification of Attached Cells*—Light or phase microscopy enhanced by colorimetric means, such as fluorescence with image analysis has been used for counting remaining attached cells. Image analysis software can quantitate changes in cell shape and other relevant morphological parameters. Other methods include, radioactive-labeled cells and bioluminescent-based ATP assays. The user is referred to Appendix X1 for additional information on sources of variation resulting from the cells in cell detachment assays.

7.2 *Modes of Detachment*—The following is a partial list of modes of detachment that can occur. Cell tethering occurs when part of the cell membrane attaches to the biomaterial surface and under shearing conditions becomes pulled out into a long membranous process or tether. Peeling refers to the breaking of ligand-receptor bonds per unit time for the cell to peel completely from the surface. Peeling requires that the shear force on the cell be maintained over a sufficient time period. Other modes of detachment involve removal by shear, whereas a number of tests of adhesion (that is, tests such as centrifugation based tests), rely on normal force being applied to the cell, with a pattern of ligand rupture being quite different from shear removal.

7.2.1 *Sources of Artifact or Error for Cell Detachment Assays*—Cell detachment assays may result in failure (rupture) of the plasma membrane prior to cell detachment. For a particular cell type, it is important to determine (either from the open literature or through controlled experiments) the force at which cell lysis versus cell detachment will occur. Cells closer to the wall (edge) of a laminar flow system may be subjected to greater turbulence than those in the center of the flow. The introduction of air bubbles into a flow system can significantly disrupt the flow and cause premature detachment of the cells.

7.3 *Issues of Interpretation of Detachment Assays*—Many cell adhesion assays measure the force needed to remove a cell or cells from a surface. The mechanism of detachment deserves consideration. Not only is the issue of peeling as opposed to tethering detachment important, as mentioned above, but the location of the adhesion failure affects the interpretation of the assay result directly. Failure of adhesive bonding as a result of both the adhesive separating from the bonded material and by mechanical failure of the adhesive or material itself need to be addressed when analyzing cell detachment assays.

7.3.1 *Cell Detachment During an Assay*—Adhesion of most cells to biomaterial surfaces involves a chain of sites of potential mechanical failure during the detachment process. The following partial list shows potential failure modes: *(1)* Failure of the adhesion of the protein layer to the biomaterial surface. *(2)* Mechanical failure within the protein layer. *(3)* Failure of the cell receptor bond to the protein ligand (this is

the failure usually assumed to occur in such studies). *(4)* Failure of the receptor to remain in the cell membrane. *(5)* Mechanical failure of the cell membrane, either in the bilipid layer or the cytoskeleton, or detachment of the bilayer from the cytoskeleton (tether formation). As with any chain, failure will occur at whichever of these locations is the weakest. Thus, some detachment assays may measure parameters unrelated to the biomaterial properties themselves, such as membrane integrity. This should not be taken to mean that the only element above worth considering is the adhesion of the protein to the biomaterial surface, since the biomaterial surface properties are known to affect the pattern of protein deposition on the material surface. The user is advised to consult the vast literature on adhesion of proteins to polymers and metals and the mechanical properties of cell membranes, as these topics are beyond the scope of this guide. The user is referred to X2.2 through X2.4 for additional information on the importance of surfaces and proteins on cell attachment.

7.3.2 The following is a non-exhaustive list of parameters a user should consider when designing detachment assays: flow rate uniformity (laminar versus turbulent flow), what is happening at the cell, dwell time, test duration, measurement method, cell density, cell passage/age, gravity, different test scenarios, detachment mechanisms, testing for cell damage, cytoskeletal reorganization, torque rather than shear, realignment with flow and image analysis/histomorphometry. The user is referred to X2.2 through X2.4 for additional information on sources of variation in cell detachment assays.

7.4 The following is a non-exhaustive list of parameters a user should consider when designing cell adhesion assays: problems with single cell variation, reduced adhesiveness, susceptibility to local material variations, cantilever calibration, very small forces, uncertainties, statistical significance and method of assessing cell detachment to include parameters such as fluid flow, gravity drop-off, vessel design, local hydrodynamics, and flow chamber design.

APPENDIXES

(Nonmandatory Information)

X1. SOURCES OF VARIATION IN CELL ADHESION ASSAYS—CELLULAR

X1.1 *Cell Type, Harvesting and Culture*:

X1.1.1 *Primary versus Tumor versus Immortalized*—Cell adhesion assays routinely employ cells of various phenotypes from non-human or non-primate sources. Cells of the appropriate phenotype for a particular application should be used (that is, osteoblasts for bone repair). The user should be aware that if a TEMP contains human cells, the appropriate assay should employ human cells of the same source. The decision to use primary (freshly isolated and usually of a natural phenotype) versus immortal cells is critical to the assay outcome. Immortal cell lines grow in an essentially clonal fashion with little change in phenotype with time and are widely available, but may not actually reflect the phenotype relevant to the biomaterial's intended application. The variable of senescence of the cells (see below) needs to be addressed. Primary cells must be well characterized prior to use, especially if the cell population contains cells of mixed type. Cell populations may also need to be enriched for cells of interest.

X1.1.2 *Passage Number and Cell Doublings*—As the cell passage number increases, the biochemical and phenotypic properties of the cell change. Cells used for adhesion assays should be maintained at a low passage number and should be used at the same passage number for each assay run. Rapidly dividing cells may adversely affect cell adhesion, resulting in cell overgrowth, loss of contact inhibition, and reduction of cell adhesion to surfaces. The optimal time of attachment prior to running the cell adhesion assay must be optimized for each cell type. For this data to be informative for a TEMP, the same cell passage and phenotypic properties should be used for these studies as are either *(1)* seeded onto the biomaterial and/or *(2)* equal to when the TEMP is implanted.

X1.1.3 *Mobility of Cells*—Some cells and cell types are highly mobile and the ability to migrate may affect how tightly the cells attach. Mobility is a function of not only cell type, but also growth conditions and length of time in culture.

X1.1.4 *Purity of Cell Type*—Adhesion studies are best made on highly purified cell types. Many of the prototype TEMPs contain mixed populations of cells, each cell type with its own adherence properties. One possible method to bypass this restriction is to use the AFM to measure single cells. However, a large sample population would be required. For flow cells, a population analysis by immunofluorescence staining may be necessary to determine the cells present and the ratio of the various cell types.

X1.1.5 *Contact Inhibition—Density of Attached Cells*—Many cells exhibit optimal phenotypic conditions when cell density is maintained below confluency. Cells grown at densities approaching confluency can exhibit loss of contact inhibition and a reduction in the ability to adhere to surfaces. 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. One additional consideration is that in the case of autologous TEMPs products, during manufacture, these cells may be seeded in a range from suboptimal density due to limited cell numbers to a maximum density when cells are plentiful.

X1.1.6 *Harvesting Conditions*—Harvesting procedures, such as trypsinization, can destroy cell membrane surface receptors and result in possible decreased adhesion. When using trypsin, or similar agents, to remove cells from surfaces, the user should be careful to minimize the exposure time to the trypsin. The cell's sensitivity to trypsin is also affected by the

age and passage number of the cells. Likewise, cell removal by techniques such as scraping can also result in damage to cell membrane surface receptors, as well as cell death. Certain non-trypsin harvesting methods (that is, EDTA, icing) may result in a more gentle cell release. However, these methods are also not without risks to the viability and functionality of the cells. Extended exposure to EDTA or icing conditions may result in loss of surface receptors and viability and may result in cell death.

X1.1.7 *Holding Conditions Prior to Testing*—Once harvested, the cells must be maintained in an environment that is conducive to maintaining cell viability. Factors which should be considered include, *(1)* cell density, *(2)* buffer (that is, contains calcium, magnesium and nutrients to maintain cell phenotype and viability), *(3)* aeration, and *(4)* holding temperature. The optimal temperature, within the holding conditions, must be defined for each cell type. In the case of TEMPs, the assay conditions chosen should reflect the conditions in TEMPs manufacturing.

X1.1.8 *Mycoplasma Contaminants in Cell Cultures*—The presence of adventitious agents, such as mycoplasma, can also affect the adhesion properties of cells. Care must be taken to maintain sterility of the cell preparation, not only during cell growth and harvesting, but also during cell holding and, ultimately, during the running of the cell adhesion assay.

X1.1.9 *Senescence in Primary Cultures*—Cells isolated from a donor source and cultured in flasks have a limited lifespan. The culture reaches senescence after a predetermined number of population doublings. In part, the senescence may be the result of the acceleration of cell division which occurs in culture. Use of early passage cell isolates may help overcome the problem. Use of early passages may not allow the generation of sufficient cell numbers for cell adhesion assays. A second consideration is that not every cell within a culture is at an identical stage of senescence. The proportion of senescent cells in the culture increases with increasing population doublings.

X1.1.10 *Assessment of Cell Behavior*—The cell behavior following running an adhesion or detachment assay may be assessed by, but not limited to, the following techniques.

X1.1.10.1 *Morphology*—Changes in cell morphology, such as aspect ratio, area, perimeter, cell surface roughness, as a function of cell adhesion should be monitored. These cell parameters can be monitored by qualitative or quantitative histomorphometry, using light, fluorescence, confocal, transmission, scanning, or atomic force microscopy or a combination of these microscopy techniques.

X1.1.10.2 *Biochemical Assays and Biological Markers*—Changes in the phenotypic properties of the cells may also be measured using biochemical analytical techniques. Adhesion may result in changes in the production of proteins (that is, production of Type I versus Type II collagen for chondrocytes), changes in matrix proteins (that is, glycosaminoglycans), DNA or RNA, cell biomarkers and cytoskeletal markers.

X1.1.10.3 *Interpretation of Image Analysis Data*—The user is referred to Guide F2603.

X1.1.11 *Method of Attachment*—Variations in the methods used to attach and grow cells can result in variations in cell attachment to biomaterial surfaces. Cells grown on porous surfaces such as sephadex beads may exhibit adhesion characteristics quite different than cells grown on plastic tissue culture dishes, spinner flasks or with mechanical strain/stress.

X2. SOURCES OF VARIATION IN CELL ADHESION ASSAYS—BIOMATERIALS

X2.1 Cell type adhesion characteristics; cell numbers; force per time effects (maintaining laminar flow for force definition; matrix material for cell growth.

X2.2 *Influences of Surfaces on Cell Adhesion*—Material surface energy and surface charge density (that is, long range non-biological physicochemical interactions such as van der Waals and electrostatic interactions) need to be considered during the initial stages of cell binding to biomaterial surfaces. When biomaterial surfaces are exposed to biological fluids containing proteins, those proteins are adsorbed onto the material surface in a particular way which affects cell adhesion to those surfaces. Biomaterial surfaces may influence cell behavior (that is, control biocompatibility), by controlling the composition and conformation of the adsorbed protein layer present on the biomaterial surfaces in contact with cells in suspension. The properties of the adsorbed protein layer need to be evaluated. For example, attaching the RGD (arginine; glycine; aspartic acid) tripeptide cell adhesion molecule improves adhesion, or increasing the hydrophilicity of the surface by, for example, coating with a layer of poly(ethylene glycol), PEG, improves biocompatibility (Brown & Gresham, 1993; Quirk et al., 2001) (**13**, **14**). Attachment of specific ligands to an adsorbed coating of PEG appears to provide some control over which proteins are adsorbed onto the layer and hence influence which cells attach. The role of surface topography and the significance of mechanical strain on protein conformation and adsorption to biomaterial surfaces should be carefully considered when selecting a biomaterial surface. Porous coatings designed to enhance tissue in-growth and, hence, integration, further increase the complexity of the surface. In such coatings, the microenvironment around the cell becomes increasingly important, adding to the topographical and chemical factors that influence cell adhesion. This microenvironment is likely to dominate cell behavior in the porous constructs or scaffolds used in tissue engineering. The time between allowing cells to attach to a biomaterial surface and measurement of adhesion must also be considered. The forces of attachment may be quite different if measured within minutes versus hours of cell attachment.

X2.3 *Cellular attachment to the extracellular matrix (ECM)*—Cell attachment to the ECM influences cell morphology and function, and influences survival, proliferation and expression of specific developmental and tissue-specific phenotypes. Adhesion can be studied by using either

morphological and biochemical methods or by qualitative and quantitative detachment methods which are classified according to the types of force applied. Difference in the experimental methods between laboratories can make comparison of adhesion results difficult. Over time cells in culture secrete their own substrates that can lead to adherence of cells to the growth matrix (that is, cell adherence to agarose).

X2.4 *Scaffolds*—The rate of degradation of the scaffold must be taken into consideration. For example, synthetic scaffolds break down over time in culture. Cells may accelerate this process. Softer scaffolds generally result in poorer adherence. One must also take into account the physical properties of scaffolds. One such consideration is how large and homogeneous are the pores and channels that are present in the scaffold or matrix.

X2.5 *Cell Binding to Biomaterial Surfaces*:

X2.5.1 Typically researchers make some measure of the force required to detach cells that have adhered to a surface, often by correlating the number of cells that become detached in response to the rate of flow of culture medium over them. The information gleaned from this type of measurement is influenced by the type of flow (that is, laminar or turbulent in the immediate vicinity of the cells), the flow rate, gravity and whether or not any measures of cell damage are made. At high flow rates the cell membrane attached to the surface ruptures releasing the main body of the cell. Some researchers use serum-free media to reduce protein interactions with the biomaterial and cells. The type of media and presence or absence of serum should be noted. Bovine serum albumin (BSA) may be used to block sites on a surface to reduce non-specific adhesion to the surface. The user should be aware that while the use of BSA may be acceptable in a cell detachment assay, regulatory and other restrictions may prevent the use of BSA in production or other applications.

X2.5.2 *Estimation of Shear Stress Close to the Surface*—Accurate determination of the shear stress requires taking the following factors into consideration: the boundary layer behavior close to the surface of the material being tested, the influence of attached cells on the flow over cells immediately downstream of them, and the shape and degree of protrusion of cells into the flowing stream. If an adhesion rather than detachment is chosen, the exact behavior of cells flowing close to a surface should also be considered. Events close to the surface in a laminar shear field are complex and not easily modeled due to the constant changes in cell shape.

X2.5.3 *Measurement of Cell Adhesion*—Studies of the process of cell attachment are typically based on atomic force microscopy measurements of the adhesive force or tack that develops over a period of time between one or more cells and a surface. Such measurements are made either by attaching one or more cells to an atomic force microscope (AFM) tip or by coating the tip with a sample of the biomaterial under investigation. As cells spread and attach to surfaces, they lay down extracellular matrix (that is, for instance, but not limited to fibrinogen, fibronectin, laminin, collagen), cell adhesion molecules, other protein factors, as well as possibly growth factors and cytokines which enhance the cell adhesion over time. The problem of mounting single cells relates to the difficulty in maintaining the desired three-dimensional orientation of the cell and causing the cell to adhere in the desired orientation. It is difficult to manipulate single cell suspensions, unless a micropipette or similar specialized instrumentation is used. When mounting multiple single cells, the adhesive strength of each cell will be different. Since the orientation of each cell is different, the adhesion markers which are available will vary between cells and the adhesive strength of the individual cells will vary. With multiple cells, the force geometry is distributed in various forms (that is, vector quantities throughout the whole matrix).

X2.5.4 *Measurement of Cell Detachment*—Studies of cell detachment usually involve measuring the force required to detach adherent cells. The force can be applied by a fluid flowing over the cells or by using the centrifugal force generated within a centrifuge. For fluid flow systems, the detachment force is typically deduced from the measured flow rate, though details of the actual forces applied to the cells is complex and in need of experimental investigation. This type of measurement is usually performed during the development of coatings or materials with controlled topographies with a view to reducing or enhancing biocompatibility. Both cell adhesion and detachment studies rely on adequate surface characterization being carried out prior to testing to determine the uniformity of the surface, both chemically and physically (S.L. James et al., 2004) (**7**).

REFERENCES

Below is a non-exhaustive list for additional information relating to adhesion. A useful review of a selection of these methods has recently been published by Missirlis, Y. F., and Spiliotis, A. D., (2002) **(15)** and Garcia, A. J., and Gallant, N. D., (2003) **(6).**

(1) Shames, I., *Mechanics of Fluids*, 2nd edition, McGraw-Hill, New York, 1982.

(2) Society for In Vitro Biology, Terminology Committee, http://www.sivb.org/edu_terminology.asp.

(3) Popov, E. P., *Engineering Mechanics of Solids*, Prentice-Hall, Englewood Cliffs, NJ, 1990.

(4) Pierres, A., Benoliel, A., and Bongrand, P., "Cell Fitting to Adhesive Surfaces: A Prerequisite to Firm Attachment and Subsequent Events," *Eur Cells and Maters*, 3, 2002, pp. 31–45.

(5) Lukas, Z., and Dvorak, K., "Adhesion Molecules in Biology and Oncology," *Acta Vet, Brno*, 73, 2004, pp. 93–104.

(6) Garcia, A. J., and Gallant, N. D., "Stick and Grip: Measurement Systems and Quantitative Analyses of Integrin-Mediated Cell Adhesion Strength," *Cell Biochemistry and Biophysics*, Vol 39, 2003, pp. 61–73.

(7) James, S. L., Mikhalovsky, S. V., Vadgamma, P., and Tomlins, P. E., "Issues Concerning the Use of Assays of Cell Adhesion to Biomaterials," Chapter in *Surfaces and Interfaces for Biomaterials*, P. Vadgamma, Editor, Woodhead Publishers, 2005, pp. 754–762.

(8) Shao, J. Y., Xu, G., and Guo, P., "Quantifying Cell-Adhesion Strength with Micropipette Manipulation: Principle and Application," *Front Biosci*., 1, 9, 2004, pp. 2183–2191.

(9) Lee, C-C., Wu, C-C., and Su, F-C., "The Technique for Measurement of Cell Adhesion Force," *Journal of Medical and Biological Engineering* , 24(1), 2004, pp. 51–56.

(10) Heneweer, C., Schmidt, M., Denker, H-W., and Thie, M., "Molecular Mechanisms in Uterine Epithelium During Trophoblast Binding: The Role of Small GTPase RhoA in Human Uterine Ishikawa Cells," *J Exp Clin Assist Reprod*., 2, 2005, p. 4.

(11) Reyes, C. D., and Garcia, A. J., "A Centrifugation Cell Adhesion Assay for High-Throughput Screening of Biomaterial Surfaces," *J. Biomed. Mater. Res*., 67(1), 2003, pp. 328–333.

(12) Huang, W., Anvari, B., Torres, J. H., LeBaron, R. G., and Athanasiou, K. A., "Temporal Effects of Cell Adhesion on Mechanical Characteristics of the Single Chondrocyte," *J. Orthopaedic Res*., 21(1-8), 2003, pp. 88–95.

(13) Brown, E. J., and Gresham, H. D., "Signal Transduction Through a Novel phagocyte Integrin," *Structure, Function and Regulation of Molecules Involved in Leukocyte Adhesion*, P. E. Lipsky, R. Rothlein, T. K. Kishimoto, R. B. Faanes, and C. W. Smith, Editors, Springer-Verlag New York, 1993, pp. 78–91.

(14) Quirk, R. A., et al., "Controlling Biological Interactions with Poly(lactic acid) by Surface Entrapment Modification," *LANGMUIR*, 17, 2001, pp. 2817–2820.

(15) Missirlis, Y. F., and Spiliotis, A. D., "Assessment of Techniques Used in Calculating Cell-Material Interactions," *Biomol Eng*., 19(2-6), 2002, pp. 287–294.

BIBLIOGRAPHY

(1) *Studying Cell Adhesion*, (hardcover) by P. Bongrand, P. M. Claesson, A.S.G. Curtis (Editor), Springer-Verlag Telos, 1995.

(2) Curtis, A., "Substratum Nanotopography and the Adhesion of Biological Cells: Are Symmetry and Regularity of Nanotopography Important?" *Biophysical Chemistry*, 94, 2001, pp. 275–283.