FINAL PROGRESS REPORT

COMBINATORIAL MEASUREMENT TECHNOLOGY FOR CELL INTERACTIONS AT POLYMER SURFACES

SUMMARY OF PROGRESS

Synthetic polymers offer tremendous promise as tissue engineering, wound healing, diagnostic, and *in vitro* cell-expansion materials. However, the structure-response relationships between cells and polymer surfaces are extremely complex due to interactions among a number of factors. The surface factors include chemistry, protein adsorption, and lateral and topographical microstructure and nanostructure. These structures, which include morphologies due to crystallization, phase separation, and mechanical / chemical etching, can have a profound influence on the responses of cultured, adhesion-dependent cells. In addition, the cell response is a complex series of spatial-temporal events, including focal adhesion formation, actin fiber formation, signaling cascades, gene transcription, protein expression, and proliferation. Cells are also responding simultaneously to both surface features as well as other cells, particularly at close contact. While it is well-known that any of these effects influences function of adherent cells, there remains a tremendous difficulty in understanding how these effects are inter-related. Experiments aimed at detecting and describing surface-cell response relationships have largely been limited to well-controlled model surfaces, as opposed to surfaces that would be used in tissue engineering and drug-delivery applications.

The overall goal of this technology development project was to implement a new combinatorial library technology for investigating the effects of both polymer chemical and physical surface features cell responses. The new technology, termed combinatorial biosurface screening (CBS) has been successful, and has resulted in 4 peer-reviewed publications, 1 in review and 1 other in preparation. The response of the biomaterials community has been positive, resulting in the introduction of new meeting symposia focused on combinatorial biomaterials discovery at the 2005 World Biomaterials Congress in Sydney, Australia; the 2004 and 2005 Gordon Research Conferences on Combinatorial Materials Development; and the recent national meetings of the American Chemical Society and the Materials Research Society. We are now in a position to offer and support the utilization of CBS technology in the biomaterials research community for discovery and characterization of complex, multifeature material surfaces. Below we summarize accomplishments related to each aim.

I have one additional note, however, before reviewing the progress. The enormous amount of data we collected, especially in Aim 3, presented challenges to efficient analysis. We anticipated this before starting the work. What was not anticipated was that even *mere detection* of relationships between cell responses and surface features required a completely new model for data analysis and visualization. This led to the development of a new methodology, which we call *local cell-feature histograms*, for analysis of images of cells adherent on surfaces. We feel that this development is the most important unexpected outcome of this project.

AIM 1. Characterize the statistical effectiveness of combinatorial library cell cultures.

1a. Computation of uncertainties on gradient combinatorial libraries. The typical uncertainty in composition and temperature gradients was measured in detail for the PCL/PDLA and PCL/PLGA model systems. In addition, we described how uncertainties in the material and cell culture properties could be calculated by using replicated libraries.

1b. Characterization of repeatability of library results relative to conventional techniques. We have completed development of a robust and repeatable system for depositing polymers onto glass or silicon substrates, such that the polymers have gradients in composition, thickness, and annealing temperature. Typical repeatability for gradient physical properties are shown in Figure 1. We have carefully defined the range of flowrate, composition, and humidity that produce repeatable, linear composition gradients.

1c. Investigation of polymer surface property gradient artifacts. Where artifacts were observed in the deposited libraries, these were examined. The primary artifacts were associated with instability of the composition gradients under certain conditions of flow and composition. We developed an optical method of detecting these flaws and determined limitations on coating conditions to minimize occurrence of these artifacts.

AIM 2 Demonstrate the scalability of results from two-dimensional combinatorial surface libraries to larger three-dimensional and porous sample geometries.

Using mouse aortic smooth muscle cells (MASMCs), we showed that there are no statistically significant differences between the results of physical and biological assays on combi chips compared to uniform, three-dimensional scaffolds prepared at compositions and temperatures found on the libraries. This work was performed in collaboration with Dr. Zorina Galis, Emory University (presently at Lilly Corporation), and was funded in part by NIH-HK072039-01 (Galis P.I.).

AIM 3 Utilize combinatorial surface libraries for detailed characterization of cell response to surface features in the following technologically relevant systems:

3a. Phase-separating biodegradable polymer blends. Two distinct material systems were developed. Blends of PCL and PDLA were screened against a rat osteoblast cell line (MC3T3-E1) and blends of PCL and PLGA were screened against two difference cell types, both the MC3T3-E1 line as well as primary MASMCs. We characterized several unique features of cell response to the phase separated patterns, discussed further in significant results.

3b. Patterned surface chemistry modification Specific Aim 3b was addressed by developing 2 new polymer blend model systems, each with enhanced chemical contrast between micropatterned phases. Our ultimate aim here was to control chemical effects separately from physical ones. One approach involved siloxane capping of the PLGA phase, and the other approach introduced a new material that has reduced cell adhesion, polyurethanes based on poly(ethylene oxide). MC3T3-E1 osteoblasts were cultured and screened for attachment and proliferation.

SIGNIFICANT RESULTS

- **Combinatorial biomaterial libraries (CBLs) enable repeatable display of 1000 distinct physical and chemical surface features on a microscope slide. Chemical reactions can also be carried out reliably on CBL surfaces**

Figure 1 below provides a schematic of the two processes used for preparing CBLs: a coating procedure for preparing binary gradients in composition and a thermal gradient heating / cooling system for preparing linear gradients in temperature. Composition gradients enable the exploration of composition-dependent surface chemistry and patterning effects, such as liquid-liquid phase separation of incompatible polymers poly(D,L-lactide) and poly(caprolactone) (lower right corner, Fig. 1). Thermal gradients allow differences in annealing temperature, which controls reaction rate in chemically-reacting systems, or controls 'ripening' kinetics for phase separation, indicated in the micrographs in Figure 1. Generally, composition and temperature gradients can be combined on one sample, enabling two-dimensions of variation in adjustable parameters. Based on a 500 μm region of interest and a spacing of 1 mm center-to-center between these regions, there are 1250 regions on a 25 x 50 mm^2 sample. Generally not all of these are sampled, usually about 100 to 200 of the regions are sampled in a given experiment. However, having the continuously-graduated regions available allows for examination of intermediate compositions on the same sample, if and when an interesting structure-response is observed.

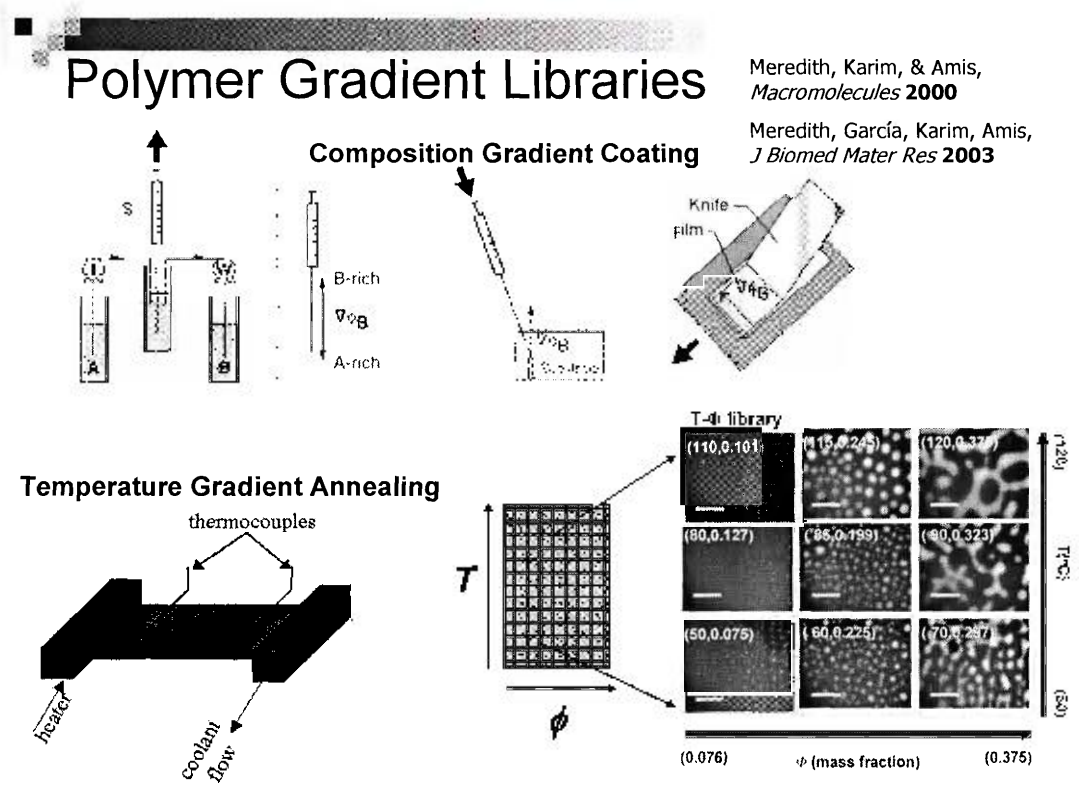

Figure 1. Gradient library preparation and images from PDLA/PCL library.

Figure 2 shows typical uncertainties for composition libraries before and after annealing, compared to the linear predicted gradient based upon the mixing process. There are no significant deviations from linearity and no significant changes in the composition gradient after 2 hours annealing at temperatures exceeding the melting point and glass transitions of PDLA and PCL.

Figure 3 illustrates typical physical properties indicative of surface microstructure that can be measured rapidly on CBLs. The bar graphs in the figure show quantitative values for the lateral size of PCL microstructural islands and the roughness induced by the PCL islands. Uncertainties are the standard error of the mean for various repeated libraries, with the number of library repetitions varied from n=2 to n=6.

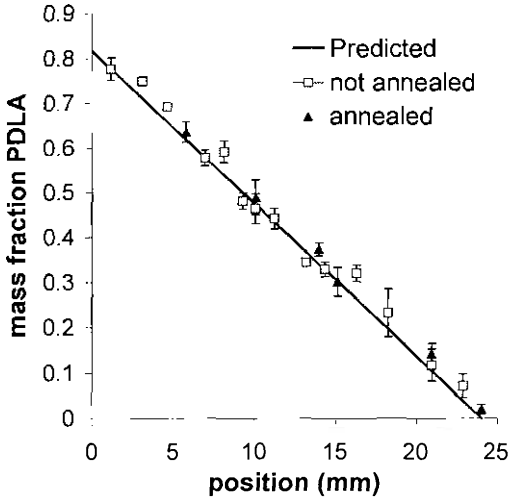

Figure 2. Comparison of predicted vs. measured Composition gradients on annealed and non-annealed libraries of PDLA/PCL blends. Measurements taken from 3 positions on 2 independent chips.

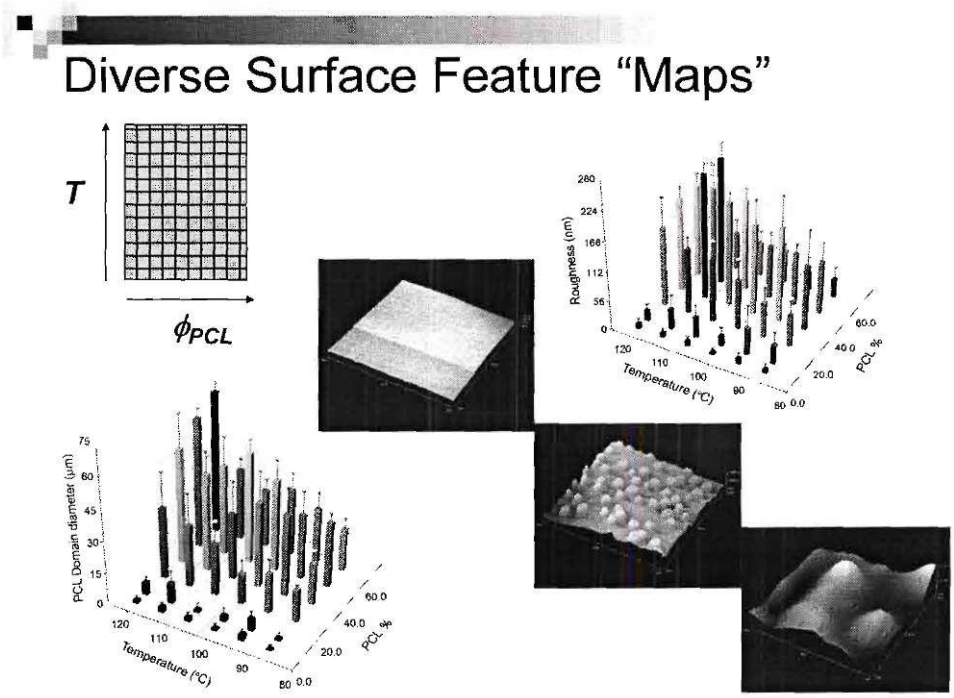

Figure 3. Sample of surface features and uncertainties from PDLA-PCL library.

- **Combinatorial biomaterial surfaces enable robust discovery of materials structure – biological response relationships.**

Figure 4 provides typical results encountered for measurement of cell function over the surface of a CBL. Shown is the fraction of proliferated MC3T3-E1 osteoblast cell-line, measured using a BrdU assay and immunofluorescent staining, on a PLDA/PCL library. Results are presented as a function of preparation conditions for the library: composition PCL and annealing temperature. Proliferation various widely across the library depending on the underlying polymer surface. The uncertainties in Figure 4 are relatively large, compared to other assays and experiments performed during this work, such as in Figure 5. Still, the reproducibility is quite good and is certainly capable of indicating trends and outliers. Figure 4 bottom shows a different type of visualization for the same data as in the bar graph. The contour plot lacks the error bars but does a better job illustrating the spot of proliferative activity that occurs on this library. Generally, spots of activity occurring at specific ranges in composition and preparation temperature are common for adhesion, attachment, population, proliferation, cell shape, and protein expression assays. As cell culture progresses, it is important to note that the proliferation drops and becomes uniform across the library by day 8, as expected for confluent cells beginning to differentiate.

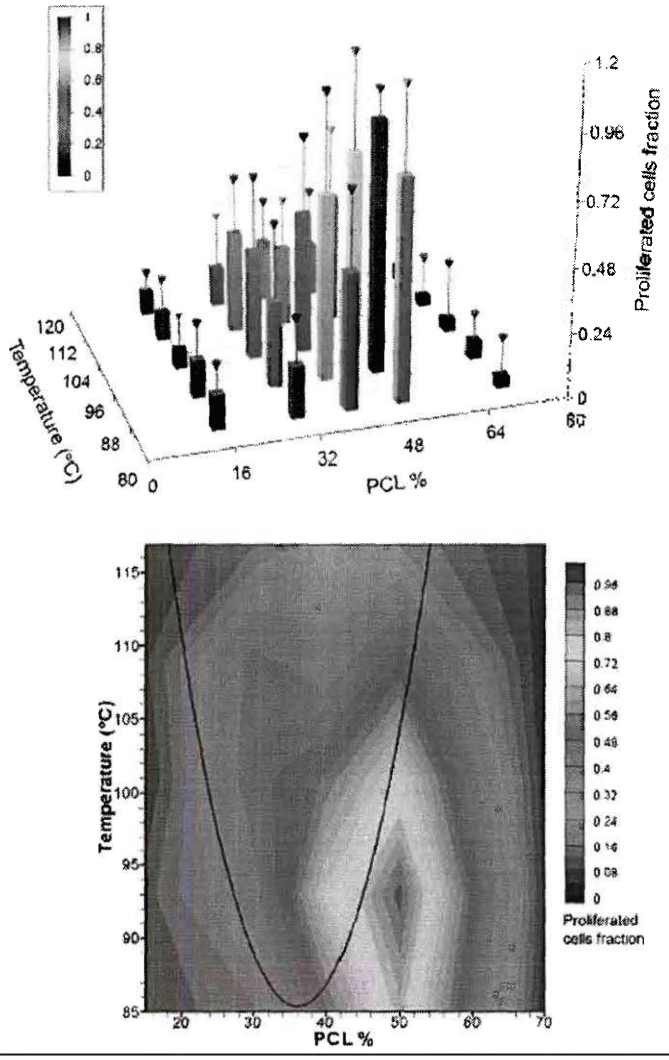

Figure 4. Top: proliferated cell fraction for MC3T3-E1 on a PDLA/PCL library at 3 days. Uncertainties are standard error of the mean with n=6 (blue), n=5 (gray), n=4 green, n=3 violet, and n=2 (red). Bottom: Another way of visualizing the data, as a contour map with proliferated fraction indicated by color.

In addition to the osteoblast cell line, we also initiated a collaboration with Dr. Zorina Galis at Emory University, Cardiology Department, for culturing a primary harvested cell line, mouse aortic smooth muscle cells, MASMCs. This work was supported in part by an R21 (PI: Galis)

from NHLBI. Figure 5 shows a selection of results from this study, which has been published as two papers in Biomaterials (see publications list below). Figure 5 shows results of 7-day proliferation for mouse aortic smooth muscle cells on PLGA/PCL phase-separated libraries, along with one of the underlying physical features, the PCL island diameter. Not only was it important to show that primary cells could be effectively cultured on CBLs, but also we note that robust detection of cell responses to the underlying surface was possible. In fact the uncertainties in Figure 5 are smaller than for the MC3T3-E1 cell line (Figure 4), and it was possible to distinguish significant differences in response on the library at both 0.05 and 0.01 levels. It was with the MASMC cells that we performed an in depth comparison between two-dimensional libraries, two-dimensional uniform cell cultures, and three-dimensional porous scaffolds. These results showed that trends were generally repeatable going from 2D to 3D samples, but that absolute values and kinetics of cell growth were not replicable. However, the 2D to 3D results were consistent with one another, and the 2D library to 2D uniform cultures were in close agreement on cell response (proliferation) to surface variations.

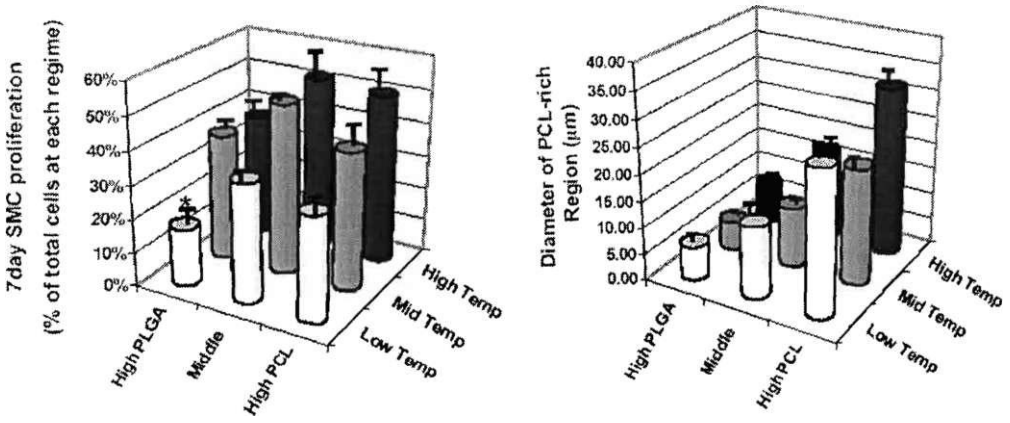

Figure 5. MASMC proliferation and population at 7 days (left) and diameter of PCL-rich islands on underlying surface (right).

We focused primarily on assays of early cell activity, such as attachment, shape, presence of focal adhesion proteins (vinculin), actin structure, proliferation, and viability. These assays are relatively simple, compared to long-term culture (7 + days) assays in which cells become confluent and cell-cell signaling events begin to compete with the cell-surface interactions. We believe that significant care must be taken when interpreting results from gradient libraries once cells have reached confluency and begin to express proteins that can modify the extra-cellular matrix or signal cells far away at other library positions. In this sense, the CBL method presented herein is most easily interpreted for early cell events, leading to the formation of hypotheses that can be tested in longer-term, more conventional uniform surface cultures.

- **Patterned surfaces generated by simple phase separation processes can be used to control complex cell functions in a repeatable, robust manner**

While most of the significant results mentioned here concern the technology developed herein, we also include one major biological finding. As illustrated in the images above (and below), adherent cells are very sensitive to phase-separated microstructure. While the sensitivity of cell adhesion and function to topography and microstructure are not new observations, what is new here is the manner in which surface microstructures were generated. Almost without exception, previous studies of cell response to physical surface features have utilized two-dimensional surfaces with regular, repeated patterns generated with microlithographic techniques. In addition, a large number of studies have examined cell behavior when cultured on surfaces roughened via physical or chemical means. These methods are inherently limited to two-dimensions, and present two extremes in ability to control the size and scale of features. Phase separation, on the other hand, occurs in three-dimensions throughout a polymer mixture, and can pattern the surfaces of internal pores. In addition, phase separation will produce features that can be tightly- or loosely-controlled in size and dispersion, depending on polymer chemistry. The polymers used here do not differ significantly in chemistry, and in fact osteoblasts cultured on the pure PDLA and PCL surfaces show little differences in cell adhesion and proliferation. However, when these materials are combined and phase separate to form microstructures, cells respond in a profoundly different manner, depending on the scale, size, and shape of the microstructures. Not only does this open up new opportunities for scientific investigation, but it also may lead to new concepts for practical biomaterials design.

- **Local cell-feature histogram analysis (LCFA) is a new approach for robust detection of structure-response patterns in cell-surface images. LCFA has the potential to become a disruptive methodology in interpretation of biomaterials experiments, whether they are traditional or combinatorial.**

The key question in all of the experiments performed here, and arguably in biomaterials science as a whole, is 'what is the relationship between surface features and cell response'? The enormous amount of data available from the surface pattern libraries required high-throughput, automated data analysis, a fact that was anticipated before starting the work. What was not anticipated was the even mere detection of relationships between cell responses and surface features required new data analysis and visualization approaches. In our libraries, cells are exposed simultaneously to changes in multiple surface features, not simply one property changed holding others constant. This, combined with the noise inherent to cell assays, leads to extreme difficulty in discovering the controlling variables, as illustrated in Figure 6. Overcoming these obstacles led to the development of a new methodology for analysis of image data for cells adherent to surfaces: LCFA. We feel that this development is the most important unexpected outcome of this project.

When we applied the conventional accepted approach of measuring summary statistics (counts and means) of object properties in each library image (cell #, proliferating fraction, island size, etc.), we found that we could identify one most significant factor relatively well. We term this the 'global' approach, since properties of all cells and features in an image are lumped together to create a data point. The total number of points then equals the number of images (~100 for

Figure 6). While we can easily identify with this standard approach that the PCL mean island diameter is the only significant factor below level a = 0.05, no other features fall below a significance level of 0.1. Hence, we have identified only one significant feature that may be controlling cell proliferation response. A plot of proliferation fraction versus mean PCL diameter, shown also in Figure 6 (left side) leads less than satisfactory results. Even though the PCL diameter is significant, the nature of the relationship with proliferation remains unknown, either lost in the noise or confounded by hidden, 'lurking' factors, we have not accounted for yet.

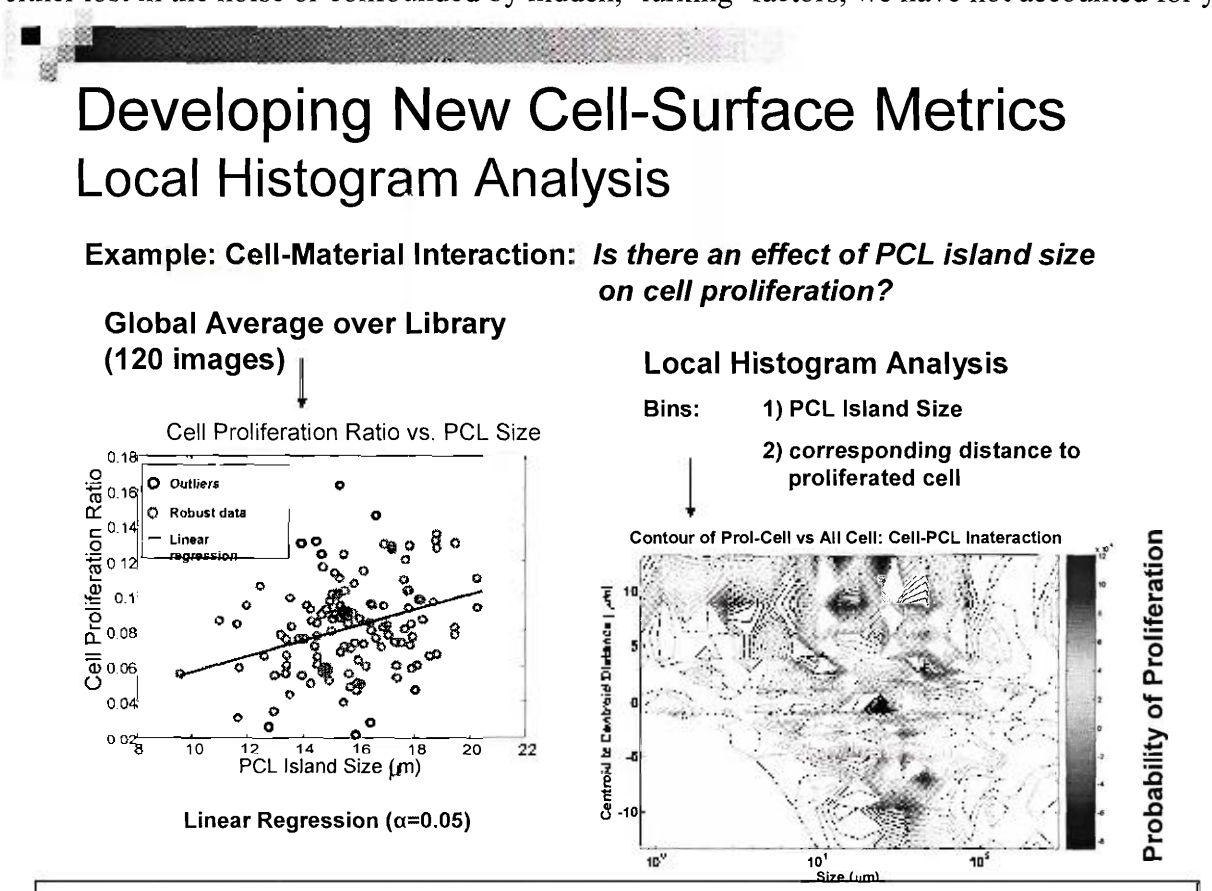

Figure 6. Illustration of local histogram analysis applied to discover cell-surface property relationships in the dataset for MC3T3-E1 on the PLGA-PCL libraries. Global regression analysis (left) is biased by the assumption of linearity and confounding or lurking factors are hidden. Local histogram analysis (right) assumes nothing about linearity and discrete, non-linear responses are revealed easily. Red areas on local histogram contour plot of proliferation illustrate regions of PCL size, and distance to cell from those islands, where proliferation is enhanced relative to random chance.

However, if we change the point-of-view of the analysis, from global to a local, the sensitivity improves considerably. This is accomplished in the following manner (described in an upcoming publication (1) in list below): All of the object positions are sorted according to pre-defined types like proliferated cell, non-proliferated cell, PCL island, etc. Then the distance between these objects is measured from the already existing database of image analysis results. These distances are then sorted into a histogram that describes the frequency of observing the pairing, for example probability of finding a proliferated cell as function of distance from any other proliferated cell. In Figure 6, right side, we show a contour plot of the probability of

observing a proliferated cell (MC3T3-E1) as a function of distance to a PCL islands of a certain size. This is actually a two-dimensional histogram that shows that it is not PCL island size alone that determines cell response. Rather, PCL island size and the distance from that island to cells determines the cell response. When the local distance information is 'averaged' together (as in left side plot Fig 6), the correlation is lost in the resulting noise. There are actually two distinct regions in Figure 6 (right) where proliferation is enhanced: large PCL islands (40-50 mm) very close to the cell's centroid, and smaller islands 1-10 mm far from the cell centroids. Examining the actual images one can see very clearly this relationship: focal adhesions are formed on the small PCL islands but the cells avoid and 'wrap around' larger microstructures. We note also that the LCFA metrics are useful for decoupling cell-cell interactions from the cell-surface effects, which is another one of the complexities facing biomaterials science. Figure 7 shows how LCFA metrics are applied to identify the well-known effect of contact inhibition of proliferation. While contact inhibition could not be identified with global statistics (Fig. 7 top), the local analysis (Fig. 7 bottom) detects the effect quite easily.

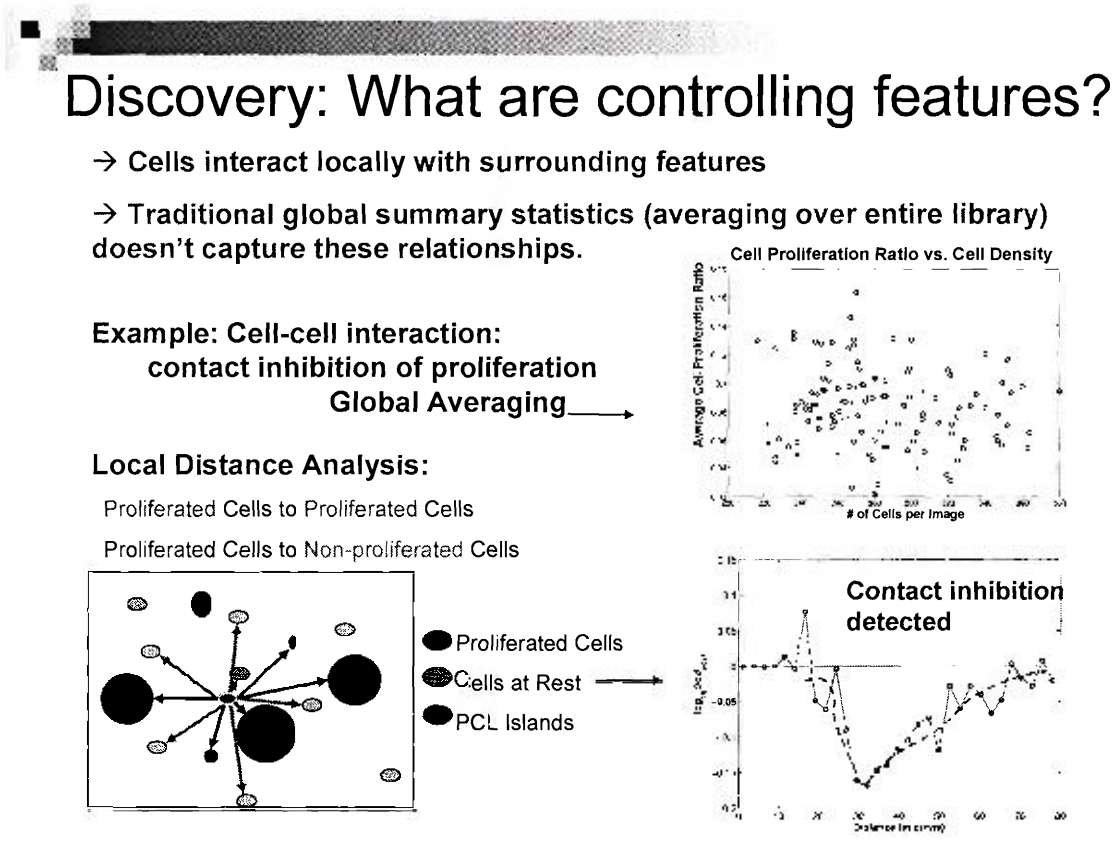

Figure 7. Illustration of inadequacy of global summary statistics to detect cell-cell contact inhibition on library (top). Local histogram analysis of cell-cell distances allows detection of the contact inhibition of proliferation from the same data set (bottom).

PUBLICATIONS

1. J. Su, J.C. Meredith, "Local Feature Histogram Analysis of Cell-Material Interactions" **2006**, in preparation.

2. P. Zapata, J. Su, A.J. García, J.C. Meredith, "Quantitative Measurement of Osteoblast Attachment, Spreading, and Proliferation on Combinatorial Polymer Libraries" *J. Biomed. Mat. Res. Applied Biomaterials*, **2006**, to be submitted 10/2006.

3. H.J. Sung, J. Su, J.D. Berglund, B.V. Russ, J.C. Meredith and Z.S. Galis, "The use of temperature–composition combinatorial libraries to study the effects of biodegradable polymer blend surfaces on vascular cells" *Biomaterials*, **2005**, *26*, 4557-67.

4. H-J. Sung, J. C. Meredith, C. Johnson, Z. S. Galis, "Degradation-rate dependent effect of biodegradable polymers on three-dimensional cell growth and angiogenesis for the engineering of vascular scaffolds" *Biomaterials*, **2004**, *25*, 5735-42.

5. J. C. Meredith, "A Perspective on High-Throughput Polymer Science" *Journal of Materials Science*, **2003**, *38*, 4427-37.

6. J. C. Meredith, J. L. Sormana, B. Keselowsky, A. Garcia, A. Tona, A. Karim, and E. J. Amis "Combinatorial Characterization of Cell Interactions with Polymer Surfaces" *Journal of Biomedical Materials Research*, **2003**, *66*, 483-490

INCLUSION OF GENDER, MINORITY AND CHILDREN STUDY SUBJECTS

The nature of this instrumental and methods development project does not involve the use of human subjects of study. Hence, no reports on inclusion of gender, minority, or student study subjects are included.

MATERIALS AVAILABLE FOR SHARING

Instrument design for gradient coating, temperature gradient annealing custom equipment developed here is available by contacting the PI, Carson.meredith@chbe.gatech.edu.

Software for the informatic screening of the large combinatorial datasets is also available through contacting the PI. All images and relative information were organized and stored in an Oracle® 10g (Oracle, CA) database for further image processing and data analysis. The software driving the system and is based on the .NET framework™ and written in Visual C#™ (Microsoft Cooperation, WA) and integrates the acquisition hardware (robotic stage and digital camera) with the database. LCFA algorithms in MATLAB™ are also available.