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3D Material Cytometry (3DMaC): A Very High-replicate, High-throughput Analytical Method using Microfabricated, Shape-specific, Cell-material Niches

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Abstract

Studying cell behavior within 3D material niches is key to understanding cell biology in health and disease, and developing biomaterials for regenerative medicine applications. Current approaches to study these cell-material niches are low throughput and can only analyze few replicates per experiment resulting in reduced measurement assurance and analytical power. Here we report 3D Material Cytometry (3DMaC), a novel high-throughput method based on microfabricated, shape-specific 3D cell-material niches and imaging cytometry. 3DMaC achieves rapid and highly multiplexed analyses of very high replicate numbers (“n” of 10^4 - 10^6) of 3D biomaterial constructs. 3DMaC overcomes current limitations of low “n”, low-throughput, “noisy” assays, to provide rapid, simultaneous analyses of potentially hundreds of parameters in 3D biomaterial cultures. The method is demonstrated here for a set of 85,000 events containing twelve distinct cell-biomaterial micro-niches along with robust, customized computational methods for high-throughput analytics with potentially unprecedented statistical power.

Introduction

In vitro 3D systems of synthetic or ECM-derived biomaterials containing cells or organoids have become essential for understanding of cell physiology and pathology, unravelling complex disease processes, and developing new biomedical materials to restore function in diseased tissues or deliver therapeutic cells¹⁻⁸. However, such approaches suffer from the lack of high-throughput analytical methods that allow rapid measurement of many cell parameters inside 3D niches in a non-destructive manner. Biological assays also inherently suffer from high “noise” that limits experimental reproducibility, and current analytical methods have low statistical power and confidence. Together, these limitations result in incomplete and often poorly reproducible data, and potentially skewed results.

Cells, in particular, are highly sensitive to manipulations and changes to their microenvironment. Thus measurements of their properties and behavior require high

replicate numbers to generate high statistical power, reduce false positives, and identify subtle, yet critical differences. Unfortunately, existing methods are time-intensive, low-throughput, destructive, or limit further sample manipulation^{1, 4, 9-15}. 3DMaC enables very high replicate, high-throughput, rapid, and non-destructive analyses of 3D cell-biomaterial niches or organoids which can be easily adopted by cell biologists, clinical scientists, engineers, and material scientists. Additionally, the method is flexible, easily allowing either single cells, aggregates of a few cells, or entire multi-cellular niches to be observed in the context of the encapsulating biomaterial. The 3DMaC analysis platform can be applied to a wide range of materials and cell systems.

In 3DMaC, very high “n” is achieved by using thousands of cell-biomaterial micro-niches, fabricated using simple lithographic techniques, as input to the analysis method. Specifically, shape- and size-specific micro-hydrogels are created to mimic specific cellular microenvironments, e.g. to probe niche-dependent stem cell differentiation or tumor biology within the immune-microenvironment. We used microgel shape, size and fluorescence (SSF) to uniquely barcode each cell-biomaterial niche. This barcoding is a unique aspect of 3DMaC and the use of niche-shape as a novel barcoding variable increases the multiplexing capability of this method many times higher than current analytical approaches. Although the micro-niches in this study are demonstrated using poly(ethylene glycol)-based materials, many other materials have been used in similar microhydrogel schemes and could be used with 3DMaC. In addition, rapid and high-throughput analyses of barcoded cell-biomaterial microgels is enabled in 3DMaC through the use of imaging flow cytometry and multi-parametric data analytics which dramatically increases the quantity and quality of data collection compared to existing methods of analyzing 3D cell-material constructs.

Despite the tremendous impact that flow cytometry has had on biological assays, the technique has been primarily restricted to population based analyses of cells in isolation and its application to biomaterials has not been realized. Here, we adapted imaging flow cytometry (ImageStreamX, ISX) to analyze 3D cell-biomaterial niches and show how robust bioinformatics methods can be used for biomaterial research. Current flow cytometry methods record data on hundreds of individual events per second in tens of fluorescence channels at once. Imaging flow cytometers enable direct imaging of each event, thus allowing shape to be a distinguishing parameter in analysis, in addition to size and fluorescence. Additionally, the new image-based data-analytics methods for cell-material niches reported here (both within the ImageStream software and as an independent algorithm using the images collected in the ImageStream) enables the identification of rare samples within the data set as each event can be selected individually.

Multiple previous studies regarding fabrication methods for microhydrogels suggest that a major gap in the field is high-throughput analysis techniques for microscale cell-material niches and methods that enable rapid, simple, and high throughput measurements based on commercially available systems, would be critical to develop. For examples, the Khademhosseini group has reported microhydrogel platforms to study cell behavior and stem cell differentiation in a variety of systems^{14, 16-18}. However, analysis were restricted to slow, low throughput microscopic methods. The Doyle group has worked extensively on a “stop-flow” lithography system that produces microhydrogels at a high rate in user-defined

shapes, and they have applied this technique to a range of applications including mRNA sensors¹⁹, microparticle arrays¹², and cell encapsulation²⁰. However, only their barcoded sensor system, has a high-throughput analysis method, but does not incorporate cell-based assays and also relies on an internally developed read-out platform, that is not readily available to the broader community²¹. Similarly, the Koh group has used shaped microgels for bioassays based on low-throughput microscopic analysis, but their technique has not been applied to 3D biomaterial-cell constructs^{22, 23}.

Other groups have used microscopic analysis to obtain image data on a larger than average number of biological replicates within a biomaterials context. This includes work from the Burdick group, where single cell or multi-cell units were imaged and masked to analyze the impact of N-cadherin-mimetic peptide on hMSCs²⁴, and work from the Gartner group using DNA-programmed assembly of cells to build specific cellular microenvironments which were analyzed in a 3D bulk hydrogel array²⁵. Neither of the systems was suspension-based which limited their replicate number, a major advantage of 3DMaC; however, these demonstrate that there is a need for a high-throughput way to analyze biomaterial-cell interactions in the context of the encapsulating material for both single cells and multi-cellular constructs.

3DMaC is a new method that brings together cell-material constructs with imaging flow cytometry and robust data analytics. It can be used with any microhydrogel systems or similar constructs, with or without cells, and achieves high replicate numbers (“n”) as well as high-multiplexing through a combinatorial barcoding of shape, size and fluorescence. It harnesses the throughput advantage, multiplexing capabilities, and ease-of-use of flow cytometry while enabling image-processing based separation of shapes to increase throughput-potential and assay capabilities beyond current capabilities. To the best of our knowledge, the application of shape-multiplexed flow cytometry to biomaterial constructs for screening cell responses has not been reported. The 3DMaC method is therefore a novel analysis platform that could be extended to study a large number of current biomaterials questions in the community.

Experimental

Materials

PEG-based microgels consisted of 20% poly(ethylene glycol) diacrylate (Mw 700) for non-cell-containing conditions. Cell-containing microgels consisted of 15% poly(ethylene glycol) diacrylate (Mw 3400) and 5% 4-armed poly(ethylene glycol) thiol (Mw 10,000) or 30% poly(ethylene glycol) diacrylate (Mw 3400). The photoinitiator used was lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP). Solutions were prepared in sterile PBS (non-cell-containing) or supplemented media (cell-containing) and filtered before use with a 0.2 µm syringe filter. 2% fluorescein o-acrylate or fluorescent nano/microparticles (FluoroSphere) were added for fluorescent barcoding. SU-8 2010, SU-8 2050, and SU-8 developer were used for wafer patterning.

PDMS Mold Fabrication

AutoCAD was used to design arrays of desired shapes. In this study there were twelve tested microshapes consisting of three different cross-sectional sizes (20, 40, and 60 μ m lengths) and four different shapes (square, circle, equilateral triangle, and right triangle). Silicon wafers were spincoated with photoresist according to the manufacturer's instructions. The wafers were exposed through a quartz photomask to 365nm light and excess unexposed photoresist was removed with developer. PDMS was degassed, poured over the wafers, and cured at 65°C. Cured PDMS was removed from the wafers, cut into individual arrays, and placed on glass slides. Arrays were stored with tape protecting the molded face and molds were sterilized with ethanol before use.

Cell Culture Conditions and Viability Staining

The A20 B lymphoma cell line (A20s), human mesenchymal stem cells (hMSCs, RoosterBio), and mouse mesenchymal stem cells (D1) were used as model cell types. All human cell research was performed according to Institutional Review Board (IRB) guidelines of the Georgia Institute of Technology, Atlanta, GA. The hMSCs used in this work are a commercial product without any identifying information and were only used for in-vitro cell culture experiments, and therefore are exempt as human subjects research. hMSCs were cultured according to manufacturer's instructions and expanded twice before use. D1 cells were expanded in DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin. Dendritic cells (DCs) were differentiated from bone marrow progenitor cells isolated from C57BL/6 mouse tibias and femurs, and cultured for 7 days in RPMI media supplemented with 10% FBS, 1% penicillin-streptomycin, 100mM sodium pyruvate, 1% non-essential amino acids, 0.1% 2-mercaptoethanol, 20ng/mL GM-CSF, and 10ng/mL IL-4. Media was changed every two days. T cells were isolated from C57BL/6 mouse spleens using the MACS Pan T cell kit and used immediately.

A20 cell-containing microhydrogels were cultured in RPMI media and stem cell-containing microhydrogels in DMEM media, both supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cell-microgel constructs were maintained in a humidified incubator at 37°C in 5% CO₂. Live/Dead staining was performed with a solution of 4 μ M calcein AM and 4 μ M ethidium homodimer in PBS. Adherent cells were used as a live control and cells treated with 0.1% Saponin-O for 30 minutes were used as a dead control.

Molded Cell-Microgel Constructs

Prepolymer formulations were based poly(ethylene glycol), and fabricated using methods adapted from Khademhosseini et al^{16-18, 26}. Prepolymer was prepared from 20% poly(ethylene glycol) diacrylate (Mw 700) in PBS for non-cell-containing conditions. Microhydrogels in Figure 2 were prepared from 15% poly(ethylene glycol) diacrylate (Mw 3400) and 5% 4-armed poly(ethylene glycol) thiol (Mw 10,000) in supplemented media for cell-containing conditions. Microhydrogels in Figure 4 were prepared from 30% poly(ethylene glycol) diacrylate (Mw 3400). Cells were centrifuged and suspended in prepolymer at the desired concentrations. Each PDMS mold was treated with oxygen plasma for at least one minute to reduce the water contact angle and prepolymer solution was immediately spread on the surface. A pipette tip was used to brush any bubbles from the

surface and excess was scraped away with a straight edge. Then the mold was sealed with hydrophobic PDMS and pressed firmly to isolate prepolymer and cells into individual microgels. PEG-based microgels were cured by exposure to 365nm light for 30 seconds through the top PDMS layer and released by pipetting in media. Constructs were cultured in 50 mL tubes or silanized wellplates. Cell-containing formulations were filtered through 100 μ m cell strainers.

Cell Function Measurements

Reactive oxygen species (ROS) staining was performed on hMSC-containing microhydrogels immediately after crosslinking. Microgels were rinsed with PBS and stained with 5 μ M dihydrorhodamine 1,2,3 dye (DHR) for 30 minutes. This dye is oxidized in the cell and indicates the presence of ROS by green fluorescence in the mitochondria. A LionheartFX Automated microscope was used to image microgels which had been formed as an array. Microhydrogels were also collected in suspension, stored on ice, and analysed using the ISX.

Antibody staining was performed on hMSC-containing microhydrogels which were fixed for 30 minutes with 4% paraformaldehyde immediately after crosslinking. Microgels were blocked with 0.5% BSA in PBS overnight, incubated overnight with DAPI and anti-humanCD105-PE in 0.5% BSA + 0.1% Triton-100, and rinsed for two hours in blocking buffer again. A Zeiss 700 laser scanning confocal fluorescence microscope was used to image microgels which had been formed as an array. These microhydrogels were then suspended in PBS and imaged using the ISX to confirm cell labelling could be seen in flow cytometry.

Multiplexed SSF Barcoding

To determine the total number of possible multiplexed barcodes, shape, size, and fluorescence were considered separately in the context of the ImageStream. Four shapes were demonstrated here and the remaining shapes are promising additions based on how the analyses distinguish shapes. Three sizes of microgel were demonstrated here, a fourth size could be added within the camera view of the ISX.

Here, fluorescence was shown in eight of the possible 10 channels. Fluorescent signals could be interpreted as “on” or “off” binary states based on an area or signal strength feature in IDEAS. Each of these ten channels could then be combined together to code 2^{10} different materials. However, even with rigorous fluorescence-minus-one (FMO) compensation for spectral overlap, it may be difficult to discern this large number of barcodes. Instead, a more realistic estimate is 500 different fluorescence barcodes based on the claims of the commercially available Luminex system. Together, these barcoding variables can yield up to 16,000 (from $8 \times 4 \times 500$) barcodes.

Microgel Fabrication Scale-Up

Fabrication throughput was scaled up via direct spincoating of prepolymer onto silicon wafers and exposure through a photomask (Supplementary Figure 2A). Using a 10cm wafer (a common larger size) and arrays that cover the whole wafer, replicate number can be

increased. This increase will enable higher levels of significance (Supplementary Figure 2B). For these calculations, the standard deviation of the mean²⁷ was set to $p = (0.5 * (1 - 0.5)/n)$, and p was calculated for each known n .

Flow Cytometer Data Acquisition

Microgels were collected by centrifugation and suspended in 100-200 μ L of PBS. Samples were run on an ImageStreamX Mark II flow cytometer at the lowest fluidics speed and images were analysed with IDEAS software. There were few images showing more than one shape passing the interrogation point at the same time (“doublets”), these occurred primarily in the beginning of the analysis period and were eliminated during analysis. However, future analyses may be able to incorporate these images. Some cross-contamination was detected between sample runs (slightly decreasing the calculated accuracies for the test dataset) and is estimated by the manufacturer at <0.5% carryover during use of the AutoSampler.

IDEAS Analysis

A training population of at minimum 25 images was defined by eye for each of the twelve tested SSF barcodes. ImageJ was used for cell viability and loading calculations. Based on feature comparisons, six features defined by IDEAS (Aspect Ratio, LobeCount, Symmetry2, Symmetry3, Symmetry4, and Circularity) were selected as the most effective at distinguishing shape. A seventh feature, Area, was selected to separate sizes within a shape population.

The DHR was clearly visible in the second channel with a 488 nm laser voltage of 20 mW. To measure the ROS activity quantitatively, two additional features calculated by IDEAS (“Modulation” and “H Homogeneity Mean”) were used to identify stained cells. A new gate was created to separate empty constructs from cell-containing constructs.

Images with desirable orientation relative to the camera were gated by graphing Area vs. Aspect Ratio and collecting the events with sufficient size for a microhydrogel and a high aspect ratio. Microparticles tilted relative to the camera would not be distinguishable by shape and were discarded during collection based on low aspect ratio (Supplementary Figure 3A).

Centroid-Contour Distance Image Analysis

The images transferred from IDEAS were analyzed by centroid-contour distance image analysis. Given a binary image of an object (i.e., a microgels), first the centroid of the object was computed. From the centroid of the object, if one were to move along the direction of the horizontal axis (i.e. 0 degree direction), the distance to the edge of the object represents the “radius” of the object along the 0-degree direction. The described distance was calculated for all directions from 0 to 359 degrees, and a vector of length 360 representing the “radius” of the object along all directions was obtained. Further the centroid-contour distance vector was converted into another vector of the same length using Fast Fourier Transform (FFT), which represents the edge of the object in the frequency domain.

Results and discussion

Fabrication of Microhydrogels

Biomaterials are defined in part by their ability to interact with cells in a variety of ways (Figure 1A) and PEG-based materials are popular for their ease of handling, modification, and favorable *in vivo* responses. Therefore PEG-based polymers were used to fabricate twelve test populations of barcoded microhydrogels using soft lithographic methods (Figure 1B)^{16-18, 20}. Both alginate and poly(ethylene glycol)-based hydrogels, were tested to demonstrate method versatility as described by previous papers, however, only PEG-based microgels are shown here (Figure 1C). These materials enabled rapid microgel fabrication, liquid suspension handling, and are easily chemically modified to mimic specific tissues.

High “n” was achieved by using arrays of $>10^4$ molds per 2 μL of precursor; tens of thousands of replicate microgels were produced and harvested (overall process, Figure 1D). For each starting volume of 2 μL of prepolymer, the average number of collected microgels was $\sim 3,500$. When scaled to a 20 μL starting volume, this number of microgels jumped to $\sim 49,200$, showing that collection efficiency was slightly higher with higher areas since mold edges were left empty to prevent film formation. Notably, the same shape/size/fluorescence (SSF) barcoding could be expanded further^{28, 29} (Supplementary Figure 1) to non-imaging flow cytometers which are more widely available, have more fluorescent color channels, and can physically sort populations of interest. As imaging flow cytometers become more commonplace and more powerful, 3DMaC can similarly increase in multiplexing capacity to accommodate studies of even more materials and therefore is a promising way to improve biomaterials design. Supplementary Figure 2 shows how the number of replicates for each microgel type (barcode) can be increased further to $>10^6$ using conventional whole wafer printing.

Cell Processing and Functional Measurements

Biomaterial microgels containing tumor and tumor-supporting cells, and stem cells were chosen for exploratory studies. Both cell types have heterogeneous population characteristics and a behavioral dependence on encapsulating material^{3, 30}; and would therefore benefit from population-level statistics gathered from individual events. In stem cell research, the starting population can demonstrate a range of responses to identical biomaterial cues, and in cancer research *in vivo* behavior is best mimicked when tumor cells are co-cultured with supporting cells in an appropriate biomaterial niche^{3, 31}. Therefore, while this method can easily be applied to single cell systems (similar to traditional FACS analysis), this method is unique in that it can also analyze entire cell niches within a biomaterial. While shape has important implications for 2D studies³², no such dependence has been reported in 3D microgel culture. However, this remains an interesting biological question for future studies and would need to be studied for each specific cell-biomaterial system of interest.

A wide range of cell concentrations could be achieved in the selected microhydrogel geometries, which is an important variable in cell-niche interaction studies (Figure 2A). Lower cell concentrations (10^5 cells/mL) mimic single cell-biomaterial interactions within a niche and higher cell concentrations (10^6 - 10^7 cells/mL) could be used to mimic organoids or

tissue microenvironments. For each of the stated concentrations the number of cells per microhydrogel was quantified using fluorescence microscopy and ImageJ analysis of more than 185 wells. Figure 2B shows the low concentration case as a number average to demonstrate the proportion of wells with at least a single cell. In this lower concentration case, the majority of microgels contain either zero cells (which can be discarded during analysis) or a single cell in the context of the encapsulating biomaterial. For the higher concentration case (Figure 2C), a weighted average is shown instead to demonstrate the likely multi-cell community size.

Therefore, the researcher can control the cell loading based on starting concentration depending on the needs of the experiment. In all cases, DAPI staining of the nuclei can be used to provide a direct quantification of the number of cells included in each microhydrogel. This additional variable can then be used to sort the data points in IDEAS such that each type of cellular community is studied in context. Fluorescent images of stained cells were collected and ImageJ used to quantify live and dead cells (for a total of more than 550 cells). This confirmed that cells remain viable post-gelation (Figure 2D) with approximately 79.9% $\pm$ 1.1% viability 24 hours after crosslinking and no difference between viability in square and circle microhydrogels.

Cell Function in Microhydrogels

Two example assays were performed on arrays of microhydrogels to demonstrate the versatility of the system. Similar assays have been shown by other groups and could be adapted for 3DMAc studies.^{16, 18, 20, 22, 23} In this example, circle-shaped microhydrogels consisted of 15% poly(ethylene glycol) diacrylate (Mw 3400) and 5% 4-armed poly(ethylene glycol) thiol (Mw 10,000) in supplemented media. Square-shaped microhydrogels had the same starting polymer supplemented with 0.66 μ M RGD peptide. Arrays were formed on PDMS via the same process described previously, however, in this case the top layer of PDMS was applied gently rather than tightly pressed. This leaves a thin layer of polymer between microhydrogels such that a large number of replicates in an array format can be easily imaged using microscopy.

Reactive oxygen species are oxygen radicals that can interfere with intracellular reactions, and a good measure of stress in the cell. In particular, ROS can be generated via free-radical crosslinking mechanisms which may impact cell viability and function. ROS activity is therefore an example of a short-term assay that could be performed on encapsulated live cells to determine (1) if different materials provide protection from ROS activity or (2) if external culture conditions such as drug concentration lead to cellular stress. The second method shows how traditional flow cytometry assays based on antibody staining are still applicable in the microhydrogel system. Microhydrogels can be fixed and permeabilized such that fluorescently-labelled antibodies can be incubated with the constructs such that they bind cell surface or intracellular markers.

To measure ROS activity, hMSC-containing microhydrogels were stained with DHR for 30 minutes immediately after crosslinking, rinsed, and imaged (Figure 2E). These microhydrogels showed that the cells were actively converting the dye and therefore that they were experiencing ROS activity, which is to be expected after the crosslinking. A

separate set of arrays were also imaged the next day (not shown) and fluorescence was reduced, indicating that the cellular stress was not a function of the encapsulating material.

To demonstrate antibody staining, hMSC-containing microhydrogels were fixed for 30 minutes immediately after crosslinking, then blocked with BSA and permeabilized with Triton-X100. Microhydrogels were incubated for one day with antibodies and DAPI to label CD105 and the cell nucleus respectively. CD105 is a constituent surface marker of hMSCs and therefore could be expected to have high expression and good staining. Previous characterization of the cells used here has been done by our group to confirm high CD105 expression (data not shown). Images of staining microhydrogel arrays were collected on a confocal scanning fluorescent microscope (Figure 2F). The anti-CD105-PE antibody used here was designed for flow cytometry studies, but can still be seen in the confocal microscope (DAPI staining not shown). Staining was present on all visible cells as was expected. These microhydrogels were imaged on the ISX to confirm that labelling would be seen in the flow cytometer (Figure 2G). Brightfield images show that the circle and square shapes could be distinguished, and that the DAPI and PE staining were visible in fluorescence channels.

ImageStream Data Collection and Barcoding

Representative brightfield images collected by the ISX for each of the test barcodes are shown (Figure 3A). The pre-defined shape and size barcodes are clearly identifiable in collected images and undesirable images were discarded based on area and aspect ratio (Supplementary Figure 3A). To quantify the barcodes computationally, masks were drawn on the brightfield images (Figure 3B) and greater than 75 features calculated for each image. To further increase the degree of multiplexing, fluorescent dyes visible in up to ten different channels (the maximum of the ISX) were added to the prepolymer. Microhydrogels were tagged in various fluorescent combinations to demonstrate, as proof of concept, six possible codes that could be layered with the shape/size signature (Figure 3C).

Based on the sensitivity of the ISX to SSF barcoding, the estimated number of barcodes available would be greater than 300 (Table 1). By using the 10 fluorescent channels in combination, a theoretical 2^{10} fluorescent barcoding indices are possible with the application of established compensation protocols³³, which would potentially expand the number of barcodes significantly. However, a more reasonable estimate is the 500 combinations already demonstrated by the commercially-available Luminex system. Each additional color used in barcoding removes a fluorescence channel that could be used for detection, but adds another multiplexing variable. Similar to a Luminex system, relative fluorescence intensity could further expand the number of available barcodes. Estimates are also given for a non-imaging flow cytometer with a larger number of potential fluorescent barcodes and possible untapped potential to distinguish shape.

IDEAS Data Analysis

Data were analyzed using the IDEAS software from EMD Millipore which is designed to analyze ImageStream data. An analysis template was defined based on microgels of known SSF barcode ("truth populations") and data collection rates were shown to be similar

(Supplementary Figure 3B). Similar to traditional gating methods, data could be plotted according to user-chosen parameters, subpopulations identified, and the data further defined. For each shape, true subpopulations were compared to extract features which give the greatest separation between groups. Similar to the shape and size gating, fluorescence signatures could be quantified using several of the IDEAS values. From these measurements, the Area and Max Pixel Intensity variables were selected for the highest accuracy separation of the fluorescent barcodes. Additionally, calibration beads were used as a proxy for cells and run at different concentrations to develop a spot counting template. The selected features for the beads were (in order) Symmetry2, Aspect Ratio Intensity, Major Axis Intensity, and Symmetry4. However, when counting actual CFSE-labelled cells with DAPI-labelled nuclei, instead the principle components were (in order) SpotCount, Major Axis Intensity, Perimeter Threshold, Compactness, Shape Ratio, and Symmetry2. IDEAS also defaulted to using the strong CFSE staining over the relatively weaker DAPI staining to count and define the different number of cells.

This template was applied to a test dataset of over 85,000 events and graphed. Both triangular shapes separate on two features but the squares and circles are overlaid (Figure 4A). Additional gating was applied to generate Figure 4B and Figure 4C (full scheme, Supplementary Figure 4) For each shape subpopulation, an area histogram shows three distinctly sized subpopulations gated such that the twelve barcodes are defined in the dataset (Figure 2c). Using the described sorting scheme, ~9000 of the images were gated into one of the twelve final populations. The IDEAS gating achieves an average accuracy of 82% for all twelve populations and an average of 88% for the 40 and 60 μ m populations (Figure 5A). Once designed, this sorting template can be applied directly to new datasets for automated identification of barcoded populations similar to traditional flow cytometry. Therefore, IDEAS-based analysis is scalable with regards to dataset size, and a single experiment could be performed and analyzed within a single day.

Next, this analysis template was applied to cell-containing microhydrogels. D1 cells were suspended in 30% poly(ethylene glycol) diacrylate at a concentration of ~60 million cells/mL for 60 μ m circles and 60 μ m squares. In this experiment, 60 μ m Squares and Circles were considered as they were the most challenging shapes to separate in IDEAS. Future studies will expand the cell template to additional shapes and sizes. Here, Circles were generated with 30 seconds of UV exposure (previously selected for optimal cell viability and crosslinking); Squares were exposed for 2 minutes and therefore 4 $\times$ the exposure. Given that free-radical polymerizations can generate harmful levels of ROS, DHR dye was selected to measure the resulting ROS activity in the context of the two microenvironments. Cells were stained for 60 minutes, washed with PBS, and stored on ice before analysis in the ISX.

As in the previous analysis, two truth populations were collected of only Circles (~48,000 events) and only Squares (~26,000 events). The template developed for non-cell-containing microhydrogels was applied and the Circle and Square populations were measured. The template still identified the two true populations with reasonable accuracy (Figure 4D). However, the rate of mis-formed hydrogels was higher in the cell-containing prepolymer as cells disrupted edge features and so throughput was lower in the cell-containing system.

There was also overlap into the Right Triangle gate which would need to be addressed in future by adding more feature gates to the template (Supplementary Figure 10). This experiment showed that free cells, which are often included in analyzed population due to the thin layer of oxygen-inhibited prepolymer in PDMS molding, were collected in the 20 μm gates and so this barcode would need modification in the template. Therefore the data in this assay was not gated by size, only shape. Because the amount of swelling will vary by polymer composition, size gates may need to be adjusted for each tested material.

After initial sorting, the data from the pure populations were computationally combined to analyze the difference in the two conditions. Constructs were gated into the desired populations using the above template with an additional gating layer for fluorescence signal to remove empty gels from analysis (Supplementary Figure 11). The fluorescence intensity of each pure population was divided by the fluorescent area (to account for cell number). Feature values for those populations were then exported and averaged. Based on this example study, the Circles had an average of 309.95 $\pm$ 169.53 and the Squares had 342.07 $\pm$ 151.91. Therefore there was no significant difference between the two groups either due to lowered accuracy in the sorting template (Supplementary Figure 11A) or insufficient biological differences between the groups tested.

Centroid-Contour Distance Analysis

Since the IDEAS software is designed to analyze cells, not the geometric microgels used in this study, we explored an alternative analysis pipeline using frequency domain features of the centroid-contour distance function³⁴, Random Forest³⁵, and t-Distributed Stochastic Neighbor Embedding (t-SNE)³⁶ for visualization. A custom pre-processing pipeline was developed to remove low-quality images (Supplementary Figures 5 and 6). Fast Fourier Transform (FFT)³⁷ of the centroid-contour distance function is used to derive frequency domain representations of the shapes, which are invariant to translation and rotation. Random Forest³⁵ was performed on a manually selected set of high-quality images, to identify features information for classifying the microgels into various shape and size categories. FFT0 represents the size of the microgels, and the shape is represented by FFT1~11 normalized by FFT0. As shown in Supplementary Figure 7, the normalized frequency features of perfect shapes exhibit strong zero/non-zero patterns. Normalized FFT1~11 of the perfect circle are all zero, whereas for squares and triangles, only subsets of the FFTs are non-zero.

Based on the zero/non-zero patterns of the normalized frequency representation, we derived three features to separate the circles, squares and triangles (Supplementary Figure 8). The nearest neighbor approach was taken for classifying the right triangles as their normalized FFT do not exhibit a distinctive zero/non-zero pattern. Euclidean distances between the normalized FFT of each image and the perfect right triangle were calculated, and a threshold was applied to identify the right-triangle microgels (Supplementary Figure 9). The first row of Figure 5B shows that the derived features achieved high precision shape classification. Figure 5C visualizes the images classified into the four shapes, in the context of the entire dataset. In principle component analysis (PCA), the two types of triangles are separated, while the circles and squares overlap slightly. In nonlinear visualization derived by t-SNE,

the four different shapes are clearly separated, providing an opportunity to classify the shapes by manual gating. Figure 5D shows four gates manually drawn on the t-SNE visualization. If the shapes were classified by the manual gates on the t-SNE visualization, the accuracy would be comparable to thresholding the features derived from the normalized FFT representation (Table S1).

After classifying the microgels by shapes, each shape group was separately classified into three sizes based on FFT0. By fitting Gaussian distributions for each size group, thresholds were set based on the intersections of the distributions, similar to Figure 4C. The performance of size classification was evaluated (Figure 5B), and the accuracy were comparable to manual gating of the features derived from the IDEAS software (Figure 5A), but the centroid distance and frequency analysis were able to classify a larger number of images to maintain higher overall image throughput.

Conclusions

This work demonstrates for the first time a high replicate, high-throughput system for biomaterial analysis using imaging flow cytometry and micro-fabricated cell-biomaterial niches, and its application to a biomaterials problem. Using the 3DMaC barcoding and analysis method, greater than 85,000 events were collected and gated with high accuracies into the constituent twelve test barcodes of shape and size. The image analysis based on radius-basis and frequency domain feature distinguished the convex shapes used in this study and the nonlinear t-SNE visualization of the frequency features provides an excellent opportunity to classify shape via manual gating. As in traditional flow cytometry gating schemes, a balance is struck between accuracy and throughput; generally one route is chosen depending on the needs of the experiment. In the future, if new shapes are included in the population, the analysis can be extended. The classification performance can be further improved by enhancing the preprocessing pipeline's ability to exclude low quality images.

By combining all samples into a pooled population, both material- and cell-based variables should be better standardized to reduce variation between experimental conditions. Already we have shown that 3DMaC collects enough individual replicates to produce data with high statistical power for each biomaterial of interest. Based on the sensitivity of the ImageStreamX, the estimated total number of barcodes that could designate different material compositions would be greater than 300. Each additional color used in barcoding would occupy a fluorescent channel that could otherwise be used for detection, but adds another multiplexing variable. Similar to the Luminex labelling system, relative fluorescence intensity could further expand the number of available barcodes to achieve unmatched numbers of biomaterials in a single assay.

In this study, the method was applied to biomaterial-based scaffolding microshapes but is equally applicable to other high-throughput screening targets such as analyte-sensing constructs^{19, 22, 23}. Most excitingly, existing non-imaging flow cytometers have the ability to physically sort microparticles (FACS) based on user-designed gating schemes such that individual events are separated out of the population and collected for further manipulation and analysis. Biomaterials screening could then be applied to translational work as

encapsulated cells are cultured, analyzed, and then only the highest performing constructs selected for implantation. As imaging flow cytometer technology continues to improve³⁸ and as imaging flow cytometers enter wider circulation, the 3DMAc method could help answer many questions of interest in the biomaterials community.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. microhydrogel fabrication and testing (a) cells interact with the surrounding microenvironment via receptors for solutes, scaffold-bound ecm-mimetics, and can perform mechanosensing. receptors also enable cell-cell communication and intercellular exchange. these interactions occur on the micro- and nanoscale. (b) soft lithography is used to produce arrays of pdms molds with wells of designed shape/size/height. a dime is shown to represent the mold area needed for >104 replicates. (c) plasma activation renders the pdms surface hydrophilic and prepolymer is sandwiched between the molds and crosslinked using uv light. these are then hydrated in media to facilitate collection. scale bar is 300 μm (d) an example 3dmac experimental process flow starts with cells, four prepolymer materials, and four activated molds. all molds shown are $\sim 600 \mu\text{m}$ in length and $\sim 480 \mu\text{m}$ in height. these shapes then barcode the microgel corresponding to the encapsulating biomaterial. collected microgels are cultured and analysed using flow cytometry. the scatterplot shows representative data where each color corresponds to a shape barcode (square-blue, circle-red, equilateral triangle-green, right triangle-purple) and each point is a separate microgel measurement.

Figure 2.

cell incorporation into microhydrogels (a) microenvironments constructed with sparse or dense cell packing to model different microenvironments. three cell populations were pre-stained with celltrace dyes to show tumor microniche cell distribution (t cells-green, dendritic cells-blue, a20s-red). (b) cell number was counted in sparsely populated microhydrogels and fit to a one-phase decay curve. (c) cell number was counted for the densely populated microhydrogels and the weighted average was graphed with a gaussian fit (d) viability of encapsulated cells after 24hr shown with live (green) and dead (red) staining. (e) phase contrast and fluorescence images of array-fixed microhydrogels containing hmscs incubated with dihydrorhodamine 1,2,3 to show ros activity in the cells immediately after crosslinking. the fluorescence image was colored green using imagej (f) phase contrast and fluorescence images of array-fixed microhydrogels containing hmscs that were fixed and stained in the gel with anti-human cd105-pe antibody. scale bars are 50μm (a) and 100μm (d), microhydrogels in (e, f) were formed in 60 μm molds.

Figure 3. imagestream microhydrogel barcoding. (a) composite image of microhydrogel barcodes captured by the ixz with desired orientation relative to camera. these twelve images would constitute good representatives of each test population and show the twelve barcodes based on shape and size. (b) composite image of masking view defines the areas used to calculate features and can be designed in ideas (mask views from left: none, default “object”, human-refined “object”, and human-generated “membrane”). masks can also be drawn in the fluorescence channels (c) ten fluorescent channels provide additional multiplexing variables.

shown are six different fluorescent barcodes demonstrated in several shapes and sizes. the isx has ten fluorescence channels which correspond to six filters (430-505, 480-560, 560-595, 595-640, 640-745, and 745-800). all scale bars in this figure are 20 μm and were added in ideas.

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Figure 4.

ideas data analysis. plots of true populations used to define the four shapes and three sizes of microhydrogels (colors correspond to user-defined data labels: squares 20 μm -aqua, 40 μm -periwinkle, 60 μm -blue; equilateral triangle 20 μm -yellow, 40 μm -neon green, 60 μm -green; right triangles: 20 μm -pink, 40 μm -magenta, 60 μm -purple; circles: 20 μm -gold, 40 μm -orange, 60 μm -red). (a) preliminary shape gating using two features shows three populations with the triangular populations separated and the squares and circles overlapped. (b) application of an additional two features defines all four shapes present in the system. (c) histograms of the area feature for each shaped population can be gated by size to define all twelve barcodes as distinguished by color within each panel. (d) clustering of shaped, cell-containing microhydrogels into circles (left) and squares (right) with the true circle

population displayed in red and the true square population in blue. the circle gating collected 2043 events and the square gating collected 597 events.

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Figure 5. imagestream data analysis. (a) evaluation of ideas sorting accuracy for a test template. percentages represent the number of events from the true run divided by the total number of events in a gate. (b) evaluation of centroid distance analysis. in the first row, percentages represent the proportion of images with correct known shape among the classified images. the remaining rows represent the performance after subsequent classification based on size. (c) pca plot. right triangles and other three shapes are well separated by pca-1. the two types of triangles are separated from circles and squares according to pca-2. however, circles and

squares overlap. (d) t-sne map. each dot represents an image of a certain shape. the gray dots correspond to images that cannot be classified with high confidence, and colored dots correspond to images can be confidentially classified as one of the four shapes with each color representing one of the four shapes. all four shapes are well separated. the black convex hulls are manually drawn gates that can be used to identify the four shapes.

Table 1

System	Parameters			Total Multiplexing
	Cross-section	Size	Fluorescence	
ImageStreamX (ISX)	Square, Circle, Right Triangle, Equilateral Triangle, Rectangle (2×, 3×, 4×), Pentagon	20, 40, 60, 80µm -> 4	10 Channels	Over 300
			500 Compensated Combinations	~16,000
BD LSR Fortessa (LSR)	Square, Circle, Triangle, Rod	25-75µm -> 3	18 Channels	Up to 216