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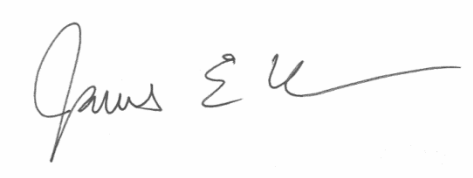
Dear Dr. Cao:

Thank you for the thoughtful reviews of our manuscript. We believe we have addressed all of the concerns raised with this resubmitted version.

Thank you for your consideration, we look forward to hearing from you.

Sincerely,



James Korkola, Ph.D.

TITLE:

Using Microarrays to Interrogate Microenvironmental Impact on Cellular Phenotypes in Cancer

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microenvironment, microarray, MEMA, breast cancer, cellular phenotype, proliferation

SUMMARY:

The purpose of the method presented here is to show how microenvironment microarrays (MEMA) can be fabricated and used to interrogate the impact of thousands of simple combinatorial microenvironments on the phenotype of cultured cells.

ABSTRACT:

Understanding the impact of the microenvironment on the phenotype of cells is a difficult problem due to the complex mixture of both soluble growth factors and matrix-associated proteins in the microenvironment in vivo. Furthermore, readily available reagents for the modeling of microenvironments in vitro typically utilize complex mixtures of proteins that are incompletely defined and suffer from batch to batch variability. The microenvironment microarray (MEMA) platform allows for the assessment of thousands of simple combinations of microenvironment proteins for their impact on cellular phenotypes in a single assay. The MEMAs are prepared in well plates, which allows the addition of individual ligands to separate wells containing arrayed extracellular matrix (ECM) proteins. The combination of the soluble ligand

with each printed ECM forms a unique combination. A typical MEMA assay contains greater than 2,500 unique combinatorial microenvironments that cells are exposed to in a single assay. As a test case, the breast cancer cell line MCF7 was plated on the MEMA platform. Analysis of this assay identified factors that both enhance and inhibit the growth and proliferation of these cells. The MEMA platform is highly flexible and can be extended for use with other biological questions beyond cancer research.

INTRODUCTION:

Culturing of cancer cell lines on plastic in two-dimensional (2D) monolayers remains one of the major workhorses for cancer researchers. However, the microenvironment is increasingly being recognized for its ability to impact cellular phenotypes. In cancer, the tumor microenvironment is known to influence multiple cellular behaviors, including growth, survival, invasion, and response to therapy[1,2]. Traditional monolayer cell cultures typically lack microenvironment influences, which has led to the development of more complex three-dimensional (3D) assays to grow cells, including commercially available purified basement membrane extracts. However, these purified matrices are typically complicated to use and suffer from technical problems such as batch variability[3] and complex compositions[3]. As a result, it can be difficult to assign function to specific proteins that may be impacting cellular phenotypes[3].

To address these limitations, we have developed the microenvironment microarray (MEMA) technology, which reduces the microenvironment down to simple combinations of extracellular matrix (ECM) and soluble growth factor proteins[4,5]. The MEMA platform enables identification of dominant microenvironmental factors that impact the behavior of cells. By using an array format, thousands of combinations of microenvironment factors can be assayed in a single experiment. The MEMA described here interrogates ~2,500 different unique microenvironment conditions. ECM proteins printed into well plates form growth pads upon which cells can be cultured. Soluble ligands are added to individual wells, creating unique combinatorial microenvironments (ECM + ligand) on each different spot to which the cells are exposed. Cells are cultured for several days, then fixed, stained, and imaged to assess cellular phenotypes as a result of exposure to these specific microenvironment combinations. Since the microenvironments are simple combinations, it is straightforward to identify proteins that drive major phenotypic changes in cells. MEMAs have been used successfully to identify factors that influence multiple cellular phenotypes, including those that drive cell fate decisions and response to therapy[4–7]. These responses can be validated in simple 2D experiments and can then be assessed under conditions that more fully recapitulate the complexity of the tumor microenvironment. The MEMA platform is highly adaptable to a variety of cell types and endpoints, provided that good phenotypic biomarkers are available.

PROTOCOL:

NOTE: An overview of the entire MEMA process, including estimated time, is outlined in the flow diagram shown in **Figure 1**. This protocol details the fabrication of MEMAs in 8-well plates. The protocol may be adapted for other plates or slides.

1. Preparation of protein, diluent, and staining buffers

1.1. Equilibrate vials of ECMs, ligands, and cytokines to room temperature (RT) and briefly centrifuge. Add the appropriate volume of the appropriate RT buffer as indicated on the product data sheet. Follow manufacturer's recommendation for stock concentrations.

NOTE: A full list of the ligands and ECMs with their stock and final concentrations are provided in **Table 1** and **Table 2**. Both ligands and ECMs are typically used at the highest concentration of the range recommended by the manufacturer that elicits a biological effect in standard 2-d culture assays. Handle proteins gently and in biosafety cabinets under laminar flow to avoid contamination.

1.2. Incubate vials with gentle rocking at RT for 1 h. Do not vortex proteins as this can cause them to denature.

1.3. Aliquot proteins for long term storage so that all aliquots are single use only to avoid degradation with repeated freeze/thaw cycles. Store lyophilized proteins at -80 °C (unless otherwise specified) until needed. Take care to collect all metadata for future reference, such as: (i) protein name, (ii) date prepared, (iii) lot/batch number, (iv) supplier, (v) catalog number, (vi) concentration, (vii) volume, and (viii) preparer.

1.4. Prepare diluent buffer containing 20% (v/v) glycerol, 10 mM EDTA, 200 mM Tris-HCl, pH 7.2, and filter sterilize. Keep this buffer sterile and store at RT.

1.5. Prepare staining buffer containing 2% (w/v) BSA, 1 mM $MgCl_2$, and 0.02% NaN_3 in phosphate-buffered saline (PBS). Filter and store at 4 °C.

2. Preparation of an ECM source plate

2.1. Remove aliquoted stocks of ECM proteins to be printed and thaw on ice. Record all lot numbers for metadata tracking.

2.2. Flick tubes of thawed proteins gently to ensure proper resuspension and spin down in a centrifuge.

2.3. Make ECM print mixtures (EPMs) and a fluorescent fiducial to be used by a liquid handling robot that will create the randomized 384-well source plates.

NOTE: The 384-well source plates will be used by a touch pin array printer to create the printed arrays in 8 well plates.

2.3.1. Label 1.5 mL microcentrifuge tubes for each EPM and the fiducial.

2.3.2. Prepare each EPM by combining 125 µL of diluent buffer (see step 1.4) with the appropriate volume of ECM stock and bring the mixture up to a total volume of 250 µL with PBS. The final concentrations in each EPM tube will be 1× ECM protein, 5 mM EDTA, 10% glycerol, and 100 mM Tris.

2.3.3. Prepare a fluorescent fiducial by dissolving it in the appropriate buffer specified by the manufacturer and transfer 250 µL to a labelled fiducial tube.

3. Creation of the source plate using a liquid handler

3.1. Design a 384-well plate layout that randomizes the positions of the ECMs and is optimized for the array printer pin head being used. Design the placement of the fiducial so that it will be printed in the row 1, column 1 position of each well to assist in array orientation.

NOTE: A total of 14−15 replicates of each ECM are used to ensure robust data. Include additional replicates of collagen or another ECM that yields robust attachment for assessment of uniformity of binding. The layout may need to utilize multiple 384-well plates depending on the number of ECMs of interest.

3.2. Transfer EPM tubes to a liquid handler, keeping tubes at 4 °C either with a cooled tube rack or by using a liquid handling robot located in a cold room.

3.3. Using the liquid handler's software, run a program to transfer 15 µL of each EPM and the fiducial to the predesignated wells within the 384-well source plate(s).

3.4. Pipet PBS into any unused wells to increase humidity and guard against desiccation during the printing process.

NOTE: See **Figure 2** for an example of a 384-well source plate set that is optimized for a 4 x 7 pin head and includes a collagen I block and PBS.

3.5. Seal plate(s) and keep at 4 °C until ready to print.

4. Printing MEMAs using an array printing robot

NOTE: The following part of the protocol specifically describes the preparation and use of MEMA to investigate the impact of different microenvironment proteins on the growth and proliferation of MCF7 cells. However, the protocol can easily be adapted to use different ligands, ECMs, and cells to study other cell lines and endpoints of interest.

4.1. Using a touch pin printer, print EPMs and fiducial spots into 8 well plates. Print multiple replicates of each ECM condition to ensure reproducibility.

NOTE: Other plate formats or slides can be used for printing, but buffer optimization may be required to achieve optimal spot formation.

4.1.1. Print the ECMs for the MEMA using 350 µm diameter pins arranged in a 4 × 7 print head configuration. Print the arrays in the 8-well plates as 20 columns by 35 rows, for a total of ~700 spots. Larger arrays are possible in these plates but come with a trade-off of increased edge effects in both cell binding and staining.

4.2. After printing, store plates in a desiccator for a minimum of 3 days prior to use.

5. Creation of ligand treatment plates

5.1. Design a 96-well plate layout including ligands of interest. To facilitate treatment of many MEMA plates at once, design this plate with spacing that allows for the use of a multi-channel pipet with 4 spaced tips to transfer liquids between the wells of 8-well MEMAs and a 96-well plate.

NOTE: In this protocol, the full set of ligands listed in **Table 2** are utilized.

5.2. Thaw ligands on ice. Briefly flick and spin down each tube.

5.3. Dilute ligands to a 200x working stock using the manufacturer's recommended buffer (typically PBS).

5.4. Pipet 10 µL of each 200x ligand stock into the corresponding well within the 96-well plate.

5.5. Seal and store plates at -20 °C.

NOTE: Make ligand treatment plates in batches, capturing all metadata for downstream analysis.

6. Culturing cells on MEMAs

6.1. Block MEMAs for 20 min with 2 mL per well of non-fouling blocking buffer containing 1% non-fouling blocking agent (**Table of Materials**) in double-distilled water (ddH_2O).

6.2. Aspirate blocking buffer and triple rinse wells with PBS. To prevent desiccation, leave final volume of PBS in wells until ready for cell plating.

NOTE: It is extremely helpful to have two bench workers for cell culture steps on MEMAs. One bench worker can perform aspiration steps, while the second performs addition steps. It is recommended to use a 1 mL multichannel pipet with tips spaced to match the 8-well plate for pipetting and a Y-splitter with two Pasteur pipettes to aspirate multiple wells at once.

6.3. Seed 2 x 10^5 MCF7 cells per well in 2 mL of Dulbecco's modified Eagle's medium (DMEM) medium containing 10% fetal bovine serum (FBS).

NOTE: Prior to a full MEMA experiment, perform a cell titration experiment to optimize cell numbers such that MEMA spots have high cell numbers (but are not confluent) at the end of the desired experimental duration.

6.4. After 2–18 h of adhesion, aspirate medium and replace with 2 mL of reduced-growth medium (DMEM with 0.1% FBS).

NOTE: Reduced serum (e.g., 0.1% FBS) or growth factor-depleted conditions can be used at this time to isolate the stimulatory impact of specific ligands.

6.5. Thaw a ligand treatment plate on ice. Centrifuge thawed plate at 200 x g for 1 min.

6.6. Transfer 200 µL of medium from each well in the culture plate to the appropriate well in the treatment plate. Pipet up and down to mix ligand volume with medium and transfer this mixture back to the appropriate well in the MEMA plate.

6.7. Lightly rock by hand and return MEMA plates to the incubator. Culture for the duration of the experiment in the presence of the ligand/ECM combination at 37 °C and 5% CO_2.

NOTE: A typical MEMA experiment runs for 72 h; longer duration experiments may require replacement of medium and re-treatment with ligand.

6.8. Pulse MEMA wells at 71 h with 100x 5-ethynyl-2'-deoxyuridine (EdU) for a final concentration of 10 µM. Incubate in experimental conditions with EdU for 1 h at 37 °C and 5% CO_2.

NOTE: Other live cell treatments may also be used at this time.

7. Fixing and staining MEMAs

7.1. After 72 h and any live cell treatments, aspirate wells. Fix MEMAs in 2 mL per well of 2% paraformaldehyde (PFA) for 15 min at RT.

7.2. Aspirate PFA. Permeabilize with 2 mL per well of 0.1% nonionic surfactant for 15 min.

7.3. Aspirate the nonionic surfactant and wash with 2 mL per well of PBS. Aspirate PBS. Wash with 2 mL of PBS with 0.05% polysorbate 20 (PBS-T).

NOTE: The MEMA surface is hydrophobic, and failure to wash with PBS-T before stain and antibody incubation will result in the formation of voids in wells during incubation steps and give rise to staining artifacts.

7.4. Aspirate PBS-T. Add EdU detection reaction reagents. Incubate for 1 h at RT, rocking and protected from light. After 1 h incubation, quench reaction with the provided commercial quench buffer.

NOTE: EdU detection and staining/antibody steps may be performed in 1.5 mL per well to reduce cost.

7.5. Aspirate the quench buffer and wash with PBS-T prior to incubating with stains or antibodies.

7.6. Incubate MEMA wells with antibodies against histone H3K9me3 (1:1000) and fibrillarin (1:400) in staining buffer containing 2% (w/v) bovine serum albumin (BSA), 1 mM $MgCl_2$ and 0.02% NaN_3 overnight at 4 °C.

NOTE: Perform antibody titrations to determine optimal concentrations prior to using them on a full MEMA set.

7.7. Following primary antibody or stain incubation, wash wells 2x with PBS and once with PBS-T.

7.8. Add secondary antibodies (donkey anti-mouse IgG and donkey anti-rabbit IgG, both 1:300) and 0.5 µg/mL 4' 6-diamidino-2-phenylindole (DAPI). Incubate for 1 h at RT in the dark.

7.9. Wash wells 2x with 2 mL per well of PBS, leaving them in the final 2 mL PBS.

7.10. Proceed to imaging or store stained MEMAs for later imaging in PBS at 4 °C protected from light.

8. Imaging of MEMAs

8.1. Image MEMA on an automated imaging system with appropriate fluorescent detection channels.

8.2. Output resulting image data to an image management system. Segment cells and calculate intensity levels using CellProfiler[8].

9. Data analysis

NOTE: Data analysis consists of normalization, variation correction, and summarization of the raw CellProfiler derived data. In this instance, the R-environment with custom code is used to perform all the steps. However, any statistical environment or software program can be utilized to perform the equivalent actions. An example of the open source custom code for the R environment for analysis is available at: https://www.synapse.org/#!Synapse:syn2862345/wiki/72486.

9.1. Preprocess and normalize the segmented image data.

9.2. Determine spot cell count using the DAPI stained nuclei.

9.3. Auto-gate EdU intensity to label cells as EdU^+. Measure proliferation using the proportion of EdU^+ cells in each spot.

9.4. Median summarize cytoplasmic stains and nuclear morphology measurements on the spot level.

9.5. Perform removal of unwanted variation (RUV) normalization on the data to improve data quality[9].

NOTE: This approach is applied to each intensity and morphology signal independently as a matrix with arrays using the rows and spots as the columns as described previously[9].

9.6. Apply bivariate loess normalization to the RUV normalized residuals using the array row and array column as the independent variables to correct for spatial or intensity related effects.

9.7. Once normalization is completed, median summarize the replicates for each microenvironment condition for reporting and further analysis.

REPRESENTATIVE RESULTS:

To simplify microenvironmental impacts on cell growth and proliferation and to identify conditions that promoted or inhibited cell growth and proliferation, the breast cancer cell line MCF7 was seeded on a set of eight 8-well MEMAs as described in the protocol. This assay exposed the cells to 48 different ECMs and 57 different ligands, for a total of 2736 combinatorial microenvironmental conditions. After 71 h in culture, cells were pulsed with EdU, fixed, permeablized, and stained with DAPI, the reaction for EdU detection, an anti-fibrillin antibody, and an anti-H3K9me3 antibody. Cells were imaged on a high content microscope. The images were uploaded to an Omero server[10], segmented using CellProfiler[8], and normalized and analyzed in R[9]. The results described below focus on the DAPI and EdU signals.

The image analysis platform of MEMAs yields some results similar to those available from flow cytometry approaches, such as DNA content plots showing 2N and 4N fractions for cells treated with a given ligand (**Figure 3A**), based on the DAPI intensity and area. These plots provide evidence for conditions that promote active cell cycling versus as indicated by clear bimodal peaks corresponding to cells in G1 or G2 phases vs. growth arrested cells, which would show changes in the peaks compared to control conditions. We use the cell number and staining intensity data to summarize the data, where the impact of the microenvironment (ligands on one axis, ECM on the second axis) on both cell number (**Figure 3B**) and EdU incorporation (**Figure 3C**) can be more easily seen as changes in heatmap color and intensity. As seen from these plots, many of the effects are ligand-driven, as the ECM condition did not strongly impact cell number or EdU positivity. Nidogen-1 is a clear exception, as the presence of this ECM molecule inhibits

cell binding and growth of MCF7. Ligands such as FGF6 and NRG1α (NRG1.1 on plots) enhance cell number and have high rates of EdU incorporation, while ligands such as AREG and NRG1-smdf (NRG1.10 on plots) inhibit cell binding and/or growth of cells. These findings are supported by the images of the cells growing on the spots, where a clear difference in cell number and EdU positivity is evident (see example in **Figure 3D**).

Since the MEMA platform is a newer technology, results were validated in separate assays. MCF7 cells were seeded into 24-well plates coated with collagen I in DMEM medium with 10% FBS. After 18 h, media were exchanged for reduced growth medium (DMEM with 0.1% FBS) and cells were treated with NRG1α, FGF6, or AREG and cultured for 72 h. EdU was added 1 h prior to fixation. Cells were stained with DAPI and for EdU incorporation, imaged, segmented, and analyzed. Similar to the results obtained from the MEMA platform, FGF6 and NRG1α both gave rise to higher cell numbers (**Figure 4A**) and EdU incorporation rates (**Figure 4B**) compared to AREG treated cells, validating our observations in the original MEMA experiments.

FIGURE AND TABLE LEGENDS:

Figure 1: Flow chart showing the workflow and timeline for the different phases of a typical MEMA experiment. Once the MEMAs are printed, they can be stored at room temperature desiccated for several months prior to use. Typically, the experimental phase lasts 3−4 days, but some slow growing primary cells have been cultured on MEMAs for up to 2 weeks.

Figure 2: ECM source plate layout for array printing. The collagen block is printed onto MEMA as a grid, which provides a highly repetitive set of conditions that allow for more robust normalization between wells. The PBS-filled wells provide humidity to aid in prevention of evaporation during the printing process.

Figure 3: Examples of data generated from a typical MEMA experiment. (**A**) Cell cycle profiles of binned DAPI intensity values versus cell counts from one 8-well plate treated with different ligands, showing biphasic DAPI intensity staining indicating cells in G1 versus G2 cell cycle phase. (**B**) Heatmap showing normalized spot cell counts clustered by similarity using hierarchical clustering. Red indicates higher cell number, and blue is lower cell number. Ligands are on the x-axis, ECMs are on the y-axis. (**C**) Heatmap showing normalized EdU incorporation, with red indicating higher and blue indicating lower EdU incorporation. Ligands are on the x-axis, ECMs are on the y-axis. (**D**) Example of MCF7 cells growing on a MEMA spot treated with NRG1-α showing high rates of EdU incorporation (pink nuclei). Green stain is cell mask and blue is DAPI.

Figure 4: Validation of MEMA results in cell culture. (**A**) Quantification of cell number resulting from treatment of MCF7 with different ligands. Equivalent numbers of MCF7 cells were plated into multiwall plates then treated with either AREG, FGF6, or NRG1α. Wells treated with AREG had significantly fewer cells than those treated with FGF6 (****** indicating student's t-test p-values less than 0.01) or NRG1α (***** indicates a p-value of 0.05) at 72 h post ligand treatment. (**B**) Quantification of the level of EdU incorporation in MCF7 due to treatment with different ligands, as in panel **A**. AREG treatment results in a significantly lower proportion of cells incorporating

EdU than cells treated with FGF6 (**, $p < 0.01$) or NRG1α (***, $p = 0.01$). Error bars represent standard deviation.

Table 1: The full list of ligands used for the MEMA experiments. The uniprot ID, stock concentrations, and final working concentrations are provided.

Table 2: The full list of ECM proteins and conditions that are used in the MEMA experiments. The uniprot ID, stock concentrations, and final working concentrations are provided. In some instances, the printed condition represents a protein complex or a combination of multiple proteins, which is indicated in the Notes column.

DISCUSSION:

The importance of "dimensionality" and context has been a motivating factor in the development of in vitro culture systems as tools in the characterization of cancer cells through their interaction with the microenvironment[11], and the ability of in vitro culture systems to mimic the in vivo environment is a driving force behind the quest to improve those culture systems. In vitro systems, however, remain significant tools of cancer research precisely because of their ability to distill the complex in vivo situation down to a simplified model[12].

Although 2D systems can include ECMs and ligands, they have traditionally lacked the throughput capacities to interrogate a wide panel of combinatorial pertubagens. Popular commercial basement membrane extracts allow for culturing in 3D, but lack the provenance of a carefully defined panel of proteins. The commercial extracts typically suffer from incompletely defined composition, which can confound analysis and result in significant batch-to-batch variation[3,13]. The MEMA platform overcomes these barriers, allowing for the study of alterations in cellular phenotypes, metabolic activity, differentiation status, and variations in cell growth and proliferation as they are modulated by specific and defined endogenous factors.

The MEMA platform is a powerful, medium- to high-throughput approach to assess the impact of the microenvironment (both ECM and soluble factors) on the phenotype of cells. The platform shows great flexibility for the types of assays and cells for which it can be utilized. We can observe effects from both soluble ligands and the ECM proteins to which the cells are exposed. Indeed, we recently found that ligands were a major driver of resistance to HER2-targeted inhibitors, but that these effects could be modulated by the ECM[5]. A variety of cells, including primary cells and cell lines derived from different cell types including lung, bladder, prostate, breast, and pancreas, as well as induced pluripotent stem (iPS) cells, have been successfully cultured on the MEMA platform (see examples in references[5,7,14]). The use of different stains allows for the readout of multiple cellular endpoints, including cell growth, differentiation, and metabolism. Other researchers have extended the platform to interrogate the impact of stiffness or elastic modulus, adding an additional dimension to the MEMA platform[15]. Finally, the platform is amenable to performing drug screens for identification of microenvironment conditions that either enhance or inhibit drug efficacy, as we and others have recently reported[5,14,15].

Perhaps the most critical step to the success of a MEMA experiment is optimizing the cell plating density. Optimizing the density of the cells ensures that enough cells are present to provide robust data, but not so many that the spot becomes overly confluent. Confluent spots can significantly confound results, particularly if proliferation is used as an endpoint, making it impossible to determine if low proliferation rates are a result of interactions with microenvironmental factors or due to contact inhibition from high cellular density. Cell titration experiments can reveal these problems, as average cell numbers per spot will demonstrate a linear increase with increasing numbers of cells plated, but will eventually plateau. The optimal cell number should be chosen in the linear range of the curve.

As mentioned above, the MEMA platform is flexible and can be prepared on a variety of substrates with different surfaces. These include glass slides and multiwall plate formats. In our experience, not all surface chemistries are amenable to MEMA printing, as we have observed spot detachment on some surfaces due to poor adhesion properties and the inability to block cell adhesion on other high adhesion surfaces. Furthermore, changing between different substrates necessitates optimization of buffer conditions, as the performance of the printing with the same print buffer can vary depending on surface chemistry.

The diameter of the printed ECM spots plays an important role in the quality of the data. In general, we recommend using the largest diameter print pins available for the arrayer in use (we currently use 350 μm diameter pins). Larger diameter spots allow a greater number of cells to occupy a spot, which tends to result in more robust data than are generated with smaller diameter pins. Since binding of the cells is a stochastic process, there does tend to be a high degree of variability in the data that is related to the number of cells originally attached to each spot. Thus, we recommend printing a large number of replicates for each ECM condition. We print 10−15 ECM replicates in each well with our current print conditions to ensure robust statistics.

We have noted in our past experiments that for the most part, ligand effects tend to dominate over ECM effects. This may be in part due to our decision to add collagen I to all ECM spots, which ensures robust cell binding. However, we believe that this may also homogenize the ECM effects, as most spots tend to behave in a manner highly similar to collagen I. Altering the spot composition to exclude collagen I may result in differential cell behavior as a result of the interaction with the ECM, but also significantly impacts cell binding, resulting in many more unoccupied spots. Users should tailor their ECM composition keeping these differences in mind, particularly those interested in stem and progenitor cells and differentiation, where the matrix can have a significant impact[16].

We typically perform the MEMA assays for relatively short periods of time (e.g., 72 h maximum). This is because the cells are constrained to the spots (the blocking buffer does not allow for growth outside the spots in our experience). With rapidly dividing cells, growth longer than 72 h will lead to overgrowth of the spot, which in turn complicates image segmentation as cells become crowded and pile up on each other, and can also impact data as growth arrest can occur with contact inhibition. We have performed longer treatments with very slow growing primary

cells (10−14 days), but care must be taken in these assays to change the media and replenish ligands every 3−4 days.

Continuing efforts to develop the MEMA platform are focused on two areas of interest, maximization of the optical quality for imaging and optimization within smaller culture vessels. Optical quality becomes a crucial factor when researchers require higher resolution microscopy to identify subcellular localization of their markers of interest. Initial screens can be performed at lower resolution on high-throughput microscopes followed by imaging of specific spots of interest on higher resolution instruments, but image quality can be compromised if the optical properties of the substrate are poor. Improvement of the optical properties of the substrate would allow researchers to perform the initial screens on high resolution imaging systems without the need to reacquire selected images at higher resolution. Finally, the ability to perform MEMAs in smaller culture vessels, such as 96-well plates, would allow a reduction in treatment volume and an expansion of interrogated ligands and replicates. This transition requires the optimization of substrate-buffer-protein interactions and array printing within new culture vessels. Such ongoing efforts will improve the MEMA platform and expand upon its powerful capabilities to identify relevant microenvironmental proteins that alter cellular phenotypes for a variety of cell types, which can then be subsequently investigated in confirmatory assays.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure1

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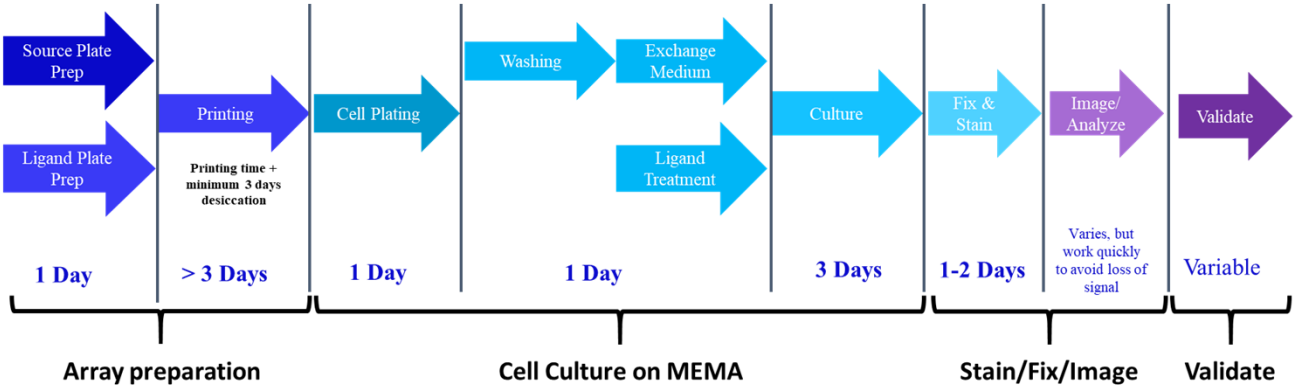

Figure2

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Figure3

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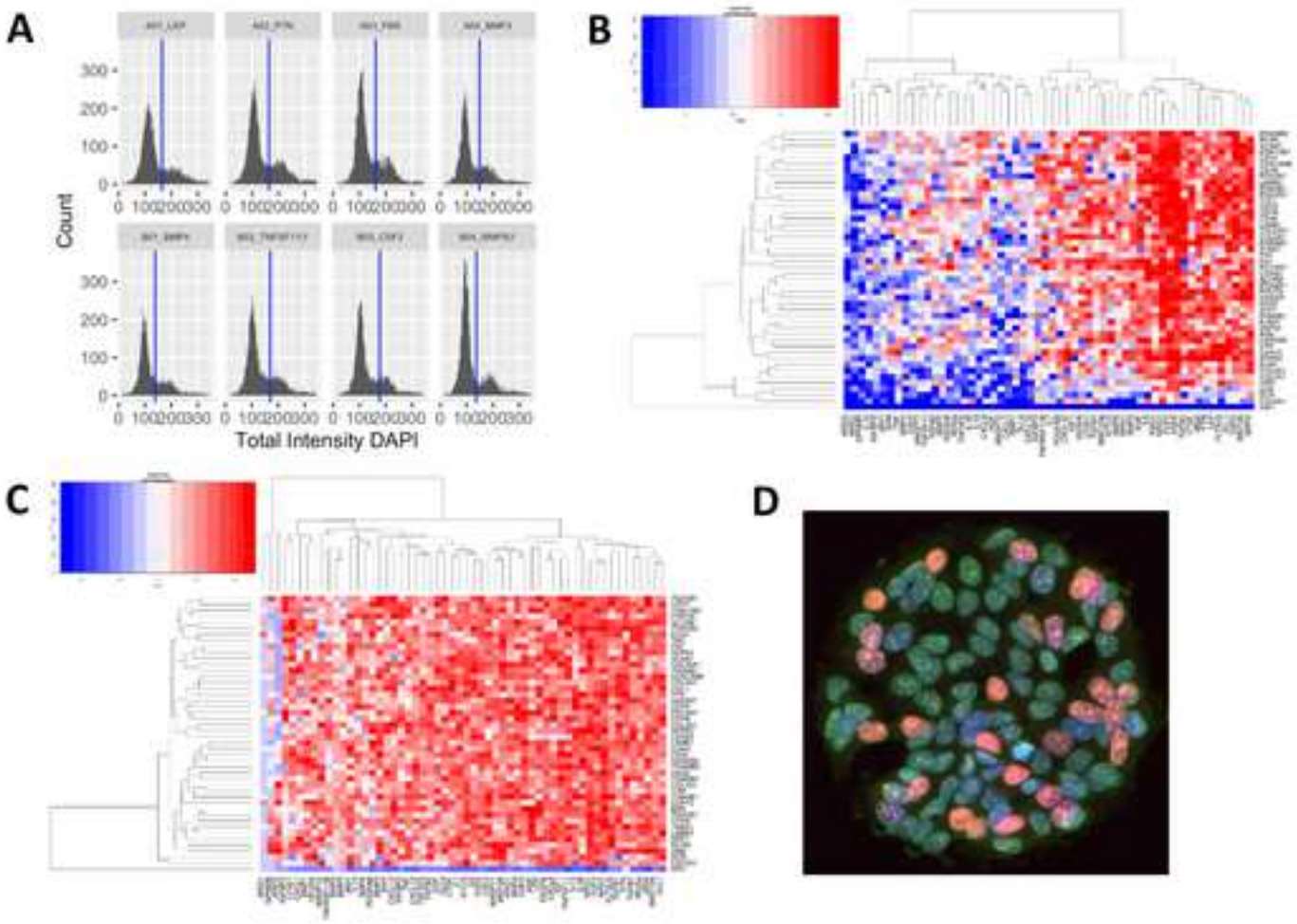

Figure4

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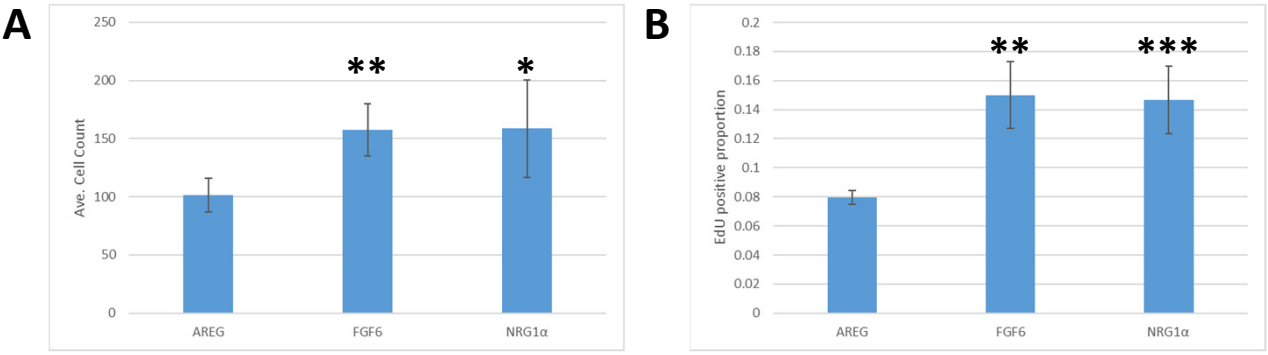

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Protein Name	Uniprot ID	Stock Concentration (µg/mL)	Final Concentration (µg/mL)
ANGPT1|1	Q15389|1	100	0.04
ANGPT2|1	O15123|1	100	0.2
AREG	P15514	100	0.02
BMP2	P12643	100	0.1
BMP3	P12645	1000	0.1
BMP4	P12644	100	0.1
BMP5|1	P22003|1	100	0.1
BMP6	P22004	100	0.1
BMP7	P18075	100	0.1
CSF2	P04141	100	0.02
CTGF|1	P29279|1	100	0.05
CXCL12|Alpha	P48061|2	100	0.01
CXCL12|Beta	P48061|1	100	0.03
CXCL1	P09341	100	0.004
CXCL8|1	P10145|1	100	0.3
DLL1|1	O00548|1	500	0.5
DLL4	Q9NR61	200	0.6
EGF|1	P01133|1	500	0.01
FASLG|1	P48023|1	10	0.02
FGF2|3	P09038|2	100	0.01
FGF6	P10767	100	0.01
FLT3LG|1	P49771|1	50	0.001
GPNMB|1	Q14956|1	100	0.5
HGF|1	P14210|1	50	0.04
IGF1|1	P05019|1	200	0.01
IGFBP2	P18065	100	0.05
IGFBP3|1	P17936|1	100	0.1
IL13	P35225	100	0.01
IL15|IL15S48AA	P40933|1	50	0.01
IL1B	P01584	25	0.001
IL6	P05231	100	0.01
IL7|1	P13232|1	100	0.01
JAG1|1	P78504|1	200	0.5
JAG2|Long	Q9Y219|1	100	0.5
KITLG|1	P21583|1	100	0.005
KNG1|HMW	P01042|1	100	0.2
LEP	P41159	1000	0.002
LYVE1	Q9Y5Y7	100	0.05
NRG1|10	Q02297|10	100	0.01
NRG1|1	Q02297|1	100	0.05
NRG1|6	Q02297|6	100	0.01
PDGFAB	go1990265	100	0.05

PDGFB|1	P01127|1	100	0.05
PTN	P21246	100	0.5
SHH	Q15465	100	0.5
TGFB1||Cterminus	P01137|Cterminus	20	0.01
TGFB1||LAP	P01137|LAP	100	0.15
TGFB2|A	P61812|1	20	0.01
THPO|1	P40225|1	50	0.002
TNFRSF11B	O00300	100	0.02
TNFSF11|1	O14788|1	100	0.01
TNF	P01375	100	0.01
VEGFA|VEGF206	P15692|1	100	0.01
WNT10A	Q9GZT5	100	0.1
WNT3A|1	P56704|1	200	0.1
Wnt5a|1	P22725|1	100	0.1

Table 2

ECM Protein	UniprotID	Stock Concentration (µg/mL)	Final Concentration (µg/mL)	Notes
ALCAM|1	Q13740|1	100	30	
CDH20	Q9HBT6	300	80	
CDH6|1	P55285|1	100	40	
CDH8	P55286	100	20	
CD44|1	P16070|1	100	30	
CEACAM6	P40199	100	30	
COL1A1	P02453	5000	200	multiple subunits with multiple uniprot ids
COL2A1|2	P02458|2	1000	200	
COL3A1|1	P02461|1	1000	200	
COL4A1|1	P02462|1	1000	200	multiple subunits with multiple uniprot ids
COL5A1	P20908	1000	200	
COL23A1|1	Q86Y22|1	200	80	
DSG2	Q14126	100	30	
CDH1|1	P12830|1	100	40	
ECM1|1	Q16610|1	100	40	
FN1|1	P02751|1	1000	200	
GAP43|1	P17677|1	158	40	
HyA-500K		1000	200	LOR-0005
HyA-50K		1000	200	LOR-0007
ICAM1	P05362	400	80	
ALCAM|1	Q13740|1	100	30	
CDH20	Q9HBT6	300	80	
CDH8	P55286	100	20	
CD44|1	P16070|1	100	30	
CEACAM6	P40199	100	30	
DSG2	Q14126	100	30	
CDH15	P55291	100	20	
VCAM1|1	P19320|1	1000	200	
LAMA1	P25391	500	200	multiple subunits with multiple uniprot ids
LAMA3|2	Q16787|2	130	40	
LUM	P51884	200	80	
CDH15	P55291	100	20	
NID1|1	P14543|1	100	9.3 µg/mL Nid, 1	+COL4 and laminin
OMD	Q99983	100	40	
SPP1|A	P10451|1	100	40	
CDH3|1	P22223|1	100	40	
PECAM1|Long	P16284|1	150	40	
TNC|1	P24821|1	500	200	
VCAM1|1	P19320|1	1000	200	
VTN	P04004	100	40	
BGN	P21810	100	40	
DCN|A	P07585|1	300	80	

POSTN|1	Q15063|1	100	40
SPARC	P09486	100	40
THBS1|1	P07996|1	100	40
BCAN|1	Q96GW7|1	100	40
ELN|3	P15502|3	1000	200
FBN1	P35555	254	80

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Aushon 2470	Aushon BioSystems	
Nikon HCA	Nikon	
BioTek Precision XS liquid Handler	BioTek	
Trizma hydrochloride buffer solution	Sigma	T2069
EDTA	Invitrogen	15575-038
Glycerol	Sigma	G5516
Triton X100	Sigma	T9284
Tween 20	Sigma	P7949
Kolliphor P338	BASF	50424591
384-well microarray plate, cylindrical well	Thermo Fisher	ab1055
Nunc 8 well dish	Thermo Fisher	267062
Paraformaldehyde 16% solution	Electron Microscopy Science	15710
BSA	Fisher	BP-1600
Sodium Azide	Sigma	S2002
Cell Mask	Molecular Probes	H32713
Click-iTEdU Alexa Fluor	Molecular Probes	C10357
DAPI	Promo Kine	PK-CA70740043
ALCAM	R & D Systems	656-AL
Cadherin-20 (CDH20)	R & D Systems	5604-CA
Cadherin-6 (CDH6)	R & D Systems	2715-CA
Cadherin-8 (CDH8)	R & D Systems	188-C8
CD44	R & D Systems	3660-CD
CEACAM6	R & D Systems	3934-CM
Collagen I	Cultrex	3442-050-01
Collagen Type II	Millipore	CC052
Collagen Type III	Millipore	CC054
Collagen Type IV	Sigma	C5533
Collagen Type V	Millipore	CC077
COL23A1	R & D Systems	4165-CL
Desmoglein 2	R & D Systems	947-DM
E-cadherin (CDH1)	R & D Systems	648-EC
ECM1	R & D Systems	3937-EC

Fibronectin	R & D Systems	1918-FN
GAP43	Abcam	ab114188
HyA-500K	R & D Systems	GLR002
HyA-50K	R & D Systems	GLR001
ICAM-1	R & D Systems	720-IC
Laminin	Sigma	L6274
Laminin-5	Abcam	ab42326
Lumican	R & D Systems	2846-LU
M-Cad (CDH15)	R & D Systems	4096-MC
Nidogen-1	R & D Systems	2570-ND
Osteoadherin/OSAD	R & D Systems	2884-AD
Osteopontin (SPP)	R & D Systems	1433-OP
P-Cadherin (CDH3)	R & D Systems	861-PC
PECAM1	R & D Systems	ADP6
Tenascin C	R & D Systems	3358-TC
VCAM1	R & D Systems	ADP5
vitronectin	R & D Systems	2308-VN
Biglycan	R & D Systems	2667-CM
Decorin	R & D Systems	143-DE
Periostin	R & D Systems	3548-F2
SPARC/osteonectin	R & D Systems	941-SP
Thrombospondin-1/2	R & D Systems	3074-TH
Brevican	R & D Systems	4009-BC
Elastin	BioMatrix	5052
Fibrillin	Lynn Sakai Lab OHSU	N/A
ANGPT2	RnD_Systems_Own	623-AN-025
IL1B	RnD_Systems_Own	201-LB-005
CXCL8	RnD_Systems_Own	208-IL-010
IGF1	RnD_Systems_Own	291-G1-200
TNFRSF11B	RnD_Systems_Own	185-OS
BMP6	RnD_Systems_Own	507-BP-020
FLT3LG	RnD_Systems_Own	308-FK-005
CXCL1	RnD_Systems_Own	275-GR-010

DLL4	RnD_Systems_Own	1506-D4-050
HGF	RnD_Systems_Own	294-HGN-005
Wnt5a	RnD_Systems_Own	645-WN-010
CTGF	Life_Technologies_Own	PHG0286
LEP	RnD_Systems_Own	398-LP-01M
FGF2	Sigma_Aldrich_Own	SRP4037-50UG
FGF6	RnD_Systems_Own	238-F6
IL7	RnD_Systems_Own	207-IL-005
TGFB1	RnD_Systems_Own	246-LP-025
PDGFB	RnD_Systems_Own	220-BB-010
WNT10A	Genemed_Own	90009
PTN	RnD_Systems_Own	252-PL-050
BMP3	RnD_Systems_Own	113-BP-100
BMP4	RnD_Systems_Own	314-BP-010
TNFSF11	RnD_Systems_Own	390-TN-010
CSF2	RnD_Systems_Own	215-GM-010
BMP5	RnD_Systems_Own	615-BMC-020
DLL1	RnD_Systems_Own	1818-DL-050
NRG1	RnD_Systems_Own	296-HR-050
KNG1	RnD_Systems_Own	1569-PI-010
GPNMB	RnD_Systems_Own	2550-AC-050
CXCL12	RnD_Systems_Own	350-NS-010
IL15	RnD_Systems_Own	247-ILB-005
TNF	RnD_Systems_Own	210-TA-020
IGFBP3	RnD_Systems_Own	675-B3-025
WNT3A	RnD_Systems_Own	5036-WNP-010
PDGFAB	RnD_Systems_Own	222-AB
AREG	RnD_Systems_Own	262-AR-100
JAG1	RnD_Systems_Own	1277-JG-050
BMP7	RnD_Systems_Own	354-BP-010
TGFB2	RnD_Systems_Own	302-B2-010
VEGFA	RnD_Systems_Own	293-VE-010
IL6	RnD_Systems_Own	206-IL-010

CXCL12	RnD_Systems_Own	351-FS-010
NRG1	RnD_Systems_Own	378-SM
IGFBP2	RnD_Systems_Own	674-B2-025
SHH	RnD_Systems_Own	1314-SH-025
FASLG	RnD_Systems_Own	126-FL-010

Comments/Description
Arrayer robot system used in the protocol
High Content Imaging system designed around Nikon Eclipse Ti Inverted Microscope
liquid handling robot used in the protocol
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Article Title: Microenvironment microarrays: a method for interrogating microenvironmental impact on cellular phenotypes

Signature: James Ek Date: 8/16/18

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Dear Dr. Cao

We wish to thank the editors and reviewers for their thoughtful review of our submitted manuscript JoVE58957R1 "Using microarrays to interrogate microenvironmental impact on cellular phenotypes in cancer." We have addressed the concerns of the editors and reviewers, which we describe on a point by point basis below.

Editors comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have thoroughly reviewed the manuscript, and to the best of our knowledge, there are no typographical or grammatical errors remaining.

2. Please provide an email address for each author.
We have now added email addresses for each co-author.

3. Lines 85-86: Here it is indicated that the protocol is for 8-well plates; however in the protocol 384 or 96 well plates are used. Is this contradictory?
This is not contradictory- the ECM libraries are prepared in 384 well plates, but the MEMA themselves are printed into 8-well plates using the ECM libraries in 8 well plates. We have added several indications in the protocol to clarify this to the readers.

4. Please list approximate volumes for all buffers and stock solutions to be set up.

5. 1.1, 2.3: Are ECMs, ligands, cytokines, buffers, and fiducial all purchased? If so, please include them in the Table of Materials.
Most of these reagents are purchased, and are already listed in the Table of Materials. We have highlighted in the notes column when the reagent is either a ligand or ECM for printing.

6. 1.2: Please specify the proteins used in this protocol.
All of the proteins are currently listed in the Table of Materials. At the request of Reviewer #2, we have now added two tables that list the ECM and ligand proteins used, their uniprot ID, the stock concentrations, and the final concentration of the protein in the experiment.

7. 3.1 and 3.2: Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have changed these two sections to remove the personal pronouns.

8. 2.3.2: What is the composition of diluent Buffer? If it is purchased, please cite the Table of Materials.
The diluent buffer is defined in step 1.4. We have added a note to clarify this. The reagents used to make the buffer are listed in the table of methods.

9. Please note that your highlighted protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. There should be enough detail in each step

to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please provide specific values to be used. We cannot film a generalized protocol; we need specific settings of a specific experiment. Some examples:
10. 5.2, 5.3: Please provide the ligands and corresponding buffer used in this protocol.

11. 6.1: Please provide the composition of the non-fouling blocking buffer.
We have corrected this to provide the composition of the non-fouling blocking buffer.

12. 6.3: Please specify the cells and medium used in this step.
We have specified that we used MCF7 and DMEM medium.

13. 6.5: Please provide centrifugation parameters (centrifugal force in x g and time).
We have specified time and force for the centrifugation step.

14. 6.7, 6.8: Please specify incubation conditions.
We have specified the incubation conditions.

15. 7.2: Please specify the temperature.
We have now specified the temperature.

16. 7.5: Please provide the composition of PBS-T.
We have now provided the composition of the PBS-T.

17. 7.8, 7.10: Please specify the stains and/or antibodies, and corresponding blocking buffers used.
We have now provided the specific staining that was performed for the MCF7 experiment.

18. 7.6: Please replace commercial language Click-iT with a generic term.
We have replaced the Click-IT commercial language.

19. Section 9: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).

The data analysis section is not driven by a software package, but rather by custom code that runs in the R-environment. We have clarified this in the data analysis section, and have provided a link to an example of the custom code that is utilized to perform analysis of the data.

20. Figure 4: Please define error bars in the figure legend.
We have indicated that the error bars represent standard deviation in the figure legend.

21. References: Please do not abbreviate journal titles.
We have edited the references to include the full journal title.

Reviewers' comments:

Reviewer #1:
This is a very interesting and well written manuscript. It will assist many scientists working in the complex field of microenvironment analyses.

Reviewer #2:

The manuscript describes a method for the high-throughput investigation of the effects of combinations of microenvironmental signals on cancer cell phenotypes.

Major Concerns:

1. Minimal details are provided regarding the commercial microarrayer utilized in the described approach. Specific details regarding the microarray instrument and arraying parameters should be provided.
We have now made it clear in the Materials Table that the Aushon 2470 printer is the arrayer that we used. We also have provided additional details regarding the pin size, print head configuration, and array size.

2. The authors mention that there is flexibility for printing on plates or slides. If so, are there distinct parameters that should be adjusted for optimal arraying on glass or plastic surfaces?
We have added a section in the Discussion to expand on this point.

3. It is also unclear if there is an optimal array spot diameter, and what range of spot diameters are possible. In particular, for the 8-well format described, what number of spots are possible within each well? Also, based on the authors experience and the design of this platform, how many replicate array spots (and wells) are typically used to obtain robust cell phenotype measurements?
We have addressed the spotting concerns (e.g., spot diameter, array size, and replicates) in both the protocol and discussion sections now.

4. In regards to the ECM protein arraying, what concentrations of ECM proteins are typically used? How does this concentration compare to protein adsorption methods in bulk?
We have now added tables that give the concentrations of the ECMs and have indicated that the concentrations used are typically equivalent to those used in standard 2-d cell culture assays.

5. The authors mention that a non-fouling blocking buffer is used, most likely to restrict cell adhesion to the arrayed spots. From the material list it appears that Kolliphor P338 is used, however, this is not explicitly stated in the protocol. More details regarding this treatment are needed.
We have clarified this in the protocol and referred the user to the Materials Table.

6. It is currently unclear how stable the arrayed spots are in regards to maintaining localized cell attachment. If cultured longer, as the authors describe, will the cells migrate and proliferate between the array spots? Can the the non-fouling treatment be adjusted to mitigate this issue, or are there other

parameters that could be optimized to improve array stability?
We have added a section in the discussion to address these issues.

Minor Concerns:

1. The ECM and soluble factors used in the representative dataset are included in the list of total materials. The manuscript would be improved if the ECM proteins and soluble factors were included in separate tables that accompanied the description of the representative data.
We have now added these as separate tables.

1b. In addition, the image resolution in Figure 3 (data heatmaps) is very low and makes it difficult to resolve representative conditions.
We have improved the resolution of the heatmaps in Figure 3.

1c. Further, the presentation of the heatmaps implies that clustering has been performed, but this clustering is not mentioned. Overall, more details regarding the analyses presented within the representative data figures is warranted.
We have indicated that we performed clustering using hierarchical clustering to display the data, and have expanded our explanations regarding the analyses presented. Furthermore, we have provided the R code for analysis to enable other users to perform similar analyses.