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14. ABSTRACT

This portion of the work proposed focused on employing and validating microtechnology for in vitro studies of primary mammary gland cell characteristics. Specific attention has been paid to developing more quantitative methods for analyzing microfluidic cell cultures using In Channel Westerns. Also, understanding how the microfluidic culture platform differs from traditional macro-scale techniques is critical and was explored in more depth than previously done. By thoroughly understanding how this culture platform affects the cellular baseline first, better and more efficient data collection can be performed, thus requiring fewer primary cells.

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Introduction:

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Body:

Training Program:

All requirements for the PhD in Biomedical Engineering have been met. I successfully defended my preliminary examination proposal, executed what was proposed and defended my thesis work. I attended seminars in cancer biology and engineering, along with meetings with my thesis committee that provided very useful insight into what work would be most critical to do to better prepare other members of the labs who may carry on my project. I attended a University of Wisconsin Comprehensive Cancer Center in early 2008, where my work was presented in poster form by one of my advisors, David Beebe (the Center requires that only PIs present work). Also, an abstract has been accepted to the Micro-Total Analysis Systems and will be presented by a coworker in the fall of 2008.

I received quite a bit of training in immunocytochemistry, Western blotting, and In Cell Western techniques. I was able to work independently using these techniques and even begin to validate in depth what an In Cell Western truly measures and the limitations of the technique from an engineering standpoint as well as a biological one.

Task 1:

The mice and reagents needed to perform the in vivo labeling of stem and progenitor cells that have become quiescent have still not proved to be accurate and well defined enough to perform the experiments we proposed. The mice that had been described in the proposal would ideally expressed a GFP labeled Histone 2b conditionally while exposed to doxycycline. This would allow us to expose mice during early development to doxycycline as a pulse, then to later analyze the population for percentages of GFP-labeled cells after a chase period. These label retaining cells would have been quiescent during the chase period and that number could be compared with the mathematical model we've produced. While some cells of the glands in these mice do express GFP-Histone 2b, only ~30% of the cells are positive in virgins treated with a continuous pulse of doxycycline rather than 100% as would be expected. Due to the uneven

expression of MMTV (mouse mammary tumor virus) in the virgin glands, the Tet transactivator that is under it suffers from heterogeneity within the glands as well. The mice were also on a mixed strain background (FVB/CD1), so the CD1 mice were recently backcrossed onto a FVB background. The development of these mice is not part of my training program, so I was unfortunately still unable to use the intended mouse system.

Beyond the mathematical modeling I performed prior to the last status report, nothing more can be done with this aim due to these limitations.

Tasks 2 and 3:

Because of the limitations due to the inability to acquire the necessary labeled cells for the assays, members of the Alexander lab (the mentor on this grant) began to plate non-labeled sorted fractions in microchannels to evaluate whether even the normal sorted stem and progenitor fractions may be used in microchannels. Very poor plating was seen (down to nearly 20%), and cells did not survive well as compared to the macroscale cultures (cultures using traditional techniques in 96 well plates).

My work focused on being able to determine what was limiting cell growth, plating efficiency and what other effects may be preventing us from using primary mammary cells in microfluidic channels. In order to do this, I found after trial and error with a few difference cell types that the mouse mammary fibroblasts that were isolated by a member of Caroline Alexander's lab from a p16/INK4a knockout mouse plated in the same amount of time as that in macrocultures (96 well plates). All other cell types tried (primary, unsorted mammary epithelial cells, a mouse epithelial cell line called NMuMGs, and finally HEK293 cells) all either suffered from a long delay in attachment solely seen in microchannel cultures (up to 16 hours of delay), or as in the case of HEK293s, did not survive at all.

To better understand what the limitations and artifacts of microfluidic culture were on these cells that was causing such a large discrepancy in cell behavior and survival in microcultures, a more quantitative method for analyzing cell behavior was needed. A benefit of the microfluidic cell culture chambers we aim to use is the low sample size required per replicate of an assay. However, current types of biological readouts that can be used with such small samples are limited and this lack of suitable readouts for microfluidic cultures has become a significant limitation of the technique for cell based assays. A thorough discussion of the limitations and benefits of microfluidic devices for cell based assays was written as a review for BioEssays and has been accepted for publication (it is included as Appendix 1).

Thus, to remedy the lack of potential readouts, the In Cell Western (ICW) technique was validated for use in microfluidic devices. The ICW technique uses quantitative immunocytochemistry and a laser scanner to provide an *in situ* measure of protein quantities in

cells grown in microfluidic channels of arbitrary geometries. Validation of the use of ICWs in microfluidic channels was performed by a detailed comparison with current, macroscale methods and shown to have excellent correlation. Transforming growth factor-b induced epithelial-to-mesenchymal transition of an epithelial cell line was used as an example for further validation of the technique as a readout for soluble factor based assays performed in microfluidic channels. The use of passive pumping for sample delivery and laser scanning for analysis opens the door to high throughput quantitative microfluidic cell-based assays that integrate seamlessly with existing high throughput systems infrastructure. This data is all included in Appendix 2 and will be submitted to Nature Biotechnology after additional data from the high throughput platform is obtained by my coworker.

With the adaptation and validation of In Cell Westerns (ICWs) to microfluidic devices, I began to look in depth at a variety of cellular responses to microcultures from metabolic signaling responses to evidence of DNA damage. Appendix 3 and 4 are a two part series that will be submitted to Integrative Biology very soon which describe several important differences in signaling pathway activation and expression levels between cells cultured in traditional macroscale cultures and in microfluidic cultures. The panel of stress assays applied in Appendix 3 aimed to provide insight into how microfluidic cultures are different, from a cellular perspective than the corresponding macroscale cultures (results summarized in Figure 1).

Readout	Total Protein (Micro/Macro)	Phosphorylated Protein (Micro/Macro)	Ratio (Phospho/Total) (Micro/Macro)
AMPKα	1.3x	1.7x	1.5x
S6	1.1x	0.84x	0.75x
ERK	2.0x	1.8x	0.91x
Hsp70	2.4x	N/A	N/A
BiP	1.6x	N/A	N/A
γH2A.x	N/A	1.0x	N/A

Figure 1: Summary of readout results comparing macro and microscale cultures for total protein, the phosphorylated protein (if applicable) and the ratio of phosphorylated to total for each applicable readout. Several of these proteins exhibit approximately 2 fold (or more) changes in expression in only 24 hours of microculture, indicating that the influences of microculture are significant and relatively rapid.

Significant differences in AMP kinase and S6 phosphorylation indicate that perhaps the reduced media volumes results in nutrient depletion (resulting in increased phosphorylation of

AMP kinase) or growth factor depletion (resulting in reduced activation of S6). Other readouts showed significant differences in microcultures than macrocultures such as the upregulation of ERK1/2, BiP and HSP70. These proteins are sensitive to a wide variety of potential stressors, thus it is unclear what specific characteristic of microfluidic culture is causing each of the responses seen. However, these differences do reflect that the microenvironment in microcultures is truly different and results in different levels of activation and expression of key proteins involved in basic cell functions like attachment, growth, and protein folding/production. Differences in levels of γH2A.x were not seen, indicating that significant differences in rates of DNA damage between the scales are not likely. This also suggests that reductions in proliferation seen in microcultures is not due to delays for DNA repair, nor that widespread apoptosis is occurring in these cultures. These differences indicate that the cellular baseline may be substantially altered in microcultures, which will be critical to understand fully prior to integrating microfluidic devices for cell based assays.

Appendix 4 describes a more detailed analysis of the metabolic and growth factor signaling occurring in microcultures as compared to traditional techniques. It was determined that one of the materials extensively used in the microfluidic field to fabricate the bodies of the microchannels can actually significantly influence cell behavior and survival, perhaps in a cell type specific manner.

Key Research Accomplishments:

- Validated In Cell Western (ICW) techniques for use in microfluidic devices
- Tested the ICW technique with a model assay (TGF-b induced EMT) in microfluidic and macrocultures
- Applied In Cell Westerns to a panel of stress responses to mouse mammary fibroblasts in microfluidic and macroscale cultures
- Determined the extent and types of influences of microchannel culture on cell behavior by doing a detailed analysis of proliferation, stress responses and glucose uptake assays
- Better understand why primary cell cultures in microchannels were less successful than when traditional cultures were used

Reportable Outcomes:

- Accepted publication in BioEssays (Appendix 1).[1]
- In prep publication to be submitted to Nature Biotechnology once final data has been obtained by a collaborator (Appendix 2).[2]
- A two part publication/tutorial review in prep that will soon be submitted to a new journal called Integrative Biology (Appendix 3,4).[3,4]
- Two conference submissions for poster presentations[5,6]

- Preliminary examination passed
- Successfully prepared, defended my thesis and graduated (May 2008)

Conclusion:

With the integration of ICWs to high throughput microfluidic assays a panel of stress assays could be reproduced for a wide variety of cell types and could be expanded to include more aspects of cellular function. This tool could be used to validate and troubleshoot microfluidic cultures for cell based assays to better understand the cellular baseline for specific cell types of interest prior to large assays being run. Also, the ability to do quantitative studies of signaling cascades *in situ* in microfluidic devices expands the available readouts for microfluidic assays. The activation or inhibition of signaling pathways in response to drugs or other stimuli can now be screened using microfluidic devices, with all of the resource benefits that they provide (in cell sample sizes and reagent costs).

While additional work more closely related to the aims proposed could not be performed due to limitations of the mouse model system, it is quite likely that the results from microfluidic assays with them may not have been completely accurate. The work that was done to validate a readout for microfluidic cell cultures, use this readout to assay microcultures for a range of stress responses will provide a baseline for understanding how to better apply microfluidic to cell based assays. It is clear that future work with microfluidic devices will require a substantial amount of biological validation and careful implementation in order for artifacts caused by the devices themselves to not cause erroneous data.

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4. Paguirigan A, Beebe DJ.In Prep. Part 1: From the cellular perspective: Differences in the cellular baseline in microfluidic cell cultures. Integrative Biology.
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6. Domenech M, Paguirigan A, Puccinelli J, Regehr K, Su X, Warrick J, Alexander C, Beebe DJ. Applying in situ analysis techniques and high throughput microfluidic methods to studying biological responses to soluble factors.; 2008 2/2/08; Madison, WI.

Appendices:

1. Bioessays review
2. Publication in prep for Nature Biotechnology
3. Part 1 and,
4. Part 2 of a publication for Integrative Biology

Microfluidics, meet Cell Biology: Bridging the Gap by Validation and Application of Microscale Techniques for Cell Biological Assays

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Microfluidics, meet Cell Biology: Bridging the Gap by Validation and Application of Microscale Techniques for Cell Biological Assays

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SUMMARY

Microscale techniques have been applied to biological assays for nearly two decades, but haven't been widely integrated as common tools in biological laboratories. The significant differences between several physical phenomena at the microscale versus the macroscale have been exploited to provide a variety of new types of assays (such as gradient production or spatial cell patterning). However, the use of these devices by biologists seems to be limited by issues regarding biological validation, ease of use, and the limited available readouts for assays done using microtechnology. Critical validation work has been done recently which highlight the current challenges for microfluidic methods and suggest ways in which future devices might be improved to better integrate with biological assays. With more validation and improved designs, microscale techniques hold immense promise as a platform to study aspects of cell biology, not possible using current macroscale techniques.

AN INTRODUCTION TO MICROFLUIDICS FOR CELL BIOLOGY ASSAYS

Various influences determine the phenotype of cells in vivo and contribute to their coordinated responses to stimuli. These influences include interactions with neighboring cells (e.g. epithelia-stromal), interactions with the extracellular matrix (ECM), and systemic factors (e.g. hormones). Yet, these interactions are not easily replicated or controlled in traditional formats. Current methods (petri dish, microtiter plates, which are in general macroscale techniques, with dimensions in centimeters and larger) afford a limited degree of microenvironmental control. Approaches that aim to

recapitulate aspects of *in vivo* microenvironments are often laborious (e.g. Dunn chamber for soluble gradients and chemotaxis), expensive (e.g. 3D gel culture) or both (transwell membrane inserts for migration, co-culture or invasion assays). Microfluidic technologies for cell based assays have the potential to increase the biological relevance of cell culture models while maintaining or increasing the throughput of current methods.

Microscale techniques for cell biology (aka, those using devices that have dimensions ranging from micrometers to millimeters), range from single cell analyses and flow cytometry-like techniques,[1] to treating fields of cells in gradient generating devices[2], patterned 3-dimensional cultures,[3,4] to microscale versions of more traditional assay types such as cell culture (via perfusion[5,6], or static cultures[3,7-9]). These microfluidic devices typically provide unique functionalities beyond traditional techniques either by controlling the cellular microenvironment in ways not previously possible, by allowing existing assays to be performed on significantly smaller samples (down even to the single cell level) or by using many fold less costly reagents. Microfluidic systems enable spatial patterning of molecules and cells[10] as well as both passive [11] and active cell handling and environmental control. Temporal and spatial control on the micrometer scale (0.1-100 μm) has been used in fundamental studies from the subcellular[12] to the organismal[13] level in studies of cell division axis orientation[14] and geometric influence on cell survival[15]. Thus it is clear that at its core, microfluidics has the potential to have a great impact in cell biology as many of the leading questions in cell biology are well suited to study using these functionalities.

Although the applications of microtechnology to cell biology have been considered for nearly two decades,[16-18] the field continues to progress via a plethora of demonstrations that provide glimpses of the potential impact of microtechnology on the methods used for cell biology and the types of data that can be obtained. To date, however, microfluidics and "lab on a chip" techniques have not made a large impact on cell biology either academically or commercially. The relative lack of integration of

microtechnology in biological laboratories could be due in part to a disconnect between the engineers who design and fabricate the devices and the biologists who would ultimately use them. This disconnect has resulted in devices that, while functional and potentially useful, are often technically challenging to use and obtain reliable, biologically meaningful data from. Meanwhile, the lack of biological validation of data obtained in microscale devices has potentially hindered the process as well. Finally, when new types of data are obtained using these new technologies, the challenge of interpreting the data in the context of what is currently known (but obtained using traditional techniques) can create yet another hurdle to presenting data to either field.

In this essay, we will illustrate how some existing microfluidic methods have been applied to biological assays and begun to be validated. These examples highlight the steps required to move from demonstration to utility and to more closely integrate microtechnology with traditional cell biology techniques. By understanding how physics affects the microenvironments found in microfluidic devices, we can better predict and understand on a general level the strengths and limitations of doing biological assays with microfluidic devices. We will briefly review some of the critical physical phenomena that will or in some cases have already been shown to affect the biological outcome of an assay performed in microfluidic devices. Ideally, this will provide some insight into how to interpret data obtained using these methods and how experiments can be designed to maximize the unique capabilities of microtechnology.

Current Cell Biological Applications of Microfluidics

To date, much of the work in microfluidics for cell biology based applications has focused on assays of cell behavior in the presence or absence of specific soluble factors. The application of controlled gradients of soluble factors has highlighted microfluidics' potential for expanding current techniques to include new assays, or providing a platform for simplifying and improving current techniques.

EXAMPLE: MICROFLUIDIC GRADIENT GENERATION DEVICES

Stimulating a field of cells with a controlled gradient of a soluble factor is a unique type of microfluidic assay that can effectively produce different microenvironments in a single device.[2] Few traditional techniques for gradient production, such as the Zigmond chamber,[19] have been able to produce as defined, controlled and repeatable gradients as those produced using microfluidic techniques. Precisely defined chemical gradients in microfluidic devices have been applied to many biological systems, such as to stimulate migratory cells (e.g. neutrophils, bacteria, sperm cells) using chemoattractants,[20-26] investigate cancer cells responding to a drug or growth factor,[27-29] or to stimulate the differentiation of embryonic stem cells.[30] This class of microfluidic devices has the potential to improve the sensitivity and complexity of experiments studying cellular responses to gradients beyond what is currently possible via traditional techniques.

One general class of gradient producing devices is based on the mixing of chemical species between two streams of fluid in laminar flow solely due to diffusion rather than convection (Figure 1a). Flowing streams allow diffusively created gradients of species to be formed at their interfaces, which then can be flowed over cells of interest to expose them to the gradient formed. Often microfluidic chemotaxis assays include a gradient of a single chemoattractant or growth factor but some devices have incorporated more complex combinations of factors.[22,31] The temporal and spatial control over defined gradients of soluble factors or immobilized factors (on surfaces) provided by flow based microfluidic devices are a significant improvement over the widely available methods. Laminar flow based systems facilitate quantitative correlations between environmental cues and observed cellular behavior which may provide insight into the mechanisms that affect signaling cascades and expression.

The limitations of these systems are primarily due to practicality issues, cost and potential biological artifacts. From a practical point of view these systems require very stable fluid flow and therefore complicated fluid handling setups, which rely

on specialized pumps and tubing not typically found in most laboratories. Also, to maintain a stable gradient in a continuously flowing system, relatively large total volumes of reagents must be used and experiments become increasingly costly the longer the time course for the response of interest. In these systems continuous flow constantly renews nutrients and chemoattractants, while also depleting waste products and intercellular signaling molecules, resulting in temporally uniform concentrations of media and experimental components. Although this uniformity is beneficial in that the response of cells over time to a uniform stimulus or the effects of rapid changes in a defined stimulus can be determined, the contribution to the response of any soluble cell-cell communication is obscured. By disrupting cell-cell communication, the location and migration behavior of nearby cells may not influence a cell's response to the stimulus as it might *in vivo*. The effects of flow alone on neutrophils has been addressed and mechanical activation by shear from laminar flow in microchannels was demonstrated.[32] Walker and colleagues have also shown that the flow rate used to create gradients can affect and therefore bias the migratory behavior of these cells.[33]

In situations where cell-cell communication plays an important role in modulating cellular response, the continuously flowing streams necessary to maintain the chemical gradients make laminar flow based methods unsuitable for probing cellular responses. Generation of gradients in static fluid preserves paracrine signaling, while still providing gradients of factors defined in both time and location (Figure 1b).[20] By allowing diffusion between a source and a sink along a thin channel, passively generated gradients can be formed and kept intact for long periods of time (over 24 hours). The source-sink concept can be used to create stable or temporally varying gradients along length of a channel. The gradient profile can then be controlled by adjusting the input concentration, distance from source to sink, or by changing the geometry of the channel (e.g., uniform width versus expanding or contracting), allowing for a range of linear or non-linear gradient profiles which may more accurately mimic *in vivo* gradients.

While flow based gradient generation devices often rely on more

complicated designs and fluid handling systems, many static gradient devices are much simpler to use. Because these devices typically require no additional equipment beyond common laboratory supplies (pipettes, microscopes, etc), they have the potential to be integrated more easily than more complicated designs. Also, with no fluid flow required for gradient maintenance, a more coordinated response of a population of cells can be observed by allowing paracrine signaling, and minimal total reagent is consumed for even long experiments. However, without constant flow, steps must be taken to minimize evaporation, which can be a significant factor for microfluidic devices with small volumes, and the time required to set up the gradients is often rather long (on the order of hours rather than seconds). Additionally, if the cell population requires more media renewal than that occurring via diffusional mixing between the channel and the source/sink (which both serve as a source for other media components), then artifacts may occur.

By treating cells with gradients of soluble components in microfluidic devices, a wide range of new assays can be performed that are more challenging or impossible to perform as accurately with traditional methods. However, the potential artifacts introduced by these devices will be important to establish for further integration of these techniques into biological research. Also, close collaboration between engineers and biologists will aid in developing devices that are more user friendly, a critical step in enabling these devices to become more accessible methods for experimentation. While microfluidics could provide a wealth of new information, it's unclear how data from microfluidics assay might fit in with data obtained using traditional methods without a baseline for comparison.

Example: Cell Culture in Microfluidic Devices

Another application of microfluidics that, while seemingly simple, holds immense promise is cell culture.[34,35] Microfluidic devices for cell culture provide a platform for higher throughput analyses of cellular responses to soluble stimuli with a variety of cost and resource benefits. Because each assay can be performed on a smaller

total number of cells, more different assays can be performed with the same sample size when done in microfluidic cultures. This is particularly beneficial for rare or expensive cell types such as stem cells, or flow sorted cell populations. Additionally, typical microfluidic cultures require far less media and potentially costly inhibitors, growth factors or other reagents than even 96 well plates.

Many dominant phenomena in microfluidic devices are unique to the microscale and by leveraging either the scalability (or lack thereof) of specific designs, more control and flexibility of the microenvironments the cells are exposed to can be obtained allowing new and different assays to be performed. Stimulating cultures of cells with drugs,[27,36,37] or other components that induce differentiation[38] in microfluidic platforms allow the replication of traditional tissue culture analyses in smaller volumes with fewer cells allowing expensive assays to be performed using minimal resources.

Typical volume densities in cultures using traditional techniques can be quite small (large media volumes for very few cells), and often are much higher in static microfluidic culture devices (Figure 2). The effects on cellular behavior of volume density alone can be significant[39,40] but have not been addressed for many microfluidic cell culture systems and seem to be cell type dependent. The lack of flow in static cultures results in less disturbance of the soluble cellular microenvironment than traditional macroscale cultures where bulk fluid flows result in convective mass transport being dominant (and thus eliminate local concentration gradients set up via diffusion to or from a cell). These devices generally do not require any equipment beyond pipettes for fluid handling and microscopes for visualization and analysis, both common laboratory equipment. The ease of use of these types of devices and the integration with existing automated fluid handling (or manual pipeting) has allowed this type of microfluidic device to be easily integrated into biological laboratories without the need for additional specialized equipment.[3]

Microfluidic devices also have optical benefits over smaller multiwell

plates such as 96 well plates. Visualizing cells via either phase or fluorescence in a 96 well plate is nearly impossible due to the meniscus of fluid in the well (interferes with phase contrast) and thickness of the plastic bottom (not suitable for high magnification fluorescence images). Microfluidic devices for culture keep fluid menisci at the ends of a channel only, allowing easy visualization via phase contrast along the length of the channel. Additionally, most devices can be placed on any substrate, allowing glass to be used when necessary for fluorescent detection and analysis.

Meanwhile, continuously perfused microfluidic cultures can provide continuous supply of nutrients and allow for longer-term cultures as compared to static cultures, but at the expense of effective volume density and soluble cell-cell communication.[6] These culture conditions employ laminar flow for continuous transport of solutes and thus are convection based devices that limit diffusive transport of solutes to and from cells in a culture.

Perfusion cultures of murine embryonic stem cells in microchannels at flow rates orders of magnitude apart showed improved morphology after several days with higher flow.[41] Experiments that benefit from or require flow such as studying how cells respond to shear stresses (such as endothelial cells[42]) are potentially well suited to microfluidic assay because very precise control over flow rates and channel dimensions can provide accurate shear stresses at the surfaces. However, perfusion systems have the potential to impart a range of artifacts to the culture, due to aspects such as continuous flow (e.g. elimination of soluble cell-cell communication, constant concentration of all components in the media) and low effective volume densities (e.g., the total volume perfused rather than the channel volume, divided by the number of cells in the culture area). Just as flow based gradient systems do, these perfusion culture devices also require additional fluid handling setups, not commonly found in biological laboratories.

Finally, membrane based culture devices employ a membrane to allow only diffusional mass transport between the static fluid in a channel, with flowing media

in source channels (convective flow), which allow longer term cultures to be performed without sacrificing soluble cell-cell communication. Nutrient exchange via convection at the boundaries of hydrogels seeded with cells has also been shown to effectively culture cells in microchannels.[43] Conceivably, these types of devices could also produce an intermediate effective volume density but are more challenging to fabricate and use than typical static culture devices.

While many cell types have been shown to be compatible with a wide variety of microdevices, proliferation kinetics are not always the same in microculture versus macrocultures.[7,9,40] Differences in the responses of cells to the engineered microenvironments of microfluidic devices to those in macro-scale techniques hasn't only been reflected in proliferation, but has also been assayed via microarray. A notable study done to analyze the artifacts imparted by a microfluidic culture chamber via the analysis of cellular expression profiles by DNA microarray[44,45] showed significant differences between the profiles of macro and micro scale cultures, though most were less than 3-fold induction or reduction. Comparisons between macro- and microscale cultures on a variety of engineered surfaces were performed to study any differences in baseline expression of cells in microfluidic assays. This work is the most comprehensive analysis of the differences in cellular behavior (in this case expression) in microfluidic devices to date.

EXAMPLE: A UNIQUE DEVICE

In contrast with these more widely applied areas of microfluidics for cell based assays, a notable example of a device with great potential, that was not as widely applied is Takayama, et al's device for subcellular domain treatments.[46] This device took advantage of diffusion based mass transport between streams of liquid in laminar flow to create regions within a device that could be treated with a compound independently from the rest of the device. The resolution was so fine that portions of single cells could be stimulated without altering the microenvironment of the rest of the cell (see Figure 3).

This device illustrates the incredible potential that microtechnology holds. No other traditional technique could control both temporally and spatially the stimulation of a cell with soluble factors as precisely as this microfluidic device. Thus, the data produced by the device could provide access to previously inaccessible cellular responses. Paradoxically, in cases like this the methods are so novel and thus the data so unique, the challenge arises of interpreting the data. No other method can be used to verify the data as a check for the validity of the assay. Unfortunately, this device likely also suffered from not being simple to operate and integrate into biology research labs. Because of the difficulties associated with obtaining data and having that data be widely accepted, often very novel and potentially useful devices go unused.

UNDERSTANDING HOW THE PHYSICS AFFECT THE MICROENVIRONMENT

When the scale of the culture device is reduced, the dominant physical phenomena that define how materials behave and how fluid and molecules move change as well. Surface effects and material interactions can substantially change microenvironment composition due to increases in surface area to volume ratios as the scale of the culture is reduced. Purcell provided a very useful account of what environments are like when dominated by diffusion and laminar flow (low Reynolds number), such as those found in typical microfluidic devices.[47] Since then, engineers have identified many of the major physical differences between macro- and microscale environments[48], some of which will be discussed briefly here in new contexts, along with other phenomena that are beginning to be more thoroughly examined.

MATERIALS

Most macroscale cultures are performed in polysytrene (or glass bottomed) tissue culture flasks, dishes and plates. While many microfluidic cultures are performed with similar substrates as macroscale cultures by adding micropatterned channel materials[49] onto tissue culture substrates, new materials are used to fabricate the body of the devices. Understanding how the materials and processes used to fabricate the

devices impact cellular behavior and the readouts of assays will be important in order to analyze the data produced by microfluidic culture systems.

While many new materials are being integrated into microfluidic devices for cell based assays, the limitations of these materials are also being evaluated. Often the materials cells interact with are considered to be "inert" with respect to their effects on cellular behavior and are largely ignored unless they are designed specifically to be bioactive. However, many reagents used during common processes in cellular assays such as fixation and permeabilization or staining and labeling do interact with these polymers.

Recent work has shown for a common polymer used for microfabrication, poly(dimethyl siloxane), or PDMS, that the partitioning of hydrophobic molecules into the polymer bulk can result in significant changes in the solution concentrations (Figure 4).[50] This issue becomes particularly important when compounds used to stimulate or block cellular processes or pathways are both small and hydrophobic such as many small molecule inhibitors or other compounds used in drug screening. When basic procedures such as fixation and staining are performed in microfluidic devices, the possibility for fluorescent reagents to leach into the bulk must be addressed via very simple, no-cell reagent only controls. Additionally, titrations of compounds used for screening or controls that may potentially interact with the materials used can be done to determine whether or not this might be a significant issue for the molecules/materials of interest.

EVAPORATION

Recent work has brought to light many important phenomena whose effects can become quite influential as the scale of the cultures are reduced, a prime example is evaporation. Evaporation dynamics of fluids is a very complex phenomenon, as it depends on many environmental variables that often change even as fluid is evaporating. Because of thermodynamic factors involved in the phase change between a liquid and a gas, evaporation is very temperature, pressure, and humidity dependent.

Most materials used in traditional culture, such as polysytrene, are relatively impermeable to water and air and only suffer evaporation from between the lids and the substrates. Many commercially available multiwell plates now have "low evaporation" lids to further minimize the surface area in which water can escape the cell culture chamber. For macroscale cultures where media volumes often range from 20mL in a large flask to 200µL in the wells of a 96 well plate, the level of humidification in a typical incubator (approximately 70-90% relative humidity) is sufficient to prevent appreciable evaporation.

However, when microfluidic cultures are performed, typical volumes can range from 5-10µL down to nanoliter volumes and additional means are required to prevent significant evaporation and subsequent concentration of the media.[51,52] However, evaporation is often a limitation of microfluidic cell culture platforms, and many ways have been proposed to combat the loss of water from culture areas. The most common of these include using continuously perfused chambers, employing additional local sacrificial water reservoirs beyond those typically found in standard incubators,[42,53] covering exposed media with oil,[54-56] or submerging the entire chamber in water,[57] though how well these methods limit evaporation is generally unknown.

One group has recently analyzed how evaporation through PDMS affects the osmolarity of the cellular microenvironment.[54] Heo, et al tested the osmolarity of 50µL of media in a well with a 200µm thick PDMS membrane under it (and mineral oil on top) over time. The osmolarity of the media increased by ~18% over 48 hours while the control culture conditions (an organ culture dish), showed very little increase. The authors also show distinct effects of this increase in osmolarity on the maturation of embryos and the survival of an endothelial cell line (HDMECs). In these examples, definite phenotypic changes result from shifts in osmolarity, although in other systems, the sensitivity of the cells of interest may not be so easily observed. Thus, evaluation of the means of preventing evaporation used for each specific device will be critical to ensure that during the assays performed, cells are not simultaneously undergoing osmotic

shock, potentially altering the results.

Fluid Flow and Mass Transport

The most striking difference between the physical environment in microfluidic culture devices and traditional macroscale culture and also the most well understood is the dominance of diffusional mass transport and laminar flow.[48] Briefly, at very small length scales (milli- or micrometers typically) and low flow rates such as those found in most microfluidic culture devices, fluid flow becomes laminar (smooth, streamlined) rather than turbulent. With little unsteady fluid flow to mix the contents of the fluid, diffusion can become a significant mechanism for soluble components to move through the culture volume (e.g., diffusion between flowing streams as in flow-based gradient devices).

Alternatively, in traditional macroscale cultures, the larger volumes and longer length scales allow for more chaotic flow, obscuring any mixing due to diffusion, resulting in more rapid homogenizing of the media due to improved mixing efficiencies. With better control of fluid flow and mass transport mechanisms, microfluidic techniques can provide temporal and spatial patterning of soluble factors or cells not otherwise possible. These capabilities enable a wide variety of new functionalities such as gradient generation discussed previously to be integrated with cell based assays using microfluidic based devices.

NEXT STEPS FOR MICROFLUIDIC CELL BIOLOGY ASSAYS

On the Topic of Validation

The results of biological assays are very sensitive to variations in both intrinsic cellular factors and extrinsic environmental factors. Sources of extrinsic variation common to currently accepted tissue culture techniques range from factors such as lot-to-lot variation of reagents (e.g. fetal bovine serum components, or the degree of

hydrophilicity of plastic tissue culture-ware), to pipetting error or other experimenter errors (e.g. differences in reagent concentrations, exposure times or temperatures such as during fixation and staining), to environmental differences (e.g. the temperature and humidity fluctuations in the incubator). Thus, positive and negative controls, and multiple replicates are crucial for verification that results seen in an experiment are truly due to the variables of interest and not the artifacts from any of the multitude of experimental factors which are inadvertently being altered each time an experiment is run.

For example, it's well known that proteins spontaneously adsorb onto polystyrene, the most common tissue culture-ware component. For a culture in which soluble secreted factors are important for the cellular response of interest, this adsorption results in loss of functional, active protein. Because protein adsorption per unit volume is dependent on the surface area to volume ratio in a culture, while a $75cm^2$ flask, 6 well and 96 well plates all share the same bulk material and are generally used interchangeably, 6 well plates have 6% higher surface area to volume ratio than the $75cm^2$ flask, and 96 well plates have 30% more for typical media volumes. While these different formats are similar because the cell culture surfaces and materials are the same, each type of culture-ware may impart its own artifacts to a culture.

Though this simple difference between these culture options could alter a sensitive assay, rarely is the format of the culture from which the data are obtained in discussed in the literature. This is because re-validation of existing laboratory techniques and materials is being done constantly. Titrations of relevant reagents to determine the best dilution for the cells, assay, or materials used are performed as a first step in any new assay. Positive and negative controls give the experimenter a way to troubleshoot experiments gone wrong and provide a comparison for the data of interest to existing data. Multiple replicates are done with different stock cell suspensions in case differences in the numerous sources of variation present in any biological assay significantly affect the outcome. All of these measures are standard measures which

serve the purpose of validation of all of the culture conditions.

Understanding how the microenvironments found in microdevices for biological assays affect both the cellular baseline and responses to stimuli will be key to better understanding the context of any future assays. The challenge posed to novel methods for cell based assays is substantial. For instance, when a Western blot is presented in a paper, the limitations and caveats inherent in the assay are widely understood: proteins are not in the native conformation (for non-native, SDS-PAGE), antibodies may not be completely specific, loading controls of housekeeping proteins or total amounts of a protein of interest control for variations in loading, etc. However, when data are presented obtained using new techniques, the understanding of its limitations and strengths is simply not there and in some cases, not even known at all. If the specific caveats of an assay or technique aren't well understood, it can be challenging for readers and reviewers (and experimenters) to accurately interpret the results. To overcome this challenge, it is more important for authors to include additional background data to support the novel data to make it more accessible to the reader.

Thus, while new culture techniques such as microfluidic methods and materials do require some degree of validation before they can truly be integrated as a tool in a biological laboratory, this isn't a particularly new process. Performing the typical validation steps described above in microfluidic devices is critical for initial validation. Also, understanding the unique limitations and benefits of the microfluidic systems in use for biological assays will provide insight into what controls will be necessary to more fully validate the results in context of current techniques. As the platforms are validated, a better understanding of how to interpret data produced by them will result.

Ease of Use and Appropriate Design

Clearly, microfluidic devices have shown utility in cell biology research by allowing for new ways of controlling the cellular microenvironment both spatially and

temporally, with a variety of potential cost and resource benefits. By using the unique physical phenomena that dominate in these devices, we can expand the types of assays and cellular responses we can study *in vitro* beyond what is currently available. While the demonstrated utility is clear, ease of use and integration with existing laboratory techniques, resources and equipment is an often overlooked issue that can present a significant barrier to use by biologists. Because microfluidic devices have often been relatively complex to fabricate and use (compared to using petri dishes or multiwell plates), the potential gain from the additional functionalities have not always outweighed the extra work required to use the devices. Thus, an ongoing challenge is to design devices and methods of using the devices that are well suited for cell biology. Close collaborations between the potential end users and the engineers who design the devices can address this challenge.

By constraining designs to operate with commonly available (biological) laboratory tools and analysis equipment (e.g. pipettors, microscopes, plate readers), devices will be significantly more accessible. Focusing microfluidic device designs to provide their unique capabilities without excess complexity will be crucial for them to be easy to use and to become common tools in biological laboratories.

The previous examples of applications of microfluidics to cell biology illustrate why simply designed devices are more likely to be incorporated into labs and widely used than their more complex counterparts. Many flow based gradient generation devices require complex fluid handling systems, (including extensive tubing, syringe pumps and associated electronics). But if simpler passive gradient generation systems (such as that in Figure 1b)[20] can provide a suitable gradient for studying the response of interest but only require a few pipeting steps, they are much more likely to be used. Similarly, the application of plate readers for microfluidic proliferation assays[8] provides a simple, fast, accessible readout for a microfluidic culture rather than devices that rely upon frame by frame microscopy analysis, or manual cell counting. The introduction of microfluidic cultures to automated liquid handling systems has also highlighted the

potential for a high-throughput microfluidic platform for cell culture, which could be easily integrated and widely used and can provide throughput beyond manual pipette loaded microfluidic channels for cell based assays.[3] In all of these cases, a strength of the devices beyond the benefits provided by the microdevices, is the smaller hurdle from the user's perspective because the devices are simpler to use or employ methods that are both familiar and more widely available. Ultimately, microfluidics will only have a significant impact on current experimental methods if they are widely accepted and used by the end users, cell biologists.

EXPANDING THE AVAILABLE ASSAYS

Much of the initial work in integrating cell culture into microfluidics focused on cell survival alone, as many of the original fabrication components and processes were cytotoxic or not compatible with mammalian cell culture. Thus, with the development of more biocompatible polymers, improved methods of spatially patterning proteins and cells and better understanding of the microenvironments the cells are exposed to in these devices, additional assays have been incorporated.[58]

Cell proliferation is a common readout from a microfluidic culture, as often entire culture areas can be imaged and analyzed via imaging software and total adherent or nonadherent cell numbers per channel can be obtained and tracked over time.[7,40] Recent work has integrated microfluidic cultures into a format which can be analyzed by a standard plate reader for cell enumeration purposes.[8]

Integration of current microfluidic culture techniques with existing biological analysis technology (such as DNA arrays), will allow us to further study the effects of microscale cultures on cellular behavior at the molecular level and widen the available range of cellular readouts for microfluidic biological applications. The integration of the research being performed in both engineering and biology has the potential to provide new methods and technologies that may allow biology to be studied in different ways. New assays and new ways of researching biological phenomena will

come from the use of technologies that provide novel functionalities. Future studies integrating cell biological assays with microfluidic cultures will rely upon well designed studies with correct and thorough positive and negative controls for validation purposes.

While some traditional techniques can already be easily integrated into microfluidic devices, some of their characteristics can provide access to new cellular assays, previously inaccessible via traditional methods (e.g., due to improved control over fluid flow and reagent delivery). These will need new methods for quantitation of phenomena that previously weren't an issue (e.g. quantifying cell migration in complex gradients). Also, other characteristics of these devices can result in different results from the same assay performed in a macroscale culture (e.g. differences in cell proliferation due to volume density differences). It will be important to understand these factors when interpreting the data produced in these devices and to address any differences in results as they too may provide insights into the mechanism being studied. These differences might then be leveraged to provide new ways to assay cellular responses by comparing macro- and microscale assays.

More types of assays and readouts need to be adapted into microfluidic cultures. Currently, traditional assays that require significantly more cell numbers or cell lysate than a typical microfluidic culture device would produce cannot be easily integrated into microfluidic assays. Commonly used methods such as performing cell separations/flow cytometric analyses or gel electrophoresis based techniques (Westerns, Northerns, Southerns) have yet to be well integrated into microfluidic culture devices in a user friendly manner. Improving and altering the protocols for these types of readouts to compliment the techniques used for microfluidic assays will be important to better provide accessible and accurate microfluidic versions of existing technology or enable new assays to be performed due to the unique capabilities of microfluidic devices.

CONCLUSION

Microfluidic devices for cell based assays have provided new types of

microenvironments and new methods for controlling and observing the cellular responses to them. The field has begun to analyze the biological effects of the physical differences of microfluidic devices for cell based assays, ranging from evaporation in static microfluidic cultures to flow induced artifacts in gradient generation devices. Nonetheless, the relative lack of quantitative biological analysis techniques that have been interfaced with microfluidic devices has prevented more facets of cellular function beyond viability or proliferation to be analyzed in them. Without a better understanding of the effects of the micro-environments present in microdevices from a cellular perspective, it will continue to be challenging to integrate work done in microdevices with biological data obtained via traditional methods. As more microfluidic devices for cell biology are developed and implemented that address the current roadblocks such as ease of use, biological validity, and limitations in readouts, the unique strengths of these devices will become more accessible to the general biology community as common laboratory tools.

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* Figure 1 adapted (from): (a) With kind permission from Springer Science+Business Media: Biomedical Microdevices, A parallel-gradient microfluidic chamber for quantitative analysis of breast cancer cell chemotaxis, 8, 2006, page 109-118, Saadi W, Wang SJ, Lin F, Jeon NL, Figure 1, and (b) from Abhyankar VV, Lokuta MA, Huttenlocher A, Beebe DJ. 2006. Characterization of a membrane-based gradient generator for use in cell-signaling studies. Lab Chip;6(3):389-93. *Reproduced by permission of The Royal Society of Chemistry,* http://dx.doi.org/10.1039/*b514133h*

**Figure 3 reprinted from Chemistry and Biology, 10/2, Takayama S,

Ostuni E, LeDuc P, Naruse K, Ingber DE, Whitesides GM, *Selective chemical treatment of cellular microdomains using multiple laminar streams*., 123-130, Copyright 2003, with permission from Elsevier.

*** Figure 4 adapted from Toepke MW, Beebe DJ. 2006. PDMS absorption of small molecules and consequences in microfluidic applications. Lab On A Chip;6(12):1484-1486. *Reproduced by permission of The Royal Society of Chemistry* http://dx.doi.org/10.1039/*b612140c*

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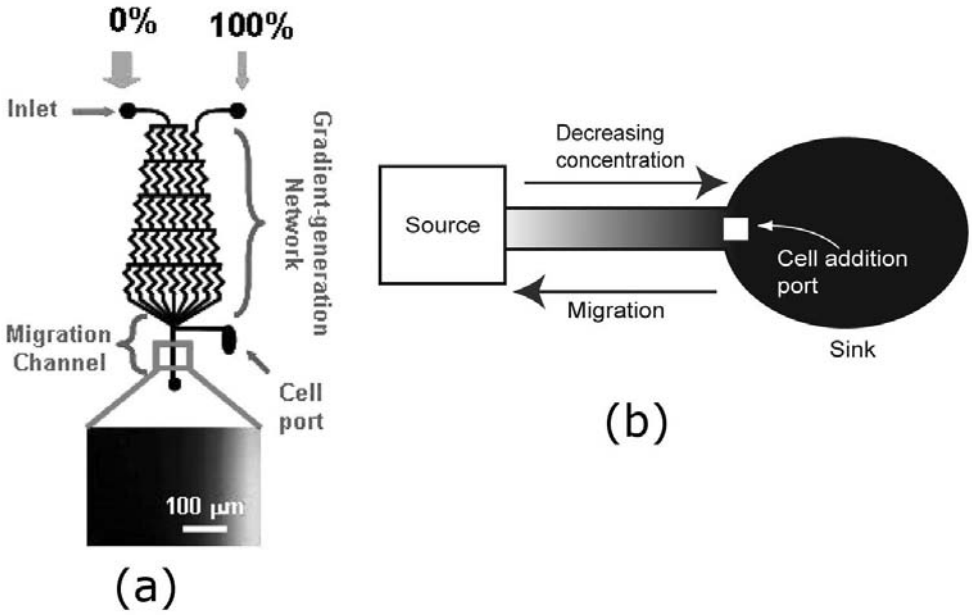

Figure 1: Examples of microfluidic gradient production devices. Flow based gradients like that shown in (a) are based on diffusional mixing solely at the interface between fluid streams. Here two solutions with different concentrations of the solute of interest (0% and 100% of the desired final concentration in this case) are introduced to the inputs of a gradient generation network. Diffusional mixing occurs at the interfaces of the fluid streams and creates a gradient of a defined profile (dependent on input concentrations) at the point labeled Migration channel where cells are treated with the flowing gradient of interest. Static gradient systems like that shown in (b) can be used to create stable gradients in a static fluid, by addition of fluid of the maximum concentration at the Source and allowing the solute to diffuse to the sink, thus exposing cells in the channel to a gradient of the factor. *

110x70mm (300 x 300 DPI)

(a)

	Surface Area per Culture Area (cm^2)	Media Volume per Culture Area (mL)	Surface Density ($cells/cm^2$)	Volume Density ($cells/cm^3$)
6-well Plate	9.5	2-4	40,000	95,000-190,000
Microchannel	0.038	0.004	40,000	380,000
Fold difference (of 6 well over microchannel)	**250x**	**500-1,000x**	**1x**	**1/4-1/2x**

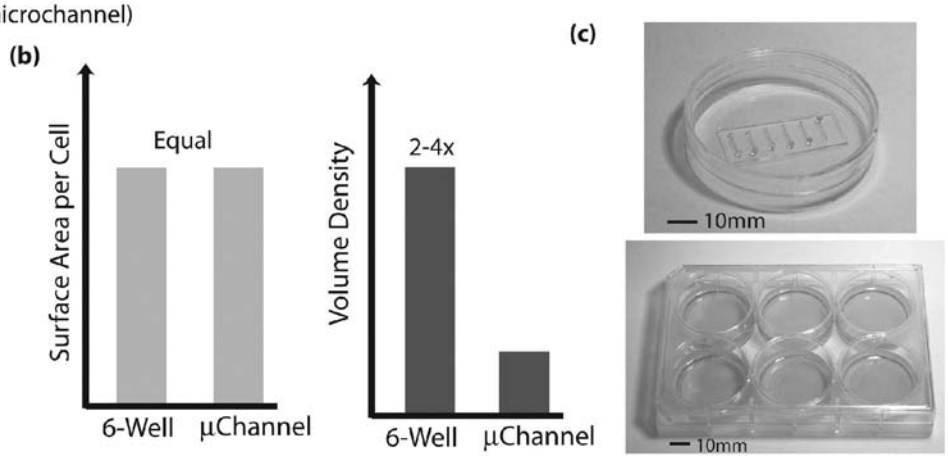

Figure 2: A comparison between volume densities of culture conditions in traditional, macro-scale culture in 6-well plates and in micro-scale, microchannel culture (a,b). Given the same cell surface density, even a rather large microchannel (750µm wide, 5mm long, and 250µm tall) can provide a volume density 2-4 times that of a traditional well in a 6-well plate, use 250 times fewer cells, and 500-1000 times less media and costly reagents. A typical microfluidic channel array with the same replicate number as a 6 well plate are considerably smaller in both media volumes and total space required (c).
152x113mm (300 x 300 DPI)

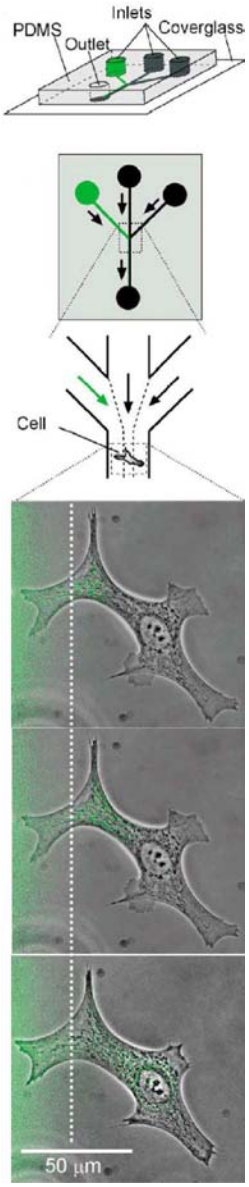

Figure 3: Illustration of a microfluidic device capable of treating subcellular domains with specific reagents while leaving the rest of a cell or region unaffected. Schematics of the device are shown at the top in which the green channels represent the channel in which dye is included. Fluid from the three inputs flows alongside one another and only mix via diffusion, allowing part of the cell shown below to be stained prior to significant mixing of the reagent. In this case, BCE cells are shown in the lower panels after being labeled with MitoTracker Green for 5, 11 and 35 minutes of exposure to the dye, from top to bottom respectively.**

41x203mm (300 x 300 DPI)

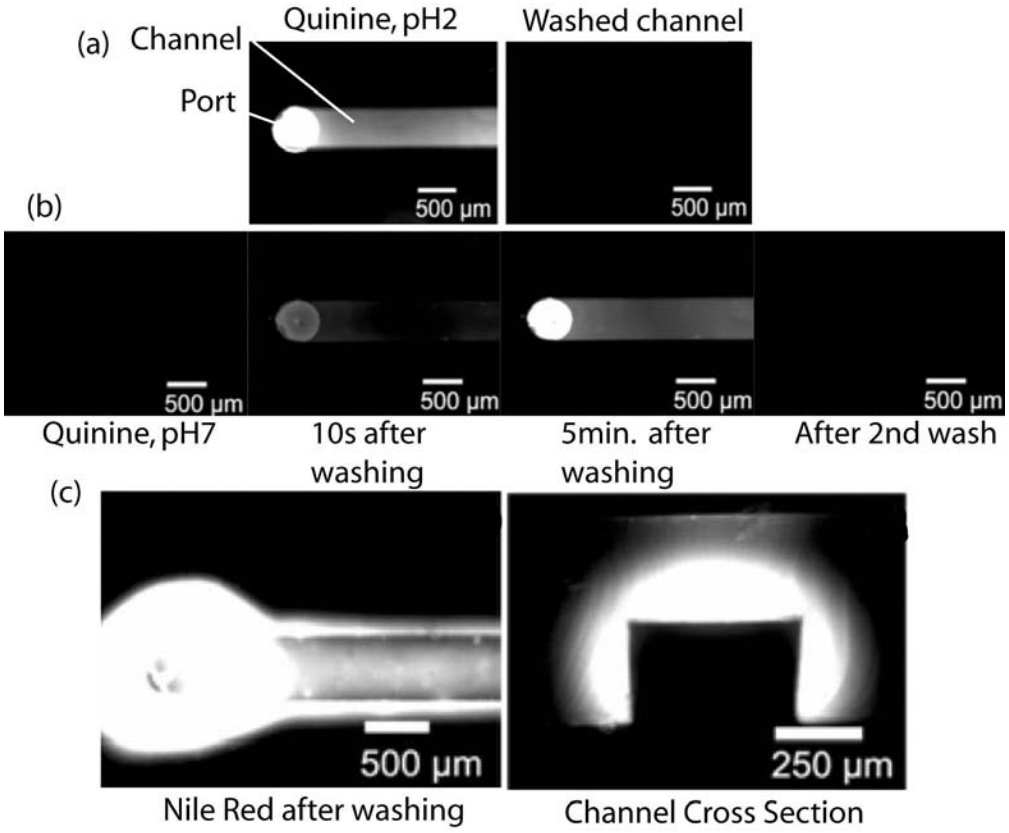

Figure 4: Microfluidic channels fabricated from poly(dimethyl siloxane), (PDMS) have been shown to absorb small hydrophobic molecules. Quinine (fluoresceces at pH2, but not at pH7) was put into a channel and then washed out with pH2 water and fluorescence images of the channel taken (a). If quinine is incubated for 5 minutes in pH7 water in the channel no fluorescence is seen (b) but after the channel is washed with pH2 water, quinine begins to leach back into solution from the PDMS channel walls and remains until it is washed again. A similar phenomenon was shown for Nile Red, as even after the channel is washed with detergent and water, significant fluorescence indicates that the Nile Red was absorbed into the walls of the channels (c).***

130x108mm (300 x 300 DPI)

CHAPTER 2: EXPANDING THE AVAILABLE ASSAYS:

ADAPTING AND VALIDATING IN CELL WESTERNS IN MICROFLUIDIC DEVICES FOR CELL BASED ASSAYS

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ABSTRACT

Microfluidic methods for cellular studies have the potential to provide a significant reduction in costs due to reduced reagent and biological specimen requirements than many traditional culture techniques. However, current types of readouts are limited and this lack of suitable readouts for microfluidic cultures has become a significant limitation of the technique for cell based assays. The In Cell Western (ICW) technique uses quantitative immunocytochemistry and a laser scanner to provide an *in situ* measure of protein quantities in cells grown in microfluidic channels of arbitrary geometries. Validation of the use of ICWs in microfluidic channels was performed by a detailed comparison with current, macroscale methods and shown to have excellent correlation. Transforming growth factor-β induced epithelial-to-mesenchymal transition of an epithelial cell line was used as an example for further validation of the technique as a readout for soluble factor based assays performed in microfluidic channels. The use of passive pumping for sample delivery and laser scanning for analysis opens the door to high throughput quantitative microfluidic cell-based assays that integrate seamlessly with existing HTS infrastructure.

INTRODUCTION

Microfluidic methods for cellular studies are wide ranging from basic cell culture to 3-D tissue engineering. Cell based assays can be performed in microfluidic devices with minimal cell numbers and reagent usage, typically with 250+ fold less total cell number in each channel than each well of a 6 well plate and with 500-1,000 times less reagent volume (Figure 1). These reductions are particularly important for assays run on expensive or rare cell types (such as primary cells, stem cells or FACS sorted subpopulations), and for experiments involving expensive reagents (such as growth factor studies or drug screening). More experimental conditions can be tested with more replicates with the same sized sample, and with less reagents when done in microfluidic cultures rather than 6 or even 96 well plates.

While the designs and functionalities of microfluidic devices are diverse, methods for analysis of cellular responses to microenvironments and experimental conditions are not as numerous. A main issue that prevents microfluidic assays from being integrated as a tool for *in vitro* assays is the lack of simple, quantitative readouts. Staining for viability using dyes such as calcein AM/propidium iodide or trypan blue are common, but require each image to be analyzed by hand or via complicated image processing.[1-5] Similarly, markers of differentiation or other immunocytochemical (ICC) methods must be analyzed manually, frame by frame (or slice by slice in 3D cultures) via microscopy.[2,6] These readouts are subject to experimenter bias in choosing a "representative field", or determining what is "positive" and what is "negative" staining for a protein of interest. They also are only applicable to a small subset of experiments in

which viability or the complete absence or presence of a specific protein are useful readouts.

While some degree of experimenter bias is removed when automated microscopy image processing methods are used, they too have significant caveats and limitations (e.g, non-uniformity of the excitation intensity within a single field of cells, inaccuracies in determining the edge of individual cells).[5] Using a plate reader to determine cell numbers in channels enables improved automation, reduced experimenter bias and the ability to assay the entire population rather than just a few representative images.[7] However this method requires specific channel geometries, limiting the types of designs that can be analyzed this way.

Simply designed microfluidic culture devices themselves could be used in biological laboratories with no requirement for extra, specialized equipment beyond that typically found in a lab equipped to do macroscale tissue culture (pipettes, Petri dishes, cell culture reagents, immunofluorescence reagents). Also, integration of these devices with current liquid handling systems could allow for rapid, high throughput cell based assays to be performed with the benefits of low volumes and small total sample size. However, the traditional techniques used for quantitative readouts in macroscale cultures require significantly larger samples than typically found in microfluidic cultures (Figure 1).

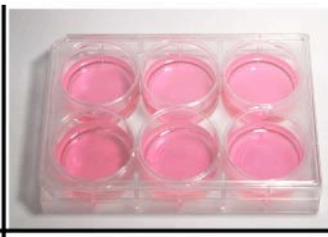
Per Well or Channel Values			Fold Macro over Micro
Surface Area	9.6 cm^2	0.038 cm^2	250x
Volume of Media/Reagents	2-4 mL	4 µL	500-1,000x
Surface Density	40,000 cells/cm^2	40,000 cells/cm^2	1x
Cell Number Per Replicate	384,000 cells	1,500 cells	250x
Avg. Total Protein in Lysate	200-800 µg	0.84-3.33 µg	250x
Protein in Lysate per unit Area	21-83 µg/cm^2	21-83 µg/cm^2	1x
Protein per Lane	20µg	20µg	1x
Lanes Filled with 10 µg of Protein	10-40 Lanes	1/24-1/6 Lane	250x

Figure 1: Calculations relating cultures in 6 well plates to those in microchannels. Because surface areas and media volumes in microchannels are significantly smaller than in 6 well plates, for the same cell surface density, fewer cells per independent environmental condition are required per replicate in microchannels. However, with cells seeded at the same surface densities in 6 well plates or in microchannels, sufficient lysate can be obtained in a single well of a 6 well plate for 10-40 lanes of a gel, while microchannels will only provide up to 1/6 the amount needed for one lane of a gel for a Western blot. While the reduction in sample sizes is a strength of microfluidic cultures, such small samples makes many traditional readouts nearly impossible.

Immunostaining techniques such as Western blotting are common practice in traditional biology laboratories, but the amount of cell lysate obtained to perform a typical assay is 250 fold more than a microfluidic culture normally provides (Figure 1).

Even if only one lane of a gel is filled with lysate from one microfluidic device, at least 6 times more cell lysate would be required to put the comparable amount of protein per lane of a gel as typically done. The applicability of microfluidics for Western blotting becomes even worse for cell types that produce very little protein per cell, or if the protein of interest is particularly rare, and requires more protein per lane to be loaded in order to detect it (e.g, 100 μg+ of total protein per lane). While low cell numbers precludes the possibility of performing traditional Western blots easily and efficiently from microfluidic cultures, it makes performing a Western type assay *in situ* beneficial. Additionally, because microfluidic cultures allow for more replicates or more conditions to be tested with the same cell sample, Western blotting techniques aren't as amenable to high throughput analyses as an *in situ* type assay would be.

Recently, techniques for performing In Cell Westerns (ICWs) using a laser scanner or plate reader have been applied to a variety of analyses.[8-10] To perform an ICW, cells are grown in monolayer cultures using typical tissue culture protocols then fixed and stained just as for immunocytochemistry using fluorescent secondary antibodies. Using commercially available laser scanners, the total fluorescent signal from a well of a 96 well plate is determined and normalized to a loading control such as β-actin or DNA content. With the appropriate controls needed to correct for issues like background fluorescence, and careful image processing, a quantitative measurement of the changes in relative levels protein expression between conditions can be determined. This technique does not rely on obtaining and processing sufficient amounts of cell lysate, performing gel electrophoresis and blotting procedures as is the case for a

traditional gel-based Western, nor is it subject to the level of experimenter bias that occurs when using microscopy based ICC methods. Also, this type of readout lends itself to automation and high throughput analyses well, providing a method to perform many Western-type assays at once, in a much more user-friendly manner.

To test and validate the ICW technique in microfluidic cultures, a well established cellular response was chosen to illustrate the technique. Epithelial to mesenchymal transition (EMT) is a transdifferentiation process involving a variety of short- and long-term cellular responses.[11-14] Mouse mammary epithelial cells undergo this transition in response to transforming growth factor β (TGF-β), and the responses of these cells to this growth factor have been studied for over a decade.[15] Specifically, the NMuMG cell line (normal murine mammary gland epithelial cells) has been shown to respond to TGF- β and undergo EMT *in vitro*.[16] The characteristic changes that occur during this transition include a growth inhibitory effect along with changes in morphology and expression. These cells typically have a cobblestone morphology, express and maintain large amounts of E-cadherin on their surfaces and lack N-cadherin. These characteristics change to a long, motile fibroblastic morphology, with a loss of E-cadherin and gain of N-cadherin after EMT occurs. Distinct actin stress fibers begin to form in the transformed cells as well. The changes in expression of E and N cadherin in response to TGF- β were used for validation of the ICW technique and validation that the complete set of effects occurred were verified via microscopy.

METHODS

Cell culture and seeding

Cells used were normal murine mammary epithelial cells (NMuMGs), a cell line from ATCC. Also tested to ensure more broad applicability, were mouse mammary fibroblasts (MMFs) isolated from C57BL/6, Ink4a knock out mice (from the Alexander lab). Both cell types were passaged in high glucose DMEM with 10% serum, with the addition of 10μg/mL insulin for NMuMGs. Cells were passaged by washing with DPBS without calcium and magnesium briefly, then incubated at 37°C with trypsin (0.05% trypsin with EDTA) for 2 minutes. The trypsin was diluted with medium, and the diluted cell suspension was then counted and centrifuged for 3-5 minutes at 200 rcf. The supernatant removed and the pellet resuspended in medium to the desired concentration. New flasks were seeded at a surface density of 40,000 cells/cm^2 and a volume density of 200,000 cells/mL, and passaged after approximately 2 days when 70-80% confluent.

To seed cells into 96 well plate wells and microchannels, media was first added to both (2μL to channels and 184μL into wells) and put into the incubator while the cells were being prepared. When the desired stock concentrations were obtained, 2μL of cell suspension was added to the microchannels, and 16μL added to the corresponding wells giving equal surface densities, but 6.25 times higher volume densities in microcultures than macro cultures. These were then placed in the humidified incubator either as is (in the case of the 96 well plate), or in a tray containing DPBS for the microcultures to minimize evaporation and osmolarity changes.

For linearity assays, the number of cells in the channels or wells needed to be known, so cells were seeded at different numbers of cells per well or channel, then fixed after attachment, but prior to significant proliferation. Serial dilutions of cell suspension was seeded into both 96 well plates and microchannels (at least 4 wells per dilution and at least 6 channels per dilution) from the same stock solution. For NMuMGs, cells were fixed at 24 hours after seeding to ensure attachment (attachment occurs between 12 and 16 hours post seeding in microchannels, and somewhat faster in 96 well plates) and for MMFs due to their more rapid attachment kinetics (attached between 2-3 hours post seeding regardless of scale), cells were fixed at 4 hours post seeding.

Fixation and Staining

Cells were fixed by first washing with PBS quickly. To fix, 4% paraformaldehyde in PBS was added and incubated on ice for 20 minutes. Cells were then washed with three sequential washes with 0.1% Triton X-100 in PBS for 7 minutes each wash, at room temperature. Blocking buffer (Licor Biosciences, #927-40000) was then added for 60 minutes at room temperature. The primary antibodies were then diluted into blocking buffer, added to the channels/wells and incubated overnight at 4°C. This solution was then washed out with PBS with 0.1% Tween-20 twice, and the secondary antibodies diluted into blocking buffer were added for 1 hour at room temperature. The channels/wells were then washed with PBS once, and then To-Pro-3 (Molecular Probes) was added at 1:1,000 in PBS, and allowed to incubate at room temperature for 10 minutes (DAPI was used for immunocytochemistry with the same dilution and incubation, from

Molecular Probes). Cultures were washed twice with PBS, all liquid removed and allowed to dry. PDMS channel tops were removed prior to scanning.

TGF- β exposure

Assays of the response of NMuMGs to TGF-β were performed by seeding cells in both macro- and microscale cultures at 40,000 cells/cm^2. Cells will be allowed to plate for 24 hours, and the media was changed to TGF-β containing media or control media (TGF-β was from Sigma). After 48 hours of exposure to 100pM TGF-β or control media (with a media change at 24 hours for microscale cultures), cells were fixed and stained for E-cadherin and N-cadherin for ICW analysis, or imaged using phase contrast microscopy (for morphology) and fluorescence microscopy (for actin staining and E and N-cadherin ICC). Actin staining was performed on cells seeded in chamber slides after fixation by incubating cells with 1:1,000 dilution of Alexa 488 conjugated phalloidin and 1:1,000 dilution DAPI for 20 minutes at room temperature after only one triton wash, then washed with PBS and mounted.

Western blotting

Cells were seeded into 60mm dishes at 40,000 cells/cm^2 and allowed to plate for 24 hours. Media was exchanged at 24 hours to either control or 100pM TGF- β containing media. After 48 hours of exposure cells were washed briefly with PBS on ice and subsequently lysed with RIPA buffer containing a protease inhibitor cocktail (Roche, Complete Mini tablets, # 11836153001). Lysates were sonicated briefly and kept frozen at -80ºC until ready for use. Lysates were defrosted, SDS tris-glycine sample buffer and

2% β-mercaptoethanol added, boiled for 5 minutes, centrifuged and the supernatant removed. The samples were run on 8% tris-glycine gels, with molecular weight markers for IR detection (Licor Biosciences, #928-40000). After wet transfer to nitrocellulose membranes, the blots were blocked (blocking buffer from Licor) for 60 minutes at room temperature, then incubated overnight at 4˚C with primary antibodies to E-cadherin (1:2,500) or N-cadherin (1:1,250), and a primary antibody to β -actin (1:1,000).

Blots were then washed 4 times with PBS with 0.1% Tween-20, for 5 minutes each, then put into blocking buffer with two secondary antibodies, one for mouse with the 800CW IR dye conjugation (to detect E or N-cadherin) and one for rabbit with the 680 IR dye conjugation (to detect actin) both at 1:15,000. After incubation with shaking at room temperature, in the dark for 45 minutes, blots were washed 4 times with PBS/0.1% Tween-20 and allowed to dry in the dark. The dry blots were then scanned on the IR scanner with the intensity of the 700 channel (the signal for actin) lower than that of the 800 channel (the signal for either E or N-cadherin).

Immunocytochemistry reagents

The primary antibody for β–actin was obtained from Cell Signaling (#4970) and used at a 1:1,000 dilution in Western blots and at 1:100 for ICWs. E-cadherin (#610182), N-cadherin (#610921) primaries were obtained from Transduction Labs. A second E-cadherin was obtained from Cell Signaling and used only for the Western blot shown in the supplement, not for any immunocytochemistry or ICWs (#3195). Secondary antibodies were from Rockland, Inc (#610-131-003, goat anti-

mouse, 800CW IR dye, and #611-131-122 Goat anti Rabbit, 800CW IR Dye conjugated), or Licor (#611-130-122-IRDye 700DX conjugated goat anti-rabbit). Alexa 488 conjugated Phalloidin, ToPro3 and DAPI were from Molecular Probes.

RESULTS

Initial validation of the ICW technique in microfluidic channels was performed by comparing results from microchannels with those found in 96 well plates using established protocols using two different cell types (NMuMGs shown here and isolated mouse mammary fibroblasts shown in the supplementary information). Subsequently, an application of the In Channel Western technique was tested using the well-established readouts resulting from EMT in NMuMGs in cultures of both scales to determine if comparable results could be obtained. The responses in both macro- and microscale cultures was verified via morphology and actin staining in addition to ICWs for E-cadherin and N-cadherin after 48 hour exposure to 100pM TGF-β or control medium. These results were then validated by microscopy and immunocytochemistry, and also via traditional Western blots and shown to be quantitatively consistent with ICW results for E and N cadherin.

LINEARITY AND ACTIN ICWS

To verify that the nuclear staining control for cell number was in fact linear with cell number, cells were seeded at a range of surface densities likely to be found in cultures, fixed after attachment but prior to any significant proliferation, fixed,

stained and scanned. Phase contrast images of the cells prior to fixation are shown in Figure 2a for NMuMGs showing the degree of confluence for each density seeded. The measured integrated intensity of a channel or well from the nuclear stain was then compared to the cell density seeded, and the linear correlation determined (Figure 2b for NMuMGs). Two cell types were used for validation to ensure the applicability of the technique for multiple cell types, and data for the second cell type, mouse mammary fibroblasts (MMFs), are shown in the supplementary information, Figure S1. Both cell types were tested at a range of typical surface densities appropriate to each cell type between 12.5 and 150 K cells/cm^2 (lower densities for MMFs and higher densities for NMuMGs).

Results showed very close, linear correlation between cell number and nuclear integrated intensity for both culture scales for two different cell types (R^2 values of between 0.9759 and 0.9981). Linearity was confirmed for both macro and microscale cultures, indicating that the nuclear integrated intensity measured can be used as a measure of cell number.

Not only does nuclear control staining need to be linear with cell number in cultures of both scales, but also immunocytochemistry based results would have to be consistent between them as well (the signal to be quantified from proteins of interest). ICWs have been performed in 96 well plates, but because the volumes used in microchannels are so much lower but have different washing efficiencies,[17] it is important that the same results are obtained with the protocol regardless of scale. If

comparisons are to be made between protein levels in macro- and microscale cultures, then the technique must give the same normalized integrated intensity (normalized to the nuclear intensity, the control for cell number) for a protein that is expressed at the same level in cells in both culture scales (such as β-actin, a typical loading control in traditional Western blots).

An ICW for β-actin was performed in order to demonstrate that the measured amount of actin per cell is consistent between macro and microscale cultures (Figure 2c for NMuMGs, Figure S1c for MMFs). The data indicate that the normalized actin intensity is in fact the same regardless of culture scale for each cell type as no significant difference was observed in actin levels (p values of 0.39 for NMuMGs and 0.52 for MMFs). This data indicate that normalized integrated intensities determined in either 96 well plates or microchannels are consistent and can be compared directly.

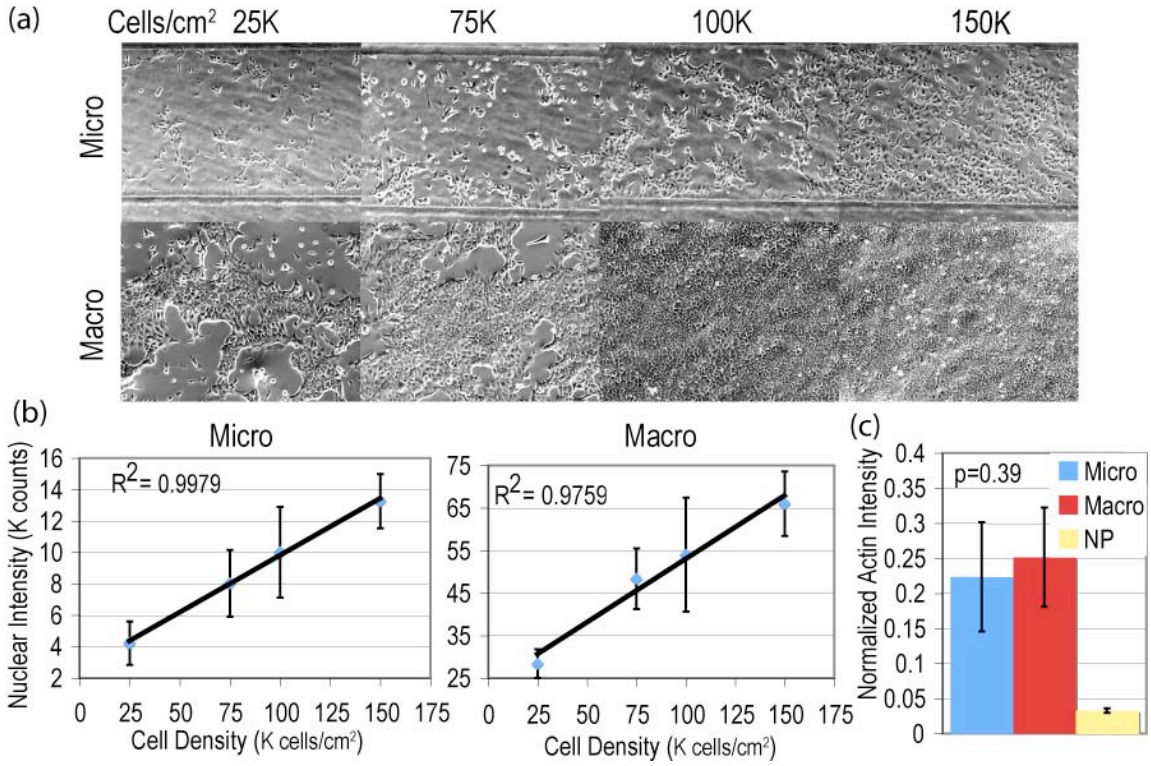


Figure 2: Phase images of NMuMGs prior to fixing (a), linearity assays (b), and actin ICW results (c). NP is the no primary control which corresponds to the level of background due to nonspecific staining.

MORPHOLOGICAL ANALYSIS OF TGF-β INDUCED EMT IN MICROSCALE CULTURES

The morphological responses of NMuMGs to a 48 hour exposure to 100pM TGF-β in microscale cultures were tested to ensure that the responses seen in cultures of either scale were similar to those established for macroscale cultures. Figure 3 shows the morphological changes of NMuMGs from the typical cobblestone morphology to elongated fibroblastic morphology in microscale cultures (a for phase and b for actin/nuclear staining). Also ICC assays for both E- and N-cadherin (Figures 3c, 3d, respectively) in both control and transformed cells were visualized via microscopy. Using the same exposure time for either treated or untreated conditions, it could be seen

qualitatively that E-cadherin is reduced and N-cadherin is increased in transformed cells as expected.

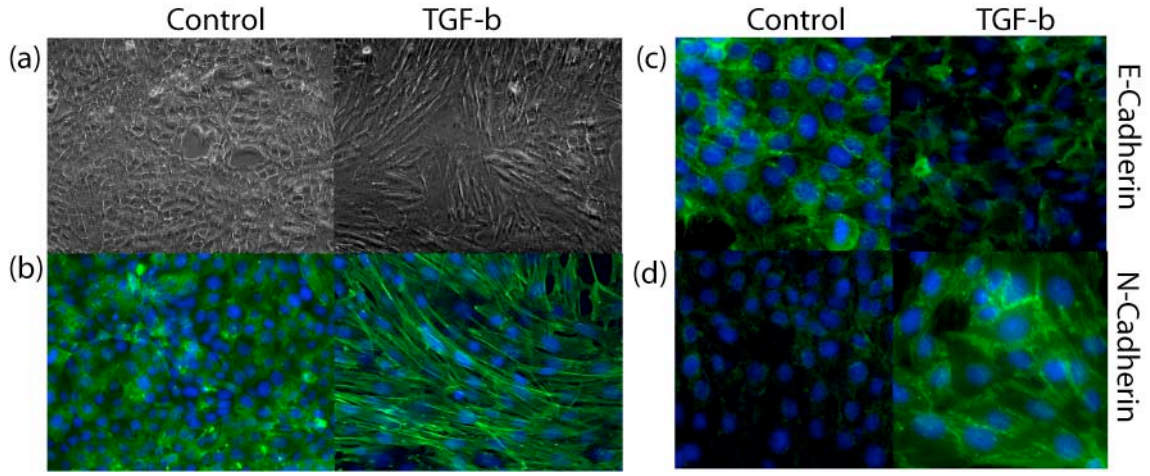


Figure 3: NMuMGs in microfluidic cultures undergo the morphological changes typically seen in macroscale cultures, changing from cobblestone to fibroblastic morphology shown in phase in (a). Also, the organization of actin changes from mesh-like localization in control cells to significant levels of stress fibers in treated cells (b) occurs as expected, (blue is nuclei, green is actin). E-cadherin expression is high in control cells and decreases after TGF-β treatment (c, blue is nuclei, green is E-cadherin). N-cadherin expression increases significantly after TGF-β exposure (d, blue is nuclei, green is N-cadherin).

E-CADHERIN AND N-CADHERIN EXPRESSION IN NMUMGS

To quantify these changes in E- and N-cadherin expression in NMuMGs after TGF-β exposure, ICWs were performed in both macro (96 well plates), and microcultures. The data shown in Figure 4 indicate that similar magnitude changes occur in E-cadherin regardless of culture scale (1.55-fold decrease in macro, and 1.46-fold decrease in micro), while the increase in N-cadherin in microcultures is nearly half of that seen in macrocultures (0.61 for macro, and 0.31 for micro). These differences may indicate a difference in sensitivity to TGF-β transformation in cultures of different scales.

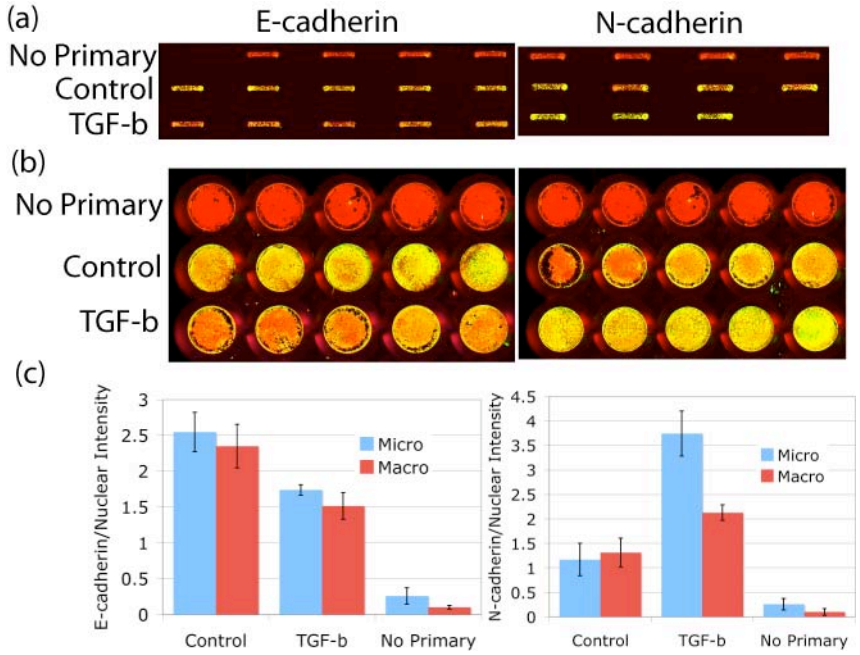
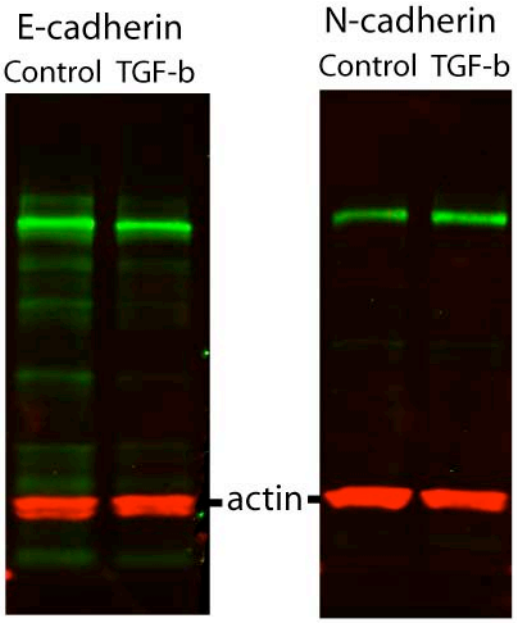


Figure 4: Summary of all readouts for E- cadherin (left) and N-cadherin (right) in control and TGF-β transformed cells. Raw images obtained from ICWs in microchannels (a), with corresponding data from 96 well plates (b). Red is ToPro3 stained nuclei, overlayed with green signal from E- or N-cadherin. Quantification of the integrated intensities from the ICW images (c). E-cadherin and N-cadherin fluorescence has been normalized to the nuclear fluorescence as a control for cell number for micro (microchannels), and macro (96 well plate).

The success of the ICW technique relies upon good antibody specificity, but also the ability of the antibodies to recognize the antigen in the non-reduced, non-denatured conformation, but instead the modified (by paraformaldehyde and triton) conformation found in fixed and permeabilized cells. Recognition of the fixed conformation can be verified by immnocytochemistry and microscopy as demonstrated in Figure 3. Changes in the levels of a specific protein in response to a control treatment known to cause changes in expression or localization can provide an idea of the specificity of the antibody beyond just verification of the expected localization in untreated cells.

Because ICWs do not provide any molecular weight information like a Western blot can, non-specific staining cannot be eliminated by only analyzing bands of the expected molecular weight. Because of this, the signal that is expected from an ICW would likely be more similar to the total intensity of all the bands in a Western blot than just the specific band. Despite the differences in protein conformation in a Western blot, antibody specificity can be evaluated by showing only one band in a traditional Western. However, it does not necessarily guarantee specificity in ICWs, but when combined with ICC data with appropriate controls, this data can be useful in validating the accuracy of an antibody.

Figure 5 shows Western blots analyzed using the same set of antibodies used in the ICWs and ICC data. With this type of analysis, the normalized intensity from both the specific bands and the total lanes can be obtained (green) and normalized to the normalized intensity of the signal from actin (red). The fold change in E or N cadherin between control and treated cells (100pM TGF-β for 48 hours) was analyzed from both the specific bands (labeled Specific), and also for the total lane, (labeled Expected). For both readouts, the ICW fold changes are more similar to the fold change in the expected signals than the specific signals from the Westerns. Two different E-cadherin antibodies were also evaluated in this manner in order to determine if either was more appropriate for use in ICWs (see Figure S2 in supplement).



Fold Induction:	Specific	Expected	Specific	Expected
Western	1.19	1.60	0.45	0.57
	Macro	Micro	Macro	Micro
ICW	1.55	1.46	0.62	0.31

Figure 5: Western blots and quantification for both E-cadherin and N-cadherin in control and TGF-β transformed cells. In these Western blots red bands are actin loading controls, while green bands are what is detected by the cadherin antibodies (either E or N-cadherin), contrast has been enhanced to show all staining including non-specific staining. The fold inductions (control over TGF-β) were calculated from Western blots either for the specific band corresponding to E or N-cadherin, or the entire lane (expected) along with ICW results for micro and macro.

ADAPTATION TO A HIGH THROUGHPUT SYSTEM

The ICW technique was performed in an array of microchannels designed to interface with an automated liquid handler. A dose response curve was obtained for the E-cadherin readout in NMuMGs after 48 hour exposures to doses between 100pM and 0pM. Initial data (Figure 6) showed a much larger change in E-cadherin expression than previously seen in manually loaded microchannel ICWs (fold decrease in E-cadherin was 4.7). However, the dose response curve does show the expected trends indicating that this type of readout can be performed in a high throughput microfluidic system.

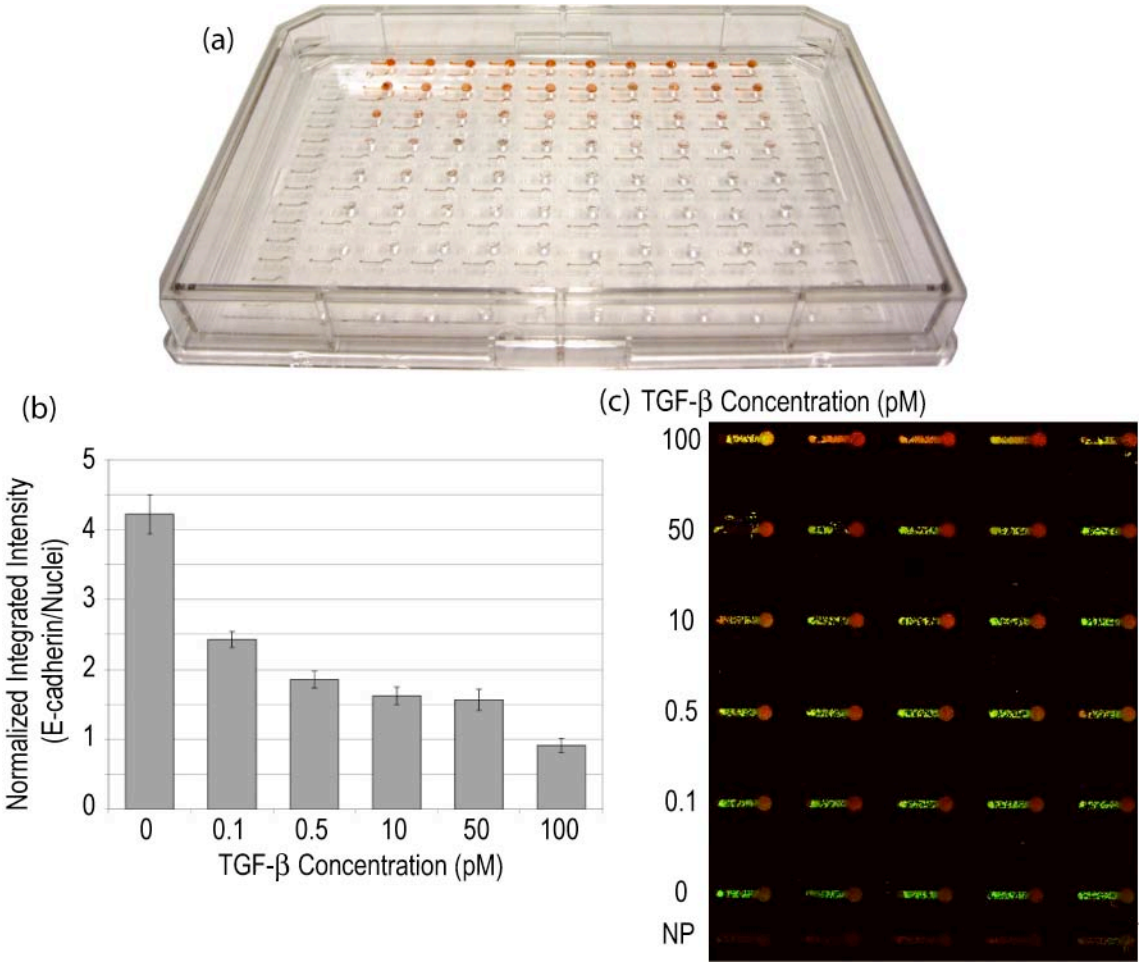


Figure 6: ICWs were performed in a high throughput microfluidic device which integrates with an automated liquid handling system. An example of the device is shown in (a) with food coloring diluted to illustrate how the dose response is arranged in the channels (dark orange corresponds to high TGF-β concentrations). The quantification of the ICW for E-cadherin after TGF-β exposure is shown in (b), and the raw image from the scanner with the doses of TGF-β listed beside each row (c). In one device, the size of a 6 well plate, a dose response with 6 doses of TGF-β can be performed for two independent readouts (each with reps of 5), with completely automated fluid handling. * High throughput adaptation data was contributed by John Puccinelli

DISCUSSION

While Western blotting cannot be easily adapted to microfluidic cultures (unless large numbers of channels are pooled), ICW techniques can be and provide a straightforward means for not only quantifying protein levels, but also doing so *in situ*. Due to the low reagent volumes in microfluidic cultures, significantly less volume of costly reagents (antibodies and buffers) are required as compared to traditional Western blotting, or even macroscale ICW techniques. By doing the assay *in situ*, less handling and therefore likely less sample corruption, contamination and loss occur than using traditional Westerns which becomes particularly critical for very small volume samples.

ICW based microfluidic assays can be done rapidly due to the relatively short protocols for fixation and staining, and require no other laboratory items (such as gels, buffers, membranes) than typical immunocytochemistry reagents and pipettes. Using commercially available laser scanners (often found in many shared laboratory facilities), entire channels with arbitrary geometries can be imaged quickly and analyzed easily. This flexibility could simplify and expand the potential readouts from cocultures of different cell types, cells grown in gradients of soluble factors or in other novel assays performed in microfluidic devices. Using a laser scanner also allows entire populations to be assayed rather than the experimenter choosing a "representative" field for immunocytochemistry type readouts, and ensures that an accurate, unbiased, quantitative measurement can be taken.

With this technique in hand, the simple microfluidic channels used for this

validation study were used with a robotic fluid handling system to provide a higher throughput expression assay while also reducing reagent costs by 8 fold (12.5% of the cost of the same assay done in a 96 well plate). Alternatively, using these simple channels with manual pipettes provides both the flexibility to look at 8 different readouts with the same amount of cell suspension as used in a 96 well plate, or perform more replicates of the same assay and also can be done in any biological laboratory.

Integration of current microfluidic culture techniques with existing biological analysis technology, will allow us to further study the effects of microscale cultures on cellular behavior and widen the available range of cellular readouts for microfluidic biological applications. Currently, many traditional assays require significantly more cell numbers or cell lysate than a typical microfluidic culture device would produce and cannot be easily integrated into microfluidic assays (such as flow cytometry, Northerns, Southerns, etc). Improving and altering the protocols for other types of readouts to compliment the techniques used for microfluidic assays will be important to better provide accessible and accurate microfluidic versions of existing technology or enable new assays to be performed due to the unique capabilities of microfluidic devices.

SUPPLEMENTARY INFORMATION:

* *To be included as a supplement for publication*

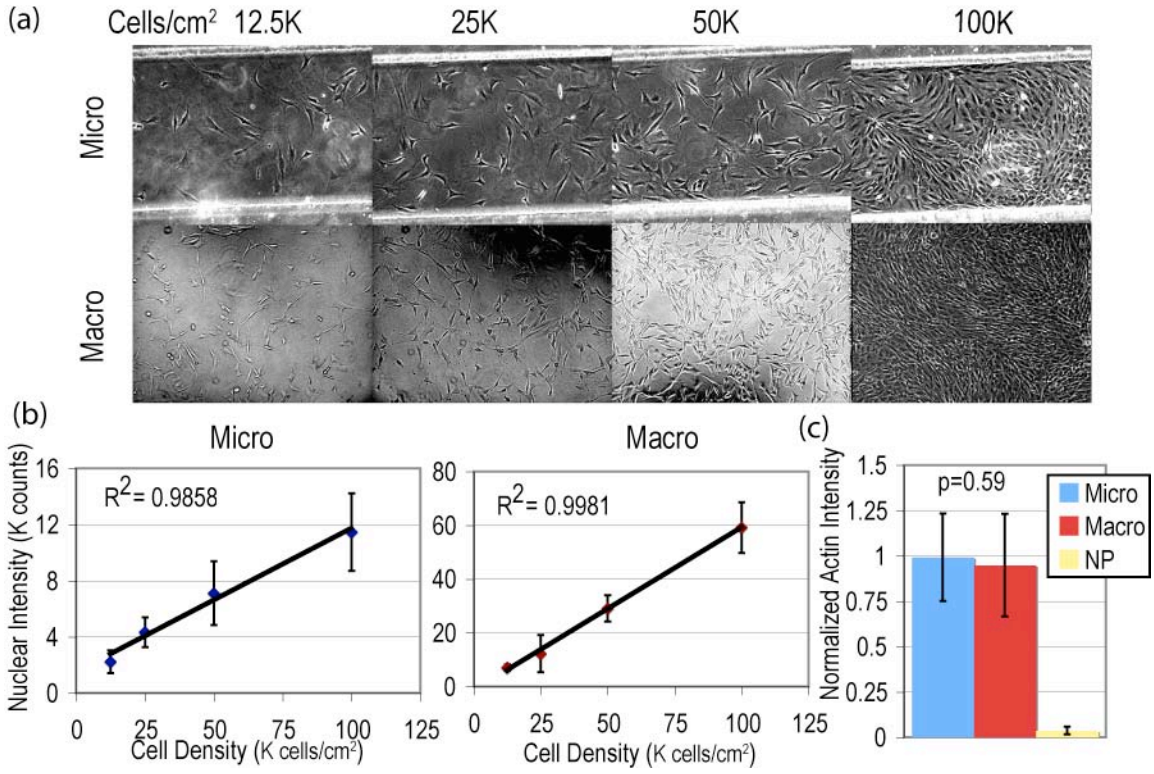
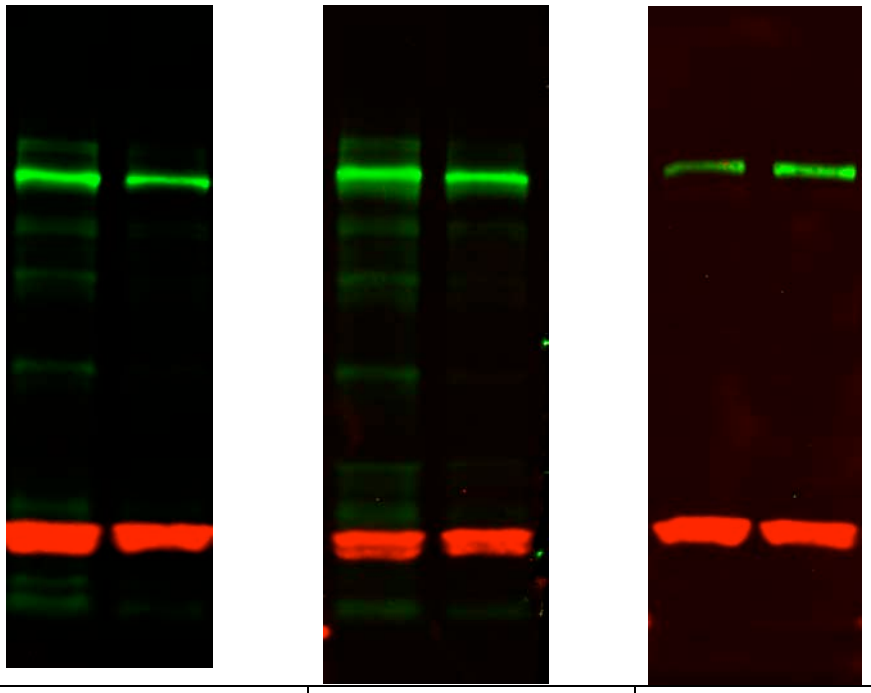


Figure S1: Phase images of MMFs (a), linearity assays (b), and actin ICWs (c).

WESTERN BLOT EVALUATION OF ANTIBODIES

Two different E-cadherin antibodies were tested with Western blots to determine whether there is a significant difference between their abilities to function in an ICW. Western blots were performed using two E-cadherin antibodies from different companies and shown in Figure S2 with the specific and non-specific signals analyzed (with the same analysis done for the N-cadherin antibody used). The bottom row of the table shows the percentage of the total lane signal that is made up by the specific signal for each condition and antibody. The E-cadherin antibodies from Cell Signaling and from Transduction Labs show different specificities. The Transduction Labs antibody (middle) proved to have higher specificity, as for both control and treated cells, the percentage of the total lane made up by the specific signal is larger than that for the CST antibody. Because of this, the Transduction Labs antibody was used for the ICWs shown above.



	CST E-cadherin Control on left, 100pM TGF- β for 48 hours on right		BD E-cadherin Control on left, 100pM TGF- β for 48 hours on right		BD N-cadherin Control on left, 100pM TGF- β for 48 hours on right	
Fold increase Control over TGF-b	Specific	Total	Specific	Total	Specific	Total
	1.32	1.82	1.19	1.60	0.45	0.57
Specific to total signal ratio	Control	TGF-b	Control	TGF-b	Control	TGF-b
	37%	51%	50%	67%	59%	75%

Figure S2: Analysis of different antibodies for E and N cadherin. The fold increase of control levels of each readout versus that in the TGF- β treated lysate when only the specific bands are considered or the total lane intensity. Also, the percentage of the total signal made up by the specific signal is listed for each condition and each antibody.

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SUPLEMENTARY INFORMATION FOR:
PART 1: FROM THE CELLULAR PERSPECTIVE:
DIFFERENCES IN THE CELLULAR BASELINE IN MACROSCALE AND MICROFLUIDIC CULTURES

The success of the ICW technique relies upon good antibody specificity, but also the ability of the antibodies to recognize the antigen in the non-reduced, non-denatured conformation, but instead the modified (by paraformaldehyde and triton) conformation found in fixed and permeabilized cells. Recognition of the fixed conformation can be verified by immnocytochemistry and microscopy. Changes in the levels of a specific protein in response to a control treatment known to cause changes in expression or localization can provide an idea of the specificity of the antibody beyond just verification of the expected localization in untreated cells.

Because ICWs do not provide any molecular weight information like a Western blot can, non-specific staining cannot be eliminated by only analyzing bands of the expected molecular weight. Because of this, the signal that is expected from an ICW would likely be more similar to the total intensity of all the bands in a Western blot than just the specific band. Despite the differences in protein conformation in a Western blot, antibody specificity can be evaluated by showing only one band in a traditional Western. However, it does not necessarily guarantee specificity in ICWs, but when combined with ICC data with appropriate controls, this data can be useful in validating the accuracy of an antibody.

For ICWs that provided somewhat small changes in protein levels, or (as in the case for BiP), unusual results were obtained in ICWs, the corresponding ICC and Western blots were performed to get a better idea of how the antibodies were functioning. The following figures includes ICC and Western blot data for many of the readouts listed as validation for the responses seen. Each set of ICC images (between images for

phospho-proteins with positive and negative controls, and also between those and the no primary controls) were taken with all the same exposure and magnification parameters and all further image processing steps (to convert the formats suitable for publication) were kept the same to maintain a semi-quantitative comparison in the images. Also, all ICC data were done in macroscale cultures. In all of the Western blot images, after scanning on a laser scanner, the contrast was increased in order to see the nonspecific staining as well as the band of interest to better understand how specific the antibodies were in Western format. In each, the entire lanes are shown from approximately 15kDa (at the bottom), to 150kDa (at the top).

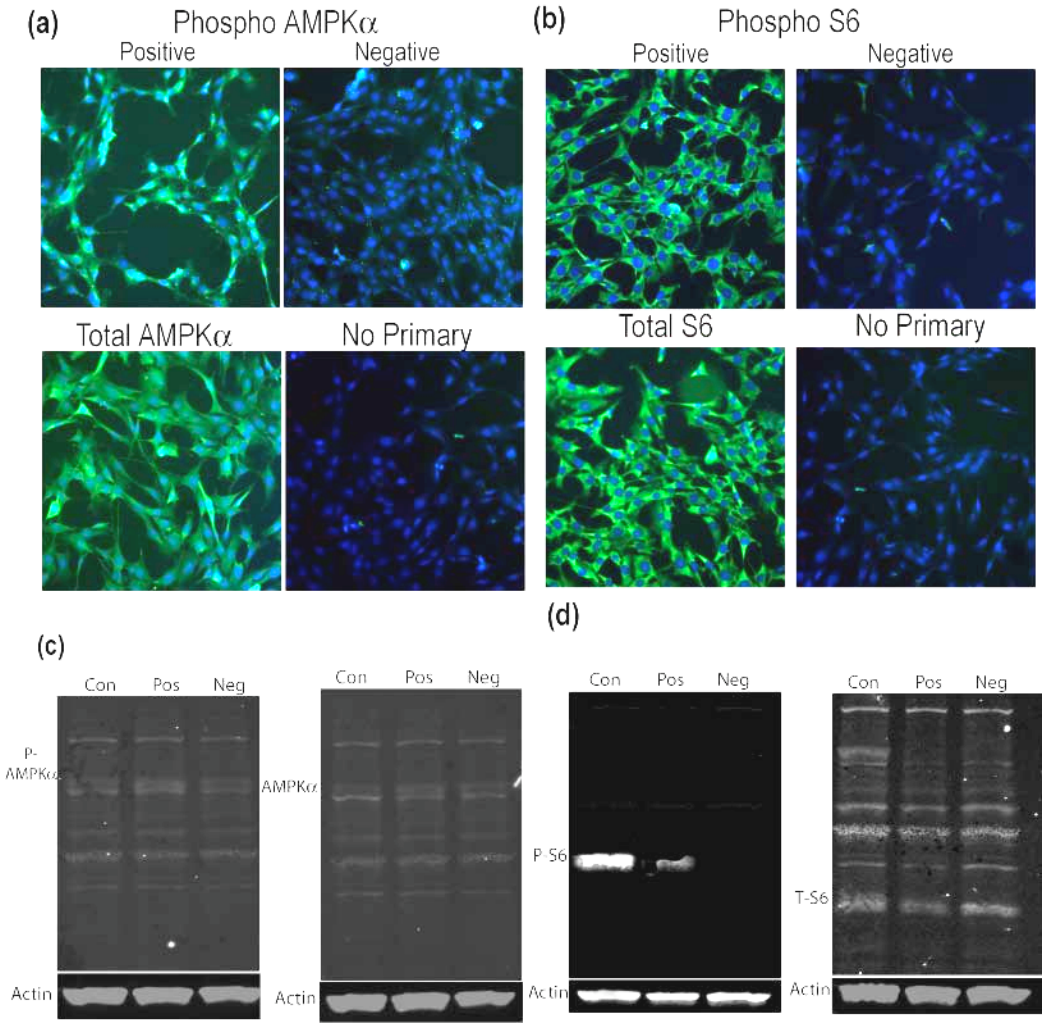

Figure S1: ICW results were checked by the corresponding immunocytochemistry (a, AMPK and b, S6). Western blots were done from macroscale cultures (c, AMPK and d, S6) as well. The responses were very clearly different in the ICC results, although the Western blots seemed less specific.

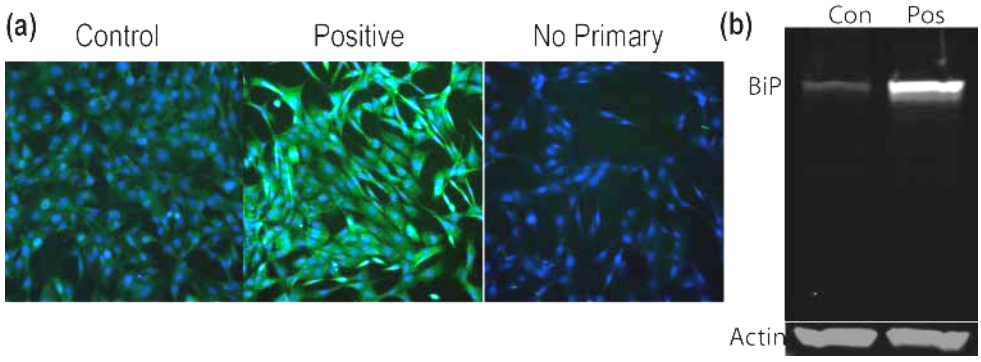

Figure S2: BiP ICC was done in macroscale cultures with the positive control having 300nM Thapsigargin for 16 hours prior to fixing. Careful fixing was done in macroscale cultures to prevent washing the cells off the surfaces, as occurred in the ICWs. Also, the Western blot showed a very large upregulation in BiP, and proved to be very specific. These data suggest that despite the cell lift off issue in macroscale cultures, the BiP ICW for control conditions comparing cultures in either scale are likely still accurate, as the antibodies function as expected.

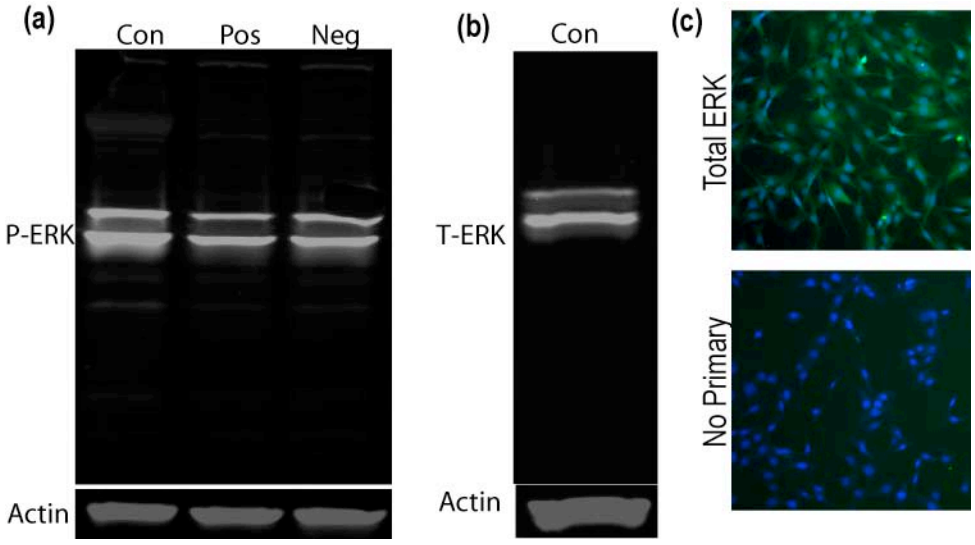

Figure S3: ERK Westerns including phosphorylated ERK with positive and negative controls (a). Due to the serum starve done for the positive and negative controls, the levels of phospho-ERK in these actually are lower than in the control cultures, but this matches what was seen in the ICWs. Blots for total ERK were done as well (b) just to check the specificity of the antibody. ICC for

total ERK (c) was done to verify that the somewhat smaller values seen in ICWs were accurate. In fact the ICC results showed weaker staining with the same protocol than what was observed for many other readouts, though results were always significantly above background levels.

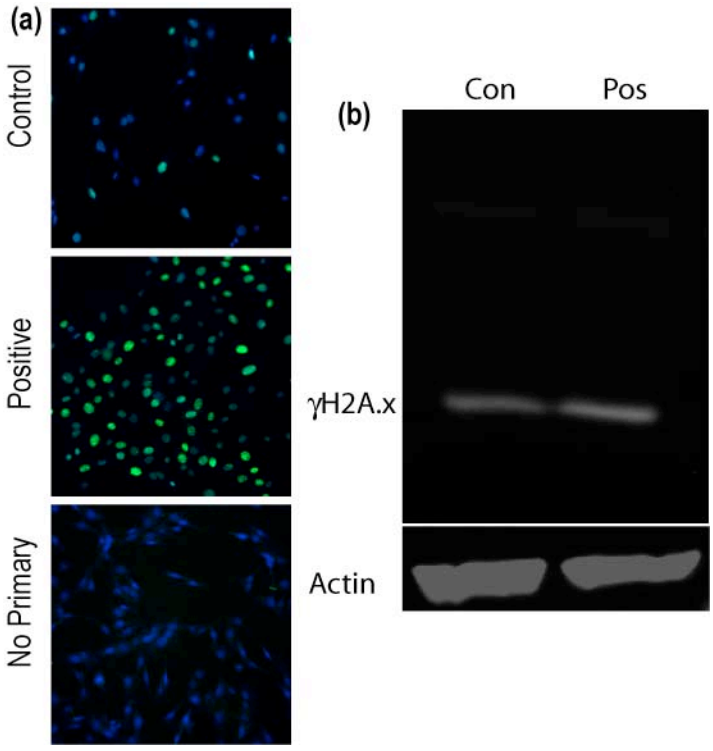

Figure S4: H2a.x staining was done (a) along with the Western blot (b) to ensure that localization was correct in the ICC results. An ICC verification for this readout was important because this was the only nuclear protein included in the panel, and was also a phosphorylated protein, both which may cause ICC/ICW methods to fail due to the antigens not being very accessible or easily degraded.

PART 1: FROM THE CELLULAR PERSPECTIVE: DIFFERENCES IN THE CELLULAR BASELINE IN MACROSCALE AND MICROFLUIDIC CULTURES

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ABSTRACT

Microfluidic devices for cell based assays can provide new types of microenvironments and new methods for controlling and quantifying cellular responses to them. However, without a better understanding of the effects of the micro-environments present in microdevices from a cellular perspective, it will continue to be challenging to integrate work done in microdevices with biological data obtained via traditional methods. To date however, very few quantitative biological techniques have been interfaced with microfluidic devices which has prevented more facets of cellular function to be analyzed and compared to those in macroscale cultures, beyond simple proliferation and viability assays. With the adaptation and validation of In Cell Westerns (ICWs) to microfluidic devices, we can begin to look in depth at a variety of cellular responses to microcultures from metabolic signaling responses to evidence of DNA damage. Here we describe several important differences in signaling pathway activation and expression levels between cells cultured in traditional macroscale cultures and in microfluidic cultures. These differences indicate that the cellular baseline may be substantially altered in microcultures, which will be critical to understand fully prior to integrating microfluidic devices for cell based assays.

INTRODUCTION

Microscale experimental techniques have been applied to biological assays for nearly two decades, but microfluidic devices for cell based assays have not been widely integrated as common tools in biological laboratories. The significant differences between several physical phenomena at the microscale versus the macroscale have been exploited to provide a wide variety of new types of assays (such as gradient production or spatial cell patterning) not previously possible using macroscale techniques. Although microfluidics holds enormous potential to provide a platform for new and more relevant cellular assays, more in depth investigation of the engineered microenvironments will be required for this potential to be fully realized. Aspects of these engineered microenvironments that are new and different from the more traditional issues associated with traditional cell culture and treatment now must be appreciated and understood.

One application of microfluidics that, while seemingly simple, holds immense promise is cell culture. Microfluidic devices for cell culture provide a platform for higher throughput analyses of cellular responses to soluble stimuli with a variety of cost and resource benefits.[1-3] Because each assay can be performed on a smaller total number of cells when done in microfluidic devices, more assays can be performed with the same sample size. However, in several cell types, differences in various aspects of cell behavior and functioning have been observed in microcultures from the phenotypes seen in macroscale cultures.[4-8] It is not clear to what extent or why these microfluidic environments seem to influence cell behavior, nor whether these effects are device- or cell type-specific (or both).

Cell proliferation is a common readout from a microfluidic culture, as often entire culture areas can be imaged and analyzed via imaging software and total adherent or nonadherent cell numbers per channel can be obtained and tracked over time.[19,41] Recent work has integrated microfluidic cultures into a format which can be analyzed by a standard plate reader for cell enumeration purposes.[42] While many cell

types have been shown to be compatible with a wide variety of microdevices, proliferation kinetics are not always the same in microculture versus macrocultures.[4,5,8,9]

Differences in the responses of cells to the engineered microenvironments of microfluidic devices to those in macroscale techniques hasn't only been reflected in proliferation, but has also been assayed via microarray. A notable study done to analyze the artifacts imparted by a microfluidic culture chamber via the analysis of cellular expression profiles by DNA microarray showed significant differences between the profiles of macro and micro scale cultures, though most were less than 3-fold induction or reduction.[6,7] Comparisons between macro- and microscale cultures on a variety of engineered surfaces were performed to study any differences in baseline expression of cells in microfluidic assays. This work is the most comprehensive analysis of the differences in cellular behavior (in this case expression) in microfluidic devices to date.

Gradient generating microfluidic devices are another type of microfludic device that illustrate why understanding of the effects of specific microfluidic devices from a cellular perspective is critical for further implementation of the devices into biological research. Stimulating a field of cells with a controlled gradient of a soluble factor is a unique type of microfluidic assay that can effectively produce different microenvironments in a single device.[10] Few traditional techniques for gradient production, such as the Zigmond chamber,[11] have been able to produce as defined, controlled and repeatable gradients as those produced using microfluidic techniques. Precisely defined chemical gradients in microfluidic devices have been applied to many biological systems, such as to stimulate migratory cells (e.g. neutrophils, bacteria, sperm cells) using chemoattractants,[12-18] investigate cancer cells responding to a drug or growth factor,[19-21] or to stimulate the differentiation of embryonic stem cells.[22]

This class of microfluidic devices has the potential to improve the sensitivity and complexity of experiments studying cellular responses to gradients beyond what is currently possible via traditional techniques. However, many of these devices rely upon continuous flow of the exogenous compound for gradient formation. The

effects of flow alone on neutrophils has been addressed and mechanical activation by shear from laminar flow in microchannels was demonstrated.[23] Walker and colleagues have also shown that the flow rate used to create gradients can affect and therefore bias the migratory behavior of these cells.[24] The validity of cell based assays done in microfludic devices will rely upon addressing these types of issues, such as flow induced bias in migratory cells, that are inherent to each specific microfluidic device design used.

An existing roadblock to further validation and understanding of the microenvironments in microfluidic devices is the relative lack of methods for quantitative analysis of more complex cellular functions applicable to devices of this scale (beyond simple viability assays). While a strength of microfluidic devices is the small sample sizes, this is also a limiting factor for many different traditional assay types (such as Western blots, flow cytometry, etc), that could potentially be used to better understand the cellular response to microculture. Without the ability to probe more complex aspects of cellular responses beyond viability or proliferation assays, the adaptation and implementation of more cell based assays to microfluidic devices will be challenging. Additionally, the relative lack of validation of the biological responses to microculture is a sizeable hurdle to the integration of these devices into current cell biology methods.

Understanding how the microenvironments found in microdevices for biological assays affect both the cellular baseline and responses to stimuli will be key to better understanding the context of any future assays performed in them. The In Cell Western technique was shown to accurately quantify protein expression or content in microfluidic cultures, *in situ*.[25] Here, we applied this technique in conjunction with several known readouts of stress to better understand whether cells cultured in microfluidic channels may be under stress, or have significantly different baseline levels of signaling and expression as compared to macroscale cultures. A range of universal readouts from energy and metabolism to DNA damage were tested and compared to macroscale cultures to identify changes in important signaling pathways that may influence the results of assays done in each culture type.

METHODS

Cell culture

Mouse mammary fibroblasts (MMFs) isolated from p16/INK4a knockout mice were cultured in DMEM with 10% serum, and 1% P/S (passage numbers ranged from 20-35). When not specified, high glucose medium was used, containing 4.5g/L glucose, otherwise no glucose DMEM was used and glucose added to the specified concentration. Cells were passaged every 2 days with approximately a 1:5 dilution, (initial confluence was approximately 20-30% and at 2 days was approximately 70-80%). MMFs were seeded at the same surface density (approx. 90-100 K/cm^2) in microchannels and in 96 well plates and allowed to plate and proliferate for 24 hours (initial seeding densities of approx 50-60% reaching 70-80% at 24 hours in macroscale cultures). This results in a 5 fold increase in volume density in microchannels versus typical macroscale culture with the same surface density. At 24 hours after seeding, positive and negative control treatments were performed as described for each readout and then cells were fixed and stained for ICWs.

In Cell Westerns

For ICW's, cells were fixed and stained for either phosphorylated and/or total protein. Cells were washed briefly with PBS, then fixed with 4% PFA in PBS on ice for 20 minutes with the addition of phosphatase inhibitor cocktail at 1:100 dilution (Pierce, Halt Phosphatase inhibitor cocktail, #78420). To permeabilize, two washes with 0.1% Triton X-100 in PBS, 7 minutes each were done then cells were blocked in Licor blocking buffer (Licor Biosciences, #927-40000) for 90 minutes at room temperature. Primary antibodies were added to Licor blocking buffer with 0.1% Tween-20 and incubated overnight at 4ºC.

Primary antibodies were used at the following dilutions; 1:50 dilution for phospho-AMPKα (Cell Signaling, #2535, rabbit monoclonal), AMPKα (Cell Signaling, #2603, rabbit monoclonal), and phospho-S6 ribosomal protein (Cell Signaling, #2211, rabbit monoclonal), 1:100 for S6 ribosomal protein (Cell Signaling, #2217, rabbit

monoclonal), 1:250 for Ki67 (Transduction Labs, #610968 mouse), 1:200 for phospho-ERK1/2, (Cell Signaling, #4370, rabbit monoclonal), 1:100 for total ERK1/2 (Cell Signaling #4695, rabbit monoclonal), 1:100 for BiP (Cell Signaling, #3177 rabbit monoclonal), 1:50 for HSP70 (Cell Signaling, #4876 rabbit polyclonal), and 1:500 for γH2A.X (abcam #ab2893 rabbit polyclonal). Cells were then washed 3 times with PBS with 0.1% Tween-20 for at least 7 minutes each wash at room temperature. IR dye conjugated secondary antibody was then added to Licor blocking buffer with 0.1% Tween-20 at 1:200 dilution (Rockland Inc., #611-731-127, IRDye 800CW conjugated donkey anti rabbit) for 45 minutes at room temperature in the dark. Secondary antibody was washed out with two washes with PBS with 0.1% Tween-20, 7 minutes each wash, then cells were incubated with ToPro3 (Molecular Probes) at 1:500 dilution for 10 minutes in PBS at room temperature, in the dark. Cells were then washed twice with PBS and allowed to dry prior to scanning on an infrared laser scanner (Odyssey, Licor Biosciences).

Western blotting

Cells were seeded at the same density as done previously (approx. 90-100 K/cm^2) in 6 well plates the same positive and negative control conditions performed at 24 hours before cells were lysed for Western blots. After positive and negative control treatments, cells were washed briefly with PBS on ice, then lysed in RIPA buffer with a protease inhibitor cocktail (Roche, Complete Mini tablets, # 11836153001), and for phosphorylated proteins, a phosphatase inhibitor cocktail at 1:100 dilution (Pierce, Halt Phosphatase inhibitor cocktail, #78420). Cells were homogenized via sonication, then tris-glycine SDS sample buffer was added with 4% β-mercaptoethanol, and boiled for 5 minutes. Lysates were loaded onto either 12% or 8% tris-glycine gels depending on the molecular weight of the protein of interest (Invitrogen), and run in tris-glycine SDS running buffer (Invitrogen) with molecular weight markers suitable for infrared detection (Licor Biosciences, #928-40000). Protein was transferred to nitrocellulose membranes and subsequently blocked in Licor blocking buffer for at least one hour at room temperature, in the dark. Primary antibodies to the proteins of interest were diluted into

Licor blocking buffer with 0.1% Tween-20 (all at 1:1000, antibody specifications given above), along with a primary antibody to actin (either mouse monoclonal to α-actin from MP Biomedicals, #69100 at 1:10,000, or rabbit monoclonal to β-actin from Cell Signaling, #4970 at 1:1000).

Blots were incubated with the primary antibodies overnight at 4°C in the dark. Membranes were then washed 3 times with PBS with 0.1% Tween-20 for 10-12 minutes each wash, then were incubated with secondary antibodies in Licor blocking buffer with 0.1% Tween-20 for 45 minutes at room temperature, in the dark, with shaking. Secondary antibodies were used at 1:20,000 and were from either Rockland Inc., (#611-731-127-IRDye 800CW conjugated donkey anti-rabbit, #610-131-121-IRDye 800CW conjugated goat anti-mouse, #611-130-122-IRDye 700DX conjugated goat anti-rabbit), or Licor Biosciences (#926-32220, IRDye 680 conjugated goat anti-mouse). Blots were then washed 3 times with PBS with 0.1% Tween-20 for 10-12 minutes each wash and allowed to dry prior to scanning. Blots were scanned using the Odyssey laser scanner, and integrated intensities of the bands of interest were normalized to the actin signal as a loading control.

Immunocytochemistry

For ICC, cells were seeded into glass chamber slides at 90-100 K/cm^2, and after 24 hours positive and negative controls for each readout were performed. The cells were fixed and stained using the same protocol as described above for ICWs, with the exception of using a secondary antibody labeled with Alexa 488 (Molecular Probes, either goat anti-rabbit or anti-mouse), and the cells were mounted instead of dried. Cells were then imaged via microscopy. All images of paired positive and negative controls were taken with the same exposure length, intensity and objective to ensure a quantitative relationship between them. Additionally, controls without primary antibodies were imaged with the same exposure length to determine the levels of background due to non-specific staining of the secondary antibody. Any image processing (e.g. exporting in formats suitable for publication) was done exactly the same for these paired images as

well to maintain image consistency and validity.

RESULTS AND DISCUSSION

In these experiments, assays for the levels of each protein, or protein modification of interest were done to determine if there are significant differences in activation levels of various stress related signaling pathways in macro- and microscale cultures. Biologically relevant readouts were chosen that relate to metabolic and growth factor signaling processes, heat shock protein/ER stress, important cellular signaling pathways (mitogen activated protein kinases (MAPKs)), and DNA damage (summarized in Figure 1). This panel of stress responses provides an overview of the basic types of cellular functions that are nearly universal, and could be applied to any mammalian cell type of interest to begin to understand what types of effects a specific microfluidic device may have on the cellular baseline functions. For these experiments, mouse mammary fibroblasts (MMFs) were seeded at the same surface densities in microchannels and in macroscale cultures (in this case 96 well plates), and assayed at 24 hours before large differences in confluency were obtained, to prevent the effect of density on cellular functions from obscuring those due to macro- or microculture. In the following sections, specific controls are listed for each readout, along with an introduction to the function and sensitivities of each, and the results obtained via ICWs.

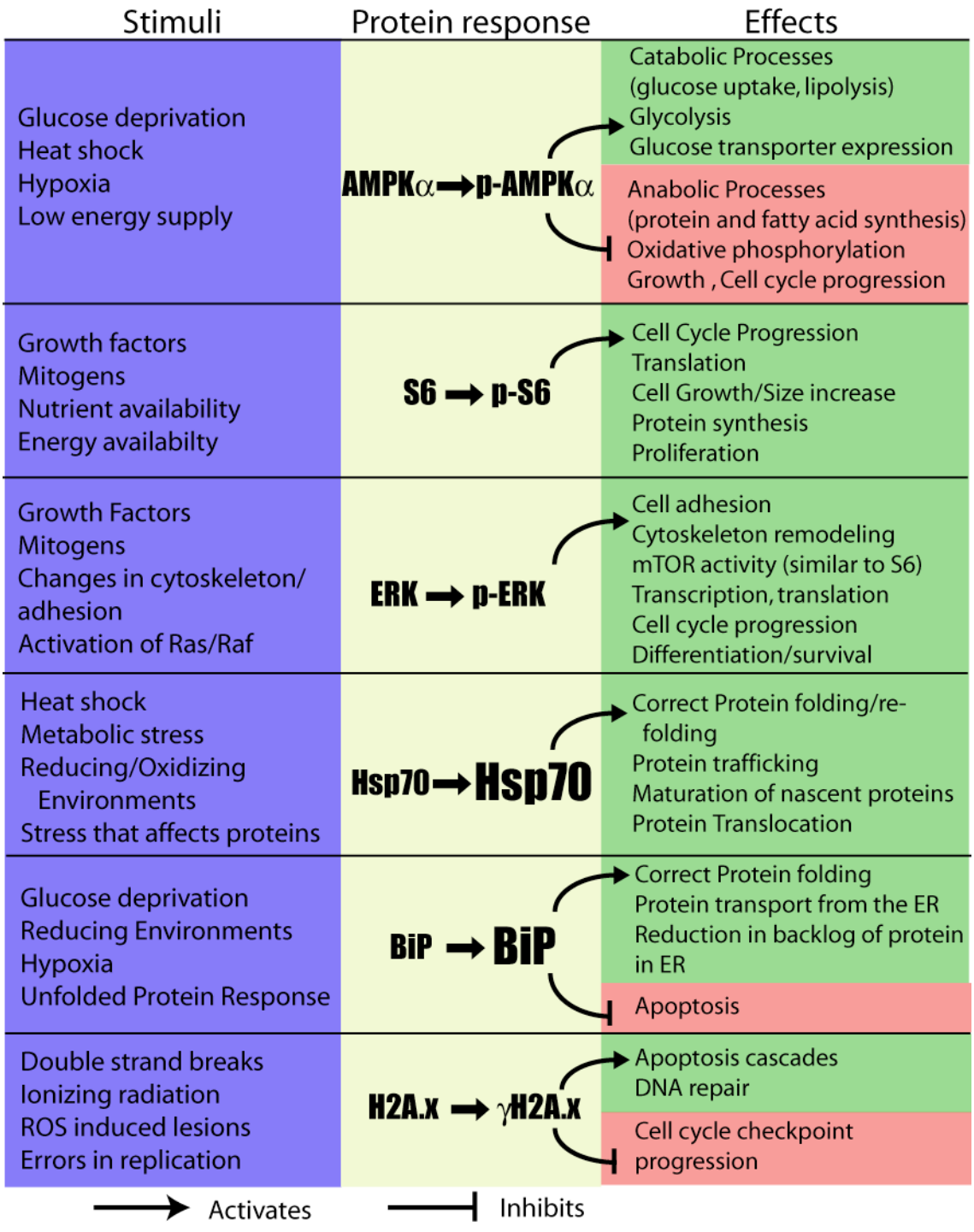

Figure 1: Summary of the readouts tested in microscale cultures. From left to right, columns show which stimuli result in the changes in phosphorylation or increases in expression (described in the center column) and what the subsequent activity of the protein is. Arrows indicate that the protein activates the processes listed, while blunt arrows indicate inhibition.

METABOLISM AND GROWTH FACTOR SIGNALING

Microchannel culture reduces the total nutrient availability per cell due to the increase in volume density, but whether this reduction results in nutrient restriction from a cellular point of view has not been shown definitively. In addition, the total amount of growth factors per cell (from FBS used in many media) is reduced as volume density increases as well. Either of these factors may cause a significant change in cellular behavior and proliferative decisions in microcultures.

Several important growth factor and metabolic cellular signaling pathways are integrated to produce a coordinated cellular response to their environment – namely, cellular energy status sensing, cell size/growth control and proliferation pathways. An important sensor of the energy status of a cell is AMP activated protein kinase (AMPK), which responds to changes in intracellular AMP/ATP availability. This pathway is directly affected by the AMP/ATP ratio in a cell and responds to changes in glucose and energy availability. The integration of many different growth factor signaling pathways occurs with the mTOR (mammialian target of rapamycin) pathway, resulting in the activation of S6 ribosomal protein downstream of mTOR. This readout provides an overview of the cells' growth factor signaling which would respond to changes in growth factor availability, and also change if nutrient depletion was occurring as it is directly related to cell growth and cell size regulation.

The cellular energy status is sensed by the AMP activated protein kinase (AMPK) pathway and can respond to a variety of stimuli which alter the availability of ATP in the cell. To determine if microcultures result in energy restriction, the level of activation via phosphorylation of AMP activated protein kinase (AMPK) will be determined. This pathway is an energy-sensing pathway and is highly conserved. In yeast, the major function of the AMPK pathway is to sense and respond to glucose starvation, whereas in mammals, the pathway responds to many types of cellular stress, which may result in changes in levels of AMP or ATP (e.g. glucose starvation, heat shock, and hypoxia).

The function of AMPK is to regulate the balance between anabolic and catabolic cellular functions in response to the microenvironment.[26,27] When it is activated, catabolic pathways, which create ATP, are activated, while anabolic pathways, which consume ATP, are inhibited. Catabolic pathways include glucose uptake and glycolysis, which are required for basic cellular respiration and function. Anabolic pathways include protein and fatty acid syntheses and are required for cell growth and proliferation.

When ATP availability is reduced, either due to a decrease in ATP production or an increase in ATP consumption, the ADP:ATP ratio will get larger. This increase is amplified by the activity of adenylate kinases and results in an even larger change in the cellular AMP:ATP ratio. Adenylate kinases in eukaryotic cells serve to maintain the reaction $2ADP \leftrightarrow ATP + AMP$ near equilibrium, which, in turn, causes the AMP:ATP ratio to vary as the square of the ADP:ATP ratio. The sensitivity of AMPK activation to levels of AMP and ATP in addition to the sensitivity of the AMP:ATP ratio to changes in energy usage in a cell makes the activation of AMPK an attractive method for analyzing the metabolic status of cells in culture.

The AMPK pathway and many other growth factor sensitive pathways all share tuberous sclerosis 2 (TSC2) as a signaling component. Activation of this signaling molecule via phosphorylation results in the inhibition of another pathway, the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway is responsible for regulation of cell size and translation rates, and integrates signaling cascades in response to growth factors and nutrient availability.[28] As part of the mTOR complex 1 (mTORC1), mTOR activates downstream targets such as S6 ribosomal protein (S6) via phosphorylation. S6 activation correlates with increases in protein translation levels and also expression of cell cycle related proteins. The levels of phosphorylated S6 will provide a view of the collaborative activation status of several growth and metabolism regulating pathways in response to many different cellular stressors that may be present in microcultures.

AMP Kinase activated protein kinase and S6 Ribosomal protein

Results for ICWs for both AMPKα and S6 are shown in Figure 2. Positive controls for phosphorylated AMPKα (the catalytic subunit of AMPK) were MMFs incubated with 25mM 2DG (2-deoxy-D-glucose) in 10% FBS medium, while negative controls were incubated with 10g/L glucose in 10% FBS medium each for 30 minutes, then fixed and stained. For S6 ICWs, positive and negative controls were incubated with 1% serum for 1 hour, with the addition of 200nM Rapamycin for negative controls. Media was then replaced in both positive and negative controls with 5μg/mL insulin in serum free medium for 15 minutes, then fixed and stained. AMPK and S6 ICW results were consistent with both the Western blot data from macroscale cultures, and also the ICC images taken after the same positive and negative control treatments (Fig. S1).

In microcultures, phosphoylated AMPKα is nearly two-fold higher than macroscale ($p=0.0006$), while the levels of total AMPKα are not significantly different regardless of scale. Interestingly, the levels of phospho AMPKα in microscale cultures were not significantly different from that of the positive controls in either scale. Meanwhile, the levels of phospho AMPKα in macroscale cultures were not significantly different from that of the negative controls in either scale. These data suggest that energy availability may be significantly less in microcultures than what is found in macrocultures. Perhaps the reduction in levels of glucose available to cells in microcultures is sufficient to affect the AMPK signaling pathway. For S6, macroscale cultures did have significantly higher levels of phosphorylated S6 than microcultures ($p=0.04$), though the total levels were unchanged. These data suggest that perhaps microcultures have fewer growth promoting signaling pathways activated in these culture conditions.

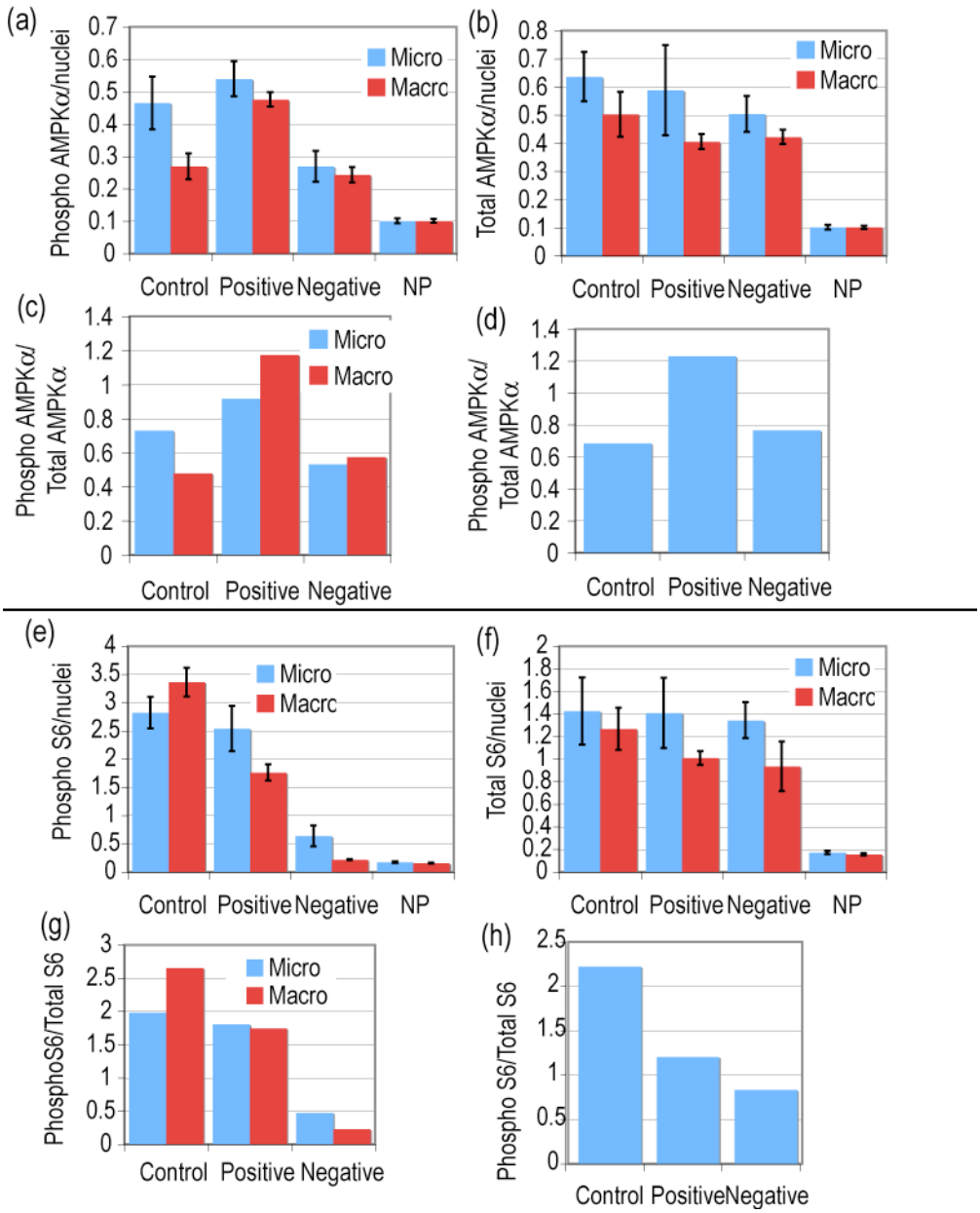

Figure 1: Integrated intensities of either phospho-AMPKα (a) or total-AMPKα (b) were normalized to nuclear intensities for each condition in each scale. The values for phospho AMPKα were then normalized to total AMPKα (c). Results from macroscale cultures via traditional Western blots gave similar results to that of the ICWs (d). A similar analysis was performed for S6, with phospho-S6 (e) or total-S6 (f) normalized to nuclear intensities for each condition in each scale, the values for phospho S6 normalized to total S6 (g) and finally, results from macroscale cultures via traditional Western blots (h). Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

HEAT SHOCK PROTEINS/CHAPERONES AND ER STRESS

Heat shock proteins (HSP) function as important protein quality control and protein folding mechanisms in the cell. These proteins function in normal, unstressed cells to aid in correctly folding nascent proteins and repairing misfolded proteins. Generally, HSP activity is ATP-dependent and occurs in a large complex of accessory proteins. The responses of both heat shock proteins and resident endoplasmic reticulum proteins to stress are critical for cell survival and apoptosis. Environmental changes that result in protein misfolding include glucose starvation, exposure to reducing agents, low pH, hypoxia, or a variety of drugs. These types of stimuli will increase the number of heat shock proteins available to deal with the unfolded proteins.

Heat shock protein 70 (HSP70) family members are found mainly in the cytosol, but can also be located in mitochondria and the endoplasmic reticulum. This family serves to fold proteins and respond to a wide range of cellular stressors that adversely affect protein function (e.g., thermal, oxidative, or metabolic stressors). HSP70 binds hydrophobic patches of unfolded or incorrectly folded proteins and, via an ATP dependent mechanism, refolds the proteins or assists in targeting them for degradation.[29,30] Increased levels of HSP70 tend to be anti-apoptotic and is a potential marker for identifying cellular stress present in microcultures.

The endoplasmic reticulum (ER) is the cell's main protein and lipid production organelle. Proteins are synthesized in the ER with resident organelle proteins aiding in folding, targeting and quality control processes. ER stress can occur as a result of large amounts of unfolded proteins, reductive environments, low glucose, or lowered pH, among others.[31,32] Several of these conditions could exist in microculture due to the small volumes, thus analysis of whether ER stress is occurring in the cells is critical.[32,33]

Immunoglobulin binding protein (BiP) is an ER resident HSP70 family member that aids in retaining nascent proteins in the ER until they are properly folded or assembled with other subunits. The Unfolded Protein Response (UPR) pathway serves to increase both ER resident proteins such as BiP and lipid biosynthetic enzymes that make

more ER membrane. When unfolded proteins levels increase in the ER, higher levels of BiP are required in order to properly fold or target for degradation the backlog of protein. BiP activity is triggered by reducing agents, overexpression of proteins, or blockage of the exit from the ER.[32] As a cell survival protein, the loss of or underexpression of BiP in response to stress induces apoptosis.

Interestingly, BiP was originally known as glucose regulated protein 78 (GRP78) since it was discovered in cells grown *in vitro* after glucose starvation,[31] which may be the case in microcultures. Thus, there are several potential sources of ER stress and glucose restriction in microculture; to assess the contribution of ER stress to microscale culture phenomena, the levels of BiP as compared to macroscale culture were analyzed.

Hsp70 and BiP

Data for both HSP70 and BiP are included in Figure 3. The positive control for Hsp70 ICWs was heat shock at 42°C for 30 minutes then 45 minutes at 37°C as a recovery, (with no negative control as this is a ubiquitously expressed protein). The positive control used for BiP ICWs was the addition of thapsigargin at the specified concentrations for 16 hours (with no negative control as this is a ubiquitously expressed protein as well). Thapsigargin causes ER stress by inhibiting the pumping of calcium into the ER, thus raising the intracellular calcium concentration. Two doses were used as the MMFs used in this experiment tended to adhere very poorly to the surface after Thapsigargin treatment, and could be easily washed off during fixation and staining. Figure 3b, shows the reduction in cell number remaining after fixation in macro and microscale cultures, indicating that macroscale cultures suffered the most cell loss due to more flow during fluid exchange (though confluence in macroscale cultures treated with thapsigargin was similar to untreated cells prior to fixation).

Figure 3a shows that in microcultures, BiP is significantly upregulated in microcultures ($p=0.04$). The Western blot data from macroscale cultures (Fig. S2)

showed a much larger increase in BiP expression, which indicates that the ICW result in macroscale cultured treated with thapsigargin are likely lower than the true value due to cell loss during fixation (which is not in issue for traditional Western blots). Additionally, cells were fixed very carefully in macroscale cultures for immunocytochemistry and proved to have a visible increase in BiP fluorescence after Thapsigargin treatment (Fig. S2). However, the control conditions can be compared between macro and microscale cultures as no cell loss occurred without the addition of Thapsigargin. Expression of HSP70 was 2.4 fold higher in microcultures than in macrocultures.

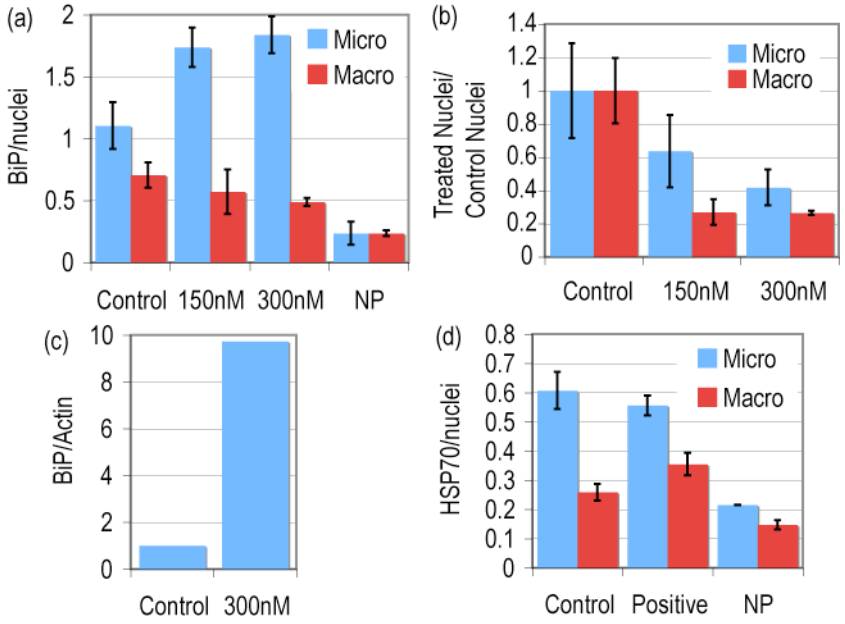

Figure 3: Integrated intensities of BiP were normalized to nuclear intensities for each condition in each scale, with two different doses of thapsigargin for 16 hours (a). Due to the differences in fluid handling between the culture scales, cells treated with thapsigargin in macroscale cultures did not remain attached to the substrate during fixation and staining. The quantification of the signal from the nuclei alone normalized to the levels in control conditions is shown in (b). Results from macroscale cultures via traditional Western blots showed significant upregulation of BiP, indicating that the ICWs treated with thapsigargin likely underestimate the degree of upregulation due to cell detachment (c), however the untreated conditions are still accurate. Heat shock protein 70 ICW results showed a significant increase (over two-fold) in HSP70 levels in microcultures than in macrocultures (d). Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

Mitogen Activated Protein Kinases (MAPKs)

The members of mitogen activated protein kinases (MAPKs) mitigate a wide variety of cellular responses to stimuli.[34-36] These kinases are part of a complex system of signaling cascades, which ultimately serve to translate signals from cell surface receptors into a cellular response. While each has a separate main activation mechanism, a complex network of activation exists due to the variety of MAP kinases and MAP kinase kinases and the different degrees of each kinase's substrate specificity. Each MAPK responds to different forms of cellular stressors and is involved in regulating different cellular adaptive response to stressors.

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are MAPKs involved in cell attachment, migration, proliferation, and differentiation.[34] ERK1/2 are found in a variety of locations in the cell, resulting in a variety of different functions. ERK1/2 are found at adherens junctions and focal adhesions and as much as half the total cellular ERK1/2 is bound to microtubules throughout the cell altering their polymerization.[34] Unphosphorylated ERK1/2 is typically are found in the cytoplasm and only translocate to the nucleus when activated. ERK1/2 are activated upon phoshorylation of their activation loop tyrosine and threonine residues by the MAP kinase kinases MEK1 and MEK2 . Activated ERK1/2 then translocates to the nucleus and stimulates the transcription of a wide variety of target genes.

ERK1/2

The ICW results for ERK1/2 are shown in Figure 4. For controls, media was replaced with 1% FBS DMEM with the addition of a cell permeable ERK activation inhibitor peptide (Calbiochem, #328005) at 30 μM for 1 hour for the negative controls. Media with 20% serum was then added to both positive and negative control cultures for 20 minutes prior to fixation and staining.

Microscale cultures had nearly double the levels of phosphorylated ERK1/2 than macrocultures ($p=0.01$). In addition to upregulation of phosphorylated ERK1/2, total amounts were upregulated in microcultures as compared to macrocultures (Fig. 4b, $p=0.02$), with the resulting ratio of phosphorylated to total protein being approximately the same regardless of scale. Western blots and ICC for total ERK and Westerns for phosphorylated ERK are included in Figure S3.

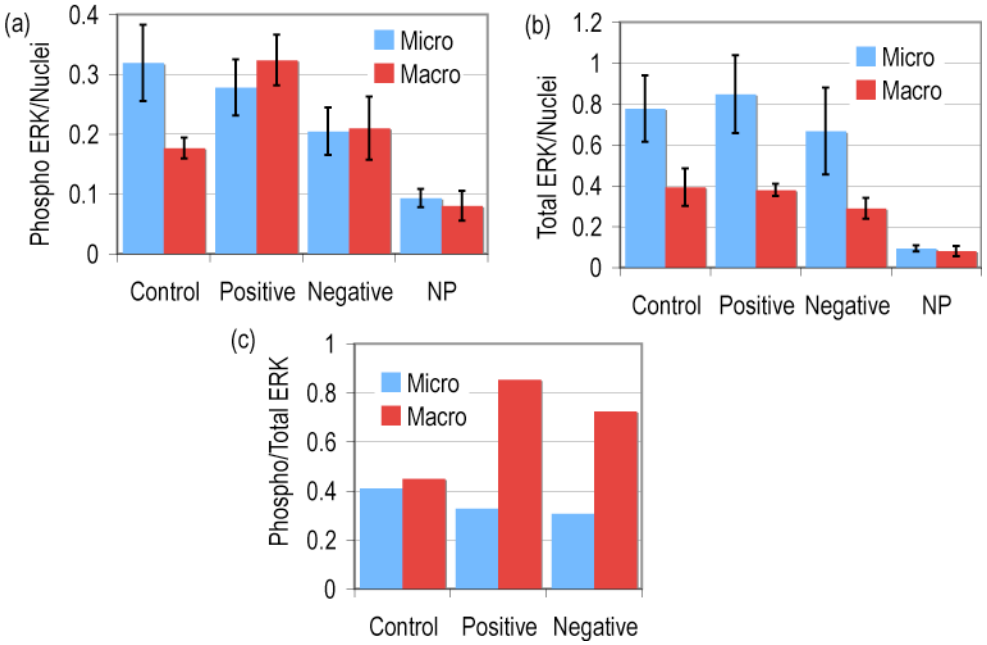

Figure 4: ICWs for phospho (a) and total (b) ERK1/2 showed consistent upregulation in micro-cultures, while the normalized amounts were consistent (c). ICC results for total ERK verified that a signal could be obtained, but was relatively weak (d). Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

DNA Damage

There are a variety of sources of DNA damage such as oxidizing environments, radiation (ionizing or UV), as well as errors in the cell's own DNA replication machinery; damage ranges from double strand breaks to lesions formed by alteration of the nucleotide bases. If DNA damage is detected, cell cycle checkpoints serve to arrest the cell cycle in order to provide the cell time to either repair the damage or undergo apoptosis (if the damage is too extensive).

Variations in respective microenvironments and behavior of cells in macro- and microscale cultures could potentially cause significant differences in levels of DNA damage (although it is not readily apparent which culture type is at higher risk). The reduction in proliferation seen previously in microcultures could be due to higher rates of DNA damage, resulting in cells spending more energy and time repairing these

defects rather than entering and finishing the cell cycle. Additionally, potentially higher rates of apoptosis in microcultures could be due to either higher levels or a higher sensitivity to DNA damage. To determine if DNA damage is occurring at a higher level in microcultures versus macrocultures, staining for important components of the DNA damage response pathway will be performed.

A marker for DNA damage and activation of the ATM/ATR pathways will be the response of histone H2A.x. H2A.x represents from 2-25% of total H2A and is phosphorylated (γH2A.x) as a result of double strand breaks by ATM and ATR.[37] H2A.x foci appear in the nucleus at sites of DNA damage. DNA fragmentation during apoptosis also results in large levels of phosphorylated H2A.x[37]; assays for levels of γH2A.x will therefore provide insight into the degree of damage present in surviving and whether apoptosis is occurring frequently in either culture scale.

γH2A.x

Figure 5 shows results of ICWs for γH2A.x, indicating that no significant difference is seen between macro- and microcultures. The positive control used for γH2A.x was 30 minutes of exposure to UV in a laminar flow hood, followed by 15 minutes of recovery at 37°C in the incubator. The same fixing and staining protocol used for all other ICWs was followed except for the fixation step. Fixing was done with 2% PFA in PBS at room temperature for 10 minutes, without phosphatase inhibitor cocktail then the standard protocol was followed. ICC and Western blot validation was done as well and is shown in Figure S4.

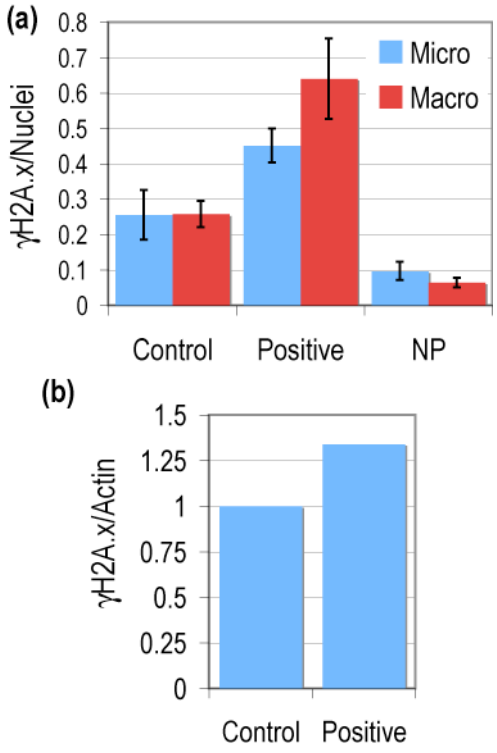

Figure 5: Integrated intensities of γH2A.x were normalized to nuclear intensities for each condition in each scale (a). Results from macroscale cultures via traditional Western blots showed similar results (b). Immunocytochemistry for each condition also verified that localization and treatments result in significant differences in levels of γH2A.x (c). Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

CONCLUSIONS

This panel of stress assays aimed to provide insight into how microfluidic cultures are different, from a cellular perspective than the corresponding macroscale cultures (results summarized in Figure 6). Significant differences in AMP kinase and S6 phosphorylation indicate that perhaps the reduced media volumes results in nutrient depletion (resulting in increased phosphorylation of AMP kinase) or growth factor depletion (resulting in reduced activation of S6). Other readouts showed significant differences in microcultures than macrocultures such as the upregulation of ERK1/2, BiP and HSP70. These proteins are sensitive to a wide variety of potential stressors, thus it is

unclear what specific characteristic of microfluidic culture is causing each of the responses seen. However, these differences do reflect that the microenvironment in microcultures is truly different and results in different levels of activation and expression of key proteins involved in basic cell functions like attachment, growth, and protein folding/production. Differences in levels of γH2A.x were not seen, indicating that significant differences in rates of DNA damage between the scales are not likely. This also suggests that reductions in proliferation seen in microcultures is not due to delays for DNA repair, nor that widespread apoptosis is occurring in these cultures.

Readout	Total Protein (Micro/Macro)	Phosphorylated Protein (Micro/Macro)	Ratio (Phospho/Total) (Micro/Macro)
AMPKα	1.3x	1.7x	1.5x
S6	1.1x	0.84x	0.75x
ERK	2.0x	1.8x	0.91x
Hsp70	2.4x	N/A	N/A
BiP	1.6x	N/A	N/A
γH2A.x	N/A	1.0x	N/A

Figure 6: Summary of readout results comparing macro and microscale cultures for total protein, the phosphorylated protein (if applicable) and the ratio of phosphorylated to total for each applicable readout. Several of these proteins exhibit approximately 2 fold (or more) changes in expression in only 24 hours of microculture, indicating that the influences of microculture are significant and relatively rapid.

With the integration of ICWs to high throughput microfluidic assays this panel of stress assays could be reproduced for a wide variety of cell types and could be expanded to include more aspects of cellular function. This tool could be used to validate and troubleshoot microfluidic cultures for cell based assays to better understand the cellular baseline for specific cell types of interest prior to large assays being run. Also, the ability to do quantitative studies of signaling cascades *in situ* in microfluidic devices expands the available readouts for microfluidic assays. The activation or inhibition of signaling pathways in response to drugs or other stimuli can now be screened using

microfluidic devices, with all of the resource benefits that they provide (in cell sample sizes and reagent costs).

Microfluidic devices for cell based assays have provided new types of microenvironments and new methods for controlling and observing the cellular responses to them. The field has begun to analyze the biological effects of the physical differences of microfluidic devices for cell based assays, ranging from evaporation in static microfluidic cultures to flow induced artifacts in gradient generation devices. Nonetheless, the relative lack of quantitative biological analysis techniques that have been interfaced with microfluidic devices has prevented more facets of cellular function beyond viability or proliferation to be analyzed in them. The results shown here indicate that from a cellular perspective, the microenvironment in microfluidic cultures can be significantly different from those in traditional macroscale cultures even for simple microfluidic cell culture.

It is possible that when more complex functions such as flow, gradient introduction, growth factor or drug stimulation, etc are incorporated into microfluidic devices that perhaps these too may affect the cellular baseline. A better understanding of how the microenvironment in microfluidic devices for cell based assays affects basic cellular functions will be critical for future work. Also, understanding the unique limitations and benefits of the microfluidic systems in use for biological assays will provide insight into what controls will be necessary to more fully validate the results in context of current techniques. These differences might also be leveraged to provide new ways to assay cellular responses by comparing macro- and microscale assays. Future studies integrating cell biological assays with microfluidic cultures will rely upon well designed studies with correct and thorough positive and negative controls for validation purposes.

ACKNOWLEDGMENTS

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SUPPLMENTARY INFORMATION TO:

PART 2: UNDERSTANDING MICROFLUIDIC ENVIRONMENTS' EFFECTS ON THE METABOLISM AND PROLIFERATION OF MICROFLUIDIC CELL CULTURES

The degree of confluence at 48 hours after seeding cells in three different culture types and 4 different glucose concentrations, and the morphology was analyzed via phase microscopy, shown in Figure S1. The morphology of cells in no glucose media in all culture types is altered, and it is clear that this cell type does not survive well in no glucose medium regardless of scale (similar results were seen in 3% FBS medium, data not shown). The density of microchannel cultures in any of the media is consistently lower than either of the two culture types, even microwell cultures, however the degrees of confluency at 3 hours post seeding were indistinguishable in all three culture types. The confluence at 48 hours post seeding seen in these conditions were consistent with the proliferation rates observed.

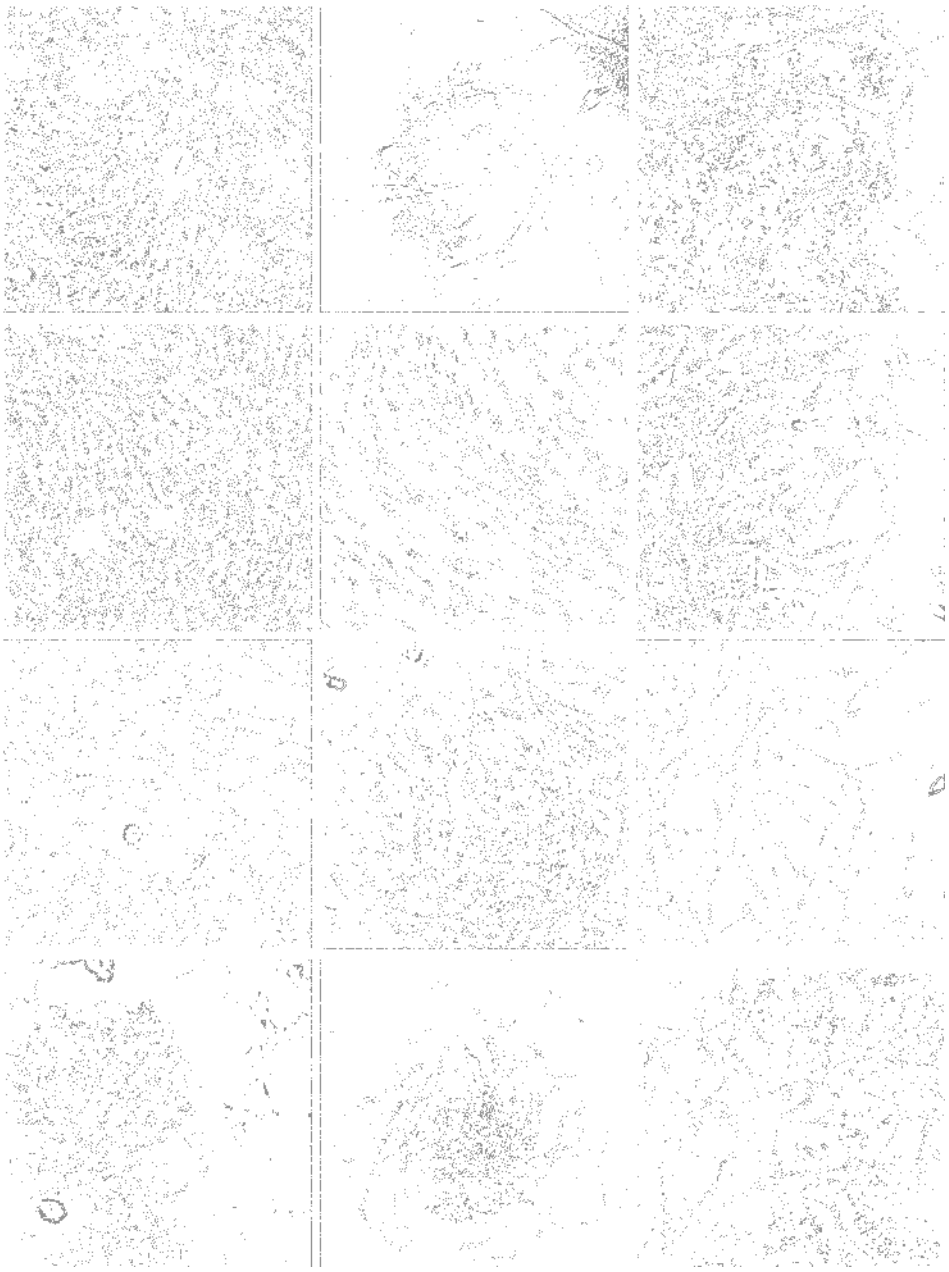

Figure S1: Phase images of microchannels, microwells and macroscale cultures at 48 hours post seeding for each glucose concentration.

The glucose concentration in the media at 3 timepoints for each culture

type is shown in Figure S2 for 9 g/L (a), 4.5 g/L (b), and 1.8 g/L (c) glucose medium. A small amount of glucose is present in 0 g/L glucose medium contributed by the serum, but the amount was below the limit of detection of the assay. Macroscale cultures showed significant glucose depletion of the medium in only the 1.8 g/L medium condition, while all microscale cultures showed significant depletion even in 9 g/L glucose medium. Microwells showed consistently more depletion than microchannels likely due to the higher levels of proliferation in these cultures.

The amount of available glucose per cell was calculated for each culture type for all media except the 0 g/L glucose medium and is shown at 3 hours post seeding and at 48 hours post seeding. In all cases, the glucose per cell at 48 hours is many times higher in macrocultures than in microcultures of the same media type even with the significant increase in cell number in macrocultures. Despite the availability of glucose in macrocultures, the per cell glucose consumption rate in microcultures with 1.8g/L glucose was larger than that of macrocultures in media with higher glucose concentrations (4.5 and 9 g/L).

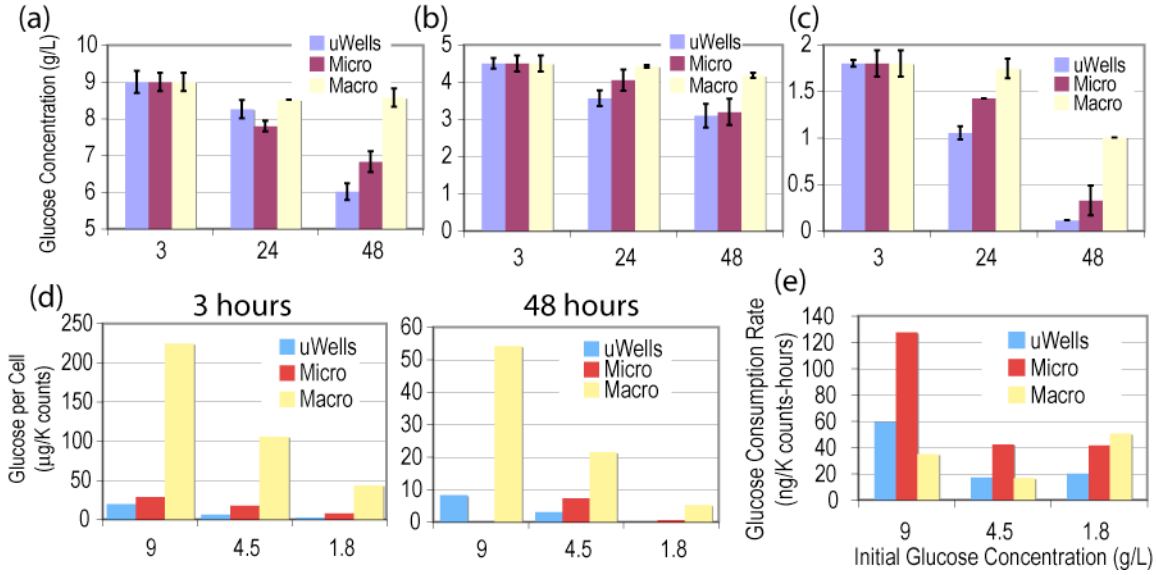

Figure S2: Cells seeded in macroculture (Macro), microchannels (Micro), and microwells (uWells) in media with a range of glucose concentrations were tracked over time for glucose concentration. (a) 9 g/L glucose media at 3, 24 and 48 hours after seeding, (b) 4.5 g/L media, and (c) 1.8 g/L media. From this data the glucose per cell at 3 hours and at 48 hours (d) show that in all conditions the amount of glucose per cell in macrocultures is significantly higher than in either microculture, even after 2 days of culture. From the proliferation data and the glucose concentration information, we can calculate the average per cell glucose consumption rate over the 2 day culture period (e).

Cells were seeded into microwells (labeled "uWells"), microchannels ("Micro") and 96 well plates ("Macro") in media with a range of glucose concentrations (9, 4.5, 1.8 and 0 g/L glucose) and serum compositions (20, 10 and 3%). The proliferation over the 2 day culture period is shown as the fold increase in nuclei at 48 hours versus 3 hours for each glucose concentration (Figure S3).

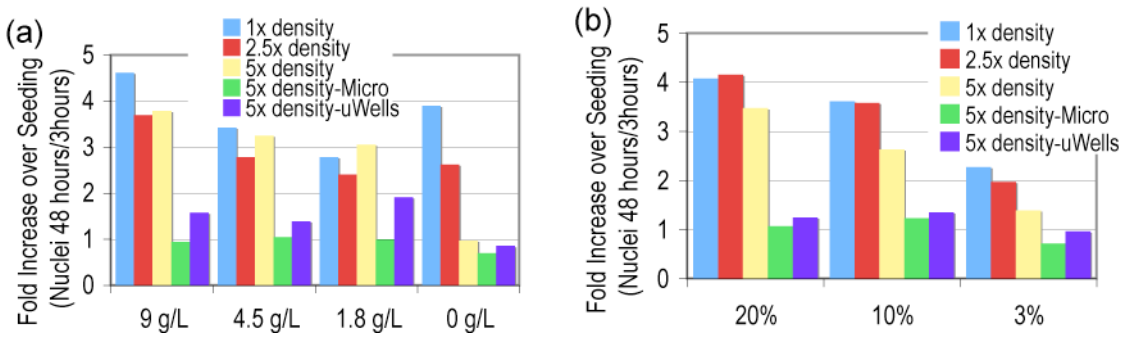

Figure S3: Proliferation was tracked in four media glucose concentrations (a), 3 serum compositions (b) in all three different culture types, and is presented as the fold increase in nuclei at 48 hours vs 3 hours after seeding.

PART 2: UNDERSTANDING MICROFLUIDIC ENVIRONMENTS'

EFFECTS ON THE METABOLISM AND PROLIFERATION OF MICROFLUIDIC CELL CULTURES

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ABSTRACT

Differences between cell behavior in microfluidic devices from cells cultured in macroscale culture conditions have been shown to be substantial and wide ranging; from proliferation reduction to expression changes via microarray to signs of stress via ICW. However, the specific characteristics of the microenvironments in microfluidic devices that cause these differences is not clear. Issues such as differences in nutrient and growth factor availability or waste accumulation due to higher volume densities, and interactions between fabrication materials and media/cells are likely sources of these differences, though may be a small sampling of the differences that exist. We have evaluated further what factors may be influencing cell behaviors related to metabolism and proliferation in microcultures in simple microfluidic culture channels. Specifically, the issues of glucose starvation, growth factor restriction, volume density and effects of interactions with poly(dimethyl siloxane), (PDMS), were addressed from a cellular perspective. Changes in glucose metabolism, growth factor signaling pathway activation, and reduced proliferation as the contact with PDMS increases. This knowledge of cell behavior in response to different culture conditions provides a more clear understanding of which characteristics of microfluidic culture are most influential to cellular behavior.

INTRODUCTION

Microfluidic devices have provided a host of new types of experimental conditions for cell biology research. Techniques range from single cell analyses and flow cytometry-like techniques,[1] to treating fields of cells in gradient generating devices[2], to microscale versions of more traditional assay types such as cell culture (via perfusion[3,4], or static cultures[5-8]). These microfluidic devices typically provide unique benefits over traditional techniques either by controlling the cellular microenvironment in ways not previously possible, by allowing existing assays to be performed on significantly smaller samples (down even to the single cell level) or by using many fold less costly reagents.

With the integration of these devices into biological research, we begin to face the issues regarding biological validation of these devices. While many devices have been used for cell based assays in microfluidics, relatively little has been done to investigate, in depth, the characteristics of microcultures which influence the behavior and phenotypes of the cells in they aim to study. Reduced proliferation[5,7,9], reduced seeding efficiencies or plating delays, changes in sensitivities to soluble factors[10], microarray analysis of expression[11,12] and differences in the baseline signaling and protein expression[13] versus macroculture have all been shown in microcultures. These data have made it clear that microfluidic cell culture alone may impart a range of influences on the behavior of the cells to be analyzed in them. From proliferation inhibition, to changes in metabolic and growth factor pathway activation, to increases in protein expression related to protein folding and ER stress, the effects of microculture may be more wide ranging than previously thought.

If results from cell based assays performed in microfluidic devices are to be incorporated into current work using traditional techniques, it will be important to know that the culture conditions alone do not predispose cells to specific responses. If significant differences in cellular responses occur in microfluidic devices, knowing what the specific effects on the cellular responses are for the device and cell types of interest to

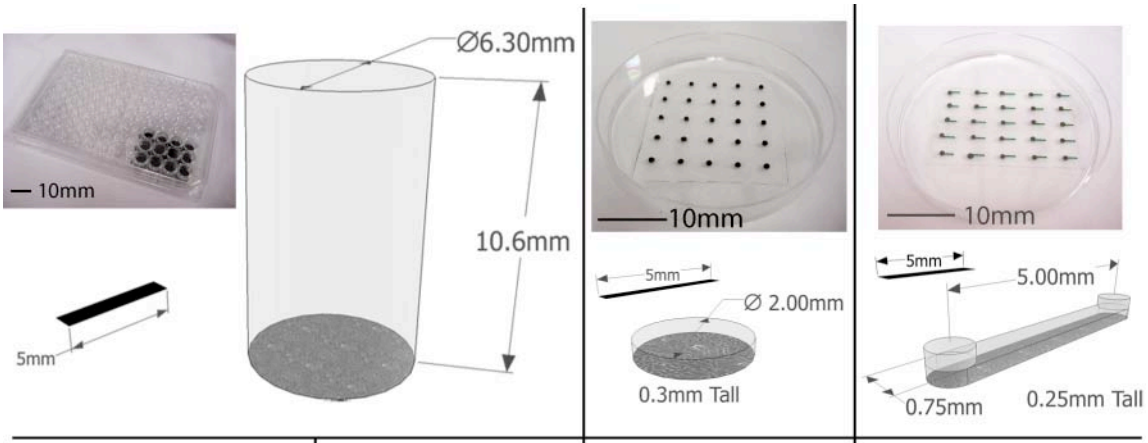
an assay will be a first step in validating new device designs.

There are a range of physical phenomena that are known to be substantially different as the scale of the device is reduced. Some of these are particularly of interest to devices that are to be used for cell based assays such as evaporation, surface interactions and material properties. In this paper, we will address a few of these phenomena from a cellular perspective (summarized in Figure 1), specifically:

1) Increased volume densities – Microcultures often use significantly less reagents, with the result being a higher volume density (more cells for each unit of media). In turn, this results in reduction of nutrients/growth factors, buffers and presumably more rapid buildup of waste products available per cell. Effects of volume density and microchannel height have shown to be important modulators of cell proliferation in microdevices[6,7,9].

2) Reduced total volume – Smaller volumes makes surface area to volume ratios with polymers involved much larger, resulting in a higher sensitivity to any surface interactions that may influence cell behavior. Also, with larger surface area (that is available for water loss) to volume ratios, evaporation induced shifts in media osmolarity (and thus media component concentrations) can become a significant limitation[14-16].

3) New polymers and material properties – Less well known, though commonly used polymers, such as poly (dimethyl siloxane), (PDMS) are used in fabrication and functional parts of devices, with unknown effects on media composition, and potential cytotoxicity. When combined with the high surface area to volume ratio, these effects may be multiplied. Initial work as suggested that PDMS in particularly may not be as "inert" as it has been previously considered[17], though its' effects on cell behavior are largely unknown.



Per Well or Channel Values	Macroscale	μWells	Microchannels
Volume of Media	200 μL	4 μL	5 μL
Total Surface Area/ Volume Ratio	0.79 mm^{-1}	1.3 mm^{-1}	2.3 mm^{-1}
PDMS Surface Area/Volume Ratio	0 mm^{-1}	~0.5 mm^{-1}	~2 mm^{-1}
Volume Density	80 cells/μL	400 cells/μL	400 cells/μL

Figure 1: Important differences between micro and macroscale cultures range from total media volume, surface area to volume ratios and volume densities. Macroscale cultures represent what is typically used in biological laboratories and have low volume densities, and surface area to volume ratios, with well studied polymers (typically poly(styrene)). Microwells (μWells) are a PDMS stencil placed on tissue culture plastic similar to microchannels. They provide the same small volumes and high volume densities, but with less contact with PDMS and total surface area to volume ratios than microchannels. These three culture devices will be used in this paper and cell behavior in each will be compared to illustrate the impact of these various characteristics (volume density differences, effects of small volumes, and interactions with PDMS).

Increases in volume densities

Volume density is not a commonly addressed variable in cell culture, although in traditional macroscale cultures volume densities are typically not largely different between different culture platforms (e.g., 96 well plate vs Petri dish). As the scale of the culture is reduced and confined in a microfluidic device, the volume densities can increase substantially (5 fold or more depending on device size). The effects of these changes are not well understood, although an increase in volume density conceivably would reduce the total amount of all media components available to each cell, including

growth factors, nutrients, amino acids, sugars and buffers. Additionally, the rate of accumulation of waste products and signaling molecules produced by the cells would be higher when less fluid is available per cell. A complete idea of the effects on cellular functions of these changes isn't clear, but current media formulations have been developed using macroscale techniques, and could potentially be less appropriate for the specific needs of microscale cultures.

Reduced Total Volume

Although microfluidic devices do provide the benefits of low reagent volumes, as the scale of the culture is reduced, the susceptibility of the culture volume to evaporative losses increases as well as the total surface area to volume ratios increase. For example, in these conditions, loss of 1uL of media volume in a macroscale culture results in a 0.5% shift in osmolarity, while the same loss in a microchannel culture will result in a 33% increase. While most incubators are humidified, often humidity levels are approximately 80% relative humidity, with wide variability (e.g. when the door is opened); while this may be sufficient to limit significant concentration of macroscale cultures, it often is not sufficient for microdevices[15,16]. Recently, evidence of changes in cell behavior and morphology as a result of evaporative losses and subsequent concentration of the media in a microdevice as compared to a macroscale culture was shown[14]. While the issue of evaporation is beginning to be addressed for cell culture systems (despite it being a very common issue), the influence of the increased surface area to volume ratios are less well understood. Conceivably, surface interactions with media components either via leaching of components into the polymer bulk or vice versa, and the interactions of proteins with surfaces may become an important factor in controlling the microenvironment cells are exposed to in these devices.

New polymers and material properties

Most macroscale cultures are performed in polysytrene (or glass bottomed) tissue culture flasks, dishes and plates. While many microfluidic cultures are performed with similar substrates as macroscale cultures by adding micropatterned

channel materials[18] onto tissue culture substrates, new materials are used to fabricate the body of the devices. While many new materials are being integrated into microfluidic devices for cell based assays, the limitations of these materials are also being evaluated. Often the materials cells interact with are considered to be "inert" with respect to their effects on cellular behavior and are largely ignored unless they are designed specifically to be bioactive.

Recent work has shown for a common polymer used for microfabrication, poly(dimethyl siloxane), or PDMS, that the partitioning of hydrophobic molecules into the polymer bulk can result in significant changes in the solution concentrations.[17] This issue becomes particularly important when compounds used to stimulate or block cellular processes or pathways are both small and hydrophobic such as many small molecule inhibitors or other compounds used in drug screening, but also may be important for basic cell culture itself. Additionally, titrations of compounds used for screening or controls that may potentially interact with the materials used can be done to determine whether or not this might be a significant issue for the molecules/materials of interest. However, if PDMS does significantly interact with the basal media components in a culture, it will be challenging to determine specifically which components are being affected and even moreso to determine the extent of the effects on the range of cellular processes occurring in cultures.

In order to better understand what the most influential characteristics of microcultures are, an analysis of glucose and growth factor availability was performed and the responses of the cells in macro- and microcultures were compared. Additionally, experiments which give insight into the potential effect of PDMS and volume density were performed to begin to understand if these may be limiting factors in microfluidic cell based assays. These results are an examination of the specific effects of microculture on cellular behavior, with a focus on metabolism, growth factor stimulation, proliferation and cell cycle progression.

METHODS

Cell culture

Mouse mammary fibroblasts (MMFs) isolated from p16/INK4a knockout mice were cultured in DMEM with 10% serum, and 1% P/S (passage numbers ranged from 20-35). When not specified, high glucose medium was used, containing 4.5g/L glucose, otherwise no glucose DMEM was used and glucose added to the specified concentration. Cells were passaged every 2 days with an approximately 1:5 dilution, (initial confluence was approximately 20-30% and at 2 days was approximately 70-80%). MMFs were seeded at the same surface density as typically done for normal passage in flasks (approx. 40-50K/cm^2) in microchannels, microwells and in 96 well plates. This results in a 5 fold increase in volume density in microchannels versus typical macroscale culture with the same surface density.

Proliferation Assays

For proliferation assays, channels and wells were sacrificed at each timepoint (5 or more channels or microwells for microcultures, 3-5 wells for macrocultures), and fixed and stained for nuclei with ToPro3. Cells were washed briefly with PBS, then fixed with 4% PFA in PBS for 10 minutes at room temperature, then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. ToPro3 (Molecular Probes) was diluted 1:500 in PBS and incubated for 10 minutes at room temperature, then washed twice with PBS and dried prior to scanning.

Glucose concentration

Glucose concentration was determined by taking media samples from cultures prior to fixing, adding 500μL of the glucose assay reagent (BioAssay Systems, QuantiChrom Glucose Assay Kit) and boiling for 8 minutes. The cooled samples were transferred to a 96 well plate, analyzed via plate reader colorimetric assay at 630nm and compared to a standard curve.

In Cell Westerns

For ICW's, cells at each timepoint were fixed and stained for either phosphorylated or total protein. Cells were washed briefly with PBS, then fixed with 4% PFA in PBS on ice for 20 minutes with the addition of phosphotase inhibitor cocktail at 1:100 dilution (Pierce, Halt Phosphatase inhibitor cocktail, #78420). To permeabilize, two washes with 0.1% Triton X-100 in PBS, 7 minutes each were done then cells were blocked in Licor blocking buffer (Licor Biosciences, #927-40000) for 90 minutes at room temperature. Primary antibodies were added to Licor blocking buffer with 0.1% Tween-20 and incubated overnight at 4˚C. Primary antibodies were all from Cell Signaling at the following dilutions; 1:50 dilution for phospho-AMPKα (#2535, rabbit monoclonal), 1:50 dilution for AMPKα (#2603, rabbit monoclonal), 1:50 dilution for phospho-S6 ribosomal protein (#2211, rabbit monoclonal), 1:100 for S6 ribosomal protein (#2217, rabbit monoclonal). Cells were then washed 3 times with PBS with 0.1% Tween-20 for at least 7 minutes each wash at room temperature. IR dye conjugated secondary antibody was then added in Licor blocking buffer with 0.1% Tween-20 at 1:200 dilution (Rockland Inc., #611-731-127, IRDye 800CW conjugated donkey anti rabbit) for 45 minutes at room temperature in the dark. Secondary antibody was washed out with two washes with PBS with 0.1% Tween-20, 7 minutes each wash, then cells were incubated with ToPro3 at 1:500 dilution for 10 minutes in PBS at room temperature, in the dark. Cells were then washed twice with PBS and allowed to dry prior to scanning on an infrared laser scanner (Odyssey, Licor Biosciences).

RESULTS AND DISCUSSION

VOLUME DENSITY AND MEDIA SUPPLEMENTATION

Previous work has suggested that by culturing cells in microchannels proliferation rates are reduced as compared to the same cell type cultured in macroscale cultures. Additionally, evidence of stress related to metabolism and growth have been shown such as altered levels of readouts for energy availability (increased AMPKα

phosphorylation) and growth factor signaling (decreased S6 phosphorylation) (see REF to part 1). Because volume densities are 5+ fold higher in microchannel cultures than typical macroscale cultures, it is possible that simply the reduction in the amount of media components such as glucose or growth factors per cell is significantly affecting cell behavior. To analyze the potential sources of these changes in microcultures, a more in depth analysis of proliferation and glucose consumption were analyzed and compared to macroscale cultures. Also, because media compositions have been developed for use in macroscale cultures, it is possible that perhaps simply by supplementing media for use in microcultures, we may be able to affect how cells function in microcultures.

To determine the rates and dynamics of proliferation in cultures of each scale cells were seeded into microchannels and into 96 well plates and fixed and stained with a nuclear dye at regular intervals. The channels and wells were then scanned on a laser scanner, and the integrated intensities for each channel or well were determined for each timepoint. The nuclear intensities of all channels/wells of several timepoints over two day cultures were normalized to the average nuclear intensity at 3 hours post-seeding, giving the fold increase in nuclear intensity, or equivalently cell number, at each timepoint (Fig. 2a). Additionally, the concentration of glucose in the media throughout the two day culture period was measured (Fig. 2b).

From these measurements, the glucose available per cell (more specifically, per K counts of nuclear intensity) at each timepoint can be calculated (Fig. 2c). While the concentration of glucose in microcultures decreases substantially, because very little proliferation occurs, the per cell amount of glucose is reduced by only 38% at 48 hours versus 3 hours post-seeding. Macrocultures showed less of a change in concentration, but the depletion that was present combined with significant cell proliferation results in a 76% decrease in glucose per cell at 48 hours. The total glucose depletion rate occurring in each culture scale for each time period was also calculated (the total drop in glucose available normalized to the hours elapsed) (Fig. 2d), and remains fairly constant in microcultures. In macrocultures, the larger total depletion rates

occur when most of the cell proliferation is occurring, tapering off during the last time period, when density constraints begin to inhibit cell proliferation.

If the proliferation curves for each culture scale are integrated over the culture period to determine the approximate number of cell-hours, the total decrease in glucose can be used to determine the average glucose consumption rate per cell, per hour (in this case per K counts of nuclear intensity). This calculation is shown in Fig. 2e and is over 4 times higher in microcultures than in macrocultures. This data suggests that while less proliferation is seen in microcultures, that metabolic rates are actually higher in microcultures with respect to glucose metabolism, suggesting that perhaps glucose restriction is not a limiting factor for these cells.

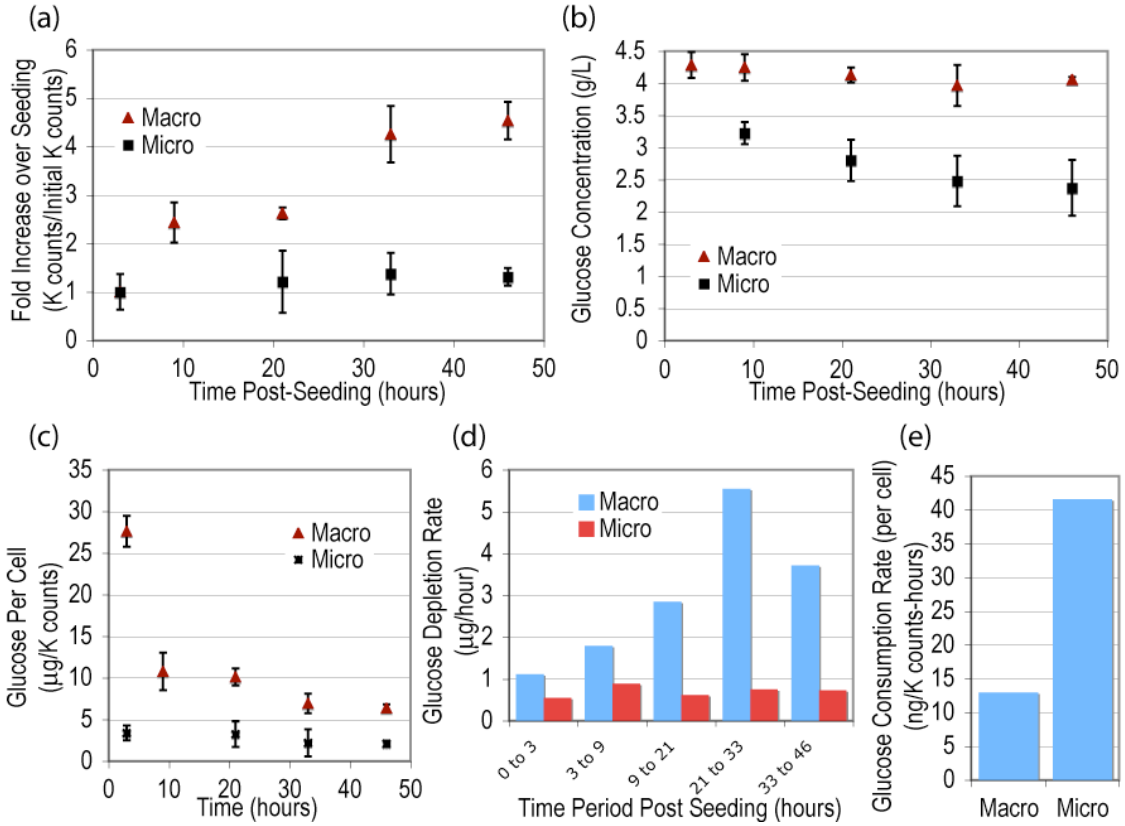


Figure 2: Proliferation over time for macro and micro scale cultures from 3 hours after seeding to 48 hours normalized to the 3 hour timepoint for each culture scale (a). The media glucose concentration throughout the culture period showed larger decreases in microcultures than macro despite having significantly less proliferation (b). The amount of glucose normalized to the cell number in the cultures is shown in (c). Using the concentration data in (b), the average population rate of glucose depletion can be determined for each culture period (d). Integrating the proliferation curve and using the total decrease in glucose, the average per cell glucose consumption rate over the two days can be determined (e). Error bars are one standard deviation, n of 4-6 for micro, 3 for macro for proliferation data, and n of 3 for all glucose concentration measurements.

Energy restriction: Glucose Supplementation or Restriction

Cells can sense their energy status via a pathway involving AMP activated protein kinase (AMPK). The function of AMPK is to regulate the balance between anabolic and catabolic cellular functions in response to the microenvironment.[19,20] When it is activated, catabolic pathways, which create ATP, are activated, while anabolic pathways, which consume ATP, are inhibited. Catabolic pathways include glucose uptake and glycolysis, which are required for basic cellular respiration and function.

Anabolic pathways include protein and fatty acid syntheses and are required for cell growth and proliferation.

When ATP availability is reduced, either due to a decrease in ATP production or an increase in ATP consumption, the AMP:ATP ratio will get larger. When the intracellular AMP:ATP ratio is high, AMPKα (the catalytic subunit) is phosphorylated, resulting in downstream changes that alter metabolic behaviors of the cell and glucose uptake, allowing cells to adapt their metabolic processes to their microenvironment. If cultures are energy deprived, it would be expected that levels of phospho-AMPKα would be higher than cultures with sufficient energy supplies.

Because cells do not only rely upon glucose for their energy supply, the increase in glucose metabolism rates seen in microcultures may be due to a more general energy restriction due to the loss of some other energy source (e.g, fatty acid oxidation). To determine if microcultures were in fact experiencing energy restriction due to differences in glucose availability, cells were seeded in microchannels or 96 well plates in 10% serum with 9, 4.5, 1.8 or 0 g/L glucose DMEM and in cell Westerns (ICWs) for phospho and total AMPKα were performed at 24 and 48 hours after seeding (Figure 3). Cells cultured in microcultures with 9 g/L glucose are provided with approximately the same per cell amount of glucose initially as macrocultures with 1.8 g/L glucose. If the total amount of glucose per cell is important for cellular behavior, then these two culture conditions would likely provide similar results.

For these cells, none of the culture conditions resulted in a significant change in phosphorylation levels with respect to either the same culture medium in the other scale, or between cultures of different glucose concentrations. However, a larger fraction of AMPKα is phosphorylated at 48 hours than at 24 hours in all culture scales and conditions suggesting that energy status does change over the culture period. These data suggest that microcultures are not functionally energy deprived with respect to macroscale cultures. However, the rate of glucose consumption in microcultures was 4

fold higher than in macro, suggesting that for the same baseline energy levels to be obtained in microcultures, a substantially larger amount of glucose must be consumed, despite the lack of proliferation seen.

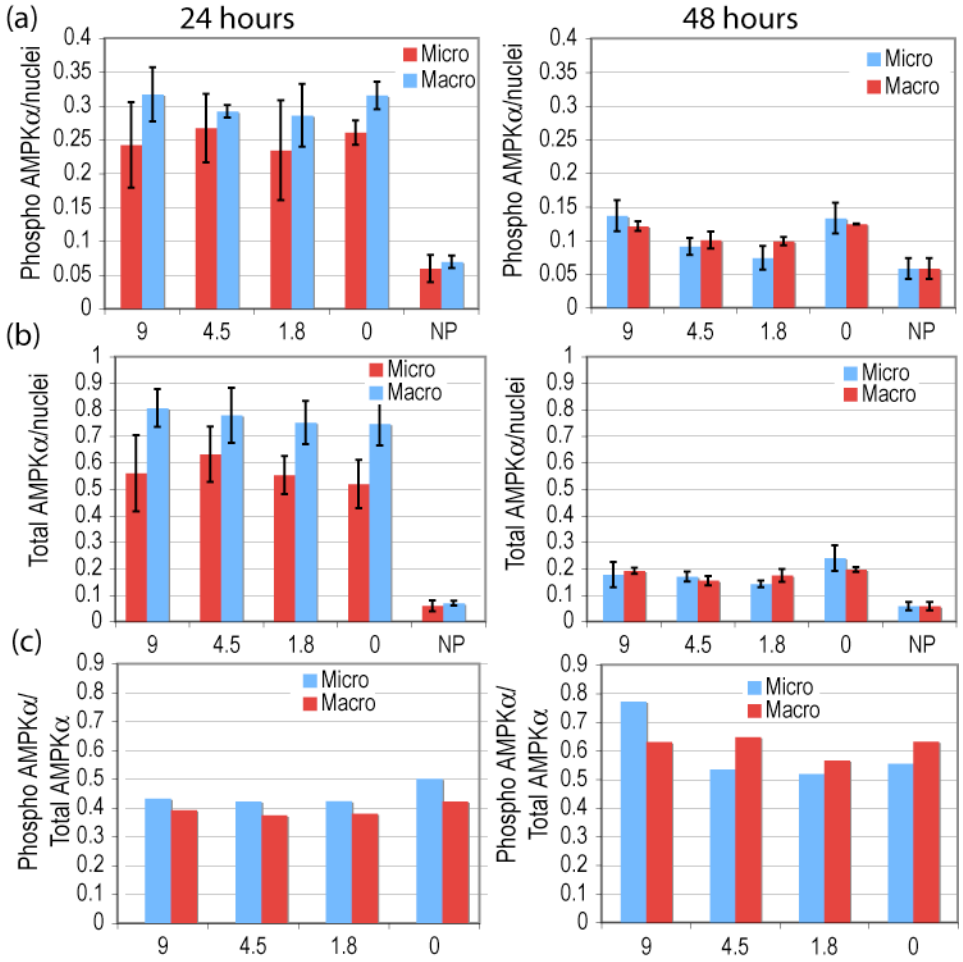


Figure 3: ICWs for phopho and total AMP kinase α at different dilutions of glucose for macro and microscale cultures at 24 (left) and 48 (right) hours post-seeding. The integrated intensity normalized to the nuclear control are shown for (a) phosphorylated AMP kinase α, (b) total AMP kinase α, and (c) the ratio of phosphorylated to total AMP kinase α. Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

Growth factor restriction: Serum Supplementation or Restriction

When the volume density of a culture is increased as in microcultures, not only is the total amount of glucose per cell reduced, but also the total amount of growth factors per cell and other components of the serum. A pathway that is responsible for cell growth and proliferation decisions that can be affected by changes in growth factor signaling is the mTOR pathway (mammalian Target Of Rapamycin). This pathway integrates signals from a wide variety of signaling pathways such as the AMPK pathway discussed above and the insulin and Wnt signaling pathways. mTOR activation results in increases in protein translation and cell growth (such as that prior to cell division). When mTOR is activated by growth signals, a downstream target, S6 ribosomal protein is phosphorylated serving as a single readout for many growth promoting signals.

To determine if growth factor/serum restriction was occurring in microcultures, cells were seeded in microchannels or 96 well plates in 4.5 g/L glucose with 20, 10, or 3% FBS in the media and ICWs were performed at 24 and 48 hours post seeding for phospho and total S6 (Figure 4). Cells seeded in microcultures with 20% serum would be provided approximately the same per cell amount of growth factors as cells in macrocultures with 3% serum due to the difference in volume density. If the per cell amount of growth factors/serum available is critical for cell behavior, then these culture types might be expected to behave similarly.

At 24 hours post-seeding, levels of phospho S6 in both 20 and 10% serum media were nearly 6 fold higher in microcultures than in macrocultures. In 20% serum cultures total S6 was 3 fold higher in microcultures than in macrocultures. Also, in both the 10 and 20% serum media, the relative amount of phospho to total S6 was between 2.5-3.5 fold higher than in macroscale cultures. However, by 48 hours, levels of phospho-S6 in microcultures were reduced nearly to levels in macrocultures, but total S6 in microcultures remained over twice the macroscale levels in 10 and 20% serum.

These data suggest that despite the lack of proliferation in microcultures, that pro-growth signaling via S6 ribosomal protein is occurring at a much higher level at

24 hours post seeding than in macrocultures, when nearly all of the proliferation that is seen in microcultures has occured. By 48 hours post seeding, levels of phosphorylated S6 and total S6 are reduced and are much closer to the macroscale levels. The reduction in proliferation seen in microcultures is likely not due to growth factor restriction, as microcultures have even higher levels of S6 phosphorylation than macrocultures.

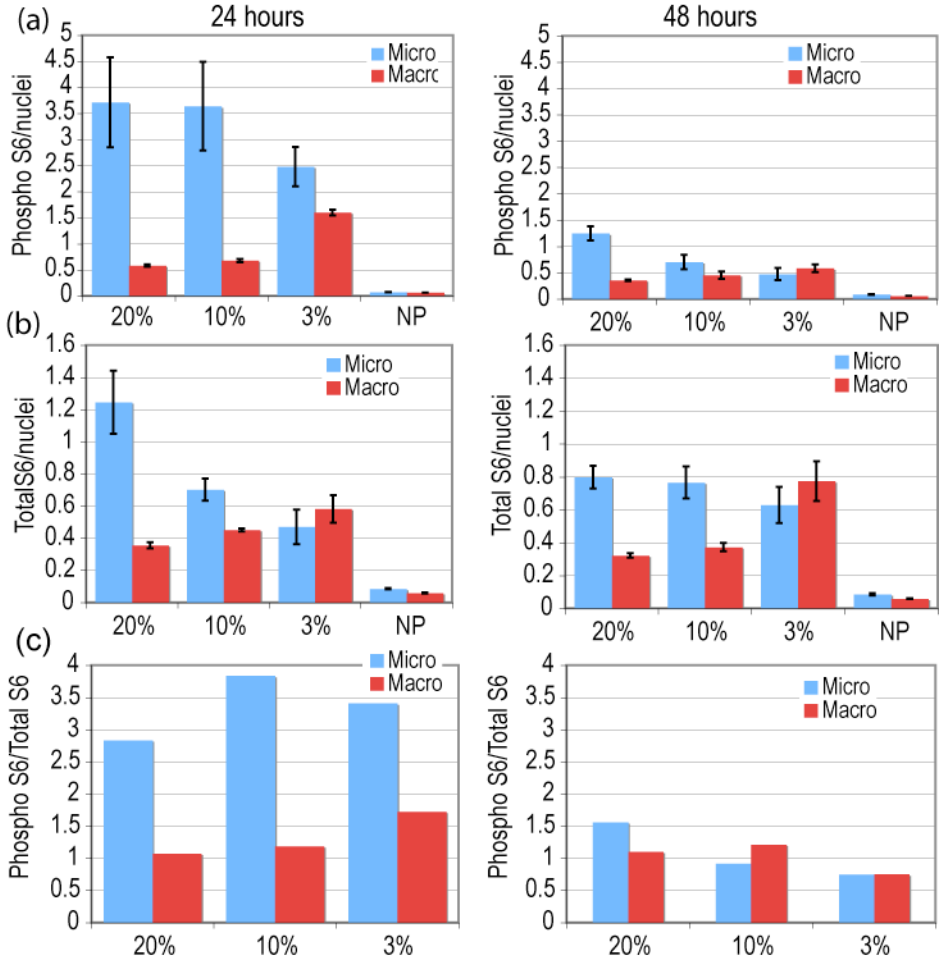


Figure 4: ICW results for cells seeded with different dilutions of FBS for macro and microscale cultures at 24 (left column) and 48 (right column) hours post seeding, for phospho-S6 (a), total-S6 (b) and the ratio of phospho to total S6 (c). Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

Finally, to determine if any of these media supplementation conditions resulted in changes in proliferation, the fold increase in cell number after 48 hours of culture (with respect to that plated at 3 hours) was determined and shown in Figure 5. No condition resulted in any significant change in proliferation of microcultures, and no corresponding macro and microscale cultures (macroscale 1.8g/L glucose/microscale 9g/L and macroscale 3% serum/microscale 20% serum) behaved similarly with respect to either S6 levels or phosphorylation status, or proliferation. Also, glucose consumption rates in 9g/L glucose microscale cultures were 3 fold higher than the corresponding macroscale culture with 1.8g/L glucose (Figure S2e), and depletion rates were consistently more rapid in microcultures regardless of the initial concentration of glucose (Figure S2a-c).

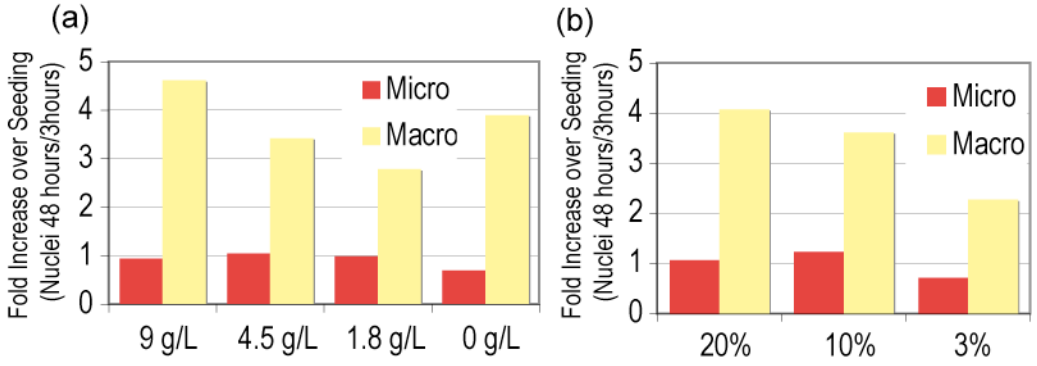


Figure 5: Proliferation of cultures with a range of media glucose dilutions (a) or FBS concentrations (b) in macro and microchannel cultures. These data suggest that simple supplementation will not increase proliferation in microchannels.

From these data, we can see that microscale cultures do not have significantly different functional levels of intracellular energy availability, but that the rate of glycolysis required in these cultures is much higher than that seen in macrocultures, regardless of the amount of glucose available. Additionally, when glucose is supplemented (or limited somewhat) in macrocultures, the glucose consumption rates were not largely different. However, in microcultures, as more glucose was present in the medium, the higher the rates of glucose consumption. Despite the apparent need to consume much more glucose per cell per hour in microcultures, pro-

growth signaling as determined by S6 phosphorylation is much higher than in macroscale cultures at 24 hours post seeding, regardless of the amount of serum in the medium. Finally, even with sufficient energy supplies and growth promoting signaling, cells in microcultures did not proliferate more in any media condition in which either glucose or serum were supplemented. These data show that simply supplementing the media used for microcultures, similar metabolic rates, pro-growth signaling or proliferation with macroscale cultures does not occur. Likely volume density increases causing glucose or growth factor depletion are not a major influence on these cells' behavior in these microchannel cultures.

Reduced Total Volume and Material Properties

If reduced volume is the key influence causing changes in cellular behabior, then evaporation or interactions with surfaces would be dominant. For these cultures, we have optimized the means of combating evaporation by using sacrificial water sources. The volumes of each type of culture type were measured after 48 hours in a humidified incubator and compared to the initial volumes to ensure that no significant evaporation was occurring (data not shown).

With evaporation under control, the effects of the increased surface area to volume ratios of the media can be better evaluated. For all subsequent analyses, a third culture type was used, microwells, (uWells) which is described in Figure 1. Additionally, to eliminate any influences of volume density, we cultured macroscale cultures with reduced media volumes to allow equal volume density cultures in various scales and culture types to be compared. In these conditions, the macroscale and microwell cultures have very similar total SA/V ratios (Figure 6c, SA/V_{total}), and are analogous to the differences between a 6 well plate and a 96 well plate (which typically provide indistinguishable results), with the exception of a low amount of contact with PDMS (SA/V_{PDMS} of ~0.5 mm^{-1}) in the microwell cultures.

If SA/V_{total} is critical, then cultures in microwells and macroscale with the

same volume densities would be expected to proliferate similarly, while microchannels would be much more affected because they have nearly double the total SA/V. However, if SA/V_{PDMS} was more significant, it would be likely that despite the equal total SA/V of microwells and macroscale, that the proliferation and behavior of cells in microwells would fall somewhere in between macro and microchannels.

To elucidate these issues with respect to proliferation, cells were seeded in macorscale cultures (96 well plates), microwells and microchannels with a range of volume densities in the standard medium (4.5g/L and 10% serum). Cells were seeded into 96 well plates at 1x, 2.5x and 5x the typical volume densities (the same cell numbers/surface densities were used, but with less medium in each well). Similarly, cells were seeded as typically done in microchannels and microwells at 5x volume density (5x as compared to the typical volume density in macroscale cultures), and allowed to attach to the substrate for 3 hours, when some microchannels/microwells were supplemented with extra medium, bringing the volume densities in those cultures to 2.5x that of macrocultures. The proliferation and glucose consumption rates in each of these culture scales is shown in Figure 6.

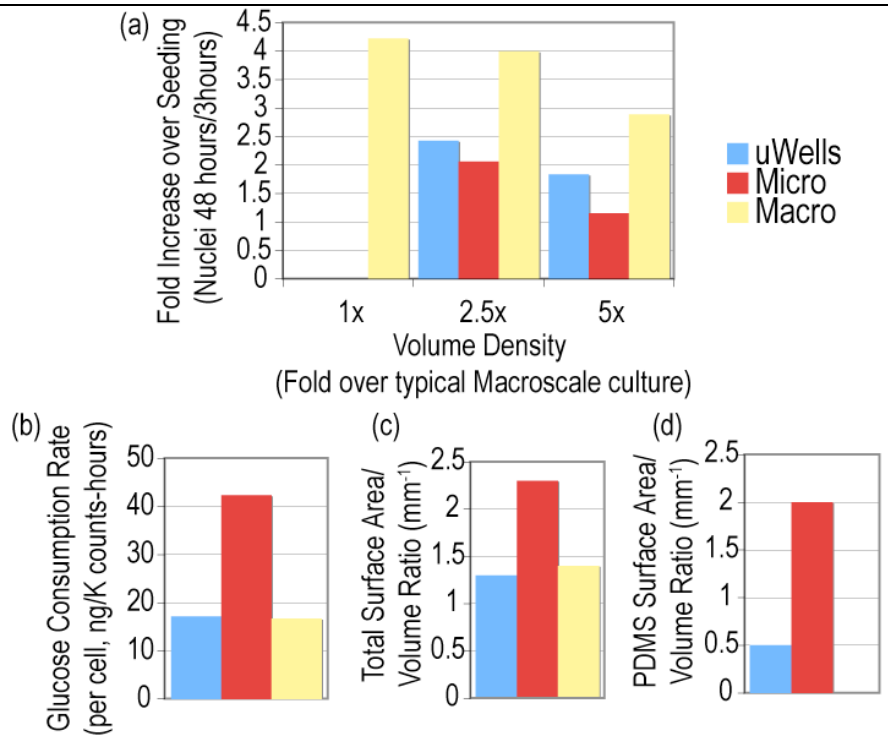


Figure 6: The proliferation (fold increase in cell number with respect to plating) in microwell, microchannel and macroscale cultures is shown in (a) for a range of volume densities in each culture type. Additionally, the glucose consumption rates of 5x density microcultures and 1x density macrocultures are shown in (b). The total surface area/volume ratio of each culture type is shown in (c) and that specifically for the contact with PDMS is shown in (d). The legend for (a) applies to all panels.

Proliferation in microwells in typical medium (and even in a range of media glucose concentrations and serum compositions, see Figure S3) was always intermediate to that seen in microchannels and macroscale cultures. However, glucose consumption in microwell cultures was consistently closer to macrocultures than microchannels (Figure 6 and Figure S3). The proliferation seen can be increased by decreasing the volume density by adding supplemental media to any culture type. However, all the fold increases in proliferation between 2.5x density cultures and 5x cultures with the standard medium were similar regardless of scale (~1.3 for macro/microwells, 1.8 for microchannels), the total SA/V or that of just the PDMS, suggesting that those increases are likely only due to increases in volume density.

Neither microculture type showed proliferation similar to that seen in macrocultures of any volume density in any media composition (besides 0g/L glucose and 3% FBS in which cell survival in any culture type was very low, Figure S3), suggesting that the contact with PDMS is likely more influential on proliferation than volume density or total SA/V ratio.

NUCLEAR SIZE AND CELL DIVISION

Microchannel cultures seem to consistently show a maximum of one population doubling over 2 days, typically in the first 24 hours after seeding, while macrocultures go through an average of 2 population doublings (for a total of 4 fold increase in cell number). Because media supplementation and volume density reductions did not show significant rescue of proliferation in microcultures, further investigation of the dynamics of any cell proliferation was warranted.

The reduction in proliferation in microchannels could be due to cells not entering the cell cycle and staying senescent. To determine if cells in all of the culture types were capable of entering the cell cycle, the presence of cells in mitosis via microscopy was verified (Figure 7). Cells in all three culture types were seeded in the standard media (4.5 g/L glucose and 10% serum), and after 48 hours fixed and stained for nuclei and imaged via microscopy. Qualitatively, the frequency of cells actively dividing (cells with condensed chromosomes) was lower in microchannels vs microwells and 96 well plates, but was evidence of cells entering the cell cycle in all culture types, (the exact frequency of cells in active phases of the cell cycle in each culture scale is difficult to determine accurately by visualization).

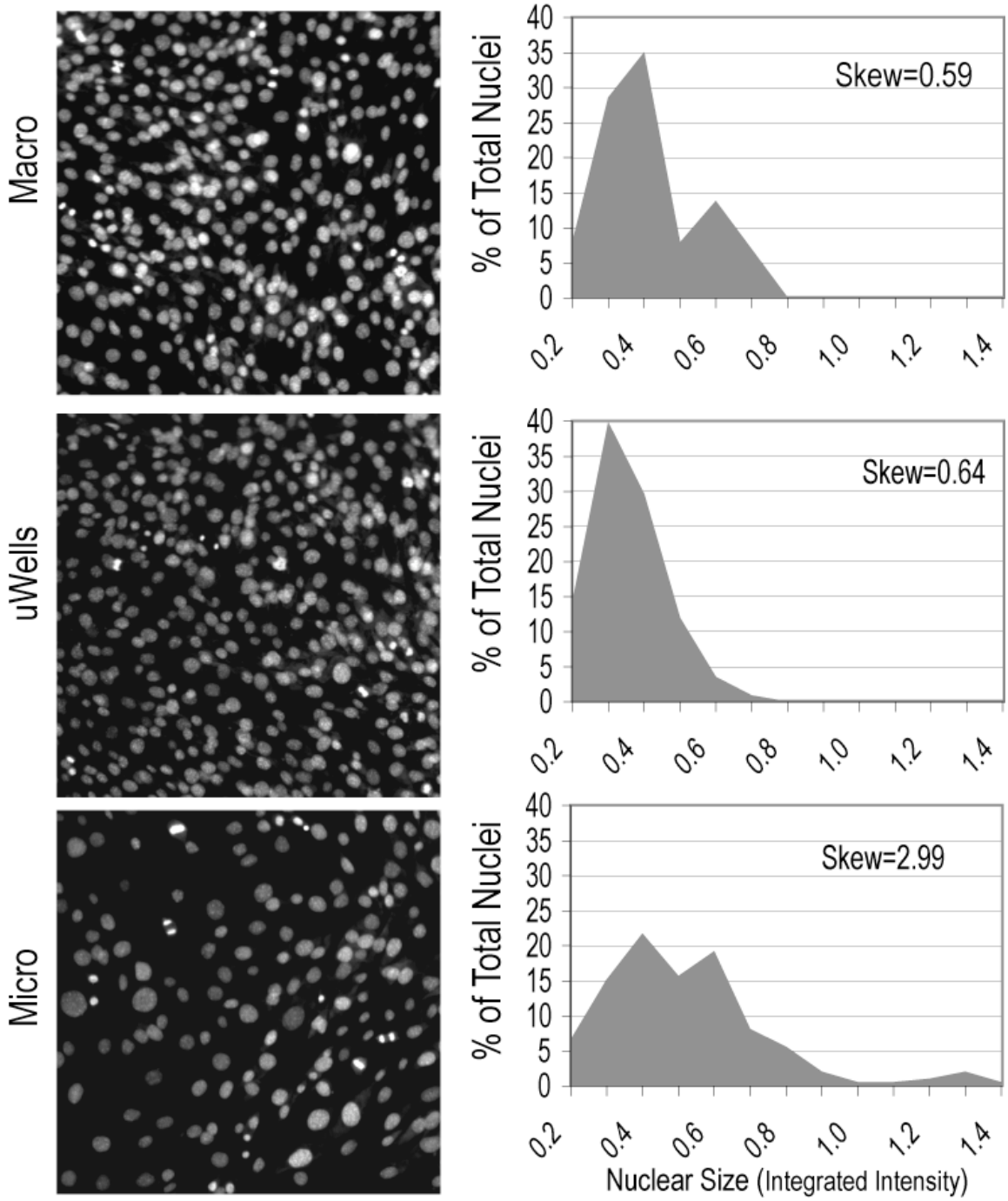


Figure 7: Representative images of the nuclei of macroscale cultures (top, Macro), microwell cultures (middle, uWells) and microchannel cultures (bottom, Micro) taken with the same exposure time and magnification. When images were analyzed by determined the integrated intensity of a sample of nuclei (minimum of 250), distributions of the nuclear size as a percentage of the total nuclei were determined, shown to the right of the images for each culture type.

Because cells in microchannels were clearly entering the cell cycle, whether the cells were successfully dividing was then considered. Qualitatively, microchannels contained much more frequent nuclei with very large area (images in Figure 7 are of the same magnification). To begin to quantify this observation, the center of 3 wells, microchannels or microwells were imaged with the same excitation intensity and magnification and the integrated intensity of the nuclei in each image was quantified using NIH Image (sample sizes were a minimum of 200 nuclei per culture type per well or channel). Nuclei which were actively dividing and visibly in some phase of mitosis were not included (determined only by the presence of any condensed chromatin).

The distributions of the integrated intensity of the nuclei in each scale are plotted in Figure 7 beside representative images of nuclei in each culture type at 48 hours after seeding. In both macroscale cultures and microwells, the distributions were fairly close to normal distributions, with very low skewness (the degree of asymmetry of a distribution around its' mean, zero being a symmetric, normal distribution), near 0.6. However in microchannel cultures, the skewness was nearly 3, and positive, indicating a large degree of skew towards the right, or nuclei with more DNA.

Upon further observation, images from microchannels showed several different cell cycle progression problems occurring that could not be found in cells growing in other culture types. An image showing several cell division defects commonly found in microchannel cultures at 48 hours after seeding is shown in Figure 8.

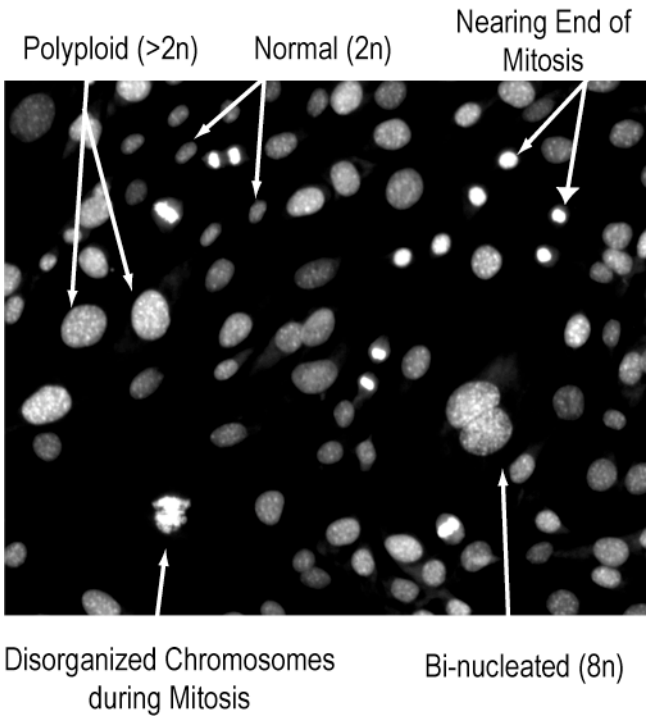


Figure 8: Defects in cell division and ploidy were common in microchannel cultures, but could not be identified in other culture types.

Specifically, many large polyploid nuclei could be found in microchannels which were verified to have more than the typical 2n amount of DNA via quantification of integrated intensity. These nuclei were noticeably larger than the nuclei in macroscale cultures or microwells, which were typically the same size as those labeled "Normal" in the image (for a reference, images in Figure 7 show all three culture types with the same magnification). It is possible that many of these nuclei were in S or G2 phase of the cell cycle, although at this stage it would be expected that the nuclei would have approximately double the DNA as a normal, nondividing nuclei rather than much more (e.g., the equivalent of 6n or more). In these analyses, nuclei with over ~0.8 fluorescence units would be expected to have more DNA than typical for 2n or 4n cells in normal phases of the cell cycle.

Examples of more extreme defects such as disorganized chromosomes in mitotic cells such as the cell labeled were fairly common. The sample cell in Figure 8 in

particular likely is polyploid as well (approximately 8n when the intensity is compared to the normal nuclei), and the extra chromosomes cannot be properly aligned during mitosis resulting in what looks like several axes forming and more DNA than the other examples of dividing cells in M phase in the image (verified to have the expected 4n amount of DNA or 2n per individual nuclei for those that were in later phases of separation). This cell, had it been able to continue to try to divide, might have ended up bi-nucleated such as the one labeled. This bi-nucleated cell not only has two distinct nuclei (verified via phase microscopy to be one continuous cell), but each nuclei contains twice the normal amount of DNA, resulting in the total integrated intensity of the total nucleus being 4 times higher than the average value from macroscale cultures (which would presumably be 8n).

These data suggest that in microchannel cultures, these cells can successfully enter the cell cycle, but have a much higher frequency of either arrest in the S/G2 phases of the cell cycle, unsuccessful division and/or accumulation of nuclear DNA. The wide distribution of nuclear sizes is not seen in microwell cultures, nor macroscale cultures, suggesting that perhaps the larger surface area to volume ratio of PDMS in the microchannels may have a more significant effect on the cultures than volume density or total SA/V. If cells in microchannels were inhibited from finishing a round of cell division, they would generally remain senescent afterwards (as perhaps for MMFs, 4n cells are inhibited from initiating another round of cell division or cells are arrested in S/G2), which would be consistent with the proliferation kinetics seen in these cultures. One round of cell division may be initiated in the first 24 hours when proliferation is generally seen in these cultures, but after this point, very little further division occurs because the first round was unsuccessful in so many cells.

CONCLUSIONS

In no case in which media was supplemented with glucose or FBS was proliferation increased (to macroscale levels) in either microculture type employed for

these experiments. These data indicate that simply changing the media formulation in microcultures is not sufficient to allow similar proliferation, glucose consumption, along with S6 levels and phosphorylation status as typically seen in macrocultures. With these differences in cell behavior in cultures of different scales and with different interactions with PDMS, a cell based assay in microchannels would not necessarily be expected to provide the same response to the same stimulus as that seen from cells in macrocultures.

In these experiments, the cells used were isolated from p16 knockout mice. p16 functions to regulate the entrance of cells into S phase of the cell cycle, and over or under expressing it results in errant cell cycle control or arrest. While for these cells, the effects of microfluidic channel culture have resulted in the accumulation of nuclear DNA, other effects, potentially more or less disruptive to cellular functions may be more dominant for other cell types. Thus, validating environments for specific cell types of interest will be critical to ensuring that microculture alone is not significantly altering the cells' basic functions.

While the data presented suggests that PDMS may be causing artifacts in microfluidic cell culture results, it is still unclear whether the interactions between the media and cells and PDMS is due to molecules leaching into or out of the PDMS. It is possible that uncrosslinked monomer is leaching from the PDMS into the medium, and would likely partition into the hydrophobic portions of the cells such as the plasma membrane, ER or nuclear envelope. Likewise, it is possible that hydrophobic growth factors or lipids from the cell culture medium and serum are being depleted by diffusion into the PDMS bulk. If lipid metabolism was an important source of energy for these cells, the loss of lipids to the PDMS in microcultures might explain why their use of glucose as an energy source increases by several fold over the rate in macrocultures or microwells. Either of these potential explanations would explain why cultures supplemented with extra medium proliferate more than those with less (either the concentration of the monomer in the medium is less, or larger amounts of lipid/growth factors are provided replacing some lost to the bulk PDMS). Also, these factors would be

consistent with the increase in growth in microwell cultures with respect to microchannel cultures, but not equal to that of macrocultures, as they have an intermediate surface area to volume ratio of PDMS.

Regardless of which effect is primarily responsible for the differences seen in microchannel cultures, these data illustrate an interesting change in phenotype depending on the type of microenvironment the cells are exposed to. In microchannel cultures (versus those in macroculture), glucose consumption rates increased, as did phosphorylation levels of S6, both indicative of activation of pathways involved in cell growth. Meanwhile cell proliferation was inhibited. While cell growth is required for cell proliferation, it is not sufficient to cause it, as cell growth can occur without resulting in proliferation. In vivo, most cells (particularly those in stroma, as these mammary fibroblasts would be) are not rapidly proliferating, but instead grow and function cooperatively in a tissue. Indeed, in tissues in which morphology and multicellular organization is required (such as the mammary tree), proliferation is often a somewhat orthogonal process with formation of organized tissue.[21] In these cases, rapid proliferation causes disruption of correct morphogenesis of a tissue structure. Each process is coordinated temporally and spatially in such a way to form both tissue that is both sufficient in size and also functional. Thus, while proliferation is inhibited in microchannels, this may more accurately represent the in vivo condition where cells are typically more quiescent than what is seen in tissue culture.

Additionally, p16/INK4a functions as a tumor suppressor by limiting aberrant cell proliferation by inhibiting entry into S phase of the cell cycle. The loss or downregulation of p16/INK4a has been shown to occur in many tumors, along with age-dependent increases in expression as cells become more and more quiescent[22]. The cells studied here were isolated from a p16/INK4a knockout mouse, thus presumably would be

more susceptible to errors in cell cycle regulation. Ideally, the effects of the loss of p16 would be seen in culture if in vitro studies of the in vivo system was to be done with these cells. With these issues in mind, the true phenotype of how these cells function in vivo may more accurately be represented by the cells cultured in microchannels (poor cell cycle control, but not rapid proliferation like that seen in macrocultures).

For each cell type studied using microchannels, some effect may be seen due to the differences in the microenvironment. If PDMS the primary source of the biological differences in these cells then material choices must be addressed. But if these devices actually mimic the *in vivo* condition and the "artifacts" are actually resulting in more *in vivo*-like cell behavior, then these devices may be extremely valuable as a model system for cellular studies.

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