Single cell functional analysis of multiple myeloma cell populations correlates with diffusion profiles in static microfluidic coculture systems

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Microfluidic cell culture systems are becoming increasingly useful for studying biology questions, particularly those involving small cell populations that are cultured within microscale geometries mimicking the complex cellular microenvironment. Depending on the geometry and spatial organization of these cell populations, however, paracrine signaling between cell types can depend critically on spatial concentration profiles of soluble factors generated by diffusive transport. In scenarios where single cell data are acquired to study cell population heterogeneities in functional response, uncertainty associated with concentration profiles can lead to interpretation bias. To address this issue and provide important evidence on how diffusion develops within typical microfluidic cell culture systems, a combination of experimental and computational approaches were applied to measure and predict concentration patterns within microfluidic geometries, and characterize the functional response of culture cells based on single-cell resolution transcription factor activation. Using a model coculture system consisting of multiple myeloma cells (MMCs) and neighboring bone marrow stromal cells (BMSCs), we measured concentrations of three cytokines (IL-6, VEGF, and TNF- α) in conditioned media collected from separate culture compartments using a multiplex ELISA system. A 3D numerical model was developed to predict biomolecular diffusion and resulting concentration profiles within the tested microsystems and compared with experimental diffusion of 20 kDa FITC-Dextran. Finally, diffusion was further characterized by controlling exogenous IL-6 diffusion and the coculture spatial configuration of BMSCs to stimulate STAT3 nuclear translocation in MMCs. Results showed agreement between numerical and experimental results, provided evidence of a shallow concentration gradient across the center well of the microsystem that did not lead to a bias in results, and demonstrated that microfluidic systems can be tailored with specific geometries to avoid spatial bias when desired. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4958982>]

INTRODUCTION

Microfluidic systems are increasingly used in cell biology studies to examine cellular behavior and function. A main reason for this application is the ability of these systems to precisely control microscale physical phenomena, such as the transport of molecules, which plays an important role in biochemical signaling and cell-cell communication. Molecular transport typically occurs via two mechanisms: convection and diffusion. Some systems rely on more dynamic approaches to achieve this control, such as the use of perfusion,^{1,2} electrolysis,³ and valve-based methods.⁴ Other platforms employ simpler operation to improve ease of use,^{5,6} but often do not have the same level of gradient stability as perfusion-based systems. Both simple

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and complex microfluidic systems are becoming useful tools for biology research and provide unique advantages to their users.

One type of system that combines ease of use with controlled transport at the microscale is passive pumping-based microfluidics. Passive pumping-based microfluidic systems rely on the use of dispensed liquid droplets to deliver, displace, and manipulate fluids.⁷ These systems are simple to operate, require only a micropipette, handle small volumes of reagents, and are amenable to automation and high-throughput applications with robotic liquid handlers. Numerous passive pumping-based microfluidic systems have been developed and have found particular utility in cell culture studies.^{8–13} Young *et al.* designed a microfluidic multi-cell culture system that offers many of these features.¹⁴ The purpose of that particular system was to enable simultaneous culture of non-adherent and adherent cell types in physically separate compartments, and allow cell-cell communication between these cell types via paracrine signaling, specifically in a manner that decouples paracrine effects from juxtacrine signaling effects arising from physical contact between cells (Fig. 1). The device was originally developed to study drug resistance in multiple myeloma (MM), an incurable hematologic malignancy of the plasma B cells in the bone marrow.^{15–17} Using the device, Young and co-workers demonstrated that coculture of MM cells (MMCs) with surrounding bone marrow stromal cells (BMSCs) in neighboring compartments predictably activated the STAT3 pathway, presumably by interleukin-6 (IL-6) interactions.¹⁸ Recently, Pak and co-workers applied the same system as an “intra-patient” chemosensitivity and resistance assay (CSRA) to test and predict the sensitivity of primary patient CD138⁺ MMCs to bortezomib (BTZ), both in the absence and presence of CD138[−] cells, and discovered that intra-patient co-cultures are better at predicting clinical responsiveness compared to monocultures.¹⁹ The significance of this particular study was that many CSRA have been used clinically in the past without much success, and that perhaps the success observed using the microscale coculture device was due to the inherent proximity between cell populations (i.e., hundreds of microns) offered by microfluidic geometries that had not been used previously. One concern regarding this system, however, is that it is currently unknown whether cells near the middle of the center well are exposed to the same concentration of biochemical factors as those near the diffusion ports, and whether this uncertainty skews the interpretation of the population distributions obtained in the studies. To ensure the system provides accurate, unbiased data that is properly interpreted, and to guide future designs of microfluidic cell culture systems, including the proper selection of feature dimensions and geometries, it is critical that we carefully elucidate how biomolecule transport occurs within the system.

The objective of this study was to characterize the transport of biomolecules under static culture conditions within this microfluidic system to investigate whether there is a spatial bias within the center well during culture that would potentially lead to a bias in data interpretation. Specifically, soluble factor diffusion was analyzed both experimentally and numerically to determine the shape of concentration profiles within the center well. Asymmetric co-culture configurations as well as asymmetric treatment applications (Fig. 1(c)) were used to study different diffusion conditions, and to provide experimental evidence supporting whether cells were exposed to shallow or steep concentration gradients of soluble factors across the center well. A numerical model was developed using IL-6 as the biomolecule of interest to predict diffusion across this system, and compare theoretical predictions to experimental results. With this study, we aimed to further demonstrate the capabilities of the system, to better understand the impact of spatial arrangements of BMSCs on diffusion, and to develop a numerical modeling tool for predicting diffusion profiles for use in future experiments and device designs. Ultimately, shallow concentration gradients were observed across the center well both numerically and experimentally, and it was demonstrated that these shallow gradients did not lead to a spatial bias in this system with its current geometry.

MATERIALS AND METHODS

Device fabrication

Devices were fabricated as described previously,¹⁴ using soft lithography to generate SU-8 master molds which were then used to create poly(dimethylsiloxane) (PDMS) layers. A cell

culture channel layer, an inlet/outlet port layer, and a glass slide were bonded together via plasma treatment to form a completed device consisting of an array of 18 microfluidic cell culture systems (referred to as “microsystems” below) (Fig. 1(a)). Each microsystem consisted of a central circular well chamber for suspension MMCs (called “center well” below; 3 mm diameter by 500 μm deep), and two side chambers for adherent stromal cells (1.5 mm wide $\times$ 200 μm

FIG. 1. (a) Photograph of the microfluidic device, consisting of 18 microscale coculture systems in a staggered array. Scale bar = 10 mm. (b) A detailed view (“Detail X” from Panel (a)) of a single coculture system illustrating features including the center well, top and bottom side chambers, diffusion ports connecting the center well to the side chambers, and an outlet well for containing a droplet of media that connects the 3 outlet ports. (c) Cross section (“Section Y-Y” of Panel (b)) of a microsystem illustrating the three bonded device layers: an inlet/outlet layer, a cell culture layer and a glass slide as the underlying bottom substrate. The illustration depicts a typical asymmetric coculture experiment where neighboring adherent bone marrow stromal cells (BMSCs) cultured in the top side chamber (left, green cells) secrete soluble factors that diffuse through the connecting diffusion ports into the center well containing non-adherent multiple myeloma cells (MMCs, center, blue cells).

deep) (Fig. 1(b)). All three chambers are connected by a series of diffusion channels (or “diffusion ports,” $100\ \mu\text{m} \times 20\ \mu\text{m}$ cross section) designed to allow paracrine signaling between cells in separate compartments (Figs. 1(b) and 1(c)). This design was based on previous work,¹⁴ with minor modifications to improve operation.

Cell preparation

RPMI8226 (human B lymphocyte plasmacytoma cells, ATCC CCL-155) and HS-5 (human bone marrow stromal cells, ATCC CRL-11882) cell lines were obtained from Cedarlane Labs (Burlington, ON, Canada). Both cell lines were cultured at 37°C and 5% CO_2 in DMEM with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S), and 1% HEPES buffer. RPMI8226 cells were seeded in T-25 culture flasks at a density of 7.5×10^5 cells/ml and were passaged every 2 to 3 days. HS-5 cells were seeded in T-25 culture flasks at a density of 5×10^5 cells/ml and passaged every 2 to 3 days. RPMI8226 and HS-5 cells were used in experiments up to a maximum of 20 passages.

Device preparation and cell seeding

Each microsystem within the device was disinfected with 70% ethanol for 10 min, and flushed with 3 volume replacements (VRs) of Dulbecco’s Phosphate-Buffered Saline (DPBS), where one VR is equal to approximately $5\ \mu\text{l}$ in the center channel, and approximately $2\ \mu\text{l}$ in the side chambers. 5 VRs of DMEM media were flushed through each microsystem, and care was taken to ensure a final DMEM media volume of $30\ \mu\text{l}$ in the outlet reservoir. Importantly, after filling the outlet reservoir, the three outlet ports (one for each compartment) were connected by a small fluid droplet, which equilibrated the pressure at the outlet of all three culture channels. This setup minimized any pressure gradients through the diffusion ports, thus eliminating flow of cells through the diffusion ports, and removing any convective transport between compartments. After filling, the device was placed in a Nunc OmniTray with sterile Kimwipes pre-soaked in DPBS. The OmniTray was placed in a bioassay dish with additional sterile DPBS-soaked Kimwipes, and this entire setup was placed in an incubator. For cell experiments, $5\ \mu\text{l}$ of an RPMI8226 cell suspension was seeded in the center well of each microsystem at a density of $\sim 1.2\text{--}1.3 \times 10^6$ cells/ml. For co-culture experiments, $2\ \mu\text{l}$ of HS-5 cell suspension was seeded in one or both of the side compartments at an approximate density of 2×10^6 cells/ml for asymmetric or symmetric coculture conditions, respectively.

Microfluidic cell culture

Experiments using exogenous IL-6 were performed by first preparing a device as described in “*Device preparation and cell seeding*,” and then seeding MMCs and culturing them for 24 h. After 24 h, $6\ \mu\text{l}$ of IL-6 diluted in culture media was added to the top side chamber to establish an IL-6 diffusion profile. Care was taken to ensure that outlet ports were all connected by a $30\text{-}\mu\text{l}$ droplet of media to equilibrate the pressure between culture channels, and that minimal pipette pressure was applied during passive pumping of IL-6 into the channels. This was important to ensure minimal convective transport of IL-6 into the center well. Exogenous IL-6 (R&D Systems, MN, USA) was reconstituted at $10\ \mu\text{g/ml}$ in DPBS containing 0.1% bovine serum albumin (BSA), aliquoted, and then stored at -80°C . IL-6 was serially diluted in DMEM media and warmed to 37°C . To test effects of varying concentration, IL-6 at concentrations of 1, 10, 100, and $1000\ \text{ng/ml}$ were added to the top side chamber and left for 12 h in the culture before the MMCs were fixed with $15\ \mu\text{l}$ of 10% neutral buffered formalin (NBF). Additionally, to study cell response over time, IL-6 at $100\ \text{ng/ml}$ was added to the top side chamber and left for 4, 8, 12, and 24 h. For co-culture experiments, BMSCs were first added to the side chambers of the microsystems (either in the top only, the bottom only, or both) and allowed to culture for 24 h. The “top only” and “bottom only” conditions were both included to provide a control for geometric variations in the device. After 24 h, MMCs were added to the center well and allowed to culture for an additional 24 h, after which cells were immediately fixed with 10% NBF.

Luminex multiplex ELISA assay

Co-culture experiments between RPMI8226 (i.e., MMCs) and HS-5 cells (i.e., BMSCs) were performed as described in the “Microfluidic cell culture” section, with one minor modification to the procedure for experiments involving media collection for ELISA. MMCs were allowed to settle for ~ 30 min, at which time media was aspirated from the outlet well only to minimize sample dilution when media samples were collected at the end of the experiment. Removing media from the outlet well was observed to not disturb MMCs. After 24 h of co-culture, a 2- μ l sample of media was collected from the top and bottom side channels, respectively, and a 5- μ l sample was collected from the center well. After collection, the media was stored at -80°C until the assay was to be performed. In preparation for the assay, samples were diluted 1:50 in fresh cell media. Soluble factors were quantified in the collected media using a 3-plex ELISA on the Luminex MAGPIX platform (Luminex Corporation, Austin, TX, USA). The 3-plex assay kit was used to measure the concentrations of IL-6, tumor necrosis factor alpha (TNF- α), and vascular endothelial growth factor (VEGF), and was executed following a standard procedure used for all multiplex assays.

Numerical simulation

A three-dimensional (3D) model of the fluid domain within the microsystem was created using Solidworks (Dassault Systèmes, MA, USA), and then exported into COMSOL Multiphysics® version 4.3 b (COMSOL, MA, USA) to create a discretized 3D model to numerically simulate diffusion within the microsystem. The model consisted of $\sim 6.0 \times 10^5$ free tetrahedral elements, and several model refinement methods (i.e., varying mesh properties, time intervals) were used to ensure model accuracy and proper convergence. Our molecule of interest, IL-6, has an estimated diffusion coefficient ranging from $D = 2.70 \times 10^{-11} \text{ m}^2/\text{s}$ to $1.46 \times 10^{-10} \text{ m}^2/\text{s}$, based on previously published values calculated using the Einstein-Stokes equation.^{20–22} Simulations were performed considering this range of diffusion coefficients (see “Statistical analysis” section for details).

Diffusion experiments

Devices were prepared as described in “Device preparation and cell seeding” up to the step where media was added. For diffusion experiments microsystems were flushed with deionized water giving the outlet reservoir a final volume of 30 μ l, and imaged prior to the start of an experiment to determine background intensity. 20-kDa FITC-Dextran (Sigma Aldrich, MO, USA) was reconstituted in deionized water at a concentration of 100 mg/ml, and aliquoted and stored at 4°C as stock. FITC-Dextran was serially diluted to 0.5 mg/ml and warmed to 37°C prior to being added to the microsystem. 6 μ l of 0.5 mg/ml solution of FITC-Dextran was added to the top side chamber of each system. Care was taken to ensure FITC-Dextran was only dispensed into the top side chambers, and that convection into the central well was minimized by limiting any unnecessary applied pressure to the micropipette during the dispensing step. Images were captured and analyzed at $t = 0, 4, 8$, and 12 h, and a standard curve for FITC-Dextran fluorescence intensity vs. concentration was generated using seven unique 20-kDa FITC-Dextran concentrations ranging from 0 mg/ml to 2.0 mg/ml.

Immunocytochemical staining

Mouse monoclonal IgG STAT3 antibody (124H6, #9139, Cell Signaling Technologies, MA, USA) was diluted at 1:400 in 3% BSA, and AlexaFluor-488 goat anti-mouse secondary antibody (A11001, Life Technologies, Thermo Fisher Scientific) was diluted at 1:500 in 3% BSA. Hoechst 33342 nuclear dye (Molecular Probes, CA, USA) was diluted at 1:1500 in 3% BSA. A standard procedure consistent with that previously reported by Young *et al.* was executed for fixation, permeabilization, blocking, and staining, consisting of sequential VRs of reagents passively pumped through system microchambers.¹⁴

Single cell image analysis

Fluorescence images were obtained with an EVOS[®] FL Auto Cell Imaging System (Thermo Fisher Scientific, MA, USA) with a 2× objective (LPlan, NA = 0.06, Thermo Fisher Scientific) for diffusion experiments with 20-kDa FITC-Dextran, and with a 20× objective (LPlanFL PH2, NA = 0.40) for all other experiments. All images were captured using a monochrome high sensitivity interline CCD camera attached onto the EVOS FL Auto System. Images were analyzed for nuclear translocation of STAT3 within single cells as described previously.¹⁴ Briefly, STAT3 and Hoechst-stained cell images were collected and processed (Fig. 2), and nuclear translocation in each cell within an image was determined by calculating the ratio of the average nuclear intensity per pixel over the average cytoplasmic intensity per pixel of STAT3, using the following equation:

$$IR = \frac{\bar{I}_n}{\bar{I}_c} = \frac{I_n/A_n}{I_c/A_c}, \quad (1)$$

where I_n is the total nuclear intensity, A_n is the nucleus area, I_c is the cytoplasm intensity, A_c is the cytoplasm area, $\bar{I}_n$ is the mean nuclear intensity, and $\bar{I}_c$ is the mean cytoplasmic intensity. The entire image analysis procedure was automated and performed using J'Experiment, a database management software package (J'Experiment or JeX: <http://jexperiment.wikidot.com>). IR values were then summarized as cell population distributions, either for the entire center well of MMCs or split into two distributions for the top half and bottom half of the center well, respectively (Fig. 2(a)).

Statistical analysis

For numerical studies, independent simulations were completed for each of the three diffusion coefficient values calculated. The results of these three simulations were combined, and the mean and standard error (SE) were calculated. The mean and SE were determined at various locations across the microsystem. For all cell-based experiments, experimental conditions were tested (at least) in duplicate per experiment, and all experiments were repeated three times ($n = 3$). For population distributions, the distributions represent the total combined cell populations from all duplicates and experiments for a given experimental condition (i.e., time and concentration). The shapes of all these combined distributions are representative of the individual distributions obtained for each independent experiment. Concentrations measured by the Luminex platform are presented for each of the three culture wells/chambers in a microsystem, and for each co-culture condition. Concentrations were averaged across 6 measurements that

FIG. 2. (a) Schematic of a typical asymmetric monoculture experiment. Exogenous IL-6 diluted in DMEM was added to the top side chamber and allowed to diffuse through the diffusion ports and into the center well over time. The center well containing MMCs was divided into a top half (red) and bottom half (blue) to compare STAT3 activation as a function of distance from an IL-6 source. (b) STAT3 and (c) Hoechst stains in MMCs were imaged across the entire center well of each system. Scale bars = 40 μm .

included 3 unique experiments ($n=3$) with duplicates for each test condition. Luminex ELISA data were statistically analyzed by ANOVA followed by Tukey's *post hoc* procedure to determine which populations were significantly different.

RESULTS

To characterize diffusion across the microsystem (Fig. 1(b)), numerical and experimental approaches were used, and their results were compared. First, a numerical model was created to simulate diffusion across the system to determine whether (a) three different soluble factor concentrations measured by ELISA correlate with predicted concentrations at different locations within the microsystem, and (b) the diffusion of a fluorescent molecule with a molecular weight similar to IL-6, 20-kDa FITC-Dextran, correlates with predicted concentration profiles across the center well. To verify these models, conditioned media collected from co-culture experiments was measured using a multiplex ELISA to determine concentrations of different relevant soluble factors in different culture compartments within the microsystem. Next, diffusion experiments were performed to characterize the diffusion of 20-kDa FITC-Dextran. Once numerical and experimental results were in agreement, cell culture experiments were performed to study IL-6-induced STAT3 nuclear translocation in MMCs across the center well. By quantifying STAT3 activation, we hoped to determine if the presence of a slight IL-6 concentration gradient across the center well resulted in a significant shift in STAT3 activation. Understanding diffusion characteristics to this degree will improve data interpretation and help guide improved device design and control.

Luminex assay and numerical results

Conditioned media from coculture experiments were collected to measure the concentrations of IL-6, TNF- α , and VEGF. IL-6 was selected due to its involvement in activating the STAT3 signaling pathway,¹⁸ TNF- α was selected because MMCs are known to secrete TNF- α leading to an increase in IL-6 secretion in BMSCs,²³ and VEGF was selected because it is also known to stimulate IL-6 production in BMSCs.²⁴ Overall, all three proteins are important known contributors to MMC survival and disease progression.

Figure 3 illustrates concentration measurements determined by the Luminex platform. Compartments containing BMSCs were expected to have the highest concentrations of soluble factors due to local secretion, lower compartment volume, and higher cell seeding density. Indeed, concentrations were found to be consistent with this expected outcome. In addition, there was a consistent trend where highest concentrations were always found in side chambers containing BMSCs, lowest concentrations were always found in the side chambers with no BMSCs (furthest away from the side chamber with BMSCs), and low to intermediate concentrations were found in the center well, as expected from the direction of the concentration gradient. Statistical analysis between all compartments for each co-culture condition showed expected trends and statistically significant differences. For example, in the coculture condition where both side chambers contained BMSCs, $p > 0.3$ when we compared concentrations of all three soluble factors between bottom chamber vs. top chamber (B vs. T) (Table I). Furthermore, in the coculture condition where only the top side chamber contained BMSCs, $p > 0.1$ when we compared concentration of all three soluble factors between the bottom chamber vs. the center well (B vs. CW), as expected. An exception was the result for TNF- α , with both side chambers containing BMSCs, where B vs. CW and CW vs. T showed $p = 0.06$, which followed our expected trend. Note that the monoculture condition served as negative control and led to soluble factor concentrations that were negligible.

Based on results from the 3-plex ELISA assays, we sought to understand in more detail the concentration profiles of IL-6 over an entire 24 h time period using computational methods. Numerical simulations in our 3D model were performed for a 24 h time period over which IL-6 diffusion was studied and concentration calculated. Representative symmetric and asymmetric coculture experiments were simulated, generating concentration profiles across the entire system. A constant IL-6 concentration of 475 ng/ml was chosen as a boundary condition in the

FIG. 3. Concentrations of soluble IL-6 (row 1), VEGF (row 2), and TNF- α (row 3) in conditioned media collected from the separate culture compartments (B, CW, T) for three different coculture configurations: (i) BMSCs in bottom side chamber only (column 1), (ii) BMSCs in both side chambers (column 2), and (iii) BMSCs in top side chamber only (column 3), as obtained by Luminex MAGPIX assay. B = bottom side chamber, CW = center well, T = top side chamber. Error bars represent standard error (SE) for three independent experiments ($n = 3$) for each configuration. Statistical significance at three levels (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was determined by ANOVA followed by Tukey's post-hoc procedure.

side chambers where BMSCs were cultured to simulate the scenario where secretion and uptake rates of IL-6 are balanced. The value of 475 ng/ml was chosen based on the Luminex results obtained above. Results showed that IL-6 concentration decreased by $\sim 60\%$ from one edge of the center well to the other in an asymmetric coculture configuration (Fig. 4). The most drastic change in concentration, however, was observed across the diffusion ports, where the concentration changed by at least one order of magnitude. The discrepancies observed between measured

TABLE I. P-values from Tukey's *post hoc* comparisons with $P > 0.05$.

BMSC culture location	Protein	Compartments compared	P-value
Bottom only	TNF- α	CW vs. T	0.16
	IL-6	B vs. T	0.99
	VEGF	B vs. T	0.32
Both	TNF- α	B vs. T	1.00
	TNF- α	B vs. CW	0.06
	TNF- α	CW vs. T	0.06
Top only	IL-6	B vs. CW	0.96
	VEGF	B vs. CW	0.51
	TNF- α	B vs. CW	0.10
	TNF- α	CW vs. T	0.36

FIG. 4. (a) Isometric view of microsystem modeled in COMSOL showing diffusion of IL-6. (b)–(d) Numerically predicted concentrations of IL-6 diffusion across the entire system after 24 h in (b) a symmetric culture configuration and (c) an asymmetric culture configuration. The graph in (d) illustrates a symmetric “U”-shaped curve for the symmetric configuration in (b) (red), and an asymmetric curve for the asymmetric configuration in (c) (blue), with sharp gradients at diffusion port locations. (e)–(g) Numerically predicted concentrations of IL-6, focused on the center well region between $x = 2.5$ and 5.5 mm. (e) Symmetric configuration and (f) asymmetric configuration. (g) Graphs for symmetric (red) and asymmetric (blue) configurations, respectively. Error bars represent standard error (SE) as discussed in Methods section.

and simulated IL-6 concentrations (Table II) were large because the numerical simulations represented ideal conditions that did not account for differences in IL-6 secretion and uptake rates, or pressure fluctuations during experimentation that could induce minor convection of soluble factors.

Diffusion experiments and numerical results

We performed diffusion tests with 20-kDa FITC Dextran over a 24-h period (as described in “Materials and Methods”) and compared the experimental diffusion profiles to numerical simulations. Experimental results (Fig. 5, blue lines) showed that Dextran concentrations were stable across the center well of the system over 12 h, and that the numerical simulation results (Fig. 5, red lines) were in close agreement with the experimental results up to 12 h, particularly in the center well region. Some discrepancy between experimental and numerical plots were observed in the top side chamber, where the experimental concentration decreased faster than the numerical prediction because of inherent transient effects at the initial dye loading stages of the experiments. Numerical results extended to 24 h also predicted that shallow concentration gradients were maintained across the center well (Fig. S1), confirming that minimal concentration bias existed in the device over time.

Based on these results and taking all factors discussed here into consideration, we expected MMCs in the center well to respond in a manner that reflected these shallow concentration profiles in the center well, with minimal changes in STAT3 activation from one edge of the well to the other.

MMC culture and STAT3 activation

Cell culture experiments in the microsystems were performed either with MMCs in monoculture exposed to exogenous IL-6 or with MMCs cocultured with neighboring BMSCs to study whether STAT3 activation of single cells in the MMC population was dependent on spatial location within the well. For all cell culture experiments, STAT3 activation was measured in all MMCs across the center well by calculating the IR value for each cell (see “Materials and Methods”). For monocultures, exogenous IL-6 was added to the top side chamber of a system (Fig. 2(a)) and allowed to diffuse through the diffusion ports and into the center well. IR distributions for both top-half and bottom-half cell populations within the center well were compared. Again, based on our numerical results, we expected top- and bottom-half populations to have very similar IR distributions because of the shallow concentration gradients across the center well.

First, we varied exogenous IL-6 concentration at 1, 10, 100, and 1000 ng/ml, and found that top-half and bottom-half cell populations had IR distributions that completely overlapped (Figs. 6(a)–6(e)), suggesting that there is no spatial bias between top and bottom halves of the well, even with increasing IL-6 concentration. When we overlapped the IR distributions for the different IL-6 concentration tests, we observed a clear shift of the distributions to the right with

TABLE II. Comparison of measured and simulated IL-6 concentrations in microsystems.

Asymmetric (BMSCs—Top)	Measured by Luminex (ng/ml)	Simulated by COMSOL (ng/ml)	Discrepancy (%)
Top chamber (T)	363.3	475.0	–30.8
Center well (CW)	27.5	38.5	–28.6
Bottom chamber (B)	15.6	3.4	78.2
Symmetric (BMSCs—Both)	Measured by Luminex (ng/ml)	Simulated by COMSOL (ng/ml)	Discrepancy (%)
Top chamber (T)	474.5	475.0	–0.1
Center well (CW)	132.6	77.1	41.9
Bottom chamber (B)	471.2	475	–0.8
Asymmetric (BMSCs—Bottom)	Measured by Luminex (ng/ml)	Simulated by COMSOL (ng/ml)	Discrepancy (%)
Top chamber (T)	14.5	3.4	76.6
Center well (CW)	163.0	38.5	76.3
Bottom chamber (B)	474.5	475.0	–0.1

FIG. 5. (a)–(c) Images captured at (a) 4 h, (b) 8 h, and (c) 12 h, from experiments examining diffusion of 20-kDa FITC-Dextran. White dashed lines show system geometry. Red dashed lines show regions analyzed for fluorescence intensity (scalebar = 600 μm). (d) Top view of system showing region where fluorescence intensity was analyzed from $y = 0$ to $y = 5.2$ mm, for both numerical (red) and experimental (blue) studies. (e) Plots of numerical (red) vs. experimental (blue) concentrations for $t = 0, 4, 8,$ and 12 h. (f) Top view of system showing region where fluorescence intensity is plotted in (g) from $y = 2.2$ to $y = 5.2$ mm, for both numerical (red) and experimental (blue) studies. (g) Plots of numerical (red) vs. experimental (blue) concentrations for $t = 0, 4, 8,$ and 12 h focused in the region from $y = 2.2$ to $y = 5.2$ mm. Note that the plots illustrate a shallow concentration gradient across the center well at all time points. Error bars represent standard error (SE) for $n = 3$ independent experiments.

increasing IL-6 concentration (Fig. 6(f)), indicating an increase in STAT3 activation, as expected. Interestingly, the shift that appeared to be largest was between the 100 ng/ml and 1000 ng/ml distributions. This is in agreement with previously published results suggesting that noticeable increases in MMC STAT3 activation are induced by IL-6 concentrations >40 ng/ml.^{25,26}

Second, we performed a timecourse experiment at 4, 8, 12, and 24 h where 100 ng/ml exogenous IL-6 treatments were applied to monocultured MMCs. Results showed that for all time-points, there was no difference between the top- and bottom-half IR distributions, as observed by their overlap (Figs. 7(a)–7(d)). When the combined IR distributions for the four timepoints were overlapped (Fig. 7(e)), the IR distributions shifted to the right with increasing time, indicating increasing STAT3 activation with time.

Third, we performed coculture experiments that involved four different culture configurations: (1) MMC monoculture as control (Fig. 8(a)), (2) MMCs in coculture with BMSCs in the top side chamber only (Fig. 8(b)), (3) MMCs in coculture with BMSCs in the bottom side chamber only (Fig. 8(c)), and (4) MMCs in coculture with BMSCs in both side chambers (Fig. 8(d)). Consistent with the first two cell culture experiments involving exogenous IL-6 and consistent with our experimental and numerical data showing shallow concentration gradients

across the center well, we observed top-half and bottom-half IR distributions that completely overlapped, indicating negligible differences in STAT3 activation between the cell populations in their respective halves.

DISCUSSION

Using a combination of numerical modeling and multiple experimental techniques, we showed that no spatial bias existed across the center well of the system during either co-culture conditions or treatment with exogenous factors. A 3D computational model was found to agree with experimental measurements for soluble factor concentration across the system. We experimentally confirmed these numerical predictions using different concentrations and co-culture conditions, and showed that over a 24-h period, the shallow concentration profile did not lead to spatial bias in STAT3 activation of MMCs in the center well. This is an important finding because it was previously unknown whether a significant concentration gradient would be present, how large such a gradient would be, or whether the gradient would lead to biased interpretation of single cell analysis performed across the cell population in the center well. The previous assumption was that a gradient would indeed be present given the experimental setup, and that cocultured BMSCs would secrete various factors that diffused inward toward the well. However, our results proved that with the current geometry, concentration gradients were very shallow ($\sim 3\%$ of the source concentration), and that there is no spatial bias when analyzing single cell IR values in the cell population. This outcome validated the appropriateness of the single cell nuclear translocation assay used in previous studies.¹⁴

The numerical model developed as part of this study has not only provided a theoretical prediction for IL-6 diffusion across our specific system but also has provided a useful tool to study changes to our system geometry, evaluate future design modifications, and predict biomolecular diffusion of other important soluble factors of interest. To demonstrate the usefulness of our numerical model, we studied several modified geometries with the model to explore changes to the system, and its effects on soluble factor diffusion (see [supplementary material](#)). We examined the effects of increasing and decreasing the cross-sectional area of the diffusion ports and studied several other soluble factors besides IL-6. Three different cross sections were compared: (i) the current diffusion port cross section design (100 μm height, 20 μm width), (ii) a cross section with double the current area (100 μm height, 40 μm width), and (iii) a cross section that is half the current area (100 μm height, 10 μm width). As cross-sectional area increased, the flux of IL-6 diffusing through the ports also increased, as expected. However, we also found that the concentration gradient across the center well increased with increasing diffusion port cross-sectional area, suggesting that cross-sectional area must be properly constrained to ensure the gradient across the center well does not increase beyond the point where the concentration across the center well may lead to a spatial bias under single cell analysis.

Next, diffusion of several different soluble factors were studied, including IL-6, IL-8, and VEGF, to span a range of molecular weights and to focus on factors associated with MM.²⁷ Diffusion coefficients used in the model were estimated for each soluble factor using the Stokes-Einstein equation, resulting in the following values: IL-6 $\approx 1 \times 10^{-10} \text{ m}^2/\text{s}$,^{20–22} IL-8 $\approx 1.6 \times 10^{-10} \text{ m}^2/\text{s}$,²⁸ and VEGF $\approx 0.75 \times 10^{-10} \text{ m}^2/\text{s}$.²⁹ We found that as the diffusion coefficient increased, soluble factor diffusion increased, as expected. We also found that for soluble factors with lower molecular weights (and thus higher diffusion coefficients), the gradient observed across the center well was much lower in magnitude than for soluble factors with higher molecular weights (and thus lower diffusion coefficients) (see [supplementary material](#)). This result again suggests that care must be taken when designing diffusion ports for coculture studies involving the biomolecular transport of high molecular-weight species. An improperly sized diffusion port coupled with a slower diffusing soluble factor of interest may lead to biased single cell data across the center well.

Interestingly, soluble factor concentrations measured in the current study from cocultured microsystems were found to be higher than previously reported values obtained from cocultures in Transwell inserts, particularly for IL-6 and TNF- α (Table III). This increase in soluble factor

FIG. 6. Single cell analyses of STAT3 activation in MMCs, triggered by addition of exogenous IL-6 to the top side chamber at various concentrations over 12 h. (a) No treatment, (b) 1.0 ng/ml, (c) 10 ng/ml, (d) 100 ng/ml, and (e) 1000 ng/ml. (a)–(e) For all concentrations, population distributions of IR values for MMCs in the top half (red distribution) overlapped the IR distributions for MMCs in the bottom half (blue distribution), with no shift in STAT3 activation, suggesting cells in the well were exposed to IL-6 in a manner leading to negligible differences in cellular response. (f) Total population distributions of IR values (top and bottom distributions combined) for all tested concentrations, showing that IR distributions shifted to the right with increasing IL-6 concentration. All distributions shown represent cumulative cell responses across 3 independent experiments ($n = 3$). Each independent experiment showed similar distributions and shifts as the set of cumulative distributions shown in (f). (RF = relative frequency; IR = intensity ratio).

concentration (i.e., two orders of magnitude larger) observed in our microsystems was previously discussed in other studies involving microfluidic devices³⁰ and was largely attributed to lower culture volumes, and higher cell-to-volume ratios, leading to soluble factors becoming more concentrated in microsystems than in conventional Transwell inserts.

Concentration changes across the system were observed to occur most drastically across the diffusion ports regardless of coculture configuration. The diffusion ports create a “bottleneck,” limiting the diffusive flux of soluble factors, and lowering the concentration in the center well by an order of magnitude. Despite such a significant drop in concentration, the IL-6 concentration present in the center well was within the range where changes in STAT3 nuclear translocation could be detected. Other studies have shown that detectable changes in STAT3 nuclear translocation were observed when IL-6 concentrations were on the order of ~ 10 – 100 ng/ml.^{25,26}

Young *et al.* previously observed “shoulders” in their IR distributions to the left of the main peak,¹⁴ and it was suggested that these features represented signs of bimodal distributions,

FIG. 7. Single cell analyses of STAT3 activation in MMCs, triggered by addition of exogenous IL-6 to the top side chamber at 100 ng/ml measured at different timepoints: (a) 4 h, (b) 8 h, (c) 12 h, and (d) 24 h. (a)–(d) For all time points, population distributions of IR values for MMCs in the top half (red distribution) overlapped the IR distributions for MMCs in the bottom half (blue distribution), with no shift in STAT3 activation. (e) Total population distributions of IR values (top and bottom halves combined) for all time points, showing that IR distributions shifted to the right over time. All distributions shown represent cumulative cell response across 3 independent experiments ($n = 3$). Each independent experiment showed similar distributions and shifts as the set of cumulative distributions shown in (e). (RF = relative frequency; IR = intensity ratio).

although the source of this bimodality was not determined. In this current study, the presence of two peaks is much more distinct. Preliminary observations suggest that the left peak is composed of primarily apoptotic cells (seen as cells containing irregularly shaped nuclei characterized by chromatin condensation), although further analysis is required to confirm this hypothesis. Because this subset of cells comprises $\sim 10\%$ – 20% of the entire population, traditional cell-averaged assays (i.e., electrophoretic mobility shift assays) would completely mask the presence of the subpopulation and lower the reported STAT3 activation.

The microscale coculture system studied here has a wide range of potential applications. By providing a platform with potential to create a more complex cellular microenvironment with improved physiological relevance, the system offers an advance over conventional Transwell inserts. The system can help further our knowledge of MM pathobiology by enabling

FIG. 8. Single cell analyses of STAT3 activation in MMCs induced by cocultured BMSCs in neighboring side chambers with different configurations: (a) no BMSCs, (b) BMSCs in top side chamber only, (c) BMSCs in bottom side chamber only, and (d) BMSCs in both side chambers. All culture configurations showed overlapping top and bottom half IR distributions, suggesting no spatial bias. All three coculture configurations (b)–(d) showed a rightward shift of the second peak, from peak IR = 1.60 for monoculture to peak IR = 1.88 for all cocultures. All distributions shown represent cumulative cell response across 3 independent experiments ($n = 3$). Each independent experiment showed similar distributions and shifts as the set of cumulative distributions. (RF = relative frequency; IR = intensity ratio).

TABLE III. Comparison of measured protein concentrations in current and other studies.

Protein	Current study (ng/ml)	Other studies (ng/ml)	References
IL-6	350–450	0.5, 1.7	31–33
VEGF	2–4	1.5	31
TNF- α	0.13–0.15	0.003	33

studies on the communication between multiple cell types within the MM bone marrow micro-environment, which is not well understood, but critical to understanding disease progression.^{15,34} The system could also aid in the pre-clinical evaluation of new MM therapies, or be used as a chemo-sensitivity resistance assay (CSRA) device to identify the most appropriate treatment for individual MM patients, as demonstrated recently.¹⁹ The current study provides strong evidence that soluble factor concentration is nearly uniform across the center well, verifying the integrity of the single-cell data obtained from this system, and further validating the usefulness of the platform as a low-cost, high-throughput tool for advancing MM biology research and drug treatment selection for personalized medicine applications.

CONCLUSIONS

In summary, we employed both experimental and computational approaches to characterize concentration profiles within cell culture compartments of a model microfluidic device, and correlated functional response of cultured cells (via single-cell nuclear translocation measurements) to the concentration patterns found within the device. We found shallow concentration gradients

within the center well that matched the response of the population distributions within the well. In the process, we developed a 3D numerical model to predict biomolecular diffusion and concentration gradients within the microfluidic device and found that the model was in good agreement with experimental diffusion of a model Dextran molecule. The model can be used as a tool for developing future microfluidic cell culture systems, particularly those for studying communication between multiple cell types, as well as for evaluating the treatment response of clinical samples.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for additional data on Dextran calibration curves, numerical modeling results, and guidelines for choosing microfluidic geometry dimensions.

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