MICROFLUIDIC SUSPENSION CELL CULTURE PLATFORM FOR STUDYING POPULATION HETEROGENEITY IN NF-KB SIGNALING

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ABSTRACT

We describe a user-friendly scalable microfluidic cell culture system that can be combined with advanced image analysis to yield an accessible technique for studying single-cell nuclear translocation events in small cell populations (~10^3 cells). The system enables complete culture, treatment, staining, and image analysis of adherent and suspension cells – all on the same platform – while only requiring a micropipette and fluorescence microscope. We demonstrate that the system can be applied to investigate NF-κB signaling in hematologic cancer cells, and examine population heterogeneity in a manner not achievable with conventional assays such as electrophoretic mobility shift assays (EMSAs).

KEYWORDS

Microfluidics, cell culture, nuclear translocation, assay, immunostaining, NF-κB, cell heterogeneity, blood, cancer

INTRODUCTION

Progress in translational cancer research is currently hindered by a lack of practical tools for studying cell function and signaling mechanisms associated with chemotherapy and drug resistance. Conventional assays for studying signal transduction events (e.g., electrophoretic mobility shift assays, or EMSAs) require large quantities of cell sample per condition (>10^5 cells), but provide only population-averaged data with no information on single cells or on heterogeneity within a cell population. Furthermore, conventional in vitro cell-based assays often do not accurately recapitulate complex in vivo microenvironments [1]. Thus, there is a critical need for new and innovative methods to study signaling mechanisms of small cell populations in more physiologically relevant microenvironments, while analyzing single cells to achieve deeper insight on population heterogeneity [2].

We have developed a microfluidic cell culture system that enables complete culture, treatment, staining, and image analysis of adherent and suspension cells in a single all-in-one platform, while only requiring a micropipette and fluorescence microscope (Figure 1). The microfluidic system uses passive pumping, which relies on surface tension of dispensed droplets to move fluids, obviating the need for external pumps and tubing. In previous work, we described the design and fabrication of the microdevice and validated the platform by examining cell viability and NF-κB nuclear translocation of a human multiple myeloma (MM) cell line (RPMI8226) treated with varying concentrations of cytokines and drugs [3]. Here, we describe for the first time the application of the technology to investigate NF-κB signaling events in mixed cell populations (RPMI8226 cells and a B-cell lymphoblast cell line, 70Z). The ability to analyze nuclear translocation of NF-κB at a single cell level enables examination of population heterogeneity, with potential to elucidate effects from subpopulations within the whole population. In addition, the microscale dimensions enables studies of samples with low cell numbers, such as those obtained from clinical patients.

MATERIALS AND METHODS

Microchamber arrays were fabricated in poly(dimethylsiloxane) (PDMS) by multilayer soft lithography methods [3,4] and bonded to 3" x 2" glass slides (Figure 1). Three different populations were tested to validate our assay for its ability to measure population heterogeneities: (1) 100% CD138+ RPMI8226s, (2) 100% CD138- 70Zs, and (3) a mixed population of 50% RPMI8226s and 50% 70Zs.

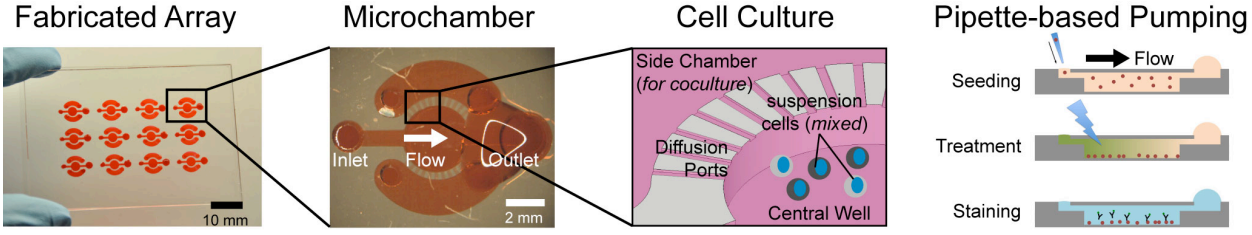

Figure 1. Microfluidic suspension cell culture system, where each fabricated microdevice consists of an array of independent microchambers. Suspension cells are cultured in the central well, and are connected via diffusion ports to side chambers for co-culture with other cell types. Passive pumping of dispensed droplets from inlet to outlet is used for cell seeding, drug or cytokine treatment, and immunocytochemical staining.

RPMI8226s were cultured and maintained in high-glucose DMEM containing 10% FBS and 1% penicillin/streptomycin (P/S), while 70Zs were cultured and maintained in RPMI-1640 containing 10% FBS, 1% P/S, and 50 μM β-mercaptoethanol. Microchambers containing mixed populations contained a 1:1 mix of the two media types. Cells were seeded and cultured at ~5,000 cells per microchamber, treated with 10 ng/mL TNF-α for 20 min (or not treated for control), fixed and stained with Hoechst 33342 nuclear dye, rabbit polyclonal antibodies for RelA (subunit of NF-κB, Santa Cruz, sc-372, 1:200) bound to goat anti-rabbit AlexaFluor 488 (Invitrogen, A11029, 1:400), and mouse monoclonal antibodies for CD138 (RPMI8226 cell surface marker, Santa Cruz, sc-65337, 1:50) bound to goat anti-mouse Texas Red (Invitrogen T-862, 1:400). Images of all stains (blue=nucleus; green=RelA; red=CD138) were acquired with an inverted fluorescence microscope (Nikon Ti Eclipse), and analyzed using custom ImageJ algorithms. CD138 images were used to identify cells as either RPMI8226 or 70Z. Hoechst and RelA images were used to produce a binary cytoplasmic mask that was then used to determine fluorescence intensities in the cytoplasm and nucleus of each cell. The ratio of nuclear and cytoplasmic signals (i.e., intensity ratio, IR) for each cell was calculated, and IR values across whole populations were analyzed to examine response of each population.

RESULTS AND DISCUSSION

The three different cell populations (pure RPMI8226s, pure 70Zs, and mixed) were cultured, treated with TNF-α, stained (Figure 2A-F), and analyzed for nuclear translocation of NF-κB. CD138 was found to be an appropriate cell surface marker for distinguishing between RPMI8226s and70Zs. (Figure 2C-D). Using the Hoechst and RelA images, individual IR values for the different experimental populations were calculated, and the results were analyzed as IR distributions (Figure 2G). The pure population of RPMI8226s (red bars) responded via RelA nuclear translocation to 20 min treatment of 10 ng/mL TNF-α, as seen in a shift of the distribution to higher IR values. The pure population of 70Zs (gray bars), as expected, did not respond, as seen in the similarity between IR distributions of the treated and untreated populations. Importantly, the mixed population (green bars) showed a partial response where a fraction of the population appeared to shift to higher IR values, as seen by the subtle bimodality of the distribution for the treated mixed population. When the population averages of the IR distributions were calculated and plotted (Figure 2H), the average IR of the TNF-α–treated mixed population was found to be clearly higher than the average IR of the pure 70Z population, and lower than the average IR of the pure RPMI8226 population. Thus, a population-averaged readout would correctly reveal the partial response of the mixed population, but would mask the bimodality of the distribution that suggests a role for population heterogeneity.

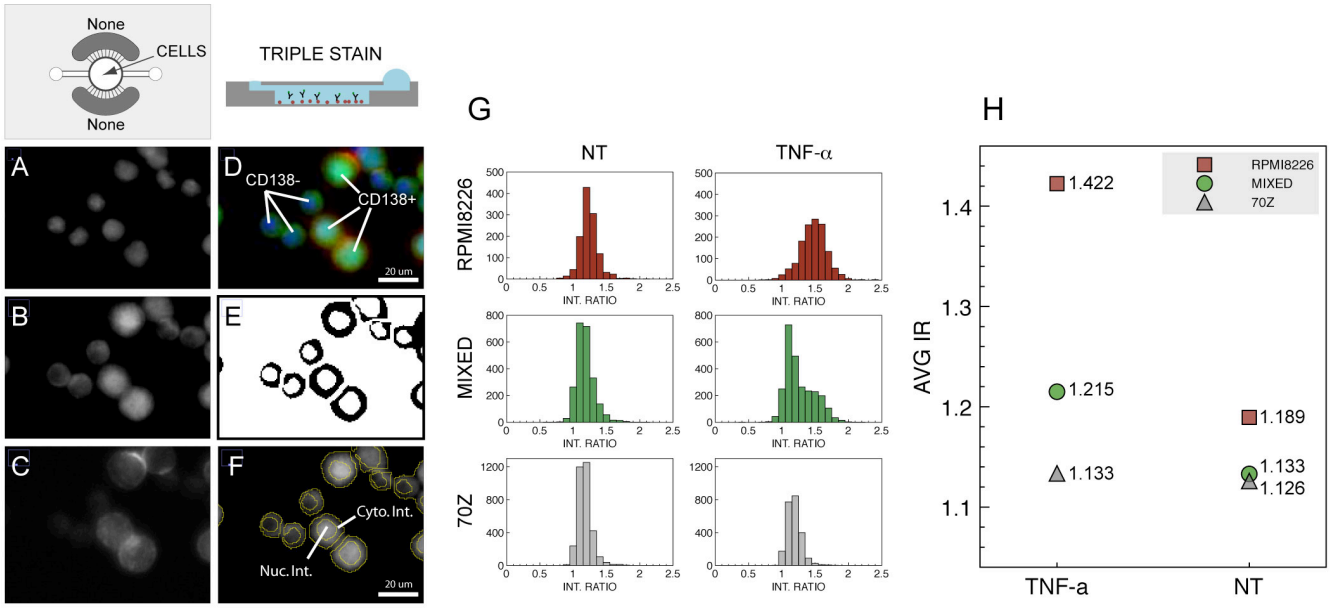

Figure 2. Triple immunostaining of cell populations for (A) Hoechst, (B) RelA, and (C) CD138 (images from mixed population). (D) Overlay of triple stain shows that CD138 is an appropriate cell surface marker for distinguishing 70Zs and RPMI8226s. (E) A binary cytoplasmic mask can be generated by subtracting the nuclear image from the RelA image, revealing a cytoplasmic region (black ring) and a nuclear region (white hole) for each cell. (F) The cytoplasmic mask (yellow outlines) can be laid over the original RelA image to divide the signal into cytoplasmic and nuclear fractions. (G) IR distributions for different treated and untreated cell populations. (H) Average IR values of the distribution in (G).

The CD138 image was used to distinguish between RPMI8226s and 70Zs in the mixed populations to measure their independent responses to TNF-α treatment (Figure 3). The subpopulation identified as CD138+ RPMI8226 cells responded to TNF-α (blue distributions) in a similar manner to the pure RPMI8226 population, while the subpopulation identified as CD138- 70Z cells did not respond to TNF-α (yellow distributions), as expected. When the distributions were overlapped, it was apparent that the bimodality seen in the total mixed population was attributable to the CD138+ population alone. Thus, the CD138 cell surface marker was important to separate the effects of different subpopulations.

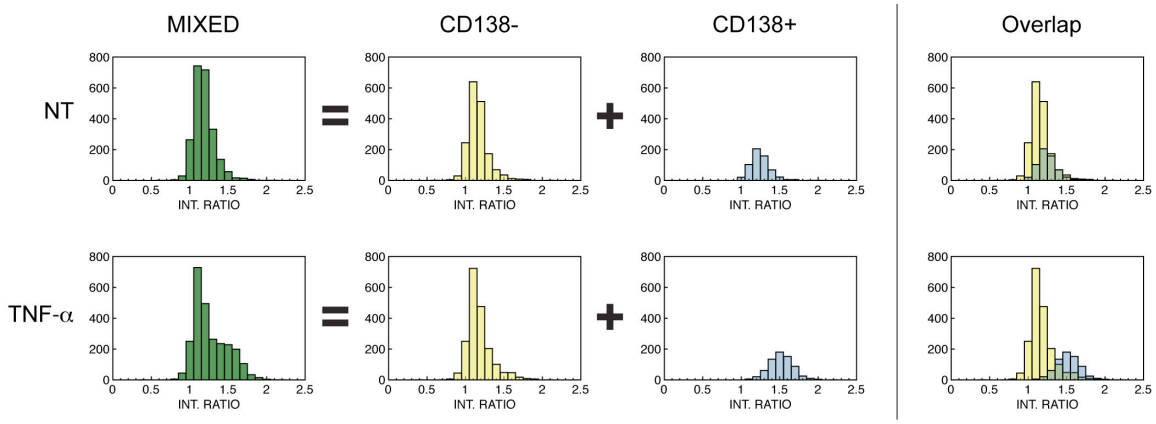

Figure 3. Treated and untreated mixed populations of RPMI8226s and 70Zs (green, from Figure 2G) analyzed with CD138 image to distinguish between the two cell types. CD138- distributions (yellow) represented 70Zs only, with no response to TNF-α. CD138+ distributions (blue) represented RPMI8226s only, with significant increase in IR value in response to TNF-α, as seen in the shift of the distribution to higher IR values. Overlapping the distributions showed that the bimodality of the total population was primarily due to the CD138+ population.

Because the microfluidic system requires only a micropipette and a fluorescence microscope for operation, it is immediately accessible to most biology research laboratories without the need for additional equipment or infrastructure. The system is scalable to larger arrays, which can be interfaced with automated liquid handlers if desired [5]. The system can be used to study cell-cell communication between adherent and suspension cells in coculture by culturing neighboring cell types in the side chambers [3]. Furthermore, because the system is designed for culture of small populations of cells, it is appropriate for examining limited samples such as those acquired from clinical patients. Currently, the system is being applied to study cell viability and NF-κB signaling of primary patient MM samples upon treatment with various drugs, and to examine nuclear translocation of NF-κB essential modulator (NEMO) induced by a DNA damaging agent (etoposide) used in chemotherapy. We envision this platform being useful for myriad cell biological questions, but particularly useful in translational research of hematologic cancers for examining molecular signaling and population heterogeneity in primary samples with extremely low cell numbers.

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