

Application Note

A comprehensive workflow for screening and real-time analysis of cell migration

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Introduction

Cell migration is directed by the interaction of cells with the extracellular matrix (ECM), neighboring cells, and biomolecules present in the milieu. Cell migration contributes to both normal physiological events and pathological aberrations. Understanding the mechanisms underlying migration is essential to the development of a variety of therapeutics including those for wound healing, tumor metastasis and augmentation of immune responses. Therefore, techniques for the quantification and dynamic visualization of migrating cells have become central to life science research.

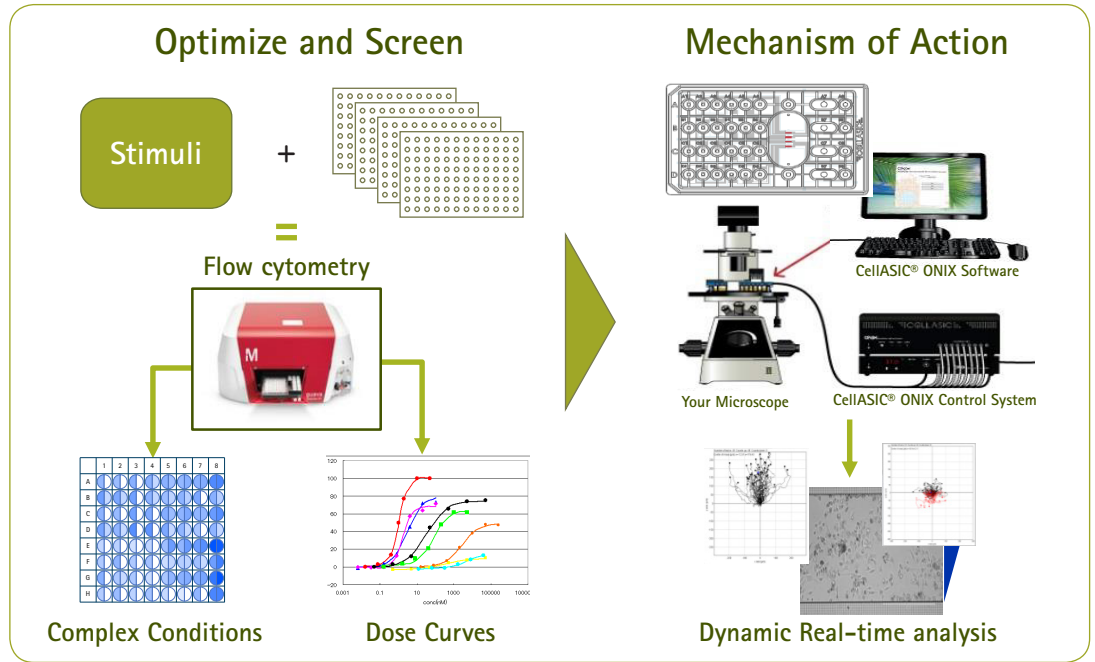
The most widely accepted method for analyzing cell migration is the Boyden chamber assay. While amenable to semi-quantitative "screening" assays, these systems suffer from the inability to establish continuous gradients; in fact, very steep gradients initially form along a single axis perpendicular to the membrane, but ultimately, due to the lack of active pressure, simple equilibrium diffusion results in the establishment of lower than expected differences in concentration between the two chambers. As a result, this method is not well-suited for correlating specific cell responses with particular gradient characteristics (i.e. slope, concentration, temporal development/change) or for studying multi-gradient

signal integration. Moreover, such gradients cannot be stably maintained under static culturing conditions, precluding longer-term real-time cell analysis. Lastly, these platforms are not strictly designed for microscope-based visualization and thus cannot be employed for dynamic mechanistic studies through imaging analysis at the inter- and intra-cellular levels.

This application note describes a two-tiered platform for migration analysis of cell populations present in peripheral blood (Figure 1). Cell culture insert plates (96-well capacity), when paired with flow cytometry, provide the ideal tools for screening functional responses (migration) and toxicity in heterogeneous samples. For selected compounds, mechanistic studies were then performed using a microfluidic-based system. The platform consists of a microfluidic culture plate and environmental control system; the latter regulates media perfusion, the establishment of stable continuous concentration gradients within the plate, as well as temperature and gas control. Most significantly, the plate can be paired with an inverted microscope enabling dynamic analysis of cell migration in real-time. Fluorescent visualization further permits selective discrimination of unique cell types in heterogeneous samples as well as changes in expression patterns of labeled proteins.

Figure 1:

The combined workflow platform (Figure 1) provides a framework for studying the effects of cytokines, growth factors and other secretory molecules on the migratory propensities of cells in tumors, immune responses, and other biological systems defined by active cell movement.



Methods

Reagents

Viability was determined using the ViaCount® reagent (MilliporeSigma). Cell frequencies were determined by flow cytometry using the following monoclonal antibodies: anti-human CD3- FITC, CD4-PE, CD-PECy7, CD19-APC, CD14-APCeFluor780, CD45RA-APC, and CD45RO-APCeFluor780 (Affymetrix). All antibodies were titrated prior to use. All stimuli (IL2, IL4, IL6, TNF α , IFN γ , MIP1 β) were purchased from Affymetrix.

Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized healthy blood by Ficoll density centrifugation. CD8+ and CD19+ cells were purified using a CELLlection™ biotin binder kit (Thermo Fisher) according to the user guide. Yield, purity (relative content), and viability were determined by flow cytometry; positive fractions were >95% pure. For certain CellIASIC® ONIX experiments, positive fractions were labeled with CellTracker™ Green (CD19+) or Deep Red dyes (CD8+) (Thermo Fisher) according to the reagent user guides.

Millicell® Migration Assays

Millicell® migration assays were performed in 96-well inserts containing 5 μ m polycarbonate membranes (Figure 3). A total of 200,000 live PBMCs were seeded per insert; cytokines were dispensed into basolateral wells. Assays were carried out for various times; cells were then harvested from both compartments and assayed for viability (ViaCount® Reagent) or lineage-specific migration (anti-CD antibodies). Samples were acquired on a Guava® easyCyte cytometers and analyzed via InCyte™ software.

Migration Assays Using the CellIASIC® ONIX system

The M04G plate is a 4-chamber device designed for use with the CellIASIC® ONIX microfluidic system for perfusion-based, dynamic live imaging analysis (Figure 2). In addition to media perfusion, the system also regulates temperature and gas control for stable, long-term cell culture. Each chamber has four switchable upstream solution channels permitting formation of stable spatial gradients. The chamber is bracketed by perfusion barriers; gradients are established by simultaneously flowing media of different compositions through upper and lower channels.

For these experiments, all steps (chamber priming, cell loading, media perfusion, and gradient formation) were performed according to the user guide.

Images

Images were acquired using the EVOS-FL Cell Imaging System (ThermoFisher) at 4X magnification using bright field, FITC, and Texas Red filters. Time-lapse imaging was performed during gradient application; images were captured at 10 minute intervals. Images were analyzed using ImageJ software in combination with Manual Tracking and Chemotaxis tools (NIH). In each case, 50 representative cells were tracked over 18 time points.

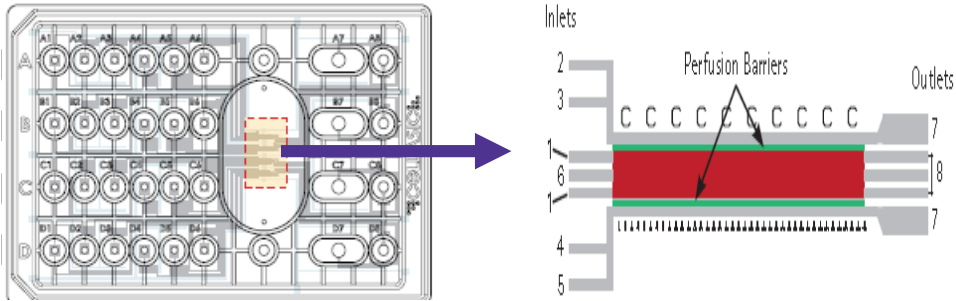


Figure 2: Schematic of the CellASIC® ONIX M04G microfluidic gradient plate. (A) Plate layout with the four individual cell chambers highlighted. (B) Close-up view of a single cell chamber.

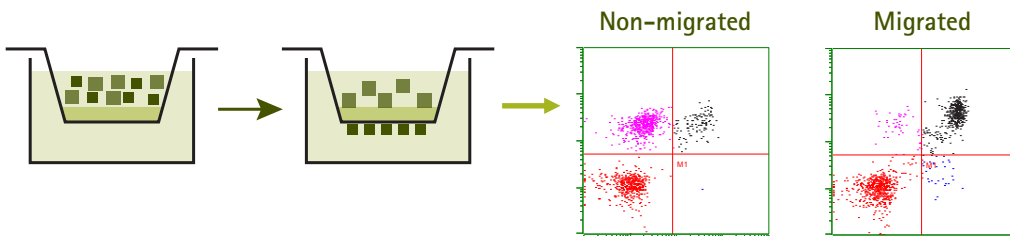


Figure 3: For migration assays, PBMCs were seeded in the upper chamber of Millicell® insert devices. Following exposure, % migration for each subset was determined by multidimensional flow cytometric analysis of cells harvested from the insert (un-migrated) and basolateral (migrated) compartments.

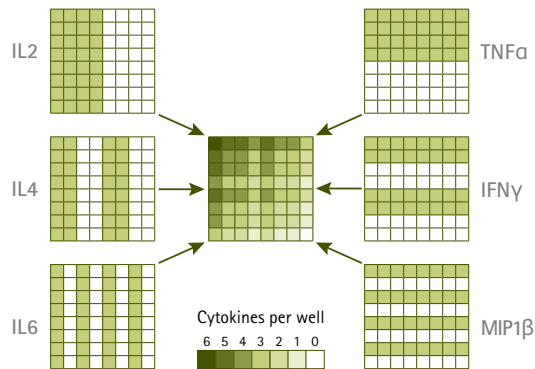


Figure 4: PBMCs were pre-cultured in serum-free media for 24 hours prior to assays; for migration, the basolateral wells contained all combinations of six cytokines (64 wells in total per assay). All white squares denote absence of the particular cytokine. The center square reflects the total contents of the 64 wells; the darkness of color representing the number of different cytokines added to the well.

Results: Optimization and Screening

Culture Conditions – % Viability

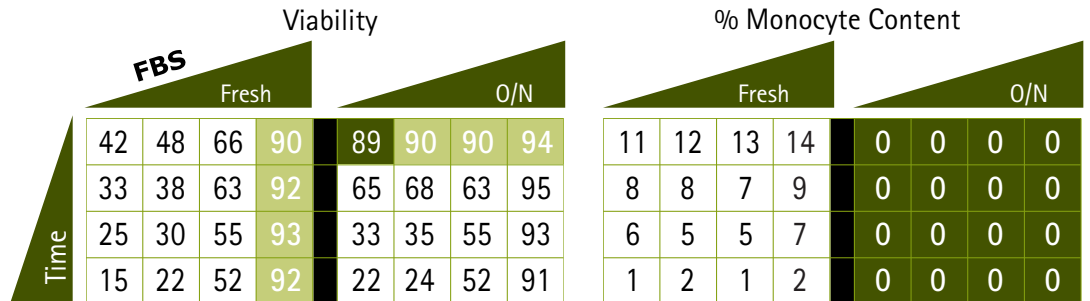
Freshly isolated PBMCs provide a number of challenges when cell-based functional assays are the desired readout. They can be variably fragile (depending on the elapsed time between blood draw and processing) and contain a significant monocyte/granulocyte fraction. Three condition sets (fresh vs. overnight pre-culture; media FBS content, culture duration) were analyzed for viability (using ViaCount® Reagent, which differentially stains viable and non-viable cells) and monocyte content (CD14 Ab). Results shown in Figure 5 represent an average of three independent blood samples (different draws and days). Fresh PBMCs were extremely sensitive to fetal bovine serum-content independent of culture time. Also, overnight culture was required to eliminate monocytes through adherence but also improved viability in low serum for short term cultures (<6 hours). Subsequent migration experiments were carried out for six hours under serum-free conditions following overnight culturing.

Cytokine Dose Response – % Migration

Using the culture conditions outlined above, cytokine titration experiments were performed to identify concentrations that elicited migration across the 5 µm pore membrane of the Millicell® 96 culture inserts. Following exposure, cells were harvested from both compartments, stained for surface markers, and analyzed by flow cytometry.

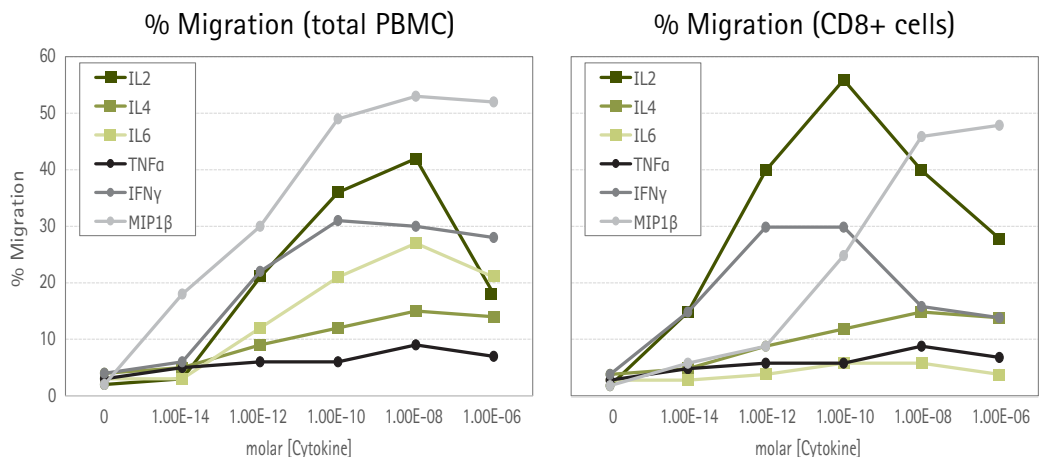
Figure 6 summarizes the results. Each cytokine elicited a unique response with regard to concentration dependence and cell type influenced. Specifically, IL2 and MIP1β had more and less pronounced effects, respectively, on the migratory propensity of CD8+ cells than the general PBMC population. At high concentrations, certain cytokines were refractory and promoted cell death. It should also be noted that there was baseline cell movement due to the fact these cells were grown in suspension.

Figure 5: Viability (% live) and monocyte content (% CD14+ cells) of PBMC cultures grown under different conditions.



Starting CD14+ fraction was 16%

Figure 6: Migration patterns of PBMCs in response to cytokine dose (dose range = 10⁻⁶ – 10⁻¹⁴ M). The graphs depict % migration for total PBMCs and CD8+ cells.



Multi-cytokine Grid – Heterogeneity in Response

During immune responses, multiple and varying combinations of cytokines are present at the regional lymph node and sites of infection. Distinct cytokine combinations induce changes in cell phenotype and function that differ dramatically from the effects exerted by each cytokine alone. Culturing strategies such as the cytokine grid enable simplified interrogation of this complex biological process (see Figure 4). Addition of multi-color flow cytometry further permits analysis in heterogeneous samples.

InCyte™ software was designed specifically to expedite assessment of plate level flow cytometry data sets through parameter-specific heat-maps.

From this limited study, it is apparent that T cell migration was strongly influenced by IL2. IL2+ wells (Purple) showed more pronounced movement of CD8+ cells (Figure 7A). By contrast, both cell types demonstrated a dual

responsiveness to MIP1β (Pink). This bifunctional effect was more pronounced for CD8 cells (refer to Figure 4 for well-to-well cytokine content).

Independent regulation of naïve (CD45RA+) and memory (CD45RO+) CD4 T cell migration is shown in Figure 7B. Within each well, the percentage of RO+ and RA+ cells is compared across all wells. Certain combinations (Purple) resulted in equivalent response. In contrast, two combinations, IL2+/TNFα- (Pink) and IL2-/IL4- /IL6- (Blue), favor differentiation stage-specific migration.

In summary, demonstrations of synergy, dominance, and substitution amongst multiple cytokines in our experimental system may reflect actual paradigms underlying such complex processes as the T cell homing and maturation.

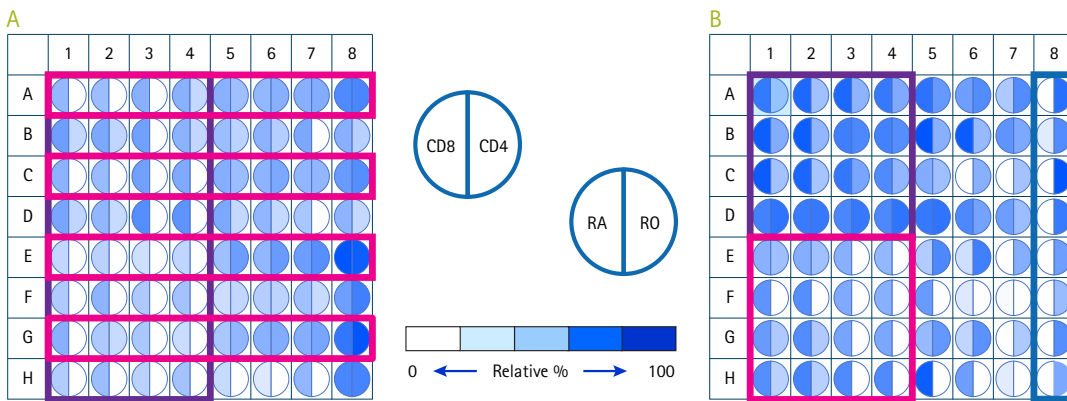


Figure 7: Guava® InCyte™ plate-based flow cytometry data analysis using InCyte™ software. Within each well of the InCyte™ plate-maps, the % migrated for CD8 and CD4 cells (left and right half of well, respectively) (A) or CD45RA and CD45RO (B) is compared across all 64 wells of the plate (cytokine grid) and relative values are displayed as gradations of blue color (see color bar).

Functional studies

Multicolor Fluorescent Labeling and Dynamic Live Cell Imaging

Imaging cells in culture in real time enables far greater interrogation of the mechanisms underlying physiological processes. Through addition of fluorescent "tags," specific sub-fractions can be analyzed in the context of their native heterogeneous cell mixture. This allows for more *in vivo*-like studies as potential cross-talk between cell types is retained. The schematic depicted in Figure 7 outlines the specific isolation and fluorescent labeling of CD8+ T (Red) and CD19+ B (Green) cells from human blood. Shown below are images derived from a single CellASIC® ONIX gradient plate chamber containing both labeled cell types. With the particular dyes chosen for these experiments, fluorescent labeling was stable for up to 48 hours.

Gradient Formation and Migration Analysis

The aim of this work was to demonstrate the combined utility of the CellASIC® ONIX Microfluidic System and

M04G Microfluidic Plates for real-time live cell analysis during exquisitely controlled cell migration studies. The M04G plate is designed to maintain stable diffusion gradient in cell culture. Continuous flow between an infinite source and infinite sink acts to preserve the established profile for days. The content and direction (UP/DOWN) with respect to the chamber are tightly regulated by perfusion controls.

Using ImageJ software in this experiment, we tracked the migratory properties of individual PBMCs exposed to an IL2 gradient. In each case, 50 cells were monitored for a total of 6 hours; the results are presented in the X/Y-plots of Figure 9B. CD8+ cells showed a strongly directed movement within the established gradient; by contrast, the gradient had little impact on the directionality of CD19+ B cell movement. While not shown here, the open source software module can also be used to derive additional chemotactic parameters such as directionality and velocity.

Figure 8:

(A) Workflow schematic for bead-based isolation and fluorescent labeling of unique PBMC subsets. (B) Images were derived from overnight culturing in the CellASIC® ONIX system of a PBMC sample reconstituted with labeled subsets. Representative Brightfield (Total), Texas Red (CD8-specific), and FITC (CD19-specific) filtered images are displayed (4X Mag).

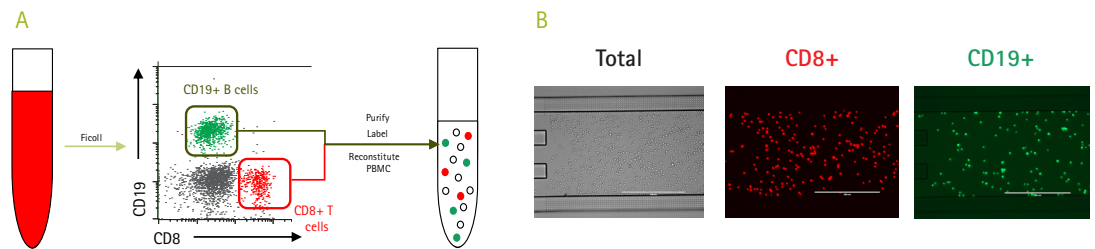
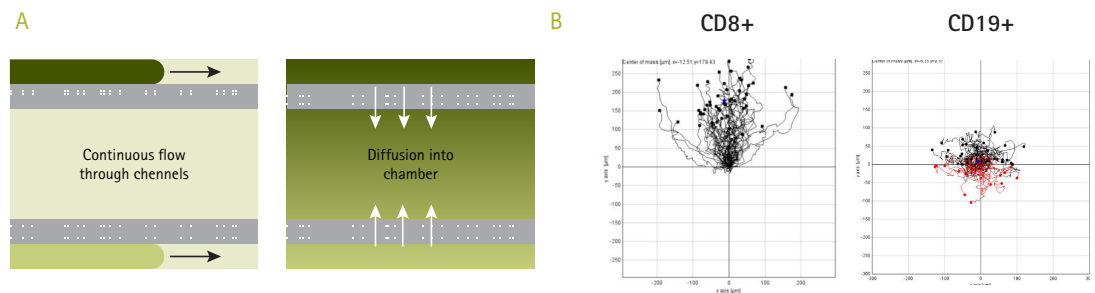


Figure 9:

(A) Spatial gradients are formed by flowing through the upper and lower inlet channels simultaneously. While flowing through the channels, solutions will diffuse across the barriers and into the chamber. The gradient profile will be linear in the y-axis with no variation in the x-axis and will be stable for 48 hours. (B) X/Y migration plots highlight the impact of an IL2 gradient on CD8+ and CD19+ cell movement. Black and red lines specify cells that possessed a net upward or downward movement relative to the Y-axis.



Conclusions

- Millicell® culture inserts provide an ideal tool for screening and culture optimization upstream of performing CellASIC® ONIX platform experiments.
- Multicolor flow analysis using a Guava® easyCyte HT cytometer greatly expands on the information content derived from migration assays particularly for complex samples such as PBMC.
- InCyte™ software provides simplified plate-based analysis of flow data for screening applications.
- The CellASIC® ONIX culture system that pairs precise and stable gradient control with live cell imaging enables the interrogation of the cellular and molecular mechanisms underlying processes such as chemotaxis.



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