

High Content Analysis of Neutrophil Phenotype During Chemotaxis



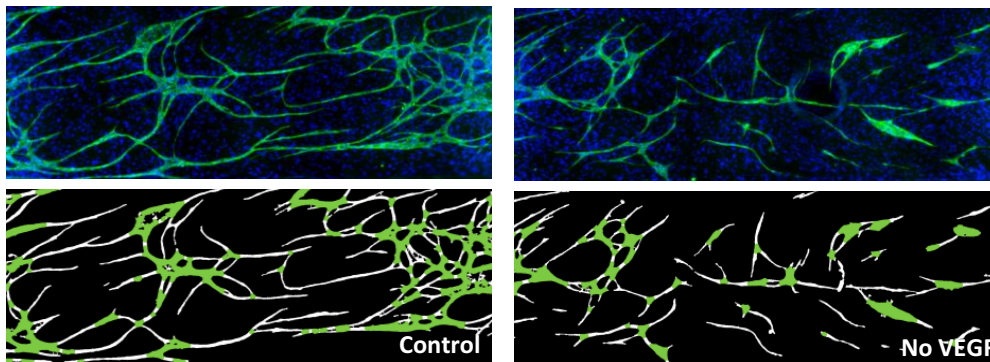
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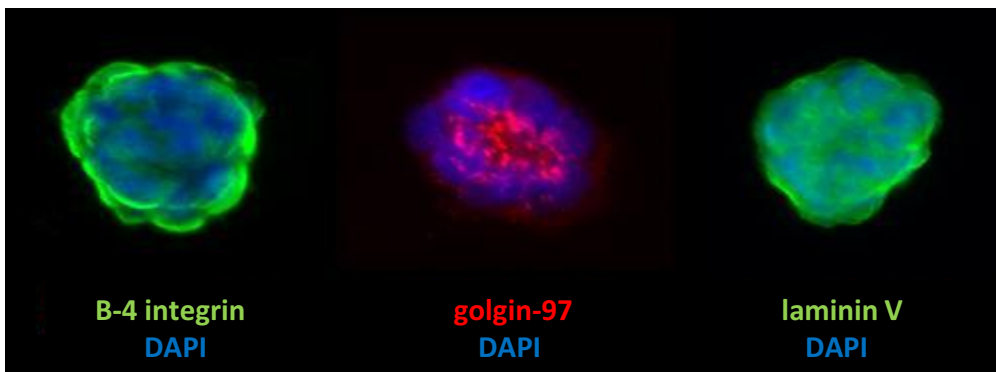
- Many aspects of human biology cannot be replicated in model organisms.
- Bellbrook Labs is focused on building biological models from the “bottom-up”.
- Bottom-up approach is to take the essential elements: ECM, cells, dissolved factors, etc., and combine them in a way that is biologically meaningful.
- Goal is to make models compatible with automated liquid handling and imaging to provide efficient, information-rich, high content assays.

Bottom-Up Cell Culture Models

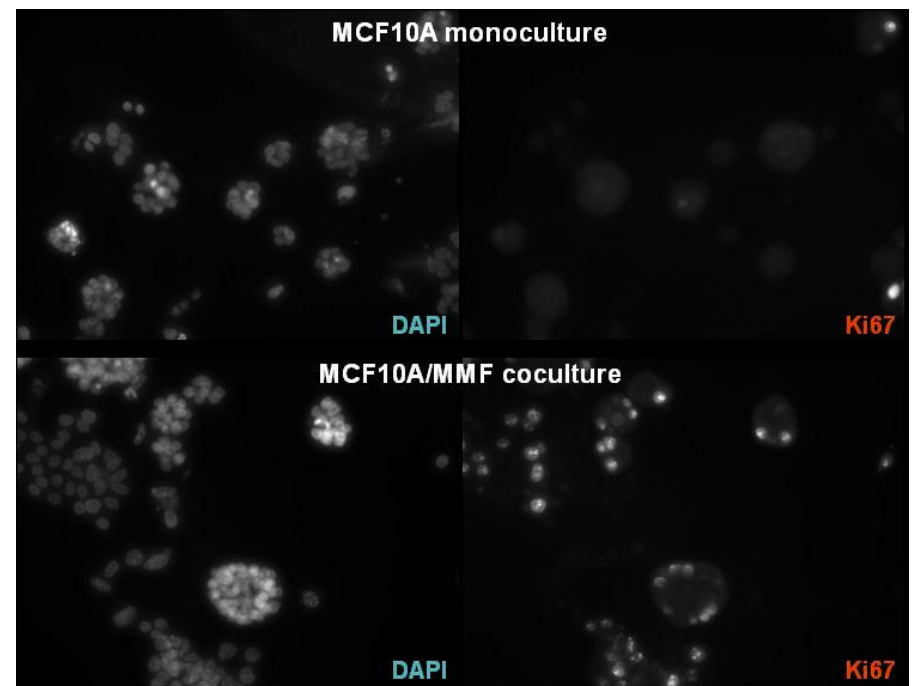
Angiogenesis



Mammary Organoids



Co-Culture



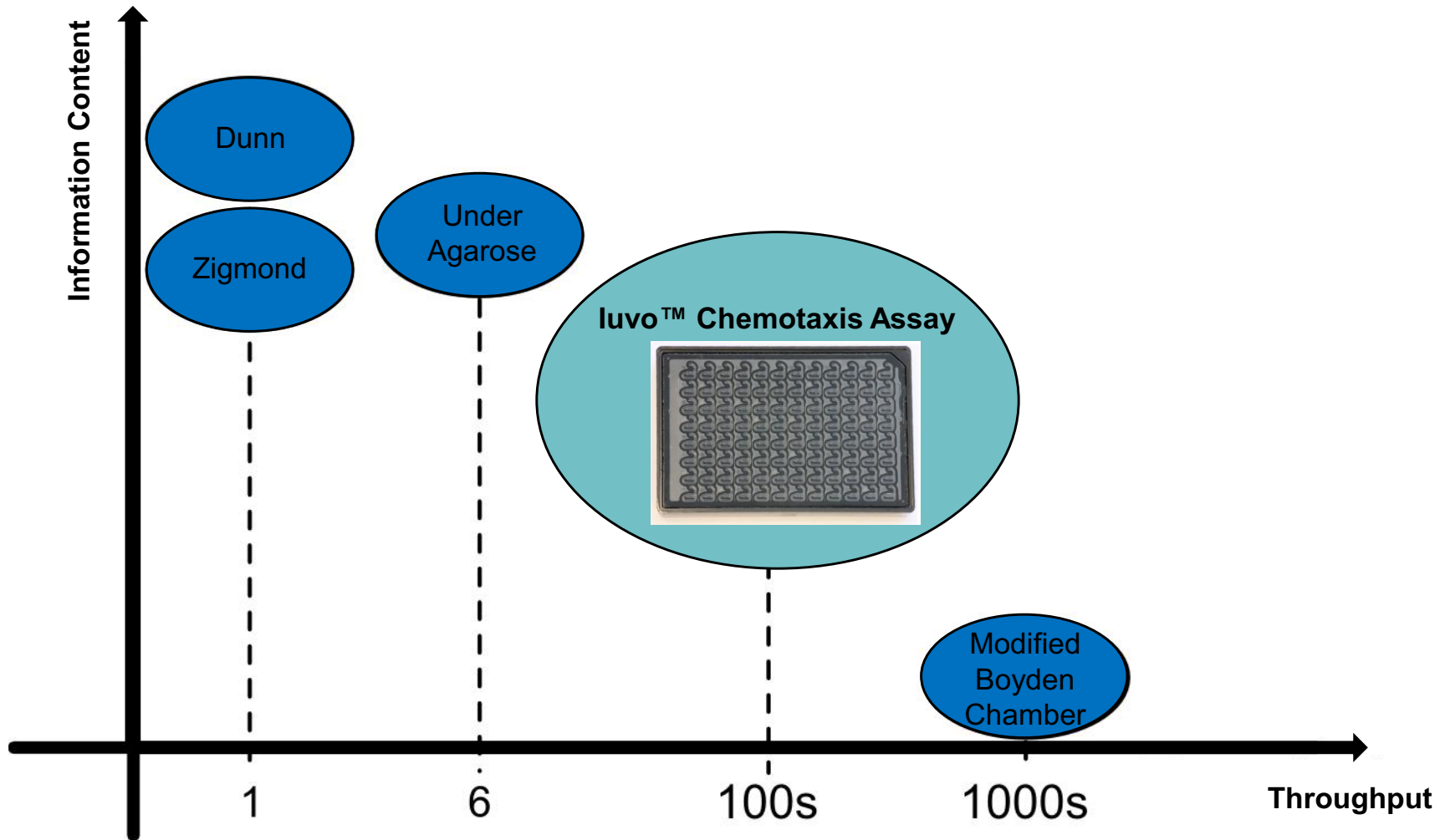
Automated Chemotaxis Assay Options

- Modified Boyden Chamber - current standard for automated chemotaxis work.
- Modified Boyden Chamber Overview:
 - chemoattractant dispensed into microtiter plate wells.
 - cells are placed on membrane and lowered into well.
 - chemotaxis is quantified on the basis of cells that move across the membrane.
- Modified Boyden Chamber Limitation: inability to observe cell shape during chemotaxis via normal microscopy.
- In Contrast, Direct Viewing Devices provide visual observation, but limited throughput.



Throughput vs. Information

iuvo™ Chemotaxis Assays approach the throughput of modified Boyden chambers while obtaining much of the information content available from direct viewing devices, such as Dunn Chambers and Under Agarose Assays.

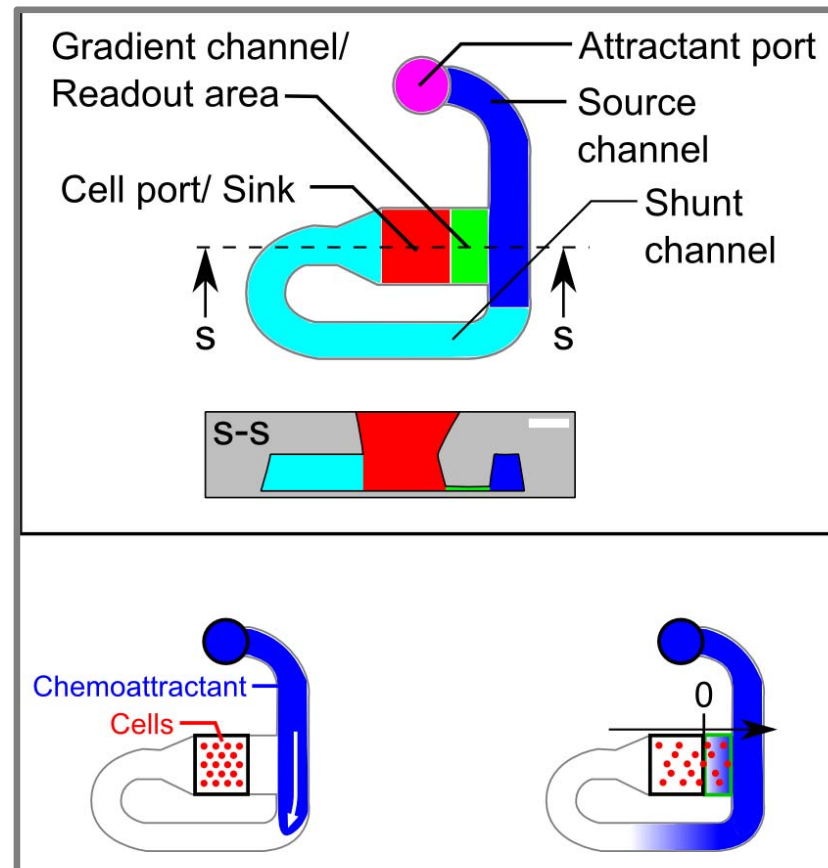


Chemotaxis Assay: Overview

- New approach using the iuvo™ Microconduit Array Platform, which utilizes microfluidics.
- Chemical gradient is formed in a microchannel and remains stable for several hours.
- Designed for use with automated liquid handling and HCA platforms, iuvo™ allows highly parallel experiments to study dose-dependent stimulation and inhibition of chemotaxis, as well as chemoattractant and inhibitor effects on cell morphology.

Assay Overview

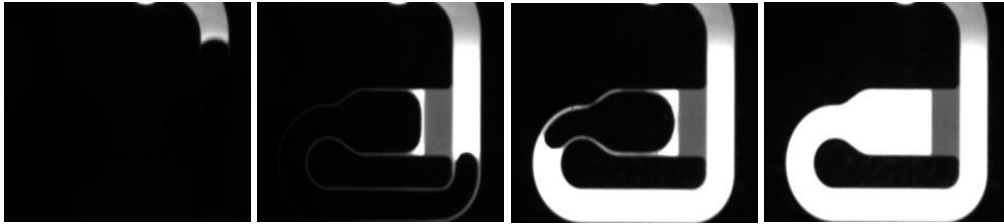
1. Cells are loaded into the Cell Port (red region) and incubated with test compounds.
2. A chemoattractant is added to the Source Channel (blue region).
3. The attractant diffuses through the Gradient Channel (green region) and forms a gradient (Note: green region is initially cell-free).
4. As the cells start perceiving the tail-end of the gradient, they respond by migrating into the Gradient Channel (green region) towards the higher concentration.



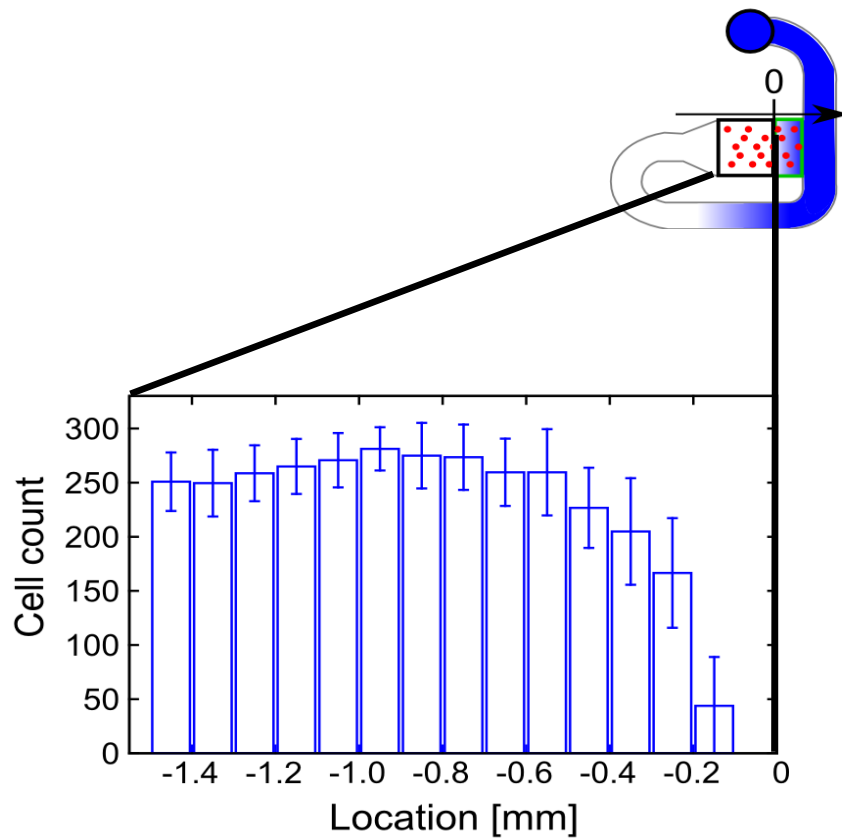
Meyvantsson, I., J. Immunol. Methods (2011), doi:10.1016/j.jim.2010.12.017



Chemotaxis Assay: Cell Loading



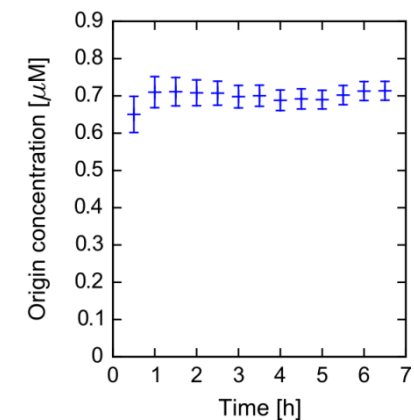
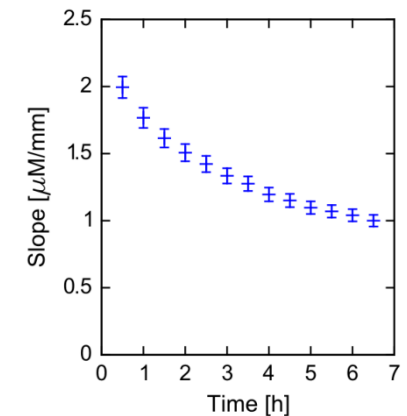
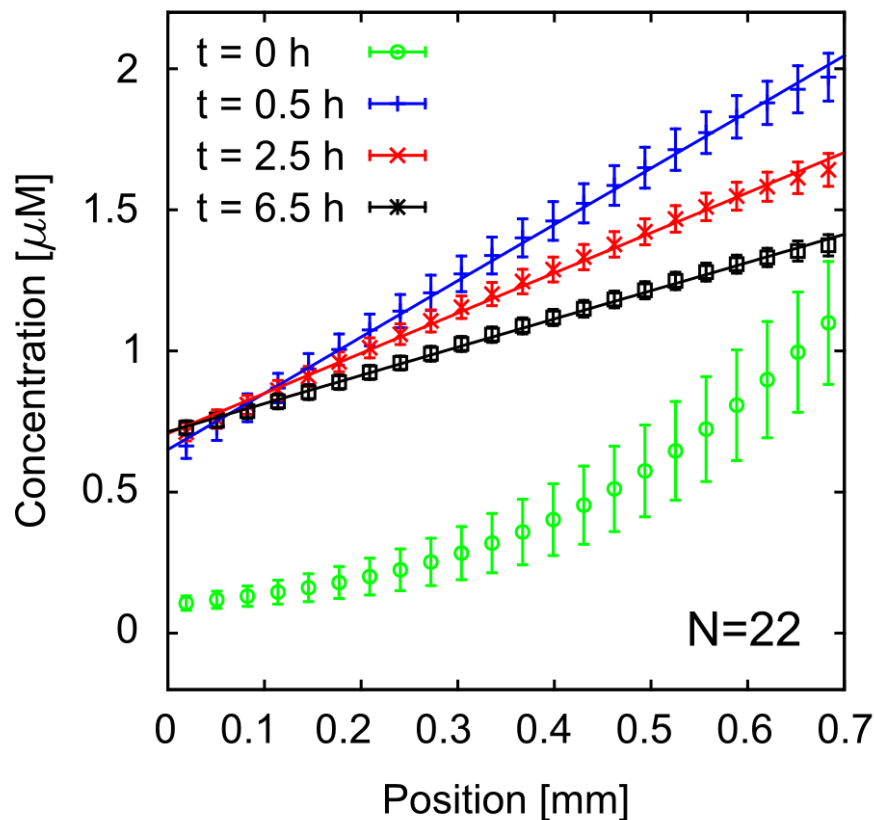
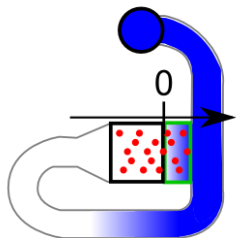
Time Sequence of Media Flow (shown in white) into iuvo™ Chemotaxis Assay Plate via Capillary Action.



Optimized Cell Dispersion Protocol Provides Repeatable Cell Distribution in the Cell Port.

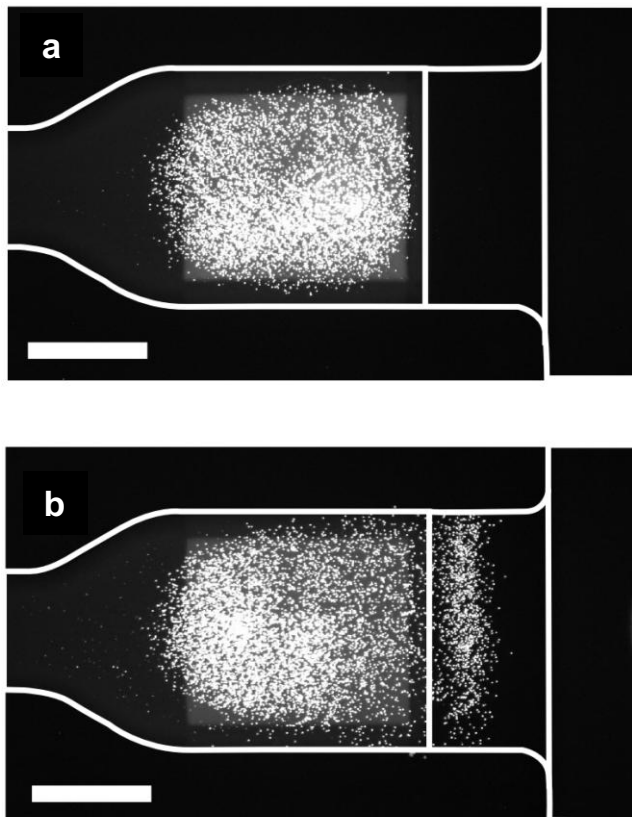
Chemotaxis Assay: Gradient Formation

- AlexaFluor® 594 (10 μM) used to characterize gradient profile as a function of time.
- Device produces a time-evolving linear gradient.
- Slope is initially 2 $\mu\text{M}/\text{mm}$ – Slope drops to 1.4 $\mu\text{M}/\text{mm}$ over a 2.5 hour assay.
- Concentration at the junction between the gradient channel and cell port remains stable at $\sim 7\%$ of the Source Concentration.
- This type of gradient is very suitable for the study of neutrophil chemotaxis.



Dose-Dependent Stimulation and Inhibition

- Dose response to Interleukin-8 (IL-8) is bi-phasic; the response grows with increasing concentration, peaks, and then decreases, eventually to zero.
- The box-whiskers analysis reveals an order of magnitude lower EC_{100} compared to cell count.
- Primary neutrophils are heterogeneous and show different sensitivities to the chemoattractant.
- Above the K_D of the receptor, cells stop being able to sense the gradient and no cell moves beyond that point in the gradient profile.

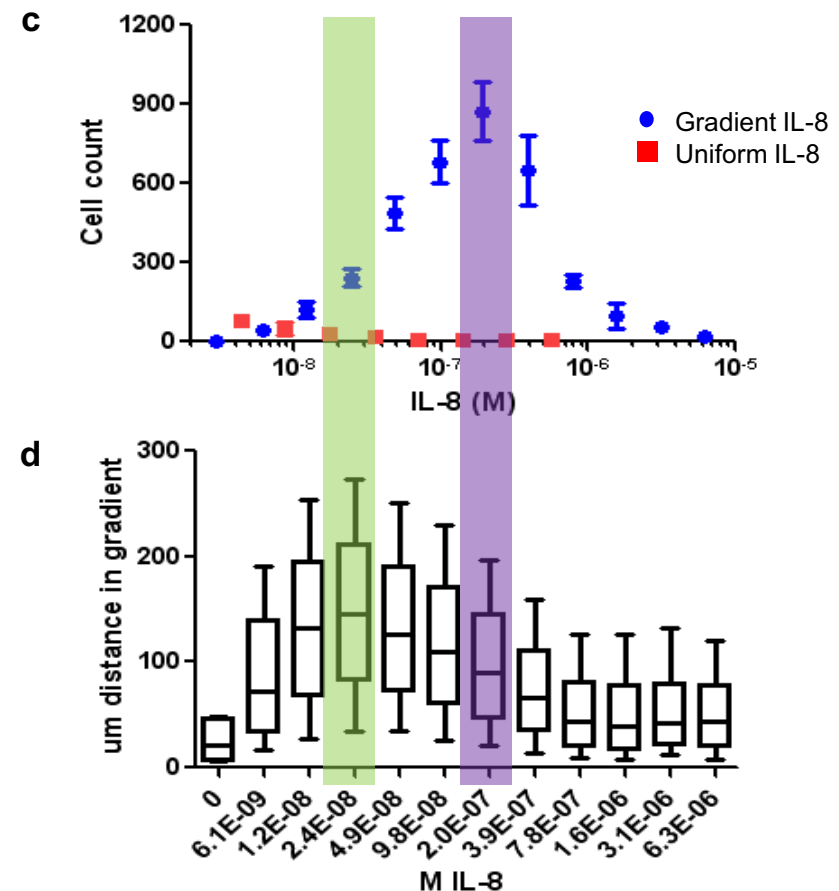


Dose-Dependent Stimulation & Inhibition of Chemotaxis

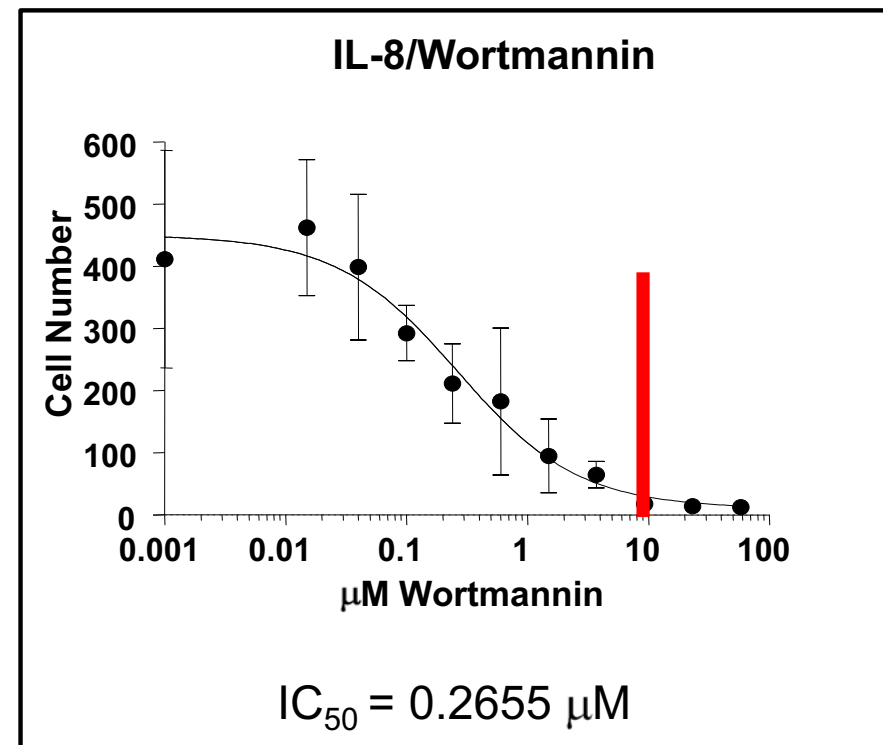
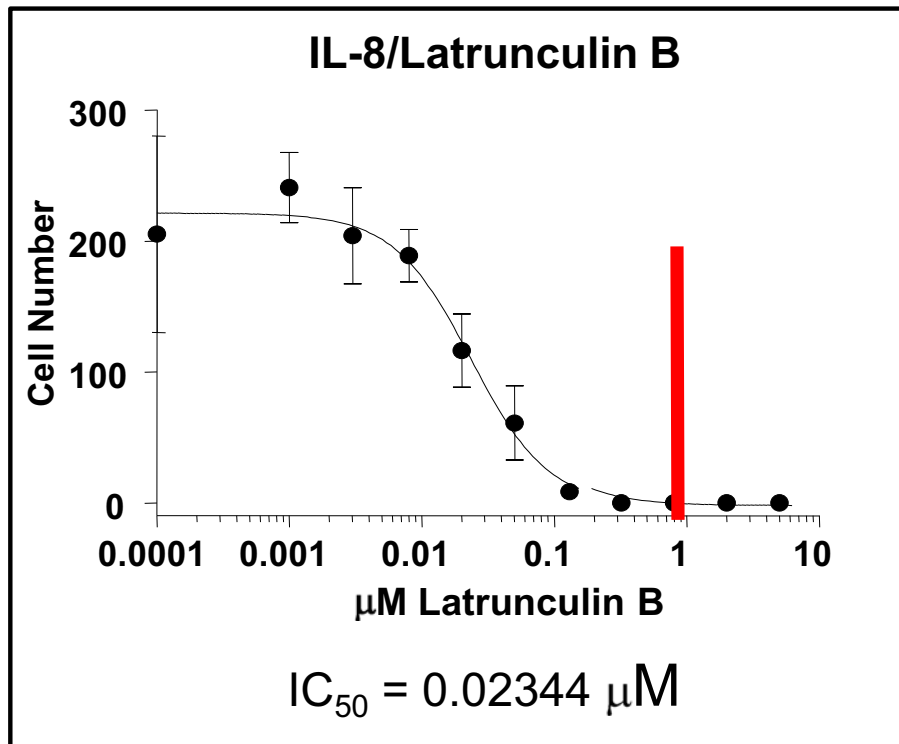
Cell distribution at end of the assay
a) no chemoattractant control.
b) 500 nM interleukin-8 (IL-8) gradient.

Dose dependant response to IL-8 gradients

c) graph shows the mean and standard deviation for replicates of 4, except otherwise indicated
d) A box-whiskers plot (10%, 25%, 50%, 75%, 90%) indicating the distribution of cell positions at the assay endpoint as a function of IL-8 gradient source concentration.



- Neutrophils were exposed to the Wortmannin (phosphoinositide 3-kinase inhibitor) and Latrunculin B (actin monomer stabilizer) in 500 nM IL-8 gradients.
- IC_{100} values were determined to be 10 μ M, and 1 μ M for Wortmannin and Latrunculin B, respectively.
- In the following slides, inhibitors at IC_{100} will be used to study cells under conditions where chemotaxis is completely inhibited.

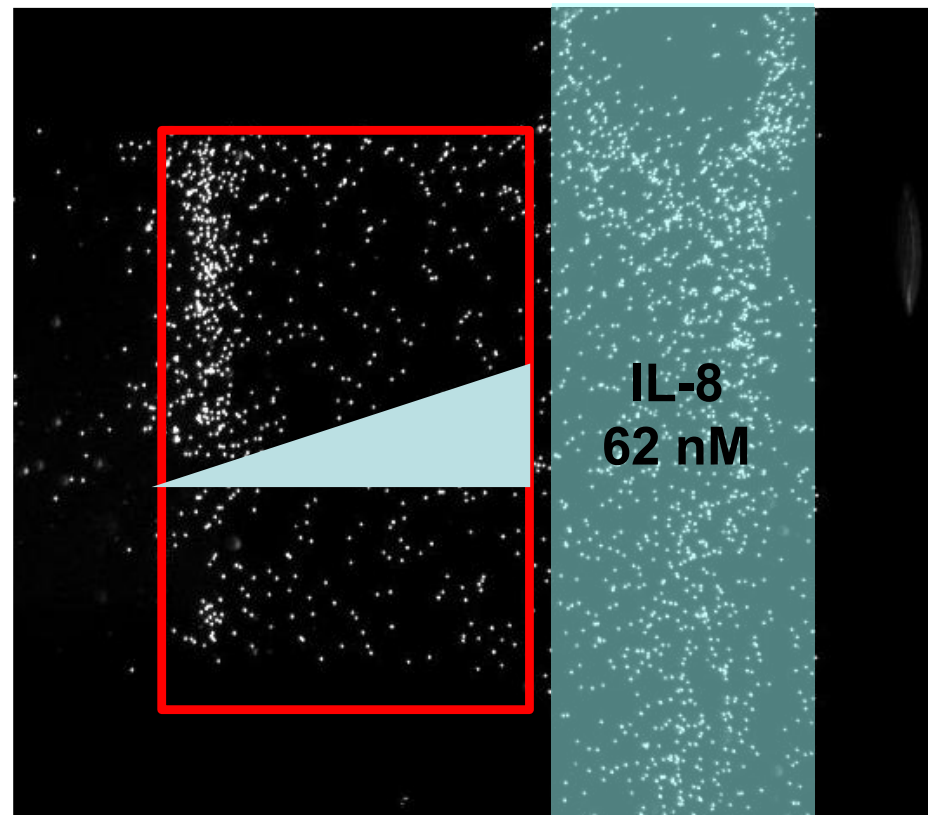


Morphology Assay: Overview

- During chemotaxis, neutrophils undergo pronounced changes in morphology.
- Cells take on an elongated appearance when they attach to the substrate, spread out, and form protrusions (at the front edge) and narrow appendages (at the trailing edge).
- Inhibitors will influence cell shape based on their mechanisms.

Assay Overview

1. Cells are loaded directly into the Gradient Channel (red box) and incubated with test compounds for 30 minutes.
2. The attractant diffuses through the Gradient Channel and forms a gradient; plate is incubated for additional 1 hour.
3. As the cells start perceiving the gradient, they respond by migrating towards the higher concentration.



Morphology Assay: Cell Shape Analysis

- Cells were analyzed for *elongation* (ratio of distance between foci to major axis) and cell *area*.
- CellProfiler, a high-content analysis software (www.cellprofiler.org), was used to quantify the shape of neutrophils under various conditions.

IL-8
62 nM

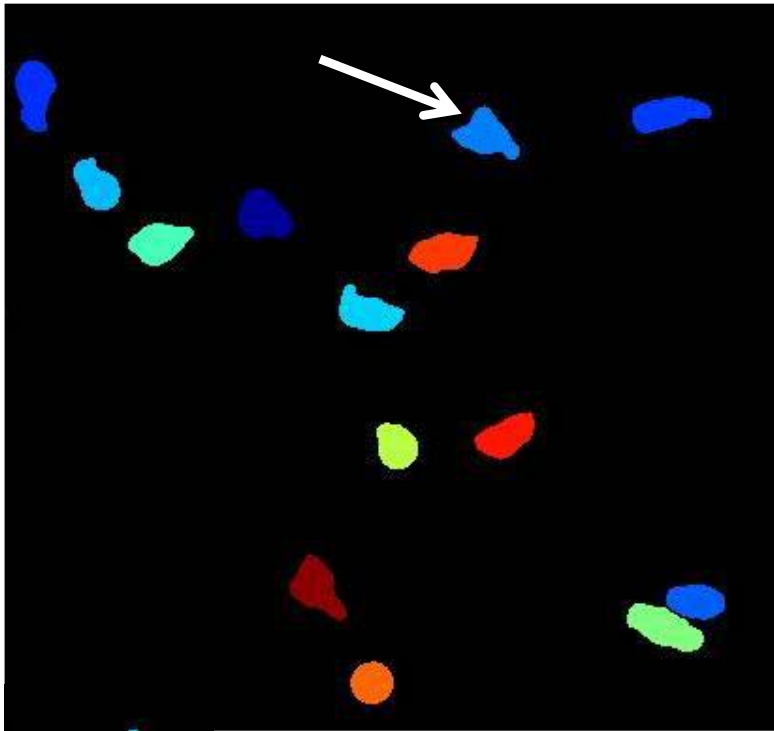
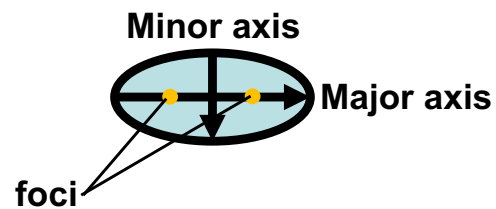


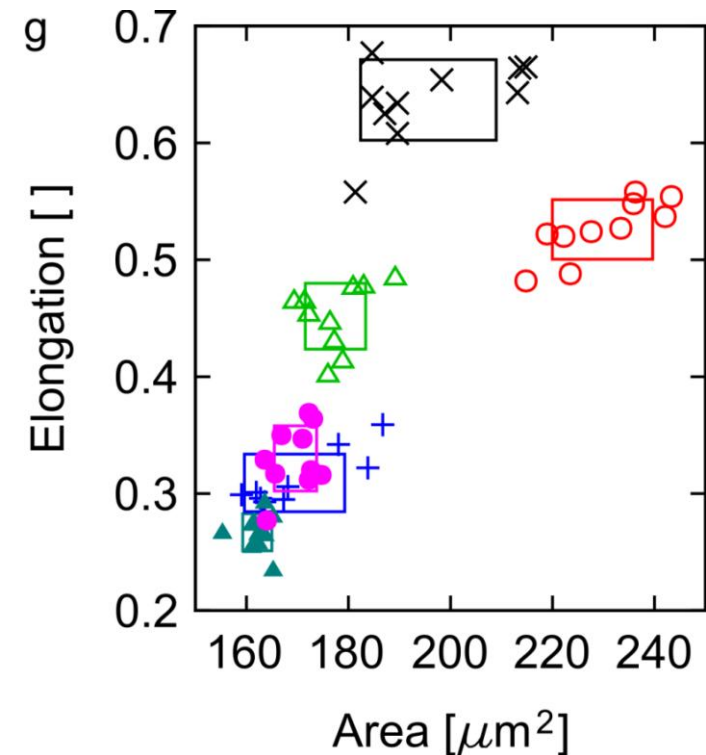
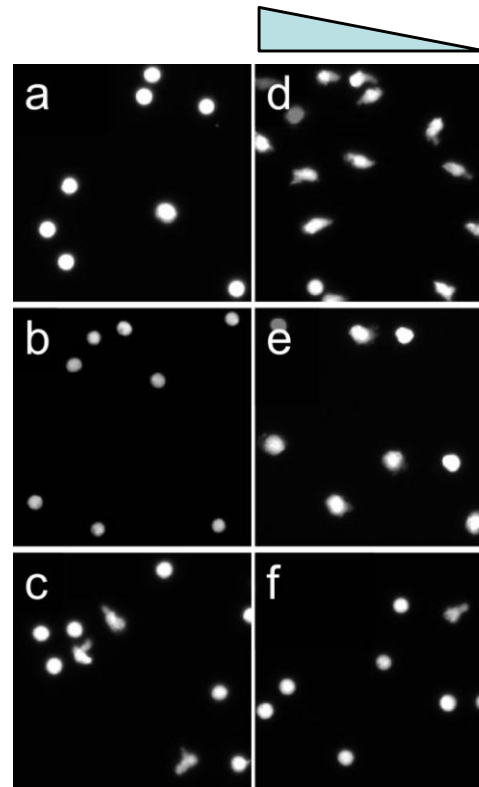
Image of a neutrophil chasing a bacterium. In this famous video, the neutrophil weaves through the other cells and eventually captures the bacterium and engulfs it (David Rogers, Vanderbilt University, www.biochemweb.org).



Effect of Gradient & Inhibitors on Cell Shape

Observations & Results

- In the absence of IL-8 (control), cells remained round with a small average area, while in the presence of IL-8 they acquired the flattened and elongated morphology characteristic of migration to varying degree.
- With increasing stimulus a greater fraction of cells responded - a higher uniform concentration around the K_D of the receptor showed a significant increase in elongation.
- Cells exposed to an IL-8 gradient had ~ 2X the elongation of control cells.
- When cells exposed to the same gradient were treated with Wortmannin, a signaling inhibitor, they were indistinguishable from the control.
- In contrast, Latrunculin B, the actin monomer stabilizer, caused a marked increase in area and reduction in elongation compared to the cells experiencing a gradient and no inhibitor.

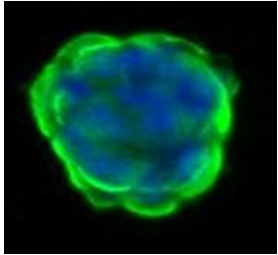


Images: Neutrophils were placed under experimental conditions with various concentration profiles of IL-8: a) none, b) uniform 0.4 nM, c) uniform 4 nM, or d–f) gradient with 62 nM source, and with d) no inhibitor, e) 10 μM Wortmannin, and f) 1 μM Latrunculin B.

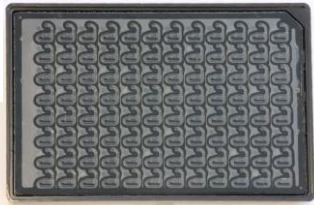
Graph: Scatter plot showing area and elongation. Conditions shown are: control (+), uniform 0.4 nM IL-8 (\blacktriangle), uniform 4 nM IL-8 (\triangle), 62 nM IL-8 gradient (\times), gradient and 10 μM Wortmannin (\bullet), gradient and 1 μM Lat B (\diamond), $N=10$ for each condition.

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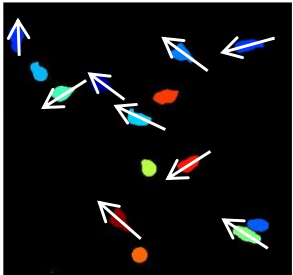
Summary



BellBrook utilizes bottom-up cell culture models to take essential elements and combine them in a way that is biologically meaningful to create information-rich, high-content assays.

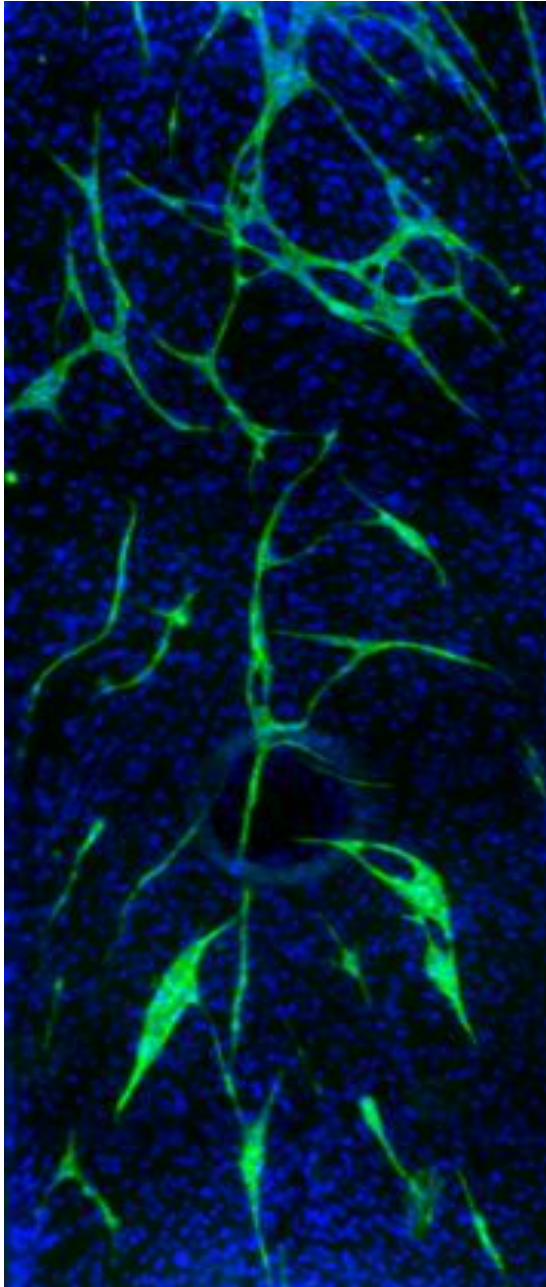


The iuvo™ Chemotaxis Assay provides both automation and direct viewing, which enables image-based readouts.



The innovative format of the iuvo™ Chemotaxis Assay enables high-content, phenotypic analysis of cells during chemotaxis events.

Acknowledgements



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