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Overview

A new format for neutrophil chemotaxis studies has been developed that enables automation friendly assays with a high-content readout. The iuvo Gradient 1000 is a microconduit array plate that facilitates horizontal chemotaxis in the presence of a defined gradient. The plate is designed to be adaptable to instrumentation found in the screening lab, including liquid handlers and high-content analysis platforms. We show that the gradient plate is compatible with the TTP LabTech Acumen™ eX3. Included in our studies are chemoattractant and inhibitor titrations. To demonstrate the speed advantage of using a laser scanning acquisition method with on-the-fly data analysis, we perform a side by side comparison to an automated microscope-based method using a common software package for analysis. The two acquisition methods show corresponding data results, with the laser scanning approach affording an approximate four-fold time savings from acquisition to data output. The combination of this new assay format and fast laser scanning acquisition method puts high throughput chemotaxis screening within reach.

iuvo™ Gradient 1000

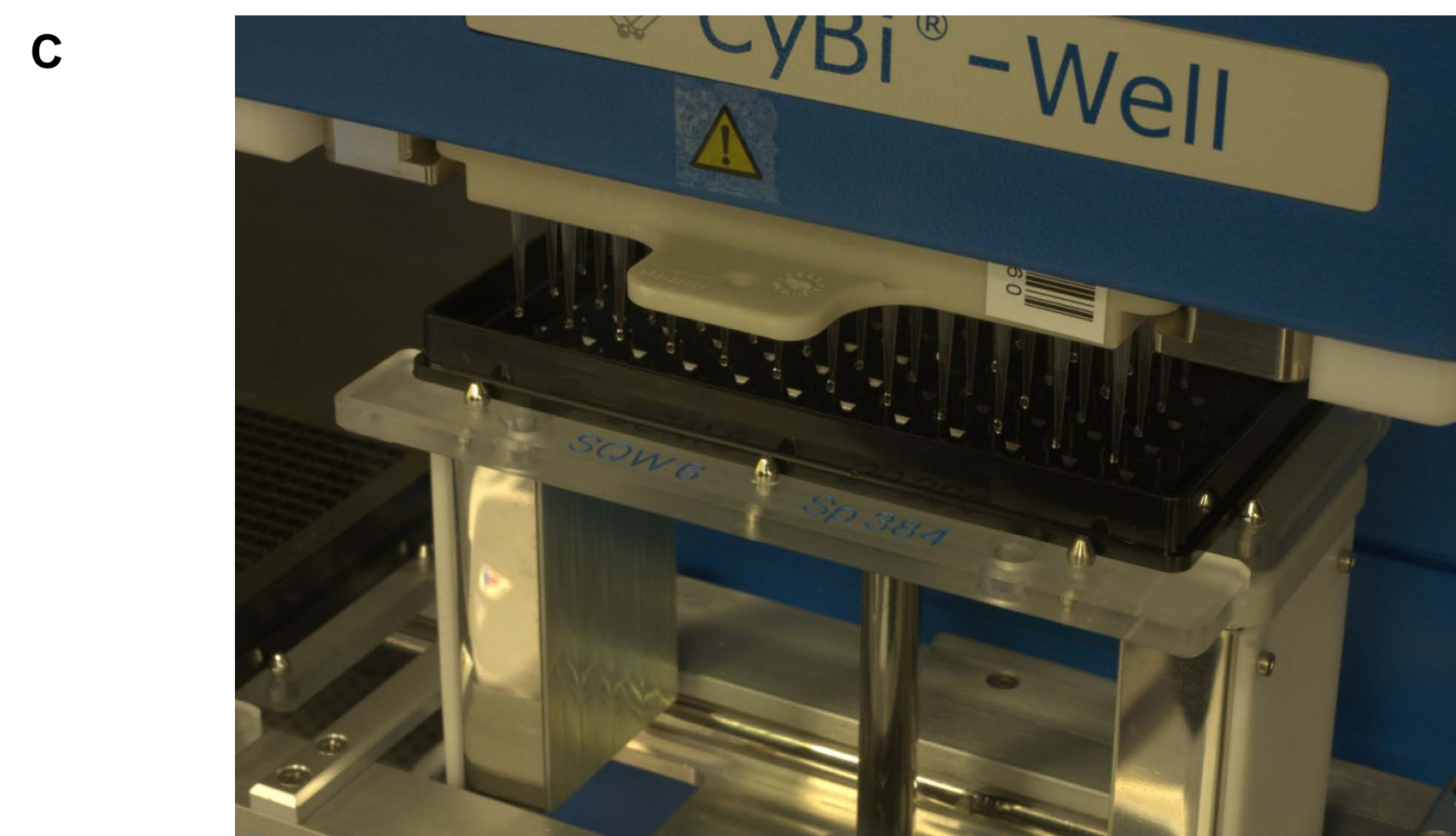
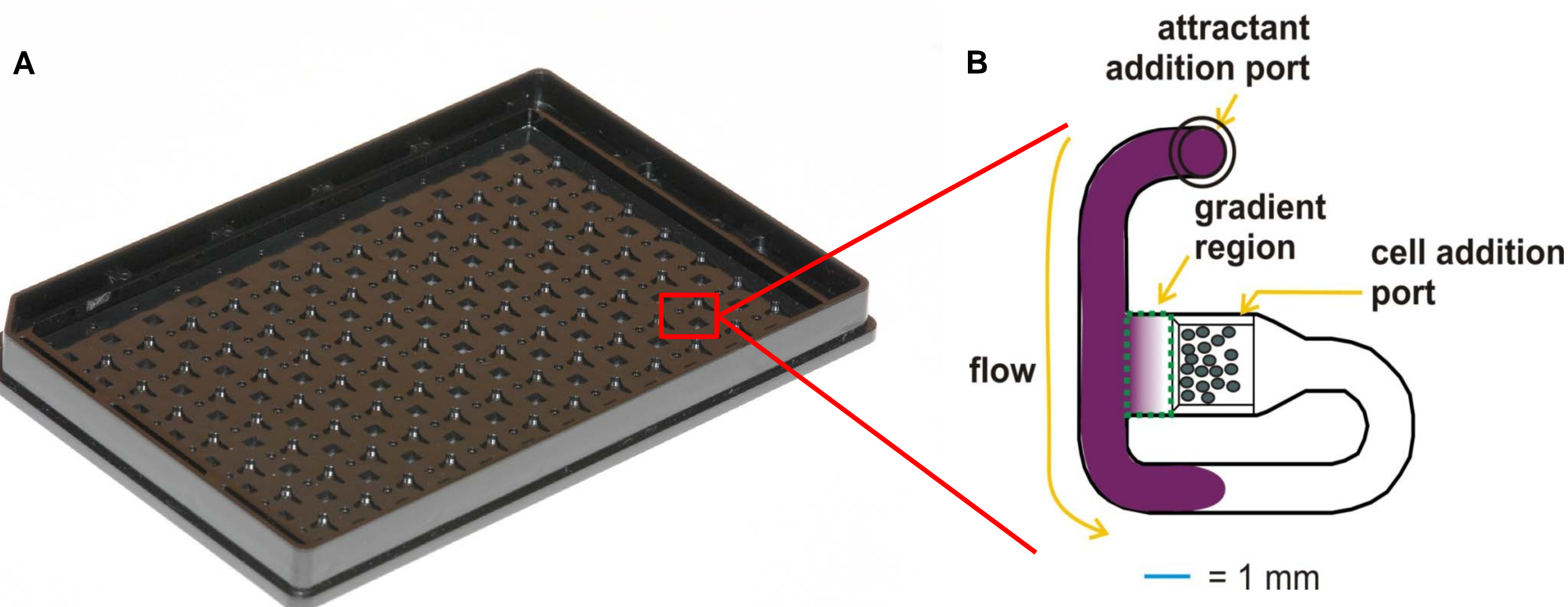


Figure 1. The Gradient 1000 plate is a microconduit array compatible with laboratory automation. A) The Gradient 1000 is composed of 96 microchannels (12 columns by 8 rows), with a footprint that complies with SBS/ANSI microplate standards. A chemotaxis assay is assembled using droplet based additions through two addition ports located on top of the plate. The readout region (gradient region) is located underneath the plate. B) Each microchannel is constructed as shown here. The two ports are for medium, chemoattractant, and cell additions. The channels are constructed in a manner that precisely controls fluid flow, allowing for a controlled cell patterning and a defined gradient of chemoattractant with stability ≥ 3 hours. To establish the gradient, chemoattractant is added to the attractant addition port. As chemoattractant flows into the channel, the molecule diffuses into the gradient. Thus, the entrance to the gradient where the attractant flows in is considered the "source" and diffusion occurs across the gradient region to the cell addition port "sink." Cells added to the channel move in a horizontal fashion across the surface of the channel toward highest concentration of chemoattractant. C) The Gradient 1000 is designed to interface with standard liquid handling instrumentation, allowing the user to take advantage of microfluidic technology without the need to purchase additional laboratory equipment. Shown here is a CyBio CyBi®-Well 96 pipetting to the plate.

The Acumen® eX3

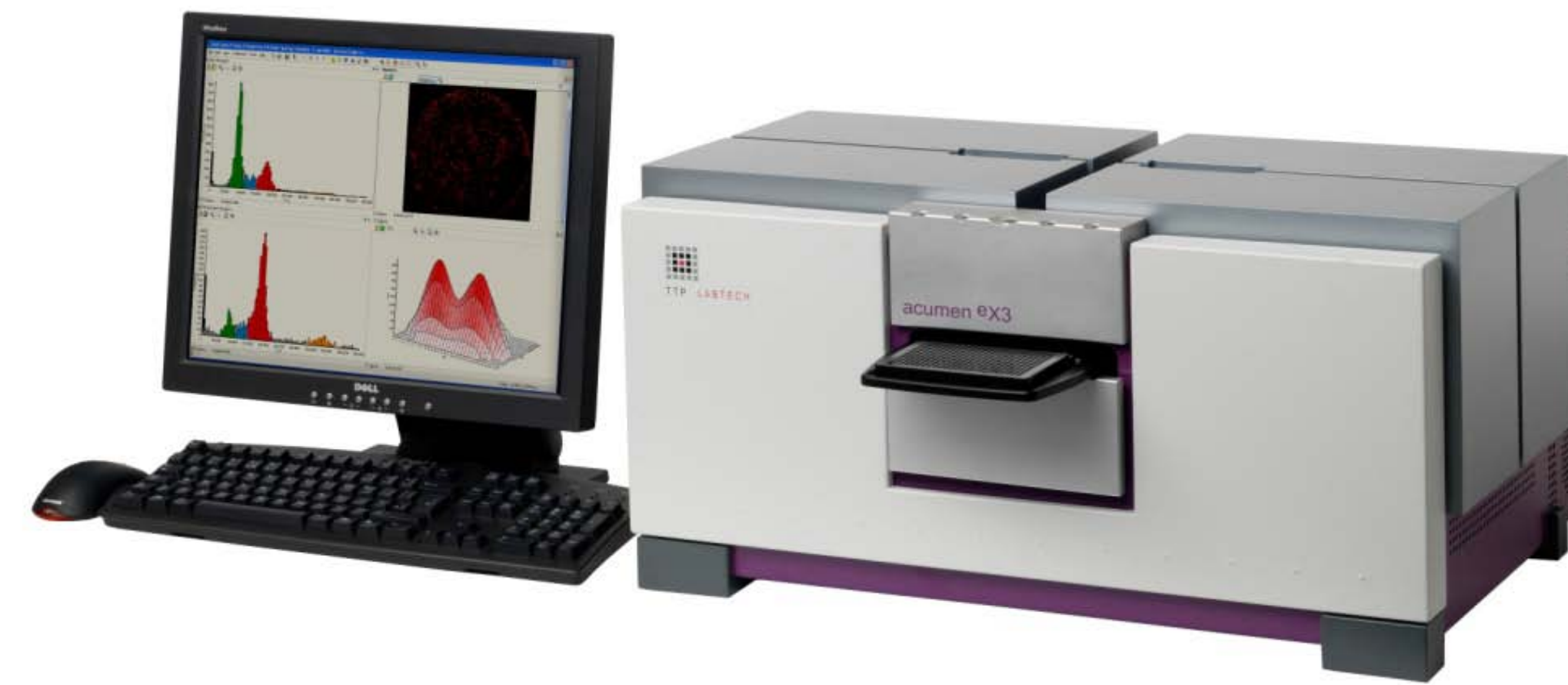


Figure 2. Features of the Acumen eX3. The Acumen eX3 technology is based on cytometry principles rather than image-based acquisition. The instrument can be equipped with up to three lasers with simultaneous four color detection for each laser. The on-the-fly analysis feature provides fast data acquisition, enabling increased throughput. For this study, we used a 488 nm laser that was compatible with our calcein-AM cell label. Only the gradient region from each channel was scanned. For object identification, we used a ratiometric approach to approximate cell number based on the total area to mean area of single cells.

We compared the laser-based, integrated analysis approach from TTP Labtech to an image-based acquisition method with off-line data analysis. An inverted Nikon Eclipse TE2000U (not pictured) was used for image acquisition. The microscope was equipped with an automated stage, 1.5X objective and FITC filter set. We used a Metamorph software package from Molecular Devices for image analysis, and the count nuclei algorithm for object identification.

Chemotaxis assay protocol

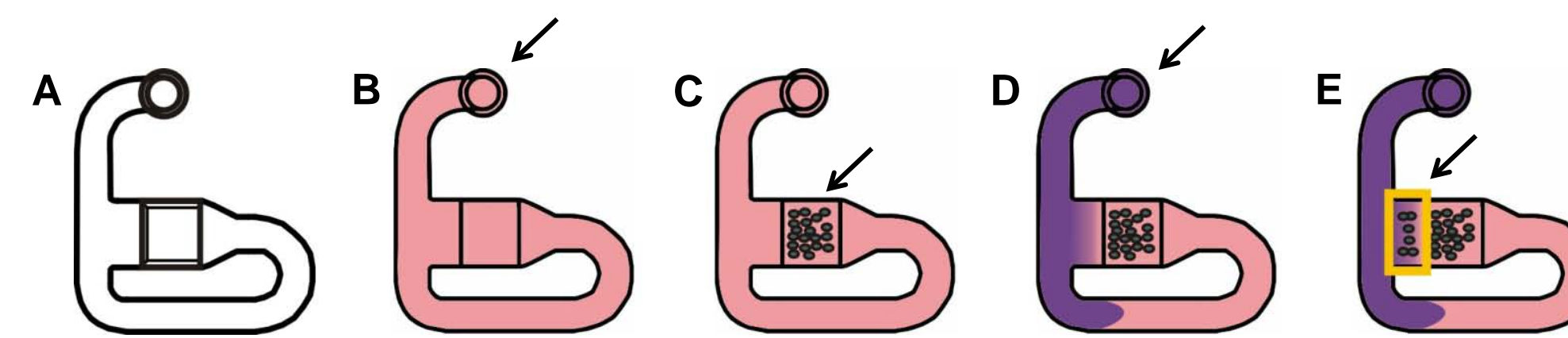


Figure 3. Performing a chemotaxis assay. Chemotaxis assays are conducted by a series of addition steps to the microchannels. A.) the plate is provided with empty channels. B.) 20 μ L of assay medium ($\pm 1X$ concentration inhibitor) is added to the attractant addition port. The assay medium used in this study consisted of RPMI 1640 + 10 % FCS. C.) 3 μ L of cells is added to the cell addition port, allowed to settle at room temperature for 10 minutes, then the plate is incubated at 37°C / 5% CO₂ for 30 minutes. If no inhibitor is being tested, the 30 minute incubation at 37°C is not needed. We used 12,000 neutrophils per channel for this study. D.) 3 μ L of chemoattractant is added to the attractant addition port. The plate is further incubated at 37°C / 5% CO₂ for 2.5 hours. The gradient of chemoattractant establishes within 30 minutes. E.) The gradient region, highlighted by the yellow box, is then imaged with an inverted microscope or other high content instrument.

Titration of Interleukin-8

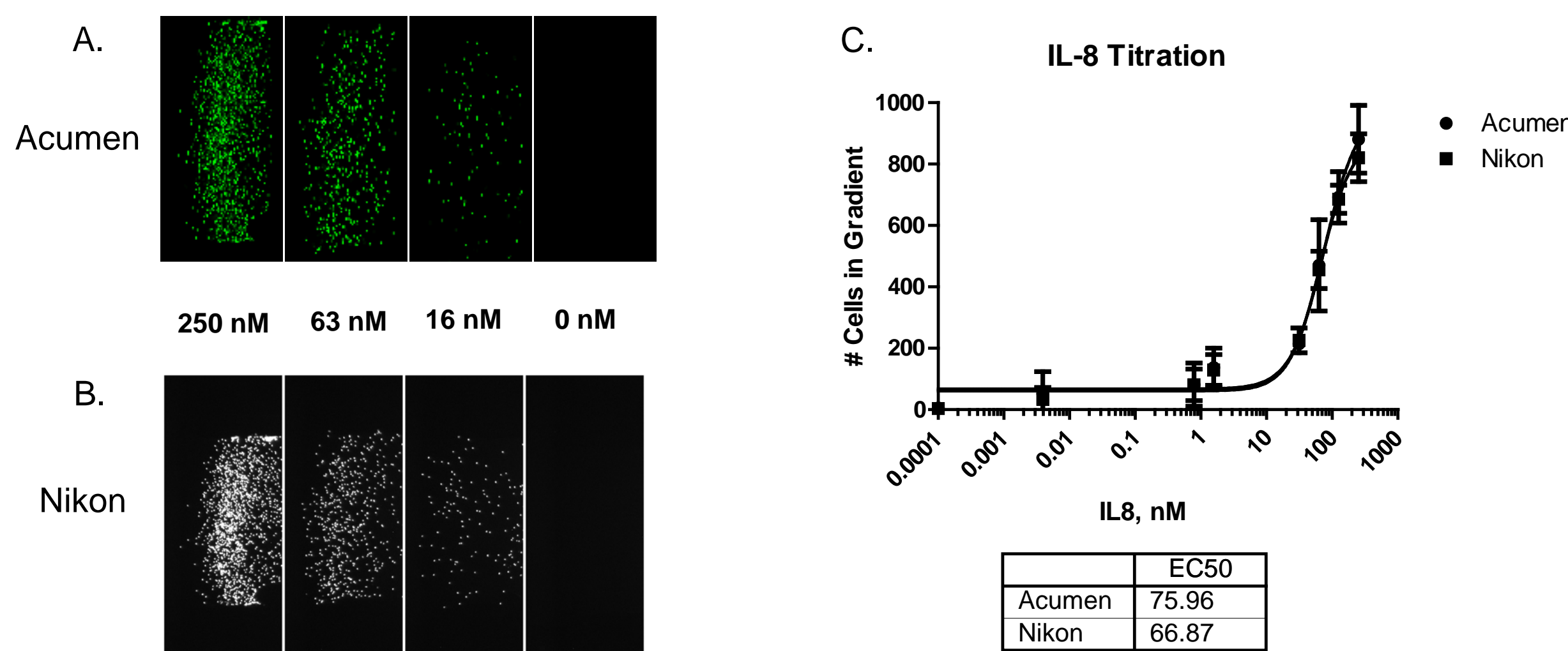


Figure 4. Interleukin-8 titration yields comparable EC₅₀ between the two acquisition methods. IL-8 was serially titrated 1:2 in assay medium and added according to the chemotaxis protocol. Following the 2.5 hour incubation, the plate was scanned or imaged. A.) and B.) Corresponding scans and images from select concentrations of IL-8 are shown for the Acumen and Nikon, respectively. The number of cells in the fields of view are comparable between the two methods. For the Nikon images, the gradient region was automatically cropped from the full image and used for data analysis. C.) A full titration curve shows that the counted cells in the gradient region are comparable between the two methods, suggesting that the ratiometric estimation of cell number used by the Acumen is comparable to the object counting algorithm used for the image-based approach. Each point on the curve represents n=4.

Inhibitor dose response curves

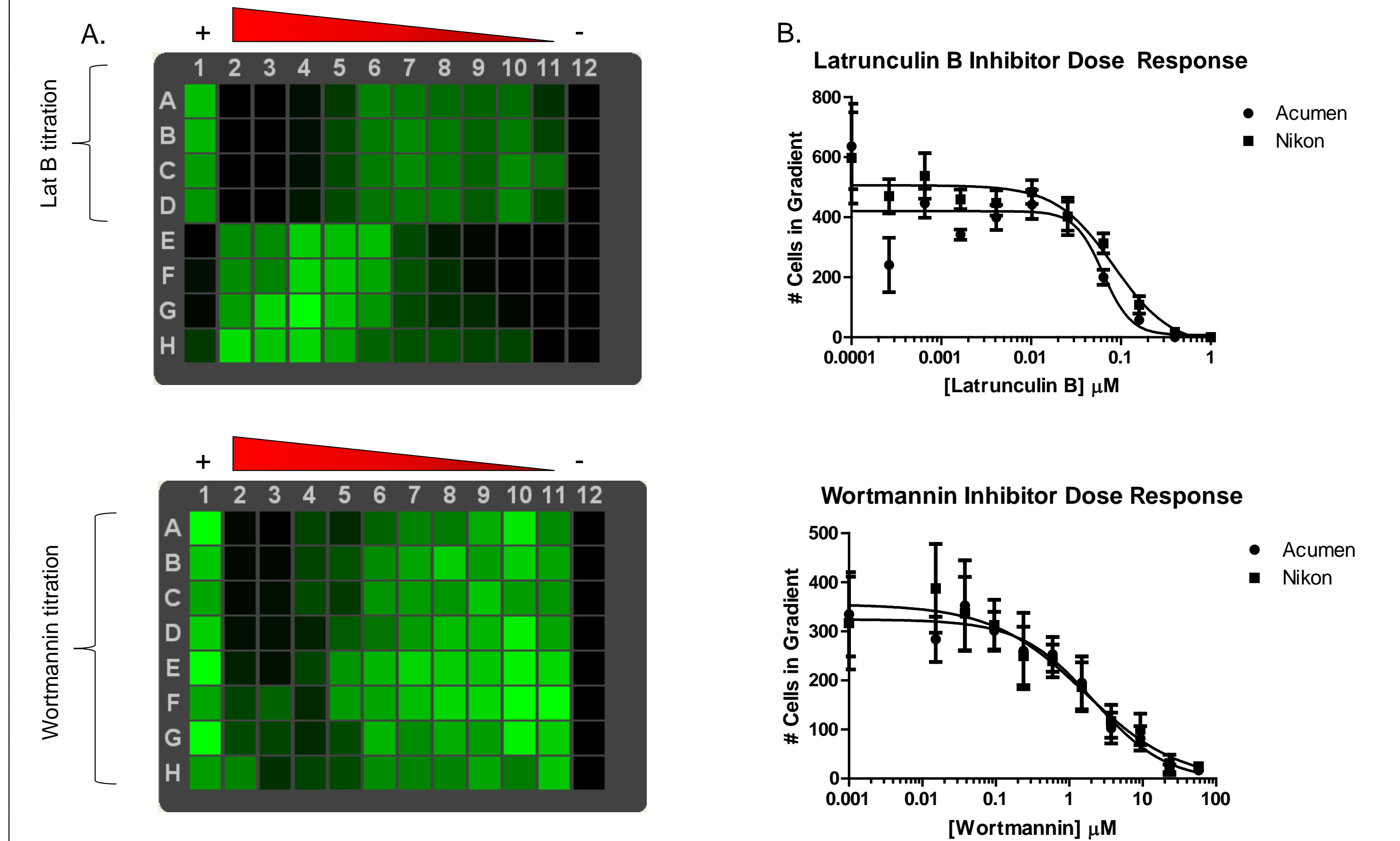


Figure 5. Chemotaxis inhibitor potencies are comparable between the image and laser-based acquisition methods. Latrunculin B, an inhibitor of cytoskeletal rearrangement, and Wortmannin, a PI3 kinase inhibitor, were serially titrated 1:2 in assay medium and added to the plate according to the chemotaxis assay protocol. 12,000 calcein-AM labeled neutrophils were pre-incubated with inhibitor, followed by the addition of 62 nM IL-8. Positive and negative controls represent chemoattractant only, or no chemoattractant, respectively. The assay was allowed to incubate in a 37 °C/5 % CO₂ incubator for 2.5 hours. The plates were then imaged or scanned. A.) Object counts from the Acumen are shown on a heat map in real-time as the plate is being scanned. B.) The ratiometric cell number estimation method used on the Acumen, and the count nuclei object identification method used with the Nikon/Metamorph method, show comparable cell counts and potencies for both compounds.

Approximately four-fold time savings using laser scanning method

Acquisition Method	Acquisition Time	Off-line data analysis	Total time per plate	Total time for 10 plates
Laser Scanner (Acumen)	5 min 30 sec	N/A	5 min 30 sec	55 min
Automated Microscope (Nikon)	5 min 30 sec	15 min	20 min 30 sec	205 min

Figure 6. The integrated data acquisition and analysis used by the Acumen affords an approximate four-fold time savings per plate. The off-line data processing required for using the automated microscopy method includes importing images into Metamorph software, the application of an analysis region to the image stack, and running the count nuclei object identification algorithm with automated data export to Excel. Although the acquisition time between the two methods is identical, the additional time needed to process the images with the automated microscopy method decreases throughput. This is particularly evident when extrapolating the total time that would be required to process 10 plates: in less than one hour, 10 chemotaxis plates could be processed on the Acumen, whereas those same 10 plates would take over 3 hours with the Nikon/Metamorph combination.

Conclusions

- The iuvo Gradient 1000 is a high-content, automation friendly plate for primary neutrophil chemotaxis assays.
- The Gradient 1000 plate is compatible with the Acumen eX3.
- Data results are comparable between the laser-based scanning method used by the Acumen eX3, and an image-based method used by an automated inverted Nikon microscope.
- The coupled data acquisition and analysis on the Acumen eX3 provides a nearly four-fold time savings to get to a quantified result.

Acknowledgements

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