

The coupling of TTP Labtech's acumen<sup>®</sup> eX3 with BellBrook's iuvo<sup>™</sup> chemotaxis assay plate provides a solution for the high-content screening analysis of neutrophil chemotaxis assays.

### chemotaxis, its role in human disease

Chemotaxis is the process in which cells migrate towards or away from a chemical stimulant and it is fundamental to a number of biological processes including the formation of embryonic patterns and migration of immune cells to sites of inflammation and infection. Chemotaxis also plays a major role in the metastasis of malignant cancers.

In the immune system, neutrophils comprise a key component of the innate immune response and are the initial responders to bacterial infection and other inflammatory stimuli. During infection neutrophils migrate towards the site of inflammation in response to chemoattractants and in turn release further cytokines and signals, which stimulate the adaptive immune response.

The chemotactic response of neutrophils is affected by a number of disease states including lentivirus infection, HIV and a number of autoimmune and allergic disorders. The study of the inhibition of neutrophil chemotaxis is valuable in the development of new drugs and can also be of interest during the assessment of their potential side effects.

### the study of the chemotactic response for drug discovery

In the modern drug discovery industry, the development of cell-based assays for high-content screening (HCS) is necessary for the efficient screening of compound libraries. Until recently, assays for the analysis of chemotactic responses were not amenable for use in high-content screening cell-based assay approaches. However, recent developments have enabled the investigation of chemotactic responses to be applied in cell-based high-throughput screening (HTS) assays.

Studies on the effect of chemical compounds on cell motility have been carried out using techniques based largely on the Boyden chamber (1). In this system, cells migrate in response to a chemoattractant from an upper chamber to a lower chamber separated by a filter. Chemotactic responses are assessed directly, by microscopic analysis or indirectly, by assaying cell lysates.

More recently, screening experiments for chemotaxis have been designed using modified Boyden transmembrane assays in multi-well formats. These assays, however, only provide basic information on the migration of cells in response to chemokine gradients and do not allow a more detailed understanding of cell health, morphology and the nature of the chemokine gradient. Furthermore, these assays are not only time consuming (requiring lengthy incubation, cell fixing and screening steps) but require large cell numbers increasing cost and preparation time.

The introduction of direct-viewing devices, such as that developed by Kanegasaki et al. (2), where cells migrate on a horizontal surface enabled the measurement of a number of parameters in addition to cell migration such as cell appearance and shape. Direct-viewing approaches as described by this research group have particular advantages for qualitative studies, allowing the study of perturbation of function by drug candidates and the use of lower cell numbers per assay.

### high-content screening of chemotaxis assays

In 2010, BellBrook Laboratories, Madison, USA released a 96 well direct-viewing assay plate to enable highly parallel studies of chemotaxis (3). The iuvo<sup>™</sup> Chemotaxis Assay plate incorporates microchannel arrays to enable precise chemokine gradients to be set up and allows direct viewing of cell migration into a defined gradient zone. Furthermore, the use of these microconduit arrays reduces both the cell number and amounts of valuable compound required per assay.



The iuvo<sup>™</sup> Chemotaxis Assay plate was designed for high throughput screening assays, being compatible with liquid handlers and appropriate imaging equipment such as TTP Labtech's acumen<sup>®</sup> eX3

The acumen<sup>®</sup> eX3 employs cytometry principles rather than image-based acquisition, providing rapid high-content readout and analysis of multiple wells simultaneously. Its simultaneous data acquisition and analysis feature provides fast data acquisition, enabling increased throughput and the generation of statistically robust data from representative cell populations.

This application note describes the use of an iuvo<sup>™</sup> Chemotaxis Assay plate in combination with TTP Labtech's acumen<sup>®</sup> eX3 high-content imager for the study of neutrophil migration. We present data comparing image acquisition and cell response to both chemoattractant and inhibitors using an inverted Nikon Eclipse TE2000U microscope and the acumen<sup>®</sup> eX3 imager. It was seen that the acumen<sup>®</sup> eX3 laser scanning approach resulted in four-fold time savings from acquisition to data output.

# methods

## iuvo™ plate design

The iuvo™ chemotaxis assay plate consists of 96 individual microfluidic units and is described in detail by Meyvantsson et al. 2011 (Figure 1). Briefly, each unit consists of 5 components, an attractant port, a source channel where chemoattractant is provided, a gradient channel into which the chemoattractant diffuses to create a gradient, a cell addition port and readout area, and a shunt channel that helps ensure a stable gradient by diverting flow around the gradient channel.

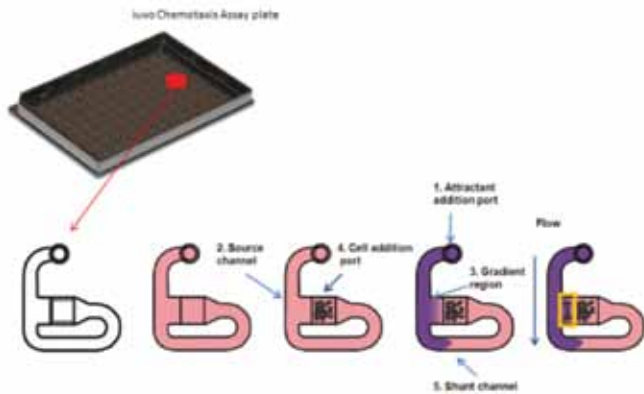


fig. 1. Diagrammatic representation of BellBrook's iuvo™ Chemotaxis Assay plate. Each well consists of a microfluidic unit with 5 components as shown above. 1. an attractant port, 2. a source channel, where chemoattractant is provided 3. a gradient channel into which the chemoattractant diffuses to create a gradient, 4. a cell addition port and readout area and 5. a shunt channel that helps ensure a stable gradient by diverting flow around the gradient channel

## neutrophil preparation

Polymorphonuclear (PMN) leukocytes (expected to be at least 90% neutrophils) were isolated from healthy donor blood (protocol approved by an Institutional Review Board) using Polymorphprep (Accurate Chemical and Scientific Corp.). Cells were re-suspended in RPMI 1640 (Sigma) containing 10% heat inactivated FCS (Gibco) and labelled with 5µM calcein AM (Life Technologies). Following incubation with calcein, cells were washed and resuspended to a concentration of  $4 \times 10^6$  /mL.

## chemoattractant and chemoinhibitors

Neutrophil activity was assessed by stimulating with IL-8, which is a powerful neutrophil chemotaxin. 62nM Human Recombinant IL-8 (R&D systems), was added in a 3µL volume to the attractant port of the iuvo™ Chemotaxis Assay plate and cells were incubated for 2.5 hours at 37°C in 5% CO<sub>2</sub>. Inhibition of chemotaxis was assessed using wortmannin (MP Biomedicals), a P1 3-kinase inhibitor. Wortmannin was added to assay medium in serial dilutions from 64µM to 1µM.

## chemotaxis assay plate set up

Chemotaxis assays were conducted by a series of addition steps to the microchannels. Assays were run in RPMI 1640 containing 10% heat-inactivated FCS. 20µL of assay medium in the presence or absence of inhibitor was added to the attractant addition port, filling the source, gradient, and shunt channels via capillary action (Figure 1). 3µL of cells ( $4 \times 10^6$  /mL) were added to the cell addition port and were allowed to settle at room temperature for 10 minutes.

Cells were then pre-incubated with the inhibitor at 37°C / 5% CO<sub>2</sub> for 30 minutes and 3µL of human-recombinant IL-8 chemoattractant (62nM) was added to the attractant port. The plate was then incubated for a further 2.5 hours before chemotactic activity was assessed.

## image acquisition

Image acquisition was obtained using either an inverted Nikon Eclipse TE2000U or the acumen eX3. The Nikon Eclipse TE2000U microscope was equipped with an automated stage, 1.5X objective and FITC filter set. For image analysis, the Metamorph software package (Molecular Devices) was used, employing the count nuclei algorithm for object identification. A 488 nm laser was employed for acumen eX3 that was compatible with the calcein-AM cell label and only the gradient region from each channel was scanned. For object identification, a ratiometric approach was used to determine cell number based on the total area to mean area of single cells.

# results

## interleukin-8 titration yields comparable EC50's between the two acquisition methods.

In order to compare the acquisition of chemotactic response data using either the acumen eX3 or the automated Nikon microscope, the migratory response of calcein-labelled human neutrophils to the chemoattractant, IL-8 was studied. In this study, a titration curve of serial dilutions of human recombinant IL-8 was established according to the described protocol and following incubation, the plate was scanned and imaged. Cell images taken within the gradient area over a range of dilutions of IL-8 are shown in Figure 2. The numbers 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.

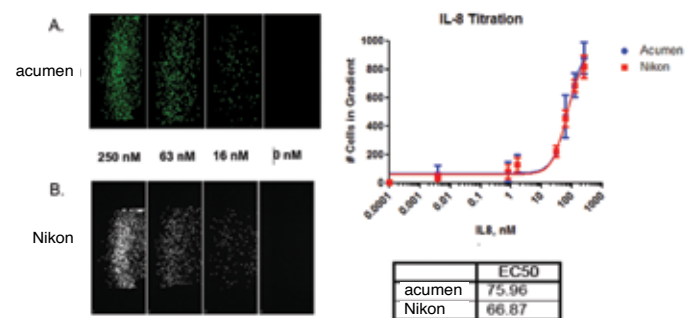


fig 2. Comparable images of calcein-labelled neutrophils in the presence of varying concentrations of IL-8 were obtained using either acumen eX3 (A) or a Nikon microscope (B). For the Nikon images, the gradient region was automatically cropped from the full image. (C) The number of cells which had migrated into the gradient area in the presence of varying concentrations of IL-8 were counted using either the acumen eX3 or Nikon microscope. It can be seen that comparable cell numbers of calcein-labelled neutrophils in the gradient of the iuvo™ Chemotaxis Assay plate are obtained with comparable EC<sub>50</sub>'s of IL-8 concentration.

It was seen from the full titration curve 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 eX3 is comparable to the object counting algorithm used for the image-based approach.

## analysis of inhibitor based potencies

In a further study, the effect of a chemotaxis inhibitor was assessed. Serial dilutions of wortmannin were pre-incubated with calcein-AM labelled neutrophils. Following addition of 62nM IL-8, the plate was incubated for 2.5 hours and a ratiometric estimation of cell number was assessed using the acumen eX3. The count nuclei object identification method was used with the Nikon/Metamorph® method. Figure 3 demonstrates that comparable cell counts are achieved using both methods of analysis of wortmannin inhibition.

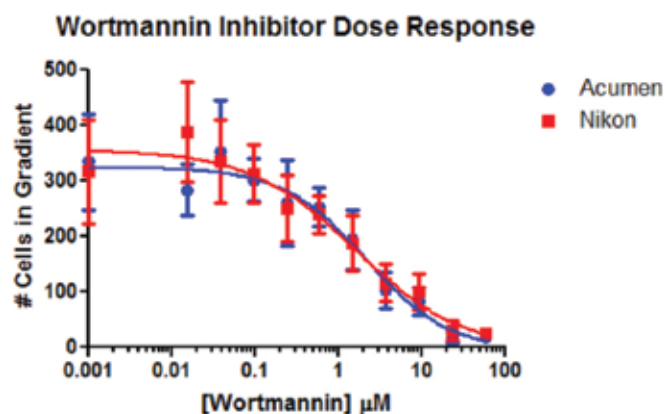


fig 3. Chemotaxis inhibitor potencies are comparable between the image and laser-based acquisition methods. Wortmannin was serially titrated 1:2 in assay medium and added to the plate according to the chemotaxis assay protocol. The inhibitory effect on cell migration in the presence of IL-8 was assessed using both acumen eX3 and Nikon acquisition methods. The ratiometric cell number estimation method used on the acumen eX3 and the count nuclei object identification method used with the Nikon/Metamorph method show comparable cell counts and potencies for wortmannin.

## data analysis

A comparison of the time taken for acquisition and data analysis of a chemotactic response experiment was carried out using either the acumen eX3 or the Nikon/Metamorph method. Table 1 shows that although the acquisition time between the two methods is identical. The simultaneous data acquisition and analysis performed by the acumen eX3 provides a significant advantage over processing time compared to the “off-line” analysis method using automated microscopy.

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

table 1. The integrated data acquisition and analysis capability of the acumen eX3 affords an approximate four-fold time saving per plate. Although the acquisition time is identical for both the acumen eX3 and the automated Nikon microscope, the additional time needed to process the images “off line” with the automated microscopy method significantly decreases throughput. This is particularly evident when extrapolating the total time that would be required to process 10 plates.

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 then being exported to Excel. The length of time involved in this process is particularly evident when extrapolating the total time that would be required to process 10 plates. Using acumen eX3, these 10 chemotaxis plates could be processed in less than one hour, whereas those same 10 plates would take over three hours with the Nikon/Metamorph combination. In conclusion, acumen eX3’s integrated data acquisition and analysis affords an approximate four-fold time savings per plate.

## discussion

This study highlights the ability to couple the novel iuvo™ chemotaxis assay technology from BellBrook Labs with the highly sensitive and rapid readout capabilities of TTP Labtech’s acumen eX3. Incorporating the miniaturisation of the iuvo™ chemotaxis assay with the “simultaneous data acquisition and analysis” analysis feature of the acumen provides fast data acquisition, thereby enabling increased throughput.

acumen eX3’s easy set up, alongside its simple template and software format eliminates the need for the definition of complicated algorithms used by automated microscope methods, resulting in a four-fold time saving per plate. acumen eX3’s unique optics provides the opportunity to add more dyes to enhance multiplexing within the same plate read time. These capabilities nicely complement the reduction in required cells and reagents accomplished by using the microconduit channels of the iuvo™ chemotaxis assay. The coupling of the iuvo™ chemotaxis assay with the TTP Labtech’s acumen eX3 high content imager provides a simple and efficient method for studying neutrophil chemotaxis significantly enhancing high-content screening analysis of chemotaxis assays.

## references

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- Kanegasaki, S., Nomura, Y., Nitta, N., Akiyama, S., Tamatani, T., Goshoh, Y., Yoshida, T., Sato, T. & Kikuchi, Y. (2003) A novel optical assay system for the quantitative measurement of chemotaxis. *J. Immunol. Methods.* 282: pp1-11.
- Meyvantsson, I., Vu, E., Lamers, C., Echeverria, D., Worzella, T., Echeverria, V., Skoien, A. & Hayes, S. (2011) Image-based analysis of primary human neutrophil chemotaxis in an automated direct-viewing assay. *J. Immunol. Methods.* 374: pp 70-7

# specifications

TTP Labtech's acumen® eX3 is a bench-top system which has been integrated with a wide array of other laboratory instrumentation, ranging from simple stacking robots through to complete plate preparation solutions to achieve walk-away operation in many application areas.

<b>Detection technology:</b>	Laser scanning imaging cytometry
<b>Laser excitation:</b>	Up to 3 solid state lasers in a single instrument (choice includes 405, 488, 561, 633 nm)
<b>Detection:</b>	4 colours simultaneously per laser using photomultiplier tubes
<b>Resolution:</b>	Equivalent to a 20x microscope objective
<b>Sample format:</b>	96, 384, 1536 and 3456 SBS-format microplates; slides
<b>Throughput:</b>	Typically 8 minutes regardless of plate type
<b>File size:</b>	Down to 50 KB per plate in HTS mode (CSV file)
<b>File export:</b>	Explorer® plate files, CSV, FCS and open source TIFF (8- & 16-bit)
<b>PC operating system:</b>	Microsoft® Windows® XP (Professional)
<b>Configurations:</b>	Stand-alone, self-maintained workstation or fully integrated
<b>Laser safety:</b>	Class 1 laser product
<b>Dimensions:</b>	670 mm x 504 mm x 350 mm (26 x 20 x 14") (w x d x h)
<b>Net weight:</b>	Approx 50 kg (110 lbs)
<b>Services:</b>	110/230V single phase 47/63 Hz 800W

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