Polymorphonuclear Cell Chemotaxis Assay Protocol: 
iuvo™ Chemotaxis Assay Plate
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## 1.0 Introduction

iuvo is a new technology platform with some similarities and also significant differences from the standard multiwell plate methods that you are accustomed to using. We have spent many hours in optimizing the methods for liquid dispensing, assay development and imaging in our own laboratory and in collaboration with field testers. Your success with the iuvo Chemotaxis Assay Plate requires close adherence to the methods defined in this protocol, so please read and follow them carefully and as always, we invite you to contact BellBrook Labs for personalized help should you have any questions.

This document provides instructions for using the iuvo Chemotaxis Assay Plate to investigate polymorphonuclear (PMN) cell chemotaxis. The term PMN generally refers to the population of leukocytes that have a multi-lobed nucleus, with neutrophils typically making up over 90% of the PMN population. The microfluidic channels are operated by a series of dispensing steps, and provide a stable gradient for at least 3 hours. Cells are placed at the tail end of the gradient, and movements occur along a horizontal surface into the gradient channel. The assay is read using fluorescence microscopy or high content analysis instruments. This protocol should be used as a guideline for primary cell chemotaxis studies as optimization of cell number, chemoattractant concentration, and cell labeling may be required for your cell type. For general liquid handling and imager optimization guidelines, please see Appendix B.
2.0 Evaporation Control

Follow these recommendations to avoid evaporation:

1. When working with the Chemotaxis Assay Plate, keep the air flow in the area to a minimum. If you are working in a hood, always have the laminar blower off.

2. Make sure to fill the saddle wells and perimeter moat area (see Figure 2) with 1X PBS prior to setting up your assay.

3. Limit as much as possible the time working with the device with the lid off (no more than 2 minutes).

4. Always store liquid filled devices in a humidified bioassay dish (see Figure 1) when not in use to prevent evaporation. We recommend a bioassay dish from Corning (catalog # 431111) lined with paper towels soaked with 1X PBS.

5. Minimize image acquisition time. When choosing an objective for imaging, we recommend not exceeding 5X magnification in order to minimize time the plate remains in the instrument. Longer acquisition times can promote evaporation, especially if the instrument runs warm.

Figure 1. Humidified bioassay dish.
3.0 Materials, Important Notes, and Pre-Assay Preparations

3.1 Materials
- iuvo Chemotaxis Assay Plate

The following materials are required, but not provided:
- Purified PMNs from human peripheral blood in assay medium
- Fluorescent live-cell label: calcein-AM (Invitrogen # C3100MP)
- Assay medium: RPMI 1640 (Sigma R7509), 10% heat-inactivated FBS (Gibco # 10100-147)
- 245 mm X 245 mm Bioassay dish (Corning # 431111)
- 1X PBS (w/o Ca^{2+} and Mg^{2+}) and paper towels
- 37˚C/5% CO_{2} incubator
- Chemoattractant: IL-8 (R&D Systems # 208-IL) or fMLP (Sigma # 47729), diluted in assay media
- Inhibitors: Latrunculin B (Calbiochem # 428020) or Wortmannin (MP Biomedicals # 195690)

3.2 Important Notes

Cell Handling
1. Handle PMNs gently and always centrifuge cells with NO brake to avoid activation.

Cell Labeling

*Important: It is best to purify and label cells just prior to use. Do not exceed 4 hours between blood draw and loading of cells into the plate.*

1. Centrifuge (at 300 x g) and resuspend cells in 1X PBS (w/o Ca^{2+} and Mg^{2+}) for calcein-AM labeling. We recommend resuspending at 1-4 x 10^6 cells/mL. Never resuspend cells at a higher concentration than 5 x10^6 cells/mL.
2. Add calcein-AM at a final concentration of 5 µM and invert to gently mix.
3. Incubate at 37˚C/5% CO_{2} for 15-20 minutes.
4. Centrifuge and gently resuspend the cells in assay medium.

Gradient Set-Up
1. The time required for formation of a stable gradient depends on the molecular weight of the chemoattractant.
2. For ligands in the 0.5-8 kDa range, such as IL8 and fmlp, it takes ~30 minutes to establish a stable gradient.

3.3 Pre-assay Preparations

See Figure 2 for schematic of the Chemotaxis Assay Plate and channel close-up.

1. To limit evaporation, prepare a humidified bioassay dish by saturating four paper towels with 1X PBS. Fold and place the towels in the bioassays dish in such a manner that they surround the entire perimeter of the dish at about a 2 inch width, leaving room for up to two plates. See Figure 1 for an example of a humidified bioassay dish.

2. Fill the moat located along the perimeter of the tray by pipetting 1X PBS until it reaches the top of the moat, but creating a negative meniscus.

3. Immediately before beginning the assay, fill saddle-shaped reservoirs located between the channels with 12 µL 1X PBS.

4. Resuspend pre-labeled PMNs in the assay medium at 4x10⁶ cells/mL.

Figure 2: Schematic of iuvo Chemotaxis Assay Plate and chemotaxis channel.
4.0 Chemotaxis Assay Protocol

1. Add 20 µL of assay medium (with or without 1X inhibitor) by rapidly dispensing (20 µL/sec) to the attractant addition port of the channel. The pipet tips should be as close to the port opening as possible; touching the tip to the port is OK. **Do not introduce bubbles into the channel.**

2. Add 3 µL of labeled PMNs (1 µL/sec) to the center of the cell addition port, lowering the tip slightly under the top of the liquid interface when dispensing. (Do not lower tip to the bottom of the port). **Do not introduce bubbles to the channel.**

3. Place the plate back into the bioassay dish. Allow cells to settle in the dish at room temperature for 10 minutes.

4. If testing inhibitor, place the bioassay dish into a 37°C/5% CO₂ tissue culture incubator for 30 minutes. This incubation serves as a pre-treatment with inhibitor before the addition of chemoattractant.

5. Add 3 µL of chemoattractant (1 µL/sec) to the attractant addition port, and immediately replace plate lid.

6. [optional] Image t=0 time point (only if t=0 images are desired).

7. Place the plate back into bioassay dish and place in 37°C/5% CO₂ incubator for 2.5 hrs.

8. Image cells in the gradient region (see Figure 4). Should you wish to normalize cell migration to cell number, see Appendix A for recommendations on image acquisition and object tracking strategies.
5.0 iuvo Chemotaxis Assay Plate Measurements

Figure 5. Chemotaxis Assay Plate measurements in millimeters. Top: Top view of the assay plate. Bottom: cross section view of the plate. The height of the gradient region is 90 µm. The refractive index of the COP material is 1.53.
6.0 Troubleshooting / FAQs

1.) The background / no ligand control is high (too many cells in the gradient region)
   a. The most frequent cause of high assay background is excessive evaporation. Ensure that the perimeter moat and saddles are filled with 1X PBS prior to setting up your assay.
   b. Incubate the assay plate in a secondary humidified container, such as a bioassay dish from Corning (part # 431111) containing PBS-soaked towels around the perimeter.
   c. Ensure the entire 20 µL of medium has been added to all channels. The liquid level in the cell port should be equal among all channels. There should be no liquid on the surface of the plate around the attractant port following the medium addition step.
   d. During the assay incubation, do not remove the assay plate from the incubator, nor remove the plate from the bioassay dish.
   e. After the assay incubation, proceed directly to imaging. Do not spend time manually observing the cells under the microscope prior to collecting the images.

2.) There is medium pooling on the surface of the plate, around the attractant addition port.
   a. Make sure pipette tips are centered as best possible in the center of the attractant port, as close to the port opening as possible.
   b. Use a flow rate of 20 µL/sec for medium addition to the channel. Keep the flow rate at 1 µL/sec for all other addition steps.

3.) Medium is wicking up the pipette tip and not going into the channel.
   a. Minimize tip wetting when aspirating from the source. Assay medium components, such as serum, can cause a protein coat on the tip if submerged too low, creating a wicking effect up the tip when liquid is dispensed to the channel.
   b. Set the dispense height as close to the port as possible. For the attractant addition port, the tip should be centered and may even go into the port slightly.

4.) What objective do you recommend using with the plate?
   a. To minimize evaporative effects, we recommend keeping the image acquisition time as short as possible. The use of 4X-5X magnification or less is recommended. If only 10X or greater magnification is available, try to minimize the number of fields required to capture chemotaxis in the gradient region.

5.) My chemotaxis response is variable.
   a. Do not introduce bubbles into the channels during liquid dispensing.
   b. Evaluate cell patterning in the port following cell dispensing. Cells should be evenly distributed across the channel, with a wall of cells at the border of the cell port:gradient
juncture. We have obtained the best neutrophil patterning by dispensing cells directly into the cell port, below the liquid meniscus, approximately 1 mm from the bottom of the channel. For larger, less buoyant cell types, a preformed droplet of cells touched off to the cell port, has provided the best patterning. If there is any liquid on the surface of the plate around the attractant addition port, this can impact the pumping of ligand into the channel, affecting gradient formation. Optimize liquid dispensing to eliminate pooling liquid around the attractant addition port.

c. Ensure that the proper evaporation control methods are in place.
d. Z’ data quality can be improved by normalizing the number of cells responding to the gradient to the number of cells present in the cell addition port within 700 µm of the cell port:gradient junction. See Appendix A to learn more about data normalization.

6.) How can I improve my data quality?
   a. Optimize cell patterning as described previously.
   b. Normalize the chemotaxis response as described in Appendix A. Data normalization has provided improved results in terms of Z’ and has no effect on EC_{50} values.

7.) How can I stop a chemotaxis assay?
   a. We have not yet developed a solution to stop a chemotaxis assay by adding a fixative, for example, to the channel.
   b. If image acquisition time is kept to a minimum, it is not necessary to stop the chemotaxis assay. Using 4X magnification and an image acquisition time of 10 minutes (per plate), we have not observed any changes in the chemotaxis response from the first imaged channel to the last.

8.) How stable is the assay?
   a. Plates can be removed from the incubator and stored at room temperature for one hour prior to imaging without affecting data quality. Z’ was shown not to be affected when imaging one hour after the plate was removed from the incubator and left at room temperature in a bioassay dish in comparison to a plate that was imaged immediately after removal from the incubator. On occasion we have noticed an increase in assay background with the additional one hour incubation at room temperature.
7.0 Appendix A: Data Normalization

Introduction
Chemotaxis can be quantified in the iuvo Chemotaxis Assay Plate by simply counting the number of cells in the gradient region. However, initial cell patterning in the cell port proximal to the gradient affects the number of cells that have the potential to migrate into the gradient region. Repeatability of cell patterning may depend on the cell type as well as the cell dispenser. If repeatability of cell patterning is questionable, normalizing data to the number of cells proximal to the gradient region may improve data quality. Below is a recommendation for this type of normalization.

Materials
Chemotaxis Assay Plate
Reagents and cells used in desired chemotaxis assay
Software that can log X-coordinates of cells.

Methods
1) After chemotaxis assay is complete, acquire images of cells in the entire gradient and cell port. (Figure 6).
2) Log X-coordinates of cells in gradient and cell port.
3) Normalize chemotaxis by:

\[
\frac{\text{# cells in gradient}}{\text{# cells in gradient} + \text{# cells 700 µm into the cell port}} \quad \text{(see Figure 6)}
\]

Note: 700 µm was determined empirically to increase data quality

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Figure 6: Polymorphonuclear (PMN) cell chemotaxis in the presence of IL-8. Shown is an example image of the gradient and cell port area acquisition required to normalize chemotaxis.
Note:
1) Presence of a chemoattractant may alter the number of cells present 700 µm into the cell port in comparison to cells with no chemoattractant. This can be avoided by normalizing based on images acquired at T=0. However, the data below strongly suggest that normalization by image acquisition only at T=2.5h will give comparable data to normalization to T=0.

Figure 7: A comparison of PMN chemotaxis to IL-8 dose response curves generated by normalization to cells 700 µm in the cell port at T=0 vs. T=2.5h.

A. Gradient Cell Count

<table>
<thead>
<tr>
<th>chemo</th>
<th>avg</th>
<th>stdev</th>
<th>cv</th>
<th>Z</th>
</tr>
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<tbody>
<tr>
<td>-</td>
<td>0.29</td>
<td>0.53</td>
<td>187%</td>
<td>0.42</td>
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<tr>
<td>+</td>
<td>784</td>
<td>152</td>
<td>19%</td>
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B. T0 Normalization

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<th>cv</th>
<th>Z</th>
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</thead>
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C. T2.5h Normalization

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<th>stdev</th>
<th>cv</th>
<th>Z</th>
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Table 1. Data quality comparison of PMN chemotaxis to IL-8. Data was analyzed by A) counting cells in the gradient, B) T=0 normalization, or C) T=2.5h normalization.
8.0 Appendix B: Instrument Integration Guidelines

Introduction

- This protocol provides guidelines on how to set up liquid handling and imaging instrumentation for BellBrook Labs’ iuvo Chemotaxis Assay Plate.
- The protocol uses mock materials for setting up a chemotaxis assay and to find the region of interest in the plate. Please see the iuvo Chemotaxis Assay Protocol that contains detailed instructions for performing the assay with cells. Please note that various instruments and software operate differently; this document does not contain instrument-specific instructions, but general guidelines that can apply to all platforms.

Note: This protocol can also be used as a guideline for those evaluating the plate manually, without liquid handling instrumentation. In those cases, we recommend the use of a manual single channel pipettor for addition of 20 µL volumes, and an electronic single channel repeater pipette (Matrix or equivalent) for all 3 µL liquid dispensings.

Evaporation Control

Working with the Chemotaxis Assay Plate requires dispensing of low-volume droplets that are more sensitive to evaporation than assays in a well format. Follow the instructions in section 2.0 for Evaporation Control.

Materials Required for Instrument Optimization

- Empty iuvo Chemotaxis Assay Plate
- Assay medium containing a fluorescent dye and fluorescent beads or labeled cells. If your assay medium contains serum or BSA, be sure to use it for this optimization protocol. As an example, this protocol uses FITC beads at 1x10^6 beads/mL in 10% serum containing medium with 1 µM Fluorescein.
- Bioassay dish (or equivalent) from Corning (part # 431111), lined with moistened paper towels (see Figure 6)
- Liquid handler able to delivery volumes from 3 µL – 20 µL with a % CV ≤ 10%
- Imaging instrumentation with precise plate positioning, and the ability to capture an area 1 mm (width) x 2 mm (length)

Liquid Handling

The following tasks will take you step-by-step through the liquid handling process for an automated chemotaxis assay. Five main tasks will be addressed: tip positioning within the ports of the plate, dispensing PBS to the saddles, and dispensing mock assay components for initial medium addition, chemoattractant addition, and cell addition.

Tip Positioning

Use an empty iuvo Chemotaxis Assay Plate.
Refer to figures 2 and 5 for Chemotaxis Assay Plate naming conventions and plate measurements.
1. **Saddle positioning:** The saddles are located at the quadrant 2 location on a 384-well grid. Center the tips within the saddle region as much as possible. Note: as there are 84 saddles, the top row of tips must be removed for saddle filling, or leave all tips on and use a reservoir lacking PBS at row A location.

2. **Attractant addition port positioning:** The attractant port is located at the quadrant 1 position on a 384-well grid. Modify XY position, if needed, to center tips above the attractant addition ports. It is normal for some tips to be off-center due to imperfect tip alignment.

3. **Cell addition port positioning:** The cell port is located at the quadrant 3 position on a 384-well grid. Modify XY position, if needed, to center tips in the cell addition ports.

4. **Attractant port addition dispense height:** Lower tips into the attractant addition ports. It is normal for some tips to not enter the port due to imperfect tip alignment. To determine if tips are inside of the port, gently push the tips with your finger; if the tips are inside of the ports, they will move slightly when pushed, but stop when they hit the edge of the port. If the tips are outside of the ports, they will move freely when gently pushed.

5. **Cell addition port dispense height:** Lower tips into the cell addition port approximately 1 mm from the bottom of the cell port. When the channel is filled with liquid, the tips should submerge below the liquid level, but not touch the bottom of the port.

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**Important considerations when dispensing to iuvo**

1. Before you begin dispensing liquid to the iuvo Chemotaxis Assay Plate, fill the moat and saddle regions of the plate as instructed in section 3.3 Pre-Assay Preparations.

2. We recommend first using water to test pipetting heights for the following dispense steps. You can remove the water and re-use the plate by completely aspirating the channel contents from the attractant port, then place the plate in a high air flow environment (ie grate of the tissue culture hood) for 20 minutes to allow for complete drying of the channel. Once water dispensing looks good, dry the channels and proceed with the addition of mock assay components. **We do not recommend re-using the plate once serum-containing media has been added.**

3. Using a minimized tip wetting technique, such as liquid level tracking or sensing, when aspirating from the source plate helps prevent droplet wicking up the pipette tip when dispensing to the plate. This is especially important if the source liquid contains serum. Preventing droplet wicking helps ensure that the entire volume of liquid is delivered to the channel.

**Dispensing to iuvo**

The goal is for 100 % dispensing success at each step:

1. Dispense 12 µL of 1X PBS at a speed of **1 µL/second** to the center of the saddle region.

2. To mimic medium addition in the assay: Dispense 20 µL of diluted FITC beads in 10% serum-containing medium with 1 µM fluorescein at a speed of **20 µL/second** in the center of the attractant addition port at the Attractant Port Addition height previously configured. **Do not introduce air bubbles to the channel.**
3. To mimic chemoattractant addition in the assay: Dispense 3 µL of diluted FITC beads in 10% serum-containing medium with 1 µM fluorescein at a speed of 1 µL/second in the center of the attractant addition port at the Attractant Port Addition height. Do not introduce air bubbles to the channel.

4. To mimic cell addition in the assay: Dispense 3 µL of diluted beads in 10% serum-containing medium with 1 µM fluorescein at a speed of 1 µL/second to the center of the cell addition port at the Cell Port Addition height previously configured. Do not introduce air bubbles to the channel.

5. Replace lid and place plate into room temperature humidified bioassay dish.

6. Allow beads to settle for 15 minutes. Image the plate.

**Imaging**
The presence of the dye and beads in the channel will help with visualization of the channel borders and with focusing, respectively.

1. Set the exposure to a level that allows you to visualize the fluorescent dye in the channel.
2. Locate the gradient region (figure 4) to the left of the cell addition port. The edge of the cell addition port should appear brighter than the gradient region. See figures 8 and 9 below.
3. Use a low power objective, or a higher power objective with image stitching, to acquire the 1 mm (width) x 2 mm (length) region of interest.

**Figure 8.** Gradient region acquisition using 1X objective (~90% of the full channel in view), FITC detection, 250 ms exposure.
Figure 9. A portion of the gradient region captured with a 10X objective, FITC detection, 100 ms exposure.

Gradient region: Cell port

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