INSTRUCTIONS

iuvo™ Chemotaxis Assay Plate

6006 2389.0

Number	Description
6006	**iuvo Chemotaxis Assay Plate,** 1 × 96 microchannel plate

Storage: Upon receipt store product at room temperature. Product shipped at ambient temperature.

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Introduction

The iuvo Chemotaxis Assay Plate is a microconduit array plate compatible with automated liquid handling and high-content screening (HCS) and analysis (HCA) platforms. All Thermo Scientific high-content platforms, including the Thermo Scientific ArrayScan VTI HCS Reader and CellInsight Image Cytometer, are optimized for use with the iuvo Plates, providing a simple workflow with sophisticated image analysis to deliver multi-parameter data on cell function. Constructed as a single piece with no filters or membranes, each plate is compliant with the SBS/ANSI standard for microplates.

Chemotaxis is the natural movement of cells in the direction of a chemical gradient. The iuvo Plates form a stable chemical gradient inside the microchannel for a minimum of 3 hours. Microscopic imaging of cell movement into the gradient region enables quantitative data analysis on cell migration and morphology. A common use for the iuvo Chemotaxis Assay Plate is investigating polymorphonuclear (PMN) cell chemotaxis.

Procedure Summary

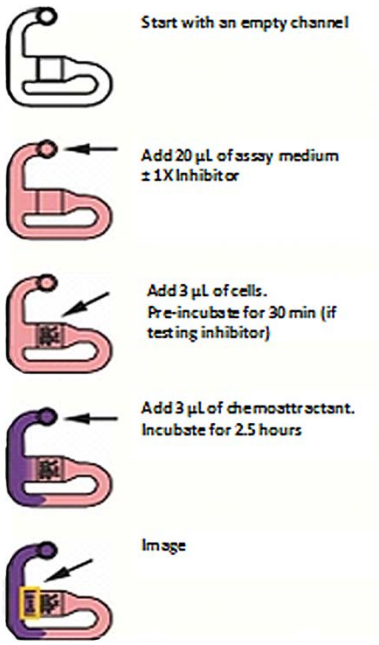

Figure 1. Schematic of assay protocol using a single plate channel.

Important Product Information

- Use this protocol as a guideline for primary cell chemotaxis studies. Optimization of cell number, chemoattractant concentration and cell labeling may be required for each cell type. For general liquid handling and imager optimization guidelines, see Appendix B: Instrument Integration Guidelines.
- Purify and label cells immediately before use. Do not exceed 4 hours between blood draw and loading of cells into the plate.
- Handle PMNs gently. Centrifuge cells with **NO** brake to avoid activation.
- The time required for stable gradient formation is dependent on the chemoattractant molecular weight. Approximately 30 minutes is required to establish a stable gradient for ligands of 0.5-8kDa (e.g., IL8, FMLP).
- For calcein-AM labeling:

  1. Centrifuge cells at 300 × *g* and resuspend in 1X phosphate-buffered saline (PBS) to a cell density of 1-4 × 10^6 cells/mL. Do not resuspend cells at a concentration > 5 × 10^6 cells/mL.
  2. Add calcein-AM to the cells at 5µM final concentration and invert tube to gently mix.
  3. Incubate at 37˚C in 5% CO_2 for 15-20 minutes.
  4. Centrifuge and gently resuspend the cells in assay medium.

Evaporation Control

NOTE: WARNING REGARDING EVAPORATION. READ THE FOLLOWING SECTIONS CAREFULLY.

- Use caution when handling any iuvo Plate. The plate holds extremely low volume droplets, which are far more sensitive to evaporation than assays in a well format.
- Evaporation of media causes undesirable flow within the channel, which may result in increased background. The moat and saddle features of this plate are designed to prevent evaporation.

  Note: Use the moat and saddle features as well as a secondary humidified container to avoid evaporation.

- When working with the plate, minimize air flow in the working area. Turn off the laminar blower when working in a hood.
- Fill the saddle wells and perimeter moat area (Figure 2) with 1X PBS before setting up the assay.

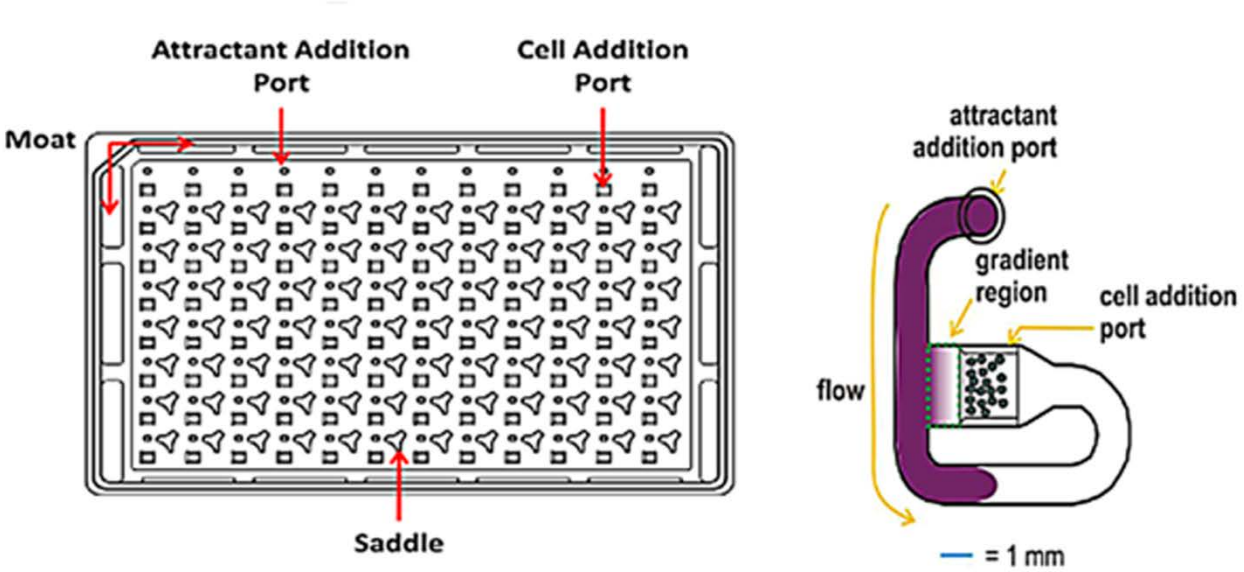

Figure 2. Overview of the iuvo Chemotaxis Assay Plate and detail of an individual chemotaxis channel.

- Reduce plate-handling time to < 2 minutes whenever the plate is uncovered.
- To prevent evaporation when not in use, store the liquid-filled plate in a secondary humidified container with a lid that allows for gas exchange. Surround the inside perimeter of the dish with clean paper towels saturated with sterile PBS (Figure 3). Bioassay dishes (e.g., Corning No. 431111) are optimal as secondary containers.

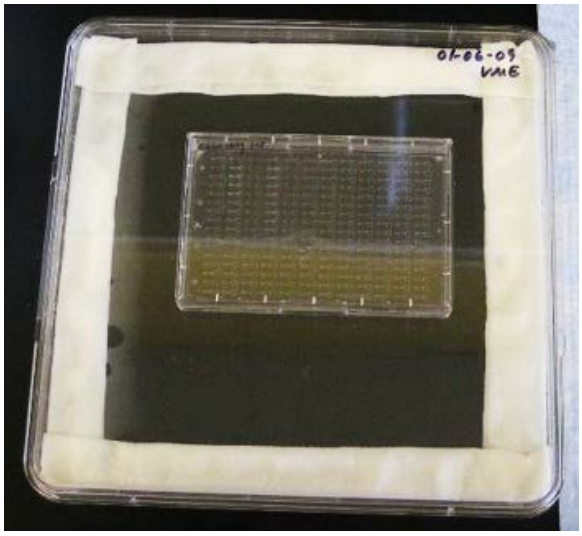

Figure 3. Lidded secondary container holding the microchannel plate and PBS-saturated towels.

- Minimize image acquisition time. When imaging cells on the plate, do not exceed a 5X magnification objective lens to minimize the image acquisition time of the instrument.

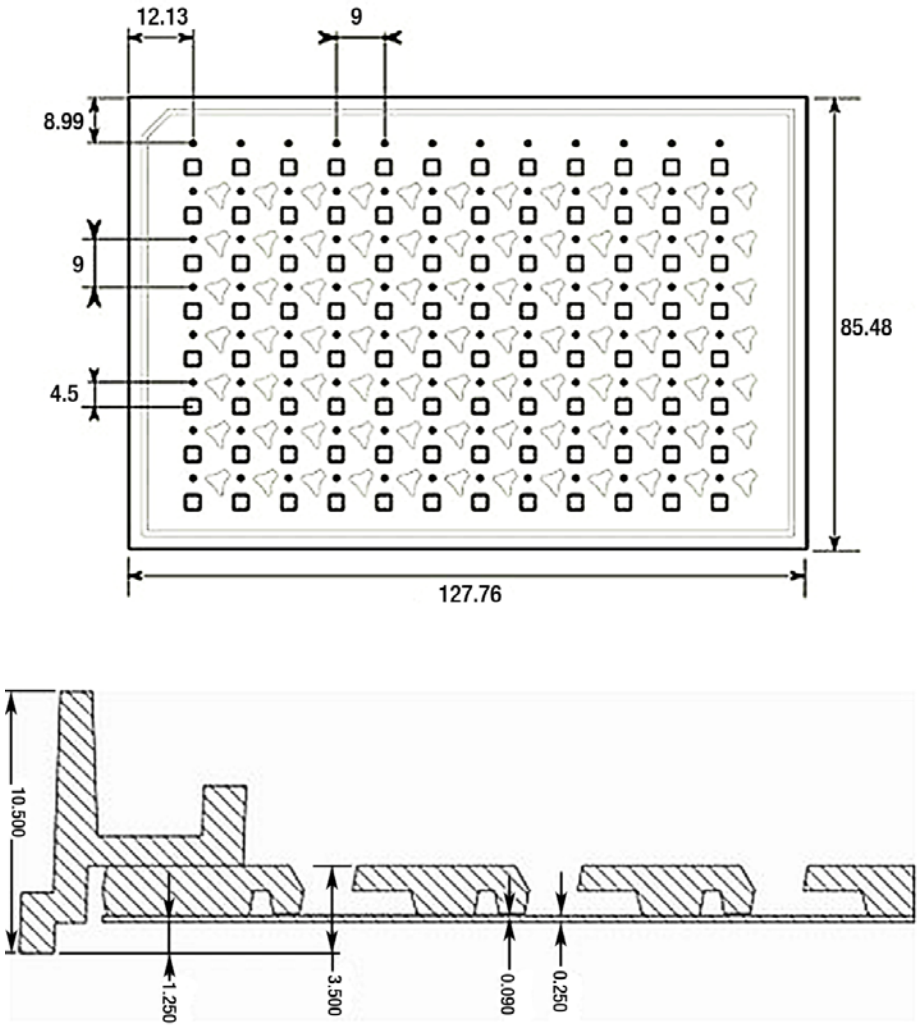

Figure 4. Top view and cross-sectional side view of the chemotaxis plate with measurements in millimeters. The height of the gradient region is 90µm. The refractive index of the COP material is 1.53.

Table 1. Plate form factors for chemotaxis assays using the Thermo Scientific ArrayScan VTI HCS Reader.

Parameter	Text or Value to Enter*	Parameter	Text or Value to Enter*
Description	iuvo Plate for 2D, 3D culture	**Well Shape**	Square
Item Number	0	**Well Pitch X**	8954
Columns	12	**Well Pitch Y**	8954
Rows	8	**First Well Center X**	-116518.5
Physical Width	85480	**First Well Center Y**	14340.5
Physical Length	127760	**First Well Center Z**	-2724.191667
Physical Height	14720	**Last Well Center X**	0
Well Width	1000	**Last Well Center Y**	0
Well Height	2000	**Last Well Center Z**	0

* For correct form factors on different equipment, consult your equipment manual or manufacturer.

Additional Materials Required

- Purified PMNs from human peripheral blood in assay medium
- Conical tube
- Fluorescent live-cell label: calcein-AM (Invitrogen No. C3100MP)
- Assay medium: RPMI 1640, 10% heat-inactivated FBS
- 245mm × 245mm bioassay dish (Corning No. 431111)
- 1X PBS (containing no Ca^{2+} and Mg^{2+})
- Paper towels
- 5% CO_2 incubator capable of 37°C
- Chemoattractant: IL-8 or fMLP; diluted in assay media
- Inhibitors: Latrunculin B or Wortmannin

Chemotaxis Assay Protocol

Note: For optimal results, follow the preparation steps below before beginning the assay.

- To limit evaporation, prepare a humidified bioassay dish by saturating four paper towels with 1X PBS. Fold and place the towels in the bioassay dish so the towels surround the entire perimeter of the dish at ~50mm width; leave room for up to two plates. See Figure 3 for an example of a humidified bioassay dish.
- Fill the moat located along the perimeter of the tray with 1X PBS until it reaches the top.
- Before beginning the assay, fill the saddle-shaped reservoirs located between the channels with 12µL of 1X PBS.
- Resuspend pre-labeled PMNs in the assay medium at 4×10^6 cells/mL.

1. Add 20µL of assay medium (with or without 1X inhibitor) to the attractant addition port of the channel (Figure 1) by dispensing rapidly (20µL/sec). Ensure pipette tips remain close to the port opening; touching the tip to the port is acceptable. Do not introduce bubbles into the channel.
2. Add 3µL of labeled PMNs (1µL/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 an inhibitor is tested, place the bioassay dish into a 37˚C/5% CO_2 tissue culture incubator for 30 minutes. This incubation serves as a pre-treatment with the inhibitor before the addition of chemoattractant.
5. Add 3µL of chemoattractant (1µL/sec) to the attractant addition port and immediately cover the plate with the lid.
6. Image the t = 0 time point (optional).
7. Place the plate back into the bioassay dish in the 37˚C/5% CO_2 incubator for 2.5 hours.
8. Image cells in the gradient region (Figure 5). If cell migration to cell number normalization is desired, see Figure 6.

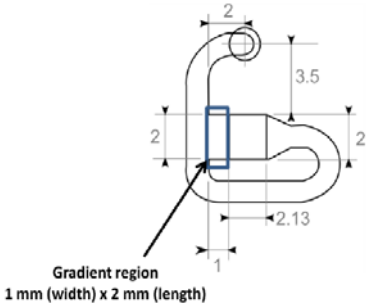

Figure 5. Gradient region dimensions for imaging.

Troubleshooting

Problem	Possible Cause	Solution
High background	Excessive evaporation	Ensure perimeter moat and saddles are filled with 1X PBS before setting up the assay
		Incubate assay plate in a secondary humidified container, such as a bioassay dish (Corning No. 431111) containing PBS-soaked towels around the perimeter
		Ensure the entire 20µL of media has been added to all channels
		Confirm liquid levels in the channel cell ports are equal
		Following media addition, ensure there is no liquid on the plate surface around the attractant port following the media addition step
		During assay incubation, do not remove the assay plate from the incubator or bioassay dish
		After assay incubation, proceed directly to imaging; do not manually observe cells under microscope before collecting the images
Media pools on plate surface near the attractant addition port	Tip was positioned off-center	Position pipette tips at the center of the attractant port, directly over the port opening
	Dispensing flow rate was not optimal	Use a flow rate of 20µL/sec for media addition to the channel; maintain flow rate at 1µL/sec for all other addition steps
Media wicks up pipette tip and not into the channel	Tip submerged too low in assay media (e.g., serum), causing a protein coat to form on the tip	Minimize tip wetting when aspirating from the source
	Tip positioning was off-center	Set 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
Variable chemotaxis response	Bubbles were in the channels	Avoid introducing bubbles into the channels during liquid dispensing
	Cell distribution was not uniform across the channel	Evaluate cell patterning in the port following cell dispensing **Note:** Optimal neutrophil patterning occurs by dispensing cells directly into the cell port, below the liquid meniscus, ~1mm from the bottom of the channel. For larger, less buoyant cell types, a droplet of cells touched to the cell port provides optimal patterning
	Liquid surrounded the attractant addition port	Optimize liquid dispensing to eliminate pooling liquid around the attractant addition port
	Evaporation occurred	Ensure proper evaporation control methods are in place
	Variable cell number affected the Z´ data quality	Improve Z´ data quality by normalizing the number of cells responding to the gradient to the number of cells present in the cell addition port within 700µm of the cell port: gradient junction (see Appendix A: Data Normalization)
Poor data quality	Uneven cell patterning	Optimize cell patterning as previously described
	Variation in Z´ data	Normalize chemotaxis response (see Appendix A: Data Normalization) **Note:** Data normalization improves Z´ data; however, it does not affect EC_{50} values

Appendix A: Data Normalization

Quantify chemotaxis in the iuvo Plate by counting the number of cells in the gradient region; be aware that initial cell spreading 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 spreading is dependent on cell concentration, cell type and cell dispenser. If cell-patterning repeatability becomes uncertain, then normalizing data to the number of cells proximal to the gradient region may improve data quality.

Data Normalization Method

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

Note: The 700µm region is empirically determined to increase data quality.

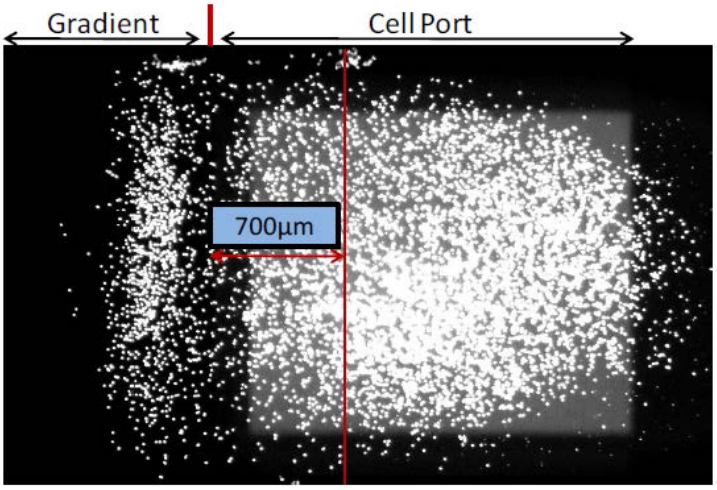

Figure 6. Polymorphonuclear (PMN) cell chemotaxis in the presence of IL-8. Shown is an example image of the gradient and cell port areas required to normalize chemotaxis.

Note: Chemoattractant cell migration may alter the number of cells present in the 700µm region of the cell port when compared to the cell number in that region with no chemoattractant. Avoid this alteration by normalizing with images acquired at t = 0. The data below strongly suggest that normalization by image acquisition only at t = 2.5 hours will give comparable data to normalization to t = 0 (Figure 7 and Table 1).

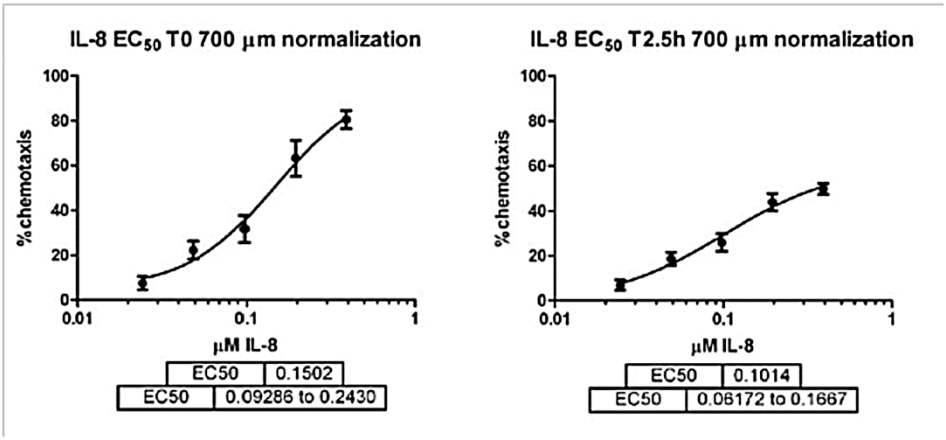

Figure 7. Comparison of PMN chemotaxis to IL-8 dose response curves generated by normalization to cells in a 700µm area in the cell port at t = 0 vs. t = 2.5 hours.

Table 1. Data quality comparison of PMN chemotaxis to IL-8. Data were analyzed by A. Counting cells in the gradient, B. t = 0 Normalization, or C. t = 2.5 hours Normalization.

A. Gradient Cell Count

Chemoattractant	Average	STDEV	CV	Z'
–	0.29	0.53	187%	0.42
+	784	152	19%	

B. t = 0 Normalization

Chemoattractant	Average	STDEV	CV	Z'
–	0%	0%	135%	0.60
+	75%	10%	13%	

C. t = 2.5 hours Normalization

Chemoattractant	Average	STDEV	CV	Z'
–	0%	0%	206%	0.57
+	37%	5%	14%	

Correlation Coefficient: 0.96

Appendix B: Instrument Integration Guidelines

Use these protocols as general guidelines for setting up high-content liquid handling and imaging instrumentation for the iuvo Plate. Follow the specific instrument and software instructions for the experimental equipment used. Mock materials are initially used to set up a chemotaxis assay and find the region of interest in the plate (see the Chemotaxis Assay Protocol).

Note: Different instruments and software may require optimization.

Note: This protocol is also a guideline for manually evaluating plates without liquid handling instrumentation. Use a manual single channel pipette for addition of 20µL volumes and an electronic single channel repeater pipette (Matrix or equivalent) for 3µL liquid dispensing.

A. Materials Required for Instrument Optimization

- Empty iuvo Plate
- Assay medium containing a fluorescent dye and fluorescent beads or labeled cells. If assay medium contains serum or BSA, use for this optimization protocol. For example, this protocol uses FITC beads at 1×10^6 beads/mL in 10% serum-containing medium with 1µM fluorescein
- Bioassay dish (or equivalent) (Corning No. 431111) lined with moistened paper towels (Figure 2)
- Liquid handler capable of delivering 3-20µL volumes with a %CV ≤ 10%
- Imaging instrumentation with precise plate positioning and the ability to capture an area of 1mm (width) × 2mm (length)

B. Liquid Handling

The following protocols go through the liquid handling process for an automated chemotaxis assay. Five main tasks are addressed: tip positioning within the ports of the plate, dispensing PBS to the saddles, dispensing mock assay components for initial medium addition, chemoattractant addition, and cell addition.

Tip Positioning

Note: Use an empty iuvo Plate. Refer to Figures 2 and 4 for iuvo Plate naming conventions and plate measurements.

1. **Saddle Positioning:** Center the tips within the saddle region as much as possible.

   Note: Do not fill the top row of the plate or leave all tips on and use a reservoir lacking PBS at the row A location.

2. **Attractant Addition Port Positioning:** 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:** 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 ~1mm 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.

Important Considerations for Dispensing to iuvo Chemotaxis Assay Plates

1. Before you begin dispensing liquid to the iuvo Chemotaxis Assay Plate, fill the moat and saddle regions of the plate as instructed in the Evaporation Control Section.
2. Use ultrapure water to test pipetting heights for the following dispense steps: Remove the water and reuse the plate by completely aspirating the channel contents from the attractant port. Place the plate in a high air flow environment (i.e., grate of the tissue culture hood) for 20 minutes to allow for complete drying of the channel. If water dispensing appears optimized, dry the channels and proceed with the addition of mock assay components.

   Note: Do not reuse the plate after adding serum-containing media.
3. Use a minimized tip-wetting technique (e.g., liquid level tracking or sensing) when aspirating from the source plate to prevent droplet wicking up the pipette tip during dispensing. This is particularly 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 the iuvo Device

1. Dispense 12µL of 1X PBS at a speed of 1µL/second to the center of the saddle region.
2. To mimic medium addition in the assay: Dispense 20µL of diluted FITC beads in 10% serum-containing medium with 1µM fluorescein at a speed of 20µL/second in the center of the attractant addition port at the Attractant Port Addition height previously configured. **Do not introduce air bubbles into the channel.**
3. To mimic chemoattractant addition in the assay: Dispense 3µL of diluted FITC beads in 10% serum-containing medium with 1µM fluorescein at a speed of 1µL/second in the center of the attractant addition port at the Attractant Port Addition height. **Do not introduce air bubbles into the channel**.
4. To mimic cell addition in the assay: Dispense 3µL of diluted beads in 10% serum-containing medium with 1µM fluorescein at a speed of 1µL/second to the center of the cell addition port at the Cell Port Addition height previously configured. **Do not introduce air bubbles into the channel**.
5. Replace lid and place plate into room temperature, humidified bioassay dish.
6. Allow beads to settle for 15 minutes.
7. Image the plate.

Imaging

Note: Dye and beads in the channel help visualization of the channel borders and focusing, respectively.

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

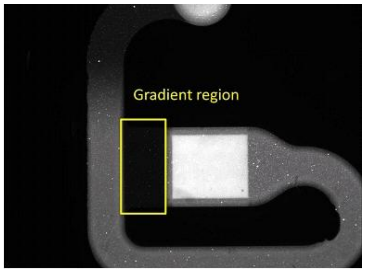

Figure 8. Gradient region acquisition using a 1X objective (~90% of the full channel in view) with FITC detection and a 250ms exposure.

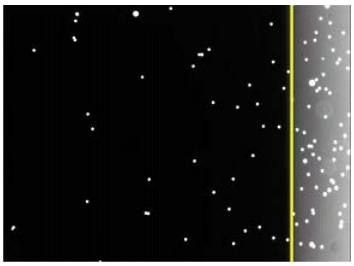

Figure 9. A portion of the gradient region captured with a 10X objective with FITC detection and a 100ms exposure.

Related Products

6002 **iuvo Microchannel 5250 Assay Plate,** 1 × 192 microchannel plate

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