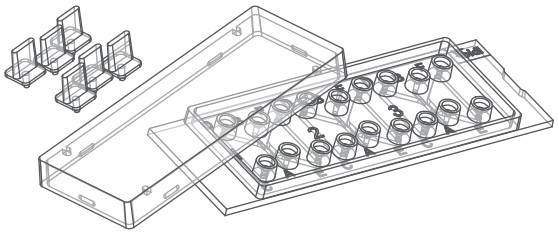
µ-Slide Chemotaxis

Instruction Manual



The ibidi labware comprises a variety of µ-Slides, µ-Dishes, and µ-Plates, which have all been designed for high-end microscopic analysis of fixed or living cells. The high optical quality of the ibidi Polymer Coverslip is similar to that of glass, enabling a variety of recommended microscopy techniques with uncompromised resolution and choice of wavelength.

The µ-Slide Chemotaxis is designed for observing the chemotactic response of cells exposed to chemical gradients. It works for adherent cells on a 2D surface as well as for cells being embedded in a 3D gel matrix.

This document applies to the following products:

80326	**µ-Slide Chemotaxis ibiTreat**
80322	**µ-Slide Chemotaxis Collagen IV**

Contents

1 General

1.1 Material

The µ-Slide Chemotaxis is made of a polymer that has the highest optical quality. The ibidi Polymer Coverslip bottom exhibits extremely low birefringence and autofluorescence, similar to that of glass. It is not possible to detach the bottom from the upper part. The slide is intended for one-time use and is not autoclavable, since it is only temperature-stable up to 80 °C/175 °F. Please note that gas exchange between the medium and the incubator's atmosphere occurs partially through the polymer coverslip, which should not be covered.

Optical Properties of Polymer Coverslip	
Refractive index (589 nm)	1.52
Abbe number	56
Thickness	No. 1.5 (180 µm)
Material	Polymer

WARNING – The ibidi Polymer Coverslip is compatible with certain types of immersion oil only. A list of suitable oils can be found in the Section "Immersion Oil".

1.2 Shipping and Storage

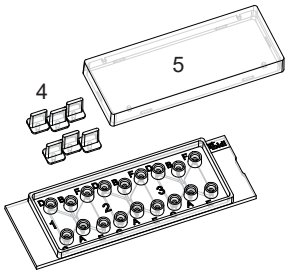
This product is sterilized and sealed in a gas-permeable packaging. The shelf life under proper storage conditions (in a dry place, no direct sunlight) is outlined in the following table.

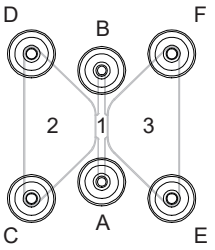
Conditions	
Shipping conditions	Ambient
Storage conditions	RT (15–25 °C)

Shelf Life	
ibiTreat	36 months
Collagen IV	18 months

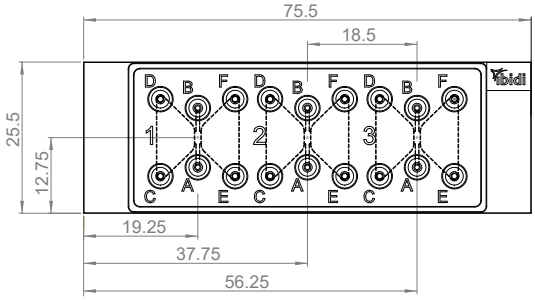
1.3 Geometry

The µ-Slide Chemotaxis provides a standard slide format according to ISO 8037/1.





1) Observation area
2) Left reservoir
3) Right reservoir
4) Plugs
5) Cultivation lid
A–F) Filling ports



Specifications	
Outer dimensions (w × l)	25.5 × 75.5 mm²
Chambers	3
Volume per chamber	
- Full chamber	130 µl
- Observation area only	6 µl
Chemoattractant volume	30 µl
Observation area	2 × 1 mm²
Coating area per chamber	
- Full chamber	3.5 cm²
- Observation area only	0.27 cm²
Growth area per chamber	
- Full chamber	1.24 cm²
- Observation area only	0.06 cm²
Distance between chambers	18.5 mm
Height with plugs	12 mm
Bottom	ibidi Polymer Coverslip

1.4 Surface

The µ-Slide Chemotaxis is available with either an ibiTreat or a Collagen Type IV-coated surface.

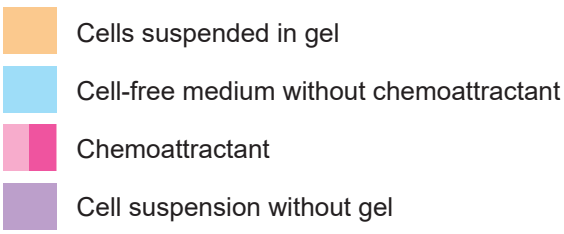
The tissue culture-treated, hydrophilic ibiTreat surface of the ibidi Polymer Coverslip is ideal for culturing adherent cells. It ensures excellent cell adhesion without the necessity for any additional coatings. Nonetheless, extracellular matrix (ECM) protein coatings can be applied to the ibiTreat surface without any restrictions, if required.

The Collagen Type IV-coated surface enhances the adhesion and growth of various cell lines. A high-quality Collagen IV solution (Corning #356233) is used to pre-coat the µ-Slide Chemotaxis. Unlike when using collagen gels, this coating is limited to the surface of the observation area, specifically designed to support cell adhesion in 2D chemotaxis experiments.

1.5 Principle

The µ-Slide Chemotaxis creates a stable, linear chemoattractant gradient between two side reservoirs that diffuse into a central observation channel. Cells are placed in this central area, either on a 2D surface or in a 3D gel. After filling the reservoirs with medium containing different chemoattractant concentrations, a defined gradient forms and remains stable for over 48 hours. This design enables reliable time-lapse imaging and precise analysis of directed cell migration.

1.6 Color Code



Color codes in this document.

NOTE – Please follow all of the steps in this document carefully to ensure correct handling.

NOTE – Please read the Troubleshooting Section 5 before starting your experiment.

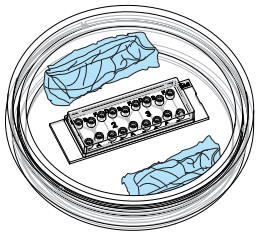
NOTE – Please also read the Application Chapter “Chemotaxis” before starting your experiment.

2 Before the Experiment

2.1 Required Materials and Equipment

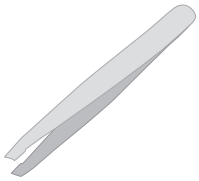
When conducting chemotaxis experiments with the µ-Slide Chemotaxis, it is essential to use the following materials and equipment:

- Cells: For information on suitable cell types, please refer to the “Questions to Ask” section in the Application Chapter “Chemotaxis”. Additional examples include chemotaxis with HUVECs in Application Note 34: Chemotaxis of HUVEC in 2D and 3D, with non-adherent cells in Application Note 23: 3D Chemotaxis Protocol with bovine Collagen I Gel for Dendritic Cells, and with HT-1080 cells in Application Note 24: Chemotaxis of HT-1080 cells in 2D and 3D.
- Gel matrix compatible with the cells (optional): Recommended gel matrices include Collagen I gels (bovine or rat tail), Matrigel®, or similar hydrogels. For detailed protocols on Collagen I gels, refer to Application Note 26: Preparation of Collagen I Gels.
- Humid chamber: Use a 10 cm Petri dish containing a lint-free moistened tissue to prevent evaporation during incubation. The humidity of the incubator alone is not sufficient.



Humid chamber with wet lint-free tissue.

- Optimal conditions for live cell imaging (e.g., stage top incubator with temperature and CO_2 control)
- Inverted microscope (phase contrast or fluorescence)
- Time-lapse video equipment (camera and image acquisition software)
- Motorized stage and autofocus (x, y, z) (optional) to observe all three chambers in parallel.
- Slant-tip tweezers for plug handling: Always handle the plugs with the recommended tweezers to prevent contamination or damage.

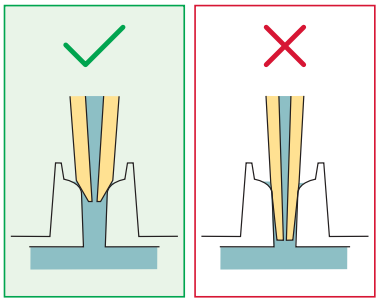


Example of slant tweezers.

- Pipets (e.g., Gilson P-20); serviced and calibrated routinely.
- Compatible, beveled 10–200 µl pipet tips:

Supplier	Ordering Number
Axygen	T-200-C, TR-222-C, TR-222-Y, or related tips
STARLAB	TipOne S1111-1816, S1111-1710, S1120-1840, or related tips
Sorenson BioScience	MultiFit Tip 10520, 10590, or related tips

Example of a compatible, beveled pipet tip.

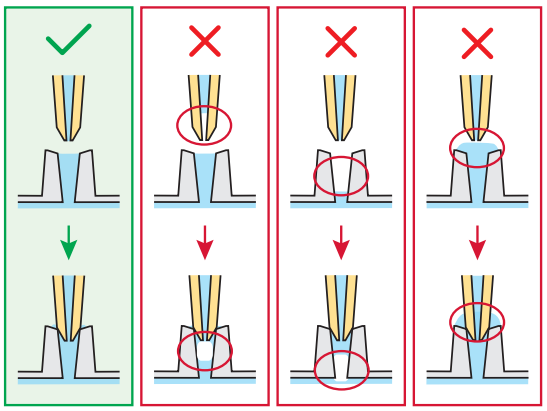


Correct pipet tips (left) fit properly into the top area of the filling port, seal completely, and can be removed easily. Incorrect tips (right) fit too tightly and may become stuck.

NOTE – Start by using a free sample together with the included food coloring. Do not use food coloring with a high sugar concentration, as it is not suitable for visualizing gradient formation.

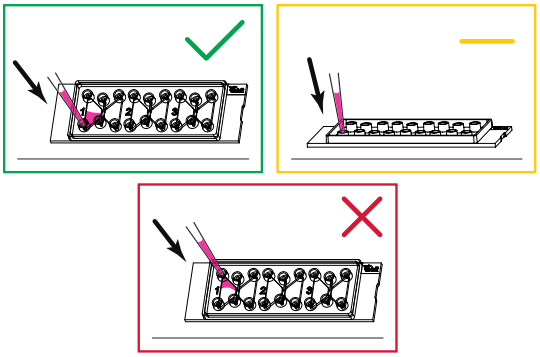
2.2 General Slide Handling

- To handle the slides properly and avoid air bubbles, always ensure that filling ports are completely filled—without overfilling—and avoid leaving them unfilled. Be cautious of trapped air bubbles in the pipet tip, and remember that sealing an empty filling port with a plug can trap air inside.



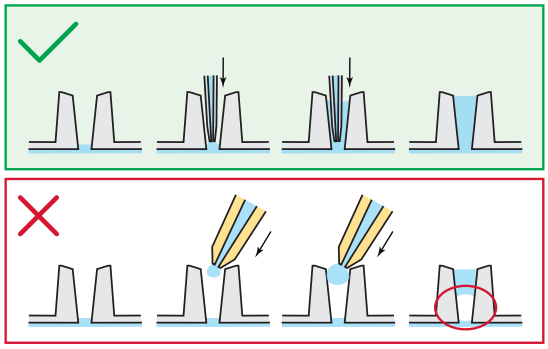
Correct slide filling: fill ports fully without overfilling, and avoid trapping air bubbles.

- Avoid trapping air by filling empty reservoirs with the slide inclined. Always inject the liquid into the lower filling port to allow lighter air to escape through the Filling Port above.



Proper filling technique to prevent air trapping.

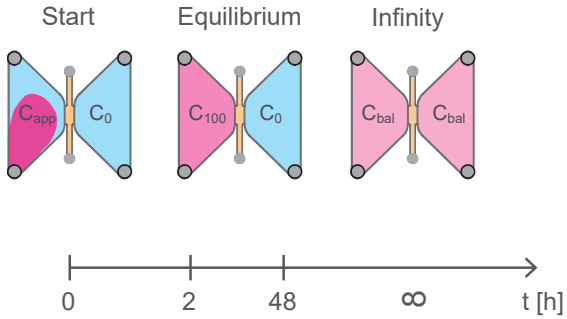
- Filling ports that are accidentally emptied or left empty can be refilled using a 10 µl pipet with a very thin tip. Never use standard-sized pipet tips for this procedure, as they may trap air bubbles.



Proper technique for refilling the filling ports.

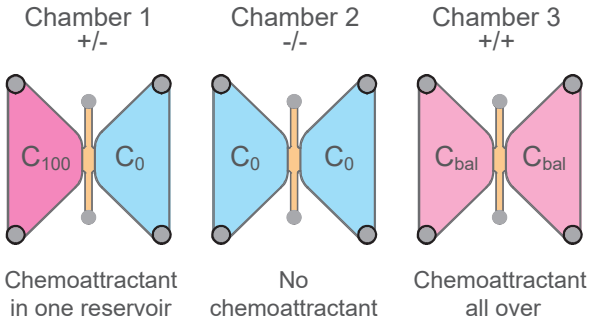
2.3 Control Experiments

Upon loading reservoirs with different chemoattractant concentrations, diffusion generates a linear gradient across the observation area. A stable steady-state gradient is established within approximately 2 h, maintained for up to 48 h, and gradually flattens as concentrations equilibrate.



Schematic representation of gradient formation in the µ-Slide Chemotaxis.

For optimal results, we recommend two control experiments: a +/+ control, in which the entire chamber is filled with the chemoattractant solution, and a -/- control, in which a neutral solution is used throughout. These controls help distinguish whether a tested compound is affecting directed cell movement (chemotaxis) or random migration (chemokinesis).



Schematic representation of three experimental chamber configurations for chemoattractant exposure.

The main concentration terms used to describe the chemoattractant setup are defined below.

C_{100}: The concentration in stable gradient equilibrium — this is the maximum concentration that reaches the cells on one edge of the observation area.

C_0: The initial concentration in the region without (or with a very low amount of) chemoattractant.

$C_{applied}$: The concentration applied in the chamber, which has a volume of 30 µl.

$C_{applied} = C_{100} \times 2$

C_{bal}: The balanced concentration of chemoattractant after infinite time, calculated using the total chamber volume of 130 µl.

$C_{bal} = C_{applied} \times 30\ µl / 130\ µl$
$C_{bal} = C_{applied} \times 0.23$

2.4 Gas Equilibration

Air bubbles either (1) emerge over time due to non-equilibrated media and slides, or (2) are introduced into the chamber by the user. Both disturb the diffusion-driven concentration gradient through convection and must be avoided.

Gas equilibration of the labware and media is absolutely necessary before starting the assay. The day before seeding the cells, place the cell medium, the slide, and caps/plugs into the incubator for equilibration. The slide and caps/plugs may remain sealed in their sterile packaging during this process. The medium should be put into a slightly opened vial to ensure gas exchange. Incubate overnight. This will prevent the liquid inside the slide, and the slide itself, from forming air bubbles during the incubation time.

2.5 Coating

Detailed information about coatings is provided in Application Note 08: Coating Protocols for ibidi Labware. If you do not require any special adhesion molecules for your application, the best choice will be ibiTreat, a tissue culture-treated surface.

In short, specific coatings are possible following this protocol:

1. Prepare your coating solution according to the manufacturer's specifications. Depending on whether you coat the entire chamber or only the observation area, use the corresponding coating volume as shown in the following table, and then adjust the concentration of the coating accordingly.

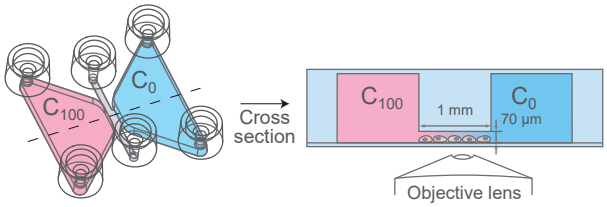
Coating Coverage	Coating Volume	Coating Area
Full chamber	130 µl	3.5 cm^2
Observation area only	6 µl	0.27 cm^2

2. Apply the corresponding coating volume to the selected coating area, and incubate at room temperature for at least 30 minutes.
3. Aspirate the solution and wash with the recommended protein dilution buffer.
4. Remove as much water or buffer as possible. Allow the chambers of the slide to dry completely before cell seeding.
5. Perform gas equilibration of the slide again after coating.
6. The slide is ready to be used.

3 The Chemotaxis Experiment

3.1 2D Chemotaxis Assay

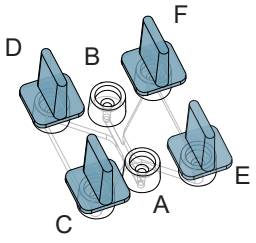
In 2D chemotaxis experiments (without the use of a gel), cells adhere directly to the surface of the observation area.



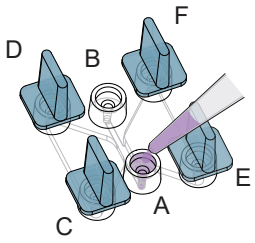
NOTE – Ensure that gas equilibration (see Section 2.4) is done before beginning the experiment.

3.1.1 Seeding Cells

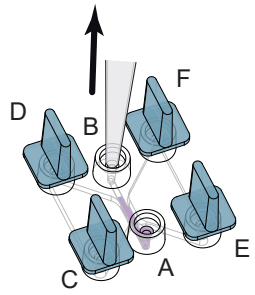
1. Unpack the µ-Slide Chemotaxis and place it into a sterile 10 cm Petri dish containing a moistened tissue.
2. Prepare your cell suspension as usual. Use a cell suspension with a concentration of approximately 2–3 $\times 10^6$ cells/ml. A high cell concentration is necessary due to the shallow observation area.
3. Close filling ports C, D, E, and F with the plugs.



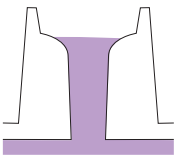
4. Use a 20 µl pipet to apply 6 µl of cell suspension to the top of filling port A, leaving space between the tip and the port. Direct injection is not recommended.



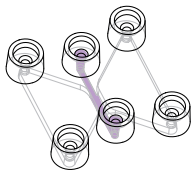
5. Immediately afterwards, using the same pipet settings (6 µl), aspirate air from the opposite filling port B. Press the pipet tip directly into the port. This will drive the liquid from filling port A through the channel, ensuring it fills completely and homogeneously. Continue aspirating until the cell suspension reaches the pipet tip.



6. Leave both filling ports A and B filled with liquid. If necessary, level out the liquid levels in both filling ports, as illustrated in the cross-section.



7. Gently remove all plugs from filling ports C, D, E, and F. Close the slide with the cultivation lid.



8. Keep the slide in the Petri dish containing a moistened tissue and incubate at 37 °C

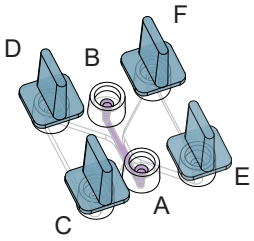
and 5% CO_2. To control evaporation, frequently check the liquid levels in the channel and in filling ports A and B.

9. Monitor cell morphology using a microscope during and after cell attachment. Cell attachment should occur within the same timeframe as in standard culture conditions.

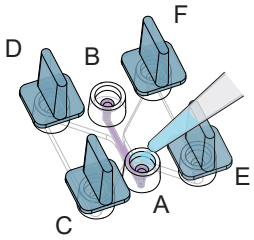
3.1.2 Removing Non-adherent Cells

After cell attachment, a washing step is recommended to remove non-adherent cells and the seeding medium. However, this step could be skipped when working with cells that are only weakly adherent.

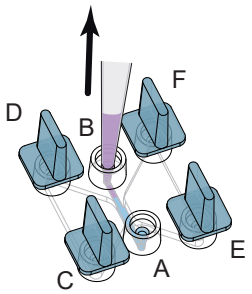
1. Close filling ports C, D, E, and F with plugs.



2. Add 10 µl of cell-free medium onto filling port A. Avoid trapping air bubbles and do not inject the medium directly.



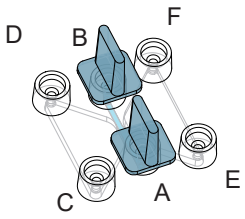
3. Aspirate the same volume of liquid (10 µl) from filling port B.



4. Repeat steps 2 and 3 to perform an additional washing cycle.

5. Ensure both filling ports A and B remain filled with liquid. If necessary, level out the liquid heights in both filling ports.

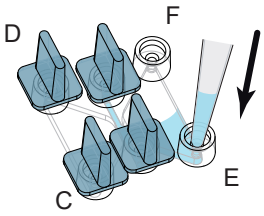
6. Gently remove all plugs from filling ports C, D, E, and F. Close filling ports A and B with plugs.



3.1.3 Chemoattractant Filling

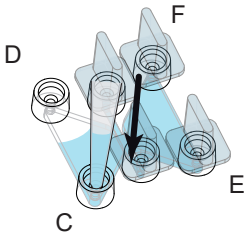
1. Gently close filling ports C and D (chemoattractant side) with plugs.

2. Fill the first reservoir by injecting 65 µl of chemoattractant-free medium through filling port E using the recommended pipet tips. Ensure that filling ports E and F are completely filled, but do not overfill.

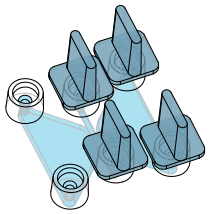


3. Transfer the two plugs from filling ports C and D to filling ports E and F to close the chemoattractant-free side.

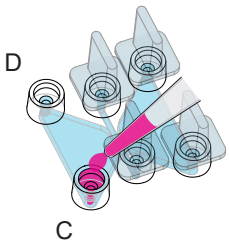
4. Fill the empty reservoir by injecting 65 µl of chemoattractant-free medium through filling port C using the recommended pipet tips.



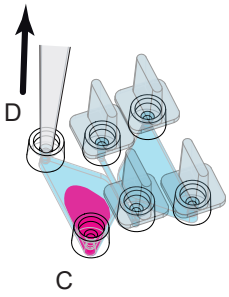
5. Now the chamber is completely filled with chemoattractant-free medium, and cells will grow only in the observation area. Monitor your cells using a phase contrast microscope.



6. Using a 20 µl pipet, apply 15 µl chemoattractant to the top of filling port C, as shown. Do not inject directly.

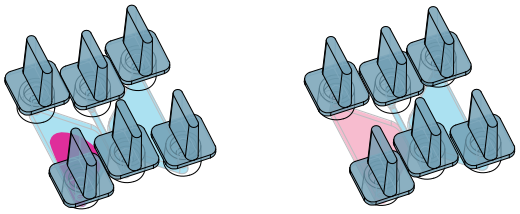


7. With the same pipet settings, aspirate 15 µl of liquid from the opposite filling port D. Press the pipet tip directly into filling port D. This will flush the chemoattractant from port C inside, filling the reservoir.



8. Repeat steps 6 and 7, in order to fill 30 µl inside the reservoir. The resulting concentration in the reservoir is half the injected concentration, since 30 µl of chemoattractant is diluted with an equal volume of neutral solution.

9. Close the filling ports C and D with plugs.



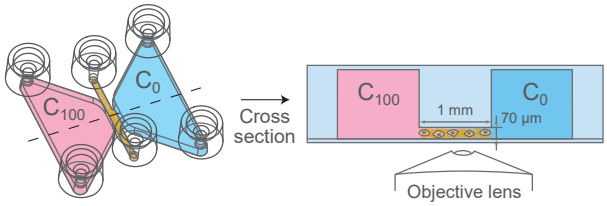
10. Start the time-lapse imaging described in Section 3.3 immediately after injecting the chemoattractant.

NOTE – Another method to fill the reservoir is the “Fast Method”, in which a large reservoir is completely filled with the chemoattractant. For the detailed protocol, see Section 3.2.3.

3.2 3D Chemotaxis Assay

In 3D experiments, the observation area is filled with a gel matrix containing embedded cells. The gel minimizes convective flow, allowing the two large reservoirs to be independently filled with either neutral medium or chemoattractant. This setup enables the formation of a stable chemical gradient across the gel.

Common aqueous gels, such as collagen gels, are generally considered permissive to diffusion and do not impede the formation of chemical gradients. In contrast, stiff hydrogels with pore sizes similar to the size of the diffusing molecule can block diffusion and prevent effective gradient formation.



NOTE – Ensure that gas equilibration (see Section 2.4) is done before beginning the experiment.

NOTE – In 3D chemotaxis experiments, ensure that for the +/+ control, the gel matrix in the observation area is prepared using the chemoattractant solution at concentration C_{bal}.

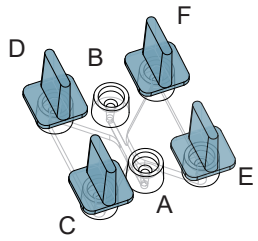
3.2.1 Seeding Cells

1. Unpack the µ-Slide Chemotaxis and place it into a sterile 10 cm Petri dish containing a moistened tissue.
2. Close filling ports C, D, E, and F with the plugs.
3. Prepare your cell suspension as usual.
4. Prepare your gel matrix according to the manufacturer's specifications.

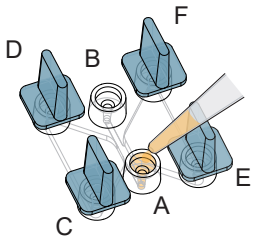
TIP – As a starting point, we recommend using a 1.5 mg/ml collagen type I gel. Detailed protocols can be found in Application Note 26: Fabrication of Collagen I Gels.

NOTE – To avoid any inhomogeneities in the gel matrix, please refer to the Troubleshooting Section 5.5.

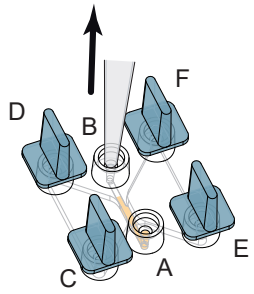
5. Add the cells to the gel matrix to get a final concentration of 2–3 $\times 10^6$ cells/ml. Mix well. A high cell concentration is necessary due to the shallow observation area.



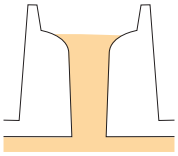
6. Use a 20 µl pipet to apply 6 µl of gel mixture to the top of filling port A, leaving space between the tip and the port. It is not recommended to inject the gel directly.



7. Immediately afterwards, using the same pipet settings (6 µl), aspirate air from the opposite filling port B. Press the pipet tip directly into the port. This will drive the liquid gel from filling port A through the channel, ensuring it fills completely and homogeneously. Continue aspirating until the gel reaches the pipet tip.

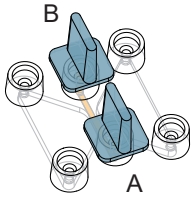


8. Leave both filling ports A and B filled with gel. If necessary, level out the liquid levels in both filling ports, as illustrated in the cross-section.



NOTE – The gel mixture may also be directly injected into the channel. After injecting a small volume of gel, the channel becomes completely filled. Afterwards, the gel must be carefully aspirated from the opposite filling port until the fluid levels at both ports are equalized. This procedure requires particular caution to prevent accidental overflow of the gel mixture into the large reservoirs. When properly performed, this method is especially advantageous when working with high-viscosity liquids.

9. Gently remove all plugs from filling ports C, D, E, and F. Close filling ports A and B with plugs.



10. Keep the slide in the Petri dish containing a moistened tissue and incubate at 37 °C and 5% CO_2. To control evaporation, frequently check the gel volume in the channel and in filling ports A and B.

11. Monitor cell morphology using a phase contrast microscope during and after gelation.

NOTE – In a 3D chemotaxis experiment, cells should be fully embedded within the gel matrix. Over time, adherent cells may sink and attach to the underlying 2D surface due to durotaxis. Be sure to clearly distinguish between cells adhered to surfaces—typically spread out—and cells suspended in 3D, which usually appear spherical or spindle-shaped.

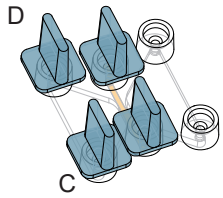
3.2.2 Chemoattractant Filling

In the following steps, both large reservoirs are first filled with neutral solution. The chemoattractant is then added without directly contacting the observation area. This results in a brief delay in gradient formation, preventing immediate receptor saturation in sensitive cells. The final concentration in the reservoir is half of the injected concentration, as 30 µl of chemoattractant is diluted with an equal volume of neutral solution.

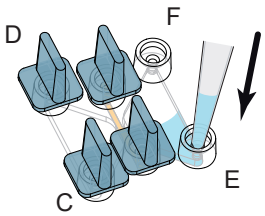
NOTE – Control the quality of the gel and cells after each filling step using a phase contrast microscope.

NOTE – Handle the liquid carefully to avoid damaging or detaching the gel matrix due to excessive pressure.

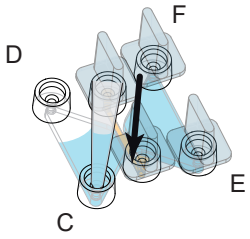
1. Gently close filling ports C and D (chemoattractant side) with plugs.



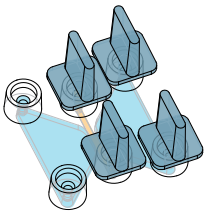
2. Fill the first reservoir by injecting 65 µl of chemoattractant-free medium through filling port E using the recommended pipet tips. Ensure that filling ports E and F are completely filled, but do not overfill.



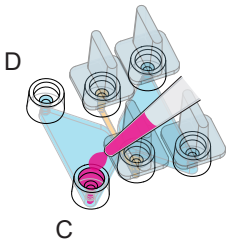
3. Transfer the two plugs from filling ports C and D to filling ports E and F to close the chemoattractant-free side.
4. Fill the empty reservoir by injecting 65 µl of chemoattractant-free medium through filling port C using the recommended pipet tips.



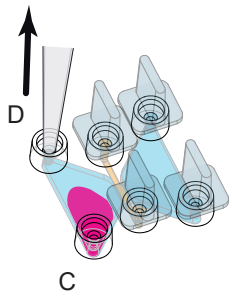
5. Now the chamber is completely filled with chemoattractant-free medium, and cells will grow only inside the gel in the observation area. Monitor your cells using a phase contrast microscope.



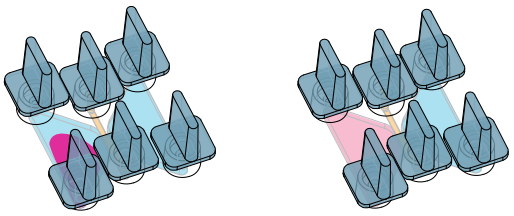
6. Using a 20 µl pipet, apply 15 µl chemoattractant to the top of filling port C, as shown. Do not inject directly.



7. With the same pipet settings, aspirate 15 µl of liquid from the opposite filling port D. Press the pipet tip directly into filling port D. This will flush the chemoattractant from port C inside, filling the reservoir.



8. Repeat steps 6 and 7 once more to fill a total of 30 µl inside the reservoir. The resulting concentration in the reservoir is half the injected concentration, since 30 µl of chemoattractant is diluted with an equal volume of neutral solution.
9. Gently close all filling ports.



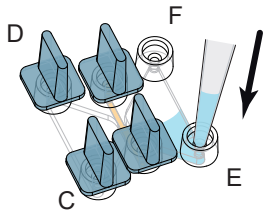
10. Start the time-lapse imaging described in Section 3.3 immediately after injecting the chemoattractant.

3.2.3 Chemoattractant Filling—Fast Method

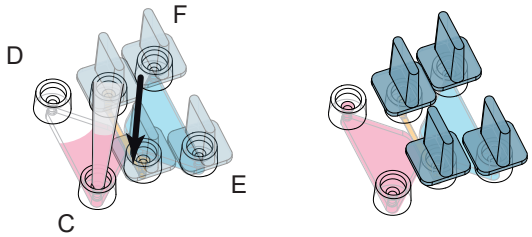
With the “Fast Method”, one large reservoir is completely filled with the chemoattractant. While this approach is more convenient, it may introduce inconsistencies by allowing the chemoattractant to flush directly into the observation area. A key drawback is that sensitive cells may initially be saturated with the chemoattractant. The chemoattractant will immediately touch the observation area and establish a linear concentration profile across the cells.

NOTE – The chemoattractant is not diluted when using the “Fast Method”.
In this case, $C_{applied} = C_{100}$.

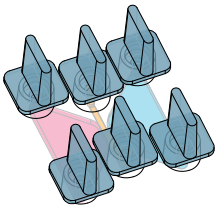
1. Gently close filling ports C and D (chemoattractant side) with plugs.
2. Fill the first reservoir by injecting 65 µl of chemoattractant-free medium through filling port E using the recommended pipet tips. Ensure that both filling ports E and F are completely filled, taking care not to overfill them.



3. Transfer the two plugs from filling ports C and D to filling ports E and F to close the chemoattractant-free side of the chamber.
4. Inject 65 µl of chemoattractant into filling port C. Do not trap any air bubbles. The chamber is now completely filled and the cells are confined to the gel within the observation area.



5. Close the filling ports C and D with plugs.



6. Start the time-lapse imaging immediately after injecting the chemoattractant.

3.3 Time-Lapse Imaging

Time-lapse imaging is essential when working with the µ-Slide Chemotaxis. Only with time-lapse recordings is it possible to analyze directed cell migration and quantify chemotaxis effects.

1. Prepare your inverted microscope and stage top incubator at least 60 minutes before starting the time-lapse recording. Depending on your cells' requirements, heating and incubation devices—such as the ibidi Stage Top Incubator—may be necessary. Working at room temperature with cells typically cultured at 37 °C and 5 % CO_2 is not recommended, as it may affect cellular behavior.

2. Mount the slide on the microscope stage and allow approximately 20 minutes for temperature equilibration before starting image acquisition. In the meantime, configure the microscope or imaging settings as described in the following steps.

   NOTE – For reliable migration data, we recommend performing an initial test experiment to determine the optimal imaging parameters for your specific cell type.

   NOTE – To prevent focus drift, please refer to the Troubleshooting Section 5.1.

3. Select an objective lens based on your experimental focus. For optimal tracking of at least 20–40 cells throughout the entire observation period, we recommend starting with a 5× or 10× objective. Higher magnifications can be used if the experiment prioritizes high-resolution microscopy over population-level chemotaxis analysis.
   Microscopy magnification overview:

   - 4×/5×: Lowest magnification and resolution; captures the most cells in one focal plane—ideal for overviews.
   - 10×: Medium magnification and resolution; most cells remain in one focal plane.
   - 20×: Medium magnification and resolution; cells appear in different focal planes.
   - 40×/100×: Highest magnification and resolution; only a few cells are visible. One cell may not lie entirely within one focal plane.

4. Configure the camera for time-lapse image acquisition. The interval between frames should be adjusted according to the migration speed of the cells. For fast-moving cells, use an interval of 0.5–1 minute per frame. For slower cells, an interval of 3–10 minutes per frame is appropriate. Ensure that each moving cell appears with sufficient overlap between consecutive frames, which is critical for accurate cell tracking throughout the experiment.

5. Monitor cell movement inside the observation area for an appropriate total duration. The imaging period should be long enough to allow cells to migrate at least ten cell diameters on average but short enough to keep most cells within the microscopic field of view. For fast-moving cells, this may require 1–4 hours of observation, while slow-migrating cells may need 12–36 hours.

6. Start the time-lapse acquisition as soon as the temperature is stable.

7. After the time-lapse, export your images as single files (e.g., TIF, JPG, PNG, BMP).

For fluorescence microscopy and for storage of fixed and stained samples, ibidi provides mounting media that are optimized for ibidi labware:
Cat. No. 50001: ibidi Mounting Medium
Cat. No. 50011: ibidi Mounting Medium with DAPI

4 Tracking and Analysis

4.1 Tracking Cells

After the experiment, track the cells using appropriate software. For cells migrating through a 3D matrix, we recommend tracking them as a 2D projection in the xy plane. This simplifies analysis and is considered to be correct for channels with an aspect ratio of 10:1 (length:height) or greater.

4.1.1 Manual Tracking

For manual tracking, we recommend the ImageJ plugin “Manual Tracking”. This plugin can quantify the movement of objects between frames of a temporal stack.

- ImageJ is available here: https://imagej.net/ij/download.html
- The Manual Tracking plug-in (including a PDF documentation) by Fabrice Cordelières is available here: https://imagej.net/ij/plugins/track/track.html
- Download “Manual_Tracking.class” into the plugins folder of ImageJ on your computer and restart ImageJ. Make sure you have administrator rights.

Here is a quick guide for tracking with the Manual Tracking tool:

1. Import a movie as single-page image files (e.g., TIF, JPG, PNG, BMP) using `File →Import →Image Sequence`.
2. Open the plugin `Manual Tracking`.
3. Select `Add Track`.
4. Follow the first cell through all time points by clicking on the cell’s midpoint. After the first click, the software creates a results table in a separate window. This table is filled with the x/y data of each cell at each time point.
5. Save the data table after tracking is completed (as a tab-separated `.xls` file).
6. The data table contains all tracked cells (tracks) and time points (slices) with x, y positions.
7. You can also export `Overlay Dots & Lines` as a movie file (`.avi`).

For tracking, we recommend printing out the first image of each image stack before watching the movie for the first time. At least 40 cells should be uniformly marked on this printout to (1) ensure homogeneity and (2) avoid tracking the same cell twice. At least 20–40 cells in the observation field need to be tracked throughout the entire experiment. Cells that are lost due to cell death, cell division, or movement out of the field of view should be excluded from the analysis.

4.2 Analyzing Chemotaxis

4.2.1 Chemotaxis Tool

ibidi provides a free software tool for plotting and analyzing tracked data. To download the software and read the instructions, please visit Chemotaxis and Migration Tool.

NOTE – Track and analyze each time section separately. Check for a time-dependent chemotaxis effect or time-dependent cell behavior.

1. Import the data table from `Manual Tracking` (tab-separated `.xls` file).
2. Initialize your dataset using the initialization menu in the lower right corner of the software:
   a) Select the number of slices (i.e., the number of images used for tracking). This information can be found in your original data table (`Show original data`).

b) Calibrate the software by setting the `x/y calibration` value. This corresponds to the length of one pixel in µm. The pixel size can be determined by following Application Note 22: Determination of the Pixel Size of Microscopy Images.

c) Calibrate the `time interval`, which corresponds to the time between consecutive images in your video.

3. Press `Apply settings` after changing values and parameters.
4. Create a `trajectory plot`, then export it as an image.
5. Export values of both Forward Migration Indices and the p-value from the Rayleigh test.
6. Convert Forward Migration Index x and y into FMI parallel ($FMI_{\parallel}$) and FMI perpendicular ($FMI_{\perp}$), based on the position of your chemoattractant. This simplifies the analysis and presentation of the FMI data.
7. Perform a two-tailed unpaired Student's *t*-test (e.g., using MS Excel) with independent variances for the single values from step 5. Compare the chemotaxis experiment (+/-) against the two control experiments (-/- and +/+) to determine statistical significance.

4.2.2 Chemotaxis Criteria

An experiment is considered to demonstrate a chemotaxis effect if the following statistically significant criteria are met:

Experimental Condition (+/-):

- $FMI_{\parallel}$ significantly greater than $FMI_{\perp}$
- Rayleigh test: $p < 0.05$

Control Conditions (+/+ and -/-):

- $FMI_{\parallel}$ and $FMI_{\perp}$ approximately zero
- Rayleigh test: $p > 0.05$

4.2.3 Presenting Results

Here are some recommendations for presenting the results (e.g., in talks or publications):

- Include the original time-lapse movie of the cells.
- Show the trajectory movie with overlaid cell tracks.
- Present a static trajectory plot (e.g., overlay of all cell paths).
- Display a table or bar chart summarizing FMI values and Rayleigh test results.
- Report the outcome of the two-tailed unpaired Student's *t*-test.

For an example of how results can be presented, see Application Note 24: Chemotaxis of HT-1080 cells in 2D and 3D.

5 Troubleshooting

5.1 Focus Not Stable

Focus drift is a common issue in time-lapse experiments, often caused by mechanical shifts or temperature instabilities. Follow these tips to keep your cells in focus:

- Keep the room temperature as stable as possible. Air conditioning should run continuously or be turned off completely.
- Do not change the room temperature during the experiment. Avoid opening doors or windows, as this can cause rapid temperature shifts.
- Eliminate all sources of mechanical vibrations. Use a vibration-damped table for the microscope.
- Use an autofocus system.

5.2 Chamber Not Filling Properly

Here are some general tips and tricks for filling the chamber:

- Always use chambers that are completely dry, especially after coating. Even small amounts of residual liquid can hinder proper filling.
- Use a pipet that is routinely serviced. Over time, pipets can lose pressure during pipetting. While they may work fine for general use, they can cause problems when used with the µ-Slide Chemotaxis.
- When filling the large reservoirs, incline the slide so that the liquid is beneath air. This positioning allows the lighter air to escape through the filling port above.

5.3 Cells Die in the Gel Matrix

pH and salt concentrations can significantly affect gel matrices. Therefore, first test your gel protocol with your cells in an easy-to-access open well environment. We recommend using small open wells, such as the µ-Slide 18 Well or the µ-Slide 15 Well 3D, where the cells can be conveniently embedded in a gel. This setup allows easy access and enables identification of the optimal matrix composition for your cells.

5.4 Cells Do Not Migrate in the Gel

To help identify the cause, consider the following questions:

- Are the cells seeded in an optimal growth medium?
- Is the pH within the optimal range for the cells?
- Can the cells degrade the gel matrix?
- Is the gel concentration too high or the gel too stiff?
- Are suitable binding motifs available for cell attachment?

5.5 Gel Matrix Not Homogeneous

Inhomogeneities in the gel matrix (inconsistent gel or fibers with preferred orientation) must be avoided to ensure consistent migration data. In general, mix the gel thoroughly, taking sufficient time. Vigorous pipetting up and down is necessary, combined with stirring using the pipet tip. After preparing the gel, mix it thoroughly with the cell suspension. Only use freshly aliquoted and non-expired components.

Two key parameters are critical for collagen gels: the initial homogeneity of the mixture and the temperature during chamber filling. Mix the viscous collagen solution thoroughly—unlike standard solutions, the collagen-cell mixture does

not homogenize by diffusion. As gelation progresses over time, preferred fiber orientations may form, particularly in small channels. This can be prevented by minimizing working time and/or performing the procedure on ice to slow down the gelation process.

5.6 Cells Do Not Attach Properly (2D Assay Only)

Due to the specialized geometry of the chamber and the low volume used, the cell seeding step is critical. To improve cell attachment, ensure the following conditions are met:

- Optimal medium: Adherent cells will not attach properly in starvation medium. Use full growth medium with all required supplements.
- Humidity: Place the slide in an extra-humid chamber. Evaporation during cell attachment is a major issue. Use a 10 cm Petri dish with moist tissue to maintain high humidity.
- Incubator access: Avoid frequent incubator door openings during the cell attachment phase. Fluctuating humidity can impair attachment. Use a dedicated incubator if possible.

5.7 Inhomogeneous Cell Distribution (2D Assay Only)

Uneven cell distribution, particularly elevated cell densities near the observation zone, can obscure directed migration. Carefully follow the seeding instructions to avoid spilling the cell suspension into the large reservoirs. Do not inject the cell suspension directly into the filling ports during seeding.

5.8 Low Reproducibility of Results

Based on our extensive experimental experience, three independent experiments with approximately 40 cells each are sufficient for statistically significant and reproducible results. If chemotactic parameters such as Forward Migration Index (FMI) or velocity differ significantly across identically conducted experiments, consider the following factors:

- Homogeneous gel matrix: Consistency in the gel matrix is critical in 3D chemotaxis. Refer to the Troubleshooting Section 5.5 for tips on achieving a uniform gel.
- Reproducible protocol: Small deviations in gel preparation or handling may affect outcomes. For some protocols, the pipetting order during gel preparation is crucial. The sequence of applying plugs can also impact results. For highest reproducibility, we do not recommend the “Fast Method” for chemoattractant filling.
- Correct substance concentrations in gel and reservoirs: In chemotaxis assays, the final concentrations of salts, buffer, and serum should be consistent across the gel and the C_0 solution. In +/+ control experiments, ensure that the chemoattractant concentration in the gel matches that in the reservoirs. Otherwise, competing gradient fields are created, which may disturb directed movements of cells.
- Homogeneous cell population: Cells prepared via transfection, differentiation, or special treatment protocols may yield heterogeneous populations and inconsistent migration behavior. Use well-characterized, homogeneous cell populations whenever possible.

6 Chemical Compatibility

The following table provides some basic information on the chemical and solvent compatibility of the µ-Slide Chemotaxis. For a full list of compatible solvents and more information on chemical compatibility, visit ibidi.com/chemicals.

Chemical / Solvent	Compatibility
Methanol	Yes
Ethanol	Yes
Formaldehyde	Yes
Acetone	Yes, without lid
Mineral oil	No
Silicone oil	Yes
Immersion oil	See Section “Immersion Oil”

7 Immersion Oil

WARNING – When using oil immersion objectives with the ibidi Polymer Coverslip, use only the immersion oils specified in the table below. The use of any non-recommended oil could damage the ibidi Polymer Coverslip. The resulting leakage may harm objectives and microscope components. All immersion oils that are not listed in the table below should be considered non-compatible.

Company	Product	Ordering No.	Lot Number	Test Date
ibidi	ibidi Immersion Oil 2	50102	24-07-04	07/2024
Cargille	Type A	16482	100592	01/2017
Cargille	Type HF	16245	92192	01/2017
Carl Roth	Immersion oil	X899.1	414220338	01/2017
Leica	Immersion Liquid	11513859	n.a.	03/2023
Leica	Immersion Liquid Type G	11513910	n.a.	04/2024
Nikon	Immersion Oil F2 30cc	MXA22192	n.a.	01/2020
Nikon	Silicone Immersion Oil 30cc	MXA22179	20191101	01/2020
Olympus	Silicone Immersion Oil	SIL300CS-30CC	N4190800	01/2017
Zeiss	Immersol 518 F	444960-0000	220211	03/2023
Zeiss	Immersol 518 F (30 °C)	444970-9010	220816	03/2023
Zeiss	Immersol 518 F (37 °C)	444970-9000	220302	03/2023
Zeiss	Immersol W 2010	444969-0000	101122	04/2012
Zeiss	Immersol Sil 406	444971-9000	80730	03/2023
Zeiss	Immersol G	462959-9901	211117	03/2023

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Further information can be found at ibidi.com. For questions and suggestions, please contact us by e-mail at info@ibidi.com or by telephone at +49 (0)89/520 4617 0.