3D Chemotaxis Protocol with Collagen I Gel for Dendritic Cells

1. General information

This is a specialized protocol for analyzing chemotaxis of murine dendritic cells embedded in a 3D collagen matrix. More general information is provided in the Application Note 17 "3D Chemotaxis Assays using µ-Slide Chemotaxis ^{3D} ".

2. Equipment needed

For this protocol the following equipment and instruments are necessary:

- Cell culture incubator (high humidity, 37 °C, 5 % CO_2)
- Inverted microscope with 10x objective lens (phase contrast) and time lapse function
- Stage top incubator (37 °C, 5 % CO_2)
- Optional: Motorized stage and autofocus (x,y,z) to observe all 3 chambers in parallel.
- Computer with ImageJ plugin "Manual Tracking" and "Chemotaxis and Migration Tool" from ibidi.

3. Cell culture of dendritic cells

One day before experiment:

- To inhibit the development of bubbles degas your slides and media in an incubator (37 °C) for 24 h.
- Dendritic cells (on day 8 – 10) are activated over night with 200 ng/ml LPS (in cell culture medium as described in table 1).

	Reagent/Material	Concentration	Company	Order No.
Cell culture	Dendritic cells (murine)	-	Self-prep*	-
	RPMI 1640	1x	Invitrogen	31870025
	FCS	10%	Invitrogen	10270106
	L-Glutamin	5%	PAA	M11-004
	Penicillin/Streptomycin	5%	PAA	P11-010
	GM-CSF	10-20 ng/ml*	Peprotech	315-03
Cell activation	LPS	200 ng/ml	Sigma-Aldrich	L4516

Table 1. Material and reagents needed for cell culture of dendritic cells.

* as published in:

Lutz, M. B. *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* **223**, 77–92 (1999)

4. Gel preparation, Collagen I, bovine, 1.6 mg/ml

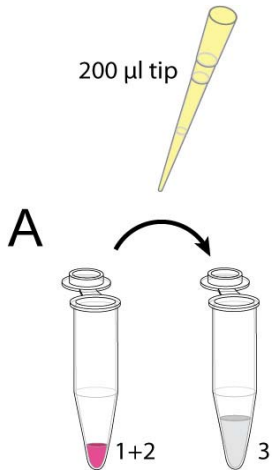
The following reagents and material is necessary for the chemotaxis experiment with dendritic cells:

	Reagent/Material	Concentration	Company	Order No.
Cells	Dendritic cells (murine)	9 x 10^6 c/ml	Self-prep	-
Gel preparation	Collagen I, bovine	3 mg/ml	Nutacon	5005-B
	$NaHCO_3$	7.5 %	Sigma-Aldrich	S8761
	10x MEM	10x	Sigma-Aldrich	M-0275
Chemoattractant	CCL19	1.25 µg/ml	R&D Systems	361-MI-025
µ-Slide	µ-Slide Chemotaxis 3D	-	ibidi	80326

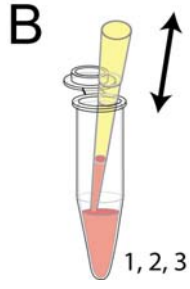
Table 2. Material and reagents needed for chemotaxis experiment with dendritic cells.

V_{total} = 270 µl		
No.	**Component**	**Amount**
1	10x MEM	20 µl
2	$NaHCO_3$	10 µl
3	Collagen I, bovine	150 µl
4	Cell suspension	90 µl
	∑	**270 µl**

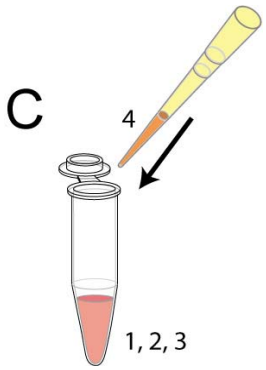
Table 3. Preparation of a 1.6 mg/ml gel



1. Prepare a cell suspension of 9 x 10^6 cells/ml in the cell culture medium mentioned in table 1.
2. Carefully mix the components 1 and 2 in a 1.5 ml tube avoiding air bubbles.
3. Prepare 150 µl collagen (component 3) in a 1.5 ml tube.
4. Transfer the 30 µl mix of Nr. 2 into the collagen tube. Use a 200 µl pipet tip for immediate mixing of a larger volume (see A).
5. Mix well, but carefully (see B). It is important to avoid air bubbles during mixing!
6. Add 90 µl of the cell suspension (Nr. 1) into the mix (see C).
7. Mix well, but carefully (see D). It is important to avoid air bubbles during mixing!

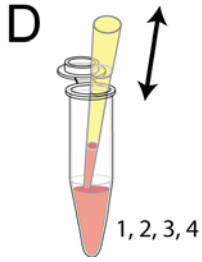


8. Immediately after that fill the mix into the channels as described in Application Note 17.
9. Place the slide into the incubator for 30 – 35 min for gelation. After that collagen fibers become visible.



Notes:

- Let bubbles in the pipette ascend to the top of the solution before pipetting into the slide.
- Avoid bubbles in your gel. They may destroy collagen-fibers, influence time-lapse recordings and optical properties of the gel. Don't use a vortex mixer.
- After making the collagen-mix, there are ca. 5 min left to pipette the gel before gelation starts.
- In case of pipetting after gelation, big damages in collagen-fibers must be expected.
- A color change will be observed (indicator turns into pink) when 10xMEM is added to sodium bicarbonate - this is not the final chemical equilibrium. Thus, the mixture must be mixed gently, while adding to collagen.



5. Chemotaxis experiment

Chemoattractant: CCL19 (1.25 µg/ml in RPMI 1640 / 10%FCS)
Chemoattractant-free medium: RPMI 1640, 10% FCS

1. After gelation of the gel, fill up the reservoirs with either chemoattractant-free or chemoattractant-containing medium.

2. For the chemotaxis experiment itself (+/-) fill one reservoir with 60 µl chemoattractant-containing medium and the other with 60 µl chemoattractant-free medium.

3. For a control experiment (-/-) fill both reservoirs with 60 µl chemoattractant-free medium.

4. Close the slide according to Application Note 17. The slide is now ready for video microscopy.

5. Put the slide into the stage top incubator.

6. Focus into the center of the gel and acquire images for 4 hours with a time lapse interval of 2 min.

Observation in the micrsocope: Because of the 3D environment, not all cells will be perfectly in focus depending on the used objective.

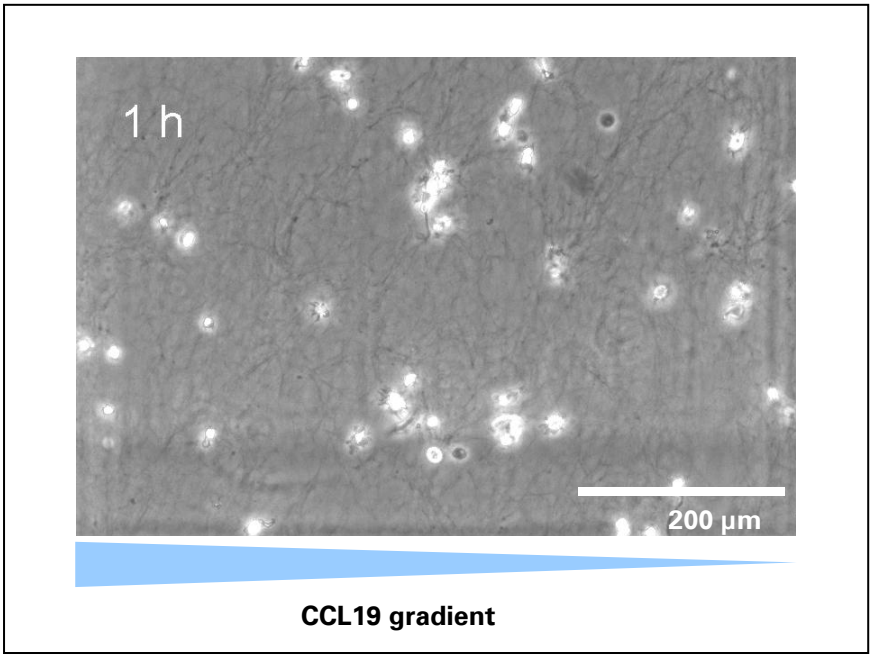
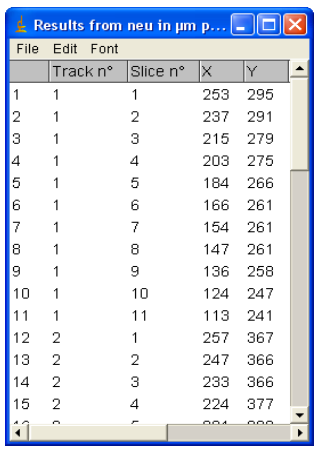


Figure 1. Bright-field image during time-lapse microscopy 1 hour after experimental set-up.

6. Manual cell tracking

1. After the experiment, track the cells with appropriate software. We suggest e.g. Manual Tracking, an ImageJ plugin able to quantify movement of objects between frames of a temporal stack.
2. ImageJ is available here: http://rsb.info.nih.gov/ij/
3. The Manual Tracking plug-in (including a pdf documentation) by Fabrice Cordelières, Institut Curie, Orsay, France is available here: http://rsbweb.nih.gov/ij/plugins/track/track.html
4. Download Manual_Tracking.class into the plugins folder of ImageJ on your computer and restart ImageJ. Make sure you have administrator rights.
5. Quick guide for tracking with Manual Tracking:
   - Import movie as single page .tif files by using „File/Import/Image_Sequence".
   - Open plugin "Manual Tracking".
   - Select "Add track".
   - Follow the first cell over all time points by clicking on it. After the first click the software creates the results table in an extra window. This table is filled with the x/y data of each cell at each point of time.
   - Save the data table after tracking is complete.



	Track n°	Slice n°	X	Y
1	1	1	253	295
2	1	2	237	291
3	1	3	215	279
4	1	4	203	275
5	1	5	184	266
6	1	6	166	261
7	1	7	154	261
8	1	8	147	261
9	1	9	136	258
10	1	10	124	247
11	1	11	113	241
12	2	1	257	367
13	2	2	247	366
14	2	3	233	366
15	2	4	224	377

6. The data table contains all tracked cells (=tracks) and time points (=slices) with (x,y) positions.
7. Track cells uniformly in order to ensure homogeneity. Avoid tracking the same cell twice. At least 30 cells need to be tracked for statistical reasons. Some cells will be lost through cell death. Do not use such cells for data analysis.
8. Optionally, export "Overlay dots & lines" as .avi.

7. Data evaluation with the Chemotaxis and Migration Tool:

The Chemotaxis and Migration plugin for ImageJ is available here:
http://www.ibidi.de/applications/ap_chemo.html
For data interpretation the Forward Migration Indices* (FMIs) of the chemotaxis experiment and the control experiment are compared. In case of chemotaxis all FMI components of the control experiments (-/-) and the perpendicular $FMI^{\perp}$ of the chemotaxis experiment (+/-) should be around 0. Value of $FMI^{\parallel}$ of the chemotaxis experiment (+/-) significantly different from 0 represent a chemotaxis effect.
Additionally, the p-values of the Rayleigh test** of all experiments are compared to judge for homogeneous angular distributions of the cell endpoint with respect to origin. p-values larger p = 0.05 indicate an homogeneous cell endpoint distribution and, therefore, non-directed migration.
Moreover, migration parameters such as directness and velocity can be used for analysis.

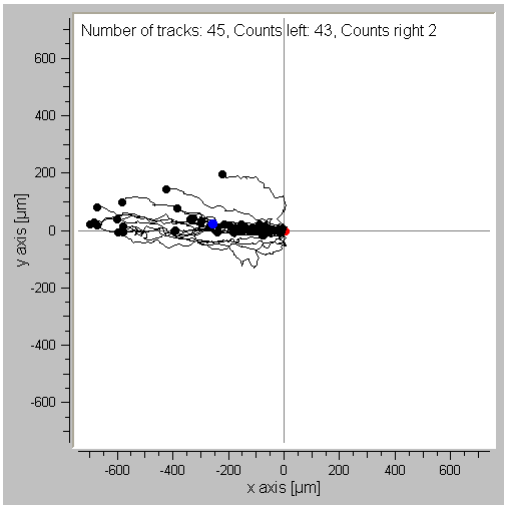
*Foxman et al. Integrating conflicting chemotactic signals. The role of memory in leukocyte navigation, *J Cell Biol* ***147***, 577-588 (1999)

**Fisher, N. I. Statistical Analysis of Circular Data, Cambridge University Press, New York (1993)

Results:

Chemotaxis experiment (+/-) (n = 40 cells)

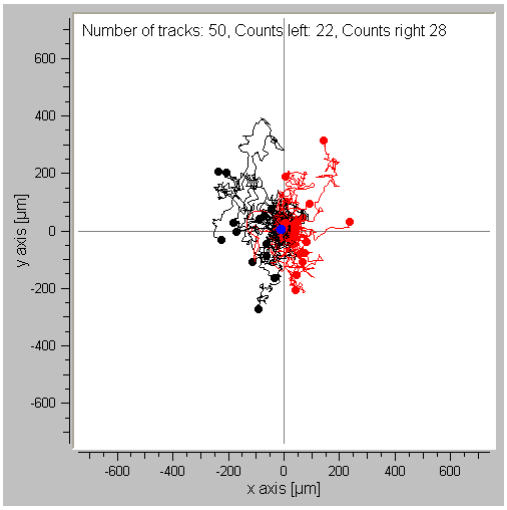
$FMI^{\parallel}$	-0.646586
$FMI^{\perp}$	0.0656136
Directness	0.66121
Velocity (µm/min) ± SD	2.87924 ± 1.13038
p-value (Rayleigh test)	8.87631×10^{-16}



→Directed migration towards chemoattractant

Control experiment (-/-) (n = 50 cells)

$FMI^{\parallel}$	0.00371511
$FMI^{\perp}$	0.0493364
Directness	0.153527
Velocity (µm/min) ± SD	2.39685 ± 0.956164
p-value (Rayleigh test)	0.680899



→ Non-directed migration

8. Notes:

- Lower magnification objectives (4 x - 10 x) provide a larger depth of focus and are therefore advantageous.

- Automated cell tracking:
  The company Wimasis is developing a solution for automated cell tracking of phase contrast image stacks. More information on automated cell tracking is available here www.wimasis.com.