JoVE submission JoVE60750
Manuscript title: Time-lapse imaging of mouse macrophage chemotaxis

Response to Editorial comments

Thank you for the guidelines and suggestions.

1. Done
2. Text revised
3. Key words added
4. Protocol modified
5. Excess text removed
6. Surgical tool(s) specified
7. Text for video: highlighted yellow
8. Complete sentences highlighted
9. Figures provided as .tiff
10. Materials are now sorted alphabetically by the material name

Response to Revierwer #1

Thank you for reading the manuscript and for your many helpful suggestions.

Minor concerns:
(Lines refer to originally submitted manuscript)

Lines 46-50: Changes made, except we deleted "particularly . . ." in response to related comments from with Reviewer #2.

Lines 56-57: Neutrophils and *D. discoideum* are commonly used models of chemotaxis and the assays are typically performed using a point-source of chemoattractant. However, this approach is unsuitable for slow moving macrophages. Much of our knowledge and theories of chemotaxis have come from experiments using *D. discoideum* and, thus, we would prefer to mention both models in this sentence.

Line 62: The relative clause has now been deleted.

Line 66: Done.

Introduction: The second paragraph of the Introduction now describes various chemoattractants, including the chemokine family and its classification into structurally related peptides.

Lines 88-89: I have changed the text and added a linking sentence.

Lines 99-100: The sentence has been modified.

Note that transwell or transendothelial migration assays do not provide a possibility to monitor cell navigation in a chemotactic gradient since the cells only move across a structure no more than one cell diameter thick.

We do not seek to strictly emulate *in vivo* conditions with this particular mouse macrophage 2D chemotaxis assay, although the notion is attractive. The next obvious step would be to perform 3D chemotaxis assays using a physiological matrix, such as collagen type I matrices, or perhaps after seeding the chemotaxis chamber with mesothelial cells to simulate the lining of the peritoneal cavity. We used, for example, 3D collagen matrices in the past to study the chemotaxis of human monocytes using time-lapse fluorescence microscopy, which worked very well. We also tested mesentery (parietal peritoneal) patches, isolated with the help of 6 mm (inner diameter) ring magnets, as potential *ex vivo* structures to study macrophage motility in a natural environment, but these preparations proved unsuitable, at least in our hands. In the end, our current mouse macrophage 2D chemotaxis assay has proven to be the most helpful approach to study macrophage motility and chemotaxis. In fact, it

is difficult to find an equivalent assay for macrophages in the literature. A great deal of knowledge on cell motility and chemotaxis has been gained using 2D chemotaxis assays, for example, the large body of work from Peter N. Devreotes and many others is derived from 2D motility assays.

Line 130: The reference to Figure 1A has been moved to another sentence in Section 1.1.

Line 177: The sentence containing Neubauer has been modified to include other counting devices.

Lines 178-180: The following sentence has been added: "The round-bottom tube allows the supernatant to be fully decanted and reduces cell clumping."

Line 261: Done.

Lines 288-290: Fluorescence labeling of cells for flow cytometry or immunofluorescence imaging is a standard procedure. A description of the labeling would create a digression from the main protocol, a chemotaxis assay using time-lapse, phase-contrast microscopy. The cell labeling and fluorescence imaging serves to underscore that two major populations of cells ($F4/80^+$ cells and $CD19^+$ cells) are obtained following peritoneal lavage. This point should be stressed in the protocol, which is specific for mouse resident peritoneal macrophages.

Lines 290-291: It is important to stress that F4/80 is a specific marker for mouse macrophages and mouse peritoneal B cells are $CD19^+$. That is, the cells are not identified by relative intensities, such as $F4/80^{hi}/CD19^{lo}$ or $CD19^{hi}/F4/80^{lo}$ etc.

Lines 298-299: This is a good point. Patent Blue V has a molecular weight of about 0.6 kDa, whereas recombinant mouse complement C5a is about 9 kDa. Thus, Patent Blue V has a higher diffusion coefficient than complement C5a. Nevertheless, using Patent Blue V it is possible to visualize how the chemoattractant-containing medium is drawn into the reservoir and how it diffuses until the reservoir is uniformly blue. In the following 1-2 days it is hard to detect the blue dye in the other (opposing) reservoir by visual inspection. This underscores the effectiveness of the system. That is, one can "see" that a gradient has been generated across the narrow connecting area. Even better, the chemoattractant lags behind the smaller blue dye, so that the complement C5a gradient is even longer lasting. Notably, it is good that the gradient is dynamic, steadily growing, because this is how gradients occur *in vivo*, that is, gradients are not static, but dynamic. In the past, to take into account molecular weight differences, we have measured, or approximated, the kinetics of the complement C5a gradient using a fluorescent dye with similar weight to complement C5a, such as 10 kDa fluorescent dextran.

Line 304: Thanks for spotting this. The reference has been added.

Lines 355-356: The following sentence was added: "Macrophages in the lower half of the observation area and showing displacement (movement) of at least one cell width over 6 h were randomly selected for analysis."

Lines 369-370: Chemicals, such as lipopolysaccharides or other Toll-like receptor ligands, may stimulate motility (chemokinesis) without having chemotactic activity.

Line 389: Thioglycolate would induce sterile inflammation (peritonitis) which would attract various immune cells, including neutrophils, followed by monocytes. After 1-2 days, the peritoneal cavity will potentially contain a heterogenous population of cells, including resident peritoneal macrophages, monocyte-derived macrophages and neutrophils, although an enriched population of macrophages can be obtained in the appropriate time window etc. The intraperitoneal injection of thioglycolate also introduces ethical considerations since a proinflammatory procedure is performed on a living animal. Moreover, more resident peritoneal macrophages are obtained than are required for filling 6, 12 or even more chemotaxis slides, each of which only requires 10 µL of cell suspension with a concentration of 10×10^6 cells/mL. The weakly adherent B cells are generally washed away during the filling procedure.

Line 435: That is correct, although we had written in the next sentence: “However, the time-lapse 2D chemotaxis assay described herein can be adapted to a 3D chemotaxis assay and both assays are suitable for phase-contrast and/or fluorescence microscopy and can be adapted for different cell types.”
The summary (last paragraph of the Discussion), which included the above text, was rewritten in response to suggestios from Reviewer #2. The summary now reads:
“In summary, we describe a real-time chemotaxis assay which allows the visualization of cells navigating in a chemotactic gradient over a period of six or more hours. Herein the assay has been customized for macrophages, which play major roles in inflammatory diseases, but have traditionally been circumvented in favor of faster moving cells like neutrophils and *Dictyostelium* amoebae.”

Response to Reviewer #2

Thank you for your detailed input and suggestions to improve the manuscript.

(Lines refer to originally submitted manuscript)

1. Line 46: Now deleted.
2. Line 52-53: Good point. We have interposed a new paragraph which elaborates on chemokines (as suggested by Reviewer #1) and makes a link to the next paragraph.
3. Abstract: We have extended the last sentence of the Abstract to highlight the advantage of the system. We have, for example, used this system together with knockout mouse models to identify the roles of G protein subunits in chemotaxis (manuscript complete).
4. Line 72: The reference was added to Line 73 of the originally submitted manuscript.
5. Start of Protocol Section: Good idea. A workflow diagram is now included.
6. Line 157: Reviewer #1 made a similar suggestion. The reply to Reviewer #1 is pasted below.
“Thioglycolate would induce sterile inflammation (peritonitis) which would attract various immune cells, including neutrophils, followed by monocytes. After 1-2 days, the peritoneal cavity will potentially contain a heterogenous population of cells, including resident peritoneal macrophages, monocyte-derived macrophages and neutrophils, although an enriched population of macrophages can be obtained at the appropriate time window etc. The intraperitoneal injection of thioglycolate introduces ethical considerations since a proinflammatory procedure is performed on a living animal. Moreover, more resident peritoneal macrophages are obtained than are required for filling 6, 12 or even more chemotaxis slides, each of which only requires 10 µL of cell suspension with a concentration of 10×10^6 cells/mL. The weakly adherent B cells are generally washed away during the filling procedure.”
7. Lines 165-166: Good point. We presume that *in vivo* resident peritoneal macrophages weakly adhere to the mesothelium lining the peritoneal cavity and/or are simply in suspension in an inactivated state. Nominally Ca^{2+}-free HBSS was used since the low [Ca^{2+}] may reduce integrin-dependent adhesion. As far as we are aware, Ca^{2+}-free, bicarbonate-free RPMI 1640 Hepes medium is not a standard, readily available formulation.
8. Line 173: For economy, in accord with the Editorial comments, we deleted some text, part of which included reference to abdominal massage. In any case, we gently massage the abdomen for about 10 s.
9. Lines 177-178: The typical number of cells obtained per mouse has been added.
10. Lines 183-184: Positive or negative selection methods would introduce more interventions. We prefer to minimize the handling of the cells.
In the originally submitted manuscript, we wrote in the Discussion:
“Fluorescence microscopy can be substituted for phase-contrast microscopy, which offers advantages for automated cell tracking, since fluorescently labeled cells can be

readily distinguished from the background. Another advantage is that specific populations of immune cells can be selectively tracked after labeling surface markers with fluorescent antibodies. We used this approach for imaging human peripheral blood $CD14^+$ cells (monocytes) migrating in a chemotactic FMLP (N-formylmethionine-leucyl-phenylalanine) gradient[36]."

We now added the sentence:

"Similarly, fluorescent anti-F4/80 antibodies could be used to image mouse macrophages migrating in a chemotactic complement C5a gradient."

11. Line 188: The mixing procedure has been elaborated:
"After pipetting the cell suspension up and down five times with the pipette volume set at 100 µL (or set at half the suspension volume) to reduce clumping … ".
12. Good point. The order of figures was based on the Results section. We have now based the order on the main text (Protocol + Results) and accordingly changed the order of the figures.
13. Lines 225-228: The paragraph has been rephrased.
14. Line 244: The figure reference has been added (Fig. 2 has become Fig. 3 in the revised manuscript).
15. Line 247: See response number 10 above. Indeed, fluorescence labeling would be more reliable than morphological criteria.
16. Line 256: The analysis section has been modified. The link between iTrack4U and ImageJ was misleading and the text has been reworded accordingly. Both programs are Java-based, but iTrack4U is a stand alone program for automated tracking and analysis. The first author of the paper describing iTrack4U (Fabrice P. Cordelières) also produced the ImageJ plugin Manual Tracking. The manual tracking datasets can be automatically analyzed by the ImageJ plugin Chemotaxis and Migration Tool. This is now clearly stated with references.
17. Figures 3D and 3E are now combined into Figure 3D. It would have been more logical to present the migration plot above the box plots of velocity and FMIs, but we wanted to have the original tracks and the migration plot side-by-side, as it is in the figure.
18. Line 307: See response number 17 above.
19. Line 354-359: We have included a box plot of x-FMI values.
20. Line 385-387 and Figure 3D: We suggested an alternative method in the originally submitted manuscript, i.e. by skipping the prefilling step the risk that medium flows into a reservoir is reduced:
"Prefilling, though, increases the likelihood that medium will partially flow into one or both of the flanking reservoirs, which will promote the seeding of cells beyond the observation area. Alternatively, the cell suspension can be directly pipetted into a dry observation area, but unwanted air bubbles cannot be subsequently expelled."
The flow of a small amount of cell suspension into the reservoirs during cell seeding is not solely disadvantageous since the pool of cells can be recruited into the observation area.
21. Line 436-449: The summary has been modified.
22. Discussion: We added the sentences: "Moreover, the assay is suitable for mouse bone marrow-derived macrophages or macrophages derived from conditionally immortalized myeloid precursor cells[39,40] We have previously used Teflon bags with luer adapters to culture bone marrow cells and derive macrophages[34]. The advantage of Teflon bags is that the cells can be readily resuspended, ready for use, after placing a bag on ice for 20-30 min."
23. Materials: The software used for acquiring images and the image analysis software are now provided in the tabulated Materials section.
24. Figure 3D: Fig. 3D is now Fig. 2D. The x-axes are now labeled.

25. Figure 3A: Good point. The “end” of each migration track was indicated by a filled circle, albeit it was too small to readily recognize. We have now enlarged the filled spots so that the end of each track is now clear. This is now indicated in the legend.
26. Figure 3A: An approximate scale bar has been incorporated.
27. Materials: The tabulated Materials section is an Excel (.xls) file with four columns: Name of Material/Equipment, Company, Catalog number and Comments/Description. Catalog numbers have been provided where appropriate.