**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Done √

2. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.Done √

3. Figures 2 and 3: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.Done √

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

Done √. We re-wrote the Introduction a bit to answer both this comment and one below. We re-ordered the paragraphs, emphasized the main goal of the protocol, and removed some redundant phrasing.

5. Please spell out each abbreviation the first time it is used.Done √.

6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.Done √.

7. Please use centrifugal force (x g) for centrifuge speeds.Done √.

8. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Done √.

9. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: GentleMacs, Miltenyi, Microsoft Excel, Ibidi, etc.

Done √. We removed all references to commercial products and put them in the Excel table of materials. We left in references to computer software that is free: ImageJ and its plugins, and the Ibidi chemotaxis tool. “

10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.Done √.

11. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.Done √.

12. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.Tried to do: we removed some notes, but others seemed important and we had a hard time moving them. Please advise if the remaining Notes are acceptable. If not, we will delete or move to the Discussion.

13. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples: Tried to do.  
For culture media and buffer, please spell out at first use and provide composition. If they are purchased, please cite the materials table. Done √.

Line 102: Please specify the filter type, pore size, etc.

Done √. New line 99: We specified “70 µm cell strainer mesh” in the text and put the specific company and order number in the Excel table.

Line 103: Additional 5 mL of what? Please specify.Done √. New line 103.  
What happens after centrifugation? Is supernatant discarded? Please specify throughout.Done √.

1.3: Is 2 min set for incubation or the whole lysis process? Please specify.Done √. New line 109.

1.3.1 and 1.3.2: Please specify the buffer used in these steps.Done √. New lines 132, 136.

1.4, 1.5: Please ensure that the protocol here can stand alone. As currently written, users must refer to another protocol and refer back and forth in order to complete this protocol.Done √. We rewrote the “MZB purification” section to remove mention of the Miltenyi kit and added a more complete description of the procedure in steps 1.3.1 to 1.3.5. We now describe it a “commercially available kit” (new line 123).

1.5.2: Please describe how analyze using flow cytometry.Done √. New lines in section 1.4.1 describing how to gate on the MZBs and added a figure (Figure 1B) to show the FACS gating strategy.

14. Lines 146-150: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.), or move the solutions, materials and equipment information to the Materials Table.Done √.

15. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.Done √.

16. Please remove the weblink and use a superscripted numbered reference instead. Done √.

17. Lines 311-323: Please remove these commands from the protocol and instead include them in a supplemental file.Done √.

18. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.Done √.

19. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.Done √. We downloaded and used the Endnote Styles filter for JoVE.

20. References: Please do not abbreviate journal titles.Done √.  
  
**Reviewers' comments:**  
  
Reviewer #1:  
  
Application of interest to a broad area of scientists......could be improved by adding suggestions of complementary applications (ie effect of added chemokines/ blocking reagents etc...).....data if available....

Done √. We added this sentence (Line 80-83): “These values can be used to determine the effects of migration inhibitors, cell stimulators, chemokines, and other migration-affecting chemicals on the shear-flow induced migration in order to understand the forces controlling immune cell movement in vivo.”.  
  
could use CD1d as an additional MZ B cell marker.....LFA-1, ICAM\_1 expression?

Done √.We “or any other combination of fluorophores for distinguishing between follicular B cells and MZBs” (Line 152).  
  
please mention source of ICAM protein....

Done √. We listed the ICAM-1 in the Excel Materials table.  
  
  
  
  
Reviewer #2:  
  
Manuscript Summary:  
In this manuscript, Tedford et al. describe a method for visualizing and quantifying shear flow-induced cell migration (flowtaxis) using a simple flow chamber method. The authors use marginal zone B cells as a cell model for testing this method given the fact that the lab's previous research elegantly showed MZ B cell migration against flow in vitro and in vivo. The manuscript is well-written and detailed, and likely to be useful to a broader scientific community. A few issues and minor typos were found throughout the manuscript that should be corrected.  
  
Major Concerns:  
None  
  
Minor Concerns:  
I wouldn't call it concern but there are a few minor details that need to be clarified. These are described below:  
Line:  
67 Authors should describe cell isolation methods and may mention a particular kit but the manuscript should avoid over-advertisement of specific companies. For example, authors may write "cells were magnetically enriched with commercially available kits".  
Done √. We rewrote the “MZB purification” section to remove mention of the Miltenyi kit in section 1.3. We now describe it a “commercially available kit” (new line 121).

69-74 Authors may leave the details for the main protocol and just mention that they will be explaining the use of an automated tracking tool (M2track) vs manual tracking in Image J .Done √. We re-wrote the Introduction a bit to address this comment and the one above. We re-ordered the paragraphs, emphasized the main goal of the protocol, and removed some redundant phrasing. We removed the reference to the manual tracking plugin from the introduction as it is not critical to the protocol.  
  
130 The last step of the magnetic isolation is not well described. This reviewer had to look up the protocol online to understand which fraction they were referring to.

Done √. We rewrote the “MZB purification” section and added a more complete description of the procedure in steps 1.3 to 1.3.5. We specified which cells are in the labeled and unlabeled fractions in the two sorting steps.

133/134 When explaining antibody stains use quantity of antibodies/ X number of cells, not volumes or dilutions of antibodies. Different vendors may sell antibodies at different concentrations.Done √. Lines 149-152: We added the necessary information. The sentence now reads: “Add a 10 % volume (5 µl) of Fc block to 10,000 cells in 50 µl and incubate for 5 min at 4°. Add 50 µl of Cell Buffer containing 0.3 µl each of B220-FITC (0.15 µg), CD21-PE (0.06 µg), CD23-APC (0.06 µg), in a total volume of 105 µl (each antibody diluted 1:350).”  
  
139 Describe ages for mice used.

Done √. Line 97: We added this: “…a mouse aged 8-16 weeks old...”  
  
163 Correct ICAM-1 concentration to 400μg/mL.Done √. New line 179.  
  
163 This is not a 100% clear. Do I understand correctly that only 1 chamber of the IBIDI slide is coated? It would be helpful to have a schematic of the flow chamber with the IBIDI slide as an additional figure (maybe even if it is shown clearly in the movie). I assume that only one chamber is used for the experiment and all reagents (coating, blocking, cells) are applied to this one chamber.

191 As mentioned above, a schematic representation of the flow chamber slide and the fluidics setup would be very helpful in addition to the video.

Done √. New line 179-181: We clarified that the slides have 6 chambers each and the researcher can coat as many as there are planned movies: “…and add 30 µl of it [ICAM-1] to one chamber on a flow slide for every migration movie required. Each slide contains 6 chambers and a researcher can easily record up to 8 movies in one session.” We also added a new Figure 1A that shows an image of the flow chamber slide with tubing attached to one chamber, up close in a high magnification image, as well as an image of the pump, fluidics unit, and attached slide.  
  
222 Which resolution is used? What are the pixel dimensions in micrometer?

Done √. New line: 324. We added “..For MZB, a 10x objective was used, giving a pixel size of 1.14 µm.”. Also in Figure Legends, new line 389: “..Calibration settings shown include the pixel resolution of 1.14 µm per pixel and a time interval of 5 sec (0.083 min) per movie frame.”  
  
237 Correct typo. Done √.   
  
276 How exactly is the threshold determined? If automated thresholding is used in ImageJ, which algorithm is the automated thresholding based on?

Done √. We re-wrote section 3.2.1, “Threshold images from the cell migration movie:” to explain how to turn the grayscale or color image of migrating cells into a black and white movie. We write that we simply boosted the contrast of the images to the maximum setting to get white cells on a black background. We then used the threshold command to convert this to black cells on a white background, as this is required by the MTrack2 plugin.  
  
292 What is the size distribution of the MZB cells? It may be useful to show a histogram of MZB size as an additional figure.

We wrote that the MZB sizes are fairly uniform and they would therefore all be contained within the range we chose (from 1 to 20-30 pixels). We made a histogram of MZB size for about about 600 thresholded MZBs in pixels (see below). The sizes range from about 2 to 26 pixels, therefore the value chosen in MTrack2 for the upper size limit (30 pixels), is appropriate. We did not add this additional figure to the manuscript for 2 reasons: one is that the sizes are not precise outlines of the MZBs because they are measured on cells that are slightly defocused and therefore a bit larger; two, we think it may be sufficient to specify the values we used in the text (1-30 pixels) for MZBs, and a researcher using the protocol for a different kind of cell would in any case measure the sizes of those cells. However, we can add the figure to the manuscript if necessary.

C:\Users\tedford\Desktop\Histogram of Data 1.tif

Figure 1R: Size distribution of MZBs used in the movies shown in Figure 3B. 1 Pixel = 1.14 µm.

401 Close-ups of the microscopy images should be shown - it difficult to see the cells and to judge the quality of the binary image. Scale bars are missing.

We now include macro views of a portion of the microscope images and scale bars for all images in Figures 3 and 4.  
  
404 I don't think this figure 2B is necessary if the macro is already included in the protocol.

Agreed, we removed the figure. We will upload the macro text as a separate file.  
  
407 A colored frame could be used to highlight the window / or the values in the screenshot that are mentioned in the figure legend.  
We added a bright green boxes to Figure 3b to the three values specified in the legend.

410 What are the red tracks in comparison to the black tracks? Please state in the figure legend.

We added a sentence to the legend for Figure 4b explaining that the red tracks terminate below the horizontal axis while the black tracks terminate above the axis. The color difference is to help to roughly determine the direction of the migrating cells with a quick glance.  
  
416 Which statistical test was used here?

We added the missing information: Student’s *t*-test.  
  
423 Is VCAM1 blocking migration against the flow in the presence of ICAM1? Please state this more clearly.  
We apologize for not stating this clearly enough. The MZBs under flow on ICAM-1 can migrate relatively long distances up the flow but on VCAM-1 they are mostly stuck fast. On ICAM-1 alone, about 80% of MZBs move more than a cell length but on VCAM-1 only 30% of MZBs move more than a cell length. We believe that if the flow is strong enough, the weak MZB migration on VCAM-1 translates to being pushed down the flow. Otherwise, the MZBs stand fast on VCAM-1, and not because it specifically blocks them from migrating up the flow, rather that it just does not support migration up the flow. We now state this in the legend for Figure 1A, the model of MZB shuttling (New Line 366).

For the discussion of the method, it is important to discuss how comparable the in vitro measured data is to the in vivo situation. For example, how does 4 dyne/cm2 apply to fluid flow in the MZ 3-D environment?  
 It is difficult to discuss the in vivo flow situation in terms of specific shear stress numbers and to compare it to the 4 dyn cm-2 flow that we used in vitro. The early publications that described the existence of flow through the marginal zone, using corrosion casts or injections of labelled particles, did not determine specific numbers for pressure or flow rate in this area. However, it is clear that the blood flows through the marginal zone. We observed flow using injections of labeled antibodies, and the Cyster group observed flow using 2-photon microscopy showing fast movements of B cells caught in flow streams in the marginal zone. There are studies on the rates of blood flow entering the spleen in humans and mice but to our knowledge there is nothing known on the rates of blood flow or pressure specifically in the marginal zone. It’s possible that it’s not easy or technically feasible to measure flow just in this area.

What we can say, is that we showed that on ICAM-1, MZBs migrate in all directions at a flow rate of 1 dyn cm-2 but migrate directionally at a flow rate of 3 dyn cm-2. They maintain the directional migration up to 15-20 dyn cm-2 without much change in their behavior, but we have not investigated what happens at higher shear flow levels. We therefore assume that the flow through the marginal zone is equivalent to at least the 3 dyn cm-2 flow that we used, but may be even higher.