**Point-by-point answers to the reviewers’ comments**

We want to thank the editor and all the Referees for the constructive comments that definitely allowed us to improve our article and contents. We have now addressed the questions pointed out by the referees.

**Reply to the editor comments**

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

Reply: We have been through the manuscript, and fixed outstanding spelling and grammar issues

**2. Figure 2: Please include a space between numbers and their units (i.e., 37 °C).**

Reply: This has been corrected throughout the text and figures

**3. Figure 3: Please define the scale bars in the figure legend.**

Reply: The scale bars have now been defined in the figure legends.

**4. Figure 6: Please define the asterisk symbols in the figure legend.**

Reply: The asterisk symbols used to denote significance are now defined in the figure legend.

**5. Supplemental Movies 4-7: The scale bar in the movie is defined as 30 µm while the figure legend says 50 µm. Please revise to be consistent.**

Reply: We thank the editor for notifying us of this error. We have now corrected this error and defined the scale bar in figure legends as 30 µm, which is the correct definition.

**6. Please provide an email address for each author.**

Reply: an email address has now been provided for each author on the submission website.

**7. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to.**

Reply: We have now included in the manuscript a new summary section that describes the protocol and its applications.

**8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.**

Reply: This is done throughout the manuscript.

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

Reply: A space has now been included between all numbers and the corresponding units throughout the manuscript and figures.

**10. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).**

Reply: The centrifuge speeds have now been changed into centrifugal force throughout the manuscript.

**11. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.**

Reply: This has now been corrected in the manuscript.

**12. 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: Falcon, Accuri C6, BD Bioscience, Cell Tracker, Ficoll, Miltenyi Biotec, AutoMACS, etc..**

Reply: Generic terms are now used throughout the manuscript. All information that was available has now been included in the table of Materials and reagents.

**13. Lines 123-127: Please break up into sub-steps. What happens after centrifugation? Is supernatant discarded or not? Please specify throughout.**

Reply: The steps are now more detailed and include this information.

**14. Lines 128-130: Does the supernatant come from step k (lines 120-122)? Please specify.**

Reply: This has now been clarified, and can be found on lines 128-131.

**15. Lines 184-189, 190-196, 266-268: Please break up into sub-steps.**

Reply: These steps have now been split into sub-steps that can be found on lines 199 to 288.

**16. Lines 247-250, 269-275: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a “Note.”.**

Reply: Every sentence in the protocol has now been written in the imperative tense. All commentaries are now placed in the Note sections.

**17. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.**

Reply: The equivalent of about 2.75 pages of text is now highlighted in yellow in the manuscript.

**18. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense..**

Reply: The relevant text has now been highlighted, and constitutes a cohesive story of the protocol.

**19. Please include all relevant details that are required to perform highlighted steps. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.**

Reply: the highlighted text contains all the relevant details needed to perform the experiments. Thus, the sub-steps involved were also highlighted.

**20. Line 374: Should B be D instead?.**

Reply: This has been corrected within the figure legend.

**21. Table of Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available. Please use SI abbreviations for all units, include a space between all numbers and their corresponding units. Please use subscripts in chemical formulae to indicate the number of atoms, e.g., CaCl2, MgCl2, etc.**

Reply: ALl trademark and registered symbols have been removed. The available details for each product has now been provided in the table of materials file.

**22. References: Please do not abbreviate journal titles.**

Reply: The full names of all journal title have now been provided in the references.

**Reply to the reviewers’ comments**

**Reviewer #1:**

**Manuscript Summary:**

**Monocytes play a critical role in the pathophysiological functions including inflammation, tissue damage and autoimmune processes. They are also important markers during some of these processes and may also aid in regeneration mechanisms under ischemic insult. The current manuscript by Ropraz et al. describes a novel method that can identify and visualize monocyte subpopulations using a confocal based imaging method. It should be highlighted that this method can differentiate adherent and transmigrated monocytes, which is key for pathological state of the disease/condition that will be helpful in understanding the molecular signaling cascade that may be triggered.**

Reply: We thank the reviewer for this clear summary of the content and the scope of our manuscript.

**Minor Concerns:**

**The review has to be revised for grammatical errors. The terminology used in the manuscript have to be consistent through out. For instance and not limited to, usage of TNFA, alfa has to be denoted by a symbol.**

Reply: We have been through the manuscript and corrected all outstanding grammatical errors. Symbols are now correctly used in the manuscript to specify the exact proteins.

**Reviewer #2:**

**Manuscript Summary:**

**The article entitled "Simultaneous study of the recruitment of monocyte subpopulations under flow in vitro" by Ropraz et al. discusses detailed procedures used for isolation of human venular endothelial cells and monocytes, and quantification of monocyte recruitment in vitro. This is a well written protocol for a fairly complex experimental setup that demonstrates the authors' expertise in the area..**

Reply: We thank the reviewer for this clear summary of our manuscript and for the comment. We agree with the referee that the protocol proposed has been written to make the experiments simple to perform.

**Major Concerns:**

**1. Improve the quality of the text within figures.**

Reply: We have been through the whole manuscript and rewritten the parts we thought required changes according to this recommendation. We think this renewed version of the manuscript now contains the information in sufficient detail to perform the experiments.

**2. Can CD14 and CD16 cell markers be used at the** **same time to differentiate between the three different populations of monocytes? Perhaps show a figure demonstrating this.**

Reply: We thank the reviewer for this interesting suggestion. The double staining with the anti-CD14 and anti-CD16 is theoretically possible to differentiate between the three populations of monocytes. However in our hands, the fast bleaching of fluorescence in far red rendered the double staining of CD14 and CD16 difficult. Thus this limitation would hamper the investigation of the three monocyte subpopulations if used simultaneously.

**Minor Concerns:**

**1. Spell out less known acronyms the first time they are used in the text.**

Reply: All the acronyms are now spelled out with the first time of use, as suggested.

**2. Write "silicone tubing" instead of "silicon tubing" throughout section 4 and in figure 2.**

Reply: We thank the referee for this notification, we have now changed this throughout the manuscript and in the figures.

**3. For monoclonal antibodies, state the clone number or indicate if polyclonal antibodies are used.**

Reply: The monoclonal antibodies used in the manuscript are associated with their clone information in the material table.

**Reviewer #3:**

**Manuscript Summary:**

**This is a protocol for studying and comparing human monocyte transmigration under flow. Confocal fluorescence microscopy is used as a read-out and this has advantages: 1. it is possible to simultaneously investigate several different fluorophore-labelled cell types, 2. greater spatial resolution and contrast can be easily achieved especially in the z-direction compared with non confocal methods. I think that the method is in general interesting and useful to the field and as stated in the intro could easily be adapted to other cells.**

Reply: We thank the referee for this summary, which we are in total agreement.

**However, there isn't too much info on how the scope could be extended. Unfortunately, as it stands, the protocol isn't very well written, several steps are confusing (beyond the many typographical errors that would fall under 'copy-editing') and important information is missing. The protocol will need substantial work before publication.**

Reply: We apologize if some steps of the protocol appeared to be confusing to the referee. We have made changes within the protocol to make the message clearer. We feel that there is now all required information to perform the experiment and analyze the results.

**Major Concerns:**

**See above for general comments, specifically:**

**-HUVEC flow chamber experiments are widely used and there are many sources with protocols. The strength here is the fluorescence / confocal imaging and analysis, however, much of the detail on how this is done is missing. Detailed protocols for the analysis necessary to get the quantitative representative data should be included, particularly as freely-available software has been used.**

Reply: As stated above, we think that we have now added enough information to perform the experiment. We have provided the extra details for cell staining, image acquisition under flow and now, as suggested by the referee, we have also explained how to analyze the results. For the analysis, we used ImageJ and the cell count plugin to count the cells. This is now explained. However, we are now developing an easier method of analysis by automating this process.

**-Some of the images and videos are unclear, feint cells are visible in the 'apical' images that look similar to transmigrated cells in 'basal' images, this should be explained.**

Reply: We agree with the referee that the nuclei of leukocytes in one plane may also be slightly visible in other focal planes. This is due to the size of the pinhole and the laser intensity used in the experiment leading to an overspill of the fluorescence signals. Actually, transmigrated leukocytes present flat nuclei affecting the fluorescence detection. Thus the laser intensity and the pinhole of the microscope are set to be able to detect the leukocyte nuclei through all the layers. What is important to distinguish between crawling and transmigrated cells, is the appearance of an unstained area (in the endothelial cell detection channel) around the leukocyte nucleus as now shown with the orthogonal projection (figure 4B or figure 5E). This constitutes a critical criteria for identifying transmigrated leukocytes.

**Following the suggestion below would show both apical and basal sides of the endothelium simultaneously for instance.**

Reply: We agree with the referee that for illustration purposes, it is important to show both the apical and the basal sides of the endothelium simultaneously. This is what we recommend now in the analysis section of the manuscript, as it appears in the figures as well.

**-Section on purity of monocytes affecting transmigration efficiency. This section is confusing. Do the authors mean that the percieved rate is influenced by counting non-monocyte cells, or do they mean the other leukocytes are affecting the actual transmigration rate? This should be more carefully written.**

Reply: We apologize that the result section on the importance of the purity of monocytes for a reliable analysis of transmigration, appeared confusing to the referee. What we point out in this section is that if monocytes are not properly purified, the contaminant cells would be falsely counted as monocytes leading to factual inaccuracies in the result. We have now added a sentence at the end of this section to precise. This is now clear in the text.

**-The importance of strict criteria for analysis is commented on in the discussion, but this information is missing from the protocol.**

Reply: We respectfully don’t agree with this comment from the referee. The criteria for identification of transmigrated leukocytes are different between assays based on phase-contrast microscopy and confocal microscopy. As we commented on in the discussion, the similar appearances of transmigrated and strongly adherent monocytes in phase-contrast microscopy render the analysis difficult. Conversely, as shown here in the manuscript for the confocal microscopy, the appearance of the monocyte nucleus underneath endothelial cells and creating a black hole around its nucleus is clearly an unambiguous criteria for defining leukocyte transmigration. We agree that this was not properly stated in the previous version of our manuscript. We have now clearly stated the criteria for defining monocyte transmigration.

**-In the discussion using 'cell shadows' as a robust signature of transmigration is discussed. This section seems biased, how would this compare with membrane staining / projecting the confocal stacks 'side-on' perpendicular to the imaging plane to simultaneously see both sides of the endothelium. This comment is also relevant to the one above regarding feint cells in the apical images, or is this what the authors mean? This section should be more clearly re-written.**

Reply: We are not sure if we have correctly understood this comment. If it is about the position of the monocyte in the z-axis, and the space occupied by transmigrated monocytes underneath endothelial cells in the abluminal space. To illustrate this, we have now included in both figure 4B and figure 5E orthogonal projections to show that the black holes corresponding to the cell bodies of transmigrated monocytes are unambiguously underneath endothelial cells.

As explained above, the pinhole size, the laser intensity as well as the high z-steps (0.5 µm/step) leads to an overspill of the fluorescence signals rendering the z resolution weak, particularly for monocyte nucleus. This is a technical limitation of the instrumentation used for timelapse imaging. The message of the manuscript is to explain how to perform such experiments with the potential to produce better image quality using different image capture set-ups. However, the proposed protocol in the study will allow for the production of images good enough for analysis.

**Minor Concerns:**

**Representative data, why not include 'xzy' 'side on' projections to illustrate the localisation of the monocytes during transmigration?.**

Reply: As stated above, we have now included in figure 4B and Figure 5E the orthogonal projection of the recruitment of monocytes. For example on the z-projection of figure 5E, we can now see that at 10 min, the CD16+ monocyte (pink) is above endothelial cells (in green). However at 15 min, the CD16+ monocytes are positioned underneath the endothelial cells.

**Protocol 1:**

**c) this step is very hard to interpret, no doubt the video will help, but e.g. what 'wire'?**

Reply: We have rewritten this section of the text, which we hope has clarified this point.

**d) empty the vein how?**

Reply: This was performed by simply pulling the syringe plunger, at one end of the cord. This is now stated in the text.

**i) how confluent?**

Reply: about 80-90% confluence. This is now indicated in the manuscript.

**Protocol 2:**

**several places including e), what temperature?**

Reply: We have now corrected this throughout the text. The temperature of all incubations is indicated.

**h) What is meant by tilt the slide at 45degC and "...take the same volume from the other."?**

Reply: We apologize for stating “45°C” when discussing the tilt of a slide, which we agree is meaningless. We have now corrected this error.

**Protocol 3:**

**b) doesn't make sense, missing word**

Reply: We thank the referee for noticing this missing word. This has been corrected now.

**Protocol 5**

**d) incomplete fixing protocol?**

Reply: The Protocol describing fixing has been written in a complete form.

**Reviewer #4:**

**Manuscript Summary:**

**The manuscript written by Ropraz et al. describes a very elegant and robust method to investigate leukocyte trafficking using an in vitro flow chamber assay in combination with confocal microscopy. Although the authors specifically focus on monocyte endothelial transmigration under inflammatory conditions, this method can easily be extended towards the analysis of other recruitment steps as well as to other leukocyte subtypes. The protocol is very well structured and very precise, each step is clearly explained, and critical topics are highlighted. This holds true for the performance of the experiments as well as for the analysis of the videos. Therefore, I am very confident that this method can easily be adapted in other labs and described results will certainly be reproducible. This manuscript clearly demonstrates the expertise of the authors in this field.**

Reply: We thank the referee for this summary of our work and comments.

**Minor Concerns:**

**Summary of minor suggestions, corrections and questions (point by point):**

**Chapter protocol:**

**1.**

**A) Coating at which temperature?**

Reply: The coating was performed at 37 °C. This is now indicated in the manuscript where necessary.

**L) Why is anti-PECAM-PE not used in the assay? This would define endothelial cell borders and would enable - in combination with the CMFDA staining - the differentiation between trans- and para-cellular transmigration. The authors should discuss this.**

Reply: We agree that the use of a junctional marker has the advantage of indicating a cell-cell junction. As we showed (Bradfield et al Blood 2007, Sidibé et al Nat Comm 2018), in previous transmigration studies, monocytes exclusively migrate into the abluminal compartment via a paracellular route. The use of VE-cadherin has confirmed this observation. Our confocal microscopy studies are consistent with these observations. The transition of migrating monocytes from the luminal to abluminal compartment always occurred between endothelial cells. For these studies, we did not use anti-PECAM1-PE because the antibody is known to affect the integrity of the endothelial cell monolayer. However, we have already used JAM-C labeling in another protocol, and confirmed using confocal microscopy that monocyte transmigration occurred exclusively at cell-cell junctions. We thank the referee for this suggestion, and we have now included in the discussion the possibility to use junctional staining to visualize the endothelial cell junctions during paracellular transmigration.

**2.**

**A) Which Ibidi flow chambers are used? 0.1 or 0.4? Do the authors degas the chambers 24h prior to the start of the experiment (as recommend by the manufacturer)?**

Reply: We use 0.4 µ slides. This has now been indicated in the protocol. To clarify, we do not degas the chambers prior to the start of an experiment.

**H) Typo? “Tilt the slide at 45°C…”. Please correct to 45°.**

Reply: This has now been corrected in the manuscript.

**3.**

**I) Typo? Using anti CD14-FITC to label PBMC makes no sense because of the fluorochrome spectra overlap with CFMDA green tracker labelled HUVECS. On page 9, line 323 the authors use anti-CD14-PE.**

Reply: We agree with the referee and apologize for this error. We meant to state CD14-PE, and not CD14-FITC. This has now been corrected throughout the manuscript.

**5.**

**B) Multi position imaging using distant FOVs (field of views) could be critical because stage movement can induce additional flow/ turbulence. What is here the threshold? How far apart should the FOVs be at max to avoid this?**

Reply: We agree that the use of very distant fields of views could affect the stability of the slide and the focus over time. Nowadays, confocal microscopes are equipped with the perfect focusing system that could help to maintain focus. In our experiments, we usually defined the fields of views within 1 cm radius without experiencing any focusing or experimental problems. As we did not purposefully investigate the effect of long distance between fields of view, we did not generate any threshold or maximum values. However, in order to not generate any ambiguity, we have now indicated in the protocol that chosen fields of views should be within a 1 cm radius.

**E) (including Movie 6/7) Reverse Transmigration occurs rarely but also under physiological/ inflammatory conditions in vivo (see papers e.g. by Sussan Nourshargh). In the movies, not only transmigration from apical to basal plane is occurring but also reverse transmigration. Please refer to the cell in the upper right corner of the movies time point 5:30 to 17:00 min. This cell even re-enters the abluminal site of the HUVECS at last. How often does this happen under these in vitro conditions? Have the authors quantified it?**

Reply: We agree with the referee that leukocyte reverse transmigration can also be observed in vitro under flow, and even quantified by this method. By using phase-contrast microscopy, monocyte reverse transmigration has been studied by Bradfield et al (Blood 2007). By using phase-contrast microscopy, we found that about 5 % of monocytes reverse transmigrate after 25 min and this can go up to 10 % after 60 min of transmigration assay under flow. However, we did not analyze monocyte reverse transmigration by the method in this manuscript as we wanted to focus on the transmigration. However as this has been pointed out by the referee, we have now indicated the possibility of analyzing monocyte reverse transmigration using this method.

**Chapter representative results:**

**-HUVEC activation (page 8)**

**Line 282: Please include reference for ICAM-1/VCAM-1 induction upon TNFα stimulation of HUVECS. Since primary human cells are reacting highly variable, what is the threshold regarding HUVEC activation below which the authors define activation between individual experiments cannot be compared to each other anymore because of too big differences?**

Reply: We have now included references for the induction of ICAM/VCAM1 upon TNF stimulation.

We agree that the diversity of primary cells can potentially lead to a high variability in the expression level of adhesion molecules as well. We think that this is a very good control of HUVEC activation when compared to unstimulated cells and that it could serve for qualifying individual experiments with the same batch of HUVEC. The expression level of adhesion molecules depends on many parameters including the sensitivity of the detection, thus it is extremely difficult to define a threshold. And if one had to define such threshold, it would also depend on which aspect of leukocyte recruitment one is investigating. It is almost impossible to globally define a threshold of difference for each individual experiment. However, the expression level must, by definition, be increased compared to unstimulated cells. In addition, such expression data could accompany the extent of leukocyte recruitment to have a better understanding of the leukocyte recruitment conditions. . All these aspects are now discussed in the manuscript.

**Do the authors always perform control experiments with anti-ICAM1 blocking antibodies? The authors should comment on this and include this information.**

Reply: As now discussed in the manuscript, for mechanistic investigations, it is important to use a negative control in which an essential molecule involved in leukocyte recruitment is targeted. We agree that an anti-ICAM1 blocking antibody could serve for this purpose. This is now discussed in the manuscript. However, during a typical recruitment assay experiment, we don’t systematically use an anti-ICAM1 blocking antibody although we can monitor for HUVEC activation by simple observation. For mechanistic studies, we use different negative controls, including anti-LFA1, anti-beta1 integrin or the pertussis toxin according to the study context. The context of using such blocking antibodies is now discussed in the manuscript.

**-Monocyte transmigration (page 9)**

**It is not exactly clear how precisely transmigration is defined/ quantified? Black hole as reference is intuitive but what is the criterion to be used to distinguish between a cell being just in the process of transmigration and a cell which has almost completely passed through. What is the exact cut-off? Furthermore, I would suggest including (besides the scheme in figure 4A) representative images of orthogonal views to visualize distinct state to make it clear.**

Reply: Monocytes are classified as fully transmigrated when they are underneath the endothelial cells, and the black hole appears around the monocyte nucleus. Only the monocytes that display both these criteria qualify as being transmigrated in this study. In addition, this transition only occurs with migration between endothelial cell-cell junctions. As suggested by the referee we have included representative images of orthogonal views presenting non-transmigrated and transmigrated version of the same monocyte (figure 4B and figure 5E).

**-Angiogenic factor driven inflammation**

**Do the authors check for potential effects of antibody labeling on monocyte transmigration? In other words, did the authors perform control experiments with unlabeled cells and analyzed transmigration rate at the end of the experiment? The authors should discuss this.**

Reply: In our recent study (Sidibé et al Nat Comm 2018), we have shown that monocyte labeling with anti-CD16 for example did not affect the recruitment capacity of the monocytes. However, for new leukocyte subtypes or new antibody it is important to assess any effect of the labeling. This is now discussed in the manuscript, as suggested by the referee.

**-Figure 4:**

**To demonstrate that the assay is robust, and results obtained are reproducible, the authors need to include biological replicates (n=3) and perform statistics. (Figure 4C/D) please include results obtained at timepoint 25 min. Error bars equal what, SD or SEM?**

Reply: Leukocyte recruitment under flow is a robust assay that is clearly established and has been shown to generate reproducible results published in several papers, from our lab and others. In this manuscript, we present an extension of the classical leukocyte recruitment assay under flow by replacing the phase-contrast microscopy with confocal microscopy with timelapse imaging. Our results are absolutely consistent with what has been observed in phase contrast microscopy. Thus the method is robust, and constitutes an essential improvement of the classical methods based on the phase-contrast microscopy. However as suggested by the referee, we have now included biological replicates of recruitment under flow by the confocal microscopy. The error bars are SD and are indicated on the revised figures.

**-Figure 5C (10 min):**

**A monocyte nucleus of the same cell is visible in the apical and basal layer at the same time. Is this a cell which is right in the process of transmigration or is it just an "overspill" of the fluorescence signal due to the limitation in z-stack resolution? Deconvolution of the confocal videos should increase spatial resolution in z and therefore help to separate different cells in distinct steps within the process of transmigration more precisely. The authors should comment on this.**

Reply: We agree with the referee. This was due to the laser intensity and the pinhole size which led to an overspill of the fluorescence signals. We agree that a deconvolution of the pictures might be helpful to improve the quality of the images. This is now discussed in the manuscript.

**-Movies:**

**Movies are running too fast. Please reduce the frame rate.**

Reply: As suggested by the referee, we have now reduced the frame rate to allow easier visualization of the recruitment events over each timeframe.

**Reviewer #5:**

**Manuscript Summary:**

**The manuscript 'Simultaneous study of the recruitment of monocyte subpopulations under flow in vitro', by Ropraz et.al, presents a method for imaging the dynamics of leukocyte transmigration across the endothelium. This is no doubt an important topic for understanding the inflammatory process and diverse inflammatory pathologies. Here the authors describe preparation of a primary human endothelial cell monolayer, isolation of primary human monocytes and methods for combining the two under physiologic shear flow and imaging the subsequent monocyte adhesion and transmigration. The method is focus on simultaneously imaging of monocytes subsets by labeling them either with fluorescent anti-CD14 or anti-CD16 together with a nuclear stain while the endothelial cells are labeled with a cytoplasmic dye (Cell Tracker CMFDA). The presence of displaced Cell Tracker CMFDA on endothelium co-localized with monocyte nuclei imaged by confocal z-stacks allows for determination of adherent, transmigration and transmigrated monocytes, where as the presence or absence of either CD14 or CD16 fluorescent signal around each nucleus allows for the simultaneous imaging of either the CD14+ and CD14- or the CD16+ and CD16- sub-populations. In this way the investigators examine the differential responses of monocyte subsets while share an identical experimental micro-environments, with emphasis on effects of inflammatory cytokines versus VEGF. The method is generally well described and has some interesting and useful features.**

Reply: We thank the referee for this fair description of our work and the comments.

**Major Concerns:**

**None.**

**Minor Concerns:**

**General Comments:**

**1) By focusing on advantages of the current fluorescence imaging methods compared to phase-contrast methods, the discussion gives the false impression that this method is the first to apply fluorescence and confocal imaging to study transmigration dynamics. In fact, many investigator have done so using a range a fluorescent markers and methods over the past ~10 years. A statement of this nature and indicating some of the advantages of the current approach to previous fluorescence methods would be more balanced.**

Reply: We agree with the referee that fluorescence, as well as confocal microscopy, were used previously in studies of leukocyte recruitment. However, the modality of use is novel, as our technique allows the investigation of different subpopulations of leukocytes and their migratory behavior. As suggested by the referee, we have now added a statement to balance and discuss the novelty we have brought to the manuscript.

**2) The discussion notes that one of the advantages of the method is that allows for automated analysis. Was automated analysis in fact described in this method?**

Reply: We have pointed out the possibility to automate the analysis of leukocyte transmigration using our method. This statement is based on the fact that the black hole surrounding the nucleus of a transmigrated leukocyte can be easily recognized by image analysis software. However, we did not use the automated analysis here though we are developing one in our laboratory for such analysis. We manually counted adherent cells as well as transmigrated ones to calculate the transmigration rate. This is now described in the methods.

**3) Shape change as an indicator of endothelial activation, should be presented with the caveat that while our experience has shown this generally to be true for HUVEC (macrovascular endothelial cells), microvascular endothelial cells (that more appropriately model the settings of leukocyte trafficking) do not typically show major shape change following activation.**

Reply: We thank the referee for this comment. We included this precise detail in the discussion now.

**Specific Comments:**

**1) The Method begins with description of HUVEC isolations. It seems to jump into instructions without appropriate background or orientation. It would seem worth mentioning that this was the original method for isolating primary endothelial cells, in part because umbilical vein is a readily available source of discarded human tissue. Moreover a large vessel umbilical vein offer potential to collect with many endothelial cells through a convenient methods that basically involves filling the lumen of this vessel with collagenase to elute the innermost layer of cells (i.e., the endothelial cells). Also, if you wish to describe this method here, a schematic should be included. Though in principle an easy method, to the uninitiated a visual aid would be very useful.**

Reply: Previously, we wanted to focus on the leukocyte recruitment modalities. However, following the referee’s suggestion, we have now included an introduction on HUVEC isolation. However as this isolation will not be part of the video, we did not provide a schematic. We thank the referee for the suggestion.

**2) There are some easily fixed ambiguities. For example Section 1, step m. 'Discard the supernatant…' What supernatant? Since the supernatant in question was not from the proceeding step, it is much more clear to simple state: 'Discard the supernatant derived from step k, above'.**

**There is a similar issue is 2i. What pellet are we washing?**

Reply: This has been rewritten to avoid ambiguity. Thus if the instruction is not for an element coming from the preceding step, we have been more precise, as suggested by the referee.

**3) 4f. Plonge or plunge?**

Reply: It is “plunge” instead. We thank the referee for all this careful reading and suggestions.