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Essen, March 25th, 2019

Dear Dr. Nam Nguyen, dear Reviewers

First, we would like to thank the Editor and Reviewers for taking their time and for a thorough revision of our manuscript JoVE59807 entitled: "Transfer of manipulated tumor-associated neutrophils into tumor-bearing mice to study their angiogenic potential *in vivo* ".

We carefully addressed all the reviewer concerns and questions. Please find enclosed the revised version of our manuscript and below a point-by-point response to all comments. We feel that the revised version of our manuscript is significantly improved and should be now suitable for the publication in the JoVE.

With best regards,
Yours,

Jadwiga Jablonska

Point to point response:

Question	Answer
Editorial board	
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.	Thank you for the comment, we corrected this accordingly
2. Please revise lines 58-59, 197-199, 201-203, 207-209 to avoid previously published text.	done
3. Are any figures reprinted from a previous publication?	There are no figure reprinted from the previous publication
4. Keywords: Please provide at least 6 keywords or phrases.	The 6th keyword is added
5. Introduction: Please expand to include the advantages of the presented method over alternative techniques with applicable references to previous studies, description of the context of the technique in the wider body of literature and information that can help readers to determine if the method is appropriate for their application.	done
6. Please define all abbreviations before use.	done
7. Please abbreviate liters to L (L, mL, µL) to avoid confusion.	done
8. 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. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Zeiss AxioObserver.Z1, Neomount, Matrigel, etc.	done
9. 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.	done
10. 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. See examples below.	done
11. Line 84: How to disinfect the mouse? Please describe.	done
12. Line 88: Please describe how to control tumor growth, sacrifice the mouse and remove tumors.	done
13. Line 89: Please provide the composition of the DMEM complete. What container is used?	done

14. Lines 92-94: Are the tumors from one mouse or multiple mice? What container is used for digestion? In 1 ml of what? It is unclear. What volume of digestion solution is used?	done
15. Line 95: Is the residue left on the filter discarded? Please clarify. How many filters/tubes are used per mouse?	done
16. Line 96: Add PBS to what? The 15 ml tube? What volume of PBS is used?	done
17. Line 104: 5 □l of DAPI or Ly6G-PE? It is unclear.	done
18. Line 106: What volume of PBS is added?	done
19. Line 107: What volume of DMEM complete is used? What does it mean by "sort into cold DMEM complete"?	done
20. Line 108: Is the sample analyzed by FACS?	done
21. Line 110: How to check the purity of the sorted neutrophils?	done
22. Line 115: Please list an approximate volume to prepare.	done
23. Line 125: Please provide the composition of the endothelial cell growth medium.	done
24. Line 129: Please describe how these are actually performed and specify all surgical tools used.	done
25. Line 141: Please describe how to prepare melanoma cells. What does mln mean?	done
26. Line 142: What does p.4.6 mean? Also p.6.2, p.1.8?	done
27. Line 160: At what temperature are the sections fixed?	done
28. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.	done
29. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.	done
30. After you have made all the recommended changes to your protocol (listed above), 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.	We have highlighted essential steps
31. 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. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.	done
32. Please include all relevant details that are required to perform the step in the highlighting. 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.	done
33. Figure 3: Please include a space between the numbers and their corresponding units of the scale bar. Please describe panel A and B in the figure legend.	done

34. Figure 4: In panel A, please make the number "3" in "mm3" a superscript. Please describe the right panel in the figure legend and probably label it as panel C.	done
35. Figure 5: Please include a space between the numbers and their corresponding units of the scale bar.	done
36. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.	done
37. 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. Please do not abbreviate journal titles. See the example below: Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).	done
Reviewer #1	
-It seems Fig 4A and Fig 4B were already used in their recently published papers (Fig4b in References 14).	Yes, the data were published in our previous manuscript, since the story is based on this manuscript. We have modified the figure so that it does not resemble IJC figure.
-Numbering is wrong. My guess it happens after removal of part 1. It looks like it was not updated.	done
-Step 5.7 is not a repeat of step 5.1-5.4 as described by the authors.	Thank you for the comment, we corrected this
-On page 2 / 6, line 88: it is better to give a tumor size at which the mice need to be sacrificed, rather than a day.	The day reflects the stage of the tumor development, tumors should not exceed 15 mm in diameter according to the animal care regulations. We sacrifice mice for this experiment at day 14. Tumor size at this time depends on mice and treatment
-On page 2 / 6, line 104: although the figure 2 shows CD11b staining, this antibody is not included here. Please add it.	done
-On page 2 / 6, line 116: In which volume do you seed these TAN ? Also specify how many TAN per well and not merely "into 2 equal parts" as you might obtain different amounts of TAN depending on the tumor. Your inhibitor might be titrated differently if you don't use the exact same number of TAN per well every time.	The reviewer is right, we corrected this
-On page 2 / 6, line 118: although the quantity of DMSO is low after the dilution of the inhibitor, still some DMSO should be added to the control well not just DMEM.	The reviewer is right, we corrected this mistake
-On page 2 / 6, line 125: do TAN survive in this endothelial cell growth medium, without serum neither cytokines?	TANs due to their activation in the tumor milieu survive in this medium up to 3 days.

	During this time, TANs release cytokines and growth factors that stimulate endothelial cells and initiate angiogenic processes. Our experience shows that their longer presence is not needed.
-On page 3 / 6, line 151: with this 2nd injection i.v. this time, most neutrophils will go to the lungs, liver and some in the spleen, but very few in the tumor (which is not even palpable at 1-4 days). So I'm not really sure what could be achieved here.	Growing tumors secret neutrophil-specific chemotactic factors, as the accumulation of these cells is observed in the place where tumor cells were injected already at day 1. It happens long before tumors are palpable. Probably, such released chemokines attracts iv injected neutrophils, since our previous experiments demonstrate their homing to the site of injected tumors.
-On page 3 / 6, line 155: This formula is accurate for ultrasonography measurements but not for calipers. For calipers, it is probably better to use V = (l2 x L)/2, where l and L are the shortest and longest diameters (in mm) of the tumors, respectively.	Our observations show that the shortest diameter = depth. Therefore, we use this formula in our publications. However, since we mainly compare growth of tumors between different conditions or mouse strains the formula that we use is not critical.
-On page 4 / 6, lines 178-179: Here, neutrophils were injected twice in the flank while earlier (page 3 / 6, line 151), the second injection was i.v. What is accurate?	The second injection was i.v. – we corrected this now
Reviewer #2	
While all the steps are listed, it would be appropriate to add clarifications and rationale to the different steps at various points so the researchers understand why specific steps are performed the way they are and understand the importance of these steps.	Thank you for the comment. The reviewer is right, we corrected this accordingly
It would be important to change the wording on the animal use (see comments below) as they are determined by country/organization/animal care committee	done
It would be important to provide a table or graph that outlines the yield per tumor or tumor size so that researchers can plan their experiments accordingly. It is also likely that combining several tumors would result in relatively less loss/higher yields than individual tumors.	Thank you for the comment, we agree with that. We corrected this accordingly
The figure with the timeline is not very clear. There are 2 groups of donor mice that provide neutrophils for the recipient on 2 different days. The aortic ring assay is likely to be run in parallel, but the abstract suggests sequential order (not likely that the neutrophils will be alive after the 12 days sitting in a well)	Thank you for the comment. The reviewer is right. These two experiments are run in parallel. First, controls the angiogenic capacity of neutrophils *in vitro*, second one *in vivo*. We corrected the

	figure and hope that it is clear now
Line 83: is there a specific reason why female mouse are used. It would be good if the authors indicate whether this would work in male mice as well	It is an interesting question the reviewer raises here. Despite of the fact that male and female innate and adaptive immune responses are comparable, we observe in our experiments that female mice grow bigger tumors. Moreover, males due to their aggressiveness are prone to infractions of the tumor, which increases inflammation and influences tumor growth. Therefore, to obtain comparable results, we always stay with the same gender.
Line 85 presumably the B16F10 is injected s.c.	The reviewer is right, corrected
Line 88: letting tumors grow by days rather than size might be in violation of many animal care standards and protocols. Please indicate a maximum tumor size that you work with and describe the consistency of the tumor. At this time point it is likely that the tumor has become necrotic in the middle and might fall apart/rupture upon harvest.	The reviewer is right. We stick to animal care standards and we indicated reviewer concerns in the text.
Line 89 please clarify DMEM complete	done
TAN isolation, Please indicate at the top that except for the 1st step the cells should be kept cold to improve viability and yield (so the reader will know that it is not an option)	done
Please indicate at the beginning that this will be flow sorting (if the authors have ever tried another method (such as magnetic beads it would be good to indicate, even if it is to warn people against it).	The reviewer is right, we included this into the manuscript
Indicate what machine you are using as there can be large differences in "fragile cell" viability using jet-in-air vs cuvette systems.	The information about sorter is included into M&M table
Line 108 : For the sorting, please provide a note indicating that the neutrophils will have high FSC and SSC and that the gating strategy involves these large gates.	The reviewer is right, it is shown on the Figure 2, which displays our sorting strategy (R2).
Line 109 : Please indicate why the "tail" of the Ly6C CD11b cells is not included in the gating strategy	We think the reviewer means Ly6G "tail". Such gating excludes monocytes that are present in the tail.
Line 110 : please add that the checking for purity is done by re-running it through the flow cytometer.	done
Please indicate something about expected yield/tumor, viability, the effect of combining tumors or multiple tumors on the yield. Please indicate something about your preferred manner of counting since the numbers are so low and it is	done

likely that flow sorting and the manipulations will affect the numbers.	
NAMPT inhibition in vitro Line 116: At point 2 indicate how many cells/well (please provide a not if you have seen any effects on viability with different seeding densities	done
Line 125 : At point 6, this step is for the angiogenic assay and not for the transfer into vivo	done
Line 129 : Please explain why the male aorta is used	done
Line 134 please proved more information on the embedding (pushed down? More Matrigel added? The originally cast Matrigel should be relatively sturdy by now	done
For the figure 3, it would be good provide the scoring system for the branching or provide the reference with the scoring system	This is an interesting idea. We will try to include it into our next studies. For the need of this protocol counting of branches was sufficient
Adoptive transfer Line 141 : please indicate the fluid used (PBS?)	The reviewer is correct, done
Line 143 : please indicate the fluid used (PBS?)	done
Line 149 : housing will be dependent on the specific animal facilities and can vary widely between facilities/countries). If there is a need to do small group housing, please elaborate.	There is no need for special housing, females can be kept according to our animal care regulations
Line 150 : presumably step 1 is not repeated. Please indicate why there is a repeat step and what would happen if this is done on a different day (this will tell the researchers how much flexibility they have in the planning)	Neutrophils have the maximal effect during the initiation of angiogenic processes in the early stages of the tumor development (0-3 days). Depletion or transfer of these cells later have no effect on the tumor vascularization
Tumor growth Line 156: please indicate that harvest would be determined by tumor size/ restrictions of the animal welfare guidelines	done
Line 168 please provide some information on the quantitation of the images (or provide a reference)	done
Reviewer #3	
-Although the protocol concerned is one that was used in the IJC paper, effort should be made to make the commentary, etc., more in keeping with this protocol and not reiterate the findings of the IJC paper too much. Critical assessment of this protocol and how it compares with others could be expanded and this would help to make it distinct from the IJC paper.	Thank you for this comment. We tried to address this in the manuscript
It is not always clear whether vehicle controls are to be used and their formulation.	Thank you for this comment, we addressed it in the manuscript
Although reasonably clear, the paper does need considerable copy editing to make it suitable for publication.	We went through the manuscript and edited it accordingly. We hope the reviewer is satisfied

Final or stock concentrations of all Ab should be listed not just their dilution factor.	We included this information into M&M table, when provided by the manufacturer