Dear Editor,

We thank the reviewers for reviewing our manuscript and for their valuable comments. We have edited the manuscript to address their concerns. You will find below our point-by-point responses.

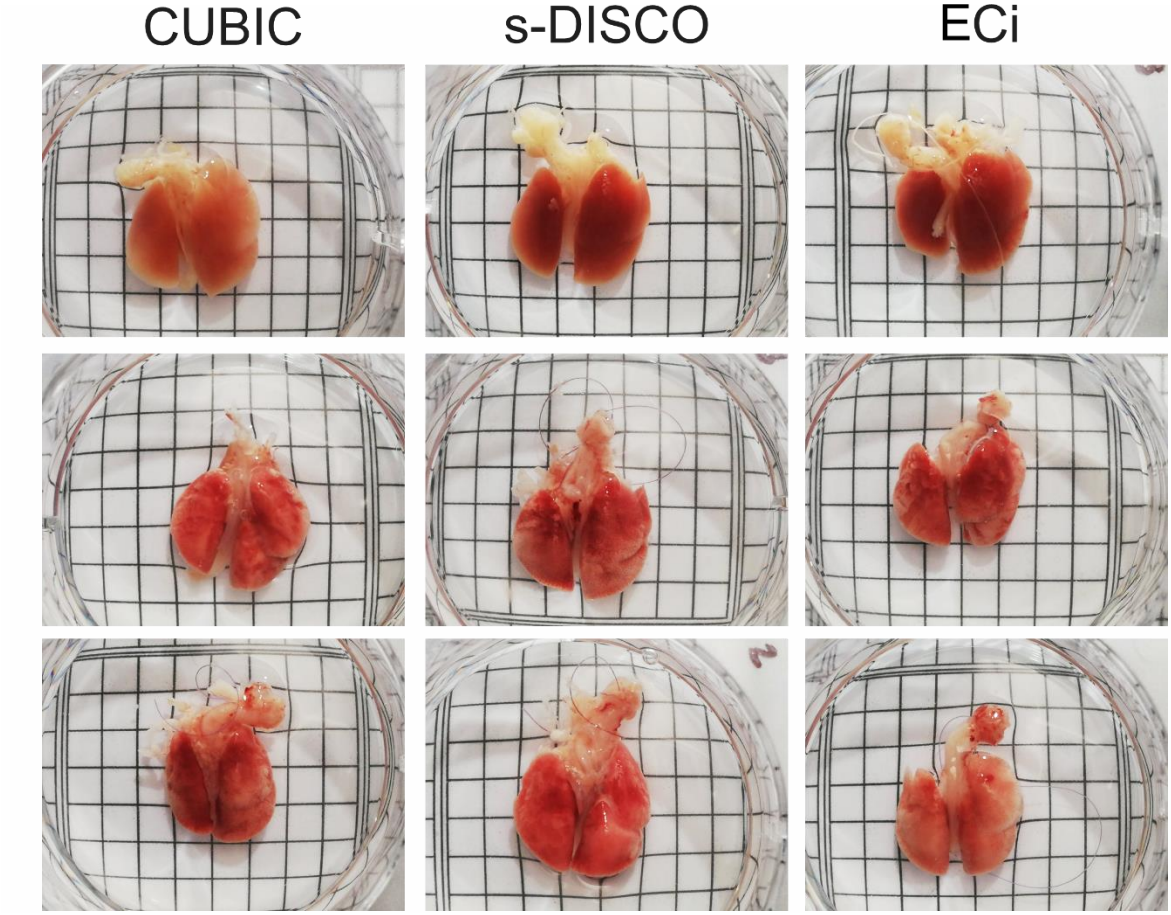
Reviewer 1:

1. Figure 1a is not necessary at all.

   We agree that this figure is not vital. However, we believe that it helps the reader to understand the cellular tools used for the study and how they can be detected *in vitro* and *in vivo*. We therefore prefer that the figure is retained.

2. The lungs before clearing for each group (CUBIC, s-DISCO, ECi) are obviously different; for instance, the lung for CUBIC-group looks whiter than the other two, leading to the injustice of the comparison. Furthermore, the calculation method and description in Figure 2d are confusing: why the value of size change in the 'Before' group is between 0.2 and 0.4, there is no clear explanation in the text. More importantly, it is hard to conclude from Figure 2e that "tissue morphology remains unaffected by all the clearing protocols". Additionally, the scale bars are lacking in Figure 2e.

   The lungs before clearing are different due to the variability of results after perfusion. This was taken into consideration and each clearing protocol was tested using lungs representing this variability as seen in the figure below. To avoid confusion and allow a better comparison, Figure 2b in the manuscript has been modified to show comparable images of the lungs before clearing.



A description of the calculation method in figure 2d has been added in the methods section (lines 459-461: *"The area (cm^2) of each single lung lobe was calculated by delineation using the ROI tool and the average of all samples was set as the before value for all comparisons*"). The "before" value is the average area in cm^2 of a single lung lobe. This has also been added in the methods section (line 459) and in the figure legend (line 129).

Regarding figure 2e, we agree that the phrase "tissue morphology remains unaffected by all the clearing protocols" is inaccurate. What we meant was that the gross morphological structures of the lung remain detectable after the completion of the 3 protocols as demonstrated by the annotation of blood vessels and airways. This has been modified in the text (line 145-146: "*Overall, this indicates that the characteristic lung structures remain detectable after all the clearing protocols (figure 2e).*") and in the figure legend (lines 131-133: *"CUBIC, s-DISCO and ECi cleared lungs show that the characteristic lung structures such as large blood vessels [indicated by asterisks (*)], and airways such as bronchi (indicated by arrowheads) are preserved after optical tissue clearing").*

3. The quality of the images shown in Figure 3 is hard to read. What are the valid fluorescence signal in the grayscale Figure 3a and 3b?

We agree that the small size of the images makes it hard to read and we have now enlarged them. The tdTomato signal in figure 3a after clearing with s-DISCO and ECi is undetectable given the level of tissue

autofluorescence. In figure 3b the Evans blue signal appears overexposed given that the tissue shrinkage results in an increase in the fluorescent molecules/field of view. The same exposure times and display settings were used to acquire and visualize the images to reflect the change in the fluorescence preservation accurately.

4. What does the red and white indicate in Figure 4? And the authors only stained 500μm-thick lung sections instead of the intact lung or lung lobe; it is far from enough to study the biodistribution of MSCs in lungs. The challenges of whole-mount immunostaining should be discussed.

Red indicates the tdTomato labelled hUC-MSCs while white indicates the human mitochondria staining. This information is now added to the figure legend (line 196: "*tdTomato hUC-MSCs (red)*"). 500 µm sections were used given that the confocal microscope used in this study did not allow us to image thicker samples. We appreciate that the whole lung was not used but a 500 µm section yields much more information than using thin sections standard in histological studies. Thus, we believe that 500 µm sections provide a better representation of the biology of the administered hUC-MSCs and this information could be extrapolated to the whole organ.

The challenges of whole organ labelling and imaging were added in lines 341-349: "*Immunofluorescent staining of whole organs enables studying biological processes at single-cell resolution while maintaining tissue integrity as well as structure and cell localization. Nevertheless, staining thick tissues poses challenges that need to be considered when opting for this method, such as non-specific binding, poor antibody diffusion, long incubation times, high background, and long working distance objectives [40]. Although we did no stain the whole lung, 500 µm thick lung sections provide a better representation of the biodistribution of the administered hUC-MSCs in comparison with traditional thin sections standard in histological studies, while saving costs and reducing the need for specialized microscopy equipment".*

5. Figure 5c shows the biological distribution of HUC-MSC after tissue clearing. There should be more detailed descriptions for this result.

The description of the data in 5c has been expanded (lines 215-222:*" Subsequent confocal imaging of lung lobes revealed that the cells distributed throughout the tissue and did not home preferentially to any site as they can be found dispersed within the lung 2h after administration (figure 5c, left; movie S1). Ex vivo imaging of CUBIC-cleared lungs collected 24 h after cell administration confirmed that most of the cells had been cleared from the lungs as shown by the reduction in size of the cell clumps (figure 5c, right). The overall cell distribution remained similar as on the injection day, with the hUC-MSCs localizing evenly throughout the organ (figure 5c, right; movie S2)."*

An animation of these data can be viewed below and has been added to the supplementary materials.


2 h cell biodistribution.mp4


24 h cell biodistribution.mp4

6. This manuscript mentioned,'The vasculature was labelled with the CD31 endothelial marker, which showed that the hUC-MSCs appear to be retained in the pulmonary microvasculature as no cells were detected in the interstitium (Figure 5d).' The images shown in Figure 5d cannot support this conclusion.

We agree that this statement is difficult to support based on a 2D Maximum intensity projection. Thus, we've generated a 3D animation (video S1 and video S2) where the colocalization between the CD31 stained microvessels and the tdTomato labelled cells is more easily appreciated. This can be viewed below.

CUBIC lung vasculature 2h after IV hUCMSCs.mp4

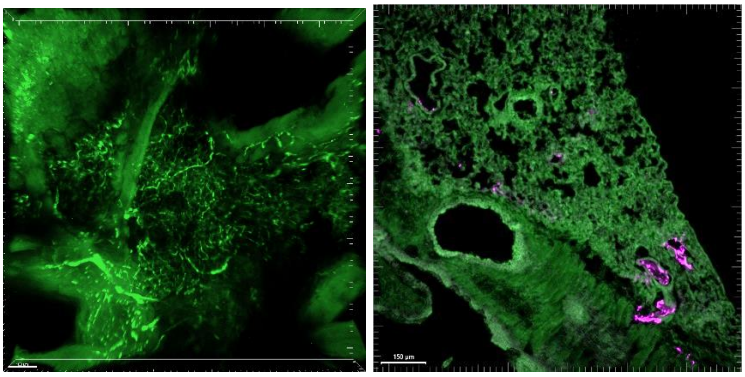
CUBIC lung vasculature 24h after IV hUCMSCs.mp4

Moreover, we have changed the statement to: *"The vasculature was labelled with the CD31 endothelial marker, which showed that the hUC-MSCs appear to be retained in the pulmonary microvasculature as no cells were detected within large blood vessels (figure 5d) at 2 h (movie S3) or 24 h (movie S2)" (lines 236-239).*

7. The authors tried to explain why Evans blue and antibody could not be used simultaneously in their work. Obviously, Evans blue is not an optimal choice in this case. There are multiple dyes that can be used to label the vasculature instead of Evans Blue. It is strongly suggested to consider another choice.

We agree that alternative staining strategies can be used to label the vasculature. Indeed, we tested perfusion with fluorescently labelled dextran of different molecular weights at different concentrations, as well as perfusion with Cy7 fluorescent dyes and did not observe better performance than with Evans Blue, which performed best in our hands. An example of the results obtained using the aforementioned approaches is below:

Dextran Cy7-PEI



8. In the original protocol of s-DISCO, s-DISCO can preserve tdTomato for up to a year. However, the author did not observe fluorescence preservation after s-DISCO clearing. However, in the original s-DISCO or FDISCO paper, tdTomato could be preserved well. The authors owed this inconsistency to the chemical purity. Actually, this problem is easy to be resolved by carrying out the experiments strictly according to the protocol of the original article. But the authors did not do that. In my opinion, they just failed this experiment.

The original s-Disco protocol is complex and requires expertise in chromatographic methods for the purification of explosive substances. Our aim was to find a method that was easily accessible to the broad biology research community without specific expertise in chemistry. We thus modified the s-Disco protocol by using 1-propanol as the dehydration agent instead of tetrahydrofuran and adding propyl-gallate to DBE without previous purification of the reagent to evaluate whether removing the reactive groups in the refractive index matching solution would be enough to preserve fluorescence. The manuscript has been modified to reflect this intention (lines 309-314: "*This protocol is complex and requires specific expertise to purify the dehydration and RI matching reagents to eliminate all peroxide and aldehyde contents before adding the propyl gallate to prevent their regeneration [36]. We aimed to test whether simplifying the protocol to make it more accessible to the wider research community, by preventing peroxide formation in DBE, would suffice to preserve the fluorescence of tdTomato").*

9. None of the three tissue clearing methods described in this manuscript are sufficient to complete the study, so why not use a different method?

We do not agree with the statement above, as we aimed to use optical tissue clearing methods to image thick lung slices in 3D to track MSCs labelled with the genetic reporter tdTomato and study their biodistribution within the host's lung. Although one single protocol did not permit to stain the vasculature, preserve protein fluorescence and perform immunostaining for cells of the immune system, we achieved these goals individually by using a combination of clearing protocols and staining strategies. Nevertheless, we agree that since starting this project, new methods have been developed, and could have been tested yet the variety of clearing protocols can constitute an overwhelming jungle to navigate and we wanted to share our findings using classical well established protocols as a starting point to guide researchers in this fast-paced area. To address this concern, we've discussed alternative methods that could be tested in the future (lines 350-363: "*The field of optical tissue clearing is moving at a fast pace with new protocols becoming available. Here, we did not find a single protocol that allowed us to preserve the fluorescence of tdTomato, stain the vasculature and perform immunostaining simultaneously highlighting the challenges of establishing an optical tissue clearing method for a new application. Alternative protocols that could be tested in the future include Ce3D, OPTIClear, and MACS [14], [40], [41]. The Ce3D protocol has been tested for lung tissue and is compatible with immunofluorescent staining of immune cells while preserving fluorescence of reporter proteins [14]. OPTIClear preserves fluorescence proteins, is compatible with lipophilic labels and most fluorescent dyes. Although this method decreases the brightness of some dyes, is incompatible with some primary antibodies and long term storage in the clearing solutions decreases fluorescence, it might be explored for the purpose of cell tracking in the lungs [40]. Finally, MACS has also been validated for lung tissue and is compatible with fluorescent proteins as well as with lipophilic dyes that have been used to stain vascular structures, and antibody labelling, making it a promising approach [41].).*

10. They should not use both 'S-DISCO' and 's-DISCO' in the context.

We appreciate this detailed observation and have corrected this issue.

Reviewer 2

We thank reviewer 2 for their positive remarks

Reviewer 3:

1. To acquire 3D distribution of fluorescent-labeled MSCs in mouse lung, the authors compared the performance of three tissue optical clearing methods. However, given that there are many excellent tissue clearing methods already established (e.g. Ce3D (Li et al, PNAS, 2017, DOI:10.1073/PNAS.1708981114), OPTIClear (Lai et al, Nat. Commun., 2018, DOI:10.1038/s41467-018-03359-w) and MACS (Zhu et al, Adv.Sci., 2020, DOI:10.1002/advs.201903185)), many of which may possess superior ability than the three methods selected in this study. Why did the authors choose these three methods for comparison? According to the results demonstrated, the authors seem not find a best method that totally meet their demands in the three methods. Therefore, the author may perform additional experiments for more clearing methods to ensure that their choice for clearing methods is optimal, or at least discuss.

   The field of optical tissue clearing is moving at a fast pace. The study was performed when some of the methods suggested for consideration by the reviewer were not available. We trust that sharing the results of the methods tested will help the research community to navigate this complex and constantly evolving area and highlights the considerations required to establish an optical clearing method for a new application. Nevertheless, we appreciate the suggestions and we have included them in the discussion as protocols that could be considered in a future study (lines 350-363 quoted above).

2. The authors displayed the clearing results for the three clearing methods and stated that CUBIC methods outperformed other two methods. However, the reviewer felt that the s-DISCO and ECi methods were not performed in an optimal manner. The time for dehydration each step is not enough for whole lungs, leading to insufficient transparency. The authors may prolonged the dehydration time from 0.5 h to 2-4 h, and the clearing performance would be better.

   Although we agree that longer dehydration times would result in increased transparency, the protocol followed was based on (Ertuk, A. et al 2012) [1]. As indicated in table 1 in their paper (below) 30 minutes is an appropriate time to clear whole lungs. Moreover, the goal of the study was to preserve fluorescence, which as demonstrated in supplementary figure 3 could be achieved by reducing dehydration time, albeit compromising tissue transparency.

Table 1 Clearing protocols for different tissues.

From: Three-dimensional imaging of solvent-cleared organs using 3DISCO

Reagents	Mammary gland, lymph node	Spinal cord, lung, spleen	Brain stem	Brain	Brain (long protocol)
50% (vol/vol) THF	20 min	30 min	1 h	1 h	12 h
70% (vol/vol) THF	20 min[a]	30 min[a]	1 h[a]	1 h	12 h
80% (vol/vol) THF	20 min	30 min	1 h	1 h	12 h
100% (vol/vol) THF	3 × 20 min	3 × 30 min	2 × 1 h	1 h, overnight, 1h	3 × 12 h
DCM	15 min	20 min	45 min	—	—
DBE	≥15 min	≥15 min	≥30 min	≥3 h	1–2 d

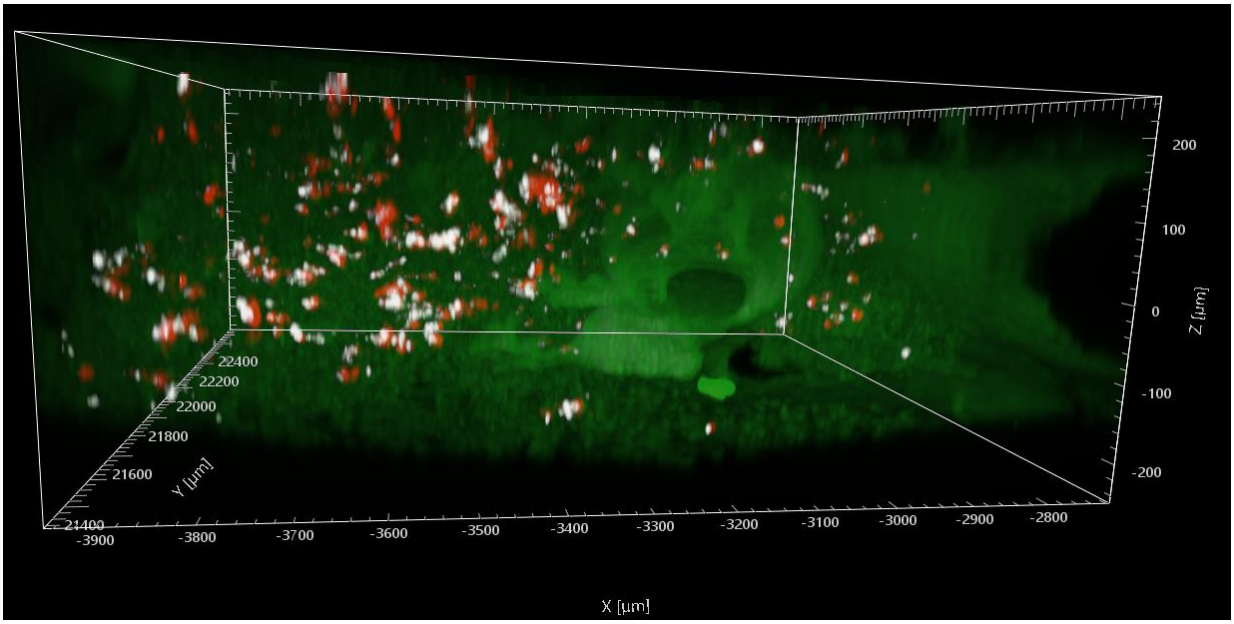
[a]For small tissues, the 70% (vol/vol) THF step can be skipped to save time. Changing the clearing solutions about two or three times during the total incubation times of 100% THF and DBE helps to get better results. For example, for spinal cord and brain clearing, refresh DBE every 10 min and 12 h, respectively. To optimize the clearing for different tissues, follow the protocol for the tissue having similar size and composition presented above and further optimize it if needed by changing incubation times by about 50% (50% increase if the clearing is insufficient or 50% decrease if the clearing is too much).

[1] Ertürk et al., 'Three-Dimensional Imaging of Solvent-Cleared Organs Using 3DISCO'.

2. The image quality for immunofluorescence in Figure 4 is not sufficient. The reviewer could not judge the compatibility of these methods with immunolabeling based on such results.

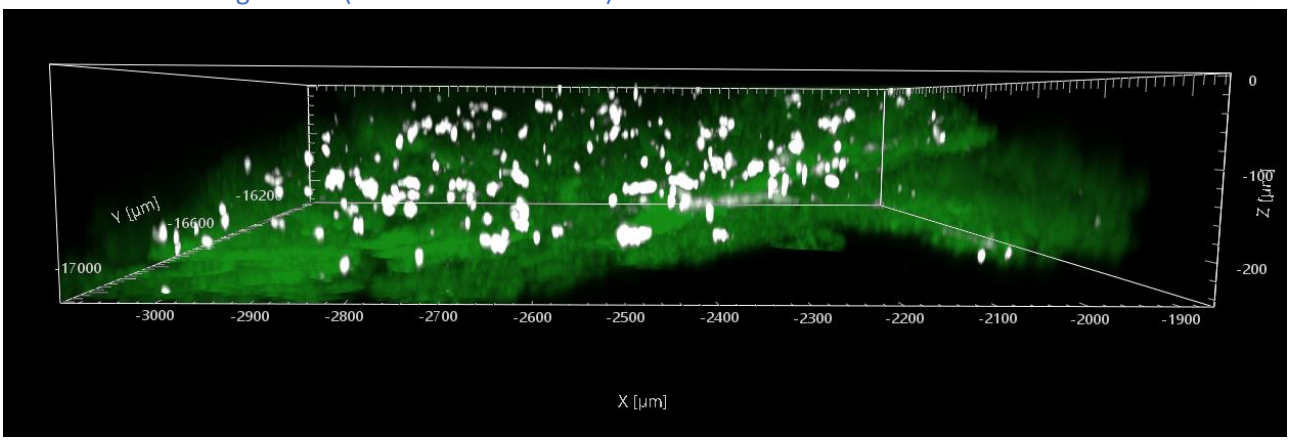
We provide enlarged versions of the 3D representations in figure 4 below, clearly showing that the human mitochondria antibody signal (white) was detectable after the completion of all clearing protocols. Moreover, we have generated animations showing that the antibody signal could be detected throughout the whole sample.

CUBIC stained lung. White (human-mitochondria), red (hUC-MSCs)



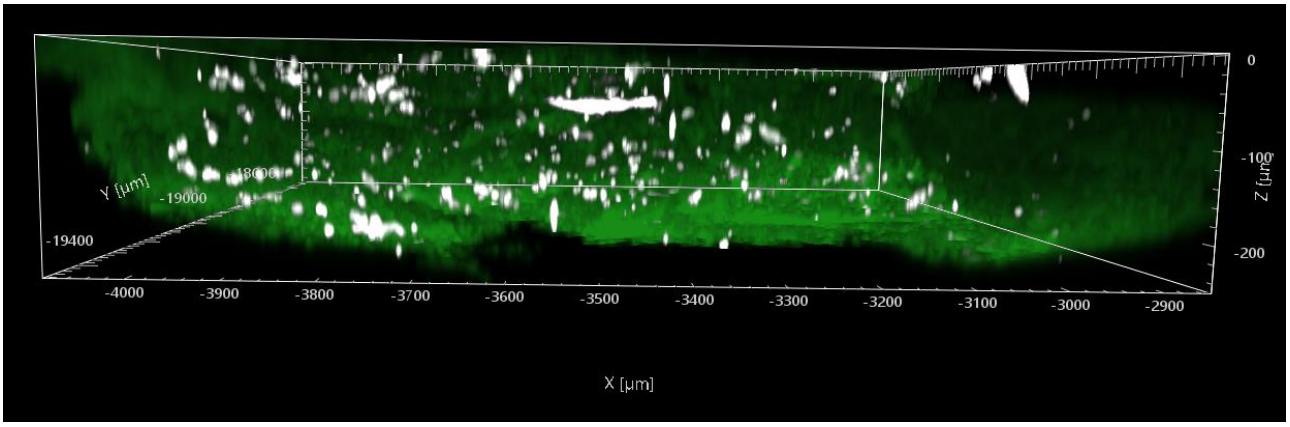
CUBIC immunostaining.mp4

s-DISCO stained lung. White (human-mitochondria)



s-Disco immunostaining.mp4

ECi stained lung. White (human-mitochondria)



ECi immunostaining.mp4

3. As for a comparative study, quantitative results in this manuscript is lack. For example, quantitative comparison of fluorescence preservation in Figure 3 should be added. Statistical analysis of 3D hUC-MSC distribution in Figure 5 should be performed.

We agree that having quantitative results for fluorescence preservation would have been desirable. We, indeed initially tried quantifying these images. However due to the high tissue autofluorescence in the tdTomato channel, it was impossible to obtain meaningful and robust data. For Evans Blue, the results showed a striking increase in the fluorescence of this dye (as can be appreciated from Figure 3).

Statistical analysis of 3D hUC-MSC distribution in Figure 5 is not straightforward as despite having acquired the images using the same imaging parameters, the 3D rendering parameters do not perform equally across images, making the comparison difficult.

4. The imaging depth in Figure 4 seem to be ‘um’ but not ‘nm’. Scalebars are missed in Figure 2b, 2e

The legend in figure 2b mentions “Each line of a square represents 2 mm.”, which serves as a scale bar. Otherwise, we appreciate this detailed observation and have corrected this issue.

We hope that our modified manuscript is now suitable for publication in IJMS,

Kind regards,

Patricia Murray

On behalf of all authors