Peer Review File

Article information: https://dx.doi.org/10.21037/atm-21-2441

First External Peer Review

Comment 1:

First of all, the title does not present what they performed in the submitted manuscript. There is no direct evidence to demonstrate antihyperandrogenism and insulin sensitizing therapy. They simply performed experiments to show the effect of Diane-35 and metformin in a rat model of DHT-induced PCOS. The title of the manuscript should cover the contents of the data directly so that the title should be changed to more relevant and direct one to present what they have in the manuscript.

Reply 1:

Thank you for your suggestion. We edited the topic which are more directly related to the content.

Changes in the text:

We changed the title to "Diane-35 and Metformin Therapy in DHT-exposure Endometrial Lesion" (page 1, line 3 to 5).

Comment 2:

The authors insisted that their PCOS model have endometrial hyperplasia. However, there is no direct evidence that DHT-induced PCOS model has endometrial hyperplasia. In fact, the uterine size was shrinked in PCOS model compared to control and Diane-35 and metformin treatment groups. Without data for endometrial hyperplasia, they should not use term, endometrial hyperplasia in the submitted manuscript.

Reply 2:

Thank you for your advise. We change the term "Endometrial Hyperplasia" to "Endometrial Lesion" [(page 3, line 40) and (page 8, line 135)].

We induced a relative hyperplasia in endometrium. Although the whole uterine size shrinked, the relative size of endometrium increased. In other words, the ratio between volume of endometrium and size of myometrium enlarged (Figure 5 D). We demonstrated this phenomenon in (page 16, line 299 to 297).

Changes in the text:

We change the term "Endometrial Hyperplasia" to "Endometrial Lesion" [(page 3, line 40) and (page 8, line 135)].

We demonstrated this phenomenon in (page 16, line 299 to 297).

Comment 3:

Technically, the quality of imaging and histology in the submitted manuscript is not good enough to demonstrate what they suggest. In fact, tissue clearance imaging is supposed to provide better resolution compared to 2-dimension histological analyses. However, the images in all figures are relatively poor in the submitted manuscript.

Reply 3:

Thank you for your advice. There are two reasons for the low resolution. One is that Lightsheet Z1 microscopy uses a relatively low numerical aperture to achieve wide fields of view, in detriment of its resolution. Another is due to the restriction of two-dimensional picture. As can be found in Extending data vedio 1 to 3, after zoom in by Imaris, the data show better resolution which allow to be analyzed.

Comment 4:

In Figure 5, the authors are strongly recommended to use myometrium-specfic antibodies, such as alpha-SMA, for the accuracy of volume measurement of the myometrium. In addition, desmin, a stromal cell marker, and E-cadherin, an epithelial cell marker, need to be used as well, respectively.

Reply 4:

Thank you for your suggestion. Due to the restriction of Lightsheet Z1 which only has three channels. It only allows we use Dapi and other two immunolabelling markers. From Tong Ma et al. (PMID: 30405019), Tong X et al. (PMID: 32547407), they use Dapi to distinguish ovarian follicles of different stages by Dapi since the morphological difference of these ovarian follicles. As can be found in Extending data vedio 1 to 3, Dapi can directly show three different layer of uterus due to the different density of cells and tissue appearance.

Comment 5:

In Figure 6, the authors should have explained in detail how the gland was identified and evaluated. If the author did not stain with an antibody specific for glandular epithelium, they should have described how the area marked as red were calculated.
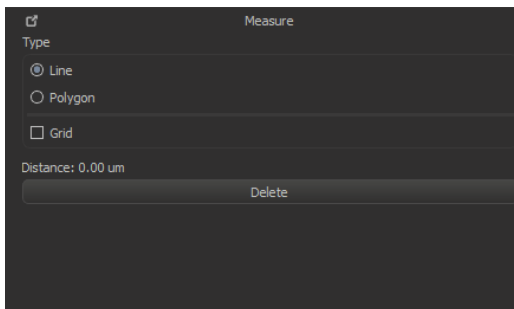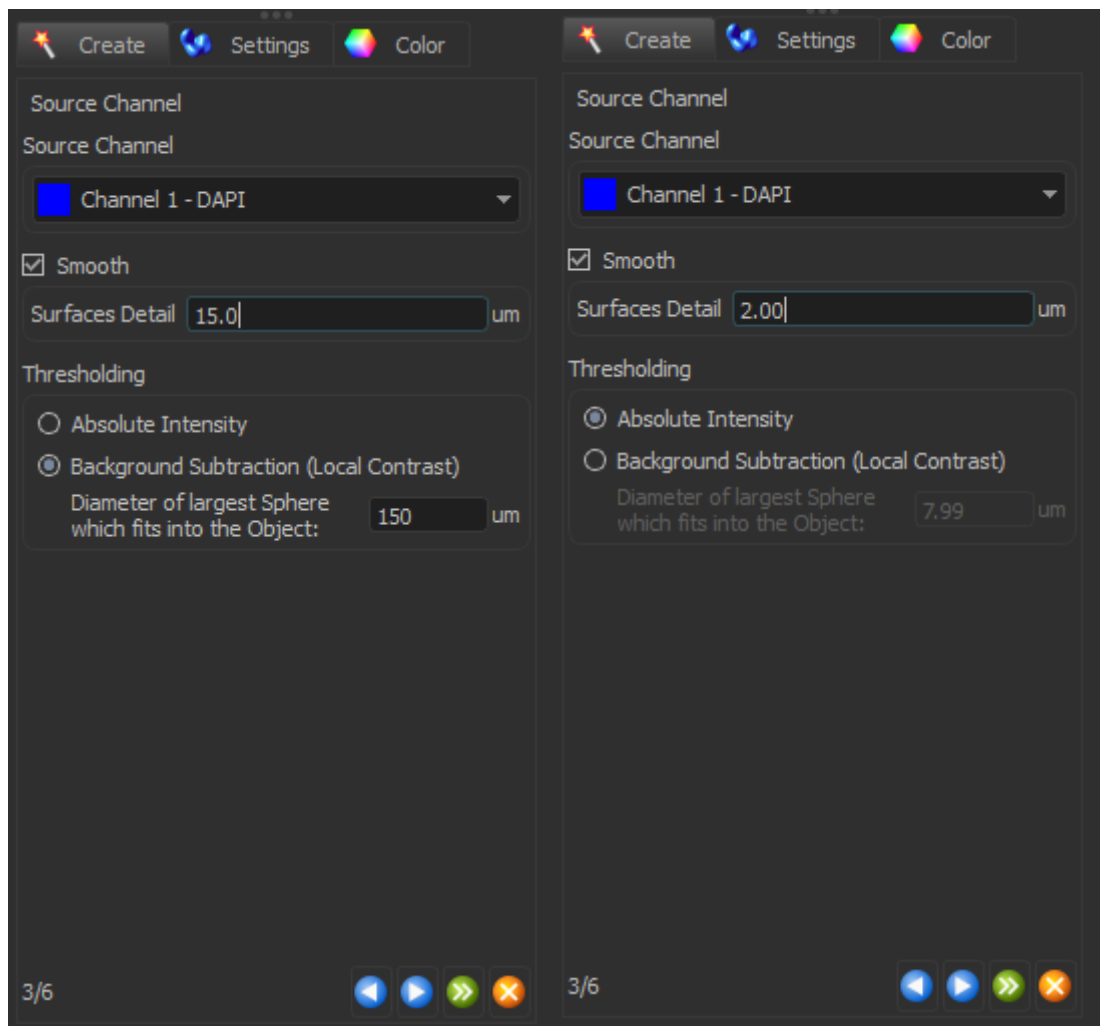
Reply 5:

Thank you for your advise.

Firstly, in 2D mode, the structures of glands, endometrium and myometrium are very clear.
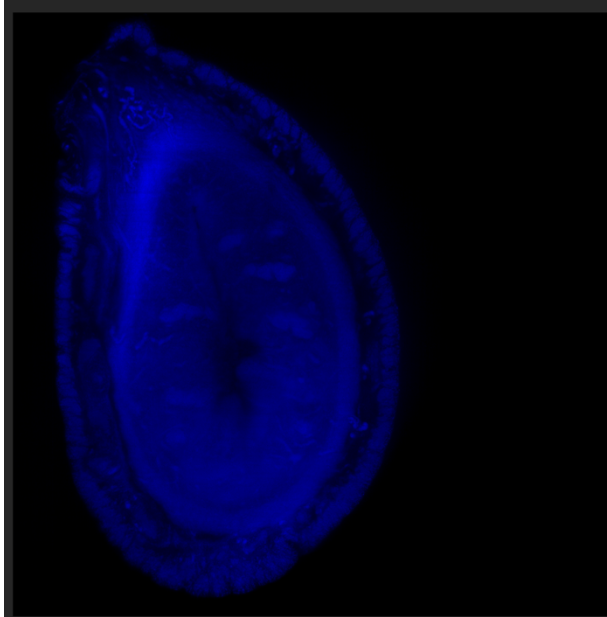
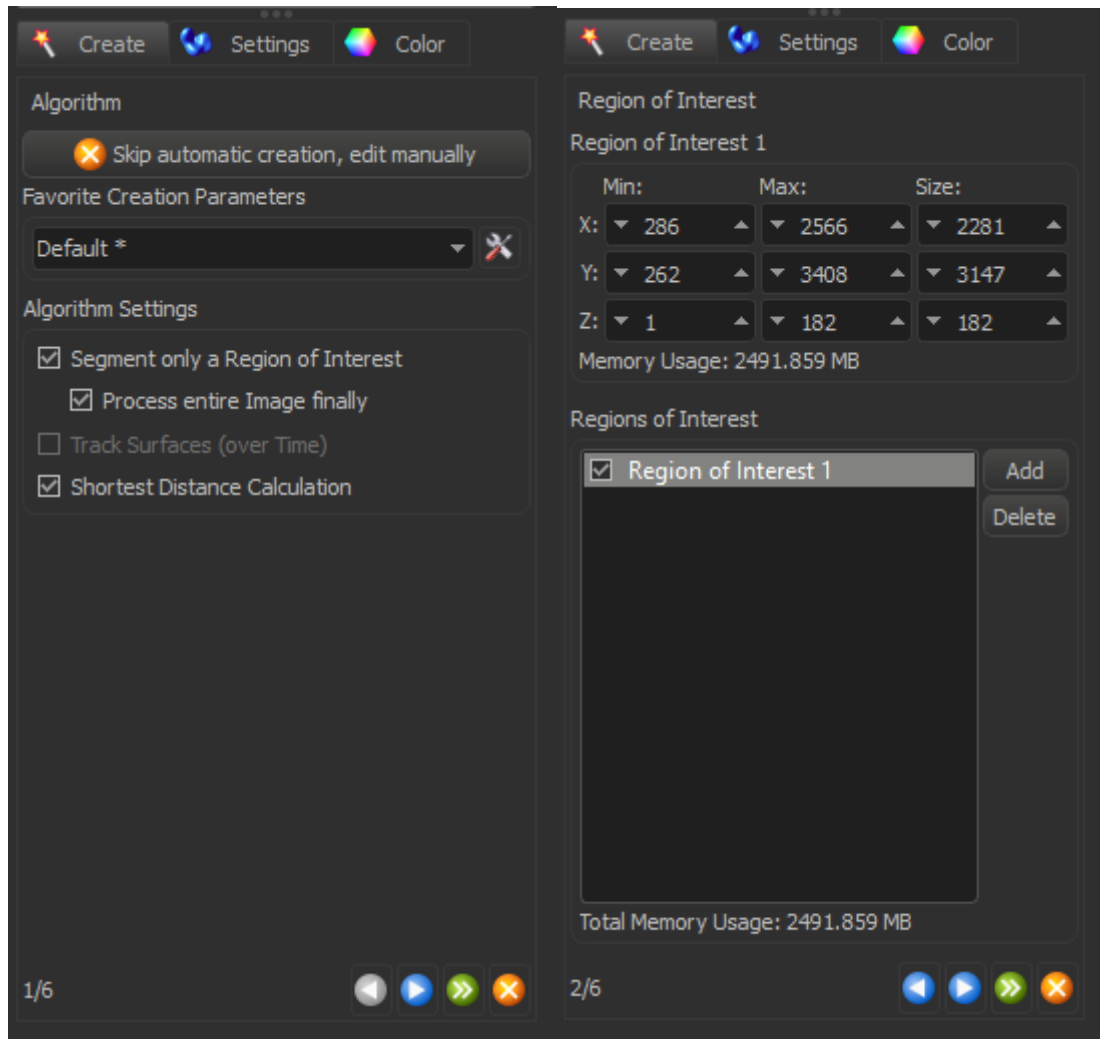Secondly, the diameter of the labeled object (15μm for gland diameter and 2μm for target protein fluorescence spot diameter) was obtained by measuring tool in 2D mode for automatic recognition in Surface mode
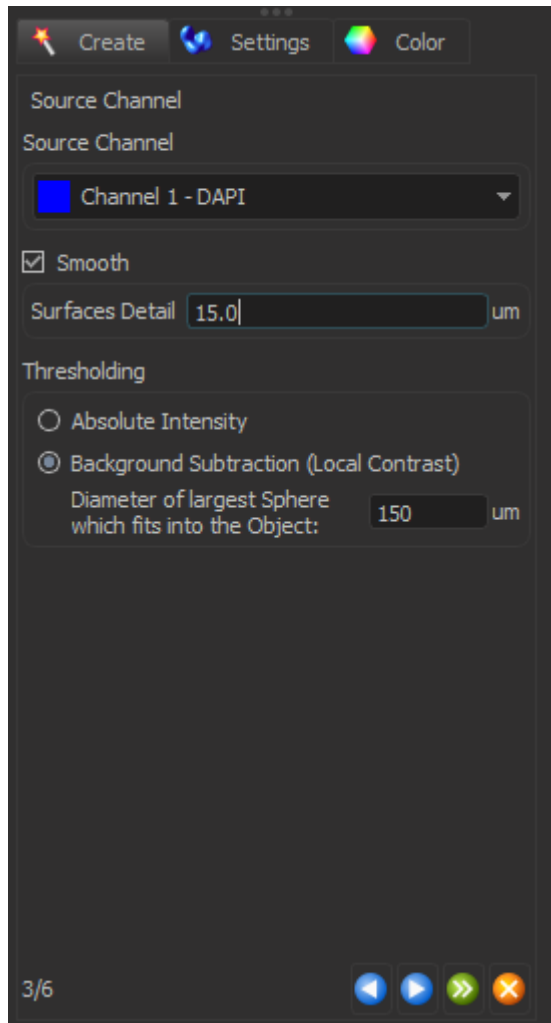
Thirdly, Select the Surface mode

Fourthly, After the region of interest (ROI) being identified, the channel-labeled object in the ROI area will be automatically identified

Left Panel

Create   Settings   Color

Algorithm

❌ Skip automatic creation, edit manually

Favorite Creation Parameters

Default *

Algorithm Settings

☑ Segment only a Region of Interest
   ☑ Process entire Image finally
   ☐ Track Surfaces (over Time)
☑ Shortest Distance Calculation

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Right Panel

Create   Settings   Color

Region of Interest

Region of Interest 1

	Min:	Max:	Size:
X:	286	2566	2281
Y:	262	3408	3147
Z:	1	182	182

Memory Usage: 2491.859 MB

Regions of Interest

☑ Region of Interest 1   Add
                            Delete

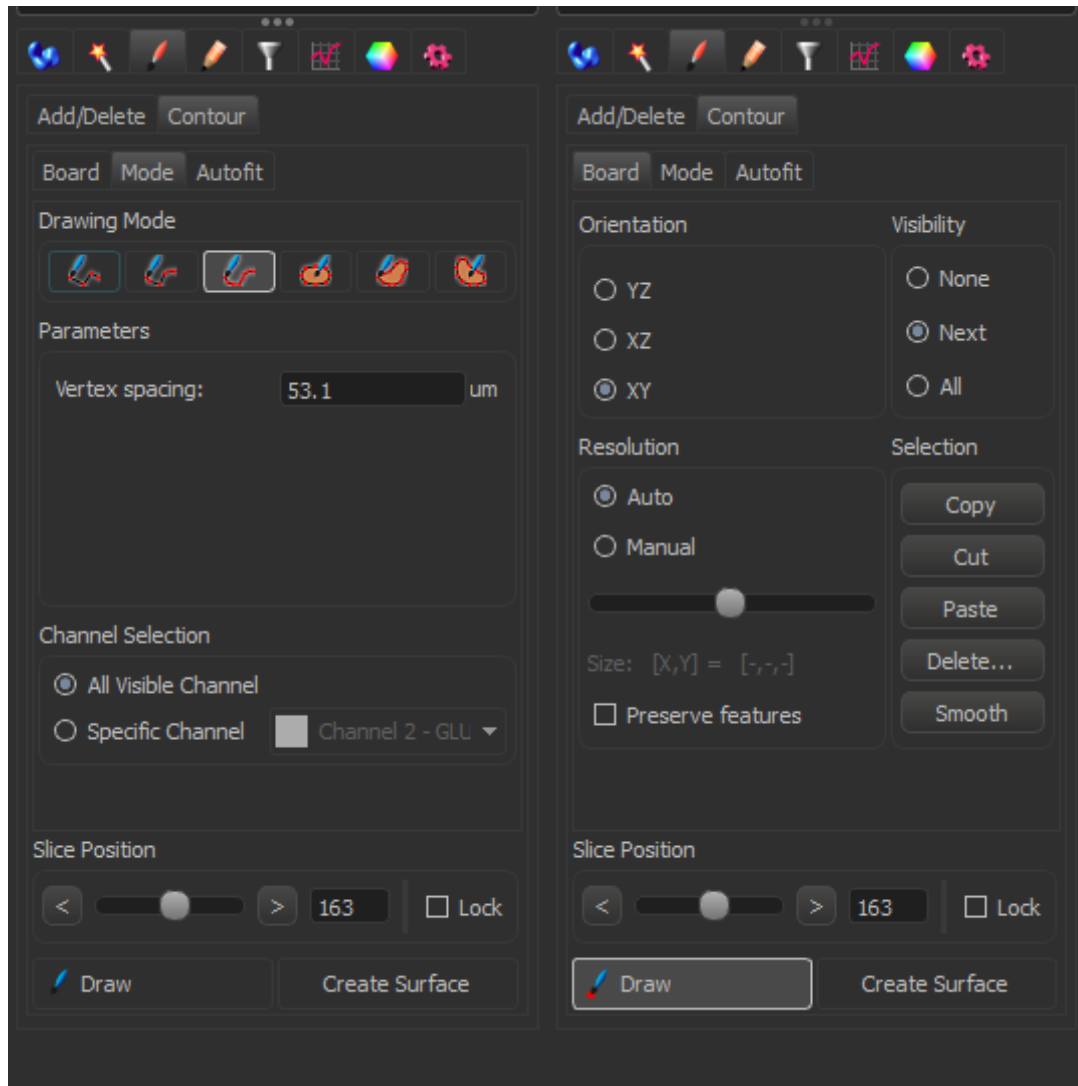Total Memory Usage: 2491.859 MB

2/6

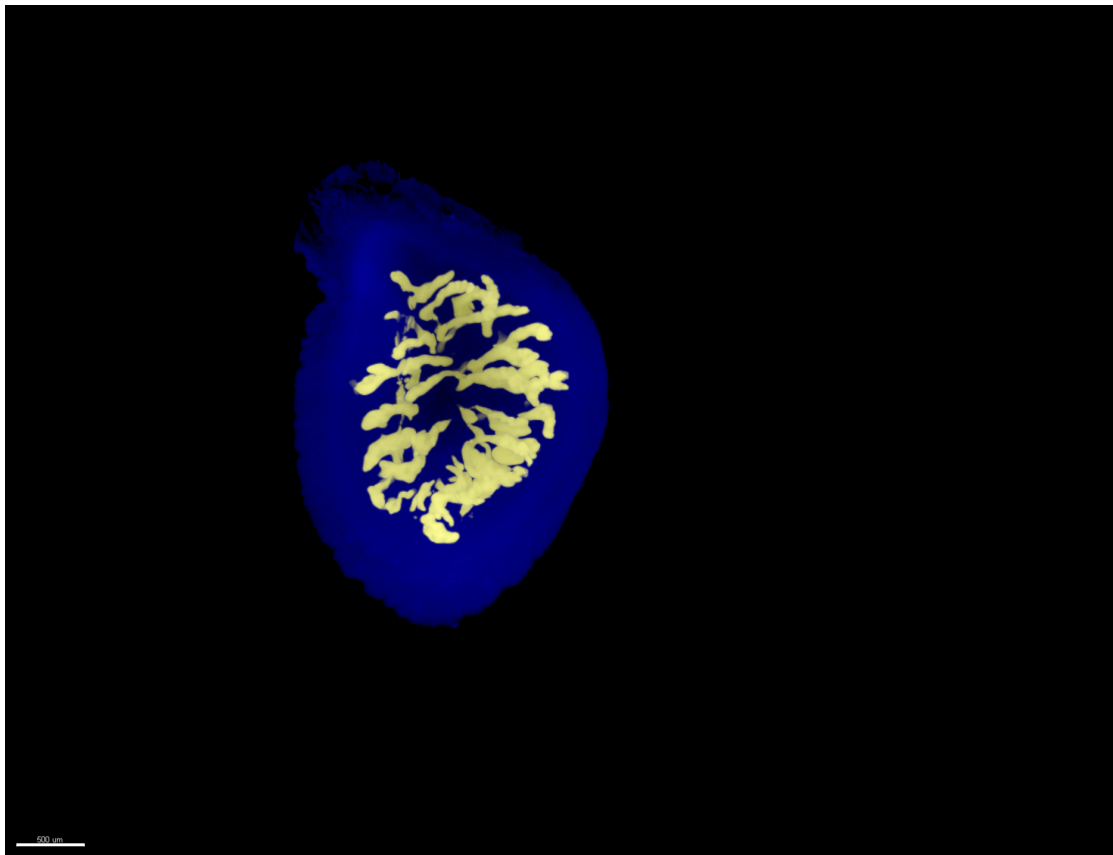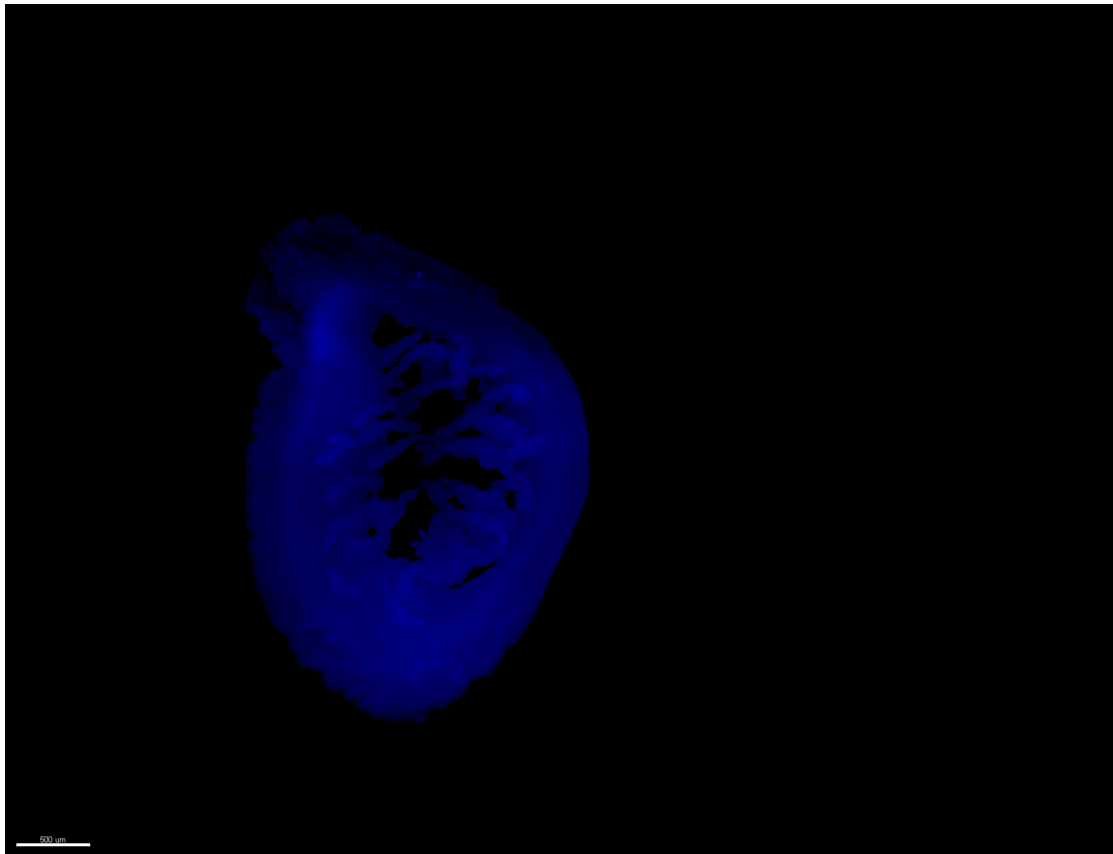Fifthly, set up the channel and object diameter

Sixthly, after automatic identification, a manual tool can be used to fine-tune
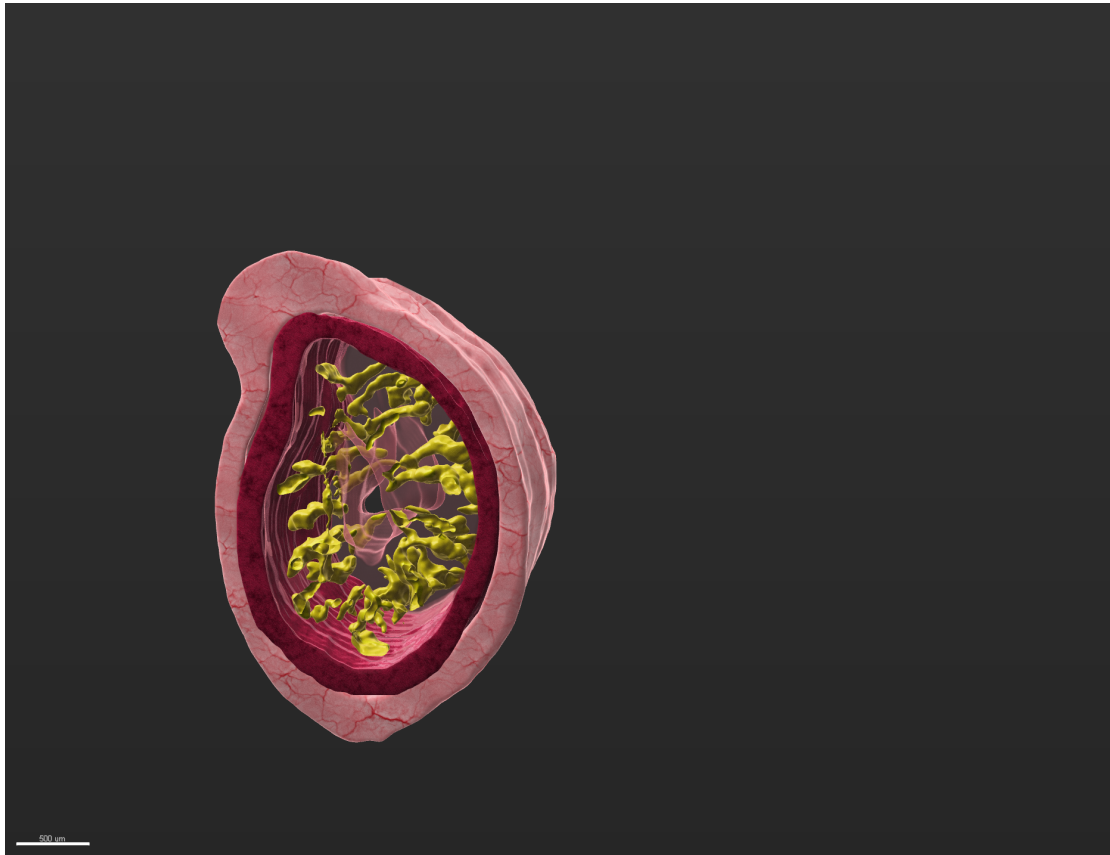
Seventhly, automatic identification is complete

Eighthly, click the identified gland (golden yellow part), and the volume and fluorescence intensity in the identified area can be automatically derived in the

statistical mode

Changes in the text:

We add the description of analysis to method (page 14, line 243 to line 246).

Comment 6:

In Figure 7, the fluorescence intensity of Figure 7B-D (GLUT4) and E-G (AR) is based on Figure 7A. But the figures and graph do not seem to match each other. For example, both AR and GLUT4 expression seem to be increased in the "DHT" and "DHT+Metformin" groups compared to the control in Figure 7A, but not in the graph. Thus, if the quantitative data in graph is correct, the representative image should be provided accordingly. In addition, it is necessary to quantify the relative amounts of gene expression using qPCR rather than fluorescence intensity.

Reply 6:

Thank you for your advise. We aim to evaluate the content of AR and GLUT4 protein expression in different tissues of endometrium so we choose immunolabeling method. The expression of AR and GLUT4 only was calculated in endometrium area (light and transparent pink) and gland area (golden yellow). Another reason to explain this phenomenon the data is derived three-dimensional imaging however due to the restriction of the two-dimensional picture, the fluorescence intensity displayed in the figure may be influenced by superposition. Besides, the total fluorescence intensity

depends not only on unit fluorescence intensity but also on volume.
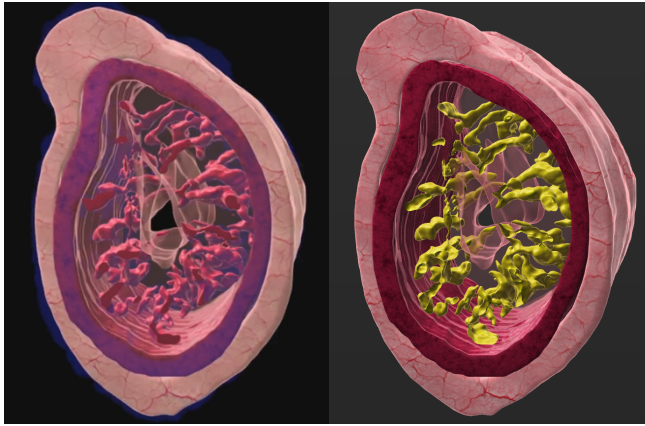
Reviewer A

The manuscript addresses a very important issue, providing thorough insight into the understudied effect of antiandrogen-supplemented birth control pills on glucose intolerance management in individuals with PCOS. Using animal model the authors have presented the development of PCOS-like phenotype under the conditions of high androgen levels, and the gradial effect of antiandrogen-supplemented birth control pill Diane-35 alone and in combination with anti-diabetic pill metformin on the developed PCOS phenotype. The features measured were estrous cycle phases, body weight, blood glucose and sex hormone levels, anti-hyperandrogenism, insulin-sensitization, numbers of cystic follicles in ovaries, morphology of reproductive tissues and endometrial hyperplasia, all of them being recognized clinical markers of PCOS. The study is well designed, the study question is clearly formed, the methods comply with the study goals and are up-to-date. The results are presented clearly and the discussion upon them is easy to follow. The authors emphasize that the manuscript is the first study to observe the rat uterus under 3D conditions by novel CUMIC method, which gives the study its elevated value.

The results show the adverse effect of Diane-35 and metformin on hyperandrogenism. Metformin showed a slight antiandrogenic effect of the changes that occurred after DHT treatment, and more dramatic effect was showen after the treatment with antiandrogenic pill Diane-35 (which was expected). The strongest antiandrogenic effect was observed in the samples co-treated with Metformin and Diane-35. It would be worth to discuss what effect would Metfomin and Diane-35 alone and their co-treatment present in the subjects that did not receive DHT treatment.

Also, we suggest some factual adjustments to be done, e.g. Introduction Line 88, whether the projecterone-based therapies result in resistance to progesterone or reveal it. The style and language should be editer as proper.

Comment 1: It would be worth to discuss what effect would Metfomin and Diane-35 alone and their co-treatment present in the subjects that did not receive DHT treatment.
Reply 1: Thank you for your suggestions! Since Metformin is one of the most important insulin sensitizer and Diane-35 is one of the most commonly used oral contraceptive. There are a lot of researches focus on their role in human (Cinthia et al PMID: 28760367; Christos et al PMID: 30048979) or rats (Xu et al PMID: 30466344; Pan et al PMID: 34923086; Zhang et al PMID: 32493421). Overall, metformin

perform an anti-tumor function in endometrial cancer and improve the function of endometrium of polycysticovary cancer. Although there are limited research focus on the single function of Diane-35, from our unpublished data, cyproterone acetate inhibit endometrial cancer cells' proliferation while ethinylestradiol plays converse role. Since we must use DHT treatment in rats to imitate the endometrial enviroment of PCOS patients, we did not use two medicine or their combined treatment in rats without DHT-tube implantation. Therefore, we move forward our next step by using metformin, cyproterone acetate and ethinylestradiol (two component of Diane-35) to treat human endometrial cancer cells and endometrial cancer animal models.
Changes in the text: None

Comment 2: Also, we suggest some factual adjustments to be done, e.g. Introduction Line 88, whether the projecterone-based therapies result in resistance to progesterone or reveal it. The style and language should be editer as proper.
Reply 2:   Thank you for your suggestions! It seems like this sentences leads to some misunderstanding: at first, endometrial cancer patients can response to progesterone-based therapy such as progestin. However, after long-term treatment or the progression of cancer, patients gradually become chemoresistant to progesterone as described by Kim et al. (PMID: 20104432).
Changes in the text: We have revised the sentence to avoid misunderstanding (see Page 5, line 74 to 76)

Reviewer B

It is a great paper that should be published in ATM after some major changes. The authors made a lot of effort to perform the study. Impressive methodology including 3D tissue imaging was used. There are, however, some minor English language mistakes - please read the paper carefully one more time and try to make corrections. In my opinion, some corrections should be performed before publication:
1. Change title. Please add information what kind of endometrial pathology You treat and that You perform study on rats.
2. Introduction section should include more data on pathogenesis of PCOS, endometrial hyperplasia and endometrial cancer. Similarly, some more information regarding the use of Diane-35 and metformin should be presented, especially on mechanisms of action of both drugs.
3. In section Introduction: lines 104-113 should be moved to Conclusions section.
4. Sections Materials and methods, Results, and Conclusions are satisfactory for me.

Comment 1: Change title. Please add information what kind of endometrial pathology

You treat and that You perform study on rats.

Reply 1: Thank you for the suggestion. We describe the experimental subjects are rats in the title. In our previous version of manuscript, we described the endometrial pathology as comparative hyperplasia since from our data, the overal volume of uterus in DHT-treated groups decreased compared with control group which is similar to the results of Zhang et al (PMID: 30800111). To avoid misunderstanding, we changed the description from "hyperplasia" to "lesion", which was defined from Line 316 to Line 324.

Changes in the text: We have modified our text as advised (see Page 1, line 4 to 5) by adding "rats'"

Comment 2: Introduction section should include more data on pathogenesis of PCOS, endometrial hyperplasia and endometrial cancer. Similarly, some more information regarding the use of Diane-35 and metformin should be presented, especially on mechanisms of action of both drugs.

Reply 2: Thank you for your suggestions! We discussed the pathogenesis of diseases and mechanism of treatments in the discussion part   (see Page 19, line 356 to 382). and added details in the introduction part.

Changes in the text: We have modified our text as advised (see Page 6, line 85 to 90).

Comment 3:   In section Introduction: lines 100-109 should be moved to Conclusions section.

Reply 3: Thank you for suggesion!

Changes in the text: We have modified our text as advised (see Page 20, line 382 to 391)

Comment 4: Sections Materials and methods, Results, and Conclusions are satisfactory for me.

Reply 4: Thank you very much for your encouragement!

Changes in the text: None