**66681\_screenshot\_1**

3.2.1.(Launch ImageJ software.) 0:00-0:03

3.2.2.(Import indicated images to ImageJ, then proceed to load and select them. Split the channels accordingly. Load a file in ND2 format for both DAPI and IB4 channels.) 00:04-00:11

3.2.3.(Navigate to Analyze > Set Scale > Configure the Image Parameters as 0.65 µm/pixel (depending on the photography parameters) > Global > OK.) 00:12-00:29

3.2.4.(Set the same display range to adjust the image presentation effect. Select Image> Adjust > Brightness/Contrast > Set. Set display range for each of the two channels separately, IB4: 0-10000; DAPI: 0-20000.) 00:30-00:47

3.2.5.(To eliminate the influence of background signals, proceed with the following steps: Click Process > Math > Subtract; Subtract the background signal based on the signal values detected by the Magic Wand tool on the background, IB4: 1000; DAPI:200.) 0:31-01:20

3.2.6.(To select the threshold value that best aligns with the original image, go to Image > Duplicate.) 01:21-01:34

3.2.7. (IB4 channel: Select both original and duplicate Image > Type > 8 bit.) 01:35-01:44

3.2.8.(IB4 channel: Select an appropriate threshold value to precisely identify the lung microvasculature. Click on Image > Adjust > Threshold > Intermodes (choose the most realistic threshold against the original diagram), tick > Dark Background > Apply.) 01:45-01:58

3.2.9.(IB4 channel: Eliminate IB4 positive signals from large blood vessels in the mouse lung, including arteries and veins: Use the Magic Wand tool to select larger blood vessels compared to the duplicated image, then click Delete (repeat that until the IB4 positive signals in the endothelial cells of larger blood vessels are removed)) 01:59-02:08

3.2.10. (IB4 channel: Count the number of target signals, click Analyze > Set Measurements, and tick Area, Limit to Threshold, and Display Label. Then select Analyze Particles and set Size: 0-infinity; Circularity: 0.00-1.00; Show: Overly Masks; tick Summarize and click OK.) 02:09-02:24

3.2.11. (DAPI channel: Select both original and duplicate Image > Type > 8 bit.) 02:25-02:45

3.2.12.(DAPI channel: Select an appropriate threshold value to precisely identify the lung microvasculature. Click on Image > Adjust > Threshold > Moments (choose the most realistic threshold against the original diagram), tick > Dark Background > Apply.) 02:46-03:18

3.2.13. (DAPI channel: Count the number of target signals, click Analyze > Set Measurements, and tick Area, Limit to Threshold, and Display Label. Then select Analyze Particles and set Size: 0-infinity; Circularity: 0.00-1.00; Show: Overly Masks; tick Summarize and click OK.) 03:19-03:26

3.2.14. (Choose Summarize Results > File > Save as.) 03:27-03:39

**66681\_screenshot\_2**

3.3.1. (Match and organize the number of IB4 positive foci and DAPI positive foci, respectively, at the same image into a unified table.) 0:00-00:31

3.3.2. (Summarize the total number of IB4 positive foci and DAPI positive foci for each lobe in indicated young or age groups.) 00:32-00:46

3.3.3. (Launch the data analysis software and create a new column table.) 00:47-01:05

3.3.4. (Sequentially name them from Group A to Group J as "Young cranial lobe", "Old cranial lobe", "Young middle lobe", "Old middle lobe", "Young caudal lobe", "Old caudal lobe", "Young accessory lobe", "Old accessory lobe", "Young left lobe", "Old left lobe". Then, input the corresponding IB4 positive foci (%) into the table.) 01:06-03:34

3.3.5. (Utilize unpaired t-tests for pairwise comparison to assess significant differences in the corresponding regions between the two age groups. Conduct a total of five comparisons for five lobes.) 03:35-04:58

3.3.6. (Create a graph for result visualization. Opt for a bar chart and include statistical analysis in the figure.) 04:59-07:29