

The evaluation method of new bone formation

The images were taken and analyzed by LAS V4.8 and Image-pro plus 6.0.

The images were imported in Image-pro plus 6.0 software (Fig.1).

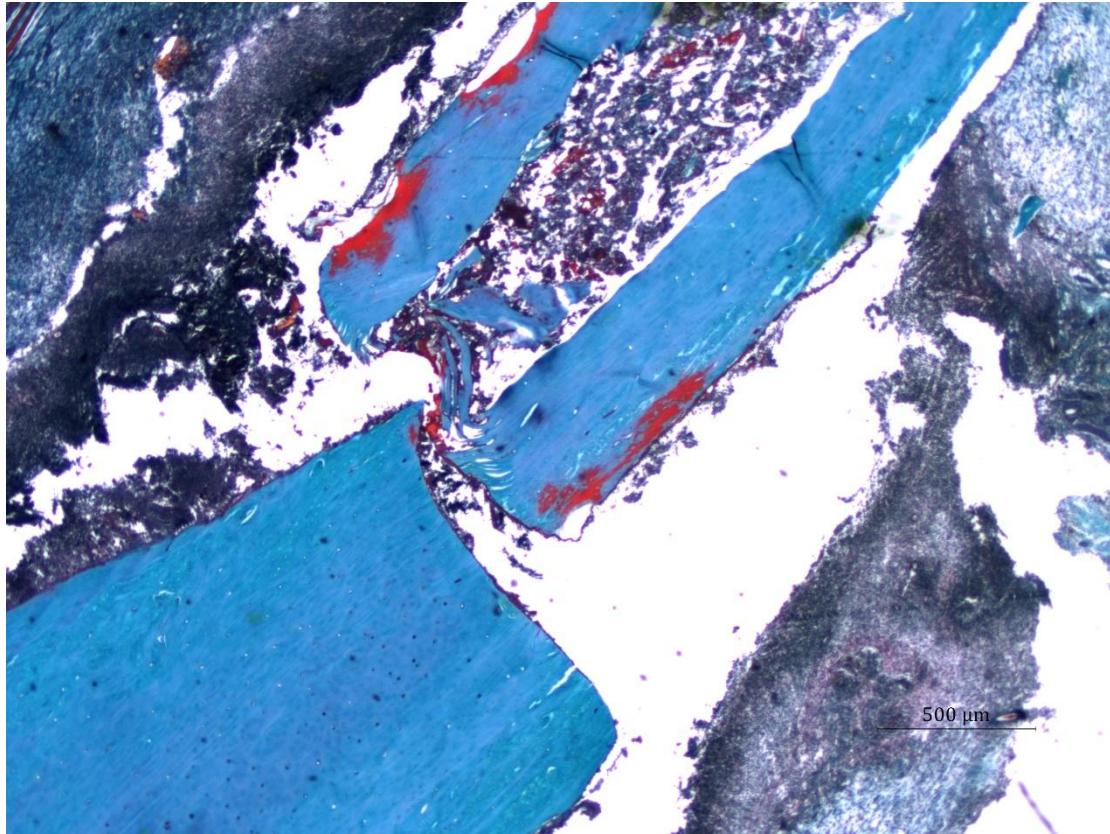


Fig.1 the images of CGS21680 treatment group at 7 days (Goldner trichrome staining).

Firstly, we checked the image information and got the pixel of the image that was 2560×1920 (Fig.2).

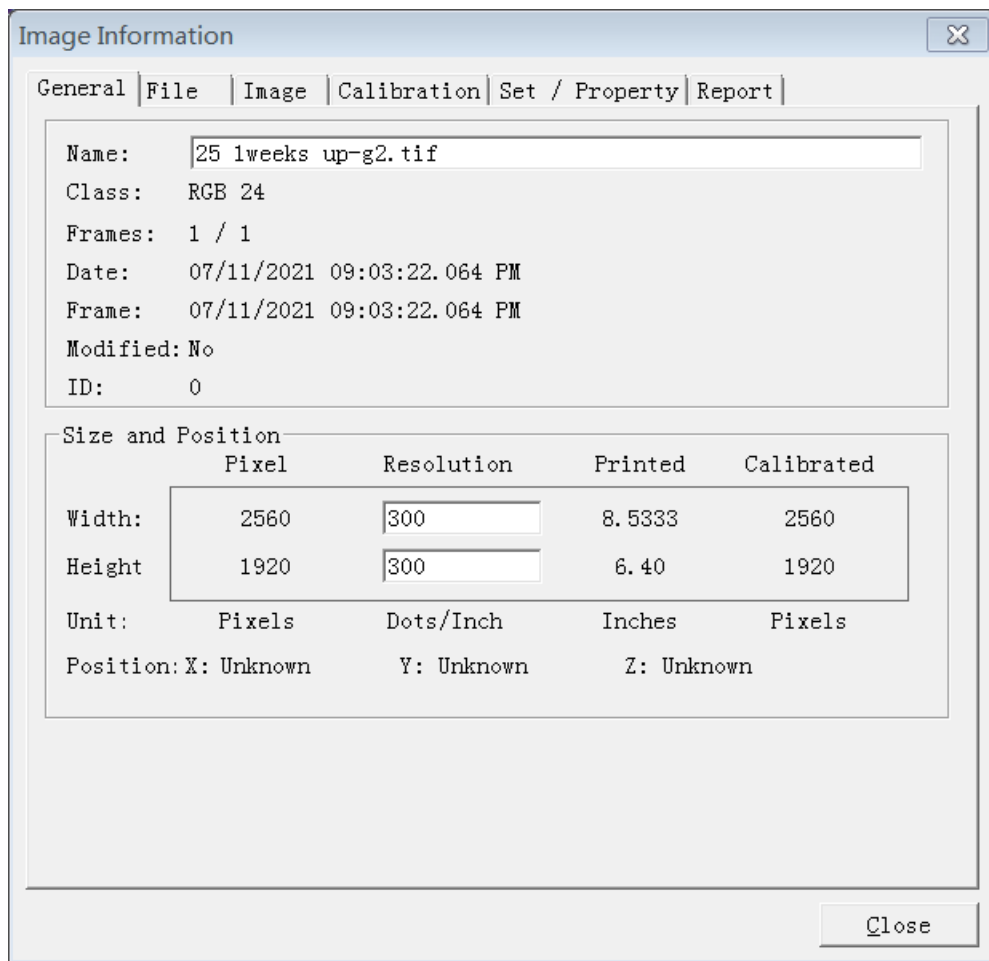


Fig.2 the image information. The width and height of image were 2560 and 1920.

Secondly, we selected the new bone formation area (red area in image) and turned it to green (Fig.3).

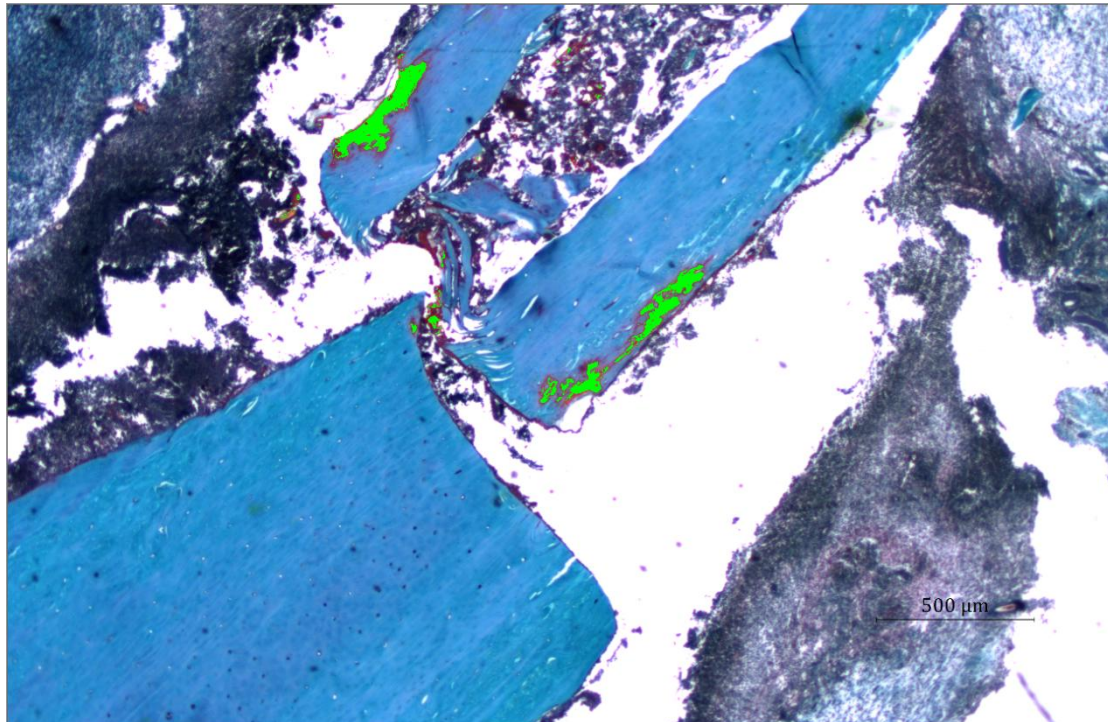


Fig.3 the new bone formation area turned into green.

Thirdly, we calculated the pixel of green area that was 22573 (Fig.4).

Statistics	
File	
☒ Locate the object. ☐ Scroll to the object.	
Stats	Area
Min	12
(Obj. #)	17
Max	9717
(Obj. #)	14
Range	9705
Mean	618. 22498
Std. Dev	1808. 3813
Sum	22573
Samples	40

Fig.4 the green area pixel was calculated by software statistics.

Finally, we used $22573/(2560 \times 1920)$ and got the new bone area/all area at 7 days.

The evaluation method of callus area

The images were taken and analyzed by LAS V4.8 and Image-pro plus 6.0.
The images were imported in Image-pro plus 6.0 software (Fig.5).

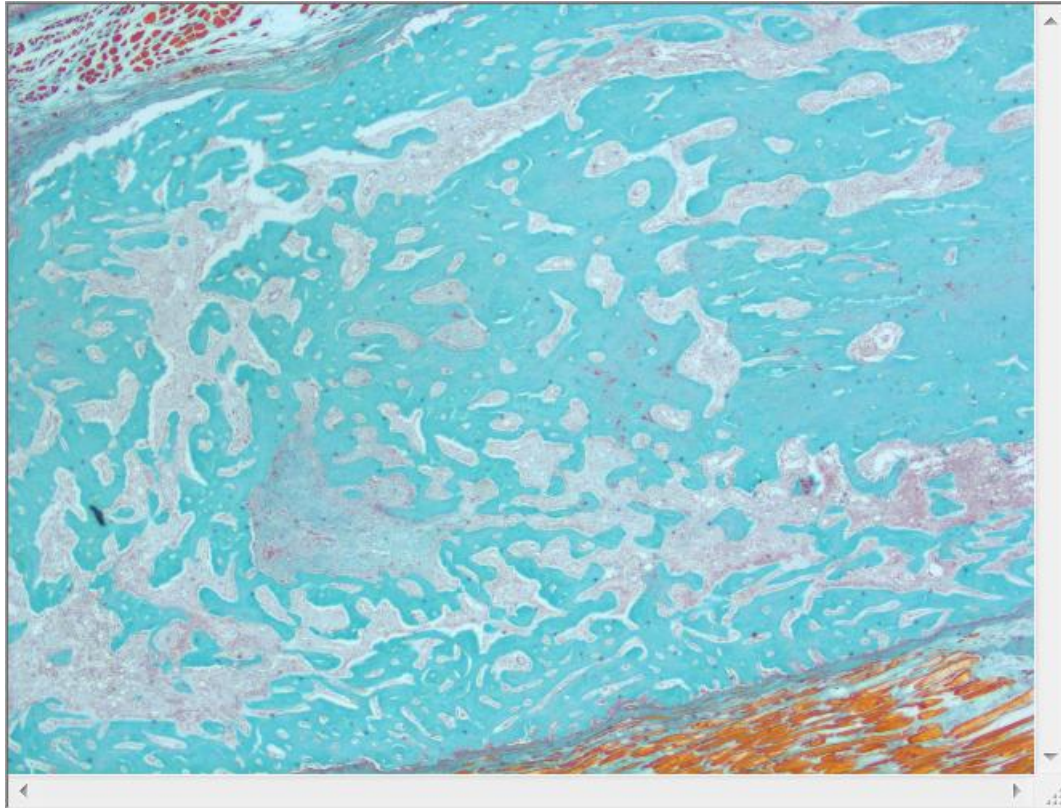


Fig.5 the images of CGS21680 treatment group at 28 days (Goldner trichrome staining).

The operation steps were same with the evaluation method of new bone formation. Firstly, we checked the image information and got the pixel of the image that was 2560×1920. Then, we selected the callus area (blue area in image) and turned it to green (Fig.6).

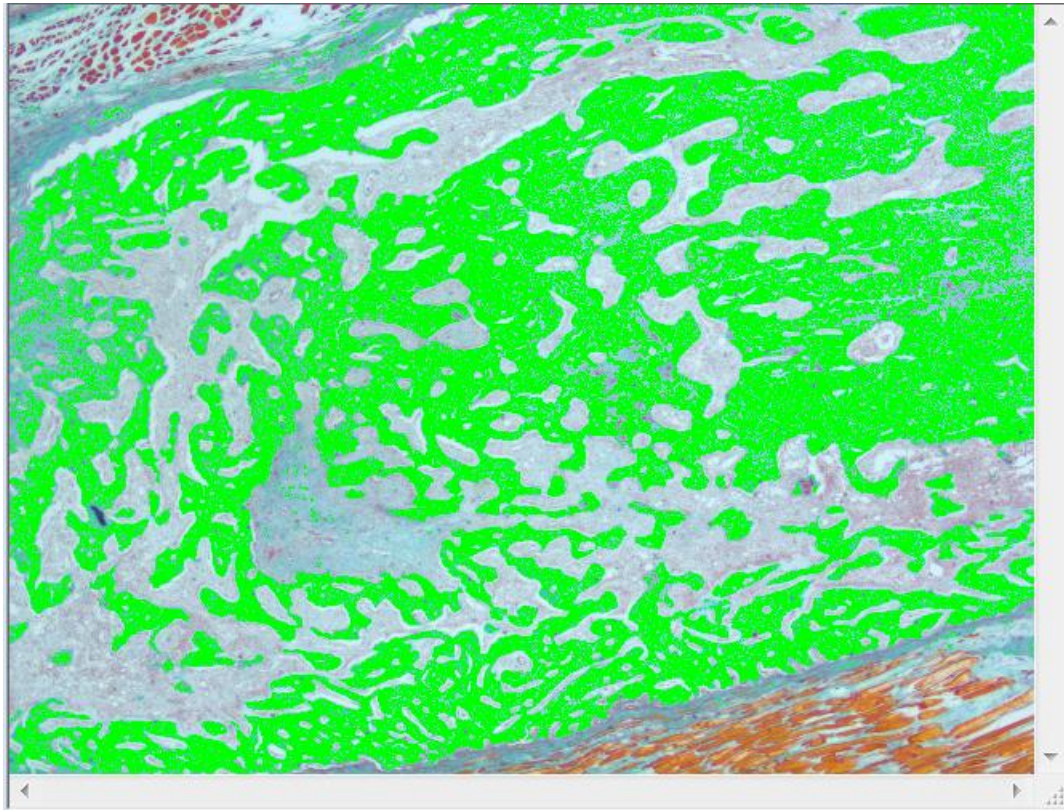


Fig.6 the callus area turned into green.

Thirdly, we calculated the pixel of green area. Finally, we used $(\text{the pixel of green area}) / (2560 \times 1920)$ and got the callus area/all area at 14 days or 28 days.

The evaluation method of CD31 positive area.

The immunofluorescence images were taken and analyzed by OLYMPUS cellSens Standard and Image-pro plus 6.0.

The operation steps were same with the evaluation method of new bone formation. Firstly, the images were imported in Image-pro plus 6.0 software. The below image was CD31 and DAPI merged image (Fig.7). We imported only CD31 staining images in software, like Fig.8.

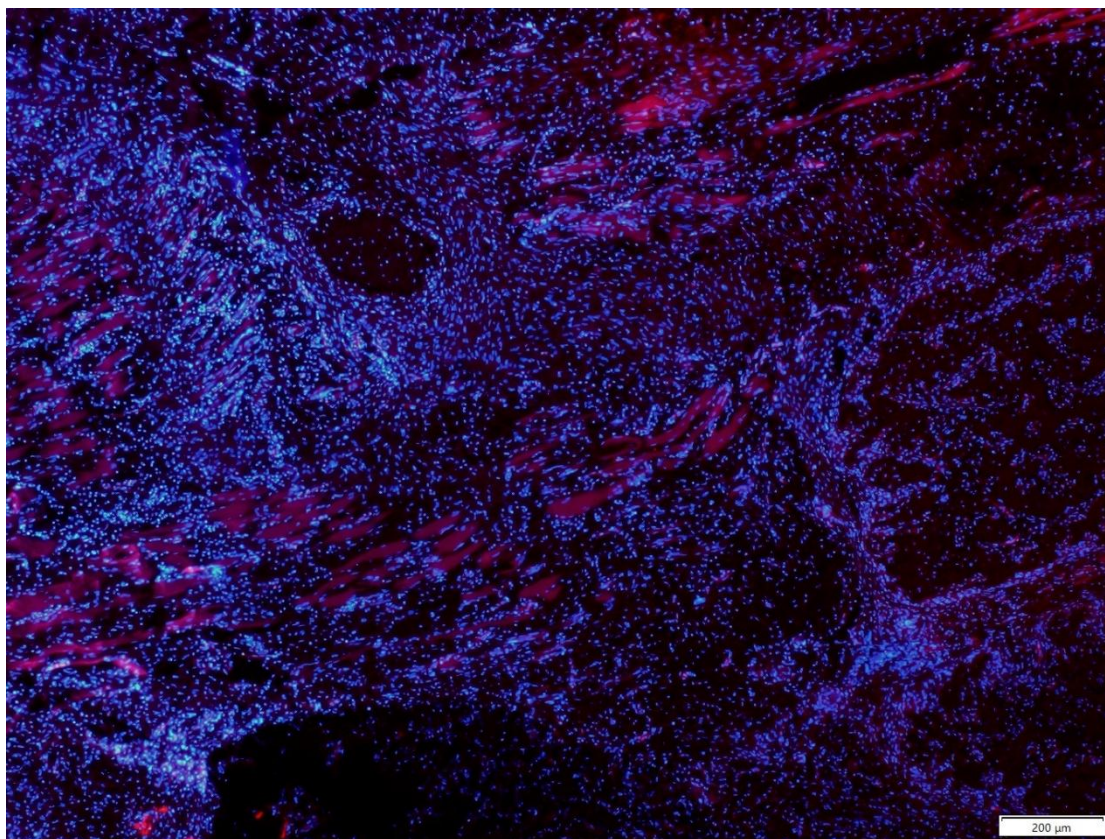


Fig.7 CD31 and DAPI merged image.

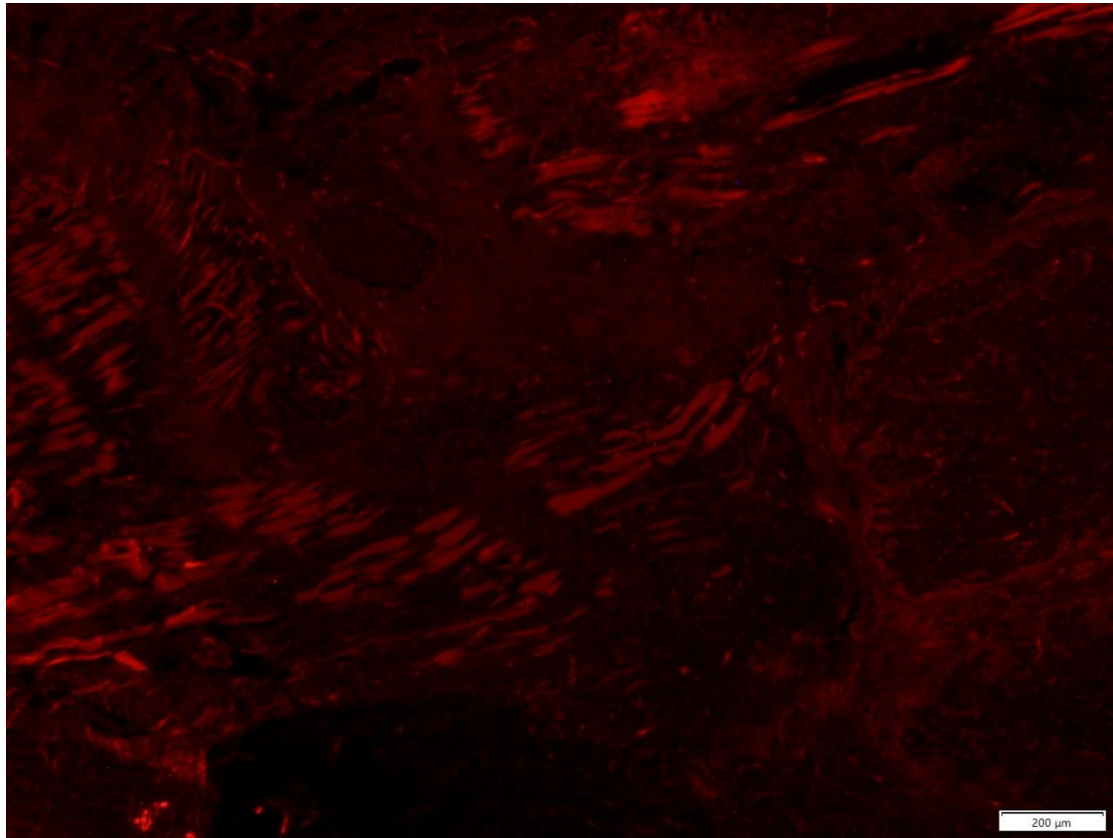


Fig.8 only CD31 staining image.

The operation steps were same with the evaluation method of new bone formation. Firstly, we checked the image information and got the pixel of the image. Then, we selected the staining area (red area in image) and turned it to green (Fig.9).

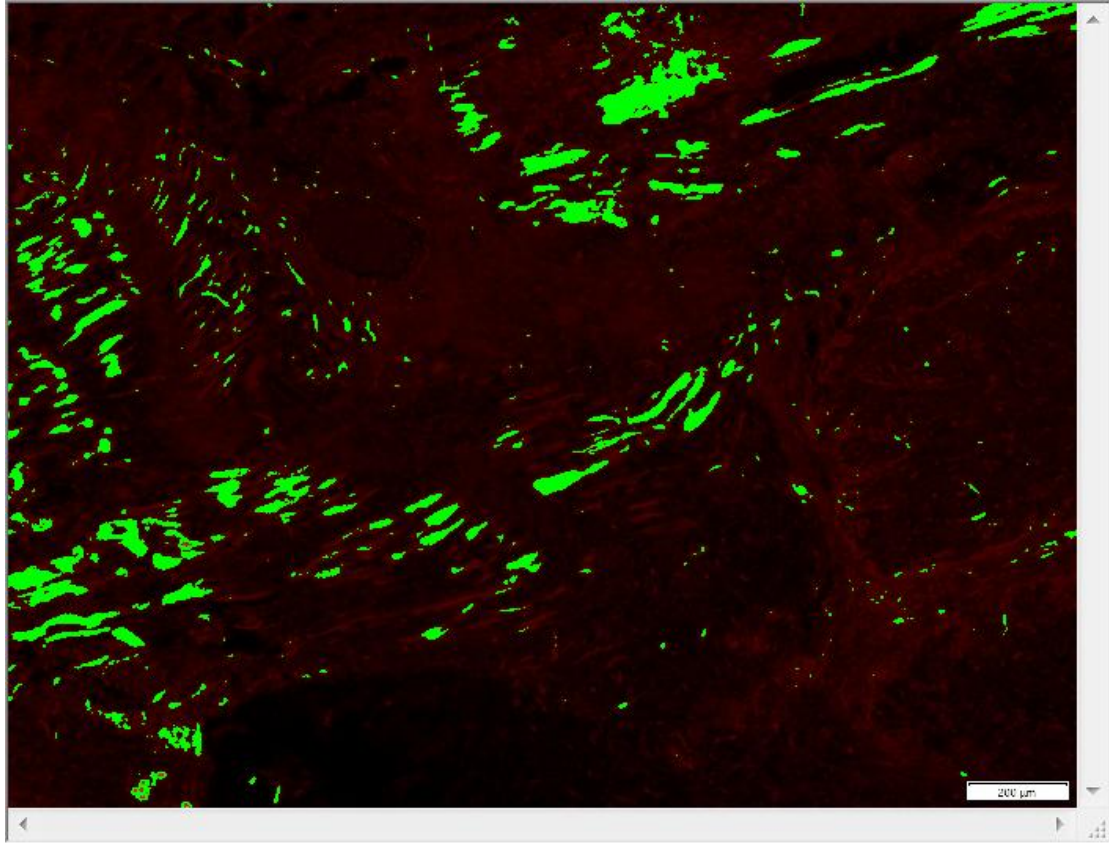


Fig.9 the staining area turned into green.

Thirdly, we calculated the pixel of green area. Finally, we used $(\text{the pixel of green area})/(\text{image pixel})$ and got the CD31 positive area/all area.

The evaluation method of vWF positive area.

The operation steps were same with the evaluation method of new bone formation. The images were imported in Image-pro plus 6.0 software (Fig.10) and we got the image pixel (Fig.11).

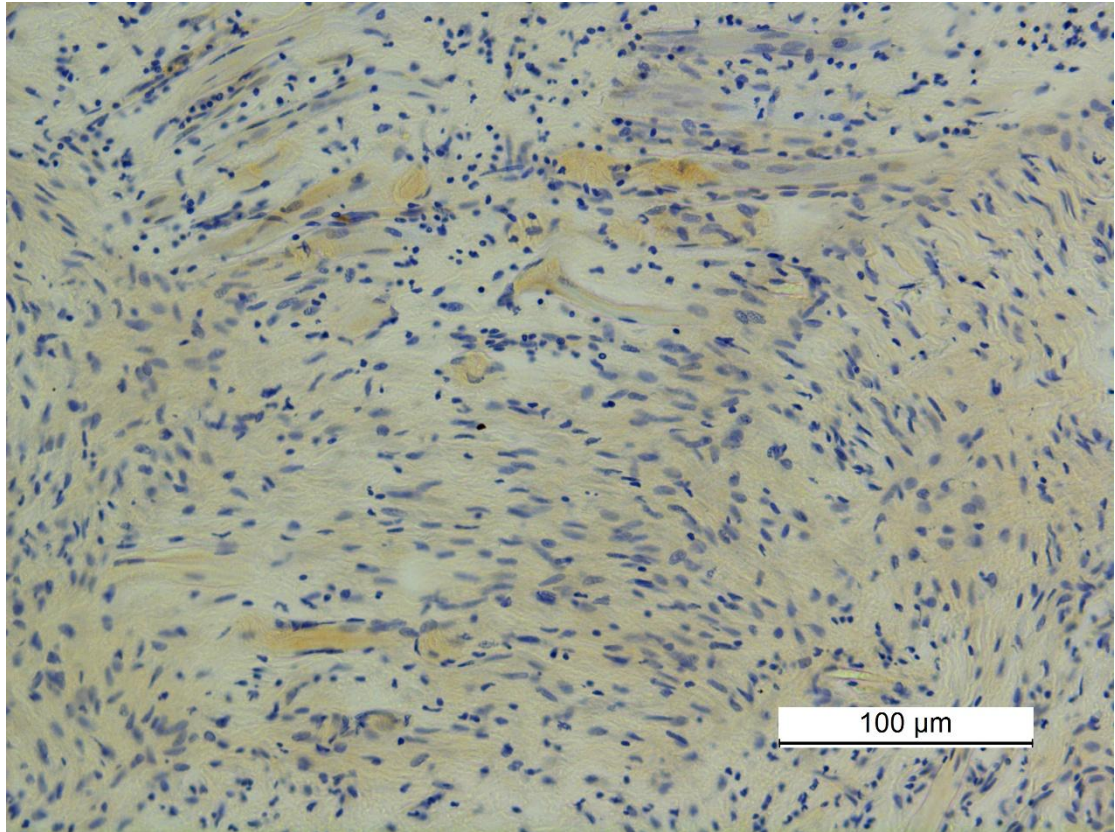


Fig.10 vWF staining image.

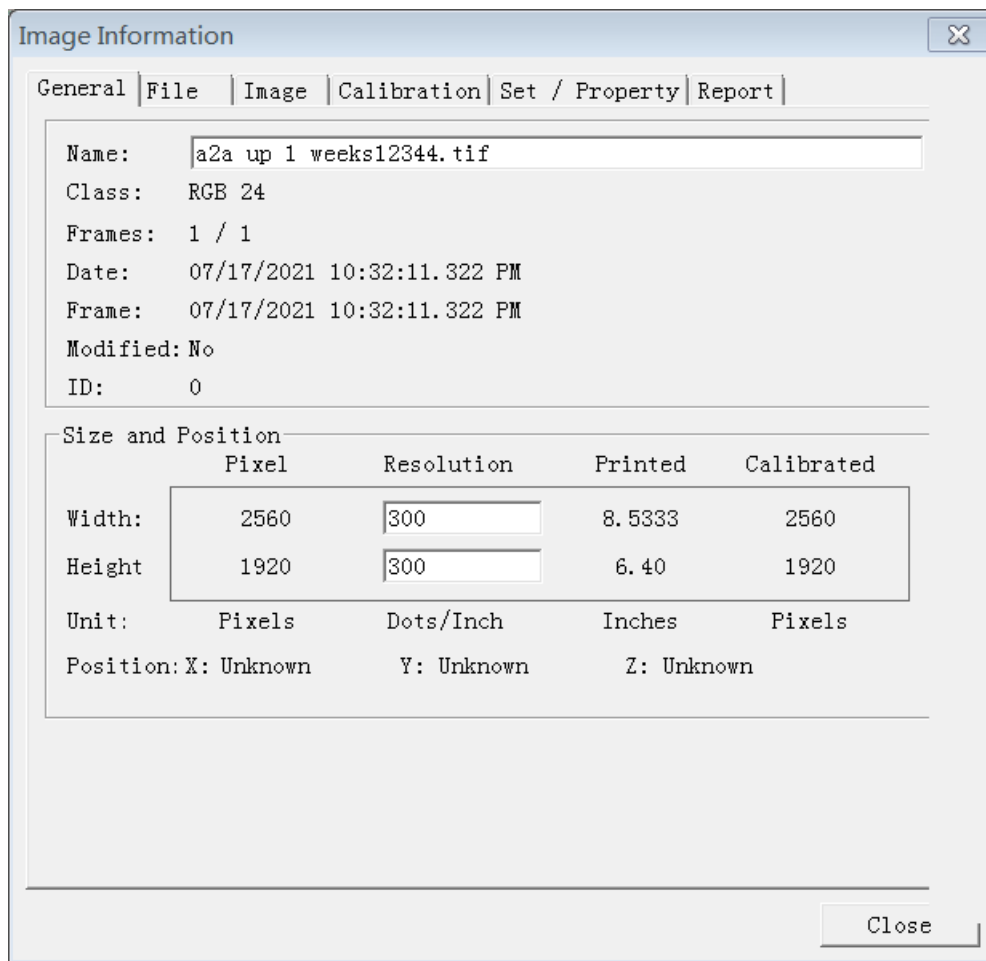


Fig.11 the image pixel was 2560×1920.

Then, we selected the staining area (brown area in image) and turned it to green (Fig.12).

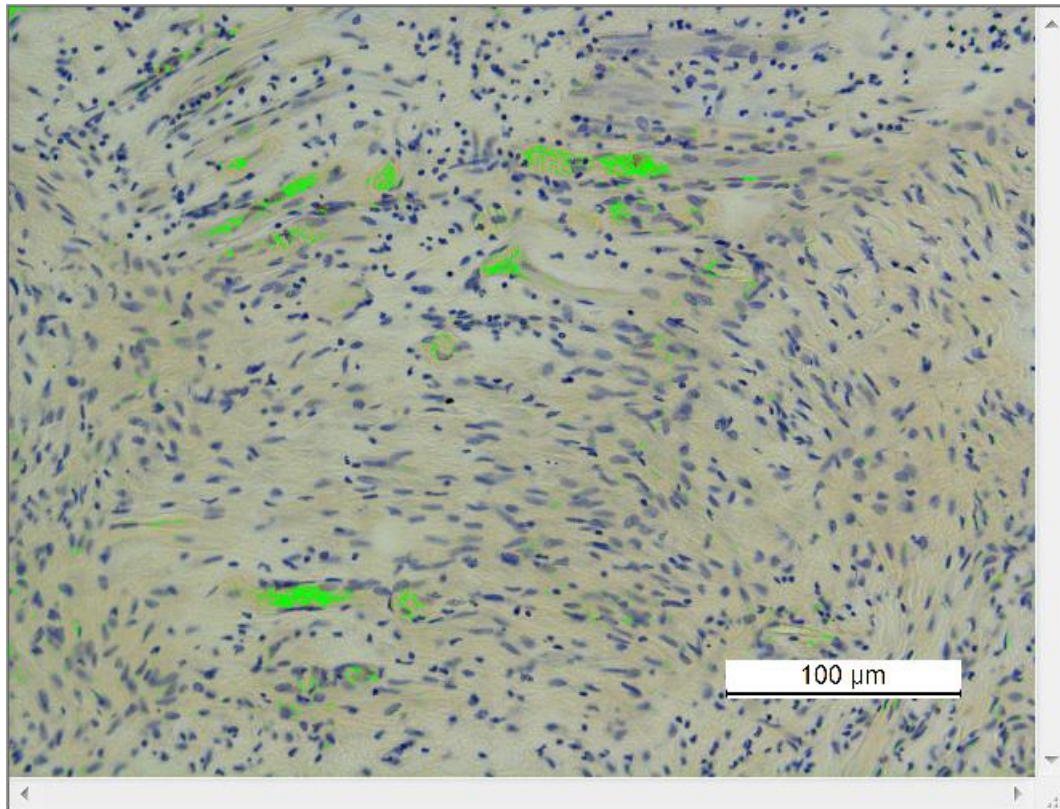


Fig.12 the staining area turned into green.

Thirdly, we calculated the pixel of green area. Finally, we used $(\text{the pixel of green area})/(\text{image pixel})$ and got the vWF positive area/all area.

The evaluation method of transwell results.

The images were imported in ImageJ software (Fig.13) and adjusted to 8-bit (Fig.14).



Fig.13 the transwell result of CGS21680 treatment group at 48h.



Fig.14 the image adjusted to 8-bit.

Then, we selected the threshold between 0 to 100 (Fig.15) and the image was adjusted to show only cells (Fig.16).

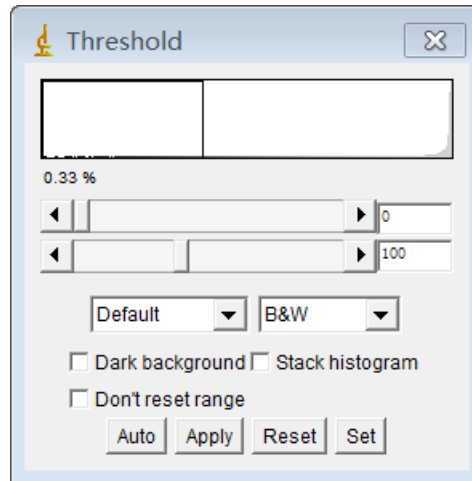


Fig.15 the threshold was adjusted between 0 to 100

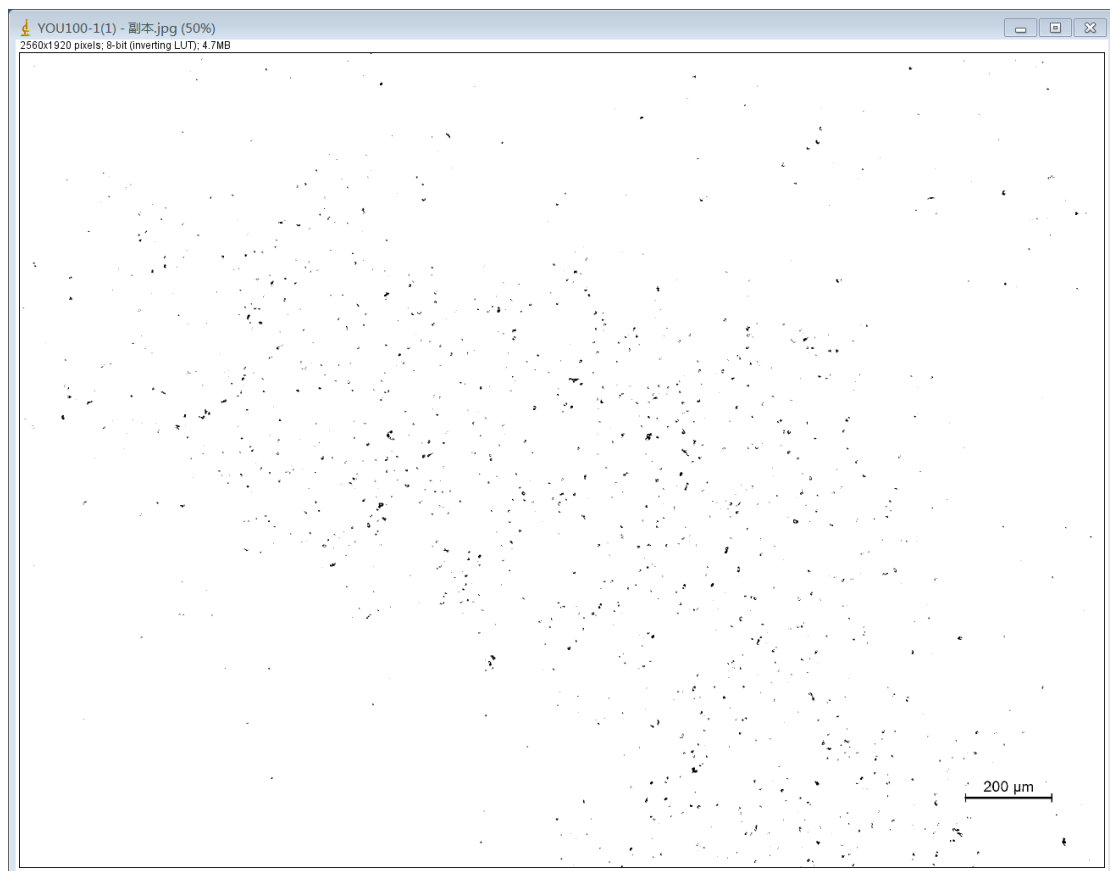


Fig.16 the image was adjusted to show only cells

Then, we used the analysis particles function to calculated the cell number (Fig.17-18).

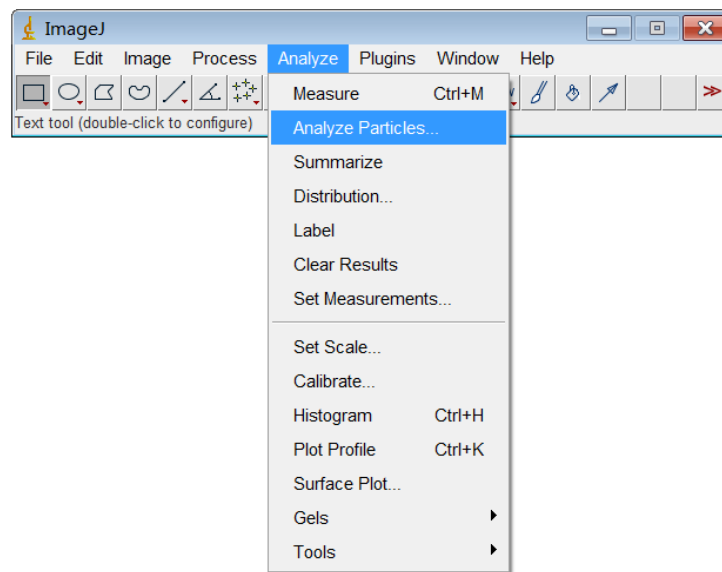


Fig.17 the analysis particles module.

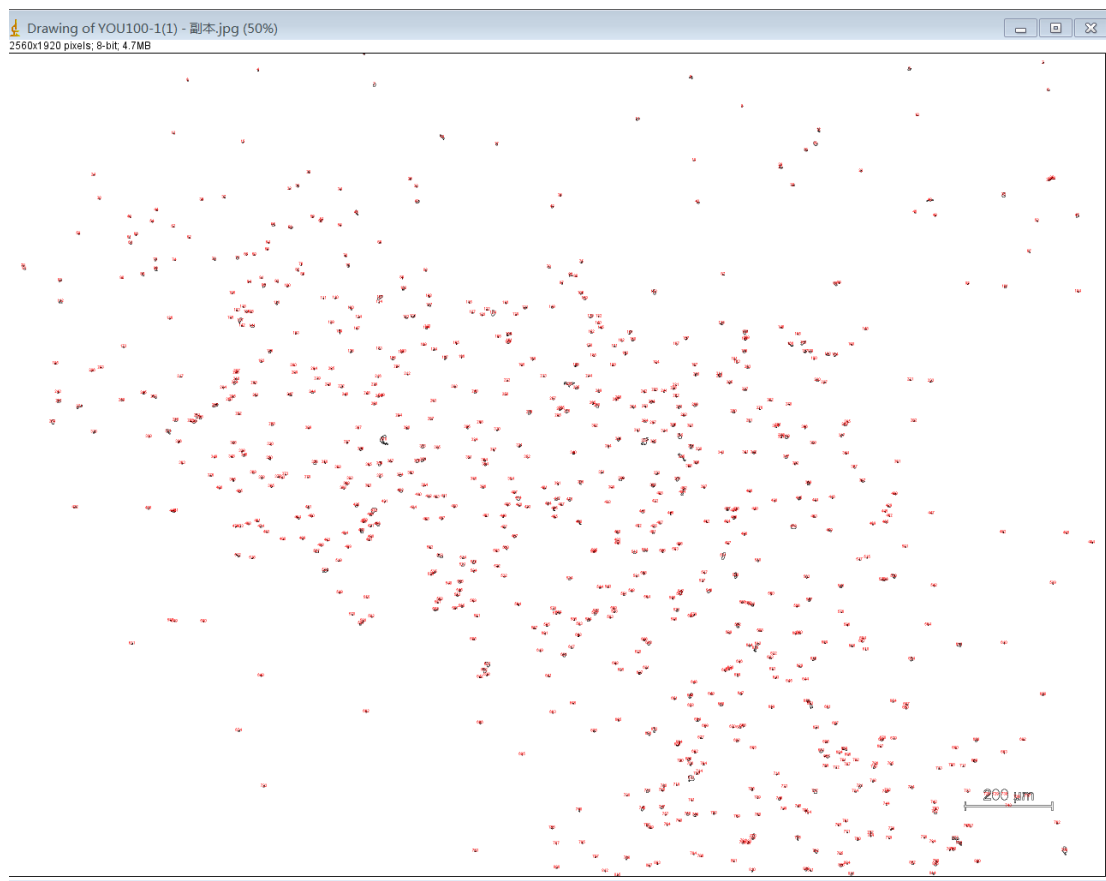


Fig.18 the outlines of the calculated cells.

The evaluation method of scratch assay results.

Firstly, the image of scratch assay was adjusted into 640×480 pixel by photoshop software (Fig.19).

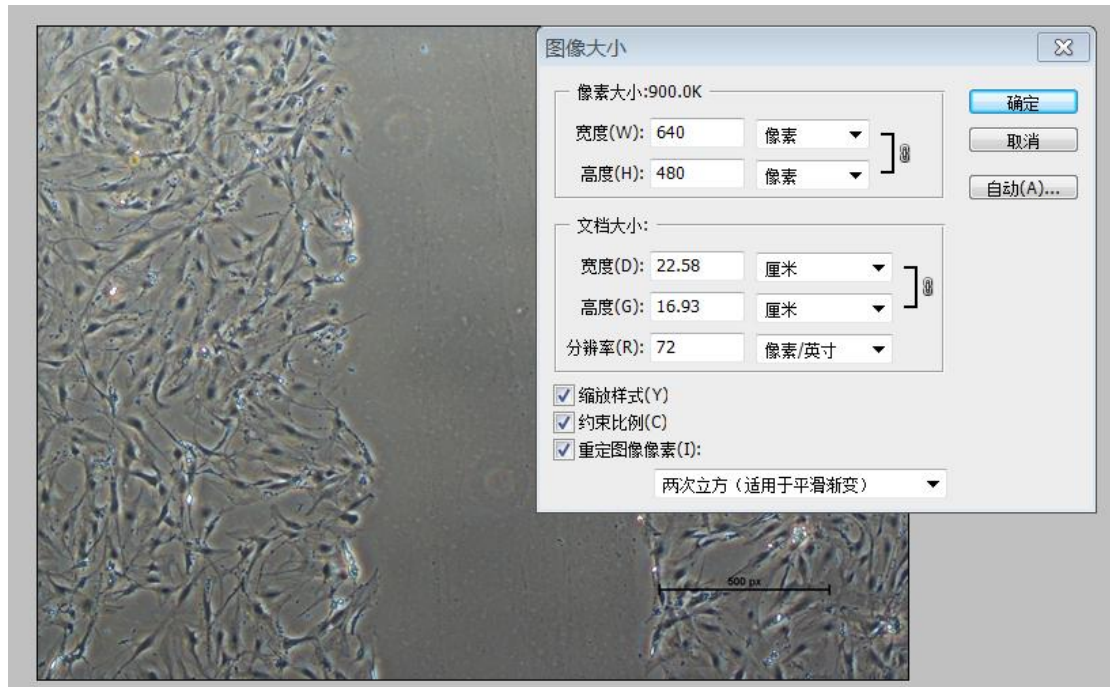


Fig.18 the image adjusted into 640×480 pixel.

Then, the image was imported in ImageJ and adjusted to 8-bit. We used process module to enhance contrast and set normalize saturated pixel 0.3% (Fig.19-20).

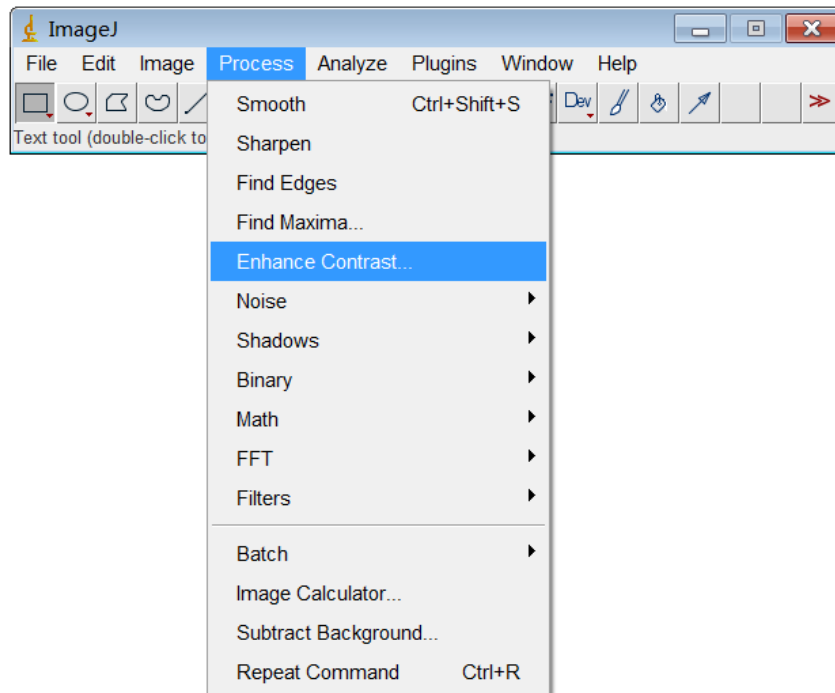


Fig.19 the enhance contract in process module.

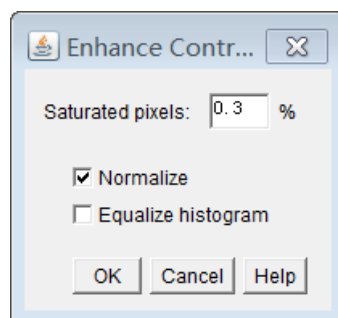


Fig.20 the normalize was selected and saturated pixels adjusted to 0.3%.

Then, we selected smooth to adjust the image (Fig.21).

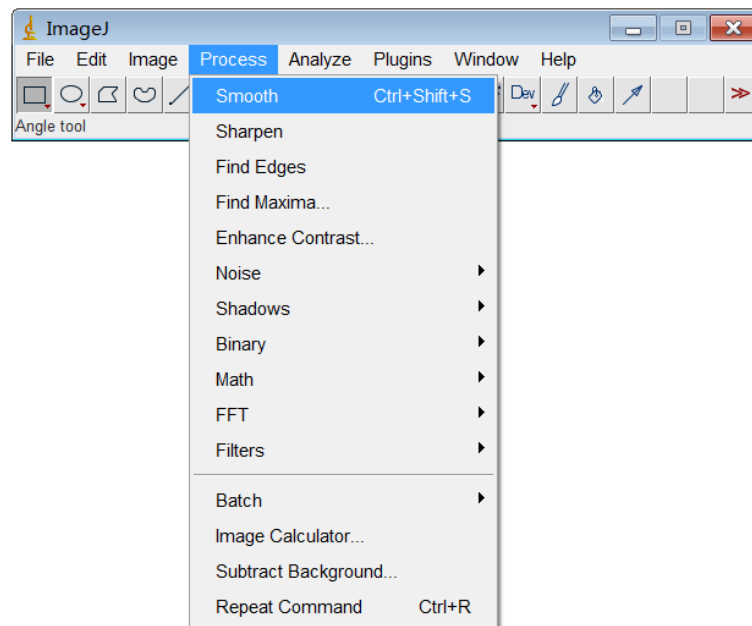


Fig.21 the smooth module.

Then, we used the “find edges” to show the edge of scratch (Fig.22-23).

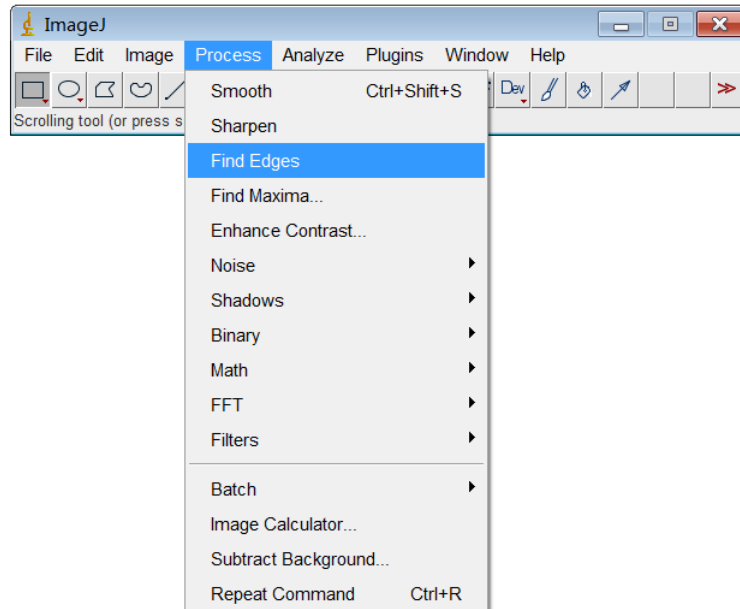


Fig.22 the find edges module.

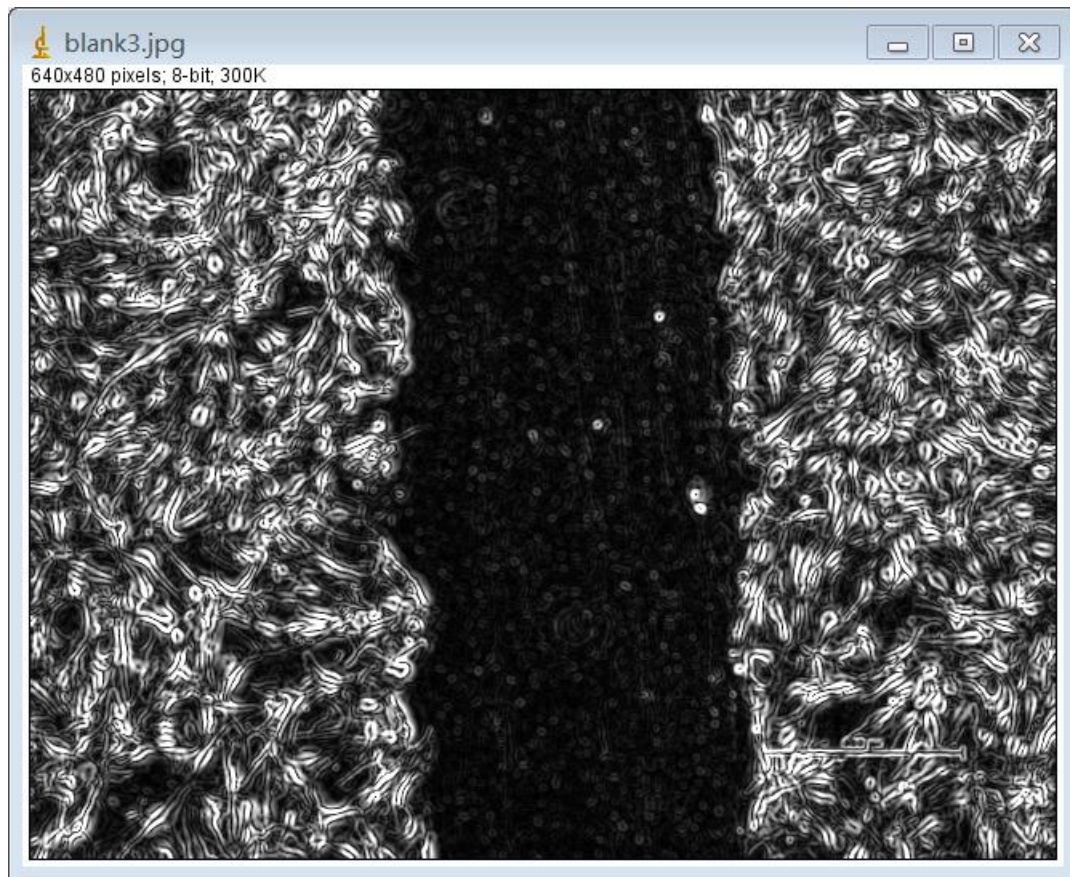


Fig.23 the images under "find edges". We can see the edges of scratch.

We used magic stick to show the area of scratch (Fig.24).

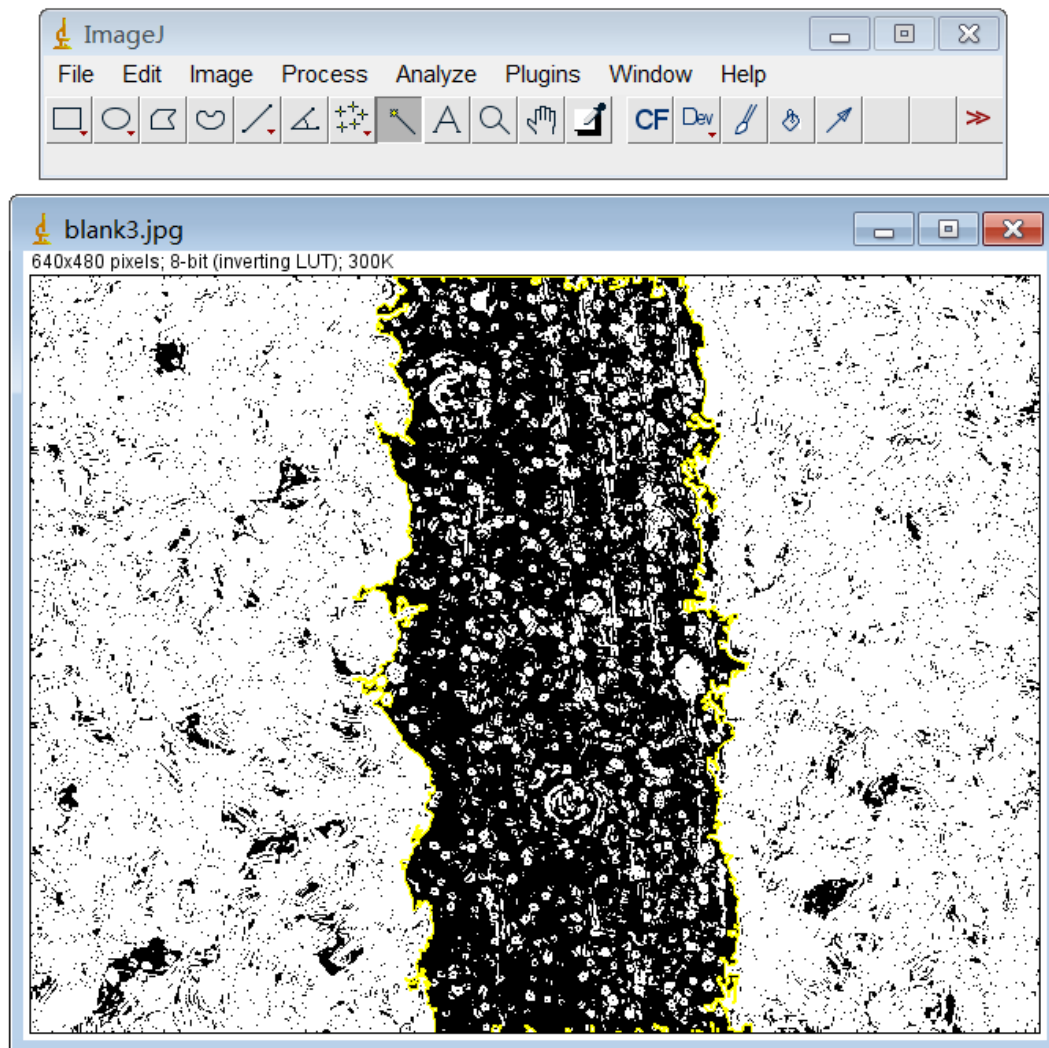


Fig.24 the area of scratch (region in yellow frame).

We used the measure module to calculate the area of scratch area (Fig.25).

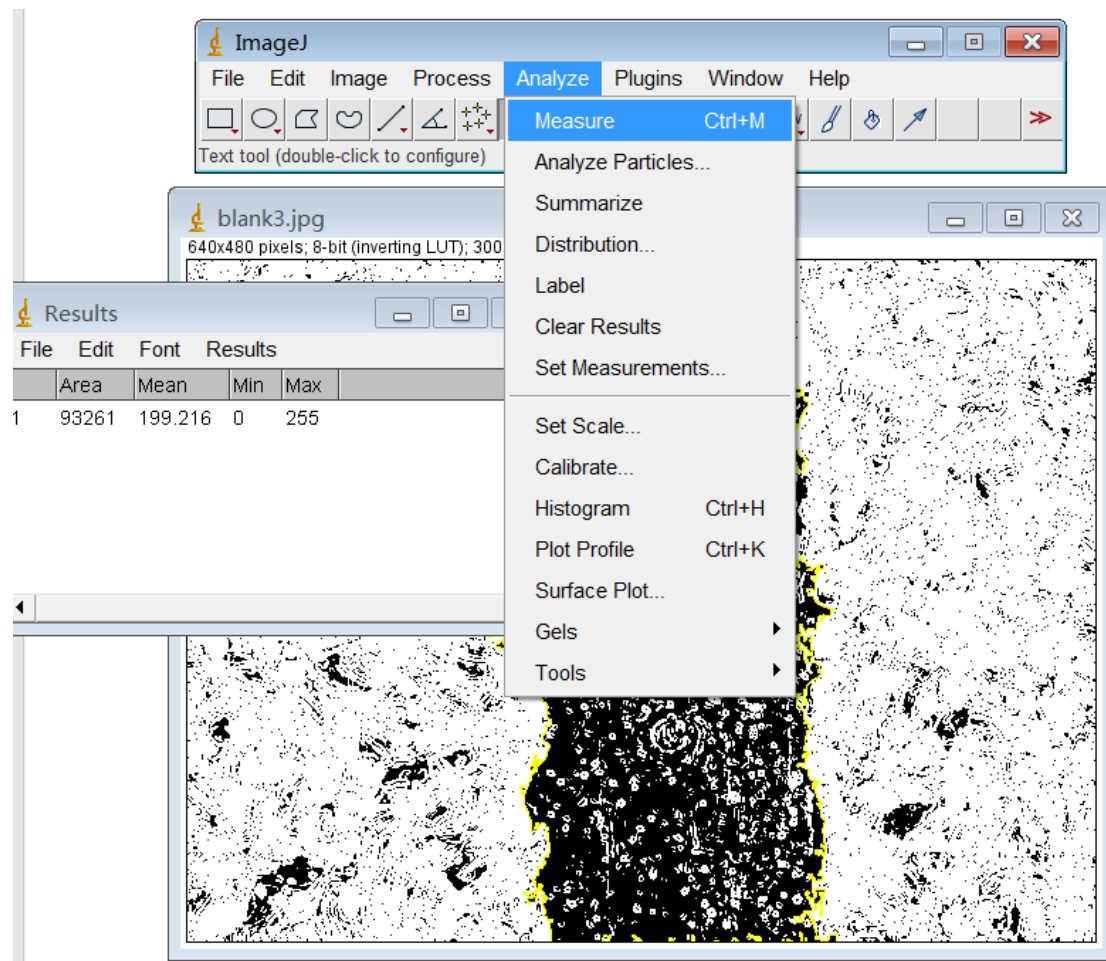


Fig.25 the area of scratch area that was 93261 pixels.

In the same way, we calculated the scratch area for 24 hours and 48 hours. Then, the formula is used to get the percent wound closure:

percent wound closure at 24h= (scratch area for 48h)/(scratch area for 0h)

percent wound closure at 48h= (scratch area for 48h)/(scratch area for 0h)

The evaluation method of tube formation.

Firstly, the image was adjusted to 640×480 pixels by photoshop software (Fig.26).

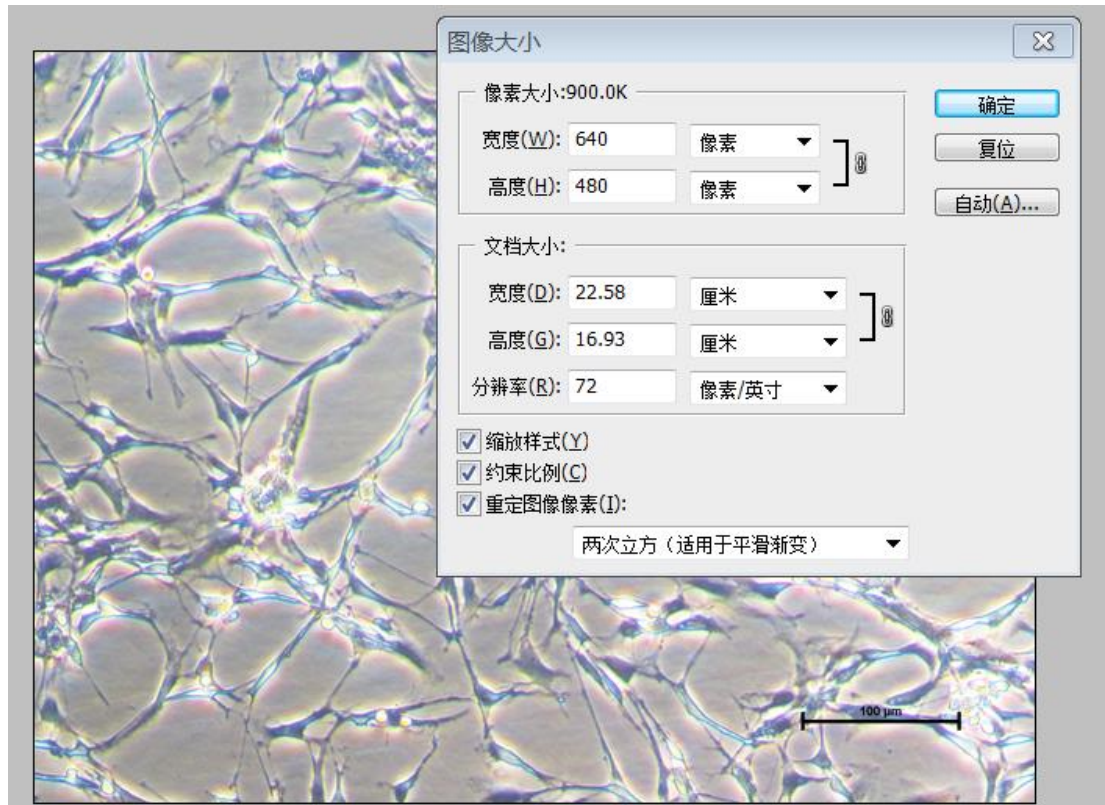


Fig.26 image was adjusted to 640×480 pixels.

Then, the image was imported in Image-pro plus 6.0 software and converted to gray scale 8 image (Fig.27-28).

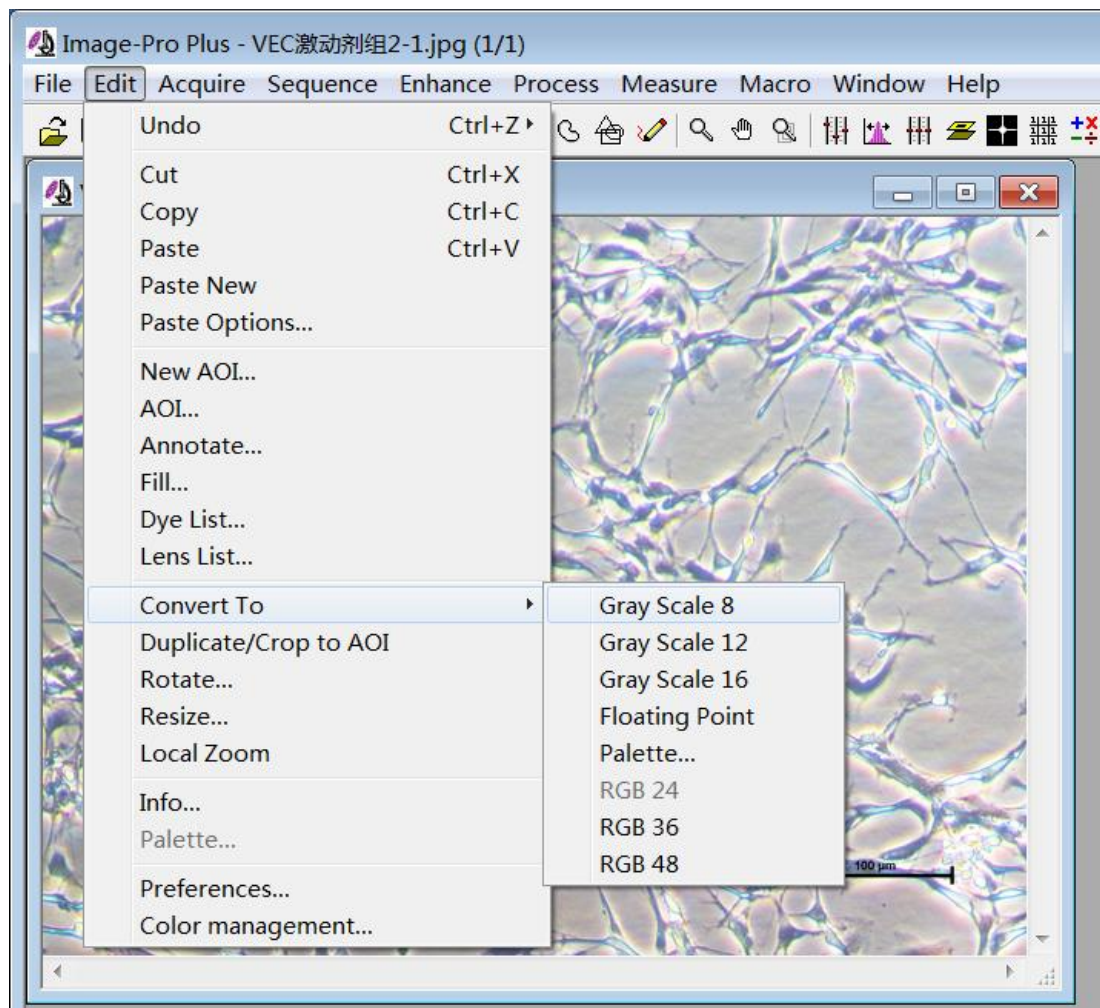


Fig.27 image was converted to gray scale 8 image.

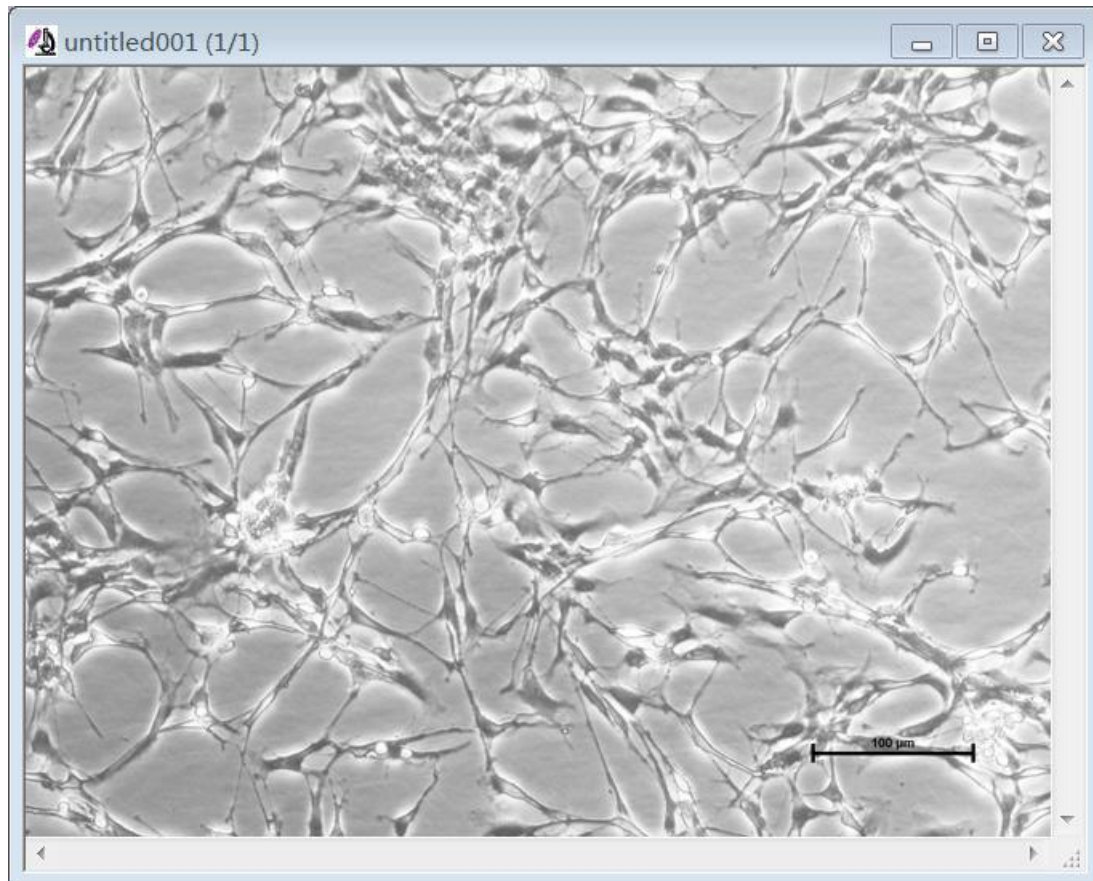


Fig.28 the gray scale 8 image.

Then, we used filters module to adjust image. Firstly, flatten was used as feature width set 20 pix. Secondly, sharpen was used and strength set 10. Thirdly, despeckle was used (Fig.29-30).

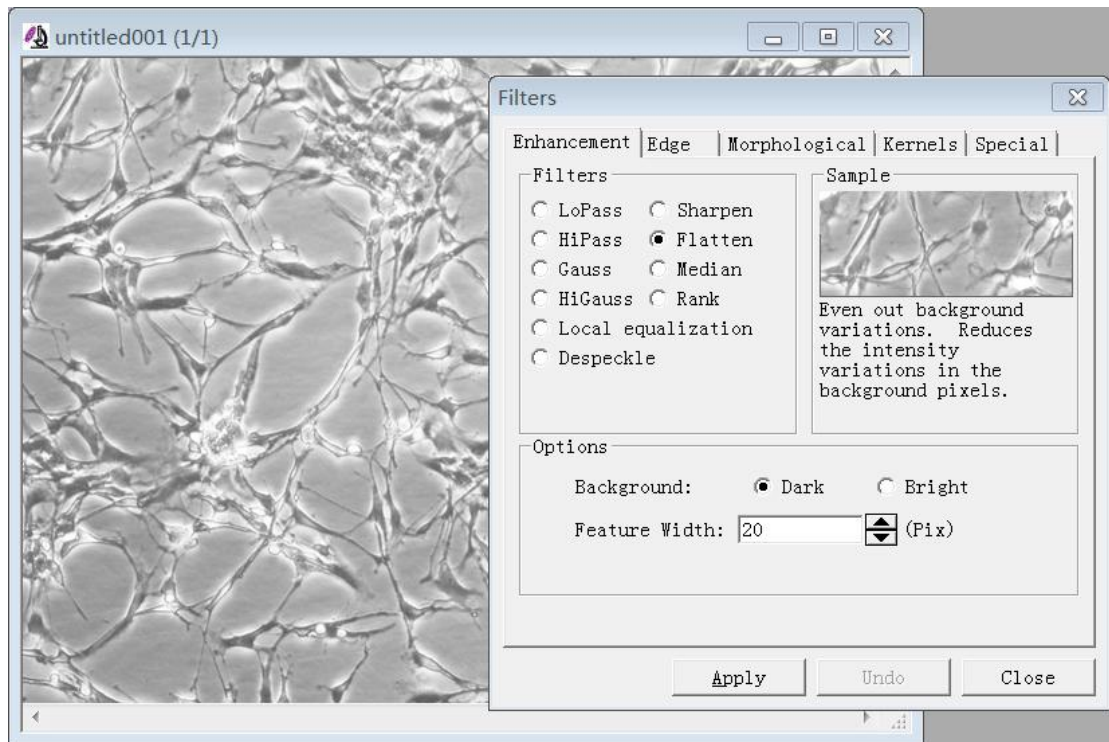


Fig.29 the filters work interface.

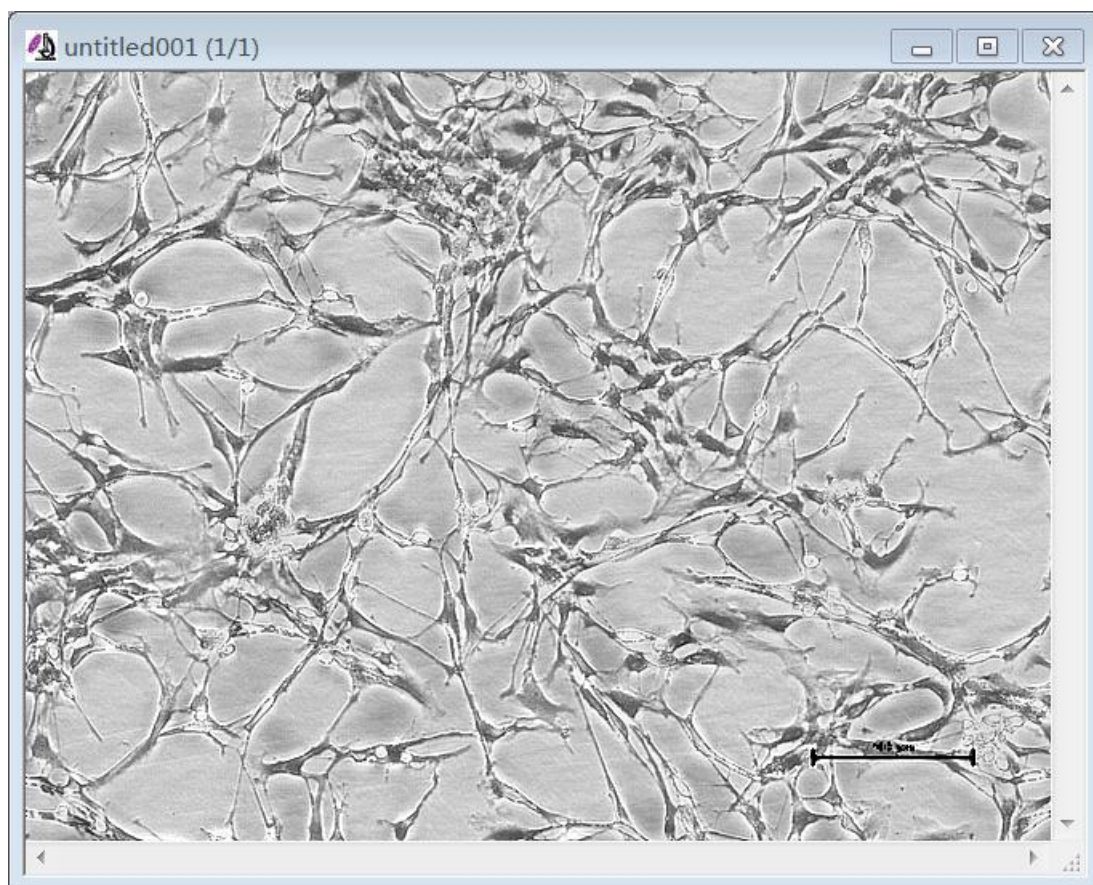


Fig.30 the adjusted image after despeckle used.

Then, we selected enhance -> equalize ->best fit and saved the adjusted image (Fig.31).

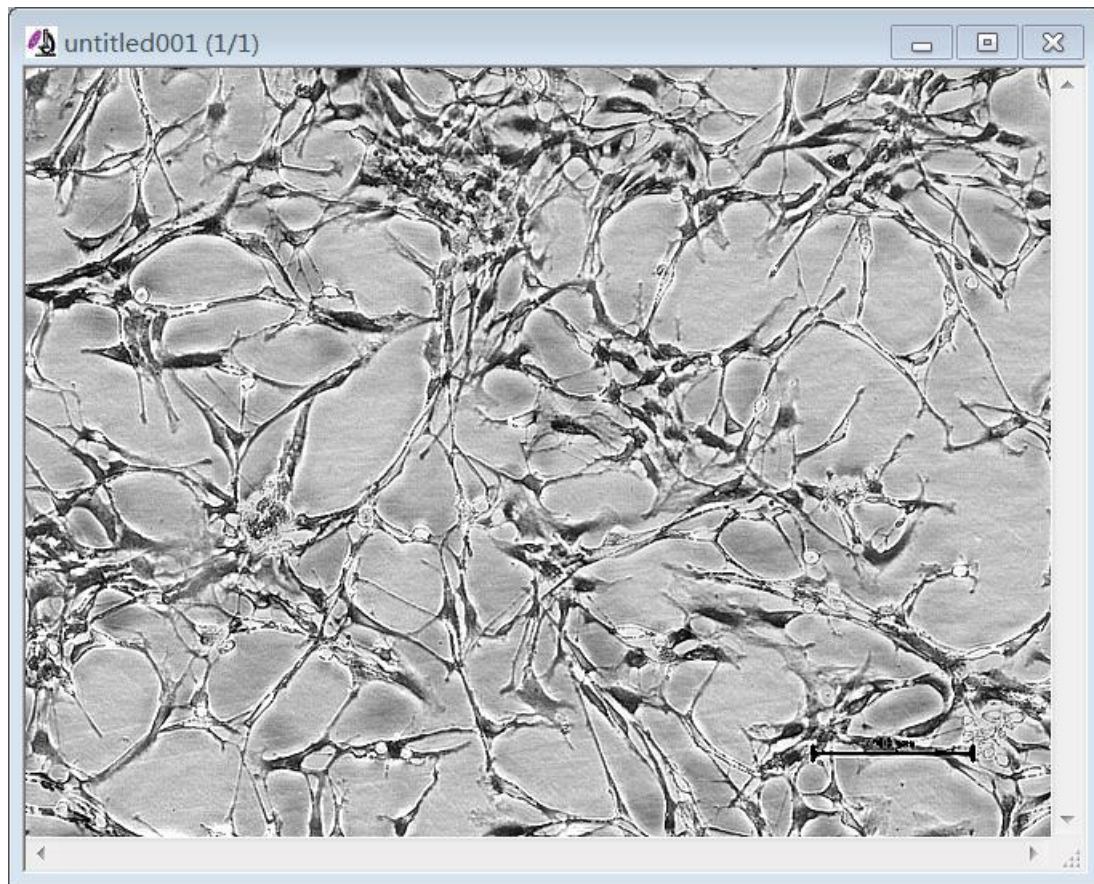


Fig.31 the adjusted image after best fit used.

The adjusted image was imported in ImageJ software and converted to RGB color model (Fig.32).

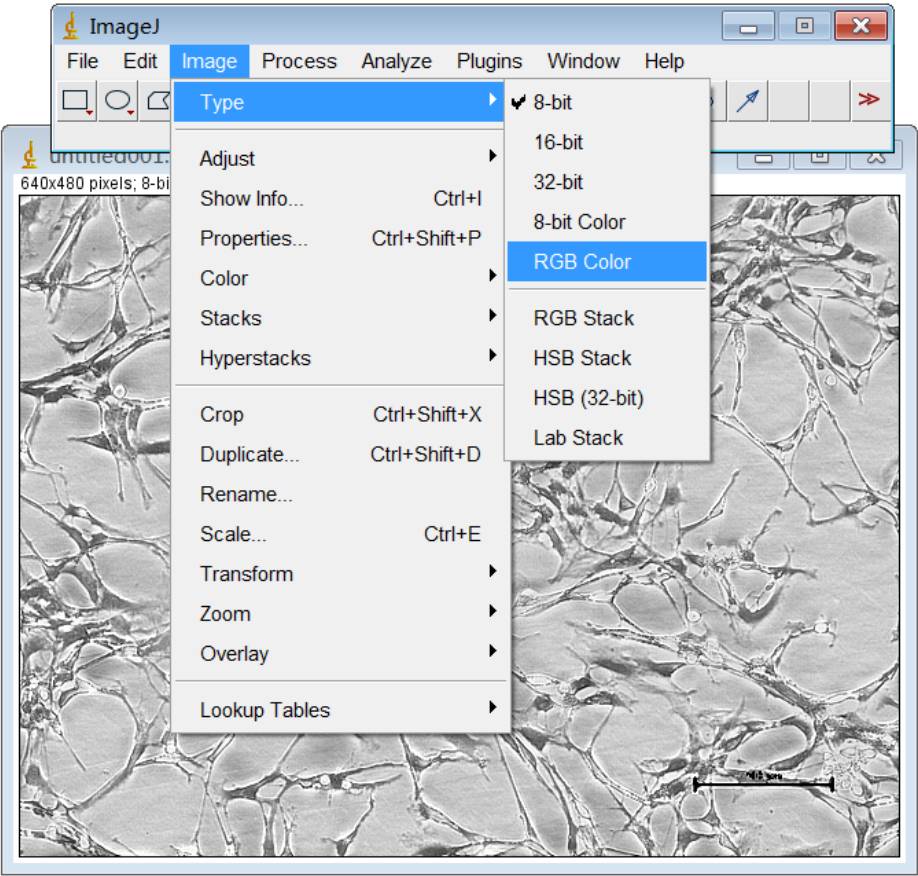


Fig.32 the image converted to RGB color model.

Then, the analyze HUVEC phase contrast module was used. We waited about 3 minutes and software would calculate the capillary length and branching points (Fig.33-34).

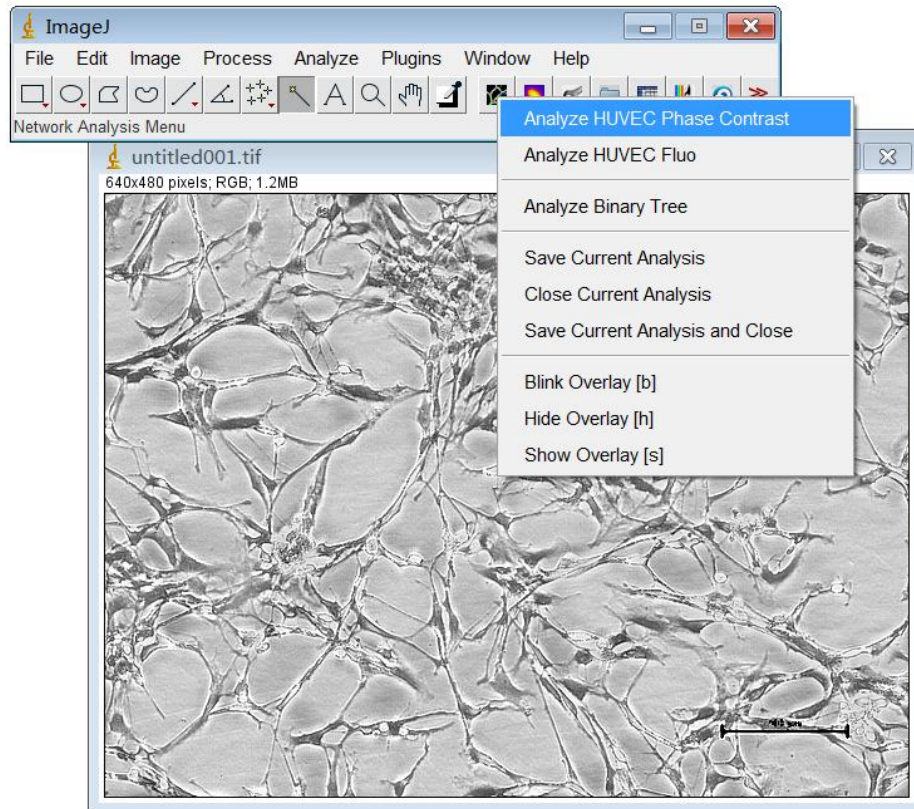


Fig.33 the analyze HUVEC phase contrast module.

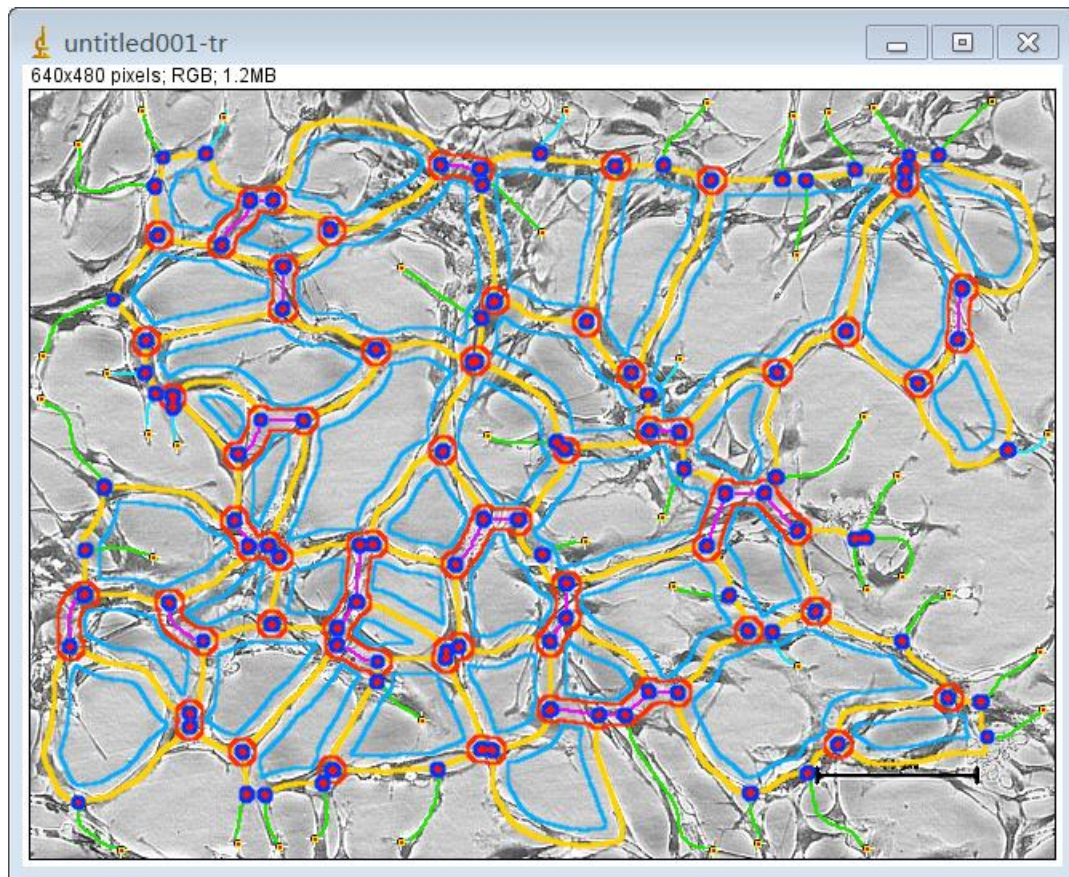


Fig.34 the capillary and branching points in image.