Indirect Immunofluorescence (CD31 and MHC IIa).v2

This assay allows for measurement of capillary density around type IIa and other fibers for fresh frozen mouse skeletal muscle sections

Procedure

(Every step is in plastic jars unless specified)

1. Prepare ice-cold 4% paraformaldehyde/PBS.
2. Take slides out of –80°C freezer and place them on ice.
3. Fix the sections in ice-cold 4% paraformaldehyde/PBS on ice for 10 min.
4. Wash in PBS for 5 min x 1.
5. Permeabilize sections in 0.3% Triton/PBS on ice for 5 min x 2.
6. Wash in PBS for 5 min x 2, WIPE.
7. Block non-specific binding with ~125 µl of 5% Normal Goat Serum/PBS for 30 min at RT on slide.
8. Apply Rat anti-CD31 antibody (Serotec MCA1364, 1:25) and Mouse anti-MHC IIa (SC71 in tissue culture supernatant, 1:25) diluted in 5% Normal Goat Serum/PBS on slide and incubate overnight at 4°C covered with seranwrap. Skip anti-MHC IIa antibody and anti-mouse antibody if you do not want to have fiber type specific analysis.
9. Wash in PBS for 5 min x 3, WIPE.
10. Apply Goat anti-mouse-IgG-Cy5 and Goat anti-rat-IgG-RRed diluted 1:25 in 5% Normal Goat Serum/PBS for 30 min at RT on slide.
11. Wash in PBS for 5 min x 3, WIPE.
12. Coverslip with VECTASHIELD mounting media.
13. Acquire images under the confocal microscope using 20X lens in the area that best represent the overall capillary density.

Reagent

10% Triton X-100: Triton X-100 5 ml, ddH_2O 450 ml.
0.3% Triton X-100/PBS: 10X PBS 50 ml, 10% Triton X-100 15 ml, ddH_2O 435 ml.
5% Normal Serum/PBS: Normal serum 50 ul, 1X PBS 950 ul.

Analysis

Manual

1. Open Word software and insert CD31 confocal images individually into a Word file following Insert>Picture>From File.
2. Copy and paste the image into Photoshop and save it as a tif file in a folder on the desktop.
3. Use “Auto level” and/or “Auto contrast” to optimize the images for analysis and save.
4. Generate another new layer in “Normal” mode and save the whole merged image, and now you are ready to count (now you have 2 layers).
5. While you select the new layer (the last layer you generated in “Normal” mode), use brush or pencil tool size 9 and select the “green color” (the color selection is at the bottom of the tools).

6. Count the endothelial cells by making a dot on top of the CD31 positive cells.
7. If the muscle section does not complete occupy the image, use Scion Image to measure the surface area (See Fiber Type Analysis for detailed procedures).

Scion Image and Photoshop

1. Open Word software and insert CD31 confocal images individually into a Word file following Insert>Picture>From File.
2. Copy and paste the image into Photoshop and save it as a tif file in a folder on the desktop.
3. Use "Auto level" and/or "Auto contrast" to optimize the images for analysis and save.
4. Open the image using Scion Image. Process the tif image as following:
   Option>Threshold
   Process>Binary>Make Binary
   Process>Binary>Erode and repeat this step once more
   Process>Binary>Dilate
5. Select all and copy this new image.
6. Past this new image into a new file of Photoshop. Save it as a different file tif, such as filename-count.
7. Choose Magic Wand Tool and click on one of the endothelial cell dot. This will mark all the CD31 positive cells.
8. Go to Window and check "Measurement log".
9. Click the "Record Measurements" button in the Measurement log window to obtain the total number of CD31 positive cells in the image.
10. Go to Scion Image to measure the surface area of the image.
11. To calculate the capillary density, divide the total cell number by the cross-sectional area of the muscle section.