Immunofluorescence Fiber typing.v2

This assay allows for measurement of all four myofibers in mouse skeletal muscle on a fresh frozen sections.

Procedures

1. Prepare ice-cold 4% paraformaldehyde (PFA)/PBS (Store at 4°C and keep from light. Do not use if it is older than 1 month).
2. Take slides out of –80°C freezer and place them on ice in a plastic jar.
3. Fix in ice-cold 4% PFA/PBS on ice for 10 min.
4. Wash in ice-cold PBS for 5 min x 1 on ice.
5. Permeabilize in ice-cold 0.3% Triton/PBS on ice for 10 min.
6. Wash in PBS for 5 min x 2 at RT. Wipe and circle the tissue using a Wax pen.
7. Block non-specific binding with ~100 µl of freshly prepared 5% normal goat gerum (NGS)/PBS for each section for 60 min at RT on slide.
8. Apply mouse anti-MHC IIb antibody (BF-F3, 1:25) diluted in 5% NGS/PBS on slide and incubate for 2 hrs at RT or overnight at 4°C in a humid chamber.
9. Rinse in PBS twice, WIPE.
10. Apply goat anti-mouse-IgM-FITC diluted 1:25 in 5% NGS/PBS for 60 min at RT on slide, and keep in the dark.
11. Wash in PBS for 5 min x 3. WIPE.
12. Fix in 4% PFA on ice for 2 min.
13. Wash in PBS for 5 min x 2. WIPE
14. Apply mouse anti-MHC I antibody (BA-F8, 1:25) diluted in 5% NGS/PBS on slide and incubate for 2 hrs at RT in a humid chamber kept in the dark.
15. Rinse in PBS twice. WIPE.
16. Apply goat anti-mouse IgG-Rho diluted 1:25 in 5% NGS/PBS for 60 min at RT on slide, keep in dark.
17. Wash in PBS for 5 min x 3. WIPE.
18. Fix in 4% PFA on ice for 2 min.
19. Wash in PBS for 5 min x 2. WIPE
20. Apply mouse anti-MHC IIa antibody (SC-71, 1:25) diluted in 5% NGS/PBS on slide and incubate for 2 hrs at RT in a humid chamber kept in the dark.
21. Rinse in PBS x 2. WIPE.
22. Apply goat anti-mouse IgG-Cy5 diluted 1:25 in 5% NGS/PBS for 60 min at RT on slide, keep in the dark.
23. Wash in PBS for 5 min x 3. WIPE.
26. Coverslip with VECTASHIELD mounting media and seal with nail polish.

Note

1. Samples must be fresh frozen. Fixed sample will not work.
2. Always start with MHCIIb first since goat anti mouse IgM crossreacts with IgG.
3. Antibodies for MHCs are in hybridoma cell culture medium stored in 1 ml-aliquots at -80°C. Thaw one vial at a time and store the unused at 4°C for several months.

Analysis

Fiber type composition analysis (% fiber number)

1. Open each of the individual confocal image files (tif files) of different fiber types (type I, IIa, and IIb).
2. Change one of the file from "Index color" to "RGB" and save as a different file, such as So-and-So-merged.
3. Go to a different fiber type file and select all and copy.
4. Go back to the merged file and generate a new layer in "Screen" mode and paste the image over (the merged image has multiple layers now).
5. Follow step 3 and 4 again for the last fiber type (now you have 3 layers).
6. Go to each of the layers to do "Auto level" and "Auto contrast" to optimize the images for analysis.
7. Generate another new layer in "Normal" mode and save the whole merged image, and now you are ready to count (now you have 4 layers).
8. While you select the new layer (the last layer you generated in "Normal" mode), use brush or pencil tool size 9 and select the "white color" (the color selection is at the bottom of the tools).
9. Count the type IIx fibers (negative fibers) first by making a dot on top of the fibers. Please use the "view" function of type IIa to make sure what fibers should be counted as type IIx.
10. Following step 8 for type IIa, I and IIb by using "light blue", "yellow" and "red" colored brush, respectively. For type IIb fibers, if the boundary of the fibers are not clear, try to use the sizes of nearby separated fibers as reference to help.

Fiber type composition analysis (% fiber area)

1. Go to the merged muscle images (3 colors) and use fixed crop box (1.5 x 1.5 inch) to select an area that represent the average fiber composition of the total muscle section. Crop the image, flatten the images and save as a different file, such as So-and-SO.crop (a tif file).
2. Open the cropped image using Scion Image.
3. Use the selection tool (top third on the right side of the tools) to enclose every fiber of your interest (say type I fibers).
4. Go to "Option" and choose "Threshold".
5. Go to "Process" and choose "Binary" to "Make binary".
6. Go to "Analyze" and "Set scale" to change the unit from "Centimeters" to "Pixels".
7. Go to "Analyze" again to "Measure".
8. Go to "Windows" to choose "Info" to show the results in pixels.
9. If the fibers of interest are far apart, you could measure some fibers at a time and repeat step 2-8 to obtain the data and add them together.
10. Count the number of the fiber of interest in the cropped image for calculation of average fiber size (surface area of the fiber of interest divided by number of fibers).

The calculation of the surface area is as following:
For 4X images, 893968 pixels = 1.0046×10^7 u^2

For 6X images, 893968 pixels = 4.4649×10^6 u^2
For 10X images, 893968 pixels = 1.6137×10^6 u^2
For 20X images, 893968 pixels = 4.0263×10^5 u^2
For 40X images, 893968 pixels = 1.0066×10^5 u^2
Total image size should be around 893968 pixles. If your total area is different, adjust the calculation accordingly.