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Protocol for ABC Immunohistochemistry and Quantifying Nerves

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Protocol status: Working
We use this protocol and it's working

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Abstract

Cardiac tissue samples were obtained from human donors and sent to East Tennessee State University for processing. At ETSU, the tissue was dissected, sectioned with a cryostat, and stained for neuronal markers using the ABC IHC method. Pictures of the sections were obtained and analyzed for nerve density across different regions of the heart.

Troubleshooting

Tissue Fixation

1 Tissues fixed in 4% paraformaldehyde in PBS at 4°C for 24 hrs

Tissue Preparation

2 Tissues shipped to ETSU at 4°C in a PBS solution containing 20% sucrose + 0.02% sodium azide

3 Remove tissue samples from solution and dissect as needed, to isolate specific regions

4 Freeze tissues on dry ice and store at -80°C until ready to be sectioned

Tissue Sectioning using Cryostat

5 Remove tissues from -80°C freezer and mount onto specimen plate using Tissue-Tek® O.C.T. Compound (Sakura Finetek USA, Cat. No. 4583)

6 Cut tissues into 30µm sections at -20 to -25°C using a Leica CM3050S cryostat (Leica Microsystems Inc., Bannockburn, IL, USA)

7 Collect sections on charged slides in a sequence that yields at least 4 sets of tissue sections per region of the heart, with each set spanning the entire thickness of the specimen

8 Store sets of tissues in slide boxes wrapped in aluminum foil at -20°C until further processing

Immunostaining Day 1

9 Stain sets 1, 2, and 3 of each tissue region with a different primary antibody: sheep anti-tyrosine hydroxylase (TH, Milipore Cat. No. AB1542, 1:500) or rabbit anti-tyrosine hydroxylase (TH, Pel-Freez Biologicals, Cat. No. P40101-150, 1:500), rabbit anti-vesicular acetylcholine transporter (VAChT, Synaptic Systems, Cat. No. 139103, 1:500), and rabbit anti-protein gene product 9.5 (PGP9.5, Abcam Cat. No. ab108986, 1:2000), respectively.

10 Leave set out to thaw at RT for 20 mins 20m

11 Using the PAP Pen, carefully draw a water barrier circle around the tissue sections on the slide — allow this circle to dry for several seconds or up to approximately one minute.

12 Rinse slides by placing them into a Coplin jar filled with PBS (pH 7.3 - 7.4): 4 × 5 min each 20m

13 Place slides in 0.5% BSA + 0.4% Triton X-100 in PBS: 1 × 10 min 10m

14 Place slides in 1.0% H_2O_2 in PBS: 1 × 15 min. (2 ml of 30% H_2O_2 in 60 ml of PBS) 15m

15 Place slides in PBS: 4 × 5 min each 20m

16 Place slides in 0.5% BSA + 0.4% Triton X-100 in PBS: 1 × 10 min 10m

17 Remove slides one at a time and use a clean Kimwipe to wipe around the tissue sections to dry the slide. Keep the sections wet.

18 Place the slides into a black, covered slide incubation box/humidity box

19 Cover the tissue sections with blocking buffer (150µl of normal goat serum (Jackson ImmunoResearch Laboratories, Cat. No. 005-000-121) OR normal rabbit serum (Jackson ImmunoResearch Laboratories, Cat. No. 011-000-120) + 10 mL of 1.0% BSA + 0.4% Triton X-100 in PBS)

20 Allow the sections to remain in blocking buffer for 1.5 - 2 hours at room temperature 2h

21 Pour off the blocking buffer

22 Replace with primary antibody solution (antibody of choice diluted in the same blocking buffer used above)

23 Incubate overnight at room temperature

Immunostaining Day 2

6h 6m

24 Place slides in PBS: 4 × 5 min each 20m

25 Place slides in 0.5% BSA + 0.4% Triton X-100 in PBS: 1 × 10 min 10m

26 While rinsing slides above, prepare the biotinylated secondary antibody solution. (Blocking buffer = 150µl of normal goat serum (Jackson ImmunoResearch Laboratories, Cat. No. 005-000-121) OR normal rabbit serum (Jackson ImmunoResearch Laboratories, Cat. No. 011-000-120) + 10 mL of 1.0% BSA + 0.4% Triton X-100 in PBS. Add 50µl of biotinylated secondary antibody to the blocking buffer solution.)

27 Remove slides one at a time and use a clean Kimwipe to wipe around the tissue sections to dry the slide. Keep the sections wet.

28 Cover the tissue with the secondary antibody solution and incubate for 2 hours at RT in the humidity box 2h

29 Prepare the ABC reagent from the Vector Kit at least 30 min prior to using. ABC reagent is made by adding 1 drop of solution A + 1 drop of solution B to 2.5 mL of solution containing 1.0% BSA + 0.4% Triton-X 100 + PBS. Add solutions
A and B to the buffer in that order. Let this sit at RT while performing the following rinses.

30 Pour off the secondary antibody solution

31 Place slides in PBS: 4 × 5 min each 20m

32 Place slides in 0.5% BSA + 0.4% Triton X-100 in PBS: 1 × 10 min 10m

33 Remove slides one at a time and use a clean Kimwipe to wipe around the tissue sections to dry the slide. Keep the sections wet.

34 Cover the tissue with the ABC reagent and incubate 1-1.5 hrs. in the humidity box 2h

35 Prepare 50mM Tris buffer (Trizma, pH 7.6) for the next rinse stage — (1.49 g Trizma (pH 7.6, Sigma T7943) + 200 mL dH_2O)

36 Pour the ABC reagent off the slides

37 Place the slides in a Coplin jar with the Tris buffer: 2 × 10 mins. 20m

38 During the last Tris buffer rinse, prepare the VIP development solution — must be at RT for proper use (Vector ImmPACT VIP Kit, SK4605). Use 5 mL VIP diluent + 3 drops of each of solutions 1, 2, 3, and 4 from the kit.

39 Place the slides onto a light-colored background to monitor color development.

40 Add the VIP color development solution to the tissue sections.

41 Monitor development of color, which should take from 2 to 20 min to reach maximum intensity. (Use a microscope to monitor.) 20m

42 Stop color development by submerging slides in dH_2O in a Coplin jar

43 Rinse with dH_2O: 2 × 2 min each 4m

44 Dehydrate the tissue

44.1 rinse in 50% EtOH: 1× 2 min

2m

44.2 rinse in 75% EtOH: 1 × 2 min 2m

44.3 rinse in 95% EtOH: 2 × 2 min 4m

44.4 rinse in 100% EtOH: 2 × 2 min 4m

44.5 rinse in Xylene: 2 × 5 min 10m

45 Attach coverslips using Cytoseal XYL (Thermo Scientific, Cat. No. 8312-4)

46 Allow to air dry overnight

Imaging 6h 6m

47 Image tissue sections using Olympus BX41 microscope equipped with an Olympus DP74 digital camera and cellSens software (Olympus America Inc., Center Valley, PA; RRID:SCR_016238)

48 Load slide onto stage and collect representative Z-stack images of each slide at 20X magnification. The Z-stack range is defined by the researcher as the furthest depth at which some nerves are still in focus, and the step size is determined by the program.

49 Process each slide using EFI (extended focus image) to enhance Z-stack image.

50 Save images as JPGs in groups sorted by specimen, region, and stain

Image Analysis 6h 6m

51 Open desired JPG image within the ImageJ program

52 Isolate and "fill" any high-contrast anomalies that should not be included in calculations

53 Change image type to "8-bit."

54 Adjust the threshold of the image until only the desired regions containing nerves are highlighted in red

55 Record the nerve density percentage shown into a spreadsheet. Repeat the above adjustment until you have three consistent readings

56 Calculate the average of the three readings

Statistical Analysis

57 Input the average nerve density data (% area) into GraphPad Prism 8 for statistical analysis. Data should be separated by donor, heart region, and stain

58 Use the "descriptive statistics" function of the software to find the mean and standard deviation of each region