Detection of Rat Macrophages, an Immunoperoxidase Method ($DAB-NiCl_2$ with Methyl Green Countestain)

Don C. Wilson, University of Washington Medical Center

I. Slide Preparation

1) Using microtome, cut 5 mm thick tissue sections.
2) Place tissue sections on SuperFrost/Pius Fisher brand slides.
3) Place slides in a 45° C drying oven overnight.
4) Deparaffinize slides in 3 changes of xyiene for 5 minutes each.
5) Rehydrate the slides in the following order:
   a) 100% ethyl alcohol - 3 changes, 5 minutes total
   b) 95% ethyl alcohol - 2 changes, 4 minutes total
   c) 70% ethyl alcohol - 1 change, 1 minute
6) Rinse slides in distilled water.
7) Place slides in 3% hydrogen peroxide for 5 minutes (save 5 mL for DAB solution).
8) Rinse slides in distilled water.
9) Rinse slides in PBS twice. Microwave antigen retrieval:
10) Place slides in a plastic rack (no metal). Rack must always be full. Load with blank slides if necessary.
11) Place slides in 200 mL of 10 mM citrate buffer, cover with a lid loosely and place at edge of microwave tray.
12) Microwave at high power setting for 10 minutes.
    NOTE: Do not let fluid level evaporate below level of tissue. Stop microwave every 3 minutes and add distilled water.
13) Remove slides from microwave and let sit in citrate at room temperature for a full 30 minutes.
14) Rinse slides in phosphate buffered saline (PBS) and one change of PBS/Triton for a total of 5 minutes.

II. Antibody Application

15) Incubate specimens (humidified chamber, room temperature) with 10% bovine serum albumin (BSA) in PBS for 20 minutes.
    Use sufficient reagent to cover the specimen (usually 50-75 mL).
16) Wash slides in 2 changes of PBS and I change of PBS with BSA/Triton for a total of 5 minutes.
17) Prepare primary antibodies by diluting:
    a) Control antibody (Mouse IgG) to 0.4 µg/mL In 1% BSA + PBS,
    b) (Serotec ED-1) anti-rat macrophage antibody 1:100 in 1% BSA + PBS.
18) Apply an adequate amount (usually about 75 mL) of the primary antibody to cover the entire tissue. Include at least 1 slide as negative control (apply control mouse IgG).
19) Incubate the slides for 60 minutes in a moist chamber at room tempera ture.
    At this time, prepare the secondary antibodies, (biotinylated anti-mouse IgG diluted to 6 µg/mL). and streptavidin/peroxidase complex. These solutions should be made up in 1% BSA + PBS.
    Thaw the appropriate number of DAB aliquots.
    Warm 175 mL of 0.05 M Tris buffer in a beaker (foil covered) in a water bath at 37° C. Warm 200 mL of 0.05 M Tris buffer in a slide boat in a water bath at 37° C.
20) Wash slides in I change of PBS and 1 change of PBS with BSA/Triton for a total of 5 minutes.
21) Incubate specimens with 10% horse serum* in PBS for 20 minutes.
    Use sufficient reagent to cover the entire specimen (50-75 mL).
22) Wash slides in 1 change PBS.
23) Apply the biotinylated secondary antibody (anti-mouse IgG) and incu-

bate for 30 minutes in a moist chamber at room temperature.

24) Rinse with 3 changes of PBS.

25) Cover sections with peroxidase labeled streptavidin (Avidin:Biotinylated enzyme Complex [ABC] solution) and incubate for 30 minutes in a moist chamber at room temperature.

26) Wash slides in 1 change of PBS and 1 change of 0.05 M Trls buffer (at 37° C In a water bath) for a total of 5 minutes.

III. Color Development

NOTE: DAB may be carcinogenlc. Use rubber gloves and prepare under a fume hood. DAB is light sensitive and should be prepared in the dark and just prior to use.

27) We prepare a stock DAB (3,3'-diamlnobenzldine (3,3',4,4'tetraminobiphenyl) tetrahydrochloride) solution by mixing 5.0 g DAB in 132 mL Tris buffer. We then divide it into 4 mL aliquots, freeze them at -70° C, and use them to prepare the working DAB solution as described. The 4 mL aliquots of stock solution are enough to make 180 mL of the working solution which will stain 20 slides at a time.

28) Preparation of working DAB solution:
NOTE: Always add reagents in the following order:
   a) Add 4 mL of thawed DAB to the warmed Tris buffer.
   b) While stirring, add I mL of 8% $NiCl_2$.
   c) Add 12 drops of 3% hydrogen peroxide with a Pasteur pipette.
   d) Mix solution thoroughly.

29) Incubate slides in the DAB solution for 10 minutes.

30) Wash slides in distilled water for 1 change.

31) Counterstain slides in methyl green for 5 minutes.

32) Dehydrate slides in the following order:
   a) 95% ethyl alcohol - 1 quick dip (methyl green is extremely soluble in water).
   b) 100% ethyl alcohol - 2 changes for a few quick dips.
   c) Leave the slides in the last change of 100% ethyl alcohol for 5 minutes.

33) Rinse slides in 3 changes of xylene for a total of 5 minutes.

34) Apply coversups with Permount.

*Obtained from normal animals, heat treated, centrifuged and filtered through a .45 micron filter.