DAB on Human Hepatocytes

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Neutrophil myeloperoxidase standard 3,3'-Diaminobenzidine Tetrahydrochioride (DAB) procedure:

When staining neutrophils, a cell suspension would be preferred, but a finely minced buffy coat can be used, if you thick section and locate the stained cells before thin sectioning. Staining should be carried out as soon as possible after fixation. The myeloperoxidase (MPO) activity falls off such that we process samples within 2-3 weeks of fixation.

Note that fixation is for 1 hour. Enzyme activity may be destroyed if tixed for longer periods.

Chemical inhibition of some peroxidatic enzymes is possible, but inhibition is not 100% specific or effective. Chemical inhibition works best when an inhibition step (inhibitor and buffer) occurs before staining with the DAB solution which also contains the inhibiting agent.

The literature shows that it is difficult to assess different peroxidatic enzymes when they are colocalized using cytochemical techniques.

Always be sure to include appropriate controls (*i.e.*, a morphological control as well as cytochemical ones).

The DAB-stained cells are viewed without uranyl acetate-lead citrate (UALC) counterstaining, or with lead citrate (LC) staining only. Counterstains may confound the granule analysis.

We place our DAB incubation in the dark (in a cabinet).

This procedure stains neutrophil primary granules for myeloperoxidase activity [0.01% H_2O_2].

Procedure:

1) 3%. Glutaraldehyde in 0.1 M Cacodylate Buffer, pH 7.35 @ 40° C (on ice) 1 hour.

2) Rinse x 3 in 0.1 M Cacodylate Buffer + 7% Sucrose, pH 7.35. Store overnight in rinse buffer.

3) Rinse x 1 in DAB solution without H_2O_2.

4) DAB solution with H_2O_2 at room temperature 30 minutes. [Control: Use DAB solution without H_2O_2 for 30 minutes.]

5) Rinse x 1 in 0.05 M Tris Buffer, pH 7.6.

6) Rinse x 2 in 0.1 M Cacodylate Buffer + 7% Sucrose, pH 7.35.

7) 1% OsO_4 in 0.1 M Cacodylate Buffer, pH 7.35, at room temperature 1 hour.

8) Dehydrate and embed

Reagents:

DAB solution:

3,3'-Diaminobenzidine tetrahydrochloride (4 HCl) 5 mg.
0.05 M Tris Buffer pH 7.6 10 mL.
3% H_2O_2 33 µL.
pH should be in the range of 7.0-7.6. When the buffer is fresh it is around 7.3-7.4.

DAB solution with 10 mM KCN:

3,3'-Diaminobenzidine 4 HCl 5 mg.
0.05 M Tris Buffer, pH 7.6. with 10 mM KCN 10 mL.
3% H_2O_2 33 µL.

0.05 M Tris buffer, pH 7.6:

Tris-(hydroxymethyl)-aminomethane [99.0-99.5%] 6.06 gm.
make up to 1000 mL with distilled H_2O.
Adjust pH to 7.6.

3% H_2O_2 (Working Solution):

50% H_2O_2 (Commercial Stock Solution) X mL.
0.05 M Tris Buffer, pH 7.6 make up to 10 mL.

See "Verification" section below to determine the value of "X".

Note: 3% H_2O_2 working solutions should be made fresh before use using a concentrated stock solution which has been checked for the actual concentration. See the verification procedure for doing this spectrophotometrically.

10 mM potassium cyanide (KCN) [in 0.05 M Tris Buffer, pH 7.6]:

Potassium cyanide 65 mg.

Make up to 100 mL with 0.05 M Tris Buffer, pH 7.6

pH to 7.6 with 1 N HCl

MODIFICATIONS:

DAB with cyanide inhibition (10 mM KCN):

This procedure inhibits cytochrome oxidase and myeloperoxidase. 4-aminobenzhydrazide inhibits MPO.1,2,3-Aminotriazole inhibits catalase.

For catalase localization use Tris buffer at pH 9.5-10.0.

For inhibition studies, always recheck the pH of the buffer-inhibitor solutions and pH them properly.

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Verification of $[H_2O_2]$:

$E_{240} = 43.6\ m^{-1}\ cm^{-1}$ (extinction coefficient at wavelength 240)

MW= 34.01 g/mole

50% H_2O_2 = 1.2 g/mL (density, rho)

Determine whether concentration is by weight or volume because serious mIscalculations are possible.

Calculation for the molar concentration of:

50% by wt => (60g)/120 g = (60g)/100 mL

(60g)/100 mL x 1/(34.01 g/mole) = (1.761 mole)/100 mL => 17.61 M

Stock solution must be diluted to measure the Absorbance (A_{240})

l = length of light path in cm

C = concentration (moles/liter)

$A_{240} = E_{240}\ l\ C => C = [A_{240} / (E_{240}\ l)]$ x dilution factor

e.g., dilution of 1/1000 A_{240} = 0.6375 => 14.6 M

(14.6 M x MW)/10 = (49.6 g)/100 mL = (49.6 g)/120 g => 41.4%

Use this percentage in making up the 3% H_2O_2 working solution.

Since the 50% H_2O_2 (17.61 M) was actually 41.4% (14.6 M) it follows that:

$V_1C_1 = V_2C_2$ => 14.6 x = 1.06 (10 mL) => x ml = 10.6/14.6 = 0.728 mL

Use 0.726 mL in making the 3% H_2O_2 working solution. ■