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We use this protocol and it's working

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DAB staining protocol for subsequent stereological cell counting

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ABSTRACT

This protocol describes the steps for performing a double chromogen staining using DAB and SK- 4700. Stained sections can subsequently be imaged and used for stereological cell quantification with Stereo Investigator software from MBF Bioscience or other Stereology software.

ATTACHMENTS

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MATERIALS

Materials:

- Pre-cut brain sections (30-50 µm thick)
- 0.1 M PB
- Triton-X100 (detergent)
- Methanol (e.g. from Sigma-Aldrich)
- Ethanol absolute (e.g. from Sigma-Aldrich)
- 30% H_2O_2 (e.g. from Merck)
- Xylene (e.g. from J.T.Baker)
- Na-hypochlorite (e.g. Sigma-Aldrich)
- Normal donkey serum (S30-100ML, Sigma-Aldrich)
- Primary antibodies (e.g. Anti-ChAT AB144P, Merck; anti-TH ab113, abcam; anti-p62, ab109012, abcam; anti-alpha-synuclein(phosphoS129), ab51253, abcam)
- Biotinylated secondary antibody
- Vectastain® Elite® ABC HRP Kit (Vector Laboratories)
- 3,3'-diaminobenzidine (DAB) (e.g. from Serva)
- SK-4700 SG Peroxidase Kit (Vector Laboratories)
- 12-well-plates with netwell inserts (e.g. Corning Costar Netwell)
- Quick-hardening Mounting Medium (e.g. EukiS, Sigma Aldrich)
- Microscopy slides

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- Glass coverslips
- Designated container for storage of microscopy slides
- Orbital shaker (e.g. Heidolph Duomax 1030)
- Plajorm shaker (e.g. Heidolph Unimax 1010)
- Microscope system configured for brightfield (and multi-channel fluorescent work) with Stereo Investigator software from MicroBrightField (MBF) (e.g. Zeiss Axio Imager.M2 with MBF extension modules needed for Stereo Investigator software)

Recommended PPE:

- Lab coat/disposable gown
- Safety goggles
- Examination gloves
- FFP2 mask/respirator

Primary antibodies:

Goat anti-ChAT antibody **Merck Millipore (EMD Millipore) Catalog #AB144P**

Anti-Tyrosine Hydroxylase antibody **Abcam Catalog #ab113**

Recombinant Anti-SQSTM1 / p62 antibody [EPR4844] - Autophagosome Marker **Abcam Catalog #ab109012**

Recombinant Anti-Alpha-synuclein (phospho S129) antibody **Abcam Catalog #ab51253**

DAB staining - Before the procedure:

12h 47m

1 Prepare [M] 0.1 Molarity (M) PB (PB).

2 Prepare PBT ([M] 0.1 Molarity (M) PB with 0.3% Triton X-100).

3 Prepare a quenching solution (for 40 mL solution: 32 mL [M] 0.1 Molarity (M) PB, 4 mL

methanol (100%), 4 mL H_2O_2 (30%)).

4 Prepare NDS solution (5% normal donkey serum diluted in PBT).

DAB Staining Procedure

12h 47m

5 Place brain sections (30 µm - 50 µm) in 12-well-plates with netwell inserts (up to 6-10 sections per netwell insert, depending on the size of the sections).

6 Place 12-well-plates on platform shaker and wash sections for 3x 5 min in [M] 0.1 Molarity (M) PB. Exchange PB solution in between washing steps.

6.1 Place 12-well-plates on platform shaker and wash sections for 00:05:00 in [M] 0.1 Molarity (M) PB. Exchange PB solution in between washing steps (1/3). 5m

6.2 Place 12-well-plates on platform shaker and wash sections for 00:05:00 in [M] 0.1 Molarity (M) PB. Exchange PB solution in between washing steps (2/3). 5m

6.3 Place 12-well-plates on platform shaker and wash sections for 00:05:00 in [M] 0.1 Molarity (M) PB. Exchange PB solution in between washing steps (3/3). 5m

7 Quench sections for 00:15:00 at Room temperature in quenching solution in 12-well-plate plajorm shaker. 15m

Note

We recommend 4 mL solution per well for good results.

8 Thereafter, wash sections for 4x 5 min in [M] 0.1 Molarity (M) PB on platform shaker. Exchange PB solution in between washing steps.

8.1 Thereafter, wash sections for 00:05:00 in [M] 0.1 Molarity (M) PB on plajorm shaker. Excha 5m
PB solution in between washing steps (1/).

8.2 Thereafter, wash sections for 00:05:00 in [M] 0.1 Molarity (M) PB on plajorm shaker. Excha 5m
PB solution in between washing steps (2/4).

8.3 Thereafter, wash sections for 00:05:00 in [M] 0.1 Molarity (M) PB on plajorm shaker. Excha 5m
PB solution in between washing steps (3/4).

8.4 Thereafter, wash sections for 00:05:00 in [M] 0.1 Molarity (M) PB on plajorm shaker. Excha 5m
PB solution in between washing steps (4/4).

9 Block sections for 01:00:00 at Room temperature in NDS solution in 12-well-plates. 1h

Note

We recommend 4 mL solution per well for good results.

10 For incubation with primary antibody (e.g. anti neuronal nuclei (NeuN), Merck Millipore, MAB377, 1:100 2h
transfer sections in a new 12-well-plate but without netwell inserts. This allows better shaking
Overnight. Solution for incubation with primary antibody should contain NDS solution and the respective primary antibody diluted according to manufacturer recommendation. Incubate sections on

orbital shaker with gentle shaking at 4 °C Overnight .

Note

We recommend at least 1 ml solution for each well.

11 On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections 4x 5 min in PB on a platform shaker at Room temperature . Change washing solution after each washing step.

11.1 5m On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections for 00:05:00 in PB on platform shaker at Room temperature . Change washing solution after each washing step (1/4).

11.2 5m On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections for 00:05:00 in PB on platform shaker at Room temperature . Change washing solution after each washing step (2/4).

11.3 5m On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections for 00:05:00 in PB on platform shaker at Room temperature . Change washing solution after each washing step (3/4).

11.4 5m On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections for 00:05:00 in PB on platform shaker at Room temperature . Change washing solution after each washing step (4/4).

12 1h Next, transfer sections back in 12-well-plate without netwell inserts for incubation with biotinylated secondary antibody (e.g., biotinylated donkey anti-mouse, Jackson ImmunoResearch, 715-065-151, 1:500) diluted in NDS solution. Incubate for 01:00:00 on orbital shaker at Room temperature .

Note

We recommend at least 1 ml solution for each well.

13 Next, without washing, incubate sections in 12-well-plate without netwell inserts on orbital shaker at 1h Room temperature for 01:00:00 in avidin-biotin-peroxidase solution using the Vectastain ABC Kit, diluted according to manufacturer's recommendation.

14 Prepare 5% DAB working solution fresh before color reaction (for 40 ml solution: 35.2 mL [M] 0.1 Molarity (M) PB, 4 mL DAB solution (5 undetermined), 0.8 mL H_2O_2 (1%)). Wear an FFP2/N95 mask or a respirator for this step and all further steps in which you work directly with DAB. Only carry out this work under a fume hood.

15 Without washing, transfer brain sections back in 12-well-plates with netwell inserts and start DAB colo 6m reaction by incubating sections in DAB working solution for 3- 00:06:00 at Room temperature under fume hood, depending on desired color intensity. Gently shake sections during incubation to avoid sections sticking together.

16 Stop color reaction by washing sections 4x 5 min. in [M] 0.1 Molarity (M) PB on plajorm shaker. 5m Exchange PB solution in between washing steps. Use a fresh 12-well-plate which was not previously in contact with DAB solution. While washing use diluted Na-hypochlorite solution to neutralize DAB solution on used 12-well-plate and inserts. Let plastics neutralize in diluted Nahypochlorite solution Overnight .

16.1 Stop color reaction by washing sections for 00:05:00 in [M] 0.1 Molarity (M) PB on plajorm 5m shaker (1/4).

16.2 Stop color reaction by washing sections for 00:05:00 in [M] 0.1 Molarity (M) PB on plajorm 5m shaker (2/4).

16.3 Stop color reaction by washing sections for 00:05:00 in [M] 0.1 Molarity (M) PB on plajorm 5m shaker (3/4).

16.4 Stop color reaction by washing sections for 00:05:00 in [M] 0.1 Molarity (M) PB on plajorm 5m shaker (4/4).

17 Block sections again for 01:00:00 at Room temperature in NDS solution in 12-well-plates on orbital shaker. 1h

Note

We recommend 4 mL solution per well for good results.

18 For incubation with primary antibody (e.g. anti-tyrosine hydroxylase (TH), Merck Millipore, AB152, 1:1000), transfer sections in a new 12-well-plate but without netwell inserts. This allows better shaking Overnight. solution for incubation with primary antibody should contain NDS solution and the respective primary antibody diluted according to manufacturer recommendation. Incubate sections on orbital shaker with gentle shaking at 4 °C Overnight. 2h

Note

We recommend at least 1 mL solution for each well.

19 On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections 4x 5 min in PB on platform shaker at Room temperature. Change washing solution after each washing step.

19.1 On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections for 00:05:00 in PB on platform shaker at Room temperature. Change washing solution after each washing step (1/4). 5m

19.2 On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections for 00:05:00 in PB on platform shaker at Room temperature. Change washing solution after each washing step (2/4). 5m

19.3 On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections for 00:05:00 in PB on platform shaker at Room temperature. Change washing solution after each washing step (3/4). 5m

19.4 On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections for 00:05:00 in PB on platform shaker at Room temperature. Change washing solution after each washing step (4/4). 5m

20 Next, transfer sections back in 12-well-plate without netwell inserts for incubation with biotinylated secondary antibody (e.g., biotinylated donkey anti-rabbit, Jackson ImmunoResearch, 711-065-152, 1:500) diluted in NDS solution. Incubate for 01:00:00 on orbital shaker at Room temperature. 1h

Note

We recommend at least 1 mL solution for each well.

21 Next, without washing, incubate sections in 12-well-plate without netwell inserts on orbital shaker at Room temperature for 01:00:00 in avidin-biotin-peroxidase solution using the Vectastain ABC Kit, diluted according to manufacturer's recommendation. 1h

22 Prepare Peroxidase Substrate Kit (SG Peroxidase Substrate Kit, Vector Labs, SK-4700) as recommended by the manufacturer. Wear an FFP2/N95 mask or a respirator for this step and all further steps in which you work directly with SK-4700. Only carry out this work under a fume hood.

23 Without washing, transfer brain sections back in 12-well-plates with netwell inserts and start SK-4700 color reaction by incubating sections in prepared peroxidase substrate solution for 3- 00:06:00 at Room temperature under fume hood, depending on desired color intensity. Gently shake sections during incubation to avoid sections sticking together. 6m

24 Stop color reaction by washing sections 4x 5 min. in [M] 0.1 Molarity (M) PB on platform shaker. 5m

Exchange PB solution in between washing steps. Use a fresh 12-well-plate which was not previously in contact with SK-4700 solution. While washing use diluted Na-hypochlorite solution to neutralize SK-4700 solution on used 12-well-plate and inserts. Let plastics neutralize in diluted Na-hypochlorite solution Overnight.

24.1 Stop color reaction by washing sections for 00:05:00 in [M] 0.1 Molarity (M) PB on platform shaker (1/4). 5m

24.2 Stop color reaction by washing sections for 00:05:00 in [M] 0.1 Molarity (M) PB on platform shaker (2/4). 5m

24.3 Stop color reaction by washing sections for 00:05:00 in [M] 0.1 Molarity (M) PB on platform shaker (3/4). 5m

24.4 Stop color reaction by washing sections for 00:05:00 in [M] 0.1 Molarity (M) PB on platform shaker (4/4). 5m

25 After washing, mount sections on microscopy slides using a fine brush.

26 Let sections dry Overnight . 5m

27 Immerse dried slides in a series of ethanol (70%, 96%, 100%) 30 sec. each for stepwise dehydration. Work under a fume hood.

28 Let slides rest for 00:10:00 in 100% xylene solution. Work under a fume hood. 10m

29 Next, quickly apply a small amount of hard-drying mounting medium (e.g., EukiS) sufficient to cover the

sections. Carefully avoid the formation of air bubbles. Gently apply a coverslip over the sections and the mounting medium. Work under a fume hood.

DAB staining – After the procedure:

30 Let slides cure for 2 days under a fume hood.

31 Dispose of waste and excess reagents/solution according to institutional guidelines.

32 Clean tools/working station.

33 Microscopy slides should be stored in a designated container at room temperature until time of observation.

Stereological cell counting – Before the procedure:

34 Turn on microscope and computer according to specific manuals/instructions.

35 Turn on MBF Stereo Investigator software.

Stereological cell counting – Procedure:

36 Load microscopy slides in the designated stage.

37 Use brightfield settings for imaging.

38 Carefully follow all steps of the Stereo Investigator Workflow according to the manual. The workflow is step-by-step and very intuitive.

39 We typically use a 60x oil immersion lens for imaging.

40 After cell counts have been collected export counting data into an excel sheet for further data analysis.

Stereological cell counting – After the procedure:

41 Clean immersion objectives with a lens wipe and the appropriate cleaning solution.

42 Turn off software/microscope/laser according to specific instructions.