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Immunohistochemical staining for GAD67 and TH in FFPE sections. V.2

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We use this protocol and it's working

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Abstract

Immunohistochemistry protocol to identify dopamine and GABA neurons in FFPE midbrain human sections.

Materials

- FFPE tissue blocks
- Microscope slides
- D-Limonene (Hurst Scientific, Histo-5LCTN)
- Ethanol (100%, 95%, 70%)
- Distilled H2O
- Antigen retrieval solution (Invitrogen, 00-4956-58)
- Antigen retrieval cooker (Aptum Biologics Ltd, UK, Retriever 2100)
- Tris-buffered saline (TBS, Sigma-Aldrich, 94158-10TAB)
- Endogenous alkaline phosphatase (AP)
- Hydrogen peroxidase (HRP)
- BLOXALL blocking solution (SP-6000-100)
- Normal horse serum blocking solution (2% horse serum, 1% BSA in 0.01M TBS)
- Antibodies to tyrosine hydroxylase (TH, rabbit polyclonal IgG, AB152 Sigma-Aldrich, RRID:AB_390204)
- Antibodies to GAD67 (mouse monoclonal IgG3, sc-28376 Santa-Cruz Biotechnology, RRID:AB_627650)
- ImmPRESS™-alkaline phosphatase (AP) horse anti-mouse IgG polymer detection kit (MP-5402 Vector Laboratories, RRID:AB_2336535)
- ImmPRESS™-horseradish peroxidase (HRP) horse anti-rabbit IgG polymer detection kit (MP-7401 Vector Laboratories, RRID:AB_2335333)
- SignalStain® Vibrant Red AP Substrate Kit (76713 Cell Signaling)
- ImmPACT® DAB EqV HRP Substrate Kit (SK-4103 Vector Laboratories)
- Cresyl violet (HB5608 Hello Bio)
- Xylene
- Eukitt® quick-hardening mounting medium (03989 Sigma-Aldrich)

Paraffin removal and section rehydration

1 After standard human brain tissue processing (10.17504/protocols.io.8epv55dxdv1b/v1), sections were deparaffinized by incubation in 60°C oven for 1 hour.

2 Sections were submerged in D-Limonene (Hurst Scientific, Histo-5LCTN) for 2 × 7 minutes.

3 Sections were rehydrated in decreasing ethanol concentrations (100% ethanol for 2 × 3 minutes, 95% ethanol for 3 minutes, 70% ethanol for 3 minutes).

4 Sections were placed in distilled H2O for 3 minutes.

Antigen Retrieval

5 Sections were incubated in 1x antigen retrieval solution (HIAR) (Invitrogen, 00-4956-58) in a programmable antigen retrieval cooker (Aptum Biologics Ltd, UK, Retriever 2100).

6 After reaching peak temperature at ~121°C, sections were gradually cooled for 2 hours.

Blocking

7 Sections were washed in tris-buffered saline (TBS, Sigma-Aldrich, 94158-10TAB) for 5 minutes.

8 Sections were washed with TBS with Tween-20 (TBST) (Sigma, P1379) for 5 minutes.

9 After removing liquid from the slides hydrophobic circles were carefully drawn with A-PAP pen (Daido Sangyo) without touching the tissue sections.

10 Sections were blocked with BLOXALL blocking solution (SP-6000-100) for 30 minutes to quench endogenous alkaline phosphatase (AP) and hydrogen peroxidase (HRP).

11 Sections were blocked again with in-house made normal horse serum blocking solution (2% horse serum, 1% BSA in 0.01M TBS) for 1 hour at room temperature.

Primary antibody incubation

12 A primary antibody cocktail of tyrosine hydroxylase (TH, rabbit polyclonal IgG, AB152 Sigma-Aldrich, RRID:AB_390204, diluted 1:200) and GAD67 (mouse monoclonal IgG3, sc-28376 Santa-Cruz Biotechnology, RRID:AB_627650, diluted 1:50) were made in the same normal horse serum blocking solution.

13 Sections were incubated with this primary cocktail for 4 nights at 4°C.

Washing

14 Sections were washed in TBST 3× 10 minutes.

Secondary antibody incubation

15 Secondary antibody cocktail was made by adding equal volumes of ImmPRESS™-alkaline phosphatase (AP) horse anti-mouse IgG polymer detection kit (MP-5402 Vector Laboratories, RRID:AB_2336535) and ImmPRESS™-horseradish peroxidase (HRP) horse anti-rabbit IgG polymer detection kit (MP-7401 Vector Laboratories, RRID:AB_2335333).

16 Sections were incubated in the secondary antibody solution for 90 minutes at room temperature.

Secondary AP antibody development

17 Sections were washed 3× 5mins in TBST.

18 Sections were placed in distilled H2O for 5 minutes and the SignalStain® Vibrant Red AP Substrate Kit (76713 Cell Signaling) solution was prepared during this time for AP secondary visualisation.

19 2 drops of reagent A and 2 drops of reagent B were added in 5ml of diluent of the SignalStain® Vibrant Red AP Substrate Kit (76713 Cell Signaling).

20 The SignalStain® Vibrant Red AP solution was added to sections for approximately 15 minutes.

Secondary HRP antibody development

21 Stained sections were placed in distilled H2O to stop the reaction.

22 Sections were placed under running tap water for a few minutes and placed back into distilled H2O.

23 The ImmPACT® DAB EqV HRP Substrate Kit (SK-4103 Vector Laboratories) was prepared during this time.

24 Equal volumes of ImmPACT® DAB EqV reagent 1 and ImmPACT® DAB EqV reagent 2 were added and mixed.

25 The ImmPACT® DAB EqV solution was added to sections for approximately 1-2 minutes.

26 Sections were placed in distilled H2O to stop the reaction. Sections were then placed under running water, and after a few minutes returned to distilled H2O.

Cresyl violet counterstaining

27 Sections were placed in 0.5% cresyl violet (HB5608 Hello Bio) for 10 minutes.

Dehydration

28 Sections were dehydrated by incubation in increasing ethanol concentrations.

29 Sections were submerged for 3 minutes in 70%, 3 minutes in 90% and 2× 3 minutes in 100% ethanol.

Clearing and mounting

30 Dehydrated sections were cleared by submerging in xylene 2× 3 minutes.

31 Sections were mounted with Eukitt® quick-hardening mounting medium (03989 Sigma-Aldrich).