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Version 2

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).