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Immunofluorescence protocol for FFPE human post-mortem brain sections to detect alpha-synuclein and tau pathology

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We use this protocol and it's working

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Abstract

This protocol details the method for preparing and staining human formalin-fixed, paraffin embedded post mortem brain tissue to detect alpha-synuclein and tau pathology using immunofluorescence.

Materials

Materials

- HistoChoice® Clearing agent - *Sigma* #H2779
- Series 2 adhesive microscope slides - *Trajan*, #472042491
- Sodium borohydride - *Sigma* #71320, Lot #STBJ4218
- Human FC blocker - *BD Pharmingen* Human BD Fc Block, clone Fc1, #564220
- TrueBlack® Background Suppressor- *Biotium* #23012A
- TrueBlack® Lipofuscin Autofluorescence Quencher - *Cell Signaling Technology* #23007)
- Biotium's CoverGrip™ Coverslip Sealant - *Biotium* #23005)
- EverBrite™ Mounting Medium - *Biotium* #23001
- 100%, 95%, 70% ethanol
- 70% formic acid
- 0.1 M sodium citrate buffer pH 6 (house made)
- 0.1 M PBS (house made)

Antibodies

	A	B	C
	SNCA (Gt)	R&D, AF1338	1:200
	Ubq (Rb)	Abcam, ab7780	1:100
	P62 (Ms IgG1)	BD #610833	1:100

Midbrain and cortical primary antibody details

	A	B	C
	Dn anti Gt-AF800	Thermo, #A32930	1:250
	Dn anti Rb-AF647	Thermo, #A31573	1:200
	Dn anti Ms-AF488	Thermo, #A21202	1:200
	Hoescht-405	Sigma, #B2261	1:500

Midbrain and cortical secondary antibody details

	A	B	C	D
	Midbrain conjugated antibody			
	AT8(Ms, IgG1)	Thermo, #MN1020	Zenon ™ Ms IgG1 Labeling Kits (AF594)	Thermo, #Z25007
	TH (Rb)	Merck, AB152	Zenon ™ Rb Labeling Kits (AF532)	Thermo, #Z25003

Zenon ™ midbrain conjugated antibody details

	A	B	C	D
	Cortical conjugated antibody			
	AT8(Ms, IgG1)	Thermo, #MN1020	Zenon ™ Ms IgG1 Labeling Kits (AF594)	Thermo, #Z25007
	HuD (Rb)	Abcam ab302514	Zenon ™ Rb Labeling Kits (AF532)	Thermo, #Z25003

Zenon ™ cortical conjugated antibody details

Hardware

- Leica HM325 rotary microtome
- Aptum Bio Retriever 2100, Aptum Biologics Ltd, UK

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet) for each of the raw materials used.

Before start

Formalin-Fixed Paraffin-Embedded (FFPE) sections of the human midbrain and cortical regions were cut at 6um with a Leica HM325 rotary microtome and mounted on Series 2 adhesive microscope slides.

Day I - Tissue Prep

1

1. Place slides with FFPE human brain tissues on a slide rack and bake in a 60 °C oven for 01:00:00

1h

De-paraffinize

2

2. Continue using the slide rack, submerge slides in the following solution to de-paraffinize:
   a. HistoChoice: 2 x 00:07:00
   b. 100% ethanol: 2 x 00:03:00
   c. 95% ethanol: 00:03:00
   d. 70 % ethanol: 00:03:00

19m

3. Submerge in MiliQ/ultrapure water for 00:03:00 .

4. Tap gently sideways on flat surface covered with paper towels to remove excess water.

Antigen retrieval

3

5. Submerge slides in 70% Formic Acid for 00:20:00 .

2h 31m

6. Wash in MiliQ/ultrapure water for 2x 00:05:00 .

7. Remove slides from the rack and submerge slides directly into [M] 0.01 Molarity (M) sodium citrate buffer (pH 6) and incubate sections in a programmable antigen retrieval cooker

8. Let the pressure cooker reach its peak of 121 °C before gradually cooling for a total of 02:00:00 .

9. Wash with MiliQ/ultrapure water for 00:01:00 , followed by washing with 1 X PBS for 00:05:00 .

Quenching aldehyde group

4 10. Prepare 0.1% Sodium borohydride ($NaBH_4$) in 1xPBS for 00:30:00 of quenching. The solution must always remain chilled in ice. 35m

11. Wash in 1x PBS for 2x 00:05:00

Human Fc blocker treatment

5 12. Add Human FC blocker in 1X PBS with a ratio of 1:50, incubate slides with Human FC blocker at Room temperature for 00:05:00 5m

Background Suppressor system treatment

6 Component A (Background Suppressor) may become turbid or form a gel at 4 °C this does not affect performance. Warm the buffer to room temperature or 37 °C until clear (light blue) and completely
liquid before use. 1h 30m

13. Add enough TrueBlack® Background Suppressor to completely cover sample.
Room temperature 00:30:00

14. Remove the Background Suppressor and add IF Blocking Buffer (home-made).
Room temperature 01:00:00

Primary antibody incubation

7 5. Prepare 150 ul per sample of primary antibody solution consisting of selected primary antibody diluted in home-made blocking buffer. 2d

	A	B
	SNCA (Gt)	1:200
	Ubq (Rb)	1:100
	P62 (Ms IgG1)	1:100

Primary antibody table and dilutions

16. Prepare humidified chamber and remove Blocking buffer by tapping on paper towel.

17. Add primary antibody diluted in Blocking buffer and incubate for 48:00:00 in 4 °C

Day 4 - Secondary antibodies

8

1. Wash slides in PBST 3x 00:10:00 . Slides must be protected from light from this step. 2h 20m

2. Prepare 150 ul per sample of secondary antibody solution consisting of selected secondary antibody diluted appropriately in home-made blocking buffer.

	A	B
	Dn anti Gt-AF800	1:250
	Dn anti Rb-AF647	1:200
	Dn anti Ms-AF488	1:200
	Hoescht-405	1:500

Midbrain and cortical secondary antibodies and dilutions

3. Incubate sample in secondary antibody in Room temperature for 02:00:00

4. Wash slides in PBST 3x 00:05:00 , followed by 1x PBS 1x 00:05:00

Conjugated antibodies

9

5. Prepare conjugated antibodies using Zenon™ labeling kits within 00:30:00 before applying the antibodies. 2h 35m

	A	B
	AT8(Ms, IgG1)	Zenon‰ Ms IgG1 Labeling Kits (AF594)

	A	B
	TH (Rb)	Zenon‰ Rb Labeling Kits (AF532)

Midbrain conjugated antibodies

	A	B
	AT8(Ms, IgG1)	Zenon‰ Ms IgG1 Labeling Kits (AF594)
	HuD (Rb)	Zenon‰ Rb Labeling Kits (AF532)

Cortical conjugated antibodies

6. Incubate sample in conjugated antibodies in Room temperature for 02:00:00

7. Wash slides in 1x PBS 3x 00:05:00

Post-treatment with TrueBlack® Lipofuscin Autofluorescence Quencher

10 8. Prepare 150 ul per sample of 1xTrueBlack® Lipofuscin Autofluorescence Quencher in 70% ethanol. 5m

9. Tap slides to paper towels to remove excess washing solution and then place in humidified chamber.

10. Add diluted TrueBlack® solution and incubate for 30-60 seconds.

11. Rinse slides in 1xPBS 3x 00:05:00

Mounting and cover slipping

11 12. Remove as much moisture without drying tissue using Kimwipes

13. Retrieve Mounting with EverBrite™ Mounting Medium to warm at Room temperature before dispensing approximately 10 - 20 ml on slides.

14. Place coverslip gently on the slides and wait for the Mounting Medium to dry before applying the perimeter of the coverslip with Biotium's CoverGrip™ Coverslip Sealant.

15. Store in a protected slide boxaway from light at 4 °C