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Multicolor fluorescence in situ hybridization and analysis

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We use this protocol and it's working



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Abstract

Antipsychotics are known to induce the expression of several immediate early genes (IEGs) via D2R antagonism, which then leads to changes in critical signaling pathways in iSPNs. To define the impact of LRRK2 kinase activity on the expression pattern of *IEGs* in the iSPNs, we used multicolored single-molecule fluorescence in situ hybridization.



Fresh Frozen Tissue Prep

- 1 Place a piece of aluminum foil into a small Styrofoam container containing dry ice.
- 2 Mice were euthanized with carbon dioxide, decapitated, their brains rapidly removed, and placed immediately on foil on dry ice
- 3 Pour ~50 mL of 2-methylbutane into the container. Keep brain in container for 2 minutes. 2m
- 4 When the brain is completely frozen, carefully remove it from the foil with forceps and place it in the OCT mold.
- 5 Pour ~50 mL of 2-methylbutane into the container. Keep brain in container for 3 minutes. 3m
- 6 Cover the top of the mold with tin foil and store the brain in -80°C freezer. Place the brain into -20°C freezer 20 mins before cryosection.
- 7 collect the region of interest with 20µm slices and mount by turning the slide upside down and pressing against the slice on the platform.
- 8 Place slides in slide box and store slides in -80°C freezer.

Fix and Dehydrate slides

- 9 Prepare 200 mL of 4% PFA and chilled to 4°C:
- 10 Place slides in the staining tank containing chilled 4% PFA in fridge (4°C) for 30mins 30m
- 11 Wash slides in 1X PBS by moving the rack gently up and down for 2 minutes and repeat with fresh 1X PBS (room temperature) 2m
- 12 Place slide rack in new staining tank containing 200 mL of 50% EtOH for 5 minutes at room temperature. Gently agitate slides by moving rack up and down 2-4 times 5m



13 Place slide rack in new staining tank containing 200 mL of 70% EtOH for 5 minutes at room temperature. Gently agitate slides by moving rack up and down 2-4 times.

5m

13.1 Turn on the HbyEZ oven and prepare the humidity contral tray by applying ~40 mL of MilliQ water to paper towels.

14 Place slide rack in new staining tank containing 200 mL of 100% EtOH for 5 minutes at room temperature. Gently agitate slides by moving rack up and down 2-4 times.

5m

14.1 Allow Protease IV to come to RT (normally stored at 4°C)

15 Place slide rack in new staining tank containing 200 mL of 100% EtOH for 5 minutes at room temperature. Gently agitate slides by moving rack up and down 2-4 times.

5m

15.1 Warm probes for 10-15 minutes at 40°C

15m

16 Practice making hydrophobic barriers using ImmEdge pen

17 Remove probes from oven, allow to come to RT

Pretreat samples with Hydrogen Peroxide and Protease

48m

18 Load the slides into the EZ-Batch holder, and add 5 drops of RNAscope Hydrogen Peroxide to each section (completely cover the sections). incubate at RT for 10 minutes.

10m

19 Place the EZ-Batch holder into the wash tray containing 200ml distilled water.

2m

20 Repeat the wash step with fresh water.

2m

21 Add 5 drops of RNAscope Protease IV to each section (completely cover the sections). incubate at RT for 30 minutes.

30m

22 Place the EZ-Batch holder into the wash tray containing 200ml PBS



2m

23 Repeat the wash step with fresh PBS

2m

24 Allow slides to dry. Collect excess PBS with Kimwipe

Multicolor fluorescence in situ hybridization

2h

25 Preparing probes by ratio 50:1:1 for channels 1, 2, and 3

26 Add mixed probes to each section. Apply ~100 μ L of probe combination to each section (completely cover the sections) and incubate at 40°C for 2hrs.

2h

26.1 30 minutes before the end of incubation: remove amps from the fridge and allow amps to come to RT

30m

27 Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes

2m

28 Repeat with fresh 1X Wash Buffer in fresh wash buffer

2m

29 Remove excess liquid

30 Apply ~3 drops of Amp 1 to each section (completely cover the sections) and incubate at 40°C for 30 minutes.

30m

31 Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes

2m

32 Repeat with fresh 1X Wash Buffer in fresh wash buffer

2m

33 Remove excess liquid



34	Apply ~3 drops of Amp 2 to each section(completely cover the sections) and incubate at 40°C for 30 minutes	30m
35	Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes	2m
36	Repeat with fresh 1X Wash Buffer in fresh wash buffer	2m
37	Remove excess liquid	
38	Apply ~3 drops of Amp 3 to each section(completely cover the sections) and incubate at 40°C for 15 minutes	15m
39	Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes	2m
40	Repeat with fresh 1X Wash Buffer in fresh wash buffer	2m
41	Remove excess liquid	
42	Apply ~3 drops of HRP-C1 to each section(completely cover the sections) and incubate at 40°C for 15 minutes	15m
43	Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes	2m
44	Repeat with fresh 1X Wash Buffer in fresh wash buffer	2m
45	Remove excess liquid	
46	Apply 150µl of Opal 520 to each section(completely cover the sections) and incubate at 40°C for 30 minutes	30m
47	Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes	



		2m
48	Repeat with fresh 1X Wash Buffer in fresh wash buffer	2m
49	Remove excess liquid	
50	Apply ~3 drops of HRP blocker to each section(completely cover the sections) and incubate at 40°C for 15 minutes	15m
51	Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes	2m
52	Repeat with fresh 1X Wash Buffer in fresh wash buffer	2m
53	Remove excess liquid	
54	Apply ~3 drops of HRP-C2 to each section(completely cover the sections) and incubate at 40°C for 15 minutes	15m
55	Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes	2m
56	Repeat with fresh 1X Wash Buffer in fresh wash buffer	2m
57	Remove excess liquid	
58	Apply 150µl of Opal 570 to each section(completely cover the sections) and incubate at 40°C for 30 minutes	30m
59	Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes	2m
60	Repeat with fresh 1X Wash Buffer in fresh wash buffer	2m



- | | | |
|----|--|-----|
| 61 | Remove excess liquid | |
| 62 | Apply ~3 drops of HRP blocker to each section(completely cover the sections) and incubate at 40°C for 15 minutes | 15m |
| 63 | Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes | 2m |
| 64 | Repeat with fresh 1X Wash Buffer in fresh wash buffer | 2m |
| 65 | Remove excess liquid | |
| 66 | Apply ~3 drops of HRP-C3 to each section(completely cover the sections) and incubate at 40°C for 15 minutes | 15m |
| 67 | Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes | 2m |
| 68 | Repeat with fresh 1X Wash Buffer in fresh wash buffer | 2m |
| 69 | Remove excess liquid | |
| 70 | Apply 150µl of Opal 650 to each section(completely cover the sections) and incubate at 40°C for 30 minutes | 30m |
| 71 | Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes | 2m |
| 72 | Repeat with fresh 1X Wash Buffer in fresh wash buffer | 2m |
| 73 | Remove excess liquid | |



- 74 Apply ~3 drops of HRP blocker to each section(completely cover the sections) and incubate at 40°C for 15 minutes 15m
- 75 Flick off excess liquid and wash slides in 1X Wash Buffer (200ml) for 2 minutes 2m
- 76 Repeat with fresh 1X Wash Buffer in fresh wash buffer 2m
- 77 Remove excess liquid
- 78 Apply ~3 drops of DAPI to each section(completely cover the sections) and incubate at RT for 1 minutes 1m
- 79 Flick off excess liquid and mount the slides with Prolong Diamond Antifade mountant.
- 80 Allow slides to dry in protected and covered area overnight. store slides in the dark at 4°C

Confocal images capture

- 81 Sections were imaged with the Nikon A1 laser microscope system using a 60X 0.75NA objective, to capture 3 z-stack images across 4 channels:

Image analysis with Cell Profiler

- 82 DAPI channel was enhanced with Enhance Or Suppress Features module, with feature size set at pixel size 30.
- 83 Identify Primary Objects module was implemented on identified nuclei, object pixel unit was set for Min 5, Max 50, with threshold strategy set as Global and thresholding method set as Otsu
- 84 *Drd1* or *Drd2* channel was enhanced with Enhance Or Suppress Features module, with feature size set at pixel size 10
- 85 *Nr4a1* or *Arc* channel was enhanced with Enhance Or Suppress Features module, with feature size set at pixel size 5.



- 86 Identify Primary Objects module was implemented on identified *Drd1* or *Drd2* signal puncta, object pixel unit was set for Min 3, Max30, threshold strategy for Global and thresholding method set as Manual
- 87 Nur77 or Arc object pixel unit was set for Min 1, Max10, threshold strategy for Global and thresholding method set as Manual
- 88 Masked D1 positive nuclei and Masked D2 positive nuclei were generated with RelateObjects
- 89 The number of objects were measured with the Measure Object Intensity module.