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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-methylbutance 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-methylbutance 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 theplatform.

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 µL 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.