Jan 22, 2024

v2 RNAscope

Cell reports

DOI
dx.doi.org/10.17504/protocols.io.14egn3odml5d/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.14egn3odml5d/v1

External link: https://doi.org/10.1016/j.celrep.2024.114483

Protocol Citation: Thomas Hnasko 2024. v2 RNAscope. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.14egn3odml5d/v1

Manuscript citation:
Ryan MB, Girasole AE, Flores AJ, Twedell EL, McGregor MM, Brakaj R, Paletzki RF, Hnasko TS, Gerfen CR, Nelson AB (2024) Excessive firing of dyskinesia-associated striatal direct pathway neurons is gated by dopamine and excitatory synaptic input. Cell reports 43(8). doi: 10.1016/j.celrep.2024.114483

Protocol status: Working
We use this protocol and it's working

Created: January 19, 2024

Last Modified: January 22, 2024

Protocol Integer ID: 93827

Keywords: v2 rnascope, frozen mouse brain tissue, v2 protocol, similar to the v2 protocol, advanced cell diagnostic, mouse brain, sample preparation, steps for sample preparation

Abstract

This is a protocol for performing RNAscope® in situ hybridization analysis on fixed-frozen mouse brain tissue using the RNAscope® Multiplex Fluorescent v2 kit (Advanced Cell Diagnostics; ACD). It is similar to the v2 protocol provided by ACD; places where it deviates from ACD's protocol have been indicated. This protocol provides steps for staining sections through dorsal striatum of mouse brain, but can also be applied to other brain areas. It also has steps for sample preparation, including cryosectioning.

Materials

RNAscope® H2O2 and Protease Reagents (REF 322381)
- RNAscope® Hydrogen Peroxide (REF 322335)
- RNAscope® Protease III (REF 322337)

RNAscope® Target Retrieval Reagents (REF 322000)

RNAscope® Wash Buffer Reagents (REF 310091)

RNAscope® Multiplex Fluorescent Detection Reagents (REF 323110)
- RNAscope® Multiplex FL v2 AMP 1 (REF 323101)
- RNAscope® Multiplex FL v2 AMP 2 (REF 323102)
- RNAscope® Multiplex FL v2 AMP 3 (REF 323103)
- RNAscope® Multiplex FL v2 HRP C1 (REF 323104)
- RNAscope® Multiplex FL v2 HRP C2 (REF 323105)
- RNAscope® Multiplex FL v2 HRP C3 (REF 323106)
- RNAscope® Multiplex FL v2 HRP Blocker (REF 323107)
- RNAscope® Multiplex FL v2 DAPI (REF 323108)

ProLong™ Gold Antifade Mountant (REF P36930)

Troubleshooting

Cryosectioning, sample preparation, and storage

1 Collect sections spaced 20 µm apart between Bregma = +1.0–0.0 in RNase-free PBS.

2 Immediately mount sections onto Superfrost® plus microscopy slides. Mount sections spaced 120 microns apart 4 per slide (Note that this is different from the ACD protocol, which recommends mounting only 1 section per slide). This amounts to a total of 8 sections between Bregma = +1.0–0.0 mounted onto 2 slides.

3 Allow slides to dry for **60 minutes** at **-20°C**, then, immediately store at **−80°C** in a slide box placed inside of a Ziploc bag with drierite desiccant until the day of the RNAscope procedure.

Pretreatment of fixed-frozen tissue samples

4 **Baking.** Bake slides for **60 min** at **60°C** in the HybEZ™ oven. Note that this is longer than the time recommended in the ACD v2 protocol.

5 **Post-fixation.** Post-fix the slides by immersing them in 4% PFA in 1X PBS for **30 min** at **4°C.** Note that this post-fixation time is longer than the time recommended in the ACD v2 protocol.

6 **Serial dehydration.** Slides are then rinsed once in ddH2O and then serially dehydrated using an ethanol series in the following order: **50%**, **70%**, two times **100%** ethanol (**5 min** each).

7 **Hydrogen peroxide treatment.** Take the dehydrated slides and add ~5–8 drops of RNAscope® Hydrogen Peroxide to cover the sections on each slide. Treat sections with RNAscope® Hydrogen Peroxide for **10 min** at **RT.**

7.1 Rinse slides with ddH2O. Repeat with fresh ddH2O water.

8 **Manual target retrieval.** Perform manual target retrieval using RNAscope® Target Retrieval Reagents as described in **Appendix B** of the ACD v2 protocol.

8.1 Prepare 700 mL of fresh RNAscope 1X Target Retrieval Reagents by adding 630 mL ddH2O to 1 bottle (70 mL) 10X Target Retrieval Reagents in a clean 1 L beaker. Mix well with stir bar on stir plate.

8.2 Place the beaker containing RNAscope 1X Target Retrieval Reagents on the hot plate. Cover the beaker with foil, and turn the hot plate on high until 1X Target Retrieval Reagents reaches **98–102°C.**

8.3 With a pair of forceps, slowly submerge a Tissue-Tek Slide Rack containing the slides into RNAscope 1X Target Retrieval Reagents solution. Cover the beaker with foil and boil the slides for **5 min.**

8.4 Wash slides 3–5 times by moving the Tissue-Tek Slide Rack up and down in the distilled water. Then Transfer the slides to **100%** ethanol for **3 min.**

9 Slides are then air dried for 5 min and a hydrophobic barrier is drawn around sections using an ImmEdge™ Pen (H-4000; Vector Laboratories, Inc.).

10 Incubate slides with RNAscope® Protease III reagent for **30 min** at **40°C** in the HybEZ™ oven.

10.1 Wash slides 2x with ddH2O.

Probe hybridization

11 **Prepare probe mixture.** C1 probes are 1X; C2 and C3 probes come as 50X solutions and therefore must be diluted 1:50 with the C1 probe.

12 **Add probe mixture to slides.** Refer to **Appendix C.** Reagent Volume Guidelines to determine the volume of probe mixture to add to each slide.

13 Insert into HybEZ™ oven for **2 hrs** at **40°C.**

14 (Optional stopping point). You can store the slides in 5X SSC **overnight** at **RT.**

Amplification

15 **Hybridize AMP 1.** Add RNAscope® Multiplex FL v2 AMP 1 to each slide.

15.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

15.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

16 **Hybridize AMP 2.** Add RNAscope® Multiplex FL v2 AMP 2 to each slide.

16.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

16.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

17 **Hybridize AMP 3.** Add RNAscope® Multiplex FL v2 AMP 3 to each slide Incubate in the HybEZ™ oven for **15 min** at **40°C.**

17.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

17.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

Develop HRP signals

18 **Develop HRP-C1 signal.** Add RNAscope® Multiplex FL v2 HRP-C1 to slides.

18.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

18.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

19 Add 150-200 µL of diluted fluorophore to label the C1 probe.

19.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

19.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

20 Add RNAscope® Multiplex FL v2 HRP Blocker to slides.

20.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

20.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

21 **Develop HRP-C2 signal.** Add RNAscope® Multiplex FL v2 HRP-C2 to slides.

21.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

21.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

22 Add 150-200 µL of diluted fluorophore to label the C2 probe.

22.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

22.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

23 Add RNAscope® Multiplex FL v2 HRP Blocker to slides.

23.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

23.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

24 **Develop HRP-C3 signal.** Add RNAscope® Multiplex FL v2 HRP-C3 to slides.

24.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

24.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

25 Add 150-200 µL of diluted fluorophore to label the C3 probe.

25.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

25.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

26 Add RNAscope® Multiplex FL v2 HRP Blocker to slides.

26.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

26.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

Counterstaining and mounting

27 Add enough DAPI to cover each section and incubate for **30 sec** at **RT.**

28 Remove DAPI and place 1-2 drops of ProLong™ Gold Antifade Mountant on each slide

29 Carefully place a 24 mm x 50 mm glass coverslip over the tissue section.

30 Dry slides 30 min to overnight in the dark and store slides in the dark at **2-8°C.**