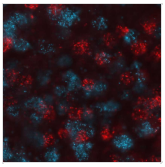

Feb 17, 2021

Fluorescence In Situ Hybridization (FISH - RNAscope) in mouse brain sections

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bq2imyce

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SUBMIT TO PLOS ONE

ABSTRACT

Here we describe a protocol to perform fluorescence in situ hybridization (FISH) in thin sections from frozen murine brain tissue. The protocol can be divided in several days or can be completed in two successive days: one day for extracting and slicing the brain and a second one for the FISH.

DOI

dx.doi.org/10.17504/protocols.io.bq2imyce

PROTOCOL CITATION

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KEYWORDS

FISH, RNAscope, Fluorescent In Situ Hybridization, Mouse, Brain Sections, Neuroscience

IMAGE ATTRIBUTION

Oriol Pavón Arocas

CREATED

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Feb 17, 2021

PROTOCOL INTEGER ID

45866

GUIDELINES

Ensure any animal work is carried out following the legislation and protocols in place at your home institution.

MATERIALS TEXT

Materials for brain extraction and slicing:

- Embedding Moulds, Peel-A-Way Truncated - T12 (Cat. No. 18986-1, Generon, £177).
- Embedding Moulds, Peel-A-Way, Square - S22 (Cat. No. 18646A-1, Generon, £177).
- Box for slides - Cork-lined Slidebox (Cat. No. HS15989C, Heathrow Scientific, or also available at SLS as MIC3566 or Fisher as 11334135, £4).

RNAscope® Fluorescent Multiplex Assay to detect up to 3 targets simultaneously requires the following items:

- RNAscope® Fluorescent Multiplex Reagent Kit (Cat. No. 320850, Bio Techne) (includes Pretreatment Kit, detection kit, and wash buffer, but can also be bought separately as below).
- RNAscope® Multiplex Fluorescent Detection Kit, 20 tests (Cat. No. 320851, Bio Techne, £1,140).
- RNAscope® Protease IV Reagents, 20 tests (Cat. No. 322336, Bio Techne, £124).
- RNAscope® Wash Buffer (Cat. No. 310091, Bio Techne, £124).
- RNAscope® Target Probes (Catalog or Made-to-Order C1 to C3 Probes, £650) - you should be able to find any gene on any channel on https://acdbio.com/catalog-probes
- RNAscope® Probe Diluent (Cat. No. 300041, £96).
- Species specific RNAscope® 3-plex Positive Control Probes (Cat. No 320881, £108).
- RNAscope® 3-plex negative control probes (Cat. No. 320871, £108).
- Immedge® Hydrophobic Barrier Pen (Cat. No. 310018, £55).
- HybEZ™ Hybridization System.
- Fluorescent microscope with detection capability to detect DAPI, AF488, Atto550, and Atto647N.

SAFETY WARNINGS

This protocol includes work with paraformaldehyde (PFA), make sure to follow all safety protocols in place at your institution and wear appropriate personal protective equipment (PPE)

DISCLAIMER:

This work was funded by a Wellcome Senior Research Fellowship (214352/Z/18/Z) and a Sainsbury Wellcome Centre Core Grant from the Gatsby Charitable Foundation and Wellcome (090843/F/09/Z) (T.B.), a UCL Wellcome 4-year PhD Programme in Neuroscience Fellowship (203729/Z/16/Z) (O.P.A.), a A*STAR National Science Scholarship (PhD; Y.L.T.), and the SWC PhD Programme (Y.L.T.).

BEFORE STARTING

Make sure you have all the materials in advance - some probes and reagents can take a few days or weeks to arrive.

Day 0 | Brain extraction 15m

1 [1. Preparation ~ 15 min]

You can extract and freeze several brains on the same day and then store them at -80°C until slicing. You can do the brain extraction either in the PFA perfusion room (with tools from there) or in the slice electrophysiology area where you prepare acute brain slices (with clean tools). NEVER bring tools contaminated with PFA to the electrophysiology area.

1.1 You will need:

- A small bucket or ice pan with dry ice.
- A bigger tray with wet ice.
- A plastic bag for the carcass.

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- Tools for decapitation and brain extraction: big scissors for decapitation, medium scissors to cut through skin, small scissors to cut skull, forceps to separate skull, spatula to remove the brain, brush to help position the brain.
- One mould for each brain (this is where you will freeze the brains in OCT to make a little cube you will later slice on the cryostat).
- 2-methylbutane (from the chemicals cabinet in the Histology area of Level 1) - It speeds up freezing in the dry ice.
- A ~250ml glass bottle with 1x PBS **per brain**.
- Filter paper.
- Glassware: a ~150mL beaker where you will put PBS (to clean the blood from the brain), a glass petri dish where you will put PBS (to clean the blood from the brain).

2 [2. Decapitation under terminal anaesthesia and Brain extraction ~ 15 min per brain]

You can do this following two paths: after terminal anaesthesia via IP injection of euthatal, or after terminal isoflurane anaesthesia (as we do for acute brain slices).

2.1 *[Common path]*

- Label each mould with the animal ID, name of experimenter, and date beforehand. Label in a consistent manner, so you know where the top, down, right, and left of the brain are.
- Fill the moulds with OCT, very carefully to avoid bubbles. Keep them flat on ice on the tray.
- Put a bit of dry ice in the ice pan. Don't put too much as you will need to make a pocket for the mould to stand flat in the floor of the ice pan.
- Fill beakers with cold PBS and also leave on ice on the tray.

2.2 *[Bifurcation - PATH A] - in Fume Hood of Perfusion Room*

Overdose by IP injection of euthatal - Decapitation - Brain extraction

- First, get and prepare the drugs you will need from the surgery prep room: we use euthatal diluted in saline. Always follow the most recent animal welfare guidelines of your home institution. Mix in a bijou vial and load on an insulin needle syringe.
- Wear appropriate PPE for the perfusion room (hair net, lab coat, gloves, mask, have fume hood on).
- Once everything is ready, proceed to scruff and inject the mouse. Wait until the anaesthesia has its effect and check reflexes to confirm the animal is fully anaesthetised.
- Move to the hood: put the tray in the hood. Double check your tools are ready (specific for perfusion room, don't mix with the clean slicing ones).
- Decapitate under the hood and extract the brain as in for slicing. Briefly, drop the head in a beaker with PBS to clean the blood, cut through the skull, extract the brain, and drop the brain in the small petri dish with PBS to wash the blood away.

2.3 *[Bifurcation - PATH B] - in Slice Electrophysiology Area*

Terminal anaesthesia with isoflurane - Decapitation - Brain extraction

- Wearing appropriate PPE, proceed to anaesthetise the mouse with isoflurane, decapitate, and drop the head in PBS.
- Move to bench to extract the brain. Drop brain in the small petri dish with PBS to wash the blood away.

2.4 *[Back to common path]*

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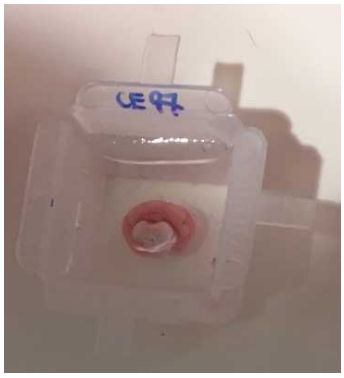
- Carefully lift the brain with the spatula from the PBS and transfer it to a piece of filter paper.
- With the help of a brush and the spatula, dry as much PBS from the brain as possible - this is important to ensure that the OCT sticks to the brain directly. **[!] Careful** - the brain will be soft and will tend to stick to the filter paper when dry, do not over dry it as you will risk damaging the tissue.
- Once dry, transfer the brain to the mould and immerse it in OCT.
- Be consistent when placing the brain in the mould: we always put the olfactory bulb down, with the dorsal part of the brain facing upwards (with respect to the written edge). In the moulds we use, there are two tiny ridges on one of the walls - we make that the dorsal wall, the one where cortex points to.



Example of a brain immersed in OCT

- Use the small forceps to very carefully arrange the brain as flat and as centered as possible (it is very important to displace as little OCT as possible - and avoid creating bubbles!).
- Finally, add a bit of 2-methylbutane on the dry ice (this will speed up freezing) and make a bit of space for the mould. Transfer the mould to the dry ice, making sure the mould sits flat on the bottom of the ice pan and is surrounded by dry ice.
- You can now proceed to extract the next brain - by the time you are ready to freeze it, the previous mould will already be frozen and you will be able to move it to a corner of the ice pan.
- Once you are done and all the brains are frozen in their moulds with OCT, you can put them in a labeled plastic zip bag and store them at the -80°C freezer until slicing.

2.5

[!] PAUSE point - once the brain is frozen and kept at -80°C you can slice it the next day, week, or month.

Day 1 | Cryostat Slicing 3h

3 [3. Cryostat Slicing ~ time depending on number of brains]

[!] Make sure you book the cryostat in advance if shared with others. Some of the following steps will vary depending on the cryostat model in your institute.

3.1 [Preparation - tools]

First, collect your brains from the -80°C freezer and leave the moulds at the cryostat to equilibrate to -20°C for one hour.

In the meantime, get what you will need:

- SuperFrost slides - Usually have 3 series (labelled A1, B1, C1) so that you add one section to each to

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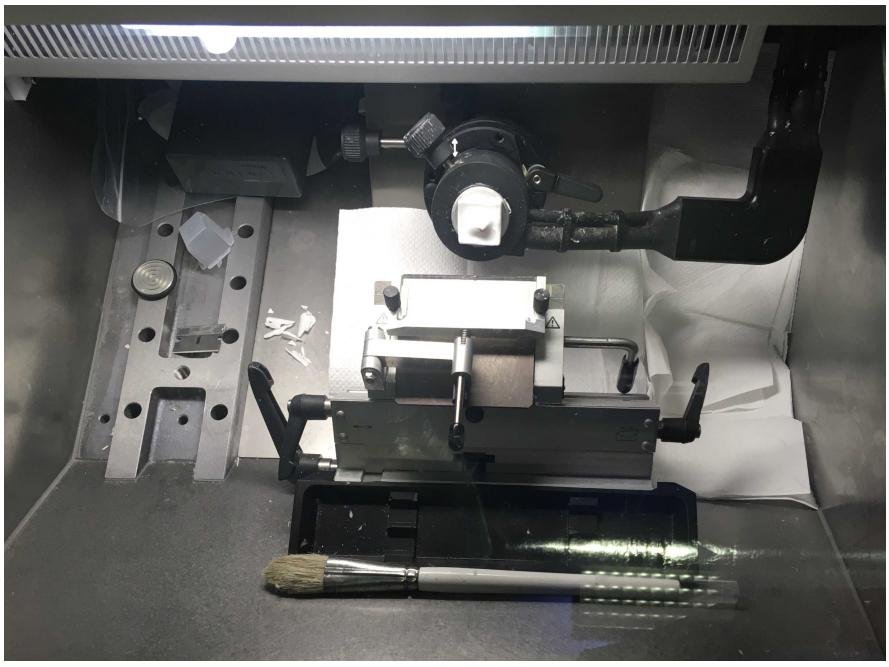
end up with every third section in any given slide.
- Write key information with pencil (Animal, date, etc.).
- Cryostat blade (reusable, usually kept in a falcon tube).
- OCT from histology area.
- A slide box (for storage at -80°C).
- 2 small brushes and 1 big brush (from the cryostat top).

3.2 [Preparation - cryostat]

- Set the cryostat temperature at -16°C for CT and -17°C for OT - this may very depending on your model and room temperature.
- You may have to play with the temperature during cutting: if sections break, then the temperature is too low and you can try to bring it up by opening the sliding cover a bit more.
- When you are done leave at -14°C to save energy.
- If screen/controls don't work or say Locked, press the Key/Lock button for several seconds until it unlocks.

3.3 [Mount brain for slicing]

- Place the blade on the holder.
- Given that we reuse them, try to offset it a bit so you don't always blunt the same region.
- Put some tissue paper beneath the blade to make it easier to clean up.
- Get the round holder and add some OCT in the center.
- Break the molds, take the frozen brain out of the mold and press the cube of frozen OCT with the brain against the blob in the round holder: the OCT will spread out and act as glue.
- Put it inside the cryostat and leave to freeze.
- Once frozen, mount the holder with the brain on the arm of the cryostat.
- Unlock it and adjust so that it is flat ("zero it").



Brain mounted on the cryostat's arm, ready to slice.

3.4 [Trim until you reach the area of interest]

- Set the cutting thickness to 30-40 µm and the trimming one to 100 µm.
- On trimming mode, remove the "lock" from the wheel on the side and start cutting and keep

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adjusting so the sections come out flat and even.

3.5 [Slice]

- When you reach your area of interest, use a razor blade to cut out as much OCT as possible so that when you mount them on the slide the OCT doesn't take extra space. It is a trade-off and you need to be careful - if you remove too much of it the sections will not hold.
- **[IMP!]** For FISH it is critical that the tissue touches the glass directly, if it ends up on top of a bit of OCT, you will loose the sections during the washes of the protocol.
- Slice carefully, don't go fast.
- Collect the sections making sure they don't wrinkle/fold on themselves.
- Once on the slide, keep outside so the OCT melts and then dries out.
- When you are done collecting sections or fill up one slide, leave it out so the OCT dries out, then put in the box and keep in the chamber at -20°C until the end.

3.6

[!] PAUSE point - At the end of the slicing, once all the slides are dry and in the storage box, store the box at -80°C until you are ready to do the FISH protocol.

Day 2 | FISH: fixation and dehydration 1h 30m

4 [4. Fixation and Dehydration ~ 1h30]

In preparation for this step, defrost 4% PFA (you need ~50 ml to fill one glass slide holder) to 4 degrees or prepare fresh 4% PFA from 16% PFA vials (dilute 12.5 ml and bring to 50 ml with PBS).

In addition, think of which and how many slides you will need for each experiment, and always take an extra one for the positive and negative controls.

This step includes work with paraformaldehyde (PFA), make sure to follow all safety protocols in place at your institution.

4.1 [Fixation ~ 1 hour]

- Take the selected slides out of the -80°C freezer and immediately place into PFA (put the slides into the glass slide holder and then pour PFA along the sides).
- **[IMP!]** always pour the solution (be it PFA, PBS or wash buffer) along the sides that the sections are not facing, or you may accidentally remove them from the slide.
- Fix at 4 degrees for 1 hour.
- Remove the PFA (dispose appropriately) and perform **2x quick washes in PBS** by adding PBS, moving slides up and down 3-5 times (washes should be quick), and repeating after replacing PBS with fresh one.

4.2 [Dehydration ~ 20 min]

- In three different falcon tubes, freshly prepare 50 ml of 50% ethanol, 50 ml of 70% ethanol, and 50 ml of 100% ethanol by diluting in pure ddH20.
- Place slides in 50% ethanol for 5 min at Room Temperature (RT) - i.e. pour out the PBS from the glass slide holder and pour in the 50ml of ethanol.
- Repeat with 70% ethanol, 5 min, RT.
- Repeat with 100% ethanol, 5 min, RT.

4.3

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[OPTIONAL - Store at -20°C for up to 1 day]

This step is optional - you can ignore this step and proceed to subsequent steps directly.

- Remove the ethanol from the holder and add 50 ml of fresh 100% ethanol to cover the slides.
- Wrap the glass slide holder with cling film (or parafilm) to prevent ethanol from evaporating and store at -20°C for up to 1 day (it is best to start subsequent steps by the next day).

Day 3 | FISH: staining and mounting 4h 40m

5 [5. FISH protocol ~ 4h40]

Before starting this part, make sure you have planned the combination of probes and channels you will use (which will determine the colors to image - see note at the end).

5.1 [Bake slides at 40 degrees ~ 15-20 min]

- **Preheat** the RNAscope oven to **40°C.**
- Remove slides from slide glass holder and **let ethanol evaporate**. Wipe the bottom side with a clean wipe.
- While drying, **cut parafilm to exactly the size** of each of your sample areas. The parafilm will prevent evaporation of the reagents and ensure they evenly cover all the brain sections.
- Place slides directly on tray (without rack).
- **Bake slides at 40 degrees** for **15-20 min** (the longer you bake them the better the sections will stick to the slide).
- In the meantime, **warm water bath to 40°C** and bring **Protease IV** vial to **RT**.
- **[!]** Make sure you take Protease IV (and not III).

5.2 [Draw hydrophobic barrier ~ 10 min, depending on number of slides]

- Use the **Immedge pen** - lines should be blue when drawn (do not use the PPAP pen, which draws green lines).
- **[!]** Draw on *multiple layers* - it is very important to ensure reagents stay on the slide.
- Draw on three of the sides (leave one blank to remove excess liquid).

5.3 [Apply Protease IV at RT and prepare probe mixtures ~ 30 min]

- Place the slides on the black slide tray from Sigma (this is only so that you can see the sections - you can use the baking tray or tissue paper if you don't have it).
- Add **~4-5 drops/slide of Protease IV** (ensure there is enough and always err on the excess side - you don't want the slides to dry out).
- Place **parafilm on**, ensuring there are no bubbles (remember you previously cut the parafilm to exactly the size of your sample area). **[! VERY IMPORTANT STEP !]**

While waiting during the 30 min, prepare the reagents for the first hybridization step:

- Dilute 20 ml of 50X Wash Buffer by adding ddH2O up to 1 L.
- **Warm up probes** (entire bottle) for **10 min at 40°C** in the water bath, then let cool to RT.
- Briefly **spin** probe bottles.
- **Prepare probe mixture** in 2 ml eppies - 50:1:1 ratio of C1:C2:C3 probes (you will need to plan the

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combination in advance - see note at the end).

- Use **150µl : 3µl : 3µl** per slide until you are very confident with the protocol, then reduce to 100:2:2 per slide.
- **Warm** the HybEZ **slide rack** in the oven to **40°C**.

After 30 min, lift the parafilm and shake off excess reagent (there should be excess reagent to shake off!! Else insufficient reagent was applied or barrier was not done well).

- Dab the parafilm dry - make sure you keep the same piece for the same slide, especially if you use different probe/control combinations.
- Perform **2x quick washes in PBS** by moving slides up and down 3-5 times (washes should be quick).
- Dab excess liquid off (blot onto tissue from side and back of slide).
- Place some wet tissue paper on the bottom of the HybEZ slide rack to keep it humid for the next steps.

5.4 [Hybridize probes at 40 degrees ~ 2 hours]

- **Hybridize probes at 40°C** for **2 hours** - Add ~150 µl/slide and place parafilm without bubbles.
- First place the slides on the HybEZ slide rack. Then add the probe mixtures to the corresponding slides and cover with parafilm without bubbles. Only then secure the slides on the rack. Finally, put the tray in the oven and leave to incubate for 2h. Repeat this procedure on the following steps.
- Once the time is up, lift parafilm, dab excess liquid off, and shake off excess reagent from the slide (there should be excess reagent to shake off!! Else insufficient reagent was applied, barrier was not done well, or chamber was not humid enough).
- Perform **2x 2 min washes in Wash Buffer at RT** (normal washes from now on, not the quick ones). After the second wash is done, shake off excess liquid (blot onto tissue from side and back of slide) and dry the bottom of the slides before proceeding to the next step.

5.5 [Hybridize Amp1-FL at 40 degrees ~ 30 min]

- **Hybridize Amp1-FL at 40°C** for **30 min** - Add ~4-5 drops/slide and place parafilm without bubbles.
- Once the time is up, lift parafilm, dab excess liquid off, and shake off excess reagent from the slide (there should be excess reagent to shake off!! Else insufficient reagent was applied, barrier was not done well, or chamber was not humid enough).
- Perform **2x 2 min washes in Wash Buffer at RT** (normal washes, not the quick ones). After the second wash is done, shake off excess liquid (blot onto tissue from side and back of slide) and dry the bottom of the slides before proceeding to the next step.

5.6 [Hybridize Amp2-FL at 40 degrees ~ 15min]

- **Hybridize Amp2-FL at 40°C** for **15 min** - Add ~4-5 drops/slide and place parafilm without bubbles.
- Once the time is up, lift parafilm, dab excess liquid off, and shake off excess reagent from the slide (there should be excess reagent to shake off!! Else insufficient reagent was applied, barrier was not done well, or chamber was not humid enough).
- Perform **2x 2 min washes in Wash Buffer at RT** (normal washes, not the quick ones). After the second wash is done, shake off excess liquid (blot onto tissue from side and back of slide) and dry the bottom of the slides before proceeding to the next step.

5.7 [Hybridize Amp3-FL at 40 degrees ~ 30 min]

- **Hybridize Amp3-FL at 40°C** for **30 min** - Add ~4-5 drops/slide and place parafilm without bubbles.
- Once the time is up, lift parafilm, dab excess liquid off, and shake off excess reagent from the

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slide (there should be excess reagent to shake off!! Else insufficient reagent was applied, barrier was not done well, or chamber was not humid enough).
- Perform **2x 2 min washes in Wash Buffer at RT** (normal washes, not the quick ones). After the second wash is done, shake off excess liquid (blot onto tissue from side and back of slide) and dry the bottom of the slides before proceeding to the next step.

5.8 [Hybridize Amp4-FL at 40 degrees ~ 15 min]

- **Hybridize Amp4-FL at 40°C** for **15 min** - Add ~4-5 drops/slide of the **correct [!]** Amp4 (either A, B, or C) depending on your chosen color combination.
- Once the time is up, lift parafilm, dab excess liquid off, and shake off excess reagent from the slide (there should be excess reagent to shake off!! Else insufficient reagent was applied, barrier was not done well, or chamber was not humid enough).
- Perform **2x 2 min washes in Wash Buffer at RT** (normal washes, not the quick ones). After the second wash is done, shake off excess liquid (blot onto tissue from side and back of slide) and dry the bottom of the slides before proceeding to the next step.

5.9 [DAPI at RT ~ 3 min]

- Apply DAPI at RT for 3 min.
- Or, alternatively, use mounting media with DAPI and skip this step.

5.10 [Mount slides ~ 15min, depending on number of slides]

- Dry excess DAPI off (without washing!).

Mount slides:
- **[!] Let them dry completely. Otherwise possible artefacts can appear when imaging.**
- Once dry, add mounting media (without DAPI), place coverslip, and apply nail polish.
- Keep in a box at 4°C until imaging.

Choosing your probe combination 15m

6 [Choosing your channels appropriately]

[IMP] Make sure you plan the combination of probes and channels you will use in advance (which will determine the colors to image and the Amp4 you will need to use).

*Source: https://acdbio.com/rnascope%C2%AE-fluorescent-multiplex-assay.

6.1 Channel selection for multiplexing:

Multiplex Fluorescence Color Module	C1 probe	C2 probe	C3 probe
Amp-4A			
Amp-4B			
Amp-4C			

Guidelines for Fluorophore Assignment:

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Microscopy Channel	Pros	Cons	Recommendations
Fluorescein - Green For Alexa Fluor 488	Visible with naked eye	Least distinct from most tissue autofluorescence	High Expressor
Cyanine 3 - Orange For Atto 550	Visible with naked eye	None	Low Expressor or Expression Level Unknown
Cyanine 5 - Far Red For Atto 647N	Easily differentiated from autofluorescence	Not visible to the naked eye	Low Expressor

7 [Positive and Negative Controls]

*Source: *https://acdbio.com/control-slides-and-control-probes-rnascope*.

Control Probes for Manual Assays - RNAscope Multiplex Fluorescent Reagent Kit:

- Species-specific positive probes and negative control probes are needed to run with human or mouse control slides for quality control check of the technique and with your sample for the Sample or RNA quality control check. ACD provides positive control probes that target species-specific housekeeping genes and negative control probes that target the bacterial DapB gene.
- Careful selection of the right positive control is recommended. The expected expression level of your target RNA is the key criteria to consider as your positive control probe should have a similar expression profile.
- When working with RNAscope Multiplex Fluorescent Reagent Kit, you will have ultimate flexibility and can use with one, two or three target RNAs.
- When using this RNAscope Multiplex Fluorescent Reagent Kit with three target RNAs, you will work in channels 1, 2 and 3 so you will also need control probes for channel 1, channel 2 and channel 3. These species-specific positive control probes and negative control probes for channel 1 when processed with your sample will enable you to assess your sample/RNA quality and serve as control for your channel 1 target probe etc. ACD offers a 3-plex Negative Control Probe which contains probes for channel 1, 2 and 3. We also offer 3-plex RNAscope® Positive Control Probes for Human, Mouse and Rat where POLR2A probe is available in channel 1 and PPIB probe in channel 2 and UBC probe in channel 3.

7.1 Positive control:

- Control probes are already mixed in a bottle labelled "Positive Control Probe 3-plex", ref 320881, website link: https://acdbio.com/search/site/%252A320881%252A/cms/probes.
- RNAscope Mouse positive control probe for RNAscope Multiplex Fluorescent Assay: Polr2a (C1 channel) and PPIB (C2 channel), UBC (C3 channel).
- Note: In terms of relative expression levels, UBC is the highest, PPIBis considered a moderate-high, and POLR2A is moderate-to-low expressor target.

The positive control will allow you to identify whether the lack of signal for a probe is due to the target not being expressed or due to a problem in the reagent kit you used: if the empty channel is both in your "test" sections and your "positive control" sections, then it is likely that one (or more) of the reagents or steps did not work.

7.2 Negative control:

- Control probes are already mixed in a bottle labelled "3-plex Negative Control Probe", ref 320871, website link: https://acdbio.com/search/site/%252A320871%252A/cms/probes.
- RNAscope Negative control probeDapB(of Bacillus subtilis strain) for RNAscope Multiplex Fluorescent Assay.

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