**Pre-week experiment checklist:**

* Is there enough of each probe that I will need?
* Are all the probes channel compatible?
* Do I have my sections in one place?
* Is there enough opal dye & enough TSA diluent to dilute the dyes?
* Are there enough of the ancillary reagents (eg: AMP1-4)?
* Did I book the FISH bench for two back-to-back days?

**Day 1 checklist:**

**\*** at minimum 200mL needed; can set up on cart night before experiment

* RNAse free 1X PBS\*
* 4% PFA RNAse free\*
* 50% Ethanol\*
* 70% Ethanol\*
* 100% Ethanol (at minimum 400mL)
* Target Retrieval\*
* RNAscope H2O2
* RNAscope protease
* Probes that you will be using
* Hydrophobic pen
* 5X SSC (100mL)

**Day 2 checklist:**

* Prepare Opal dye mixes (1:1500 dilution)
* Equilibrate reagents to room temp (AMP1, AMP2, AMP3, HRP-C1, HRP-C2, HRP-C3, HRP-C4, HRP blockers).

***FISH Day 1: dehydrating sections, target retrieval, protease, probe hybridization.***

|  |  |  |  |
| --- | --- | --- | --- |
| **Steps** | **Reagents needed** | **Time** | **Completed?** |
| 1. Turn on the oven – set to 60°C bake option
 |  | ~15 mins – while oven warms up |  |
| 1. Set up PBS, PFA, Ethanol, Target retrieval, and water wash buckets
 |  |  |
| 1. Wash the slides in PBS for 5 mins
 | PBS | 5 mins |  |
| 1. Post-fix the slides by immersing them in 4% PFA (at room temp, don’t want to shock tissue)
 | 4% PFA | 15 mins |  |
| 1. 50% Ethanol wash – 5 mins
 | 50% Ethanol | 5 mins |  |
| 1. 70% Ethanol wash – 5 mins
 | 70% Ethanol | 5 mins |  |
| 1. 100% Ethanol wash #1 – 5 mins
 | 100% Ethanol | 5 mins |  |
| 1. 100% Ethanol wash #2 – 5 mins
 | 100% Ethanol | 5 mins |  |
| 1. Air dry slides - meanwhile switch oven to 40°C and set up humidity tray & turn on steamer with Target retrieval and distilled water bins inside.
 |  | 10 mins |  |
| 1. Add Hydrogen Peroxide to each section (ensure that each section is fully covered). Incubate for 10 mins at RT.
 | H2O2 | 10 mins |  |
| 1. Remove Hydrogen Peroxide, immediately submerge in distilled water (wash #1).
 | dH2O | 5 mins |  |
| 1. Second water wash (wash #2).
 | dH2O | 5 mins |  |
| 1. Check that target retrieval is at 99°C. Place slides in target retrieval for 5 mins.
 | Target retrieval | 5 mins |  |
| 1. Remove slides from target retrieval, wash in hot water 2-3 times.
 | dH2O (in steamer) | 15 seconds |  |
| 1. Transfer the slides to 100% alcohol for 3 mins.
 | 100% Ethanol | 3 mins |  |
| 1. Dry at RT for 15 mins.
 |  | 15 mins |  |
| 1. (Make sure sections are fully dry before this step) Draw a hydrophobic barrier around the section. Make sure it doesn’t touch the tissue (you will get autofluorescence), but try and make the circle as small as possible. Make sure the barrier is completely dry before proceeding to the next step (you can make your probe mix while it dries). **NOTE: This is an optional stopping point. Can leave slides to dry O/N at RT.**
 | Hydrophobic pen (from RNAscope) | 20 mins |  |
| 1. Load slides onto EZ-batch slide holder – add protease **III** to each section – make sure it is fully covered. Place in oven with humidity tray at 40°C for 30 mins.
 | Protease III | 30 mins |  |
| 1. Remove from oven, wash tray in distilled water (wash #1).
 | dH2O | 2 mins |  |
| 1. Repeat wash in distilled water with fresh water (wash #2).
 | dH2O | 2 mins |  |
| 1. Add 50mL of probe mix (if you have more than 1 probe mix, make sure you have (A) pre-determined which section gets which mix, (B) PAY ATTENTION! Make sure each section is covered. Place tray in oven (40°C, with humidity tray) - incubate for 2 hours. (Make SSC during this time and reagents for next day FISH.)
 | Probe mixes | 2 hours |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Place slides in 5X SSC – cover with parafilm – seal with tape, write DO NOT TOUCH. Leave O/N.
 | 5X SSC | 10 mins |  |
| **Estimated total time** | **5.5 hours** |

***FISH Day 2: hybridizing AMPs & developing signals.***

|  |  |  |  |
| --- | --- | --- | --- |
| **Steps** | **Reagents needed** | **Time** | **Completed?** |
| 1. Warm oven to 40°C and set up humidity tray. Wash slides in wash buffer (wash #1).
 | Wash buffer | 30 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Incubate with Amp 1 for **30 mins.**
 | **Amp 1** | 35 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Incubate with Amp 2 for **30 mins**
 | **Amp 2** | 35 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Incubate with Amp 3 for **15 mins**
 | **Amp 3** | 20 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Add HRP-C1 – **15 mins** at 40°C.
 | **HRP-C1** | 20 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Add 50mL of **Opal 520** to each section. 30 mins at 40°C.
 | **Opal 520** | 35 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. HRP blocker - **15 mins** at 40°C.
 | **HRP blocker** | 20 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Add HRP-C2 – **15 mins** at 40°C.
 | **HRP-C2** | 20 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Add 50mL of **Opal 570** to each section. 30 mins at 40°C.
 | **Opal 570** | 35 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. HRP blocker - **15 mins** at 40°C.
 | **HRP blocker** | 20 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Add HRP-C3 – **15 mins** at 40°C.
 | **HRP-C3** | 20 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Add 50mL of **Opal 620** to each section. 30 mins at 40°C.
 | **Opal 620** | 35 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. HRP blocker - **15 mins** at 40°C.
 | **HRP blocker** | 20 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Add HRP-C4 – **15 mins** at 40°C.
 | **HRP-C4** | 20 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Add 50mL of **Opal 690** to each section. 30 mins at 40°C.
 | **Opal 690** | 35 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. HRP blocker - **15 mins** at 40°C.
 | **HRP blocker** | 20 mins |  |
| 1. Wash slides in wash buffer (wash #1).
 | Wash buffer | 2 mins |  |
| 1. Wash slides in wash buffer (wash #2).
 | Wash buffer | 2 mins |  |
| 1. Remove excess liquid, let sections dry off enough before adding mounting media with DAPI. (If not using Vectashield, add DAPI as per RNAscope protocol, adds ~20 mins to protocol.) Mount sections, make sure you have recorded which probes were on which sections.
 | Mounting media with DAPI or DAPI and mounting media | 30 mins |  |
| **Estimated total time** | **8 hours** |

**Probe mixes used:**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Probe mix** | **Total volume** | **Channel** | **Probe** | **Amount** | **Added?** |
| **Probe mix A****(MOL subtype mix)** | **50mL \* 20 sections = 1mL****(I have 100uL left) 🡪 900uL** | **C1** | **Hapln2** | **800mL** | **Yes** |
| **C2** | **Wdfc18** | **16mL** | **Yes** |
| **C3** | **Ptgds** | **16mL** | **Yes** |
| **C4** | **C030029H02Rik** | **16mL** | **Yes** |
| **Probe mix B (VGLUT-KO expression mix)** | **50mL \* 8 sections = 400mL** | **C1** | **Gad2** | **400mL** | **Yes** |
| **C2** | **Olig2** | **8mL** | **Yes** |
| **C3** | **Zdhhc9** | **8mL** | **Yes** |
| **C4** | **Slc17a7** | **8mL** | **Yes** |

**Sections used + probe mixes assignation:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Genotype | Animal # | Anterior | Middle | Posterior |
| Zdhhc9-KO | 1067347 | **Mix A** | **Mix A** | **Mix B** |
| Zdhhc9-KO | 1066909 | **Mix A** | **Mix A** | **Mix B** |
| Zdhhc9-KO | 1067345 | **Mix A** | **Mix A** | **Mix B** |
| Control | 1067348 | **Mix A** | **Mix A** | **Mix B** |
| Control | 1067346 | **Mix A** | **Mix A** | **Mix B** |
| ? | 1109140 | **Mix A** | **Mix A** | **Mix A** |
| ? | 1108432 | **Mix A** | **Mix A** | **Mix A** |
| ? | 1108431 | **Mix A** | **Mix A** | **Mix A** |
| ? | 1109139 | **Mix A** | **Mix A** | **Mix A** |
| Zdhhc9-VGLUT-KO | 881271 | **Mix B** | NA | **Mix B** |

**Notes:**

* Animal #1067347 had mixing of Middle section Mix A and Posterior section Mix B probe – be skeptical when imaging, might have to void both animals.