**Experiment overview:**

Infect L2-5 of the posterior and anterior cortex with Syn-GFP (medially) and Syn-mCherry (laterally). 2 weeks following infection, perfuse brains, vibratome (thick sections m and m). For corpus callosum crossing and cortical coverage analysis, utilize m thick sections. **Objective:** Determine axon crossing abnormalities (dorsal vs ventrally, % coverage in CC) as well as axon projection deficits in cortices.

**Reagent identifiers:**

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| --- | --- | --- | --- | --- | --- | --- | --- |
| **1°/2°** | **Antibody** | **Species** | **Company** | **Ref #** | **Lot #** | **Dilution** | **Storage** |
| 1° | GFP | Chicken | Invitrogen | A10262 |  | 1:500 | 4° DELI (box next to rocker) |
| 1° | mCherry | Rabbit | AbCam | Ab167453 | GR339637-1 | 1:500 | 4° fridge Ab box B |
| 1° | MBP | Rat | Novus Biological | NB600717 |  | 1:100 | -20° Ab freezer 1: Zdhhc9 box (MBP/Opalin/NF200) |
| 2° | chicken | goat | Thermo Fisher | A11039 | 1937504 | 1:500 | 4° fridge |
| 2° | rabbit | goat | Thermo Fisher | A11011 | 2500544 | 1:500 | 4° fridge |
| 2° | rat | goat | Thermo Fisher | A21247 | 219156 | 1:500 | 4° fridge |
| Stain | DAPI | ----- | Invitrogen | D1306 | 2680173 | 1:10000 | 4° DELI (vial covered in aluminum foil on rack next to left door) |

**Pre-start checklist:**

* Is there enough antibody aliquots for all antibodies and DAPI?)
* PBS-TX (check needed % TX, GFP-chicken Ab requires 0.5% TX) [~1L]
* Brains sectioned? Reverse engineer date from perfusion to sectioning to determine earliest date you may do immuno.
* Have you booked imaging time for after your immuno?

**General notes:**

* When suctioning between washes, use a glass pipette that has a polished (blunt) tip. Be very careful during washes that you don’t damage the tissue with the tip or suction the section up. Especially important with thin sections (< 40m thick)
* Can streamline process by making sure your reagents for the next step are prepared, often have time to do this during washes (check notes in step descriptions)
* Prior to mounting, you want to let your sections dry a bit on slide, make sure you are consistent across experiments. I typically dry ~10 mins (sections should be opaque by end). Make sure to avoid bubbles, ESPECIALLY ON REGIONS OF INTEREST. Check slide at end of mounting.

**Recommended slide naming:**

|  |  |
| --- | --- |
| Day, Month, Year  Animal #  Antibody A -   Antibody B -   Antibody C -   DAPI  Slide # Initials | +  + |

**Slide mounting tips:**

* Make sure to use superfrost + slides
* Write label in pencil (always)
* \*\*VERY IMPORTANT\*\*: Do not place coverslip OR brain section too close to edge (++ edge) – this will cause serious issues when imaging (you will not be able to focus on your section).

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| **Steps | DAY 1** | **Estimated duration** | **Completed?** |
| 1. Screen and select sections with confirmed fluorescence (wet mount) | 2-4 hours (ideally do day before) |  |
| 1. 3 x 5 min washes in PBS (thaw NGS aliquots during this step) | 20 mins |  |
| 1. Block sections in PBS with triton-X (0.5%) in 5% NGS – room temperature on rocker for 2 hours (prep antibody mix during this step) | 2 hours |  |
| 4. Incubate sections for 48 hours at 4° – total incubation volume per well = 1000ul. Make sure to cover wells with parafilm to minimize antibody evaporation. Antibodies to add to the blocking buffer include:  i. GFP anti-body (2ul in 1ml)  ii. mCherry anti-body (2ul in 1ml)  iii. MBP anti-body (10ul in 1ml) | 48 hours |  |
| **END OF DAY 1** – 6 hours if selecting sections same day | 3 hours if not | |  |

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| **Steps | DAY 2** | **Estimated duration** | **Completed?** |
| 1. Wash 3x5 min in PBS-TX (0.5%) (Prep 2°antibody mix during this step) | 20 mins |  |
| 1. Incubate 2° antibody for 2 hours in PBS-TX (0.5%) at room temperature on rocker (covered to protect from light exposure) 2. 488: goat-anti chicken – 1:500 3. 568: goat-anti rabbit – 1:500 4. 647: goat-anti rat – 1:500 | 2 hours |  |
| 1. 3x5 min wash in PBS-TX (0.05%) (Prep DAPI mix during this step) | 20 mins |  |
| 1. Counterstain with DAPI 1:10,000 in PBS-TX (0.5%) for 10 mins (covered, RT, on rocker) | 10 mins |  |
| 1. 3 x 5 min wash in PBS | 20 mins |  |
| 1. Mount and coverslip with Pro-long gold (150L min for thicker than 100m sections). Leave coverslips horizontally in coverslip box (properly labelled) overnight at room temperature (important for the curing of Pro-long). Switch to 4° for long term storage day after. | ~30 mins depending on how many sections |  |
| **END OF DAY 2** – ~ 3.5 hours | |  |