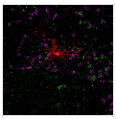

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HCR of patched/recorded Cerebellar Molecular Layer Interneurons

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1 **Imaging and analysis**

2 Neurons were filled with 100 µM Alexa-594 via patch pipette to visualize their morphology using 2-photon imaging. After completion of the electrophysiological recordings the patch electrode was retracted and the cell resealed. We used a custom 2-photon laser-scanning microscope with a 40x, 0.8 numerical aperture (NA) objective (Olympus Optical, Tokyo, Japan) and a pulsed 2-photon laser (Chameleon or MIRA 900, Coherent, Santa Clara, CA;). Excitation of Alexa 594 was achieved with 800 nm excitation. Z-stacks of each MLI including L-DIC images were acquired at the end of each experiment and locations of each cell within the slice were recorded. 2-photon images were further processed in ImageJ.

3 **Tissue fixation of acute slices**

4 After recording and imaging, cerebellar slices (200 - 300 um thick) were transferred to a well-plate and submerged in 2-4% PFA in PBS (pH=7.4) and incubated overnight at 4 °C. Slices were then washed in PBS (3x5min) and then kept in 70% Ethanol in RNAse-free water until HCR was performed. HCR was performed within 3-5 days without apparent loss of signal.

5 **Hybridization Chain Reaction Day 1**
-> beginning from after EtOH wash step above, slices are currently in EtOH.
-> some samples may be still attached to a circular cover slip or bottom of well-plate, gently nudge off the cover slip with the metal spatula.

6 The slices contain patched cells, backfilled, imaged and cataloged as to thier positions in the cerebellum. Following HCR you will have to find those cells so be really gentle with the slices. Use only a 1ml pipette with the tip cut open diagonally with a razor blade so that it has a 3.5-4.0 mm diameter opening. Never press on the slices. When moving the slices from well to well, swirl around the dish or pipette up and down nearby so that the slice floats to the surface, then lift out the slice within the enlarged pipette tip. Never press the slice against the side of the well plate or squash it to lift it out. Expel slice gently into new location (well) or if placeing on slice expel with extra volume and then remove excess liquid from around slice with a fine (200ul) pipette. Never pipette directly onto the slice, always around it. Keep everything covered in tin foil or under a box during processing. Save the slices not used by storing them in their plates at 4^{o}C.

7 **Permeabilize:**

8 Make ~30mLs 8% SDS in 1x PBS

When you're making the 8% SDS, the PBS might cause a white precipitate to form. If this happens, vortex the solution until the white ppt. resoluabilizes. The SDS solution should be clear.

9 Get a fresh well plate, fill up 4 wells with the SDS solution

10 Use the cut-open pipette tip to gently move the slices from EtOH into the SDS solution

11 Incubate at RT for two hours in the dark with agitation.

12 **Wash:** instead of foil, find a box that will fit over the well plate for quicker exchanges.

13 Prepare 100mLs 2X SSC in RNase-free water

14 Aspirate out the SDS solution, taking care not to rough-up the slice with your pipette.

15 Fill up the wells with slices with 2X SSC, repeat for a total of three quick washes to rinse out SDS.

16 After the third quick wash, aspirate, fill up well with more 2X SSC.

17 Cover in foil and wash for one hour at RT with agitation.

18 Asprate 2X SSC, fill with fresh 2X SSC, cover in foil, wash for one hour at RT with agitation.

19 Aspirate 2X SSC, fill with fresh 2X SSC, cover in foil, wash for one hour at RT with agitation.

20 Now you are ready for HCR. Instead of HCR on cryosection slides, here we'll perform it in the small well plates (ones with ~700uL volume) as "free floating" HCR. All of the steps will basically be the same, except you'll do the HCR in the wells, similar to free floating immuno, and you'll use the larger incubators for the 37°C steps.

21 **Free floating HCR**

22 **Hybridization Day 1:**

23 Pre-warm the 30% probe hybridization buffer to 37°C

24 Add 700uL of warmed hyb buffer to four wells in a fresh well plate

25 Transfer the slices to this new plate, relabel.

26 Incubate for 5 mins at 37°C

27 Make probe mix for samples: 700uL each,1:100 RNase inhibitor included. Each probe is added at 1.4 ul to 700ul of pre-warmed hyb buffer. Each matching hairpin set will be added later at the amplification steps, so make sure you record/coordinate the probed samples with thier matching hairpins.

28 Aspirate initail 700ul of probe hyb, buffer, and add the 700ul of probe mixes to your samples. .

29 Fill up 4 of the empty wells halfway with water to humidify the plate.

30 Wrap edges tightly with parafilm to keep in moisture.

31 Wrap with foil.

32 Incubate overnight at 37°C.

33 **Washing Day 2:**

34 Washes: instead of foil, find a box that will fit over the well plate for quicker exchange.

35 Pre-warm the 30% probe wash buffer to 37°C

36 Aspirate and re-fill the well with warm probe wash buffer.

37 Wash for 15 mins at 37°C. Repeat this aspirate and 15 min. 37°C wash step two more times for a total of 3 washes.

38 Aspirate and re-fill the well with 2X SSC. Incubate for 5 min RT

39 **Amplification:**

Aspirate and re-fill the well with amplification buffer. Incubate for 30 min RT.

40 During the 30 minute pre-amp, prepare the hairpin(s) amplification cocktail using the snap cool protocol as usual: Add 14ul of each hairpin(s) to 700ul of amplification buffer for each sample. Make certain to coordinate the proper hairpin set with the appropriately probed samples.

41 Aspirate pre-amp buffer and add the 700ul of amplification cocktail to the free-floating slices.

42 Incubate in amplification cocktail for a minimum of 4 hours at RT. Then place at 4°C overnight.

43 **Post Amplification Washes:**

44 Aspirate amplification cocktail and fill wells with 2x SSC. Wash for 10 minutes at RT. Repeat for a total of three washes. Washed samples are stable in 2x SSC for a few days, but we like to perform microscopy as soon as possible to maximize signal.

45 **Microscopy:**

46 If your cerebellar slices are of known orientation (i.e. you know which side was the top of the 200-300 um acute slice during recording and filling, and are thus sure that the patch-filled cells can be located at the coverslip interface) you can mount samples on a glass slide. If you do not know the orientation of the slice/filled cells then you will have to mount between two coverslips. It is certainly more convenient to know the orientation because finding the filled cells is thus accelerated by 1/2 (don't have to search both sides of slice).

47 Using the cut pipette tip transfer method, gently transfer the accute slice either face up to to a glass micro-slide or randomly onto a glass 24x50mm no.1 coverslip. Remove the excess 2x SSC with a 200ul pipette and place a few drops of ProLong glass antifade mountant (invitrogen P36981) on the slice before it dries out at all. Either coverslip the slide or create a coverslip sandwich making sure to not incorporate bubbles into the mount. This will be stable if stored in the dark for a number of days.

48 Images are collected on an Andor CSU-X spinning disk confocal system on an Nikon Eclipse Ti microscope equipped with an Andor iKon-M camera. The images were acquired by an oil immersion objective at 60x. The Alexa 594 channel plus associated HCR probe/hairpin channels were projected through a 10-20 micron thick z-series so that an unambiguous determination of the association between the patch-filled cell and it's HCR gene expression could be made.