Protocol of Passive-CLARITY Immunohistochemistry

- A major factor for successful immunostaining is the complete removal of lipids during clearing
- High antibody concentrations (1:50~1:100) are usually required for effective immunostaining to ensure deep penetration into tissue.
- Whole mouse brain staining will need significantly longer incubation times and antibodies may still not be able to fully penetrate to the core of the sample
- Prevent from light if necessary

Procedure

1. 2 mm thickness of hydrogel brain slices cleared with 50ml 10% SDS/borate buffer for one week at 39℃ with shaking. Refresh 10% SDS/borate buffer every 2 days. (**Note:** The SDS/borate buffer needs to be refreshed once the pH goes below 7.5 or clearing efficiency will drop.)

   * **SDS/borate buffer** (10% SDS and 200mM boric acid in dH_2O, pH 8.5)
2. Wash with 50ml of 0.2% PBST 2 times, >12h/time, at 37 ℃ with shaking.
3. Wash with 50ml of PBS 2 times, >12h/time, at 37 ℃ with shaking. (**Note:** washing step is quite important to remove the remaining SDS or white precipitate will form after blocking buffer treatment!)
4. Keep the sample brains in 5ml of blocking buffer on an orbital shaker or rocker at 4℃ for 3 days. Refresh blocking buffer every day.

   ***Blocking buffer** (10% normal goat serum, 0.2% Triton-X 100, and 0.05% sodium azide in PBS)
5. Incubate the specimen with 3ml primary antibody (beginning with 1:50 dilutions) on an orbital shaker or rocker at 4℃ for one week. Refresh primary antibody every 2~3 days. (**Note:** antibody incubations at 4℃ can reduce non-specific

binding.) As with any antibody staining procedure, it is important to systematically optimize staining conditions (detergent, temperature, concentrations and so on) for the particular antibody used.

***Ab dilution buffer** (1% normal goat serum, 0.2% Triton-X 100, and 0.05% sodium azide in PBS)

6. Wash off the primary antibody with 50ml washing buffer at RT with shaking for 2 times, 6~12 h/time. Then keep the specimen in fresh 50ml washing buffer at 4℃ for 1 day with shaking. (**Note:** washing step is quite important for immunostaining!)

   ***Washing buffer** (3% NaCl and 0.2% Triton-X 100 in PBS): Store this nonhazardous buffer at 4℃.

7. Incubate the specimen with 3ml of desired secondary antibody (1:50~1:100) on an orbital shaker or rocker at 4℃ for 4 days. Refresh primary antibody every 2 days. (**Note:** a nuclear labeling dye, such as DAPI (1 μg/ml), can also be added at this step.)
8. Wash off the secondary antibody with 50ml washing buffer at RT with shaking for 2 times, 6~12 h/time. Then keep the specimen in fresh 50ml washing buffer at 4℃ for 1 day with shaking.
9. Post-fix the specimen in 5ml 4% paraformaldehyde solution on an orbital shaker or rocker for 2 hr at RT.
10. Wash with PBS at RT with shaking for 3 times, 30 min/time.
11. Transfer the specimen into **RapiClear-CS solution** (refractive index, 1.45nD; SunJin Lab Co., Taiwan), and periodically check the visual clearing of the sample over the next few hours. Clearing time should be no longer than 24 hours for sample will start to swell if it keeps incubating in RapiClear-CS solution. (**Note:** before RapiClear-CS solution, the specimen may be stored indefinitely in 0.2%

PBST with 0.05% sodium azide at 4℃.)

12. The cleared sample is then directly embedded with the **RapiClear-CS gel** reagent (refractive index, 1.45nD; SunJin Lab Co., Taiwan). The preparation of embedded samples requires a container suitable for confocal observation. The simplest approach is to use iSpacer (SunJin Lab Co., Taiwan).

    12-1. RapiClear-CS gel reagent is a gel with the melting temperature in approx. 70 ℃, gelling temperature below 30 ℃.

    12-2. Place the RapiClear-CS gel in hot-water bath or heating block (75 ℃) for 10 min.

    12-3. Once molten, the RapiClear-CS gel is left to cool to 40 ℃ in a water bath or on a heating plate. It is very important to ensure that the RapiClear-CS gel is at 40℃ before use. (**Note:** users can aliquot RapiClear-CS gel to 5~10ml tubes for later use. Label and store at 4 ℃. In this case, each aliquot can be liquefied using a heating block and then transferred to a heating block at 40 ℃).

    12-4. Sample is introduced to the container with blunt-ended forceps. iSpacer with 24 x 40 mm coverslip is used as a container (http://www.sunjinlab.com/modules.php?name=introduction&op=demonstration).

    12-5. Place the molten RapiClear-CS gel to the container. (**Note:** avoid bubble formation during handling is quite important)

    12-6. Place a 24 x 40 mm coverslip and seal with clear nail polish or Neo-Mount (Merck, cat# 109016). (**Note:** coverslip must be at a 45 degree angle, and must be lowered slowly to prevent air bubbles.)

    12-7. Let the RapiClear-CS gel polymerize at RT for 1 hour. Two advantages of sample embedded in the RapiClear-CS gel: prevents sample floating as well

as swelling from happening. (**Note:** do not place sample at 4 ℃ when polymerization)

13. Confocal imaging. (**Note:** select a long working distance as well as high NA value of objective lens for confocal scanning)