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 3 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℃.)

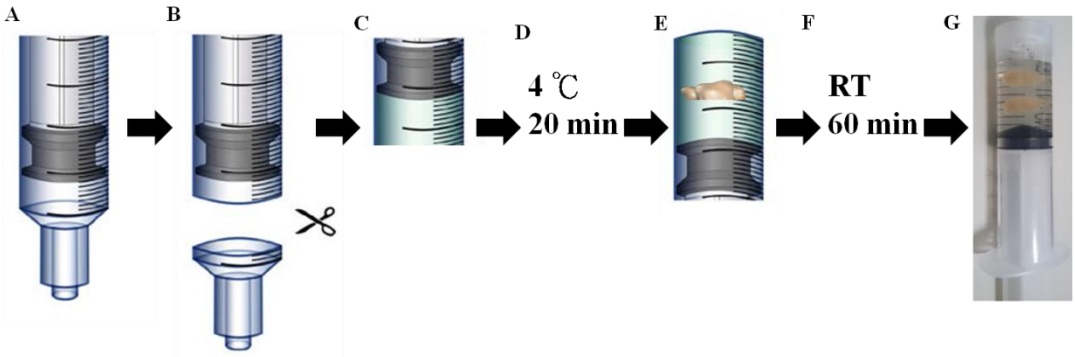
12. The cleared sample is then directly embedded with the **RapiClear-CS gel** reagent (refractive index, 1.45nD; SunJin Lab Co., Taiwan). The preparation of embedded samples requires a container suitable for light sheet microscope (LaVision BioTec Inc., Germany) observation. The simplest approach is to use a syringe as container.

    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 a 60ml tip-off syringe with blunt-ended forceps. Place the molten RapiClear-CS gel to the syringe, as shown in following figure. (**Note:** avoid bubble formation during handling is quite important)



Sample embedding container with a 60ml syringe

The tip of a syringe is cut away (A, B), RapiClear-CS gel can be easily pumped in using the

plunger (C), and place in 4 ℃ for 20min polymerization (D). 1ml gel is then added into the syringe to form a thin gel for fixing sample orientation. Sample placed into the gel with a blunt-ended forceps as soon as possible. Polymerize at RT for 30min. Then add 3ml RapiClear-CS gel into the syringe till sample is submerged. Polymerize again at RT for 30min. This process will avoid floating of the sample upon applying all gel at once. Seal the syringe with parafilm (E~G). The RapiClear-CS gel should be kept at 40 ℃ through the application processes to avoid air bubbles.

12-5. 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 ℃ upon polymerization)

12-6. Embedded sample can be pushed out of the syringe and cut to an appropriate length for imaging.

12-7. Apply (smear over) a proper amount of the melting RapiClear-CS gel on the platform surface of sample holder. Then quickly place sample on the platform. Polymerize at RT for 20min. (**Note:** this step can prevent sample floating from happening after immerse in RapiClear-CS solution.)

12-8. Add 150ml **RapiClear-CS solution** to the imaging chamber. (**Note:** avoid bubble formation during handling is quite important.)

12-9. Place sample holder into imaging chamber.

13. Ultramicroscope imaging.

14. After imaging, remove the RapiClear-CS solution from chamber and wash out RapiClear-CS solution from objective lens and chamber as soon as possible with dH_2O. Then, clean the lens following LaVision cleaning protocol (**Note:** washing step is quite important for maintain ultramicroscope imaging performance!)

15. RapiClear-CS solution applied on ultramicroscope imaging can be reused if stored at 4℃ and properly sealed.