**CLARITY Staining Tips 6-15-13**

**General**

-Begin with 37°C and decrease as needed if high background or overstaining occurs

-Cover plate wells with an adhesive sealing film to prevent evaporation and subsequent concentration changes (E&K Scientific product number T796100).

-Keep plate on a shaker for all staining and washing steps.

-Begin with a 1:50 primary and secondary dilution for 1mm blocks; adjust as needed.

**Antibody Elution and Multiple Staining Rounds**

1) After imaging the first round, must remove FocusClear (or other immersion medium) from the tissue. Place into 50 mL of PBST on a shaker at room temperature for one day.

2) Once the sample is washed, replace PBST with clearing solution. Incubate sample at 60°C overnight.

3) Remove the section from clearing solution and replace with PBST to wash the sample. Incubate on a shaker at room temperature for one day.

4) Proceed with your next round of primary antibodies.

For samples not perfused with hydrogel monomer solution before fixation, incubate in the monomer solution for one week before attempting degassing/embedding. Do this step in a 24 well plate with a ThermalSeal film cover (as with staining) in 4°C on a shaker (shaker is optional, but helpful) to allow enough time for monomers to diffuse in.

For samples too thin to withstand removal from a rigid hydrogel, make polymer viscous instead of solid by removing bisacrylamide from the hydrogel monomer solution (replace with dI water). You will still be degassing the sample in a 50mL conical tube and polymerizing for 3 hours at 37°C as usual. These samples are also too thin to require ETC clearing; they can be cleared passively by shaking at 37°C in clearing solution for 1-2 weeks (check for visual cues). The clearing solution should be changed periodically. A 1:100 antibody dilution is a better starting point for this size.

A vibratome can be used to create 500um blocks from larger samples of brain tissue. Prior to cutting, embed in 2-3% agarose/PBS and cut out the block as close to the tissue as possible without doing any damage. Use higher (closer to 3%) concentrations of agrose with more fragile tissues. A large petri dish can be used to embed. Section at highest amplitude and lowest speed for cleanest cut as a rule of thumb. For softer tissue use slower forward speed and higher amplitude for preservation of finer structures.