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CLARITY protocol

Developed by the Chung lab

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CLARITY is a tissue-clearing method that transforms intact tissue into a nanoporous hydrogel-hybridized form (crosslinked to a three-dimensional network of hydrophilic polymers) that is fully assembled but optically transparent and macromolecule-permeable. CLARITY enables intact-tissue in situ hybridization, immunohistochemistry and antibody labelling. This allows fine structural analysis of clinical samples, in a form suitable for probing the underpinnings of physiological function and disease.

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Reagents

Reagent	Vendor
Beuthanasia-D	Schering-Plough Animal Health Corp.
32% Paraformaldehyde (PFA)	Electron Microscopy Sciences, 15714-S
40% Acrylamide solution	Bio-Rad, 161-0140
Azo-initiator	Wako, VA-044
10X PBS	Invitrogen, 70011-044
Ultrapure distilled water	Invitrogen, 10977-015
Boric acid	Sigma-Aldrich, B7901
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, L3771
Lithium hydroxide monohydrate	Sigma-Aldrich, 254274
N-methyl-D-glucamine	Sigma-Aldrich, M2004
Diatrizoic acid	Sigma-Aldrich, D9268
60% Iodixanol	Sigma-Aldrich, D1556
Triton-X (TX)	Sigma-Aldrich, T8787
Sodium azide	Sigma-Aldrich, S2002
1X PBS	Invitrogen, 10010-023

Equipment

Transcardial perfusion of fixatives and hydrogel monomers	
Equipment	**Vendor**
Dissection board (Styrofoam lid is fine)	–
20 ml syringes with luer lock ends	Fisher Scientific, 14-820-19
1 ml syringes	Terumo, SS-01T
Winged infusion sets	Terumo, SV-25BLK
Needles	Fisher Scientific, BD 305109
Absorbent pads	VWR, 56616-032
50 mL Falcon tubes	BD Falcon, 352070
Guillotine, for sacrificing larger animals	Kent Scientific, DCAP
Surgical scissors	Fine Science Tools, 14130-17
Fine scissors	Fine Science Tools, 14137-10
Hemostats	Fine Science Tools, 13011-12
Forceps	Fine Science Tools, 11050-10, 11251-10
Spatula	Fine Science Tools, 10092-12

Hydrogel-tissue hybridization	
Equipment	**Vendor**
Desiccator with 3-way stopcock	VWR, 24988-197
Vacuum pump	Buchi, 071000
Compressed nitrogen tank	AirGas, NI UHP300
Compressed gas tank pressure regulator	AirGas, Y11215B580
Teflon tape	McMaster-Carr, 4591K11
3/8" tubing	McMaster-Carr, 5155T36
3/8" to 1/4" barbed tubing connector	McMaster-Carr, 5463K633

ETC clearing system	
Equipment	**Vendor**
Buffer Filter with Light-Blocking Blue Bowl	McMaster-Carr, 4448K35
Platinum wire with 0.5mm diameter	Sigma, 267201
Bottle for Chamber fabrication	Nalgene via Amazon, 2118-0002
Nalgene Straight Side Jar – Poly, 32oz	Nalgene via Amazon
Single barbed tube fitting (7/16" hex for 1/4" tubing)	McMaster-Carr, 5463K245
Tube to tube coupling for 3/32" to 1/16" tubing	McMaster-Carr, 5117K51
3M Duo adhesive dispenser	McMaster-Carr, 7467A43
3M Duo adhesive-mixing applicators	McMaster-Carr, 7467A12
3M Duo adhesive cartridges	McMaster-Carr, 746A17
Sample holder	BD Falcon, 352340
Bio-Rad HC PowerPac System	Bio-Rad, 164-5052
Banana to Large Alligator Test Lead Set	Elenco, TL16
Clear 1/4" tubing	McMaster, 5155T26
Clear 5/8" tubing	McMaster, 5155T46
1/4" wye connector	McMaster, 53055K155
4x Chemical resistant stopcock 1/4" to 1/4"	McMaster, 48285K24
5/8" to 1/4" tubing connection	McMaster-Carr, 2974K271
Elbow connection 1/4" male pipe to 1/4" barbed fitting	McMaster-Carr, 5463K489
Elbow connection 1/4" barbed fitting	McMaster-Carr, 5463K594
Rubber grounding plug	Leviton via Amazon, L00-515PR-000
Magnetic water pump	Pan World via Premium Aquatics, NH-10PX

Imaging	
Equipment	**Vendor**
KWIK-SIL	World Precision Instruments, KWIK-SIL
Willco-dish	Ted Pella, 14032-120
Blu-tack reusable adhesive	Blu-Tack via Amazon

Tissue

In principle, any tissue type from any animals of any age with or without fluorescence can be used. In the previous paper[1], we demonstrated that CLARITY is compatible with whole adult mouse brain, whole adult zebrafish brain and extensively formalin-fixed post-mortem human brain section (without the perfusion step and further optimization in this case).

Tissues with strong fluorescent protein expression can undergo CLARITY processing described in this protocol and then can be directly imaged; tissues without fluorescent proteins can be labelled with antibodies or RNA probes[1] for subsequent imaging.

Solution preparation

Hydrogel monomer solution

Keeping all reagents on ice, prepare a 10% stock solution of initiator solution by dissolving 1 g of azo-initiator in 10 mL UltraPure water. Then prepare the following solution:

Reagent	Volume
UltraPure water	26 mL
40% Acrylamide solution	4 mL
Initiator solution	1 mL
10X PBS	4 mL
32% PFA	5 mL

For each tissue sample being processed, 80 mL will be needed. Always add water first to keep the other components dilute, and add the reagents in the order listed here. The solution can be stored at -20°C indefinitely.

CAUTION: Make sure to keep all reagents and the final solution on ice at all times. The hydrogel polymerization reaction is triggered by heat.

SDS clearing solution

Adjust the following solution to pH 8.5 using boric acid.

Reagent	Concentration
Lithium hydroxide monohydrate	20 mM
SDS	200 mM

PBST

Make a solution consisting of 0.1% Triton-X and 0.1% Sodium Azide using 1X PBS.

Reagent	Volume
1X PBS	500 mL
TX	500 µL
Sodium azide	500 mg

Optical clearing solution (PROTOS)

This solution consists of 23.5% (w/v) N-methyl-D-glucamine, 29.4% (w/v) diatrizoic acid, and 32.4% (w/v) iodixanol in water. Use a stir bar (or shake if necessary) to fully dissolve the powders at each step. Do not use heat when mixing the solution, as this will cause a color change.

This solution should be stored carefully to ensure that no water is lost, as just a small amount of evaporation will result in precipitation. Teflon tape can be used to increase the security of the bottle's seal, and parafilm can be used around the cap.

It may be necessary to use a 60% iodixanol solution (see reagents list) rather than iodixanol powder, as it is not cheaply available. The optical clearing solution in the case would look as follows:

Reagent	Volume
47% Iodixanol solution in water*	10 mL
N-methyl-D-glucamine	3.39 g
Diatrizoic acid	4.24 g

*(To create this, add approximately 2.75 mL water to every 10 mL of 60% iodixanol solution)

Note: reagents should be added in order

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Equipment setup

Hydrogel-Tissue Hybridization

- Mount the nitrogen tank with an appropriate tank bracket and attach the regulator to the tank outlet using Teflon tape if necessary to prevent leaking.

- Run 3/8" tubing from the regulator outlet to the stopcock of the desiccator using a 3/8" to 1/4" barbed tube fitting.

- Connect the vacuum pump to the desiccator by simply connecting the supplied tubing to the barbed fitting on the stopcock.

ETC System

- Create the measurement reservoir in a similar manner using a 32 oz Nalgene bottle and 1/4" barbed elbow connectors.

  Be sure to place the connections on opposite sides of the bottle, and angle them slightly to maximize mixing in the chamber.

- Apply epoxy to both the inside and outside parts of the connection and allow to dry overnight.

  Caution: Don't allow any epoxy to enter the tubing connectors, as this will impede flow within the system.

- Create two more holes in the lid of the bottle, large enough for insertion of a pH probe and a thermometer for data acquisition. These holes should be left unsealed.

- Create a heat exchange module by measuring out two pieces of around 2 ft of 1/4" tubing.

- Connect these to the system in parallel using wye connectors and submerge in water.

- Connect the water filter to the system using 1/4" male pipe thread to 1/4" tubing elbow connectors.

- Tube all the components of the system together using 1/4" tubing, though 5/8" tubing will be needed for the pump connection.

  A reducing fitting should be used to connect this larger tubing to the rest of the system.

– **Critical step**: the system should be connected in the following order (in the direction of flow): Pump, Filter, ETC Chamber, Measurement Reservoir, Heat Exchanger.

  Placing the measurement reservoir direction after the ETC chamber allows for direct readouts of the temperature and pH as they are in the ETC chamber. It is also important that the reservoir is only separated from the pump inlet by tubing, as the reservoir is necessary to start the system. If desired, drain valves can be created using wye connectors and stopcocks and placed between any elements of the system.

  Additionally, when connecting the system with 1/4" tubing, 1/4" stopcocks should be added to the system on either side of the ETC chamber, so that it can periodically be isolated from the system to check the samples without draining the entire system.

Method

Perfusion and Tissue Preparation

1. Make a fresh batch of hydrogel monomer solution, or thaw frozen stock solution at 4^0C or on ice. After the solution is completely thawed and transparent (but still ice-cold), gently invert to mix. Ensure no precipitation or bubbles are seen in the solution.

2. Deeply anesthetize an animal with beuthanasia-D (0.5 mL per 1 kg of body weight intraperitoneally) and surgically open the chest cavity with a midline abdominal incision that bifurcates rostrally into a Y-shape. Punch a small hole in the right atrium and insert an injection needle into the left ventricle to allow perfusion.

   Caution: experiments involving animals must be conducted in accordance with governmental and institutional regulations. Animals must be fully anesthetized before making incisions: deep anesthesia can be confirmed by absence of corneal reflex (eye blink) or by any other overt signs of response to physical stimuli.

3. Prepare two syringes filled with ice-cold PBS and hydrogel monomer solution, respectively, each with winged needle sets for each solution.

   In the case of mice, perfuse first with 20 mL of ice-cold PBS at a rate of less than 5 mL/min, carefully take the needle out and perfuse with 20 mL of the ice-cold hydrogel monomer solution. Rats require about 200 mL of each solution at the rate of 20 mL/min.

   Critical step: maintain a slow rate of perfusion: we found that injecting less than 5 mL per minute for both solutions in the case of mice yields better results. Use extreme caution not to introduce bubbles to the vasculature (especially when introducing needles), as this decreases the quality of perfusion.

4. Carefully harvest the organs of interest and place them immediately in a 50 mL conical tube containing 20 mL of the ice-cold hydrogel monomer solution for both post-fixation and even infiltration of monomers. Keep this on ice until it can be transferred to a 4^0C refrigerator.

Caution: always keep the temperature low to prevent thermal initiation of the hydrogel-formation reaction.

5. Incubate the sample for one day at 4^0C to allow for further distribution of monomer and initiator molecules throughout the tissue.

   Caution: if the sample contains fluorophores, cover the tube containing the sample in aluminum foil to prevent photobleaching.

   Caution: If the tissues are left in the hydrogel solution for more than one day, enough protein will diffuse out of the tissue to act as a cross-inker, causing rigid gel to form around the sample. This will result in a slower rate of lipid clearing.

   Critical step: Uniform penetration of monomers throughout the tissue is critical for 1) even polymerization throughout the tissue and 2) keeping the macro- and microstructure intact. Parts of the region of cellular structures that are not infiltrated with monomers may not be bound to the hydrogel mesh even after hybridization, and subsequent electrophoresis will result in loss of the unbound biomolecules. Furthermore, uneven distribution of monomers may cause anisotropic expansion and reduction in volume during the electrophoretic tissue clearing and refractive index matching steps.

Hydrogel Tissue Embedding

6. After the tissues have been allowed to incubate in the hydrogel monomer solution for one day, move the samples to 10 mL of fresh hydrogel monomer solution. The tubes that the tissues are transferred to should have Teflon tape applied to them before the solution is added.

7. Place the conical tubes in a desiccation chamber on a tube rack and unscrew the caps about halfway. The desiccator should have a three-way stopcock. Removal of oxygen is necessary for hydrogel-tissue hybridization because oxygen radicals may terminate the polymerization reaction.

   Critical Step: if the caps are not unscrewed, there will be no gas exchange in the desiccator and oxygen will not be removed from the conical tubes.

8. Connect nitrogen gas and a vacuum pump to the desiccator via the three-way stopcock. Open flow in all three directions and turn on the nitrogen gas. Allow the gas to flow for about five seconds. This step is necessary to flush oxygen from all the tubing in the system.

9. Without turning off the nitrogen flow, turn on the vacuum pump and adjust the stopcock so that flow is only open to the desiccator and the vacuum pump. Allow the vacuum pump to run for at least ten minutes. The nitrogen should not be shut off because the tubing is gas-permeable. If nitrogen flow is stopped, oxygen will diffuse back into the tubing.

10. Turn the stopcock VERY SLOWLY so that flow is only open to the nitrogen gas and the desiccator, and then turn off the vacuum pump. Allow the desiccation chamber to fill with nitrogen gas.

11. Very quickly, lift the lid of the desiccator and tighten the caps of the conical tubes inside. It helps to have two people – one to hold the lid slightly open and another to close the tubes. The nitrogen gas can now be shut off.

Critical step: if the lids are not closed quickly enough, oxygen will re-enter the conical tubes and impede the polymerization reaction. If at this stage you find that the lids were already closed, open them slightly and repeat the de-gassing procedure.

12. Gently shake the samples in a 37°C warm room for two hours. This temperature will trigger radical initiation by the azo-initiator.

13. To remove unreacted PFA, wash the samples in 50 mL of clearing solution at 37°C for 24 hours, with gentle shaking. Do this a total of three times.

    Caution: this clearing solution with PFA must be discarded as hazardous waste according to government and institutional regulations.

    Pause point: tissues may be stored in clearing solution indefinitely following this step. If the sample contains fluorescence, be sure to cover with aluminum foil.

Electrophoretic Tissue-Clearing

14. At this point, you should have already constructed an ETC system as detailed in the section equipment setup. Add the sample to the ETC chamber and close the lid. Connect any remaining unconnected tubing.

15. Fill the system with clearing solution by first filling the measurement reservoir and placing it on a surface a few inches higher than the level of the heat exchanger and pump. This will allow buffer to fill the tubing. Start the pump and add more clearing solution to the measurement reservoir as needed to fill the system.

16. Connect the electrodes to the lead cables and start the power supply. Use around 40 V.

    Caution: never start the power supply unless you have confirmed that the flow rate is satisfactory. The flow rate can be adjusted by slightly turning one of the stopcocks that surrounds the ETC chamber. A high flow rate may result in physical damage to the tissue, whereas low flow rate may result in inadequate cooling and damage the sample. Be sure to stop the voltage before stopping the pump when you shut the system off.

    Caution: pH below 7 and temperatures above 37°C can result in loss of fluorescence and damage to the tissue. Be sure to check the system regularly to ensure that the temperature is not too high and that the pH has not dropped below about 7.3. If the pH is low, drain the current buffer and add new clearing solution. If the temperature is too high, lower the voltage to reduce resistive heating.

17. Check the samples regularly to determine that the system is working properly and that clearing is progressing. The entire process should take several days.

18. Remove the cleared samples from the ETC system and wash them twice with PBST for 24 hours each.

    Pause point: samples can be stored indefinitely in PBST at room temperature.

19. Place the sample in a volume of optical clearing solution that is sufficient to cover the tissue completely and allow it to incubate for two days. After the first day, move the sample to a container of fresh optical clearing solution.

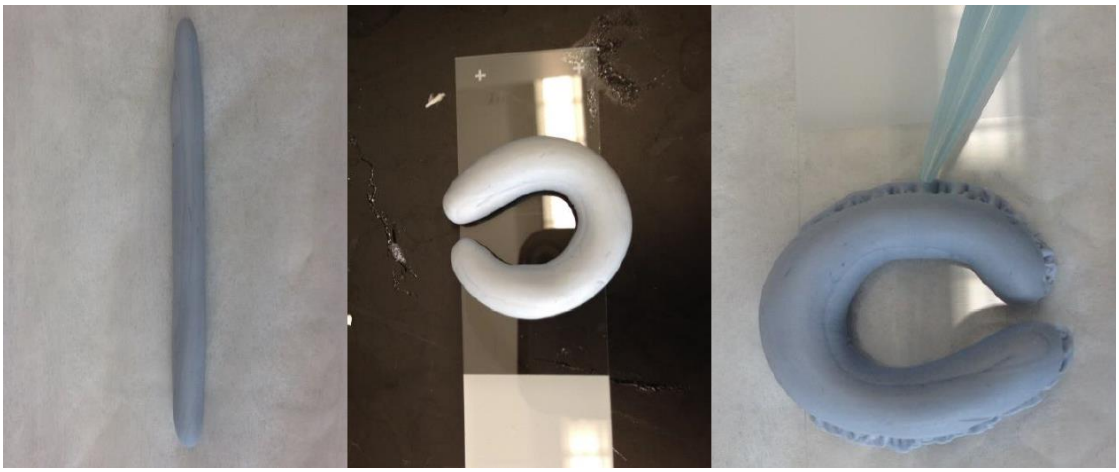
20. Caution: Make sure that the container holding the sample and optical clearing solution is completely sealed and air-tight. Evaporation of water from optical clearing solution will cause the refractive index of the solution to change and will thus lower the effectiveness of optical clearing.

21. To image the cleared sample, it must be mounted between a glass slide and a black Willco dish.

    - Roll up a piece of Blu-Tack adhesive into cylinder shapes of a thickness slightly more than the thickness of your sample.

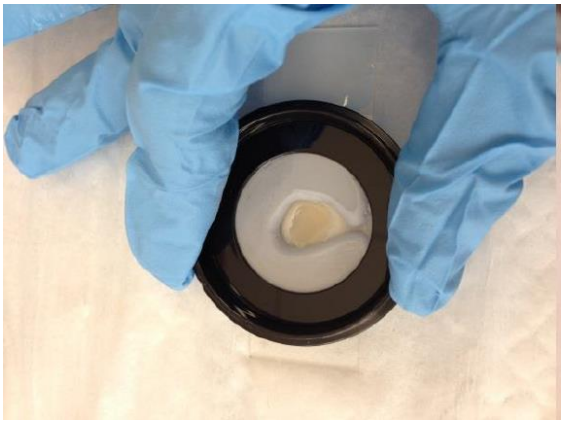
    - Place them horizontally on the glass slide.

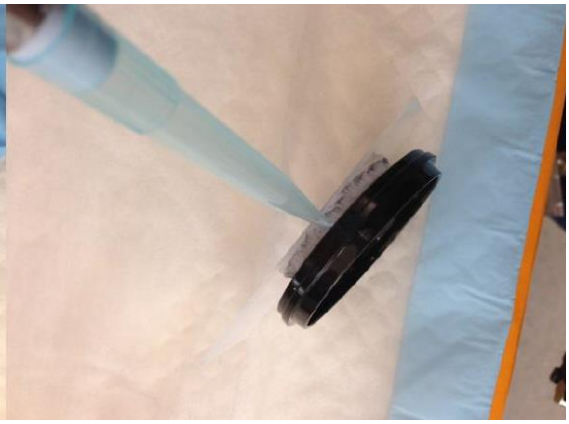
    - Press down the edge of Blu-Tack to close up the gap between the Blu-Tack adhesive and the glass slide (Shown in pictures).



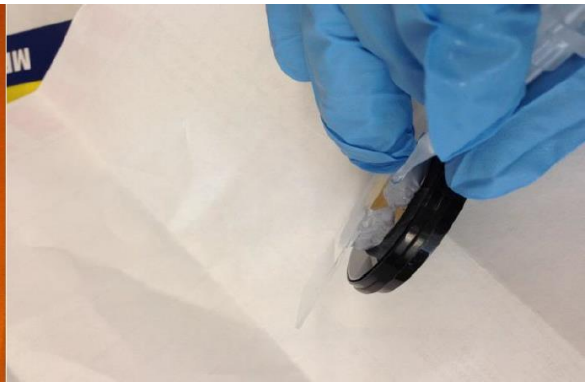
22. Carefully place the sample in between the Blu-Tack pieces and add about 20 µL of **optical clearing solution** to the sample.

22. With the lipped side facing up, firmly press a Willco dish down onto the adhesive until it just comes into contact with the sample. Using a pipette, add more optical clearing solution to the gaps between adhesive until the imaging chamber is filled.





23. KWIK-SIL is an adhesive that cures rapidly. Carefully add it to the gaps between the Blu-Tack to build a wall and seal in the sample. Take care not to introduce any bubbles, and make sure the chamber is completely filled with optical clearing solution.



24. Cover this construction with aluminum foil and store it away safely to cure. After about 20 minutes, the sample is ready for imaging.

References

1. Chung, K. et al. Structural and molecular interrogation of intact biological systems. Nature 497, 332–7 (2013).