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CLARITY Protocol with LifeCanvas Devices

ORIGINAL ARTICLES

Sung-Yon Kim*, Jae Hun Cho*, Evan Murray, Naveed Bakh, Heejin Choi, Kimberly Ohn, Sara Vassallo, Luzdary Ruelas, Austin Hubbert, Meg McCue, Philipp Keller and Kwanghun Chung. Stochastic electrotransport selectively enhances the transport of highly electromobile molecules, ***PNAS***, 2015 Nov 17: 112(46): E6274-83. doi: 10.1073/pnas.1510133112. Epub 2015 Nov 2. PubMed PMID: 26578787; PubMed Central PMCID: PMC4655572.

Kwanghun Chung, Jenelle Wallace, Sung-Yon Kim, Sandhiya Kalyanasundaram, Aaron Andalman, Tom J. Davidson, Kelly A. Zalocusky, Joanna Mattis, Sally Pak, Viviana Gradinaru, Hannah Bernstein, Julie Mirzabekov, Charu Ramakrishnan, and Karl Deisseroth, Structural and molecular interrogation of intact biological systems, ***Nature***, 2013, 497, 332-337

RELEVANT ARTICLES

Taeyun Ku*, Justin Swaney*, Jeong-Yoon Park*, Alexander Albanese, Evan Murray, Jae Hun Cho, Young-Gyun Park, Vamsi Mangena, Jiapei Chen, and Kwanghun Chung. Multiplexed and scalable super-resolution imaging of three-dimensional protein localization in size-adjustable tissues, ***Nature Biotechnology***, 2016, doi:10.1038/nbt.3641.

Evan Murray*, Jae Hun Cho*, Daniel Goodwin*, Taeyun Ku*, Justin Swaney*, Sung-Yon Kim, Heejin Choi, Jeong-Yoon Park, Austin Hubbert, Meg McCue, Young-Gyun Park, Sara Vassallo, Naveed Bakh, Matthew Frosch,, Van J. Wedeen, H. Sebastian Seung, and Kwanghun Chung. Simple, scalable proteomic imaging for high-dimensional profiling of intact systems, *Cell,* Dec 3:163(6): 1500-14. doi: 10.1016/j.cell.2015.11.025. PubMed PMID: 26638076.

EQUIPMENT

Hydrogel-Tissue Hybridization
EasyGel (LifeCanvas Technologies, EG-1001)

Tissue Clearing with Stochastic Electrotransport
SmartClear II (LifeCanvas Technologies, STM-SC2A)

RI Matching and Washing
EasyClear (LifeCanvas Technologies, EC-1001)

Imaging
KWIK-SIL (World Precision Instruments, #KWIK-SIL)
Willco-Dish (Ted Pella, #14032-120)
Blu-Tack Reusable Adhesive (Blu-Tack via Amazon)

REAGENTS

Anesthetics
Beuthanasia-D (Schering-Plough Animal Health Corp.)

Hydrogel Monomer Solution
32% Paraformaldehyde (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, MilliQ Water)

PBST (Only needed if you intend to stain tissue after clearing)
10X PBS (Invitrogen, #70011-044)
Triton-X 100 – Amresco, 0694

Washing Buffer
20mM sodium borate buffer, pH 9.0 containing 200mM SDS

Wash buffer is used to remove PFA and residual hydrogel chemicals before active clearing using SmartClear. It can also be used for passive lipid clearing using EasyClear.

Rapid Lipid Clearing Solution with SmartClear
SmartClear Clearing Buffer A, B (LifeCanvas Technologies, SC-B2001)

Optical Clearing Solution
EasyIndex (LifeCanvas Technologies, EI-Z1001/EI-Z1011)

REAGENT SETUP

PBST (Only needed if you intend to stain tissue after clearing)
To PBS, Add Triton-X 100 (TX) to a final concentration of 0.1% (v/v). This is achieved by adding 1 mL of TX to 1 L of PBS.

Hydrogel Monomer Solution Preparation
Keeping all reagents on ice, prepare a 10% stock solution of initiator by dissolving 1 g of initiator in 10 mL UltraPure water. Using this stock, prepare a solution (w/w) of 3-4% acrylamide, 0.1-0.25% initiator, 1X PBS, and 4% PFA in UltraPure water. For each tissue sample being processed, 40 mL will be needed. Always add water first to keep the other components dilute. 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 activated by heat.

Tissue

In principle, any tissue type from any animals of any ages with or without fluorescence can be used. CLARITY is compatible with whole adult mouse brain, whole adult zebrafish brain and even extensively formalin-fixed postmortem 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 labeled with antibodies or RNA probes (Chung et al., 2013) for subsequent imaging.

PROCEDURES

Perfusion and Tissue Preparation

If you are using a previously fixed tissue sample, proceed to Step 5.

1. Make a fresh batch of hydrogel monomer solution, or thaw frozen stock solution at 4°C 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.

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 mouse, perfuse first with 20 mL of ice-cold phosphate-buffered saline (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 solution. Rats require about 200 mL of each solution at the rate of 20 mL/min.

Caution: Maintain a slow rate of perfusion: we found that injecting less than 5 mL per minute for both solutions in the case of mouse 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.

5. Immediately place the tissue 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.

For the brain, we recommend to remove the dura membrane as it may interfere with clearing and fluorescence imaging. Keep this ice until it can be transferred to a 4°C refrigerator.

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

6. Incubate the sample for 1-2 days (depending on the quality of perfusion) at

4°C in the **hydrogel monomer solution** to allow for further distribution of monomer and initiator molecules throughout the tissue. If the tissue was previously fixed and without the use of the **hydrogel monomer solution**, you will need to incubate the tissue in the **hydrogel monomer solution** for 4-7 days (sample size dependent).

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

Caution: It is not recommended to keep the tissue in the **hydrogel monomer solution** for more than 7 days. With longer incubation times a thin hydrogel film can form on the outside of the tissue and can decrease clearing efficiency. If this thin film is seen, it can carefully be removed.

Caution: 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 tissue clearing and refractive index matching steps.

Hydrogel Tissue Embedding with EasyGel

7. Set the temperature of the slots in the **EasyGel** module to 37°C. Place the conical tubes in the slots and screw on the vacuum lids. Switch on the vacuum valve and wait for 1-2 hours. The elevated temperature initiates the crosslinking and hydrogel formation.

8. After formation of the hydrogel, unreacted PFA, initiators, and monomers need to be removed. Incubate the samples in 40-50 ml of **Washing Buffer** at 37 °C for 24 hours, 1/2~max shaking using the **EasyClear** module.

Note: If clearing will not be done immediately and the tissue needs to be stored, it is best to store after hydrogel formation and washing. Tissue can be stored in **Washing Buffer** at 37 °C for short term storage (<2 weeks) or at 20 °C for long term storage (>2 weeks). If the sample contains fluorescence, be sure to cover with aluminum foil.

Caution: The solutions with PFA (hydrogel monomer and used **Washing Buffer**) must be discarded as hazardous waste according to government and institutional regulations.

Rapid Tissue-Clearing (SmartClear II / II Pro)

9. After washing the sample, carefully place the tissue in the sample holder and put it in the SmartClear II System. Use the gentle clearing setting for fluorescent protein expressing samples. The fast clearing setting is ideal for SWITCH-processed samples (Murray, Cell, 2015). The settings are carefully optimized to achieve the most effective clearing. Altering the settings could result in slow clearing or tissue damage. Carefully follow instructions for the **SmartClear II** module.

10. After clearing is complete (1-4 days),
    - For samples that have endogenous fluorescence, do not require staining, and will

be imaged within 2-3 days, wash off the **SmartClear Clearing Buffers** with PBS (40 mL for a whole mouse brain) using the **EasyClear** module (37°C, 1/2~max shaking, lay out the tubes horizontally, overnight for small tissues, 1-2 days for a whole mouse brain).

- For samples that will be stained, wash off the **SmartClear Clearing Buffers** with PBST (40 ml for a whole mouse brain) using the **EasyClear** module (37°C, 1/2~max shaking, lay out the tubes horizontally, overnight for small tissues, 1-2 days for a whole mouse brain).

- For samples that will be index matched and imaged at a later date, the samples should be stored in PBS with 0.02% sodium azide at room temperature or 4ºC. The day before imaging, proceed to Step 11.

Index Matching

11. Incubate the stained and fully washed sample in **EasyIndex** with proper shaking at 37 °C. (optimal shaking can be achieved using the **EasyClear** module). Use 500 µl, 1 ml, or 40 ml of **EasyIndex** for clearing 100 µm-thick tissue slice, 1 mm-thick tissue, or entire intact mouse brain, respectively. The sample is now ready for imaging.

Caution: The sample may have to be incubated in **EasyIndex** as long as needed depending on the thickness. 100 µm slice can be cleared within 10 minutes, whereas intact mouse brains require overnight incubation. The required incubation time is highly dependent on the shaking speed. After **EasyIndex** index matching, the sample should be clear enough to see through easily. If the solution immediately surrounding the sample seems inhomogeneous, it suggests that the sample has not yet fully equilibrated with the solution. The sample must be further incubated in a fresh **EasyIndex** solution with proper shaking until it reaches complete equilibrium.

Caution: Samples should not be stored in EasyIndex for more than 2-3 days. If a sample will not be imaged until a later date, one of the storage options listed above should be used. If as sample has been index matched and needs to be recovered and saved, the sample should be incubated in PBS at 37 °C with gentle shaking overnight. After this the sample should be transferred to PBS with 0.02% azide for storage.

<EasyIndex Storage Tip>
EasyIndex works best when its chemical composition is preserved. Water evaporation will not only change the clearing effect of EasyIndex, but also cause
chemical precipitation. Please seal the cap tightly after usage. For long-term storage, we advise sealing the cap with Teflon tape.

Imaging

12. To image the cleared sample using conventional confocal or two-photon microscope, it must be mounted properly. Roll up a piece of Blu-Tack adhesive into cylinder shapes of a thickness that is slightly thicker than 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.

13. Carefully place the sample in between the Blu-Tack pieces and add about 20 µL of EasyIndex to the sample.

14. 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 EasyIndex to the gaps between adhesive until the imaging chamber is filled.

15. KWIK-SIL is an adhesive that cures rapidly. Carefully add it to the gaps between the BluTack 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 EasyIndex.

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