CHUNG LAB

SWITCH protocol

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ORIGINAL ARTICLES

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.

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.

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

REAGENTS

- 32% paraformaldehyde – Electron Microscopy Sciences, 15714-S
- 50% glutaraldehyde – Electron Microscopy Sciences, 16310
- Potassium hydrogen phthalate – Sigma-Aldrich, P1088
- Acetamide – Sigma-Aldrich, A0500
- Glycine – Sigma-Aldrich, G7126
- Sodium azide – Sigma-Aldrich, S2002
- Triton-X 100 – Amresco, 0694
- Sodium Dodecyl Sulfate – Sigma-Aldrich, L3771
- Sodium Sulfite – Sigma-Aldrich, S0505
- DiD – ThermoFisher, D7757
- N-methyl-d-glucamine – Sigma-Aldrich, M2004
- Diatrizoic acid – Sigma-Aldrich, D9268
- 60% Iodixanol – Sigma-Aldrich, D1556
- EasyIndex (refractive index matching solution, LifeCanvas Technologies)

EQUIPMENT

Tissue clearing and destaining

- EasyClear (LifeCanvas Technologies) or 37°C, 70°C shaking water baths.
- Optional, SmartClear (LifeCanvas Technologies) or custom-built ETC device.

Sample mounting for confocal microscopy

- Slide Glasses (Fisherbrand Cat. No. 12-550-14G)
- Cover Glass (VWR Cat. No. 48393070)
- BluTack putty (Bostik)
- WillCo dish (WillCo Wells GWSB-5030)

REAGENT SETUP

Perfusion solution

Create a solution with a final concentration of 1X PBS, 4% paraformaldehyde (PFA), and 1% glutaraldehyde (GA). As 40 mL of this solution is necessary for each perfusion, a typical recipe is: 4mL 10X PBS, 5 mL 32% PFA, 0.8 mL 50% GA, and 30.2 mL water. This solution should be made fresh immediately prior to performing perfusion and kept on ice at all times. It is recommended to chill all of the separate ingredients before mixing the components.

Fixation-OFF solution

Titrate a bottle of PBS to pH 3 using HCl. Create solutions of 0.1 M HCl in water and 0.1 M potassium hydrogen phthalate (KHP) in water. Finally, mix these solutions in a ratio of 2:1:1 (pH 3 PBS):(0.1 M HCl):(0.1 M KHP). To this new solution, add a stock solution of GA to make a final concentration of 4% GA. Ensure that this solution stays cold at all times. It is recommended to chill the solution before adding GA.

Fixation-ON solution

Add a stock solution of GA to PBS (pH 7.4) to make a final concentration of 1% GA. Ensure that this solution stays cold at all times. It is recommended to chill the PBS before adding GA.

PBST

To PBS, Add Triton-X 100 (TX) to a final concentration of 0.1% (v/v). Also, add sodium azide to a final concentration of 0.02% (w/v). Practically, this is achieved by adding 1 mL of TX and 0.2 g of sodium azide to 1 L of PBS.

Inactivation solution

To PBS, add acetamide to a final concentration of 4% (w/v) and glycine to a final concentration of 4% (w/v).

Thermal clearing solution

To water, add sodium dodecyl sulfate (SDS) to a final concentration of 200 mM, sodium sulfite to a final concentration of 20 mM, sodium hydroxide to a final concentration of 10mM. Titrate the solution to pH9 using boric acid. This solution should be made fresh frequently, as the sulfites tend to degrade over time in solution.

DiD-OFF solution

To PBS, add SDS to a final concentration of 10 mM. Dissolve 1mg of DiD powder per 200 µL. This solution should be kept protected from light. Note: molecules similar to DiD can be used if other excitation/emission wavelengths are desired, so long as the molecule is sufficiently lipophilic.

Antibody-OFF solution
To PBS, add SDS to a final concentration of 0.5 mM. This is most easily accomplished by diluting a stock solution of SDS. When adding large proportions of antibody to this solution (say, >1:10), care should be taken to account for the resulting change in SDS concentration.

Optical clearing solution
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. Therefore, an example recipe for the optical clearing solution is as follows: Dilute 60% iodixanol solution to 47% iodixanol. To achieve this, roughly 2.75 mL water should be added for every 10 mL of 60% iodixanol solution. Then, for every 10 mL of the resulting solution, 3.39 g n-methyl-d-glucamine and 4.24 g diatrizoic acid should be added in order. Be sure to take into account that the final volume will be significantly larger than the starting volume.

In addition to our optical clearing solution (termed PROTOS), there are proprietary optical clearing solutions available as well as other published recipes that other groups have developed for their tissue clearing protocols. The proprietary formulations are prohibitively expensive (RapiClear and FocusClear) and at least FocusClear is known to result in the formation of precipitate within samples during long-term storage. PROTOS is the most cost-effective option for high quality optical clearing, which is an absolute must for thick tissue imaging. CUBIC-mount (Lee, 2016) and sRIMS (Yang, 2014) are cheaper than PROTOS, but they are noticeably less effective.

Solution	Recipe	Cost per 500 mL
PROTOS	29.4% diatrizoic acid 23.5% n-methyl-d-glucamine 32.4% iodixanol	$565.80
RIMS	88% w/v histodenz 0.1% tween-20 0.01% sodium azide 0.02 M PB	$1,562
RapiClear	proprietary	$6,000
FocusClear	proprietary	$11,520

sRIMS	70% w/v sorbitol 0.01% sodium azide 0.02 M PB	$7.63
CUBIC-mount	50% sucrose 25% urea 25% N,N,N',N' –tetrakis (2-hydroxypropyl) ethylenediamine	$29.81

Premade PROTOS is available from LifeCanvas technologies (EasyIndex, http://www.lifecanvastech.com/)

PROCEDURES

All samples must be preserved through use of either procedure 1a or 1b below and then inactivated through procedure 2 and cleared through procedure 3 in order. Procedures 4a and 4b are optional, but it is not recommended to perform both in the same round of staining. Samples thicker than 50-100 µm must undergo procedure 5 in order to be imaged fully, but it is optional for very thin samples. After procedure 6, you may go back to procedure 4a or 4b to complete another round of staining.

Processing times at each step will vary depending upon the tissue type and size of the sample. Unless otherwise noted, the parameters given below were optimized for adult mouse brain samples.

1a. Perfusion

If it is possible, perfusion is the preferred method of tissue preservation. Using the perfusion technique of your choice, first perfuse 20 mL of ice-cold PBS through the beating heart of an anesthetized mouse, followed by 20 mL of the ice-cold **perfusion solution** described above. Take care not to introduce any bubbles during the procedure, and use a flow rate slow enough to avoid damage to the vasculature or brain sample (<5 mL/min). After both solutions have been perfused, carefully remove the brain from the skull using any technique you are comfortable with. The dura membrane should also be removed during the process. Place the sample into 20 mL of **perfusion solution** and incubate at 4 ˚C with gentle shaking for 3 days.

1b. SWITCH-mediated tissue preservation

If perfusion is not possible, the sample must be preserved using SWITCH. The sample should be first fixed with PFA for several days before proceeding. Incubate the sample in 40 mL **fixation-OFF solution** at 4 ˚C with gentle shaking for 2 days. The sample should then be moved to **fixation-ON solution** at 4 ˚C with gentle shaking for an additional 2 days. NOTE: the timing for the Fixation OFF and ON steps is dependent on the sample size and may need to be optimized from these starting values on a case-by-case basis. We found that these parameters worked well for banked human samples of roughly 0.5-1.0 cm thickness.

2. Fixative inactivation

After fixation via either perfusion or SWITCH, the sample must be washed in **PBST** to remove unbound fixative molecules. For mouse brains, 2 washes of 6 hrs each at RT with gentle shaking was sufficient. To inactivate remaining fixative molecules, the sample must then be washed in **inactivation solution** at 37 ˚C O/N using a water bath or EasyClear. If the solution turns yellow, the **inactivation solution** should be replaced with fresh solution and the sample incubated for several more hours. Note: if the sample needs to be cut, this should take place now before the sample is cleared.

3. Thermally-assisted lipid clearing

Inactivated samples must next be incubated in **thermal clearing solution** to wash away remaining **inactivation solution** and to distribute sodium sulfite through the sample. After 2 washes of 6 hrs each at 37 ˚C, the samples should be placed in a tube of fresh **thermal clearing solution**, which should then be placed in a water bath heated to 70 ˚C or in EasyClear. Other temperatures or methods of consistent heating may be used, but samples may deteriorate over time at higher temperatures. If a sample contains fluorophores that were genetically-encoded, introduced through viral injection, etc., then the sample may be cleared at 37 ˚C to preserve this fluorescence. The clearing process will take longer at this low temperature, but temperatures higher than this will result in loss of fluorescence during clearing.

4a. SWITCH-mediated myelinated fiber labeling

After a sample has been clearing, SWITCH-mediated labeling is possible. Myelinated fibers can be readily visualized with the lipophilic DiD fluorescent molecule. The sample should be equilibrated in a solution of 10 mM SDS in PBS in order to distribute SDS molecules throughout the sample. The sample should then be placed in a volume of **DiD-OFF solution** just large enough to cover the sample and incubated at 37 ˚C with gentle shaking for 12 hrs to 7 days depending on the size of the sample (1 mm-thick section to whole mouse brain). The sample should then be moved to 40 mL of **PBST** and incubated at 37 ˚C for 12 hrs to 2 days. We have also observed that tomato lectin and nuclear stains such as DAPI or Syto16 can be used with this SWITCH approach.

4b. SWITCH-mediated immunolabeling

After lipid clearing of a sample, SWITCH-mediated immunolabeling is possible. The sample should be equilibrated in **antibody-OFF solution** in order to distribute SDS molecules throughout the sample. The sample should then be placed in a fresh volume of **antibody-OFF solution** just large enough to cover the sample, and then antibodies should be added in the desired proportions. The sample should then be incubated at 37 ˚C with gentle shaking for 12 hrs to 7 days depending on the size of the sample (1 mm-thick section to whole mouse brain). The sample should then be moved to 40 mL of **PBST (antibody-ON solution)** and incubated at 37 ˚C for 12 hrs to 2 days to initiate antibody binding and wash out unbound antibodies. If secondary antibody labeling is required after primary staining, incubate the sample in a fresh volume of PBST with secondary antibodies. It is important to use enough secondary antibodies to saturate all primary antibodies within the sample.

5. Optical clearing

After labeling, the sample must be equilibrated in PROTOS, refractive index-matching solution, or in EasyIndex to achieve maximal optical clearing. Incubate the stained and fully washed sample in PROTOS with proper shaking at 37°C using EasyClear or shaking water bath. Use 500 µl, 2 ml, 25 ml, 50ml of PROTOS for clearing 100 µm, 1 mm, intact mouse hemisphere, intact mouse brain, respectively.

The sample may incubate in PROTOS or EasyIndex longer as needed depending on the thickness. 100 µm slice can be cleared within 10 min, whereas intact mouse brains require overnight incubation. After optical clearing, the sample should be clear enough to easily see through by eye. 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 PROTOS solution with proper shaking until it reaches complete equilibrium.

6. Sample Mounting

1. Form a blue tack “worm” only slightly thicker(~0.1mm) than the samples. Lay this “worm” in a circle around the center of a slide glass **(Fig. 1A)**.

2. Use a pipet tip to seal the blue tack and slide glass surface by pressing down the blue tack along the outside edge of the circle **(Fig. 1B)**.
□CRITICAL STEP Sealing blue tack can prevent PROTOS from slipping between the blue tack and slide glass, and also from the evaporation and drying during storage.

3. Place slices on the center of the slide glass inside this circle **(Fig. 1C)**. Ensure that the slices are wet with PROTOS. The entire circle does not need to be filled with protos, unless samples are to be stored for an extended period of time
□CRITICAL STEP if long term storage of the sample is intended, see the end of this procedure for details.

4. Place a clean “covering dish”(wilco dish) on top of the blue tack circle, pressing down until it comes into contact with the surface of the samples **(Fig. 1D)**.

5. Place the slide under the microscope’s water objective and fill the “covering dish” with enough water so that a column of water forms between the objective and dish upon lowering the objective **(Fig. 1E)**.
□CRITICAL STEP Ensure no bubbles are formed between the objective and dish, because they may distort images.

Note: This set up works for microscopes with low NA→ these microscopes allow changes in medium between the objective and final sample. In this case, a 10X water objective on a confocal microscope was used to image 1mm slices.

Note: For extended sample preservation- Leave the circle of blue tack disconnected and place the samples at the center of this circle. Seal the blue tack to the slide glass surface, except for a small opening as described above. Press on the “covering dish” until it makes contact with the samples. Use a pipet to fill the space between the slide glass and “covering dish” with PROTOS through the small opening. Use glue to seal this opening. These samples can now be stored for an extended time.

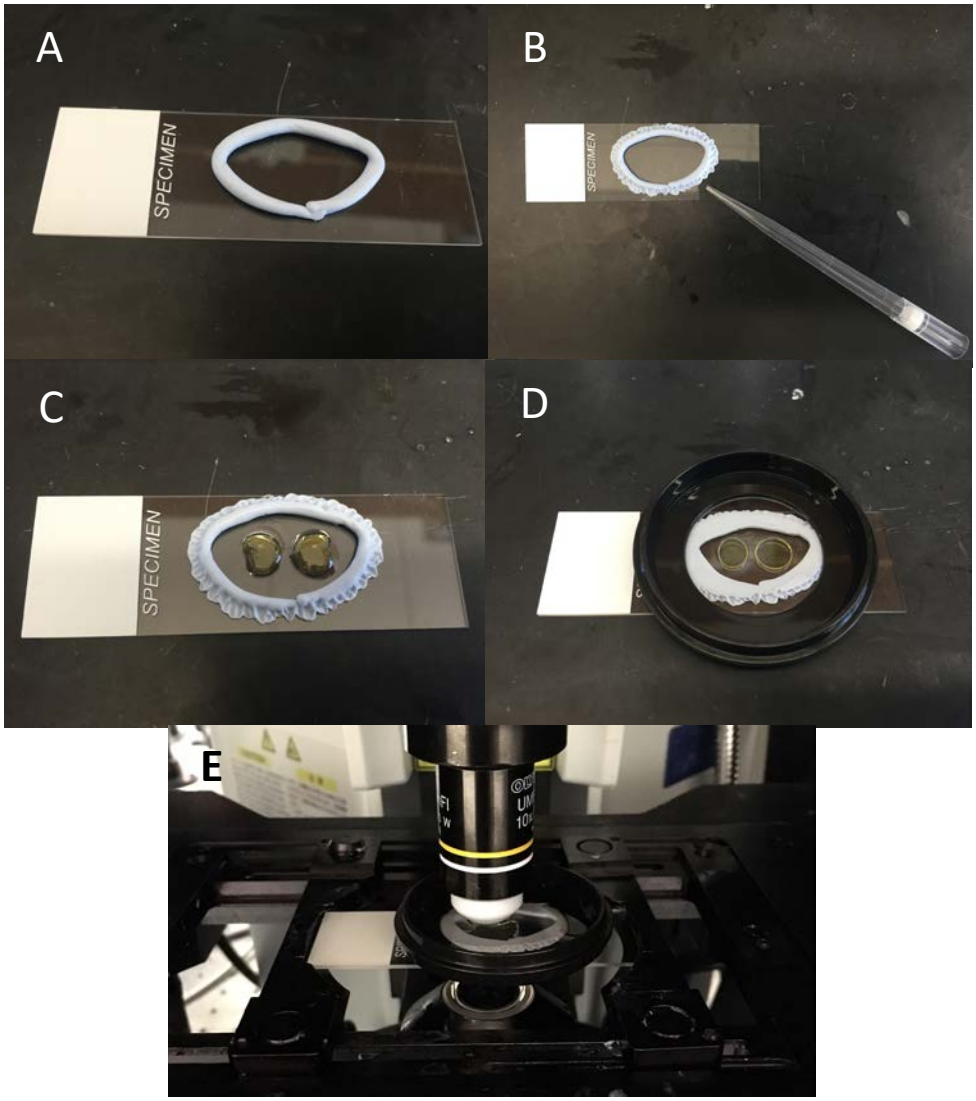

Fig. 1 Sample Mounting Diagram. A) Place a blue tack ring slightly thicker than the sample on a glass slide. B) Seal the blue tack to the slide using a pipet tip. C) Place the samples in the center, keeping them wet with PROTOS. D) Press on a covering dish until it comes in contact with the sample surface. E) Place the slide under the water objective, filling the covering dish with water until it forms a column of water with the lowered objective.

7. Molecular probe elution

After imaging, the **optical clearing solution** should be washed out of the sample with **thermal clearing solution**. After the sample has equilibrated, the sample should be placed in a 70 ˚C water bath or EasyClear for 2 hrs to O/N depending on the size of the sample. Labeling can proceed again after this step is completed