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LifeCanvas Technologies
1035 Cambridge St, Suite 6
Cambridge, MA 02141,USA
www.lifecanvastech.com

SWITCH Protocol with LifeCanvas Devices
Based on Chung Lab's SWITCH protocol

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

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EQUIPMENT

Tissue Clearing and Destaining

EasyClear (LifeCanvas Technologies)
SmartClear (LifeCanvas Technologies)

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)

REAGENTS

Anesthetics

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

Perfusion Solution

32% paraformaldehyde – Electron Microscopy Sciences, 15714-S
50% glutaraldehyde – Electron Microscopy Sciences, 16310
10X PBS (Invitrogen, #70011-044)
UltraPure Distilled Water (Invitrogen, #10977-015, MilliQ Water)

Fixation-Off Solution

10X PBS (Invitrogen, #70011-044)
Potassium hydrogen phthalate – Sigma-Aldrich, P1088
50% glutaraldehyde – Electron Microscopy Sciences, 16310

Fixation-On Solution

10X PBS (Invitrogen, #70011-044)
50% glutaraldehyde – Electron Microscopy Sciences, 16310

PBST

10X PBS (Invitrogen, #70011-044)
Triton-X 100 – Amresco, 0694
Sodium azide – Sigma-Aldrich, S2002

Inactivation Solution

10X PBS (Invitrogen, #70011-044)
Acetamide – Sigma-Aldrich, A0500
Glycine – Sigma-Aldrich, G7126

DiD-Off Solution
10X PBS (Invitrogen, #70011-044)
Sodium Dodecyl Sulfate – Sigma-Aldrich, L3771
DiD – ThermoFisher, D7757

Antibody-Off Solution
10X PBS (Invitrogen, #70011-044)
Sodium Dodecyl Sulfate – Sigma-Aldrich, L3771

Washing Buffer
20mM sodium borate buffer, pH 9.0 containing 200mM SDS
*-Wash buffer is used to wash away remaining **inactivation solution** and to distribute SDS through the sample 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

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 of mouse, 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).

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.

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 2 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 PFA-fixed 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 room temperature with gentle shaking for an additional day. 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 12 hrs each at RT with gentle shaking were sufficient. To inactivate remaining fixative molecules, the sample must then be washed in **Inactivation Solution** at 37°C O/N using 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. Lipid clearing

Inactivated samples must next be incubated overnight in 40ml of **Washing Buffer** at 37°C for 24 hours, ~½ max shaking using the EasyClear module. After wash, use **SmartClear**. If a sample contains fluorophores that were genetically-encoded, introduced through viral injection, etc., then the sample may be cleared using gentle clearing condition in SmartClear to preserve this fluorescence. If the sample does not contain fluorescent proteins, fast clearing option in SmartClear can be used.

4a. SWITCH-mediated myelinated fiber labeling

After a sample has been cleared, 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. Index Matching

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.

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)**.

Note: Sealing blue tack can prevent EasyIndex 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 EasyIndex. The entire circle does not need to be filled with protos, unless samples are to be stored for an extended period of time

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 EasyIndex through the small opening. Use glue to seal this opening. These samples can now be stored for an extended time.

4. Place a clean "covering dish"(Willco 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)**.

Note: 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.

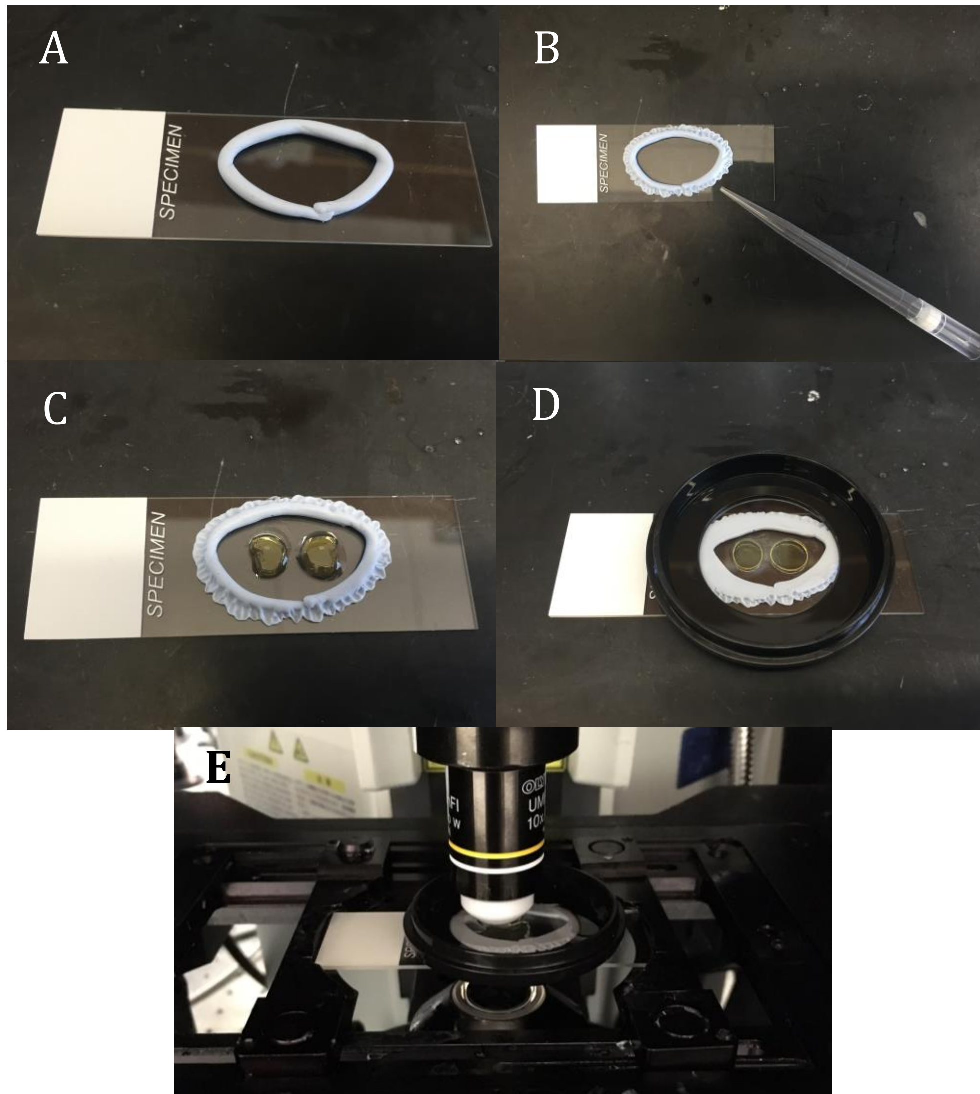

Fig. 1 Sample Mounting Diagram. A) Place a blue tack ring slightly thicker than the sample on a glass slide. B) Seal the blue tack to the slide using a pipet tip. C) Place the samples in the center, keeping them wet with EasyIndex. D) Press on a covering dish until it comes in contact with the sample 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** at RT or 37C. After the sample has equilibrated, the sample should be placed in a 70°C EasyClear for 2 hrs to denature and dissociate the imaged probes. After 2 hrs at 70°C, the sample should be incubated at 37C O/N if the sample is thicker than 1-mm to allow the dissociated probes to diffuse out. For the next round of labeling, go to step #4b.