CHUNG LAB

eMAP protocol for brain slice samples

Epitope-preserving Magnified Analysis of Proteome (eMAP) utilizes similar reagents and protocol as the original realization of MAP, but it significantly improves upon the preservation of epitopes by employing a purely physical hybridization with marked enhancements in overall performance, reproducibility and compatibility.

Related reports

1. Joha Park, Sarim Khan, Dae Hee Yun, Taeyun Ku, Katherine L. Villa, Jichen E. Lee, Qiangge Zhang, Juhyuk Park, Guoping Feng, Elly Nedivi, Kwanghun Chung (2021) Epitope-preserving magnified analysis of proteome (eMAP). *Science advances* 7.46 (2021): eabf6589.
2. Taeyun Ku, Justin Swaney, Jeong-Yoon Park, Alexandre Albanese, Evan Murray, Jae Hun Cho, Young-Gyun Park, Vamsi Mangena, Jiapei Chen & Kwanghun Chung (2016) Multiplexed and scalable super-resolution imaging of three-dimensional protein localization in size-adjustable tissues. *Nature Biotechnology* 34:973–981.
3. Taeyun Ku, Alexandre Albanese & Kwanghun Chung (2017) Advanced Magnified Analysis of Proteome (MAP) for superresolution mapping of biological tissues. Neuroscience 2017 (Washington, DC).

	MAP	**eMAP**
Proposed principle	Chemical fixation of biomolecular network to a dense hydrogel	Physical hydrogel-tissue hybridization of a dense hydrogel to a fixed biomolecular network
Linear expansion ratio	3.3×–4.0×	3.8×–4.0×, 10× with recursive embedding
Compatible tissue type	Organs perfused with hydrogel chemicals (e.g., mouse organs)	Any fresh or fixed/stored samples (e.g., 4% formaldehyde fixed mouse tissue, banked human tissue)
Antibody compatibility	+++	+++++
Post-expansion signal level	+++	++++
Reproducibility	++	+++++
Scalability	++++	+++++

Reagents

- 10× phosphate buffered saline (PBS)
- 32% paraformaldehyde (PFA) — Electron Microscopy Sciences, 15714-S
- Acrylamide (AAm) — Sigma-Aldrich, A3553
- 2% bisacrylamide (bis) — Bio-Rad, 161-0142
- Sodium acrylate (SA) — Sigma-Aldrich, 408220
- VA-044 initiator — Wako, VA-044
- Sodium dodecyl sulfate (SDS) — Sigma-Aldrich, L3771 or 75746
- Monosodium phosphate
- Disodium phosphate

- Sodium sulfite — Sigma-Aldrich, S0505
- Sodium azide — Sigma-Aldrich, S2002
- Triton X-100

Other materials

- Blu-Tack — Bostik, Blu-Tack Reusable Adhesive 75g
- Slide glasses (1 mm-thick)
- Cover slips #1
- Willco Dishes — Willco Wells, HBSB-5030
- Nitrogen gas

Equipment

- Shaker (at 37 °C)
- Heating device or water bath (up to 95 °C)
- Custom-built gelation device or EasyGel (LifeCanvas Technologies)
- Peristaltic pump (for perfusion only)

Solutions

- (for fresh animals) 1× PBS
- (for fresh animals) **Perfusion solution** — 4% PFA in PBS
- **eMAP solution** — 30% AAm (w/v), 0.1% bis (w/v), 10% SA (w/v), 0.03% VA-044 (w/v) in PBS
  - ✓ **Initiator stock solution** — 10% VA-044 (w/v) in DI water
    - ➢ Aliquot in Eppendorf tubes and freeze them at −20 °C until use.
  - ✓ **eMAP stock solution** — eMAP solution without VA-044
    - ➢ Initiator must be freshly added to eMAP stock solution for each use. To account for this, eMAP stock solution is slightly more concentrated than the final concentration (100/99.7). eMAP stock solution can be stored at 4 °C protected from light (e.g. covered with aluminum foil). Validated for up to one year of storage.
    - ➢ Quality of SA have been inconsistent in the past and is known to affect the quality of eMAP experiments. eMAP solution prepared with poor quality SA may have yellow coloration, oily phase separation, and/or black particles due to impurity. For best results, purchase multiple bottles of SA and check their quality first by dissolving some in DI water. If the solution shows yellow coloration, discard the solution and use another SA bottle. After making the eMAP stock solution, vacuum-filter the solution to remove dust. If the solution has an oily phase after filtration, centrifuge the solution (or set aside for at least two days until the top layer turns clear) and collect the clear supernatant for use.

  To prepare eMAP solution from stock solutions, thaw 10% initiator stock solution aliquot and add 0.3% volume to 99.7% volume of eMAP stock solution.
- **Clearing solution** – 6% SDS (w/v), 0.1 M phosphate buffer, 50 mM sodium sulfite, 0.02% sodium azide (w/v) in DI water. Titrate the pH to 7.4.
- **PBSN** — 0.02% sodium azide (w/v)
- **PBST** — 0.1% Triton X-100 (w/v), 0.02% sodium azide (w/v) in PBS
- **Expansion buffer** —0.01X PBS (or just DI water)

Initial sample preparation

- eMAP processing is validated with mouse brain, marmoset brain tissues, and formalin-fixed human brains from a brain bank; however, eMAP processing will likely be compatible with most biological tissues.
- If you are starting with live animals, you can perfuse them with your typical perfusion method. Here we describe our typical mouse perfusion method for completeness. If you are starting with previously fixed samples, skip the perfusion step.
- In this protocol, we describe how to eMAP-process brain slices prepared with a vibratome.

Experimental steps

i. (for live animals) Perfusion
ii. Sectioning
iii. Hydrogel monomer incubation
iv. Mounting
v. Gel embedding
vi. Additional sectioning
vii. Clearing and denaturation
viii. Immunostaining
ix. Expansion, mounting, and imaging

i. (for live animals) Perfusion

Following perfusion protocol is for mouse. For other animals, consult appropriate literature.

1. Keep **PBS** and **Perfusion solution** on ice.
2. Perfuse an anesthetized mouse transcardially with ice cold **PBS.** We use 5 mL/min flow rate and 25 mL **PBS**.
3. Switch to **Perfusion solution**. We use 25 mL of **Perfusion solution**.
4. After perfusion, extract the brain and put it in a 50 mL conical tube with 30 mL of **Perfusion solution**.
5. Incubate for 2 days at 4 °C with gentle shaking.
6. Wash with **PBSN** overnight at 4 °C.
7. Exchange the **PBSN** and wash overnight at RT.

ii. Planning your eMAP experiment/Sectioning

Proper planning for sectioning is important for achieving successful results with eMAP. There are several factors you should consider while designing your eMAP experiment.

- Location and orientation of your biological signal of interest (e.g., Do you want to image a whole neuron? Reconstruct a specific brain region?).
    - eMAP tissues are typically processed as 170 μm or 1 mm thick sections. It may be convenient to orient your sections in a way that contains your biological signal of interest in the fewest number of slices.
- Imaging capability of your microscope (e.g., what is the working distance of your objective, etc.).
    - Most high magnification objectives with high numerical aperture (NA) has limited working distance (e.g., 63X 1.2NA objective we use has 300 μm working distance. The thickest contiguous piece of tissue that this objective can image is then ~75 μm accounting for the 4× linear expansion. Approximately 150 μm if you are to flip the tissue over to image both sides.).
- Antibody labeling (i.e. thicker tissue will be harder to label uniformly throughout its depth).

- Larger tissues are harder to label uniformly and will consume more antibodies.

- Mounting setup for gelation.
  - The mounting setup described in this protocol is designed to minimize the gel layer on the top and bottom of the slice, which can impede molecular probe penetration. Thickness of 170 µm and 1 mm are chosen reflect the spacers we use for mounting (cover slip and slide glass respectively). The mounting method is described in detail in a later section.

Above factors will inform your decision on how best to trim and section your tissue for eMAP processing.

Note that eMAP process tissues can be sectioned further either after the **Gel embedding** step or the **Clearing and denaturation** step.

In general, if compatible with your experiment design, eMAP processing a larger block of tissue first, then sectioning it after the hydration step leads to better results. This approach ensures that your eMAP-processed slices do not have excess gel layers that could interfere with clearing and labeling (except the top-most and bottom-most slices).

iii. Hydrogel monomer incubation

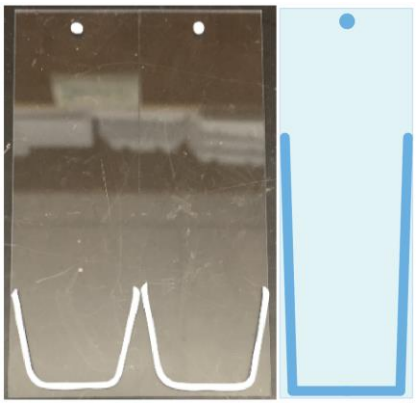
eMAP solution should be light-protected during all steps.

1. Add freshly thawed **Initiator stock solution (VA-044)** to **eMAP stock solution** to prepare **eMAP solution**.
2. Incubate samples in the **eMAP solution** at 4 °C. We incubate thin slices or a hemisphere in a 15-mL conical tube with 12 mL **eMAP solution** and a whole brain in a 50-mL conical tube with 35 mL solution. Based on our experience, incubate 1-mm-thick or thinner samples for overnight, 2–3-mm-thick samples for 2 days, 5-mm-thick (e.g., mouse hemisphere) samples for 5 days, and 7-mm-thick (e.g., whole mouse brain) for 1 week.

iv. Mounting

An excess gel layer formed on top and bottom surface of the tissue hinders lipid clearing and antibody penetration. If you are mounting/embedding a sample that will be cleared/labeled without further sectioning, it is crucial to minimize the excess gel layer while mounting.

The general idea is to form a cassette with controlled thickness using two slide glasses, Blu-Tack, and spacers.



Pic 1. Blu-Tack well on a slide glass. Deeper wells are also acceptable.

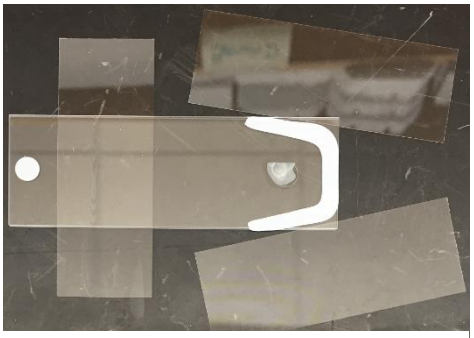
1. Prepare materials for mounting: Two 1-mm-thick slide glasses per sample, spacers (cover slips #1 for tissue slices of 170 µm thickness and additional 1-mm-thick slide glasses for 1-mm thick slices), Blu-Tack, and a fine paint brush for tissue handling.

   Note that you can stack multiple cover slips #1 to work with tissue slices with thickness that is of multiples of 170 µm (e.g. 340 µm, 510 µm etc.).

2. Shape a piece of Blu-Tack using gloved hands into a long and thin tube (roll and then pull). The thickness should be roughly 1.5–2x of the sample thickness. Form a well on one side of the slide glass (**Pic 1**). Add a small Blu-Tack ball on the other side. Use a razor blade to trim excess Blu-Tack. When mounting millimeters-thick sample, make sure that the Blu-Tack is firmed attached. The solution can easily leak with poor cassette preparation, which leads to failure of the eMAP sample preparation. Deeper well may be prepared based on preference. Make sure that Blu-Tack stays dry during the mounting steps.

3. If processing multiple samples, prepare multiple cassettes at once.

4. Wet the center of the well on the slide glass using a tiny volume of **eMAP solution** (i.e., the sample incubation solution) with a clean paint brush then place the tissue slice. Avoid generating bubbles.
5. Wet the top surface of the slice using a paint brush. This can help prevent trapping any bubbles while covering the sample in the next step.
6. If you are mounting a 170-µm-thick slice, place three cover slips as spacers (**Pic 2**). If you are mounting a 1-mm-thick slice, place three glass slides. *If you plan to trim/slice your sample again after gel-embedding, spacers are not strictly necessary, as you can section off the extra gel layer.*
7. Place a slide glass in parallel on top of the bottom slide glass.
8. Apply even pressure on the top slide glass to seal the Blu-Tack well. Using a flat object such as another slide glass may be useful. Avoid trapping bubbles within the tissue. This can be a tricky step that might require some practice.
9. Continue to apply pressure until the glass slides are flush with the spacers. Some resistance should be felt from all the three spacers inserted. Ideally, tissue should stay in place when you hold the cassette upright. *If you are mounting an irregular piece of tissue without spacers, press until the sample is slightly compressed.*
10. Remove the glass spacers, if used.
11. Carefully inspect the cassette to check that Blu-Tack has formed a good seal throughout. The cassette will need to be prepared again if **eMAP solution** leaks during the next step.



Pic 2. Sandwiching a brain section with the aid of three spacers. (Thinner wall than pictured here is preferable.)

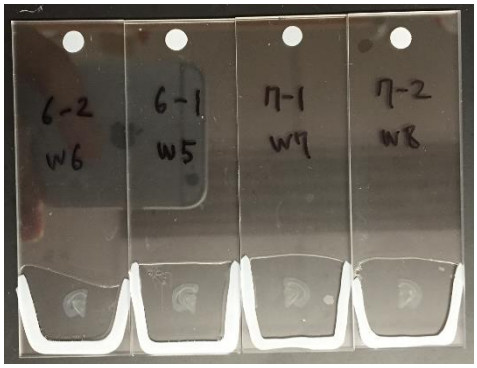
Pic 3. Completion of mounting by filling the well with eMAP solution

12. Hold the cassette upright then carefully add **eMAP solution** between the slide glasses to fill up the Blu-Tack well using a micropipette (**Pic 3**). To avoid trapping bubbles while mounting thin slices, add a small amount of solution first and use gentle taps to cover the inner wall of the well before filling the well with more solution.
13. Once the mounting is finished, quickly proceed to the **Gel embedding** step.

v. Gel embedding

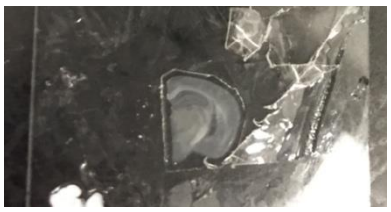
For eMAP gelation, you need an airtight container that can maintain positive nitrogen gas pressure and controlled temperature (at a range of 33 °C to 37 °C). Our setup includes a 50 mL conical tube heater and a customized 50 mL conical tube cap with a tube connection to a nitrogen gas tank and pressure gauge/modulator). Constant purging of the conical tube with nitrogen gas may suffice. Vacuuming is unnecessary. An EasyGel device (LifeCanvas Technologies) can be used for this procedure.

1. Prepare a 37 °C heating environment. Use a lower temperature for thicker samples: 35 °C for 2 mm thick, and 33 °C for 3 mm thick samples. This is to account for the exothermic gelation step. We recommend that you optimize the temperature yourself for eMAP processing thick samples.
2. Insert the cassette prepared in the **Mounting** step into a 50 mL conical tube.
3. Set a nitrogen gas pressure to 10 psi, and purge the tube with a nitrogen gas for at least 15 seconds or longer. If you are gelling thick samples (e.g., >1 mm), cover the top opening of the cassette with tape to prevent splashing any solution during the purging step.
4. Cap the tube. Maintaining a positive nitrogen gas pressure is preferred.



Pic 4. After embedding

5. Place the tube in the heating environment. If the conical tube needs to be placed horizontally, adjust the setup to prevent any leakage from the cassette.
6. Incubate for 2–3 hours.
7. Stop the nitrogen supply, disconnect the tube from the gas line, and retrieve the cassette.
8. Carefully split the slide glasses. You may use a razor blade. Proceed slowly and be careful not to rip the tissue during this step.
9. Cut out the excess gel around the tissue using a razor blade (**Pic 5**). *The excess gel laterally surrounding the tissue restricts a tissue expansion since the pure gel has a lower expansion rate. Therefore, it is recommended to remove the gel. The excess gel can be removed at different steps and depends on your choice of protocol optimization.*



Pic 5. Excess gel around an embedded slice trimmed using a razor blade

vi. Hydration/Additional sectioning

Gel embedded samples must be hydrated before proceeding to the next steps. Additional sectioning may be performed at this point depending on your experiment design.

1. To hydrate your gel embedded sample, incubate it in **PBSN** at 37 °C for several hours (thin sections) or overnight (millimeters-thick samples).
2. After hydration, the sample expands approximately 1.7× linearly. The sample may expand up to 2.0× linearly during this hydration step if you incubate longer (e.g., 2 weeks).
3. At this point, the extra gel, if any, can be removed. The extra gel attached to the original brain surface can be more easily removed by gentle separation using a pipette tip and/or a razor blade. The gel attached to a cut tissue surface may be more difficult to remove. Such gel may be easier to remove after the **Clearing and denaturation** step.
4. Proceed to the **Clearing and denaturation** step if additional sectioning is not required.
5. If desired, embed your sample in an agarose gel for more stable sectioning.
6. For sectioning hydrated eMAP processed tissue using a vibratome, we recommend following operation parameters: feed rate of <0.3 mm/s, amplitude of >0.7 mm and frequency >7 (Leica VT1000S).
7. Hydrated tissues are at 1.7× expansion and it will expand further up to 4×. Keep these factors in mind when choosing the sectioning thickness.
8. Section your sample and collect one or more slices according to your experimental plan.
9. *While the current step after **Gel embedding** and hydration is the optimal point for additional sectioning, eMAP tissues can be further sectioned at a later step (i.e., after clearing and denaturation; even after staining and imaging). In such a case, even slower feed rate and higher vibration frequency may need to be used. Also consider that sectioning an already thin tissue can be challenging and will lead to loss of tissue depending on your mounting method.*

vii. Clearing and denaturation

1. Incubate the hydrated eMAP-processed sample in **Clearing solution** in a conical tube. Note that fully-processed sample will reach up to 2× linear expansion compared to the original size. Choose an appropriately sized container to account for this slight expansion. The **Clearing** step uses detergents to remove lipids from cell membranes to improve macromolecule permeability and optical transparency. For thin (original thickness 100 ~ 200 μm) samples, clear at 37 °C for 6 hours. 1 mm to 2 mm-thick samples (original thickness) can be cleared at 37 °C, which may take several days. For a whole mouse brain, we typically clear at 56 °C for approximately nine days. Clearing time and temperature can be further optimized depending on your purpose. Fully cleared sample should be completely translucent without any opaque regions when inspected on backlight. Periodic inspection should help

you gauge the clearing progress if you are unfamiliar with clearing/delipidation process. *If you want to preserve endogenous fluorescence such as eGFP, avoid clearing temperature above 37 °C.*

2. The 95 °C **Denaturation** step helps achieve a higher and more isometric expansion ratio. The **Denaturation** step quenches endogenous fluorescent proteins, which may or may not be desirable. For thin sections, preheat 17.5 mL of **Clearing solution** to 95 °C then incubate the sample for 10 minutes. Promptly retrieve the sample and move it to **Clearing solution** at RT. Additional optimization may be necessary for thicker samples. *If you want to preserve endogenous fluorescence, skip this denaturation step.*
3. Wash the sample with **PBST** at 37 °C. SDS will interfere with subsequent immunostaining step. To remove large detergent/lipid micelles inside the tissue, wash the sample thoroughly. For thin sections, wash for at least 6 hours with multiple solution exchanges. For larger samples (up to a whole mouse brain) we typically wash up to 2 days with multiple solution exchanges.
4. *After clearing and denaturation, you may try to remove any extra gel if it still exists. An extra gel surrounding the original brain surface can be more easily removed by a gentle separation using a pipette tip and/or a razor blade, but any gel bound to a cut tissue surface may need to be cut by a razor blade.*

viii. Immunostaining

We describe our typical passive immunostaining protocol for thin samples here. You may use your own immunostaining protocol, but we have included several considerations for designing immunostaining experiment for eMAP processed tissues. Uniform labeling of thick (1 mm or thicker) tissue will likely require a specialized protocol. As with all immunostaining experiments, optimizations maybe necessary. All incubation steps are done with gentle shaking.

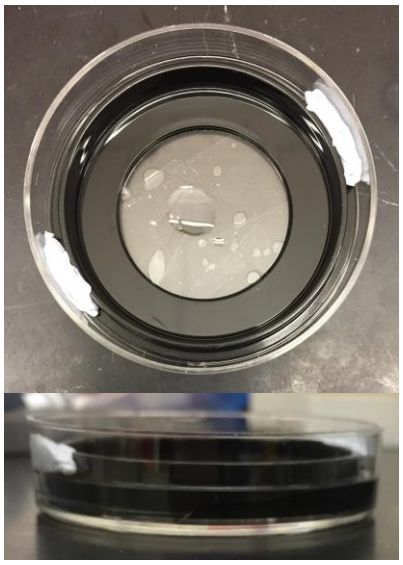
- ✓ Based on our experiments, blocking had no appreciable effect on immunostaining of eMAP tissues.

1. For small, thin samples, either conical tubes or a well plate can be used based on your preference.
2. We typically use 200 µL **PBST** for samples in a 48-well plate, 300 µL **PBST** for samples in a 24-well plate, and 300 µL **PBST** or more for large sample so as to fully immerse the sample.
3. Incubate your samples in **PBST** with appropriate dilution of primary antibody overnight at 37 °C. We have found that for many synaptic targets, a large amount of antibody is necessary to uniformly label eMAP tissue through its entire thickness. Longer incubation may be necessary if you are looking to achieve uniform labeling. This may be due to increased number of antigens accessible for labeling from both improved epitope preservation and slight expansion of the tissue. Also note that there is a 4-fold dilution of fluorescent signal when imaging a fully expanded tissue; therefore, high labeling density is desired.
4. Wash the sample with **PBST** at 37 °C for at least 6 hours with at least three solution exchanges.
5. If using dye-conjugated primary antibodies, proceed to **Expansion, mounting and imaging** step.
6. Incubate your sample in **PBST** with appropriate dilution of secondary antibody for at least 6 hours at 37 °C. Typically molar ratios of 1:2 between primary antibody and secondary antibody is recommended if using full IgG secondary antibodies.
7. Wash the sample with **PBST** at 37 °C for at least 6 hours with at least three solution exchanges.
8. (Optional) If you find that your immunostaining result has excessive non-specific signal, you may opt to use this optional fixation step after primary antibody labeling. Note that this step should not be used if you are performing multi-round immunostaining.
9. After primary antibody staining and washing, incubate your sample in **4% formaldehyde in PBS** for 30 minutes at RT.
10. Wash the sample with **50mM Tris in PBS** for 1 hour.
11. Wash the sample with **PBST** for 1 hour.
12. Proceed to secondary antibody labeling.

ix. Expansion, mounting, and imaging

The expansion solution can be chosen based on your purpose and the stability of your antibodies. While **DI water** is the ideal solution for maximal expansion of eMAP tissue, antibodies may slowly dissociate overtime, which is problematic for long imaging sessions. Using **0.01× PBS** as the **expansion buffer** offers better antibody stability in exchange for slightly reduced expansion ratio. Mounting of eMAP samples for imaging discussed here is for an upright confocal microscope

1. Prepare a petri dish that is large enough for fully expanded tissue and fill with **Expansion buffer**.
2. Place your sample in a petri dish and wait 10 minutes for thin samples. For thicker samples, longer incubation at 37 °C is recommended. Note that it can take up to two days for an eMAP-processed whole mouse brain to fully expand.
3. Exchange the **Expansion buffer** at least once. For thicker samples, multiple exchanges are recommended.
4. For a quick imaging of thin samples, you may transfer the expanded sample to a slide glass with a clean paint brush. Take care when handling expanded tissue as they can be quite fragile.
5. For any longer imaging session, we recommend the following mounting setup utilizing a 60-mm-diameter petri dish and a Wilco dish. This setup enables large-volume scanning which can hours or even days depending on the microscope, objective, and tissue size. Position the fully expanded tissue at the center of the 60-mm-diameter petri dish and position stacked coverslips that match the height of your expanded sample. For small samples we position 18 mm x 18 mm, #1 coverslips in a triangular position. Carefully lower the Wilco dish on to the sample and the spacers. Slowly add 1 mL of **Expansion buffer** to the gap between the side of the Wilco dish and the petri dish, taking care to avoid bubbles. Gently press the Wilco dish down and apply Blu-Tack to the gap to establish a stable mount. Do not press directly on the glass of the Wilco dish as it can easily break. (**Pic 6**)
6. An upright microscope will be used for a sample mounted on a petri dish and a Willco dish. Choose a right microscope, an objective, and light sources for your experimental design. You may need to design an alternate mounting method for use with an inverted microscope.
7. Properly mounted sample should apply very slight compression to the sample to prevent drifting of the sample during imaging. Expanded eMAP tissue could be described as slippery, and it slowly drift without stable mounting. The drifting is especially noticeable under high magnification, high numerical aperture objective. For improperly mounted sample, it might take around half an hour to stop drifting. If your sample keeps moving or drifting during a setup for a long imaging session, check if your expansion buffer has evaporated. Sealing the gap between the petri dish and the Wilco dish with an ointment may be necessary in such case.
8. After imaging, shrink the sample back in **PBST** for storage. The shrinking step is much quicker than the expansion step. If your sample is thick, such quick shrinkage can cause inhomogeneous shrinkage speed between tissue regions, causing sample to crack and be broken. To prevent such damage, shrink your sample with multiple steps using serial dilution of **PBST**. For a thin sample, few minutes in **PBST** is sufficient. For reference, shrinking expanded whole mouse brain takes 1.5 days with four separate solution exchanges with serially diluted **PBST**.



Pic 6. A mounting setup for stable imaging.

Notes on recursive embedding

eMAP processed tissue can be embedded multiple times with the same protocol (steps **v** and **vi**) to achieve a higher total expansion ratio of up to 10× linear expansion at 4-fold embedding. Since each additional embedding adds an extra layer of gel, trimming the tissue after each round of embedding can help with overall reproducibility. Please contact the lab for additional details.