

abcam

Array tomography protocol

Overview

Find out how to perform ultra-high resolution array tomography to uncover previously unseen details of brain molecular architecture.

Array tomography is an ultra-high resolution imaging technique originally developed at Stanford University by Kristina D. Micheva and Stephen J. Smith in order to study neural circuits. Resin-embedded tissue is cut into ordered arrays of ultra-thin serial sections that are mounted onto microscope slides and stained with fluorescent antibodies. The arrays can be eluted and re-stained multiple times in order to reconstruct a three-dimensional image of antigen distribution.

Array tomography offers multiplex, ultra-high resolution volumetric imaging with depth-independent immunofluorescent staining.

Contents

1. Tissue fixation, dehydration and embedding

Discover how to fix, dehydrate and embed tissue for use with array tomography. Tissue fixation immobilizes antigens, whilst preserving molecular architecture. Fixed tissue is dehydrated through immersion in increasing concentrations of ethanol and the water replaced with an acrylic resin. The acrylic resin allows ultra-thin sectioning and repeated immunostaining.

2. Preparation of arrays

Learn how to prepare arrays of serial sections from resin-embedded tissue. Coverslips are treated with a subbing solution to adhere serial sections. The embedded tissue is cut into ultra-thin sections, which are transferred onto coverslips and mounted onto microscopy slides for repeated immunostaining.

3. Immunostaining, elution and data analysis

Read about antibody staining of arrays, repeated elution and data analysis. Arrays are blocked and incubated with fluorescent antibodies before being imaged and analyzed. This process can be repeated multiple times to build a three dimensional image of antigen distribution within single tissue specimens.

[Watch our on-demand webinar](#), presented by Kristina D. Micheva for an introduction to immunofluorescent array tomography.

All protocols are courtesy of Kristina D. Micheva.

1. Array tomography: Tissue fixation, dehydration and embedding

Tissues are fixed, dehydrated and embedded in preparation for array tomography. Find out how to complete the process.

Tissue fixation immobilizes antigens while preserving molecular architecture. Once fixed, tissue is dehydrated using ethanol and the water is replaced with an acrylic resin suitable for ultra-thin sectioning and repeated immunostaining.

Fixation

Tissue can be processed on the bench or using a microwave. The fixative should be prepared on the same day and kept at room temperature.

1. Dissect out the brain and put into a 35 mm Petri dish containing fixative solution.
2. Isolate the region of interest.
3. Transfer the tissue into a 20 ml scintillation vial with fixative solution.

If using a microwave, use approximately 1 ml of fixative per vial, or just enough to cover the tissue. Excessive volume will cause overheating.

4. Microwave

- I. Microwave for 1 min at 100–150 W, stand for 1 min then microwave again for 1 min at 100–150 W.

After microwaving check the glass vial for overheating. If solutions are getting too warm (>37°C), decrease the amount of liquid added.

- II. Microwave three times using a cycle of 20 seconds on - 20 seconds off - 20 seconds on at 350-400 W.
- III. Incubate the tissue in fixative at room temperature for 2–3 h.

5. Bench

- I. If a microwave is not available, fix first at room temperature for 2–3 h and then leave overnight at 4°C. The volume of fixative isn't important for bench top processing, provided it covers the tissue.

Dehydration and embedding

Tissue can be processed on the bench or using a microwave.

1. Wash the tissue twice in wash buffer for 5 min each at 4°C.
2. Transfer the tissue into a 100 mm Petri dish, cover with wash buffer and dissect into smaller pieces (<1 mm in at least one dimension).
3. Return the tissue to the 20 ml scintillation vial and wash twice in wash buffer, for 15 minutes each at 4°C.
4. Microwave

- I. Replace the wash buffer with 50% ethanol at 4°C and microwave samples for 45 s at 350 W.

If using a microwave, use approximately 1 ml of liquid per vial, or just enough to cover the tissue. Excessive liquid volume will cause overheating.

- II. Replace the 50% ethanol with 70% ethanol at 4°C and microwave for 45 s at 350 W.

Stop here if processing samples with GFP or other fluorescent proteins (see below).

- III. Replace the 70% ethanol with 95% ethanol at 4°C and microwave for 45 s at 350 W.
- IV. Replace the 95% ethanol with 100% ethanol at 4°C and microwave for 45 s at 350 W.
- V. Replace the 100% ethanol with 100% ethanol:LR White resin (1:1 mixture) at 4°C . Microwave for 45 s at 350 W.
- VI. Replace the 100% ethanol:LR White resin (1:1 mixture) with 100% LR White resin at 4°C and microwave for 45 s at 350 W. Repeat twice more with fresh resin.

For preservation of GFP fluorescence omit the 95% and 100% ethanol steps. Instead, add 70% ethanol again and then place samples into a mixture of 70% ethanol:LR White resin (1:3; if it turns cloudy add one to two extra drops of LR White resin) and microwave for 45 s at 350W. Replace with 100% LR White resin and microwave for 45 s at 350 W; repeat this twice more.

5. Bench

- I. If a microwave isn't available, perform steps 4.1 to 4.6 by incubating in the indicated solutions for 5 minutes at 4°C.

6. Replace with fresh 100% LR White resin and leave overnight at 4°C.

At this point, if needed, the sample can be left for several days at 4°C in LR White resin.

7. Using a fine paintbrush, place tissue pieces at the bottom of gelatin capsules (paper labels can also be added inside the capsule, Figure 1). Fill to the rim with 100% LR White resin.

8. Close the capsules securely and place into a capsule mold.

Oxygen inhibits LR White resin polymerization, which is why gelatin capsules that completely exclude air are used. The little bubble of air that will remain at the top of the capsule will not interfere with the polymerization.

9. Put capsules into an oven at approximately 53°C for 24 h.

If the oven temperature falls below 50°C, the resin may not polymerize. It is better to set the oven temperature just above 50°C (~53°C) to ensure consistent polymerization.



Figure 1: Gel capsules with paper labels in a gel capsule mold.

Materials and reagents

- Dissection equipment
- 0.02 M PBS (2x)
- 8% Paraformaldehyde (EMS, 157-8)
- Sucrose
- Glycine
- Ethanol (200 proof/absolute)
- LR white resin (hard grade, SPI supplies, 2645)
- Petri dishes (35 mm and 100 mm)
- 20 ml glass scintillation vials
- Gelatin capsules, size 00 (EMS, 70010)
- Capsule holder (EMS, 70161)
- Oven (set at ~53°C)

Optional: PELCO 3451 laboratory microwave system with a ColdSpot set at 12°C (Ted Pella)

Preparation

Fixative: (prepare the same day, keep at room temperature): 4% paraformaldehyde, 2.5% sucrose in 0.01 M PBS.

4 ml: 2 ml 8% paraformaldehyde
 2 ml 0.02 M PBS (2x)
 0.1 g sucrose

Wash buffer: 3.5% sucrose and 50 mM glycine in 0.01 M PBS, use at 4°C (can be prepared in advance and stored at 4°C for up to one month; discard if appears cloudy).

50 ml: 25 mL 0.02 M PBS (2x)
 25 mL H₂O
 1.75 g sucrose
 187.5 mg glycine

Ethanol (keep at 4°C):

50% ethanol
 70% ethanol
 95% ethanol

2. Array tomography: Preparation of arrays

Tissue embedded in acrylic resin is cut into arrays of serial ultra-thin sections, which are transferred to coverslips and mounted onto glass slides in preparation for immunostaining.

Coverslip preparation

1. Place coverslips into a staining rack (ensure coverslips are clean and dry before use).
2. Prepare subbing solution by dissolving 1.5 g gelatin in 290 mL ultrapure water and heating to <math><60^{\circ}\text{C}</math>. Do not overheat. Dissolve 0.15 g chromium potassium sulfate in 10 ml ultrapure water. Combine the two solutions, filter and pour into a staining dish. Use fresh.
3. Immerse the coverslips in the subbing solution for 30–60 s, with gentle agitation.
4. Remove coverslips and drain off excess liquid. Leave in a dust-free place until dry.
5. The dry, subbed coverslips are stored in dust-free boxes until they are needed.

If the coverslips will be used for more than four antibody elutions, they should be carbon-coated using a carbon-evaporator. Carbon-coating will ensure better adhesion of the sections to the coverslips. Aim for a light grey color. After carbon-coating, coverslips are very hydrophobic so leave them several days before use.

Preparation of arrays

1. Trim the block around the tissue to form a pyramid with a small trapezoid-shaped blockface. The trapezoid-shaped blockface is most effective when it has a width of 1 mm or less and its width is greater than its height (Figure 2A).
2. Cut semi-thin sections until you reach the tissue. Trim the block again at this point to ensure that the blockface has not become too large and the leading and trailing edge remain parallel. The Cryotrim 45 diamond knife is very good for this purpose.
3. Using a paint brush, apply diluted Weldwood contact cement (dilute with xylene~1:2) to the leading and trailing sides of the block pyramid. Blot away any excess glue with a tissue.
4. Fill the knife boat of the Jumbo Histo diamond knife with water and insert a coverslip. A stainless steel rod helps prevent the water from receding (Figure 2B).

Carbon-coated coverslips are more hydrophobic so if using these add 0.005% Tween-20 to the water in the knife boat. Keep the water level lower to avoid it jumping onto the blockface. The Tween-20 may dissolve the glue causing ribbon breakage. If this occurs decrease Tween concentration by adding a few drops of water to the knife boat and /or add two coats of glue to the sides of the blockface.

5. After the glue has dried (~2 min), start cutting ribbons of serial sections (60–200 nm) with the Jumbo Histo diamond knife. Thinner sections will adhere better to the coverslip.
6. When the desired length of the ribbon is achieved, carefully detach it from the knife edge using an eyelash probe. Remove the stainless steel rod and gently

push the ribbon towards the coverslip so that the edge of the ribbon touches the glass at the interface of the glass and the water. The edge of the ribbon will then stick to the coverslip.

- Using a syringe, slowly lower the water level in the knife boat until the entire ribbon sticks to the coverslip. Take out the coverslip. The position of the ribbon can be indicated using a permanent marker (but only mark the opposite side of the coverslip to the sections).
- Lay the coverslips flat to dry before placing them on a slide warmer (~55°C) for 30 minutes. The coverslips can be stored at room temperature for at least 3 months.

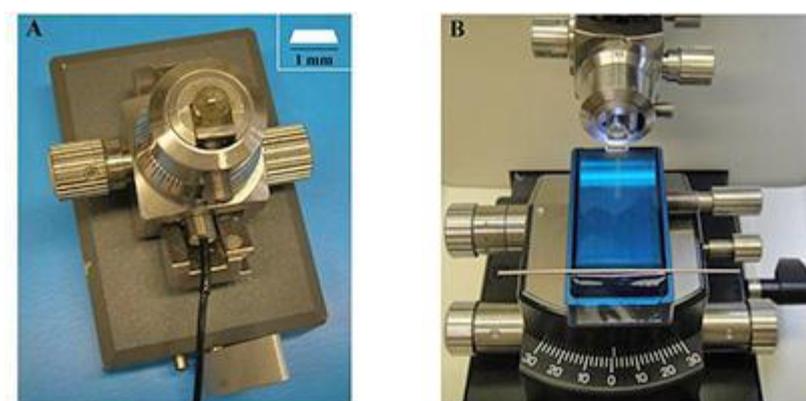


Figure 2: Sectioning of the embedded tissue. (A) The block is trimmed to form a pyramid with a small trapezoid-shaped blockface. The width of the trapezoid should be 1 mm or less with a width greater than its height. (B) Serial sections of 60–200 nm are cut using the Jumbo Histo diamond knife. The stainless steel rod helps to prevent the water receding.

Materials and reagents

- VWR Micro Cover Glasses (No. 1.5, 24 x 60 mm), or for quantitative studies use Bioscience Tools High Precision Glass Coverslips (CSHP - No. 1.5 - 24 x 60 mm)
- Gelatin (300 Bloom)
- Chromium potassium sulfate (chrome alum, $\text{KCr}(\text{SO}_4)_2 \cdot 12(\text{H}_2\text{O})$)
- Staining rack and dish
- Jumbo Histo diamond knife (Diatome)
- Cryotrim 45 diamond knife (Diatome) - optional
- Ultramicrotome
- Weldwood Contact Cemen
- Xylene
- Thin paint brush
- Slide warmer
- Eyelash probe
- Stainless steel rod
- Syringe and filter

Additional materials and reagents for carbon-coated coverslips:

- Tween-20
- Carbon rods (e.g. Ted Pella #93010)
- Carbon evaporator (e.g. Cressington carbon-coater 308R)

3. Array tomography: Immunostaining, elution and data analysis

Find out about antibody staining of arrays, repeated elution and data analysis in array tomography.

The section arrays are labeled with fluorescent antibodies or other fluorescent stains and imaged to generate ultra-high resolution 3D images. The sections can be restained multiple times to analyse large numbers of antigens within a single tissue specimen.

Preparation

- 50 mM glycine in TBS: 4 mg glycine in 1 ml of TBS.
- Blocking solution (0.05% Tween-20 in TBS): Make a 1% stock of Tween-20 (10 μ l Tween in 1 ml of ultrapure water). Then add 50 μ L of the 1% Tween stock solution to 0.94 ml TBS.
- Elution solution (0.2 M NaOH and 0.02% SDS in ultrapure water): To prepare, add 200 μ L of NaOH (10 N) and 10 μ L SDS (20%) to 10 ml of ultrapure water. Store at room temperature for up to six months.

Immunostaining

1. Encircle the sections with a PAP pen, leaving space at the ends of the ribbon.
2. Put the coverslips in a Petri dish or box and put wet KimWipes around the edges to prevent evaporation of solutions. Keep the dish closed during incubation times.
3. Pipette approximately 150 μ l 50 mM glycine into the circle drawn by the PAP pen and incubate the sections for about five minutes at room temperature. Glycine quenches autofluorescence and also helps to block non-specific antibody binding.
4. Remove the glycine and apply approximately 150 μ l blocking solution for about five minutes (no need to wash in between).

After this step, it is important not to let the sections dry out.

5. Dilute primary antibodies in blocking solution. This is usually 1:50 to 1:100 from a 1mg/ml stock. Spin down the antibody solution at 13,000 rpm for 2 min before applying to sections.
6. Remove blocking solution and add approximately 150 μ l diluted primary antibodies to the sections (there is no need to wash in between). Incubate for 2 h at room temperature or overnight at 4°C (incubation time depends on the antibody being used and will require optimization).
7. Wash the sections with TBS. Washing is achieved by creating a continuous flow of buffer across the sections; pipette TBS onto one end of the sections and remove it with another pipette from the other end. Do this repeatedly for 15 min, for 10–15 s each time (Figure 3).
8. Dilute secondary antibodies in blocking solution. This is usually 1:150 from a stock of 2 mg/ml. Spin down the antibody solution at 13,000 rpm for 2 min before applying to sections.

9. Add approximately 150 μL diluted secondary antibodies to the sections and incubate for 30 min at room temperature. Keep in the dark.
10. Wash with TBS as described in Step 7.
11. Wash the sections with filtered ultrapure water. Wash the sections once as described in Step 7, then wash the entire coverslip by holding under a stream of ultrapure water expelled from a syringe with a filter.

At this point it is very easy for the sections to dry out, so be careful to always leave some water behind.

12. Mount the coverslip onto a glass microscope slide. Remove some, but not all of the water, then add a couple of drops of mounting medium to one end of the array. The mountant will repel any remaining water and it can be removed from the other end. Aim for just enough mounting medium to cover the array of sections.
13. Turn the coverslip over and slowly lay over a glass slide. If bubbles form, the coverslip can be removed, washed with water and mounted again. If there is too much mounting medium (it comes out from the edges and the coverslip slides around) then blot away with a tissue.
14. Once mounted, carefully clean any dust (this will be visible under the microscope) from the coverslip and slide by wiping with 70% ethanol.

Image as soon as possible after staining or at least the same day. For some antigens, the staining may be very weak and not visible with low magnification objectives. Use a high magnification objective (e.g. 63x or 100x) and longer exposures (up to several seconds) for some antigens if necessary.

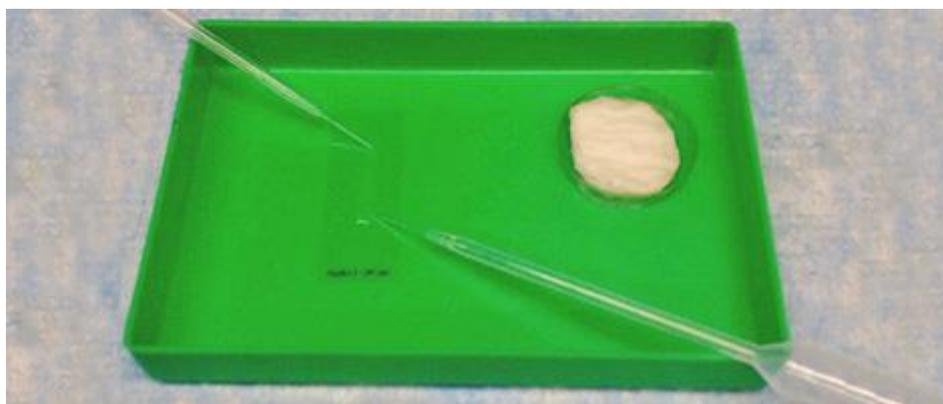


Figure 3: Washing of sections is achieved by creating a continuous flow of buffer across the sections; pipette TBS onto one end of the sections and remove it with another pipette from the other end.

Elution

1. Add water around the edge of the coverslip to detach it from the microscope slide. Wait about one minute.
2. The coverslip will float up on the water; pick it up with tweezers and wash away mounting medium with ultrapure water.

Ideally, perform this step immediately after imaging. Leaving the sections too long in mountant will decrease the quality of subsequent immunolabeling. After washing off the mountant, the sections can be left in TBS until the elution step.

3. Apply the elution solution for 20 min at room temperature (add the solution gently to the sections, do not wash with the elution solution; if the sections are not well attached to the glass they may start detaching at this point). The elution time may vary for different antibodies; it can be tested by applying only the secondary antibody after elution and checking for remaining fluorescence.
4. Wash with TBS for 15 minutes, as described in Step 7 of the Immunostaining section. The initial wash should be slow.
5. Rinse the entire coverslip with water, as described in Step 11 of the Immunostaining protocol.
6. Once the water has dried, place the coverslip on the slide warmer (55°C) for 30 min.
7. Antibody incubations can now be performed as before in the Immunostaining section of this protocol.

Data analysis

For a description of the recommended software for data analysis, please refer to the following references:

- Micheva KD, Busse B, Weiler NC, O'Rourke N and Smith SJ (2010). Single-Synapse Analysis of a Diverse Synapse Population: Proteomic Imaging Methods and Markers. *Neuron* 68, 639–653.
- Micheva KD and Smith SJ (2007). Array Tomography: A New Tool for Imaging the Molecular Architecture and Ultrastructure of Neural Circuits. *Neuron* 55, 25–36.

Materials and reagents

- PAP pen
- Tris-buffered saline (TBS)
- Glycine
- Bovine serum albumin
- Primary antibodies
- Secondary antibodies: the appropriate species of Alexa Fluor 488, 594 and 647, IgG (H+L), highly cross-absorbed (Invitrogen)
- Samco™ fine tip transfer pipettes (Thermo Scientific 13-711-31)
- SlowFade Gold antifade mountant with DAPI (Invitrogen)
- Gold Seal™ Rite-On™ Frosted Microslides (Thermo Scientific)
- Ultrapure water
- 50 ml syringes
- Syringe filters
- NaOH (10 N)
- 20% SDS