

# Array tomography protocol: immunostaining, elution and data analysis

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

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## Overview

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  $\mu$ l Tween in 1 ml of ultrapure water). Then add 50  $\mu$ 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  $\mu$ l of NaOH (10 N) and 10  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ L 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