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Synaptic Evaluation and Quantification by Imaging Nanostructure (SEQUIN) for Excitatory and Inhibitory Synaptic Loci in Mouse Cortex

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Saroj Sah^{1,2}, Thomas Biederer^{1,2}

¹Department of Neurology, School of Medicine, Yale University, New Haven, CT 06510, USA;

²Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20815, USA



Maria Matos

Yale University

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We use this protocol and it's working

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Abstract

SEQUIN is an imaging and analysis platform for the quantification and characterization of synaptic structures in the mouse brain, developed by Sydney J. Reitz, Andrew D. Sauerbeck, and Terrance T. Kummer (STAR Protocols 2.1, 2021: 100268). It enables quantification of synaptic loci and the separation of pre- and post-synaptic proteins. We have optimized the SEQUIN method to analyze synapse subtypes, including excitatory and inhibitory synaptic loci, specifically in the mouse prefrontal cortex.



Materials

Reagents

- PBS
- normal goat serum (Jackson Immuno Research)
- VGluT1 (Synaptic Systems, Cat. No. 135 304)
- VGLUT2 (Millipore, Cat. No. AB2251)
- Homer1 (Synaptic Systems, Cat. No. 160 003)
- VGAT (Synaptic Systems, Cat. No. 131 003)
- Gephyrin (Synaptic Systems, Cat. No. 147 111)
- Triton X-100
- sodium azide
- Alexa dyes 488, 568, and 647 (Thermo Fisher)
- DAPI
- Tris-MWL 4-88 (Electron Microscopy Sciences #17977-150, Hatfield, PA, USA)
- AF300 (Electron Microscopy Sciences #17977-25)

Equipment

- ZEISS LSM 900

 VGLUT1 antibody **Synaptic Systems Catalog #135 318**

 Anti-VGluT2 Antibody **Merck MilliporeSigma (Sigma-Aldrich) Catalog #AB2251-I**

 Homer1 antibody **Synaptic Systems Catalog #160 003**

 VGAT antibody cytoplasmic domain **Synaptic Systems Catalog #131 003**

 Gephyrin antibody **Synaptic Systems Catalog #147 111**

 Citifluor Mwl4-88 **Electron Microscopy Sciences Catalog #17977-150**

 Citifluor Af300 **Electron Microscopy Sciences Catalog #17977-25**



Staining with antibodies

1d 13h 50m

1 Select brain sections for staining.

1.1 Fill Petri dish with PBS and select brain slices using a paintbrush.

1.2 Transfer selected sections (3 sections per well) into a new well in a 24-well plate containing PBS.

2 Wash sections in PBS.



2.1 Wash sections in PBS for  00:05:00 on a shaker at a lower speed. (1/3)

5m

2.2 Wash sections in PBS for  00:05:00 on a shaker at a lower speed. (2/3)

5m

2.3 Wash sections in PBS for  00:05:00 on a shaker at a lower speed. (3/3)

5m

3 Incubate sections in blocking solution ( 350–500 μ L per well) for  01:00:00 at  Room temperature .

1h



- Blocking solution in PBS contains: 20% normal goat serum (Jackson ImmunoResearch).

4

- After blocking, drain off the blocking solution and incubate the sections with the primary antibody ( 350 μ L per well) for 24–48 hours at  Room temperature .



Note

We have optimized SEQUIN for VGluT1 (Synaptic Systems, Cat. No. 135 304, dilution 1:200), VGLUT2 (Millipore, Cat. No. AB2251, dilution 1:300), and Homer1 (Synaptic Systems, Cat. No. 160 003, dilution 1:250) to analyze excitatory synaptic loci, as well as VGAT (Synaptic Systems, Cat. No. 131 003, dilution 1:250) and Gephyrin (Synaptic Systems, Cat. No. 147 111, dilution 1:250) to analyze inhibitory synaptic loci. Inhibitory synaptic proteins primary antibodies were incubated for  36:00:00 at  4 °C .

4.1

1d 12h

- Antibody solution in PBS contains Normal goat serum, Triton X-100 and Sodium azide as mentioned below and antibodies at appropriate dilution, mix well, centrifuge the mixture at $10,000\text{--}17,000 \times g$ for 5 min and incubate the sections in this antibody solution.

A	B
Normal goat serum	10%
Triton X-100	0.3–0.45%
Sodium azide	0.02–0.05%

Note

A higher concentration, 0.45% Triton X-100, may enhance the penetration of the VGLUT1 antibody for VGLUT1 / Homer staining.

4.2 Cover the wells with parafilm to stop evaporation during incubation.

5 Wash sections in PBS.



5.1 Wash sections for  00:05:00 in PBS. (1/3)

5m

5.2 Wash sections for  00:05:00 in PBS. (2/3)

5m

5.3 Wash sections for  00:05:00 in PBS. (3/3)

5m

6 Incubate sections with secondary antibodies conjugated to Alexa dyes 488, 568, and 647 (Thermo Fisher, dilution 1:250) diluted to a working concentration in antibody solution ( 350 μL per well).



Note

Secondary antibody incubation can be done at  Room temperature for

 04:00:00

or at

 4 °C

 Overnight .

- Antibody solution in PBS contains

A	B
Normal goat serum	10%
Triton X-100	0.3–0.45%
Sodium azide	0.02%

6.1 Cover the plate with parafilm and then aluminum foil to stop evaporation and protect from light.

7 Wash sections in PBS.



7.1 Wash sections for  00:05:00 in PBS. (1/3)

5m

7.2 Wash sections for  00:05:00 in PBS. (2/3)

5m

7.3 Wash sections for  00:05:00 in PBS. (3/3)

5m

8 Incubate sections in DAPI to counterstain cellular nuclei for 5–10 mins.



9 Wash the section with PBS for  00:05:00 .

5m



10 Mount sections on charged slides. Once the PBS adhered to slices dries, dunk slide in distilled water 5X quickly to remove salt residue. Allow to dry again in dark, flat location.

- 11 Mount the mounting media freshly prepared by mixing Tris-MWL 4-88 (Electron Microscopy Sciences #17977-150, Hatfield, PA, USA) with AF300 (Electron Microscopy Sciences #17977-25) in a 9:1 ratio using 1.5H coverglass (cleaned with ethanol, followed by water, and dried before mounting). Keep the slides flat at  Room temperature for 3-5 days to allow the mountant to cure and be ready for imaging.

Image Acquisition & Analysis

- 12 Turn on ZEISS LSM 900 with Airyscan 2 confocal microscope with 63× 1.4 NA oil objective.
- 13 Open Zeiss ZEN software and navigate to Smart Setup followed by airyscan detection mode and "+" select the dye/dyes to image from the list.
- 14 In imaging setup, set up desired airyscan channels, and adjust emission range and laser power in "Live" mode for the sample to be imaged.
- 15 In acquisition mode, set crop area (zoom) greater or equal to 3.1X, set speed to maximum, and bidirectional. Choose the "SR" button to choose image pixels that are optimal for Airyscan.
- 16 In channel setting, choose the "Live" button to see an image of sample, adjust the Master Gain (up to 800-850) and the laser power until 1/3 of the dynamic range of pixel intensity is occupied, as represented by the histogram in the "Display" tab.
- 17
 - Select a region of interest with the brightest signal.
 - In the Acquisition window, turn on Continuous mode, check only one channel of interest in the Channel window and go with Continuous mode. While the continuous mode runs, open the Airyscan view (honeycomb icon at the bottom of the software).
 - A display of the 32 detectors is depicted. The brightest detector should be the central element. If not, give the scope several moments to auto-adjust.
 - Do not proceed until the brightest detector is centered with a green signal confirming airyscan is configured optimally.
- 18 Within the Z-stack window, set the step size ("Interval") to 120 nm, and set the number of optical sections ("Slices") to 35 in "Live" mode.
- 19 Adjusting all the image acquisition parameters, select "begin experiment" to begin the scan and save the image once scanning is done.

Image pre-processing

- 20 Select "Processing" tab within Zeiss ZEN software and choose "Airyscan Process" from dropdown menu.
- 21 With the image of interest open, choose "Select Image."
- 22 Determine Airyscan strength for many images in Zeiss Zen (lowest auto-determined Airyscan strength may be 6) and apply empirically determine this value for a given system and experiment.
- 23 Choose "3D" option and click "Apply."
- 24 Save the new image as CZI.

Channel alignment of airyscan processed images

25

Note

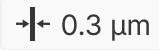
Different channels may not align for the same punctum in a few microscopes with different Alexa dyes, so it is necessary to determine the channel alignment value.

- Perform staining using a primary antibody that provides the best signal with minimal background.
 - These slices should then be co-stained with two secondary antibodies conjugated to different dyes, for which channel alignment is required.
 - Perform imaging and pre-processing.
- 26 Turn on the "Processing" tab within Zen Black software.
 - 27 Choose "Channel Align" from the dropdown menu in the left sidebar.
 - 28 Open previously acquired and Airyscan Processed channel alignment image, followed by "Select Image" in the left sidebar.
 - 29 Unselect "Fit."

- 30 Offset one channel in Y such that channels are no longer overlapping to facilitate visualization of chromatic aberration.
- 31 View the orthogonal projection of this image and focus on one dual-labeled punctum. Manually shift the Z value for one of the channels until the channel images are fully aligned. Record the Z value offset.
- 32 Once the image has been aligned, confirm the suitability of adjustment value on multiple puncta throughout the image.
- 33 Open the previously acquired and Airyscan processed image and choose "Select Image" in the left sidebar, unselect 'FIT', input the recorded Z.
- 34 Click "Apply" to create a new image and save as new CZI.

Puncta detection

- 35 Place all airyscan processed and channel-aligned images in a single folder.
- 36 Open Imaris.
- 37 Click "Observe Folder" and navigate to the folder of CZI images.
- 38 Choose "Select Folder", folder will be added to list of "Observed Folders."
- 39 Open the folder in Imaris (double-click).
- 40 Select all images in the folder, right-click, and select "convert to IMS."
- 41 Select "New Batch" in top menu.
- 42 When a preview of the image appears, select "Next" to begin object creation.

- 43 To create spots, click "Spots" button. Create 2 spots (one for pre, and another for postsynaptic protein) for analysis.
- 44 Set parameters for channel, size, and boundaries of spot creation in the "Create" window.
 - 44.1 "Algorithm Settings": leave all unchecked. Advance to the next screen.
 - 44.2 "Source Channel": choose the source channel for spot detection.
 - 44.3 Under "Spot Detection" (same screen). Check "Model PSF-elongation" along Z-axis and "Background Subtraction."
 - 44.4 "Estimated XY Diameter" for punctum is generally set to 0.2 μM .
 - 44.5 "Estimated Z Diameter" for punctum is generally set to 0.6 μM .
 - 44.6 Navigate to the next screen.
 - 44.7 Create two filters.
 - 44.8 Highlight one filter and change it to "Distance to Image Border XY Img=1" above automatic threshold.
 - 44.9 Set the leftmost value of this filter to  (half XY diameter from above) and ensure rightmost value is not activated.
 - 44.10 Change the second filter to "Position Z."
 - 44.11 Set the left and right values so they are  (half Z diameter from above) from the extremes.

**Note**

Ex: an image that ranged from 0.06 to 4.14 in Z would be filtered between 0.36 and 3.84.

- 44.12 Click the green arrow to complete the setup process for this analysis.
- 44.13 Repeat all the steps for the second Spot/channel.
- 45 When both spots have been created and edited, choose the "OK" button in the Objects window.
- 46 Name the batch.
- 47 Right click the new batch and select "Run Batch" to run on all images in the folder. After the batch finishes, export the results in the CSV file format.

Pre-to-postsynaptic puncta separation analysis

- 48 Download the necessary MATLAB codes required for SEQUIN analysis (<https://github.com/KummerLab/SEQUIN>).
- 49 Analyze separation distances.
- 49.1 Open MATLAB.
- 49.2 Within the MATLAB "Current Folder" window, edit the "Analysis parameters.m" file to reflect all needed parameters, such as, designated channels in Imaris for Spot1 and Spot2, folder name of Imaris outputs and the frame size of the image, intensity interval for spot selection ([80 100] for VGLUT1 and VGAT positive synaptic analysis, and [40 100] for VGLUT1 positive synaptic loci) and hit "Run" button at the top of the window. This script will analyze the data, which will appear in a newly created "data output" folder.





- 50 Compile Pre-to-Postsynaptic Puncta Separation Analysis data into one workbook.
- 50.1 Within the MATLAB "Current Folder" window, navigate the compileExceldocsparemeter.m code.
- 50.2 Edit it to reflect all needed parameters, and press enter.
- 50.3 This will compile all Pre-to-Postsynaptic Puncta Separation Analysis outputs into a single file in a newly created "compileddata" folder.

Data representation

- 51 Retrieve generated pre-to-postsynaptic separation distances.
- 51.1 In the newly created compileddata, find the Excel file, and navigate the 'Flip Sub Zeros Frequency' worksheet which will contain frequency distributions of paired pre-post synaptic loci sorted into bins of 20 nm for each image analyzed. Each column represents different images analyzed.
- 52 **Graph data**
- 52.1 Plot the frequency distribution of synaptic loci data found on the "Flip Sub Zeros Frequency" tab in the GraphPad prism against a geometric series "10 nm, 30 nm, 50 nm, [...]".

Note

The Gaussian curve can be fitted to the Flip Sub Zeros Frequency and the peak value of the Gaussian curve represents the mean pre-postsynaptic separation of the synaptic loci.

- 52.2 Sum all the synaptic loci falling within the Gaussian curve and determine the density of synaptic loci (VGluT1 / Homer, VGAT / Gephyrin, VGLUT2 / Homer).



Protocol references

Reitz, S. J., Sauerbeck, A. D., & Kummer, T. T. (2021). SEQUIN: An imaging and analysis platform for quantification and characterization of synaptic structures in mouse. *STAR protocols*, 2(1), 100268.

Sauerbeck, A. D., Gangolli, M., Reitz, S. J., Salyards, M. H., Kim, S. H., Hemingway, C., ... & Kummer, T. T. (2020). SEQUIN multiscale imaging of mammalian central synapses reveals loss of synaptic connectivity resulting from diffuse traumatic brain injury. *Neuron*, 107(2), 257-273.

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