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Fluorescence immunohistochemistry for quantitative imaging and analysis

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We use this protocol and it's working

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Abstract

Quantitative imaging and analysis of fluorescently stained tissue sections is sensitive to multiple factors. Two critical ones are endogenous autofluorescence and careful preparation of antibodies. This protocol provides methods for reducing the impact of autofluorescence through high-intensity broad wavelength light exposure. Additionally, it outlines a stepwise protocol for creating primary antibody Master Mixes which are then subsequently combined together, or with buffer, to ensure consistency in antibody concentrations across all conditions.

Materials

Reagents:

	A	B	C
	Reagent	**Vendor**	**Cat#**
	10x Stock PBS	Corning	MT-46013CM
	Normal Goat Serum	Vector	S-1000-20
	Glycine	Sigma Aldrich	G7126-1KG
	Acetamide	Sigma Aldrich	A0500-500G
	Sodium Azide	Sigma Aldrich	S2002-100G
	Ethylene Glycol	Sigma Aldrich	102466-1L
	Sucrose	Fisher Chemical	S5-500
	10000K Finisher Bulb	HTG Supply	LAM-AM24T5AK-B
	Mowiol 4-88	Electon Microscopy Sciences	17977-150
	AF-300	Electon Microscopy Sciences	17977-25
	1.5H coverglass	Marienfeld	107242
	DAPI	Invitrogen	D3571

- 10X Phosphate-Buffered Saline **Corning** **Catalog #**46-013-CM
- Normal Goat Serum **Vector Laboratories** **Catalog #**S-1000-20
- Glycine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G7126
- Acetamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A0500-500G
- Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S2002-100G
- AgroMax 2 Foot T5 Finisher Bulb – 10000K **HTG Supply** **Catalog #**LAM-AM24T5AK-B
- Citifluor Mwl4-88 **Electron Microscopy Sciences** **Catalog #**17977-150
- Citifluor Af300 **Electron Microscopy Sciences** **Catalog #**17977-25
- DAPI **Invitrogen - Thermo Fisher** **Catalog #**D3571

Light Treatment and Blocking

2d 1h 10m

1 Remove formaldehyde fixed brain cryosections sections from storage plates containing cryopreservation solution (30% Sucrose + 30% Ethylene Glycol + 0.02% Sodium Azide in 1X PBS).

2 Rinse tissue sections three times in 1X Phosphate Buffered Saline (PBS) for 00:05:00 each. 5m

3 Transfer sections to 48 well tissue culture plate containing 1x PBS.

4 Carefully remove all PBS and replace with 'Light Treatment' buffer (1x PBS + 4% Glycine + 4% acetamide + 0.05% Sodium Azide).

5 Ensure all sections are flat on the bottom of each well.

6 Seal wells containing tissue sections with clear Duck Brand EZ-Start tape.

7 Turn on high-intensity light bulbs (AgroMax T5 Finisher Bulb – 10000k) and ensure that the intensity of light at the surface of each bulb is greater than 400 Lux. Re-measure Lux every 24:00:00 .

8 Position the tissue plate such that each section is positioned directly above the bulb.

9 Expose the tissue sections to >400 Lux light for 40:00:00 - 48:00:00 at Room temperature . 2d

10 Rinse tissue sections three times in 1X PBS for 00:05:00 each.

11 Block tissue sections for 01:00:00 at Room temperature with 20% NGS + 0.3% TritonX-100 + 0.02% Sodium Azide in 1X PBS. 1h 5m

- Blocking buffer must be centrifuged at 17500 x g, 00:05:00 before use.

Primary Antibody Preparation

3d 0h 10m

12 Make 'Master mix' of each primary antibody such that the antibody concentration is 3x greater than the final target working concentration. Combine Master mixes with each other or primary antibody buffer to create antibody cocktails for Experimental tissue sections and No Primary controls. Primary
antibody buffer is 10% normal goat serum (NGS) + 0.3% TritonX-100 + 0.02% Sodium Azide in 1X PBS .

13 Thoroughly mix all solutions using brief pulses on a bench top vortex.

14 Centrifuge all final antibody mixes at 17500 x g, 00:05:00 . 5m

15 Remove all 1X PBS from wells with tissue sections and replace with primary antibody mixture.

16 Seal wells with tissue sections with clear Duck Brand EZ-Start tape.

17 Place tissue sections on shaker at Room temperature , protect from light, and incubate primary antibodies for 64:00:00 - 72:00:00 . 3d

18 Rinse tissue sections three times in 1X PBS for 00:05:00 each. 5m

Secondary Antibody and DAPI Staining

1d 0h 55m

19 Create mixture of secondary antibodies in 10% NGS + 0.3% TritonX-100 + 0.02% Sodium Azide in 1X PBS.

20 Centrifuge all final secondary mixes at 17500 x g, 00:05:00 . 5m

21 Remove all 1X PBS from wells with tissue sections and replace with secondary antibody mixture.

22 Seal wells with tissue sections with clear Duck Brand EZ-Start tape.

23 Place tissue sections on shaker at Room temperature, protect from light, and incubate secondary antibodies for 18:00:00 - 24:00:00. 1d

24 Rinse tissue sections three times in 1X PBS + 0.3% TritonX-100 for 00:15:00 each. 15m

25 Remove PBS + TritonX-100 and replace with DAPI in 1X PBS at a concentration of 1:50,000.

26 Incubate tissue sections in DAPI for 00:10:00 - 00:20:00. 20m

27 Rinse tissue sections two times in 1X PBS for 00:15:00 each. 15m

Mounting

28 Mount tissue sections on charged histology slides in 1X PBS.

29 Remove excess PBS from around tissue sections and then allow slides to dry at a 45-degree angle protected from light.

30 Immediately when sections have fully dried, quickly dip the sections 5 times in deionized water to rinse off excess salts.

31 Remove excess water from around tissue sections and then allow slides to dry at a 45-degree angle protected from light.

32 Immediately when sections have fully dried, apply Mowiol 4-88 mounting media that has been freshly prepared the day of staining. The Mowiol mounting is made by mixing

Mowiol 4-88 with AF-300 in a 9:1 ratio. The mixture must be thoroughly vortexed and then centrifuged to remove all bubbles. Apply 300 µL - 600 µL of mountant mixture to the slide.

33 Apply thoroughly cleaned 1.5H coverglass with gentle pressure to expel excess Mowiol from underneath the coverglass.

34 Allow the slides to dry horizontally protected from light for at least 24:00:00. The mountant will continue to harden over time and 3-7 days of curing is recommended to ensure solid curing and improved refractive index matching.

Protocol references

Sauerbeck AD, Gangolli M, Reitz SJ, Salyards MH, Kim SH, Hemingway C, Gratuze M, Makkapati T, Kerschensteiner M, Holtzman DM, Brody DL, Kummer TT. SEQUIN Multiscale Imaging of Mammalian Central Synapses Reveals Loss of Synaptic Connectivity Resulting from Diffuse Traumatic Brain Injury. Neuron. 2020 Jul 22;107(2):257-273.e5. doi: 10.1016/j.neuron.2020.04.012. Epub 2020 May 8. PMID: 32392471; PMCID: PMC7381374.