

Cellular Localization of Innate Immune Proteins with Glial Cells in Paraformaldehyde-fixed Paraffin-Embedded Brain Tissue Sections --Manuscript Draft--

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PROTOCOL TITLE:

Cellular Localization of Innate Immune Proteins with Glial Cells in Paraformaldehyde-fixed Paraffin-Embedded Brain Tissue Sections

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KEYWORDS:

Astrocytes; Glioma; Innate Immunity; Microglia; NLRs, NLRX1

PROTOCOL OVERVIEW:

The central nervous system (CNS) relies on glial cells, including astrocytes and microglia, to maintain homeostasis and support neuronal activity. Microglia are the resident immune cells of the CNS. Microglia comprise approximately 10% of all cells in the CNS, with regional differences¹. They originate from the yolk sac, regulate neurogenesis, support neuronal survival, and take part in synaptic pruning during early development^{2,3}. Astrocytes are multi-functional, star-shaped glia that regulate the blood-brain barrier, cerebral blood flow, neurotransmitter cycling, extracellular fluid homeostasis, neuronal metabolism, and synaptic functions⁴⁻⁶. The percentage of astrocytes in the human brain ranges from 17% to 61 %, depending on the region⁷. Astrocytes and microglia are key regulators of innate immunity in the CNS. Astrocytes and microglia can also present antigens and regulate the production of various cytokines and chemokines⁸. By regulating immunity, microglia and astrocytes play a crucial role in various neuroinflammatory diseases, including brain cancer⁹. Thus, studying innate immune signaling in astrocytes and microglia is necessary to understand the physiology of various CNS diseases.

This protocol presents a method for assessing the cellular localization of innate immune proteins in astrocytes and microglia in paraformaldehyde-fixed, paraffin-embedded human brain tissue sections. Here, we demonstrate the cellular localization of the innate immune protein NLRX1 in microglia and astrocytes (**Figure 1**). NLRX1 is an NLR (Nucleotide-binding domain, Leucine-rich repeat containing protein) family member, regulating diverse aspects of innate immune signaling¹⁰. A major strength of this protocol is its ability to simultaneously visualize NLRX1 and cell-specific markers within the native tissue architecture, enabling assessment of spatial distribution and cellular localization¹¹. The protocol is compatible with both paraffin-embedded and cryopreserved tissue sections following appropriate optimization of antigen retrieval and staining conditions^{12,13}. Furthermore, the approach can be adapted to investigate the

colocalization of NLRX1 with other cellular markers. The protocol is most useful for studies examining neuroinflammation, innate immune signaling, mitochondrial biology, and glial cell responses in neurological disorders^{12–14}. It is particularly suitable when cell-type-specific localization of NLRX1 within brain tissue is of interest.

ETHICS DISCLAIMER:

The paraffin-embedded paraformaldehyde-fixed glioma (grade 2, Astrocytoma) and normal brain tissue were obtained with approval from the Internal Review Board and the Ethics Committees of AIIMS, Jodhpur, and Tata Memorial Cancer Hospitals. Informed consent was acquired for the use of tissue samples for experiments from human participants. All experiments were performed in accordance with the ethical guidelines and regulations of All India Institute of Medical Sciences, Jodhpur, and Indian Institute of Technology Jodhpur.

PROTOCOL:

The reagents and the equipment used are listed in the **Table of Materials**.

1. Procedure for fluorescent colocalized staining of NLRX1 and microglia

1.1 Preparation

1.1.1 Ethanol: Prepare 90% and 75% ethanol in 1x-PBS (200 mL) and store at room temperature (RT).

1.1.2 Permeabilization buffer: Prepare permeabilization buffer by adding 20 μ L of 0.1% Triton X-100 to 1x-PBS (20 mL) and store at RT.

1.1.3 RCA (Ricinus Communis Agglutinin) solutions: Prepare RCA dilution (1:500) in permeabilization buffer. Prepare freshly for each experiment.

1.1.4 Blocking buffer: To make a blocking buffer, add 5% Fetal Bovine Serum (FBS, 0.5 mL) to the permeabilization buffer (10 mL) and store at 4 °C. Prepare freshly for each experiment.

1.1.5 Primary antibody dilutions: Prepare anti-NLRX1 rabbit primary antibody dilution (1:1000) in blocking buffer. Prepare freshly for each experiment.

1.1.6 Secondary antibody dilutions: Prepare anti-rabbit fluorophore-conjugated secondary antibody ($\lambda_{\text{excitation}}$ 594 nm) dilutions (1:1000) in blocking buffer. Prepare freshly for each experiment.

1.2 Staining

1.2.1 Wash the tissue sections twice with Xylene for 20 min.

88 NOTE: Paraformaldehyde-fixed paraffin-embedded glioma (Astrocytoma, grade II) and normal
89 brain tissue sections (5 μ m thickness) were used for the staining protocol. Normal tissue sections
90 were taken from different parts of the central nervous system, including the corpus callosum,
91 ventricular section, and sensory cortex.

92

93 CAUTION: Xylene is harmful upon direct contact with skin or eyes and long-term exposure.

94

95 1.2.2 For rehydration of tissue sections, wash them with a series of decreasing ethanol
96 concentrations. Start the tissue section washing with 100% ethanol (2x), followed by 90% (2x),
97 and 75% ethanol (2x). Each wash lasts 8 min.

98

99 1.2.3 Rinse the tissue sections thrice for 5 min each in 1x-PBS (~500 μ L).

100

101 1.2.4 Wipe the PBS around the tissue sections using lint-free tissue paper.

102

103 1.2.5 Mark around the tissue section using a hydrophobic marker or wax pencil and add a few
104 drops of 1x-PBS to prevent the tissue sections from drying.

105

106 1.2.6 Aspirate PBS from the tissue sections using a pipette. Immerse the tissue section in a
107 permeabilization buffer (~500 μ L) and incubate for 15 min at room temperature (RT).

108

109 1.2.7 Immerse the tissue sections in RCA solution (~500 μ L) and incubate at 37 °C for 1 h.

110

111 1.2.8 Take out the RCA solution and rinse the tissue sections thrice for 5 min each in 1x-PBS
112 (~500 μ L).

113

114 1.2.9 Add blocking buffer (~500 μ L) to the tissue and incubate at 4 °C for 1 h and 30 min in a
115 humidified chamber (prepared by lining a closed container with moistened tissue paper).

116

117 NOTE: Blocking incubation can be changed depending on the type of tissue section and/or the
118 background noise associated with antibody incubation.

119

120 1.2.10 Aspirate blocking buffer from the tissue section. Immerse the tissue sections in primary
121 antibody solution (~500 μ L) and incubate for 13–15 h at 4 °C in a humidified chamber.

122

123 NOTE: Primary antibody incubation can vary depending on the type of antibody and tissue used
124 for the experiment. Do not add the primary antibody to the tissue section for primary antibody
125 control. Add the blocking buffer instead.

126

127 1.2.11 Take out the primary antibody solution and rinse the tissue sections thrice for 5 min each
128 in 1x-PBS (~500 μ L).

129

130 1.2.12 Immerse the tissue sections in secondary antibody solution (~500 μ L) and incubate for 1
131 h at 37 °C in a humidified chamber.

132

133 NOTE: Add secondary antibody in the primary antibody control tissue, too.

134

135 1.2.13 Take out the secondary antibody solution and rinse the tissue sections thrice for 5 min
136 each in 1x-PBS (~500 μ L).

137

138 1.2.14 Remove excess PBS using lint-free wipes. Add mounting media (~40 μ L) to the tissue
139 sections and place a clean coverslip at the top of the tissue section. Leave the slides at RT for 1 h
140 and store them at 4 $^{\circ}$ C.

141

142 NOTE: Do not let the slide dry before mounting; otherwise, it will cause salt deposition, resulting
143 in an elevated background autofluorescence signal. To prevent tissue dehydration, keep the
144 tissue sections soaked in a small amount of 1 $\times$ PBS.

145

146 1.2.15 Image the tissue sections using an automated multimode microplate reader with
147 integrated cellular imaging equipped with a 40x objective.

148

149 NOTE: DAPI, Alexa Fluor 488, Alexa Fluor 594, and MitoTracker Green signals were captured using
150 the corresponding fluorescence filter cubes. Exposure times were optimized to avoid signal
151 saturation and were maintained consistently across all samples within an experiment. Images
152 were acquired under identical illumination and acquisition settings for comparative analyses. The
153 negative control should show little to no signal relative to the experimental sample, indicating
154 that the observed staining is specific and not due to non-specific antibody binding or background
155 fluorescence.

156

157 2. Procedure for fluorescent colocalized staining of NLRX1 and astrocytes

158

159 2.1 Preparation

160

161 2.1.1 Ethanol: Prepare 90% and 75% ethanol in 1x-PBS (200 mL) and store at RT.

162

163 2.1.2 Permeabilization buffer: Prepare permeabilization buffer by adding 20 μ L of 0.1% Triton X-
164 100 to 1x-PBS (20 mL) and store at RT.

165

166 2.1.3 Blocking buffer: To make a blocking buffer, add 5% FBS (Fetal Bovine Serum, 0.5 mL) to the
167 permeabilization buffer (10 mL) and store at 4 $^{\circ}$ C. It should be freshly prepared for each
168 experiment.

169

170 2.1.4 Primary antibody solution: Prepare primary antibody solution by mixing anti-NLRX1 rabbit
171 primary antibody (1:1000) and anti-GFAP mouse primary antibody (1:1000) in a blocking buffer.
172 It should be freshly prepared for each experiment.

173

174 2.1.5 Secondary antibody solution: Prepare secondary antibody solution by mixing anti-rabbit
175 fluorophore-conjugated secondary antibody ($\lambda_{\text{excitation}}$ 488 nm) (1:1000) and anti-mouse

fluorophore-conjugated secondary antibody ($\lambda_{\text{excitation}}$ 594 nm) (1:1000) in blocking buffer. It should be freshly prepared for each experiment.

2.2 Staining

2.2.1. Repeat steps 1.2.1 to 1.2.6.

2.2.2. Take out the permeabilization buffer and add a blocking buffer (~500 μL) to the tissue. Incubate the tissue sections with a blocking buffer at 4 °C for 1 h and 30 min in a humidified chamber.

2.2.3. Aspirate blocking buffer from the tissue section. Immerse the tissue sections in primary antibody solution (~500 μL) and incubate for 13–15 h at 4 °C in a humidified chamber.

2.2.4. Remove the primary antibody solution and rinse the tissue sections thrice for 5 min each in 1x-PBS (~500 μL).

2.2.5. Cover the tissue sections with secondary antibody (~500 μL) solution and place them at 37 °C for 1 h in a dark condition in a humidified chamber.

2.2.6. Take out the Secondary antibody solution and rinse the tissue sections thrice for 5 min each in 1x-PBS (~500 μL).

2.2.7. Remove excess PBS using lint-free wipes. Add Mounting media (40 μL) to the tissue section, then place a clean coverslip at the top. Leave the slides at RT for 1 h and then store them at 4 °C.

REPRESENTATIVE RESULTS:

Paraffin-embedded paraformaldehyde-fixed glioma and normal brain tissue sections (5- μm thickness) were stained for colocalized staining of NLRX1 with microglia and astrocytes¹⁵ (**Table 1**). Microglia were stained using RCA lectin, astrocytes were stained using antibodies specific to astrocyte marker GFAP, and NLRX1 was labeled using an NLRX1-specific antibody. In the colocalized staining of NLRX1 with microglia, red puncta represent NLRX1 as it was stained with a red fluorophore-conjugated secondary antibody, and cells with green puncta represent microglia as RCA fluoresces in a green channel (**Figure 2A**). Upon overlapping the images from green and channels, a yellow signal appears, indicating colocalization of RCA and NLRX1 staining and confirming the presence of NLRX1 in microglia. In the colocalized staining of astrocyte (GFAP) and NLRX1, green puncta represent NLRX1, and red puncta represent GFAP, as they were stained with green and red fluorophore-conjugated secondary antibodies, respectively (**Figure 3A**). An overlay of the images from both channels results in a yellow signal confirming the colocalization of GFAP staining (red) and NLRX1 staining (green). Thus, leading to the conclusion of the cellular localization of NLRX1 in astrocytes. Immunopositive NLRX1-expressing astrocytes and microglia with an observable DAPI-stained nucleus were counted blindly twice. Cell counts are averages of at least seven fields (**Figure 2B** and **Figure 3B**).

Brain tissue sections without anti-NLRX1 and anti-GFAP primary antibody treatment were taken as the negative control for the NLRX1 and GFAP-specific primary antibodies. Isotype antibody controls may be included as needed.

FIGURES AND TABLES:

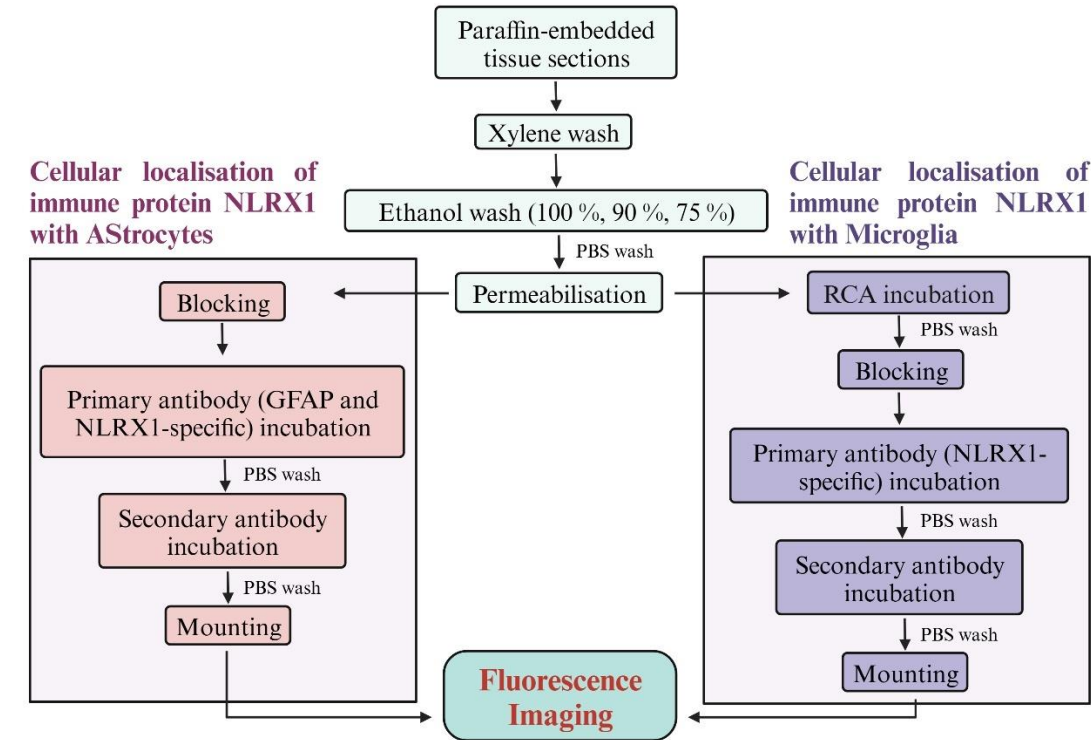


Figure 1

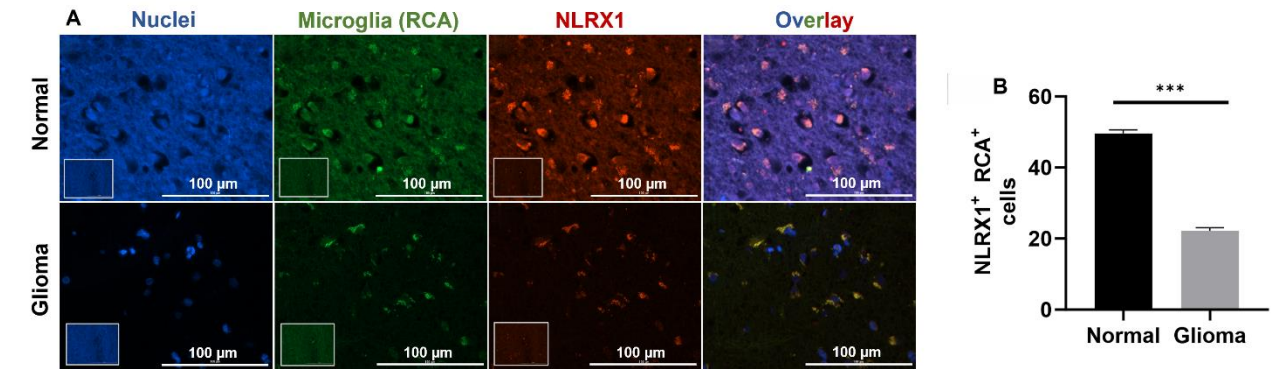


Figure 2

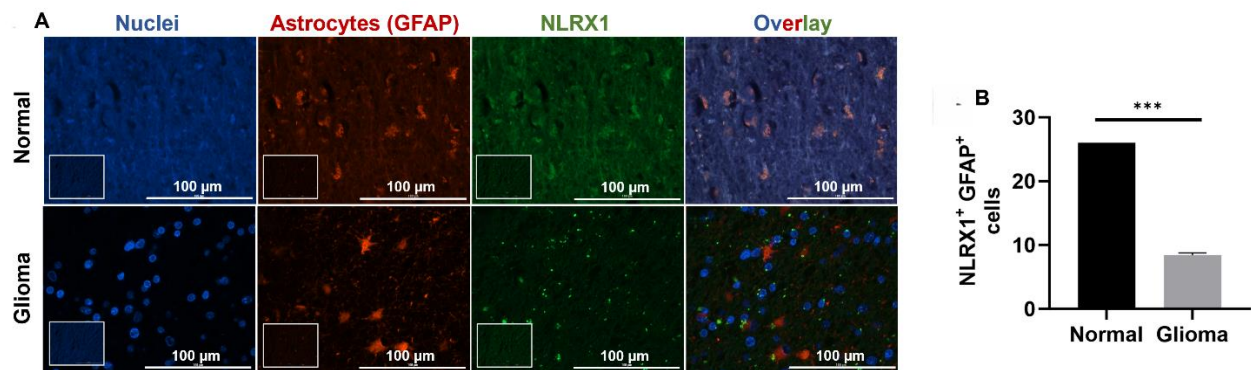


Figure 3

Sample type	Experimental use	Disease status
Glioma sample	Colocalized staining of NLRX1 with Microglia	Astrocytoma (WHO grade II glioma)
Glioma sample	Colocalized staining of NLRX1 with Astrocytes	Astrocytoma (WHO grade II glioma)
Normal brain (Sensory Cortex)	Colocalized staining of NLRX1 with Microglia	Normal
Normal brain (Corpus Callosum)	Colocalized staining of NLRX1 with Astrocytes	Normal
Normal brain (Ventricular section)	Anti-NLRX1 antibody control	Normal
Normal brain (Corpus Callosum)	Anti-NLRX1 and anti-GFAP antibody control	Normal

Table 1

FIGURE AND TABLE LEGENDS:

Figure 1: Flowchart of the staining protocol. Paraffin-embedded paraformaldehyde-fixed tissue sections were washed with xylene and rehydrated with ethanol. For microglia and NLRX1 staining, after PBS wash, tissue sections were permeabilized and stained with RCA. After RCA incubation, tissue sections were blocked and then treated with a primary antibody specific for NLRX1. For astrocyte and NLRX1 staining, after tissue permeabilization, tissue sections were blocked and then treated with a primary antibody specific for GFAP and NLRX1. After primary antibody incubation, a secondary antibody was added. The tissue sections were mounted using mounting media containing DAPI and imaged using a fluorescence microscope.

Figure 2: Cellular localization of innate immune protein NLRX1 in microglia of patient-derived normal brain and glioma tissue sections. (A) Paraffin-embedded paraformaldehyde-fixed glioma and normal brain tissue sections (5 µm thickness) were obtained from AIIMS Jodhpur. Through immunohistochemistry, nuclei were stained with DAPI (blue), microglia were stained with RCA (green), and NLRX1 (red) was stained by NLRX1-specific immunolabelling. The scale bar is 100 µm. Images of the top panel are taken from the normal brain tissue section, and images of the bottom panel are taken from the glioma tissue section. Images were captured using a 40x objective lens. (B) The expression of NLRX1 is quantified across the microglia of normal and glioma tissue sections by having unbiased readers, who were blinded to the sample identities, manually count the NLRX1-expressing microglia. Data are represented as mean ± SEM. Error bars indicated SEM. *** $p < 0.005$ (Student's *t*-test). This figure has been modified from Meena et al.¹⁵.

Figure 3: Cellular localization of innate immune protein NLRX1 in astrocytes of patient-derived normal brain and glioma tissue sections. (A) Paraffin-embedded paraformaldehyde-fixed glioma and normal brain tissue sections (5 µm thickness) were obtained from AIIMS Jodhpur. Through immunohistochemistry, nuclei were stained with DAPI (blue), astrocytes were stained with GFAP-specific immunolabelling (red), and NLRX1 was stained with NLRX1-specific immunolabelling (green). The scale bar is 100 µm. Images of the top panel are taken from the normal brain tissue section, and images of the bottom panel are taken from the glioma tissue section. Images were captured from a 40X objective lens. (B) The expression of NLRX1 is quantified across the astrocytes of normal and glioma tissue sections by having unbiased readers, who were blinded to the sample identities, manually count the NLRX1-expressing microglia, astrocytes, and macrophages. Data are represented as mean ± SEM. Error bars indicated SEM. *** $p < 0.005$ (Student's *t*-test). This figure has been modified from Meena et al.¹⁵.

Table 1: Details of Patient-derived paraffin-embedded tissue samples.

DISCUSSION:

This protocol presents a method for performing immunofluorescence microscopy to assess the localization of the immune protein NLRX1 in microglia and astrocytes in paraffin-embedded tissue sections⁵. For staining microglia, RCA (Ricinus communis agglutinin) or antibodies such as IBA-1 and TMEM119 may be used^{6,17}. RCA is a lectin that binds to β-D-galactose residues and labels microglia. One advantage of using RCA instead of antibodies is that it stains microglia quickly (in an hour; conversely, antibodies may require overnight incubation¹⁸). However, the limitation of the RCA stain is that it can also stain endothelial cells. Several well-known and widely available astrocyte markers are expressed as membrane, intracellular, or secreted factors proteins¹⁹. Among them are structural proteins, membrane channels and transporters, transcription factors, and energy metabolism markers. One of the most abundantly reported astrocyte markers is GFAP (Glial Fibrillary Acidic Protein). GFAP is an intermediate filament type III protein found on astrocytes and present in different isoforms (α, γ, δ/ε, and κ in the human brain, β additionally in rodents). GFAP supports the BBB structure mechanically. For GFAP staining, a GFAP-specific primary antibody is required.

The protocol starts with xylene washes of tissue sections, which are necessary to remove paraffin, as xylene dissolves it. It is important to perform a xylene wash for an optimized duration, as prolonged exposure may damage cellular structures and lead to excessive dehydration. Conversely, an improper or too-short xylene wash can lead to incomplete paraffin removal or poor tissue clearing²⁰. After paraffin removal, tissue sections were washed with a decreasing ethanol grade, followed by PBS to rehydrate the tissues, as xylene dehydrates them. The tissues were permeabilized using Triton X-100, a detergent that compromises the cell membrane. This allows blocking buffers, dyes, and antibodies from entering the cells. For colocalization of microglia and NLRX1, microglia were first stained with RCA, followed by staining for NLRX1 with an NLRX1-specific primary antibody. After RCA staining, blocking is required for the specific staining of NLRX1. Thus, before primary antibody treatment, tissue sections were incubated in a blocking buffer containing serum proteins that bind and block antigenic sites on the cells. This aims to improve the specificity of primary binding.

Furthermore, to assess colocalization of astrocytes and NLRX1, GFAP- and NLRX1-specific primary antibodies were used. After primary antibody incubation, PBS washes were given to remove the unbound antibodies. Next, a secondary antibody conjugated with a fluorophore and specific to the primary antibody was added to the cells. The secondary antibody binds to the primary antibody and, upon excitation, fluoresces. PBS wash was performed to reduce the risk of non-specific binding of secondary antibodies. After that, cells were mounted in mounting media containing DAPI. Mounting helps protect the sample for a longer period, and DAPI stains the cell nuclei. The imaging of these stained tissue sections was performed using a multimode plate reader (Fluorescence microscope). Images were captured in four filters, including Brightfield, DAPI, GFP, and Texas Red, to capture the different fluorescence emitted from the fluorophore-conjugated NLRX1, astrocytes, and microglia. At least 7 frames were imaged per sample. To quantify colocalization of signals, immunopositive NLRX1-expressing astrocytes and microglia were counted twice, in a blinded manner.

TABLE OF MATERIALS:

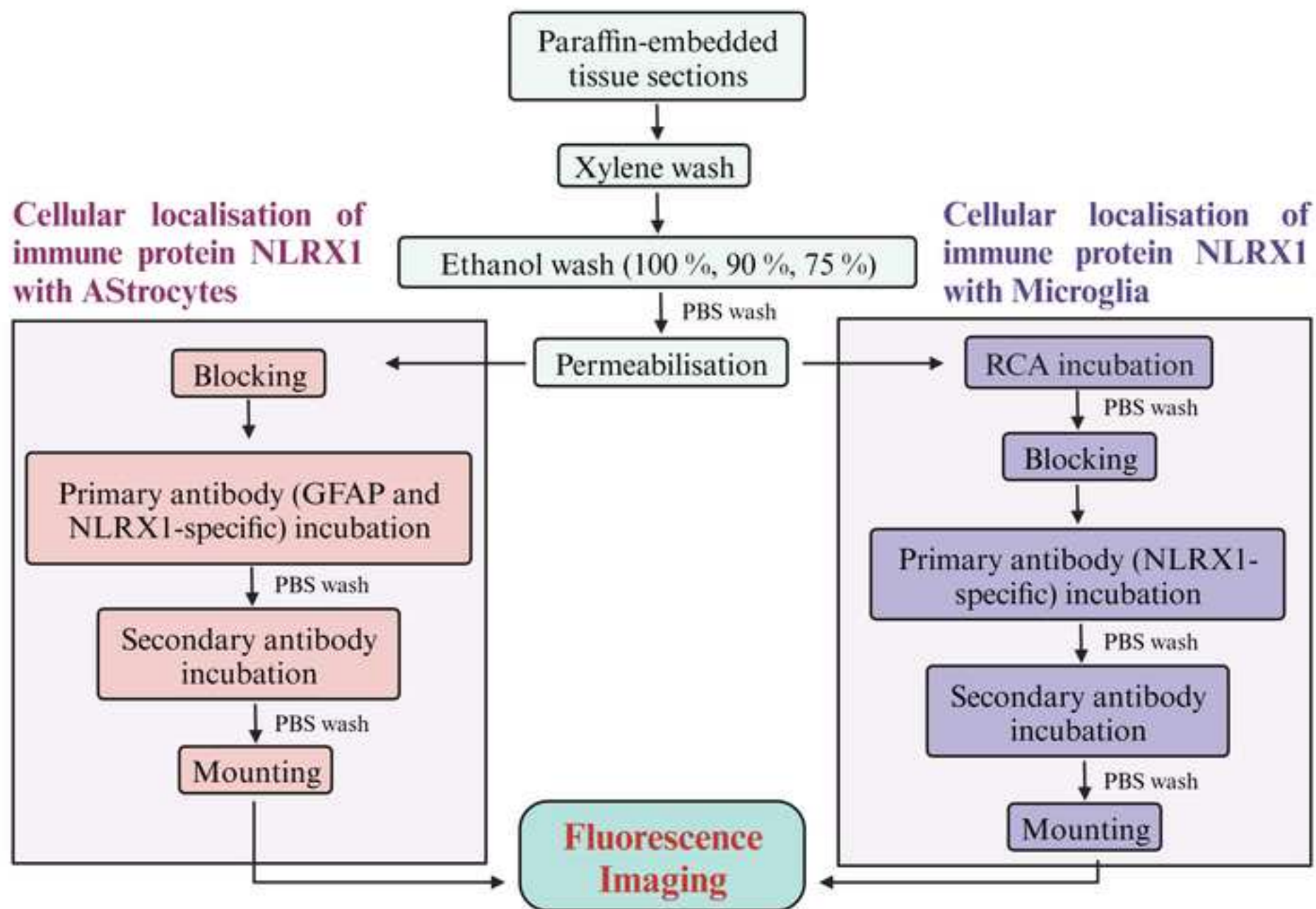
Name of Material/ Equipment	Company	Catalog Number
10x-PBS	Himedia	ML023-500ML
Alexa Fluor 488 nm anti-rabbit antibody	Invitrogen	A11008
Alexa Fluor 594 nm anti-mouse antibody	Invitrogen	A11005
Alexa Fluor 594 nm anti-rabbit antibody	Life Technologies	A11012
Anti-GFAP Primary Antibody	Cell-signaling technology	3670S
Anti-NLRX1 Primary Antibody	Novus Biologicals	NBP1-76287
Ethanol		
FBS	Cell Clone	CCS-500-SA-U

Fluorescein Ricinus Communis Agglutinin	Vector laboratories	FL-1081
Fluorescence Microscope	BioTek	
Fluoroshield TM with DAPI	Sigma	F6057-20ML
ImmEdge Pen	Vector laboratories	H-4000
Kim Wipes		
Microscopic Coverglass	Blue Star	
Triton-X-100	Sigma	1001723790
Xylene	Fisher Scientific	35417

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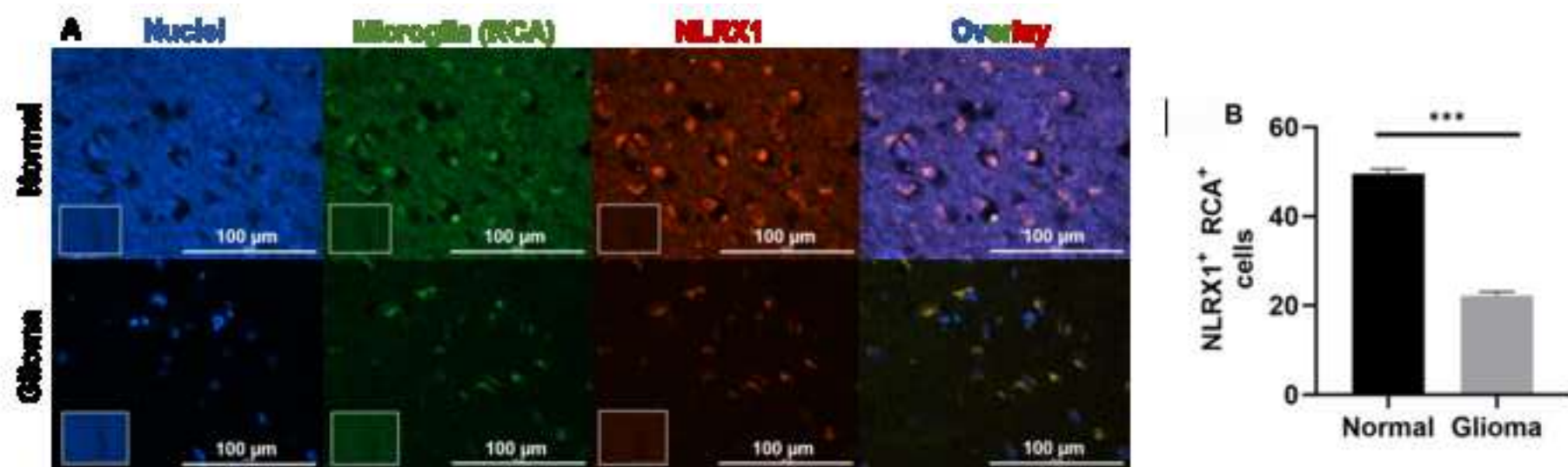
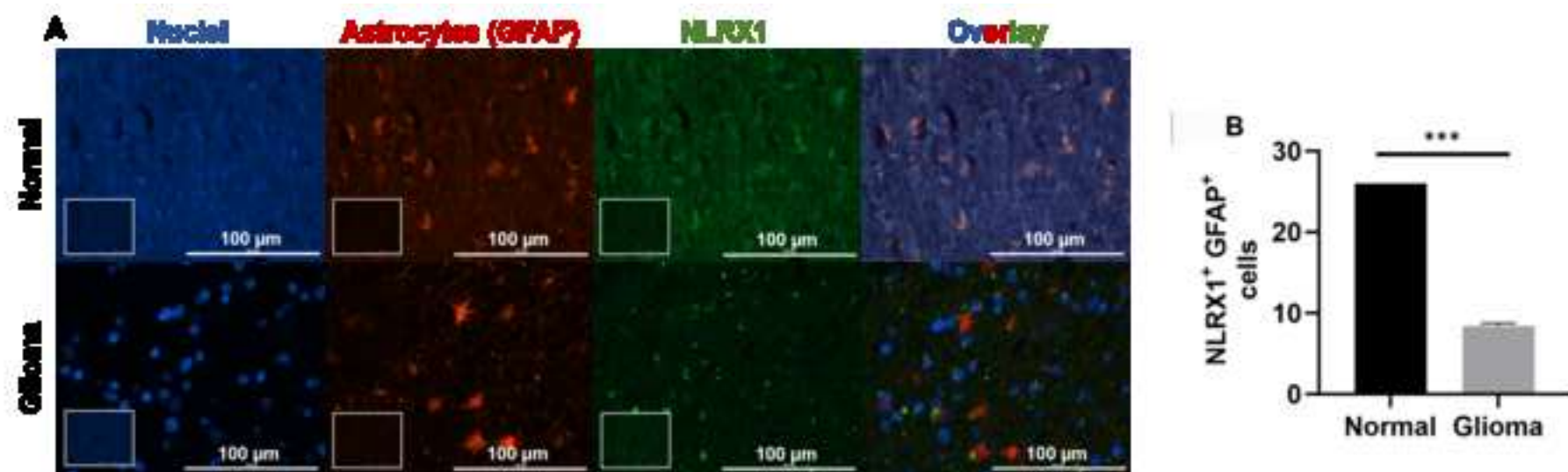


Figure 3

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Normal brain (Ventricular section)	Anti-NLRX1 antibody control	Normal
Normal brain (Corpus Callosum)	Anti-NLRX1 and anti-GFAP antibody control	Normal

Name of Material/ Equipment	Company
10x-PBS	Himedia
Alexa Fluor 488 nm anti-rabbit antibody	Invitrogen
Alexa Fluor 594 nm anti-mouse antibody	Invitrogen
Alexa Fluor 594 nm anti-rabbit antibody	Life Technologies
Anti-GFAP Primary Antibody	Cell-signaling technology
Anti-NLRX1 Primary Antibody	Novus Biologicals
Ethanol	
FBS	Cell Clone
Fluorescein Ricinus Communis Agglutinin	Vector laboratories
Fluorescence Microscope	BioTek
Fluoroshield TM with DAPI	Sigma
ImmEdge Pen	Vector laboratories
Kim Wipes	
Microscopic Coverglass	Blue Star
Triton-X-100	Sigma
Xylene	Fisher Scientific

Catalog Number
ML023-500ML
A11008
A11005
A11012
3670S
NBP1-76287
CCS-500-SA-U
FL-1081
F6057-20ML
H-4000
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