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Application of the NeuN Neuronal Marker to Study the Development of the Human Fetal Cortex

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Abstract—Objective: This study presents a method for separating NeuN-stained neurons and glial cells in the human fetal cerebral cortex using flow cytometry. NeuN neuronal nuclear antigen is widely used as a universal neuron-specific marker. Antibodies against this protein allows to specifically identify neurons, since this protein is not found in glial cells. The application of this marker allows precise determination of the cellular composition in different brain regions. **Methods:** This study uses protocols NeuN-based immunohistochemical neuronal quantification for flow cytometry to distinguish neurons and glial cells in different regions of human fetal cerebral cortex. **Results:** Immunohistochemical staining was optimized to establish a validated antibody concentration for subsequent flow cytometry. Then two parahippocampal cortical sample suspensions stained with DAPI and NeuN were analyzed. We acquired three subpopulations: $DAPI^{-}/AF488^{+}$, $DAPI^{-}/AF488^{-}$, and $DAPI^{+}/AF488^{-}$ with variable intensities. Fluorescence microscopy validation detected nuclei matching all cytometry subpopulations. **Conclusion:** Flow cytometric approach offers single-nucleus resolution for detecting rare populations. Along with immunohistochemical staining, flow cytometry enhances quantitative and qualitative analysis of neural populations and allows to calculate glia-neuron index which is a critical metric for understanding brain organization, development, and plasticity.

Keywords: immunohistochemistry, flow cytometry, neocortex, human, prenatal development, NeuN, glia-neuron index

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INTRODUCTION

Immunohistochemical markers represent a significant advancement in neurohistology, offering superior structural and cellular differentiation compared to classical histochemical techniques (Nadia, 2008; Tanapat, 2019). This is particularly evident when contrasting immunohistochemistry with Nissl staining—where toluidine blue, thionin, or cresyl violet often render neurons and glial cells morphologically indistinguishable (Christopherd et al., 2016), complicating histological interpretation.

Modern neuroscience employs neuron- and glia-specific markers to resolve distinct cellular populations: neuronal markers NeuN (Fox-3/Rbfox3), β-III-tubulin, MAP2 (Gusel'nikova and Korzhevskiy, 2015); glial markers GFAP (astrocytes), lig2 (oligodendrocytes) (Middeldorp and Hol, 2011).

The neuronal nuclear antigen NeuN serves as a pan-neuronal marker. NeuN antibodies targeting R3HDM1 (R3H domain-containing protein) reliably identify post-mitotic neurons, exhibiting no detectable expression in glial cells. With the exception of a small group of specialized neurons (Purkinje cells, Cajal-Retzius cells, etc.), positive results of staining of neuronal nuclei were shown in various groups of vertebrates—fish, amphibians, birds, mammals. (Mullen et al., 1992; King, 2004; Tochinai and Yoshino, 2004; Scott et al., 2005; Gusel'nikova and Korzhevskiy, 2015).

While stereological methods remain standard for cortical cell quantification in small, homogeneous

regions with clear cytoarchitectonic borders (Schmitz and Hof, 2005; Kolomeets and Uranova, 2015), they prove inadequate for large cortical areas. The isotropic fractionation technique overcomes this limitation, enabling rapid whole-tissue cell counting by staining nuclei suspension for NeuN to distinguish neurons from glia (Herculano-Houzel and Lent, 2005). This method allows to quantify stained neuronal nuclei by counting a homogeneous nuclear suspension aliquot in a Neubauer counting chamber under a fluorescence microscopy. Total cell counts are extrapolated to the full suspension volume using the established formula (Herculano-Houzel and Lent, 2005). Crucially, the neuron-specific nuclear marker NeuN enables discrimination between neuronal and glial nuclei in this protocol.

For even larger specimens, flow cytometry offers high-throughput quantification of DAPI-stained nuclei, with NeuN fluorescence resolving neuronal populations (Young et al., 2012). Flow cytometry measures fluorescence and light scattering parameters from individual nuclei traversing a laser beam within a hydrodynamically focused fluid stream. The DNA intercalating dye, such as DAPI (4′,6-diamidino-2-phenylindole), binds double-stranded DNA, enabling total nuclear counts, while NeuN immunolabeling specifically identifies neuronal nuclei.

Despite extensive animal studies (Herculano-Houzel and Lent, 2005; Steinhausen et al., 2016), human data remain scarce: which creates a critical gap given their relevance to neuropathology (Pelvig et al., 2008; Andrade-Moraes et al., 2013). Technical challenges persist, however, particularly rapid postmortem protein degradation in archival human tissue that precludes standard NeuN immunohistochemistry protocols. Consequently, conventional NeuN antibody protocols and standard dilutions are not readily useful in this context due to epitope degradation.

This study adapts flow cytometry to quantify $NeuN^+$ neuron-to-glia ratios in human fetal cerebral cortex, addressing methodological barriers in archival tissue analysis.

MATERIALS AND METHODS

Materials

Temporal and parahippocampal cortex specimens from 4 human fetuses (24–25 gestational weeks) obtained from the Department of Pathological Anatomy, Saint Petersburg State Pediatric Medical University (SPbSPMU; St. Petersburg, Russia) were used for protocol optimization. All cases lacked neuropathology per autopsy reports. Tissues were fixed within 24 h postmortem (to preserve immunogenicity) in 4% paraformaldehyde/0.1M phosphate buffer (PB, pH 7.4, contain only Na_2HPO_4 and NaH_2PO_4). Phosphate-buffered saline, PBS, pH 7.4, may also be used in all case.

Immunofluorescent Staining

Right hemispheres were placed in 20% sucrose/PB for cryoprotection until blocks sunk to the bottom of a vial, sectioned at 30 μm thickness using a CM3050S cryostat (Leica microsystems, Germany). Left hemispheres were paraffin-embedded, with 15 μm frontal sections cut on a SM2010S microtome (Leica microsystems, Germany). Rat brain frontal sections (20 μm thick) served as positive controls for immunofluorescent (IF) staining.

Sections were stained by indirect IF-staining protocol using primary mouse anti-NeuN antibodies (MAB377; Millipore, Germany; RRID:AB_2298772), secondary Alexa Fluor 488 goat anti-mouse antibodies (A-21121; Thermo Fisher, USA; RRID:AB_2535764) and DAPI (Life Technologies, USA) as a nuclear counterstain.

NeuN primary antibodies were titrated (1 : 50, 1 : 100, 1 : 250, 1 : 500) in PB (pH 7.4) consisting of 1% normal goat serum (NGS) and 0.25% Triton X-100. Secondary antibody concentration was manufacturer-recommended (1 : 250). Per concentration, 5 human specimens were stained: 2 paraffin, 2 frozen, 1 negative control (primary omitted). Two rat brain sections per dilution served as positive controls.

For paraffin-embedded sections, tissues were first deparaffinized in xylene (2 × 5 min) and rehydrated through a graded ethanol series (96% ethanol, 2 × 5 min), then washed three times for 5 min in phosphate buffer (PB). For non-paraffinized sections, the protocol began at this point. Antigen retrieval was performed by autoclaving the slides in Tris-EDTA buffer (pH 9.0) at 120°C for 5 min, followed by slow cooling to prevent boiling. Tissues were blocked for 120 min at room temperature in PB containing 10% normal goat serum (NGS) and 0.25% Triton X-100, then incubated with anti-NeuN primary antibodies overnight at 4°C. After three 5-min PB washes, sections were incubated with secondary antibodies (Alexa Fluor 488, 1 : 200), and DAPI (1 : 1000) in PB with 1% NGS, and 1% Triton X-100 for 40 min at 37°C, followed by final PB washes (3 × 5 min). Slides were mounted with 80% glycerol and visualized using an SP5 confocal

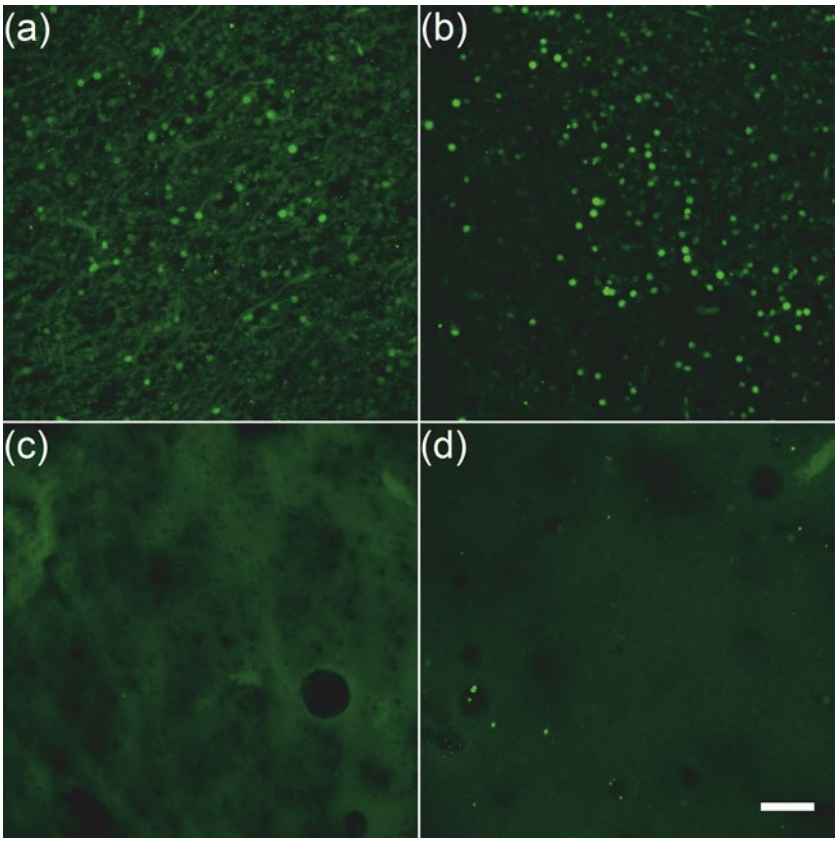

Fig. 1. NeuN antibody titration in human cerebral cortex. (a) 1 : 50 Dilution: intense neuronal labeling with high background (arrows). (b) 1 : 100 Dilution: optimal signal-to-noise ratio. (c) 1 : 250 Dilution: weak specific signal. (d) 1 : 500 Dilution: signal indistinguishable from autofluorescence. Imaging: Leica SP5; objective 40× (NA 1.3); 488 nm laser; 510–540 nm detection. Scale bar: 50 μm.

microscope (Leica, Germany) with standardized settings. Negative control was done by omitting the primary antibodies during the IF-staining protocol.

Imaging was done by SP5 confocal microscope (Leica, Germany) with standardized acquisition settings across samples.

Flow Cytometry

Tissue preparation according to Herculano-Houzel and Lent (2005): parahippocampal cortex from 2 fetuses (24–25 gestational weeks) was homogenized in citrate buffer/1% Triton X-100 using glass Dounce and Potter-Elvehjem homogenizers with PTFE pestle (Sigma-Aldrich, Germany), followed by centrifugation (4000 *g*, 4°C). NeuN immunodetection employed indirect immunofluorescence with commercial antibodies at the optimized dilution (1 : 100) established previously. Primary antibody: mouse anti-NeuN (MAB377; Millipore, Germany; RRID:AB_2298772). Secondary antibody: Alexa Fluor 488 goat anti-mouse (A-21121; Thermo Fisher, USA; RRID:AB_2535764). DAPI (D1306; Thermo Fisher, USA) served as a pan-nuclear counterstain.

Indirect staining protocol: **Day 1**: washes (PBS pH 7.4, 3 times for 5 min) with centrifugation after each wash; antigen retrieval (Tris-borate pH 9.0, boiling, 5 min); NeuN (1 : 100), NGS 1%, Triton X-100 1% in PB overnight at 4°C. **Day 2**: DAPI (1 : 1000, 5 min) with subsequent centrifugation; Alexa Fluor 488 (1 : 100, NGS 1%, Triton X-100 1% in PB 120 min at 37°C); washes (PBS, 3 times for 5 min) with centrifugation after each wash.

Nuclear staining quality was confirmed using a DM4000 microscope (Leica, Germany) with standard filter sets: A4 for DAPI (excitation 360/40 nm, emission 470/40 nm) and YFP for Alexa 488 (excitation 500/20 nm, emission 535/30 nm). Stained suspensions were analyzed on a BD FACS Aria III flow cytometer (USA)

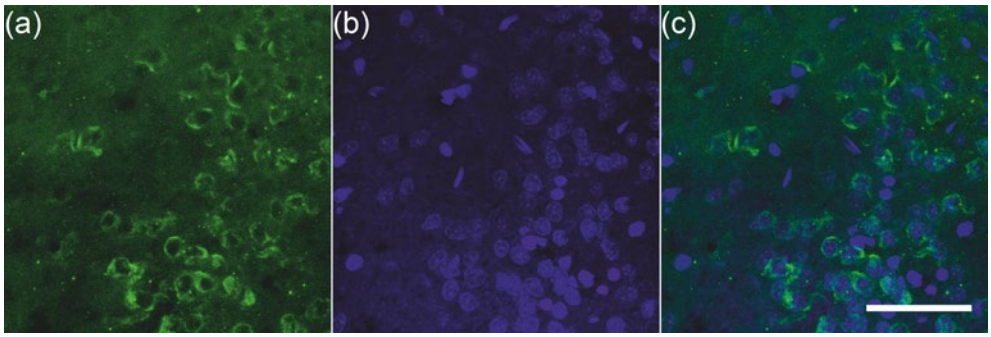

Fig. 2. Rat cortex positive control (1 : 100 NeuN dilution). (a) NeuN-Alexa 488 signal. (b) DAPI nuclear counterstain. (c) Merged channels showing neuronal specificity. Imaging Leica SP5; oil objective 100× (NA 1.47); 405/488 nm lasers; 420–450/510–540 nm detection. Scale bar: 50 μm.

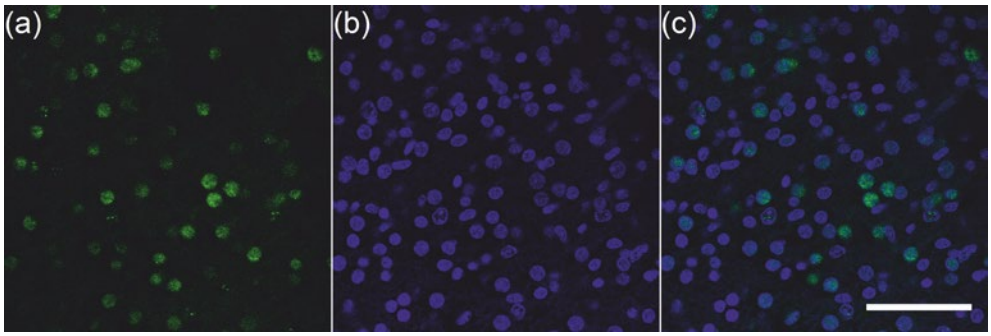

Fig. 3. Human fetal cortex labeling (1 : 100 NeuN dilution). (a) $NeuN^+$ neurons (Alexa 488). (b) Total nuclei ($DAPI^+$). (c) Merged image: $DAPI^+/NeuN^{+/-}$ cells. Imaging: identical parameters to Fig. 2. Scale bar: 50 μm.

configured as follows: DAPI: 405 nm laser, 430–470 nm bandpass filter; Alexa 488: 488 nm laser, 515–545 nm bandpass filter. Data was acquired and analyzed with BD FACSDiva Software v8.0.1.

RESULTS AND DISCUSSION

Immunohistochemistry Optimization

Immunohistochemical staining was optimized to establish a validated antibody concentration for subsequent flow cytometry. Confocal microscopy analysis revealed that NeuN-specific binding generated sufficient signal contrast at 1 : 50 and 1 : 100 dilutions. While the 1 : 50 dilution produced higher background intensity—indicative of non-specific binding—the 1 : 100 dilution achieved optimal signal-to-noise ratios. Higher dilutions (1 : 250–1 : 500) yielded insufficient signal to overcome inherent tissue autofluorescence (Fig. 1). Parallel validation in rat brain sections confirmed 1 : 100 as the ideal concentration for neuronal visualization (Fig. 2). Specificity was further demonstrated through clear discrimination between $NeuN^+/DAPI^+$ neuronal nuclei (green and blue light) and $NeuN^-/DAPI^+$ glial nuclei (blue only; Fig. 3).

Flow Cytometry Analysis

Two parahippocampal cortical sample suspensions stained with DAPI and NeuN were analyzed; unstained sample was used as a negative control. We acquired forward scatter area (FSC-A), side scatter area (SSC-A), forward scatter width (FSC-W), DAPI fluorescence (450/40 nm; DAPI-A), and Alexa Fluor 488 fluorescence (530/30 nm; AF488-A). Nuclear events were first gated using an FSC-A threshold (>1000 AU), followed by singlet isolation via FSC-A vs. FSC-W (confirmed by uniform DAPI-W distribution). Within singlets, a minor population (0.3%) exhibited $AF488^+/DAPI^-$ fluorescence, suggesting technical artifacts. The dominant

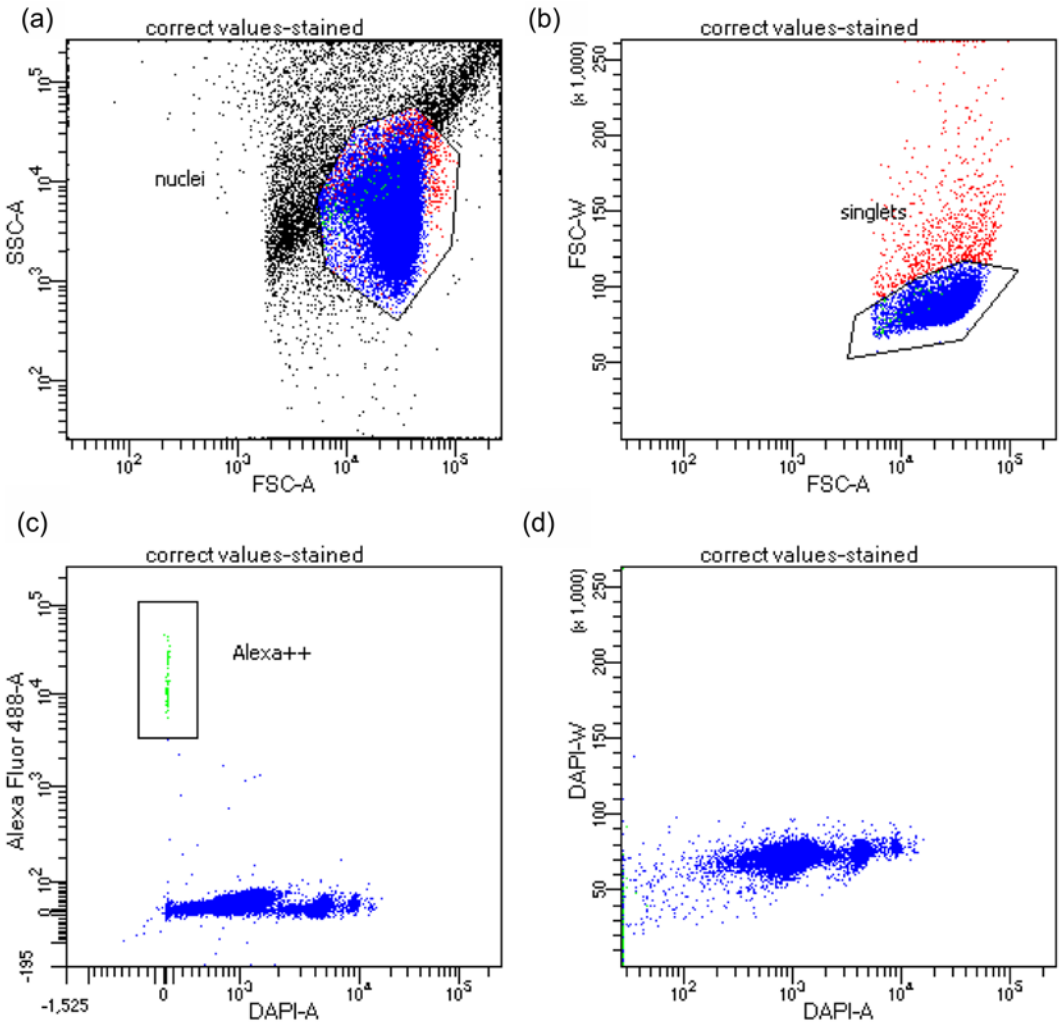

Fig. 4. Flow cytometry gating strategy for NeuN$^+$ neuronal quantification. (a) FSC-A vs. SSC-A: initial nuclear threshold (>1000 AU). (b) FSC-A vs. FSC-W: singlet isolation. (c) Alexa Fluor 488-A vs. DAPI-A: NeuN$^+$/DAPI$^+$ neurons (Q2; 2.7%) and anomalous NeuN$^+$/DAPI$^-$ events (Q1; 0.3%). (d) DAPI-W vs. DAPI-A: DNA content validation. Instrument: BD FACS Aria III; Lasers: 405 nm (DAPI), 488 nm (Alexa 488); Filters: 450/40 nm (DAPI), 530/30 nm (Alexa 488).

AF488$^-$ population segregated into three DAPI-intensity subgroups: P1 (DAPI$^-$ debris), P2 (diploid; G_0/G_1 phase), and P3 (tetraploid; G_2/M phase), with P3 showing twofold higher DAPI intensity than P2 (Fig. 4).

Fluorescence microscopy validation detected nuclei matching all cytometry subpopulations: DAPI$^-$/AF488$^+$, DAPI$^-$/AF488$^-$, and DAPI$^+$/AF488$^-$ with variable intensities. While this methodology enables neuronal quantification in human fetal cortex, the anomalous DAPI$^-$/NeuN$^+$ population necessitates further protocol refinement.

CONCLUSION

This study validates the efficacy and specificity of NeuN antibody (targeting R3HDM1 protein) for neuronal labeling in archival human fetal brain tissue. Through systematic optimization—first on histological sections, then in parahippocampal cortical cell suspensions—we established robust protocols for identifying post-mitotic neurons despite extended fixation. These findings enable precise neuronal quantification via flow cytometry and enhance the accuracy of neurodevelopmental mapping across cortical regions.

ABBREVIATIONS AND NOTATION

AF488—Alexa Fluor 488;

FSC-A—forward scatter area;

FSC-W—forward scatter width;

IHC—immunohistochemistry;

NGS—normal goat serum;

NeuN—neuronal nuclei;

PB—phosphate buffer;

PBS—phosphate-buffered saline;

SSC-A—side scatter area.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The research was approved by the Ethics Committee of Saint Petersburg State Pediatric Medical University (SPbSPMU). Procedures adhered to Russian Federal law no. 8-FZ, Ministry of Health Order no. 354 (June 6, 2013) with ethical approval (SPbSPMU protocols no. 6.5 dated June 10, 2014 and no. 2/5 dated February 6. 2019).

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

AUTHOR CONTRIBUTION

Conceptualization, writing, histology: K.N.F. Flow cytometry and data analysis: A.E.R. Sample preparation: I.V.E. All authors reviewed the final version of the manuscript.

REFERENCES

Andrade-Moraes, C.H., Oliveira-Pinto, A.V., Castro-Fonseca, E., da Silva, C.G., Guimaraes, D.M., Szczupak, D., Parente-Bruno, D.R., Carvalho, L.R.B., Polichiso, L., Gomes, B.V., Oliveira, L.M., Rodriguez, R.D., Leite, R.E.P., Ferretti-Rebustini, R.E.L., Jacob-Filho, W., Pasqualucci, C.A., Grinberg, L.T. and Lent, R., Cell number changes in Alzheimer's disease relate to dementia, not to plaques and tangles, *Brain*, 2013, vol. 136, pp. 3738–3752. https://doi.org/10.1093/brain/awt273

Erö, C., Gewaltig, M.-O., Keller, D., and Markram, H., A cell atlas for the mouse brain, *Front. Neuroinform.*, 2018, vol. 12, Art. ID: 84. https://doi.org/10.3389/fninf.2018.00084

Gusel'nikova, V.V. and Korzhevskiy, D.E., NeuN as a neuronal nuclear antigen and neuron differentiation marker, *Acta Naturae*, 2015, vol. 7, pp. 42–47. https://doi.org/10.32607/20758251-2015-7-2-42-47

Herculano-Houzel, S. and Lent, R., Isotropic fractionator: a simple, rapid method for the quantification of total cell and neuron numbers in the brain, *J. Neurosci.*, 2005, vol. 25, pp. 2518–2521. https://doi.org/10.1523/jneurosci.4526-04.2005

King, C., Lacey, R., Rodger, J., Bartlett, C., Dunlop, S., Beazley, L., Characterisation of tectal ephrin-A2 expression during optic nerve regeneration in goldfish: implications for restoration of topography, *Exp. Neurol.*, 2004, vol. 187, pp. 380–387. https://doi.org/10.1016/j.expneurol.2004.02.009

Kolomeets, N.S. and Uranova, N.A., Oligodendrocyte clustering deficits in schizophrenia's inferior parietal cortex correlate with onset age, *Psychiatry (Russ.)*, 2015, vol. 67, pp. 52–57. https://doi.org/10.30629/2618-6667-2022-20-3-6-15

Middeldorp, J., and Hol, E.M., GFAP in health and disease, *Prog. Neurobiol.*, 2011, vol. 93, pp. 421–443. https://doi.org/10.1016/j.pneurobio.2010.10.023

Mullen, R.J., Buck, C.R., and Smith, A.M., NeuN, a neuronal specific nuclear protein in vertebrates, *Development*, 1992, vol. 116, pp. 201–211. https://doi.org/10.1242/dev.116.1.201

Nadia, P., Matthew, B., Sofoklis, P., Revers, D., and Martine, H., A rapid method combining Golgi and Nissl staining to study neuronal morphology and cytoarchitecture, *J. Histochem. Cytochem.*, 2008, vol. 56, pp. 539–550. https://doi.org/10.1369/jhc.2008.950246

Pelvig, D.P., Pakkenberg, H., Stark, A.K., and Pakkenberg, B., Neocortical glial cell numbers in human brains, *Neurobiol. Aging*, 2008, vol. 29, pp. 1754–1762. https://doi.org/10.1016/j.neurobiolaging.2007.04.013

Schmitz, C. and Hof, P.R., Design-based stereology in neuroscience, *Neuroscience*, 2005, vol. 130, pp. 813–831. https://doi.org/10.1016/j.neuroscience.2004.08.050

Scott, B.B. and Lois, C., Generation of tissue-specific transgenic birds with lentiviral vectors, *Proc. Natl. Acad. Sci. USA*, 2005, vol. 102, pp. 16443–16447. https://doi.org/10.1073/pnas.0508437102

Steinhausen, C., Zehl, L., Haas-Rioth, M., Morcinek, K., Walkowiak, W., and Huggenberger, S., Multivariate meta-analysis of brain-mass correlations in eutherian mammals, *Front. Neuroanat.*, 2016, vol. 10, Art. ID: 91. https://doi.org/10.3389/fnana.2016.00091

Tanapat, P., Neuronal cell markers, *Mater. Methods*, 2013, vol. 3, p. 196. https://doi.org/10.13070/mm.en.3.196

Tochinai, S. and Yoshino, J., Phylogeny and ontogeny of regeneration in vertebrates, in *Neo-Science of Natural History: Integration of Geoscience and Biodiversity Studies*, Mawatari, S.F. and Okada, H., Eds., *Proc. Int. Symposium on "Dawn of a new natural history – integration of geoscience and biodiversity studies,"* March 5–6, 2004, Sapporo, pp. 45–51. http://hdl.handle.net/2115/38490

Von Bartheld, C.S., Bahney, J., and Herculano-Houzel, S., The search for true numbers of neurons and glial cells in the human brain: a review of 150 years of cell counting, *J. Comp. Neurol.*, 2016, vol. 524, pp. 3865–3895. https://doi.org/10.1002/cne.24040

Young, N.A., Flaherty, D.K., Airey, D.C., Varlan, P., Aworunse, F., Kaas, J.H., Collins, C.E., Use of flow cytometry for high-throughput cell population estimates in brain tissue, *Front. Neuroanat*., 2012, vol. 6, Art. ID: 27. https://doi.org/10.3389/fnana.2012.00027