Journal of Neurological Disorders

J Neurol Disord
Volume 11:6, 2023

Research Article

Open Access

Zebrafish Whole Mount Staining Using Luxol Fast Blue Stain

Waseem Hasan[1], Mazen Shafea[1], Doua Abdulrahman[1] and Sahar Daas[1,2*]

[1]Department of Human Genetics, Sidra Medicine, Qatar
[2]Department of Health and Life Sciences, Hamad Bin Khalifa University, Qatar

Abstract

Background: Neurological disorders prevalence burdens the healthcare system. These neurological disorders are defined as functional impairments that affect the nervous system; the clinical neuropathological manifestations can present structural, biochemical, or electrical abnormalities.

Zebrafish disease models have been used extensively to study human neuropathological and behavioral disorders due to the unique *in vivo* live real-time visualization of the developing nervous system within the transparent model.

Myelin is a lipoprotein of a fatty multilayered membrane that surrounds the axons of the nerve cells. The myelination of the axons increases the electrical impulse speed rate through the nervous system and indicates the proper function of the nervous system.

In zebrafish model, histological procedures are employed to study the nervous system. However, the proper orientation and serial sections should be obtained the neuroanatomy of the brain at the cellular level. Consequently, it is challenging to examine the structural alterations in real dimensional developing brain.

Luxol fast blue is used to stain myelin sheath which is rich in phospholipids. This stain is used to differentiate white matter (rich in myelin) from the grey matter in brain tissue sections.

Results: Here, we developed a new method of whole-mount staining that optimize the penetration of staining regents of luxol fast blue into the white and gray matter of zebrafish larvae developing brain with decrease non-specific binding and background. Our result showed the clear anatomical structures of the central nervous system as a utility for experimental neuropathology in zebrafish model.

Conclusion: Our developed method provides three-dimensional visualization of the developing central nervous system of zebrafish; this is useful to characterize neuron demyelination in corresponding disease model.

Keywords: Zebrafish • Luxol blue • Wholemount • Disease model

Introduction

The global burden of the neurological disorder has been emphasized by world health organization as the greatest threat to public health, which is one of the major causes of childhood morbidity and mortality [1]. Based on the Global Burden of Disease Study, these disorders were responsible for 6.39% of neurological disease in 2015 and predicted to increase to 6.77 in 2030 [1].

Since some of these neurological disorders are linked to a genetic mutation in the paediatric population, it is crucial to explore the associated abnormalities at early stages of life. To validate and understand how these neurological disorders affect neuronal structure and function, animal models are excellent toolkit for modelling human diseases in a way to reveal the mechanisms generating neurodevelopmental defects [2]. These models will facilitate the investigation of genetic variation on cellular, tissue, organ, and whole animal levels.

Recently, Zebrafish (*Danio rerio*) has become increasingly used for investigating wide range of human diseases [3]. It is considered as a robust model system for studying gene function and signaling pathways during development [4]. Zebrafish has several advantages over other models that make it a versatile animal model. Their external fertilization, large reproductive capacity, and embryo transparency facilitate genetic manipulation at the early stages of embryonic development [5-7]. Interestingly, zebrafish share 70% genetic similarity with humans and 82% of associated genes with human disease, making it one of the best animals for disease modeling [8]. In this regard, zebrafish model has been successfully used to establish models of human neurodegenerative disorders, such as Parkinson's, Huntington's, and Alzheimer's diseases [9].

The evaluation of myelination abnormalities is essential to evaluate the impact of neurological disorders in the central nervous system. In order to understand myelination process during development, several zebrafish transgenic reporter lines are currently in use [10].

The immunostaining protocols provide a significant advantage in terms of identifying differences in certain cellular, whole tissue or organ structures. This enables *in vivo* analysis of anomalies as an effect of genetic variation related to neurodevelopment [11].

Several techniques have been developed to stain the peripheral nerve fibers. Among these, Luxol Fast Blue (LFB) staining is one of the most reliable stain used to demonstrate the axonal degeneration and demyelination in histological studies. LFB staining method was developed by Kluver and Barrera to commonly stain myelin sheath in rat brain in paraffin-embedded sections [12]. Since myeline is a lipoprotein, the copper phthalocyanine chromogen component of LFB soluble in hydrophobic solvent such as alcohol makes it useful to stain myelin sheaths [12]. To visualize the three-dimensional structure of the developing zebrafish brain, whole-mount staining techniques can be applied to assess the neuroanatomy composition without any impact on the original structure [13].

Here, we have developed a whole-mount staining protocol using LFB,

***Address for Correspondence:** Sahar Daas, Department of Human Genetics, Sidra Medicine, Qatar, Email: sdaas@sidra.org*

Received: 04-December-2023, Manuscript No. jnd-23-121926; **Editor assigned:** 06-December-2023, PreQC No. P-121926 (PQ); **Reviewed:** 20-December-2023; QC No. Q-121926; **Revised:** 25-December-2023; Manuscript No. R-121926 (R); **Published:** 01-January-2024, **DOI:** 10.4172/2329-6895.11.6.570

staining myelin and nerve cells, in zebrafish model. We have optimized the use of LFB to penetrate the cellular components of central nervous system within a whole organism. Our developed staining method successfully highlighted the neurological anatomy of the zebrafish developing brain. A flowchart of the LFB myelin staining procedure using zebrafish larvae is presented as (Figure 1).

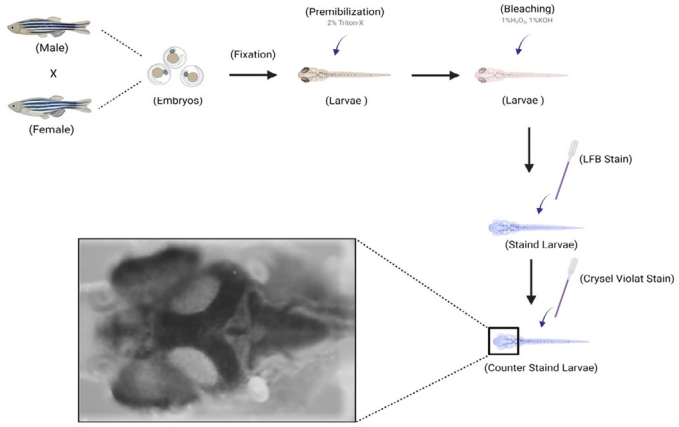

Figure 1. Zebrafish brain whole mount staining using Luxol fast blue stain flowchart. Flowchart of the general procedure steps for Luxol Fast Blue staining steps using zebrafish model

Methods

Ethical statement

All protocols used in this report were approved by the local Animal Care and Use Committee and conform to the Zebrafish Policy published by the Qatar Ministry of Public Health that follows the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Experiments performed on zebrafish were approved by the IACUC Office of Qatar Foundation (QF-IACUC, EVMC-2020-006) under MOPH guidelines (IACUC-SIDRA-2020-0006).

Zebrafish housing, husbandry, and care

Zebrafish (*Danio rerio*) wildtype (AB strain) were obtained from European Zebrafish Resource Center (EZRC, Germany). The optimal condition of housing density of 3-5 fish per liter in a recirculating aquaculture system under standard environmental conditions of temperature at 27°C, conductivity at 800 µs, and pH at 7.5 with 14 hours light starting and 10 hours dark cycle. The health of the zebrafish was monitored on a daily basis.

Study design and sample size

For zebrafish experiments, embryos were collected in N-phenylthiourea (PTU). A total number of 10-15 embryos were used per experiment and done in triplicate. Embryos were raised at 28 incubator. Larvae at the selected stage were fixed at 3 and 5 days post-fertilization (dpf) for staining.

In this technical report, we aimed to visualize the brain anatomy of zebrafish developing central nervous system in a three-dimensional view. Therefore, our modified protocol achieved whole mount staining to differentiate gray and white matter in the developing zebrafish brain.

Materials and reagents

Luxol fast blue staining kit were purchased from Electron Microscopy Sciences (Cat# 26681); phosphate buffered saline was obtained from Gibco (Cat# 10010031), Ethanol was obtained from VWR (Cat# 20821.321), and 12 NetWell Insert was obtained from Corning (Cat# 07-200-211). All the following chemicals were purchased from Sigma-Aldrich, paraformaldehyde (Cat# P6148-1 KG), Tween (Cat P2287-100 ML), Triton-X100 (Cat# X100), 30% hydrogen peroxide (Cat# H1009), Potassium hydroxide (Cat# P1767), Methylcellulose (Cat# M0387).

Part 1: Embryo collection

- Wildtype zebrafish were maintained overnight in breeding tanks filled with system water, where males were separated from females.
- Next day, the dividers were pulled to allow the zebrafish to mate, without any disturbance during the mating time.
- Using a mesh strainer, embryos were collected in egg H_2O, at the end of the day, and exchanged with egg H_2O containing 0.003% of 1-phenyl 2-thiourea (PTU) at the gastrulation phase, to prevent pigmentation within the zebrafish developing model.
- Embryos were incubated at 28.5°C until they reach the desired developmental stage. For this developed protocol, embryos were raised until 3 and 5 dpf.

Part 2: Larvae fixation

- Zebrafish larvae were euthanized using 1 ml of buffered (PH=7) tricaine (4 mg/ml) for at least 10 min.
- Euthanized zebrafish larvae were transferred to 1.5 mL microfuge tubes using a transfer pipet and washed 3 times with phosphate buffered saline supplemented with 0.2% tween (PBS-T) for 5 minutes (min) each.
- Zebrafish larvae were treated with Proteinase-K (10 ug/ml in PBST) for 20 min, then washed 3 times PBS-T for 5 min each.
- Zebrafish larvae were fixed in 1.5 ml of 4% phosphate-buffered paraformaldehyde (PFA) at 4°C overnight with gentle rocking.

Part 3: Zebrafish samples preparation

- Fixative was removed, and the zebrafish larva were washed 3 times PBS-T for 5 min each.
- The fixed zebrafish samples were then permeabilized using PBS containing 2% Triton-X100 for 2 hours on a rotating platform at room temperature and then washed 3 times with PBS-T for 5 min each.
- The zebrafish samples were bleached using a solution containing 1% hydrogen peroxide (H_2O_2) and 1% potassium hydroxide (KOH) in PBS for 20 min. The bleaching was performed under a stereomicroscope with constant monitoring until the zebrafish eye became sufficiently translucent.
- The samples were washed 3 times with PBS-T for 5 min each.
- The samples were dehydrated through serial incubations in Ethanol (ETOH) at room temperature:

a) Incubation in 25% ETOH/75% 1X PBS-T for 5 min, rocking

b) Incubation in 50% ETOH/50% 1X PBS-T for 5 min, rocking

c) Incubation in 75% ETOH/25% 1X PBS-T for 5 min, rocking

d) Incubation in 95% ETOH/5% 1X PBS-T for 5 min, rocking.

Part 4: Luxol fast blue staining

- Samples were incubated directly in Luxol Fast Blue Solution (Cat# 26681-01) at 56°C with gentle rocking in the hybridization oven overnight.
- on the same tube, the excess stain was rinsed using 1 ml of 95% ETOH, and then rinse the samples by invert with 1 ml of PBS-T, 3 times until the solution color became clear.
- Samples were transferred carefully into the Net Well insert using a transfer pipette.
- Color differentiation for the stained brain structures was performed as follows: Inserts containing the embryos were differentiated in the

well containing Lithium Carbonate Solution (Cat# 26681-04) for 3 min-5 min. Note: Carefully watch the differentiation step to avoid over differentiation and to avoid losing the luxol fast blue stain intensity.

- The differentiation was continued in the well containing 70% ETOH and observed under the stereomicroscope for approximately 30 min or until high contrast between the white brain matter (blue) and the grey brain matter (pale blue or colorless) of the zebrafish brain is observed. Note: Step 5 can be repeated if necessary.
- To stop the differentiation process, samples were transferred to a well containing PBS-T.

Part 5: Cresyl violet counterstaining

- Prepare the cresyl violet counterstain working solution: Add 75 ul of Acetic Acid, 10% (cat#26681-03) to 10 ml of cresyl violet acetate, 0.1% (cat#26681-02).
- Samples were counterstained for 2 min in freshly prepared cresyl violet acetate working solution
- Stained samples were placed in 2 washes of 95% ETOH for 5 min each.
- Stained samples were transferred and stored in 100% ETOH until imaging.

Part 6: Imaging

- Stained samples were mounted in 3% methylcellulose in a depression glass slide.
- Samples were imaged using a ZEISS stereomicroscope (Model #, Lumar v.12 stereo) equipped with a Nikon camera (Model#, DS-Ri1).

Results

Whole mount staining of zebrafish white and grey matter of the developing brain

Successfully, we have developed a whole-mount staining protocol using LFB to stain the zebrafish model myelin and nerve cells. We have optimized the use of LFB to penetrate the cellular components of the central nervous system within a whole organism. Our developed staining method successfully highlighted the neurological anatomy of the zebrafish developing brain. A flowchart of the LFB myelin staining procedure using zebrafish larvae is presented as Figure 1. Furthermore, our modified protocol achieved the whole mount staining of the zebrafish brain structures without comprising the sample structures. We used 4% paraformaldehyde, a routine fixative, for zebrafish specimens. The zebrafish samples were treated with Proteinase K before fixation process. Proteinase K treatment significantly improves the stain penetration into the whole mount specimen. After that, proteinase K treatment the zebrafish samples placed in the fixative overnight at 4°C. For additional permeabilization we used Triton X-100 detergent to improve the stain penetration without affecting the structural morphology of the fixed specimens. A staining process of zebrafish specimen using luxol fast blue and cresyl violet were shown in (Figure 2).

Visualization of zebrafish-stained brain structure by imaging with light microscopy

Several techniques have been developed to stain the peripheral nerve fibers. The LFB staining is one of the reliable stains used to demonstrate the axonal degeneration and demyelination in histological studies. LFB staining method was developed by Kluver and Barrera to commonly stain myelin sheath in paraffin-embedded sections of rat brain [12]. Since myeline is a lipoprotein, the copper phthalocyanine chromogen component of LFB is useful to stain myelin sheaths [13]. Additionally, to enhance specimen clarity, after embryo incubation and growing in PTU to prevent pigmentation, we performed bleaching step in the fixed specimens by incubating them in a solution containing 1% hydrogen peroxide (H_2O_2) and 1% potassium hydroxide (KOH). Our procedure resulted in sufficiently translucent and optically clear whole mount specimens. This optical clarity allowed the contrast visualization of the different brain tissues, the white-grey matter. Myelin is a fatty multilayered membrane that surrounds the axons to increase the electrical impulse speed rate [14], which is generated by oligodendrocytes of the Central Nervous System (CNS) and the Schwann cells of the Peripheral Nervous System (PNS) [15].

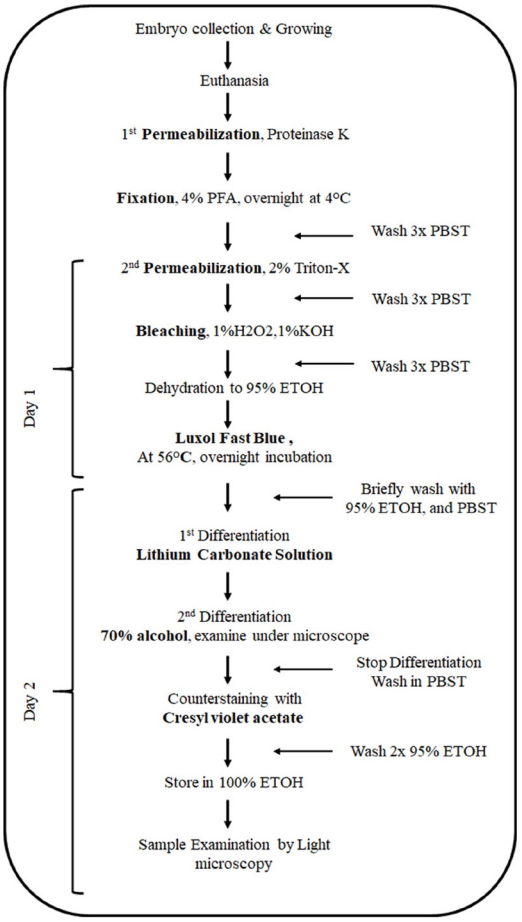

Figure 2. Luxol Fast Blue Staining Process: (A) Dorsal views of zebrafish specimen after overnight incubation with luxol fast blue. (B) White brain matter (blue) and the grey brain matter appearance after differentiation steps. (C) Brain structural components after counter staining using creysl violet stain at 5 days post-fertilization (dpf)

Effectively, our LFB whole-mount staining protocol achieved a clear contrasted visualization of the myelinated brain regions in zebrafish larvae. Thus, we were able to visualize a complete stain of the developing zebrafish brain. Images of a dorsal view of the brain were obtained and representative images for stained zebrafish larvae at 3 dpf and 5 dpf are shown in (Figure 3). We were able to visualize the myelinated zebrafish brain regions, including forebrain (FB), midbrain (MB), Cerebellum (CRB), and hindbrain (HB). Our method emphasized the anatomical structure of the zebrafish developing brain and demonstrated the cellular sub-regions of brain white-grey matter. Overall, our developed protocol assisted the evaluation of the zebrafish brain that can be used to investigate brain anomalies and axonal demyelination disorders.

Survey 1

By week 4, all 6 patients (100%) had positive scores (scores 4 and 5) for feelings of relaxation (Q2), positive thoughts (Q3), enjoying using the device (Q9), and wanting to keep the device (Q11). Regarding feeling better about oneself (Q6) and reduction in PTSD symptoms (Q10), 5 out 6 patients (83%) showed positive scores. Regarding memory (Q1), concentration (Q4), and irritability (Q5), 4 patients (67%) had positive scores. Half of the patients had improved thinking ability (Q7). Patients exhibited the lowest

scores regarding using less alcohol and/or drugs (Q8), with 2 out 6 (33%) having positive scores, and the remaining had neutral scores. Of note, no individual patient reported scores of 1 or 2 for any of the questions by week 4, suggesting that no patients disagreed with improved satisfaction scores, and either felt improved or at least neutral.

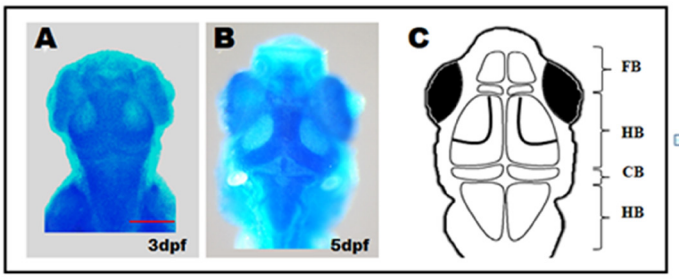

Figure 3. Luxol Fast Blue Stained Zebrafish Specimens: Images of Luxol Fast Blue stained zebrafish specimens dorsal view. (A) Zebrafish specimen imaged at 3 days post-fertilization (dpf). (B) Zebrafish specimen imaged at 5 dpf. (C) Schematic drawing of larval zebrafish developing brain depicting the brain area at 5 dpf: Forebrain (FB), Midbrain (MB), Cerebellum (CB) and Hindbrain (HB). Scale bar 10 μm

The pooled median score by week 4 was 4 (IQR: 4-4), and ranged from 3 to 5, suggesting a typically positive outcome and significantly improved overall score over time ($p<0.001$) (Table 3). The question that had the largest upward trend from week 1 was regarding improved memory (Q1), showing a statistically significant improvement by week 4 ($p<0.001$). Other individual questions did not demonstrate significant time-dependent improvements. Patients had the lowest upward trend regarding thinking ability (Q7) and use of drugs/alcohol (Q8), exhibiting slight, albeit not significant, reductions in score on week 4 compared to week 1.

Survey 2

By week 4, all 6 patients (100%) had positive scores (0 and 1) for having flashbacks (Q1) and negative emotional state (Q5). Regarding stressful events (Q4), losing interest in activities (Q6), being super alert (Q7), being easily startled (Q8), and being irritable or angry (Q9), 5 out 6 (83%) had positive scores, while 1 patient had moderate severity. No individual patient reported adverse scores of 3 or 4, suggesting no severe symptoms by week 4 (Table 4).

Of the individual survey questions, patients had statistically significant improvements regarding feelings of being emotionally upset (Q2: MD=-1.5, $p=0.031$) and being overly alert (Q7: MD=-3, $p=0.031$; Table 5). Although not statistically significant for all individual questions, all patients uniformly had numerically improved scores by week 4. The composite score was substantially improved from baseline, with an overall median score of 3 (IQR: 2-4) at baseline to 1 (IQR: 0-1) by week 4 (MD=-2 [95% CI: -3; -2], $p<0.001$). Overall, the predicted probability of obtaining the best outcome (score=0) was 2% at baseline vs. 43% by week 4. Conversely, the predicted probability of obtaining the worst outcome (score=4) was 39% at baseline vs. 2% at week 4. The overall cumulative odds ratio was 38.2 ($p<0.001$), suggesting that on average, the odds of moving from one score to a lower (improved) score at week 4 compared to the baseline are 38.2 times higher. The significant decrease in symptoms based on Survey 2 over time is illustrated in Figures 1 and 2.

Discussion

The global burden of the neurological disorder has been emphasized by world health organization as the greatest threat to public health, which is one of the major causes of childhood morbidity and mortality (1). Based on the Global Burden of Disease Study, these disorders were responsible for 6.39% of neurological disease in 2015 and predicted to increase to 6.77 in 2030 [1]. We have employed LFB stain that binds to the lipoproteins component of the myelin sheath for whole mount staining of zebrafish specimens [16].

Our developed protocol achieved a whole mount contrasted staining of the brain structure in three-dimensional perspective. Fixed zebrafish larvae were incubated in LFB for overnight at 56°C, to allow the stain penetration of the specimen's tissue. Removal of the excess non-specific stain was achieved using a weak alkaline solution (Lithium bicarbonate), which impair net protein charges to be negative. This step, in turn, aided to get rid of anions from the hydrophilic part of the proteins and maintained the lipid-rich myelin sheaths (hydrophobic proteins) stain [17]. This first differentiation step was followed by a treatment with 70% Ethanol that provides the second differentiation, where enough LFB stain that will identify the myelinated brain region of the brain white matter [18-20]. Finally, subsequent staining with CV stain was applied as a counter stain for the neuronal cell bodies and Nissl substance. The differentiation steps in our developed protocol is essential to achieve the desired color intensity of the LFB. Therefore, CV stain intensity can be optimized for over stained samples by washing the specimen carefully using 70% ETOH [21-28].

Conclusion

Neurological human disorders are sometimes associated with brain anomalies demyelinating disorders such as leukodystrophies, acute demyelinating encephalomyelitis, multiple sclerosis, and cerebral palsy. The degeneration of brain white matter in particular, the myelin sheath leads to axonal demyelination disorders. The evaluation of myelination abnormalities is essential to evaluate the impact of neurological disorders on brain development. To validate and understand how the brain structure is affected, zebrafish model provides an excellent toolkit to understand myelination process during development.

The immunostaining protocols provide a significant advantage in terms of identifying differences in certain cellular, whole tissue or organ structures. This enables *in vivo* analysis of brain anomalies as an effect of genetic variation related to neurodevelopment.

Various techniques have been used to study myelin in CNS and PNS by evaluating the morphological parameters in the myelin sheath, among them. Staining is useful for determining the distribution of myelinated fibers in gray/white matter. The conventional LFB method has been used widely to evaluate the morphological characteristics and to examine the nerve structure in animal models. Although some studies proposed innovative approaches for screening whole mount zebrafish brain, many come with a great financial cost and the need for high technical specialization. Other studies applied on rates tissue suggested that, combining LFB with other staining methods could be useful for the analysis of both axonal integrity and myelination. Nevertheless, the studies on zebrafish whole mount staining are scars and still under development.

Therefore, in the current study, we have developed a new method for whole-mount zebrafish staining that optimizes the penetration of staining regents of LFB into the white/gray matter, with minimal non-specific binding and background. Our developed method achieved clear anatomical three-dimensional visualization of the central nervous system as a utility for experimental neuropathology in zebrafish model.

Acknowledgement

We thank Sidra Medicine Research Branch for providing a research and development budget.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. World Health Organisation. "Neurological Disorders: Public Health Challenges." 2006.
2. Zhao, X and Bhattacharyya A. "Human Models are Needed for Studying Human Neurodevelopmental Disorders." *Am J Hum Genet* 103(2018):829-857.
3. Lieschke, GJ and Currie PD. "Animal Models of Human Disease: Zebrafish Swim into View." *Nat Rev Genet* 8(2007):353-367.
4. Saleem, S and Kannan RR. "Zebrafish: An Emerging Real-time Model System to Study Alzheimer's Disease and Neurospecific Drug Discovery." *Cell Death Discov* 4(2018):45.
5. Cooper, MS, D'Amico LA and Henry CA. "Analyzing Morphogenetic Cell Behaviors in Vitally Stained Zebrafish Embryos." *Methods Mol Biol* 122(1999):185-204.
6. Himabindu, A, Siddiqui AN and Jeevani T. "Genetic and Degenerative Neurological Disorders-an Emphasis on Alzheimer's, the Mystery." *J Genet Syndr Gene Ther* 2(2011):1-5.
7. Spitsbergen, JM and Kent ML. "The State of the art of the Zebrafish Model for Toxicology and Toxicologic Pathology Research--Advantages and Current Limitations." *Toxicol Pathol* 31(2003):62-87.
8. Howe, K, Clark MD, Torroja CF and Torrance J, et al. "The Zebrafish Reference Genome Sequence and its Relationship to the Human Genome." *Nature* 496(2013):498-503.
9. Xi, Y, Noble S and Ekker M. "Modeling Neurodegeneration in Zebrafish." *Curr Neurol Neurosci Rep* 11(2011):274-282.
10. D'Rozario, M, Monk KR and Petersen SC. "Analysis of Myelinated Axon Formation in Zebrafish." *Methods Cell Biol* 138(2017):383-414.
11. Kogata, N and Howard BA. "A Whole-mount Immunofluorescence Protocol for Three-dimensional Imaging of the Embryonic Mammary Primordium." *J Mammary Gland Biol Neoplasia* 18(2013):227-231.
12. Kluver, H and Barrera E. "A Method for the Combined Staining of Cells and Fibers in the Nervous System." *J Neuropathol Exp Neurol* 12(1953):400-403.
13. Yanqing J, Yeung JLH, Lee JH and An J, et al. "Visualization of 3D White Adipose Tissue Structure Using Whole-mount Staining." *J Vis Exp* 17(2018):e58683.
14. Waehneldt, TV. "Phylogeny of Myelin Proteins." *Ann N Y Acad Sci* 605(1990):15-28.
15. Desmazières, A, Sol-Foulon N and Lubetzki C. "Changes at the Nodal and Perinodal Axonal Domains: A Basis for Multiple Sclerosis Pathology?" *Mult Scler* 18(2012):133-137.
16. Gregorios, JB. "Histophathologic Techniques: Goodwill Trading Co." 2006.
17. Kiernan, JA. "Why is Lithium Carbonate used for Myelin Staining with Luxol Fast Blue?" *Biotech Histochem* 85(2010):325.
18. Rhein, V, Song X, Wiesner A and Ittner LM, et al. "Amyloid-beta and Tau Synergistically Impair the Oxidative Phosphorylation System in Triple Transgenic Alzheimer's Disease Mice." *Proc Natl Acad Sci USA* 106(2009):20057-20062.
19. Schmued, L, Slikker WJr. "Black-gold: A Simple, High-Resolution Histochemical Label for Normal and Pathological Myelin in Brain Tissue Sections." *Brain Res* 837(1999):289-297.
20. Schmued, LC, Swanson LW and Sawchenko PE. "Some Fluorescent Counterstains for Neuroanatomical Studies." *J Histochem Cytochem* 30(1982):123-128.
21. McNally, KJ and Peters A. "A New Method for Intense Staining of Myelin." *J Histochem Cytochem* 46(1998):541-545.
22. Lee, SC. "Histology for Pathologists: Third Edition." 2007.
23. Savaskan, NE, Weinmann O, Heimrich B and Eyupoglu IY. "High Resolution Neurochemical Gold Staining Method for Myelin in Peripheral and Central Nervous System at the Light and Electron-microscopic Level." *Cell Tissue Res* 337(2009):213-221.
24. Tolivia, J, Navarro A and Tolivia D. "Differential Staining of Nerve Cells and Fibres for Sections of Paraffin-embedded Material in Mammalian Central Nervous System." *Histochemistry* 102(1994):101-104.
25. Lempereur, S, Machado E, Licata F and Buzer L, et al. "ZeBraInspector, A Whole Organism Screening Platform Enabling Volumetric Analysis of Zebrafish Brain White Matter." *Develop Biol* 490(2022):1-10.
26. Sajadi, E, Raoofi A, Abdi S and Azimi H, et al. "The Modified Method of Luxol Fast Blue for Paraffin-embedded Myelin Sheath Staining." *Int J Morphol* 38(2020):1197-1200.
27. Carriel, V, Campos A, Alaminos M and Raimondo S, et al. "Staining Methods for Normal and Regenerative Myelin in the Nervous System." *Methods Mol Biol* 1560(2017):207-218.
28. Moszczynski, AJ, Volkening K and Strong MJ. "Neurofilament Immunohistochemistry Followed by Luxol Fast Blue, for Staining Axons and Myelin in the Same Paraffin Section of Spinal Cord." *Appl Immunohistochem Mol Morphol* 28(2020):562-565.

How to cite this article: Hasan, W, Shafea M, Abdulrahman D and Daas S. "Zebrafish Whole Mount Staining Using Luxol Fast Blue Stain." *J Neurol Disord*. 11 (2023):570.