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https://doi.org/10.1369/0022155418778543

Hu, D., Nicholls, P.K., Yin, C., Kelman, K.R., Yuan, Q., Greene, W.K., Shi, Z. and Ma, B. (2018) Immunofluorescent Localization of Non-myelinating Schwann Cells and Their Interactions With Immune Cells in Mouse Thymus.
Journal of Histochemistry & Cytochemistry

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Immunofluorescent localization of non-myelinating Schwann cells and their interactions with immune cells in mouse thymus

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Keywords

Non-myelinating Schwann cells (NMSC), Thymocyte, Dendritic cell (DC), Thymus, Immunofluorescence staining

Running headline:

NMSCs and NMSC-immune cell interaction in thymus

Abstract

The thymus is innervated by sympathetic/parasympathetic nerves fibers from the peripheral nervous system (PNS), suggesting a neural regulation of thymic function including T cell development. Despite some published studies, data on the innervation and nerve-immune interaction inside the thymus remain limited. In the present study, we used immunofluorescent staining of glial fibrillary acidic protein (GFAP) coupled with confocal microscopy/3D reconstruction to reveal the distribution of non-myelinating Schwann cells (NMSCs) and their interactions with immune cells inside mouse thymus. Our results demonstrate (1) the presence of an extensive network of NMSC processes in all compartments of the thymus including the capsule, subcapsular region, cortex, cortico-medullary junction, and medulla; (2) close associations/interactions of NMSC processes with blood vessels, indicating the neural control of blood flow inside the thymus; (3) the close "synapse-like" association of NMSC processes with various subsets of dendritic cells (DCs; e.g., $B220^+$ DCs, $CD4^+$ DCs, and $CD8^+$ DCs), and lymphocytes (B cells, $CD4^+$/$CD8^+$ thymocytes). Our novel findings concerning the distribution of NMSCs and the associations of NMSCs and immune cells inside mouse thymus should help us understand the anatomical basis and the mechanisms through which the PNS affects T cell development and thymic endocrine function in health and disease.

Introduction

As an essential primary lymphoid organ, the function of the thymus includes the maturation and selection of antigen-specific T cells[1-3]. The thymus is divided into two microanatomically defined regions, namely the cortex and the medulla, each of which contains several different thymic epithelial cell (TEC) subtypes. T-cell precursors enter the thymus through postcapillary venules (PCV) at the cortico-medulla junction and then begin a highly ordered differentiation programme[1-2]. Therefore, several types of thymocytes are located in spatially restricted regions of the thymus. The maturation process consists of the development of T-cell antigen receptors with high diversity that can bind to different antigens, whereas the selection process consists of eliminating self-reactive T cells. Thymic epithelial cells can produce self-hormones (e.g., thymulin, thymosin, thymopentin, and thymic humoral factors), which participate in immune cell maturation and selection[4]. In addition, the thymus can also synthesize hormones such as melatonin, neuropeptides, and insulin[4].

Recent studies have demonstrated the bidirectional crosstalk/communication of the peripheral nervous system (PNS) and the lymphoid tissue/organs including the lymph node, spleen, thymus, and gut-associated lymphoid tissue (GALT) [5-13]. The thymus is innervated by various types of nerve fibers from PNS, suggesting neural modulation of thymic functions including T cell development[10-13]. Sympathetic innervation originates from postganglionic neurons in the superior cervical and the stellate ganglion of the sympathetic chain[10]. In addition, the thymus might also be innervated by parasympathetic fibers from branches of the vagus nerve according to some reports[13].

Although the central nervous system (CNS) has relatively few unmyelinated nerve fibers, many nerve fibers or parts of nerve fibers (approximately 80%) in PNS are non-myelinated[14]. These non-myelinated nerve fibers consist of the Group C nerve fibers (sensory/efferent), the postganglionic sympathetic fibers, some of the preganglionic sympathetic/parasympathetic fibers, and the motor nerve terminals at neuromuscular junctions[14]. The Schwann cells of these

non-myelinated nerve fibers, namely the non-myelinating Schwann cells (NMSCs), have many axonal lengths embedded within grooves of their plasma membrane[14]. The Remak fibers, whose Schwann cells form the main populations of NMSCs, have small axons that can extend longitudinally for 50-100μm[14]. In addition to neural regulation and repair, Schwann cells (including NMSCs) can also function as immunocompetent cells and play essential roles in the immune response and its regulation[15-17]. For example, like microglia, Schwann cells are able to recognize exogenous/endogenous danger signals through pattern recognition receptors. Upon activation, Schwann cells can initiate and regulate local immune reactions by antigen presentation and by secreting cytokines/chemokines[15-17]. Furthermore, Schwann cells have been demonstrated to interact with lymphocytes, dendritic cells (DCs), and macrophages under healthy conditions and in disease states[18-20].

In view of the location/functions of NMSC and their associations with nerve fibers[14], we believe that NMSCs are highly suitable for studying the local interactions/communications of PNS and primary lymphoid tissues/organs. By using immunofluorescent staining and confocal microscopy/three-dimensional (3D) reconstruction, we have investigated the distribution of NMSCs and NMSC-immune cell associations/interactions in the mouse thymus to improve our understanding of the microanatomical basis of the interaction of PNS and thymus.

Material and Methods

Animals

C57BL/6 female mice (8-10 weeks old) were purchased from the Animal Resources Centre (Perth, Australia). All animal experiments were carried out in accordance with the Australian code for the care and use of animals for scientific purposes at Murdoch University, Perth, Australia, with local animal ethics committee approval. In total, eight mice were used for the study.

Section preparation

Microscope slides (27mm×75mm) were obtained from Thermo Fisher Scientific (Scoresby, Australia). After a brief wash with 70% ethanol, slides were coated with 0.01% poly-L-lysine solution (PLL, Sigma) for 10 minutes followed by air-drying overnight at room temperature. Mice were killed by carbon dioxide followed by cervical dislocation. Their thymuses were then quickly removed, embedded in Tissue-Tek® O.C.T. COMPOUND (ProSciTech, Kinwan, Australia), and snap-frozen in liquid nitrogen. Cryosections (20 µm) were prepared by using a Leica CM1850 UV Cryostat (Leica Biosystems, Nussloch, Germany) and mounted on the above-mentioned PLL-treated microscope slides.

Antibodies

The specificities and sources of antibodies are described in Table 1.

Immunofluorescent staining

Sections were fixed in 4 °C methanol for 3 min and in 4 °C acetone for 3 min. All the following steps were performed at room temperature. The sections were air-dried for 1 h and then rehydrated for 10 min in phosphate-buffered saline (PBS). All washes (3x10 minutes) between stages were performed in PBS. After the sections had been permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 5 minutes, potential non-specific binding sites were blocked with antibody dilution buffer (2% goat serum (Sigma) and 1% IgG-free bovine serum albumin (Sigma) in PBS) for 20 minutes at room temperature. Sections were then incubated with primary antibodies overnight at 4 °C. After being washed, the sections were then incubated with secondary antibodies for 1 hour at room temperature. Following the final washing step, sections were mounted with Fluorescence Mounting Medium (DAKO).

Confocal microscopy

Confocal microscopy was performed with a Nikon Instruments C2 plus Confocal Microscope (Nikon Instruments, Melville, NY) equipped with three lasers (excitation wavelength at 488nm, 561nm, and 633 nm). A Plan Apochromat λ 40x objective lens was used for imaging.

For some micrographs, Tile Scan was performed to scan a large area of the thymus. After their acquisition, the images were adjusted and analyzed by using NIS-Elements Advanced Research (AR) of the confocal system. Maximal intensity projection of a Z-stack was performed by using the "Maximal intensity projection" function of the NIS-Elements AR.

Image processing

The images obtained from confocal microscopy were exported as BMP files and further processed (adjustment of brightness and contrast)/edited (cropping and labelling) in Jasc Paint Shop Pro 9 (Corel Corporation, Ottawa, Canada).

Results

In our previous studies[20], we have utilized glial fibrillary acidic protein (GFAP) as a reliable marker for localization of NMSCs in lymph node. We also used this antibody for immunofluorescent staining of a variety of tissues including brain, lung, trachea, skin, and intestine and observed brightly stained cells with classical Schwann cell morphology. We also tested other NMSC markers such as p75 neurotrophin receptor ($p75^{NTR}$)/S100beta and observed staining patterns similar to that of GFAP (data not shown here). In addition, close associations of GFAP and other neuronal markers (such as protein gene product 9.5 (PGP9.5) and neurofilament) have also demonstrated that GFAP is a reliable marker for NMSCs associated with Remark fibers (Fig.1).

To reveal the distribution of NMSCs in the thymus, 20-μm-thick thymic cryosections from C57BL/6 female mice were labeled with anti-GFAP together with other two antibodies, namely anti-CD11c and anti-B220. The results are shown in Fig.2 and Fig. 3. An extensive meshwork of NMSCs and NMSC processes was observed in the various thymic compartments, including the capsule/subcapsular region (Fig. 2), cortex (Fig. 3A), and medulla (Fig. 3B). The cortex possessed a few B cells/DCs, some of which had close associations with NMSCS processes (Fig. 3A). The GFAP staining in the medulla was much stronger, and NMSC processes were

thicker than those of the cortex (Fig. 3B). Some cell bodies of NMSC were also observed in the medulla (Fig. 3B). In addition, in the medulla, the NMSC processes exhibited close associations with a large number of DCs ($B220^+$ and $B220^-$) and some B cells (Fig. 3B).

Thymocytes are classified into a few distinct maturational stages based on the expression of cell surface markers[1]. The earliest thymocyte stage is both $CD4^-$ and $CD8^-$ and the next major stage is $CD4^+$ $CD8^+$. The final stage of maturation is the single positive stage ($CD4^+$ or $CD8^+$). To reveal the relationship of NMSC and thymocytes in the thymus, we stained NMSCs, $CD4^+$ thymocytes (revealed by anti-CD4 staining), and DCs. The results are shown in Figs. 4 and 5. In the cortex (Fig.4, Fig. 5A, and 5B), most of the thymocytes were $CD4^+$, and many of them had a close association with NMSC processes. In the medulla region (Fig.4, Fig. 5C, and 5D), NMSC processes were thicker than that of the cortex, and some of them had close associations with blood vessels. We also observed a large number of DCs (mostly $CD4^+$), some of which exhibited close appositions to NMSC processes (Fig. 5C and 5D).

We then analyzed the distribution of NMSCs, $CD8^+$ thymocytes (revealed by anti-$CD8a^+$ staining), and DCs. The results are shown in Fig. 6. In the cortex (Fig. 6A), most of the thymocytes were $CD8^+$, and many of them exhibited close apposition to NMSC processes. In the medulla (Fig. 6B), we observed that the NMSCs processes had close associations with $CD8^+$ thymocytes, $CD8^+$/$CD8^-$ DCs, and thymic corpuscles.

To understand the spatial relationship between NMSCs and blood vessels/DCs, triple-immunolabelling with antibodies against GFAP, CD31, and CD11c was performed on thymic cryosections. The results are shown in Fig. 7, S Video 1, and S Video 2. The NMSCs processes were seen to be closely associated with blood vessels (including capillaries and PCV) in the cortex, cortico-medullary junction, and medulla of the thymus (Fig.7).

Discussion

In the present study, immunofluorescent staining and confocal microscopy/3D reconstruction were utilized to reveal the distribution of NMSCs and NMSC-immune cell

(thymocytes/lymphocytes and DCs) associations *in situ* in the mouse thymus. Our findings can help us understand the microanatomical basis of the PNS-thymus interaction. We believe that this is the first work that shows these kinds of local contact/interactions of NMSC and DCs /lymphocytes in mouse thymus.

The immune system and PNS are anatomically and functionally interconnected through the dense innervation (mainly sympathetic) of the primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid tissues/organs[20-25]. As has been demonstrated in some studies and across a wide variety of species including human and mouse, the thymus is innervated by various types of nerve fibers from the autonomic nervous system[10-13,24]. The thymus receives a substantial sympathetic innervation from cervical and upper thoracic sympathetic chain ganglia, and some neuroanatomical evidence has also been presented for parasympathetic or sensory input to the thymus [10,24]. In addition, phrenic nerve might additionally contribute to the innervation of thymus[11].

By using an anti-GFAP antibody as a reliable cellular marker for NMSCs[20], we have analyzed the distribution of NMSCs in the thymus. We have observed an extensive meshwork of NMSC/NMSC processes in the various thymic compartments, including the capsule, subcapsular region, cortex, cortico-medullary junction, and medulla. Since NMSCs have been reported to be closely associated with nerve fibers including PGP9.5^{+} nerve fibers, tyrosine hydroxylase (TH) $^{+}$ sympathetic nerve fibers, and neurofilament (NF) $^{+}$ nerve fibers[23,25], the extensive distribution of NMSCs also indicates the presence of dense nerve fibers, especially Remak nerve fibers, inside the mouse thymus. This kind of dense innervation is clearly linked with the neural control of thymocyte development and endocrine function of the thymus.

In the thymus, noradrenergic fibers enter with nerve bundles and plexuses around blood vessels, travel into the cortex from subcapsular plexuses and with the vasculature, and branch into the parenchyma of the thymic cortex[26]. The vasculature and parenchymal regions of both the outer and deep cortex are innervated by these fibers. Compared with some published results

showing only sparse nerve fibers, which are distributed through trabecula and mainly associated with/near blood vessels, our results demonstrate a much denser innervation inside each compartment of the thymus. In addition, the GFAP staining in the medulla is much stronger, and NMSC processes are thicker than those of the cortex.

We have further shown that these afferent nerve fibers are closely associated with blood vessels (including capillaries in cortex/medulla and PCV in cortex-medulla region), indicating the neural control of blood flow inside the thymus. In our previous study of the lymph node[20], we also observed this type of close association of NMSC and blood vessels (including high endothelial venules (HEV) and other blood vessels) [20]. Therefore, the neuronal regulation of the blood flow and vascular permeability of blood vessels might affect T-cell trafficking, i.e., both the entry of T cells into the thymus and their exit from the thymus as mature T cells.

The thymic microenvironment consists of diverse stromal cells, nerve fibers/neuropeptides, and a sophisticated extracellular matrix (ECM), which govern the directed migration and maturation of the developing T cells, together with various chemokines[2]. T-cell development in the thymus depends mainly on sequential interactions between the thymocytes and this distinct microenvironment in cortex and medulla of the thymus[3]. T cells undergo positive and negative selection in the thymic cortex and medulla, respectively. These cells are derived from hematopoietic stem cells that are found in the bone marrow. The progenitors of these cells migrate to and colonize the thymus. The developing progenitors within the thymus, also known as thymocytes, undergo a series of maturation steps that can be identified based on the expression of different cell surface markers. T cells differentiate into $CD4^+$ or $CD8^+$ cells in the cortex. Interactions between the sympathetic innervation and the immune cells inside thymus have been reported, both anatomically and functionally, in some studies[10,20,26-28]. Despite these published studies, detailed microanatomical studies of nerve-immune cell interactions remain rare. Therefore, we have investigated the NMSC-immune cell interactions *in situ* inside the mouse thymus to address this question. In our study, we have observed a very extensive

interaction of NMSCs with $CD4^+$ or $CD8^+$ thymocytes in the cortex(for positive selection), cortico-medullary junction, and medulla (for negative selection), indicating the potential neural control of T cell development in the thymus through Remark fibers. In addition, NMSCs also have close apposition with thymic B cells (about 0.3% of whole thymic cells), which can present antigen and induce negative selection[3].

The thymic medulla, which is mainly composed of medullary mTECs and DCs, provides a specialized microenvironment dedicated to the establishment of central T-cell tolerance[29]. Three distinct subsets of DCs are located mainly in the medulla: resident conventional DCs ($CD11c^{hi}CD11b^{-}$ $CD8\alpha^{hi}Sirp\alpha^{-}$), migratory conventional DCs ($CD11c^{hi}CD11b^{+}CD8\alpha^{lo}Sirp\alpha^{+}$), and plasmacytoid DCs ($CD11c^{int}B220^{+}PDCA\text{-}1^{+}$)[29]. In this study, we have investigated the distribution of DCs and their associations with NMSCs. In the cortex, we observed some DCs, most of which were located in the medullary region. We also observed that NMSCs were closely associated with some $B220^+$ DCs, $CD4^+$ DCs, and $CD8^+$ DCs, indicating potential neural control of negative selection inside the thymus. Although further studies of the molecular mechanism need to be carried out *in vitro* and *in vivo*, our findings provide a reliable microanatomical basis for the NMSC/nerve fibers-DC communications inside the thymus.

At the cellular level, the NMSC-immune cell contact described in our study has a “synapse-like” morphology, which has been described in our previous reports[9,20]. Instead of being a random event, this kind of cell-cell contact is very extensive inside the thymus and indicates potential PNS’ effect on thymic functions including T cell development and the secretion of hormones. Since the NMSCs or nerve fibers are relatively static, and as immune cells such as DCs, macrophages, and lymphocytes might be mobile, this type of contact/communication should be dynamic under healthy conditions and in disease. Further studies should be carried out to uncover the mechanisms of this type of cell-cell contact. For example, noradrenaline

released from nerve terminals plays a role in the maturation of thymocytes through the activation of β-adrenoceptors[30-31]; In addition, noradrenaline has been reported to inhibit the proliferation of thymocytes and promote their differentiation *in vitro*[30-31]. Furthermore, the direct production of noradrenaline, dopamine, and other catecholamines can also regulate several immune activities such as differentiation, apoptosis, and cytokine production in an autocrine/paracrine manner [32-33].

In summary, our novel findings concerning thymic NMSC distribution and their interaction with thymocytes/lymphocytes/DCs should provide new insights into the bidirectional communications of primary lymphoid tissue/organs and the PNS. Further *in vivo* and *in vitro* functional studies need to be carried out to reveal the molecular mechanisms of these kinds of cell-cell communications. Since the sympathetic and peptidergic innervation of thymus seems to have a role in the pathogenesis and progression of some neuroimmune (e.g., multiple sclerosis) and immune diseases (e.g., allergy, autoimmune diseases, and immunodeficiency) [10, 34], a better understanding of PNS-immune system interaction might benefit the development of therapeutic strategies for diseases that involve altered neuroimmune crosstalk[10,35].

Acknowledgements

The authors would like to thank Wayne Rasband for the ImageJ programme.

Competing Interests

The author(s) declare they have no competing interests.

Funding statements

None.

Author Contributions:

DH, WKG, PKN, ZS, and BM conceived the study, designed the experiments, and wrote the paper. DH, CY, KK, QY, and BM performed the experiments and analyzed the data. All authors discussed the results, provided comments, and reviewed the manuscript.

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Figure legends

Figure 1 Distribution of GFAP and neuronal markers (PGP9.5 and neurofilament) in the cortex of C57BL/6 mouse thymus. Antibodies against GFAP (green) and CD31 (blue) label mainly NMSCs and blood vessel endothelial cells, respectively. Neuronal markers (PGP9.5 and neurofilament) were labelled with red colour. Objective lens: 40x; Scale bar: 20 µm.

Figure 2 Distribution of NMSCs, B cells, and DCs in the C57BL/6 mouse thymus. Antibodies against GFAP (green), B220 (red), and CD11c (blue) label mainly NMSCs, B cells, and DCs, respectively. M: Medulla; C: cortex; CMJ: cortico-medullary junction; CP: capsule; Objective lens: 40x; Scanning mode: tile scan (3x3); Scale bar: 100 µm.

Figure 3 Distribution of NMSCs, B cells, and DCs in the cortex (A) and medulla (B) of C57BL/6 mouse thymus. Antibodies against GFAP (green), B220 (red), and CD11c (blue)

label mainly NMSCs, B cells, and DCs, respectively. The white arrows indicate a $B220^-$ $CD11c^+$ DC with a close association with NMSC processes. The yellow arrows indicate a $B220^+$ $CD11c^+$ DC with a close association with NMSC processes. The pink arrows indicate two NMSC cell bodies that have a close association with $CD11c^+$ DC. Each image is a maximal intensity projection of a Z-stack. BV: blood vessel; C; cortex; Objective lens: 40x; Scale bar: 20 µm; Stack size: 6.0 µm; optical slice interval: 0.50 µm.

Figure 4 Distribution of NMSCs, $CD4^+$ thymocytes, and DCs in the C57BL/6 mouse thymus. Antibodies against GFAP (green), CD4 (red), and CD11c (blue) label mainly NMSCs, $CD4^+$ thymocytes, and DCs, respectively. M: medulla; C: cortex; CMJ: cortico-medullary junction; CP: capsule; BV: blood vessel; PCV: postcapillary venule; Objective lens: 40x; Scanning mode: tile scan (3x3); Scale bar: 100 µm.

Figure 5 Distribution of NMSCs, $CD4^+$ thymocyte, and DCs in the cortex (A-B) and medulla(C-D) of C57BL/6 mouse thymus. Antibodies against GFAP (green), CD4 (red), and CD11c (blue) label mainly NMSCs, $CD4^+$ thymocyte, and DCs, respectively. M: medulla; C: cortex; CMJ: cortico-medullary junction; BV: blood vessel; The yellow arrows indicate a $CD4^+$ $CD11c^+$ DC with a close association with NMSC processes. Each image is a maximal intensity projection of a Z-stack. Objective lens: 40x; Scale bar: 20 µm; Stack size: 6.0 µm; optical slice interval: 0.50 µm.

Figure 6 Distribution of NMSCs, $CD8^+$ thymocyte, and DCs in the cortex (A) and medulla (B) of C57BL/6 mouse thymus. Antibodies against GFAP (green), CD8 (red), and CD11c (blue) label mainly NMSCs, $CD8^+$ thymocyte, and DCs, respectively. CMJ: cortico-medullary junction; CP: capsule; TC: thymic corpuscle (Hassall's corpuscle). The yellow arrows indicate a $CD8^+$ $CD11c^+$ DC with a close association with NMSC processes. Each image is a maximal intensity projection of a Z-stack. Objective lens: 40x; Scale bar: 20 µm; Stack size: 6.0 µm; optical slice interval: 0.50 µm.

Figure 7 Distribution of NMSCs, blood vessels, and DCs in the cortex (A), cortex-medulla (B), and medulla(C) of C57BL/6 mouse thymus. Antibodies against GFAP (green), CD31 (red), and CD11c (blue) label mainly NMSCs, blood vessels, and DCs, respectively. Each image is a maximal intensity projection of a Z-stack. M: medulla; C: cortex; PCV: postcapillary venule; Objective lens: 40x; Scale bar: 20 µm; Stack size: 6.0 µm; optical slice interval: 0.50 µm.

Supplementary videos

S Video 1 Distribution of NMSCs, blood vessels, and DCs in the medulla of C57BL/6 mouse thymus. Antibodies against GFAP (green), CD31 (red), and CD11c (blue) label mainly NMSCs, blood vessels, and DCs, respectively. The arrow indicates NMSC, which has close associations with DCs. This video is an animation of 13 optic slices. Objective lens: 40x; Scale bar: 10 µm; Stack size: 6.0 µm; optical slice interval: 0.50 µm; frame rate: 4 fps (frames per second).

S Video 2 Distribution of NMSCs, blood vessels, and DCs in the medulla of C57BL/6 mouse thymus. Antibodies against GFAP (green), CD31 (red), and CD11c (blue) label mainly NMSCs, blood vessels, and DCs, respectively. The arrow indicates NMSC, which has close associations with DCs. This video is generated by a 3D reconstruction of 13 optic slices in S video 1. Objective lens: 40x; Scale bar: 10 µm; Stack size: 6.0 µm; optical slice interval: 0.50 µm; frame rate: 4 fps.

Table 1 Sources of primary and secondary antibodies

Target	Conjugate	Species and isotype	Cells labelled	Company
CD11c		Armenian hamster monoclonal IgG	Dendritic cell (DC)	STEMCELL Technologies (Tullamarine, Australia)
B220 (CD45R)		Rat monoclonal IgG	B cell	Australian Biosearch (Karrinyup, Australia)
CD31		Rat monoclonal IgG	Blood vessel endothelial cell	Australian Biosearch
CD4		Rat monoclonal IgG	$CD4^+$ thymocyte	Australian Biosearch
CD8a		Rat monoclonal IgG	$CD8^+$ thymocyte	Australian Biosearch
protein gene product 9.5 (PGP9.5)		Chicken polyclonal	Neuronal marker	Abcam Australia (Melbourne, Australia)
160 kD Neurofilament Medium(NF-M)		Chicken polyclonal	Neuronal marker	Abcam Australia
Glial fibrillary acidic protein (GFAP)		Rabbit polyclonal	NMSCs	DAKO (North, Sydney, Australia)
Rabbit IgG H&L	DyLight® 488	Goat polyclonal F(ab')2		Abcam Australia
Armenian Hamster IgG H&L	Alexa Fluor® 647	Goat polyclonal		Abcam Australia
Rat IgG H&L	Alexa Fluor® 555	Goat polyclonal		Abcam Australia
Rat IgG H&L	Alexa Fluor® 647	Goat polyclonal		Abcam Australia
Chicken IgG	Alexa Fluor® 555	Goat polyclonal		Abcam Australia