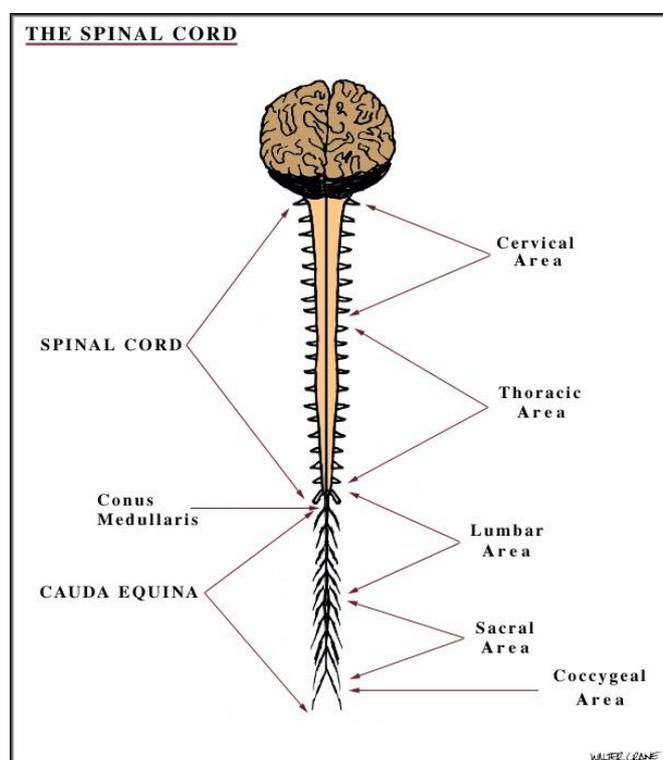


## CHAPTER 1: INTRODUCTION

### 1.1 REVIEW ON SPINAL CORD

#### 1.1.1 SPINAL CORD ANATOMY

Spinal cord, together with the brain, forms the central nervous system (CNS) of vertebrates (Edwards et al., 2002). Peripheral nervous system (PNS), on the other hand, is composed of the spinal nerves and cranial nerves.



**Figure 1.1:** Human spinal cord (Hamal, 2010)

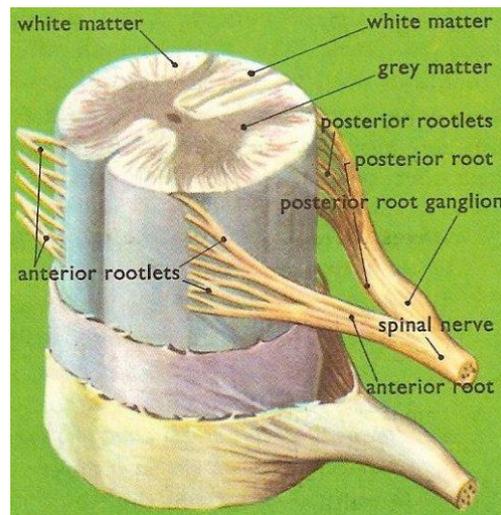
Spinal cord is an elongated cylindrical mass of nerve cells (Parker, 2007) with its rostral end connected to the medulla oblongata (Figure 1.1). Playing important roles in our daily lives, it is protected by the vertebral column and three layers of meningeal membranes from physical knocks (Chusid and McDonald, 1964).

### 1.1.2 FUNCTIONS OF SPINAL CORD

Spinal cord plays vital function as bridge for information transmission between the brain and body. Ascending pathway in the spinal cord allows the impulses to travel up to the brain to be processed. Motor impulses, on the other hand, travel through the descending pathway from the brain to the spinal cord. These impulses will be transmitted by motor neurons to the effector organs such as muscle, skin and glands. Besides that, the spinal cord also mediates reflex activity (Edwards et al., 2002).

### 1.1.3 SPINAL NERVES

The spinal cord depends a lot on the spinal nerves to carry out its functions as they will go directly to the particular muscle to gather and send information.

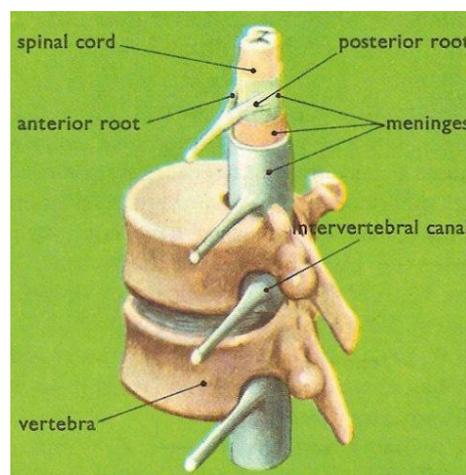


**Figure 1.2:** Connections of the spinal nerve roots to the spinal cord (Darling, 1999)

As demonstrated by Figure 1.2, a spinal nerve refers to a mixed nerve which is a combination of dorsal and ventral roots (or posterior and anterior roots). The dorsal root is made up of sensory nerve fibers that go into the dorsal horn of the spinal grey matter. The posterior root ganglion is caused by the cell bodies of the nerve fibers (Edwards et al., 2002).

The ventral root, on the other hand, consists of motor nerve fibers that come from the ventral horn. These nerve fibers are responsible to send motor impulses to the effector organs such as skin, glands and muscle. The word “innervations” in the title stands for the nerve supply to certain body parts.

Spinal nerves branch out from the spinal cord to reach different parts of the body through gaps between vertebrae (Figure 1.3). They divide and enter the back and front of the spinal cord as spinal nerve roots, each composed of many rootlets (Parker, 2007).



**Figure 1.3:** Spinal nerves pass through the intervertebral canals (Darling, 1999)

#### **1.1.4 CELLULAR BASIS OF SPINAL CORD**

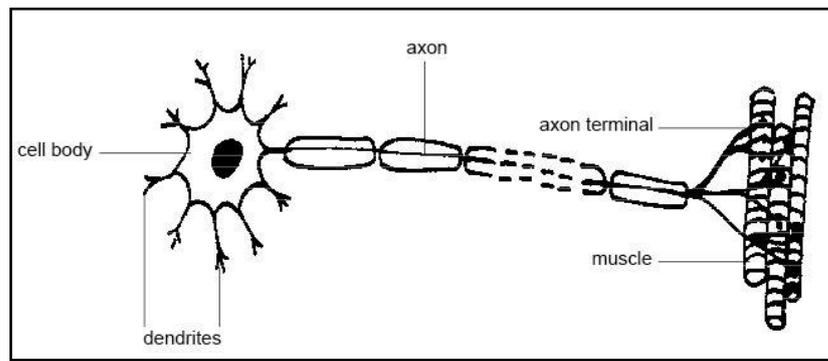
Spinal cord houses millions of neurons in a network of glial cells which form its structural and functional basis. Neurons are highly specialized to conduct impulses to other responsive cells at a distance (Tortora, 1986). They exist in great variety of shapes and sizes. Glial cells, on the other hand, do not generate electrical signals like the neurons. They are smaller in size and outnumber neurons by 5 or 10 times. There are various types of glial cells with different functions but they generally provide support and protection to neurons.

In retrospect, cell theory, which states that cells are the structural units of all living matter and the basic elements of all tissue and organs, fit in every part of the body

except the brain and spinal cord. A nerve cell covers more area compared to other cells due to its very complex shape and long processes (Kandel and Schwartz, 1985). This leads to some scientists to come up with the currently defunct reticular theory which stated that nervous system is a continuous structure. Ramon y Cajal, who first presented the histological sections of the nervous system, demonstrated the neurons as individual units, which was in contrast to the reticular theory (Frixione, 2009). As has been proven, all neurons are not continuous but they are connected in such a way that certain pathways are established to permit transmission of information.

#### **1.1.4.1 MOTOR NEURON**

Motor neurons are specific nerve cells involved in the motor system of nervous system. Each region of motor neuron has distinctive signaling functions. Like any other cells, motor neuron has a cell body with centrally located nucleus that acts as the metabolic center. Once stimulated, the highly-branched dendrites at one end of the neuron would carry impulse towards the cell body (Tortora, 1986). Impulses are then carried away from the cell body by an elongated and fine extension arising from the cell body called axon. Blocks of myelin sheath are distributed along the axon and the gap between two blocks is called Node of Ranvier. It enables impulses to travel even faster along the axon through saltatory conduction.



**Figure 1.4:** Morphology of a motor neuron (Lawson, 2007)

#### **1.1.4.2 AXOPLASMIC TRANSPORTATION OF MOTOR NEURON**

Back in the late eighteenth century, it was believed that there was flow of “animal spirits” from the brain to the muscles. However, this theory was scientifically proven to be wrong in 1948 when Weiss conducted an experiment which led to the finding that there were substances originating in the cell body transported along the axon towards its peripheral end. Neurotransmitters that get transported are released at synapses between the neurons and other cells, i.e. other neurons or cells like muscle and fibers.

Now it has been established that substances in the neuron can be transported in two ways, either anterogradely or retrogradely. Anterograde transport moves substances away from the cell body. There are fast and slow transport in the axon (Waxman et al., 1995). Slow transport serves to renew the axoplasm continuously in growing or regenerating nerves (Ottoson, 1983) by constantly transporting protein, amino acid and nutrients needed by axon (Muhamad, 1995). Fast anterograde transport, on the other hand, is always associated with particulate and organelles rather than soluble fractions of proteins, glycoproteins, phospholipids and membrane-bound enzymes. It requires adenosine triphosphate (ATP) and oxygen for the process to be carried out (Ottoson, 1983, Waxman et al., 1995).

In contrast to this, retrograde transport returns membrane constituents and proteins to the cell body. In addition, it always acts as the pathway for polio, herpes and rabies viruses to reach the CNS. The retrograde transport can be exploited histologically in identifying specific neuronal control over a certain group of muscle.

#### **1.1.5 RESEARCHES ON SPINAL CORD**

Galen was the first ever man who successfully uncovered the knowledge on anatomy of spinal cord (Clarke and O'Malley, 1996). He gave detailed description on the vertebral column and spinal cord, as well as the nerve roots (Marketoss and Skiadas, 1999). Gerard Blasius then illustrated the H shape of the grey matter in a cross-section of spinal cord (Tandheelkd, 1988). The fragments of the spinal cord's anatomical jigsaw puzzle were then slowly yielded. Subsequent findings on the spinal cord anatomy include arrangement of the fibre bundles, substantia gelatinosa, as well as the pathways or tracts in the cord (Pearce, 2008).

Researchers and scientists, then, started to relate the anatomy of the spinal cord with its physiology. It is noted that researches on spinal cord within the past 20 years emphasis mainly on spinal cord regeneration research (Kwon et al., 2002), owing to the fact that spinal cord injuries cause paralysis and disabilities that deprives mobility of thousands of people each year. Mammals such as monkeys, rabbits, cats and mice are always used as the experimental subjects to investigate the spinal cord, anatomically and physiologically in general, or specifically in searching for treatments to heal spinal injuries (Chiken et al., 2001; Lange and Leonhardt, 1978; Crowe et al., 1997; Trowell, 1943). However, fish, too, is a popular experimental subject which has its spinal cord investigated (Funakoshi et al., 2004). Spinal cord has direct control over trunk muscles and fin muscles which are being used for its movements.

## **1.2 REVIEW OF FISH**

### **1.2.1 FISH CENTRAL NERVOUS SYSTEM**

Behaviour is always a mirror of morphological and physiological capabilities of the nervous system structure, both the brain and spinal cord. Although fish do not have the superior functions in human (Rose, 2002) such as consciousness and complicated thoughts, they do need some intelligence to lead their daily lives. The brain makes it possible for them to learn and memorize, to initiate as well as coordinate movement, and in some species to recognize specific individuals (Goodson, 2005; Portvella and Vargas, 2005). The spinal cord, as in other vertebrates, connects the brain with the body. All information and decisions made are brought to the body through the spinal cord. All motor impulses are transmitted by the spinal specialized cell (motor neurons) to the effector organs, such as muscle limbs for terrestrial vertebrates and fins for aquatic vertebrates. The fins probably act as “reduced appendages” to the fish.

### **1.2.2 FISH FINS**

Fins are a very particular characteristic of fish (Bond, 1979). Generally, they are folds of skin which have broad surface and supporting structures (Harder, 1975), which are of different shapes and sizes. While vertebrates like amphibians, reptilians and mammals have limbs that are capable of great motility and enabling movement, fins are the appendages of similar functions for fishes. It is interesting to note that scientists have great interest in determining whether the limbs of tetrapods evolved from fish fins.

Tracing through the evolutionary history of fishes, it can be observed that gradual swift of locomotion nature results in thousands of fin designs (Bond, 1979). The most primitive fish were heavy and clumsy. Thus, their fins were used primarily to crawl on underwater floor and also to anchor a resting position. When time progressed and

evolution took place, the fishes left the underwater floor and began to swim. That was when some of the fins were converted into organs of propulsion (Harder, 1975).

There are two main categories of fins, median and paired fins. Median fins, which are also known as unpaired fins, are located in the medial plane. Dorsal fin, anal fin, caudal fins and adipose fin are all median fins. Paired fins, on the other hand, which include pectoral fins and pelvic fins are symmetrically arranged, one on each side of the body. Both median and paired fins work together to aid in movement and to control direction.

A fish has several fins and what they do with them depends on their species. Fish fins can perform a wide range of functions such as self protection, touching, sensing, enabling fish to move on land and allowing fish to rest on them (Bond, 1979). Fish fins are undoubtedly the most important organ in assisting movement in water.

Fish locomotion revolved around two main aspects, which were forward movement and directional control. Forward movement was generally initiated by muscle contraction while directional control depended largely on the fins (Lagler, 1977).

Lauder and Drucker (2004) proposed that dorsal fin acted as propulsor, stabilizer and also help in direction maneuvering. For adipose fin, Reimchen and Temple (2004) had recently suggested that it might generate thrust on its own and acted as a flow sensor. Information on anal fin function was lacking but Standen and Lauder (2005) suggested that anal fin acted as bilge keels to straighten and balance the body position of fish. Caudal fin always acted as the important forward thrust generator (Lauder, 1989). Although most of the researches directed the role of the caudal fin as the propeller, it was also proved to be important in maneuvering the directional change (Liao and Lauder, 2000).

Pectoral fins were used extensively for propulsion (Lauder and Drucker, 2004). Some fishes could even swim steadily with only the pectoral fins (Drucker and Jensen, 1996). Besides, some fish made use of the pectoral fins to hover and maintain stationary position (Lauder and Drucker, 2004). The pelvic fins were said to act as vertical “rudders” (Schmalhausen, 1916) and as bilge keels (Breder, 1926). However, Harris (1938) stated that the slight differences observed in swimming behaviour after pelvic fins amputation confirmed the small importances of the pelvic fins in swimming locomotion.

In real-life situation, forward movement and directional control of the fish were indeed inseparable. There are some overlapping of the functions performed by the fins, meaning that it was not necessary for one swimming movement to be governed strictly by only one fin. As a matter of fact, all the fins worked together closely to perform swimming locomotion. The knowledge on complicated propulsion and maneuvering could not be attained if the individual fin were to be studied in isolation. Evidences of such collaboration among fins could be found in some researches. Breder (1926) had demonstrated that median fins acted together in braking mechanism. The act was performed by curving of dorsal and anal fins to one side opposite to the caudal fin. Besides that, Lauder and Drucker (2004) had mentioned that median and paired fins must always work together to stabilize the body position during steady rectilinear swimming locomotion. They also stated that pectoral fins and dorsal fins acted collectively to maneuver the movement.

There are a number of researches investigating the fish fins; some of them focus on the locomotion, kinematics, mechanics and hydrodynamics aspects (Walker and Westneat, 2002; Wilga and Lauder, 1999; Nauen and Lauder, 2002; Gibb et al., 1994). Besides that, quite a number of papers can be found describing the functions of different fins (Standen and Lauder, 2005; Wilga and Lauder, 1999; Drucker and Jansen, 1996;

Lauder, 1989; Harris, 1938; Schmalhausen, 1916)). It is important to note that researches which relate nervous system to the fish, in specific spinal motor innervations, are similar to this study. The diverse local freshwater fishes available allowed this study to focus on selected representatives so that comparison can be done between the two groups of fish with reference to the dorsal fin.

Differences in the distribution of median fins enable the fish to be classified into two categories, which are fish with (i) long and continuous dorsal fin, and (ii) short and non- continuous dorsal fin.

### 1.2.2.1 FISH WITH LONG AND CONTINUOUS DORSAL FIN

Two fishes selected to represent the group of fish with long and continuous dorsal fin are *Channa micropeltes* (*toman*) and *Clarias* sp. (*keli*).

#### 1.2.2.1.1 *Channa micropeltes* (Valenciennes and Cuvier, 1840)



**Figure 1.5:** Lateral view of *Channa micropeltes* (juvenile)

Kingdom	: Animalia
Phylum	: Chordata
Subphylum	: Vertebrata
Class	: Actinopterygii
Order	: Channiformes
Suborder	: Channoidei
Family	: Channidae
Genus	: <i>Channa</i>
Species	: <i>micropeltes</i>
Common name	: <i>Toman/ Snakehead</i>

**Figure 1.6:** Taxonomy of *Channa micropeltes*

*Channa micropeltes* (Figure 1.5, Figure 1.6) has different local name in different places. For example, it is known as “ikan toman” in Malaysia, “pla chado” in Thailand, and “gabus tobang” in Kalimantan. On the other hand, the juveniles are commonly known as red or redline snakehead.

The fish had a snake-like head, which was depressed and rather pointed. It had an elongated body with its lower jaw protruding. The body and head were covered by scale. The most prominent feature of the fish is two longitudinal black stripes with a bright yellow intermediate area which extend behind the eyes until the end of the body.

In Asia, it is distributed in Mekong and Chao Phraya, Peninsular of Malaysia, and the islands of Sumatra and Borneo (Talwar and Jhingran, 1991). In Malaysia, they inhabit all parts of Peninsular Malaysia. Its habitat is usually in lakes, big rivers and streams (Mohsin and Ambak, 1990), as it prefers standing or slowly flowing water (Talwar and Jhingran, 1991).

All fishes in the order of Channiformes have scales on the head and body trunk. Besides, these fishes are able to breathe through suprabranchial organ which is an airbreathing organ. *Channa micropeltes* fulfills the criterias of fish classified under suborder channoidei where the pelvic fins are located at subabdomen area, and that the dorsal and anal fins are not spinous. Besides that, like any other fish from the family Channidae, *Channa micropeltes* has snake-like head and is carnivorous (Mohsin and Ambak, 1990). A significant feature of juvenile *Channa micropeltes* is the two black stripes on the body.

1.2.2.1.2 *Clarias* sp.



**Figure 1.7:** Lateral view of *Clarias* sp. (juvenile)

Kingdom	: Animalia
Phylum	: Chordata
Subphylum	: Vertebrata
Class	: Actinopterygii
Order	: Cypriniformes
Suborder	: Siluroidei
Family	: Clariidae
Genus	: <i>Clarias</i>
Species	: -
Common name	: <i>Keli/Catfish</i>

**Figure 1.8:** Taxonomy of *Clarias* sp.

*Clarias* sp. or catfish is more familiarly named as “ikan keli” in Malaysia (Figure 1.7, Figure 1.8).

The fish had a flattened head with an elongated body. It had small eyes and four pairs of barbels. No scale was found on the body but as was covered with a smooth scaleless skin. The dorsal and lateral parts of the body were grey in colour while the belly was whitish.

This fish is commonly found in Thailand, Vietnam, Indo-china and Philippines (Mohsin and Ambak, 1990). Their habitat ranged from open and shallow water to medium and large-sized rivers. They are also found in muddy area where they can bury themselves for a lengthy period. In Malaysia, their favourite spots are rice fields and swampy ditches in Perlis, Kedah and Perak (Mohsin and Ambak, 1990).

In the order of Cypriniformes, pectoral fins of the fishes are located at the abdomen and they are not spinous. However, first dorsal fin or pectoral fin might be spinous. Fishes from this order that do not have scales are classified as suborder Siluroidei. Fishes from suborder Siluroidei is characterized by small eyes and whisker-like barbels which give them the name “catfish”. Normally, there is one hard spine on each of the pectoral fins which allow them to attack other animals. Fishes classified in family Clariidae have long dorsal fin, four pairs of barbels, and an airbreathing organ.

*Clarias* sp. are carnivorous and feed on small bottom dwelling animals, aquatic insects, young shrimps and small fishes (Mohsin and Ambak, 1990). Besides that, when kept as livestock, they can also feed on zooplankton, rice bran, kitchen refuse, fish meal and formulated feeds.

### 1.2.2.2 FISH WITH SHORT AND NON-CONTINUOUS DORSAL FIN

Two fishes selected to represent the group of fish with short and non-continuous dorsal fin include *Mystus nemurus* (baung) and *Pangasius* sp. (*patin*).

#### 1.2.2.2.1 *Mystus nemurus* (Valenciennes and Cuvier, 1840)



**Figure 1.9:** Lateral view of *Mystus nemurus* (juvenile)

Kingdom	: Animalia
Phylum	: Chordata
Subphylum	: Vertebrata
Class	: Actinopterygii
Order	: Cypriniformes
Suborder	: Siluroidei
Family	: Bagridae
Genus	: <i>Mystus</i>
Species	: <i>nemurus</i>
Common name	: <i>Baung/Catfish</i>

**Figure 1.10:** Taxonomy of *Mystus nemurus*

*Bagrus nemurus* and *Macrones nemurus* are the synonyms for *Mystus nemurus* (Figure 1.9, Figure 1.10). From the Greek, “nema” means thread while “oura” gives the meaning tail, which refers to the filamentous extensions on the dorsalmost principal caudal rays.

This fish had a pointed head and a humped back. The mouth was big and located at low position, with the upper jaw slightly longer. It had 4 pairs of barbels all together, the distinctive one being the longest pair of maxillary barbels extending to the anal fin. No scale was found on the body and it was covered with mucous. It had a very nice-looking shiny skin distributed throughout the body, with darker colour at the head part, and light greyish yellow at the body. Ventral side of the body was milky white in colour.

They are widely distributed throughout the Southeast Asia, especially in Mekong, Thailand Waters, Jawa Sumatera, Singapore and Borneo. In Malaysia, they are found in certain areas such as Sungai Klang and Tanjung Karang in Selangor, Sungai Terengganu and Sungai Nerus in Terengganu, Sungai Keratong in Pahang, as well as Bukit Merah in Perak (Mohsin and Ambak, 1990). They do not like habitat which is too clean or too muddy. Thus, the favourite spot for them are tidal area.

*Mystus nemurus* and *Channa* sp. are from the same suborder. *Mystus nemurus* was distinguished into family Bagridae by the features of the nostrils where they are separated apart and there is a pair of barbells located at the posterior nostrils.

*Mystus nemurus* has dorsal and pectoral fins serrated caudally. Its adipose fin is shorter than anal fin. Besides that, the upper jaw is slightly longer as compared to the lower jaw.

This fish is carnivorous as they feed only on prawns, insects, crabs and small fishes in natural habitat (Mohsin and Ambak, 1990). They are nocturnal, that is, they are active during night time (Bond, 1979).

1.2.2.2.2 *Pangasius* sp.



**Figure 1.11:** Lateral view of *Pangasius* sp. (juvenile)

Kingdom	: Animalia
Phylum	: Chordata
Subphylum	: Vertebrata
Class	: Actinopterygii
Order	: Cypriniformes
Suborder	: Siluroidei
Family	: Schilbeidae (Pangasiidae)
Genus	: <i>Pangasius</i>
Species	: -
Common name	: <i>Patin</i> /catfish

**Figure 1.12:** Taxonomy of *Pangasius* sp.

*Pangasius* sp. had a flat and broad head. Its mouth was wide and located at a position which was quite low. The body was shark-liked and characterized with deep curved belly. The body was scaleless and covered with mucous. It was shiny black at the dorsal portion of the fish and light gray at the lateral side while the belly was whitish.

Worldwide, *Pangasius* sp. (Figure 1.11, Figure 1.12) can be found in India, Bangladesh, Burma, Jawa and Thailand (Wong, 2005). In Malaysia, this fish is usually found in Perak and Pahang (Mohsin and Ambak, 1990). They live in high estuary (freshwater tidal zone) as juveniles, moving to brackish water as sub-adults, and finally as adults to river mouths and inshore areas.

Fishes in the family of Schilbeidae (or more commonly known as Pangasidae) have two to three pairs of barbells. They have scaleless and elongated body. Adipose fin is located opposite to the anal fin. A vital characteristic of fishes under this family is that one sharp spine could be observed at pectoral and dorsal fins.

The fish are omnivorous as they feed on snails, other mollusca and also plants.

## **1.3 REVIEW OF TECHNIQUES**

### **1.3.1 HISTOLOGY**

The word histology is derived from the Greek. “Histo” means “tissue” while “logos” mean “study or science of” (Ham, 1965). More precisely, histology is the study of composition and structure of plant and animal tissues in relation to their specialized function. It is accomplished by producing extremely thin slices of tissue, using dye to colour the cellular components, and then examining it under the microscope.

In this study, histological techniques employed on spinal cord enable the accessibility to the information on how spinal tissue is organized at all structural levels, its functional unit, and also the cell types. By employing different staining techniques, different information could be retrieved from the histological slides.

Immunohistochemistry is another popular technique practiced in neurobiology to detect specific neurotransmitters or substances. Such information will contribute to understanding of spinal cord organisation at cellular level (Adli et al., 1988).

In order to study certain type of neurons like motor neurons, dyes can be injected into muscle tissues to be transported to the targeted neuron. This method would be exploiting the retrograde axoplasmic transport. When processed histochemically, the injected dyes could be viewed in the tissue. With the help of this technique, we can thus precisely identify and investigate certain neurons.

## **1.4 RESEARCH PROBLEM**

Fish fins are appendages which are extensions attached to the body just as the limbs are in higher vertebrates. To date, most of the studies looked at the innervations of limbs especially in mammals, such as rats, cats and monkeys. On the contrary, understanding on the innervations of the fish fin is still at its infancy as studies on the relevant field are relatively scarce. Hence, this study aimed to fill the knowledge gap, i.e. to investigate the organisation of spinal motor neuron in relation to the distribution of the median fins.

In addition, data obtained from this study would contribute to the fish database which is an ongoing project in the research group.

## **1.5 RESEARCH OBJECTIVES**

To effectively achieve all the objectives as listed before, this study is divided into a few main experiments. Each experiment was carefully planned and designed to accomplish one or more objectives. Some of the experiments may contain some subtopics. This study first focused on morphology of fish and functions of the fins. Then, the correlation between motor neuronal organisation and the distribution of fins will be investigated. Finally, this study zoomed into the spinal motor innervations of the caudal fin. The specific objectives of the each experiment are as shown below.

### **Experiment 1 (Chapter 2): Fish external morphology**

- To study the external morphology of selected fishes involving differently distributed fins.

### **Experiment 2 (Chapter 3): Organisation of motor neurons innervating fish body trunk**

- To study the gross morphology and general histology of spinal cord of fishes with differently distributed fins.

### **Experiment 3 (Chapter 4): Protocol establishment on spinal motor innervations of caudal fin muscles**

- To determine appropriate protocols for study on spinal motor innervations of caudal fin muscles

### **Experiment 4 (Chapter 5): Spinal motor innervations of caudal fin muscles**

- To identify the motor neurons innervating caudal fin muscles and to characterize the organisation of these motor neurons.

## **CHAPTER 2: FISH EXTERNAL MORPHOLOGY**

### **2.1 INTRODUCTION**

Human observers are always astonished by the fish movements, which succeeded over the evolution and resulted in a rich diversity. Fish differ from other animal species by being able to live successfully in the water medium. The absence of limbs does not hinder their locomotion as their movement is made perfect by the fins. Fishes perform different mode of swimming, which depends largely on the body shape and fins design that suit their living habitat.

Fish moves forward in the water by means of two primary methods, which are (1) body movement caused by alternate expansion and contraction of myomeres, and (2) movement of the fins. The third method is by exhalant jets of water from the gill-opening during respiration process. Each of these methods can be employed differently but most of the time they are inter-related (Greenwood, 1931).

Although there are quite a number of existing publications on fish locomotion (Nauen and Lauder, 2002; Drucker and Jensen, 1996; Gibb et al., 1994; Lauder 1989), the works that delve deep into the role and function on each fin system are still ongoing as it is a vast field. To date, the fin function is frequently investigated from two distinct scientific angles. Understanding the kinematics and hydrodynamics of the fins by mathematics and calculations may elucidate how the fins function. Amputation of fins is another popular way that helps researches to figure out the functions on fins in assisting locomotion.

### **2.2 OBJECTIVE**

- To study the external morphology of selected fishes involving differently distributed fins.

## **2.3 METHODOLOGY**

### **2.3.1 OBSERVATION ON FISH EXTERNAL MORPHOLOGY**

Fishes used were solely obtained from two established aquariums:

(a) Kok Aquarium

(20, Jln 2/21, Seksyen 2, 46000 Petaling Jaya, Selangor)

(b) Irene Aquarium

(8-10, Jalan 21/22, Sea Park, 26300 Petaling Jaya, Selangor)

The fish was immersed in anesthetic agent, i.e. tricaine methanesulfonate (MS222) until it was deeply anaesthetized. The fish external morphology was carefully examined with emphasis on the fish fins. The shape of the fins and their distribution were observed.

#### **2.3.1.1 ANESTHETIZATION**

MS222, or tricaine methanesulfonate (which is sometimes known as Tricaine-S) is a popular anesthetic agent used on cold-blooded animals. The anesthetics can produce several stages of anesthesia, depending on dosage used and time of exposure. For observation of fish external morphology, the fish reached the state of surgical anesthesia, which is total loss of equilibrium and no reaction to touch stimuli.

0.17% MS222 solution or a concentration of 170mg/L was sufficient to anesthetize the fish to the desirable state. Usually 3L of MS222 solution was prepared, by adding 510mg of MS222 in powder form into the distilled water. The MS222 solution used was freshly prepared every time before use. The fish was then brought to immersion in the MS222 solution. About 15 minutes of exposure time was needed for the specimen to reach the desired state of anesthesia.

## **2.4 RESEARCH FINDINGS**

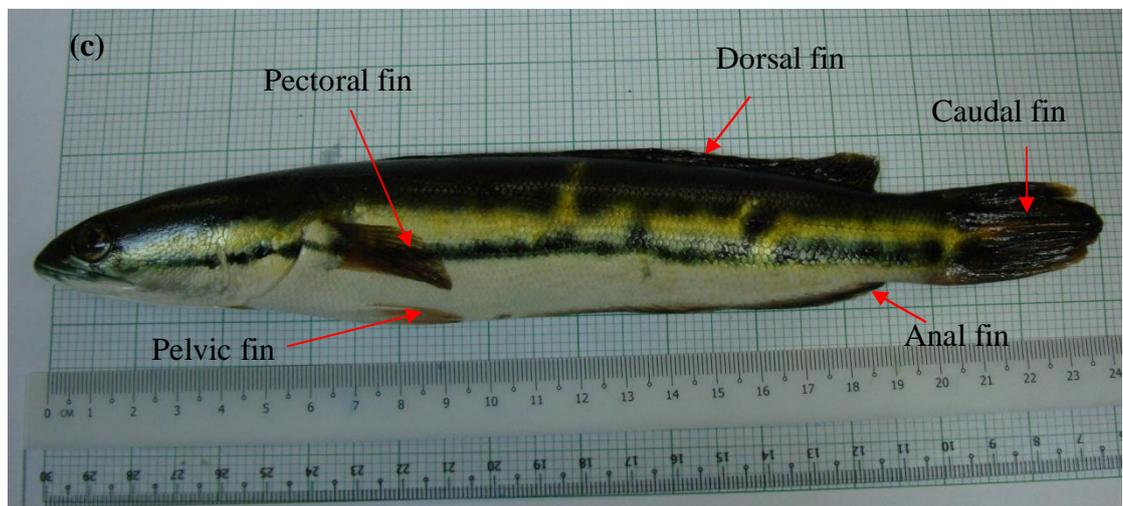
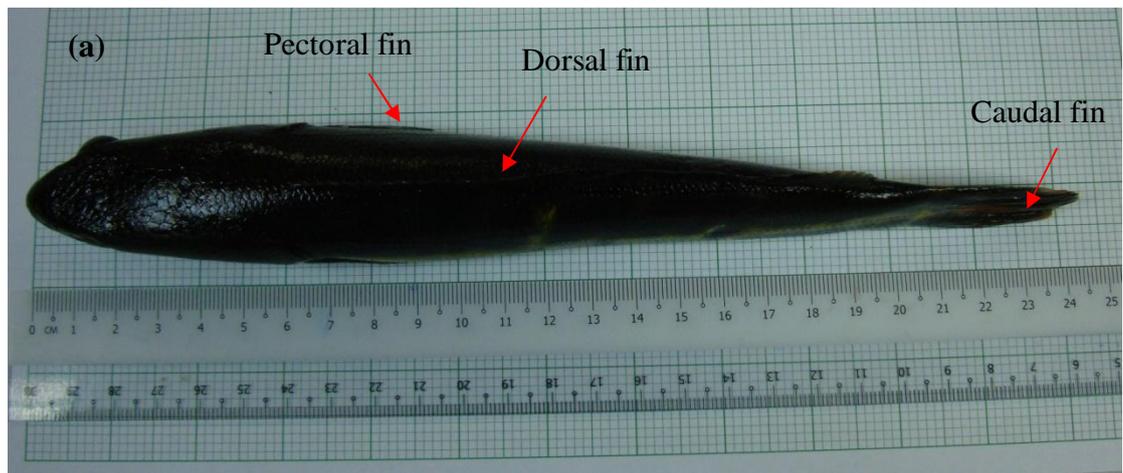
### **2.4.1 OBSERVATION ON FISH EXTERNAL MORPHOLOGY**

The description is on the overall shape and the fins of each type of fish.

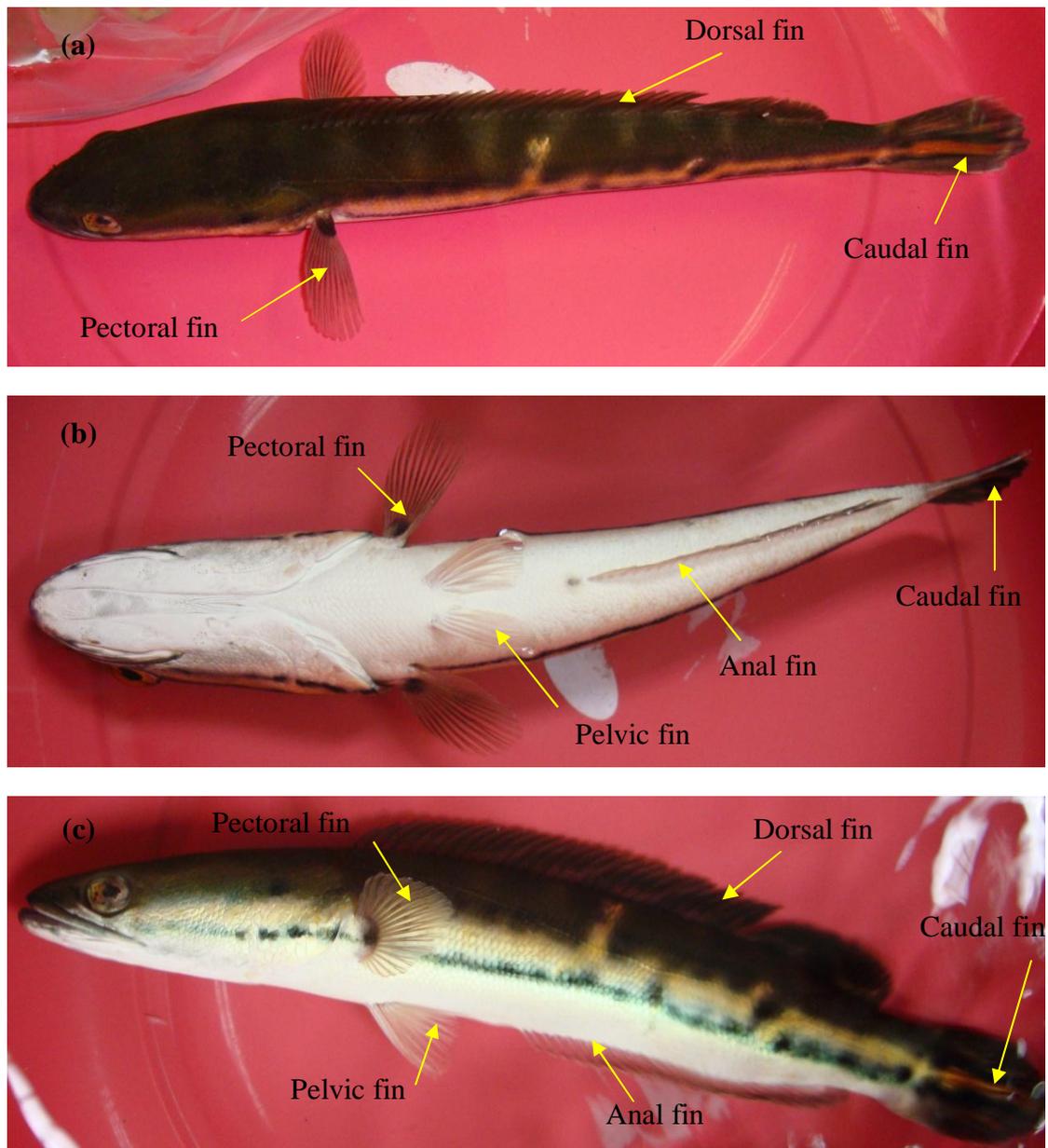
#### **2.4.1.1 FISH WITH LONG AND CONTINUOUS DORSAL FIN**

##### **2.4.1.1.1 *Channa micropeltes***

The dorsal and anal fins which composed of soft fin rays were long and continuous (Figure 2.1, Figure 2.2). The dorsal fin started right above the pectoral fin while anal fin started behind the anus. Both dorsal and anal fin ended before the caudal fin. The caudal fin was rounded. A pair of transparent rounded pectoral fins located high up at the lateral side of the body behind the gill cover. Pelvic fins, which had slightly smaller size compared to the pectoral fins, were situated at the fish belly before the anal fin.



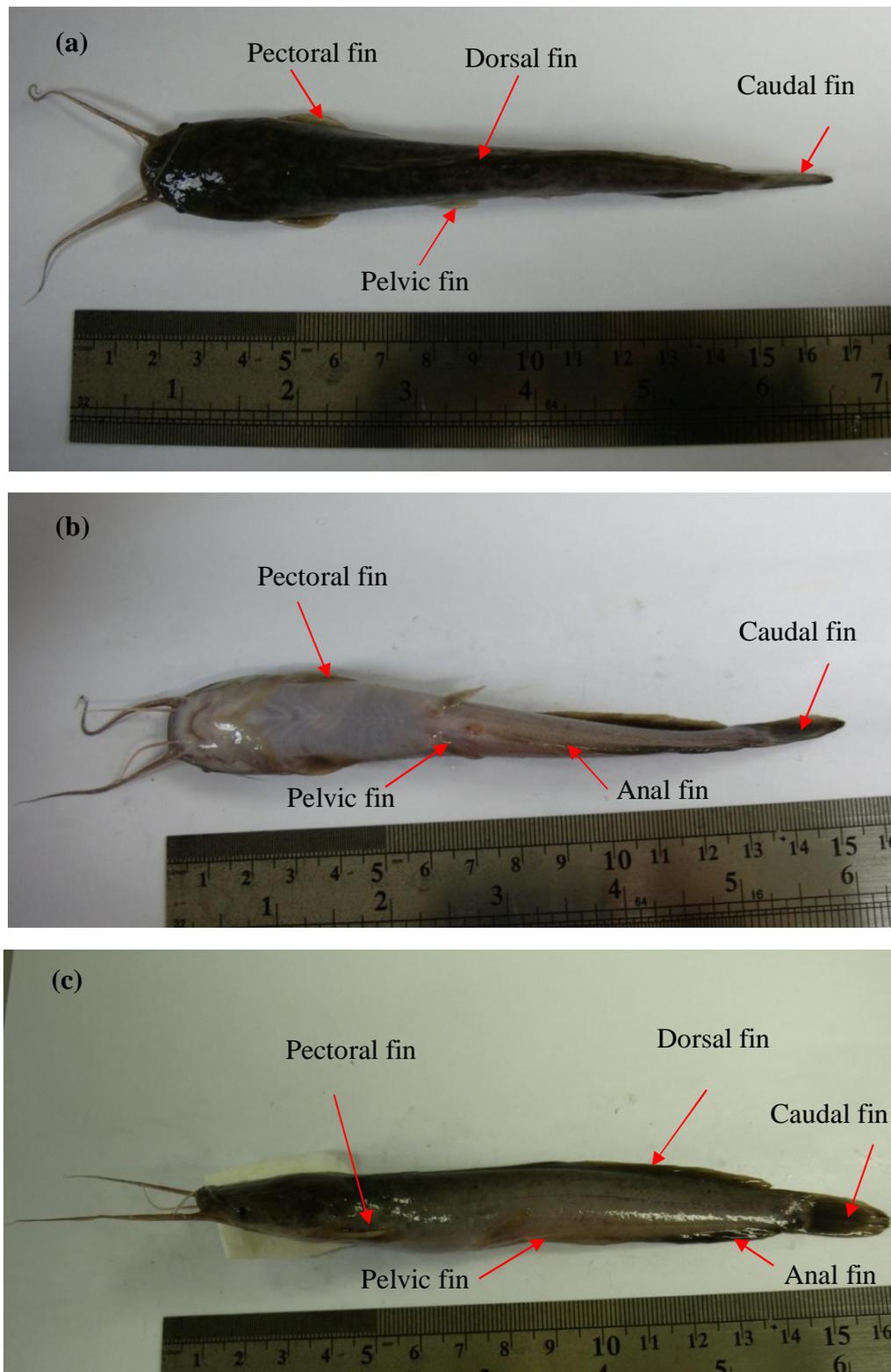
**Figure 2.1:** External morphology of *Channa micropeltes* outside water: (a) Dorsal view, (b) Ventral view and (c) Lateral view. Long and continuous dorsal fin was observed.



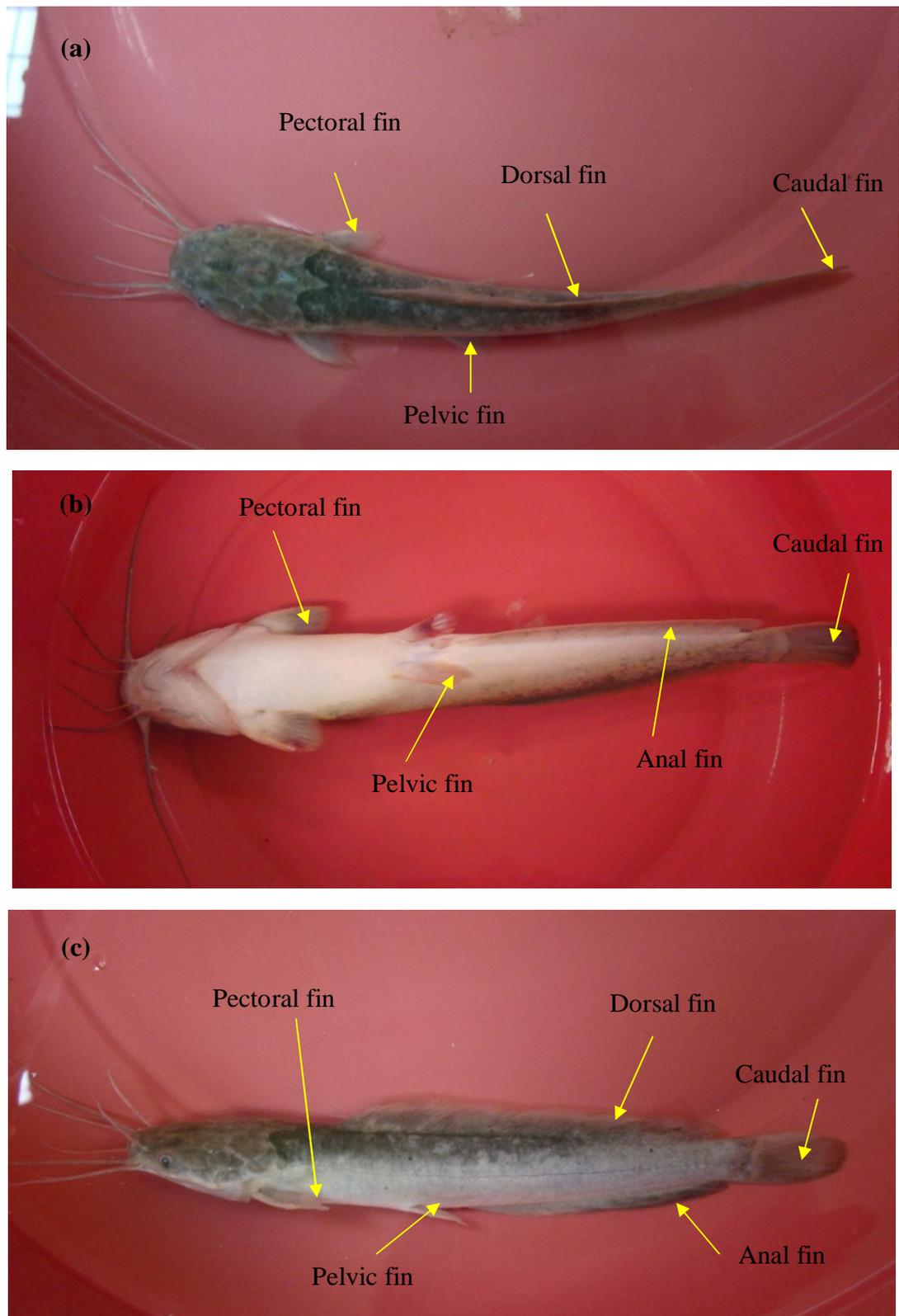
**Figure 2.2:** The views of *Channa micropeltes* in water: (a) Dorsal view, (b) Ventral view and (c) Lateral view. Long and continuous dorsal fin was more clearly observed in water.

#### **2.4.1.1.2**      *Clarias* sp.

It had long and continuous dorsal and anal fins (Figure 2.3, Figure 2.4). Dorsal fin extended after the head along the body length. It was important to note that they were discontinuous with the rounded caudal fin. Anal fin, on the other hand, started right after the anus and stopped before the caudal fin. Both dorsal and anal fins composed of only soft fin rays. A pair of pectoral fins located at the lateral side of the body near the belly. It consisted of one hard fin rays and a few soft fin rays. Pelvic fins had similar shapes and were slightly smaller than pectoral fins. However, it consisted of only soft fin rays. All its fins were blackish except the pelvic fin which was white in colour.



**Figure 2.3:** External morphology of *Clarias* sp. outside water: (a) Dorsal view, (b) Ventral view and (c) Lateral view. Long and continuous dorsal fin extended along the body length.

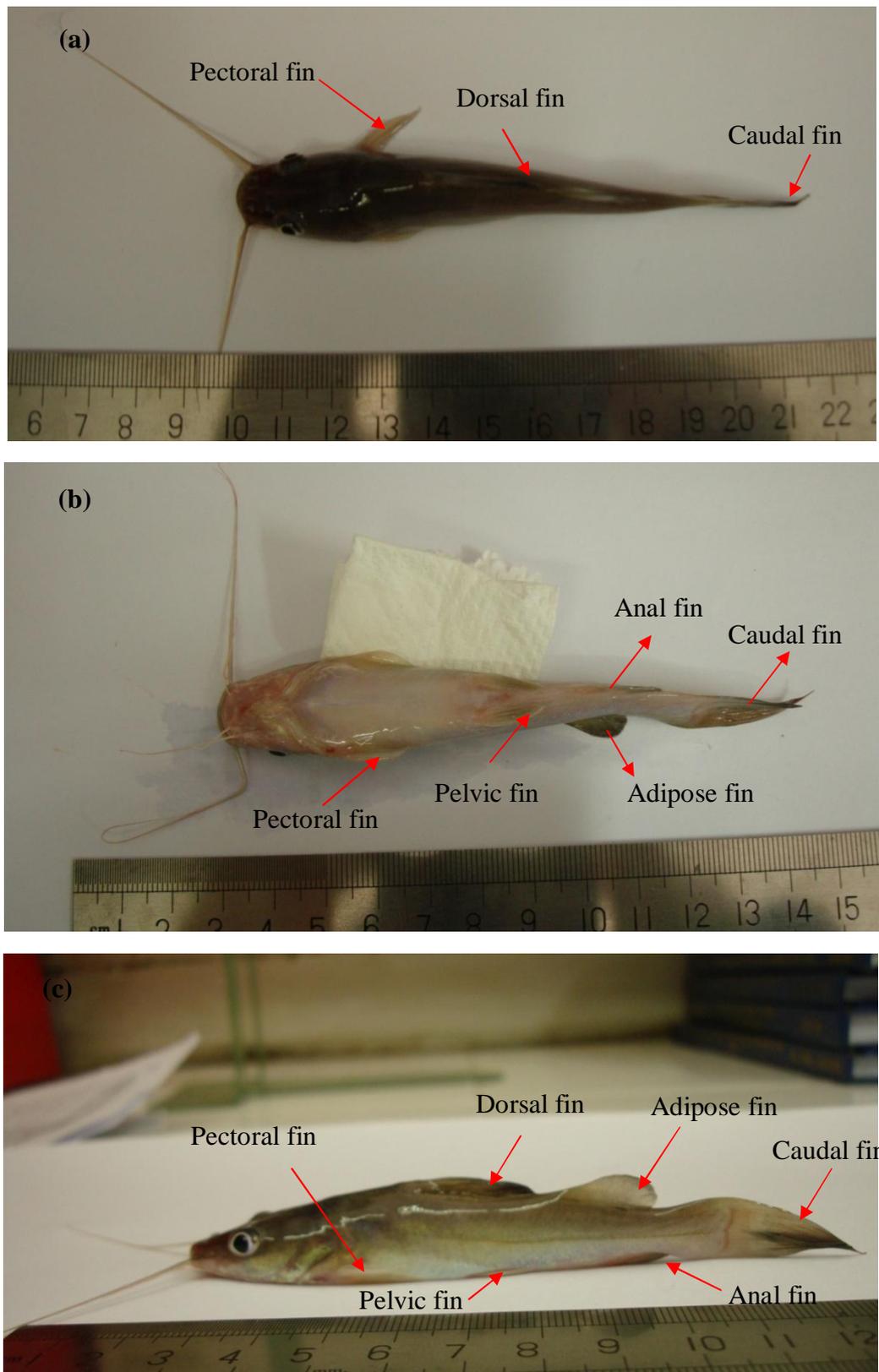


**Figure 2.4:** The views of *Clarias* sp. in water: (a) Dorsal view, (b) Ventral view and (c) Lateral view. Long and continuous dorsal fin was clearly observed in water.

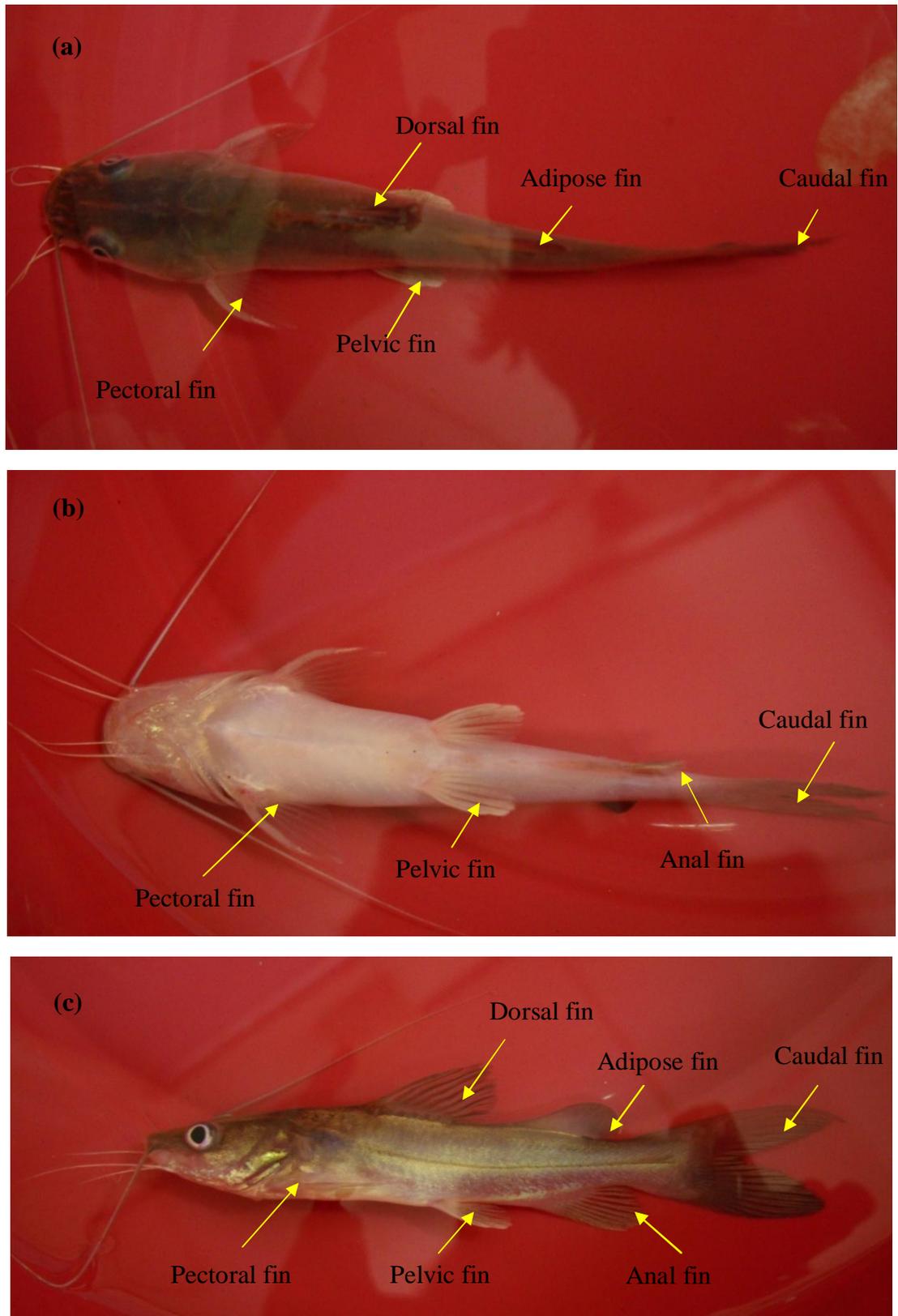
## **2.4.2.1 FISH WITH SHORT AND NON-CONTINUOUS DORSAL FIN**

### **2.4.2.1.1 *Mystus nemurus***

Short and non-continuous fins distributed quite evenly throughout the fish body (Figure 2.5, Figure 2.6). Dorsal fin which was located on the hump consisted of one spine and a few soft fin rays. Adipose fin was present and its size was quite big. A big and forked caudal fin was located at the end of the body. Anal fin was directly below the adipose fin, and both having similar size and length. However, anal fin consisted of a few soft fin rays while adipose fin had no fin ray at all. Pectoral fins were located low right behind the gills. Each pectoral fin had one spine and a few soft fin rays. A pair of pelvic fins could be observed on the abdomen of the fish. All the fins were yellowish grey and projected towards the caudal fin.



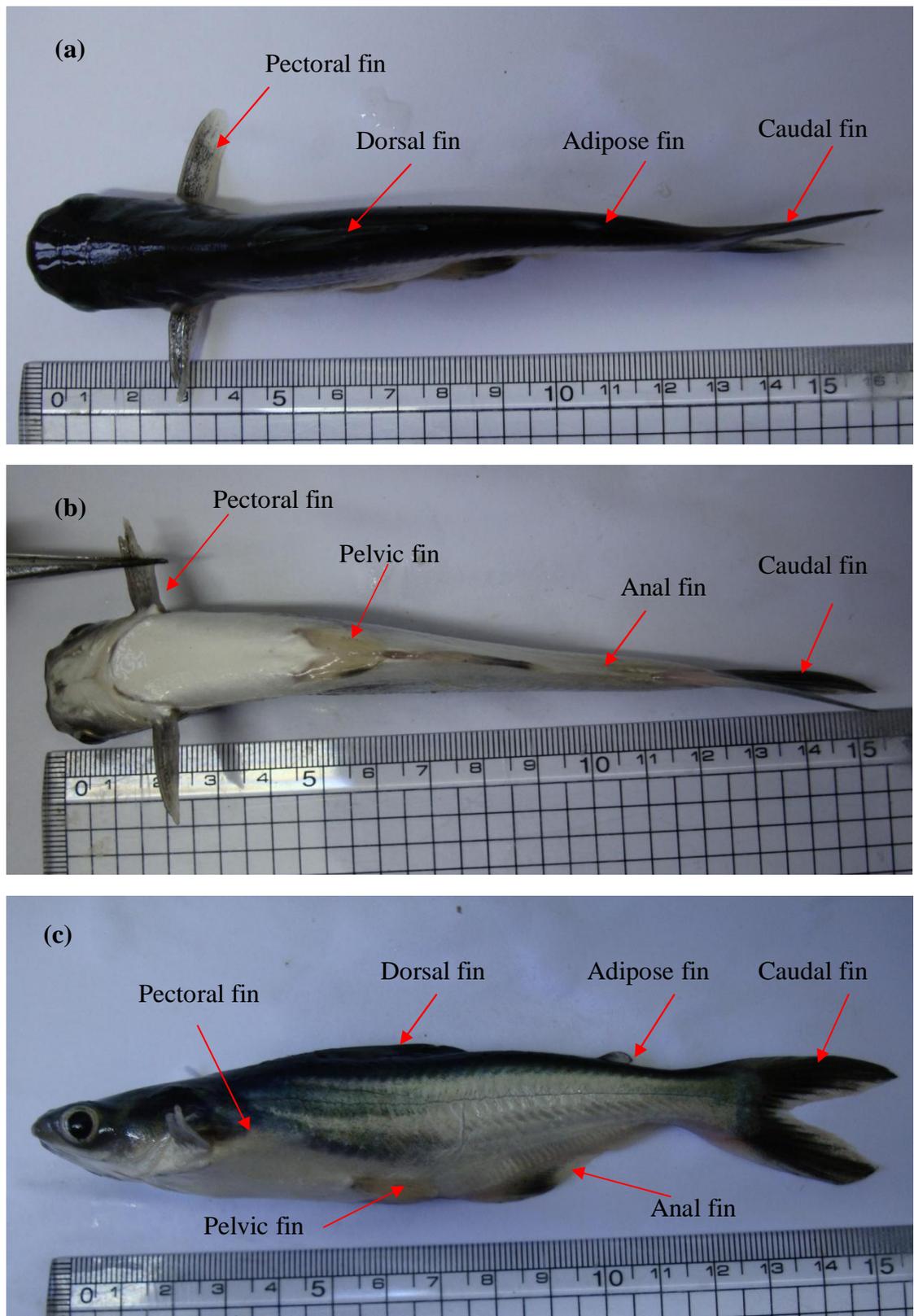
**Figure 2.5:** External morphology of *Mystus nemurus* outside water: (a) Dorsal view, (b) Ventral view, and (c) Lateral view. It has short and non-continuous median fins made up of dorsal fin and adipose fin.



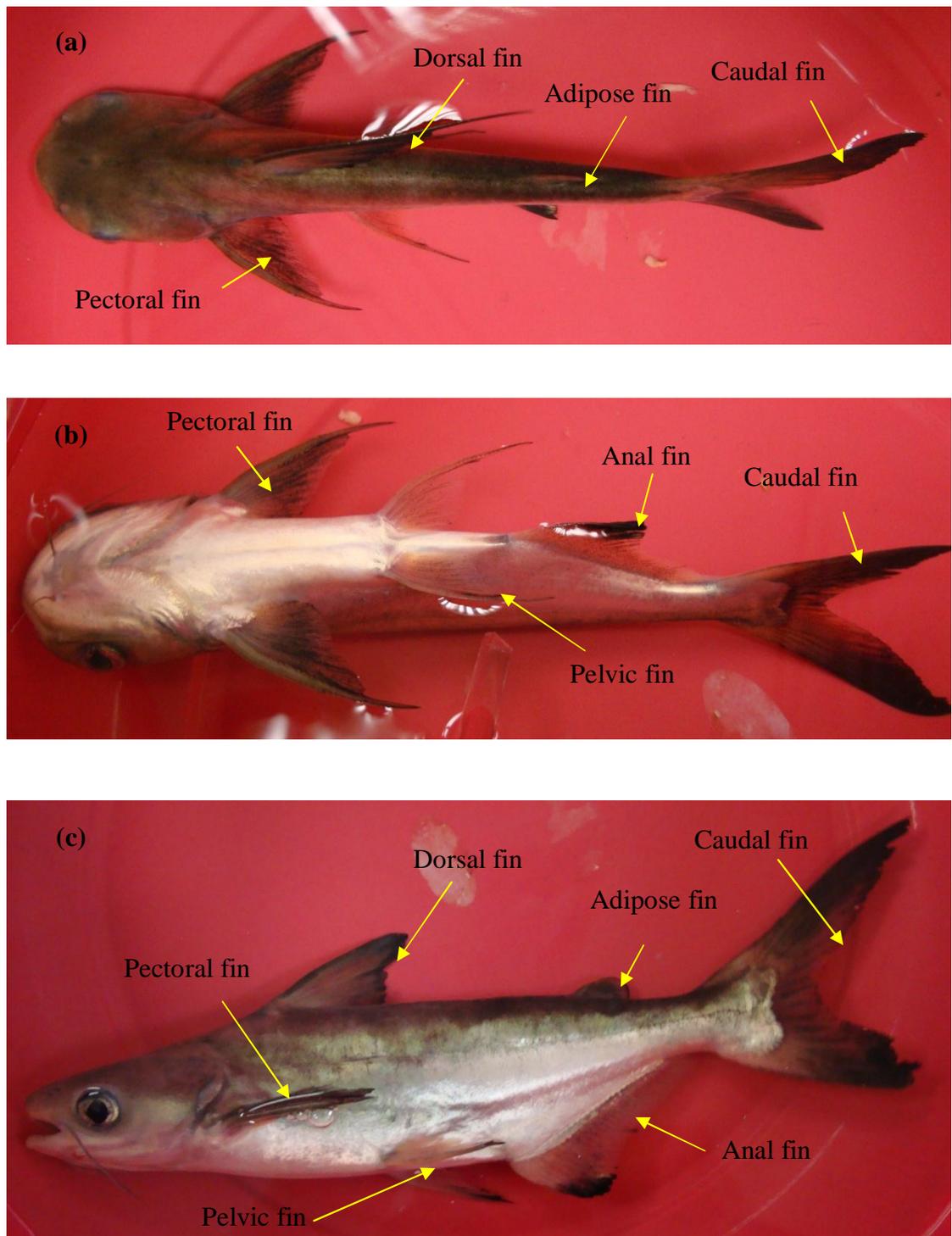
**Figure 2.6:** The views of *Mystus nemurus* in water: (a) Dorsal view, (b) Ventral view and (c) Lateral view. Short and non-continuous median fins made up of dorsal fin and adipose fin were clearly observed in water.

#### **2.4.2.1.2**      *Pangasius sp.*

The fins were short and non continuous (Figure 2.7, Figure 2.8). The dorsal fin, which was near to the head region, composed of one hard fin ray and a few soft fin rays. Also on the dorsal side was adipose fin which was small and has no fin ray. Dorsal, pectoral and pelvic fins were prolonged. The anal fin, on the other hand, had hard and soft rays while the former was longer than the latter. The caudal fin was isocoercal. A pair of transparent and rounded pectoral fins was located behind the gill cover. The colour of all the fins was dark except for the pelvic fins which were translucent.



**Figure 2.7:** External morphology of *Pangasius* sp. outside water: (a) Dorsal view, (b) Ventral view and (c) Lateral view. The median fins made up of dorsal fin and adipose fin were short and non-continuous.



**Figure 2.8:** The views of *Pangasius* sp. in water: (a) Dorsal view, (b) Ventral view and (c) Lateral view. It had short and non-continuous median fin. The dorsal fin composed of one hard fin ray and a few soft fin rays while adipose fin was located between dorsal fin and caudal fin.

## 2.5 DISCUSSIONS

### 2.5.1 OBSERVATION ON FISH EXTERNAL MORPHOLOGY

The body shape for all four fishes were “torpedo-shaped”. It was also known as fusiform body shape, which the body was compressed at the sides and tapered more at the tail than at the head. This body shape actually allowed the fish to glide more easily through the water.

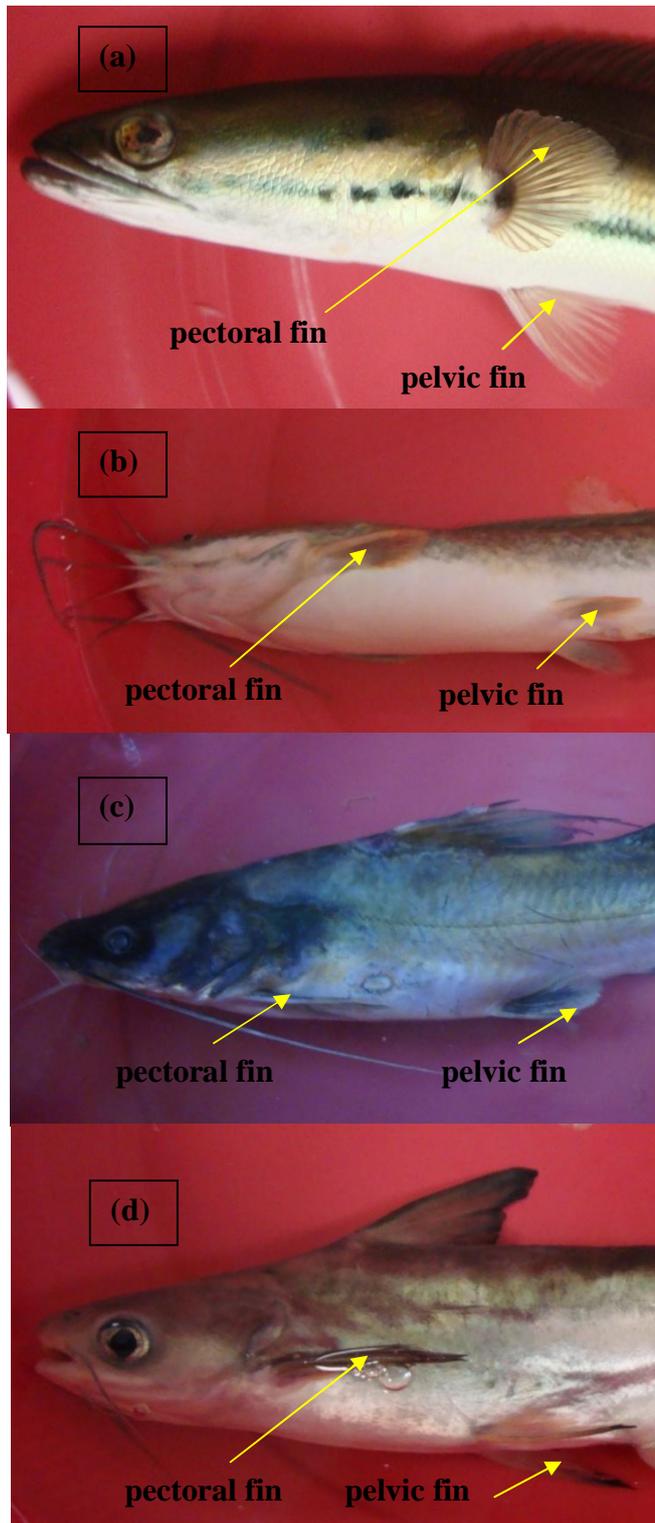
The major difference that split the four fishes above into two groups was the distribution of median fins. Both *Channa micropeltes* and *Clarias* sp. had median fins including dorsal and anal fins extending along the body length while *Mystus nemurus* and *Pangasius* sp. had short and non-continuous median fins. The distribution of dorsal fin which displayed significant difference in the fishes was given emphasis in this study.

In fish with long and continuous median fins, the dorsal and anal fins were composed of soft and transparent fin rays. The fins were long and extended along the medial plane of fish body and stopped right before the caudal fin. Besides the dorsal fin, it must also be mentioned that both the two fishes had rounded caudal fin.

However, there were some differences between the two fishes. The pectoral fins of *Channa micropeltes* located higher up at the body behind the gills while *Clarias* sp. had the pectoral fins located near the belly. Harris (1938) suggested that if the pectoral fins were located higher up the body towards the dorsal surface, the pelvic fins tend to move forward. This statement was true when being applied to the fishes in this study. *Channa micropeltes* had its pelvic fins located more rostrally located compared to all three other fishes (Figure 2.9). Such fin design was to avoid the rising of the head when the pectoral fins act to stop forward movement (Harris, 1938).

On the other hand, representatives of the group of fish with short and non-continuous median fins had relatively short dorsal and anal fins compared to the other group of fish. In both fishes, there was one spine supporting the dorsal and pectoral fins. Besides that, both fishes had a forked caudal fin.

One significant difference between *Mystus nemurus* and *Pangasius* sp. was that the former had a bigger adipose fin which was almost similar to the length of its anal fin.



**Figure 2.9:** Location of pelvic fins in relation to the pectoral fin. (a) *Channa micropeltes*, with its pelvic fins located higher up the body than (b) *Clarias* sp. (c) *Mystus nemurus*, and (d) *Pangasius* sp., had a pair of more rostrally located pectoral fins

## 2.5.2 TECHNIQUES AND PROTOCOLS

It was very vital to note that observation on fish external morphology must be carried out when the fish was in a relaxed state. Fish easily experienced stress by handling and transport, physical injury or even death. Under this condition, there might be a change in the fish coloration. It was observed that during the fish amputation process where the fish became lighter in colour. Chromatophores were the cells in charge of the pigments. When the fish was frightened or under stress, chromatophores aggregated under the influence of adrenaline and consequently caused rapid colour change in fish (Moyle and Cech, 1988).

## 2.5.3 GENERAL DISCUSSIONS

Varieties of fin design could be observed in the world of fish. However, the fin design of each fish was unique. Be it the shape or the location of the fin, they were well suited to the habitat and living behaviour of the fish. Hence, body shape and morphology of the fish could provide information such as its way of living.

The group of fish with long and continuous fish fins, which were *Channa micropeltes* and *Clarias* sp., was characterized by elongated bodies, blunt-shaped head and rounded tails. The dorsal and anal fins were long while paired fins that present were small. They thus fulfilled the characteristics of eel-like fish. Eel-like fish were not capable of performing high-speed swimming locomotion. They were in fact adapted for entering small crevices, for making way through beds of aquatic plants and for burrowing into soft bottoms and mud (Moyle and Cech, 1988). The living behaviour was compatible with their habitats, which were lakes, streams and paddy fields.

For eel-like fish, dorsal and anal fins acted together as propulsor by sending waves of movement down the fish. These fishes were equipped with rounded caudal fin as they

do not need rapid movement in moderately swift water. The reduced pelvic fins found on the fish allow them to squeeze through tight places.

*M. nemurus* and *Pangasius* sp. have flattened head, humped back and enlarged pectoral fins. They could thus be classified as bottom rover, which was a combination of rover predator and bottom fish. The fins design made them good swimmers. Thus, they were bottom dwellers which practice food hunting by capture through pursuit. To accomplish this, they were equipped with well-developed barbells while fins were evenly distributed on the body for better stability and maneuverability (Moyle and Cech, 1988).

For rover predator, dorsal and anal fins were elongated so that they could provide stability to the fish while they were swimming. Since the fish hunted for prey, forked caudal fin was very important in providing the fish swimming locomotion with speed. The pelvic fins, on the other hand, allowed the fish to steer and halt.

## **2.2 CONCLUSIONS**

Based on the distribution of the fins, the fish used in this study were generally divided into two groups, i.e. (a) fish with long and continuous dorsal fin and (b) fish with short and non-continuous dorsal fin.

In conclusion, fish body morphology had an effect on the swimming behaviour which was under great influence of the habitat. Observation on the body morphology of fishes in this study was compatible with the swimming behaviour and their living habitat.

## **CHAPTER 3: ORGANISATION OF MOTOR NEURONS**

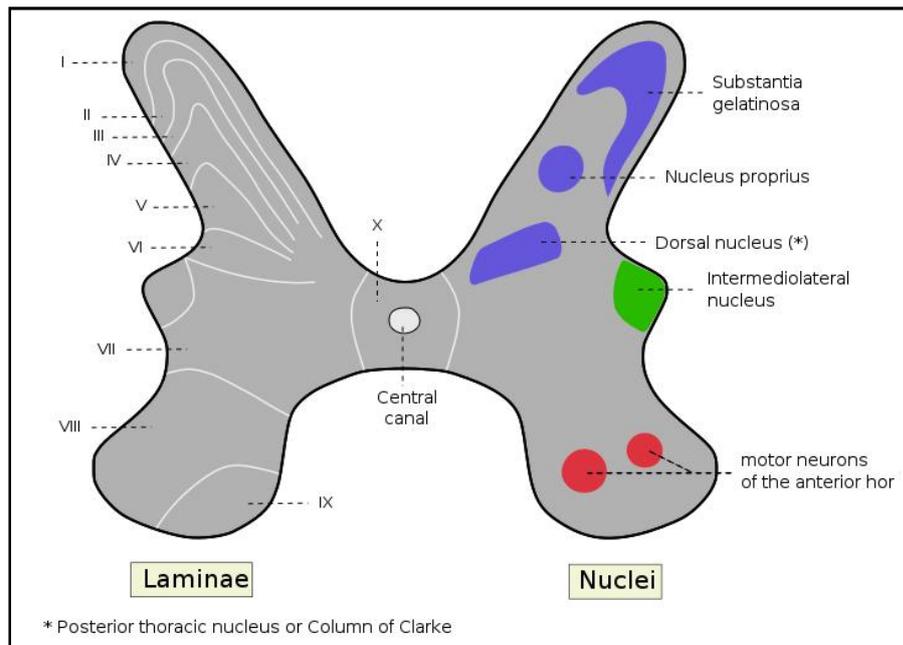
### **INNERVATING FISH BODY TRUNK**

#### **3.1 INTRODUCTION**

Spinal cord acts as a link that bridges efferent information transmission between the brain and body, innervating effector organs with regards to the internal and external environments (Witherspoon, 1984). This function is the results of its neuronal activities supported by the neuroglial cells. Electrical impulses travel through the ascending and descending fiber tracts, converging on spinal motor neurons, the “final common pathway”, which innervate effector organs according to impulses from the central sources.

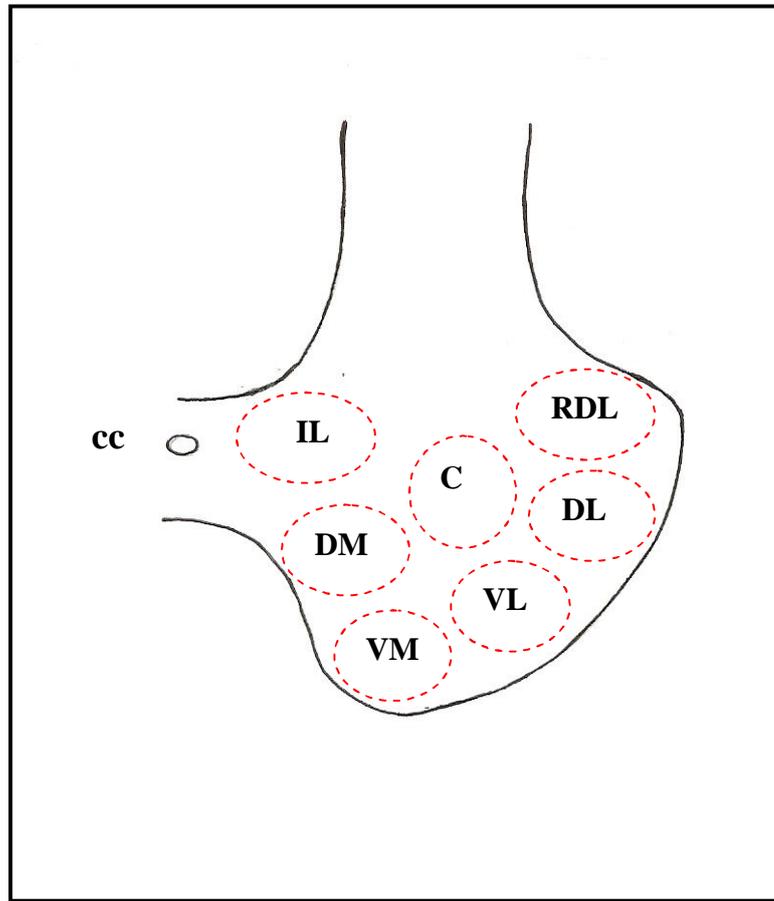
Anatomical organisation of the spinal cord is best presented by a cross section of histological slide. The spinal cord is basically made up of grey matter core surrounded by white matter with a tiny central canal located at the centre. Grey matter can be further divided into dorsal and ventral horns. Motor neurons housed in the ventral horns of the spinal grey matter are commonly described using nomenclatures Rexed lamina or cell columns. These nomenclatures are ultimately important for scientists to outline the organisation the motor neurons.

The Rexed laminae (Figure 3.1) were identified in the early 1950s by Bror Rexed to label portions of the spinal cord, since the distribution of cells and fibers within the grey matter of the spinal cord exhibits a pattern of lamination. Rexed laminae comprise a system of ten layers of grey matter (I-X) (FitzGerald, 1985). The cellular pattern of each lamina is composed of various sizes or shapes of neurons (FitzGerald, 1985).



**Figure 3.1:** Rexed laminae (adopted from <http://nba.uth.tmc.edu>, 2007)

Apart from that, the ventral horn can be classified into cell columns (Figure 3.2). The ventral horn is first divided into three main regions, which are medial, central, and lateral. The area nearer to the central canal is considered medial and the one being furthest away is regarded as lateral, while the area between them is central. The whole ventral horn can be divided into seven cell columns, namely intermediolateral, dorsomedial, ventromedial, central, ventrolateral, dorsolateral and retrodorsolateral (FitzGerald, 1985). The cell columns mentioned above are in an order where intermediolateral (IL) cell column is the one most medially located while those that follow are located progressively lateral.



**Figure 3.2:** Distribution of cell columns on the ventral horn

(CC: Central canal; IL: Intermediolateral; DM: Dorsomedial; VM: Ventromedial; C: Central; VL: Ventrolateral; DL: Dorsolateral; RDL: Retrodorsolateral)

Extensive studies on the fish spinal cord are presented in quite a number of papers with focus on different stages of fish, ranging from embryo to adults (Yoshida et al., 1999; Nishikawa et al., 1991; Myers, 1985; Coghill, 1913). Nieuwenhuys's review on fish spinal cord provides detailed information of past findings (Nieuwenhuys et al., 1998). Besides looking at cytoarchitectonic features of the grey matter, scientists had also researching on the ascending and descending pathways. After decoding the general morphology of the spinal cord, scientists then zoomed in to carry out investigation on specific cells and neurons. Most of these studies utilize histological techniques and immunohistochemistry procedures.

The specimens used in this study were group of fish with long and continuous dorsal fins and group of fish with short and non continuous median fins. This study aims to reveal the correlation between motor neuronal organisation and the fins distribution.

### **3.2 RESEARCH OBJECTIVE**

- To study the gross morphology and general histology of spinal cord of fishes with differently distributed fins.

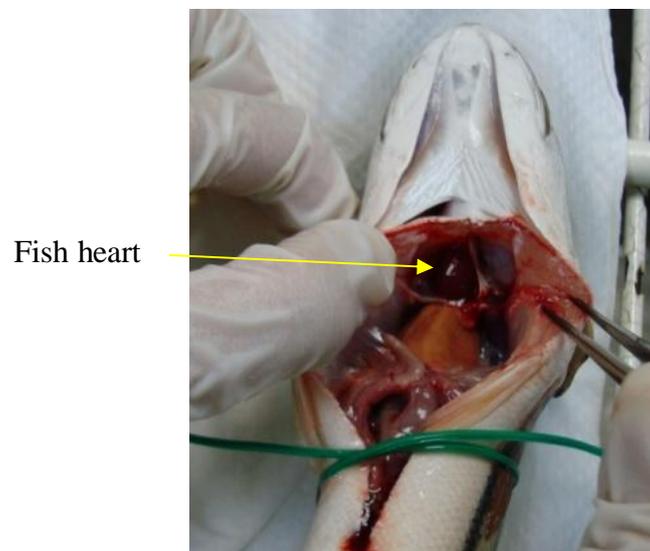
### 3.3 METHODOLOGY

#### 3.3.1 INTRACARDIAC PERFUSION

The fish was anaesthetized using MS222. Intracardiac perfusion was carried out under deep sedation (please refer to 2.2.1.1 for detailed protocol of anaesthetization).

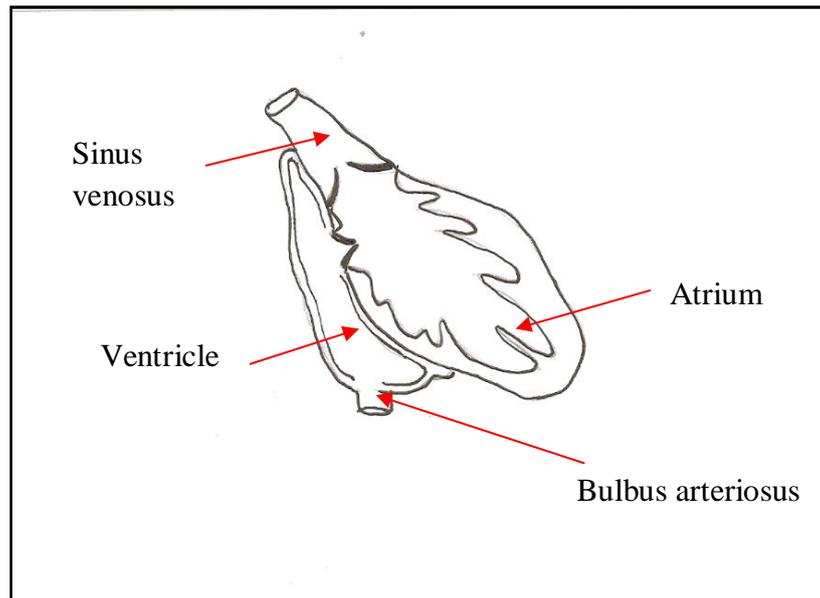
The perfusion pump was set up readily before use. All solutions needed including 0.6% saline and 10% formalin was prepared beforehand. The tube with the fine needle attached to it was cleaned by pumping distilled water and then saline through it. All air bubbles in the tube were removed.

The fish was laid on a wire tray with its ventral side facing upwards. To reduce its movement during the perfusion process, it was tied with wire on the tray. A cut was made upwards from the anus to a position between the left and right gills in order to expose the heart (Figure 3.3). Part of the gills and muscle could be cut away.



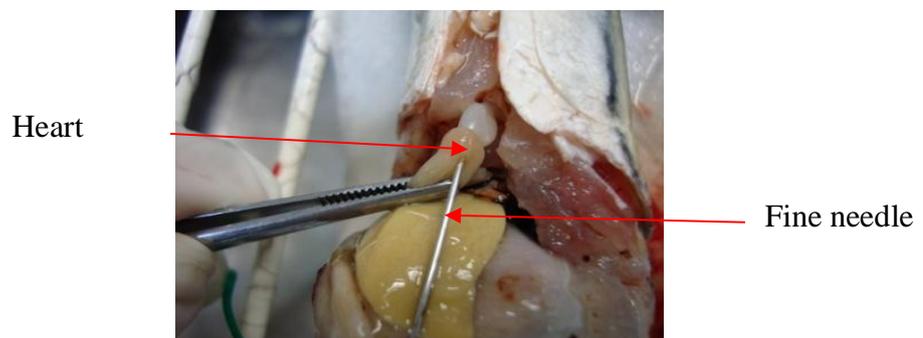
**Figure 3.3:** The ventral side of fish body was cut open to expose the heart

The structure of the fish heart must be fully understood in order to carry out a successful perfusion.



**Figure 3.4:** Structure of the fish heart

As shown in Figure 3.4, the fish heart is consisted of one atrium and one ventricle. The ventricle is connected to conus arteriosus which resembles aorta in human (Figure 3.5). The fine needle attached to the tube was inserted through the ventricle into the conus arteriosus. A puncture was made at the ventricle to allow flowing out of the fluids.

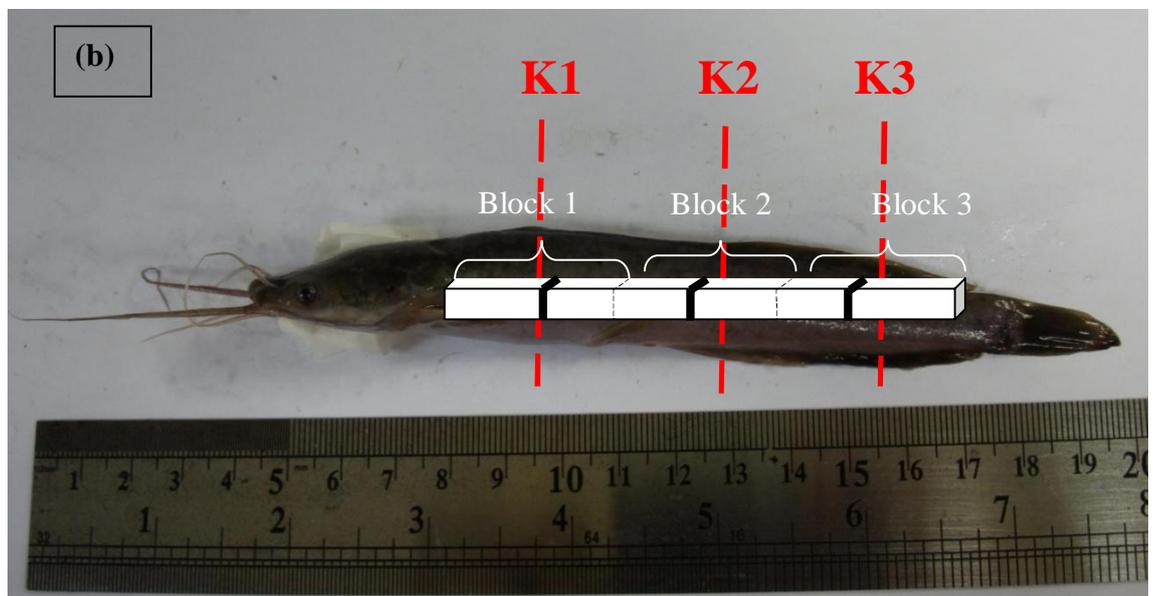
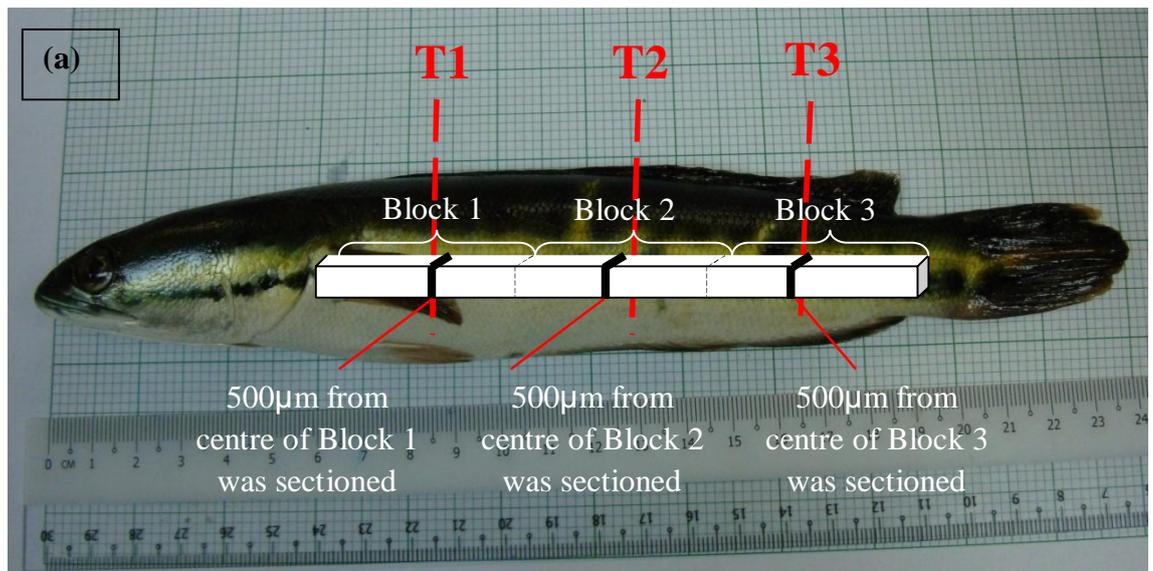


**Figure 3.5:** Fine needle was inserted into the fish heart during intracardiac perfusion

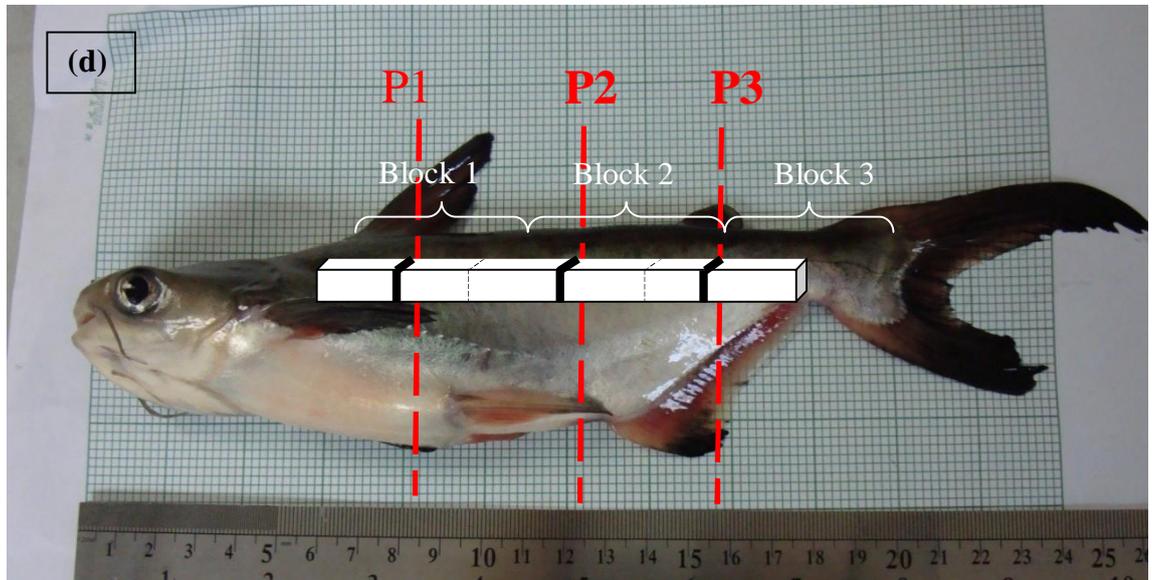
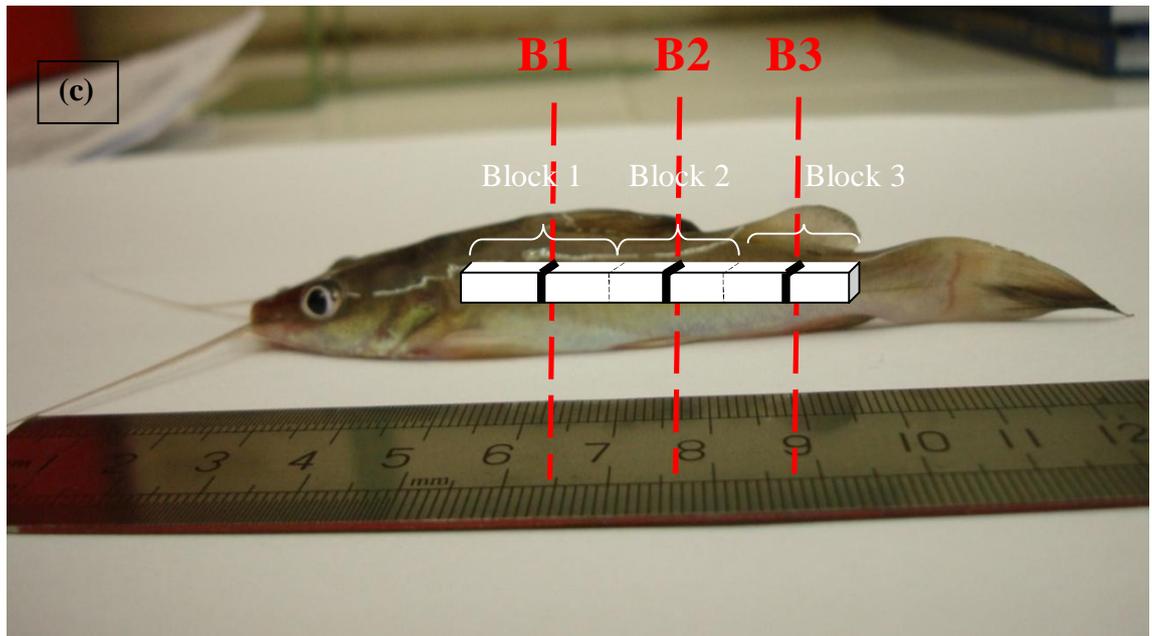
Saline was pumped into the heart until blood flowing out was gradually replaced by clear solutions. Saline was then replaced by 10% formalin. Perfusion ended when the fixatives reached the whole fish which was signaled by the jerky movement of the fish. A successful perfusion could lead to hardened fish body.

### **3.3.2 SPINAL CORD ABSTRACTION AND POST FIXATION**

Specific spinal cord segments were collected, which would be discussed in details below. Fish body trunk was divided into three equal segments, resulting in three blocks of spinal tissue of equal length. Spinal tissue with the length of 500µm from the middle part of each block was processed histologically and sectioned. Hence, based on the method mentioned above, for *C. micropeltes* and *Clarias* sp. from the group of fish with long and continuous dorsal fin, spinal cord tissues were obtained from body segments along the dorsal fin [Figure 3.6 (a) and (b)]. However, for group of fish with short and continuous median fins which included *M. nemurus* and *Pangasius* sp., spinal cord tissues were harvested from three particular segments, which were from the dorsal fin, trunk, and adipose fin [Figure 3.7(a) and (b)]. Spinal tissues obtained from different segments were labeled accordingly. To ensure the spinal tissues were collected precisely from the specified regions, spinal cord abstraction was done immediately after perfusion, while the muscles were still intact.

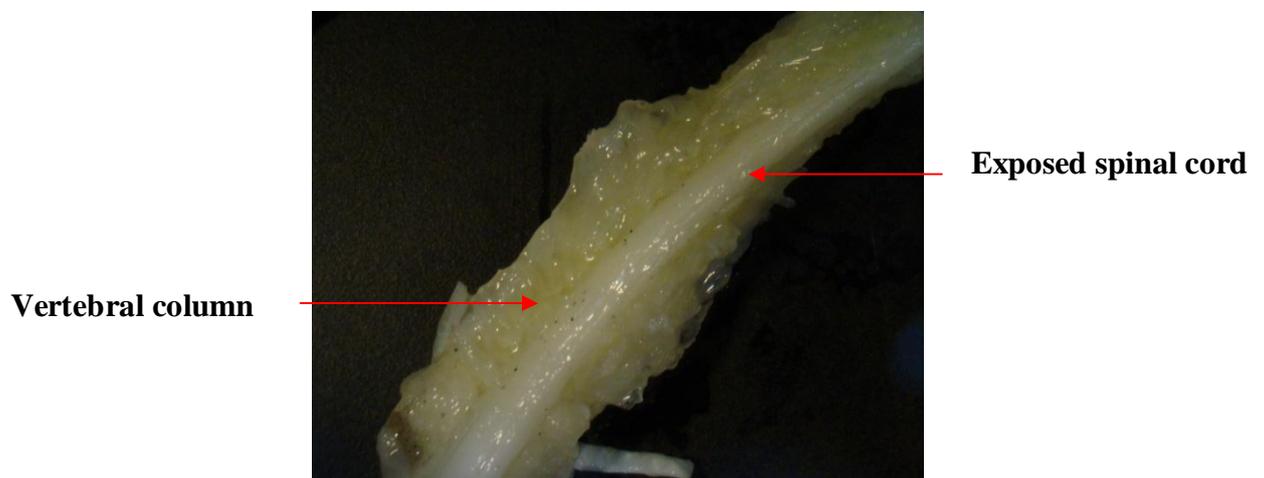


**Figure 3.6:** Spinal cord tissue was harvested from different segments in fishes with long and continuous dorsal fins: (a) *Channa micropeltes*, and (b) *Clarias* sp.



**Figure 3.7:** Spinal cord tissue was harvested from different segments in fishes with short and non continuous median fins: (a) *Mystus nemurus*, and (b) *Pangasius* sp.

When the perfusion was completed, the particular segments mentioned above containing the spinal cord were taken out. Its muscles were quickly removed to expose the vertebral column containing the spinal cord. The vertebral column was post-fixed in 10% formalin for at least 24 hours. Prior to that, a few holes were punched on the vertebral column to allow the fixatives to seep in more quickly to fix the spinal cord. To dissect out the spinal cord, the dorsal part of vertebral column was cut open (Figure 3.8) with extra care and then the spinal cord was removed with ease. The spinal tissue was then ready for histological procedures.



**Figure 3.8:** Spinal cord was exposed following the excision of dorsal part of vertebral column

### **3.3.3 DEHYDRATION**

The spinal cord was passed through a series of alcohol with increasing concentration. It was first transferred into a scintillation vial filled with 70% alcohol for at least 24 hours. After that, the 70% alcohol was poured out and replaced by alcohol of different concentrations, starting with 85% alcohol, then two changes of 95% alcohol (95% Alcohol I and II), and two changes of absolute alcohol (Absolute Alcohol I and II). Each immersion was for 45 minutes.

### **3.3.4 CLEARING**

Clearing agent, in this case toluene, served to clear away the alcohol. This process was accomplished by two changes of toluene (Toluene I and II) and one mixture of toluene and paraffin (1:1). Each immersion was for 45 minutes.

### **3.3.5 INFILTRATION AND EMBEDDING**

The spinal cord was immersed in melted paraffin (60°C) for three times (Paraffin I, II and III) in order for the paraffin to permeate through the tissue. Each immersion was for 45 minutes.

After paraffin III, the tissue was embedded in a mould of hot wax. After the tissue was aligned properly, the mould was left to be cooled off until the wax solidified.

### **3.3.6 SECTIONING AND MOUNTING OF THE TISSUE SECTIONS**

The tissue block, after being removed from the mould, was trimmed into a suitable size and attached on a block holder. The paraffin block was then sectioned using a microtome at 10µm to produce a paraffin ribbon consisting of individual sections.

Selected sections were mounted onto the glass slide. Before mounting, diamond tip pen was used to scratch the left side of the slides with information on the specimen for identification and to identify the correct sides of the slides. A tiny drop of Mayer's Albumin was placed in the centre of the slide and smeared with a clean finger. Sufficient amount of distilled water was then dropped and spread on the slide.

A few paraffin sections were laid slowly on the slide to prevent any trapped air bubbles between the slide and the section. The mounted slide was then placed on the hot plate for a few minutes to allow the paraffin to expand. It also permitted the slide to dry up.

### **3.3.7 STAINING**

In order to view the tissue sections for microscopic study, the tissue sections were stained using dye. It was essential to note that different staining methods gave rise to different structural view of the tissue.

In this study, three different staining techniques were applied on the spinal tissue, which included haematoxylin and eosin staining (H&E), Nissl staining, and fiber staining.

### **3.3.7.1 HAEMATOXYLIN AND EOSIN (H&E) STAINING**

Haematoxylin and eosin staining, or more commonly known as H&E staining, was one of the most widely used staining techniques in histology. It provided basic information on the tissue as it stained both the nucleus and cytoplasm of a cell.

### **3.3.7.2 NISSL STAINING**

In this study, Nissl staining using cresyl violet as dye was applied on the spinal tissue. This stain demonstrated Nissl substances in the neuronal somas. Nissl substances were primarily composed of rough endoplasmic reticulum. Since they were only found in the neuronal somas or cell bodies of neurons, this stain highlighted the grey matter which was packed with neuronal somas.

### **3.3.7.3 FIBER STAINING**

Lillie's variant of Weil-Weigert method, was the chosen fiber staining to demonstrate the outline of the grey matter from the white matter. The structural basis of grey and white matters was different, as the former consisted mostly of cell bodies while the latter made up of mainly axon wrapped by myelin sheaths. This stain highlighted the myelin sheaths, giving the white matter purplish blue colour as compared to the red colour of grey matter.

### **3.3.8 COVERSLIPPING**

Coverslipping is a process where the portion of the slide with the tissue sections is covered up using a thin glass coverslip. This would make the slide permanent and to prevent the tissue from drying up. Appropriate amount of mounting medium was placed on the coverslip. It was important to note that different mounting medium was needed for a specific staining technique, e.g. Canada Balsam was used for H&E staining while DPX was meant for Nissl staining. The coverslip was then slowly lowered towards the slide until it touched the surface. They were then kept in the oven at 60°C to dry.

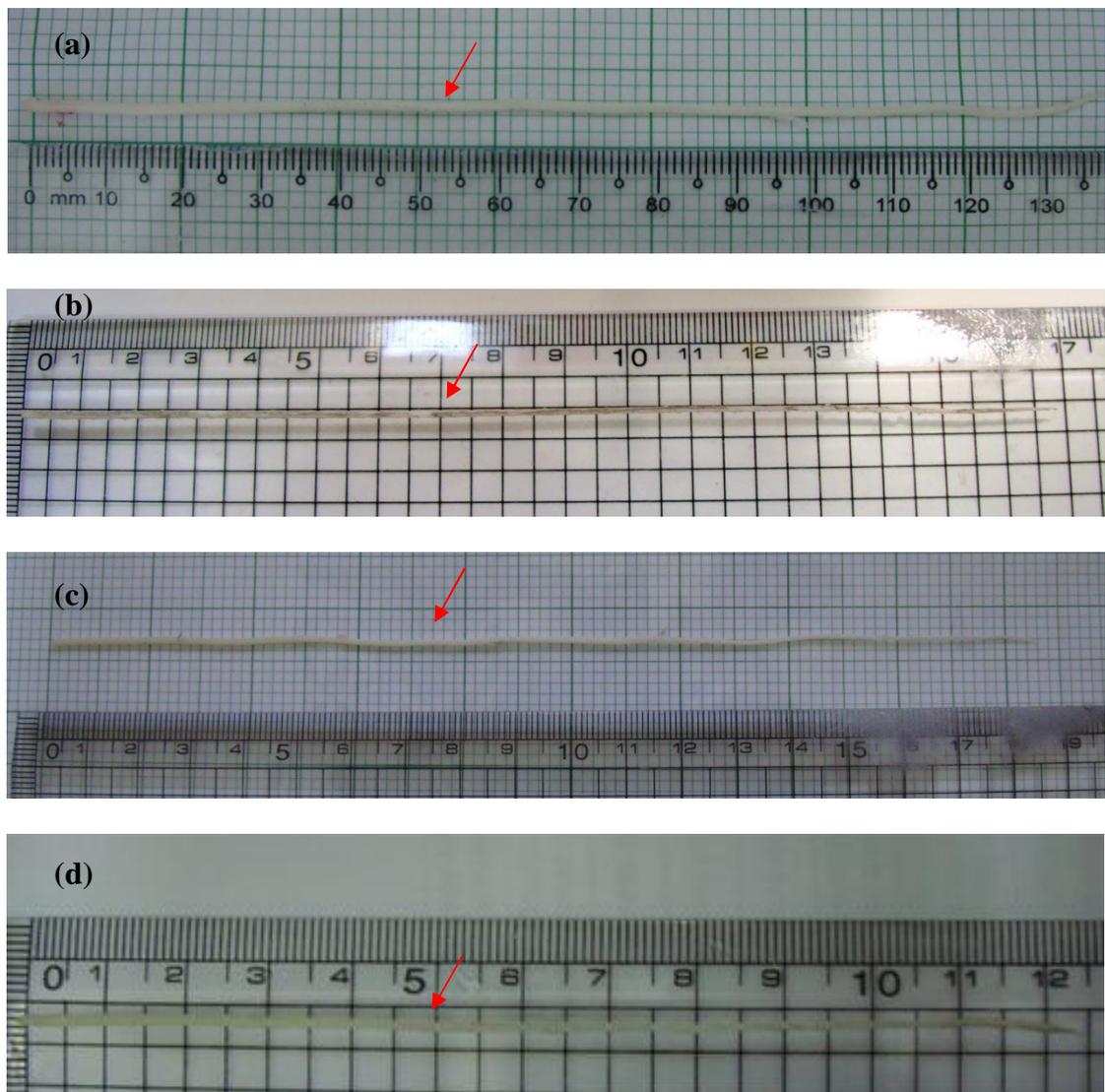
### **3.3.9 DATA ANALYSIS**

All the histological slides produced were observed using light microscope BX51 (Olympus, Tokyo, Japan) under the magnification powers of 4X, 10X and 40X. Data were collected by capturing microphotographs from the light microscope using LifeScience Research Analysis Software (Olympus, Munster, Germany).

### 3.4 RESULTS AND DISCUSSIONS

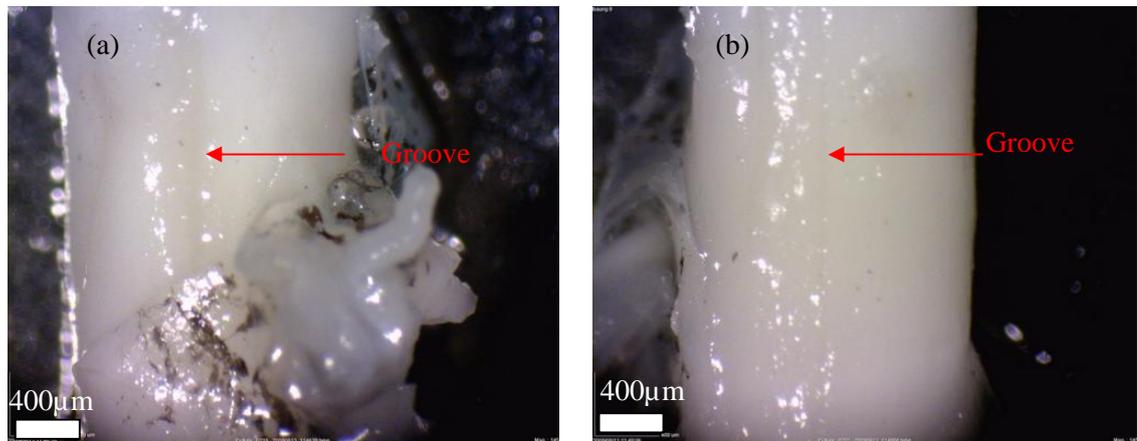
#### 3.4.1 GROSS MORPHOLOGY OF SPINAL CORD

Spinal cord of all four fishes displayed similar gross morphology (Figure 3.9). They were all soft and whitish in colour. In all four fishes, the spinal cord extended to the full length of vertebral column. No enlargement was observed on the spinal cord. The size of the spinal cord gradually decreased from rostral towards caudal, resulting in tapered end.



**Figure 3.9:** Spinal cord of (a) *Channa micropeltes*, (b) *Clarias* sp., (c) *Mystus nemurus* and (d) *Pangasius* sp. (indicated by arrows)

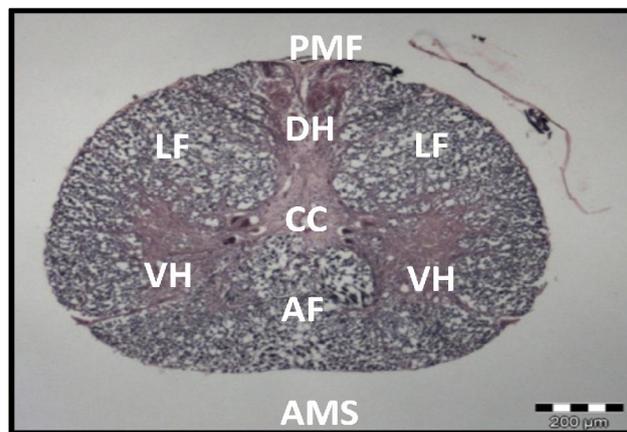
Additional information was obtained when the spinal cord was observed under stereo microscope. Midline rooves could be observed at both dorsal and ventral sides of the spinal cord, respectively. The groove at the dorsal side, the posterior median sulcus, was more obvious compared to the one at ventral side, the anterior median fissure. Spinal cord of *M. nemurus* was displayed below as representative to demonstrate the midline grooves (Figure 3.10).



**Figure 3.10:** Midline grooves on the spinal cord of *Mystys nemurus* at the (a) dorsal and (b) ventral side

### 3.4.2 GENERAL HISTOLOGY OF SPINAL CORD

Generally, the spinal cord architecture of the selected fishes in this study was similar. In cross sections, they appeared to be rounded in shape. As shown in Figure 3.11, the core of the spinal cord was the grey matter in an inverted-Y shape, surrounded by the peripherally located white matter. A tiny central canal was observed at the centre of the spinal section. Grey matter consisted of two dorsal horns and ventral horns. A distinctive feature of the fish spinal cord in this study was the inseparable dorsal horns. The dorsal horns were thin and started right from the dorsal edge of the spinal cord. At the ventral side, the grey matter broke into two broad columns of ventral horns. The ventral horns were connected by a line formed by the extensions from the ventromedial position of the horns. White matter was divided by the grey matter into anterior and lateral funiculi. The posterior funiculus, the small column of white matter between the spinal dorsal horns of vertebrates was lacking.



**Figure 3.11:** Spinal cord of *Channa micropeltes* stained by myelin staining as representative to demonstrate the general spinal cord architecture of the selected fishes in this study

[AF: anterior funiculus; AMS: anterior median sulcus; CC: central canal; DH: dorsal horn; LF: lateral funiculus; PMF: posterior median fissure; VH: ventral horn]

The anterior median fissure and posterior median sulcus were the midline grooves which could be observed from the gross morphology of the spinal cord. Under the stereo microscope, a midline groove could be found at the dorsal side of the spinal cord and another less obvious one at the ventral side. In cross sections, the midline grooves were seen as anterior median fissure and posterior median sulcus. Interestingly, a human spinal cord had a deeper and longer posterior median sulcus, which gave rise to a split between the two halves of spinal cord. However, all three stains failed to highlight the posterior median fissure, especially to give detailed information on its length.

Posterior funiculus, which was part of the white matter, was a bundle of nerves that served as the ascending and descending tracts for information transmission. In human, the broad posterior funiculus could be divided into fasciculus gracilis and fasciculus cuneatus. Both fasciculus gracilis and fasciculus cuneatus acted to carry sensory information, the former transmits information from lower body part while the latter carries information from upper body part such as arms. The histological appearance of the fish spinal tissue showed that where the posterior funiculus barely existed and this tally with the fact that the fish was not equipped with limbs.

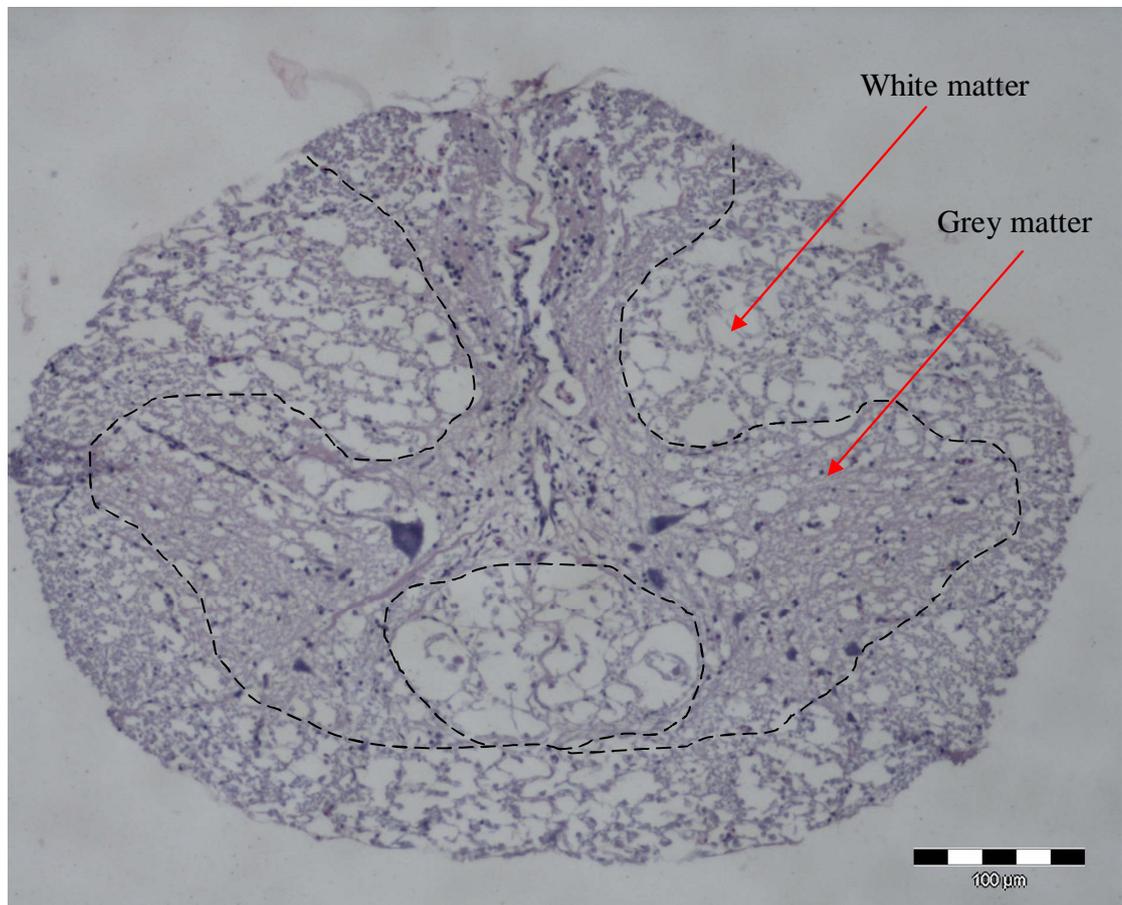
The central portion of spinal cord, which is the grey matter in inverted-Y shape, is composed of cell bodies of neurons. As in other vertebrates, the peripherally oriented white matter is primarily made up of the neuronal axons, which give rise to the ascending and descending fiber tracts (Pearce, 2008). Glial cells were found in both grey matter and white matter.

Dorsal and ventral horns of the spinal grey matter were different in function. The dorsal was sensory in function while the latter coped with motor function. Sensory neurons located in the dorsal horns received all sensory information including touch, proprioception, light and vibration. On the other hand, ventral horns strictly govern the effector organ such as muscle and internal organs. The functional units in ventral horns were motor neurons. The intermediate region between the dorsal and ventral horns contained interneurons which connected afferent and efferent neurons in neural pathways.

The fish spinal cord, which did not display much difference rostral-caudally, was different from spinal cord of limbed vertebrates that exhibited significant features at four different levels. The enlargements of spinal cord of limbed vertebrates are due to the increase in cell bodies at cervical and lumbar levels to control the limbs. Although fish fins are the appendages for movement, they are not able to perform delicate movement like the fingers which needs high control of relevant muscles. Therefore, no enlargement existed in the fish spinal cord.

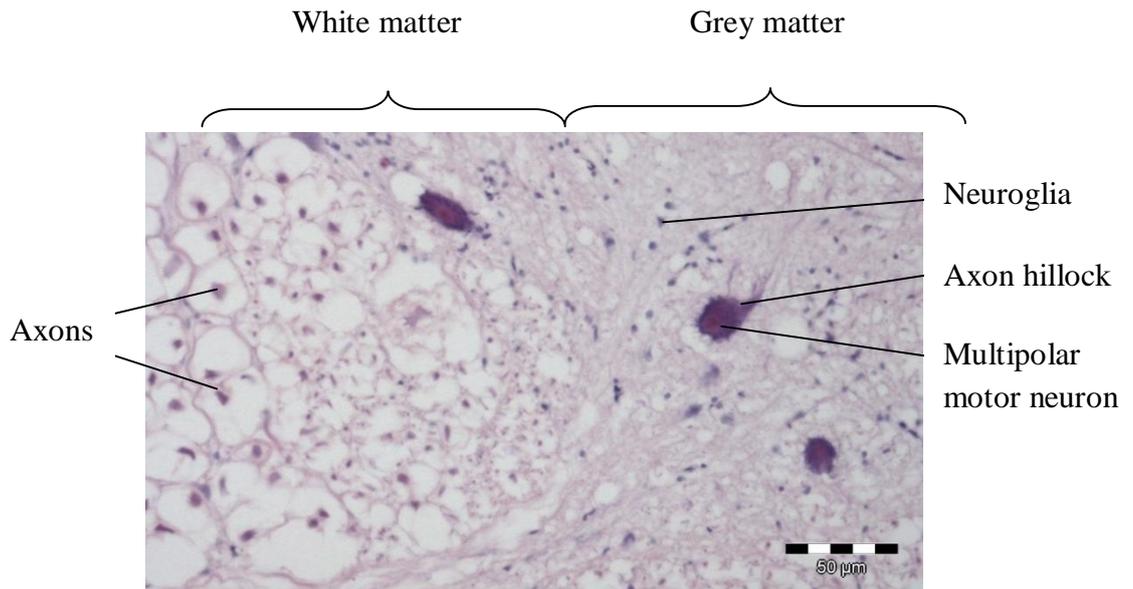
### 3.4.2.1 HAEMATOXYLIN AND EOSIN (H&E) STAINING

H&E was selected as it is one of the simplest stains. This stain was able to outline the basic structure of the fish spinal cord (Figure 3.12).



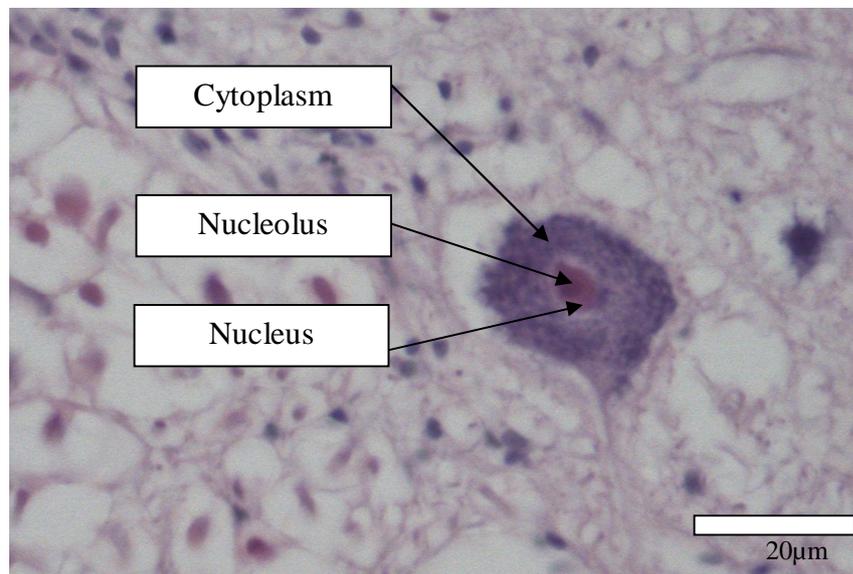
**Figure 3.12:** Spinal cord of *Pangasius* sp. stained with H&E, 100X. A spinal cord cross section showed the inverted-Y shaped grey matter surrounded by white matter.

Dorsal and ventral horns making up the grey matter could be differentiated from the white matter (Figure 3.13). However, the delineation of the grey matter from the white matter was not distinctively obvious, as in mammalian spinal cord, although sufficient viewed at higher magnifications.



**Figure 3.13:** Delineation on grey and white matters by H&E staining. Axons were seen on white matter while motor neurons were located in grey matter.

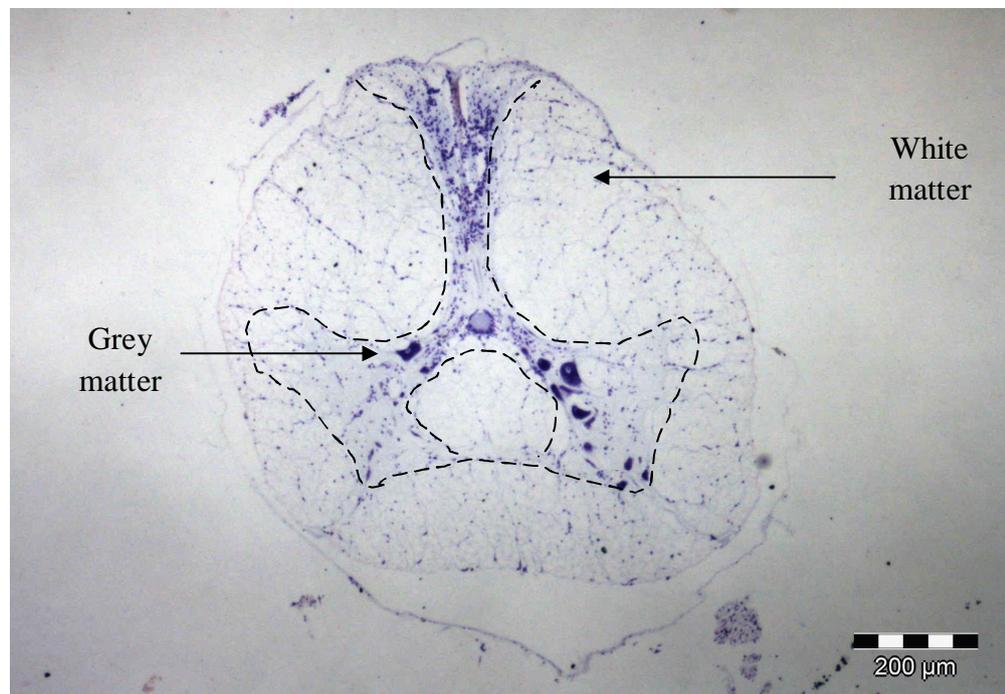
Haematoxylin stained the nuclei in purplish blue while eosin coloured cytoplasm in pink. A dark red nucleolus was observed in the centre of the nucleus (Figure 3.14).



**Figure 3.14:** Motor neuron stained by H&E staining. The motor neuron was made up of nucleus and cytoplasm.

### 3.4.2.2 NISSL STAINING

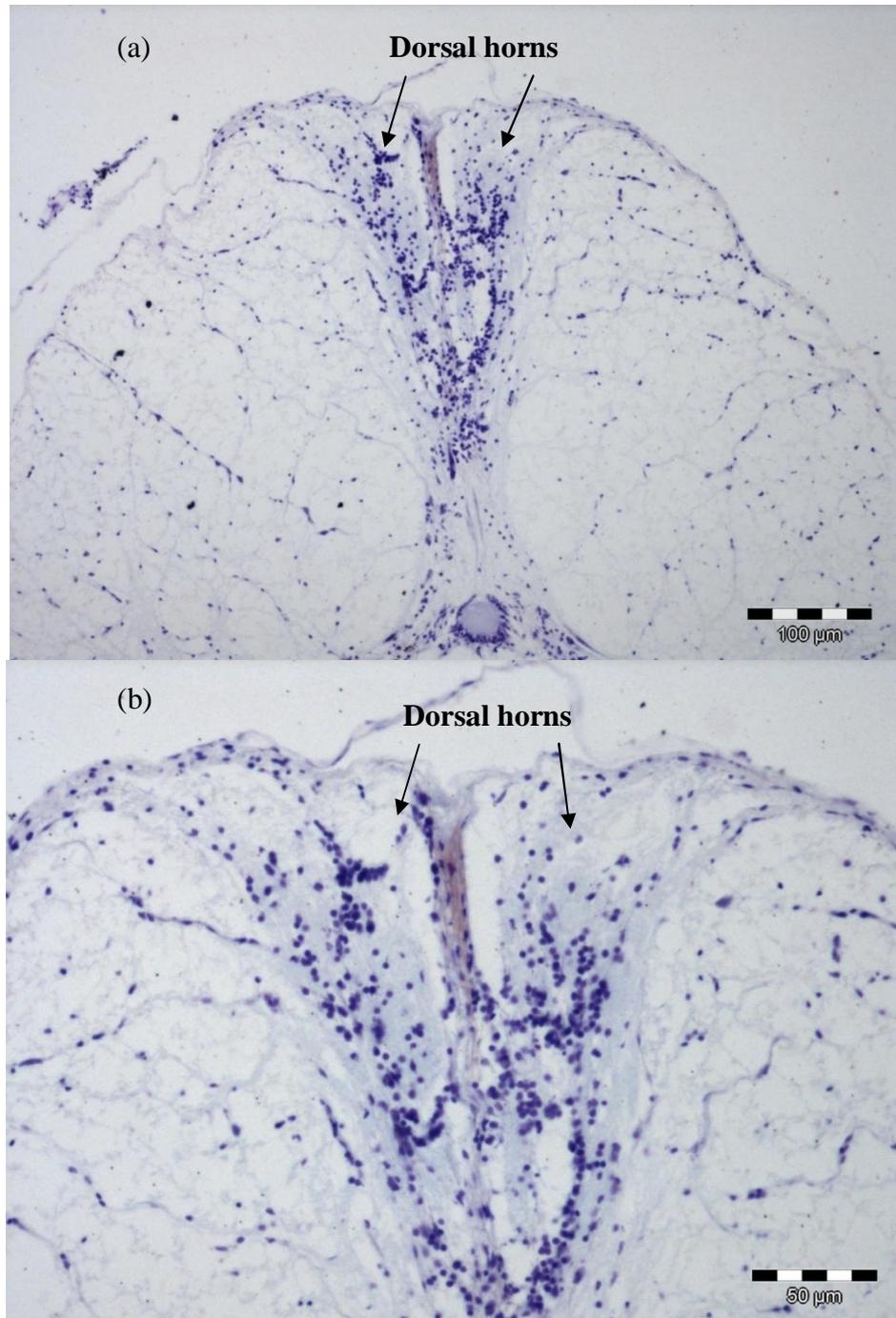
The cross sections of spinal cord with Nissl staining displayed a slight delineation of the grey and white matter (Figure 3.15). Although the grey matter was coloured with very slight violet and the white matter was left unstained, the delineation of grey and white matters was well defined. Grey matter was more compact than the white matter. It gave superb highlight on the neuronal somas, especially the ones located at the ventral horn of spinal grey matter.



**Figure 3.15:** Cross section of *Channa micropeltes* spinal cord stained with Nissl stain using cresyl violet as dye, 40X. The grey matter was coloured with slight violet and the white matter was left unstained.

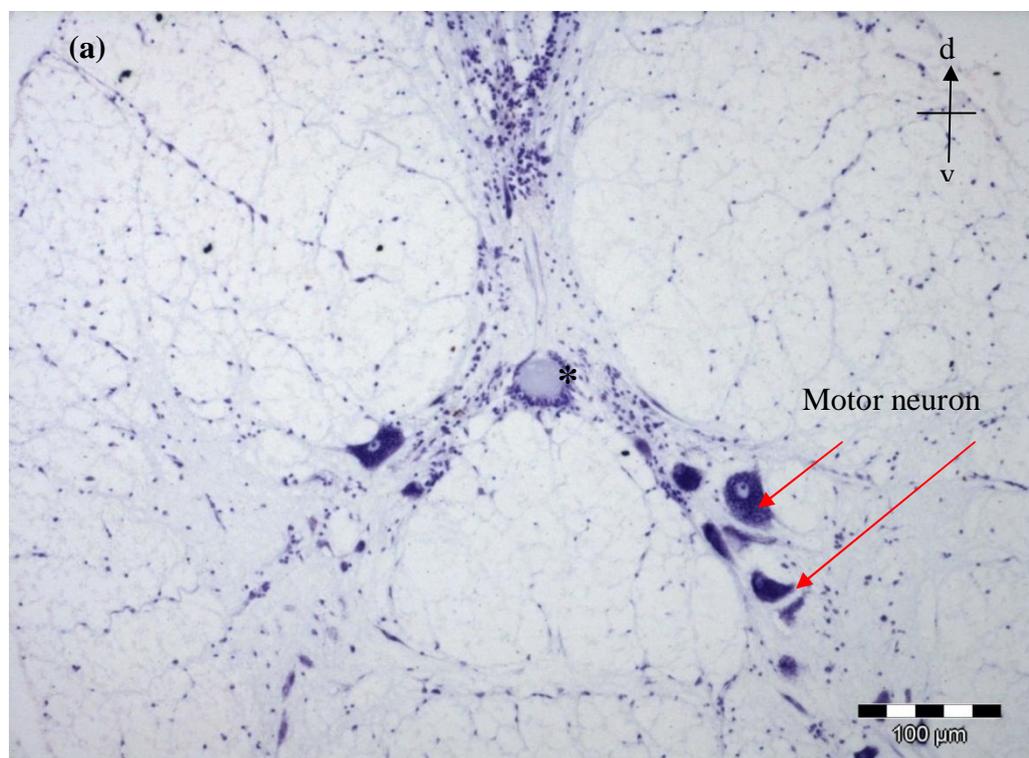
More information was revealed when the tissue was inspected at higher magnification. For example, it was apparent that posterior funiculus was absent (Figure 3.16). The two dorsal horns were joined together and only separated by posterior median fissure at the most dorsal area. No white matter was found in between the two dorsal horns.

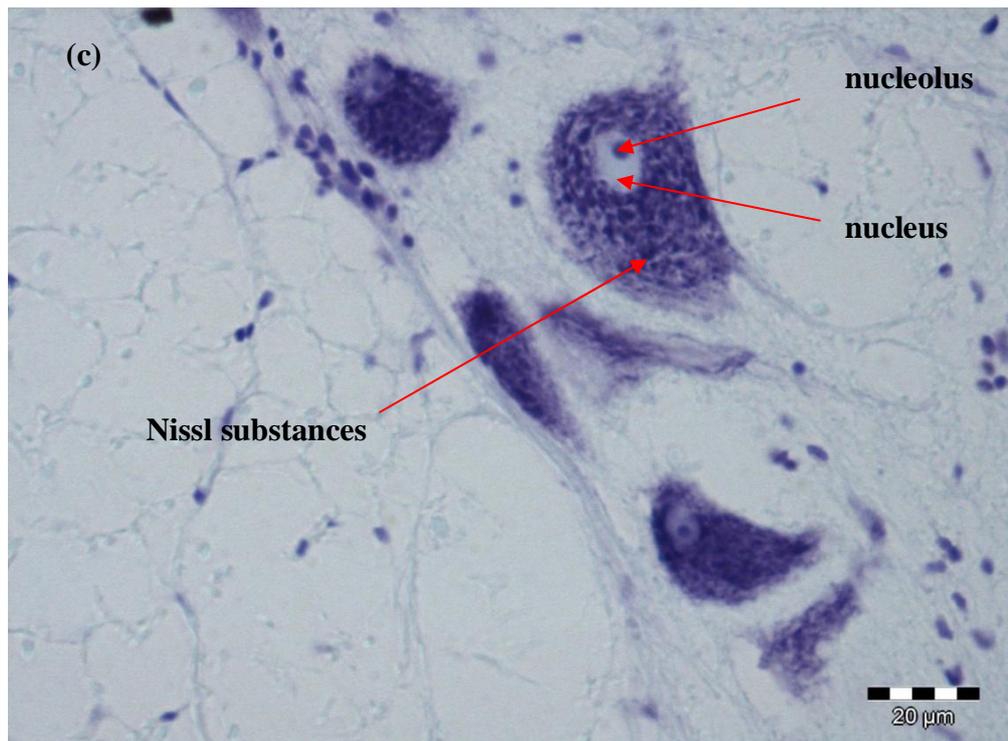
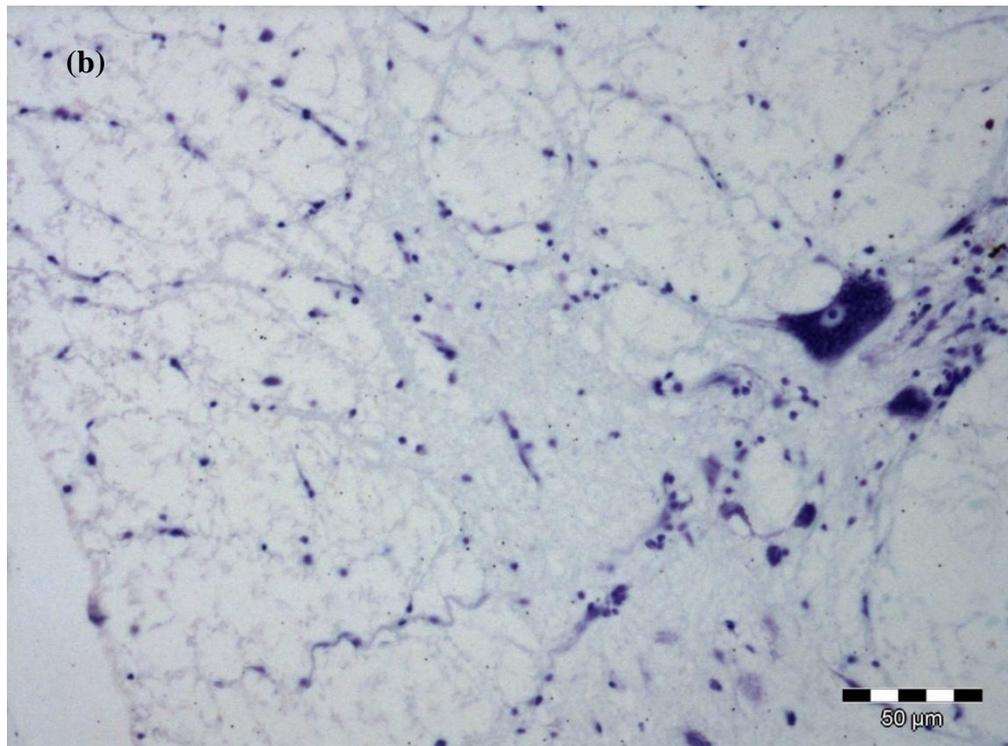
Besides neurons, numerous small blue dots representing glia cells were seemed distributed throughout the spinal cord section. They were found in both grey and white matters. The ones lining the central canal were portrayed well.



**Figure 3.16:** Dorsal part of the spinal cord (*Channa micropeltes*) highlighted by Nissl stain. (a) 100X, (b) 200X. The two dorsal horns were joined together and only separated by posterior median fissure at the most dorsal area.

The cellular pattern of motor neurons was readily observed at low magnification but the details of these nerve cells were better presented when viewed using higher magnifications (Figure 3.17). The motor neurons positioned within the grey matter were of various size and shape. Generally, motor neurons which were stained in bright violet strikingly stood out from the background. In the sections where the motor neurons were cut right through the middle, it was observed that both the cytoplasm and nucleolus were stained, while the nucleus was left unstained. The cytoplasm was clearly illustrated to be made up of Nissl granules in deep blue colour.

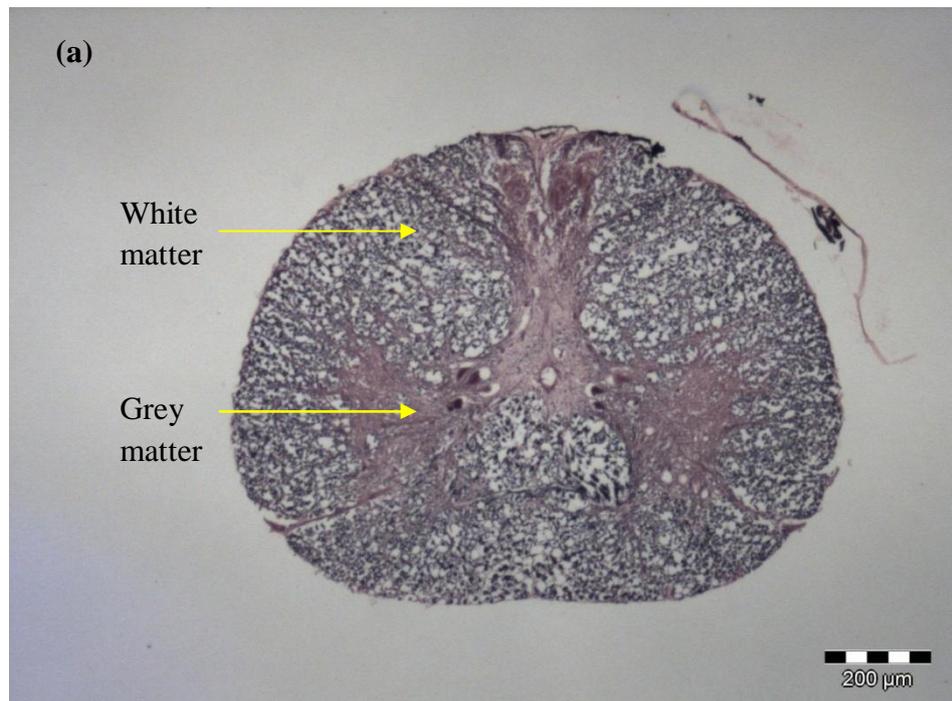


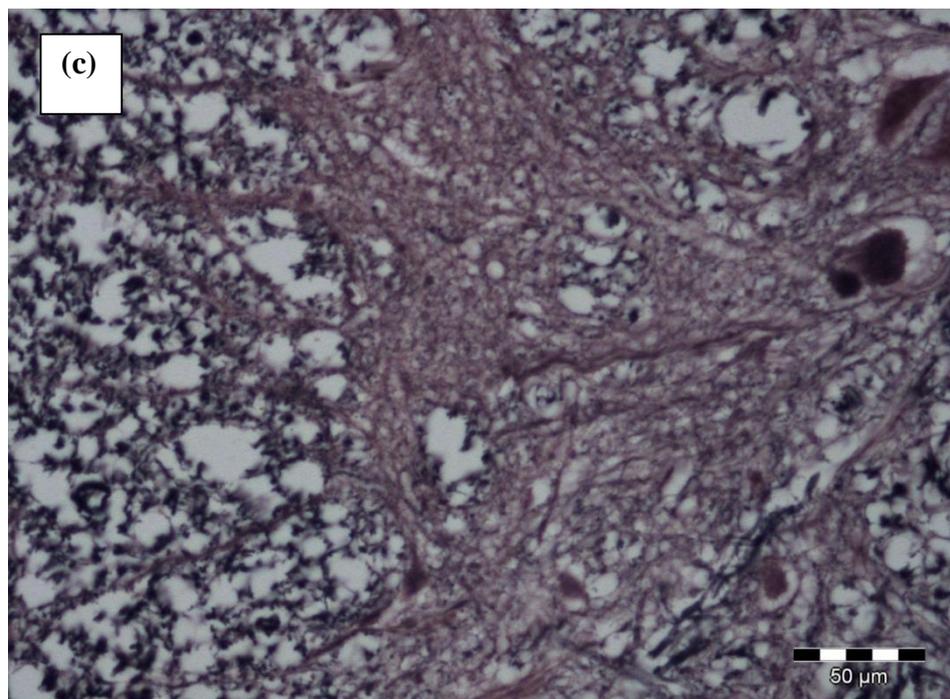
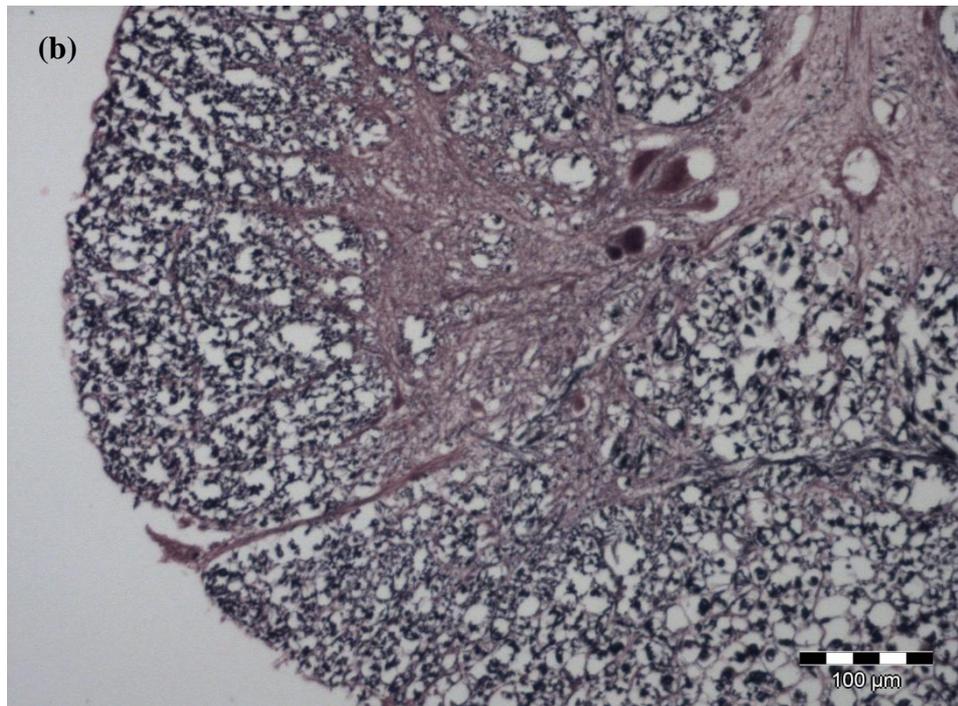


**Figure 3.17:** Distribution of motor neurons in the ventral horns at (a) 4x, (b) 20X and (c) 40x magnification (\*: central canal; d: dorsal; v: ventral). The motor neurons were highlighted as the Nissl granules in the cytoplasm were stained in deep blue colour.

### 3.4.2.3 FIBER STAINING

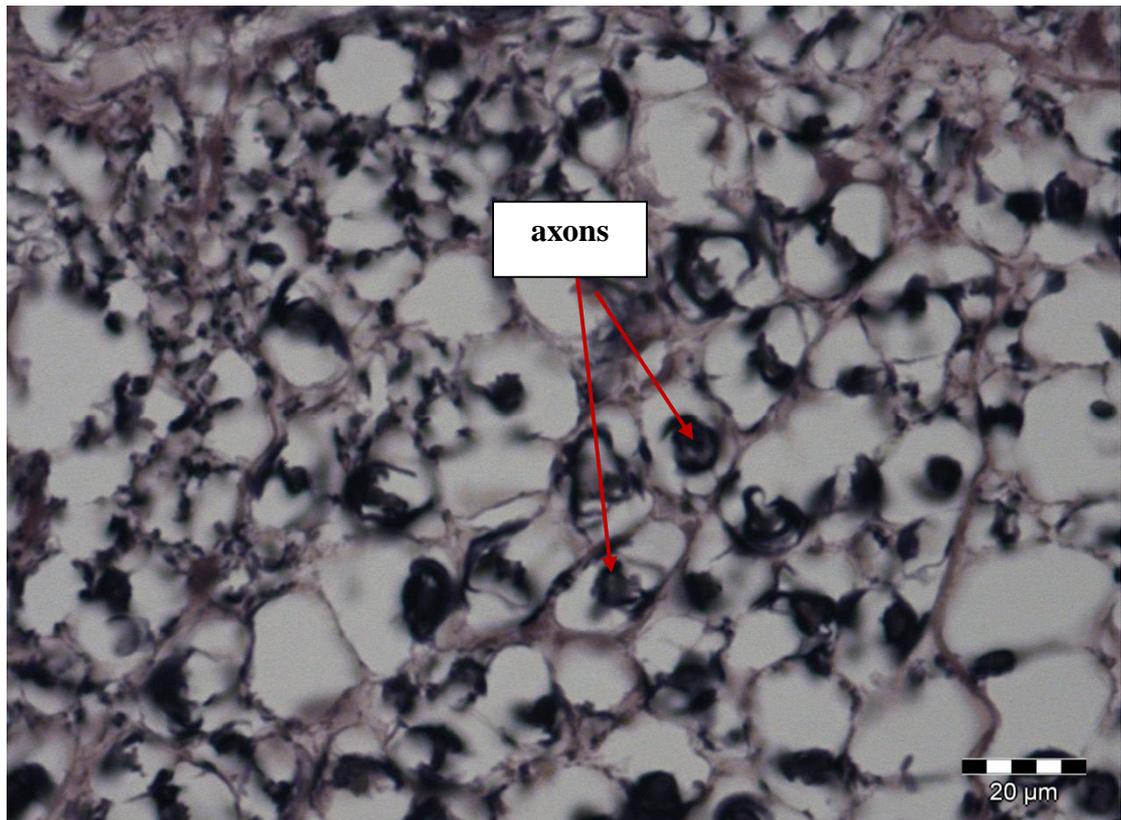
With fiber staining, which in this case stained the myelin, grey matter was delineated from the white matter (Figure 3.18). Grey matter was stained red, in contrast to white matter which was coloured blue. A closer look at higher magnifications revealed the shape and extent of grey matter better.





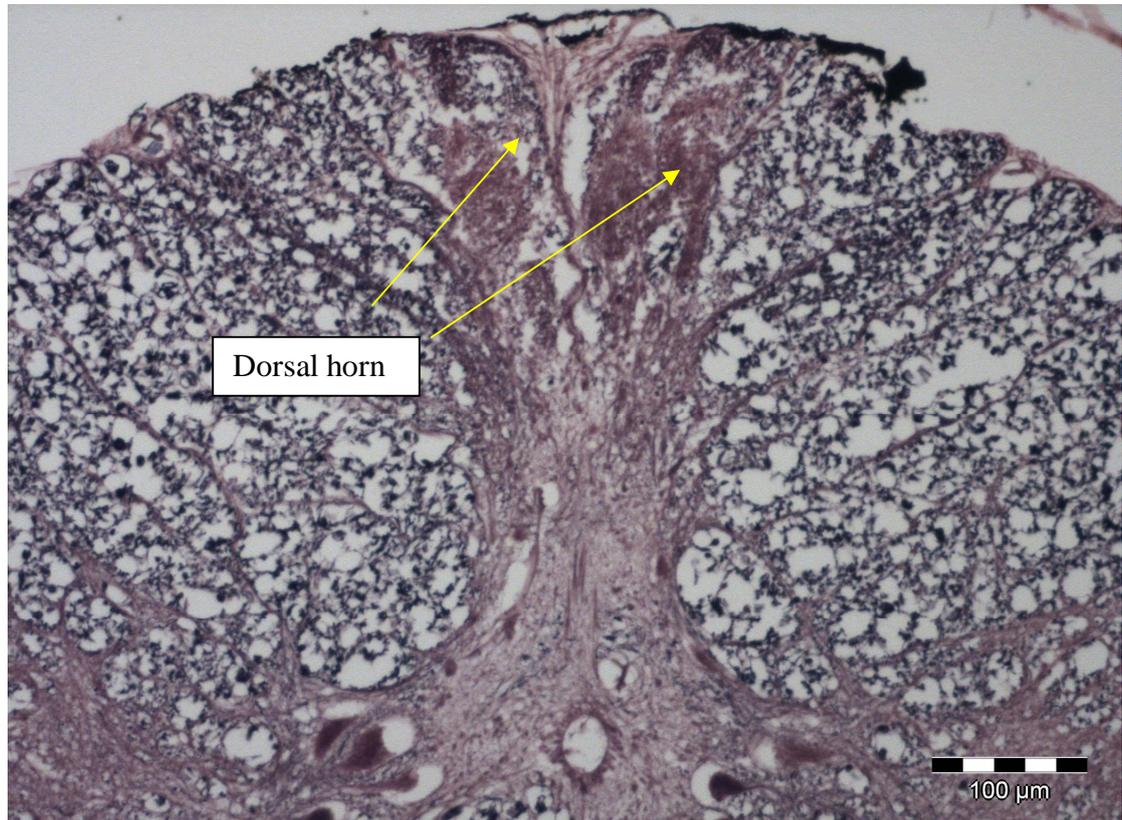
**Figure 3.18:** Distinct delineation of grey matter from the white matter at (a) 40X, (b) 100X and (c) 200X magnifications.

Grey matter consisted of nerve cell bodies while the white matter was made up of nerve fibers, or axons. These axons serve as ascending and descending tracts which convey impulses to and from the brain. They were enveloped by myelin which appeared blue in a spinal tissue stained by myelin stain. In a cross section, these axons could be observed as blue dots distributed throughout the white matter (Figure 3.19).



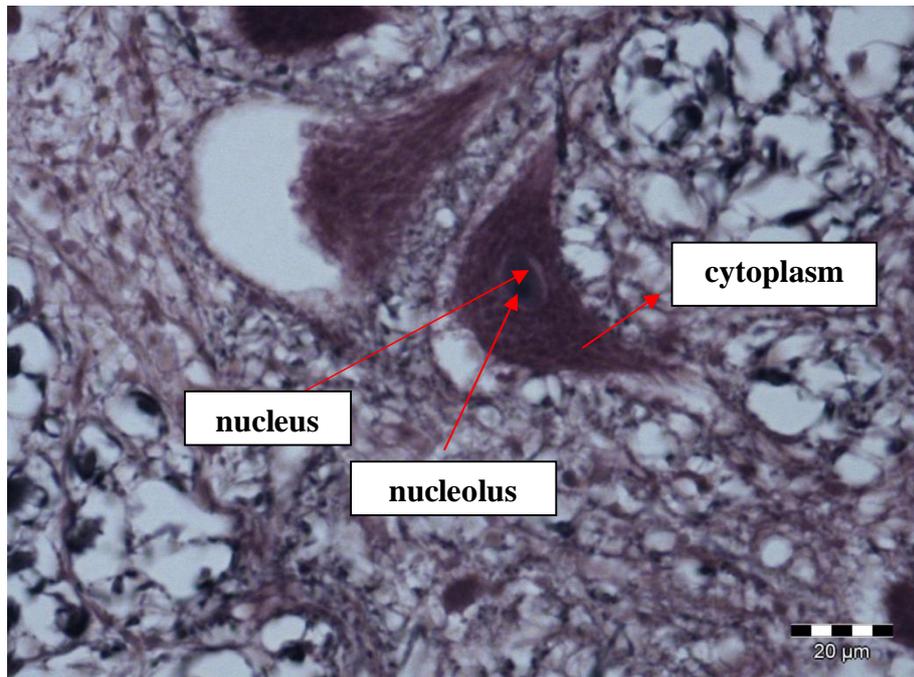
**Figure 3.19:** Axons enveloped by myelin distributed throughout the white matter.

Besides that, the lack of posterior funiculus as a result of joint dorsal horns was once again confirmed by this stain as presented in Figure 3.20.

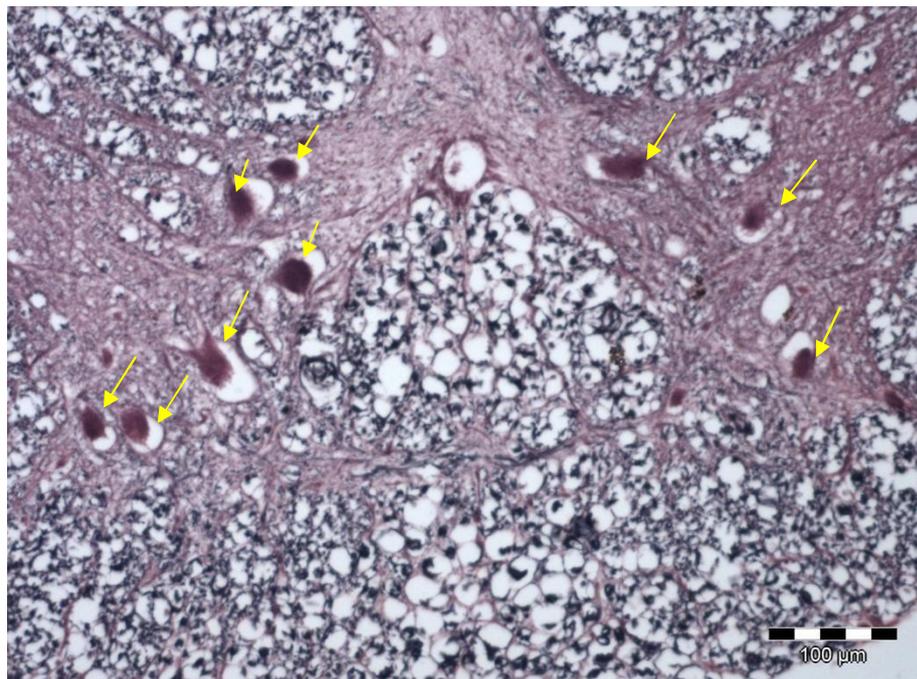


**Figure 3.20:** Joint dorsal horns as illustrated by myelin stain, 10X.

Although this stain was not meant to highlight the motor neurons as the Nissl stain did, it still gave clear and distinct cellular pattern of motor neuronal to the spinal tissue. The cytoplasm of the motor neuron was stained deeper red than the grey matter, while the nucleus was coloured deep purple (Figure 3.21). A dark prominent circle represented the nucleolus was located in the nucleus. Given the delineation of grey and white matters was defined (Figure 3.22), classification of motor neurons into cell columns could be done in an exact way. Also visible in Figure 3.22 was the extension from the ventromedial position of the left and right ventral horns, forming a line connecting both ventral horns.



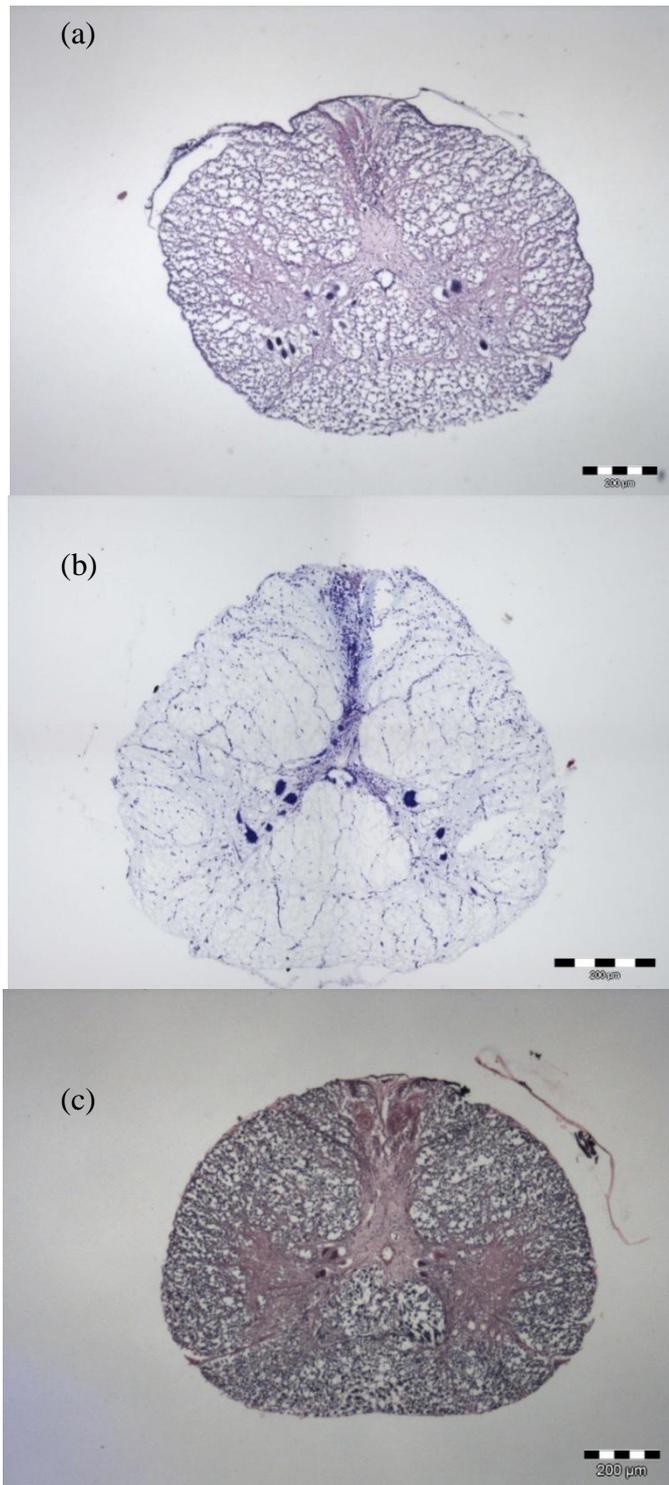
**Figure 3.21:** Motor neurons coloured by myelin stain; cytoplasm in deep red while nucleus in purple with a prominent dark nucleolus in it.



**Figure 3.22:** Definite cellular pattern of motor neurons (indicated by arrows) in a well-defined border of grey matter.

#### **3.4.2.4 SUMMARY OF DIFFERENT STAINING TECHNIQUES**

Results of three different staining techniques had been discussed in detail in the previous sections. In sum, the stains used in this study were capable of presenting highlights on different spinal structures. Photo micrographs of spinal cord stained by the three different techniques were aligned together for comparison purpose (Figure 3.23). H&E stain provided information on the general tissue organisation of the spinal cord. Nissl stain was excellent in outlining the cellular pattern of motor neurons as it gave superb highlight on neuronal somas. Myelin stain, on the other hand, demonstrated distinct delineation of grey and white matters, thus showing the shape and extent of ventral horns which could not be clearly demonstrated by two other stains. Calculation of motor neurons for inspection on cellular pattern was possible in tissue sections stained by all three techniques.



**Figure 3.23:** Cross sections of *Channa micropeltes* spinal cord (4X) stained by (a) H&E, (b) Nissl stain and (c) fiber staining techniques.

### **3.4.3 SPINAL CYTOARCHITECTURE AT DIFFERENT SEGMENTS**

#### **3.4.3.1 GENERAL HISTOLOGY OF SPINAL CORD**

Going down the spinal cord from rostral towards the caudal end, as shown by the histological sections obtained from three different levels, it was observed that the size of the spinal cord gradually decreases. This fact was illustrated by the decreasing diameter of spinal cord from segment 1 to segment 3 (Table 3.1). It was important to note that no enlargement was found at any level of the spinal cord. In addition, no lateral horn was observed positioned between the dorsal and ventral horns of the spinal grey matter.

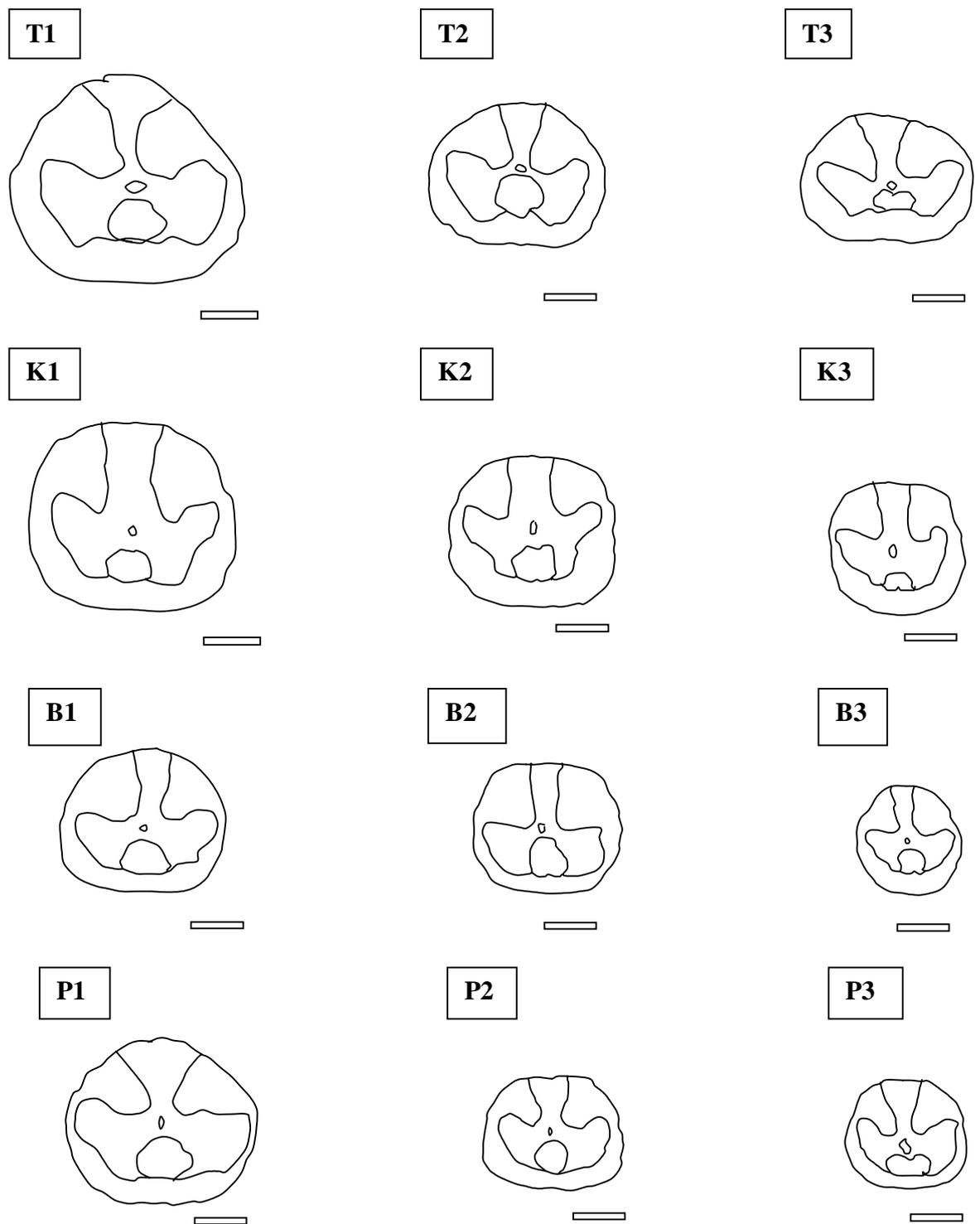
The size of the spinal cord decreased gradually towards the caudal end in correspondence to the steadily reduction of both sensory and motor control over the body (Figure 3.24). It was essential to note that a certain spinal cord level was in charge of specific body segment. The rostral part of the spinal cord had a role to play on the control of body musculature and internal organs. However, the spinal cord at the caudal end might only need to take care of the corresponding caudal body parts.

Moving down the cord, the amount of white matter relative to the grey matter decreased. In rostral spinal cord, there were ascending tracts grouped and descending tracts which had not peeled off. However, in caudal spinal cord, axons ascending were just about to gather while most of the descending fibers had just peeled off, leaving only those innervating that particular level.

From Table 3.1, it was observed that there was correlation between the size of the specimen with the diameter of its spinal cord. The data below demonstrated the fact that there was a positive correlation between the size of a specimen and the diameter of its spinal cord. Commonly, the specimen grows in size along with its age. Thus, the age of the specimen also has positive correlation with the spinal cord diameter.

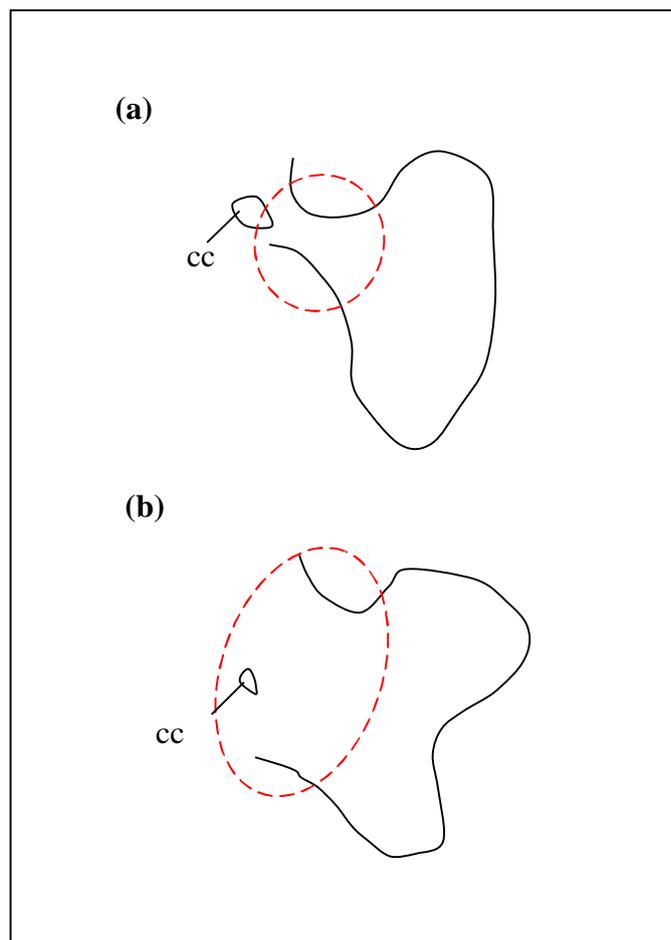
**Table 3.1:** Diameter of spinal cord at three segments for the four fishes used in this study

Fish (n=6)	Whole Body Length (cm)	Weight (g)	Diameter of Spinal Cord ( $\mu\text{m}$ )		
			Segment 1	Segment 2	Segment 3
<i>Channa micropeltes</i>	22.67 $\pm$ 0.77	93.14 $\pm$ 3.11	878.18 $\pm$ 1.27	706.98 $\pm$ 0.69	667.51 $\pm$ 1.23
<i>Clarias</i> sp.	15.38 $\pm$ 1.44	20.45 $\pm$ 2.36	698.78 $\pm$ 1.45	570.54 $\pm$ 2.03	446.89 $\pm$ 1.98
<i>Mystus nemurus</i>	12.45 $\pm$ 1.35	15.39 $\pm$ 1.07	601.19 $\pm$ 1.11	543.30 $\pm$ 0.98	419.62 $\pm$ 1.54
<i>Pangasius</i> sp.	13.33 $\pm$ 1.26	19.26 $\pm$ 1/56	623.29 $\pm$ 1.79	495.49 $\pm$ 1.65	399.08 $\pm$ 1.77



**Figure 3.24:** Illustration of spinal cord cross sections obtained from three different segments of *C. micropeltes, toman* (T), *Clarias sp., keli* (K), *M. nemurus, baung* (B), and *Pangasius sp., patin* (P); scale bar = 200 $\mu$ m (Refer nomenclature T1-T3 and K1-K3 from Figure 3.6 and B1-B3 and P1-P3 from Figure 3.7.)

For each fish, the shape of grey matter did not differ much from rostral towards the caudal end, as observed from spinal cord acquisition site 1 to site 3. The shapes of spinal grey matter for *Channa micropeltes*, *Mystus nemurus*, *Pangasius* sp., as displayed in Figure 3.25(a), had thin areas of extension protruding outwards from central canal before becoming dorsal and ventral horns. Such characteristic was not observed in *Clarias* sp. [Figure 3.25(b)].

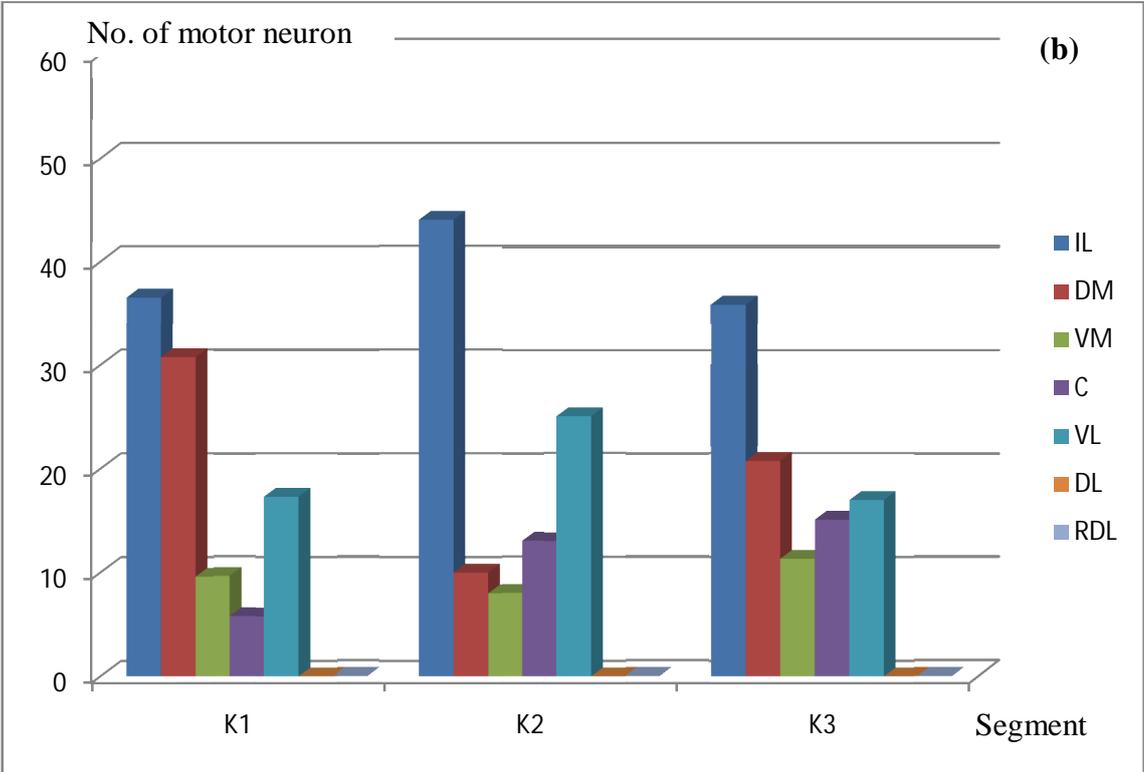
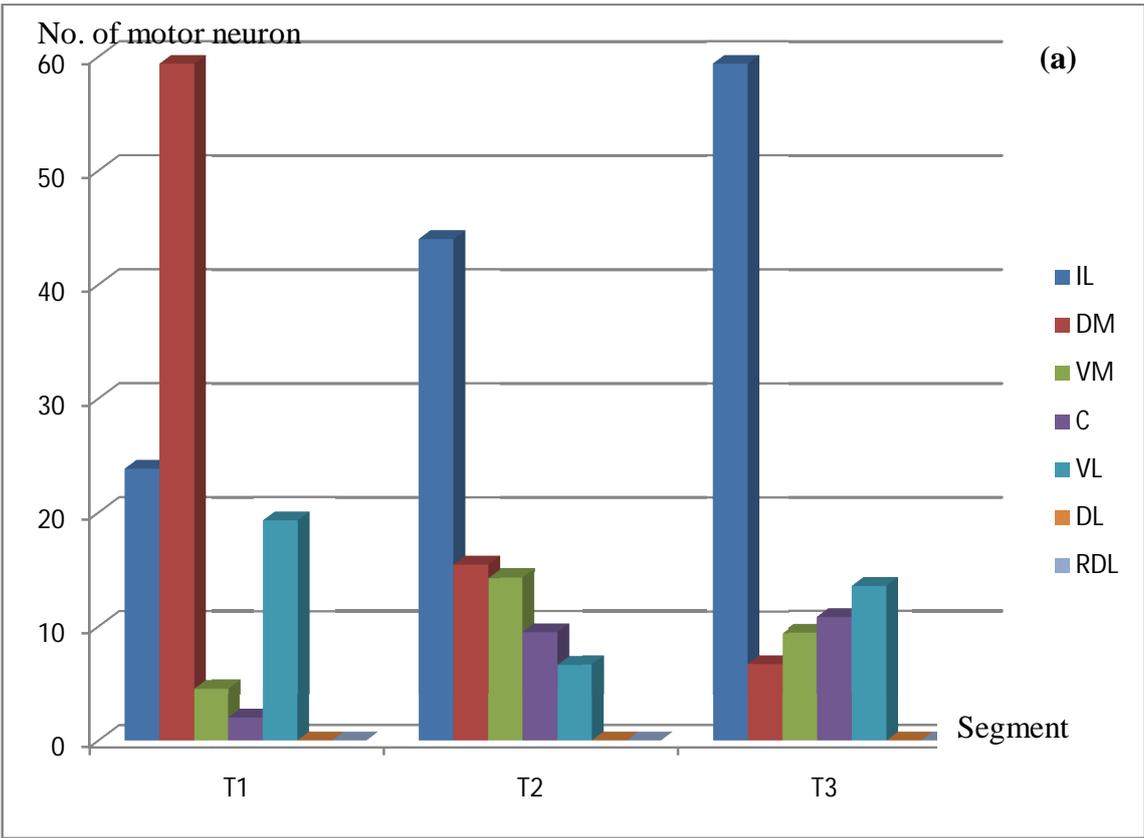


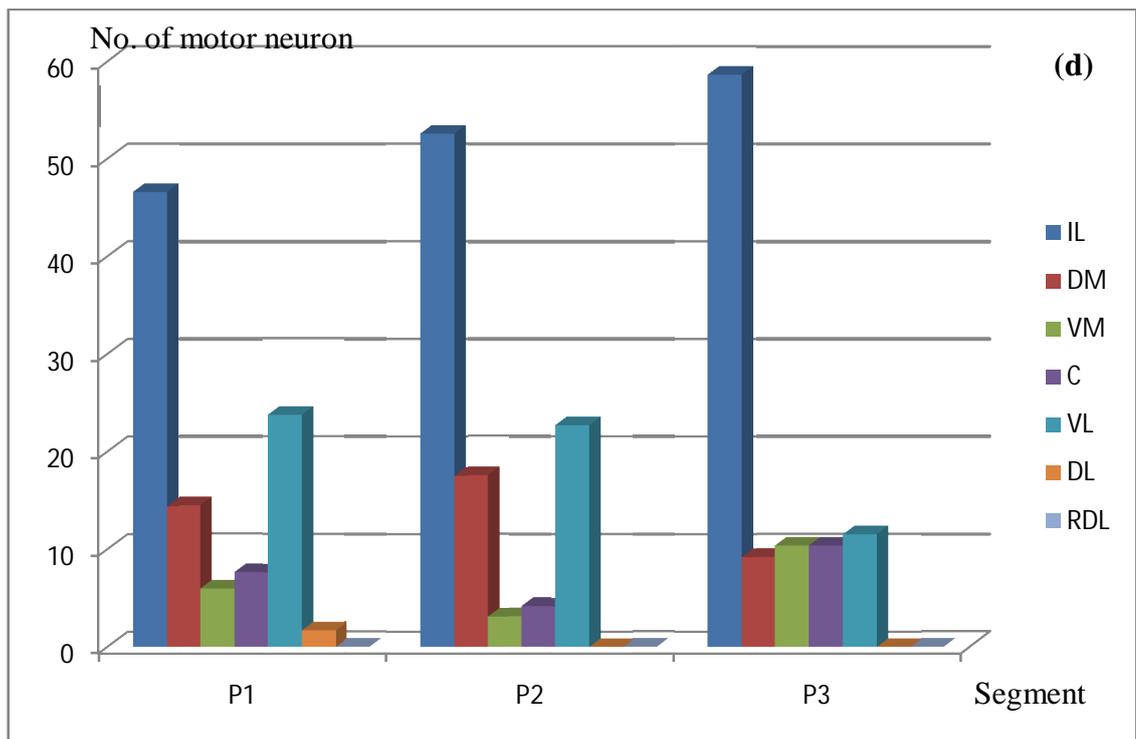
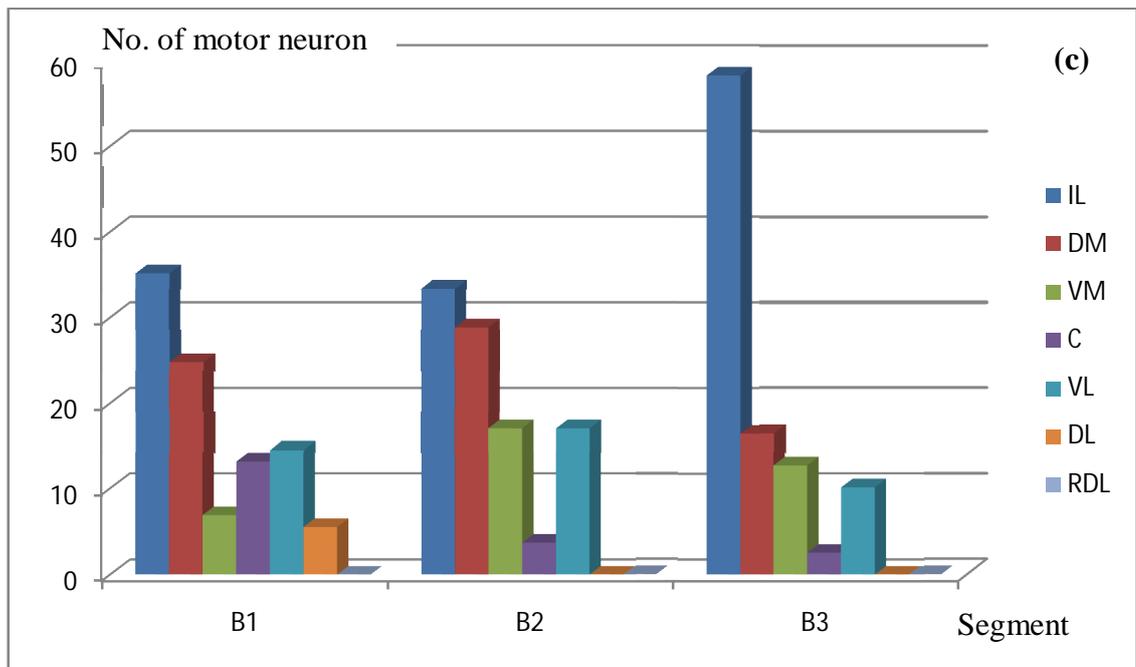
**Figure 3.25:** Shapes of spinal grey matter in (a) *Channa micropeltes*, *Mystus nemurus*, *Pangasius* sp., and (b) *Clarias* sp.; cc: central canal. The different shapes of grey matter were circled in red, where (a) had thin and (b) had broad areas of extension protruding outwards from central canal before becoming dorsal and ventral horns.

### **3.4.3.2 MOTOR NEURONAL ORGANISATION**

Numerous motor neurons could be observed at the ventral horns of the spinal grey matter. No discreet motor neuron pool was observed. Instead, motor neurons of various shapes and sizes were found singly, in pairs, or in small clusters

The motor neurons, when organized into cell columns, presented the result in the following pages.





**Figure 3.26:** Organisation of motor neurons into cell columns for (a) *Channa micropeltes*, (b) *Clarias* sp., (c) *Mystus nemurus*, and (d) *Pangasius* sp. The organisation would be explained in details in the following text. (IL: Intermediolateral; DM: Dorsomedial; VM: Ventromedial; C: Central; VL: Ventrolateral; DL: Dorsolateral; RDL: Retrodorsolateral)

For evaluation purpose, grey matter of the ventral horn was divided into seven cell columns, namely intermediolateral (IL), dorsomedial (DM), ventromedial (VM), central (C), ventrolateral (VL), dorsolateral (DL) and retrodorsolateral (RDL). For the group of fish with long and continuous dorsal fin, motor neurons were seen clustered into the first five cell columns mentioned. On the other hand, for the group of fish with short and continuous median fins, motor neurons were found in an extra column, which was dorsolateral cell column. By referring to Figure 3.26, dorsolaterally located motor neurons were found in P1 and B1 segments. From the perspective of fish external morphology, P1 and B1 corresponded to where dorsal fins were located. In other words, these motor neurons might be innervating the dorsal fin muscles. However, it should also be taken into consideration that the motor neurons found dorsolaterally were probably innervating the pairs of pectoral or pelvic fins which were also located at those areas.

From previous researches (Trujillo-Cenoz and Bertolotto, 1988; Fetcho, 1986a), it was a known fact that medially located motor neurons innervate trunk muscles while those laterally located innervate distal body appendages. In this study, highest percentage of motor neurons was distributed at the medial columns (IL, DM and VM) as compared to lateral columns (VL, DL and RDL). IL and DM columns, with few exceptions, contained the most number of motor neurons. According to FitzGerald (1985), motor neurons of these two columns were in charge of the trunk myotomal muscle. It was important to note that all four fishes used in this study moved mainly by undulation of trunk muscles with assistance of fins, as mentioned in Chapter 2. These facts suggested that the high percentage of motor neurons in IL and DM columns were indeed important to cope with the need of myotomal motor neurons as the main movement generator.

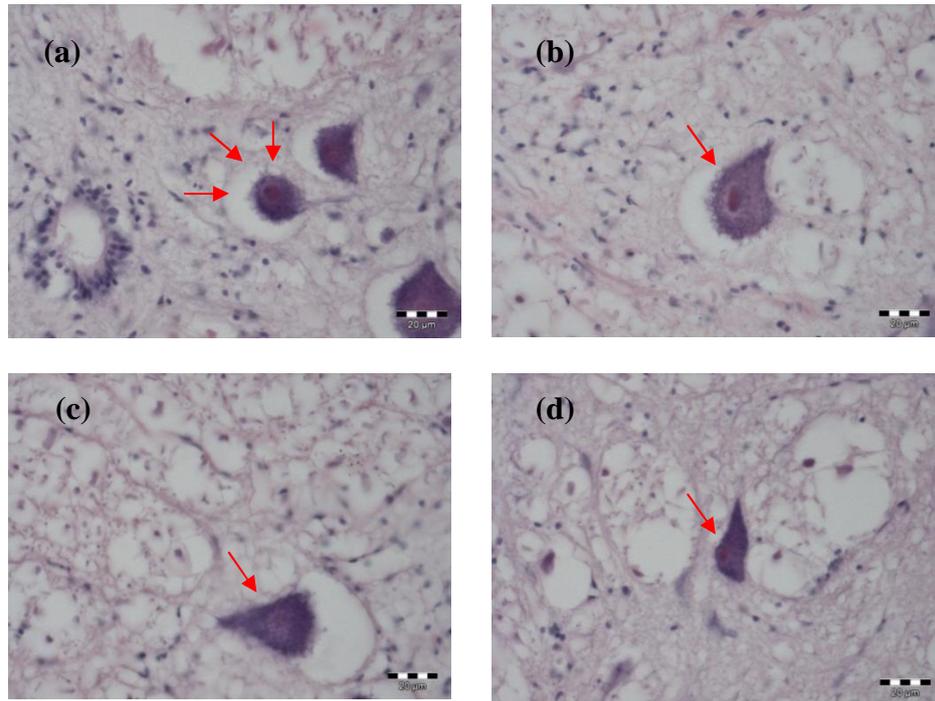
In higher vertebrates such as mammals and reptiles, motor neurons located in DL and RDL innervate distal organs, that is, the limbs (Table 3.2). These motor neurons allow precise control over the delicate movement of the extremities. In this case where the fish has no limbs, the fins act as the appendages that assisted in locomotion. However, the fins are not as developed as the limbs. In addition, fish fins are only thin layer of skin extended from the body. Hence, fin muscles are most probably innervated by motor neurons located at the ventrolateral cell columns alone. Hence, dorsoventral and retrodorsolateral cell columns do not exist as there are no distal organs to be innervated.

**Table 3.2:** Cell columns and corresponding muscles innervated (FitzGerald, 1985)

CELL COLUMN	MUSCLES
<i>Medial</i>	
Ventromedial	Erector spinae
Dorsomedial	Intercostal, avdominals
<i>Lateral</i>	
Ventrolateral	Arm/thigh
Dorsolateral	Forearm/leg
Retrodorsolateral	Hand/foot
<i>Centre</i>	
Central	Diaphragm

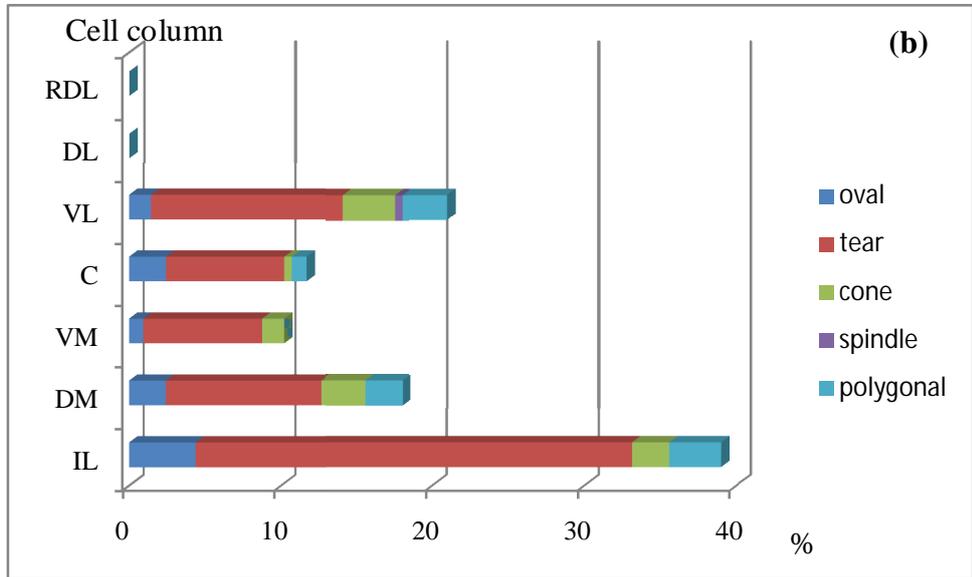
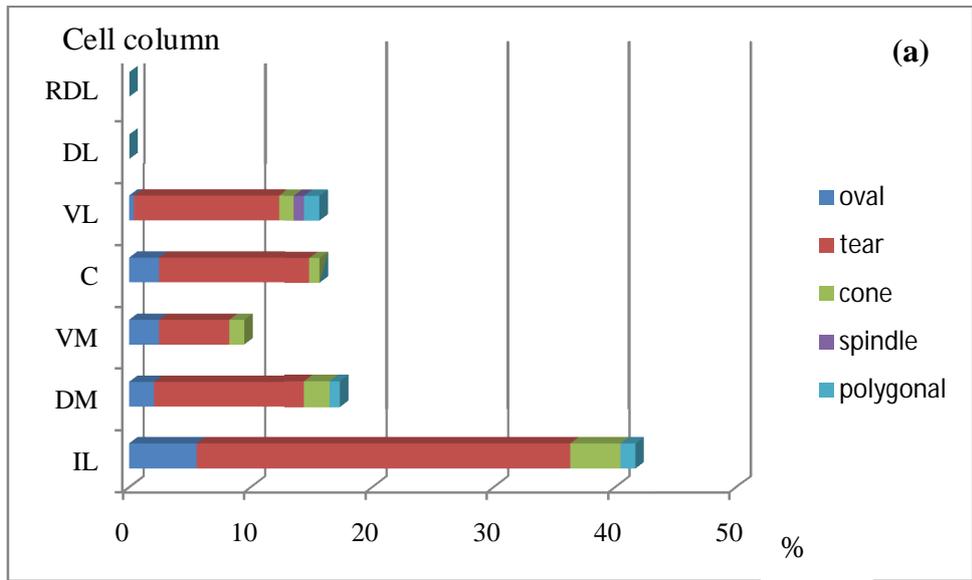
Although the number of neurons within each cell column throughout the length differed for each species, IL for both fishes had the most neurons compared to the other cell columns.

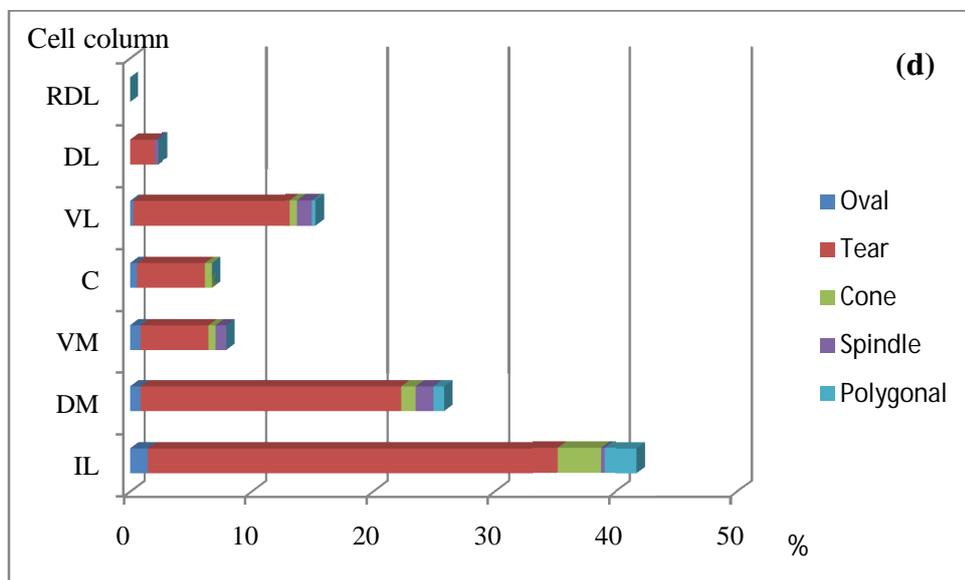
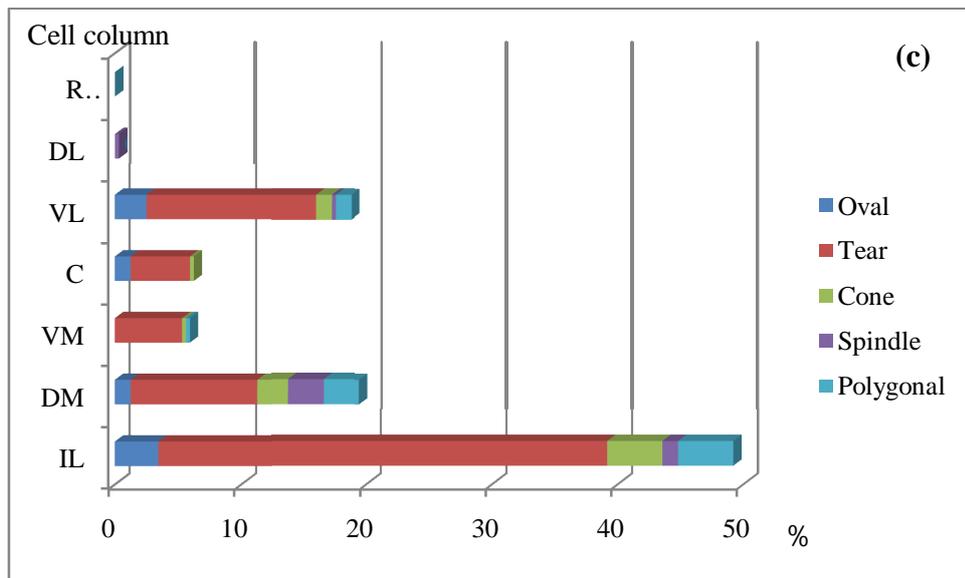
### 3.4.3.2.1 MOTOR NEURONAL SHAPE



**Figure 3.27:** Diverse motor neuronal shapes in this research which include (a) oval (b) tear, (c) cone and (d) spindle shapes (indicated by arrows).

Motor neurons came in various shapes but could generally be classified as cone, oval, spindle or tear shapes (Figure 3.27). Those that did not fit into these categories were considered as having polygonal shape. There is no standard classification on motor neuronal shape. The shape of the motor neuron was best confirmed with the presence of the axon and nucleolus, which showed that the motor neuron was sectioned right at the center, and thus demonstrating its real shape.





**Figure 3.28:** Motor neuronal organisation of different cell shapes into each cell column for (a) *Channa micropeltes*, (b) *Clarias sp.*, (c) *Mystus nemurus* and (d) *Pangasius sp.*

Cell migration during development gives rise to motor neurons of different shapes (Rockerfeller University, 2009). When a neuron migrated away from its site of origin, the dendrites will stay anchored. The dendrites get longer while the position of cell body moves further away. The shape of the neuron is formed when cell migration completes.

There is no standard classification of the motor neuronal shapes. In studies concerning motor neuronal shapes, researches categorize them based on their

appearances. This study utilised classification which includes tear, oval, cone, spindle and polygonal shapes.

Tear-shaped somas of motor neurons were dominant while motor neurons of round, cone and polygonal shapes were distributed in all cell columns in a very low percentage (Figure 3.28). Spindle-shaped somas of motor neurons seemed to be a unique feature of VL cell column in fishes with long and continuous median fins, which included *C. micropeltes* and *Clarias* sp. However, in fishes with short and non continuous median fins, which in this case *M. nemurus* and *Pangasius* sp., spindle-shaped motor neurons were found in both medial and lateral columns.

Researches on the motor neuronal shapes in salamanders (Nishikawa et al., 1991; Roth et al., 1988; Wake et al., 1988; Roth and Wake, 1985) are far more complete than in other animals. It was found that tear-shaped and spindle-shaped neurons were mostly found in medial and lateral columns, respectively (Nishikawa et al., 1991). However, to date, there is no research which outlines the correlation between the function of a motor neuron and its shape.

This experiment had provided information on the correlation of motor neuronal organisation and the function of body parts, for example the fin function. However, motor neurons which precisely innervate the fins could only be identified using retrograde techniques, which would be discussed in the next chapter.

### 3.4.3.2.2 SIZE OF MOTOR NEURONS

**Table 3.3:** Range of cell size

Fish	Range ( $\mu\text{m}$ ) of cell size						
	IL	DM	VM	C	VL	DL	RDL
<i>Channa micropeltes</i>	7.57-34.15	9.61-22.91	7.33-21.26	11.26-18.55	6.78-16.74	-	-
<i>Clarias</i> sp.	4.36-32.61	5.02-16.65	5.52-14.51	5.62-21.26	4.83-16.51	-	-
<i>Mystus nemurus</i>	3.27-16.69	3.62-12.04	3.21-12.37	3.21-10.72	2.31-11.86	3.22-11.15	-
<i>Pangasius</i> sp.	2.78-25.47	2.88-11.17	3.93-9.89	3.28-11.72	2.21-14.65	8.57-9.77	-

Medially located motor neurons were bigger than those located laterally (Table 3.3). The biggest and smallest motor neurons of each species were located in IL and VL cell columns, respectively.

Large motor neurons located at IL columns are most probably primary motor neurons. Other motor neurons are secondary motor neurons, which differentiate later than the primary motor neurons. Primary motor neurons are vital for rapid movements while secondary motor neurons are meant for slower and weaker movements (Fetcho, 1987).

The diversity of motor neuronal sizes is most probably affected by the animal age and animal strain (Jacob, 1988).

### 3.5 CONCLUSIONS

The spinal cord of all four fishes used in this study exhibited similarity from the aspects of gross morphology and general histology. The soft, whitish spinal cord extended to the full length of vertebral column, with the size reducing from rostral towards the caudal end.

Histological sections of the spinal cord revealed its general architecture and organisation. The inverted-Y shaped of the grey matter was enclosed by the peripherally located white matter. A prominent figure of the spinal grey matter was the joint dorsal horns resulting in the lack of posterior funiculus. Besides that, the ventral horns were connected by a horizontal line formed across the midline by the extension from the ventral horns.

For all fishes, inspection of spinal cord on rostral, middle and caudal sites revealed that the shapes of ventral horn remained similar. However, it is important to note that the shape of ventral horn of *Clarias* sp. was different from *C. micropeltes*, *M. nemurus* and *Pangasius* sp.

Dorsolaterally located motor neurons were only observed in the rostral body part (B1 and P1) of *M. nemurus* and *Pangasius* sp. Tear-shaped somas of motor neurons were dominant while motor neurons of round, cone and polygonal shapes were distributed in all cell columns in a very low percentage. Last but not least, medially located motor neurons were bigger than those located laterally.

**CHAPTER 4: PROTOCOL ESTABLISHMENT ON**  
**SPINAL MOTOR INNERVATIONS OF CAUDAL FIN MUSCLES**

**4.1 INTRODUCTION**

Histological techniques carried out on non-mammalian neural tissue normally do not yield results easily as on mammalian neural tissue. Hence, the retrograde labeling protocol had to be first established at preliminary stage. Each part of the protocol especially tracer administration and enzymatic reaction could possibly contribute to failures of getting results. Therefore, experiments below were very vital in establishing the protocol step-by-step

**4.2 OBJECTIVE**

- To determine appropriate protocols for study on spinal motor innervations of caudal fin muscles

**4.3 METHODOLOGY**

*Clarias* sp. was used as the experimental subjects for protocol establishment except for section 4.2.1.3 where both *Clarias* sp. and *Pangasius* sp. were used. *Clarias* sp. was chosen as it was a tougher fish than *Pangasius* sp. and most of them survived after the HRP administration protocol. It also made better recovery after anaesthetization.

**4.3.1 HORSERADISH PEROXIDASE (HRP) HISTOCHEMISTRY**

The most commonly used dye for such study is horseradish peroxidase (HRP). HRP is an enzyme found in the roots of the horseradish plant. It is a popular dye chosen for such procedure because it is small in size (MW= 40000 Daltons), and thus tends to be taken up by the nerve cells. It yields precipitation which can be easily visualized when reacts with a substrate and hydrogen peroxidase.

#### **4.3.1.1 COMPATIBILITY OF REAGENTS**

A quick test was performed to test the compatibility of the reagents used. HRP dye and chromogen 3, 3'-Diaminobenzidine (DAB) was prepared according to protocol given. HRP solution was prepared by dissolving HRP powder (Sigma type VI, USA) in Tris-buffered saline (TBS). For DAB Enhanced Liquid Substrate Kit (Sigma D3939, USA), one drop of Solution A (DAB chromogen) was mixed with 1ml of Solution B (buffer solution containing H<sub>2</sub>O<sub>2</sub>).

One drop of HRP solution was placed on the filter paper and immediately after, one drop of mixture solution from DAB Enhanced Liquid Substrate Kit was placed at the same spot. The test was repeated with different concentrations of HRP solution.

#### **4.3.1.2 HRP UPTAKE IN FISH NERVE**

Skinning was initiated 1.5cm from the caudal fin. With skinning completed, superficial muscle layer just anterior to caudal fin was exposed. Muscles at the midline were removed in order to expose spinal nerves.

The exposed spinal nerves were transected. 5µl of HRP solution was pipetted around the transected nerve. With the muscle around the ventral root removed, HRP solution formed a reservoir around the cut nerve, enabling it to be taken up by the nerve.

The fish was sacrificed after two days. The spinal cord was abstracted from the vertebral column and fixed by immersion in 10% neutral buffered formalin (NBF) for two hours. The caudal part of spinal cord was processed as whole mount and frozen sections, in order to decide the better protocol to study motor neuronal organisation.

##### **4.3.1.2.1 WHOLE MOUNT**

The Spinal cord block was first immersed overnight in TBS to clear away all remnants of formalin. It was then reacted with the chromogen by soaking it into 1ml

mixture solution from DAB Enhanced Liquid Substrate Kit. Immediately after that, the spinal cord was dehydrated in ascending concentration of alcohol solutions, cleared in methyl salicylate, and mounted in Canada Balsam.

#### **4.3.1.2.2 FROZEN SECTIONS**

The spinal cord block was immersed in 30% sucrose overnight, sectioned using cryostat and mounted on tefflon-coated slides. The slides were washed with TBS before they were incubated in the mixture of solution from DAB Enhanced Liquid Substrate Kit. After the reaction, the slides were washed in TBS again and coverslipped using glycerol.

A few frozen sections were counterstained with haematoxylin. The protocol started with rinsing of slides in TBS, prior to immersion in distilled water for 5 minutes. The slides were then immersed in haematoxylin for 30 seconds. Immediately after that, the slides were rinsed in distilled water before they were immersed in 0.2% ammonia solution for 10 seconds in order to make the sections turn blue. After rinsing the slides in distilled water, they were dried and coverslipped with glycerol.

#### **4.3.1.3 HRP ADMINISTRATION AND TIME OF SURVIVAL**

HRP administration could be done in a variety of ways. In order to decide the most appropriate way of HRP application for this study, a few methods which included pressure injection, placing HRP in powder form, and soaking of transected nerve in HRP solutions were experimented.

The specimens were kept for three selected survival periods (24 hours, 48 hours and 72 hours) to decide the most appropriate time of survival. Stained motor neurons in the transverse sections were calculated.

#### **4.3.1.3.1 PRESSURE INJECTION**

The fish was anaesthetized using MS222. 5µl of 50% HRP solution was injected using microsyringe into the caudal fin muscles located just anterior to the caudal fin rays. Injections were made repeatedly to dorsal, medial and ventral regions of the caudal fin muscles.

#### **4.3.1.3.2 PLACING TISSUE PAPER SOAKED WITH HRP ON THE MUSCLE**

Crystals of tracer, when placed on the targeted muscle, ensured a constant release of the substance to be taken up by nerve. However, HRP crystal and micropipette were not easily available. As a replacement, tissue paper soaked with HRP solution placing at the transected nerve ends for constant release of dye.

Skinning was performed on the anaesthetized fish to expose the caudal fin muscles. Tissue paper (measured 3mm X 3mm) soaked with 50% HRP solution was inserted into the muscle bundle. The wound was closed by applying tissue adhesives.

#### **4.3.1.3.3 EXPOSING TRANSECTED NERVES TO HRP SOLUTION PLACED ON A PARAFILM SHEET**

The anaesthetized fish was skinned and the superficial muscle layer of caudal fin muscles was removed to expose motor nerves. The nerves were transected and the cut ends were placed on a parafilm sheet. 5µl 50% HRP solution was placed at the transected ends for 30 minutes. After that, the wound was closed by using tissue adhesives.

#### **4.3.1.3.4 SOAKING OF TRANSECTED NERVES IN HRP SOLUTION**

Skinning and removal of superficial muscle layer was as described in 4.2.3.3. The transected nerves were massively soaked in 5µl of 50% HRP solution for 30 minutes. The wound was then closed by wrapping parafilm around it.

#### **4.3.1.4 METHOD OF FIXATION**

Followed HRP administration and the determined time of survival, the fish was sacrificed. The spinal cord was fixed by one of the two methods described below. After the tissue was treated with enzymatic reactions, number of stained motor neurons was calculated.

##### **4.3.1.4.1 FIXATION BY IMMERSION**

The vertebral column containing spinal cord was immersed in 10% NBF (neutral buffered formalin) for 30 minutes. A few holes were punched on the vertebral column to facilitate the process of fixation. After that, the abstracted spinal cord was post-fixed in 10% NBF for two hours before it was stained.

##### **4.3.1.4.2 INTRACARDIAC PERFUSION**

The anaesthetized fish was perfused intracardially (as described in 3.2.1). The vertebral column containing the spinal cord was dissected out and the dorsal part of the vertebral column was removed prior to immersion in 10% NBF. After 30 minutes, the abstracted spinal cord was post fixed in 10% NBF for two hours. The spinal tissue was then treated with enzymatic reactions.

##### **4.3.1.5 TISSUE THICKNESS**

After fixation, the tissue was immersed in 30% sucrose solution that acted as cryoprotector to reduce the formation of ice crystal in spinal tissue. The spinal cord block was mounted on the stage holder using OCT compound. It was then sectioned at 20 $\mu$ m and 50 $\mu$ m thick using a cryostat.

#### **4.3.1.6 SLIDES STORAGE**

The frozen sections were left to be air-dried completely before they were kept in the fridge at -20°C. The slides were divided into three groups where they were kept for 1 day, 3 days and 1 week before they were treated with enzymatic reaction.

#### **4.3.2 TRIAL ON FLUORESCENT DYE**

Lucifer Yellow is known as a dye with low molecular weight which makes it an excellent dye to mark nerve cells. Because of its fluorescent properties, every step in the protocol had to be cautiously carried out in order to avoid the exposure of the dye and tissue to the light.

##### **4.3.2.1 LUCIFER YELLOW ADMINISTRATION**

The fluorescent dye was administered into the spinal nerves as described for HRP administration. The transected spinal nerves were exposed to 10% solution of Lucifer yellow for duration of 30 minutes.

##### **4.3.2.2 TISSUE PROCESSING**

The vertebral column containing spinal cord of the related segment was immersed in 10% formalin to start initial fixation. Spinal cord was then abstracted and post-fixed in 10% formalin for 2 hours, followed by dehydration in alcohol solutions of ascending concentrations (see appendix). The spinal cord was cleared in methyl salicylate before it was mounted using Canada balsam. Throughout the whole process described above, the scintillation vial containing the spinal cord tissue was covered by foil paper in order to avoid exposure to light.

##### **4.3.2.3 SLIDES VIEWING**

The slides were inspected using Confocal microscope.

## **4.4 RESULTS AND DISCUSSION**

### **4.4.1 HRP HISTOCHEMISTRY**

#### **4.4.1.1 COMPATIBILITY OF REAGENTS**

In the test performed, when HRP solution was reacted with mixture of solutions from DAB Enhanced Liquid Substrate Kit, precipitation was immediately observed on the filter paper. When tested with HRP of different concentrations, precipitation with different colour intensity showed up. The more concentrated the HRP solution, the darker was the colour of the precipitation.

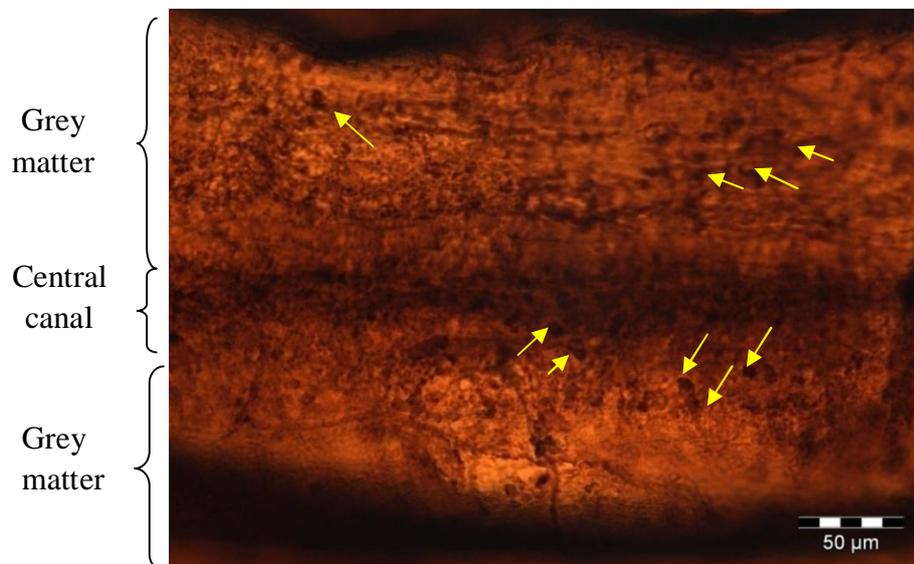
The test had demonstrated two facts. First, the reagents used which included HRP and DAB Enhanced Liquid Substrate Kit, were compatible in their reactions. The various concentrations of the different solutions had a big role to play in yielding result. For example, diluted HRP reacted with DAB to produce light coloured precipitation which could not be observed easily, especially when applied to fish neural tissue sections. It was also important to note that too high a concentration might lead to necrosis. Recommended concentration of HRP solution in the published journals ranged from 10-50% (Van der Want et al., 1997; Anderson et al., 1985; Denizot et al., 1983; Kristensson and Olsson, 1973). To yield result, this study utilised 50% HRP solution.

#### **4.4.1.2 HRP UPTAKE IN FISH NERVES**

It is an established fact that retrograde transport is possible in axon making up a nerve (Winer, 1977). In order to test the possibility of HRP uptake in fish nerve, the most direct and efficient approach was through HRP application to the ventral roots. Ventral roots contain motor axons originating from somas located in the spinal cord. In other words, HRP taken up would be guaranteed to be transported by retrograde axoplasmic flow into the neuronal soma of motor neurons (Kristensson and Olsson, 1973).

Due to the fish anatomy, the easiest approach to administer HRP through the ventral roots is at the caudal region of the fish body. However, care had to be taken since there were numerous delicate whitish lines, both motor nerves and tendons which were not easily differentiated. Although they were all observed projecting towards the caudal fin ray, most of the motor nerves, if not all, were accompanied by blood vessels. Furthermore, although both were fragile, motor nerves were easily broken. Tendons could be further verified by a simple check. If the tendon was connected to the fin ray, a gentle pull could cause movement of the particular fin ray. Extra precaution should be taken as accidentally injured nerves could result in unspecific staining.

For whole mount, with the presence of HRP in the spinal cord, the spinal cord rapidly turned to deep brown colour. This proved the sensitivity of DAB Enhanced Liquid Substrate Kit to HRP. It was important to highlight that brownish precipitation could be observed as the product of the reaction. It was the indicator of a successful HRP-DAB reaction.



**Figure 4.1:** Ventral view of longitudinally oriented spinal cord wholemount, with arrows showing some of the brown precipitations (200X).

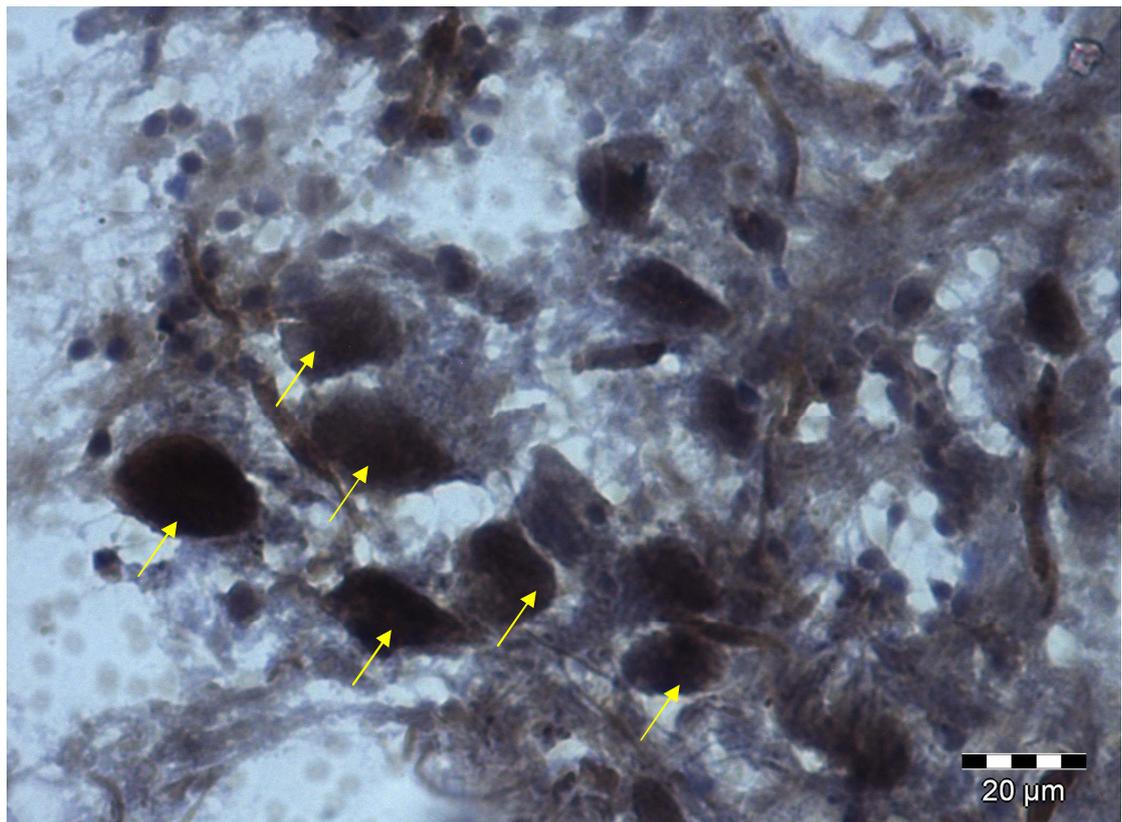
Tiny brownish dots representing brown precipitations were observed in the area of grey matter (Figure 4.1). However, due to the thickness of the spinal cord whole mount and high background staining, motor neurons could not be detected. In addition, as a result of peroxidase-diaminobenzidine reaction, blood vessels and red blood cells were also stained; thus increasing the difficulty of identifying stained motor neurons. Therefore, whole mount of spinal cord was not suitable for the investigation on motor neuron organisation. It was impossible to determine the location of motor neurons based on cell column organisation.



**Figure 4.2:** Spinal cord section with stained motor neurons at 40X magnification.

For the transverse section of spinal cord, labeled motor neurons and its location could be easily identified. Even without any counter staining, grey and white matters were delineated (as demonstrated by dashed line in Figure 4.2). It was sufficient to determine the location of motor neurons based on cell column organisation. Although

red blood cells were also stained, they could be easily differentiated from stained motor neurons based on size and shape. Motor neurons were bigger in size and had a defined neuronal shape. Counterstained sections demonstrated darkly stained motor neurons (Figure 4.3) for visualization purposes but they were not used since it was difficult to portray these motor neurons with HRP-DAB precipitation well from the background.



**Figure 4.3:** A counterstained section with darkly stained motor neurons as indicated by arrows, 400X

In sum, this experiment suggested that transverse sections were more appropriate to demonstrate labeled motor neurons based on cell column organisation. Besides that, counterstaining was not necessary as delineation of grey and white matters were demonstrated in sections stained only by mixture solution of DAB substrate kit.

#### **4.4.1.3 HRP ADMINISTRATION AND TIME OF SURVIVAL**

##### **4.4.1.3.1 PRESSURE INJECTION**

Compared to other tracer application methods such as iontophoresis and the use of HRP gelfoam or crystals, pressure injection was simpler but there was risk where the diffusion of tracer near the injection site might happen (Van der Want et al., 1997).

In this study, pressure injection did not result in any stained motor neurons.

##### **4.4.1.3.2 PLACING TISSUE PAPER SOAKED WITH HRP ON THE MUSCLE**

The tissue paper soaked with HRP solution which was placed at the transected nerve ends for constant release of dye became unmanageable and it was difficult to place it on a particular muscle. In addition, tissue adhesives hardened the tissue paper when it was applied to close the wound, hence probably blocking the release of the tracer. This protocol did not result in any stained motor neurons.

##### **4.4.1.3.3 EXPOSING TRANSECTED NERVES TO HRP SOLUTION PLACED ON A PARAFILM SHEET**

With failure of the previous protocols, this protocol aimed to increase direct exposure of the nerves to HRP in order to increase the possibility of dye uptake by the nerves. However, no stained motor neurons were observed. One of the problems encountered was that the drop of HRP solution on the parafilm sheet tended to evaporate.

##### **4.4.1.3.4 SOAKING OF TRANSECTED NERVES IN HRP SOLUTION**

Stained motor neurons were observed in the transverse sections.

Time of survival was indeed a critical factor for this experiment (Orsini and Pollock, 1991). If the animal was sacrificed too soon after HRP injection, the dye might not reach the neuronal somas in the spinal cord yet. However, if time of survival was too long, the HRP might have been degenerated. Time of survival depended on size of specimen and distance between site of dye administration and the targeted location.

In this study, it was found that highest number of stained motor neurons was observed in survival time of 48 hours as compared to 24 and 72 hours (Table 4.1).

**Table 4.1:** Number of motor neurons at different time of survival

Time of Survival (hours)	Number of Motor Neuron	
	<i>Clarias</i> sp.	<i>Pangasius</i> sp.
24	120	143
48	138	169
72	96	142

**Table 4.2:** Researchers utilizing retrograde tracing techniques

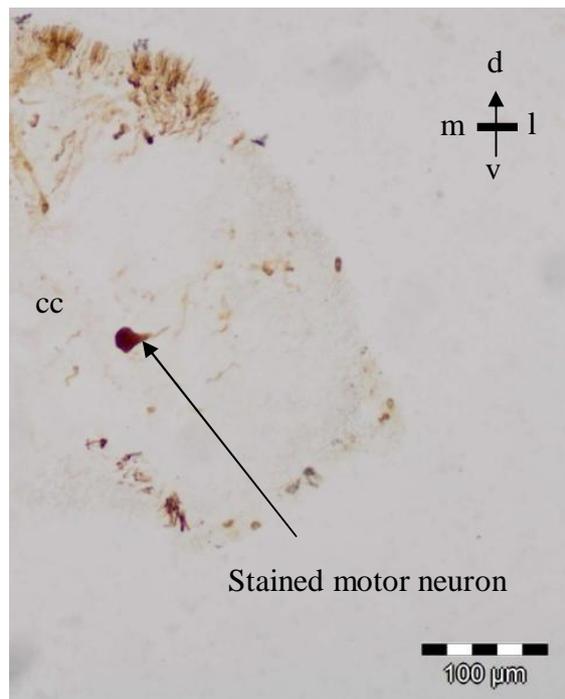
Fish	Length (mm)	Dye Injected	Labeling Site	Survival Time	Reference
Zebrafish ( <i>Danio rerio</i> )	Embryonic and larval	HRP	Spinal cord	1-5 hours	Bernhardt et al., 1990
	Adult (30-35)	Neurobiotin	Dorsal and caudal fin muscle	1-4 days	Schneider and Sulner, 2006
Goldfish ( <i>Carassius auratus</i> )	80-170	HRP	Axial musculature	4-7 days	Fetcho, 1986
Mudpuppies ( <i>Necturus maculosus</i> )	180-220	HRP		2-4 weeks	
Gadid Fish ( <i>Ciliata mustela</i> )	110-240	Carbocyanide dyes	Facial and skin nerve	3 weeks – 3 months	Kotrschal et al., 2004
<i>Sternarchus albifrons</i>	125-210	HRP	Electric organ	2-3 days	Anderson et al., 1985
<i>Gymnarchus niloticus</i>	300-800	HRP	Electric organ and lateral muscle	3-5 days	Denizot et al., 1983

According to this study (Table 4.1) and works done by others (Table 4.2), it was reasonable to select two days of survival for both *Clarias* sp. and *Pangasius* sp.

#### 4.4.1.4 METHOD OF FIXATION

Fixation must be sufficient enough to prevent the nervous tissue from degeneration, but at the same time, must not affect the performance of the tracer (Van der Want et al., 1997).

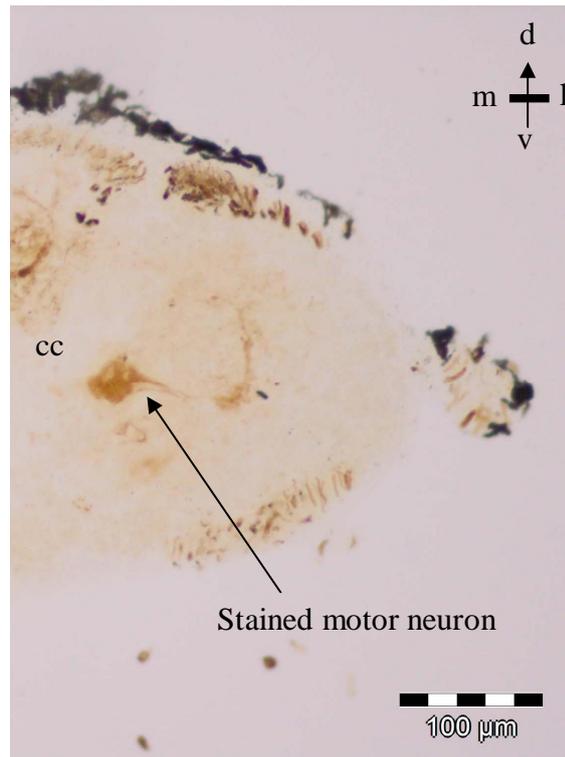
##### 4.4.1.4.1 FIXATION BY IMMERSION



**Figure 4.4:** Non-perfused spinal tissue, 100X  
(cc: central canal; d: dorsal; m: midline; l: left; v: ventral)

It was true that perfusion enabled the fixatives to reach the spinal tissue encased in the vertebral column before cell degradation was initiated by the death of specimen. However, fixation by immersion was possible in this study as the thin caudal spinal cord allowed fixatives to diffuse into tissue before degradation took place.

#### 4.4.1.4.2 INTRACARDIAC PERFUSION



**Figure 4.5:** Perfused spinal tissue, 100X  
(cc: central canal; d: dorsal; m: midline; l: left; v: ventral)

It should be noted that perfused spinal tissue (Figure 4.5) contained motor neurons which appeared to be lesser in number and lighter in colour as compared to non-perfused tissue (Figure 4.4). Other than that, non-perfused tissue did not differ much from tissue fixed by perfusion. Perfusion eliminated brownish staining of red blood cells and blood vessel formed by peroxidase-diaminobenzidine reaction since the blood was remained from the tissue before fixation process. However, in non-perfused transverse sections, these non-specific labeling did not interfere in the calculation of motor neurons. Motor neurons could still be easily differentiated from the red blood cells by its bigger size, neuronal shape and location

It should be emphasized that perfusion was more important in longitudinal sections and wholemount where the non-specific labeling looked similar as the stained axon outlining the pathways and connection of the motor neurons.

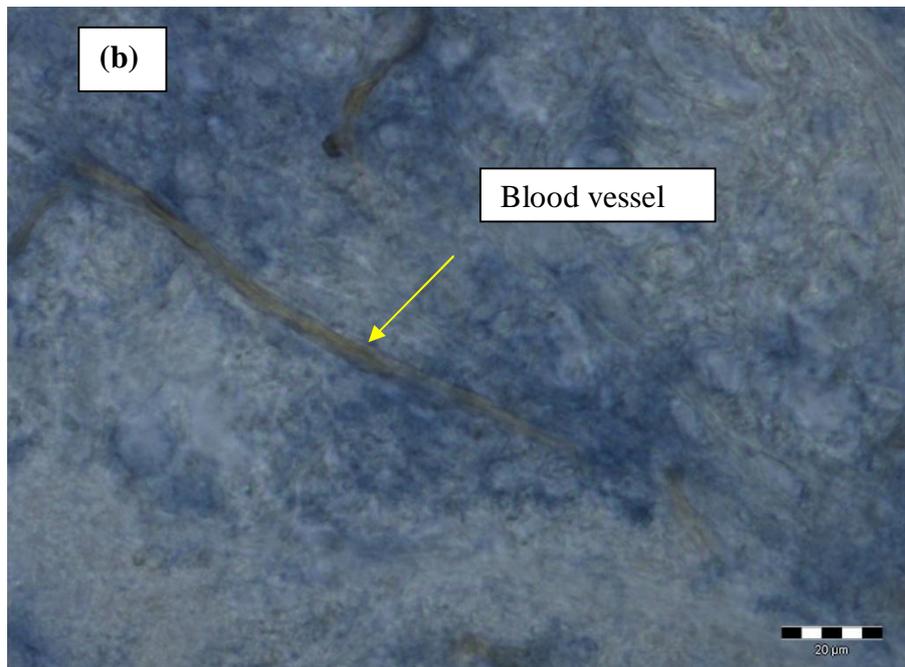
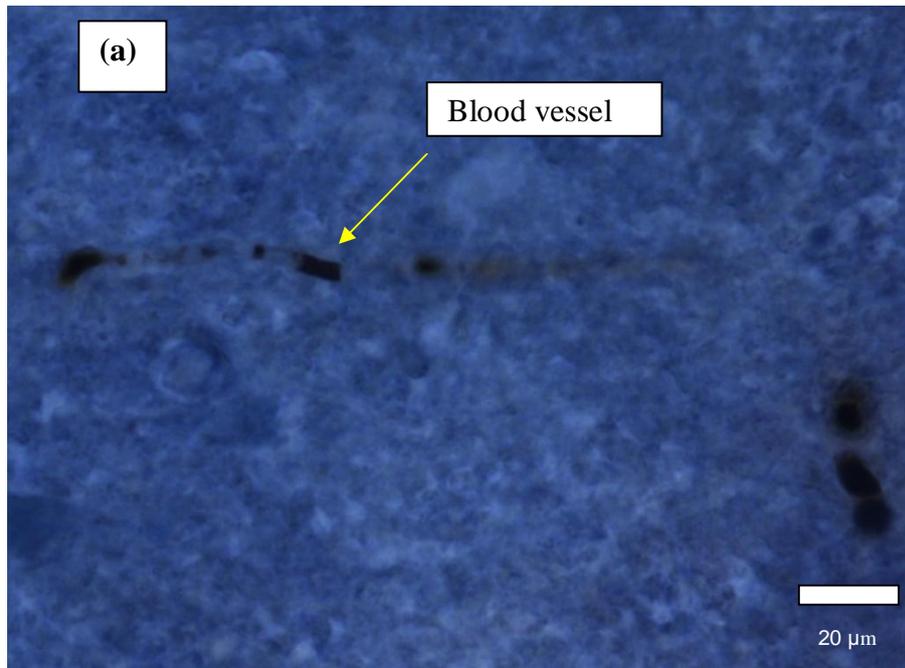
After fixation, the tissue was immersed in 30% sucrose solution. It was important to

emphasize that immersion in sucrose should be brief to prevent inactivation of HRP (Adams, 1977). It was recommended to soak the tissue until it sank in sucrose solution. In this context, the thin block of fish spinal cord usually took only 30 minutes to sink.

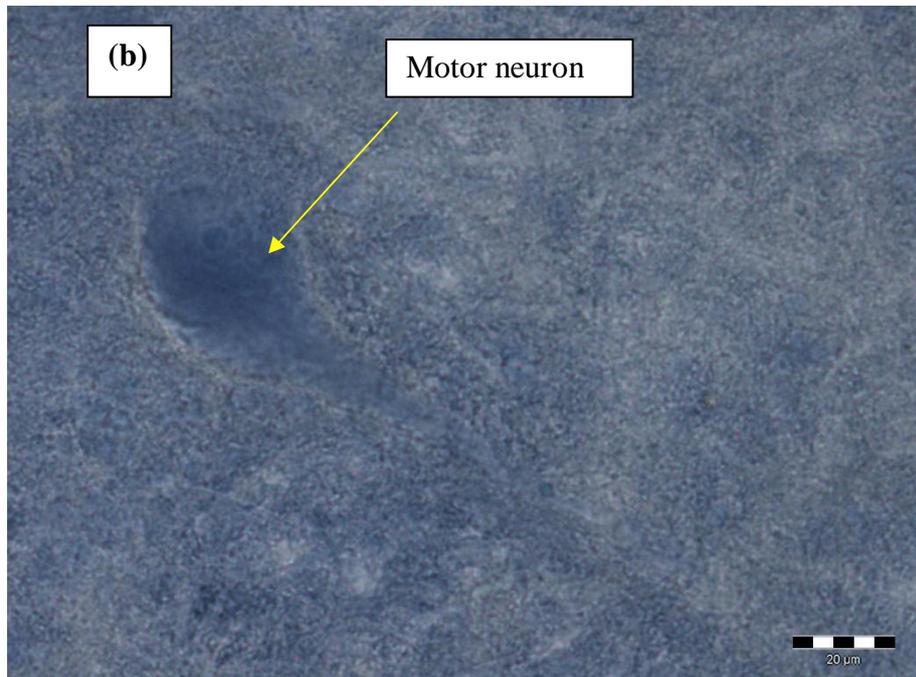
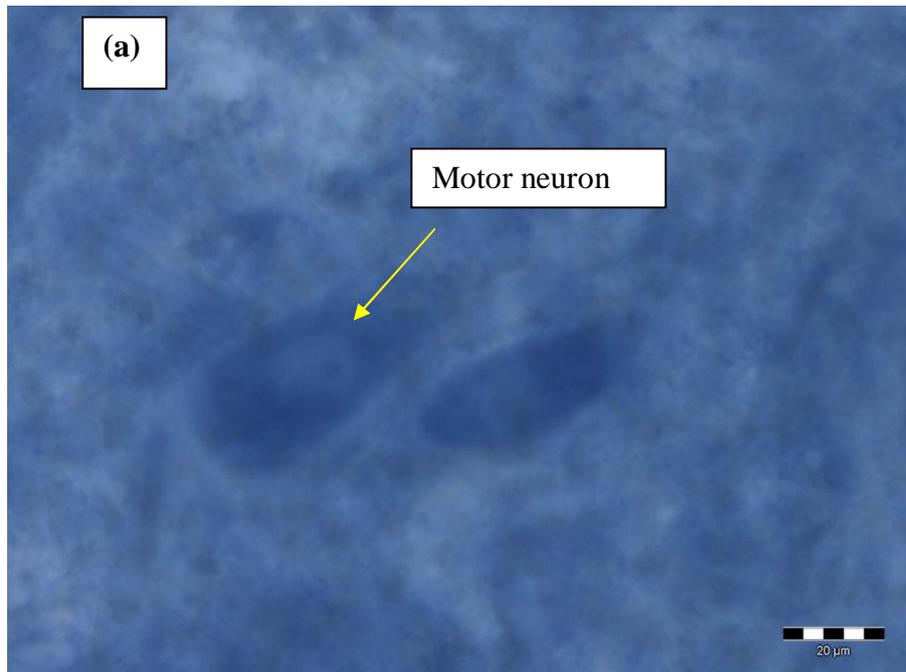
In conclusion, fixation by immersion technique was more suitable for this study taking into considerations the difficulty in perfusion technique as well as the quality and quantity of motor neurons demonstrated by it.

#### **4.4.1.5 TISSUE THICKNESS**

Various published works recommended the cryostat section thickness in the range of 20-50 $\mu$ m. It was true that the thicker the tissue sections, the more manageable they were. At 50 $\mu$ m, the sections would not curl up from the sides and thus could be mounted very nicely. However, it was difficult to focus on the thick tissue sections using microscope. The image captured was not sharp especially at high magnification (Figure 4.6, Figure 4.7). In comparison, thin sections could provide more histological information. It was important to note that too thin a section would not be possible for mounting on the slide. This might cause the loss of some important sections, especially when serial sections were needed. 20 $\mu$ m thick section was chosen in this study since at this thickness motor neurons were clearly visible.



**Figure 4.6:** The brownish blood vessel was used as indicator to demonstrate the sharpness of the picture at (a) 50 μm and (b) 20 μm, 400X



**Figure 4.7:** Motor neurons focused at 400X magnification in (a) 50 μm- and (b) 20 μm- thick tissue sections.

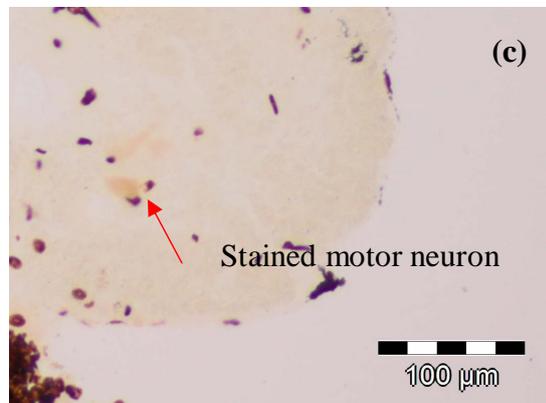
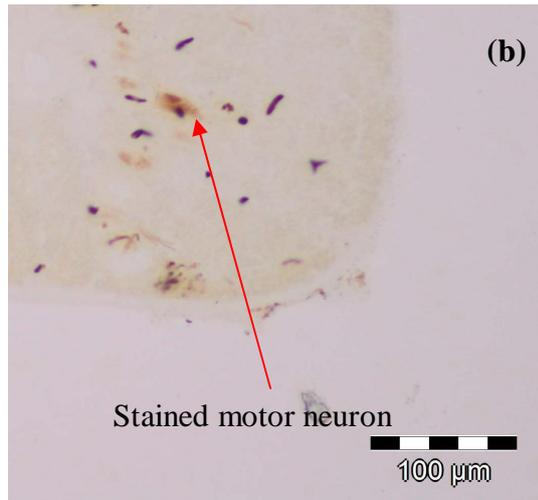
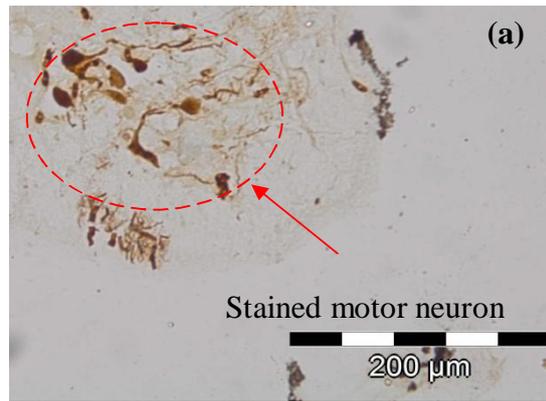
#### **4.4.1.6 SLIDES STORAGE**

The intensity of brownish precipitation in spinal sections gradually decreased after one day, three day, and one week storage (Figure 4.8).

In cases where the sections were stained immediately the next day after sectioning, the sections were well stained and brown HRP-DAB products were clearly visible.

After three days of storage, some of the brownish products remained apparent while others were less evident. Number of stained motor neurons decreased while grey matter delineation was less defined. Besides that, most of the soma shape of stained neurons could not be determined.

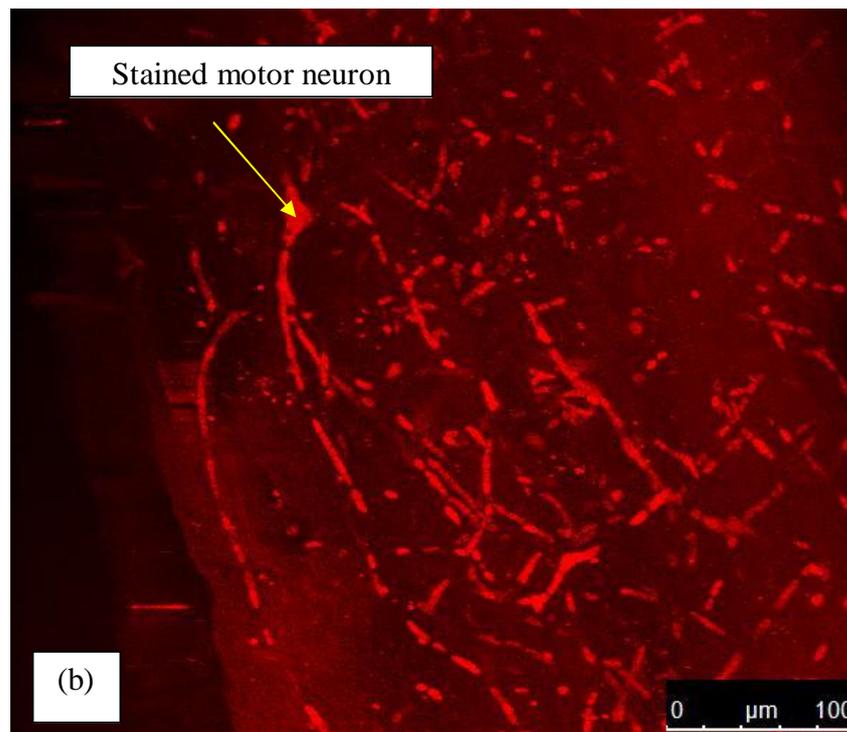
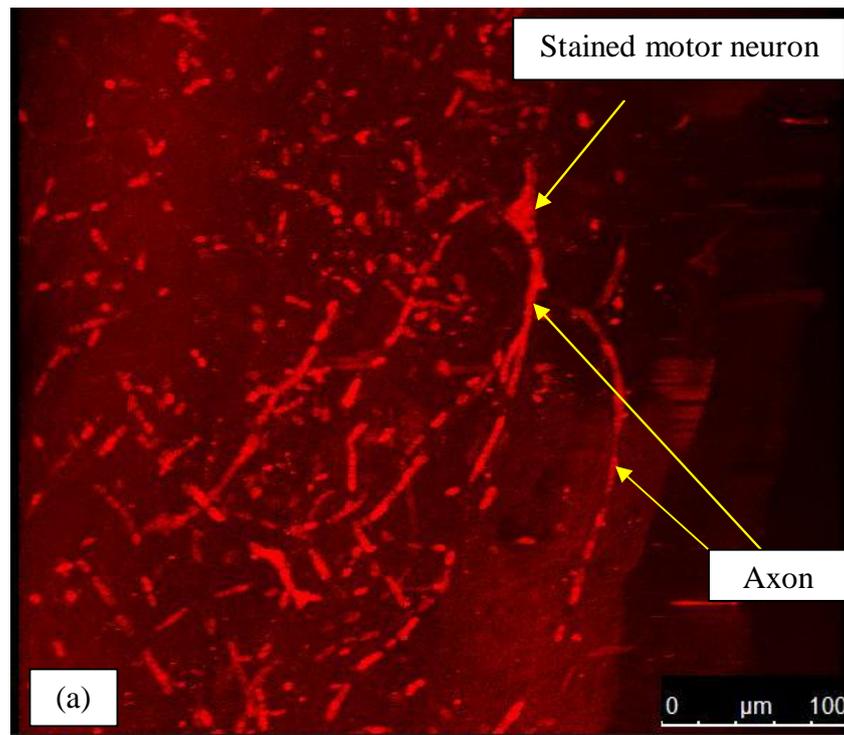
One week after the slides were stored, the number of stained neurons decreased significantly, with the neurons and grey matter delineation bearing very faint stain.



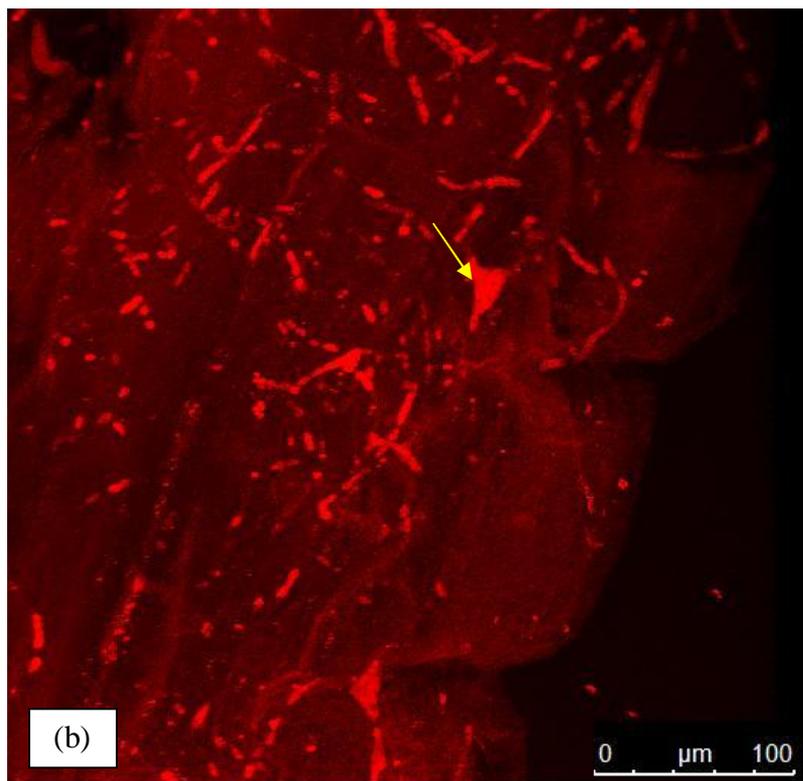
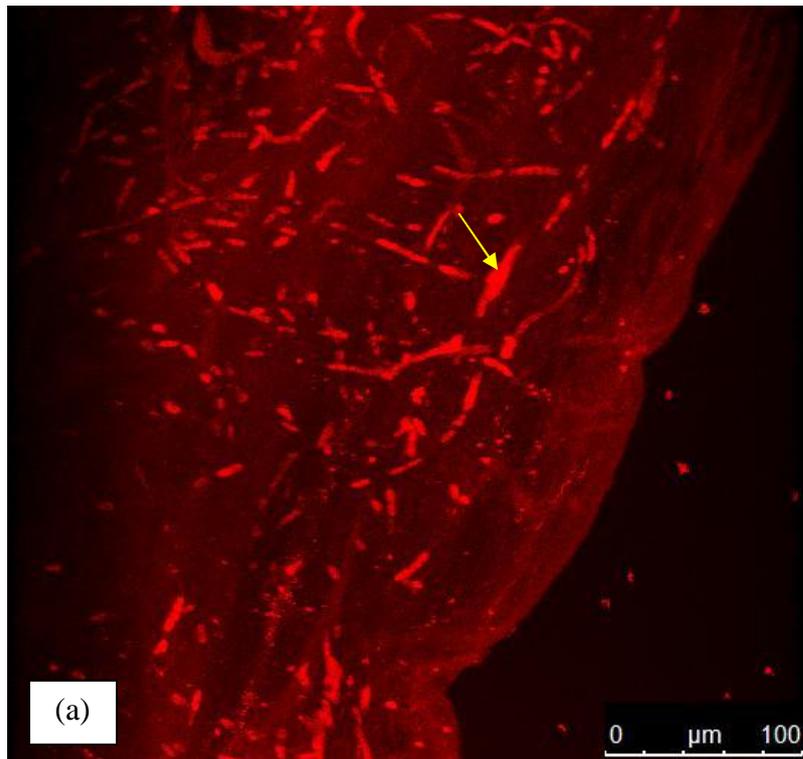
**Figure 4.8:** (a) Normal slides (40X) and gradually decreasing intensity of brownish precipitation in spinal sections after (b) 3 days and (c) 1 week storage, 100X.

#### **4.4.2 TRIAL ON FLUORESCENT DYE**

Confocal microscope allowed inspection of a particular cell from multi dimensions. A video recording of the tissue from different planes following 360° rotation was produced. In other words, the cell shape was revealed in a three-dimensionally manner with its cytoplasmic extensions as well as connection with other cells clearly outlined. Figure 4.9 and 4.10 were print screened from the videos.



**Figure 4.9 (a) and (b):** Motor neuron labeled by Lucifer Yellow viewed at different angles.



**Figure 4.10 (a) and (b):** Motor neuronal shape (as indicated by arrow) was revealed when viewed at different angles.

In this trial experiment utilizing fluorescent dye, only the morphology of the motor neurons was highlighted. The projections of the motor neuron which included the axon and dendrites were not outlined.

It was important to note that fluorescently labeled motor neurons were not solely highlighted in the spinal cord. Dotted lines that took up the fluorescent stain at the background were artifacts. To improve this, intracellular injection was recommended to enable absolute penetration of dye into the neuronal soma as well as its cytoplasmic extensions. 3-D reconstruction of motor neuron could be implemented using confocal microscope in further investigate the morphology of the cell.

Confocal microscope enabled absolute inspection on particular cells in a certain region of tissue. Hence, the technique is not suitable for this study where a large part of the spinal cord needs to be studied. Furthermore, it is difficult to determine the location of motor neurons using cell column organisation.

## 4.5 CONCLUSIONS

Protocols determined for this study are as follow:

1. HRP (Sigma type VI, USA) in powder form was used as dye while DAB Enhanced Liquid Substrate Kit was used in enzymatic reaction.
2. HRP was administered by soaking transected nerve ends into 5 $\mu$ l of 50% HRP solution for 30 minutes.
3. The fish treated with HRP was kept for two days of survival.
4. Spinal tissue was fixed by immersion using 10% NBF.
5. Spinal tissue was sectioned using cryostat at 20  $\mu$ m thick.
6. All slides must be stained after at most one day of storage.
7. HRP histochemistry was a better method for this study as compared to fluorescent technique.

## **CHAPTER 5: SPINAL MOTOR INNERVATIONS OF CAUDAL FIN MUSCLES**

### **5.1 INTRODUCTION**

Studies on innervations provide the insight into the neural basis of locomotion, taking account of the characterization and organisation of the motor system. The definition of “innervations” is the nerve supply to a specific body part. Although much has been known, scientists are still interested to delve deep into the innervations of appendages for movement. In mammals, scientists are able to identify neuronal pathway of a particular muscle with precision (e.g. Vanderhorst and Holstege, 1997; Gordon et al., 1991; Jenny and Inukai, 1983). However, researches on non-mammalians also play equally vital role in contributing knowledge to this field.

Voluntary movement, such as the fin movements of fish, is strictly governed by the nervous system. To initiate movement, impulses will travel down the axons of upper motor neurons from somas located in the brain down to the spinal cord. The electrical impulses through descending pathways then converge on specific motor neurons of the spinal ventral horn, the “final common pathway”, which innervates effector organs. New impulses generated from these lower motor neurons will initiate specific response of the fin.

Investigation on spinal motor innervations has been made easier by the application of dye in defining neuron projections and pathway. In other words, motor neurons which innervate certain muscle or organ can be conveniently traced by employing dye. To understand the control of nervous system on the movement coordination in limbed vertebrates, retrograde labeling technique was applied to the axon terminals of motor neurons innervating the locomotor appendages. Injected dye into the target muscle would be taken up by the nerve endings of motor neurons and transported retrogradely

by the axons towards the cell somas. The origin of the neuronal pathway was thus made clear.

Browsing through the researches done in the past (Vanderhorst and Holstege, 1997; Gordon et al., 1991; Stephen and Holder, 1985; Landmesser, 1978), HRP histochemistry is undoubtedly a popular technique which works on various animals. Some instances of researchers involving fish investigating the innervations of axial musculature, fin muscles, and electric organ of fish are displayed below (Table 5.1).

**Table 5.1:** Selected list of publications on motor innervations in fish

Fish	Innervations	Reference
Zebrafish ( <i>Danio rerio</i> )	Dorsal and Caudal fin muscle	Schneider and Sulner, 2006
Goldfish ( <i>Carassius auratus</i> )	Axial musculature	Fetcho, 1986
Mudpuppies ( <i>Necturus maculosus</i> )		
Gadid Fish ( <i>Ciliata mustela</i> )	Facial and skin nerve	Kotrschal et al., 1993
<i>Sternarchus albifrons</i>	Electric organ	Anderson et al., 1985
<i>Gymnarchus Niloticus</i>	Electric organ and lateral muscle	Denizot et al., 1983

This study aims at more precisely delineating the organisation of motor neuron innervating fish caudal fin muscles by using the cell column nomenclature. Besides, *Clarias* sp. with rounded caudal fin and *Pangasius* sp. with forked caudal fin would be used for comparison purpose in this study in order to investigate the correlation of motor neuronal organisation with the morphology of caudal fins.

## 5.2 OBJECTIVE

- To identify the motor neurons innervating caudal fin muscles and to characterize the organisation of these motor neurons.

## **5.3 METHODOLOGY**

### **5.3.1 HRP EXPERIMENT**

#### **5.3.1.1 HRP ADMINISTRATION**

The fish were slightly anaesthetized using tricaine methansulfonate (MS222), where anaesthetization was indicated by the lost of balance and cease of fins movement. The fish was immersed in a container filled with MS222 from the head to the gills throughout the surgery. Upon skinning and removal of superficial muscle layer, motor nerves supplying the caudal fin were exposed. These nerves were transected to facilitate maximal exposure of the nerves to the retrograde tracer later. The nerves were massively soaked in 50% HRP solution (Sigma type VI, USA) for 30 minutes to allow the uptake of tracer. Subsequently, parafilm was used to wrap around the wound and the fish was returned to the aquarium.

#### **5.3.1.2 TISSUE PROCESSING**

After 2 days of survival, overdosed anaesthetization was carried out to sacrifice the fish. The caudal part of the vertebral column containing spinal cord was excised from the tissue and fixed in 10% neutral buffered formalin (NBF) for an hour. The spinal cord was then abstracted from the vertebral column for further fixation, which took 2 hours before it was immersed in 30% sucrose solution that acted as cryoprotector to reduce the formation of ice crystal in the tissue sections. The spinal tissue was freeze-sectioned into 20 $\mu$ m-thick serial sections with a cryostat. The sections were mounted on teflon coated slides treated with poly-l-lysine and were air dried before they were kept at -20°C for enzymatic reaction on the following day.

### **5.3.1.3 ENZYMATIC REACTION**

Before the staining process, the slides stored in the freezer were first brought to room temperature. The slides were then rinsed with two changes of TBS by agitation for 10 minutes. The surrounding area of every well was blotted dry using filter paper. All steps were carried out in the incubation chamber.

For the enzymatic reaction, the 3, 3'-Diaminobenzidine (DAB) Enhanced Liquid Substrate Kit (Sigma D3939, USA) was used according to the protocol given. One drop of Solution A (DAB chromogen) was mixed with 1ml of Solution B (buffer solution containing hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>). The mixture of Solution A and B was pipetted into each well of teflon coated slides to expose the tissue sections to the mixture for one hour.

After that, the slides were rinsed as before with TBS to stop the peroxidase activity. All slides were left to dry thoroughly before they were coverslipped with glycergel.

### **5.3.1.4 NUMBERING OF SPINAL SEGMENTS**

Spinal cord segments were numbered according to their vertebral columns. For comparison purpose, the same numbering was applied on both fishes where the last segment was named V-1 and the rostral segments were named using gradually increasing numbers. The segments were measured and the spinal cord was sectioned according to the length of each segment.

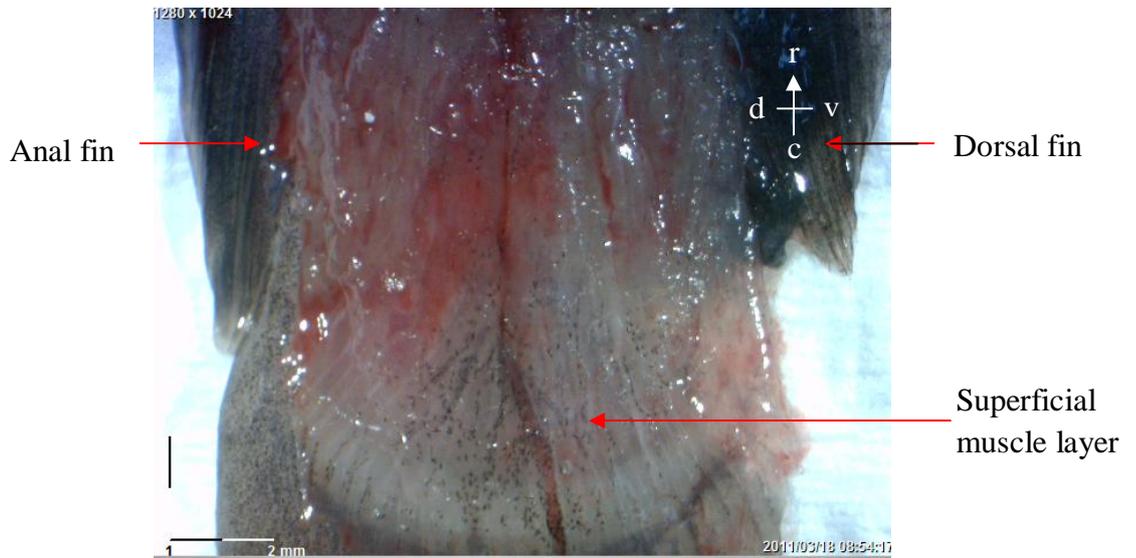
## **5.4 RESULTS AND DISCUSSIONS**

### **5.4.1 ANATOMY OF CAUDAL FIN MUSCLES**

In this study, the caudal fin muscles were named following the nomenclature used by Schneider and Sulner (2006) as well as Lauder and Drucker (2004). Like any other bony fish, the caudal fins of both *Clarias* sp. and *Pangasius* sp. were made up of superficial and deep muscle layers, which made precise control of tail movement possible (Lauder and Drucker, 2004). However, the muscle organisation of each layer was different. The organisation of caudal fin muscles was not clearly seen in photo micrograph. However, the muscle bundles could be felt and identified using the forceps. Schematic drawings would be used to illustrate the organisation of caudal fin muscles.

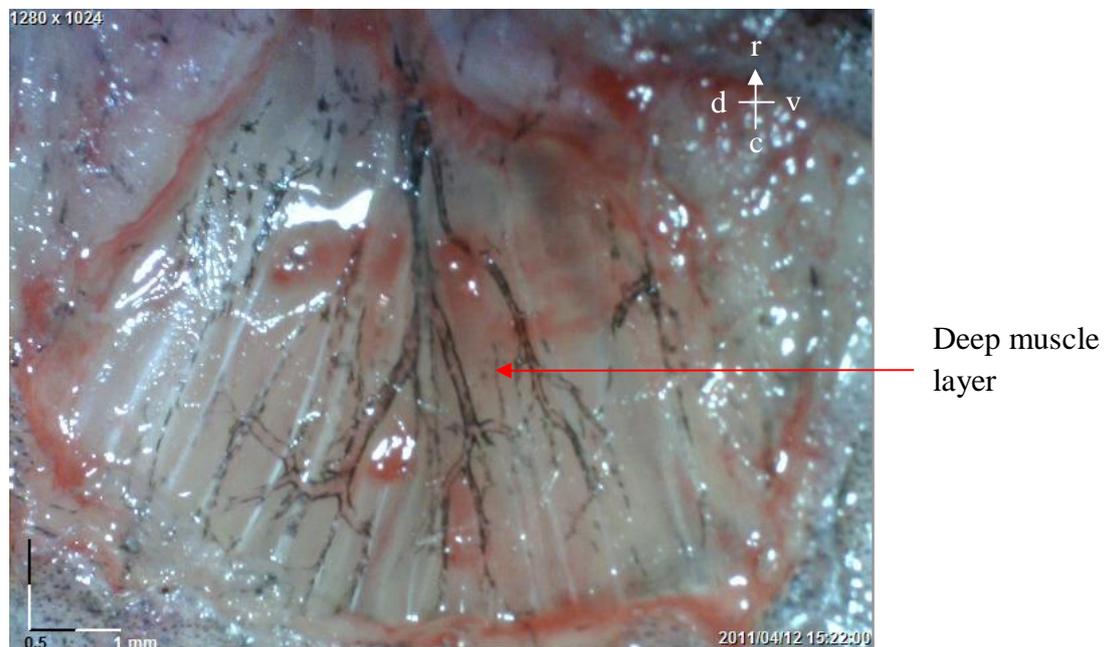
#### **5.4.1.1 *Clarias* sp. (Keli)**

At the tail region of *Clarias* sp., the lateral myotomal trunk muscles at superficial muscle layer [Figure 5.1, Figure 5.3(a)] were flattened into a thin longitudinal layer of *lateralis superficialis*, which anchored at the caudal fin rays. Numerous tendons were found at the superficial muscle layer. Two blocks of muscles at the dorsal and ventral sides of the caudal fin were known as *epaxial* and *hypaxial* muscles, respectively. They extended beyond *lateralis superficialis* and anchored at the posterior ends of caudal fin rays. Besides that, *interradialis* muscles that interconnected the caudal fin rays were also observed.

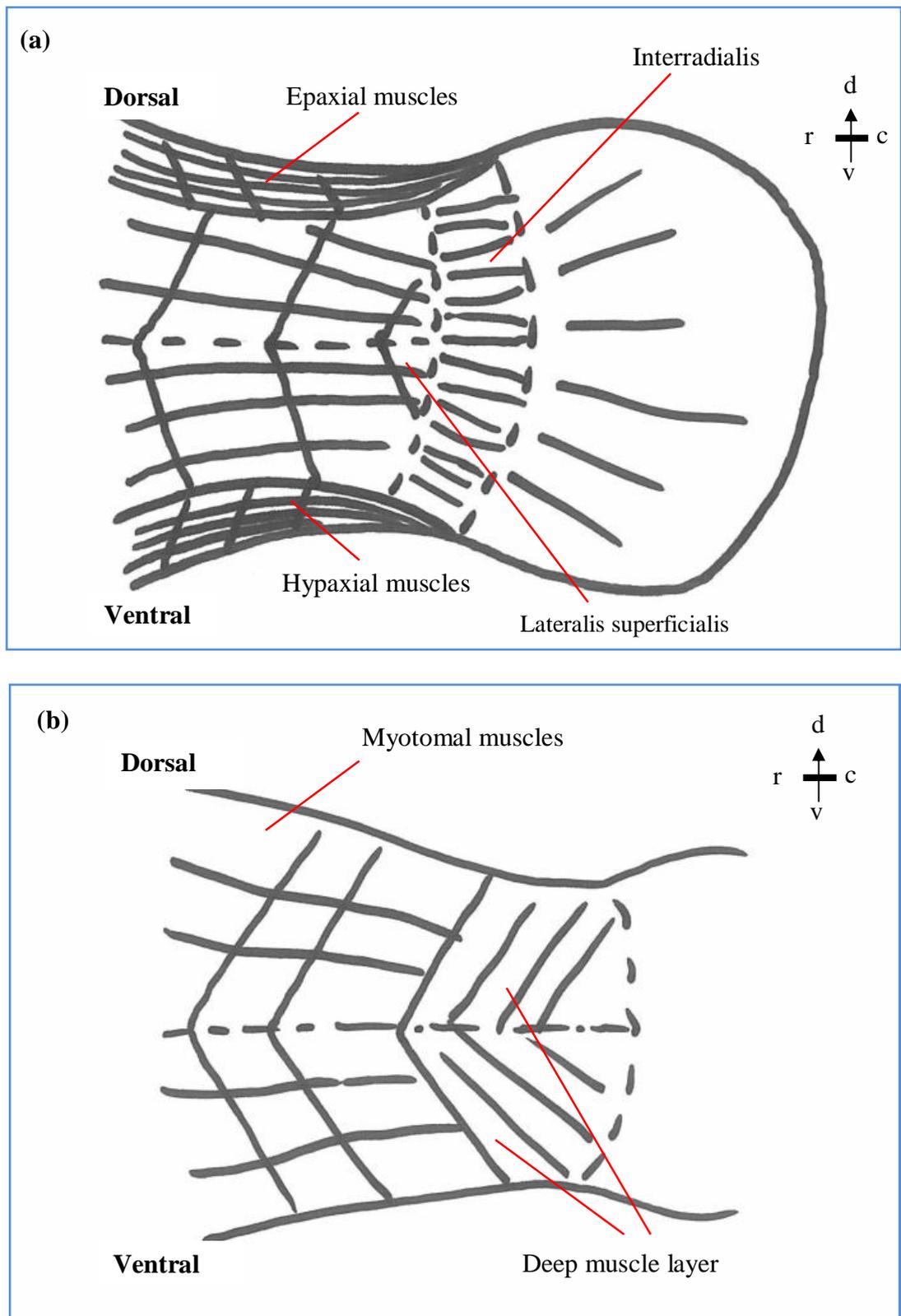


**Figure 5.1:** Superficial muscle layer of *Clarias* sp. caudal fin, 1.943X

Upon removal of superficial muscle layer, the deep muscle layer (Figure 5.2) was exposed. In the deep layer musculature, the muscles originated from vertebral column were attached to the caudal fin rays. However, this muscle layer was hardly distinguishable into muscle bundles (Figure 5.3b).



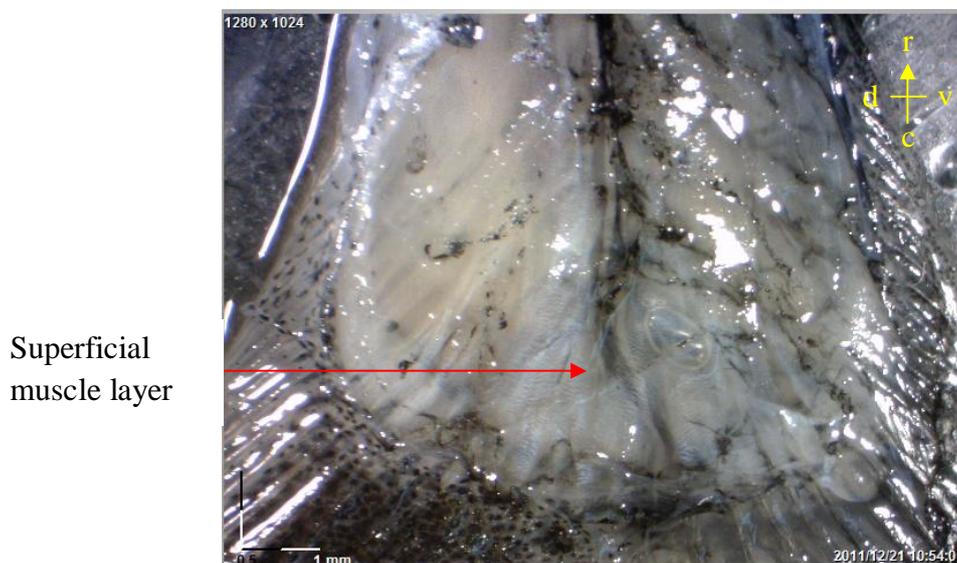
**Figure 5.2:** Deep muscle layer of *Clarias* sp. caudal fin, 2.9X



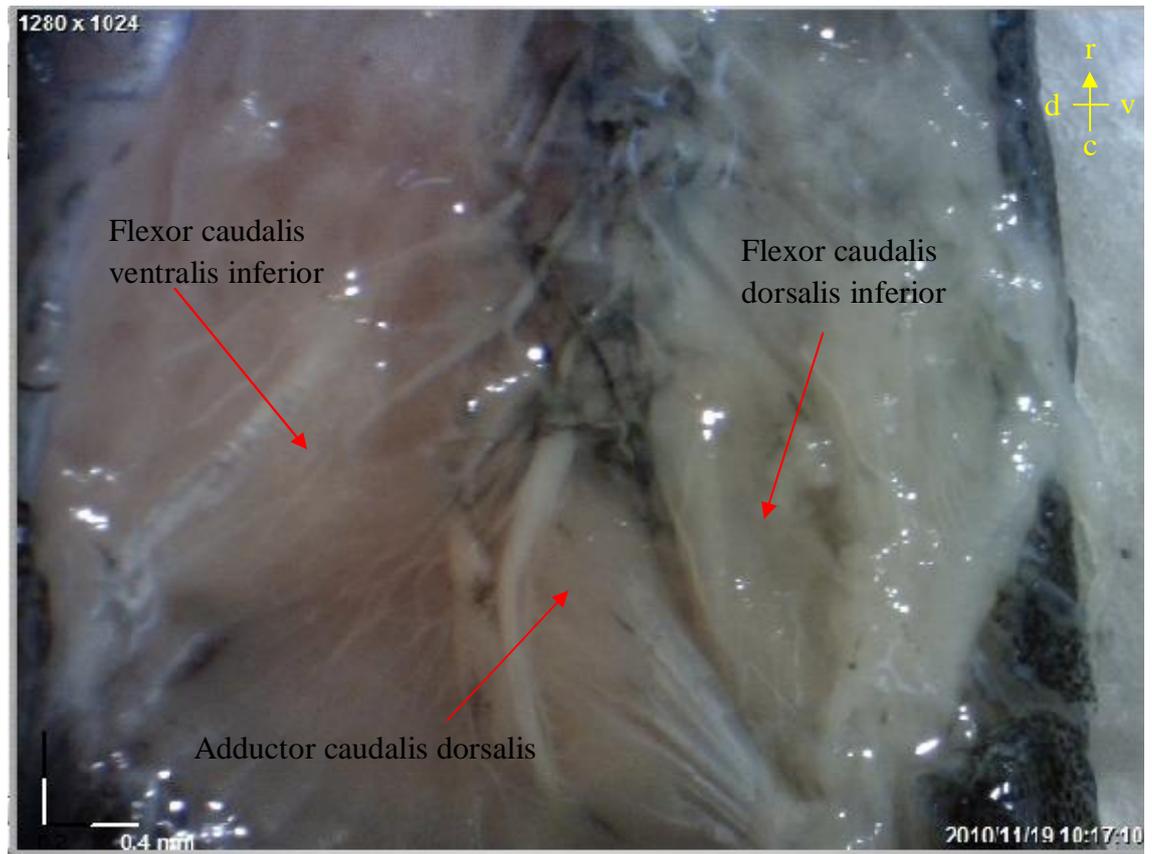
**Figure 5.3:** Schematic drawings of *Clarias* sp. caudal fin. (a) Superficial muscle layer; (b) Deep muscle layer (c: caudal; d: dorsal; r: rostral; v: ventral)

#### 5.4.1.2 *Pangasius* sp. (*Patin*)

Compared to *Clarias* sp., the musculature of *Pangasius* sp. caudal fin was more complicated. At the superficial muscle layer [Figure 5.4, Figure 5.6(a)], two blocks of muscle bundles, namely lateralis profundus dorsalis and lateralis profundus ventralis, were observed at the dorsal and ventral sides of the tail region. Their locations were similar as the epaxial and hypaxial muscles in *Clarias* sp., but they were discrete muscle bundles, which were anchored at the anterior tips of the caudal fin rays. Lateral myotomal muscles narrowed towards the caudal fin and finally attached to the caudal fin rays in the interior of lateralis profundus dorsalis and lateralis profundus ventralis. Also visible were the lateralis superficialis dorsalis and lateralis superficialis ventralis, which had a narrow insertion site beside the myotomal muscles. Interradialis muscles were also observed at caudal fin rays.

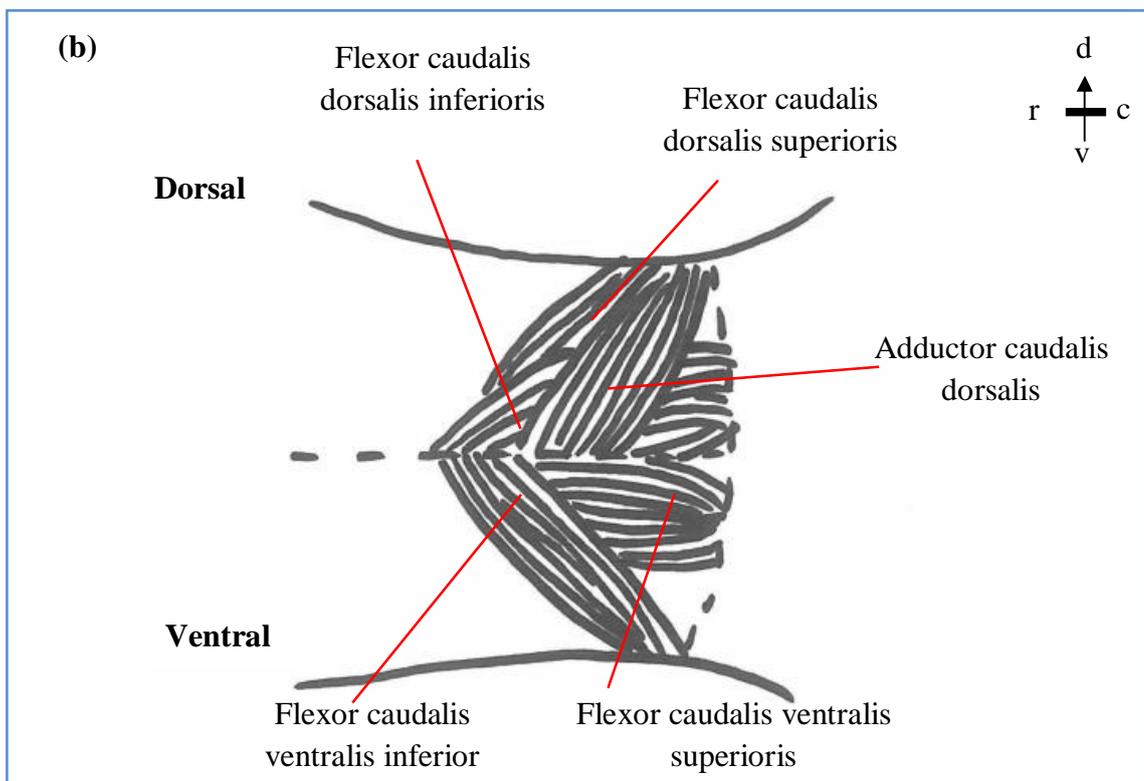
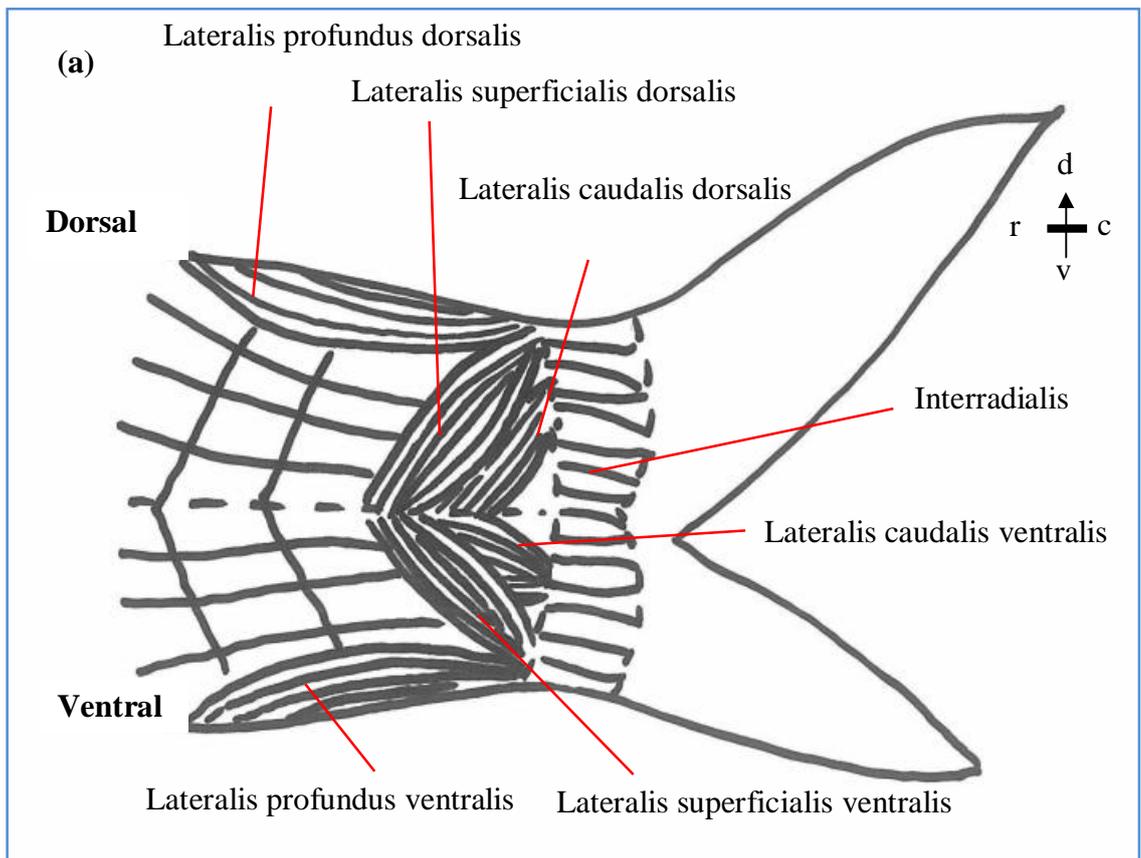


**Figure 5.4:** Superficial muscle layer of *Pangasius* sp. caudal fin, 1.943X



**Figure 5.5:** Deep muscle layer of *Pangasius* sp. caudal fin, 2.9X

Deep musculature dissection of *Pangasius* sp. [Figure 5.5, Figure 5.6(b)] revealed several distinct muscle bundles. A significant muscle bundle (adductor caudalis dorsalis) originated from the caudal end of vertebral column rested horizontally to attach to the dorsal side. Two other muscle bundles were located at the dorsal side of the caudal fin: (i) flexor caudalis dorsalis inferioris and (ii) flexor caudalis dorsalis superioris. The former had a broad attachment site while the latter narrowed towards the extreme dorsal side. At the ventral side, the flexor caudalis ventralis superioris narrowed towards the tips of caudal fin rays. Lateral to this muscle bundle was the flexor caudalis ventralis inferioris, which had a broad attachment site.



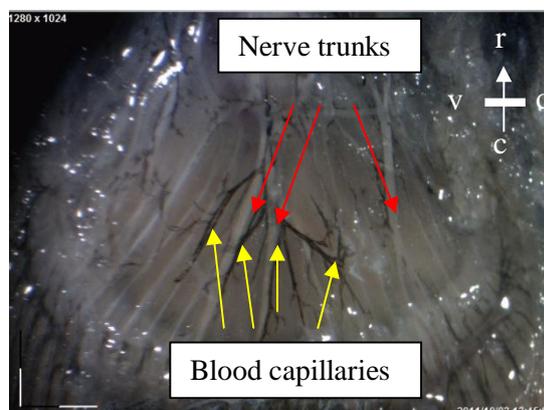
**Figure 5.6:** Schematic drawings of *Clarias* sp. caudal fin. (a) Superficial muscle layer; (b) Deep muscle layer (c: caudal; d: dorsal; r: rostral; v: ventral)

## 5.4.2 ORGANISATION OF MOTOR NERVE IN CAUDAL FIN

In both *Clarias* sp. and *Pangasius* sp., only lateral line nerve could be observed at superficial muscle layer. Removal of this muscle layer led to exposure of motor nerves supplying the caudal fin muscles. At the tail region, the nerves were arranged in such a way that rostral spinal nerves innervate lateral area while caudal spinal nerves innervate medial region. In other words, from rostral towards caudal end, spinal nerves extended from the vertebral column first project towards the lateral area and gradually projecting towards the medial region. However, it is important to note that the nerves innervating the ventral area of caudal fin located more rostral than the nerves innervating the dorsal area.

### 5.4.2.1 *Clarias* sp. (*Keli*)

Spinal nerves projected from vertebral column towards dorsal, medial and ventral region of caudal fin muscles (Figure 5.7). The nerves branched and form web-like organisation throughout the caudal fin muscles to maximize its coverage for innervations purpose. Most of the nerves travelled caudally and some of them anchored at the tips of caudal fin rays. It is important to note that the sole centrally located nerve supplying the medial region projected from the vertebral column at the most caudal region.

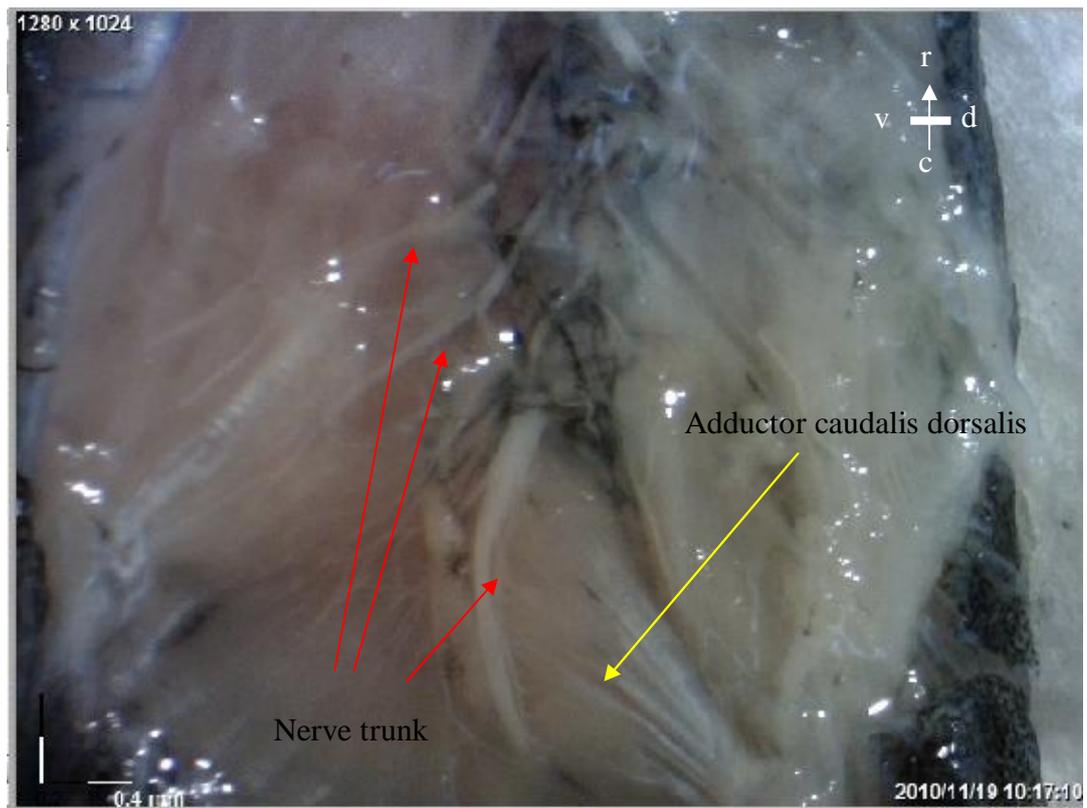


**Figure 5.7:** Organisation of motor nerves supplying *Clarias* sp. caudal fin (c: caudal; d: dorsal; r: rostral; v: ventral)

#### 5.4.2.2 *Pangasius* sp. (*Patin*)

Generally speaking, motor nerves were observed extending from vertebral column to dorsal, medial and ventral regions of the caudal fin muscles.

It is interesting to note that the only nerve supplying the medial region was bigger in size as compared to other nerves (Figure 5.8). It passed through adductor caudalis dorsalis and travelled right to the caudal fin rays. Instead of travelling straight down, it was seen extended out from the caudal vertebral column, forming a curve which twisted slightly towards the dorsal caudal fin. When observed at high magnification using stereo microscope, the nerve could be seen sending out branches into adductor caudalis ventralis and ventral area of caudal fin.

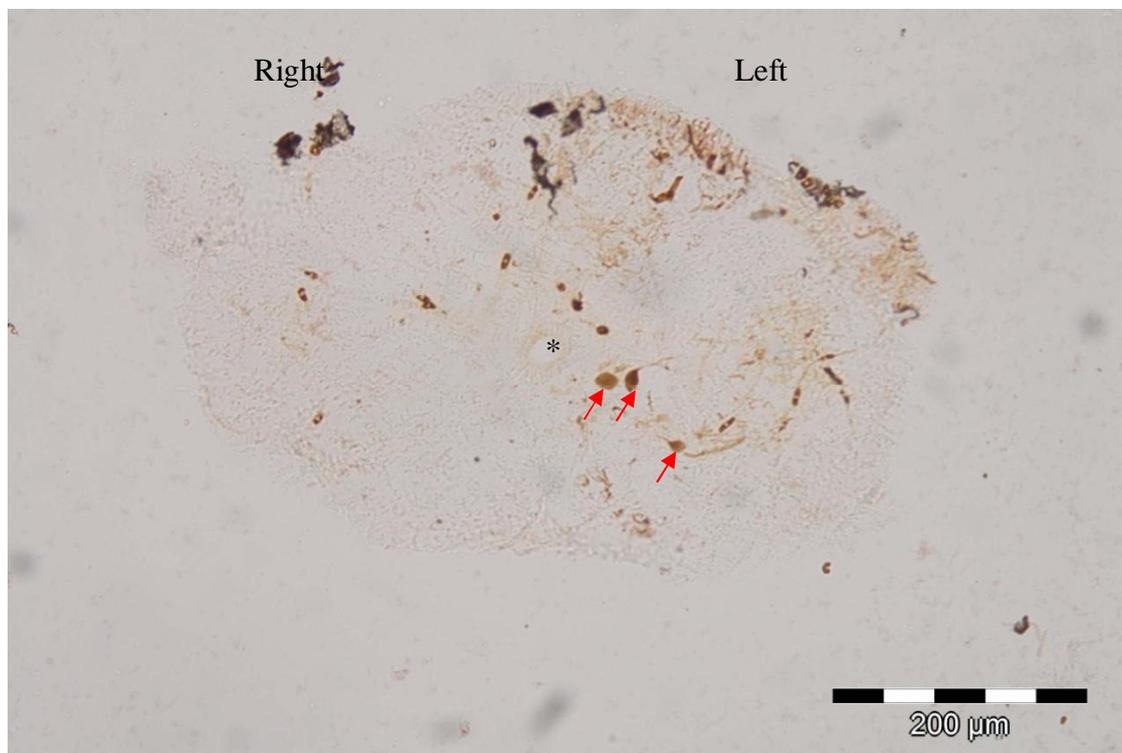


**Fig 5.8:** Nerve trunk innervating medial region of *Pangasius* sp. caudal fin, 8.7X (c: caudal; d: dorsal; r: rostral; v: ventral)

### 5.4.3 ORGANISATION OF CAUDAL FIN MOTOR NEURONS

#### 5.4.3.1 GENERAL VIEW

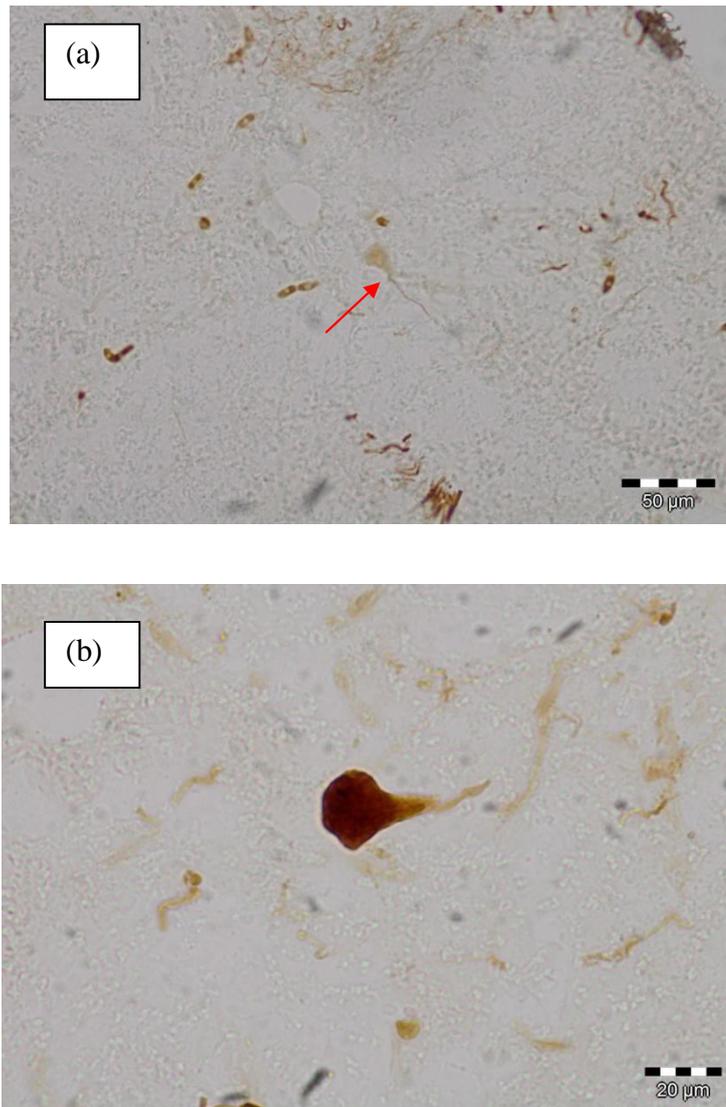
HRP-labeled motor neuronal somas were identified by the brownish product from the enzymatic reaction of HRP and chromogen. Application of HRP to the left side of the caudal fin muscles resulted in ipsilateral labeling of motor neuronal somas at the ventral horn of spinal cord (Figure 5.9). This illustrated that the axons projecting to the caudal fin muscles originated from the specific perikarya of motor neuron at the ventral horn and the axons did not cross over to innervate the other side of the body.



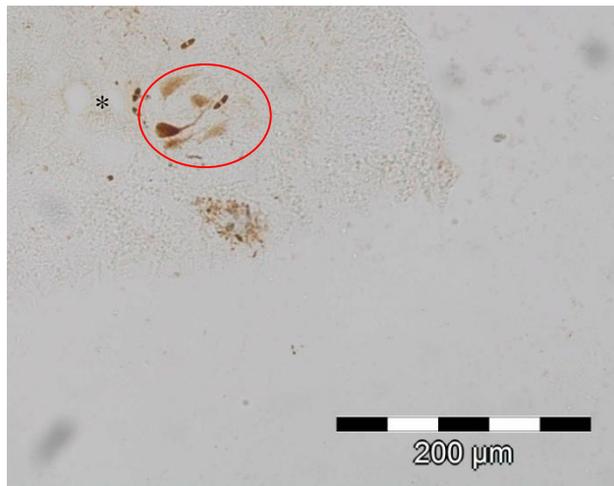
**Figure 5.9:** Ipsilateral labelling of motor neurons (indicated by arrows) where only the motor neurons at left ventral horn were stained. (\*:central canal)

The colour of HRP-DAB precipitation ranged from light to dark brown (Figure 5.10). The colour intensity varied even in a single section (Figure 5.11). In some labeled motor neurons, brownish dots representing the granules of reaction product were clearly observed. It was essential to note that all these motor neurons contributed to the

number of motor neurons innervating caudal fin muscles. In general, motor neurons located in the rostral segments especially V-5 and V-6 were always faintly stained. On the other hand, more heavily stained motor neurons were frequently found in the caudal segments (V-1 and V-2). This indicated that the innervations of the caudal fins is done by motor neurons found all the way rostrally

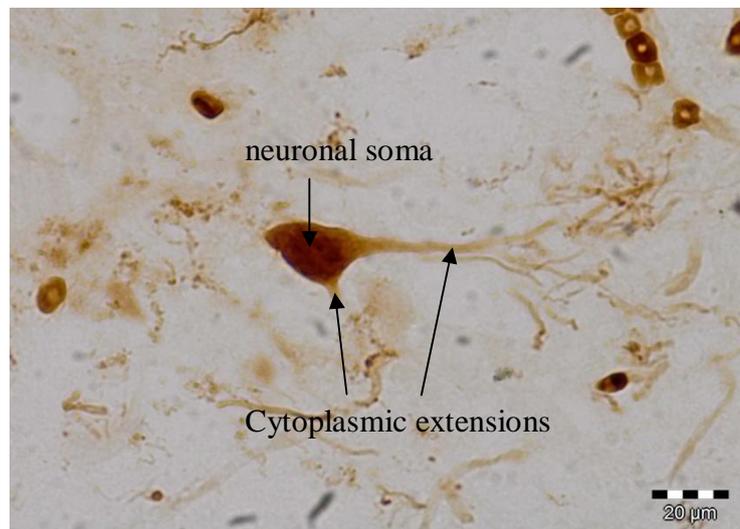


**Figure 5.10:** (a) Faint (as indicated by arrows, 200X) and (b) heavy (400X) labeling of motor neuron by HRP-DAB precipitation



**Figure 5.11:** Spinal cord section bearing HRP-labeled motor neurons (highlighted in circle) with different intensity of brown precipitation, 40X (\*:central canal)

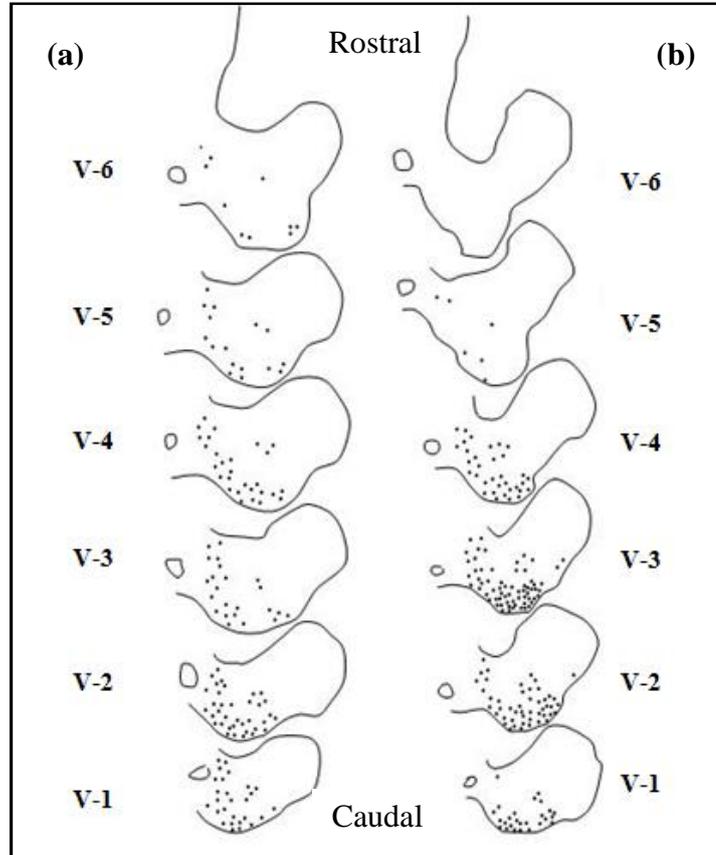
In some sections, the HRP-DAB reaction product gave good delineation on the morphology of a motor neuron. Besides the stained soma, the cytoplasmic extensions were also observed (Figure 5.12). Nevertheless, cytoplasmic extensions filled with HRP-DAB reaction product were not observed in all labeled motor neurons and thus could not contribute information on dendritic morphology.



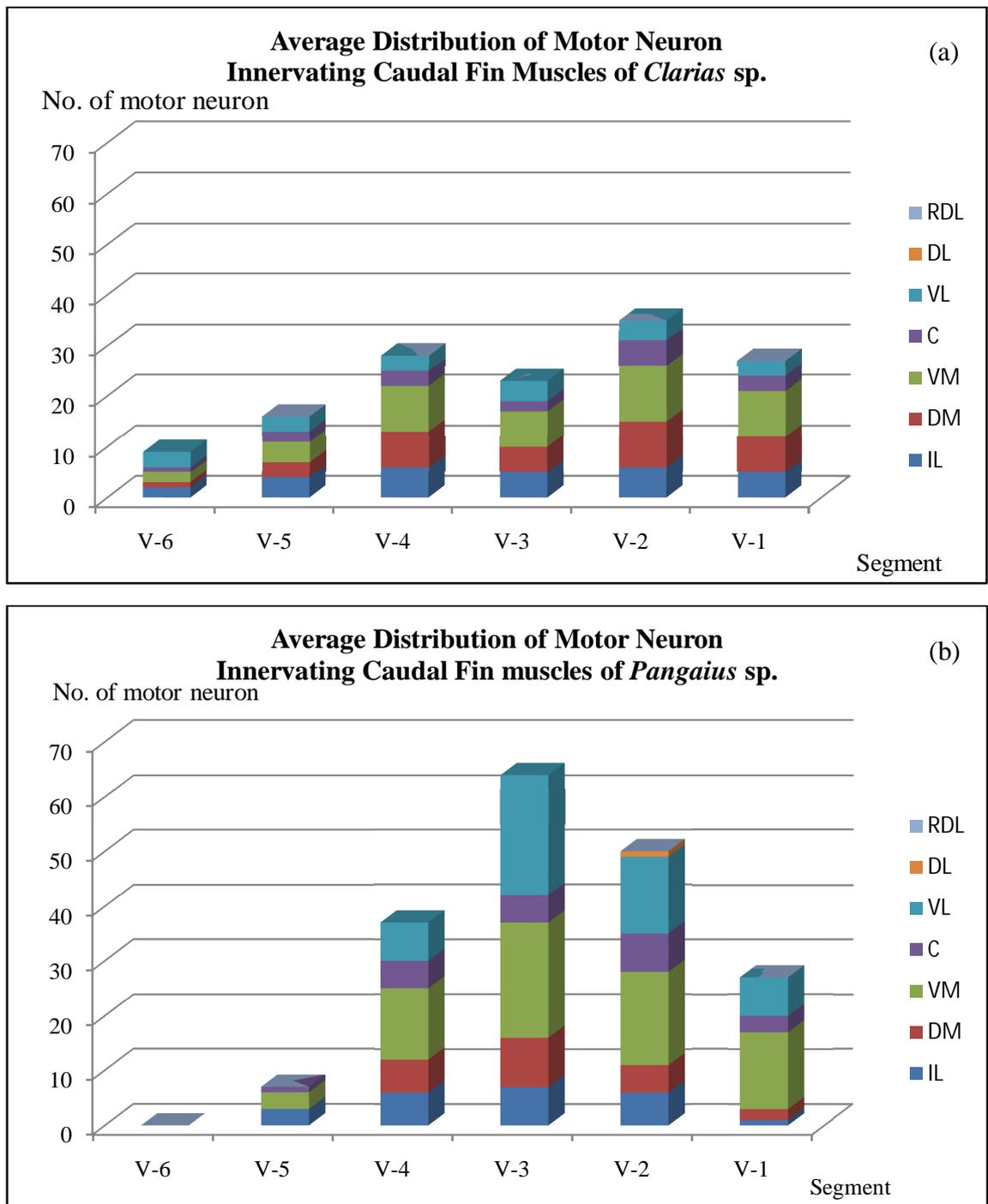
**Figure 5.12:** Cytoplasmic extensions of a motor neuron delineated by HRP-DAB reaction product, 400X

**5.4.3.2 DIFFERENCES OF CAUDAL FIN MOTOR NEURONS IN *Clarias* sp. AND *Pangasius* sp.**

**5.4.3.2.1 AVERAGE DISTRIBUTION OF MOTOR NEURON**



**Figure 5.13:** Average distribution of motor neurons innervating the caudal fin muscles of (a) *Clarias* sp. and (b) *Pangasius* sp. (n=6) at different levels (Each dot represented one motor neuron)



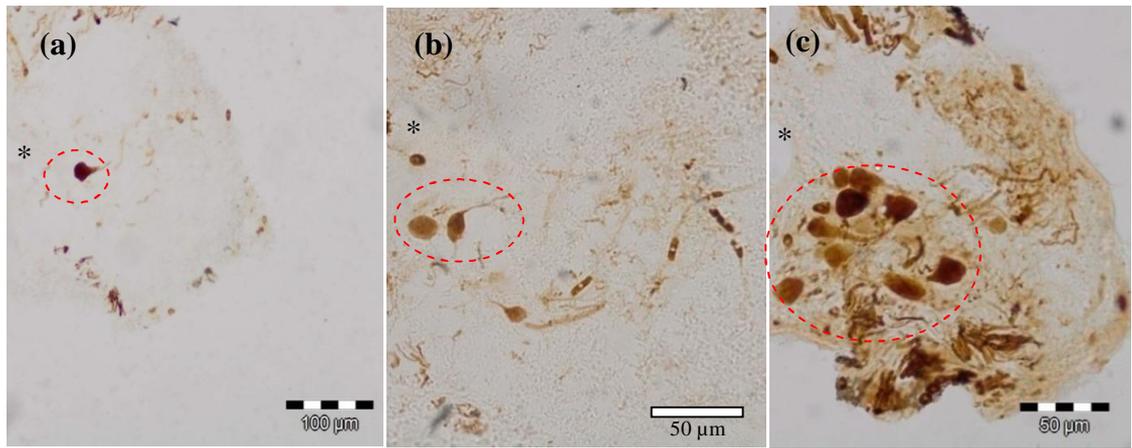
**Figure 5.14:** Average distribution of motor neurons innervating (a) *Clarias* sp. and (b) *Pangasius* sp. caudal fin muscles in accordance to cell column

Data of successful cases were obtained from six specimens for *Clarias* sp. (SL= 13.5cm, sd=0.58) and *Pangasius* sp. (SL= 13.69cm, sd= 0.44). Generally, *Clarias* sp. caudal fin was innervated by motor neurons in the last six segments of spinal cord (Figure 5.13). Two segments which were more rostrally located (C-6 and C-5) demonstrated low appearance of HRP-labeled motor neurons. Average number of motor

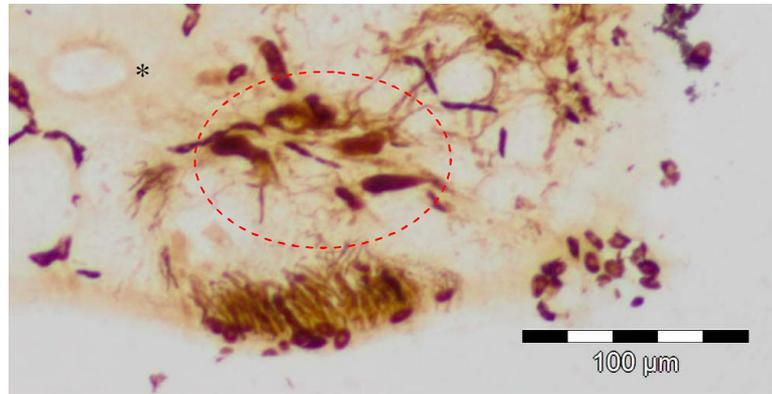
neurons innervating *Clarias* sp. caudal fin were 142.33 or approximately 142. In terms of motor neuronal organisation based on cell column classification, it was apparent from Figure 5.14(a) that most of the HRP-labeled motor neurons were located in the medial columns (IL, DM and VM) as compared to the central (C) and lateral columns (VL). For medially located cell columns, motor neurons in VM slightly outnumbered those positioned in IL and DM. Centrally located motor neurons were observed in all six segments. Besides that, a small number of motor neurons were present in VL cell column.

Motor neurons innervating *Pangasius* sp. caudal fin, on the other hand, were located in the last five spinal segments (Figure 5.13). Segment C-3 contained most number of stained motor neurons, followed by segments V-2 and V-4. Average number of motor neurons innervating the caudal fin of *Pangasius* sp. was 176.5 or approximately 176. Motor neurons innervating the caudal fin of *Pangasius* sp. were observed in all medial, central, and lateral cell columns. In segments V-1 to V-4, ventrally located (VM and VL) motor neurons far exceeded medially (IL and DM) located motor neurons. From Figure 5.14(b), it was apparent that RDL motor neurons were observed only in segments V-2 and V-3.

Motor neurons were observed to be distributed in a random way. HRP-labeled motor neurons appeared singly, in pairs or in clusters (Figure 5.15). Clusters of motor neurons were frequently observed at the caudal region of spinal cord. It is interesting to note that most of the motor neuron clusters at the caudal region of *Pangasius* sp. spinal cord were ventrally located (Figure 5.16).

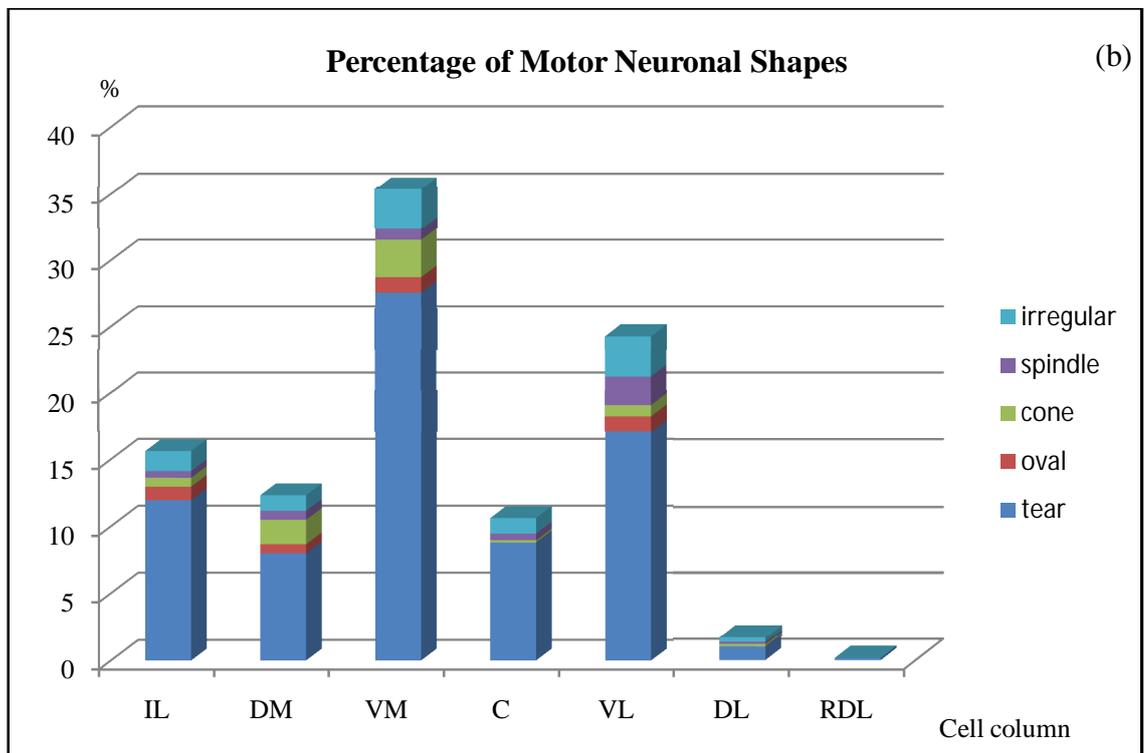
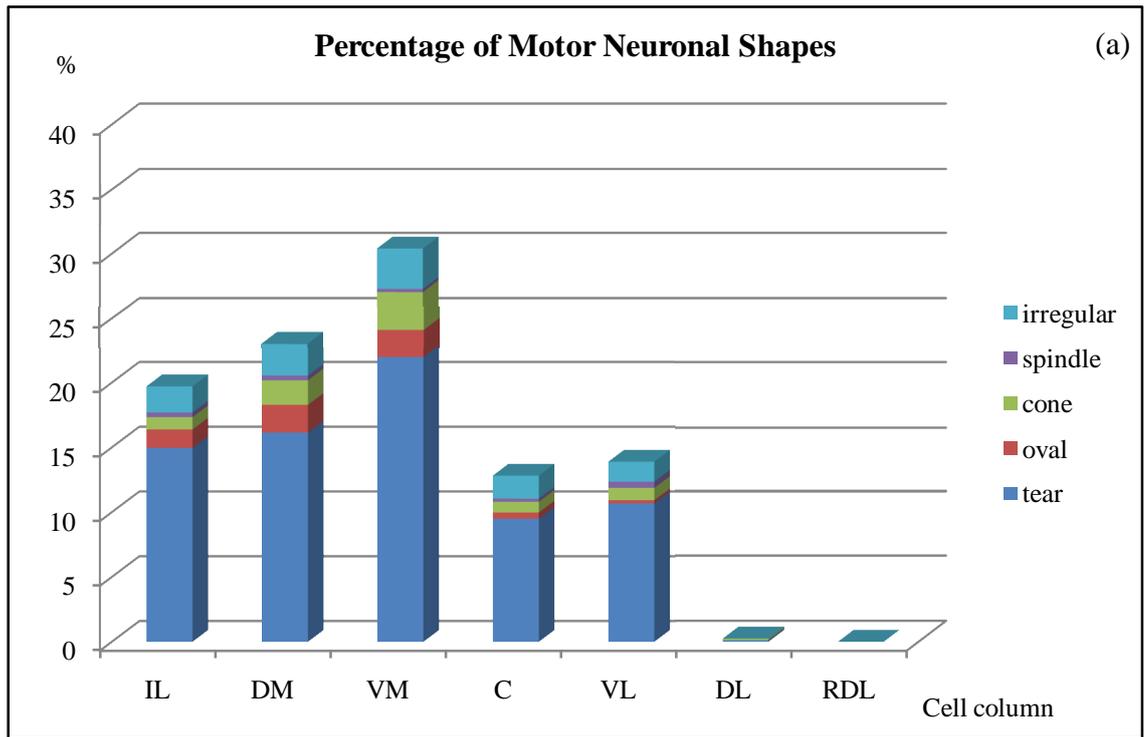


**Figure 5.15:** Motor neurons innervating *Clarias* sp. appeared (a) singly (100X), (b) in pair (200X) and (c) in cluster (200X) (\*: central canal)



**Figure 5.16:** Cluster of motor neurons in caudal region of *Pangasius* sp. spinal cord located ventrally at the ventral horn, 100X

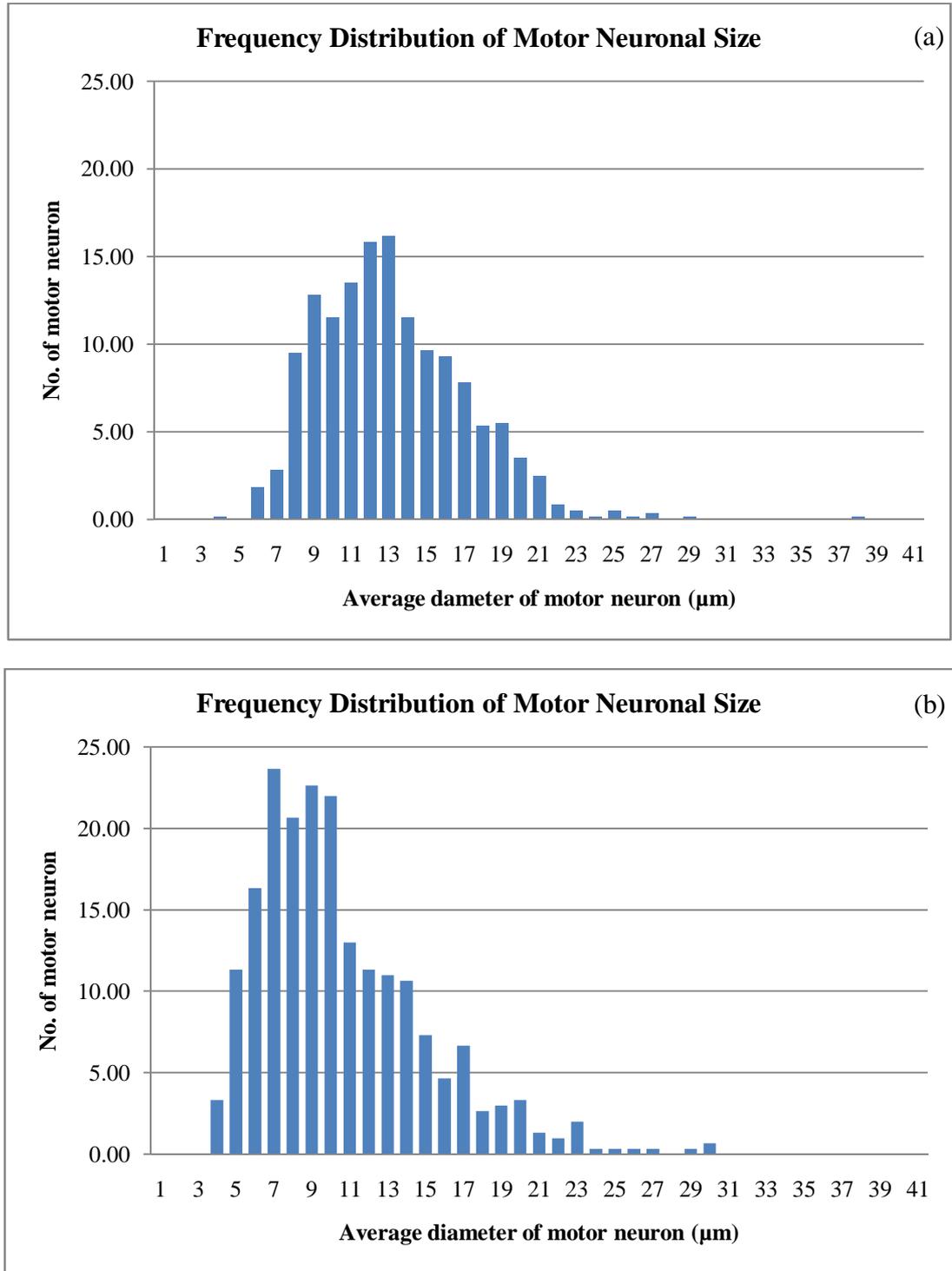
5.4.3.2.2 MOTOR NEURONAL SHAPE



**Figure 5.17:** Shapes of motor neurons innervating (a) *Clarias sp.* and (b) *Pangasius sp.* caudal fin found in different spinal cell column

For both fishes, tear shape was the dominant shape among all motor neuronal shapes (Figure 5.17). Other motor neurons e.g. oval, cone and spindle shapes, distributed in relatively low percentage at different cell columns. It is interesting to note that for *Clarias* sp., all tear shape made up the DL column. In addition, it is observed that oval-shaped motor neuron did not make up DL motor neurons at all in *Pangasius* sp.

5.4.3.2.3 SIZE OF MOTOR NEURON



**Figure 5.18:** Frequency distribution of the caudal fin of (a) *Clarias* sp. and (b) *Pangasius* sp. motor neuronal size (n=6)

From Figure 5.18(a), it is observed that the size of motor neurons innervating *Clarias* sp. caudal fin peaked at the range of 8-17 $\mu$ m, with the dominating diameter size at 13 $\mu$ m. The largest and smallest sizes of motor neurons observed in were 4  $\mu$ m and 38  $\mu$ m, respectively. Large motor neurons (>20  $\mu$ m) existed in very low quantities.

As shown in Figure 5.18(b), the motor neurons innervating *Pangasius* sp. caudal fin peaked at the range of 6-10 $\mu$ m. The largest motor neuron observed was 30  $\mu$ m while the smallest one was at 4 $\mu$ m. Only a small number of large motor neurons (>20  $\mu$ m) were observed.

The difference in motor neuronal size indicated that most of the motor neurons innervating caudal fin of *Clarias* sp. were large medial motor neurons while those innervating caudal fin of *Pangasius* sp. were small ventral motor neurons. In addition, animal age and species might be the contributory factors to the mentioned differences (Jacob, 1998).

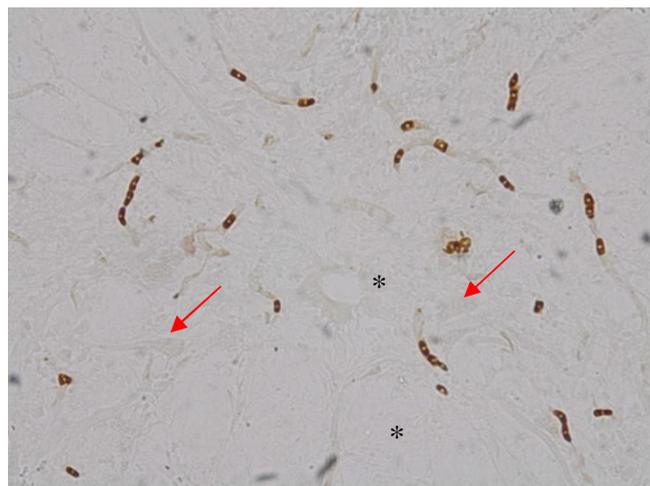
#### 5.4.3.4 NEGATIVE CONTROL

Negative control played extremely important role in confirming the validity of the results obtained from this protocol.

There were two negative controls (Negative Control I and Negative Control II) in this experiment. Negative Control I involved spinal cord tissue obtained distal from the site of HRP application. The control slides were reacted accordingly to the chromogen, like all experimental slides.

No reaction product was observed in the control slides. Motor neurons appeared to be clear from any brownish precipitation. It should be noted that the spinal cord section shown in Figure 5.19 was taken from specimen that did not undergo intracardiac perfusion. The endogenous activity of red blood cells reacted with DAB to form obvious brown precipitation. These precipitations stood in contrast to the clear colourless motor neurons.

Negative Control II involved spinal tissue in between experimental sections, but being immersed only in distilled water. The spinal tissue appeared colourless as no brownish precipitation was observed in the motor neuron as well as the red blood cell.



**Figure 5.19:** Negative Control I at 200X magnifications, with red arrows indicating the motor neurons. (\*: Central canal)

#### 5.4.4 GENERAL DISCUSSIONS ON CAUDAL FIN MOTOR NEURONS

From previous researches, scientists had successfully classified motor neurons into two categories, which included motor neurons that innervate (i) axial musculature and (ii) fin musculature. For example, axial musculature of electric fish, *Gymnotus carapo*, was innervated by large (50-75µm) periependymal motor neurons, which formed continuous columns extending along the body length and ended before the caudal region (Trujillo-Cenoz and Bertolotto, 1988). In another study where HRP was applied to the myotomal muscles of goldfish, labeled motor neurons including large dorsal cell (representing IL and DM cell columns) and small ventral cells (representing VM cell column) were observed throughout a large proportion of the medial column of ventral horn (Fetcho, 1986a).

Fin musculatures, on the other hand, were generally innervated by motor neurons that originating from the ventral portions (VM and VL cell columns) of the spinal grey matter. The dorsal fin motor neurons of lamprey were reported to be located at the lateral column (VL column) (Shupliakov et al., 1992). Similar organisation was observed in the innervations of dorsal fin of zebrafish, *Danio rerio* (Schneider and Sulner, 2006), and anal fin muscles of *Gymnotus carapo* (Trujillo-Cenoz and Bertolotto, 1988).

Pectoral fins as appendicular musculature appeared to be similar in location as forelimbs of mammals. They displayed unique skeletal musculature as compared to the median fins. Limb muscles were innervated by motor neurons in lateral column. However, the pectoral fins of *Gymnotus carapo* were innervated by motor neurons located in the ventral most region, namely VM and VL cell columns (Trujillo-Cenoz and Bertolotto, 1988).

This study demonstrated that caudal fin muscles were innervated by both medial and lateral motor neurons. This is most likely attributed to the unique musculature of caudal

fin, which comprises two muscle layers.

Motor neuronal organisation could also be investigated from the types of muscle fibers that they innervate. To date, there is no a standard classification of caudal fin muscle into red and white fibers. However, for axial musculature, it is a known fact that the superficially located red muscles are functionally different from the white muscles located underneath (Winterbottom, 1974). The red muscles facilitate slow movement while the white muscles are involved in fast swimming movement. Topographical distribution of motor neurons innervating these muscles was also different; the white muscles were innervated by large dorsal motor neurons and small ventral motor neurons (Fetcho, 1986).

Although the nerve trunks applied with HRP were located in the deep muscle layer, branches from these major nerve trunks also travelled to the superficial layer. Therefore, the labeled motor neurons in this study represented motor neurons innervating the caudal fin at both muscle layers.

#### 5.4.5 DISCUSSIONS ON DIFFERENCES OF CAUDAL FIN MOTOR NEURONS IN *Clarias* sp. AND *Pangasius* sp.

Caudal fin motor neurons of *Clarias* sp. and *Pangasius* sp. exhibited some differences which included their distribution into cell columns. Caudal fin motor neurons of *Clarias* sp. heavily resided in the medial cell columns (IL, DM, and VM) while high density of motor neurons innervating caudal fin of *Pangasius* sp. were viewed in ventral cell columns (VM and VL).

To clarify the differences, the fishes could be studied in terms of the caudal fin musculature and tail movement.

Caudal fin muscles of *Clarias* sp. were hardly distinguishable into muscle bundles while two muscle layer of *Pangasius* sp. caudal fin was made up of discrete muscle bundles. Judging from the musculature differences of both *Clarias* sp. and *Pangasius* sp., it is possible that the two caudal fin muscle layer of *Pangasius* sp. had undergone further differentiation. It is important to note that motor neurons occupied the ventral horn in an 'inside-out' sequence as proposed in previous studies (Yoshida et al., 1999; Myers, 1985; Van Raamsdonk et al., 1983). Primary motor neurons first occupied the dorsal region of ventral horn near the central canal, followed by secondary motor neurons located ventrally to the primary motor neurons. Muscle differentiation may cause the existing motor neurons to break into different cell columns, resulting in more ventromedially and ventrolaterally located motor neurons.

Caudal fin of *Clarias* sp. moved with undulation of the body trunk with the soft fin rays of caudal fin enabling flexible movement, such as expanding and minimizing the tail. *Pangasius* sp. caudal fin was not as flexible but its lateral movement was able to generate powerful forward propulsion. In cases where the fish needed to counter act with the water, only the inner part of the caudal fin was moved. Besides that, *Pangasius*

sp. was able to tilt the caudal fin to the left or the right.

*Clarias* sp. moved mainly by body undulation and its caudal fin helped in generating propulsion. Hence, the simple organisation of its caudal fin musculature was sufficient to enable the caudal fin to move along with body undulation. Large number of medial motor neurons at the caudal fin region was to cope with the need of body undulation until the caudal part of the body trunk.

On the other hand, the caudal fin of *Pangasius* sp. was the main propulsion generator. Ventrally located motor neurons were vital in controlling fine movement of the discrete muscle bundles at the caudal fin.

## **5.4.6 DISCUSSIONS ON TECHNIQUES AND PROTOCOLS**

### **5.4.6.1 ANAESTHETIZATION**

All anaesthetization is risky and could be fatal.

The fish seemed to be very sensitive to the anesthetization. A few of the fishes experienced haemorrhage after they recovered from anaesthetization. Bleeding under the skin was observed to start from the caudal fin and gradually spread towards the head. Apart from that, obvious bleeding could also be observed at the base of the fin ray. This was most possibly due to the overdose of the anaesthetic. The haemorrhage may be due to congealed blood clogging the gills. When this happened, the fish usually had problem recovering and would eventually die.

In order to reduce the risk of fatality in anaesthetized fish, stress for the fishes was reduced. For example, handling of the fish was reduced to the minimum. A highly stressed fish had higher resistance to anaesthetic agent, thus requiring more MS222 to achieve desired state of anaesthetization, and thus could lead to overdose of the fish.

### **5.4.6.2 CARCINOGENICITY OF DAB**

DAB is a potential carcinogenic agent and thus was treated with care. Basic protections such as gloves must be worn throughout the protocol. All glassware, instruments and disposables that came into contact with DAB must be neutralized by rinsing them with Clorox. To avoid contamination of microscopes, all slides, after reacted in DAB, must be rinsed in TBS to stop DAB enzymatic reaction before proceeding on with staining protocols.

## **5.4.7 TROUBLESHOOTING**

### **5.4.7.1 TISSUE TEARING**

Tissue tearing was a common problem encountered when sectioning was done by cryostat. This problem must be minimized as fine tissue morphology was vital in data analyzing. The morphology of the tissue sectioned using cryostat was not as good as paraffin sections. The bigger the tissue, the easier it was torn when freeze-sectioned. In this context, fish spinal cord with diameter of about 20  $\mu\text{m}$  had remained intact after being sectioned.

Another critical factor contributing in getting a good section is the temperature. -20°C was the most ideal temperature for fish spinal tissue sectioning.

### **5.4.7.2 FALLING OF TISSUE SECTIONS**

Falling of tissue sections might cause the loss of some important information. To overcome this problem, the adhesion of tissue sections to the slide must be ensured.

Poly-l-lysine had effectively increased the adhesion of fish spinal tissue to teflon-coated slides. Immersion of slides in poly-l-lysine for 15 minutes was sufficient to achieve satisfactory result. Immersion of slides for prolonged period would not increase tissue adhesion.

In this study, a few cross sections of the spinal tissue were placed in each well of the slide to save the usage of slides and chemicals. In order to secure the sections onto the slides, the spinal tissue must come into contact with the slide, while overlapping of the OCT compound was allowed.

### **5.4.7.3 FALSE LOCALIZATION OF HRP-DAB REACTION PRODUCT**

Identification and calculation of HRP-labeled motor neurons were carried out with extra care to avoid mistakenly taking into consideration the motor neurons that shows false positive result. In some sections which exhibited false positive result, motor neurons demonstrated very faint brownish colour. It should be noted that at the early stage of protocol establishment, the ependymal cells lining the central canal exhibited the same brownish colour as the motor neurons.

This phenomenon took place via two routes. During the fixation process, unfixed HRP may diffuse from its site of origin to adjacent tissues (Bancroft and Gamble, 2002). Secondly, when the slides were washed in TBS before they were reacted with the chromogen, some HRP residues on the sections could possibly spread to other sections via TBS.

False localization of HRP could be overcome by fine-tuning the fixation and washing process. In this study which utilised fixation by immersion method, the fixation process was done in a rapid and efficient manner.

## 5.5 CONCLUSIONS

Motor neuronal organisation in fish did not agree with those seen in higher vertebrate as no discrete motor pools were observed. Motor neurons of *Clarias* sp. and *Pangasius* sp. caudal fin muscles occupied both medial (IL, DM, and VM) and ventral cell columns (VM and VL). Most of the motor neurons innervating *Clarias* sp. rounded caudal fin occupied the medial region while those innervating *Pangasius* sp. forked caudal fin occupied the ventral region. The different locations of motor neurons innervating *Clarias* sp. and *Pangasius* sp. caudal fin muscles may be attributed to the topographic organisation in relation to the migration of motor neurons innervating the differentiated caudal fin muscles during development.

## **CHAPTER 6: GENERAL DISCUSSIONS**

### **6.1 GENERAL DISCUSSIONS**

The behavior and morphology of the organism could act as the mirror of the organisation of its nervous system. This study utilised two selected groups of fishes with different distribution of fins to investigate the distribution and organisation of motor neurons. First group of fish, having long and continuous dorsal fins, were *C. micropeltes* and *Clarias* sp. while the second group of fish with short and non continuous dorsal fins included *M. nemurus* and *Pangasius* sp.

A distinctive feature of the first group of fish was the dorsal and anal fins which extended along the body trunk. *C. micropeltes* and *Clarias* sp. moved by body undulation to generate forward propulsion. Given the location of the long dorsal fin was along the medial plane of body trunk which was thrown into a successive series of S-shape for forward propulsion, thus, this fin had a role to play in creating thrust. Besides that, the rounded caudal fin was believed to be also involved in contributing on more thrust.

Another group of fish shared the similarity of having short and non continuous median fins which consisted of a high dorsal fin and an adipose fin. *M. nemurus* and *Pangasius* sp. exhibited carangiform locomotion, which employed caudal part of the body for propulsion. The big forked caudal fin was responsible to create powerful stroke to shift forward.

Myotomal muscles made up the fish body trunk. Therefore, spinal cord acquisition from rostral, medial or caudal part of the fish body resulted in high percentage of motor neurons was distributed at the medial columns (intermediolateral, IL; dorsomedial, DM; and ventromedial, VM) as compared to lateral columns (ventrolateral, VL; dorsolateral,

DL; and retrodorsolateral, RDL). This was compatible with the previous findings (Trujillo-Cenoz and Bertolotto, 1988; Fetcho, 1986) that medially located motor neurons innervate trunk muscles while those laterally located innervate distal body appendages. Besides that, as previously mentioned above, all fishes used in this study moved mainly by the means of alternating myotomal muscles contraction. Hence, high percentage of motor neurons in medial cell columns was to cope with the needs of myotomal motor neurons as the main movement initiator.

A number of large dorsal motor neurons in IL and DM cell columns resembled primary motor neurons. According to Trujillo-Cenoz and Bertolotto (1988), primary motor neurons were the population of early differentiated spinal motor neurons subserving the trunk-tail movement (Herrick and Coghill, 1915; Coghill, 1913). With the emergence of appendicular appendages, another population of motor neurons differentiated at the ventral region of ventral horn, forming secondary motor neurons (Youngstorm, 1940). Primary motor neurons were differentiated from secondary motor neurons by its size and location. Large motor neurons in this study ranged from 20-37.11 $\mu\text{m}$ .

From muscle fibres intracellular recording and EMG recording, it was learnt that primary and secondary motor neurons co-activate to generate swimming locomotion. Firing of primary motor neurons activated synchronously muscle fibers from a large area while secondary motor neurons provided more localized control. Besides that, primary and secondary motor neurons were found to be involved in fast and slow swimming movement, respectively (Liu and Westerfield, 1988).

Motor neurons located in dorsolateral (DL) cell column were only observed in the spinal cord from the region of *M. nemurus* and *Pangasius* sp. dorsal fin. Both fishes had high-raised dorsal fin with the presence of a spine. In limbed vertebrates, laterally

located motor neurons innervated limb muscles with the more dorsal column (RDL) innervating extremities. As compared to fish with long and continuous dorsal fins, DL motor neurons could possibly be related to the morphology and movement that could be performed by the dorsal fin.

Motor innervations were further investigated by employing HRP retrograde tracing technique at rounded caudal fins of *Clarias* sp. and forked caudal fin of *Pangasius* sp. In addition to being different in morphology, they were also different in muscle organisation. It was revealed that caudal fin motor neurons of *Clarias* sp. heavily resided in medially located cell columns (IL, DM, and VM columns) while most of the motor neurons innervating *Pangasius* sp. caudal fin were viewed in ventral cell columns (VM and VL columns).

Besides motor neuron innervating body trunk and fin muscles, retrograde studies on *Gymnarchus niloticus*, the weakly electric fish had revealed another types of motor neurons which innervate the fish electric organ. The electromotoneurons differed from motor neurons innervating lateral muscle in terms of its location and morphology (Denizot et al., 1983). They were positioned centrally but dorsally to the central canal, in contrast to ventrolaterally located motor neurons innervating lateral muscle. Such motor neurons were not observed in this research. In addition, they were spherical in shape, with no dendritic processes.

Another type of motor neurons, the sonic motor nucleus (SMN) which innervates sound-producing-sonic muscle in Red Piranha was located at the dorsal zone and ventral area of the ventral horn (Onuki et al., 2005). Their position was thought to have associated with the synchronous or asynchronous contraction patterns of the bilateral sonic muscles (Ladich and Bass, 1998).

According to the existing findings stated above, Fetcho (1987) proposed that there was no evident topographic organisation of axial musculature motor neurons observed in anamniotes, such as fish. Instead, the motor neuronal organisation was in association with functional subdivisions. Fetcho had also put forward the idea that motor neurons in amniotes, on the other hand, were topographically organized. For example, lateral motor columns in ventral horn of rat spinal cord were distinct and well organized. According to Kitamura's classification (Kitamura et al., 1980), the ventral horn was divided into medial, ventrolateral and dorsolateral nuclei where each of ventrolateral and dorsolateral nuclei was subdivided into four groups. By assembling data from previous researches, motor neurons were somatotopically arranged such that those innervated the trunk, superficial back and chest, shoulder, arm and hand were arranged in order from medially to ventrolaterally and then dorsolaterally (Jameie et al., 2005). Similar topography and somatotopic organisation had been reported in avian (Landmesser, 1978), axolotl the salamander (Stephens and Holder, 1985), and mammals such as cat (Vanderhorst and Holstege, 1997; Gordon et al., 1991) and monkey (Jenny and Inukai, 1983).

The topographic and somatotopic organisation was credited to the origin of the muscles being innervated. It was reported by Fetcho (1987) that in mammals (Romanes, 1951) and amphibian (Lamb, 1976) that medial motor neurons innervated muscles derived from embryonic ventral muscle mass while lateral motor neurons projected to muscles derived from embryonic dorsal mass.

The major difference of motor neuronal organisation in anamniotes and amniotes, as discussed above, suggested that topographical organisation of motor neurons was absent in early vertebrates. Such organisation might occur in conjunction with the origin of amniotes vertebrates (Fetcho, 1987).

The usage of vertebrate embryos in the researches is vital as their spinal cords are relatively simple to define all neuronal elements (Roberts and Clarke, 1982) and demonstrate their connectivity. Neuroscience researches on various developmental stages are equally important. For example in angelfish larvae, its swimming locomotion was characterized by simple wriggling and twitching movement (Yoshida et al., 1996), which was governed primarily by the body axial muscles. Apparently, primary motor neurons innervate these movements as they were the only motor neurons stained at this stage (Yoshida et al., 1999).

It is however essential to emphasize that the insights into how exactly motor neurons play their parts in firing swimming locomotion requires more than just researches relating motor neuronal organisation to the organisation of muscles they innervate. The control of complex swimming movements could be looked into by multidimensional approaches. Intracellular injections into motor neurons of lamprey trunk myotomal (Wallen et al., 1985) and dorsal fin muscles (Shupliakov et al., 1992) allowed excellent illustrations of dendritic tree. This piece of information permitted researchers to reveal their input during locomotion. Based on the dendritic morphology, dorsal fin motor neurons were further categorized into Type I neurons with ipsilateral dendritic tree and Type II neurons with ipsi- and contro-lateral dendritic arborizations. Nerve terminals of *Gymnotus carapo* were studied to serve as platform to explore functional properties of motor neurons (Trujillo-Cenoz and Bertolotto, 1988).

According to Schneider and Sulner (2006), studies on the organisation and function of interneurons in correlation to the control of fish swimming locomotion were vital. This was owing to the fact that abundant reticulospinal interneurons served as an indicator that descending pathways were critical in regulating the swimming movement.

With the completion of this study, another piece of puzzle on motor neuronal organisation had been revealed. Position of motor neurons innervating fish myotomal muscles and different fins were identified. However, it is important to emphasize that developmental research ranged from motor neuronal organisation of embryo to adult fish would certainly give a clue on the cause of such organisation.

Future researches should go beyond the limitations of this research. A map of the organisation of nerves in the caudal fin muscles is to be generated in order to describe the origin and projection of the nerve in accordance to the location of muscle bundles. Further work on HRP experiment could be carried out such that the innervations of a single muscle bundle could be investigated and identified.

## **CHAPTER 7: CONCLUSIONS**

### **7.1 CONCLUSIONS**

Morphology of the fish body and the fins distribution had undoubtedly influenced the mode of swimming locomotion. Fishes with long and continuous dorsal fin which extended along the body trunk moved mainly by body undulation. On the other hand, fishes with short and non continuous median fins moved by carangiform locomotion, which utilised caudal part of the body to propulse. Function of each fin varied in different fishes, depending on the habitat and living behaviour of the fish. In all four fishes selected for this study, dorsal fin played the roles to balance the fish body and to smoothen turning movements. In addition, in the group of fish with long and continuous fish fins, dorsal fin was also needed for generating forward propulsion.

In terms of gross morphology and general histology, the spinal cords of all four fishes used in this study were similar. Diameter of the soft and whitish spinal cord gradually decreased from rostral towards the caudal end. When processed histologically, the cross section of the fish spinal cord was observed. A prominent figure of the spinal grey matter was the joint dorsal horns resulting in the lack of posterior funiculus. The ventral horns were connected by a line formed by the extensions from the ventrolateral position of the horns.

By classifying the ventral horn into seven regions which included intermediolateral, (IL), dorsomedial (DM), ventromedial (VM), central(C), ventromedial (VL), dorsolateral (DL) and retrodorsolateral (RDL) cell columns, it was found that DL motor neurons were only found in spinal cord from the dorsal fin area. Other than that, motor neuronal organisation of all four fishes was similar, with more motor neurons located in the medial column as compared to the lateral column.

HRP retrograde study enabled precise identification of motor neurons innervating the caudal fin. HRP histochemistry experiments using fish nervous tissue did not yield result as easy as mammal neural tissue. The protocol was set up step by step to determine the most appropriate protocol that yield the best result. Most of the motor neurons innervating the rounded caudal fin of *Clarias* sp. distributed at the medial column, namely IL, DM and VM cell columns. However, larger proportion of motor neurons innervating the forked caudal fin *Pangasius* sp. was located at the ventral region of the ventral horns, which included VM and VL cell columns.

Fetcho (1987) proposed that there is no evidence of topographic organisation of axial musculature motor neurons observed in anamniotes, such as fish. Instead, the motor neuronal organisation was in association with functional subdivisions for this muscle. The findings of this study encompassed innervations of the appendage muscles, specifically the caudal fins. Motor neurons innervating the simple and rounded caudal fin of *Clarias* sp. remained in the medial column, while those innervating the more extended forked caudal fin of *Pangasius* sp. were more ventrolaterally located.