Electron tomography of muscle cross- bridge by regulatory light chain labelling with APEX2

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**ELECTRON TOMOGRAPHY OF MUSCLE CROSS-
BRIDGE BY REGULATORY LIGHT CHAIN LABELLING
WITH APEX2**

MUFEEDA CHANGARAMVALLY MADATHUMMAL

SCHOOL OF BIOLOGICAL SCIENCES

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MUFEEDA CHANGARAMVALLY MADATHUMMAL

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of Doctor of Philosophy

2019

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ABBREVIATIONS

A	Actin
A.M.ADP complex	Actin-myosin adenosine diphosphate complex
A.M.ADP.Pi complex	Actin-myosin-adenosine diphosphate-inorganic phosphate complex
A-band	Anisotropic band
ADP	Adenosine diphosphate
AM complex	Acto-myosin complex
Amp	Ampicillin
APS	Ammonium persulfate
APEX2	Genetically engineered Ascorbate peroxidase
Asn	Asparagine
ATP	Adenosine triphosphate
BDM	2,3-butanedione monoxime
BME	B-mercaptoethanol
BSA	Bovine serum albumin
Ca²⁺	Calcium ions
cDNA	Complementary DNA
Chl	Chloramphenicol
CIP	Calf intestinal phosphatase
DAB	Diaminobenzidine
dNTP	Nucleoside triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELC	Myosin essential light chain
EM	Electron microscopy
ET	Electron Tomography
F-actin	Filamentous actin
FHC	Familial hypertrophic cardiomyopathy
G-actin	Globular actin
GFP	Green fluorescence protein
Glu	Glutamic acid
H₂O₂	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMM	Heavy meromyosin
HRP	Horseradish peroxidase

I-band	Isotropic band
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
K⁺propionate	Potassium propanoate
KPO₄	Potassium phosphate
Leu	Leucine
LCD	Lever arm domain (Light chain domain)
LMM	Light meromyosin
M	Myosin head
M.ADP.Pi complex	Myosin-adenosine diphosphate-inorganic phosphate complex
M.ATP	Myosin-adenosine triphosphate complex
Mg	Milligram
Mg²⁺	Magnesium cation
Mg²⁺acetate	Magnesium acetate
MgATP	Adenosine 5'-triphosphate magnesium salt
MgCl₂	Magnesium chloride
miniSOG	Mini-singlet oxygen generator
ml	Millilitre
M-line	Mittelscheibe line
mM	Millimolar
MyBP C	Myosin-binding protein-C
Myosin S-1	Myosin subfragment-1
Na₂ATP	Adenosine 5'-triphosphate disodium salt
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NMR spectroscopy	Nuclear magnetic resonance spectroscopy
OsO₄	Osmium tetroxide
PBS	Phosphate-buffered saline
PBST	Phosphate Buffered Saline with Tween 20
PCR	Polymerase chain reaction
Pi	Inorganic phosphate
PMSF	Phenylmethylsulfonylfluoride
PVDF membrane	Polyvinylidene difluoride membrane
RLC	Myosin regulatory light chain
ROS	Reactive Oxygen Species
RZ value	Reinheitszahl value
SDS	Sodiumdodecylsulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEM	Transmission electron microscopy

TEMED	Tetramethylethylenediamine
TFP	Trifluoperazine
Tm	Melting temperature
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
Val	Valine
μg	Microgram
μl	Microliter
μM	Micro molar
Z-line	Zwischenscheibe line

ABSTRACT

Muscle contraction results from the cyclic interaction of myosin and actin by coupling energy of adenosine triphosphate (ATP) hydrolysis. During muscle contraction myosin and actin interact with each other through a structure called “cross-bridge”. Despite intense structural studies, the organisation of myosin cross-bridges on myosin filaments is not well understood. Myosin regulatory light chain (RLC) is one of the prominent proteins present at the lever arm domain of cross-bridges. Phosphorylation of RLC modulates cellular functions including muscle contraction. Moreover, RLC mutation is associated with cardiomyopathy. To understand the role of RLC in muscle contraction, high-resolution structure in its sarcomeric environment is required. Currently available high-resolution structure of myosin is inadequate to understand the role of RLC. A novel electron microscopy (EM) labelling technique based on APEX2, an engineered variant of soybean ascorbate peroxidase (APEX) protein is a promising technique to resolve the structure of specific protein in its native environment. In this study, the visualisation of the muscle cross-bridge organisation using APEX2 was attained. APEX2 provides the contrast EM by oxidation of di-aminobenzidine (DAB) substrate. For that purpose, fully functional RLC-APEX2 fusion protein was exchanged into muscle fibre. RLC-APEX2 exchanged muscle prepared in the relaxed state showed good ultrastructural preservation and good EM contrast upon chemical fixation. The presence of RLC-APEX2 in myosin cross-bridges permitted us to visualise structural features by electron tomography (ET) in relaxed state. Our standardised APEX2 based EM labelling protocol could be a promising tool for the labelling of other sarcomeric proteins in diseased states, thus permitting the direct visualisation and ultrastructural organisation of muscle proteins by ET.

1. INTRODUCTION

Forty per cent of animal body weight consists of the muscular system (Frontera & Ochala, 2015). In the muscular system, striated muscle which includes skeletal muscle and cardiac muscle is responsible for body movements, posture maintenance and functioning of the circulatory system. Besides movements, skeletal muscle is vital for maintaining body temperature by metabolic activity and stores energy in the form of sugars (Wolfe, 2006).

Skeletal and cardiac muscles which are sometimes referred to as striated muscle are made up of regularly arranged contractile protein filaments to form a highly ordered structure. This highly ordered structure appears as characteristic transverse light and dark banding (striated) under light microscopy. Moreover, this highly organised structure is vital for the performance of striated muscle. While performing work, striated muscle shortens, thus achieving its function. Furthermore, muscle shortening is an ‘energetically efficient process’ (He *et al.*, 1999). The striations arise from the organisation of two types of longitudinally arranged and alternating filaments, predominantly consisting of myosin and actin. A repeating unit of filaments in each muscle cell is known as a sarcomere. During shortening, the actin and myosin filaments slide past each other and interact through a structure called “cross-bridge” or “myosin head” present in myosin, driven by ATP hydrolysis (Hodge *et al.*, 1954, Huxley & Niedergerke, 1954, Huxley, 1957, Huxley, 1969, Squire, 2016). These cross-bridge structures are formed by the interaction of myosin head and actin filament (Geeves, 1991). The ‘higher degree of structural organisation’ of muscle proteins is also crucial for the regulation of muscle contraction and force production (Månsson *et al.*, 2015). Elucidating the ultrastructural organisation of prominent proteins present in high ordered structural unit of striated muscle will help to determine their function. Over the past six decades, the macromolecular assembly of striated muscle has been studied using X-ray diffraction, electron microscopy (EM), and other microscopy techniques, producing insight into muscle contraction mechanism by furnishing near-atomic resolution structure (ultrastructure). Ultrastructure of muscle aims to answer one of the greatest quests in biology that is the molecular mechanism of muscle contraction (Geeves & Holmes, 2005, Sweeney & Houdusse, 2010) in healthy and diseased muscles. Many of the mutation in muscle proteins are associated with myopathies. To understand the role of protein in muscle contraction ultra-structure of each protein in its sarcomeric environment is required. With the current state of imaging, it is challenging to visualise the

3-D structure of specific small proteins present in the muscle in their sarcomeric environment. However, advancements in EM technology for imaging ultrastructure of organelle or protein compartment in their cellular environment at nanometer resolution by tagging proteins of interest is a promising approach. EM labelling technology aims to understand the dynamic process of proteins inside the cell (Tsien, 1998, Brama *et al.*, 2015, Yi *et al.*, 2015). Myosin regulatory light chain (RLC) is one of the smallest abundant protein presents in the cross-bridge structure. It is non-covalently bound to a region of the myosin molecule known as the lever arm of the molecular motor. The myosin protein binds to actin and produces movement in muscle by rotating its lever arm structure. Moreover, mutations in RLC protein are known to be associated with cardiomyopathy (Moore *et al.*, 2012). It was anticipated that by standardising EM labelling technique in muscle fibre and elucidating the ultrastructure of RLC in cross-bridge will help to identify their significance in muscle contraction and the control of contraction.

1.1. Skeletal muscle structure

Body movements are assisted by skeletal muscles, attached to the skeletal system by a collagenous tissue tendon. Skeletal muscle architecture is characterised by a precise and clear-cut arrangement of specialised multinucleated cells called ‘myocytes’ or ‘muscle fibres’ (Figure 1.1) to perform their specialised function - force production. Muscle fibres are long cylindrical cells varying in diameter and length of 20-100 μm and 2-50 mm respectively (Squire *et al.*, 2005). Muscle fibres are covered by a plasma membrane called sarcolemma and contain cellular organelles like mitochondria, transverse tubules (t-tubules), sarcoplasmic reticulum, and sarcoplasm. The space between muscle fibres is filled with capillaries, nerve fibres, collagen matrix, and interstitial fluid. The well-defined organisation of muscle filaments is illustrated in Figure 1.1.

A single muscle fibre is composed of thousands of myofibrils. Muscle fibres are arranged in a parallel manner to form muscle fibre bundles (2-5 μm diameter). Myofibrils are made up of filamentous muscle proteins, thick (myosin) and thin (actin) filaments, which are arranged in a regular repeating functional unit, the sarcomeres (Figure 1.1). The microscopic view of the alternating dark and light bands in the sarcomere are formed

primarily by myosin and actin respectively (Figure 1.2). The appearance of dark bands in sarcomere is caused by myosin molecules, which has a high refractive index; these bands are called A-bands (anisotropic bands). Light bands are made up of less refractive index protein, actin, and these bands are called I-band due to its isotropic property (Squire, 2016). During the muscle shortening, the A-bands maintain their length, but the I-bands become shorter.

Muscle contraction in these striated muscles is activated by neurotransmitters, principally by acetylcholine, binding to the receptors located in the post-synaptic membrane of the neuromuscular junction. Sodium influx results in a propagated action potential along the muscle fibres. The action potential results in depolarisation of the t-tubules. The depolarisation which reaches into the centre of the muscle cells causes calcium ion release from sarcoplasmic reticulum. Calcium binds to troponin-C, a protein associated with actin filament which triggers a conformational change in the muscle thin filaments. This results in the interaction of the myosin motor with actin, forming the “cross-bridge” structure. Under appropriate physiological conditions, the filaments slide past one another through the cross-bridge interactions, resulting into muscle shortening, limb movement and force production.

Figure 1.1: Typical vertebrate skeletal muscle organisation.

Skeletal muscles are attached to the skeletal system by fibrous collagenous tissue called tendon. Each muscle fibre is made up of thousands of myofibrils which run along the muscle bundle. Muscle fibres contain other cellular organelle -mitochondria, sarcoplasm, t-tubules. Muscle fibrils are made up of the longitudinal repetition of the basic contractile unit called Sarcomere. One sarcomere consist area between two successive Z-lines. Actin (thin filament) and myosin (thick filament) are the major components of sarcomere. Adapted from- www.britannica.com

1.2. Sarcomere structure and organisation

The sarcomere is the structural and functional unit of muscle. One sarcomere is 2-2.5 μm long and 1-2 μm in diameter at resting state, changes in dimension during muscle contraction-relaxation cycle (Ferenczi, 2000, Squire *et al.*, 2005), and encompasses the area between two successive Z-lines (Z-bands) as shown in Figure 1.2A. The Z-lines bisect the light bands, I-bands. Under electron microscopy, Z-lines are seen as distinct dark lines, due to high protein density, that runs continuous across the longitudinal section of the muscle fibre. (Figure 1.2B).

In the sarcomere, repeating units of bipolar thick filaments are symmetrical around the M-line. Thin filaments emerge from either side of the Z-line, pointing in the opposite direction. The arrangement of thick and thin filaments gives the banding pattern to the sarcomere (Figure 1.2A and 1.2B). Actin filaments of the adjacent sarcomere are connected at the Z-line by a structural protein, α -actinin. These structural proteins help in transmitting force along the muscle through the Z-line. The M line is located at the central region (bare zone) of the thick filaments, A-band, and consequently bisecting the sarcomere besides A band (Luther & Squire, 2014). The M-line's accessory proteins (e.g., Myomesin) have a role in stabilising and aligning the muscle structure by cross-linking myosin filaments (Agarkova & Perriard, 2005, Squire *et al.*, 2005).

Huxley et al. was the pioneer to observe three zones (I-band, A-band, and H-zone) with different degree of contrast between Z and M lines under the electron microscope (Hanson & Huxley, 1953). These contrast differences were formed by the assembly of proteins with varied protein density. Protein composition of different bands is explained in section 1.3. Sarcomeric proteins.

Longitudinal and cross-sectional views of each band are shown in the Figure 1.2B and 1.2C respectively, I-band (isotropic band), present on either side of the Z-line is the actin-containing regions devoid of myosin (thick) filament. The A-band (anisotropic) mainly consists of myosin filaments, accounting for its higher protein density, but in most of the A-band, there is overlap between the myosin and actin filaments. In cross-section, each thick filament is surrounded by six thin filaments giving a double hexagonal lattice

arrangement, where the outer hexagonal ring is formed by myosin and inner hexagonal formed by actin (cross-sectional view Figure 1.2. C). The region of the A-band close to the M-line is a region normally devoid of actin filaments, and is denoted as the H-zone. In the H-zone a simple hexagonal pattern is seen because of the myosin filament organisation, and they are spaced 450 Å apart (Huxley, 1953). Thick filaments in the H-zone appear as a thick shaft, but on either side of the central shaft a small structure called “cross-bridges” are visible in the A-band. The cross-bridges are formed by the interaction between myosin heads and actin filament. These cross bridges are shown to be key to muscle contraction and force production (Lymn & Taylor, 1971, Huxley, 1974, Geeves & Holmes, 2005, Vandenboom, 2011). So, the project is based on the interest in the visualisation of these cross-bridges by a new labelling technique for use in electron microscopy as explained later.

Figure 1.2: Structural organisation of sarcomere.

(A) Schematic representation of single sarcomere is shown. Different bands including I-band, A-band, H-zones are marked (Squire, 2016) (B) Longitudinal view of sarcomere by electron microscopy: similar to the schematic representation all the major bands are shown. A-band of sarcomere has more contrast than I-band due to the presence of high refractive index protein myosin. I-band contains less refractive index protein actin. One sarcomere unit is represented by the area between two consecutive Z-lines. (C) The cross-sectional view of major zones: because of the different organisation of myosin and actin in different bands in sarcomere it gives different view in cross-sectionally (Squire, 2016).

1.3. Sarcomeric proteins

Actin and myosin are the two major proteins of the sarcomere chiefly constituting the thin and thick filaments, respectively, and are responsible for the movements and force production in muscle. Regulatory proteins - troponin complex and tropomyosin along with other proteins like myosin-binding protein-C (MyBP C), titin, and nebulin contribute to the sarcomere structural organisation, maintaining filament length and regulation of muscle contraction (Squire *et al.*, 2017, McNamara & Sadayappan, 2018).

1.3.1. Thin filament

Thin filaments are made up of actin monomers joined to form a helical structure, together with two regulatory proteins - troponin and tropomyosin as shown in Figure 1.3A (Tobacman, 1996). The length of each thin filament is remarkably constant in skeletal muscle at approximately 1.0 μm (Huxley, 1953, Huxley & Hanson, 1957), but there is more length variation in cardiac myocytes.

1.3.1.1. Actin

Straub established that actin is the backbone of the thin filament (Straub, 1942). Filamentous actin (F-actin) of muscle is formed by the polymerisation of monomeric G-actin (globular actin) at a high ionic concentration (Straub, 1943, Straub & Feuer, 1950, Pardee & Spudich, 1982). The F-actin was first visualised by Hanson and Lowy by negative staining EM (Hanson & Lowy, 1963). In some low-resolution representations, the actin filament appears as two strings of beads intertwined with each other (helical structure) at a repeating interval of 349 Å. F-actin is a left-handed helix with six left-handed turns (Huxley & Brown, 1967). Each turn consists of 13 globular monomers, which are arranged at a regular interval of 56.5 Å. The actin monomer consists of two domains, one large and one small, and each is further divided into two subdomains, giving subdomains 1-4 (Figure 1.3B). The small domain encompasses both N and C- termini, while the interface of the small and large domains make ATP and calcium-binding pockets (Kabsch *et al.*, 1990).

Subdomain 1 has the myosin binding site buried internally and subdomains 3 and 4 interact with the complementary subdomain of the adjacent monomer (Holmes *et al.*, 1990).

1.3.1.2. Troponin and tropomyosin

Excitation-contraction of striated muscle occurs when the intracellular calcium concentration changes, this phenomenon is regulated by a molecular switch mediated by the troponin complex (Ebashi *et al.*, 1967). While tropomyosin has a significant role in actin structure stabilization in addition to the regulation of muscle contraction (Gordon *et al.*, 2001). The troponin complex is a heterotrimeric complex (Figure 1.3C) composed of troponin I (TnI), which can constrain the muscle contraction by inhibiting actomyosin ATPase activity (Greaser & Gergely, 1971). Troponin T (TnT), which binds to tropomyosin, and troponin C (TnC), which reversibly binds Ca^{2+} ions at physiological concentration (Greaser & Gergely, 1971). The troponin complex is a globular complex of 200 Å with an extended tail region. Troponin requires tropomyosin for the interaction with actin filament (Ebashi & Kodama, 1965). The tropomyosin monomer (33 kDa and 400 Å) connects to the adjacent monomer in a head to tail fashion forming an α -helical chain as shown in Figure 1.3D (Phillips Jr *et al.*, 1986, Whitby *et al.*, 1992). The tropomyosin molecules are bound to the TnT of the troponin complex. The tropomyosin and troponin complexes are situated in the helix groove of the actin filament. The localisation of these two regulatory proteins in the thin filament was determined by fluorescently-labelled proteins (Endo, 1966), and by immune-EM (Ohtsuki, 1975). Each strand of thin filaments contains one troponin complex at a repeated interval of 385 Å on an actin filament (Huxley & Brown, 1967) and is associated with one tropomyosin molecule (Figure 1.3A).

Figure 1.3: Thin filament and its components.

(A) Schematic model of thin filament showing all its components- F-actin, Troponin complex and tropomyosin filament (B) Structures of F-actin filaments (PDB:3G37) (left) Crystal structure of the Actin monomer, where subdomains 1, 2, 3, and 4 are shown. ATP binding cleft is marked (PDB: 1NWK). (C) Structure of troponin complex from skeletal muscle, containing troponin C (TnC; blue (MW 20 kDa)) and of troponin I (TnI; green (MW 24 kDa)) and troponin T (TnT; red (MW 37 kDa)) (PDB: 1J1D). (D) Crystal structure of tropomyosin showing two α -helical chains bound to each other (blue) and its 3-D surface view (magenta) (PDB:1C1G).

1.3.2. Thick filament

Hugh Huxley was the pioneer in showing the presence of the myosin molecule (long-rod shaped with globular ends) in thick filament (Huxley, 1963). The myosin motor protein belongs to a large protein superfamily. It possesses a highly conserved ATPase domain that binds to actin and moves along the actin filament (Lyubimova & Engelhardt, 1939, Mermall *et al.*, 1998, Sellers, 2000). There are at least 18 classes of myosin proteins identified in the animal and plant kingdoms (Thompson & Langford, 2002). The human genome encodes 12 different classes of myosins, and each one is designated for different cellular functions (Heissler & Sellers, 2014, Masters *et al.*, 2016). Myosin in striated muscles (skeletal and cardiac muscles) are class II myosins (Figure 1.4 A), a hexameric protein complex with two heavy chains and two pairs of light chains (Cooper, 2000). Thick filaments are formed by the anti-parallel assembly of myosin molecules. The diameter of one thick filament is 300 Å, and its average length in one sarcomere is 1.6 µm (Huxley, 1953, Huxley & Hanson, 1957, Huxley, 1963). However single myosin filament has an approximate length of 150 nm (Slayter & Lowey, 1967, Lowey *et al.*, 1969). The C-terminus of the heavy chain forms a coiled-coil helical rod and the N-terminus forms a globular head region that is associated with light chains (Figure 1.4A). Tryptic digestion of a single myosin molecule produces two fragments, *heavy meromyosin (HMM)* and *light meromyosin (LMM)*. LMM is responsible for myosin backbone filament formation, whereas actin interaction sites and ATPase activity resides in HMM (Perry, 1951, Szent-Györgyi, 1953, Cohen *et al.*, 1961, Lowey & Cohen, 1962, Margossian & Lowey, 1973, Margossian & Lowey, 1973). The actin binding property and ATPase activity of HMM are maintained in a chymotryptic digest called the myosin S-1 region (~120 kD), or subfragment-1, forming the head region, also known as the cross-bridge (Mueller & Perry, 1962). A biochemically active and soluble ATPase functional unit consists of one myosin heavy chain S1-fragment associated with two light chains and interacting with thin filaments. An alternative soluble proteolytic fragment is HMM (heavy meromyosin) which consists of two myosin heads and a somewhat longer fragment of the lever arm, together with two sets of two myosin light chains. The HMM fragment associates with actin filaments and displays actin-activation of the ATPase activity. This fragment has been widely used in *in vitro* motility assays to

investigate various myosin families, particularly myosin that do not form thick filaments (Yanagida *et al.*, 1984, Kron & Spudich, 1986) of cross-bridge cycle.

The 3-D structure of the myosin filaments was studied in different muscle types of various organisms to understand their role in the muscle contraction (Luther *et al.*, 1996, Squire *et al.*, 2005, Al-Khayat *et al.*, 2006, Zoghbi *et al.*, 2008, Al-Khayat *et al.*, 2013). These studies have found that invertebrates have a greater number of myosin strands (single myosin molecule is considered as single strand which contains a pair of head and long tail) in thick filaments, thus exerting more tension as compared to the vertebrates (Al-Khayat *et al.*, 2009, Woodhead *et al.*, 2013). Besides the fact that the number of strands varies in different species, myosin shares common structural features including the helically-ordered arrangement of myosin heads on the filament backbone (Figure 1.4B.). The surface helical net (arrangement of myosin head pair on the backbone of the thick filament) of myosin filament varies in different species based on the number of strands in the filament. The number of myosin molecules (myosin strand) that contribute myosin heads to each crown level defines the rotational symmetry of the filament. The vertebrate striated muscle, each thick filament has three myosin strands (molecule) forming a right-handed helical structure. Three myosin molecules that contribute three pairs of myosin heads to each crown level and hence has a three-fold rotational symmetry. Each strand has nine pairs of myosin head per full turn of 1287 Å. The arrangement of heads on myosin strands appears like a projection from the backbones commonly referred as 'crowns of heads' (Al-Khayat *et al.*, 2013). As shown in Figure 1.4C the heads are arranged at regular intervals on the myosin backbone with a repeat distance of 143 Å (Huxley & Brown, 1967). This spacing may have advantages for facilitating actomyosin cross-bridge interactions since actin symmetry is also conserved across species (Galkin *et al.*, 2002).

Figure 1.4: Thick filament and its constituents.

(A) Schematic representation of class II myosin molecule containing one heavy chain and two light chains. Light chains are associated at myosin S1 or head region. (B) The number of different myosin single molecules are associated to form thick filament in which myosin rod are associated to form central shaft region (green) and heads are arranged in a helical manner (yellow). (C) Arrangement of myosin heads are known as “crowns of head” are arranged at regular intervals on myosin backbone with a repeat distance of 143 Å. (D) Single myosin thick filament has bipolar arrangement where heads are arranged either side of central barren rod.

The electron microscopy shadow casting technique revealed that the bipolar thick filaments are formed by the tail-to-tail assembly of myosin molecules to constitute a central bare zone, and further addition of myosin molecules resulting in myosin heads projecting from the backbone on either end of the rod as shown in Figure 1.4D (Huxley, 1963). This arrangement is important for giving directionality to the contractile apparatus, ensuring that thin filaments are pulled towards the centre of the sarcomere from both ends. These globular heads are the N-termini of myosin where the S-1 region resides (Elliott & Rome, 1969). S-1 region forms the motor domain and a neck region (also known as lever arm) of myosin; it is connected to the myosin backbone by a hinge region. The motor domain holds the ATP binding site and the actin binding site. Hence it acts as the catalytic domain of the myosin molecule, driving muscle contraction (Butler *et al.*, 2001). Following the motor domain, an extended α -helical region represents the lever arm. The lever arm is associated with two light chains, ELC (essential light chain) and RLC (regulatory light chain) as shown in Figure 1.4A. (Uyeda *et al.*, 1996). The lever arm is the 'regulatory domain' and has a central role in muscle movement and mechanical force production during the working stroke of the cross-bridge.

1.3.2.1. Myosin light chains

As briefly described earlier, the lever arm region is stabilised by two non-covalently bound light chains, projecting from the ATPase catalytic site toward the C-terminal tail of the myosin as shown in Figure 1.5A (Tsao, 1953). In vertebrate skeletal muscle, the myosin molecule is associated with one RLC of 18 kDa and either isoform of ELC with a molecular weight of 21 kDa or 16 kDa (Lowey & Risby, 1971, Weeds & Lowey, 1971, Heissler & Sellers, 2014). Both light chains belong to the calmodulin family, containing ‘helix-loop-helix motif’ called EF-hand, responsible for calcium binding (Grabarek, 2006, Hong *et al.*, 2009). Even though ELC has different functions in different muscle types, its conformation is similar in all myosin II molecules, irrespective of species. From the crystal structure of myosin S-1 and the ELC complex, ELC was identified to bind the conserved IQ motif of the extended α -helix or the neck region of myosin (Bähler & Rhoads, 2002). The molecular structure of the myosin head-ELC complex suggests that interaction of ELC with the heavy chain may couple ATP hydrolysis energy to the lever arm rotation (Borejdo *et al.*, 2002, Ushakov, 2008).

Figure 1.5: Thick filament and its constituents.

(A) Molecular structure of Myosin S1 or head is resolved by X-ray crystallographic technique. The structure shows that myosin head has two domains called motor domain (catalytic domain) where actin binding region and ATPase activity resides. Next to catalytic domain an extended alpha-helix region (red) is present called as lever arm region where two light chains are associated. RLC (green) and ELC (blue) is shown (PDB: 1QVI) (B) Molecular structure of RLC. RLC has similar folds like all other calmodulin family proteins where Ca^{2+} binding site and phosphorylation sites reside.

Myosin regulatory light chain

The regulatory light chain (RLC) was first identified in 1969 (Weeds, 1969). Subsequently, several functional studies have shown that RLC has a major role in the structural stabilisation and modulation of striated muscle and the regulation of smooth muscle contraction (Szent-Györgyi *et al.*, 1973, Barron *et al.*, 1979, Hartshorne & Mrwa, 1982). From the crystal structure of chicken smooth muscle myosin S-1, RLC was shown to be localised at the head-rod junction (lever arm region) of myosin (Xie *et al.*, 1994) as shown in Figure 1.5B. This study shows that RLC and myosin heavy chain interact through a non-covalent interaction. The N-terminus of RLC interacts with Asn-825 and Leu-842 residues in the heavy chain C-terminus while the C-terminus of RLC interacts with Glu-808 and Val-826 residues of myosin heavy chain (Rayment *et al.*, 1993, Szczesna-Cordary *et al.*, 2004). The N-terminal region of RLC shares common structural features with EF-hand Ca^{2+} -binding family of proteins (Rayment *et al.*, 1993). A cation (Ca^{2+} or Mg^{2+}) binding site is present in the first helix-loop-helix motif of RLC. Also, phosphorylation sites are found in close proximity to the cation binding site, and these sites aid RLCs in modulating striated muscle function.

1.3.3. Other filament proteins

Besides actin and myosin filament proteins, there are several associated proteins in the sarcomere with important roles.

Titin

Titin is an essential structural protein that runs along the centre of myosin filament at M-band then through the I-band to the Z-band. Titin acts as a molecular spring that is responsible for some aspects of the mechanical behaviour of muscle. Moreover, during sarcomere development titin acts as a scaffold for sarcomeric protein assembly (Trinick, 1996, LeWinter & Granzier, 2010, Tskhovrebova & Trinick, 2010).

Myosin binding protein-C

Myosin Binding Protein-C (MyBP-C) is a sarcomeric protein that associates with the thick filament and plays a major role in structure and function of muscle (McNamara & Sadayappan, 2018). MyBP-C is a part of the thick filament, and interacts with the myosin tail region and RLC, (Moos *et al.*, 1975, Colson *et al.*, 2010, Ratti *et al.*, 2011)). Mutations in RLC phosphorylation sites have shown to affect the phosphorylation state of MyBP-C (Scruggs *et al.*, 2009).

1.4. Regulation of muscle contraction

Vertebrate muscle contraction is activated by calcium ions. Actin-myosin interaction in vertebrate striated muscles is regulated at the level of actin filaments by calcium-sensitive regulatory proteins (troponin complex) (Gordon *et al.*, 2000). The regulatory unit in striated muscle consists of seven actin monomers, one troponin complex and one tropomyosin (Ebashi & Endo, 1968).

Regulation of the thin filaments in striated muscle contraction is explained with the help of structural changes occurring to the filaments when troponin binds to the Ca^{2+} ions (Ca^{2+}). At low cytoplasmic calcium ion concentration ($<1 \mu\text{M}$) corresponding to the relaxed state of skeletal muscle, troponin is largely calcium-free. Upon activation by a nerve action potential triggering an action potential in the myocyte plasma membrane and a depolarisation wave into the t-tubule system, calcium from the sarcoplasmic reticulum is released into the cytoplasm, rapidly raising its concentration to approximately $10 \mu\text{M}$. At this calcium concentration level, troponin molecules bind calcium. Binding of Ca^{2+} to the troponin-C affects the location of tropomyosin on actin filaments. At low concentrations of calcium ion tropomyosin blocks the myosin binding site on actin and inhibits myosin cross-bridge association. At high Ca^{2+} , calcium binding to the troponin-C induces a structural change in the troponin complex; consequently, this leads to a change in the tropomyosin location on the actin helical groove. Hence the tropomyosin shifts centrally towards the actin helix groove and exposes the myosin binding site (Xu *et al.*, 1999). During the

interactions with Ca^{2+} , actin also undergoes two conformational changes. During the ‘off state’ of actin, with no bound Ca^{2+} , myosin binding sites are covered by tropomyosin and the affinity of myosin for actin is greatly reduced. In the presence of Ca^{2+} , binding of Ca^{2+} to the troponin leads to the exposure of myosin binding site on actin and this conformation is called the ‘on state’ (Lehman *et al.*, 1994, Lehman *et al.*, 1995, Galinska-Rakoczy *et al.*, 2008, Lehman, 2016) with a strong affinity of myosin for actin.

However, in smooth muscle, muscle contraction happens only after phosphorylation of RLC at the Ser 20 residue by Myosin light chain kinase (MLCK). MLCK is Ca^{2+} -calmodulin dependent kinase (Small & Sobieszek, 1977, Sobieszek & Small, 1977, Hartshorne & Mrwa, 1982) that also phosphorylates striated muscle RLC. Unlike in smooth muscle, the function of this phosphorylation in striated muscle affects diverse physiological properties such as Ca^{2+} sensitivity of isometric tension, force development and cross-bridge cycling rate (ATPase) (Crow & Kushmerick, 1982, Sweeney & Kushmerick, 1985, Metzger *et al.*, 1989, Sweeney *et al.*, 1993, Szczesna-Cordary, 2003). In striated muscle RLC phosphorylation enhances the isometric force production, power output, shortening speed, and shortening velocity (Sweeney *et al.*, 1993, Szczesna-Cordary, 2003, Toepfer *et al.*, 2013). The boosting or diminishing effect in physiology by muscle protein modification are considered as modulation effect. Whereas signal factors that are controlling or initiating the muscle contraction are considered as regulatory effect. For example, in smooth muscle, muscle contraction is activated by RLC phosphorylation. Thus, the phosphorylation of RLC by smooth muscle specific myosin light chain kinase (smMLCK) regulate muscle contraction by initiating the signal (Pearson *et al.*, 1984). RLC isoforms in cardiac muscle have been seen to have a different degree of phosphorylation (Morano, 1999). These studies collectively highlight that RLC plays a modulatory role rather than a regulatory one at this point in time, the mechanism of RLC modulation in striated muscle is not well understood.

1.5. Historical perspectives on muscle contraction mechanism studies.

The muscle contraction field set a good paradigm in cell biology by demonstrating that the combination of biochemical and structural approaches can lead to a detailed understanding of a cellular function. Current knowledge regarding muscle contraction and

its mechanism is available following biophysical, biochemical and structural studies of muscle and its constituents. On evolving understanding of the structure, constituents, and biochemical process in muscle, different representation formulated. An early representation is the sliding filament hypothesis which has been complimented in more recent years by the power stroke and swinging cross-bridge concept.

1.5.1. Biochemical studies for establishing actin-myosin interaction

Mechanical insight into muscle contraction became available after the myosin protein was extracted by high salt solution in 1864 by Kühne (Kühne, 1864). It was proposed that this viscous protein is responsible for generating the muscle rigor state (contracted state). Reporting ATPase activity of myosin was another key milestone finding in the muscle biochemistry field (Lyubimova & Engelhardt, 1939). Subsequent biochemical studies showed that Kühne's myosin molecule consists of two proteins 'actin and myosin' and that the myosin's viscosity and flow birefringence were changed on the addition of the ATP (Banga & Szent-Györgyi, 1942, Needham, 1942, Straub, 1943, Szent-Györgyi, 1943). However, convincing evidence for muscle contraction as a result of actomyosin interaction with ATP was shown by physiological studies of glycerol extracted psoas muscle by Szent-Gyorgyi (Szent-Gyorgyi, 1949). Since then glycerinated psoas muscle, with some modification, is still being used for the structural and biochemical studies.

1.5.1.1. Swinging cross-bridge cycle

Several biochemical studies have helped to elucidate the kinetic mechanism of ATP turnover by myosin and actin, and this is extensively reviewed in Geeves and Holmes 2005 (Eisenberg & Moos, 1968, Lymn & Taylor, 1971, Taylor & Trenlham, 1979, Greene & Eisenberg, 1980, Ma & Taylor, 1994, Geeves & Holmes, 2005). Of the several proposed mechanism, the swinging cross-bridge mechanism was a major breakthrough in muscle kinetic studies. This mechanism explains the role of the by-products (ADP and Pi) of ATP hydrolysis and how the chemical energy is coupled to mechanical energy resulting in force generation and muscle contraction. The foremost visualisation of cross-bridges was reported by Huxley (Huxley, 1957, Huxley, 1957) using EM of ultra-thin sections of the

muscle. Soon after, the mechanical cross-bridge cycle was proposed by Huxley (Huxley, 1969). However, the role of the cross-bridge in tension generation was first experimentally tested in isometrically contracting semitendinous muscle from *Rana temporaria* (Huxley & Simmons, 1971). Biochemical changes in the cross-bridge during ATP hydrolysis and subsequent force productions was studied using HMM and actin by Lymn and Taylor (Lymn & Taylor, 1971) as shown in Figure 1.6.

Figure 1.6: Cross-bridge cycle mechanism.

When an action potential arrives at neuro-muscular junction it stimulates muscles, cause the release of Ca^{2+} ions from sarcoplasmic reticulum, the Ca^{2+} bind to troponin: the troponin changes shapes and the tropomyosin moves in order to expose the myosin binding site. The myosin is then able to bind to actin, forming a cross-bridge as shown (1). The myosin heads tilt, causing the actin to be pulled along so overlap more with myosin filament. ADP and Pi are released (2). This is power stroke. New ATP attached to the myosin head as the cross-bridge has broken (3). The ATP is hydrolysed, providing enough energy to force the myosin heads to release the actin (4). They tip back to their previous condition and are then able to repeat the process, forming a cross-bridge further along the filament.

The current model considers that one ATP molecule is hydrolysed per actin-attachment/detachment cycle of the cross-bridge and the energy derived from ATP hydrolysis is coupled to contraction. Furthermore, it states that in the absence of ATP, the myosin head (M) tightly binds to the actin (A) at an acute angle of $\sim 45^\circ$ relative to the actin filament, forming a rigor state or acto-myosin complex (AM). But when an ATP molecule binds to the myosin in this state, a rapid dissociation of myosin heads from actin ensues, forming a bimolecular complex with ATP (M.ATP). This is followed by rapid hydrolysis of ATP to ADP and inorganic phosphate (Pi) by the intrinsic ATPase activity of myosin. ATP hydrolysis products bound to myosin head form an M.ADP.Pi complex. As a result of ATP hydrolysis, the myosin head conformation changes from 45° to 90° followed by binding to the closest actin binding site on the thin filament giving rise to a third state that resembles the muscle's relaxed state. In the fourth state, myosin heads in complex with ADP and Pi bind to the actin at an angle of 90° , forming the A.M.ADP.Pi complex. Following hydrolysis there is a tendency of the actin-bound myosin heads to rotate to the 45° state to generate muscular force which is thought to be accompanied by the release of Pi, forming the force-bearing A.M.ADP state. Subsequently, ADP is released from the actin-attached cross-bridge to return to the initial conformation, i.e. to an angle of 45° and for the affinity for ATP to rise. These stages will be repeated during muscle contraction and shortening in the presence of calcium. It is believed that the conformational changes in the myosin head result in the pulling of actin towards the centre of the sarcomere. Evidence for angle change during active contraction is still speculative even though there is clear evidence of a difference in cross-bridge angle between rigor and relaxed states in skeletal muscle (Greene & Eisenberg, 1980, Crowder & Cooke, 1987, Mello & Thomas, 2012, Montes et al., 2018). The details of the shape change in the actomyosin complex during the force-generation cycle remain unclear.

In summary, myosin reversibly undergoes a conformation change during the cross-bridge cycle: firstly, when ATP is hydrolyzed, and then when hydrolysis products are released from the myosin head, as shown in Figure 1.6.

1.5.2. Early structural studies

The study of the mechanism of muscle contraction was one of the factors driving the onset of biophysics, a sub-field of cellular biology. The establishment of biophysics field involved techniques such as interference microscopy, phase contrast microscopy and 2D-EM (conventional EM) and helped in revealing structural features of the muscle contraction-relaxation cycle (Huxley, 1957, Huxley & Hanson, 1957, Hanson & Lowy, 1963). Moreover, for the first time, 2D- EM images of an ultra-thin section of muscle established evidence for the muscle cross-bridges, a structural feature required for muscle contraction (Huxley, 1957). With the visual evidence from the relaxed and contracted muscle 2D-EM and interference microscopy, the first muscle contraction theory was framed, the ‘sliding filament model’.

1.5.2.1. Sliding filament theory

The sliding filament theory in contracting muscle was proposed by A F Huxley, Niedergerke and H E Huxley, Hanson independently (Huxley, 1953, Huxley & Niedergerke, 1954). They observed that the sarcomeric A-band remain constant in length whereas the I-band shortens in length during contraction. The observed variation in the total length of sarcomere without any change in length of thin or thick filaments led them to propose that muscle contraction occurs as a result of relative sliding movement of thick and thin filaments, which interacts with each other through the cross-bridge structures and when ATP bind to myosin head (Figure 1.7A and 1.7B).

Figure 1.7: Sliding filament mechanism.

Sliding filament mechanism is explained in (A) Schematic representation and in (B) electron microscopy image. During muscle shortening sarcomere contracts as a result of cross-bridge cycle causing the Z lines to come closer and reducing the length of sarcomere. During this process I band becomes smaller (region between A and B in schematic diagram and less dark area in electron microscopy image). The A band stays the same width (region between B and C in schematic diagram) and dark region in the electron micrograph.

1.5.3. Impact of high-resolution EM and X-ray studies on building the structural model for muscle contraction

Transmission electron microscopy (TEM), X-ray diffraction, and crystallography were used to reveal the ultrastructural details of muscle since the early 1950s. But the resolution of conventional EM is limited due to the 30-100 nanometer thickness of plastic sections. Improvements in sample preparation, TEM imaging and software improved the resolution limit of micrograph further down. Some of the high-resolution EM techniques currently used for elucidating the molecular structure of muscle include heavy metal staining, cryo-negative staining, unstained vitrified cryo technique and single particle analysis (Huxley, 1967, Slayter & Lowey, 1967, Vibert & Craig, 1985, Liu *et al.*, 2006, Behrmann *et al.*, 2012, Von der Ecken *et al.*, 2015, Wagenknecht *et al.*, 2015, Hu *et al.*, 2016, Chou & Pollard, 2019)

Heavy metal staining has significant advantages over plastic sections; isolated myofibrillar components provide more structural details when they are stained with heavy metals and prepared on carbon-coated grid. Important structural detail such as the bipolar organisation of thick filament and the 3-D organisation of actin in the thin filament was first revealed using negative staining of filaments (Huxley, 1963). Soon, Slayter and Lowey revealed the architecture of single myosin molecule by negative stain EM which was shown to have that two globular heads (Slayter & Lowey, 1967). Later this technique was adopted to illustrate the structural changes in thick filament due to the binding of calcium or myosin phosphorylation (Vibert & Craig, 1985, Levine *et al.*, 1991). However, one major limitation of the negative staining technique was that filament structures of muscle fibres are poorly preserved. However, the above limitation was overcome by using tannic acid as a post fixative before negative staining (Stewart & Kensler, 1986). Advancements in EM techniques improved the resolution. With negative staining technique, visualisation of the steric blocking model of thin filament regulation in the presence and absence of calcium ion was achievable (Lehman *et al.*, 1995). Another EM staining technique, namely metal shadowing, was used for the visualisation of invertebrate smooth muscle thick filament. Metal shadowing was used to enhance EM contrast (Elliott, 1974).

Even though conventional EM is still being used in muscle structural studies, there are some major drawbacks to this technique. Conventionally, muscle samples are processed by chemical fixation using glutaraldehyde or paraformaldehyde. During chemical fixation sub-cellular structures are preserved by intra and intermolecular crosslinking actions of aldehyde (Huang & Yeung, 2015). Subsequently, alcohol dehydration and plastic embedding were done to preserve the ultrastructural details. However, these chemical processes can alter the molecular arrangements or length of muscle filament as compared to the *in vivo* sample (Page & Huxley, 1963). These artefacts can be greatly minimised or entirely eliminated by cryo-fixation. During cryo-fixation samples were fixed at -180°C . During cryo-fixation, water molecule present inside the cells were vitrified without forming ice-crystals. Subsequently samples were processed by freeze-substitution and plastic embedding. Early cryo-EM studies of the muscle filament showed a well-preserved helical order of myosin filaments (Menetret *et al.*, 1990). Thus, using this method the muscle filament structure can be preserved to near *in vivo* state. Our current understanding of thin filament and its regulation resulted from cryo-EM studies (Xu *et al.*, 1999). Importantly, cryo-EM helped to interpret and map the structures of the myosin head, ELC, RLC and titin by 3-D reconstruction of human heart myosin filament (Al-Khayat *et al.*, 2013).

Alongside EM, X-ray studies also helped to further our knowledge of muscle structure (Irving & Maughan, 2000, Al-khayat *et al.*, 2003, Perz-Edwards *et al.*, 2011). X-ray diffraction had a major advantage over EM as it can be done in living cells (Bergh *et al.*, 2008). X-ray diffraction studies in the 1970s laid a milestone for the actin switching mechanism (Parry & Squire, 1973). The difference in X-ray diffraction pattern intensities of resting and active muscle helped to propose the mechanism of actin switching. Based on the diffraction pattern, it is suggested that during the relaxed state myosin binding sites are blocked by tropomyosin (Vibert *et al.*, 1972, Parry & Squire, 1973). A further milestone discovery by X-ray diffraction in muscle includes the elucidation of the molecular orientational changes in myosin head responsible force generation (Irving *et al.*, 1992). An additional advantage is that it is a non-invasive technique to study the cross-bridge structural-state under different conditions (Tsaturyan *et al.*, 1999, Spudich, 2001, Ma & Irving, 2019)

Despite the fact that X-ray diffraction can give the molecular organisation of muscle close to its *in vivo* state, application of this technique is limited due to the difficulties in interpretation of results (Squire, 1975). The structural information requires careful interpretation as it depends on the current understanding of the shape and order of cross-bridges obtained by X-ray crystallography and EM technique.

Besides cryo-EM and X-ray diffraction studies, X-ray crystallography also helped to understand muscle molecular architecture. The atomic structure of G-actin solved by X-ray crystallography provided a major advancement in the muscle contraction field (Kabsch *et al.*, 1990). X-ray crystallography and high angle X-ray diffraction data together helped to understand the organisation of monomeric G-actin to form F-actin filament (Holmes *et al.*, 1990, Kabsch *et al.*, 1990). Another milestone was reached when crystallography helped solve the structure of myosin (Rayment *et al.*, 1993, Rayment *et al.*, 1993). The myosin head was shown to be a remarkably interesting structure, consisting of a motor domain (catalytic domain), where ATP binding and actin binding sites reside. In close proximity to the catalytic domain an α -helical region also called the converter domain. A light chain domain (LCD) resides where RLC and ELC wrap around a single alpha-helical, 20nm-long section, this region is also called the lever arm region. From the crystallography structure of the actin-myosin head complex, the catalytic domain was found to be firmly attached to actin during muscle contraction whereas LCD of myosin acts as a lever arm that amplifies the movement. The evidence collected from the X-ray crystallographic structure of actin-myosin head helped to propose a novel mechanism for muscle contraction known as "lever arm hypothesis" (Holmes, 1997).

1.5.3.1. Swinging lever arm hypothesis

As previously discussed, the cross-bridge cycle model suggests that myosin heads can interact with actin filaments and nucleotides. To understand the myosin head conformation during the different states in the cross-bridge cycle, the myosin head complex bound with different nucleotides (ATP, ADP, ADP.Pi, MgATP) was studied by X-ray crystallography (Rayment *et al.*, 1993, Dominguez *et al.*, 1998, Houdusse *et al.*, 2000). It was observed that in all states, the structure of the motor domain remained unchanged

whereas the lever arm conformation was different (Rayment *et al.*, 1993). It was found that the lever arm could move or swing axially relative to the motor domain (Figure 1.8). These results lead to a new mechanism of muscle contraction known as the swinging lever arm hypothesis, which intends to explain the movement of the head structure during ATP hydrolysis and filament movement. This model proposes that changes in the conformation of the myosin head or movement of the cross-bridge is due to the swinging of the lever arm, rather than the movement of the whole head during ATP hydrolysis and ADP/pi release (Irving *et al.*, 1995, Uyeda *et al.*, 1996, Geeves & Holmes, 2005). According to this model, during muscle contraction, the 3-D structure of the motor domain remains the same, but the changes in the lever arm conformation are sufficient to pull the actin filament towards the M-band at the centre of the sarcomere. The arm translates the product release (associated with large free energy changes) into large conformational changes while the myosin head remains docked in the same position. Crystal structure studies helped the cross-bridge model to evolve into the swinging lever arm model (Figure 1.8). The distance of swing, based on crystallographic studies using S1, was shown to be $\sim 12\text{nm}$, (Dominguez *et al.*, 1998) comparable to the actin filament displacement length measured with optical tweezer technique (Finer *et al.*, 1994, Simmons *et al.*, 1996).

Figure 1.8: Hypothetical model for lever arm mechanism (Brunello et al., 2014)

The catalytic domain of myosin (purple) binds to actin filament (yellow) in the conformation phase before generation force, placing the lever arm in position "lifting" (red). After the generation of power, the motor adopts a rigid conformation in the low position with the lever arm (blue).

1.5.4. Spectroscopical structural studies

Even with high-resolution structures obtained by x-ray crystallography, and cryo-electron microscopy (cryo-EM), the structural details provided for the myosin light chain domain (LCD; lever arm) under ambient conditions in *vertebrate* muscle are inadequate. Electron paramagnetic resonance (EPR) spectroscopy is an innovative method developed for mapping protein domain orientations in the cellular environment (Cooke *et al.*, 1982, Barnett & Thomas, 1989, Zhao *et al.*, 1996, Thompson *et al.*, 2015, Nogara *et al.*, 2016, Savich *et al.*, 2019). Moreover, this approach can complement high resolution in vitro methods like x-ray crystallography and NMR by providing structural information from protein domains in the native environment. EPR has been used to study the angular distribution muscle proteins by attaching spin labels to the proteins, such information allow changes in domain orientations to be studied in larger macromolecular complexes under physiological conditions (Arata, 1990, Hambly *et al.*, 1991, Hambly *et al.*, 1992). The spin label provides rigid and stereospecific attachment of the probe to an α -helix (Wilcox *et al.*, 1990, Fleissner *et al.*, 2011, Sahu *et al.*, 2013), thus providing unambiguous information about helix orientation, distance, and rotational motion (Arata *et al.*, 2003, Binder *et al.*, 2015). Irvin group has done remarkable work and shown the light chain orientation by labelling with bifunctional rhodamine in skinned muscle fibres. These studies have provided the in situ orientation information about the ELC (Knowles *et al.*, 2008) and RLC domains (Brack *et al.*, 2004, Romano *et al.*, 2012, Fusi *et al.*, 2015). The bifunctional rhodamine approach was initially applied to the myosin regulatory light chain (RLC) in skeletal muscle fibres (Corrie *et al.*, 1999, Hopkins *et al.*, 2002). These studies have determined the changes in the RLC orientation during different experimental conditions and helped to comprehend the mechanism of myosin motor domain.

1.6. Novel labelling techniques for Transmission electron microscopy (TEM) and electron tomography (ET)

As an imaging technique, EM can complement other high-resolution imaging techniques like X-ray crystallography and NMR spectroscopy. EM produces structural insight into the macromolecular organisation at sub-nanometre to near-atomic resolution

(Castón, 2013). As a result, EM techniques are extensively used for understanding the macromolecular structure of tissue, cells and macromolecular assemblies at 1 nm resolution.

Regardless of the high-resolution cellular architecture obtained by EM, fluorescence microscopy is still chiefly applied to identify specific proteins inside the cell owing to its comparatively ease in labelling the proteins. Conventional protein labelling in EM was done by gold labelled antibodies (De Mey *et al.*, 1982, Lucocq, 1994, Mayhew *et al.*, 2003, Mayhew *et al.*, 2004, Giepmans *et al.*, 2005, Mayhew *et al.*, 2009, Mayhew & Lucocq, 2015). For instance, immune-gold labelling was used early in 1990 to map the troponin complex position in insect flight muscle (Reedy *et al.*, 1994). However, the popularity for antibody labelling started to wane due to artefacts, namely the lack of specificity in fixed cells and poor cellular ultrastructural preservation.

A relatively recent breakthrough in cellular protein labelling in EM was the development of genetically engineered protein tags that can be employed for the visualisation of proteins and their localisation inside cells at high resolution (Sartori *et al.*, 2007, Sosinsky *et al.*, 2007). To this end, proteins of interest are labelled in situ with genetically fused probes, and the resulting fusion proteins have an enhanced property to withstand strong chemical fixation and produce high EM contrast (Sosinsky *et al.*, 2007, Vicidomini *et al.*, 2010). Since these probes bring such important benefits, it was believed that they can be widely applied to study protein localisation using EM (Gaietta *et al.*, 2006). Lucifer yellow (Hanani, 2012), ReAsH (Gaietta *et al.*, 2002), HRP (Li *et al.*, 2010), Mini-SOG (Shu *et al.*, 2011) and APEX (Martell *et al.*, 2012) are some of the examples for genetically encoded tags for EM.

1.6.1. Electron tomography

Conventional EM can only provide ultrastructural information in two-dimensions (2D), whereas electron tomography (ET) offers information on cellular structures in 3D (Subramaniam *et al.*, 2003). ET has repeatedly provided structural details of cellular

architecture, protein organisation in macromolecular complexes (Mattei *et al.*, 2016, Dodonova *et al.*, 2017, Sastri *et al.*, 2017, Hutchings & Zanetti, 2018)

The principle of ET is illustrated in Figure 1.9. ET produces 3D information of an object of interest from a set of 2D-images attained at different viewing angles (tilt series) (Crowther *et al.*, 1970). Tilting is done by rotating the sample holder in the TEM column. The specimens are tilted around a fixed eucentric axis in the range of $+70^\circ$ to -70° as shown in Figure 1.9A. The maximum attainable tilt angle is typically $\pm 70^\circ$ due to the restriction in holder tilting and sample thickness (Lučić *et al.*, 2005, Lučić *et al.*, 2013). Each electron micrograph acquired at different angles represents the structural information of the object obtained for that particular direction of the electron beam. Subsequently, tilt series images are perfectly aligned and combined by software to produce 3D reconstructed image (tomogram) as shown in Figure 1.9B and 1.9C. In electron tomography, stacks of Z-axis images are produced by computational extraction of the reconstructed single tomogram, rather than acquiring images in Z-plane as in confocal microscopy. For a tomogram, the resolution is limited by the thickness of the sample and the number of projections (Crowther *et al.*, 1970). As mentioned earlier the tilt angle for a tomography holder is restricted to $\pm 70^\circ$ resulting in incomplete sets of projections. The reconstruction of the incomplete set of projection cause distortion due to the missing information at higher angles, which is known as the “missing wedge” due to its characteristic shape in Fourier space (Lučić *et al.*, 2005). To reduce the distortion due to the missing wedge, dual-axis tomography can be performed. Generally, dual-axis tomography is done by acquiring a second tilt series images perpendicular (90°) to the first tilt series. Until now electron tomography has been used to furnish 3-D architecture of muscle Z-band (Burgoyne *et al.*, 2015), thin filament (Burgoyne *et al.*, 2008), cardiomyocyte (Rog-Zielinska *et al.*, 2016), calcium-activated thick filament (Taylor *et al.*, 1999) and other cellular organization in muscles (Gherghiceanu & Popescu, 2011, Wagenknecht *et al.*, 2015). Here we combined APEX2 labelling and ET to visualise RLC in permeabilised muscles and hypothesis that resultant tomogram can give good resolution thus RLC organisation in muscle fibre.

Figure 1.9: Working principle of electron tomography.

(A) A set of tilt series were acquired around a single axis using tomogram holder which can rotate maximum angle of $\pm 70^\circ$. (B) Acquired images were aligned and combined with the help of computer software. (C) Tilt series images are back projected using computer software and 3D structure was reconstructed. Adapted from K Grünwald 2002 (Grünwald *et al.*, 2002).

1.6.2. APEX2, a protein tag to visualise proteins by EM

APEX2, a 28 kDa genetically engineered monomeric peroxidase from plant (ascorbic acid peroxidase, APEX), is the protein tag we chose to use in EM labelling (Martell *et al.*, 2012, Lam *et al.*, 2014, Ludwig *et al.*, 2016). APEX2, an engineered plant peroxidase, was introduced to overcome the limitations of other genetic tags such as miniSOG and HRP. Compared to other peroxidases, APEX is active at high calcium concentrations which provides a major advantage in studying muscle contraction. Additionally, the APEX2 protein can withstand strong chemical fixation and produce a discrete electron-dense stain with diaminobenzidine (DAB), providing 5-10 nm resolution in EM (Lam *et al.*, 2014). The working principle of APEX2 is illustrated in Figure 1.10. Singlet oxygen generated by APEX2 locally polymerises DAB into a precipitate that is stainable with Osmium and therefore can be readily imaged at high resolution by EM. Since APEX2 is a versatile label for EM of genetically tagged proteins in cells, tissues, and organisms, it has proven itself to be a valuable tool for EM. Another advantage of APEX is that labelling is performed in situ, allowing the protein of interest to be studied in 3D by ET.

RLC-fused to APEX2 will be a new tool for the visualisation of the muscle-cross bridge by EM. For our experiment RLC-APEX2 was expressed in bacteria, purified and exchanged against native, un-labelled RLC into permeabilised muscle fibres. These muscle cell fragments are then fixed and overlaid with a solution of DAB and H₂O₂, and subsequently post-fixed with Osmium tetroxide (OsO₄) to produce contrast in EM. The intention of this project is to visualise RLC in the cross-bridge by 3D ET to study the molecular changes that occur to cross-bridges during the contraction-relaxation cycle. With this approach, understanding of the physiological roles of RLC during muscle contraction can advance.

Figure 1.10: Working principle of APEX2.

Scheme showing how APEX2 is used as reporters to generate contrast for EM DAB (diaminobenzidine). Conjugated APEX2 protein converts H_2O_2 into single reactive Oxygen. The singlet Oxygen helps in the polymerization of DAB into an osmophilic compound at the site of polymerization, later stained with Osmium tetroxide (OsO_4) to visualize by EM.

1.7. Aim of the thesis and significance of RLC labelled EM structure

Michael Barany, one of the eminent scientists in muscle physiology, suggested that ATP-dependent muscle force production is the result of structural changes in the protein domain or subdomains four decades ago (Bárány *et al.*, 1975). Since then our understanding of muscle contraction and molecular mechanism significantly advanced by physiological and structural studies.

All above stated structural techniques advanced muscle contraction field from sliding filament theory to swinging cross-bridge hypothesis, but still, there are many unresolved questions. One of the fundamental questions regarding the architecture of the thick filaments is how RLCs are organised in the muscle at the different stage of contractions. And, mechanistic insight into how the lever arm domain (LCD) or proteins associated with LCD (RLC and ELC) operate at different stages of muscle contraction in intact muscle fibre is lacking. As explained earlier, according to the swinging lever arm hypothesis, myosin head movement along actin is the result of LCD domain movement. This suggests the importance of proteins located at LCD (RLC) and their structure during muscle contraction. It is believed that RLC can be effectively studied at the ultrastructural level (Ling *et al.*, 1996, Kampourakis & Irving, 2015, Kampourakis *et al.*, 2015). The role of myosin regulatory light chain (RLC) as a regulatory protein in smooth muscle is well documented but its role in striated muscle is not yet well established (Kendrick-Jones *et al.*, 1970, Szent-Györgyi *et al.*, 1973, Szczesna-Cordary, 2003). It is anticipated that with the help of the new EM labelling technique, organisation of RLC in muscle cross-bridge can be elucidated at different stages of contraction and this will improve the understanding of the muscle contraction mechanism.

The major goal of this thesis is to standardise the APEX2 labelling method to visualise the muscle cross-bridges by electron microscopy. More specifically, we aim to do this by labelling the myosin regulatory light chain (RLC) protein present in the lever arm domain of muscle cross-bridges by exchanging native RLC with recombinant RLC-APEX2.

1.8. Research gaps and hypotheses

Various structural models for F-actin complexed with myosin head have been proposed. Each has provided insightful structural details of the acto-myosin complex (Holmes *et al.*, 2003, Lorenz & Holmes, 2010, Behrmann *et al.*, 2012). Recent Cryo-EM structure of rigor complex at 3.9 Å has improved the currently available structural details (von der Ecken *et al.*, 2016). However, the above structural details are insufficient to understand the organisation of lever arm domain. This is because the actin-myosin complex in the above structural studies contains only partial myosin motor protein and F-actin at its complex but not in intact muscle.

However, X-ray diffraction, conventional EM and fluorescence polarisation studies were used to understand the conformational changes in the cross-bridge or lever arm during rigor state in intact muscle fibre (Lombard *et al.*, 1995, Dobbie *et al.*, 1998, Knowles *et al.*, 2008).

Moreover, RLC was shown in crystal structures of chicken skeletal S1 and scallop myosin (Rayment *et al.*, 1993, Xie *et al.*, 1994), these structures lack the RLC phosphorylation domain due to difficulty in crystallisation. However above-mentioned phosphorylation studies point out the requirement of fully functional RLC structure in intact muscle. In addition to the RLC phosphorylation domain, the location of RLC on Myosin S1 structure is also equally important. RLC is localised at the lever arm domain or head-rod junction of myosin heavy chain (Rayment *et al.*, 1993). According to the ‘swinging lever arm’ hypothesis, the movement of the myosin head along actin is the result of the rotational movement of the lever arm domain. These structural details indicate that RLC is an ideal candidate for labelling the myosin cross-bridge so as to investigate its ultrastructural details.

Electron microscopy is a potential technique that helps in revealing subcellular ultrastructure (Devan *et al.*, 2018, Pfeffer & Mahamid, 2018, Smith & Starborg, 2018). Owing to developments in EM such as direct electron detector technology and image processing software (RELION, Iterative Helical Real Space Reconstruction (IHRSR)). These advancements improved the capture high-resolution molecular images of

myofilament components- actin, myosin (Zoghbi *et al.*, 2008, AL-Khayat *et al.*, 2010, Al-Khayat *et al.*, 2013, Hu *et al.*, 2016). With the help of myosin S1 and actin complex, it was possible to dissect the ATP hydrolysis pathway, turnover and coupling of its hydrolysis energy for force production in the cross-bridge. Recent high-resolution cryo-EM studies using the complex (actin-myosin) helped to advance our understanding of the cross-bridge greatly by revealing myosin force sensing mechanism (Mentes *et al.*, 2018). The limitation was that myosin S1 was not attached to myosin thick filament in all these structural studies, i.e. the complex does not recapitulate the native myosin-actin complex in the intact muscle. Thus, myosin S1-actin complex is not an ideal model for understanding the cross-bridge mechanism. In order to have a profound understanding of the role and mechanism of the muscle cross-bridge during contraction of the vertebrate striated muscle, visualisation the cross-bridge in the muscles native form becomes a compelling prerequisite.

The use of fluorescence or phosphorescence probes tagged to the myosin head improved the structural and dynamic details of the muscle cross-bridge (Thomas & Cooke, 1980, Hambly *et al.*, 1992, Tanner *et al.*, 1992, Ostap *et al.*, 1995, Sabido-David *et al.*, 1998, Brack *et al.*, 2004). Tagging of fluorescence probes to lever arm proteins like RLC and ELC in polarization studies validated the lever arm rotation at different states of muscle contraction (Irving *et al.*, 1995, Hopkins *et al.*, 2002, Knowles *et al.*, 2008). However, the angular changes noticed were significantly different from those obtained from X-ray crystallography. This disagreement in cross-bridge orientation calls for a robust method to effectively validate the outcomes from different systems.

APEX2-based EM labelling has improved visualization of the 3D organization of specific proteins or subcellular structures (Martell *et al.*, 2012, Lam *et al.*, 2014, Ludwig *et al.*, 2016). Based on the current APEX2-based EM technology, a hypothesis was postulated to label one of the prominent proteins (RLC) in the lever arm and so study the organization of the cross-bridge in the relaxed and rigor states.

New advancements in labelling technologies for EM may help to locate a specific protein of interest. APEX2 based protein labelling could reveal the ultrastructure of RLC bound to cross-bridges in muscle by standardising the method for muscle fibre.

Consequently, this method might help to understand the molecular mechanism of muscle contraction by obtaining a high-resolution structure of RLC in muscle fibre. Based on the literature review it is hypothesized that by labelling RLC with APEX2 in intact muscle, it could help to visualise the distribution of cross-bridge around the actin or myosin filament in some extended detail.

1.9. Myosin Regulatory light chain exchange process

Reversible extraction of native RLC in muscle fibre is possible under mild extraction conditions. This technique had been used for extracting and replacing RLC in different muscle fibres (Moss *et al.*, 1982). More recently, the light chain exchange technique was used for the replacement of native RLC with RLC extracted from different tissue or different species (Yang & Sweeney, 1995). Previously, exchange was performed in the presence of ATP and EDTA. EDTA is a chelating agent and can remove the Ca^{2+} ions which serve to stabilise RLC binding to myosin and reversibly dissociate it from the myosin head. Although 80% of skeletal RLC exchange was achieved in the presence of ATP and EDTA, it required 80-fold molar excess of RLC protein (Trybus & Chatman, 1993). To counter the former limitation, a new exchange protocol was developed based on a property of trifluoperazine (TFP). This chemical can induce conformational changes in calmodulin resulting in the exposure of its hydrophobic surface, causing loss of interaction with its target enzyme (Cook *et al.*, 1994, Vandonselaar *et al.*, 1994). The use of TFP in exchange buffer improved the exchange efficiency close to 100% even with 10-fold molar excess of RLC (Yang & Sweeney, 1995). Additionally, TFP has also been used to extract troponin C (similar to RLC structure and belongs to the calmodulin family) from its complex (Putkey *et al.*, 1991). The protocol is explained in detail in Materials and Method 2.2.3.4.

2. MATERIALS AND METHODS

2.1. Materials

Table 2.1: Competent E. coli strains used for the gene amplification and protein expression

Competent cells	Use
NEB10 β , (NEB)	Plasmid amplification and cloning
BL21(DE3)	Protein expression

Table 2.2: Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) Gel constituents and markers

Gels and ladders	Composition
12% SDS-PAGE resolving gel (5 ml)	1.5 M Tris (pH 8.81) 3 ml, 30% Acrylamide 2 ml, 10% SDS 0.05 ml, 10% APS 0.05 ml, water 1.6 ml, TEMED 0.002 ml
4%SDS-PAGE stacking gel (2 ml)	0.5 M Tris (pH 6.80) 5 ml, 30% Acrylamide 0.25 ml, 10% SDS 0.02 ml, 10% APS 0.02 ml, water 1.2 ml, TEMED 0.004 ml
1 Kb ladder	Thermo scientific
100 bp ladder	NEB
Page Ruler Plus Pre-Stained Protein Ladder 10-250 kDa, Precision Plus Protein™ Dual Colour	Thermo Scientific, Bio-Rad

Table 2.3: Buffers and composition

Buffers	Composition
Relaxing solution (1X)	70 mM K ⁺ propionate, 8 mM Mg ²⁺ acetate, 5 mM K ₂ EGTA, 7 mM Na ₂ ATP, 6 mM imidazole, 10 mM PMSF (serine protease inhibitor), 50 mg/L trypsin inhibitor, 4 mg/L leupeptin (thiol
Skinning solutions	1X Relaxing solution + 1% triton-x-100
RLC-APEX2 Lysis buffer	25% Sucrose, 50 mM Tris-HCl (pH 8), 0.1 M NaCl, 0.2 M EDTA, 1 mM DTT, 5-10 mg Lysozyme
RLC-APEX2 pellet wash buffer	50 mM Tris, 5 mM EDTA, 1 mM DTT (pH 8)
Inclusion body solubilising buffer/ IMAC column binding buffer for RLC-APEX2	25 mM HEPES (pH 8), 500 mM NaCl, 1 mM MgCl ₂ , 1 mM DTT, 20 mM imidazole, 6 M urea
IMAC washing buffer for RLC-APEX2	25 mM HEPES (pH 8), 500 mM NaCl, 1 mM MgCl ₂ , 1 mM DTT, 50 mM imidazole, 6 M urea
IMAC elution buffer for RLC-APEX2	25 mM HEPES (pH 8), 500 mM NaCl, 1 mM MgCl ₂ , 1 mM DTT, 500 mM imidazole, 6 M
Dialysis buffer A	25 mM HEPES (pH 8), 250 mM NaCl, 1 mM MgCl ₂ , 1 mM DTT, 4 M urea

Dialysis buffer B	25 mM HEPES (pH 8), 250 mM NaCl, 1 mM MgCl ₂ , 1 mM DTT, 2 M urea
Dialysis buffer C	25 mM HEPES (pH 8), 250 mM NaCl, 1 mM MgCl ₂ , 1 mM DTT
APEX2 lysis/ IMAC column binding buffer	50 mM Tris (pH 8), 50 mM NaCl, 10 mM imidazole
APEX2 IMAC wash buffer	50 mM Tris (pH 8), 50 mM NaCl, 20 mM imidazole
APEX2 IMAC elution buffer	50 mM Tris (pH 8), 50 mM NaCl, 250 mM imidazole
DEAE – ion exchange column binding buffer	20 mM KPO ₄ , pH 7.5
DEAE – ion exchange column elution buffer	500 mM KPO ₄ , pH 6.5
10X SDS running buffer	250 mM Tris-base, 1.92 M glycine, 1%SDS
10X TBE	890 mM Tris-borate, 890 mM boric acid, 20 mM BME

2.2. Methods

2.2.1. Molecular biology methods

2.2.1.1. DNA analytical methods

Plasmid and PCR product concentrations and purity was determined by spectrophotometry at 260 nm Nanodrop 2000 (extinction coefficient $50 (\mu\text{g/ml})^{-1} \text{ cm}^{-1}$ for double stranded DNA) (Thermo scientific). Cloned constructs were validated by restriction enzyme digestion with suitable restriction enzymes later confirmed by sequencing by 1st First BASE Laboratories Sdn Bhd (Singapore). Sequencing results were analysed by Bioedit (Ibis Biosciences, USA), and MultAlin (<http://multalin.toulouse.inra.fr/multalin/>) alignment software. Agarose gel electrophoresis was performed in 1 % TAE-agarose gels at constant 100 V with ethidium bromide in 1X TAE buffer. DNA samples were mixed with 5X DNA loading dye before sample loading in the gel.

2.2.1.2. Competent E. coli cells for the transformation of pET-3D-RLC-APEX2 and pET-3D-APEX2 plasmids

Chemically competent NEB10 β and BL21 cells were prepared by the CaCl_2 method Sambrook and Russell 2001 (Sambrook *et al.*, 2001). Cells from glycerol stock were revived by streaking onto an agar plate and growing overnight (8-16 hr) at 37 °C. A single colony was inoculated in LB media and grown overnight in INNOVA 44R incubator shaker (New Brunswick Scientific) at 37 °C, 220 rpm. The overnight grown culture was used to inoculate 1 litre of media and grown to mid-log phase ($\text{OD}_{600} = 0.5$) at 37 °C, 220 rpm. After chilling the cells on ice for 20 min, the culture was centrifuged for 30 min at 4000 rpm at 4 °C, and pelleted cells were resuspended gently in 20 ml ice-cold 0.1 M CaCl_2 , 15 % glycerol (autoclaved). The stock culture was prepared as stated above and stored at -80 °C until further use.

2.2.1.3. E. coli Transformation for introducing recombinant plasmid into bacteria

For transformation, 100 µl of chemically competent cells were thawed on ice and mixed with ~100 ng plasmid DNA. After incubation on ice for 30 min, cells were heat-shocked at 42 °C for 90 s and allowed to cool down before 400 µl recovery media (LB media without antibiotics) were added. Cells were incubated with shaking at 37 °C and 1200 rpm for 45 min to 1 hr. Cells were spun down at 5000 rpm for 30 sec, 400 µl supernatant media was discarded and the pellet was resuspended in the remaining supernatant to spread on to selection plates, i.e. LB-Agar plates supplemented with respective antibiotics (100 µg/ml Ampicillin) and incubated for overnight at 37 °C. A single colony of the transformants was picked and inoculated in liquid medium for further analysis.

2.2.1.4. Plasmid and oligonucleotides to generate recombinant vector: pET-3D-RLC-APEX2 and pET-3D-APEX2

Oligonucleotides used in this study were purchased from First BASE Laboratories Sdn Bhd (Singapore). To amplify RLC and APEX2, cDNAs primers were designed in such a way that the RLC primers were incorporated with NcoI and BamHI restriction sites as shown in Table 2.4. The annealing temperature for the primers was calculated using online software Oligo Calculator (Thermo Fisher®). A point mutation was incorporated into the RLC reverse primer to mutate the stop codon (TAG to GCG). APEX2 primers were included with BamHI sites on either side to construct pET-3D-RLC-APEX2 plasmid. An additional forward and reverse primers with BamHI, NcoI site respectively was designed to construct pET-3D-APEX2 plasmid as a control for our studies.

Table 2.4: Primers

Name		Sequence	T _m (°C)
RLC	for	CGAGCC <u>CCATGG</u> CACCTAAGAAA	58.8
	rev	TAGC <u>GATCCC</u> CGCGTCCTTCTCTTCT	58.6
APEX2 (for RLC-APEX2 construct)	for	GTC <u>GATCCC</u> GGTGCTGCTATGGGAAAGTCTTACCCAAGT	53
	rev	CCAC <u>GATCCC</u> TAGGCATCAGCAAACCCAAG	52.4
APEX2 (for APEX2 construct)	for	GAGCC <u>CCATGG</u> GAAAGTCTTACCC	60.8
	rev	CCCAC <u>GATCCC</u> TAGGCATCAGCA	60.8

2.2.1.5. Polymerase chain reaction (PCR) for amplification of RLC and APEX2 cDNAs

RLC, APEX2 cDNA fragments amplification was achieved by site-directed mutagenesis and PCR using high fidelity polymerase with proofreading capacity, the Pfu polymerase (ThermoScientific, Singapore). Plasmid DNA was used as the template for PCR, set up in a total volume of 50 μ l on ice with the appropriate concentration of reagents mentioned below (Table 2.5). All reagents were pipetted into a PCR tube kept on ice all the time; reagents were mixed by a short spin. The temperature cycles used for amplification is shown in Table 2.6. Primers used are listed in Table 2.4.

Table 2.5: PCR reaction mix with Pfu polymerase.

Components	Amount
Pfu 5x buffer	10 μ l
10 μ M dNTP	1.5 μ l
(1 U) Pfu Polymerase enzyme	0.5 μ l
template DNA	1 ng
10 μ M forward primer	1.5 μ l
10 μ M reverse primer	1.5 μ l
Total volume	50 μ l

Table 2.6: Temperature cycles for DNA by PCR

Temperature (°C)	Time (minutes)	Cycle(s)
95	3	1
98	20 seconds	25
55	30 seconds	
72	30 seconds	
72	1	1
4	∞	-

2.2.1.6. Vector preparation

A modified pET-3D vector, which contains a His tag (six Histidine residue) and a TEV cleavage site near NcoI site (Figure 2.1), was used for generating RLC-APEX2 and APEX2 plasmids.

Figure 2.1: pET-3D plasmid map.

Digested PCR products were cloned in between NcoI and BamHI site (marked in red). Vector containing Ampicillin resistant gene as selection marker.

2.2.1.7. Small-scale plasmid isolation from bacteria for RLC-APEX2 and APEX2 gene cloning

Plasmids were purified from transformed NEB- β *E. coli* strain using a Miniprep DNA purification kit (GE Healthcare) as described by the manufacturer. For plasmid isolation, 5 ml of bacterial cells were grown overnight with an antibiotic (100 mg/ml ampicillin) at 37 °C and at shaking speed of 220 rpm. Bacterial cells were harvested by centrifuging at 5000 rpm for 10 minutes (3000 g). The bacterial cell pellet was resuspended in 250 μ L cell resuspension buffer and lysed with 250 μ L cell lysis buffer by incubating the mixture at room temperature for 5 minutes. The lysed cells were neutralized with 300 μ L neutralization buffer. The cell lysate including genomic DNA, cell debris and proteins were precipitated by centrifuging at 11,000 rpm for 10 minutes (or 12,000 g). The supernatant containing the plasmid DNA was added into a spin column and centrifuged at 11,000 rpm at room temperature for 1 minute (12,000g). The flow-through was discarded, and the spin column was washed with 750 μ L washing buffer, centrifuged again as described above and this washing procedure was repeated twice. Bound DNA was eluted using 100 μ L deionised water. The isolated plasmid was digested with the above-mentioned restriction enzymes and purified by agarose electrophoresis to remove residual nicked and uncut DNA.

2.2.1.8. Cloning strategy for bacteria to produce recombinant bacteria

Cloning strategy used for RLC-APEX2 and APEX2 is explained in detail.

2.2.1.9. Restriction digestion and ligation of RLC and APEX2 into pET-3D to produce pET-3D-RLC-APEX2 and pET-3D-APEX2 vector

0.5 -3 μ g DNA was digested with the respective restriction enzymes in 20 μ L reaction volume of buffer according to the manufacturer's recommendations. The reaction was incubated for 3 hours at 37 °C (Table 2.7). Digested pET-3D plasmid vector DNA was treated with calf intestinal phosphatase (CIP) to dephosphorylate the plasmid for 1 hour at 37 °C before further use. Linearized plasmids and PCR products were separated by 1%

agarose gel electrophoresis and further purified using the GE DNA Purification kit according to the manufacture's instruction. Purified PCR products and plasmid DNA were eluted in deionized water and were used for later ligation. DNA concentrations were measured using spectrophotometry at 260 nm (extinction coefficient $50 (\mu\text{g/ml})^{-1} \text{ cm}^{-1}$) (Nanodrop).

Table 2.7: Restriction digestion of pcr products

Reagents	Vector	Insert (RLC)	Insert (APEX2)
DNA	30 μl	50 μl	50 μl
Enzyme1 (BamHI) (5-10 units/ μg)	0.5 μl	0.5 μl	0.5 μl
Enzyme2 (NcoI) (5-10 units/ μg)	0.5 μl	0.5 μl	0.5 μl
NEB Buffer (10X)	5 μl	6 μl	6 μl
100X BSA	0.5 μl	0.6 μl	0.6 μl
Milli-Q water	13.5 μl	2.4 μl	2.4 μl

Ligation of purified DNA fragments (insert and vector) was carried out at 37 °C for 4 hours according to the manufacturer's instructions. Approximately 25 ng of digested plasmid DNA and 75 ng of digested PCR product were incubated with T4 DNA ligase in a total reaction mix of 20 μl . After 4 hours of incubation, the entire ligation mixture was transformed into *E.coli* NEB- β cells and plated on LB agar (ampicillin) plates. Two reaction

mixture were prepared for constructing RLC-APEX2 and APEX2, one reaction mixture containing digested pET-3D and RLC; other one containing pET-3D and APEX2. Positive clones were confirmed by colony PCR and restriction digestion. Ligation of the DNA fragments was performed using a molar vector: insert ratio of 1:3 according to the following formula: $\left(kb\ of\ \frac{insert}{vector}\right) \times ng\ of\ vector = ng\ of\ insert\ needed\ for\ a\ 1:3\ molar\ ratio$

2.2.1.10. Cloning of RLC-APEX2 to for gene amplification

pET-3D-RLC and APEX2 PCR product were digested with BamHI restriction enzyme. Ligation of APEX2 into pET-3D-RLC was carried out as described above. The ligation mixture was transformed into NEB- β and plated on ampicillin LB plate (100 μ g/ml ampicillin) positive colonies containing insert was inoculated into 6 ml LB medium supplemented with ampicillin. This culture was grown overnight in a shaking incubator at 37 °C, 220 rpm. The plasmids were purified using Miniprep DNA Purification System (GE healthcare), and their concentrations were measured by spectrophotometry using Nanodrop 2000 Spectrophotometer (Thermo-scientific, Singapore) at 260 nm (extinction coefficient 50 (μ g/ml)⁻¹ cm⁻¹ for double stranded DNA)(Positive clones were confirmed by restriction digestion and DNA sequencing (1st Base Singapore).

2.2.2. Protein Biochemistry to produce quantitative amount of recombinant RLC-APEX2 and APEX2 protein

In this study, the pET-3D vector, which contains an N-terminal hexahistidine tag (His6), was used for the heterologous expression of RLC-APEX2 and APEX2 fusion proteins in *E.coli*. His-Tag proteins were purified by immobilised metal-ion affinity chromatography (IMAC). For protein expression, RLC-APEX2 and APEX2 plasmids were transformed into *E.coli* BL21-DE3. These clones were used for further expression studies. For making glycerol stocks, single colonies of transformed cells were grown in 2 ml of LB media with ampicillin antibiotics overnight at 37 °C and 220 rpm. 500 μ l of overnight cell

suspension and 500 µl of 80% glycerol were mixed/vortexed and frozen with liquid nitrogen and stored at -80 °C.

2.2.2.1. Protein quantification

Protein concentration was measured by their absorbance at 280 nm by spectrophotometry (Nanodrop 2000), and the concentration was calculated using the extinction coefficients listed in Table 2.8. Extinction coefficients of the proteins were calculated with the online program ProtParam (<http://web.expasy.org/protparam/>) (Gasteiger *et al.*, 2005). The final concentration of the protein was calculated using the extinction coefficient and molecular weight.

C-concentration of the protein in mg/ml

A280-absorbance measured by Nanodrop at 280nm

ϵ – Extinction coefficient $M^{-1}cm^{-1}$

Table 2.8: Extinction coefficient and Molecular weight.

Protein	Extinction coefficient ($M^{-1}cm^{-1}$)	Molecular weight (kDa)
RLC-APEX2	20400	48.5
APEX2	17420	29.29

2.2.2.2. SDS-PAGE gel electrophoresis

In order to separate and determine the approximate molecular weight of proteins, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used. Gels were prepared as described in Table 2.2. Protein samples were mixed with 6X SDS-sample buffer and heated for 5 min at 95°C. Gel electrophoresis was carried out in Mini Protean (Bio-rad) electrophoresis chambers at a constant voltage of 100 V for 20 min followed by 140-160 V for the rest of the run.

2.2.2.3. Coomassie blue staining of SDS-Gels

For visualisation of protein in an SDS-Gel Coomassie blue staining was used as a standard method. Coomassie blue dye interacts with protein and gives a visible blue colour to the protein bands in the gel. Protein bands were fixed and stained by incubating the gels for 30 minutes with Coomassie blue staining solution. Background staining was removed by destaining for 1 hr with methanol-acetone based destaining solution. Acetic acid in the destaining solution is useful to fix the protein whereas methanol removes the unbound dye and thereby reduces the background.

2.2.2.4. Test expression of proteins

Glycerol stocks of the RLC-APEX2 and APEX2 plasmids were inoculated in LB supplemented with 100 µg/mL ampicillin (LB plate/amp) at 37 °C in a shaker incubator at 220 rpm shaking condition overnight for the expression of the respective proteins. 50 µl of this overnight culture was inoculated in 5 ml LB media supplemented with antibiotic and grown at 37 °C to an optical density of ~0.6 at a wavelength of 600 nm (OD₆₀₀). The RLC-APEX2 cultures were subsequently shifted to the temperature to be tested (23 °C and 37 °C), whereas APEX2 was shifted to 23 °C. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation (4000x g, 10 minutes) at different time points after induction for RLC-APEX2. Cells were resuspended in 1 ml respective lysis buffers (25% Sucrose, 50 mM Tris-HCl (pH 8), 0.1 M NaCl, 0.2 M EDTA, 1 mM DTT, 5-10 mg Lysozyme for RLC-APEX2 and 50 mM Tris (pH

8), 50 mM NaCl, 20 mM imidazole for Apex2) and incubated on ice for 30 minutes. Before sonication, 1 mM of phenyl methyl sulfonyl fluoride (PMSF) was added to the cells. Cells were lysed on ice by sonication at 30% amplitude for 5 minutes using 15 seconds pulses alternated with 45 seconds of rest. 20 µl of the lysate was added to 5µl of 5X SDS sample buffer for analysis of total protein expression. The samples were denatured by incubation at 95 °C on a heat block and centrifuge for 2 minutes before loading on 12% SDS polyacrylamide gel. 5 µl of a pre-stained marker (PageRulerPlusPrestained Protein Ladder 10-250 kDa; Thermo Scientific) was loaded alongside the samples. Gel electrophoresis was performed at a starting voltage of 120 V for 20 minutes followed by 160 V for the rest of the run.

2.2.2.5. Expression of His6-fusion constructs

Unless otherwise stated all protein, purification procedures were carried out at 4 °C. RLC-APEX2/ APEX2 were inoculated in 100 ml LB with respective antibiotics for large-scale expression and purification. RLC-APEX2 and APEX2 cultures were cooled down to 23 °C before induction; both were induced by 1mM IPTG. Following induction, both were kept at 23 °C overnight at 220 rpm. After incubation, *E.coli* cells were harvested by centrifugation in JA-10 centrifuge with JA 10 rotor (Beckman Coulter) at 5000 rpm for 20 minutes at 4 °C. Cell pellets were either stored at -80 °C or directly lysed with lysis buffer as described in 2.2.2.4.

2.2.2.6. Purification of His6-fusion constructs

2.2.2.6.1. RLC-APEX2 purification

His6-fusion constructs were purified by immobilised metal affinity chromatography (IMAC). Cells were harvested by centrifugation, resuspended in lysis buffer (25% Sucrose, 50 mM Tris-HCl (pH 8), 0.1 M NaCl, 0.2 M EDTA, 1 mM DTT. 5-10 mg Lysozyme). PMSF was added to the resulting suspension and stirred for 15-20 minutes at 4 °C to reduce protein degradation. The cells were lysed by sonication (7-8 cycles of 15 seconds on and 45 seconds off) and centrifuged at 14000 rpm for 20 minutes at 4 °C (17,000 g). The pellets

were washed with 50 mM Tris, 5 mM EDTA, 1 mM DTT (pH 8) and centrifuged. The resulting inclusion bodies were solubilized in 25 mM HEPES, 500 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 20 mM imidazole. The insoluble material was removed by centrifuging at 9000 rpm for 15 minutes (10,000 g), and the soluble component was used for further purification procedure. Protein molecular weight and purity was checked on 12% Gel by SDS-PAGE.

2.2.2.6.2. APEX2 purification

The cell pellet was resuspended in lysis buffer containing 50 mM Tris (pH 8), 50 mM NaCl and 10 mM imidazole. PMSF was added to the resulting suspension and stirred for 15-20 minutes at 4 °C. The cells were lysed by sonication as mentioned for RLC-APEX2 and centrifuged at 24000 rpm for 20 minutes at 4 °C to remove debris (40,000 g). The soluble component was used for the further purification procedure.

2.2.2.7. Ni-NTA purification

The supernatant was bound in batch to an affinity column packed with Ni-NTA resin (Thermo Scientific) equilibrated with respective binding buffers (Table 2.3) 3 times (4 hours/binding). The column was washed with 5 column volumes of IMAC washing buffer containing HEPES based buffer for RLC-APEX2 and Tris-based buffer for APEX2 (composition in Table 2.3). The His6-fusion protein was eluted in 1.5 ml Eppendorf tubes using elution buffer (25 mM HEPES (pH 8), 500 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 6 M urea for RLC-APEX2 and 50 mM Tris (pH8), 50 mM NaCl, 250 mM imidazole for Apex2). Samples from Ni-NTA chromatography were analysed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) to determine the presence of the protein of interest and its purity. Fractions were pooled together and RLC-APEX2 was dialysed overnight against dialysis buffer A (25 mM HEPES (pH 8), 250 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 4 M urea) successively followed by Buffer B (25 mM HEPES (pH 8), 250 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 2 M urea) for 8 hours, then finally 8 hours with Buffer C (25 mM HEPES (pH8), 250 mM NaCl, 1 mM MgCl₂, 1 mM DTT).The purified RLC-APEX2 and APEX2 were concentrated by ultrafiltration using an amicon15 membrane

(Millipore). RLC-APEX2 and APEX2 proteins were used for haeme reconstitution, followed by a second purification using an anion exchanger DEAE column.

2.2.2.8. Haeme reconstitution

Concentrated RLC-APEX2 and APEX2 proteins were used for the reconstitution experiments. Haeme is required for the activity of peroxidase. Reconstitution was carried out as published for APEX2 (JD Martell 2012). RLC-APEX2 and APEX2 purified by Ni-NTA column were dialysed extensively in 20 mM KPO₄ (pH 7.5) three times at 4 °C. To prepare haeme stock (as haeme cofactor source), 50 mg of hemin-Cl (Sigma) was diluted in 2.0 mL of 10 mM NaOH extensively until most of the haeme powder dissolved. Subsequently, haeme solution was diluted to working stock concentration using 8.0 mL of 20 mM KPO₄ (pH 7.5). The extensively mixed mixture was centrifuged at 7,500g for 8 minutes at 4 °C to remove insoluble hemin or aggregates (10,000g). The supernatant was transferred to a new tube, and the centrifugation was repeated for two times. The final supernatant was collected in a fresh tube and used as the working haeme stock. 600 µL of haeme stock (7.6 mM) was added to 400 µL of respective proteins (400 µM) in 100 µL increments over a period of 20 minutes. Both haeme and protein samples were wrapped in aluminium foil to prevent photodamage. The haeme-protein mixtures were gently agitated at 4 °C for overnight and then centrifuged at 7,500g for 15 minutes at 4 °C to remove aggregates. The supernatant was loaded onto a HiTrap DEAE Fast-flow column (GE Healthcare).

2.2.2.9. Anion Exchange Chromatography to purify reconstituted RLC-APEX2 and APEX2

A Diethylaminoethyl cellulose (DEAE) column was utilized with an Akta Purifier system (GE Healthcare Life Sciences, UK). Proteins were eluted into fractions using a salt gradient by gradual substitution of 0 to 100% Purification Buffer A (20 mM KPO₄ (pH 7.5)) with Purification Buffer B (500 mM KPO₄ (pH 6.5)). The column had been pre-equilibrated with low salt (20 mM KPO₄, pH 7.5) at 4 °C until the ionic strength of the column became stable. 1ml of RLC-APEX2 or APEX2 and haeme mixture was loaded to the pre-

equilibrated column at the rate of 1 ml/minute. The column was washed with 5 column volumes of 20 mM KPO₄ (pH 7.5), followed by elution with 5 column volumes of 250 mM KPO₄ (pH 6.5). At this ionic strength and pH, APEX2 was eluted from the column, whereas excess haeme remained bound. APEX2 typically eluted over a volume of ~5mL. Fractions containing reconstituted APEX2 were identified by absorbance at 280 nm, and these fractions were used for western blot to confirm the presence of fusion protein.

2.2.2.10. Western blotting and immunodetection

After SDS-PAGE, proteins were transferred to an activated Polyvinylidene difluoride (PVDF) membrane using transfer buffer (50 mM Tris-HCl (pH 8.0), 20% methanol, 192 mM glycine) in a semi-dry western blot apparatus (Bio-rad). The membrane was activated by incubating with 100% methanol for a few seconds. Activated membranes and SDS-PAGE gels were pre-incubated with Phosphate buffered saline (PBS) with Tween-20h transfer buffer. Gels and PVDF membranes were assembled in a transfer tray as illustrated by the manufacturer. The transfer was performed at 25 volts for 30 min. The membranes were blocked with 100% methanol for a few seconds. Primary antibodies, anti-His (rabbit polyclonal) and anti-RLC (goat polyclonal) were diluted 1:1000 in 1X Phosphate buffered saline with Tween-20 (PBST) with 5% w/v non-fat dry milk. The primary antibody was incubated with the membrane for one hour at room temperature and subsequently washed three times with PBST (PBS, 0.1% (v/v) TWEEN 20) with 5 minutes incubation time between each wash. The secondary antibody, horseradish peroxidase (HRP) conjugated, anti-mouse and anti-goat were diluted 1:8000 in 1X PBST with 5% w/v non-fat dry milk (to reduce non-specific binding) and incubated with the membrane for 1 hr at RT followed by another three times of wash. The HRP coupled antibody was detected by enhanced chemiluminescence (ECL) staining with a 1:1 mix of ECL detection reagent 1 and 2 (Millipore). ECL detection reagent mixture was poured on the membrane and incubated for 1 minute. Excess reagent was removed. Blots were developed on X-ray film with a Kodak Imaging System.

Reconstituted APEX2 and RLC-APEX2 were concentrated at 4 °C and equally divided into two fractions, one fraction was exchanged into PBS buffer (3 × 4 mL) for

enzymatic assay, and remaining fraction was exchanged into exchange buffer for further EM experiment using an Amicon Ultra-4 filter with an Ultracel-10 membrane (Millipore). Reconstituted APEX2 RLC-APEX2 samples were flash-frozen in liquid Nitrogen for storage at -80°C until needed for further test.

2.2.2.11. Enzymatic activity assay of reconstituted RLC-APEX2 and APEX2

Activity assays were performed by spectrophotometry in an 1800 UV-vis spectrophotometer (Shimadzu) using a cuvette reader (Martell *et al.*, 2012, Lam *et al.*, 2014) at 470 nm $\epsilon_{470} = 22 \text{ mM}^{-1} \text{ cm}^{-1}$. Equal concentrations of purified, haeme-bound holoenzyme were used for each experiment. Concentrations of RLC-APEX2 were calculated based on holoprotein content, as determined Bradford assay. HRP (Sigma) (which was used as a positive control for the assay) was dissolved into PBS (pH 7.4) from a lyophilized form, and enzyme concentration was calculated using the Bradford assay. Guaiacol (Sigma), a standard substrate for peroxidase was diluted in room temperature PBS (pH 7.4) to yield a concentration of 100 mM. Solutions were agitated at 37°C for at least 5 minutes and then vortexed thoroughly to ensure the guaiacol was dissolved entirely. H_2O_2 (Sigma) was diluted from stock solutions to the final concentrations indicated in each result figure (Figure 3.8). 100 μL reactions were assembled in quartz cuvettes by adding guaiacol solution, then APEX2 fusion protein corresponding to each reaction. Reactions were initiated by addition of H_2O_2 . Conversion of guaiacol to tetraguaiacol was measured at 470nm over a period of time until the absorbance reached a stable value. The maximum absorbance value was noted for each reaction and plotted against the concentration of H_2O_2 to compare the maximum product conversion by APEX2 fusion protein, APEX2 and HRP. The rate of conversion of H_2O_2 into reactive oxygen was measured by the conversion of substrate guaiacol into a coloured product at 470nm for the initial 30 seconds.

2.2.3. Rabbit dissection

New Zealand white ex-breeder rabbits was sacrificed via intravenous injection of 2 ml sodium pentobarbital into the shaved ear. Psoas major muscle was used for the electron microscopic studies. The psoas major muscle in rabbits is a well-developed muscle,

consisting of regularly arranged relatively uniform parallel fibres. This enables easy dissection and combined with relatively homogenous myosin isoform, makes psoas an ideal candidate as a source of muscle fibres. Rabbit dissections were done by myself under the supervision of Dr. Haiyang Yu.

To dissect psoas muscle fibres, the rabbit carcass was placed on supine position and an incision, approximately 20 cm in length, was made down the centre of the abdomen with a scalpel. The skin and superficial layers were folded back exposing the intestine, which was moved to one side to reveal the psoas muscle.

Using angled Rabbit dissection forceps, the muscle fascia was pulled away. Muscle surface was bathed with a relaxing solution to prevent drying. In the central region of the muscle one pair of forceps was used to pierce through a few millimetres of tissue. Gentle lateral movement, running along the direction of the muscle fibres, separated a bundle of fibres- around 5 cm in length. Two surgical silk sutures were passed beneath the bundle, and an applicator stick was placed on top. Double sutures were made at each end, securing the bundle to the stick but without cutting into fibres. Once tied, the bundle was cut at each end releasing it from the body. This process ensured the samples were taken with sarcomere at their resting length. Once dissected, the bundles were placed in a beaker of relaxing solution on ice. Approximately 10-12 bundles were removed from each animal.

The bundles were transferred into a beaker of a fresh, relaxing solution and incubated at 4 °C for 90 minutes. This was followed by three more incubation stages where the glycerol content was gradually increased from 0 to 50%, see Table 2.9 for schedule. Once the process had been completed the bundles were stored individually at -20 °C in 15 ml tubes containing 50% glycerol solution. Bundles were stored and used for up to 6-12 weeks.

Table 2.9: Rabbit muscle fibre glycerol treatment for long term storage (at 4 °C)

End of dissection	0 minute
Change into fresh 0% glycerol relaxing solution	90 minutes
Change into fresh 12.5% glycerol relaxing solution	90 minutes
Change into fresh 25% glycerol relaxing solution	Overnight
Change into fresh 50% glycerol relaxing solution	4 hours at 4 °C
Store at -20 °C	Upto 6 weeks

2.2.3.1. Muscle fibre preparation for electron microscopy and fluorescent microscopy

A small segment of the muscle sample was cut out from the stored rabbit muscle segment and placed in a glass dish containing relaxing solution. The glass dish was cooled to 4 °C on the stage of a dissecting microscope connected to a refrigeration system. Using forceps, the fibres were separated into either individual fibres or small bundles of two-three fibres under the dissecting microscope, epi-illuminated through the platform. Care was taken to minimise damage to the fibres, handling them only at the ends. Once a suitable fibre had been isolated, damaged ends were removed with a pair of micro-scissors and ~ 4 to 5 mm in length fibre segments were used for the EM experiments.

T-clips, small t-shaped pieces of aluminium with a hole in the main arm were introduced to the bath, and the shorter arms of T were folded up. Each end of the dissected fibre was then placed in a T-clip and folded over the ends securing the fibre by crimping

(as shown in Figure 2.2). T-clip attached fibres were transferred to a glass dish containing skinning buffer (composition in table 2.3) to remove the membrane.

Figure 2.2: T-clip attached muscle fibre

2.2.3.2. Chemical permeabilisation of fibres

Chemical permeabilisation of fibres - a process which perforates the cellular membrane of the cells to enable free diffusion of solutes into and out of the fibre - is carried out by incubating muscle fibres in relaxing solution containing 1% Triton-X-100 approximately for 1 hour. Soluble proteins and membrane-bound proteins will diffuse out of the fibres following this detergent treatment. The plasma membrane, as well as the mitochondria and sarcoplasmic reticulum, are disrupted in chemically permeabilised fibres, removing residual ATPase activity from Ca^{2+} pumps (West T G 2004). Moreover, permeabilisation allows the exchange of small filament-bound proteins, for example to replace endogenous RLC with labelled RLC.

Our lab has been using chemically permeabilised rabbit psoas muscle as a model for biophysical studies. The benefit of permeabilised muscles fibre is that they allow for the

study of the contractile machinery, namely the sarcomeric protein filaments without disrupting the integrity of their structure and position in a cell. Moreover, permeabilised muscle fibres lack the cell membrane and associated intracellular signalling components (Figure 2.3), which make easy to manipulate the system at different physiological conditions by providing exogenous resources for metabolic energy (e.g. Mg_2ATP) and chemical reconstitution of the intracellular solution in buffered media. Permeabilization has been used successfully to investigate the physiological properties of skeletal muscle (He *et al.*, 1999, Bershitsky *et al.*, 2010). Permeabilised muscle fibres are effectively used for the exchange of endogenous small proteins like myosin light chains which are non-covalently bound to filament backbones of muscle, with fluorescently or chemically labelled proteins for various studies (Ushakov, 2008, Caorsi *et al.*, 2011). The light chain exchange method, a well-established technique in our lab is extensively exploited for understanding the role of phosphorylation in RLC. Furthermore, this method is used for the exchange of ELC with fluorescently- labelled protein for structural dynamic studies. Published results show that 60-70% of the native protein can be exchanged with recombinant protein with the little altering of the mechanical function of the muscle (Caorsi *et al.*, 2011).

Figure 2.3: Chemical permeabilisation of rabbit psoas muscle fibre.

Rabbit muscle fibres were treated with triton-X-100 detergent to remove the membrane and associated proteins. Electron microscopy images of 60 nm thin section of rabbit muscle were shown before (lower) and after (upper) permeabilisation. Increased contrast in chemical skinned fibre is due to the use of tannic acid as post fixative.

2.2.3.3. RLC-APEX2 and APEX2 labelling with rhodamine

Concentrated RLC-APEX2 was exchanged into labelling buffer (NaH_2PO_4 (pH 7.2), 0.1 M NaCl) using Amicon centrifuge tube. About 2.5 ml of 2 mg/ml RLC-APEX2 protein was incubated with 10 mg/ml N-hydroxysuccinimide-rhodamine ester (NHS-rhodamine was dissolved in DMSO) for 2 hr on ice in the dark. Excess dye was removed using Zeba desalting spin columns (Thermo scientific). An SDS-gel was run to check the conjugation of dye to the protein and to further confirm the absence of excess dye in the protein mixture.

2.2.3.4. RLC-APEX2 exchange into skinned muscle

Light chain exchange is used for the replacement of native light chains (RLC or ELC) with recombinant light chains or extracted from different tissue or different species in permeabilised muscle. Light chain exchange protocol has shown that the contractile physiology of exchanged muscles is preserved and is effective due to structural homology of light chain isoforms in different species or tissue (Yang & Sweeney, 1995). Rhodamine labelled or reconstituted RLC-APEX2 were exchanged into ‘exchange buffer’ (buffer composition 150 mM potassium propionate, 10 mM KH_2PO_4 , 5 mM DTT, 5 mM ATP, 5 mM EGTA, 5 mM EDTA and 0.5 mM trifluoperazine (pH 6.5)) using Amicon ultra-centrifuge tubes for recombinant protein exchange into permeabilised muscle fibres. A slightly modified version of Borejdo, J et al (2001) RLC exchange protocol was used for our studies. T-clipped skeletal muscle fibres were mounted between two hooks on the experimental stage, where one of the fibre ends is attached to the force transducer and other end attached to a motor via the T-clips (as shown in Figure 2.4). Before initiating the exchange process, the sarcomere length was adjusted to 2.4 μm using a diffraction pattern generated by a 532 nm He-Ne laser transmitted through the fibre (Bershitsky *et al.*, 1996). The exchange process was carried out at 20 °C. Fibres were incubated with 0.5 mg/ml or 0.25 mg/ml RLC containing exchange buffer with 0.5 mg/ml TnC, this process was repeated three times for 15 minutes each. Non-incorporated or excess RLC was washed out with relaxing buffer three times. Exchanged muscle was further used for subsequent fluorescent microscopy and EM sample preparation immediately after the process.

A

B

Figure 2.4: Schematic diagram for RLC exchange

(A) Muscle fibre force measurement experimental setup (B) Schematic illustration of recombinant protein exchange into the muscle fibres. skinned muscle fibres were immersed in exchange buffer containing recombinant RLC protein for 15 minutes and three times at 20 °C. during this process trifluoperazine, a chemical present in the exchange buffer help to exchange recombinant protein with native protein in muscle.

2.2.3.5. Fluorescence microscopy imaging of rhodamine labelled RLC-APEX2 exchanged muscle

Localization of RLC in muscle fibre was examined by confocal fluorescence microscopy. Exchanged muscles with rhodamine-labeled RLC-APEX2 or rhodamine-labeled APEX2+ RLC were fixed with 1% paraformaldehyde for 15 minutes before taking images. Post fixing, the muscle samples were washed thoroughly (3 times for 15 minutes) with PBS buffer to avoid background fluorescence. Fixed samples were mounted on a glass slide with a mounting solution. Images were recorded at 555 nm.

2.2.3.6. Biophysical experiments

To assess the physiological properties of RLC-APEX2 exchanged muscle, we tested the isometric force production (Experiments were done with the help of Dr. Haiyang Yu). A single permeabilised muscle fibre was mounted between two hooks (similar apparatus used for RLC exchange). Isometric force for the exchanged muscles and non-exchanged fibre were calculated (Toepfer *et al.*, 2013). At the beginning of the experiment, sarcomere length was set to 2.1 μm using diffraction of helium-neon laser light (633 nm) to visualize the first-order diffraction pattern spacing. Skeletal muscles were taken through a sequence of solution changes using a two-stage platform system and moved horizontally using a stepper motor. Each platform had two pedestals, which carried 25 μl of solution on their surface. The platform was moved so that the skeletal fibre was submerged in these solutions in the following order: relaxing, pre-activating, and relaxing. The relaxing solution contains MgATP but no added calcium, thus maintaining the fibre in the relaxed state. The pre-activating solution was similar to relaxing solution except that the concentration of the calcium buffer, EGTA, is much reduced to accelerate the rate of increase of calcium concentration in the core of the fibre when the fibre is transferred from pre-activating to activating solution. The activating solution contains 32 μM calcium strongly buffered with EDTA, as well as MgATP (5 mM). Following transfer to activating solution, the fibre develops force to reach the isometric plateau. The fibre was then shifted to relaxing buffer where force returned to the relaxed level. Throughout the process, force was recorded. The final concentration of reagent and ionic strength was calculated by a computer program.

Table 2.10: Relaxation-Activation buffer

Composition (mM)	Relaxing (pH7.1)	Pre-activating (pH7.1)	Activating (pH7.1)
TES	100	100	100
MgCl ₂	7.8	6.8	6.5
Na ₂ ATP	5.7	5.7	5.7
EGTA	25	0.1	0
GLH	20	20	20
HDTA	0	24.9	0
CaEGTA	0	0	25

2.2.3.7. Chemical fixation, dehydration and plastic embedding of non-exchanged muscle in relaxed or rigor state

After permeabilisation, the psoas muscle was kept in relaxing or rigor buffer for preparing muscle in relaxed or rigor state. For preparing muscle in rigor, muscle fibres were washed with a series of buffer as mentioned in table 2.11 to remove all the substrate ATP and get muscle in rigor state.

Table 2.11: Rigor preparation buffer (Ionic strength was adjusted to 150 mM with potassium propionate)

Composition (mM)	Relaxing (pH 7.1)	Pre-Rigor (pH 7.1)	Mg ²⁺ free Rigor	Mg ²⁺ free Rigor	Ca ²⁺ free Rigor
TES	60	60	60	60	60
ATP (Na ₂ ATP)	5	0.1	0.1	0	0
Mg ²⁺ (MgCl ₂)	5	0.1	0.1	0	0
EGTA (K ₂ EGTA)	30	30	30	30	30
EDTA	0	0	10	10	0
Ca ²⁺	0	0	0	0	0

During the above-mentioned process, muscle fibres were pinned to Sylgard gel in a Petri dish (before pinning, muscle fibres were straightened). Following rigorisation, fibres were fixed with 2% glutaraldehyde for 1 hour. For relaxed state fibres were fixed with 2% glutaraldehyde in relax buffer for 1 hour, followed by a second fixation with 2% glutaraldehyde + 0.2% tannic acid (w/v) in 0.1 M cacodylate buffer (CB buffer) on ice for 30 minutes on ice. Subsequently, all the procedures were done on ice unless otherwise mentioned. After rinsing with cacodylate buffer, the muscle was post-fixed with 1% aqueous osmium tetroxide (OsO₄) approximately for one hour (until muscle fibre became black in colour). The muscle was then dehydrated in a graded ethanol-series (20, 50, 70, 90 %), each for 15 minutes, and finally with 100% ethanol for 15 minutes at room temperature two times. Samples were then infiltrated in epoxy resin overnight and embedded in epoxy resin. Polymerization of the resin was carried out at 64 °C for 24-48 hours.

2.2.3.8. EM sample preparation for RLC-APEX2 exchanged muscle

Exchanged muscle fibres were pinned to a sylgard plate as mentioned for non-exchanged muscle sample preparation. Fibres were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (0.1 M sodium cacodylate pH 7.2) either in Relaxing buffer or Rigor buffer. After the addition of the glutaraldehyde solution, fibre-containing plates were immediately transferred on ice and incubated for one hour. Further procedures were done on ice until dehydration with 100 % ethanol. After one-hour incubation, excess or un-reacted glutaraldehyde was quenched by using blocking solution containing glycine (50 mM glycine in 0.1 M CB buffer) for 10 minutes and three times. Fibres were washed with cacodylate buffer 5 minutes 5 times before DAB- peroxide reaction. 1.4 mM of DAB free base was prepared in 0.1 M HCl. Properly dissolved DAB solution was diluted with chilled 0.1 M CB buffer in a ratio of 0.5 ml DAB in 4.5 ml of CB buffer. 3 mM of H₂O₂ was added to DAB mixture just before adding to cells and incubated for 5 minutes. The further reaction was stopped by rinsing with CB buffer 5 minutes, 5 times. Further procedure including post-fixation with OsO₄, dehydration, resin infiltration, and plastic embedding was done as previously mentioned in 2.2.3.7.

2.2.3.9. Ultramicrotomy

For tomography and 2-D EM imaging (longitudinal and cross-sectional sectioning), thin ~100 nm and 60 nm sections were cut with a diamond knife using a Leica ultramicrotome (Leica EM UC7). For longitudinal sectioning sample was aligned in a way that cutting direction was perpendicular to the fibre axis to reduce the damage to sample by compression as shown in Figure 2.2. Sections were picked up on thin bar 200 mesh copper grids. Non-APEX2 samples were stained with Reynolds lead citrate for 8 minutes. After staining sections were rinsed with Milli-Q water three times, tomography sections were coated with 2.5 nm thick carbon on both sides. These sections were then glow discharged for 80 seconds on both sides and dipped into a solution of 10 nm colloidal gold particles in 0.1% BSA for 30 seconds and air-dried.

2.2.3.10. Electron microscopy and tomography:

Electron tomography is a technique that utilises the transmission electron microscope (TEM) to record a series of 2D projections of an object (specimen) from various angle by incrementally tilting that object in a multiple directions with respect to the electron beam and uses these projections to generate a 3–D reconstruction (tomograms) of the object (Midgley & Weyland, 2003, Frank *et al.*, 2012). Electron tomography follows the same principles as that used in other imaging modalities (such as X-ray computed tomography). Projections of the same object are taken at a range of angles surrounding the object (Franke *et al.*, 2019). This is done in the electron by tilting the specimen as the beam is fixed. The three-dimensional reconstruction is then based on the central slice theorem which states that ‘a projection at a given angle is a central section through the Fourier transform of that object’ (Midgley & Weyland, 2003) hence, performing an inverse Fourier transform on the collated Fourier transformed projections results in the reconstruction of the object in three dimensions.

Essentially, electron tomography consists of four main steps, which are schematically shown in Figure 2.5. In the first step, a series of tilt images is acquired from the same region of interest over a tilt range in angular increments of typically 1 or 2 degrees. The obtained tilt series of images is aligned to a common tilt axis in order to eliminate relative shifts and rotations between the successive images. A reconstruction is calculated using a mathematical algorithm applied to the aligned series of images (Leary *et al.*, 2012). In the last step, a quantification of the tomogram is carried out for further analysis.

Figure 2.5: Schematic diagram for electron tomography experiment

Single-axis/ dual-axis tilt acquisition

Single-axis tilt acquisition is the most common acquisition method followed in tomography. It is based on tilting the specimen around the eucentric axis of the microscope stage with constant angular tilt increment over a large tilt range. To collect all the information to create an accurate 3D reconstruction, the sample needs to be tilted ideally over 180° in the microscope. Because of the limited spacing between the pole pieces of the objective lens of the microscope and the tilt limit of the microscope goniometer, it is not always possible to cover the full range. The limited angular range leads to missing projections at the high tilt angles and results in a ‘missing wedge’ of information in Fourier space and thereby induces artifacts in the 3D reconstruction. Dual-axis tomography helps to solve the missing wedge information to some extent. Dual-axis tomography acquires second tilt series images, at 90° to the first axis and combines two tilt series to produce better resolution image (Mastronarde, 1997).

Alignment

When acquiring the images at the different tilt angles, the specimen is tracked into the field of view at each angle, which results in possible local shifts between the different images. In order to obtain a 3D reconstruction of the tilt series, these local shifts need to be compensated for. To compensate for these shifts, we search for the common tilt axis of all images. The alignment procedure in general is done either by fiducial marker tracking or by cross-correlation (Kremer *et al.*, 1996). The fiducial marker tracking alignment requires that markers are added to the sample. The alignment is then done by measuring the coordinates of the fiducial markers through the entire tilt series. Typically, gold nanoparticles are used as fiducial marker.

When using cross-correlation for the alignment, there is no need for markers. The method is based on calculating the cross-correlation between two successive projection images (Winkler & Taylor, 2006). The cross-correlation image is formed by multiplying the Fourier transform of the first projection with the complex conjugate of the Fourier transform of the second projection. The actual cross-correlation image is the inverse Fourier transform of this product. The position of the maximum intensity in the cross-correlation

presents the relative shift between the two projections. The correlation will not coincide completely because the two projections are acquired at different tilt angles, therefore the peak determination is done iteratively and cannot be solved exactly. To simplify the determination of the peak position of the cross-correlation image, a band pass filter is often used. The band pass filter is applied on both projections before the cross-correlation image is obtained, which improves the quality of the peak determination. Here, in this thesis the alignment was done based on cross-correlation.

Reconstruction

The purpose of the tomographic reconstruction is to obtain the 3D structure of the specimen from the aligned tilt series. There are several approaches for the reconstruction. The 3D structure can be reconstructed by an analytical method, such as Weighted back-projection (WBP). This is the most common reconstruction method used in ET due to its simplicity and fast (Midgley *et al.*, 2007). These analytical methods are based on the central slice theorem. Back projection is based on evenly distributing the intensity in each projection image over computed back-projection rays. This back-projecting process is repeated for all the projection images, which results in intersecting rays at certain positions. The intersection reinforces the intensity at points where mass is present in the original structure. The resulting reconstruction yields blurring in real space due to the uneven sampling of the spatial frequencies in Fourier space, which is solved by applying a weighted filter. The solution to the reconstruction problem is found based on an iterative method.

These iterative reconstruction algorithms are developed to improve the quality of the reconstruction in comparison to the quality of the reconstruction when using weighted back projection. The latter quality is far from perfect because of the limited sampling in Fourier space. The improvement of the iterative algorithms is based on the fact that each projection is a perfect reference of the object. Nowadays, the most popular iterative technique is the simultaneous iterative reconstruction technique (SIRT). SIRT is based on reprojecting the current reconstruction and comparing the reprojections to the original projections (Chen *et al.*, 2016). By taking the difference projection, a difference reconstruction can be obtained to correct the previous reconstruction. This procedure is performed iteratively until an adequate solution is reached.

IMOD

IMOD is a set of image processing, modelling and display programs used for tomographic reconstruction and for 3D reconstruction of EM serial sections and optical sections. The software contains tools for assembling and aligning data within multiple types and sizes of image stacks, viewing 3-D data from any orientation, and modelling and display of the image files. IMOD was developed by David Mastronarde, Rick Gaudette, Sue Held, Jim Kremer, Quanren Xiong, and John Heumann at the University of Colorado.

2.2.3.10.1. RLC-APEX2 exchanged muscle tomography reconstruction

Here in this thesis, muscle sections were examined under FEI T12 TEM electron microscope at 120 kV and photographed using a CCD camera (4k x 4k). For acquiring 2D images of RLC-APEX2 exchanged muscle sample, 60 nm thick sections were photographed at different magnification. For tomography, images were recorded for 100 nm sections at 120 kV using FEI T12 TEM. Dual-axis tomography tilt-series were done for the sample to improve the resolution. The image acquisition was fully automated as previously described using the software SerialEM, Boulder Colorado (Medalia *et al.*, 2002, Majorovits *et al.*, 2008). The areas selected for tomography were baked for 30 min at lower magnification to stabilize the section due to the high-intensity beam and to minimize shrinkage of the section thickness during the tilt series and reduce the effects of within-tilt-series variation on the subsequent back-projection. Pre-irradiating the section in this way subjected the specimen to the steepest portion of the nonlinear shrinkage curve before data were collected (Kizilyaprak *et al.*, 2015). All images were binned to 472 x 472 pixels and photographed at 23,000x magnification. Images from muscle samples were recorded at 2 μm under focus and electron dose of 15 $\text{e}/\text{\AA}^2$. Flat field correction was done to gain normalization. Tilt series about 2 orthogonal axes were recorded from -64 to +64 degrees in steps of 1 degree automatically using the software. The dual-axis tilt series images were aligned and back-projected using IMOD 4.9 software (Boulder Laboratory for 3-D Electron Microscopy of Cells, Colorado, USA). The image processing is explained briefly. In order to reconstruct the dual axis single tomogram, the files were changed to 'mrc' format and opened in IMOD software. With the help of software, X-ray and other flaws in CCD camera was removed to

reduce the artefacts due to jerky movements in the tomogram. Subsequently, alignment was done manually by identifying 10 nm gold fiducial marker on both axes. Before the final alignment and merging two tilt series, each series was binned for $2 \times 2 \times 2$. These micrographs were back-projected and combined to generate the tomogram.

3. RLC-APEX2 FUSION PROTEIN FOR LABELLING CROSS-BRIDGES IN RABBIT STRIATED MUSCLE

3.1. RLC-APEX2 gene construct for exchange with native rabbit skeletal muscle RLC

In order to visualise the muscle cross-bridge in the relaxed and rigor states with APEX2-labelled RLC, the RLC-APEX2 fusion protein was expressed in bacteria and subsequently exchanged with the endogenous protein. To express the RLC fusion protein in bacterial expression system, we generated the gene construct containing human ventricular RLC fused with APEX2 at its C-terminus as well as asAPEX2 alone as a control.

The protein sequences of human ventricular RLC and isoforms of rabbit skeletal RLCs (Figure 3.1A) show 73% sequence similarity. The reported phosphorylatable serine residues and calcium-binding domains (marked in square in Figure 3.1A.) are highly conserved in the sequences.

As mentioned earlier (method 2.2.3.4) RLC can be exchanged between different types of tissues or animal without affecting muscle physiological properties. So, the aim was to express ventricular RLC fused with APEX2 and subsequently exchange this with native rabbit skeletal RLC.

Since the N-terminus of RLC is needed for the Ca^{2+} dependent conformation of myosin head during its actin-activated ATP hydrolysis (Nieznanski *et al.*, 2003), so APEX2 was tagged to the C-terminus of RLC. APEX can be fused to either the N-terminus or C-terminus of a protein of interest without altering the protein structure and function (Martell *et al.*, 2012). Thus, a gene construct was designed in which APEX2 was fused to the C-terminus of RLC (Schematic representation of gene construct is shown in Figure 3.1B.) Studies have shown that interaction of RLC with myosin head through the N-and C-termini affect the flexibility and stiffness of the myosin lever arm (Hopkins *et al.*, 1998). Burghardt et al. showed in their GFP-tagged RLC studies that the use of a smaller linker sequence (8 amino acid) helped in the production of the folded protein (Burghardt *et al.*, 2007). Hence, a linker sequence was added between APEX2 and RLC comprising six amino acids for connecting the different domains of the two proteins for their independent folding (Figure 3.1C).

The RLC-APEX2 gene construct (Figure 3.1A) was used for further expression and subsequent experiments. To validate the kinetic activity of the recombinant RLC-APEX2 fusion protein, the APEX2 gene construct was also generated. For both gene constructs, six histidine residues were added at their N-termini to permit purification by metal affinity chromatography.

3.2. Construction of the RLC-APEX2 and APEX2 in bacterial expression vector

To obtain RLC-APEX2 and APEX2 gene constructs for expression studies, RLC and APEX2 cDNA were cloned into a modified pET-3D vectors containing the His-Tag in a sequential manner as explained in Materials and Methods (2.2.1.9 and 2.2.1.10). PCR amplification products of RLC and APEX2 cDNAs are shown in Figure 3.2A. A 500 bp DNA band in lane 1 and ~750 bp band in lane 2 correspond to the PCR amplified cDNA of RLC and APEX2 respectively. The verified PCR products were digested with respective restriction enzymes (Table 2.7). To clone the amplified PCR products into the pET-3D vector, the vector was also digested with the same restriction enzyme (Figure 3.2B). A band proximately 5 kb represents the digested pET-3d vector. A small band of 500 bp in the same lane corresponding to an unrelated gene previously cloned into the vector, showing successful digestion of the vector with the respective enzymes. The 5 kb size band (digested vector) was eluted from the gel by agarose gel purification method. The purified digest of RLC PCR product was cloned into pET-3D vector to generate the pET-3D-RLC plasmid. RLC clones were confirmed by digestion with previously employed restriction enzymes, as shown in Figure 3.2C. Digestion of RLC insert from the vector indicates successful cloning (cut lane Figure 3.2C). This plasmid was used for transformation into *E. coli NEB-β10*

To create pET-3D-RLC-APEX2, BamHI digested APEX2 product was cloned into pET-3D-RLC digested with the same enzymes. The correct orientation of the APEX2 insert was confirmed by PCR using RLC forward primer and APEX2 reverse primer (indicated in methods) (Figure 3.2D). As predicted, the resulting PCR product was 1.5 Kb in size as seen in line 2 of Figure 3.2D. This confirms the presence of RLC-APEX2 fusion gene construct. As a control for the experiment, APEX2 alone was cloned into pET-3D vector digested with Bam HI and NcoI (result not shown). Both plasmids were sequence-verified. pET-3D-RLC-APEX2 and pET-3D-APEX2 were transformed into *E.coli BL21* for protein expression studies.

Figure 3.2: Construction of the RLC-APEX2 in expression vector.

(A) Restriction digestion of the PCR products of cDNAs: RLC digested with BamHI and NcoI (lane2) and APEX2 digested with NcoI (lane3) respectively. (B) pET-3D plasmid double digestion- lane 2 undigested and lane 3 shows digested plasmid with BamHI and NcoI. A small band at lower side shows some other clones in the parental pET-3D vector. (C) Confirmation of pET-3D-RLC clone by restriction digestion. Lane2 undigested pET-3D-RLC clone and lane3 digested pET-3D-RLC clone. (D) Confirmation of RLC-APEX2 clone by PCR- Lane 2 negative control (pET-3D vector alone), lane 3 pET-3D-RLC-APEX2 clone respectively.

3.3. Expression and purification of RLC-APEX2 and APEX2 proteins

For labelling the cross-bridge structure of muscle, the RLC-APEX2 fusion protein has to be expressed in bacteria, purified, reconstituted and exchanged with endogenous RLC. In order to test the expression of RLC-APEX2 and APEX2 as well as their stability under different conditions, small-scale expression studies in 5 mL LB broth with ampicillin antibiotic were carried out. Both fusion proteins were expressed in *E. coli* BL 21-DE3. Test expression studies at different temperatures and various concentrations of IPTG showed that RLC-APEX2 and APEX2 fusion proteins exhibit an optimal level of expression at 1 mM IPTG and at 22 °C and 37 °C respectively. These conditions were used for large-scale expression and purification of RLC-APEX2 and APEX2. Successful expression of 48 kDa RLC-APEX2 and 28 kDa APEX2 after induction with 1 mM IPTG at 22°C overnight and 37°C for 3 hr respectively was seen using SDS-PAGE analysis (Result is not shown). However, RLC-APEX2 and APEX2 protein expressed at 37°C was found to be degraded in small scale purification, we opted for overnight expression at the lower temperature of 22°C to increase the yield of full-length protein and to avoid degradation.

RLC-APEX2 and APEX2 were expressed as His-tagged fusion proteins in *E. coli* BL21-DE3. Similar to other studied RLC fusion proteins, the RLC-APEX2 fusion protein was found in inclusion bodies (Burghardt *et al.*, 2007, Toepfer *et al.*, 2013). The protein was solubilised by 6M urea and separated from the soluble proteins as outlined in the Materials and Methods section (Materials and Method 2.2.2.6 and 2.2.2.7). His-tagged RLC-APEX2 fusion protein was eluted from Ni-NTA column with 500 mM imidazole in HEPES buffer (pH 8). APEX2 protein was expressed as a soluble protein and eluted with 300 mM imidazole in Tris buffer (pH 8). Eluted fractions were analysed using SDS-PAGE and the fractions with respective bands at 48.5 kDa for RLC-APEX2 (Figure 3.3A) and 28 kDa for APEX2 (Figure 3.3B) were pooled and concentrated. Concentrated proteins were subsequently used for the reconstitution experiment to obtain fully functional peroxidase enzyme.

Figure 3.3: IMAC column purification of the RLC-APEX2 and APEX2 proteins from total cell lysate.

(A) RLC-APEX2 purification profile by Ni-NTA beads: Lane 1: marker, lane2- uninduced control, lane 3: induced crude lysate, lanes 4, 5 and 6 are lysate, soluble fraction and pellet. Lanes 7-15: 10 μ L of each eluate fractions loaded onto each well. Sharp bands at ~48 kDa denote purified RLC-APEX2 protein. **(B)** APEX2 purification profile by Ni-NTA beads: Lane 1: marker, lane 2- uninduced control, lane 3- induced crude lysate, lane 4- unbound. Lanes 5-13: 10 μ L of each eluate fractions loaded onto each well. Sharp bands at ~26 kDa denote purified APEX2 protein.

3.4. Production of holoenzymes by reconstructing RLC-APEX2 and APEX2 with haeme.

APEX is a class I peroxidase enzyme which requires haeme for the removal of H_2O_2 that is produced in plant cells as a result of photosynthetic electron transport and photorespiration (Nakano & Asada, 1981, Raven & Dunford, 2015, Smirnov, 2018). Production of fully functional recombinant haeme protein is challenging in *E. coli* BL21 strain due to low incorporation of the cofactor haeme into the proteins. Expressing the protein in vector in bacteria and supplementing the growth media with 5-aminolevulinic acid showed full incorporation of haeme into the protein (Lam *et al.*, 2014). One limitation of the pTRC vector is the reduced compatibility in *E. coli* DH5 or BL21 strain and reduction in protein yield. To achieve a high yield of APEX2 holoenzyme, fusion proteins were expressed in the pET-3d vector, purified, and subsequently reconstituted with haeme cofactor.

In order to obtain a fully functional APEX2 holo-enzyme, purified RLC-APEX2 and APEX2 were incubated overnight with hemin-Chloride at 4°C with shaking. Incubation with hemin-Chloride aids haeme incorporation to the binding pocket of APEX2 (Patterson & Poulos, 1995, Mandelman *et al.*, 1998). The reconstituted protein was separated from excess haeme by Anion exchange chromatography. The proteins were loaded to the column equilibrated with 20 mM KPO_4 pH 7.5 and was eluted with a linear gradient of 20-500 mM KPO_4 pH 6.5. APEX2 protein was eluted at a concentration of 100 mM KPO_4 while fusion protein RLC-APEX2 was eluted at 200 mM KPO_4 . The difference in the salt concentration required for the elution could be attributed to the difference in their isoelectric point calculated from protparam (pI value of 5.43 for RLC-APEX2 and 5.85 for APEX2). SDS-PAGE analysis was done for the eluted fractions to assess protein purity (Figure 3.4A and 3.4B). Bands at ~48.5 kDa and 28 kDa indicated the presence of purified RLC-APEX2 and APEX2 recombinant proteins respectively.

Figure 3.4: Purification of reconstituted RLC-APEX2 and APEX2 by anion exchange chromatography.

(A) SDS-PAGE analysis of reconstituted RLC-APEX2 purification. Lane 1- marker, lane2 (IN/input) - reconstitution mixture of RLC-APEX, lane 3 (FT/flow-through) - unbound fraction of reconstituted RLC-APEX2 mixture, lane 4 (W) - wash, lane 5-10 purified fraction sharp bands ~ at 48 kDa in gel signify the presence of reconstituted RLC-APEX2.

(B) SDS-PAGE analysis for reconstituted APEX2 protein. Lane 1- marker, lane2 (IN/input) - crude APEX2 reconstitution mixture, lane 3 (FT/flow-through) - unbound fraction, lane 4 (W) - wash, lane 5-7 peak 1 (unspecific protein bound), lane 8-14 purified fraction bands ~ at 26 kDa indicating purified reconstituted APEX2.

Moreover, the identity of RLC-APEX2 protein in the elution fractions was confirmed by Western blotting. Western blotting with anti-His showed that purified RLC-APEX2 migrates at a molecular weight of about 48 kDa (Figure 3.5). In addition, no degradation of RLC-APEX2 was observed in purified fraction. Degradation product was eliminated during column wash procedure. Fractions containing RLC-APEX2 and APEX were pooled, concentrated and stored in -80°C until further analysis.

Next, we determined if reconstituted and purified APEX2 fusion proteins had haeme incorporated. Determination of haeme incorporation into peroxidases (APEX2 and RLC-APEX2) was then tested by calculating the RZ (Reinheitsszahl) value explained in method (Chau & Lu, 1995)). The RZ value gives the amount of hemin content in the holoenzyme which is measured as the ratio of absorbance at 403 and 275 nm (Adak & Datta, 2005). An RZ value of greater than 3 indicates full incorporation of hemin. A study with APEX2 showed that an RZ value of equal to 2 corresponds to ~ 60 % haeme incorporation (Lam *et al.*, 2014).

In most of our preparations, the RZ values for APEX2 and RLC-APEX2 was found to be greater than 2, save for a few cases in which the values were found to be lower (mean 2.5 ± 0.08). In such cases, haeme reconstitution was performed once again with haeme-chloride followed by purification, and this resulted in an improvement of haeme incorporation more than 60 %. Batches of purified proteins with RZ values greater than 2 were chosen for future experiments.

Figure 3.5: Western blot analysis for the reconstituted RLC-APEX2 ion-exchange chromatography purified fractions

Western blot analysis for the reconstituted RLC-APEX2 purified fractions with His-antibody shows a single band confirming the purity of the reconstituted protein. IN- represent for the crude reconstitution input mixture, FT- unbound protein, W-wash fraction, and purified fractions.

3.5. *In vitro* Functionality test for RLC-APEX2 and APEX2

Next, we determined if the RLC-APEX2 and APEX2 are enzymatically active. The mechanism of APEX2 for EM labelling is explained in chapter 1 (Introduction 1.6.2). Briefly, the peroxidase activity of APEX2 converts H_2O_2 into Reactive Oxygen Species (ROS). Singlet oxygen helps polymerization of Diaminobenzidine (DAB) into an osmophylic compound that provides the required contrast for the visualization by EM (Martell *et al.*, 2012). However, because of the insolubility of the DAB polymer in water (Seligman *et al.*, 1968) a colourimetric method for DAB polymerisation assay is not well established. Instead guaiacol was used as the substrate to measure peroxidase kinetics.

Activity toward guaiacol generally correlated well with robust polymerization of DAB *in vitro* (Martell *et al.*, 2012).

Here a standard colourimetric based peroxidase activity assay for APEX2 was used. The assay is based on the conversion of the substrate guaiacol into a coloured product tetraguaiacol (Hiner *et al.*, 2000). The reaction mechanism for the conversion of guaiacol to its product is given below.

The activity of the peroxidase is measured as the change in the absorbance at 470 nm due to the formation of the coloured product tetraguaiacol (Martell *et al.*, 2012, Lam *et al.*, 2014).

Kinetic assays (Materials and Methods 2.2.2.13) were performed in a UV-Vis spectrophotometer (SHIMADSU) using 100 ul volume cuvettes. The protein concentrations of reconstituted RLC-APEX2 and APEX2 were determined by the Bradford assay using Bovine Serum Albumin (BSA) as standard. In our estimation of peroxidase activity, Horseradish peroxidase (HRP) was used as a positive control and reconstituted APEX2 was used to compare the enzyme kinetics of RLC-APEX2. Published data with recombinant APEX2 has shown that assay conditions of 20 nM APEX2 and 1.4 mM guaiacol substrate is sufficient to determine the initial rate (Martell *et al.*, 2012). Hence, I have performed the assay with 20 nM APEX2 and 0.01 or 0.1 mM H₂O₂. However, all of the H₂O₂ was consumed so quickly that the initial rate of guaiacol turnover could not be captured on our spectrophotometer as the reaction was already saturated (data not shown). A similar effect was also observed for RLC-APEX2 when we used 20 nM enzyme concentration (data not shown). However, we noted an increase in the absorbance value as the concentration of H₂O₂ increases. This showed that RLC-APEX2 is active but highlighted the need to standardise the enzyme concentration for determining the initial rate. Therefore, I tried various concentrations for both enzymes.

At 200 nM enzyme concentration the initial rate of turnover was maintained for several seconds and could be readily measured by spectrophotometry (Figure 3.6A and B). Hence 200 nM enzyme was used for further kinetic studies at 0.01 mM H₂O₂ the enzyme didn't show any activity (Figure 3.6A), but at a concentration of 0.1 mM H₂O₂ I observed a steep increase in activity. However, at 1 mM H₂O₂ there was no significant difference in kinetic activity compared to 0.1 mM. When the concentration increased from 1 mM to 10 mM the activity decreased. This could be due to irreversible inhibition of APEX2 enzyme by H₂O₂ as previously reported (Nicell & Wright, 1997, Lam *et al.*, 2014)). At 100 mM H₂O₂ no enzyme activity was recorded due to complete inhibition. Overall the results suggested that H₂O₂ concentration between 1-10 mM is ideal for measuring APEX2 activity. So, the same H₂O₂ concentrations were tested for RLC-APEX2 (Figure 3.6B). However, 2.5 mM guaiacol was used instead of 1.4 mM as I was not able to detect the initial rate at 1.4 mM guaiacol concentration as the substrate was consumed too quickly.

Figure 3.6: Enzyme activity of RLC-APEX2 and APEX2 at 200 nM concentration.

(A) Reconstituted APEX2 was incubated with 1.4 mM guaiacol. 0.01 mM to 100 mM H₂O₂ concentration was used to determine the enzyme activity. (B) 2.5 mM guaiacol substrate was used for RLC-APEX2 enzyme kinetic assay. 1 mM to 20 mM H₂O₂ were used. Both enzymes show increased activity as substrate concentration increases, until it reaches its maximum activity. At higher concentration of H₂O₂ both enzymes were inhibited.

The enzyme activity increased for the tested H_2O_2 concentration between 1 mM to 5 mM. But the activity decreased with increasing H_2O_2 concentration. Thus, the enzymes rate of reaction was seen to reach its maximum at 5 mM H_2O_2 (Figure 3.7A and B). Interestingly the concentration of H_2O_2 to be used in the EM is also around 5mM H_2O_2 (SS Lam 2015). However, Sandin's lab has found that 0.1 mM H_2O_2 concentration is ideal for their EM and proximity labelling experiments. So, I decided to use 200 mM RLC-APEX2 for our initial rate assay as it would give highest activity when 5 mM H_2O_2 is used.

The initial rate of reaction was calculated for the first 30 seconds by plotting the absorbance at 470 nm against time (Figure 3.7A and B). Initial rates determined for APEX2 and RLC-APEX2 showed that enzyme activity is inhibited at higher H_2O_2 concentration. This property is not observed for horseradish peroxidase (HRP) enzyme (Figure 3.7C). From the result it is concluded that H_2O_2 concentration above 5 mM cause RLC-APEX2 inhibition so decided to use 3 mM H_2O_2 for our subsequent EM studies.

Figure 3.7: Initial rate of peroxidase enzymes.

Compared to HRP, APEX2 and RLC-APEX2 shows substrate inhibition. **(A)** APEX2 shows a fast-initial rate as the concentration of substrate (H₂O₂) increased, but became inhibited at higher concentration of substrate. **(B)** RLC-APEX2 also shows similar behaviour. As the concentration increases initial rate increased, but above 5 mM H₂O₂ concentration enzyme was inhibited. **(C)** HRP was not inhibited by substrate. (n=4 or 5)

3.6. RLC-APEX2 exchanged rabbit skeletal muscle

We commenced a pioneer effort to exchange RLC-APEX2 into skeletal muscle for subsequent EM imaging. To evaluate the degree of exchange and to reveal its localization within the muscle, RLC-APEX2 was fluorescently labelled with rhodamine (labelling the cysteine residues). In order to exchange RLC-APEX2 fusion protein into skeletal muscle, I used a well-established TFP based protocol. Over the past decade our lab regularly achieved 80% exchange efficiency for ELC and RLC by employing the TFP-based protocol (Toepfer *et al.*, 2012). The exchange efficiency was determined by comparing the fluorescence intensity of rhodamine labelled light chain to non-exchanged muscle. Moreover, the exchanged muscle showed no physiological changes compared to the native muscle based on isometric force analysis (Caorsi *et al.*, 2011, Mansfield *et al.*, 2012, Toepfer *et al.*, 2012).

To perform the exchange of RLC-APEX2, muscle fibres were chemically permeabilized (as described in the Material and Method 2.2.3.2). Visualizing the RLC-APEX2 fusion protein (in our case with rhodamine labelling) in the exchanged muscle would evaluate the efficiency of the exchange process. Two different sets of experiments were carried out to visualize RLC-APEX2 localization. In the first experiment, rhodamine labelled RLC-APEX2 protein was used for the exchange. In the second experiment RLC (non-labelled) and rhodamine labelled APEX2 proteins were used. The exchange process was initiated by adjusting the sarcomere length of permeabilized fibres to 2.1 μm by a He-Ne laser light diffraction grating set-up followed by incubation with the exchange proteins. It is known that troponin C (TnC) is removed during TFP based exchange process along with RLC (Putkey *et al.*, 1991). Since removal of TnC could impair muscle physiology, TnC was added into the exchange buffer and wash buffers to compensate for the protein loss. Post-exchange treatment, the fixed muscle fibres were imaged under confocal microscopy. The results from the rhodamine labelled RLC-APEX2 and APEX2 exchange is shown in Figure 3.8. Confocal images of rabbit skeletal muscles exchanged with rhodamine labelled RLC-APEX2 showed that fluorescence was confined to the A-band (Figure 3.8A marked with arrow). No fluorescence was observed in the I-band as expected. These results indeed indicate successful exchange of RLC-APEX2 protein and its localization to the myosin lever arm region. On the other hand, exchange with RLC and

rhodamine labelled APEX2 (Figure 3.8C) showed no fluorescence in A-bands but only fluorescence from the periphery of muscle fibres. This indicates that APEX2 alone does not localize in the muscle structure.

Figure 3.8: Confocal micrographs of rabbit skeletal muscle exchanged with fusion proteins labelled with rhodamine.

(A) Confocal microscopy image of rhodamine labelled RLC-APEX2 exchanged muscle fibre shows the RLC-APEX2 localization at A-bands (arrows). (B) Transmitted light microscopy image of RLC-APEX2 exchanged muscle fibre. (C) Confocal microscopy image of rhodamine labelled APEX2 + RLC protein exchanged muscle fibre shows no specific localization of APEX2 in muscle fibre. (D) Transmitted light microscopy image of rhodamine labelled APEX2 + RLC exchanged muscle fibre.

3.7. The effect of RLC-APEX2 exchange on the force production of permeabilised rabbit skeletal muscle

To investigate if RLC-APEX2 produced any physiological changes to the exchanged muscle fibre, isometric force was determined and compared with the non-exchanged fibre. Light chain (RLC or ELC) exchange studies with fluorescent or spin probe tagged RLC showed no adverse effects in muscle physiology or in the structure (Hambly *et al.*, 1991, Hambly *et al.*, 1992, Sabido-David *et al.*, 1998, Brack *et al.*, 2004). In addition to the fluorophores, GFP-tagged RLC has also shown successful localization into the lever arm domain without affecting the physiology of the muscle (Burghardt *et al.*, 2007). Since RLC fused to GFP did not show any adverse effect, it is presumed that tagging RLC with APEX2 (which is similar in size to GFP) will not affect the physiological properties of a muscle. To confirm our hypothesis, we have decided to analyse the muscle force production during isometric contraction during activation-relaxation cycles. Isometric force is the measure of the force produced by a muscle fibre held at constant length using energy from ATP. Schematic representation for the typical isometric force trace is shown (Figure 3.9A). Initially force will rise as the muscle fibre get activated (force increasing phase) and it reaches the maximum attainable force (isometric plateau).

The isometric force calculated for the RLC-APEX2 exchanged muscles were slightly reduced (Figure 3.9C) compared to non-exchanged muscle fibres (Figure 3.9B). However, from force trace data, I observed that RLC-APEX2 exchanged muscle fibre were able to reach the isometric force plateau (Figure 3.9C) similar to the non-exchanged muscle fibre. Also, this was similar to the earlier reported phenotype of RLC exchanged muscle fibre (Toepfer *et al.*, 2013). Teopfer et al. have shown that with 0.5 mg/ml RLC in exchange buffer would help to exchange 50 % of native RLC with exogenous RLC protein. I believe that in our study 50 % of RLC was exchanged with RLC-APEX2. Additionally, this result is comparable to the muscle fibres exchanged with rhodamine labelled RLC (Brack *et al.*, 2004). These results conclude that rabbit skeletal muscle exchanged with RLC-APEX2 fusion protein has similar physiological properties as non-exchanged muscle fibre.

These results suggest that APEX2 tagged RLC may serve as a potential strategy to localise RLC within muscle cross-bridge with high spatial precision using EM. Genetic tag for EM-based experiments where APEX2 protein will not hinder the physiology hence the structure.

Figure 3.9: Representative isometric force trace for the rabbit skeletal muscle.

(A) Schematic representation of an isometric force trace is shown. During activation stage muscle force gradually increases (force rising phase), and reaches to the maximum (isometric plateau). (B) Force trace for the non-exchanged muscle fibre was recorded when fibre was activated by a series of solutions – (pre-activating, activating and relax buffer). Force was increased over the period of time and reached its isometric plateau level in which solution. (C) Similar force trace was recorded for the RLC-APEX2 exchanged muscle fibre. Compared to non-exchanged fibre force was reduced to ~ 33 % but reached its isometric plateau after few seconds.

3.8. Summary

In this chapter, results have shown that RLC-APEX2 expressed as insoluble protein can be refolded. The refolded protein can be used for in vitro reconstitution to produce fully functional APEX2 enzyme.

Results have also shown that APEX2 tagged with RLC is functionally active by demonstrating that it can robustly convert substrate-guaiacol into a coloured reaction product similar to the APEX2 protein. Our data clearly shows that at higher concentration of H_2O_2 , RLC-APEX2 exhibits irreversible enzyme inhibition.

Rhodamine labelled RLC-APEX2 was exchanged into skinned rabbit skeletal muscle and analysed the localisation of the fusion protein by confocal microscopy. Here, found that irrespective of APEX2 tagging, RLC is localised to the A-band, suggesting specific incorporation into myosin cross-bridge.

Isometric force calculated for the RLC-APEX2 exchanged muscle shows ~ 33% reduction in force. However, muscle fibres were able to undergo relaxation-contraction cycles. This shows that the physiological property of muscles is not affected by the APEX2 tag. This result illustrates that exchanged muscle can produce force similar to the non-exchanged muscle by cross-bridge mechanism. Reduction in the force production in exchanged muscle might be due to low exchange and inactive cross-bridge affected by exchanged RLC.

Having characterised the basic biochemical and physiological properties of RLC-APEX2 and muscle fibres, the next aim was to visualise the muscle cross-bridge at different stage of muscle contraction. With the initial aim to visualise the muscle cross-bridges in the relaxed state, we standardised different sample preparation conditions to preserve the ultrastructure of muscle in relaxed state. This work is described in next chapter.

4. RLC-APEX2 EXCHANGED MUSCLE IN RELAXED STATE BY ELECTRON MICROSCOPY IMAGING

The idea of direct visualization of the muscle cross-bridge at different stages of muscle contraction captivates muscle biologists. By visualizing the cross-bridge organization in detail, it would be able to speculate about the mechanism of muscle contraction.

To validate one of our hypotheses- labelling of one of the prominent proteins (RLC) in muscle by APEX2 method- I have for the first time established APEX2 labelling in isolated and permeabilised rabbit skeletal muscle fibres for the direct visualisation of the muscle cross bridge.

4.1. Poor ultrastructural preservation of skeletal muscle by standard APEX2 based sample fixation method.

In order to visualize RLC in skeletal muscle in the relaxed state, I used the standard APEX2-based sample preparation method developed by JD Martell et al. The APEX2-based EM labelling method had hitherto only been applied to cell lines (Martell *et al.*, 2012, Lam *et al.*, 2014). As a novel application, published method (Martell *et al.*, 2012) was adopted to label the RLC protein in isolated muscle fibres.

In initial test experiments, I used the standard APEX2 labelling procedure published by SS lam 2015 et al. I refer to this procedure as the conventional APEX2 method (as explained in chapter 2.2.4.1). Studies with APEX2 have shown that sample fixation with strong chemical fixative is required to minimise the diffusion of osmophilic diaminobenzidine (DAB) polymer (Martell *et al.*, 2012, Ariotti *et al.*, 2015). In our specific experimental setup, I incubated the permeabilised rabbit skeletal muscle in relax buffer for 15-20 minutes. Immediately after the removal of relax buffer, muscle fibres were fixed with 2% glutaraldehyde in cacodylate buffer for one hour at 4 °C.

EM of the muscle prepared by conventional APEX2-based chemical fixation showed poor structural preservation of the rabbit skeletal muscle (Figure 4.1A). Generally, a muscle sample is well preserved if their longitudinal sections present the essential features, namely the foremost criterion is a straight and continuous Z-line across the section. Also, the major components of muscle- actin and myosin should be visible as separate bands

in the electron micrograph of relaxed muscle. In our preparation, however, a curved Z line was observed instead of a continuous alignment suggesting uneven distortion and contraction. Overall, we failed to observe details from the ultrastructure of the sample (Figure 4.1A). Additionally, EM of cross-sections (Figure 4.1B) did not display a regular hexagonal arrangement of actin and myosin filaments, as expected from a well-preserved specimen. Therefore, the conclusion is that muscle fixation in 2 % glutaraldehyde in cacodylate buffer alone does not preserve the muscle's ultrastructure, in spite of the fibres being fixed immediately after shifting from relax buffer. These results imply that for improving the ultrastructural preservation of muscle fibres, optimization of the sample preparation protocol is vital. Given the above results, various fixatives was tested and sample preparation conditions that had previously been established for preserving the ultrastructure of muscles in diverse systems.

Figure 4.1: Poor ultrastructural preservation of skeletal muscle by standard APEX2 based sample fixation method.

(A) Electron micrograph of 60 nm thick longitudinal section of rabbit psoas muscle fixed with glutaraldehyde in cacodylate buffer shows poor structural preservation. Sarcomere do not appear in a straight line and Z-lines are wavy. Distinctive A-bands and I-bands are not visible. (B) Cross-sectional view of fibre shows distortion in myofilament arrangements and absence of hexagonal packing.

4.1.1. Glutaraldehyde in relax buffer fixation method offers high quality structural preservation-Longitudinal section of rabbit psoas muscle

Chemical fixation followed by plastic embedding is a method that has been used for decades. It has helped to resolve muscle ultrastructure at 5 nm resolution (Sader et al. 2007). To preserve muscle fibres in their close-to-native relaxed state (or at least minimise artefacts), we tried diverse chemical fixation conditions. I was inspired by the seminal work of Thomas Burgoyne and his colleagues. This method has shown successful preservation of the ultrastructure by glutaraldehyde in relax buffer and plastic embedding. In this method, 3% glutaraldehyde Krebs buffer was used as the fixative (to maintain the relax state of cardiac muscle) for visualizing the ultrastructure of Z-bands by electron tomography (Burgoyne *et al.*, 2015).

As extensive exposure to fixatives and dehydration chemicals could cause severe deterioration in the muscle ultrastructure and alter the length of filaments (Page & Huxley, 1963, Sjöström & Squire, 1977). I decided to optimize the duration of each stage of the sample preparation with the aim to reduce artefacts. Prolonged alcohol dehydration has been shown to affect the cross-bridge periodicities (Reedy *et al.*, 1983), so we first attempted to optimise the dehydration time. I analysed electron micrographs under different sample preparation conditions and found that samples fixed with glutaraldehyde for 1 hr in relax buffer and alcohol dehydrated for 15 minutes at 4 °C showed good structural preservation.

Permeabilised rabbit psoas muscles were fixed with glutaraldehyde in relax buffer for one hour on ice. A previous study showed that use of tannic acid as a post fixative reveals fine details of myofilaments (Geissinger *et al.*, 1983). Tannic acid is used in electron microscopy to increase the contrast of the specimen by acting as a mordant (Núñez-durán, 1980, Singley & Solursh, 1980). Therefore, tannic acid was included as post-fixative after glutaraldehyde fixation to visualise myosin filaments and to increase contrast. Subsequently, post-fixation was done with osmium tetroxide (OsO₄). Samples were then dehydrated with a series of ethanol solutions (20%, 50%, 70% and 90%) of increasing concentrations until 100%. Plastic embedded samples were subsequently cut using an

ultramicrotome (Leica UC7) with a diamond knife where the knife edge was aligned perpendicular to the muscle fibre axis to obtain 60 nm thin sections.

To validate if structural features are preserved in our samples and to analyse the quality of the sample, EM images were compared with published results (Burgoyne *et al.*, 2015). A representative EM image of muscle fibres used to validate our method is shown in Figure 4.2.1.1. The representative published image highlights well-preserved sarcomeres with characteristic I-bands, A-bands and a continuous Z-line across the longitudinal section. Additionally, myosin and actin filaments are parallelly arranged (Figure 4.2A, (Burgoyne *et al.*, 2015)). Similar to the published result, the EM image of our sample also showed the characteristic features of well-preserved muscle sarcomeres. However, some spaces were noticeable between the myofibres, resulting in discontinuity of the Z-line. Moreover, thin filament and A-band edges appeared to be less straight this may be due to artefact from chemical fixation (Figure 4.2B). I presume this might be due to the permeabilization of the muscle fibre. During permeabilization (membrane removal), membrane-associated organelles are removed without affecting the myofilament structure. Our electron micrographs showed much higher contrast than the published image, which might be attributed to the use of tannic acid during sample preparation. Overall our sample preparation method shows that it is appropriate for skeletal muscle sample preparation for EM.

Figure 4.2: muscle fixed with glutaraldehyde in relax buffer provide structural preservation and quality EM images.

(A) Published result shows well-preserved sarcomere with characteristic I-band, A-band and continuous Z-line across the longitudinal section (Burgoyne *et al.*, 2015). (B) Electron micrograph of 60 nm thick longitudinal section of rabbit psoas muscle fixed with glutaraldehyde in relax buffer shows high quality and detailed EM image comparable with published result. However thin filaments appear less straight, and the A-band edges also be less straight due to artefacts from chemical fixation.

Muscle samples with improved ultrastructural preservation allowed us to visualize the distinctive features of the muscle myofilaments. In order to assess structural preservation in the relaxed state, I have examined longitudinal sections in detail. A representative electron micrograph of a longitudinal section of the psoas muscle in the relaxed state is shown in Figure 4.3 (Figure 4.2 is an enlarged view of Figure 4.3A). These EM images were used for further morphological analysis due to its good quality image). Myofibrils, which are largely made up of two types of filaments, actin and myosin, were evident in the electron micrographs (Figure 4.3B). The denser band between two Z-lines represents the A-band, which is composed of myosin filaments. The less-dense band on either side of the dark area represents the I-band, which consists of actin filaments. These filaments are arranged parallel to the fibre axis in the longitudinal section (Figure 4.3B).

Structural features of the sarcomere in the relaxed state are described in Figure 4.3C. Image analysis of six electron micrographs from longitudinal sections showed that sarcomeres were $2.5 \pm 0.05 \mu\text{m}$ in length (standard deviation for $n=6$). This is in agreement with the maximum length of a sarcomere, $2.5 \mu\text{m}$ in its relaxed state as reported by A F Huxley (Huxley & Niedergerke, 1958). 25 different areas from six different micrographs were selected for measurements of A and I band lengths. A-bands and I-bands were $1.6 \pm 0.04 \mu\text{m}$ and $0.9 \pm 0.03 \mu\text{m}$ in length ($n=25$), respectively. I-bands depend on sarcomere length. Overall, these data indicated that I was successful in producing high quality and detailed EM images with our newly improved chemical fixation method, similar to the published result (Burgoyne *et al.*, 2015).

Figure 4.3: Electron micrograph of glutaraldehyde fixed rabbit psoas muscle in longitudinal section

(A) Electron micrograph of 60 nm thick longitudinal section of rabbit psoas muscle showing myofibrils arranged in parallel. (B) Magnified view of a small area showing a single sarcomere unit with different banding patterns (Magnification x6.8 k). (C) Example of one sarcomere unit used for the image analysis. Image showing different components of the sarcomere, denser A-band surrounded by less dense I-band on either side bisected by Z-line (Similar image as Figure4.2B). (Scale bar 1 μm).

4.1.2. Preservation of cross-sectional morphology muscle in relaxed state

Our previous results from longitudinal sections showed that our muscle sample was preserved in the relaxed state. To confirm the preservation of the relaxed state of the muscle I analysed cross-sections by EM. A well-preserved chemically-fixed muscle fibre should display a regular hexagonal pattern of myosin filaments in A and H-bands (Huxley, 1953). Based on our previous results, I speculated that our glutaraldehyde-fixed and plastic-embedded sample would show characteristic features in A and H bands.

Electron micrographs of cross-sections of rabbit psoas muscle fixed with 2% glutaraldehyde and 0.2% tannic acid for 1 hr in relaxing buffer and post-stained with OsO_4 displayed regular hexagonal patterns of myosin filaments in the H (Figure 4.4) and A bands (Figure 4.5). The observed pattern was similar to previously reported results by Huxley (Huxley, 1953). A typical electron micrograph of a cross-sectional view of the H-band showed regularly organised myosin filaments (Figure 4.4A). A well-preserved H-band of relaxed muscle should not have actin present. Due to the lack of actin filaments, the H-band has a simple hexagonal lattice arrangement of myosin filaments (Figure 4.4B). An example of a magnified view illustrating the single hexagonal pattern is shown in Figure 4.2.2.1C. From the plot profile analysis of five different sets of hexagonal patterns, it was calculated that the filaments are spaced by $255 \pm 36 \text{ \AA}$ (Figure 4.4D). This value is consistent with the spacing between myosin filaments observed by H E Huxley (200-300 $\text{\AA}$) (Huxley, 1953). However, the spacing of myosin in the relaxed state of intact muscle as observed by X-ray diffraction is around 450 $\text{\AA}$ (Huxley & Brown, 1967). This difference is likely to be due to shrinkage of the muscle fibre during dehydration (Page & Huxley, 1963).

Figure 4.4: Preservation of cross-sectional features of glutaraldehyde fixed muscle in relaxed state in H-band.

(A) Electron micrograph of 60 nm thin section showing H-band where the hexagonal pattern of myosins are seen (B) Enlarged view of a small area (marked in red box) clearly showing the hexagonal pattern are marked. (C) Single hexagonal pattern (red line indicates the distance measured between myosin) (D) Plot profile data for the myosin spacing in H-band.

Our next aim was to analyse the cross-sectional view of A-bands, in which both myosin and actin filaments are present (Figure 4.5A). The presence of both filaments gives the A-band its characteristic double hexagonal pattern (Figure 4.5B). The double hexagonal lattice arrangement of the actin and myosin filaments manifests as a single myosin filament (large dot) surrounded by six thin filaments (small dots), a well-known characteristic feature of A-bands (Figure 4.5B). Filament spacing in the A-bands was measured in ImageJ. Actin spacing in the A-band was 157.17 ± 20 Å (calculated from five different double hexagonal patterns) and myosin filaments were spaced by 264 ± 18 Å (analysed from six double hexagonal patterns). Again, these measurements are in agreement with earlier EM studies (Huxley, 1953). Notably, myosin extensions (the cross-bridge) were also seen in some areas of the image (Figure 4.5C marked in red arrows). Overall, these results established that our modified glutaraldehyde/osmium protocol preserves ultrastructural features of the muscle. Numerous repetitions using the same experimental conditions consistently reproduced the above results. We are confident, therefore, that the above-mentioned method maintains the structural morphology reported in earlier publications and hence be appropriate for APEX2-based EM labelling experiments in the relaxed state.

Figure 4.5: Preservation of cross-sectional features of glutaraldehyde fixed muscle in relaxed state in A-band.

Transverse section 60 nm thick section of psoas muscle stained with 2% lead citrate. **(A)** Electron microscopy image of A-band (13,000X magnification) shows the regular double hexagonal arrangement of thick and thin filaments. **(B)** Enlarged view of area marked in Figure **(A)**. Thick filaments (larger dots) and thin filaments (smaller dots) are clearly seen. **(C)** One example of double hexagonal pattern is shown which was used for calculation of filament spacing. Arrows showing cross-bridges are extending from thick filaments.

4.2. RLC-APEX2 exchanged muscle fibre gives visible electron microscopy contrast in cross-sectional view of A-bands without heavy metal staining

Next, we aimed to test whether RLC-APEX2-exchanged muscle produces specific EM contrast using the previously standardised conditions. APEX2 has been successfully exploited as a tag in cultured cell lines, Zebrafish and mammalian tissues to obtain EM contrast (Martell *et al.*, 2012, Lam *et al.*, 2014, Ariotti *et al.*, 2015, Hatani *et al.*, 2018, Hirabayashi *et al.*, 2018). The principle is that the peroxidase activity of APEX2 catalyses the polymerization and precipitation of DAB. This creates contrast for EM after OsO₄ fixation which enables visualization of the structures in which APEX2 is expressed. Since APEX2 produces localised EM contrast, we wanted to exchange APEX2 fused to RLC (RLC-APEX2) protein into muscle fibres to visualize muscle cross-bridges. One limitation of this approach is that APEX2 requires the cofactor haem for its activity. Availability of endogenous haem in cell lines and tissues is sufficient to confer high APEX activity for most studies (Lam *et al.*, 2014, Ariotti *et al.*, 2015). The lack of haem in the RLC-APEX2 expressed *E. coli* required us to perform *in vitro* reconstitutions of haem to APEX2 prior to exchanging into the muscle. Although the reconstituted APEX2 was enzymatically active in solution (Result section 3.5), I needed to validate its activity for EM in the exchanged muscle.

To test our hypothesis, I analysed cross-sectional electron micrographs of RLC-APEX2 exchanged rabbit psoas muscle fibre in the relaxed state. Samples were prepared as outlined in the Materials and Methods chapter (2.2.3.8). Muscle fibres were fixed with glutaraldehyde for 1 hr, and subsequently bathed in cacodylate buffer containing DAB and 3 mM H₂O₂. The DAB reaction was carried out for a maximum of 5 minutes, as a longer duration may result in the diffusion of the polymerised DAB product. Negative controls were treated identically except that H₂O₂ was omitted. I used 3 mM H₂O₂ for our reaction because our biochemical studies showed that higher concentrations would inhibit the enzyme (Chapter 3.5). Muscle fibres treated with DAB and H₂O₂ for 5 minutes turned into a dark brown colour due to the DAB reaction products, and this colour change was observable by light microscopy and with the naked eye. I have noticed that samples that turned light brown always produced less contrast in EM (data not shown). Variations across

experiments might be explained by the extent with which RLC-APEX2 fusion protein is exchanged, or by variations in enzymatic activity of the reconstituted RLC-APEX2 fusion protein. APEX2 reacted samples were stained with OsO_4 to enhance the EM contrast of the DAB product. After osmium staining, samples were dehydrated and embedded in plastic resin in order to acquire thin sample sections from which structural features could be visualized.

I decided to examine cross-sections of RLC-APEX2 exchanged muscle fibre because the cross-bridge can be seen in A-bands as a connecting bridge between myosin (thick filament) and actin (thin filament) (Figure 4.5C). I have analysed 60 nm thin sections of plastic embedded muscle sample at different magnifications. Typical cross-sectional views of myofibrils from RLC-APEX2 exchanged muscles in the relaxed state treated with DAB alone (control) or with DAB+ H_2O_2 are shown in Figures 4.6.A and C, respectively. Images were taken at different magnification to compare the contrast. At 13 k magnification and higher magnifications I observed clear difference in the contrast. All images were taken at -2 μm defocus to increase the contrast for the unstained samples. As expected, I have observed a characteristic contrast difference in DAB+ H_2O_2 treated samples (Figure 4.6C) compared to the negative control sample treated with DAB only (Figure 4.6A). This enhanced EM contrast is due to the peroxidase activity of APEX2 fused to RLC. The A-band region of the sarcomere was seen in all images shown. Figure 4.6D and B show magnified views of the marked areas in Figure 4.6C and A (red box) respectively. From the magnified image, connections between myosin and actin filaments (cross-bridges) are clearly visible in the DAB+ H_2O_2 treated sample (Figure 4.6D marked with black arrows). This indicates that the RLC-APEX2 protein provides characteristic contrast detectable by EM. Overall the hexagonal packing of myosin filaments was regular but, in some areas, hexagonal packing was distorted, which might be a result of chemical fixation or sectioning (cutting angle with respect to fibre axis). Such artefacts are also seen in earlier studies (Huxley, 1953). The control sample (treated only with DAB) showed weak EM contrast (as seen in Figure 4.6A and B). A speculation was made that less contrast in H_2O_2 samples is due to absence of peroxidase reaction. In order to verify that the contrast enhancement observed in cross-sections was caused by RLC-APEX2, I analysed longitudinal sections.

Figure 4.6: EM contrast in RLC-APEX2 exchanged muscle due to APEX2 and peroxide reaction.

Cross-section of 60 nm thick section of RLC-APEX2 exchanged rabbit skeletal muscle. (A) Electron microscopy image of A-band (13 k magnification) without H_2O_2 shows less contrast. (B) Enlarged view of A-band shows the regular arrangement of myosin filament in the RLC-APEX2 exchanged muscle. (C) RLC-APEX2 exchanged muscle overlaid with H_2O_2 and DAB mixture shows contrast due to peroxidase activity of APEX2 in the presence of H_2O_2 . (D) Enlarged view of A-band shows arrangement of myosin filaments. Connecting bridge between actin and myosin (cross-bridges) are seen in peroxide treated sample (black arrows).

4.3. Longitudinal sections of RLC-APEX2 exchanged muscle fibres show characteristic EM labelling contrast in A-bands.

To further evaluate our RLC-APEX2-exchanged muscle sample, I analysed 60 nm thin longitudinal sections. One advantage of longitudinal sections over cross-sections is that they can provide a clear view of both A and I bands in a single muscle fibre even when viewed at lower magnification (Hall *et al.*, 1946, Draper & Hodge, 1949, Rozsa *et al.*, 1950). Either band in transverse sections should give characteristic contrast due to the difference in protein density (Chapter 1.2). I expected that RLC-APEX2-exchanged muscle fibres would produce more contrast in A-bands, which can be easily detected in longitudinal sections. Moreover, the longitudinal section gives a clear view of the myofibrillar arrangement which helps in assessing the quality of the sample (Burgoyne *et al.*, 2015).

Using longitudinal sectioning, I intended to analyse the contrast difference between the A and I-bands and evaluate the structural preservation of the band alignment. Figure 4.7 shows electron micrographs of muscle fibres exchanged with RLC-APEX2 and a comparison of the EM contrast produced in RLC-APEX2-stained and control samples. Continuous myofilaments are seen in both DAB and DAB+H₂O₂ treated fibres. Ultrastructural details are well preserved in the sections. Z-lines and M-bands are in a straight line and continuous. This indicated that no structural artefacts were introduced by the DAB+H₂O₂ reaction.

As expected, DAB+H₂O₂ samples (Figure 4.7D) showed more EM contrast in the A-band compared to samples in which H₂O₂ was omitted (Figure 4.7B). This could be due to the conversion of DAB into an osmophilic DAB polymer by the peroxidase activity of APEX2 conjugated to RLC.

By contrast, I-bands exhibited comparable (similar) contrast. This was expected since the I-band is completely devoid of muscle cross-bridges, and, hence should not contain RLC-APEX2 fusion protein. Therefore, the contrast produced in the I-band can be attributed to actin. By contrast, the enhanced contrast in the A-band is due to the localisation of APEX2 tagged RLC protein at the muscle cross-bridges. Remarkably, H₂O₂ overlaid muscle fibres (figure 4.7C and D) typically show less dark M-line than the A-band due to the lack of muscle cross-bridges or RLC. Moreover, compared to H₂O₂ omitted samples (Figure 4.7A and B) H₂O₂ overlaid samples (Figure 4.7C and D) has shown well-defined sarcomere features due to the labelling reaction.

Figure 4.7: EM contrast in RLC-APEX2 exchanged muscle is localised to the A-band.

Longitudinal section of 60 nm thick section of RLC-APEX2 exchanged rabbit skeletal muscle. **(A)** Electron microscopy image of A-band (4.8 k magnification) without H₂O₂ shows less contrast. **(B)** Single sarcomere units are shown at 13 k magnification. Parallel arrangement of myofilaments is seen. **(C)** H₂O₂ and DAB treated RLC-APEX2 exchanged muscle shows higher EM contrast due to localization of RLC-APEX2 to myosin cross-bridges or in the A-band. **(D)** Enlarged view of A-band shows arrangement of myosin filaments.

In addition to visible contrast observed in the electron micrograph between H₂O₂ treated and non-treated samples, I did plot analysis for the images at 18.5 K magnification (Figure 4.8 B and C). Plot analysis represents the 2D graph of the pixel intensities along a line within the image. An example for the plot analysis is shown in the Figure 4.8A. The x-axis represents the distance along the line and the y-axis represents the pixel intensity. From the graph the length of one sarcomere was calculated to be 2.1 μ m for without peroxide and 2.5 μ m for peroxide treated fibre. This measurement corresponds to the sarcomere length of the non-exchanged fibre (section 4.1.1). Thus, exchange of RLC-APEX2 into fibres does not alter the sarcomere length.

Figure 4.8B shows a fibre without H₂O₂ addition on the left and its plot analysis result on the right. Figure 4.8C shows an electron micrograph of H₂O₂ treated muscle fibre on the left and its scale plot result on the right side. The contrast difference observed in the samples in the presence and absence of H₂O₂ (Figure 4.8B left, C left) is also reflected in the in their plot analysis results of the same (Figure 4.8B right, C right). This result shows increased pixel intensity in the A-band region for the H₂O₂ treated sample (Figure 4.8B) than the H₂O₂ omitted sample (Figure 4.8C) due to peroxides activity of RLC-APEX2. This result confirms our hypothesis that APEX2 fused with muscle protein is a promising genetic tag for muscle studies.

Figure 4.8: Plot profile for RLC-APEX2 exchanged muscle shows increase in contrast in A-band.

Longitudinal section of 60 nm thick section of RLC-APEX2 exchanged rabbit skeletal muscle. **(A)** An example for the plot analysis. Plot analysis represents the 2D graph of the pixel intensities along the line (yellow line) within the image. The x-axis represents the distance along the line and the y-axis represents the pixel intensity **(B)** Electron microscopy image of A-band (23 k magnification). Sample prepared without H_2O_2 (left) and its plot profile analysis result (right) **(B)** **(C)** Electron microscopy image of A-band. Samples were prepared with H_2O_2 +DAB (left) and its plot profile analysis result (right).

4.4. Requirement of high contrast sample for RLC-APEX2 exchanged muscle tomography studies

In an attempt to visualise the 3D organisation of muscle cross-bridges stained with RLC-APEX2, I prepared freshly exchanged muscle fibres using 0.5 mg/ml RLC-APEX2 during the exchange. From above result, I had observed that 3 mM H₂O₂ treated 0.25 mg/ml RLC-APEX2 exchanged sample is not good enough for tomography due to less EM contrast. I tested 3 mM and 0.5 mM H₂O₂ for 0.5 mg/ml RLC-APEX2 exchanged samples. I assumed that 3 mM H₂O₂ concentration might be inhibiting peroxidase activity of APEX2 in 0.25 mg/ml RLC-APEX2 exchanged sample.

Interestingly, EM images of samples treated with 0.5 mM H₂O₂ showed less EM contrast than the ones treated with 3 mM H₂O₂. Previous work in cell lines carried out in the Sara Sandin lab suggested that at 3 mM H₂O₂, RLC-APEX2 protein might be inhibited, but instead, I observed better EM contrast at 3 mM H₂O₂ compared to 0.5 mM H₂O₂. Therefore, I conducted all subsequent experiments with 3 mM H₂O₂.

As a standard procedure, 2D images had to be analysed to ensure structural preservation. Fibres prepared in the relaxed state exhibited uniform sarcomere length, and are straight and aligned laterally (Figure 4.9). Samples exchanged with 0.5 mg/ml of RLC-APEX2 showed close packing of myofibrils, and myofibrils were continuous. Z-bands and M-lines were also continuous (Figure 4.9). No structural deformations were visible from the 2D electron micrographs. This indicated that our samples were suitable for 3D tomography.

Successful 3D reconstruction of a tomography tilt series depends upon the good contrast and signal-to-noise ratio of the unstained samples; this implies the requirement of high contrast for visualizing observable features. When I compared the samples prepared with 0.5 mg/ml (Figure 4.10A) to those prepared with 0.25 mg/ml RLC-APEX2 (Figure 4.10B), I noticed increased contrast in the former. In some areas of 0.25 mg/ml RLC-APEX2 exchanged sample, barrel distortion of sarcomere was observed (marked with a black oval, Figure 4.10A). However, less barrel distortion was seen in the 0.5 mg/ml RLC-APEX2 exchanged sample (Figure 4.10B). In general, these results imply that samples prepared with 0.5 mg/ml RLC-APEX2 indicates that APEX2 reaction method is able to give good contrast. Moreover, glutaraldehyde in relaxed buffer preserved the ultrastructural details. Improvement in image quality (Figure 4.9A and Figure 4.10A) shows that our EM images are good enough to provide the ultrastructural details, similar to the published result (Thomas Burgoyne 2015). Earlier, with 15-30 nm thin sections, Huxley et al., showed the single myofilament layer and individual cross-bridges (Huxley, 1963). Inspired from that work, attempt was made to cut 30 nm thin sections to visualise APEX2 tagged RLC, but the attempt was unsuccessful.

Figure 4.9: Preservation of RLC-APEX2 exchanged muscle in relaxed state; sample used for tomography.

Longitudinal section 60 nm thick section of psoas muscle exchanged with 0.5 mg/ml RLC-APEX2 at 1.7 k magnification. Chemical Fixed samples were overlaid with DAB and H₂O₂ solution to get the EM contrast. Electron microscopy image longitudinal sections show sarcomere are straight, well aligned and uniform in length. Sarcomere length is 2.1 μm .

Figure 4.10: Enhanced contrast in RLC-APEX2 exchanged muscle in relaxed state; sample used for tomography

Longitudinal section 60 nm thick section of psoas muscle exchanged with **(A)** 0.5 mg/ml RLC-APEX2 shows enhanced EM contrast than **(B)** 0.25 mg/ml RLC-APEX2 exchanged muscle fibres at 1.7 k magnification. Chemically-fixed samples were overlaid with DAB and H₂O₂ solution to get the EM contrast. Barrel distortion was observed for the 0.25 mg/ml RLC-exchanged muscle fibre (oval marking).

4.5. Electron tomography of longitudinal sections

Due to the difficulty in visualising the cross-bridge by 2D EM, the attempt was done to use electron tomography. Studies have shown that changes in cross-bridge shape are related to functional states and these have been detected in EM images of longitudinal sections (Hirose et al., 1994). In 2D EM images, superimposed signals produced by the protein of interest in the crowded cellular environment complicate interpretation of the 3D organization. This can be overcome by dual-axis tomography (explained in chapter 1, section 1.6.1), which involves collecting two orthogonal tilt series. Due to the increase in Fourier information collected in dual-axis tomography, there is a reduction in artefacts in the reconstructed tomogram (reduced missing wedge). This improves resolution (Mastronarde, 1997, McEwen & Marko, 2001).

With 0.5 mg/ml RLC in exchange buffer, Töpfer et al. showed 50% replacement of native RLC with recombinant protein (Toepfer *et al.*, 2013). To avoid overcrowding of DAB polymer or unwanted effect of higher concentration of APEX2, I initially started with 0.25 mg/ml of RLC-APEX2 for our exchange experiments. But when I performed the tomography for 100 nm thick sections of same sample, I was not able to visualize RLC or cross-bridge due to low contrast. To overcome this, we conducted another set of exchange with 0.5 mg/ml of RLC-APEX2 protein.

I speculated that tomography of the A-band can give more detailed information regarding the organization of the cross-bridge on the thick filaments during different states of the muscle contraction cycle. For this purpose, tomography was performed on 100 nm thick longitudinal sections prepared with DAB+H₂O₂ (Figure 4.11A). The areas selected for tomography comprise half a sarcomere, which contains A-bands rich in thick filaments and I-bands devoid of thick filaments (Figure 4.11B). Two tilt series perpendicular to each other were recorded using a tilt scheme of $\pm 64^\circ$ in increments of 2° . Images were taken at -2 μm under focus and an electron dose of $13.7 \text{ e}/\text{\AA}^2$. Tomograms were generated by filtered back projection and the dual-axis tilt series combined using IMOD software. Gold particles of 10 nm diameter were used for alignment of the tilt series (Figure 4.11A). Figure 4.11B-D shows a series of tomographic slices showing the parallel arrangement of thick filaments. Each slice is about 0.18 nm thick. Supporting tomography video is included in the APPENDIX.

Figure 4.11: Electron tomography of rabbit skeletal muscle A-band

(A) Electron microscopic images of rabbit skeletal muscle 100 nm thick sample used for tomography at 23 K magnification. A-band has high contrast compared to other bands, I-band on either side of A-band and M-line which is dissecting the A-band is due to the localization of RLC-APEX2 protein in cross bridge. 10 nm gold particles used as fiducial markers can be seen in the image. (B, C and D) 2D slices of tomogram out of 130 slices. Thickness of each slice is about 0.18 nm. Parallel arrangement of myofibrils can be seen.

Tomography images were taken at 30 K magnification, electron dose 13.7 e/Å. -2 µm under focus, Scale bar 100 nm.

To study the structural arrangement of cross-bridges in the A-bands and analyse their features in the relaxed state, I averaged 30 consecutive slices from the tomogram series to produce 5.4 nm thickness section (Figure 4.12A and 4.13A). However, I was not able to visualise the helical periodicities of the cross-bridge in the relaxed state as previously reported by Thomas D Lenart et al. (Lenart *et al.*, 1996). Interestingly, I noticed some novel structural features (dot-like appearance on thick filament), which were not reported previously on the relaxed thick filament (Figure 4.12B red arrows). Moreover, these structural features are single (red arrows) or two dots very close to each other (red circle). I believe that these might be single cross-bridges or single head of myosin fibres. These structural features are probably due to RLC-APEX2 fusion. Thus, these features could be myosin cross-bridges. Although these structural features are disordered, they are present on myosin filaments (Figure 4.12A black box), further supporting our idea that these could be cross-bridges. The regular arrangement of cross-bridges is not visible in our tomogram. The disorder of the myosin head on thick filaments might be due to their labile nature in the relaxed state as previously observed (Wray, 1987, Wakabayashi *et al.*, 1988). In the non-overlap (H) zone, few myosin heads projected out from the surface of the thick filaments (Figure 4.12A white arrow).

To confirm the presence of cross-bridges, I analysed the optical diffraction pattern from the averaged images (Figure 4.12A and Figure 4.13A) but I was unable to obtain a Fourier transform. Surprisingly, I noticed some abnormal structural features in the I-band (Figure 4.13A (black box), Figure 4.13B (red arrow)). Although this band is known to be devoid of cross-bridges or RLC, the noted structures might represent RLC binding non-specifically to I-bands.

Overall these images indicate that APEX2-tagged RLC labelling is a promising tool for the visualisation of myosin cross-bridges.

Figure 4.12: RLC-APEX2 organisation on myosin thick filament by ET.

(A) Representative of stack of 30 consecutive images. Arrangement of muscle cross-bridge on thick filaments are seen (black box). Similar structural features in H-band (white arrow). (B) Enlarged view of area from figure (A) shows arrangement of muscle cross-bridge like structures on thick filaments (red arrows- single dots) and more than one dots close to each other (red circle).

Figure 4.13: Ultrastructural details of muscle cross-bridge

(A) Representative averaged stack of 30 consecutive images from 130 tomographic slices (thickness 5.4 nm). Arrangement of muscle cross-bridge on thick filaments are seen. Non-specific structural features in I-band (black box). **(B)** Enlarged view of area from Figure **(A)** shows muscle cross-bridge in I-band (red arrows).

4.6. Summary

Following the characterisation of recombinant RLC-APEX2 in chapter three, I intended to visualise the cross-bridge organization in relaxed muscle. Our biochemical assays showed that RLC-APEX2 is enzymatically active in PBS buffer. Moreover, the localization studies with rhodamine-labelled RLC-APEX2 showed that the protein is incorporated preferentially into the A-band. We went on to establish protocols that would permit EM labelling of the RLC-APEX2 fusion protein in exchanged muscle fibres. It was found that muscle fibres fixed with 2% glutaraldehyde in relax buffer preserve the muscle's structural morphology. These conditions were also appropriate to assess EM contrast produced by APEX2 peroxidase activity in exchanged muscle fibre. It is further found that increasing the RLC-APEX2 concentrations during the exchange offers a means to produce sufficient EM contrast for tomography experiments. More interestingly, we were able to visualise novel dot-like structural features of muscle cross-bridge in the RLC-APEX2 exchanged muscle tomogram. These structural features might be RLC-APEX2 fusion protein. This might signify the myosin cross-bridge. To confirm and validate our result we require to obtain the RLC-APEX2 muscle fibre in rigor state, where helical periodicities of myosin cross-bridges are easily identifiable. Overall our results suggest that APEX2 based labelling in muscle is well-suited for ultrastructural studies. I envision that further development and refinement of the results presented in this chapter will provide useful information to wide range of workers interested in the ultrastructural details of other proteins in muscle.

5. RLC-APEX2 EXCHANGED MUSCLE IN RIGOR STATE

At the end of the power stroke, dissociation of the product of ATP hydrolysis from myosin head results in strong actin-myosin interaction which lasts until cross-bridges dissociate from actin by binding a fresh molecule of ATP. In the absence of ATP, these strongly bound cross-bridges dominate, resulting in a muscle state termed the rigor state because it corresponds to the high stiffness state that of cadaveric muscle. In the rigor state, the strong binding of cross-bridges means that they adopt the helical order corresponding to the spacing of myosin binding sites on actin. This highly ordered state may reveal details about the organisation of the RLC in the cross-bridges that may not be detectable in the more disordered relaxed state of rabbit muscle.

X-ray diffraction studies and fluorescence polarisation have shown regular ordered cross-bridge structure along the filament in rigor state. However, the orientation of the lever arm or associated RLC is not well understood (Thomas & Cooke, 1980, Crowder & Cooke, 1987). To overcome this, fluorescently labelled RLC protein was used to determine the lever arm orientation (Ling *et al.*, 1996, Burghardt *et al.*, 2007). Some of the unresolved questions regarding rigor state are, for instance, the number of cross-bridge attached to the actin in real-time and the different conformational states of actin-myosin interactions.

As explained earlier, due to direct visualisation of structure in EM, EM is a robust technique compared to the interpretation of diffraction pattern or fluorescence signals. Moreover, advanced EM labelling technique may help provide the ultrastructural details of the particular protein at nm resolution (Giepmans *et al.*, 2005).

In our previous chapter, results are shown that labelling RLC with APEX-2 has helped us study the ultrastructural details of the muscle in the relaxed state. The same strategy will be used to study the structure of cross-bridges in the rigor state.

5.1. Standardization psoas muscle sample preparation in rigor-state

In order to visualise the cross-bridge in rigor state, I had to standardize the sample preparation in rigor state. Rigor state can be induced in muscle fibre (in vitro) by depletion of substrate MgATP which results in strong actin myosin interaction (Bremel & Weber, 1972, Dos Remedios *et al.*, 1972, Bendall, 1973). To prepare muscle in rigor state,

permeabilised rabbit psoas muscle were bathed in relax solution followed by several washes in EDTA containing buffer to reduce the MgATP concentration (pre-rigor and magnesium-free rigor buffer as mentioned in method 2.2.3.7 were used to remove the ATP substrate and ligands). At low MgATP concentration, force of the fibre is affected by binding of the calcium from the buffer (Kawai & Brandt, 1976) which results in distortion of the lattice. Thus, calcium was removed to reduce the force production. Finally, muscle fibres were bathed in rigor buffer without EDTA for 15-30 minutes and fixed in the same with 2% glutaraldehyde at 4 °C. The rest of the procedure until plastic embedding was similar to relaxed sample preparation.

Fixed, dehydrated and plastic-embedded rigor muscle samples were sectioned to 60 nm thin sections and picked on 200 mesh copper grids. These samples were stained with lead citrate. Sections were imaged under 120 kV TEM microscopy at different magnifications. In longitudinal section, the sarcomeres have the conventional striation pattern -A-bands, I-bands, H-zone together with continuous M and Z-lines (Figure 5.1). The sarcomere was calculated to have a uniform length of $2.3 \pm 0.01 \mu\text{m}$ across the whole fibre.

Figure 5.1: Structural preservation of non-exchanged muscle in rigor state by glutaraldehyde fixation.

Electron micrograph of 60 nm thick longitudinal section of rabbit psoas muscle shows the ultrastructural preservation in rigor state. Myofibrils are arranged in parallel, denser A-band surrounded by less dense I-band on either side bisected by Z-line. Continuous Z-band across the filaments. (Scale bar 1 μm).

Moreover, well-aligned myofilaments in rigor sample imply good structural preservation. In the longitudinal section some periodic structures are prominent in the A-band (Figure 5.2A red box). These structural features were prominent in magnified EM image (Figure 5.2B white arrows) and not seen in relaxed muscle sample. I presume that it could be due to the regular helical arrangement of the cross-bridge in rigor state as reported earlier (Reedy *et al.*, 1965, Reedy & Reedy, 1985, Taylor *et al.*, 1993, Schmitz *et al.*, 1996).

Presence of left-handed cross-bridges was reported in EM images of 25 nm thin sections (Reedy, 1968, Heuser & Cooke, 1983). According to their study, a 25 nm thin section contains only a single myosin-actin (Myac) layer which makes it possible to visualise the cross-bridge in 2D (Reedy, 1968). However, I was unsuccessful in acquiring 25-30 nm thin sections. In future, I will attempt to make 25-30 nm sections by repeated sectioning. Reedy used insect muscle which has a much better-ordered structure than mammalian muscle (Reedy *et al.*, 1983), this might be another reason for our unsuccessful attempt.

A

B

Figure 5.2: Periodic structural features observed in rigor state.

(A) Longitudinal electron micrograph of 60 nm thin section shows the half sarcomere. Periodic structural features are shown in the A band region. (B) Enlarged area from Figure (A) clearly shows the periodic structures (marked with white arrows).

5.2. Tomography of RLC-APEX2 exchanged muscle fibre in rigor state

From the previous chapter, tomography of relax muscle has shown the less characteristic helical arrangement of myosin cross-bridge. To extend our studies to rigor state I prepared 0.5 mg/ml RLC-APEX2 exchanged muscle. Similar sample preparation was performed as relax sample. Muscle fibres were pinned on sylgard and fixed with 2% glutaraldehyde in rigor buffer at 4 °C. The excessive fixative was washed prior to DAB and H₂O₂ treatment. During the DAB and H₂O₂ reaction, fibres were observed for the colour change. After 1-2 minutes it was noticed that fibre started turning into brown colour. The reaction was continued for five minutes to make sure that DAB and H₂O₂ diffused well into muscle fibre and gave uniform contrast throughout the muscle. The reaction was stopped by washing with cacodylate buffer, and the sample preparation procedure was continued as the conventional plastic embedded method. 100 nm thin sectioned for the tomography are shown in Figure 5.3 at 13k magnification. Surprisingly, the images showed less contrast compared to the observed relax samples (Figure 4.9). When I performed the reconstruction of relax sample tomogram with less contrast, I was not able to visualise any structural feature. This is because for tomogram high contrast samples are required. Possibly the lesser contrast may be due to the lesser activity of APEX protein or other conditions. Taking these into account, I decided to repeat the experiment to get higher contrast images in order to pursue tomography.

Figure 5.3: Less EM contrast observed in RLC-APEX2 exchanged muscle in rigor state.

Longitudinal section of 60 nm thick section of psoas muscle exchanged with 0.5 mg/ml RLC-APEX2 shows less EM contrast than relax sample (Figure 4.9). Chemically-fixed samples were overlaid with DAB and H_2O_2 solution to get the EM contrast.

6. DISCUSSION

In this project, an APEX2-based labelling procedure for direct visualisation of muscle cross-bridges by electron microscopy was established. The muscle cross-bridges on myosin thick filaments were able to observe in dual-axis tomograms of fixed, and relaxed state muscle fibres. This electron tomography demonstrates the APEX2 labelling of RLC proteins in muscle fibres. To compare the muscle-cross bridge organisation in the rigor state, I standardised the chemical fixation conditions. I attempted to visualise muscle cross-bridges in the rigor state by APEX2-RLC labelling protocol. However, I was not able to achieve the necessary EM contrast in the sample for tomography in the rigor state. The tomography for rigor state will be carried out in the near future. In this chapter the advantages of APEX2 labelling for muscle fibres over other techniques is summarised: X-ray diffraction and fluorescence imaging. Also, the prospects of APEX2 in muscle biology tomography shall be discussed.

6.1. Ultrastructure of myosin cross-bridge organisation in relax and rigor state

According to the ‘swinging lever arm hypothesis’, the movement of myosin along the actin filament is the result of the rotational motion of myosin’s lever arm (Geeves & Holmes, 2005). Different lever arm conformations were observed in the crystal structure of the myosin head domain in the presence (Dominguez *et al.*, 1998) and absence of ATP (Rayment *et al.*, 1993). These structural features represent the *in vivo* relax and rigor state of muscle contraction. However, these structures were determined with incomplete myosin heads. This limits our understanding of the molecular organisation of cross-bridges in intact muscle.

Diversity in cross-bridge arrangements in relaxed and rigor states are studied to some extent in intact muscle by 2D-EM, X-ray diffraction and polarisation microscopy (Huxley *et al.*, 1980, Huxley *et al.*, 1981, Huxley *et al.*, 1982, Heuser & Cooke, 1983, Tsukita & Yano, 1985, Tsukita & Yano, 1986, Amemiya & Wakabayashi, 1991, Harford *et al.*, 1991, Wakabayashi *et al.*, 1991, Lenart *et al.*, 1996). These studies indicate that cross-bridges display a range of different angles with respect to the myosin filaments in rigor and relaxed states of skeletal muscle. However, angular changes of the lever arm domain in the cross-bridge during active contraction is still putative (Crowder & Cooke, 1987, Mello &

Thomas, 2012, Mendes *et al.*, 2018). Cross-bridges are known to shift their position on the myosin backbone during their interaction with actin as determined by x-ray diffraction (Huxley & Brown, 1967, Haselgrove & Huxley, 1973). To complement the atomic structure produced by x-ray diffraction, we require direct evidence of the cross-bridge or lever arm organisation. With Cryo-EM to probe the ultrastructure of myosin heads, the scope to acquire direct evidence was broadened (Walker *et al.*, 1994, Walker *et al.*, 1995, Hu *et al.*, 2016). However, due to the labile nature of cross-bridges on single myosin molecule, these high-resolution structures have limited value to understand their organisation in intact muscle. These studies indicate the requirement of further direct evidence for the ultrastructure of muscle cross-bridge organisation in intact muscle.

The direct visualisation of muscle cross-bridges by EM makes it more convenient than X-ray diffraction techniques. However, until the recent development of specially designed molecular tag for EM (Gaietta *et al.*, 2002, Shu *et al.*, 2011, Martell *et al.*, 2012, Lam *et al.*, 2014), visualisation of a specific structure in the crowded cellular environment was not possible. To visualise the cross-bridge organisation, the established EM tag protein-APEX2 was exploited. The lever arm domain of myosin cross-bridge is stabilised by myosin regulatory light chain (RLC), and this protein is required for the efficient work production. Moreover, mutations to RLC are associated with cardiac diseases. This makes RLC an interesting candidate to label for ultrastructural studies and to understand their role in muscle contraction. With the same intention, RLC was extensively utilised in spectroscopic studies (Hopkins *et al.*, 1998, Brack *et al.*, 2004, Burghardt *et al.*, 2007). Here APEX2 was tagged to the C-terminal of RLC to directly visualise the muscle cross-bridge organisation at different states of muscle contraction.

6.2. APEX2, a molecular tag for muscle electron microscopy and its limitations in muscle biology

APEX2 molecular tag was used for the first time for the direct visualisation of muscle cross-bridge. The standard trifluoperazine (TFP) based chemical exchange method was followed to exchange native RLC with APEX2 tagged RLC. For one set of light chain exchange experiment, approximately 30 μM RLC in 50 μl is required. To achieve such a

high concentration, large amount of protein is required. In order to produce such a high amount of protein, RLC-APEX2 was produced by recombination in *E. coli* (BL21-DE3). The haeme cofactor is necessary for functional APEX2. But unavailability of haeme in *E. coli* (BL21-DE3) necessitates the addition of a haeme reconstitution procedure (*in vitro*). Nevertheless, this limitation is easily overcome for APEX2 in cultured cell lines and *in vivo* studies due to the availability of endogenous haeme. Poor haeme incorporation into APEX2 might hinder its peroxidase activity. Consequently, this would lead to low DAB polymerisation and thus resulting in poor EM contrast (Martell *et al.*, 2012). Moreover, reduced haeme incorporation to the APEX2 results in low protein stability (Mandelman *et al.*, 1998).

During our purification procedure, unstable or degraded proteins are removed (Figure 3.4A). DEAE-Sephadex anion exchange chromatography for the reconstituted RLC-APEX2 shows that degradation products are weakly bound since they were removed during the wash process while stable reconstituted RLC-APEX2 was bound to the column and eluted with increasing salt concentration. More than 50 % of full-length RLC-APEX2 was weakly bound to the column due to saturation and eluted with other non-specific proteins (Figure 3.4A). This result was similar when the DEAE column was changed to the strong anion exchange columns (Q Sepharose High Performance). The typical yield of reconstituted RLC-APEX2 protein was 10 % (Table 6.1) after the reconstitution. Low yield of protein was a major setback for the RLC-APEX2 based EM labelling experiments for *in vitro* studies. However, the eluted fraction showed ~ 80 % purity for the reconstituted protein as observed by SDS-PAGE and western blot (Figure 3.4A and Figure 3.5).

Table 6.1: Protein yield of reconstituted RLC-APEX2

From (4L) culture	Amount of protein /4L	% of yield
From inclusion bodies	80 mg	100 %
After haeme reconstitution	12 mg	15 %
After buffer exchange	8 mg	10 %

Since no one has attempted the exchange of native RLC with RLC-APEX2, the functionality of RLC-APEX2 in muscle is not documented. An ideal molecular probe should not affect the structure and function of muscle. A similar sized GFP tag is commonly used in muscle for determining the cross-bridge orientation with respect to RLC (Burghardt *et al.*, 2009, Burghardt *et al.*, 2011, Burghardt & Sikkink, 2013, Burghardt *et al.*, 2016) with minor effect on muscle performance.

It is known that removal of RLC affects the myosin lever arm stability and consequently muscle movements (Lowey *et al.*, 1993). This was speculated to be due to head-to-head aggregate formation resulting in myosin head distortion (Flicker *et al.*, 1983, Trybus, 1994).

However, during the light chain exchange process when the native RLC is replaced by exogenous RLC the functionality of muscle is unaffected. According to Teopfer *et al.*, 50 % of native RLC can be replaced by the exogenous RLC when 0.5 mg/ml of RLC was used for the exchange process. Using a similar protocol in our study should result in 50 % RLC-APEX2 exchange. However, in our study, replacement of RLC by RLC-APEX2

resulted in ~30-33 % reduced maximum isometric force (Figure 3.9). Reduction in force was noted in numerous RLC exchange processes where 60-70% native RLC was replaced with exogenous RLC (Ling *et al.*, 1996, Brack *et al.*, 2004). Reports have shown that isometric force reduction is a non-specific effect of RLC exchange procedure. However, RLC-APEX2 exchanged muscle fibres were able to reach an isometric plateau. Those fibres also were able to undergo relaxation-activation cycles (data is not shown). Thus, muscle cross-bridges function is maintained after RLC exchange. They were able to bind ATP substrate and subsequently undergo contraction-relaxation cycle through the cross-bridge cycle. Similar results were obtained by RLC-GFP exchanged muscle fibre. Since GFP tagging with RLC has not shown major differences in functionality it is considered as an ideal fluorescent probe for muscle studies (Burghardt *et al.*, 2011, Burghardt & Sikkink, 2013). The contraction-relaxation function seen with RLC-APEX2 support the view that APEX2 is a suitable EM tag for muscle ultrastructural studies.

Restored force production by RLC-APEX2 exchanged muscle fibre indicated incorporation of RLC-APEX2 to myosin lever arm domain thus stabilising the structure. Subsequently, incorporation of rhodamine labelled RLC-APEX2 into muscle fibres confirms the successful incorporation of RLC-APEX2 (Figure 3.8A). Rhodamine fluorescence was restricted to the A-bands in exchanged muscle indicating its localisation to the myosin region. Replacement of native RLC with EM probe (APEX2) tagged RLC on the lever arm aided in the direct visualisation of muscle cross-bridges by EM.

Initially the attempted was performed to standardise RLC labelling experiment with an EM tag called miniSOG (mini Singlet Oxygen Generator). This requires high intense light to generate singlet oxygen (Shu *et al.*, 2011). However, this requirement for high intensity light could be a limiting factor for using miniSOG in the relatively large and thick samples such as intact muscle fibre. Remarkably, APEX2-based EM labelling method is relatively straightforward as it requires only DAB and H₂O₂ compared to intense light for miniSOG. So, APEX2 was preferred over mini-SOG for our muscle cross-bridge visualisation studies.

Cells or tissue samples which express APEX2 tagged protein overlaid with DAB and H_2O_2 for few minutes gave the required EM contrast due to its peroxidase activity (Martell *et al.*, 2012). Since DAB and H_2O_2 are small enough to diffuse rapidly into the permeabilised muscle fibre lattice, and EM contrast can be generated within the muscle. So, this makes the method aptly suitable for muscle biology. Although APEX2 is a suitable EM tag, its enzyme activity is inhibited with high concentration of H_2O_2 (Martell *et al.*, 2012, Lam *et al.*, 2014). This similar phenomenon was observed in our enzyme kinetic studies (chapter 3.5).

In the same way, APEX2 tagged to RLC has also shown similar inhibition kinetics, implying that fusion of other protein to APEX2 does not affect its inhibition by a high concentration of H_2O_2 (Figure 3.6B). The consequence of inhibition might result in low EM contrast and subsequently poor samples for tomography analysis.

Compared to APEX2, HRP might serve as a good tool for EM labelling studies. HRP's activity was not inhibited by H_2O_2 (Figure 3.7C). However, HRP requires post-translational modification for its activity. This might be challenging in exchange studies, where the protein has to be expressed in bacteria for high yield. Moreover, HRP shows reduced activity in calcium abundant environments (Hopkins *et al.*, 2000). But, APEX2 has no such limitation at high calcium concentrations. These properties make APEX2 superior over other peroxidases studying muscle contraction.

Our calculated H_2O_2 concentration for maximum activity of RLC-APEX2 was the same as reported for APEX2 by JD Martell *et al.* and SS Lam *et al.*, namely 5 mM H_2O_2 concentration. However, in Sara Sandin's lab just 0.1 mM H_2O_2 concentration seems ideal for the EM labelling and proximity labelling experiments. It implies that the APEX2's activity at this H_2O_2 concentration is sufficient to achieve the required EM contrast. So far, the reason for achieving ideal APEX2 activity by using H_2O_2 at a lower concentration than it is required for the maximum activity is unknown. This drove the requirement for standardisation of H_2O_2 for defining the ideal activity of APEX2 for our EM studies. Later it is found that 3 mM H_2O_2 would be an optimum concentration for the RLC-APEX2

exchanged muscle. This is close to Martell's value of 5 mM. Maximum activity at different concentration is unknown yet.

EM contrast in the sample is a function of the protein (RLC-APEX2) and H₂O₂ concentration used for the sample preparation (Figure 4.10). From our sample preparation standardisations, it is found that EM contrast improved by increasing RLC-APEX2 concentration in the exchange process and subsequently improving tomography results. However, one EM sample preparation requires an ample amount of time (one sample preparation for EM takes approximately two weeks). Also, only a few of the many standardisation conditions can be tested at a time. Overall experiments take several months to observe the improvements in results. Time investment is another limiting factor for APEX2 based EM studies.

However, with all the above difficulties I was fortunate to observe visible EM contrast difference in A-band of RLC-APEX2 exchanged sample (Figure 4.8). The EM contrast was specifically localised to the A-bands where myosin cross-bridges can be seen. This EM contrast is due to the peroxidase activity of APEX2 tagged with RLC protein localised at the region. Since APEX2 has shown to be functional in muscle fibre, it can be considered as a novel EM tag for muscle studies but with some limitations such as expression in bacteria and requirement of heame for its activation.

6.3. Myosin cross-bridge organisation in relaxed state by APEX2 labelling and electron tomography

As a promising approach electron tomography has the potential to produce 3D information of macromolecules in cells. From reconstructed relax state tomogram small dot-like structures was visualised on the thick filaments (Figure 4.12B). These dot-like structures tentatively mark cross-bridges or myosin heads. However, one might argue that these structural features may be due to the diffusion of DAB polymerised products. We know that in EM sample prepared at 4 ° C, diffusion of DAB products is localised to the site of production (Ariotti *et al.*, 2015). So, the structure that is visualised is due to the DAB polymerisation at RLC-APEX2 in lever arm.

In rabbit skeletal muscle the myosin cross-bridges are distributed at a fixed interval of 13.5 nm (Haselgrove, 1980). Based on Toepfer et al. 0.5 mg/ml recombinant protein would provide 50 % of native RLC replacement with recombinant RLC (Toepfer *et al.*, 2012). If our sample incorporates 50 % exchange, then we should be able to visualise cross-bridges periodicities due to the presence of RLC-APEX2. However, we were not able to determine any periodicities in our tomogram (Figure 4.12A and Figure 4.12B) or from the Fourier transform. This might be due to the low exchange fraction with the APEX2 tagged.

Next, we explored the presence of helical patterns of myosin heads on the thick filaments. However, I was not able to visualise any helical pattern in our tomogram. The helical periodicity of cross-bridges in the relaxed state was reported from x-ray diffraction studies of live muscle fibre from various vertebrates (Elliott, 1964, Huxley & Brown, 1967). In all the studied organisms, the periodicity of the cross-bridge was maintained but the interval varied for different organisms. As mentioned earlier, myosin cross-bridges in rabbit skeletal muscle have a radial distribution of 13.5 nm. Less accurate helical structural preservation was noted in the insect flight muscle fixed with chemical fixatives for EM (Reedy *et al.*, 1983). However, there is no clear evidence that these artefacts are induced due to chemical fixation.

Interestingly rabbit muscle has shown diversity in myosin head organisation at different temperatures in the relaxed state (Malinchik *et al.*, 1997, Xu *et al.*, 1999). Helically ordered myosin heads are more prominent at or above 20 °C. However, as temperature decreases a drastic transition of ordered to disorder state for myosin head was seen in X-ray diffraction (Wray, 1987). Later, a similar phenomenon was also observed using electron microscopy of rabbit skeletal muscle prepared at different temperatures (Kensler *et al.*, 1994).

Temperature effects on myosin head organisation are the result of the nucleotide bound to it (Wray, 1987). Only in the relaxed state are myosin heads bound with nucleotides. These myosin heads can bind to the ATP in the two forms, either ATP or ADP.Pi in the relaxed state. At low-temperature myosin head and ATP forms an M.ATP

complex. This complex results in open state for myosin head. This open state favours the disordered array of myosin heads (Tsaturyan *et al.*, 1999, Xu *et al.*, 2003).

However, at higher temperature myosin and ATP results in the formation of M.ADP.Pi complex due to the ATP hydrolysis. This complex facilitates the helically ordered state of myosin (Xu *et al.*, 2003). This result was consistent when myosin filaments were treated with blebbistatin, a myosin inhibitor in the presence of ADP (Xu *et al.*, 2009). Based on the above evidence it suggest that during our sample preparation at 4 °C myosin head would have undergone an ordered-disordered transition as reported by Wray *et al.* (Wray, 1987) rather than the artefacts produced by chemical fixation.

I was able to visualise single myosin cross-bridges (single dots in Figure 4.12B) and two cross-bridges (two dots in Figure 4.12B) close to each other. I presume that two cross-bridges very close to each other might be two myosin heads of the single myosin cross-bridges. The tomogram shows a greater number of cross-bridges in the middle of the A-band, which gradually reduced at the edge of the A-band. A similar organisation was noticed in GFP-tagged RLC exchanged muscle study (Burghardt *et al.*, 2007).

Brenner B *et al.* reported that skinned rabbit muscle fibre prepared in the relaxed state at low temperature (5 °C) and low ionic strength (~ 20 mM) without Ca²⁺ will favour the myosin cross-bridge and actin attachment to a significant extent (Brenner *et al.*, 1982). However, these actin-attached state of myosin cross-bridges is seldom found at physiological ionic strength (~150 mM) (Huxley, 1968, Thomas & Cooke, 1980). In Future it will be able to visualise the helical periodicity in RLC-APEX2 exchanged rabbit muscle prepared at low temperature by markedly reducing the ionic strength.

I was able to standardise APEX2 based sample preparation in skeletal muscle and visualise the myosin cross-bridge on thick filaments in a relaxed state. I envision that APEX2 will be suitable as an EM tag to extend our study to the rigor state.

6.4. Direct visualisation of muscle cross-bridge in rigor state: immediate goal

With the help of x-ray diffraction and 2D EM in insect flight muscle, Reedy et al. (Reedy *et al.*, 1965) have confirmed the existence of different cross-bridge organisation in relaxed and rigor state. Since I have successfully standardised EM labelling condition for visualising muscle-cross bridges in the relax state, I used similar sample preparation condition for rigor state. The rigor state sample shows ultrastructural preservation by chemical fixation (Figure 5.1A). Moreover, in 2D EM images it is not able to visualise the characteristic structural features (Figure 5.2A and B) which were not present in relax sample 2D EM (Figure 4.3). However, RLC-APEX2 exchanged rigor sample prepared for tomography showed less EM contrast (Figure 5.3A) than the relax sample (Figure 4.9) though the samples were prepared in similar conditions. The reason for the lower contrast in rigor sample is not understood yet. It could be the result of RLC-APEX2 exchange into muscle fibre and/or the activity of the enzyme. Since there is room to improve these conditions I hope by repeating the rigor state sample good EM contrast sample for tomography can be achieved. Once successful this will help us to compare cross-bridge organisation in relax and rigor. This would push forward the understanding of the major changes occurring to cross-bridges during the muscle contraction.

6.5. Towards understanding the helical order in the relaxed state

Electron micrograph of muscle in relaxed and rigor preparation preserves the ultrastructural details and morphological features in longitudinal and cross-sectional relaxed state muscle. These results were consistent with other published results (Table 6.2).

Figure 6.1: Diagram showing the cross-sectional view of A and H bands where the lattice measurements are taken in our experiments.

(A) Spacing between myosin in A-band Double hexagonal lattice. **(B)** Spacing between myosin in H-band simple hexagonal lattice

Table 6.2: Comparison between our data and published results

Method (EM/X-ray)	EM (current thesis data)	EM	X-ray diffraction	X-ray diffraction	X-ray diffraction
Muscle source	Rabbit psoas	Rabbit psoas	Live muscle	Mouse skinned toe muscle	Plaice fin muscle
Reference	This study	(Huxley, 1953)	(Huxley, 1967)	(Matsubara <i>et al.</i> , 1984)	(Harford & Squire, 1986)
Spacing between myosin in Double hexagonal lattice in A-band (Å) (A)	255 ± 36	200 - 300	440	408	-
Spacing between myosin in H-band (Å) (B)	264 ± 18	200 - 300	-	-	470 ± 2

Even though morphological features were consistent with the previous reports, I was not able to visualise the helical periodicity in relaxed state tomogram. This might have resulted from low temperature as mentioned earlier. But rapid freezing and subsequent

freeze-substitution process have shown good preservation of the helical array close to the native state (Kensler & Levine, 1982, Ip & Heuser, 1983, Cantino & Squire, 1986, Stewart & Kensler, 1986). During high-pressure freezing- freeze substitution (HPF-FS) ultrastructure of macromolecule are preserved to close to its native state. During this process structures were preserved by instant immobilisation of molecules by rapid fixing at cryo-temperature. And Subsequent dehydration at low temperature will help to avoid ice-crystal formation, thus reducing artefacts due to chemical fixation and dehydration. However, this method works well with native muscle samples and cannot be used with APEX2 exchanged samples until recent development in APEX2 based method.

Conventionally in high-pressure freezing and freeze-substitution (HPF-FS), process water molecules are replaced by organic solvents during the dehydration process (Steinbrecht & Müller, 1987). In general, organic solvent environments are not suitable for the DAB polymerisation and APEX2 requires an aqueous environment for its enzymatic activity. Very recent development of the novel cryo-chem method in 2018 solves the environment problem by replacing the organic solvent with an aqueous solvent. Thus, restoring the APEX2 activity and making it compatible with freeze-substitution samples (Tsang *et al.*, 2018). This gives a new hope to visualise the muscle ultrastructure close to its native state by APEX2 labelling. So, I propose that RLC-APEX2 exchanged muscle prepared using cryo-chem and HPF-FS would enable us to visualise the helical organisation of muscle in relaxed state.

X-ray diffraction model has suggested that two myosin heads of single cross-bridge are arranged in a tilted angle position, but in the opposite direction (like “V”) (Malinchik *et al.*, 1997). Based on the x-ray diffraction model, it should be able to visualise a similar organisation by RLC-APEX2 exchanged muscle fibre prepared by high-pressure freezing and freeze substitution technique. Improving the exchange of RLC-APEX2 to 50 % or more will facilitate the visualisation of a greater number of cross-bridges in the tomograms. Subsequently, identification of the different intermediate population of cross-bridges by HPF-FS may also be possible.

6.6. Perspective of RLC-APEX2 to understand the role of RLC phosphorylation in vertebrate striated muscle

Although it has been shown that RLC phosphorylation can affect the Ca^{2+} sensitivity of the ATPase activity in the reconstituted fibre (Szczena-Cordary, 2003), the mechanism is still elusive. The role of RLC phosphorylation in striated muscle activation is still unknown because no crystal structure of RLC or EM studies has resolved the serine-19 of phosphorylation domain (PD) (Wendt *et al.*, 2001, Alamo *et al.*, 2008). Also, the structural changes of the cross-bridge during RLC phosphorylation in muscle contraction cycle is still elusive. However, by exchanging the phosphorylated RLC-APEX2 into the cross-bridge and with the resulting high-resolution 3-D structures, we can understand some of its roles.

6.7. Medical Science outlook

The role of the myosin regulatory light chain in striated muscle is not well understood. Prevalence of familial hypertrophic cardiomyopathy (FHC) in the young population is about 1 in 500; this can cause sudden cardiac death in patients (Maron, 1997). Mis-sense mutations in RLC are associated with FHC (Seidman & Seidman, 2001). 2% of genetic mutations in FHC are associated with ventricular RLC (Alcalai *et al.*, 2008). The severity of RLC mutation on physiology is continuously being studied to understand the mechanism of disease pathology.

Phosphorylation of mutated RLC acts as an attenuation mechanism to nullify the physiological outcomes of FHC mutation (Szczena *et al.*, 2001). Our lab has also reported that RLC phosphorylation improves cardiac mechanical functions (Toepfer *et al.*, 2013). The RLC phosphorylation levels were seen to be lower in heart failure patients compared to normal conditions. In addition, studies in rat heart trabeculae have shown that extended levels of RLC phosphorylation have an added effect on force and power output.

Recently Yuan *et al.* have confirmed that phosphorylation/pseudo phosphorylation of RLC in FHC is required for the rescue from the disease state (Yuan *et al.*, 2015). It has been shown that recombinant light chain with the mutation can be exchanged into the

muscle fibre (Sweeney, 1995). As an extension to this study, we could use APEX2 tagged with FHC associated RLC to understand the attenuation mechanism by RLC phosphorylation or Ca^{2+} binding. Moreover, we can shed lights on the modulatory mechanism of RLC by exploring the ultrastructure of RLC by ET in intact muscle at different disease conditions.

During organogenesis, cardiomyocytes are one of the preliminary cells in the embryo to become the functional heart. Isolated cardiomyocytes were used to understand the myofibrillogenesis during heart development (Chacko, 1973, Dabiri *et al.*, 1999). With the help of TEM some of these questions related to myofibrillogenesis were addressed (Fischman, 1970, Peng *et al.*, 1981). Other than TEM, immunofluorescence microscopy was used to understand the distribution of myofibrillar proteins (Bennett *et al.*, 1979). In a recent study, APEX2 was successfully expressed in induced pluripotent stem cell-derived cardiomyocytes. These cells showed no negative effect on differentiation and proliferation (Hatani *et al.*, 2018), thus, demonstrating that APEX can be a highly valuable tool in proteomics and structure-function studies. This development will open many avenues to understand the unknown mechanisms of various proteins in muscle. As one such example, APEX2-tagged sarcomere proteins can be expressed in cardiomyocytes and explored to understand the muscle protein localisation and interaction during myofibrillogenesis and sarcomere assembly.

In summary, APEX2 is a versatile molecular tag which can be applied widely in muscle studies to understand the 3D organisation of proteins *in vitro*, for example in exchange studies in cardiomyocytes.

7. APPENDIX

Tomographic slices of 0.18 nm thickness through the reconstruction of relaxed muscle fibre. All the tomographic slices of this reconstruction are shown in the attached video. Cross-bridges or myosin regulatory light chain are seen as black dots on the myosin filaments due to the peroxidase activity of APEX2 attached to the RLC.

supplimentary
video_tomography_

8. REFERENCES

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