Chapter 5

Isolation, culture and morphometric characterisation of primary skeletal myoblasts

5.1. Introduction

Skeletal muscles in vivo are highly organized tissues consisting of structural and functional units called myofibers. Each myofiber is a long cylindrical cell with multiple numbers of nuclei, sarcomeres, and mitochondria. Muscle fibre harbours muscle-specific stem cells known as satellite cells (J. B. Dixon, Drum, and Weatherwax 2016). These cells lie between the plasma membrane and basal membrane of muscle fiber (Mauro 1961). These mitotically quiescent cells are responsible for growth and repair of muscle. Several markers, e.g., CD34, c-Met, syndecan-4, M-cadherin, integrin α7, and integrin β1 have been used to ascertain the presence of satellite cells in vivo and on muscle fibres in vitro (Z. Yablonka-Reuveni et al. 2008). Primarily, the cell is characterized by the expression of paired box transcription factor Pax7 as it is an essential requirement for adult muscle regeneration throughout life (P. Seale et al. 2000; Peter S. Zammit, Partridge, and Yablonka-Reuveni 2006, 20; Sambasivan et al. 2011). These cells undergo activation usually during exercise, injury, and diseased conditions leading to proliferation (Fu, Wang, and Hu 2015). Proliferation involves division of satellite cells to form premature muscle cells or myoblasts. During this stage, some of the satellite cells undergo quiescence to form reserve stem cells (Olguin and Olwin 2004; Peter S. Zammit et al. 2004; Yoshida et al. 1998). The myoblast cells initially are recognized by the expression of markers such as Pax7 and MyoD. Subsequently, myoblasts undergo differentiation characterized by the expression of MyoD and myogenin. Differentiated myoblasts under the influence of MyoD express p21 protein for cell cycle exit and fuse to form multinucleated myotubes (Zipora Yablonka-Reuveni 2011; Andres and Walsh 1996). Myoblast fusion is an intricate process involving steps with significant consequences. Firstly, migration of differentiated cells takes place in response to chemotactic factors released by other myoblasts (G.U. Bae et al. 2008). Tightly regulated migration process moderates the interactions between differentiated myoblasts leading to their fusion

(Knudsen and Horwitz 1977; Wakelam 1985). Chemokine molecules and protein receptor pairs aid in recognition between the differentiated myoblasts (Horsley et al. 2003). A similar process occurs between myotubes and myoblasts that requires a protein receptor pair of integrin VLA-4 and its receptor VCAM-1 (Rosen et al. 1992). The recognition follows the formation of cell-cell contact points by lamellipodia and filopodia (Hollnagel et al. 2002; Radice et al. 1997; Schwander et al. 2003). Such contact projections form with the help of adhesion proteins such as M-cadherin, N-cadherin and integrin proteins. Alignment of actin and non-muscle myosin 2A leads to localisation of focal adhesion assemblies at the terminal ends and thereby forming a bipolar cell morphology of myoblasts (Peckham 2008). The myoblasts align themselves in an end to end manner with their characteristic bipolar or teardrop morphology. Close apposition of the myoblast membranes primes the gap formation in actin fibres with vesicle accumulation at the site. Such situation leads to fusion pore formation and cytoplasmic continuity (Shilagardi et al. 2013; Nowak et al. 2009; R. Duan and Gallagher 2009). The fusion of these myoblasts leads to the creation of nascent myotubes that further fuse with other nascent myotubes to form myotubes (Abmayr and Pavlath 2012; Rochlin et al. 2010). Myotubes develop a dense myofibril network and achieve contractile nature (Chal and Pourquié 2017). During this process, nuclei present within the new cell experience movement towards the periphery marking the characteristic feature of the skeletal muscle cells (W. Roman et al. 2017). This peripheral positioning of the nuclei is of great significance, as the appearance of centrally aligned nuclei in myotubes are associated with musculopathological conditions in vivo (Folker and Baylies 2013; Roman and Gomes 2017).

In light of the above, the role of satellite cells in adult myogenesis and regeneration is indispensable. Although, use of secondary myogenic cell lines has been a successful approach for initial myogenesis studies. Variability in the results obtained from cell lines

exists due to the origin, culture conditions and passage number. In addition, these cells may show side effects due to their immortalized nature. Due to these disadvantages of secondary cells and the physiological relevance of primary skeletal muscle cells, the usage of primary cells has been preferred for myogenesis studies (Hindi et al. 2017).

Studies in satellite cell myogenesis have progressed due to the application of a range of methods for isolation of primary skeletal muscle cells. Earliest efforts to isolate primary skeletal muscle cells from rat skeletal muscle dates back to 1974. The technique employed enzymatic digestion of skeletal muscle to release satellite cells (Bischoff 1974). Thenceforth, the cell isolation protocol has undergone various improvements and modifications. However, the primary steps involved in the isolation procedure have remained the same. Significant steps involved in the isolation procedure include dissection of the skeletal muscle, mincing, enzymatic digestion, and repeated mechanical trituration to release the satellite cells from their surrounding extracellular matrix (Baquero-Perez et al. 2012). Further, methods like differential preplating, percoll density gradients, fluorescence activated cell sorting (FACS), and magnetically activated cell sorting (MACS) are applied for purification of culture from non-myogenic cells like fibroblasts and immune cells (Shahini, Vydiam, et al. 2018).

This chapter deals with the protocols and procedures used for the isolation, culture, and morphometric characterization of primary myoblasts from rat hind limb muscles. Isolation and culture of cells was adopted from the procedure described by Danoviz et al. with minor modifications (Danoviz and Yablonka-Reuveni 2012). First of the steps involves dissection of tibialis anterior and gastrocnemius muscles from rat hind limb. Further, the hind limb muscles were minced and subjected to mechanical trituration leading to the isolation and plating of the satellite cells on gelatin coated cell culture substrates. The cultured cells were characterized to be satellite cell by using characteristic

markers like Pax7 and Myogenin. The satellite cells were cultured on standard culture surfaces and various bright field based morphological observations were made. It was observed that the cells proliferated to increase their number after which they fused to form myotubes. The cells were assessed for the cytoskeletal changes taking place by fluorescence staining of the actin. The myoblasts formed were further characterized at different stages of differentiation for their calcium dynamics. The spontaneous twitching behavior was observed in the myotubes, which was characterized by using a kymograph-based approach.

5.2. Materials and method

5.2.1. Materials

Gelatin Type B [Himedia, Mumbai, India], Horse serum [Invitrogen, Bengaluru, India], Pronase [Sigma Aldrich, Bengaluru, India], Hank`s Balanced Salt Solution with (HBSS) [Himedia, Mumbai, India], Dulbecco Modified Eagle`s Medium (DMEM) [Himedia, Mumbai, India], Fetal Bovine Serum (FBS) [Himedia, Mumbai, India], Phosphate Buffered Saline (PBS) [Himedia, Mumbai, India], Paraformaldehyde [Himedia, Mumbai, India], Fluo-8 AM [AAT Bioquest, Sunnyvale, California, USA], Triton X-100 [Himedia, Mumbai, India], Bovine serum albumin (BSA) [Himedia, Mumbai, India], Poly Vinyl Alcohol 1,4-diazabicyclo[2.2.2]octane (DABCO) [Sigma Aldrich, Bengaluru, India], PhalloidinTetramethylrhodamine Conjugate [AAT Bioquest, Sunnyvale, California, USA], 4',6-diamidino-2-phenylindole (DAPI) [Himedia, Mumbai, India].

5.2.2. Dissection of lower hind limb muscles tibialis anterior and gastrocnemius

Dissection media consisting of DMEM+1% penicillin and streptomycin was warmed at 37°C and kept at room temperature for future use. Four-week-old Charles foster rats were sacrificed as per the guidelines of the animal ethical committee of the Institute of Medical

Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India. A younger aged rat was preferred for the study in order to obtain a high yield of cells (Day et al. 2010).

The dissection board and the autoclaved dissection tools were sprayed and surface sterilized using 70% ethanol. The sacrificed rat was sprayed with 70% ethanol. In order to isolate the tibialis anterior muscle, the rat was pinned on the dissection board in a supine position (face up position). The entire length of the lower hind limb was cut through by using straight operating scissors starting from the portion opposite to Achilles tendon towards the knee. During the procedure, removal of the skin was ensured to expose the underlying muscles. Next using fine scissors, a thin line of fascia was cut through (moving again from distal end from the knee towards the knee) and removed without damaging the underlying muscle tissue.

Further, tibialis anterior muscle`s distal tendon was exposed and loosened and cut through using another pair of straight scissors. The tibialis anterior was held at the already cut distal tendon and pulled towards the knee using forceps. The proximal tendon (near knee) of the tibialis anterior muscle was cut to ensure removal of the skeletal muscle as shown in Figure 5.1(a-f). The removed muscle was further placed in a 60 mm plate containing dissection media. A similar procedure was repeated for the other hind limb before proceeding with the dissection procedure for gastrocnemius. For gastrocnemius muscle, the rat was placed in a prone position or face down position, and the body was pinned appropriately. Using a pair of straight scissors, the proximal hind limb muscles were cut off covering the proximal portions of gastrocnemius muscle. Similar to the Tibialis anterior dissection, the distal achillies tendon can be next identified connected with gastrocnemius muscle. Fine tip forceps was placed below the achillies tendon and moved back and forth to loosen the gastrocnemius muscle. Using straight scissors, the Achilles tendon was cut. The cut end of the muscle was held and pulled towards the proximal end

using forceps. The gastrocnemius muscle was next cut at the proximal end (Figure 5.1(g-k)) and transferred to a 60 mm Petri dish containing dissection medium. The isolated skeletal muscle was washed in a dissection medium. The washed muscles were taken in a dissection medium to observe under a stereo zoom microscope. Using fine straight and curved forceps undesirable tissues like tendons, blood vessels, and fats were removed from the muscle tissue.

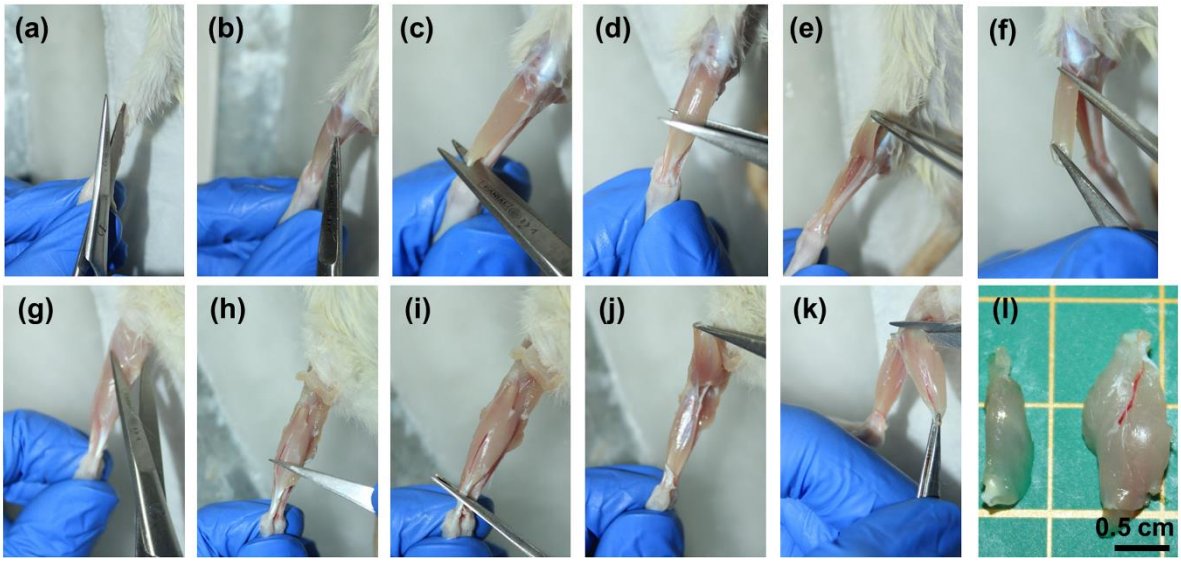

Figure 5.1 Figure depicts the sequence of steps involved in dissection of rat hind limb skeletal muscles tibialis anterior (a-f) and gastrocnemius muscle (g-k). (l) Represents the isolated skeletal muscles tibialis anterior (left) and gastrocnemius (right).

5.2.3. Enzymatic digestion of dissected skeletal muscles pieces

The cleaned muscles were cut into pieces ranging nearly 2-3 mm for facilitating enzymatic digestion. The muscle pieces along with the dissection medium were next transferred to a 15 mL centrifuge tube and subjected low speed centrifugation at 200 g for 4 minutes. The supernatant was discarded and 2 mL of dissection media was added to the centrifuge tube. The contents of the centrifuge tube were next transferred to 35 mm Petri plate. Before enzymatic digestion, a 1% pronase solution in dissection media was freshly prepared and sterilized by passing it through a 0.22 μm syringe filter. The 35 mm

Petriplate was added with 0.7 mL of dissection media along with 0.3 mL of 1% pronase enzyme. The Petri plate was incubated for 1 h in a CO_2 incubator at 37°C.

5.2.4. Mechanical trituration of the digested muscle

After enzymatic digestion, the muscle pieces with pronase solution were transferred to 15 mL centrifuge tube and centrifuged at 400 g for 5 minutes. The supernatent was discarded without disturbing the enzyme digested muscle pellet. The enzyme digested muscle pellet was added with 5 mL trituration medium consisting of 89% DMEM, 10% horse serum (HS) and 1% penicillin-streptomycin. The first round of trituration was performed by using 10 mL plastic Pasteur pipette by forcibly passing the muscle fragments for 20 times. The muscle fragments were allowed to settle down under gravitational forces and the supernatant was collected in a fresh 15 mL centrifuge tube. The settled muscle fragments were added with a fresh 5 mL of trituration media and the second round of trituration was performed by using 1 mL micropipette for 30 times. The supernatant was aspirated and collected and pooled to the supernatant from previous trituration step.

5.2.5. Coating gelatin on standard culture grade Petri plates

2% gelatin type B solution was firstly prepared in phosphate buffer saline at pH 7.4 and sterilized by autoclaving. For coating, standard cell culture grade 35 mm Petri plates were added with 400 µL of 2% gelatin solution. The Petri plates were incubated in cell culture hood at room temperature for 1 h. Subsequently, the gelatin solution was removed and the Petri plates were allowed to incubate at room temperature for a minimum of 30 minutes until further use.

5.2.6. Plating of the cells on gelatin coated Petri plates

The cell suspensions collected after trituration steps were further passed through a 40 µm cell strainer to remove cell debris. The strained cell suspension, collected within a 50 mL centrifuge tube was centrifuged at 1000 g for 10 minutes. The supernatant obtained was

discarded, and the cell pellet was resuspended in 10 mL growth medium consisting of DMEM, 20% FBS, 10% HS and 1% penicillin-streptomycin. This cell suspension was transferred to a 100 mm standard culture grade Petriplate for 1 h under standard CO_2 incubation (Gharaibeh et al. 2008; Jankowski, Deasy, and Huard 2002)**.** Preplating eleminates the non-myogeneic cell population. The unadhered cells were finally seeded on 2% gelatin-coated culture grade Petri plates at a density of $2.5x10^4$ cells /8 cm^2. The growth medium was changed once every 3 days. A schematic representation of the isolation and culture of cells is as shown in Figure 5.2.

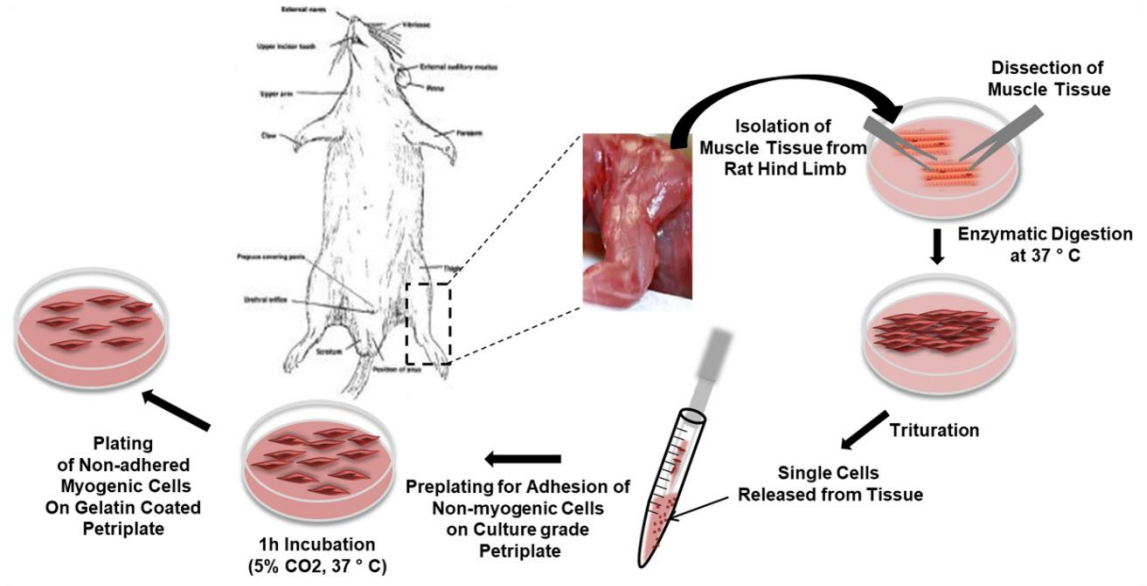

Figure 5.2 Figure depicts the scheme adopted for isolation of primary myoblasts from rat hind limb muscles. The general steps involve dissection of muscles, enzymatic digestion, trituration, pre-plating of cells for separation of non-myogenic cells and plating of myogenic cells.

5.2.7. Bright field and immunofluorescence microscopy procedures

Routine microscopic observations were carried out using bright field images captured with a Nikon TiU microscope. For immunofluorescence microscopy, the cells cultured on cell culture grade Petri plates were fixed with 4% (v/v) paraformaldehyde for 20 minutes at a room temperature, permeabilised with 0.1% (v/v) Triton-X 100 detergent for 10 minutes at a room temperature and blocked with 1% (w/v) BSA in phosphate buffered

saline (PBS) for 30 minutes at room temperature. Further, the cells were incubated with mouse monoclonal primary antibodies of anti Pax7 antibody (2.5µg/mL) or anti myogenin antibody (2.5µg/mL) [Developmental Studies Hybridoma Bank (DSHB)] overnight at 4˚C followed by incubation with secondary antibody Alexa flour^{TM} 488 goat anti-mouse [Thermofischer scientific] (1:1000) for 2 hours at room temperature. F-actin and nuclei were stained for 1 hour and 30 minutes, respectively, using Phalloidin Tetramethylrhodamine Conjugate (1:1000) and 4',6-diamidino-2-phenylindole (DAPI) (1µg/mL). The cultured glass substrates were further treated with PVA-DABCO and visualised under the fluorescence microscope.

5.2.8. Assessment of purity of myoblasts in the culture

In order to assess the purity of the culture, the cells cultured on 2% gelatin coated cell culture grade Petri plates for 3 days were subjected to Pax7/DAPI staining and the percentage of Pax7 positive cells was quantified from a set of 6 images from three different experiments. The quantification involved manual counting of Pax7 positive cells by using Image J (Rueden et al. 2017). The results were expressed as mean ± standard deviation (SD).

5.2.9. Live cell imaging of the myoblasts

Live cell imaging was performed using a custom-built cage incubator for Nikon Ti-U microscope. The incubator chamber was supplied with humidified 5% CO_2 while maintaining the microscope stage temperature at 37°C.

5.2.10. Calcium dynamics of myoblasts and myotubes

A concentration of 5 µM Fluo-8 AM dissolved in Hank`s balanced salt solution (without calcium and magnesium) (HBSS) with 0.02% pluronic F-127 was freshly prepared. Prepared Fluo-8 AM solution was added to the culture plates, previously washed with PBS followed by 1 h incubation at a room temperature succeeded by an exchange of the

dye solution with HBSS (with calcium). Time series of images were recorded using Nikon Ds Qi-2 monochrome camera attached with a Nikon Ti-U fluoroscence microscope integrated with the appropriate excitation and emission filters.

5.2.11. Determination of twitch frequency of spontaneous contractions by kymograph based approach

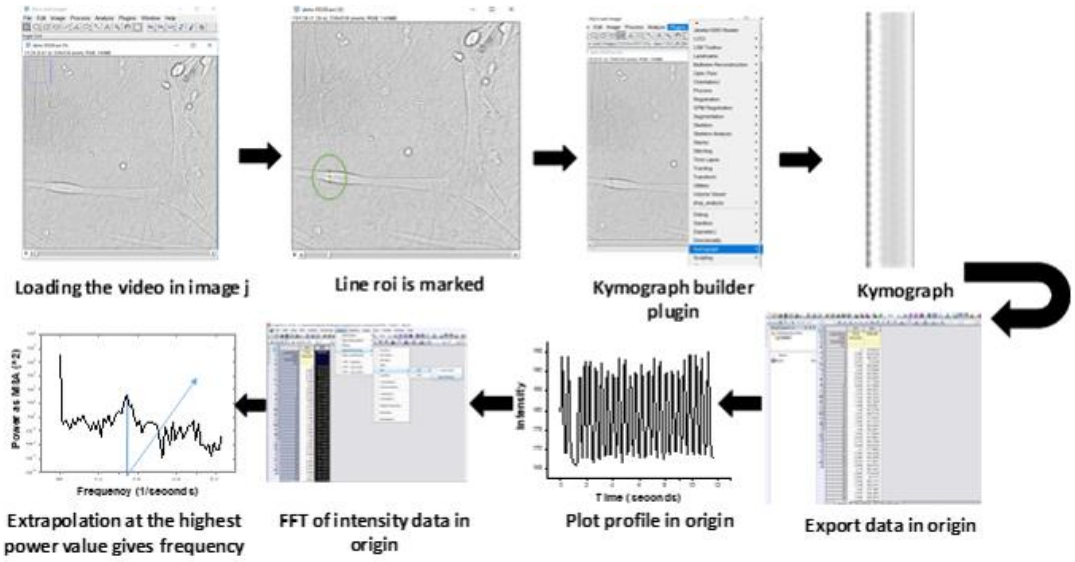

Figure 5.3 Figure depicts the scheme adopted for determination of principal twitch frequency for spontaneous contractions in myotubes.

A video of the spontaneously twitching myotube was captured using a microscope. The video was taken as input in ImageJ. A line ROI was drawn at the location of maximum movement on the myotube. A kymograph of the captured video was generated using kymograph builder plugin in Fiji application, an open source image processing software. Further, an FFT function was applied to the gray intensity values of the kymograph using OriginLab software in order to generate a power versus frequency plot that finally can be used to interpret the level of principal frequency of spontaneous contraction of a myotube.

5.3. Results and discussion

5.3.1. Isolation and culture of skeletal muscle

The rat hind limb muscles, i.e., tibialis anterior and gastrocnemius muscles were successfully isolated, enzymatically digested and triturated to obtain skeletal muscle-derived cells. The procedure used for isolation involving digestion with pronase enzyme and mechanical trituration followed by differential plating has been well proven for isolation of muscle satellite cells (Zipora Yablonka-Reuveni 2011; Danoviz and Yablonka-Reuveni 2012; Bischoff 1974). In this case, the isolated cells showed a myoblast like behavior as shown in Figure 5.4. Figure 5.4 shows the morphological changes in the cells at various stages of culture in vitro. Plating of cell suspension in a conventional polystyrene Petri dish followed by uninterrupted culturing of cells in the growth medium for continuous three days resulted in adherence and alignment of cells. After three days in vitro, the morphology of the adhered cells was found to be polygonal and bipolar in shape; the cells approaching 6 DIV exhibited a remarkable increase in polygonal cells compared to bipolar owing to the fresh medium. Addition of fresh serum rich growth medium provides a proliferative environment, which leads to increase in the cells with polygonal morphology. The appearance of an elongated morphology of myotube in their nascent form and with a larger width compared to that of the bipolar shaped cells was observed by 9 DIV. Likewise, after a period of 12 DIV cells metamorphosed spontaneously and 56% of them demonstrated the elongated myotube morphology with the corresponding decrease in bipolar and polygonal cell percentages to 29% and 15%, respectively. These observations were used as a basis to estimate the percentage of varying forms of cell population manifested in culture over the days (Figure 5.4B). These observations suggest that the isolated cells have a myogenic origin. Once satellite cells undergo activation, they go through the division to form myoblasts (Collins

et al. 2005). Tear drop morphology and alignment observed within a group of cells were identified as characteristics of myoblast cells (Clark et al. 2002; Peckham 2008). These myoblast cells further proliferated, increased their number, differentiated and eventually fused to form premyotubes (multinucleated cell formed due to fusion of few myoblasts) (Abmayr and Pavlath 2012). All the above said observations reveal that satellite cells are highly dynamic in nature and that myogenesis proceeds from the stages of adhesion and proliferation to the appearance of bipolar myoblasts and finally differentiation into myotubes through alignment and fusion of the myoblasts. Various studies support that such transitions play a crucial role in satellite cells-based muscle regeneration (Collins et al. 2005; Day et al. 2007; Fujimaki et al. 2016; Lepper, Partridge, and Fan 2011).

5.3.2. Pax7 based immunofluorescence staining of the isolated cells confirms satellite cell origin

Pax7 transcription factor is one of the characteristic markers for identification of satellite cells. In order to verify the origin, the isolated cells were cultured on gelatin coated surface for 3 days and stained with anti Pax7 antibody, as shown in Figure 5.5. The purity of the isolated cells was determined by manual counting of Pax7 positive cells (cells with nucleus stained in green in Pax7 staining). A total of 65.13±3.32 percentage cells were Pax7 positive indicating the satellite cell origin of the myogenic cells. Pax7 staining along with the results form bright field microscopy confirm that the isolated cells are proliferating myoblasts with satellite cell origin.

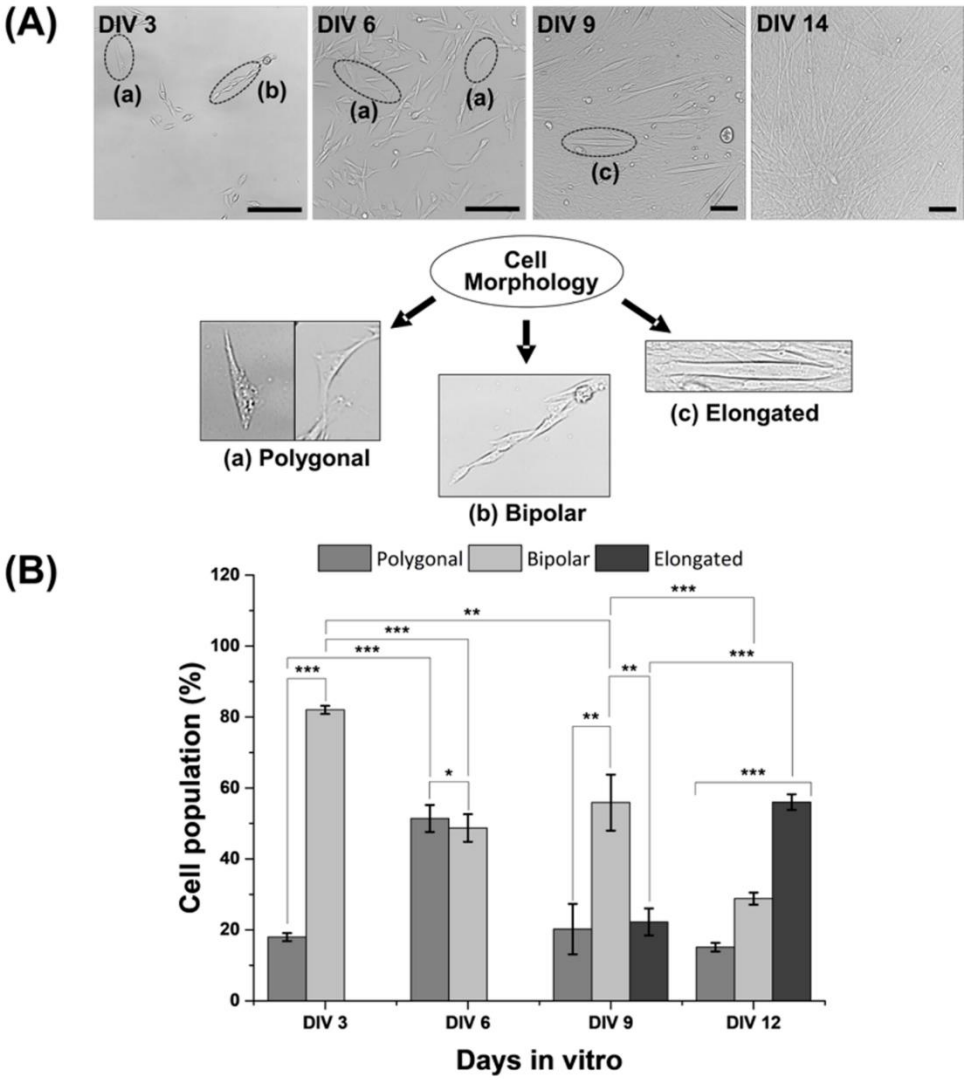

Figure 5.4 Figure depicts bright field images of primary skeletal muscle derived cells cultured on 2% gelatin surface. The cells over the course of the culture adhere, fuse and form myotubes (A) various stages of cell morphology during myogenesis at different time points on gelatin-coated standard culture plates (Scale bar: 100 µm) (B) morphology-based cell population dynamics of skeletal muscle-derived cells over a period of 12 days. Significance levels are presented as ***p<0.001, **p<0.01 and *p<0.05.

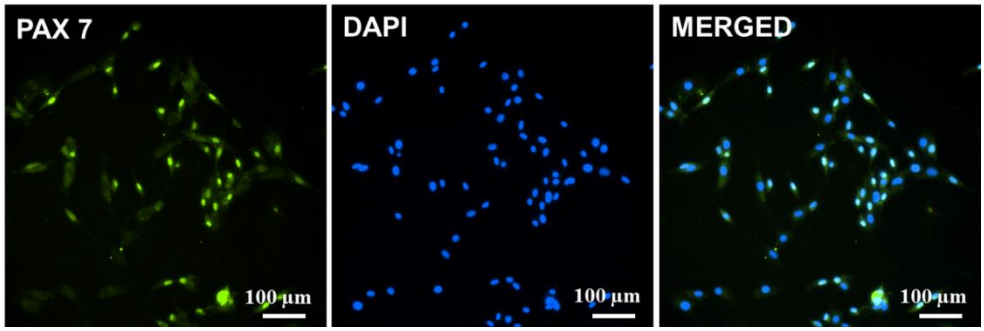

Figure 5.5 Figure depicts merged fluorescence image of isolated cells cultured on 2% gelatin surface stained by using anti PAX7 antibody (green-PAX7 transcription factor, DAPI (nucleus-blue) respectively. This confirms the presence of myogenic cells with satellite cell origin.

5.3.3. Live cell imaging and myoblast dynamics

Real time imaging of the isolated cells on DIV 6 showed many events of cell division taking place indicating the proliferating nature of the myoblasts. Figure 5.6 shows a representative time series of images showing myoblast cell division. During live cell imaging of cells at DIV 9, the event of myotube – myoblast fusion can be observed as shown in Figure 5.7. These observations indicate that the isolated myoblasts undergo fusion to form more mature myotubes. Thus, the live cell imaging of the myoblasts at DIV 6 and DIV 9 undergo proliferation and fusion.

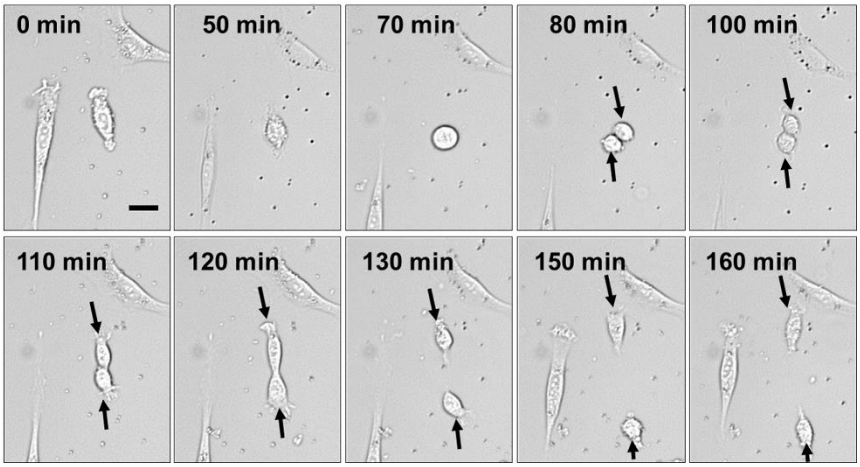

Figure 5.6 Figure depicts the proliferating nature of isolated myoblasts on 2% gelatin surface. Time lapse images of the cell division process acquired by cultured live cell imaging after 6 days of culture on standard tissue culture plates (Scale bar: 25 µm).

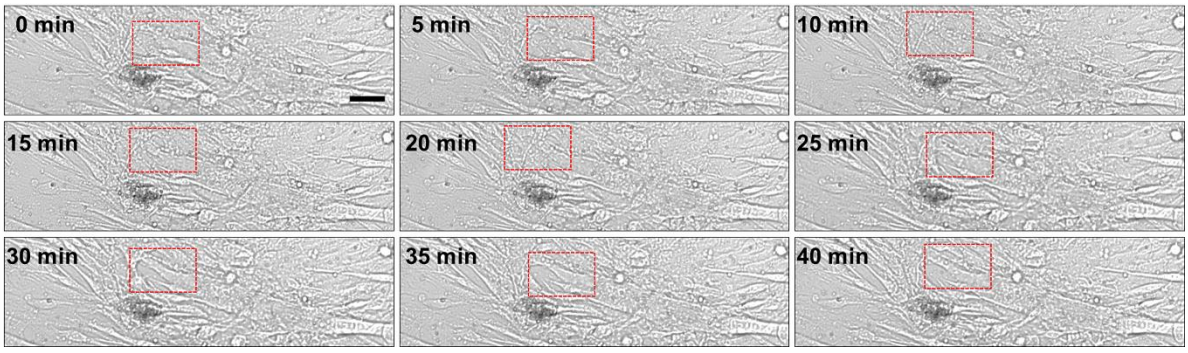

Figure 5.7 Figure depicts a time lapse sequence of images showing myoblast-premyotube fusion process acquired by live cell imaging on 9 DIV of culture on a standard tissue culture plate (Scale bar: 50 µm).

5.3.4. Cytoskeletal actin staining of myoblasts and myotubes

Actin is the most abundant and conserved protein in eukaryotes. Actin proteins expressed in muscle and non-muscle cells include three main isoforms of α, β and γ. According to the muscle of expression the "α" isomers have three sub-isomers including skeletal, cardiac and smooth (Dominguez and Holmes 2011). Actin protein is involved in a myriad of cellular functions including cell adhesion, reorganization of cell shape, cell motility and for intracellular transportation. Such dynamic functions require an equilibrium of polymerizing and depolymerizing globular actin units (G-actin) to form helical filamentous actin (F-actin) (Pollard and Cooper 2009; Ballestrem, Wehrle-Haller, and Imhof, 1998). Role of actin within skeletal muscle is of great significance as the proteins are involved in the processes of myoblast migration (G.U. Bae et al. 2008), fusion (Shilagardi et al. 2013; S. Kim et al. 2007) and formation of multinucleated myotubes with functional sarcomeres. The migration and recognition process in differentiated myoblast require the formation of lamellipodia and filopodia which is made posssible by dynamic actin remodelling (Ohtake 2006). Prior to myoblast fusion process, the myoblasts undergo morphological changes from an unorganized fibroblast like morphology to a spindle shaped bipolar morphology characteristic of a differentiated myoblast. The importance of actin`s role becomes magnified when it was observed that any interference in actin remodeling by using Cytochalasin D or Latrunculin A negatively affected the myoblast migration and myoblast fusion (Nowak et al. 2009). In order to understand the changes taking place in actin cytoskeleton of the isolated myoblasts. The myoblasts were stained with fluorescent dye rhodamine phalloidin, which binds to the actin. The cells at DIV 7 the organization of actin filaments and nuclei present within a group of myoblast cells at 7 DIV (Figure 5.8). The images illustrate the polarised actin filaments in the cells (left image); Also, (right image) the myoblast cells located in close

proximity exhibit a characteristic sarcomeric actin patterning indicating the differentiating status of the myoblasts (R. Duan and Gallagher 2009).

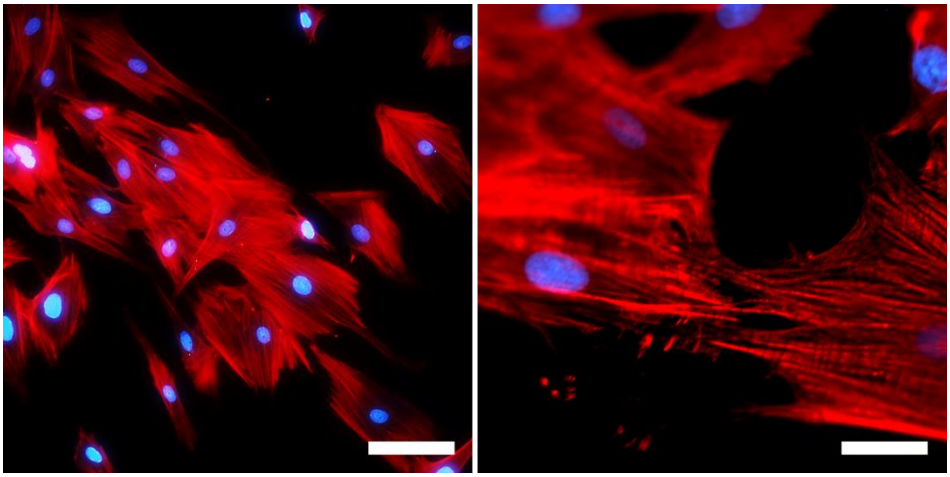

Figure 5.8 Figure depicts fluorescence images of myoblast cells stained for actin filaments (red) and nuclei (blue) presenting a group of aligned myoblasts before fusion (Scale bar: 50 µm); right side panel image shows closely apposed myoblasts with sarcomeric patterned actin filaments in (red) and nuclei (blue) (Scale bar: 6 µm).

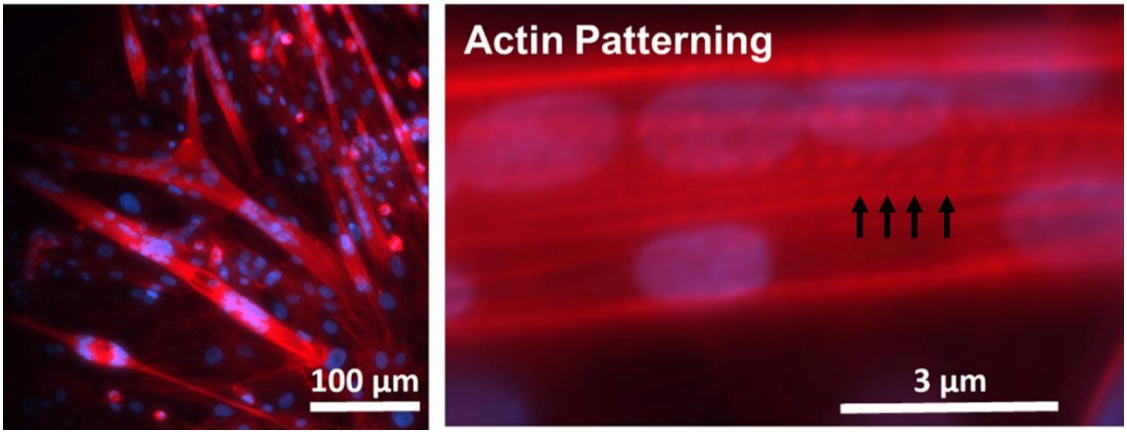

Figure 5.9 Figure depicts the merged fluorescence images of multinuclear differentiated primary skeletal muscle myotubes cultured on 2% gelatin surface under DAPI (nucleus) and TRITC (actin filaments) channels.

These observations could be correlated with dynamic reorganization of actin filaments within myoblasts, which is one of the necessary prerequisites for myotube formation (Nowak et al. 2009; Peckham 2008). The fusion of differentiated myoblasts leads to formation of myotubes. The staining of myotubes reveals the multinucleated nature of the myotubes at 14 DIV. Fluorescent images also reveal the expression of sarcomeric actin

within the myotubes (Figure 5.9). Thus, leading to an observation that the myotubes formed have mature contractile units for force production.

5.3.5. Calcium dynamics in myoblasts and myotubes

Role of calcium as a cell-signalling molecule is universal due to its versatile nature. The cells use calcium signals in a variety of combination in order to generate spatio-temporal signals for regulating numerous cellular functions, including fertilisation, cell proliferation, cell differentiation, apoptosis, cancer, etc. (Berridge, Lipp, and Bootman 2000). In particular, the role of calcium signalling in skeletal muscle is indispensable as calcium dynamics is necessary for skeletal muscle development, homeostasis and regeneration (Michelle K. Tu et al. 2016). Excitation contraction function due to calcium signalling is a well characterized phenomenon. In order to study the dynamics of intracellular calcium, fluorescence-based calcium indicators have widely been used (Bootman et al. 2013). In this study, the basal level of cytosolic calcium dynamics was investigated using a fluorescent calcium sensitive dye, Fluo-8 AM. The fluorescent images representing the dynamics of cytosolic calcium are shown in Figure 5.10. The corresponding plots show modulation in the concentration of cytosolic calcium with respect to time as observed in myoblast and myotube stage of satellite cells cultured for 14 DIV. Observation of transient calcium has been found to be essential for the regeneration of muscles (Michelle Kim Tu and Borodinsky 2014; Lorenzon et al. 1997). Calcium transients act through various effector molecules such as calcium/calmodulin dependent phosphatases (i.e., calcineurin) and kinases; mitogen activated protein kinases (MAPKs) and various calcium dependent transcriptional factors. Maintenance of the basal levels of cytosolic calcium is known to be associated with store operated calcium channels (SOCE) that are governed by the various proteinaceous factors such as sensor of internal calcium stores, stromal interaction molecule 1 (STIM1), stromal interaction

molecule 2 (STIM2) and the SOCE channels i.e., ORAI1 and Transient Receptor Potential Canonical (TRPC) channels (Stiber and Rosenberg 2011; Michelle K. Tu et al. 2016). In particular, the expression of STIM1 and ORAI1 is essential in differentiation of human myoblasts (Darbellay et al. 2009). TRPC channels along with fibroblast growth factors have been implicated in the activation of satellite cells-based regeneration (Y. Liu and Schneider 2014). Calcineurin has been found to have critical roles in satellite cell activation through regulating myogenic regulatory factors, e.g., MEF2, myogenin, and MyoD; that in turn controls the skeletal muscle differentiation (Friday et al. 2003).

Recently Tu *et al.* have implicated the need for studies to gain further insights into calcium dynamics related to specific cell types involved in repair and regeneration of skeletal muscles (Michelle K. Tu et al. 2016). In this study, tests were performed to understand the basal levels of cytosolic calcium transients in the various stages of myoblasts. Signalling patterns observed at a single cell level indicated a significant rise in the spikes of intracellular calcium as the cells proceeded through the stages of differentiation, i.e., from proliferating myoblast to myotube stage (Figure 5.10). Average time per calcium spike indicating the frequency; for myoblasts was found to be 1.4 s (Figure 5.10A), that further increased to 2.53 s for myotube (Figure 5.10B), respectively. Average peak amplitude for myoblast and myotube was found to be 1.086 and 1.40, respectively. These values indicate that the amplitude and frequency of the calcium spikes increase with the differentiation status changing from proliferating myoblast to myotube. In case of the myotube, both the frequency and amplitude of these repetitive calcium oscillations have increased. Myotubes being the most mature contain increased number of well-developed sarcoplasmic reticulum contributed by the fused myoblasts. Therefore, it is likely that they show highest levels of calcium transients in comparison to myoblasts. Such difference in basal level of calcium signalling between the evolving stages of

skeletal muscle i.e., according to their state of maturity has been reported previously (Constantin, Cognard, and Raymond 1995).

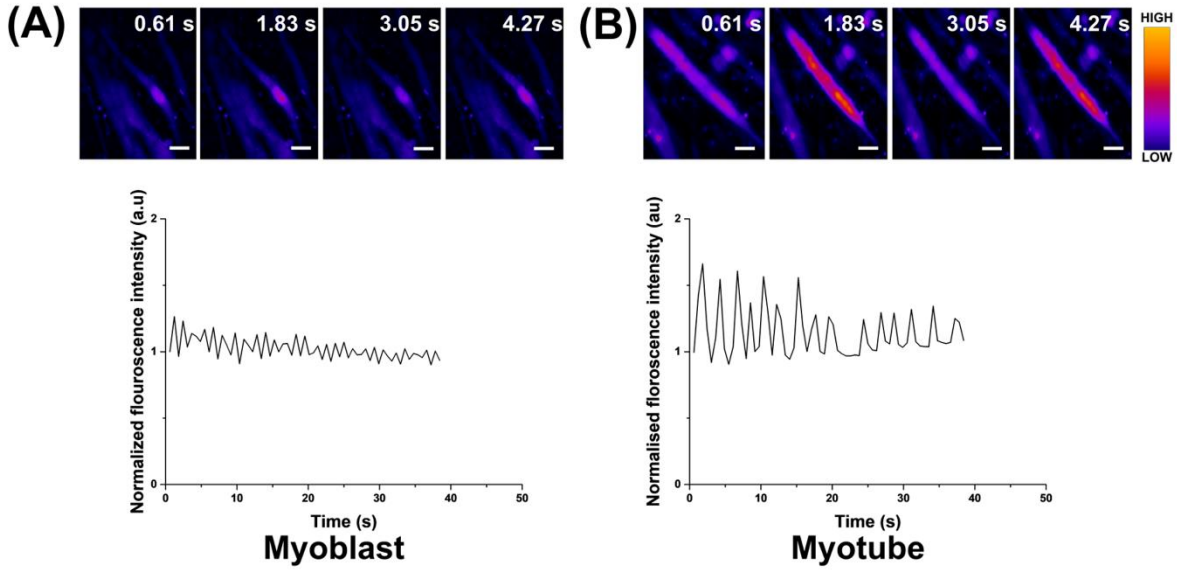

Figure 5.10 Figure depicts the calcium dynamics occurring within (A) myoblast and (B) myotube under standard culture conditions (Scale: 25 µm).

The reason for such a difference has been implicated to the mechanism of calcium uptake; in premature stages of myoblasts plasma membrane bound voltage-gated channels and sarcoplasmic reticulum are involved whereas in myotubes only sarcoplasmic reticulum release is responsible (Sebille et al. 2017).

5.3.6. Determination of spontaneous twitch frequency of the myotubes by using kymograph approach

The isolated satellite cells formed myotubes which showed spontaneous contractile behaviour. A previous study by bajaj et al. applied the kymograph based approach to characterize the twitch response of myotubes under controlled electrical stimulation (Bajaj et al. 2011a). In this study, same method was applied for characterizing the twitch frequency of spontaneous contraction occuring within the myotubes formed by fusion of satellite cells. A kymograph is a simplified way of representing the dynamics of the twitching process on a single image. It is a space versus time plot that represents intensity change along the line region of interest (ROI) with change in image frames of the video.

Each time point gives an intensity line along the X-axis of the kymograph plot. These intensity lines are stacked along the y axis for all the frames of the video. Thus, the obtained plot of change of intensity in space along x-direction with varying time along y-direction represents the dynamic process of myotube twitch. The results prove that the method can be succesfully applied to express the dynamic activity in the form of a graph of amplitude vs time. Subsequently applying FFT (Fast Fourier transform) to the amplitude signal provides a way to characterize the principle twitch frequency of spontaneously twitching myotubes (Figure 5.11).

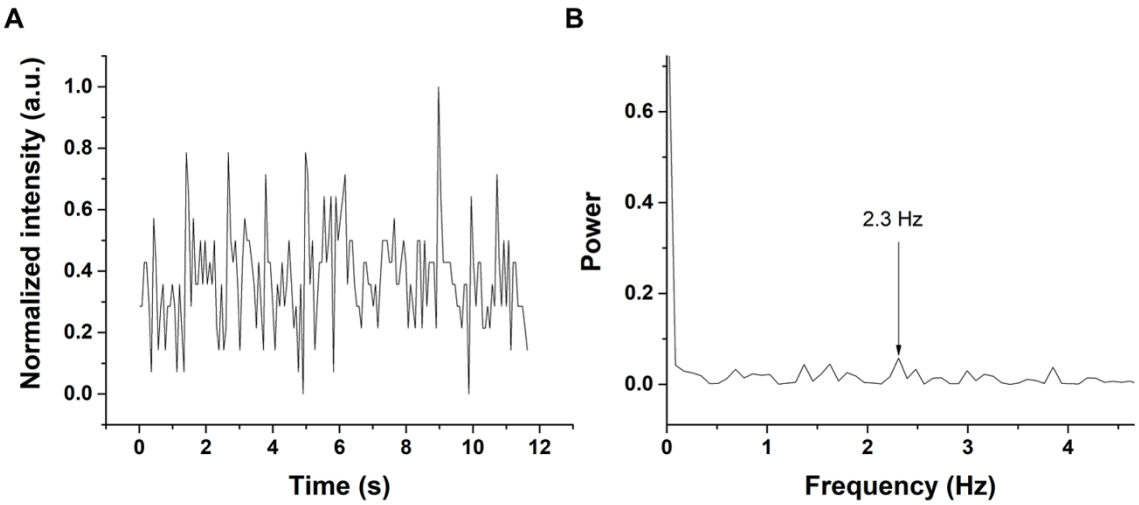

Figure 5.11 Figure depicts the results of kymograph-based approach for representing dynamics of spontaneous myotube twitching (A) amplitude vs time results (B) Principal twitch frequency determination after application of fast Fourier transform.

Isolation of primary satellite cells and their analysis has been explored by various other (Zipora Yablonka-Reuveni 2011; Danoviz and Yablonka-Reuveni 2012; Bischoff 1974; Hindi et al. 2017). In this study, satellite cells were isolated from rat hind limb skeletal muscles and were characterized. The characterization of the isolated cells by morphometric based population analysis of the cells in the form of polygonal, bipolar and elongated cell morphologies over a period of 12 days of in vitro culture, has not been shown by others. In addition to it the study presents the basal calcium

characterization of various stages of maturation attained by the satellite cells including myoblasts and myotubes. The study also presents the application of kymograph-based approach for characterization of spontaneous twitching behavior of the myotubes.

5.4. Summary

Satellite cells were isolated from rat hind limb muscles, characterized at the morphology and molecular level, and cultured on gelatin coated substrates to explore the events and progression of myogenesis process. Pax7 immunofluorescence characterized the isolated cells to be satellite cells ($Pax7^+$). Nearly 65 % of the cells were found to be $Pax7^+$ indicating the purity of isolated cells. During the in vitro culture period, the cells undergo a transition in morphology from a polygonal to bipolar and bipolar to elongated morphology, which corresponds to differentiated myoblasts, and myotube morphology. The cytoskeletal actin staining of the cells in bipolar morphologies showed the differentiated status of the cells. The cytoskeletal actin staining of the myotubes showed the multi-nucleated nature confirming fusion of the myoblasts. Characteristic sarcomeric actin patterning confirms the maturity of the myotubes. The live cell imaging shows that the cells undergo proliferation and fusion. Twitching behavior of the myotubes indicates the formation of functional contractile proteins. The calcium studies show an increase in basal calcium dynamics with the progression of myogenesis of the cells.