ARCTURUS

APPLICATION NOTE #2

Laser Capture Microdissection of muscle fiber populations and expression analysis by RT-PCR

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Abstract

To locate gene products in distinct muscle fiber populations of different muscle allotypes, a novel protocol was established. A rapid method to distinguish muscle fiber types using histochemistry and immunohistochemistry was used to provide criteria for their selective isolation by laser capture microdissection while preserving messenger RNA (mRNA). Polymerase chain reaction showed a differential expression pattern of muscle specific genes in different muscle fibers in laser captured material.

Introduction

While vertebrate limb muscles have traditionally been the paradigm for studies of skeletal muscle myogenesis, a few atypical muscle groups such as extraocular muscles (EOMs) have distinct functional specializations and patterns of gene expression[1]. Differences between muscle groups (called "allotypes") have been the subject of research for the past decades. The limb and EOM allotypes differ in their pattern of innervation. All limb fibers and 80% of EOM fibers are singly innervated fibers (SIFs) which have only one neuromuscular junction. About 20% of EOM fibers are multiply-innervated fibers (MIFs): they have multiple neuromuscular junctions[2]. In the past, immunohistochemistry and in situ hybridization were mainly used to study differences between muscle fiber populations[3,4], While useful, these techniques are limited in their capability to assess multiple gene products from a mixed muscle fiber population. Moreover, the mixed fiber populations of these muscles make it impossible to study the composition of individual fiber types. To overcome these problems, laser capture microdissection was used to isolate different muscle fiber populations and analyze multiple gene products by polymerase chain reaction. The muscle fiber populations in EOM and limb muscle were distinguished by a combination of the histochemical acetylcholine esterase stain by Karnowsky and Roots[5] and immunohistochemical staining with a mouse-monoclonal slow myosin heavy chain (MyHC) antibody (NOQ7-5-4D)[6]. SIFs show a large, C-shaped neuromuscular junction after acetylcholine esterase staining and do not react with the anti-slow MyHC antibody. The neuromuscular junction of MIFs is smaller and more circular and all MIFs are positive for slow MyHC by immuno-staining. Since mRNA is sensitive to degradation, total time from thawing the slides to dehydration by xylenes was reduced to 32 minutes by combining the histochemical and immunohistochemical reactions and the direct coupling of the antibody to Alexa Fluor® 488 by Molecular Probes Zenon™ technology.

This protocol describes a way to isolate and visually distinguish muscle fiber population from two different muscle allotypes and characterize different muscle fibers by multiple RT-PCRs.

Equipment and Reagents

This protocol requires the following reagents:

- ❑ RNase Away® (Invitrogen, Cat. # 10328-011)
- ❑ Zenon™ Alexa Fluor® 488 Mouse IgG1 Labeling Kit (Molecular Probes, Cat. # Z-25002)
- ❑ PicoPure™ RNA Isolation Kit (Arcturus, Cat. # KIT0204)
- ❑ RiboAmp™ RNA Amplification Kit (Arcturus, Cat. # KIT0201)
- ❑ Slow Myosin MyHC Antibody NOQ7-5-4D
- ❑ PCR Primer (Invitrogen, custom-made)
- ❑ Goat Serum (Sigma, Cat. # G-9023)
- ❑ Ethanol (Pharmaco, Cat. # 111ACS200)
- ❑ Hydranal®-Xylenes (Riedel-de Haen, Cat. # 37866)
- ❑ 2-Methylbutane (Fisher, Cat. # A-03551-4)
- ❑ Colorfrost® /Plus Microscope Slides (Fisher, Cat. # 12-550-19)
- ❑ Acetylthiocholine Iodide (Sigma, Cat. # A-5751)
- ❑ Sodium Acetate (Sigma, Cat. # S-2889)
- ❑ Sodium Citrate (Fluka, Cat. # 71402)
- ❑ Cupric Sulfate (Sigma, Cat. # C-1297)
- ❑ CapSure® HS LCM Caps (Arcturus, Cat. # LCM0214)
- ❑ FastStart Taq DNA Poymerase (Roche, Cat. # 2 032 926)
- ❑ dNTP Mix PCR Grade (Invitrogen, Cat. # 18427-013)
- ❑ UltraPure™ Agarose (Invitrogen, Cat. # 15510-027)
- ❑ UltraPure™ 10x TAE (Invitrogen, Cat. # 15558-026)
- ❑ Ethidium Bromide (Sigma, Cat. # E-1510)
- ❑ Tissue-Tek® OCT Compound (Sakura, Cat. # 4538)
- ❑ DEPC – Treated Water (Ambion, Cat. # 9920)

Equipment and Labware

General equipment necessary:

- ❑ Disposable gloves
- ❑ Flow hood
- ❑ Dry ice
- ❑ Liquid nitrogen
- ❑ Ice or cold block
- ❑ Nuclease-free, aerosol resistant tips
- ❑ RNase-free microcentrifuge tubes
- ❑ Scale
- ❑ Kimwipes™ or similar lint-free towels

The following laboratory equipment is required to microdissect muscle fiber properly:

- ❑ Cryostat
- ❑ Cryostat knife
- ❑ Arcturus PixCell® II Laser Capture Microdissection System with fluorescence package
- ❑ QImaing™ Retiga 1300 cooled mono 12-bit digital camera

The following laboratory equipment is required for RNA isolation and analysis by RT-PCR:

- ❑ Incubation oven
- ❑ Microcentrifuge
- ❑ Arcturus alignment tray
- ❑ Thermo Cycler with heated lid
- ❑ Horizontal gel chamber
- ❑ Power supply
- ❑ Gel Doc 2000™ Documentation System (Bio-Rad, Cat. # 170-8615)

RNase-free Technique

In addition to the usual precautions listed below, some special precautions were taken connected to the rapid staining protocol:

1. Use RNase® AWAY according to the manufacturer's instructions on laboratory bench surfaces, cryostat, cryostat knife, PixCell® II Laser Capture Microdissection System and Arcturus alignment tray.
2. Disposable gloves are to be frequently changed and RNase-free plasticware used.
3. Heat inactivate serum.
4. Use DEPC-treated water for the preparation of all staining solutions and for washing.
5. Use chemicals in the highest grade available.
6. Staining and washing time is to be reduced to a minimum, while still preserving the capability to distinguish different muscle fiber populations.

Method

1. After dissection, limb muscle and EOMs are covered in OCT compound and successively frozen in cooled 2-methylbutane (30 seconds) and liquid nitrogen (10 minutes). The muscle can be stored at -80ºC.

2. The muscle is cut into 10 µm sections with the cryostat. During the cutting process, the slides with 4 sections each are stored on dry ice and are afterwards stored at -80ºC.

3. For the acetylcholine esterase stain by Karnowsky and Roots, the following solution is prepared in the following order:
   - 5 mg of acetylthiocholine iodide are dissolved in 6.5 ml of 0.1 M sodium acetate buffer pH 5.2, followed by:
   - 0.5 ml 0.2 M sodium citrate,
   - 1.0 ml of 30 mM cupric sulfate,
   - 1.0 ml DEPC-treated water, and
   - 1.0 ml of 5 mM potassium ferricyanide.

   Larger quantities of the four solutions can be prepared and stored at 4ºC. But the staining solution has to be prepared fresh since the acetylthiocholine starts to precipitate out of solution after 2 hours.

4. To stain slow MyHC positive fibers rapidly Molecular Probes's Zenon™ antibody-labeling technology is used. 20 µl of NOQ7-5-4D are mixed with 5 µl of Component A (Zenon™ mouse labeling reagent), incubated for 5 minutes. Followed by 5 minutes of incubation with Component B (Zenon™ mouse blocking reagent).

5. After adding Component B to NOQ7-5-4D, the slide for laser capture microdissection is placed in DEPC-treated water with 2% heat inactivated goat serum for 5 minutes to block non-specific binding sites.

6. The Alexa Fluor® 488 conjugated NOQ7-5-4D antibody is diluted 1:40 in the prepared acetylcholine esterase staining solution.

7. The blocked slide is now incubated

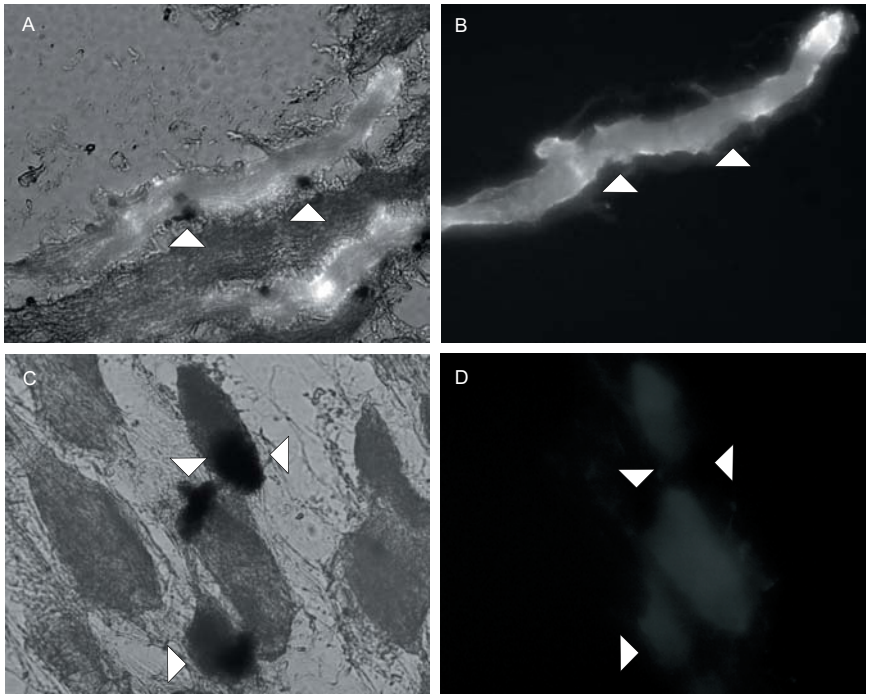

Figure 1. Laser Capture Microdissection of Muscle Fiber Populations.
A multiply innervated muscle fiber is shown before (A) and after (B) laser capture mircodissection. The fiber shows strong immunohistochemical staining for slow myosin and the two grappe-like neuromuscular junctions are marked with an arrow.
Figures C and D show the laser capture microdissection process of a population of singly innervated muscle fibers. This fiber population is negative for slow MyHC immuno-staining and show comparable large neuromuscular junctions which are again marked with an arrow. Figures A and C are taken with the fluorescence and dimmed visual light on and Figures B and D show the fiber after microdissection on the laser microdissection cap with only fluorescence light on. Some autofluoresence is detectable in Figure D, but the signal is weaker than in Figure B.

with the prepared staining solution for 20 minutes.

8. After 20 minutes, the slide is washed twice for 3 minutes in DEPC treated water.

9. The slide is dehydrated in 75%, 90%, and 100% ethanol for 30 seconds respectively, followed by 5 and 7 minutes in fresh xylenes. After 1 minute in a flow hood the slide is ready for laser capture microdissection.

10. The Arcturus PixCell® II Laser Capture Microdissection System with fluorescence package is switched on and the blue filter cube is set. The QImaing™ Retiga 1300 cooled mono 12-bit digital camera is activated.

11. The microdissection laser is set to 7 µm size, 70 mW power and 850 µs pulse duration. The last two values are adjusted to the lowest values possible to still perform laser capture microdissection. This adjustment to a minimal setting enables to actually dissect single muscle fibers.

12. SIFs were dissected based on their large neuromuscular junction stained by Karnowsky and Roots's acetylcholine esterase stain and their lack of slow MyHC staining (Figure 1C). MIFs were dissected based on their smaller neuromuscular junction and a positive signal for slow MyHC (Figure 1A). Additionally, muscle fibers were dissected which were positive or negative for slow MyHC, but didn't show a site of innervation based on Karnowsky and Roots's acetylcholine esterase stain. To dissect single fibers, it can be advantageous to search for fibers on the edge of a fiber bundle as they prove to be easier to dissect without picking up undesired fiber populations.

13. To avoid cross-contamination by other cell types, every cap is scanned visually after the dissection by placing the cap on a fresh slide and viewing it at lowest power. This is followed by a scan at the highest power to make sure that only the desired tissue was captured based on the fluorescence signal (Figures 1B and 1D). The cap is discarded, if unwanted tissue was present. Monochrome pictures of samples were taken using the QImaing™ Retiga 1300 cooled mono 12-bit digital camera.

14. Each cap is then placed in an ExtracSure™ device. The RNA from approximately 25 fibers of each population is pooled and RNA extraction is performed with the PicoPure™ RNA Isolation Kit as described in its protocol within 2 hours of the dehydration with xylenes.

15. After the RNA is extracted, the RiboAmp™ RNA Amplification Kit is used according to the manufacturer's instructions to linearly amplify the sample RNA and transcribe the aRNA back to single stranded cDNA. One round of linear amplification was performed for all samples, typically yielding 300 - 600 ng of aRNA.

16. To study expression of muscle specific genes, 50 ng cDNA are amplified with FastStart Taq DNA Polymerase for 40 cycles with conditions and reagents as presented in Table I. The epsilon- and gamma- subunit of the acetylcholine receptor and slow MyHC are amplified. The housekeeping gene GAPDH was amplified as a reference for the amount loaded and the quality of the cDNA. All primers (Table II) are intron spanning to produce a cDNA specific PCR product. To avoid false positive results by PCR product carry-over, PCRs are setup in a flow hood.

17. 15 µl of each PCR product were loaded on a 2% agarose gel and separated by 120 V for 25 minutes. Gels are stained with ethidium bromide and visualized using the Gel Doc 2000™ Gel Documentation System.

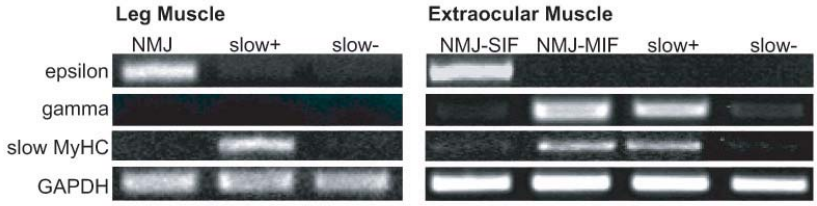

Figure 2. Semi-quantitative RT-PCR of different muscle fiber populations.
Gel pictures of polymerase chain reaction for epsilon- and gamma- subunit of the acetylcholine receptor, slow myosin heavy chain (MyHC), and GAPDH.
Muscle fibers were distinguished by their reactivity to the acetylcholine esterase stain by Karnowsky and Roots and to Alexa Fluor 488 conjugated slow MyHC antibody. In the leg, three different populations were analyzed: (NMJ), the neuromuscular junction region of fibers negative for slow MyHC; (slow +), non innervated region of fibers positive for slow MyHC; (slow -), non innervated region of fibers negative for slow MyHC. In the EOMs, fibers were again distinguished by their reactivity to anti-slow MyHC antibody and their staining for acetylcholine esterase. (NMJ-SIF), the neuromuscular junction region of fibers negative for slow MyHC - these are all singly innervated; (NMJ-MIF), the neuromuscular junction region of fibers positive for slow MyHC - these are all multiply-innervated; (slow +), non innervated region of fibers positive for slow MyHC - i.e. MIFs; (slow -), non innervated region of fibers negative for slow MyHC-i.e. SIFs.

Results

The data presented in this application note demonstrates that it is possible to isolate a single muscle fiber type or a distinct muscle fiber population and analyze from it the expression of muscle specific genes by RT-PCR. Figure 1 demonstrates the isolation of an individual fiber type by LCM. The Zenon™ technology based immunohistochemical staining of slow MyHC positive fibers gives a strong, unambiguous signal in less than 35 minutes. The acetylcholine esterase stain of Karnowsky and Roots allows us to assess rapidly the innervation pattern of muscle fibers. When used alone, this esterase stain can provide results within 5-10 minutes. Hence, rapid identification of distinct fibers

based on their immunohistochemistry and innervation pattern allows us time to process tissue via LCM and isolate mRNA without significant time for degradation.

Using this type of analysis on limb muscle confirms data previously obtained by other means[4]. Hence, we can apply the technique to another muscle allotype, the EOM, and be sure that this protocol and the Arcturus PixCell II Laser Capture Microdissection System is sufficient to distinguish muscle fiber types and give new insight into the differences among muscle allotypes.

References

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Table I. PCR Cycling Conditions, Reaction Components and Volumes Used

FastStart PCR Master Mix		**Step**	**Thermal Cycler programming**	
Water	x µl	1	94°C	4 minutes
10x Buffer	5 µl	2	94°C	30 seconds
dNTP	1 µl	3	46 / 57°C	30 seconds
Forward primer	1 µl	4	72°C	60 seconds
Reverse primer	1 µl	5	Go to Step 2	39 times
Sample	y µl	6	72°C	7 minutes
Total	50 µl	7	End	

Table II. PCR Primer Sequences and Annealing Temperatures

	Product size (bp)	**Forward primer (fwd) Reverse primer (rev)**	**Annealing temp.**
γ	114	fwd 5'-GGGTCCGCAAGGTGTTTTTG	
		rev 5'-AGGAAGAGCCATTCTGGAGTCG	57°C
ε	215	fwd 5'-TGTTTGAGGGACAGAGGCATCG	
		rev 5'-CACCAACGCTGCCCAAAAAC	57°C
Slow MyHC	497	fwd 5'-CGGGATCCAGCANGAGCTGGANGAG	
		rev 5'-GCGAATTCGTGTTTCTGCCTGAAGGT	46°C
GAPDH[1]	195	fwd 5'CCATGGAGAAGGCTGGGG	
		rev 5'CAAAGTTGTCATGGATGACC	57°C

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