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## Multi-step immunofluorescent analysis of vitamin D receptor loci and myosin heavy chain isoforms in human skeletal muscle

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### Abstract

Vitamin D receptors have been shown to be present in human skeletal muscle using different techniques. We developed a multi-staining immunofluorescent method to detect vitamin D receptor expression and co-localize it with myosin heavy chain isoform expression in skeletal muscle biopsies in older female subjects. Serial sections were cut from frozen samples obtained by needle biopsy of the vastus lateralis. Samples were probed with a primary vitamin D receptor monoclonal antibody and then re-probed with a type IIa myosin heavy chain isoform-specific antibody. Independent

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unfixed sections followed a similar protocol and were probed with type IIx and type I myosin heavy chain isoform-specific antibodies. Immunohistochemistry and fluorescent microscopy co-localized vitamin D receptor loci and myosin heavy chain isoforms in whole skeletal muscle sections. We quantified intranuclear vitamin D receptor staining patterns and number of individual muscle fiber subtypes within a muscle section. Immunohistochemical staining of the vitamin D receptor was confirmed by Western blot using the same monoclonal antibody. This multi-staining immunofluorescent technique allows for measurement of intranuclear vitamin D receptor expression in the context of the specific muscle fiber type profile in a single section. This method can thus be a useful approach to study potential relationships between muscle fiber subtypes and vitamin D receptor expression.

## Keywords

Vitamin D receptor; Immunohistochemistry; Skeletal muscle

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## Introduction

Recent data suggest that vitamin D supplementation reduces the risk of falls (Bischoff-Ferrari et al. 2009) and improves measures of muscle performance (Pfeifer et al. 2000, 2009; Bischoff et al. 2003) in older adults with low vitamin D status. Muscle biopsies in adults with profound vitamin D deficiency reveal atrophy of type II (fast-twitch) muscle fibers, which are the first fibers to be recruited when preventing a fall (McComas 1996). While the actions of vitamin D on skeletal muscle are not well-understood, current research suggests that the effects of vitamin D may, at least in part, be mediated through the vitamin D receptor (VDR). The VDR is a ligand-activated transcription factor and a member of the superfamily of nuclear receptors for steroid hormones (DeLuca 1988). The active form of vitamin D,  $1\alpha,25$ -dihydroxyvitamin D [ $1,25(\text{OH})_2\text{D}$ ], is believed to bind to a nuclear VDR which, in turn, modulates the expression of genes related to the regulation of calcium transport and cell proliferation and differentiation (Boland et al. 1985; Simpson et al. 1985). The nuclear VDR has been identified in human skeletal muscle tissue using different techniques (Costa et al. 1986; Bischoff et al. 2001). Prior published localization techniques, however, have not had the capability of studying potential relationships between degree of intranuclear receptor expression and muscle fiber subtype.

The aim of this study was to co-localize VDR-positive myonuclei and specific skeletal muscle fiber subtypes within a human skeletal muscle section. To accomplish this aim, we developed a multiple immunofluorescent staining technique that identifies the VDR and muscle fiber subtypes by incubating a muscle section with a monoclonal antibody to the VDR and then myosin heavy chain (MHC) isoform-specific antibodies directly labeled with distinct fluorophores. The intranuclear VDR signal detected using a monoclonal antibody was verified by Western blot and compared to two alternative primary VDR antibodies.

## Methods

### Subjects

Eight muscle biopsy specimens used to develop the method were obtained from four healthy postmenopausal female participants, age 65–85. The Tufts Medical Center-Tufts University Health Sciences Campus Institutional Review Board approved the study, and written informed consent was obtained from each participant.

## Muscle biopsy

Eight muscle biopsies were obtained from the vastus lateralis at the level of the mid-thigh under local anesthesia (xylocaine 1%) with a 5-mm Duchenne biopsy needle and suction (Bergström 1975). The specimens were mounted in a vinyl cryomold (Tissue-Tek, USA) and secured using a viscous mounting medium (O.C.T., Tissue-Tek, USA) and then frozen in isopentane/liquid nitrogen slurry.

## Immunohistochemistry

Eight muscle tissue specimens were cut in seven  $\mu\text{m}$  sections at  $-23^{\circ}\text{C}$  with a cryostat microtome (Leica CM1850, Leica Microsystems, Germany), placed onto microscope slides, and left to air-dry at room temperature for at least 15 min. Samples were washed with phosphate buffered saline (PBS) for 5 min, fixed in 3% neutral buffered formalin for 10 min, and then blocked in PBS/2% goat serum for 20 min. Slides were probed overnight ( $4^{\circ}\text{C}$ ) with a primary mouse/anti-human VDR/NR1H1 monoclonal antibody (clone H4537, Perseus Proteomics, Inc.; Table 1). Subsequently, sections were probed with goat/anti-mouse IgG<sub>2A</sub> Alexa Fluor-568<sup>®</sup> secondary antibody conjugate (Table 1). Samples that were probed with the VDR antibody, were then re-probed with a type IIa (N2.261 IgG<sub>1</sub>) MHC isoform-specific antibody (Table 1). Unfixed sections followed a similar protocol to identify other muscle fiber subtypes. Mouse antibodies against human type I (A4.951 IgG<sub>1</sub>; A4.840 IgM) and IIx (212F IgG<sub>1</sub>) MHC were used (Table 1). A rabbit anti-human antibody (IgG) raised against laminin was used to facilitate identifying individual muscle fibers. Specific goat anti-mouse and anti-rabbit Alexa Fluor<sup>®</sup> secondary antibodies were used for detection of MHC and laminin primary antibodies, respectively (Table 1). Slides were mounted with 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium to stain myonuclei (Vectashield H-1500; Vector Laboratories, Inc). Control sections were processed independently as described above, without primary, secondary or both antibodies (blank control). No staining signal higher than the natural self-fluorescence of the sections was observed (results not shown).

Digital imaging was performed through 100 $\times$  and/or 400 $\times$  final magnification. Adobe Photoshop<sup>®</sup> CS3, Nikon NIS-AR (3.01) and NIH Image J software (1.37v) were employed for data acquisition and data analysis.

## Immunoblotting

To confirm VDR expression, three samples were prepared by homogenization in lysis buffer (50 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol (v/v), 1% Triton-X (v/v), 50 mM NaF, 5 mM Na Pyrophosphate) and then by centrifugation at 10,000 rpm/ $4^{\circ}\text{C}$ . Supernatants were stored at  $-80^{\circ}\text{C}$ . Total protein concentrations of muscle extracts were measured using the Pierce 660 nm protein assay (Thermo Fisher Scientific). To detect VDR, 30  $\mu\text{g}$  of whole cell muscle lysates were loaded onto and resolved by SDS-PAGE using 10% mini-gels (150 V/60 min with the BioRad Mini-Protean Tetra Cell System) and electrophoretically transferred (350 mA/120 min) to 0.45  $\mu\text{m}$  nitrocellulose membranes. Five  $\mu\text{g}$  of 293T lysate (Santa Cruz Biotechnology, Inc.) were used as control. Membranes were blocked in 5% non-fat dry milk (NFM)/Tris-Buffered Saline-0.05% Tween 20 (TBS-T) solution, washed with 0.05% TBS-T, and probed with three different commercially-available primary antibodies to the VDR (VDR/NR1H1 monoclonal antibody, clone H4537 [Perseus Proteomics, Inc.]; VDR D-6 monoclonal antibody [sc-13133 Santa Cruz Biotechnology, Inc.] and VDR 333C6a [sc-81423 Santa Cruz Biotechnology, Inc.] Table 1) in 5% BSA/TBS-T (diluted 1:1,000) overnight. Membranes were then washed with 0.05% TBS-T and incubated with different HRP-conjugated secondary antibodies (Table 1) in 1% NFM/TBS-T (diluted 1:2,000) solution for detection using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

## Results

Immunohistochemistry and fluorescent microscopy co-localized VDR and MHC isoforms in whole skeletal muscle sections. Figure 1 illustrates incubation of muscle cryosections with directly labeled antibodies to types I, IIa, and IIx MHC isoforms and laminin. The emission wavelength of each different secondary antibody (Table 1) employed on the MHC isoform staining allows simultaneous identification of the muscle fiber subtypes in two muscle sections overlaid to create a single multi-colored image (Fig. 1). We measured the relative number of individual muscle fiber subtypes within a cryosection, which in the Fig. 1 section revealed a predominance of type II fibers (Table 2).

In each sample, we randomly selected ten higher magnification (400×) fields, which were representative of the whole section. In Fig. 1, the ten fields comprised 46% of the muscle fibers in the whole section and had a similar type I to type II muscle fiber type ratio (32% to 68%) to that of the whole section (30% to 70%). The 400× images were captured using fluorescent (Fig. 2a/b) and bright field microscopy (Fig. 2c). Figure 2a shows DAPI staining. Figure 2b illustrates co-localization of VDR-positive nuclei with DAPI. Bright field images (Fig. 2c) were used to assign the myonuclei to a particular fiber. We measured the relative number of VDR-positive myonuclei within each muscle fiber subtype in all 10 fields. Table 3 lists the ratio of VDR-positive myonuclei to total myonuclei by muscle fiber subtype from 10 fields from Fig. 1. Of note, VDR immunohistochemical staining was not solely associated with clearly identified intranuclear staining as shown in Fig. 2b (arrows).

To confirm identification of VDR by immunohistochemical staining in these cryosections, we performed Western blot analysis using the same VDR monoclonal antibody (VDR/NR1H1). The immunoblot was run alongside two other commercially-available primary antibodies to the VDR (D-6 and 333C6a) to confirm the accuracy of the VDR antibody used in the immunohistochemical stain (Fig. 3). The molecular weight band at approximately 50 kDa is consistent across all three lanes incubated with a VDR antibody (Fig. 3).

## Discussion

This study describes a multiple immunofluorescent staining technique which co-localizes the VDR and muscle fiber subtypes in a single human skeletal muscle section. Similar to a prior study in human skeletal muscle specimens (Bischoff et al. 2001), we identified VDR myonuclear staining using a human monoclonal antibody to the VDR. Unlike prior studies, however, our technique provides information on VDR expression in the context of muscle fiber subtype and its distribution. Using type I, IIa and IIx MHC isoform-specific antibodies directly labeled with distinct fluorophores, we were able to identify hybrid fibers which are of particular interest when studying factors such as vitamin D supplementation that may have effects on muscle morphology (Sorensen et al. 1979; Boland 2005; Sato et al. 2005). Our technique could be utilized in future studies to provide information on whether the VDR has differential expression based on muscle fiber subtype. Although we cannot generalize on a pattern of VDR expression by fiber subtype in this study, a more extensive sampling of muscle specimens in a larger study would allow for such an analysis. If VDR has a direct role on muscle as proposed, this knowledge may help to explain the morphological changes noted in vitamin D deficiency (Boland 2005) and in vitamin D repletion (Sorensen et al. 1979; Sato et al. 2005) demonstrating a potentially selective effect on type II muscle fibers.

Notably, our technique stained peripheral areas of the muscle fiber that did not appear connected with a myonucleus within these 7  $\mu$ m sections. This preliminary finding will need to be explored further to determine whether this signal may be representative of the putative membrane-associated VDR believed to activate rapid, non-genomic, second messenger

intracellular signaling cascades that influence muscle intracellular calcium regulation, muscle contractility and myogenesis (Boland 2005). Recent animal studies identified VDR in isolated membrane fractions of both chick intestinal cells and chick embryonic skeletal muscle cells (Capiati et al. 2002; Huhtakangas et al. 2004).

The strength of this method is that, by incubating a single muscle section with different specific primary antibodies to different MHC isoforms, it successfully reduces the amount of muscle tissue, reagents, and time needed to perform these analyses. A limitation in this study was that we did not verify the VDR signal in muscle using an inhibitor or knockout model; however, we confirmed VDR expression in muscle sections by Western blot analysis using the same antibody and compared it to other antibodies to further corroborate our findings.

In summary, this technique co-localizes VDR using a human monoclonal antibody and type I, IIa, and IIx muscle fibers using specific antibodies to MHC isoforms in a sample obtained from a human skeletal muscle biopsy. The method identifies and quantifies VDR-positive myonuclei in skeletal muscle, identifies specific MHC isoforms and VDR positive myonuclei within individual muscle fiber subtypes, and identifies peripheral VDR staining patterns that need further investigation. Use of this technique in a large sample of muscle specimens would provide patterns of VDR expression and its associations with MHC iso-forms in human muscle.

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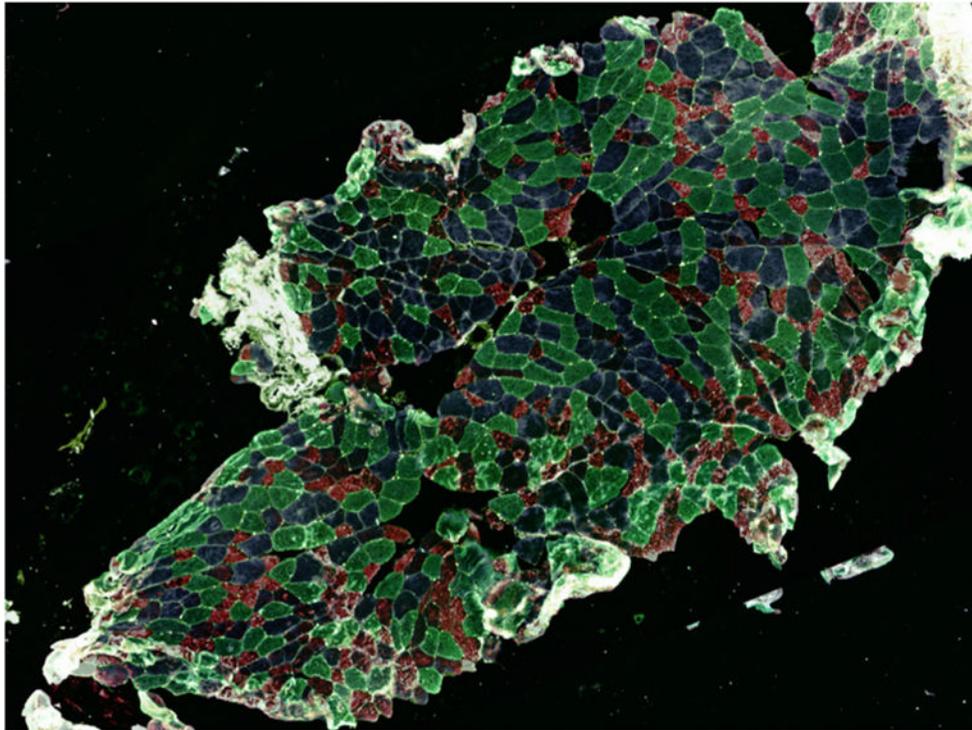
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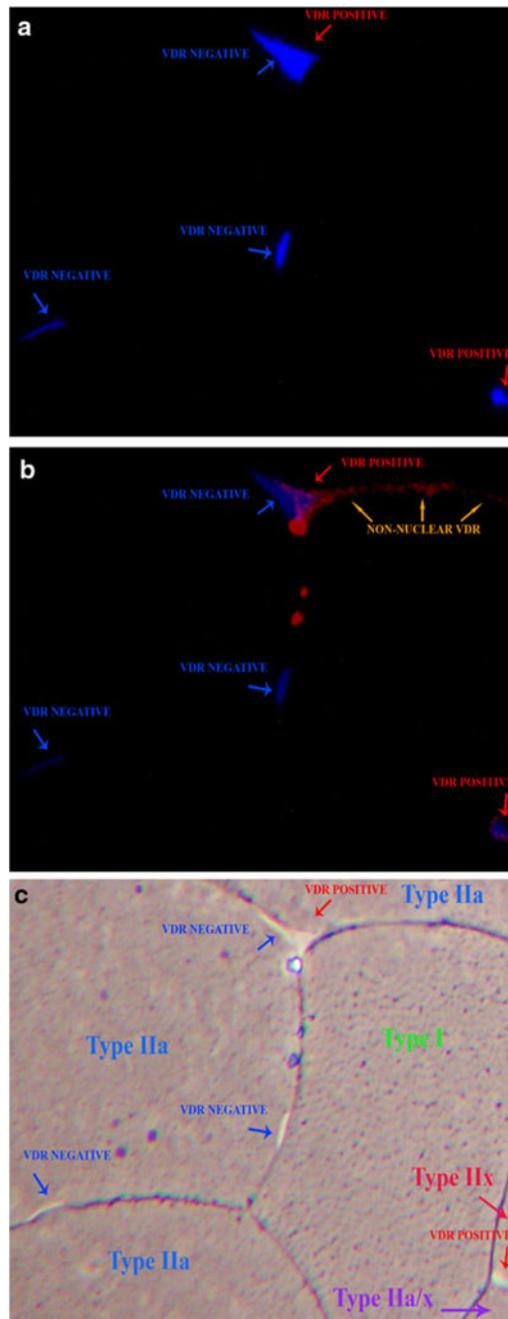
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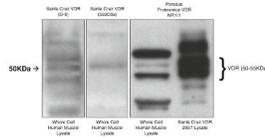
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**Fig. 1.** Composite image (100 $\times$ ) of multi-immunofluorescent staining of MHC isoforms in a human skeletal muscle tissue section. Colorization patterns are: *dark green* = type I, *green/blue* = hybrid I/IIa, *blue* = type IIa, *red* = type IIx, *purple* = hybrid IIax, and *light green* = laminin



**Fig. 2.** VDR-positive myonuclei in skeletal muscle section at 400× magnification showing: **a** DAPI staining myonuclei; **b** co-localization of VDR-positive myonuclei with DAPI; **c** bright field microscopy



**Fig. 3.** VDR expression in whole cell lysate of human skeletal muscle is shown using three commercially available primary antibodies. From left to right are VDR (D-6), VDR (333C6a) and VDR (NR1H1). VDR expression was verified by use of human VDR transfected 293T cell lysate from Santa Cruz Biotechnology, Inc

Table 1

List of primary and secondary antibodies used in immunohistochemistry and immunoblotting

Antibody	Host	Reactivity	Clone	Dilution	Vendor
<i>Primaries</i>					
Anti-VDR (IH/WB)	Mouse	Anti-human IgG <sub>2A</sub>	H4537	1:40 (IH) 1:1,000 (WB)	R&D systems
Anti-VDR (WB)	Mouse	Anti-human IgG <sub>2A</sub>	D6	1:1,000 (WB)	Santa Cruz Biotechnology
Anti-VDR (WB)	Mouse	Anti-human IgG <sub>1</sub>	333C6a	1:1,000 (WB)	Santa Cruz Biotechnology
Anti-MHC-I	Mouse	Anti-human IgM	A4.840	1:250	DSHB
Anti-MHC-I	Mouse	Anti-human IgG <sub>1</sub>	A4.951	1:250	DSHB
Anti-MHC-IIa/neonatal I	Mouse	Anti-human IgG <sub>1</sub>	N2.261	1:333	DSHB
Anti-MHC-IIx	Mouse	Anti-human IgG <sub>1</sub>	212F	Undiluted (cell culture supernatant)	Produced in-house**
Anti-Laminin	Rabbit	Anti-human IgG	Polyclonal	1:250	Sigma-Aldrich
Antibody	Host	Reactivity	Dilution	Emission wavelength	Vendor
<i>Secondaries</i>					
Alexa Fluor® 350	Goat	Anti-mouse IgG <sub>1</sub>	1:250	442 (Blue)	Invitrogen Corp.
Alexa Fluor® 430	Goat	Anti-rabbit IgG	1:250	539 (Light Green)	Invitrogen Corp.
Alexa Fluor® 488	Goat	Anti-mouse IgG <sub>1</sub>	1:250	519 (Green)	Invitrogen Corp.
Alexa Fluor® 568	Goat	Anti-mouse IgG <sub>1</sub>	1:250	603 (Orange Red)	Invitrogen Corp.
Alexa Fluor® 568	Goat	Anti-mouse IgG <sub>2A</sub>	1:250	603 (Orange Red)	Invitrogen Corp.
HRP-Labeled	Horse	Anti-mouse IgG	1:2,000	n/a	Cell Signaling
HRP-Labeled	Goat	Anti-mouse IgG <sub>1</sub>	1:2,000	n/a	Invitrogen Corp.

VDR Vitamin D receptor, IH immunohistochemistry, WB Western blot, MHC myosin heavy chain, DSHB Developmental Studies Hybridoma Bank

\*\* From hybridomas generously donated by Dr. Peter Merrifield, University of Ontario, Canada

**Table 2**

Muscle fiber type profile of Fig. 1

Muscle fiber type	Number of fibers	Percent (%)
Type I	200	28.6
Hybrid I-IIa	9	1.3
Type IIa	196	28.0
Type IIx	198	28.3
Hybrid IIa-IIx	96	13.7
All type I <sup>a</sup>	209	29.9
All type II <sup>b</sup>	490	70.1
Total	699	100

<sup>a</sup>Type I fibers consist of type I and hybrid I-IIa

<sup>b</sup>Type II fibers consist of type IIa, type IIx and hybrid IIa-IIx

**Table 3**

Relative number of VDR-positive myonuclei in 10 randomly selected 400 × fields

Muscle fiber type	Number of VDR- positive nuclei	Total number of nuclei	Ratio (VDR/Total)
Type I	165	178	0.93
Hybrid I-IIa	14	14	1.00
Type IIa	155	180	0.86
Type IIx	145	170	0.85
Hybrid IIa-IIx	70	83	0.84
All type I <sup>a</sup>	179	192	0.93
All type II <sup>b</sup>	370	433	0.86
Total	549	625	0.88

<sup>a</sup>Type I fibers consist of type I and hybrid I-IIa

<sup>b</sup>Type II fibers consist of type IIa, type IIx and hybrid IIa-IIx