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Visualisation of Collagen in fixed skeletal muscle tissue using fluorescently tagged Collagen binding protein CNA35

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ABSTRACT

Detection and visualisation of Collagen structure are important to understand the relationship between mechanical behaviour and microstructure in skeletal muscle since Collagen is the main structural protein in animal connective tissues, and is primarily responsible for their passive load-bearing properties.

In the current study, the direct detection and visualization of Collagen using fluorescently tagged CNA35 binding protein (fused to EGFP or tdTomato) is reported for the first time on fixed skeletal muscle tissue. This Technical Note also establishes a working protocol by examining tissue preparation, dilution factor, exposure time etc. for sensitivity and specificity. Penetration of the binding protein into intact mature skeletal muscle was found to be very limited, but detection works well on tissue sections with higher sensitivity on wax embedded sections compared to frozen sections. CNA35 fused to tdTomato has a higher sensitivity than CNA35 fused to EGFP but both show specific detection. Best results were obtained with 15 μm wax embedded sections, with blocking of non-specific binding in 1% BSA and antigen retrieval in Sodium Citrate. There was a play-off between dilution of the binding protein and time of incubation but both CNA35-tdTomato and CNA35-EGFP worked well with approximately 100 μg/ml of purified protein with overnight incubation, while CNA35-tdTomato could be utilized at 5 fold less concentration.

This approach can be applied to study the relationship between skeletal muscle micro-structure and to observe mechanical response to applied deformation. It can be used more broadly to detect Collagen in a variety of fixed tissues, useful for structure-functions studies, constitutive modelling, tissue engineering and assessment of muscle tissue pathologies.

1. Introduction

Collagen is the main structural protein in connective tissues in animals and while Collagen types I, III, IV, V, VI, XII and XIV occur in skeletal muscle extra cellular matrix (ECM) (Listrat et al., 1999, Nishimura et al., 1998), Collagen types I and III are the major components (Bailey and Light, 1989, Light et al., 1985). Collagen is primarily responsible for the passive load-bearing properties of tissues, for example Collagen fibre reorientation in muscle ECM in response to external loading has been observed by several authors (Billiar and Sacks, 2000, Purslow, 1989, 2010, Purslow and Trotter, 1994). Thus detection and visualisation of Collagen structure are important to understand the relationship between mechanical behaviour and micro-structure in skeletal muscle, which researchers have addressed principally through microscopy and mechanical modelling (Purslow, 2002, 2010, Purslow and Trotter, 1994, Takaza et al., 2014, Krahn et al., 2006, Nishimura, 2010, Sharafi and Blemker, 2010).

The overall structure of Collagen-rich tissues can be visualised by commonly used histological stains such as Hematoxylin and Eosin (H & E) (e.g. Pietsch et al. (2014)) or various forms of trichrome or von Gieson. Picrosirius red staining has been frequently used as it can differentiate Collagen (red) from muscle (yellow) and it can be coupled with polarized light birefringence to distinguish different Collagen compositions (Junqueira et al., 1979, Rich and Whittaker, 2005, Vidal et al., 1982, Takaza et al., 2014), but histological stains do not specifically detect Collagen or allow visualization of Collagen fibril structure. Other visualisation methods based on intrinsic Collagen properties such as autofluoresence (Voytik-Harbin et al., 2001) or second harmonic generation (Cox et al., 2003) also suffer from low intensity signal and limited contrast, resolution and specificity since other tissues can also produce signal. Methods based on specific Collagen binding, especially coupled with strong fluorescent probe

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detection, allow higher resolution analysis of Collagen fibril organization, for example using confocal microscopy.

Detection of Collagen by specific binding can be achieved using antibodies raised against individual Collagen types but with the existence of multiple Collagen types, this requires a panel of different antibodies to analyse complex tissue types such as skeletal muscle. Alternatively an adapted form of a natural Collagen recognition protein called CNA35 can be used; the original binding protein is produced on the cell surface of the bacterium Staphylococcus aureus, isolated from patients with bacterial arthritis (Patti et al., 1994, Xu et al., 2004). Two soluble domains of the protein recognize the Collagen triple helix (Patti et al., 1994, Rich et al., 1999, Zong et al., 2005) and have been developed as a Collagen detection tool, first rendered fluorescent by labeling with Oregon Green 488 (CNA35-OG488) (Krahn et al., 2006). The fluorescent CNA35 "probe" has advantages over other methods for Collagen detection including its ability to act as a highly specific, pan-Collagen probe for skeletal muscle: Krahn et al. (2006) showed specific binding to a variety of fibril forming Collagens including I, III and IV with very little cross reactivity with non-Collagenous extracellular matrix proteins. Thus, CNA35 has the advantage of being a pan detector for all Collagens with one tool, it does not require expensive antibodies specific for each collagen type and avoids the need for 2nd fluorescently labeled antibodies. It also allows for using confocal microscopy to visualise collagen fibrils at high resolution. This makes CNA35 particularly valuable in examining fibril organization. CNA35-OG488 has been used in a number of applications (reviewed in Aper et al. (2014)) including examining the effect of mechanical and biochemical cues on extracellular matrix organization in vivo and in tissue engineering constructs (e.g. De Jonge et al. (2013), Foolen et al. (2008)). Moreover, fluorescently labelled CNA35 can be used to visualise the Collagen from very small fibrils to mature Collagen fibres (Boerboom et al., 2007, Krahn et al., 2006, Aper et al., 2014).

The CNA35 probe offers a number of other advantages over antibody detection of Collagen. Collagen has a high density of CNA35 binding sites, so sensitivity is high. Fluorescent antibodies are costly, require a two-step process and have a limited penetration capability in dense tissues, and their high affinity binding to Collagen may affect tissue function in live tissues (Kumar and Rudbeck, 2009, Krahn et al., 2006). CNA35 is five times smaller than antibodies facilitating tissue penetration, it does not affect Collagen properties and, when directly fluorescently labelled, it can bind and be detected in one step (Krahn et al., 2006, Aper et al., 2014, Boerboom et al., 2007, Foolen et al., 2008). For applications where the overall Collagen organisation is of interest, the broad specificity to multiple fibril forming Collagens is an advantage (Krahn et al., 2006). To further expand the usefulness and accessibility of CNA35 Collagen probes, Aper et al. (2014) described the production of an array of further genetically engineered versions fused to six commonly used fluorescent proteins including EGFP (Enhanced Green Fluorescent Protein), tdTomato and mCherry. Because the fluorescent tag is genetically encoded and fused to the probe, it can be easily produced in high amounts through expression in *Eschericia coli* bacteria, purified using an added His tag, and can be widely shared with the research community as DNA constructs. These fluorescent fusions of CNA35 promise to be powerful tools for Collagen detection in a wide variety of applications, particularly for studying the biomechanics of Collagen rich tissues and bioengineering of constructs.

Aper et al. (2014) demonstrated the use of fluorescently fused CNA35 probes to visualise Collagen in live tissues, using two-photon confocal imaging of human skin and engineered cardiac cells. While CNA35 shows clear advantages for use on live or unfixed tissues, some experimental situations necessitate the use of fixed tissue. For example, current visualisation methods do not permit observation of Collagen reorientation in muscle ECM under externally applied deformation without tissue fixation to "hold" the deformation effect and due to limited stain/probe penetration in mature muscle tissue which therefore may require fixation and sectioning (Takaza et al., 2014). Since Collagen detection by CNA35 has never been reported on fixed tissue, here we investigate specific binding following Paraformaldyhyde (PFA) treatment, which is commonly used to preserve muscle structure (e.g. Briguet et al. (2004), Iwasaki et al. (2013)). This Technical Note also establishes a working protocol (examining section type, determining dilution factor, exposure time etc.) for Collagen detection and visualisation in fixed skeletal muscle tissue using CNA35-EGFP and CNA35-tdTomato. Confocal microscopy is then used to assess the ability to visualise Collagen organisation. This approach will be valuable in future studies examining the relationship between Collagen conformation and biomechanics. In particular it can be applied to study the relationship between skeletal muscle micro-structure and observed mechanical response to applied deformation and hence constitutive modelling, tissue engineering and assessment of muscle tissue pathologies.

2. Methods

2.1. Sample preparation

Porcine and chicken skeletal muscles from 3-month old females were used. The chicken samples were harvested from pectoralis muscle of Ross 508 chickens (Annyalla Chicks Ltd. Co Kildare, Ireland) and the porcine samples were excised from the biceps femoris of an outbred pig (Perma Pigs Ltd. Co Louth, Ireland; cross between PIC line 337 and mixed breed female; 50% Large White, 25% Duroc, 25% Land Race) by trained personnel using ethically approved protocols.

2.2. Wax embedding of tissue samples

Immediately post mortem, cubic blocks of muscle tissue with a nominal size of 10×10×10 mm were immersed in 4% paraformaldehyde (PFA) in PBS for 48 h as preliminary tests showed 24 h led to incomplete fixation at the core of the block. The samples were then washed in phosphate buffer saline (PBS) for 24 h and a further two brief washes to remove PFA. The fixed tissues were cut into 0.5 mm cubes to ensure complete dehydration and a more manageable size for sectioning (initial fixation of a larger sized block was carried out to accommodate future experiments where muscle deformation will be applied). The samples were dehydrated using the following graded alcohol series; 70% in H2O, 90%, 100% and then immersed in Histoclear™, which clears and prepares the tissue for wax penetration in subsequent exchanges in molten paraffin at 60 °C (step details given in Table 1). The tissues were then placed in plastic moulds filled with molten wax and allowed to harden on a cold plate. The paraffin wax blocks were mounted on a Leica RM2255 automated microtome and 8, 15, and 20 μm thick sections were cut. The sections were then floated out on a 50 °C water bath and mounted on superfrost microscope slides. They were baked for 10 min at 60 °C and left overnight to increase adherence of sections. Prior to exposure to CNA35 probes,

Table 1
The steps and timing for dehydration and clearing of muscle samples for wax embedding.

Solution	Time	Temperature
70% EtOH	1 h	Room temp
90% EtOH	1 h	Room temp
100% EtOH	1 h	Room temp
100% EtOH	1.5 h	Room temp
100% EtOH	1.5 h	Room temp
100% EtOH	2 h	Room temp
50% Histoclear™:50% EtOH	10–30 min	Room temp
Histoclear™	1 h	Room temp
Histoclear™	1 h	Room temp
Histoclear™/wax mush (1:1)	1 h	In oven @ 60 °C
Pure fresh wax(1)	1 h	In oven @ 60 °C
Pure fresh wax(2)	2 h	In oven @ 60 °C