# Three-dimensional fine structures in deep fascia revealed by combined use of cryo-fixed histochemistry and low-vacuum scanning microscopy

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INTRODUCTION: Recent physiological studies have shown that the deep fascia has received much attention concerning clinical medicine. Myofascial trigger point injection therapy was popularized in the 1950s and is still used worldwide for the treatment of medical conditions. In addition, deep fascia can be treated and operated on in daily orthopedic surgery, rehabilitation, and reconstructive surgery. Myofascial release has also received significant attention in sports medicine as a treatment of deep fascia. Despite the growing interest in fascia, there is still a lack of comprehensive anatomical and histological descriptions of this structure. In this study, we aimed to clarify and visualize the structure of the deep fascia by taking advantage of cryofixation techniques and low-vacuum scanning electron microscopy.

METHODS: Male Wistar rats (10 weeks) were used in this study. All animal procedures were carried out according to protocols approved by the University of Miyazaki Animal Research Committee (Approval number: 2019-513-2), and all experiments were performed according to the institutional guidelines of the Animal Experiment Committee. The front of the thigh was excised from the skin to the rectus femoris muscle by 1 $cm^2$, and fixed by 4% paraformaldehyde (conventional chemical fixation). Secondly, small pieces of sequential sections from the skin to the rectus femoris muscle and the deep fascia to the rectus femoris muscle were excised by 1$cm^2$ and fixed by plunge freezing followed by freeze-substitution method. Finally, the deep fascia and rectus femoris muscle were held with two hooks and fixed using grasping forceps to maintain the original length and structure of the deep fascia (hook-holding procedure) *in vivo* (Fig.1). The 1 $cm^2$ specimen around the two hooks was carefully excised, and fixed by plunge freezing followed by freeze-substitution method. We performed histological analysis and low-vacuum scanning electron microscopy. To verify the morphological changes, a comparative histological analysis of two groups was performed: with and without the hook-holding procedure. The thicknesses of the deep fascia, first layer, second layer, third layer, and between the second and third layers were measured using imaging software and we calculated the mean ± standard deviation. Statistical comparisons were conducted for the two groups using the Student's t-test (JMP Pro version 16). Statistical significance was set at $p < 0.05$.

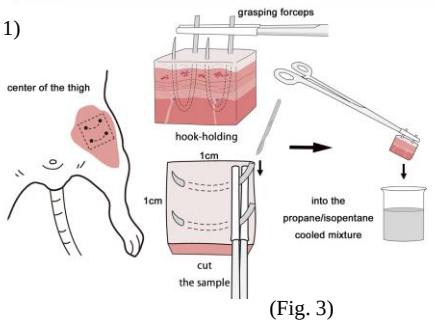
RESULTS SECTION: The ultrastructural observations revealed three-dimensional stratification of the deep fascia composed of three layers: the first superficial layer consisting of collagen fibers extending in various directions with blood vessels and peripheral nerves; the second intermediate layer formed by a single thick collagen bundle with flexibility; and the third deepest layer, consisting of relatively straight and thin collagen fibers. The thickness of the deep fascia, as well as the first and third layers did not differ from those obtained without the hook-holding procedure (Fig. 3). However, the thickness of the second layer was significantly lower than that obtained without the hook-holding procedure ($p < 0.05$). Similarly, the thickness ratio of the second layer to the deep fascia was significantly lower than that obtained without the hook-holding procedure ($p < 0.05$).

DISCUSSION: We found that the deep fascia is composed of three layers: the first superficial layer was composed of vessels and nerves with collagen fibers extending in various directions; the second intermediate layer was formed by thick collagen bundle and may be capable of changing form, inducing flexibility against extension and contraction; and the third deepest layer was consisting of straight thin collagen fibers, which differs from the epimysium that connects to the perimysium (Fig. 2). Further application of these techniques and structural findings to clinical experiments including functional analysis may be required to elucidate deep fascia-associated disorders. The present morphological approach paves the way to visualize three-dimensional ultrastructures for future biomedical studies including clinical pathophysiology.

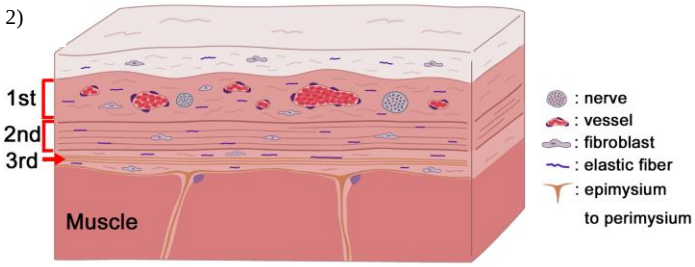
SIGNIFICANCE/CLINICAL RELEVANCE: (1-2 sentences): The deep fascia is composed of three layers: the first superficial layer consisting of collagen fibers extending in various directions with blood vessels and peripheral nerves; the second intermediate layer formed by a single thick collagen bundle with flexibility; and the third deepest layer, consisting of relatively straight and thin collagen fibers.

IMAGES AND TABLES:

(Fig. 1)




(Fig. 2)




(Fig. 3)