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Automated high-content morphological analysis of muscle fiber histology

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ABSTRACT

In the search for a cure for many muscular disorders it is often necessary to analyze muscle fibers under a microscope. For this morphological analysis, we developed an image processing approach to automatically analyze and quantify muscle fiber images so as to replace today's less accurate and time-consuming manual method. Muscular disorders, that include cardiomyopathy, muscular dystrophies, and diseases of nerves that affect muscles such as neuropathy and myasthenia gravis, affect a large percentage of the population and, therefore, are an area of active research for new treatments. In research, the morphological features of muscle fibers play an important role as they are often used as biomarkers to evaluate the progress of underlying diseases and the effects of potential treatments. Such analysis involves assessing histopathological changes of muscle fibers as indicators for disease severity and also as a criterion in evaluating whether or not potential treatments work. However, quantifying morphological features is time-consuming, as it is usually performed manually, and error-prone. To replace this standard method, we developed an image processing approach to automatically detect and measure the cross-sections of muscle fibers observed under microscopy that produces faster and more objective results. As such, it is well-suited to processing the large number of muscle fiber images acquired in typical experiments, such as those from studies with pre-clinical models that often create many images. Tests on real images showed that the approach can segment and detect muscle fiber membranes and extract morphological features from highly complex images to generate quantitative results that are readily available for statistical analysis.

1. Introduction

To address the need for quick and objective analysis of muscle fibers to develop novel therapies, we present, in this paper, an image processing approach for microscopic images to segment and analyze cross-sections of muscle fibers. In the search for treatments for the large populations with many types of muscular disorders like muscular dystrophy (MD), researchers have to manually examine and analyze the morphology of muscle fibers to identify important biomarkers about the fibers, such as the restoration of lost membrane proteins and presence of endomysial fibrosis or whether they are degenerative or regenerative. Indeed, morphological features of muscle fibers are important biomarkers of muscle health and indicators of success of therapeutic treatments. Manual analysis, however, is time-consuming and error-prone, given that it is subject to inter-observer variations; therefore, our quantitative analysis approach is a needed replacement.

Specifically, we developed algorithms to measure muscle fiber morphologies in a high-throughput high-content manner and tested it on images acquired from a preclinical model of Duchenne muscular dystrophy (DMD), which is the most common and severe form of MD [1] that affects 1 in 3500 newborn boys. The method was tested on microscopic images of the tibialis anterior (TA) muscles of *mdx* (C57BL/10ScSn-Dmd^{mdx}/J) mice, and we show that it achieved high accuracy in identifying muscle fibers, quantifying their parameters, and exporting quantitative results for further statistical analysis. Despite microscopic images of cross-sections of muscle fibers being often challenging to analyze because not only must cross-sections be

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segmented but individual cross-sections must be identified to measure perimeters, areas, and other features, our image processing approach provided a quick, objective, and quantitative tool to analyze highly complex muscle fiber images. As each image consists of hundreds to thousands of muscle fibers, an image processing method should be highly automatic and robust to handle cross-sections of muscle fibers of different signal intensities, shapes, and sizes. In addition, an automated image processing approach needs to identify areas that did not belong to valid muscle fibers to exclude them from measurement. As a single experiment may create hundreds microscopic images of muscle fibers, it is not suited to manual analysis that cannot keep up with large numbers of images and that involves a human observer who is often forced to manually click points on a computer screen to mark the boundary of a muscle fiber. Given that two observers are unlikely to mark the boundary in the same way, this process is highly subject to inter-observer variation. What's more, the high complexity of muscle fiber images makes it very difficult, if not impossible, to extract morphological features such as areas, diameters, and elongations. Therefore, there is an urgent need to develop a computerized analysis approach to model and quantify muscle fiber images as part of an overall more efficient and effective process to find new treatments.

To process and analyze complex images like cross-sections of muscle fibers, several steps are generally required, including pre-processing, segmentation, and morphological analysis. Pre-processing aims to correct uneven illumination of the images, remove artifacts, and improve image contrast. Segmentation typically focuses on identifying valid objects or extracting signal components from the background. Over the years, many segmentation methods have been proposed to suit various scenarios of image processing. In general, segmentation methods can be categorized as global thresholding or pixel-wise classification. Representative global threshold techniques include Ostu's method [2] that maximizes inter-class variance of the segmentation results, and *k*-means segmentation [3] that clusters pixels into two classes such that each pixel belongs to the nearest cluster. Pixel-wise segmentation techniques include watershed segmentation [4], active contours [5], and graph cut [6], and their variations and improvements. For example, to overcome its well known over-segmentation problem, many techniques have been developed to restrain the watershed process by placing seed points into regions to limit the number of final partitions [7]. Active contour-based methods minimize an energy function that includes both internal energy that constrains the deformation of the contour in terms of its first and second order derivative, and external energy that is minimized when the contour deforms to the boundary of the object, e.g., a high gradient value is encountered. These methods, however, may cause leakage in deformation when there are no obvious gradient changes in the images. An improvement is made by the Chan–Vese model that detects objects whose boundaries are not clearly defined by their gradients by minimizing an energy function set up as a minimal partition problem [8] Graph cut is another type of energy minimization approach to segment an image into the foreground and background by searching for a max flow/min cut partition of the image into two disjoint sets, such that the dissimilarity between the two sets, measured as the weight of edges that have been removed, is minimized [9]. In many cases, segmentation of an image is set up as an optimization problem that searches for a solution to achieve a balance between a data fidelity term and a pre-set term that constrains the segmentation result. For example, an area-constrained segmentation method has been proposed by Niethammer and Zach for soft selections of segmentation solutions that counteracts the effect of shrinking bias encountered in many techniques [10]. Bergeest and Rohr developed a segmentation technique based on active contours by using level sets and convex energy functional, i.e., the functional has only one a minimum to reach a global solution that avoids local minima [11].

After segmentation, post-processing is often employed to quantify the results and extract morphological features that are of biological and medical significance. For instance, in muscle fiber analysis, the number of muscle fibers in a unit area and the perimeters and areas of muscle fibers are often used to evaluate the health status of the muscle as well as to identify the type of muscle fibers, e.g., degenerative or regenerative. In identifying muscle fiber centers, Liu et al. proposed a learning-based method to find the geometric centers of muscle fibers and then used a snake model to obtain the boundaries [12]. Mula et al. developed a multiple step approach to first enhance the boundaries of muscle fibers and then search for seed points inside each fiber to drive a deformable model to delineate each fiber [13].

In this paper, we present an image processing approach that is able to segment cross-sections of muscle fibers in very challenging cases and extract quantitative features for in-depth analysis, the aim of which is to provide computer-aided measurements of morphologies of muscle fibers for researchers to use to develop novel insights on the cellular mechanisms of musculoskeletal diseases. Ultimately, they will be able to more objectively evaluate their experimental approaches and reduce the time needed to analyze large numbers of muscle fibers acquired in experiments.

2. Materials and methods

2.1. Animals and experimental treatment

Animal experiments were carried out under the guidance and approval of the Institutional Animal Care and Use Committee of Harvard Medical School. Male *mdx* mice at the age of 4–8 weeks were purchased from Jackson Lab. To assess how many of the myoblasts might survive post transplantation, the mice were transplanted with wild type myoblasts from C57BL6 mice to assess how many of the myoblasts might survive post transplantation. In preparation for myoblasts transplantation, the hind legs of the mice were given 18 Gy irradiation 3 days in advance. Then 1×10^5 myoblasts suspended in 10 μl HBSS (Hank's Balanced Salt Solution) were injected into each tibialis anterior (TA) muscles at 3 positions. The mice were then maintained for various periods of time before they were euthanized for tissue harvest. The mice were fed with standard pelleted rodent chow and kept in a 12-h light/12-h dark cycle.

2.2. Histology and image acquisition

When the TA muscles were harvested, the mice were deeply anesthetized by intraperitoneal injection of Ketamine (100 mg/kg) and Xylazine (10 mg/kg) and then intracardially perfused with physiological saline and periodate/lysine paraformaldehyde (4%) solution. After postfixation and dehydration, the muscles were frozen in OCT embedding compound and sectioned coronally at 12 μm thickness from the mid-portion of the muscles. For immunohistochemistry, slides were washed with PBS (Phosphate Buffered Saline) and blocked with 5% goat serum, and muscle sections were incubated with a rabbit anti-dystrophin antibody (Sigma, 1:500) followed by incubation with a goat-anti-rabbit Cy3-conjugated secondary antibody (Jackson Lab, Bar Harbor, ME, USA). The images used were acquired by an Olympus IX-70 microscope equipped with a CCD (Charge-Coupled Device) camera. A single image typically has a size of 800 × 600 pixel, with a pixel size of 0.7 μm. Approximately 20 to 30 images were collected with a slight overlap in four directions to cover the whole cross-section of the TA muscle. The individual images were then merged to form a mosaic picture.