PE1.38 Image Analysis of the intramuscular connective tissue 260.00

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***Abstract*—Intramuscular connective tissue (IMCT) plays a functional role on patterning muscle development and ageing. The distribution of both IMCT and muscle fibers are highly age-related but their interaction is not comprehensive interpreted. This short paper aims to introduce an automatic image analysis application of mouse *Gastrocnemius-Soleus* (GM-S) muscle. First IMCT and muscle fibers were visually distinguished by Sirius Red staining. And then images were captured and processed by Visilog 6.7 following to be analyzed by R-statistical program. Results packed up various morphological parameters, next to employ principle component analysis (PCA) for demonstrating the degree of correlation between those properties and providing a profitable approach to identify the significant contributors among those parameters in different ages.**

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***Index Terms*—IMCT, PCA, skeletal muscle fiber**

I. Introduction

Intramuscular connective tissue (IMCT) was significant to serve the determination of meat texture [1]. Its general structure, morphology and composition have been studied [2, 3]. On the whole, each piece of skeletal muscle is encased in epimysium; fascicles (bundles of skeletal muscle fibers) are delineated by perimysium and individual muscle fibers are surrounded by endomysium. Those IMCT structures (epimysium, perimysium and endomysium) varied in composition [2] but overall fibrillar collagen acted as the major component [4]. Since Sirius red was introduced to access the area and percentage of IMCT structures by mainly staining fibrillar collagen because it reacts with sulphonic acid groups on collagen molecule [5]. Some previous studies of IMCT by image analysis [6, 7] or scanning electron microscopy [8] provided measurements between muscles of different maturity or from distinct parts of the body. However, improved research on architecture of IMCT is possible to open up. This paper presents the image analysis application to test the objectivity and reliability of studying multivariate data on mouse IMCT, in order to detect the morphology varying among different ages.

II. Materials and Methods

Gastrocnemius-Soleus (GM-S) muscles of twelve C57BL6 male mice (2, 8, 11 and 22 months, obtained from Laboratory of Animal Centre of National University of Singapore and raised in Laboratory of Animal Facility of Nanyang Technological University, Singapore) were dissected immediately after animal termination and fast-frozen in isopentane. Samples were preserved at -80^{o}C till cryo-sliced into 10µm transverse sections (5mm x 5mm). Those sections were incubated by acetone for 60mins before being stained and fixed with picro-formalin (90% ethanol, 1.3% picric acid and 12.5% formaldehyde, Sigma). After sections were rinsed, and stained with picro-sirius red (0.1% Sirius red in saturated picric acid, sigma) for 60mins, they were immersed in 0.01 mol/L HCl, rinsed, dehydrated (serial ethanol) and finally cleared by xylane (Labonord) and mounted (Labonord).

Three images (1280 x 960 pixels representing a 780 x 585 µm^2 field) per each animal were captured in bright field by Sony DFW-SX900 camera coupled to a Nikon Laboratoire II microscope at magnification of x125. All the images were processed through a home-made visual basic program developed under Visilog 6.7 software (Noesis, France) and principle component analysis (PCA) was performed by FactoMiner package [9] in the R-Statistical program (version 2.8.1).

III. Results and Discussion

Series images (Fig 1 to Fig 5) were generated during acquisition and processing. Firstly, **TIFF-RGB color images** (Fig 1) were acquired in 3 randomized fields per animal by fixed condition (exposure time: 4ms and setting of white balance: Bal U=217, Bal V=81). IMCT stained by Sirius red appeared in red while muscle fibers were yellow. **Positive OD greyscale images** (Fig 2) was obtained after selecting the green component which contained high-contrast in original

RGB image. This greyscale images were converted to positive optical density (OD) with background subtracted. **Binary images** (Fig 3, zoom in from ROI in Fig 2) were generated by thresholding OD greyscale images. IMCT (in blue) refers to 1 while background is 0, respectively. Area ($\sum$ pixel $_{value=1}$ / $\sum$ pixel) of IMCT was calculated. These binary images were also helpful to mask OD images and calculated the volume of IMCT (sum of pixel intensity unmasked by binary images). To underline thick IMCT (possible perimysium), 3 times opening operation were processed to reduce the thin portion. Each time the area and volume were measured. **Skeleton** (shown in Fig 4, width= 1 pixel) was obtained from binary images of IMCT. Total length was directly measured, and then connection points ([A] in Fig 4, common link of 3 muscle fibers) were detected and use to divide each segment ([B] in Fig 4, randomly colored). The number, mean length and intensity of segments could be obtained along with the number of connection points.

Individual muscle fibers (randomly colored in Fig 5) corresponded to the negative binary image of IMCT. Incomplete fibers on border were discarded while others were characterized by number, mean area, perimeter and shape factor (Shape factor = Perimeter x Perimeter / (4 x PI x Area)).

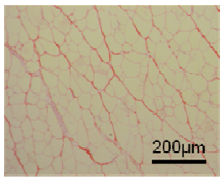


Fig 1 (left): TIFF-RGB original age

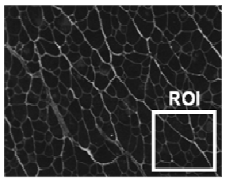
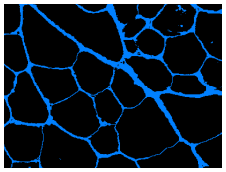


Fig 2 (right): Positive OD greyscale image, ROI refers to one representative region of interest.



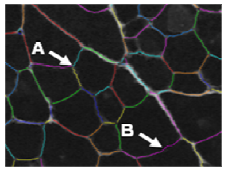


Fig 3 (left): Binary image shown IMCT (in blue), generated by thresholding and binary transformation of the ROI in OD greyscale

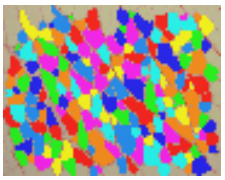


Fig 4 (above right): Skeleton of IMCT displayed in overlay of the ROI. [A]connection point; [B]segment, defined by connection points and randomly colored

Fig 5 (bottom right): Individual fibers (randomly colored) displays in overlay of the original image

After image processing, 17 parameters (Table 1) were recorded, subsequent to PCA which was employed to understand the link and variation of those parameters (consider as variables). Eleven dimensions (described by variables and their correlation coefficient) were originated by default but only the top 3 were selected for interpretation due to there significance (Dimension 1: 51.47%, Dimension 2: 29.85% and Dimension 3: 12.52%).

Table 1: Variables recorded by image processing and analyzed by PCA. They are corresponded in Fig 6[A] and grouped based on the internal angle.

Name	Description	Group
Seg.L	mean length of segments	G1
Fib.A	mean area of muscle fibers	G2
Fib.P	mean perimeter of muscle fibers	
Seg. I	mean intensity of segments	G3
Fib.S	mean shape factor of muscle fibers	
IMCT.V0	original volume of total IMCT	G4
IMCT.V1	volume of IMCT after 1st opening	
IMCT.V2	volume of IMCT after 2nd opening	
IMCT.V3	volume of IMCT after 3rd opening	
IMCT.A0	original area of total IMCT	G5
IMCT.A1	area of IMCT after 1st opening	
IMCT.A2	area of IMCT after 2nd opening	
IMCT.A3	area of IMCT after 3rd opening	
Seg.N	number of segment	G6
Con.N	number of connection points	
Ske.L	mean length of skeleton	
Fib.N	number of fibers	G7

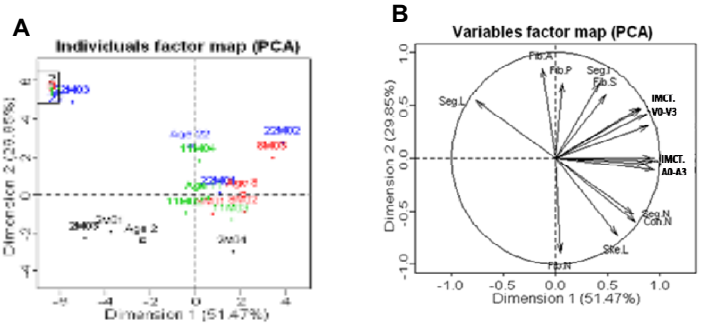


Fig 6: [A] Variables graph and [B] individual graph under Dimension 1 (51.47%) and 2 (29.85%). Variables in [A] are interpreted in Table 1; Individual mouse (o) marked and mean characterization per age ( )

Overview of variables was assessed under Dimension 1 and 2 (Fig 6 [A], the max 2 dimensions, present 81.32% of total variance). Grouped variables (combining variables at a low angle) showed positive correlation (at an angle below 90°), noncorrelation (cross at right angles) or negative correlation (meet an angle of 180°). This **variables graph** (Fig 6 [A]) also indicated the reliability of each variable, which was reflected by the length of the vector (the vector ceased near the centre area would be eliminated). Each mouse was localized in **individual graph** (Fig 6 [B]). Unfortunately, combination of Dimension 1 and 2 have not adequately distinguished different ages, maybe

because of data redundancy.

The variables of G2 and G7 (Table 1) were negatively correlated since their product kept stable (near to the total area of each image). Neither G2 nor G7 was correlated to G5 (total IMCT area), which proved that in unit muscle section, swelled muscle fibers would not result in the shrunk IMCT. On contrary, the interaction between G4 (IMCT volume) and G2 (G7 respectively) pointed out that the change of G2 or G7 might come from the variations of IMCT structure (endomysium and perimysium). Similar variation (thickness of endomysium and perimysium) was reported by Alnaqeeb in 1984[10].

Classifications of G2 and G6 can be illustrated mathematically. Variables of G4 and G5 shared highly homology. However, there was not superficial reasons for the composition of G3 (segments intensity and shape factors). For searching the potential linking, other assemble of dimensions were detected.

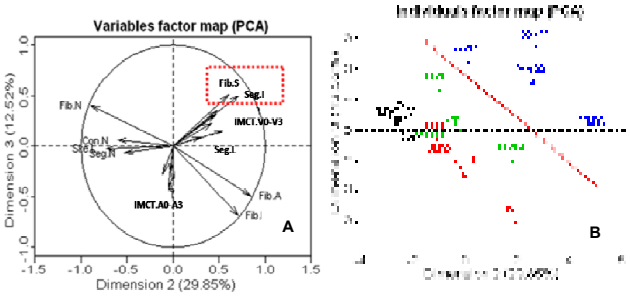


Fig 7 showed the **variables and individual graph** of Dimension 2 and 3. Although it contained less variance (41.47%), muscle from 22 month mice were successfully separated from others (Fig 7 [B]) and this division was significantly attributed to G3 (Fig 7 [A]). Measurements of segments intensity (Fig 8) confirmed it increased significant within old animals, partly meaning IMCT structure varying. Correspondingly, shape factors share the similar trend, referred to the muscle fibers become irregular.

Fig 7 (left): [A] Variable graph and [B] individual graph of Dimension 2 (29.57%) and 3 (12.52%). Red dashed line in [B] indicates 22 months mice (blue) are separated. Their region (top right corner) is superimposed on region highlighted by red box in [A], proved that variables (Fib.S-fiber shape factor and Seg.I-segments intensity) in this red box are the main contributors of variations occurring in 22 months mice. Other variables (IMCT.V and Seg.L) slightly determined this separation due to their weak coefficient (presented as short length in [A]).

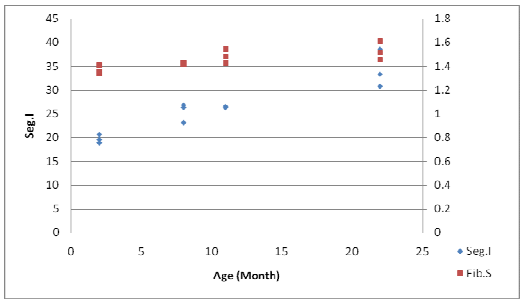


Fig 8 (left): Age-related variations of Fib.S (fiber shape factor) and Seg.I (segments intensity). Seg.I and Fib.S shared highly similar trend and Seg.I increased significantly in 22 months, also showed strengthened from 2 to 8 months

IV. Conclusion

Previous image analysis studies mainly focused on demonstrating the detailed performance of particular parameters. However, potential variables and their interaction may also determine muscle architecture. This short paper provides an effective approach by automatically diversified data schemes in order to discovery the interrelations. It is not only beneficial on supplementary comprehension of IMCT affecting muscle morphology; but also offers informative pattern to verify different ages from multivariate data.

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