Area determination of cooked single muscle fibres with confocal laser scanning microscopy

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Background and Objectives

Measuring meat texture on a single fibre level provides an important tool to enlarge our understanding of mechanical properties of meat (Purslow, 1996). In particular, the structural observations of their fracture behaviour during tensile tests add to our perceptions how this muscle component resists mechanical stresses. However, proper quantification of these properties is required to construct accurate models which describe the observed behaviour mathematically. Since single muscle fibres differ in size and shape, a standardisation of their response to applied forces is necessary. This would normally be done by expressing the results in terms of tensile stress i.e. the force devided by the area over which the force acts. Therefore, an accurate measurement of the fibre cross-sectional area is required. Due to the limitations of ordinary light microscopy, a direct measure of the cross-sectional area has been very difficult in the past. Only information about the diameter of single fibres has been routinely achievable. Based on these measurements, the cross-sectional area was then calculated assuming a circular fibre area. Recent developments in microscopy and image analysis now enable us to improve these approaches. Confocal laser scanning microscopy facilitates a direct optical cross-sectioning of tissues.

The image method used in our investigations, as in a large proportion of confocal microscopy, is in essence similar to conventional fluorescence microscopy. However, the excitation of fluorescent emission at all depths through a specimen, and not just at the plane of focus, results in a out-of-focus flare confusing the image formed in conventional fluorescence microscopy. This presents difficulties when trying to map the three dimensional topography of structures. In the confocal microscope the rejection of all light other than coming from the plane of focus results in a cleaner "optical slice" being obtained. By collecting many planar (xy) images at various depths (z direction) through the specimen, an accurate three-dimensional map of the fluorescently labelled structure is obtained. A comprehensive description of the principles of the confocal technique is given by Pawley (1995).
The objective of this was to quantify the level of error in the traditional means of estimating muscle fibre cross-sectional area from apparent diameter measurements. The approach taken was to compare the estimated area from apparent diameter measurements to the true cross-sectional area measured on the same fibres by three-dimensional image constructions using confocal microscopy.

Methods

Samples of the *M. longissimus thoracis and lumborum* from a variety of different breeds were cooked for 1 h at 80 °C in a water bath. Individual muscle fibres were dissected under a stereomicroscope (Carl Zeiss, Jena) in the filtered exudate from the cooked muscles. Fibres were fixed in glutaraldehyde (2.5% in 0.1 M phosphate buffer pH 5.6) for 5 min, washed in buffer and then mounted with glycerol. Due to the autoflourescence of glutaraldehyde, a relatively uniform flourescent signal was given by all part of the muscle fibre, and no further staining was necessary. Slides were prepared for mounting by constructing a chamber on the slide to prevent pressure distortion of the fibre. For this purpose, 120 µm thick coverslips were cut into small stripes and glued on the slides. On each fibre, measurements were taken randomly at three positions along the fibre. At each position a zx-scan was performed followed by an xy-scan allowing visualisation of the photobleached line caused by the zx-scan. A total of 200 fibres were measured.

The confocal laser scanning microscope (TCS4d, Leica Laser Technik GmbH, Heidelberg, Germany) was equipped with an argon/krypton laser which emits at 488, 568 and 647 nm. The settings were as follows: objective: 40x plan fluotar/1.00-0.50 oil, excitation line: 568 nm, main beamsplitter: DD 488/568, barrier filter: OG 595 nm longpass, pinhole size: 70 (180 µm). Laser power was constant during all measurements. Voltage was adjusted for the specific recording. Each line-scan was averaged eight times. The format of recorded images was 512 x 512 pixels representing an area of 125 x 125 µm.

Image analysis was performed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) on the recorded images from the confocal microscope. Fibre diameters were measured from the xy-scans at the position of the zx-scan, which was determined from the photobleaching. Estimates of areas were made for all three diameters measured per fibre, before being averaged to give the final *estimated fibre area*. Real fibre areas were calculated on the cross-sectional images from the zx-scan and the three measurement were again averaged to give the final *area*.

Statistical analysis was executed using Microcal Origin (Microcal Software, Inc., Northhampton, MA, USA).

Results and Discussion

Confocal images from an xy-scan (figure 1) and a zx-scan (figure 2) of the same fibre show that the cylindrical appearance of the fibre as viewed conventionally (fig. 1) is deceptive; the cross-section is usually anything but circular (fig. 2). The results shown for this fibre are typical for the 200 fibres examined in this study. The substantial deviation of the cross-sectional shape from a true circle tends to produce significant errors in the estimation of fibre are from diameter measurements. If the apparent diameter is large compared with the depth of the fibre then the estimated area will be erroneously high; if the fibre is then rotated through 90° so that the apparent diameter is small compared with its depth then the estimated cross-section will be erroneously low. It may be argued that a uniform, linear relationship between estimated and true fibre cross-sectional area of fibres could result from a larger number of measurements on

fibres at random orientations, where under- and over-estimates of fibre area from apparent diameter measurements must be expected to cancel out to some extent. Figures 3 and 4 show that, in practical terms, this does not occur. Fibre areas measured from zx-scans range from 630 μm^2 to 4299 μm^2, whereas estimates of fibre areas based on apparent diameter measurements range from 436 μm^2 to 8836 μm^2. Figure 3 shows the distribution of fibre areas by the two methods. From this figure it can be seen that estimations of the fibre area on the basis of diameter measurements leads to an underestimation of the areas of small fibres as well as an overestimation of the areas of big fibres. The relative error from the actual fibre area is greater for large fibres than for small fibres.

In figure 4 the two sets of area measurements are plotted against each other to show that there is in practice a non-linear relationship between them. The regression line shown was fitted from a 2nd order polynomial fit of the data, where the relationship between the true and estimated area is linear and residuals from the regression line are random. The equation for this non-linear fit is:
True area = 803.37 + 0.55 (est. area) -2.54 x 10^{-5} (est. area) ^{2} ; $R^2 = 0.56$.
The straight line drawn through the data with unity slope is the expected fit in the data, if the estimation of fibre area from diameter measurements is an accurate estimate of the true fibre area. This line does not provide even an approximate fit to the data.
The non-linear relationship between true and estimated area is most probably due to the fact, that in practice, isolated fibres tend to settle down on glass slides with their longest dimensions parallel to the glass surface, simply out of gravitational stability.

Conclusions

On an individual fibre basis, estimation of cross-sectional area by apparent diameter measurements gives unacceptably high errors, as can be seen by examination of single data points on figure 4. Over the 200 fibres measured in this study, this error did not "average out"; the estimated fibre area for the whole population of fibres is statistically higher than the true value. It is therefore concluded that accurate measurements of the cross-sectional area of muscle fibres should only be obtained by techniques such as the confocal zx image analysis used here.

Acknowledgement

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References

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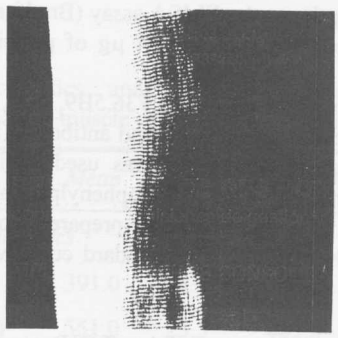

Fig 1: Xy-scan of a single muscle fibre obtained by confocal laser scanning microscopy

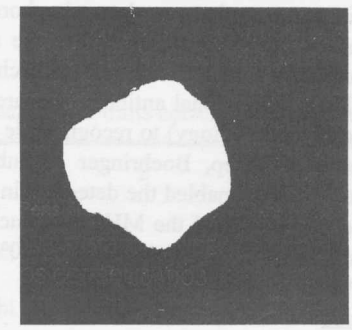

Fig 2: Binarized image of a single muscle fibre zx-scan obtained by confocal laser scanning microscopy

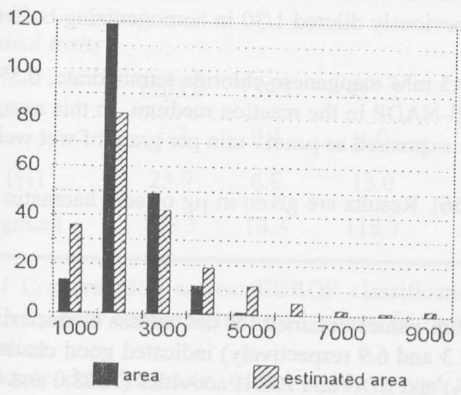

Fig. 3: Distribution of fibre cross-sectional areas as estimated from diameter measurements and as measured from confocal zx-scans

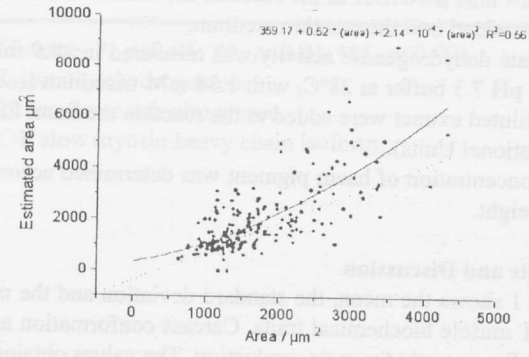

Fig.4: Comparison of fibre cross-sectional areas as estimated from diameter measurements and as measured from confocal zx-scans