SOME CONFOCAL GLIMPSES OF MEAT - A TRANSMISSION LIGHT MICROSCOPIAL STUDY

SARAFIS,V. and TINGLE, J.

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SUMMARY

Confocal microscopy provides a number of tools to investigate thick biological structures. Confocal transmission microscopy has been used here to study samples of meat from lamb obtained from several muscles. Transmission microscopy reveals the three dimensional structure of small samples of meat (up to 100 microns in thickness). In order to increase the sensitivity of the transmission mode we have utilised crossed polarisers placing the sample between them and aligning it so that the muscle fibres lie at 45° to the polars. This reveals the A bands of the sarcomeres with greater clarity than in unpolarised light. The use of cross circular polarisers in confocal mode was applied as an improvement resulting in non-azimuth dependent imaging. Evidence of collagen fibrils could be seen in polarised light confocal microscopy due their intrinsic birefringence. Fluorescence confocal microscopy in epifluorescence mode was used with the following dyes, Eosin (a general protein stain) and a fluorescent labelled phallacidin (Bodipy Phallacidin Fl from Molecular Probes Co., Eugene, Oregon, USA) which is a specific actin stain. The use of these two fluorochromes revealed the generalised protein distribution in the case of Eosin (particularly the A bands) and the actin within the I bands was visualised with a phallacidin probe. While the I bands are clearly stained diminution in the intensity of fluorescence was noted in the vicinity of the Z discs which were lightly stained. The reason for this staining pattern is yet to be elucidated.

Introduction

The microscopic imaging of meat has been approached in a number of different ways. Primarily the methods used involved fixation and processing of the tissue before imaging. The methodologies used may have resulted in artefacts due to preparation methods. Recently the technique of confocal laser scanning microscopy has been applied to imaging thick specimens avoiding the need for processing and sectioning for moderate thickness of tissues (approximately 100 microns). Confocal laser scanning microscopy (CLSM) has normally used the epi-illumination mode and only rarely has transmission microscopy been attempted. The use of transmission CLSM allows one to use three dimensional visualisation software and allows a higher resolution to be obtained. In this paper the use of transmission CLSM has been used both in simple bright field as well as in polarisation mode. In addition fluorescence confocal microscopy has been carried out as a control. Delaquis, et al, (1991), investigated pork muscle fibres confocally and pointed to the usefulness of confocal microscopy in studying muscle fibres in particular the measurement of sarcomere lengths. Sheppard and Gu (1993), modelled several aspects of imaging in CLSM in 3-D and arrived at the conclusion that it was safe to image thick muscle fibres due to the virtual absence of spherical aberration. Thus to a first approximation the imaging experiments reported here would seem to give a reliable image free to a large degree of that artefact.

Materials and Methods

A table scanning CLSM (Oxford Optoelectronics) was used for the imaging experiments. It was equipped with infinity corrected UPlanApo 100x objectives corrected for a 0.17 mm coverslips from Olympus. The NA of the objectives was adjustable using an iris diaphragm from 0.5 to 1.35. In addition a long working distance plan achromat lens 20x 0.4 NA with correction collar for different slide thickness was used. Polarisers used were HN22 linear polarisers from Polaroid Corporation. Circular polarisers were constructed by adding $\lambda/4$ retarders from Polaroid Corporation. Muscle material was obtained from lamb longissimus muscle from a chilled carcass from a commercial butcher. Muscle was dissected by using tungsten electro sharpened needles in saline under a dissecting microscope and mounted in 20% bovine serum albumin made up in 0.6% sodium chloride solution in distilled water. Some preparations were mounted directly in saline solution. For fluorescence studies staining was conducted either using a 1% Eosin solution in saline for 5 mins and then

washed with saline until no dye was eluted from the tissue or using the actin specific Bodipy Phallacidin Fl. The latter stain was made up as per the protocol of Molecular Probes Inc. viz, a 300 unit solution was prepared by adding 1.5 ml of methanol and make up stock. 1-10 dilution in bovine serum albumin as described above was used to stain the muscle preparation for 30 mins, washed twice with bovine serum albumin and mounted in bovine serum albumin solution. The preparations were examined in transmission in bright field, between linear crossed polarisers or crossed circular polarisers. When sample was placed for examination between crossed linear polarisers the muscle fibres were oriented at 45° to the polarisers axes. Fluorescence CLSM excitation was carried out using the 476 and 482 lines of a Ion Laser with a filter set consisting of 460 to 500 nm excitation filter, a dichroic filter with cut off at 505 nm and a barrier filter of 510-560 nm. The interference filter set was obtained from Chroma Technology Corporation, Vermont, USA. The pin hole in the photomultiplier detection mode was chosen to give true confocal imaging occupying approximately one third of the Airy disc impinging upon it from a long focal lens placed in the path of the fluorescence beam.

Results and Discussion

Muscle fibres examined in bright field transmission CLSM show a well defined striated appearance due to the repeating sarcomeres in the myofibrils. The unstained muscle used gives an image of high contrast and clearly shows the slippage of component myofibrils leading to mis-registration of sarcomeres across the muscle fibre (figure 1). The contrast is enhanced when material is examined under crossed linear polarisers. Here the A bands come into prominence when the muscle fibres are oriented diagonally (figure 2). In order to assess the appearance of muscle fibres in aggregates and to verify the relative displacement of myofibrils in post mortem muscle fibres scanning along the horizontal and vertical line was carried out and confirmed the changes in registration of myofibrils observed in confocal layer scanning in the X Y dimensions. Attempts to observe this in standard bright field microscopy of the same samples did not produce an image with clearly defined changes at different focal planes. Changes of appearance through focus due to optical artefacts in standard techniques of optical transmission microscopy have been noted by various authors summarised in Hanson and Huxley, (1953), Huxley and Hanson, (1960) and Ross and Casselman (1960).

When examined under crossed circular polarisers the sarcomere brightness seen in the A bands is uniform irrespective of muscle fibre orientation thus avoiding the need to orient muscle fibres diagonally. In both cases it is possible to build a three dimensional representation of the construction of the muscle fibres.

To confirm the structures as seen by transmission CLSM in polarisation mode material was also examined using Eosin staining and observed in fluorescence excitation. This reveals the distribution of protein in the muscle fibres along the myofibrils. In order to confirm the sarcomeric structure as observed using polarisation (which emphasises the A bands in the muscles examined) staining with Bodipy Phallacidin Fl was carried out. This stain is actin specific and reveals the I bands leaving the A bands unstained. This stain confirmed the interpretation shown in figure 2. However, some staining is noted in the M line region and in some preparations there appears to be some diminution of staining towards the Z disc. This needs to be investigated further in order to ascertain aspects of the specificity of this stain.

In addition it is possible to observe the endomysial collagen due to its birefringence.

Conclusion

Confocal microscopy is introduced here as a powerful technique for the investigation of small meat samples. In extending this work to the future the introduction of transmission confocal microscopy offers a complementary technology to the more commonly used fluorescence techniques in CLSM.

References

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Legends for Figures

Figure 1 Transmission CLSM image of teased unstained beef muscle. Scanning Magnification 5000x

Figure 2 Transmission CLSM image of teased unstained beef muscle placed between crossed linear polarisers at 45°. Note the bright appearance of the A bands. Scanning Magnification 5000x