Analysis of the Cellular Structure
and Biochemistry of Keratin Fibres

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School of Materials
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ΔHₙ</td>
<td>Denaturation Enthalpy</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>CMC</td>
<td>Cell membrane complex</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscope</td>
</tr>
<tr>
<td>ET</td>
<td>Electron Topology</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier Transform</td>
</tr>
<tr>
<td>IFs</td>
<td>Intermediate Filaments</td>
</tr>
<tr>
<td>KAP</td>
<td>Keratin-Associated Proteins</td>
</tr>
<tr>
<td>LM</td>
<td>Light Microscopy</td>
</tr>
<tr>
<td>MDSC</td>
<td>Modulated DSC</td>
</tr>
<tr>
<td>O1</td>
<td>Orthocortical cells type1 or Ortho-1</td>
</tr>
<tr>
<td>O2</td>
<td>Orthocortical cells type2 or Ortho-2</td>
</tr>
<tr>
<td>OFDA</td>
<td>Optical Fibre Diameter Analyses</td>
</tr>
<tr>
<td>P</td>
<td>Paracortical cells</td>
</tr>
<tr>
<td>PM</td>
<td>Projection Microscopy</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>Tₜ</td>
<td>Denaturation temperature</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
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</table>
Abstract

Wool, which is classed as keratin fibre, is the most important animal fibre in textile industries. Cortical cells of wool, which is the majority component, have been studied in various aspects. However, there are little researches focused on the relationship between wool diameter and cortical cells included ortho-, meso-, and paracortical cells. Moreover, in the past three decades, studies of cortical cells have been restricted to qualitative analysis and the interpretation of a single analytical technique. Thus, this study set out to fill this gap of knowledge. Merino wool fibers with various diameters were analysed by three methodologies based on thermal (differential scanning calorimetry; DSC), optical (light microscopy), and scanning probe microscopy (atomic force microscope; AFM) analysis. Additionally, this study also provided a large scale of quantitative analysis of the relationship between wool diameter and main cortical cells percentages including ortho- and paracortical cells by deconvolution of DSC curve using three Gaussian distributions of equal width.

Unlike previous finding, we found that the fine and coarse wools have a different characteristic of denaturation temperature. Only coarser wools (diameter larger than 25 µm) show a linear relationship between wool diameters and denaturation temperature. Interestingly, a combined thermal and optical methodological approach showed that the denaturation temperature difference between para- and orthocortical cells drop when wool diameters increase. The decrements in temperature difference between para- and orthocortical cells might then due to a much lower distinguished between ortho- and paracortical cells that observed in the methylene blue staining pattern of coarser wools. However, the relationship between wool diameters and cortical cell percentages is not observed by both thermal and optical analysis. Significantly, the optical analysis confirms the quantitative analysis of cortical cells estimation by DSC deconvolution. Thus, apart from conventional microscopy techniques, DSC deconvolution is a feasible technique to estimate cortical cells percentages.

While Janus green staining was used to determine percentage areas of ortho- and paracortical cells, the successful modified methylene blue staining provided the new evident that mesocortical cells might not distinguish enough to classify as a minority of cortical cell types. The AFM image observed also confirm expectations based on earlier staining patterns of Janus green and methylene blue observations. Additionally, all three techniques support that mesocortical cells did not exist. To sum up, the importance of this research is in raising awareness about the issues of employing a multidisciplinary approach to investigate the cellular structural structure and finding the relationship between wool diameter and cortical cells. It is expected that these findings will contribute to a better understanding of the biological and structural basis of wool fibre such as human hair.
Declaration

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Throughout this process without my family I would not be where I am today. Thank you for their love and support. Extended love and gratitude to all my friends who have supported me throughout my PhD, there are too many to name them all. It has been a pleasure to have them with me on this journey. Finally, I would like to express my gratitude to the Royal Thai Government for the funding.
Chapter 1 Introduction and Research Objective

1.1 Thesis Outline

Keratin fibres are one of the biomaterial that has high degree of complexity. Over the past century, the studies of keratin fibres have been a surge of interest in various aspects. Cortical cells of wools are one of the research topics that have gained interested all the time. The reason is that cortical cells are the majority of keratin fibres (around 90%), so the properties of fibres are directly associated with cortical cell structure such as chemical composition and the arrangement. Additionally, wool and human hair have similar structure of cortical cells. Therefore, better understanding of wool cellular structure and biochemistry might extend knowledge and be applied for human hair and other keratin fibres researches.

Throughout, this thesis, the study of cortical cells of keratin fibres will be investigated by two main techniques, which are thermal and optical analysis. There are various types and sources of keratin fibres. In this research, only hard α- keratin fibre is studied. Merino wools with various sources and diameters were applied as a model of keratin fibres. This thesis is composed of seven themed chapters. The first chapter will give a brief introduction for each chapter. Additionally, a background of protein, keratin and wool fibres, including fibre formation and chemical composition will be explained. The significant technical terms such as main cortical cells, cortex and cuticle cells will be gave a definition and explanation in “The morphology of wool fibre” section. More importantly, a research question and the objective of this study will be explained at the end of in this chapter.

This thesis, wool fibres were classed according to their sources and diameter ranges. Thus, Chapter 2 was introduced in order to provide an introduction of wool diameter measurement methods and diameter grading systems. This chapter will involve with the sample characterisation. Sources of wool samples and diameter measurement process will be explained in this section. Additionally, at the end of Chapter 2, the code of all the samples will be named depending on the source and diameter and this code will be used for the whole thesis.

The following four chapters are the experimental parts, which are the main body of this thesis. Qualitative and quantitative analyses of the relationship between wool cortical cells and
wool diameters will be discussed. The results of thermal analysis and optical analysis of wool cortical cells will be explained in Chapter 3 and 4, respectively. DSC was applied for thermal analysis, while microscopy technique was applied for optical analysis. The relationship between both techniques will be discussed in Chapter 5. Atomic force microscope (AFM) is another analytical technique that was applied in this study and the results of AFM will be explained in Chapter 6. The relationship between light microscopy and AFM will be also reported in this chapter. Lastly, Chapter 7 will provide the conclusion and future works. The purpose of the final chapter is to reflect on the key finding and the extension of wool cortical cells knowledge in this study.

1.2 Introduction to Protein and Keratin Fibres

1.2.1 Protein

Protein is one of the biological polymers, which has amino acid as a monomer (NH₂-CHR-COOH) (Kuffner and Popescu, 2012). This polymer appears in diversity of living organisms and performs different functions depend on their chemical properties and structures. Proteins are produced by condensation polymerisation as shown in Figure 1.1. After this polymerisation reaction, water (H₂O) is eliminated while peptides boned (-CO-NH-) are created to link between two amino acid groups. Side-groups (-R) of polymer chains are important factors that control diversity of proteins (S.J.Eichhorn et al., 2009). According to the side-chains, amino acids are classified into five groups: “acidic” amino acids, “basic” amino acids, amino acids with hydroxyl groups, sulfur-containing amino acids, and amino acids with no reactive groups in the side chain (Popescu and Hocker, 2007).

The examples of amino acid for each type are shown in Table 1.1. Proteins consist of complex structure. However, in generally, the final proteins compose of four levels layering together. Protein molecular is the preliminary structure. This molecular is build up by joining each atom by covalent bond. The secondary structure, α-helix and β-sheet are created by link primarily hydrogen bonding between the atom. The third structure is significant to, protein function because
the process of building up globular protein is in this structure. The last structure, two protein are connected together for particular function (Bischof and He, 2005).

**Figure 1.1** The reaction of condensation polymerization of amino acids to form protein chains

(LibreTexts, 2019)

**Table 1.1** The examples of amino acids according to five of the side-chains (Bryson et al., 2009, Wortmann, 2009, Kuffner and Popescu, 2012)

<table>
<thead>
<tr>
<th>Group</th>
<th>Amino acid name (molecular formula of side chain)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acidic</strong></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid (–CH₂–COOH)</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid (–(CH₂)₂–COOH)</td>
<td></td>
</tr>
<tr>
<td>Asparagine (–CH₂–CONH₂)</td>
<td></td>
</tr>
<tr>
<td><strong>Basic</strong></td>
<td></td>
</tr>
<tr>
<td>Arginine (–(CH₂)₃–NH–C(NH₃)=NH)</td>
<td></td>
</tr>
<tr>
<td>Lysine (–(CH₂)₄–NH₂)</td>
<td></td>
</tr>
<tr>
<td><strong>Hydroxyl</strong></td>
<td></td>
</tr>
<tr>
<td>Serine (–CH₂–OH)</td>
<td></td>
</tr>
<tr>
<td>Threonine (–CH(CH₃)–OH)</td>
<td></td>
</tr>
<tr>
<td><strong>Sulphur containing</strong></td>
<td></td>
</tr>
<tr>
<td>Cysteine (–CH₂–SH)</td>
<td></td>
</tr>
<tr>
<td>Thiocysteine (–CH₂–S–SH)</td>
<td></td>
</tr>
<tr>
<td>Cystine (–CH₂–S–S–CH₂–)</td>
<td></td>
</tr>
<tr>
<td><strong>amino acids without reactive groups (Inert)</strong></td>
<td></td>
</tr>
<tr>
<td>Glycine (–H)</td>
<td></td>
</tr>
<tr>
<td>Alanine (–CH₃)</td>
<td></td>
</tr>
<tr>
<td>Leucine (–CH₂–CH(CH₃)₂)</td>
<td></td>
</tr>
</tbody>
</table>
The fibrous and the globular are the major groups of proteins. These groups are classified according to their physical forms. The structural comparison between globular and fibrous is shown in Figures 1.2a and b. Globular proteins are “spherical (globe-like) protein”. A polypeptide chain of this protein shows a compact and round shape. These proteins are a group of protein that have water-soluble property. In X-ray diffraction this group present amorphous halo (M. SUNDE and BLAKE., 1998). Enzymes and haemoglobin in blood are examples for globular proteins (Wang et al., 2016). In contrast, fibrous proteins are insoluble in water. Fibrous proteins are distinguished from globular proteins by their elongated form with parallel arrangement of polypeptide chains. This structure results in more stable structure than globular proteins. Keratin, collagen and elastin are the example of proteins in the group (Kuffner and Popescu, 2012, Wang et al., 2016).

![Figure 1.2](image)

**Figure 1.2** Three-dimensional structures of a) globular protein and b) fibrous protein (G.Bagler and S.Sinha, 2005)

### 1.2.2 Keratin Fibres

Keratins are made up by the fibrous group, so the word “keratins” refers to a group of insoluble and filament-forming proteins (Popescu and Hocker, 2007). Keratin is found the most in epithelial cells. The role of keratin is structural proteins. This group of protein is the most important biopolymer in animals (McKittrick et al., 2012, Wang et al., 2016). Unlike other biological materials, following Figure 1.3, keratin presents high toughness and high modulus among other biomaterials (Wegst and Ashby, 2004).
According to the X-ray diffraction result, keratin is classified into two groups: α-keratin and β-keratin (Fraser et al., 1972, Gillespie, 1990) (see Figures 1.4a and b). Generally, the feather pattern represents β-keratin, while amorphous pattern has been considered as α-keratin (R.D. Bruce Fraser and Parry, 2011). The most important feature that distinguishes α- and β-keratins is the molecular structure, shown in Figures 1.4c and d. The α-keratin proteins are organised as coiled coils, while the β-keratin proteins are organised as a pleated sheet structure (Gillespie, 1990, Alibardi L et al., 2006). At nanoscale, a similarity of nanoscale between β- and α-keratin is found. Both of them show a fine filament-matrix structure, but the chemical composition is different. In β-keratin, there are no different types of proteins between filament and matrix (Fraser et al., 1972); the filament and matrix are incorporated into one single protein (R.D. Bruce Fraser and Parry, 2011). On the other hand, α-keratin, “several kinds of low-sulphur proteins compose the IFs (Gillespie, 1990), while the matrix consists of high-sulphur” (Fraser et
al., 1972, Wang et al., 2016). Other different characteristics between β- and α-keratin are summarised in Table 1.2.

**Figure 1.4** Differences between α- and β- keratin; X-ray diffraction patterns a) α-keratin, and b) β-keratin (Fraser et al., 1972). Ball-and-stick model of the polypeptide chain structure c) α-keratin, and d) β-keratin (Wang et al., 2016).

**Table 1.2** The comparison of basic structures between α- and β-keratins adopted by (Wang et al., 2016)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>α-keratins</th>
<th>β-keratins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameters of the filaments (nm)</td>
<td>IFs: ~ 7</td>
<td>β-keratin filaments: 3–4</td>
</tr>
<tr>
<td>X-ray diffraction patterns</td>
<td>Equatorial reflection with spacing 0.98 nm and a meridional reflection with spacing 0.515 nm</td>
<td>Axial repeat of 0.31 nm reflection and the equatorial reflection ~ 0.47 nm</td>
</tr>
<tr>
<td>(Fraser et al., 1972, Richard S. Bear and Rugo, 1951)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Characteristic structure</td>
<td>Based on α-helical structure 40–68 kDa</td>
<td>Based on β-pleated sheet structure 10-22 kDa</td>
</tr>
<tr>
<td>Molecular mass (Alibardi L et al., 2006)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

β-keratin is found in birds while α-keratin is found in mammal. In α-keratins, they can be categorized into soft and hard α- keratin. Hard α- keratin is found in nail, hoof, hair, and wool, while epidermis and other epithelial parts of the body are composed of soft α-keratin. Wool and
human hair can be classified into the hard α-keratin fibres (Wang et al., 2016). So, in this study, only hard α-keratin will be focused.

The Structural formula of two peptide chains is illustrated in Figure 1.5. There are significant five interactions of amino acid side-chains in order to stabilise its structure which is summarised following Table 1.3. The hydrogen bonds inside the helix chain (Figure 1.5, No.2,) plays an important role for the stabilisation of the α-helical structures in keratin, this interaction causes the chain to twist and exhibit a helical shape (TJ McMurry et al., 1989, DAD, 1996). Additionally, the disulphide bond (Figure 1.5, No.4) plays an important part in fibre properties such as its relatively high wet strength, insolubility and thermal stability (Popescu and Hocker, 2007, Zahn H et al., 2003).

![Structural formula of peptide chains to illustrate five important interactions between amino acid side-chains in α- keratins](image)

**Figure 1.5** Structural formula of peptide chains to illustrate five important interactions between amino acid side-chains in α- keratins (Wortmann, 2009, Zahn H et al., 2003).
Table 1.3 The summary of five important interactions between amino acid side-chains in α-keratins

<table>
<thead>
<tr>
<th>No</th>
<th>Cross-link position</th>
<th>Interaction/ bonding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Between phenyl rings and phenyl rings</td>
<td>Apolar interact</td>
</tr>
<tr>
<td>2</td>
<td>Between residue of asparagine and serine</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>3</td>
<td>Between residues of glutamic acid and arginine</td>
<td>Electrostatic interact</td>
</tr>
<tr>
<td>4</td>
<td>Between residues of cysteine and cysteine</td>
<td>Disulphide bond</td>
</tr>
<tr>
<td>5</td>
<td>Between amino group of a lysine residue and the carboxyl group of glutamic or aspartic acid</td>
<td>Isopeptide cross link</td>
</tr>
</tbody>
</table>

In term of molecular structure and formation of the intermediate filaments (IFs), α-keratin fibres consist of “individual polypeptides of IFs are elongated molecular with an extended α-helical domain that forms a parallel coiled coil with another monomer”(Alberts et al., 2002). The monomeric unit of the IF and model of IFs construction are illustrated in Figures 1.6a and b, respectively. The monomeric unit of the keratin IF have specific sequence of chemical structure. Each end of α-helical central rod is non-helical N-and C-terminal domains that have a high amount of cystine. These two ends will bond with other IFs and matrix. The central rod region (about 46 nm in length) contains with two almost identical helical segments (1 and 2), which consist of shorter α-helical coiled coil segments (A and B). All helical segments are linked with non-helical linkers, named L1, L1, 2, L2, respectively (see Figure 1.6a) (PA. Coulombe and Omary., 2002, S.J.Eichhorn et al., 2009, Wang et al., 2016).

The formation of IF was simplified and illustrated in Figure 1.6 b. Firstly, from the left to right, the dimer (45 nm long) is formed by disulphide cross links between two isolated right-handed α-helix chains. This linkage forms a left-handed coiled-coil. A dimer combines with another dimer end-to-end and stagger side-by-side via disulfide bonds to form a protofilament (about 2 nm diameter) (C-C Chou and Buehler, 2012). Then, these protofilaments (octamer) bind together to form protofibril. Then, IFs are formed by doubling of the previous structure. The IF (about 7 nm diameter) is formed by combining protofibril. Lastly, the keratin fibres are form by
embedding these IFs into an amorphous matrix proteins, which are sulfur-rich keratin (See Figure 1.6c) (R.D.B.Fraser et al., 1988).

![Diagram of IF structure](image)

**Figure 1.6** Schematic drawing of detailed structure of IFs a) the molecular of IF monomeric unit shown chemical sequence: The non-helical N- and C-terminal α-helical coiled coil segments (1A, 1B, 2A, 2B), and short links (L1, L12 and L2) adapted from (PA. Coulombe and Omary., 2002, Jones LN et al., 1997). b) the IFs formation process: “α-helix chains twist to form the dimers, which assemble to form the protofilament. Four protofilaments organise into the intermediate filament” (Wang et al., 2016), and c) A two-phase model of keratin fibres. The IFs are embedded in an amorphous keratin matrix (Baias et al., 2009).
1.3 Introduction to Wool Fibres

1.3.1 The Formation of Wool Fibres

Wool is known as animal fibres. It can be classified as the hard α-keratin fibres. There are many sources of wools such as angular, lama, alpaca, sheep, and goat. In this study, the term of wool only refers to the sheep wool. Sheep wool has traditionally been viewed as the representative mammalian keratin fibre for the purposes of describing morphology and protein composition (Thomas et al., 2012). This fibre consists of two parts, as shown in Figure 1.7. The first part is follicle, which locates below the surface of the sheep skin. The initial fibre is produced from this part. Along the follicle consists of five different zones, which can be categorised in term; “zone 1 – bulb zone (proliferation and differentiation); zone 2 – elongation (fibril formation); zone 3 – pre-keratinisation (lateral aggregation); the first three zones a Zone 4 – hardening or keratinisation; the formation of disulphide bonds between cysteine residues are formed at this point, which result in the occur of the process of crosslinking of the protein chains. Then, the fibre is stabilised and dehydrated in the last zone (zone 5), which is post-hardening (hard keratin)” (R. C. Marshall et al., 1991, Kuffner and Popescu, 2012).

Another part is shaft (wool), which grows from the follicle and is composed mainly of the fibrous protein. Different breeds of sheep produce different physical characteristics of wool fibres. In general, cross section of wool fibres is in elliptical cylinder, with diameter ranged from 15 -100 μm, while the staple length depends on the breed of wools (Ammayappan, 2013). Although, the variation of wool fibres properties is high, from chemical point of view, all of wool fibres consist of carbon, hydrogen, oxygen, nitrogen, and sulphur as primary elements (Kuffner and Popescu, 2012). The fraction of each element is summarised following Table 1.4. From a chemical point of view, wool is a polymer made of amino acids. It is composed of “eighteen different amino acids in which the importance amino acids are cysteine (13.1%), glutamate (11.1%) and serine (10.8%)” (Simpson et al., 2002, Ammayappan, 2013). In the view of morphology, wool fibres have three distinct morphological parts; cuticle cells, cortex, and medulla. The cortex and cuticle cells are the main components of wool fibres, while a medulla tends to be found in coarser wools only (M. Feughelman, 1997, Plowman, 2003). The details morphology of wools will provide following;
Figure 1.7 Schematic diagram of a fibre follicle showing various growth of 5 zones; For explanation of zones, see Text (Kuffner and Popescu, 2012).

Table 1.4 Elemental composition of wool fibres (water-free) (Zahn H et al., 2003).

<table>
<thead>
<tr>
<th>Element</th>
<th>Weight, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>50.5</td>
</tr>
<tr>
<td>Oxygen</td>
<td>22</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>16.5</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>6.8</td>
</tr>
<tr>
<td>Sulphur</td>
<td>3.7</td>
</tr>
<tr>
<td>Ash</td>
<td>0.5</td>
</tr>
</tbody>
</table>
1.3.2 The Morphology of Wool Fibre

The molecular structure and morphology of wool fibres is complex. The schematic diagram of morphology of wool fibre is illustrated in Figures 1.8. Cuticle and cortical cells are the two main categories of cells in wool fibres. From the left hand side, α-helical is the initial unit of the fibre. α-helical are combined together and formed microfibrils or intermedia filaments (IFs). IFs are low sulphur content. These microfibrils are embedding in intermicrofibrillar matrix or matrix, which is high cysteine content, and form macrofibrils. The macrofibrils are the main component of cortical cells. The differences of proportions between IFs and matrix are directly associated with different types of cortical cells. These cortical cells are held together by the cell membrane complex (CMC). Holding cortical cells together make up the cortex, which holds the largest area in fibre. The cortex is protected by the cuticle cells, which is the outmost layer (Leon, 1972, Popescu and Hocker, 2007).

Figure 1.8 Schematic of the α-keratin fibre under various magnifications (Popescu and Hocker, 2007).
The cuticle cells are an external layer of wool fibre. Around 10% of wool fibres consist of cuticle cells (Leon, 1972). It consists of roughly rectangular flattened cells overlapping each other, which form a protective layer (see Figure 1.9a). Cuticle cells compose of four different layers, which are shown in Figure 1.9b. The outermost layer is epicuticle followed by A-layer, exocuticle, and endocuticle, respectively. CMC is found between each layer in cuticle cells to bind overlapping cuticle cells together (Popescu and Hocker, 2007). Although, exocuticle and endocuticle are the majority of cuticle cells, the hydrophobic property of wools is influenced by the outermost layers (epicuticle). This layer consists of lipid, which provide a protective layer and hydrophobicity for the fibre. It has good resistance against acid, oxidizing agent, reducing agent, enzyme and alkali (Negri et al., 1993, Popescu and Hocker, 2007, Ammayappan, 2013). Moreover, the epicuticle layer consists of high amount of 18-methyleicosanoic acid which gives lower surface tension than water (Leon, 1972, Negri et al., 1993). Alcoholic alkaline or chlorine treatment can be applied to remove this layer for increasing wettability of wool fibres (Negri et al., 1993). A significant difference of these layers is cysteine concentration. High cysteine content can be found in A-layer and exocuticle, while endocuticle has the lowest cysteine content (Negri et al., 1993). A high cross-link density of disulphide bond results in chemically resistant of this layer (Popescu and Hocker, 2007).

Cortex is the internal cells of wool fibres. These cells are wrapped up in the cuticle cells. Around 90% of wool fibres are occupied by this cell, so cortex is the predominant component in wool structure and has the biggest influence on wool's physical and chemical characteristics (Popescu and Hocker, 2007, Ammayappan, 2013). Cortex is the aggregation of cortical cells. Each cortical cell is composed of macrofibrils, which consist of microfibrils and matrix. According to TEM investigation, the cortex of merino wools compose of three different cortical cells types; para-, ortho-, and mesocortical (Bryson et al., 2009, Popescu and Hocker, 2007). They are separated from each other by a cell-membrane complex (M. Feughelman, 1997, Plowman, 2003, R. C. Marshall et al., 1991). However in other hairs fibres, which are coarser than Merino wools, tend to consist of orthocortical cells only (Popescu and Hocker, 2007).
Figure 1.9 Scanning electron micrographs of wool cuticle cell a) Tipped of wool; which is build the protective property for the fibres (Kuffner and Popescu, 2012). b) Schematic side view (Top), and scanning electron micrograph of the four layered structure of cuticle cells (Below); EPI, epicuticle; a, a-layer; EXO, exocuticle; ENDO, endocuticle; C.M., cell membrane (Popescu and Hocker, 2007).

In case of Merino wools, ortho- and paracortical cells are the main cortical cell types. 60-90% of cortex consists of orthocortical cells, while 40-10 are the percentage of paracortical cells in cortex. However, there are many arguments for the mesocortical cells percentage. This cell is reported as a minority of cortical cells. Basically, mesocortical cell was found in low –crimp Merino wools (Ammayappan, 2013). The differences of cortical cells types have been studied intensively. Ortho and para cortical cells differentiate each other in various aspects including morphology, chemical, and physical properties.

In view of morphological and structural of macrostructure, the arrangement of IFs and the ratio between IFs/matrix are significant indicator to differentiate between ortho – and paracortical cells. Figures 1.10 illustrate the cross section of three different segments in wool fibres.
IFs arrangement in orthocortical cells is helical structure with a high density of IFs, while highly ordered of IFs arrangement in horizontal way and parallel to the fibre axis is investigated in paracortical cells. Although mesocortical cells shows randomly IFs arrangement, hexagonal or pseudo hexagonal pattern are often found (R. C. Marshall et al., 1991). Another significant differentiation is the ratio between matrix and IFs. According to high density arrangement of IFs in orthocortical cells, this cell has lower amount of matrix than paracortical cells. Therefore, orthocortical cells have lower sulphur proteins than paracortical cells (Plowman et al., 2007). Moreover, macrofibrils of each cortical cell shows specific feature. Smaller and more separation macrofibrils are found in orthocortical cells. On the other hand, macrofibrils in paracortical cells are big and interlock each other (Rogers, 1959, Popescu and Hocker, 2007).

**Figure 1.10** The cross sectional images of Romney wool observed by TEM a) A total Cross-sectional area showing the three different types of cells, b) Orthocortical, c) Mesocortical and d) Paracortical cells: P, paracortex, O, orthocortex and M, mesocortex (Plowman et al., 2007).
In the view of chemical composition, generally, the orthocortical cells are more reactive with basic dyes while paracortical cells are lightly dyed with basic dyes (Donald F.G. Orwin et al., 1984, Hiroshi Sakabe, 1986). Numerous studies have indicated that the higher concentration of cysteine express on the paracortical cells, while orthocortical cells consist of proteins high in glycine and tyrosine (Plowman et al., 2007). According to these distinctions of cortical cells, they influence on the properties of wool fibres. For instance, there are extensively reported that cortical cells arrangement influences on a crimp property of wools (Ammayappan, 2013). The studies of effect of cortical cells arrangement on wool properties will be discussed further in Chapter 4.

1.4 Objective

So far, there are numerous of studies on wool cortical cells in various aspects. The fundamental study on fibre characteristic of wools provides reliable data on chemical compositions and morphology of the fibre. Further studies on cortical cells have explored the relationship between cortical cells and fibre properties. There have been considerable interested in the relationship between cortical cells and crimp of wool. Various theories of crimp formation and the relationship have been reported.

The diameter of wools is one of the important factors for wool industry. Qualities of end products, and production process are directly associated with the diameter. However, little works have been conducted on the relationship between cortical cells and wool diameters. Moreover, to date, no large-scale studies have been performed to investigate this relationship. Thus, the aim of this research is set from this research gap. This thesis would like to investigate the relationship between cortical cells and wool diameter. A combined qualitative and quantitative methodological approach was used in this study. In order to achieve this aim, the following objectives are listed;

• Study the relationship between wool diameters and main cortical cell percentages (ortho- and paracortical cells) by thermal (DSC), optical (LM) and AFM analysis techniques in both quantitative and qualitative views.

• Investigation the relationship between thermal and microscopic analytical techniques.
• Investigation the possibility of using the deconvolution of DSC peaks for the estimation of orho- and paracortical cells percentages.

• Investigation the relationship between light microscopy and scanning probe microscopy techniques (AFM).
1.6 References


Chapter 2 Sample Evaluation

2.1 Diameter of wool

The diameter of fibres is one of the important wool properties. This parameter has been used to identify, classify and grading the difference between sheep wool and other animal fibres (LUNNEY, 1983). For instance, Merino wools have the finest fibres when compared with other breeds of sheeps. Additionally, in the textile industry, wool diameter is primary importance determining wool value. Lighter weight materials are produced by the fine diameter of wools whereas larger diameter fibres produce heavier weight materials. Additionally, softer, denser, and better handle properties of garments can be achieved by using finer yarns which are produced by fine fibre (Botha and Hunter, 2010). Therefore, an accurate and reliable wool diameter measurement method is important. This chapter will include a brief review of numerous methodologies of wool diameter measurements and some influential parameters.

Moreover the importance of wool diameter is clearly seen by many grading systems have been invented in order to describe the fineness of wool fibres. Some grading system such as United States Department of Agriculture (USDA) classify wool diameter into four ranges, which are fine (<17.70 – 22 µm), medium (22.05 – 30.99 µm), coarse (31.00 – 36.19 µm), and very coarse wools (36.20 – 40.20 µm). On the other hand, more sensitivity grading system such as English or Spinning Count Grade classes wool diameters into 16 ranges. The relationship between American, English and micron systems of grading wool was illustrated in Table 2.1 (Kott, 1993). In this study, USDA standard was applied to describe the ranges of wool diameters.
Table 2.1 Comparison of different wool grading systems (Kott, 1993).

<table>
<thead>
<tr>
<th>USDA Grade</th>
<th>Old Blood Grade</th>
<th>English or Spinning Count Grade</th>
<th>Limits for Average Fibre Diameter (µm)</th>
<th>Variability Limit for Standard Deviation Maximum (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fine</td>
<td>Fine</td>
<td>Finer than 80’s</td>
<td>&lt;17.70</td>
<td>3.59</td>
</tr>
<tr>
<td>Fine</td>
<td>Fine</td>
<td>80’s</td>
<td>17.70-19.14</td>
<td>4.09</td>
</tr>
<tr>
<td>Fine</td>
<td>Fine</td>
<td>70’s</td>
<td>19.15-20.59</td>
<td>4.59</td>
</tr>
<tr>
<td>Fine</td>
<td>Fine</td>
<td>64’s</td>
<td>20.60-22.04</td>
<td>5.19</td>
</tr>
<tr>
<td>Medium</td>
<td>1/2 Blood</td>
<td>62’s</td>
<td>22.05-23.49</td>
<td>5.89</td>
</tr>
<tr>
<td>Medium</td>
<td>1/2 Blood</td>
<td>60’s</td>
<td>23.50-24.94</td>
<td>6.49</td>
</tr>
<tr>
<td>Medium</td>
<td>3/8 Blood</td>
<td>58’s</td>
<td>24.95-26.39</td>
<td>7.09</td>
</tr>
<tr>
<td>Medium</td>
<td>3/8 Blood</td>
<td>56’s</td>
<td>26.40-27.84</td>
<td>7.59</td>
</tr>
<tr>
<td>Medium</td>
<td>1/4 Blood</td>
<td>54’s</td>
<td>27.85-29.29</td>
<td>8.19</td>
</tr>
<tr>
<td>Medium</td>
<td>1/4 Blood</td>
<td>50’s</td>
<td>29.30-30.99</td>
<td>8.69</td>
</tr>
<tr>
<td>Coarse</td>
<td>Low 1/4</td>
<td>48’s</td>
<td>31.00-32.69</td>
<td>9.09</td>
</tr>
<tr>
<td>Coarse</td>
<td>Low 1/4</td>
<td>46’s</td>
<td>32.70-34.39</td>
<td>9.59</td>
</tr>
<tr>
<td>Coarse</td>
<td>Common</td>
<td>44’s</td>
<td>34.40-36.19</td>
<td>10.09</td>
</tr>
<tr>
<td>Very coarse</td>
<td>Braid</td>
<td>40’s</td>
<td>36.20-38.09</td>
<td>10.69</td>
</tr>
<tr>
<td>Very coarse</td>
<td>Braid</td>
<td>36’s</td>
<td>38.10-40.20</td>
<td>11.19</td>
</tr>
<tr>
<td>Very coarse</td>
<td>Braid</td>
<td>Coarser than 36’s</td>
<td>&gt;40.20</td>
<td>--</td>
</tr>
</tbody>
</table>

2.1.1 Diameter Measurement Technique

Normally, human eyes can roughly identify and separate the differentiation of fibre diameter. However, there is a limitation when the differentiation is less than 2.5 µm (Bryant, 1957). Therefore, the technology of diameter measurement is required. Many researchers have focused on improving the measurement methods in order to increase the precision of mean diameter. During 1930 – 1950, gravimetric method was widely used in which the definite fibres
length and amount of fibres are weighed and calculated in cross-section area. This method assumes that wool cross section is a circular and the density of every wool fibre is similar and constant during the measurement process. Although this technique is less time consumption, the precision is questionable. In contrast better accuracy and precision of the measurement can be achieved by microscopy method. Projection microscope (PM) was then developed to improve accuracy of wool diameter measurement and this technique has been used since 1777 (Baxter, 2015, Botha and Hunter, 2010). In this study, PM was applied as a measurement method. The principle and details of this method will explain in Section 2.1.2.

Apart from microscopy methodology, airflow (AF), optical fibre diameter analyses (OFDA), and laser scan are important techniques of measuring the mean diameter. The principle of AF method relies on the relationship between fibre diameters and surface areas. In AF process, air will flow through a bulk of fixed mass fibres and fixed pressure system so the velocity of the output air is affected by the surface area. Finer wools have lower flow rate than coarser wool as fine wool have a higher surface area (Baxter, 2015). OFDA and laser scanner are the newest techniques for diameter determination and these two methods were developed in order to reduce human error. However, the principles behind these techniques are different.

OFDA utilise the image analysis techniques while laser scan is non-microscopically method. The OFDA technique uses a video camera to produce electronic images of magnified fibres distributed over a horizontal glass slide. The diameters of fibre snippets are automatically measured by the software (Baxter et al., 1992, Baxter, 2015, Materials, 2010). The laser scan technique operates by light scattering. The proportion of scattered light intensity and the diameters is the principle behind this technique. The fibre diameters are measured by dispersing snippets in a solution of isopropanol and put in the glass chamber. Fibres are transported through this chamber where intersects a circular laser beam. The light detector measures the change of the signal generated when the shadow cast by the fibre snippet. The signals are recorded electronically and analysed almost instantaneously by computer (Botha and Hunter, 2010). The advantage and disadvantages of these three techniques are summarised following Table 2.2.
Table 2.2 The Summary of advantage and disadvantage of different wool diameter measurement methods (Botha and Hunter, 2010).

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantage</th>
<th>Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF</td>
<td>- Fast</td>
<td>- Indirect method</td>
</tr>
<tr>
<td></td>
<td>- Measure large sample size</td>
<td>- Inability to measure fibre distribution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Need calibration</td>
</tr>
<tr>
<td>OFDA</td>
<td>- Fast</td>
<td>- Sensitive to fibre strength</td>
</tr>
<tr>
<td></td>
<td>- Provide diameter variability, mean fibre curvature (MFC), and comfort factor</td>
<td>- Expensive</td>
</tr>
<tr>
<td></td>
<td>- More accurate than AF</td>
<td>- Require calibration</td>
</tr>
<tr>
<td>Laser scan</td>
<td>- Fast</td>
<td>- Sensitive to fibre strength</td>
</tr>
<tr>
<td></td>
<td>- Provide diameter variability, mean fibre curvature (MFC), and comfort factor</td>
<td>- Expensive</td>
</tr>
<tr>
<td></td>
<td>- More accurate than AF</td>
<td>- Require calibration</td>
</tr>
</tbody>
</table>

2.1.2 Projection Microscope (PM)

A projection microscope is one of the light microscopes (LM) or optical microscopes. Similar to other light microscopies, lens is an important element in projection microscope. The structures of a light microscope and a projection microscope are similar, both of which include a light source, a condenser, and objective lens. X-Y stage and circular screen are the extra components of a projection microscope. The objective lenses of both microscopes are similar, but the second lens is different. In a light microscope, an eyepiece is employed; while a projecting lens is applied in a projecting microscope (D.J.Rawlin, 1995, B.Bracegirdle, 1998, Savile, 1995).

The main difference between these microscopes is the type of a final image. In a projection microscope, the real image of the object is highly enlarged and displayed on screen, whereas light microscope shows a virtual image (Savile, 1995). The difference in the image formation process of these microscopes is illustrated in Figure 2.1.
There are two ways to measure wool diameter using projection microscope. First method is cross-section, where wool fibres are cut at right angle to its length and the real diameter of the fibres is measured. However, bad cutting processes such as burrs and imperfection right angle cutting impact to the accuracy of the measurement results. Therefore, another technique, is gain more importance in term of fibre diameter measurement. Profile method is the second technique; in which the transverse diameter of fibres is measured. Fibres are cut in a short length, and then the distance between both edges of fibre is measured. Moreover, there are standard test methods for transverse diameter determination (Andreson, 1955, Baxter, 2015).

Although projection microscopy method is more time consuming, labour intensive and high operation cost, compared to other methods, projection microscopy method is a significant method to product a reference standard for calibration (LUNNEY, 1983). IWTO-8 standard is one of the wildly used standards in this technique. Another exist standard for projection microscope is ASTM D-2130 standard. Another benefit of this technique is that it provides both of mean fibre diameter and fibre distribution (Baxter, 2015, C. C. Kritzinger, 1964, LUNNEY, 1985).
2.1.3 Error Parameters in Wool Diameter Measurement by PM

As the diameter of the wool fibres can vary along, mean fibre diameter is used. There are many factors that can influence the accuracy of measurement through the measurement process such as preparation process, mounting medium, microscope focusing procedure, and human error. Temperature and humidity are the two important factors that can change the diameter of wool fibres (Wortmann, 2009). Therefore, the procedure of sample preparation has been suggested by IWTO.

According to IWTO-8 standard, wool fibres should be conditioned and cut in standard atmosphere (65 ± 2% relative humidity and 20 ± 2°C). Wool fibres should put in this condition at least 4 hours in open space before the measurement (Andreson, 1955, IWTO, 2011). However, improper snippets length can reduce the accuracy of measurement. This was demonstrated by S. Parthasarathy and team in 1981. They reported that wool fibre with mean diameter around 20-28 μm was used. They found that mean diameter of fibre is influenced by the snippet length. The greater effect was found in coarser fibres. The lengths of 0.6-1.4 mm give the less variation of mean fibre diameter. If too shorter length snippets are cut, a biased result might be found (Andreson, 1955, Browne and Hindson, 2008).

Mounting medium selection is one of the concerned factors. The suitable mounting medium should have a refractive index around 1.53-1.43 in order to prevent the reflective effect between wool sample and the medium. The medium should have an appropriate viscosity so the snippets can be well dispersion. Another significant factor is water absorbability of the medium. A suitable medium should be hydrophobic. Immersion oil, cedar wood oil and liquid paraffin are examples of suitable mounting mediums that have been applied for wool diameter measurement (Andreson, 1955, Savile, 1995, IWTO, 2011).

In addition, human effect has been showed to affect the precision of measurement (C.C. Kritzinger, 1964). This process is operator dependent and the variation between each measurement can range between 0.69 to 0.58 μm. Moreover, there was “day-to-day variation” from the same operator in different day. The bias of variation is around 0.4 μm. The Image focusing procedure is also an important factor that involve in the accuracy of the measurement.
According to this fact, IWTO-8 standard recommend that diameter measurement process should be carried by two operator (C. C. Kritzinger, 1964, IWTO, 2011).

Another parameter that can influence measurement accuracy is a number of tests. IWTO-8 recommends that under projection microscope to perform 600 measurements per one sample is the recommendation (300 measurements per operator). The variation between number of tests and percentage accuracy of the mean is illustrated in the following Table (see Table2.3). This percentage accuracy refers to the upper and lower limits within the bulk mean (LUNNEY, 1983, Andreson, 1955, IWTO, 2011). It has been reported that standard deviation (SD) and coefficient of variation (CV) increases when mean diameter of wool increase (Baxter, 2015, LUNNEY, 1983).

Table 2.3 The effect of measurement number on the percentage of accuracy.

<table>
<thead>
<tr>
<th>Percentage accuracy</th>
<th>Numbers of measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>2500</td>
</tr>
<tr>
<td>2%</td>
<td>600</td>
</tr>
<tr>
<td>3%</td>
<td>300</td>
</tr>
<tr>
<td>5%</td>
<td>100</td>
</tr>
</tbody>
</table>

2.2 Objectives

As mentioned in Chapter1, study the relationship between wool diameters and cortical cells is the aim of this thesis. Thus, wool diameter profile is a primary factor that influence to the rest of interpretation. This chapter is set for characterisation wool samples, especially diameter. Moreover this study is focus on large scale study so the ranges of wool diameters should cover from fine to very coarse wools. The aim of this chapter was to examine the characteristic of sample diameters. In order to achieve this aim the following objectives are introduced:

- Investigation and characterisation mean fibre diameter and diameter distribution of each sample group by projection microscopy technique.
- Wether the diameter of sample covered four diameter ranges according to USDA grade.
2.3 The Methodology of Wool Diameter Evaluation

2.3.1 Wool Samples

In this thesis, standard reference Merino wools top, where wool fibres were collected from many sheep, were used as a representative of keratin fibres. All of the specimens were provided by International Wool Textile Organisation (IWTO). Specimens were passed cleaning, combing, carding and removing short fibres process by IWTO before sending to researcher. Additionally, IWTO classed the specimens into 8 groups according to fibre mean diameter.

There are two sources of Merino wools: New Zealand and Australian Merino wools. New Zealand Merino wools (Set (1)) are the mostly used in the thesis. Thus the profile of mean diameter and fibre dispersion was re-measured again with IWTO - 08: 2011 standard test methodologies in order to confirm IWTO’s data sheet. According to the standard method, the projection microscopy was introduced as a technique for mean diameter measurement. All 8 groups of specimens from Set (1) were coded and divided into 3 subgroup, named a, b, and c as summarised in Table 2.4. 100 of snippet fibre images were randomly selected from each sub-sample. Then they were photographed and measured. This process made 300 measurements in total for each group.

Another set of specimen was Merino wools from Australian (Set (2)). These Australian Merino wools were introduced as a support set of sample in order to extend the scale of sample size. Additionally, the influence sources which might link with nutrition might be examined by compared the results from both sets. Thus, the provided mean diameters of specimen Set (2) from IWTO, were directly used in this study. The mean diameters of Set (2) were summarised in the following Table 2.5.
Table 2.4 Summary of the number of New Zealand Merino wools (Set (1)) specimen for wool diameter measurement.

<table>
<thead>
<tr>
<th>No</th>
<th>IWTO Sample name</th>
<th>Sub-sample name (Numbers of randomly selected snippets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-0</td>
<td>a (100)       b (100)       c (100)</td>
</tr>
<tr>
<td>2</td>
<td>1-1</td>
<td>a (100)       b (100)       c (100)</td>
</tr>
<tr>
<td>3</td>
<td>1-2</td>
<td>a (100)       b (100)       c (100)</td>
</tr>
<tr>
<td>4</td>
<td>1-3</td>
<td>a (100)       b (100)       c (100)</td>
</tr>
<tr>
<td>5</td>
<td>1-4</td>
<td>a (100)       b (100)       c (100)</td>
</tr>
<tr>
<td>6</td>
<td>1-5</td>
<td>a (100)       b (100)       c (100)</td>
</tr>
<tr>
<td>7</td>
<td>1-6</td>
<td>a (100)       b (100)       c (100)</td>
</tr>
<tr>
<td>8</td>
<td>1-7</td>
<td>a (100)       b (100)       c (100)</td>
</tr>
</tbody>
</table>

Table 2.5 Summary of Australian Merino wools (Set (2)) specimen data sheet provided by IWTO.

<table>
<thead>
<tr>
<th>No</th>
<th>IWTO Sample name</th>
<th>Mean diameter (µm)</th>
<th>Type of wool (USDA Grade)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-0</td>
<td>17.61</td>
<td>Fine</td>
</tr>
<tr>
<td>2</td>
<td>2-1</td>
<td>19.24</td>
<td>Fine</td>
</tr>
<tr>
<td>3</td>
<td>2-2</td>
<td>21.34</td>
<td>Fine</td>
</tr>
<tr>
<td>4</td>
<td>2-3</td>
<td>23.74</td>
<td>Medium</td>
</tr>
<tr>
<td>5</td>
<td>2-4</td>
<td>26.54</td>
<td>Medium</td>
</tr>
<tr>
<td>6</td>
<td>2-5</td>
<td>28.47</td>
<td>Medium</td>
</tr>
<tr>
<td>7</td>
<td>2-6</td>
<td>31.50</td>
<td>Coarse</td>
</tr>
<tr>
<td>8</td>
<td>2-7</td>
<td>37.11</td>
<td>Very Coarse</td>
</tr>
</tbody>
</table>
2.3.2 Sample Preparation

Wool fibres are sensitive to humidity and temperature (Wortmann, 2009). Therefore, IWTO-08:2011 standard highly recommends that wool sample should be conditioned in standard atmosphere (65 ± 2% relative humidity and 20 ± 2°C) before starting the measurement process. In this study, one day before the experiment, wool samples were conditioned in standard atmosphere for 8 hours. Fibres were then kept in zip lock plastic bag and placed in a desiccator for overnight. On the day of experiment, samples were placed in standard atmosphere again for 4 hours. After conditioning, the fibres were cut by a blade stamp to produce samples with uniform snippet length and the length can be controlled by the gap between the blades. So, the length of snippet during 1 – 1.5 mm was made by applied this cutting process. Liquid paraffin was used as a mounting medium.

To prepare the slide, a few drops of liquid paraffin were added to a glass slide. The glass slides size 26x76 mm and 1mm thickness were used in this experiment. The wool snippets were then placed onto the slide and a sewing needle were used to disperse the snippets evenly. The snippets were stirred in a circular motion to ensure they were distributed evenly. Next, a coverslip with the size of 24x24 mm with 0.13-0.17 mm of thickness was carefully lower on the preparation slide to prevent the air bubble.

2.3.3 Wool Diameter Measurement Procedural and Data Analysis

Before the experiment, PM was calibrated by stage micrometers. The magnification 200 times was applied and PIA 4000 programmed was used to transfer the images from the microscope screen to computer screen. The slide was moved in the direction as illustrated in Figure 2.2 in the measuring process to avoid remeasuring the same segment of fibre. After the prepared slide was loaded on to the microscope, the projection microscope was adjusted to focus on the top left corner of the coverslip (point A) and the image was transferred to the computer. Next, the slide was moved in Y direction by 1.00mm (point B) and then transversely by 1.00 cm until the edge of coverslip (point C). The slide was then moved transversely in Y direction again.
Each fibre image, which was displayed on computer screen, was focused individually. If both edge of fibre cannot be focused, the focusing should be adjusted to one edge is in focus and the other edge shows a white black line as shown Figure 2.3a.

**Figure 2.2** Path taken across slide during measurement according to IWTO-08: 2011 (IWTO, 2011)

**Figure 2.3** The example of focused restriction of individual fibre a) correct focuses, b) and c) incorrect focus (IWTO, 2011)
Additionally, in order to improve the accuracy of measurement, a number of excursion criteria were introduced. The fibre snippets were excluded from measurement if;

- a. Images with more than half their width outside the central circle or images with widths not wholly within the boundary of measuring field.

- b. Images that end 2.5 cm from the point of measurement.

- c. Images that cross another image within 2.5 mm from the point of measurement.

- d. Images of fibres that are damaged.

Data was analysed using Image J software program. First, scale was calibrated in pixels using the known distance. The known distance was made by PIA 4000 programmed in PM. Next, a straight line was drawn right angle to the width of fibre. Each fibre was measured individually. The measurement of width is made from the focused edge to the inside of the white line. Afterwards, the mean fibre diameter, standard deviation, coefficient of variation, and standard error were calculated.

### 2.4 Results and Discussion

The mean diameters of all samples from Set (1) were summarised in the following Table 2.6. Sample 1-0 has the finest mean diameter, which is 15.70 μm, whereas sample 1-7 has the largest mean diameter of which 37.83 μm. According to USAD grade (see Table 2.1), the diameters of sample Set (1) are cover four ranges of wool diameters (fine, medium coarse and very coarse wools). The majority of wool samples show normally distributed apart from sample 1-1 and 1-3. The most interesting aspect of this Table 2.6 is the positive relationship between mean diameter and standard deviation. Also, similar trend is found in the relationship between mean diameter and fibre distribution. These results are in agreement with previous studies (Baxter, 2015, C. C. Kritzinger, 1964).
Table 2.6 The summary of New Zealand Merino wool (Set (1)) means fibre diameter evaluation by projection microscope under IWTO-8 standard.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Mean (µm)</th>
<th>SD (µm)</th>
<th>CV %</th>
<th>σSE (µm)</th>
<th>Diameter Range [µm] (width)</th>
<th>Most Frequency [µm] (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-0</td>
<td>15.70</td>
<td>1.64</td>
<td>10.44</td>
<td>0.16</td>
<td>9-33 (24)</td>
<td>16(46)</td>
</tr>
<tr>
<td>1-1</td>
<td>18.68</td>
<td>3.62</td>
<td>19.38</td>
<td>0.21</td>
<td>11-34 (23)</td>
<td>18(35),19(35)</td>
</tr>
<tr>
<td>1-2</td>
<td>21.58</td>
<td>2.42</td>
<td>11.19</td>
<td>0.14</td>
<td>12-41 (29)</td>
<td>21(25)</td>
</tr>
<tr>
<td>1-3</td>
<td>24.99</td>
<td>2.69</td>
<td>10.78</td>
<td>0.16</td>
<td>13-43 (30)</td>
<td>21(30), 23(30)</td>
</tr>
<tr>
<td>1-4</td>
<td>26.98</td>
<td>6.15</td>
<td>22.8</td>
<td>0.36</td>
<td>13-45 (32)</td>
<td>26 (26)</td>
</tr>
<tr>
<td>1-5</td>
<td>28.98</td>
<td>7.16</td>
<td>24.7</td>
<td>0.41</td>
<td>12-50 (38)</td>
<td>26 (25)</td>
</tr>
<tr>
<td>1-6</td>
<td>35.62</td>
<td>8.98</td>
<td>25.22</td>
<td>0.52</td>
<td>17-63 (46)</td>
<td>32 (19)</td>
</tr>
<tr>
<td>1-7</td>
<td>37.83</td>
<td>7.38</td>
<td>19.51</td>
<td>0.43</td>
<td>19-63 (44)</td>
<td>37 (22)</td>
</tr>
</tbody>
</table>

A relationship between the mean diameter of the distribution of fibre reveals that larger fibre diameter have greater variation of wool diameter within the group. The widest fibre distribution range is over 40 which are found in Set 1-6 and 1-7 which are classed as coarse and very coarse wools, respectively. Then the distribution decreases to 24 in the fibres with the finest mean diameter (Set 1-0). Further statistical analysis is presented in the following Figure 2.4. A clear distinctive trend of fibre distributions between fine and coarse wools is seen when the histograms of the largest and the smallest mean diameter are compared. On the right hand side, coarse wool shows the highest degree of variability in wool diameter. The mean diameter of the coarsest wool is 37.83 µm however; the coarsest fibre from the same sample can have a diameter of up to 63µm. The opposite trend of fibre distribution is presented on the left hand side which is fine wool. This profile of fibre distribution might be used for further investigation to explain the reasons behind higher standard deviation of coarser wools results. Moreover, the higher standard deviation of results might relate with nonhomogeneous of wool sample.
Figure 2.4 Comparison of fibre distributions between sample 1-0 (finest mean diameter) and sample 1-7 (coarsest mean diameter).

As mentioned before, the mean diameter of sample Set (2) obtained from the IWTO’s data sheet. Similar with Set (1), Set (2) consist of the four ranges of wools. A comparison between Table 2.5, which is Set (2) mean diameter and Table 2.6 reveals that the mean diameter of each group from sample Set (2) is similar with Set (1). Thus, it could be assumed that the characteristic of fibres distribution in Set (2) is similar with fibre Set (1). The diameter of wool fibre is more diverse in coarser wools compared to fine wools. The mean diameters of specimens from Set (1) and (2) are summarised following Table 2.7. In addition, all 16 groups of specimens were systematically coded. The first digit in bracket refers to the source of Merino wools; (1) is New Zealand, and (2) is Australia Merino wool while the second digit reflects to the mean diameter.
Table 2.7 The summary of mean fibre diameters and the sample code from both sets of specimens.

<table>
<thead>
<tr>
<th>No</th>
<th>Sample Name</th>
<th>Mean diameter (µm)</th>
<th>Sample Code</th>
<th>Type of wool (USDA Grade)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-0</td>
<td>15.7</td>
<td>Set(1,16)</td>
<td>Fine</td>
</tr>
<tr>
<td>2</td>
<td>2-0</td>
<td>17.61</td>
<td>Set(2,18)</td>
<td>Fine</td>
</tr>
<tr>
<td>3</td>
<td>1-1</td>
<td>18.68</td>
<td>Set(1,19)</td>
<td>Fine</td>
</tr>
<tr>
<td>4</td>
<td>2-1</td>
<td>19.24</td>
<td>Set(2,19)</td>
<td>Fine</td>
</tr>
<tr>
<td>5</td>
<td>2-2</td>
<td>21.34</td>
<td>Set(2,21)</td>
<td>Fine</td>
</tr>
<tr>
<td>6</td>
<td>1-2</td>
<td>21.58</td>
<td>Set(1,22)</td>
<td>Fine</td>
</tr>
<tr>
<td>7</td>
<td>2-3</td>
<td>23.74</td>
<td>Set(2,24)</td>
<td>Medium</td>
</tr>
<tr>
<td>8</td>
<td>1-3</td>
<td>24.99</td>
<td>Set(1,25)</td>
<td>Medium</td>
</tr>
<tr>
<td>9</td>
<td>2-4</td>
<td>26.54</td>
<td>Set(2,27)</td>
<td>Medium</td>
</tr>
<tr>
<td>10</td>
<td>1-4</td>
<td>26.98</td>
<td>Set(1,27)</td>
<td>Medium</td>
</tr>
<tr>
<td>11</td>
<td>2-5</td>
<td>28.47</td>
<td>Set(2,28)</td>
<td>Medium</td>
</tr>
<tr>
<td>12</td>
<td>1-5</td>
<td>28.98</td>
<td>Set(1,29)</td>
<td>Medium</td>
</tr>
<tr>
<td>13</td>
<td>2-6</td>
<td>31.5</td>
<td>Set(2,32)</td>
<td>Coarse</td>
</tr>
<tr>
<td>14</td>
<td>1-6</td>
<td>35.62</td>
<td>Set(1,36)</td>
<td>Coarse</td>
</tr>
<tr>
<td>15</td>
<td>2-7</td>
<td>37.11</td>
<td>Set(2,37)</td>
<td>Very Coarse</td>
</tr>
<tr>
<td>16</td>
<td>1-7</td>
<td>37.83</td>
<td>Set(1,38)</td>
<td>Very Coarse</td>
</tr>
</tbody>
</table>
2.5 Conclusion

This chapter was set in order to characterise the sample diameter and distribution. Two sets of Merino wool were used in this study. Set (1) is New Zealand Merino wools, which is the mostly used in the thesis. Australian Merino wools (Set (2)) were used as a support specimen. Each set of sample was classed into 8 groups according to fibre mean diameter. The mean diameters of Australian Merino wools provided by IWTO data sheet were directly used. On the other hand, the mean diameters of New Zealand Merino wools were re-measured and characterised. Projection microscopy technique was selected as a measurement method, in accordance to IWTO-8 standard. Other measurement conditions such as number of snippet images and solution medium were controlled strictly according to IWTO-8 standard.

The results show that both sets of samples have similar range of mean diameters. Mean diameters of wool samples from New Zealand range from 16 -38 µm while Australian Merino wools show mean fibre diameter during 18 – 37 µm. In These ranges of diameter cover four ranges of wool diameter according to USDA grade including fine, medium, coarse and very coarse wool. The majority of sample is in fine and medium ranges of wool diameter. Additionally, fibre characterisation also reveals a positive relationship between mean fibre diameter and fibre distribution profile. Thus, coarse wools tend to show more diverse of wool diameters than fine wools. At the end of this chapter all 16 groups of samples were codes. The code reflects the sources of wool and the mean diameter of each group.
2.6 References

ANDRESON, S. L., DANIELS, R. C., WILDMAN, R. C. 1955. fibre measurement. Wool research testing and control UK: Wool industries research association


IWTO 2011. METHOD OF DETERMINING FIBRE DIAMETER DISTRIBUTION PARAMETERS AND PERCENTAGE OF MEDULLATED FIBRES IN WOOL AND OTHER ANIMAL FIBRES BY THE PROJECTION MICROSCOPE. IWTO.


Chapter 3 Thermal Analysis of Wools

Thermal analysis (TA) is “the study of the relationship between sample properties and its temperature as the sample is heated or cooled in a controlled manner” (Lever et al., 2014). There are many TA techniques such as thermogravimetry (TG), differential thermal analysis (DTA), thermomechanical analysis (TMA), dynamic mechanical analysis (DMA) and evolved gas analysis (EGA). One of the most widely used TA techniques is Differential Scanning Calorimeter (DSC) (Haines, 2012, Lever et al., 2014). In a brief review the DSC principle and its applications in keratin fibres, especially wool, will be explained.

3.1 Differential Scanning Calorimetry (DSC)

3.1.1 The Principle of DSC

DSC is a significant technique for TA analysis because of its advantages. This technique requires small amount of sample and supports many forms of samples. Moreover, the sample preparation process is not complicated. Additionally, a fast measurement process and comprehensive data are the biggest benefit from this technique. This technique can be employed for the determination of changing heat capacities, transition temperatures, and enthalpies. In addition, the phase transition of materials including melting, glass transitions, phase changes, and curing can be investigated (Gabbott, 2008).

According to the International Committee for Thermal Analysis (ICTA) nomenclature committee, DSC is described as a thermal technique “in which the heat flux (power) to the sample is monitored against time or temperature while the temperature of the sample, in a specified atmosphere, is programmed” (Groenewoud, 2001). This refers to the principle of DSC. A samples and a reference, which is empty pan, are heated, cooled or held isothermally. Then the differences of heat flow changing in both pans are measured. Heat capacities are observed as changes in the heat flow. The DSC curve is the output data from this technique (Gabbott, 2008, Lever et al., 2014).
A typical or conventional DSC is classified into two systems according to a number of furnaces. A heat flux DSC, which is used for this research, is a single furnace design. On the other hand, a power compensation DSC has two small identical furnaces, one for a sample and another for a referent pan. Moreover, a power compensation DSC varies energy input in order to maintain less temperature difference (nearly zero) while constant rate of heating input is the working procedure of the heat flux DSC. Thus, a difference of temperature arises during the operation of heat flux DSC (Groenewoud, 2001, Bunjes and Unruh, 2007, Gabbott, 2008). The difference of these two DSC designs is shown in Figure 3.1.

![Figure 3.1 The schematic diagrams of different DSC designs: heat flux DSC (Top) and power compensation DSC (bottom) (Bunjes and Unruh, 2007)](image)

A modulated temperature differential scanning calorimeter (MDSC or MTDSC) is an improved method to overcome the limitations of traditional DSC. The limitations of standard DSC increase when the thermograms contain complex or weak transitions due to the difficulty of the heat flow interpretation. Therefore MDSC has been introduced to increase the sensitivity of measurements. In MDSC, two simultaneous heating rates are introduced, while only one heating rate is conducted in traditional DSC. As illustrated in Figure 3.2, a first heating rate is linear, which is similar to conventional DSC. A second heating rate is a sinusoidal moderation on top of a constant heating rate (Gill et al., 1993, Verdonck et al., 1999, Knopp et al., 2016).
In MDSC, two measurements are made; average and amplitude. First, similar to conventional DSC, the average value of the modulated heat flow signal is calculated. This signal is associated with the total heat flow of all thermal events in a sample. At the same heating rate, this value is equivalent with the heat flow from conventional DSC. The yield of this heat flow is referred as total heat capacity ($C_p^T$) which is a function of temperature according to Equation 3.1. Another measurement is the heat flow amplitude which is only created in MDSC experiment.

$$C_p^T = \frac{1}{(\beta m)} \frac{dQ}{dt} \tag{3.1}$$

When, $\beta = \frac{dT}{dt}$ is the heating rate

$t = \text{time}$

$T = \text{absolute temperature}$

$m = \text{sample mass}$

$\frac{dQ}{dt} = \text{heat flow}$
In Figure 3.3, the raw data of modulated heat flow and modulated heating rate signals are shown. The heat capacity of MDSC is calculated from the amplitude of these two signals by the Fourier Transform analysis.

![Figure 3.3](image)

**Figure 3.3** The raw data signals of modulated heating rate and modulate heat flow from MDSC for quench cooled PET (Verdonck et al., 1999)

Reversing and non-reversing heat flows are the additional signals that are obtained after the total signal is separated (see Figure 3.4). The reversing heat flow is the fraction of the total heat flow while the non-reversing heat flow is the different signal that obtained after subtracted the reversing heat flow from the total heat flow signal. The correlation of these three signals is simplified and shown in equation 3.2 (Schawe and Höhne, 1996);

\[ C^T_p = C^R_p + C^NR_p \]  

(3.2)

When, \( C^T_p \) = total heat capacity

\( C^R_p \) = reversing heat capacity

\( C^NR_p \) = non-reversing heat capacity
The result of MDSC are three signals: total, reversing and non-reversing heat flow (HDPE:PC:PENT blend) (Verdonck et al., 1999)

The non-reversing heat flow reflects kinetic components such as enthalpy relaxation, crystallization, evaporation and decomposition. Moreover the benefit of MDSC is a baseline, which is \( C_p^R \) underlying the total heat capacity curve. Therefore, only the non-reversing signal will be focused in this study. Non-reversing heat capacity reflects the endothermic peak of thermal denaturation. Peak of the cure reflect the denaturation temperature \( T_D \). The area under this curve or denaturation enthalpy \( \Delta H_D \) is determined by applied linear baseline from the start \( T_1 \) and the end temperature \( T_2 \) of the peak. This denaturation enthalpy is summarised following equation 3.3 (Gill et al., 1993, Schawe and Höhne, 1996, Verdonck et al., 1999).

\[
\Delta H_D = \int_{T_1}^{T_2} C_p^{NR} dT = \int_{t_1}^{t_2} C_p^{NR} \beta dt
\]

(3.3)
3.1.2 Thermal Stability of Proteins and Keratin Fibres

Protein fibres have complex structures of polypeptides chains. When these structures are affected by heat, their structures are lost and stability is poor as a result of chemical bond breaking and re-arrangement of protein structures. This phenomenon is called denaturation. Less ordered arrangement, increasing flexibility and more open polypeptide structures are examples of the results of protein denaturation. Interestingly, the covalent polypeptide backbones of proteins are not rearranged in this process. Only the hydrogen bonds are broken or rearranged in a less-order form. This denaturation is simplified and illustrated in Figure 3.5 (Bischof and He, 2005).

![Figure 3.5](image.png)

**Figure 3.5** The simplified descriptive diagram of the denaturation process within one protein of α-helical secondary structure; the nature stage (left) with hydrogen bonds and polypeptide backbone. The denature stage (right) with unravelled of polypeptide backbone and broken hydrogen bonds (Bischof and He, 2005).

Compared to other proteins, the fibrous group of hard α-keratin, which is found in wool and human hairs, can withstand higher temperature than others, so the denaturation temperature of α-keratin fibre is higher. It has been reported that the denaturation of α-helical soluble proteins was found around 100°C (F. J. Wortmann et al., 2012). In the context of wool fibres, the denaturation process is the associated with unfolding of α-helical material in the IFs to random coils. However, the sulphur bond content in the matrix has an influence on the unfolding of the IFs because this disulfide bond density controls the viscosity of matrix around the IFs. Therefore it kinetically prohibits the denaturation process in the IFs (F. J. Wortmann and Deutz, 1993, Bischof and He, 2005, F. J. Wortmann et al., 2012).
Activation energy (kinetic) and enthalpy (total heat absorption or release) are the energies involved in the denaturation process. The denaturation can occur when energy input overcomes the activation energy. Therefore, temperature and time are influencing factors of this process. The process can, in principle, be both of exothermic or endothermic. In case of regeneration such as coagulation, aggregation and gelation, the energy state after denaturation is lower than the initial state. In contrast, a higher energy state is investigated, when an endothermic reaction occurs. This process can be reversible or irreversible. Irreversible processes of proteins fibres occur more often when the temperature is above 45ºC (Bischof and He, 2005). The summary of thermodynamic diagram of denaturation process is illustrated in Figure 3.6.

**Figure 3.6** Energy states of protein a denaturation process (Bischof and He, 2005)
3.1.3 DSC Investigation of Keratin Fibres

DSC is one on the well-known thermal analysis techniques that has been used for investigation various keratin materials including wool fibres. Different morphologies and damage of keratin fibres can be investigated by DSC. Various results from DSC analysis have been achieved by different methods in the experiments. The effects of ambient condition have been reported by many researchers. Dry stage and wet stage DSC are the two major approaches of the DSC measurement, that have been employed for α-keratin fibres especially wools and hairs (Jinan Cao et al., 1997, Popescu and Gummer, 2016, Lima et al., 2018)

3.1.3.1 Dry- and Wet-DSC Investigation

Under the dry condition of DSC, the water are evaporated with the raising of temperature. On the other hand, DSC under the wet condition allows fibres to absorb water before the experiment which is maintained during the heating (Lima et al., 2018). The difference of the DSC results from these two methodologies is the position of denaturation peaks. In wool fibres, although the positions of the denaturation peaks between the two approaches are different, both of them tend to show a bimodal endothermic peak due to the difference of cross-link density between ortho- and paracortical cell (F. J. Wortmann and Deutz, 1993, Cao, 1997, F. J. Wortmann and Deutz, 1997, Lima et al., 2018). For hair fibre, numerous studies applied DSC in the wet state to determine the effects of cosmetics on cellular structure and hair repair, while the effect of daily thermal hair styling tool was investigated by dry-DSC (Popescu and Gummer, 2016, Lima et al., 2018)

The dry-DSC investigation of Lincoln wool was studies by Spei and Holzem (1987) and the results showed the first peak during 230-240°C followed by the second peak at 250-260°C (Spei and Holzem, 1987). However, dry-DSC investigations tend to show unclear results because the pyrolysis reaction after the evaporation of water. This pyrolysis occurs around 210-250°C, which is the same range as the denaturation peak (F. J. Wortmann et al., 2002). According to the drawback of DSC in dry state, therefore, DSC investigation in wet condition was applied. The effect of water on the reduction of the denaturation temperature was shown by Wortmann and
Deutz (1993). The effect of amount of water was further confirmed by Cao et al (1997). In this study, silicone oil was applied on merino wool before the experiment in order to keep a certain amount of water in fibres. The lower endothermic peaks were investigated, which were located at 170°C and 185°C respectively, because water in the fibres was preserved (Cao, 1997). Up to now, several studies have detected the decreased of endothermic peak temperatures of wool fibres from approx. above 200 °C in dry stage to 130-150°C in wet stage DSC (Haly and Snaith, 1968, F. J. Wortmann and Deutz, 1993, F. J. Wortmann et al., 2007, F. J. Wortmann et al., 2012). The reason behind is that water acts as a plasticiser in the matrix. Additionally, DSC of wool in water was found to be a better procedure to analyse the morphological components (F. J. Wortmann et al., 2007).

3.1.3.2 The Connection between DSC Results and the Cellular Structure of Keratin Fibres

According to Feughelman’s (1959) model, generally, the morphology of wool and hair is two-phases, which consist of a crystalline and an amorphous phase. The crystalline region consists of α-helical intermediate filaments (IFs). These IFs are embedded in an amorphous region which is a matrix of IF-associated proteins (IFAP), (Feughelman, 1959, F.J. Wortmann, 2009). Commonly, the transition and thermal stability in the cortex can be investigated by thermal analysis (F. J. Wortmann et al., 2012). Therefore, there are numerous thermal analysis studies including DSC that focus on the denaturation process (Sparrow et al., 1988, F. J. Wortmann and Deutz, 1993, F. J. Wortmann et al., 2002). By applying MDSC, the non-reversing heat flow reflects the denaturation process of wool fibres. The peak location of the DSC curves is the denaturation temperature while the area under the DSC peak is the denaturation enthalpy (Wortmann, 2009).

The effect of the viscosity of the amorphous matrix material on the denaturation temperature by DSC investigation has been investigated (F. J. Wortmann and Deutz, 1997). A strong increased of denaturation temperature when the cross link density of the matrix increases has been reported. Furthermore, the inter-relation between denaturation temperature and cross link density is described by linear model. This mean the denaturation temperature is attributed to
the disulphide linkage or cysteine content in the matrix (F. J. Wortmann and Deutz, 1997, F. J. Wortmann et al., 2007). A decrease of peak temperatures may indicate structural changes and chemical damage, which are associated with the extension or break of disulphide and hydrogen bonds (Popescu and Gummer, 2016). This point is supported by the fact that the denaturation temperature of hair increases when hydrogen bonds are stabilized in an acid environment. On the other hand decreasing denaturation temperatures were observed when hydrogen bonds were ruptured in alkaline treatments (Istrate et al., 2013, Torres et al., 2013).

Further studies of the connection between denaturation enthalpy and cellular structural in the cortex showed that the unfolding of α-helical material in the Ifs, is probably reflected by the denaturation enthalpy. However, the correlation between denaturation enthalpy and α-helixes is not clear. Therefore, denaturation enthalpy is material invariant (F. J. Wortmann and Deutz, 1993, F. J. Wortmann et al., 2002, Monteiro et al., 2005). Similar to the denaturation temperature, the denaturation enthalpy increases in acidic environment while it decreases in alkaline environment. This result is supported the fact that the amorphous matrix kinetically controls the denaturation process (Istrate et al., 2013). Concluding, previous research has established that the denaturation temperature is kinetically controlled by cross linkages density of the matrix while the helix-coil transition in the Ifs is related to the denaturation enthalpy.

3.1.3.3 DSC Peak Separation and Deconvolution Hypotheseses

DSC peak separation is an important process in DSC results interpretation. Up to now, there are two predominant hypotheses of peak separation, which are the helix/matrix and the ortho/para-cortical hypothesis (Spei and Holzem, 1987, Haly and Snaith, 1968, Hole, 1985, Crighton, 1990). The helix/matrix hypothesis attributes the first peak, which is located at lower temperature, to α-helical denaturation while the second peak reflects the matrix denaturation (Spei and Holzem, 1987). The ortho/para hypothesis, however, attributes the lower temperature peak to the denaturation in orthocortical cells while the denaturation of paracortical cells is related to the higher temperature peak or shoulder (Haly and Snaith, 1968).

The first evidence that supported the ortho/paracortical cells hypothesis is the difference of peak temperatures. The temperatures are around 4-7°C apart due to differences in sulphur
content between paracortical and orthocortical cells. Paracortical cells have a higher content of disulphide bonds than orthocortical cells, so denaturation temperature of paracortical cells is higher than orthocortical cells (F. J. Wortmann and Deutz, 1993). The ortho/para hypothesis is further confirmed by Deutz (1997). Ortho- and paracortical cells were isolated from merino wool and examined by DSC in water. They reported that the denaturation peak of isolated orthocortical cells was located at 138°C while isolated paracortical cells showed a denaturation peak at 144°C (F. J. Wortmann and Deutz, 1993). The higher denaturation temperature of paracortical cells can be explained by the macrofibrils structure. Paracortical cells consist of higher proportion of matrix, which is rich in disulphide bones content than orthocortical cells (Maclaren and Milligan, 1981).

Recently, the ortho/para hypothesis was applied by Tonin et al. (2004). The effect of industrial processes on structural modification of wool was explained (Tonin et al., 2004). Furthermore, this hypothesis was applied for DSC peak deconvolution (Zhang, 2013).

As mention before, keratins fibres are two-phase materials. Their DSC denaturation curves overlap each other especially wool fibres. The DSC peak deconvolution processes have been applied in order to separate this overlapping. Moreover, the fraction of ortho- and paracortical cell groups can be estimated by this process (F. J. Wortmann and G. Wortmann, 2018). Freire et al. (1978) reported the usefulness of the deconvolution technique to study thermodynamic transitions in macromolecules. The correlation between the thermodynamics protein stability and structural properties of the macromolecule can be investigated by this process. Therefore the DSC deconvolution was applied for many forms of proteins, including hard α-keratin like hair and wool fibres. (Freire and Biltonen, 1978a, Freire and Biltonen, 1978b, Murphy and Freire, 1992, Toledo-Nunez et al., 2016).

Not surprisingly, several methods have been developed for keratin peak deconvolution. In case of wools and hairs which consist of helical proteins in IFs, the Gaussian function is applied to fit the DSC curves (Zhang, 2013). The reason behind this is that this function can fit the bell-shape of the first derivative result from a sigmoid conversion curve, which is the assumption for α-helical proteins deconstruction. Additionally, the deconvolution analysis approach and peak indication are based on the results from optical analysis such as electron microscope and staining under the light microscope. Two peak separations were applied for alpaca fibres while the
assumption of three peaks was introduced for hair and wool fibres (Shim, 2003, Zhang, 2013, F. J. Wortmann and G. Wortmann, 2018). In a recent review on wool DSC deconvolution, F. J. Wortmann has pointed out the possibility of nonexistence of mesocortical cells. The three peak separation hypothesis was still applied. The middle and the third peak are identified as the fraction of orthocortical cell fraction and paracortical cell. However, the first peak which is located at lowest denaturation temperature is identified as a lower sulphur sub group of ortho-type cells. The peak interpretation is based on the electron microscopic analysis from Bryson W. et al. (2009) (Bryson et al., 2009, F. J. Wortmann and G. Wortmann, 2018).

### 3.2 Objectives

In Chapter 3, MDSC in wet conditions was applied. The thermal analysis is performed on Merino wool top of various diameters and obtained from different sources. The aim of this study is to study the relationship between thermal properties and wool diameter. In order to achieve this aim, the following objectives are introduced:

- Investigation of the relationship between wool diameter and thermal properties: denaturation temperature and enthalpy.
- Estimation of cortical cell percentage by MDSC peak deconvolution, including consideration whether deconvolution is a feasible approach for quantitative analysis of cortical cells.
- Determination of the relationship between mean fibre diameters and MDSC deconvolution results in both quantitative and qualitative analysis.
3.3 Experimental

The top wools from both sets were investigated by DSC. All DSC investigations were conducted on a modulated heat flux DSC (DSC Q10, TA-Instruments). Pressure-resistant (25 bar) stainless-steel large-volume capsules were used in this study. The experiments were carried out under excess water conditions in order to eliminate the effect of pyrolysis (F. J. Wortmann et al., 2002). Firstly, specimens were stored overnight under standard room condition (20°C, 65% relative humidity) to ensure invariant water content. Then, fibre snippets were prepared using an electric shaver. After that, snippets of wool were weighed at 5-7 mg into large volume capsules followed by adding 40 µL of distilled water. The capsules were sealed with lids containing O-ring rubber seals using the sample encapsulating press. At least 3 capsules per sample were prepared. Finally, capsules were left at ambient room temperature overnight before measured by DSC in order to reach equilibrium water content and distribution. All of DSC measurements were conducted with a 3°C/min heating rate and a modulated heating rate of 1°C/min with a temperature range 80-180°C. An empty capsule was applied as a reference pan.

The corresponding heat flow was recorded and DSC raw data were analysed by using the TA Instruments Universal Analysis 2000 Program. There are two data analysis approaches which will be described in the following parts.

3.3.1 DSC Thermal Analysis of Virgin Wool in Water

The typical MDSC curves were processed by TA universal analysis. MDSC curves show total, non-reversing and reversing heat capacity. In order to determine denaturation temperature and enthalpy, only the non-reversing curve was considered. As shown in Figure 3.7, a baseline which is drawn from start to the end of the peak is required. From this curve, denaturation temperature can be directly found from the peak while denaturation enthalpy is determined by the calculation of area under the peak. To ensure the reproducibility of the DSC data, each sample was repeated between 3 to 10 times, depending on the complexity and reproducibility of the DSC curve. Further data analysis included mean, standard deviation and standard error were calculated using Equations 3.4a - c, respectively.
- Mean:

\[ \bar{X} = \frac{1}{n} \sum_{i=1}^{n} x_i \]  

(3.4a)

With \( x \) = measured values

\( n \) = number of measurements

- Standard Deviation:

\[ \sigma = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}} \]  

(3.4b)

- Standard Error:

\[ \sigma_{SE} = \frac{\sigma}{\sqrt{n}} \]  

(3.4c)

Figure 3. 7 Example for MDSC non-reversing heat capacity curve analysis in “TA Instruments Universal Analysis 2000” showing results of the denaturation temperature (T_D) and the denaturation enthalpy (\( \Delta H_D \)) of wool set (1,16).
3.3.2 DSC-Deconvolution of Merino Wools

The MDSC curves in water were collected for Merino wool with various mean diameters. The same MDSC data processing as described in section 3.3.1 was applied as the first step. In order to separate the overlapping peaks, a deconvolution process was introduced. According to the well-established deconvolution procedure from previous work, the Gaussian function and model-base boundary conditions were applied in this study (Freire and Biltonen, 1978a, Zhang, 2013, F. J. Wortmann and G. Wortmann, 2018). As mentioned before, the thermal denaturation of α-helical proteins is assumed to follow a sigmoid conversion curve for which a bell-shaped curve is the first derivative. Hence, the Gaussian distribution function is suitable against this background.

Similar to previous analyses, only the non-reversing heat capacity curve was used for the deconvolution process (see Figure 3.8a). Afterward, the DSC baseline was corrected to fit into the Gaussian curves by defining the temperature at the start and the end of the denaturation process as shown Figure 3.8b. Then, the DSC data from TA universal analysis program was transferred to Excel software.
Figure 3.8 DSC curve for untreated Merino wools in water at capacity MDSC curve of wool in water (Sample Set (1, 16)). a) the typical non-reversing heat analysis in “TA Instruments Universal Analysis 2000”. b) Base line correction process on the $C_p^{NR}$ curve between the two chosen points; determination of starting (T1) and ending (T2).
Figure 3.9a illustrates the new curve which was created by subtracting the non-reversing heat capacity with its new slope of baseline. After this MDSC-curve preparation process, the SOLVER-function of Excel (Microsoft) with least-squares criterion was introduced as the approach for the deconvolution of all fits. The principles underlying this approach are discussed by Brown (Brown, 2001). As mention before, the principle behind this peak separation is the Gaussian function which is given in Equation 3.5. The heights, peak positions and width of each peaks for three peaks, result in nine parameters.

\[ f(x) = ae^{-\frac{(x-b)^2}{2c^2}} \]  

(3.5)

When  
   a is the height of peak  
   b is the centre position of the peak  
   c is the width of the curve

A novel method was developed to deal with the identification of boundary conditions of the deconvolution. According to the microscopy investigation (TEM), it is generally agreed that Merino wool cortex consists of three types of cortical cells, which is ortho-, para-, and mesocortical cells. This result was applied for the first restriction. The three sub-curves fitted by deconvolution were imposed. The second restriction was introduced under the assumption that the helical material in the IFs of all cortical cell types has the same denaturation processes. The similar shape of deconvolution cures is the result due to this assumption. This means all of Gaussian peaks have the same standard deviation or the width of curve. The third restriction is related to the position of the peak and cortical cell type. According to the sulphur content, the highest denaturation temperature peak is associated with the highest sulphur content in cortical cells, which is paracortical cells followed by orthocortical cells and the lowest denaturation are the unassigned. The final restriction is based on Orwin (1984) work. They reported that orthocortical cells are the majority component in the cortex (Donald F.G. Orwin et al., 1984). Therefore, the proportion of orthocortical cells should be higher than paracortical cells, resulting in the restriction of higher height of second peak than the third peak. The final result of MDSC deconvolution under all of these restrictions is shown in Figure 3.9 b.
Figure 3.9 Summary of deconvolution process of the non-reversing heat capacity curve ($C_p^{NR}$) into three Gaussian distributions (Set (1, 16)). a) Result of the corrected MDSC peak, b) Result of the MDSC-curve deconvolution into three Gaussian distributions each peaks are assigned to the three types of cortical cells.
3.4 Results and Discussion

Denaturation of wool is an irreversible process. This process depends on components in the cortex of wools. Therefore, the measurement of wool thermal stability is related to the cellular structure of wool fibres. In this study, the non-reversing heat capacity was used to determine the denaturation temperature ($T_D$) and denaturation enthalpy ($\Delta H_D$). The peak of this curve indicates $T_D$ and the peak area with the determined baseline indicates $\Delta H_D$. The results in this chapter are divided into two parts. The first part will focus on the relationship of wool diameter to the denaturation temperatures and the denaturation enthalpy. Another part will show the results of the MDSC-curve deconvolution. In this part, deconvolution is employed to investigate whether cell composition of Merino wools can be resolved into the three Gaussian distributions. Further discussion will focus on the relationship between deconvolution results and mean wool diameters.

3.4.1 DSC on Wool of Various Diameters in Water

The relationship between wool diameters and denaturation temperatures from wet stage DSC is shown in Figure 3.10. In this graph, the results of both sets of samples are compared. It shows that the range of denaturation temperatures for both sets is during 135°C to 140°C. The highest denaturation temperature of Set (1) (139.5°C) is observed for the largest mean diameter wool. For Set (2), the highest denaturation temperature is observed for the largest mean diameter, which is 140.2 °C. A similar denaturation temperature is also observed for the smallest mean diameter. This result might indicate chemical differences between the two sets of specimens.

When the denaturation temperatures of both sets are compared, the results further confirm that both sets of samples have their own characteristics. Though samples in Set (1) and (2) have similar mean diameters, the denaturation temperature is different. Poor relationship between denaturation temperature and wool diameters are found for both sets. This finding is contrary to previous studies which suggested that coarser wool tends to have higher denaturation temperatures because of higher proportions of paracortical cells. These paracortical cells contain higher cross link densities of disulfide bonds that increase the denaturation temperature of

Figure 3.10 The relationship between denaturation temperature and wool diameters. The data present mean denaturation temperature with standard error for Merino wool set (1) and (2) at various mean diameters.

Interestingly, fine and coarse wools present different relationship between denaturation temperature and diameter. More distinctive positive linear relationship is observed in coarser ranges of diameters (see Figures 3.11). This relationship is consistent with previous study. The linear correlation coefficient ($R^2$) of Set (1) increases from 0.29 to 0.91 after the separation of fine wools. The DSC results of Set (2) also present the similar trend. The results of coarse and very coarse wool suggest that very coarse wools tend to consist of higher Sulphur content. Both sets of sample show lowest denaturation temperature at the similar diameter range. During 24-29 µm the denaturation temperatures from both sets drop to the lowest point, then the temperature rebound at finer and coarser ranges of wool diameters. In fine wools, more fluctuation is observed. In Set (2), the relationship between the denaturation temperatures and wool diameters seems to be a negative relationship. Unlike the relationship from Set (1), the almost constant relationship is observed. This variation of relationship might suggest that finer wools are rich in sulphur content like very coarse wools. However, cortical cells structure might involve in this difference.
Figure 3.11 The linear relationship between denaturation temperature and diameter of coarse and very coarse wools. The mean values of denaturation temperatures were presented with standard error a) Set (1), b) Set (2).

The correlation between wool diameters and denaturation enthalpy is also studied. In Figure 3.12, the relationship between denaturation enthalpy and wool diameter was illustrated. This graph indicates that there is not significant change between denaturation enthalpy and wool diameters. In addition, both sets of samples show similar trend. Unlike the denaturation temperature, coarser wools are not present any obvious relationship between denaturation temperature...
enthalpy and wool diameters. Hence, the denaturation enthalpy of specimens is a material invariant. This result is good agreement with previous works from F. J. Wortmann (F. J. Wortmann and Deutz, 1997, F. J. Wortmann et al., 2002). Other keratin fibres such as hair, alpaca and horn also support that the denaturation enthalpy is material independent properties (F. J. Wortmann and Deutz, 1993, Shim, 2003).

![Figure 3.12](image)

**Figure 3.12** The relationship between denaturation enthalpy and wool diameters of Merino wool. The data presented mean value with standard error.

### 3.4.2 DSC Peak Deconvolution of Untreated Merino Wools

In this study, the deconvolution results are interpreted according to the ortho/para hypothesis along with the microscopic analysis results from previous studies. The MDSC curves are obtained from different mean diameters of Merino wools. The MDSC peak deconvolution is conducted using Gaussian distributions. Three sub-curves with equal width (standard deviation) are assumed for deconvolution. The MDSC deconvolution curves have excellent fits, which is reflected in the coefficients of determination, $R^2 > 0.98$ (Freire and Biltonen, 1978a, Zhang, 2013, F. J. Wortmann and G. Wortmann, 2018). The denaturation temperatures and fractional peak
areas, which are related to the amounts of α-helical in each cortical cell types, can be determined by the deconvolution process.

### 3.4.2.1 The Deconvolution Curve Analysis

In the previous studies by Zhang (2013), mesocortical cells were counted as a third component in the cortex, while, in this study, only paracortical cells and orthocortical cells are identified as the main components. The significant assumption for this deconvolution interpretation is based on microscopic analyse and the ortho-para hypothesis. All microscopy studies have supported this due to ortho- and paracortical cells being the major cell fractions in wool. This supports the observation of bimodality or a shoulder to the high temperature side in the DSC curve of wool (Horio and Kondo, 1953, Donald F.G. Orwin et al., 1984, F. J. Wortmann and Deutz, 1997, F. J. Wortmann et al., 2012). According to the TEM results, a mesocortical cells type is classified as the third minor cell fraction in the cortex. This cell type is classified as a low-sulphur version of paracortical cells. However, the clear definition of mesocortical cells and differentiation between mesocortical cells and the other two cortical cells could not be shown (Whiteley and Kaplin, 1977, Bryson et al., 2009, Thomas et al., 2012). In addition, the position of the curves is the denaturation temperature which relates to the disulphide content of each component. Therefore, paracortical cells should be located at the highest denaturation temperature followed by meso- and orthocortical cells, respectively. It can be seen in the following Figure 3.13 that the highest denaturation peak (peak III) represents the paracortical cells or para-cell type (P). According to light microscopic investigations, orthocortical cells are found to have the largest fractional area; subsequently the peak II represents the orthocortical cells (Donald F.G. Orwin et al., 1984). If mesocortical cells are distinctly separate enough, mesocortical cells should be located between these two curves. However, this deconvolution cannot further confirm the present of mesocortical cells as a distinctive cortical cell types.

Recently, four types of cortical cells were reported (Type A, B, C and D) (Bryson et al., 2009). They reported that Type A and B are similar to orthocortical cells, while Type C and D are similar to paracortical cells. Cell Types B and C are the majority of cortex. It can be said the Type A and D are the ortho-like and para-like cortical cell, respectively. The minority of cortex is Type
A, while Type D cells were the least common cell type (Bryson et al., 2009). This finding leads to the peak interpretation that the peak with lowest denaturation temperature (Peak I) might correspond with cell Type A, which is Ortho-like cortical cell. So the middle peak (Peak II), which is the highest fractional area, associates with cell Type B. and Peak III, which has highest denaturation temperature represent the paracortical cells (P). Therefore, the percentage areas of orthocortical cells (O) are represented by the two curves; included ortho1-cell type (O1) and ortho2-cell type (O2) (see Figure 3.13). This interpretation is also in line with the fact that orthocortical cells are the majority component in the cortex of merino wool (F. J. Wortmann and G. Wortmann, 2018). The results of MDSC peak deconvolution are shown in Table 3.1 and 3.2.

![Figure 3.13 DSCw curves in water for Merino wool; Set (1, 16). The curves are deconvoluted using three Gaussian distributions of equal width (standard deviation). For each of the peaks, labelled as I - III with increasing temperature. Each peak represented different cortical cell types. Paracortical cells are located at Peak III followed by ortho2 and ortho1. The areas under the curves imply cortical cell percentages.](image-url)
### Table 3. 1 Denaturation temperatures (T\textsubscript{D}) for the assumed three components of the DSCw curves in various diameters, as determined through curve deconvolution under Gaussian equation. Coefficients of determination are in all cases R\textsuperscript{2} > 0.9.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Mean O1</th>
<th>Standard Error of O1</th>
<th>Mean O2</th>
<th>Standard Error of O2</th>
<th>Mean P</th>
<th>Standard Error of P</th>
</tr>
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<tr>
<td>Set(1,16)</td>
<td>130.1</td>
<td>0.77</td>
<td>136.3</td>
<td>0.58</td>
<td>139.7</td>
<td>0.50</td>
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<td>Set(2,18)</td>
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<td>140.1</td>
<td>0.45</td>
<td>143.1</td>
<td>0.17</td>
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<td>136.0</td>
<td>0.49</td>
<td>140.3</td>
<td>0.47</td>
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<tr>
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<td>131.1</td>
<td>0.36</td>
<td>137.1</td>
<td>0.15</td>
<td>141.9</td>
<td>0.29</td>
</tr>
<tr>
<td>Set(2,21)</td>
<td>131.3</td>
<td>0.93</td>
<td>136.1</td>
<td>0.30</td>
<td>141.4</td>
<td>0.29</td>
</tr>
<tr>
<td>Set(1,22)</td>
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<td>1.26</td>
<td>136.9</td>
<td>1.02</td>
<td>140.6</td>
<td>0.45</td>
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<tr>
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<td>0.93</td>
<td>137.3</td>
<td>0.30</td>
<td>141.4</td>
<td>0.29</td>
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<td>Set(1,25)</td>
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<td>134.5</td>
<td>0.05</td>
<td>139.6</td>
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<td>140.4</td>
<td>0.15</td>
</tr>
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<td>0.96</td>
<td>135.7</td>
<td>0.24</td>
<td>140.4</td>
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<td>135.8</td>
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<td>139.5</td>
<td>0.55</td>
<td>140.0</td>
<td>0.50</td>
</tr>
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</table>

### Table 3. 2 Fractional enthalpies (cortical cell percentages) for the assumed three components of the DSCw curves in various diameters, as determined through curve deconvolution under Gaussian equation. Coefficients of determination are in all cases R\textsuperscript{2} > 0.9

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Percentage area of cortical cells (%)</th>
<th>Mean O1</th>
<th>Standard Error of O1</th>
<th>Mean O2</th>
<th>Standard Error of O2</th>
<th>Mean P</th>
<th>Standard Error of P</th>
</tr>
</thead>
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3.4.2.2 The Relationship between Denaturation Temperature of Each Cortical Cell and Wool Diameters

The data in Table 3.1 shows that both sets illustrate the same trend. Paracortical cells have the highest denaturation temperature followed by O2 and O1, respectively. As mentioned before, higher denaturation temperature of paracortical cells associate with higher concentration of cysteine in the matrix. Further analytical results on the relationship between denaturation temperatures of each cortical cell type and mean diameter are shown in Figures 3.14a - c. It is clear to see that there is no significant relationship between denaturation temperatures of each cortical cell type and wool diameter. Only weak relationship are observed in P Set (2), and O2 Set (1). The $R^2$ is 0.3 and 0.4, respectively. The paracortical cell show negative relationship between increasing of wool diameter and denaturation temperature, while positive relationship is found in orthocortical cells. However, the p-values of both relationships are greater than 0.05, which mean that the relationships are not significant enough to support the changes.

Although, the specimens with fine diameters range are separated, the relationship between denaturation temperature of each cortical cell type and wool diameter are not found. This finding is contrary to previous studies in section 3.4.1. The finding suggests that the positive relationship between denaturation temperature of the whole fibre and mean diameters can be found in wool diameter 24.0 - 38.0 µm. The reason behind this could be referred to as the ortho/para hypothesis. The significant different within each cortical cell is less difference than whole fibre. Therefore, the significant change cannot be investigated when compared with the whole fibre.
Figure 3.14 The relationship between each cortical cell denaturation temperatures and wool diameters. The data present mean value of denaturation temperature, which obtained from MDSC peak deconvolution, with the standard error a) Paracortical cells, b) Orthocortical cells 2, c) Orthocortical cells 1.
3.4.2.3 The Relationship between Fractional Enthalpy and Wool Diameters

Another result that obtained from the MDSC peak deconvolution is the fractional enthalpies which are connected with the main fraction of cortical cells or cortical cells percentages. The data in Table 3.2 were plotted and illustrated in Figures 3.15a and b. It is clear to see that the vast majority component of both sets is the O2 followed by the paracortical cells and O1, respectively. These results from the deconvolution process are in line with the result from microscopical investigation (Donald F.G. Orwin et al., 1984). In Set (1), the percentage of O2 is around 53.6 - 68.4 %. The results between Set (1) and (2) are compared and reveal that more variation of data tends to be found in Set (2). A wider range of O2 percentage is observed in sample Set (2), which has the O2 percentages around 48.7 - 72.6 %. Like O2, more fluctuation trend of P and O1 percentage are found in samples Set (2). Although, almost all of the specimens from Set (2) reveal higher percentage area of the third peak than the first peak, the outliers of data are found. Percentage areas of O1 (first peak) is higher than P (third peak) are determined in samples Set (2, 18) and Set (2, 32). Additionally, the higher of standard error in Set (2) is another evident to confirm that samples Set (2) are more nonhomogeneous than sample Set (1).

The relationship between cortical cells percentages and wool diameters is shown in Figures 3.16. The results suggest that there is no significant relationship between the diameters and percentages of cortical cells. This trend does not agree with previous study by Orwin et al. (1984). In the past, Orwin and his team investigated the relationship between orthocorical cells percentages and wool diameters. They reported that increasing of wool diameter have either log-linear or linear regressions with orthocortical cells percentages (Donald F.G. Orwin et al., 1984). However, the results from DSC deconvolution investigation cannot indicate any relationship between increasing of mean diameters and changing of cortical cells type.

The reasons behind this variation could be due to three main reasons. The first reason is investigation techniques. In this study, DSC was applied. The single point of DSC data represent more than 100 snippets of fibres. So the variation is higher than single point of data obtaine from microscopic analysis. The further study to confirm the estimation of cortical cells percentages by deconvolution is required. Another reason could be related to the source of material. Although Orwin and this work applied wool fibres as a specimen, Orwin used wools from individual sheep.
On the other hand, wool tops, which consist of fibres from more than one sheep, were applied in this work.

**Figure 3.15** The fractional enthalpies obtained from MDSC peak deconvolution with standard error a) Set (1) b) Set (2)
Figure 3.16 The relationship between mean percentage areas of cortical cells types and diameter. The data obtained from MDSC peak deconvolution. The graph presents the mean value with the standard error a) paracortical cells, b) orthocortical cells 2, c) orthocortical cells 1.
Furthermore, following Figure 3.17, the linear relationship might be driven from the one point of data, which is smallest cortex diameter. In Drysdale, without the data from the smallest diameter, there is no significant change between diameter and percentage of orthocortical cells. According to the DSC results, the fine wools tend to show more fluctuation trend. So it might be another possibility that the data at the smallest diameter might be different if a size of sample increases.

![Figure 3.17](image)

**Figure 3.17** The relationship between percentage of orthocortical cell and cortex diameter obtained from Orwin (1984) • Drysdale, ▲ Perendale. The graph showing fitted lines for the linear relationship for two sheep (Donald F.G. Orwin et al., 1984).

### 3.4.2.4 The Relationship between Denaturation Temperature and Cortical Cells Percentages

Interestingly, the results of deconvolution reveal that the denaturation temperatures of wool fibres and the cortical cell percentages are independent to each other. From the previous studies, there is general agreement that the denaturation temperatures relate to density of crosslink in matrix, which is mainly found in paracortical cells. Against this background, higher percentage area of paracortical cells would be expected when the denaturation temperatures of wools increase. However, following Figure 3.18a, a prominent trend between the denaturation temperatures and paracortical cells is not observed. Although samples from Set (2) present negative relationship between O2 percentages and the denaturation temperature of wool, the significant level is low. Therefore, the conclusion of the relationship between fractional O2-cell
type area and denaturation temperature cannot be accepted (see Figure 3.18). The data between Set (1) and (2) are compared and the results show that data from Set (2) are more variation than set (1). So, these results might reflex the variation of chemical component in the main cortical cell types and further confirm the non-homogeneous properties of sample Set (2).

**Figure 3.18** The plot of denaturation temperature of whole fibres and fractional area of main cortical cells with standard error. The cells fractional areas obtained from MDSC peak deconvolution process under three fitted of Gaussian distributions with equal width in wet condition a) Paracortical cells, b) Orthocortical cells.
3.4.2.5 The Relationship between Denaturation Temperature Differences and Wool Diameter

The relationship between wool diameters and deconvolution results is observed. Following Figures 3.19, the denaturation temperatures of P and O2 are plotted against wool diameters. The gap between the denaturation temperatures of P and O2 seem to reduce when wool diameter increase. Both sets of samples show a similar trend. This hypothesis was further investigation and the result is shown in Figure 3.20.

Figure 3.19 Compared the mean denaturation temperature between para-cell type and ortho2-cell type with standard error, the data obtained from MDSC peak deconvolution process under three fitted of Gaussian distributions with equal width in wet condition a) Samples Set (1), b) Samples Set (2).
The denaturation temperature differences between P and O2 of fine wool is around 3-4°C, while the difference drop to ~ 1°C in very coarse wool. Linear regression was applied to estimate the relationship. Both sets show negative relationship with a coefficient determination (R²) of around 0.5 with high significant level (p-value < 0.01). These results suggest that the difference of sulphur content between ortho- and paracortical cells decrease in coarser wools. The further detail is requested to further confirm this relationship and the DSC results. Cross sectional analysis by microscopy technique and the relationship between optical analysis and thermal analysis will be explained in Chapter 4 and 5.

Figure 3.20 The relationship between wool diameters and denaturation temperature differences of main of cortical cells (P-O2); mean value with standard error, the data obtained from MDSC peak deconvolution process under three fitted of Gaussian distributions with equal width in wet condition.
3.5 Conclusion

The thermal analysis by MDSC in excess water was applied to investigate the denaturation enthalpy and denaturation temperature of Merino wools with different diameters. The denaturation enthalpy of wools is individual to the materials properties. This finding is consistent with previous studies. Interestingly, the results from the various mean diameters provided a deeper insight into the relationship between denaturation temperature and wool diameter. Although, the liner relationship between denaturation temperature and wool diameter is investigated, it does not cover the whole ranges of mean diameters. Only, wools with mean diameter ranges from 24.0 to 39.0 µm, also named as coarse wools, reflect this liner relationship. This linear relationship of coarse wools is found in both sets of sample. However, fibres with fine diameter range (10.0 - 22.0 µm) have a different character of thermal stability from coarse wools. In addition, fine wools from both sets showed different relationship between denaturation temperature and wool diameter.

The deconvolution of MDSC in water of Merino wools shows the excellent fit under the three fitting of Gaussian function. The presence of a low-sulphur orthocortical cells type (ortho-like, O1) is confirmed by DSC peak deconvolution under the ortho/para hypothesis and optical analysis results. However, the presence of mesocortical cells could not be confirmed by this experiment. According to the excellent fit and large number of fibre snippets, it can be concluded that MDSC deconvolution method might be a feasible method to estimate main cortical cell percentages. However, integration between DSC results with optical analysis results will provide better reliability of this deconvolution results. Although significant relationship between wool diameters and cortical cell percentages that obtained from DSC deconvolution is not observed, the negative linear relationship between wool diameters and temperature difference of main peaks (Peak III - II) is observed. Further study is request to confirm this relationship.

According to the thermal properties that obtained from DSC and DSC-deconvolution process, the results suggest that samples Set (2) are more non-homogenous than Set (1). Thus, only wool fibres from Set (1) were selected as a specimen for further experiments in order to reduce the effect of nonhomogeneous on further analytical results.
3.6 References


Chapter 4 Optical Analysis of Wool Cortical Cells

4.1 The Study of Wool Cortical Cells

As mentioned in the previous parts, cortical cells are the majority of component in wool fibres. Therefore, studies of cortical cells arrangement, proportion of cortical cells, and chemical components in each cortical cell have gained interest over the last decade. Additionally, there have been many attempts to investigate relationship between the cortical cells and fibres properties. An optical technique using microscopic analysis is one of the significant techniques for wool cortical cells study. The predominant advantage of this technique is the cellular structure and cells arrangement in the cortex can be visualised. Both light microscope (LM) and electron microscopy including Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) have been applied.

This section will give a brief overview of the previous studies of optical analysis on the cellular structure of keratin fibres, mainly wool fibres. The first part of this section will review the staining techniques for light microscope. Cortical cells studies by different microscopy techniques will be reviewed in the remaining parts.

4.1.1 Staining Techniques

4.1.1.1 Introduction

In general, cross section of wool under microscope is clear oval or circular shape (McCloghry and Uphill, 2010). Thus, a staining process is used to visualise the difference of cortical cells types. Dyes or stains reagents are colorful substances that bind to biological cells or tissue as a result of chemical attractions (Council, 2006). After staining, the cellular structure of wool can be investigated. Selection of staining technique and types of dyes depend on microscope types and target cell areas (Thorsen, 1958). In addition, the optimum point of each dye condition depends on wool breed and diameter of wool.

Basic and acid dyes are mostly used for wool fibres staining under light microscope. In the past, sodium plumbite, gold chloride, lead sulfide and other dyes with lead ions were applied
to stain paracortical cells. Nickelous nitrite and bromine were reported to stain orthocortical cells successfully. Various staining conditions have been applied in order to increase the efficiency of dye technique. For instance, Thorsen (1958) found that applied urea and monothioglycol could increase the intensity of orthocortical cells staining in nickel solution (Thorsen, 1958). By using acid dyes, there have been a considerable number of disagreements among different studies over interpretation of results obtained by different staining procedures. Some studies reported that paracortical cells were stained by acid dye (Fraser and Roger, 1954). JH Dusenbury argued that orthocortex was preferentially stained by both acid and basic dyes (Dusenbury and Coe, 1955). On the other hand, there were agreements on interpretation of using basic dye. Evidences from a number of experimental studies showed that orthocortical cells were stained by using basic dye (Fraser and Roger, 1954, Snyman, 1963b). Therefore, basic dyestuffs and reagents such as Janus green and Methylene blue have been widely used to study the cellular structure of wool fibres (Snyman, 1963b).

Embedding technique is also important factor in order to get an accurate measurement of the relative areas of ortho- and paracortical cells segments (Snyman, 1963b). Suitable embedding techniques have been studied extensively. Collodion, Ned-O-Lac, JB-4 Resin, gelatin were the examples of embedding mediums have been used for wool cross-section(Dusenbury and Coe, 1955, Orwin et al., 1984). Butyl methacrylate, and resin were the most commonly used embedding medium for fibre cross section preparations (Snyman, 1963b). However, the preparation process was time consuming because of slow setting time. Therefore, molten paraffin was introduced to solve this problem. By using molten paraffin, moreover, a large bundle of wool (around 200 fibres) can be cut into cross section in one time while collodion embedding technique can cut small bundle (70-80 fibres) at a time (McCloghry and Uphill, 2010).

4.1.1.2 Janus Green Staining

Janus green is a cationic dye, commonly known as basic dye (Zollinger, 2003). This dye has been applied for many biological studies including wool fibres (Gurr, 2012). Ohara (1938) was one of the first to stain wool fibres by Janus green. The partial staining of wool fibres was observed and this staining pattern was named bilateral staining (Ohara and Melliand, 1938).
Fifteen years later, Horio and Kondo confirmed Ohara’s finding by using the same dye (Horio and Kondo, 1953). They also found that the darker area was always observed on outer portion of the crimp curvature as shown in Figure 4.1. Later on, data from several studies have identified the stained area as orthocortical cells while less dye intensity area has been associated with paracortical cells (Mercer, 1954, Thorsen, 1958, Rogers, 1959). The significant benefit of Janus green staining is to interpret the coiling nature of wool fibre which will be explained in session 4.1.2.2.

There are various Janus green staining conditions. Temperature, staining time, and dye solution are varied to enhance the optimum contrast between ortho- and paracortical cells (Horio and Kondo, 1953, Dusenbury and Coe, 1955, Ito et al., 1986). Although numerous dye bath conditions of Janus green were applied, the result of a partial staining pattern with a darker stained area towards the outside of curvature was always observed. Unlike other dyes such as Ponceau 2R, no differential staining occurs when the pH of the solution is lower than 6.0 or greater than 8.0. Later on, JH Dusenbury (1955) explained that “Janus green is relatively independent method used to produce” (Dusenbury and Coe, 1955). Notable observation was made by Horio and Kondo, who found that the partial staining pattern occurred on dyed fibres both in form of fibres and cross sections. Moreover, the staining contrast was enhanced by washing the fibers with a very dilute acid immediately after staining (Horio and Kondo, 1953).

Figure 4.1 A bilateral staining of Merino wool by Janus green staining technique (Horio and Kondo, 1953) a). Part of a wool fibre staining differentially b) Wool cross section staining (Magnification, 1500 x). DA-Dye stands for a dye accessible, and Non-DA is Non-dye-accessible.
4.1.1.3 Methylene Blue Staining

Methylene blue is a synthetic basic dye. This dye is widely used as biological staining especially nucleic acids, such as DNA and RNA because the deepest shade of blue are shown when in contact with acids (Council, 2006, IARC, 2016). The application of methylene blue is also applied in wool cortical cells study. Clarke & Maddocks (1965) developed the methylene blue staining technique and this staining method is well-known and extensively applied for wool and hair fibres (Clarke and Maddocks, 1965). The methylene blue staining method is opposed to other dye stuffs including janus green. By using methylene blue, oxidation process is a necessary step before dyeing while janus green stain is a direct method (Orwin et al., 1984, Plowman et al., 2007). The result from methylene blue staining also shows differential staining intensity between main cortical cells types. However, the result interpretation is different. The darker stained area is paracortical cells while lighter stained area is orthoecocytic cells (Orwin et al., 1984). The retention of methylene blue in the paracortical cells is due to conversion of cysteine to cysteic acid by oxidation process (Ito et al., 1986, Plowman et al., 2007). Therefore, an oxidation process is one of the important steps for cortical cells study using methylene blue staining.

Up to now, there have been a number of studies to investigate the effect of oxidising agents and conditions on wool fibres. In fact, as first reported by Arthur L. et al. (1936), wools were oxidised by hydrogen peroxide. The results revealed that the disulphide group of the cystine was the main point of attack during oxidation. (Smith and Harris, 1936, Blackburn and Lowther, 1951). Later on, peracetic acid was used as an oxidising agent because it was more stable in water (Alexander et al., 1950). In the following year, however, S. Blackburn and A.G. Lowrher demonstrated that performic acid oxidise cystine of wool faster than hydrogen peroxide and peracetic acid (Blackburn and Lowther, 1951). Alexander (1950) explained that performic acid probably had faster diffusion rate in the fibre due to larger extent of fibre swelling (Alexander et al., 1950). A comparison of performic and peracetic acid was further studied. By using peracetic acid, incomplete oxidation of wools was investigated and this partial oxidation in wool could be further oxidised by using performic acid. Therefore, they concluded that “performic acid is the more powerful oxidizing agent for wool” (Thompson and O’donnell, 1959).
Methylene blue staining procedures have been developed to enhance an identification accuracy of relative ortho- and paracortical cells areas. The optimal condition depends on many factors such as temperature, concentration, pH, staining time and quantity of adsorbent (Khan et al., 2005). Numerous studies on staining procedures have been reported to investigate the optimum differentiation of staining intensity between ortho- and paracortical cells regions. For instance, the loss of differentiation of methylene blue staining was developed by J. G. Snyman (1963). Shorter time of staining (around 40 second) in boiling condition successfully improved differential staining intensity of main cortical cells types (Snyman, 1963b). One well-known early study that is often cited and applied in research on methylene blue staining is the technique from Clarke and Maddocks (1965)(Orwin et al., 1984, McCloghry and Uphill, 2010). In this procedure, fibres were cross-sectioned and dried on glass slides. Performic acid was applied as an oxidising agent. The oxidation process was took place on the cross section for 60 minutes (Clarke and Maddocks, 1965). This oxidation process increased the dye affinity to the cells; therefore, frequent washing with water did not remove the dye(Fraser and Roger, 1954). After oxidation process, the slides were rinsed in distilled water, and stained with methylene blue for 5 min (Clarke and Maddocks, 1965).

4.1.2 Cortical Cells Study by Optical Analysis

4.1.2.1 Electron Microscopy Technique

Electron microscope is an important technique in cortical cells investigation and plays a key role in the study of ultrastructural characterisation of wool cortical cells types. Morphological arrangement of macrofibril can be visualised by using electron microscopy technique, especially TEM. Currently, there are numerous studies using TEM to investigate the morphology of wool and the results are in good agreement (Rogers, 1959, Orwin et al., 1984, Caldwell et al., 2005, Plowman et al., 2007). TEM studies indicate that there are three types of cortical cells; ortho-, para- and mesocortical cells. Figure 4.2a illustrated TEM image of cross-sectional distribution of three cells types in wool. Moreover, a significant finding from TEM technique is the ultrastructure of microfibrillar arrangement, as shown in Figure 4.2 b – d. The difference of these three cells
types is the ultrastructure arrangement of their IFs and IF/matrix ratio, which was already explained in Chapter 1 (Caldwell et al., 2005, Plowman et al., 2007).

Without staining, in general, the chemical component of each cortical cell can be reflected by the brightness of TEM image. The matrix regions is brighter than microfibrils due to the greater electron density which has been attributed to amount of disulfide bonds (Kaplin and Whiteley, 1978). The iodine staining technique was introduced to investigate tyrosine region. The mapping results showed higher density of iodine in orthocortical cells region than paracortical cells, which mean that orthocortical cells are rich in tyrosine (Plowman et al., 2007).

**Figure 4.2** TEM of a transverse section a fine Romney wool fibre with high fibre curvature a) Two-dimensional micrographs of fibre cross section showing the three different types of cells: Paracortical cells (P), Orthocortical cells (O) and Mesocortical cells (M). b) –d) higher magnification images showing the different packing modes b) Paracortical cells with loosely packed, parallel IF arrangement c) Mesocortical with regular hexagonally packed, parallel IF arrangement d) Orthocortical with helical arrangement of IFs(Caldwell et al., 2005)

4.1.2.2. Light Microscopy Technique

In contrast electron microscopy technique, fibres need to be stained before being investigated under the light microscope. The difference of dye affinity between ortho- and paracortical cells enable us to visualise the cortical cells structural arrangement within fibre cross-sections. Thus, several studies have applied light microscopy technique to study the arrangement of cortical cells types. Ohara (1938) was the first to mention the bilateral staining on Merino wool by using basic dyes staining (Ohara and Melland, 1938). This finding has gained attention to the
study of cortical cells arrangement. A bilateral and radial structural arrangement of cortical cells types were observed by using methylene blue staining. The comparison of these staining patterns is illustrated in Figure 4.3a and b.

Figure 4.3 Light micrograph of cross sections of Merino wool fibres dyed with methylene blue showing the different cortical cells arrangement. The densely-dyed portion is orthocortex (O) and the other is paracortex (P). a) Bilateral arrangement and b) Radial arrangement (Rogers, 1959).

Furthermore, these staining patterns tended to be associated with fibre diameter (Rogers, 1959, Orwin et al., 1984). The bilateral arrangement tended to be found in fine wool, whereas the radial arrangement with orthocortical cells in the centre of fibre could be observed more frequently in coarser wool (Menkart and Coe, 1958, Rogers, 1959). Moreover, the segregation of bilateral arrangement between ortho- and paracortical cells was more difficult to justify when
diameter increased. Thorsen (1958) also reported the disappearance of segmentation at diameter above 35 µm. This finding brought to the conclusion that the disappearance of segmentation was more related to fiber diameter than breed of wool (Thorsen, 1958, Orwin et al., 1984). Other staining patterns also had been observed and the results were summarised in Figure 4.4 (Plowman et al., 2019).

![Diagram](image)

**Figure 4.4** “Cell distribution classification scheme for wool transverse sections, extended from that defined by Orwin et al. (1984)”, adopt from (Plowman et al., 2019)

The correlation between cellular arrangement and crimp of wool is the significant finding from light microscopy technique. By using Janus green staining, Horio and Kondo (1953)
observed that the bilateral arrangement in cross section had direct relationship to the occurrence of crimp in wool fibres. The orthocortical cells, which were more accessible to basic dyes than the other, always located on the outside of the crimp wave. On the other hand, paracortical cells located inside of the crimp wave (see Figure 4.1). Moreover, the unequal expansion of the ortho- and the paracortical cells result in the changes of crimp shape (Horio and Kondo, 1953, Mercer, 1954). The correlation was further confirmed by Orwin et al. (1984) study. They reported that distinctness of bilateral arrangement between ortho- and paracortical cells became less with decreasing wool crimp (Orwin et al., 1984).

Although most research on light microscope has been carried out in qualitative analysis aspect, there were some quantitative studies using light microscopy results. The earlier study by Snyman (1963) showed that higher proportion of paracortical cells tended to be found with increasing in wool diameter and larger percentage area of paracortical cells was also found in high crimp wool than in low crimp wool (Snyman, 1963a). However, a significant quantitative analysis on cortical cells study was presented by Orwin et al. (1984). The methylene blue stained and unstained areas were measured by free hand drawing and the percentage area of each cortical cell was calculated. Contrary to the result of earlier work (Snyman, 1963a), Orwin et al. (1984) demonstrated that the orthocortical cells was the majority of component in the fibre cross sections and percentage areas of paracortical cells decreased with increasing in wool fibre diameter. The relationship between wool diameter and orthocortical cells were described by a log-linear and a linear correlation. In some breeds of wools, these relationships might be affected by nutritional stress or season (Orwin et al, 1984).

**4.2 Objective**

In Chapter 4, the cellular structure of main cortical cells types was studied using optical analysis. So far, limited comparisons between the results of Janus green and methylene blue staining, which are the predominance staining technique for wool optical analysis has not been discussed. Moreover the evidence showing that the percentage areas of main cortical cells types are associated with wool diameters is inconclusive. No large-scale studies have been performed to investigate this relationship. Thus, the extension of qualitative and quantitative study based on
Orwin et al. (1984) work will help address this research gap. The aim of this study is to investigate the relationship between cellular structure and wool diameter. In order to achieve this aim, this research was designed to address the following objectives;

• Investigation the relationship between cellular structural obtained from Janus green and methylene blue staining and wool diameter.

• Comparison of the results from Janus green and methylene blue staining techniques.

• Estimation the percentage areas and investigation the relationship between main cortical cells types and wool diameters by Janus green staining.

4.3 Experimental

According to the DSC results, only the specimen from set one was introduced in order to eliminate the effect of chemical variation on the cellular arrangement. In this study, wool cross sections were stained by two different techniques; Janus green and methylene blue staining and the cross sections were visualised by light microscope. The experimental procedure was divided into three phases; staining, cross –sectional preparation, and image analysis. The appropriate staining condition and cross-sectional process were improved to increase the accuracy of the staining results. After that the stained cross sections were investigated individually by a light microscope. Magnification was set at 400 times. The image files (.jpeg) size 1280x 1024 pixels were then imported into an image J software to measure the cortical cells areas and calculate the percentage area of ortho- and paracortical cells. The further details of each step will be described as follows;
4.3.1 Staining Techniques

4.3.1.1 Janus Green Staining

Although the Janus green dye condition was reported by M. Horio (1953), this condition could not be applied in coarse wools (Horio and Kondo, 1953). In order to optimise the staining condition, the optimal reaction time, temperature and solid: liquid ratio was investigated. The variable conditions are shown in Table 4.1. All fibres were dyed in 0.1% concentration of Janus green solution. The dyed fibres were put in 1% acetic acid bath for 5 minutes, and then washed with distilled water for 1 minute 5 times. Dyed fibres were left overnight to dry at the room temperature. A bundle of wools from each sample was pull through 0.5 centimetre diameter plastic tube until it was completely filled. This resulted in about 100 fibres lying parallel with each other in each tube. This tube was embedded in paraffin wax and make 5 µm cross section by using microtome. The embedding and cutting process will be explained in session 4.3.2.

Table 4.1 The summary of various Janus green staining conditions

<table>
<thead>
<tr>
<th>No</th>
<th>Reaction time (hours)</th>
<th>Reaction temperature (°C)</th>
<th>Solid: Liquid ratio (g : mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>60</td>
<td>1:300</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>80</td>
<td>1:300</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>60</td>
<td>1:300</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
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<td>1:600</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>80</td>
<td>1:300</td>
</tr>
</tbody>
</table>

4.3.1.2 Methylene Blue Staining

Similar to Janus green staining, only specimens from set (1) was focused in this staining. However, only samples from set (1, 16), set (1, 27), and set (1, 38) were selected as a representative of the four ranges of wool diameters. The staining process of methylene blue was different from the Janus green staining. The oxidation process was used in methylene blue staining while direct dye method was applied for Janus green. Commonly, the standard methylene blue staining by Clark and Maddocks (1965) started with embedding and making a cross section. Afterwards the cross-section slides were oxidised by performic acid for 5 minutes.
(Clarke and Maddocks, 1965). However, in this study, the whole fibres of wool were oxidised by freshly prepared performic acid first (65% distilled water, 25% formic acid [99%], 10% hydrogen peroxide [30%] v/v) (McCloghry and Uphill, 2010). Wool fibres were immersed in performic acid for various times without stirring. The effect of oxidation time was studied under these variations: 1, 2, 5, and 24 hours. All oxidation processes were carried at normal room temperature (20 ± 5°C).

After the oxidation process, fibres were rinsed with distilled water and left overnight to dry at room temperature then a bundle of oxidised wool was pulled through 0.5 centimetre diameter plastic tube. This tube was embedded in paraffin wax and 5µm cross section was cut by microtome. After going through wax process (see 4.3.2.2), cross sections were stained with methylene blue. The methylene blue solution was prepared using the following standard procedure; Polychrome methylene blue (1 g) and potassium carbonate (1 g) in 400 ml of distilled water, boiled for 30 min, then cooled. Concentrated acetic acid (3 ml) was added and the mixture was agitated until any precipitate was dissolved. The solution was boiled gently for another 5 minutes and cooled before use (McCloghry and Uphill, 2010). The effect of staining time was studies under these variations; 5, 10, and 30 minutes. After the staining completed, slides were then rinsed with distilled water and the areas of orthocortical cell were decolourised in 1% hydrochloric acid (1% hydrochloric acid [cone], 99% ethanol [70%] v/v) for 30 seconds. Finally, the sections were dehydrated by 1 minutes in 100 ethanol, followed by 95, 85, and 70% ethanol/water (v/v), respectively. Finally, a slide was washed with xylene for 1 minutes and cover with glass coverslip No.1.

4.3.2 Cross-sectional Process

4.3.2.1 Embedding

A bundle of prepared wool fibres was pulling through a plastic tube (0.5 centimetre diameter and 4 centimetres long). Next, this plastic tube was cut into 0.5 to 1 centimetre long. In order to prevent the same fibre measurement, one tube was cut once and put in hot wax bath. The paraffin wax was selected as an embedding agent because of many reasons such as less time consumption, prevented fibres damage from polishing process, and handle easier in cross
sectional process. The embedding time in hot paraffin wax bath was around 10 to 20 minutes. After that specimen was placed and oriented in a molded then the molded was filled with hot paraffin. Finally, the specimen “block” was solidified on a cold surface. When it set, the molded was removed. This process provided a stable base for clamping in the microtome.

4.3.2.2 Cross Section Process

A paraffin block of wool bundle was cut by microtome (RM2145, Leica) using fine steel blades to make a thin cross section. The thickness of each cross section is 5 µm. The beginning of the cut sections were stuck together edge-to-edge, forming a “ribbon”, so this ribbon was put in hot water bath (temperature around 60 °C) to unfold it. These float sections were mounted on a super force microscope slide. Next, the sections went through heat-fixed process in which the cross-section slides were put in the oven at 60°C for 30 minutes. From this step, the fibre cross sections were adhered to the slide, so they were not washed off during staining and de-wax process. The first step of the de-wax process began with putting slides into a xylene bath for 5 minutes with two baths change. The section was dehydrated through 1 min changes of 100, 95, 85, and 70% ethanol/water (v/v), and then put in distilled water for rehydration. Lastly, a few drops of liquid paraffin were applied before covered the sections by a glass coverslip No.1.

4.3.3 Quantitative Method Evaluation and Image Analysis

This part of the experiments was designed to investigate the percentage area of ortho- and paracortical cells. The algorithm of image analysis was split into three phases, namely, taking cross-sectional images, measurement of cortical cells area, and analysis of the percentage areas. Cross-sectional image collection was based on IWTO 8: 2011 standard (IWTO, 2011). Subjective and computerised measurement methods were evaluated to determine the test method. The conditions of result verification were introduced to verify the measurement results before determine the relationship between wool diameter and main cortical percentage areas. The details of each phase will be explained as follows:

4.3.3.1 Taking Cross-sectional Image Procedure
All of cross-sectional images, which were stained by Janus green, were taken under the light microscope with 400 times magnification. Each cross section was focused individually before capturing the image to provide a clear circumference. 100 cross-sectional images of each sample were photographed. Therefore, the total number of samples for Janus green and methylene blue staining was 800 and 300 fibres, respectively. In this work, two regulations were applied to prevent taking the same cross sectional image and reduce the edge error effects. The first restriction was microscope stage movement which was recommended by IWTO 8, 2011 (IWTO, 2011). The pathway is shown in Figure 4.5 and the details of this pathway were already explained in Chapter 2. Another restriction was applied to improve the accuracy of circumference measurement. Cross sections with the damage on edge, broken cross sections, unclear circumference or cross sectional images that crossed another image were excluded from the analysis eliminated (see Figures 4.6 a-d). The cross-sections intersected and cut off by the image frame were also. This images collection process provided suitable cross sections images for measurement.

Figure 4.5 The regulations of pathway taken across slide during taking cross-sectional images (IWTO 8: 2011)
4.3.3.2 Measurement of Cortical Cells Area and Quantitative Methods Evaluation

All cross sectional images were processed by Image J software to measure the stained (orthocortical cells) and unstained (paracortical cells) areas. The calibration by stage micrometer (100 x 0.01 = 1 mm) was the first step for image analysis. The scale was set by calibrating the known distance into pixels using the “set scale” function in the analysis tool menu. Each cross-sectional image was measured in three areas; total cross sectional area, stained, and unstained areas. The computerised method (automatic threshold setting) and subjective method (hand drawing) were evaluated and compared. Both techniques were examined in order to determine the best method for quantitative analysis. 45 cross sectional images from set (1, 16) were used as a specimen for this quantitative methods evaluation. Each cross-sectional image was measured twice using these two methods. Finally, the relationship between computerised and subjective method were tested using linear regression analysis.

The computerised method relies on the contrast between stained and unstained areas. Figures 4.7 a – e illustrate the overview of the computerized procedure. First, each image was
converted to 32-bit greyscale (see Figures 4.7a and b). This grayscale image analysis method takes advantage of its ability to eliminate the adjusting hue and saturation effect of original colour (Wang et al., 2011, Borsa and Van Zeghbroeck, 2018). The threshold adjustment only depends on the brightness contrast of the image. Additionally, following Figure 4.7c, the background was subtracted to reduce the noise during threshold selection. At this point, the total cross sectional area (TA) was measured. Figures 4.7d and e show the final step of image analysis by computerised procedure. Ortho- and paracortical cells were measured according to their grey level. The “threshold” function was applied to find the region of interest. The selected areas were shaded in red then “Particle analysis” function was used to measure the shaded areas. An aggregate between ortho- and paracortical cells areas is cortex area (CA).

Another method is subjective which has been used for long time to measure cortical cells area. The “Freehand tool” function was used to draw a circumference surrounding the region of interest. Then “measure” function was used to measure the selected area. Generally, a primary concern of a subjective method is human error such as the bias of operator. Therefore, the effect of human error on the reliability of the measurement was also investigated. 50 cross sectional images from set (1,16) and (1,27), which made 100 cross section in total, were measured. Each cortical cells area measurement was repeated for 5 times then the variation and accuracy of these 5 measurements were analysed using statistical tool including mean, standard deviation and standard error. Finally, the effect of human error on the reliability and accuracy was evaluated by testing the relationship between the distribution of standard error and wool diameters.
Figure 4.7 Summary of the area measurement process using automatic threshold setting method
a) The original image captured by digital camera, b) Conversion image to grayscale c) Elimination the background effect, d) and e) Selection the region of interest from automatic threshold setting
d) Orthocortical cell area, and e) Paracortical cell area

4.3.3.3 The Verification and Analysis of the Results

The area of total cross section, ortho- and paracortical cells ($\mu m^2$) from 800 cross sections were measured of this experiment. The results were verified before applying the data to investigate the relationship between wool diameter and percentage of cortical cell areas. Cortex and cuticle cells proportions were used as the standard to verify the measurement results. Assuming the percentage area was equal to the percentage of a cortex component in a fibre, about 90% of wool fibres consist of the cortex and 10% is cuticle cells (see Chapter 1). So the cortex area, which includes ortho- and paracortical cells areas, should be slightly smaller than total cross sectional area
because total cross sectional area included the cuticle cell. According to this background, the results were verified using the following criteria.

1. Cross-sectional images with cortex area larger than total cross sectional area were excluded
2. The difference of area between the cortex and total area or cuticle cell area should not be larger than 10%

The different percentage areas between cortex and total area was calculated using Equation 4.1. After all results were verified, the area of each cortical cell was converted into percentage area using Equation 4.2 and 4.3. Finally, the relationship between each cortical cell and wool diameter was analysed using linear regression and significant test, using SPSS software.

Cuticle cell area(%) = \( \frac{TA - CA}{TA} \times 100 \) (4.1)

Orthocortical cell area (%) = \( \frac{SA}{TA} \times 100 \) (4.2)

Paracortical cell area (%) = \( \frac{UA}{TA} \times 100 \) (4.3)

Where;

TA = Total cross sectional area
CA = Cortex area
SA = Stained area
UA = Unstained area
4.4 Results and Discussion

The cellular structure of Merino wool was studied by using two staining techniques; Janus green and methylene blue. Both staining techniques were modified to enhance the staining contrast. Both qualitative and quantitative approaches were used in this study. In this section, the first two parts will focus on the qualitative analysis of cortical cells by both staining techniques. The cellular arrangement and variation of the staining patterns will be discussed. The results from Janus green and methylene blue staining will be also compared to examine the advantages and limitation of both techniques. The final part will describe the image analysis approach and the results of quantitative analysis. The investigation of relationship between main cortical cell percentage areas (orthocortical cell percentage areas, and paracortical cell percentage areas) and wool diameter will be discussed in this part.

4.4.1 Cortical Cells Study by Janus Green Staining

4.4.1.1 The Optimal Staining Condition

The well-known Janus green staining procedure was suggested by M. Horio and T. Kondo (Horio and Kondo, 1953). All of 8 samples from set (1) were introduced. Unfortunately, this condition is not able to stain coarse and very coarse wools. The result after applying Horio’s staining condition is illustrated in Figure 4.8. There are several transparent cross-sectional images or unstained fibres especially fibres with large diameters. Therefore, an optimum dyeing condition for this study is examined by varying reaction temperatures, reaction times and the solid: liquid ratios (see Section 4.3, Table 4.1).

The staining results reveal that temperature is the most influential factor to increase the accessibility of Janus green dye in coarser wools. Although reaction time and solid: liquid ratios are increased, almost all large fibre cross sectional areas are unstained. On the other hand, the ortho- and paracortical cells regions can be differentiated from each other when reaction temperature is increased from 60°C to 80°C. However, the over staining is observed when reaction time continues for 3 hours at 80°C. The over staining and optimum staining results are compared and shown in Figures 4.9a and b, respectively. Loss of differentiation between ortho-
and paracortical cells region is the result from over staining and this over staining is more often to be observed in fine wools. The optimum of Janus green staining condition is summarised as follow;

- Reaction time 2 hours

- Reaction temperature 80°C

- Solid: liquid ratio 1:300

- Dye concentration 0.1%

Figure 4.8 The light micrograph of the cross section of Merino wool fibres (set1, 38) stained with Janus green by using Horio’s staining condition; 60°C, 0.1% dye concentration of Janus green, 1:300 (g/ml), 2 hours.
Figure 4.9 The light micrograph of the cross section of Merino wool fibres (set1,16) stained with Janus green shows the effect of reaction time a) Over-stained condition; 3 hours, b) Successive staining condition on wool cross sections; 2 hours (other conditions; $80^\circ$C, 0.1% dye concentration, 1g of wool: 300 ml of dye solution).
4.4.1.2 The Study of Cellular Structural Arrangement by Janus Green Staining

As previously mentioned, the dye distribution in cortical cells is due to their differential dyeing behaviour. Thus, staining patterns can be used to investigate cellular structure in wool fibres. For Janus green staining, the orthocortical cells are shown by darker stained area while lighter stained area refers to the paracortical cells (Horio and Kondo, 1953). The Janus green staining results show various staining patterns and there is more diversity of staining patterns in coarse and very coarse wools than fine and medium wools as shown in Figures 4.10 a and b. Figure 4.10 a illustrates the staining result of sample set (1, 16) which is fine wools. The majority of staining pattern in this sample is bilateral arrangement, which reflects the arrangement of ortho- and paracortical cells in fine wools. In contrast, Figure 4.10 b shows the result of staining pattern from sample (1, 36), which is very coarse wools. It is clear to see that diversity of staining pattern can be observed. Not only bilateral stained, but faded colour, mono-colour, and non-continuous (island) stained patterns are also observed.

![Figure 4.10](image)

**Figure 4.10** Comparison the diversity of staining pattern associated with wool diameters; Light micrograph of the wool cross section stained with Janus green. The darker area is orthocortical cells, and the lighter area is paracortical cells. a) Fine wools; set (1, 16) with almost bilateral arrangement pattern and b) Coarse wool; set (1, 36) with variety of staining pattern

Although bilateral staining pattern is observed in coarse wools, less clear separation between main cortical cells types is observed when wool diameter increases. The diversity of bilateral arrangements, which associate with wool diameter are illustrated in Figures 4.11 a - c.
Apart from the bilateral arrangement, several stained patterns are more likely to be found in coarser wools as shown in Figures 4.12 a - d. Moderate dye uptake is more often found in cross section of coarser wools. This moderate dye uptake results in less distinctiveness between ortho- and paracortical cells (see Figure 4.12 a). Some studies indicated this moderate stained as mesocortical cells (Orwin and Bailey, 1990). Many cross-sectional images cannot be identified the cortical cells types because the stained affinity is the same (see figure 4.12 b and c). Interestingly, for very coarse wool, some cross-sectional images show the cord of orthocortical cells with random arrangement (see Figure 4.12 d).

Comparison of the findings with other studies confirms that the cellular arrangement of cortical cells types associate with diameter of wools. The bilateral arrangement is the majority of staining pattern in fine wools and this result is in line with the previous staining results by Janus green and other basic dyes and the segregation (Horio and Kondo, 1953, Dusenbury and Coe, 1955, Orwin et al., 1984). The segregation between ortho- and paracortical cells becomes less distinctness with increasing wool diameter (Orwin et al., 1984). However, the radial or annular arrangement which was revealed by methylene staining cannot be investigated by using Janus green staining (Rogers, 1959). The undefined cortical cells region of coarse and very coarse wools indicates the limitation of Janus green staining. Therefore the second staining technique, which is methylene blue, is introduced.

Figure 4.11 The segregation of bilateral arrangement associate with wool diameters; Wool cross section stained with Janus green a) Fine, b) Medium and c) Coarse wools
Figure 4.12 Staining patterns of coarser wools; Light micrograph of the coarse and very coarse wool cross section stained with Janus green a) Moderate staining between light and heavy stained areas b) and c) Uniform stain d) Cord of orthocortical cells with random arrangement.

4.4.2 Cortical Cells Study by Methylene Blue Staining

4.4.2.1 Effect of Modified Method on Staining Results

Three samples from Set (1) are selected as a representative for 4 ranges of wool diameter. Fine and medium wools are represented by sample set (1, 16) and set (1, 27), respectively. Coarse and very coarse wools can be represented by set (1, 38) according to the wide distribution of fibre diameter (see Chapter 2 for sample characterisation). Clark and Maddock’s staining procedure was modified in order to enhance staining contrast (Clarke and Maddocks, 1965). The performic acid oxidation, as the first step in this process, plays a central role in fibre preparation. In this modified method, fibres were oxidised in the fibre form while Clark and Maddock’s protocol applied oxidising agent on cross sectional fibres. According to the modified process, longer oxidation time can be applied and this results in a better control of fibre
cross sectional density on a glass slide because it prevents the wash off and falling effect during oxidation on a glass slide.

The effect of oxidation time on optical results is investigated. In general, the staining results indicate that the optimum of oxidation time depends on wool diameters. Figures 4.13a - c show the staining results obtained from set (1, 16), which are fine wools. All of them show the bilateral arrangement. When comparing Figure 4.13a and b, it is clearly to see that oxidation time affects to optical results. Although main cortical cells types can be differentiated by using 1 hour oxidation time (see Figure 4.13 a), more distinctive segregation of main cortical cells types is observed when oxidation time increases from 1 to 5 hours (see Figure 4.13b). Increasing oxidation time over 5 hours might result in the cuticle cells damaging (see Figure 4.13c), but the cellular arrangement in cortex is not affected. The bilateral arrangement can be also observed by using 24 hours oxidation time that of Joseph H. (1995) who found cuticle cells are not associated with cellular arrangement in the Merino wool cortex (Dusenbury and Coe, 1955). Although cuticle cells do not affect to the cellular arrangement of wools, an unclear edge is effects on equivalent diameter measurement. Therefore, oxidation time around 5 hours is an optimum condition for samples (1, 16). Interestingly, the optimum oxidation time of sample (1, 27) is similar with sample (1, 16).
Figure 4.13 The effect of oxidation time on fine and medium wools; the light micrograph of sample set (1, 16) cross sections stained with methylene blue for 10 minutes a) 2 hours b) 5 hours c) 24 hours oxidation time. The darker area is paracortical cells, and the lighter area is orthocortical cells.
Figures 4.14a - d display the staining results of coarse and very coarse wools which are sample (1, 38). Although oxidation time around 5 hours is sufficient for fine and medium wools to differentiate the region between ortho- and paracortical cells, unstained cross-section is observed under the same oxidation time. This unsuccessful of 5 hours oxidation condition in coarser wools is illustrated in Figures 4.14a and b. Thus, a longer oxidation time is required for coarse and very coarse wools. Figures 4.14c and d show the staining results of coarse and very coarse wools after pass 24 hours oxidation. It is clearly to see that dye intensity on the cross sections are increased and better staining contrast between main cortical cells types can be observed. Thus, the oxidation time around 24 hours is an optimum condition for samples (1, 38). From these results, it can be concluded that, to enhance the staining contrast by methylene blue staining, different oxidation times should be applied because the oxidation time depends on wool diameter.

Figure 4.14 The effect of oxidation time on coarse and very coarse wools; the light micrograph of unstained and stained cross-section of sample set (1, 38) stained with methylene blue for 10 minutes a) and b) 5 hours c) and d) 24 hours oxidation time.
The effect of staining time is also investigated and the results are shown following Figures 4.15a - e. Compared Figures 4.15a and b reveals that at the same oxidation time (2 hours), increasing staining time from 10 to 30 minutes does not impact to the staining results in fine wools. It is clearly to see that there is not a remarkable difference between Figures 4.15a and b. On the other hand, following Figures 4.15a and c, better contrast between para- and orthocortical cells regions are enhanced by increasing oxidation time. The insignificance of increasing staining time over 10 minutes is also confirmed by coarser wools staining results. Figures 4.15d and e show the overview result of sample (1, 38) after wools were stained for 10 and 30 minutes, respectively. The majority of wool cross sections in Figure 4.15e are unstained although longer staining time was applied. However, 10-minute staining time is sufficient to differentiate between ortho- and paracortical cells when longer oxidation time was applied (see Figure 4.15d). The effect of staining time on wool cross section is supported by previous work from J. Menkar and A.B. Coe (1958) who reported that under the light microscope, the Merino wool cross sections were visually complete in 10 minutes at room temperature (Menkart and Coe, 1958).

Figure 4.15 Compared the effect of staining time and oxidation time on staining results; Light micrograph of wool cross sections stained with different methylene blue staining conditions. a) 2 hours oxidation time, 10 minutes staining time b) 2 hours oxidation time, 30 minutes staining time c) 24 hours oxidation time 10 minutes staining time. d) 24 hours oxidation time 10 minutes staining time e) 5 hours oxidation time 30 minutes staining time.
The results reveal that increasing oxidation time is more significantly improving staining results than increasing staining time. Therefore, the best contrast between ortho- and paracortical cells regions are achieved by increasing oxidation time and 10 minutes staining time is sufficient. Three sets of samples were oxidized with different time but all of samples were used the same staining time. The ortho- and paracortical cells arrangement of coarse and very coarse wool can be differentiated by increasing oxidation time. The optimum staining conditions of each sample are summarised following Table 4.2.

**Table 4.2** The summary of methylene blue staining conditions for 4 ranges of wools

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Wool diameter range</th>
<th>Oxidation time (Hours)</th>
<th>Staining time (Minutes)</th>
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<tr>
<td>(1,16)</td>
<td>Fine</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>(1,27)</td>
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<td>10</td>
</tr>
<tr>
<td>(1,38)</td>
<td>Coarse and very coarse</td>
<td>24</td>
<td>10</td>
</tr>
</tbody>
</table>

### 4.4.2.2 The Study of Cellular Structural Arrangement by Methylene Blue Staining

Similar to Janus green staining, lighter and darker staining areas are investigated and staining patterns can be used to reveal the cellular structure of wools. However, the interpretation is different. By using methylene blue, the orthocorical cells are represented by lighter stained areas while darker stained area refers to the paracortical cells. The methylene blue staining results of fine wools; Set (1, 16) show the bilateral arrangement as a majority of cortical cells arrangement. The similar trend had been observed with Janus green staining results which had shown in Figure 4.10a. Interestingly, the over-staining is rarely found by using methylene blue staining. This result imply that specificity of methylene blue staining is higher than Janus green staining.

The methylene blue staining of medium range of wools (set (1, 27)) was investigated under the low magnification. The result shows similar trend to Janus green staining of medium wools. The bilateral arrangement is the majority of cellular arrangement. However, by using higher magnification, the darker staining areas of methylene blue staining reveal more details about biochemistry behind the bilateral structure than Janus green dyes. The comparison
between methylene blue and Janus green staining results of medium wools is illustrated in Figures 4.16 a – d.

Figure 4.16 Comparison the effect of staining agent on staining results; Light micrograph of medium wool cross sections set (1, 27) with diameter range around 23-27 µm a) and b) Methylene blue staining, c) and d) Janus green staining.

Figure 4.16a and b are a cross section of medium wool fibres prepared using methylene blue stain. The stained area with blue stain represents the paracortical cells and the unstained area represents the orthocortical cells. Within the paracortical cells, the uptake of methylene blue dye varies across the cross section, which reflects the distribution of cysteine within the cells. The area with the highest cysteine concentration has the darkest shade of blue on the cross section. Figure 4.16c and d is a cross section of medium wool fibres prepared using Janus green stain. The green area represents the orthocortical cells and the unstained area is the paracortical cells of wool fibre. When comparing these two staining techniques, the different compartments of the wool fibre cross section are better visualised with methylene blue stain. With Janus green staining, it is difficult to see the outline of the paracortical structure and the distribution of cysteine concentration on wool cross sections cannot be observed. Interestingly, Janus green fading
effect, which is more often to be observed in medium range of diameter, might associate with methylene blue moderate staining areas.

The significant differences of staining results between both techniques are observed in the staining result of coarse and very coarse wools (set (1, 38)). As mentioned in the previous part, almost all large cross-sectional images of Janus green staining show uniform staining for the entire cross section (see Figure 4.12b and c). On the other hand, this uniform staining is rarely found when methylene blue was applied. Various staining patterns of coarse and very coarse wools can be observed by using modified methylene blue staining technique. The examples of methylene blue staining results are illustrated following Figures 4.17a – f. The results show that the intensities and staining patterns of coarse and very coarse wools are more widely various than fine wools. The majority of coarser wools staining pattern is illustrated following Figure 4.17a – c. Generally, the pseudo-random distribution of heavy stained areas and low staining contrast between main cortical cells types are observed. Radial symmetry (annular) and two cords of paracortical cells on the opposite side (Multilobate) are also observed in this sample (see Figures 4.17 d and e). Unlike fine wools, a bilateral arrangement is the minority of staining pattern for coarse wools. The comparison of bilateral arrangement between fine and coarse wools reveals that bilateral arrangement becomes less distinctiveness with increasing wool diameter. Additionally, the staining intensity across cross sectional area is different. In general, bilateral arrangement of fine wools shows the separation between light (unstained) and heaviest staining areas (see Figure 4.13c). However, at the same oxidation time, the separation between moderate and heaviest staining is observed for bilateral arrangement in coarse wools (see Figure 4.17f). This moderate staining in bilateral arrangement provides some tentative evidence that cells staining with intermediate intensities might not directly represent mesocortical cells.
Figure 4.17 Staining patterns of coarse and very coarse wools; Light micrograph of wool cross sections Set (1, 38); stained with Methylene blue a) Coarse wool fibre with a random distribution of small light and dark staining areas, b) and c) Very coarse and coarse wool fibre with low staining contrast between main cortical cells, d) Coarse wool fibre with radius distribution, e) Coarse wool fibre with both sides staining f) Coarse wool fibre with low contrast of bilateral segregation.
Until now, a weak to moderate staining intensity is the difficulties of microscopic cell type differentiation and has long been a question for cellular structural analysis. The gold standard interpretation of this moderate staining hasn’t been concluded. In the past, numerous studies reported that the moderate staining on wool cross section is a representative of mesocortical cells. This interpretation is based on the TEM investigation which is significant technique to confirm the existence of mesocortical cells. As mentioned in Chapter 1, this cell was described as a hybrid between ortho- and paracortical cells. The cysteine content of mesocortical cells is higher than orthocortical cells but less than paracortical cells and this cell type are classified as a minority of cortical cells types (Orwin et al., 1984). In this study, however, the moderate staining area is not the minority compartment in the cross-sectional area. The moderate stained area is observed more than 50% of total cross-sectional areas; especially coarse and very coarse wools (see Figures 4.17a - c). Therefore, this moderate staining might not directly associate with the mesocortical cells. This moderate staining might occur from loss certain regions between ortho- and paracortical cells. This mean the cortical cells structure of coarse and very coarse wools might be similar to human hair which is no distinguish regions between ortho- and paracortical cells (Popescu and Hocker, 2007).

To sum up, both staining results suggest that the dye uptake for fibre stained with methylene blue increase remarkably compare with Janus green. Generally, Janus green staining is bimodal staining system, while methylene blue staining shows various staining intensities. Therefore, better visualisation of the cortical cells arrangement of coarse and very coarse wool is achieved by using modified methylene blue staining technique. This better staining result of methylene blue confirms the fact that coarser wools tend to compose of higher proportion of cysteine content than finer wool (Orwin et al., 1984, Wortmann and Wortmann, 2018). In addition, the result suggests that the distribution of cysteine concentration can be observed by modified methylene blue staining method. Although Janus green is not as sensitive as methylene blue staining technique, especially for coarser wools, the staining results from all ranges of wool diameters indicate that both techniques reflect a similar trend. The staining contrast between main cortical cells generally decreases associated with increasing wool diameter which is consistent with previous observations (Orwin et al., 1984, Plowman et al., 2019). These results indicate the relationship between wool diameter and cortical cells arrangement.
4.4.3 Quantitative Analysis of Wool Cross Section

4.4.3.1 Effect of Method Selection and Human Error Evaluation

In this part, two image analysis methods were applied to determine a more precise and accurate way of measuring wool cortical cells. 45 cross-section images were measured twice, using subjective and automatic threshold methods, respectively. Set (1, 16) were stained with Janus green stain. Three different areas of cross section were measured total cross sectional, ortho- and paracortical cell areas, using image J software. See Section 4.3 for more details about the measuring methods. Figures 4.18a - c show the results of total cross-sectional area, ortho- and paracortical cells areas obtained using both subjective and automatic threshold methods. There is a strong positive relationship between the measurements of all three areas obtained using subjective and automatic threshold methods, with a linear coefficient ($R^2$) greater than 0.95. When measuring the total cross-sectional areas and paracortical cell areas, both methods produce very similar results (see Figures 4.18a and b). Whereas there is a significant difference in the results of orthocortical cell areas (see Figure 4.18 c). The y-intercept of this graph is higher than the previous two graphs, which implies that the results of orthocortical cell areas measured using automatic threshold setting method is significantly larger than the subjective method.

One of the explanations that could account for this is that the cuticle cells, which are also stained by Janus green, have a similar threshold for staining as orthocortical cells. Thus, by using the automatic threshold setting method, the cuticle cells areas are measured as part of the orthocortical cells area. This hypothesis is further confirmed by comparing the total cross-sectional areas with the cortex areas (paracortical cell area + orthocortical cell area). The majority of the total cross sectional areas from the automatic threshold setting method is smaller than the cortex areas. The mean percentage of the total area is smaller than the cortex area. The average ($\pm$ SD) percentage differentiation of these two areas is 2% ($\pm$ 3.8). On the other hand, the average result ($\pm$ SD) from subjective method show larger area of total cross sectional area than cortex area around 7 $\pm$ 3.7 %, which is more reasonable. These findings suggest that automatic threshold setting is not able to distinguish between the cuticles cells and orthocortical cell. Moreover, unreliable results might increase when staining contrast decreases. This is the major
disadvantages of utilizing automatic threshold setting. Therefore, it is reasonable to say that subjective method is a more superior measuring method for this study.

**Figure 4.18** Comparison of measurement results between subjective and automatic threshold setting method for sample (1,16), N = 45; a) Total cross sectional area, b) Paracortical cells area, c) Orthocortical cells area.
However, there are still some concerns about human errors in using subjective approach to measure wool fibres. There is a concern subject to the operator-based measuring approach. In order to determine whether subjective measurements could be used to differentiate the main cortical cells types, the uncertainty from human error is examined. The impact of human error on measurements accuracy of this study is evaluated using statistical analysis. The error was assessed by drawing the same cross section three times. In order to determine whether human error or operator bias account for the inaccuracy of measurements, we plot the dispersion of standard error after eliminating the effect of area against mean wool diameters, as shown in Figure 4.19. Random error is observed in the dispersion of human errors. This random error is a statistical fluctuation in the measured data and can be reduced by a large number of observations. Instead of re-measuring the sample multiple times (as conducted in this session), therefore, the sample size of the study is increased. A wider range of wool fibres can be included and this could increase the validity of the study. Wool fibres with eight different mean diameters are included in the study. 100 cross sectional images are obtained from each group, yielding 800 images in total.

![Figure 4.19](image_url)  
**Figure 4.19** Standard errors of subjective measurements after normalising the effect of area against mean wool diameters.
4.4.3.2 The Relationship between Cortical Cells Types and Wool Diameter

In this study we used Janus green staining to differentiate ortho- and paracortical cells. Stained cross sections with various diameters were investigated by light microscope and images captured by digital camera. Image analysis was performed by image J software using operator-based method to measure the interested areas. The data reveals that from the total of 800 cross sectional images, the verified result is round 67% (see Section 4.3 for results verification method). The diameters of the wool fibres range from 9 to 54 µm. 83% of verified results are fine and medium wools, followed by coarse and very coarse wools, respectively. This suggests that there is less segregation between para- and orthocortical cell as the wool diameter increases, which is consistent with qualitative analysis results of this study.

The data are plotted and shown in Figures 4.20a and b. These illustrate the relationship between wool diameters and percentages of ortho- and paracortical cells areas, respectively. Although wool fibres are from different sets of samples, a similar trend for cortical cell proportions is found when wools with the similar diameter are investigated. Therefore, the relationship between diameter and cortical cells areas might depend on fibre diameter rather than sets of samples. The vast majority of fibres (around 90%) consist of greater percentage area of orthocortical cells than paracortical cells, especially fine wools. Small numbers of fibres were found to consist of > 50% paracortical cells. Coarse and very coarse wools tend to show the greater percentage area of paracortical cells than fine wool. However, there is no clear relationship between wool diameter and percentage areas of main cortical cell types. Figures 4.20a and b show weak linear relationship between wool diameters and percentage area of main cortical cells types, with a linear coefficient of determination (R²) around 0.2. This result is statistically significant (p-value < 0.01). However, the change of cortical cell percentage is small compared to the size of sample. Thus, the p-value may reduce according to the large sample size. Since the trend is weak, it might be too early to conclude that there is a relationship between percentage areas of main cortical cells types (ortho- and paracortical cells) and wool diameters.
Figure 4.20 The relationship between percentage areas of main cortical cells types and wool diameters obtained from image analysis a) Orthocortical cells and b) Paracortical cells.

This finding is inconsistent with Orwin’s study (Orwin et al., 1984), which demonstrated a strong linear correlation between wool diameter and cortical cell proportion. They found that as wool diameter of Merino sheep’s increases, the orthocortical cells proportion also increases. A log-linear correlation between increasing proportions of orthocortical cells and increasing fibre diameters was also reported in other breeds of wools such as Drysdale and Romney (Orwin et al., 1984). One of the possible explanations for this might be most likely attribute to wool sample.
The specimen in Orwin’s study was collected from single sheep, whereas wool tops is used in this study.

In addition, different experimental methods including the staining technique and interpretation might account for these contradicting findings. Orwin (1984) used Methylene Blue staining to differentiate between ortho- and paracortical cells. (Orwin et al., 1984). Although the sensitivity of Methylene Blue is better than Janus Green staining, especially in coarse and very coarse wools, various intensities of dye uptake might cause a bias from operators. This might affect to the accuracy of the measurements. Furthermore, the interpretation of the relationship between wool diameter and cortical cells proportions was based on the mean value. Samples were classed according to their diameter. Orwin used the mean values of ortho- and paracortical cells areas were used for each range of diameters to determine the correlation. Therefore, it is difficult to compare Orwin’s (1984) results directly with the result from this study due to the different approaches to interpretation. Further investigation and experimentation into the relationship between wool diameter and cortical cells proportions is strongly recommendation. Using a broader range of analytical techniques or integration the result from different analytical technique might fill this gap of question.
4.5 Conclusion

In this chapter, light microscopic analysis of stained Merino wool cross sections was conducted. The main goal is to gain a better understanding of the relationship between the cortical cell structure and wool diameters, both qualitative and quantitative. This research has led to the successful modification of staining methods to enhance the visualisation of cortical cell areas. Increasing reaction temperature was the key factor to enhance the Janus green staining contrast for coarser wools. The optimum staining temperature was determined at 80°C. Meanwhile, oxidation time was the key factor to enhance the methylene blue staining contrast. As mentioned in Section 4.4.2, the optimum oxidation time for fine and medium wools was 5 hours while coarse and very coarse wools was 24 hours. After the acid oxidation process, the best methylene blue contrast between main cortical cells types was achieved by staining at room temperature for 10 minutes.

Both modified staining methods allowed us to see the cellular structural arrangement of the main cortical cells types. The qualitative analysis under the light microscope was able to observe differences of cellular arrangement between finer and coarser wools. The majority of cellular arrangements for fine and medium wools are the bilateral arrangement. Less segregation between ortho- and paracortical cells was observed with increasing wool fibre diameters. These staining results were observed for both staining techniques. Meanwhile, various staining patterns such as two cords of paracortical cells and radial are only observed by using methylene blue staining technique.

The comparison of these two staining results shows significant differences of staining results between Janus green and methylene blue. Better staining results were observed in coarse and very coarse wools. The modified methylene blue staining was able to determine differences of cellular arrangement in coarser wool better than modified Janus green staining method. In addition, different dye uptake of methylene blue varies across the stained areas was observed. This is suggested to reflect the distribution of cysteine within cross sections. Thus, the results suggest that this moderate staining might not directly be associated with the mesocortical cells. However, this moderate staining areas is the further question related to this microscopy analysis.
For the quantitative study, repeatability, precision and accuracy of the image analysis methods were investigated. It was found that inaccuracy of orthocortical cell areas was observed by using the automatic threshold setting method. Stained areas of cuticle cells were counted as part of the orthocortical cell areas. Due to this result, the subjective, that operator-based method was shown to be the test approach to take forward for image analysis. The most interesting finding to emerge from this study was that there was an unclear relationship between percentage areas of main cortical cells types and wool diameters. This result was contrary to a previous study that shown linear correlation between orthocortical cell proportions and Merino wool diameters ($R^2 = 0.95$) (Orwin et al., 1984). However, with a small sample size and wool sample from individual sheep, caution must be applied, as the Orwin’s finding might not be generally applicable. In addition, several factors could explain this inconsistency including, staining procedure and different approaches of data analysis.

In summary, this study appears to be one of the first attempts to compare the advantage and limitations of Janus green versus methylene blue staining. The results suggested that both staining method could be applied for wool cortical cells study in different ways. According to the better sensitivity of methylene blue staining, this dye was more suitable for the qualitative analysis such as cellular structural arrangement study. On the other hand, Janus green staining might be the better method for the operator-based, quantitative analysis. The simple interpretation of Janus green staining might reduce the bias from operators. There were a few remaining questions that would require further studies. Firstly, the presence of mesocortical cells as a distinct cell group. Cross sections which show moderate methylene blue staining areas may represent the transition between ortho- and paracortical cells. Thus, mesocortical cell might unable to be confirmed by light microscopy technique. Another remaining question was the relationship between the proportion of cortical cells and wool diameter. More research is required to develop a deeper understanding of this relationship. Using a broader range of analytical techniques or integration of results from different analytical technique might address the questions raised by this study.
4.6 References


Chapter 5 The Study of Relationship between Thermal and Optical Analysis of Merino Wool Cortical Cells

5.1 The Study of Keratin Fibres by Multiple Analytical Techniques

5.1.1 The Study of Keratin Cortical Cells Morphology by Multiple Optical Analysis Tools

Most research on cortical cells morphology of keratin fibres carried out in transmission electron microscopy (TEM). Differences between the cells types presented in the cortex of keratin fibres have long been recognised using this technique. Numerous studies had attempted to explain and classify a cellular structural arrangement of each cortical cells type. Up to date, ortho- and paracortical cells are in generally agreement of the main cortical cells in wools (Rogers, 1959, D. F. G. Orwin and Bailey, 1988, Caldwell et al., 2005, Harland et al., 2011). According to reliable of TEM data, this microscopic results were integrated with other optical techniques to support and provide more reliable results (Donald F.G. Orwin et al., 1984, Bryson et al., 2009), as shown in Figure 5.1a and b. As mentioned before, a well-known study by Orwin reported the relationship between TEM and light microscopy (LM) technique of wools. TEM data is a good indicator of methylene blue staining. The deepest methylene blue intensity areas of oxidised wools on a light micrograph correspond to paracortical cells region on TEM. Moderate and unstained areas reflect the meso- and orthocortical cells, respectively (Donald F.G. Orwin et al., 1984).

Recently, the connection between TEM and fluorescence light microscopy (FLM) of human hair cortical cells was reported (Bryson et al., 2009). Fluorescence stain colour was observed to connect to the cell types, as determined from TEM. Paracortical cells were stained by Fluorescein sodium (FS) dye, which is green. Orthocortical cells and sub-group of orthocortical cells show red sulforhodamine 101 (SR) stained (Bryson et al., 2009). In addition, FLM micrographs showed bilateral arrangement and annular cortical cells distribution patterns in curved and straight hairs, respectively. This finding from FLM further confirmed differences of cortical cell arrangement between curved and straight hairs, which were also observed by using
methylene blue and Janus green staining (Rogers, 1959, Horio and Kondo, 1953, Donald F.G. Orwin et al., 1984). This combination between TEM and other LM techniques suggest more comprehensive aspect of cortical cell arrangement because of the different working principle. TEM differentiated cell types relied on their macrofibrils appearance and IF arrangements, while LM and FLM distinguished cell types on basis of the reaction between matrix proteins and dyestuffs.

![Figure 5.1](image)

**Figure 5.1** The relationship of cortical cells observation from variation of microscopy techniques. a) the relationship between TEM and LM; “TEM of a transverse section of a Merino wool fibre stained by reduction and osmication” An Inserted imaged on left corner shows an adjacent section of methylene blue staining from the same fibre as seen by LM; heavily stained is the paracortical cell (P), lightly stained is mesocortical cells (M), and unstained is the orthocortical cells(O) (Donald F.G. Orwin et al., 1984), b) and c) the relationship between TEM and FLM (Bryson et al., 2009).
Although TEM studies over the past two decades provided important information in terms of morphological features on microstructures of cortical cells, the TEM data reflect on limited to that on the surface of fibre cross sections (Kajiura et al., 2005). Several studies extended a classification system of cortical cells types based on structural parameters such as IF helical angles and IF–IF centre to centre spacing rather than on morphological analysis (TEM) (Dobb, 2009, Plowman et al., 2019). The integration of low-angle electron-diffraction and microscopic analysis was one of the first attempts (Dobb, 2009). Scanning microbeam small-angle X-ray scattering (SAXS) was another technique that was integrated with microscopy technique to investigate macrofibrils ultrastructure, which was an important criterion for cortical cells classification (Kajiura et al., 2005). Recently, a combination of TEM and electron tomography (ET) techniques used in cortical cells study was another significant approach to addressed TEM limitation and allowed better understanding of IF arrangement in each cortical cell type (Bryson et al., 2009, Caldwell et al., 2005, Harland et al., 2011, Harland et al., 2014, Plowman et al., 2019).

ET is “a method of generating 3D images from multiple 2D projection images” (Roque and Antony, 2010). According to enhancement of computer-controlled stages on the TEM and more reliable Charge-coupled device (CCD) cameras, ET technique become a useful tool to model three-dimensional (3D) orientation and arrangement of IFs within different cortical cell types (Bryson et al., 2009, Hall et al., 2012). Several studies of 3D IFs arrangements of wools revealed a consistent result with 2D TEM observation (Caldwell et al., 2005, Harland et al., 2011). In orthocortical cells of wools, IFs arrange helically along the macrofibrils (whorl-like). While meso- and paracortical cells IFs are predominantly aligned parallel to the macrofibril axis (Caldwell et al., 2005, Harland et al., 2011).

IF helical angles and IFs spacing between macrofibrils are able to be determined by ET method. This leaded to broader perspectives of IFs arrangement within different cortical cell types. A strong relationship between IF helical angle and distance from the macrofibril center was observed in orthocortical cells followed by para- and mesocortical cells, respectively (see Figure 5.2) (Caldwell et al., 2005, Harland et al., 2011). In Romney wools, IF–IF center to center spacing of orthocortical cells showed significantly smaller than the spacing in the meso- and paracortical cells (Caldwell et al., 2005). Modeled IFs arrangement from mesocortical cells
showed more regularly and tightly packed than paracortical cells (Caldwell et al., 2005, Harland et al., 2011). However, there was no significant difference in IF spacing between the meso- and paracortical cells (Caldwell et al., 2005). Importantly, tomography is able to reveal a dissimilarity of macrofibrils arrangement between sheep breeds. Although the macrofibrils tilting of orthocortical cells in Romney and Merino wools appeared to be predominantly tangential rather than radial direction, a helical turn was different. Romney wools showed right-handed helical turn (Caldwell et al., 2005). In contrast, left-handed helical turn was found in majority of Merino wools (Harland et al., 2011, Harland et al., 2019). In addition, in orthocortical cells, Merino wools, which was higher average curvature, showed greater values of IF increment data for orthocortical cells than Romney wools (Caldwell et al., 2005, Harland et al., 2011). This difference in orthocortex IF increment angles between sheep breeds may associate with fibre curvature (Harland et al., 2011, Harland et al., 2014).

When comparing TEM results between wools and human hairs, more hardly distinguished cell types is found in human hairs, which is a significant difference between both fibres. Modelled IFs arrangements of hairs showed varying architecture within a single cell (Harland et al., 2014). Recently, the Japanese hairs examined with TEM showed the evident that cortical cell types would be more than three types (Bryson et al., 2009). At high-magnification, cortical cells were distinguished into four types (Type A, B, C and D) (Bryson et al., 2009). Type A and B cortical cells exhibited macrofibrils and IFs arrangements similar to orthocortical cells of wools, while Type C and D cells contained fused macrofibrils arrangements similar to the meso- and paracortical cells (Bryson et al., 2009). Type B and C were found as a predominant cortical cell types. According to this finding, the conventional definitions of wool cortical cell types by TEM investigation were not sufficient and were incompatible with observations of Japanese hair cortical cells (Bryson et al., 2009, Harland et al., 2014, Plowman et al., 2019). Importantly, integration of TEM data with tomography technique could differentiate between Type A and B. Type A cells were composed “entirely of double-twist macrofibrils with a clear ‘whorl pattern’ and high IF increments (called high-intensity)”, while Type B showed mixture of low- and high intensities (Harland et al., 2014).
Figure 5.2 The results of 3D images of IFs arrangement: (A–C) Model of IFs from the ortho-, para-, and mesocortex, respectively. Tomographic regions are displayed on the left, with corresponding modelled representations of IFs arrangements on the right. In each case, the model in the plane perpendicular to the macrofibrils axis is tilted in 45° increments. (D–F) Model rows of IFs from the ortho-, para-, and mesocortex, respectively. In each case, the model is rotated around the macrofibril axis in 30° increments. Dimensions at the bottom of the figure are in nm measured from the macrofibrils centre (Caldwell et al., 2005).
Fast Fourier Transform (FFT) power spectrum was another analytical method that combined with TEM and ET to investigate a different arrangement of macrofibril within different cortical cells types (Harland et al., 2011, Harland et al., 2014, Kadir et al., 2017). FFT results relied on the average data from measuring tens or hundreds of individual IFs within a macrofibril (Harland et al., 2011). The Merino wool study by Duane P. Harland et. al, (2009) is one of the significant works that applied FFT method and the results were shown in Figure 5.3. They found that the significantly greater IF center-to-center distance was observed in para- followed by meso and ortho- macrofibrils, respectively (Harland et al., 2011). Proportion of matrix determined for all three cortical cell types using FFT method confirm expectations based on earlier studies. Paracortical cells consisted of the highest proportion of matrix volume fractions followed by meso- and orthocortical cells, respectively (Donald F.G. Orwin et al., 1984, Harland et al., 2011).

Although combined ET and FFT in cortical cells study could reveal more details of macrofibrils arrangement, these methods based on TEM images. In TEM sample preparation processes, fibres are necessary to pass strong chemical modification processes such as chemical fixation, and impregnated with heavy metals (Kajiura et al., 2005, Harland et al., 2011). In addition, the fibres need to be in a very dry stage. Thus, it should be noted that TEM sample preparation processes might affect to IFs arrangement. This affects IF angle and IF spacing may differ from fibre under normal environmental conditions. Modelling and interpretation process are limitation of this TEM based method. It was reported that in high complexity of cortical cells structure such as human hair, the thickness of section affect to the interpretation of result (Harland et al., 2014, Plowman et al., 2019). In addition, several studies reported the difficulty of selected tomographic regions between meso- and paracortical cells. This resulted in the reliability and clarity of the modelled IFs arrangement of these two cell types (Caldwell et al., 2005, Browne and Hindson, 2008, Plowman et al., 2019).
5.1.2 Integrating Various Techniques for Keratin Fibre Studies

According to limitations of each analytical technique, there is a necessary for researcher to involve a greater variety of techniques in order to clarify and broaden the knowledge of keratin fibre. In the view of cellular structure of cortical cells, using microscopy techniques are able to show the IFs arrangement and the morphology of cortical cells. On the other hand, identify specific proteins expressed in different cortical cell types were achieved by using proteomic techniques. Isoelectric focusing-2DE (IEF-2DE) and mass spectrometry (MS) are one of the proteomic techniques that were applied for keratin fibre study. For example, MS and IEF-2DE were used to identify specific proteins expressed between ortho- and paracortical cell of Merino wool (Plowman JE et al., 2009, Thomas et al., 2012, Plowman et al., 2019). Using proteomic techniques, Plowman et. al. (2009) found the higher concentrations between the present of keratin associated proteins 3 (KAP3) family and paracortical cells. This finding provided an explanation for the higher cysteine content reported in the paracortex. In addition, this protein might have an influence on fibre crimp (Plowman JE et al., 2009). Further investigation using
combination between microscopic and proteomic techniques also suggested that protein profiles may link with the changes in morphology of cortical cells and fibre properties (Thomas et al., 2012). Recently, unexpected result was presented by integrating three largely different tools for investigating keratin fibres, namely TEM, solid-state NMR and Transient Electro-Thermal Technique (TET). This finding revealed new evidence that matrix is not amorphous phase (Kadir et al., 2017).

Thermal analysis techniques, especially DSC, were another technique that has been employed in several keratin studies, especially in hair fibres. A corroboration of DSC with other techniques such as X-ray diffraction, tensile stress–strain and Fourier transform infrared spectroscopy (FTIR) provides more robust and reliable results (Popescu and Gummer, 2016, Leite and Maia Campos, 2017, Lima et al., 2018). For example, the DSC investigation of oxidative damage keratin fibres treated with low pH solution shown an increase of enthalpy and a shift of the peak temperature towards higher temperature. This unexpected finding was further confirmed by amino-acid analysis, X-ray diffraction, Raman spectroscopy and tensile strength observations (Istrate et al., 2013). DSC and microscopy techniques could be combined to investigate structural changing of cuticle cells. After chemical modification, lower denaturation enthalpy was observed by DSC. This reflected the degradation of hair fibre. The DSC investigation was further confirmed by the appearance of damage cuticle cell, which was observed by atomic force microscopy (AFM)(Monteiro et al., 2005).

In the view of cortical cell study, the relationship between the DSC curve characteristics and cortical cell types of alpaca fibres was reported (Shim, 2003). Huacaya fibres showed a bimodal endothermic peak, while Suri fibres displayed a single endothermic peak. These DSC results related to their cortical cell types distribution which was observed by SEM. The SEM images revealed that Suri fibre had only paracortical cells, but both ortho- and paracortical cells were observed in Huacaya fibre. This finding suggested that both DSC and microscopy techniques are reliable techniques to distinguish Huacaya and Suri alpaca fibres (Shim, 2003). Recently, another connection between thermal analysis and microscopy images was reported by Wortmann (F. J. Wortmann and G. Wortmann, 2018). The appearance of morphological arrangement of cortical cell types, obtained by TEM, associated with the characteristics of DSC-
peak deconvolution. They purposed that the lack of contrast between cell types in fibre cross sections in the TEM might related to the temperature difference between the main cell types (Peak III and II) (See Figure 5.4). In wools, which is a high contrast between ortho- and paracortical cells, the temperature difference of both main peaks was 6°C. This temperature difference of hair, which lack of contrast between main cell types, was found around 3 – 4 °C (F. J. Wortmann and G. Wortmann, 2018). These obtained results from previous studies showed that DSC is an interesting alternative technique to combine with microscopy technique. A combination of these data may enlarge the knowledge of the keratin fibre study.

![Figure 5.4](image.png)

**Figure 5.4** The relationship between DSC deconvolution curves and cross section of TEM. “The DSC curves in water are deconvoluted using three Gaussian distributions of equal width (standard deviation). For each of the peaks was labelled as I-III with increasing temperature” (F. J. Wortmann and G. Wortmann, 2018). a) Merino wool with bilateral arrangement, and b) hair with less contrast of cortical cell types.
5.2 Objectives

In Chapter 5, microscopy and DSC analysis techniques were applied to study cellular structure of main cortical cells types. Both analytical techniques were performed on Merino wool top with various diameters. The aim of this chapter is to examine relationships between cortical cells appearance in light microscope and the DSC deconvolution results. A combination of quantitative and qualitative approaches was used in the data analysis. In order to achieve this aim, this research was designed to address the following objectives;

• Comparison of the cortical cells proportions results obtained from DSC deconvolution and light microscopy analysis. Do both analytical techniques show the similar trend of a relationship between cortical cells proportions and wool diameters?
• Investigation the relationship between Janus green staining patterns and DSC-deconvolution curves results.
• Investigation the possibility of using DSC deconvolution as a complement method to the microscopic methods.

5.3 Experimental

The integration of results based on thermal (DSC) and optical analysis (LM) were applied in this chapter. The data of DSC and microscopic analysis of wool cortical cells obtained from Chapter 3 and 4, respectively. It should be noted that the samples for DSC investigation were Set (1) and (2), while only fibres from Set(1) was applied for microscopy technique. A combination of quantitative and qualitative approaches was used in this data analysis. In term of quantitative analysis, the results of cortical cell percentages from both techniques were compared. A general linear regression models and t-test were applied to determine the relationships between wool diameter and cortical cell percentages.
5.4 Results and Discussion

5.4.1 The Comparison of Different Analytical Techniques for Cortical Cell Percentages Determination

As mentioned in Chapter 3 and 4, both thermal and microscopy techniques were applied to find the cortical cell percentages. The relationship between wool diameters and cortical cell percentages was also examined. As an overview, a trend of the relationship between wool diameters and cortical cell percentages, obtained by both techniques is similar, although both techniques are based on different working principle. The results, obtained by DSC deconvolution, reveal that there is no relationship between wool diameters and cortical cell percentages. The results obtained by image analysis show a weak relationship between wool diameters and cortical cell percentages.

Figures 5.5a and b show the comparison of cortical cell percentages that are obtained from DSC-deconvolution and image analysis of Janus Green staining. The range of paracortical cell percentages investigated by DSC deconvolution is during 5 – 44%. Using image analysis method, wider distribution of paracortical cell percentages is observed (5 – 91%). However, both show the majority of the paracortical cell percentages during 21 – 40%. In addition, almost of the paracortical cell percentages, obtained using DSC deconvolution, fit in with the results of paracortical cells percentages from image analysis investigation. However, the deconvolution results have one outlier, which is Set (2, 32). This outlier might relate to different sources of sample. Since these outliers are the data from Set (2), while the image analysis results consist of the data from Set (1).

In view of orthocortical cells, the orthocortical cell percentages using DSC deconvolution refer to the summation of ortho-like (O1) and typical orthocortical cells (O2). Unlike the thermal technique, the microscopy technique cannot distinguish between O1 and O2. Thus, all staining areas refer to orthocortical cells in image analysis methods. Figure 5.5b displays a comparison of orthocortical cell percentages obtained with these two methods.


Figure 5.5 Comparison of cortical cell percentages results obtained with two methods; DSC deconvolution, and Image analysis of Janus green staining, for assessing cortical cell proportions

a) Paracortical cell proportions  
b) Orthocortical cell proportions.
Similar to paracortical cell percentages, image analysis gives a wider distribution of orthocortical cell percentages than DSC peak deconvolution method. A few outliers of DSC deconvolution results are observed. Almost of the outliers data are from Set (2) and all of these outliers show greater value of orthocortical cell proportions than the results using image analysis estimation. Interestingly, the majority of data between both methods is different. The majority of data obtained using DSC-deconvolution is during 61 – 80%. While, using image analysis method, the majority of orthocortical cell percentages are during 51 – 70%. These results suggest that the orthocortical cell percentages results using DSC peak deconvolution tend to be greater than the percentages that are measured by image analysis method. There might be two likely causes for the differences between these analytical results. First, this level of difference in values obtained with the two methods probably is representative of measurement noise. It has to be noted that the Image analysis measurement was set 10% lose from the cuticle cell, so this measurement condition might be one of the possible reasons. Another possibility is the difference of working principle. Each data point of image analysis reflects the data of single fibre while more than 100 snippets are involved in each data point of DSC deconvolution. The greater orthocortical cell percentages using DSC deconvolution might be affected by variation of snippets.

In summary, both techniques are the feasible approaches for the quantitative analysis of cortical cells. The percentage areas of main cortical cell types can be measured by both techniques and the results from both techniques are similar. In addition, in term of the relationship between wool diameter and cortical cells, the results from both techniques are inconsistent with Orwin's study (Donald F.G. Orwin et al., 1984).
5.4.2 The Relationship between Staining Patterns and DSC Deconvolution Curves

It has demonstrated previously that the staining patterns of wools, especially methylene blue staining, corresponded to wool diameters (See Chapter 4). The majority of fine wools shows the bilateral arrangement with clear separation between ortho- and paracortical cells. The lack of contrast between the bilateral arrangement is found when wool diameters increase. Coarse and very coarse wools show various staining patterns, but the majority of staining pattern is the pseudo-random distribution of heavy stained areas with low staining contrast between the main cortical cell types. These staining results are in line with those of previous studies (Donald F.G. Orwin et al., 1984, Plowman et al., 2019). In view of DSC deconvolution, the characteristic of the deconvolution curves between fine and very coarse wools is different and this trend is observed in both sets of samples. Figures 5.6a and b show the deconvolution curves of fine and very coarse wools, respectively.

The relationship between DSC deconvolution curves characteristic and staining pattern is observed. The results suggest that finer wools with strong bilateral arrangement conform to the clear separation of DSC deconvolution curves (Peak II and III). On the other hand, DSC deconvolution curves of very coarse wools are more scarcely distinguishable. This characteristic curve shows a relationship with low staining contrast between the main cortical cell types that observed as a majority pattern in coarser wools. In addition, a segregation of deconvolution curve is more hardly in coarser wools. These results might suggest that single endothermic peak tends to be found in coarser wool, while DSC peak of finer wools is the bimodal endothermic peak.

As mentioned in Chapter 3, the analysis of relationship between the temperature differences between ortho- and paracortical cells and wool diameters indicates the negative linear relationship. Both sets of samples show a linear coefficient of determination ($R^2$) around 0.5 with statistically significant (p-value < 0.01). Following Figure 5.7a, it is clear to see that the temperature difference between the main cell types (peak III and II) for fine wools is about 4°C while it drops to 2°C or less than 1°C in very coarse wools. The reason behind a drop of the temperature difference between main cell types in DSC deconvolution might relate with the difference of sulphur content.
Figure 5.6 The relationship between DSC deconvolution curves and methylene staining patterns. DSC deconvolution curves of Merino wool in excess water. The curves are deconvoluted using three Gaussian distributions of equal width (See Chapter 3 for detail of method). The inserted image in the left corner is methylene blue staining pattern of wools. a) Fine wools; Set (1,16) and b) Very coarse wools; Set(1,38).
Further investigation is performing by combined this negative relationship with the staining patterns, as shown in Figures 5.7a - c. We found that clear separation of staining contrast between ortho- and paracortical cells in fine wool (see Figure 5.7a and b) link with higher denaturation temperature difference between ortho- and paracortical cells. Additionally, Less distinguish of main cortical wells in coarse wools (see Figure 5.7a and c) is observed when the denaturation temperature differences of the main cortical cells decrease. This finding is further supported by previous investigation from Wortmann (F. J. Wortmann and G. Wortmann, 2018). They found that the denaturation temperature difference between ortho- and paracortical cells corresponded to the lack of contrast between cell types in hair, which is observed by TEM (F. J. Wortmann and G. Wortmann, 2018). So, it might be concluded that the relationship between the denaturation temperature difference of main cortical cells and the staining pattern is observed.

Up to now, several optical analysis studies are in agreement about the relationship between crimp of wool and wool diameter. Finer wools present higher crimp than coarser wools and this hypothesis is clearly observed in Merino wool (Popescu and Hocker, 2007). Moreover, there are good agreements that cortical cells arrangement influence to the crimp of wools. The staining pattern of high crimp wools tends to present bilateral arrangement, which clear separation between ortho- and paracortical cells while less distinguish of main cortical cells tends to be found in low crimp wools. According to these optical analysis agreements, the relationship between crimp of wool and denaturation temperature between ortho- and paracortical cells is further confirmed. Higher crimp of wools tend to show greater denaturation temperature difference between ortho- and paracortical cells.
Figure 5.7 The relationship between the thermal analysis (using DSC deconvolution) and optical analysis (using methylene blue and investigated by light microscope). a) The influence of wool diameter and drop of para- and orthocortical cells denaturation temperature difference ($T_{D,III} - T_{D,II}$), b) and c) Cross sectional images of fine and coarse wool, respectively with different arrangement of cortical cells showing the relationship between cortical cells arrangement and wool diameter. b) Strong bilateral separation between para- and orthocortical cells implied higher denaturation temperature difference between para- and orthocortical cells in fine wools, and c) less contrast between para- and orthocortical cells arrangement implied lower denaturation temperature difference between para- and orthocortical cells in coarse wools.
5.5 Conclusion

In this chapter, the ortho- and paracortical cell percentages were measured by two analytical techniques; DSC deconvolution and microscopy techniques. The DSC investigation is a large-scale study. More than thousands of wool snippets were investigated by this technique. Thus, results from this technique are generally applicable. On the other hand, microscopy technique is limited on sample size and time consumption. However, this technique is conventional method for measuring cortical cell areas. The integration between both techniques provides more reliable results on the relationship between wool diameter and cortical cells.

Generally, the results from both techniques present a similar trend in quantitative and qualitative analysis. At similar diameter, the ortho- and paracortical cell percentages obtained by both techniques are similar. Both techniques showed that the majority of cortical cells is orthocortical cells. The relationship between ortho- and paracortical cell percentages and wool diameters obtained from image analysis and DSC deconvolution also shows the similar trend, which is inconsistent with Orwin’s report. However, in this study, quantitative analysis is based on large-scales study, which is different from Orwin (1984). More than thousands of wool snippets were investigated by the DSC technique. Additionally, this relationship is further supported by microscopy technique, which is the conventional technique. Thus, it can be conclude that there is no significant relationship between wool diameter and main cortical cell percentages.

In the view of qualitative analysis, two significant relationships are found. First, the negative linear relationship between wool diameters and the denaturation temperature differences of main cortical cells types (ortho- and paracortical cells) is observed. Another relationship is the relationship between staining patterns and denaturation temperature difference between ortho- and paracortical. A drop of denaturation temperature difference between ortho- and paracortical cells link with less distinguish of staining contrast of the main cortical cell types that is observed as a majority of staining pattern in coarser wools. On the other hand, in fine wools, a strong bilateral arrangement conforms to the greater difference of denaturation temperature between ortho- and paracortical cells.
Integrated these two relationships might found that high denaturation temperature difference of main cortical cells link with high crimp of wools according to the general agreement that high crimp wools tend to be found in finer wools and they have a strong bilateral arrangement between orho- and paracortical cells. Moreover, these relationships might reveal the difference of sulphur content between fine and coarse wool. Thus the difference of sulphur content between ortho- and paracortical cells in coarse wools might less than fine wools. This assumption is in line with methylene blue staining results.

In summary, both techniques can be applied for cortical cells study. The relationship of results between DSC deconvolution and microscopy technique are observed in both quantitative and qualitative aspect. The deconvolution of DSC peak is one of the feasible approaches to estimate the ortho- and paracortical cells percentages apart from the conventional technique such as optical analysis techniques. Less time consumption and larger scale of sample size are the outstanding benefit from the DSC deconvolution technique. Moreover, DSC deconvolution might be a feasible method to predict the diameter and crimp of wool fibres by using the denaturation temperature difference of main cortical cells types.
5.6 References


Chapter 6  Atomic Force Microscopy Investigation of Wool Cortical Cells

6.1 Introduction and Literature Reviews

Atomic Force Microscope (AFM) is relatively new technique for surface science. This technique has several advantages over conventional electron microscopy techniques. Primarily, it is possible to make a real time measurement. Moreover, the significant advantage by using AFM is that the sample can be measured in nature condition and be able to measurements in air or fluid environments. In contrast, conductive sample preparation process is required and the high vacuum environment is applied by using electron microscopy techniques. In addition, AFM sample sizes are less restriction than TEM and SEM (Jagtap and Ambre, 2006, Kaur, 2012, da Gama et al., 2017). Less sample damage in comparison with the electron-beam techniques is also another advantage. According to highly adaptable with probes, this technique allows us to study other properties of materials such as mechanical properties, chemical composition and photo-oxidative degradation (Hillbrick, 2012, Lim et al., 2019). According to numbers of advantages, the AFM technique has gained attention and used widely for biological materials study.

This section consists of three parts. The first two parts will introduce a basics AFM working principle. Overview literatures on AFM application for keratin fibres will be briefly reviewed in the last part.

6.1.1 Introduction to AFM

AFM is one of the most significant techniques in scanning probe microscopes (SPMs) family, which include scanning tunnelling microscope (STM) and scanning near field optical microscope (SNOM) (Bowen and Hilal, 2009, Kaur, 2012). Difference from other microscopy techniques, SPM techniques generates an image by “feeling” rather than “looking” (Morris et al., 1999, Hillbrick, 2012). This technique uses a very sharp probe to scan across a sample surface. The interaction between probe and sample surface is used to produce an image while other microscopes including LM and EM images are generated by visualisation.
The resolution of STM depended on the technique and sharpness of the probe tip (Bowen and Hilal, 2009). In the case of the AFM, which also refer as the scanning force microscope (SFM), high-resolution three-dimensional topographic image of the surface with nanometer resolution could be produced (Hillbrick, 2012). This resolution is similar to commercial the electron microscopes, but the AFM operated under the experimental conditions familiar to the light microscopes. The summary of a comparison of AFM and other optical techniques that have been used for keratin study is given in Table 6.1 (Jagtap and Ambre, 2006).

**Table 6.1** The comparison of AFM and other optical techniques (Jagtap and Ambre, 2006)

<table>
<thead>
<tr>
<th>Technique</th>
<th>Features</th>
<th>Radiation damage</th>
<th>Best Resolution</th>
<th>Sample preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEM</td>
<td>Surface topography</td>
<td>Rarely serious</td>
<td>4 nm</td>
<td>Easy</td>
</tr>
<tr>
<td>TEM</td>
<td>Internal morphology, lamellar, and crystalline structure</td>
<td>Severe</td>
<td>0.2 nm</td>
<td>Difficult and time consuming</td>
</tr>
<tr>
<td>AFM</td>
<td>Surface topography</td>
<td>None</td>
<td>0.3 nm</td>
<td>Easy</td>
</tr>
</tbody>
</table>

Figure 6.1 shows a schematic of AFM basic set-up (Maver et al., 2016). AFM consists of three main compartments; a probe, which is assembly of a sharp tip attached to a flexible cantilever, a laser beam and a photodetector. AFM working principle is scanning a surface of sample with the sharp probe and measures the interaction between probe and surface. The movement of cantilever is in z direction (up and down), which is controlled by z-scanner. At the same time, the movement of sample is in X and Y direction. This allows the position of the probe to be moved in relation to the surface. Any detection of the cantilever produces a change in the position of the reflected laser beam on the photo detector (da Gama et al., 2017). The position of detector then tracks and records those beam changes. The feedback loop from detector drives the piezocrystal in Z direction to maintain a constant deflection. By raster-scanning motion this probe across a surface and simultaneously monitoring through a laser beam reflected off the cantilever, the topographic features of the sample can be produced (Bowen and Hilal, 2009, Hillbrick, 2012).
6.1.2 Principle of AFM Operation Modes

In AFM, three image modes namely contact, intermittent contact (tapping), and, non-contact are the most commonly AFM imaging operates. The difference of each mode is the interaction between tip and sample surface (Bowen and Hilal, 2009). These different interactions correspond to change of potential energy at various particle positions, which can be explained by the Lennard-Jones and Morse potentials (Kaur, 2012, Maver et al., 2016). These models are applied to represent the force regime curve of tip-sample interaction, as illustrated in Figure 6.2.
The contact mode or static mode is an original imaging operator in AFM. This image mode is mainly in the repulsive regime. As its name says, the probe remains in contact with the sample at all times (Bowen and Hilal, 2009, Hillbrick, 2012). Since the contact is made, strong repulsive forces cause the cantilever to bend to accommodate changes to topography. The constant force and constant height modes are two types of measurements in contact mode (Hillbrick, 2012). The working principle of constant force mode based on keeping deflection constant by move the sample or the tip up and down by using the feedback loop to control the Z scanner. In contrast, constant height mode, the forces are changed to maintain a constant height. Both constant high and force measurement methods can produce surface topographies, but constant force measurement can be applied for wider ranges of samples. The constant height measurement, generally, is applied for quick scans of samples with small height differences (Maver et al., 2016). Although, the advantage of AFM contact mode is the relative simplicity of its technology, the samples can be destroyed because of the frictional forces. The disadvantage of this mode is the probe contamination and damage of a sample surface. Also, the quality of image can be reduced as a result of the tip contamination and damage during the experiment (Bowen and Hilal, 2009, Maver et al., 2016, Jagtap and Ambre, 2006). Thus sample with a hard and relatively at surface is suitable for AFM contact mode.

The second AFM imaging mode is intermittent contact or tapping mode. Tapping mode is developed to overcome the drawback of contact mode. In tapping mode, the probe oscillates close to the surface. When the oscillation value of the cantilever is close to its resonant frequency, the probe is repeatedly brought into and out of contact with a surface (Bowen and Hilal, 2009). As the probe approaches to the surface, the interaction between the tip and the surface causes the oscillation amplitude of the cantilever. The topography is produced by detecting the change of oscillatory amplitude of the cantilever. Similarly, the tapping mode used feedback loop to alter the Z-height of piezocrystal (Bowen and Hilal, 2009, Maver et al., 2016). The benefit for this mode is less damage on sample because the applied force is lower than the contact mode. In addition this tapping mode produces phase imaging alongside of topographic image, which allows recognizing changes in material properties. The limitation of this tapping mode is complexity of analysis, especially quantitative analysis and time consumption. Soft
samples such as biomaterial are suitable for this imaging mode (Bowen and Hilal, 2009, Maver et al., 2016).

Non-contact mode is the third AFM imaging mode in AFM. Unlike the contact and tapping mode, the tip is brought just nearly the surface of sample, but the tip does not touch the surface. The cantilever is again oscillated as in tapping mode, but at much smaller amplitude than in tapping mode (Bowen and Hilal, 2009). The tip is just above the contamination layer. Thus, the forces between the tip and sample are low (van der Waals forces) (Jagtap and Ambre, 2006). “The detection scheme base on measuring changes to the resonant frequency amplitude of the cantilever as the interaction between the tip and sample dampen the oscillation”. The significant advantage of this mode is non-damage of sample surface. This imaging mode is preferable for easily deformable and damaged samples. However, the limitation of noncontact mode is slower scan speed and lateral resolution is lowest comparing with contact and tapping mode (Bowen and Hilal, 2009, Jagtap and Ambre, 2006). The comparison of three AFM modes of operation is summarised in Table 6.2 (Jagtap and Ambre, 2006, Marrese et al., 2017).

**Table 6.2** Summary of AFM modes of operation (Marrese et al., 2017)

<table>
<thead>
<tr>
<th>AFM Modes of Operation</th>
<th>Working Principle</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
</table>
| **Contact Mode**       | - Physical contact between the tip and the surface | - High scan speeds  
- High resolution | - Damage to soft sample  
- Later forces may produce image artefacts |
| **Tapping Mode**       | - Intermittent and short contact between the sample and the tip | - High resolution  
- Minimal damage to sample | - Slower scan speed if compared with contact mode |
| **Non-contact Mode**   | - No contact between the tip and the sample | - Low resolution  
- No damage to sample | - Slower scan speed if compared with both contact and tapping mode |
6.1.3 AFM Topography of Keratin Fibres Study

In the last decade, AFM technique has been applied for the surface imaging of keratin fibre, especially hair. AFM has been used extensively to image the surface of hair cuticle cell. Almost all of them apply AFM for longitudinal section study. Numerous studies applied AFM for investigation the cosmetic effect on hair cuticle cell. Contact and tapping mode are two imaging modes for nanoscale level have been used for wools and hair studies (Hillbrick, 2012).

Figures 6.3a - c are the comparison of AFM topography of cuticle cell with different levels of damage (Clifford et al., 2012). In virgin hair (non-damage), AFM topography shows a rich and well-defined structure of cuticle step. The cuticle structure was also observed on moderately damaged, but they show greater height difference than non-damage hair. On the other hand, severely damaged hair topography, the structure of cuticle cells cannot be observed (Clifford et al., 2012). An exposed of a cortex on a damage hair was also observed by AFM as shown Figure 6.3d (J. ALAN SWIFT and SMITH, 2000). Additionally, AFM image is the feasible technique to study physical properties of human hair. The parameter such as the cuticle step height, cuticle density and roughness were defined for a quantitative characterization of AFM images. These parameters were applied to study the effect of bleaching on human hair (S. P. GURDEN et al., 2004).

Additionally, the comparison between TEM image and AFM image is another research topic that has been widely reported. Several researchers have concentrated on comparison AFM with scanning electron microscopy images (Clifford et al., 2012). G. Poletti et.al, (2003) studied the similarity and the differences between SEM and AFM techniques on cuticle cells investigation (Poletti G et al., 2003). Longitudinal section of human hair was investigated and they reported that “The differences in the surface details between two images become greater as the dimensions of the region involved are decreased” (Poletti G et al., 2003).

However, in the view of fibre cross section, TEM and AFM techniques provide similar results (Parbhu A.N. et al., 1999, N. Chen and Bhushan, 2005, Clifford et al., 2012). In human hair, a relationship between TEM and AFM was reported (, N. Chen and Bhushan, 2005). In addition, using AFM, sub cellular structure of cuticle cells (A-layer, exocuticle, and endocuticle)
are easily observed because of “their distinct stiffness and viscoelastic properties” (N. Chen and Bhushan, 2005).

Figure 6. 3 The studies of damaged human hair by using AFM technique showing different levels of damaged cuticle cells a) AFM topographic images of untreated hair, b) AFM topographic images of moderately damaged hair and c) AFM topographic images of severely damaged hair(Clifford et al., 2012). d) AFM image of 250 mm from the hair’s root end; cortex (COR) exposed at bottom left. Image size 20 × 20 μm² (J. ALAN SWIFT and SMITH, 2000)
The relationship between cortex topography from AFM and TEM image was also found. The cortex region shows a very fine circular structure of about 50 nm in size, which represents the transverse face of the macrofibrils and matrix. At this scale, no intermediate filament structure can be revealed (see Figure 6.4) (N. Chen and Bhushan, 2005). Recently, the roughness value for cortex was studied (Samanta et al., 2016). They found that for virgin hair, the roughness increases from the cortex to the medulla region. This result was also found in bleached and colour treated hair (Samanta et al., 2016).

In view of wool cross sectional, L.A. Titcombe et. al (1999) is one of the initial work that applied AFM on wool cross-section. They reported that cellular component of merino wool can be observed by AFM (L. A. TITCOMBE et al., 1996). The excellent relationship of the AFM and TEM images was also reported, as shown in Figures 6.4 a-d (Parbhu AN et al., 1999). At high resolution (Figures 6.4 c and d), cellular arrangement of the cuticle, cortical cells, and the cell membrane complex (CMC) are clearly observed in both TEM and AFM images. The higher features on AFM image (lighter region) associated with the heavy stain on TEM image (darker stained) (Parbhu AN et al., 1999, R. C. Marshall et al., 1991).

**Figure 6.4** The relationship between AFM and TEM on wool cross sectional image. Wool fibre showing its internal cellular structure; Cuticle cells (Cu), cortical cell region (Co). a) TEM image at low magnification, b) AFM image at low resolution, c) TEM image at high magnification, d) AFM image at high resolution (Parbhu AN et al., 1999)
6.2 Objective

In Chapter 6, the cellular structure of the main cortical cells types was studied using AFM technique. So far, numerous AFM studies of keratin fibres have been focused on cuticle cells. There are limited studies on cortical cells. Almost of the cortical cells studies use AFM as a complementary method to SEM and TEM morphologies. The relationship between light microscopy and AFM is rarely reported. As mentioned in previous section, AFM resolution obtained is nanoscale which is better than light microscopy technique. Thus, corroborating staining results with AFM topographies might help to address the limitation of light microscopy analysis and extend the knowledge of cortical cells of wools. The aim of this study is to investigate the relationship between light microscopy and AFM technique. In order to achieve this aim, this research was designed to address the following objectives;

- Investigation wether the AFM is the feasible technique for differentiate ortho- and paracortical cells.
- Study the relationship between the AFM topographies and staining patterns under optical analysis.

6.3 Experimental

6.3.1 Sample Preparation

Samples from Set (1) were applied as a specimen for AFM investigation. According to USDA standard, the specimens are classed into 4 sub-groups, which is fine, medium, coarse and very coarse wools (see Chapter 2). In order to differentiate between ortho- and paracortical cells areas, wool fibres were stained by Janus green and methylene blue following the modified staining procedure (see Chapter 4, section 4.3). Also, the embedding and cross-sectional process on glass slides were prepared similarly with the preparation for optical analysis. However, for AFM, the cover slide wasn’t applied at the end. The slides were put in ambient condition (25±2°C, 60±5% relative humidity) at least 12 hours before the AFM investigation.
6.3.2. AFM Topography of Stained Wool

The AFM topography images were acquired in tapping mode using “SCANASYST-AIR" model for the cantilever. This cantilever is a single V-shaped silicon nitride ($\text{Si}_3\text{N}_4$), length 115 µm, and spring constant (kN) 0.4 N/m. AFM topography images were processed using “ScanAsyst". All AFM topography experiments were performed under the standard amplitude condition. A movable platform of microscope allowed moving the probe and position over the desired sample area. Once the probe was positioned on the desired areas, samples were scanned at rate 0.5-1 Hz. The slower scan rate was selected automatically if the area is too rough for the tip. 512 samples per line were applied with scan size 3-10 µm. Finally, the AFM topography was produced by Nanoscope analysis V1.4 program.

6.4 Results and Discussion

AFM was used to examine the surface topography of wool cross section by tapping mode. AFM images of Merino fibres cross sections were obtained in ambient environment. In fine wools, topographical structure of cross section shows a clear different regions, reflecting ortho- and paracortical cells. The differences of macrofibrils arrangement, nuclear remnant and CMC are observed. Paracortical cells show larger and coarser pack of macrofibrils than orthocortical cells. The CMC between paracortical cells are more readily distinguishable from the intermacrofibrillar cement than orthocortical cells. This AFM topography is consistent with the morphology image of Merino wool obtained by using TEM (see Figure 6.5a and b).
Figure 6.5 The images of wool cross section obtained from TEM and AFM techniques: a) TEM image; morphology of cross section of a fine crimpy merino wool fibre stained by the thioglycollate-OsO₄ method (Rogers, 1959), b) AFM topography of fine Merino wool cross section (Set (1,16)).

The Janus green stained cross sections with various staining patterns were investigated by AFM tapping mode. The similarity and difference of optical results between these techniques is the aim for this study. Generally, AFM topographies and Janus green staining patterns show excellent correspond, as shown in Figures 6.6 and 6.7. The topography of cross sections with strong bilateral staining pattern show clear separation regions between para- and orthocortical cells. Darker stained area of Janus green shows the characteristic of orthocortical cells topography, while topography of paracortical cells is observed in lighter stained area. The boundary of main cortical cells separation from both techniques is similar (see Figures 6.6). In coarse and very coarse wools (diameter larger than 30 µm), the majority of a Janus green staining pattern shows low staining contrast between the main cortical cell types or mono-staining pattern. Not surprisingly, a good accordance between this staining pattern and AFM topography is also observed. Although the working area was reduced (better resolution), segregation region of main cortical cells types is not observed (See Figures 6.7).
Figure 6.6 The correlation between Janus green staining pattern and AFM topography of Merino wool cross section (Set (1, 16). a), and b) Light microscopic images of the cross section stained by Janus green showing bilateral arrangement. c) AFM topography of the same cross section (6.6b) showing clear separation between ortho- (Top part) and paracortical cells (bottom part).
Figure 6.7 The relationship between Janus green staining pattern and AFM topography of Merino wool cross section (Set 1, 27). a), and b) Light microscopic images of the cross section stained by Janus green showing low contrast of staining intensity; (mono-staining). c) and d) AFM topographies of the same cross section (6.7b) with different size of working area. Both topographic images show an unclear separation of main cortical cells types.
Generally, following Figures 6.8, the AFM topography of coarse wool shows the majority of area is similar to orthocotical cells. Macrofibrils arrangement of paracortical cells (larger and coarser pack) are also observed as a minority. These coarser packs of macrofibrils randomly appear on the topography of cross section of coarse wool.

![Figure 6.8](image)

**Figure 6.8** The AFM topography of coarse wool; Set (1,38). a), and b) Light microscopic images of the cross section stained by Janus green showing low contrast of staining intensity; (monostaining). c) AFM topography of coarse wool show mixed macrofibrils arrangement between ortho- and paracortical cells. The red circle indicate the coarser pack of macrofibrils (paracortical cells)

Over straining is the limitation for light microscopy technique. An over-stained pattern is sometime more often happening in fine wool, which has a significant impact on interpretation of results. This over-staining might refer to a lack of paracortical cells, in the case of Janus green staining. However, a staining process might result in over-staining of the cortical cells. Using AFM, the cortical cells arrangement behind this overstrained area or loss of staining contrast is revealed. The cross section of fine and very coarse wools with over and heavy stained are shown in Figures 6.9 and 6.10, respectively. In fine wool, ortho- and paracortical cells of over stained cross section can be distinguished by using AFM (see Figures 6.9).
Figure 6.9 The AFM topography of fine wool with over-stained pattern; Set (1,16). a) and b) Light microscopic images of cross section stained by Janus green. The white arrow and red square indicated the direction and working area of AFM scanning. c) and d) AFM topographies of the same cross section (6.9b) show two regions of main cortical cells types.

On the other hands, in coarse wool, the distinguished between main cortical cells types is not clearly observed. According to light microscopic image (see Figures 6.10a and b), the bleary transition between light and dark areas was selected for the working area. Following Figures 6.10c and d, low and high resolutions of topographic images, the boundary between ortho- and paracortical cells cannot be observed. Further investigation on lighter and darker staining areas was carried on and the results are shown in Figures 6.10e and 6.10f, respectively. At high resolution, the topography of both areas is similar. The topography of orthocortical cells might be applied to explain the characteristic of these topographies. So, in this cause, the result from AFM
technique suggests that there is higher possibility that, over-staining of coarser wools indicated the single type of cortical cells which is orthocortical cells. Additionally, the results of AFM investigation of over-staining pattern suggest that the sensitivity and accuracy of AFM is higher than Janus green staining. Thus, the corroborated between these two techniques can extend the knowledge that limited from staining technique.

**Figure 6.10** The AFM topography of coarse wool with over-stained pattern; Set (1,38), a), and b) Light microscopic images of the cross section stained by Janus green showing over-stained. c) – f) AFM topography of same wool cross section (6.10b). c) and d) Transition area between lighter and darker stained areas: c) Low resolution, b) High resolution. e) Topography of lighter staining area, f) topography of darker staining area.
The relationship between methylene blue staining patterns and AFM topography are also investigated. Like Janus green staining patterns, good relationship between staining patterns and AFM topographic images is observed. Different regions between ortho- and paracortical cells are easily seen when cross section showed bilateral arrangement. Darker staining area of methylene blue show paracortical cells topography and lighter stained area show topography of orthocortical cells (see Figures 6.11). Undefined cortical cell regions are observed for coarser wool with less staining contrast of methylene blue (see Figures 6.12). Although, the staining patterns from both staining methods show excellent correspond with AFM topographic images, there are some different points.

At similar diameter, topography of methylene blue cross section is less clear than Janus green in the views of the segregation between main cortical cells types (see Figures 6.11 and 6.6). In addition, topography of methylene blue cross sections shows less details of macrofibril arrangement than Janus green. CMC and nuclear remnant are not observed on topography of methylene cross section. Interestingly, on surface of methylene blue cross sections show randomly small embossed dots all over the cross section. Orthocortical cell areas tend to have in higher density of these dots than paracortocal cells area. Moreover more flatten and well arrangements of macrofibrils seem to be present on Janus green cross sections. However, it should be noted that these small embossed dots might be confirmed. Small pit on the surface is another possibility of this surface topography. According to the limitation of sample preparation, height sensor cannot be applied to further investigation (see Chapter 7 for details).
Figure 6.11 The relationship between methylene blue staining pattern and AFM topography of Merino wool cross section (Set 1, 16). a), and b) Light microscopic images of the cross section stained showing bilateral arrangement; Darker is paracortical cells, lighter is orthocortical cell. c) and d) AFM topography of the same cross section (6.11b) showing clear separation between ortho- (right part) and paracortical cells (left part). e) AFM topography of paracortical cell, and f) Topography of orthocortical cells.
Figure 6.12 The relationship between methylene blue staining pattern and AFM topography of Merino wool cross section (Set (1, 38). a), and b) Light microscopic images of the cross section stained showing pseudo random arrangement c) and d) AFM topography of the same cross section (6.12b) showing undefined regions of main cortical cells types.
6.5 Conclusion

In this chapter, the similarity and the differences between AFM and light microscopy techniques are studied. Merino wool cross sections were stained by two different staining techniques; Janus green and methylene blue. Cortical cells arrangements and the regions of ortho- and paracortical cells were initially indicated by staining patterns. Then stained cross sections were investigated by AFM tapping mode. The AFM topographies of wool cross sections showed the arrangement of macrofibrils. Nuclear remnant and CMC were also observed on the topography of Janus green cross sections. Topography of paracortical cells regions showed coarser pack of macrofibrils than orthocortical cells. Additionally, nuclear remnant was clearly observed in topography of paracortical cells. This study showed consistent AFM topography with TEM image of wool cross section.

In view of the relationship between AFM and light microscopy, there was good correspondence between topography and staining patterns obtained from Janus green and methylene blue. For bilateral staining pattern, AFM topography showed bilateral arrangement. A similar boundary of main cortical cells types was observed between AFM and staining pattern. The boundary between the two segments was more hardly to be observed when wool diameters increase. The cross sections with pseudo random arrangement showed characteristic of AFM topography like orthocortical cells. Moreover, surface damaged of methylene blue cross section was observed by AFM. Topography of cross sections stained with methylene blue did not show CMC and nuclear remnant components. The reason behind this might be the staining process. The results revealed the effect of oxidation process on the cortical cells. Further investigation about this methylene blue topography will help to answer this hypothesis.

Interestingly, the difference between these two techniques was observed, when over stained pattern of Janus green is investigated. This result suggests that the sensitivity and accuracy of AFM was higher than Janus green staining. The corroborated between these two techniques might extend the knowledge that limited from staining technique. Thus, in term of optical analysis, AFM is complementary technique for traditional optical microscopy techniques (light microscopy and Electron microscopy: SEM and TEM), especially qualitative analysis.
6.6 References


Chapter 7 Conclusions and Future Work

7.1 Conclusion

7.1.1 Advances in Experimental Techniques

This research led to many advances in experimental techniques for DSC and light microscopy techniques. Firstly, a novel method was developed to deal with the identification of boundary conditions of the deconvolution. DSC curves were deconvoluted under the Gaussian distribution. Three sub-curves fitted under the assumption of equal width of three curve (same standard deviation) quite satisfactorily fit the DSC curve of all Merino wools ($R^2 > 0.9$). Other restrictions include the position of three peaks and the assumption for a majority cortical cell also improves the accuracy of the fitted. The deconvolutions of DSC curves under these restrictions suggest that this method might be feasible to estimate the proportion of main cortical cell types and roughly estimate wool curvature. This deconvolution method is faster than optical analysis and the greater bulk of sample observations can be applied.

Previously, Janus green and methylene blue staining techniques had been carried out without much consideration about the different advantages and limitations; this research provides an in-depth study of these staining processes. The staining methods were modified and the results suggest that increasing temperature is the key factor to enhance the Janus green staining. This modified method can be applied for coarser ranges of wool diameters and give better contrast than the staining condition from M. Horio and T. Kondo (1953). The result of the modified methylene blue staining process suggests that oxidation time is a more influential factor to enhance the staining contrast than staining time. The optimum oxidation time depends on wool diameter. In this study, the optimum oxidation time for fine and medium ranges of wool diameters was measured to be 5 hours, while overnight oxidation time was the optimum time for coarse and very coarse wools. By using this modified method, a better differentiate of cortical cell types in coarse and very coarse wools can be revealed. In previous studies, cross sectional form was used in oxidation process while, in this study, the oxidation process is applied on the fibre form. The better control of fibre cross sections on the glass slide and prevention of wash off effect are the results from this modification.
The comparison between Janus green and methylene blue staining results suggest the suitable application for each staining technique. The modified methylene blue staining method provides a high sensitivity of staining pattern, while the bimodal is the staining results obtain from using Janus green staining. Thus, the methylene blue staining technique is the suitable technique for qualitative analysis including the cortical cell arrangement investigation. On the other hand, simplicity of Janus green staining results makes it more robust and reliable technique for cortical cell proportion based on operator.

### 7.1.2 Thermal and Optical Analysis of Wools Cortical Cells

Cortical cells of Merino wool top of various diameters and obtained from different sources were studied using thermal and optical analysis. Combinations of quantitative and qualitative approaches were used in the data analysis. The DSC in excess water was applied for thermal analysis of wools and the results indicated that the denaturation enthalpy of wool is material independent. This result is consistent with previous studies. However, the relationship between denaturation temperature and wool diameter is different from previous studies. We found that fine and coarse wools have different thermal properties. The positive linear relationship between denaturation temperature and wool diameter is observed in coarser wool, while the opposite trend is found in finer wools. Thus, in this study, only the denaturation temperature for coarser wools is in line with previous studies. This result might indicate that chemical composition of finer and coarser is different, and the developing of keratinization could affect the sulphur content.

The thermal analysis was further carried out by the deconvolution process. The DSC curve was deconvoluted into three-sub curves that were Gaussian distributions of equal width. Each curve implied each cortical cell type. The curve located at the highest denaturation temperature referred to the paracortical cells and the orthocortical cells by the middle curve. Interestingly, this result could not distinguish mesocortical cells from the main cortical cell types. However, this deconvolution approach confirmed the existance of ortho-like cortical cells, which was investigated by (Bryson et al., 2009). Additionally, estimations of the denaturation temperature of each cortical cell type and the cortical cell percentages can be achieved by this deconvolution process. The results indicated that there is no relationship between wool diameters
and the denaturation temperature of each cortical cell type. Also, the relationship between the cortical cell percentages and wool diameter cannot be investigated. The negative relationship between temperature difference between main cortical cell types and wool diameter was the only relationship that can be observed by DSC deconvolution.

The optical analysis was carried out by two different staining techniques. The relationship between staining patterns and wool diameter was found and both staining techniques presented the similar trend. The bilateral arrangement is presented in the majority of staining patterns for fine wools. Less discrimination between the boundaries of ortho- and paracortical cell tend to be observed more often when wool diameters increase. In coarser wools, more variation of staining patterns is observed. The majority of staining pattern is pseudo-randomly distributed. This result is in agreement with the previous. Interestingly, the methylene blue staining presented new evident that the moderate stained areas may not directly refer to mesocortical cells. In the view of quantitative analysis, the cross sections stained by Janus green were measured to calculate the percentage of the area covered by ortho-and paracortical cells using image J. The results revealed the weak linear relationship between wool diameters and cortical cell percentages ($R^2 = 0.2$, p-value < 0.01).

7.1.3 The Relationship between Different Analytical Techniques

Almost all previous studies of the cortical cells structure and arrangement relied on optical techniques, especially TEM. Although TEM is a high resolution and sensitivity technique, limitations of TEM based techniques are the number of samples and time consumption. A few numbers of fibres were involved in the experiment. Similarly with other optical analysis techniques, the sample size that can be involved in the experiment is small. Thus, with a small sample size, caution must be applied, as the findings lack variation and the result might not be able to reflect the reality. Difference from previous studies, this research applied differences analytical techniques including DSC, light microscopy and AFM techniques. The DSC allows us to apply a larger sample size than optical analysis. More than 100 snippets are involved in each data point of DSC. However, the limitation of DSC is that the cortical cell arrangement cannot be visualised. This limitation is overcome by using a light microscopy technique. In addition, both
quantitative and qualitative analysis can be done by this technique. AFM is an analytical technique of high sensitivity. The AFM resolution is similar to TEM, which means this technique provides a better resolution than light microscopy technique. Thus, in this research, AFM was applied to confirm the accuracy of staining technique.

Both DSC deconvolution and staining under the light microscopy investigation were applied to investigate the cortical cell proportions. Generally, the results from both techniques were consistent. The cortical cell percentages obtained by DSC deconvolution were confirmed by optical analysis technique. The majority of cortical cells were orthocortical cells. Additionally, both techniques present the similar trend of the relationship between cortical cell percentages and wool diameters. DSC deconvolution showed that there is no relationship between wool cortical cell percentages and wool diameters. The weak linear relationship between cortical cell percentages and wool diameters was found to be statistically significant using optical analysis. These results are different from the previous study by Orwin (1984), who reported the linear relationship between wool diameters and the orthocortical cell percentages. It should be noted that Orwin applied a small sample size and the source of sample is obtained from individual sheep wool, the finding might not see the variation. Additionally, the difference is attributed to the experimental technique and the result interpretation. Thus, according to the result from both techniques, it is possible to conclude that, there are no relationship between wool diameter and cortical cells.

In the view of qualitative analysis, the corroborating results from deconvolution curves investigation with methylene blue staining patterns further confirm the negative linear relationship between temperature difference of main cortical cells and wool diameters. Finer wools with strong bilateral arrangement is associated with the greater difference temperature. In contrast, in coarse wool, the lack of staining contrast implies the pseudo random arrangement of main cortical cells types. This staining pattern is associated with a low difference in temperature between main cortical cell types. The results suggest that, in coarser wools, the difference of sulphur content is less than finer wools. In addition, the integration of these results suggests that the deconvolution might be a feasible method to estimate the crimp of wool fibres. Moreover, the integration between AFM and light microscopy technique provides more robust optical results. By using the
AFM, the accuracy of the Janus green and methylene blue staining patterns is further confirmed. Almost all of the staining results were consistent with the AFM topographies. The distinguished regions between mesocortical cells and main cortical cell types also cannot be observed by using AFM. AFM can be used for further investigation of the irregular and specific staining patterns such as over stain and mono staining pattern in fine wool, according to the more sensitive AFM technique.

In summary, this study raises the possibility that mesocortical cell might not be the distinctive region in cortex. The DSC deconvolution is the feasible technique to estimate the cortical cell percentages. In addition, The DSC deconvolution and AFM techniques can be used as the complement methods to support the traditional microscopy analysis. Less time consumption and able to use with large sample size are the benefit from the DSC technique. On the other hand, high resolution and sensitivity are provided by AFM investigation.

7.2 Future work

According to the DSC results, the thermal property between samples Set (1) and (2) is different. More fluctuation trend was found in sample Set (2). This result will be confirmed by the protein analysis results. The protein analysis techniques might provide more robust results for DSC. The analysis of sulphur content will help to explain and confirm the DSC results in various aspects. Additionally, the comparison between sulphur content between fine and coarse wool might be applied to explain the different trend of denaturation temperature between fine and coarse wools. The development of DSC deconvolution and image analysis approach are also an interested aspect for the future enhancement for cortical cell study.

The deconvolution results suggested that the difference of sulphur content between main cortical cell types of coarser wool is lower than fine wool. This result might be further confirmed by using microanalysis technique such as Energy Dispersive X-ray (EDX). TEM-EDX will be one of the techniques that can be applied. This technique provides the visualisation of wool cross-section and sulphur content mapping. Additionally, the TEM-EDX is more reliable in term of quantitative analysis than SEM-EDX.
The development of image analysis program or developing Math-lab coding will help to enhance the accuracy of measurement. Moreover, the complexity of modified methylene blue staining patterns might be measured by the improvement of image analytical program. The majority of staining wool samples in this research is fine and medium wools. The extension of diameter range will be requested to prove the assumption that coarser wool might have similar characteristic with human hair.

As mentioned in Chapter 6, the height sensor could not be applied in this study, according to tilted and non-smooth of cross sectional surface. This height sensor might help to investigate the damage on surface. Thus, the development of sample preparation is necessary. The topography of methylene blue staining showed special characteristic on the surface. In methylene blue cross section, there are numerous of holds, which randomly dispersion on the cross sectional surface. In this study, the assumption of these holds was the effect of oxidation process. To prove this assumption, TEM-EDX might be applied. However, AFM might be the better feasible approaches because of less restriction of sample preparation. Similar with TEM-EDX, AFM provide both optical and chemical investigation at the same time. The conductive probe or tailoring the AFM probe with chemical group might be applied. The quantitative measurement of Young’s modulus and other mechanical properties is challenging and interesting aspect for the future work. The stained cross section of Janus green might be used for the guidance of working area.