Image Analyzing with Polarized Light Microscope

I. INTRODUCTION
Humans are primarily visual creatures and not all animals depend on their eyes, as we do, for 99% or more of the information received about their surroundings. Image processing is used for two somewhat different purposes:

a) improving the visual appearance of images to a human viewer and
b) preparing images for measurement of the features and structures present.

The application of imaging techniques in science has been confined in the 1970s. The highly specialized and automatic image analysis systems have applications in various fields including biology, agriculture, medicine and industry. Image processing and analysis can be defined as the "act of examining images for the purpose of identifying objects and judging their significance". Image analyzing (IA) involves to study in detecting, identifying, classifying, measuring and evaluating the significance of physical and cultural materials, their patterns and spatial relationship. IA can be made by polarized light microscope (PLM).

II. POLARIZED LIGHT MICROSCOPE
Polarized light improves the quality of the image obtained with birefringent (or doubly-refracting) materials. PLMs have a high degree of sensitivity and can be utilized for both quantitative and qualitative studies at a wide range of birefringent materials. Image contrast arises from the interaction of polarized light with a birefringent material to produce two individual wave components that are each polarized in mutually perpendicular planes. There is a large number of identifying characteristics of particles that can be seen or ascertained with the aid of a PLM, the most important of which include: Morphology, size, surface texture, hardness, reflectivity, magnetism, pleochroism (change in color), dispersion staining, transparency, birefringence (numerical difference between refractive indices), extinction angle, melting point, chemical composition (elements, ions, functional groups make up the particle), fluorescence and the others.

1) Imaging on Polarized Light Microscope
Polarized light microscopy is used to distinguish between singly refracting (optically isotropic) and doubly refracting (optically anisotropic) materials. In PLM, plane polarized light (light that vibrates in a single direction only) is allowed to impinge on the material. If the material contain anisotropic or birefringent structures (i.e., structures capable of rotating the plane polarized light), the emerging light beam will be altered by twisting and partially extinguished. Anisotropic substances have different optical properties, such as uniaxial or biaxial crystals and oriented polymers or liquid crystals, generate interference effects in the PLM, which result in differences of color and intensity in the image as seen through the eyepieces and captured on film or as a digital image. Anisotropic materials, which include 90% of all solid substances, have optical properties that vary with the orientation of incident light on the axes. They have a range of refractive indices depending both on the propagation direction of light through the substance and the vibrational plane coordinates. Anisotropic materials act as beamsplitters and divide light rays into two orthogonal components. Polarization colors by the anisotropic material result from the interference of the two components of light split. Figure 1 illustrates conoscopic images of uniaxial crystals observed at the objective. Interference patterns are formed by light rays traveling along different axes of the crystal being observed. Uniaxial crystals (Figure 1) display an interference pattern consisting of two intersecting black bars that form a Maltese cross-like pattern. On the other hand, isotropic substances have only one refractive index.
and will not rotate plane polarized light. Isotropic materials, which include a variety of gases, liquids, unstressed glasses and cubic crystals, demonstrate the same optical properties. These materials have only one refractive index and no restriction on the vibration direction of light passing through them.

PLM is a contrast-enhancing technique that improves the quality of images obtained with birefringent materials in comparison to other techniques such as dark field and brightfield microscopic illumination in addition to providing qualitative and quantitative information about the optical properties of the material. In non-polarized white light, light waves vibrate at right angles to the direction of propagation with all vibration directions being equally probable. In polarized light, there is only one vibration direction. The human eye-brain system has no sensitivity to the vibration directions of light, and polarized light can only be detected by an intensity or color effect but these reflected colors are not original colors. Polarized light is most commonly produced by absorption of light having a set of specific vibration directions in a medium. Any device capable of selecting polarized light from natural (unpolarized) white light is now referred to as a polar or polarizer.

Figure 1. Images of uniaxial crystals observed at the objective of PLM

Polarized light microscopy can be used both with reflected and transmitted light. A bright field LM can be converted into a polarization microscope by inserting a polarizing prism or filter above the objective lens (the analyzer). If two plates (polarizer and analyzer; called Nicol prisms) are ranged parallel to one another, plane polarized light is transmitted through to the eye. When anisotropic material is placed between crossed prisms, it will rotate some of the plane polarized light and thus be visible. Each object position and information from the grey (brightness, contrast) or color image can be evaluated.

2) Applications of Polarized Light Microscope

Polarized light microscopy is perhaps best known for its applications in the geological sciences, which focus primarily on the study of minerals in rock thin sections. However, a wide variety of other materials can readily be examined in polarized light, including both natural and industrial minerals, cement composites, ceramics, mineral fibers, polymers, wood, urea, foods and biological macromolecules and structures. The technique can be used both qualitatively and quantitatively in the material sciences, geology, chemistry, biology, metallurgy, medicine and food analysis.

The hygiene and safety risks, and high labor and social costs limit the use of human workers in the food sector, especially as food safety regulations are becoming more stringent. Recruiting, training and retaining skilled butchery staff is becoming difficult and costly. Cost calculations can only be made on an individual basis, but many generic drivers are quoted by the food industry for the introduction of machine image analyzing. PLM has many applications in the study of food structure and morphology. The image analyzing can be applied in the following areas of food industry: (i) Image processing methodology: images and image processing shape analysis, feature detection and object location, texture, three-dimensional processing, pattern recognition and the others. (ii) Application to food production: Inspection and inspection procedures, inspection of baked products, cereal
grain inspection, image processing in agriculture, vision for fish and meat processing, system design considerations, food processing for the millennium and the others.

IA systems are applied to various foods for quality assurance purposes: poultry carcass inspection, detection of defects on foods, beef marbling and color, prediction of beef qualities, color grading of beef fat, the color of eggshells, relative antibrowning potency of oxalic acid on banana and apple slices, color inspection of potatoes and apples, shape grading of potatoes, grading of mushrooms, quality inspection of bakery products, classification of cereal grains, computer-assisted sensory evaluation of meals, quantification of features of almonds and the others. IA can also quantify appearance, surface texture; color attributes, a modification of its geometry and appearance (color) during processing such as for fruit shrinkage during air dehydration, more accurate definition of volume surface and/or thickness of a food piece, area, length, width, volume; shape, dimensions, number of holes and the others. PLM can not directly determine material crystal structure or chemical composition.

Some advantages of image analyzing: the generation of precise descriptive data; quick and objective operation; reduction of tedious human involvement; automation of many labor intensive processes, consistency, efficiency and cost effectiveness; the non-destructive natures; the easy of permanent records; the composition and three-dimensional structure of a variety of samples; information about thermal history and stresses; useful information in manufacturing and research; and analysis relatively inexpensive and accessible investigative. Disadvantages include the need for defined and consistent lighting, calibration requirements; and the difficulties encountered with overlapping objects when both sides of a food need to be evaluated.

Figure 2. Polarized light microscope configuration

3) Basic Properties of Polarized Light Microscope

A PLM, illustrated in Figure 2, is equipped with all of the standard recommended parts for examination of birefringent materials under polarized light. Although similar to the common brightfield LM, the PLM includes additional components. These include the polarizer, analyzer, strain-free objectives, condenser, a circular graduated stage capable of 360-degree rotation and an opening in the PLM body. The polarizer and analyzer are the essential components of the polarizing microscope, but others are desirable features.
Removal of the polarizer and analyzer from the light path, while maintaining the other component configuration, results in an instrument equivalent to a typical brightfield microscope.

- **Polarizing filters.** There are two polarizing filters in a polarizing microscope: polarizer and analyzer. The polarizer is equipped with a rotating polarizer assembly that is directly attached to the bottom of the condenser. The polarizer provides vibration in the left-to-right or East-West direction, although most of these elements can be rotated through 360 degrees. The analyzer is usually aligned with a vibration direction oriented North-South and can be moved in and out of the light path as required. When both the analyzer and polarizer are inserted into the optical path, their vibrations are positioned at right angles to each other. In this configuration, the polarizer and analyzer are said to be crossed.

- **Lamp.** PLM has an illuminator which utilizes a 20 to 100-watt high-energy tungsten-halogen lamp. A transformer providing direct current (DC) voltage to the lamp is built directly into the microscope base and is controlled by a potentiometer positioned near the lamp switch in the microscope body (the lamp voltage control). A collector lens, field iris diaphragm and surface of mirror are components to control the light distribution, size and intensity of the illumination field.

- **Observation tube.** The microscope is equipped with a binocular observation tube mounted on the standard intermediate attachment, which houses the selectable and focusable Bertrand lens, as well as a 180-degree rotatable analyzer, allowing both conoscopic and orthoscopic examination of birefringent matters.

- **Stage.** The circular rotating stage provides 360-degree rotation of stage to facilitate orientation studies with centration of the objectives and to make the center of rotation with the center of the field of view for material. Stage can be fixed in any position using a knurled locking screw. An optional mechanical stage adapter provides accurate specimen positioning along two orthogonal axes (x-y translation). Stage rotation angle can be measured to an accuracy of 0.1 degree.

- **Objectives.** Suitable objectives for polarized light microscopy are available with varying degrees of optical correction, and include achromats, plan achromats, and plan fluorites. The objectives (typically ranging in magnifications of 4x, 10x, 20x, 40x and 100x) are mounted in specialized adapters that allow each to be individually centered. The circular 360-degree rotatable stage can be clamped at any rotation position. The performance of an objective is limited by several factors: the anti-reflection coatings used on lens surfaces, and the refractive properties due to angle of incident light on the front lens.

- **Revolving nosepiece.** PLMs are equipped with a specialized nosepiece between the nosepiece and objective that allow each objective to be independently centered on the instrument optical axis. This enables each objective to be centered with respect to the stage and microscope optical axis so that matter features remain in the center of the view-field when the stage is rotated through 360 degrees.

- **Condenser.** Polarized light microscopy requires a condenser that is similar to that used in conventional brightfield microscopy with a numerical aperture between 0.90 and 1.35. Condensers top lens provides adequate illumination and numerical aperture provides conoscopic observation with high-numerical-aperture objectives. The condenser is centerable and focusable, with a numerical aperture specification of 1.25 for oil immersion and 0.90 in air.

- **Eyepieces.** PLM eyepieces are fitted with a cross wire reticle to mark the center of the field of view. The cross wire reticle assists in focusing the specimen and
composing images with a set of frames bounding the area of the view-field to be captured either digitally or onto film.

- **Bertrand lens.** This lens presents in an intermediate tube. A Bertrand lens projects an interference pattern formed at the objective rear (back) focal plane into focus at the microscope image plane. The lens is designed to enable easy examination of the objective rear focal plane, to allow accurate adjustment of the illuminating aperture diaphragm and to view interference figures.

- **Retardation plates.** PLMs contain a slot to allow the insertion of retardation plates between the crossed polarizers, which are used to enhance optical path differences in the specimen. This slot is placed in the microscope intermediate tube. Compensation plates inserted into the slot are then situated between the specimen and the analyzer.

- **Nosepiece.** Revolving nosepiece turret facilitates rapid objective changes during material observation. The nosepiece supports up to four objectives and its plane of rotation is inclined in order to minimize interference between the objectives.

- **Focusing mechanism.** It is a rack and pinion assembly that provides a total stage movement of 25 mm, controlled through concentric coarse and fine focus knobs located on either side of the lower microscope body. The right hand fine-focus knob is marked with graduations around its full circumference, with the minimum interval corresponding to a stage movement of 2.5 micrometers.

- **Intermediate tube.** Light that is diffracted, refracted and transmitted by the material converges at the rear focal plane of the objective, and is then directed to an intermediate tube, which houses a second polarizer, typically termed the analyzer. The intermediate tube also provides the attachment of observation and photographic systems that form an image by transformation of the diffraction information.

- **Observation tubes.** Two binocular observation tubes are available, one having a field number of 20, and one with a wider view-field corresponding to a field number of 22.

### III. SOME EXAMPLES FOR APPLICATION OF IMAGE ANALYSIS

Careful specimen preparation is essential for good results in polarized light microscopy. The method chosen will depend on the type of material studied. The standard thickness of material or amount in preparation is important. The final preparation should have a cover glass cemented with an optically transparent adhesive. Softer materials can be prepared in a manner similar to biological samples using a microtome. Slices between one and 40 micrometers thick are used for transmitted light observations. These should be strain-free and free from any knife marks. Biological and other soft specimens are mounted between the slide and the cover glass using a mounting medium whose composition will depend on the chemical and physical nature of the specimen.

1) **Food starches.** Figure 3 and 4 compare a micrograph of corn and soybean dusts, respectively, with the same field seen with polarized light. Starch grains are identified by the Maltese cross (equal arms of cross) formation. This comparison shows the large percent of starch particles in the sample. Staining of the dust with iodine solution stained the rounded particles deep blue, also identifying them as starch. Figure 3 and 4 also compares an optical micrograph with the polarized light micrograph of the same field. Comparison of Figure 3 with Figure 4 indicates that the starch content of the corn dust is greater than that of the soybean dust. Particle analysis measurements are carried out on an image analysis system, equipped with a PLM, to determine relative amounts of starch in the corn and soybean samples. The field area fraction of particles considered to be starch, based on shape and polarized light characterization, was 43.5% for corn and 27.4% for
soybean. These percentages were calculated as total area of starch particles divided by total area of particles. Also, the starch gelatinization can be followed by this technique. As gelatinization proceeds, the starch granules lose their crystal structure and hence their birefringence (Figure 5).

2) **Muscle fibers.** They observed in a bright field LM exhibit transverse striation. When viewed under polarizing light, these structures can be seen with alternating bands of anisotropic muscle proteins. (Figure 6a). Meat quality is often related to the degree of muscle contraction and the distance between repeating bands (sarcomere length) can be measured by PLM.

3) **Microstructure fats.** The microstructure of fats and emulsions can be studied by PLM. Because these materials contain crystalline triglycerides that occur in three major polymorphic forms, it is possible to differentiate them with the aid of PLM (Figure 6b,c).

4) **Minimum inhibitory concentration of chemicals.** The microbiological minimum inhibitory concentration (MIC) assay can be measured from the zones of inhibition on agar plate. In most antimicrobial assays, microorganisms give zones with a fuzzy edge and reading such a variable zone boundary by eye will generally give rise to some inconsistencies. By using image analyzer, the zone boundary can be set at a particular grey level, thus increasing the reliability of the zone measurement and the assay. Occasionally, the zone will not be a true circle, and then the average of a series of diameter measurement must be taken with eye visual. With an image analyzer, the total area of the zone can be rapidly measured, and reduced to an average zone diameter by a computer (Figure 7). Before image analyzing, the standard curve can be prepared from known concentration of antimicrobial agent vs. zone diameter or area.

5) **Acrylamide.** Figure 8 shows total amount of acrylamide and color changes in chips depending on frying time.

6) **Asbestos.** Asbestos is a generic name for a group of naturally occurring mineral fibers, which have been widely used as insulating materials, brake pads and to reinforce concrete. These materials can be harmful to the health when inhaled and it is important that their presence in the environment be easily identified. Specimens are commonly screened using polarizing microscopy with a quick and easy distinguishing between asbestos and other fibers (Figure 9a), and between the major types asbestos (such as chrysotile, crocidolite, and amosite). From a health care point of view, it is believed that the amphibole asbestos derivatives (crocidolite and amosite) are more harmful than the serpentine and chrysotile. Asbestos fibers are anisoscopic and will be affected by rotation under plane-polarized light. Chrysotile asbestos fibers may appear crinkled, like perm or damaged hair, under plane-polarized light, whereas crocidolite and amosite asbestos are straight or slightly curved due to different refractive index value. Chrysotile has a refractive index of about 1.550, while that of amosite is 1.692, and crocidolite has the highest, with a value of 1.695. Identification of the asbestos fiber types depends on shape, refractive indices, pleochroism, birefringence, and fast and slow vibration directions.

7) **Medical materials.** One of the most common medical applications for polarized light microscopy is the identification of gout crystals (monosodium urate. Gout is an acute, recurrent disease caused by precipitation of urate crystals (problems in the elimination of uric acid result with formation of urate crystals) and characterized by painful inflammation of the joints, primarily in the feet and hands. In practice, several drops of fresh synovial fluid are sandwiched between a microscope slide and cover glass and sealed with nail polish to prevent drying. After the sample has been prepared, it is examined in PLM (Figure 9b). Gout can also be identified with PLM in thin sections of human tissue. Polarized light is also useful in the medical field to identify amyloid, a protein created by metabolic deficiencies and subsequently deposited in several organs (spleen, liver, kidneys, brain), but not observed in normal tissues.
8) **History of rock formation.** As well as providing information on component minerals, an examination of geological thin sections using polarizing light microscopy can reveal a great deal about how the rock was formed. Phyllite, a metamorphic rock, clearly shows the alignment of crystals under the effects of heat and stress (Figure 9c). The crossed polarizers image reveals that there are several minerals present, including quartz in gray and whites and micas in higher order colors. Oolite, a light gray rock composed of siliceous oolites cemented in compact silica, is formed in the sea. Oolite forms in the sea when sand grains are rolled by gentle currents over beds of calcium carbonate or other minerals (Figure 9d). These minerals build up around the sand grains and subsequent cementation transforms the grains into coherent rock.

9) **Natural and synthetic polymers.** During the solidification of polymer melts, there may be some organization of the polymer chains, a process that is often dependent on the annealing conditions. When nucleation occurs, the synthetic polymer chains often arrange themselves and the solidified regions. These can be seen in crossed polarized illumination as white regions, termed spherulites, with the distinct black extinction crosses of natural polymers (Figure 10a). Nucleation in polymer melts can take place as the result of accidental contamination or contact with a nucleating surface, and can lead to substantial weakening of the product. Identification of nucleation using PLM can be a valuable aid for quality control. Both biological and synthetic polymers can undergo a series of lyotropic or thermotropic liquid crystalline phase transitions, which can often be observed and recorded in PLM (Figure 10b). Other polymers may not be birefringent (evidenced by the polycarbonate specimen, Figure 10c). Nylon fibers reveal refractive index differences (Figure 10d).

![Figure 3. Optical micrograph (a) and polarized light micrograph (b) of corn dust](image-url)
Figure 4. Optical micrograph (a) and polarized light micrograph (b) of soybean dust.

Figures 5. Partially (a) and fully (b) gelatinized starch in PLM

Figure 6. Muscle fibers (a), milkfat (b) and anhydrous milk fat (c) in PLM

Figure 7. Displayed image of inhibition zone on an agar plate. The inhibition zone consists of a bright circle of clear agar surrounded by a dark area of bacterial growth.
Figure 8. Acrylamide in chips: Total area equals to the total amount of acrylamide

Figure 9. Aspestor fiber (a), gout crystals (b), phyllite (c) and oolite (d) in PLM

Figure 10. Natural (a), synthetic (b) and other (c) polymers, and nylon (d) in PLM

IV. MATERIALS AND METHODS
A) Materials
B) Method
1) Uses of Polarized Light Microscope
   Learning the sample analysis on optical and polarized light microscope

2) Analysis of starch gelatinization during cooking time at 85°C
   In this experiment, starch gelatinization will be controlled and analyzed by using Image Analysis on polarized light microscope.
   1. During the cooking, the sample will be collected at each 5 min.
   2. The kernel will be cut using razor-blade.
   3. The centre of the kernel will be analyzed using Image Analyzer.
   4. Collect the data as indicated on Table 1.
   5. Calculate percent gelatinization (cooked).
   6. Draw a calibration curve by comparing cooked starch % versus cooking time using cooking data.
   7. Standard deviation and a linear model will be derived.

Table 1. Starch gelatinization during cooking at 85°C (1 cm=239.73 pixels)

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<th>% Cooked area ((c/a) x 100)</th>
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Table 2. Calculation of percent starch gelatinization using diameter during cooking at 85°C

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