Module -2:

Dispersion staining Microscopy - phase contrast microscopy – differential interference contrast microscopy

2.1. Dispersion Staining Microscopy

All liquid and solid materials experience change in optical properties with variation in the wavelength of the light employed to measure them. This variation in properties as a function of wavelength is called the dispersion of the optical properties. The dispersion curve is obtained by plotting optical property of interest as a function of the wavelength at which it is measured. This technique can also be employed for identification or characterization of unknown materials.

In light microscopy, dispersion staining is an important tool based on the variations in dispersion curve of an unknown material with respect to a reference material. The dispersion curve of the reference or standard material is compared with that of the sample under investigation. The dispersion curve changes because of the refractive index of the investigating material. These variations manifest themselves as colors when the two dispersion plots intersect for some visible wavelength. It is an optical staining method which does not require any stain or dye for producing colors. The most popular application of this technique is for confirming the presence of asbestos in construction materials.

Dispersion staining can be achieved via five basic optical configurations of the microscope:

1. Colored Becke` Line (Maschke, 1872; Wright, 1911)
2. Oblique Illumination (Wright, 1911)
3. Darkfield (Crossmon, 1948)
4. Phase Contrast (Crossmon, 1949)
5. Objective Stop (Cherkasov, 1958)

All the configurations listed above offer some advantages and disadvantages of their own. (1) & (2) were proposed for the first time by F.E. Wright in 1911, in United States, and were based on the work of O. Maschke in Germany during 1870s.

Sample preparations steps are similar in all these techniques. These are summarized below:

1. An intimate contact must be established between the sample and the standard/known reference material, i.e., clean solid sample should be mounted in the reference liquid. Thus, there should be close contact of one mineral phase with the other, or a consistent liquid must contain reference material in solid form. Solid mounted in the reference liquid (or the mounting medium) is the most common type.
2. For producing dispersion colors, both materials must have equal value of refractive indices over some wavelength range in visible region, and also, the dispersion curve for refractive indices must be very different for these materials.
3. Great care must be taken while mounting the sample under the coverslip such that undesired optical effects can be minimized.

2.1.2 **Exemplar Materials**:

Class 1 Fiber evidence:

Textile Fiber evidence is common trace evidence recovered at crime scenes. Fibers are used and may be found everywhere; therefore, fibers have a crucial role in crime investigation to link the suspect, crime scene and victim. When viewed with the microscope, many textile fibers look similar to each other. Traditionally, morphology and optical properties are used to differentiate textile fibers. Once the birefringent fibers have been identified to be present, microscope is adjusted for dispersion staining mode. The dispersion staining central stop objective is inserted and the color of scattered light is observed.

Class 2 Soil minerals:

Forensic soil examination is well known and used because of its ability to match a perpetrator or victim to the crime location (Raymond &Tedrow, 1991). Soil evidence is valuable because there are an unlimited number of soil varieties and soil compositions change relatively quickly from each location, even if the separation is a short distance. The discriminating power of soil examination depends on the mineral components in soil. The purpose of the examination is to identify the minerals contained in a sample of soil from a certain location.

Class 3 Illicit Drugs:

In developing countries, identification of illicit drugs is one of the major problems where the drug analysis must be efficient and inexpensive. Drug detection is a major problem in a provincial area due to the limited budget and personnel. With the high number of cases related to illicit drugs, the analyst in a remote laboratory needs to have a method that can detect and analyzed drugs rapidly and reliably.

2.1.3. Application of Dispersion Staining Microscopy

The applications of dispersion staining technique are:

1. Differentiating the components present as mixture.
2. If the components in the mixture exhibit no distinguishable morphological features, and also possess very slight difference in the refractive indices, these components appear very similar via conventional microscopy techniques. Dispersion staining can be used to differentiate them by forcing them to assume different colors.

2.2. Phase Contrast Optical Microscopy

When light traverses through a medium, its amplitude and phase change due to interactions with the medium. These changes depend upon the properties of the traversing medium. Scattering and absorption of light results in change in amplitude, which depends on wavelength and it gives rise to colors. On the contrary, phase changes are imperceptible without having special arrangements made on the condition required. Phase-contrast microscopy is an optical-microscopy technique which works on the principle of converting phase shifts in light to variations in the brightness of the image. In this way, the invisible phase shifts become visible when observed as brightness changes. This technique is most suitable for transparent specimens.

One of the most benefited application of phase-contrast microscopy is biology, where it can be used to image several cellular structures which are not observable via simple bright-field microscopy. For visualizing these structures, researchers often tried to stain them, which involved sometimes elaborate sample preparation steps, often damaging cells. Phase-contrast microscopy has allowed studying cells in their natural states.

2.2.1. Working Principle-

Phase-contrast microscopy separates incident light of the source from the specimen scattered light, and manipulates the two differently.

Condenser annulus focuses the incident light onto the sample resulting in scattering of some light by the sample while the remaining light, left unaffected, forms the background light. The scattered light is very weak in case of unstained biological samples, and is phase shifted by −90° with respect to the background light.

Image contrast can be enhanced either by creating constructive interference between scattered and background lights; or by reducing background light reaching the image plane.

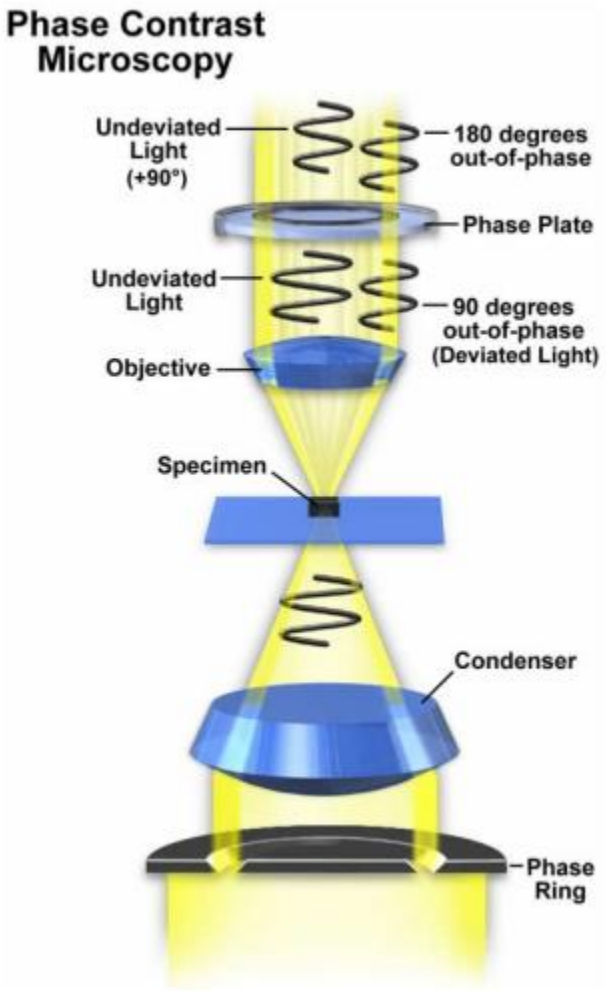

Figure 1 Phase Contrast Optical Microscopy.

The background light is phase shifted by +90°, making it 180° out of phase, with respect to the scattered light. Images with a lighter background and a darker foreground are formed by subtracting scattered light from the background light.

2.2.2. Advantages-

- The ability to examine living cells in their natural state can give more information than other microscopy techniques where the specimens are killed, fixed or stained in order to investigate.
- This technique provides high-contrast and high-resolution images.
- Standard technique to study and interpret thin samples.

- Present phase contrast microscopes are integrated with CCD or CMOS and can therefore, capture photo and video images.

2.2.3. Disadvantages-

- Rings limit the aperture, thereby decreasing the resolution.
- Thick organisms and particles cannot be studied as they appear distorted.
- Images appear grey or green depending on whether white or green light is used for illumination. This leads to poor imaging.
- Bright areas generally surround the images resulting in 'Halo effect'.

2.1.1. Applications-

This technique is used in biological and medical research, such as cytology and histology. It can be employed to study living cells, tissues, and microorganisms which remain transparent in bright field illumination.

Investigations involving crystallography, mineralogy, and polymer morphology can also be carried out by phase contrast technique. Colorless micro crystals, powders, particulate solids, and crystalline polymers, with slightly different refractive index from that of the surrounding immersion liquid, can be readily detected by this technique.

2.2. Differential Interference Contrast Microscopy

Differential interference contrast (or DIC) microscopy is employed to enhance contrast in transparent and unstained specimens. The principle of interferometer is used in DIC to obtain information of the optical path length of a specimen and its invisible characteristics. This is also termed as Nomarski Interference contrast microscopy after its discoverer Georges Nomarski in 1952.

A polarized light source is divided into two orthogonally polarized mutually coherent parts which are spatially displaced (sheared) at the sample plane, and recombined before observation. The interference of the two parts at recombination is influenced by the optical path difference (i.e. the product of refractive index and geometric path length) between them. By adding an adjustable offset phase to determine the interference at zero optical path difference in the sample, the contrast is proportional to the path length gradient along the shear direction, giving the appearance of a 3D physical relief corresponding to the variation of optical density of the sample, emphasizing lines and edges though not providing a topographically accurate image.

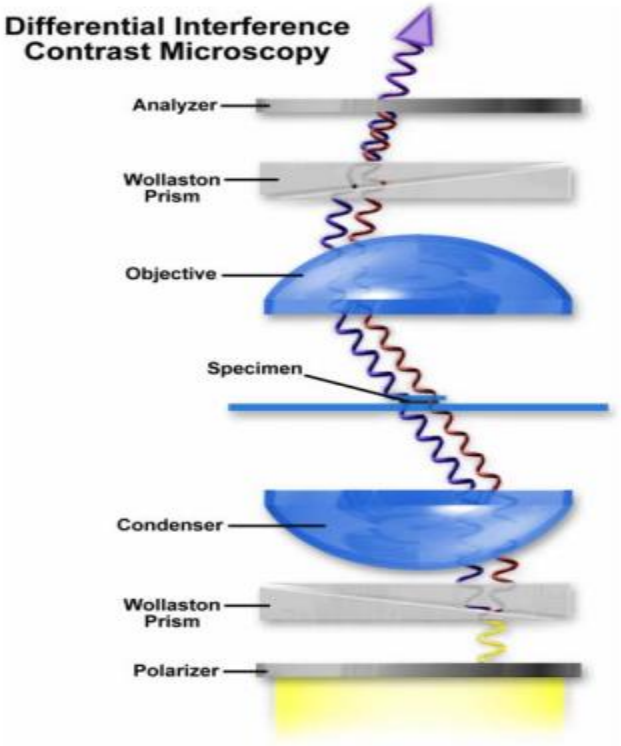

Figure 2 Schematic illustration of microscope configuration for differential interference contrast.

2.3.1 The Light Path-

1. Unpolarized light enters through microscope and is polarized at an angle of 45°.In this type of microscopy, generally polarized light is used.

2. The polarized light then passed through the first Wollaston prism which divides the light into two rays i.e. the sample rays and the reference rays. Both the rays are polarized at 90˚.

3. Condenser focuses both the rays onto the sample so that they pass via two adjacent points (~0.2 μm apart) in the specimen

4. The rays traverse through adjacent areas of the specimen with a separation similar to the resolution of the microscope. Different optical lengths are experienced by these rays because the of difference in refractive index, as refractive index gradients are different for different areas of the sample.

5. The rays are then passed through the objective lens.

6. The second two layered modified Wollaston prism focuses the rays.

7. Second prism recombines the two rays into one 135° polarized ray resulting in interference patterns, brightening or darkening of image according to the optical path difference experienced by the rays.

2.3.2. Working-

In this type of interference contrast microscopy, contrast is obtained by visual display of the gradients in refractive indices of various areas of the sample. The process starts with light from the source passing via a polarizing filter. Polarizing filter is located between the light source and the condenser. A double layer modified Wollaston prism splits the beam into two parts, spatially separated by a distance similar to resolution of the objective lens. One beam is directed to the sample while the other passes via background. These beams are then again recombined by another Wollaston prism positioned above the objective lens.

2.3.3. Advantages-

An advantage of DIC is that sample appears bright with a dark background. The technique has the advantage of using full width condenser with aperture setting, whereas the original slit condenser produced a thin vertical beam of light, thereby limiting the amount of light focused on the sample surface. The full condenser compensates for the phase differences of the emitted light resulting in a brighter image. Better resolution and high definition images can be obtained by combining this technique with digital imaging systems.

Do you know?

- **Dispersion staining Microscopy:** **The first paper documenting dispersion effects seen through the microscope was written in 1872 by O. Maschke in Germany. This paper discussed the occurrence of colored Becke` lines when a particle was in a liquid of matching refractive index. Prior to this paper these colors were thought to be the result of the microscope lenses (chromatic aberration) and not the result of the slide mounted subject and the medium in which it was**

Phase contrast microscopy

- **Frits Zernike**, a Dutch physicist and mathematician, built the first phase contrast microscope in 1938.
- It took some time before the scientific community recognized the potential of Zernike's discovery; he won the Nobel Prize in 1953 and the German-based company Zeiss began manufacturing his phase contrast microscope during World War II.

Review your learning

Questions

1. Phase Contrast microscopy
    a) Continuously changes the phase of the incident light from the condenser to improve contrast in the specimen.
    b) Uses circular filters in the condenser and objective to give contrast to parts of the cell with different refractive indices.
    c) Uses special lenses to distinguish between solid and liquid phases of the cell.
    d) Uses special lenses to change the color of light passing through them.
2. Differential Interference Contrast microscopy
    a) compares two identical specimens on the same microscope.
    b) illuminates the specimen with light of two different colors.
    c) illuminates the specimen with light of two different phases.
    d) illuminates the specimen with both reflected and transmitted light.
3. Which of the following statements is most correct about the differential Gram stain?
    a) Gram's iodine differentially stains Gram positive cells
    b) Crystal violet differentially stains Gram positive cells
    c) Safranin differentially stains Gram negative cells
    d) Acetone/Alcohol differentially destains Gram negative cells.
4. What type of microscopy allows for the visualization of internal components within live, unstained specimens?
    a) Bright-field
    b) Dark-field
    c) Phase-contrast
    d) Fluorescence
5. All of the following are examples of special stains except:
    a) Negative stains
    b) Endospore stain
    c) Ziehl-Neelsen acid-fast stain
    d) Flagellar stain
6. The order of reagents used in the Gram stain are
    a) Alcohol, crystal violet, iodine, safranin
    b) Iodine, crystal violet, safranin, alcohol
    c) Crystal violet, iodine, safranin, alcohol
    d) Crystal violet, safranin, alcohol, iodine

7. What are the applications of Dispersion Staining Microscopy?
8. Briefly explains the working of Phase Contrast Optical Microscopy?
9. List the advantages and disadvantages of Phase Contrast Optical Microscopy.
10. Briefly explains the working of Differential Interference Contrast Microscopy?