Chapter 10
Phase Contrast

Phase contrast makes living, unstained microscopic structures visible. Normally the difference in refractive index between a living microscopic structure and its surrounding environment is so small that the structure refracts very little light. Light is, however, diffracted by the specimen. Diffracted light is, on average, slowed down by 1/4 of a wavelength relative to undiffracted light. Undiffracted light is referred to as direct light. In the absence of any color contrast resulting from differential absorption, contrast can be created from the interference of diffracted and direct light. Phase contrast is a method of enhancing this interference.

Types of Specimens for Phase Contrast

Phase contrast is especially useful for living biological specimens. Today, cell cultures are a primary specimen for phase contrast. Phase contrast is useful for specimens that produce very little refraction; that is, their refractive index is not much different from their surrounding medium. Phase is also useful for specimens that possess little or no color of their own and which have not been artificially colored. In addition to cell and organ cultures, such specimens include bacteria, aquatic invertebrates, blood, and other body fluids.

Historical Background of Phase Contrast

Frits Zernike, a physicist at the University of Gröningen, Holland, discovered the phase principle in 1932. He described its use in microscopy in 1935. He won the Nobel Prize in physics in 1953 for this work. Zernike separated direct light from the specimen from diffracted light from the specimen by use of a special disk in the condenser. He increased the phase difference between the direct and diffracted light by use of a special plate in the back focal plane of the objective lens. The resulting increase in interference between the direct and diffracted light in the intermediate image plane produced amplitude contrast that the microscopist could see.

F. Zernike

Properties of Light, Lenses, and the Specimen in Phase Contrast

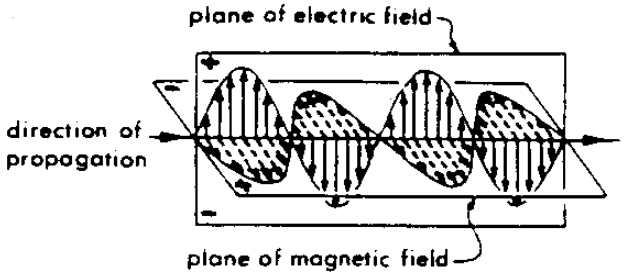
Our discussion of phase contrast must begin with several ideas about the physical nature of light, how light is affected by the specimen and subsequently by the objective lens before we can consider the effect of the phase contrast apparatus. Here these ideas are reviewed. After this introduction, the effect of the phase apparatus can be easily understood.

The Electromagnetic Nature of Light

James Clark Maxwell in 1864 described the mathematical nature of electromagnetic fields of which light is one. According to his theory, light consists of an electric vector and a magnetic vector. Both vectors are transverse to the direction in which the light is traveling and they are at right angles to one another. Figure 10.1 illustrates this. Only the electric vector is important when considering the propagation of light through optical systems. The electric vector is our light wave.

In these notes I sometimes refer to light as a wave and sometimes as a ray. These terms have specific meanings in wave and geometric optics. For our purposes however, picture a light wave as a wave on a pond that results from dropping in a stone. Close to the stone (i.e., the source) the wave is highly curved while very far from the source the wave is nearly linear. Picture a ray as a line drawn from the source across the crests of the waves in the direction the waves are traveling. Close to the source, rays can be drawn which point in 360 degrees away from the source. Very far from the source, however, any two near-by rays would be nearly parallel lines. The microscope's condenser produces nearly parallel waves / rays of light.

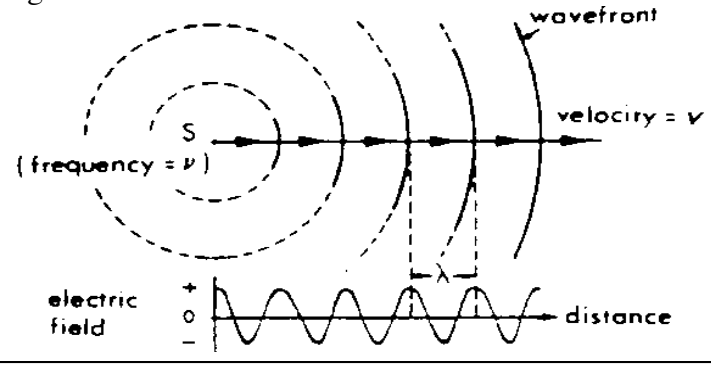
Figure 10.1



The Frequency of Light

Frequency is the number of complete vibrations per second. The source of the light wave determines frequency. For example, a particular electron transition in an excited iron atom releases a photon with a frequency of 5.7 X 10^{10} cycles per second (which is green light). Frequency is a constant regardless of the medium through which the light wave travels. Frequency determines the color of light. Figure 10.2 illustrates the relationship of frequency and the

Figure 10.2



electric vector. Figure 10.6 demonstrates that the wavelength of light is different in media of different refractive indices, but that frequency remains the same. This is because the velocity of the wave is different in different media. This difference in velocity is important in phase contrast.

The Wavelength of Light

Wavelength is the distance from one wave crest to the next. The velocity of the wave sets wavelength in a particular medium. Wavelength is velocity divided by frequency. Light travels slower in denser media (higher refractive index) than in rarer media (Figure 10.6).

The Light Wavetrain

Light originates with an electron transition from an outer to an inner orbital shell of an atom. The electron gives up energy in very discrete amounts during this transition and some of this energy is in the form of visible light. The time required for the electron transition is about 3 X 10^{-8} seconds. The speed of light in air is about 1 X 10^{8} meters per second. So, a light **wavetrain** or quantum or photon is about 3 meters long. A light wavetrain has a beginning and an end. It has a **direction of propagation**, a **vibration frequency** (that is dependant on the energy released and that is represented by a discrete number of up and down transitions in the wavetrain) and a **vibration direction** or **azimuth** that is at right angles to the direction of propagation. The vibration direction can be at any angle around the direction of propagation.

Polarization of Light

Polarization describes the spatial plane in which the **electric vector** of a light waverain oscillates. Unpolarized light consists of zillions of light wavetranes at all possible vibration angles of the electric vector perpendicular to the direction of propagation. Figure 10.3 represents unpolarized and polarized light with the light coming at you. Light in which all but a single electric vector has been eliminated is **plane polarized**. Such light has an electric vector that oscillates in a single plane.

Figure 10.3

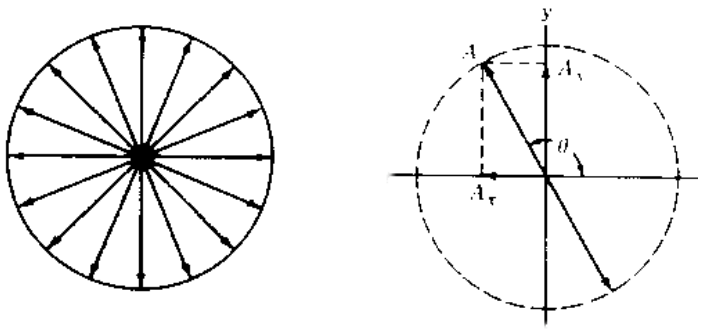
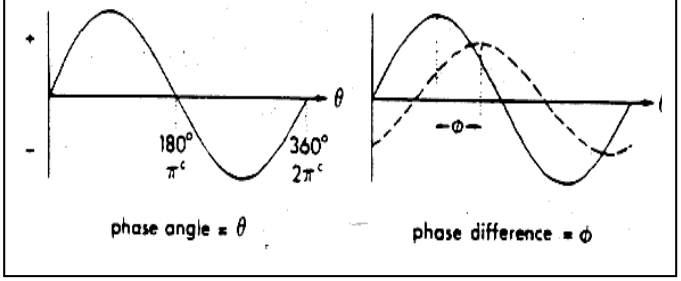


Figure 10.4



Phase of Light

Phase refers to the instantaneous position in space of a sinusoidal wave. The phase angle

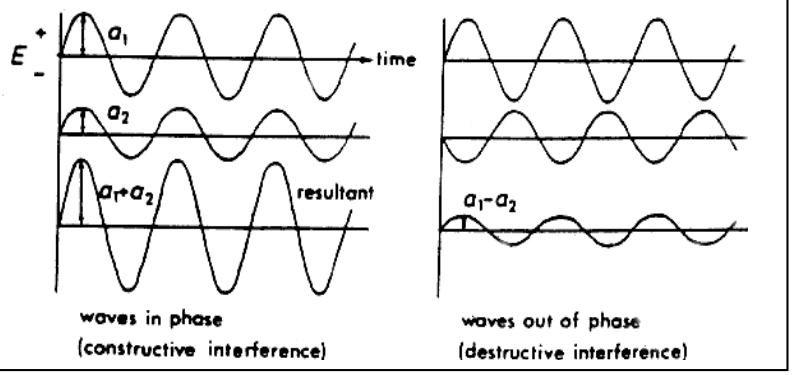
of a sinusoidal wave is the sin of the angle of the electric vector at any point in time. The phase angle Θ ranges from 0 to 360 degrees. (The electric vector is calculated as: E = a sin Θ where E is the electric vector and a is amplitude.) Phase difference (ϕ) between two waves of the same frequency is the difference in their phase angles (figure 10.4).

The important thing in all this is as follows: Two wavetrains of the same frequency and polarization angle are brought together. If their maximum and minimum peaks do not coincide, they are out of phase by an amount equal to the horizontal distance between any two corresponding points on the waves. **These wavetrains can interact with one another by an amount that depends on the phase difference, creating a resultant wavetrain that is increased or decreased in amplitude (i.e. in intensity or brightness). This interaction produces contrast and is what makes phase contrast possible.**

The Amplitude of a Light Wave

Amplitude is the height or maximum displacement of a wave. It is related to the **intensity** of the light and to the energy in the wave. The relationship is like this: the greater the amplitude the more intense the light and the greater the energy of the wavetrain.

Figure 10.5



Interference of Light Waves

If two wavetrains are brought together that are in phase and have the same polarization angle, they will interfere constructively to produce a single wavetrain with greater amplitude. If the two wavetrains are out of phase, they will interfere destructively, resulting in a single wavetrain of smaller amplitude. Figure 10.5 illustrates this.

Coherence of Light

Spatially coherent light wavetrains have the same frequency, direction, and polarization. Temporally coherent light wavetrains have exactly the same phase and speed. Laser light is both spatially and temporally coherent. Light in phase contrast microscopy is partially coherent.

Effect of Refractive Index Differences

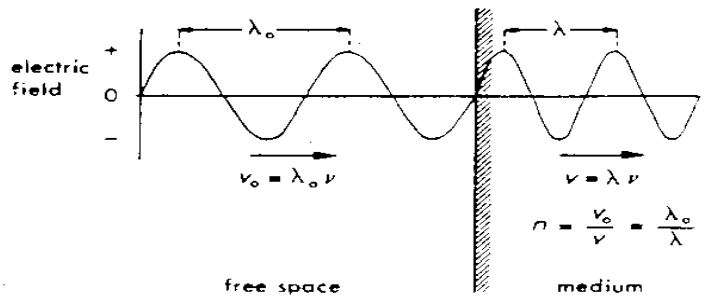
There are several effects associated with refractive index (figure 10.6):

1) A light wavetrain moves slower through a medium of higher refractive index than through a medium of lower refractive index.

2) Suppose a specimen has a higher refractive index than the surrounding medium. A light wavetrain passing through the specimen will be retarded in phase relative to a wavetrain passing through the medium. The specimen is a <u>phase retarding specimen</u>.

3) Suppose a specimen has a lower refractive index than the surrounding medium. A light wavetrain passing through the specimen will be advanced in phase relative to a wavetrain passing through the medium. The specimen is a <u>phase advancing specimen</u>.

Figure 10.6



The change in phase produced by the difference in refractive index between specimen and surround can be as small as 1/20th of a wavelength and still produce phase contrast.

Effect of the Specimen on the Wave

Think of the "specimen" as consisting of a set of narrow slits. The specimen has the following effects on a light wave (here think of a light wave as a single up and down portion of a wavetrain – like a wave on water):

1) The specimen will retard or advance part of a light wave depending on the difference in refractive index between the specimen and its surroundings.

2) The specimen will diffract some part of a light wave passing through it while some other part of the wave will pass through directly without being diffracted.

3) A light wave that does not pass through the specimen will not be diffracted.

4) **Many new diffracted and direct wavetrains are <u>created</u> from a <u>single</u> wavetrain when it passes a structure in the specimen. The new diffracted and direct light wavetrains have the same frequency and polarization angle, but not the same phase or direction. They are partially coherent. The diffracted light is <u>retarded</u> by approximately 1/4 wavelength. This is true for most biological specimens.**

Effect of the Objective Lens on the Diffracted and Direct Light

The objective lens affects light in several ways:

1) Direct light enters the objective as parallel rays or waves. The rays come to a focus at the objective's back focal plane. From here they spread out to cover the entire intermediate image plane.

2) Diffracted light enters the objective as diverging rays. The rays over spread the objective's back focal plane. From here they are focused at the intermediate image plane.

Intermediate Image Plane

Diffracted and direct light waves that were created from a single wavetrain recombine and interfere in the intermediate image plane.

1) Even though unpolarized white light is used to illuminate the specimen, interference is possible because, for any given incident light wavetrain that passes through a specimen structure the resulting diffracted and direct wavetrains will be partially coherent.

2) **Interference of diffracted with direct light in the intermediate image plane produces amplitude contrast. However, the 1/4 wavelength difference in phase between the direct and diffracted part of a wavetrain that is introduced by the specimen produces insufficient contrast for human vision. Additionally, there is a great deal more direct light than diffracted light. The intensity of the direct light overwhelms any contrast resulting from interference. The phase apparatus overcomes these two insufficiencies.**

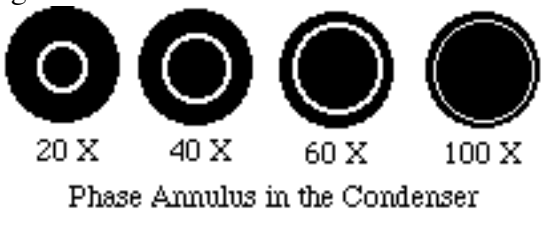
Phase Apparatus

Unlike dark field in which a homemade apparatus will give an acceptable result, phase contrast requires precision optical elements in both the condenser and the objective. These elements must be matched to one another. No one condenser element can be used with all objective elements. The phase contrast condenser will have a selection of phase annuli. These will correspond to the phase plates in the phase objectives.

Phase Annulus in the Condenser

The **Phase Annulus** is a clear ring in an otherwise opaque disk (figure 10.7). Its purpose is to produce a circle of light in the front focal plane of the condenser. The condenser thus produces a hollow cone of illumination that is focused on the specimen plane. Different objectives require different phase annuli for as the objective's NA increases so must the diameter of the annulus. Strictly speaking, a phase annulus is not required if the phase plate in the objective is made to modify only the 0 order diffraction spot in the objective's back focal plane. It has been

Figure 10.7



Phase Annulus in the Condenser

Phase Plate in the Objective Lens

found, however, that the use of a ring of illumination and a complementary circular phase altering ring in the objective back focal plane gives the best result.

Phase Plate in the Objective

The **Phase Plate** is a clear plate with a circular ring in it (figure. 10.7). The ring is usually a groove cut into the plate and filled with a phase advancing or phase retarding material (usually a dielectric). The ring may appear darker or lighter than the rest of the plate due to the addition of some material that alters the amplitude of light (a neutral density material usually a thin film of evaporated metal). The position of the ring lies over the image of the annulus in the condenser. The ring is **conjugate** with the annulus. The rest of the plate is **complementary** to the annulus and to the phase ring. Thus the phase plate has conjugate and complementary areas. The phase ring may be made as part of a lens in the objective rather than as a separate plate.

Phase Telescope

The phase telescope is a special eyepiece that focuses an image of the back focal plane of the objective onto a human's retina. Observing the objective's back focal plane is necessary for aligning the phase annulus to the phase ring. On some microscopes a built in Bertrand lens takes the place of the phase telescope.

Green Filter

The design of a phase contrast system is based on green light (Fraunhofer E line at 525 nm). Manufacturers use the green line to determine the amount of material to use in the phase ring for phase advancement or retardation. The green filter provides this illumination. Phase contrast however works well enough in white light.

Centering Tools

The phase annulus in the condenser must coincide exactly with the phase ring in the objective. Tools are provided for moving the phase annulus relative to the phase ring while observing the two with the phase telescope.

Effect of the Phase Apparatus

Each peace of the phase apparatus produces a specific effect. The combination of all these effects results in phase contrast. Different types of phase contrast may be produced depending on the design of the apparatus.

Phase Annulus in the Condenser

The annulus creates a hollow cone of illumination that comes to a focus on the specimen. Unlike the dark field hollow cone, this ring of direct light enters the aperture of the objective lens. The direct light forms a ring of illumination at the back focal plane of the objective. The size of this ring of light just matches the size of the phase ring in the phase plate. In the specimen plane, the ring of light is at a focus and thus is a solid circular patch of illumination. The specimen diffracts part of this light and the diffracted

light spreads out over the entire back focal plane of the objective and thus over the entire phase plate (including over the phase ring).

Phase Plate in the Objective

The phase ring has a specific thickness different from the rest of the phase plate, such that it changes the phase of the direct light by 1/4 wave relative to the diffracted light (usually advancing the phase of the direct light). The amplitude of the direct light is also changed because the phase ring is made darker than the rest of the phase plate. It is made darker because there is always a great deal more direct light than diffracted light. This direct light could swamp any amplitude contrast created by interference. On average, a specimen retards the diffracted light's phase by 1/4 wave. The additional 1/4 wave advancement given to the direct light by the phase ring causes the direct and diffracted light to be about 1/2 wave out of phase. This is just right for maximum interference in the intermediate image plane.

Types of Phase Contrast

Several types of phase contrast are possible depending on the construction of the phase plate in the objective as listed below and illustrated in figure 10.8.

1) In "positive" phase contrast the direct light is advanced 1/4 wave in phase (- type). This produces destructive interference and creates dark details on a light background. This is the most common form of phase contrast (figure 10.13)

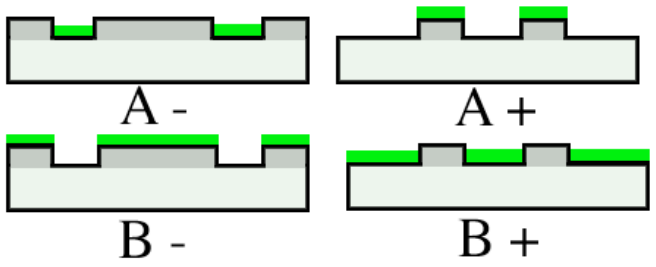


Figure 10.8 Cross sections of different types of phase plates. Gray = longer path length, Green = light absorbing material.

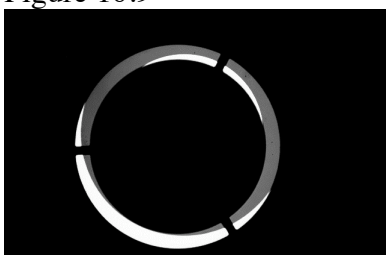
2) In "negative" phase contrast the direct light is retarded 1/4 wave in phase (+ type). This produces constructive interference resulting in light details on a dark background.

3) In either positive or negative phase contrast, the phase plate may be one of two types. Either the direct light can be absorbed (A type) or the diffracted light can be absorbed (B type). The most common type is A -. Both A and B type plates are rated by the percentage transmission of the ring area, 20% is most common.

Setting Up Phase Contrast

The phase apparatus must be aligned properly for phase contrast to work. Following are the steps necessary for correct phase alignment:

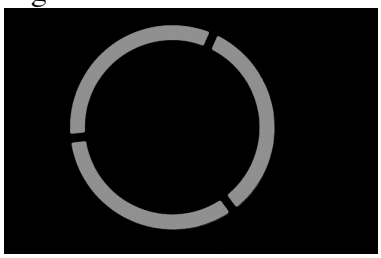
Figure 10.9



1) Align the microscope for Köhler illumination. Phase contrast requires that the condenser produce nearly parallel light waves in the specimen plane. Köhler illumination provides this by placing an image of the light source in the front focal plane of the condenser. Once this is done, fully open the condenser iris.

2) Focus on a specimen with the low power phase objective.

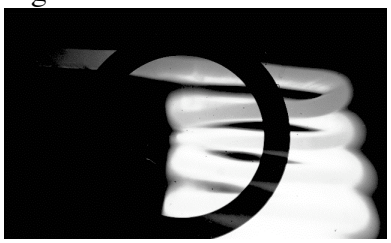
Figure 10.10



3) Insert the proper phase annulus for the selected objective. Remove an eyepiece and replace it with the phase telescope and focus the telescope on the phase annulus (or insert and focus the Bertrand lens).

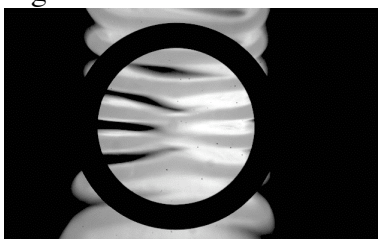
4) Align the phase ring to the phase annulus using the alignment tools. Figure 10.9 illustrates a phase annulus (white) with an overlying phase ring (gray). The two are not properly aligned. Figure 10.10 illustrates proper alignment.

Figure 10.11



5) Repeat steps 1 - 4 for each phase objective and its corresponding phase annulus. Once aligned, the phase annuli should remain aligned for long periods of time. One possible cause for a shift in alignment is the repositioning of objectives on the nosepiece.

Figure 10.12



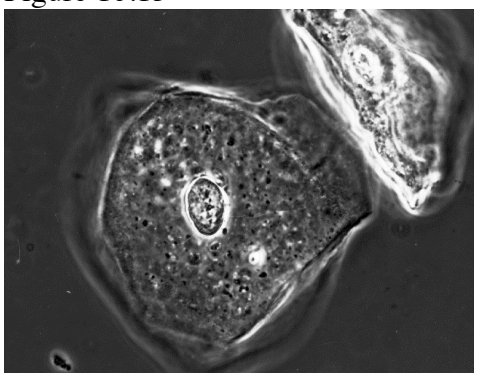
6) Align the filament image as follows: put condenser in bright field, use a 10X or 20X phase objective, remove the illuminator's diffuser, and observe the back focal plane of the objective using the phase telescope. Align the filament by using the illuminator's adjustments. Figures 10.11 and 10.12 illustrate improper and proper filament alignment respectively. The filament should be of a tightly coiled type. The coils should be centered on and completely filling the phase ring.

7) Insert the green filter. The thickness and material of the phase ring are calculated to advance (or retard) green light by 1/4 wave.

8) Put back the eyepiece, select the correct phase annulus for the phase objective, and enjoy.

Appearance of Phase Contrast Images

Figure 10.13



Ideally the appearance of a phase contrast image represents differences of refractive index and thickness of microscopic structures in the specimen. Phase contrast is often said to reveal edges. Figure 10.13 illustrates the most common type of phase contrast in which structures appear dark as compared with their surrounding medium. Unfortunately there are two artifacts that are common to all phase contrast images. These are the result of the apparatus used to create the image in the first place. First, some of the light that is diffracted by the specimen enters the phase ring and is thus changed by it. Second, some of the direct light passes through the complementary area of the phase plate. The combination of these phenomena results in a phase image having the following two artifacts.

Phase Halo

Phase halo is a diffuse ring that is opposite in intensity from the specimen and that surrounds the specimen and substructures. In negative phase contrast, a dark specimen will have a bright halo on a light background (note the nucleus of the cheek epithelial cell in figure 10.13). In positive phase contrast a light specimen will have a dark halo on a darker background.

Shading Off

Shading off effect occurs within the specimen itself. If the specimen is dark there will be a central bright region that shades off into the dark body of the specimen; if the specimen is light there will be a central dark region that shades off into the light body of the specimen. The shading-off effect is difficult to illustrate simply for it presents a complex pattern of light and dark areas within the specimen.

Problems in Phase Contrast

There are two areas in which phase contrast can be limiting.

1) Phase contrast is excellent for thin, colorless, nearly transparent specimens. However if the specimens are very thick a confusing phase image will result do to the superposition of phase altering structures.

2) The phase plate does limit the objective's NA. Some loss of resolution occurs because the phase ring occupies part of the objective's aperture. Never-the-less, most phase objectives are perfectly suitable for bright field work.

Exercises

1) Make a note of the phase objectives on your microscope. Phase objectives are usually marked with "Ph" (usually in green letters) followed by a number. If you are not sure if an objective is phase, observe it in bright field without the eyepiece in place to see if there is a phase plate in the lens. Write down all the information written on these objectives.

2) Determine which phase annuli match your phase objectives. Make a note of it. How can you figure this out without an instruction book?

3) Observe the hollow cone pattern of light created by the phase contrast condenser by placing a white card on edge on top of the condenser with a phase annulus in place. This is more easily done on an upright than on an inverted microscope.

4) Set your microscope up for phase contrast. Observe a slide of soap bubbles. Briefly describe their appearance noting the halo and shading-off artifacts. Observe a cheek cell slide and note the phase contrast image plus the artifacts.

5) Experiment with a bright field objective lens and the phase annuli to produce a uni-directional dark field effect. Rotate the annulus slightly out of position to achieve the effect. Which lens – annulus combinations work? This method is known as oblique illumination.