Phase Contrast Microscopy

Secondary article

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Phase contrast microscopy is a method that enables us to see very transparent objects, which are otherwise almost invisible by ordinary light microscopy, in clear detail and in good contrast to their surroundings. This is achieved optically, without altering the specimen by staining or other processing.

Article Contents

Introduction

The human eye perceives only differences in wavelength (as colour) and amplitude (as brightness) of the light reaching it. The eye cannot see differences in the phase relationship between different beams of light. Classically, biological specimens have usually been viewed as stained slices of material by bright-field transmitted-light microscopy (**Figure 1f**). Stains are used to alter both the colour and brightness of the light passing through the specimen, and so increase contrast in the image. Cellular function is understood better by studying the actual motion, growth, reproduction and exchange of cell constituents of living cells than it is by drawing conclusions from artificially manipulated dead ones. Since cells are generally transparent structures, they are almost invisible to the eye by bright-field microscopy (**Figures 1b**,**g**).

The phase contrast microscope exploits the interaction of the illuminating beam of light with the specimen to convert an image of an invisible specimen (or one of very low visibility) into an image that the eye can detect. Other forms of contrast enhancement, such as darkground microscopy, differential interference contrast microscopy and Hoffmann modulation contrast microscopy, have also been developed. For further details, see Bradbury and Evennett (1996). Perhaps the most widespread use of phase contrast microscopy in biology is for cytology and tissue culture, admirably lending itself to quick checking of live cell cultures (**Figure 1a**).

How Phase Contrast Works

Light can be considered as a wave. When light encounters glass, or other optically transparent material denser than air, it is slowed down, and the number of waves increases in proportion to both the density (as determined by the refractive index) and thickness of the material. Consider a second identical beam (from the same source), which moves wholly in air parallel to the first without entering the glass. The first beam will have travelled a greater distance than the second. Furthermore, the two beams, which started out in synchrony, or in phase, are now out of phase; this difference is referred to as the phase difference between the two beams. All specimens diffract, or scatter, light, and these diffracted beams carry the information about the structure of the object (Oldfield, 1994; Plášek and Reischig, 1998). An image of the object is formed at the primary image plane owing to interference between those beams diffracted by the specimen and the undiffracted (zero order) beam. With a stained specimen there is a half-wavelength ($\frac{1}{2}\lambda$) phase difference between the undiffracted beam and those diffracted by the specimen. Interference of the two sets of beams leads to overall differences in amplitude, which can be detected by the eye as differences in brightness. Coloured objects are merely amplitude specimens that selectively absorb light of certain wavelengths within the visible spectrum.

Thin transparent objects such as cells introduce a phase difference between the two beams of only one-quarter wavelength ($\frac{1}{4}\lambda$). For a theoretical discussion of why this is so, using vector treatment, see Pluta (1989) or Bradbury and Evennett (1996). To render these specimens visible, it is necessary to have an artificial means of introducing an extra $\frac{1}{4}\lambda$ phase difference between the diffracted and undiffracted beams. There would then be $\frac{1}{4}\lambda$ phase difference contributed by the specimen and a further $\frac{1}{4}\lambda$ phase difference by the microscope.

If the beam illuminating the field of view is constrained by an annulus in the first focal plane of the condenser, it will form an annular image in the back focal plane of the objective (**Figure 2**). Suppose that a circular 'trench' (or, alternatively, a ridge) matching the image of the condenser annulus is placed within the back focal plane of the objective. The optical path traversed by the undiffracted beam alone can now be selectively advanced (or retarded) and the necessary extra $\frac{1}{4}\lambda$ phase difference between the two beams introduced before they recombine to form the image at the primary image plane of the microscope. This 'trench' (or ridge) is called the phase ring and it is carried on the phase plate. The phase ring carries an absorbing layer that reduces the amplitude of the undiffracted zero order beam, reducing its brightness to match that of the weaker beams diffracted by the specimen.

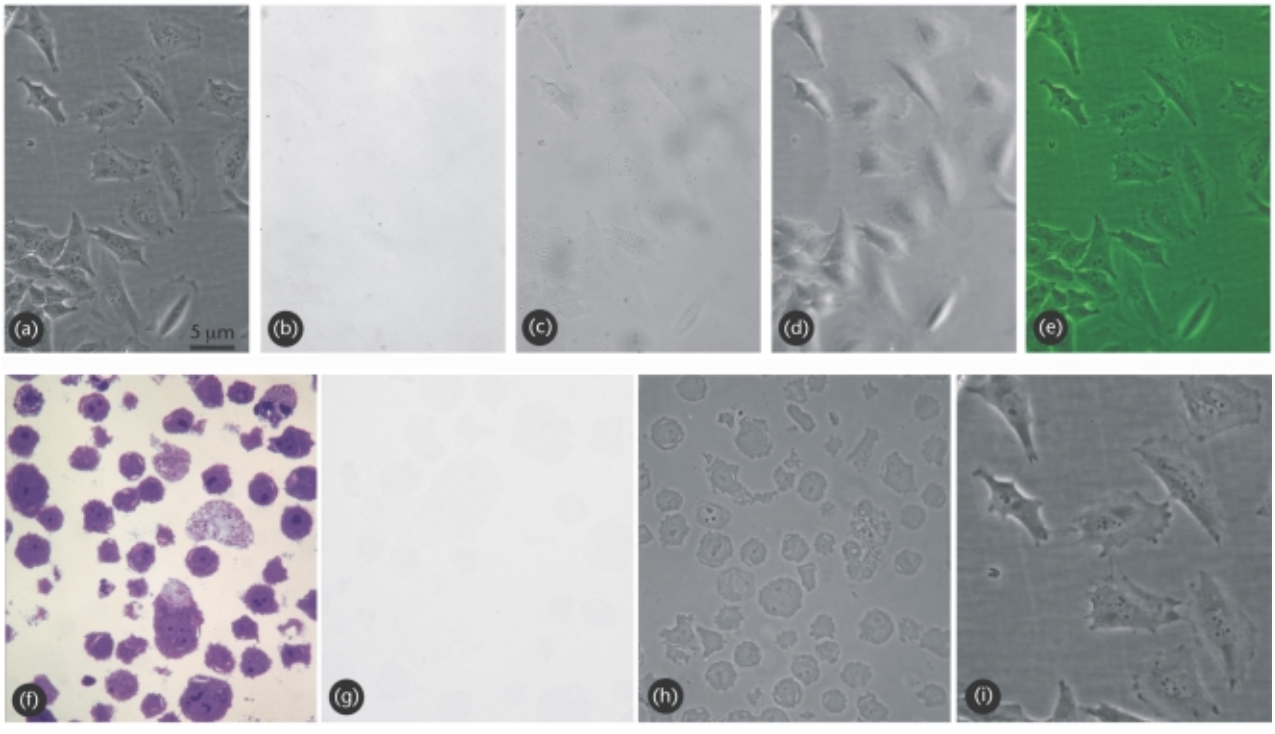

Figure 1 All parts of this figure show the same field of view of living HeLa cells (a–e) and fixed, embedded HeLa cells in thin section (f–h).

(a) Living HeLa cells in culture by phase contrast. (b) The same cells by transmitted-light bright-field microscopy. (c) In bright-field mode, without phase contrast, closing the condenser diaphragm will enhance contrast to some degree, but at the expense of resolution in the image. This method is to be avoided. (d) Same image as (a), but the image has been taken with the annulus and phase plate out of alignment (see also Figures 3e,f). (e) The use of a green filter improves the quality of the phase contrast image. (f) Stained HeLa cells, together with the bright-field image (g) for comparison with the phase contrast image (h).

Parts (h) and (i) are included for comparison of phase contrast images of living cells with those that have been fixed, embedded and sectioned thinly. The manner in which cells and tissues are fixed (if at all) and prepared will influence the resulting phase contrast image. The living cells (h) exhibit high contrast, where there is a relatively high difference of refractive index between the cells and the watery medium they are contained in. The sections of cells embedded in resin in (i) exhibit lower contrast. This is because there is a smaller difference of refractive index between the cell constituents and the background resin. Likewise, cells fixed in methanol, an extracting fixative, exhibit a higher contrast image than those fixed in paraformaldehyde, a crosslinking fixative that retains more of the cytoplasm.

Figures (a)–**(e)** were taken using a Zeiss Axiovert 25, inverted microscope for tissue culture using a 32×NA 0.5 long working distance objective. Figures (f)–(h) were taken using a Zeiss Axiophot microscope equipped with a Plan Neofluar 40×NA 1.30 oil immersion phase contrast objective.

Construction of the Phase Contrast Microscope

A special set of objectives, fitted with phase plates, is normally needed for phase contrast microscopy. Manufacturers generally provide several different sizes of annuli in the condenser to match objectives of differing magnification and numerical aperture (**Figure 3d**). These annuli can normally be rotated within the condenser housing, and brought onto the optical axis of the microscope as required (**Figures 3a,b**). Provision is usually made for centring each annulus with respect to the optical axis of the condenser.

Interpreting the Phase Contrast Image

Provided that the undiffracted and diffracted beams are out of phase with one another by $\frac{1}{2}\lambda$ overall, they will interfere to form a visible image, and it does not matter whether the diffracted beams are retarded or advanced by $\frac{1}{4}\lambda$ with respect to the undiffracted beam. Two forms of phase contrast microscopy are therefore possible; these are referred to as positive and negative phase contrast. Positive phase contrast refers to the most widely used system where the phase plate is constructed with a 'trench', so that the diffracted beams (passing outside the phase ring) travel one-quarter of a wavelength further than the zero order beams. Structures with a refractive index higher than their

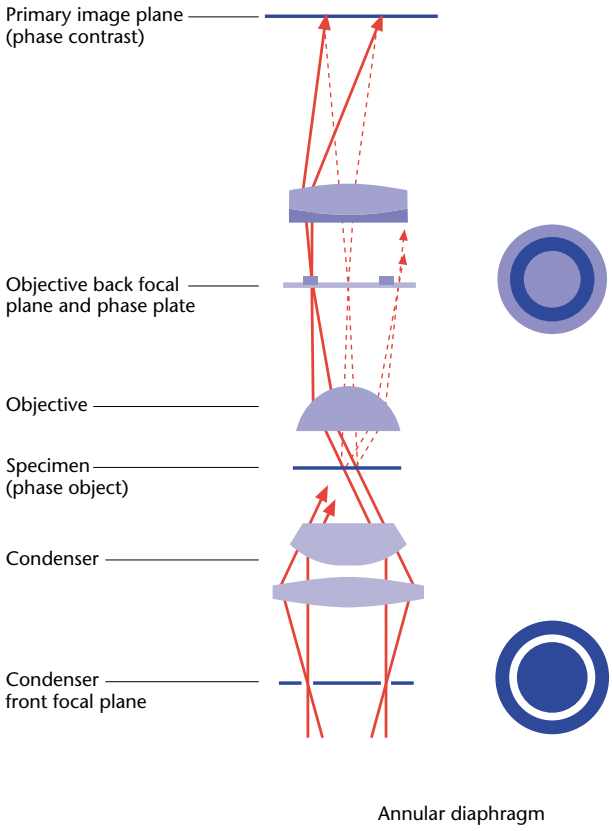

Figure 2 Ray diagram of the phase contrast method. The heavy lines represent the undiffracted beams, while the diffracted beams are shown by dashed lines. Adapted with permission from Plášek and Reischig (1998).

surroundings give rise to diffracted beams retarded by one-quarter wavelength, and these more highly refracting areas will thus appear darker in the final image, against a lighter background (**Figures 1a,h**). Positive phase contrast is responsible for the commonly recognized appearance of a cell, with the nucleus, lysosomal compartments and the cell membrane appearing darker than their surroundings.

The phase contrast effect is maximal at regions of sudden change in optical path difference ('edges'), and is less pronounced where the change in optical path difference between adjacent areas is not so abrupt ('wedges'), a phenomenon known as 'shading-off'. As a consequence, the centre of one structure may appear the same shade of grey as that of another of quite different refractive index. Phase contrast is better suited to structures with an 'edge' rather than structures with 'wedge' boundaries.

The Halo Artefact

Most beams diffracted by the specimen will not pass through the phase ring. However, the phase ring has an appreciable width and some diffracted rays will inevitably pass through it, causing the haloes that are a familiar part of phase contrast images. In positive phase contrast objects of refractive index higher than the background form an image in which these dark structures are surrounded by a bright halo, and lined internally with a darker halo. In negative phase contrast, the situation is reversed. Phase contrast is not suited for making precise linear measurements: it is difficult to assess accurately the precise position of an edge in the image owing to the halo artefact.

Setting Up the Phase Contrast Microscope

Set the microscope up, in proper adjustment for Köhler illumination for bright-field microscopy, using a well-stained specimen. Ensure that the condenser is set at the correct height, and is centred. If in doubt, refer to Bradbury and Bracegirdle (1998) or Oldfield (1994). Without altering the focus, replace the stained specimen with the transparent one. Open the condenser aperture fully. Swing in a low-power ($10\times$ or $20\times$) phase contrast objective; the specimen will probably not be visible. Insert the correct annulus; an indication of the appropriate annulus is usually marked on the barrel of the objective in green script (e.g. Ph3).

Remove an eyepiece and insert a centring-telescope (sometimes called a 'phase telescope'), or insert a Bertrand lens system into the optical path to image the back focal plane of the objective through the eyepieces. Whichever device is used, focus on the phase plate within the objective. The image of the annulus in the condenser (which is conjugate with the objective's phase plate) will also be in focus.

Using the centring adjustments provided for the annuli, and without disturbing the normal centre position of the condenser itself, superimpose the image of the condenser annulus precisely over that of the objective phase ring (**Figure 3e**). The centring screws used for this superimposition (usually set at 90° or 120° on the condenser housing) are not those used for Köhler illumination. They are either captive on the condenser (**Figure 3d**), or may be recessed hexagonal screws at the rear of the condenser, requiring an Allen key for adjustment. If in doubt on this point, refer to the manufacturer's instructions. Once adjusted, the annuli in the condenser should remain centred over a lengthy period; it should not be necessary to recentre each time the microscope is used. Remove the centring-telescope and replace the eyepiece, or remove the Bertrand lens. For an inverted microscope the alignment procedure is usually the same.

Although in practice the phase contrast system works over the full spectrum of white light, it must necessarily be manufactured for illumination of one wavelength,

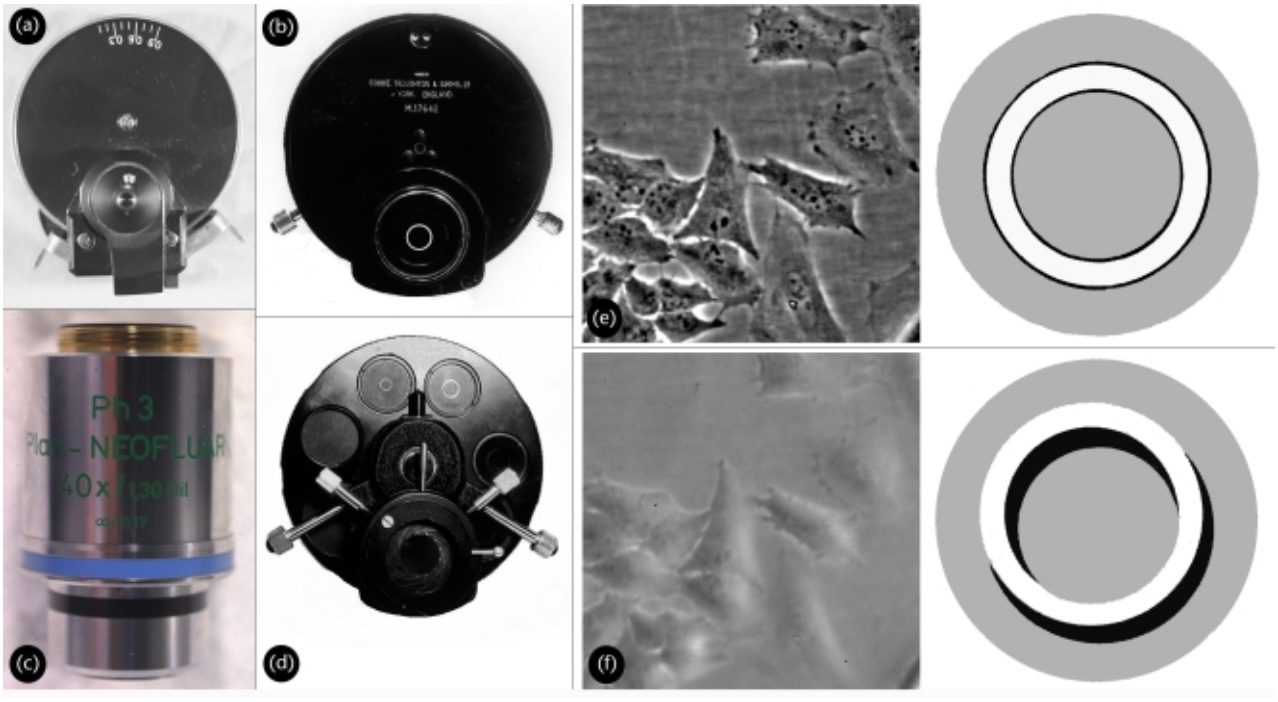

Figure 3 (a) and (b) show the top view of different types of phase contrast condenser, in which the various annuli are contained within a housing. This permits them to be changed quickly and efficiently as required. (c) The commonly encountered green inscription engraved on the barrel of a phase contrast objective. The correct annulus to use is denoted, shown here by the designation Ph3. (d) The underside of the condenser in (b), revealing the separate controls for centring the condenser onto the optical axis during alignment of the microscope, and those for independently aligning the annulus with the phase ring. The different sizes of annuli can also be seen. (e) and (f) show the effects on the phase contrast image of not having the annulus and phase ring in absolute alignment.

generally selected as 550 nm. This is chosen because the eye is most sensitive to green light and objectives are best corrected for spherical aberration at this wavelength. Hence, for optimum contrast, a green filter should be used in the illuminating light path (**Figure 1e**). If a satisfactory phase contrast image is not obtained (e.g. **Figure 1d**), first check that the microscope is correctly set up for Köhler illumination, and then that the condenser is correctly centred and set at the right height. If this fails to remedy the situation, check that the image of the annulus is of the correct size and its image is precisely superimposed over the phase ring.

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