bibliography for more details. Only a few of those available will be discussed here.

Gram's stain. By far the most important in terms of use and application is the Gram stain, developed by Christian Gram in 1884 and subsequently modified. The fixed film of bacteria is flooded initially with a solution of methyl violet. This is followed by a solution of Gram's iodine, which is an iodine–potassium iodide complex acting as a mordant, fixing the dye firmly in certain bacteria and allowing easy removal in others. Decolourization is effected with either alcohol or acetone or mixtures of the two. After treatment some bacteria retain the stain and appear a dark purple colour and these are called Gram positive. Others do not retain the stain and appear colourless (Gram negative). The colourless cells may be stained with a counterstain of contrasting colour, such as 0.5% safranin, which is red.

This method, although extremely useful, must be used with caution as the Gram reaction may vary with the age of the cells and the technique of the operator. For this reason, known Gram-positive and Gram-negative controls should be stained alongside the specimen of interest.

Ziehl–Neelsen's acid-*f*ast stain. The bacterium responsible for the disease tuberculosis (*Mycobacterium tuberculosis*) contains within its cell wall a high proportion of lipids, fatty acids and alcohols, which render it resistant to normal staining procedures. The inclusion of phenol in the dye solution, together with the application of heat, enables the dye (basic fuchsin) to penetrate the cell and, once attached, to resist vigorous decolourization by strong acids, e.g. 20% sulphuric acid. These organisms are therefore called acid fast. Any unstained material can be counterstained with a contrasting colour, e.g. methylene blue.

Fluorescence.microscopy

Certain materials, when irradiated by short-wave illuminations, e.g. UV light, become excited and emit visible light of a longer wavelength. This phenomenon is termed fluorescence and will persist only for as long as the material is irradiated. A number of dyes have been shown to fluoresce and are useful in that they tend to be specific to various tissues, which can then be demonstrated by UV irradiation and subsequent fluorescence of the attached fluorochrome. Coupling antibodies to the fluorochromes can enhance specificity, and this technique has found wide application in microbiology. As with the staining procedures described above, this technique can only be applied to dead cells. The three following techniques have been developed for the examination of living organisms.

Dark-ground.microscopy

The usual function of the microscope condenser is to concentrate as much light as possible through the specimen and into the objective lens. The dark-ground condenser performs the opposite task, producing a hollow cone of light that comes to a focus on the specimen. The rays of light in the cone are at an oblique angle, such that after passing across the specimen they continue without meeting the front lens of the objective, resulting in a dark background. Any objects present at the point of focus scatter the light, which then enters the objective to show up as a bright image against the dark background.

Specimen preparation is critical, as very dilute bacterial suspensions are required, preferably with all the objects in the same plane of focus. Air bubbles must be absent from both the film and the immersion oil, if used. Dust and grease also scatter light and destroy the uniformly black background required for this technique. With this technique it is not possible to see any real detail but it is useful to study motility.

Phase-contrast.microscopy

This technique allows us to see transparent objects well contrasted from the background in clear detail and is the most widely used image enhancement method in microbiology. In essence, an annulus of light is produced by the condenser of the microscope and focused on the back focal plane of the objective where a phase plate, comprising a glass disc containing an annular depression, is situated. The direct rays of the light source annulus pass through the annular groove and any diffracted rays pass through the remainder of the disc. Passage of the diffracted light through this thicker glass layer results in retardation of the light. This alters its phase relationship to the direct rays and increases contrast.

Differential-interference..contrast.microscopy

This method uses polarized light and has other applications outside the scope of this chapter, such as