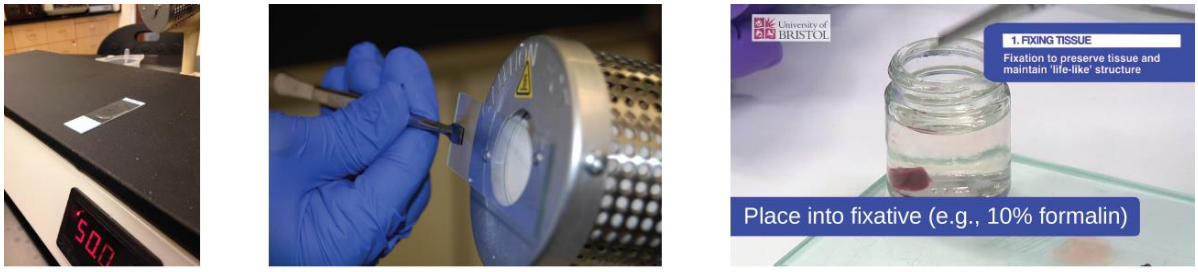
Preparing Specimens for Light Microscopy

In clinical settings, light microscopes are the most commonly used microscopes. There are two basic types of preparation used to view specimens with a light microscope: wet mounts and fixed specimens.

The simplest type of preparation is the **wet mount**, in which the specimen is placed on the slide in a drop of liquid. Some specimens, such as a drop of urine, are already in a liquid form and can be deposited on the slide using a dropper. Solid specimens, such as a skin scraping, can be placed on the slide before adding a drop of liquid to prepare the wet mount. Sometimes the liquid used is simply water, but often stains are added to enhance contrast. Once the liquid has been added to the slide, a coverslip is placed on top and the specimen is ready for examination under the microscope.

The second method of preparing specimens for light microscopy is **fixation**. The "fixing" of a sample refers to the process of attaching cells to a slide. Fixation is often achieved either by heating (heat fixing) or chemically treating the specimen. In addition to attaching the specimen to the slide, fixation also kills microorganisms in the specimen, stopping their movement and metabolism while preserving the integrity of their cellular components for observation.

To heat-fix a sample, a thin layer of the specimen is spread on the slide (called a **smear**), and the slide is then briefly heated over a heat source (Figure 2.31). Chemical fixatives are often preferable to heat for tissue specimens. Chemical agents such as acetic acid, ethanol, methanol, formaldehyde (formalin), and glutaraldehyde can denature proteins, stop biochemical reactions, and stabilize cell structures in tissue samples (Figure 2.31).



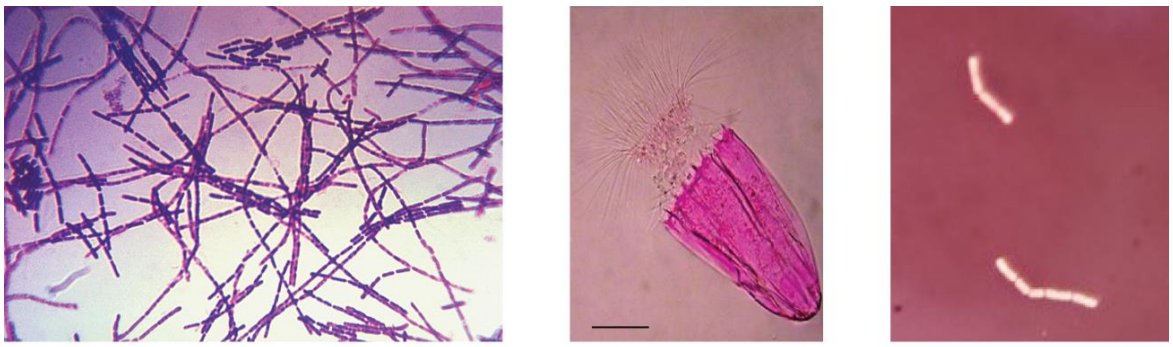
(a) (b) (c)

Figure 2.31 (a) A specimen can be heat-fixed by using a slide warmer like this one. (b) Another method for heat-fixing a specimen is to hold a slide with a smear over a microincinerator. (c) This tissue sample is being fixed in a solution of formalin (also known as formaldehyde). Chemical fixation kills microorganisms in the specimen, stopping degradation of the tissues and preserving their structure so that they can be examined later under the microscope. (credit a: modification of work by Nina Parker; credit b: modification of work by Nina Parker; credit c: modification of work by "University of Bristol"/YouTube)

In addition to fixation, **staining** is almost always applied to color certain features of a specimen before examining it under a light microscope. Stains, or dyes, contain salts made up of a positive ion and a negative ion. Depending on the type of dye, the positive or the negative ion may be the chromophore (the colored ion); the other,

uncolored ion is called the counterion. If the chromophore is the positively charged ion, the stain is classified as a **basic dye**; if the negative ion is the chromophore, the stain is considered an **acidic dye**.

Dyes are selected for staining based on the chemical properties of the dye and the specimen being observed, which determine how the dye will interact with the specimen. In most cases, it is preferable to use a **positive stain**, a dye that will be absorbed by the cells or organisms being observed, adding color to objects of interest to make them stand out against the background. However, there are scenarios in which it is advantageous to use a **negative stain**, which is absorbed by the background but not by the cells or organisms in the specimen. Negative staining produces an outline or silhouette of the organisms against a colorful background (Figure 2.32).



(a) (b) (c)

Figure 2.32 (a) These *Bacillus anthracis* cells have absorbed crystal violet, a basic positive stain. (b) This specimen of *Spinoloricus*, a microscopic marine organism, has been stained with rose bengal, a positive acidic stain. (c) These *B. megaterium* appear to be white because they have not absorbed the negative red stain applied to the slide. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by Roberto Danovaro, Antonio Pusceddu, Cristina Gambi, Iben Heiner, Reinhardt Mobjerg Kristensen; credit c: modification of work by Anh-Hue Tu)

Because cells typically have negatively charged cell walls, the positive chromophores in basic dyes tend to stick to the cell walls, making them positive stains. Thus, commonly used basic dyes such as basic fuchsin, crystal violet, malachite green, methylene blue, and safranin typically serve as positive stains. On the other hand, the negatively charged chromophores in acidic dyes are repelled by negatively charged cell walls, making them negative stains. Commonly used acidic dyes include acid fuchsin, eosin, and rose bengal. Figure 2.40 provides more detail.

Some staining techniques involve the application of only one dye to the sample; others require more than one dye. In **simple staining**, a single dye is used to emphasize particular structures in the specimen. A simple stain will generally make all of the organisms in a sample appear to be the same color, even if the sample contains more than one type of organism. In contrast, **differential staining** distinguishes organisms based on their interactions with multiple stains. In other words, two organisms in a differentially stained sample may appear to be different colors. Differential staining techniques commonly used in clinical settings include Gram staining, acid-fast staining, endospore staining, flagella staining, and capsule staining. Figure 2.41 provides more detail on these differential staining techniques.

Gram Staining

The **Gram stain procedure** is a differential staining procedure that involves multiple steps. It was developed by Danish microbiologist Hans Christian Gram in 1884 as an effective method to distinguish between bacteria with different types of cell walls, and even today it remains one of the most frequently used staining techniques. The steps of the Gram stain procedure are listed below and illustrated in Figure 2.33.

1. First, crystal violet, a **primary stain**, is applied to a heat-fixed smear, giving all of the cells a purple color.
2. Next, Gram's iodine, a **mordant**, is added. A mordant is a substance used to set or stabilize stains or dyes; in this case, Gram's iodine acts like a trapping agent that complexes with the crystal violet, making the crystal violet–iodine complex clump and stay contained in thick layers of peptidoglycan in the cell walls.
3. Next, a **decolorizing agent** is added, usually ethanol or an acetone/ethanol solution. Cells that have thick peptidoglycan layers in their cell walls are much less affected by the decolorizing agent; they generally retain the crystal violet dye and remain purple. However, the decolorizing agent more easily washes the dye out of cells with thinner peptidoglycan layers, making them again colorless.
4. Finally, a secondary **counterstain**, usually safranin, is added. This stains the decolorized cells pink and is less noticeable in the cells that still contain the crystal violet dye.

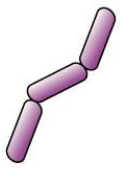
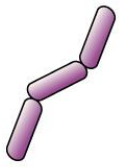
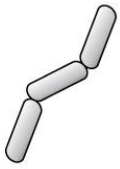
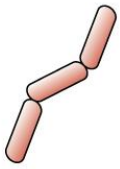
Gram stain process			
Gram staining steps	**Cell effects**	**Gram-positive**	**Gram-negative**
Step 1 **Crystal violet** *primary stain added to specimen smear.*	Stains cells purple or blue.		
Step 2 **Iodine** *mordant makes dye less soluble so it adheres to cell walls.*	Cells remain purple or blue.		
Step 3 **Alcohol** *decolorizer washes away stain from gram-negative cell walls.*	Gram-positive cells remain purple or blue. Gram-negative cells are colorless.		
Step 4 **Safranin** *counterstain allows dye adherance to gram-negative cells.*	Gram-positive cells remain purple or blue. Gram-negative cells appear pink or red.		

Figure 2.33 Gram-staining is a differential staining technique that uses a primary stain and a secondary counterstain to distinguish between gram-positive and gram-negative bacteria.

The purple, crystal-violet stained cells are referred to as gram-positive cells, while the red, safranin-dyed cells are gram-negative (Figure 2.34). However, there are several important considerations in interpreting the results of a Gram stain. First, older bacterial cells may have damage to their cell walls that causes them to appear gram-negative even if the species is gram-positive. Thus, it is best to use fresh bacterial cultures for Gram staining. Second, errors such as leaving on decolorizer too long can affect the results. In some cases, most cells will appear gram-positive while a few appear gram-negative (as in Figure 2.34). This suggests damage to the individual cells or that decolorizer was left on for too long; the cells should still be classified as gram-positive if they are all the same species rather than a mixed culture.

Besides their differing interactions with dyes and decolorizing agents, the chemical differences between gram-positive and gram-negative cells have other implications with clinical relevance. For example, Gram staining can help clinicians classify bacterial pathogens in a sample into categories associated with specific properties. Gram-negative bacteria tend to be more resistant to certain antibiotics than gram-positive bacteria. We will discuss this and other applications of Gram staining in more detail in later chapters.

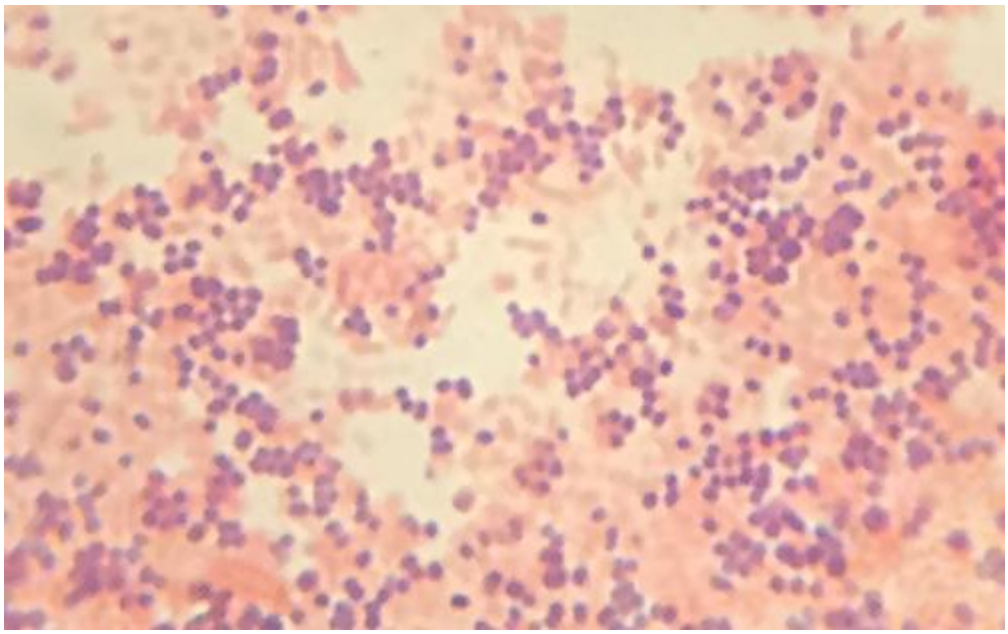


Figure 2.34 In this specimen, the gram-positive bacterium *Staphylococcus aureus* retains crystal violet dye even after the decolorizing agent is added. Gram-negative *Escherichia coli*, the most common Gram stain quality-control bacterium, is decolorized, and is only visible after the addition of the pink counterstain safranin. (credit: modification of work by Nina Parker)

Acid-Fast Stains

Acid-fast staining is another commonly used, differential staining technique that can be an important diagnostic tool. An **acid-fast stain** is able to differentiate two types of gram-positive cells: those that have waxy mycolic acids in their cell walls, and those that do not. Two different methods for acid-fast staining are the **Ziehl-Neelsen technique** and the **Kinyoun technique**. Both use carbolfuchsin as the primary stain. The waxy, acid-fast cells retain the carbolfuchsin even after a decolorizing agent (an

acid-alcohol solution) is applied. A secondary counterstain, methylene blue, is then applied, which renders non–acid-fast cells blue.

The fundamental difference between the two carbolfuchsin-based methods is whether heat is used during the primary staining process. The Ziehl-Neelsen method uses heat to infuse the carbolfuchsin into the acid-fast cells, whereas the Kinyoun method does not use heat. Both techniques are important diagnostic tools because a number of specific diseases are caused by acid-fast bacteria (AFB). If AFB are present in a tissue sample, their red or pink color can be seen clearly against the blue background of the surrounding tissue cells (Figure 2.36).

Using Microscopy to Diagnose Tuberculosis

Mycobacterium tuberculosis, the bacterium that causes tuberculosis, can be detected in specimens based on the presence of acid-fast bacilli. Often, a smear is prepared from a sample of the patient's sputum and then stained using the Ziehl-Neelsen technique (Figure 2.36). If acid-fast bacteria are confirmed, they are generally cultured to make a positive identification. Variations of this approach can be used as a first step in determining whether *M. tuberculosis* or other acid-fast bacteria are present, though samples from elsewhere in the body (such as urine) may contain other *Mycobacterium* species.

An alternative approach for determining the presence of *M. tuberculosis* is immunofluorescence. In this technique, fluorochrome-labeled antibodies bind to *M. tuberculosis*, if present. Antibody-specific fluorescent dyes can be used to view the mycobacteria with a fluorescence microscope.

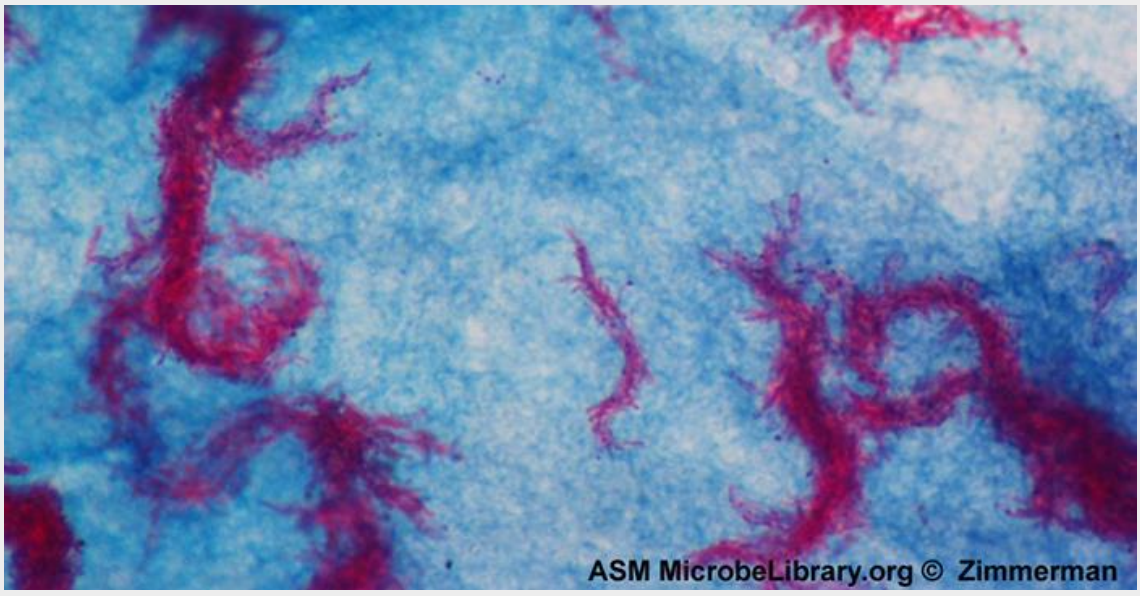
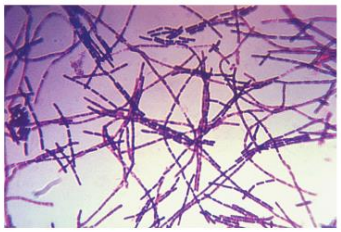
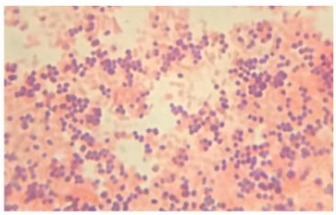


Figure 2.36 Ziehl-Neelsen staining has rendered these *Mycobacterium tuberculosis* cells red and the surrounding growth indicator medium blue. (credit: modification of work by American Society for Microbiology)

SIMPLE STAINS				
Stain Type	**Specific Dyes**	**Purpose**	**Outcome**	**Sample Images**
Basic stains	Methylene blue, crystal violet, malachite green, basic fuchsin, carbolfuchsin, safranin	Stain negatively charged molecules and structures, such as nucleic acids and proteins	Positive stain	
Acidic stains	Eosin, acid fuchsin, rose bengal, Congo red	Stain positively charged molecules and structures, such as proteins	Can be either a positive or negative stain, depending on the cell's chemistry.	
Negative stains	India ink, nigrosin	Stains background, not specimen	Dark background with light specimen	

DIFFERENTIAL STAINS				
Stain Type	**Specific Dyes**	**Purpose**	**Outcome**	**Sample Images**
Gram stain	Uses crystal violet, Gram's iodine, ethanol (decolorizer), and safranin	Used to distinguish cells by cell-wall type (gram-positive, gram-negative)	Gram-positive cells stain purple/violet. Gram-negative cells stain pink.	
Acid-fast stain	After staining with basic fuchsin, acid-fast bacteria resist decolorization by acid-alcohol. Non acid-fast bacteria are counterstained with	Used to distinguish acid-fast bacteria such as *M. tuberculosis*, from non–acid-fast cells	Acid-fast bacteria are red; non–acid-fast cells are blue.