Different staining techniques in microbiology pdf

I'm not robot!

Be careful of aerosols when transferring bacteria from your loop to the slide. The loop is very flexible and it is easy to zing off a loop-full of organisms. Do not assume your organism is dead. Heat or methanol fixation is not guaranteed to kill the organism. Dispose of your completed slides in the disinfectant bucket at your bench. General Considerations You are striving for a light suspension of cells that will leave a faint cloudy deposit on your slide. You have lots of room on your slide; use it! It helps to initially draw a circle on the bottom of the slide so you know where to look for your smear. It is very easy to get confused which side of the slide your smear is on. Be sure to label the far edge of the slide. Do this consistently on the same end of the slide to help orient your slide. Be patient and take the time to let your slide air dry before proceeding with adhering it to the slide. If your slide is wet and you heat fix it, the bacteria will boil and the cellular morphology will be lost. If your slide is wet and fix it in methanol, it will most likely wash off the slide. Smears that are too thick will most likely wash off the slide regardless of the fixation method. Smear from Broth Broth cultures are usually easier to work with because the cells are already diluted in the broth. Be sure to carefully mix the culture tube to suspend the bacteria in the broth. Label your slide. Aseptically transfer a loop-full of organism onto the center of your slide. Use the flat part of the loop to smear the broth drop around the slide. Use a spiraling, circular motion to spread out the drop. Because the broth is full of protein, the smear will usually stay spread out and not bead up on the surface of the slide. Set the slide aside to air dry. This will take several minutes at least. Do not rush this step. Smear from Plate You can scoop a lot of organisms off with your loop. You may want to use an inoculating needle to transfer your organism to the slide. Be sure to use sterile water to dilute your samples. Regular tap water or the de-ionized water in your rinse bottles are often contaminated with bacteria. Label your slide. Aseptically transfer a loop-full of sterile water to the center of the slide. This serves to both dilute your bacteria and give you something to spread around. Pick a well-isolated colony. Prick it with your sterile needle, or slightly scoop the edge of the colony with your sterile loop. Place your needle/loop in the center of the drop and with a spiraling circular motion spread the bacteria on the slide. Set the slide aside to air dry. This will take several minutes at least. Do not rush this step. Fixation The fixation procedure is the same regardless of smear source, plate or broth. There are two methods of adhering your bacteria to the slide, heat fixation or methanol fixation. Heat fixing is only used with BSL1 organisms. The organisms we will be working with are BSL2, so you will need to use the methanol fixation technique. Heat fixing the slide can create aerosols and with BSL2 organisms, we need to prevent this as much as possible. Methanol fixation causes fewer changes in cellular morphology and creates no aerosols. Please be careful when working with the methanol, if you forget you have fixed it with methanol and your slide isn't totally dry, the remaining methanol will catch on fire. Methanol Fixing (BSL2) Be sure your slide is totally dry. Set it on the staining rack over the sink. Carefully flood the slide with 95% methanol. Let it sit for two minutes. Tilt the slide and pour off the methanol. Touch the edge of the slide to a paper towel to wick off the excess methanol. Set the slide aside to air dry before staining. Simple Stain Simple stains are just that - add one stain to a fixed smear slide, let it sit, rinse it off, let it dry, and view. It is a quick procedure for determining the presence and morphology of bacteria in clinical samples such as stool and discharges. Methylene blue is used to determine the morphology of fusiform and spirochetes in oral infections. It is also the stain of choice for identifying the metachromatic granules in Corynebacterium diphtheriae. The granules will stain a distinctly deeper blue than the surrounding blue bacteria. Other species of Corynebacterium do not have the metachromatic granules. Any basic dyes, such as methylene blue, crystal violet, malachite green, or safranin work well. Basic (cationic or positively charged) dyes bind to negatively charged components in the cell membrane and cytoplasm. Materials Methylene blue Safranin Crystal violet Malachite Green Staining racks Micro tool boxes Prepared smear slides General Considerations Staining is part art and part science. There are no hard and fast rules for staining and rinsing times. The times listed are suggestions that usually work well. You will need to experiment with what works for the bacteria you have and the techniques you use. It is essential that you record exactly what you do and the results you observe in your lab book. You will be repeating these stains later in the semester and you don't want to waste your time re-inventing your successful staining procedure. It would be useful for each lab bench member to pick a different stain so you can see what they all look like. Simple Stain Procedure Place your carefully prepared fixed smear slides on the stain rack over the sink. Do one slide at a time. Cover the smear with any of the basic dyes available to you. You only need enough dye to cover the smear. The stain should not drip off the slide. Let the stain sit for 1-5 minutes. Using the clothespin, grab the long end of the slide, tilt the slide over the sink and rinse the stain off with a stream of water from the wash bottle.Be sure to spray above the smear and let it dribble down. If you spray directly on the smear you are liable to wash the smear off the slide. Rinse till the water runs clear or is only slightly colored. Touch the edge of the slide to a paper towel to remove excess water. You can now let it air dry. Alternately you can dry it with blotting paper by placing it in the blotting paper book and pressing lightly. While this method is quicker, you can also blot off a poorly adhered smear. View your slide under oil immersion and record your observations in your lab book. Discard your used stained slides in the disinfectant bucket in the sink. Negative Stain Negative stains are even simpler than simple stains because you do not have to make a smear. A drop of cells is spread on a slide and viewed without fixation. The stain is a suspension of carbon, found in India ink or nigrosin. The carbon particles are negatively-charged, as is the cell membrane. The background looks black or sepia colored and the cells remain clear, since they repel the dye. Some positively charged inclusion bodies, such as sulfur, may stain. This stain gives accurate information on cell morphology and capsule presence because the cells are not fixed. Cell size appears slightly larger because any extracellular coatings or secretions on the outside of the cell membrane also do not stain. Negative stains are useful for rapid determination of the presence of Cryptococcus neformans, the causative agent of cryptococcisis, in cerebral spinal fluid. This technique is also used when you stain for endospores and capsules. Materials Nigrosin dye Assorted cultures General Considerations Just as in preparing a smear, you only need a small amount of organism. If you have too many organisms, you won't be able to see the morphology of individual cells. It is also important not use too much nigrosin. If it is too thick, the background will have a cracked appearance similar to mud puddles drying in the sun. You want to get a light film. Your instructor will demonstrate this technique for you. Negative Staining Procedure Label your slide. If you are working from a broth culture, place a loop-full of organisms about three fourths of the way on the left side of the slide. If you are working from a plate culture, add a drop of sterile water to the slide and dilute your organism in the drop without spreading the drop. Put one or two drops of nigrosin on another slide. Use your sterilized loop to pick up a loop-full of nigrosin. Carefully mix it in with the drop of cells, without spreading the drop too much. Hold the right end of the slide in your right-hand; with your left–hand take another slide at a 45° or less angle to the first slide, just past your nigrosin/cell drop. Scoot the angled slide back along the surface of the first slide till it just touches the drop of nigrosin and cells. Wait for capillary action to draw the liquid along the leading edge of the angled slide. Push the angled slide across the surface of the flat slide. Most of the nigrosin should still be left on the original spot. Discard the slide in the disinfectant bucket. Set the stained slide aside to air dry before observing it under oil immersion. Be sure to start examining your slide in the area with the faintest gray background. Record your observations in your lab book. Discard your used stained slide in the disinfectant bucket. Special Note Nigrosin comes off the slide and onto your oil immersion lens very easily. Be sure to thoroughly clean your oil lens when you are finished. Then clean it again. Once it dries on the lens it is very difficult to remove and will impair your ability (and the other micro students using that scope) to see clearly out of the lens. Gram Stain The Gram stain is the most common differential stain used in microbiology. Differential stains use more than one dye. The unique cellular components of the bacteria will determine how they will react to the different dyes. The Gram stain procedure has been basically unchanged since it was first developed in 1884. Almost all bacteria can be divided into two groups, Gram negative or Gram positive. A few bacteria are gram variable. Trichomonas, Strongyloides, some fungi, and some protozoa cysts also have a Gram reaction. Very small bacteria or bacteria without a cell wall, such as Treponema, Mycoplasma, Chlamydia, or Rickettsia do not have a gram reaction. The characterization of any new bacteria must include their gram reaction. Typically a differential stain has four components; the primary stain, a mordant that sets the stain, a decolorizing agent to remove the primary stain, and a counter stain. In the Gram stain, the primary stain is crystal violet. This gives the cell an intense purple color. The mordant, iodine, forms a complex with the crystal violet inside the cell wall. The cell is then washed with either Gram's de-colorizer or 95% ethanol. Gram positive cells will retain the dye complex and remain purple. The dye rinses out in gram negative cells. The counter stain, safranin, is used to color the cells that lost the primary stain, other wise they would remain colorless and you wouldn't be able to see them. The large iodine-crystal violet complex is retained within the cell walls of gram positive cells because of the molecular structure of the many layers of peptidoglycan in the cell wall. There are lots of cross-linked teichoic acids and the iodine-dye complex cannot physically get out. There is also less lipid in the membrane and the decolorizing agent cannot get to it as well. Gram negative cells have an outer membrane and only one layer of peptidoglycan, with more lipid. The crystal violet dye is easily washed out. General Considerations The accuracy of the Gram stain is dependent on the integrity of the bacterial cell wall. There are a variety of things that can influence the cell wall integrity; old cells (i.e. cultures over 24 hours old), the sample is from someone treated with antibiotics that target that cell wall such as penicillin, the cells have been roughly handled or you over heat fixed them. Under these conditions, gram positive cells will come out as gram-negative. If you de-colorize too long, Gram-positive cells will look like Gram-negative cells. Conversely, if you do not decolorize enough, Gram-negatives will look like Gram-positives. The only way you can trust your results it to always run a known Gram-positive and a known Gram-negative on the same slide. If they stain as predicted you can be pretty sure the result of your unknown sample is reliable. The Gram staining takes practice to get right. Do not expect to get a good Gram stain on your first try. It is a good idea to hold your slide with a clothespin; your gloves will get pretty psychedelic as will everything you touch! Gram Stain Procedure Label your slide. Prepare your smears on a slide with a Gram negative on the left, your unknown in the middle, and a Gram positive on the right.Don't forget to methanol fix your slide! Laying your slide on the staining rack, cover the smears with crystal violet for 1 minute.Use just enough stain to fully cover the smear but not so much that it runs or drips off the slide. Tilt the slide and pour the crystal violet off and briefly rinse with water from your wash bottle.Remember not to spray directly on the smears or you will wash them off. Flood the slide with iodine mordant for 1 minute. Tilt the slide, pour off the excess iodine and gently decolorize with Gram's de-colorizer until it just begins to run clear. Tilt your slide on some paper towels to remove excess de-colorizer. Flood your slide with the safranin for 1 minute. Tilt the slide to pour off the excess safranin and gently rinse with water until it runs clear. Let the slide air dry Observe under oil immersion. The Gram positive control should be purple and the Gram negative control should be pink. Discard your used slide in the disinfectant bucket. Capsule Stain (Klebsiella) Congo Red Capsule Stain The Congo Red Capsule stain is a modification of the nigrosin negative stain you may have done previously. The bacteria take up the congo red dye and the background is stained then with acid fuchsin dye. The capsule or slime layers, highly hydrated polymers, exclude both dyes. The background will appear blue, the bacterial cells will appear pink, and the clear halos are the capsules. Clinically, the capsules of some highly pathogenic bacteria (i.e.: pneumococci, Haemophilis influenzae, and meningococci), can be distinguished with the use of antisera specific for that type of capsule. The bacteria are suspended in the antisera and then mixed with methlyene blue. In the antisera staining procedure, the bacteria will appear blue surrounded by a clear halo and then surrounded by a thin blue line where the antisera have attached to the capsule. Materials Congo Red stain Acid fuchsin stain Acid alcohol Klebsiella pneumoniae culture Enterobacter aerogenes culture Congo Red Capsule Stain Procedure Place a loop-full of Congo Red on a slide Mix a small amount of your organism into the drop of Congo Red.Spread the organism/dye suspension well on the slide Let the slide thoroughly air dry. Fix the dried slide with acid alcohol for 15 seconds. Rinse with distilled water and cover the slide with acid fuchsin for 1-5 minutes. Rinse with water and allow to air dry. Examine the slide under oil immersion.Cells stain red/pink, and the capsules appear as colorless halos against a dark blue background. Wirtz's Endospore Stain Endospore formation is characteristic of Clostridum and Bacillus spp. The ability to concentrate and coat their protoplasm allows them to survive the adverse environmental conditions they experience in their soil habitat. This also allows the spores to resist staining. The "live" organisms are easily visualized with simple stains and Gram's stains. Endospores are typically highly refractile, light striking them is deflected. Many Bacillus species have inclusion bodies that are highly refractile. These inclusion bodies may look like endospores with regular staining. The presence of endospores must be confirmed with endospore specific stains. The presence, and characteristic shape and position of endospores require special procedures to permeate the endospore coat. Most endospore stains involve heating the slides while keeping them continually moist with the dye. While quicker, it produces volatile chemicals and is just a big mess. The same results can be obtained by letting the dye sit on the slide for 30 minutes. It is a good idea to start this slide first and work on another stain while you are waiting for the dye to permeate the endospore. General Considerations You will be using a Bacillus species for the endospore stain. The shape and position of B. cereus spores are very similar to those of B. anthracis. Bacillus does not start forming spores until it runs out of food. If the cultures are too young, you will mostly see just the pink rods of the bacteria. If the cultures are too old, you will mostly see just the small green ovals of the endospores. Ideally, you should see the green oval bodies of the endospore surrounded by the pink vegetative bacterial cell. Select a sample from the middle of a colony with the straight inoculating needle for the best results. The edge of the colony is still actively growing and will have few endospores. Materials Malachite Green Stain (5%) Safranin (0.25%) - counter stain Bacillus spp. plate Wirtz's Endospore Stain Procedure Make a smear of Bacillus and methanol fix. Flood the smear with malachite green stain. Allow the stain to sit for at least 30 minutes.Add more stain if it starts to dry out. Rinse the slide with distilled water. Flood the slide with safranin (0.25%) for 1-5 minutes. Rinse the slide with distilled water and let air dry. Observe under oil immersion.The endospores are aqua and the bacterial cells are pink.

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