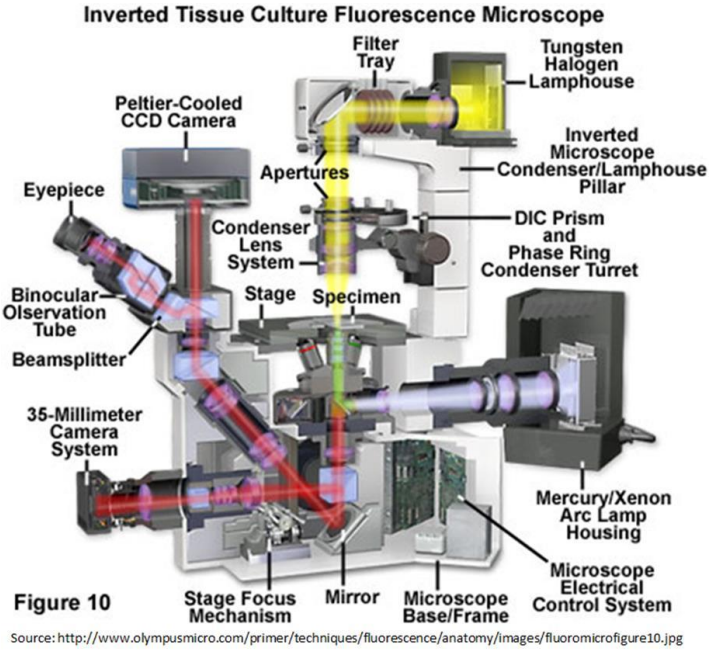
A Primer on Microscopy **(email sagarsin@scarletmail.rutgers.edu for additional questions):**

Microscopy is an important technique to visualize structures which are too small to see normally, as well as ascertaining protein localization. Basic microscopes contain a light source, a condenser lens to focus light, a viewing eye-piece, and an objective lens (4x, 10x, 20x, etc.) to magnify the image.

Prior to imaging, samples usually have to be stained to either: 1.) increase contrast between structures to make identification easier, and/or 2.) identify specific proteins in the sample (e.g. through immunolabeling).

Three types of microscopy are commonly used: **bright-field microscopy, phase-contrast/differential interference contrast microscopy, and fluorescence microscopy.**

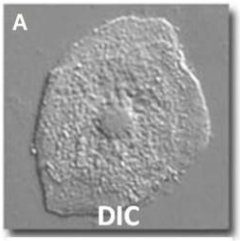


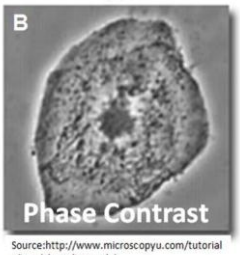
Source: http://www.olympusmicro.com/primer/techniques/fluorescence/anatomy/images/fluoromicrofigure10.jpg

Bright-Field Microscopy: White light (usually from a halogen lamp) illuminates the sample from the bottom and the resolved image is observed from above. As the light illuminates the sample, some of it is absorbed by dense parts of the sample, which helps create contrast in the viewed image. Bright field microscopy is the simplest form of microscopy and is used to visualize our sample before employing other forms of microscopy. The limitations of bright-field microscopy are low contrast, low optical resolution, and samples must be stained before being viewed.

Bright field is used when:

- You want a general idea of where your sample is on your slide/well plate
- Your sample has intrinsic colors (e.g. chloroplasts)
- Your sample is stained with a non-fluorescent contrasting agent (e.g. H&E dyes)





Source:http://www.microscopyu.com/tutorials/java/phasedicmorph/

Phase-Contrast Microscopy: Certain cellular structures are difficult to see without shifting the phase of entering light. Phase-contrast works similarly to bright-field, but the background light is shifted by 90° which reduces the contrast between the background and scattered light, causing objects in the foreground (i.e. your sample) to increase in contrast.

Phase contrast is used when:

- You want to image some larger structures but do not want to stain the sample
- You want to image living samples

Differential Interference Contrast (DIC) Microscopy: Similar to Phase-Contrast, incident light passes through a polarizing filter prior to reaching the specimen. The difference is the light passes through a second filter, called a Normarski Filter, which captures the pathlength differences from light reflected from the sample. Thus, a surface with no features will appear grey, but a surface with features will appear lighter or darker depending on the pathlength difference.

Fluorescence Microscopy: Some structures are naturally fluorescent or have binding affinity to certain fluorophores which cause them to emit a certain wavelength of light when they absorb another (smaller) wavelength of light. In this mode of microscopy, light (usually from a mercury bulb) travels through an excitation filter cube, which allows a certain wavelength of light to pass through. The single wavelength of light passes through the objective, where it is absorbed by

the sample. Depending on the fluorophore, the sample emits a lower wavelength of light which passes through the objective and back to the detector or eye-piece. Some common fluorophores are DAPI (absorbs near-UV, emits blue), GFP/FITC (absorbs blue, emits green), RFP/TRITC (absorbs green, emits orange), and Cy-5 (absorbs orange, emits red/near IR).

Fluorescence is used when:

- Your sample is stained with a fluorescent dye
- You are staining for multiple proteins/structures each with a different fluorophore in a single sample and want to identify each one separately
- You are staining your sample with primary and secondary antibodies

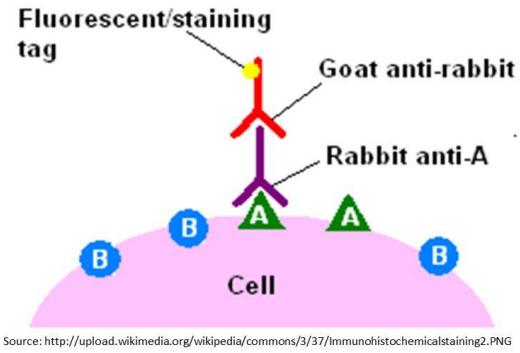
Cell Labeling (email sagarsin@scarletmail.rutgers.edu for additional questions):

Suppose you have a sample of cells or tissue and you're interested in finding whether a particular protein is expressed in said sample. Or perhaps you want to quantify the appearance of a certain structure in your sample (like cell nuclei). It becomes important to know how to label the specific features you are looking for. Several labeling techniques can be used:

- Dyes or contrasting agents: useful for gross structures (e.g. H&E stain, OsO_4 stain)
- Immunolabeling: useful for finding specific proteins
- Live/Dead assays: a common test (and usually the first one employed) for cell viability for a given condition

The steps for each vary but generally speaking, almost all labeling techniques require **fixation which kills the cell/tissue**.

Immunolabeling: Given the high specificity of antibody labeling (which can pinpoint specific proteins), this technique is used most often in labeling cells or tissue. Below are the general steps that need to be followed when doing immunohistochemistry:



Source: http://upload.wikimedia.org/wikipedia/commons/3/37/Immunohistochemicalstaining2.PNG

1. **Fix your sample with formalin or paraformaldehyde**. This creates crosslinks in the proteins of your sample, effectively creating a "snapshot" of your cells/sample.
2. **Wash your sample with buffer**. Commonly used buffers include PBS (Phosphate Buffer Saline) or 'Immunobuffer' (PBS with bovine serum albumin and Triton X-100, a permeabilizing agent). These washes help remove any unused fixing agents (or unreacted antibody)
3. **Incubate with a blocking buffer**. A blocking buffer is usually composed of serum from a certain species of animal, X (goat and donkey are commonly used), mixed in PBS. The sample is incubated with this mixture for about an hour (or overnight depending on the protocol). The goal of this blocking buffer is to prevent nonspecific binding of your antibodies to any other epitopes (proteins) on your sample.
4. **Incubate with the primary antibody**. Next, the sample is incubated with your primary antibody. Primary antibodies are usually labeled as Y anti-Z, where Y refers to the species of animal the antibody was extracted from, and Z is the protein of interest (e.g. mouse anti-neurofilament). Primary antibodies can either be monoclonal (from a single parent immune cell), or polyclonal (multiple immune cells). Note that some proteins which are especially conserved might have antibodies which are not species-specific (e.g. mouse anti-neurofilament can work in rats, chickens, and humans, as well as mice).
5. **Incubate with the secondary antibody**. Following incubation with the primary, we incubate with a secondary antibody with a fluorophore or identifying agent attached. Secondary antibodies are usually labeled as X anti-Y: #, where X and Y are animal species, and # is the excitation wavelength for the fluorophore. It's important to pay attention here. The secondary antibody needs to be compatible with the animal species used for the primary, as well as the blocking buffer. For instance, if we used a goat serum for blocking, and mouse for the primary, we'd need a GOAT anti-MOUSE secondary.
6. **Wash and image**. Once the secondary antibody is bound to your primary antibodies, your sample is washed a few more times to remove any excess antibody solution and is ready for imaging!