What is Fluorescence?
Fluorescence results when molecules called fluorophores absorb light, which briefly raises their energy level to an excited state. They emit fluorescent light as they decay from this excited state (known as Stoke’s Shift). In general, a fluorophore will be excited by high frequency light (wavelengths in the ultraviolet, violet, or blue region of the spectrum), and emit light at slightly lower frequencies (wavelengths in the green or red region of the spectrum). See Figure 1.

Fluorophores come in a range of colors that span the visible spectrum (traditionally red, green, and blue fluorophores are used). Three of the most common fluorophores used are DAPI (emits blue), FITC (emits green), and Texas Red (emits red) see Figure 2, which can be used simultaneously to “triple stain” a sample because they do not have overlapping excitation and emission spectrums.

Figure 2 Three Common Fluorophores

Hoechst (DAPI): Excitation at ~350-360nm (UV)  Emission at ~455-465nm (Blue)
FITC: Excitation at ~488nm (Blue)  Emission at ~530-540nm (Green)
Texas Red: Excitation at ~595nm (Green)  Emission at ~630-640nm (Red)
**Fluorescence Microscopy**
A fluorescence microscope is much the same as a conventional light microscope with added features to enhance its capabilities.

- The conventional microscope uses **visible light** (400-700 nanometers) to illuminate and produce a magnified image of a sample.

- A fluorescence microscope uses higher intensity **ultra violet light** that **excites** fluorescent molecules in the sample of interest. Following excitation, the fluorescent molecules will then **emit** a longer wavelength light which produces the magnified image of the sample. See Figure 4.

**Figure 4** Compound Fluorescent Microscope        Inverted Fluorescent Microscope

A typical fluorescence microscope setup used in fluorescence microscopy applications consists of three filters: excitation, emission, and the dichroic beam splitter. See Figure 5

**Filter #1: Excitation Filters**
The excitation filter is placed within the illumination path of a fluorescence microscope. Its purpose is to filter out all wavelengths of the light source, except for the excitation range of the fluorophore under inspection.

**Filter #2: Emission Filter**
The emission filter is placed within the imaging path of a fluorescence microscope. Its purpose is to filter out the entire excitation range and to transmit the emission range of the chosen fluorophore.

**Filter #3: Dichroic Filter or Beam splitter**
The dichroic filter or beam splitter is placed in between the excitation filter and emission filter, at a 45° angle. Its purpose is to reflect the excitation signal towards the fluorophore under inspection, and to transmit the emission signal towards the detector.

**Figure 5 Fluorescent Filter Setup**
Fluorescent Labeling of Biological Samples

Fluorophores can be used to “tag” subcellular structures like organelles, cytoskeleton fibers, chromosomes, and cytosolic or membrane bound proteins.

Fluorophores are often conjugated onto antibodies, which are proteins that are designed to recognize and bind to specific target proteins (antigens) on a cell. Staining cells with fluorescent-conjugated antibodies is an experimental technique called immunocytochemistry (ICC). Figure 6 below demonstrates the use of two different antibodies to detect a cellular target protein (at left) and an example of a completed ICC sample (at right) using three fluorophores: DAPI, FITC, and Texas Red.

Figure 6

Primary and Secondary Antibodies
Used in ICC Protocol

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>FITC-labelled Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>binds to target protein</td>
<td>binds to primary antibody</td>
</tr>
<tr>
<td>antigen (I)</td>
<td>antigen (II)</td>
</tr>
</tbody>
</table>

Triple Stained Cells
(DAPI, FITC, Texas Red)

Other fluorescent stains bind selectively to specific cellular structures (example: Hoechst dye binds to DNA), including the fluorescent Gram stain technique described below.

LIVE BACLight Bacterial Gram Stain: The fluorophores used are nucleic acid stains (so no antibody conjugation is needed). The protocol utilizes two stains. The first is green fluorescent SYTO 9 (emits green) which will stain both live Gram positive and negative cells. The second stain is red fluorescent hexidium iodide (emits red) which will stain only Gram positive cells. So in a mixed culture, Gram positive cells will initially stain green. However, the red stain will displace the green; so ultimately, Gram negative cells will be green while Gram positives will be red. Dead bacteria may appear variable, so it is important to use a log phase culture of bacteria.

References
3. http://www.jic.ac.uk/microscopy/more/T5_7.htm
**LIVE BacLight Bacterial Gram Stain Protocol:**

Component A: SYTO 9 (emits green)
Component B: hexidium iodide (emits red)

1. Culture prep: Grow a culture of bacteria to late log phase, approximately 24-48h in growth media (TSA for example). We will grow separate gram positive and gram negative cultures and then mix them together.

2. Add 100ul (in our case, first mix 50ul of each of the gram positive and gram negative cultures together) of the bacterial culture to 1ml of filter-sterilized water (to prevent impurities and particles from staining and interfering with the assay) in a microcentrifuge tube.

3. Centrifuge the culture, to concentrate the bacteria in the tube, for 5 min. at 10,000xg. Make sure to balance the centrifuge with a microcentrifuge tube with a volume of 1.1ml of water.

4. Decant the supernatant and resuspend the pellet in 1ml of filter-sterilized water.

5. Combine Component A and B in a 1:1 ratio in a microfuge tube and mix well (enough to make at least 3ul/1ml bacteria).

6. Add 3ul of the dye mix to the 1ml bacterial suspension.

7. Mix thoroughly and incubate at RT in the dark (place in a box, or cover with foil) for 15 min.

**To observe:**

8. Place 5ul of the stained suspension on a cleaned slide and cover with a coverslip. If planning to view with oil immersion, seal the preps with paraffin.

9. Observe in a fluorescent microscope. Live gram negative bacteria should fluoresce green, gram positive red. Dead cells may fluoresce either color and any debris may also obscure the results.