NGB WMD-CST
Microscope Specialty Training Program:
Fluorescence Microscopy
v3.2
Students in this course will become proficient in techniques of fluorescence microscopy for investigation of biological samples, particularly for the identification of microbes in the environment. The course consists of demonstration, plus hands-on training in the practice of sample collection, preparation, and observation using the technique of fluorescence microscopy. After completion of this course students will have gained experience in designing protocols using fluorescence microscopy, and in implementing those protocols for the investigation of lab and/or field samples.

Most of what is required to complete the workshop successfully will be presented in the lectures and this handout. For more detailed information, however, the students are directed to:


Rost, FWD. 1995. Fluorescence Microscopy. Cambridge ; New York : Cambridge University Press,
# SCHEDULE

<table>
<thead>
<tr>
<th>Day</th>
<th>Morning</th>
<th>Topic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Course introduction; introduction to the theory and practice of epifluorescence microscopy, microscopes for fluorescence, filter sets (identifying, purchasing, installing), light sources (replacing and aligning burners), cleaning, alternate light source for ALS.</td>
<td>The practice of fluorescence microscopy: Labs 1–3: Sample preparation. The epifluorescence microscope, survey of microscopic organisms, cytochemical fluorescence microscopy.</td>
</tr>
<tr>
<td>3</td>
<td>Labs 11 &amp; 12: Bacteria viability, fluorescent identification of Bacillus endospores.</td>
<td>Investigation of five (5) unknown samples. Problem Solving. Final exam</td>
</tr>
</tbody>
</table>
FLUORESCENCE MICROSCOPY

A fluorescence microscope is a conventional compound microscope that has been equipped with a high-intensity light source (usually an arc lamp) that emits light in a broad spectrum from visible through ultraviolet (UV). Most conventional fluorescence microscopes utilize incident illumination to illuminate the sample from above. In this way the objective lens is used as both the illumination condenser and the fluorescent light collector (Figure 1). The arrangement of optical components that permits illumination from above the specimen is termed epifluorescence illumination, epi-illumination, or reflected light illumination.

The wavelength of illumination (excitation; Ex) is set by placing after the light source a filter that limits light transmission to a narrow range of wavelengths. The light then impinges on a dichroic beam splitter (dichroic mirror, Fig 1) and is reflected down through the objective lens and onto the sample. Pigment molecules within the specimen (either intrinsic or applied) absorb the light and re-emit the energy at a longer wavelength (this is “fluorescence”). The objective lens collects this emitted fluorescent light (emission; Em), and transmits it back into the microscope and through the dichroic mirror. Any reflected excitation light is blocked by a third filter (barrier, or emission filter). Thus, only light emitted from fluorescent molecules within the specimen are observed. The arrangement of three optical filters is referred to as a fluorescence filter cube, since they are almost always mounted together in a cubic metal mount. The user selects different wavelengths by selecting different filter cubes. The mechanical arrangement in a typical microscope may be either a sliding or rotating filter cube holder.

![Diagram of fluorescence microscope](image)

Figure 1 Arrangement of optics in an epifluorescence microscope. A: Incident (excitation) light path; B: reflected path of fluorescent light emission.

LIGHT SOURCES

Excitation light for fluorescence microscopy must have two qualities: extreme brightness and breadth of spectrum. Brightness is required because most of the emitted light is rejected by the excitation filter. Spectrum breadth is required to supply any wavelength necessary for scientific investigation. While incandescent lights, for example, can be exceedingly bright, they lack emission in the blue and UV range. Thus these sources are inappropriate for fluorescence microscopy. Generally, there are three types of light sources used in fluorescence microscope applications: mercury, mercury metal halide, and xenon. Their spectra are shown in Figure 2. Mercury burners last 200–400 hours including “starts”. Consider each “start” as burning 3h of bulb life. Mercury

---

1 The dichroic mirror is designed to reflect light shorter than a certain wavelength and transmit light of longer wavelengths.
metal halide and Xenon burners last 1500–2000 hours. There is no time penalty for starting these light sources. The approximate costs are $150 for Hg and $650 for Xe/Hg-metal halide burners.

![Graph](image1.png)

**Figure 2:** Light sources for fluorescence microscope applications. Left: Mercury/Hg-Metal halide. Right: Xenon

**ALIGNING THE LAMP**

To increase the intensity of illumination, most arc lamps have a mirror behind the lamp to reflect back to the specimen light that would otherwise be lost. The goal in alignment is to position the lamp and mirror to create two focused spots of light (direct and reflection) that are centered in the field of view but not overlapping. Xe and Metal Halide burners require no alignment.

1. Start with the lamp OFF, and the lamp housing disconnected from the microscope.
2. While looking straight into the collector lens adjust the collector focus until you can see the lamp electrodes. You should also see the inverted mirror image, but it might not be in focus.
3. Change the lamp height and lateral adjustments until the lamp electrodes (not the mirror image) are just slightly off center in the field of view. If achieving this is impossible, then disassemble the lamp housing and reinsert the lamp fully into the base.
4. Mount the lamp housing on the microscope and turn on the lamp.

*The light output of an arc lamp is intensely bright. Wear good sunglasses for all following steps to protect your eyes.*

5. If the microscope has an alignment objective, mount this to the objective nosepiece. If the microscope has an integrated alignment port observe the arc here. Otherwise, remove an objective and observe the arc projected onto a piece of paper or a business card. Use an epifluorescence excitation line that is less intense (*e.g.*, blue or UV).
6. Focus the collector lens until the image of the arc (not mirror image) forms a sharp oval.
7. Change the arc lamp adjustment screws (vertical and/or horizontal) until the image of the arc is just slightly to the left of center within the field of view.
8. Adjust the three mirror controls until a focused, oval spot of light is approximately the same size and shape as the arc itself and positioned just to the right of center. You will have to adjust the mirror focus, then the vertical and/or horizontal positions multiple times to achieve this desired positioning.
9. Stop off the arc light and insert an objective or swing the nosepiece into an objective position, then focus on a piece of fluorescent paper (a business card works well).
10. Focus on the paper surface in epifluorescence mode and repeat steps 5–7 until there exists two centered ovals of light, the arc and image of the arc when viewed through the microscope eyepiece.
11. Defocus the collector lens until both spots of light merge and fill the entire field of view. At this point the arc lamp is adjusted correctly.
12. Let the lamp “burn-in” for 1–3 h.

**EXCITATION AND EMISSION SPECTRA OF FLUORESCENT DYES**

Fluorescent molecules absorb and subsequently emit photons throughout a range of energies (wavelengths). If the emission of light is nearly instantaneous ($<10^{-9}$ sec), the phenomenon is called fluorescence. The spectrum of absorbed light is called the Excitation spectrum (Ex). Emitted light follows a similar spectral profile and is called the Emission spectrum (Em). The separation between the excitation and emission peaks is called the Stokes Shift (compare the DAPI spectrum to that of Syto 11, Figure 3).

![Excitation and Emission spectra of two fluorescent probes for DNA: DAPI (A) and Syto 11 (B). Note the difference in the Stokes shift between these two dyes.](image-url)
FLUORESCENCE APPLICATIONS IN SPECIMEN ANALYSIS

INTRINSIC FLUORESCENCE

Most plant and some animal tissues exhibit intrinsic fluorescence (“autofluorescence”) attributed to the presence of secondary metabolites. Usually an obscuring phenomenon, intrinsic fluorescence may be used as a diagnostic tool for the identification of organ anatomy, cell constituents, or pathogenic activity.

Table 1 Intrinsic Fluorescence May Be Used as a Diagnostic Tool to Identify or Localize Certain Cell Components

<table>
<thead>
<tr>
<th>Compound or tissue component</th>
<th>Ex</th>
<th>Em</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll (plants)</td>
<td>Blue (430-450 nm, but up to 550 nm)</td>
<td>Red (Em_{max} = 685 nm)</td>
</tr>
<tr>
<td>Cutin (plants)</td>
<td>UV (365)</td>
<td>Blue/white</td>
</tr>
<tr>
<td>Guard cells (plants)</td>
<td>Violet (450)</td>
<td>Green (520)</td>
</tr>
<tr>
<td>Hair</td>
<td>UV (365)</td>
<td>Blue (440)</td>
</tr>
<tr>
<td>Clothing fibers</td>
<td>UV (365)</td>
<td>Blue/White (&gt;420)</td>
</tr>
<tr>
<td>Lignin (plants)</td>
<td>UV (365)</td>
<td>Blue (450–480) Green (510–520)</td>
</tr>
<tr>
<td>pollen grains (Sporopollenin)</td>
<td>UV (365)</td>
<td>Yellow-red</td>
</tr>
</tbody>
</table>

*Ex: excitation; Em: emission in nm.

INDUCED BACKGROUND FLUORESCENCE

Aldehyde fixation, especially glutaraldehyde, induces a tissue fluorescence that spans the visible spectrum, although it is especially bright in the yellow/red range. This induced fluorescence may obscure the localization of a fluorescent probe and may lead to false positive identification. When possible use a fluorescein-like probe (fluorescein, Oregon Green, Cy2, BODIPY Fl, etc.) since its fluorescence emission may be distinguished from background. Formaldehyde-induced-fluorescence (FIF) has been used as a diagnostic tool to distinguish certain cell components and compounds. Treating tissue with either aqueous or heated formaldehyde vapors induces autofluorescence that is often a different fluorescent color from endogenous and/or applied fluorescent probes.

INDUCED SPECIMEN FLUORESCENCE

Some dyes can be applied to tissue sections or whole mounts to induce a generalized fluorescence, which can then be used in histochemical investigations. For example Eosin Y can be used on mammalian tissues to identify fibers and other structures via fluorescence. Safranin O, Aniline Blue, the Acridine and fuchsin dyes, and myriad other general purpose dyes can be used for fluorescence investigations.
CYTOCHEMICAL FLUORESCENCE MICROSCOPY

Cytochemical fluorescence microscopy uses fluorescent molecules as reporters for specific properties of the material under study. These properties include organelle identity, ion concentration, presence of an enzyme activity, and location of macromolecules including nucleic acids and lipids. Fluorescent dyes have been used successfully to localize cellular organelles in both plants and animals. The chemical structure (hydrophobic, hydrophilic, planar) or the fluorescent properties of certain dyes (pH- or calcium-dependent fluorescence, esterase susceptibility) have led to their wide use in cell biology. Table 2 lists some of the most widely used fluorescent dyes. It is designed to introduce the student to the techniques of fluorescence histochemistry, and is by no means an exhaustive list of possible probes.
### Table 2  A Few Common Fluorescent Dyes and Their Targets

<table>
<thead>
<tr>
<th>Target</th>
<th>Dye</th>
<th>Solvent</th>
<th>Stock</th>
<th>Dilution</th>
<th>Ex</th>
<th>Em</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleus</strong></td>
<td>Acridine Orange</td>
<td>Water</td>
<td>0.1mg/ml</td>
<td>1:1000</td>
<td>480</td>
<td>520-650</td>
</tr>
<tr>
<td></td>
<td>Bisbenzimide (Hoechst 33258, 33342)</td>
<td>Water</td>
<td>1 mg/ml</td>
<td>1:500</td>
<td>352</td>
<td>461</td>
</tr>
<tr>
<td></td>
<td>DAPI (4',6-diamidino-2-phenylindole, dihydrochloride)</td>
<td>Water</td>
<td>0.1 mg/ml</td>
<td>1:100</td>
<td>358</td>
<td>461</td>
</tr>
<tr>
<td></td>
<td>Propidium iodide</td>
<td>Water</td>
<td>1 mg/ml</td>
<td>1:1000</td>
<td>493</td>
<td>630</td>
</tr>
<tr>
<td></td>
<td>SYTO 11–17 (permeant)</td>
<td>DMSO</td>
<td>5 mM</td>
<td>1:5000</td>
<td>488-621</td>
<td>509–634</td>
</tr>
<tr>
<td></td>
<td>SYTOX Green (impermeant)</td>
<td>DMSO</td>
<td>5 mM</td>
<td>1:300</td>
<td>504</td>
<td>523</td>
</tr>
<tr>
<td><strong>Endoplasmic reticulum</strong></td>
<td>DiIC₆,* DiIC₁₈, Rhodamine 6G</td>
<td>DMSO, mineral oil</td>
<td>1 mg/ml</td>
<td>1:100</td>
<td>547</td>
<td>571</td>
</tr>
<tr>
<td></td>
<td>DIOC₇*</td>
<td>EtOH</td>
<td>0.5 mg/ml</td>
<td>1:500</td>
<td>484</td>
<td>507</td>
</tr>
<tr>
<td><strong>Golgi</strong></td>
<td>NBD Ceramide</td>
<td>DMSO (100%)</td>
<td>1 mg/ml</td>
<td>1:400</td>
<td>460</td>
<td>534</td>
</tr>
<tr>
<td><strong>Mitochondria</strong></td>
<td>CMXRos** (<em>MitoTracker</em>)</td>
<td>DMSO (100%)</td>
<td>200 μm</td>
<td>1:400</td>
<td>594</td>
<td>608</td>
</tr>
<tr>
<td></td>
<td>Rhodamine 123</td>
<td>DMSO (100%)</td>
<td>1 mg/ml</td>
<td>1:400</td>
<td>505</td>
<td>534</td>
</tr>
<tr>
<td></td>
<td>DIOC₆*</td>
<td>EtOH</td>
<td>0.1 mg/ml</td>
<td>1:100</td>
<td>482</td>
<td>511</td>
</tr>
<tr>
<td><strong>Viability</strong> (esterase activity, membrane integrity)</td>
<td>fluorescein diacetate</td>
<td>DMSO</td>
<td>1 mM</td>
<td>1:250–1:1000</td>
<td>489</td>
<td>514</td>
</tr>
<tr>
<td></td>
<td>Calcein AM</td>
<td>DMSO</td>
<td>1 mM</td>
<td>1:250–1:1000</td>
<td>496</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>BCECF AM</td>
<td>DMSO</td>
<td>1 mM</td>
<td>1:250–1:1000</td>
<td>508</td>
<td>531</td>
</tr>
<tr>
<td><strong>Callose</strong></td>
<td>Aniline Blue</td>
<td>PO₄ buffer, pH 9</td>
<td>0.1 mg/ml in DI or buffer</td>
<td>1:1000</td>
<td>UV</td>
<td>Blue</td>
</tr>
<tr>
<td><strong>Cell wall</strong> (may also stain chitin)</td>
<td>Calcofluor White M2R</td>
<td>PO₄ buffer, pH 7</td>
<td>0.1 mg/ml</td>
<td>1:1</td>
<td>UV</td>
<td>Blue</td>
</tr>
<tr>
<td><strong>Chromosome banding</strong></td>
<td>YOYO-1</td>
<td>Water or PBS</td>
<td>1 mM</td>
<td>0.5 μM</td>
<td>491</td>
<td>509</td>
</tr>
<tr>
<td></td>
<td>SYTOX Green, Methyl Green</td>
<td>Water</td>
<td>0.4 mg/ml</td>
<td>1:1</td>
<td>381</td>
<td>470</td>
</tr>
<tr>
<td><strong>Primary and secondary amines</strong></td>
<td>fluoresceamine</td>
<td>Acetone, acetonitrile</td>
<td>0.4 mM</td>
<td>1:1</td>
<td>381</td>
<td>470</td>
</tr>
<tr>
<td><strong>pH indicators</strong></td>
<td>SNAFL-1 (acid) (basic)</td>
<td>Water</td>
<td>NA</td>
<td>10–50 μm</td>
<td>479, 508</td>
<td>537</td>
</tr>
<tr>
<td></td>
<td>SNARF-1 (acid) (basic)</td>
<td>Water</td>
<td>NA</td>
<td>10–50 μm</td>
<td>518, 548</td>
<td>579</td>
</tr>
<tr>
<td></td>
<td>BCECF AM</td>
<td>Water</td>
<td>NA</td>
<td>10–50 μm</td>
<td>508</td>
<td>531</td>
</tr>
<tr>
<td><strong>Calcium indicators</strong></td>
<td>Fura-2, Fura-PE3</td>
<td>Water</td>
<td>NA</td>
<td>10–50 μm</td>
<td>340</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>Indo-1, FFP18</td>
<td>Water</td>
<td>NA</td>
<td>10–50 μm</td>
<td>350</td>
<td>405, 480</td>
</tr>
<tr>
<td></td>
<td>Fluo-3</td>
<td>Water</td>
<td>NA</td>
<td>10–50 μm</td>
<td>490</td>
<td>525</td>
</tr>
<tr>
<td></td>
<td>Calcium Orange</td>
<td>Water</td>
<td>NA</td>
<td>10–50 μm</td>
<td>550</td>
<td>580</td>
</tr>
<tr>
<td></td>
<td>Calcium Green</td>
<td>Water</td>
<td>NA</td>
<td>10–50 μm</td>
<td>490</td>
<td>525</td>
</tr>
<tr>
<td><strong>Green Fluorescent Protein (GFP)</strong></td>
<td>smGFP</td>
<td>Water</td>
<td>NA</td>
<td>NA</td>
<td>397 (480)</td>
<td>495</td>
</tr>
<tr>
<td></td>
<td>smRS–GFP</td>
<td>Water</td>
<td>NA</td>
<td>NA</td>
<td>385</td>
<td>488</td>
</tr>
<tr>
<td></td>
<td>smBFP</td>
<td>Water</td>
<td>NA</td>
<td>NA</td>
<td>385</td>
<td>488</td>
</tr>
<tr>
<td><strong>Antifade solution</strong></td>
<td>DABCO</td>
<td>Water</td>
<td>50μg/ml</td>
<td>1:10</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

§§

### Notes:
- *DiIC₆* and *DiIC₁₈* are probes for monitoring the endoplasmic reticulum.
- *DIOC₆* is used for monitoring the Golgi apparatus.
- *CMXRos* is a mitochondrial probe.
- Fluorescein diacetate and Calcein AM are membrane integrity indicators.
- Aniline Blue and Calcofluor White M2R are used for callose detection.
- YOYO-1 and SYTOX Green are used for chromosome banding.
- Fluoresceamine is used for detecting primary and secondary amines.
- SNAFL-1 and SNARF-1 are pH probes.
- Fura-2 and Indo-1 are calcium indicators.
- smGFP and smRS–GFP are green fluorescent proteins (GFP) for monitoring primary and secondary amines.
- DABCO is an antifade solution.
Workshop in Fluorescence Microscopy

Figure 4 Molecular structures of common fluorescent molecules.

**PREPARING DYE SOLUTIONS**

As shown in Table 2, fluorescent dyes for biological investigation are usually used in aqueous solutions in the \( \mu \text{M} \) range of concentration. Since most dyes are sold in powder form, dyes should be prepared as stock solutions at 1000x working concentration. Dissolve solid dyes in DMSO or DMF (dimethyl formamide) and store in small aliquots at -20°C. Dyes are photolabile, so take care to limit exposure to light. Prepare the working concentration of dye immediately before use, and mix with the solution in which the sample is mounted (e.g., PO₄ buffer, pH 7, water, PBS, etc.).
INTRODUCING DYSES INTO CELLS AND TISSUES

Some fluorescent probes such as DAPI (a DNA dye) generally are not transported through the plasma membrane. Thus, introduction of a dye, or dye loading, can pose a problem. Often addition of a detergent (saponin, Tween-20, DMSO, etc.) can be used to facilitate introduction of the dye into living cells. Chromatin dyes can be introduced, and the tissues stabilized, by a pretreatment of 30–50% EtOH or by freezing on dry ice (good for bacteria). This treatment, however, kills the cells. For plant tissue, DAPI pretreatment with EtOH is almost always necessary.

Addition of a charged moiety to a fluorophore (fluorescent portion of the molecule) often renders the fluorescent dye permeable. Addition of an acetate to fluorescein (to make fluorescein diacetate, FDA), or a methyl ester group to any of a number of dyes can make a useful dye for living systems. Endogenous esterases cleave acetoxymethyl ester (AM) groups, leaving a non-permeable, but fluorescent dye. These types of dyes are good indicators of cell viability.

Summary:

- Detergent treatment can facilitate introduction of dyes.
- Ethanol (30–50%) can make cells permeable, facilitating dye entry.
- A slight vacuum treatment can be used to remove air pockets, and facilitate dye introduction.
- Gentle abrasion of surfaces.
- Excising and utilizing very small samples; thus increasing surface to volume.
- Rapid freeze, then thaw

PHOTOBLEACHING AND ANTIFADE COMPOUNDS

When illuminated by light, fluorescent molecules (fluorophore) absorb light energy and reirradiate it at a lower energy and longer wavelength. It is possible, however, for a fluorophore to absorb more energy than can be emitted. When this occurs, the molecule can be physically damaged (covalent bond breakage) by the absorption of excess light energy and be rendered nonfluorescent. This phenomenon, called photobleaching, is common to all fluorophores and occurs more readily in some than others. Fluorescein, for example, is one of the most commonly used fluorescent dyes but is also one of the most rapidly photobleaching. Fading of the fluorescent dye is always a problem, with the amount of fading proportional to the intensity of the excitation light and the duration of illumination. Adding additional compounds to the sample medium can increase the amount of usable time before fading of the fluorophore.

It is desirable to decrease the amount of photoinduced damage to a fluorescent probe. This can be achieved (to a limited degree) by adding an energy scavenging compound to the mounting medium. In this context, these molecules are referred to as antifade compounds and the resultant medium as an antifade solution. Choose from among the following:

- 5 μg/ml 1,4-diazobicyclo(2,2,2)octane (DABCO); but not for SYTO dyes.
- 10–50 mg/ml n-propyl gallate.
- 1 mg/ml p-phenylenediamine.
- You may also use commercial antifade solutions such as Vectashield (Vector Laboratories) or SlowFade or Prolong Antifade; both from Molecular Probes, Inc.
MULTIPLE FLUORESCENT DYES AND OVERLAPPING SPECTRA

When a sample is investigated with more than one fluorescent probe, there will exist a high probability of overlapping emission spectra. That is, fluorescence of one dye can pass through the emission filter of the other dye. A common example is the overlap of emission of two common dyes Fluorescein and Texas Red (Figure 5).

![Figure 5](image)

Figure 5 Overlapping emission “tails” from Fluorescein (A) and Texas Red (B) requires the use of separate selective emission filters.

LONG PASS EMISSION FILTERS

A “standard” Fluorescein filter set includes a “Long Pass” filter as the emission (or barrier) filter (Figure 6). This filter passes fluorescence from Fluorescein, but would also pass the red fluorescence from Texas Red. The resulting “bleedthrough” could confuse interpretation.

![Figure 6](image)

Figure 6 Basic Rhodamine filter set (Chroma Corp). The emission filter (A) is a long pass filter that passes all wavelengths longer than 590 nm.
BAND PASS EMISSION FILTERS

A more appropriate filter for visualizing Fluorescein is one that has a Band Pass emission filter in place of the LP shown in Figure 6. Figure 7 shows the spectra of a more discriminating filter set for Fluorescein. The band pass emission filter passes the green fluorescence of Fluorescein, but blocks longer wavelength emission from other (red) fluorescing dyes such as Texas Red, or from autofluorescence.

![Figure 7](image)

Figure 7  Fluorescein/GFP filter set with a Band Pass emission filter. The emission filter passes a discrete window of wavelengths (A).

MULTIPLE WAVELENGTH FILTER SETS

Aside from wavelength overlap and the resulting bleed through, visualizing multiple fluorescent probes introduces another problem. Each filter set consists of three individual glass components, and six refractive surfaces. Samples visualized through a filter set, therefore, produce a refracted image that is almost always offset relative to an image of the same sample but through a different filter cube. It is nearly impossible, therefore, to maintain image registration when merging digital images or making multiple film exposures of a sample when using two or more filter cubes. Interpretation of colocalized fluorescent probes is equivocal at best under these experimental conditions.

A simple solution to the registration problem is through the use of multiple wavelength filter sets. These sets consist of the standard three pieces of glass. However, instead of passing one wavelength slice, these sets pass two or more (Figure 8). Since each probe’s fluorescence passes through the same glass, registration is maintained between signals, and probe colocalization can be interpreted correctly.
Figure 8  Multiple wavelength filter set for Fluorescein and Rhodamine. Two excitation peaks excite fluorescein and rhodamine. Two emission peaks pass fluorescence from these two families of dyes.
**FLUORESCENCE MICROSCOPY AS AN INVESTIGATIVE TOOL**

**Introduction**

The technique of fluorescence microscopy will be examined in this laboratory exercise. Numerous fluorescent dyes are specific enough in their cellular binding characteristics that they can be used in cytochemical experiments as indicators of the presence or location within the cell of organelles or molecular components. In addition, many dyes can be introduced into the living cell, and thus be utilized to study the dynamics of cellular processes such as pH, metabolic activity, metal ion concentration, organelle movement, and cellular rearrangement. In this laboratory you will be using a few common fluorescent dyes to identify cellular components using epifluorescence microscopy.

*Note: The dyes used in this exercise are at the least irritating and possibly carcinogenic (especially DNA dyes). Use appropriate caution!*

**MAKING SLIDE PREPARATIONS**

All experimental samples must be placed on a microscope slide before you can look at them using the microscope. The general procedure for fresh samples is this:

1. Clean glass slides in 95% EtOH. Wipe dry with a KimWipe.
2. Place an EtOH-cleaned microscope slide, frosted or label side up, on a paper towel or other clean surface.
3. Add a small drop of immersion medium (water, buffer, or glycerol solution).
   1. If the sample moves too much (Brownian movement or active movement), consider immobilizing it:
      i. Use a very small amount of immersion medium to cause the coverglass to stick tightly to the slide.
      ii. Stick the sample to the slide with adhesive tape or Vaseline. Be certain however, that you will not image through the tape as the optics will be poor.
      iii. Use 50–100% glycerol as the mounting medium. The high viscosity will slow movement.
      iv. Use charged glass slides for microbial agents (e.g., poly-l-lysine treated, silane-treated, or Fisher “Probe-On Plus” slides).
      v. Embed the sample in a very thin layer of agarose (1-2%), gelatin (10-15%) or acrylamide.
      vi. Microbial samples can be dried onto the surface of a clean glass slide (42°C for 5 min).
4. Add the sample:
   • For large samples cut a small piece with a razor blade
   • For small samples apply and/or crush the sample in the immersion medium
   • For microbial samples use a toothpick to transfer a small amount of sample to the immersion medium.
   • For swabs, apply the swab directly to the immersion medium and agitate a bit to dislodge part of the sample into the medium
5. If you plan to do fluorescence imaging, add a small amount of fluorescent probe (10μl or so).
6. Apply the coverglass gently so as to not introduce bubbles. Put one edge down first, then lower the opposite edge.

Seal the edges of the coverglass with fingernail polish or use Invitrogen “Secure-Seal™ spacer”. Once the coverglass is affixed, the slide surface may be sterilized with 10% bleach solution.
Alternate method for microbial samples

If the sample doesn’t need to be kept alive, microbial samples may be dried onto the slide before probing:

1. Add aqueous microbial sample (usually 5–7μl) to an EtOH-cleaned slide
2. Add fluorescent probe (5–7μl)
3. Dry at 42°C (5–10 minutes)
4. Add 10–15μl microscope immersion oil
5. Apply coverslip
6. Seal (fingernail polish or SecureSeal)

EXPERIMENTAL SYSTEMS

- **Various non-biological specimens.** Use as described.
- **Cheek epithelial cells.** Use a wooden toothpick and gently scrape the inside of your cheek. Apply the extracted epidermal cells to a small drop of water plus fluorescent dye.
- **Throat swab.** Use a QTip to take a palate swipe. Apply the sample to a small drop of water/dye solution on a microscope slide. Apply a coverslip and secure with fingernail polish.
- **Elodea leaf:** This common aquarium plant is an excellent sample for light microscopy. The cells contain an array of cytoplasmic strands, nuclei, and visible organelles. In addition, there is a rich population of epiphytes growing on the leaf surface. Gently place a leaf into a small drop of water, probe, or fluorescent dye on a microscope slide. For cell wall staining a 5 sec dip in Hexane helps dye penetration.
- **Plants:** Use a razor blade to cut small sections of plant material. The sections should be <3mm on a side, and as thin as possible. A common sample is the inner epidermis of onion. Cut a deep square in an onion, pull out three layers (storage leaves), cut a small square on the surface of the inner leaf. Peel it off with a forceps and place immediately in a drop of water with probing dye.
- **Microbial specimens:** Simply place the collected sample in a small drop of water, probe, or fluorescent dye. Possible collection methods:
  - Soil sample
  - Surface sample (using toothpicks or tape)
  - Biofilm samples from sink drains
  - Throat/Tongue swabs
  - Tooth tarter samples
  - Yogurt or cheese. Good source of microorganisms *Lactobacillus acidophilus*, *L. bulgaricus*, and *Streptococcus thermophilus*.
  - ProGreens (mixture of bacteria and cyanobacteria). Mix with water, incubate >1h, centrifuge. Use the supernatant.
### FLUORESCENT PROBES

All probes for class are prepared ready to be diluted with their particular solvent. You will not be required to weigh anything.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Working concentration</th>
<th>Target</th>
<th>Ex/Em</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine Orange</td>
<td>0.1 μg/ml</td>
<td>Bacteria vegetative cells and endospores, nuclei (when used at high concentration)</td>
<td>Blue/Green</td>
</tr>
<tr>
<td>Antifade solution</td>
<td>5 μg/ml (Do Not use any antifade for SYTO BC)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Calcein AM</td>
<td>1 μM in water (stock is 1 mM in acetone)</td>
<td>Viability test for animal cells. Fluoresces if cell is alive.</td>
<td>Blue/Green</td>
</tr>
<tr>
<td>Calcofluor M2R (Fluorescent Brightener)</td>
<td>2 mg/ml in water for soil 0.1 μg/ml in water for cultures</td>
<td>Plant and fungal cell walls in soil and as cultured cells</td>
<td>UV/Blue</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>(intrinsic)</td>
<td>Chloroplasts in plants and algae</td>
<td>Green/Red</td>
</tr>
<tr>
<td>DAPI</td>
<td>1 μg/ml in water (dilute stock 1:1000)</td>
<td>DNA (nuclei)</td>
<td>UV/Blue</td>
</tr>
<tr>
<td>DiOC₇</td>
<td>1 μg/ml</td>
<td>Mitochondria</td>
<td>Blue/Green</td>
</tr>
<tr>
<td>DTAF</td>
<td>0.2 mg/ml</td>
<td>Bacteria in soil samples</td>
<td>Blue/Green</td>
</tr>
<tr>
<td>Fluorescein diacetate (FDA)</td>
<td>10 μg/ml</td>
<td>Viability stain for plants. Fluoresces if cell is alive.</td>
<td>Blue/Green</td>
</tr>
<tr>
<td>MitoTracker Green</td>
<td>5 nM</td>
<td>Mitochondria</td>
<td>Blue/Green</td>
</tr>
<tr>
<td>Nile Red</td>
<td>1 μg/ml in DMSO</td>
<td>Lipids</td>
<td>Blue/Yellow</td>
</tr>
<tr>
<td>Phloxine B</td>
<td>1 μg/ml</td>
<td>Bacteria (membranes)</td>
<td>Green/Red</td>
</tr>
<tr>
<td>Propidium Iodide</td>
<td>1 μg/ml</td>
<td>Viability test. Fluoresces if cell is dead.</td>
<td>Green/Red</td>
</tr>
<tr>
<td>Rhodamine B</td>
<td>1 μg/ml</td>
<td>Eukaryotic mitochondria; Bacteria</td>
<td>Green/Orange</td>
</tr>
<tr>
<td>SYTO mixture (“BC”)</td>
<td>(as per kit)</td>
<td>Bacterial DNA</td>
<td>Blue/Green</td>
</tr>
</tbody>
</table>
QUESTIONS FOR EACH SAMPLE

1. What does the sample look like in brightfield? Sketch it on a piece of paper to force your eye to really look at the sample.

2. If your microscope has contrast-enhancing optics (Phase, DIC) switch to that and observe the sample further.

3. Is there variability in the size, color, or shape of the components of your sample?

4. What are the differences in the sample?

5. Is anything moving?

6. Switch to Epifluorescence mode and try the Three Major Fluorescence filter sets (UV, Blue, Green excitation)

7. Does it autofluoresce?

8. What is the color of the fluorescence? What does this tell you about the sample?

9. Does the sample photobleach?

10. Choose a fluorescent dye to probe the sample. Base your decision on what you wish to localize and what background fluorescence there is (if any).

11. Does the sample label with specific probes? DAPI or SYTO are always good dyes to start with as DNA is found in all biological samples. The best probe has a fluorescence spectrum that does not overlap with endogenous fluorescence of the sample. You might have to treat the sample with EtOH before adding fluorescent probe (espec. When probing with DAPI).

12. Are the cells alive (assuming that there are cells there).
GENERAL OBSERVATIONS AND OBJECTIVES

1. Autofluorescence is almost always useful, and can be diagnostic for some organisms or structures.

2. The components of a complex sample may be characterized using a few, highly specific fluorescent probes.

3. All biological material contains a common set of easily distinguishable components. These include fats, oils, nucleic acids, and pigments. These can be identified using either autofluorescence or fluorescent probes.

4. Photosynthetic organisms (plants, algae) fluoresce when excited by green light. The cherry-red fluorescence is diagnostic for chlorophyll.

5. Be certain you have a working knowledge of the size of common objects. This includes plant and animal cells, bacteria, cellular organelles (e.g., chloroplasts), spores and seeds.

6. Bacteria may be characterized by their size (usually <1μm), shape (round, rod, spiral), and the presence of DNA.

7. Plant and fungal spores are almost always larger than bacteria. Fungal spores and plant pollen are often multicellular.

8. Very few (if any) biological specimens are angular. Look at the shape in your samples and evaluate whether it may be non-biological.
## FLUORESCENT DYE MATRIX FOR THE OLYMPUS BX51

<table>
<thead>
<tr>
<th>ALS BX51</th>
<th>Standard Designation</th>
<th>UV/365nm</th>
<th>Blue/480nm</th>
<th>Green/540nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>“WU”</td>
<td>UV</td>
<td>DAPI</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcofluor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>“WB”</td>
<td>FITC</td>
<td></td>
<td>Acridine Orange</td>
<td>Calcofluor AM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DIOC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DTAF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FDA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nile Red</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SYTO</td>
<td></td>
</tr>
<tr>
<td>“WG”</td>
<td>TRITC</td>
<td></td>
<td></td>
<td>Pl, Phloxine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chlorophyll</td>
</tr>
<tr>
<td>“TRITC”</td>
<td>Triple</td>
<td>DAPI &amp; Calcofluor (may be too bright)</td>
<td>All green-fluorescing dyes</td>
<td>All red-fluorescing dyes</td>
</tr>
</tbody>
</table>
LABORATORY EXERCISES

1) THE EPIFLUORESCENCE MICROSCOPE: INTRINSIC FLUORESCENCE, D1A

To familiarize you with the epifluorescence microscope you will examine a number of biological and non-biological samples. Some have artificial pigments that fluoresce (e.g., currency), other samples have “natural”, or intrinsic, fluorescent molecules (e.g., chlorophyll). Regardless of the source, fluorescence may be used as a means of identifying sample, or a sample’s constituent components. For example, cells from photosynthetic organisms contain molecules that function to capture light energy for photosynthesis. While there are a number of light-harvesting molecules, the major one is the green pigment chlorophyll. When given “enough” light, chlorophyll fluoresces, and thus it acts like a probe for photosynthetic organisms. Fluorescence of chlorophyll is diagnostic for plants.

Examine the following samples using both transmitted and epifluorescent light microscopy. In some cases the sample will be opaque, and cannot be observed in transmitted light. In this case look only in fluorescence mode. When you do the investigation, think about the “Questions for Each Sample” on page 15.

Questions:

1. For each sample make sure you know what excitation and emission wavelength(s) you use.
2. Is the fluorescence bright or dim? Is there a wavelength dependency to emission intensity?
3. What color or colors is the endogenous fluorescence.
4. Does the sample photobleach?

Examine the following samples using both transmitted and epifluorescent light microscopy:

<table>
<thead>
<tr>
<th>New $20 bill (or $10, $5)</th>
<th>Fresh vs. Used cigarette filter</th>
<th>Willamite mounted in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insect parts</td>
<td>Feather</td>
<td>ProGreens (Cyanobacteria, pollen)</td>
</tr>
</tbody>
</table>
2) **SURVEY OF ORGANISMS. OBSERVATION OF SIZE AND INTRINSIC FLUORESCENCE, D1A**

**Introduction**
This exercise is designed to familiarize you with two concepts of biological fluorescence microscopy: relative specimen size and presence or absence of intrinsic fluorescence. In this exercise you will examine a range of organisms using pre-prepared sample slides. Specimens range from large mammalian cells to tiny bacteria.

- Observe each sample slide using *transmitted white light* at two or three different objective magnifications, starting at the lowest power. On your handout sheets draw what you observe, noting size, shape, and any distinguishing features.
- After you observe the specimen in transmitted light, switch the microscope to epifluorescence mode using the appropriate excitation wavelength (noted next to sample name). Record your observations on the worksheet.

**Examine the following samples (from large to small)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Excitation color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant leaves (a fern)</td>
<td>Green</td>
</tr>
<tr>
<td>Mammalian cells (cheek epithelial cells)</td>
<td>Blue</td>
</tr>
<tr>
<td>Fungi (Neurospora hyphae)</td>
<td>UV</td>
</tr>
<tr>
<td>Multi-cell alga (Oscillatoria)</td>
<td>Green</td>
</tr>
<tr>
<td>Cyanobacteria (Anabaena)</td>
<td>Green</td>
</tr>
<tr>
<td>Agapanthus Pollen</td>
<td>Green</td>
</tr>
<tr>
<td>Baker’s Yeast</td>
<td>UV</td>
</tr>
<tr>
<td>Single-cell green protist (Euglena)</td>
<td>Green</td>
</tr>
<tr>
<td>Bacteria (Bacillus subtilis)</td>
<td>Blue</td>
</tr>
</tbody>
</table>
3) **CYTOCHEMICAL FLUORESCENCE MICROSCOPY—AN OVERVIEW OF FLUORESCENCE PROBING, D1A**

**Introduction**

The technique of fluorescence microscopy will be examined in this laboratory exercise. Numerous fluorescent dyes are specific enough in their cellular binding characteristics that they can be used in cytochemical experiments as indicators of the presence or location within the cell of organelles or structures. In this lab exercise you will be using a few common fluorescent dyes to identify cellular components or metabolic state using epifluorescence microscopy.

*Use all dyes with all materials.*

**EXAMINE THE FOLLOWING SAMPLES**

<table>
<thead>
<tr>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheek cells</td>
</tr>
<tr>
<td>Hair root</td>
</tr>
<tr>
<td>Onion leaf epidermal cells</td>
</tr>
</tbody>
</table>

**FLUORESCENT INDICATOR OF VIABILITY—CALCEIN AM OR FLUORESCEIN DIACETATE**

Calcein AM and Fluorescein diacetate (FDA) are membrane-permeant non-fluorescent dyes that can be introduced into cells via incubation. Once inside the cells they are hydrolyzed by endogenous esterase (plus ATP) into the negatively charged fluorescent calcein or fluorescein. Thus, these dyes can be used as indicators of metabolism, and therefore cell viability.

**Procedure for animal cells:**
1. Incubate material in calcein AM working solution (dilute stock with DI 1:1000) for 10–20 minutes.
2. Observe at 488 nm excitation (blue light)
3. Esterases in living cells release calcein that fluoresces yellow/green.

**Procedure for plant cells**
1. Incubate material in freshly prepared FDA (made from dry FDA powder, add a drop of acetone to dissolve, then add 10ml DI to make the working solution. Use that day.)
2. Observe at 488 nm excitation (blue light)
3. Esterases in living cells release fluorescein that fluoresces yellow/green.
**FLUORESCENT DYE FOR CHROMATIN—PROPIDIUM IODIDE (PI)**

Propidium Iodide is a membrane-impermeant nucleic acid intercalator. The dye is commonly used to selectively stain dead cells in a cell population and also used as a counterstain for nuclei in multicolor fluorescent imaging.

**Procedure:**

1) Incubate a small section of the onion epidermis in 70% EtOH for 10 minutes to permeabilize the cell.
2) Wash tissue and transfer to PI solution (1 μg/ml) and incubate for >5 minutes.
3) Observe with green (543 nm) epi-illumination. Nuclei fluoresce orange/red when stained with PI.
4) Look particularly for surface bacteria. They will be fluorescent and <1μm.

**FLUORESCENT INDICATOR OF BACTERIA—PHLOXINE B**

Phloxine B, a red acid dye, is a derivative of fluorescein with distinctly bluish shade; used for disinfection and detoxification of wastewater through photo-oxidation (it binds to bacteria). It is used as an intermediate for making photosensitive dyes and drugs. It is used as a cytoplasm stain in histology. Phloxine stains bacteria in mixed samples. Phloxine photobleaches easily.

**Procedure:**

1) Place the tissue segments on a microscope slide in a drop of Phloxine B working solution (1μg/ml).
2) Place and seal a coverslip. Antifade solution should not be used.
3) Observe with epi-illumination at 525nm (green light). Bacteria surrounding the cell and mitochondria within the cell fluoresce bright orange/red.
4) **PLANT AND FUNGI CELL WALLS, D2M**

The cell wall of these organisms consists of the polymer of β-linked glucans. The glucan found in plant cell walls is **cellulose**. The glucan found in fungi is **chitin**. Cellulose and chitin are not natively fluorescent, however the fluorescent probes Calcofluor M2R and Acridine Orange may be used to locate and identify these components, and indirectly cell walls. Examine the following samples using epifluorescence light microscopy. As usual, look first at autofluorescence of a control sample, then observe the probed sample.

**Look for patterning: edges, shapes and sizes while making your visual evaluation.**

**Stain solutions:**

- a) Probe for fungi: 1μg/ml Calcofluor M2R dissolved in water.
- b) Probe for plants: Acridine Orange (AO; 0.7mg/ml)

**AO solution: for 10 mls**

- 30mg sodium acetate
- 10 ml DI water
- 95 μl glacial acetic acid
- 70mg Acridine Orange
- mix well, store at -20°C
- pH should be 4.0

**Procedure for Fungi**

1. Add sample to a drop of Calcofluor M2R stain solution
2. Incubate **more** than 10 minutes.
3. Observe using UV excitation. Calcofluor fluoresces blue/white.

**Procedure for Plants**

1. Make a epidermal peel of plant of interest
2. Place peel on slide with 3 drops of AO solution
3. Add coverslip
4. Wait 2 minutes at room temp
5. Use KimWipe on left side of coverglass, add water to right side of coverglass.
6. Observe using FITC or Rhodamine filter set. Under these high concentrations, AO dimerizes and fluoresces in both channels.

**Examine the following samples using both transmitted and epifluorescent light microscopy.**

<table>
<thead>
<tr>
<th>Blue cheese (take a “blue” sample)</th>
<th>Mulch (look for the white, fuzzy component)</th>
<th>Elodea leaf. Look for epiphytes on the surface.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton boll fibers</td>
<td>Brie rind</td>
<td>Onion leaf peel</td>
</tr>
</tbody>
</table>
5) LIPIDS, D2M

All living organisms contain lipids (usually sequestered within organelles). Lipids generally do not fluoresce however, so these components are candidates for simple fluorescence probing using the fluorescent dye Nile Red. This dye is lipophilic and thus will be localized to lipid-containing organelles (liposome) in cells. When excited by blue light, Nile Red fluoresces yellow/gold. As with all investigations using fluorescence test your unstained sample first under several different excitation regimes to identify possible intrinsic fluorescence.

Note the Shape and Size of stained objects. Lipid drops will be perfectly round, cells rarely are.

**Stock solution:** Dissolve 1mg/ml in DMSO or acetone.

**Stain solution:** Dilute 1:1000 in water or buffer.

**Procedure:**

1. Check autofluorescence in a control (no stain) sample
2. Add sample to a drop of Nile Red solution
3. Incubate >5 minutes

Examine the following samples using both transmitted and epifluorescent light microscopy. If your sample is a liquid (e.g., oils) place a drop on a dry microscope slide and apply the coverslip.

| Thumbprint on a glass slide | Cheek cells | Onion epidermal cell (make a “peel”) |
6) **IDENTIFYING BACTERIA IN EUKARYOTIC CELL PREPARATIONS, D2M**

Eukaryotic cells (animal and plant) can be used to gain a familiarity with the morphology, staining ability, and size of bacterial cells. These cells have DNA-containing nuclei, so DAPI and/or PI are good fluorescent probes to use. These dyes may also be used to identify the bacteria cells in the surrounding neighborhood.

**Note the size and shape difference between bacteria and eukaryotic cells.**

**Procedure:**

1. Use the flat end of a wooden toothpick and scrape the inside of your cheek.
2. Make two slide preparations: one for PI staining and the other for DAPI staining.
3. On the appropriate slide, place the cells in a drop of water plus DAPI or PI (1μg/ml).
4. Observe with transmitted light using either Phase Contrast or DIC.
5. Switch to epifluorescence microscopy and excite the sample with UV light (DAPI) or Green (PI).
6. Look for blue/white (DAPI) or Red (PI) fluorescent nuclei.
7. Look for mouth bacteria. They will be very small, fluorescent objects surrounding the epithelial cells.
8. Use the same dyes to look at other putative microbial samples.

<table>
<thead>
<tr>
<th>Cheek cells</th>
<th>Throat swab</th>
<th>Tongue scraping</th>
</tr>
</thead>
</table>


7) IDENTIFYING BACTERIA USING A SPECIALIZED FLUORESCENT PROBE, D2M/A

One of the initial tests in an investigation of an unknown sample is to determine the presence of bacteria. Fluorescence probing for DNA is one positive method of identifying the presence of bacteria. Molecular Probes’ Bacteria Counting (SYTO BC; B-7277) stain is a high-affinity nucleic acid stain that easily penetrates both gram-positive and gram-negative bacteria and results in an exceptionally bright green fluorescent signal. The Molecular Probes kit is designed for flow cytometric analysis of bacterial populations, however, the SYTO BC dye is useful for microscopy investigations.

The criteria used for microscopic identification of prokaryotes include cell shape, size, grouping, Gram-stain reaction, and motility. Bacterial cells almost invariably take one of three forms: rod (bacillus), sphere (coccus), or spiral (spirilla and spirochetes). Bacilli may occur singly or form chains of cells; cocci may form chains (streptococci) or grape-like clusters (staphylococci); spiral shape cells are almost always motile; cocci are almost never motile.

Staining solution: Dilute SYTO BC stock solution 1:1000 with water

Procedure:

1. If you have a suspension (e.g., ProGreens powder, dust) centrifuge 0.5ml in a 1ml Eppendorf tube for 60s. Use the supernatant.
2. Add approximately 5μl supernatant to a clean glass slide.
3. Add 5μl Syto BC stain solution.
4. Mix gently then apply a coverslip.
5. Observe with blue excitation using fluorescence microscopy. Note the size difference between bacteria and other organisms in these samples.

Examine the at least six (6) of the following samples using epifluorescent microscopy. Make drawings and/or notes on your worksheets.

<table>
<thead>
<tr>
<th>Brie cheese</th>
<th>Half-and-half</th>
<th>Biofilm from a sink drain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProGreens supernatant</td>
<td>Air duct dust</td>
<td>Tooth tarter sample</td>
</tr>
<tr>
<td>Aquarium water</td>
<td>Cottage Cheese</td>
<td>Throat or tongue swab</td>
</tr>
<tr>
<td>Soil sample</td>
<td>Baker’s Yeast</td>
<td>Laundry sludge</td>
</tr>
</tbody>
</table>
8) TESTING LIQUIDS FOR BACTERIAL CONTAMINATION, D2A

In this experiment you will be testing liquids for bacterial contamination using a disposable filtration unit. The unit fits onto a syringe. Aqueous solutions, with putative bacteria is pushed through a small (13mm) 0.2μm glass filter, which catches the bacteria, supported by a larger paper filter. The glass “Anodisc” filter is recovered and mounted on a glass microscope slide. It is small enough to fit under a standard 22mm square microscope coverglass. Bacteria will be stained with the fluorescent nucleic acid dye SYTO BC to make them visible on the filter surface.

Procedure:

1. Unscrew Millipore Swinnex filter holder syringe unit. Note the syringe only fits on one side of the unit, which we will refer to as part A. The other half we will refer to as part B.

2. Place a drop of sterile water on the filter support grid on part B. Place the Whatman 0.2μm 13mm Anodisc filter on drop in the middle of the grid. Indicate the TOP surface with a pencil mark. Note that these filters are glass and are very easily damaged.

3. Place a drop of sterile water on top of the Anodisc filter. Place the Whatman 25mm #1 paper circle on the drop of water.

4. Put the O-ring into part A. Make sure the O-ring is seated.

5. Turn part B upside down. The filters should stay on because of the water. Screw part B into part A. This will help keep the O-ring in place. Tighten the assembly.

6. These can be made up in advance. Store until needed.

7. Before use, test with sterile water to be sure filter assembly doesn’t leak.

8. Load syringe with 50 ml of aqueous sample and some air. Do not burp the syringe. Do not use a smaller syringe or the pressure will rupture the filter.

9. Push the sample through the filtration unit you have assembled. Collect the filtrate for disposal. Push the air through last to make sure the Millipore Swinnex filter holder syringe unit is empty of liquid.

10. Open Millipore Swinnex filter holder syringe unit. Remove the Whatman 25mm #1 paper circle for disposal. Remove the Whatman 0.2μm 13mm Anodisc filter and place on aluminum weigh boat.

11. Heat filter on aluminum weigh boat 42°C for 6–10 minutes to dry filter.

12. Place a 10μl water on a glass slide then apply the filter, top side up. (Water keeps the filter from sliding away).

13. Add a 10μl SYTO BC working solution to the filter and apply coverslip. Seal with nail polish.

14. Observe with the 100x objective using Blue excitation light. Bacteria will fluoresce green.

Examine samples in the following order. Use the same filter apparatus, but change filters.

1. Unopened bottled water, filter the full volume
2. Supplied water sample
3. Gatorade
9) IDENTIFYING BACTERIA ON SURFACES, D2A

Identifying bacteria on surfaces is a procedure that can be performed quickly and easily using fluorescent probes previously discussed. The general procedure is to determine the intrinsic fluorescence of the surface in question, then choose a dye with a fluorescence spectrum that differs from background. Probe the sample with SYTO BC to localize the bacteria by fluorescence microscopy.

Procedure:

1. Cut a small (<2mm²) piece of sample and place on a microscope slide.
2. Add a drop of SYTO BC working solution onto the sample surface.
3. Add a coverslip and seal.
4. Observe using Blue excitation. Look for Green-fluorescing bacteria. **Look at the edges of the sample to see the stained bacteria.**

Examine the following samples.

| Vegetable leaves | Cloth sample |
10) INSTA-FLUOR SLIDES, D2A
This is the method for preparing dry, storable slides for fluorescent probing for bacteria. You will use these slides tomorrow.

Materials:
- 100% ethanol
- KimWipes
- P20 Pipetteman
- 2x SYTO BC solution
- Pipette tips
- Sharpie marker or Secure-Seal™
- 37°C hot block
- Slide box

 Protocol:
1. Dip glass slides in 100% ethanol, dry with a KimWipe.
2. Using a Sharpie, draw a circle the size of a nickel on the middle of the slide, or attach a SecureSeal from Invitrogen.
3. Put 10 μl of fluorescent probe onto the slide in the middle of the circle.
4. Using the pipette tip, smear the probe to the size of a dime.
5. Dry slides on 42°C heated block. This should take about 5 minutes.
6. Store slides in slide box at room temperature. The slide box should dark enough to prevent photobleaching.
7. When ready to use, remove tape covering from the SecureSeal and add 10μl of sample to be tested. Avoid adding more much or the seal will leak.
8. Add coverglass and observe using blue excitation light.

These have been tested to last up to one year at RT, desiccated.
11) **Determining Bacteria Viability Using Fluorescence Microscopy, D3M**

Bacterial cell viability can be determined using fluorescence microscopy, and the fact that in living cells certain dyes are excluded by an intact outer cell membrane. In this experiment you will use two DNA dyes: SYTO and Propidium Iodide (PI) to determine the state of the plasma membrane, and thus whether the cells are living or dead. Both dyes enter a dead cell (having a disrupted or absent membrane), whereas PI is excluded by the membrane of a living cell. SYTO freely crosses the membrane and stains DNA in living cells. When used in combination, living cells fluoresce green because they are stained only with SYTO, whereas dead cells fluoresce red when stained by both dyes. The red fluorescence of PI quenches green fluorescence of SYTO.

**Staining solution:**
- SYTO BC working solution (dilute stock 1:1000)
- Propidium Iodide (PI) solution (1μg/ml)

**Procedure:**

1. Prepare a toothpick-end-size sample suspension in 1 ml water. Mix thoroughly.
2. Take 10μl of this sample solution and apply to a microscope slide.
3. Add 10μl stain working solution, add a coverslip, and seal with nail polish.
4. Observe with blue excitation (for SYTO BC) or green excitation (for PI).

Examine the following samples using both transmitted and epifluorescent light microscopy.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Additional Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brie cheese</td>
<td>Plated bacteria sample</td>
</tr>
<tr>
<td>ProGreens supernatant</td>
<td>Environmental sample of choice (prepare at least one)</td>
</tr>
<tr>
<td>Biofilm from a sink drain</td>
<td>Add directly to the slide, do not dilute.</td>
</tr>
<tr>
<td>Tooth tarter sample</td>
<td>Add directly to the slide, do not dilute.</td>
</tr>
</tbody>
</table>
12) **BACTERIAL ENDOSPORE STAINING, D3M**

Certain bacteria genera (*Bacillus, Clostridium*) develop resistant endospores during periods of environmental stress. These spores are highly resistant to desiccation and UV damage, and because of this they also are resistant to the normal methods of fluorescence staining. In this experiment you will use a method adapted from cytology experiments in plants to modify the endospore cell wall sufficiently to introduce the fluorescent dye Acridine Orange.

**Required items:**

- Glass slides
- Kimwipes
- 100% ethanol
- P20 Pipetteman
- Pipette tips
- Hot block at 42°C
- MAA: 3 parts methanol, 1 part Glacial acetic acid. **Prepare just before use.**
- Cargille’s FF immersion oil
- Coverglass, 22x22 #1
- Fluorescence microscope
- 2 Coplan jars
- Acridine Orange 1μg/ml staining solution with 5μg/ml DABCO antifade. **Prepare just before use.**

**Procedure:**

1. Clean a glass slide with 100% ethanol and KimWipes.
2. Put 10 μl of sample to be tested on cleaned slide.
3. Using the pipette tip, smear the sample into a circle the size of a dime.
4. Place the slide on the 42°C heat block to dry. This should take about 3 minutes.
5. Put the slide into a Coplan jar with 40 ml ice cold MAA. Let sit for exactly 5 min.
6. Put the slide into a Coplan jar with 40 ml ice cold 100% ethanol. Let sit for 1 min.
7. Remove slide from jar and wipe the bottom surface with a KimWipe—NOT the sample side.
8. Place slide on 42°C heat block to evaporate the ethanol.
9. Add 10μl of Acridine Orange + DABCO stain. Smear the stain to cover the dried sample (about the size of a dime).
10. Add coverslip and seal with nail polish.
11. Observe with microscope using 100x objective. Excite with blue light. Spores will stain as green ovals. Vegetative cells will be solid green staining.

**Examine the following samples using both transmitted and epifluorescent light microscopy.**

- *Bacillus subtilis* endospore preparation. Centrifuge, then take 10μl of supernatant.
- *Bacillus thuringiensis* from *Mosquito Dunkers*. Add a pencil eraser-size sample (supplied) to 10ml water, shake vigorously, soak 10 min, use 10μl supernatant to test for spores.
- *Bacillus atrophaeus* endospore preparation. Centrifuge, then take 10μl of supernatant.
# APPENDIX

## ALS Fluorescence Microscopy Kit

Here are some standard components required for basic fluorescence microscopy. Use this list as a starting point for your investigative kit.

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epifluorescence microscope</td>
<td>Objectives: 10×, 20×, 40×. 100× oil immersion</td>
</tr>
<tr>
<td>Immersion oil (Cargille Type FF)</td>
<td>Spare HBO 100w burner or spare Metal Halide burner</td>
</tr>
<tr>
<td>Filter sets. A basic set consists of three excitation wavelengths: UV (365nm), Blue (480nm), Green (520nm)</td>
<td>Fluorescent probes: DAPI, SYTO BC, PI, Calcein AM, Calcofluor M2R, Acridine Orange</td>
</tr>
<tr>
<td>Cotton</td>
<td>Wooden applicator sticks</td>
</tr>
<tr>
<td>Lens paper</td>
<td>KimWipes</td>
</tr>
<tr>
<td>Frosted end microscope slides</td>
<td>Glass Coverslips, (#1)</td>
</tr>
<tr>
<td>Razor blades</td>
<td>Forceps: Dumont #5</td>
</tr>
<tr>
<td>Coplan jars</td>
<td>Nail polish, clear</td>
</tr>
<tr>
<td>Bleach</td>
<td>Invitrogen Secure-Seal</td>
</tr>
<tr>
<td>“Hot Block” (42°C)</td>
<td>Toothpicks, wooden</td>
</tr>
<tr>
<td>EtOH, 100%</td>
<td>Swinnex Filter Holder (Millipore)</td>
</tr>
<tr>
<td>DABCO antifade solution (5μg/ml)</td>
<td>Anodisc 0.2μm 13mm diameter filter (Whatman)</td>
</tr>
<tr>
<td>Syringe, plastic disposable (50ml)</td>
<td>Filter paper circle 25mm (Whatman #1)</td>
</tr>
<tr>
<td>Acetic acid, Glacial</td>
<td>MeOH, 100%</td>
</tr>
</tbody>
</table>
# IMPORTANT WEBSITES

<table>
<thead>
<tr>
<th>Company</th>
<th>Notes</th>
<th>URL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CST website from UC Berkeley</strong></td>
<td>Site for this course. Updated regularly.</td>
<td><a href="http://microscopy.berkeley.edu/cst">http://microscopy.berkeley.edu/cst</a></td>
</tr>
<tr>
<td><strong>Bacteriology site</strong></td>
<td>Good basic site for information about bacteria.</td>
<td><a href="http://www.textbookofbacteriology.net/">http://www.textbookofbacteriology.net/</a></td>
</tr>
<tr>
<td><strong>Molecular Expressions</strong></td>
<td>The best website for everything microscopy</td>
<td><a href="http://micro.magnet.fsu.edu/primer/index.html">http://micro.magnet.fsu.edu/primer/index.html</a></td>
</tr>
<tr>
<td><strong>Biocompare</strong></td>
<td>Web-based buyers guide for scientists. Good research tool for Immunologicals</td>
<td><a href="http://biocompare.com/">http://biocompare.com/</a></td>
</tr>
<tr>
<td><strong>Chroma Technologies</strong></td>
<td>High quality filters for fluorescence microscopy</td>
<td><a href="http://www.chroma.com">http://www.chroma.com</a></td>
</tr>
<tr>
<td><strong>Semrock, Inc</strong></td>
<td>High quality filters for fluorescence microscopy</td>
<td><a href="http://www.semrock.com">http://www.semrock.com</a></td>
</tr>
<tr>
<td><strong>Omega Optical</strong></td>
<td>High quality filters for fluorescence microscopy</td>
<td><a href="https://www.omegafilters.com/">https://www.omegafilters.com/</a></td>
</tr>
<tr>
<td><strong>HyTest Ltd</strong></td>
<td>Immunologicals</td>
<td><a href="http://www.hytest.fi/">http://www.hytest.fi/</a></td>
</tr>
<tr>
<td><strong>Molecular Probes</strong></td>
<td>Best source for fluorescent probes</td>
<td><a href="http://probes.invitrogen.com">http://probes.invitrogen.com</a></td>
</tr>
<tr>
<td><strong>Nikon Microscopy Site</strong></td>
<td>Good background information for fluorescence microscopy</td>
<td><a href="http://www.microscopyu.com/articles/fluorescence/index.html">http://www.microscopyu.com/articles/fluorescence/index.html</a></td>
</tr>
<tr>
<td><strong>Ocean Optics Inc</strong></td>
<td>Interesting company for detection kits and devices</td>
<td><a href="http://www.oceanoptics.com/Products/eds.asp">http://www.oceanoptics.com/Products/eds.asp</a></td>
</tr>
<tr>
<td><strong>Olympus FluoView site</strong></td>
<td>Good background information for fluorescence microscopy</td>
<td><a href="http://www.olympusfluoview.com/applications/index.html">http://www.olympusfluoview.com/applications/index.html</a></td>
</tr>
<tr>
<td><strong>Sigma-Aldrich</strong></td>
<td>Fluorescent probes</td>
<td><a href="http://www.sigmaaldrich.com/Brands/Fluka__Riedel_Home/Analytical/Fluorescent_Probes.html">http://www.sigmaaldrich.com/Brands/Fluka__Riedel_Home/Analytical/Fluorescent_Probes.html</a></td>
</tr>
<tr>
<td><strong>TefLabs</strong></td>
<td>Fluorescent probes</td>
<td><a href="http://www.fluorescentprobes.com/">http://www.fluorescentprobes.com/</a></td>
</tr>
<tr>
<td><strong>EXFO X-Cite fluorescence illumination system</strong></td>
<td>Fluorescence illuminator and light guide system</td>
<td><a href="http://www.exfo-lifesciences.com/products/X-Cite120_PC.html">http://www.exfo-lifesciences.com/products/X-Cite120_PC.html</a></td>
</tr>
</tbody>
</table>
**INVESTIGATING UNKNOWN SAMPLES**

You will be given five unknown samples. Use your knowledge of fluorescence microscopy and the use of fluorescent probes to evaluate these samples. **Score each sample for the presence of organisms.** Add drawings, comments, or both in the final column.

Complete the following table:

<table>
<thead>
<tr>
<th>Unknown Sample #</th>
<th>Organism categories present</th>
<th>Comments/drawings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Appendix

<table>
<thead>
<tr>
<th>Unknown Sample #</th>
<th>Organism categories present</th>
<th>Comments/drawings</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>