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## 7.13 Experimental Microbial Genetics

Fall 2008

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## Digital Optical Microscopy

The invention of the microscope revolutionized biology in the 1700's by permitting scientists to explore the world of microorganisms and details of cell structure. In the 1990s the microscope has continued to be a powerful tool for biologists. We can watch subnanometer movements of cells or single molecules in cells with digital CCD cameras, measure piconewton forces exerted by motor proteins with optical traps formed by IR lasers, reconstruct the 3D structure of a cell using powerful image analysis algorithms, follow the chemical changes in a cell using fluorescent dyes, or determine the precise location of various gene products that are tagged with antibodies, enzymes, or light emitting (fluorescent or luminescent) proteins. The dynamics of cellular responses captured in a microscope tell us about the lifestyle and life span of a structure. The purpose of the microscope module in Project Lab is to familiarize you with the operation of a state-of-the-art digital microscope. The microscope system consists of a Nikon Optiphot 2 microscope, a Photometric ImagePoint CCD camera, and a MacIntosh G3 PowerPC computer for data acquisition and image analysis. With this system, you will observe the dynamics of fluorescently tagged proteins in cultured CAD cells. The manual will illustrate how to set up a microscope for imaging with phase contrast, differential interference contrast, or fluorescence optics, and how to analyze the images using Spot or Photoshop software packages.

### Parts of a Microscope

The microscope is divided into three parts: an illumination system, an optical system, and an image viewing system. The illumination system consists of the lamp, shutters, filters, condenser, and field diaphragm. These elements deliver and focus the light onto the specimen. The optical system consists of the objective lens and various prisms, filters, and magnifiers. They focus the image onto the oculars or camera. A CCD camera captures a digital image and the image is stored on a computer.

### The Microscope

A microscope consists of an illumination system, an imaging system, and a detecting system. These systems focus light onto a specimen, collect light from the specimen, and record the magnified image. The specimen is mounted on a transparent glass slide and positioned on the movable stage of the microscope. Light from a bright source is focused by the collector and condenser lenses onto the specimen. Light transmitted through the specimen is collected by the objective lens and focused on the focal plane of the objective lens, creating a magnified image of the specimen. The image is then further magnified and projected by the ocular lens (eyepiece) onto a detector, either the human eye, a piece of photographic film, or imaging chip of a CCD camera. The condenser diaphragm and the other apertures restrict the amount of light entering or leaving a lens.

The total magnification is a product of the magnification of the individual lenses: if the objective lens magnifies 100-fold (100X) and the ocular lens magnifies 10X, the final magnification recorded (by the eye) will be 1000X. The magnification recorded on the film depends on what lenses are present in the path to the camera (and not on the ocular lens). The resolution of a microscope lens is the minimum distance,  $D$ , between two

distinguishable objects that can be resolved from each other: the smaller the value of **D**, the better the resolution. **D** depends on three parameters: the angular aperture, **a**, or halfangle of light entering the lens from the specimen; the refractive index, **N**, of the air or fluid medium between the specimen and the objective lens; and the wavelength, **l**, of incident light:

Decreasing the value of **l** or increasing either **N** or **a** will decrease the value of **D** and thus improve the resolution.

- 1) The angular aperture depends on the width of the objective lens and its distance from the specimen. Moving the objective lens closer to the specimen will increase  $\sin a$  and reduce **D**.
- 2) The refractive index measures the degree to which a medium bends a light ray that passes through it; the refractive index of air is defined as  $N = 1.0$ . One way to decrease **D** is to use immersion oil as the medium between the specimen and the objective lens. Since the refractive index of such oils is 1.5, the resolution will be improved 1.5-fold.
- 3) The shorter the wavelength of incident light, the lower the value of **D** and better the resolution.

Under the best conditions, with violet light (wavelength,  $l = 0.4 \mu\text{m}$ ) and  $N \sin a$  of 1.4, a limit of resolution of just under  $0.2 \mu\text{m}$  can theoretically be obtained under the light microscope.

### **Types of Light Microscopy**

A typical living cell is transparent, thus nearly invisible to the eye. To “see” a cell we must either stain the specimen with a colored or fluorescent dye (described in sample preparation) or manipulate light so that interference caused by the cell generates contrast.

#### Transmitted light microscopy

- Bright field
- Phase Contrast
- DIC/Nomarski

#### Epifluorescence light microscopy

**Bright field** microscopy can only be used to view stained specimens since light is usually not absorbed by unstained specimen. Histological stains such as hemotoxin and eosin are used to image cytoplasmic and nuclear structures in a cell. Because enzymes within cells can remain active under precisely controlled conditions, we can take advantage of this activity to locate the enzymes within the cell. This is done by adding a substrate which yields a colored product when the enzyme acts on it. However, without artificial means to generate color contrast, brightfield microscopy has limited usefulness.

**Phase-contrast** microscopy is used to give detailed views of transparent, live, unstained cells and tissues. The brightness in a region of the sample depends on the refractive index of that region. Because light moves more slowly in a medium of higher refractive index,

the part of a light wave that passes through a specimen will be refracted and consequently will be out of phase with the part of the wave that does not pass through the specimen. When the two parts of the light wave are recombined, the resultant light will be brighter if they are in phase and less bright if they are out of phase. Thus, dense or thick regions of the cell will appear darker than their surroundings.

Incident light passes through an annular diaphragm, which focuses a ring of light on the sample. Light that passes unobstructed through the specimen is focused by the objective lens onto the phase-retarding gray phase ring (on the surface of the objective lens), which absorbs some of the direct light and alters its phase by one-quarter of a wavelength. If a specimen refracts or diffracts the light, the phase of some light waves is altered and the light waves are redirected through the thin, clear region of the phase plate. The refracted and unrefracted light are recombined at the image plane. Structures within a cell are highlighted by the light and dark fringes generated by constructive and destructive interference.

**Nomarski, or Differential Interference Contrast (DIC)** microscopy, generates a three-dimensional or "relief" image. The "shadow" represents a difference in refractive index rather than literal thickness. Contrast is very good and the condenser aperture can be used fully open, thereby reducing the depth of field and maximizing resolution. A prism splits a beam of incident polarized light into two parallel beams: one passes through one region of a specimen and the other passes through a closely adjacent region. After passage through the specimen, the beams are reunited by a similar prism in the objective. In a homogeneous specimen, there is no difference between the two beams, and no contrast is generated. However, near a refractive boundary (e.g. a nucleus within the cytoplasm), the path difference between the normal and the reference beam will generate a relief. A path difference of 0 results in no transmitted light; maximum light is transmitted if the path difference is half the wavelength. By these means, different regions of the specimen appear bright or less bright in contrast to the mid-gray background.

Phase-contrast microscope is useful in examining the structure and movement of larger organelles, such as the nucleus and mitochondria, in live cultured cells. The disadvantage of this technique is that it is suitable for observing only single cells or thin cell layers. The Nomarski technique, in contrast, only defines the outlines of the large organelles; however, thicker objects can be observed by combining this technique with optical sectioning.

**Fluorescence microscopy** relies on emission of light by the sample when excited by higher wavelength light. Unlike a transmitted light microscope, a fluorescence microscope illuminates the sample through the objective lens (epi-illumination). A specific wavelength of ultraviolet light from a mercury lamp source is selected by the excitation filter. The excitation radiation is reflected by the dichromatic filter and focused by the objective lens onto the sample. Fluorescent molecules in the sample absorb the light and emit light (fluoresce) at a longer wavelength. This light is collected and focused by the objective lens. An emission filter blocks any residual excitation light. Because most of the excitation light passes through the specimen, epi-illumination allows us to

detect very low levels of fluorescent light, making possible the detection of single molecules.

### **Instructions for using the microscope and video camera with Spot software**

#### **Cleaning the lenses**

The optical cleanliness of the microscope lenses must be scrupulously maintained to obtain clear, high contrast images. If the image appears blurred, low contrast, or spotty, then the system is dirty. Furthermore, dirt on the surfaces of lenses is a sure-fire method to scratch the lens. Most dirt is found on the microscope slide or cover slip. If you see diffuse spots or circles when the sample is in focus, then check the glass surface of the cover slip or microscope slide for fingerprints, immersion oil, or dried tissue culture media. If the spot moves, then check for air bubbles in the oil or in the liquid surrounding the specimen.

- Water or grease on the objective lens will cause the image to appear dull or of low contrast and blurry at best focus. Clean the objective lens as described below.
- Dirt in the plane of focus is usually on the surface of the field diagram lens (or on a glass surface in the same focal plane).
- Check the surface of the eyepiece. Grease from eyelashes will obscure the image when viewed through the oculars but not when viewed through the CCD camera.

The objective lenses should be routinely checked for cleanliness before use. Be extremely careful to handle the objectives by their barrel and do not touch the lens surface with fingers. Dropping an objective will destroy it. (And lenses are VERY expensive)

1. Moisten a piece of lens paper and gently touch it to the lens surface. Repeat with a dry piece of paper.
2. Grease and immersion oil may be removed by moistening a piece of lens paper with water, and gently touching the lens surface. Repeat with a dry piece of paper.
3. If still dirty, repeat 2 with ethanol; then repeat again with water. Notify the instructor.

#### **Turning on the Microscope**

**Always turn on the mercury lamp first** because arcing of the bulb generates an electromagnetic pulse (EMP) which can blow the computer. A corollary to this rule is to **turn on the computer last**. If you want to view the image using the CCD camera or capture the image onto the computer, then you must turn on the camera, monitor, and computer.

1. mercury lamp (for fluorescence microscopy, **ALWAYS TURN ON FIRST**)
2. Power strip: This will give power for microscope illumination (in the back, on the right) and the CCD camera
3. Computer (turn on **LAST**) When turning things off, turn off the computer first, then power strip and then UV lamp. Please don't forget the power strip. Otherwise, the video camera is still on.

#### **Finding and Focusing**

**A.** Start with the 10X objective and put your cells in focus.

- Carefully rack down the stage. The objectives should be swung out to the side.
- Place specimen into the specimen holder.
- Swing the objective of choice into place.

NOTE: The easiest way to find your cells is by using DAPI fluorescence

Once you have the cells in focus, just close the shutter to the UV lamp and turn up the white light. If you still can't see them, adjust contrast. The easiest way to see cells under white light is by using the following filter combination (these apply to PHASE objectives only):

- DIC filter beneath stage pushed in; DIC filter near eyepieces pulled out
- Use Phase 3 filter in dial or Phase 4 in dial (depending on what objective you use)
- May need to increase illumination

**B.** Set up for Koehler illumination. For optimal imaging, the microscope lens and specimen slide must be clean and the system set up for Koehler illumination. Under these conditions, the specimen is brought into focus and the condenser lens is aligned with the objective lens and positioned to fully illuminate the specimen.

- Adjust the condenser to illuminate the specimen with an optimal amount of light
- Adjust the intensity of the lamp with the slider controls at the front of the microscope base. A middle setting should be adequate for most applications.
- Close the aperture (under stage) completely.
- Bring the aperture leaves into focus (a decagon image) by using the condenser focus (knob below stage on left).
- Align the decagon to the center with the alignment knobs. Centering can be checked by opening up the field diagram. This step centers the condenser lens within the optical and illumination path.
- Open up the condenser such that the edges of the decagon are just inside the edge of the tube. This ensures the specimen is fully illuminated but prevents excess illumination.

NOTE: Every time you change the objective lens, the condenser may be put out of focus. You may need to focus the condenser each time you change the objective.

**C.** To use higher magnification, just rotate the appropriate objective into place. If the objective requires oil, then place a drop of oil on top of the specimen before placing the objective. Remove air bubbles from the oil. Be careful not to get oil on dry (<60X) objectives.

**D.** To use fluorescence:

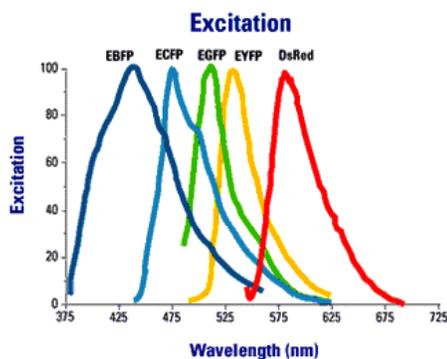
- DIC filter in condenser (beneath stage) pushed in; DIC filter near eyepieces pulled out (otherwise it will cut the fluorescence signal). It doesn't matter about anything below the stage under fluorescence, since all illumination is coming from above through the objective.
- Dim white light and open shutter to UV lamp

When looking through phase objectives (10x, 40x, 100x), the DIC filters should not be engaged (filter ring to ph3 or ph4, not DIC 1.4).

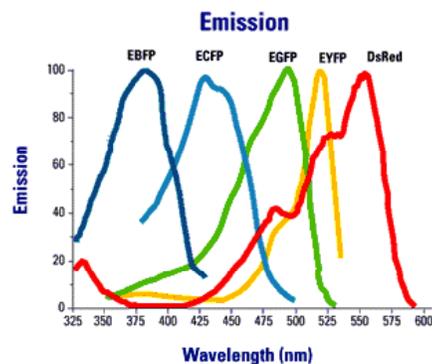
\*\*The GFP fluorescence is the most important feature of your cells, so you should focus on that, then acquire all three of your images (i.e., GFP, DAPI, DIC) in the same plane of focus.

To adjust illumination from the mercury bulb: slide in/out the neutral density (ND) filters in the back. The filters can be used in any combination. Try to minimize illumination to the specimen and close the shutter (or slider) when not examining the specimen to avoid bleaching.

### Excitation and Emission Spectra for Living Colors™ Fluorescent Proteins



TOP



Select the filter set of choice.

Type	Ex. wavelength(EX)	Barrier wavelength (BA)
a) DAPI	340-380	435-485
b) FITC	465-495	515-555 for GFP (EGFP)
c) yellow GFP	490-510	520-550 for yellow GFP (EYFP)
d) cyan GFP	426-446	469-500 for cyan GFP (ECFP)
e) BV-2A	400-440 (nm)	470 (nm) for rhodamine

Only 4 of these filter modules can be mounted in the scope. If you need to use one that is not mounted, ask a TA about changing.