Microscopy Tutorial

Scientific Imaging Shared Resource
Fred Hutchinson Cancer Research Center

$$d = \frac{\lambda}{2n \sin \alpha}$$

This tutorial is still under construction. We plan to release an updated and illustrated version in the future. To help us improve it, please send your comments, suggestions and criticisms to imaging@fhcrc.org.

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Introductory Notes.

The main goals of this tutorial are:

• To introduce the basic concepts of image formation on the light microscope
• To understand the design and operation of the microscopes in Scientific Imaging
• To help users optimize image collection
• To discuss some of the techniques used for the enhancement, display and quantitative analysis of complex microscopy images.

In the appendix, we have included manuals for the different microscopes available at the FHCRC Scientific Imaging Shared Resource, as well as various Tables and other practical data that may be of general interest.

Please feel free to make suggestions about additional data, information, etc…, that you may deem useful to include in future upgrades. Our Email is: imaging@fhcrc.org.

Part I: The Fundamentals.

Chapter1. Important concepts in microscopy.

The optical microscope uses light as a probe to obtain structural information about small objects. Contrary to common belief, magnification is not the primary goal of the microscope. For instance, a high degree of magnification could be achieved by taking a photographic image of a sample, and enlarging the image. However, beyond a certain limit, no further spatial information would be achieved with this method (empty magnification). The primary task of the microscope is to **resolve** small structural detail in the samples. Magnification is a second step designed to make such detail visible to our eye, or to other image recording devices. In a compound microscope, the objective performs both tasks of resolving and magnifying small detail, while the eyepiece magnifies the primary image formed by the objective to make small detail visible to our eye. A microscope, therefore, is basically a device designed to extend the limit of resolution of the human eye.

The following parameters are very important in light microscopy:

• Resolution

The capability to reveal small detail. Resolution of a microscope is primarily determined by the **Numerical Aperture** of the objective and condenser (objective only in epifluorescence), and by the wavelength of light, but other optical components, as well as the imaging device, may alter the final resolution. It is important to note that resolution is not the ability to see small objects, but rather to separate very close objects. The ability to see small objects is mainly limited by contrast. Given enough contrast, single molecules can be imaged with the light microscope. More precise definition of resolution will be given below.

• Contrast

The difference in intensity or color between two objects, or between object and background. A certain contrast threshold is necessary for our eyes to separate two objects. In optical microscopy, where samples are generally nearly transparent, contrast enhancing techniques are necessary to reveal detail.

• Magnification

Optimal magnification is achieved when it matches the resolution of the microscope system to the resolution of the imaging device (e.g. eye or CCD camera). In the optical microscope, the useful magnification range is about 500-1000 times the Numerical Aperture of the objective. If magnification is too low, details resolved by the objective are not revealed to the eye or recorded by the imaging device (undersampling). The Nyquist sampling theorem establishes that the smallest resolvable detail should be sampled 2-3 times (that is should be recorded over 2-3 pixels on a CCD device, or 2-3 photoreceptor cells on the retina)

• Sensitivity

In fluorescence microscopy, signals can be extremely faint (several orders of magnitude fainter than the signal of a distant star). This requires extremely sensitive imaging devices or photodetectors. Since such signals could be as small as a few photons, imaging devices also need to have extremely low noise (see paragraph on digital imaging).

Chapter 2. The Physical Nature of Light.

Light is an electromagnetic radiation composed of two orthogonal electric and magnetic fields propagating together trough space and a variety of media. Most properties of light, such as image formation in the microscope, can be explained by the wave theory. Some other properties, however, are best explained by assuming that light is composed of material particles (photons). For example, light rays from distant stars are bent by the strong gravitational attraction of the sun. This duality of light has not been totally reconciled.

The most important attributes of an electromagnetic wave are:

Wavelength λ
Frequency ν
Speed in vacuum c
Amplitude A
Energy E

Wavelength (or frequency) is perceived as color by the human eye
Amplitude is perceived as brightness (intensity)

The following equations apply to light:

$\mathbf{E= h\nu}$ (where h is the Planck constant)

The energy carried by a light wave is directly proportional to the wave's frequency

$\mathbf{c= \lambda\nu}$

Speed of a light wave equals its wavelength multiplied by its frequency.

Note: when light travels through different media (such as vacuum, air, water, glass), speed and wavelength change. Frequency remains constant.

Qualities of light :

Polychromatic:	contains many different wavelengths (or frequencies)
Monochromatic	contains a single, or a narrow range of wavelengths
Polarized:	all waves vibrate in a well-defined plane
Non-polarized	waves vibrate in a single plane
Coherent:	all waves are in phase (aligned with respect to wavelength)
Non-Coherent	Waves are not in phase
Collimated:	beam is parallel
Divergent	

Interactions of light with matter (and their role in microscopy):

Transmission/Absorption

Transparent substances have 100% transmission. Objectives are designed with special glasses and coatings to maximize transmission of light in specific wavelengths such as visible, UV, or IR. Fluorescence filters are designed to specifically absorb or transmit defined wavelengths.

Reflection

Mirrors reflect all, or specific wavelengths (polychroic mirrors). Mirrors are used to deflect light to various imaging devices inside the microscope.

Refraction

When light rays enter a transparent medium of different refractive index, they are bent proportionally to the difference of refractive index of the two media according to Snell's law: $n1 \times \sin a1 = n2 \times \sin a2$; the change in direction is due to a difference in the speed of light in the two media. The refractive index of a medium (n) is defined as the ratio of the speed of light in vacuum (c) to the speed of light in the medium (v): $n=c/v$. The refractive index of diamond is about 2.4; light in diamond travels at about 40% its speed in vacuum. Generally, short wavelengths are refracted more than longer wavelengths. This is responsible for the well-known effect of dispersion of white light by a prism. Lenses and objectives focus light by refraction.

Diffraction

The bending of light waves by sharp edges or small objects. This is a very different mechanism from refraction. The shadow of a sharp edge is not a sharp boundary, but a series of sinusoidal ripples. Similarly, a beam of light passing through a small pinhole is not projected as a discrete point, but rather as a larger disc surrounded by concentric rings of decreasing intensity (Airy pattern). These rings are generated by interference of the different diffracted rays. A similar effect is observed when trying to image a point source of light with an objective. The size of the Airy disc is always larger than the size of a point light source, thus imposing a limit to the maximum achievable resolution.

The amount of bending is proportional to the wavelength of light (red bends more than blue), and inversely proportional to the diameter of the pinhole, or the size of the object (small objects bend light more than large objects). Therefore, better resolution is achieved with blue light than with red light.

Size of Airy disc generated by a pinhole:

Diffraction angle alpha proportional to wavelength, inversely proportional to diameter
For the first minimum (=Airy disc diameter): $\alpha=1.22\ \lambda/d$.

The resolving power of the human eye is limited by diffraction because of the small size of the pupil. In bright sunlight, the pupil is smaller, and resolution is poorer than in dimmer lighting (in dimmer lighting, however, image contrast is lower, also limiting resolution to some extent).

Diffraction is the main limiting factor in image formation in a well-corrected light microscope.

•Polarization

Natural light is generally not polarized, i.e. light is composed of waves that vibrate in every possible orientation. Laser light is strongly polarized. A polarizer filters light by only letting through the waves or wave components that vibrate in a specific plane. Two polarizers oriented at right angles will not pass any light. Polarization is used for contrast enhancing in microscopy (e.g. polarization microscopy, DIC).

Fluorescence

Fluorescent molecules absorb light of a specific wavelength, and reemit part of the energy absorbed as light of a longer (lower energy) wavelength. Fluorescence is due to the interaction of light waves with the electronic shells of molecules. Electrons are affected by electromagnetic waves, including light waves. The electric field of a traveling light wave can momentarily displace an electron to a different energy state (quantum state) in a molecule, such that the difference in energy of the two states matches the energy carried by the light wave (according to the equation $E=h\nu$). After a brief instant (about a nanosecond), the electron falls back to its normal state, and releases the extra energy as a new light wave. Part of the accumulated energy is dissipated during the process; hence, the emitted light always has lower energy (longer wavelength).

Phosphorescence involves a similar mechanism. However, the delay can be much longer (e.g. seconds).

A few definitions:

Stokes shift: the difference in wavelength between the absorption and emission of a fluorescent molecule.

Fluorescence lifetime: The lag between absorption and emission.

Molar Extinction Coefficient: the ability of a molecule to absorb photons

Quantum Efficiency: the percentage of absorbed photons that are re-emitted.

Chapter 3. The Human Eye.

The eye is an optical device that focuses the image of objects onto a light sensitive surface made of light-sensitive cells, the retina. The focusing elements are the cornea and the lens. The amount of light is controlled by the iris, a muscular diaphragm that regulates the diameter of the pupil.

The retina is composed of two types of cells:

Cones: respond maximally to light at 555 nm (green-yellow), and are responsible for daylight (photopic) and color vision.

Rods: are about one thousand times more sensitive than cones, and are maximally sensitive at 507 nm (blue-green light). Rods are responsible for night-time (scotopic) vision and do not provide color vision.

The eye can detect light and adapt to intensities over a very wide range (dynamic range of about a billion). During night vision, the eye can detect as little as about 100-150 photons.

Resolution of the eye:

The resolution of the eye is limited by the diffraction of light that passes through the small pupil aperture, and by the size of the photoreceptor cells.

A small object viewed through the pupil of the eye will give rise to an image on the retina similar to an Airy disc. The angular size of the Airy disc is determined by the diameter of the pupil and the wavelength of light. For a diameter of 4mm and λ=600 nm, the angle α between the center of the Airy disc and the first minimum is equal to

$$\alpha=1.22\lambda/d = 0.00018 \text{ radians}$$

For a distance pupil-retina of 2 cm, an angle of 0.00018 radians corresponds to a distance of approximately 3.7 microns on the retina. This distance is equal to the diameter of the Airy disc at half peak intensity. In brighter light, with a pupil of 2 mm, the Airy disc is twice as large (diameter of Airy disc about 7 microns).

Rayleigh's criterion states that for two points to be resolved, the center of the second Airy disc must not be closer than the first minimum of the first Airy disc (in other words, the center to center distance of the two Airy discs must be equal or greater than the diameter

of the largest Airy disc). Therefore, two points can be resolved by the human eye if the distance between the center of their images on the retina is greater than ~4 microns.

The Nyquist sampling theorem states that for a periodic signal to be resolved, it must be sampled at twice the frequency. Intuitively, it seems reasonable to think that for two points to be resolved by the human eye, their images (Airy discs) must fall on two non-adjacent photoreceptor cells on the retina. If the images fall on adjacent receptors, it is not possible to know if there is one, or two objects. The size of a photoreceptor cell is about 2-3 microns. The distance between two non-adjacent photoreceptors, therefore, is about 6 microns, a distance greater than the maximum resolution of the eye. The eye, therefore, roughly satisfies the Nyquist criterion for sampling, and has matched the size of its photoreceptors to its resolving power!!

What is the maximum resolution of the eye?

As seen above, the angular resolution of the eye, as limited by diffraction (and by the size of the photoreceptors), is about 0.00018 radians, or about 1/100 degree. At the closest focusing distance of the eye (~250 mm), this angle corresponds to a maximum lateral resolution of D= 0.00018x250= 0.045 mm, or about 1/20 mm. This number approximately matches the actual resolution of the eye as determined experimentally.

Chapter 4. Microscope Design.

There are two types of compound microscopes, upright and inverted. They function according to the same principles.

Optical components:

Illuminator:

Contains the light source for transmitted light (generally a tungsten filament incandescence lamp), a reflector and a collector lens to maximize light collection, a field stop (diaphragm) to define the illuminated area. May also include heat filters, color balancing filters, diffusers and neutral density filters for light intensity attenuation.

Condenser:

Used to provide bright and even illumination onto the sample. Condenser is equipped with a condenser diaphragm that controls resolution and contrast of the image (see Koehler illumination). One should never use the condenser diaphragm to adjust light intensity. In transmitted light, the Numerical Aperture of the condenser contributes to the resolution of the microscope.

Specimen stage:

Holds and can be used to orient the specimen. Stages can be motorized (x,y scanning stages, and z-scanning stages).

Objective:

Collects light from the sample, and accounts for the actual resolution and magnification of the microscope. In older microscopes, objective forms an intermediate image in the microscope tube. In modern "infinity" systems, the objective projects an image at infinity (i.e. does not form an image inside the microscope tube). Rather, a tube lens must be used to form the primary image. The Numerical Aperture describes the light-gathering ability of an objective, and determines objective resolution. Immersion media (such as water, glycerol, or oil) are used to increase the Numerical Aperture of high-performance objectives.

Binocular head with eyepieces:

The eyepiece provides additional magnification that makes small detail visible to the eye.

Accessory imaging devices:

Digital cameras, video cameras, and standard photographic cameras can be used to record microscopy images.

Optional components:

DIC (polarizer/Condenser prism/Objective prism/analyzer)

Phase contrast (Phase Contrast objective/Phase ring in condenser)

Fluorescence (Arc lamp/fluorescence filter sets)

Definitions:

Parfocal distance: distance between sample and base of objective nosepiece. Modern systems should be parfocal, i.e. once an objective is focused, all objectives should be focused.

Free working distance: distance between the objective and the coverslip, when the objective is focused on the closest part of the object (i.e. just beneath the coverslip).

Image formation in the light microscope.

There are two sets of conjugate planes that define an image forming path and a pupil beam path.

The image forming path contains four conjugate planes: the illumination field diaphragm, the sample or object plane, the intermediate image plane, and the final image plane (surface of retina or imaging device). These four planes are conjugate. This means that when you look through the eyepiece, any object you see can be in any of those four planes (although generally you hope that you only see the object. Any extraneous object, however, could be present on the glass plate at the illumination field stop, your sample, a glass surface in the eyepiece near the intermediate image plane, or even your retina).

These conjugate planes make it possible to calibrate the magnification of a microscope by simultaneously observing a stage micrometer (in the object plane) and an eyepiece reticle (in the intermediate image plane). The first steps of Koehler illumination are designed to

bring the field illumination stop and the object into conjugate planes (that is into the same plane of focus).

The Pupil beam path also contains four conjugate planes. These are the lamp filament, the condenser aperture diaphragm, the objective back focal plane (back aperture) and the iris diaphragm of the eye. These planes control the amount of light that forms the final image. During normal microscope operation, these planes are not directly visible (they are maximally de-focused). These planes, principally the condenser aperture and objective back focal plane, control the resolution of the image (and also the contrast). The second part of Koehler illumination is designed to adjust these planes to obtain optimal resolution and contrast.

Koehler Illumination.

Koehler illumination is a method for providing optimal illumination of the sample (i.e. bright and uniform). Koehler illumination is also required to ensure optimal image formation, and to obtain the highest resolution allowed by the microscope components.

The operations performed to achieve Koehler illumination are designed to align the microscope optical paths (illumination and pupil paths), and to properly adjust the two sets of conjugate planes. The basic procedure is summarized below for transmitted light with an internal light source.

I. Adjust the illumination.

Turn ON the illumination. Fully open the field diaphragm (the opening through which light comes out of the illumination module). Fully open the condenser aperture diaphragm. If a frosted glass (diffuser) is present in the light path, remove it.

Place a white paper sheet on top of the field illumination aperture. Make sure the illumination is bright and centered. If not, center the lamp using centering screws.

II. Align the condenser and field diaphragm.

Bring a low power objective (i.e. 10x) into the light path by rotating the nosepiece. Place a specimen on the stage and focus it carefully by using the coarse and fine focus dials. It is best to bring the specimen very close to the objective (but not touching) while looking at both, and then focus by moving the specimen away from the objective while looking through the eyepiece)

On binocular microscopes, adjust the eyepieces at this stage to have best focus on both eyes with diopter correction ring. Also, adjust the interpupillary distance.

Center and focus the field diaphragm: Slowly close the field diaphragm to its smallest setting. The field diaphragm may be way out of focus. Focus the edges by moving the condenser up or down. Center the field diaphragm by moving the centering screws on the condenser. Both operations may have to be carried alternatively until the field diaphragm is maximally closed, centered, and focused. Color fringes at the edges of the field diaphragm indicate poor chromatic correction of the condenser. When done, open the field diaphragm until it just contains the field of view (i.e. until it just disappears out of the field of view).

III. Adjust the condenser aperture diaphragm.

This step requires viewing the back aperture of the objective. On microscopes equipped with a Bertrand lens, this is achieved by engaging the Bertrand lens and viewing normally through the eyepiece. On other microscopes, one needs to remove one of the eyepieces and to look down the microscope tube. An eyepiece telescope may help focus on the objective back aperture,

As you move the condenser aperture lever, you will see the outline of the condenser aperture in the back focal plane (aperture) of the objective. Adjust the condenser aperture so that approximately 75% of the objective back aperture is illuminated. This is an empirical optimal setting. Opening the condenser aperture more will slightly increase resolution, but reduce contrast. Closing the condenser aperture will increase contrast but reduce resolution.

Replace the eyepiece, or disengage the Bertrand lens. Your microscope is now properly adjusted for Koehler illumination.

When a new objective is used, it is important to readjust the field aperture to the size of the new field of view, and to readjust the condenser aperture to fill ~75% of the objective back aperture. These two steps only take a few seconds. Note that Koehler can not be performed with low power objectives (e.g. 5x or less). In this case, adjust for Koehler with 10x before using 5x. If condenser has a swing-out element, flip it out to reduce the Numerical Aperture of the condenser and allow full illumination of the field of view for low-power, low-NA objectives.

Note: if the image of the condenser aperture is not centered inside the objective back aperture, the condenser or objective needs realignment. Follow instructions in the microscope manual, or consult staff.

Critical (Nelsonian) illumination.

In some types of microscopes (generally older microscopes with external light source), a different type of illumination is used. In critical illumination, a homogeneous light source (such as a candle with a diffuser screen, or a large frosted light bulb) is focused (or just barely defocused) directly on the sample plane. This simpler and cheaper method provides reasonably bright and moderately even illumination depending on the light source, without losing too much resolution. It is nowadays mainly used for low-end, or older microscopes.

Chapter 5. Resolution.

In light microscopy, resolution is limited by lens aberrations, and by the laws of physics (mainly diffraction). Lens aberrations can be corrected to a large degree. However, diffraction imposes strict limits to the resolving power of the microscope.

Major lens aberrations :

•Chromatic (axial and lateral) (corrected in Achromatic, Apochromatic, Fluar or Fluorite objectives)

•Spherical (also corrected in the lens types above)

•Field curvature (corrected in Plan or Planatic objectives)

•Coma, Astigmatism, Barrel and Pincushion Distortion (Corrected in any "decent" lens).

Resolution is limited by diffraction.

The two-dimensional image of a point light source in a light microscope is an Airy disc.

As seen in earlier chapters, the size of the Airy disc generated by a pinhole is proportional to wavelength and inversely proportional to size of pinhole: $\alpha=1.22\ \lambda/d$ (for first minimum, where α is the angle between the center and the first minimum of the Airy disc). Similarly, the image of a point light source in microscopy is an Airy disc.

Definition:

The Numerical Aperture (NA) of an objective is equal to $n \sin\alpha$, where n is the refractive index of the imaging medium (Typically air, oil, water, or glycerol), and α is the angle of the cone of light gathered by the objective (or rather half that angle). Since $\sin \alpha$ can be 1 at most, maximum NA in air (n=1) is 1, and max NA in oil (n=1.5) is 1.5.

An objective forms an image of small objects by collecting light rays diffracted by those objects. Therefore, similar laws to those that describe the diffraction of light by a pinhole will apply to image formation in the microscope. As a result, formulas that describe the resolution of a microscope are very similar to those that describe the dimensions of an Airy disc formed by a pinhole.

In the late 1800's, Ernst Abbe, working in the Carl Zeiss factory, developed a modern theory of image formation in the microscope based on diffraction.
One of his important formulas states that for self-luminous objects (e.g. fluorescent beads, or samples illuminated by plane (non-focused) light, the lateral resolution of a lens, d, only depends on the wavelength of light and the NA of the objective:

$d = 1.22\ \lambda/2xNAobj$

When the sample is illuminated with a properly adjusted condenser of Numerical Aperture N.A.cond, resolution is given by:

$d = 1.22\ \lambda\ /N.A.cond + N.A.obj$

The two formulas above allow us to calculate the size of the Airy disc generated by a tiny object in epifluorescence and transmitted light, respectively. This is the same as the resolution, since for two points to be resolved, their Airy discs must not overlap, as seen above. Note that sometimes the 1.22 factor is rounded to 1, and 0.61 to 0.5 depending on various illumination and imaging parameters.

In confocal microscopy, illumination of the sample is restricted to a focused point of light generated by the objective, and the lateral resolution is generally taken to be:

$d = 0.5\ \lambda\ /N.A.obj$

Axial (longitudinal) resolution.

The axial resolution of a microscope, Zmin (also called depth of field), is also primarily determined by the Numerical Aperture of the Objective (and condenser) and the wavelength of light. The calculation of the axial resolution is rather complex. Zmin can be approximated with the formula:

$Zmin = n\ \lambda\ /\ (N.A.obj)^2$

Where n is the refractive index of the medium between objective and coverslip. Depending on the optical set-up and illumination, Zmin could be twice as much as the formula above.

It is important to note that the axial resolution is generally about half as good as the lateral resolution, at best. Furthermore, since lateral resolution varies with the NA, while axial resolution varies with the square of the NA, they do not vary proportionally. Similarly, the NA of the objective will have a much greater impact on axial resolution. For instance, an NA of 1.4 will give 40% better lateral resolution than NA of 1, but about

twice as good axial resolution. These considerations are particularly important for confocal and deconvolution microscopy, where obtaining 3-D images is critical. Knowing the axial and lateral resolution values for your favorite lens is important for determining optimal sampling, and getting the best images and 3-D reconstructions.

A few notes about objectives:

•There is (approximately) an inverse relationship between NA and Working Distance

• There is generally a loose direct correlation between magnification and NA

• The brightness of an objective is proportional to the square of the NA. (Actually, in epifluorescence, brightness varies with the fourth power of NA, because light goes through the objective twice). This means that an objective of NA=1.4 is almost twice as bright than an objective with NA=1. In fluorescence, the ratio is almost four!!! Therefore, always chose the highest possible NA, especially in fluorescence where light is limiting (in addition, higher NA gives better resolution too!)

• The brightness B of an objective is inversely proportional to the square of the magnification M.

In transmitted light:

$B \sim NA^2/M^2$

In epifluorescence:

$B \sim NA^4/M^2$

Therefore, especially in fluorescence, always chose the highest possible NA and lowest possible magnification.

A final note:

Most objectives are designed to work with a very specific type of coverslip. The standard is # 1.5, or 0.17 mm coverslips. Some objectives are designed to work without coverslip, and a few objectives do not care (mainly objectives with very long working distance or very low NA.

Also, objectives are designed to work with specific immersion media (generally air, oil, water or glycerol). It is really important to use the right coverslip and the right immersion medium (or absence thereof), to obtain optimal results. Using the wrong coverslip or

immersiom medium will result in loss of resolution, loss of image brightness, and image degradation due to spherical aberration. A wrong coverslip (value: ~ 1 cent) can totally waste the extra resolution and image quality obtained by a $ 10,000 objective!!

Chapter 6. Contrasting Methods in Transmitted Light.

The human eye perceives differences in intensity (brightness) and wavelength (color). It does not perceive differences in polarization or phase. Therefore, for us to perceive an object, either its intensity (brightness) or color must be different from that of the background. The relative difference in intensity of two objects is called contrast.

6.1. Contrast

If Io is the intensity of an object and Ib the intensity of the background, then contrast C is defined as C=(Io-Ib)/Ib. or ΔI/Ib

The contrast threshold necessary to see an object depends on many factors, including the size of the object and the average illumination intensity. In bright light, this threshold may be as little as 5%, while in dim light, as much as a factor of 2-5 may be required. In microscopy, a factor of 0.2 or 20% is a reasonable estimate. This is the value that was used to establish Rayleigh's criterion for resolution (two Airy discs can be resolved when the Maximum of the first Airy disc overlaps the first minimum of the second; under these conditions, the intensity of the valley between the two peaks is about 80% of the max peak intensity, and can be perceived by the eye).

Biological samples, in general, are relatively transparent, therefore providing little natural contrast. As a result, various contrast-enhancing methods have been developed for light microscopy.

Generally, our eye is more sensitive to differences in wavelength (hue or color) than to differences in intensity. This property is used in the choice of staining methods for microscopy samples, or the choice of look-up tables (pseudocoloring) to detect small gradients in intensity in microscopy images.

6.2 Staining

The most common method to enhance contrast based on differences in intensity is the histological staining of biological samples. Histological stains function by binding to specific biological structures and differentially absorbing light, generally in a small part of the visible spectrum, therefore enhancing contrast by amplifying differences in intensity, or color.

6.3. Dark Field.

Small biological objects such as those studied in microscopy are basically transparent. Therefore, most of the light that illuminates them goes through unperturbed and generates a bright background. A small fraction of the light is diffracted, and will generate the actual image of the object. Because the fraction of the light that is diffracted is very small (e.g. $<5\%$), contrast of the object will be very poor. The Dark Field technique enhances contrast by eliminating most of the non-diffracted light (i.e. the light that goes through the specimen unperturbed), and therefore generating a dark background. Only diffracted light is collected, providing a high-contrast image.

Dark Field illumination uses a normal objective with low N.A., and requires a Dark field Annulus in the condenser, to illuminate the sample with a hollow cone of light. It also requires that $N.A._{cond} \geq N.A._{obj}$, so that the illuminating cone of light is wider than the N.A. of the objective, and therefore is not collected. (certain objectives have a variable aperture that can be stopped down for dark field)

6.4. Phase contrast.

Changes in the refractive index of the sample induce shifts in the phase of the light waves that traverse it. Those shifts can be amplified by interference with the unmodified waves that have been attenuated.

Phase contrast requires a phase ring in the condenser to illuminate the sample with a hollow cone of light, and also requires a specialized phase objective with an internal phase annulus near the back focal plane. The phase rings in the condenser and objective need to be matched and adjusted (centered). Contrary to dark field, the illuminating cone of light is collected by the objective, but its intensity is attenuated by the phase annulus in the objective. The phase of the illuminating light is also shifted by the phase annulus. When the diffracted rays that form the image meet the undiffracted rays, they will interfere and generate changes in amplitude (intensity) that our eyes can perceive. Those changes will be proportional to the degree of phase shift generated by the sample, i.e. its refractive index.

The devices used for phase contrast microscopy reduce the axial resolution of the microscope. Phase contrast is thus most effective for relatively thin samples.

6.5 Polarization contrast.

This method uses polarized light for illumination (generated by placing a polarizer in front of the light source). A second polarizer (analyzer) located past the objective and oriented perpendicular to the first polarizer (analyzer) filters the transmitted light, so that no light goes through. If an object in the sample modifies the polarization plane of the incident light, this light will pass through the analyzer and generate an image.

6.6 Differential Interference Contrast

DIC is a technique that converts differences in refractive index into differences in amplitude (intensity). DIC, in fact, detects gradients in refractive index.
Several techniques exist, including the one known as Nomarski interference contrast.

An important advantage of DIC is that it does not significantly alter resolution, and therefore can work on thicker samples. DIC also does not require special objectives.

Components of a DIC system:

DIC requires four optical components: two polarizing filters (polarizer and analyzer), and two DIC prisms (condenser prism and objective prism).

Principle: a polarized light ray is split in two components by a DIC prism, generating two perpendicularly polarized rays that travel in parallel and are separated by a small distance (generally ≤1/2 wavelength). When these two parallel rays encounter two different structures (with different refraction indices), their phase will change differentially. Another prism recombines the two rays. If the phase shift of the two rays was the same, nothing happens. If the phase shift was different for the two rays, the polarization plane of the resulting ray will be different from the original. The recombined rays then pass through an analyzer (another polarizing filter orientated perpendicularly to the illumination polarizer). Polarizer filters all but the altered rays, generating a high contrast image of the object.

Therefore, DIC converts specimen-induced differences in phase (not visible to the human eye) into differences in intensity (visible).

Note: each objective requires a matching set of prisms, one in the condenser, and one behind the objective. Some microscopes use a single, adjustable objective prism.

To perform DIC optimally requires the proper adjustment of the polarizer and analyzer (at right angles to maximally attenuate transmitted light), and also the proper alignment of matching DIC prisms. Consult microscope manual for instructions on how to properly adjust the microscope for DIC.

Part II: Advanced Topics.

Chapter 7. Fluorescence Microscopy.

Fluorescence is the "ultimate" contrast enhancing method. It is highly sensitive, highly selective (because one can label specific structures with appropriate probes), versatile (antibodies to label proteins, probes for nucleic acids, probes for a variety of cellular components, fluorescent proteins for live studies) and can potentially provide superb contrast because of self-luminous objects in dark background).

Limitation: because the amount of light emitted by a fluorescent molecule is very small, and because there may be only very few labeled molecules in a sample, highly sensitive detectors are required, e.g. photomultipliers or CCD cameras.

7.1. Principle.

A fluorescent molecule can absorb the energy carried by a photon (or more accurately by an electromagnetic wave) to displace electrons to a different energy state (excitation). Within a short period (about a nanosecond), it will release most of the stored energy in the form of another photon of longer wavelength and lower energy (emission). Depending on its specific electronic configuration, a fluorescent molecule will optimally absorb and emit at well-defined wavelengths. The absorption and emission properties of fluorescent molecules may vary depending on environmental factors (solvent, pH, etc).

Some definitions:

Stokes shift: difference between the excitation and emission maxima. Fluorophores with a large Stoke's shift (i.e. with a good separation between excitation and emission) can be imaged more optimally.

Molar extinction coefficient: The propensity of a fluorophore to absorb light

Quantum efficiency: the percentage of absorbed photons that are reemitted.

In general, the best fluorophores are those with the highest product of Molar extinction x QE.

Quenching: apparent decrease in fluorescence due to absorption of the emitted photons by surrounding molecules.

Photobleaching: temporary or permanent decrease in fluorescence due to photodamage (changes in chemical structure of the fluorescent molecule induced by exposure to light)

7.2 Configuration of a fluorescence microscope.

The main distinctive elements of a fluorescence microscope are the illumination source, and special optical filters to separate excitation and emission. Generally, highly sensitive detectors are required for the practical acquisition of fluorescence images.

Fluorescence microscopy can be performed in transmitted mode or in the reflected mode (epifluorescence). In the latter case, which is the most common in biology, the objective is used both as a condenser to focus the excitation light on the sample, and as a collector for the emitted light. This will determine the choice of objectives to those with the highest NA, and the lowest practical magnification. Good chromatic correction is also essential. It is equally possible, however, to collect the emitted light in transmission mode.

7.2.1 The light source.

For fluorescence microscopy, very intense illumination is critical. The two most common illuminators for fluorescence are high voltage, high pressure arc lamps (e.g. Mercury or Xenon), or monochromatic lasers.

Mercury arc lamps give very intense, but uneven illumination. The intensity is distributed in spikes at well-defined wavelengths. Common fluorophores have absorption spectra that match the spikes of Hg lamp illumination. Xenon arc lamps are more even, but less intense illumination sources.

Arc lamps use very high voltages, and have a limited life span (generally 200-300 hours). They need to be carefully aligned when installed. The very high voltages used (mostly during ignition) generate strong electromagnetic fields that can be damaging to nearby electronic components. It is therefore important to shield those components, or place the arc lamp and/or power supply in a remote location (and use a fiber optic for instance to pipe the light to the microscope). Alternatively, whenever possible, one should follow the rule “first ON, last OFF” for arc lamps.

Lasers provide very intense, monochromatic illumination, and are used mainly for confocal microscopy. Laser light is polarized.

7.2.2 Filters for fluorescence.

In conventional (widefield) fluorescence microscopy, specific wavelengths have to be delivered to the sample (excitation). The emitted light needs to be separated from the reflected excitation light, and specific wavelengths have to be selected for imaging. Such separation of light is achieved with fluorescence filters and other optical devices.

Fluorescence filters.

a. Excitation filters provide a barrier in front of the illumination source to select a portion of the light spectrum, typically a narrow band of wavelengths.

b. Dichroic or polychroic mirrors (beam splitters) separate excitation from emission light. These function by reflecting the excitation light, but transmitting the emission light.

c. Emission filters allow specific wavelengths to reach the detectors. Long-pass filters are designed to collect as much emitted light as possible. Band-pass filters collect less light (only a narrow band of wavelengths) but are better able to discriminate between different emitters.

In addition, attenuators such as density filters may be used to reduce the illumination intensity, or to block potentially damaging wavelengths such as UV or IR (heat filters).

In a fluorescence microscope, all three components (a, b, c) may be grouped together into a filter cube (e.g. our Nikon E800), or can be independent (e.g. Olympus DeltaVision).

Note: the ratio of intensities between excitation and emission light may be as small as 1 in a billion. Therefore, the filter set must be able to attenuate the amount of excitation light that goes through by at least that much, while transmitting emitted light as efficiently as possible.

Other devices.

Confocal microscopes use monochromatic light from various lasers to excite fluorescent molecules. Laser light is very intense, but can be attenuated by reducing laser power, by the use of density filters, or by electronic attenuators such as Acousto Optical Tuning Filters (AOTF), Acousto Optical Modulators (AOM), or Electro Optical Modulators (EOM). The advantage of such devices is that they can be switched to filter different wavelengths very quickly, allowing fast sequential imaging of different dyes. They can also be used to illuminate freely-defined regions of the sample, e.g. for photobleaching.

Monochromators.

In monochromators, white light from the illuminator (for instance a Xenon lamp) is dispersed by a prism or other device. A slit is then used to select a specific band of wavelengths for excitation, therefore eliminating the need for excitation filters.

Spectral detectors.

Emitted light can also be dispersed into its components by a prism or a diffraction grating. This approach also allows to collect specific wavelengths without the need for specific emission filters, resulting in more flexibility. As an example of spectral detection, the Leica SP series of confocal microscopes uses a dispersion prism and a movable slit to direct specific wavelengths to the photodetectors. The Zeiss META confocal microscope uses a dispersion diffraction grating to project the entire spectrum of emitted light onto an array of 32 photodetectors (META detector).

Acousto Optical Beam Splitters.

This device, introduced by Leica, functions as a programmable tunable band-pass filter. It consists of a crystal that can be modulated electronically to transmit or deflect specific wavelengths, thereby allowing much faster switching between different channels.

7.2.3. Special considerations.

• Chose the best fluorophores:
    match fluorophores to existing filter sets
    chose fluorophores with high extinction coefficient and Q.E.
    use fluorophores with high resistance to photobleaching
    use well-separated fluors for multiple labelings (excitation and/or emission)

• Use attenuation of the excitation light when possible to reduce bleaching. Block the illumination beam when not necessary. Use field stop to minimize the illuminated area.

• When possible, use band pass filters for best separation, long pass filters for best sensitivity.

• Use anti fading agents. Different anti fading agents work best with specific dyes, so it may be necessary to tes various combinations to find the ones that work best. Classic anti fade agents include p-phenylene-diamine (best for FITC; OK for Rhodamine), DABCO (FITC), n-propylgallate (Rhodamine), 2-mercaptoethyleneamine (DNA stains such as propidium Iodide, acridine orange, chromomycin A3). In addition, a variety of commercial products is also available.

Chapter 8. Electronic Imaging.

One very important aspect of modern microscopy is the ability to collect digital images. Digital imaging devices provide much enhanced sensitivity compared to standard photographic film (100x-1000x), and offer the convenience of generating digital images that can be used for further processing, quantitative analysis, and display on a variety of media.

The main types of imaging devices used in microscopy are Charge Coupled Devices (CCDs) and Video Cameras for digital widefield microscopy, and photomultiplier tubes (PMTs) for confocal microscopy. CCD cameras can also be used with spinning disc confocal microscopes.

8.1 The CCD

At the heart of the CCD is a silicon chip containing a two-dimensional array of picture elements (pixels). When a photon strikes a pixel, an electron jumps out of its normal location in the silicon matrix, generating an electron/electron hole pair. These electrons/electron holes are stabilized by a voltage differential and can be accumulated over time (integration), until they are read sequentially by the CCD electronics and associated PC.

Compared to photographic film, CCDs offer similar resolution, much greater sensitivity (up to 1000 times more sensitive than standard 100 ASA film), and offer a linear response over a much greater range of light intensities. In many ways, they are the best imaging devices currently available, especially for quantitative microscopy. One major limitation of CCDs compared to video cameras and photomultipliers was their slower read-out speed. This gap is slowly being filled with video or near video rate CCD cameras.

8.1.1. Types of CCD designs.

There are three main types of CCD designs: full-frame, frame transfer, and interline transfer CCDs.

The simplest CCD are full-frame CCDs. All the pixels are used to collect an image. After image collection, the chip is read, row by row and pixel by pixel.

Frame transfer and interline CCDs can collect an image while the previous image is being read. This substantially increases the speed of the camera, but also increases the cost, because twice as many pixels are necessary. In frame transfer (e.g. the Kodak KAF series chip) half of the CCD collects an image. The image is transferred to the other half of the CCD, which is read while the first half collects a new image.
In interline CCDs (e.g. the Sony ICX series chip), every other row of pixels is used for imaging. Data from each row is transferred to the neighbor row, which is read while the imaging row collects a new image.

8.1.2 Characteristics of CCD cameras and definitions.

Pixel size: Typically in the 6-20 micron range. Larger pixels give greater sensitivity (because they can accumulate more electrons), but lower resolution.

Pixel number: Typically around 1,000,000 ("Megapixel") for scientific grade CCDs; up to about 6,000,000.

Well size: the number of electrons that can be accumulated in each pixel; generally in the 20,000 to 60,000 range.

Quantum efficiency: The percentage of photons striking the CCD that are converted to electrons/electron hole pairs (and therefore counted). Typically around 50% for scientific grade CCDs.

Spectral response: QE of a CCD as a function of Wavelength. Typically, best in the green-red part of spectrum. Different cameras have different spectral responses, and can be optimized for UV or IR.

CCD Noise: spurious electron/electron hole pairs generated in the CCD or by the electronics. Noise can be:

- thermal noise: CCDs are sensitive to light, but also to heat. Can be reduced by cooling the CCD.

- electronic noise: Readout (pre-amplifier) noise and bias noise.

- Photon noise (shot noise): statistical noise equal to the square root of the signal.

Dynamic range or Signal-to-Noise ratio: Full well capacity of a CCD divided by Noise.

Example: Full well capacity: 40,000 electrons
Noise: 10 electrons

Dynamic range: 4,000 (12 bit)

From the above, it can be seen that cooling a CCD will improve its dynamic range, by decreasing thermal noise. Collecting more photons will also increase the dynamic range, by proportionally reducing the shot (statistical) noise.

Caution: Often manufacturers use the Dynamic range to describe the full well capacity only, therefore leading to misleading (and optimistic) estimates of the performance of the CCD.

Readout rate: CCD cameras are relatively slow, and can collect images at rates of 1-30 frames per second. Readout rate depends on CCD architecture and electronics.

Binning: pooling of pixels. Increases sensitivity and readout rate. Reduces resolution and image size. For example, a CCD with 1024x1024 pixels can be used at bin=2 to generate an image with 512x512 pixels, where each pixel is the sum of four original pixels. This increases the sensitivity, and reduces image size by a factor of four. However, all other things being equal, resolution is also reduced.

8.1.3. Color CCD cameras.

CCD cameras, by default, are monochrome (they just count photons). Color CCD cameras, however, can be manufactured. These use one of a few types of designs:

• Standard monochrome CCD with external filter wheel. A filter wheel with Red, Green and Blue filters is used to take three sequential exposures, therefore generating a color RGB image with appropriate software. Preserves the resolution of the camera, but slows down the imaging procedure.

• Three-chip design: Light is split by a prism or other device into three beams. Each beam is directed to a separate CCD chip (or portion of a CCD chip) masked by a color filter. Preserves resolution and speed of acquisition. Increases cost because of the need for three chips (or a larger chip).

• Color mask. A mask with pixel sized color filters (Bayer mask) is placed in front of the chip. Fixed masks generate a color image that is a mosaic with each pixel collecting only one color (similar to laptop screen) where the actual resolution for each color has been reduced (faster and cheaper, but not too good). Alternatively, the mask can be moved to collect all color channels on each pixel (still cheap because uses only a single chip; and preserves full resolution, but slower).

8.1.4. CCD cameras and microscope resolution.

• Nyquist theorem of sampling:

The Nyquist rule states that to sample a signal of a given frequency (or resolution), the imaging device must have 2-3 times the resolution of the signal (this principle was already used to establish the resolution of the eye)

How does this apply to microscopy?

The resolution of an objective is equal to the diameter of the Airy disc of a point source imaged by that objective (Abbe's theory and Rayleigh's criterion). For conventional fluorescence, that size is given (approximately) by the rule:

$d = 0.61\ \lambda / N.A._{obj}$

For an objective of N.A. = 1.4, at 600nm, d= 0.26 microns.

If the objective has a magnification of 100x, the size of the Airy disc, as it is projected on the surface of the CCD camera, will be 0.26 x 100 = 26 microns.

To take full advantage of the resolution of the objective, the pixel size of the CCD has to be 13 microns or smaller (Nyquist rule).

Since the size of the Airy disc at the CCD depends both on the N.A. and magnification of the objective, it will vary for each objective. On the other hand, pixel size on a CCD has a fixed value. Images produced by certain objectives will be over sampled, while others will be under sampled. Scientific grade CCD cameras often come with pixel sizes of about 6-8 microns. They are often best matched for the high N.A. 60x/1.4 objective (image size of the Airy disc at 600 nm = 16 µm). In many cases, special optics are incorporated to match objective resolution and camera pixel size. Generally, the 100x/1.3-1.4 N.A. objective will be over sampled, due to the larger image size. In this case, binning may be used, since it will increase sensitivity, decrease image and file size, without significantly altering resolution. For low power/high N.A. objectives, the Airy disc image may be smaller. In this case, a 1.6 x tube lens may be used to magnify the image on the CCD and take advantage of the full resolution of the objective.

Knowing the pixel size of the CCD and the resolution of the objective will allow you to chose the most optimal images in terms of resolution, sensitivity and speed. When speed and photobleaching are not an issue, it may be useful to oversample (project the Airy disc

over 3-5 pixels). This will allow for images than can withstand some degree of croppind and enlargement (but will generate larger files).

8.2. The Photomultiplier Tube.

Photomultipliers or PMTs are very sensitive light detectors used principally in single-point laser scanning confocal microscopy. Contrary to CCDs, PMTs are not imaging devices. The image on a scanning confocal microscope is reconstructed computationally from the sequential reading, pixel by pixel, of light intensities over the entire object.

A PMT is an electronic tube with a thin glass or quartz window placed in front a photocathode. Photons entering this window strike the photocathode, where their energy is utilized to release electrons. These electrons are multiplied and accelerated by a series of photodynodes under high voltage, and the resulting current, which is proportional to the original number of photons striking the photocathode, is read by the PMT electronics. The quantum efficiency (number of photons entering the PMT's window that are actually counted, is ~20-40%, about 50% lower than that of a high-grade CCD camera. However, because the signal is amplified, and because the read-out noise can be kept very low, PMTs are capable of detecting signals generated by a few photons. Furthermore, PMTs do not store charge, and the read-out time is on the order of a few nanoseconds.

These properties make PMTs the detector of choice for point scanning confocal microsopy, where individual pixels have to be read very fast, and where the photon counts per pixel are extremely low (often less than 10 photons).

Chapter 9. 3-D Microscopy: Deconvolution and Confocal Microscopy.

Until recently, light microscopy has been used most effectively for the imaging of thin biological samples. Such samples may include smears, thin sections, or single cells or cell monolayers in culture. Imaging of thicker samples is difficult because of image degradation due to out-of-focus blur and spherical aberrations.

More recently, the trend has been to image live or fixed samples under conditions that preserve their three-dimensional structure. Two major developments in microscopy have made this possible:

• the existence of computer-controlled motorized microscopes that allow the serial optical sectioning of biological samples by precise focusing of the microscope (with a motorized stage or motorized objective nosepiece).

• the development of techniques for the removal of out-of-focus haze. These techniques fall in two broad categories: deconvolution widefield microscopy, and laser confocal scanning microscopy.

9.1 Three-dimensional microscopy: special considerations.

• Lateral resolution of an objective: $d = 0.61\ \lambda\ /N.A.$ (equal roughly to half the wavelength of light)

• Axial (vertical) resolution of an objective: $Z = n\ \lambda\ /N.A.^2$

where n is the refractive index of the medium between lens and object. (this is an approximation; depends on many factors).

The axial (vertical) resolution of the light microscope is about half the lateral resolution. Besides, lateral and axial resolution do not vary proportionally.

• Airy disc and Point Spread Function (PSF).

As seen earlier, in 2-D microscopy, the image of a luminous point source is an Airy disc. In 3-D microscopy, the image of a point source is called the Point Spread Function or PSF (some sort of three dimensional Airy disc). A typical PSF will be shaped like a

vertical football. As a result, realistic 3-D images of biological samples are almost impossible to obtain!!

9.2. Deconvolution.

The advent of motorized, computer-controlled microscopes has made possible the acquisition of image stacks of three-dimensional biological objects. Because biological samples are basically transparent, each individual z-section is contaminated by the out-of-focus light originating from the sections immediately above and below. This out-of-focus light leads to reduced resolution and contrast of the images.

Deconvolution is a computational approach for the removal of such out-of-focus haze. The operation is performed after the acquisition of 3-D stacks on the microscope.

Principle:

A biological object (sample) can be interpreted as being composed of a large number of sub-resolution point sources. When imaged through a microscope, each point source gives rise to a three-dimensional image, the PSF. Therefore, the final 3-D image (I) can be thought of as the convolution of the object (O) by the PSF (a 3-D array of PSFs):

$I = O*PSF$

The PSF can be estimated from the optical properties of the objective, or can be measured experimentally by imaging a sub-resolution point source, such as a 0.1 micron fluorescent bead.

Since we know the final result (image) and the PSF, it is possible to determine the actual structure (or at least an approximation) of the object by performing the reverse operation, or deconvolution.

In practice, several deconvolution techniques have been implemented. Constrained iterative deconvolution, for example, starts with a guess for the object, and tests the similarity between the actual image and the product of the guess by the PSF. After each iteration, the guess is refined, until the result is sufficiently similar to the experimental image.

Advantages and limitations of deconvolution.

Advantages:

Can be performed with a standard widefield microscope equipped with an accurate computer-controlled stepper motor and a CCD.

Completely automated image acquisition, including z-stacks and time-lapse

Uses sensitive cooled CCD cameras as detectors; advantageous for live-cell imaging with minimal photobleaching; images can be integrated on the CCD chip for long periods of time, therefore allowing the imaging of very dim signals

Limitations:

Best results obtained with relatively thin samples.

Deconvolved image is not obtained immediately, but requires further processing of the original image after acquisition.

Aberrations due to objectives or sample may generate deconvolution artifacts.

9.3. Confocal Microscopy.

Confocal microscopy uses optical methods, as opposed to computational methods, and very specialized hardware, to remove out of focus haze from thick samples. In confocal microscopy, a deblurred image is obtained directly during image acquisition.

A special chapter will be devoted to confocal microscopy.

Chapter 10. Advanced Techniques.

10.1 FRET.

Fluorescence Resonance Energy Transfer, or FRET, is a technique that allows to visualize molecular interactions between fluorescent molecules.

Principle:

Fluorescent molecules absorb photons over a wide range of wavelengths, and emit photons over a wide range of wavelengths, with the emission peak shifted towards the red part of the spectrum (longer wavelength, lower energy).

If two fluorescent molecules are present, such that the emission peak of the first overlaps the excitation peak of the second, the second molecule may absorb the photon emitted by the first molecule. For instance, CFP absorbs in the violet and emits in the blue, while YFP absorbs in the blue and emits in the green-yellow. If the two molecules are present, it is possible to excite CFP with violet light. The emitted blue light will be absorbed by YFP, which will then emit in the yellow. For this event to happen, however, the molecules have to be very close to each other (<10 nm). The technique, therefore, is useful to determine close association between two molecules (e.g. proteins), or to detect changes in conformation of proteins (e.g. the “chameleon” calcium indicator).

In practice, FRET can be measured by exciting molecule A, and detecting emission from molecule B. However, substantial overlap of the spectra may make this approach difficult to interpret, and stringent controls are needed.

Another approach is to collect images of both molecules. After control images have been acquired, molecule B is destroyed by photobleaching. This will result in an increase in fluorescence of molecule A, since the photons it emits can no longer be absorbed (quenched) by molecule B (acceptor bleaching method). This method is more robust, since it provides its own internal control.

10.2 FLIM

FRET is a difficult technique that requires careful controls. A more robust technique is called FLIM, or fluorescence lifetime imaging.

Principle:

A fluorescent molecule reemits an absorbed photon within a very small time interval after excitation. This small time interval is called the fluorescent molecule's lifetime, and is very specific for a given molecule. The lifetime can be determined by giving a brief excitation pulse and detecting the emission within a specific (very short too!) time window. During FRET, the two molecules, donor and acceptor, will generally have different lifetimes. Therefore, the FRETting of two molecules can be determined by measuring not the changes in emission intensity, but the change in lifetime of the emitted light.

FLIM is more robust than FRET, but requires additional hardware and software.

10.3 FRAP, FLIP and FCS.

Fluorescence Recovery After Photobleaching, or FRAP, is a method to analyze the trafficking of molecules inside living cells.

Principle:

A labeled molecule is distributed inside a cell or organelle. An intense illumination beam is used to bleach the molecule within a small area (or volume). Over time, labeled molecules from outside the bleached region will redistribute in the bleached area. The rate of fluorescence recovery gives an indication of the diffusion rate, or motion rate, of the molecules.

Alternatively, it is also possible after photobleaching a specific region to measure the decrease in fluorescence intensity over another region (due to the redistribution of bleached molecules). This technique is called FLIP, for Fluorescence Loss In Photobleaching.

A more robust and sophisticated technique for measuring molecular motion is Fluorescence Correlation Spectroscopy (FCS). The technique excites fluorescence in a very small volume, and counts the number of emission signals over time. Statistical analysis provides an estimate of the diffusion rates of the molecules within that volume. FCS requires specialized hardware and software.

10.4 Photoactivation and uncaging.

Photoactivation and uncaging allow to specifically track a population of fluorescent molecules over time.

Principle:

Certain fluorescent molecules may not naturally fluoresce when excited at a given wavelength, but may be induced to do so by exposure to intense illumination at a different wavelength (photoactivation). For example, photoactivatable variants of Green Fluorescent Protein have been generated. Alternatively, the fluorescence of a specific compound may be prevented by the presence of an inhibitory moiety. Exposure to intense light at a specific wavelength may cleave or inactivate the inhibitory moiety, therefore liberating the fluorescent moiety (uncaging).

Photoactivatable or caged compounds provide a more elegant alternative for FRAP experiments, since only the desired structures (population of molecules, organelles, cells) will be labeled.

Part III. Image Processing

Chapter 11. The Basics of Image Processing.

Image processing is a complex topic. This section is intended to give an overview of the basic concepts, and provide guidelines for image acquisition, manipulation, analysis and display. More advanced topics, such as volume rendering, segmentation, colocalization studies, etc, are best studied through practice. If in doubt, or for specialized tasks, always consult a manual or ask someone experienced. It is way too easy to do the wrong thing in image processing!

10.1. Digital images.

Natural images are composed of a continuous spectrum of intensities and colors. Analog imaging devices, such as photographic film and analog video cameras display such images through a wide range of tonal values that appear continuous to the human eye.

Digital imaging devices sample the continuous-tone image and reproduce it with a limited number of tonal intensities. For example, an 8-bit monochrome device will provide an image with $2^8 = 256$ levels of grey. As a result, a continuous tone 2-D image composed of an infinite number of points will be translated into a digitized image composed of a finite 2-D array of pixels, where each pixel can assume a certain value representing the sampled intensity of the digitized image.

The process of digitization therefore always results in a loss of spatial resolution (defined by the pixel size), and a loss of resolution of the signal (represented by the dynamic range of the digitized image).

A properly sampled image should record the original image without apparent loss for the human eye. In microscopy, this is achieved by using the Nyquist sampling theorem, where the pixel size should be equal to, or less than half the size of the smallest resolvable distance (half the size of the Airy disc for any given objective). Similarly, a digital image should display tonal values in a scale that appears continuous to the human eye. Images with a minimum of 8-bit resolution (256 grey levels) or 24-bit color (256 levels for each red, green and blue channels) meet this requirement.

10.2. Image file formats.

The “standard” file format for continuous tone grey or color images is TIFF (Tagged Image File Format). 8-bit TIFF files display images in 256 levels of grey. 24-bit RGB TIFF files display images in color, using a red, green and blue channel (RGB) with 256 intensity levels each. 16-bit TIFF files contain 65,536 levels of grey. Digital images generated during microscopy also often contain 12-bit (4096 levels of grey). These are generally saved in 16-bit format (with much of the intensity range of the 16-bit file not being used).

The output of many imaging systems is often a TIFF file (e.g. Leica confocal microscope). Other systems may generate proprietary file formats that consist basically of a TIFF file containing additional information (such as a file header with image acquisition and display information for a specific software). Zeiss confocal files, DeltaVision files, and Photoshop files belong to this category.

TIFF files can be fairly large, because the pixel intensity information is stored for every pixel. Compression schemes have been devised to store images in smaller file formats. One popular compressed file format is JPEG. Such file formats are convenient, and adequate for producing low or medium quality prints. However, when a TIFF file is converted to JPEG or other compressed file format, some of the original information is irretrievably lost, and the original data can no longer be recovered. Such formats are not acceptable for quantitative studies. As a matter of fact, even when converting a 16-bit TIFF file to an 8-bit format (e.g. with Photoshop), some of the original information may be lost. Therefore, always be careful with file format conversion, and always keep the original file.

Note on file size:

A bit is the smallest unit of binary information used in computer technology, and is represented by one of two values: 0 or 1. A byte is a string of 8 bits, and therefore can have one of 256 values (00000000, 00000001, 00000010, etc…). Much information in computer memory is stored in 8-bit format.

An 8-bit greyscale TIFF image needs 1 byte of memory per pixel. Therefore, a 512x512 pixel 8-bit image needs 262,144 bytes of memory, or 262Kbytes. A 1 million pixel (megapixel) image will need 1 Mbyte. A 1 megapixel 24-bit RGB TIFF image will need 3 Mbytes (i.e. 3 bytes per pixel for red, green and blue information). The same image, in JPEG format, will require substantially less memory space.

10.3 Sampling frequency.

Sampling is a necessary step to transform an analog image in a digital form. The quality of a digital image (image resolution) is determined by the number of pixels and the range of intensities of those pixels. While the number of pixels of the final image is often used

to describe image resolution (e.g. one million, or 512x512), this number more accurately describes image size. Image resolution is more accurately described as the size of the smallest resolvable object, and therefore is related to how the image was sampled on the imaging device (e.g. CCD). Image resolution therefore should be expressed in units of distance per pixel (e.g. 0.2 microns/pixel) and is generally called “pixel size” in image processing software. It is important to note that this refers to the actual size of an object in the image that spans one pixel, not to the actual physical size of the pixel in the CCD camera. While the physical size of a pixel is fixed, image “pixel size” depends on a variety of factors such as objective magnification, presence of a specific tube lens, pixel binning, physical pixel size, and geometry of the optics that link CCD camera and microscope. In confocal microscopy, where detection is done through a non-imaging device (PMT), pixel size will also depend on the scan rate and zoom factor of the confocal microscope.

While this may appear complex, the bright side is that most imaging software will display the image pixel size based on the current settings of the microscope. Users will generally only need to verify that this pixel size is appropriate and matches the resolution of the objective that is being used.

What is the optimal pixel size?

The lateral resolution of an objective (d) can be approximated by the following formula:

$d = 0.61 \lambda /NA$ (in confocal microscopy $d = 0.5 \lambda /NA$)

The Nyquist sampling criterion suggests to use a pixel size that is equal to d/2, or smaller (some would say smaller than d/2.5). Note: the same criterion should be used when collecting 3-D data, where the spacing of the z-sections should be half the axial resolution of the objective).

Example: for a 60x objective with NA= 1.4, at 550 nm, d = 0.24 microns. Pixel size should be 0.12 microns or less.

A table providing the lateral and axial resolution for the objectives of the microscopes in the imaging facility are provided at the end of this tutorial, and are displayed near the microscopes. These should help you chose he optimal settings.

Note: proper spatial sampling is necessary not only to preserve the full resolution of the objective, but also to avoid sampling artifacts (aliasing). This is even more critical when sampling periodic signals, where aliasing may generate patterns that do not exist in the original image.

How do I set the optimal pixel size?

Both the resolution of an objective, and the physical size of the pixels in a CCD camera are fixed. Therefore, how can one adjust the image pixel size?

In most widefield (conventional) microscopes, CCD camera and microscope optics (such as camera adaptor) have been chosen so that one particular type of objective is well-matched with the CCD. Often, the match is best for the 60x/NA1.4 objective. For some objectives, image pixel size will be smaller than necessary (e.g. 100x/NA1.4, which has the same resolution as the 60x/NA1.4, and generates a similar Airy disc, but projects a 66% larger image of the Airy disc on the CCD). In this case, a binning of 2 (the combination of 4 (2x20 pixels) is recommended.

In other instances, e.g. for low magnification, high numerical aperture objectives (such as 40x/NA 1.4), image pixel size will be insufficient to provide adequate sampling. The 40x/1.4 objective has the same resolution, but projects an image that is 60% the size of the image projected by the 60x lens. In this case, it may be useful to use a 1.6x tube lens to provide optimal sampling.

In the confocal microscope, scanning and zoom parameters can be used to achieve the same goals.

Note:

The procedures above are designed to provide optimal sampling of the image, and generate the smallest possible file size that fully preserves the resolution of the objective. In many cases, you may want to depart from these guidelines. For instance, when performing time-lapse microscopy on live samples, it may be preferable to sacrifice resolution in order to gain sensitivity, to achieve shorter imaging times (faster imaging and less photodamage).

On the other hand, with robust samples, you may want to use very small pixel sizes (oversampling). This will provide "higher-resolution" images than can be cropped and enlarged while preserving image quality.

10.4 Background subtraction.

Ideally, it is expected that variations in intensity in the image faithfully represent variations in intensity in the object. This condition is not always fulfilled. For instance,

the illumination may be uneven, or individual pixels may show variations in intensity due to noise. Correcting the image from these variations is called background subtraction.

Background subtraction may be performed during, or after image acquisition. The most common strategies are outlined below.

10.4.1. Background subtraction during image acquisition.

This approach requires the acquisition of a dark image (no exposure of the CCD camera to light) and the acquisition of one, or a series of, bright images where the CCD camera records the image of an even test slide exposed to light. The dark image will reveal "bad" pixels that have higher than normal dark current noise (signal in the absence of light). The bright images will record unevenness in the illumination field, and detect pixels whose response to light deviates from the average. These parameters are then recorded and applied during acquisition of subsequent images. A set of dark and bright images needs to be recorded for each set of imaging conditions (e.g. objective, wavelength, etc…).

10.4.2. Background subtraction after image acquisition.

Two approaches can be used, depending on whether control images are available or not. In the first case, a bright background image is collected as above, but is not applied automatically. After the experimental images have been acquired, background images can be subtracted (or divided) from the experimental image. Subtraction is preferred for imaging devices with a linear response, while division is preferred for detectors with a logarithmic response.

In the second approach, the background is estimated from the image itself. Different software packages use different algorithms. A robust algorithm lets the user select points across the image that are defined as background. The software then fits a curve to these points, and uses linear algebra to subtract the interpolated background from the original image.

Background subtraction, when properly applied, greatly improves image appearance without otherwise altering image values. Background subtraction is recommended for quantitative studies, and greatly facilitates operations such as thresholding or automatic feature identification (segmentation).

10.5 Noise removal.

Noise in digital images can have many sources (thermal noise, statistical or shot noise, amplifier noise, etc…). Noise can generally be reduced by averaging, or summing,

several images of the same object. This approach is often used during acquisition in confocal microscopy (Kalman averaging).

If sampling was done properly, the smallest visible objects will be represented by an Airy disc that spans several pixels. Any smaller features (usually single bright or dark pixels) that appear on the image can be safely considered to represent noise.

Such pixel noise can be effectively removed by applying a digital filter to the image. An efficient filter for pixel noise removal is the median filter. With a median filter, a small box (e.g. 3x3 pixels) is scanned across the image. Within the box, the central pixel value is replaced by the median value of all the pixels in the box. A related approach will replace the value by the mean value of the neighbors. This approach effectively removes pixels that are significantly different from their neighbors. A mean pixel generates an image that better resembles the original image, but is more sensitive to high noise. Mean and Median filters are examples of the popular Photoshop smoothing filters, and result in a small degree of image blurring. They can, however, generate substantially more pleasing images for printing or display, and may improve quantitation when judiciously applied.

Other digital filters.

A large number of digital filters have been developed that function on a principle similar to the median and mean filter. Such filters are called convolution filters and typically replace the value of one given pixel by some sort of combination of the values of the pixels in the neighborhood. Such filters can perform specific functions, such as enhancing contrast, smoothing or sharpening the image, detecting and sharpening edges, etc.

One filter of special importance is the fourier transform filter. This filter replaces a given image by its fourier transform. The fourier transform replaces a display of the spatial domain by the frequency domain. Periodic features across the entire image, for instance, will appear as a small set of spots in the fourier transform. It is then possible to apply standard filters on the fourier transform to specifically alter specific frequencies. This technique can be very powerful, and allows the removal, for instance, of scanner lines or printer dot patterns from an image, without otherwise altering the image.

10.6 Image dynamic range and histogram adjustment.

The range of pixel intensities in an image is represented in a histogram where pixel intensities are displayed on the x axis and the number of pixels with a given intensity is displayed in the y axis.

Ta take full advantage of the detector's dynamic range and collect optimal images, the collection parameters (e.g. exposure time, PMT gain, etc…) should be adjusted so that the histogram fills most of the intensity scale, but shows no cut-off at low or high intensities. If there is cut-off at either end of the histogram, pixel values outside the min and max values are clipped, and linearity between pixel value and image intensity is lost. On the other hand, if the histogram is too narrow, the dynamic range of the imaging device is not fully exploited. When choosing the imaging parameters, a representative section should be used that contains the lowest and highest pixel values one is to expect in the entire sample. If this is not possible, one should allow some margin, in order to accommodate darker or brighter pixels.

Note: when photobleaching is an issue, or when trying to maximize collection speed, one may want to sacrifice dynamic range by collecting images with lower pixel intensities. Typically, these images will have a lower signal to noise ratio and will be more grainy.

Adjusting the histogram after collection.

It is not always possible to collect a perfect image. A situation where the range of intensities in the object is greater than the dynamic range of the image will show clipping of the histogram: all the pixels below a certain threshold will be set to "0", and all the pixels above a certain threshold will be set to the max value (e.g. 255). In these situations, the image is saturated, and information about low and high intensities has been irretrievably lost (no quantitative information about those values can be obtained).

In the opposite case, the range of intensities in the object does not fill the dynamic range of the detector, and an image file with a narrow histogram is generated. In this case, the information content of the image (the dynamic range) will be reduced. Such images will show poor signal to noise and poor contrast, but are useable for quantitative studies.

The contrast of such images can be improved by readjusting the histogram to occupy the full dynamic range of the image. However, since the number of grey values in the original image is reduced, and since the software can not interpolate intensity values to fill the gaps, stretching the histogram too much will result in images that show posterization (discontinuous variations in the grey levels). The new histograms will have a spiky appearance, with many values of grey level that are not represented. However, histogram stretching may improve the image for printing or display. Keep in mind that nothing will compensate for a poor initial image. Therefore, it is essential to optimize the imaging parameters to obtain the best possible histogram.

Note about file format conversion.

Often, image files will need to be converted. For instance, a 16-bit confocal image may need to be converted to 8-bit for processing with Photoshop. It is important to understand

the concept of histogram during these conversions. Typically, a well-balanced 16-bit image will be to a satisfactory 8-bit image. However, if the histogram is very narrow (for instance when a 12-bit image from a CCD camera is stored as a 16-bit file, and therefore occupies only a narrow portion of the file's dynamic range), the entire dynamic range of the file, not the image, will be compressed into the 8-bit format. As a result, the actual data will occupy a narrow portion of the final 8-bit image, with most of the dynamic range of the image being wasted. The image may then appear mostly white (or black). Stretching contrast at this stage will partially restore the image, but may result in severe posterization. It is therefore recommended to adjust the histogram in the original file before converting it to 8-bit. Generally, it is not useful or recommended to do the opposite conversion (8-bit to 16-bit).

10.7. Deconvolution and image restoration.

These techniques are generally applied to 3-D images, but can sometimes be used to enhance 2-D images as well.

Deconvolution and image restoration describe a set of algorithms designed to "restore' image quality from the degradation imposed by out of focus information, and by imperfections in the imaging system (e.g. spherical aberration).

As seen previously, the point spread function or PSF describes the 3-D image of a light point source. The PSF is determined by the numerical aperture of the objective, the wavelength of light and the imaging medium. Typically, the PSF of a small, spherical point source, is a larger asymmetrical object, elongated along the vertical axis. The final 3-D image is the sum of all the PSFs of all the points in the object. By nature, this image will substantially depart from the original object.

Deconvolution attempts to reconstitute a realistic image of the object, through the knowledge of the PSF of the optical system. By doing so, it provides a sharper, more realistic, and more quantitative image, and improves the resolution of the image. The approach is generally used with images collected on a widefield microscope (Deconcolution microscope), but can also be used to improve confocal images. Major limitations of deconvolution are the absence of well-defined standard criteria to decide when to stop the iterative deconvolution process, and the extra time required for the process. Its benefits, however, usually outweigh its limitations, especially for widefield 3-D microscopy.

10.8. Image segmentation.

Segmentation allows the identification and isolation of specific features from a digital image. These features can then analyzed, for instance to determine their size, integrated intensity, number,etc. Segmentation is therefore an important step for quantitative image analysis.

Our eyes and brains are generally well adapted to identify features and patterns in images. This is generally not so for computers and software. Therefore, only optimal images with good contrast and low noise, will be optimal for automatic segmentation. This is why images for segmentation should be collected under optimal conditions, de-noised with appropriate filters, and background subtracted.

Typically, segmentation is done by thresholding the image, i.e. by selecting a range of intensities. For multi-channel fluorescence images, thresholding is best performed on individual channels. Color images (such as those obtained from transmitted light imaging of histological preparations) may benefit from thresholding procedures based on hue or saturation. To achieve this, the image is split into its component channels (such as red-green-blue, or hue-saturation-intensity). The channels that show the best separation of the features of interest are then selected for intensity thresholding.

More sophisticated techniques involve the use of watershed algorithms or other intensity comparison that isolates structures based on the relative intensity of these structures compared to their immediate neighborhood (as opposed to a fixed threshold). Finally, “classifiers” can be used to define both a range of intensities, dimensions, and sometimes shape, to identify features of interest.

Once a segmentation scheme has been successfully applied, most image analysis software packages will provide a list of statistics such as the feature count, size, integrated intensity, position of the center of mass, and other useful data. Such data can often be exported to a spreadsheet for further analysis. In addition, masks can be saved and combined, e.g. to perform comparisons between sets of features (such as for co-localization studies). Masks can also be used to extract features from the original image for display.

10.9. Volume rendering and display of time-lapse data.

Three-dimensional and time lapse data require special techniques to optimally display the information. For 3-D data, projections can be made to show specific sections of the sample on a single 2-D image. Volume rendering algorithms can be used to display the data as viewed from different angles. Such display can be interactive, or can be saves as a Quick Time or other movie format. Finally, stereopairs can be generated from projections

of volume rendered data. Such stereopairs will provide 3-D images when appropriately viewed (e.g. with reg/green glasses).

Time-lapse data can be displayed as a sequence of single sections or 2-D projections of multiple sections over time (i.e. as a movie). Finally, sophisticated imaging software has now the capability to generate 3-D volume renderings of individual 3-D data stacks, and to display the 3-D renderings as a time-sequence.

10.10. Image analysis software.

A list of the different software packages available in the scientific imaging shared resource, along with a brief descriptive, is given in the appendix.