

Polarized Light Microscopy

©Robert Bagnell 2012

I have included a chapter on polarized light in these notes primarily because polarized light is required for Differential Interference Contrast, so some understanding of polarized light is helpful in understanding DIC. Polarized light is a very powerful technique in its own right. My laboratory has only simple polarizing equipment thus making the demonstration of advanced polarization techniques impossible. In my notes, I try to convey the power of this method while being, myself, only a beginning user.

Polarized light responds to the molecular organization of biological samples in ways that enable a microscopist to interpret that organization. This is true even though the organizing elements are far below the resolution of the light microscope. Take a look at the Inoue PolScope movie (appendix and <http://www.molbiolcell.org>) in which the spindle fibers of a living dividing cell are shown clearly even though the cell is unstained. Shinya Inoue (1) relates an interesting story in which he tried to convince Keith Porter (the eminent electron microscopist) in the 1950's that the spindle of dividing cells was made of filaments, based on its **sign of birefringence** as indicated by polarized light. Porter kept insisting the spindle was made of membranes; that is up until the 1960's when Sabatini perfected the use of glutaraldehyde for fixing biological samples for electron microscopy. Using this fixative, Porter demonstrated that the spindle region was filled with microtubules. He even used Inoue's polarization results to back up his claim! The sign of birefringence is one of several types of information available from polarized light microscopy. This chapter is an introduction to this fascinating and powerful technique.

The Nature and Use of Polarized Light

Light originates with an electron transition from an outer to an inner orbital shell of an atom. The electron gives up energy in very discrete amounts during this transition and some of this energy is in the form of visible light. The time required for the electron transition is about 3×10^{-8} seconds. The speed of light in air is about 1×10^8 meters per second. So, you might say, a light **wavetrain** or quantum or photon is about 3 meters long. A light wavetrain has a beginning and an end. It has a **direction of propagation**, a **vibration frequency** (that is dependant on the energy released and that is represented by a discrete number of up and down transitions in the wavetrain) and a **vibration direction** or **azimuth** that is at right angles to the direction of propagation (fig 15.1). The vibration direction can be at any angle (or azimuth) around the direction of propagation (fig 15.2 left). Light that we see coming from an ordinary light source is composed of zillions of these light wavetranses at all possible vibration directions and with a range of frequencies that is characteristic of the light source atoms. This is called **natural or unpolarized light**. Light that vibrates in only one azimuth is called **plane polarized light** (fig.15.2 right).

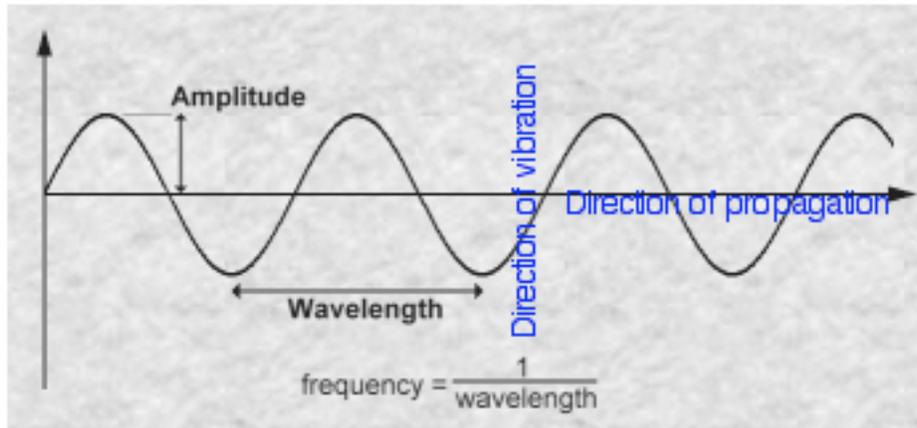


Figure 15.1 Directions of vibration and propagation of a light wave.

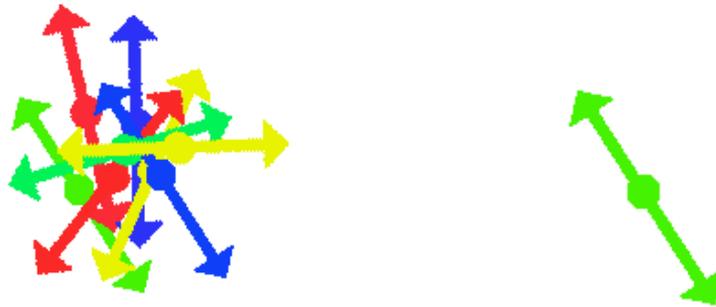


Figure 15.2 Vibration Direction of wavetrains looking into a beam of Unpolarized (left) and Plane Polarized (right) light.

(Note that the concept of a light wavetrain is different from the concept of light as a sequence of waves as waves on the surface of water. Here we approach the double conception of light as a discrete particle and light as a continuous wave. Each has its place in describing how light behaves in optical systems.)

How can unpolarized light become polarized? Light can be partially plane polarized by reflection off a smooth dielectric (i.e. non-conducting) surface such as glass or the surface of a highway. This was discovered by E.L. Malus in 1808 as he looked through a piece of quartz at light reflected off the windows of the Luxembourg Palace in Paris. As he rotated the quartz, the intensity of the reflected light diminished and was totally extinguished at certain angles of rotation. How come? Glass will reflect, i.e. re-emit, wavetrains whose vibration angle is parallel to the plane of the glass and will absorb all other wavetrains. Quartz is a **birefringent** crystal that will block all wavetrains except those that are parallel to its transmission axes. By rotating the quartz, Malus was sequentially allowing and blocking passage of the polarized light reflected from the windows. Light can be polarized by dichroism, which is the partial absorption of light in all but one azimuth of vibration (such as Polaroid sunglasses do). Finally, light can be polarized by **birefringence**. Many biological structures are birefringent and it is by measuring various aspects of this birefringence that we can learn about the molecular nature of the specimen.

Birefringent materials have the ability to create two plane polarized wavetrains of light from an unpolarized light wavetrain. The azimuths of vibration of these two polarized wavetrains are at right angles to one another so they do not interfere with one another.

Birefringence occurs in materials that show a distinct symmetry in their organization. This symmetry can be "**intrinsic**" such as the atomic arrangement of the material, as in calcite. The symmetry can be "**form**" as in a collection of aligned fibers, such as collagen. The symmetry can be "**flow**" as is created by the organization of subcellular materials during cell or chromosome movement. The symmetry can also be do to "**strain**" that is introduced into an otherwise homogeneous material, such as glass or plastic, by mechanical or thermal forces.

Birefringent materials have an "**optic axis**" which, for biological materials, can be presumed to be the axis along which the material appears to be organized, such as down the length of a group of aligned fibers or across the width of a cell membrane with its lipid bilayer structure. I say "presumed" because the actual optic axis may be otherwise, as in DNA in which the organization is across the molecule rather than down its length.

Of the two wavetrains created by birefringence, one has its azimuth of vibration perpendicular to the optic axis no matter what its direction of propagation through the material. This wave is called the "ordinary" or O ray and it travels at the same speed in the material no matter what its direction of propagation. The refractive index that it "sees" is designated n_o . The other wavetrain's azimuth of vibration is not perpendicular to the optic axis. It is called the "extraordinary" or E ray and its speed through the material varies depending on its direction of propagation. The refractive index that it sees is designated n_e . Some materials are more birefringent than others. The amount of birefringence of a material is the difference between n_e and n_o . One of the wavetrains travels more slowly through the material than the other and this could be either the O ray or the E ray depending on the material. So there develops a phase difference between the wavetrains that increases with the thickness of the material - the thicker the material the longer the waves stay in it and so the phase difference increases. This difference in phase is called the **retardance** of the material.

Let's summarize all this:

The difference in n_e and n_o is the birefringence ($B = n_e - n_o$).

Birefringence multiplied by the thickness of the material is the retardance (gamma G) also called the optical path difference (OPD).

If n_e is greater than n_o , the **sign of birefringence** is positive (+).

If n_e is smaller than n_o , the sign of birefringence is negative (-).

The sign of birefringence is a result of the atomic or molecular arrangement of the birefringent material. It can be determined using polarized light microscopy.

For collagen and the spindle apparatus, the sign of birefringence is positive. In general, the sign of birefringence is positive for rod like structures (see the DNA exception). For calcite, DNA, and cell membranes the sign of birefringence is negative. In general, the sign of birefringence is negative for plate like structures. Shinya Inoue had measured the birefringence of the spindle apparatus and found it to be positive. This is why he knew that the spindle apparatus contained filaments and not membranes, contrary to what Keith Porter originally thought.

Polarized light microscopy gives us the ability to determine the optic axis {with the specimen between crossed polars, the specimen orientation that produces extension at all rotation angles is such that the specimen is being observed parallel to its optic axis} (or we can guess at the optic axis based on the form of the material) and to determine the sign of birefringence by use of a first-order red plate {the presumed optic axis of the specimen is aligned with the slow axis of the red plate; if the specimen color becomes blue the sign is +, if it becomes yellow the sign is – Slater pg 331}. It allows us to measure the retardance by use of a compensator {the red plate is a compensator either advancing or retarding by 550 nm so by observing the color, noting that color's retardance on a Michel Levy Chart and adding or subtracting 550nm the specimen retardance can be obtained; also use of a ¼ wave plate compensator and crossed polarizer and analyzer with specimen vibration set at 45 degrees to polarizer, analyzer is rotated to position of extension of specimen, retardance is read off of analyzer – one full 360 degree turn being equivalent to one wavelength for any utilized illumination wavelength so retardance = 180 / analyzer reading X wavelength}. If the thickness (t) of the specimen is known or can be guessed at then n_e and n_o can be determined.

$$B = n_e - n_o = G / t$$

In the Inoue movie, the above calculation has been performed automatically using a computer and a specially designed Pol microscope. The resulting movie shows contrast that is a result of the instantaneous birefringence of the specimen at every pixel at every frame regardless of the orientation of the specimen. Information about the polymerization state of the actin fibrils as well as the number of fibrils at any point in time can be deduced from the movie. This movie contains a wealth of molecular information at a spatial resolution beyond the capability of a light microscope.

The Polarized Light Microscope

The simplest polarizing light microscope is one that has a polarizer between the light source and the condenser. The polarizer, by convention, is oriented so that its maximum transmission direction is “east – west”, that is, horizontally across the front of the microscope. Thus plane polarized light of known vibration direction illuminates the specimen. If a birefringent specimen is observed in this light it will be seen to increase

and decrease in contrast as it is rotated, with a maximum and minimum occurring at 90 degree intervals. If the refractive index of the mounting medium is the same as the lower refractive index of the specimen, the specimen will seem to disappear at a certain rotation angle.

The next simplest polarizing light microscope is one that has an east - west polarizer between the light source and condenser and a north – south “analyzer” between the back focal plane of the objective lens and the eyepiece. When the polarizer and analyzer are “crossed” like this, no light will be seen in the microscope in the absence of a specimen. If a birefringent specimen is placed on the microscope and rotated, it will be seen to reach a maximum intensity on a black background at some particular angles. The intensity will gradually decrease as the specimen is rotated until it completely disappears. The specimen will also show polarization colors (Newton’s colors). These colors appear because of the partial cancellation of some colors from the white light source do to destructive interference of some wavelengths as a result of a change in optical path difference of the O and E rays of those wavelengths in the birefringent parts of the specimen. This common setup of a polarizing microscope can be used to determine if polarizing materials are present in a biological specimen such as amyloid, uric acid crystals and different collagen types. If the thickness of the sample is known, the polarization colors can be used to determine the specimen’s birefringence by reference to a Michel-Leve chart showing color vs. thickness vs. birefringence.

In a more advanced polarizing microscope, an additional optical element is introduced after the specimen but before the analyzer and oriented at 45 degrees to the polarizer and analyzer. This element, called a compensator, is designed to either increase or decrease the OPD and is often calibrated so that retardance can be read off directly. Some compensators are even controlled by and read by computer. A common type of compensator is one that will cause a complete cancellation of green (550 nm) light thus giving the field of view a red tint. This is called a first-order red plate and is very helpful in increasing the visibility of weakly birefringent materials as are found in biological samples. Other modifications to the polarizing microscope include a calibrated rotating stage, objective lenses that can be individually centered on the optical axis, strain free lenses, and a depolarized light source.

Exercises

- 1) If your microscope is equipped for polarized light or for DIC, then you already have a polarizer and an analyzer. See if you can arrange the optical system so that only the polarizer and analyzer are in the optical path. Check the objective lens back focal plane for the maltese cross and adjust the orientation of the analyzer or polarizer to produce the best cross polars. Skip to 2f below.
- 2) Add polarization to your microscope:

- a) Obtain a sheet of Polaroid from a hobby store or from Edmund Scientific. Cut one piece large enough to fit over the light port and one large enough to fit over an eyepiece.
- b) Determine the vibration axis of the Polaroid by looking through it at light reflected off a shiny floor and rotating the Polaroid to the angle that gives maximum light transmission. At this angle the vibration angle of the Polaroid is horizontal. Mark this on the Polaroid. This is so because light reflected from the floor is partially plane polarized in the plane of the floor. Only wavetranses whose electric vector is horizontal are completely reemitted; all others are partially absorbed by the floor.
- c) Align the microscope for Kohler illumination. Place one sheet of Polaroid over the microscope's light port with the vibration axis going "East and West" across the front of the microscope.
- d) Place the other sheet of Polaroid over one of the microscopes eyepieces with the vibration axis going North and South. This Polaroid is referred to as the Analyzer.
- e) With a blank slide in the microscope and the light turned up bright, adjust the rotation of the Polaroid over the eyepiece to achieve the darkest field possible. This position may not be North and South because of the effect of the prisms in the binocular head.
- f) Put a birefringent specimen on the stage. Rotate the specimen around and observe what happens. A bit of Kimwipe in water is a good specimen. This shows form birefringence due to the arrangement of the cellulose in the fibers, and it will show Newton's colors.
- g) The best location for the upper polarizer (analyzer) is above the objective lens and prior to the intermediate image plane. If your microscope has a slot in this position, try making an analyzer that will work there instead of over the eyepiece.

3. Determine the sign of birefringence of an aligned fibrous material:

Place a slide of the material on your microscope between crossed polars. Note the color of the material. Insert a first order red plate and note if the material's color becomes more blue, in which case the sign is positive, or more yellow, in which case the sign is negative.

4. Measure the retardance (OPD) of the above sample:

Rotate the sample between crossed polars to get the brightest possible image. This places the fibers at 45 degrees to the polarizer and analyzer. Insert a green filter of 550 nm wavelength. Insert a $\frac{1}{4}$ wave plate. Rotate the analyzer until the image is extinguished. Read the number of degrees of rotation off the analyzer and calculate retardance as $180 / \text{analyzer reading} \times 550\text{nm}$.

(1) Inoue, Shina, Windows to dynamic fine structures, then and now. FASEB Journal Vol. 13 Supplement 1999, S185 - S190.