

Photonics-Enabled Technologies

Diagnostic Applications of Lasers



OP-TEC

Optics and Photonics Series

Diagnostic Applications of Lasers

Photonics-Enabled Technologies

OPTICS AND PHOTONICS SERIES

**OP-TEC: The National Center of Optics
and Photonics Education**

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OP-TEC 

National Center for Optics and Photonics Education

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PREFACE

This module addresses the role of optics and lasers in the field of biomedicine. OP-TEC treats biomedicine as a *photonics-enabled* technology. The current OP-TEC series on photonics-enabled technologies comprises modules in the areas of manufacturing, biomedicine, forensic science and homeland security, environmental monitoring, and optoelectronics, as listed below. (This list will expand as the OP-TEC series grows. For the most up-to-date list of OP-TEC modules, visit <http://www.op-tec.org>.)

Manufacturing

Laser Welding and Surface Treatment

Laser Material Removal: Drilling, Cutting, and Marking

Lasers in Testing and Measurement: Alignment Profiling and Position Sensing

Lasers in Testing: Interferometric Methods and Nondestructive Testing

Environmental Monitoring

Basics of Spectroscopy

Spectroscopy and Remote Sensing

Spectroscopy and Pollution Monitoring

Biomedicine

Lasers in Medicine and Surgery

Therapeutic Applications of Lasers

Diagnostic Applications of Lasers

Forensic Science and Homeland Security

Lasers in Forensic Science and Homeland Security

Infrared Systems for Homeland Security

Imaging System Performance for Homeland Security Applications

Optoelectronics

Photonics in Nanotechnology

The modules pertaining to each technology can be used collectively as a unit or separately as stand-alone items, as long as prerequisites have been met.

For students who may need assistance with or review of relevant mathematics concepts, a review and study guide entitled *Mathematics for Photonics Education* (available from CORD) is highly recommended.

The original manuscript of this module, *Diagnostic Applications of Lasers*, was prepared by Dr. Fred Seeber and Dr. Raman Kolluri. Formatting and artwork were provided by Mark Whitney and Kathy Kral (CORD).

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Diagnostic Applications of Lasers

INTRODUCTION

Since the discovery of the laser in 1959, lasers have become an indispensable part of many technologies, including surgical and other medical procedures. As laser technology has improved over the years, many new lasers have been discovered with extensive applications in the medical field. Lasers now are available with output wavelengths ranging from deep ultraviolet to far infrared. Lasers whose wavelengths coincide with the absorption wavelengths of human tissue components are used extensively in almost all areas of surgery.

New procedures are being developed around the unique characteristics of laser radiation. Pulsed lasers allow the laser beam to be manipulated and produce very short pulses with different energy densities. This allows surgeons to perform the surgery at specific places without affecting the surrounding areas. This module and the companion module *Therapeutic Applications of Lasers* provide an overview of the uses of lasers in medical and surgical diagnostic and therapeutic applications.

PREREQUISITES

The student should be familiar with the following before attempting to complete this module.

1. High school mathematics through intermediate algebra and basics of trigonometry
2. CORD's Optics and Photonics Series Course 1, *Fundamentals of Light and Lasers*
3. CORD's Optics and Photonics Series Course 2, *Elements of Photonics*

Module 2-1: *Operational Characteristics of Lasers*

Module 2-2: *Specific Laser Types*

Module 2-3: *Optical Detectors and Human Vision*

OBJECTIVES

Upon completion of this module, the student should be able to do the following:

- Understand the principles and applications of optical and laser scanning microscopes including confocal microscopy.
- Describe the interaction of light with matter and describe image characteristics.
- Understand terms such as *magnification*, *resolution*, and *contrast*.
- Explain the principle of confocal laser scanning, multiphoton, and near field microscopes.
- Understand the concepts of laser applications in optical coherence tomography, Doppler velocimetry, and medical tomography.
- Explain the basic concepts and applications of holographic imaging, ballistic photon imaging, photo acoustic imaging, and terahertz imaging.
- Describe the use of principles of photo chemical methods such as fluorescence imaging and laser Raman spectroscopy.
- Understand the use of photo biological techniques such as immuno fluorescence and optical biopsies to diagnose diseases.

SCENARIO

In high school, John Taylor always had a special interest in the biology, physics, and biomedicine-related courses he took. He also visited nearby hospitals and clinical centers as a part of a team that wanted to learn more about career programs related to medical and diagnostic applications of physics and, in particular, lasers. Upon completion of high school, John decided to enroll at a community college, where he studied various aspects of laser applications in diagnostic medicine. He was able to study the purpose, principles, and clinical applications of many kinds of lasers such as argon, krypton, and Nd:YAG. John also had hands-on training in operating and troubleshooting laser equipment. After receiving an associate degree with a specialization in medical laser applications, John was able to find many employment opportunities that offered both challenging career prospects and competitive salaries. He joined a clinic as a medical diagnostic technician where he received training on medical lasers and their applications in clinical diagnostics. John is now involved in maintaining and repairing laser equipment used in surgery and collecting data for diagnosing medical problems. These data are extremely critical to surgeons in selecting medical options for patients. He also has kept in constant touch with laser manufacturers, other clinical institutions, and community colleges to stay updated on new developments in this area. John is extremely excited to be working in medicine as a laser technician. His work is challenging, meaningful, and both financially and intellectually rewarding.

PHOTO PHYSICAL APPLICATIONS

The term *photo* relates to light. In this module we introduce techniques and instruments that use light sources such as compound microscopes, optical microscopes, and fluorescent imaging. Lasers in conjunction with microscopes are used extensively in clinical diagnostics. We will study the working principles of microscopes such as magnification, resolution, and contrast. These are extremely important in the interpretation and analysis of medical imaging data. In this module, we also introduce the techniques of fluorescent imaging and its uses in medical diagnostics.

Microscopy

A microscope is an instrument for viewing objects that are too small to be seen by the unaided eye. The science of investigating microscopic objects using this instrument is called *microscopy*. The microscope is an important tool used in scientific research and has extensive applications in the practice of medicine.

A compound microscope consists of refractive glass or plastic lenses that focus light into the eye or some other type of optical detector. Microscopes can magnify an object to approximately 1500 times its normal size. This means that objects that are as close as 0.2 micrometers can be seen distinctly as two separate objects. Specialized techniques (e.g., scanning confocal microscopy) may exceed this magnification.

Magnification, Resolution, and Contrast

Several important concepts determine how effectively a microscope operates. In this section we will address the concepts of magnification, resolution, and contrast.

Magnification

Magnification increases the dimension of the object. In a typical microscope, the magnification can range between 500 and 1500 times the actual size of the object. Another important consideration in magnification is “sensitivity.” For instance, the optical signal that comes from a fluorescence microscope can be extremely faint. Thus, any detector used with this microscope must be sensitive enough to capture this signal and process it so that the magnified object can be viewed.

For a microscope, the angular magnification is given by

$$M = M_o * M_e \quad (1)$$

where M_o is the magnification of the objective lens and M_e the magnification of the eyepiece. Figure 1 illustrates how light rays emitted by the object, O , are processed by these two lenses to produce a magnified and inverted image, I_2 .

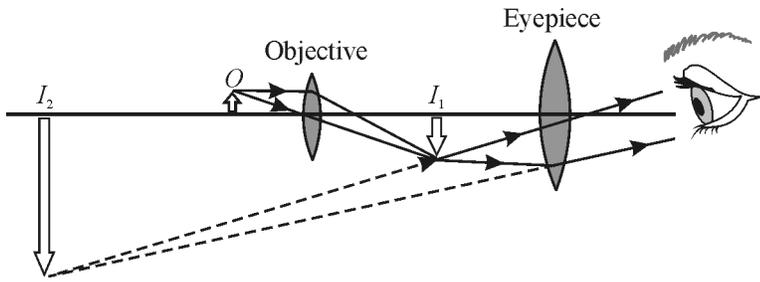


Figure 1 Magnification of a compound microscope

Resolution and limits of resolution

Resolution is the ability of an optical system (like a microscope) to distinguish, detect, and/or resolve the physical details of an object under observation. Several factors affect resolution. These include the numerical aperture of the system, an optical phenomenon called *diffraction*, and defects in lenses called *aberrations*.

Numerical aperture

The numerical aperture (NA) is a dimensionless quantity given by

$$NA = n \sin\alpha \quad (2)$$

where n is the refractive index of the imaging medium and α is the half angle of the maximum cone of light that can enter or exit the lens (see Figure 2).

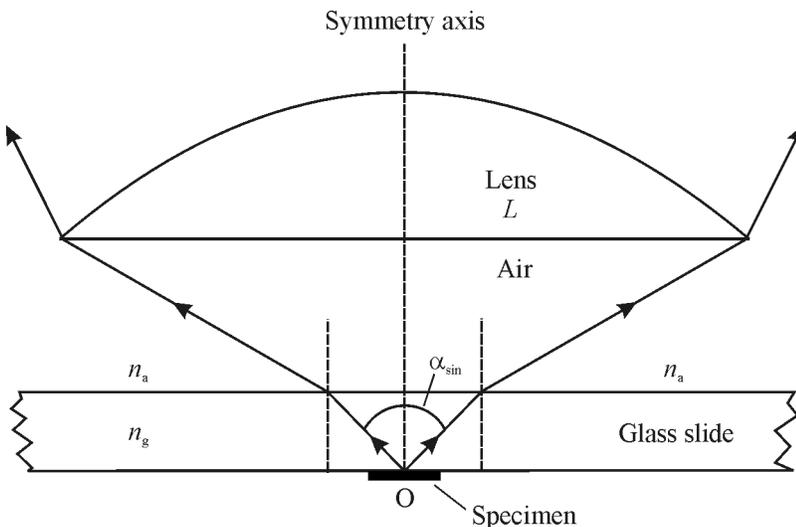


Figure 2 Microscope objective illustrating the light-gathering power of a lens

Example 1

Calculate the numerical aperture of the objective if $n=1.5$ and $\alpha=38^\circ$.

Solution

$$NA = 1.5 * \sin(38^\circ) = 0.923$$

The resolving power of a microscope is proportional to λNA , where λ is the wavelength of the light being emitted by the object under observation. An objective lens with a larger numerical aperture will be able to visualize finer details than one with a smaller numerical aperture. Lenses with larger numerical apertures also collect more light and will generally provide a brighter image.

Diffraction

A more technical description of resolution was defined by Lord Rayleigh in 1879. He demonstrated that the resolution limit of a pure optical system is determined by diffraction. The phenomenon of diffraction occurs when light passes by the edge of an aperture. The light wave is disrupted by the edge and dispersed into a multitude of angles. This effect can be seen as a softening of the edges around a shadow. As light passes through a circular aperture diffraction generates a pattern called an Airy disc (Figure 3).

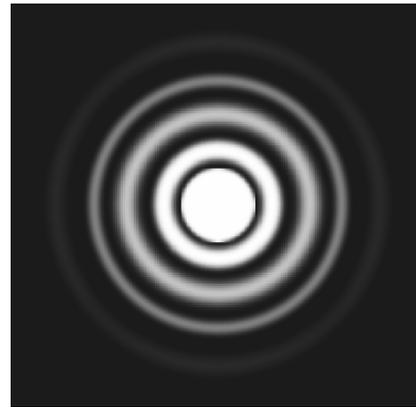


Figure 3 Airy disc diffraction pattern produced by light passing through a circular aperture

Optical systems are typically limited by a circular aperture since lenses are fabricated based on spherical surfaces. Light passing through the system undergoes diffraction and produces an Airy pattern with a disc at the center called the Airy disc. No matter how high quality or large the lens becomes, the lens can never produce a single point of light. The image will always have the characteristic ring pattern seen in Figure 3. The Airy pattern dimensions are governed by the focal length and diameter of the limiting aperture of the system. All lenses can be expressed in terms of their f -number, $F/\#$. The $F/\#$ is calculated by dividing the focal length, f , of a lens by the diameter of the limiting aperture of the system, D .

$$F/\# = \frac{f}{D} \quad (3)$$

The diameter of the Airy disc formed by a lens can be expressed as:

$$d = 2.44\lambda F/\# \quad (4)$$

where λ is the wavelength of the radiation coming from the object.

A *diffraction-limited* lens is a lens that produces an Airy pattern. Taking into consideration Rayleigh’s criterion and the fact that “real” lenses are not perfect, the diameter of the Airy disc gives a good approximation of the minimum distance two objects can be separated and still be resolved. The following equation defines this resolution:

$$\text{Resolution} = 2.44\lambda F/\# \quad (5)$$

Example 2

Given: A lens with a focal length of 25.4 mm and a limiting aperture of 50 mm used at a wavelength of 632.8 nm.

Find: The limiting resolution of this lens.

Solution

$$\text{Resolution} = 2.44\lambda F/\# = 2.44\lambda \frac{f}{D} = 2.44(632.8 \times 10^{-9}) \frac{25.4 \times 10^{-3}}{50.0 \times 10^{-3}} = 0.784 \times 10^{-6} \text{ m} = 0.784 \text{ } \mu\text{m}$$

Because of imperfections in optical systems, most systems image points of light as small circular patterns with diameters larger than the diameter of the Airy disc. This circular pattern is referred to as the *blur spot*. The blur spot radius is always greater than the Airy disc radius. Thus, Equation 5 calculates the theoretical minimum of resolution. In the real world the resolution is typically greater than this calculated value.

Aberration

As just mentioned, a blur spot is the result of imperfections in the optical design called *aberrations*. These aberrations can be caused by simple misalignments in the optics or more complicated imperfections inherent in the surface shape of the lens. The major classes of aberrations are categorized as first-order and second-order. The first-order aberrations are the result of mechanical problems in the system: tilt and defocus. The second-order aberrations are caused by the surface shape of the lens and depend on where the object is located with respect to the optical axis of the system. The second-order aberrations, also called *Seidel aberrations*, are listed below in Table 1.

Table 1. Second-Order Seidel aberrations

Seidel Aberration	Effect
Spherical	On-axis rays are focused at a different point than off-axis rays.
Coma	Rays passing through the center of lens are focused to a different plane than rays passing by the edge of the lens.
Astigmatism	Rays in the vertical axis are focused to a different point than rays in the horizontal axis.
Distortion	Rays are non-uniformly distributed on the focal plane.
Curvature	Rays are focused on a curved focal plane.
Chromatic	Rays with different wavelengths are focused to different focal points.

As aberration sources in an optical system increase, so does the diameter of the resulting blur-spot. As the size of the blur-spot increases, the performance of all imaging systems, including microscopes, degrades and their ability to resolve objects decreases.

Contrast

Contrast is defined as the relative difference in intensity or color between an object and its background. In the context of color, contrast is the ability to distinguish different colors from the background. Here, either the intensity or color must be different from that of the background. When samples are transparent, contrast-enhancing techniques are necessary to reveal details.

Since microscopes are widely used in medicine, selecting the proper one for a specific application is important. The concepts you have just studied are critical in making this selection. Magnification, resolution, and contrast are key parameters in matching a microscope to a specific medical procedure.

Fluorescence Imaging Microscopes

Introduction

The microscopes studied in the last section used light that was reflected off an object or passed directly through it. In this section, we will study microscopes that use light that radiates from the object through fluorescence.

The absorption and subsequent re-radiation of light by organic and inorganic materials depends on either *fluorescence* or *phosphorescence*. The emission of light in fluorescence is simultaneous with the absorption of the excitation light. Once the excitation light is removed, fluorescence stops. Fluorescence lasts less than a microsecond. Contrast this to phosphorescence, where light continues to be emitted long after the excitation source is removed.

Fluorescence occurs when a *fluorophore* interacts with an incident photon. (A fluorophore is a component of a molecule that causes it to be fluorescent.) Absorption of the photon causes an electron in the fluorophore to rise from its ground state to a higher energy level. When the electron returns to its ground level, a photon (fluorescence emission) is released with a wavelength that depends on the difference in energy between the ground state and the excited state of the electron. A given fluorophore may emit at a single wavelength or multiple wavelengths. Each fluorophore has a specific emission spectrum with the fluorescence emissions always having a longer wavelength than that of the excitation light.

The fluorescence emission depends on the absorption and emission characteristics of the fluorophore, its concentration in the specimen, and the optical path length of the specimen. The fluorescence produced (F) is given by the equation:

$$F = \sigma \cdot Q \cdot I \quad (6)$$

where σ is the molecular absorption cross-section, Q is the quantum yield, and I is the intensity of the incident light. For a type of fluorophore called *fluorescein*, the absorption cross-section (σ) is $3 \times 10^{-16} \text{ cm}^2$ per molecule, Q is about 0.99, and $I = 3.37 \times 10^{20} \text{ W/cm}^2$. This gives a value

of 100,000 photons per second per molecule. The efficiency and resolution of the system are determined by how many of the emitted photons can be detected and how long the emission rate continues.

Care must be taken in using fluorophores. Fluorophores lose their ability to fluoresce when they are over-illuminated. This process is called *photobleaching*. Care must be taken to prevent photobleaching through the use of chemicals or by minimizing illumination. Illuminating a fluorophore can also cause photo toxicity, which can cause the death of cells being observed.

For the fluorescence to be observed, the contrast between the specimen and background should be high. This is achieved by labeling the area of interest with a fluorophore such as GFP (green fluorescent protein). A xenon or mercury arc discharge lamp is used as the exciting source.

Most fluorescence microscopes are *epifluorescent*, that is, they direct the excitatory light onto, rather than through, the specimen. Since most of the excitatory light is absorbed and not reflected, the light returning to the optical detector is primarily composed of the fluorescent emissions emanating from the specimen. Elimination of this reflected light allows the specimen to be more accurately analyzed. Another way to reduce unwanted light is through the use of filters.

Fluorescence filters

Fluorescent imaging typically involves the collection of light from fluorophores or other fluorescent sources associated with the specimen under study. This light has a characteristic wavelength or band of wavelengths. The environment in which this imaging is done also contains similar and different wavelengths of light that come from other sources such as indoor lighting or sunlight. To ensure that fluorescent images are of the highest quality, environmental or background light must be eliminated or reduced during the imaging process. Fluorescent filters provide a means for significantly reducing background light.

There are several different types of fluorescent filters (Table 2). A laboratory in this module also provides a means for exploring how these enhance fluorescent images.

Table 2. Fluorescent filter types and functions

Filter Type	Function
Emission	Allow specific wavelengths to reach the detector
Long-pass	Allow all wavelengths to reach the detector
Band-pass	Allow only specific wavelengths to reach the detector
Dichroic	Allows a small bandwidth of wavelengths to reach the detector
Neutral density	Keeps certain bands of wavelengths, like UV or IR, from entering the detector

Another way to ensure that the maximum amount of light from a specimen is observed is to make certain that the light emanating from the specimen has a wavelength that is different from those of environmental or background sources. Some optical detectors can differentiate between wavelengths and only allow those from the specimen to be observed. One way to take advantage of this scheme is for a fluorophore to exhibit a *Stokes shift*.

Stokes shift

When a molecule or atom absorbs light, it enters an excited electronic state. The Stokes shift occurs because the molecule loses a small amount of the absorbed energy before re-emitting the rest of the energy as luminescence or fluorescence at wavelengths longer than those of the absorbed light. The amount of wavelength shift depends on the time between absorption and emission. Fluorophores with a large Stokes shift are available. Figure 4 demonstrates the Stokes shift.

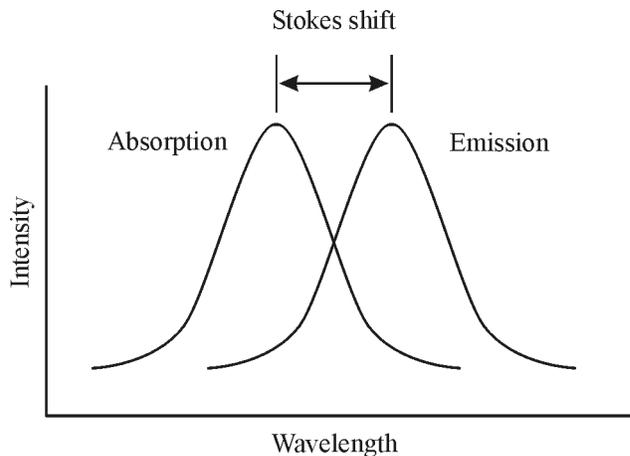


Figure 4 Schematic diagram showing Stokes shift

While elimination of unwanted environmental or background light is important, it is equally important to collect as much of the specimen light as possible. *Image brightness* is a measure of how effectively a microscope performs this task.

Image brightness

Image brightness (a dimensionless quantity) is governed by the light-gathering power of the objective. Image brightness, a function of numerical aperture and its magnification, is defined by the equation:

$$\text{Image brightness for epifluorescence} = F_{(\text{epi})} = 10^4 \frac{NA^4}{M^2} \quad (7)$$

Note that in fluorescence microscopy, brightness is inversely proportional to the objective magnification squared. Thus, for objective lenses of identical magnification, image brightness of both the illumination field and the fluorescence image increases dramatically with the objective lens numerical aperture. This is one of the primary reasons that manufacturers produce objectives designed with very high numerical apertures for fluorescence microscopy.

Diagnostic applications of fluorescence microscopes

- Identify tissue changes caused by cardiovascular diseases
- Detect the presence of atherosclerotic changes
- Detect atherosclerotic coronary artery segments

- Image tumors
- Access coronary blood flow and perfusion of lung, heart, or kidney

Ratio Fluorescence Imaging

Fluorescence microscopy has long been used for qualitative characterization of subcellular distributions of proteins, lipids, nucleic acids, and ions. However, quantifying those distributions is complicated by a variety of optical, biological and physical factors. *Ratio fluorescence* imaging is a technique for determining these distributions.

The ratio fluorescence technique can be used to identify the location of intracellular free calcium in living cells, measure the concentration of intracellular ions and pH, and monitor the changes in intracellular ions. The ion-sensitive fluorescent probes, or dyes, are useful in identifying and measuring biologically significant, nonfluorescent substances.

Fluorescent dyes that are specific to calcium, sodium, magnesium, and potassium are available. When they bind to ions, their fluorescent properties are altered. Through quantitative study of the changes in fluorescent properties, the concentration of the ion being investigated can be measured. Calcium ions are the most widely studied because of the availability of a number of fluorescence probes or indicators, such as Indo-1, Fura-2, and SBR1, that can label the Ca^{2+} ion.

Fluorescence indicators are fluorophores that have specific medical applications. Several important processes, including signal transmission in nerve cells and muscle fiber concentration, are associated specifically with calcium ion activity. These Ca^{2+} indicators are chemicals in which the fluorescent emission is wavelength-shifted by binding to a particular metabolite, in this case, calcium ions. This is shown in Figure 5.

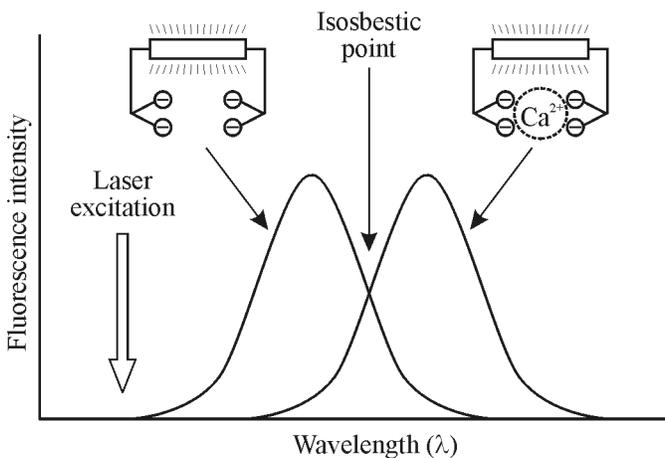


Figure 5 Presence of free calcium ions (Ca^{2+}) shifts the fluorescence-emission spectrum of a calcium indicator. At the isosbestic point, the total intensity remains constant, which is useful for ratioing or normalizing.

Changes in metabolic concentration and distribution can be studied by introducing one or more of these indicators into a living cell. A microscope is then used to acquire a sequence of fluorescent images at a single wavelength. Changes in calcium ion concentration show up as an increase or decrease in image intensity in different parts of the cell. In principle, absolute

calcium concentrations and concentration differences can be obtained by calculating the ratio of image intensities at two different wavelengths. This requires use of an ultraviolet (UV) source.

The use of fluorescence ratios for measuring ionic concentrations such as intercellular Ca^{2+} and pH are well known. The same methodology can be used to observe and measure a variety of other cellular properties such as catabolism of internalized proteins, distribution of cellular lipids, trafficking of membrane proteins, and membrane fusion events. Using these methods, it is possible to follow biochemical and ionic changes, single organelles, or small regions of the cytoplasm in living cells. The range of applications is constantly expanding as new fluorescent probes are developed and new uses of existing probes are devised.

IMAGING TECHNIQUES

Multiphoton and Near-field Microscopy

Two-photon excitation microscopy (also known as non-linear, multiphoton or two-photon laser scanning microscopy) is an alternative to confocal microscopy that provides a distinct advantage in 3D imaging. In particular, multiphoton excitation excels in the imaging of living cells such as brain slices, embryos, and whole organs.

As mentioned in the last section, the absorption of light can lead to photobleaching and phototoxicity. Two-photon excitation provides 3D optical sectioning without absorption. This technique offers greater depth of penetration than confocal microscopy.

Principle of multiphoton excitation

Two-photon excitation mechanism—Two-photon excitation takes place when a material simultaneously absorbs two photons. Since the energy of a photon is inversely proportional to its wavelength, the two absorbed photons must have a wavelength twice that required for one photon excitation. Figure 6 illustrates one- and two-photon excitation schemes in the UV and IR spectral regions.

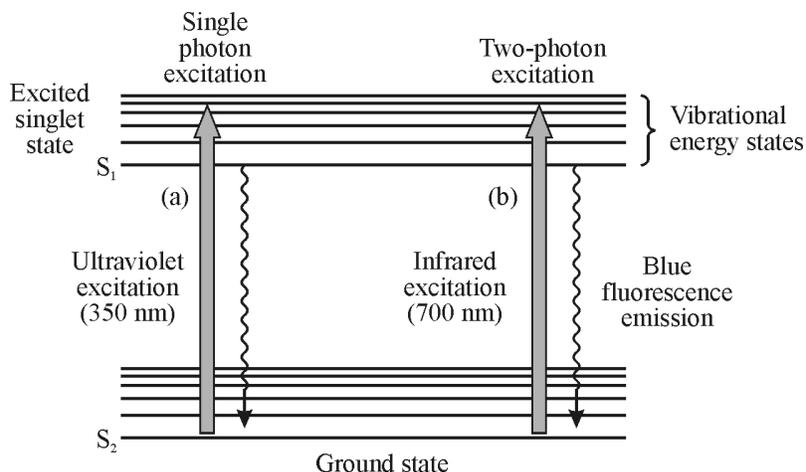


Figure 6 Schematic showing two-photon excitation mechanism

From Figure 6(a) and (b), it can be seen that a fluorophore that normally absorbs UV radiation at about 350 nm can also be excited by two photons in the near IR region (about 700 nm), provided both reach the fluorophore at the same time (within an interval of about 10^{-18} sec). In order to produce a significant number of two-photon absorption events, the flux of photons causing this absorption event must be at least a million times that required to generate the same number of one-photon absorptions. This requires a high-powered laser.

Principle of operation

Two-photon absorption is accomplished with a laser scanner using a Ti-sapphire pulsed laser with a pulse repetition rate of about 80 MHz, a pulse width of 100 femtoseconds, and output radiation in the IR region. This type of laser generates the high-photon flux required for two-photon absorption.

The most commonly used fluorophores in two-photon absorption processes have excitation spectra in the 400–500 nm range, whereas the laser used to excite the fluorophores lies in the 700–1000 nm (infrared) range. If the fluorophore absorbs two infrared photons simultaneously, it will absorb enough energy to raise it to an excited state like that shown in Figure 6. The fluorophore will then return to a lower energy level by emitting a single photon whose wavelength will depend on the type of fluorophore used.

Two-photon fluorescence requires a highly focused laser beam. This focus is accomplished using lenses that focus the laser light on a small segment of the sample. The fluorescence emanating from this segment is collected by a highly sensitive detector. This fluorescence becomes the intensity of one sample point or pixel in the overall image of the specimen. By scanning the laser over the specimen, one can assemble a detailed image of the specimen's features from the individual pixels.

Problems encountered in two-photon microscopy include the following:

- The two-photon absorption spectrum of a molecule may vary significantly from its one-photon counterpart, and wavelengths greater than 1400 nm may be significantly absorbed by the water in living tissue.
- Pulsed lasers are generally expensive.
- Special optics are required to withstand the intense pulses of the laser.

Three-photon excitation mechanism—In this case, three photons interact simultaneously with the fluorophore to produce emission. The photon flux required for three-photon excitation is only ten-fold greater than the flux needed for two-photon absorption. Three-photon excitation can be used to extend the region of useful imaging into the deep UV. For example, 720-nm radiation can be used to excite a fluorophore that normally absorbs at 240 nm. As demonstrated in the last section, the shorter the wavelength of light, the greater the resolution. So, examining the fluorescents from a fluorophore radiating in the UV region can offer more detailed imaging of the specimen.

Diagnostic applications of multiphoton excitation microscopy

Two-photon excitation reduces photo-toxicity, increases tissue imaging depth, and can initiate localized photochemistry. Areas where two- and three-photon excitation microscopy are used include the following:

- Time lapse imaging of hamster embryo development
- In-situ imaging of human skin
- Imaging the naturally occurring reduced pyridine nucleotides [NAD(P)H] as an indicator of cellular respiration
- Quantifying of glucose-stimulated insulin secretion (GSIS)
- Mapping the microcircularity in animal brain slices

Near-field scanning optical microscopy (NSOM)

Principle—In *near-field scanning optical microscopy* (NSOM), a subwavelength light source (i.e., the aperture diameter is smaller than the wavelength of light passing through it) is used as a scanning source. These sources are transmitted through a fiber optic cable that terminates in a conical tip. The specimen is attached to an X-Y positioner with nanometer movement. Using this positioner, the specimen is moved across the light source to illuminate the areas of interest. During this scan, the light source is kept at a constant distance from the specimen to ensure uniform sampling.

Illumination of a specimen with the “near-field” of a small light source enables construction of optical images with resolutions of about 50 microns. This technique is particularly useful in studying nondestructive, in-vitro, and in-vivo techniques for characterizing biological samples.

Optical Coherence Tomography (OCT)

Optical coherence tomography (OCT) is an interferometric, noninvasive technique for imaging subsurface tissue structures with ultrahigh spatial resolution. OCT is based on the measurement of reflected light from tissue discontinuities such as the epidermis-dermis junction. Depths of 1–2 mm can be imaged in turbid tissues such as skin or arteries; greater depths can be achieved in transparent tissues such as the eye. OCT is a widely accepted imaging technique in ophthalmology.

OCT complements other imaging techniques used to image subsurface tissue structures, such as ultrasound and confocal microscopy. Figure 7 shows resolution versus penetration depth of OCT compared with standard clinical imaging techniques.

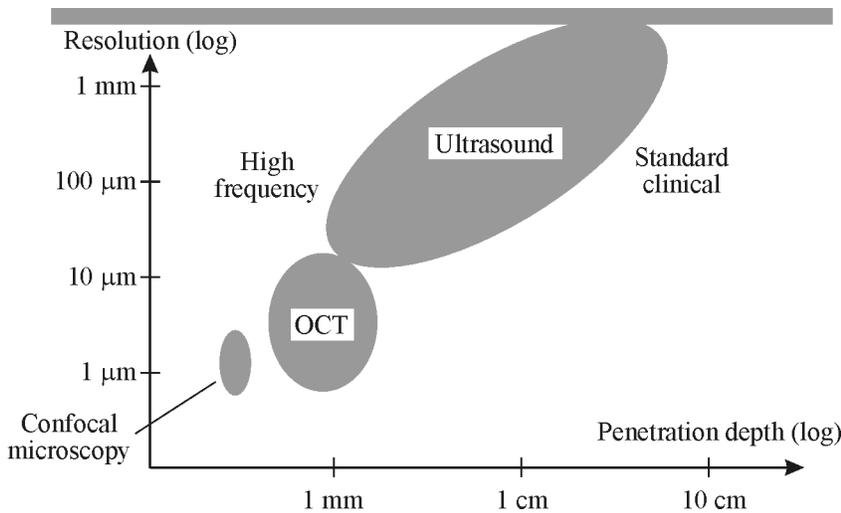


Figure 7 Comparison of OCT with standard imaging techniques (adapted from Peter Andersen, Riso National Laboratory, Denmark)

Equation 8 shows how the optical bandwidth ($\Delta\lambda$) and wavelength (λ) of an OCT probe light are related to the depth of resolution (ΔL)

$$\Delta L = \frac{0.44\lambda^2}{\Delta\lambda} \quad (8)$$

When this equation is used with typical OCT values for $\Delta\lambda$ and λ , the depth at which details on an object can be resolved falls in the range of 20–30 μm. This calculation gives insight as to where OCT can be best applied.

Diagnostic applications

OCT has been employed in a variety noninvasive, diagnostic applications. Some of them are:

- In ophthalmology, OCT is used in diagnosing and monitoring retinal diseases such as glaucoma, macular edima, macular hole, central serous chorioretinopathy, epiretinal membranes, optic disc-pits, and choroidal tumors. Using OCT, parameters such as eye length can be accurately measured. Cross-section images of the retina give a clear and quantifiable assessment of retinal separation and macular degeneration.
- In dermatology, OCT is used to diagnose skin diseases and detect skin cancer. OCT can be used to image the morphology of normal skin layers and components, and disorders such as psoriasis.
- In cardiovascular applications, OCT is used for detection of vulnerable plaque. This involves the study of how fluids, such as blood, travel through the body. Because these lesions are difficult to detect using conventional radiological techniques, OCT can be used to perform intracoronary imaging to identify high-risk *atherosclerotic plaques*. Although OCT's penetration is limited to a few millimeters, its resolution represents a 25-fold improvement over high-frequency ultrasound, MRI, and computer tomography.
- In combination with endoscopy, OCT is useful in the study of gastroenterology.

Light Scattering Spectroscopy (LSS)

Light scattering spectroscopy (LSS) is an optical technique for measuring the power spectral density of surface cellular structures and morphology. It permits in-vivo, non-invasive detection of structures in biological tissues at the intercellular level. LSS can provide quantitative measurements of parameters in real time without requiring tissue removal. This technique is widely used in the early detection of colon cancer.

Working principle—The angular and wavelength distributions of light scattered by a cell nucleus depend on its size and refractive index. Thus, if light, directly back scattered from nuclei, is observed, the size and refractive index of these nuclei can be obtained from the spectral variations. However, for biological samples, the single scattering events can be masked. The light scattered back from a tissue specimen consists of two components—one due to a single-scattering event, the other due to diffused background light. In order to study a single-scattering event, the scattered light must be distinguished from the diffusive background.

Polarized light provides a means for distinguishing scattered light from background light. This is based on the fact that linearly polarized light incident on a turbid medium such as biological tissue loses its polarization as it traverses the medium and undergoes multiple scattering events. However, light that undergoes just a single scattering event will retain its polarization. When the polarized light (single scattered) and unpolarized light (multiple scatters) reach the optical detector of the LSS, their differences in polarization will allow them to be separated. Thus the single-scattered light spectrum that contains the information needed can be analyzed without the corrupting influence of the unpolarized light. This analysis can yield the size distribution of nuclei, their population density, and their refractive index.

In LSS, the light source is an arc lamp. Light is collimated by an achromatic lens. A broad band polarizer, a narrow-band wavelength-selecting filter, beam splitter, and mirror combination are used. The spatial distribution of light emerging from the tissue surface is imaged onto an optical detector called a charged couple device (CCD) (Figure 8).

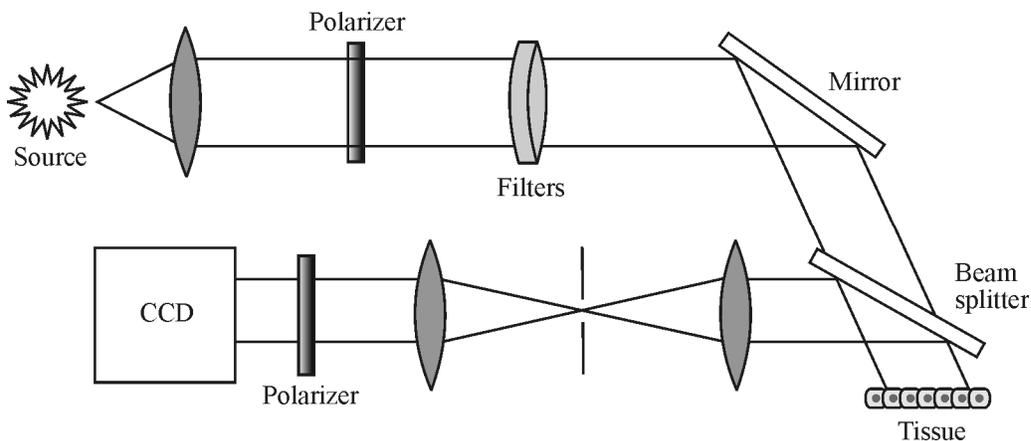


Figure 8 Schematic of a light scattering spectroscopy

Diagnostic applications

- LSS imaging can be used to detect precancerous lesions in optically accessible organs such as the colon, cervix, and oral cavity.
- LSS-based imaging provides quantitative images of increased chromatic content of cells or tissues.
- LSS can provide early detection of cervical neoplasia in-vivo.
- LSS used in conjunction with fluorescence and reflectance spectroscopy can characterize plaque.

Doppler Velocimetry

The *Doppler effect* is the change in frequency and wavelength of a wave that is perceived by an observer moving relative to the source of the wave. This effect is demonstrated by the change in frequency of the sound a train makes as it approaches a person and then moves away.

Laser Doppler velocimetry

Laser Doppler velocimetry (LDV) optically measures the velocity of a fluid without interfering with the fluid itself. The process measures the Doppler shift in wavelength of the laser radiation scattered by the moving particles. A monochromatic laser beam is directed at the target and the reflected light is collected. The change in wavelength of the reflected radiation is a function of the object's relative velocity. This velocity is given by:

$$v = \frac{f\lambda}{2 \sin \theta} \quad (9)$$

where λ is the wavelength of the laser, v is the particle velocity, θ is the beam angle, and f is the frequency of the laser light that is reflected from the moving object. By measuring this frequency, the flow velocity can be measured. LDV typically uses a He-Ne or Argon ion laser with a power of 10–20 mW.

Planar Doppler velocimetry (PDV)

Construction of a flow map using point measurement techniques such as LDV requires sequential measurements over a planar array of positions within the flow. This type of flow mapping can be performed using *planar Doppler velocimetry*. This technique determines the flow velocity by measuring the Doppler frequency shift of the light scattered by particles in the planar array. It utilizes a pulsed-injection, seeded Nd:YAG laser, one or two CCD cameras, and a molecular iodine filter. The laser illuminates a plane of the flow. The Doppler-shifted scattered light is then split into two paths using a beam splitter and imaged onto one or more cameras.

Diagnostic applications

PDV is used in many diagnostic applications. Some of them are:

- Producing accurate assessment of blood flow and the velocity of blood in an echocardiogram
- Making velocity measurements of blood flow used in obstetric ultrasonography and neurology
- Determining circulation-associated fetal hypoxemia

Medical Tomography

Tomography is a technique used to show a single plane, or slice, of an object. There are several forms of tomography. Three of the most widely used diagnostic techniques are discussed below.

Computer tomography (CT)

Computer tomography is an imaging method that uses X-rays to create cross-sectional, 2D images of the body. Images are acquired by rapid rotation of an X-ray tube 360° around the patient. The transmitted radiation is then measured by a ring of detectors surrounding the patient. The final image is generated from a reconstruction of multiple X-ray projections.

Early CT scanners acquired images one slice at a time (sequential scanning). More modern CT scanners use a spiral CT in which the X-ray tube rotates continuously in one direction while the table on which the patient is lying is moved through the X-ray beam. The transmitted radiation takes the form of a helix. Instead of being acquired one slice at a time, information is acquired as a continuous volume of slices.

Some CT scanners have multi-slice or multi-detector capabilities. They employ the spiral CT technique but incorporate multiple rows of detector rings. These machines can acquire multiple slices per tube rotation. This increases the area of the patient covered for a given time of X-ray exposure. Figure 9 shows a normal CT scan of the head.



Figure 9 *CT scan of the head showing the cerebellum, the orbits, the ethmoid sinuses, and a small portion of each temporal lobe*

Computer tomographic angiography (CTA)

In *computer tomographic angiography* (CTA), intravenous images are acquired in the arteries and reconstructed and displayed as 2D or 3D images. This technique is commonly used for imaging the aorta and renal and cerebral arteries. Contrast between different tissues of the body is improved by the use of various contrast media. These are high-molecular-weight substances that increase the opacity of the organ. CTA rapidly creates detailed pictures of the body, including the brain, chest, and abdomen. It is used in biopsies and to study blood vessels and identify masses and tumors.

Positron emission tomography (PET)

Positron emission tomography is a technique that allows measurements on distinct areas of the human brain while the patient is comfortable, conscious, and alert. While an X-ray or CT scan shows only structural details within the brain, PET gives a picture of the brain at work. To get these detailed pictures, the radiation from the PET must interact with different substances. Oxygen-15 can be used for the study of oxygen metabolism, carbon monoxide for the study of blood volume, or water for the study of blood flow in the brain. Fluorine-18 can be attached to the glucose molecule to study the brain's sugar metabolism.

Working principle—A positron is an anti-electron. Positrons are given off during the decay of specific radioisotope nuclei. When matter collides with its corresponding antimatter, both are annihilated. When a positron meets an electron, the collision produces two gamma rays having equal energy, but going in opposite directions. The gamma rays leave the patient's body and are detected by the PET scanner. The scanner provides information to a computer that creates a complex picture of the patient's working brain.

The PET scanner uses radiation emitted from the patient to develop images. Each patient is given a minute amount of a radioactive pharmaceutical that closely resembles a natural substance used by the body. One example of such a pharmaceutical is 2-fluoro-2-deoxy-D-glucose (FDG), which is similar to a naturally occurring sugar, glucose, with the addition of a radioactive fluorine atom. Gamma radiation produced from the positron-emitting fluorine is detected by the PET scanner and processed to show the metabolism of glucose in the brain.

Single-photon emission computed tomography (SPECT)

Single-photon emission computed tomography (SPECT) is a nuclear medicine tomographic imaging technique that uses gamma rays to form images. It provides true 3D information on a specimen. This technique is employed in tumor imaging, infection imaging (leukocyte), thyroid imaging, and bone imaging. It is also used to provide information about localized functions in internal organs such as functional cardiac or brain imaging.

Working principle—SPECT imaging is performed by using a gamma camera to acquire multiple 2D images (also called projections) from multiple angles. The source of the gamma radiation detected by this camera is radionuclides that are contained in radiopharmaceuticals that are ingested by the patient. These pharmaceuticals are chosen on the basis of their ability to concentrate at locations within the body that must be examined. The gamma camera produces images of the distribution of these radionuclides. These images are processed by using a tomographic reconstruction algorithm that yields a 3D data set. This data set may then be

manipulated to show thin slices along any chosen axis of the body, similar to those obtained from other tomographic techniques such as MRI, CT, and PET. Because SPECT acquisition is very similar to planar gamma camera imaging, the same radiopharmaceuticals may be used.

To acquire SPECT images, the gamma camera is rotated around the patient. Projections are acquired at defined points during the rotation, typically every 3–6 degrees. In most cases, a full 360-degree rotation is used to obtain an optimal reconstruction.

Holographic Imaging Techniques

Holography is an imaging technique that uses lasers. Holographic imaging consists of two steps. In the first step, the interference pattern from an object is recorded on a photographic plate. This creates a hologram. In the second step, the hologram is reconstructed.

X-ray holography

X-ray holography can be used for examining samples in aqueous solutions. The resulting hologram has a high resolution. To generate this hologram, the sample is illuminated with an X-ray beam. Holograms permit real-time observations, which are useful in the analysis of biological specimens. Resolutions of 1.3 μm have been obtained using holographic techniques.

Endoscopic holography

Endoscopic holography provides a tool for non-contact, high-resolution 3D imaging and nondestructive measurements inside natural cavities of the human body, or in any difficult-to-access environment. It combines features of holography and endoscopy. The ability to record 3D images with large focal depths and high resolutions allows detailed study of internal organs and tissues.

Holography in ophthalmology

Any retinal detachment or intraocular foreign body can be detected using a holographic image of the eye. Holography is used for the measurement of corneal topography and crystalline lens changes. It is also used for the study of surface characteristics of both the nerve head and the cornea. The major advantage of holographic techniques is that they provide the ultra-high precision and resolution necessary to measure the elastic expansion of the cornea.

Holography in dentistry

Holograms are used for storing orthodontic study models that can be retrieved for accurate 3D measurements. They are also used as training aids in the disciplines of dental anatomy and operative dentistry.

Ballistic Photon Imaging

When light is transmitted through a turbid medium, such as translucent biological tissues, some photons do not scatter but pass through. These are called *ballistic photons*. They follow the

shortest path through the medium. Other photons in the light beam are scattered and move through the medium on paths that take them longer to transverse it. These photons are called *snake photons*. In biological tissue, scattering-absorption interactions complicate image reconstruction. The ability to separate out the ballistic and snake photons can lead to higher-quality images.

Working principle—Ultrashort laser pulses that are used for optically scanning tissue have opened up the whole field of optical tomography. The basis for these techniques is the detection of *photons that travel ballistically* through tissue without scattering. Since they travel in a straight line, they arrive earlier at the detector than do the scattered photons. With sufficiently short pulses (around 30 ps), the ballistic photons can easily be separated from the slower, scattered ones. Ballistic photons can be used with optical tomography to scan the body in search of tumors or other abnormalities. They can also be used for spectral studies.

Advantages of ballistic photon imaging technique

In ballistic imaging, photon trajectories are easily traceable back to their points of origin. Image reconstruction is straightforward. Spatial resolution is very high, limited only by diffraction and coherence, and image quality is uniform throughout the sample.

Photoacoustic Imaging

Photoacoustic imaging works by flashing a near-IR laser at low energy on a target. The near-IR wavelength helps the light both to scatter and penetrate deep into the body. As the light is absorbed into the body, it expands the tissue through thermoelastic expansion. This expansion creates ultrasonic acoustic waves that can be picked up by very sensitive ultrasound detectors. Photoacoustic imaging is used to detect vascular disease, skin abnormalities, and some types of cancer.

Diagnostic applications

The high-resolution capability of the photoacoustic system makes it particularly well suited to clinical dermatological applications that require visualization of blood vessel networks and healing wounds. It is also useful in the study of molecular imaging in cardiovascular studies, neurology, and oncology. Hemoglobin (and its various oxygenated states) provides strong optical contrast at near-IR and visible wavelengths, making the technique well suited for imaging blood vessels.

Terahertz Imaging

Electromagnetic waves at terahertz frequencies are known as *terahertz radiation* or *terahertz waves* (also called *T-rays*, *T-lux*, *Terahertz light*, *T-light*, and *THz*). This region of the EM spectrum is between 300 gigahertz (3×10^{11} Hz) and 3 terahertz (3×10^{12} Hz). Unique properties of THz radiation allow it to “see” deeper and with more detail than imaging methods such as ultrasound, X-rays, and radar. T-rays effectively tooth cavities, image skin burns, and skin cancer.

Terahertz radiation is nonionizing and can penetrate a wide range of nonconducting materials. It can pass through cardboard, clothing, wood, paper, plastic, masonry, and ceramics. Table 3 identifies different ways terahertz radiation can be generated.

Table 3. Techniques for Generating Terahertz Radiation

	Optically pumped terahertz lasers	Time domain spectroscopy	Backward wave oscillators	Direct multiplied sources	Frequency mixing
Average power	>100 mW	-1 μ W	10 mW	Milliwatts to microwatts (decreasing w/ increasing frequency)	Tens of nanowatts
Usable range	0.3 -10 THz	-0.1-2 THz	0.1-1.5THz	0.1-1THz	0.3-10THz
Tunability	Discrete lines	N/A	200 GHz	~10 – 15% of center frequency	Continuous
Continuous wave pulsed	CW or pulsed	Pulsed	CW	CW	CW
Turnkey systems available	Yes	Yes	No	Yes	No

Clinical applications

Terahertz radiation is nonionizing; it does not damage DNA. At some frequencies, terahertz radiation can penetrate several centimeters of tissue and reflect. Terahertz radiation can also detect differences in tissue density and water content. Because of these special properties, terahertz imaging is used to detect epithelial cancer (noninvasively), take 3D pictures of teeth, and image samples that are opaque in the visible near-IR regions.

LASER APPLICATIONS

Lasers in conjunction with microscopy are used extensively in medicine and related fields. In ophthalmology, lasers adapted to slit lamps deliver laser radiation to treatment sites in the human eye. Argon, dye, krypton, and Nd:YAG lasers are used extensively in these type of procedures. As seen from this example, surgical microscopes provide a stable platform for the precise application of laser radiation.

Laser Scanning Microscopy (LSM)

Laser radiation has advantages over ordinary light because of its high directionality and monochromaticity. An additional advantage is the laser’s ability to reach very small beam diameters and extremely high powers. A highly coherent laser beam can be focused down to its diffraction limit—only a few hundred nanometers.

Laser microscopy couples a laser to a microscope in a number of configurations and imaging modes. These configurations generate microscopic images by laser scanning. The laser beam on the sample excites fluorescence, which is collected. This fluorescence is then imaged onto a

pinhole, which in a confocal geometry eliminates unwanted light that could obscure the desired image.

In this section the word *confocal* will be used several times. When *confocal* is used, it simply means that the focal point of the objective lens forms an image at the pinhole aperture. This is important because this optical arrangement increases the quality of the final image. Here is how: If the object we want to view is at the focal point of the objective lens, the vast majority of the fluorescents emanating from it will pass through the aperture. Light that comes from other objects, not located at the focal point, will be mostly blocked by the pinhole. Thus, the pinhole will pass through light from the object we want to examine and block light from objects of no interest.

LSM and *confocal LSM (CLSM)* can achieve high resolution and a high signal-to-noise ratio. They permit a wide range of quantitative measurements. These include topography mappings, extended depth focus, and 3D visualization. Some of the important LSM techniques include:

- Fluorescence microscopy, which involves the excitation of the sample at one wavelength and observation at another wavelength
- Fluorescent micro-thermal imaging (FM), which uses the fluorescent emissions from a specimen to provide high-resolution spatial mappings of temperature distributions across surfaces
- Excimer laser microscopy, which uses illumination in the deep UV to perform material removal functions through laser ablation
- Multi-photon excitation spectroscopy, which uses two or more photons of different wavelengths to interact with a material and produce excitation spectra in a specified wavelength region

Applications of LSM and CLSM

Working principle—In CLSM, a fluorescent specimen is illuminated by a point laser source. The laser has a finite beam diameter that defines the spot size that scans the specimen. This spot size establishes the limit to how small a volume can be sampled on the specimen. When the laser scans the specimen, it illuminates a sample volume and causes it to fluoresce with a discrete intensity. The detector in the imaging system captures and stores the location of the volume and its fluorescent intensity. These data are then processed by a computer to create an image of the specimen.

The resolution of a CLSM is dependent on its beam size and ability to penetrate into the specimen. These two factors are called the *lateral resolution* and *axial resolution*. The lateral resolution (Δx) for CLSM and other confocal microscopes is given by:

$$\text{Lateral resolution} = (\Delta x) = \frac{0.50\lambda}{NA_{\text{obj}}} \quad (10)$$

The axial resolution defines the thickness of the probed slice or section thickness. It is evaluated by the ratio $\frac{\lambda}{NA^2}$. This ratio is used in Equation 11, which defines the axial resolution for CLSM and other confocal microscopes.

$$\text{Axial resolution} = \Delta Z = \frac{1.4n\lambda}{NA_{\text{obj}}^2} \quad (11)$$

In this equation, n is the refractive index of the objective lens immersion system.

Example 3

Calculate the axial resolution and the lateral resolution for a CLSM with an objective numerical aperture of 1.2 for an illuminating wavelength of 1.06 μm . Assume air as the medium between the objective and the specimen.

Solution

$$\text{Axial resolution} = \Delta Z = \frac{1.4n\lambda}{NA_{\text{obj}}^2} = \frac{1.4 * 1.0 * 1.06 \mu\text{m}}{(1.2)^2} = 1.03 \mu\text{m}$$

$$\text{Lateral resolution} = (\Delta x) = \frac{0.50\lambda}{NA_{\text{obj}}} = \frac{0.50 * 1.06 \mu\text{m}}{1.2} = 0.44 \mu\text{m}$$

CLSM operation

Figure 10 shows the schematic diagram of a CLSM. A laser provides the excitation light. An objective lens focuses the beam onto the specimen, where it excites fluorescence. The fluorescent light is collected by the objective lens and is directed onto the detector through a beam-splitter. The required range of the fluorescence spectrum is selected by an emission filter that also acts as a barrier blocking the excitation laser line. A pinhole is placed in front of the detector at the focal plane of the objective lens to suppress stray light, thus enhancing image contrast.

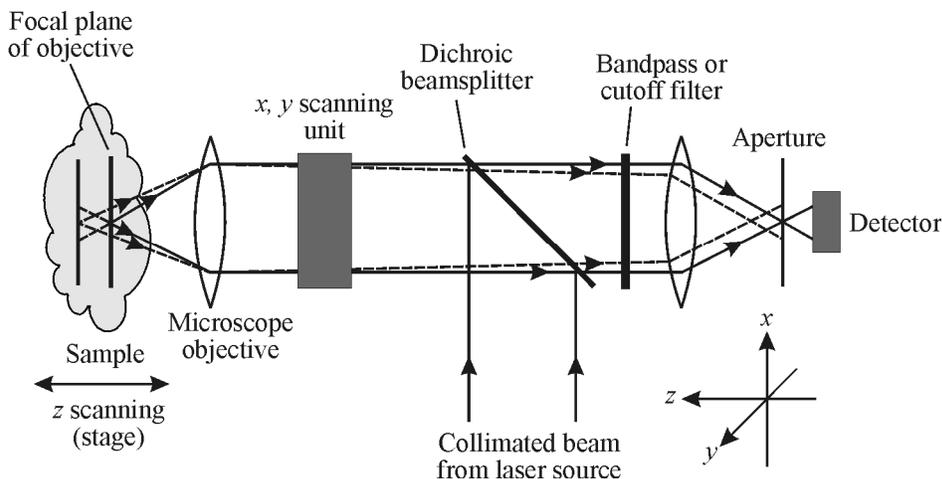


Figure 10 Schematic of a confocal laser scanning microscope

The detector is attached to a computer that builds up the image, one pixel at a time. A computer can generate a 3D image of the specimen by assembling a stack of 2D images from successive planes.

Since a CLSM depends on fluorescent emissions, the specimen often must be treated with a fluorescent dye. However, the actual dye concentration must be very low so that it does not impact the biological effect being measured.

Clinical uses of CLSM

Confocal microscopy is clinically used in the following situations:

- In evaluating various eye diseases and, in particular, imaging *endothelial cells* of the cornea
- For localizing and identifying the presence of *filamentary fungal elements* in the *corneal stroma*, which are associated with keratomycosis
- For determining tumor margins in *basal cell carcinomas*
- For identifying proliferative and inflammatory skin disorders
- For finding superficial tissue layers in the bladder and organs of living rats
- For generating freshly excised head and neck specimens
- For detecting drug delivery and distribution
- For determining the distribution of melanin and cell-to-cell interactions such as *diapedesis* and *apoptosis*

Laser Raman Spectroscopy

Raman spectroscopy is used to study rotational, vibrational, and other low-frequency modes in molecular systems. It depends on the inelastic scattering of photons by molecules that cause a frequency shift and, consequently, an energy shift of the photon. This shift provides information about the system.

Raman microspectroscopy

Raman microscopy generates very high spatial resolutions. This is because the objective lenses of the microscope focus the laser beam to a very small diameter, creating high photon fluxes. These photons cause Raman spectra to be generated in very small regions of the sample. These Raman spectra contain information for measuring the properties of cells, proteins, organs, and erythrocytes and for studying biological samples such as DNA, peptides, and protein.

Diagnostic applications

Raman microscopy is used extensively in monitoring blood gases under anesthetic conditions and observing skin cancers and other skin diseases. It is a noninvasive, nondestructive technique. It can be used to differentiate between subtle changes in tissue and to identify bio-terrorism agents such as anthrax. Determining chemical concentrations in biofluids, identifying teeth bacteria, and studying individual immune cells are other areas in where Raman microscopy is used.

PHOTO BIOLOGICAL TECHNIQUES

Immuno Fluorescence

Immunofluorescence involves the use of fluorescent dyes to label antibodies or antigens. Antibodies are Y-shaped proteins found in bodily fluids, including blood. The immune system uses antibodies to identify and neutralize foreign objects such as viruses and bacteria. An antigen is a molecule that stimulates an immune response. Immunofluorescence is often used to visualize subcellular distributions of biomolecules.

Immunofluorescent-labeled tissues are examined using fluorescence microscopes or confocal microscopes. Ultraviolet light can be used to make the antigen-antibody combination visible. Antibody identification is performed on blood (serum) in the diagnosis of conditions such as myocarditis, syphilis, and toxoplasmosis, among others.

Fluorescent labeling is the process of covalently attaching a fluorophore to another molecule, for example, a protein or nucleic acid. This is typically accomplished using a reactive derivative of the fluorophore that selectively binds to a functional group contained in the target molecule. The most commonly labeled molecules are antibodies, which are then used as probes to detect particular targets. Fluorescent labels are detected by fluorescence microscopes or flow cytometers, which can identify targets embedded within cells.

Diagnostic applications of immuno-fluorescence

Medical applications of this technique include the detection of the following:

- Colorado tick fever
- Focal segmental glomerulosclerosis
- Infectious mononucleosis (CMV)
- Infectious mononucleosis (EB)
- Lyme disease

Fiber Optic Sensors

A fiber optic sensor is a thin optical fiber that carries light. Fiber optic sensors are ideal for probing in remote or hostile locations where miniature sensors are required, such as in the body or where extreme sensitivity is required. A detailed description of fiber optic sensors is given in the module entitled *Therapeutic Applications of Lasers*.

Optical Biopsy Techniques

A biopsy is a medical test that involves the removal of tissues or cells for examination. The tissue is often examined under a microscope and can be analyzed chemically. When only a

portion of the tissue is removed, the procedure is called an *incisional biopsy* or *core biopsy*. When an entire area or lump is removed, the procedure is called an *excisional biopsy*.

An *optical biopsy* is performed by illuminating target tissue with light of a specific wavelength and then spectroscopically evaluating the light reflected or emitted by the tissue. Figure 11 shows a schematic diagram of an optical biopsy.

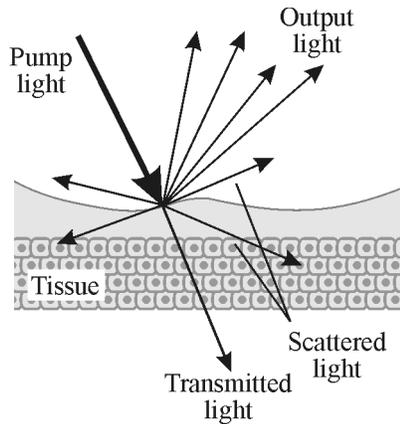


Figure 11 Schematic illustrating the mechanism of optical biopsy

In an optical biopsy, a beam of light consisting of several colors is used. Each color is sequentially sent through an optical fiber as a pulse and directed onto the test tissue. Each pulse excites certain chemicals in the tissue. The chemicals instantly respond by emitting characteristic fluorescent flashes that the cable transmits back to a measuring device.

The morphology of cancerous tissue affects light differently from normal tissue. The imaging instrument collects and analyzes scattered light and the natural fluorescence from the tissue for a number of different illumination wavelengths. (This technique is known as *hyperspectral imaging*.) It enables a detailed analysis of tissue in situ without the need for surgical excisional biopsies.

Optical biopsy technique

The optical biopsy system includes a laser, an optical fiber, and a computer that analyzes the light given off by tissue. For instance, in a bowel examination, the physician inserts the optical fiber through an instrument (sigmoidoscope or colonoscope) and points it at a growth. The growth absorbs the laser light and returns light back through the optical fiber to the computer, which analyses it. The system's computer then indicates whether or not the growth is malignant.

Benefits of optical biopsies

- Noninvasive with no tissue removal required
- Rapid measurements with real time results
- High spatial resolution to detect small tumors
- Fluorescence imaging to clearly define tumorous regions

- Has the potential to detect precancerous conditions
- Can provide differential diagnoses of cancers (bladder and breast cancer)
- Can provide a diagnosis of an internal organ's blood supply
- Can provide a diagnosis of atherosclerosis

Quantum Dots as Biological Fluorescent Markers

A *quantum dot* (QD) is a semiconductor nanostructure that restricts the movement of valence band holes, conduction band electrons, or excitons (bound pairs of conduction band electrons and valence band holes) in all three spatial directions. Quantum dots are composed of many, many atoms that behave like a huge single atom. Quantum dots have the ability to absorb incident light and emit light at a different frequency.

The material used for the quantum dot must be a semiconductor, i.e., a material whose conductivity is somewhere between that of an insulator and a conductor. A key feature of quantum dots is that the color can be adjusted to any visible wavelength by changing the dot's size. Also called *nanocrystals*, these dots are typically 2–8 nm in diameter. Unlike molecular fluorophores, which typically have very narrow excitation spectra, semiconductor nanocrystals have the ability to absorb light over a broad spectral range. For this reason, it is possible to optically excite a broad spectrum of quantum dot “colors” using a laser. This allows simultaneous probing of several markers in biosensing and assay applications.

Quantum dots have three useful characteristics:

- They can be designed to emit a specific frequency of light (specified by the user).
- They can be configured to attach to any material through the use of an outside coating.
- Their fluorescent light radiates much brighter than traditional fluorescent labels. This makes them superior markers for biological experiments.

Quantum dots are useful in a variety of diagnostic applications. One example is the development of a fluorescent immunoassay using antibody-conjugated quantum dots. Several protein toxins have been successfully detected using this system. In another example, quantum dots embedded in polymer microbeads have been used for DNA hybridization studies.

Quantum dot conjugation

Quantum dots are insoluble in water and are not easily attached to biological entities. When they are prepared, layers of organic ligands are coated onto their surfaces, making them hydrophobic. Various attempts have been made to make them water soluble by replacing these ligands with others that have hydrophilic ends, but this often degrades their optical properties. Polymer-coated quantum dots fulfill the promise of fluorescent semiconductor nanocrystals for both in-vivo and in-vitro studies. Figure 12 illustrates a biocompatible conjugate QD.

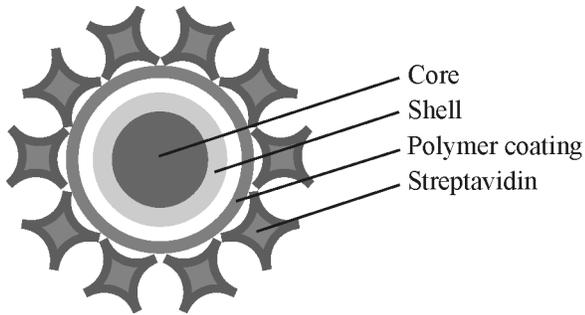


Figure 12 Schematic of a QD conjugate

Diagnostic applications

- Antibodies labeled with quantum dots are a quick and extremely efficient way to detect bovine spongiform encephalopathy, otherwise known as mad cow disease.
- Allows bio-labeling of detection reagents for microscopy, flow cytometry, and immunoassay
- Creates a platform for multiplexed assays such as proteomics, genotyping, and gene expression
- Allows live-cell and in-vivo imaging

EXERCISES

1. What is a laser microscope?
2. Define the terms *magnification*, *resolution*, and *contrast*.
3. Define the terms:
 - a. *Numerical aperture* and *diffraction*
 - b. What are the limitations of a microscope?
4. Calculate the magnification of a microscope if $M_o = 3$ and $M_e = 10$.
5.
 - a. Calculate the NA of the objective if $n = 1.33$ (water) and $\alpha = 30^\circ$.
 - b. Use answer from part a to calculate the resolution limit if the wavelength of the light used is 550 nm.
6.
 - a. What is fluorescence imaging?
 - b. List five diagnostic applications of fluorescence imaging.
7. Calculate the brightness for an epifluorescent image if, for the objective, $NA = 0.85$ and $M = 10$.
8.
 - a. Explain two-photon excitation mechanism.
 - b. Explain three-photon excitation mechanism.

9. a. List three diagnostic applications of multi-photon excitation microscopy.
- b. What is NSOM?
10. a. What is OCT?
- b. Specify its range of penetration.
11. a. Calculate the depth resolution (ΔL) for an OCT instrument with $\Delta\lambda = 8$ nm and $\lambda = 700$ nm.
- b. List three diagnostic applications of OCT.
12. a. Explain the meaning of *LSS*.
- b. List three diagnostic applications of *LSS*.
13. a. What is Doppler effect?
- b. What are the purposes of LDV and PDV techniques.
- c. List three clinical applications of Doppler velocimetry.
14. Define the following terms.
 - a. *Computer tomography* (CT)
 - b. *Positron emission tomography* (PET)
 - c. *Single photon emission computed tomography* (SPECT)
15. What is holographic imaging?
16. a. What is ballistic photon imaging?
- b. State advantages of ballistic photon imaging.
17. a. Define *photoacoustic imaging*.
- b. State its clinical advantages.
18. What is terahertz imaging?
19. What is a confocal microscope?
20. Calculate the axial and lateral resolution for a confocal microscope having $NA = 1.5$ for an illuminating wavelength of 2.50 μm , assuming air as the medium between the objective lens and specimen.
21. a. What is Raman spectroscopy?
- b. Why is this technique clinically important?
22. a. What is immunofluorescence?
- b. How is fluorescence labeling of biological tissues performed?
23. a. With the help of a sketch, explain the meaning of optical biopsy.
- b. List the medical benefits of optical biopsy.
24. a. What is a quantum dot?
- b. State three characteristics of quantum dots.

25. a. What is quantum dot conjugation? Explain briefly with the help of a sketch.
b. List important diagnostic applications of quantum dots as biological markers.

LABORATORIES

The following two laboratories give students an opportunity to gain a hands-on experience in understanding the principles underlying filters and lenses. These two areas are keys to mastering the material presented in this module.

Laboratory 1: Absorption and Transmission of Optical Filters

Sunglasses and brake light filters are both examples of optical filters. They both absorb and transmit a portion of incident light. The difference is that sunglasses are independent of wavelength. They equally absorb and transmit all wavelengths. A brake light filter is wavelength dependent: It transmits only red light and absorbs all other colors. The absorption of light through a material is based on the exponential law of absorption given by the following equation:

$$E = E_0 e^{-\alpha x}$$

where E = transmitted irradiance,
 E_0 = incident irradiance,
 α = absorption coefficient cm^{-1} , and
 x = thickness in cm.

The transmission (T) can be calculated using the following equations:

$$T = E/E_0 = e^{-\alpha x} \text{ (no units)}$$

The absorption coefficient can be found by:

$$\alpha = -(\ln E/E_0)/x$$

A plot of the transmitted light versus filter thickness is shown in Figure L1. The slope of the curve is the absorption coefficient. The absorption coefficient is a constant based on the material. The material can be wavelength dependent.

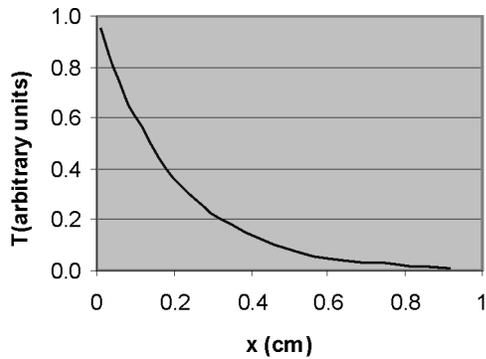


Figure L1 *Transmitted light versus filter thickness*

Objectives

- Measure the transmission of optical filters as a function of filter thickness.
- Calculate the absorption coefficient α of each filter type using the exponential law of absorption

Equipment

HeNe laser

Optical power meter

Broadband plastic filters

Lab jack

Filter mount

Neutral density filters

Diverging lens

Micrometer

Lens mount

Procedure

1. Plug in laser and carefully place on lab jack.
2. Holding only the edges of the diverging lens, insert into lens holder
 - a. Place lens holder into the path of the laser approximately 5 cm from the aperture of the laser.
3. Observing safety precautions, turn on laser.
 - a. Make sure the reflected beams from the lens are directed back into the aperture of the laser.
 - b. The laser beam must be incident on the center of the lens for proper alignment.
4. Turn on power meter.

- a. Set $\lambda = 633 \text{ nm}$. In the wavelength (λ) calibration mode, the number on the meter will indicate the λ in nm. After exiting this mode, the number will indicate power ranging from μW to mW depending on range chosen.
 - b. Press λ button to exit wavelength calibration mode and initially set range to 3 mW
 - c. Press zero on meter (negates background light).
5. Measure the power (P_o) and calculate the irradiance (E_o) at a distance where the beam fills the area of the detector. Record in Data Table L1. The irradiance is calculated using the following formula: $E_o = \frac{P_o}{A_d}$
 P_o = measured power
 A_d = area of the detection
 6. Measure thickness of red filter with micrometer at two corners holding the filter by the edges. Record the average value in Data Table L2. Optical filters are delicate. Handle them with care. Ensure that the micrometer does not come in contact with a central portion of the filter. Do not get fingerprints on filter surfaces
 7. Insert filter into filter holder. Carefully place filter in the path of the beam **near** the detector. Refer to Figure L2. Ensure that the reflected beam from the filter is directed back into the laser aperture.
 8. Measure power (P) and record in Data Table L2.
 9. Insert a second red filter into filter holder and record power (P) in Data Table L2. Assume thickness is the same as the first filter. Range setting may need to be adjusted as transmitted irradiance drops. Do not adjust range below $100 \mu\text{W}$. Power levels that cannot be read by the $100 \mu\text{W}$ range can be considered 0.
 10. Repeat step 9 with up to six filters or where power drops to 0.
 11. Calculate the irradiance and absorption coefficient (using the equation given in the introduction) and transmission, for each reading and thickness. Record in Data Table L2.
 12. Repeat steps 6–11 with a set of blue filters and neutral-density filters.
 13. Include in lab report a plot of transmission versus thickness for both red and neutral-density filters.

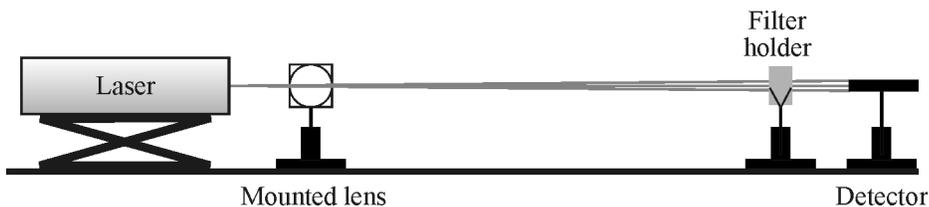


Figure L2 *Laser setup*

Laboratory 2: To study the characteristics of image formed by a converging lens

The word *lens* comes from the Latin word for lentil, a seed whose shape is similar to that of a common lens. An optical lens is made from some transparent material, most commonly glass. One or both surfaces usually have a spherical contour. If both spherical surfaces are convex (bulge outwards), the lens is called the *biconvex* or *converging lens* (also called a *positive lens*). The properties of lenses are due to refraction of light passing through them. When light rays pass through a lens, they are refracted, or deviated from their original paths, according to the law of refraction.

A biconvex lens is a converging lens. Incident light rays parallel to the optic axis of the lens converge at a focal point on the opposite side of the lens. When light travels inside a lens, it is refracted and displaced laterally. A lens with spherical geometry has for each lens surface a center of curvature, a radius of curvature, a focal point, and a focal length. The focal points are at equal distances on either side of a thin lens. Opposite sides of a lens are generally distinguished as the *object side* and the *image side*.

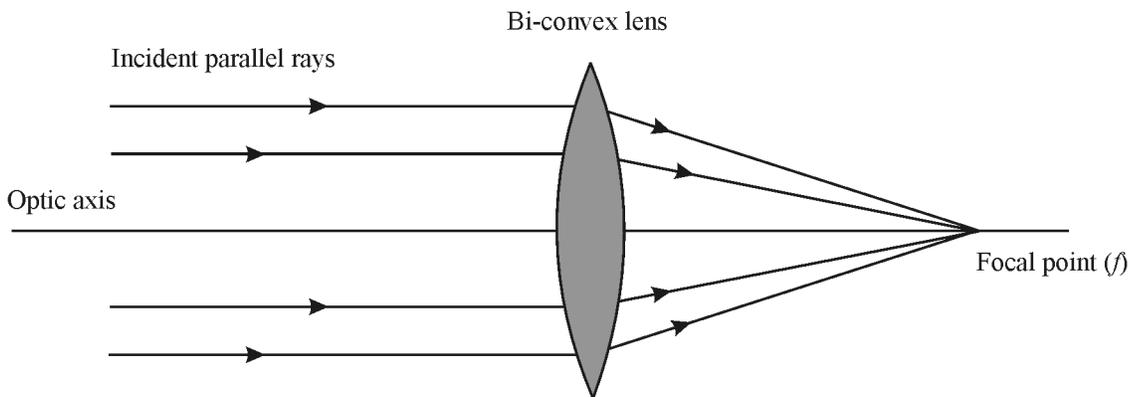


Figure L3 Rays converging at the focal point (f)

For a lens, the image of an object is *real* when formed or projected on the side of the lens opposite the object's location (on the image side) and *virtual* when formed on the same side of the lens as the object's location (on the object side). The characteristics of the image formed by a converging lens depend on the distance of the object in front of the lens. For a convex lens, the object is located within one of the three regions defined by the focal point f and twice the focal point $2f$ or at one of these two points, as shown in Figure L4.

If p is the object distance from the lens, for $p > 2f$, the image is real, inverted, and reduced. For $2f > p > f$, the image will also be real and inverted but enlarged or magnified. For $p < f$, the image will be virtual, upright, and enlarged. For $p = f$, the image forms at infinity. For $p = 2f$, the image is real, inverted, and the same size as the object. Figure L4 shows the image characteristics formed by a convex lens with respect to various object distances from the lens.

The image distances and image characteristics for a lens can be found analytically. The **thin-lens equation** (lens thickness is much smaller than the focal length) is

$$1/p + 1/q = 1/f \quad \text{or} \quad f = pq/(p + q)$$

The magnification is:

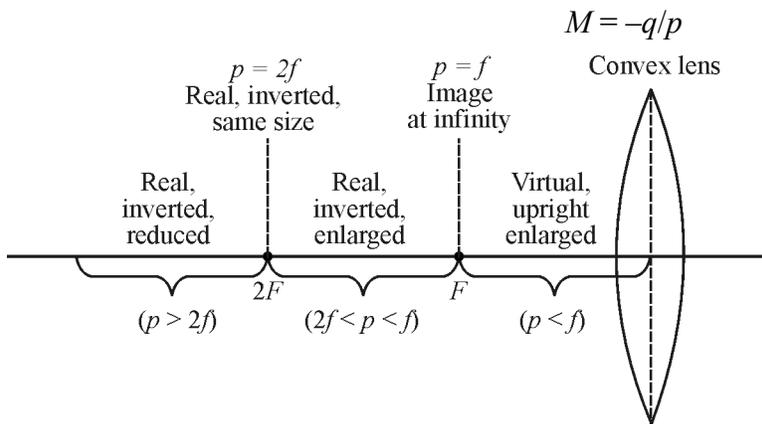


Figure L4 Characteristics of image formed by a converging lens

Objective

To find the focal length of a converging lens and describe image characteristics in each of three cases:

Case 1: $2f > p > f$

Case 2: $p = 2f$

Case 3: $p > 2f$

Equipment

Ruler

Screen

Light source with pattern (object)

Lens/screen holders

Three lenses of known focal length

Optical bench

Procedure

The experimental arrangement is shown in Figure L5. Use the following procedure in the sequence shown.

1. Measure the height of the object (vertical arrow) and record in Data Table L3.
2. Place the lens in the lens holder.
3. Check to make sure the optic axis is aligned straight.
4. Check to make sure the object is illuminated by the light source and remains fixed in position at one end of the measurement bench as shown.
5. The object distance p is the distance between the object and the lens.

6. The image distance q is the distance between the lens and the screen.
7. Choose the object distance $p = f$ and obtain a sharp image of the object on the screen. Record p in Table 1.
8. Measure the image in the vertical direction and calculate M_{measured} .
9. Use the object and image distance to solve for the experimental focal length. Record in Table 1.
10. Repeat steps 1 through 7 for object distance equal to $2f$ and for an object distance greater than $2f$.
11. Repeat this procedure for the next two lenses provided to obtain p and q experimentally.
12. Find magnification M and describe image characteristics.
13. Calculate the *percent error* between measured and the actual values of f using, $\% \text{ error} = (f_{\text{actual}} - f_{\text{exp}})/f_{\text{actual}}$.
14. Record observations and data in Table 1.

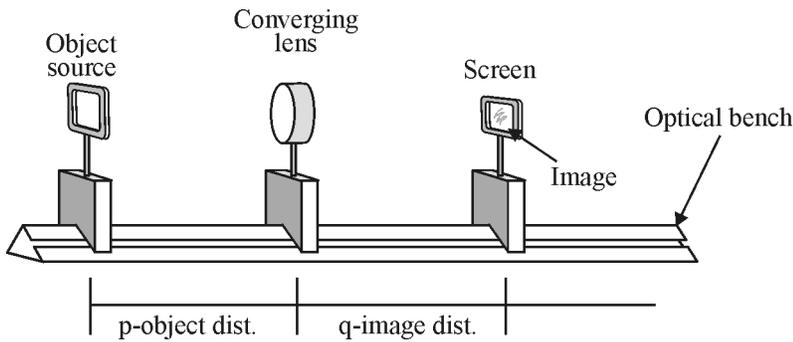


Figure L5 Arrangement for measuring image characteristics using a converging lens

Data Table L3

f_{actual} (cm)	f_{meas} (cm)	p (cm)	q (cm)	h' (cm)	$M_{\text{meas}} = h'/h$	$M_{\text{actual}} = -q/p$	Image characteristics f % error
10	9.76	15	28	4.5	-1.5	-1.86	Magnified, inverted, real 2.4
10							
10							
15							
15							
15							
20							
20							
20							

Object height (h) = _____ (cm)

Equations

$$f = q \cdot p / (q + p)$$

$$M = -q/p$$

$$M_{\text{measure}} = \text{Object height/Image height}$$

REFERENCES

Physics for Scientists and Engineers; Serway and Jewett; 6th ed, 2004, pg. 1161

Fundamentals of Light and Lasers, Course 1, CORD.

http://en.wikipedia.org/wiki/Stokes_shift

Laser Diagnostics of the Future will be more than skin deep; Laser Focus World, January 1993, pg. 100

<http://www.microscopyu.com/articles/fluorescence/multiphoton/multiphotonintro.htm>

Peter E. Andersen; Riso National Laboratory; Denmark; extracted from PowerPoint presentation; contact: peter.andersenEE@risoe.dk

<http://www.aip.org/tip/INPHFA/vol-9/iss-4/p27.html>

Laser Diagnostics of the Future will be more than skin deep; Laser Focus World, January 1993, pg. 100

http://en.wikipedia.org/wiki/Raman_spectroscopy

“What Is a Biopsy?” *Netdoctor.co.uk*. May 6, 2004 (May 27, 2004).

<http://nanobioforum.org/files/kBarovsky.pdf>

Medical Applications of Lasers by D. R. Vij, K. Mahesh, Kluwer Academic Publishers, 2002

Lasers in Medicine by Ronald W. Waynant, CRC Press, 2000

Lasers in Medicine and Surgery, STEP II, CORD, www.cord.org