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The Basics of Confocal Microscopy

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1. Introduction

Confocal microscopy is a powerful tool that creates sharp images of a specimen that would otherwise appear blurred when viewed under a conventional microscope. This is achieved by excluding most of the light from the specimen that is not from the microscope's focal plane. The image thus obtained has less haze and better contrast than that of a conventional microscope and represents a thin cross-section of the specimen (Diaspro, 2002; Hibbs, 2004; Matsumoto, 2002; Muller, 2002; Paddock, 1999; Pawley, 1995). Laser scanning confocal microscopy has become an invaluable tool for imaging thin optical sections in living and fixed specimens ranging in thickness up to 100 micrometers. In fact the confocal microscope is often capable of revealing the presence of single molecule (Peterman, Sosa et al., 2004). Modern confocal microscopes have kept the key elements of Minsky's design: the pinhole apertures and point by point illumination of the specimen. During the 1990s advances in optics and electronics afforded more stable and powerful lasers, high-efficiency scanning mirror units, high-throughput fiber optics, better thin film dielectric coatings, and detectors having reduced noise characteristics. In addition, fluorochromes that were more carefully matched to laser excitation lines were beginning to be synthesized (Mason, 1999). Coupled to the rapidly advancing computer processing speeds, enhanced displays, and large-volume storage technology emerging in the late 1990s, the stage was set for a virtual explosion in the number of applications that could be targeted with laser scanning confocal microscopy. Modern confocal microscopes can be considered as completely integrated electronic systems (Spring and Inoue, 1997) where the optical microscope plays a central role in a configuration that consists of one or more electronic detectors, a computer (for image display, processing, output, and storage), and several laser systems combined with wavelength selection devices and a beam scanning assembly. In most advanced systems, integration between the various components is so thorough that the entire confocal microscope is often collectively referred to as a digital or video imaging, capable of producing electronic images. These microscopes are now being employed for routine investigations on molecules, cells, and living tissues that were not possible just a few years ago (Goldman and Spector, 2005).

Modern instruments are equipped with **1)** 3-5 laser systems controlled by high-speed acousto-optic tunable filters (AOTFs), which allow very precise regulation of wavelength and excitation intensity; **2)** Photomultipliers that have high quantum efficiency in the near-ultraviolet, visible and near-infrared spectral regions, these microscopes are capable of

examining fluorescence emission ranging from 400 to 750 nanometers; **3)** Spectral imaging detection systems which further refine the technique by enabling the examination and resolution of fluorophores with overlapping spectra as well as providing the ability to compensate for autofluorescence; **4)** Spatial pinhole that eliminate out-of-focus light in specimens that are thicker than the focal plane thus increases optical resolution and contrast of a micrograph using point illumination. (Murphy, 2001; Wilson and Carlini, 1988; Pawley, 2006) Besides allowing better observation of fine details, it is also possible to build three-dimensional (3D) reconstructions of a volume of the specimen by assembling a series of thin slices taken along the vertical axis using confocal microscope (Al-Kofahi, Can et al., 2003). In the recent years confocal microscopy has gained a tremendous popularity in the scientific and industrial communities owing to its great number of applications in life sciences, semiconductor inspection and material science (Conn, 1999; Diaspro, 2002; Gu, 1996; Hibbs, 2004; Mason, 1999; Masters, 1996; Matsumoto, 2002; Muller, 2002; Paddock, 1999; Pawley, 1995; Sheppard and Shotton, 1997; T.R.Corle and G.S.Kino, 1996; Wilson, 1990).

Principle

Current instruments are highly modified from the earliest versions, but the principle of confocal imaging that was developed by Marvin Minsky is employed in all modern confocal microscopes (Minsky 1961; 1988). The image in a confocal microscope is achieved by scanning one or more focused beams of light, usually from a laser or arc-discharge source, across the specimen. This point of illumination is brought to focus in the specimen by the objective lens, and laterally scanned using some form of scanning device under computer control. The sequences of points of light from the specimen are detected by a photomultiplier tube (PMT) through a pinhole (or in some cases, a slit) (Fellers and Davidson, 2007), and the output from the PMT is built into an image and displayed by the computer.

Although unstained specimens can be viewed using light reflected back from it, most of the times specimens are labeled with one or more fluorescent probes. A laser is used to provide the excitation light (in order to get very high intensities). The laser light reflects off a dichroic mirror hits two lenses mounted on motors. These mirrors scan the laser across the sample, dye in the sample fluoresces and the emitted light gets descanned by the same mirrors that are used to scan the excitation light from the laser. The emitted light passes through the mirror and is focused onto the pinhole. The light that passes through the pinhole is measured by a detector, i.e. a photomultiplier tube. Thus there is never a complete image of the sample at any given instant; only one point of the sample is viewed. The detector is attached to a computer which builds up the image, one pixel at a time.

Earlier, a 512x512 pixel image formation could be done probably 3 times a second due to the limitation in the scanning mirrors. Subsequently, to speed up scanning a special Acoustic Optical Deflector (AOD) was used in place of one of the mirrors. AOD uses a high-frequency sound wave in a special crystal to create a diffraction grating, which deflects the laser light. By varying the frequency of the sound wave, the AOD changes the angle of the diffracted light, thus allowing a quick scan leading to 512x480 pixel images 30 times per second. If one looks at a smaller field of view, then the process can be even faster (up to 480 frames per second).

The basic difference between the two techniques are as follows: (Lichtmann, 1994; Murray, 2005; Sandison and Webb, 1994; Swedlow, Hu et al., 2002; White, Amos et al., 1987; Wilson, 1989; Wright and Wright, 2002).

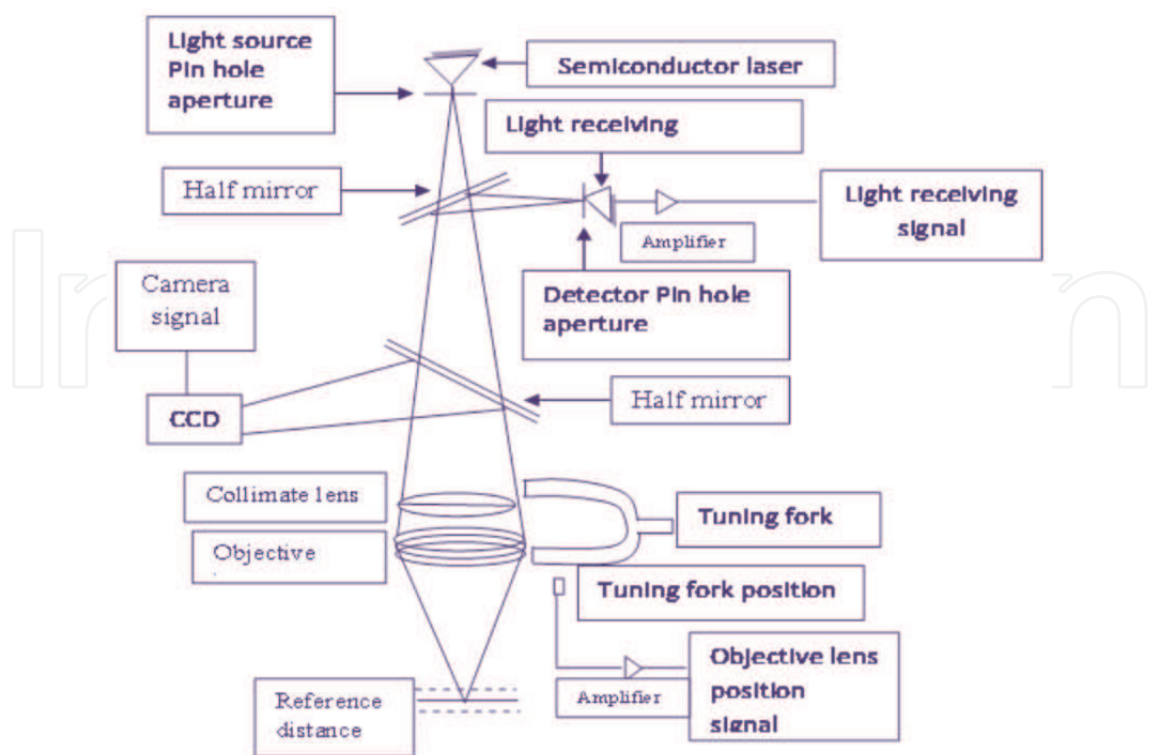


Fig. Schematic representation of basic principle of confocal microscope

Sl. No.	Characteristics	Widefield fluorescence microscope	Confocal microscope
1.	Fundamental setup	Comprises of fluorescence light Source, solid-state charge-coupled devices (CCDs) and fluorescence filter cube.	Comprises of laser, light source pinhole aperture, photomultiplier detector, detector pinhole aperture and dichrome mirror
2.	Principle	The entire specimen is bathed with light.	Only a single point is illuminated at a time to avoid unwanted scattering of light.
3.	Resolution	Low	High
4.	Regions of out-of-focus information	Blurred and large	Reduced
5.	Optical resolution in z	2-3 μm	0.5 μm
6.	Signals from optical sections	Many signals cannot be seen separately.	Improved z-resolution allow for more accurate signal discrimination.
7.	Acquisition of 3-D data	Not possible	Possible
8.	Cost	Relatively inexpensive	Expensive
9.	Hazards	No any	High intensity laser irradiation is hazardous.

Components of confocal microscope

1. Light source (laser system)
2. Filters
3. Acousto optical devices
4. Scanner
5. Detector (PMT, APD)
6. Pin hole

2. Laser systems

LASER- Light Amplification by the Stimulated Emission of Radiations can be defined as materials with altered distribution of atoms is such that there are more excited atoms, ready to emit energy. The process of altering the energy distribution of the atom so that most of them are in a high energy state, population inversion. Lasers did not become a reality, until methods to achieve population inversion become feasible in 1950s. Mercury and xenon light sources, the most common light sources for microscopes are found to be too weak for confocal system, hence it uses different type of lasers.

Laser types

Depending on the materials used as the medium lasers, are grouped into three categories:

- Gas lasers
- Solid state lasers
- Semi-conductor lasers

Laser safety

The two major concerns in safe laser operation are exposure to the beam and the electrical hazards associated with high voltages within the laser and its power supply. While there are no known cases of a laser beam contributing to a person's death, there have been several instances of deaths attributable to contact with high voltage laser-related components. Beams of sufficiently high power can burn the skin, or in some cases create a hazard by burning or damaging other materials. But the primary concern with regard to the laser beam is potential damage to the eyes, which are most sensitive to light.

3. Filtering devices

In fluorescence microscope filtering devices are used to separate light beams on the basis of their wavelengths. Four different types of filters are used to selectively transmit or block a desired range of wavelengths.

- a. Short pass filters - they cut off wavelengths longer than a certain wavelength e.g. heat filters are used to exclude infra red light to reduce specimen heating by illumination.
- b. Long pass filters -e.g. fluorescent filters that transmit light of longer than a certain wavelength.
- c. Band pass filter- that transmit light only between a cut-on and cut-off wavelength, especially useful when one is trying to image signals from more than one fluorochrome simultaneously.
- d. Dichrome mirrors -that separates the emitted light from the excited light.

Now-a-days these filters are rapidly replaced by active optical devices such as acousto-optical devices.

4. Acousto-optical devices

These devices work on the principle of acousto-optic effect. In general, acousto-optic effects are based on the change in refractive index of a medium due to the presence of sound waves in that medium. Sound waves produce a refractive index grating in the material and this grating is seen by the light wave. These variations in the refractive index, due to the pressure fluctuations, may be detected optically by refraction, diffraction, interference and reflection effects. The acousto-optic effect is extensively used in the measurement and study of ultrasonic waves. However, the growing principle area of interest is in acousto-optical devices for the deflection, modulation, signal processing and frequency shifting of light beams. This can be attributed to the increasing availability and performance of lasers, which have made the acousto-optic effect easier to observe and measure. Technical progresses in both crystal growth and high frequency piezoelectric transducers have brought valuable benefits to acousto-optic components' improvements. Some materials displaying acousto-optic effect include fused silica, arsenictrisulfide, tellurium dioxide and tellurite glasses, lead silicate, mercury(I) chloride, lead (II)bromide and other materials (Wachmann, 2000; Wachmann, Niu et al., 1996).

Various acousto-optic devices used now-a-days are as follows:

a. Acousto-Optic Tunable Filters (AOTFs)

The integration of optoelectronic technology into confocal microscopy has provided a significant enhancement in the versatility of the spectral control for a wide variety of fluorescence investigations. AOFT is an adjustable quartz filter that works at frequencies as high as sound i.e. "Acousto". Light that passes the AOFT is diffracted depending on its own wavelength and the wavelength of the ultrasonic wave field. The ultrasonic wave field can be modulated so that the intensities of different laser lines can be changed between 0% to 100% by the software even during the scanning process (Chang, 1995). Several benefits of the AOTF combine to enhance the versatility of the latest generation of confocal instruments, and these devices are becoming ever-increasing popular for control of excitation wavelength ranges and intensity. The primary characteristic that facilitates nearly every advantage of the AOTF is its capability to allow the microscopist control the intensity and/or illumination wavelength on a pixel-by-pixel basis while maintaining a high scan rate. This single feature translates it into a wide variety of useful analytical microscopy



Fig. The AOTF enables you to select the wavelengths (laser lines on/off)

tools, which are even further enhanced in flexibility when laser illumination is employed. The rapid intensity and wavelength switching capability of the AOFT enables sequential line scanning of multiple laser lines in which each excitation wavelength can be assigned a different intensity in order to balance various signals for optimal imaging (Sharma, 2001). The AOFT speed and versatility controls the wavelength and intensity of light that simultaneously or sequentially scan each sample at sufficient speed to accurately monitor dynamic cellular processes (Chen, Mills et al., 2003). The AOFT functions allow the selection of small user-defined specimen areas termed as regions of interest. (ROI) can be illuminated with either greater or lesser intensity and at different wavelengths for precise control in photobleaching techniques, or spectroscopic measurements (Chen, Mills et al., 2003; Day, Periasamy et al., 2001; Wallrabe and Periasamy, 2005). The illumination intensity can only be increased in selected regions for controlled photobleaching experiments but can be attenuated in desired area in order to minimize unnecessary photobleaching. When the illumination area is under AOFT control, the laser exposure is restricted to the scanned area by default and the extremely rapid response of the device can be utilized to provide beam blanking during the fly-back interval of the galvanometer scanning mirror cycle there by limiting unnecessary specimen exposure (Claxton, Fellers et al.). When the AOFT is combined with multiple lasers and software that allow time course control of the sequential observations, time-lapse experiments (4-D imaging) can be designed to acquire data from several different areas in a single experiment, which might, for example, be defined to correspond to different cellular organelles.

Thus development of AOFT has ultimately provided substantial additional versatility to techniques such as FRAP (Lippincott-Schwartz, Altan-Bonnet et al., 2003; Klonis, Rug et al., 2002), FLIP (Phair and Misteli, 2002) as well as uncaging i.e. localized photoactivated fluorescence (Politz, 1999).

b. Acousto-optic Beam Splitter (AOBS)

The AOBS is actually an AOFT used in an imaginative manner to replace the dichoric mirror which is usually used to separate the illumination and detection paths in confocal microscope. In practice, AOBS is programmed so that it leaves most of the fluorescent light undetected and only deflects light at specific laser lens.

The advantages of AOBS over systems that use dichoric mirrors as the main beam splitter are its high efficiency, its lack of moving parts and its flexibility in terms of using new laser lines simply by changing the software. While proper operation of the AOBS depends on the light input being parallel as it passes through the crystal light originating above or below the plane of the focus, one may see slightly different transmission characteristics. Fortunately in confocal operation most of the out focus light is removed at the pin hole.

c. Acousto-optic modulator (AOM)

Optical wave can be modulated by varying the parameters like amplitude, phase, frequency and polarization etc. Besides the acousto-optic interaction also makes it possible to modulate the optical beam by both temporal and spatial modulation. A simple method of modulating the optical beam travelling through the acousto-optic device is by switching the acoustic field on and off. When off, the light beam is undiverted, the intensity of light directed at the Bragg diffraction angle is zero, when switched on Bragg diffraction occurs, the intensity at the Bragg angle increases. Hence, acousto-optic device modulates the output along the Bragg diffraction angle by switching it on and off. In confocal microscope the device acts as a modulator for keeping the acoustic wavelength (frequency) fixed and thereby varying the drive power to vary the amount of light in the deflected beam. There are several limitations associated with

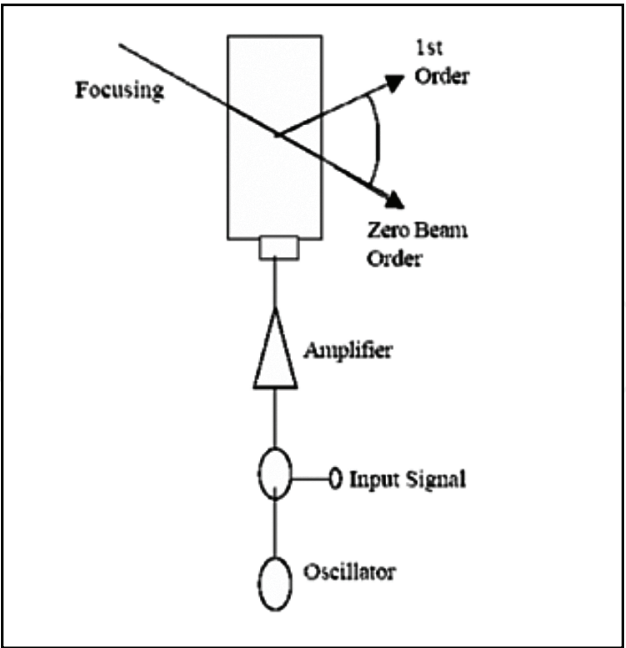


Fig. Schematic representation of AOM

the design and performance of acousto-optic modulators. The acousto-optic medium must be designed carefully to provide maximum light intensity in a single diffracted beam. The time taken for the acoustic wave to travel across the diameter of the light beam limits the switching speed and hence the modulation bandwidth. So to increase the bandwidth the light must be focused to a small diameter at the location of the acousto-optic interaction.

d. Acousto-optic deflectors (AOD)

AODs are essentially the same as acousto-optic modulators (AOMs). In an AOM, only the amplitude of the sound wave is modulated (to modulate the intensity of the diffracted laser beam), whereas in an AOD, both the amplitude and frequency are adjusted (Hosada, Seya et al.). AOD can scan a laser beam at up to 1000 KHz compared to about 500-1000Hz for linear galvanometer scanner and about 4-8 KHz for resonant galvanometer. However, since AOD produces deflection by diffraction and the scan angle depends on the wavelength of light beam, the longer wavelength fluorescent light will be deflected by a different amount on its way back through the crystals and will therefore fail to pass through the pin hole which

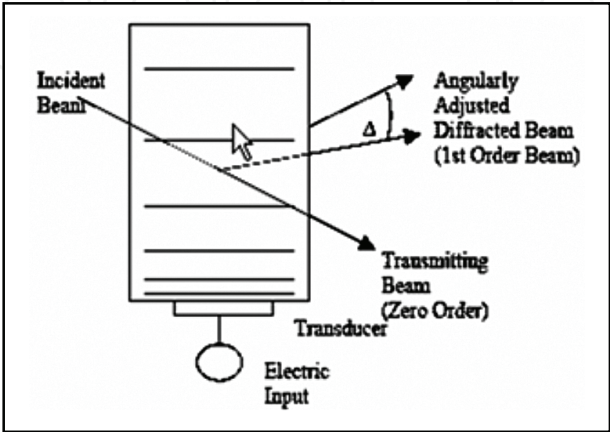


Fig. Schematic representation of AOD

restricts its use. Although this dispersion effect can be theoretically compensated by a chromatic corrections, but they are not practical in case of confocal. However, to overcome this limitation of AOD, a confocal microscope is designed to descanned only along the vertical direction (controlled by slow galvanometer), followed by collection of light using a slit rather than a pin hole (Brakenhoff, Blom et al., 1979). The penalty is the reduction in the amount of optical sectioning and a very slight distortion in image caused due to loss of circular symmetry. Nevertheless, it is possible to obtain high quality image using slits (Keller, 1995).

5. Scanners

Confocal imaging relies upon the sequential collection of light from spatially filtered individual specimen points, followed by electronic signal processing and eventually the visual display as corresponding image points. The point-by-point signal collection process requires a mechanism for scanning the focused illuminating beam through the specimen volume under observation which is achieved by scanning the stage or the beam. Thus, the principle scanning variations commonly employed to produce confocal microscope images includes:

- Scanning a laterally translating specimen stage coupled to a stationary illuminating light beam (**stage scanning**)
- Scanning light beam with a stationary stage (**beam scanning**). In modern confocal microscope, two fundamentally different techniques for beam scanning have been developed.
 - Single beam scanning (Wilhelm, Grobler et al., 2003) uses a pair of computer controlled galvanometer mirror to scan the specimen at the rate of approximately 1 frame/sec.
 - Multiple beam scanning confocal microscope that are equipped with a spinning Nipkow disk containing an array of pinholes (Ichihara, Tanaami et al., 1996; Inoue and Inoue, 2002; Nokano, 2002), here the light source is arc-discharge lamp instead of lasers to reduce specimen damage and enhance detection of low fluorescence level during real time image collection. Apart from this multiple beam microscope can readily capture image with an array of detectors, such as CCD camera system, PMT etc. (Chong, Coates et al., 2004).

Scanning the stage has the advantage that the optical system is spatially invariant but beam scanning can be faster and avoids the problem of moving the sample. However, beam scanning has certain limitations as it is usually performed using a feedback controlled galvano-scanner mirror. So, in this case the light from the sample must be de-scanned so that it does not move relative to the pinhole (Murphy, 2001; Webb, 1996). Each technique has performance features that make it advantageous for specific confocal applications, but that limits their usefulness in others.

6. Detectors

As light emitted by fluorophores in the sample is mainly unpolarized, polarization sensitive beam splitters can only separate half of it from polarized excitation light. Instead, the emitted light is either sequentially split by a combination of short pass or long pass dichroic mirror before being passed through a band pass or long pass emission filters to detectors or it strikes a dispersive element and then can be projected to detector. In confocal microscopy fluorescence

emission is directed through a pinhole aperture positioned near the image plane to exclude light from fluorescent structures located away from the objective focal plane, thus reducing the amount of light available for image formation. As a result, the exceedingly low light levels most often encountered in confocal microscopy necessitate the use of highly sensitive photon detectors that do not require spatial discrimination, but instead respond very quickly with a high level of sensitivity to a continuous flux of varying light intensity. Thus in confocal microscopy the collection and measurement of secondary emission gathered by the objective is accomplished by several classes of photosensitive detectors, including photomultipliers (PMT) and assigning pseudocolors detector (APD) (Spring, 2001).

- a. **Photomultiplier detector:** It measures intensity without spectral information. It actually outputs an electrical impulse to the position logical circuits.

Principle of signal amplification:

- Conversion of photons into electrons
- Multiply electron
- Signal read out

- b. **Assigning pseudo colors detector:** For multiple-channel acquisition it is helpful to assign indexed colors to different grey-scale-images. The detector signals are adjusted by gain and offset such that maximum number of grey level is included in the resulting image (output).

- **Gain:** Amplifies the input signal by multiplication which results in a higher gray level value; bright features are brought closer to saturation, general image brightness is increased.
- **Offset:** sets the gray level of a selected background to zero; adjust the darkest features in the image to black.

7. Pinhole

The optical sectioning (the rate at which the detected intensity drops off in the axial direction) capability of a confocal microscope depends on pinhole and its capability to reject out-of-focus light rays i.e. the strength of the optical sectioning depends strongly on the size of the pinhole. Thus one can assume that making the pinhole as small as possible is the best way to enhance optical sectioning. However, as the pinhole size is reduced a large number of photons that arrive at the detector from the specimen is blocked. This may lead to a reduced signal-to-noise ratio. To offset the weaker signal more fluorescence is needed from the specimen but this can be done to a limit (by raising the intensity of the excitation light) on the contrary high intensities can damage the specimen, and in the case of fluorescence, also degrade the fluorophore. Moreover, it has been shown that optical sectioning does not improve considerably with the pinhole size below a limit, which approximates the radius of the first zero of the Airy disk. Thus, a fine approximation is to make the pinhole about the size of the Airy disk. Confocal fluorescence imaging using the pinhole along with the earlier optics significantly reduced the intensity of the emission that reaches the detector. With the advancement in technology, the use of photo detectors for capturing light the sensitivity of confocal imaging is enhanced. Thus, the detector's sensitivity and noise behavior are vitally important. The sensitivity is characterized by the quantum efficiency. That is, the accuracy of the measurement is improved by increasing the number of photons arriving at the detector.

Confocal working

Confocal imaging is accomplished by using a two-step process. First, excitation light that is focused on the specimen by the objective is initially passed through a small aperture, often a slit or pinhole. Alternatively, a very narrow beam of laser light can be introduced into the system via an optical fiber. By conditioning the excitation light this way, the amount of fluorescence not in focus can be controlled or minimized. Second, fluorescence emissions that originate from above or below the plane of focus are blocked by a second aperture or slit in front of the detector. The smaller this second opening, the higher the rejection rates of out-of-focus light and the thinner the optical section. These thin optical sections have greatly improved contrast and axial resolution, but they are obtained at the expense of overall specimen brightness.

Fluorophores

The choice of fluorophores is the most important aspects of fluorescence, confocal microscopy. It is typically influenced by several factors:

- The fluorophores should tag the correct part of the specimen.
- It must be sensitive enough for the given excitation wavelength.
- For living specimens it should not significantly alter the dynamics of the organism
- The specimen should not affect the fluorophore as its chemical environment can affect the position of the peaks of the excitation and emission spectra.

A major problem with fluorophores is that they fade (irreversibly) when exposed to excitation light, a phenomenon known as photobleaching. Although this process is not completely understood, it is believed in some instances to occur when fluorophore molecules react with oxygen and/or oxygen radicals and become nonfluorescent. The reaction can take place after a fluorophore molecule transitions from the singlet excited state to the triplet excited state. Although the fraction of fluorophores that transitions to the triplet state is small, its lifetime is typically much longer than that of the singlet state. This can lead to a significant triplet state fluorophores population and thus to significant photobleaching. Several strategies have been developed to reduce the rate of photobleaching. One method is to merely reduce the amount of oxygen that would react with the triplet excited state. This can be done by displacing it using a different gas. Another method is by the use of free-radical scavengers to reduce the oxygen radicals. Shortening the long lifetime of the triplet excited state has also been shown to be effective. Still other ways include using a high numerical aperture lens to collect more fluorescence light and thus use less excitation light. While photobleaching makes fluorescence microscopy more difficult, it is not always undesirable. One technique that takes advantage of it is fluorescence photobleaching recovery (FPR) or fluorescence recovery after photobleaching (FRAP). It involves exposing a small region of the specimen to a short and intense laser beam, which destroys the local fluorescence, and then observing as the fluorescence is recovered by transport of other fluorophore molecules from the surrounding region. Quantities such as the diffusion co-efficient of the dyed structures can then be determined.

Fluorophores for Confocal Microscopy

Biological laser scanning confocal microscopy relies profoundly on fluorescence as an imaging mode due to its high degree of sensitivity, coupled with the ability to specifically target structural components and dynamic processes in chemically fixed as well as living cells and tissues. In confocal microscope this is achieved by fluorophores. Recent advances in

Sl. No.	Fluorophores	Excitation (nm)	Emission (nm)
1.	5-Hydroxytryptamine (HAD)	370-415	520-540
2.	Acridine yellow	470	550
3.	Acridine orange	500	530
4.	Alexa Fluor488	494	519
5.	Alexa Fluor532	530	555
6.	Alexa Fluor546	554	570
7.	Bodipy 500/510	508	515
8.	Bodipy 530/550	534	554
9.	Cascade Blue	375	410
10.	Coumarin	384	470
11.	CY2	489	506
12.	CY3	548	562
13.	CY5	650	670-700
14.	Dansyl	340	520
15.	DAPI	345	458
16.	DPH	354	430
17.	Erythrosin	529	554
18.	Ethidium Bromide	510	595
19.	FITC	494	518
20.	Fluorescein	495	517
21.	FURA-2	340/380	500/530
22.	GFP	395/489	509
23.	Hoechst 33258	365	480
24.	Hoechst33342	355	465
25.	Laurand	364	497
26.	Lucifer yellow CH	428	535
27.	Nile Red	485	525
28.	Oregon Green 488	493	520
29.	Oregon Green 500	503	522
30.	Oregon Green 514	511	530
31.	Prodan	361	498
32.	Pyrene	341	376
33.	Rhodamine 110	496	520
34.	Rhodamine 123	505	534
35.	Rhodamine 6G	525	555
36.	Rhodamine B	540	625
37.	SITS	336	438
38.	SNARF	480	600/650
39.	Stilbene SITS, SITA	365	460
40.	Texas Re	589	615
41.	TOTO-1	514	533
42.	YOYO-1	491	509
43.	YOYO-3	612	631

Table 1.

fluorophore design have led to superior synthetic and naturally probes, including fluorescent proteins and quantum dots, which exhibit a high level of photo stability and target specificity. Synthetic probes are constructed around synthetic aromatic organic chemicals designed to bind with a biological macromolecule or to localize within a specific structural region, such as the cytoskeleton, mitochondria, golgi apparatus, endoplasmic reticulum, and nucleus. Various other probes are employed for monitoring dynamic processes and localization of environmental variables, including concentrations of inorganic metallic ions, pH, reactive oxygen species, and membrane potential. Still others are useful in monitoring cellular integrity, endocytosis, exocytosis, membrane fluidity, protein trafficking, signal transduction, and enzymatic activity. Besides, fluorescent probes have been widely applied to genetic mapping and chromosome analysis in the field of molecular genetics.

Excitation and Emission Wavelengths of some well-known fluorophores

A list of excitation and emission wavelengths of probes utilized in fluorescence research and applications is listed in Table 1. The positions of the maxima depend upon the solvent utilized in the measurements. The table is only intended to provide an indication of the wavelength range covered by each fluorophores.

Choosing fluorophore combinations for confocal microscopy

During multiple label fluorescence staining protocols, be it be wide-field and confocal microscopy, the sensible choice of probes is major criteria for obtaining the best target signal while simultaneously minimizing bleed-through artifacts.

Spectral bleed-through artifacts

The spectral bleed-through of fluorescence emission (often termed **crossover** or **crosstalk**), that occurs due to very broad bandwidths and asymmetrical spectral profiles exhibited by many of the common fluorophores, is a fundamental problem that must be addressed in both wide-field and confocal microscopy. The phenomenon is usually manifested by the emission of one fluorophore being detected in the photomultiplier channel or through the filter combination reserved for a second fluorophore. Bleed-through artifacts often complicate the interpretation of experimental results while working with a combination of fluorophores, mainly if sub-cellular co-localization of fluorophores is under investigation or quantitative measurements are necessary, such as in resonance energy transfer (**FRET**) and photobleaching (**FRAP**) studies.

Signal-to-Noise considerations

Confocal microscope can also be used for quantitative estimation. For quantitative assessment of imaging modes utilizing digital microscopy techniques, the effect of signal sampling on contrast and resolution must be considered. The measured signal level values do not directly represent the number of photons emitted or scattered by the specimen, but are proportional to the number of photons. Furthermore, each individual sample of signal intensity is only an estimation of the number of collected photons, and will vary with repeated measurement. This variation is referred to as noise which imparts an uncertainty in the quantification of intensity and therefore in the contrast and resolution of the image data. Thus for better quantitative estimation signal-to-noise ratio must be taken into consideration.

Resolution

Confocal microscopy has inherent resolution limitations due to diffraction. Resolution can be defined as the ability to recognize two closely associated points as two distant points. As

discussed in confocal microscope the point source (LASER) is used to produce a point of light on the specimen. In fact it appears in the focal plane as an Airy disk, whose size depends on the wavelength of the light source and the numerical aperture of the objective lens. The Airy disk limits the maximum resolution that can be attained with the confocal microscope, the best resolution is usually about 200nm. Ideally, the image of a point would just be a single intense point right at radius $\frac{1}{4} 0$. However, the finite size of the Airy disk sets the scale for which details can be resolved. According to the Rayleigh criterion, the minimum separation between two Airy disks for which they are distinguishable is equal to their radius. This corresponds to the maximum of one Airy disk superimposed on the minimum of the other. Resolution along the optical axis is also limited by diffraction effects. As in the lateral direction there is a periodic, but elliptical distribution of intensity in the shape of an Airy disk.

Types of confocal microscope

Two types of confocal microscopes are commercially available:

- **Confocal laser scanning microscopes**
- **Spinning-disk (Nipkow disk) confocal microscopes**

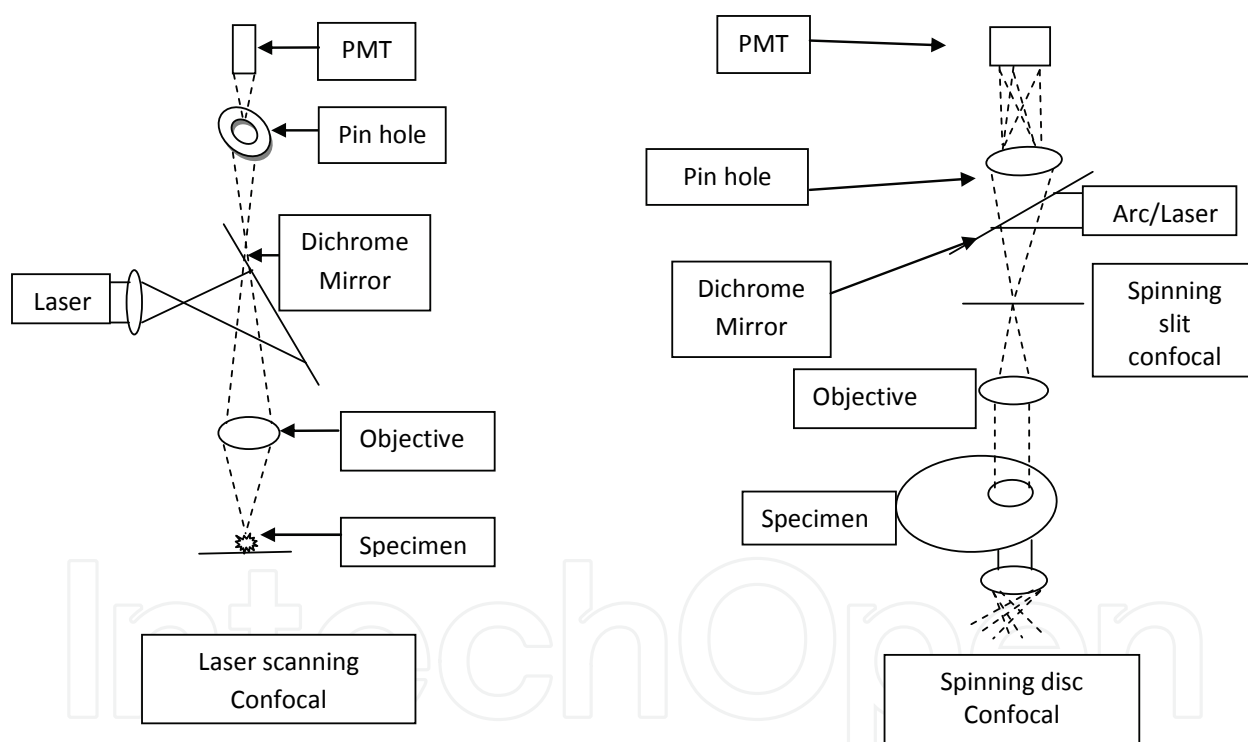


Fig. Schematic representation of the working principle of different types of confocal microscope

Each of these classes of confocal microscope has particular advantages and disadvantages. Most systems are either optimized for resolution or high recording speed (i.e. video capture). Confocal laser scanning microscopes can have a programmable sampling density while Nipkow use a fixed sampling density defined by the camera resolution. Imaging frame rates are typically very slow for laser scanning systems (e.g. less than 3 frames/second). Commercial spinning-disk confocal microscopes achieve frame rates of over 50 per second- a desirable feature for dynamic observations such as live cell imaging.

Cutting edge development in confocal laser scanning microscopy now allows better than video rate (60frames/second) imaging, by using multiple microelectronic mechanical systems based scanning mirrors. Confocal x-ray fluorescence imaging is a novel technique that allows control over depth, in addition to horizontal and vertical aiming. For example, when analyzing buried layers in a painting.

Difference between laser scanning and spinning disc confocal microscope:

Sl. No.	Characteristics	Laser Scanning confocal microscope	Spinning-disk confocal microscope
1.	Light source	Laser	Arc or Laser
2.	Pin hole size	Small	Large
3.	Mode of scanning	Point-by-point scanning	Full frame imaging
4.	Detector used	PMT	Full-frame CCD cameras are used for image formation.
5.	Dimension analysis	It is used for the re-construction of 3-D image	It is required for 4-D(time-lapse) imaging of living cells
6.	Types	Only one type: laser scanning confocal microscope.	Two types: Nipkow disk scanning confocal and DSU disk scanning confocal.
7.	Advantages	It provides highest level of confocality and ability to do thin optical sections.	It offers speed and great transmission.
8.	Disadvantages	Compromise detectible fluorescence in order to deliver high degree of confocality. Point-by-point acquisition of image is time consuming. Any specimen movement during scan can result in jagged edges in image or poor definition of intracellular details due to both time it takes to capture the image and damaging effects of the laser.	Less confocality.

Fast Confocal Microscope

Most confocal microscopes generate a single image in 0.1–1 sec. For many dynamic processes this rate may be too slow, particularly if 3D stacks of images are required. Even for a single 2D image, slow frame rates translate into long exposure times of the specimen to intense laser light, which may damage it or cause photobleaching. Two commonly used designs that can

capture images at high speed are the Nipkow disk confocal microscope and a confocal microscope that uses an acousto-optic deflector (AOD) for steering the excitation light.

Applications of Confocal Microscope

The broad range of applications available to laser scanning confocal microscopy includes a wide variety of studies in neuroanatomy and neurophysiology, as well as morphological studies of a wide spectrum of cells and tissues. In addition, the growing use of new fluorescent proteins is rapidly expanding the number of original research reports coupling these useful tools to modern microscopic investigations. Other applications include resonance energy transfer, stem cell research, photobleaching studies, lifetime imaging, multiphoton microscopy, total internal reflection, DNA hybridization, membrane and ion probes, bioluminescent proteins, and epitope tagging. Many of these powerful techniques are described in these reviews.

CLSM is widely-used in numerous biological sciences discipline, from cell biology and genetics to microbiology and developmental biology.

FRAP (Fluorescence Recovery After Photo-bleaching)

FRAP is a technique designed to quantifying two dimensional lateral diffusion of a fluorescent probes tagged to biological samples like nucleotides and proteins in single cells or in molecularly thin film. In a FRAP experiment, a region with fluorescent molecules is irradiated or photo-bleached with laser light. This results in the fluorescent molecules inside that region to become non-fluorescent. The part recovered after a time lapse of this experiment is due to subsequent redistribution of fluorescent and bleached molecules throughout the volume. This gives information about their mobility. This technique is very useful in biological studies of the fluid mosaic model of cell membranes. It can also be used to determine if a protein is able to move within a membrane, or whether it is tethered to other structural components of the cell. FRAP allows quantitation of a number of 'dynamic parameters' such as diffusion coefficient, immobile fraction and binding or residence time.

- FRAP can be applied to one photon or multi-photon.
- Slow FRAP takes seconds and fast FRAP takes micro- to milli-seconds.
- The amount of light that recovers (returns) relative to the amount of light that was there before the photobleaching is the percent recovery.
- The speed with which the fluorescent molecules migrate back into the photobleached area is a measurement of the "diffusional mobility" which is usually called lateral mobility

FRET (Fluorescence Resonance Energy Transfer)

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. This mechanism is termed "Förster resonance energy transfer" and is named after the German scientist Theodor Förster. When both chromophores are fluorescent, the term "fluorescence resonance energy transfer" is often used instead Förster resonance energy transfer. Energy passes from the excited DONOR molecule to the ACCEPTOR molecule through nonradiative dipole-dipole coupling which then emits fluorescence. The fluorophores must be extremely close to one another for this to happen ($< 0.1\text{nm}$). Thus, if FRET occurs, the donor fluorescence

decreases (quenching) and the acceptor fluorescence increases. So the ratio of acceptor/donor goes up. When the acceptor is photobleached, donor fluorescence increases. Thus the efficiency of FRET is dependent on following parameters:

- Donor and acceptor molecules must be in close proximity (typically 10-100Å).
- The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor
- Donor and acceptor transition dipole orientations must be approximately parallel.

Some selected applications of FRET:

- Structural and conformation of proteins
- Spatial distribution and assembly of protein complexes
- Receptor/ligand interactions
- Immunoassays
- Prohibiting interactions of single molecules
- Real-time PCR assays and SNP detection
- Detection of nucleic acid hybridization
- Primer-extension assays for detecting mutations
- Automated DNA sequencing
- Distribution and transport of lipids
- Membrane fusion assays
- Fluorogenic protease substrates
- Indicators for cyclic AMP and zinc

Thus, FRET is an important technique for investigating a variety of biological phenomena that produce changes in molecular proximity.

FLIM (Fluorescence Lifetime Imaging)

The fluorescence lifetime is defined as the average time that a molecule remains in an excited state prior to returning to the ground state. The process of fluorescence emission by an excited molecule occurs like radioactivity with a characteristic half-life or decay lifetime of a few nanoseconds. The life time varies between different fluorescent molecules and for the same probe in a different environment. For example, the lifetimes of many fluorophores are altered by the presence of ions such as Ca^{2+} , Mg^{2+} , Cl^- , pH or K^+ . This allows the researcher to follow environmentally induced changes. FLIM does not require wavelength-ratio metric probes. Fluorescence lifetime can give information about colocalisation of two molecules (e.g.FRET) and separation of two emissions with same spectrum.

An advantage of lifetime imaging is that the absolute values of lifetimes are independent of the probe concentration, photobleaching, light scattering and the amount of excitation intensity. Fluorescence lifetime imaging (FLIM) thus offers several opportunities to study dynamic events of living cells.

FLIP (Fluorescence loss in photobleaching)

A part of a cell at a distance from where there is an excess of fluorescent protein is bleached with a laser at low intensity. One then images the sample just before and at constant intervals after bleaching, allowing the bleached molecules to redistribute through the cell.

FLAP (Fluorescence localization after photobleaching)

The molecule to be located has 2 fluorophores, one to be bleached, and the other to act as a reference label. One can then track the distribution of the molecule after it is bleached. The

FLAP signal was obtained by subtracting the bleached signal from the unbleached one, allowing the tracking of the labeled molecule.

FISH (Fluorescence *in situ* hybridization)

FISH involves the preparation of short sequences of cDNA complementary to the DNA sequence of interest (Daims, Ramsing et al., 2001; Schmid, Twachtmann et al., 2000; Wagner, Horn et al., 2002). These cDNA probes are labeled with fluorescent tags, and once the probes hybridize to the complementary DNA, the area of DNA fluoresces. FISH can be performed on non-dividing cells as well as actively dividing cells.

Living Cells imaging

Confocal microscopy has been used effectively for the 3D study of dynamics in living cells. However, the imaging of living specimens adds the challenge of maintaining the life and normal function of the organism. There are of course difficulties involved in preparing the sample for viewing as is the case in conventional microscopy. But while performing the experiments effect of photo-damage on the specimen caused due to focused high intensity excitation light must be taken into account. This is complicated by the repeated exposure required for tracking the cellular dynamics—a problem that has worsen 3-D data collection. Fluorescence also introduces the problem of the fluorophore influencing the cell behavior as well as the risk that oxygen molecules reacting with fluorophores in triplet excited states may generate free radicals that damage the cell. Despite the challenges, a wide variety of sophisticated fluorophores have been developed to study different aspects of cell biology. They are designed to mark specific parts of the cell interior and often can simply be introduced to the cell wall. The fluorophores molecules make their way into the cell and attach to the intracellular structures of interest such as the mitochondria and the Golgi apparatus. This is not always the case, however, as some fluorophores must be injected directly into the cell. Labeling is even applied to the study of “nonphysical” structures of the cell, some fluorophores have been developed for the measurement of dynamic processes such as membrane potentials and ion concentrations. Multicolor fluorescence to distinguish between small features such as proteins within a cell it is useful to tag them with different fluorophores and image them as separate colors. There are two ways to do this 1) fluorophores are selected to correspond with the wavelengths of a multiline laser and 2) their response to the same excitation wavelength causes emission at different wavelengths. In both cases the resulting emission is separated with appropriate filters and directed to different detectors. However, there can be cross talk between channels of the emitted light. For most of the commonly used fluorophores there is usually some overlap between their emission spectra, making perfect channel separation impossible by filtering alone. To first order this can be corrected by determining the level of overlap of emission of each individual fluorophores into the channels of the other fluorophores and subtracting it out mathematically.

Thus, confocal microscopy can serve as a valuable tool for live cell imaging.

Clinically, CLSM is used in the evaluation of various eye diseases, and is particularly useful for imaging, qualitative analysis, and quantification of endothelial cells of the cornea (Patel and McGhee, 2007). It is used for localizing and identifying the presence of filamentary fungal elements in the corneal stroma in cases of keratomycosis, enabling rapid diagnosis and thereby early institution of definitive therapy. Research into CLSM techniques for

endoscopic procedures is also showing promise (Hoffman, Goetz et al., 2006). In the pharmaceutical industry, it was recommended to follow the manufacturing process of thin film pharmaceutical forms, to control the quality and uniformity of the drug distribution (Le Person, Puigalli et al., 1998). CLSM is also used as the data retrieval mechanism in some 3D optical data storage systems and has helped determine the age of the Magdalen papyrus.

Advantages:

Confocal microscopy offers several distinct advantages over traditional widefield fluorescence microscopy-

- The primary advantage of laser scanning confocal microscopy is the ability to serially produce thin (0.5 to 1.5 micrometer) optical sections through fluorescent specimens that have a thickness ranging up to 50 micrometers or more.
- The ability to control depth of field.
- The most important feature of a confocal microscope is the capability of isolating and collecting a plane of focus from within a sample, thus eliminating the out of focus "haze" normally seen with a fluorescent sample. Fine detail is often obscured by the haze and cannot be detected in a non-confocal, fluorescent microscope
- Elimination or reduction of background information away from the focal plane (that leads to image degradation) - The most important feature of a confocal microscope is the capability of isolating and collecting a plane of focus from within a sample, thus eliminating the out of focus "haze" normally seen with a fluorescent sample. Fine detail is often obscured by the haze and cannot be detected in a non-confocal, fluorescent microscope.
- The capability to collect serial optical sections from thick specimens- this is due to the fact that with the confocal microscope, the z-resolution, or optical sectioning thickness, depends on a number of factors: the wavelength of the excitation/emission light, pinhole size, numerical aperture of the objective lens, refractive index of components in the light path and the alignment of the instrument.
- The confocal microscope has a stepper motor attached to the fine focus, enabling the collection of a series of images through a three dimensional object. These images can then be used for a two or three dimensional reconstruction.
- Double and triple labels can be collected with a confocal microscope. Since these images are collected from an optical plane within the sample, precise co-localizations can be performed.
- Confocal reflection microscopy can be utilized to gather additional information from a specimen with relatively little extra effort, since the technique requires minimum specimen preparation and instrument re-configuration. In addition, information from unstained tissues is readily available with confocal reflection microscopy, as is data from tissues labeled with probes that reflect light. The method can also be utilized in combination with more common classical fluorescence techniques. Examples of the latter application are detection of unlabeled cells in a population of fluorescently labeled cells and for imaging the interactions between fluorescently labeled cells growing on opaque, patterned substrata.
- Has the ability to compensate for autofluorescence.

The basic key to the above mentioned advantages is the use of spatial filtering techniques in confocal microscope to eliminate out-of-focus light or glare in specimens whose thickness exceeds the dimensions of the focal plane.

Disadvantages:

Co-localization of fluorophores in Confocal Microscope - Two or more fluorescence emission signals can often overlap in digital images recorded by confocal microscopy due to their close proximity within the specimen. This effect is known as **colocalization** and usually occurs when fluorescently labeled molecules bind to targets that lie in very close or identical spatial positions.

8. Summary

A confocal microscope provides a significant imaging improvement over conventional microscopes. It creates sharper, more detailed 2D images, and allows collection of data in three dimensions. In biological applications it is especially useful for measuring dynamic processes. A number of designs have been developed to achieve video-rate confocal microscopy, which enables the capture of short-timescale dynamics.

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Ever since the invention of laser by Schawlow and Townes in 1958, various innovative ideas of laser-based applications emerge very year. At the same time, scientists and engineers keep on improving laser's power density, size, and cost which patch up the gap between theories and implementations. More importantly, our everyday life is changed and influenced by lasers even though we may not be fully aware of its existence. For example, it is there in cross-continent phone calls, price tag scanning in supermarkets, pointers in the classrooms, printers in the offices, accurate metal cutting in machine shops, etc. In this volume, we focus the recent developments related to laser scanning, a very powerful technique used in features detection and measurement. We invited researchers who do fundamental works in laser scanning theories or apply the principles of laser scanning to tackle problems encountered in medicine, geodesic survey, biology and archaeology. Twenty-eight chapters contributed by authors around the world to constitute this comprehensive book.

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