Article ID: 1004-4213(2011)05-0667-6

Design and Optimization in Constructing An In-vivo Confocal Laser Scanning Microscopy

LI Zhao-hui

(Changchun Institute of Optics, fine Mechanics and Physics, Chinese Academy of Sciences, Changchun 130033, China)

Abstract: Confocal laser scanning microscopy(CLSM) is an invaluable tool for a wide range of investigations in the biological and medical sciences. A type construction of laser confocal scanning and fluorescent detection system was provided. A polygon mirror and a galvanometer scanner were used to implement *x*-*y* scan. The relay optical system was designed based on commercial lens with optimized consideration to achieve small scan spot size, large Field-of-View and high efficiency. Fluorescence was detected by a photomultiplier tube with excellent signal-to-noise ratio. The scan system control and image acquisition were designed with an optimized scan velocity and sample clock. All these measures could reduce the blurring effect in the whole imaging process and improve the resolution. The analysis results show that the design of the CLSM is reasonable and all components achieve an optimized consideration for in-vivo scanning imaging; this type of CLSM are easily accessible and can be upgraded according to optical requirements; the performance is comparable to available commercial products, but is superior in many aspects of cost, flexibility and versatility.

Key words: Confocal Laser Scanning Microscopy(CLSM); Optical scanner; Scanning and data acquiring control

CLCN: TP702 **Document Code**: A **doi**: 10.3788/gzxb20114005.0667

0 Introduction

Confocal Laser Scanning Microscopy (CLSM) has been applied in many disciplines ranging from biology and medicine to materials science. By rejection of out-of-focus blur and scattering light, the confocal microscopy permits sharp depth imaging for thick specimens, and provides increasing resolution and SNR as to the conventional microscopy. Although commercial laser scanning systems are available, there are several advantages for custom-built CLSM such as being cost-effective, flexible to multimode imaging needs, and scalable, etc[1-2].

Modern CLSM commonly has reflectance type and fluorescence type. For these two models, the illuminated focused diffraction-limited spot scan a transverse *x*-*y* plane of the sample, scattered light or resulting fluorescence in the illuminated region is detected.

Different scanning mechanisms yield different laser scanning microscopes. Of these, only five ones have found widespread application: 1) scanning pinhole disk systems; 2) use of acousto-optic devices (AOD) for rapid *x*-scanning of a laser spot; 3) slit-scanning systems; 4) use of a resonant galvanometer mirror allowing *x*-scanning of a laser spot at video rate (8 kHz); 5) use of polygon mirror.

Each of these approaches have their relative technical advantages and limitations for special applications. For our case, in order to make the study cell reaction to the stimulation executed by optical tweezer intuitively and accurately, we need to combine the fast 3D imaging system to visualize the measuring process. After comparing different scanning imaging systems in performance, restricts of special application, and budget etc., we selected a polygon mirror and a galvanometer scanner implementing *x*-*y* scan. This system constitution offers excellent performances of high-speed scanning and cost-effective, however, it has fundamental drawbacks, for example, introducing off-axis aberrations in *x*-scan process, wobbling of polygon scanner, scan uniformity, reliability, etc. Careful consideration and trade-off should be made

Foundation item: The National Natural Science Foundation of China (XXXX) and

Author: LI Zhao-hui (1969-), male, associate research fellow, Ph. D. degree, mainly focuses on optical instrument design. Email: Lizh_ciom@yahoo.com.cn

Received date: 2010-10-11 **Revised date**: 2011-01-13

to get an optimum scanning imaging system[3-4].

Each aspect in the CLSM based on polygon mirror and galvonometer scanner contributes to the image quality, such as optics aberration, scan system accuracy, electronics of control system, and data acquiring and processing, etc. Among all the parameters of a CLSM, scan speed substantively play a key role which determines the complexity and performance of an apparatus of confocal scanning imaging.

In this paper, we deal with some of the effects that are due to quickly scanning in the case of CLSM based on polygon mirror and galvonometer scanner, particularly, address the question how to remove the blurring caused by light spot moving in the period of exposure or sampling time. Our purpose in this study is to find an optimised design result compromise considering the relationship between scan optics parameters and sync control electrics to reduce the smearing effect and increase the resolution of digital images.

1 Instrumentation

1.1 Overview

The schematic of the confocal laser scanning microscopy is shown as Fig. 1[5-6].

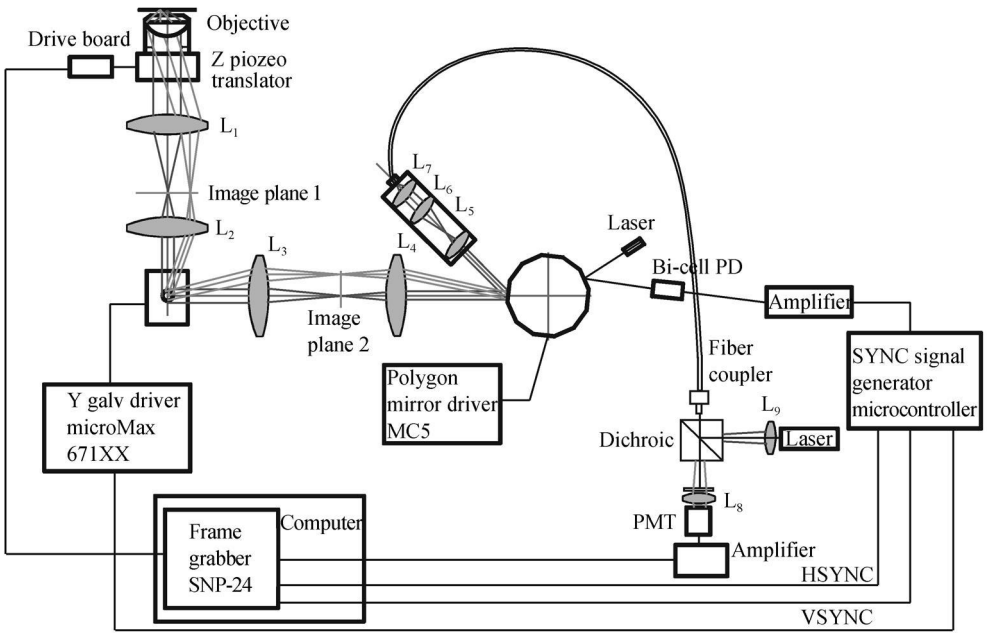


Fig. 1 Schematic of the system layout

This confocal microscope is designed for in vivo fluorescence imaging of a biological tissue. For reflectance work mode, wave plate, polarizer and polarizing beam splitter (PBS) will be needed to reject specular reflections and stray light. We can also insert the two-photon imaging module.

Excitation laser beam from fiber is scanned in 3-D with polygonal mirror, galvanometer-driven mirror, and piezoelectric translator. Emission light from the specimen is collected at PMT.

The microscope objective is an Olympus UApo 20X, 1.0 (NA), working distance 1.8 mm, infinity-corrected, water-immersion objective. An argon-ion laser operating at 488nm is used as the light source excite the sample to emit the fluorescence at about 520 nm. The laser beam is delivered through a single mode fiber (other types of optical fiber used were multi-mode optical fiber and fiber coupler) which both acts as a point source of spherical waves providing a diffraction limited spot and servers as a detected pinhole. The light passes through a beam expander that increases the diameter to satisfy the requirement of scan resolution, reflected by a dichroic filter, and illuminates one of the facets of the polygonal mirror. The 488-nm light is raster scanned horizontally (x-direction) by the polygonal mirror (SA24C model, BMG-5 controller, Lincolnlaser Corp.) and vertically (y-direction) by a galvanometer mirror (Model 6210, controller: MicroMax 67121, Cambridge Technology Inc.) across the sample. The relay lenses make the x, y scanner conjugate respectively and imaged the scanning pivot point onto the back pupil of the objective for uniform illumination of the entire field of view. To perform 3-D volume imaging, z-axis focusing is accomplished by a computer controlled piezoelectric objective translator.

1.2 Relay lenses

In order to reduce the effect of vignette arose

from scanning, the scan mirror has to pivot around the entrance aperture of the microscope objective, and retain the same degree of overfilling of the microscope entrance aperture whenever the beam is scanning. To fulfill the two requirements, the objective entrance aperture should be imaged onto the center of the two scanners, which can ensure a small tilting of the mirror around its nominal position will result in a corresponding change of only the direction of the laser beam at the position of the objective entrance aperture.

An afocal optical system is designed to couple the two scan mirrors in the experiment setup, and the scan mirror location is made conjugate to the entrance pupil of the microscope objective.

Relay lenses are constructed using two achromatic doublets (Thorlab) to make up a telescope. One telescope is inserted between the two scan mirrors and images the polygon mirror onto the galvanometer mirror with the proper magnification factor. The other telescope is between the objective and the galvanometric scanner to image the rear aperture on the galvanometer mirror.

The focal lengths of the two telescopes are f_1= 200 mm, f_2= 100 mm, f_3= 100 mm, f_4= 50 mm, respectively. The resultant transverse magnification is 4-fold. This total magnification factor should be chosen to expand the light beam to overfill the aperture of the objective, and reduce the geometric space as possible.

1.3 Scan optics

The heart of the whole confocal imaging system is the scanning subsystem.

The optimum parameters of the two scanners, such as scan angle, rotation speed and scan beam diameter, should be chosen according to image quality, sampling rate, geometric space, alignment and cost, etc.

Typically, the number of resolvable spots per scanning line at a focal plane, can be expressed as

$$N = L/d = D_{max}/ \quad (1)$$

Where a laser beam with diameter D enters the polygon facet and $_{max}$ is the total optical scan range, is the maximum wavelength applied in CLSM, and d is the focused spot size. The total number of resolvable spots for our microscanner illuminated with 488 nm light is approximately 110　82. The lateral resolution r_A = 0.61 / NA = 0.3　m (for NA = 1,　= 520 nm) require the incident light beam on the polygon D> 1.5 mm (Expanding 4-fold to a diameter of 6mm at the entrance pupil of the objective). Simultaneously, D is restricted by the microscope objective aperture, which should overfill the rear pupil after magnified by the whole optical system. Increasing light beam diameter D will improve the scan resolution, but leads to the larger polygon mirror size, which will decrease the response time of scanning for big inertia.

We determine a polygon mirror with 36 facets. The 36-faceted polygon is 70mm in diameter and 6mm in height, resulting in a single facet clear aperture of approximately 6 mm　6 mm.

In such a scanning optical system, the intersection of reflected beam with the plane of the facet mirror is displaced by a distance. That is the center of scan of the reflected beam that is no longer a stationary point but a locus(Fig. 2)[7].

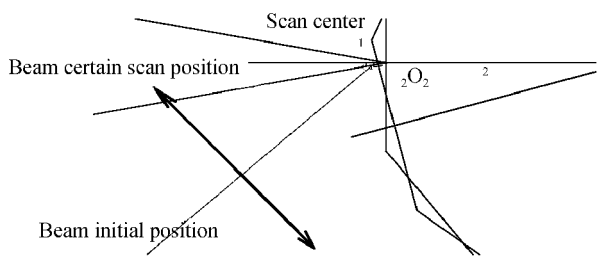


Fig. 2　Effects of polygon rotation to scan beam

In addition, manufacture error for different facet, mechanical component and alignment error all make the center of reflection wobbling in an offset range.

To decrease the amplitude of these errors, we adopt the polygon scanner itself as a master clock to synchronize the x, y-scanners, the objective translator, and the PMTs. The component to generate trigger pulse is shown in Fig. 3.

A 650 nm laser diode illuminates one facet of the polygon mirror ahead of the main beam. And its reflection scans across detected by a bi-cell photodiode (Advanced Photonix: SD3113-24-21-021). The signal from the photodiode is differentially amplified (Analog Device: AD811) before being converted to TTL levels by Schemitt trigger (74ACT14). After proper delay, the output TTL pulse corresponds to a fixed position within a scan line both servers as HSYNC signal input of dat acquisition card (SNAPPER-PCI-24) and input of Microcontroller (PIC18F458). Microcontroller acts as a programmable counter and yields VSYNC signal upon the reset condition set in software. Horizontal and vertical synchronization pulses and a pixel clock signal are

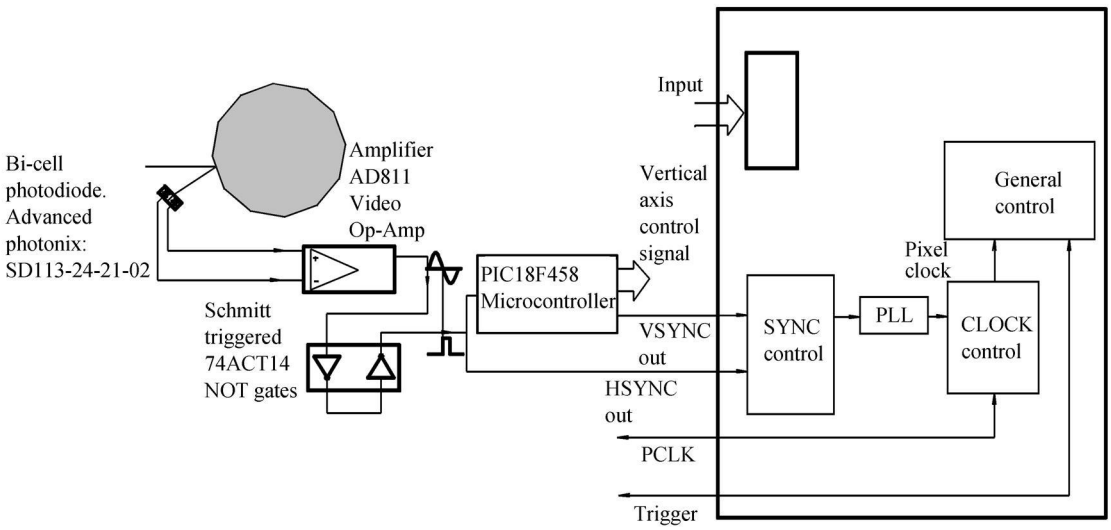


Fig. 3 Unit of generating trigger pulse

used to synchronize capture of the digital data by the data acquisition board. Control program written in Visual C++ set up the parameters of sampling and scanning, such as number of line per image, amplitude of the scan on the vertical axis, frame rate.

Finally, the pixel clock signal is generated internally on the acquisition card by frequency multiplying the HSYNC signal through a phase-locked loop.

1.4 Detection system

The focused light spot excites the biological sample to emit 520 nm fluorescence. Fluorescence emission from the specimen is collected by the same objective, descanned by the scan mirror, and, after passing back through appropriate dichroic beam splitters (NT47-412, = 506 nm, Edmundoptics. Inc) and 515nm cut-off long pass filter, is imaged by a double lens into the photomultiplier tube (PMT) detector (H7422-40, Hamamatsu), with wavelength coverage up to 850nm. The current signal from PMT is sent to transimpedance amplifier (Hamamatsu C6438-01) to convert into voltage signal, and then sampled at approximately 1MHz with a 12-bit AD converter on the frame grabber board (Active Silicon: Snapper-PCI-24).

1.5 Consideration of imaging speed of CLSM

There have been various efforts in improving image quality for CLSM. Although optical aberration, signal intensity and data processing etc. are all important issues, time specification play a much more role in the design of our rapid scan imaging system. Here emphases tradeoff consideration in the design of scanner and image sampling system. Given incident light angle of polygon mirror = 45: as for 0.3 m lateral resolution, and we sample 250 pixels, so FOV= 75 m, the duty cycle is

$$_{\text{scan}} = \arctan\left(\frac{\text{FOV}\ \frac{f_1}{f_0}\ \frac{f_3}{f_2}}{f_4}\right) = \arctan\left(\frac{\text{FOV}\ f_1}{2f_4}\right) = 8.53 \quad (2)$$

$$C = \frac{_{\text{scan}}}{} \quad 100\% = 42.65\% \quad (3)$$

If we operate the polygonal scanner at 10K RPM, for n= 36, then the active scan time(unit: s) is

$$T_{\text{scan}} = \frac{60/(10\ \ 10^3)}{36} \quad C = 0.71 \quad 10^{-4} \quad (4)$$

If we sample a 250 250 pixels image, then we get dwell time on one pixel T_{pixel} (= 0.284 s). The timing diagram for image formation is shown in Fig. 4.

The sampling rate of A/D conversion must be over than Nyquist sampling criterion, which is chosen to ensure all the signals from the incoming photons are sampled twice for every resolvable point. Since the period of Tpixel require data acquisition card have more than 7MHz sampling rate, we adopted a frame grabber with the maximum 20MHz of sampling rate.

In CLSM system, the focused light spot is always moving, even when the receiver is sampling the reflected signal. So the relative displacement will result in blur of spot image. We denote the rotate angle of polygon mirror at the period of sampling as $_x$, the corresponding spot shift on the object plane become $T_x = f\ _x/$, where is magnification of relay optics combined with the objective lens.

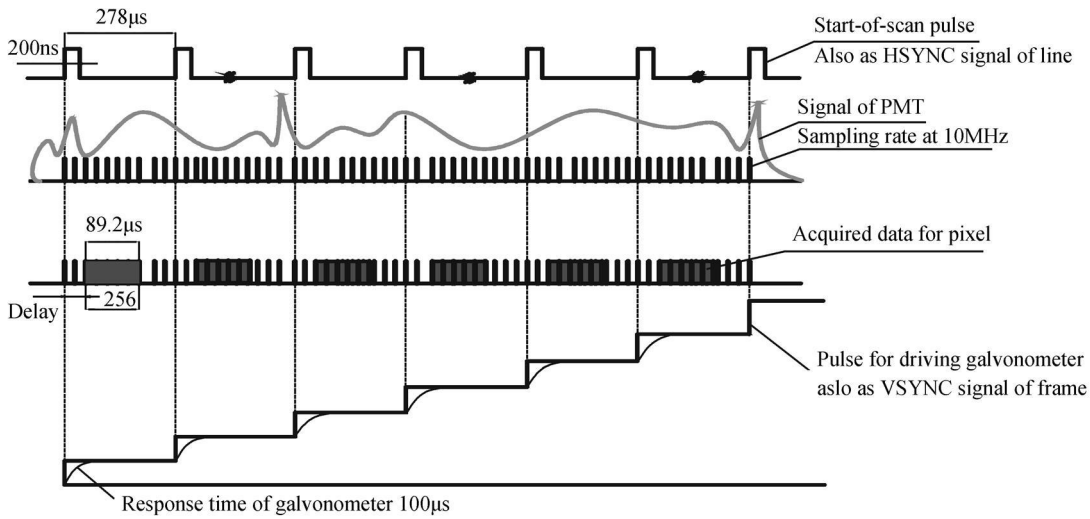


Fig. 4　Timing for data acquisition

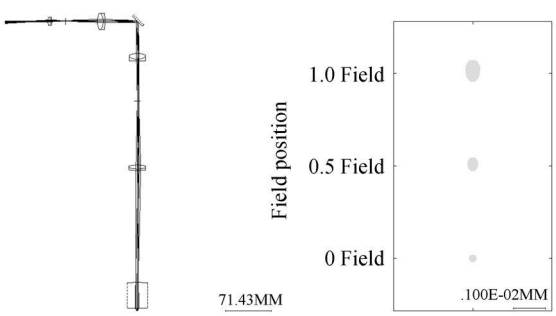
Considering signal energy level, we choose 10 MHzas sampling frequency, the translation of focused light spot is about T = 0. 05 m and 0. 1 m at spin rate of 6 Krpm and 12 Krpm respectively of the polygon mirror[8-9].

2 Performance estimate

Ideally, for a confocal system with 515 nm illumination using a 1. 0-NA water immersed objective, the lateral resolution is about 0. 3 m. The actual resolutions are much larger in that systems because of residual system aberrations in the relay optics and objective lens[10-11].

The introduction of relay lenses will degrade the image quality and limit the scan angle. To make the deterioration from the relay optics negligible, the size of the point spread function should be as small as possible.

We make a simple model for relay optics with CODE V and determine the maximum scan angle without degradation of point-spread-function, the results are shown in Fig. 5. Here the microscope objective is assumed being an ideal module without aberration.



(a) Schematic diagram of the relay optics　(b) Spot diagram analysis

Fig. 5　Scan optical system layout

At deflection angle of 2 degrees we could see that the focused spot distribution still keep symmetry and uniformity. 3 degrees deflection brings mainly coma aberration and field curvature, make the scan spot elliptic, the RMS diameter is diffused to 0. 4 m. We can see from here that it is important to design a custom relay lenses to maintain performances in the beam scanning.

So 3 degree scan angle can converts to approximately 75 m of the field of view (FOV) by the magnification factor of the microscope objective. 75 m 75 m image field which can monitor tens of cells is enough for our experiment occasion. The FOV become 10 mm at intermediate image plane, then the galvonometer should scan an angle 2. 86 degrees.

3 Conclusion

Now we are not equipped with a stage for axial scanning. The stage will make it possible to 3D image of small sized structure. Moreover, software of data acquiring and image formation will be improved for real time imaging and highest lateral resolution. Continuing efforts will be made forward small structure, portable and clinical instrument.

A benchtop prototype of fiber scanning confocal micoroscope has been constructed in which some consideration and optimization have been proposed. The tradeoff design between scanning velocity and imaging quality should be made to ensure expected resolution since the light spot will get blur at the sampling period. An intermediate optics including relay lens and scanning mirror are designed and its impacts on image formation are analysed. The simulation results show that the modulation transfer function

of the system is not varied during scanning. This CLSM may be developed for other kinds of imaging instrument for diagnosis in biomedicine imaging, modern semiconductor and nanotechnology industries.

References

[1] MULLER M, SCHMIDT J, MIRONOV S L, *et al*. Construction and performance of a custom-built two-photon laser scanning system[J]. *J Phys D: Appl Phys*, 2003, **36**(14): 1747-1757.

[2] CHOU D R, BOWER B A, WAX A. Low-cost, scalable laser scanning module for real-time reflectance and fluorescence confocal microscopy[J]. *Applied Optics*, 2005, **44**(11): 2013-2018.

[3] XI P, RAJWA B, JONES J T, *et al*. The design and construction of a cost-efficient confocal laser scanning microscope[J]. *Am J Phys*, 2007, **75**(3): 203-207.

[4] QIU Z R, QIN J, ZHANG H W, *et al*. An optimized design of a scanning confocal probe[C]. *SPIE*, 2006, **6288**: 0X01-0X09.

[5] CALLAMARAS N, PARKER I. Construction of a confocal microscope for real-time *x*-*y* and *x*-*z* imaging[J]. *Cell Calcium*, 1999, **26**(6): 271-279.

[6] LIANG C, DESCOUR M R, SUNG K B, *et al*. Fiber confocal reflectance microscope (FCRM) for *in-vivo* imaging[J]. *Opt Express*, 2001, **9**(13): 821-830.

[7] LEYBAERT L, de MEYER A, MABILDE C. A simple and practical method to acquire geometrically correct images with resonant scanning-based line scanning in a custom-built video-rate laser scanning microscope[J]. *Journal of Microscopy*, 2005, **219**(3): 133-140.

[8] IM K B, HAN S, PARK H, *et al*. Simple high-speed confocal line-scanning Microscope[J]. *Optics Express*, 2005, **13**(13): 5151-5156.

[9] LI Z, HERRMANN K, POHLENZ F. Lateral scanning confocal microscopy for the determination of in-plane displacements of microelectromechanical systems devices[J]. *Optics Letters*, 2007, **32**(12): 1473-1475.

[10] SANDERSON M J. Acquisition of multiple real-time images for laser scanning microscopy[J]. *Microscopy and analysis*, 2004, **7**(4): 17-23.

[11] BAE Y S, LEE D, MOON S, *et al*. Confocal Fluorescence lifetime imaging microscopy based on a realtime sampling method[C]. *SPIE*, 2007, **6443**: 0W-1-0W-8.

激光扫描实时共聚焦显微成像系统设计

李朝辉

(中国科学院长春光学精密机械与物理研究所, 长春 130033)

摘 要: 共聚焦扫描显微镜已成为生物医学和材料科学领域研究中非常有价值的一种工具. 本文给出了一种反射型激光扫描共聚焦显微成像系统的系统结构和具体设计. 采用多面体转镜进行水平扫描, 摆镜进行垂直扫描. 利用商品透镜设计了光学扫描中继系统, 采用光电倍增管作为激发出的荧光探测器, 同时给出了数据采集和扫描同步控制系统的组成与设计. 利用 CODE V 优化光学扫描系统以获得尽可能小的扫描光斑尺寸和较大的视场, 并综合考虑了采样频率、扫描速度和探测器对整个系统性能的影响, 从而给出了该型共聚焦显微成像系统的相互匹配的设计参量. 分析结果表明: 共聚焦扫描系统设计合理可行; 从光学扫描系统到 PMT 探测单元的各项技术指标得到优化, 满足实时探测的要求; 该系统具有适应性强, 易升级, 低成本的技术特点, 同时可达到同类商品的技术性能.

关键词: 共聚焦显微镜; 光学扫描系统; 同步采集控制