The design and construction of a cost-efficient confocal laser scanning microscope

Peng Xi[a] and Bartlomiej Rajwa
Purdue University Cytometry Laboratories, Bindley Bioscience Center, Purdue University, West Lafayette, Indiana 47907

James T. Jones and J. Paul Robinson
Weldon School of Biomedical Engineering, Purdue University, West Lafayette, Indiana 47907

(Received 2 July 2006; accepted 13 October 2006)

The optical dissection ability of confocal microscopy makes it a powerful tool for biological materials. However, the cost and complexity of confocal scanning laser microscopy hinders its wide application in education. We describe the construction of a simplified confocal scanning laser microscope and demonstrate three-dimensional projection based on cost-efficient commercial hardware, together with available open source software. © *2007 American Association of Physics Teachers.*
[DOI: 10.1119/1.2388973]

I. INTRODUCTION

Confocal scanning laser microscopy is a very successful tool that has greatly impacted biological research due to its superior axial and lateral resolution over wide-field imaging.[1–4] However, commercially available confocal systems are too expensive for most individual educators.[5] In state-of-the-art biological laboratories with a confocal system, the instrument is usually operated by a technician or is so heavily utilized by researchers that it is impossible to allow students to modify it or use it as a teaching tool.

In this paper we describe a low-cost method of constructing a confocal scanning laser microscopy system. Lateral resolution is measured with a fine-pattern slide, and axial dissection capability is evaluated by a stationary reflection measurement. We find that the low-cost system shows good capability in depth-resolved scanning and 3D reconstruction. Therefore, this system should be valuable for educators to demonstrate the principles of confocal microscopy, as well as for researchers on a modest budget.

II. FUNDAMENTALS OF CONFOCAL MICROSCOPY

Traditional wide-field imaging microscopy employs a flat (2D) detector such as a film, a CCD or CMOS sensor, or the retina of the eye. This imaging mode lets us see clearly when the target falls within the depth of field, while the targets out of the depth of field are blurred but still contribute intensity to the detector, as shown in Fig. 1(a). As a result, we cannot obtain 3D information from this configuration. Confocal microscopy is able to achieve 3D information by sacrificing the area of view first. From Fig. 1(b) we see that only one point is illuminated and that the signal from this point is exclusively collected. Then, by the relative movement of the detecting point and the target, 3D information can be collected.

Because confocal microscopy detects only one point at a time, lateral and axial resolution are enhanced compared to that of wide-field microscopy. The diffraction-limited resolution of a confocal system has been discussed extensively.[2] When the illumination and detection employ the same objective and the same wavelength, the lateral resolution can be expressed by the full width at half maximum (FWHM) of the confocal lateral point-spread function:

$$r_{xy} = \frac{0.61\lambda}{\sqrt{2}\mathrm{NA}}, \tag{1}$$

where r_{xy} is the lateral resolution, λ is the incident wavelength, and NA is the numerical aperture of the objective. The scanning angle can be calculated by

$$\theta = \pm \arctan\frac{\ell/2}{f_{\mathrm{ep}}}, \tag{2}$$

where ℓ is the scanning length at the image plane of the objective, which is overlapped with the focal plane of the eyepiece, and f_{ep} is the focal length of the eyepiece. For example, in our setup a Nikon Fluor 40×, NA 1.30 oil-immersion objective was used. For a 256×256 image, the scanning size ℓ can be set to 5.25 mm for an imaging size of 131 μm under a 40× objective. An eyepiece is commonly used in a confocal setup as the scanning lens, because the spatial aberration of an eyepiece is corrected optically. For the 10× eyepiece, in which the nominal focal length f_{ep} is 25 mm, the optical scanning angle is ±6°. Because the scanning mirrors of the galvanometer mechanically rotate 1°/V, and the optical scanning angle is twice that of the mechanical, the driving voltage should be ±3 V. The pinhole size is related to the size of the Airy disk projected onto the pinhole plane for the optimal resolution and intensity. The focal spot diameter on the detection fiber plane can be calculated from

$$d_p = 2\frac{f_{\mathrm{dobj}}}{f_{\mathrm{ep}}}M_{\mathrm{obj}}\frac{0.61\lambda}{\mathrm{NA}_{\mathrm{obj}}}, \tag{3}$$

where f_{dobj} is the focal length of the detection lens, and M_{obj} is the magnification of the objective.

In our setup the fiber core of the optical fiber plays the role of the confocal pinhole because it transmits only the light illuminated on the core. For a 10× eyepiece and detection objective with f_{dobj}=11 mm, the pinhole size can be chosen to be 10.46 μm for an objective of NA 1.30. We use an optical fiber with a core size of 9 μm. In order to compare the data with that from the Bio-Rad MRC-1024 commercial apparatus, the pinhole size of the MRC-1024 is set to 1.0 mm at 488 nm illumination. Because the MRC-1024 has a system magnification of 64, this configuration corresponds

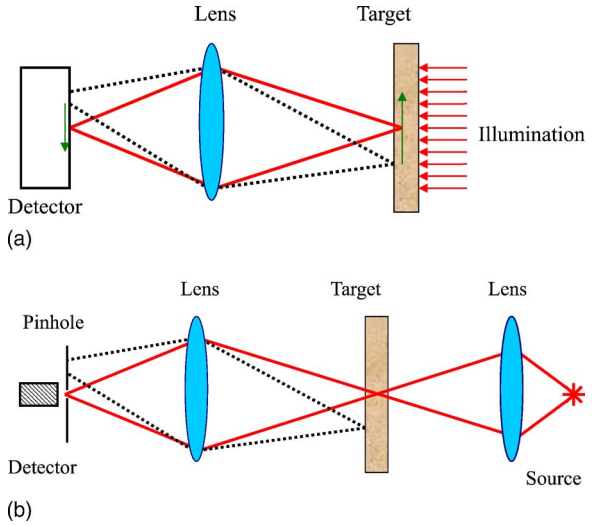


Fig. 1. Different types of microscopy: (a) wide-field microscopy and (b) confocal microscopy.

to the pinhole size of our system.[6] The ideal axial resolution can be evaluated by the FWHM of the axial point-spread function:

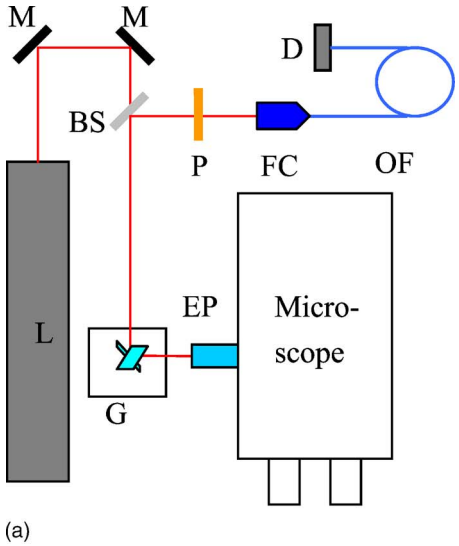
$$r_z = \frac{2n\lambda}{\sqrt{2}NA_{\text{obj}}}, \tag{4}$$

where n is the refractive index of the medium. Thus for a NA 1.30 objective, with a medium refractive index of 1.5 and an illumination wavelength of 633 nm, the resolution is calculated to be 0.8 μm. We note that the sectioning capability of a confocal system is not the result of the enhancement of the axial resolution comparing with wide-field microscopy, but is due to the sharp decrease in the detected intensity when out of focus. The optical dissection capability can be experimentally determined by stationary reflection.[7]

III. SYSTEM CONSTRUCTION

Our confocal scanning laser microscopy system is designed to work with a Nikon Diaphot 200 inverted microscope which has a side imaging port. The microscope cost about $20,000 when purchased new in 1994. An inverted microscope with a side imaging port has the advantage that the system can be set up on a breadboard.[8] The inverted microscope can probably be obtained from a biology department. Physicists could develop the microscopy system in collaboration with biologists so that it can be applied for optics and image reconstruction by physics students and state-of-the-art microscopy for biology students.

Figure 2 illustrates the system configuration. The system uses a He-Ne laser with 633 nm emission. The laser beam is guided onto a pair of galvanometers (6215H, Cambridge Technologies) for horizontal (fast axis) and vertical (slow axis) scanning. The two galvanometric mirrors are located close together and perpendicular to each other. The controlling units for the two galvanometric axes are also commercially provided for the best performance of the galvanometers. A Conix Z-axis motor (CZM, Conix Research) controls the focal depth in the sample with a precision of 0.1 μm. We use a Nikon CFWN 10× eyepiece to construct the scanning



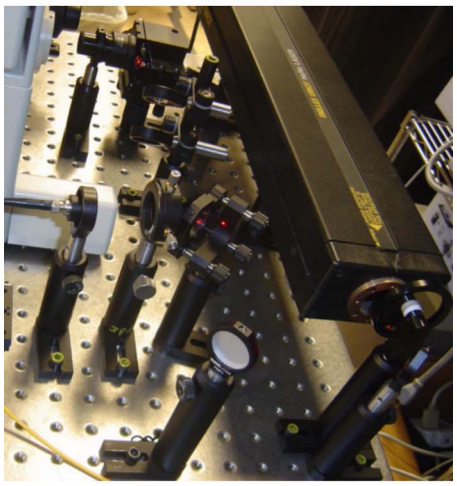


Fig. 2. Confocal scanning laser microscopy system setup: (a) schematic diagram and (b) photo. L: laser; M: reflection mirror; BS: beam splitter; G: galvanometer; EP: eyepiece; P: polarizer; FC: fiber collimator; OF: optical fiber; D: detector.

plane at the side-view port of the Nikon Diaphot 200. The laser is focused onto the sample through the microscopic objective.

The signal from the sample goes back to the galvanometers and is descanned. Thus, the return signal exactly follows the path of incidence. A beam splitter is used to reflect the return signal. A fiber collimator (FC220FC-A, Thorlabs) is used to focus the backscattered signal, and a 9 μm core-size optical fiber (S1-F2 F2-01-0020-GF, Go4Fiber) conducts the signal into a photomultiplier tube (PMT) detector (HC120-13 MOD, Hamamatsu). The application of the optical fiber further reduces the overall cost and simplifies the alignment work by eliminating the mechanical pinhole. A computer power supply is used to provide DC current for the PMT. To connect the optical fiber onto the PMT detecting window, a FC-FC fiber adapter is cut in half and mounted on the PMT window. The fiber also acts as the pinhole for confocal detection. A polarizer placed between the beam splitter and the fiber collimator blocks the specular reflection.

Two frequencies of triangular wave forms are sent to the

Table I. Listing of equipment for the confocal scanning laser microscopy system. The cost listed is for guidance only.

Item	Supplier	Part	Cost	URL
Galvanometers with amplifier	Cambridge Technologies	6215H	2875	www.camtech.com
DC power supply	Tekpower	HY3005-D3	220	www.radios4you.com
Galvanometer controlling and data collection unit	National Instruments	PCI 6251	845	www.ni.com
I/O block	National Instruments	SCB 68	295	www.ni.com
Connection cable	National Instruments	SHC68-68-EPM	95	www.ni.com
Z axis motor	Conix Research	CZM	1715	www.conixresearch.com
Laser	Melles Griot	25 LHP 151-249	770	www.mellesgriot.com
PMT detector	Hamamatsu	HC120-13 MOD	1150	www.hamamatsu.com
PMT power supply	Echo Star	ESTAR-450-N	11	www.geeks.com
Kinetic mount	China Da-heng Group	GCM-080304M (2)	79	www.cdhcorp.com
Polarizer	China Da-heng Group	GLC-050002	18	www.cdhcorp.com
Reflective mirrors	China Da-heng Group	GCC-102002 (4)	52	www.cdhcorp.com
Optical fiber	Go4Fiber	S1-F2 F2-01-0020-GF	5	www.go4fiber.com
FC-FC fiber adapter	Go4Fiber	G020001	2.3	www.go4fiber.com
Fiber collimator	Thorlabs	F220FC-A	124	www.thorlabs.com
Fiber collimator adapter	Thorlabs	AD12F	27	www.thorlabs.com
Beam splitter	Thorlabs	EBS1	27.5	www.thorlabs.com
Posts	Thorlabs	TR075, TR1 (8), TR2 (2)	56	www.thorlabs.com
Post mounts	Thorlabs	PH1-ST, PH2-ST (10)	88.4	www.thorlabs.com
Fixed optical holder	Thorlabs	FMP1 (2)	28	www.thorlabs.com
Lens mount	Thorlabs	LMR1	16.5	www.thorlabs.com
Optical bases	Thorlabs	BA1 (4), BA1S (7)	61	www.thorlabs.com

galvanometers for horizontal and vertical scanning. The driving signal for the galvanometers is provided by a National Instruments PCI 6251 data acquisition system, which also functions as the A/D converter for the photodetector to digitize the analog signal to an 8-bit digital signal for imaging. The signal-driving and data-collection program is programmed with LabVIEW 7. In our experiment the frame rate is 256×256 at 4 fps, so the scanning signals are 512 and 2 Hz, respectively. Because the galvanometers have been fine tuned to have a 142 μs delay in response in both axes, proper delay in the data collection should be added to construct the correct image. The program also controls the Conix Z-axis motor with the RS232 port of the controlling computer. The data are stored as bitmap file sequences.

After data collection, the open source software ImageJ is used to process the images and generate 3D projections of the sample.[9] The 3D projections are stored as bitmap sequences for animation generation. Because commercially available hardware and software are used together with the open source program ImageJ, the overall cost for the system is less than $8,600, only a fraction of the price of a commercial confocal system. Nevertheless, this system provides adequate 3D imaging capability and superior extendibility. The detailed parts list is given in Table I. The custom program and 3D animation can be found on EPAPS.[10]

IV. RESULTS

To demonstrate the image quality of our system, a Cyrix 6x86 CPU microchip was imaged on both a commercial Bio-Rad MRC-1024 system and our low-cost system with the same Nikon Plan 10×, NA 0.25 objective. The laser wavelength of the MRC-1024 system is 488 nm and the pinhole

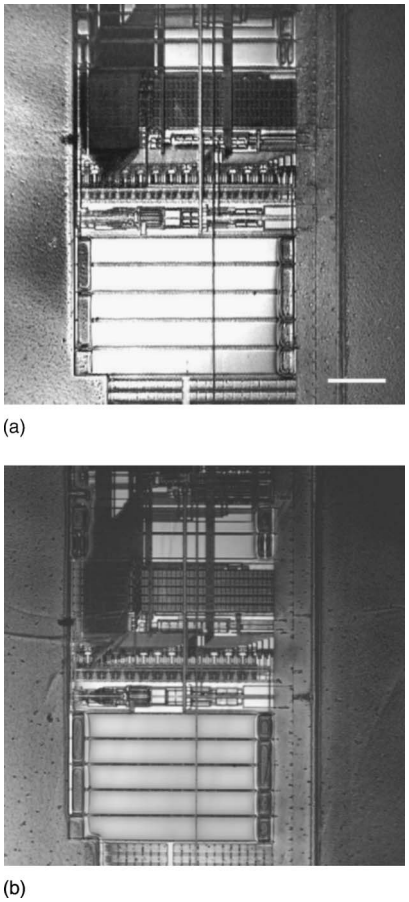


Fig. 3. The microchip image: (a) from our low-cost confocal scanning laser microscopy system and (b) from the commercial Bio-Rad MRC-1024 system. Scale bar: 50 μm.

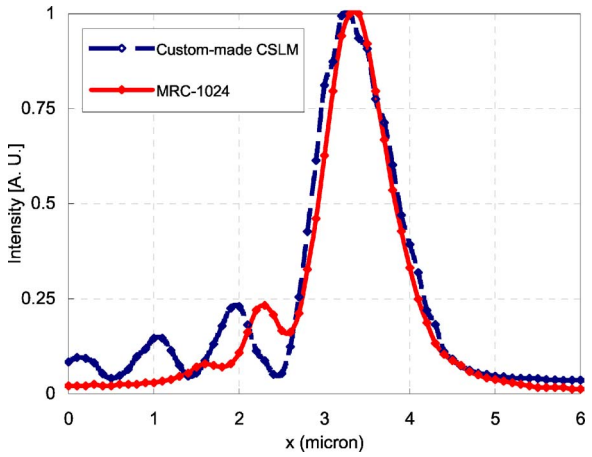


Fig. 4. Measurement of sectioning capability with the mirrored slide reflection.

size is 1.0 mm. The result is shown in Fig. 3 and demonstrates the capability of the confocal scanning laser microscopy to image a nonbiological sample. A Richardson resolution test slide (Richardson Technologies, part No. 80303) was used to calibrate the system and measure the lateral resolution. The finest resolvable pattern was 250 nm with a Nikon Fluor 40×, NA 1.30 oil-immersion objective.

A stationary reflection measurement was processed with a mirrored slide to evaluate the axial dissection capability of our system.[8] The Nikon 40×, NA 1.30 objective was used. From Fig. 4 we can see that the FWHM was measured to be 1.1 μm for our system. The measurement was repeated on the MRC-1024 with the wavelength of 488 nm and pinhole size of 1.0 mm, which corresponds to our setup, with a resulting FWHM of 0.9 μm. The result indicates that our setup is comparable to the usual configuration of a commercial confocal scanning laser microscopy system operating in backscattering light mode.

A fern spore slide was imaged using the Nikon 40×, NA 1.30 oil-immersion objective. The *Z*-axis step was 1 μm per image, and 60 successive images were collected. The driving voltage was ±3 V for both galvanometers. A slice of the depth-resolved backscattered light images is shown in Fig. 5(a). It can be seen that only the objects at the same depth are imaged in the slice. For comparison, a QImaging Retiga 1300i 12-bit digital color CCD camera was used to collect the image of the same spore with a Nikon 1× relay lens. The wide-field color image is shown in Fig. 5(b). After collection

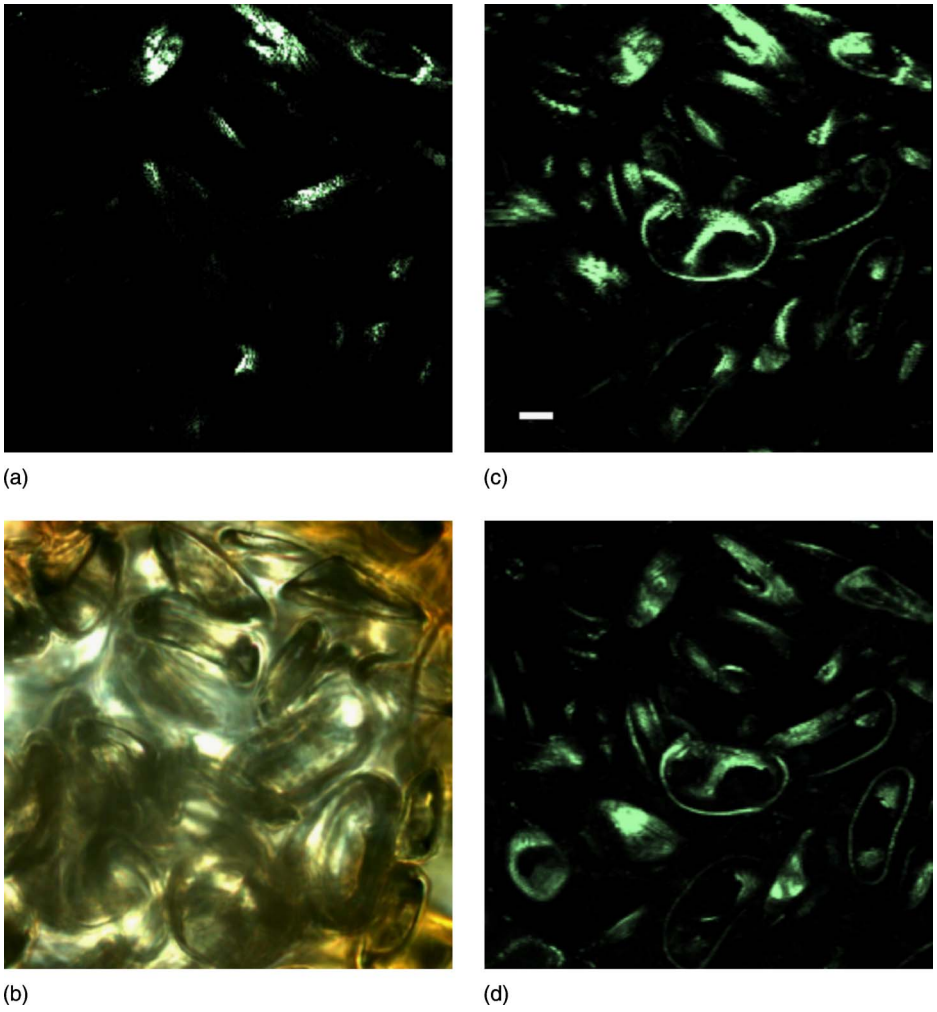


Fig. 5. Fern spore image: (a) depth-resolved backscattered light image from the low-cost confocal scanning laser microscopy instrument. (b) Wide-field image from a QImaging Retiga 1300i digital CCD camera. (c) Axial projection with the images from our confocal scanning laser microscopy setup. (d) Axial projection with the images from the Bio-Rad MRC-1024 system. Scale bar: 10 μm.

of the depth-resolved image, axial projection was performed with ImageJ as shown in Fig. 5(c). The axial projection reveals the shapes of the prothallia from the top view. 3D animations of the fern spore that clearly display the spatial structure and position of each prothallium can be found on EPAPS.[10] Figure 5(d) shows the corresponding results obtained from the Bio-Rad MRC-1024 confocal system with the same objective and pinhole size of 1.0 mm. It can be seen that our confocal scanning laser microscopy system obtains results comparable to the commercial systems.

V. CONCLUSION

We have provided a simple and affordable method for building a confocal scanning laser microscopy system on a modest budget with decent lateral resolution and 3D dissection capability comparable to that of commercial systems. The system can be an excellent tool for giving students hands-on experience in building a confocal system, which can give them a deeper understanding of optics, electronics, microscopy, and system engineering. This work also uses commercial parts and open source software to reduce the budget in areas where laser scanning, fiber coupling, data collection, or image processing is involved.

ACKNOWLEDGMENTS

Resources for the project were provided by funds from the Purdue University Cytometry Labs, the Department of Basic Medical Sciences, and the Weldon School of Biomedical Engineering.

[a]Electronic mail: pengxi@flowcyt.cyto.purdue.edu

[1] *Handbook of Biological Confocal Microscopy*, 2nd ed., edited by J. B. Pawley (Plenum Press, New York, 1995).

[2] R. H. Webb, "Confocal optical microscopy," Rep. Prog. Phys. **59**, 427–471 (1996).

[3] J. P. Robinson, "Confocal microscopy," Cytometry **42**, 128–129 (2000).

[4] J. P. Robinson, "Principles of confocal microscopy," Methods Cell Biol. **63**, 89–106 (2001).

[5] The cost of the Bio-Rad MRC 1024 was $350,000 in 1994.

[6] William Bradshaw Amos, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, private communication, May 2006.

[7] B. Rajwa, "Modern confocal microscopy," in *Current Protocols in Cytometry*, edited by J. P. Robinson, Z. Darzynkiewicz, J. Dobrucki, W. C. Hyun, A. Orfao, and P. S. Rabinovitch (Wiley, Hoboken, NJ, 2005).

[8] I. Parker, N. Callamaras, and W. G. Wier, "A high-resolution, confocal laser-scanning microscope and flash photolysis system for physiological studies," Cell Calcium **21**, 441–452 (1997).

[9] M. D. Abramoff, P. J. Magelhaes, and S. J. Ram, "Image processing with ImageJ," Biophotonics Int. **11**, 36–42 (2004).

[10] See EPAPS Document No. E-AJPIAS-75-012701 for the controlling software and the 3D projection animations of the fern spore specimen. This document can be reached via a direct link in the online article's HTML reference section or via the EPAPS homepage (http://www.aip.org/pubservs/epaps.html).