Design and construction of a confocal laser scanning microscope for biomolecular imaging

S. Arunkarthick, M. M. Bijeesh, Anand Satya Vetcha, Nishith Rastogi, P. Nandakumar and Geetha K. Varier

Confocal fluorescence microscopy has established itself now as an indispensable tool for biomedical and biomolecular research. However, the rigid design and high cost of the commercially available confocal microscopes have limited its wider usage. Here we report on the development of a confocal laser scanning microscope and demonstrate the three-dimensional sectioning capability of the microscope. The design is versatile and cost-effective, and can be constructed using components normally available in an optics laboratory with minimum amount of additional expenditure. Detailed description of the design and alignment procedure is given so that this note can serve as a primer for construction of a simple confocal microscope.

Confocal fluorescence microscopy is one of the most popular and widely used imaging techniques in biology. High sensitivity, spatial resolution and optical sectioning capability make it an ideal tool in the study of biological systems such as membranes, tissues and cells[1–5]. However, most of the commercial confocal fluorescence microscope designs are not versatile enough to be employed in different laboratory configurations or as an educational tool for students in an undergraduate laboratory setting. Further, commercial confocal microscopes are expensive and not affordable to many laboratories, especially in India. The lack of flexibility and high cost of commercial systems have prompted many laboratories to construct their own confocal microscopes optimized for specific requirements[6,7].

Here we report on our design of a simple and flexible confocal fluorescence microscope that can be used for research as well as for undergraduate teaching. An inverted microscope, data acquisition card, laser and optics, normally available in an optics laboratory, are used in the construction. An X–Y galvanometric mirror scanner working at 512 and 2 Hz respectively, is used to raster scan the sample and construct images at a maximum rate of 4 frames/sec. The design is versatile enough to be assembled by students and can be used in both reflection and fluorescence mode. The same set-up can readily be used in other microscopy applications such as photothermal or multiphoton microscopy with minimal modifications. Detailed descriptions of the alignment and calibration procedure are given so that this note can be used as a guide for constructing a confocal laser scanning microscope (CLSM).

Confocal laser scanning microscopy

In conventional wide-field microscopes the entire specimen is illuminated at the same time and the images are acquired by a CCD camera or a photodetector. Even though this helps in fast acquisition of images, it suffers from low *XY* and axial resolution. A confocal microscope makes use of a laser as the excitation source so that it can be tightly focused on the sample. Fluorescence from the focal point alone is passed to a photodetector kept at the conjugate focal plane of the objective lens and an aperture of appropriate dimension blocks all out-of-focus light from reaching the detector. This confocal detection helps in improving the axial resolution of the microscope and results in sharper images. Two-dimensional images of the sample slices are acquired point-by-point either by moving the laser beam over the sample (laser scanning) or by moving the sample (stage scanning) in the *X*–*Y* plane. The stage scanning typically requires larger time to acquire an image and is more expensive compared the laser scanning.

In this work we have used either an Ar-ion laser (488 nm) or a green He–Ne laser (543 nm) as the excitation source. A silicon photomultiplier with a transimpedance pre-amplifier (SensL, Ireland, MiniSM-30035-X08) is used as the detector and fluorescence is coupled to the detector through an optical fibre. The optical fibre also acts as the confocal aperture. The silicon photomultiplier is as sensitive as a normal photomultiplier (PMT) in the 500 nm region of wavelength and does not require a high-voltage power supply. A galvanometric mirror scanner (Cambridge Technologies Inc., USA, 6215H M40) is used to scan the laser beam over the sample and thus to acquire a two-dimensional image of the sample. A data acquisition (DAQ) card (National Instruments Corporation, USA, USB-6251) provides the voltage wave forms for X and Y scan mirrors, acquires the voltage output from the silicon photomultiplier in a synchronized manner, and constructs the image by sequentially digitizing the signal.

An Olympus IX-71 (Olympus Corporation, Japan) inverted microscope equipped with an oil immersion 60X objective of numerical aperture 1.25 is used in the construction of CLSM. A CCTV camera mounted on the right side camera port is used for initial focus adjustments. The left side optical port of the microscope is used as the input port for the excitation laser beam. The scan lens is mounted at the primary image spot, at a distance of 102 mm from the left side optical port. Optical components along with the microscope are tightly mounted on a vibration-free optical bench and the alignment of optical components is achieved with a range of adjustable mounting posts.

The overall layout of the optical system used for the construction is shown in Figure 1. The laser beam is directed to the scanning mirrors (X and Y) by a dichroic mirror (DM). Mirrors M1 and M2 help align the beam centrally through the scan mirrors and to the inverted microscope. The laser beam reflects the two scan mirrors and is focused to the conjugate plane of the microscope by the scan lens (SL). The optics of the microscope relays the scanning spot to the specimen which is appropriately fluorescently labelled. The emitted fluorescence returns along the same pathway and is

de-scanned by the same scan mirrors and transmitted to the detector through the dichroic mirror M3. Mirror M4 directs the fluorescence to a focusing lens (FC). This lens focuses the light to an optical fibre mounted on a translation stage, and the optical fibre guides the fluorescence to the silicon photomultiplier. The optical fibre also acts as the pinhole for the confocal system. One can choose the axial resolution and thereby the thickness of the optical slice using lens of appropriate focal length and fibre core diameter. An additional band pass filter is used to remove the excitation laser leaking through the dichroic mirror. For reflection mode the band pass filter is replaced with a neutral density filter. A second (probe) beam, such as the 800 nm from a Ti : sapphire laser can be introduced through the dichroic M3 so as to have photothermal or multiphoton imaging capability. Table 1 provides details of the components used in CLSM set-up and their cost.

It is important to develop appropriate imaging software for the successful implementation of confocal microscopy. Many open source software are available that can be custom-integrated for confocal microscope applications[8,9]. We have written a simple LabVIEW (National Instruments Corporation, USA)-based software program for image acquisition[10]. The software controls the galvanometric mirrors by providing the necessary voltages and acquires the voltage output of the detector. The scanning is achieved by providing a sawtooth waveform to the scan mirrors. The entire program is divided into four modules. The program in modules 1 and 2 is responsible for system initialization. Module 3 is used for generation of X–Y scanner voltage and triggered voltage measurement. Module 4 is used for post-processing of scanned images such as generation of colour table, saving the scanned image data. Figure 2 *a* shows the front panel of the LabVIEW software used for data and image acquisition along with the images of 100 nm polyester beads taken using the microscope.

Optical alignment of CLSM

It is necessary to develop a routine alignment procedure for CLSM, especially for a homemade set-up where one may need to make frequent changes for conducting experiments in different configurations. In the following we describe a step-wise procedure which will aid in easy, straightforward realignment of the system.

Step 1: First, the microscope is adjusted for uniform Kohler illumination procedure[11]. The microscope focus is fixed on any sample (e.g. standard microparticles or a strand of hair kept between the microscope cover slip and glass slide) by looking through the eyepiece using white light and the condenser lens. The scan lens is positioned approximately at a distance of 102 mm from the left side port of the microscope. Looking through the scan lens, its position is fine-tuned to have a clear, focused and centrally positioned view of the sample. This process is enabled by mounting the scan lens on a translation stage.

Step 2: The scan mirror is approximately levelled with the central axis of the eyepiece by adjusting the height of the scan mirror mounts. The X and Y scan mirrors have a height difference of 0.5 cm. The input to the microscope is at 9 cm from the ground. The optics is aligned at 8.5 cm from the tabletop before the scanner and at 9 cm after the scanner.

Step 3: The objective lens is turned to out of position, and a piece of lens-cleaning tissue paper is placed over the open aperture. The laser beam is centred onto the X-scanning mirror using the mirrors M1 and M2. Then by adjusting the scanner, the beam is reflected normally into the microscope so as to have maximum illumination at the open objective position. Subsequently, the beam location is centred on the objective turret with the adjustments on mirrors M1 and M2, and scanner is fixed.

Step 4: The scanner is activated and positioned at zero position and it is again confirmed that the illumination remains centred on the objective turret. A sawtooth waveform is applied to the X and Y mirrors to observe whether the beam moves symmetrically on the objective turret. Fine iterative adjustments of the scan mirror assembly and mirrors M1 and M2 may be needed to achieve this.

Step 5: The objective lens is placed back in its position and focused on a mirror (as a reflective object) placed on the stage. The laser light reflected from the mirror retraces the path and can be observed on the transmission side of the dichroic mirror. If the mirror is kept at the correct focus of both the microscope objective and the scan lens, then a collimated reflected beam can be observed beyond the dichroic mirror. This beam along its path is traced using the back of a business card and is directed to the mirror M4.

Step 6: The reflected light from mirror M4 is directed to a focusing lens (FC). FC focuses the light to an optical fibre mounted on a translation stage, and the optical fibre guides the signal to the detector. Intensity of the signal reaching

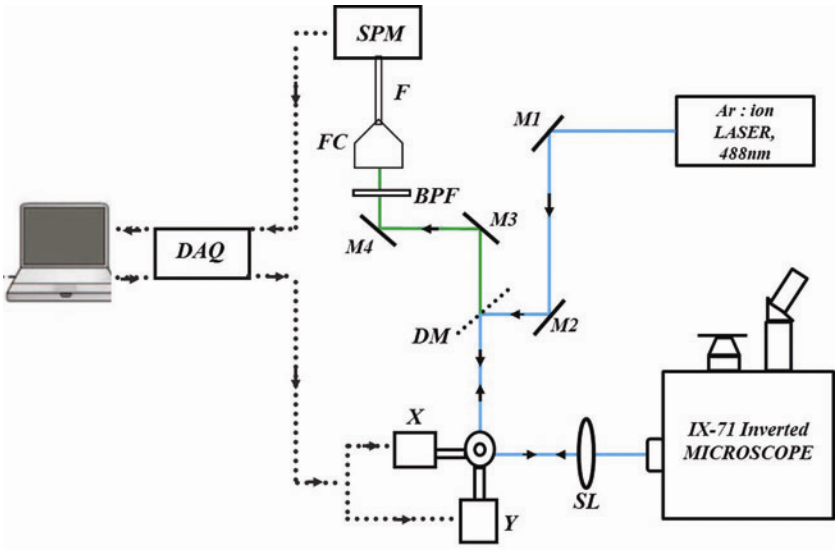

Figure 1. Experimental set-up of the confocal laser scanning microscope. SL, Scan lens; X and Y, Scanning mirrors; DM, Dichroic mirror; FC, Fibre coupler; F, Optical fibre; BPF, Band pass filter; SPM, Silicon photomultiplier; M1–M4, Mirrors; DAQ, Data acquisition card.

Table 1. Cost and components of the confocal laser scanning microscope

Component	Model	Make	Cost (INR)
Galvanometric scanner with power supply	6215H M40	Cambridge Technology Inc., USA	129,153
Microscope	IX71	Olympus Corporation, Japan	495,044
Data acquisition card	NIDAQ USB-6251	National Instruments Corporation, USA	72,443
Ar-ion laser	35-LAP-321-230	CVI – MellesGriot, USA	317,390
Silicon photomultiplier	MiniSM-30035-X08	SensL, Ireland	102,000
Fibre coupler unit	LFC1	HolmarcOpto-Mechatronics Pvt Ltd, India	10,000
Dichroic mirror	LWP-45-Rp-488	CVI – MellesGriot, USA	33,750
Filter	XLP-514.5-25.0M	CVI – MellesGriot, USA	31,275
Mirrors (5)	5108	New Focus, USA	15,075
Posts (10)	P30	HolmarcOpto-Mechatronics Pvt Ltd, India	650
Post mounts (10)	PH30	HolmarcOpto-Mechatronics Pvt Ltd, India	2,050
Mirror mounts (5)	9807 : 1″	New Focus, USA	23,175
Scan lens	WHN10X-H	Olympus Corporation, Japan	7,500
Optical bread board	LL-BB-60*60	HolmarcOpto-Mechatronics Pvt Ltd, India	18,000
		Total cost	1,257,505

the detector is monitored with an oscilloscope and maximized by iteratively adjusting the position of the optical fibre. Once the signal is maximized, the detector is connected to the DAQ card.

Step 7: The mirror is replaced and a sample containing a uniform thin layer of fluorescein or FITC dye is imaged to confirm even illumination. Standard sized fluorescent polystyrene beads are imaged to study the lateral and axial resolution and to verify image quality. Fine adjustments to the mirrors M1 and M2 may be required on a day-to-day basis to optimize the signal.

Standardization of CLSM

To calibrate the microscope against the applied galvanometer voltages, the 1951 USAF Resolution Test Target, 3″ × 3″ (Thorlab, R3L3S1N-Negative) is used. This 3″ × 3″ target has 10 groups (–2 to +7) with 6 elements per group, offering a maximum resolution of 228.0 line pairs/mm. Figure 3 shows the image of 1951 USAF test target with a triangular pulse of 3 V applied to the galvanometric scanner which has the image size of 129 μm. The measurement is repeated with different voltages on the scan mirror and scanning length on the sample per unit voltage is deduced to be 43 μm.

The lateral and axial resolution of the microscope can be determined by studying fluorescent microspheres having a size much less than the resolution of the microscope. We have used 100 nm monodisperse polystyrene beads (Sigma-Aldrich Chemicals Pvt Ltd, USA, 90517-5ML-F) for determining the resolution. The samples were prepared by labelling diluted polystyrene beads with fluorescein dye. Figure 2 *b* shows the lateral intensity profile of a single polystyrene bead having a size of 100 nm. The profile has a full width at half maximum (FWHM) of 254 nm, indicating the lateral resolution of the microscope. In order to determine the axial resolution of the microscope, one may acquire and study the cross-sectional images of a 100 nm polybead at discrete intervals. These image stacks, generally referred to as axial point spread functions, may be analysed to determine the line intensity profile along the *z*-axis. Currently, our microscope does not have automatic *z*-sectioning capability. Hence we have acquired the axial intensity profile by manually moving the microscope objective using the axial fine motion knob of the microscope. The axial intensity profile shown in Figure 2 *c* has a FWHM of 880 nm, approximately three times that of the lateral resolution.

An approximate theoretical resolution for confocal microscope according to the Rayleigh criterion can be expressed as[6,12]

$$\text{Lateral resolution} = \frac{0.61\lambda_{\text{exc}}}{\text{NA}},$$

$$\text{Axial resolution} = \frac{0.88\lambda_{\text{exc}}}{(n - \sqrt{n^2 - \text{NA}^2})},$$

where λ_{exc} is the excitation wavelength of the laser, n is the refractive index of the immersion medium and NA is the numerical aperture. Here it is assumed that the pinhole size is equal to the size of the airy disk projected onto the pinhole plane. The airy disc size on the pinhole plane, and hence the pinhole diameter can be calculated using the relation[6]

$$d_{\text{p}} = 2\frac{f_{\text{FC}}}{f_{\text{SL}}} M_{\text{OBJ}} \frac{0.61\lambda}{\text{NA}},$$

where f_{FC} is the focal length of the detection lens, M_{OBJ} the magnification of the objective and f_{SL} is the focal length of the scan lens. In these experiments we have used a lens of focal length 120 mm to focus the fluorescent beam to an optical fibre of core diameter 400 μm. For a 60X objective having a numerical aperture of 1.25 and 488 nm excitation laser wavelength, the theoretical lateral and axial resolution of the microscope can be calculated as 238 and 648 nm respectively, close to experimentally measured values.

The performance of CLSM has to be evaluated by subjectively assessing a biological test slide for image quality. These inspections are vital if one is interested in making quantitative intensity measurements on experimental biological systems. We have used a dye-labelled pollen grains test slide to study the biological applicability of the microscope.

Dye-labelled pollen grains (model no. 304264) is obtained from M/s Carolina Biologicals, USA. Different *z*-sections of the pollen grains were taken. Optical sections were gathered in approximately 2 μm steps perpendicular to the *z*-axis by moving the objective manually using the axial fine motion knob of the microscope.

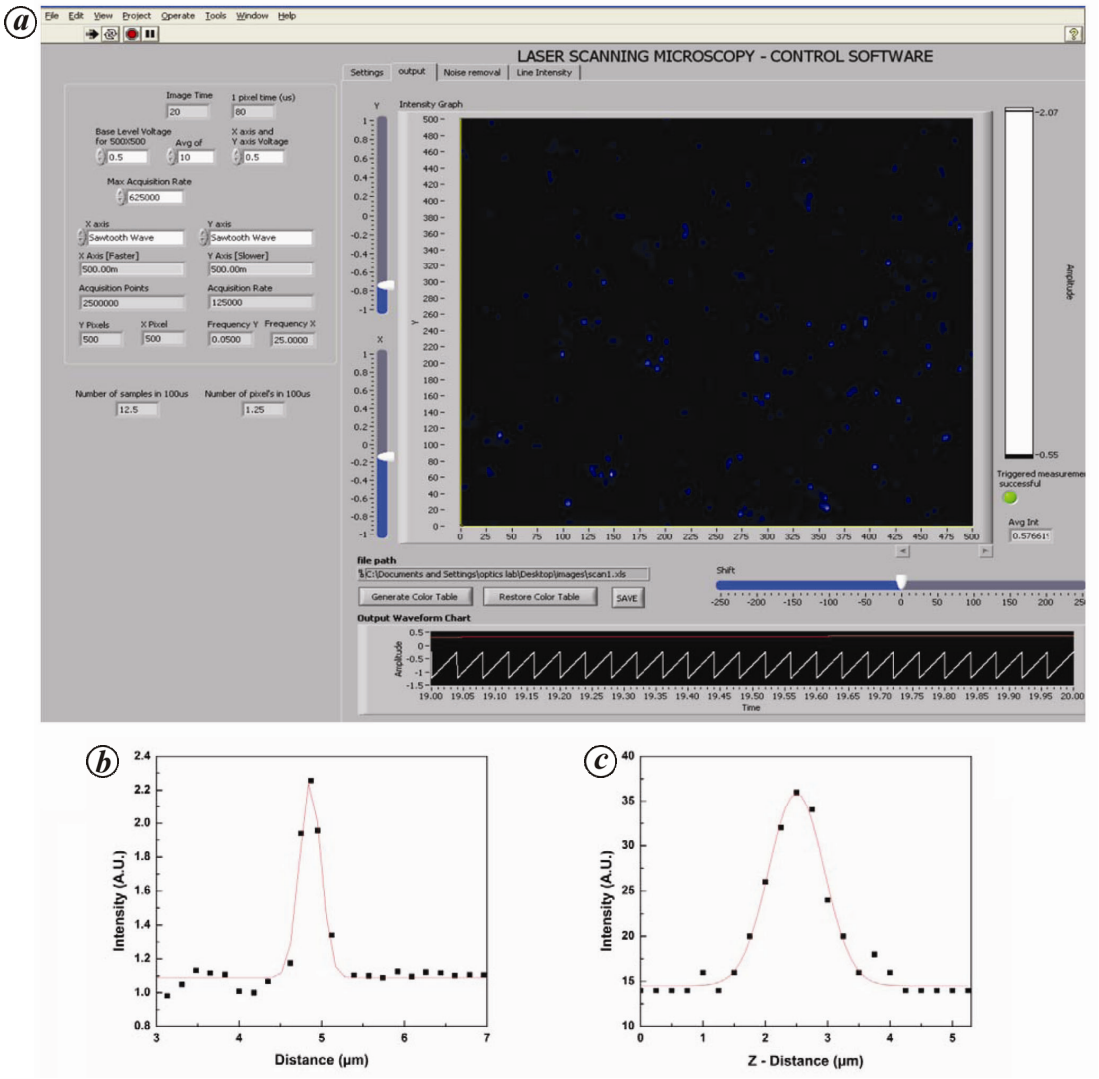

Figure 2. ***a***, Front panel of the LabVIEW software used for data acquisition along with the image of polystyrene beads having a mean size of 100 nm. ***b***, ***c***, Lateral (***b***) and axial (***c***) line intensity profiles of a single 100 nm polystyrene bead.

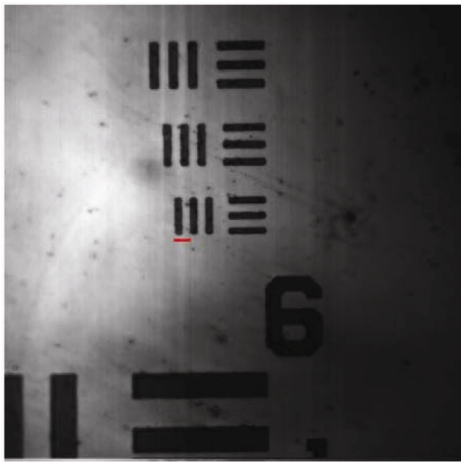

Figure 3. The 500 × 500 pixel image of 1951 USAF test target obtained by providing a saw-tooth waveform of 3V to the scan mirrors. There are 17 pixels between the smallest line pair (red line) corresponding to a specified distance of 1/228 mm. Thus, 500 pixels in the image correspond to 129 μm.

Figure 4 *a* shows ten axial cross-sections of pollen grains. The weak constant background mostly arises from electronic noise and background illumination. Figure 4 *b* shows the 3D reconstruction of the pollen grains from these optical cross-sections using ImageJ software[13]. These images are acquired using 488 nm wavelength line of an Ar-ion laser. The laser power at the objective focus plane is 50 kW cm^{-2}. Scan frequencies of 25 and 0.05 Hz respectively, are used for the X and Y scan mirrors, providing a pixel dwell time of 80 μs.

Summary

We have successfully implemented a CLSM set-up using an Olympus IX-71

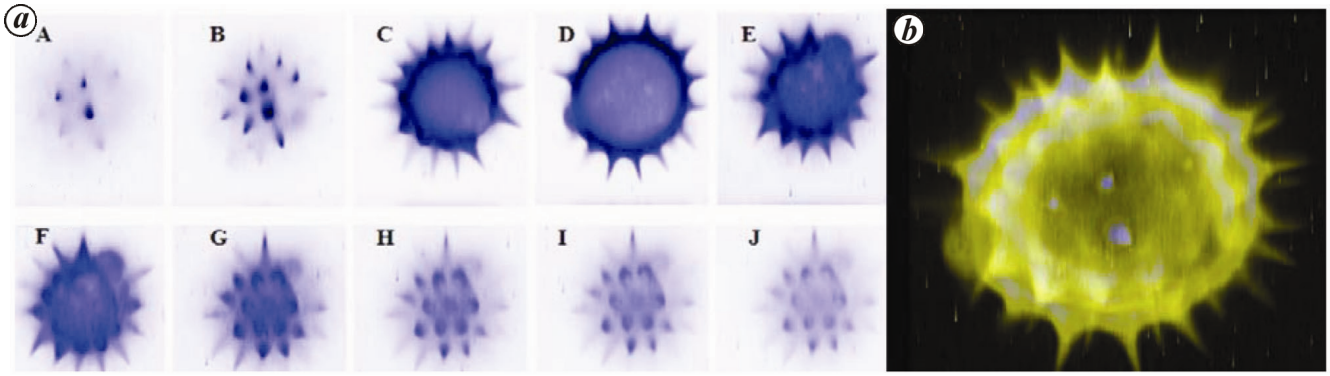

Figure 4. ***a***, Cross-sectional images of a dye-labelled pollen grain. ***b***, 3D reconstruction of the pollen grain serial optical sections.

microscope and an X–Y scan mirror system, and standardized the confocal microscope using different standard microparticles and biological samples. The total cost of components used in this construction is about Rs 12.5 lakhs. The microscope can be implemented in any standard optics laboratory with minimum amount of additional expenditure. The simple and versatile microscope design employed here can be easily optimized and configured according to specific user requirements.

1. Paddock, S. W. and Eliceiri, K. W., *Methods Mol. Biol.*, 2014, **1075**, 9–47.
2. Stephens, D. J. and Allan, V. J., *Science*, 2003, **300**, 82–86.
3. Nwaneshiudu, A., Kuschal, C., Sakamoto, F. H., Anderson, R. R., Schwarzenberger, K. and Young, R. C., *J. Invest. Dermatol.*, 2012, **132**, e3.
4. Pawley, J. B., *Handbook of Biological Confocal Microscopy*, Springer, New York, 2006.
5. Wilson, T. and Sheppard, C., *Theory and Practice of Scanning Optical Microscopy*, Academic Press, London, 1984.
6. Xi, P., Rajwa, B., Jones, J. T. and Robinson, J. P., *Am. J. Phys.*, 2007, **75**, 203–207.
7. Callamaras, N. and Parker, I., *Cell Calcium*, 1999, **26**, 271–279.
8. Koch, P. C., Seebacher, C. and Hess, M., *J. Neurosci. Methods*, 2010, **188**, 127–140.
9. Pologruto, T. A., Sabatini, B. L. and Svoboda, K., *Biomed. Eng. Online*, 2003, **2**, 13.
10. Bijeesh, M. M., Arunkarthick, S., Arvind, K., Nishith, R., Geetha, K. V., Meenal, K. and Nandakumar, P., *KIRAN–Bull. Indian Laser Assoc.*, 2011, **22**, 26–28.
11. Ernst Keller, H. and Watkins, S., *Curr. Protoc. Cytom*, 2013, **63**, 2.2.1–2.1.9.
12. Cole, R. W., Jinadasa, T. and Brown, C. M., *Nature Protoc.*, 2011, **6**, 1929–1941.
13. Schneider, C. A., Rasband, W. S. and Eliceiri, K. W., *Nature Methods*, 2012, **9**, 671–675.

ACKNOWLEDGEMENTS. We thank DBT, DRDO and DST, New Delhi for financial support. S.A. thanks CSIR, New Delhi for SRF.

Received 5 September 2014; accepted 6 November 2014

S. Arunkarthick, M. M. Bijeesh, P. Nandakumar and Geetha K. Varier are in the Department of Physics, Birla Institute of Technology and Science, Pilani-K. K. Birla Goa Campus, Zuarinagar, Goa 403 726, India; Anand Satya Vetcha lives at Flat No. B-401, SJR Brooklyn, ITPL Main Road, Kundalahalli Colony, Brookefield, Bangalore 560 066, India; Nishith Rastogi lives at 8082, Sobha Ruby, Bangalore 560 058, India.*
**e-mail: geethakv@goa.bits-pilani.ac.in*