

Development of a high speed laser scanning confocal microscope with an acquisition rate up to 200 frames per second

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Abstract: There has been an increasing interest for observing fast biological phenomena such as cell movements in circulations and action potentials. The laser scanning confocal microscopy offers a good spatial resolution and optical sectioning ability to observe various *in vivo* animal models. We developed a high speed laser scanning confocal microscope capable of acquiring 512 by 512 pixel images at 200 fps (frames per second). We have incorporated a fast rotating polygonal scanning mirror with 128 facets for the X-axis scanner. In order to increase the throughput of the Y-axis scanner, we applied a bi-directional scanning method for vertical scanning. This made it possible to scan along the Y-axis two times during each scanner motion cycle. For the image acquisition, we used a custom photomultiplier tube amplifier with a broad frequency band. In addition, custom imaging software was written for the new microscope. In order to verify the acquisition speed of the developed confocal microscope, a resolution target moving at a series of constant speeds and a sedated mouse with slight movements due to heartbeats were observed. By comparing successive frames, the frame acquisition speeds were calculated.

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OCIS codes: (170.1790) Confocal microscopy; (170.5810) Scanning microscopy; (120.4570) Optical design of instruments.

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1. Introduction

The confocal microscope was first introduced by Marvin Minsky in the 1950s in order to overcome the limitations of the wide field microscope [1, 2]. The principal of the confocal microscopy lies with the confocal detection scheme which employs a spatial pinhole in front of the photo-detector. A coherent laser source is focused to a point in the sample and the backscattered or fluorescence light from the focal point is guided back to a photo-detector. By placing a spatial pinhole at the conjugate focal point of the light source, the photo-detector would exclusively collect the light from the focal point in the sample. This exclusive detection made the 'optical sectioning' possible in diffusing biological samples. The name 'confocal' stems from the geometrical design which utilizes two conjugated focal points. In addition to the optical sectioning, the lateral resolution of confocal microscopy is superior to that of the wide field microscopy. This is because the lateral resolution of wide field microscopy is determined solely by the point spread function of the light emitted from the sample, while the lateral resolution of confocal microscopy is determined by the convolution of point spread functions of the illumination source and the emitted light.

Two dimensional scanning of the confocal microscopy has been typically implemented by two distinctive approaches. The first type is the "laser scanning confocal microscopy" in which a single laser beam is steered two dimensionally over the sample using mechanical steering mirrors such as the galvanometer and spinning polygonal mirror. The light from the sample is then detected by a single photo-detector that is equipped with a confocal pinhole. The second type is the "spinning-disk based confocal microscopy" which employs a pinning-disk with multiple pinholes. Successive sets of pinholes on the spinning disk are illuminated with a laser source so that the light passed through each pinhole is focused by the objective onto the sample surface. Multiple laser beams that correspond to individual pinholes are then illuminated on the sample simultaneously. As the spinning disk rotates, the sample is raster scanned by consecutive sets of multiple laser beams and the light from the sample is detected by array of photo detector such as CCD camera.

Two distinctive types of scanning method have their own advantages and disadvantages over the other type. The axial and lateral resolution of laser scanning confocal microscopy is superior to the spinning disk based confocal microscopy whose spatial resolution suffers by the cross-talk among pinholes and detector arrays. In terms of the imaging speed (acquired frames per second), the spinning-disk based systems is faster than the laser scanning confocal microscopy. The imaging speed of laser scanning type is mostly limited by the number of raster scans of the mechanical scanning mirror. Most commercial laser scanning confocal microscopy operates at the frame rate of less than ten frames per second [3, 4]. Home-built laser scanning confocal microscopes that had been reported in many studies (including our group's) operate at the video rate imaging (30 frames per second) [5–7].

On the other hand, a commercial spinning-disk confocal microscope can typically operate at the frame rate of a few hundred frames per seconds [8–10]. Simultaneous and multiple illuminations and detections allow the spinning disk based confocal microscopy to have a higher frame rate compared to the laser scanning confocal microscopy. If one can combine only advantages of two distinct confocal microscopy implementations, many interesting cellular dynamic processes can be observed with superior spatial and temporal resolutions.

In this study, we present a high speed laser scanning confocal microscope capable of acquiring 512 x 512 pixel images at up to 200 frames per second, approximately seven times faster than currently reported laser scanning confocal microscopy [5–7]. Our design of a high speed laser scanning confocal microscope has incorporated a high rotational speed polygonal mirror, a fast custom-made preamplifier for the photomultiplier tube detector, electronics for bi-directional scanning of a galvanometer-driven mirror, and custom-written software for image acquisition.

2. Methodology

The schematic of the constructed high speed laser scanning confocal microscope design is illustrated in Fig. 1. The newly developed confocal microscope is similar to conventional laser scanning confocal microscopes in components. We use a 405 nm diode laser as a light source. The laser beam can be scanned two-dimensionally by two different scanning mirrors on the sample plane: a polygonal mirror for fast horizontal scanning and a galvanometer-driven mirror for slow vertical scanning.

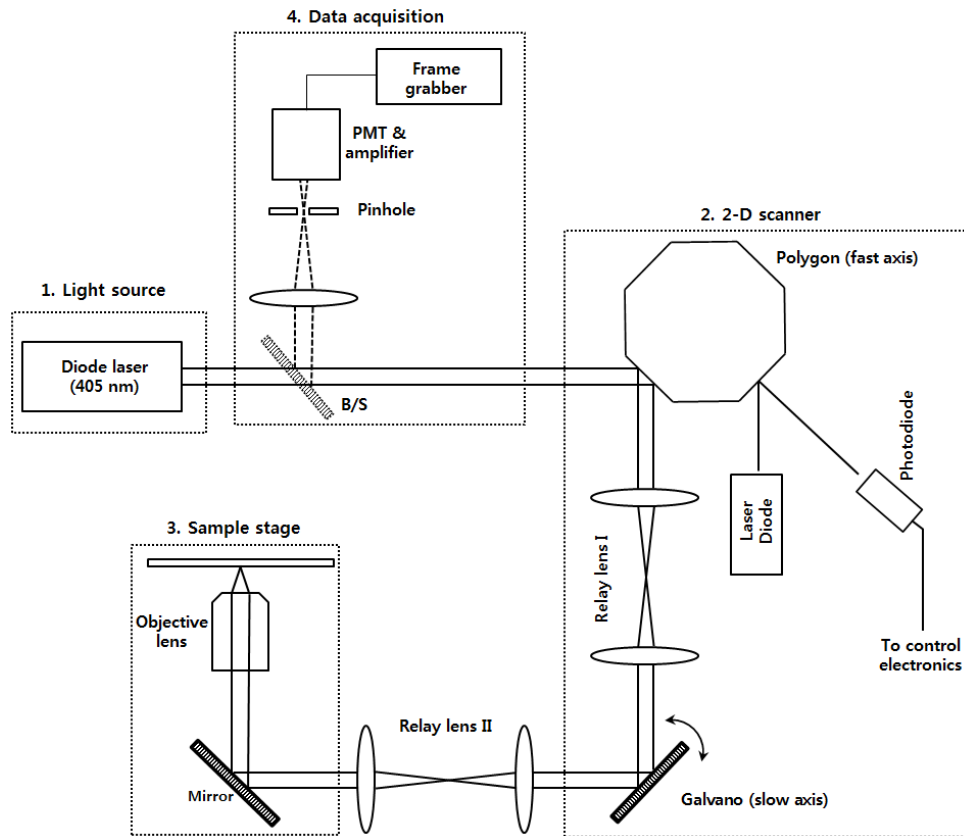


Fig. 1. Schematic diagram of high speed laser scanning confocal microscopy.

Two-dimensional scanned laser beam is coupled into an inverted microscope type. The laser beam is reflected by the mirror toward the objective lens and focused on the sample. An objective lens has 40x magnification and 1.2 NA (Carl Zeiss, Germany, water immersion).

Backscattered light from the sample goes back along the incoming beam path and is reflected by a beam splitter toward a photomultiplier tube (PMT). By using a convex lens the focused backscattered light goes through a pinhole ($D = 50 \mu\text{m}$) to reach the PMT. A small

photocurrent signal from the PMT is then converted to an input voltage signal to the frame grabber. Finally, the data acquisition software is used to display and to save acquired images.

In order to improve the acquisition speed of a typical video-rate laser scanning confocal microscope, we have made modifications for the following instrument aspects: a faster beam steering optics, wide bandwidth amplifier for the PMT detector, control electronics for bi-directional galvanometer scanning, and image acquisition and processing software. First, a straight forward path to attain a faster scanning speed than 30 fps is to replace the slow laser beam scanner with a faster one. A typical video-rate (30 fps) confocal microscope requires about 15,000 lines per second raster scanning speed for a 512 x 512 pixel image frame. However, at least 102,400 lines per second raster scanning speed is required for a 200 fps system at 512 x 512 pixel resolution. When considering front and back porch time controls and other analog synchronization processes, we estimate that the raster scanning rate for the fast scan direction (X-axis) to be 115,200 lines per second. In order to satisfy the calculated speed requirement, we have chosen a 128-facet polygonal mirror scanner running at 54,945 revolutions per minute (RPM). At the maximum rotation speed of the polygonal scanning mirror, 117,216 lines per second scanning speed can be achieved. The polygonal motor can be driven at one of four factory-preset speed settings or at a custom speed by supplying an external 5 V TTL-compatible signal to the motor controller. This new X-axis polygonal mirror scanner allows the acquisition of approximately two hundred 512 x 512 pixel images per second, resulting in about seven times faster frame acquisition rate compared with a conventional video-rate confocal microscope. However, there is one limitation with using a polygonal mirror scanner. Despite its very high speed, the field of view in the fast scan direction is fixed since the laser beam's scan angle is determined by the length of the mirror facet.

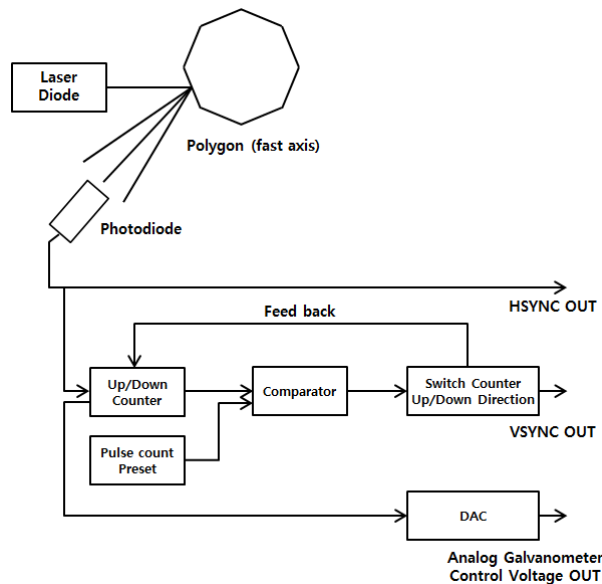


Fig. 2. The analog galvanometer mirror control voltage, HSYNC and VSYNC generation electro-circuit for bi-directional slow axis scanning.

Second, the new system design includes an immediate two-fold increase in scanning speed by bi-directionally scanning the relatively slow galvanometer in the slow direction (Y-axis). In bi-directional scanning the galvanometer scans forward and backward, thus resulting in two line scans every period of motion. Unfortunately, the bi-directional scanning has two drawbacks. The timing required for correct image constructions requires that the galvanometer should run forward and backward symmetrically. However, a real galvanometer

displays slight unsymmetrical motions at the two extreme ends of its motion when the direction of the mirror motion changes. Also the opposite scanning directions for forward and backward scans require switching the pixel sequence in reverse order for every other scan line. The above mentioned asymmetry and pixel sequence reversing force us to collect more pixels per line, for example 576 pixels for a 512-pixel line image, in order to preserve the distortion-free imaging by discarding 32 front and 32 back ends pixels. In order to achieve a bi-directional Y-axis scanning, we have designed and constructed an electronic circuit that generates a triangular galvanometer mirror control signal and TTL-compatible synchronization signals. Figure 2 and Fig. 3 show the control circuit to generate a voltage control signal for the galvanometer and required timing signals, the horizontal synchronization (HSYNC) and the vertical synchronization (VSYNC) pulses. A photodiode circuit detects the end of a polygonal mirror's facet and generates H-sync pulses.

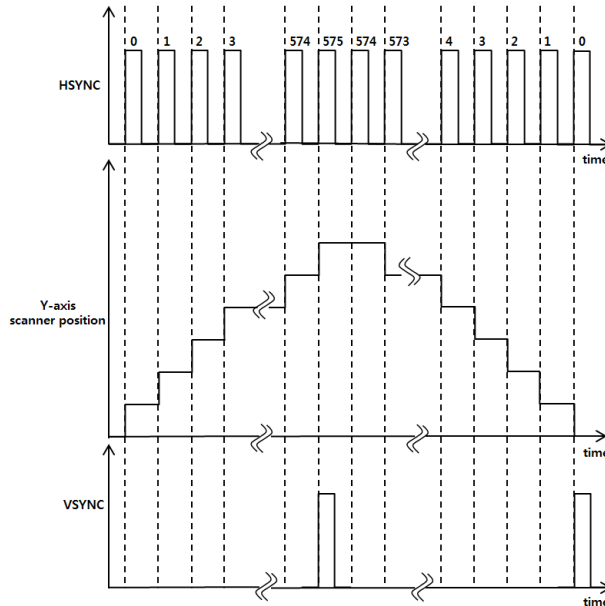


Fig. 3. The analog galvanometer mirror control voltage signal and SYNC signal timing.

Then the number of H-sync pulses are counted and compared with a preset value. When the counter value equals the preset value, the counter counts down until the counter value becomes zero. In other words, the counting part of the circuit changes the counting direction when either the preset number of HSYNC pulses is counted or the counter value has reached zero. The analog part of the circuit then generates an analog voltage signal proportional to the counter value thereby creating a triangular voltage signal with desired amplitude for the galvanometer. The circuit board also generates the necessary VSYNC signals to complete the frame acquisition. The triangular waveform is responsible for the bi-directional scanning which delivers a double-rate frame acquisition speed compared to a conventional single-direction scanning confocal microscopy. The circuit allows a user to select the HSYNC count-preset value so that a custom image size can be achieved.

Third, a fast front-end preamplifier for the PMT has been constructed using OPA847 from Texas Instruments and its reference designs. A typical 512 x 512 pixel video-rate confocal microscope can be constructed with a 10 MHz frame grabber. However, for 200 fps acquisition rate, at least 65MHz bandwidth is required for the detector electronics. Since the frame grabber (Matrox Solios eA/XA) used in the new system digitizes data at the maximum rate of 65 MHz, a preamplifier has been designed to have a bandwidth greater than 65 MHz while the transimpedance gain is set to 80,000. The selected frame grabber comes with a

comprehensive collection of software libraries for custom developing medical imaging applications.

Fourth, custom data acquisition software has been developed to address the special needs for the bi-directional scanning. The newly developed software allows variable controls for horizontal and vertical front and back porch settings. It also correctly flips the order of pixels for every other line for proper image displays.

3. Experiments and results

The performance of the constructed laser scanning confocal microscope was evaluated with a resolution test target (Edmund Optics, 1951 USAF Contrast Resolution Target). Figure 4 shows 512 x 512 pixel snap shot images of the resolution target taken at three different frame rates. Since the observed image has the field of view of 130 μm x 130 μm , one pixel corresponds to about 250 nm.

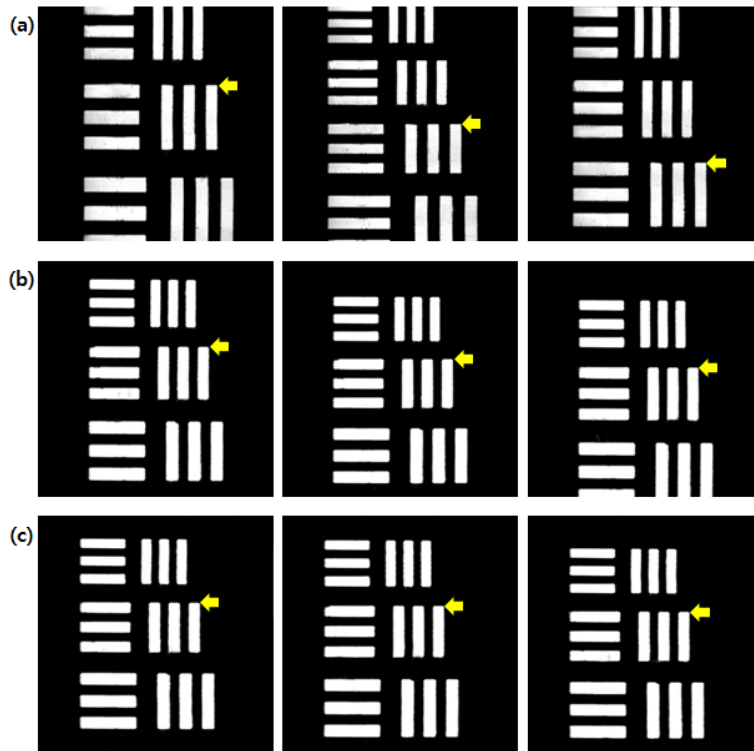


Fig. 4. High speed laser scanning confocal microscopy images of a resolution target moving at the following constant speeds: (a) 30 fps (b) 100 fps (c) 200 fps.

In order to evaluate the frame acquisition rate of our high speed laser scanning confocal microscope, we observed a target moving at a constant speed and a sedated mouse with slight movements due to heartbeats. If a resolution target moves, the location of the observed pattern from two consecutive frames will appear to be shifted in the direction of the target movement. With the moving target's speed information, the time separation between consecutive frames can be precisely calculated. For the observation of the specimen moving at a constant speed, we constructed a sample stage incorporating a motorized stage in the Y-axis (slow scan direction) scanning direction. While the motorized sample stage was moving at the speed of 500 $\mu\text{m}/\text{s}$, we observed the air-force resolution target at three different frame rates. Figure 4 shows the sequential frame images of the moving target obtained for three different frame rates. The acquired images display different amounts of shift. For a faster

frame rate, the amount of shift is smaller compared with those for lower frame rates. The observed shift between two consecutive frames corresponds to 67 pixels for 30 fps imaging speed. The relative shifts for 100 fps and 200 fps imaging rates compared to that for 30 fps are about 1/3 and 1/7 of 67 pixels, respectively.

Figure 5 shows the apparent change in lengths of a pattern on the resolution target for different frame acquisition speeds. When we compare frames obtained from moving and stationary targets, we can observe an apparent pattern length change of the resolution target due to the movement of the target. The top image in Fig. 5 shows the image of the stationary target. When the target's movement is in the slow scan direction, acquired images appear to be elongated in the slow scan direction. However, when the resolution target moves in the opposite direction as the slow scan direction, acquired images appear to be shortened in the slow scan direction. As shown in Fig. 5, the amount of length distortion is smaller for faster scan rates. We measured the resolution target appears to be elongated or shortened by about $5.1\ \mu\text{m}$ for 30 fps acquisition rate, while the distortion for the same pattern was about $0.6\ \mu\text{m}$ for 200 fps acquisition rate. It is clear that less distortion in the acquired images can be achieved with the increased scan rate.

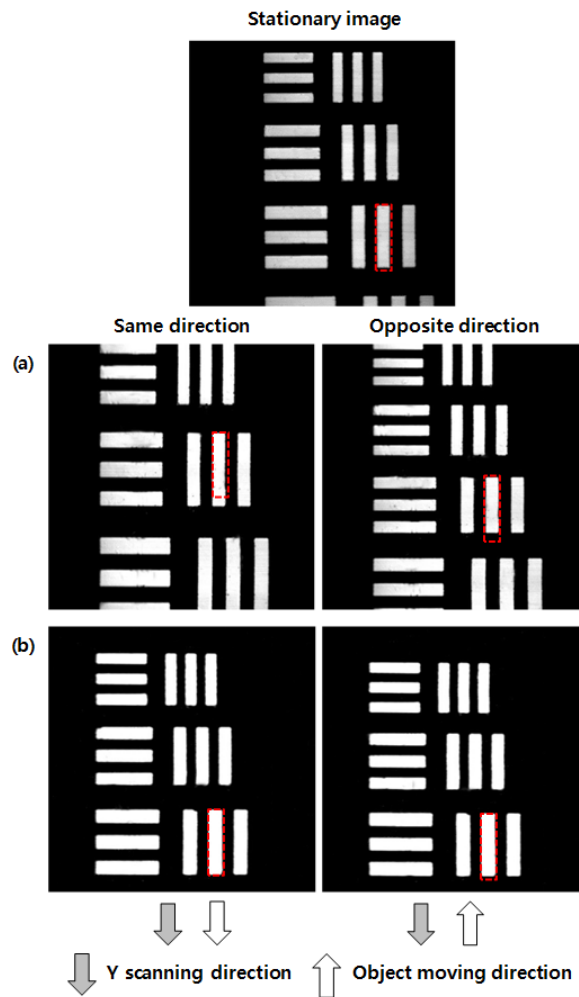


Fig. 5. Apparent elongation and shortening of a pattern on the resolution target along the Y-axis for three different frame rates. The red box indicates the non-distorted size of the pattern under investigation. (a) 30 fps, (b) 200 fps.

We also observed the ear skin tissue of a three-week old mouse sedated by intraperitoneal anesthesia. Figure 6 shows reflected light images of mouse skin obtained at three different acquisition rates. The stratum corneum and hairs are visible in all images. An anesthetized mouse shows a small body movement due to the pumping of blood by the heart. Although small, the continuous and repetitive movement of the mouse in the depth direction causes the stratum corneum to periodically appear focused and defocused. The close-up images of the skin feature in two consecutive frames clearly demonstrate our microscope's confocal sensitivity. For three different acquisition speed settings, we compared sequential image frames to measure the period for focus/de-focus time for the same feature. We have empirically found that 2, 4, and 7 frames are needed to observe the repetition in the acquired images for 30 fps, 100 fps, and 200 fps, respectively. Therefore, at a higher scanning rate more frames can be captured in a given time period revealing shorter time scale changes.

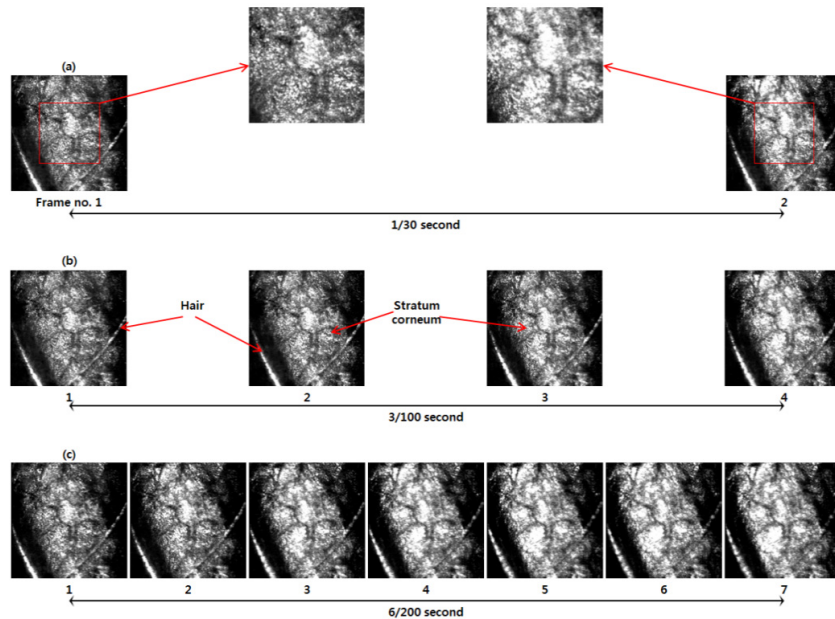


Fig. 6. *in-vivo* mouse skin showing structures and movement. (a) 30 fps, (b) 100 fps, (c) 200 fps.

4. Conclusion

In conclusion, we developed a high-speed laser scanning confocal microscope utilizing a fast polygonal scanning mirror and a bi-directionally operated galvanometer mirror scanner. Necessary high speed analog electronics and imaging software have been designed and developed as well. Confocal images of 512 x 512 pixels with a high spatial resolution can be routinely acquired at a rate up to 200 fps. The frame rate analysis was carried out using a moving resolution target demonstrating minimal distortions in the obtained images for moving samples, and the constructed confocal microscope can be applied to observe appropriately fast dynamic phenomena in clinical and biological applications.

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