

Flow cytometry using spectrally encoded confocal microscopy

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Received March 31, 2010; revised May 31, 2010; accepted June 6, 2010;

posted June 9, 2010 (Doc. ID 126326); published June 24, 2010

Flow cytometry techniques often rely on detecting fluorescence from single cells flowing through the cross section of a laser beam, providing invaluable information on vast numbers of cells. Such techniques, however, are often limited in their ability to resolve clusters of cells or parallel cell flow through large vessels. We present a confocal imaging technique that images unstained cells flowing in parallel through a wide channel, using spectrally encoded reflectance confocal microscopy that does not require mechanical scanning. Images of red blood cells from our system are compared to conventional transmission microscopy, and imaging of flowing red blood cells *in vitro* is experimentally demonstrated. © 2010 Optical Society of America

OCIS codes: 180.1790, 170.1470, 170.1530, 170.3880.

Flow cytometry is a powerful tool for automatically analyzing large populations of live cells suspended in a liquid medium [1]. In conventional flow cytometers, cells pass through a focused laser beam one after the other, where their scattering and fluorescence are measured, providing multivariate statistics of cell size, shape, and functional properties. Flow cytometry of blood samples is useful for the clinical diagnosis of various disease states, but it requires extraction and processing of blood. *In vivo* flow cytometry of cells flowing in blood vessels could open up new clinical applications with potential benefits for the diagnosis of hazardous medical conditions in real time. Specific techniques for *in vivo* flow cytometry have been demonstrated but usually rely on injection of fluorescent markers or fluorescently labeled blood cells [2]. Intravital microscopy [3] provides single-cell resolution and is often used for imaging blood cells *in vivo* for research. The use of exogenous fluorophores in these approaches could yield potential complications in clinical applications owing to the high cytotoxicity of common fluorescence markers.

Imaging flowing cells deep below the tissue surface is even more challenging owing to tissue scattering. Most commonly, label-free high-resolution imaging through thick tissue is accomplished using reflectance confocal microscopy (RCM), which uses a pinhole to reject out-of-focus scattered light [4]. In most cases, confocal microscopy requires mechanical beam scanning, which limits acquisition rates and often necessitates bulky imaging probes. Wavelength encoding techniques allow spectrally encoded confocal microscopy (SECM) [5] to instantaneously capture a confocal image of an entire line. While previous demonstrations of SECM required single-axis probe scanning to acquire an image, such scanning would not be required to image flow, by taking advantage of the unidirectional motion of the cells. Removing the need for scanning would allow high acquisition rates and potentially enable small, inexpensive imaging probes, which could also be used endoscopically through a single fiber [6]. Measuring flow profiles was recently demonstrated using interferometric spectrally encoded endoscopy (SEE) [7]. However, owing to its

low NA optics, SEE is not capable of imaging subcellular features or resolving single cells.

In this Letter, we report a concept for confocal imaging-based flow cytometry that does not require mechanical scanning. By spectrally encoding the illumination and the backscattered light, an image of a line inside a flow chamber is repeatedly acquired at high rates, allowing parallel high-resolution imaging of flowing blood cells, which reveals their size, shape, and velocity. The experimental system is schematically illustrated in Fig. 1. Light from a Ti:sapphire oscillator (Tsunami, Spectra Physics) with a center wavelength of 800 nm and spectral bandwidth of 60 nm was coupled into the input port of a single-mode 50/50 fiber coupler (fiber NA = 0.11). At the distal end of the fiber, the SECM probe consisted of a collimating lens (150 mm focal length), a transmission grating (1200 lines/mm), and an objective lens (20 \times , NA = 0.75), producing a 0.81-mm-long spectrally encoded line. The light backscattered by the flowing cells was collected by the probe's optics and analyzed by a home-built spectrometer, composed of a collimating lens (50 mm focal length), a transmission grating (1800 lines/mm), an achromatic lens (150 mm focal length), and a 2048-pixel line CCD (Aviiva EM4, e2v, Inc.). The transverse resolution was approximately 1.5 μm (FWHM), estimated by measuring the transverse line spread function of the system using a reflective USAF 1951 resolution target. The spectrometer had optical resolution (approximately 13 μm) comparable to the CCD pixel size (14 μm); thus, the spectral line at the

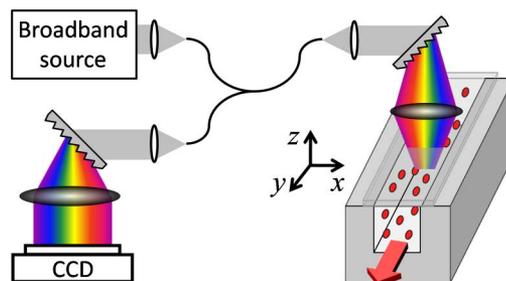


Fig. 1. (Color online) Schematic of the SECM flow cytometry system.

sample, which contained approximately 540 resolvable points, was oversampled by 1670 CCD pixels.

Reflectance images of red blood cells (RBCs), which lack a nucleus or large organelles, depend largely on the refractive index difference between the cell cytoplasm and its surrounding medium, as well as on the cells' shape and orientation in space [8]. To study the characteristics of the SECM images of RBCs in suspension, we compared them to images obtained by conventional bright field microscopy (NA = 0.45 objective). For this purpose, RBCs were immobilized for the duration of the experiment by *in situ* polymerization in a biocompatible hydrogel matrix. Cells were suspended in a solution of polyethylene-glycol (PEG) conjugated to Fibrinogen and subjected to light-activated curing [9]. This hydrogel contains 10 mg/ml of protein and its refractive index is nearly that of a saline solution. A 170- μm -thick sample was prepared by curing 20 μl of RBC-hydrogel mixture between a microscope slide and a cover glass. The sample was laterally scanned along a single axis using a piezoelectric stage with a velocity of 1 mm/s. Two-dimensional SECM images of the sample were acquired with a 2000 line/s acquisition rate (0.5 ms exposure) and then transferred to a microscope for brightfield imaging. Because the depth of field in the confocal image (approximately 2.5 μm) was much smaller than that of the brightfield image, we integrated six SECM images separated by 3 μm in the z axis [Fig. 2(a)]. SECM and brightfield images of four corresponding regions of interest are

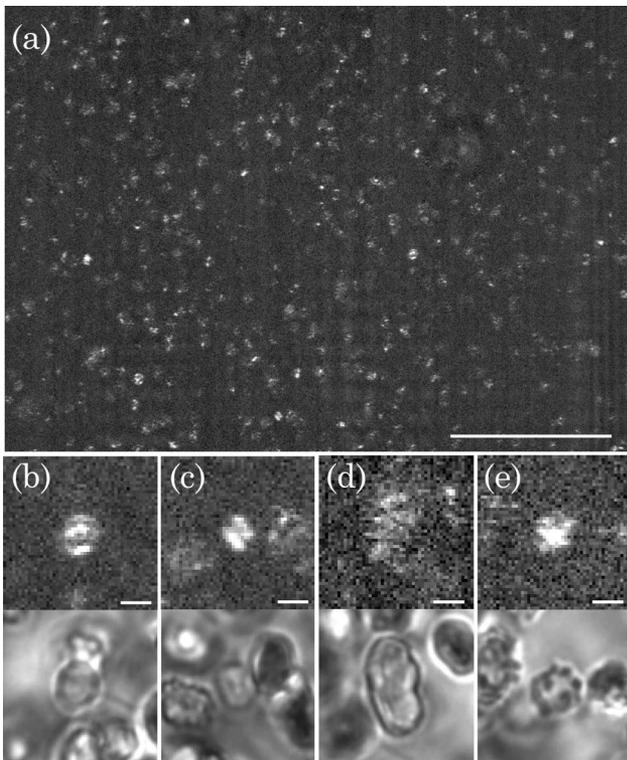


Fig. 2. Comparison of SECM and transmission brightfield images. (a) Depth-integrated SECM image of blood cells in hydrogel; scale bar represents 100 μm . (b)–(e) Magnified views of four selected regions show individual cells from the SECM image (top row) in comparison with brightfield microscopy (bottom row). (e) A characteristic crenated cell is visible both in the SECM and the brightfield image. Scale bars represent 5 μm .

shown in Figs. 2(b)–2(e), revealing some distinct differences between the two modalities. Note that most cells are not visible in the confocal image owing to the confocal gating and the small depth of field of the system, while these cells are still visible in the bright field image. The cells' size, shape, and orientation are visible in the SECM image with high contrast. Several techniques could be applied for decreasing speckle noise in SECM, including the use of higher NA objectives [10] and double-clad fibers [11].

Next, we used our system to image the flow of blood cells through a channel with a 0.55 mm \times 0.55 mm square cross section, fabricated in polymethyl methacrylate and sealed with a 160- μm -thick cover glass (Fig. 1). A few microliters of blood obtained by finger prick were diluted in phosphate-buffered saline to obtain a concentration of approximately 6500 RBCs/ μL (corresponding to a 1 : 1000 dilution ratio). A steady laminar flow was created by connecting the flow chamber between two reservoirs having a small height difference. The acquired raw data contained two-dimensional matrices in which the horizontal axis represents the spectrally encoded transverse dimension (x axis) and the vertical axis represents time. Figure 3(a) shows a typical image acquired 80 μm below the cover glass over 4 s of continuous flow. Note that, in many occasions, two or more cells were crossing the spectrally encoded line simultaneously and could still be resolved owing to the high lateral resolution. The

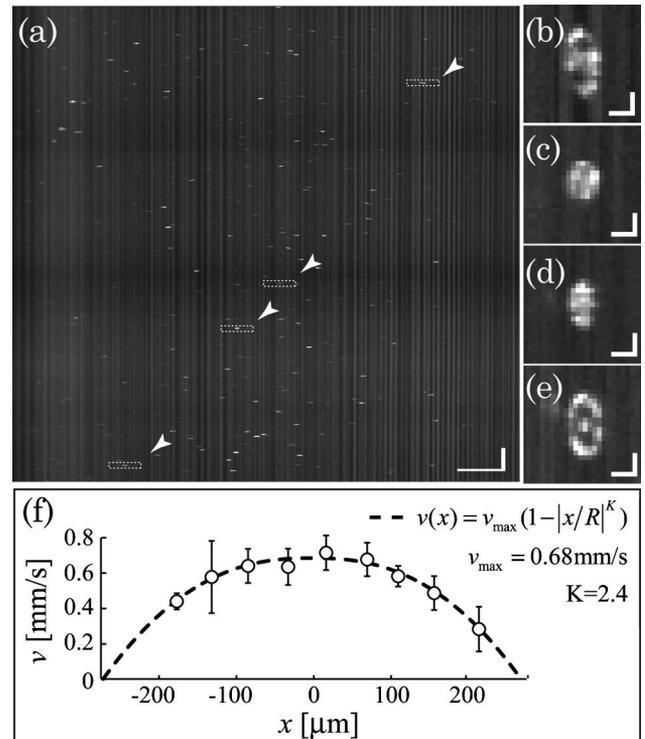


Fig. 3. Flow images of blood cells using SECM without mechanical scanning. (a) Bright spots in a 4-s-long acquisition correspond to flowing blood cells. Horizontal scale bar represents 50 μm . Vertical scale bar represents 200 ms. (b)–(e) Magnified views of the four marked rectangles in (a), which contain single RBCs. Horizontal scale bars represent 5 μm . Vertical scale bars represent 5 ms. (f) Velocity profile across the chamber obtained from measuring the averaged vertical dimensions of the cells. A power-law fit of the data is marked by a dashed curve.

visible pattern of vertical lines was caused by interference between reflections within the fiber coupler. Examples of four individual cells are shown in Figs. 3(b)–3(e), revealing the circular shape of the RBCs with differences in their length along the time axis. When cells traverse the spectrally encoded line, their average length in pixels along the flow (time) axis, N_t , is inversely proportional to their velocity according to $N_t = B \cdot l_{av}/v$, where B denotes the camera line rate, l_{av} denotes the average length of the cells along the flow direction, and v denotes their flow velocity. Using a large number of cells for averaging cell sizes and orientations, we estimated the flow velocity profile across the chamber. A plot of the nearly parabolic velocity profile is shown in Fig. 3(f). Data were captured using a 1000 line/s acquisition rate (1 ms exposure) with approximately 25 μ W illuminating each resolvable point (each flowing cell was exposed to approximately 1.5 μ J). Note that to maintain resolution, exposure time should be kept below the minimal time that is required for a cell to move across a single lateral resolution element. Error bars represent standard deviations caused mainly by the random distribution of cell orientations. Note that, in some applications, a power-law fit with exponents that differ from the value of two could be indicative of several blood rheological properties [12].

In practical use, an SECM-based flow cytometry system would have unique imaging capabilities. With no distal scanning required, the imaging probes could be made extremely compact and robust, connected to the main light source and signal acquisition system only by a single optical fiber. Using a dedicated CCD array with high sensitivity and imaging rate would improve the signal-to-noise ratio and allow resolving cells flowing at high velocities (tens of mm/s). For *in vivo* applications, an optimal choice of wavelength would be critical for providing both the required resolution and tissue penetration. A 800 nm wavelength was used to allow substantial tissue penetration while maintaining high resolution. Using light sources in the 1300 nm range would result in better penetration depths; however, resolution would

deteriorate, which would reduce the system's capability to accurately characterize the cells.

In summary, we have demonstrated the potential of SECM to collect cytometric data from a parallel flow of unlabeled blood cells using optical probes with no mechanical scanning. The flow of diluted blood was imaged with subcellular lateral resolution and sufficient temporal resolution to allow measuring the cells' shape and velocity. The presented approach could be useful for noninvasive *in vivo* flow cytometry with probes that could potentially be further miniaturized to be applied endoscopically.

The authors thank Dror Seliktar and Tanya Rosenblit for helping with the hydrogel preparation, Jonathan Shemesh for assisting with the flow setup, and Limor Minai for fruitful scientific discussions.

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