Multi-channel imaging cytometry with a single detector

Sarah Locknar[1], John Barton[1], Mark Entwistle[2], Gary Carver[1] and Robert Johnson[1]
[1] Omega Optical, Brattleboro, VT 05301
[2] Philadelphia Lightwave, Trevose, PA 19053

ABSTRACT

Multi-channel microscopy and multi-channel flow cytometry generate high bit data streams. Multiple channels (both spectral and spatial) are important in diagnosing diseased tissue and identifying individual cells. Omega Optical has developed techniques for mapping multiple channels into the time domain for detection by a single high gain, high bandwidth detector. This approach is based on pulsed laser excitation and a serial array of optical fibers coated with spectral reflectors such that up to 15 wavelength bins are sequentially detected by a single-element detector within 2.5 μs. Our multichannel microscopy system uses firmware running on dedicated DSP and FPGA chips to synchronize the laser, scanning mirrors, and sampling clock. The signals are digitized by an NI board into 14 bits at 60MHz – allowing for 232 by 174 pixel fields in up to 15 channels with 10x over sampling. Our multi-channel imaging cytometry design adds channels for forward scattering and back scattering to the fluorescence spectral channels. All channels are detected within the 2.5 μs – which is compatible with fast cytometry. Going forward, we plan to digitize at 16 bits with an A-to-D chip attached to a custom board. Processing these digital signals in custom firmware would allow an on-board graphics processing unit to display imaging flow cytometry data over configurable scanning line lengths. The scatter channels can be used to trigger data buffering when a cell is present in the beam. This approach enables a low cost mechanically robust imaging cytometer.

Keywords- imaging flow cytometry, multispectral imaging, multispectral cell sorting, multispectral cell analysis, imaging cell sorting

1. INTRODUCTION

High-content flow-cytometry data has been the norm for decades, with commercially available cell-counters able to detect cells containing up to 30 fluorescent labels nearly simultaneously.[1,2] These systems, while very versatile are also expensive, employing several detection modules in series. Each detection module consists of a laser and an array of detectors, usually separated by bandpass and dichroic filters to detect the labels of interest. Up to 7 detection modules are stacked together with the flowing cells hitting each laser excitation and detection module in turn before reaching its destination. Each laser and PMT requires hardware to obtain and process the signal (power supplies, A/D converters, etc.) which makes these systems very large and power hungry. The data signal is generally integrated over the time during which the cell remains in the beam. Information about how long the cell is in the beam (which would give some idea about size) is not processed, so errors from pairs or clumps of cells are common and treated as a single unit.

In contrast, imaging flow-cytometers have been on the market for over a decade[2] (notably the Millipore Sigma Amnis) which utilize a time-delayed integration CCD based system. The time-delayed integration synchronizes the line-readout rate to the flow rate. This allows integration to occur over a single line while the particles are moving in space, because the particle and the line are traveling at the same rate. These also use spectral splitting by employing filters to direct different wavelength bands onto different parts of the CCD chip. The size of the CCD limits the number of channels to 6-12 for a single detector.

Omega Optical has recently developed a multi-spectral detection scheme that maps spectral bins into the time domain. This system couples a nanosecond laser to a high gain, high bandwidth detector via a series of fiber-optic delay lines containing short-wavelength reflectors. The initial concept was optimized for confocal imaging of intrinsic fluorescence of cancer margins so the system is designed for low-light-level use.[3,4] The optical system is easily adapted to imaging flow cytometry by replacing one of the scan mirrors with a flow-cell and pump. In addition, we have added scattering (back and forward/side) channels commonly used in flow-cytometry.

2. METHODS

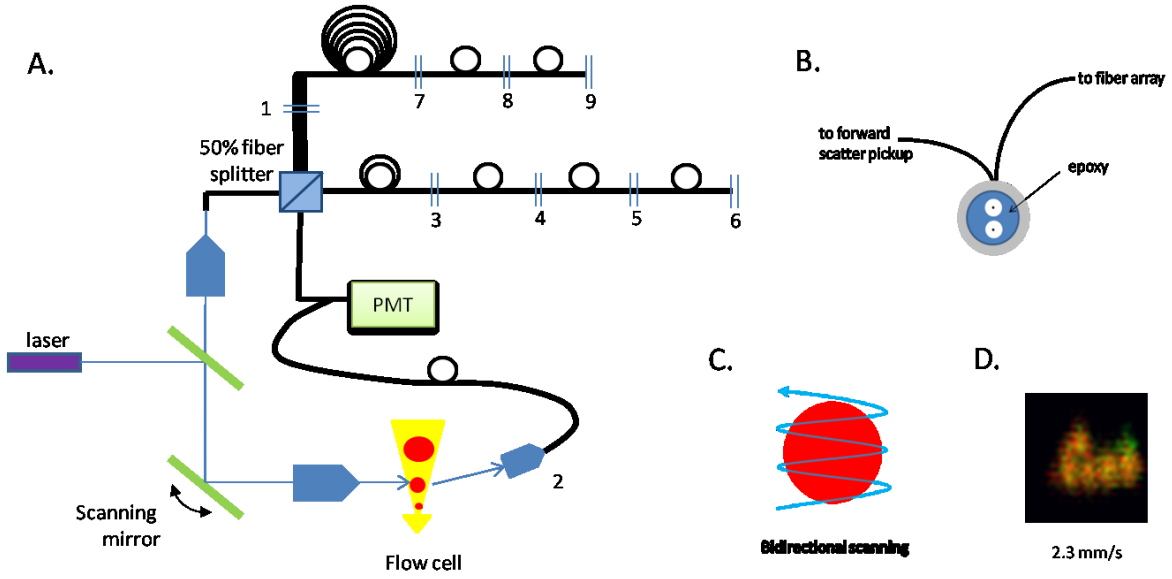

Figure 1. A. Schematic of the fast multispectral imaging system. Signals reach the detector from each wavelength segment in the order as labeled. Circles represent fiber delays and double lines represent coated fiber tips. **B**. Schematic of 2-in-one fiber ferrule. This delivers forward and backward signals to the same PMT. **C**. Imaging is performed with a bidirectional raster scan across the flowing particles. **D**. Five 2 μm red and one 1 μm green fluorescent beads moving at a linear rate of 2.3 mm/s.

Figure 1A is a schematic of the system. A 100 ns laser pulse is rastered across the flowing sample (flowing perpendicular to the optical plane, Figure 1C) and the resulting back reflectance and fluorescence is directed onto the

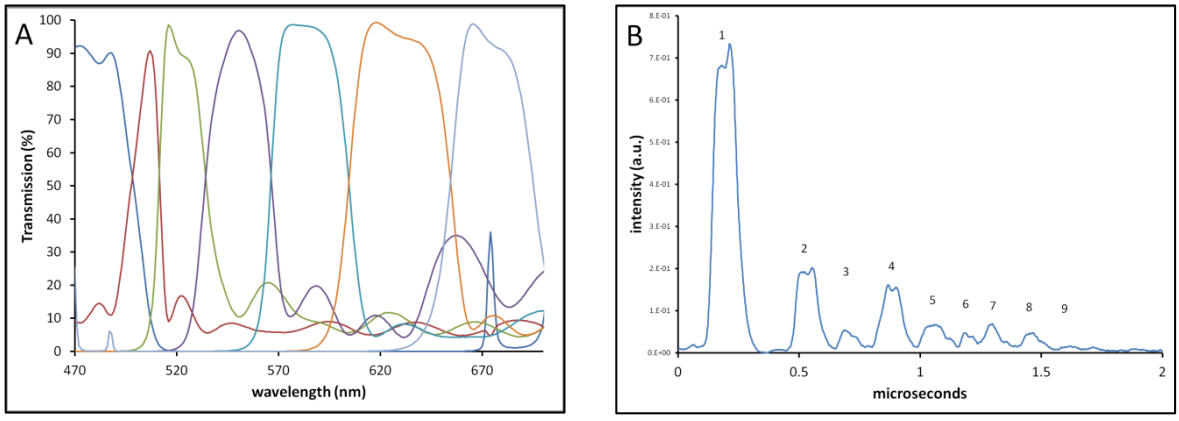

Figure 2. A. Spectral response of the assembled fiber array. The shape of each bandpass is formed by the 2 edges surrounding it. Post-processing corrections correct for low %T crosstalk ripple. The forward scatter channel is not shown. **B.** Oscilloscope trace of each wavelength bin reaching the detector during its given timeslot. bin 1 is blue light rejected from the red fiber leg and is not used for analysis.

face of a 62.5 μm core fiber which acts as a confocal pinhole. The light passes through a 50/50 splitter into blue and red fiber arrays containing increasingly red reflecting elements (double lines). Spectral bands return to the detector at defined time points, thus indexing wavelength into the time domain. The position of the cut-on wavelengths can be adjusted during the design phase to suit the needs of the user (Figure 2A). Back scattering in this implementation is achieved in bin 3 (the first bin in the blue array). The array has been designed with wider bandpasses at longer wavelengths to accommodate reduced detector sensitivity in that regime. The forward scattering channel is positioned at the desired angle (90^o for side-scatter and about 165^o for forward scatter) with a focusing lens at the proximal tip of the fiber. Forward scatter passes through the only transmissive filter in the system to isolate the laser wavelength and reaches the detector first in the time channel. We assembled a dual-core ferrule (Figure 1B) to deliver both forward and backward signals to the PMT. All signals reach the single PMT detector within 2.5 μs.

The 100 ns laser light pulse induces a pulsed emission that reaches the detector in 166 ns intervals (dictated by the length of the fiber delay lines, Figures 1A, 2B). The signal is digitized at a rate of 60 MHz with 14-bit precision. The digitization rate is fast enough that the rise and fall time of the signal hitting the PMT is captured (Figure 2B), but given the weak signals, a larger bit depth is desirable to more adequately sample the low end. A weighted average of each signal profile is performed to give an intensity value for each wavelength bin and a cross-talk correction is applied to account for light from the wrong wavelength ending up in the wrong time bin (due to filter imperfections).[3,4] In this system, each pixel contains 8 intensity values that can be further processed before saving or display.

3. DATA AND ANALYSIS

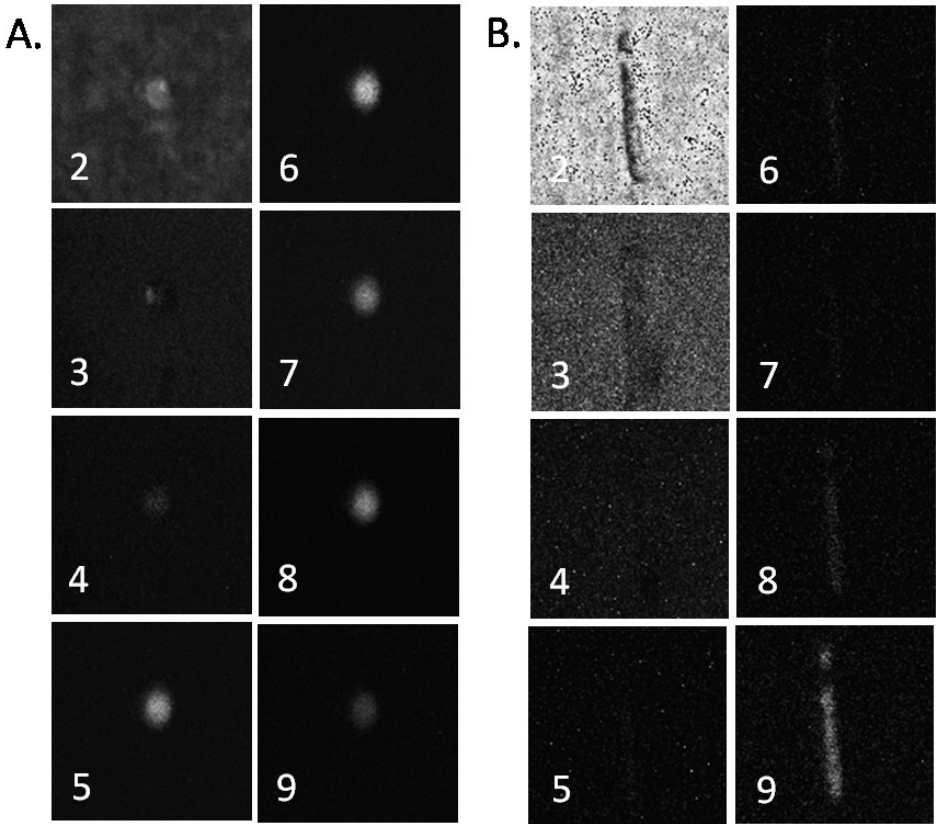

Figure 3. Example of images obtained in each wavelength bin using a 488 nm laser. Field of view is 50 x 50 μm. **A**. A 6 μm diameter Spherotech bead containing a mixture of fluorescent dyes. The image appears in most wavelength bins. **B.** An unlabelled cell of filamentous green algae. The intrinsic chlorophyll only shows up significantly in bins 8 and 9.

With the current software (256 pixels per scan line- 232 displayed), we are able to scan a line in 640 μs at a lateral resolution of just under 2 μm (Figures 1D, 3B). Most lymphocytes (a common sample for flow-cytometry) are on the order of 10-20 μm in diameter which would correspond to a throughput of several hundred cells per second. In that amount of time, 8 images are generated – one for each scattering channel, and one for each fluorescence wavelength bin (Figure 3). Previously, we have demonstrated 10 fluorescence channels[3] and the system as currently configured can detect up to 12 fluorescence channels + 2 scatter channels for a total of 14.

These data can be analyzed in the traditional flow-cytometry way (average intensity histograms), but doing so would ignore the wealth of information in the images. In an ideal situation, images would be analyzed on-the-fly and the cells would be sorted in real-time based not only on the intensities but on image parameters. Cell sorting enables further analysis to be completed- genetics, biochemical assays, etc. There are a number of shape parameters including roundness, aspect ratio, elongation, etc. that can be applied to each image in the wavelength stack. One can also count objects within a single cell, such as nucleoli, chromosomes, or other markers. Using these data in the sorting process will lead to exciting advances in biology. With suitable FPGA resources, this image-based sorting should be achievable in real-time.

In our work on intrinsic fluorescence detection, we have made extensive use of the spectral angle mapping algorithm as a fast and accurate method to characterize images. In the method, the spectrum is mapped onto an n-dimensional space (where n= number of wavelengths) and compared to a known n-dimensional vector. If the vectors overlap within an acceptable error, they are considered a match. Because flow cytometry samples are labeled with fluorescent tags, the spectra themselves are known. One should be able to include shape parameters into the reference vectors as well.

4. FUTURE DIRECTIONS

To further optimize the system for flow cytometry, a number of enhancements need to be made. Currently, the system acquires data continuously- in order to minimize the data stream, we will add triggering to the data acquisition routine. Data will be saved and processed only when a sample is present as determined by forward and back scattering channels. We will enable an adjustable scanline length that can match the sizes of cells that are being sorted and the resolution of the focused spot. We also plan hardware upgrades, including an on-board low-noise 16-bit A/D conversion for greater dynamic range and on-board FPGA based digital signal processing, both of which enable an inexpensive standard PC interface (USB2, USB3, or Ethernet) because the bulk of the data processing will take place before the data is transferred to the computer.

5. CONCLUSIONS

Herein we demonstrated a fast multi-spectral detection scheme for flow-cytomtery that uses a single PMT detector. This system has been shown to generate images with decent resolution at linear flow rates of ~2 mm/s. Further developments include changes to the hardware and software to reduce the number of pixels in the x-axis to more closely match the particle size, increase the number and location of wavelength bins, triggering, software and hardware enhancements. We anticipate the following enhancements-

- Addition of a dedicated low-noise analog-to-digital conversion device with increased bit depth to 16-bits, and optimized analog front-end to take full advantage of the improved dynamic range
- FPGA upgrade to include image acquisition triggering and control
- Firmware update to include on-chip digital signal processing of the raw data stream
    - Noise reduction
    - Contrast enhancement
    - “Region of Interest” windowing and enhancements
    - Configurable pixel field of view (FOV) and scan size
    - Configurable video processing parameters to extract image details of interest
- Standard PC data interface to host image display system (USB, or Ethernet)

6. ACKNOWLEDGEMENTS

The prototype confocal instrument was developed with funding from the National Cancer Institute through the NIH (SBIR Ph. II 5R44CA124036-03)

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