

Flow Cytometer in the Infrared: Inexpensive Modifications to a Commercial Instrument

Carleton C. Stewart,^{1*} Mitchell L. Woodring,² Edward Podniesinski,¹ and Brian Gray³

¹Laboratory of Flow Cytometry, Roswell Park Cancer Institute, Buffalo, New York

²Pacific Northwest Laboratory, Richland, Washington

³PTI Research, Exton, Pennsylvania

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Background: The application of molecules that fluoresce in the infrared (IR) region to measure cell products would be enhanced by a flow cytometer capable of measuring them. To our knowledge, none exist at this time. Accordingly, we have developed such an instrument.

Methods: A Becton Dickinson LSR flow cytometer was modified to include a small 785-nm IR diode laser the size of a C cell battery with 44-mW output power. The instrument was modified further to accommodate this laser in addition to a 405-nm solid-state laser, a 488-nm air-cooled argon laser, and a 658-nm solid-state laser. Because the IR laser is dangerous to the eye, the laser beams were viewed for optical alignment using a CCD camera and video monitor. An avalanche photodiode was used in place of a photomultiplier tube because its detection sensitivity in the IR region is superior.

Results: To assess performance, scatter and fluorescence measurements were made using microspheres that fluoresce in the IR region, and human leukocytes were stained

with CD45 biotin followed by a streptavidin conjugated with an IR dye. An avalanche photodiode was 2.3 to 2.8 times more sensitive than a photomultiplier tube for detecting IR fluorescence. Cells stained with CD45 biotin and avidin conjugated with an IR dye could easily be resolved and their fluorescence quantified; there was virtually no autofluorescence. In addition, a lipophilic membrane dye that emits in the IR region was studied. HL60 cells were stained with this dye and they exhibited bright fluorescence intensity.

Conclusion: A commercial instrument could be modified to accommodate an IR laser for exciting dyes that fluoresce in the IR region. This new capability will extend the range of fluorescence that can be measured by flow cytometry. © 2005 International Society for Analytical Cytology

Key terms: flow cytometer; measurement of infrared excitation and emission; avalanche photodiode

From the time in the early 1960s, when Mack Fulwyler first described the electrostatic method for cell sorting by flow cytometry (1), the instrumentation has become compact with increased capabilities. Flow cytometry is the only technology in which rapid single cell multiparameter analysis and sorting can be accomplished (2-8). In keeping with Mack's great contributions to flow cytometry, we present an extension of its capabilities into the infrared (IR). The increasing numbers of fluorochromes that can be excited by different wavelengths allow specific detection based on their excitation or emission characteristics. Because the data collected is correlated, the increased number of parameters can be explicitly associated with each cell present. Currently, the fluorochromes most often used are excited in the ultraviolet (UV) or visible spectrum and emit at visible or near IR wavelengths. We have added a laser operating in the infrared (785 nm) to an LSR flow cytometer to excite infrared dyes.

One advantage of IR excitation is the decreased background or biological noise that provides for increased

detection sensitivity and dynamic range. Another advantage is to extend the number of probes that can be simultaneously detected. This would be helpful in multiple laser systems in which the detected IR wavelengths would not cross over into other shorter wavelength marker excitation and emission spectra. Yet another advantage is that coaxial laser interrogation can simplify alignment and electronics by eliminating pulse delay circuitry. Further, the laser featured in this report costs less than \$600.

In addition to the modifications to the optical path, an evaluation of photomultiplier tube (PMT) detectors and

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*Correspondence to: Carleton C. Stewart, Laboratory of Flow Cytometry, Roswell Park Cancer Institute, Buffalo, NY 14263.
E-mail: stewart@rpciflowcytometry.com

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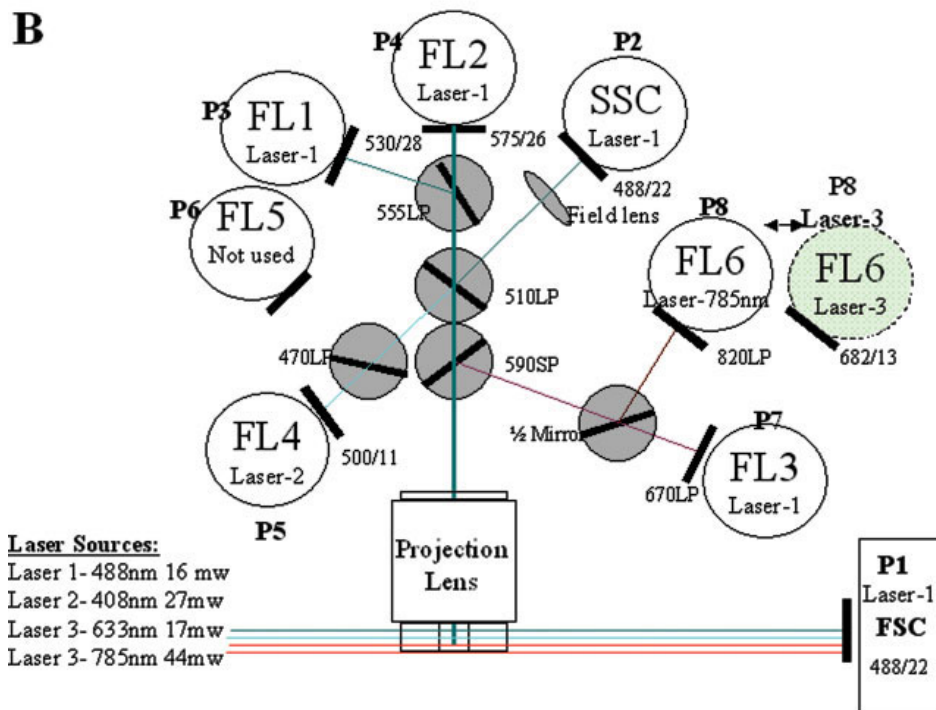
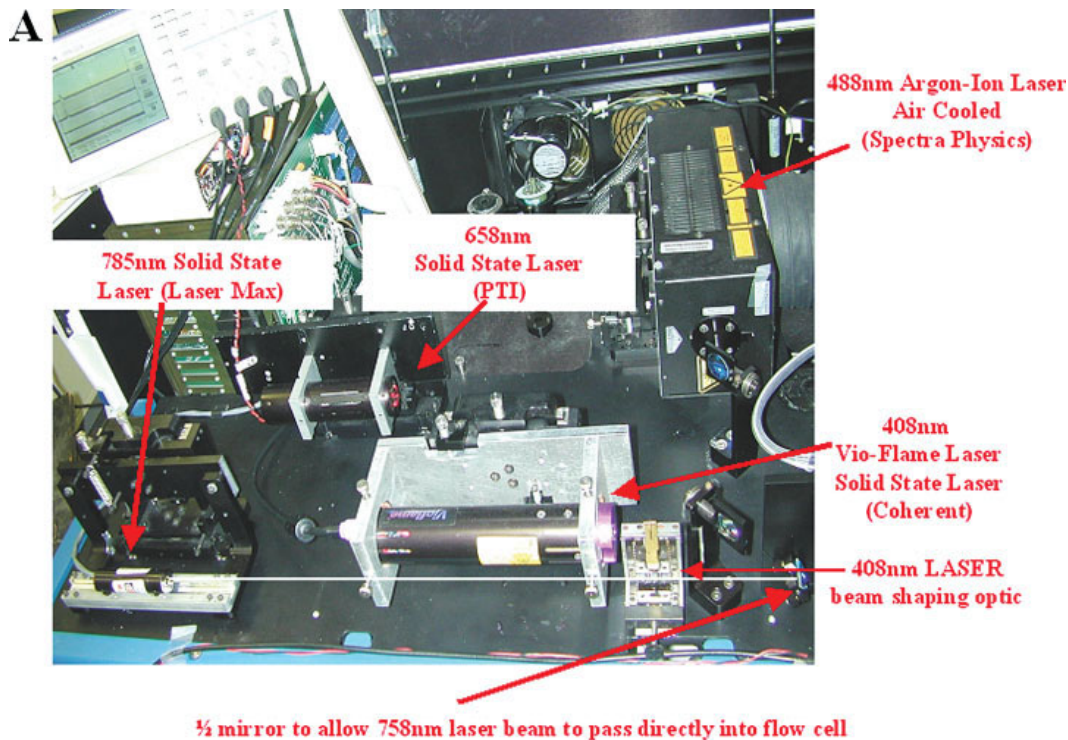


FIG. 1. Laser layout modifications to an LSR-I. **A:** The IR laser was positioned behind the 408-nm Vioflame solid-state laser (Coherent), both of which were put in place of the HeCd UV laser. A 658-nm solid-state laser (Power Technologies) was positioned where the 635-nm laser had been and the 488-nm air-cooled argon laser was not changed (for an LSR-II, no changes to the solid-state lasers supplied would be necessary). The 785-nm beam (white line) passes under and next to the 408-nm beam to a half mirror to allow it to pass directly into the excitation focus optic. The laser mounts can be obtained from Del-Tron Precision, Inc. (Bethel, CT, USA). **B:** A schematic of the optical path from the flow cell to the detectors with associated filters and mirrors is shown. No change in the physical PMT arrangement was made. The FL6 PMT was replaced with the APD after mounting on a Delrin insert machined to fit the PMT holder.

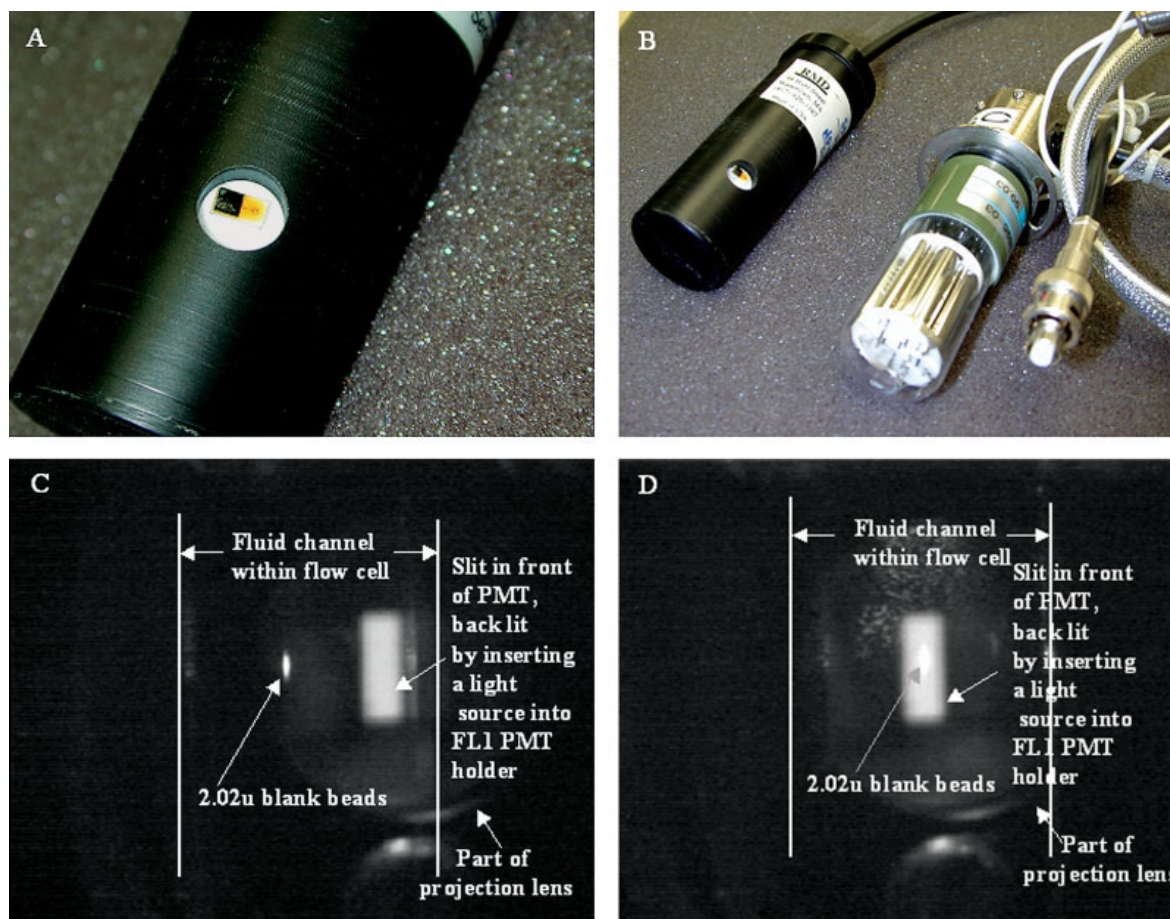


FIG. 2. APD in a PMT holder. **A:** This view shows a close up of the 5×5 -mm APD wafer mounted on the black Delrin housing. **B:** This view shows the APD assembly inserted into the PMT holder next to the Hamamatsu PMT it replaced. **C:** The IR laser intercept is not properly aligned and the $2.02\text{-}\mu\text{m}$ blank beads are seen on the left side of the detector slit. **D:** When aligned, the poorly seen beads (because of backlighting) are centered within the detector slit.

avalanche photodiode detectors (APDs) was performed. The latter device was selected because of its high quantum efficiency in the IR region. Commercially available reagents and some new IR emitting membrane dyes being developed for cell tracking were used to evaluate the IR flow cytometer. Because the laser source used for this modification is beyond detection by the human eye but can produce irreversible damage to it, a method was developed for laser alignment using a CCD camera.

MATERIALS AND METHODS

In modifying the LSR-I (Becton Dickinson Biosciences [BDB], San Jose, CA, USA), we replaced the UV laser with a 405-nm Vioflame laser (Coherent Inc., Auburn, CA), the 635-nm laser was replaced with a 658-nm laser (Power Technology Inc., Little Rock, AR), and we added a new inexpensive 44-mW IR solid-state laser operating at a nominal wavelength of 785 nm (model 780-50, LaserMax, Rochester, NY, USA). The layout of these changes is shown in Figure 1A. The 785-nm laser has a beam dimension of

1.8×0.8 mm, a median time before failure of 52,000 h, and requires 8 to 10 V DC at 156 mA to operate.

The optical layout for the detectors and associated optical filters is shown in Figure 1B. Except for P8 (FL6), which is currently used for detection using an APD, IR fluorescence excited by the IR laser, or fluorescence using a PMT that is excited by the 658-nm laser, the arrangement is essentially unchanged from the original BDB configuration. The laser intercept was adjusted into the third laser position for conventional signal processing of the IR signal by P8. A fourth laser window circuit needs to be built a means to obtain three-detector fluorescence from the 90SP dichroic optic. The three detectors would be 650-nm longpass (FL3, 488-nm excitation), 682/20 nm (FL4, 658-nm excitation), and 820-nm longpass (APD, 785-nm excitation). We operated the IR laser instead of the 658-nm laser, but it can be operated coaxially with one of the other lasers for four-laser capability.

For optimal detection of IR fluorescence, Radiation Monitoring Devices (Watertown, MA, USA) provided an

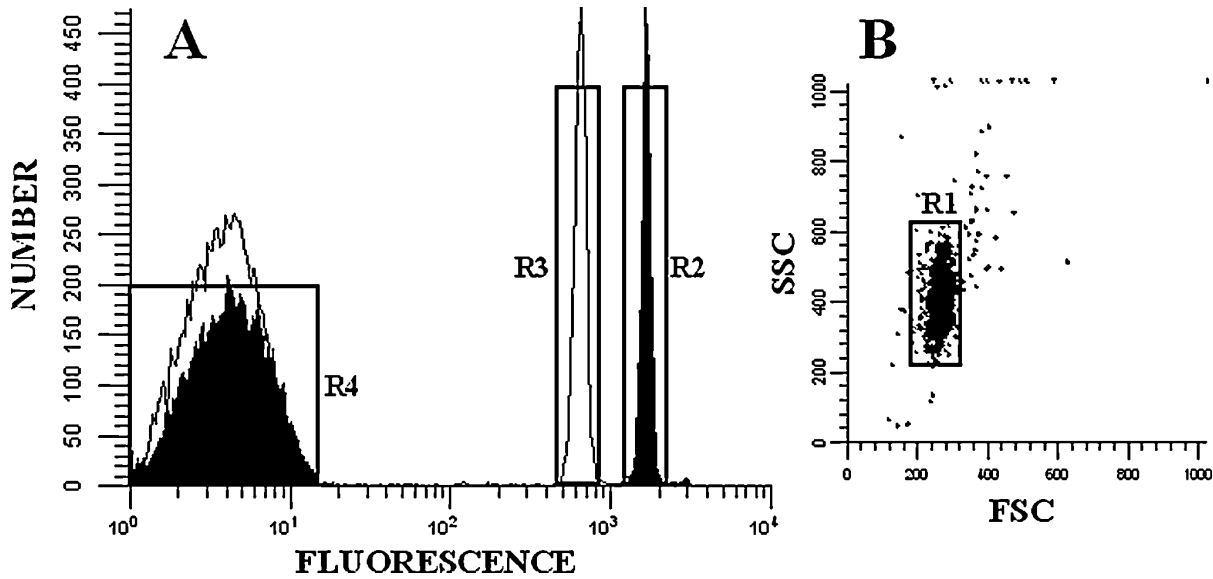


FIG. 3. Comparison of sensitivity between an APD detector and a standard PMT (Hamamatsu R-3896). Data were acquired from the same sample tube containing 5.5- μm blank beads (BCP-50-5) and 5.1- μm Jade Green IR beads (Spherotech CFP-5078-S Lot 082902-B). **A:** R2 shows the fluorescence intensity of the beads detected by the APD and R3 shows the fluorescence intensity of the beads detected by the PMT. The blank beads detected by the APD (solid histogram) and by the PMT (solid line) are also shown in R4. **B:** The forward scatter (FSC) versus side scatter (SSC) plot is shown; R1 is the region used to gate on the singlets. An 820-nm longpass filter was used.

APD. The APD was mounted on a cylinder of high-density polyethylene (HDPE) plastic (Delrin, DuPont, Wilmington, DE, USA). Delrin was chosen for its good machining and isolative properties and is shown in Figure 2A. Two small holes were drilled through the HDPE cylinder and the APD signal and power leads were fed through the holes. On the backside of the HDPE plug, the wires were separately soldered to a high-voltage/signal and ground coaxial cable interconnection. The coaxial cable was fed through the housing. The white material behind and around the APD, as shown in Figure 2A, was press-fit into the housing and the coaxial cable was secured using black silicone adhesive at the top of the housing. This provided an easy adaptation into the LSR optical bench, as shown in Figure 2B, where an APD had been inserted into the standard PMT housing next to the Hamamatsu PMT model R3896 it replaced. The actual sizes of an APD and a PMT can also be compared in Figure 2B.

Because the IR wavelengths will severely damage the human eye, it was necessary to modify the engineer's service scope to allow electronic visualization of the laser beam intercepts and the detectors for alignment. Two types of miniature cameras were evaluated to replace the viewing eyepiece in the service scope: a CMOS color camera (Startek Video, Ft. Lauderdale, FL, USA) and a black-and-white CCD camera (Ramsey Electronics, Victor, NY, USA) that was slightly more sensitive. Thus, one can choose between color and black-and-white viewing. A video monitor (Startek Video) was used to display the microspheres from the camera output for optical alignment.

The 5.1- μm IR-sensitive beads (Spherotech, Libertyville, IL, USA) were used to evaluate the modified LSR. A 488nm

excitation was used for forward and side scatter measurements and 785-nm excitation was used for exciting the IR dyes. Images from the video monitor are shown in Figures 2C and 2D and were obtained using the black-and-white CCD camera to view and hydrodynamically focus the sample core stream. Once microsphere signals were detected, conventional methods of system optimization were performed through the use of an oscilloscope and real-time computer data display.

Normal blood was stained with CD45 biotin (BDB) and Rockland S000-32 IR dye 800 conjugated to streptavidin (Rockland Inc., Boyertown, PA, USA), as previously described (9). Briefly, blood from a healthy donor was obtained in a 10-ml heparinized Vacutainer. After centrifu-

Table 1
Comparison of IR Detection Sensitivity Between an APD and a PMT^a

Marker	Geometric mean	CV	Normalized value	Sensitivity
R2 APD	1677	6.58	407	2.31
R3 PMT	650	8.2	176	
R4 APD	4.12	54.11		
R4 PMT	3.68	49.22		

^aData are from the analysis of the histograms shown in Figure 3. To get a quantitative comparison of detector sensitivities, the geometric means of the fluorescence intensities were used. For each detector, the mean fluorescence for each microsphere population (R2 or R3) was divided by the mean fluorescence of negative beads (R4) to normalize the values. The sensitivity was then calculated as a ratio of the normalized value for the APD to the PMT. The coefficient of variation (CV) for each population is also shown.

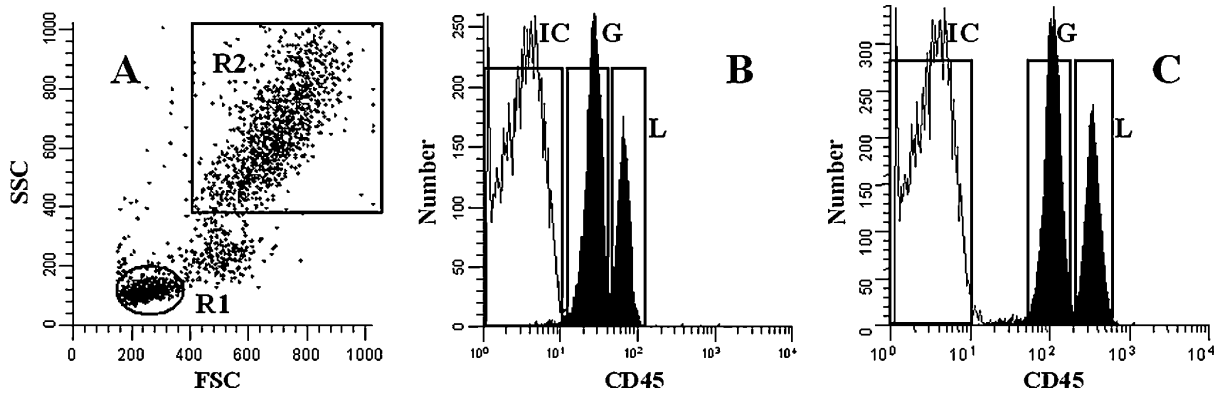


FIG. 4. Immunophenotyping with IR streptavidin. The fluorescence was evaluated at 785-nm excitation. A: The FSC versus SSC plot shows the location of R1 used to gate on predominantly lymphocytes and R2 to gate on predominantly granulocytes. B: The fluorescence emission was measured with a PMT detector (820-nm longpass) operating at 520 V DC. C: Data were acquired using an APD operating at 1,735 V DC. In B and C, isotype control (IC) cells, granulocytes (G), and lymphocytes (L) in regions for analysis of fluorescence intensity are shown. An 820-nm longpass filter was used.

ging at 1,500g, plasma was removed and cells were washed with 10 ml of phosphate buffered saline. Cells (50 μ l) were placed in 10- \times 75-mm plastic tubes in addition to 20 μ l of CD45 biotin and were incubated 15 min on ice. After washing the cells in 3 ml of lysing buffer (1.652 g ammonium chloride, 0.2 g potassium bicarbonate, .0074 g ethylenediaminetetraacetic acid [tetra sodium] made up to 200 ml with distilled water), they were incubated 15 min with 20 μ l of S000-32 IR dye 800 conjugated to streptavidin. Cells were washed in 3 ml of phosphate buffered saline and fixed in 0.5 ml of 2% ultrapur paraformaldehyde (PolySciences, Malvern, PA, USA). Controls consisted of unstained cells only and cells stained with a biotinylated isotype control.

HL-60 cells were stained with various concentrations of PTIR 273, a probe that excites optimally at 785 nm and emits at 814 nm, as previously described (10). The general cell membrane labeling procedure for PKH26 (stock PKH26-GL, Sigma Chemical Co, St. Louis, MO, USA) was used. Briefly, HL-60 cells were grown in RPMI-1640 medium containing 10% fetal calf serum in 75-cm² culture flasks at 37°C in a 5% humidified CO₂ incubator. Cells were shaken off the flask, centrifuged at 400g for 5 min, and resuspended by gentle agitation in 1 ml of Diluent C (Sigma Chemical Co.) at 2×10^7 cells/ml. Immediately before staining, PTIR 273 was prepared at $2 \times$ final concentrations with Diluent C. Then 1 ml of cells was mixed with 1 ml of the appropriate concentration of dye, resuspended by gentle pipetting, and incubated 5 min at 25°C. After incubation, 2 ml of fetal calf serum was added to stop the staining reaction. Cells were centrifuged at 400g for 5 min and washed three times with holding medium consisting of Hank's Balanced Salt Solution containing 2 g glucose per liter and buffered with 20 mM HEPES at pH 7.2.

RESULTS

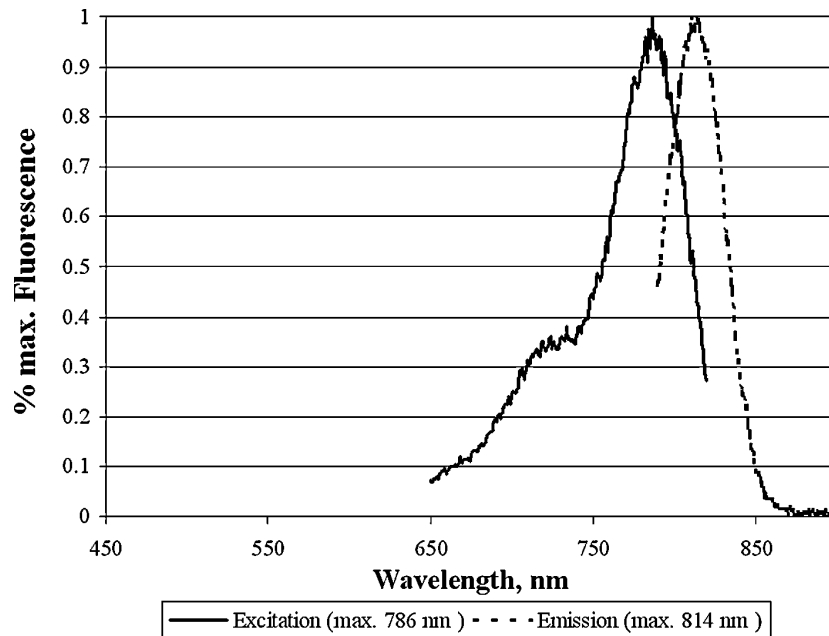
A comparison of detection sensitivities between the APD used in this modification and the PMT is shown in Figure 3. The events detected by the PMT focused on

the 488-nm laser intercept to measure forward and side scatter is shown in Figure 3B. Region R1 was used to gate the singlets. The fluorescence emission histograms from microsphere fluorescence detected by an APD (R2) and the emission detected by the PMT (R3) are shown in Figure 3A. The voltage on the two detectors was adjusted so the blank beads (R4) were in the first decade on the histogram plot. A comparison of the geometric mean fluorescence intensity and the coefficient of variation for each detector is shown in Table 1. The APD was 2.3 times more sensitive than the PMT.

We evaluated two potential applications using IR dyes to evaluate the instrument's capability: immunophenotyping and staining cells with a lipophilic membrane dye. Blood from a healthy donor was processed using standard methodology (9) by staining cells with CD45 biotin and IR dye 800 conjugated to streptavidin. A comparison of fluorescence intensities detected by a PMT with an APD is shown in Figure 4. The PMT fluorescence signal shown in Figure 4B is about 2.8 times less intense than the APD signal shown in Figure 4C. Cells stained with an isotype control are shown in the first quadrant.

Lipophilic membrane dyes can be used to measure the homing patterns of cells *in vivo* (11) and the number of divisions they undergo *in vitro* (10-19). We tested PTIR 273, a lipophilic dye recently developed by PTI Research Inc. (Exton, PA, USA) that fluoresces in the IR region. PTIR 273 is a carbocyanine derivative that has the excitation/emission spectrum shown in Figure 5 (20). The presence of two long saturated alkyl tails gives PTIR 273 stable membrane intercalating properties similar to those of the PKH dyes (10,21) and its far red emitting sister compound, PTIR 271 (13,22). The data are shown in Table 2 and Figure 6. At concentrations above 15 μ M, increased toxicity was found. Concentrations above 7.5 μ M can be used to stain cells bright enough for tracking and measuring cell divisions with good viability.

FIG. 5. Fluorescence spectra of PTIR273. The excitation and emission spectra (in 0.25 μM ethanol) is shown. The excitation maximum is 786 nm (solid line) and the emission maximum is 814 nm (dashed line).



DISCUSSION

The flow cytometer we modified was an LSR-I from BDB. Although there are features that make each model of flow cytometer unique, the general outline described in this report should be applicable to most models. For example, although not described, we have also modified a FACScan (BDB) to a single IR laser instrument by replacing the 488-nm air-cooled laser with the LaserMax IR laser and one PMT with the APD. The LSR-II is supplied with solid-state lasers, and, although the PMT arrangement is different, replacing one of the PMTs with an APD as described here should not be difficult.

Although the APD is more sensitive for detecting IR emission, the standard PMTs supplied with the instrument can still be used. It was our intent to make the comparison to see just how much more sensitive an APD might be. For the dyes used in this report, either detector is suitable; for longer wavelengths, e.g., longer than 850 nm, the sensitivity of the APD is likely to make it the device of choice.

Without question, it is mandatory that the viewing of the IR laser beam be performed electronically. For this purpose, a CMOS or a CCD camera replaced the eyepiece of the engineer's service scope. Although the CCD camera was slightly more sensitive, it provided only a black-and-white image. If a color image is desired, a CMOS camera can be used because it had adequate sensitivity in our experience.

For immunophenotyping, avidin conjugated with an IR excitable dye gave good results in our proof-of-principle experiment using CD45 conjugated with biotin. It would be expected that any biotinylated antibody could be used instead of CD45. Because we are not aware of any commercial antibodies directly conjugated with an IR excita-

ble dye, Alexa 750 (Molecular Probes, Eugene, OR, USA) could be directly conjugated to any antibody as a "home-brew." The reagent can be purchased as a kit and the instructions are clear and simple. One advantage of using fluorochromes that excite in the longer wavelength excitation region is decreased autofluorescence. This produces an increased signal-to-noise ratio and has been appreciated with the apparent increase in brightness of allophycocyanin, CY7, and allophycocyanin/CY7 fluorochromes excited at 635 to 658 nm. The increased brightness is actually the result of decreased noise from autofluorescence.

There is extensive literature on the use of lipophilic membrane dyes as cellular tracking probes (10,11,13,17-19) and in the measurement of proliferation precursor frequencies (10-16,22). The dye PTIR 273 was evaluated as a prototype IR excitable lipophilic membrane dye to illus-

Table 2
*HL-60 Cell Line Stained With Membrane Dye PTIR-273**

Dye concentration	%Recovery	%Viability	Geometric mean
20.0 μM	7	7	732
15.0 μM	68	97	377
10.0 μM	76	97	315
7.5 μM	80	94	240
5.0 μM	70	95	68
0.0 μM	100	99	3.4

*Data are from the analysis of histograms shown in Figure 6. The percentage of recovery is the number of cells per-milliliter after staining multiplied by 100 and divided by the number of cells per milliliter before staining. The percentage of viability is the number of propidium iodide negative cells multiplied by 100 and divided by the total cells. The geometric mean is the fluorescence intensity of the cells.

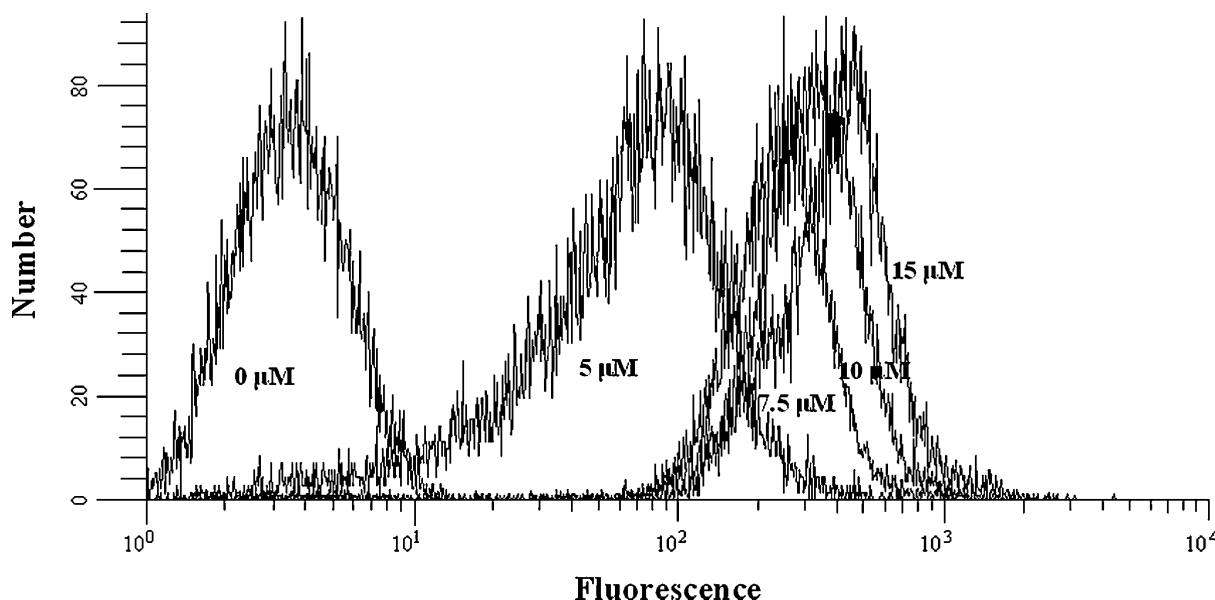


Fig. 6. HL-60 cells stained with the lipophilic membrane dye PTIR 273. The dye was excited by the 785-nm IR laser, and emission was detected with an APD. An 820-nm longpass filter was used. The histograms, derived from unstained and stained cells, have been overlaid.

trate the measurement of viable cells as an example of the instrument's capability. These new lipophilic IR dyes are in development by PTI Research Inc., and new analogs may be brighter and less toxic than the prototype used in these experiments. A detailed report on the optimization and biology of these new IR dyes is forthcoming.

With the capability of measuring probes in the IR region, a new dimension in multicolor fluorescence becomes practical. Other cellular features of interest can be simultaneously measured using fluorochromes excited and measured in the UV and visible regions combined with the IR dye in a multicolor paradigm. The capability of a commercial flow cytometer can be enhanced by modification to provide excitation and fluorescence measurements in the IR region. This new capability will provide for a whole new series of dyes for multicolor interrogation of cellular properties.

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LITERATURE CITED

1. Fulwyler MJ. Electronic separation of biological cells by volume. *Science* 1965;150:910-911.
2. Fulwyler MJ, McHugh TM. Flow microsphere immunoassay for the quantitative and simultaneous detection of multiple soluble analytes. *Methods Cell Biol* 1990;33:613-629.
3. Szollosi J, Matyus L, Tron L, Balazs M, Ember I, Fulwyler MJ, Damjanovich S. Flow cytometric measurements of fluorescence energy transfer using single laser excitation. *Cytometry* 1987;8:120-128.
4. Fulwyler MJ. Flow cytometry and cell sorting. *Blood Cells* 1980;6:173-184.
5. Fulwyler MJ. Hydrodynamic orientation of cells. *J Histochem Cytochem* 1977;25:781-793.
6. Fulwyler MJ. Status quo in flow-through cytometry. *J Histochem Cytochem* 1974;22:605-616.
7. Steinkamp JA, Fulwyler MJ, Coulter JR, Hiebert RD, Horney JL, Mulancy PF. A new multiparameter separator for microscopic particles and biological cells. *Rev Sci Instrum* 1973;44:1301-1310.
8. Fulwyler MJ, Glascock RB, Hiebert RD. Device which separates minute particles according to electronically sensed volume. *Rev Sci Instrum* 1969;40:42-58.
9. Stewart CC, Stewart SJ. Cell preparation for the identification of leukocytes. In: Darzynkiewicz Z, Crissman H, Robinson JP, editors. *Methods of cell biology*. Volume 64. New York: Academic Press; 2001. p 218-270.
10. Horan PK, Melnicoff MJ, Jensen BD, Slezak SE. Fluorescent cell labeling for in vivo and in vitro cell tracking. *Methods Cell Biol* 1990;33:469-490.
11. Wallace PK, Palmer ID, Perry-Lalley D, Bolton ES, Alexander RB, Horan PK, Yang JC, Muirhead KA. Mechanisms of adoptive immunotherapy: improved methods for in vivo tracking of tumor-infiltrating lymphocytes and lymphokine-activated killer cells. *Cancer Res* 1993;53:2358-2367.
12. Givan AL, Fisher JL, Waugh MG, Bercovici N, Wallace PK. Use of cell-tracking dyes to determine proliferation precursor frequencies of antigen-specific T cells. *Methods Mol Biol* 2004;263:109-124.
13. Barbier M, Gray BD, Muirhead KA, Ronot X, Boutonnat J. A flow cytometric assay for simultaneous assessment of drug efflux, proliferation, and apoptosis. *Cytometry* 2004;59B:46-53.
14. Givan AL, Fisher JL, Waugh M, Ernstoff MS, Wallace PK. A flow cytometric method to estimate the precursor frequencies of cells proliferating in response to specific antigens. *J Immunol Methods* 1999;230:99-112 (erratum *J Immunol Methods* 2000;237:207).
15. Boutonnat J, Muirhead KA, Barbier M, Mousseau M, Ronot X, Seigneurin D. PKH26 probe in the study of the proliferation of chemoresistant leukemic sublines. *Anticancer Res* 1998;18:4243-4251.

16. Boutonnat J, Barbier M, Rousselle C, Muirhead KA, Mousseau M, Seigneurin D, Ronot X. Usefulness of PKHs for studying cell proliferation. *C R Acad Sci III* 1998;321:901-7.
17. Teare GF, Horan PK, Slezak SE, Smith C, Hay JB. Long-term tracking of lymphocytes in vivo: the migration of PKH-labeled lymphocytes. *Cell Immunol* 1991;134:157-170.
18. Horan PK, Slezak SE. Stable cell membrane labeling. *Nature* 1989;340:167-168.
19. Slezak SE, Horan PK. Fluorescent in vivo tracking of hematopoietic cells. Part I. Technical considerations. *Blood* 1989;74:2172-2187.
20. Gray B, Askenasy N, Breslin E, Muirhead K, Ohlsson-Wilhelm B, Farkas D. Novel far red and near infrared dyes for cell-tracking in vivo. In: *Proceedings of the American Association of Cancer Research. Molecular imaging in cancer: linking biology, function and clinical applications in vivo*; Orlando; 23-27 January 2002. 2002. p A21.
21. Poon RY, Ohlsson-Wilhelm B, Bagwell CB, Muirhead K. Use of PKH membrane intercalating dyes to measure cell trafficking and function. In: Diamond RA, DeMaggio S, editors. *Living color: flow cytometry and cell sorting protocols*. New York: Springer-Verlag; 2000. p 303-552.
22. Barbier M, Laurier JF, Grunwald D, Mousseau M, Muirhead K, Gray BD, Boutonnat J, Ronot X. PKH versus PTIR membrane dyes for cell proliferation and chemoresistance analysis. *C R Acad Sci Biol* 2002; 325:1-8.