BRIEF REPORT

Visible Diode Lasers can be Used for Flow Cytometric Immunofluorescence and DNA Analysis

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This report describes a feasibility study concerning the use of a visible diode laser for two important fluorescence applications in a flow cytometer. With a 3 mW 635 nm diode laser, we performed immunofluorescence measurements using the fluorophore allophycocyanin (APC). We have measured CD8 positive lymphocytes with a two-step labeling procedure and the resulting histograms showed good separation between the negative cells and the dim and the bright fluorescent subpopulations. As a second fluorescence application, we chose DNA analysis with the recently developed DNA/RNA stains TOTO-3 and TO-PRO-3. In our setup TO-PRO-3 yielded the best results with a CV of 3.4%. Our results indicate that a few milliwatts of 635 nm light from a visible diode laser is sufficient to do single color immunofluorescence measurements with allophycocyanin and DNA analysis with TO-PRO-3. The major advantages of using a diode laser in a flow cytometer are the small size, the low price, the high efficiency, and the long lifetime. © 1994 Wiley-Liss, Inc.

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Since its introduction in flow cytometry (3), fluorescence detection has proven to be a very sensitive and versatile technique. Specific stains for DNA, RNA, and other cellular constituents (e.g., mitochondria) and functional stains for detection of Ca^{2+}, pH, and membrane potential are now well developed. Combined with the monoclonal antibody labeling technique, a very sensitive and specific research tool is available for flow cytometry. Most fluorescent dyes used for these purposes are excitable with UV, blue, or green light, which means that argon-ion or krypton gas lasers have to be used. The major disadvantages of these lasers are well known: they are very expensive, bulky, inefficient, noisy, need considerable cooling, require regular maintenance, and have a limited lifetime.

Another laser, the helium-neon (He-Ne) gas laser, is not often used even though for a number of important applications good results can be obtained with it (8,10,11). The major drawback is the fact that very few red-excitable dyes (excitation maximum above 600 nm) are commercially available (C-phycocyanin (CPC), al-lophycocyanin (APC), and indodicarbocyanin (Cy-5)), and only single color measurements can be done.

Recent developments in the field of solid state physics have led to the production of visible diode lasers with a power of a few to tens of milliwatts (depending on the exact wavelength). We have already shown that diode lasers yield good results for scattering measurements in a flow cytometer (4). Now we have investigated whether diode lasers are worthy substitutes for He-Ne lasers in flow cytometric applications. In other words, can the currently available diode laser now also

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be used for fluorescence measurements? We have tested this for two common flow cytometric applications: first, immunofluorescence measurements using the dye allophycocyanin (APC) coupled to a monoclonal antibody, and second, DNA analysis by using the DNA stains TOTO-3 and TO-PRO-3 (benzothiazolium-4-quinolinium dimer and monomer iodide, respectively).

MATERIALS AND METHODS

The visible diode laser we used was the Philips CQL 840/D (Philips, Eindhoven, The Netherlands), a ridge structure InGaAlP-based laser with a maximum optical output power of 3 mW at 635 nm. This single mode laser emits the light highly divergent; it has a 6° far-field angle (FWHM) parallel to the junction and a 35° angle perpendicular to the junction. The laser is mounted in a SOT 148D case (size: 9 mm in diameter, 5 mm in height) together with a photodiode to monitor the output power. We have mounted this laser into a small aluminum cylinder (25 mm diameter) in order to cool it passively to room temperature. The diode laser driver we used was a voltage stabilized power supply (E-018–0.6/D, Delta Elektronika, Zierikzee, The Netherlands) in series with a 150 Ω resistor (inserted to limit the current).

The optical setup (see Fig. 1) contains the diode laser and a compact disk (CD) lens (RP 036 equivalent, Philips), which collects the highly divergent emitted light and creates a collimated beam. In order to eliminate spontaneous emission we have used a 640 nm shortpass interference filter (35–5420, Ealing, Watford, England) in the excitation beam. In the case of immunofluorescence measurements, a spherical lens (80 mm focal length) was used to focus the light in the flow cell, yielding a spot of ~78 μm by 14 μm. Due to the elliptical emission profile of the laser, an elliptical spot is obtained, which has its long axis horizontal. With a sheath flow velocity of ~2.5 m/s, this results in an illumination time of ~5 μs. For DNA analysis a highly uniform illumination profile is needed, so we used (instead of one spherical lens) a 200 mm and a 100 mm cylindrical lens to create a focus of ~200 μm by 30 μm. The forward scattered light is detected with a negatively biased photodiode (PIN 10 D, United Detector Technology, Santa Monica, CA). Fluorescence is collected on one side of the quartz cuvette with a 1.2 NA gel-immersion objective and detected with a head-on photomultiplier (R 1104, Hamamatsu, Hamamatsu City, Japan). The rectangular cuvette has a flow channel of 160 μm by 400 μm, which allows the use of high numerical aperture objectives in the orthogonal direction. Two 3 mm color glass filters (RG 665, Schott, Mainz, Germany) block the scattered laser light. These filters gave enough suppression at the laser wavelength (more than 10^{-6}) and although the cutoff was 30 nm above the laser wavelength, transmission of APC fluorescence was calculated to be still about 40%. On the opposite side of the flow cell, a 0.4 NA CD lens
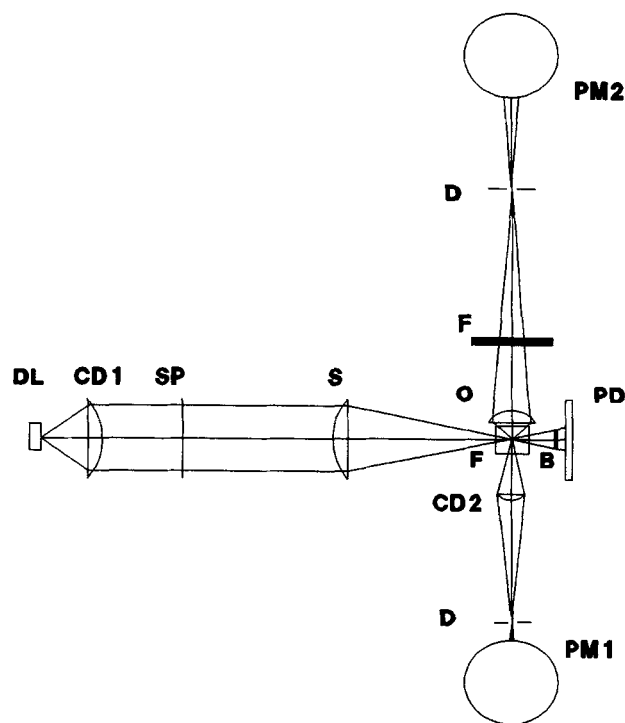

FIG. 1. Setup of the flow cytometer used for immunofluorescence measurements using a visible diode laser. Note that fluorescence and scattering is detected on opposite sides of the flow cell. DL, diode laser; CD1, CD2, Compact Disk player lenses; SP, 640 nm short pass filter; S, spherical lens; F, flow cell; O, objective; B, beam stop; PD, photodiode; F, 665 nm long pass filter; D, diaphragm; PM1, PM2, photomultipliers.

collects the orthogonally scattered light and focuses it on a side-on photomultiplier (R 928, Hamamatsu).

Signals are processed using a conventional peak detection system and a 12-bit analog-to-digital converter (2). The acquired data are stored and analyzed with a PC (1).

The immunofluorescence labeling procedure is as follows: human lymphocytes of a healthy donor were isolated by density centrifugation over Percoll (1.077 g/dm³) and labeled with antihuman CD8-biotin conjugate (Beckton-Dickinson, San Jose, CA). The second labeling step is performed with streptavidin-APC conjugate (Molecular Probes, Eugene, OR).

The DNA staining procedure: 10^6 K562 cells (an erythroblast cell line) in 1 ml RPMI were washed and suspended in 0.2 ml PBS, 1 ml Modified Vindelov's PI solution (9) without PI (0.01 M Tris pH = 8, 1 mM NaCl, 0.1% NP-40, 0.07% RNAse) was added to the cells and incubated for at least 10 min on ice. The cells were washed once and stained with 100 μl TOTO-3 iodide (10 μM in PBS) or 300 μl TO-PRO-3 iodide (10 μM in PBS) (Molecular Probes) and incubated for 30 min at room temperature and in the dark.

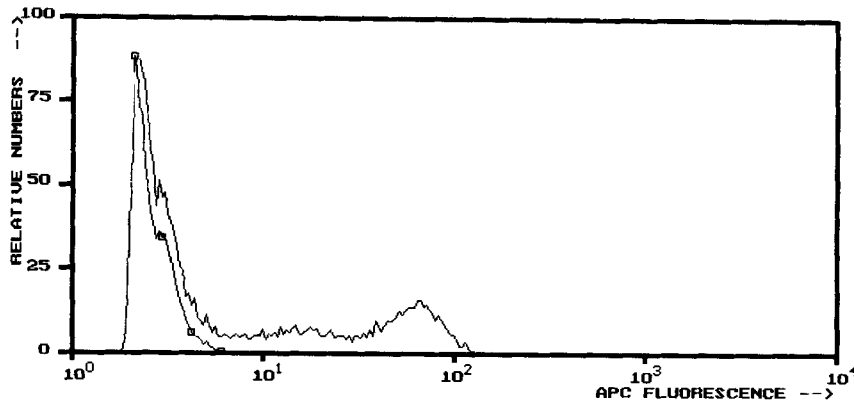Allophycocyanin calibration beads (8 μm) were ob-

FIG. 2. Histogram of fluorescence signals obtained from (13,182) lymphocytes incubated with CD8-biotin/streptavidin-APC with the diode laser-based flow cytometer. The negative control is indicated with the rectangles.

tained from Flow Cytometry Standards Corporation (cat. 901, NC).

RESULTS

The specified power of 3 mW was obtained at a laser current of ~75 mA; this current strongly depends on the ambient temperature. After the warming up period of 30 min, output power fluctuations were negligible during 1-h experiments. High frequency noise ($\geq$ 1 kHz) is very low and determined by the quality of the laser driver; in our case the noise and ripple was ~0.02%. When operated at maximum power, the temperature of the metal block was increased by one or two degrees.

In Figure 2 we show the results of an immunofluorescence measurement of human lymphocytes indirectly labeled with APC. The histogram clearly shows three subpopulations of cells: the bright positive cells (cytotoxic T cells), the dim fluorescent subpopulation (mostly NK cells), and the negative cells. The histograms of control cells (which are only incubated with streptavidin-APC) and untreated cells overlapped with that of the negative subpopulation (see Fig. 2), the nonspecific labeling can therefore be considered negligible.

In Figure 3 results are shown of measurements for different laser powers. This figure indicates that the histogram peaks of the logarithmically measured fluorescence intensity increase logarithmically with the increase of the power. It can be seen also that even with optical powers of only about 1.5 mW, adequate separation between the dim positive and negative cells can be obtained.

We found that the diode laser also emits some light of longer wavelengths than the specified wavelength, probably resulting from spontaneous (LED) emission. Scatter of this light by the cells can increase background in a fluorescence measurement. In order to eliminate this, we have inserted a short pass filter (cut off wavelength 640 nm) in the excitation beam. For very low fluorescence signals this filter proved to be necessary.
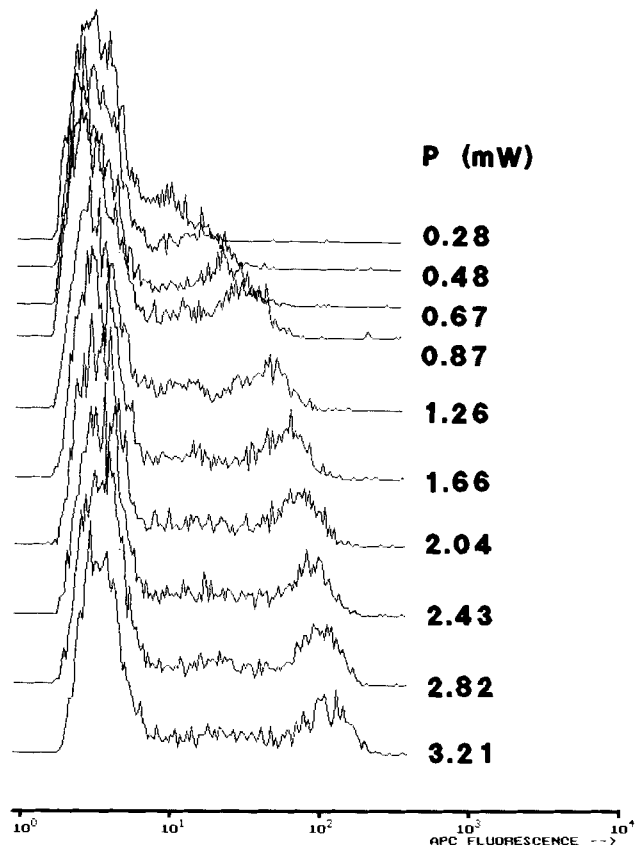

FIG. 3. Histograms of (~4,096) CD8-biotin/streptavidin-APC incubated lymphocytes obtained with different optical powers (indicated on the righthand side).

In order to evaluate the use of a diode laser for DNA analysis, we had to improve the uniformity of the illumination profile. We replaced the focusing lens by two cylindrical lenses, which resulted in a larger and more uniform focus. With the described setup we measured
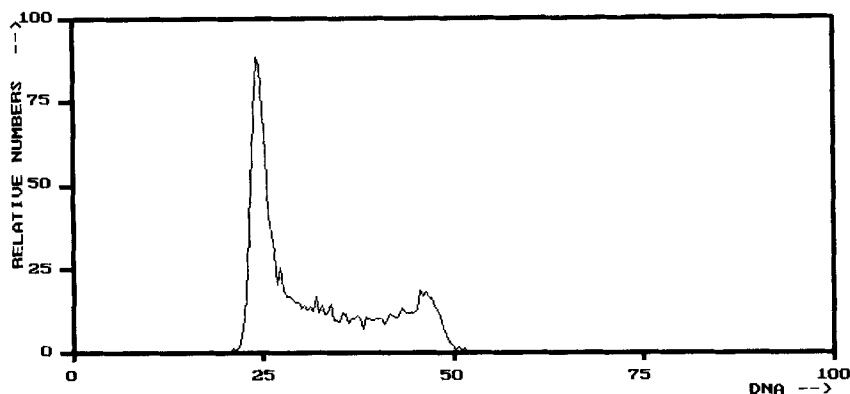
FIG. 4. Flow cytometric DNA histogram of (9,645) K562 cells stained with TO-PRO-3 using the 635 nm diode laser as light source. The CV of the diploid cell population is 3.4%.

DNA histograms of TO-PRO-3 stained K562 cells; in Figure 4 a typical example is shown. Similar results were obtained with TOTO-3 for the same cell type and experimental conditions. Also, other cell types (human NK, Epstein-Barr virus transfected human B cells, mouse L1210 cells) yielded good DNA histograms. We observed that the TO-PRO-3 fluorescence intensity is on the average 30–50% higher than that of TOTO-3. Also, the CVs of TO-PRO-3 histograms are in our measurements better than the CVs of TOTO-3 histograms, namely, ~3.4% compared to 4.1%.

In experiments to determine the optimal staining procedure, we obtained maximum fluorescence signals when ~100 μl TOTO-3 solution and 300 μl TO-PRO-3 solution was applied to 10^6 K562 cells/ml. Above 300 μl TO-PRO-3, the fluorescence intensity saturated and remained stable, whereas the TOTO-3 fluorescence displayed a decrease when the applied amount exceeded 100 μl.

The fluorescence intensity of TO-PRO-3 stained K562 cells is ~20 times higher than that of the CD8-positive lymphocytes. The intensity of APC calibration beads lies in between these two and is ~6.5 times less than that of the TO-PRO-3 stained cells.

DISCUSSION

Earlier attempts to measure immunofluorescence, using a 5 mW 670 nm diode laser for excitation of Ul-tralite 720 (Ultralite Corporation, no longer available) and a 0.6 NA objective, were unsuccessful (4). However, in this study we show that by using highly efficient fluorescence detection with an 1.2 NA objective and a 3 mW 635 nm diode laser, it is possible to perform immunofluorescence measurements and DNA analysis.

The results in Figure 2 indicate that a diode laser can be used for single-color immunofluorescence measurements. The measurement of CD8 labeled lymphocytes has about the same quality as measurements of FITC labeled cells measured in a routine flow cytom-

eter. The signal of the negative cells lies in the order of a few photoelectrons per cell and probably originates from autofluorescence.

A critical inspection of our setup shows that still some improvements can be made. First, only 60% of the excitation beam is transmitted by the short pass filter; this can easily be increased to 90%, e.g., by using a line filter centered at 635 nm. Second, only 40% of the APC fluorescence is transmitted by the long pass filters. This fraction can also be enlarged by using optimized filters. Third, our photomultiplier has a low quantum efficiency at 660 nm (~3%), whereas a photomultiplier with a GaAs photocathode (e.g., the Hamamatsu R636) can have efficiencies at 660 nm as high as 13%. Altogether, these improvements would increase the signals roughly by a factor of 10.

As a second test, we chose the measurement of DNA with the recently developed dyes TOTO-3 and TO-PRO-3. These dyes are reported to have a high fluorescence intensity and a high enhancement of the fluorescence when bound to DNA (or RNA) (6). Both stains could be used to measure DNA histograms, but with TO-PRO-3 we have obtained the best results (CV = 3.4%). At this stage it is not clear yet if the CV is determined by the instrument, the dye, or the staining procedure. It should be noted that the measured intensities are of such a low level that photon noise could also be the determining factor for the CV. The G0/G1 peak of the DNA histogram corresponds roughly with 1,000 photoelectrons, resulting in a theoretical CV of 3%. We have not found flow cytometric DNA measurements using these dyes in the literature.

In principle, optimal fluorescence detection in an orthogonal setup requires a vertical polarization (7). In our setup we used the different emission angles of the diode laser to create an elliptical spot with a horizontal polarization. By inserting a half-wave retarder, which rotates the polarization 90°, we observed marginal differences in the fluorescence signal. This was probably caused by the fact that the loss of light due to the trans-

mission factor of the retarder (~80% transmission) was compensated by more efficient detection. Furthermore, the high numerical aperture makes the detection less critically dependent on incident polarization (compare 5).

The output power of the diode laser strongly depends on the temperature. In our experiments we did not use a stabilization circuit, so these fluctuations were noticeable, but during 1-h experiments, no adjustments were necessary. For practical purposes, however, we advise a power-controlled laser driver and a simple Peltier element-based temperature stabilization unit.

The advantages of diode lasers are remarkable. Diode lasers are inexpensive (~$150), very small, efficient, have a long lifetime, and can be modulated at frequencies up to 1 GHz. At present the power is still low compared to argon-ion lasers, but it is enough for immunofluorescence. A second disadvantage is the fact that the laser emission is astigmatic, but this can be corrected for by relatively simple optics. The fact that diode lasers can easily be modulated with frequencies up to 1 GHz indicates that they can be used for fluorescence lifetime experiments (12). We can also imagine a system consisting of two diode lasers with different wavelengths, which are chopped out of phase, that can separate two fluorophores with different excitation spectra but overlapping emission spectra.

The main problem is the limited availability and exploitation of red-excitable dyes. We therefore stress the need for more red-excitable dyes to enable development of dual-color or even three-color immunofluorescence measurements.

Currently, a large effort is being put into the development of diode lasers with shorter wavelengths, so we expect a bright future for these lasers in flow cytometry.

CONCLUSIONS

We have shown that a 3 mW 635 nm diode laser can be used for two-step immunofluorescence measure-ments with streptavidin-APC as a secondary label, yielding good separation between negative, dim positive and bright positive subpopulations. Also, DNA analysis using TOTO-3 and TO-PRO-3 can readily be done with a visible diode laser.

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