Research Report

Four-Laser Scanning Confocal System for Microarray Analysis

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ABSTRACT

We have constructed a confocal scanner suitable for routine microarray analysis from commercially available parts. We have outlined the details that should be considered when designing such an instrument and listed some of the specific components comprising the system [the full list of system components is available on CD from the corresponding author (D.J.G.) at no charge]. Here, we describe the methods used to test the linearity and sensitivity of the instrument. Performance was evaluated with two commonly used dyes, fluorescein and Cy5. While the instrument had a linear correlation between the dye concentration and fluorescence intensity, the observed deviation from a slope of 1.0 underscores the importance of running multipoint calibration experiments to obtain accurate dye quantitation over the full dynamic range of the scanner. This method has utility in testing commercial instruments in addition to the scanner described here. An array with over 300 spots dyed with Cy3 was scanned with our instrument and a high-end commercial instrument. The agreement between the two instruments was very good over a 1000-fold intensity range. Our scanner is a cost-effective alternative to more costly commercial scanners with similar capabilities.

INTRODUCTION

Microarray analysis of gene expression, mutation, and single-nucleotide polymorphism detection is rapidly becoming important and is replacing other molecular biology techniques (2). Unfortunately, the relatively high prices of commercial systems may prevent some academic laboratories from making use of this innovative technology. Although our group (6) and others (<http://cmgm.stanford.edu/pbrown/mguide/index.html>) have published information on constructing robotic spotters to create microarrays, there is little published information on the construction of analyzers. An original confocal microarray scanner developed by Shalton et al. (<http://cmgm.stanford.edu/pbrown/scanner.html>) (10) used an argon/krypton laser and automated filter wheel changer, both of which tended to make the scanner relatively expensive. Also, the construction is described in the most general terms. The National Institutes of Health has also made available a description of their arrayer and scanner (<http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/aboutreader.html>) that features three lasers to provide versatility; but again, details on the construction are sparse. Somewhat more information is given about the scanner built at the Albert Einstein College of Medicine at <http://sequence.aecom.yu.edu/bioinf/microarray/reader.html>.

Several alternatives to scanners for microarray analysis and alternate designs have been described, including a proximal imaging device that seeks to achieve efficient light capture with a lens-less silicon detector placed very close to the array (3,4,7). Another involves DNA-coated sensor beads held in the etched end of a fiber-optic bundle

(5). Commercial systems based on CCD cameras are also available, although our experience with a system based on this principle (1,8) suggested that it would not have the capabilities needed. Finally, the design of a number of commercial scanning instruments was recently described (10). Despite the variety of options, we decided to use the x,y scanning confocal design in our construction of a scanning device because we felt that it was the most versatile and had adequate sensitivity. We designed it with manually selected optical filters, which is not burdensome if one does not change lasers and filters too often. Our design incorporates two photomultiplier tubes (PMTs) to acquire data at two wavelengths or two different sensitivities so that taking the intensity ratio at two wavelengths simultaneously or collecting data over a very large dynamic range would not involve re-scanning. This report describes a few important details and some of our experience building a useful, versatile, and sensitive laser-scanning confocal instrument. Our device is highly versatile and a fraction of the cost (estimated at \$25 000 with one single-wavelength laser and \$35 000 with four lasers including a multi-wavelength argon laser) of a comparable commercial instrument.

MATERIALS AND METHODS

Laser Light Sources

Figure 1 shows a general overview of the optical system. For brevity, we have provided only general information in this paper. The full details and costs of materials will be provided on CD from the corresponding author at no charge.

Four lasers providing six different excitation wavelengths are incorporated into the system. This includes a red diode laser, multi-wavelength argon laser, green diode-pumped solid-state laser, and yellow gas laser. The excitation wavelengths currently available are 458, 488, 515, 532, 594, and 635 nm, respectively.

These six excitation wavelengths enable one to use a large number of the fluorescent dyes. For example, the red laser (635 nm) is useful for Cy5, the green laser (532 nm) for Cy3, the yellow (594 nm) for Texas Red®, and the blue (488 nm) for fluorescein, YOYO-1, and others. The laser beams are co-linear and coincide with three long-pass filters (540 lp; Coherent, Santa Clara, CA, USA), which are used in place of dichroic mirrors, and several aiming mirrors used after each beam passes through a pinhole located within a spatial filter. This pinhole forms the first part of the confocal system.

Filters and Mirrors

Because of the relatively wide bandwidth of the red diode and green solid-state lasers, it is essential to use a filter (Figure 1, f1) on the excitation side of the system to prevent light from spilling into the emission band. The items labeled f1, d, and f2 (i.e., the excitation filter, dichroic mirror, and emission filter, respectively) represent the two matched sets of triple-bandpass filters. Together, these filter sets are quite useful and encompass a wide range of potential dyes. Full details are provided on the CD available from the corresponding author.

We also have a separate, interchangeable dichroic mirror (Omega XF2010; Omega Engineering, Stamford, CT, USA) that can be placed at point d (Figure 1). The mirror is well suited to the argon laser wavelengths, reflecting light in the range of 450–505 nm. We use this in combination with the narrow bandwidth emission filters

540DF10, 570DF10, 600DF10, and 620DF10 (Omega Engineering) to study the four BigDyes™ (Applied Biosystems, Foster City, CA, USA), and this particular dichroic mirror could be used with many other emission filters as well. The sliding filter arrangements are implemented by using a pair of 1/2-in round optical mounting rods that slide in sockets or one fixed rod with a sliding connector and a second rod sliding in a mounting socket. The rods and surrounding cylindrical sockets act as mounting and bearing surfaces. This arrangement has proved simple, reliable, inexpensive, and easy to adjust. Because the rods are mounted horizontally, there is no need to lock them in place to prevent filters from slipping out of position during use.

Detectors

We used two model R6358 photomultipliers, along with sockets, cables, and miniature power supplies (all from Hamamatsu, Bridgewater, NJ, USA). For a modest cost, these PMTs have excellent sensitivity and a low, dark current. Although we included a self-constructed Peltier cooling system to reduce the dark current still further, we have not had to use it for any of our studies thus far. The power supplies can be varied from 0 to 1000 V, by means of a varying low-voltage signal. We have tried several microscope objectives with this system: numerical apertures (NAs) at 40×0.65 , 60×0.85 , and 40×0.85 . The first two are relatively inexpensive lenses (Edmund Industrial Optics, Barrington, NJ, USA). The last is a Fluor 40™ (Nikon, Melville, NY, USA), which is much more expensive and, as might be expected, provides significantly better results. We prefer lower-power objectives because they have a greater depth of field and are therefore less likely to lose focus during portions of the scan (i.e., even though they do not collect as much light). The problem of keeping the slide surface to be analyzed in exact focus during a scan is very important in confocal systems and will be discussed.

Slide Holder, Stages, and Controller

The x and y stages and the controller

Figure 1. Front view of optical train, showing main components and their arrangement. Key to components: 635 nm diode laser, 1; 532 nm diode-pumped solid-state laser, 2; multi-wavelength argon laser, 3; 594 nm He Ne laser, 4; (3 and 4 are mounted on the back side of the optical breadboard); mirror, m; filter, f; filter used as dichroic mirror, fd; dichroic mirror, d; pinhole, ph; PMT, pm; stage, st; spatial filter, sf; slide holder, sh; microscope focusing body with 40× objective, mi; optical breadboard, bb; and internal light-barrier box, ib. The support structure and external light-blocking enclosure are not shown.

MICROARRAY *Technologies*

are perhaps the most crucial and expensive components of the system. They must be able to move with very low off-axis movement or vibration, reproducibly stop at well-defined positions, accelerate, and move as rapidly as possible from one position to another. The ones we chose were designed for the semiconductor processing industry (Kensington 8500; Kensington, Richmond, CA, USA), with 0.5- μm resolution, 10-in movement range in both the x and y axes, and 4 in/s maximum speed. A ruled glass scale, operating in a servo-loop, assures the high repeatability of position. Because the motors driving the stages must accelerate and decelerate at the beginning and end of each position change, it is impossible to acquire data at equally spaced intervals simply by taking data points at equal time intervals (e.g., with a software loop). To solve this problem, we tapped a signal from the stage controller (Kensington). This was processed to provide a series of pulses (currently, one pulse every 16 μm , but this could be changed to 8 or 4 μm), regardless of the speed. These pulses trigger data-capture events. With the large spot volume (5 nL) and spot diameter (500 μm) we typically use, the smaller number of data points and limited resolution are more than adequate for our purposes. Smaller sampling intervals create very large data files. We found that during the initial acceleration and final deceleration phases of movement, large currents flowed to the motors, creating unacceptable noise in the PMT signal, despite our efforts to shield all cables and circuits. This was solved simply by overscanning and discarding 100 data points at the beginning and end of each scan line. A typical time for a $1.5 \times 1.5\text{-cm}$ scan (877 969 data points) is 10.5 min. Although this is somewhat slower than one obtains with several commercial instruments, it is entirely adequate for our needs at this time.

The slide holder is mounted on two rails on the x axis, 1/4 in high and shimmed very accurately so that their top surfaces form a plane that is very highly parallel to the two axes of movement. The ends of the slide are pressed up against a cut out support plate so that its upper surface is parallel to the plane of travel. A spring-loaded bottom

plate presses up against the slide, forcing it into contact with the accurately positioned top plate. The top surface of the slide is thus reproducibly positioned at a fixed distance from the objective and accurately parallel to the two axes of movement, regardless of the slide thickness or non-parallelism of its top and bottom surfaces. This is an important consideration in the de-

sign. With the 40 $\times$ objective that we use, such parallelism is essential. As little as 2.5 μm (10^{-4} in) tilt in the top surface of the slide caused by misalignment or dust or grease on the slide surface causes poor scans. A corollary of this requirement is that poor-quality slides with waviness in their surfaces will produce poor scans. This sensitivity to tilt and focal plane also explains

Figure 2. Initial tests of linearity and sensitivity. (A) The system's response to various concentrations of (wet) spotted fluorescein. The laser power was not changed but was kept at a low nominal value. The linearity was excellent, although the slope differed slightly from unity. (B) The system's sensitivity to low concentrations of fluorescein and Cy5 dye. The 10^{-11} M Cy5 sample is estimated to represent about 10 molecules/ μm^2 of the surface area.

why some prefer low-power objectives (e.g., 20 $\times$) with a wider depth of field, even though they have a lower NA and a poorer light-gathering potential. We often compromise with a “semi-confocal” design, using a large pinhole 1–2 mm in diameter at position ph (Figure 1), eliminating most off-axis light while maximizing signal intensity. The data provided with this paper were taken with such an arrangement.

Mechanical Design

All of the optical components are mounted on two optical breadboards (Vere, New Kensington, PA, USA) each 2 $\times$ 3 ft in size. These are mounted perpendicularly to each other using structural aluminum beams (80/20, Columbia City, IN, USA) that can be purchased cut to length and are easily assembled with special bolt assemblies and a small wrench. These structural

members have an advantage in that they have built-in slots along all four sides. Although these are used primarily to join pieces together, they are also ideal for holding plywood panels to form a light-tight box. The front panels are hinged so that one can gain easy access to the optical train for adjustments and changes. An inner box constructed of heavy black posterboard (Figure 1, lb) further isolates the PMTs from stray laser light. Figure 1 shows that two of the lasers are mounted on the back side of the vertical optical breadboard. The beams are brought over the top of this support with mirrors, using appropriate shielding to prevent operator exposure to the laser light even when the front doors are open.

Electronics

Several special electronic circuits were designed for use in this system.

Figure 3. Example image obtained from an experimental microarray slide (Cy3-stained). The comparable numerical values for spot intensity for the 339 measurable spots were obtained with our system and with a commercial scanner.

These consisted of circuits to perform the following functions: (i) measure PMT temperatures and cool them thermoelectrically to minimize dark current (currently not used); (ii) control PMT high voltage, convert PMT output current to a voltage, isolate the voltage from ground (avoiding ground-loop noise), and apply an offset voltage to zero the PMT; (iii) add safety features to shut down high voltage and stop stage movement under conditions that would cause danger to the system or operator; (iv) provide power for one laser; and (v) interface with the Kensington stage controller (data capture pulse production and conditioning). The 16-bit A/D converter used to capture data and other ports for miscellaneous I/O signals were part of a multi-purpose data card (CAS-DIO1602/16; Measurement Computing, Middleboro, MA, USA) installed in a Pentium II® computer. A faster Pentium III or IV computer is recommended for new construction to minimize file saving and loading times. Also, a CD burner is desirable for archiving data. Each CD holds 650 MB permanently at very low cost and avoids filling up the hard disk with microarray data.

Software

The software to drive the stage and collect the data was written in the LabView G language (National Instruments, Austin, TX, USA). It provides point and click (on-screen) controls to move the stages in fixed size increments between 1 μm and 10 cm, change scan width and height, start and stop, pause and continue a scan, automatically save it as a Microsoft® Excel® file with a date/time code name or manually with any desired name, and load a previous scan for analysis. LabView has commands to view the scan (x,y intensity plot), expand a portion of the scanned field for closer inspection, move the viewpoint around, and position crosshair cursors on the scanned image. We have added functions to average readings in the rectangular box bound by two crosshair cursors. One can also change the gain of the displayed image between 1 and 256 and choose one of several false-color scales to represent fluorescent intensity.

Higher gains are advantageous when the image would otherwise be too weak to view and interpret (i.e., when the laser intensity and/or PMT gain has been set too low). Several on screen indicators show other details about the scan. As written, the software is useful for handling low-density arrays, but another program is needed to analyze the results for arrays of hundreds or thousands of spots. Commercial software can be used for this as well as the free ScanAnalyze program available from Dr. M. Eisen (<http://rana.lbl.gov/>). Our data can be made ScanAnalyze compatible. Although the final image does not appear on screen until the scan is complete, an additional display has been added so that each scan line can be seen as it is acquired. This enables the operator to set the laser intensity and PMT high voltage properly. This numerical intensity versus the x position plot is more useful for adjusting the instrument than an x,y plot in which the intensity is depicted only on a color scale.

RESULTS AND DISCUSSION

The PMT voltage dependence and linearity of the scanner were tested with a series of 5,6 dicarboxy fluorescein dilutions in alkaline solution. The dye was diluted in 5% glycerin in water to assure that the dye solution never completely dried out after spotting. Although the Cy dyes fluoresce well even when dry, many other dyes, including fluorescein, do not. The final concentrations were in the range of 10^{-8} to 10^{-4} M, and the solutions were spotted in an array at volumes of 100 nL/spot. Both the concentration and spot size were quite large for this experiment and were not designed to test the instrument sensitivity (solutions were hand-spotted with a standard micropipet). Excitation was at 488 nm, and emission was measured at 530 nm with the XF67 filter set. Figure 2A shows the results obtained at several different PMT voltages. Although all five concentrations of dye were scanned at each voltage, some data were either off scale or below the limit of detectability at a given voltage so that only 2–4 data points are shown for each case. Only a single data point was on scale at a voltage of 800, so this data point has

been omitted from the graph. The data covered almost the entire range from 0 to 2^{16} (65536 counts) of the A to D converter at voltages of 200 and 300. As the figure shows, the plot is linear at all voltages between 200 and 600. We observed an increase in data scatter below 200 V (the lower limit of useful voltages). The slope of these lines ranges from 0.90 to 0.93, with an average of 0.915.

Because a slope of 1.00 is expected, representing a direct proportionality between the light intensity and voltage on this log-log scale, a second experiment was conducted to test the response of the PMT and electronics. This experiment consisted of setting a low laser intensity, omitting the emission filter, and reflecting a small portion of the excitation beam back into the photomultiplier. Neutral density filters between 0.3 and 2.0 A were inserted to decrease the light intensity in a series of steps up to a 100-fold attenuation. This also resulted in a good linear plot of log voltage versus absorbance (data not shown), with a slope of -0.88. The similarity of this slope (expected value, -1.0) to that obtained in the fluorescence studies suggests that there is some systematic error in the photomultiplier, amplifier, and/or analog-to-digital converter circuitry. Although the difference between slopes of 0.92 and 1.0 sounds small, it can create a serious error if ignored. A one-point calibration at a fluorescence intensity of 100 would produce a 30% error in the estimated concentration at an intensity of 10 000 (and vice versa) using an assumed slope of 1.0. The utility of these two experiments, even for testing commercial instruments, should be apparent.

To test the sensitivity, a second series of dilutions of fluorescein were made, producing concentrations of 10^{-6} – 10^{-10} M, and these were again spotted on a slide in large 100-nL droplets. A similar slide was made with Cy5 in water (without glycerin) in the range of 10^{-5} to less than 10^{-12} M. Figure 2B shows that 10^{-10} M fluorescein and 10^{-11} M Cy5 were still detectable on our instrument. A calculation based on the spot size indicates that the latter represents less than 10 molecules/ μm^2 . Although we might have demonstrated an even higher sensitivity and some scanner

manufacturers claim ultimate sensitivities of 1 molecule/ μm^2 , this represents less than 1% of a strong hybridization signal. Our previous work agrees with that of others (11) that a good hybridized layer contains 10^3 – 10^4 molecules/ μm^2 . By using newer surfaces that provide more than a monolayer of molecules, we have obtained a dynamic data range of 10^4 on our instrument.

We have compared the Cy5 results obtained with our instrument with those from a high-performance GSI Lumonics ScanArray 5000™ (Packard Instrument, Meriden, CT, USA) on the same slide. The GSI instrument has better sensitivity (10^{-12} M was clearly visible), and its image has a less granular appearance because it is operated at a higher resolution than is currently used in our system. However, we could easily change our resolution to match or exceed theirs.

We also scanned a portion of a slide on which 432 Cy3-stained samples were spotted (Figure 3). A number of these were deliberate blanks, but 339 produced measurable spots. The slide was imaged with our system and with a GSI ScanArray 5000. Because the software we used (Microarray Suite; ScanaLytics, Fairfax, VA, USA) processes data somewhat differently than that used by the GSI 5000 when carrying out background subtraction and averaging, we compared the raw values of total spot intensities (the sum of intensities of all pixels included in the spot). These ranged from 6000 to 6 million. If the spots averaged 100 pixels, then this would represent an average intensity range from 60 to 60 000. The average deviation in the results between the two instruments was 19.7%, and the SD of the differences was 29.6%, which is a very reasonable correlation, considering the general level of reproducibility for microarray data.

In summary, we are quite pleased with the performance of our instrument and find it to be useful, rugged, and reasonably economical to construct. The greatest advantage of our instrument lies in the fact that we can substitute optical filters and make other modifications to conform to new types of samples and experiments at will, without the fear of voiding a warranty. With a potential 10×10 -in scanning field, our

scanner also could be used for large nylon membranes, microplates, and many other items. Further details on the construction, parts list, specifications, and software are available on the CD from the authors.

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