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TITLE ULTRASENSITIVE FLOW CYTOMETRIC ANALYSES

AUTHOR(S) James H. Jett, L. Scott Cram, Richard A. Keller, John C. Martin, George C. Saunders, Larry A. Sklar, and John A. Steinkamp

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Los Alamos National Laboratory
Los Alamos, New Mexico 87545

Ultrasensitive flow cytometric analyses

James H. Jett, L. Scott Cram, Richard A. Keller, John C. Martin, George C. Saunders, Larry A. Sklar and John A. Steinkamp

Los Alamos National Laboratory, Los Alamos, NM 87545
(LAS, Univ. of New Mexico School of Medicine, Albuquerque, NM 87113)

ABSTRACT

Flow cytometry as a technology has matured to the point that it is now used routinely in clinical settings. The primary clinical applications of flow cytometry are immunophenotyping and cellular DNA content analysis. Immunophenotyping is used to determine the fraction of specific white blood cell types labeled with fluorescently labeled antibodies to specific cell surface antigens. DNA content measurements are used for determination of normality in tumor diagnosis and for cell cycle distribution analyses.

New techniques and approaches to cellular analysis are being developed at the Los Alamos National Flow Cytometry Resource. These developments can be divided into those that improve sensitivity through the implementation of new measurement techniques and those that move the technology into new areas by refining existing approaches. An example of the first category is a flow cytometric system capable of measuring the phase shift of fluorescence emitted by fluorophors bound to cells is being assembled. This phase sensitive cytometer is be capable of quantifying fluorescence life time on a cell-by-cell basis as well as using the phase sensitive detection to separate fluorescence emissions that overlap spectrally but have different lifetimes.

A Fourier transform flow cytometer capable of measuring the fluorescence emission spectrum of individual labeled cells at rates approaching several hundred per second is also in the new technology category. The current implementation is capable of resolving the visible region of the spectrum into 8 bands. With this instrument, it is possible to resolve the contributions of fluorophors with overlapping emission spectra and to determine the emission spectra of dyes such as calcium concentration indicators that are sensitive to the physiological environment.

Radioimmunoassay (RIA) is a well established and very sensitive assay that has been applied to quantification of the number of soluble molecules in clinical samples. A flow cytometric based solution immunoassay has been developed that has the sensitivity of a RIA. The flow assay uses fluorescent and nonfluorescent microspheres coupled to antibodies or antigens in sandwich or competitive binding modes to give a detection limit of 10^{-14} molar for detection of soluble antigens.

The last example of new technology development is the application of single molecule detection to DNA sequencing. Flow cytometric techniques have been refined to the point that it is possible to detect individual fluorescent molecules in solution as they flow past a laser beam. This capability has lead to a rapid DNA sequencing project. The goal of the project is to develop a technique that is capable of sequencing long strands of DNA (40,000 kb) at a rate of between 100 and 1,000 bases per second.

Extensions of existing technology are being pursued in three areas: DNA fragment sizing, fast

kinetic measurements, and optical sorting. A cytometry-based assay that extends the sensitivity of conventional cytometers to size DNA fragment has been demonstrated . Preliminary results have shown well resolved, linear measurements of fragment lengths in the range of 10 to 50 kilobases.

Many important biological processes take place on subsecond time scales. Currently, flow cytometric measurements of kinetic processes can be made with an initial time resolution of a few seconds. By using stopped flow mixing technology, a system is being developed that will achieve 100 millisecond time resolution. This technology will enable the examination of early steps in signal transduction pathways at physiological concentrations of ligands.

One of the origins of the Human Genome Initiative was the ability to separate physically sufficient quantities of individual human chromosomes to construct DNA libraries using recombinant DNA techniques. Sorting technology continues to evolve with the current development at Los Alamos of an optical sorter that will be capable of sorting chromosomes at rates not attainable previously.

STATUS

The National Flow Cytometry and Sorting Resource (NFCR), funded by the National Institutes of Health National Center for Research Resources and the Department of Energy, was established at Los Alamos to advance the state-of-the-art in flow cytometry as well as to provide facilities for collaborative research and services to the biomedical community. The developmental projects of the NFCR have led the way for advances in numerous areas of flow cytometry. NFCR developments in instrumentation capabilities and in biological applications have found their way into clinical applications and commercial instrumentation. This paper highlights several NFCRinstrumentation developments that point the way towards new ultrasensitive clinical assays.

The developments can be categorized into those that improve sensitivity through the implementation of new measurement techniques and those that move the technology into new areas by refining existing approaches. The first category includes the use of phase-sensitive fluorescence detection, the application of Fourier transform interferometry to spectral analysis, the use of multiple sized microspheres in a fluorescence based soluble analyte immunoassay, and the extension of single molecule detection to rapid DNA sequencing. Developments in the second category are represented by kinetic cytometry using stopped flow techniques, DNA fragment sizing, and sorting by optically induced damage to biological particles. Many of these developments have been, or will be, described in detail at SPIE sponsored conferences and are referenced below.

Phase sensitive flow cytometry

Numerous fluorophors are used in flow cytometric analyses to tag a wide range of cellular components, and the list of applications continues to grow as new probes are identified. In multiparameter measurements, the most common technique used to separate multiple fluorescence emissions relies upon differences in emission spectra and uses color separating filters and dichroic mirrors to achieve spectral separation. However, it is not always possible to find a good combination of filters and mirrors that will cleanly separate the fluorescence emissions. An example of the overlap of emission spectra occurs when analyzing labeled antibody fluorescence

in the presence of high levels of cellular autofluorescence. In cases like this, it may be necessary to employ another approach which takes advantage of differences in fluorescence lifetimes to resolve the fluorescence emissions.

Performing such separations is one goal of the phase-sensitive flow cytometer development. To achieve the separation, the excitation laser beam is modulated at a high frequency (5 to 33 MHz), and phase sensitive detection electronics are used to separate the components of the mixed fluorescent emissions. The frequency of the modulation used depends upon the lifetimes of the fluorescence stains being detected. Steinkamp and Crissman (1,2) have reported on the development of a phase-sensitive flow cytometer that employs this approach to separate overlapping fluorescence emissions. In addition, the system can measure fluorescence lifetimes directly.

Fourier transform flow cytometry

Another approach to separating highly overlapping fluorescence emissions in a flow cytometer is to make high resolution spectral measurements. This has been accomplished by the development of a Fourier transform interferometer system by Buican (3). In this system, the fluorescence emissions are collected by a single microscope objective, passed through a laser light-blocking filter, and transmitted to an interferometer. The interferometer is constructed using three photoelastic modulators driven resonantly at 85 kHz mounted between two polarizing beam splitters. The dwell time of particles in the laser beam is increased to about 100 µsec as compared to transit times of from 2 to 5 µsec in typical flow cytometers. In this transit time, the interferometer makes more than six complete cycles through the region of spectral sensitivity which is typically 500 to 700 nm. The output of the interferometer detector is continuously digitized at 8.5 MHz with 8 bit resolution. The ADC output is passed to a custom built processor that averages the multiple passes of the interferometer during the time that a cell is present to produce a single pass interferogram. The average interferogram is then transformed by hardware to yield an uncorrected emission spectrum or deconvolved in real time to yield the intensity of the emission due to a specific dye. The coefficients used in the deconvolution are determined by training the processor using samples of cells stained with only one fluorochrome at a time.

Microsphere based fluorescence immunoassay

The development of immunoassays (4) in the 1950s revolutionized both basic biomedical research and clinical assays in which very low levels of specific molecules needed to be determined. Absorbance and fluorescence immunoassays that are analogs of the original radioactivity-based assay have now been developed. A new fluorescence-based immunoassay is a microsphere-based assay that is analyzed by a flow cytometer (5,6). This assay has achieved an detection sensitivity of 10^{-14} molar in the sandwich mode and 10^{-12} in the competitive binding mode. It is a homogeneous assay that is based on using two types of microspheres.

Large, 10 µm diameter, nonfluorescent spheres are labeled with antibody to the molecule being detected. These microspheres are incubated in the assay fluid. The antigen of interest binds to the microspheres in proportion to its concentration in the fluid.

Small, 0.1 µm diameter, fluorescently labeled microspheres are added to the the mixture. In addition to being fluorescent, the small spheres are also labeled with antibody (for the sandwich assay) or with antigen (for the competitive binding assay for soluble antigens). The antibody

labeled small spheres are bound to the large spheres at locations where the soluble antigen has bound the the large spheres. This is a direct reading homogeneous assay in that the number of small spheres bound is directly proportional to the concentration of antigen molecules in the fluid being assayed. In the competitive binding assay, the antigen-labeled small microspheres bind to the large microspheres only at sites not occupied by the free soluble antigen. This competition produces an assay in which the number of small fluorescent spheres bound to the large spheres is inversely proportional to the amount of soluble antigen in the assayed fluid.

In either case, after incubation, the assay fluid containing the mixture of large and small spheres is analyzed on a flow cytometer. Data collection is triggered by the presence of a large microsphere in the laser beam. The presence of a 10 μm sphere is easily determined by forward angle light scatter. The fluorescence is collected in the usual manner along with the light scatter signal. Although there is a sea of unbound small fluorescent particles present, they do not interfere with the measurement for two reasons. First, the detection electronics are AC coupled to remove the DC component of the fluorescence signal due to the unbound small spheres. In addition, the level of the DC fluorescence detected is reduced by limiting the probe volume that is seen by the detector. The resulting assays have the sensitivities stated above and can be used in a wide variety of applications.

Rapid DNA sequencing by single molecule detection

A long term collaboration between the Chemistry and Laser Sciences Division, the Physics Division, and the Life Sciences Division at Los Alamos has resulted in improved sensitivity for detection of fluorescent molecules in a flow cytometric-based apparatus; this improved sensitivity allows individual molecules to be detected as they pass through the laser beam. Recently, two species of molecules with different excitation and emission characteristics have been detected in a single apparatus (7). The measurement of the lifetimes of individual molecules has also been accomplished in a flowing system (8,9). Successes in detecting the presence of individual molecules have resulted in a program to sequence long strands of DNA at high rates.

This research is being described in detail (Conference #1891: Advances in DNA Sequencing) of this symposium (10). Briefly, the scheme is to synthesize a strand of DNA complementary to the strand to be sequenced using prelabeled fluorescent nucleotides. Each nucleotide type will have a unique label. The labels will be identifiable by their emission spectra and/or their fluorescence lifetimes and have photophysical properties such as high light absorption cross section, high fluorescence quantum yield, and low photodegradation that are conducive to single molecule detection. A single labeled strand of DNA will be suspended in the flow stream of a modified flow cytometer that is capable of single molecule detection. Suspension of the labeled fragment of DNA will be by attachment to a microsphere that can be manipulated or attached to a grid. An exonuclease will be added to the flow stream that is capable of cleaving the individual labeled bases at rates of between 100 and 1000 bases per second. The liberated bases will then be transported by the fluid flow through a laser beam where they will be detected and identified. The labeled base can be identified by its emission spectral properties or by its fluorescence lifetime. Significant progress has been made on all aspects of this scheme as detailed elsewhere(11).

DNA fragment sizing by flow cytometry

The second category of developments in flow cytometry includes those that refine or extend existing techniques. An outgrowth of our ability to detect single molecules is the application of sensitive fluorescence detection to the sizing of individual DNA fragments. This flow cytometric approach is capable of providing quantitative information on the size populations present in a mixture of DNA fragments in less than four minutes and with an accuracy that equals or exceeds that attainable with gel electrophoresis. In addition, the calibration curve for the system has been demonstrated to be linear (R=1.000) out to 48 kilobases. We expect that this linearity will extend to even larger fragments of DNA. The sizing information is obtained by staining the DNA stoichiometrically with a fluorescent intercalating dye. The solution of stained fragments is passed through a flow cytometer designed for high sensitivity measurements. The intensity of the fluorescence measured for each fragment is directly proportional to the fragment length. Results are being presented at this conference by M. Johnson, et al. (12) and are being published elsewhere (13).

Fast kinetic measurements

There are a multitude of kinetic processes in biological systems that have rate constants that range from picoseconds to hours. Flow cytometers offer a unique capability to perform assays of cellular ligand binding kinetics that are otherwise very difficult or impossible to perform. The small probe volume of a cytometer makes it possible to analyze the of binding of fluorescent ligands to cells in the presence of free ligand in solution (14). Several approaches to making kinetic measurements in flow have been developed (15). Watson and Dive have described (16) a continuous mixing system that is capable of recording data within one second of mixing cells with reactants. Single shot mixing systems in which data recording commences within one second after mixing has also been developed (17). Approaches using stopped flow techniques are currently underway to reduce the time between mixing and recording data to 100 milliseconds (18). This method will allow a whole new class of experiments to be performed in signal transduction systems.

High speed optical chromosome sorting

The construction of chromosome-specific recombinant DNA libraries requires the sorting of large quantities of chromosomes. To date, conventional commercial sorters and specially constructed high speed sorters have been applied to this task (19). Current trends in cloning use vectors, such as yeast artificial chromosomes, that accept large inserts. Cloning into these vectors is less efficient than cloning into vectors that accept small inserts. Thus, the demands for sorted material continue to increase. Since droplet based sorting has reached its limits in terms of speed, a totally new approach to sorting is being developed. This approach is based upon the use of a photoactivated cross-linking agent and exposure to sufficient fluxes of ultraviolet light to prevent cloning of the chromosomal DNA. The analysis of a chromosome population is performed in a conventional manner except that the rate of analysis is 10 to 20 times higher. Sort decisions are implemented by switching an ultraviolet laser beam on and off with a optical modulator. The sample stream is continuously irradiated except when a desired chromosome is present. The effluent is collected and processed for cloning. Only DNA from unirradiated chromosomes will be cloned.

SUMMARY

Several of these new flow cytometric techniques being developed at the NFCR may ultimately have clinical applications. Chromosome-specific libraries are already finding application in tumor diagnosis. The microsphere based fluoroimmunoassay can be easily adapted to quantification of analytes of clinical interest such as the levels of creatine kinase that are increased after heart attacks. DNA fragment sizing provides the basis for the development of a flow cytometric RFLP assay. High speed DNA sequencing will become more routine which will make it more available in the future.

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