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B. Research Highlights

1. Rapid Kinetics Flow Cytometry: Larry A. Sklar, John P. Nolan, Robert C. Habbersett, and John C. Martin

Overview: Among the most powerful and least exploited capabilities of flow cytometry is the ability of cytometers to discriminate free and cell-bound ligand without a wash step. Ligand-receptor binding can be monitored with a sensitivity improved by 3 to 6 orders of magnitude over static cuvette methods. Kinetic methods based on these principles have already been useful in examining ligand-receptor binding interactions with time constants in the range of seconds. However, if the technology were extended into the subsecond time frame, the power of flow analysis could be applied to ligands with affinities as low as $1 \times 10^6 \text{M}^{-1}$. These would include hormones, peptides and peptidomimetics. Since binding events occur on cells in subsecond time domains and precede cell activation, such assembly and activation events would be accessible to real-time analysis. Many other biological questions (cell activation, enzyme activities, etc.) also demand subsecond kinetic analysis. In the ongoing investigations, kinetic capabilities have been systematically introduced into flow cytometry through the development of a Rapid Mix Flow Cytometer. The performance of the device has been evaluated by physical and biological criteria. The instrumentation is being applied to biological questions involving model peptide receptor systems. An invention disclosure has been made at LANL and we anticipate that the instrumentation will be suitable for commercialization and patenting.

Approach: A flow system offers the possibility of characterizing a low affinity ligand-receptor interaction in a homogeneous assay system before ligand has a possibility to dissociate as it might in a heterogeneous radioligand assay system. Consider the example of a ligand with an affinity of $1 \times 10^6 \text{M}^{-1}$. If the ligand is a small molecule like a peptide hormone, the association rate constant would approach a limit of $-1 \times 10^7 / \text{M} \cdot \text{sec}$ and the off rate constant would be $-10 / \text{sec}$. This ligand would exhibit a dissociation halftime $\approx 100 \text{ msec}$, a time inconsistent with analysis in a heterogeneous assay system. In order to examine such interactions where equilibration is occurring in the subsecond time domain it would be necessary to mix and deliver sample in a subsecond time frame. Since flow cytometry provides the capability of single cell analysis in cell populations with the receptors present at natural abundance, this approach makes it possible to examine ligands and receptors on living cells that are actually transducing signals. To be useful in the most general range of applications, a "fast" cytometer should possess the following capabilities: 1) sample mixing and delivery in a time-frame of hundreds of msecs; 2) accurate and reproducible mixing volumes so that information pertaining to the concentrations of the reagents is known; 3) integration of sample delivery into the cytometer fluidics system so that the sample flow is stable to retain the advantage of flow analysis to discriminate free and bound ligand.

Brief Summary of Progress and Results to Date: In first year of the project, concepts to modify a commercial mixer and add a fast valve between mixer and cytometer solved a crucial problem in sample delivery, allowing the output of the mixer (several hundred uls of sample mixed in a few msecs) to be delivered into the flow cytometer at an appropriate rate of $-1 \text{ ul/sec}$. In the second year of the project: 1) We succeeded in modifying the mixing and delivery system and tested the mixer independent of the flow cytometer; 2) We integrated the mixing device with the flow cytometer and achieved mixing and delivery times in the range of 500-1000 msecs; 3) We established data acquisition capabilities to collect data vs. time and display the data. In the past year (FY'94): 1) We mounted the Biologic mixing device on a FACS 2 optical bench, which provides pressure driven fluidics and stream-in-air sample measurement. The FACS 2 also provides conventional "slow" sample delivery and two channel fluorescence detection plus FALS.; 2) We reduced the dead volume between exit valve and the sheath stream via a custom made sample nozzle; 3) Sample lines were modified for ease of sample handling by incorporating
sample injection loops. This allows reduction in the amount of sample needed per experiment and greatly decreases the time needed between experiments to flush lines and change reagents. 4) We configured computer-triggered time ramps for short (10-30 second) and longer (1-10 min.) experiments. These changes established a stable and robust instrument configuration for routine testing. We extensively tested the ability of the instrument to quantitatively proportion reagents and efficiently mix sample, achieving delivery times of under 300 msec for fluorescent microspheres (Fig. 1). This work is described in more detail in the manuscript "A Rapid Mix Flow Cytometer with Subsecond Kinetic Resolution" (Cytometry, in press). A more recent development is the capability of the instrument to perform multiple mixing steps. This is accomplished by configuring the sample lines to allow one syringe to mix the contents of two sample loops, for instance cells and ligand to initiate receptor binding. After the mixture is allowed to age for some time, it is mixed with a second reagent from the second syringe. This configuration is compatible with simple, one step mixing protocols, but also enables the execution of more complicated pulse-chase-type of experiments to allow study of complex reaction sequences occurring in or on cells.

Biological Applications: Initial emphasis is being placed on biological applications involving the formyl peptide receptor of the neutrophil, a model system for the study of G-protein coupled receptor mediated signaling. The receptor is involved in host defense and binds ligands of the type CH₅-met-leu-phe. Neutrophils express ~100,000 of these receptors and the affinities of the ligands can be 1x10⁹/M and higher. Several biological preparations (transfected cells) of the formyl peptide receptors which permit complementary cytometric and spectrofluorometric analysis of ligand-receptor interactions are available as are peptide ligands with affinities ranging from ~1x10⁵ to ~1x10¹⁰/M. Analysis of the kinetics of association and dissociation of these ligands with their receptors provides mechanistic information about cell signaling including interactions of receptors with guanine nucleotide binding proteins (G proteins), receptor desensitization, and receptor internalization and recycling. Kinetic analysis of Ca²⁺ fluxes or other downstream consequences of ligand binding can reveal information on the dynamics of the intervening signals. Examples of these types of experiments are shown in Fig. 1. Rapid binding kinetics of FITC-labeled formyl peptide (Fig. 1A) are readily measured in a single mix experiment on the RMFC. Using a double mix protocol cells are allowed to bind for a short interval (here 3-10 seconds), before excess unlabeled peptide is added to initiate ligand dissociation. When the association and dissociation kinetics are analyzed by various models of ligand-receptor-G protein-effector interactions, mechanistic information about the rates and sequence of the macromolecular interactions is obtained. When the Ca²⁺ response of cells to ligand binding is measured (Fig. 1B), further information is obtained about the events which precede the Ca²⁺ flux. Additionally, since the kinetic resolution of the RMFC (<100 msec/channel) allows the detection of cells in the middle of their Ca²⁺ flux, direct information about the mechanisms involved involved in Ca²⁺ transport.

Future Applications and Development: Analysis of ligand association and dissociation with receptor as a function of time provides information about rates and extent of receptor coupling with G proteins as well as receptor desensitization. Further information on the initial events in cell activation will be obtained by measuring rapid Ca²⁺ fluxes in response to ligand binding. Analysis of this data relies heavily on computational methods and data modeling. In addition to using the unique computational resources available at Los Alamos, we are taking advantage of Statistical Analysis and Modeling software (SAAMII) developed at the Resource for Kinetic Analysis and the SAAM Institute at the University of Washington.

Additional applications currently under development include the use of rapid dilution to detect binding of low affinity ligands, an important capability for the characterization of pharmaceuticals which target cell surface receptors, and the analysis of intracellular drug transport in multi-drug resistant and sensitive cell lines. Discussions with external collaborators concerning fluorescent RGD peptides for platelets and the assembly of transcriptional complexes on beads are ongoing.
Fig. 1. Kinetics of formyl peptide stimulated signal transduction. A) Kinetics of FITC-formyl peptide-receptor association and dissociation. Cells expressing the formyl peptide receptor were mixed with 10 nM fmel-leu-phen-phen-FITC (4PEP) to measure association kinetics in a single mix experiment (circles). Ligand dissociation kinetics were measured in a double mix experiment where excess ligand is added (arrows) 3, 5 or 10 seconds after initiation of binding (inverted triangles, squares, and triangles). Data from duplicate runs of each experiment are presented. B) Kinetics of Ca++ flux in response to formyl peptide binding. Fluo-3 loaded cells were mixed with varying concentrations of formyl peptide and the resulting Ca++ flux was measured.
3. Logarithmic and Anti-logarithmic Transformations and Statistical Analysis
Chiranjit Deka, Robert C. Habbersett, Gary C. Salzman, Larry A. Sklar, and James H. Jett

In flow cytometry both logarithmic and linear amplifiers are used extensively to amplify the signals from photo-detectors before data acquisition. Renewed interest in the quantitative statistical analysis of flow cytometric data has generated a variety of questions regarding the use of nonlinear transformations of flow cytometric data. Although transformation of raw data from one domain to another has received considerable attention, the effects of such nonlinear transformation on statistical analyses has not been studied critically. We have explored and compared the relative merits of the different statistical methods, with special emphasis on the practical considerations of they applicability of those methods to flow cytometric data.

In general, both analytical and numerical methods can be used to obtain statistical information in a given domain using distributions acquired in another domain. The analytical methods allow one to calculate central location statistics in the linear domain using the logarithmic domain arithmetic mean, standard deviation, etc. However, in order for such calculations to give meaningful results, the characteristics of the log-amplifier used in the log-domain data acquisition must be known accurately. Further, such analytical procedures are limited in the types of distributions that they can handle. We have explored these analytical methods in detail and have defined the limits of their applicability to real world flow cytometric data. As an alternative to the analytical methods, one can use the numerical method. Here, a data set is first transformed to the desired domain and statistical calculations are carried out numerically on the transformed data. Non linearly transformed distributions (linear to logarithmic, or visa versa) are, however, associated with non-uniform sampling intervals. The sampling of the log-transformed fluorescence data are biased towards the high intensities while that of the anti log transformed data are biased towards the low intensities. We have examined the effects of the biased sampling on the statistical information derived from non linearly transformed data sets. Further, it has been shown that some of the problems that result from the use of a biased, non-linearly transformed distribution may be avoided if one uses a density distribution and applies a curve fitting procedure for statistical analysis.

A paper describing the transformations and the density distribution approach will appear in Cytometry.

4. Energy Transfer Measurements of Molecular Conformation in Micelles
Larry A. Sklar and Christina Wistrom

In the Summer of 1993, David Holowka, Ph.D., of Cornell University did a sabbatical at the National Flow Cytometry Resource. David is a well-known biophysicist, who among other things, uses spectroscopic tools to examine molecular organization in membranes. He wanted to use fluorescence resonance energy transfer in flow cytometry to examine the spatial relationships between membrane components. As often happens, one thing led to another and his tips about the use of “fast d2” (1,1-dilinoleyl-3,3,3′,3′ tetramethyl indocarbocyanine perchlorate, Molecular Probes) led Christina Wistrom, a former postdoc at the NFCR, and me into an altogether new area of investigation. We had already been working for a number of years with Richard Ulevitch, Ph.D. and Peter Tobias, Ph.D. at The Scripps Research Institute on the interactions of lipopolysaccharide (LPS) with cell receptors and plasma binding proteins. Flow cytometry was being used to quantify the effects of LPS on the adhesive function of isolated leukocytes (Lynam et al. Blood, 83, 3303, 1994) as well as the physiology of leukocytes in blood (Weingarten et al. J. Leuk. Biol. 53, 518, 1993). It was our goal to characterize the physiology of cells in trauma patients. Because bacterial endotoxins (LPS), cell wall components of gram negative bacteria, are known for their involvement in septic shock there were a number of important questions about the
delivery of LPS to its cell surface receptor CD14 to be answered. For example, even though the
chemical structure of LPS is known for many bacterial strains, the supramolecular structure of LPS
fragments in the blood stream is still being investigated. We decided that it would be useful to try
to examine LPS in a monomeric form in a sulfobetaine palmitate detergent micelle system.

We began to use fluorescence resonance energy transfer (RET) to study the organization of
LPS from several different gram negative bacterial strains. LPS labeled with fluorescein
isothiocyanate (FITC-LPS) was the donor probe. As acceptor probes we selected 1,1-dilinoleyl-3,
3, 3', 3' tetramethyl indocarbocyanine perchlorate (Fast C18-Dil) and octadecyl B rhodamine
chloride (C18-Rhd) to be incorporated into the micelles in the presence of the LPS. Because of
extensive spectral overlap between FITC and the acceptors, the calculated distances of 50% transfer (Ro) for the donor - acceptor pairs were considerable. For the pairs FITC-LPS/C18-Dil and
FITC-LPS/C18-Rhd solubilized in sulfobetaine palmitate detergent micelles the distances were
68Å and 58Å, respectively. Theoretical considerations suggested to us that because these transfer
distances were larger than the diameter of the micelle (~40Å), that it would be possible to gain
some insight into the conformation of the FITC LPS. We measured energy transfer between the
FITC LPS donor as a function of the acceptor surface density. Whereas at one C18-Dil per micelle
the transfer efficiency of donor fluorescence of LPS isolated from wild type S.minnesota (MW
20K, with a long O-antigen) was 15%, a short chain mutant S.minnesota Re 595 (MW 2500,
lacking both core and O-antigen) exhibited a transfer efficiency of ~70%. Qualitatively similar
results were obtained with C18-Rhd. As a control, we showed that with either acceptor, the
transfer efficiency was essentially identical when FITC Re 595 LPS or C18-Fluorescein were the
donors. We have gone on to develop a simple model to calculate the typical distance between
FITC-LPS donor probes from the different bacterial strains and acceptor probes. The donor FITC-
LPS with short polysaccharide chain S. minnesota Re 595 and acceptor probe "Fast Dil" appears to
be close to the micelle surface. Separation distances for the longest FITC-LPS, S. minnesota were
calculated to be 1.5 Ro, (~100 Å) or farther from the micelle surface. These results suggest an
extended conformation for the longer O-antigen polysaccharide chain in the detergent model
system. A manuscript describing this work in detail has been submitted for publication to the
Biophysical Journal.

The use of RET and detergent solubilization may prove to be a general tool for the
examination of macromolecular assemblies.

5. Enrichment of Mus spretus Chromosomes by Dual Parameter Flow Sorting and Identification of Sorted Fractions by Fluorescence in situ Hybridization (FISH) onto G-banded Mouse Metaphase Spreads

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The enrichment of individual mouse chromosomes by flow cytometry (FCM) is rather
complicated due to similar size and DNA base composition of most of the mouse chromosomes.
We recently demonstrated that Mus spretus chromosomes which appear as separate peaks in a dual
ABSTRACT: We have developed a stopped-flow flow cytometer with true subsecond resolution to be used in rapid kinetic analysis of cell activation. This instrument is extending the application of flow cytometry to important biological molecules that interact with cell surface receptors. In particular, the new instrument makes it possible to examine the interaction of hormones, peptides, peptidomics, and other small molecules with receptors when the affinities of the binding are too low to be examined by other methods.

We have modified a commercial stepper-motor-driven, stopped-flow mixing system, and integrated the mixer with a flow cytometer. The new device mixes and delivers the biological sample within 250 msecs, then acquires data with 50 msec resolution. This time frame will detect ligand-receptor interactions in living cells for ligands in the affinity range of up to 1 μM. The new device is also expected to extend the use of flow cytometers to new communities of biomedical and health scientists, including pharmacologists, neuroscientists, and cell biologists.

In the previous year we have improved the system by decreasing the dead volume between the exit valve of the delivery system and the port of the flow system so as to achieve delivery times under 300 msec; applied the new instrument to cells, where we are examining rapid association and dissociation of fluorescent ligands with their cell surface receptors; and rapid cell responses. We have described
the results in an R and D 100 application.

We are beginning to modify the device to permit two sequential mixing operations, allowing analysis of the complex time-dependent biological processes that occur during the activation of living cells. Successful completion of these steps should lead to a significant demand for our technology by investigators outside the Laboratory.
To examine the physical basis of the interaction between ligand and receptor, fluorescence lifetimes and the aqueous accessibility of bound ligand are being examined for a family of fluorescent formyl peptide ligands, and native and mutant receptors expressed in a variety of cell types. We are testing several hypotheses: 1) whether the lifetime is sensitive to the environment; 2) whether the lifetime or intensity can be used to measure the accessibility of the ligand to soluble quenchers as a measure of the size of the binding pocket. Pilot studies have suggested that the lifetime is not affected by the environment in the pocket. Accessibility studies using the iodide quenching methods have shown that fluorescent probes associated with the ligand remain accessible.
to the aqueous compartment.
The aim of the project is to characterize the structural properties of formyl peptide ligands associated with formyl peptide receptors. A family of formyl peptides with fluorescein isothiocyanate conjugated at positions 2 through 7 has been generated and their binding to receptors has been characterized by flow cytometry with respect to binding and cell physiology. These studies have suggested that the family of peptides will be most appropriately used in the future to detect sites of contact by photoaffinity crosslinking. In order to facilitate further structural studies: an epitope tagged receptor has been obtained from Butcher to be used in immunopurification and identification of the crosslinking sites, Ye has agreed to assist with the molecular biology to produce
further mutant receptors, and Weinstein has agreed to assist with receptor computational modeling to assist with the interpretation of the biochemical and molecular biological results.
ABSTRACT: This project involves the application of rapid kinetic methods to determine the interactions of fluorescent guanine nucleotides with signal transducing G proteins. It involves a combination of flow cytometric and spectrofluorometric methods. At the present time, we are preparing the biological samples (cell membranes for the work) and Neubig is developing the fluorescent nucleotides. Experiments are going on at both institutions.
ABSTRACT: The kinetic analysis of ligand receptor interactions suggests that receptors and G proteins exist in rapidly coupling and slowly coupling pools in membrane surfaces. To determine whether receptors in the slowly coupling pools were physically precoupled to G proteins or coupled rapidly, we have examined a number of peptides for their ability to interfere with receptor-G protein coupling in broken cell preparations. These peptides represent portions of receptors and G proteins in regions alleged to mediate coupling during signal transduction. Although these peptides have been shown to affect the interactions between the purified proteins, they do not seem to affect the interactions of the receptors and G protein in the cell membranes. This observation leads us to suggest that the receptors and G proteins are physically precoupled, supporting the model derived from kinetic analysis.

The next phase of the project will involve a description of the underlying biochemistry of signal transduction. The kinetic methods will combine rapid mix flow cytometry with biochemistry to evaluate the evolution of receptors among different macromolecular complexes. These receptor complexes will be isolated by immunopurification of epitope tagged receptor and the transduction molecules in the complexes will be identified. We will attempt to develop a mathematical model from the kinetic methods to account for the biochemical steps in receptor activation and desensitization.
Adhesive interactions among leukocytes and between leukocytes and endothelium are central to processes of inflammation. These interactions are mediated by sequential molecular steps involving carbohydrates and selectins, and integrins and ICAMs. We are presently using flow cytometry to examine the interaction of neutrophils with cells transfected with ICAM, a counter structure for the neutrophil integrins.
Role of Selectins in Leukocyte Adhesion

We have examined, for the first time, the function of adhesive receptors on leukocytes in blood. We have compared the results to isolated cells making several novel observations. Leukocyte adhesion is both integrin and selectin dependent. The aggregation occurs at low levels of adhesive receptors and decreases as the integrin expression increases and the selectin expression decreases. In the next series of experiments we are: 1) attempting to understand the mechanism by which antibodies to selectins activate neutrophils and 2) define a counterstructure for selectin such as the mucin CD43. We have used A1 glycoprotease (Mellors) to demonstrate a role for O-glycosylated proteins in the aggregation process. We have tentatively identified an O-glycosylated protein important to the process using antibodies supplied by McEver.
The Effect of LPS on Leukocyte Physiology and Adhesion Receptor expression, internalization

4 11 17

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Bacterial endotoxins are important modulators of septic shock. We have examined for the first time the pathway of LPS action on neutrophils in blood. This pathway involves a plasma binding protein and a cell surface receptor CD14. We have shown that LPS can act directly upon the neutrophil in blood without the production of inflammatory mediators by other cell types. These studies set the stage for detailed mechanistic evaluation of the action of inflammatory mediators and regulators in physiological systems. LPS causes the modulation of important adhesive proteins on leukocytes-selectins and integrins which alter the ability of neutrophils to recognize one another and alter their compartmentation in the circulation. Ongoing studies are aimed at defining the relationship between the expression of adhesion molecules and neutrophil physiology.
We have developed protocols to assess the physiology of leukocytes in blood from trauma patients as they progress through ARDS (acute respiratory distress) and sepsis, the major cause of death in otherwise healthy adults ages 25-44. These protocols involve the analysis of cell function and the expression of surface receptors indicative of cell activation. Studies on 11 trauma patients reveal alterations in CD14, selectin, and integrin levels during the response to the traumatic injury. In the past year we have attempted to streamline protocols for analysis of blood samples. In pilot studies using whole blood exposure to LPS followed by fixation and lysis with commercial reagents, it appears that adhesive molecule expression is sensitive to the treatment conditions.
Analysis of the macromolecular assembly of the transcription complex

Flow cytometry, kinetics, protein/RNA interactions

The transcription of DNA to RNA requires an RNA polymerase and a transcription termination factor called RHO. We have successfully assembled the transcription complex on beads suitable for flow cytometric analysis. A number of strategies are being developed that will allow observation of the kinetic steps of transcription. In general, these involve binding fluorescent oligonucleotides to the complex (which makes the beads fluorescent) and allowing the oligonucleotides to dissociate during the reaction. Seed money for this project has been obtained from an NIH P20 Institutional Development Grant to UNM Cancer Center.
Analysis of the invasive properties of metastatic cells transfected with receptors for chemoattractant

Flow cytometry, cell motility, signal transduction

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Invasion of tissue by tumor cells is an essential component of metastasis. In the past year we have developed a project to define the role of cell motility in the invasive process and the ultimate localization of metastatic tumors. We have proposed as a supplement to the NFCR to transfect metastatic breast tumor cells with formyl peptide chemoattractant receptor. If the project is funded, the cells will be sorted by flow cytometry and selected on the basis of motility and invasion assays. The localization of metastases of the cell sublines will be characterized in animal models.
The goal of this project is to understand basic mechanisms of cell recognition of bacterial product LPS via the interactions of LPS, its soluble binding protein and its membrane receptor CD14. This is the basic research counterpart of the trauma studies. It involves spectroscopic and cytometric analysis of the complexes of LPS with its binding protein and has led a general new way of examining macromolecular assemblies in liposomes (manuscript submitted to Biophysical Journal). See Research Highlight
ABSTRACT: Pilot studies have been undertaken to evaluate the possibility of detecting single molecules of serum lipoproteins. These studies suggest that it may be possible to develop rapid protocols for complete analysis of the serum lipoprotein distribution in clinical samples. Pilot studies are being extended to other lipid containing systems including liposomes and several types of fluorescent dye molecules. A separate approach to lipoprotein quantitation has also been initiated using a bead based flow cytometric assay. In this assay, beads are being labelled with antibodies to low density lipoprotein (LDL). A competitive assay involves the displacement of fluorescent LDL from the beads by LDL in plasma and detection of the fluorescent beads by flow cytometry.
ABSTRACT: A phase sensitive spectrofluorometer has been used in the analysis of the fluorescence lifetimes of novel porphyrin molecules.
Fluorescence lifetimes of environmental pollutants

A phase sensitive spectrofluorometer has been used in the analysis of the fluorescence lifetimes of environmental pollutants.
Assembly of Complement and LPS

Fluorescence lifetimes

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Resonance energy transfer is being used to determine whether there are specific interactions of LPS and complement in membranes.
Detection and Analysis of Inflammatory Cells in Asthma

Eosinophils, basophils, cytokines

ABSTRACT: Eosinophils and basophils are rare blood cells that play important roles in pulmonary inflammation associated with asthma. Pilot studies have been undertaken to evaluate the possibility of detecting eosinophils in patients receiving immunotherapy who become hypereosinophilic. These studies suggest that it may be possible to identify the eosinophils using an antibody to VLA4 in mixed granulocyte populations. VLA4 is an adhesive integrin that is expressed at low levels on neutrophils, the major granulocyte population. Studies in the coming year will attempt to: 1) define the adhesive properties of eosinophils, using inflammatory mediators that have preferential effects on eosinophils rather than neutrophils; 3) identify blood basophils by staining with anti IL3 receptor antibody; 4) develop intracellular detection of cytokines expressed when these cells are challenged by exposure to inflammatory mediators.
Plans are being developed to examine families of heparin-related molecules for their ability to regulate adhesive interactions among leukocytes and to analyze these interactions by flow cytometry.
ABSTRACT: We have succeeded in devising strategies to design peptides with particular characteristics: 1) binding and dissociation rates appropriate for subsecond analysis of macromolecular assembly; 2) introduction of the fluorophore into regions of the receptor binding pocket which undergo conformational changes; and 3) high affinity antagonists to the receptor. A family of formyl peptides with fluorescein isothiocyanate conjugated at positions 2 through 7 has been generated and their binding to receptors has been examined. (Manuscript in preparation)
ABSTRACT: The pathways of cell activation important to allergy and asthma are being examined by transfecting RBL (rat basophilic leukemia) cells with formyl peptide receptors. This will permit the comparison of signal transduction pathways initiated by distinct mechanisms but leading to similar cell response. The primary pathways involve G protein mediated signals initiated by formyl peptide and receptor kinase associated signals initiated by crosslinking of the IgE receptor by antigen. At the present time transfected cells have been produced which are being characterized by flow cytometry. Several aspects of the signalling pathway and motile response have been evaluated. These include actin polymerization, phosphotyrosine kinase activation, and calcium response, all by flow cytometry. We are presently attempting to use these cells as a model for examining the activation of adhesion involving the VLA4 integrin and VCAM by flow cytometry.
Two new techniques have recently been developed: one uses a microscopic method to examine the topography of molecules on the inner surface of membranes (Wilson); the other allows receptors and G protein to be fixed in their native distribution (Domalewski). These two groups are using rat basophilic leukemia cells with transfected formyl peptide receptor to examine the topography of receptors and G proteins as it relates to cell activation. Experiments have also been performed using the fixation technique with RBL to attempt to trap receptors in their coupled and uncoupled forms and identify these forms on the basis of flow cytometric analysis of ligand affinity.
ABSTRACT: We are testing the hypothesis that actin polymerization is regulated by PIP3 kinase. The experiments involve measuring actin polymerization by flow cytometry in permeabilized neutrophils that have been stimulated with formyl peptide. To examine roles of PIP3 kinase, a number of soluble inositol phosphates which could compete with the normal substrates of the enzyme are being evaluated in the actin assays. The soluble inositol phosphates appear to have minimal affect on the actin polymerization pathway. This project has been completed with negative results.
ABSTRACT: The goals of this project are to define the molecules responsible for leukocyte-platelet interactions in blood. Pilot studies have begun in which platelet-neutrophil interactions have been detected in blood. The role of platelet selectin, neutrophil integrin, and RGD peptides are being examined. The peptides appear to enhance neutrophil-platelet interactions by blocking platelet-platelet interactions. This project is on hold pending funding of collaborators.
The goal is to develop methods for analyzing immune cell and their contribution to Hanta Virus Associated ARDS. This project is on hold pending funding of collaborators.
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ABSTRACT: The course was co-sponsored by the National Flow Cytometry Resource and the University of New Mexico, School of Medicine. It was held at Los Alamos from June 11 to June 16, 1995, with 48 students and more than 20 faculty. Instrumentation and software from commercial companies was used.
ABSTRACT: The NFCR invites speakers to Los Alamos/UNM to present seminars and to discuss Resource research and collaborative activities. Dr. Howard Shapiro of Boston MA recently presented a seminar on the analysis of bacteria by flow cytometry.
### III.D. SOURCES OF INVESTIGATOR SUPPORT

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IV. B. DISSEMINATION OF INFORMATION

The principal means of advertising the availability and capabilities of the Resource are by dissemination of information at meetings, courses, technology transfer, the NFCR Newsletter, and the Research Resources Report published by the US Department of Health and Human Services.

Literature:
The Resource brochure, Newsletter, and other materials have been widely distributed at meetings and to those who write or call requesting information about the Resource. A poster designed to illustrate the activities and availability of the Resource was presented at the XVII International Conference on Analytical Cytology at Lake Placid, NY. Copies of information sheets describing NFCR facilities, Resource-associated personnel and areas of speciality, collaborative opportunities and the upcoming annual course in flow cytometry were distributed at that meeting. A description of the NFCR is included in the Directory of Federal Laboratory and Technology Resources which is published by the National Technical Information Service of the U. S. Department of Commerce.

The NFCR Newsletter (formerly the Flow Systems Newsletter), published by the National Flow Cytometry Resource, is a widely distributed quarterly publication that reaches most of the flow cytometry community (approximately 1000 subscribers worldwide). The Newsletter format has been altered to focus mainly on the capabilities and services of the Resource. In addition to highlighting Resource projects, the newsletter continues to announce courses, conferences and workshops, list positions desired and positions available, and publish short technical notes. On occasion, a brief description will be included on published flow cytometry articles that are particularly noteworthy. It appears that the Newsletter is read and remembered. For example, a recent notice appeared on the Flow Cytometry Electronic Bulletin Board citing an old (1983) article that appeared in the Newsletter concerning construction of a constant pressure sheath delivery system.

As detailed in Part III-E of this report, the NFCR has been very active in publishing results of research. This is another form of information dissemination. In addition to authoring papers, NFCR staff is active as peer reviewers for several journals. During 1994 twelve Los Alamos investigators reviewed papers for *Cytometry*.

Meetings:

Meetings and courses attended by the Los Alamos Staff at which research carried out at the Resource was presented are listed below.

5/94  U. S. Industry Coalition: Visit to 20 biotechnology institutes in Moscow and St. Petersburg, Russia (Cram)

9/94  Clinical Cytometry Meeting, Charleston SC (Marroqe, Salzman [Invited tutorial])

10/94 XVII Congress of the International Society for Analytical Cytology Lake Placid, NY (Cram[Poster Chairman, Tutorial], Crissman, Deka, Fawcett [Tutorial], Habbersett, Jett[Commercial Exhibits Chairman], Marrone, Martin, Nolan, Roslaniec, Steinkamp)

10/94  Biomedical Engineering Society, Tucson, AZ (Sklar, Invited lecture)

10/94 University of New Mexico Conference on Defense Conversion, Albuquerque, NM (Sklar, Organizing Committee)
Technology Transfer:
The products of our research and development are available to the general scientific research community. There are two software products that have been distributed during the year. The Cluster FCS program developed by Gary Salzman for the Macintosh computers has been distributed to approximately 100 investigators as listed in Table 4 at the end of this section. The IDLYK program developed by Robb Habbersett as part of the DiDAC development has been distributed to those listed in Table 5. In addition, both of these sets of software have been demonstrated to numerous visitors to the NFCR.

Technology transfer also takes place by assisting investigators with sample preparation protocols. One area that remains particularly active is the transfer of experience with the preparation of chromosomes samples for analysis and for sorting. Table 6 lists technology transfer activities that involve investigators desiring to use NFCR developed protocols. Table 7 is a list of grant submissions for which NFCR personnel have written letters of support. Many of these investigators will become collaborators.

Technology transfer activity involving industry is another area of activity. During the year we have been actively pursuing the development of closer ties with the flow cytometry industrial community. Reciprocal visits with Becton Dickinson Immunocytometry Systems (BDIS) scientific and administrative staff have resulted in a preliminary agreement on an area of cooperation between the NFCR and BDIS. As mentioned in the training section later in this report, the flow cytometry industrial community generously supports the annual course by loaning equipment valued at close to one million dollars and providing operational assistance by installing the equipment and having assistants available during the course.

Tours and Visitors:
The NFCR hosts numerous tours and visitors at Los Alamos and at the University of New Mexico Cytometry Unit. At Los Alamos, we hosted on average more than one formal tour group a month and gave many informal tours to other visitors. These groups included the Director of the National Institute for Environmental Health and a contingent of senior investigators, high school students, and industry representatives. At UNM, numerous educational groups tour the facilities including medical students, graduate students and others.

5. C. Summary of Training Component

The NFCR offers training to high school, undergraduate, graduate, and postdoctoral students through its facilities at Los Alamos National Laboratory and the University of New
Mexico. Training of visitors is also common and will be enhanced through the new International Society for Analytical Cytometry STEP program. Because cytometric investigations involve flow and imaging systems using fluorescent probes, training encompasses instrument conception and construction, cell biology, chromosomes, and fluorescence spectroscopy. Numerous students and post doctoral fellows have been involved in training programs in the last 12 months, including 4 who have gone on to staff and faculty positions. Trainees often play a major role in projects with NFCR collaborators.

The Annual Course in Flow Cytometry:
The Annual Course in Flow Cytometry will be held in Los Alamos 11-16 June 1995. The tremendous success of the course was due to the large number of NFCR personnel who worked very hard to make it happen, the visiting faculty that gave of their time to help present the subject matter and the loan of equipment and software by six corporations. Forty eight investigators from around the world will attend the course. The course is attracting students from as far away as Hong Kong, Switzerland, Canada, Argentina, Finland, Kenya, Israel and Australia. In addition to the formal attendees, we will also have six local student assistants who will attend the lectures and guide the laboratory groups. The course will consist of 13 lectures, 6 laboratory sessions, and 3 evening sessions with short talks, commercial tutorials and discussions. In addition to the NFCR faculty, eight distinguished flow cytometrists will come to Los Alamos to share their expertise with the students. Dr. Joe Gray, Department of Molecular Cytometry, University of San Francisco, CA and Chairman of the NFCR Advisory Committee will present the special invited lecture on advances in molecular level diagnostics.

The ISAC STEP Program:
A new training program is being developed by the International Society for Analytical Cytology to make available institutional expertise for training in cytometric specialties.

The NFCR has been requested to participate in Chromosomes (Joe Fawcett, Scott Cram), Data Analysis (Jim Jett, Gary Salzman), DNA Analysis (Harry Crissman), and Ligand-receptor interactions (Larry Sklar). In addition we expect to offer training associated with cell cycle kinetics (Harry Crissman), cell adhesion (Larry Sklar), and whole blood cytometry (Larry Sklar).

Trainees moving on to faculty and staff positions:
After training with Larry Sklar at UNM, Teresa Ainsworth began a residency in Emergency Medicine at the University of Rochester

After training with Larry Sklar at LANL Christina Wistrom joined the Research Faculty at the University of California at Riverside

After training with Dick Keller and the NFCR staff at LANL, Mitch Johnson joined the chemistry department at Duquesne University

After training with Gloria Sarto, MD, PhD (an NFCR collaborator at UNM), Aki Fukushima, M.D., returned to a faculty position in Japan.

Students receiving degrees:
After training with Larry Sklar at UNM, Debora Guyer received her MS and continued in the PhD program at UNM.

After training with Larry Sklar at UNM, Christine Schammel received her MS.
Current Postdocs:
The first four postdocs, for which a synopsis of their research is provided, are most closely associated with the NFCR.

Chiranjit Deka (Ph.D., University of Central Florida, 1992)
Working with John Steinkamp and Larry Sklar the object of my research is to develop phase sensitive detection techniques in flow cytometry. It is possible to determine fluorescence lifetimes by amplitude demodulation using digital data acquisition techniques. A paper describing this approach appeared in Cytometry. Work in progress includes multi-harmonic frequency flow cytometry which will allow simultaneous phase-shift measurements at multiple frequencies, application of phase sensitive detection methods to detection and measurement of low intensity surface marker fluorescence in the presence of autofluorescence, and characterization of fluorescence self quenching of highly labeled antibodies.

John P. Nolan (Ph.D., Biochemistry, Pennsylvania State University, 1992)
Working with Larry Sklar, I am using the unique instrumentation available at the NFCR to develop new approaches to the study of cell function. Measurement of fluorescence lifetimes from individual cells on the Phase Sensitive Flow Cytometer is providing spectroscopic information on the physical interactions between the human neutrophil formyl peptide receptor and fluoresceinated ligands. Kinetic measurement capabilities, including the prototype Rapid Mix Flow Cytometer, allow precise determination of receptor-ligand binding and dissociation events occurring on the second and sub-second time scale. This combination of methods provides a novel, but potentially general, means to study receptor structure and function in intact cells. A paper describing this work is in press in Cytometry. High sensitivity fluorescence detection is being used to detect and analyze very small (<200 nm) biological particles from cells and biological fluids. Development of this approach will enable previously impossible measurements on sub-cellular structures and macromolecular aggregates.

Mary Roslaniec (Ph.D., University of California, Los Angeles, 1993)
Working with Scott Cram and John Martin, the goal of my research is to develop an understanding of photochemical processes that lead to inactivation of DNA, a process that forms the basis of the optical chromosome sorter. The method involves the photoinduction of psoralen cross-links in chromosomal DNA. When cross-linked, the chromosomes are unclonable, hence, selectively cross-linking unwanted chromosomes provides a means to select the desired chromosomes. Since I began to work at the NFCR in the fall of 1993, I have received a significant amount of assistance re: instrumentation from John Martin while Dr. Richard Reynolds is instructing me in several areas of molecular biology techniques that are necessary for the project. This supplementation to my photophysical background has been invaluable in developing this multifaceted project.

Christina Wistrom (Ph. D., University of California, Davis, 1991)
Working with Larry Sklar and Dick Keller, the goal of my research at the NFCR is to focus on using high sensitivity and single molecule detection of fluorescence associated with macromolecular assemblies and ligand-receptor interactions. Increased detection and sensitivity of flow cytometry instruments allow us to expand into an exciting area of non-invasive biological research which span from qualitative analysis of serum lipoproteins, ligand-receptor interactions to macromolecular organization in biological membranes such as topography and macromolecular domains in biological membranes. I am also developing a new method for quantification of serum lipoproteins using fluorescent labels and Single Molecule Detection in flow cytometry. With a qualitative and quantitative analysis of serum lipoproteins in flow cytometry this type of analysis can be faster and easier than current ultra centrifugation methods. High Sensitivity Fluorescence Detection will allow us to examine biological events occurring at the individual molecular level on membrane surfaces as well as in solution.
The following post doctoral fellows work in areas that are associated with the NFCR and draw on the expertise of the NFCR staff.

Anne Hall, Ph.D., works with Larry Sklar at UNM and is involved in collaborative projects in cell receptor and molecular biology with a number of NFCR collaborators.

Jay Schecker, Ph.D., works with Dick Keller, John Martin, and Jim Jett at LANL on manipulation and enzymatic cleavage of single-strands of DNA as part of the DOE supported DNA sequencing by flow cytometry development project.

Jeff Petty, Ph.D., works with Dick Keller, John Martin, and Jim Jett at LANL on applications of high sensitivity single molecule detection in flow to DNA fragment sizing as part of the internally supported development of applications of sensitive fluorescence detection to biomedical problems.

Rhett Afleck, Ph.D., works with Dick Keller, John Martin, and Jim Jett at LANL on manipulation and enzymatic cleavage of single-strands of DNA as part of the DOE supported DNA sequencing by flow cytometry development project.

Ming Wu, Ph.D., works with Dick Keller, Peter Goodwin, John Martin, and Jim Jett at LANL on the development of high sensitivity single molecule detection techniques in flow as part of the DOE supported DNA sequencing by flow cytometry development project.

David Robbins, Ph.D. works with Dick Keller and Larry Sklar on the application of high sensitivity detection to liposomes and serum lipoproteins.

Current Graduate Students (supervised by Larry Sklar at the University of New Mexico):

Terri Gilbert Houghton and Janeen Vilven work on ligand-receptor interactions and are involved with a number of NFCR collaborators including Richard Neubig at Michigan, Richard Freer at MCV and Richard Ye at Scripps.

Greg Jones collaborates on macromolecular assembly and phase sensitive detection with a number of NFCR collaborators including Richard Ulevitch and Peter Tobias at Scripps. He went to Scripps for two weeks for training in cell biology and biochemistry.

Deborah Guyer, Theresa Bennett, and Christine Schammel work on cell adhesion projects with a number of NFCR collaborators including Alan Mellors (Guelph), Bruce Edwards (Lovelace), Eugene Bucher (Stanford), Rodger McEver (Oklahoma).

Larry Seamer is working on leukocyte signal transduction in collaboration with John Nolan and the rapid mix flow cytometer. Jim Jett is also on Seamer's thesis committee.

Graduate Research Assistants at Los Alamos:

Shanna Minter is currently training with Harry Crissman in cytochemistry under the Historically Black Colleges and Universities program.

Mark Naiver, after receiving a MS in electrical engineering, has returned to the NFCR to work with the DiDAC development team. He recently accepted a electrical engineering position and will continue to contribute to the DiDAC development.

Julia Gonzales, B.S., works on the fetal cell sorting project with Babetta Marrone.

Stephanie Pendergrass, B.S., works with Babetta Marrone and Gary Salzman on the development of digital image analysis software.

Graduate Students Relying on NFCR expertise and facilities:

Mark Wall is in the UNM Chemistry Dept and receives training in fluorescence lifetime analysis.

Undergraduates:

Ralph Dilibero, UNM Biochemistry, is working on an BS honors thesis with the NFCR/UNM flow cytometry group.

Liat Shama, Williams College, worked with the NFCR/UNM Cytometry group during the
summer of 1994 using flow cytometry to measure ligand-receptor interactions

Other Notable efforts
Jim Jett presented a lecture titled "Lasers, Cells and Chromosomes" to biology classes at Capitan High School.
Gary Salzman and Jim Freyer were team coaches in the Los Alamos National Laboratory sponsored High School Supercomputer Challenge.
Table 6: Technology Transfer

Chromosome Isolation and Preparation:
Aru Muga Nathan, PhD, University of Nebraska, Lincoln, NE
Eduardo Cantu, PhD, Medical University of South Carolina, Charleston, SC
Kit Snow, PhD, Coulter Corporation, Miami Lakes, FL

Whole Blood Cytometry/Cell Aggregation Protocols:
Joanne Hom, PhD, Eli Lilly laboratories
Rodger Mc Ever, PhD, Kevin Moore PhD, University of Oklahoma
Richard Crowell, MD, Gus Hallin, MD, VA Hospital, Albuquerque
Randolph Kessler, MD, UNM School of Medicine
Bruce Edwards, PhD, The Lovelace Institutes
Cheryl Willman, MD, UNM
Buck Rhodes, PhD, RhoMed, Inc. Albuquerque, NM
Phil Marder, BS, Eli Lilly, Laboratories

Native Cell Isolation Protocols:
Ross Robinson, Cardinal Associates, Santa Fe, NM

Ligand-Receptor Interactions and Quantitation:
Alex Speers, PhD, Halifax Nova Scotia
David Royce, PhD, UCSD
Steven Lichtman, PhD, UNC

Fluorescent bead cytometric assays for soluble analytes
Louis Smith, PhD, Baylor College of Medicine
Peter Siims, PhD, Milwaukee Blood Center

Cell lines and membrane preparations
Richard Neubig, MD, PhD, University of Michigan

Resonance Energy Transfer Methodology
Carolyn Mold, PhD, University of New Mexico
Roger Wiggins, MD, University of Michigan

Assistance with data analysis questions:
Robert Chervenak, PhD, Louisiana State University, Shreveport, LA
Bert Pinsky, PhD, BDIS, San Jose, CA
TABLE 7: Letters of Support, Consultation, Collaboration by NFCR Investigators for Grant Submissions

1. Miniaturized Flow Cytometer, Perry Skeath, ITM; Agency: NIH SBIR Phase II (funded)


3. Fluorescent ligands for peptide receptors (Commonwealth Biotechnology, Richmond, Va). Agency: NIH SBIR (pending)

4. NK cell adhesion (Dr. Bruce Edwards, Lovelace Research Institute, Alb, NM) Agency: NIH R01 (pending)

5. Miniature optical devices for cell sorting (Dr. John Wood, UNM, Alb, NM) Agency: DARPA


7. Molecular Imaging Capabilities at UNM SOM (Dr. David Peabody, UNM, Alb. NM) Agency: UNM Research Allocations Committee

8. Asthma SCOR Pilot Studies at UNMSOM (Dr. R. Lyons UNM, Alb. NM) Agency: UNM Research Allocations Committee

9. Topography of Receptor G Protein Interactions (Dr. B. Wilson, UNM, Alb. NM) Agency: NIH

10. Detectors for Environmental Pollutants (Dr. G. Lopez, UNM, Alb. NM) Agency: ONR

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