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13. ABSTRACT (Maximum 200 words) We have met approximately 90% of the goals outlined in our original grant application. Fifteen papers were published acknowledging ARO funding. The work accomplished involves the exploitation of temperature, concentration, pH, and ionic strength gradients on-chip for use in heterogeneous immunoassays for ligand-receptor binding at biomembrane surfaces. The platforms we have built allow for high throughput measurements, while requiring only microliters of solution in order to obtain binding information. The signal-to-noise ratio is higher than conventional assays because time dependent sources of noise have largely been eliminated. Specifically, we have studied IgG binding to fluid membranes as a function of both hapten density and cholesterol content in the membrane. The results suggest that we can now understand the basic principles for multiple binding of analytes (proteins, toxins, viruses, etc...) at a membrane interface. In addition, we expanded our lab-on-a-chip platforms for use with whole cells in chemotaxis as well as in the employment of elastomeric proteins and polymers for temperature gradient assays.				
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(4) Statement of the problem studied.

The goal of this ARO grant, DAAD19-01-1-0346, was to explore the fundamental principles involved in the formation of a new generation of highly selective biosensors based upon the principle of multivalent ligand-receptor attachment. The specific approach was to employ supported lipid bilayers and high throughput microfluidic devices to understand at the molecular level the principles of device and platform design.

(5) Summary of the most important results

Below I will explain the progress that we have made in developing on-chip multivalent assays over the last three years. The object was to exploit our devices for obtaining binding data for ligand-receptor interactions as a function of membrane chemistry. These devices are ideally suited for this task because of their high throughput capabilities and vastly decreased reagent volume requirements. In fact, only 2 μ L of protein solution are needed to collect each data point and all the points on a binding curve can be obtained simultaneously. We have created spatially addressed bilayer arrays with different concentrations of membranes components (lipids, ligands, cholesterol, etc) at each address. A schematic example of a simple 2 x 2 bilayer array is shown below (Figure 1). Each membrane address contains 2,4-dinitrophenyl phosphatidylethanolamine

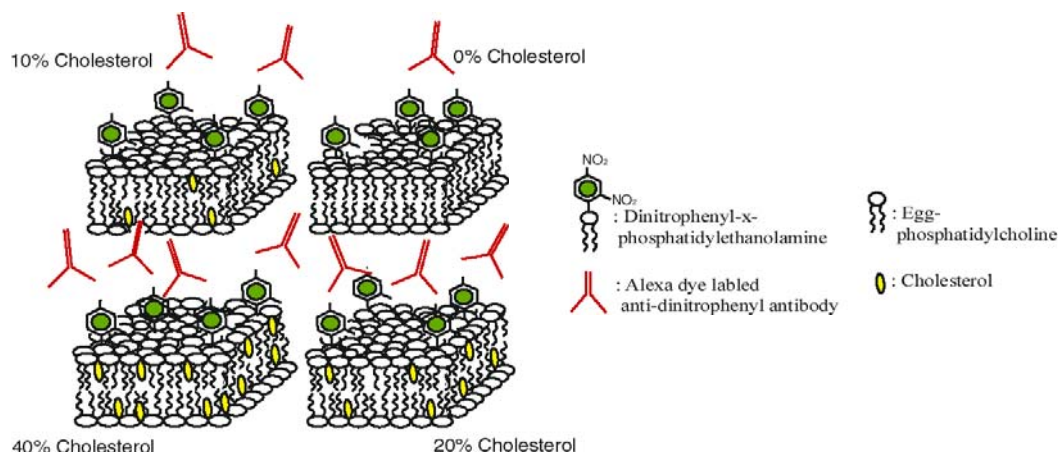


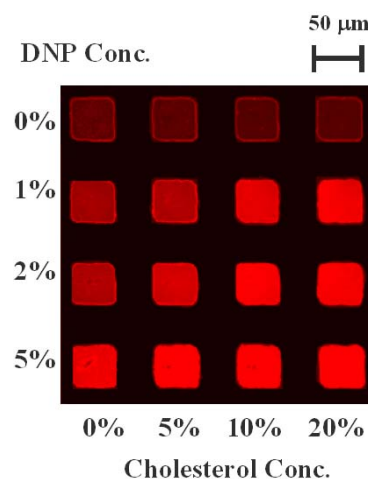
Figure 1. A spatially addressed array of lipid bilayers containing an antibody binding ligand (shown in green) and varying amounts of cholesterol at each address.

well as various concentrations of cholesterol. Anti-2,4 dinitrophenyl antibodies

(DNP-PE) as bind specifically to the DNP-PE ligands; however, the amount of cholesterol in each membrane is crucial for determining the extent of binding. As can be seen in Figure 2, antibody binding increases substantially as the cholesterol content is increased. For example, there is appropriately twice as much antibody

Figure 2. A 4 x 4 array of fluorescently labeled antibodies bound to membrane with various ligand densities and cholesterol content.

bound to the membrane at 1 mol% DNP when 20 mol% of cholesterol is present as opposed to the conditions under which the membrane contains no cholesterol. We have performed spectroscopy and microscopy experiments which demonstrated that this effect was caused by an increased availability of



the DNP-PE ligand above the membrane surface as cholesterol was added. Therefore, the exact membrane chemistry is crucial to the ligand's orientation and binding avidity.

In order to obtain quantitative binding strengths for ligand-receptor interactions, we have developed bilayer coated microfluidic devices. These lab-on-a-chip platforms allowed both the surface bound membrane as well as the aqueous solution above it to be precisely and separately controlled in each channel. A schematic diagram of a two-channel device as well as a total internal reflection fluorescence micrograph from a twelve-channel device are shown in Figure 3. A device consists of a glass support bonded to a lithographically patterned polydimethylsiloxane (PDMS) mold in order to form microfluidic channels. The channels are coated with phospholipid membranes over the entire surface (shown in green). This is done by flowing in the

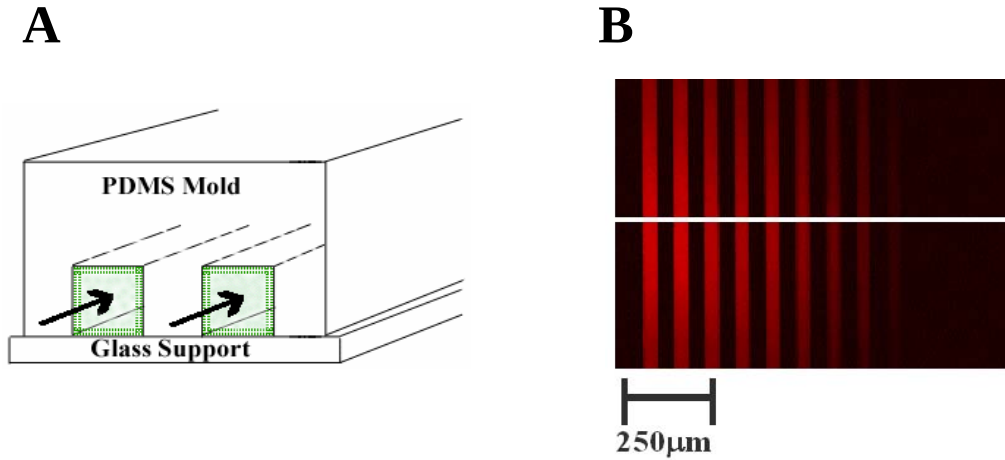


Figure 3. (a) Schematic representation of a bilayer coated microfluidic channel array. (b) A working bilayer coated array containing various concentrations of fluorescently labeled antibodies.

appropriate vesicle solutions and washing out excess materials. In the working device (3b), the bilayers contained DNP-PE ligands, which could then bind with fluorescently labeled anti-DNP antibodies that were subsequently introduced. The surface binding process was followed in all channels simultaneously by total internal

reflection fluorescence microscopy (TIRFM), a technique which discriminates between surface bound species and those in bulk solution. Control and background experiments demonstrated that the vast majority of the antibodies was indeed specifically bound to the interface under the conditions that were probed. A high concentration of antibody was added to the left most channel and a subsequently lower concentration was added to each neighboring channel (left to right). Quantitative information was obtained by plotting the intensity of surface bound antibodies in each channel against the concentration of IgG introduced (Figure 4).

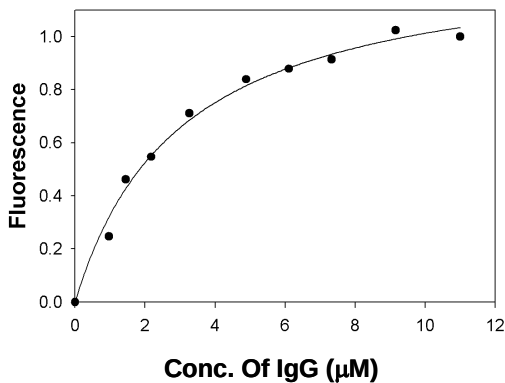


Figure 4. Plot of total fluorescence intensity from membrane bound antibodies vs. the concentration of antibodies applied in the bulk solution.

This is in essence a "one-shot" binding curved for a membrane associated ligand-receptor binding process. Fitting the curve to a

Langmuir isotherm, $\Theta = \frac{K_{Aapp}[c]}{1 + K_{Aapp}[c]}$, allows the determination of

the apparent association constant, K_{Aapp} . In this case $[c]$ represent the concentration of antibody in bulk solution while Θ is surface coverage, a value which varies between 0.0 and 1.0. A least squares fit to the data provides the value of K_{Aapp} . This value, however, is usually reported as the equilibrium dissociation constant, K_{Dapp} , which is $1/K_{Aapp}$. In the particular case shown above the value is slightly less than 2 μM, which is in excellent agreement with literature values. Significantly, this value was determined rapidly, on-chip, with just a few microliters of solution. The signal-to-noise is also very high because taking all data simultaneously eliminates time dependent fluctuations which can affect sequentially performed assays.

In order to further improve our on-chip membrane binding assays, we exploited the very regular laminar flow of microfluidics to create a concentration dilutor on-chip. This allows a single high concentration of protein to be converted into an array of concentrations in a single step (Figure 5). Thus binding curves can be achieved from a single initial protein solution injection. As a spin-off to this technology, we have now used this device to develop a new assay for bacterial chemotaxis in association with Michael Manson's laboratory.

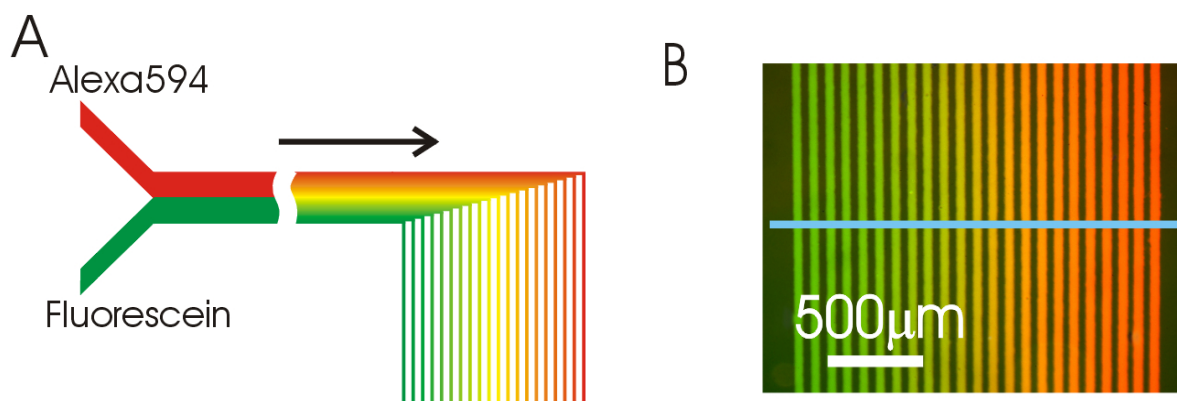


Figure 5. (a) Schematic diagram of a diffusion diluter in which two solutions are introduced, allowed to mix by diffusion as they flow downstream side-by-side, and separated into microchannels with distinct compositions. (b) A fluorescence false color image of the microchannels from a working diffusion diluter.

Similar to the concentration dependent experiments, it was also deemed desirable to be able to run many experiments simultaneously at a variety of different temperatures. To this end we developed a temperature gradient microfluidic device that allows dozens of individual nanoliter sized samples to be analyzed in parallel. This allows us to obtain one-shot DNA melting point curves, Arrhenius plots, and binding curves in a straight forward manner (Figure 6). As a result of having this bioanalytical technique we now are able to monitor protein folding and crystallization processes in a high-throughput fashion as a function of temperature.

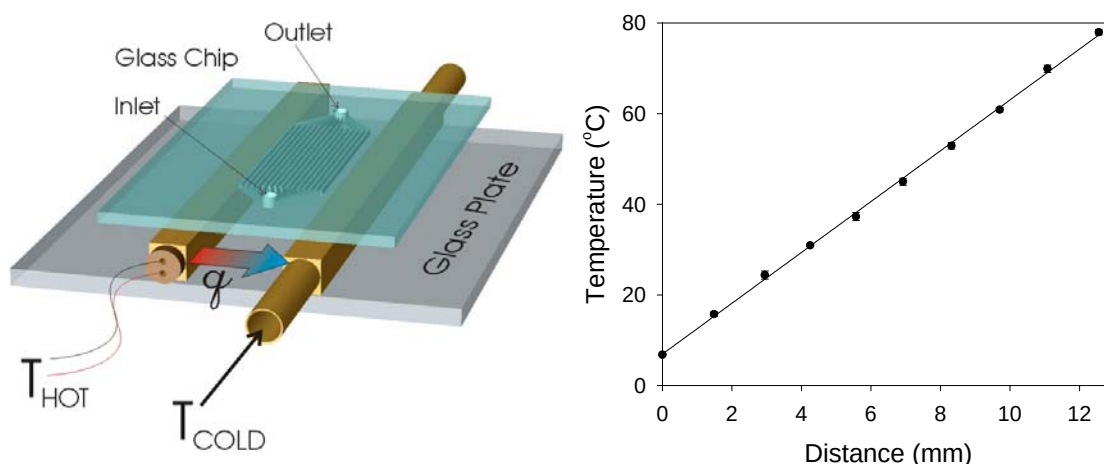


Figure 6. (a) Schematic diagram of the platform employed in temperature gradient microfluidics. The device operates by placing two brass tubes underneath a linear array of microchannels. (b) Thermocouple measurements of the temperature inside a working temperature gradient device as a function of position.

A key advance made in our laboratory was the development of a macroscope for looking at large fields of bilayers on a chip. This development will make possible the use of standard pin arrays to transfer vesicle solutions to a patterned field of hydrophilic boxes that have the identical registry. A first demonstration of the macroscope was made for a 7 x 9 array of egg PC bilayers with two different dyes patterned alternately at each address (Figure 7). Such large arrays can now be employed to rapidly screen a variety of different membrane chemistries for their effects on multivalent protein binding.

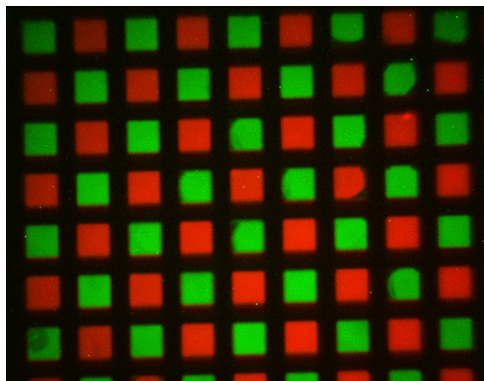


Figure 7. A fluorescence image of a spatially addressed array of 9 x 7 egg PC bilayers on chip. The center to center distance between adjacent membranes is 1 mm. Therefore the image shows approximately a 63 mm² area.

A final advance in early 2004 was the formation of air-stable fluid phospholipid bilayers. This report constituted the first time a fluid bilayer can be made on monitored in air. The advance was verified by fluorescence recovery after photobleaching and fluorescence microscopy. The process works by using a coat protein specifically bound to the bilayer via ligand-receptor interactions.. A schematic diagram of the system is shown in Figure 8.

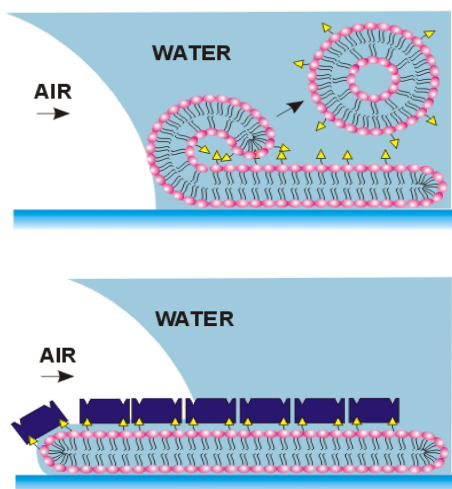


Figure 8. (top) The introduction of the air/water interface destroys a standard bilayer from the edge, peeling the membrane away in vesicle sections (note: some lipids may also form a monolayer at the air surface). When the same bilayer is protected by a close-packed and specifically bound protein monolayer (bottom), it survives the air/water interface. The proteins may serve to mechanically “pin” down the edge of the bilayer, allowing air to pass over the surface without disrupting the overall lipid ordering.

(6) Papers submitted or published under ARO sponsorship during this reporting period.

(a) Manuscripts submitted, but not published:

none

(b) Papers published in peer-reviewed journals:

Creating Fluid and Air-Stable Solid Supported Lipid Bilayers

Matthew A. Holden, Seung-Yong Jung, Tinglu Yang, Edward T. Castellana and Paul S. Cremer

J. Am. Chem. Soc. 126 (2004) 6512-6513

Patterning Enzymes inside Microfluidic Channels via Photoattachment Chemistry

Matthew A. Holden, Seung-Yong Jung and Paul S. Cremer

Anal. Chem. 76 (2004) 1838-1843

High Throughput Studies of Polymer and Solution Structure on the Phase Separation of Thermoresponsive Polymers

Hanbin Mao, Chunmei Li, Yanjie Zhang, Steven Furryk, Paul S. Cremer and David E. Bergbreiter

Macromolecules 37 (2004) 1031-1036

Probing the Mechanism of Aqueous Two-Phase System Formation for α -Elastin On-Chip

Yanjie Zhang, Hanbin Mao, and Paul S. Cremer

J. Am. Chem. Soc. 125 (2003) 15630-15635

The Vroman Effect: A Molecular Level Description of Fibrinogen Displacement

Seung-Yong Jung, Soon-Mi Lim, Fernando Albertorio, Gibum Kim, Marc C. Gurau, Richard D. Yang, Matthew A. Holden, and Paul S. Cremer

J. Am. Chem. Soc. 125 (2003) 12782-12786

Light Activated Patterning of Dye-Labeled Molecules on Surfaces

Matthew A. Holden and Paul S. Cremer

J. Am. Chem. Soc. 125 (2003) 8074-8075

A Versatile, Sensitive Microfluidic Assay for Bacterial Chemotaxis

Hanbin Mao, Paul Cremer, and Michael Manson

PNAS 100 (2003) 5449-5454

Investigations of Bivalent Antibody Binding on Fluid Supported Phospholipid Membranes: The Effect of Hapten Density

Tinglu Yang, Olga K. Baryshnikova, Hanbin Mao, Matthew A. Holden, and Paul S. Cremer

J. Am. Chem. Soc. 125 (2003) 4779-4784

Measuring LCSTs by Novel Temperature Gradient Methods: Evidence for Intermolecular Interactions in Mixed Polymer Solutions

Hanbin Mao, Chunmei Li, Yanjie Zhang, David E. Bergbreiter, and Paul S. Cremer

J. Am. Chem. Soc. 125 (2003) 2850-2851

Microfluidic Diffusion Diluter: Bulging of PDMS Microchannels under Pressure Driven Flow

Mathew A. Holden, Saurabh Kumar, Ali Beskok, and Paul S. Cremer

J. Micromech. Microeng., 13 (2003) 412-418

Generating Fixed Concentration Arrays in a Microfluidic Device

Mathew A. Holden, Saurabh Kumar, Edward Castellana, Ali Beskok, and Paul S. Cremer

Sensors and Actuators B, 92 (2003) 199-207

Reusable Platforms for High-Throughput On-Chip Temperature Gradient Assays

Hanbin Mao, Mathew A. Holden, Min You, and Paul S. Cremer

Anal. Chem., 74 (2002) 5071-5075

Designing Linear Temperature Gradients for Massively Parallel and Combinatorial Measurements On-Chip

Hanbin Mao, Tinglu Yang, and Paul S. Cremer

J. Am. Chem. Soc., 124 (2002) 4432-4435

Design and Characterization of Immobilized Enzymes in Microfluidic Systems

Hanbin Mao, Tinglu Yang, and Paul S. Cremer

Anal. Chem., 74 (2002) 379-385

Stochastic Sensing Based on Nature

H. Bayley and P.S. Cremer

Nature, 413 (2001) 226-230

(b) Papers published in non-peer-reviewed journals:

none

(c) Papers presented at meetings, but not published in conference proceedings:

Combinatorial Microfluidics for Biochemical and Biophysical Studies, Department of Chemistry, Oxford University, Oxford, UK, April 27, 2004

Investigations of Fibrinogen Adsorption at the Liquid/Solid Interface: The Vroman Effect; Using Temperature Gradient Microfluidics to Investigate Thermoresponsive Polymers and Proteins, ACS National Meeting, Anaheim, CA, March 29, 2004 and April 1, 2004

Protein-Membrane Interactions On-Chip, Pittcon, Chicago, IL, March 8, 2004

Multivalent Binding Studies on a Chip, National Institute of Standards and Technology, Gaithersburg, MD, Feb. 18, 2004

Multivalent Ligand-Receptor Binding on a Chip, 48th Annual Biophysical Society Meeting, Baltimore, MD., Feb. 15, 2004

Combinatorial Microfluidics for Biochemical and Biophysical Studies, Purdue University, West Lafayette, IN, Jan. 20, 2004

Biomembranes on a Chip, Duke University, Durham, NC, Oct. 27, 2003

Membranes on a Chip, Dept. of Chemistry, UC-Irvine, Irvine, CA, August 25, 2003

Biomembranes on a Chip, Smith College, Northampton, MA, July 18, 2003

Temperature Gradient and Combinatorial Microfluidics, General Electric Corporation, Schenectady, NY, July 17, 2003

Microfluidic and Array-Based Designs for Investigating Ligand-Receptor Interactions, Fritz Haber Institut der MPG, Berlin, Germany, July 2, 2003

Macromolecular Adsorption at the Liquid/Solid Interface and Biophysical Chemistry on a Chip, University of Heidelberg, Heidelberg, Germany, June 30 and July 1, 2003

Microfluidic and Array-Based Designs for Investigating Ligand-Receptor Interactions on Solid Supported Phospholipid Membranes, Euresco: Biological Surfaces and Interfaces, Il Ciocco, Italy, June 23, 2003

On-Chip Ligand Receptor Binding, University of Colorado, Dept. of Chemical Engineering, Boulder, CO, April 8, 2003

ACS National Meeting, New Orleans, LA, March 24, 2003

Ligand-Receptor Binding on Biomembranes: An On-Chip Approach, Columbia University, New York, NY, March 14, 2003

Ligand-Receptor Binding on Biomembranes: An On-Chip Approach, Harvard University, Cambridge, MA, March 13, 2003

Biomembranes on a Chip, Trinity University, San Antonio, TX Feb. 27, 2003

Biomembranes on a Chip, University of Florida, Gainesville, FL, Feb. 19, 2003

Combinatorial Microfluidics, 3M Corporation, St. Paul, MN, Oct. 22, 2002

Multivalent Interactions on a Chip, Department of Chemistry, University of Minnesota, Minneapolis, MN, Oct. 21, 2002

Biosensors: A Chemist's Approach, Texas A&M Commerce, Oct. 11, 2002

Design High Throughput Microfluidic Devices, 2nd German and American Symposium, Durham, New Hampshire, August, 24, 2002

Bivalent Antibody Binding on Fluid Biomembranes, ACS National Meeting, Boston, MA, Aug. 21, 2002

Designing a New Generation of Biosensors, Self-Assembly of the Future, Massa M., Italy, April 23, 2002

Investigating Ligand-Receptor Interactions at Biomembrane Interfaces, Baylor University, Waco, TX, April 12, 2002

Designing a New Generation of Biosensors, MRS Meeting, SF, CA, April 3, 2002

Role of Water in Biofilms, ARO Workshop, Research Triangle Park, NC, Feb. 26, 2002

Investigations of Ligand-Receptor Interactions at Biomembrane Interfaces, Department of Chemistry, University of California at Berkeley, Berkeley, CA, Feb. 19, 2002

Exploiting Nanofilm Coatings in Microfluidic Devices for Biosensing, Mardi Gras Nanotechnology Conference, Baton Rouge, LA, Feb. 9, 2002

Developing Microfluidic and Array-Based Designs for Investigations at Biomembrane Interfaces, Department of Physics, Texas Tech University, Lubbock, TX, Feb. 7, 2002

Microfluidic and Array-Based Designs for Investigations of Multivalent Ligand-Receptor Interactions at Biomembrane Interfaces, Department of Chemistry, The Scripps Research Institute, La Jolle, CA, Jan. 24, 2002

(d) Manuscripts submitted, but not published

none

(e) Technical Reports:

Technical reports were filled with the ARO for the project for 2001, 2002, and 2003.

(7) Scientific personnel supported by this project:

Mathew Holden, Ph.D. student and earned his degree in 2004

Edward Castellana, Ph.D. student

Hanbin Mao, Ph.D. student and earned his degree in 2003

Olga Baryshnikova, M.S.. student and earned her degree in 2002

Seung-Yong Jung, Ph.D. student and earned his degree in 2003

Dr. Tinglu Yang, Postdoctoral fellow

(8) Report of Inventions:

none

(9) Bibliography

The bibliography is identical to the section of papers published in peer reviewed journals. The work is described in the section of the summary of the most important results.

(10) Appendixes

none