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14. ABSTRACT

A platform technology for monitoring signaling pathways in single T cells using optical nanoprobes has been developed to provide a stable and biocompatible environment for the cells, and allow acquisition of additional data on cellular metabolic and physiological activity using other assays. Platform components were developed and validated to study the signaling dynamics of multiple T cells. MALDI-TOF protein detection, OCIBD IL2 quantification, and the use of quantum dots in live cell imaging were optimized for single cell applications. MALDI data showed clear activation dependence, but there was insufficient MALDI data for statistical analysis; ongoing technical developments are addressing this. Future efforts will fully integrate the microfluidic nanophysiometer, OCIBD analyte detection system, MALDI-TOF protein traps, and cell loading with quantum dots to achieve a fully-integrated, self-contained and instrumented cellular microenvironment that supports precise and timely decisions regarding when best to probe the cell invasively for more detailed intracellular signaling information. This system will allow the identification of unique patterns of signaling pathways of stimuli that can be used to identify previously unknown or maliciously engineered toxins, pathogens, antidotes, and prophylactics.

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Executive Summary

The objective of this project was to develop a platform technology that would enable the monitoring of signaling pathways in single T cells using optical nanoprobes. The platform was optimized to provide a stable and biocompatible environment for the single cells under study, and to generate additional data on the metabolic and physiological activity of the cell which will complement those data obtained through the optical nanoprobes. During the course of the funding period, the major components of a novel platform for the study of cell signaling dynamics of multiple single T cells were successfully developed and validated through proof-of-principle experiments. In doing so, many technologies, such as MALDI-TOF protein detection, OCIBD IL2 detection, and the use of quantum dots in live cell imaging were optimized for single cell applications. Current efforts are focused upon full integration of the microfluidic nanophysiometer, OCIBD analyte detection system, MALDI-TOF protein traps, and cell loading (for internalization of quantum dots) methods in order to achieve a fully self-contained system. We were unable to obtain sufficient MALDI data for statistical analysis, but are proceeding to develop improved techniques that should provide the requisite data in the near future. When fully integrated, the platform will provide an instrumented microenvironment for the cells and allow a precise and timely decision regarding when best to probe the cell invasively for more detailed intracellular signaling information. These capabilities will provide a system that will allow the identification of unique patterns of signaling pathways (*i.e.,* signaling fingerprints) of stimuli that activate the pathways of interest – of particular value in the identification of previously unknown or maliciously engineered pathogens.

Primary Personnel (Supported)

- John Wikswo, Ph.D., PI
- Franz Baudenbacher, Ph.D. – Nanophysiometer and dynamic profiling
- Shannon Faley – T-cell activation and signaling
- Jacob Hughey -- Microfluidics
- Dmitry Markov – Optical determination of protein binding
- Amanda Kussrow – Optical determination of protein binding
- Phil Samson – Microscopy, microfluidics, and cell lysing
- Michael Warnement – Q-Dot labeling of T cells

Associated Personnel (Not supported)

- Mike Ackerman – Nanophysiometer fabrication
- Darryl Bornhop, Ph.D. – Optical detection of protein binding
- Richard Caprioli, Ph.D. – MALDI-TOF and mass spectrometry
- David Cliffel, Ph.D. – Cytosensor/electrochemical electrodes
- Jeremy Norris, Ph.D. – MALDI-TOF and mass spectrometry
- Sandra Rosenthal, Ph.D. – Q-Dots
- David Schaffer – Nanophysiometer fabrication
- Ian Tomlinson, Ph.D. – Q-Dots
- Momchil Velkovsky, Ph.D. – statistical analysis

Publications

None at this time. Multiple publications in preparation.

I. Project Objectives

The primary objectives of this research were to develop a microfluidic platform for the maintenance and study of T cells, utilize optical nanoparticles to probe well-defined T cell activation states, and to optimize mass spectroscopy and interferometric techniques for analyzing T cell signaling states. The following specific aims were proposed:

- **Aim 1.** Optimize the Vanderbilt NanoPhysiometer as a platform for maintaining and controlling single cells while recording dynamical optical data regarding intracellular signaling pathways.
- **Aim 2.** Develop a standardized T-cell activation model that will allow quantitative tests of our ability to characterize T-cell signaling pathways.
- **Aim 3.** Determine the activation profile for a complementary set of four different methods of activating T cells.
- **Aim 4.** Demonstrate the suitability of our platform for recording from optical nanoprobes by detecting the time course of the appearance of particular membrane receptor proteins.
- **Aim 5.** Develop techniques for on-line optical detection of protein binding following lysing of the single cell to complement the off-line analysis of protein state obtained with MALDI-TOF.
- **Aim 6.** Develop the statistical and analytical tools required to correct for cell-cycle and cell-to-cell fluctuations, thereby allowing correlation and comparison of cell signaling data from large numbers of single cells whose individual cell states in terms of proteomics expression levels are slightly different.

II. Status of Effort

After several design iterations, we have developed an optically transparent, microfluidic nanophysiometer capable of sustaining both primary and Jurkat T cells during activation. In addition, we have demonstrated controlled cell lysing to facilitate the analysis of intracellular proteins.

We have adopted two activation protocols. The first method requires the addition of phorbol myristate acetate (PMA) and ionomycin (alone or in combination to provide three separate activation tests) to trigger the PKC and calcium pathways, respectively, that lead to the secretion of IL2 and surface expression of IL2Rα. The second method involves utilizing polystyrene beads coated with CD3 and CD28 antibodies which, when bound, signal the T cell to activate and proliferate. Both methods were successful in activating both Jurkat and primary CD4+ T cells as shown via IL2 Elisa assays as well as imaging cells labeled with anti-IL2Rα conjugated quantum dots.

MALDI-TOF analysis of T cells activated with PMA only, ionomycin only, PMA + ionomycin, or nothing (control) indicated major differences in protein profiles corresponding to the different activation states.

We can both visualize cells decorated with quantum dots and label cell surface receptors with antibody conjugated quantum dots within the nanophysiometer.

Anti-CD25 conjugated quantum dots were used to track temporal dynamics of CD25 (IL2Rα) expression in T cells during activation. Other cell surface markers such

as CD4 and CD28 were also successfully conjugated to quantum dots and used to label primary and Jurkat T cells. In addition, organic fluorophores were utilized to monitor activation (ionomycin) induced calcium spikes from primary T cells within the nanophysiometer.

OCIBD (On Chip Interferometric Backscatter Detection) experiments performed in media containing 1% FBS have yielded IL-2 detection limits within the range expected from that of a single activated T cell. Current efforts are focused on analyzing media from activated cells and correlating IL-2 concentrations obtained using OCIBD to Elisa results. In addition, we are also moving toward incorporating the OCIBD detection system with the current nanophysiometer so that we may obtain on-line, real-time IL2 production concentrations.

Feasibility has been demonstrated in all of the most important objectives: platform development, cell viability within the nanophysiometer, T cell activation, cell lysing, utilizing quantum dots as nanoprobes of cell signaling, and IL-2 detection. Issues with MALDI detection limits have refocused our efforts to obtain protein profiles using advanced laser desorption methods currently under investigation. These improvements should enable the statistical analysis of cell-to-cell variations.

III. Background

A. Motivation & Significance

Our understanding of biological phenomena is often based upon extremely restrictive data. Examples include experiments that measure the ensemble average of populations of 10^6 to 10^7 cells; or measurements of a single variable while all other variables are hopefully held constant, or recording of one variable on one cell, or averages over a few minutes to hours, or combinations of some of the above (as with a ten-liter bioreactor with fifty variables after a one-week reactor equilibration to steady state). Biologists are coming to recognize from the exponential growth of genomic and proteomic information that such limited measurements can adequately reflect neither the dynamics on intracellular signaling nor the complexity of the signaling networks therein. Attempts to develop mathematical models for cellular signaling are faced with the challenge of a very large number of system variables and a very small amount of available data. It is estimated that a single mammalian cell may require 100,000 variables and equations, and the physiology of biological tissues clearly demonstrates that cell-to-cell interactions are critical to system function. The difficulty of understanding signaling pathways is further increased by the fact that many of the intracellular and intercellular interactions are nonlinear. Because the data required for such

Table 1. Sizes, Volumes, and Time Constants

X	V, m^3	V	Tau_Diff	Example	N
1 m	1	1000 L	10^9 s	Animal, bioreactor	100
10 cm	10^{-3}	1 L	10^7 s	Organ, bioreactor	100
1 cm	10^{-6}	1 mL	10^5 s = 1 day	Tissue, cell culture	10
1 mm	10^{-9}	1 uL	10^3 s	Uenviron, well plate	10
100 um	10^{-12}	1 nL	10 s	ECM,	5
10 um	10^{-15}	1 pL	0.1 s	Cell	100
1 um	10^{-18}	1 fL	1 ms	Subspace	2
100 nm	10^{-21}	1 aL	10 us	Organelle	2
10 nm	10^{-24}	1 zL	100 ns	Protein	1
1 nm	10^{-27}	1 npL	1 ns	Ion channel	1

complicated models do not yet exist, there is a pressing need to develop experimental techniques suitable for obtaining vastly greater quantities of data from single cells and small populations of interconnecting cells. With this in mind, the Vanderbilt Institute for Integrative Biosystems Research and Education has launched a program to "instrument and control the single cell," which is directed towards the development of the advanced instrumentation, techniques, and models that will be required for the emerging field of systems biology.

The key facet of our program is based upon the fact that in diffusion-limited biological systems, such as electrochemical or remote binding measurements in cell culture, the frequency response of the system is limited by the time required for the quantity of interest to diffuse from the cell to the sensor (Table 1). Only by going to spatial scales of 10 μm or less is it possible to record cellular changes that occur in less than 1 s. The challenge addressed by this project is to devise methods that will allow us to record up to 100 cellular parameters at the spatial scale of a single cell.

Recent world events have resulted in heightened concern over the threat of chemical and biological warfare (CBW) agents and renewed interest in elucidating mechanisms of detection, prevention, treatment, and containment of large-scale disease epidemics. While detection systems and treatment protocols against naturally occurring diseases such as small pox and anthrax are already being implemented, these efforts do not address the more concerning issue of maliciously engineered biological agents. This yields the question: how do you construct a detection system with the ability to sense novel pathogens with completely unknown pathogenesis? We believe this can be accomplished by utilizing T cells as biosensors and taking advantage of the discriminatory power of their diverse intracellular signaling pathways. However, there are several issues to be addressed prior to achieving a fully functional T cell based biosensing platform. First, while T cell signaling pathways are some of the most thoroughly investigated and best understood, there is still much to learn before a T cell based system can achieve its maximum potential. Second, instead of detecting a single signal from millions of cells as is commonly done, it is desirable to detect efficiently multiple signals from single cells so that (1) rare, important signaling events are not averaged out and (2) simultaneous data points that inherently contain spatial and relational correlations with respect to the overall signaling cascade. To address these issues we proposed to develop a platform to study signaling dynamics of single T cells by utilizing technology that allows the simultaneous detection of multiple signals in live cells. Ultimately, this platform will be capable of yielding six fluorescent signals (3 internal, 3 external), metabolic signatures (pH, lactate, oxygen, and glucose), analyte detection, and intracellular protein profiles of cell lysates *from a single cell.* The experiments described in this report were designed to serve as proof-of-principle demonstrations for several individual components of the overall platform.

B. T Cell Immunology

Thymocytes (T cells) are the major signal transducer with respect to the adaptive immune response in mammalian systems. These cells accept generic information from antigen presenting cells (APCs), rapidly distinguish between dangerous and benign

peptides, then initiate signaling pathways to spark immune reaction specific to antigens perceived as threatening. Capabilities such as these make T cells an ideal target for developing cell based biosensor technologies. However, in order to utilize T cells to discriminate between or identify various stimuli, the internal signaling cascades must be well understood. Many of the experiments described within this report were designed to facilitate the ability to gain more insight into the intricacies of T cell signaling pathways by developing a platform for studying multiple independent signaling pathways in single cells. Preliminary, proof-of-principle experiments were based upon well-known signaling events occurring during T cells activation.

Antigen Processing & Immune Synapse

Antigen presenting cells, primarily dendritic cells, can be considered the sentinels of the adaptive immune system as their primary functions are to indiscriminately break down all peptides, including those of self and non-self origins, and express the peptide fragments bound to the MHC receptor. It is the MHC complex to which the T cell receptor (TCR) binds and discriminates between dangerous and non-threatening antigens. There are two classes of MHC receptor, class I MHC and class II MHC, that respond to cytosolic peptides or endosomal peptides, respectively. While not an absolute division, the MHC class I receptors present mainly viral peptides while the MHC class II bind primarily bacterial antigens. Cytotoxic T cells (CD8+) interact with class I MHC while helper T cells (CD4+) bind the class II MHC receptor.

The immune synapse is formed by the strong interaction between T cells and antigen presenting cells (APCs) during which information on a particular antigen is transmitted to the T cell. If the MHC receptor contains a self, non-dangerous peptide fragment, the binding affinity between the TCR and the MHC receptor will be too weak to transmit a signal resulting in the dissociation of the T cell and APC. When a pathogen is presented instead, the TCR and MHC bind tightly and recruit additional signaling and adhesion molecules to the synapse as shown in **Figure 1**. This results in the amplification and propagation of activation signals through intracellular pathways causing stimulatory cytokine production and clonal expansion of the reacting T cell line (details described below).

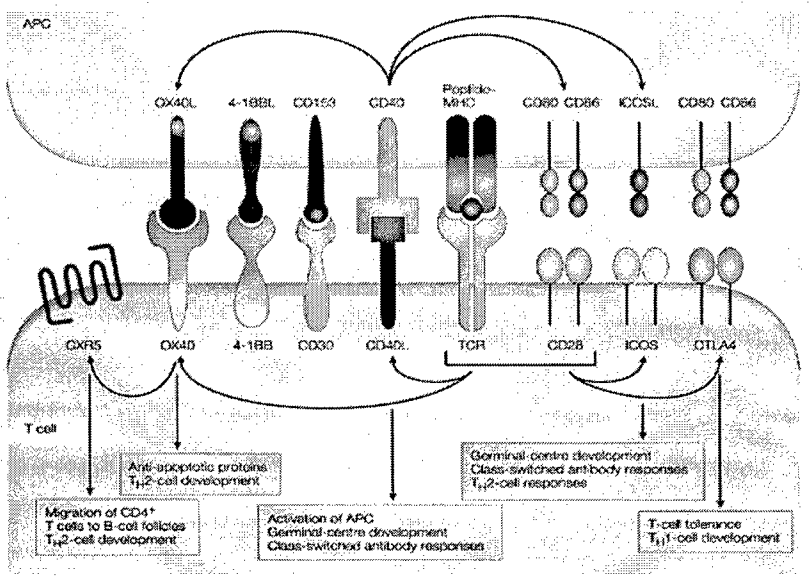

Figure 1. Illustration of the immune synapse (from Acuto and Michel, Nature Reviews Immunology, 2003.).)

T Cell Activation Pathways

Given the centrality of T cells and their activation to this project, it is useful to understand the sequence of events that occur with T cell activation. **Figure 2** illustrates the major signaling pathways leading to IL-2 and IL-2Rα production. Successful engagement of the Tcell receptor (TCR) and coreceptors by antigen presenting cells (APCs) results in a cascade of intracellular events that ultimately result in the transcription of gene products necessary for T-cell proliferation and effector functions. This cascade of events can initiate several important signaling pathways, including the DAG (diacylglycerol), IP_3 (inositol 1,4,5-triphosphate), and MAPK (mitogen-activated protein kinase) pathways. Activation of phospholipase C ($PLC\gamma1$) by adaptor proteins and ZAP-70 results in the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce IP_3 and DAG. IP_3 binds to the endoplasmic reticulum and releases calcium stores. This increase in intracellular calcium concentration activates transcription factor NFAT (nuclear factor of activation in T cells), which then moves to the nucleus. DAG recruits and activates protein kinase C (PKC) in the lipid membrane[1]. There are several calcium-dependant and -independent isoforms of PKC whose substrates are not clearly defined. However, studies have shown that PKC is integral in the activation of NFκB (nuclear factor kappa beta)[2] and AP-1[3]. In particular, the PKC θ isoform is essential for the pro-

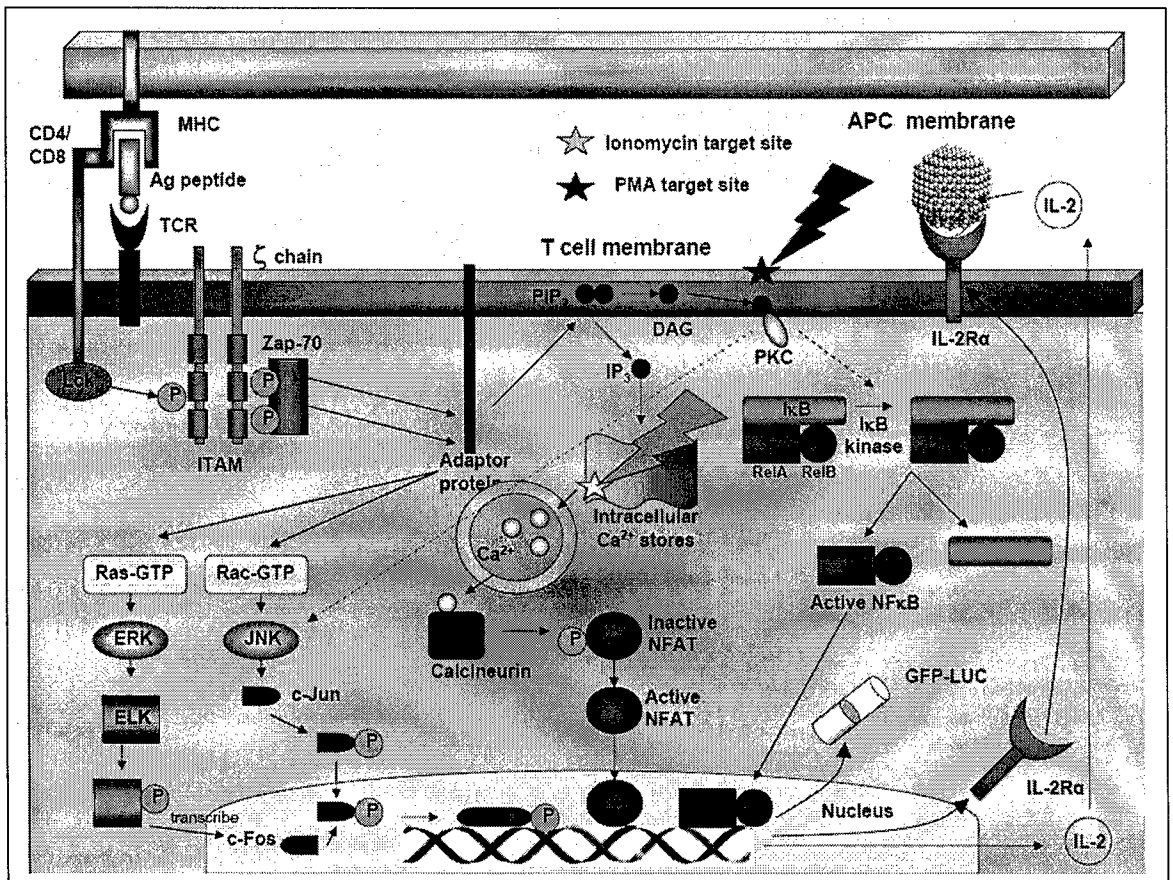

Figure 2. Major intracellular T cell activation pathways leading to the production of IL-2 and IL-2Rα.

duction of TCR-induced IL-2[4]. Once in the nucleus, the transcription factors NFAT, NFκB, and AP-1 act in concert to coordinate the activation of many genes involved in host defense and inflammatory responses, such as cytokine IL-2 to promote T cell activation and proliferation. It is estimated that together these transcription factors generate more than 250 gene products upon activation.

In vivo, T cell activation occurs by direct cell-cell contact with an APC. *In vitro*, T cell stimulation can be artificially induced by using 2 chemicals, phorbol myrastoyl acetate (phorbol ester or PMA) and ionomycin[5]. Both PKC and Ras-GRP (precursor to MAPK cascade) possess phorbol ester binding sites and are thus stimulated by PMA resulting in the activation of NFkb and AP-1[6]. Ionomycin stimulates calcium release from intracellular stores which results in the activation of NFAT. Thus, administration of these two chemicals simultaneously upregulates all three transcription factors.

NFκB: Five members of the mammalian NFκB/Rel family have been identified. The preponderant form of NFκβ in most cells consists of a heterodimer containing p50 and RelA (p65), which contains a transactivation domain[7-9]. In quiescent cells, NFκB complexes are sequestered in the cytoplasm by inhibitory proteins (IκB-α, IκB -β, IκB -ε)[10]. Stimulation of the NFκB pathway is mediated by diverse signal transduction cascades, resulting in phosphorylation of IκBs. Phosphorylated IκB-α (or IκB -β) is ubiquitinated and degraded by the 26S proteasome, allowing nuclear translocation of the NFκB complexes[11,12]. Activation of NFκB is necessary for maximal transcription of many adhesion molecules, enzymes, cytokines, and chemokines important in the initiation of inflammation and has been shown to be an essential component in signaling pathways of macrophages, dendritic cells, and T and B-lymphocytes[13,14]. Still, the independent effects of cell and tissue specific activation of this transcription factor complex are largely undefined.

NFAT: There are four members of the NFAT family (NFAT 1-4), all of which share a high degree of DNA binding region sequence homology with the NFkB/Rel family of transcription factors. NFAT 1 and 4 are constitutively expressed in T cells, while NFAT-2 transcription is induced by T cell activation. Previous studies have shown that NFAT 1,2 ,and 4 exert overlapping control in the initiation of IL-2 production in that cells may be deficient in one isoform without suffering significant decrease in IL-2 levels. However, cells deficient in 2 or more isoforms result in marked impairment of IL-2 production[15]. Under resting conditions, a phosphorylated NFAT resides in the cytosol. Increase in intracellular calcium during activation results in calcineurin activation and subsequent binding to NFAT. Within 5-10 minutes following activation, the NFAT/calcineurin complex rapidly translocates to the nucleus[15] where NFAT associates with AP-1 to initiate transcription of various gene products including IL-2, IL-4, IL-5, TNF-α, and GM-CSF[16-22]. High intracellular calcium levels are required to sustain NFAT activity in the nucleus as calcineurin dissociation results in the movement of NFAT from the nucleus back to the cytosol[15].

AP-1: Activator protein -1 (AP-1) is a potent transcription factor consisting of 2 basic region-leucine zipper proteins (bZIP), c-Jun and c-Fos[23]. Ligation of the TCR activates MAPK cascades, ERK (extracellular receptor-activated kinase) and JNK (c-Jun N-terminal Kinase), that lead to the de novo synthesis of c-Jun and c-Fos in parallel pathways, respectively [1]. A third MAPK, FRK, facilitates the dimerization of c-Jun and

c-Fos by phosphorylating the C-terminal of c-Fos. Both c-Jun and c-Fos can act as transcription factors, but are more stable as a heterodimer and thus increase their DNA-binding ability[24]. In T cells AP-1 is known to associate with NFAT and NFκB in the production of IL-2, among other gene products.

IL-2: Interleukin-2 (IL-2), also known as T cell growth factor, is the major activating cytokine in T cells. Antigen stimulated naïve T cells (both CD4+ and CD8+) generate high levels of IL-2 which acts in autocrine fashion to promote clonal expansion, expression of IL-2 receptor-alpha (IL-2Rα), and increased cytokine production [1]. Because IL-2 is not produced in naïve T cells, it is a good indicator of T cell activation. IL-2 synthesis requires transcriptional activation by NFκB, NFAT, and AP-1 and typically peaks 8 to 12 hours following antigen stimulation in primary T cells [25]. IL-2 is one of the key signals for which we are developing dynamic detection methodologies.

Jurkat T cells/ T cell hybridoma: Generating a stable cell line from T cells freshly isolated from peripheral blood samples is difficult due to their inherent volatility and relatively short lifetime. In addition, the peripheral blood will contain a very diverse population of T cells in terms of maturity and receptor type. T cell hybridomas overcome these limitations by fusing a particular normal T cell line with a lymphoma cell line. Fused cells are immortal and thus are stable over time, but also retain many of the characteristics of the normal T cell. In this experiment, in addition to primary T cells, we have used Jurkat cells (ATCC, clone E6-1), a well-studied T cell hybridoma generated from leukemia lymphoma by Schneider *et al.*[26] However, the Jurkat cell line is only a model of normal CD4 T cells. Because of their ease of use, we used Jurkat cells to optimize many of the experimental protocols, but once this was done, we conducted experiments with primary CD4+ T cells isolated from peripheral blood of human donors in order to generate more scientifically relevant results.

C. T cells as biosensors

There is much more to the activation of T cells than is described in the previous discussion of intracellular signaling following TCR ligation. The choice between T cell activation versus anergy is a function of TCR affinity for MHC + pathogenic peptide[27], presence of co-factors and adhesion proteins[28,29], cytoskeletal rearrangements[30], inflammatory cytokines[31], the activation of any cytosolic inhibitory proteins[32], as well as signals yet to be identified. Furthermore, activated CD4+ cells then differentiate into either TH1 or TH2 effector cells depending upon the interactions occurring during the immune synapse which determined whether there will be a humoral or cell based T cell response[33]. There are situations such as pregnancy and tumorigenesis that, according to the traditional model of adaptive immune response, should lead to acute inflammatory reaction and rejection, but, for reasons not entirely clear, result in immune tolerance. Conversely, a completely MHC matched donated organ suffers from acute and chronic immune rejection despite the use of immune suppressive medications. Matzinger proposed the concept that T cells are capable of not just discriminating between self and non-self, but actually go a step further and differentiate between dangerous versus non-dangerous signals[34]. Clearly, there exists a high level of complexity and discriminatory ability within the signaling pathways of T cells. These characteristics not only allow these cells to be highly effective for mediating immune response against novel patho-

gens, but also to be utilized as biosensors to gain insight into methods of detection, prevention, and treatment for biological warfare agents, the premise being that T cells exposed directly (not via APCs) to a particular toxin will subsequently yield unique signatures in the form of promotion or inhibition of particular signaling events or molecules. After toxin exposure, T cells that are subsequently activated using chemicals or artificial APCs will exhibit changes in the known signaling cascades that can be identified and used for identification and discrimination against other potential CBW agents. Studies concerning anthrax toxin conducted by Paccani *et al.* support the feasibility of this premise as they have shown that both the lethal factor (LF) and edema factor (EF) of anthrax toxin suppress T cell activation by inhibiting the MAPK dependant NFAT and AP-1 signaling pathways[35]. This work illustrates an additional benefit of using T cells as biosensors in that the information collected is not only useful for identifying pathogens, but also provides details concerning its impact upon the adaptive immune response. This is highly relevant in developing preventative and therapeutic treatments.

IV. Accomplishments/New Findings

A. NanoPhysiometer

Preliminary experiments involving the nanophysiometer were primarily devoted to optimizing device design and demonstrating the ability to trap T cells, activate and labels T cells *in situ*, and then lyse them. The first generation autoloading nanophysiometer fabricated of PDMS using soft lithography techniques shown in **Figure 3A** was designed to minimize stress on the T cells during loading and provide a means to easily isolate single cells for long-term signaling experiments. Cells are captured as they passively encounter PDMS buckets while the device is perfused with a cell suspension delivered using syringe pumps. Media perfusing the cells contain yo-pro-1, a nucleic acid stain shown to effectively label apoptotic T cells earlier than propidium iodide[36], such that dead or apoptosing cells can be

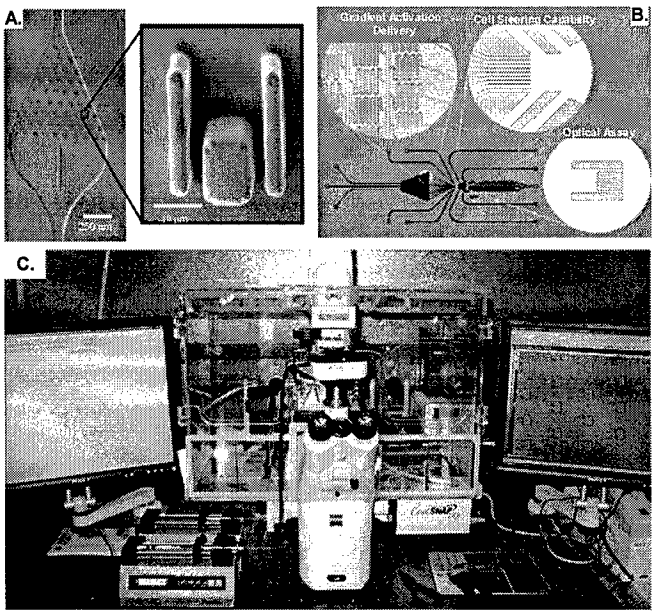

Figure 3. Autoloading Nanophysiometer. (A) First generation NanoPhysiometer contains 55 20μm cell traps that are loaded by simply flowing the cells through the device. **(B)** Second generation NanoPhysiometer includes a higher density of 20 μm cells traps, a mixer capable of delivering a gradient mixture of metabolic agents/dyes, and extra ports to accommodate sheath flow for accurate cell steering and delivery to the cell traps. **(C)** The experimental setup includes syringe pumps for reagent delivery, a microscope incubator which houses the NanoPhysiometer to maintain temperature and CO_2 levels for cell culture.

easily distinguished by fluorescence. Based upon yopro-1 fluorescence, cells appear to tolerate the loading procedure very well as long as the flow rate is kept at or below 1μl/min. Higher flow rates result in cell lysing as the high pressures compress cells against the traps.

The microscope stage supporting the NanoPhysiometer is completely surrounded with a microscope incubator that maintains the environment at 37°C and 5% CO_2. Utilizing this conformation, Jurkat cells were activated using 10nm PMA and 4 μm ionomycin incorporated in the perfusing media and maintained 36 hours in a continuous flow environment before exhibiting yopro-1 fluorescence indicative of cellular apoptosis/death. However, Jurkat cells must be maintained for 72 hours *in situ* following administration of activating agents in order to achieve maximal IL2Rα surface expression. There were several issues with this device design including a flow profile that resulted in poor flow through the central region of the device, causing low cell trapping efficiency and, most likely, inconsistent reagent delivery. In addition, the build-up of back pressure in the exit port during long-term experiments an increase in perfusate flow rate in order to prevent cells from becoming dislodged from traps. It is believed that these issues contributed to the inability to maintain cells (both Jurkat and primary T cells) during activation since the reagent and environmental needs of these cells were satisfied with the perfusate (RPMI 1640 containing 10% FBS, 10ng/ml IL-2, and 10 μg/ml Cipro) and microscope incubator.

The subsequent design and fabrication of the second generation nanophysiometer (**Figure 3B**) addressed these concerns with a redesigned exit port to promote a more even flow profile across the device, a higher density of cell traps to increase trapping probability, additional cell delivery ports to allow cell steering by adjusting the flow rate of the various perfusates, and the incorporation of a microfluidic mixer which will allow gradient delivery of chemical agents in future experiments. Experiments utilizing the new device conformation have shown the improvements to be significant as this design yields nearly 100% cell trapping efficiency (*i.e.,* all traps contain at least one cell). We have demonstrated that we can load primary cells into the nanophysiometer and labeled them *in vitro* with anti-IL2Rα conjugate 655nm QDs upon activation prior to loading in the nanophysiometer. We are able to maintain primary CD4+ T cells for 24 hours within the nanophysiometer, which is sufficient to induce IL2 secretion and IL2Rα surface expression. Current efforts are focused on maintaining activated primary cells for 2-3 days in order to monitor cell division following activation.

A major component of the platform vision is the ability to lyse cells in the NanoPhysiometer for the study of intracellular proteins using MALDI-TOF. It is important that the cells are lysed quickly (preferably less than 30 seconds) to prevent any stress induced signals from influencing the protein profile and ensure that the lysis methods do not employ MALDI incompatible reagents, including many detergents and polymer coatings. In addition, the intracellular contents must be contained within the traps in order to reserve the ability to accurately determine the protein population in a given cell. We evaluated electroporation as a method for cell lysis to determine its ability to satisfy these stipulations. Platinum electrodes were fabricated on the surface of glass slides serving as the base of the nanophysiometer. The PDMS devices were carefully aligned with the glass slides such that the patterned electrodes were oriented above and below

the cell traps. Electrodes were connected to a voltage generator (Axon Instruments) capable of controlling the peak voltage as well as pulse duration and structure. Trapped Jurkat T cells were constantly perfused with media containing 1 nM yopro-1 such that live/dead cells were easily distinguished. Beginning with a 1 second square pulse of 1V, we increased the voltage 0.5 V increments until bright field images indicated altered cell morphology or yopro-1 fluorescence was detected. A single 1 second, 10 V pulse resulted in significant membrane disruption that is evident in both bright field and fluorescent images. In addition, it appears as though the cell lysates remain contained within the cell trap. These results suggest electroporation is a feasible cell lysing method. Future experiments will include in-depth studies of optimal electroporation parameters and address questions concerning the status of the nuclear membrane following electroporation.

Another method of cell lysing currently under investigation involves rapidly compressing the top and bottom PDMS layers together using air pressure applied to a chamber above the cell-holding region. This method requires no electrodes, special wiring, or additional external equipment and, like electroporation, has the potential to be modulated in order to internalize quantum dots in the extracellular fluid. Cells perfused with media containing yopro-1 were successfully lysed by compression without visible loss of intracellular components (as determined by yopro-1 fluorescence), an important issue with respect to downstream analysis of protein profiles. Current experiments are geared towards optimizing device design in order to yield uniform and tunable pressure applications.

B. Quantum Dots as Nanoprobes

While QDs possess many desirable attributes in comparison to traditional organic fluorophores, their application to live cell assays is still in its infancy. However, in order to use QDs to probe intracellular signaling events, methods to easily modulate QD fluorescence and to efficiently deliver QDs to intracellular targets in live cells must be refined. Thus, the preliminary results presented here focus on evaluating T cell activation using cell surface markers and investigated methods of QD internalization.

Because IL2Rα is only expressed on the surface of activated T cells, it is often used to differentiate between activated and non-activated cell populations. Utilizing a commercially available QD conjugation kit (Quantum Dot Corporation), we conjugated 655 nm QDs to IL2Rα antibodies in order to label activated T cells. Primary CD4+ and Jurkat T cells were activated *in vitro* using 10nM PMA and 4 μM ionomycin. Cells were labeled by co-incubation with 5 nM anti-IL2Rα conjugated QD solution for 30 minutes at room temperature 24 hours (primary CD4+ T cells) and 72 hours (Jurkat cells) following stimulation. Unbound QDs were removed by centrifugation. We have confrimed that activated Jurkat and primary T cells exhibit high levels of IL2Rα expression, while less than 5% of the unactivated cells are labeled with the QD conjugates. This is a positive indicator that the QD conjugates are specifically binding IL2Rα rather than non-specifically adhering to the cell surface, a common problem with QDs. Another indicator that the level of non-specific binding is low is demonstrated by the lack of QD labeling of dead cells (indicated by yopro-1 fluorescence) in the activated cell populations. Finally, anti-CD4 conjugated 605 nm QDs were utilized to label the Jurkat and primary cell lines. As expected, both cell lines were labeled with nearly 100% efficiency with these conju-

gates. However, it is important to note that the control experiment involving labeling the same population of cell with an isotype control has not yet been performed. This control experiment will verify that the anti-CD4 QD conjugates are specifically labeling CD4, rather than non-specifically adhering to the cell membrane.

Confocal images of Jurkat cells labeled with anti-IL2Rα conjugated QDs were obtained to verify that QDs were indeed labeling the surface of the cells. There have been reports that cells often passively endocytose QDs. Cells were fixed with 4% paraformaldehyde immediately following QD labeling as well as 24 hours later. We determined that the QD conjugates are localized to the surface of the cells fixed immediately following labeling, indicating that passive endocytosis does not occur within the 30 minute incubation time required for QD labeling. However, cells fixed 24 hours after labeling clearly indicate internalization of QDs. This is most likely due to the cells' tendency to endocytose capped, or antibody triggered, receptors. While most studies have shown QD to be relatively bio-compatible [37,38], it will be important for future studies to evaluate whether the internalized QDs are being passed into the lysozomal pathways and subject to degradation. The release of cadmium from the QD core would have a cytotoxic effect upon the cells, but it is not clear if or how long it takes for the cells to degrade the QD coating.

We have been devoting a continuing effort towards the development of multiple conjugates targeting different surface bound receptors involved in T cell activation, as well as an investigation of the temporal dynamics of T cell activation using IL-2Rα antibody-QD conjugates. Antibody conjugation has provided a versatile, generalized approach for targeting specific biomolecules of interest. Currently, we have developed antibody conjugates specific to CD4 (a coreceptor in T helper cells), CD28 (a costimulatory molecule), and CD25 (IL2-Rα). Further development of QD probes for future multiplexed cell signaling investigations will continue rapidly using commercially available antibodies.

In order to track the temporal expression levels of IL-2Rα expression, Jurkat T cells were chemically activated with PMA and ionomycin at 12 hour intervals, and then labeled with antiCD25-QD conjugates. ELISA based IL-2 quantification from the media verified cellular activation; and fluorescent intensities increased towards later time points, indicative of the time course required for T cell activation and IL-2Rα transcription and expression. Current research is directed towards quantification of the fluorescent signal, and correlation of this signal with IL-2Rα ELISA based assays. Future experiments will involve incorporation of this detection scheme with the nanophysiometer to elucidate the temporal dynamics of receptor expression for individual cells on a massively parallel basis over the entire course of activation.

In addition to labeling cell surface markers of T cell activation, we have also begun to investigate methods of loading QDs in T cells. Specifically, a commercially available pinocytic loading method (Molecular Probes) traditionally used for loading membrane impermeable dyes was evaluated for its ability to facilitate delivery of QD into the cytosolic region of T cells without activating or harming the cells. Cells are first incubated in a hypertonic solution containing 20nM QDs. The hypertonic solution causes the cells to shrink and, in an attempt to maintain tonicity, the cells endocytose extracellular fluid that contains the QDs in solution. The hypertonic solution is then re-

placed with a hypotonic solution that causes the cells to swell and the endocytic vesicles to burst, releasing the QDs into the cytosolic region. Confocal images verified the internalization of PEG coated 605nm QDs using the pinocytic loading method.

Once it was established that the pinocytic loading method resulted in QD internalization, additional experiments were performed to assess the effects of the loading method and QDs upon cell viability. First, cells were labeled 72 hours following QD internalization with anti-IL2Rα 655nm QDs and re-evaluated using confocal microscopy to determine if the loading method resulted in activation. We showed that cells loaded with the PEG coated QDs do exhibit low levels of IL2Rα expression, indicating that the cells are at least partially activated by either the loading method or the presence of QDs. We observed that the internalized QDs are completely aggregated into several distinct regions within the cell rather than evenly dispersed.

To assess the impact of the QDs versus the loading method upon cell viability, Jurkat cells were also labeled with yopro-1 72 hours following the loading procedure and analyzed by FACS to determine a live/dead ration of cells. Controls included (1) resting cells in culture and (2) cells subjected to the pinocytic loading method, but not loaded with QDs (sham load). Several different QDs with various conjugation schemes were analyzed, including anti-IL2Ra and anti-CD4 conjugated QDs in addition to the PEG coated QDs described previously. FACS analysis showed that the sham loaded cells have nearly twice the number of non-viable cells compared to resting cells. This indicates that the loading procedure alone results in 20-25% cell loss. It is most likely that many cells burst or experience membrane blebbing during the incubation in hypotonic solution. Shortening this incubation time may increase survivability. Additional experiments are required to fully evaluate the pinocytic loading method as suitable for use in T cells. Specifically, a challenge assay is needed to ascertain if the low level of activation detected will impact cell function. It is also important to verify that the QDs do not remain sequestered in endosomes as they must be released into the cytosol in order to be utilized for functional assays. Future experiments will also address other potential methods of QD internalization, such as scrape loading, syringe loading, electroporation, and photochemical internalization.

Quantum dots do not appear to have a compounding negative effect upon the cells into which they were loaded, regardless of conjugation scheme. PEG coated QDs actually seem to increase the survivability of the loading procedure. This may be due to the ability of large molecular weight PEG to fuse cell membranes, perhaps aiding in the repair of any disruptions induced during the hypotonic lysis step. It is important to note, however, that our cytotoxicity analysis for loading cannot be used to make a global statement concerning the safety of QD for intracellular studies. There is very little regulation or quality control in the overall and batch-to-batch fabrication of QDs, even from commercially available sources, such that the properties of the QDs are highly variable between batches. Until the production of QDs becomes more standardized, toxicity data such as is presented here is relevant only to the specific batch of QDs utilized in the viability assays.

C. Calcium Analysis

T cells express peptide-specific receptors on their cell surfaces that trigger rapid and extensive intracellular events upon recognition and binding of cognate antigen. Or-

dinarily, the receptors are exquisitely selective, but they can be ligated non-selectively by special monoclonal antibodies (CD3) that are capable of bypassing the peptide-specific binding area of the TCR complex. This non-specific (or polyclonal) activation of T cells stimulates the same internal machinery of the cell, and in fact is commonly used by immunology researchers to expand T cell populations. Pharmacologic agents such as phorbal myristate acetate (PMA) and ionophores like Ionomycin can also be used to stimulate T cells. Their mechanism of action is downstream from the TCR. Other chemical means of activation include a class of molecules known as lectins (*e.g.,* phytohemaglutinin) that indiscriminately ligate surface molecules on the TCR. We have begun to develop the techniques to controllably harvest and contain small polyclonal populations of primary T cells in within the nanobioreactor microfluidic device and measure the metabolic effects of activation mediated by various means, including PMA and ionomycin and T cell membrane receptor antibodies coated onto polystyrene beads.

Primary T cells are smaller than cells of the Jurkat hybridoma line. They are suspension cells, preferentially growing suspended in culture media, but they can be adhered to specially coated surfaces. As part of the follow-on effort to this grant, we are redesigning the containment fences of the nanobioreactor to accommodate the small size of primary T cells by reducing the spacing between gaps to 3 microns (from approximately 100 microns) and decreasing the cross-sectional area of the gaps from 3 x 25 microns to 3 x 3 microns). This design will allow media an escape route around the contained T cell and reduce the tendency to push the cell through the gap. We are also testing special coatings on the glass floor of the NBR to anchor the T cells during studies. While we optimize the NBR for studies with primary T cells, we have studied the use of Ionomycin and PMA to activate single cells of the Jurkat cell line, and can readily detect the response of Jurkat cell cytosolic calcium to infusion of Ionomycin and PMA. The measurement was made on a single cell adhered to a glass-bottom, poly-lysine coated petri dish being perfused by a micro perfusion pencil and using a fiber optic bundle connected to a photomultiplier tube (PMT). The response of the cell to the chemicals is essentially immediate. Another advantage of chemical activation is that all cells in the NBR will be activated at the same time. This, combined with the precise cell-harvesting techniques described above, enables a plot of extracellular acidification rate versus number of activated cells which will help to estimate the acidification rate of a single T cell in response to activation. A similar study in which repeated 30 second dosing of a **single** Jurkat cell led to repeated fluorescence plateaus. In this study calcium chloride dihydrate was added to the RPMI media (denoted RPMI 1640C) to approximately 2 mM concentration to simulate physiologically realistic levels. The data indicate that ionomycin permeabilizes the cell membrane to calcium, intracellular organelle membranes or both, leading to an increase in cytosolic calcium reflected by the increase in fluo-3 fluorescence collected by the PMT. The decrease in fluorescence that accompanies cessation of ionomycin perfusion seems to indicate that the ionomycin can be flushed away, returning the cell membranes to normal calcium permeability. This is consistent with published findings that ionomycin can partition itself between hydrophobic and hydrophilic milieus and acts as a chauffeur for calcium across lipid membranes.

As our fourth method of stimulating T cells, we conducted studies on primary cells using the multi-trap nanophysiometer (MT-NP) and antibody coated beads. In the

follow-on to this project, we are planning to repeat these bead activation studies with the lower profile devices and quantitate the activation efficiency of commonly used antibody-coated beads. We are also conducting long-term viability studies within the devices to facilitate the doubling of T cells within traps following activation with beads.

D. OCIBD Analyte Detection

One of the most promising techniques for picoliter volume molecular interaction sensing or protein-protein binding assays is based on interferometry. The On-Chip Interferometric Backscatter Detector (OCIBD) employs a coherent light source for illumination of a microfluidic channel etched in glass or fused silica. The interaction of the laser beam with the fluid contained within the channel results in a high finesse interference pattern, which is analyzed by a phototransducer located in the direct backscatter direction.[39] OCIBD has been previously shown to work as a universal solute detector in CE[40] for non-invasive nanoliter temperature determinations[41], and as a highly accurate flow sensor[42]. As shown below, OCIBD can also be successfully used to study protein–protein interactions without the need for fluorescent or radioactive labels. These determinations have been performed within picoliter volumes using microfluidic channels molded in polydimethylsiloxane (PDMS).

We have shown that over a concentration range from $1 \cdot 10^{-10}$ M to $1 \cdot 10^{-11}$ M, the OCBID has a linear response with a 3σ detection limit of $4 \cdot 10^{-11}$ M, equivalent to 10,800 molecules or 270 ag of protein within the probe volume (490 pL). We conducted binding experiments in cell media, and obtained preliminary K_D value of 53 pM, in the appropriate range for high affinity receptors to IL-2, which ranges from 10 pM to 60 pM[43]. This experiment is currently being repeated using media (containing 10% FBS) taken from cells activated for various lengths of time (between 0 and 72 hours). The IL2 concentrations obtained using the OCIBD method will soon be compared to that obtained by Elisa assay on the same samples. Future efforts will focus on incorporating the OCIBD technology with the nanophysiometer in order to obtain on-line measurements of IL2 production by activated T cells.

E. MALDI-TOF Analysis of T Cells

MALDI experiments have been devoted to collecting essentially control data for future single cell analysis. Basically, large populations of Jurkat cells (50,000 cells/well) are activated according to four different protocols: no activation agent, PMA only, ionomycin only, or PMA and ionomycin. At specific time points during activation (0, 12, 24, 36, 48, and 72 hours for Jurkat cells) cells are lysed using 50 mM Tris (a MALDI compatible detergent) and the intracellular proteins analyzed using MALDI. The goal is to identify peaks that appear linked to T cells activation through rigorous statistical analysis of the protein spectra generated over time and thus may be of great interest to identify and analyze further. We have obtained protein spectra 0, 12, 24, 48, and 72 hours post activation in Jurkat cells. One major limitation of MALDI MS is the inability to resolve proteins of large molecular weight (>80 kD). Thus the spectra collected thus far are primarily catalogs of protein peaks below this mass threshold. In order to detect the larger proteins, the current protocol involves enzymatically digesting the cell lysates using trypsin and then detecting the fragments by MALDI. This, however, makes the analysis significantly more complex as the spectra generated contain more noise. In addition, while there are points along the proteins that trypsin is more likely to cut, the true liga-

tion sites on each protein are not known making the process of protein identification much more difficult. Because so many proteins and transcription factors known to be important to T cell activation have molecular weight well beyond the MALDI detection limits, such as NFAT, NFkb, and most membrane proteins, we are currently investigating alternative methods to detect large molecular weight proteins using mass spectroscopy. When this is accomplished, we will resume acquiring the MALDI data required for statistical analysis of protein signaling.

V. Conclusions

During the course of the funding period, the major components of a novel platform for the study of cell signaling dynamics of multiple single T cells were successfully developed and validated through proof-of-principle experiments. In doing so, many technologies, such as MALDI-TOF protein detection, OCIBD IL2 detection, and the use of quantum dots in live cell imaging were optimized for single cell applications. Current efforts are focused upon full integration of the microfluidic nanophysiometer, OCIBD analyte detection system, MALDI-TOF protein traps, and cell loading (for internalization of quantum dots) methods in order to achieve a fully self-contained system. We were unable to obtain sufficient MALDI data for statistical analysis, but are proceeding to develop improved techniques that should provide the requisite data in the near future. Once integrated, all the components will be in place for the development of a diverse cell-based detection system for the identification of previously unknown or maliciously engineered pathogens.

VI. Personnel

Primary Personnel (Supported)

- John Wikswo, Ph.D., PI
- Franz Baudenbacher, Ph.D. – Nanophysiometer and dynamic profiling
- Shannon Faley – T-cell activation and signaling
- Jacob Hughey -- Microfluidics
- Dmitry Markov – Optical determination of protein binding
- Amanda Kussrow – Optical determination of protein binding
- Phil Samson – Microscopy, microfluidics, and cell lysing
- Michael Warnement – Q-Dot labeling of T cells

Associated Personnel (Not supported)

- Mike Ackerman – Nanophysiometer fabrication
- Darryl Bornhop, Ph.D. – Optical detection of protein binding
- Richard Caprioli, Ph.D. – MALDI-TOF and mass spectrometry
- David Cliffel, Ph.D. – Cytosensor/electrochemical electrodes
- Jeremy Norris, Ph.D. – MALDI-TOF and mass spectrometry
- Sandra Rosenthal, Ph.D. – Q-Dots
- David Schaffer – Nanophysiometer fabrication
- Ian Tomlinson, Ph.D. – Q-Dots
- Momchil Velkovsky, Ph.D. – statistical analysis

VII. Publications

None at this time. Multiple publications in preparation.

VIII. Interactions/Transitions

A. Meetings, conferences, seminars

1. "Correlations Between Single Cell Signaling Dynamics and Protein Expression Profiles" presented at DARPA/DSO sponsored workshop entitled "Real Time Monitoring of Signaling Pathways in Biological Cells" held April 22, 2004 in Washington, D.C.
2. "BioMEMS for Instrumenting and Controlling the Single Cell," Workshop on Microanalytical Devices for Bioprocessing," 2004 IEEE EMBS Conference, San Francisco, CA, September 2004.
3. "Need for Cellular and Molecular Sensors and Actuators," Mini-Symposium: Biomolecular Processors through Micro- and Nanotechnology, 2004 EMBS IEEE Conference, San Francisco, CA, September 2004.

4. "Correlations Between Single Cell Signaling Dynamics and Protein Expression Profiles" presented at DARPA/ DSO sponsored meeting entitled "Simbiosys" held Oct 12-14, 2004 in Vail, CO.
5. "Integrative Systems for Biotechnology and Bioinformatics," A Workshop on Challenges and Opportunities in Integrative Macro- Micro- and Nano-Systems, Sponsored by the National Science Foundation, Arlington, VA, March 7-8, 2005.
6. "Back to the future: Systems biology as the new physiology," UCSD Center for Theoretical Biological Physics, San Diego, 22 April 2005
7. "The Technical and Computation Challenges of Merging NanoScience and Systems Biology," UT Dallas, 4 May 2005
8. "Instrumentation Challenges for Systems Biology," Keynote Lecture, Third IEEE Sensors Conference, Vienna, Austria, October 26, 2004.
9. "The Need for Dynamic Sensing and Control of Cells to Specify and Validate Systems Biology Models," Systems Biology Lecture Series, University of Michigan, Ann Arbor, MI, November 16, 2004.

B. Consultative and Advisory Functions – None at this time.

C. Transitions

We are at present working with Pria Diagnostics of Menlo Park, CA to merge their optical instruments with our electrochemical ones to provide an inexpensive, multiparameter, point-of-use cellular diagnostic device for determination of ratios of CD4+/CD8+ T cells. We are also working with Pria to further develop the OCIBD technology for protein determination.

IX. New Discoveries, Inventions, Patent Disclosures

None at this time. We anticipate filing disclosures on technology presently under development.

X. Honors/Awards

No new honors or awards during this funding period.

XI. References

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