An Evaluation of Cytokine-Capture Nanoparticle Technology:
Stepping from Bench-Space into Potential Markets

by

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ABSTRACT

The feasibility of bringing a nascent technology for detection and quantification of local cytokine concentrations on cell surfaces to market is presented in this paper. Quantum dots or fluorochrome-loaded nanoparticles are conjugated with antibodies for target analytes and with proteins that allow nanoparticle attachment to the surface of T cells. A second labeled monoclonal antibody is introduced to detect the presence of any captured-cytokines using 3D fluorescent microscopy or flow cytometry. Microscopy of DO.11 cells labeled with cytokine-capture particles have shown successful detection of exogenous IL2. A comparison of existing patents with cytokine-capture technology revealed that although each aspect of the device is covered by prior IP, the capabilities of the technology exceed the claimed uses of the individual components. A preliminary market research for cytokine-capture technology applications resulted in dismissing the immunoassay industry as a target market. However, T cell monitoring was identified as a far more lucrative industry.

Thesis Supervisor: Darrell J. Irvine
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1. Introduction

Despite the billions of dollars feeding into AIDS and cancer research, an effective cure or vaccine has yet to emerge for either disease from on-going research. The limited success of pharmaceutical drugs against the endemic diseases have led some researchers to focus on immunotherapies as solutions. Paramount to immunotherapies is the proper stimulation and activation of T lymphocytes. An integral part of proper stimulation is intercellular and autocellular communication via soluble signaling molecules, such as cytokines. Although methods of bulk quantification of cytokines exist, there are no effective methods to track and determine concentrations of these small soluble molecules at cell interfaces. The soluble-molecule-capture device is designed to overcome this obstacle by attaching fluorescent particles (polystyrene nanospheres or quantum dots) onto the surface of the T cell to "capture" any cytokines that diffuse to the T cell surface.

The potential applications of the cytokine capture device address a market demand and subsequently create value in the technology. As a researcher and/or inventor of the nascent technology, there is an opportunity to capture some of this value by bringing forth the technology into commercialization. In order to determine if the pursuit of commercialization is advisable, the next steps are to identify further potential applications of these capture devices, investigate the intellectual property yet unclaimed in the field, and examine the likelihood of success of the device given the existing and future markets.

2. Background

2.1. The immune system

2.1.1 Overview of immune system. The function of the immune system is to defend the body from harmful pathogens while avoiding destruction of healthy tissue. There are two classifications of immunity to maintain an infection-free organism: innate and adaptive immunity. Innate immunity serves as the first line of defense from external sources of infections, beginning with the body's exterior barriers, for instance the skin and mucous membranes. Adaptive immunity is the coordinated, cellular response of lymphocytes and antibodies towards the identification of and the elimination of pathogens infecting cells and tissues. The present research takes particular interest in cell-mediated adaptive immunity[1,2].

In cell-mediated immunity, professional phagocytes, known as antigen presenting cells (APCs), digest pathogens and present antigens in coordination with major histocompatibility complex (MHC) molecules on the cell surface (Figure 1). An antigen is either a unique polypeptide or polysaccharide that serves as a fingerprint for the immune system to identify invading pathogens, such as bacteria, viruses, and even cancer cells. Although most microbes are destroyed in the process, certain microbes have evolved to survive phagocytosis. A coordinated cellular response is necessary to completely eliminate an infection of intracellular microbes. T Lymphocytes, matured in the thymus, are the directors of a cell-mediated immune response. The activation of the T cell occurs when T cell receptors recognize antigen-specific peptide sequences complexed with the appropriate MHC by an APC[3]. A diagram of the T cell activation process in Figure 1.

Figure 1 Roadmap of the activation of T cells. Antigen presenting cells identify potentially infectious foreign bodies from the antigens that the pathogens produce. APC's destroy the source of the antigen, binds the antigen with the major histocompatibility complex, and presents the complex on the APC surface. The T cell with receptor specific to the antigen will undergo engage with the MHC/antigen complex, initiating T cell activation.

T cells are categorized in various subclasses by the surface molecules they express. Once activated, CD4+ T cells, also conventionally called helper T cells, proliferate and stimulate B lymphocytes. B cells manufacture antibodies against the pathogen. When soluble antibodies decorate the surface of the pathogen or infected cells, macrophages respond by eliminating labeled cells. In the later stages of immune activity, a few helper T cells differentiate into memory T cells. CD8+ T cells, also known as cytolytic T lymphocytes (CTLs) identify and kill those cells infected with pathogens. In the situation for viral infection, where viruses can multiply rapidly, CTLs can eliminate infected cells earlier than macrophages. The two subpopulations of T cells recognize different sets of MHC molecules. CTL T cell receptors bind to MHC class I molecules, which are expressed by all nucleated cells, while CD4+ T cells recognize MHC class II molecules presented on APC surfaces[1].

2.1.2 Immunological synapse. At the onset of an adaptive immune response, an immunological synapse is formed between antigen presenting cells and naive T lymphocytes[3]. The binding of an antigen on the MHC complex to a T cell receptor (TCR) triggers the activation of the T cell response through cell signaling pathways. Immediate responses from cell signaling include the activation of naive CD8+ T cells into CTLs. However, further proliferation and differentiation of T cells require longer engagements between TCR's and antigen-MHC complexes[1].

Although coupling between the TCR and its ligand pair are critical for T cell activation, physicochemical barriers reduce the stability of TCR-ligand complexes. Examples of these barriers include 1) the small sizes of the TCR and antigen molecules, 2) steric hindrance of neighboring glycoproteins, 3) the presentation of only a few MHC-complexes on APC cells, and 4) movement of T cells[3]. Adhesion molecules, present on the surfaces of both cells, are critical in stabilizing the TCR complex.

After the initial binding of TCR and the respective presented antigen on the APC, the adhesion molecules anchor the T cell onto the APC cell. During the first interactions, clusters of adhesion molecules (LFA-1 and ICAM-1) are surrounded by antigen-MHC-TCR complexes, forming the premature synapse. Once a peptide is recognized, the peptide-MHC (p-MHC) complexes bound to TCR's are transported into the center of the synapse; whereas, the adhesion molecules diffuse out to form an outer ring. The resultant mature synapse structure aids in reducing the effects of physical barriers of maintaining a synapse (Figure 2a and b)[3,4].

Figure 2 The immunological synapse: (a) a molecular diagram of the localization of various complexes of a mature synapse. LFA-1-ICAM-1 adhesion molecules holds the formation intact to permit sustained binding of TCR to p-MHC can occur. It is hypothesized that this synapse acts as a channel for cytokines and other soluble molecules to pass between the APC and T cell. (Image provided by J. Doh.); (b) formation of the immunological synapse. plain view fluorescence microscopy of the interface of a T cell interacting with a lipid bilayer model of the APC surface. Adhesion molecules are depicted in red; TCR-p-MHC's are shown in green. Although the TCR-p-MHC's initially surround a central cluster of adhesion proteins, both TCR-p-MHC's and adhesion proteins diffuse to form the structure of a mature immunological synapse after hours of engagement[4].

2.1.3 APC cytokine production. Cytokines are a common mode of communication

between all cells. An assortment of cytokines is involved in differentiation and proliferation of

cells. Initially immunologists and molecular biologists hypothesized the synapse was a structure

required to promote engagement of the TCR-antigen complex. However, studies have shown that

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the synapse is redundant for initial T-cell activation[5], and exogeneous peptide-MHC molecules

are sufficient for binding TCRs and initializing cell signaling pathways for activation.

One theory that has developed from the discovery of APC cytokine production is that the

immunological synapse serves as a channel for the passing of soluble molecules, from the APC

to the T cell, which localizes the concentration of cytokines to specific cytokine receptors on the

target T cells. Such a structure would prevent undesirable signaling from cytokines binding to

"spectator" T cells in the vicinity of the presenting APCs.

Given that cytokines are critical in the development of naïve T cells into effector T cells,

cytokines may play a significant role in the initial engagement of professional APC's to T cells

and activation of immune responses. Along with the understanding of the function of cytokines

in the initial signaling response to T cells, the external stimulation of T cells via the introduction

of exogenous cytokines could enhance the success of external immune response activation.

Using advances in tagged-antibody imaging and immunometric assays quantifying bulk

concentration of secreted analytes, observations of molecular dynamics on cell surfaces have

elucidated many mechanisms of T cell activation. However, these immunological tools are

insufficient to detect localized concentrations of secreted cytokines inside and outside of the

immunological synapse. In response to short-comings of current assays and imaging reagents,

this paper presents a technology that is designed to "capture" and "detect" soluble cytokines at T

cell surfaces at the onset of T cell activation.

The cytokine-capture detection device serves as a mobile substrate that is conjugated with

antibodies for the desired antigen or antigens and is bound onto T cell surfaces. Once the

nanodevice is in place, any cytokines that may pass through the synapse following T cell

stimulation by APCs are captured onto the fluorescent substrate and are detected by a second monoclonal antibody labeled with a fluorochrome. The activated T cells are visualized using fluorescent microscopy to observe the distribution of beads and cytokines. Flow cytometry may also be utilized to quantify the population of T cells that have been activated.

2.2 Immunoassays

Since their initial development in the 1960s, immunoassays have provided researchers the tools to investigate cellular dynamics by targeting proteins and other cellular molecules with great specificity. The technology of cytokine detection originates from these fundamental immunoassay reagents and procedures; although the technology exceeds the detection capabilities of current immunoassay reagents. In order to more effectively present the new functions of the cytokine-capture technology, the next section will provide an overview of related technologies, placing greater emphasis on the tools that are employed in cytokine-capture technology.

2.2.1 General overview of immunoassays. Antibody-fluorescence tagging is a subset of immunometric assays, which is included in the larger umbrella of immunoassays. The functionality of immunoassays is dependent on an antibody's binding specificity for an antigen. Immunoassays exploit the sensitivity, specificity, and avidity of antigen-antibody interactions. Antibody proteins have two binding pockets targeted for the appropriate analyte, as shown in Figure 3. The structure of an antigen typically contains multiple binding sites specific for an antibody. When an antigen and antibody come together and bind, multiple non-covalent bonds form[6].

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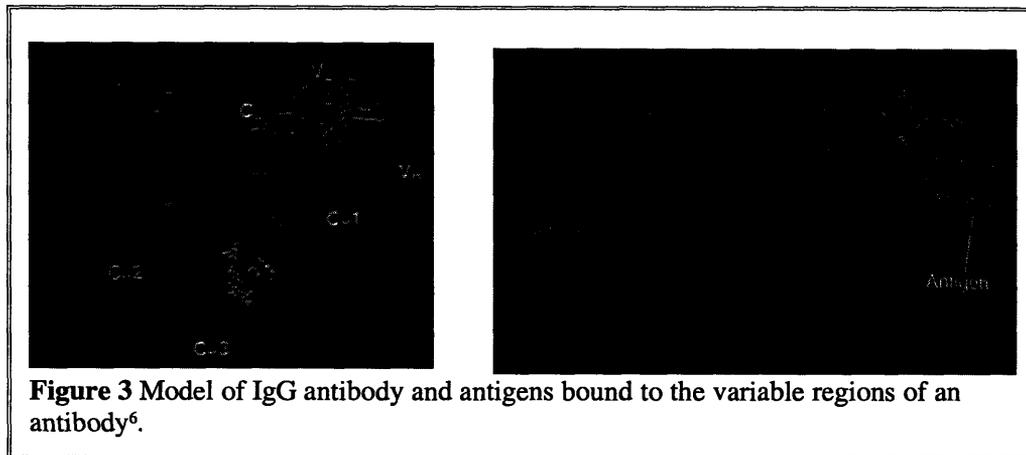
Figure 3 Model of IgG antibody and antigens bound to the variable regions of an antibody[6].

Figure 4 is a schematic of a simple immunoassay composed of a bound antibody onto a solid substrate and a secondary monoclonal antibody conjugated to any number of labels such as an enzyme of fluorescent dye. The sample is introduced to the solid substrate for an incubation period. Following incubation, the secondary antibody is added to detect any captured antigen. After a quick wash, the amount of bound antigen is quantified by detecting labeled reagents.

The affinity of the antibody probe can be as low as picomolar concentrations
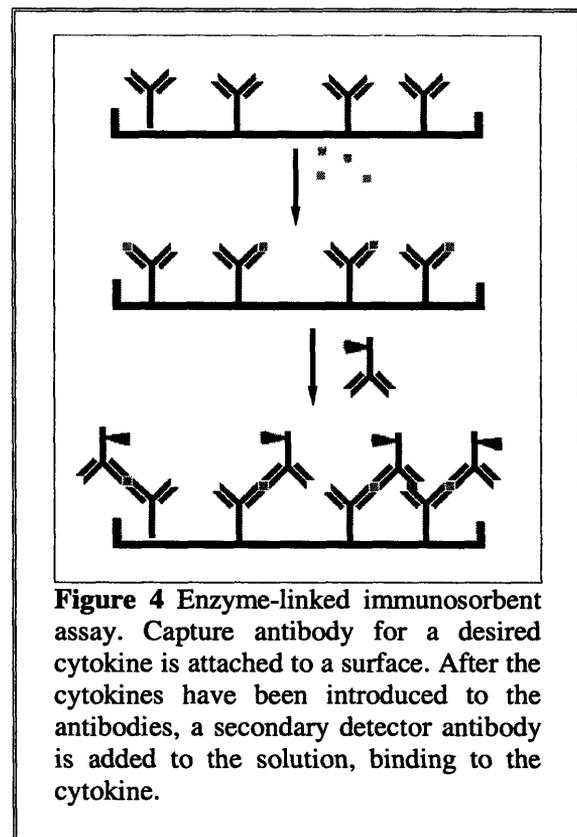
Figure 4 Enzyme-linked immunosorbent assay. Capture antibody for a desired cytokine is attached to a surface. After the cytokines have been introduced to the antibodies, a secondary detector antibody is added to the solution, binding to the cytokine.

[$K_D \sim 10^{-12}$ M]. Further experiments have indicated that the antibodies retain specificity even at such low concentrations[6]. For these reasons, immunoassays have been a valuable tool to probe

basic molecular biological problems, as well as serve as medical diagnostic assays to quantify the presence and quantity of analytes of interest.

2.2.2 Monoclonal vs. Polyclonal antibodies. The development of monoclonal antibodies has tremendously broadened the capabilities of antigen detection. Unlike polyclonal antibodies, which are heterogeneous pools of antibodies that bind to multiple sites called epitopes, monoclonal antibodies target a specific epitope on an antigen. These epitope sites are often spaced sufficiently apart to accept binding of more than one monoclonal antibody at once. Only within these conditions is it feasible to capture an antigen with one antibody and detect with a secondary antibody[1,6]. However, when employing antibodies for targeting surface protein on a heterogeneous cell population, it is critical to take care selecting suitable antibodies due to variations in antigen-antibody binding affinities.

2.2.3 Antibody labels. A wide variety of labeling methods are available for designing an immunoassay. The first detection method used was radioisotope labeling, e.g., iodine-125. However, concerns regarding exposure to radiolabels and the inherent instability of radioactive materials have promoted the development of alternative methods using non-radioactive labels. Examples include chemiluminescence, enzymes, streptavidin/avidin-biotin, microparticles, and fluorescence[6].

The preferred method of detection for the cytokine-capture nanoparticles is fluorescence labeling for several reasons. First, both quantitative and qualitative detection methods are available for fluorescent markers. The versatility of detection methods allows a small sample of cells to be imaged using fluorescent microscopy and a larger population of cells of the same

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sample can be analyzed and sorted using flow cytometry. Another benefit of choosing fluorescent dyes as labels is the flexibility to use multiple wavelength channels for simultaneous detection. The ability to tracking more than one labeled analyte is useful when the target antigen location can be compared to reference molecules.

When imaging eukarayotic cells with fluorescent microscopy, there is greater autofluorescence in the green channel than for longer wavelengths in eukarayotic cells[7]. Therefore, the red or far-red channels should be designated for the most sensitive detection needs. This is even more pertinent for single molecule detection where the signal is only detected from a single fluorescent source.

Another important consideration when designing a multi-fluorescent experiment is that commonly red fluorescent molecules can "bleed" through into the green channel upon excitation. Red fluorescent proteins are synthesized by coupling two green fluorescent proteins. Protein folding, or maturation, is critical for proper fluorescence behavior, and the maturation of red requires another step from green fluorescent protein folding. Incomplete maturation results in bleed through into the green fluorescent channel. The red bleed through into green effectively eliminates the use of the green channel in an parallel fluorescent experiement[7].

One severe limitation of fluorescent organic molecules is their poor photostability. Despite the large signal to noise ratios for organic fluorescent molecules, photoactive molecules become "quenched" rapidly. Often times, there is only sufficient fluorescence for single use excitation. For flow cytometry analysis, the quenching phenomenon is not a significant concern, because only a single strong signal is necessary for detection. However, the photobleaching behavior limits the use of fluorochromes as single molecular tracking beacons to observe cellular

dynamics in real time. There are new engineered fluorescent products available with dramatically longer excitation lifespans. These new probes will be discussed further in later sections.

2.2.4 4D Fluorescent Imaging techniques. Once cellular components are fluorescent labeled, there are several options for visualizing fluorescence within the cell. For the purposes of tracking fluorescent reagents labeling internal and surface molecules, 4D microscopy is the imaging equipment of choice. With 4D imaging microscopy techniques, researchers have the tools to image 3D cross-sections of fixed and live cells over long periods of time. Once captured, the 2D images can be spatially reconstructed to simulate a 3D model of the cell or object of interest. As cells may be imaged over a few hours to multiple days, a proper environment must be established to maintain cell viability (temperature, humidity, CO_2 concentration, etc.)[8].

2.3 Quantum dots.

As an alternative to organic fluorescent dyes, a new fluorescent marker has begun to enter into biological assays. Quantum dots were identified as a candidate for stable biolabels in 1998 by both Alivisatos group at University of California, Berkeley[9] and Nie group at Indiana University[10]. Quantum dots are semiconductor nanocrystals, clusters of a few thundered to a few thousand atoms, commonly spherical in shape, with well-ordered crystal structures[11].

Due to quantum dots' small dimensions of a few nanometers, which is within the order of deBroglie wavelength, the energy states of free electron carriers are quantized. Similar to the particle-in-a-box scenario, the allowable energy levels are determined by the size of the nanocrystal. The semiconductor material of the quantum dots is chosen to create an appropriate band gap so that an electron can be stimulated to jump the gap with light of a certain energy. If an

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electron is excited to a higher electron state, e.g. by light, when the electron falls back down to its ground state, a photon with shorter wavelength is emitted. The smaller the quantum crystal, the larger the gap between energy levels. For a smaller crystal, a photon of shorter wavelength is emitted[11].

2.3.1 Biological application. Quantum dots for biological labeling purposes have been prepared with a CdSe core semiconductor with a ZnS shell. ZnS is a higher bandgap material that confines the excited energy within the nanocrystal, improving quantum conversion into a fluorescent signal. Quantum conversions up to 70-80% have been achieved to date. These photoactive semiconductors have the advantages of 1) greatly improved photostability as compared to conventional fluorescent molecules[12], 2) narrow emission range, 3) controllable emission range for the same excitation wavelength by altering nanocrystal size[13,14], and 4) comparable or slightly lower quantum yield to organic dyes with a larger absorption range[12,13].

Sukhanova, et. al. have published the duration of fluorescence for quantum dots and a few commonly used organic fluorescent dyes. AlexaFluor® 488 molecule, the most stable organic dye available, has a half-life of 4.3 min. Standard fluorochomes, such as R-PE and FITC, show half-lives of 0.7 min and 0.4 min, respectively. In comparison, nanocrystals are found to have half-lives of over 20 hours[13]. The longevity of quantum dot fluorescence is dependent on the quantum yields, which is a function of thickness of ZnS shell and other post-processing modifications.

The emission spectrum of quantum dots are symmetrical and shown to be 23-35 nm fullwidth-half max[12]. If a conservative estimate of 40 nm is given for a single bandwidth, the number of distinctive channels available for fluorescence detection of quantum dots would be at

least 5 wavelengths, e.g. 500 nm, 540 nm, 580 nm, 620 nm, and 660 nm[15].

2.3.2 Technical barriers. In order to successfully use quantum dots as biolabels, general problems needed to be addressed: 1) the intrinsically hydrophobic nature of the nanocrystals' surfaces after synthesis, which leads to aggregation and nonspecific binding in aqueous solutions [12,13,14] and 2) conjugated with desired biomolecules without loss of quantum yield or biological function[13,14].

A significant technological barrier for the application of quantum dots as biological markers was their hydrophobic surface after synthesis. In order to prevent hydrophobic aggregation in water, a common problem of biomaterials, surface modifications were needed to make the dots soluble and independently mobile in aqueous solutions.

Much research has been directed towards creating a hydrophilic coating. Methods include silanization, mercaptocarbonic acid treatment, polymer deposition and protein adsorption. Reacting mercaptocarbonic acid to the ZnS shell yields thiol-ZnS linkages and a hydrophilic final layer. However, the thiol-ZnS bonds are dynamic, and over time the treated nanoparticle is stripped of its hydrophilic coating for QDs. A mercaptocarbonic acid with two thiol groups improves the stability of the mercaptocarbonic layer. Another surface modification approach involves silanization of the nanocrystal. Mercapto-trimethoxysilane is bound to the ZnS outer shell, and the silane groups are crosslinked together. Both chemical treatments have the disadvantage of pH sensitivity[12]. To prevent precipitation of quantum dots, coatings with exposed ionic groups or a hydrophilic polymer are desired, particularly in neutral H_2O.

2.3.3 Cell studies. In one study, quantum dots, encapsulated with a lipid vesicle, were

injected into embryonic Xenopus cells (2 x 10^9 QD/cell) and tracked through in vivo imaging.

Quantum dot fluorescence was observed up to the late tadpole stage, although there was a

significant reduction in signal after the tailbud stage. Fluorescence was also detected in the

progeny of the originally labeled embryonic cells, indicating that quantum dots do not inhibit

normal cellular growth and replication[14].

Quantum Dot Corporation has successfully conjugated these semi-conductors with streptavidin, which opens up the opportunity to use quantum dots as nanodetectors for biological processes. Wu, et. al., from Quantum Dot Corporation, demonstrated specific labeling of cell surface molecules and nuclear proteins in Sk-BR-3 cancer cells using quantum dots. The researchers targeted Her2 protein on the Sk-BR-3 cancer cells with anti-Her2 antibodies, which were detected by
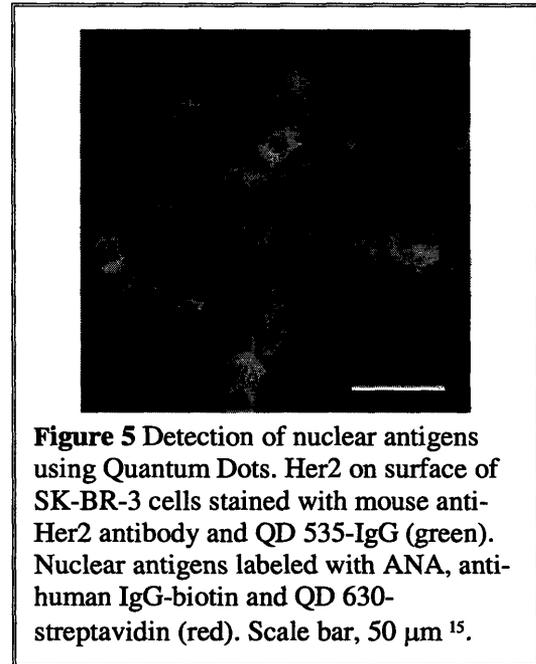
Figure 5 Detection of nuclear antigens using Quantum Dots. Her2 on surface of SK-BR-3 cells stained with mouse anti-Her2 antibody and QD 535-IgG (green). Nuclear antigens labeled with ANA, anti-human IgG-biotin and QD 630-streptavidin (red). Scale bar, 50 μm [15].

QD-535 (535 nm emission) conjugated to IgG protein. Furthermore, the authors ran an

experiment, simultaneously targeting Her2 cell surface molecules and nuclear proteins using

QD630 conjugated to streptavidin. In Figure 5, fluorescence from the red and green quantum dots

are located in the correct regions of the cells[15] . These experiments confirm the detection

application for surface and intercellular molecules; however, the intensity of the signal is

attributed from a collection of quantum dots, not single dots. Further research by Dahan, et. al.,

has demonstrated that single quantum dots can be resolved using microscopy, thus opening the

application of quantum dots for single molecule tracking methods.

2.4 Significance of Technology (Health Implications)

The immune system is equipped to identify which cells are self and to eliminate those that are foreign and infected; failure of the immune system activities is detrimental to maintaining proper health. Those pathogens which are successful have developed mechanisms to avoid detection and to *thwart* the activity of the immune system. Two well-publicized immune disabilitating illnesses will be discussed here: cancer and HIV.

2.4.1 Tumors. Although tumor cells originated from normal, healthy cells, slight deviations in normal antigens presented on the MHC molecules identify the cell as non-self. These antigens can arise from a mutated protein, overexpressed protein, product of an oncogene, or an oncogenic virus. CTLs activated against a specific antigen are sufficient for tumor cell elimination. However, in order to stimulate naive $CD8^+$ cells, signals for $CD4^+$ T cells and APCs are required. Therefore, similar to bacterial or viral antigen detection, APCs must digest tumor cells and display antigen complex on its Class I and Class II MHC molecules, as well as costimulating molecules to serve as secondary signals. The APC then can stimulate both $CD4^+$ T cells and $CD8^+$ naïve cells. Through this mechanism, a cancer patient's own immune system can work towards combating tumors[1].

Controlling tumor growth is the most effective at the early onset of unnatural cell growth. However, tumors often overwhelm the immune system due to their rapid expansion. In addition to replicating at a higher frequency than that at which CTLs can destroy malignant cells, tumors also have methods of evading antigen recognition. For example, the tumor antigens may deviate only slightly from antigens of healthy, normal cells, which elicits a weak immune response.

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There are also some tumors, called antigen loss variants, which occasionally stop presenting antigens or displaying Class I MHC molecules. Others secrete growth factors, such as TGF-β, that impede immune responses.

Current treatments for cancer, e.g. chemotherapy and radiation, damage malignant cells and healthy cells indiscriminately. New cancer research focuses on immunotherapies to treat cancer sites with higher specificity. One concept for a cure is to create a vaccine from patient's own tumor cells or antigens, such as heat shock protein vaccines. When the vaccine is exposed to the patient, the patient's immune system would process vaccine antigens and generate an immune response against the antigens of the tumor. Instead of introducing agents for APCs to process and present, another treatment approach is to expand a patient's dendritic cells and to expose them to cancer alleles or antigens in vitro. Once the expansion is complete, the APCs are reinjected into the body, ready to activate T cells[1].

2.4.2 HIV. With the rising numbers of cases of immune deficiency diseases, the most notable of which is acquired immunodeficiency syndrome (AIDS), there is continual research geared towards finding an effective cure to increase the life-expectancy of afflicted patients. However, the difficulties associated with targeting viruses for eradication, or developing a vaccine, have launched research towards producing novel therapies.

In the case for immunosuppressed patients, such as those diagnosed with human immunodeficiency virus (HIV), survival is dependent on the activity of virus-specific immune response. HIV targets activated CD4+ T cells, although APCs and other immunological cells are also infected to serve as viral reservoirs. As the virus actively synthesizes viral protein and genetic material, it destroys the host cell[1]. Peripheral blood CD4+ T cell levels have been found to
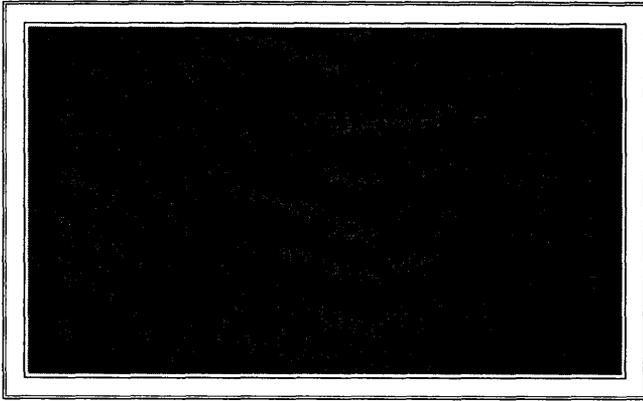
decline from normal counts of 1500 cells/mm^3 to 200/mm^3 in unhealthy patients[16]. The chart in Figure 6 tracks the viral load and CD4 T cell counts through the progression of a diseased patient. The killing of APCs and other cells causes additional damage to lymphoid organs. The combined effect of the architectural destruction and helper T cell death results in a severely compromised immune system.

Once the immune system is weakened, the patient's condition deteriorates into AIDS. However, there is evidence in the literature that supports the effectiveness of cell-mediated immunity in maintaining HIV nonprogressor status. CTLs are often found to be defective in HIV patients, even though the virus does not actively infect and destroy killer cells. The presence of active CTLs, specific towards HIV, is critical for controlling the virus[17]. Although CD8+ T cells can identify and kill infected cells once matured, CD4+ T cells assist with naïve CD8+ differentiation into CTLs. As CD4+ numbers diminish as the viral infection progresses, the CTLs are not properly activated into their cytotoxic state[1]. With proper stimulus of these T cells, it is hoped that the patient's own immune response could effectively fight off the disease. In order to engineer activation of T lymphocytes, a more thorough comprehension behind the mechanism of T cell activation must be achieved. The cytokine capture technology described here can shed new light on the role of soluble signals in T cell activation, which are believed to be important in programming T cell responses against HIV.

3. Approach (Cytokine-Capture Detection Technology)

Although labeling capabilities of proteins, DNA, and other biomolecules with organic fluorescent dyes have elucidated cellular mechanisms unable to be detected with bright field light-microscopy, little progress has been made on the study of cytokines' role in T cell activation. If cells secreted sufficient amounts of cytokine into the surrounding media, the protein can be detected through various bulk cytokine assays. However, cellular behavior, such synapse formation and auto-stimulation, indicate that smaller concentrations of highly localized cytokines are released by cells to achieve more sensitive intracellular communication. The cytokine capture approach aims to detect and to quantify local cyokine concentrations, or any soluble signaling molecule, on cell surfaces.

The mechanism of cytokine capture is similar to that of ELISA (Enzyme-linked immunosorbent assay, Figure 4), localized to cell surfaces. In order to determine the spatial distributions of cytokines (in particular IL-2, IL-12) within and outside the vicinity of the immunological synapse, fluorescent nanoparticle substrates conjugated with anti-cytokine antibodies will be positioned on T cell surfaces to form the cytokine capture-device.

To attach these nanodetectors to T cells using biotin/avidin binding, the cells are biotinylated, which allows for tight binding with the avidin-related proteins on the nanosphere surface. Biotin-N-Hydroxy succinimide molecules form a covalent bond with free amines on the cell surface, which should result in an even distribution of biotin sites over the surface of the cell. Once the nanoparticles are attached to the T cells, APC cells are stimulated to form a synapse with proximal T cells. Once cytokines are produced and secreted from the APC cells, the spheres will

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Naïve T cell

1. Biotinylation

Biotin

2. Attachment of particles

3. Introduction of APC

Neutravidin

Anti-cytokine antibody

Far-red spheres (660/680)

Dectector Anti-cytokine antibody-PE

Red fluorescence

4. Stimulation with pathogen

Far-red fluorescence

6. 3D Time-lapse fluorescent microscopy

5. Addition of detector anti-cytokine antibody

Figure 7 Schematic for Developing Cytokine Technology.

be located inside and/or outside the synapse to "capture" any cytokines present in the near vicinity. Figure 7 is a graphical representation of the proposed cytokine-capture mechanism. After a designated time, the synapse is frozen by a cross-linking reagent, and a second fluorochrome-labeled (phycoerthrin [PE], excitation/emission: 575/585 nm) detector anti-cytokine antibody is introduced to the system to bind and "read out" to any cytokines captured by the nanospheres.

The substrate of choice for the local cytokine detection assay is either fluorochromes encapsulated within polystyrene nanoparticles or quantum dots. The latter is preferable due to the minimal amount of nonspecific binding with non-polar molecules. In both examples of fluorescent nanoparticle labels, the fluorescence generated is considerably more stable than that of a single fluorochrome. The fluorescence emitted by a stimulated fluorochrome is initially

strong and easily detectable; however the signal quickly quenches over only a few exposures

(under half a minute)[13]. The signal stability of the polystyrene bead loaded with fluorochromes or

quantum dots allows for tracking molecules for many hours using intermittent exposures without

any significant signal decay.

3.1 FluoSpheres

Polystyrene spheres loaded with far-red fluorescent dyes (diameter: 20 nm,

excitation/emission: 660 nm/680 nm) have the benefit of sustaining fluorescent signal integrity,

as compared to single fluorochromes. Far red spheres were chosen, because cellular

autofluorescence is the weakest for red wavelengths or longer. Also, far-red spheres do not

exhibit bleed through into the green channel, unlike red fluorescent organic molecules.

FluoSpheres can be attached to specific cell-surface molecules or distributed evenly over

the cell surface by randomly biotinylating the surface of the cell. Biotinylation of free amine

groups on cell surface allows for attaching avidin-conjugated nanospheres to any cell. Each

sphere is bound to multiple biotin molecules. If one set of binding pair were to disengage, other

binding pairs would keep the nanodetector attached to the cell surface. Because more

complementary molecules are close in vicinity, another binding event is likely to occur, thereby

stabilizing attachment in a coordinated binding effort. However, there is a concern that the

binding of biotin molecules, linked to any surface proteins with free amine groups, could

unintentionally trigger signaling pathways or disrupt normal cellular function. This possibility

can be avoided by tagging specific molecules with antibodies as shown in Figure 8. Furthermore,

if surface molecules were selected carefully, the investigator has the additional advantage of

knowing if the spheres are being shuttled into or out of the synapse. Carboxylate functional

groups on the nanospheres are primed to attach antibodies, e.g. anti-CD4 antibody, Neutravidin or Streptavidin, onto the spheres.

3.2 Quantum dots.

For the application of quantum dots in the cytokine-detector system, a biotinylated antibody for a desired surface protein is incubated with the cell culture. After a wash to remove unbound antibody, the streptavidin conjugated quantum dots are introduced to the system and are allowed to bind to the biotinylated groups. Another biotinylated monoclonal antibody, this time targeted for the cytokine of interest, is added to the cell suspension. The second antibody would then bind to the remaining free streptavidin sites, and then T cells are ready to be activated. Figure 8 demonstrates the method of assembling quantum dot nanodetection system for cytokine detection.

Figure 8 Schematic for quantum dots cytokine-capture technology.
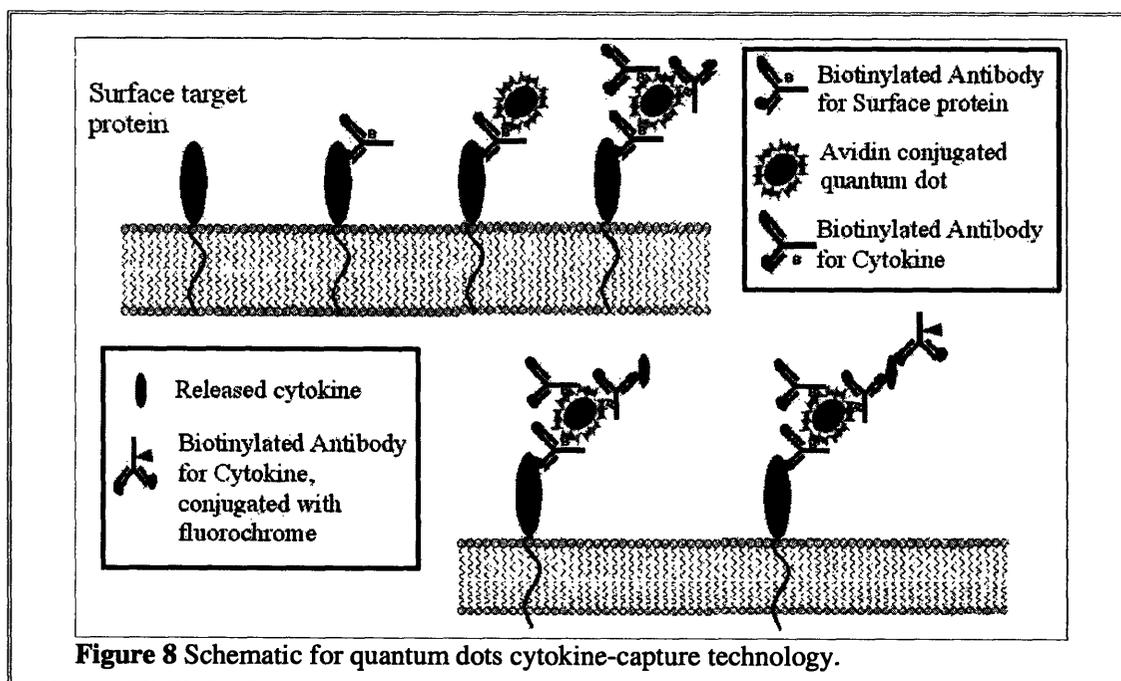
3.3 Applications of nanoparticle capture technology: T cell monitoring

One potential application of the cytokine-capture technology is in diagnostic monitoring

of antigen specific T cells. As detailed in a previous section, the immune response begins when a

T cell recognizes a presented antigenic peptide-MHC on an APC surface. The binding triggers an

array of cellular pathways, which results in differentiation and triggering of the T cell defensive

actions. The minimum requirement for a T cell response is the peptide-MHC (p-MHC)

complex[18]. Without the requirement of APC's to activate T cells, soluble p-MHC offers the

possibility of activitating a patient's immune system in the form of a fusion antigen-presenting

vaccine. In addition to the activating capabilities of p-MHC, these molecules could serve as

markers for activated T cells or antigenic-specific T cells.

Low avidity between the peptide-MHC and TCR has prevented the use of monomeric

soluble p-MHC molecules in specific T cell detection. Studies have shown that at room

temperature, the half-life of the p-MHC-TCR complex is around 10-25 s with affinities ranging

from 10^{-4} to 10^{-7} M[19]. However, these times do not offer a long enough window to utilize soluble

p-MHC as T cell labels. One solution to this deficiency is to design a detector structure that

increases the effective affinity (avidity) for detection purposes.

3.3.1 Tetramer structure. To label T cells bearing a given TCR, a technology known as

tetramer staining was developed. The tetramer technology does not deviate far from more

conventional cellular protein labeling techniques, where a cell surface protein is tagged with a

fluorescent marker. In place of singular antigenic peptide-MHC complex bound to a fluorescence

label, the p-MHC complex is manipulated to form a cluster where multiple (n>2) antigens are

assembled into a supramolecular structure. The initial design was to biotinylate the p-MHC

Figure 9 Diagram of tetramer technology
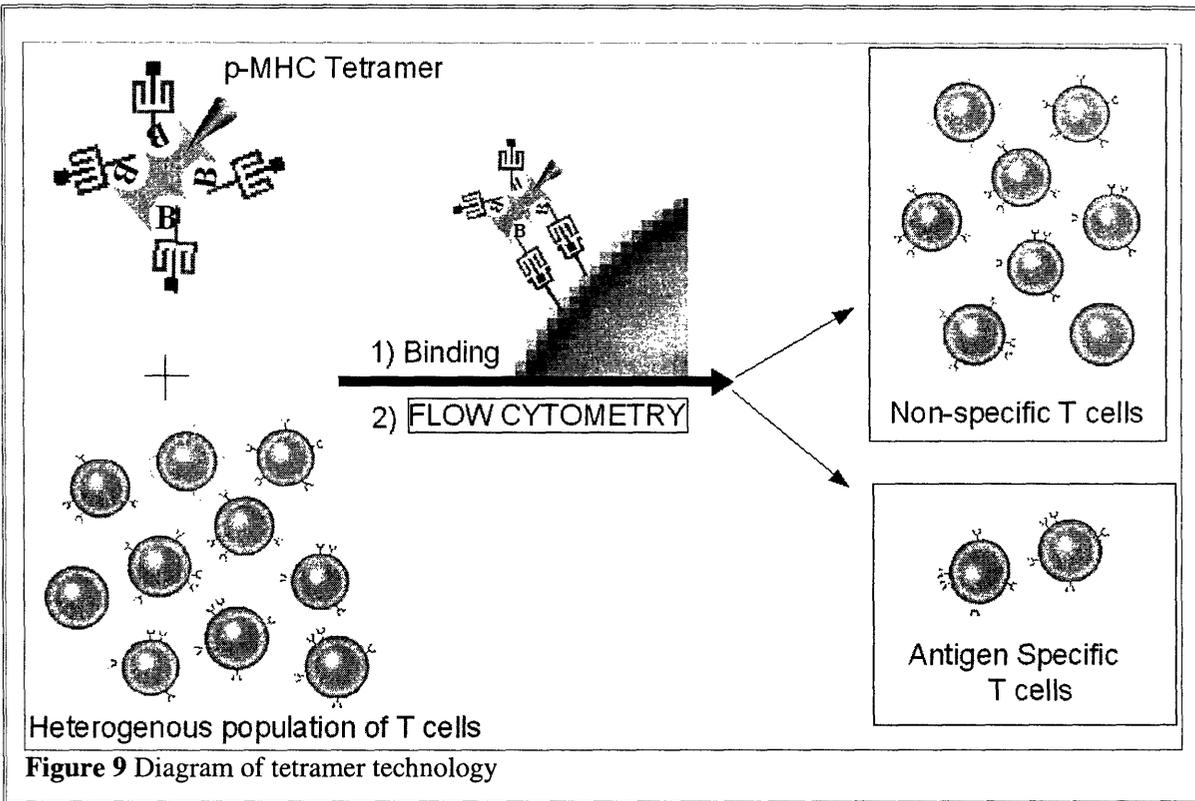
complexes and incubate the antigens with streptavidin at saturating concentrations. Each streptavidin molecule has found binding sites for biotin, creating a 4-mer, or tetramer, structure[19]. Figure 9 Other variations of antigen-peptide assembly have been proposed, such as forming dimers from two binding sites on Ig molecules or multimers on polymeric surfaces.

3.3.2 Technical complications. A few technological barriers for tetramer application include the range of avidity for polyclonal populations and low frequency of activated T cells in peripheral blood samples. Given a polyclonal population of T cells, the avidity of p-MHC varies for the spectrum of TCRs. The range of avidity for CD4+ T cells has been cited for a variety of target diseases. The antigen for influenza virus, anti-hemaggluttinins (HA), bind with relatively high affinity, as almost all T cells that exhibit viral response were tagged by HA-tetramers. In

contrast, anti-glutamic acid decarboxylase (GAD), the antigen for autoimmune diabetes, fails to bind to most TCRs due to low avidity. A general trend for human autoimmune antigens is to tend towards low avidity[18].

Besides the concern of avidity for polyclonal populations, another technological concern is the low frequency of activated T cells (CD8+ or CD4+) in peripheral blood. For an antiviral response, activated CD8+ T cells undergo a large "burst size" to concentrations of 1 in 100 to 1 in 1000 T cells. This frequency response is large enough to confidently detect antigenic-specific T cells. Fluorescent detecting thresholds tend to be in the order of magnitude of 1 in 10,000[18].

The frequency of CD4+ T cells is orders of magnitude lower, often times at the limits of the detection capabilities of flow cytometers. Investigators employ tools to amplify pre-labeled CD4+ T cell samples in vitro to achieve frequencies greater than 1 in 10,000 to 1 in 100,000 estimated of numbers in vivo. One method is to incubate the peripheral blood lymphocyte (PBL) sample with antigens to induce proliferation of the cells of interest, such as the case for tetanus or the influenza virus. If further expansion stimulus is required, the T cells are restimulated with specific p-MHC. This is the technique used for autoimmune diabetes or relapsing polychroidritis[18]. However, Class II molecules have the added difficulty of ranging in solubility and stability, resulting in differences in binding activation energies. Accessory structures (e.g. Ig domains or leucine-zippers) can aid in controlling the range of avidity[20].

By expanding T cell PBL samples, antigen positive CD4+ cells can be identified using tetramers and flow cytometry. However, the data cannot be used to infer in vivo responses due to in vitro manipulations. For example, each in vitro expansion is difficult to control and to replicate conditions. By coupling the expansion data with another cellular measurement, the investigators can obtain some qualified information of what is occurring in vivo. For example,

carboxy fluorescence diacetate succinimidyl ester is a dye that is used to quantify the number of cell divisions during in vitro expansion.

Additional complications arise when applying the use of tetramers for clinical tests. Besides selecting an appropriate peptide to activate a heterogeneous T cell population, the peptide must be functional and effective over heterogeneous patient populations. For example, even within the human HLA Class I tetramers, HLA-A2 induces a response with specificity prevalent around the world. On the contrary, HLA-B requires the careful selection of compatible subjects. For the case of HLA Class I tetramers, over 200 epitopes for HLA-DR exist and over 12 HLA-peptides are expressed in high frequency in the world population[18].

3.3.3 Patient T cell screening. A very promising application for multimers is patient monitoring of diseases by recording of T cell counts throughout the progression of a disease. This monitoring could be applied to diseases such as autoimmune diabetes, multiple sclerosis, and rheumatoid arthritis. Often case, only one or two class II alleles are expressed by a heterogeneous population. e.g. In Type I diabetes, three multimers can cover approximately 80% of the patients. For the other potential application of tetramers in vaccine screening, either patients must be pre-screened for subset of population with the same subset of HLA-peptide, or characterize and assemble a large 'library' of HLA-multimers.

3.3.4 Application of QD cytokine capture system to T cell monitoring. Here the author proposes incorporating p-MHC-conjugated Quantum Dots into patient T cell monitoring technology. The assembly of the quantum dot multimers would be a modified protocol of the cytokine-capture quantum dots synthesis. Biotinylated p-MHC complexes and anti-cytokine

antibody would be incubated with streptavidin-coated quantum dots. After a purification step, the quantum dot multimer would be introduced to harvested patient's peripheral blood lymphocytes (PBL).

Current multimers use conventional fluorescing probes, which have the limited capacity of a one-time-only detection. Although, a single excitation is all that is necessary to characterize and to sort antigenic-specific T cells, the photostability of quantum dots would make multiple screenings of T cells possible. As a result, the same sampling of T cells could be visualized using microscopy techniques, and the photoactive material would still retain enough photolumination properties for flow cytometry analysis.

Tetramer technology attempts to capitalize on T cell proliferation, which occurs at the onset of immune response. Despite the increase in a specific T cell clone population, the actual numbers of CD4$^+$ and CD8$^+$ are few. Due to the combination with the low frequency of T cells and low affinity of binding between p-MHC molecules and TCRs, it is imperative that when a multimer interacts with a presenting T cell, binding occurs and is sustained until detection.

In addition to the benefits of enhanced photostability, larger multimers could be assembled on a quantum dot surface. Similar to the cooperative binding of two p-MHC molecules in the case for tetramers, the quantum dots surface could present more p-MHC molecules to a given region on the T cell surface. Just as tetramers had increased the detection sensitivity from single p-MHC detector molecules, quantum dots coated with p-MHC soluble molecules would bind "more tightly" as the p-MHC-TCR density increases. As TCRs cluster upon T cell activation, the quantum dot would effectively cross-link the TCRs.

If the observed frequency of T cells with the desired TCR clone could be increased significantly above flow cytometry sensitivities thresholds, PBLs would not have to be expanded

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prior to sorting. By avoiding this additional step, researchers could study the in vivo behavior of the T cells with less concern of in vitro manipulation. Also, if the patient were to receive the sorted T cells as immunotherapy, the turn around time of harvesting cells would be abbreviated.

The quantum dots could also present antibodies for cytokines, in addition to p-MHC molecules. By introducing an additional cytokine-capture motif in the multimer design, the same sample of T cells could be screened for cytokine production. In this manner, T cells could first be sorted by receptor affinity for the p-MHC complexes and then be further separated based on functionality.

4. Materials and Methods

4.1 Nanosphere protein synthesis

4.1.1 *Single protein-attached Spheres*. All chemicals were purchased from Sigma-Aldrich and were used as received unless specified otherwise. Antibodies or avidin molecules were EDC coupled onto dark-red, carboxylated fluorescent nanospheres (FluoSpheres®, Molecular Probes, 20 nm, excitation/emission: 660/680 nm) by adding 20 μL N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) and 32 μL N-hydroxy succinimide (NHS) in 4.5 mL activation buffer (0.1 M 2-(N-Morpholino) ethanesulfonic acid (MES), 0.5 M NaCl, pH 6.0) with 250 μL of dark red nanospheres (1.8×10^{15} nanospheres/mL). The solution was rotated at 20°C for 15 minutes. The concentration of the activated nanospheres was determined by UV-VIS spectroscopy. The protein to be coupled, e.g. Neutravdin (4.2 mL, 1 mg/mL in PBS) was added to the activated spheres. The solution was vortexed and then rotated for 2 hours at room temperature in the dark. This coupling reaction was quenched by adding 100 mM glycine and rotating the solution for another

30 min in the dark. The spheres were dialyzed into PBS + 0.05% Tween 20 at 4°C (300K

MWCO dispodialyzer, Spectra Por) overnight.

4.1.2 *Multiple protein-attached Spheres.* Multiple proteins may be attached consecutively

or simultaneously. For simultaneous coupling, equal concentrations of proteins were attached to

the nanospheres. To ascertain that anti-IL2 antibody successfully had attached to the

nanospheres, fluorescein (FITC) labeled anti-IL2 antibody was first attached to Nav-attached

spheres. FITC-anti-IL2 antibody (0.05 mg) was introduced to 500 uL MES activation buffer.

Nav-dark-red FluoSpheres were added to antibody-MES solution and incubated at room

temperature for 15 minutes. After the incubation period, the spheres were activated with EDC

(0.2 mg) and incubated again at room temperature for 2 hours in the dark. The coupling reaction

was quenched by introducing glycine (0.5 mM) and incubating the sample solution for 30 min at

room temperature. Excess protein was removed by dialysis in PBS/Tween 20 solution over night.

4.2 Assay for Neutravidin-functionalized nanoparticle binding to biotin.

An aqueous solution of Poly (L)-Lysine (PLL, 60 – 80 μL, 30 mg/mL) was added to each

well of a Labtek® 8-well chambered coverglass (Nalge Nunc). The PLL solution was allowed to

sit for 2-5 min to absorb to the glass surface and then was washed with de-ionized water.

Negatively charged biotinylated poly(methyl methacrylate) spheres (dia. 415 nm, 100 μL, [10^8

spheres/mL]) were then added to the PLL surface and incubated for 5 min at 20°C (spheres

courtesy of J. Doh). Again these spheres were washed with water. Finally, 100 μL of the N_{AV} or

anti-IL2 –N_{AV} nanoparticles (~10^{11} spheres/mL) were introduced to the biotinylated beads and

incubated for 20-30 min and subsequently washed. Specific binding of the nanoparticles to the biotinylated beads was assessed by fluorescence microscopy.

4.3 Biotinylation and N_{AV} spheres loading onto fluorescently-labeled T cells

The T cell line EL4 was grown in RPMI media at pH 7.4 with 10% fetal calf serum (FCS), 100X non-essential amino acids, Penicillin (100 U/mL), Streptomycin sulfate (100 µg/mL), β mercaptoethanol (50 µM), G418 disulfate salt (320 µg/mL). The procedure for biotinylation of B and T cells was adapted from Wülfing, et al[21]. 0.5 - 0.75x10^6 cells were washed with Ringers Buffer (10 mM Hepes; 154 mM NaCl; 7.2 mM KCl; 1.8 mM $CaCl_2$; pH 7.4), centrifuged for 5 min at 300xg, and suspended at 10^6 cells/100 µL in Ringer's Buffer. In order to determine the specificity of binding of the nanodetectors onto biotinylated cells, half the cells were loaded with the fluorescent calcium indicator dye Fura-2 AM (Molecular Probes, excitation/emission: 340/380 nm). These cells were then incubated for 30 min at 37°C with 10 µL Fura-2 AM [2.5 µL/100 µL] in Ringer's Buffer. The cells were washed with Ringers Buffer to remove excess Fura-2 AM from the media.

Two different samples of T cells, which consisted of 1) biotinylated, Fura-2 AM loaded cells mixed with unlabeled cells and 2) biotinylated cells mixed with Fura-2 AM loaded cells, were prepared as an internal control to detect nonspecific binding to nonbiotinylated cells. Biotin-NHS at 20 µg biotin/100 µL cell suspension was added to the appropriately labeled cells (Fura-2 AM loaded or unlabeled cells), and the cells were subsequently incubated at 4°C for 30 min. The cells were washed with RPMI Imaging Media (5% FCS, no antibiotics), and the biotinylated cells and Fura-2 AM loaded cell suspensions we combined together, as well as the

biotinylated + Fura-2 AM loaded and plain cells. The fluorescent nanobeads (either Neutravidin

–attached, anti-IL2 –N$_{AV}$ attached or plain beads) were introduced to the cell mixtures at a range

of concentrations, and the nanobeads were allowed to incubate for 10 min at 4°C in the dark. The

cells were washed once more and suspended in RPMI Imaging Media at 10^6 cells/100 μL media.

4.4 Introduction and detection of IL2 on nanosphere-attached T cells

Anti-IL2 capture antibodies were coupled to Nav spheres using the protocol for protein

attachment in §4.1.2. Once the cytokine antibodies were attached to cells (see §4.3), IL2 was

exogenously introduced in concentrations of 50 ng/mL. IL2 cytokines were incubated for 15 min

at 4°C. After washing away excess IL2, anti-IL2 detector antibody (1-0.1x10^{-6} μg/ml) was added

to the IL2 solution and incubated for an additional 15 min at 4°C. After repeated washes, the cells

were resuspended in 10^6 cells/100 uL RPMI imaging media and transferred into Labtek® wells

for microscopy imaging.

4.5 Fluorescence Imaging

The fluorescent nanospheres and other fluorochrome-conjugated antibodies were detected

using the 3D timelapse fluorescent microscope. The cell images were obtained with a 40X oil-

immersed objective; whereas, the biotinylated spheres required a 100X lens for observations.

DIC bright-field images of all samples were first observed. Dark-red fluorescence was collected

with 660 nm excitation and 680 nm emission filters; FITC was observed using 488 nm excitation

and 530 emission filters; and PE fluorescence was excited at 565 nm and collected at 575 nm;

however, PE could also be excited weakly at 488 nm. Fluorescence exposure times ranged from

50ms to 400ms. During the timelapse imaging one picture, either DIC or dark-red, was taken

every 7 sec for 15 min. In order to observe the spheres attached over the full surface area of the

cells, Z sections were taken in 1 μm steps for either 60 μm depth or 25 steps. In color overlay of

images, the fluorescence from each channel of fluorescence or white light has been represented

by a respective color (red, blue, or green). When overlap between red and green images has

occurred, the resulting color appears yellow.

5. Experimental Results

5.1 Preliminary testing of N$_{AV}$-spheres using biotinylated spheres

Before beginning cell work with

N$_{AV}$-spheres, the spheres were incubated

with biotinylated polystyrene beads

(diameter 415 nm) to observe N$_{AV}$-

attached nanoparticle binding to biotin.

Figure 10 shows the result of microscopy

experiments for N$_{AV}$-attached spheres and

Anti-IL2-FITC N$_{AV}$-spheres. Fluorescence

corresponding to dark-red spheres is

Figure 10 Color Overlay of Nav-nanospheres (red) + anti-IL2-FITC antibody (green) on biotinylated spheres. Coordination of overlap between the fluorescent images corresponds to yellow hues in the upper left figure.
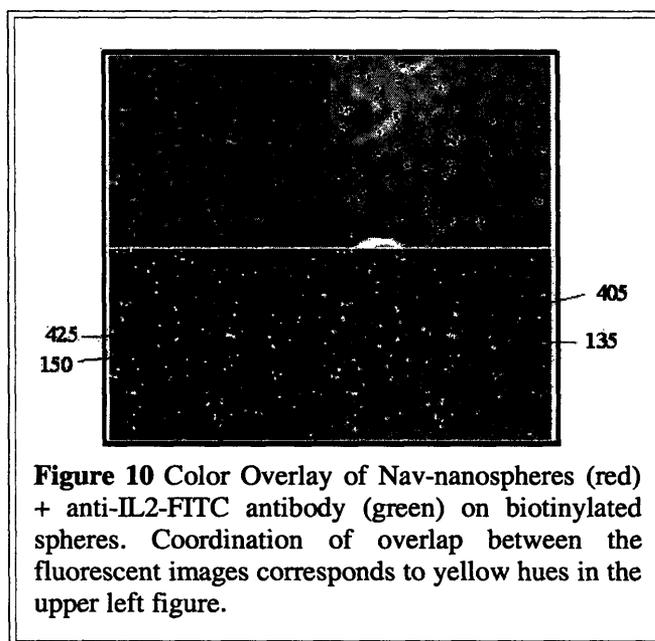
observed for all biotinylated spheres. However, PS beads appear to be dark points in the bright

field; therefore, although fluorescence is observed for nanospheres, individual detector beads

cannot be resolved. Also, the poly-lysine layer increases the non-specific binding of the spheres

to the wells due to electrostatic attraction between the positively-charged poly-lysine chains and

residual carboxylate groups on the nanoparticles. Nevertheless, because fluorescence could not

be detected from control experiments with non-biotinylated spheres or plain nanospheres (data

not shown), the alignment of dark-red fluorescence with bright field image is a result from

neutravidin-biotin coupling. The overlap of FITC fluorescence with that of far-red beads shows

that sequential protein coupling onto spheres is successful. However, fluorescence detection does

not quantify the ratio of anti-IL2 spheres to neutravidin molecules. For FITC-anti-IL2 molecule

detection, only a single conjugated protein is required for detection. As a positive control, the

number of capture antibodies is not imperative; however this is not the case for cell attachment or

capture cytokine antibodies to achieve coordinated binding.

5.2 Cell work with N_{AV}-spheres

5.2.1 N_{AV}-spheres and controls. Optimization of the sphere attachment to cells was

accomplished using Nav-attached spheres sans IL-2 capture antibody. It was found that a ratio of

1000-5000 spheres per cell yielded well-defined fluorescence for individual particles bound to

cells, as well as high contrast in signal to noise ratio. A series of z-stack image show how

particles dot the entire surface of the cell in Figure 11. Here the DIC image is held constant and

the dark-red fluorescence plane is varied. After 5 hours of incubation at 37°C, we see much more

Figure 11 Cross sections of a cell coated with Neutravidin-attached dark-red
nanospheres
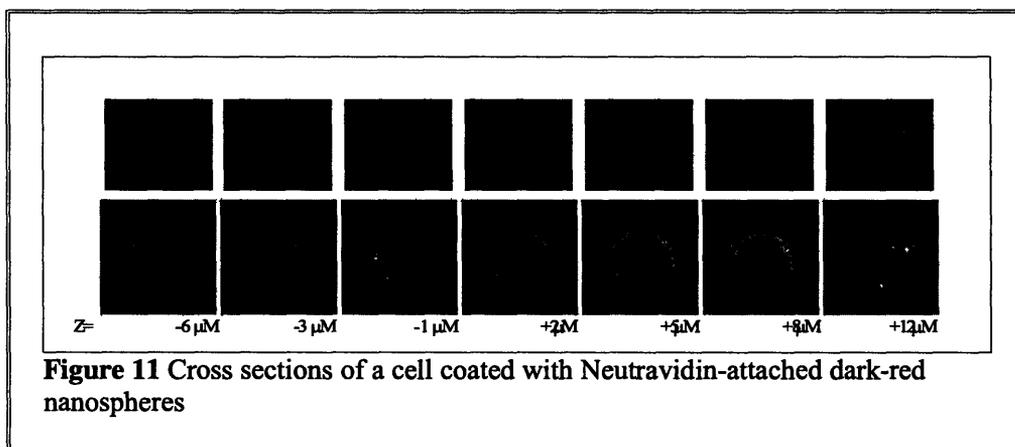
incorporation of spheres into the cell cytoplasm due to endocytosis of the particles.

5.2.2 Exogenous IL2 detection on cell surface. To test the effectiveness of capturing IL2

on T cell surfaces, exogenous IL2 is introduced to a culture of unstimulated DO.11 cells labeled

with cytokine-capture nanospheres (Figure 12). In this experiment, dark-red fluorescence

corresponds to PS nanospheres (FluoSphere 660/680nm), and the detector anti-IL2-antibody is

active in the red channel. Both the sample treated with IL2 and the control experiment show

strong fluorescence due to the presence of nanoparticles. There are a few areas where the beads

appear to be clustered together, as seen by the bright and out-of-focus patches on the surface.

Clustering of beads is undesirable in the case the beads were cross-linking or triggering surface

molecules. Cells migration could also induce polarization of beads, because cells have been

observed to pool their receptors to the acting rear during motion. Dark-red fluorescence is also

well distributed around the cell surfaces, as expected.

The red fluorescence is clearly detected in the sample with treated with IL2, whereas the

negative control exhibits no fluorescence in the red bandwidth. The optimal range of signal
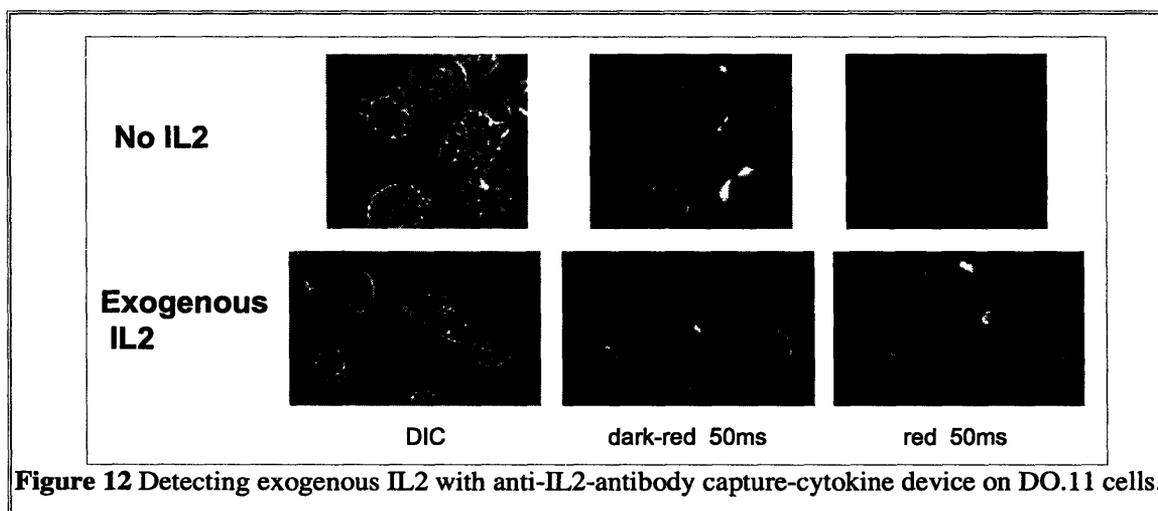
Figure 12 Detecting exogenous IL2 with anti-IL2-antibody capture-cytokine device on DO.11 cells.

intensity for the cells incubated with IL2 has been applied to the image of the control sample. Points of fluorescence from the nanospheres and the anti-IL2 detector antibody align well, indicating that IL2 is being detected on the nanosphere substrate. Discrepancies between the two fluorescent images (red vs. dark-red) can be attributed to movement of cells during the timelapses between different excitations.

The work to date has shown that IL2 can be detected on the surface of nanoparticles. However, there is more research that needs to be conducted to take this technology into production. For instance, the amount of IL2 introduced was the quantity typically used to stimulate T cells in in vitro experiments. However, this concentration may be significantly more than the quantity produced within the synapse. Certainly before any serious discussion with patent lawyers on IP protection can take place, cytokine capture must be demonstrated with naïve T cell stimulation by APCs. Nevertheless, the incubation stage of the technology is the time to begin investigating of a patents and market analysis. The discussion of the cytokine-capture technology will end here, and the rest of the paper will focus on the current status of patent in immunoassays and on important patents that may block cytokine-capture nanoparticle IP protection, followed by a look at potential markets for the technology.

6. Intellectual Property

The commercial applications of the local cytokine-capture detection device create great value in the technology, which may be secured by patenting. Once an invention has been patented, its monetary value can be tapped by licensing the IP or creating a new company based upon the technology. For these reasons, there should be strong considerations towards protecting the invention once there is proof-of-concept.

In order to analyze whether or not IP can be successfully obtained for the cytokine-capture device, the following sections will provide first a broad overview of issued immunoassay patent claims, outline common characteristics of immunoassay patents, and finally, identify trends of issued patents in the last 5 years. After the introduction of IP in immunoassays, the cytokine-detection technology and applications will be analyzed against current patents that may impede the efforts of securing IP protection.

6.1 Overview of patents in immunoassay industry

6.1.1 Patents in Immunoassay. Nearly 2,700 patents relating to immunoassays and over 16,000 patent claims relying on antigen-antibody binding have been issued since 1976[22]. The number of new patents issued annually can be seen in Figure 13. Since 1984, patents claiming the use of antigen or antibodies have increased in number exponentially until the late 1990s. The dramatic increases can be attributed to advancements in commercial detection methods and purification of antibodies. Also during this time, investors funneled large finances into the biotechnology field, spawning further research and development. However, since the explosion of issued patents in the two year span between 1996 and 1998, the numbers have plateaued with
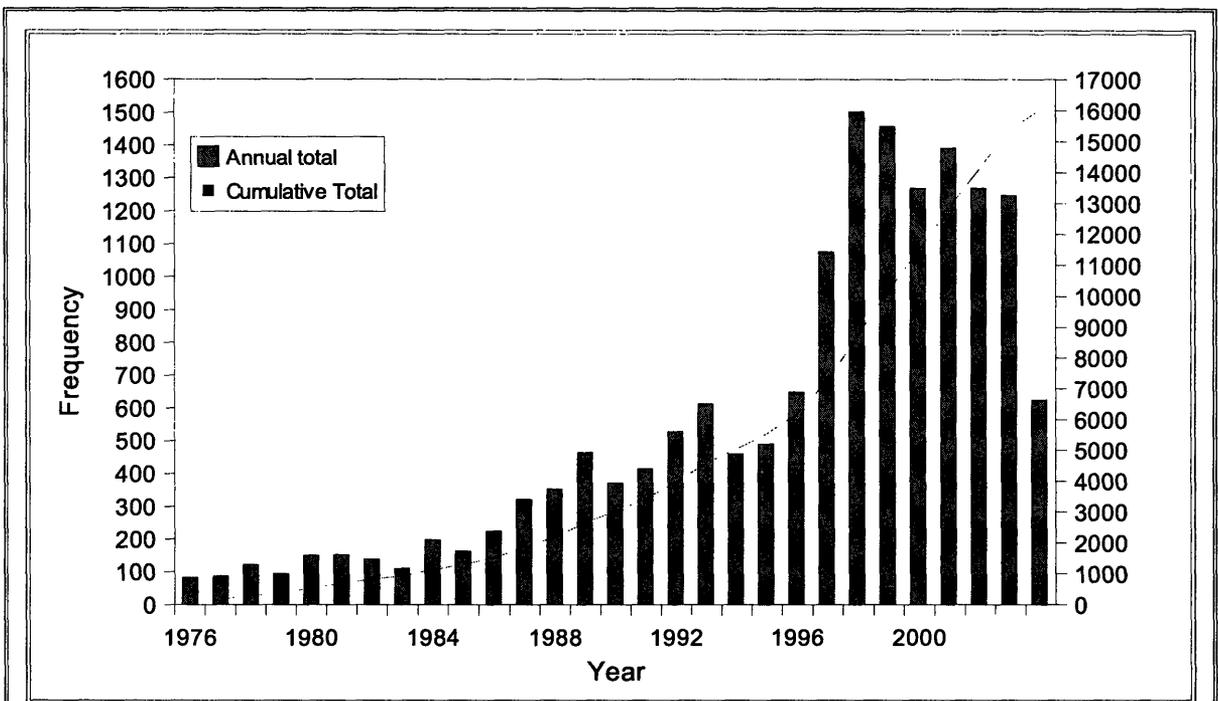
Figure 13 Annual issued patents claiming the use of antibodies or antigens.
*Data does not represent total patents issued in 2004, collected in July 2004
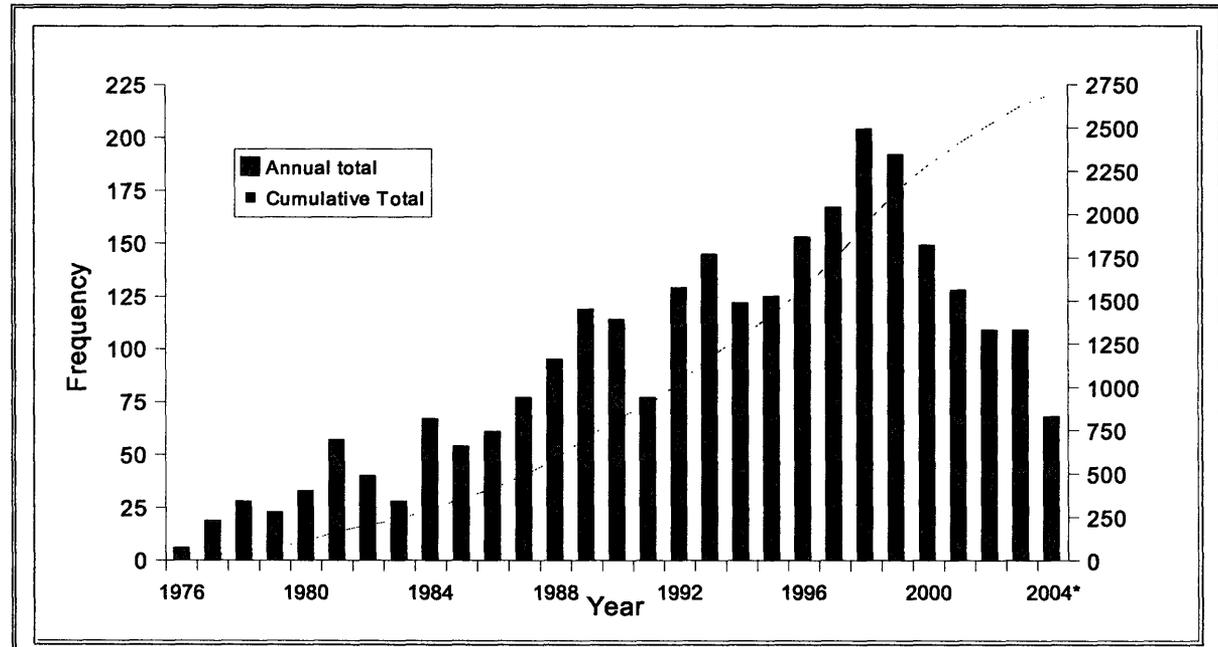
Figure 14 Annual number of issued immunoassay patents
*Data does not represent total patents issued in 2004, collected in July 2004

an average of 1,350 patents per year.

The chart of immunoassay patents licensed (Figure 14) also shows an exponential increase until a peak frequency in 1998. Within four years, the number of annual licensed immunology patents fell to half the number seen in 1998. The significant drop in issued IP for immunoassay patents, compared to the more stable numbers of patents claiming antibodies or antigens use, indicates the diverging markets of the immunodiagnostic industry and other industries employing antigen and antibody recognition, such as immunotherapy patents citing antibodies for disease treatment.

In both charts, the growth in the number of annually issued patents peaked in 1998. It is worthy to note that in 2000, investors were losing finances in the technology sector. Although the information technology and silicon industries were most severely affected, the attitude towards venture investments swung towards more conservative ventures; as a result, the cash flow into the biotechnology industries also suffered.

6.1.2 Patent overview. Before proceeding to analyze the patents relevant to the cytokine-capture device, it is useful to remark on similarities in current immunoassay patent claims. Understanding these similarities can identify which differences between claims are significant and which new inventions are worthy of patenting. Most of the broad immunoassay patents outline the method to detect the target analyte and any physical devices to aid in detection. Typically within the first few claims, the order in which an analyte and antibody are combined together are presented along with a method of detection and/or quantification. For example, in

the patent filed by David et. al. (Assignee: Hybritech, Inc.; #4,376,110; March 1983) a soluble complex with the antigen of choice is first formed with a labeled monoclonal antibody. A second monoclonal antibody that is bound to a solid carrier is introduced to the antigen-complex solution. The claims cover enzyme, fluorogenic material, and radioactive isotopes as types of labeled antibody. The solid carrier is separated from the solution, and the amount of labeled antibody is measured in each samples. The novel approach for detection lies with the solid substrate that can be precipitated and separated from the sample solution[23].

At a first glance, the uses of antibodies in immunoassays appear to have minimal variation between one method from another (e.g. labeled antibody detecting antigen or sandwich detection structure). However, immunoassays are dependent on the approach of analyte detection and quantification. Therefore, the subtle variations can result in differences of specificity or sensitivity of the assay and vary due to target antigen. Often an invention includes additional substrates, which aids in increased detection speed or collection yield. The inclusion of a novel substrate makes a patent more robust and easier to defend. In other technologies, infringements of processing patents are more complicated to prove, since two different processing techniques may still yield indistinguishable products. In the case of immunoassay patents, defending such a patent is straightforward, because method of detection is conspicuous in an immunological assay kits and evidence of transgressions can be identified by comparing the product to the claims protected in the patent.

Unlike the patents issued at the beginning of immunoassay technology swell, the majority of the recent patents issued within the last 5 years focus on detection of specific analytes pertaining to diseases. Much of of this movement is indicative of the focus of developing

immunotherapies for cancer and other disease inflicted patients. The increasing specificity of the claims of recent patents indicates a mature market, implying only small regions of unclaimed IP areas remain for immunoassays. Although this maybe discouraging information for a new company, there have been recent patents involving new devices to track analytes, such as quantum dots or fluorochrome loaded polymer nanospheres, which have been able to claim strong, broad patents.

6.2 Cytokine-detection device vs. existing patent claims

Cytokine-capture device is not a fundamentally new technology, because many of the components of the device have roots in current immunological assays. The invention integrates elements of a bulk immunometric assay to a mobile substrate that can be anchored directly to a cell surface and imaged. As a result, the functionality of the nanospheres/quantum dots covers local analyte tracking abilities of surface molecules on a cell, which cannot be achieved with the parallel use of equivalent existing materials alone, e.g. an imaging method and an immunometric assay.

Here, we will proceed with an analysis of issued patents in order to discuss the viability of securing a patent for the cytokine-capture device. The invention will be dissected into essential components and then compared to the claims of current patents and commercial products. If no patent or product is uncovered that seeks to accomplish the same the functionalities of the device, there is a high chance of success for obtaining IP for the cytokine-capture technology.

The individual components of cytokine-capture device are, as listed in Table 1, are organized into either structural or procedural constituents. The table also holds examples of patents or products that have already acquired IP for the feature of the technology described.

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Objective of Cytokine-Capture Device: to target and quantify cells with particular surface proteins and/or analytes present in the vicinity of the cell surface		
	Aspects of Device	**Examples of claims or products**
Procedural	Antibody conjugated to surface of beads	(#6,274,323B1, 2001). Method of detecting polynucleotide analytes, i.e. DNA or RNA. The invention combines sample on a solid support with a specific binding molecule, designated as avidin/biotin, fluorescein, mouse immunoglobulin, and other antibodies. The complex is then combined with a second member of binding pair, linked to a quantum dot. The detection of the quantum dot takes place within the sample. The application described in this patent simply demonstrates how quantum dots can replace fluorochromes or other encapsulated fluorochromes. BD Bioscience's BD™ Cytometric Bead Array. The assay is composed of a suspension of different-sized capture beads, each coupled with an antibody for a specific antigen. The target sample is incubated with the suspension of beads. The analyte-bound beads are first separated using flow cytometry by size and then incubated with a detection antibody. The final amount of fluorescence corresponds to the quantity of bound analytes[24].
	Attachment of beads to cells	Biotin/avidin; Antibody/surface antigen
	Stimulation of cells for analyte secretion	#5,739,001 (Brown, et. Al., Assignee: E. L. duPont de Nemours and Company.) Stimulation of cells to produce an unlabeled analyte. The analytes covered by the patent are specified as cyclic nucleotides, leukotrienes, cytokines, growth factors, nucleic acids, and enzymes. The stimulated production of the antigen is immobilized on a solid phase. The detection system employs a competitive reaction between labeled analyte with the unlabeled analyte and capture reagent.
	Add detector antibody for captured analytes	ELISA. The detector antibody used in prior experiments use ELISA detector antibodies, available from Sigma-Aldrich. Materials arrive with method for antibody attachment.
	Fixing cells with PFA	Standard method of fixing cells.
	Imaging with fluorescent microscopy	A common method of detecting fluorescent-labeled proteins and other cellular components.
	Quantify with cell analyzers	Flow cytometry
Structural	Substrate to attach antibodies	Fluorescent beads Quantum Dots #6,274,323B1, 2001
	Detector Antibody	Commercially available
	Sandwich structure	ELISA #4,376,110

Table 1 Components of cytokine capture device and existing patents

Only a few examples of already patented claims or existing technologies are provided, because a single example is sufficient to prove that an invention is repetitive.

One foreseeable difficulty in obtaining a patent for the cytokine detection technology is that all of the individual structural elements of the assay have been either already patented, such as quantum dots, or are examples of a well-known set of analytical tools, such as monoclonal or fluorescent-conjugated antibodies. Most of the listed aspects of the invention are individually covered by multiple patents, and the sum of all the patents indicated above covers all aspects of the detection process for the cytokine-capture system.

The wealth of IP blocking the design of the cytokine-capture invention is alarming. Unfortunately, this is not surprising, since all of the reagents required in such a kit is commercially available. However, all is not lost, because the integration of the various elements of these different technologies produces a new assay that exceeds the capabilities of the existing technologies. However, royalties on these subcomponent technologies could be due, which would reduce the value of the patent.

The greatest threat for blocking cytokine-capture IP is the patent issued by Assenmacher, et. al. (#6,576,428 B1; Assignee: Miltenyi Biotech GmbH). The patent describes a method to identify antigen-specific T cells by capturing molecules secreted upon T cell activation. The invention claims that T cells are to be identified by stimulating a population of T cells through the presentation of at least one antigen and by labeling cells with "a capture moiety for the product." The bound "product" is then labeled with another "label moiety." No specifications are made regarding the identity of the label. The capture antibody is attached to the surface of the T cells with a "lipid anchor[25]."

This patent clearly lays out the assembly of a device that captures analyte on a cell surface

in a similar sandwich structure as the cytokine-capture device. The primary function of the invention is to quantify and sort cells that are capable of cytokine secretion. It is not, however, comprised of the necessary components to image the location of cytokine over the cell surface. Furthermore, the capture moiety is structurally different from cytokine-capture device. First, the capture antibody complex does not serve as a tracking device for surface proteins, because it lacks a labeled marker. Second, only a single capture antibody is attached to the lipid anchor. One benefit of having multiple antibodies on a substrate is to secure the attachment of detector device for longer lengths. For these reasons, the cytokine capture technology is not obstructed by the claims of Assenmacher *et. al.* patent.

Localized detection cannot be achieved by two parallel experiments of one imaging method and one immunometric assay. As described in the technology sections of this paper, surface proteins can be tracked using fluorochromes or the more stable alternative fluorescent probes. Also, ELISAs, BD™ Cytometric Bead Array[24] or other immunometric assays can quantify levels of secreted, unlabeled antigens. However, all of these elements individually cannot accomplish local detection on a cell surface. For this reason, the cytokine-capture detection technology stands apart as a novel, patentable, assay.

6.3 Patents in Multimer Technology

Even once IP protection for cytokine-capture technology is granted by the patent office, a favorable market is required for a successful start-up. In the case where cytokine-capture detection technology cannot generate adequate revenue as a stand-alone tool—an immunoassay market analysis will follow this section—it is advisable to seek other applications of the technology that can capture a related but different market. For this reason, this section will

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discuss the IP situation in T cell monitoring technology.

Searching for relevant patents on tetramer technology is a difficult task simply because there is no consensus on a technical term for T cell monitoring by multimeric p-MHC presenting complexes. Moreover, there are only a few such patents that exist to date. The following are two different patents that are the foundational of the two commercialized products for T cell monitoring.

Tetramer technology is protected by effectively a single patent, invented by J. D. Altman, et. al. at Stanford University (#5,635,363). The patent claims the capability of detection, separation, and labeling of antigen-specific T cells. The invention is a multimeric complex composed of α and β chains and bound antigen peptide in an $(\alpha-\beta-P)_n$ configuration, where n is determined to be two to ten monomer units. The α and β chains are derived from biotinylated fusion proteins with amino acid sequences recognizable by a modifying enzyme, such as Bir A and protein kinase. The monomer units are assembled through biotin-avidin interactions and also contains a light detectable label. Separation of labeled cells takes place using flow cytometry[19]. There are no specifications of appropriate peptides in this patent, unlike later patents that focus on identifying specific fusion proteins to elicit a specific antigenic response. The breadth of the patent and the cross-licensing, which will be discussed in the next chapter, reveals the importance of the original design in the foreseeable future of T cell labeling and sorting.

Besides a multimeric p-MHC complex reliant on avidin-binding, there are a few other inventions that attempt to create a p-MHC multimer with different approaches. A second design for a multimeric peptide-MHC covalently links at least 2 chimeric proteins composed of a p-MHC and a heavy immunoglobulin chain (Schneck, *et. al.*; assignee: Johns Hopkins University;

July 2001; #6,268,411 B1) resulting in TCR-p-MHC complex that has greater avidity than that of

TCR with the peptide monomer[26]. In addition to these patents, Quantum Dot Corporation has

announced intentions of working with BD Biosciences to use quantum dots to label antigen-

specific T cells[27].

Although there are patents that claim the use of nanoparticles as a substrate to present

multiple p-MHC molecules, the cytokine capture device still has the advantage of quantitatively

measuring the number of and sorting of cells that are specific to the presented antigen peptide

complex and are capable of secreting cytokines. Furthermore, the cells bound to cytokine-capture

device could be imaged and sorted multiple times due to the nanoparticle's integrity of

fluorescent signal. The application of cytokine-capture device offers a new method of identifying

T cells specific to an antigen and testing its functionality on a single substrate. Without present

patents claiming these additional capabilities, the application is a likely candidate for IP

protection.

7. Market Analysis

The technology of cytokine-capture system strives to offer biomedical scientists and doctors a new diagnostic tool that will help reveal the mechanism of the T cell activation and potentially aid in the development of immunotherapy cures. Unfortunately, a patented invention without a lucrative target market cannot succeed, no matter how promising the fundamental technology may be. Therefore, the market value of the cytokine-capture device with respect to immunotherapy research and clinical therapies should be the driving force to determine pursuing commercialization of the technology. This final chapter will take a look at the immunoassay market and other related markets to judge if the market size is favorable for a new business to grow and succeed and then will proceed to suggest a business strategy to commercialize the cytokine-capture device.

7.1 Current Status of Immunoassay Market

7.1.1 Overview of In Vitro Diagnostic market. Immunoassays are covered under an umbrella category of in vitro diagnostics (IVD), which includes assays for glucose or scanninng blood. A 2001 market analysis, conducted by Business Communications Company, reported that the in vitro diagnostics market reached $15.5 billion. Furthermore, this industry is anticipated to grow at an annual rate of 5.1%. In 2006, BCC forecasts the market should reach $19 billion[6]. Boston Biomedical Consultants, Inc. published that IVD products in 2001 generated $21 billion revenue worldwide, $10 billion of which was from North American sales. The yearly percentage increase of the two markets were 4% and 7%, respectively[28].

Boston Biomedical Consultants, Inc.'s 2001 survey also broke down the market into smaller divisions to identify the high growth sub-markets. Figure 15 depicts the percentage of total revenue by clinical segment and by the end user. Also noted are the percent growth in each sector between 2000 and 2001 fiscal years. The growth of the $5 billion immunoassay market is slowing down significantly with 1% increase from the previous year, whereas clinical chemistry and conventional microbiology market sales have been falling since their peak in 1991. In 2001 the highest growth sectors were nucleic acid testing, diabetology, cardiac marker diagnostics, and histochemistry with growth percentages over 10%. Growth in "Unconventional infectious diseases" and some cancer testing areas was moderate in 2001. When the IVD market is divided by end users, it is clear that clinical laboratories dominate the market. However, there is a general shift in industry towards creating point-of-care testing units, which is confirmed by the 11% increase in patient self-testing sales in 2001[28].

Figure 15 Breakdown of IVD industry by clinical segment and end user[28].
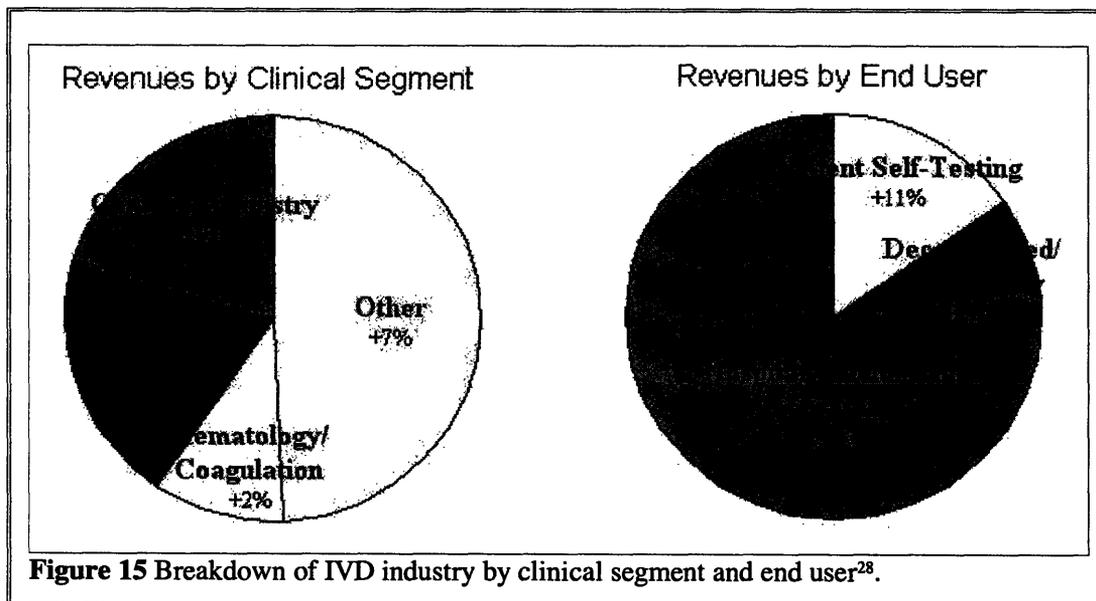
7.1.2 Dominating companies in immunoassays. The *Medical & Healthcare marketplace*

Guide 18th Ed, published by Dorland Healthcare Information, identifies the leading companies in

immunoassays as Abbott Laboratories, Atlantic Antibodies, Amersham/NycoMed, Beckman

Coulter, Becton Dickinson/PharMingen, Caltag, DAKO, R&D Systems, and Sigma-Aldrich[28].

Table 2 displays issued US patents, mergers and acquisitions, and sales in 2003 pertaining to the

above mentioned companies.

Of the companies listed above, Abbott Laboratories and Beckman Coulter are also named

one of six dominating companies in the IVD market. Added to this list would be GE Healthcare;

however, the final negotiations of the acquisition of Amersham/NycoMed had not taken place at

the 2003 publication. Besides Abbot Laboratories and Beckman Coulter, the other four

Company/Division Name	Revenue in 2003	Patents*		Parent company	Mergers/Acquisitions
Abbott Laboratories	Approx $3 B[29]	2735	363		
Amersham Biosciences GE healthcare biosciences	$550 M[30] $2.8 B[31]	663	50	GE Healthcare	Previous Mergers: NycoMed, 1997; Pharmacia Biotech, 1997 Acquired by GE Healthcare for $9.5 billion April 2004[32]
Beckman Coulter	$651 M[33]	1546	158		Coulter acquired by Beckman 1997[33]
Becton Dickinson Pharmingen	$645 M[24]	2014	259	Becton and Dickinson and Company	Acquires Clontech, Bioetric imaging, Transduction Labs in 1995[24]
Caltag	$8.2 M[31]	0		Private	
DAKO	Not in US	14	6	DAKOcytomation	DAKO and Cytomation merger 2002[34]
R&D Systems	$91 M[35]	3	0	Techne	
Sigma-Aldrich Biotechnology	$294 M[36]	114	6		
Molecular Probes	$17.9 M[31]	72	52	Invitrogen	Acquired by Invitrogen for $322 million Aug 2003[37]
Quantum Dot	Est $1.2 M[31]	8		Private	
Biocrystal, Ltd	$200,000.00[31]	22		Private	

Table 2 Immunodiagnostic Companies.

*The first column displays the total number of patents owned by the company or subdivisions. The second column is the number of patents specifically claiming antibody or antigen use.

companies are Roche Diagnostics, Johnson & Johnson, Bayer Corporation, and Dade Behring,

Inc. Each of these companies raise over $1 billion in sales and their revenues account for over

60% of the IVD market[28].

High frequencies of mergers and acquisitions within the biotechnology companies has

yielded large conglomerates such as Becton, Dickenson and Company, Beckman Coulter, Abbott

Laboratories, or the recently formed GE Healthcare. For example, in April 2004, GE Healthcare

acquired Amersham for $9.5 billion, including royalties and licensing fees[32]. Many of these

corporations have subdivisions for bioscience research and production of related products.

Revenues of the immunoassay and diagnostics divisions of each of these corporations well

exceed $100 million.

7.1.3 Evidence of Mature Market. With billions of dollars in cash flow annually in the immunoassay market, gaining a hold of only a small fraction of the market still would result in impressive revenues. For instance, 0.1% captured market correlates to $500 million in sales. Unfortunately, such a large market indicates that the market likely has attained mature status. The figure to the right (Figure 16) displays the stages of development of a technology with respect to market status for clinical chemistry and immunoassays. With the onset of au-

Figure 16 S-curve representation of immunoassay market[6].

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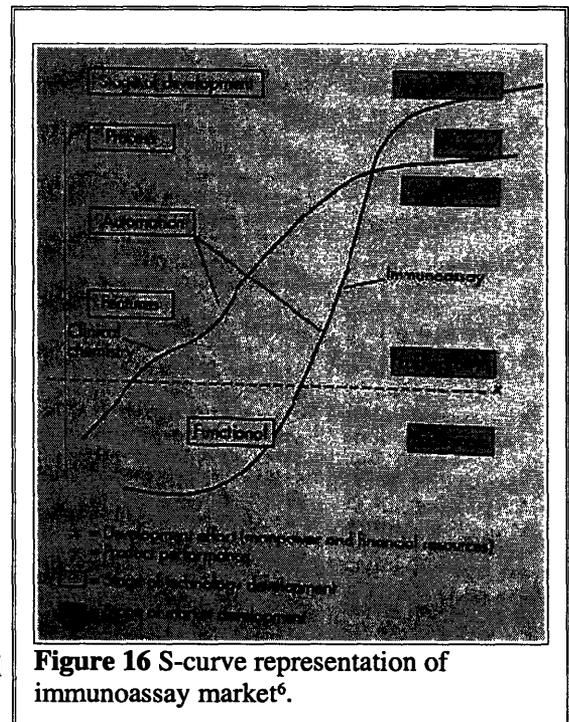
tomation of immunoassays, hospital and research laboratories have capitalized on the speed and accuracy of detecting analytes for a large variety of tests. According to the graph, this would place immunoassays in the early and late growth stages a decade ago.

The presence of large corporations in this field further suggests that the technology is well developed. Within the framework of a large corporation, a company can manufacture reagents, substrates, and other necessary products for conducting assays at high production volume, improving their net earnings, and still keep prices competitive. Immunoassay kits are only a small component of the annual sales for these large companies, and often companies supply automated immunoassay analyzers and cell sorters as well[28]. In addition to the companies listed in Table 2, there are over a hundred of smaller public and private companies also competing for market share in immunoassays.

There are many obstacles to creating a business in a mature market. Examples include initial high overhead costs in a market where well-established companies can afford to lower prices by producing products in bulk. The rising new small to medium size companies tend not to serve as antibody providers but rather offer new products for more sensitive detection. An example of such a product is Molecular Probe's Alexa Fluoro® fluorescent dyes, which have been shown to outlive the fluorescent lifespan of commonly used fluorochromes. These smaller companies focus on developing new technology that will likely replace existing products in the near future. Other companies of interest in this paper are those that are engineering new fluorescent agents that exhibit long fluorescence excitation capabilities and onto which many antibodies can be conjugated. For example, the technology available from Quantum Dot Incorporated is of great interest for cytokine-capture devices.

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7.1.4 Market for cytokine-capture device in immunometric applications. The effective

capturable market of the cytokine-capture device is significantly smaller than the total

immunoassay market, because the device as envisioned is an immunometric assay. The

immunometric assay market may not be subjected to all of the difficulties associated with a

mature market, since it is comprised of a small percentage of the total immunoassay market.

Therefore, there may be more room for new immunoassay companies, offering more robust

assays, to capture an enticing percentage of the market. Unfortunately, the proposed technology

is not relevant to many current immunometric assays, thus, the actual market field is even

narrower than that of immunometric assays..

The most likely consumers of the cytokine detection system would be researchers

investigating the localization of soluble molecules for academic interests and potentially

scientists who are interested in the effectiveness of immunotherapies. The former market group

would only comprise at most of few hundred research groups around the world, and the quantity

of cytokine detection kits demanded would only be a small fraction of the demand required to

make the cost of the assay reasonable. The latter group of researchers would also include those of

the large pharmaceutical and biotechnology industry. Given the large monetary requirement for

testing potential immunotherapies, a cheaper alternative to identify T cell responses would be

attractive for the companies. However, most likely a bulk assay would be sufficient to indicate T

cell activation. Given these arguments, even without a formal estimation of the realistic potential

market, we can conclude that the immunoassay market would not sustain a start up company

marketing cytokine capture technology products.

7.2 Current Market Status of T cell monitoring industry

Before abandoning ideas of commercializing the cytokine-capture technology, there is one area of application that may be a well-suited market with attractive yields: the T cell monitoring industry. Because the technology has evolved from an immunology laboratory, there are no particular biases towards one antigen market from another. Therefore, to maximize the captured value of technology for immune system monitoring, it is critical to find a market with a large enough population where patients require many screenings and the patient's treatment is covered by health insurance. Multiple screenings is achievable with chronic patients who live long enough due to treatments. Although a complete cure would also be a motivating factor for patients to undergo re-screenings, the monitoring per patient ensures sustainability of revenue. Another important concern is that patients are able to pay for their screenings, either out of pocket of from an insurance company. With these criterion in hand, a study of potential disease antigen candidates for the cytokine capture device should be made to identify the most applicable markets.

Wolff states that the more widespread and the more deadly a disease is, the greater the demand is for a cure and the greater the benefits for the company that can offer a cure. There are diseases where the current drug and diagnostic market has not reached its full capacity, because of the lack of effective treatments[38]. Diseases that may be potential candidates for immunotherapy are cancer, bacterial/infection, and HIV-AIDs, as previously described earlier in the paper.

Internationally, there are 40 million people living with HIV with 5 million people who have acquired HIV in 2003 alone. An estimated 95% of these people live in low to middle

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income countries. Within the United States, 380,000 people are afflicted with AIDS[39]. The chart

in Figure 17 presents the number of deaths, new cases, and the number of patients living with

AIDS as reported by the Centers of Disease Control and Prevention (CDC). The concerted efforts

to raise AIDS awareness in the US resulted in impressive decreases in the number of new AIDS

cases each year, and the implementation of highly active antiretroviral therapy (HAART) has

significantly lengthened the expected lives of HIV patients. Despite these efforts, people are

being diagnosed with AIDS at over twice the rate of the number who die annually. The result is a

growing US population living with AIDS. This corresponds to billions of dollars required to test,

treat, and monitor HIV/AIDS patients every year, which will continue to rise.

Figure 17 AIDS Cases, Deaths, and Persons Living with AIDS
by Year, 1985-2002, US[39]
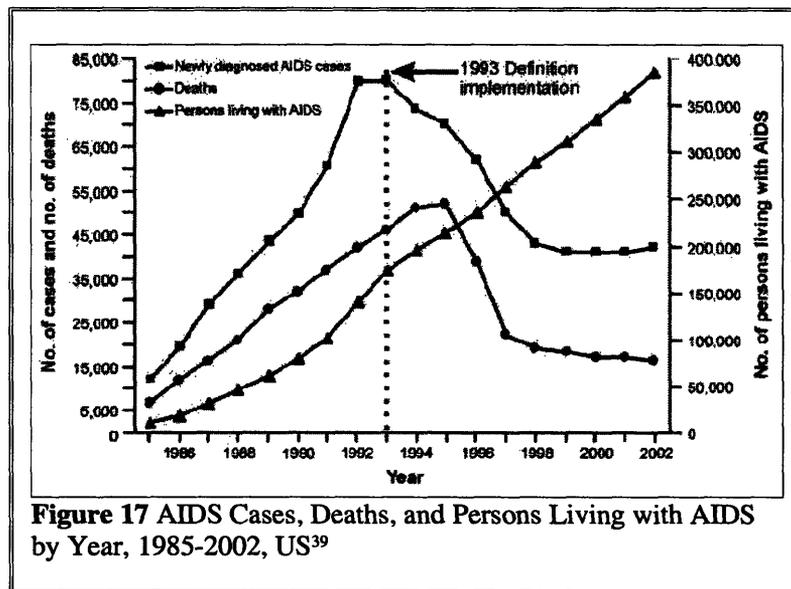
The National Institute of Cancer estimates that over 9.6 billion living Americans have had

a history of cancer. Nearly 1.4 million new cases of cancers and almost 600,000 deaths due to

cancer were reported for 2003. Approximately 65% of cancer patients are still alive after 5 years.

However, this figure does not take account of the how early the cancer was detected, which has a

high correlation with the success of controlling or eliminating the infected area. Also some patients are cancer free or in remission. NIH estimated that medical costs for cancer patients were $64.2 billion for 2003. According to a recent National Health Interview Survey, 16% Americans below age 65 have no insurance. Approximately 1/3 people over 65 have medicare insurance only[40].

Target Disease	Approximate Current Market
Bacterial/Infection	$23 billion
Cancer	$16 billion
HIV/AIDS	$4 billion

Table 3 2001 Current Market for Target Disease[38]

The total market sizes of these diseases are shown in Table 3[38]. Included in the estimates are sales from drug therapies as well as from patient diagnostics. A more suitable estimate of the potential captured market would be the revenues of clinical laboratory testing services. In 1996, Marketdata Enterprises reported the value of AIDS tests to be $287 million with an annual growth rate of 6.8%[6].

7.2.2 Foreseeable technical barriers. There are currently two commercially available reagents (for research purposes only) that exploit the increased avidity of TCR-p-MHC interactions with a multimeric presentation of antigens. BD Pharmingen has introduced DimerX products, a soluble dimer protein, for antigen-specific T cell detection using flow cytometry analysis. The second example is Beckman Coulter's Tetramer technology, based on Altman, et. al., patent discussed in §6.4. Tetramers are currently involved in 20 clinical trials in diseases, e.g. cervical cancer and renal cancer. The presence of these two products already available on the market places the cytokine capture device at a significant disadvantage, particularly with the

progress of tetramer technology through FDA trials. The time necessary to bring the cytokine

capture device to the production stage could take over 5 years, after which the device would

begin clinical trials.

A major hurdle for the cytokine capture technology is gaining FDA approval for the

device. At present, the potential application of a device towards infectious diseases with high

public health concerns are placed as Class III "high risk" devices. With Class III designations, the

entire device must be FDA approved. Once ready, safety of the device is first investigated using

ex vivo, in vitro, or in vivo systems in animal models. Preclinical trials take on average 6-6.5

years. Following the completion of preclinical trials, the device is prepared to enter clinical trials.

Clinical trials are composed of three phases and also typically take 6 years. Whitmore estimates

that for every 5,000 potential candidates that enter preclinical trials, only 5 will succeed in

beginning human trials and of the 5, only 1 will become an approved product[41].

A significant concern is the potential cost of the cytokine-capture device, and this cost

will vary depending on the substrate of choice. At present, only a few companies commercially

produce quantum dots and charge hundreds of dollars for less than 1 mL of solution. Marketing

the device for immunodiagnostics is unreasonable due to the high costs of an assay, particularly

because researchers can purchase all the necessary reagents and tailor a soluble molecule capture

device to their specific needs. However, the price of the cytokine-capture nanoparticles may be

more suitable for T cell monitoring. A more thorough cost analysis for the device is necessary to

determine if the price of cytokine-capture device would be competitive with other commercial T

cell monitoring products.

7.3 Business strategies

7.3.1 Lessons from successful start-ups: Quantum Dot Corporation. Before drafting a

business plan for a company based on cytokine capture technology, there are many valuable

lessons to learn from recent successful companies. Quantum Dot Corporation is an example of a

notable biotechnology start-up that entered into the immunoassay market. The company was

founded in 1998 and has raised $37.5 million in venture capital investments to date. Science

Magazine recognized the research conducted at QDC as one of the top ten scientific break-

throughs of the year 2003. QDC offered its first product in November of 2002, and currently it

offers the largest range of quantum dot products applicable to immunoassays[27].

The first lesson to learn from QDC's history is the power of the patent. The strength of

QDC's patent portfolio is the reason that QDC offers the most advance technology today. With

every advancement in the technology, QDC blocked off an effective method of synthesizing

quantum dots; as a result, QDC has very few competitors in the field. In the case of the cytokine-

capture detection technology, all the reagents required for the assay are commercially available.

However, the new detection capabilities sets the technology apart from current patents possessed

by QDC or Molecular Probes. With the patents for the cytokine detection device on hand, the

company would be able to block companies with fluorescent bead products from creating a

similar capture device out of their products.

The second lesson is the importance of having top researchers affiliated with the startup

company. Wolff emphasizes that top scientists in academic are intangible assets to a company

seeking investments from venture groups and collaborations with established companies. One

advantage is that fundamental research is first incubated in an academic setting with there are

minimal pressures for revenue generation. At the foundation of the company is semiconductor

nanocrytalline research conducted by four premiere chemists in the field, Dr. Paul Alivisatos

from University of California Berkeley, Dr. Moungi Bawendi from MIT, Dr. Paul Mulvaney

from University of Melbourne, and Dr. Shuming Nie from Indiana University; the first three

researchers currently serve on the company's scientific advisory board[27]. The patents filed by

these four researchers are the foremost in quantum dot technology, and their combined IPs

current make up the backbone of QDC's patent portfolio. Again, in the case of a cytokine

detection device company, the founders should recruit and form early collaborations with

immunologists, such as Mark Davis, who holds the premiere patent on tetramer technology.

The last lesson focuses on the benefit of collaboration. When a company overcomes the

technical barriers of commercializing a technology and a product is finally released, careful

collaborations with top industries is a great way of accessing important patents and associating

the new technologies with products of reputable companies. Partnerships with large companies

can also serve as credibility for the new company, which is important when seeking more rounds

of investments[38]. Prior to the first product launch in November 2002, QDC had already been

working with Genentech Inc and GlaxoSmithKline on incorporating quantum dots in their assays

for over a year. Since then, other collaborators include Matsuhita (Panasonic), SC Biosciences,

Roche Diagnostics, Agilent Technologies, and Becton Dickenson and Company[27].

Large companies also benefit from collaborations, because they can access important

emerging technology without investing in production. Corporations are more hesitant to start

own research in a field because the initial sales are not lucrative enough. For example, despite the

growing number of papers published incorporating the use quantum dots, the large bioassay

companies are not the ones leading the development of these potentially powerful and significant

biological probes. At the moment, biologically active quantum dots are at present commercially

available for research use only.

Similarly, joint partnerships in antigen-specific T cell monitoring should be pursued once

a cytokine-detection product is developed, or close to development. The reputation of the partner

company should aid in acquiring further investments, which will be critical for funding required

clinical tests. Also, the expertise found in the mature company would facilitate proceeding

through FDA clinical trials. If the T cell monitoring product were successful in obtaining FDA

approval, the demand of cytokine-capture devices would exceed the capabilities of the new

company production laboratories. At this point, the larger company's facilities would be able to

help support the volume of devices needed to capture as much of the market as possible.

7.3.2 Proposed business strategy. Now that all of the marketing concerns have been ad-

dressed, the proposed strategy for progression establishing a new business will be addressed. A

rough time-line is sketched in Figure 18.The first important step is to establish proof-of-concept

of the technology. Prior research has shown that detection of exogenous cytokines is indeed pos-

Figure 18 Cytokine-capture technology business strategy timeline
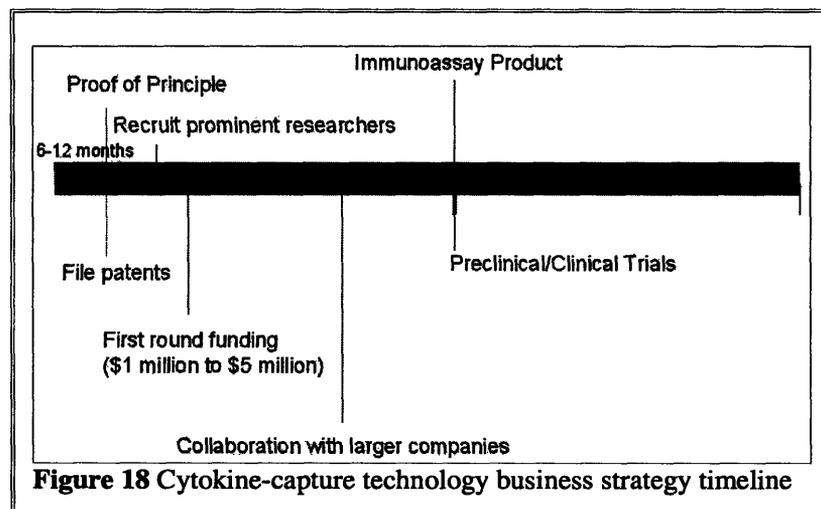
sible on fixed cells. However, applying these experiments to live cells with stimulation by APCs is still not a trivial matter. A reasonable time estimate for obtaining reproducible, optimized data is between 6-12 months. Other goals include capturing multiple analytes on a single bead surface and successful quantification of cells secreting cytokines using flow cytometry methods .

Upon gathering adequate proof for the mechanism of the cytokine-capture device, patents should be filed immediately. Although the cytokine-capture device is dependent on other commercial materials, some of which are already patented such as quantum dots or FluoSphere beads, the cytokine capture technology is not limited to these specific products. Because of the general application of quantum dots or fluorescently stable beads, a cytokine-capture patent can cover without infringing on the IP of QDC or Molecular Probes. Furthermore, the patent would be able to block these companies from commercializing a similar product, and the companies will be more conducive for collaboration or licensing the technology if they wish to have a hand in local soluble molecule detection devices.

Besides the obvious fundamental technology, applications of the technology should be filed as early as possible. In this case, the most important IP for a potential company will be the cytokine-capture device as applied on cell surfaces. However, another patent that could be filed soon there after is a T cell screening patent based on the capture technology. On average for immunoassay IP, the patent application process appears to take 1 ½ – 3 years for each patent.

Prior to seeking first round investments, more academic researchers should be contacted and consulted on the prospective of commercialization of such a device. When possible, this is the time to recruit other prominent researchers to aid in preliminary research of the technology. The participation in academic collaboration will reduce the risk of investing in a product with technical barriers that cannot be overcome and will prolong the need for immediate outside fund-

ing. At the time of exploring for venture capital firms or angel investors for first round investments, the nucleating company will have prominent researchers at the core of the company, along with issued or processing patents, and ongoing research in the academic setting.

The target sum of first round of investments will likely range between $200,000 and $2 million dollars; however this amount will depend on the status of the cytokine-capture technology. The closer the technology is to a commercial product, the greater the investment should be to speed up development and begin to prepare for production. Although in the past, venture groups would make investments as low as $350,000; however, currently the ball-park minimum is $ 1 million. If the company is seeking below 1 million dollars, it should seek the support of angel investors or small business grants. Goals at this juncture are further to develop the technology and begin acquiring relevant patents for the company's IP portfolio, begin establishing contacts and collaborations with larger companies, and move the technology towards commercialization. Completion of a product should happen 2-3 years following initial investments.

The next step is to begin preclinical trials. During the process of obtaining FDA approval, more potential collaborations should be pursued. A licensing agreement could be a source of cash flow for research. While clinical trials are on the way, the device should be sold intended for research purposes only. In this manner, more publicity can be directed at the product as studies based on the cytokine-capture device are published. However, at the expected low volumes of production, the expectation is not to generate great revenue. Success of the clinical trials would result in providing patients with a treatment against a fatal disease and in capturing a market worth hundreds of millions of dollars.

8. Conclusion

The simplicity of the cytokine probe design makes these nanodetectors a potentially powerful and robust tool for local analyte detection and quantification. Given the potential marketability of the cytokine-capture technology through T cell monitoring, it is critical to protect the cytokine-capture technology with patents once successful cytokine-capture is demonstrated. Unfortunately, because the cytokine-capture nanoparticle device relies on previously patented technology of Quantum Dot Corporation or Molecular Probes, a significant concern is the cost of royalties to the companies.

Due to the current mature market status of immunoassays, it is recommended not to enter the market as a start-up company; however some cash flow could be generated through licensing out the technology to an established company in the field. Instead, the cytokine capture technology should be specialized for T cell monitoring, where a successful device could displace current methods of monitoring immune responses to cancers and HIV. Further market research is necessary to identify other promising diseases to target, and a thorough cost analysis is imperative to determine if the cytokine-capture technology can be competitive with other rising patient T cell monitoring systems currently in development.

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