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14. ABSTRACT We have blood samples from 40 African-American men with prostate cancer and 30 ethnically-matched control healthy men with questionnaire data on demographics, general health and cancer family history. Our total accrual goal is 100 cases and 200 controls. The use of nanoscale materials and devices has enhanced the lower limits of detection of proteins and other compounds in plasma and tissues. We have investigated using nanoparticles or quantum dots conjugated to proteins of interest for early cancer detection. Although sub-ELISA levels of proteins can be detected, this technology is somewhat cost-prohibitive. We are investigating other nanotechnologies for early cancer detection. Early circulating cancer cells can be identified in plasma and are thus a potential biomarker for early detection of prostate cancer. Aptamers are modified nucleic acids with the ability to bind specifically to protein targets with very high affinity, similar to antibodies, and can be employed for cancer detection. We will employ cell-based SELEX (systematic evolution of ligands by exponential enrichment) in which aptamers are selected from a library of random sequences of synthetic DNA by repetitive binding of the oligonucleotides to target molecules (prostate cancer cell surface proteins). Two aptamers will be immobilized onto a gold nanowire biosensor surface with electrochemical detection and the sera from the cases and controls will be assayed for circulating prostate cancer cells. This study will make an impact in early prostate cancer detection.					
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A. Introduction

In this study our objective is to set up a prostate cancer case-control study in order to develop new serum biomarkers for early detection of prostate cancer using Nanotechnology. We proposed to collect blood samples from 100 African-American men with prostate cancer and 200 ethnically-matched control healthy men with questionnaire data on demographics, general health and cancer family history to investigate prostate cancer risk factors. We are accruing well now and will achieve the accrual goal by June 2009. We requested, and were approved for a no-cost extension to November 1 2009. In early prostate cancer when there are very few cells, we expect trace serum levels of proteins. Recent advances in nanotechnology mean that target biomarkers of prostate cancer can be conjugated to quantum dots, thereby enhancing detection characteristics and making it possible to detect very low levels of a particular protein. Semiconductor nanoparticles and nanomaterials (such as quantum dots-QDs and nanowires) have become a powerful tool in the research community to perform biomarking and detection. We have investigated the QD-conjugated serum biomarkers in a multiplex panel¹⁻⁵. However, we have found this technology to be cost-prohibitive when moving from the test samples to a larger sample population. We tried to dilute the QD-AB so as to reduce costs but the results are inconsistent and non-reproducible. We had been investigating other technologies such as aptamers and microfluidic devices and we had a Master student Marcus Marley who completed his degree in 2008. We are in the process of moving to this technology. And have applied for and have received local funding to continue the study. Success in the current application could translate rapidly to other cancer sites for which no good screening test is available. This project is essential in terms of expanding research on the African American populations and vital to improve early detection of prostate cancer so as to reduce the dismal mortality rate.

B. Body

Task 1. To collect pre-treatment serum samples from a cohort of African-American undergoing screening for prostate cancer (Months 1-26 according to the Timeline in the Statement of Work)

Progress:

1.1. Recruitment

Approach: In this study prostate cancer cases are defined as African American men with biopsy-proven prostate cancer. Controls are African American men with a PSA < 2.5ng/ml and no other evidence of prostate cancer by DRE or biopsy. Our case-control collection is on-going and we have collected 40 African-American men with prostate cancer and 30 African-American controls into this study so far. We have an approved no-cost extension of one year to Nov 1 2009. We will achieve our accrual goal by June 2009. Once consent is obtained, the participants have been asked to fill in a short research questionnaire and two blood samples (10ml each) are drawn. Recruitment is taking place at Lifetime Cancer Screening at the H. Lee Moffitt Cancer Center and Research Institute and Moffitt Cancer Center Hospital, the Radiation Oncology clinics in Tampa and Brandon and the 30th Street Clinic. All samples are transferred to the Tissue Procurement and the LCS laboratory at the H. Lee Moffitt Cancer Center and Research Institute and processed for serum and DNA, respectively, where they are stored. Original consents and questionnaires are stored in a locked cabinet in a locked office. The questionnaire data collected is scanned into an Access database by the Survey Methods Core. The risk factor database will be linked with the laboratory database, both are in Access.

1.2. Investigator meetings and conference calls

We continue to hold regular conference calls every 3-4 weeks usually of one hour duration including the engineers, molecular biologists and clinicians. The minutes are recorded and distributed prior to each meeting.

1.3. Addition of extra study sites for recruitment.

We added three new sites to the study in 2008. The Tampa Bay Radiation Oncology sites in Tampa and Brandon (Dr. John 'Jack' Steel) and then more recently the 30th Street Clinic (Dr. Patrick Watson). The recruitment into this study is going very well because of adding these sites to our study. In particular, the attendees of the 30th Street clinic are primarily African American.

1.4. Additional study staff

We have added Dr. John 'Jack' Steel and Dr. Patrick Watson who is the general practitioner at the 30th Street clinic. Furthermore, Dr. Yifan Huang, our biostatistician left Moffitt Cancer Center so she was replaced by Dr. Daohai Yu.

1.6. Summary: Task 1: Recruitment is on-going and going well. We will continue accrual to June 2009. We have an approved No-cost extension for the study to Nov. 1 2009

Task 2. To engineer a panel of quantum dot (QD) – antibody (AB) conjugates targeting established prostate cancer biomarkers (antigens). (Months 2-10 in the Timeline in the Statement of Work).

Approach: The experimental luminescent QDs will be composed of surface passivated II-VI semi-conductors such as ZnS, CdS, ZnSe, CdSe, or CdTe and their core/shell structures, e.g. CdSe/ZnS, CdTe/CdS, or CdS/ZnS. We will target QDs to cover emission in the broad visible to infrared spectral range to achieve optimal spectral performance. The QDs will be directly conjugated to antibodies to the selected prostate cancer biomarkers: Prostate Specific Antigen (PSA), Kallikrein 2 (KLK2), Kallikrein 14 (KLK14), Osteoprotegerin (OPG), Antip53Ab, Caveolin-1 (Cav-1) and Interleukin-6 (IL-6), with or without linker molecules, depending on the molecular structure of each antibody.

We purchased antibodies (multiple times) for the above biomarkers. Previously, standard curves were determined for each antigen/antibody using ELISA kits (CanAg, CA, USA). The lower limit of detection for this technique was approximately <0.1ug/l. Therefore we determined that the antibodies were detecting the chosen biomarker, (as was requested in the review of the annual report from 2006). As we stated last year we have successfully conjugated antibodies to the above biomarkers to quantum dots and we have gone ahead and are characterizing them according to Task 3 below.

2.1. Summary: Task 2 completed

Task 3. To define the photoluminescence signatures of bound versus unbound QDs created in Aim 2, reflecting antigen-antibody complex formation. (Months 11-25 according to the Statement of Work)

Approach: High-resolution photoluminescence (PL) spectroscopy will be used to analyze antibodies and QDs separately before and then after they have been conjugated. This permits the identification of unique spectral signatures, and is based on standard PL descriptors such as: PL peak position at 400nm – 1,700nm spectral range, full width at half maximum (FWHM) of the PL band, optical/acoustic phonon frequencies, excitation wavelength dependence (different laser sources), light polarization, temperature quenching, and PL quantum efficiency. From the PL descriptors of the optimized AB-QD conjugates we will generate a calibration curve of the PL intensity versus concentration of the conjugated QDs in serum/plasma. This allows determination of the sensitivity limit of the QD luminescence diagnostics, limits of concentration linearity and dynamic range.

3.1. Progress: Our goal is to identify a sensitive and specific panel of biomarkers employing reproducible and robust technology at a reasonable cost so it can be applied as a screening test to the general population. In the last report we have investigated the chemistry of bound versus unbound QDs in ten ways: 1. ELISA; 2. Different types of QDs; 3. Same AB, different QDs; 4. Same QD, different ABs; 5. Storage time; 6. Temperature; 7. Spectral mapping; 8. Gel electrophoresis; 9. ‘Home-made’ QDs; and exploring alternatives - 10. Novel technologies and additional biomarkers.

We have a paper in press on the application of the bio-conjugated quantum dots (QDs) for “sandwich” enzyme linked immunosorbent assay (ELISA). QD-ELISA can detect PSA antigen at concentrations as low as 0.01ng/ml which is ~50 times lower than the classic “sandwich” ELISA was demonstrated. Scanning photoluminescence (PL) spectroscopy was performed on dried ELISA wells and the results compared with the same QD samples dried on a solid substrate. We confirmed a “blue” 37 nm PL spectral shift in a case of QDs conjugated to PSA antibodies. Increasing of the “blue” spectral shift was observed at lower PSA antigen concentrations. The results can be used to improve sensitivity of “sandwich” ELISA cancer antigen detection. Currently, the threshold of PSA AG detection for “sandwich”-ELISA with organic dyes, but for many ELISA kits the threshold is 1 ng/ml [1-4], which is usually low enough for early cancer detection, but “sandwich” ELISA with QDs could possibly detect as low as 0.01 ng/ml of PSA AG. One of the current problems in QD usage for biomedical applications is that bioconjugation reactions may be incomplete and result in residual non-conjugated QDs in the same bio-conjugated solution. Our recent experiments demonstrated that PL spectra of QDs are changed by bioconjugation [5-10]. This is manifested as a blue or short-wavelength spectral shift of the PL maximum, which can be clearly observed as a color change of the dried bioconjugated sample. This unique spectroscopic feature of the bioconjugated QDs may serve as a fingerprint of the bioconjugation reaction. Ultimately, this will

dramatically improve sensitivity of biomolecules detection using QDs because a background PL from the non-conjugated QDs can be spectrally separated. In this paper, we report on PL spectroscopic study of the dried “sandwich” ELISA wells, utilizing QDs to detect PSA AG at the concentration range of 0.01 – 1.0 ng/ml. The agarose gel electrophoresis technique with the organic dye fluorescamine is employed to assure the quality of the conjugate, used for “sandwich”-ELISA technique. A “blue” spectral shift magnitude was found to be larger for ELISA “sandwiches” in comparison to the same bio-conjugated QDs, dried on a flat silicon substrate. This effect can be attributed to the elevated stress applied to the individual QD, involved into the “sandwich” formation, as well as more or less efficient, but probably not complete, nonconjugated QD elimination caused by the washing procedure. The PL signal from the bio-conjugated QDs was found in all wells, containing AG, with different concentration but was undetectable in the control well without AG. It is expected that the results can provide strong benefits for early cancer detection and forensic technology.

3.2.2. Materials and methods

3.2.1. QDs, ABs, conjugation and agarose gel electrophoresis Commercial 705 nm CdSeTe/ZnS core-shell PEGylated (with covalently coupled Polyethylene glycol molecules on top in order to produce non-ionic surfactants) QDs covered with a polymer were obtained from Invitrogen Inc. Commercial monoclonal PSA ABs and AGs were obtained from Millipore Inc. The conjugation procedure was performed using a commercially available 705 nm QD conjugation kit [2]. The main steps of this procedure are also described elsewhere [3-4]. Uncoated polystyrene ELISA wells were purchased from NUNC company (part of Thermo Fisher Scientific). All buffers in stock solutions (coating, blocking and washing) were purchased from Immunochemistry Technologies LLC. The agarose gel electrophoresis technique with fluorescamine was employed to verify the conjugation reaction was explained in our previous work [5-10].

3.2.2 The “Sandwich”-ELISA method : Wells were coated with capture PSA AB by adding 50 μ l of 5X diluted coating buffer mixed with AB in concentration 7.5 μ g/ml. The amount of coating AB was taking in excess to provide a complete coverage of bonding sites. The wells were then covered with aluminum foil to prevent light exposure and incubated for 20h at room temperature (RT) under constant slow rotation. After that coated wells were washed 3 times with washing buffer and incubated with blocking buffer (300 μ l/well) at the same conditions to ensure blocking of all unused sites on the well, available for further protein bonding. This stage was also followed with 3-times washing, and immediately proceed to AG solution addition. 50 μ l of PSA AG solution was added to wells #2- 4 in the following concentrations: well #2 – 1.0ng/ml; well #3 – 0.1ng/ml; well #4 -0.01ng/ml; and well #5 was a control – pure PBS (pH 7.4) added, no AG. Well #1 was loaded with 5 μ l pure nonconjugated 705nm QD solution and let dry on air. The wells #2 to #5 were incubated in the same conditions for 12h, washed 3 times with washing buffer, and the 50 μ l of 2X diluted AB*QD solution was added immediately, incubated for 12h at the same conditions, washed 3 times and let dry on air until the further spectroscopic analysis. The 2X dilution of a conjugate with QD incubation buffer was used in the effort to lower the expenses, associated with the experiment. QD*AB conjugated is very concentrated in ABs (300 μ g/ml of AB in the stock solution, used for conjugation, therefore, they are taken in excess even if the conjugate is diluted 2 times. QD concentration could also be a limited factor in effort to use diluted conjugate for ELISA. We assume however that further dilution is possible and research in this direction is currently in progress.

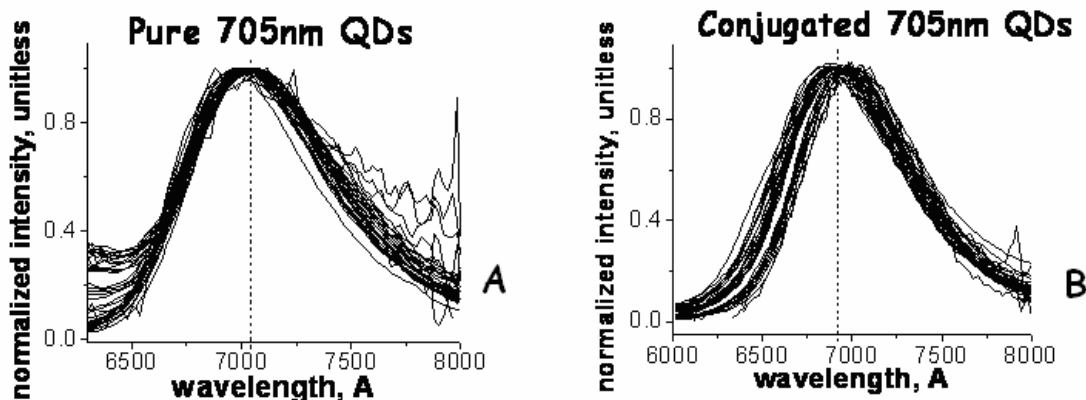
3.2.3. Samples and PL measurement After the final washing, ELISA wells were dried on air at room ambience for up to 120min, and then each well was stored in a clear plastic box in order to minimize contamination of the wells. In order to eliminate a contribution of conjugated QDs which may occasionally be attached to the walls of the well and have no relation to the “sandwich” formation, the bottom was separated from the walls using a clean heated blade which allowed an accurate cut. Spectroscopic measurements were conducted with ELISA bottoms only. We realize that this procedure is complicated and hardly to use as described in clinics, but it is quite suitable for the main purposes of this work – to research a possibility to form a “sandwich” with conjugated QDs, using lower AG concentrations, and study the scanning PL spectroscopy of bioconjugated QDs, involved in the “sandwich” formation. In all experiments, the conjugated and non-conjugated portion of same-sized QDs was used as a reference. Individual droplets of the nonconjugated and conjugated solutions of the 3 μ l volume were deposited on the unpolished surface of the crystalline Si wafer. Before the initial PL study, the droplets were dried in atmospheric ambience at room temperature forming clear spots on the silicon surface. Both ELISA sample and dried QD spots deposited on silicon were used for spectroscopic PL mapping with a smallest step of 0.5mm to produce a set of up to 100 individual PL spectra for each well and spot. The scanning PL spectroscopy was performed at room temperature using a 488 nm Ar laser with power density of 70 W/cm² as the

excitation source. ELISA samples or silicon wafers with deposited QD spots were mounted on a computer-controlled X-Y moving stage. The typical mapping area was 8mm x 8mm for ELISA wells, and 3.5mm x 3.5mm for dried QD samples. The PL spectrum was dispersed by a SPEX 500M spectrometer and recorded by a cooled photomultiplier coupled with a lock-in amplifier. A detailed schematic and description of the PL system can be found elsewhere [5-10].

3.3. Results and Discussion

3.3.1. PL spectral mapping on ELISA samples In order to accurately measure, record and analyze the PL signal across the samples, the spectroscopic mapping technique was employed. PL spectra, obtained in the process of the spectral mapping of ELISA wells, were compared with the spectra, obtained from identical batch of nonconjugated and conjugated QDs, dried on the silicon substrate. The results are shown in Figure 1.

Figure 1. Normalized PL spectra from the spectroscopic mapping on non-conjugated 705nm QDs (A) and bio-conjugated with PSA antibody 705nm QDs (B), dried on a silicon substrate. Dashed lines correspond to the PL peak positions averaged across the sample area.



It was shown in our previous work [5-10], that the “blue” spectral shift of different magnitudes on dried

bioconjugated QDs was observed on different QDs and different antibody molecules. The positive “blue” shift magnitude correlation with the antibody molecular weight was also found [29]. In this work, the spectral mapping procedure revealed a non-uniform peak position across the dried spot of the bioconjugated QDs ranging from 682 nm in the central part of the sample up to 706 nm at the periphery. This pattern was observed previously and assigned to a “plate-shape” effect. We will discuss below in more detail. The spectra, obtained from ELISA wells are shown in Figure 2.

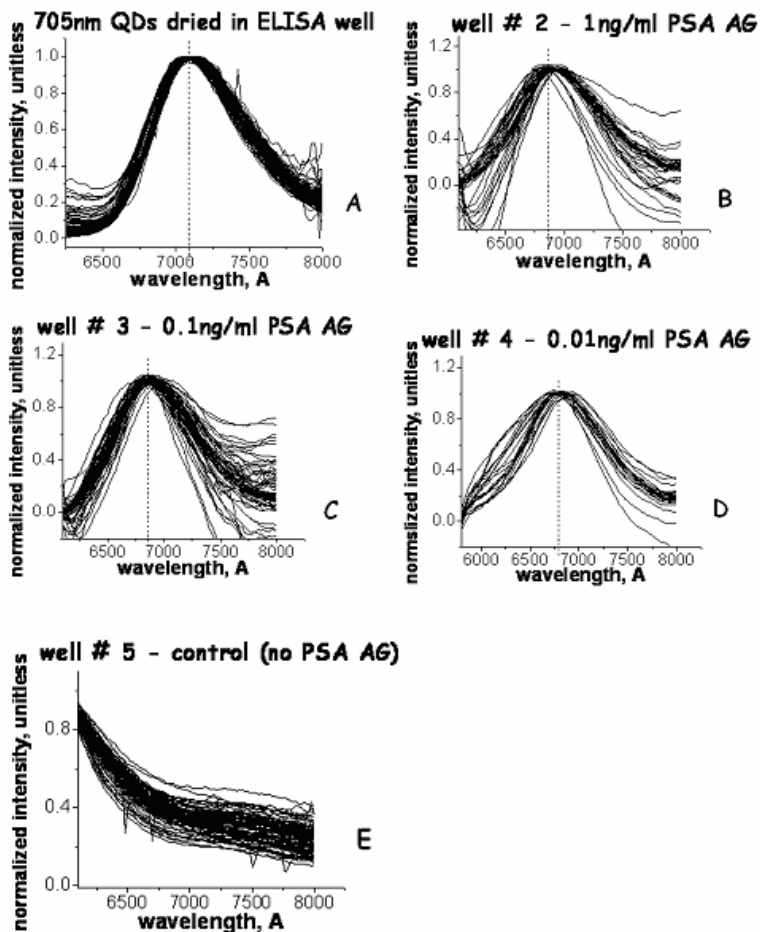


Figure 2. Normalized PL spectra measured on ELISA wells # 1-5 (A-E, respectively). Dashed lines correspond to the average PL peak positions. It is obvious that all wells, containing AG (B-D) provide the PL spectrum that matches to the QD luminescence.

In contrast, the control well # 5 without PSA AG shows a negligible PL peak intensity in the range of 575-800nm, although was loaded with the same amount of conjugated QDs and undergone identical washing regime. Clearly, the AB*QD conjugate in the well # 5 did not form a “sandwich” because of the PSA AG absence, and was therefore washed out. We point out that a residual optical signal observed in the well #5, is a spectroscopic tail of 488nm laser line,

scattered by the plastic well, and therefore has no relation to the QD luminescence. Well # 1 was included in the experiment in order to compare the “blue” spectral shift of conjugated QDs in comparison to nonconjugated. Well # 1 was precovered with primary AB in the same way as all other wells, but did not undergo any washing cycles, therefore, the PL spectral peak position of nonconjugated QDs, dried on the plastic ELISA well, could be taken from the well #1. All ELISA wells, which contained PSA AG, have a PL signal from the conjugated QDs, involved into the “sandwich” formation. It was obvious that PL intensity and PL peak spectral position are not uniform across the well area, and this nonuniformity was tracked with the PL spectroscopic mapping procedure. The spectral maps of the #2-4 ELISA wells are shown in Figure 3.

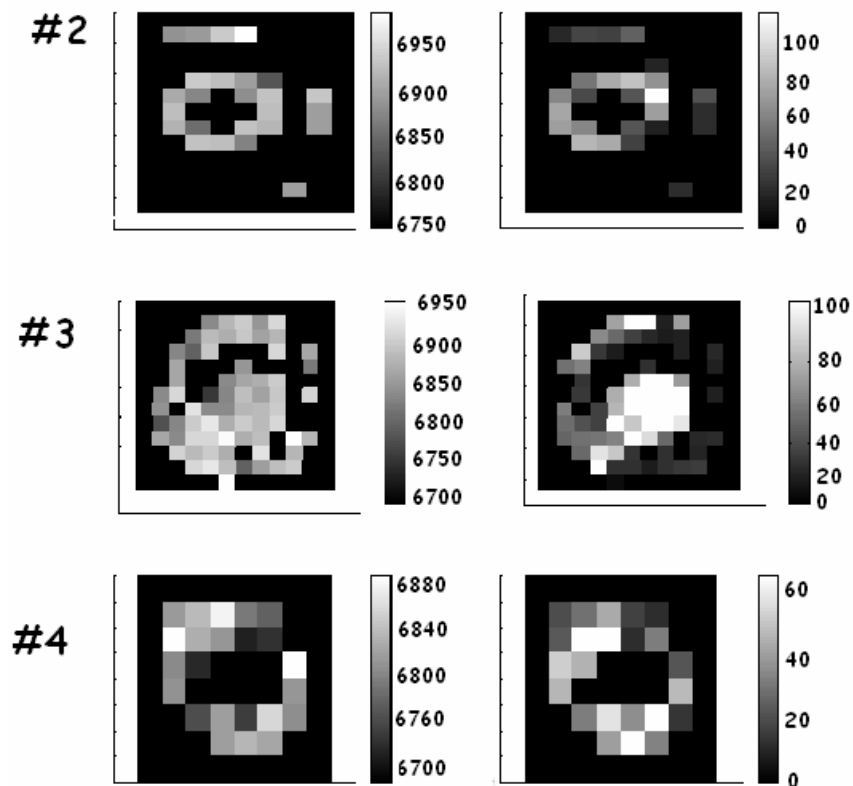
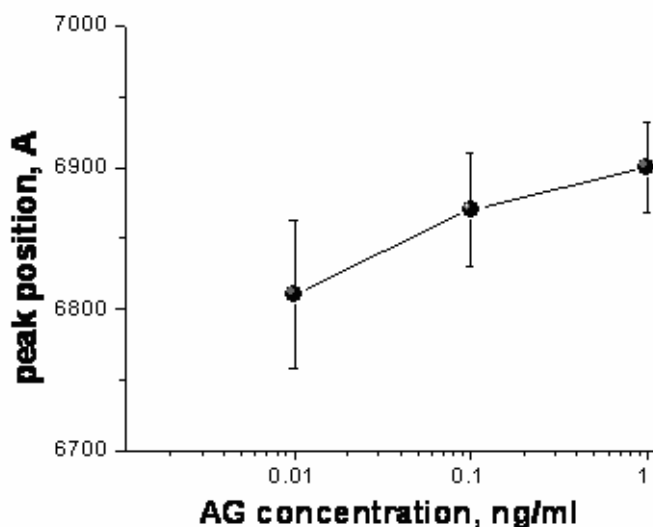


Figure 3. Spectral maps of the ELISA wells # 2-4. Left column – peak positions in A, right column – corresponding intensities [arb units]. The spectral map of the well #1 is not informative (because nonconjugated QDs were simply dried in the well), while the well #5 does not exhibit a measurable PL intensity from the 705nm PL band. The well #4 (with the smallest AG concentration of 0.01 ng/ml) shows the QD PL peaks mostly at the periphery area, the wells # 2 at the center, and the well # 3 all over the sample. This inhomogeneity can be attributed to a non-uniform capture of the PSA AG molecules by the capturing antibodies, when forming a sandwich structure.

In this experiment using the PL spectral mapping technique we observed a new effect, as a dependence of the “blue” spectral shift versus the AG concentration. In Figure 4 the average peak positions, along with the standard deviations, are presented.

Figure 4. Average spectroscopic peak (close to 705nm) positions and their standard deviations (presented as error bars) of the ELISA samples with different AG concentrations.



The average PL peak position is shifted to the short wavelengths with decreased AG concentrations, and its standard deviations are also increased with decreased AG concentrations. The average peak positions, along with their standard deviations, are as follows in wells # 1-4: 7080 (+10); 6900 (+32); 6870 (+40); 6810 (+52). Additional research in the effort to confirm this interesting effect is currently in progress.

3.3. “Plate-shape” effect and residual nonconjugated QDs As was described in our previous works [29-30], the authors found a so-called “plate-shape” effect on the QD samples dried on the solid surface (silicon). This effect was especially pronounced in conjugated samples. It means the different intensity and peak profiles across the area of a dried sample, with both intensity and peak positions being elevated in the periphery region and decreased in the center. The typical “plate-shape” effect for 705nm QD, conjugated to PSA AB, dried on the clear silicon chip, is shown on the Figure 5.

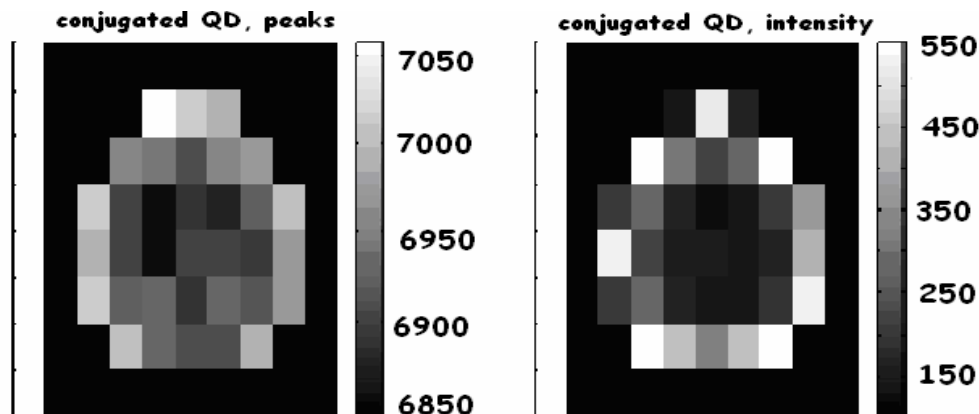


Figure 5. Spectroscopic peak position (A) and PL intensity (B) maps of 705nm QD sample, conjugated to PSA AB, dried on a clear silicon chip. Lighter areas correspond to elevated intensity/peak position values. We attributed this effect to either increased stress applied to QDs in the center, which may change their shape/size, or to the increased concentration of nonconjugated QDs in the periphery region,

which is caused by their increased mobility because of their small size in comparison to heavy and bulky conjugated QDs. In the effort to approve one of this hypothesis, an average peak position values along with their ranges were analyzed for ELISA wells #2-4 and conjugated sample, presented in Figure 5. The results are shown in the Table 1.

Table 1. Average peak positions for different conjugated samples and their magnitudes.

Sample	Average PL peak position, nm	PL peak range, nm
Conj dried on Si	694	682-706
Well #2	690	687-693
Well#3	687	675-694
Well#4	681	671-691

From the Table 1 it is clear that the upper limit of the sample, dried on the silicon surface, is 110-130A elevated in comparison to any of the ELISA wells. And what is even more important, is that the upper limit of the dried on Si chip conjugated sample surely lies on the very periphery (Figure 6A) and corresponds to the wavelength of emission of pure, 705nm QD sample, deposited on the silicon surface [5-7]. This helps to draw a conclusion that because only conjugated QDs take part in the ELISA “sandwich” formation, nonconjugated QDs, small fraction of which is always present in a conjugate, are washed away and don’t interfere with the PL from conjugated QDs. However, when a small droplet of conjugate is deposited on silicon, the nonconjugated fraction of QDs contributes to the PL signal. This observation is important, because allows to separate and eliminate the residual nonconjugation QDs, and their PL signal which may interfere with conjugated QDs and cause false positive results. It is also important, because serves as an additional proof that the PL, coming from ELISA wells, is the PL of conjugated 705nm QDs, involved into the “sandwich” formation, and not the residual QDs, got stuck into the plastic.

3. 4. Conclusions “Sandwich” ELISA bimolecular tool enhanced with conjugated 705nm QDs instead of commonly used luminescent dyes was employed to detect a PSA AG in the concentration range from 0.01 to 1.0 ng/ml. Three ELISA wells with AG concentrations 1.0, 0.1 and 0.01 ng/ml shows the PL peak, originated from the conjugated QDs in the sandwich structure with corresponding AG (PSA). The control well without AG molecules shows a negligible QD PL intensity. A short-wavelength, blue PL spectral shift was observed on all ELISA wells, utilizing conjugated QDs, and the values of this spectral shift (13-37nm) were enhanced in comparison to the same QDs, conjugated to PSA AB, dried on silicon substrate (2-26nm). This fact was attributed to effective washing of nonconjugated QDs from the ELISA wells, which are always present in a conjugate mixture and therefore contribute to an average PL of the dried QD samples. In

addition, a negative correlation was observed between an average “blue” PL spectral position and AG concentration. The origin of this effect is under investigation and presumably attributed to decreasing of elastic stress at higher AG concentrations. If confirmed, this will benefit the ultralow limits of detection for biomolecules, because “blue” spectral shift will increase with AG concentration decreasing, making it easier to distinguish between PL coming from “sandwiches” and possible traces of nonconjugated QDs or other contamination at ultralow concentrations of a target molecule. This effect will also be useful in predicting the unknown biomolecule concentration based on the spectral shift position alone, eliminating a need for PL intensity assessment. The results of this research could lead to critical improvements for *in vitro* cancer antigen detection sensitivity using the QD luminescent tagging technique.

However, the cost of QDs and antibodies together is approx. \$800. From this two conjugations can be performed. This results in only a small volume of conjugated QD-AB and we can assay only about six samples. We performed experiments with different dilutions of the QD-AB conjugate of 1:10, 1:4, and 1:2 to determine if the QD-AB solution could be diluted but would still reveal detection of the proteins. However, the results were very inconsistent. The 1 in 10 dilution did not work at all (results not shown).

3. 5. Investigation of additional nanotechnologies: In the last annual report we revealed our interest in exploring two additional nanotechnologies. Microfluidic devices and Surface enhanced Raman scattering (Zhang Lab.). We have proceeded with the former technology. We have included two very experienced and well published researchers Dr. Shekhar Bhansali who also previously integrated PSA into a microfluidic device for prostate (and other) cancer detection [11-17] and Dr. Weihong Tan who is already performing the Cell-SELEX in prostate cancer and has identified aptamers to prostate cancer cell surface proteins [18-23]. We have been awarded funds to perform the following research. This is within the scope of the Specific Aims in this proposal and we will modify the Statement of Work to reflect this new direction.

The results of these investigations may have an impact on our final choice of biomarkers and technology used in the biomarker panel. The following is funded through a local collaboration with University of Florida.

Early circulating cancer cells can be identified in plasma and are thus a potential biomarker for early detection of prostate cancer. Novel highly sensitive and specific technologies are required as the concentrations are very low. Aptamers are modified nucleic acids with the ability to bind specifically to protein targets with very high affinity, similar to antibodies, and can be employed for cancer detection. The use of nanoscale materials and devices has enhanced the lower limits of detection of proteins and other compounds in plasma and tissues.

Our **overall objective** is to detect prostate cancer cells with high specificity and sensitivity in plasma from African American men using cutting-edge technologies so as to develop an early prostate cancer screening test.

In Aims 1 & 2 we will identify aptamers to novel prostate cancer cell surface proteins and characterize the target proteins.

In Aims 3 & 4 we will develop microfluidic devices incorporating our aptamers and assay the ability of these aptamer-based devices to predict prostate cancer in plasma from cases versus controls.

Therefore our aims are:

Aim 1: Identify two aptamers specific to prostate cancer membrane proteins from cell lines from an African American male We will employ cell-based SELEX (systematic evolution of ligands by exponential enrichment) (cell-SELEX) in which aptamers are selected from a library of random sequences of synthetic DNA or RNA by repetitive binding of the oligonucleotides to target molecules (cell line derived from an African-American man with prostate cancer (ATCC cell line CRL-2422)) for the selection of a panel of prostate cancer cell-specific aptamers. A counterselection and enrichment strategy is used to collect DNA sequences that only interact with the prostate cancer target cells but not the control cells (benign prostate cell line from an African American man (cell line RC-165N)).

Aim 2: Identify and characterize the proteins captured by the prostate cancer membrane protein specific-aptamers A three step process is employed using the aptamer in prostate cancer and non cancer cell lines: i. covalent binding of the aptamer with the target via photo-crosslinking, ii. magnetic bead conjugation, magnetic extraction and enrichment, iii. mass spectroscopic analysis and confirmation analysis.

Aim 3: To develop aptamer-based microfluidic device biosensors The chosen aptamers will be immobilized onto a gold nanowire biosensor surface with electrochemical detection. Upon protein binding, changes in the electrical properties in the vicinity of an electrode are detected.

Micro-fluidics refers to a subset of micro-electromechanical systems (MEMS) designed with one or more channels featuring at least one cross-sectional dimension that is less than one millimeter. Such devices are particularly advantageous to biomedical applications for several key reasons. Currently the cost of implementing present-day immunoassay technology is much greater than the cost of using micro-fluidic devices to complete the same task. Micro-fluidics chips are phenomenally smaller and more cost-efficient than the immobile technology that exists in analytical chemistry laboratories today. Secondly, the amount of sample material (blood, serum, etc.) that is required for a micro-fluidic biosensor to perform is significantly less than the quantities needed for traditional sampling techniques.

The reduction in sample size can be attributed to the comparatively minute volume of the channels. However, reduced sample size is only one of the benefits of developing micro-fluidic devices for biomedical testing and diagnostics. These types of lab-on-chip (LOC) micro-assemblies have recently led to the development of polymer micro-fluidic diagnostic devices that tout both smart capabilities and the convenience of disposability. LOCs have facilitated the start of a technological revolution in the way point-of-care testing is administered in clinical diagnostics. These devices are capable of producing diagnostic results in a matter of hours. Currently, wait times of a day or more are rather commonplace.

In the current state of the technology, lab-on-chip assemblies are being developed for use in the diagnosis of several diseases. Some of the current studies include investigations on micro-fluidic assemblies to test for AIDS, tuberculosis, severe acute respiratory syndrome (SARS), malaria, and pneumonia, as well as several other diseases that are considered to be infectious. LOC technology also demonstrates strong potential to be the benchmark for diagnosing non-communicable diseases such as cancer, leukemia, cardiovascular diseases, stroke, diabetes, and numerous disorders related to the central nervous system (CNS). The most recognizable example of micro-fluidics benefiting the biomedical testing industry can be seen currently in the field of diabetic testing. Point-of-care diagnostic systems based on biological micro-sensors are already used widely in patient homes to determine blood glucose levels in diabetic patients. Such testing does not require the presence of a health care professional as long as the patient is able-bodied enough to prick their own finger.

The particular micro-fluidic chip intended for use in this project is an electrochemical biosensor that can be both sensitive and selective in screening for particular bio-molecules. It immobilizes the bio-molecule of interest on the surface of aligned silica (SiO_2) nanowires on Au electrodes to monitor the levels of target substance in a sample. Nanowires are ideal for such applications for several reasons: superior electrical conductivity, large surface area-to-volume ratios, small diffusion times, and the capability to host functional groups on their surfaces. High surface area-to-volume ratios are particularly important increasing the response time and capture efficiency of the electrode. SiO_2 nanowires can be covalently modified by the addition of functional groups, which are molecules that facilitate linking to specific enzymes.

Research Design

MICROCHIP MANUFACTURING

Silica (SiO_2) Nanowires

Synthesis

SiO_2 nanowires are synthesized on a silicon substrate using a deposition step, followed by an annealing process. In previous research palladium (Pd) was deposited on silicon using reactive sputtering. Growth of the nanowires was facilitated in an autoclave by the vapor-liquid-solid (VLS) growth process. The VLS growth method is a form of chemical vapor deposition where material from a vapor is incorporated onto the substrate by means of a liquid catalyst. The source of the liquid catalyst material is usually a metallic alloy with a low melting point.

In this case, Pd will be deposited on the surface of prime-grade n-type Si wafers in the same manner as found in previous research with the exception that the area of the wafer to be coated by the sputtering will be adjusted to suit this project. Likewise, the annealing process will follow the format detailed in prior research. The coated Si sample was divided into multiple pieces and heated in an open-ended quartz tube furnace with Argon (Ar) as a carrier gas (flow rate 30 sccm). Annealing is accomplished in three temperature stages. The furnace temperature is ramped to 1100 °C before the samples are introduced. Once the samples are placed in the furnace, they remain heated for one hour. Subsequently, the annealed samples are allowed to cool naturally cool to room temperature.

Surface Modification

Surface modification is the procedure by which the nanowires are cleaned, activated, and functionalized by the deposition of the capture antibody. Modification begins with a 2% solution of 3-aminopropyltrimethoxysilane (APTMS) in ethanol that initiates the activation of the nanowires. APTMS precipitates onto silica substrates to form high concentrations of reactive organic compounds known as amine groups (they form from ammonia, NH_3). The particular pedigree of amine group formed in this process is a primary amine group. Primary amine groups only replace one of the hydrogen atoms in ammonia with a hydrocarbon group, which distinguishes them from tertiary amine groups. The activation process distributes amine groups evenly across the surfaces of the nanowires. Activation is followed by deposition of the capture antibodies. Once the capture antibodies have been successfully deposited on the surfaces, the nanowires are incubated.

MICRO-FABRICATION OF DETECTION CELL

Photolithography

Photolithography is used to transfer the electrodes to the substrate. The substrate material used is silicon wafer with (100) planar crystalline surface. In this method, a photo resist chemical is centrifugally spun onto the substrate. Subsequently, UV light is passed through a mask patterned with the geometry of the electrodes. Depending on which type of resist is used (either positive or negative), the resist is either strengthened or weakened. The illustration on this page shows how positive and negative resists work.

Formation of the electrodes on the surface is performed by electron beam evaporation. Magnets are used to focus the energy of an electron beam towards the bulk material. The energy from the impact causes the formation of a vapor. Initially, a thin (300 Å) layer of chromium facilitates adhesion of the Au to the substrate. Then Cr is followed by the deposition of the thicker (1500 Å) Au. A reference electrode is formed by coating the third Au. The reference electrode is important in the processing volumes.

Lift-Off

Lift off is the chemical process of removing photoresist from the surface of the substrate. The chemical lift-off comes from its ability to remove the deposition materials from the previous e-beam deposition. As the chemicals deteriorate the photoresist, the Au and Cr adhering to the surface of the photoresist are also washed away, leaving a clean and precise electrode pattern.

Research Methods

EXPERIMENTAL PROCEDURE

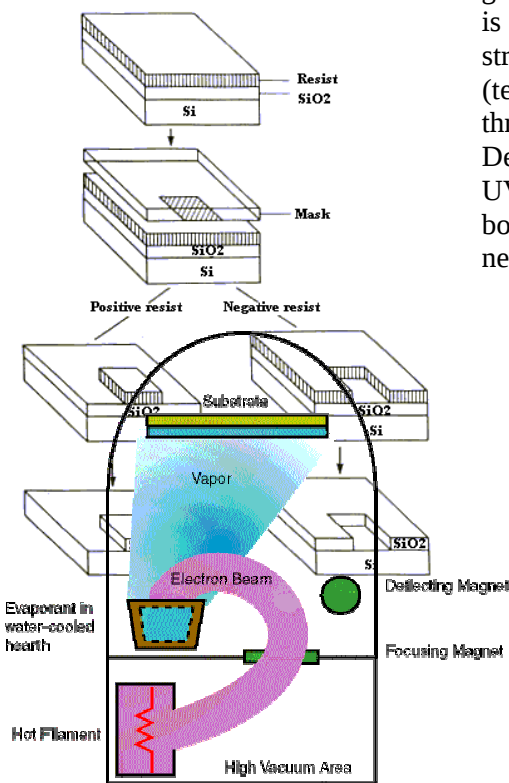
Fabrication

SiO_2 Nanowires

The n-type silicon wafers are cleaned using a standard RCA cleaning process before deposition of Pd can occur. Any contamination that exists on the Si wafer surface has to be removed by following specific processing steps in order to obtain high performance and high reliability semiconductor devices. In addition, the cleaning will also prevent contamination of process equipment, particularly the high temperature tubes and chambers used in oxidation, diffusion, and deposition.

The three steps of the RCA cleaning procedure are outlined below:

1. **Organic Clean:** removes any insoluble organic contaminants with a 5:1:1 $\text{H}_2\text{O}:\text{H}_2\text{O}_2:\text{NH}_4\text{OH}$ solution.



geometric pattern for the is a thermally oxidized structure. In this (termed “spin coating”) through openings in a Depending on which the UV light will either bonds. The illustration negative resists work.

the wafer is used to focus the to be deposited. The vapor that coats the (Cr) is deposited to deposition is layer of Au. Lastly, electrode with Ag. of small sample

the remaining utility of the unwanted deposition without

2. **Oxide Strip:** removes a thin layer of SiO₂ using a diluted 50:1 H₂O:HF solution. This discards remaining metallic contaminants from the previous step.
3. **Ionic Clean:** Removes ionic and heavy metal atomic contaminants using a solution of 6:1:1 H₂O:H₂O₂: HCl.

After RCA cleaning is complete Pd is sputtered onto the substrate at a power of 50W with a constant pre-sputtering of about 4 min. A base pressure of 1.33×10^{-4} Pa and a working pressure of about 2.66×10^{-2} Pa are maintained during deposition. For sample management purposes, the Pd-deposited Si wafer can then be cut into segments of a desired size, placed on a blank Si wafer, and annealed in an open-ended quartz tube furnace that utilizes Ar as a carrier gas (flow rate 30 sccm). There are three steps to the annealing process, and they are as follows:

1. Ramp the furnace temperature to 1100 °C
2. Insert the samples into the furnace and heat them for 1 h.
3. Allow the samples to naturally cool to room temperature.

The surfaces of the nanowires are then modified by immersing and soaking the substrate with the nanowires in a 2% solution of 3-aminopropyltrimethoxysilane (APTMS) in ethanol for 1 h. The samples are rinsed immediately afterward for a period of 5 minutes with ethanol. The capture antibody of choice is then deposited on the nanowire surfaces and incubated overnight at 4 °C.

Electrochemical Cell Manufacturing

The procedure for fabricating Au electrodes on the oxidized Si substrate wafer is outlined briefly below:

1. Photolithography
2. E-beam evaporation and lift-off: - Cr (300 Å) -Au (1500 Å)
3. Reference electrode was plated with Ag
4. Microfluidic chamber of SU-8 fabricated to hold the reagents. -Hard-baked at 180 °C for 3 min.

Aim 4: To test these novel aptamer-based probes in detecting prostate cancer cells in plasma from African-American men with and without prostate cancer. Once a test device has been developed and tested, we will mass manufacture probe devices and assay for prostate cancer cells in plasma from 100 prostate cancer cases and 200 controls. Statistical analysis will be performed to determine specificity and sensitivity of the probes by defining the area under the Receiver Operator Characteristic (ROC) Curves to determine the overall accuracy of each novel probe.

Significance: Improved and accurate prostate cancer detection in African American men would lead to a reduction in false-positive cancer results along with the subsequent unnecessary biopsy procedures, cost and anxiety placed upon the individual, and a reduction in false-negative results thereby reducing the subsequent morbidity and mortality. This study represents an important effort to effect a dramatic shift in the stage distribution of prostate cancer at the time of diagnosis and a consequent improvement in the current dismal survival rates for African-American men with this disease.

We have started the recruitment of the African-American prostate cancer case-control collection (Task 1). We have completed Aim 2 and 3 (Task 2 and 3). We have found that the QD-AB technology, can detect sub-ELISA levels of antigens such as PSA, it is very expensive and so is cost prohibitive for general population screening. So we have proceeded in a new direction still using nanotechnology and biomarkers in sera but using microfluidic devices. This will therefore change somewhat the choice of markers in Aim 3 and the overall assay infrastructure in Aim 4.

3.12. Summary: Task 3 as written is complete but we will modify the Task to include our new direction..

Task 4. To derive optimal cutpoints for the sensitivity and specificity of the QD-conjugated biomarkers developed in Aims 2 to 3 individually, and in combination, for the early detection of prostate cancer in African-American men. (Months 26-36 according to Timeline). Approach: Assay the levels of the individual biomarkers in serum samples collected during PSA/DRE screening using a nested case-control approach. We will use logistic regression and Receiver Operator Curves to define the cutpoints that collectively define the optimal performance of the entire panel.

4.1. Summary: Task 4 will be started once we have collected a significant number of cases and controls. The final statistical model will not be completed until the end of the no-cost extension in the latter half of 2009.

C. Key research accomplishments

1. Continued and consistent study recruitment.
2. Exposure in the press regarding the study and press conference at the AACR meeting. The press coverage continues and our study was highlighted in the Department of Defense annual report.
3. Achieved funding for a further year of study using aptamer technology and microfluidic devices.

D. Reportable outcomes

Press Release

Invited to attend a press conference in Disparities Research at the AACR annual 2008 meeting and the study was highlighted in the Department of Defense Prostate Cancer Research Program Annual Report and other journals and health magazines.

Publications:

1. Ganna Chornokur, Sergei Ostapenko, Yusuf Emirov, Nadezhda Korsunska, Abraham Wolcott, Jin Zhang, Catherine Phelan, Abhilasha Nagaram, Thomas Sellers. Biologically Engineered Quantum Dots for Biomedical Applications// MRS Proceedings Volume 1095E, 1095-EE08-05, 2008.
2. G Chornokur, S Ostapenko, Yu Emirov, N E Korsunska, T Sellers and C Phelan. Spectroscopic behavior of bioconjugated quantum dots// Semicond. Sci. Technol. **23** (2008).
3. G. Chornokur, S. Ostapenko, E. Oleynik, C. Phelan, N. Korsunska, T. Kryshchuk, J. Zhang, A. Wolcott and T. Sellers. Scanning Photoluminescent Spectroscopy of Bioconjugated Quantum Dots // Superlattices and Microstructures (in press) 2008.

Oral presentations:

1. February. 2008, 'Early detection of prostate cancer in African-American men.' FPCN Brother-to-Brother meetings, Municipal library, Martin Luther King Blvd, Tampa - Presenter Dr. Catherine Phelan
2. March 28-29th 2008, Gastrointestinal and Genitourinary Current Therapies and Clinical Trials Agenda 'Early detection of prostate cancer in African American men.' - Presenter Dr. Catherine Phelan
3. June 2008, BIAMS symposium, Toledo, Spain. The talk title: Scanning Photoluminescent Spectroscopy of Bioconjugated Quantum Dots. Presenter Dr. Ostapenko
4. August 7, 2008, Florida A&M University Graduate/ Ph.D. Student's Schedule. Presenter Richard Tanner, MSc
5. October 30, 2008, U. S. Department of State International Visitor Leadership Program, Moffitt Cancer Center. Presenter Dr. Catherine Phelan
6. November 2008 'Biomarkers' to Cancer Biology PhD students, Moffitt Cancer Center. Presenter Dr. Catherine Phelan

Abstracts and Poster presentations

1. March 2008, Materials Research Society Spring meeting, San-Francisco, CA. The poster title: 'Biologically Engineered Quantum Dots for Biomedical Applications.' G. Chornokur, S. Ostapenko, Yu. Emirov, A. Wolcott, J. Zhang, A. Nagaram and T. Sellers, C. Phelan.

2. April 2008, 'Nanotechnologies and early detection of prostate cancer'. American Association of Cancer Research, San Diego, April 2008. Catherine M. Phelan, Sergei Ostapenko, Anna Chornokur, Richard Tanner, Jin Zhang, Julio Pow-Sang, John Jack Steel, Thomas Sellers.
3. September 2008, 1st Annual Nanoscience Symposium "NanoFlorida 2008", Orlando, FL. The poster title: Bioconjugated QDs for early cancer detection. G. Chornokur, S. Ostapenko, Yu. Emirov, A. Wolcott, J. Zhang, A. Nagaram and T. Sellers, C. Phelan.

D4. Degrees obtained/contributed to by this study

Ganna Chornokur:

'Photoluminescence Spectroscopy of Bio-Conjugated Quantum Dots', Degree of Doctor of Philosophy, Department of Electrical Engineering, College of Engineering, University of South Florida - on-going. Main mentor: Sergei Ostapenko; Thesis committee member: Catherine Phelan

Marcus Marley:

'Microfluidic devices and their application to early cancer detection'. Completed March 2008.

Masters degree, Department of Electrical Engineering, College of Engineering, University of South Florida. Main mentor: William Lee; Thesis committee member: Catherine Phelan

D.5. Tissue or serum repositories – Frozen in Tissue Procurement. DNA, serum and plasma stored on all cases and controls.

D.6. Databases – Access database from the questionnaire and pathological data

D.7. Funding applied for based on work supported by this award

1. NIH R21 'Biologically Engineered Quantum Dots for Biomedical Applications.' PI Sergei Ostapenko (Not funded)
2. NIGMS R25 training grant for funding for minority predoctoral students. PI Bernard Batson (pending)
3. NSF 'The Application of Quantum Dots and Nanowires in Early Cancer Detection.' PI Sergei Ostapenko (Not funded)
4. NIH Health Disparities Center of Excellence P20. 'Aptamer-based microfluidic devices for early prostate cancer in African American men.' RO1 format PI – Catherine Phelan. Overall PI Lee Greene. (Under review)
5. Moffitt Cancer Center/University of Florida Joint Cancer Center Funding Opportunity Advancing the Partnership (AP) Awards. PI Catherine Phelan – funding begins January 2009 for one year - \$99,931

E. Conclusion

Implications of completed research: We are developing novel biomarkers, using cutting-edge technology, for early detection of prostate cancer in African-American men. We have characterized the chemistry of our QDs and ABs to determine the best combination for multiplexing. Unfortunately we have found this technology cost-prohibitive for use in a large population-based screening. We investigated another type of nanotechnology (microfluidic devices) and we will identify novel biomarkers specific to African American men. The samples collected in this study are essential for other studies in African-American men.

"so what section" – The novel, sensitive biomarkers generated in this study will be of vital importance for early detection of prostate cancer in African-American men to reduce the morbidity and mortality from this disease. We are applying novel technologies. Furthermore, we are applying our knowledge and these technologies for early detection of other cancer types.

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