High throughput Detection of Molecular Targets in Cancer

Using Nanoparticles: Application in Diagnostics

by

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A thesis submitted in conformity with

the requirements for the degree of

Doctor of Philosophy

Graduate Department of Biomedical Engineering

University of Toronto

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Abstract

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2008

In light of cancer as a multi-parameter disease, technological advancements are being developed to obtain automated and high throughput assessment of molecular targets in diagnostics, with the aim to reduce subjective analytical assessment of tumours and/or to integrate molecular data obtained from different tests on given tumour samples. Towards this goal, a multi-disciplinary approach is presented in this dissertation using nanotechnology. First, by combining quantum dot (QD) nanocrystals, tissue microarray, optical spectroscopy, and algorithm design, an automated and high throughput quantitative approach in the assessment of biomarker surrogates in neoplastic tissue is introduced. The validation study was performed using epidermal growth factor (EGFR) in 8 formalin-fixed paraffin-embedded (FFPE) xenografts of lung adenocarcinoma ($r^2=0.9$) (chapter 2). Furthermore, in chapter 3 a novel design of QD-based fluorescent probes, nanobeads, was presented by encapsulation of QDs in polystyrene in an emulsion polymerization reaction.
Nanobeads exhibited resistance to fluorescent quenchers such as solutions of extreme pH and colorimetric dyes of hematoxylin & eosin (H&E) and Giemsa, key standard dyes used in cancer diagnostic. Comparative studies with organic dye, phycoerythrin (PE), and QDs demonstrated significant ($P<0.001$) fluorescent quenching in H&E and Giemsa using Kruskal Wallis test. The design allowed a simultaneous consolidation of fluorescent immunoassaying, using nanobeads, and morphologic and parametric visualization of cellular features, using colorimetric stains, in a given biopsy. Sten-volmer analysis demonstrated a reduction of fluorescence resonance energy transfer among nanobead$_{610\text{nm}}$ and nanobead$_{560\text{nm}}$. The application of the QD-based methods bears potential significance in cancer diagnosis and in tumor management, especially human core biopsies of limited quantity. In chapter 4, cellular endocytosis of gold particles was shown to be size dependence, with gold particles of 50 nm demonstrating the highest uptake. The cellular response subsequent to gold uptake was assessed using 10k cDNA microarrays. Significant analysis of microarray (SAM) showed the gene expression of treated and non-treated particles to be 99.65% similar. The remaining 35 genes however, demonstrated down regulation of apoptosis, cell proliferation and cell adhesion responses in treated cells. While promising, future research and development are required to adopt nanoparticles in diagnostics.
Acknowledgements

I’d like to express my gratitude and appreciation to my supervisor Dr. Warren Chan for his support and guidance. I am also grateful for the opportunity he gave me to be a part of the exciting integrated nanotechnology and biomedical science laboratory. I gratefully acknowledge and thank Dr Ming Tsao for his co-supervision on my thesis project and advise throughout my research work. I would like to thank Dr. Chen Wang, Dr. Chris Yip and Dr. Craig Simmons for their support and for providing me with valuable comments throughout my program. As well, very special thanks to Dr. Brian Wilson and Dr. Gurmit Singh for their time and valuable critics on my thesis.

I would also like to thank my friends in the Chan lab. I am grateful to Dr Sebastien Fournier for his expertise in chemistry and his valuable collaboration on the bead project. Seb, thank you for helping chemistry make sense. My only advice, you might want to start thinking about learning French! Many thanks go to Dr Alex Lee for his expertise in algorithm writing and for being such a kind person. Thanks to Dr Travis Jennings for his computer wizardry, introducing me to the illustrator, and helpful discussions and directions on FRET and spectroscopy and his sense of humour. Travis, but I can not thank you for introducing me to joggling! Thanks to Tanya Hauck and Sawitri Mardyani for being such good friends. As well, thanks for many interesting discussions on science, nano, QDs, gold, TTC and making ginger bread house! It was fun! To
my desk-neighbors, Steve Perrault and Carl Walkey, thanks for interesting
discussions on science. Carl, I know that you are secretly happy that I am leaving
so you can use my desk! I’ would like to also thank Dr Qing Xiang and Jesse
Klostranec for their expertise and help in spectroscopy and Dr Robin Anderson
for her help with the Ninhydrin assay. Robin, thank you more for your
wonderful sense of humour. To Hans Fisher, thanks for his quantum dot
synthesis expertise. Collective acknowledgement and thank to Barbara
Alexander, Andy Hung, Wen Jiang, Dr Betty Kim, Dr Darcy Gentleman, David
Li, Jill Osborne, Karin Yaccato, Kevin Babb, Ryan Davey and Winnie Fung.
Thank You all for making my work fun and enjoyable.

I’d like to thank my friends, colleagues and wonderful people in OCI and
beyond for their help on various parts of my project: Dr. Patricia Reis, James Ho,
Dr Sarit Aviel-Ronen, Roya Navab, Keisha Warren, Lisa Leung, Jianing Zheng
and Ming Li.

I’d like to express my love and gratitude to my family, especially Majid,
who has always lovingly been by my side through times of frustration and joy.
Thanks to my happy dog, Fidel, for keeping me smiling.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AF</td>
<td>Autofluorescence</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
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<tr>
<td>AS</td>
<td>Antisense</td>
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<tr>
<td>Au</td>
<td>Gold</td>
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<tr>
<td>CCD</td>
<td>Charged-coupled device</td>
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<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<tr>
<td>CdSe</td>
<td>Cadmium Selenide</td>
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<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco Minimum Essential Media</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor</td>
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<tr>
<td>FDR</td>
<td>False Discovery Rate</td>
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<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
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<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
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<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
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<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
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<tr>
<td>FWHW</td>
<td>Full width at half maximum</td>
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<tr>
<td>G Phase</td>
<td>Gap phase</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GlyRs</td>
<td>Glycine receptors</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HAD</td>
<td>Hexadecylamine</td>
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<tr>
<td>ICP-AES</td>
<td>Inductively coupled plasma atomic emission spectrometry</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>LCM</td>
<td>Laser capture microdissection</td>
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<td>LIF</td>
<td>Laser-induced fluorescence</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>M Phase</td>
<td>Mitotic phase</td>
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<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>mRNAs</td>
<td>Messenger RNAs</td>
</tr>
<tr>
<td>NHI</td>
<td>Ninhydrin</td>
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<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NNI</td>
<td>National Nanotechnology Initiative</td>
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<tr>
<td>NOD/scid</td>
<td>Non-obese diabetic severe combined immunodeficient</td>
</tr>
<tr>
<td>PDADMAC</td>
<td>Diallyldimethylammonium chloride</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethyl methacrylate</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum dot</td>
</tr>
<tr>
<td>Q-RT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>S Phase</td>
<td>Synthetic phase</td>
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<tr>
<td>SAM</td>
<td>Significant Analysis of Microarray</td>
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<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TOPO</td>
<td>Trioctyl phosphine oxide</td>
</tr>
<tr>
<td>UHN</td>
<td>University Health Network</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>ZnS</td>
<td>Zinc Sulfide</td>
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Chapter 1

*Literature Review*
1.1 Oncology

1.1.1 Cancer and its Prevalence

The concept of cancer as a tumour was first described by Avicenna. In his book, *The Canon of Medicine* [1] [2], Avicenna noted that tumours progressively grew in size over time. He was the first to describe a surgical method for the removal of tumour mass including veins running to the tissue. The classical epidemiology study of cancer is dated back to 1775 when Sir Percival Pott [3] discovered a high rate of mortality among men who performed chimney sweeps. Pott’s observation led to two important discoveries: a potent carcinogen agent, tar, and that cancer can develop years after exposure to such carcinogen. The widespread use of the microscope was the key in shifting the study of cancer to the cellular level, a notion that led to the discovery of “tumour spread” from primary sites to lymph nodes.

Today, more than 11 million people are diagnosed with cancer each year [4]. Deaths from cancer worldwide are estimated to be rising, partly due to a longer lifetime, with a projection of 9 million people dying from cancer in 2015 and about 11.5 million in 2030 (World Health Organization) [5]. The most prevalent type of cancer worldwide has been lung cancer with 1.2 million new cases annually; followed by breast cancer, over 1 million cases; colorectal, 940,000; stomach, 870,000; liver, 560,000; cervical, 470,000; esophageal, 410,000; head and neck, 390,000; bladder, 330,000; malignant non-Hodgkin lymphomas, 290,000; leukemia, 250,000; prostate and testicular, 250,000; pancreatic, 216,000;
ovarian, 190,000; kidney, 190,000; endometrial, 188,000; nervous system, 175,000; melanoma, 133,000; thyroid, 123,000; pharynx, 65,000; and Hodgkin disease, 62,000 cases [6].

1.1.2 Molecular Etiology of Cancer

1.1.2.1 Impaired Cellular Proliferation

Where does cancer come from? What are the cellular mechanisms behind it? How do cancer cells acquire selective proliferative advantage in their microenvironment? The answer requires understanding the fundamentals of cellular functions and networks, a task that by itself is an ongoing and unprecedented challenge in science. Much of the understanding in that regard is attributed to the study of animal models used for exploring the basic cellular processes involved in the development and behavior of multi-cellular organisms.

In normal cells, homeostasis is achieved through cell cycle checkpoints whereby regulation of proliferation, growth, differentiation and apoptosis is achieved. Cellular regulation is governed during four cycles: Synthetic phase (S phase) associated with the duplication of genome and DNA synthesis, Mitotic phase (M phase) where chromosome segregation to daughter cells occurs, and Gap phases (G1 and G2) that function as inter-phases to the next cycle. In the event of extracellular signals such as mitogenic stimuli, G1 phase may be temporarily or permanently arrested, leading a quiescent G0 phase or apoptosis. The transition between phases of the cell cycle is governed by a family of proteins composed of
cyclin and cyclin-dependent kinase (CDK) molecules. The formation of a cycling-CDK complex is required for kinase activity, whose regulation is highly controlled by other mechanisms such as phosphorylation.

Increasing evidence suggests that dysregulation of the cell cycle is common during tumourigenesis [7]. Abnormal cell cycle regulation in cancer stems partly from genetic or epigenetic alterations of cyclins or CDKs [8], mutation or inactivation of their inhibitor [9], amplification or over-expression of positive regulators such as growth signals [10], or mutation of checkpoint and surveillance genes including those implicated in programmed cell death or apoptosis [11]. Indeed studies have shown that cancerous cells have a reduced ability to undergo apoptosis in response to physiological stimuli and DNA damage [7]. Through dysregulation of cell cycle and apoptosis, malignant cells acquire the ability to bypass the checkpoints imposed by normal regulatory processes, thereby promoting cellular immortalization and allowing rampant accumulation of cells with proliferative advantage.

More recently the discovery of MicroRNA (miRNA) [12] has shed light on their role in cell cycle regulation, or lack thereof in cancer. Often described as “messenger with license to kill the messenger” [13] miRNAs are 22 nt long non-coding RNAs that function in the regulation of gene expression by silencing messenger RNAs (mRNAs). The introduction of miRNAs in 2001 was immediately followed by the investigation of their potential role in cancer. Despite their low abundance in the human genome (only 1%), over 50% of
miRNA encoding genes are located within the genomic fragile sites and are associated with amplification, deletion and translocation in cancer [14]. Expression studies have demonstrated a global alteration of miRNA profile in cancer [15] [16]. More recently, Iorio et al., showed a cluster of miRNAs that were under-expressed in breast cancer tissues and that this reduced expression was significantly correlated with the pathological features of tumour stage, proliferation index, and the estrogen and progesterone receptors expression [17]. A number of studies have also shown the functional role of miRNAs as oncogenes or tumour suppressor genes in the development of cancer [18].

Johnson et al. reported the presence of multiple binding sites to let-7 miRNA in the 3’ untranslated region (UTR) of RAS and MYC oncogenes. let-7 was reported to negatively regulate the expression of the oncogenes by repressing their transcript [19]. Conversely, a different function of miRNA was discovered in lung cancer. A polycistronic cluster of miRNA, miR-17–92, was reported to be highly over-expressed in aggressive forms of small-cell lung (SCLC) cancer, and their transfection in A549 lung adenocarcinoma cell line was shown to induce cell growth, suggesting an oncogenic function for mir-17–92 miRNA [20]. The profiling of known miRNAs to specific cancer types or cancer states is an active area of research.
1.1.2.2 Models of Cancer Progression

Cancer is no longer considered a homogenous mass of cells. Indeed advancements in genomics and proteomics have uncovered the heterogeneous nature of cancer cells. But the lingering question remains: What leads to the formation of heterogeneous cell populations we collectively call cancer? The answer may lie in stem cells.

Direct evidence for the existence of cancer stem cells came from studies in which different subpopulations of epithelial tumour cells were identified. Only a proportion of these tumour cells had a unique and a highly tumourigenic phenotype (CD44+ CD24-) and exhibited the ability to form tumours in NOD/scid (non-obese diabetic severe combined immunodeficient) mice [21] [22]. These cells were also shown to have the ability to self-renew as well as give rise to other subpopulations of tumour cells with different phenotypes. This was taken to suggest that these tumourigenic cells, like stem cells, could undergo self-renewal and differentiation. Over the past decade, cancer stem cells have been identified and extensively described in pancreatic [23], colorectal [24], liver [25], brain [26], head and neck [27] cancers.

According to the “stem cell model” of cancer [22], in the process of tumour formation, normal stem cells undergo mutation resulting in the formation of “cancer stem cells”. Alternatively, somatic tumour cells may undergo differentiation to acquire “stem cell-like” properties. The abnormal stem cells, while retaining their inherent “self-renewal” ability, lose their normal
function leading to aberrant differentiation. This leads to formation of a mass of tumourigenic cells interspersed with non-tumourigenic neighboring cells (Figure 1.1). The identification and targeting of these aberrant stem cells for therapeutic purposes are active areas of research.

1.2 Cancer Diagnostics

The aim of molecular diagnosis in cancer is to relate the manifestations of disease to identifiable processes in cells, tissues, or organs. Traditionally, pathological examination of biopsies has been the key standard in the identification of tumour classifiers. In light of cancer as a polygenic, multifunctional disorder, an emerging paradigm in molecular diagnosis is the identification of biomarkers through genomics, epigenomics and proteomics approaches. The assessment of molecular signatures includes qualitative and quantitative testing, including morphological and phenotypic assessment of tumour.

1.2.1 Morphological Assessment

The current standard in clinical oncology is centered around morphological assessment of tumour biopsies, such as histopathological diagnosis, and medical imaging. In the former category, a primary classification of tumour is performed through staging; defining localization of primary tumour; measurement of the tumour size; involvement of lymph node metastasis; and assignment of tumour
Figure 1.1- Illustration of the Cancer Stem Cell Model. In the process of tumourigenesis, cancer stem cells acquire mutations while retaining their ability to self-replicate. Cancer stem cells may also give rise to progenitor cells that, similar to stem cells, have the ability to differentiate and proliferate. The aberrant function of cancer stem cells leads to increased genomic instability and phenotypic diversity among cell populations. The observation of cellular heterogeneity in cancer is consistent with the Cancer Stem Cell model. The model also elucidates the recurrence of cancer, or metastasis, due to the ineffective targeting of cancer initiating stem cells for therapy.
grade (the degree to which cancer cells resemble normal cells). At the cellular level, the morphological assessment of tumour biopsies is aided by “staining”: Colouring a previously uncoloured cellular structure, using colourimetric stains. Staining methodologies include nuclear staining and counter staining. The adjuvant function of counter stains is to differentiate between darkly stained nuclei and cytoplasm and extracellular regions. Staining augments the number of visible elements in tumours and provides an increased understanding of the biological entities. A more refined tumour grading is performed by a pathologist based on the structure and growth pattern of the cells. Grading factors vary with each type of cancer. Histological grades indicate the degree of morphological resemblance between the tumour and normal cells of the same cancer type. Nuclear grading indicates the shape and size of the nucleus and the fraction of dividing tumour cells. On a 4-scale basis: grades I, II, III and IV define well differentiated, moderately differentiated, poorly differentiated and undifferentiated cancers, respectively. The tumour stage is also determined based on the tumour size and shape, lymph node involvement and metastasis. These morphological examinations are central in the diagnosis, prognosis and determining treatment modalities in cancer. Paralleling the tumour staging of biopsies with the examination of biomarkers (where available) are essential diagnostic modalities in the assessment of cancer.
1.2.2 Proteomics

Proteomics is the study of cellular proteins expressed by a genome, their function and interactions. The aim of proteomics in oncology is to provide insight into the dynamic state of cellular transformation in cancer. This is achieved through the study of proteins implicated in cancer, their abundance, distribution, localization and interactions. The challenge however resides in identification of low abundance disease analytes at an early stage.

1.3 Emerging Cancer Detection Technologies

1.3.1 Oncoproteomics

Oncoproteomics refers to the application of proteomic technologies in oncology. Indeed technological advancements over the past decade have propelled oncoproteomics into an era of protein profiling. Jessani et al., demonstrated a rapid screening of proteins to measure the functional dynamics of protein kinase in breast cancer [28]. Global comparison of invasive and non-invasive tumours using protein chips further discovered a handful of proteases, lipases, and esterases involved in invasive melanoma [28][29]. Using DotScan™ microarray, an array of a cluster of differentiation antibodies printed as 10 nL wells on a slide, a comparison of immunophenotype from a tumour sample was performed to that of an unknown to create a library of consensus patterns. In diagnostics, DotScan™ is being developed for rapid classification of tumours based on their dot pattern, which indicates the presence or absence of known antigens [30] [31].
This method seems ideal in situations where cancer cells are presented in a limited quantity. Similarly, tissue microarrays have been invaluable in overcoming restrictions in molecular analysis of tissue specimen that include variation in diagnostic procedures and limited patient sample biopsy [32].

In diagnostics, the identification of signature makers using oncoproteomics is an active area of research. The aim is to obtain a collective profile of cancer that is suggestive of a discrete proteomic signature. The signature is, of course, more reflective of the biological phenotype of tumour than cellular heritage. It includes causal or consequential effects in the progression of tumours from a variety of origins and thus may represent attractive targets for the diagnosis and treatment of cancer.

1.3.2 Nanooncology

1.3.2.1 Definition of Nanotechnology

The term “Nanotechnology” was first coined 1974 by Norio Taniguchi [34]. In its current form, nanotechnology is a field in science that encompasses physics, chemistry, material science, electrical engineering and more recently biology. The premise of this multidisciplinary field is the manipulation of size and shape of particles on a scale of nanometers (10^{-9}m) to produce different properties associated with particles [35] [36]. The National Nanotechnology Initiative (NNI) defines nanotechnology as controlled creation of devices and systems with a dimension of 1-100 nm, that exhibit novel functions associated with their size.
The definition, therefore, contrasts particles such as DNA or monoclonal antibodies that exist in a nanometer scale but lack the “tunable” functionality.

In the past decade, the application of nanotechnology has reportedly offered remarkable potentials, ones that transcend disciplines in science and embrace, to name a few, medicine, diagnostics, tissue engineering, drug delivery, communication, chemistry, optics and yes, cosmetics [37]. The optimistic transforming view of nano application, however, is cautioned by others that attribute much of the creation of the nano Buzz to hype, fueled by institutional self-interest to garner financial support [38] or, in a lesser depreciative degree, to an effusive futuristic view of what nano can actually deliver. In medicine, notably in oncology, nano has been centered around in vitro diagnostics, therapeutics and in vivo imaging. Much needed comprehensive studies on environmental safety and bio-toxicity await further research.

1.3.2.2 Implications of Nanotechnology in Oncology

With the advancement in the field, nanotechnology manifests itself in a variety of applications in biology. Quantum dots (QDs) [36], gold particles [39], carbon nanotubes [40], liposomes [41], dendrimers [42] are examples of different nanoparticles. The small size of particles, customizability and multi-functionality are central to biological applications. Perhaps what makes nanotechnology bio-“philic” is the similarity of particle size to that of many biological entities;
hemoglobin, for instance is 5 nm in diameter and the lipid bilayer in cell membrane is 6 nm thick [43].

The advantage of particle size has shifted studies from broad to specific applications. Magnetic particles, for instance, reportedly offer a promising means to image metastatic lesions due to their ability to cross the permeable vascular epithelium [44]. As therapeutic agents, nanoparticles have been shown to penetrate the stomach-epithelium barrier or infiltrate the blood-brain barrier [45] [46] [47]. As a drug delivery platform, the size of nanoparticles enables them to escape the walls of splenic sinuses [48] or infiltrate through the fenestrae of the endothelial wall in the liver [49]. Through direct oligonucleotide labeling, QD particles have also been employed in multicolour fluorescent in situ hybridization (FISH) [50] and sub-nuclear imaging of the centromeres on interphase chromosome spread [51].

With the widespread excitement associated with the applications of nanoparticles, concerns have been raised on the potential impact of particles on cellular function. The hazardous effect of many elements or compounds used in the synthesis of nanoparticles has been known for decades. For example, cadmium, the metal element in some QD particles, has shown to have toxic and carcinogenic effects [52] [53]. From the toxicology perspective, however, particles of similar composition but different size may trigger biologically different responses. Studies indicate that inhalation of airborne particles of less than 100 nm in size, compared to coarse particles of the same composition, was associated
with enhanced level of pulmonary toxicity [54]. Other studies suggest the surface coating of nanoparticles may take precedence over their size in triggering an inflammatory response [55]. From the perspective of nanoparticle synthesis and design, the focus is to examine various surface coatings to increase biocompatibility of particles in physiological conditions [56] while providing adequate characterization and standardization of the particles properties [57].

1.4 QD Particles in Oncology

1.4.1 Composition and Optical Properties of QDs

The physical science of QD emerged in the 1980’s with the observation by Louis Brus and colleagues that nanocrystal semiconductors of the same composition exhibited different colours [58]. This led to his conclusion that "The band gap of semiconductors was experimentally a function of particle size". Indeed what differentiates QD from bulk semiconductor material in general is that the dimension of QD particles reaches the exciton Bohr radius of its constituent compound [59]. This state, defined as quantum confinement [60], is characterized by a discrete energy level for QDs of a given size (2-10 nm). As the size of QDs increases, the band gap energy associated with the particles also decreases, leading to size-dependant tunable emission spectra. Therefore, by changing the size of QDs from 2 to 10 nm, an array of colours can be detected (Figure 1.2).
The most prevalent composition of QDs is a Cadmium Selenide (CdSe) core with a Zinc Sulfide (ZnS) shell [61]. Over the past decade, the photoluminescent properties of CdSe/ZnS QD have become central in their recognition as molecular probes in biology. A notable difference between optical properties of QDs and that of their organic counterparts is their broad absorption and narrow emission profile. Organic dyes typically have narrow excitation spectra, a property that limits the excitation window to a narrow range of wavelengths. Moreover, the emission spectra of the dyes exhibit broad, asymmetric and red-tailing characteristics. The implication of this is signal overlap in the detection of multiple dyes. Conversely, QDs have broad absorption spectra, enabling excitation in a wide range of wavelengths. Furthermore, the narrow emission profile of QDs (e.g. 30 nm full width at half maximum; FWHW) allows the resolution of different emissions with minimum spectral overlap.

1.4.2 Photo-stability and Brightness of QDs

Photo-stability and brightness are other attributes of QDs. QDs are less susceptible to photo-bleaching compared to their organic counterparts [62]. This is because QDs have inorganic composition that resist “breakdown” during the processes of excitation and fluorescence emission. This property is well-suited
Figure 1.2- Size-dependence of Quantum Dot Emission. The fluorescence emission of QDs is characterized by discrete energy levels and is tunable based on the size of the particles. As the particles increase in size, their fluorescence emission shifts from blue to red in the optical spectrum. The size-tunable emission spectrum of QDs is attributed to the quantum confinement effect.
for applications where long-term imaging of biological sample is required [61]. The fluorescence lifetime of QD is 10 to 40 ns [63], longer than that of auto-fluorescence emitted from biological samples. The use of QD therefore, can lead to a significantly reduced background noise in biological imaging application.

### 1.4.3 Applications of QD Particles in Cancer

#### 1.4.3.1 Molecular Localization

Since their introduction in biological applications, QDs have been exploited extensively in the imaging of biological targets. In 2003 Science Magazine described QD bio-imaging technology as:

“One of the top 10 scientific breakthroughs of the year” and "...the most exciting new technique to emerge from the collaboration of physicists and biologists” [64].

The combination of brightness, photo-stability and multiplexing ability of QDs have made QDs ideal candidates for targeting bio-molecules. The photostability of QD is sought particularly in applications where long-term detection of a signal is required. Dubertret et al., examined the stability of QD fluorescence in tracking embryogenesis [65] [66] by injecting QD particles (5x10⁹/cell) into Xenopus embryos. The hydrophilic lipid coating of QDs rendered the particles bio-
compatible, as measured by cell size, viability and movement. QDs into blastomeres were successfully incorporated into the progeny of daughter cells and were detectable after 4 days.

The application of QD particles in bio-labeling and for molecular localization of targets has been exploited by many groups. Dahan et al., demonstrated the localization and diffusion dynamic of individual glycine receptors (GlyRs) in the neuronal membrane [67]. The use of QD enabled the acquisition of sequential images for the duration of 20 minutes and consequently characterization of three different locations of GlyRs. In other studies [68] [69], QDs bearing EGF (epidermal growth factor) ligand were used to study the receptor endocytosis of erbB/Her2 transmembrane receptor tyrosine kinase. This demonstrated that QDs were capable of binding and activating the erbB/HER2 receptor-mediated signal transduction pathway. This study further elucidated the kinetics of membrane transport and the dynamics of receptor activation and internalization.

Active in vivo tumour targeting was demonstrated by Gao et al. [70], aided by a novel design of QD particles. The QDs were encapsulated in an amphiphilic polymer that was in turn conjugated to PSMA, a prostate tumour-targeting ligand. The use of the polymer coating minimized the previously described limitation of fluorescence reduction in physiological conditions [71]. The complex was injected intravenously to mice transplanted with human prostate cancer cells. The QD appended to PSMA was shown to localize to tumour sites.
A parallel experiment with green fluorescent protein (GFP) demonstrated the brightness of QD compared to the GFP signals related to the tissue autofluorescence.

The application of QD bioconjugates in target detection was challenged recently by an immunoprofiling study, whereby the fluorescence detection of QD and organic fluorophore conjugates in oocysts staining were compared in a flow cytometry analysis [72]. The detection of cells using QD-conjugates exhibited significantly lower fluorescence intensity and higher non-specific binding compared to that of organic conjugates. This was taken to imply that, despite their advantageous optical property over fluorophores, QDs, in their current form, present significant limitations for conventional flow cytometry analysis. The direct QD-antibody conjugation to IgG in the latter study however, produced only partially successful conjugates as demonstrated by high level of non-specific binding.

The conflicting reports on the suitable application of QD in molecular detection fostered the need to consider other factors influencing QD fluorescence signal and reproducibility of the applications. Appending QDs to biomolecules is indeed a sophisticated task, one that requires meticulous expertise in the chemical and physical properties of the dots as well as the biology of conjugates. Knowledge of the parameters affecting the QD signals [73] is central in the proper application of the materials.
1.4.3.2 Multiplex Detection of Biomarkers

In light of increasing studies on the heterogeneous nature of cancer cells, multi-parameter, simultaneous analyses of many target proteins have become more appealing. The use of organic fluorophores for such multiplex analysis is limited by the relatively broad and overlapping emission spectra of dyes and the requirement for different excitation sources to account for the spectral overlap of the fluorophores [74][75][76].

QDs offer a suitable solution. Their broad absorption allows for simultaneous excitation of multiple colours, and their narrow emission facilitates the separation of signals. Many studies have exploited the properties of QDs and have demonstrated multiplexing, including imaging of hematopoietic cells [77], tumour vasculature [78], study of apoptosis [79] and gene expression analysis [80]. More recently, multicolour immuno studies [81] [82] demonstrated a significant improvement in such multiplexing capability by using a combination of QDs with fluorophores for the detection of 17 fluorescence emissions. This work led to characterization and enumeration of a population of T cells by polychromatic flow cytometry [83].

Multiplexed detection capability of QDs has extended its applications to put forward the notion of barcoding [85] [86] [87] [88] [89]. Multicolour QD barcoding is achieved by embedding QDs of different emissions into microbeads in a controlled manner. The unique features of barcoding are i) the ability to incorporate dyes with desired ratios into bead systems and ii) the
functionalization of the particle surface for facile conjugation of ligands to the surface of the beads. The encapsulation of different and controlled ratios of QD in each population of micro beads confers a unique optical signature to a collection of particles [85]. Each population of microbeads is conjugated to a specific target and detected using the spectral barcodes.

Encapsulation of organic fluorescent molecules of varying emissions has been reported in microbeads and is currently used in oligonucleotide assays [86]. The advent of QDs, however, has led to the refinement of barcoding technology. The attraction is in the ability to employ the multiplexing property of QDs and the ease of detection using one source of excitation. The encoding of QD of different emissions and intensities into microbeads has been demonstrated for detection with flow cytometry [87]. The combination of two colours and three intensities of QD have been used in the detection of high throughput, allele-specific oligonucleotides in nucleotide polymorphisms (SNPs) assays.

1.4.4 Fluorescent Energy Transfer between QDs

1.4.4.1 Fluorescence Quenching

Fluorescence quenching is a process whereby the fluorescent intensity of a given substrate is decreased via a number of processes. Dynamic quenching refers to a condition under which energy is transferred from a donor molecule to an acceptor molecule at an excited state. Förster resonance energy transfer [90] [91] is an example of such quenching. In static quenching, the donor and acceptor
bind to form a non-fluorescent complex. Planar aromatic dyes are reported to enhance static quenching due to overlaps of their empty orbital [92].

1.4.4.2 Förster Resonance Energy Transfer

Förster resonance energy transfer (FRET) [90] [91], named after Theodor Förster, is a process between two molecules, whereby energy from an excited fluorophore (donor) is transferred to an acceptor fluorophore (Figure 1.3). Three distinct conditions need to be met in order for FRET to occur: i) an overlap of the donor emission spectrum and the acceptor absorption spectrum, ii) a 10 -100 Å (1-10 nm) distance between the donor and the acceptor, iii) a parallel dipole orientation of the donor emission dipole moment and the acceptor absorption dipole moment (for optimal energy transfer). In molecular imaging, FRET is presented as an invaluable tool to measure the proximity of molecules to which fluorophores attach. In biology, FRET has been credited for monitoring intracellular ion sensing [93] and downstream events of cellular signaling pathway [94], dimerization and localization of receptor-ligand pair [95], conformation, hybridization, and automated sequencing of nucleic acids [96].

The unique broad excitation spectra and tunable narrow and symmetric photoemission of QDs has reportedly enhanced the application of FRET. This is attributed in particular to the ability to “tune” the fluorescent emission of QDs that in turn, allows for a better control of the spectral overlap with a particular acceptor in FRET assays [97]. Secondly, the ability to excite a population of QDs
at an excitation wavelength far distant (>100 nm) from their respective emissions leads to an extensive multiplexing ability for FRET studies [98].

The transfer of energy from a donor to an acceptor QD, although valuable in FRET-based studies such as demonstration of physical proximity between cellular entities, may pose limitations in the applications of QD in biology. The fluctuation of emission signal in immunofluorescence studies may lead to a false interpretation of the level of biomarkers (demonstrated in chapter 2). One plausible solution in the reduction of FRET effect is the modification to the current quantum dot design (demonstrated in chapter 3) to allow physical separation (> 10 nm) between QDs of different emissions. FRET in QDs is illustrated in Figure 1.3.

1.5 High Throughput Molecular Approaches in Clinical Diagnosis of Cancer

Science has long pondered on the existence of variation. Nearly a century ago, Sir William Osler (1849-1919) offered the following sentiment:

“Variability is the law of life, and as no two faces are the same, so no two bodies are alike, and no two individuals react alike and behave alike under the abnormal conditions which we know as disease.”
Figure 1.3 – Förster Resonance Energy Transfer (FRET) between Quantum Dots. FRET is characterized by the energy transfer from the donor chromophore in its excited state by dipole coupling mechanism to an acceptor chromophore. A condition of FRET is close proximity (typically <10nm) of the donor and acceptor. Owing to their small size, quantum dots are effective FRET inducers. Top: upon spatial proximity of quantum dots of different emissions, the energy from an excited donor quantum dot (green) is transferred to an acceptor quantum dot (red). Bottom: FRET leads to a decrease of the donor fluorescence emission (QD\textsubscript{560nm}) and an increase in the acceptor fluorescence emission (QD\textsubscript{610nm}). The shaded green and red spectrographs demonstrate the individual emissions of QD\textsubscript{560nm} and QD\textsubscript{610nm} and the grey spectrograph line represents the simultaneous emission detection of the same quantum dots and the effect of FRET.
Variation presents itself in a diverse vignette. Biodiversity is perhaps the most recognized form of diversity. In a study led by National Science Foundation in 2007, the significance of biodiversity was equated to a long-termed notion of genetic diversity: “If any one type is removed from the system, the cycle can break down, and the community becomes dominated by a single species” [99]. In the forefront of medical discoveries, variation is depicted as population polymorphism, inter-tumour heterogeneity, and finally extending itself to the smallest core of the human body, the cell, in the form of intra-tumour heterogeneity. The question becomes: how does one implement a test to discover a disease or offer remedies, given the well-founded notion of “variation”?

Recent advances in genomics and proteomics have highlighted the heterogeneous nature of cancer and presented the significance of coalescing biological information gleaned from these approaches. In fact, appreciation of the magnitude of heterogeneity in cancer has propelled molecular profiling into a high throughput assessment era. The premise is a top-down approach to facilitate identification of gross alterations. It should be noted that the definition of high throughput “molecular assessment” has evolved from and surpassed the previous notion of high throughput “screening”. The latter defines screening of a large number of biological samples in a given test. The newly founded notion of high throughput molecular assessment also includes combining different biological information obtained from on a given sample biopsy with the aim to increase the biological information obtained from the patient. More recently,
high throughput approaches such as microarrays and multiplexed RT-PCR assays have been implemented in diagnostics to monitor the activation of multiple genes in regulatory pathways [100] [101] [102]. Tissue and protein arrays have minimized a significant component of experimental variation and reduced the processing time of biological analytes [103] [104].

High throughput assessment may be central in reducing inherent sources of variations in testing regimens. In the clinic, variation exists in “analytic” and “pre-analytic” measures. The former is often discussed as inter- and intra-observer variability in the pathological assessment of tumour biopsies [105] [106]. Pre-analytic measures are conditions that take place prior to the clinical assessment of samples (i.e. during sample preparation or preservation). Profound influences of such variables on the fidelity of clinical tests are well documented. Variables such as titration and potency of antibody largely affect the distribution of chromogenic reaction as determined by immunohistochemistry (IHC) in diagnostic applications [107]. The inadequacy of this approach is exemplified by challenges facing the previously described notion by the National Institute of Health (NIH) consensus that the presence of any level of ER protein granted the classification of tumour as ER+ and warranted an endocrine therapy in breast cancer [108]. Other attributes to clinical variation include duration and nature of fixation of tumour samples; the storage period of unstained slides prior to fluorescent or chromogenic histochemistry; and the nature of epitope retrieval methods such as heat or proteolysis enzyme-
induced antigen recovery methods. Indeed, studies have reported a significant increase in immunostaining intensity of tumour samples as the duration of formalin fixation time increases [109] or an increase in the degradation of sample as the time prior to fixation increases [110]. Similarly, genetic testing is inherently subject to batch-to-batch variability in the preparation of chromosome samples; drying rate, relative humidity, and age of metaphase spreads can profoundly influence the overall success of karyotype tests [111].

With the transition to a comprehensive, real time high throughput molecular diagnosis, a novel concept in cancer is emerging as personalized medicine [112] [113] [114]. The argument is that current methods are based on predictive prognoses that encompass known clinical outcomes; identifying the classes of alterations known in cancer and determining whether the modifications correspond to a defined clinical outcome. Personalized diagnosis however, aims to survey overall differences between recurrent and remitted cancers, before and after a diseased state, or responsive and non-responsive cancers to therapeutic agents. The aim is to develop molecular classifiers, using comprehensive functional genomics and proteomics, into a score to predict an outcome such as survival, metastasis or resistance to therapy, with the ultimate goal of matching patients with a customized treatment regimen.

The obvious attractions of a high throughput approach to diagnostics are currently accompanied by significant technical limitations. Development of novel strategies and improvement of current methodologies in the examination of
biopsies are central in advancements to the field. Furthermore, the enormity of biological data generated from high throughput tests calls for effective data management and processing strategies, while the collection of comprehensive personal medical data may pose ethical concerns. The recognition of those limitations however, is an initial step in moving forward the era of molecular oncology.

1.6 Rational and Objectives

Cancer is often only diagnosed after its signs and symptoms drive the patient to the clinic. Currently the gold standard of cancer diagnosis is physical examination, imaging and pathology/molecular assessment of biopsies. Molecular diagnosis, although valuable, provides limited insight into the unique molecular profile of a given patients disease. This is further complicated by diagnostic and prognostic heterogeneity in the patient populations.

The National Cancer Institute has set a goal to eliminate suffering from cancer death by 2015. A roadmap to achieve this goal is through the development of novel cancer diagnosis and therapeutics. Developing novel techniques to detect molecular signatures in a high throughput manner may improve large scale automated detection and evaluation of biomarkers. High throughput based-studies are ideal candidates for such complex disorders, as they acknowledge the heterogeneous nature of cancer and take advantage of
unprecedented data that can be generated and used to study the progression of disease. Owing to its unique attributes, nanotechnology may enable the refinement of diagnostic techniques and the introduction of novel approaches to examine biological analytes. The purpose of this dissertation, therefore, is to examine the application of nanoparticles and develop novel designs of particles tailored to specific diagnostic modalities in pathology, with the overall move towards high throughput assessment.

This thesis is prepared into 4 chapters. Chapter 1 presents a review of cancer as it pertains to diagnostics, along with relevant terminologies. An overview of nanoparticles is given, along with specific properties of QD nanocrystals and their application in oncology. The combined use of high throughput diagnostics with the use of QD as an alternative modality is discussed. In chapter 2, the application of QD nanoparticles in combination with optical spectroscopy and tissue microarray is demonstrated in a high throughput readout methodology to measure protein abundance in tissue microarrays. Two criteria of QDs were examined in this chapter: the photostability of the dots was used to obtain stable, non degrading level of signal per analyte. The brightness of QDs was employed to achieve high signal over autofluorescent noise. Chapter 3 describes the development of a novel design of QD particles to coalesce two standard diagnostic techniques on a given sample: examination of morphology and immunophenotype of cancer biopsies. This was achieved by encapsulation of QD particles in polymer in the form of nanobeads. The structure of the
nanobead acted as an impervious coat to entrapped QDs and led to preservation of their fluorescence signal and minimized the effect of fluorescent energy transfer. In Chapter 4 the potential application of gold particles in biology is demonstrated by measuring the size dependent endocytosis of gold nanoparticles and a high throughput readout of cellular expression induced by intracellular uptake of gold nanoparticles using cDNA copy number alterations. In Chapter 5, an overall summary and discussion of future directions is presented.
Chapter 2

Application of Quantum Dot Nanocrystals in Automated High Throughput Quantification of Protein Expression in Cancer

This chapter has been partially discussed as:


This article was selected and discussed as “Brightening Tumour Analysis” in Highlights of the Recent Literature, Science 2006; 314: 5806.


Author Contribution:

The experiments in this chapter were carried out by A. A. Ghazani. The algorithms (D-noiser, Normalizer and Multiplexer) were designed by A. Ghazani and written by Dr. Alex Lee. The denoising smoothing functions were designed and written by Dr. Alex Lee. J. Klostranec and Q. Xiang helped with the set up of the laser equipment. The work was done in collaboration with Dr. M. Tsao.
2.1 Introduction

A tissue microarray (TMA) is an invaluable tool for simultaneous histological analysis of hundreds of archival tumour biopsies [115] [116]. Current research in cancer proteomics includes microscopic examination of hundreds of tissue samples and evaluation of protein expression levels. The intensity of cancer markers, as determined by visual analysis of colourimetric stains, is used to define a numerical score for staging of tumour biopsies [117]. In quantitative immunohistochemistry [118], a pathologist assigns a numerical scoring of 0 to 3 (i.e., nominal values of 0, 1, 2 or 3) for quantification of tumour antigens (Figure 2.1), with aggressive lesions being given a higher number demonstrating a higher grade [119] [120]. The assessment of antigen expression using this method is on a discontinuous scale. Moreover, the human eye is not capable of discerning subtle differences of the antigen expression level. This is particularly the case in a high and low end of the expression scale. These factors can subject scoring to inter- and intra-observer variability [121].

To circumvent or minimize the pre-analytical and analytical limitation, several automated methods have also been used to determine localization and quantification of target antigens [122] [123] [124]. The precept of these techniques is protein expression analysis through acquisition of fluorescence images obtained from conventional fluorescence dyes. However, the signal intensity and thus the accuracy of this quantification method can be compromised by the photobleaching property of fluorophores [125]. Furthermore, the sensitivity of
Figure 2.1-Examples of different intensity of immunohistochemistry (IHC) staining. The intensity of staining against epidermal growth factor receptor (EGFR) demonstrates the differential levels of EGFR in different tumour samples. The intensity of tumour cores was numerically assessed in nominal values as 0, 1, 2, and 3.
quantitative analysis of protein expression can be affected by tissue autofluorescence. In archival tumour samples, tissue autofluorescence is due to light absorption and scattering from endogenous proteins such as elastin, fibronectin, and collagen [126] [127] [128], from nucleic acids [129] and from tissue preparation processing and fixation [130]. Given the excessive photobleaching and low signal-to-noise ratios of fluorescent dyes [131] they may be poor candidates for antigen quantification studies. Thus, the feasibility of a sensitive expression profiling and in situ quantification of cancer markers in formalin fixed paraffin embedded (FFPE) biopsies, the most valuable archival tumour specimen, is restricted by the properties of fluorescent molecules and techniques currently used in the laboratory and diagnostic settings.

With the advent of nanotechnology, QD nanocrystals may hold a promising approach for the quantification of antigen-derived markers in FFPE tissue samples. QD fluorescence is less prone to photobleaching as compared to traditional fluorophores [132]. This could lead to more sensitive and accurate signal detection. In comparison to organic dyes, QDs have a molar extinction coefficient on the order of $(0.5-5) \times 10^6$ M$^{-1}$ cm$^{-1}$, about 10-50 times larger than that of organic dyes [133][134][135]. Consequently, QDs can absorb more photons at the same excitation photon flux and could yield brighter signals compared to traditional organic dyes [136], the implication of which is minimizing the autofluorescence effect in biological imaging applications. The use of QD in quantitative signal analysis has been demonstrated by others in
gene expression profiling studies [80] and high throughput genotyping assays [137].

In this chapter, we examine the application of QD in protein quantification. We further demonstrate the design of a system using QD bioconjugates in conjunction with optical spectroscopy system and tissue microarray (TMA) for automated, high throughput analysis of cancer antigens. The automation of the system was achieved by developing a series of algorithms to allow analysis of tissue autofluorescence, cellular content and multiplex antigen expression.

2.2 Materials and Methods

2.2.1 Cell lines and Xenografts
Xenografts of human lung tumour and biological information (i.e. xenograft types, and endogenous RNA) were provided by Dr. Ming Tsao Laboratory. They included FFPE xenografts from lung carcinoma cell lines of MGH7, RVH-6849, A549, H460, H1264, MGH8, H520 and H157.

2.2.2 Tissue Microarray Construction
The H&E slides for each tumour were examined and the areas of high tumour cellularity were selected for microarray construction. For each case, 3 to 4 cores were punch-extracted from formalin fixed paraffin embedded tissue blocks and mounted in linear arrays in a recipient master TMA block, using a Tissue
Microarrayer (Beecher Instruments). The blocks were then processed and cut for staining at 2µm using a microtome (HM325, Microm) and placed on microscope glass slides.

2.2.3 QD Immunostaining

Formalin fixed paraffin embedded tissue array slides were de-waxed at 60°C overnight, treated with xylene to remove paraffin and rehydrated in 100%, 95% and 70% alcohol for 5 minutes each. The slides were subsequently blocked with 1XPBS/1%BSA for 1 hour or avidin and biotin for 15 min each (Vector Laboratories). For human antibodies to E-Cadherin (1:100, Vector Lab) and pan-cytokeratin (1:200, DAKO), heat induced proteolytic epitope retrieval was conducted in 10 mmol/L citrate buffer (pH 6.0) in a pressure cooker (Nordic Ware, Minneapolis, MN, USA) for 10 minutes at 120°C. For staining with human epidermal growth factor receptor (EGFR; 1:50, Zymed), slides were treated in 0.4 % pepsin (pH 2.0) at 42°C for 5 minutes prior to PBS wash. Primary antibody incubation was performed for 1 hour or overnight at RT followed by 3X rinse in Tris-buffered saline (pH 7.6). For QD-based staining, detection was performed with 20nM QD$_{565nm}$-IgG, QD$_{605nm}$-IgG and QD$_{565nm}$-IgG conjugates (Invitrogen). Alexa 488 dye was used for positive control. The dilution of QD-IgG was performed in 2%BSA/1XPBS. The use of 2%BSA minimizes the non-specific binding of the QDs to the tissue. For staining using immunohistochemistry, the slides were rinsed after antibody incubation and visualized with 3, 3′-
diaminobenzidine solution, counterstained with hematoxylin (DakoCytomation), dehydrated and mounted with Permount mounting solution (Sigma).

### 2.2.4 Signal Detection

Fluorescent images from tissues were detected using an inverted IX71 fluorescent microscope (Olympus) and captured using a charged-coupled device (CCD) camera (Olympus). For immunohistochemistry staining, the slides were scanned by ScanScope CS (Aperio Technologies), a bright field scanner. Cores were examined with an ImageScope image viewer program (Aperio Technologies).

### 2.2.5 Spectroscopy

A 505 nm long pass filter (N41001, Chroma Technology Corp.) was used to reflect the laser light and pass the Stokes-shifted fluorescence signals. Emitted QD signal was collected by a 20 X objective lens (0.75 NA) and recorded in a spectrometer (2300i Acton/Princeton instrument). The excitation source was provided by an argon laser at 488 nm. Modification was made to the system to allow reading of the entire TMA core to account for cellular heterogeneity, by placing a 5x objective lens (0.25 NA) between the laser and the microscope and thus expanding the laser beam.
2.3 Results and Discussions

2.3.1 Assessment of QD-based Immunostaining

An advantage of the QD signal to conventional dyes is the photostability of the signal. This was experimentally tested on the FFPE tissue sections. The level of QD-immunostained signal against EGFR was compared to Alexa 488, one of the most stable fluorescent dyes [138] on the A549 lung carcinoma xenograft platform. The signal obtained from QD immunostaining showed no detectable reduction in signal intensity during continuous irradiation of a UV laser for 30 min, whereas the fluorescent signal from Alexa 488 showed a 70% reduction in signal intensity during the same exposure time (Figure 2.2). In addressing photo bleaching, the QD signal was shown to provide a more accurate means of measuring signal intensity and quantifying tumour-derived antigens in cancer biopsies.

QD-based immunostaining of membrane epidermal growth factor receptor (EGFR), pan cytokeratin, and E-cadherin in lung cancer xenografts was performed on FFPE tissue microarray slides obtained from lung carcinoma cell lines MGH7, RVH-6849, A549, H460, H1264, MGH8, H520, and H157. The
Figure 2.2- Photostability of QDs in Histological Staining. The mean signal obtained from EGFR detected by QD and Alexa 488 on A549 FFPE lung carcinoma xenograft sections. The slides were continuously exposed to a 120 W Fiber-coupled fluorescence lamp (Exfo-lifesciences, Mississauga, Canada) for 30 minutes. Pictures were taken every 5 minutes using a Zeiss Axiovert 200M inverted microscope equipped with a 20X (0.75 NA) objective lens. (Filters for QD$_{655}$ nm: Excitation = 488 nm; Emission = 650/40 nm, for Alexa 488: Excitation = 480/30 nm; Emission = 535/40 nm). Alexa 488 signal showed 70% reduction in the signal intensity after 30 minutes, whereas the signal obtained from QD showed no reduction from the initial level during the same exposure time.
validation of staining was performed by comparing the pattern of antibody expression to that obtained from an organic dye, Alexa 488 (Figure 2.3). The optimal staining condition was established by primary antibody at 1:100. Higher concentration of antibody produced over-staining and included non-specific cytoplasmic pattern. The reproducibility of the QD-immunoassaying was also confirmed by obtaining the membrane pattern staining in repeated staining experiments (Figure 2.4).

2.3.2 Measurement of Signal using Optical Spectroscopy

Optical spectroscopy was accomplished by integrating an inverted Olympus microscope, a spectrometer, and a CCD camera (Figure 2.5). The excitation source was provided by an argon-krypton laser (488 nm). A dichroic filter was used to reflect the laser light and to pass the Stokes-shifted fluorescence signals. Signals were collected for each microarray core. To account for tumour heterogeneity, no subsampling was done for each core. Instead, modifications to the spectroscopy system were made to allow the reading of the TMA core by placing a 5× objective lens between the laser and the microscope to expand the laser beam. Sensitivity can be somewhat compromised by this strategy, as the fluorescent signal is directly related to power density. However, the increased spot size allowed fluorescence signal per microarray core to be analyzed to avoid any experimental bias due to possible intratumoural heterogeneity in tumours.
Figure 2.3- Validation of QD-based immunostaining. The staining pattern of EGFR using QD$_{655}$ nm (bottom panel) in A549 lung carcinoma xenograft was compared to a parallel staining using an organic dye, Alexa 488 (top panel). QD-based immunostaining against EGFR produced a membrane staining, the typical pattern of EGFR staining. Images were taken using a 20X (0.75 NA) objective lens and a CoolSnap monochrome CCD camera. (Filters for QD$_{655}$ nm: Excitation = 488 nm; Emission = 650/40 nm, for Alexa 488: Excitation = 480/30 nm; Emission = 535/40 nm). Scale bars show 40 μM.
Figure 2.4 – Reproducibility of QD-based Immunostaining. The pattern of QD-immunostaining against EGFR demonstrates the classical membrane staining pattern in samples of lung cancer (A-C). DAPI localizes nuclei (blue) and the composite image demonstrates an overlay of EGFR and DAPI in each experiment. Images were taken using a 10X (0.25 NA) objective lens and a Coolsnap monochrome CCD camera. (Filters for QD<sub>655</sub> nm: Excitation = 488 nm; Emission = 650/40 nm. Scale bars show 40 µm.
Figure 2.5- Diagram of the Optical Spectroscopy System. Wavelength-resolved fluorescence spectroscopy was accomplished by integrating an inverted Olympus microscope with a laser excitation source, a spectrometer, and a CCD camera. The tissue sample is excited using an argon laser at 488 nm. The emission from the tissue sample is collected by a 20× objective (0.75 NA) and passed through a dichroic mirror and a 500 nm long pass (LP) filter. The signal is then dispersed into a spectrum through the spectrometer and detected by a CCD camera. A spectrum indicating the fluorescence emission is depicted.
xenograft tumours were stained separately for cytokeratin, EGFR, and E-cadherin using QDs. Initially, the spectrograph of pure QD (no staining) was taken to saturate the detector. This ensured that the readings from tissue stains were taken below the upper limit of the detector. The settings were kept constant throughout the experiment to reduce experimental variations. The fluorescence images of the A549 core tissue core on the array is depicted in Figure 2.6 A, while a corresponding spectrum obtained using the optical spectroscopy technique of the entire tissue is shown in Figure 2.6 B.

2.3.3 Analysis of Immunosignal

For accurate determination of antigen-derived signals, background autofluorescence needed to be removed and excluded from the analysis. Tissue autofluorescence is due to light absorption of endogenous fluorophores and it related to the content of tissue. The background autofluorescent signal, therefore, was obtained in each core as determined by a tissue microarray slide with no tumour-specific staining. Background signal was removed by subtracting tissue autofluorescence obtained from a given core (before staining) from the total signal (autofluorescent and antigen derived signals) of the same core (after staining). To make this process automated, we designed an algorithm, the *D-Noise*, to automatically remove the autofluorescence signal from the total signal (Figure 2.6 B).
Figure 2.6- QD-based Immunostaining and Quantification of Tumour Antigens. (A) FFPE A549 xenograft sections were labeled for E-cadherin (left), EGFR (middle), and cytokeratin (right). Images were obtained using a 20× (0.75 NA) objective lens. (Filters: excitation = 488 nm; emission = 565/40 nm, 605/40 nm, and 655/40 nm for QD<sub>565nm</sub>, QD<sub>605nm</sub>, and QD<sub>655nm</sub>, respectively.) (B) The protein expression profile of the antigens as detected by optical spectroscopy is shown in the corresponding figures. The autofluorescent signal is removed from the antigen derived signal using the D-Noise. Normalizer normalizes tumour expression values for epithelial content. (AF refers to autofluorescence).
The software uses the data points (i.e. intensity values) per wavelengths collected by the spectrometer. *D-Noise* does not apply the changes (i.e. AF removal) back to the image. It is designed to provide text files for calculations and data graphs for visualization, the same way that it can be done manually using commercially available software such as excel (parallel calculations were performed with excel for validation). An optional function of the software is to remove signal noise from the spectra. This is a more sophisticated function and is carried out by the Wavelet Shrinkage technique [192] (Appendix A). The function of Wavelet Shrinkage is thresholding the wavelet coefficients, removing small value coefficients and reconstructing the signal back with a reduced noise. This function is best used when a signal has too much noise. In our case, the application of denoising did not produce a change in signal intensity (Figure A.1). A full description of the algorithms and their functions are provided in Appendix A. Accurate tumour antigen quantification is also contingent upon isolation and selection of the signals only from the expression of tumour antigens and not from normal stroma and benign epithelial cells. A method referred to as masking, commonly used in tissue pathology using immunohistochemistry (enzyme based) methods [139], is generally performed to locate epithelial cells.
Figure 2.7- Quantification Analysis of Cancer-derived Antigens in Tissue Microarray. Tissue cores on a tissue microarray (A) were stained for EGFR, cytokeratin, and E-cadherin using QDs. The fluorescent image is a composite picture of the antigens detected by individual QD immunostaining (of different colour emission) for each of the targets. 4',6-Diamidino-2-phenylindole (DAPI) staining is used to locate nuclei in blue (B). Images were taken at 40× (0.85 NA). (Filters: excitation = 360/40 nm; emission = 565/40 nm, 605/40 nm, and 655/40 nm for QDs emitting at 565, 605, and 655 nm and 460/50 nm for DAPI, respectively). The Normalizer algorithm normalizes tumour expression values (EGFR and E-cadherin) for epithelial content (cytokeratin). The Multiplexer algorithm creates a composite profile of tumour antigen values in each core (C). Cores A1 to A8 correspond to lung cancer xenografts RVH-6849, MGH8, MGH7, H520, H460, H157, H1264, and A549, respectively.
and use cellular content to standardize tumour measurements. This is more pivotal in human tumour samples as opposed to xenografts, as tissue sections may well differ in cellular density (i.e., cell count and arrangement per area). In this study, a mask was derived from QD immunolabeling of the lung carcinoma tumour sections using pan-cytokeratin staining, a marker for epithelial cells. Normalizing each core to cellular content was performed by the Normalizer algorithm, whereby the mean signal from antigen-derived staining is divided by the cytokeratin signal. Subsequently, the Multiplexer algorithm combined the normalized values of all antigens expression in each core to create a composite graph from individually labeled probes (Figure 2.7).

2.3.4 Validation of Xenograft Study

In order to examine the accuracy of the protein quantification system, QD-based immunoassays were conducted on the microarray and analyzed for FFPE lung carcinoma xenografts of MGH7, RVH-6849, A549, H460, H1264, MGH8, H520, and H157, known to have a differential level of expression for membrane EGFR. Optical spectroscopy was performed and quantitative analysis of fluorescent signals from EGFR was obtained in a single staining experiment. Different EGFR expressions in the xenografts produced different levels of antibody binding. Since the antibodies are bound to QDs, the amount of QD in
the tumours would be reflective of the relative EGFR amount. The fluorescent signal from the xenografts was measured and analyzed using our algorithm as described above. Subsequently, the quantitative values of EGFR in xenograft tissues were compared to the endogenous RNA levels of EGFR measured by quantitative real time polymerase chain reaction (Q-RT-PCR) and showed a statistical correlation of 0.90 (Figure 2.8).

EGFR was chosen advisedly for the validation study, as the antibody is well characterized for FFPE tissue specimen in the laboratory. Moreover, molecular studies on EGFR have demonstrated the protein expression level of EGFR to correlate with mRNA expression detected by quantitative PCR [140] [141]. EGFR over-expression has been shown to be associated with high EGFR mRNA expression in squamous cell tumours [142] and with increased gene copy number in NSCLC [143] and squamous cell carcinomas (SCC) [144].

The xenograft tumours provided a suitable platform to test the feasibility of using QDs in conjunction with the spectroscopy system for the quantification of EGFR antigens in FFPE samples. More recently, other studies demonstrated the application of a similar protein quantification system using bioconjugated QDs in the profiling of HER2, ER and PR markers in MCF-7, BT-474 and MDA-MB-231 breast cancer cell lines [177] [178]. It should be noted however, that there is a challenge in applying this system, in its current form, to clinical tumour platforms. This is simply because the xenograft tumours did not adequately
account for the complexity of tumour architecture in humans. The limitation lies in the biology of tumor, mainly in the feasibility of the selection of transformed cells from non-transformed cells by an automated system (as opposed to a pathologist). The architecture of tumour is sophisticated and includes heterogeneous tumour cells infiltrating neighboring stroma. Experimental images are presented in appendix B that elucidate tumour heterogeneity and the spread of tumour cells in lung carcinoma. Studies have addressed this challenge by introducing computer-based systems for tumor identification and quantification purposes [117] [118] [124]. The premise of this method is the creation of a tumor-specific mask by thresholding the image of a target marker by an epithelial marker. This creates a binary mask (each pixel is either ‘on’ or ‘off’). Using an “AND” algorithm, pixels can be selected that are positive for both the markers for epithelial cells and the antigen of interest (i.e. hence the tumour cells). The quantification of the target marker is subsequently done in silico. The limitation of this method is that in the image acquisition process, 8-bit and 12-bit images offer a dynamic range of 0-255 and 0-1024, respectively. The upper limit of 255 or 1024 can be easily saturated in regions of tumours that show high levels of fluorescent intensity. The challenge, therefore, is in the ability to balance the antibody concentration to a low-enough level to avoid reaching the saturation of the detector. Reducing antibody concentration however, may undermine the integrity of staining (i.e. false negative reading). Future studies are required to
Figure 2.8- Validation of QD-based Immunolabeling. Data obtained from quantification of EGFR expression using QD-based immunolabeling were compared to endogenous EGFR cDNA using Q-RT-PCR (n=8). The differential expression level of protein was measured by optical spectroscopy after immunolabeling of EGFR with QD bioconjugates on lung carcinoma xenografts of MGH7, RVH-6849, A549, H460, H1264, MGH8, H520, and H157. The endogenous level of RNA transcript for EGFR was measured in the FFPE tissue sections using Q-RT-PCR. The correlation coefficient was found to be 0.90.
address these issues. Future directions for this project are discussed in detail in the *Future Directions Section* in Chapter 5.

### 2.3.5 Examination of FRET in Immunohistochemistry

The broad absorption spectra of QDs make them amenable to excitation with a single light source. We examined whether concurrent detection of antigens using QD-based multiplex staining on the same tissue can be used for quantitative analysis of protein expression. The level of antigen expression from EGFR, E-cadherin, and cytokeratin in a multiplexed simultaneous staining using QD\(_{655\text{nm}}\), QD\(_{605\text{nm}}\), and QD\(_{565\text{nm}}\), respectively, was compared to those obtained from separate staining experiments for each antigen. The same QDs were used for both the multiplex and single staining experiments to keep the comparative studies consistent. The emission spectra obtained from the multiplexed experiment showed a strong enhancement of the QD\(_{655\text{nm}}\) signals and reduction of the QD\(_{605\text{nm}}\) and QD\(_{565\text{nm}}\) signals (Figure 2.9). The values produced by multiplexed staining differed from antigen levels obtained from individual staining. The results suggest that fluorescence resonance energy transfer (FRET) may cause the inaccurate measurements in multimarker analysis of the same tissue. FRET is a dipole-dipole interaction process between excited electrons of two molecules, whereby energy from an excited fluorophore (donor) is transferred to an acceptor fluorophore without emission of a photon [90]. In organic fluorescent dyes and QDs alike, FRET is contingent upon the overlap of
Figure 2.9 Effect of FRET on QD-based Quantification of Multiplexed Protein Markers. QD immunolabeling of EGFR, E-cadherin, and cytokeratin on a FFPE A549 lung carcinoma tissue section in a multiplex staining experiment (left) and in separate staining experiments (right). The spectra in the multiplexed staining experiment show different values than the expected level of protein expression for EGFR, E-cadherin, and cytokeratin obtained from individual staining. The QD$_{655}$ nm signal from EGFR is greatly enhanced, whereas the QD$_{605}$ nm and QD$_{565}$ nm signals obtained from E-cadherin and cytokeratin, respectively, are reduced in fluorescence intensity.
the donor emission spectrum and the acceptor absorption spectrum [145]. This in
turn results in the enhancement of acceptor fluorescence and quenching of donor
fluorescence. The efficiency of FRET depends on the spatial distance between
molecules as well as fluorescent (hence antigen) concentration [146]. The former,
in quantitative measurements of tumour antigen, is unknown, and the latter is
sought to be measured. Therefore, in such a case where two unknowns are
present and the conditions of the expression level may not be uniform for all the
antigens to be assessed, an accurate quantitative analysis is restricted to one QD
color per antigen at a time. Future research pertaining to the design of QDs for
minimizing fluorescence cross-talk will be needed. In Chapter 3 a novel design of
QD is presented and the efficiency of fluorescent energy transfer is further
discussed.

2.4 Conclusion

In conclusion the quantitative analysis of antigens in TMA using the QD-
based immunoprofiling enabled measurements of tumour-derived antigen and
delineated expression profiles of FFPE xenograft specimens. The use of QD in
conjunction with optical spectroscopy provided a tool to obtain quantitative and
objective measurements on a continuous scale. This may have implications in
reducing analytical variations in the analysis of tumour biopsies. The application
of QD for a multiplexed targeting of biomarkers was hampered due to fluorescent resonance energy transfer. The algorithms provided an automated mathematical tool to remove autofluorescence, normalize tumour protein expression to cellular content, and produce a comprehensive profile of tumour derived antigen on a tissue microarray. The use of this method of quantification along with the algorithms can be expanded to encompass the study of a myriad of protein markers individually, or as a group of markers involved synergistically in carcinogenesis. Future studies are required to address the automation of “tumour identification” process in clinical biopsies.
Chapter 3

Novel Quantum Dot Molecular Probes for Simultaneous Detection of Immunophenotype and Cellular Morphology in Cancer

-This chapter has been partially prepared as the following manuscript:

A. A. Ghazani et al., Novel fluorescent nanoprobe for simultaneous detection of colourimetric and fluorescent signal in the histopathology of cancer. In Progress.

-The intellectual property of the nanobead and its applications belong to A. A. Ghazani, S. Fournier, W.C.W. Chan at the Institute of Biomaterial and Biomedical Engineering and C. Wang at Mount Sinai Hospital.

Author Contribution

The application of QD nanobeads design was proposed by A. A. Ghazani and the synthesis and chemistry modifications were carried out by Dr. S. Fournier. The synthesis of QD was carried out by D. Li. All other experiments in this chapter were carried out by A. A. Ghazani. Dr Fournier was consulted on chemistry related observations.
3.1 Introduction

The clinical diagnosis of cancer encompasses two techniques, examination of cellular morphology and immunophenotype of patient biopsies. The former is routinely performed using biological stains to make cellular structures visible under light microscopy. Methodologies include nuclear staining aided by hematoxylin or methylene blue and counter staining using eosin. The adjuvant function of counterstain is to differentiate between darkly stained nuclei (blue) and cytoplasm and extracellular regions (pink). The method aims to ascertain the presence of tumour metastasis and/or define the stage of cellular differentiation. A second class of diagnostic testing is immunostaining aimed at determining the presence, distribution and sub-cellular localization of antigens. Information obtained from both techniques is used in diagnosis, treatment and prognosis of cancer. The assay du jour in pathology is to prepare serial samples from tumour biopsies (where available) and perform immunoassays and standard colourigenic stains on different slides.

Given the clinical significance of morphological and immunophenotypic assessment of biopsies, it seems preferable to merge both techniques on a given sample. The implications of a combined assessment would be in i) medical management: the ability to link the morphological state of cancer cells to their immuno-expression could lead to an increased understanding of the biology and clinical state of the tumour, and ii) tumour management: performance of
techniques on a same biopsy minimizes the critical limitations of sample availability, specially in the case of human core biopsies. Recently many studies have demonstrated that multi-marker examination of tumours, as opposed to a single-marker examination, provides a more accurate assessment of clinical conditions such as micrometastases[147] and detection of cancer at an early stage [148][149]. In that regard, QDs offer a unique tool in allowing multiplex detection of desired antigens in the in vitro assessment of tumour biopsies [84]. However, similar to traditional fluorophores, QDs exhibit fluorescent quenching effect when placed in a variety of solutions, including biological stains.

Biological stains are dyes used to make cellular structure (i.e. nucleus and cytoplasm) visible and provide an increase in histological information. The interaction between the fluorescent molecules and aromatic dyes (e.g. conventional stains) leads to quenching of fluorescent signals [150] [151] due to aromaticity of the dyes, whereby electron transfer can act as fluorescence reducing agents [92] [150]. This is exemplified by photochemical studies on methylene blue that have characterized it as a potent oxidant in photodynamic therapy for the treatment of root canal [152] and experimental colorectal tumours [153][154].

The focus of this study is to design nanoparticles that can resist the fluorescent quenching effect of the dyes to enable simultaneous immunofluorescence and morphological assessment of samples. From the photochemical perspective, this seems feasible by protecting QDs from their
surrounding environment. We achieved this by proposing a novel design of QD-based fluorescent probes. The encapsulation of QDs in a polystyrene network provides a protective coat to environment solutions. The photo-physical, photo-chemical and photo-biological parameters of the nanobead and dye combination are described and the feasibility of their application in the simultaneous detection of immunofluorescence and cellular morphology is demonstrated in human cancer.

3.2 Materials and Methods

3.2.1 Synthesis and Functionalization of QDs

CdSe QDs covered with a ZnS passivation layer were synthesized and rendered soluble in organic solvent using TOPO (trioctyl phosphine oxide) and HDA (hexadecylamine). Such QDs were stored in chloroform solutions until further use. Functionalization of the QDs was carried out by first precipitating the QDs out of chloroform by addition of an equivalent volume of methanol followed by 5 minute centrifugation at 4000 rpm. The QD pellet was then redispersed into oleic acid at room temperature followed by heating to 150°C for 2h. This step allowed the displacement of TOPO groups with oleic acid ones onto the ZnS shell. After cooling to room temperature, the QDs were precipitated out of oleic acid by adding an equivalent amount of methanol and the dry pellet obtained after centrifugation was redispersed into styrene.
3.2.2 Synthesis and Functionalization of Nanobeads

The synthesis of the beads was carried out according to Lansalot et al. [181] with modifications. Briefly, 1 mL of hexadecane was added to 8 mL of styrene/QD solution. Simultaneously, an aqueous solution containing 120 g of water, 62 mg of sodium dodecyl sulfate and 111 mg of sodium bicarbonate was prepared. Both solution were then mixed together and stirred for 10 min at 700 rpm followed by 10 minutes of sonication. This emulsion was then incorporated into a reaction vessel preheated to 75 °C under inert atmosphere and stirred at 350 rpm. 8mL of a potassium persulfate (182 mg aqueous solution) was injected into the reaction mixture to initiate the polymerization and left stirring overnight. Functionalization of nanobeads was performed by adding 50 mL of the previous suspension diluted into 100 mL of water into a reaction flask heated to 70 °C and stirred at 100 rpm. Ammonium persulfate (0.013 g in 1 mL of water) and glycidyl methacrylate (1 mL) were both added to the reaction vessel using separate syringe pumps at a rate of 1 mL/h. The mixture was left stirring for 5 hours. The nanobeads were purified by centrifugation (3-5 times) to remove any un-reacted starting materials. To a reaction vessel containing a 150mL suspension of the previously synthesized epoxy terminated beads at 70 °C under nitrogen atmosphere, 1mL of acrylic acid in 1mL of water was added at a rate of 2mL/h in conjunction with ammonium persulfate (13mg in 1mL of water) added at a rate of 1mL/h. After the addition is complete, stirring at 100rpm of the suspension was continued for 5h before cooling to room temperature.
3.2.3 Bio-conjugation

For glycidyl based conjugation, 0.5 mg antibody or 1 mg streptavidin was mixed with 200 µl beads in 1XPBS (pH 7.4). The mixture was stirred for 1 hour at room temperature or overnight at 4 °C. The control included beads with no glycidyl modification and tubes with no proteins. The pellet was spun down and washed 3X and re-suspended in 1XPBS. For carbodiimide-based conjugation, 2 mL carboxylate-modified nanobeads were spun down and re-suspended in 500 µl MES (50 mM) buffer (pH 5). To 100 µl of the nanobead solution, 10 µl of 50 mg/mL Sulfo-NHS and 10 µl of 50mg/mL EDC were added. After 20 min incubation at RT, the pellet was washed 3X and re-suspended in 100ul MES (50 mM) buffer (pH 5). 1 mg streptavidin was mixed with amine-reactive Sulfo-NHS nanobeads and incubated for 2 hours at room temperature. The control included tubes with no EDC and tubes with no proteins. The pellet was washed 3X and re-suspended in 1X PBS.

3.2.4 Ninhydrin Assay

The validation of conjugation was done by a ninhydrin (NHI) assay. 0.5g ninhydrin was dissolved in 10 mL ethanol followed by sonication. The conjugation reactions were divided in two, one used for the test and other used
as reference for the ninhydrin assay. 1 mL of NHI solution and 1 mL of ethanol were added to the test and reference, respectively. The control tubes contained beads with no protein added (negative control) or protein (streptavidin or IgG) with no beads added (positive control) in the same concentration of the test experiments. The reactions were mixed overnight at room temperature. The ninhydrin absorbance was measured at 570 nm using a UV/VIS spectrophotometer (Shimadzu UV-160, Mandel Scientific Inc).

### 3.2.5 Detection of Optical Signal

Bright field and fluorescence imaging were carried out using an inverted fluorescent microscope (Olympus) and a CoolSnap Pro colour camera (Media Cybernetics, Inc.). Fluorescence images were taken with a Hg lamp excitation source (Olympus) and a UV excitation filter (405/20 nm, Chroma Technology Corp.) Emission of nanobead\textsubscript{560nm} and nanobead\textsubscript{610nm} were detected with a 560/40 nm (Chroma Technology Corp.) and a 600 nm long pass (Chroma Technology Corp.) emission filter, respectively. DAPI excitation and emission filters were respectively 360/40 nm and 460/50 nm (Chroma Technology Corp.). Spectroscopy was carried out using an optical spectroscopy system comprising an inverted fluorescent microscope (IX71, Olympus). The excitation source was provided by a 488 nm Argon laser. The laser power was at 25 mW. A 505 nm long pass filter (N41001, Chroma Technology Corp.) was used to reflect the laser
light and pass the Stokes-shifted fluorescence signals. Emitted QD signals were collected by a 20× objective (0.75 NA) and recorded in a spectrometer (2300i Acton/Princeton instrument).

### 3.2.6 Electron Microscopy

A 50 µL sample of the nanobeads was pelleted down and dried for transmission electron microscopy (TEM) analysis. Samples were cryosectioned, placed on a copper grid and visualized with a FEI Tecnai with Gatan Dual view camera transmission electron microscope, operating at 200 kV. The size monodispersity of samples were examined using a Hitachi S-5200 scanning electron microscope (SEM) operating at 1.0 kV.

### 3.2.7 Assessment of Fluorescence Intensity

Nanobeads were prepared (1:10 dilution) in the following solutions: water, methylene blue, hematoxylin, HCl (pH 0.0), NaOH (pH 14.0), 1X PBS pH 7.4 and 10X PBS pH 7.4. The solutions were chosen based on their involvement in pathology procedures including staining and fixation or as examples of extreme pH conditions. For slide treatment, 50 µl nanobeads were mounted on slides using cytospin and treated with sequential steps of Giemsa and H&E staining. The intensity of signal was measured using the optical spectroscopy system in section 3.2.6. Nanobeads were excited using a UV excitation source (Photonic Products) at 405/90 nm (Chroma Technology Corp.) and detection at 550 nm
long pass (Chroma Technology Corp.). Emitted QD signals were collected by a 20× objective (0.75 NA) and recorded in a spectrometer (2300i Acton/Princeton instrument).

### 3.2.8 Statistical Analysis

Fluorescence intensity data are presented as mean ± standard deviation, and were evaluated for statistical significance (GraphPad Prism, after consultation with Melania Pintilie, Biostatistics Department, University Health Network, and Toronto). One-way analysis of variance (ANOVA), followed by the Kruskal-Wallis test was performed to compare intensity values in different solutions and treatments. The Kruskal-Wallis one-way analysis of variance is a nonparametric test that compares the means of three or more unmatched groups. Statistical significance was defined as $P < 0.001$.

### 3.2.9 Preparation and Immunostaining of Histology Samples

FFPE tissue sections were treated with xylene to remove paraffin and rehydrated in 100%, 95% and 70% alcohol for 5 minutes each. The slides were subsequently blocked with 1X PBS/1%BSA for 1 hour. Primary antibody incubation was performed for 1 hour or overnight at room temperature followed by 3X rinse in Tris-buffered saline (pH 7.6). Heat-induced proteolytic epitope retrieval was conducted in 10 mmol/L citrate buffer (pH 6.0) for 10 minutes in a microwave
(Nordic Ware, Minneapolis) at 120 °C. Alexa 488 was used for the positive control study. Dilution of nanobeads was performed in 2% FCS/1XPBS to minimize non-specific binding of probes to the tissue. H&E treatment was performed by rinsing the slides in hematoxylin, HCl, ethanol, and eosin followed by dehydration the slides in 70%, 90% and 100% ethanol and xylene before mounting. H&E staining was performed using an autostainer.

3.2.10 Preparation and Immunostaining of Hematopoietic Samples

Leukocytes were separated from whole blood obtained from patients with chronic lymphocytic leukemia (CLL) using the Ficoll-Paque density gradient method. The antibody conjugates against CD5, CD19 and CD38 were used for staining cell surface markers. Bead/antibody conjugates were incubated with freshly prepared CLL cells for one hour at room temperature, followed by washing with 1X PBS for three times to remove excess antibodies. The cells were then placed on slides by cytopspin method for fluorescence microscopy examination. Slides were stained with Giemsa using an automatic hemastainer.

3.3 Results and Discussions

3.3.1 Design and Characterization of Nanobeads

Nanobeads were synthesized in an emulsion reactor where polymerization of styrene occurred in the presence of QDs of desired emission profile (Figure 3.1). Scanning electron microscopy (SEM) demonstrated that the nanobeads were 100
nm in size (Figure 3.2). The successful incorporation of QDs in the nanobeads was determined by transmission electron microscopy (TEM), elemental analysis, and optical microscopy. The contrast in TEM images showed localization of QD in nanobeads (Figure 3.3), elemental analysis revealed the presence of cadmium, zinc, and selenium in the pellet of nanobeads, and the optical microscopy showed bright fluorescent particles (Figure. 3.4). The presence of QDs in the beads was confirmed by comparing transmitted light and fluorescent signals of the beads on a slide. Conclusive determination of the exact populations of beads with QD inside was not reached. The number of QDs in beads was estimated from the TEM images, based on the diameter of bulk QD sphere in the beads and the size of QD particles. It was speculated that ~25 QDs are present/bead (i.e. the difference of volumes was 25X). However, this number is a gross estimate and depends on how well individual QDs are packed inside the QD aggregates. The number of QD particles using emulsion polymerization reaction was previously reported as 21 – 132 per various sizes of beads ranging from 100 nm to 255 nm [181]. Ultimately, the number of QD per bead depends on the initial concentration of QD in each synthesis. Furthermore, it has been reported that increasing the concentration of surfactant directly correlated with a higher polymerization rate and a higher number of particles inside the beads [181].

The surface of the nanobeads was functionalized with glycidyl groups for conjugation to antibodies [155]. The presence of glycidyl groups were confirmed by Fourier Transform Infrared Spectroscopy (FTIR) (Figure 3.5) by comparing the
beads with and without glycidyl coating. The peak at 1270 cm$^{-1}$ corresponding to glycidyl was shown present and absent in the FTIR graph, respectively.
Figure 3.1- Synthesis of Nanobeads. The beads are synthesized in an emulsion reactor (top diagram courtesy of Sebstien Fournier). The process of nanobead synthesis is depicted in the bottom. QDs were first functionalized by displacing TOPO groups with oleic acid. During emulsion reaction, the polymerization of styrene occurred in the presence of QDs of desired emission to create nanobeads. The surface of the nanobeads was functionalized with glycidyl groups for conjugation to proteins.
Figure 3.2- SEM Image of Nanobeads. SEM picture demonstrates the size of nanoparticles to be 100 nm in diameter. SEM image was taken using a Hitachi S-5200 scanning electron microscope operating at 1.0 kV.
Figure 3.3- TEM Cryosection Image of Nanobeads. The location of QD particles is demonstrated inside the nanobeads. The phase separation between QD and styrene during the emulsion reaction creates aggregates of QD upon which polymerization of styrene occurs. TEM image was taken with a FEI Tecnai with Gatan Dual view camera transmission electron microscope, operating at 200 kV. The cryosection image was obtained with the help of Dr. Fournier and Dr. Neil Coombs at the TEM facility in the department of chemistry.
Figure 3.4- Fluorescent Image of Nanobeads. The fluorescent picture depicts nanobead$_{560\text{nm}}$ and nanobead$_{610\text{nm}}$. (Filter: excitation = 405/20 nm, emission = 560/40 nm, 600 nm LP for nanobead$_{560\text{nm}}$ and nanobead$_{610\text{nm}}$, respectively). Images were taken at 40X (NA=0.85). The scale bar denotes 1 $\mu$M.
Figure 3.5 – Characterization of Nanobead Surface by FTIR. FTIR on nanobeads with and without glycidyl (also known as epoxy) groups demonstrated the presence and absence of the functionalized groups at 1270 cm⁻¹, respectively. The peak at 1730 cm⁻¹ is due to the presence of C=O, perhaps from the formation of keton from glycidyl groups during the reaction. The broad peak at 3100-3500 cm⁻¹ denotes carboxylate groups on the functionalized beads.
3.3.2 Assaying Fluorescent Stability of Nanobeads

Previous studies have demonstrated the fluctuation of fluorescence of QD-polymer bead in buffers and solutions of different pH and salt content [156], demonstrating the ability of the solutions to penetrate inside the bead structure. We examined the stability of the nanobead fluorescence by measuring the signal intensity of beads placed in extreme solutions. For this purpose, solutions of NaOH pH 14.00 and HCl pH 0.00 were selected as ultimate extreme pH solutions. Also, 1X and 10X PBS buffers (pH 7.4) were selected as biological buffers with different salt concentrations. Fluorescent spectroscopy of samples in solutions and water were taken in repeated experiments (n=19). The mean of maximum signal intensity was calculated and compared among test conditions using the Kruskal-Wallis test. The Kruskal-Wallis one-way analysis of variance is a nonparametric test that compares the means of three or more unmatched groups. No significant change \((P = 0.2156)\) in signal intensity was observed among beads treated with water, at extreme pHs, or different PBS conditions (Figure 3.6). The solutions, therefore, did not seem to penetrate inside the beads. The stability of nanobeads in low pH would make the beads amenable to intracellular applications or \textit{in vivo} milieus of various pHs (e.g. low-pH lysosomes).
Figure 3.6- Assessment of the Fluorescence Stability of Nanobeads. The fluorescence of nanobeads$_{610}$ nm were measured in water, 1X PBS, 10X PBS, NaOH (pH 14) and HCl (pH 0) (n=19). No significant ($P=0.2156$) fluctuation of QD signal was observed among solutions of extreme pH and different PBS salt contents. The graph and error bars represent the mean intensity of emission signal and +/- 1 standard deviation, respectively.
The stability of the QD fluorescence inside nanobeads is not merely a function of coating QDs with polystyrene, but may also be attributed to the specific design of the particles. Previous attempts in protecting QD signal in polystyrene microbeads demonstrated the fluctuation of QD beads fluorescence in low pH or high salt contents [156]. The key difference between the old design and what is presented here, beside the size of particles, is the incorporation of QDs into the polystyrene network. In the old design, the synthesis of polystyrene microbeads was made first followed by the incorporation of QDs into polystyrene matrix using the matrix swelling process. During this process, large channels are generated by placing microbeads into chloroform, permitting QDs to penetrate the polystyrene network. The subsequent removal of chloroform results in de-swelling of the beads and the entrapment of QD inside the particles. In that design, the fluctuation of QD inside the beads demonstrates that solutions do reach ZnS-capped CdSe QDs and quench their fluorescence through surface interactions. This could be due to incomplete incorporation of QD into the polystyrene network. Alternatively, the channels created during the swelling process may be incompletely closed when de-swelling occurs and, therefore, allow external solutions to permeate the particles where QDs reside and consequently quench them. Figure 3.7 demonstrates a TEM cross section of 300 nm beads and localization of QD on the border of polystyrene beads in the old design. What is unique in the design of nanobeads presented in the new design is that the localization of QD is different. QD is in
Figure 3.7 - TEM Cross Section Image of Beads. In this method, QDs are incorporated inside the beads by the “swelling” approach. The location of QD is in the outer edge of the polystyrene beads (300 nm in diameter). This design of QD-bead does not fully protect QD from external solution [156]. The location of QD in this design is different that the positioning of QD in the novel nanobead design presented in this chapter (image 3.3). The scale bars in the left and right images are 100 nm and 20 nm, respectively. The cross section image was obtained with the help of Dr Fournier and Doug Holmyard at the Mount Sinai Hospital, TEM facility.
the core of bead particles (Figure 3.3). The incorporation of QD into the polystyrene network occurs during the emulsion reaction process. The phase separation between QDs and styrene results in the formation of QD aggregates, upon which polystyrene network grows. The entrapment of QDs in the core of the beads provides a suitable protection from external solution.

3.3.3 Photo-physical Assessment of Nanobeads & Dyes

Although the colourimetric dyes on biopsies are examined using bright field microscopy, we set out to examine whether they pose any interference in the fluorescence detection of nanobeads. The optical parameters of the dyes indicated the excitations for eosin, methylene blue and hematoxylin were 520 nm, 570 nm and 660 nm, respectively (Figure 3.9). QD in nanobeads, however, have a broad absorption and can be excited with light at any wavelength below their emission, with optimal excitation at lower wavelength. The emission peaked at 550 nm, 670 nm for eosin and methylene blue, respectively. For hematoxylin, emitted light was recorded as background noise. The use of QD offered a unique opportunity. Owing to their broad absorption, a window of wavelengths at about 350 nm to 450 nm would excite the QDs and not the dyes. This provides minimal spectral overlap between the dyes and QDs. Excitation at higher wavelengths can introduce partial or full optical interference among the dyes and nanobead combination.
Figure 3.8- Spectrographic Profiles of Nanobeads and Biological Dyes. The normalized absorption (left) and emission (right) profiles of nanobeads are demonstrated with those of methylene blue, eosin and hematoxylin. The broad absorption of QDs in nanobeads allows for the excitation of the beads at low wavelengths (350 nm - 450 nm) with minimized spectral interference from the dyes.
A second photophysical characteristic of nanobeads that was examined was the potential of fluorescent resonance energy transfer among particles of different emissions. We have previously demonstrated that the emission level of QD signals can be compromised in multiplex assays due to FRET (chapter 2) [84] [159]. Reportedly, a minimum spatial separation of 10 nm between QDs is effective in the reduction of FRET [160] [161]. The TEM cross section of nanobeads in Figure 3.3 demonstrated the location of QDs to be in the core of the nanobeads due to a phase separation between QD and polystyrene during synthesis. Therefore, in nanobeads, QDs of different emissions are spatially separated from one another by the polystyrene structure and the effect of FRET may be reduced.

The FRET effect was experimentally demonstrated by obtaining the spectrographic emission profile of nanobead\textsubscript{610\text{nm}} and nanobead\textsubscript{560\text{nm}}. The emission profiles obtained from nanobeads in the multiplexed condition did not show the signature fluorescence fluctuation due to FRET (i.e. reduction of donor fluorescent and increase in acceptor emission) when compared to the profiles obtained from individually taken emissions of nanobeads of the same concentration (Figure 3.9). Similar experiments with QD showed a reduction of the QD\textsubscript{560\text{nm}} and enhancement of the QD\textsubscript{610\text{nm}} (Figure 3.8). This particular FRET metric is defined as the fractional decrease in donor fluorescence due to acceptor quenching. The predictive power of FRET efficiency can be modeled by Stern-Volmer expression [180]:
\[ F_0FI = 1 + K_{sv}[Q] \]

\(F_0\) and \(F\) are the fluorescent intensity in the absence and presence of quencher respectively, \(K_{sv}\) is the Stern-Volmer constant and \([Q]\) is the concentration of the quencher. The Stern-Volmer model is also applied to regimes of dilute probe concentrations, and satisfied for FRET studies by demonstrating an increase in \(F_0/F\) (usually by a decrease in \(F\)) as \([Q]\) increases [180]. To illustrate this, titrations of nanobeads\(610\ nm\) probe were prepared while keeping the concentration of nanobead\(560\ nm\) constant. Comparative studies were performed with QD\(560\ nm\) and QD\(610\ nm\). The nanobead emission profile did not show the expected Stern-Volmer model of FRET (Figure 3.10). In other words, gradual increase in the concentration of nanobead\(610\ nm\) did not lead to a decrease in the emission intensity of nanobead\(560\ nm\). Conversely, the QD emission profile showed that, although the amount of QD\(560\ nm\) was kept constant, the level of its emission signal reached an undetectable level as the concentration of the QD\(610\ nm\) acceptor increased (Figure 3.10).

The implications of FRET in multiplex immunoassay are in the sensitivity of signal detection. Specifically, when the level of antigen expression to be measured happens to be low, the emission signal may be inaccurately detected due to energy transfer between QDs of different emissions. This could create a false negative reading (similar to QD data in figure 3.10) and the diagnostic
conclusion that the antigen of interest is not present. As demonstrated in this section, nanobead fluorescence is less prone to FRET due to spatial separation of QDs of different emissions and, therefore may be a sensitive probe for the detection of low level immuno signals. Further studies focusing on the measurement of low level expression of biological samples are required to assess the sensitivity of target signal detection using nanobeads.
Figure 3.9 - The Effect of Fluorescent Energy Transfer in Nanobeads. TOP: the spectrograph emission of nanobead$_{560\text{ nm}}$ and nanobead$_{610\text{ nm}}$ are demonstrated when taken individually (shaded green and red respectively) and in a multiplexed assay (blue line). The spectroprofile of nanobeads in the multiplex assay do not demonstrate the signature fluorescent energy transfer fluctuation (i.e. reduction of the donor emission and increase in the acceptor emission). Bottom: Unlike in nanobeads, in free QDs energy from an excited QD$_{560\text{ nm}}$ is transferred to an acceptor QD$_{610\text{ nm}}$. This results in an increase of the acceptor (QD$_{610\text{ nm}}$) fluorescence and decrease in the donor fluorescence (QD$_{560\text{ nm}}$). The green and red shades represent the spectrograph of QD measured individually and the line represents the spectrograph of the same QDs when combined in a multiplex detection. The samples were excited using an argon laser at 488 nm. The emissions were collected by a 20× objective (0.75 NA) and passed through a dichroic mirror and a 500 nm LP filter. The signal was then dispersed into a spectrum through the spectrometer and detected by a CCD camera.
Figure 3.10 - Synchronous Fluorescence Spectra of Nanobeads. Top: Addition of nanobeads 610 nm at concentrations of $2.7 \times 10^{12}$ µM (blue), $5.4 \times 10^{13}$ µM (green), $1.08 \times 10^{14}$ µM (red) (λexc = 480 nm) resulted in no variation in the emission intensity of $1.5 \times 10^{12}$ µM nanobead$_{60nm}$. Note that the total volume was kept constant and the only variable was the concentration of nanobead$_{610nm}$. Bottom: similar experiment with QD$_{560}$ nm and QD$_{610}$ nm demonstrates a decrease of QD$_{560}$ nm intensity ([QD$_{560}$ nm] = $3.35 \mu$M, λexc = 480 nm) upon increasing the concentration of QD$_{610}$ nm: 1.66 µM (blue), 3.32 µM (green), 6.7µM (red). When 6.7 µM QD$_{610}$ nm is added, 83.6% of original emission signal of QD$_{560}$ nm is quenched to bring the QD fluorescence to an undetectable level. A conceptual illustration included with each graph demonstrates the spatial separation between QDs in the nanobead design and in free QD condition. The samples were excited using an argon laser at 488 nm. The emissions were collected by a $20\times$ objective (0.75 NA) and passed through a dichroic mirror and a 500 nm LP filter. The signal was then dispersed into a spectrum through the spectrometer and detected by a CCD camera.
3.3.4 Photo-Chemical Assessment of Nanobeads & Dyes

A number of studies of methylene blue have demonstrated its oxidative potential [152] [153] [154]. Indeed, aromatic dyes in general are potent static quenchers [150]. Static quenching is a ground-state quenching condition and occurs as a result of the formation of a non-fluorescent complex between fluorescent and quencher molecules [92][150]. Because the loss of fluorescence is resulted from a direct interaction, we hypothesized that preventing the interaction of dyes and nanobeads would protect the fluorescence of QD particles in nanobead structure.

The “fluorescence stability” data in Section 3.3.2 demonstrated that beads provided an impervious coat to the QD and effectively protected them from external media. Here, the photo-chemical characteristics of nanobeads were examined in the presence of dyes.

First, nanobeads were directly placed with methylene blue, hematoxylin (basic components of stains) and HCl (pH 0). The use of HCl serves two purposes: due to its low pH, it degrades the dots and provides the ultimate test for the fluorescent stability of nanobeads, QD and PE. It is also commonly used in the process of slide treatment in histology, therefore directly relates to the testing of nanobeads in biopsy preparation procedures. Fluorescent spectroscopy of nanobeads was taken in repeated experiments (n=18). The mean of signal intensity in each test was calculated and compared to the value obtained in water using the Kruskal-Wallis test. Comparative studies were conducted with commercial water-soluble ZnS-capped CdSe QD$_{655}$ nm (n=100) and organic dye
phycoerythrin (PE) (n=55). No significant change ($P = 0.7812$) in signal intensity was observed among beads treated with water, methylene blue, hematoxylin and HCl (Figure 3.10). However, there was significant loss of QD signal ($P < 0.001$) among the test conditions. Similarly, the PE emission signal was significantly ($P < 0.001$) quenched in hematoxylin (98.9% loss), methylene blue (97.4%) and HCl (97.8%) (Figure 3.11).

In addition to biological stains, the fluorescence stability of the beads was measured for all the steps of slide preparation. Beads were cytospun onto microscope slides and treated with Giemsa and H&E staining procedures as performed in diagnostic testing. This ensured that the nanobeads were treated in all the consecutive treatment steps of staining, fixation, mounting, xylene treatment, eosin and washes with ethanol and phosphate buffered saline (pH 7.0). The mean of signal intensity after treatments was compared to the mean of signal intensity before treatment using the Kruskal-Wallis test and showed to be statistically not significant ($P = 0.3916$, n=51) (Figure. 3.12). The solutions, therefore, did not seem to penetrate inside the beads.

The photo-chemical experiments with nanobeads in biological dyes were in accordance with the nanobead data presented in section 3.3.2 in demonstrating that the polystyrene nanobead structure provides an impervious coat to its QD signal. It further demonstrated the stability of nanobeads in biological stains.
Figure 3.11- Photo-chemical Assessment of Nanobeads in Biological Dyes. The fluorescence of nanobeads_{560 nm} in water, methylene blue, hematoxylin and HCl (pH 0) did not demonstrated a significant (*P = 0.7812) fluctuation. The fluorescence of QD however, showed significant (•P <0.001) quenching in biological dyes and HCl. Similarly the emission of PE was significantly (••P <0.001) affected in the same conditions. The mean intensity of emission signals in each test condition was normalized to the level of signal in water and presented in percent fluorescent intensity. The error bars are +/- 1 standard deviation.
Figure 3.12- Photo-chemical Assessment of Nanobeads in Diagnostic Staining Conditions. Nanobeads were first placed on microscope slides by the cytospin method and subjected to a stepwise treatment with the solutions used for Giemsa and H&E staining, as routinely performed in diagnostic laboratories. The fluorescence of nanobeads before and after treatment did not demonstrated a significant ($P=0.3916$) fluctuation. The graph and error bars represent the mean intensity of emission signal and +/- 1 standard deviation respectively.
3.3.5 Photo-biological Assessment of Nanobeads & Dyes

Among the components of H&E and Giemsa, eosin demonstrated fluorescence. In fact the “fluorescent-like” property of eosin is well documented [157]. Eosin is reported to stain the cytoplasmic region and not epithelial borders or cell membrane [158]. Based on the optical profile of eosin, excitation at 550 nm would excite eosin, whereas excitation at 400 nm would minimize the emission of eosin. This was also demonstrated in biological samples subjected to routine Giemsa (for cells) or H&E (for tissues) staining. They included hematopoietic cells and FFPE tissue of epithelial cancer. Excitation of the samples at 400 nm and 550 nm resulted, respectively, in minimizing and enhancing the detection of the eosin signal from the cytoplasmic regions (Figures 3.13 and 3.14). The intensity of eosin staining was shown to be sample-specific and more prominent in tissue sections. Indeed, increased tissue fluorescence has been reported in FFPE samples after eosin treatment [157].

Ultimately the goal was to detect the emission of nanobeads over photo-biological effects (i.e. the interaction of light and cells) induced by staining. They included possible reabsorption effect from blue nuclear stains. In the latter category, it was demonstrated in Section 3.3.3 that fluorescence from the beads is stable after direct treatment with nuclear stains (i.e. no significant loss of signal due to photo-chemical interactions). In this section, emission signals from nanobeads at 610 nm were collected from FFPE tumour regions and demonstrated to be higher than that obtained from biological effect.
of autofluorescence or fluorescence reabsorption after H&E staining (Figure 3.15).

3.3.6 Demonstration of Application of Nanobeads in Simultaneous Detection of Immunophenotype and Tumour Morphology in Cancer Biopsies

The feasibility of using nanobeads for a simultaneous detection of morphology and immunoexpression is demonstrated using cytology and histology biopsies. For cytology, chronic lymphoid leukemia (CLL) was used as a model. B-lymphoblastic leukaemia cells were stained against CD5 and CD38, two prominent diagnostic and prognostic markers of CLL. In a direct conjugated reaction, nanobead_{560nm} and nanobead_{610nm} were, respectively, conjugated to CD5 and CD38. The conjugates were incubated with CLL cells for staining as performed routinely in clinical laboratories. Cells were examined under a microscope before and after Giemsa treatment and confirmed to present a membrane pattern using the commercial phenotyping antibody PE. Immunostaining using the nanobeads showed homogeneous CD5+ cells (Figure 3.16) and heterogeneous CD5+/CD38+ and CD5+/CD38− lymphocytes (Figure 3.17), while enabling concurrent Giemsa staining of leukemic cells. CD38 has been recently demonstrated to have prognostic potentials in leukemia. No clinical conclusion on an association between the morphology of CLL cells and their expression state could be made. Future studies are required to examine the putative link between the morphology and expression of CD38+ CLL cells.
In terms of multiplexing ability, here only two types of nanobeads were used. In theory, the use of nanobeads can be extended to multiplex (e.g. 4 or 6 makers) studies, owing to the unique optical properties of QDs. However, the physical dimension of nanobead particles presented here is 100 nm. The multiplex application of nanobeads in immunoassays may be compromised due to steric effects imposed by the physical dimensions of the particles. The extent of this limitation is perhaps related to the density or topographical distribution of target receptors. Future design of smaller particles may be needed for such applications. Changing a number of factors such as styrene concentration, polymerization time and surfactant concentration during emulsion polymerization reaction may produce smaller particles. Alternatively, polymers such as polymethyl methacrylate (PMMA) or materials such as silica may be used to coat QDs with protective layer. This method may give a better control over the thickness of the coating and, therefore, the size of the particles. This way, the distance separation between adjacent particles may also be reduced to the minimum requirement for FRET reduction. However, the underlying factor that should be considered in alternative designs is that the coating should be able to protect QDs from extreme solutions. Materials such as silica or PMMA may not satisfy this requirement as they are hydrophilic and allow solutions to infiltrate the coating. Hydrophobic modifiers on the particles surface may circumvent this limitation.
Figure 3.13 – Photo-biological Effect of Giemsa Staining of Cells. Eosin in Giemsa is used to make the cytoplasmic region (and not the membrane or nuclear regions) of cells visible. The picture demonstrates hematopoietic cells (both A and B) stained with Giemsa (bright field). Excitation of cells at $\lambda = 550$ nm shows the cytoplasmic regions stained by eosin surrounding the nuclear region. Emission of eosin or optical interference from eosin is reduced when the same cells are excited at $\lambda = 405$ nm. Images were taken at 40 X(0.85 NA) Filter: excitation = 405/20 nm, emission = 550 nm LP and excitation = 546/10 nm, emission = 565 nm LP). The scale bar is 40 $\mu$m.
Figure 3.14 – Photo-biological Effect of H&E Staining in FFPE Tissue. Eosin counter-stain in H&E is used to make cytoplasmic region (and not the membrane or nuclear regions) of cells visible. The picture demonstrates epithelial cells in cervical cancer stained with H&E (bright field). Excitation of FFPE slide at $\lambda = 550$ nm shows the cytoplasmic regions stained by eosin surrounding the nuclear region and areas of high eosin content. Emission of eosin and its optical interference is reduced when sample is excited at $\lambda = 405$ nm. Images were taken at 10X (0.5). Filter: excitation = 405/20 nm, emission = 550 nm LP and excitation = 546/10 nm, emission = 565/LP nm, respectively). The scale bar is 40 $\mu$m.
Figure 3.15 - Detection of Nanobead Signal After H&E Treatment. Emission spectrographs of nanobeads at 610 nm were taken on FFPE tumour regions after H&E staining. The level of nanobead signals (red, peaking at 610 nm) is different in different tumour regions but higher compared to the level of autofluorescence (green) after H&E staining. The samples were excited using an argon laser at 488 nm. The emissions were collected by a 20× objective (0.75 NA) and passed through a dichroic mirror and a 500 nm LP filter. The signal was then dispersed into a spectrum through the spectrometer and detected by a CCD camera.
Figure 3.16- Demonstration of Simultaneous Detection of Immunofluorescence and Cellular Morphology. Chronic lymphoid leukemia (CLL) cells (A) were stained against CD5 using PE commercially available antibody (B) and nanobead_{610nm} (C). Nanobead stained slide was treated with Giemsa (D). Both the test and control fluorescent staining showed CLL cells to be positive for CD5. Images taken at 10 X (0.5 NA) and 20X (0.75 NA). (Filter: excitation = 405/20 nm, emission = 600 LP nm and excitation = 546/10 nm, emission = 580/30 nm for Nanobead_{610nm} and PE, respectively). The scale bars show 40 μm.
Figure 3.17- Demonstration of Simultaneous Detection of Heterogeneous Immunofluorescence and Cellular Morphology. Chronic Lymphoid Leukemia cells were stained against CD5 and CD38 using nanobead$_{560\text{nm}}$ (shown as green) and nanobead$_{610\text{nm}}$ (shown as red). Nanobead stained slides were treated with Giemsa (A). Image B is the superimposed fluorescent images of CD5 and CD38. Image C is an overlay of fluorescent and bright field demonstrating the Giemsa stained cell morphology and immunofluorescence. Image was taken at 20X (0.75 NA) (Filter: excitation = 405/20 nm, emissions = 560/40 nm and 600 LP nm for Nanobead$_{610\text{nm}}$ and nanobead$_{560\text{nm}}$, respectively).
The demonstration of the feasibility of nanobead application in FFPE tumor biopsies was also examined. FFPE lung cancer tissue were stained against the prominent membrane marker EGFR using nanobeads of 10nm and nuclear regions (using DAPI) (Figure 3.18). The EGFR membrane staining was comparable to that obtained from control experiment using traditional Alexa 488 (Figure 3.17). The treatment of the slide with H&E resulted in quenching of DAPI signal, but not nanobead staining. H&E treatment also resulted in an increase in tissue autofluorescence. Autofluorescence is due to light absorption and scattering from endogenous tissue content such as elastin, fibronectin and collagen [130] [162], nucleic acids [163] and has been demonstrated to be higher after fixation and tissue processing [130]. Our study was in agreement with the literature, as tissues after H&E demonstrated an increase in the level of autofluorescence, but the level of beads were easily detectable over the increased autofluorescence (Figures 3.15 and 3.18). It should be noted, however, that the effect of autofluorescence is tissue-specific, related to the biology of the samples. For samples with high connective tissue content, the interference of autofluorescence can be further minimized by using nanobeads with emission at higher wavelength (e.g. red and infrared).

The technical feasibility of nanobead applications was also demonstrated in a simultaneous multi-parameter assessment in FFPE tumour biopsies. Using breast carcinoma as a model, we aimed to detect the presence and distribution of the epithelial marker, CK8/18, and a proliferative associated marker, epidermal
growth factor receptor-2 (HER2/neu) concurrently with H&E staining on the same breast cancer slide. HER2/neu and CK8/18 antibodies are well characterized markers and are used to identify respectively Her-2 oncogene positive phenotype and luminal epithelial cells, cells from which breast cancer originally differentiates. CK8/18 and HER2/neu antibodies were covalently conjugated to nanobead$_{560nm}$ and nanobead$_{610nm}$, respectively. Immunostaining was performed on a breast carcinoma histology section that was subsequently treated for H&E staining. H&E functioned as an internal control for the identification of epithelial cell. The morphological assessment of cells by H&E showed proliferation of epithelial cells within the ducts of the breast, as a marker for ductal carcinoma \textit{in situ} (DCIS) (slide was read by Dr. R. Goswami) (Figure 3.19). In agreement with H&E, the sub-cellular localization of epithelial cells was demonstrated by immunofluorescence (green) to be confined to the ducts. The immunofluorescence of HER2/neu proliferative marker (shown in red) was also confined to epithelial cells (as confirmed by H&E). Immunophenotypic and morphological assessment of tumour discerned what could be a single epithelial cell in the tumour microenvironment that infiltrated the confines of the basement membrane and was partially masked by inflammatory cells. The perturbation of epithelial cells from ductal boundaries of the breast is considered a critical indicator of early stages of invasive breast carcinoma [164]. In fact, the diagnosis of microinvasion has been reported to be missed in routine H&E analysis [164]. This has been attributed to performing H&E and immuno-expression analyses
on different (i.e. separate) slides. The limitation is that identification of single epithelial cells can be masked due to presenting indiscriminant colours in H&E. Moreover, small lesions in needle biopsies are often not present in deeper sections of tumour and the serial slides cut from paraffin blocks may well differ in histological information they present [119][165][164]. More recently the significance of detecting epithelial cytokeratin positive cells in the prognosis of micrometastasis was demonstrated in cancers of breast [166], colon [167] and gallbladder [168]. The simultaneous examination of cellular morphology and immunofluorescence may be useful in the studies of microinvasion.
Figure 3. 18 - Demonstration of Simultaneous Detection of Immunofluorescence and Tissue Morphology. FFPE lung cancer tissue sections were stained against EGFR using nanobead$_{610}$ nm (left panel) and Alexa 488 (right panel). The slide stained with nanobead$_{610}$ nm was treated with H&E. H&E resulted in quenching of DAPI nuclear staining (blue in A) to an undetectable level (B). The pattern of EGFR epithelial staining is demonstrated in tests A and B and confirmed using commercially available dye, Alexa 488. The histomorphology of tumour is shown in the bright field H&E image (C). D is the bright field image of the tissue with no visible cellular structure (no H&E). E (10X, 0.5 NA) and F (20X, 0.75 NA) demonstrate the epithelial pattern of staining. (Filter: excitation = 405/20 nm, emission = 600 LP nm and excitation = 480/40 nm, emission = 535/50 nm for Nanobead$_{610}$ nm and Alexa 488, respectively). The scale bars show 40 µm.
Figure 3.19 - Simultaneous Subcellular Localization and Immunofluorescence Detection in Breast Carcinoma. An FFPE breast carcinoma tissue was stained against epithelial marker CK8/18 using nanobeads 560 nm (A, green) and HER2/neu proliferative marker using nanobeads 610 nm (A, red) and subsequently treated with H&E (B). H&E showed proliferation of epithelial cells within the ducts of the breast (blue arrows), as a marker for ductal carcinoma in situ (DCIS). Image C is the overlay of immunofluorescent (A) and H&E (B) images. The subcellular localization of epithelial cells was demonstrated by H&E and immunofluorescence stainings. They showed what could be a single epithelial cell (white arrows) in the tumour microenvironment that infiltrated the confines of basement membrane. Images were taken at 20X (0.75 NA). (Filter: excitation = 405/20 nm, emissions = 560/40 nm and 600 LP nm for Nanobead$_{610nm}$ and Nanobead$_{560nm}$, respectively). The scale bars show 40 µm.
3.3 Conclusion

In conclusion, a novel design of fluorescent sensor is presented in this chapter. The nanobeads offer unique properties of QD (i.e. wide absorption and narrow symmetrical emission) while preserving the fluorescence of the particles in extreme quenchers. Nanobeads are impervious to solutions and retain their fluorescence property after subsequent treatment with H&E in histology, Giemsa in cytology and relevant solutions for slide treatment, including extreme pH (i.e. pH 0 and 14). From the technical perspective, the feasibility of a simultaneous detection of morphology and immunofluorescence in cancer biopsies was demonstrated. Future studies are required to standardize the detection parameters (i.e. amount of antibody) for each specific diagnostic test. The use of QDs in nanobeads allowed spectral separation of QDs and colourimetric dyes and, therefore, detection of immunofluorescence without optical interference with colourimetric dyes. The design of nanobeads provides a distance between QDs of different emissions encapsulated in different beads. Therefore, the signal from nanobeads may be less prone to FRET in immunoassays as compared to QDs, providing potentially a more sensitive detection probe in multiplexing assays. The use of nanosensors in clinical diagnosis may offer a novel method to examine cancer biopsies by providing a multi-factor assessment of diagnostic markers (i.e. biomarker assessment together with routine morphological examination) of the same cells or tissue sections while allowing for preservation of valuable patient samples.
Chapter 4

Application of Gold Nanoparticles in Cancer

This chapter has been partially discussed as the following papers:


This paper was the 5th most cited paper in Nano Lett in 2006. Since its publication, it has been cited 77 times to date.


Author Contribution:

This chapter is the work of the A. A. Ghazani as part of larger projects of Dr. D. Chithrani and T. Hauck. The experiments in this chapter were performed by A. A. Ghazani with the exception of experiments in figure 4.2 that was performed by Dr D. Chithrani.
4.1 Introduction

Gold (Au) is a metal element with widespread manifestations; from a highly sought-after metal as jewelry to a decorative and edible garnish in upscale restaurants. More recently, advances in metallic nanostructures has led to specific applications in medicine, mainly as delivery vehicle for thermal therapy. As a non-viral delivery platform, gold-oligonucleotide complex was demonstrated to act as an antisense (AS) agent, capable of inducing a higher cellular uptake with no toxicity as compared to the standard transfecting agents [169]. In a thermal ablative platform [170], gold particles offer an alternative to invasive surgery by delivering lethal temperature to a localized area of tumour while sparing the normal surrounding stroma tissue [171].

More recently, gold particles were demonstrated in cancer imaging, cancer diagnostics and therapeutic applications [182] [183]. The factors of biocompatibility (i.e. the ability to penetrate cells) and toxicity are the main critical factors that will determine the extent of utility of nanoparticles in such clinical applications. However, not enough is known on the consequences of nanoparticles exposure to living systems [184]. In this chapter, the effect of gold nanoparticle size on their cellular uptake is examined. Furthermore, using high throughput microarray experiments, the gene expression profiles of HeLa cells after uptake of gold particles are assessed.
4.2 Materials and Methods

4.2.1 Cell Sample and Culture

HeLa cells were cultured in Dulbecco Minimum Essential Media (DMEM) including 10% fetal bovine serum. At 80% confluence, colloidal gold particles (150 µM) of 14 nm, 30 nm, 50 nm, 74 nm and 100 nm were added to the culture and incubated for 6 hours at 37°C in 5% CO₂. Control experiments contained no nanoparticles. Following incubation, media were removed from the wells and cells were detached from the Petri dish surface using trypsin (1 ml). Cell pellets were washed 2X with PBS and collected.

4.2.2 Cell Viability Assay

The measurement of cell viability was carried out using dye exclusion with trypan Blue. 200 µL aliquot of cells were placed in Eppendorf tubes and mixed with an equal volume of 0.4% trypan blue. The mixture was incubated at room temperature for 5 minutes. An aliquot of 10 µl of stained cells were placed on a hemocytometer and counted for the number of viable (unstained) and dead (stained) cells. The percentage of viable cells is the number of viable cells divided by the number of dead and viable cells.
4.2.3 Sample Preparation for Measurement of colloidal Au Concentration

Cell pellets and supernatants were acid-digested by nitric acid at 110°C. Elemental analysis was carried out using inductively coupled plasma atomic emission spectrometry (ICP-AES) to measure the molar concentration of gold in the samples.

4.2.4 RNA Extraction

Total RNA was isolated from both untreated HeLa cells (control) and HeLa cells treated with gold nanorods (25 µM) for 6 h (test) using the RNeasy kit (Qiagen, Canada) according to the manufacturer's instructions. The experiment was carried out with three biological replicates in each group.

4.2.5 High Throughput Assessment of Gene Expression

The expression array hybridization and analysis were carried out by the University Health Network (UHN, Canada) microarray center (www.microarray.ca). Briefly, sample RNA (10 µg) and reference RNA (10 µg; human universal reference RNA) were labeled indirectly by the aminoallyl labeling procedure with Cy5 and Cy3 (Amersham Biosciences), respectively, and subsequently hybridized onto human 10K cDNA arrays (UHN, Canada) according to manufacturer's instructions. The slides were scanned using an
Agilent G2565BA scanner (Agilent, USA). TIFF images were quantified using ArrayVision v.8.0 software (Imaging Research Inc.) to produce the array data. Normalization (LOWESS, subgrid method) and clustering were carried out using the GeneTraffic software (Iobion Informatics). SAM (Stanford University) was used to perform the statistical analysis. Thresholds of ± twofold were used to define over and under expression. Array data were subjected to SAM to compare the test and control samples and obtain a list of genes with significant differential RNA expression values.

4.3 Results and Discussion

4.3.1 Cellular Uptake of Colloidal Gold Nanoparticles of Different Size

In these experiments HeLa cells were used as a cancer cell model to establish cellular uptake of gold nanoparticles. HeLa is an epithelial cancer cell line, widely accessible and easy to grow. Gold particles of various sizes (14 nm, 30 nm, 50 nm, 74 nm and 100 nm) were added to the cell culture. The surface of gold particles contained citric acid. After incubation of gold with HeLa cells for 6 hours, cells were harvested using trypsin and prepared for cell viability assay and measurement of gold uptake. Trypan blue exclusion assay was performed on harvested cells. No significant alterations in the percent of viable cells were found compared to that of control (no gold added) after 6 hour incubation time.
In general cells show 95% to 98% viability in the test experiments. This was in agreement with previously published studies on different sized gold particles. Goodman et al. [172], demonstrated incubation of 2 nm gold particles in Cos-1 cells for 24 hour did not show significant cellular death. Connor et al. [173] measured toxicity induced by gold particles of varying size and containing a variety of surface modifiers and concluded that short term incubation did have any detrimental effect on cell survival.

The average uptake of gold particles per cell was assessed from ICP and cell count data to, respectively, show the total gold element presents in the cell pellets and the total number of cells in each experiment. Among the sizes tested, gold nanoparticles of 50 nm in diameter demonstrated the highest uptake (Figure 4.1) in HeLa cells compared to that obtained from sizes 14 nm, 30 nm, 74 nm and 100 nm. The optimal size for an effective uptake of semiconductor nanoparticles [174] and gold particles [179] has been demonstrated to be approximately 50 nm. Osaki et al. [174] concluded that the size range of 50 nm governs cellular recognition for receptor-mediated endocytosis and demonstrated a higher cellular uptake compared to smaller particles. The results from our study with gold particles demonstrated a similar trend, as the cellular uptake of 14 nm and 30 nm gold particles was lower than that of 50 nm particles. Endocytosis in this case may be mediated by non-specific absorption of serum proteins onto golds surface. Studies have demonstrated the absorbance of protein to citric acid-stabilized gold particles as a way of bio-coupling [185]. In our study, Dr Devika
Chitrani confirmed that gold incubated with DMEM containing 10% serum showed the presence of protein on the gold surface. This was demonstrated by absorption spectra, FTIR spectra and Gel electrophoresis (Figure 4.2). The possible endocytosis mediated uptake of gold was also explored by transferrin coated particles. The function of tranferrin is related to intracellular transportation of ions via receptor-mediated endocytosis [186]. Studies have also demonstrated the function of this protein in a receptor-mediated transportation of a wide range of metal ions, including therapeutic metal ions and radio diagnostic metal ions [186] [187]. The transferrin receptor-mediated endocytosis pathway has also been exploited for targeted drug delivery [188]. In our study, the cellular uptake of gold particles coated with transferrin demonstrated to be three times smaller than that of non-transferrin coated particles (Figure 4.3). The size dependence of cellular entrance however, was similar to that obtained from serum coated particles: particles of 50 nm in diameter exhibited a higher uptake as compared to the particles of smaller (14 nm and 30 nm) and larger (74 nm and 100 nm) than 50 nm. Since serum contains a diverse set of proteins, the surface of the citrate-stabilized gold nanoparticles incubated with serum probably contains a variety of proteins. Therefore, the diversity of the proteins may allow entrance into cells via multiple receptors as compared to transferrin.
Figure 4.1. Size-dependence of Gold Cellular Uptake. The uptake of gold particles is size-dependent. Spherical gold nanoparticles of 50 nm in diameter (1:1 aspect ratio) demonstrated the highest uptake in HeLa cells as compared to smaller sizes of 14 nm and 30 nm and larger sizes of 74 nm and 100 nm particles. Data published in Chithrani et al [39].
Figure 4.2. Nonspecific Adsorption of Serum Proteins on Gold Nanoparticles. (A): Absorbance spectra of gold nanoparticles before incubation with serum (red) and after incubation with serum (black). B: FTIR spectra of gold nanoparticles before incubation with serum (red) and after incubation with serum (black). C: Electrophoretic mobility of gold nanoparticles before incubation with serum (lane 1) and after incubation with serum (lane 2). The data for these experiments were collected and analyzed by Dr. Devika Chithrani. Data published in Chithrani et al [39].
Figure 4.3. Size Dependence of Transferrin-absorbed Gold Cellular Uptake. The uptake of serum protein-coated gold particles (filled circles) is higher than that of transferin coated-gold particles (filled triangles). Both coatings demonstrated a similar trend in cellular uptake. The particles of 50 nm in size demonstrated a larger uptake compared to the particles of 14 nm, 30 nm, 74 nm, and 100 nm in size. Data published in Chithrani et al [39].
4.3.3 Gene Expression Analysis

The application of gold nanorods has been demonstrated in biosensing applications and thermal ablation of cancer cell [189]. Hauck et al. [170], demonstrated that the coatings of nanorods of size 18 X 40 nm affect cellular uptake of particles in HeLa cells and that among coatings of polymers, PDADMAC (diallyldimethylammonium chloride)-coated gold rods demonstrated the highest cellular uptake. The implication of this finding is that PDADMAC-coated nanorod may be better suited for biological applications. An assessment of the potential effect of these particles however was needed to elucidate the cellular behaviour after the uptake of gold particles. Therefore, a high throughput transcriptome study of nanorod uptake was performed by gene expression analysis. HeLa cells were used as a model cell line.

Total RNA was extracted from HeLa cells incubated with PDADMAC (25 μM) nanorods after 6 hour incubation. The control experiment included HeLa cells of the same culture with no addition of nanorods. The experiments were performed in triplicates. Human Universal Reference RNA was used as a reference for all array experiments, so that they can be compared with future experiments. Test and reference RNA were differentially labeled, and hybridized to 10K human cDNA arrays (includes 10 000 well-annotated genes and expressed sequence tags. Figure 4.4 demonstrates the experimental scheme and a conceptual image of the array experiment. Significance Analysis of Microarrays (SAM) was conducted to compare expression profiles of the test and the control
HeLa cells devoid of gold particles nanorods. From 10,000 genes assessed, 35 genes (0.35% of the total genes) showed significant down regulation as compared to control (Table 3.1). The False Discovery Rate (FDR) of the SAM analysis was 1.65% (n=3 replicates). FDR is a measure of the proportion of false positives among reported significantly altered genes. The SAM Plot Sheet of Microarray Data is presented in Appendix C. Figure 4.5 illustrates the heat map of the array experiment created by GeneTraffic to facilitate visual comparison of the data. Figure 4.5 depicts the overall symmetry between the test and the control experiments. No over expression was induced by addition of gold nanorods to HeLa cells. Of the 35 genes that were down regulated, a number of genes involved in apoptosis (p53 binding protein, TNF receptor superfamily, member 6, Mitogen-activated protein kinase 1 and 3), cell adhesion (Cadherin 6), cell cycle regulation, cellular metabolism, and electron and ion transport. Apoptosis (programmed cell death) is a regulatory process of cell division during development or cell proliferation. The data suggest that nanorods may induce changes in the cell cycle regulation pathway. Apoptotic signal and cycle regulation cascades include many proteins and feedback loops. Therefore, further study is required to fully understand the relationship of nanoparticles uptake and apoptosis. The potential toxicity induce by nanorods was further evaluated by examining the expression levels of genes responsible for heat-shock proteins and heat-shock transcription factors on the array. Heat-shock proteins are produced as a consequence of the exposure of cells to cellular stress, such as
toxins [176]. The microarray data indicated no substantial change in gene expression for proteins associated with heat shock toxicity.

The result demonstrated here is reflective of partial changes induced by nanorods after only 6 hour incubation in HeLa cells. Molecular effects therefore are not a measure of a chronic cellular exposure to particles. However, a-six hour incubation reportedly provided ample time for gold particles to undergo endocytosis followed by excocytosis [190]. The uptake half-life of gold particles has been demonstrated to be 1.19 hour, 1.71 hour and 2.12 hours for gold particles of 14 nm, 50 nm and 74 nm respectively. For the same size particles, the excocytosis half-life was reportedly 0.33 minutes, 0.50 minutes, and 0.75 minutes respectively [190]. It was further demonstrated that after 6 hour incubation in HeLa cells, about 80% of nanorods have exocytosed from cells. A comprehensive time-series study is required to shed light on the molecular changes induce after longer exposure to gold particles.

Molecular effects induced by gold uptake have also been assessed by other groups. Maiti et al [184] examined transcriptional profile of gold particles of 18 nm in size after 6 hour incubation with HeLa cells. The study included a measure of 19,000 genes on cDNA arrays and 47000 transcripts on high-density oligonucleotide arrays. The study reported the transcription profile of cells exposed to 18 nm gold particles did not induce known stress response pathways including immune response, cell cycle regulation, apoptosis and external stimuli sensing under conditions used. A study by Rafailovich et al. [191], also provided
a measure of cell toxicity of gold particles by measuring not the gene expression, but cell proliferation, morphological changes, cell migration and protein synthesis. The authors used 14 nm gold particles incubated with dermal fibroblasts for 2-6 days. The presence of the particles reportedly induced abnormal actin filaments and extracellular matrix constructs. There was also a decrease of cell proliferation, adhesion, and motility as a function of particle concentration (0.1 to 0.6 mg mL⁻¹). In our study, cadherin protein was also found to be altered (down regulated). This may be indicative of early changes in cell adhesion ability.

The reported conclusions are not in agreement, partly because different parameters were used in each study. For example, the incubation time of gold particles with fibroblast cells in the study by Rafailovich et al., was 2-6 days, much longer than other studies on gold toxicity. Other factors include particle size, dose and surface chemistry. More comprehensive, high throughput studies with adequate characterization are required to establish cellular response to gold nanoparticles.
Figure 4.4. Conceptual Figure of Gene Expression Profiling Using Microarrays. The premise of cDNA array is a competitive hybridization between differentially labeled test and reference RNA onto complimentary strands on the array. In this experiment, sample RNA (HeLa cells treated and non-treated with gold) and reference RNA (human universal reference RNA) were labeled with Cy5 and Cy3 respectively and hybridized onto human 10K cDNA arrays (UHN, Canada). The slides were then scanned using an Agilent G2565BA scanner (Agilent, USA). TIFF images were quantified using ArrayVision v.8.0 software (Imaging Research Inc.) to produce the array data.
Figure 4.3 . High Throughput Analysis of Acute Gold Toxicity. Gene Expression of HeLa cells treated with PDADMAC gold (left) and untreated (right) demonstrated no significant change in overall expression profile as compared to universal human RNA. Array data were subjected to SAM analysis to compare the test and control samples and obtain a list of genes with significant differential RNA expression values. From 10k genes on the array, the overall similarity was found to be 99.65%.
<table>
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<tr>
<th>Accession number</th>
<th>Unigene (UG)</th>
<th>Fold change</th>
<th>Gene name</th>
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<td>R80235</td>
<td>Hs.567303</td>
<td>5.33</td>
<td>Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)</td>
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<td>Cytochrome c, somatic</td>
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<td>Vasodilator-stimulated phosphoprotein</td>
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<td>Anaphase-promoting complex subunit 2</td>
</tr>
<tr>
<td>AA421819</td>
<td>Hs.171054</td>
<td>1.77</td>
<td>Cadherin 6, type 2, K-cadherin (fetal kidney)</td>
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<td>Hs.64235</td>
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<td>Hs.501149</td>
<td>1.87</td>
<td>PDZ domain containing 8</td>
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<td>Hs.60235</td>
<td>3.66</td>
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<td>Hs.460109</td>
<td>1.55</td>
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<td>Cytidine deaminase</td>
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<td>Six transmembrane epithelial antigen of the prostate 2</td>
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[a] Unigene (UG) cluster.

Table 4.1. Genes Exhibiting Altered Expression after Treatment with Gold. The results of microarray analysis demonstrated a list of genes with significant down regulation of their expression.
4.4 Conclusion

This study demonstrated the cellular uptake of spherical gold particles to be dependent upon the size of particles. The nanoparticles of size 50 nm had the highest uptake rate compared to particles of size 14 nm, 30 nm 47 nm, and 100 nm. The size-dependant uptake of gold may have implications in “tuning” cellular function [39]. Microarray analysis demonstrated that the uptake of large quantities of gold nanorods did not induce a significant alteration among 99.65% of 10,000 genes assessed. However, altered expression in apoptotic and cellular proliferation genes were demonstrated. They may be indicative of early cellular changes in response to gold’s uptake. A comprehensive analysis of cellular responses to gold of various dose and size, during different incubation times will elucidate a more comprehensive collection of biological effects.
Chapter 5

Conclusions & Future Directions
5.1 Summary and Conclusions

Clinical diagnostics encompasses a broad field from anatomical pathology to the detection of cellular and molecular markers. The focus of this dissertation is related to oncoproteomics in histology and cytology (Figure 5.1). The aim is to examine the application of QD nanoparticles for specific functions (as discussed in each chapter) in oncodiagnostics. A parallel goal is the refinement of current testing modalities and the design of novel strategies towards a high throughput molecular assessment: the ability to examine many markers at a given time and/or to garner biological information obtained from a given biopsy by combining different test methodologies.

In chapter 2, QDs were used in immuno-expression studies. The application of QD was combined with high throughput tissue microarray, fluorescent spectroscopy and algorithm design. The photostability of QD allowed systemic quantification of cellular analytes over prolonged times, during which biopsies are exposed to constant UV irradiation. The method described in chapter 2 demonstrated the feasibility of QD application in an automated and objective assessment of target biomarkers. The continuous readout of antigen levels bears potential implications in developing a more refined method of biomarker quantification. In chapter 3, a novel design of nanoparticles was introduced by encapsulating QDs of desired emission into polystyrene nanobeads. The nanobead demonstrated fluorescence stability of QD signal in colourimetric dyes that are widely used in diagnostics. The novelty of the new
probe is in the ability to combine two powerful diagnostic tests of cancer biopsies: immunophenotype and cellular morphology. The advantage of this detection strategy is in 1) medical advancement: the ability to correlate different sources of biological information on the same sample and 2) tumour management, specially in cases of limited tumour availability (e.g. tumour core biopsy). The technical feasibility of nanobead application was demonstrated in a simultaneous examination of tumour immunoprofile and topographical relation of neoplastic cells in their microenvironment. In chapter 4, the intracellular uptake of another type of nanoparticles, gold particles, was shown to be size-dependent. This has implications in “tuning” the function of cell (e.g. cellular delivery) based on the size of particles. The cellular uptake of gold particles however, induced alterations in the expression profile of a subset of genes.

5.2 Future Directions

Future directions in chapter 2:

The ultimate goal of antigen quantification discussed in chapter 2 is to perform an automated and objective scoring on clinical biopsies. This requires moving from xenograft samples to clinical tumor samples that often have a complex architecture. Therefore, steps need to be taken to account for the biology of tumour that exhibits infiltration of transformed cells into stroma. The key is to be able to tease out cancer cells from normal epithelial cells. This may be achieved by 1) creating epithelial masks by cytokeratin [177] [178] and 2) using
H&E slides in parallel for the confirmation of tumor morphology. Figures depicting the complex architecture of tumor biopsies are presented in the Appendix B. The use of H&E slide as a guide is pivotal for each tumor, as it allows identification of essential regions, such as tumors, and exclusion of non-essential regions, such as necrotic cells. Future directions, therefore, need to include: tumor masking of biopsies using both QD-based immunolabelling and H&E staining on separate slides. One limitation in that regard is that serial sections of tumor biopsies are often different in tumour architecture (Appendix B). Therefore, this step of tumour identification would be ideal when performed on the same TMA slide. The use of nanobeads as opposed to QDs for staining would be more suitable for this step.

Due to the “tumor selection” need within each TMA core, modifications also required in the spectroscopy and measurement system. Two important factors need to be taken into account: 1) Measurements need to be taken from smaller regions within each core to allow the selection of tumor cells by the operator. 2) Measurements need to be taken from many tumor cells per core to account for tumor heterogeneity (i.e. expression level of a given maker may be different from cell to cell). This can be achieved by subsampling in each core. The optimal number of measurements per each core should be determined experimentally.

Ultimately, the measurements from the proposed method need to be validated. There are two main levels of validation that need to be satisfied: 1)
validation of subsampling measurement. This would be complicated, as any method of validation (e.g. transcript copy number measurement) should be also performed on the same population of tumor cells selected for spectroscopy-based measurement. Methods such as needle microdissection enable collection of a bulk of cells from TMA that can be subsequently used for analysis by other techniques. However, this technique is not refined enough to allow cell-specific selections. A more practical approach would be using laser capture microdissection (LCM) that enables the user to isolate only cells of interest from surrounding stroma. The microdissected cells can be subsequently analyzed for PCR to validate the spectroscopy-based measurements per each core. 2) The ultimate validation should be done to examine the accuracy of the readings per core. This could be achieved by performing a parallel pathology reading of the TMA slide. This method could give an overall comparison of the two scoring techniques. However, caution should be taken in the interpretation of the findings: pathology scores are based on 4 values. Excluding the score for non-transformed tissues (i.e. score of 0), only 3 values define tumours (i.e. score of 1, 2 and 3). Many tumours of intermediate state on TMA arrays are usually assigned the score of 2. Therefore, performing a simple correlation study between a various measurements obtained from spectroscopy and a non-variable score of 2 could create a false positive correlation. A more accurate way of validating the readings is to correlate findings of each method with patient data. Archival tumour samples are valuable sources for this purpose, as they allow
retrospective studies using patient-specific data. Therefore, it would be possible to not only compare the QD and pathology-based measurements in such cases, but also to examine the correlative power of each method with patient data.

**Future Directions in Chapter 3:**

The unique feature of nanobead particles is that they offer a novel method of pathological reading of tumours: simultaneous examination of immunooexpression and cellular morphology. In order to take the invention from bench to diagnostics labs, a series of technical optimizations and standardization along with extensive clinical assessments need to be performed. Future directions include:

The size of nanobead particles is 100 nm, larger than typical fluorescent proteins and dye molecules (e.g. GFP and FITC are about 10 nm). The physical dimension of nanoprobeS may pose limitations due to steric effect in multiplex applications. The extent of this limitation may be related to the density and/or topographical distribution of target receptors. Modifications to the design of particles may be able to reduce the size. Technically, changing factors such as styrene concentration, polymerization time and surfactant amount during emulsion polymerization reaction can be used as a strategy to reduce particle size. Ideally, an alternative design of the probes would have the following characteristics: 1) have smaller size, 2) demonstrate fluorescence stability in extreme solutions (i.e. colourimetric dyes) and 3) provide distance separation
between QDs for the reduction of FRET (i.e. >10 nm between QDs of different emissions). Strategies other than emulsion reaction may also be explored such as coating QD particles with polymers [193] or silica [194]. The advantage of these methods, over emulsion reaction, is that a small size of QD may be easily achieved [195]. Using these methods however, it may be a trade off between the size of particles and their ability to withstand fluorescence quenching in colourimetric dyes. For instance, hydrophilic polymers or silica coatings may produce smaller particle size and provide satisfactory distance separation between particles to reduce FRET, but may also allow penetration of solutions inside. Perhaps the use of hydrophobic modifiers on the particle surface may be tested for these methods.

Research and development should also focus on standardizing the conjugation protocol. The conjugation should meet both chemistry criteria and biological criteria (i.e. preserving the functionality of antibody). A number of methods can be used for conjugation. Surface-activated beads such as glycidyl nanobeads described in chapter 3 have the advantage of ease of conjugation, as 1) they are reactive to amine groups of antibodies, with no chemical byproduct created and 2) the conjugation can be carried out at neutral pH. The latter is important in creating a suitable environment for antibodies, as many antibodies are only functional in physiological pH. The standardization of this conjugation method may be difficult as the functional groups are subjected to time-dependent hydrolysis. Carboxylic acid functional groups are more stable, but
their conjugation to biomolecules is optimal at low pH (pH 5-6), a condition that may not be suitable for pH-labile antibodies. Perhaps a more suitable method would be achieved through functionalization of nanobeads with amine groups. Amines are modified with a cross linker to create maleimide-activated surface that in turn, is able to react with sulphhydryl groups of antibodies. This method provides a rapid binding at neutral pH and at room temperature. Modification of antibodies should also be made in this method, a condition that would perhaps require biochemical expertise of the researcher assigned to this task. Steps should be taken to quantify and fully characterize the functional groups. The amount of functional groups can be used to tailor the amount of antibody bound to the surface. A standard method for the enumeration of nanobeads should be established. As non-significant as it may seem, the exact quantification of the particles is required for antibody optimization. This was a limitation in our step, as the calculations of the particles amount were estimated, based on the amount of styrene used in the reaction. Subsequently, steps of quality control are required to adjust the amount of antibody in each test to the amount of antibody that is currently used in diagnostic labs for clinical applications.

Staining optimization need to be carried out using well-known cell lines in cytology and FFPE xenografts in histology. This is to ensure that all the test and quality controls can be done on cellular platform that do not exhibit patient-specific variations. Immuno-assessment and pathology readings should be included with each test to provide adequate diagnostic accuracy, to confirm the
pattern of antibody staining (e.g. well-recognized epithelial pattern). Subsequently, similar testing should be carried out on tumour biopsies using split-sample techniques. Diagnostic readings are required for the clinical samples stained with nanobeads and those stained with standard IHC methods to establish the proper degree of staining (e.g. to avoid a false positive clinical reading due to over staining) and an adequate diagnostic accuracy.

Regarding application, there is a specific niche in the field of diagnostic that merits exploration: we demonstrated in chapter 3 that the slides were first immuno-stained and then H&E-treated. It would be interesting to examine a reverse staining order. From the technical perspective, this would be feasible, as nanobeads resist quenching by H&E. However, future studies can examine whether H&E staining affects the binding affinity of target antigens on the slides and whether immuno-staining is possible on H&E-treated slides. If shown feasible, the application of the nanobeads can be further extended to valuable archive sample. Clinical pathology laboratories have an archive of H&E-treated FFPE slides. FFPE archival samples are the most valuable sources of biopsies in histology, as they come with patient data, associated with each samples. Combining biological information extracted from specimen (i.e. immunoprofile) with patient data would allow retrospective case-control studies or retrospective cohort studies.

The application of nanobeads can be also adopted by other diagnostic tests that require colourimetric stains other than H&E or Giemsa. Histochemistry
applications include: Safranin stain used as a counterstain for colouring cell nuclei red and detection of granules, Jenner’s stain, used in microscopy for staining blood smears, Wright’s stain including May-Grunwald stain to examine peripheral blood smears and bone marrow aspirates, Prussian blue to demonstrate iron deposits in biopsy specimens and in hemochromatosis diseases, Congo red, fast green FCF and silver salts used by various techniques to selectively stain cells and cellular components.

**Future Directions in Chapter 4:**

The application of gold particles and the work discussed in chapter 4 is relevant to ex-vivo or in vivo settings. Elucidation of intracellular behaviour and effect of gold particle should be accompanied with adequate characterizations and standardization of their physiological and chemical properties. Comprehensive studies on size, dose and surface chemistry are required to elucidate the behaviour of gold particles in cellular conditions. For the assessment of gene expression, array platforms of greater genome coverage (e.g. tiling arrays) could provide information on the status of regulatory pathways involved in cellular response. Gene discovery tools may provide comprehensive functional information of the genes and their involvement in biological pathways.
Conclusion

In conclusions, QD nanoparticles demonstrated unique applications in a high throughput molecular assessment in cancer. Proposed nanobeads are novel contrast agents that bear potential implications in changing molecular-targeting strategies. While promising, future research and development are required to fully examine and/or adopt nanoparticles in diagnostics.
Figure 5.1. Schematic of Clinical Diagnostics Assessment. The application of QD nanoparticles in this dissertation is relevant to oncoproteomics. More specifically, QDs and novel design of the nanobeads are relevant to the field of in vitro diagnostics: in immunohistochemistry studies, histological staging and combined assessment of cellular morphology and imuno-expression studies (markers as red).
Appendix A- The D-Noise, the Normalizer, and the Multiplexer Routines

Hierarchy of Software:

```
  computer_algorithm.m
   |        |
 wavelet_shrinkage.m
   |        |
  -------------------
  |        |
thresholding.m  odwt.m  oidwt.m
   |        |
sfilter.m  sfilter.m
```

**Description of the Algorithm:**
The D-nosier, Normalizer and Multiplexer routines are combined in the algorithm denoted as computer_algorithm in the hierarchy of Software diagram shown above. The overall function of the algorithm is to remove autofluorescence background, apply normalization factor and combine multiplex analysis of individually obtained spectrographs. It should be noted that autofluorescence background removal is not applied to the fluorescent image by the algorithm. Instead the aim of the algorithms is to help the user in data analysis by making the process automated. The function of the algorithm can be also done manually with commercially available sources such as excel.

An optional function of the software is the removal of the signal noise from the spectra, a more sophisticated task of the algorithm. This is a spectral denoising function and is different than the D-Noise function that removes the AF. Denoising is performed by wavelet shrinkage [192] technique. Its function is thresholding the wavelet coefficients, removing small value coefficients and reconstructing the signal back with a reduced noise. This function is best used when a signal has too much noise. In our case, the application of denoising did not produce a change in signal intensity, it was only used to remove very fine noise from the signal (Figure A.1). The denoising procedure can be summarized as follows:

1. Apply the wavelet transform to the signal. (odwt.m)
2. Estimate a threshold value. (thresholding.m)
3. Remove (zero out) the coefficients that are smaller than the threshold. (wavelet_shrinkage.m)
4. Apply the inverse wavelet transform. (oidwt.m)
5. Reconstruct the signal. (wavelet_shrinkage.m)
**Description of the algorithm:**

The algorithms are comprised of the following functions. The variables in each subcategory are also defined below:

“Background signal loading” function is used to load the background text file obtained from the spectrometer. The background files were obtained from each core before staining. The text file collected from the spectrometer produced a list of wavelengths with corresponding intensity values. Wavelengths are used as X values (denoted as XB) and fluorescent intensity values are used as Y values (denoted as YB) in the software. “Raw data loading” function is used to load the data. Both background data and raw data at this stage are considered “noisy data”. The spectral de-noising (smoothing) can be carried out at this stage by removing spectral noise in the signal. This is done by wavelet shrinkage function which is a signal denoising technique based on the wavelet transform (odwt.m) function.

“Thresholding” function (thresholding.m) is used to estimate a threshold value. The variables are defined as:

- DWT=coefficients
- th=threshold level
- method string=specifying thresholding method
- w_th=Thresholded DWT coefficients

Wavelet coefficients having small absolute value are considered by the software to encode spectral noise. These coefficients values are removed by the “wavelet shrinkage” function (wavelet_shrinkage.m). The variables are defined as:

- X=Vector of observations
- Wavelet=Character string; 'haar', 'd4', 'la8', 'la16'
- nlevels=# of levels of partial DWT
- Output=DWT wavelet coefficients
- dwc=Structure of column vectors - Wavelet coefficients by wavelet filter of each level
- dvc=Structure of column vectors - Wavelet coefficients by scaling filter of each level

The following three output vectors give information on the circularity assumption:

- L= total boundary coefficients numbers in DWT for each level
- nl=# of left boundary elements of each level in MRA
- nr=# of right boundary elements of each level in MRA
- delayh=Required time advances of each dwc to approximately align with X
delayg=Required time advances of each dvc to approximately align with X

[DW,dwc,dvc,L_,nl,nr,delayh,delayg] = odwt(X, 'd4', 6)

The “inverse wavelet transform” function (oidwt.m) is applied. The graph is reconstructed with reduced spectral noise by the “wavelet shrinkage” function (wavelet_shrinkage.m). The variables are defined as:
N=Number of original signal
Dwc= Wavelet coefficients by wavelet filter of each level
Dvc= Wavelet coefficients by scaling filter of each level
Wavelet=Character string; 'haar', 'd4', 'la8', 'la16'
N levels=# of levels of the (partial) DWT
X_h=Column vector - Reconstructed signal
D=Structure of column vectors - Details of MRA
S=Column vector - Smooth of MRA
[X_h,D,S] = oidwt(N, dvc, dwc, 'd4', 6)

Plotting data is performed after subtraction of background (AF, denoted as YBB) from total data (denoted as YDD) using the D-noise function (different than the spectral denoising function) (Figure A.2). Normalization is done by “Normalize function” to the user define values of cytokeratin (denoted as N). Multiplexing is performed by the “combined plotting function”. The detailed codes of the algorithm are listed on pages 141-154.
Figure A.1. Removal of Noise from Spectra. Denoising is performed to remove background noise from data by applying the wavelet shrinkage technique. The figure depicts spectra before denoising (left) and after denoising (right).
Figure A.2. AF D-Noise Function. The level of AF before (red) and after (blue) immunostaining was measured from each core. The function of D-noise is to subtract the AF signal from total signal (AF and antigen derived signals) as demonstrated in chapter 2.
function computer_algorithm()

% The D-Noise algorithm, the Normalizer algorithm, and the Multiplexer
% Algorithm.
%
% DWT-based denoising main routine v.1.0
%
% Denoising fluorescence spectra based on the (partial) orthogonal
% discrete wavelet transform.
%
% Author: Jeong Jin Alex Lee
% Integrated Nanotechnology & Biomedical Science (INBS) Lab.
% Center for Cellular and Biomolecular Research (CCBR)
% Institute of Biomaterials & Biomedical Engineering (IBBME)
%
clc
disp('***** Denoising, Normalization, and Multiplexer Routine ***** ')
disp(' ')

% Background signal loading
n_frame = input(' Number of background signal frames (integer): ');
while isempty(n_frame) || (n_frame <= 0) || (n_frame ~= floor(n_frame))
    disp('--- Please enter a positive integer')
    n_frame = input(' Number of frames (integer): ');
end
disp(' ')

disp(' <Note> Given sequential file names, e.g., aaa_1.txt, aaa_2.txt, ....)
disp(' the common part is aaa')
f_name = input(' Common part of background signal file name (string): ','s');
while isempty(f_name)
    f_name = input(' Common part of background signal file name (string): ','s');
end
disp(' ')

tmp = load([f_name,'_1.txt']);
xB = tmp(:,1);
yB(:,1) = tmp(:,3);
if n_frame > 1
    for k = 2:n_frame
        tmp = load([f_name,'_',num2str(k),'.txt']);
        yB(:,k) = tmp(:,3);
    end
clear tmp n_frame f_name
yBB = mean(yB(:,1:size(yB,2)),2);

% Raw data loading
n_set = input(' Number of raw data sets (integer): ');
while isempty(n_set) || (n_set <= 0) || (n_set ~= floor(n_set))
    disp(' Please enter a positive integer')
    n_set = input(' Number of data sets (integer): ');
end

disp(' ')

for k = 1:n_set
    disp([' For data set ',num2str(k),','])
    n_frame = input(' Number of raw data frames (integer): ');
    while isempty(n_frame) || (n_frame <= 0) || (n_frame ~= floor(n_frame))
        disp(' Please enter a positive integer')
        n_frame = input(' Number of frames (integer): ');
    end

    f_name = input(' Common part of data file name (string): ',s);
    while isempty(f_name)
        f_name = input(' Common part of data file name (string): ',s);
    end

disp(' ')

    tmp = load([f_name,'_1.txt']);
    xD[k] = tmp(:,1);
    yD[k](:,1) = tmp(:,3);
    if n_frame > 1
        for ka = 2:n_frame
            tmp = load([f_name,'_',num2str(ka),'.txt']);
            yD[k](:,ka) = tmp(:,3);
        end
    end
    clear tmp n_frame
    yDD(:,k) = mean(yD[k](:,1:size(yD[k],2)),2);
end

% Optional DWT-based denoising routine

DWT_option = input(' DWT-based denoising (y or [n]): ',s);
disp(' ')
if isempty(DWT_option)
    DWT_option = 'n';
elseif strcmp(DWT_option,'y')
    DWT_option = 'y';
elseif strcmp(DWT_option,'n')
    DWT_option = 'n';
else
    while ~strcmp(DWT_option,'y') && ~strcmp(DWT_option,'n')
        disp(' Please enter y or n')
        DWT_option = input(' DWT-based denoising: ',s);
    end
if strcmp(DWT_option,'y') % % Denoising routine

    disp(' ...Please wait, DWT-based denoising in progress...')
    disp(' ')
    yBB_h = wavelet_shrinkage(yBB);

    in_data = [xB yBB];
    out_data = [xB yBB_h];
    data_array = ['%f','%f
'];

    fid1 = fopen('background_before_denoising.txt','w');
    fprintf(fid1,data_array,in_data);
    fclose(fid1);

    fid2 = fopen('background_after_denoising.txt','w');
    fprintf(fid2,data_array,out_data);
    fclose(fid2);

    figure(100)
    subplot(1,2,1)
      plot(xB,yBB,'-k')
      ylim([min(yBB),max(yBB)]);
      xlim([min(xB),max(xB)]);
      xlabel('Wavelength (nm)');
      ylabel('Fluorescence intensity (a.u.)');
    title('Background before denoising','FontSize',12,'FontWeight','bold','FontName','Times New Roman','Color','k')
    set(gca,'TickDir','out')
    set(gca,'Box','off')

    subplot(1,2,2)
      plot(xB,yBB_h,'-k')
      ylim([min(yBB),max(yBB)]);
      xlim([min(xB),max(xB)]);
      xlabel('Wavelength (nm)');
      ylabel('Fluorescence intensity (a.u.)');
    title('Background after denoising','FontSize',12,'FontWeight','bold','FontName','Times New Roman','Color','k')
    set(gca,'TickDir','out')
    set(gca,'Box','off')

    saveas(gcf,'background_denoising','fig')
    saveas(gcf,'background_denoising','bmp')

    disp('>>>> Denoising complete for background signal')

for k = 1:n_set
yDD_h(:,k) = wavelet_shrinkage(yDD(:,k));

in_data = [xD[k] yDD(:,k)];
out_data = [xD[k] yDD_h(:,k)];

fid3 = fopen(['ch',num2str(k),'_before_denoising.txt'],'w');
fprintf(fid3,data_array,in_data');
close(fid3);

fid4 = fopen(['ch',num2str(k),'_after_denoising.txt'],'w');
fprintf(fid4,data_array,out_data');
close(fid4);

figure(100+k)
subplot(1,2,1)
plot(xD{k},yDD(:,k),'-k')
ylim([min(yDD(:,k)),max(yDD(:,k))]);
xlim([min(xD{k}),max(xD{k})]);
xlabel('Wavelength (nm)');
ylabel('Fluorescence intensity (a.u.)');
title(['Channel',num2str(k),' before denoising'],...
     'FontSize',12,'FontWeight','bold',...
     'FontName','Times New Roman','Color','k')
set(gca,'TickDir','out')
set(gca,'Box','off')

subplot(1,2,2)
plot(xD{k},yDD_h(:,k),'-k')
ylim([min(yDD(:,k)),max(yDD(:,k))]);
xlim([min(xD{k}),max(xD{k})]);
xlabel('Wavelength (nm)');
ylabel('Fluorescence intensity (a.u.)');
title(['Channel',num2str(k),' after denoising'],...
     'FontSize',12,'FontWeight','bold',...
     'FontName','Times New Roman','Color','k')
set(gca,'TickDir','out')
set(gca,'Box','off')

saveas(gcf,['ch',num2str(k),'_denoising'],'fig')
saveas(gcf,['ch',num2str(k),'_denoising'],'bmp')

disp(['>>>> Denoising complete for dataset: ',num2str(k),...
     ' of ',num2str(n_set)])

end

yBB_h = yBB_h;
yDD_h = yDD_h;

end
% Plotting Background Signal
figure(11)
ymin = 0;
ymax = max(yBB);
plot(xB, yBB, 'b-', 'LineWidth', 1.7)
ylim([ymin ymax*1.1])
xlabel('wavelength (nm)', 'FontSize', 16, 'FontWeight', 'bold')
ylabel('fluorescence intensity (a.u.)', 'FontSize', 16, 'FontWeight', 'bold')
title('Background Signal', 'FontSize', 18, 'FontWeight', 'bold', ...
    'FontName', 'Times New Roman', 'Color', 'k')
set(gca, 'TickDir', 'out')
set(gca, 'Box', 'off')
grid on

% Plotting and Save Channel Data
yMAX = 1.1*max(max(yDD));
for k = 1:n_set
    figure(11+k)
ymin = 0;
ymax = yMAX;
plot(xD{k}, yDD(:,k), 'b-', ...
    xD{k}, yDD(:,k)-yBB,'r-', ...
    'LineWidth', 1.7)
ylim([ymin ymax])
xlabel('wavelength (nm)', 'FontSize', 16, 'FontWeight', 'bold')
ylabel('fluorescence intensity (a.u.)', 'FontSize', 16, 'FontWeight', 'bold')
legend([  'Originall(Ch',num2str(k),')', 'AF subtracted',2]);
title([  'Channel',num2str(k),',', 'FontSize',18, 'FontWeight', 'bold', ...
    'FontName', 'Times New Roman', 'Color', 'k')
set(gca, 'TickDir', 'out')
set(gca, 'Box', 'off')
grid on
clear ymin ymax

saveas(gcf,[  'ch',num2str(k),'_plot','fig'])
saveas(gcf,[  'ch',num2str(k),'_plot','bmp'])
 fid1 = fopen([  'ch',num2str(k),'_data.txt'],'w');
 for ka = 1:length(xD{k})
    fprintf(fid1,% 5.5f    %5.5f    %5.5f\n,...
        xD{k}(ka),yDD(ka,k),yDD(ka,k)-yBB(ka));
 end
fclose(fid1);
end

% Normalization
combined = yDD - [yBB yBB yBB];
combined = combined / max(max(combined));
disp('')
N_factor = input(' Normalization factor (integer): '); while isempty(N_factor) || (N_factor <= 0) || (N_factor ~= floor(N_factor))
    disp('--- Please enter a positive integer')
    N_factor = input(' Normalization factor (integer): ');
end
disp('')
combined = N_factor*combined;

% Combined Plotting
if n_set == 3
    % combined = yDD - [yBB yBB yBB]:
    figure(11+n_set+1)
    plot(xD{1}, combined(:,1), 'b:', ...
         xD{2}, combined(:,2), 'r:', ...
         xD{3}, combined(:,3), 'k:', ...
         'LineWidth',1.7)
    ylim([0 N_factor*1.2])
    xlabel('wavelength (nm)', 'FontSize',16,'FontWeight','bold')
    ylabel('fluorescence intensity (a.u.)', 'FontSize',16,'FontWeight','bold')
    legend('Channel 1', 'Channel 2', 'Channel 3', '2');
    title('All Channels', 'FontSize',18,'FontWeight','bold', ...
          'FontName','Times New Roman','Color','k')
    set(gca,'TickDir','out')
    set(gca,'Box','off')
    %grid on
    saveas(gcf,'combined_plot','fig')
    saveas(gcf,'combined_plot','bmp')
end

disp(' ')
disp('Completed!!')
disp(' ')
disp(' ')
disp(' ')
function [X_h] = wavelet_shrinkage(X)

% Denoising based on the Discrete Wavelet Transform (DWT)
% 
% input: X Column vector - Noisy data
% output: X_h Column vector - Denoised data
% 
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% wavelet = 'd4';
% wavelet = 'd8';
wavelet = 'd12';
% wavelet = 'c12';
% wavelet = 'haar';
% wavelet = 'la8';
% wavelet = 'la10';
% wavelet = 'c6';
% wavelet = 'c12';

data_l = length(X);

if data_l < 128 % DWT depth
    nlevels = 2;
elseif data_l < 256
    nlevels = 3;
elseif data_l < 512
    nlevels = 4;
elseif data_l < 1024
    nlevels = 5;
else
    nlevels = 6;
end
J = nlevels;

% for the case that data length is power of 2
if log2(data_l) == floor(log2(data_l))
    N = data_l;
    [%[DW,dwc,dvc,L,nl,nr,delh,delg] = odwt(X, wavelet, nlevels); % only for la and c
    [DW,dwc,dvc,L,nl,nr] = odwt(X, wavelet, J);
    var = (median(abs(dwc{1}))/0.6745)^2; % a practical variance estimation
    th = sqrt(2*var*log(N)); % Universal thresholding rule

    for j=J:-1:1
        % dwcth[j] = thresholding(dwc[j], th, 'hard');
        dwcth[j] = thresholding(dwc[j], th, 'soft');
    end
end
% dwcth[j] = thresholding(dwc[j], th, 'mid');
% dwcth[j] = thresholding(dwc[j], th, 'firm');
end

[X_h,D,S] = oidwt(N, dvc, dwcth, wavelet, J);

% --------- for the case that data length is NOT power of 2
else

N = 2^floor(log2(data_l));
X1 = X(1:N);
X2 = X(data_l-N+1:end);

%[DW,dwc,dvc,L_,nl,nr,delh,delag] = odwt(X1, wavelet, nlevels); % only for la and c
[DW,dwc,dvc,L_,nl,nr] = odwt(X1, wavelet, J);
var = (median(abs(dwc{1}))/0.6745)^2; % a practical variance estimation
th = sqrt(2*var*log(N)); % Universal thresholding rule

for j=J:-1:1
% dwcth[j] = thresholding(dwc[j], th, 'hard');
dwcth[j] = thresholding(dwc[j], th, 'soft');
% dwcth[j] = thresholding(dwc[j], th, 'mid');
% dwcth[j] = thresholding(dwc[j], th, 'firm');
end

[X_h1,D,S] = oidwt(N, dvc, dwcth, wavelet, J);

%[DW,dwc,dvc,L_,nl,nr,delh,delag] = odwt(X2, wavelet, nlevels); % only for la and c
[DW,dwc,dvc,L_,nl,nr] = odwt(X2, wavelet, J);
var = (median(abs(dwc{1}))/0.6745)^2; % a practical variance estimation
th = sqrt(2*var*log(N)); % Universal thresholding rule

for j=J:-1:1
% dwcth[j] = thresholding(dwc[j], th, 'hard');
dwcth[j] = thresholding(dwc[j], th, 'soft');
% dwcth[j] = thresholding(dwc[j], th, 'mid');
% dwcth[j] = thresholding(dwc[j], th, 'firm');
end

[X_h2,D,S] = oidwt(N, dvc, dwcth, wavelet, J);

X_h = [X_h1; X_h2(2*N-data_l+1:end)];
end
function [w_th] = thresholding(w, th, method)

% Thresholding algorithm
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%
% input: w Column vector - DWT coefficients
% th real number - threshold level
% method string - specifying thresholding method
% 'hard' 'soft' 'mid' 'firm'
% output: w_th Column vector - Thresholded DWT coefficients

L = length(w);

switch method
    case 'hard'
        for i=1:L
            if abs(w(i)) <= th
                w_th(i) = 0;
            else
                w_th(i) = w(i);
            end
        end
    case 'soft'
        for i=1:L
            if abs(w(i)) < th
                w_th(i) = 0;
            else
                w_th(i) = sign(w(i))*(abs(w(i))-th);
            end
        end
    case 'mid'
        for i=1:L
            if abs(w(i)) < th
                w_th(i) = 0;
            elseif abs(w(i)) < 2*th
                w_th(i) = 2*sign(w(i))*(abs(w(i))-th);
            else
                w_th(i) = sign(w(i))*abs(w(i));
            end
        end
    case 'firm'
        th2 = 2.0*th;
end
for i=1:L
    if abs(w(i)) < th
        w_th(i) = 0;
    elseif abs(w(i)) < th2
        w_th(i) = sign(w(i))*th2*(abs(w(i))-th)/(th2-th);
    else
        w_th(i) = w(i);
    end
end

otherwise
    error('Invalid thresholding method');
end

w_th = w_th';
function [DW,dwc,dvc,L_,nl,nr,delayh,delayg] = odwt(X, wavelet, nlevels)

% Compute the (partial) orthogonal discrete wavelet transform
% 
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%
% Inputs: X Vector of observations
% wavelet Character string; 'haar', 'd4', 'la8', 'la16'
% nlevels # of levels of partial DWT
% Output: DW Column vector - DWT wavelet coefficients
% dwc Structure of column vectors - Wavelet coefficients by wavelet filter of each level
% dvc Structure of column vectors - Wavelet coefficients by scaling filter of each level
% The following three output vectors give information on the circularity assumption
% L_ Row vector - total boundary coefficients numbers in DWT for each level
% nl Row vector - # of left boundary elements of each level in MRA
% nr Row vector - # of right boundary elements of each level in MRA
% The following two output vectors are only for Symmlet and Coiflet
% delayh Row vector - Required time advances of each dwc to approximatly align with X
% delayg Row vector - Required time advances of each dvc to approximatly align with X
% [DW,dwc,dvc,L_,nl,nr,delayh,delayg] = odwt(X, 'd4', 6)

N = length(X);
J = nlevels;
% Loading filter coefficients
[g] = sfilt(wavelet);
h = fliplr(g);
for i=1:length(g)
    h(i) = h(i)*(-1)^(i-1);
end

L = length(h);
% Analysis (Forward DWT)
for t=1:N/2
    dwc{1}(t,1) = sum(h.*X(mod(2*t-[1:L],N)+1));
    dvc{1}(t,1) = sum(g.*X(mod(2*t-[1:L],N)+1));
end

L_(1) = L/2 - 1; % total # of boundary coefficients in DWT
nl(1) = 2*L_(1); % # of left boundary elements in MRA
nr(1) = L - 2; % # of right boundary elements in MRA
if wavelet(1) == 'l' % in case of LA (Symmlets)
    if L == 14
        ddh = -L/2 - 1;
        ddg = -(L-4)/2;
    elseif L == 10 | L == 18
        ddh = -L/2 + 1;
        ddg = -L/2;
    end
end
else
ddh = -L/2;
ddb = -(L-2)/2;
end
delayh(1) = ceil((abs(ddh)+1)/2 - 1);
delayg(1) = ceil((abs(ddb)+1)/2 - 1);
elseif wavelet(1) == 'c' % in case of Coiflet
delayh(1) = L/6;
delayh(1) = L/3 - 1;
end

if J > 1
    for j=2:J
        for t=1:N/2^j
dwc[j](t,1) = sum(h.*dvc[j-1](mod(2*t-[1:L],N/2^(j-1))+1));
dvc[j](t,1) = sum(g.*dvc[j-1](mod(2*t-[1:L],N/2^(j-1))+1));
        end
        L_(j) = ceil((L-2)*(1-2^(-j))); % total # of boundary coefficients in DWT
        n(j) = 2^j*L_(j); % # of left boundary elements in MRA
        nr(j) = (2^j-1)*(L-1)+1 - 2^j; % # of right boundary elements in MRA
        if L_(j) > N/2^j
            fprintf('No boundary effect free wavelet coefficients at level %d
',j);
        else
            delayh(j) = ceil((abs(ddh)+1)/2^j - 1);
delayg(j) = ceil((abs(ddb)+1)/2^j - 1);
        end
    end
end

DW = [];
for j=1:J
    DW = [DW; dwc[j]];
end

function [g] = sfilter(name)
function [X_h,D,S] = oidwt(N, dvc, dwc, wavelet, nlevels)
% Compute the orthogonal inverse discrete wavelet transform along with MRA
% %
% Author: JeongJin Alex Lee
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% % Institute of Biomaterials & Biomedical Engineering (IBBME)
% % University of Toronto
% %
% Inputs: N Number of original signal
% dwc Structure of column vectors - Wavelet coefficients by wavelet filter of each level
% dvc Structure of column vectors - Wavelet coefficients by scaling filter of each level
% wavelet Character string; 'haar', 'd4', 'la8', 'la16'
% nlevels # of levels of the (partial) DWT
% Output: X_h Column vector - Reconstructed signal
% D Structure of column vectors - Details of MRA
% S Column vector - Smooth of MRA
% [X_h,D,S] = oidwt(N, dvc, dwc, 'd4', 6)

J = nlevels;

[g] = sfilter(wavelet);
h = fliplr(g);
for i=1:length(g)
    h(i) = h(i)*(-1)^(i-1);
end

L = length(h);
% Synthesis (Inverse DWT)
idvc[J] = dvc[J];
for j=J-1:2
    for t=1:N/2^(j-1)
        atmp = upsample(dwc[j],2,1);
        btmp = upsample(idvc[j],2,1);
        idvc[j-1](t,1) = sum(h.'*atmp(mod(t-2+[1:L],N/2^(j-1))+1)) ...
                  + sum(g.'*btmp(mod(t-2+[1:L],N/2^(j-1))+1));
    end
end

for t=1:N
    atmp = upsample(dwc[1],2,1);
    btmp = upsample(idvc[1],2,1);
    X_h(t,1) = sum(h.'*atmp(mod(t-2+[1:L],N)+1)) ...
              + sum(g.'*btmp(mod(t-2+[1:L],N)+1));
end

% MRA (Multiresolution Analysis)
for j=1:J
    if j > 1
        for k=j-1:2
            % Code here
        end
    end

end

for t=1:N
    atmp = upsample(dwc[1],2,1);
    btmp = upsample(idvc[1],2,1);
    X_h(t,1) = sum(h.'*atmp(mod(t-2+[1:L],N)+1)) ...
              + sum(g.'*btmp(mod(t-2+[1:L],N)+1));
end

% MRA (Multiresolution Analysis)
for j=1:J
    if j > 1
        for k=j-1:2
            % Code here
        end
    end

end
for t=1:N/2^(k-1)
    if k==j
        atmp = upsample(dwc[j],2,1);
        D_{ini}[k-1](t,1) = sum(h'.*atmp(mod(t-2+[1:L],N/2^(k-1))+1));
    else
        btmp = upsample(D_{ini}[k],2,1);
        D_{ini}[k-1](t,1) = sum(g'.*btmp(mod(t-2+[1:L],N/2^(k-1))+1));
    end
end
end
end

for t=1:N
    if j == 1
        atmp = upsample(dwc[1],2,1);
        D[1](t,1) = sum(h'.*atmp(mod(t-2+[1:L],N)+1));
    else
        btmp = upsample(D[1],2,1);
        D[1](t,1) = sum(g'.*btmp(mod(t-2+[1:L],N)+1));
    end
end
end

V_{ini}[J] = dwc[J];
if j > 1
    for k=J:-1:2
        for t=1:N/2^(k-1)
            btmp = upsample(V_{ini}[k],2,1);
            V_{ini}[k-1](t,1) = sum(g'.*btmp(mod(t-2+[1:L],N/2^(k-1))+1));
        end
    end
end

for t=1:N
    btmp = upsample(V_{ini}[1],2,1);
    S(t,1) = sum(g'.*btmp(mod(t-2+[1:L],N)+1));
end
Appendix B – Figures of Lung Cancer TMA.

The figures on each page depict a core on TMA. Staining against EGFR is performed using QD655nm (A) and IHC (B) on different slides of the same TMA. The distribution of EGFR+ cells is somewhat different in each slide due to heterogeneity of the tumor structure. Cytokeratin+ epithelial cells (C) are detected with FITC. Intratumour heterogeneity in the expression of both EGFR and cytokeratin is seen in many samples. The infiltration of tumour cells into stroma demonstrates the complexity of the tumour architecture. Development of a successful mask for the quantification of EGFR expression requires the identification of only EGFR+/cytokeratin+ cells. All other regions need to be excluded from the analysis. Image D is an overlay of images A and C. EGFR+ and cytokeratin+ cells are demonstrated in red and green respectively. The TMA is obtained from Dr. Ming Tsao laboratory. All the slides were reviewed by Dr. M. Tsao.
Figure B.1.
Figure B.3.
Figure B.4.
Figure B.5.
Figure B.6.
Figure B.7.
Figure B.8.
Appendix C – SAM Plot Sheet of Microarray Data

Figure C.1. SAM Plot Sheet of Microarray Data. SAM analysis was performed to demonstrate genes with altered expression after treatment of cells with gold particles 35 of 10000 genes assessed showed significant down regulation. The False Discovery Rate is 1.65 %. The threshold is +/- twofold.
Appendix D - Biographical Sketch

Education Background:

2005-Present  PhD Candidate, Nanomedicine & Molecular Diagnostics, University of Toronto  
Thesis: “High throughput detection of cancer targets using nanoparticles: application in diagnostics"

2002-2005  MSc, Cancer Genetics & Cytogenetics, University of Toronto  

2002  Hon. BSc, Molecular Biology and Genetics, York University  
Thesis: “Cloning and sequencing of avian Men-1 tumour suppressor gene in v-src transformed cells”

PhD Related Publications:


This article was selected and discussed as “Brightening Tumour Analysis” in Highlights of the Recent Literature, Science 2006; 314: 5806.


This paper was the 5th most cited paper in Nano Lett in 2006.

MSc Related Publications:


Selected Presentations/Abstracts


References


[34]. Taniguchi N. On the basic concept of 'nano-technology. *Proc Intl Conf Prod Eng* 1974;


[38]. Berube DM. Nano-hype: The truth behind the nanotechnology buzz


[57]. Warheit DB. How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? Toxicol Sci 2008;101;(2):183-5


[73]. Lee JA. Toward the accurate read-out of quantum dot barcodes: Design of deconvolution algorithms and assessment of fluorescence signals in buffer, [Deerfield Beach, FL: VCH Publishers], 2007. 3113p


[75]. Roederer M. Compensation is not dependent on signal intensity or on number of parameters. *Cytometry* 2001;46;(6):357-9


[91]. Förster T. Discuss Faraday Soc 1959;27:7-17


[103]. Jain KK. Recent advances in clinical oncoproteomics. *J BUON* 2007;12 Suppl 1:S31-8


[111]. Hughes S, Beheshti B, Marrano P, Lim G, Squire JA. Comparative genomic hybridisation analysis using metaphase or microarray slides. 2007;2:


[133]. J. M. Klostranec WCWC,. Quantum dots in biological and biomedical research: Recent progress and present challenges. Adv Mater 2006;18:(15):1953-64


[145]. Chen Y, Mills JD, Periasamy A. Protein localization in living cells and tissues using FRET and FLIM. *Differentiation* 2003;71;(9-10):528-41


[150]. Sugden JK. Photochemistry of dyes and fluorochromes used in biology and medicine: Some physicochemical background and current applications. *Biotechnic and Histochemistry* 2004;79:(2):71-90


[158]. Wittekind D. Traditional staining for routine diagnostic pathology including the role of tannic acid. 1. value and limitations of the hematoxylin-eosin stain. Biotechnic and Histochemistry 2003;78:(5):261-70


[171]. Stern JM, Cadeddu JA. Emerging use of nanoparticles for the therapeutic ablation of urologic malignancies. Urologic Oncology: Seminars and Original Investigations, 2008;26:(1):93-6


