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# Fluorescent nanocrystals for use in early cervical cancer detection

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**Abstract**

*Background.* Quantum dots (qdots) are a promising alternative to organic fluorophores for biological imaging. Advantages of quantum dots over organic fluorophores include broad excitation coupled with narrow, tunable emission, high resistance to chemical and metabolic degradation, a higher photobleaching threshold and finally the ability to be modified with a targeting ligand. These many properties allow quantum dots to be used in conjunction with optical detection methods for imaging.

*Methods.* We are investigating the use of quantum dots to detect precancerous biomarkers. We have directly targeted epidermal growth factor receptors with quantum dots conjugated to anti-EGFR antibodies.

*Results.* Compared to appropriate controls, we do see specific labeling of EGF receptors.

*Conclusions.* Quantum dots provide a promising alternative to conventional organic dyes for biological imaging. Combined with optical imaging technologies, quantum dots can help visualize changes in cervical cancer at the molecular level. This ability may alert health care providers to the need for intervention before a cancer can metastasize.


*Keywords:* Cervical cancer; Quantum dots; EGFR; Fluorescent probes

## Introduction

According to the American Cancer Society, cancer has surpassed heart disease as the leading cause of death of Americans under the age of 85. Cancer kills 30% of our population and is widely acknowledged as the most devastating threat to the health of North Americans of all ages and the world as a whole. Over the last 50 years, despite tremendous advances in our understanding of the molecular and cellular processes of cancer, there has been no change in the age-adjusted mortality due to cancer [1]. In order to further reduce the morbidity and mortality due to cancer, it is necessary to improve methods for early detection and prevention (Table 1).

Advances in molecular biology and imaging technologies may be the key to reducing cancer incidence, morbidity and mortality. The ability to non-invasively monitor cellular processes at the molecular level has the potential to lead to new ways of diagnosing disease, selecting effective therapeutics and monitoring response to these treatments. Molecular imaging requires two components: a molecular-specific source of signal (typically provided through a contrast agent) and an imaging system to detect this signal. In recent years, high resolution micro-PET, MRI and ultrasound have shown promise for molecular imaging in animal studies [2]. However, these systems are expensive and do not provide sufficient resolution to image subcellular detail in real time. An alternative is optical imaging. Optical imaging can be carried out non-invasively in real time, yielding unprecedented spatial resolution (less than 1 μm lateral resolution). Optical imaging systems are inexpensive, robust and portable. Confocal microendoscopes which image fluorescent and reflected light have been used to

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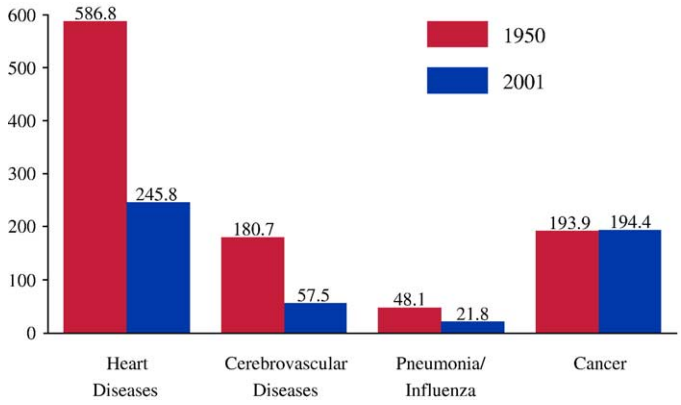
Table 1
Change in US death rate by cause, 1950 and 2001




*Age-adjusted to 2005 standard population.
Sources: 1950 Mortality Data—CDC/NCHS, Mortality Revised.
2001 Mortality Data—NVSR-Death Finale Data 2001—Volume 52, No. 3.
http://www.cdc.gov/nchs/data/nvsr/nvsr52/nvsr52_03.pdf.

image subcellular features in epithelial tissue at video rate to depths exceeding 400 μm [3–6]. Lower magnification optical microscopes can be used to image the reflectance and fluorescence of large surface areas of tissue at risk for cancer and precancer and to identify suspicious areas and tumor borders [7,8].

The cervix is attractive for optical detection because it can be accessed easily for imaging, and the changes which take place in the epithelium and stroma can be interrogated using visible and near infrared light. Additionally, the use of a contrast agent targeted to a biomarker of cancer will enable cancer to be detected at an early stage. It is believed that the vast majority of epithelial cancers begin as preinvasive lesions, usually years before a tumor is palpable. These precancerous lesions are confined to the surface epithelium (typically less than 500 μm beneath the tissue surface). The detection of epithelial cancers, and particularly preinvasive disease, is ideally suited to optical imaging technologies, which can penetrate 1–2 mm of tissue depth [9–11].

### *Quantum dots*

Imaging the molecular features associated with cervical cancer requires molecular-specific contrast agents which can safely be used in vivo. Generally, a contrast agent consists of three parts: (i) a probe molecule which provides molecular-specific recognition of cancer biomarkers conjugated to (ii) an optically interrogatable label in (iii) a mucoadhesive, permeation-enhancing formulation (Fig. 1). Recent work has explored the use of several optically active labels including metal nanoparticles, quantum dots and organic fluorophore dyes.

Quantum dots, or nanocrystals, have unique electrical and optical properties such as: high quantum yields, narrow emission bands, high absorbancy, large Stoke's shift, high resistance to photobleaching and can provide excitation of several different emission colors using a single excitation wavelength [12]. These properties are due to their nanometer scale size (1–10 nm), which is smaller than the Bohr radius [13]. Quantum dots exhibit an electronic structure intermediate between bands and bonds resulting in a direct correlation between size and band gap energy (emitted wavelength): as the size of the quantum dot decreases, the band gap energy increases. This property enables quantum dots to be designed to emit at a wavelength of interest.

The above properties of quantum dots make them an ideal choice for cellular labeling, in vivo labeling, long-term cellular studies and multispectral imaging. A high quantum yield, high absorbancy, narrow emission bands and large Stoke's shift make them easy to visualize without imposing stringent requirements on the optical systems. The narrow emission spectrum without the long tail at red wavelengths characteristic of dyes reduces or eliminates spectral cross talk in detection. The large Stoke's shift enables fluorescent signals from the qdots to easily be separated out from scattered excited light. Additionally, the high photostability of quantum dots relative to dyes allows the real-time monitoring or tracking of intracellular processes over long periods of time (minutes to hours). The fact that nanoparticles are similar in size scale (<5 nm) to common biomolecules makes them ideal for applications such as intracellular labeling and bioconjugation [14]. Of additional interest is the possibility of exciting a broad spectrum of quantum dot colors using a single excitation laser wavelength, which may enable several biomarkers to be probed simultaneously.

A final attractive quality of quantum dots is that they can be synthesized to emit within a spectral region, 600–1300 nm, which has been demonstrated as best suited for biological imaging due to high physiological transmissivity [15]. The main benefit of using near IR light is an increased

penetration depth due to the low extinction coefficients of hemoglobin, melanin, and water, three prominent skin chromophores, in the near IR region [16] as well as the ability to filter out the signal from excitation light. Other factors that influence tissue fluorescence spectra include collagen, FAD, NAD(P)H, tryptophan and other scatterers and absorbers.

Despite the many advantages of quantum dots as compared to conventionally used fluorophore dyes, their biological applications have been hampered by their inherently low solubility in water. Recently, new chemical strategies have been established for rendering the quantum dots soluble in various biological solvents enabling their use in biological imaging [17–22].

## Biological applications

### *Cellular labeling*

Semiconductor quantum dots are emerging as promising fluorescent labels for cellular imaging. In addition to the above-mentioned benefits over dyes, quantum dots have been shown to specifically and effectively label molecular targets both at cellular and subcellular levels. The main hindrances to cellular labeling have mainly been quenching due to incomplete surface passivation, non-specific binding and toxicity or disruption of cellular processes.

Several groups have successfully demonstrated the specific targeting of qdots in cellular systems as well as the ability to detect the signal from qdots in cellular labeling. Bruchez et al. have used a combination of qdots linked to a humanized anti-Her2 antibody to label Her2, a breast cancer marker, on the surface of fixed and live cancer cells. The qdot conjugates were shown to specifically and effectively label the molecular targets. Additionally, Bruchez et al. have demonstrated multi-target labeling by simultaneously labeling nuclear antigens and microtubules using quantum dots with two different emission wavelengths. Nuclear antigens were targeted using human anti-nuclear antigen (ANA), and microtubules were labeled with anti-α-tubulin antibody and visualized under a fluorescent microscope. Both colors were clearly visible and spectrally resolved [23]. Additional groups have labeled various cellular targets utilizing different types of quantum dot structures, surface passivation strategies, recognition elements (proteins, peptides, DNA) and conjugation chemistries. Chan and Nie labeled HeLa cells with CdSe/ZnS core-shell quantum dots that had been functionalized with mercaptoacetic acid and crosslinked to transferrin or IgG via carbodiimine chemistry. In the absence of transferrin, no quantum dots were observed inside the cell. When transferrin, an iron transport protein, was present, the quantum dots were recognized by the receptors on the cell surface and endocytosed [24].

### *In vivo labeling*

The ability to label in vivo with quantum dots has recently been realized. For example, quantum dots conjugated to peptides which specifically target normal lung or tumor blood vessels or for tumor lymphatic vessels were i.v.-injected into tumor-bearing mice. Specific targeting of lung or tumor vasculature using peptide-coated ZnS-capped CdSe quantum dots was demonstrated. Additionally, no acute toxicity was observed after 24 h of circulation [25]. In another study, the long-term stability and tissue deposition of quantum dots were investigated in Balb/C mice that were intravenously injected with PEG-750 qdots. The results show that even after 133 days the qdot fluorescence is still present and localized within the lymph nodes, bone marrow and spleen [25]. These studies suggest that the labeled nanoparticles are stable in the in vivo environment.

Another important factor to consider in both in vitro and in vivo cellular labeling is the cytotoxicity of quantum dots. The inherently toxic elements of the qdot core (e.g., cadmium, tellurium) have caused concern that these elements could potentially harm both cell cultures and live animals. Cadmium exposure manifests itself in liver hepatocytes where it binds to the sulfhydryl groups of critical mitochondrial proteins causing inactivation of thiol groups. This leads to oxidative stress and mitochondrial dysfunction. Although it has been shown that short-term qdot labeling in immortalized cell lines is not highly sensitive to heavy metals, it is of major concern in vitro and especially in vivo.

Bhatia et al. have investigated the effect of processing parameters and surface coatings on the cytotoxicity of qdots using extreme conditions, such as those experienced in the reticuloendothelial system in the liver, to produce worst-case scenario conditions to elucidate any problems that may occur in vivo. She also investigated the effect of qdots on normal hepatocyte functions such as albumin production, migration and differentiation over 2 weeks in a cell culture. Results of Bhatia's studies show that capping ligands or surface coatings that can prevent the diffusion of oxygen to the qdot and therefore decrease the liberation of free cadmium ($Cd^{2+}$) reduce cytotoxicity. She compared various coatings, such as mercaptoacetic acid, ZnS, DHLA, BSA and polyacrylate/streptavidin, and investigated the consequence of increasing the time period between organometallic synthesis of qdots and capping for passivation. Both BSA and polyacrylate/streptavidin-coated qdots showed the largest reduction in cytotoxicity, but some free cadmium was liberated. Therefore, cadmium liberation from the qdots must be addressed. In all cases, delay in capping caused a marked increase in cytotoxicity due to the oxidation and liberation of cadmium.

Finally, hepatocytes were co-cultivated with nonparenchymal cells that promote differentiated function of hepatocytes in vitro to demonstrate the utility of qdots as a tool for long-term live cell labeling of a sensitive model system

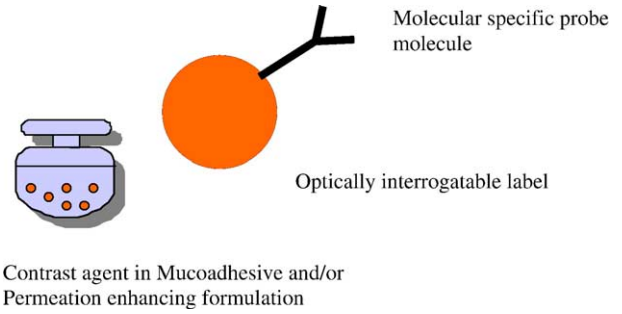



Fig. 1. Contrast agent concept.

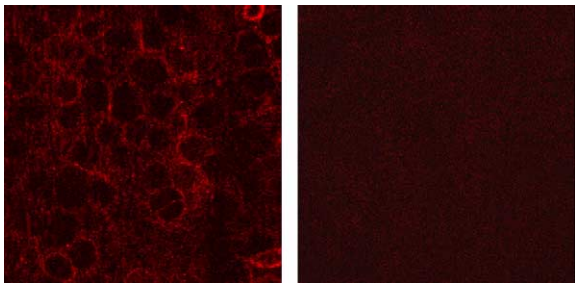


Fig. 3. SiHa tissue phantoms labeled with 655 nm qdot streptavidin conjugated to anti-EGFR antibody (left) and anti-IgG antibody (right).

of liver tissue, the site of Cd toxicity in vivo. It was shown that organically coated ZnS-capped CdSe qdots did not hinder cell viability, migration or differentiation when monitored for a 2-week period. Qdots could be similarly used to track dynamic cellular processes including stem cell progeny, cancer metastases, morphogenesis and wound healing [26].

## Nanocrystals to label cervical cancer

### *Biomarkers targeted*

In the last decade, enormous progress has been made to understand the molecular events that accompany carcinogenesis. The identification of unique molecular markers of cancer, such as EGFR, and the associated processes they modulate allow direct targeting and interrogation of these markers by optical tags. The epidermal growth factor receptor is a 170-kDa transmembrane receptor tyrosine kinase that, in binding six ligands, stimulates the proliferation of a wide variety of animal cell types [27]. EGFR is expressed ubiquitously in cells and is over-expressed in human malignancies including breast cancer, glioma and lung cancer, making it a promising biomarker [28]. There is good consensus among immunohistochemical studies in the cervix that EGFR levels demonstrate a statistically significant increase as lesion severity progresses from earlier dysplasia to invasion [29,30]. Activation of the epidermal growth factor receptor has been shown to contribute to the proliferation and spread of many different types of solid tumors. The over-expression of EGFR has been correlated with many processes related to cancer, including uncontrolled cell proliferation, autocrine stimulation of tumor growth by tumors producing their own growth factors and prevention of cellular apoptosis.

### *Ex vivo labeling of SiHa cells*

Extensive literature documenting the usefulness of quantum dots for use in labeling exists as evidenced in the above review. We have begun to use this nanotechnology to specifically label SiHa cervical cancer cells (Fig. 2)

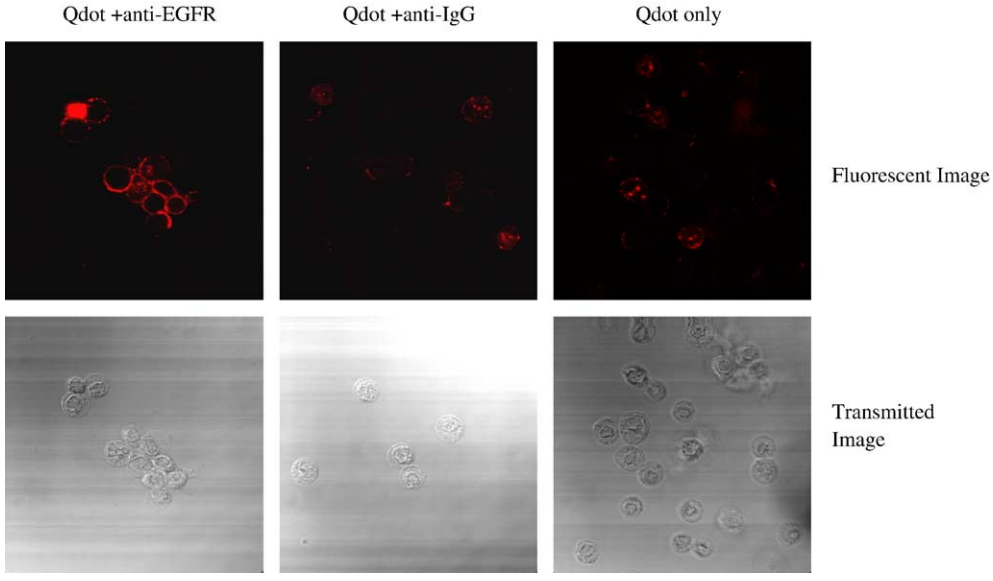



Fig. 2. Confocal fluorescence and transmitted images of SiHa cervical cells labeled with 30 nM anti-EGFR quantum dots (Qdot Corp.).

and three-dimensional tissue constructs (tissue phantoms) made with SiHa cells (Fig. 2). SiHa cells are known to have EGFR expression level between $2 \times 10^4$ and $20 \times 10^4$ receptors/cell [31]. Tissue phantoms are important in extending the labeling of cells to a more complex tissue-like system in an effort to validate the biological utility of quantum dots as labeling agents.

For cellular labeling, SiHa cells were incubated with biotinylated anti-EGFR monoclonal antibody (clone 111.6, LabVision). As controls, cells were incubated with biotinylated normal mouse IgG or with PBS only. A 10 nM solution of the 605 nm quantum dot streptavidin conjugate (Quantum Dot Corp., Hayward, CA) was prepared, and cells were incubated for 1 h. As an additional control, cells which had not been exposed to antibody or the quantum dot solution were also prepared. After final washing, imaging was performed on a laser scanning confocal microscope with 488 nm and 568 nm excitation. Images of the SiHa with the specific targeting due to anti-EGFR antibody showed significantly stronger intensity at both 488 nm and 568 nm than the controls with non-specific IgG antibody.

For phantom labeling, SiHa cells were made into three-dimensional tissue culture phantoms [32]. Phantoms of each epithelial cell line were prepared by resuspending cultured cells in a volume of buffered collagen type I to obtain a cell density of $10^8$ cells/ml. Collagen cell suspensions were plated into 6.5 mm diameter transwells and allowed to gel at 37°C. The prepared tissue phantoms were allowed to grow in DMEM plus 5% FBS for 24 h so that they formed a highly dense structure consisting of multiple layers of epithelial cells [33]. The phantoms were determined to have a thickness between 400 and 600 μm. For labeling, phantoms were incubated with biotinylated anti-EGFR monoclonal antibody (clone 111.6, LabVision) at .02 mg/ml in 10% DMSO for 45 min at room temperature. As controls, cells were incubated with biotinylated normal mouse IgG only. After rinsing the phantoms with phosphate-buffered saline (PBS), phantoms were incubated in 40 nM solution of 655 nm quantum dot streptavidin conjugate (Quantum Dot Corp., Hayward, CA) in 10% DMSO for 45 min. After final washing, cell phantoms were imaged using a Zeiss Axiovert 100M microscope modified for structured illumination in order to provide an optically sectioned image. Images of the SiHa with the specific targeting due to anti-EGFR antibody showed significantly stronger intensity than the controls with non-specific IgG antibody (Fig. 3).

## Conclusions

Currently, the clinical classification of cancer and its precursors is based on phenotypic markers, such as nuclear to cytoplasmic ratio and extent of invasion. It would be highly advantageous to be able to specifically target biomarkers of cervical cancer that are present well before metastasis thereby increasing the patient's chance of survival. Fluorescent organic dyes have been investigated for use as an optically active agent for cancer detection. However, the sensitivity of organic dyes to photobleaching may limit their utility for in vivo use.

Quantum dots are a good alternative to the use of organic dyes for biological imaging. Their resistance to photobleaching and brightness make them more attractive for use in imaging than conventional fluorophore dyes. Additionally, the ability to add targeting ligands makes them attractive for use in detecting biomarkers for cancer. Preliminary results show that quantum dots conjugated to anti-EGFR antibodies can be used in conjunction with optical technologies to visualize the molecular changes involved in cervical cancer.

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