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Quantum Dot Immuno-Conjugates Allow for Reliable Photo-Stable Detection of Intracellular Stages of Toxoplasma Gondii

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QUANTUM DOT IMMUNO-CONJUGATES ALLOW FOR RELIABLE PHOTO-STABLE DETECTION OF INTRACELLULAR STAGES OF *Toxoplasma gondii*

By

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Approved by:

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ABSTRACT

Luminescent quantum dot nanoparticles have emerged as a highly effective alternative to organic fluorescence probes in a variety of applications employing the use of immunoconjugates. In the present study, we have successfully used quantum dot technology for the immunofluorescent detection of intracellular stages of *Toxoplasma gondii* in a CV-1 mammalian cell line. This assay will allow us to investigate the question of whether selenium and other antioxidants have a direct impact on the intracellular cycle of the parasite. A Lab-Tek cell culture system was used to determine optimal concentrations of primary and secondary antibodies. Infected CV-1 monolayers were fixed in 4% formalin and cell membranes were permeabilized with detergent prior to blocking and immune-labeling. The use of a mouse anti-*T. gondii* primary antiserum followed by quantum dot – conjugated secondary antibody (Goat anti-mouse IgG) resulted in a punctuate, intensely bright fluorescent labeling of intracellular tachyzoite stages with no evidence of photobleaching or fading. In addition, the application of cover slips using Cryoseal Mounting Media 60, allowed for the long term storage of stained slide preparations.

INDEX WORDS: Immunology, Parasitology, *Toxoplasma gondii*, Quantum Dot Technology, Immuno-fluorescence

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DEDICATION

I would like to dedicate this work to my parents, my family and my husband. Your love, support and words of encouragement gave me the strength to complete my undergraduate career. Without the help of each of you this would have not been possible.

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It was a pleasure working with the many people who made this thesis possible. I would like to thank everyone who supported me and were involved in one way or another in the preparation of this thesis. With the biggest contribution to this CE/T project, I would like to express my sincere gratitude to my thesis advisor Dr. Cheryl D. Davis, for the continuous support of my undergraduate career and research experience, for her patience, motivation, enthusiasm, and immense knowledge. Throughout my thesis-writing period, she provided encouragement, sound advice, good teaching and lots of good ideas. Along with my thesis advisor, I would like to thank the rest of my thesis committee: Dr. Nancy Rice and Amy Chester, for their insightful comments and the willingness to be part of this thesis. I would also like to thank my academic advisor Dr. Kenneth Crawford, for his guidance throughout my undergraduate career and valuable advice about career choices.

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INTRODUCTION

Toxoplasma gondii is an Apicomplexan protozoan parasite that causes a disease known as toxoplasmosis. Infection is generally chronic and asymptomatic in individuals with a strong immune system. On the other hand, infection can be reactivated in individuals who are immunocompromised, causing various types of neuropathology. *Toxoplasma gondii* infection is also an important cause of miscarriage in pregnant women, and serious birth defects in newborns. Toxoplasmosis is prevalent worldwide in many mammalian species as well as birds. Cats are the only definitive host for this parasite. *T. gondii* has three stages in its life cycle: tachyzoites, present during the acute phase of infection; bradyzoites, present within tissue cysts during the chronic phase of infection; and oocysts produced during the sexual cycle within the gastrointestinal tract of cats. Tachyzoites have a crescent shape, and they do not have a visible way of locomotion such as pseudopodia, flagellum or cilia. They enter host cells by penetrating through the host cell plasma membrane or by inducing phagocytosis. After entry of tachyzoites into a host cell, there is a variable lag period before the parasite divides, and this lag phase is partly parasite strain dependent (Dubey et al, 1998; see Figure 1).

Previous studies in our laboratory determined the impact of vitamin E and selenium supplementation in a murine model of toxoplasmosis. Results showed that increased dietary supplementation with these two antioxidants resulted in trends towards increased tissue cyst number, tissue pathology, and weight loss (McCarthy and Davis, 2003). These results were surprising, since dietary supplementation with antioxidants such as vitamin E and selenium generally results in an enhancement of the immune response, resulting in increased

resistance to a variety of pathogens as well as tumors in animal models and in humans (McCarthy and Davis, 2003). An important question that emerged from our antioxidant study was whether vitamin E and selenium might have direct effects on the intracellular cycle of *T. gondii*. We decided to first investigate the impact of selenium exposure on the intracellular cycle of the parasite. My role in this investigation was to develop and optimize an in vitro cell culture model of infection that would allow us investigate this question. This study was conducted using the ME49 strain of *T. gondii* and a CV-1 (African Green Monkey Kidney) mammalian cell line. An indirect immunofluorescence assay utilizing Quantum Dot technology was also developed to allow for accurate visualization and quantification of intracellular stages of the parasite.

Quantum Dots (QD) are nanometer-sized functional particles that display crystals of semiconductor materials, which makes them well suited for visualization and tracking (Pathak et al., 2005). They posses a core-shell material, such as CdSe coated with ZnS, and are further coated by a polymer shell that allows the materials to be conjugated to biological molecules (Lee and et al., 2004). Scientists have developed hybrid functionalized QDs-liposome nanoparticles and have found that they are efficiently taken up by living cells without causing cell death. Therefore, QDs can be used as fluorescent probes for *ex vivo* cell-labeling studies with most types of water-soluble QD without further modifications (Figure 2). Luminescent quantum dot nanoparticles also have emerged as a highly effective alternative to organic fluorescence probes in a variety of applications employing the use of immuno-conjugates. Quantum Dots (QDs) are able to overcome some of the limitations commonly encountered with organic fluorophores such as fading and low signal strength (Lee and et al., 2004; Howarth et al., 2005). Brighter signals have been reported in

experiments with QD than organic dyes due to greater adsorption of the excitation light and the high photobleaching threshold in QDs (Lee and et al., 2004). Also, QDs offer advantages such as higher quantum yield, a narrow, tunable and symmetric emission spectrum, and allow *in vivo* imaging.

Quantum dot-conjugated antibodies have been successfully used for the detection of *Cryptosporidium parvum* and *Giardia lamblia* in water samples (Zhu et al., 2004). Another study conducted by Tokumasu and Dvorak (2003) demonstrated the applicability of QD technique to the study of erythrocyte membrane modifications that occur during invasion by *Plasmodium spp*, the causative agent of malaria. Quantum Dots have also been successfully used to detect *C. parvum* oocytes. The study conducted by Lee et al. in 2004, clearly demonstrated that QDs are highly photostable when compared to other organic dyes such as Alexa 488-streptavidin, FITC, and EPA. Immunofluorescent detection of *C. parvum* oocytes using QD-conjugated antibodies was shown to be a highly specific, sensitive, and photostable.

In the present study, we adapted QD technology for the immunofluorescent detection of intracellular stages of *T. gondii* in CV-1 cells. The primary goal of the present study was to develop and optimize a Quantum Dot immuno-conjugate technique that would allow for reliable, photo-stable detection of intracellular stages of *Toxoplasma gondii* in an in vitro model of infection.

Figure 1. Life cycle of *Toxoplasma gondii* (from Cambridge University Website)

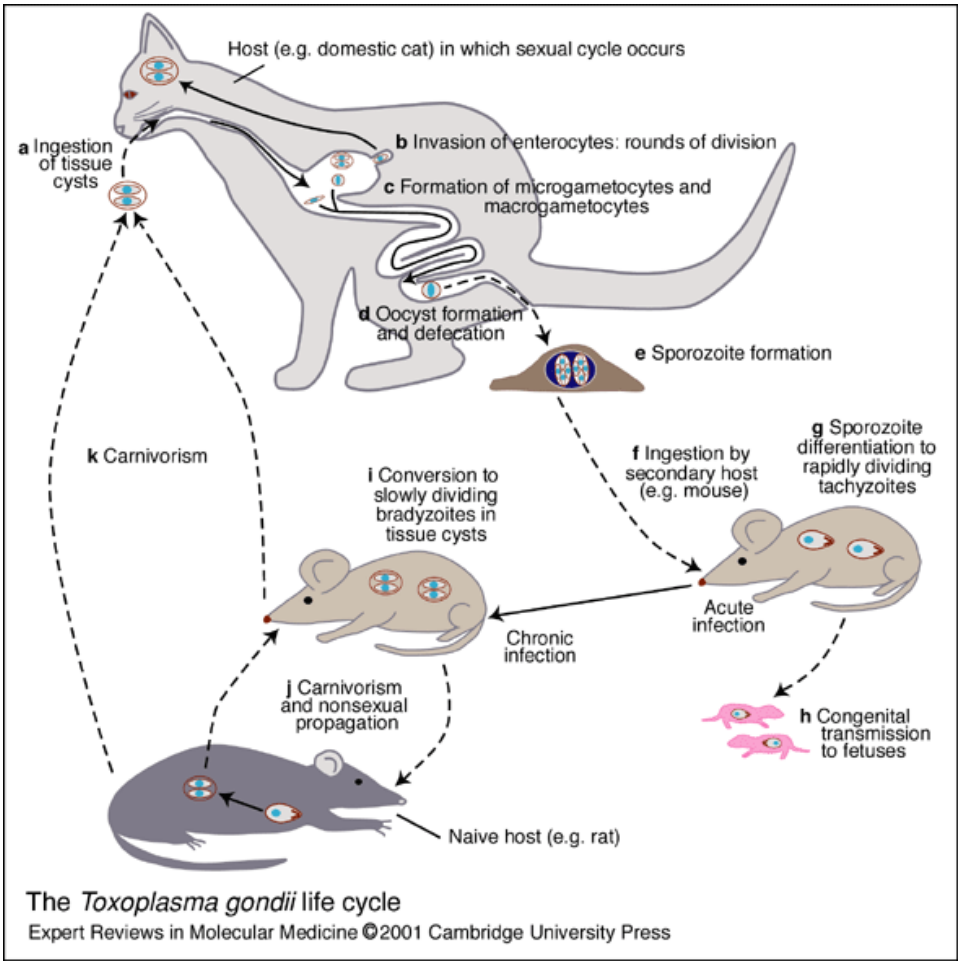
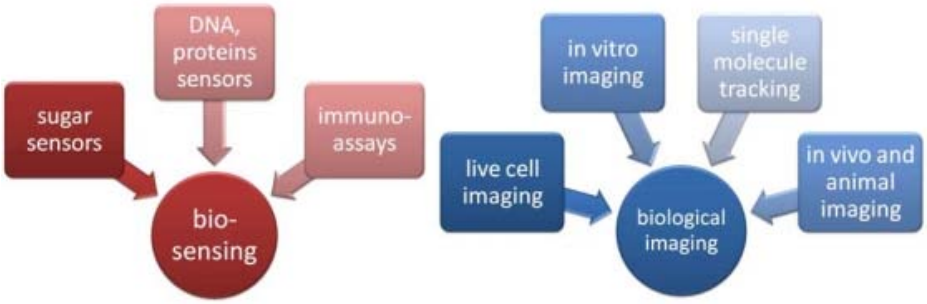


Figure 2. Examples of QDs' bioanalytical and biomedical applications. (from International Journal of Molecular Science)



MATERIALS AND METHODS

Cell cultures:

An African Green Monkey Kidney cell line (CV-1) was used for all in vitro cell culture experiments. The cells were maintained in sterile RPMI-1640 medium, supplemented with 10% newborn calf serum and penicillin-streptomycin (all culture reagents from Sigma Chemical Company, St Louis, Mo.). The cells were grown in T75 T flasks and were incubated at 37ºC with high humidity and 5% CO_2.

Parasites:

The parasite strain used in this study was the *Toxoplasma gondii* ME49 strain. The ME49 strain is maintained in our laboratory by passage in Swiss Webster mice and by infection of CV-1 cell monolayers with tachyzoite stages of the parasite.

Preparation of cells for immunofluorescence assay (IFA):

After infected CV-1 cells reached confluency, they were harvested from T flasks using sterile ethylenediaminetetraacetic acid buffer (EDTA). Culture supernatants were removed from each T flask, and cells were incubated in the presence of 5 ml of sterile EDTA for 5-10 minutes at 37°C. Once cells had visibly detached from the T flask, 5mL of complete RPMI-1640 was added to each flask, and the cells were resuspended by repeated pipetting. The resulting cell suspension was then placed into a sterile 15ml centrifuge tube and the tube was subjected to centrifugation for 5 minutes at 2000rpm. After decanting the supernatant, the cell pellet was re-suspended in 4ml of RPMI-1640. The infected cells were counted using a hemacytometer, and the concentration of cells was adjusted to 1.7 x10^6 cells/ml.

Antibody reagents:

The primary antibody used in the Quantum-dot immunofluourescence assay (Q dot assay) was mouse anti-*Toxoplasma gondii*. Mice in the chronic stages of infection with the ME49 strain of *T. gondii* were anesthetized with the inhalant anesthesia isoflurane. Blood was obtained by cardiac puncture, and was allowed to clot overnight at 4°C. Sera were then aliquoted and frozen at -20°C until use. The secondary antibody used in all Q dot assays was goat anti-mouse IgG conjugated to fluorescent Quantum dots (Invitrogen Corporation, Carlsbad, CA). The control primary antibody used in each assay was normal mouse serum.

Lab tech slides:

Lab Tek Chamber Slides (VWR Corporation, West Chester, PA) were used for all Q dot assays. In preparation for the assay, each of the 8 wells on the slide was filled with 250µl of the infected cell suspension at a concentration of 1.7×10^{6} cells/mL. Cells were cultured overnight at 37°C in a CO_2 incubator to allow the attachment of the cells to the slide.

Q dot assay:

Fixation and permeabilization

The supernatant of all wells was decanted. Wells were rinsed with 250µl of 1X phosphate buffered saline (PBS), and then decanted. Cultured cells were fixed with 4% formalin in PBS for 15 minutes. After the 15 minutes incubation, each chamber was washed twice with PBS. Cells membranes were permeabilized with 0.25% Triton X (Sigma Chemical Co.) in PBS for 15 minutes, followed by two washes with PBS. Cells were incubated in a humidity chamber with blocking buffer (5% Nonfat Dry Milk in PBS) overnight at 4°C. Wells were then

washed 3 times in PBS, with 5 minute intervals. Cells monolayers were incubated with mouse anti-*T. gondii* antiserum or normal mouse serum for 1 hour at 37°C (at dilutions ranging from 1/25 to 1/100 in blocking buffer). Wells were then washed 3 times in PBS, with 5 minute intervals. Incubation followed with Q dot conjugated secondary antibody (Quantum dot conjugated goat anti- mouse IgG) for 1 hour at 37°C (at dilutions of 1/500 or 1/1000). Chambers were removed from slides, and slides were washed 3 times in PBS, with 5 minutes intervals. Glycerol was added to each slide, and a cover slip was then mounted on the slide.

Imaging

Slides were viewed on a Zeiss microscope equipped with epifluorescence. Stained cells were viewed and photographed at various magnifications (primarily 400X and 1000X) using oil-immersion lenses with a camera mounted on the microscope.

RESULTS

In vitro cell culture model of *T. gondii* infection

The CV-1 (African Green Monkey Kidney) cell line was successfully grown in T-75 flasks and on LabTek culture slides in the laboratory, and proved to be an excellent in vitro model for monitoring the intracellular cycle of *T gondii* infection (Figure 3). The CV-1 cell line was readily infected with tachyzoite stages of *T. gondii*, and high numbers of tachyzoite stages could be obtained from culture supernatants within two weeks following infection.

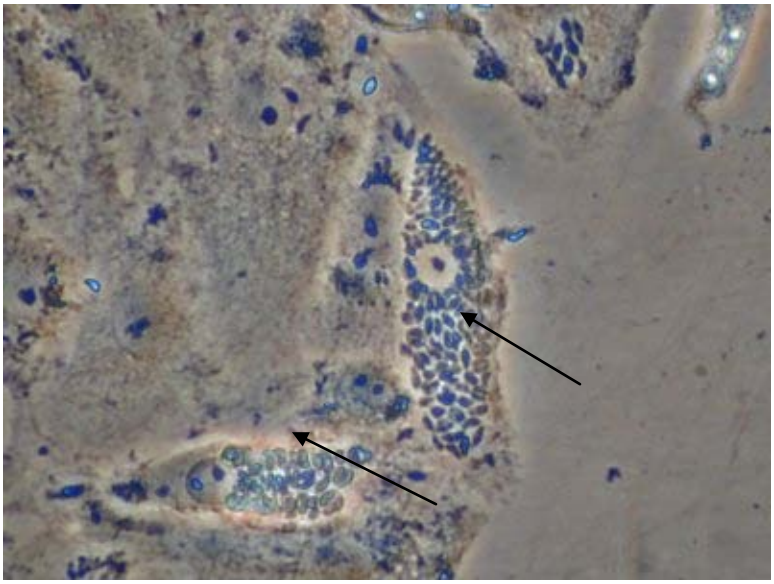


Figure 3. CV-1 cells infected with tachyzoite stages of *T. gondii*. Note the numerous tachyzoite stages indicated by arrows. (400X)

Control immunofluorescence assays

Initial assays were performed without primary antibody to determine if there would be non-specific binding of the Quantum Dot conjugated secondary antibody. No non-

specific binding of secondary antibody was observed at dilutions of 1/500 or 1/1000 in the absence of primary antiserum. (see Figure 4).

Immunostaining of intracellular tachyzoite stages of *Toxoplasma gondii*

Different concentrations of both primary and secondary antibodies were tested in indirect immunofluorescence assays (IFAs) to find the optimal conditions for staining intracellular tachyzoite stages. As described above, CV-1 cells infected with tachyzoite stages of *T.gondii* were grown in Lab-Tek culture slides with eight chambers. In the first test series, a two-fold dilution of primary antibody (mouse anti-*T*. *gondii* or normal mouse serum) was used ranging from 1/50 to 1/400. Cells in the top row of four chambers were exposed to normal mouse serum. Cells in the bottom row of four chambers were exposed to the same dilutions of mouse anti-*T*. *gondii* antiserum. Duplicate Lab-Tek slides were then exposed to dilutions of either 1/500 or 1/1000 of the QD-conjugated secondary antibody. In a second assay, a two-fold dilution of primary antibody (mouse anti-*T*. *gondii* or normal mouse serum) was used ranging from 1/20 to 1/100, with QD-conjugated secondary antibody dilutions at 1/500 and 1/1000. Dilutions that showed the greatest fluorescence intensity and specificity, with the lowest background staining were the dilutions with 1/50 or 1/100 primary antiserum and 1/500 or 1/1000 QD-conjugated secondary antibody. These dilutions of primary and secondary antibodies yielded intensely bright, punctate, fluorescent staining at both 400X and 1000X magnification without background fluorescence. Representative IFA assay results are shown in Figures 5-8.

The Quantum Dot Immunoconjugate allowed for the reliable, photo-stable detection of intracellular stages of *Toxoplasma gondii*. Fluorescent staining of tachyzoite stages

remained highly photostable even after several minutes of continuous illumination. No evidence of photo-bleaching was observed in our assays, even in the absence of anti-fade reagents. In addition, fluorescence intensity remained strong even when viewing slides that had been stored for a period of several weeks. Quantum dot fluorescence appears to be stable for weeks – months.

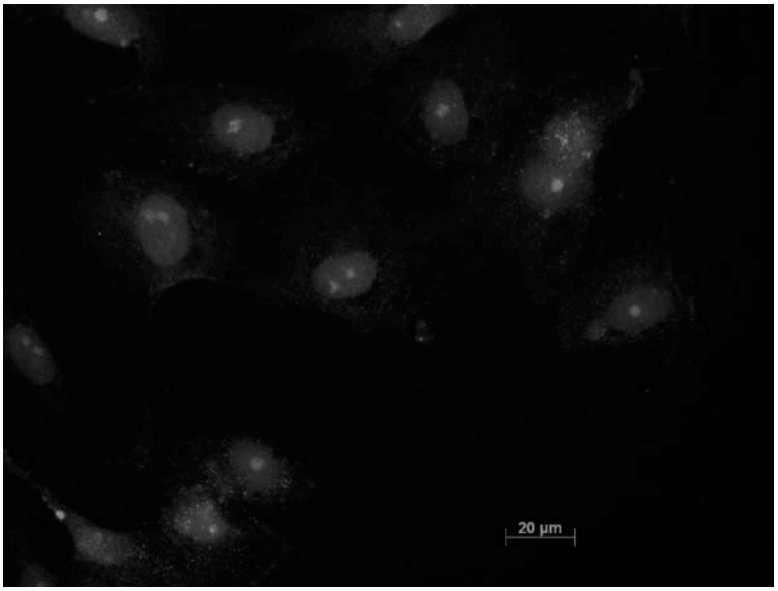


Figure 4. Control Immunofluorescence Assay, CV-1 cells infected with *T. gondii*. No primary antibody, 1/500 dilution of secondary antibody (Goat anti-mouse Ig – Quantum Dot conjugated). Slides were viewed and photographed using a Zeiss fluorescent microscope. Image shown in black/white.

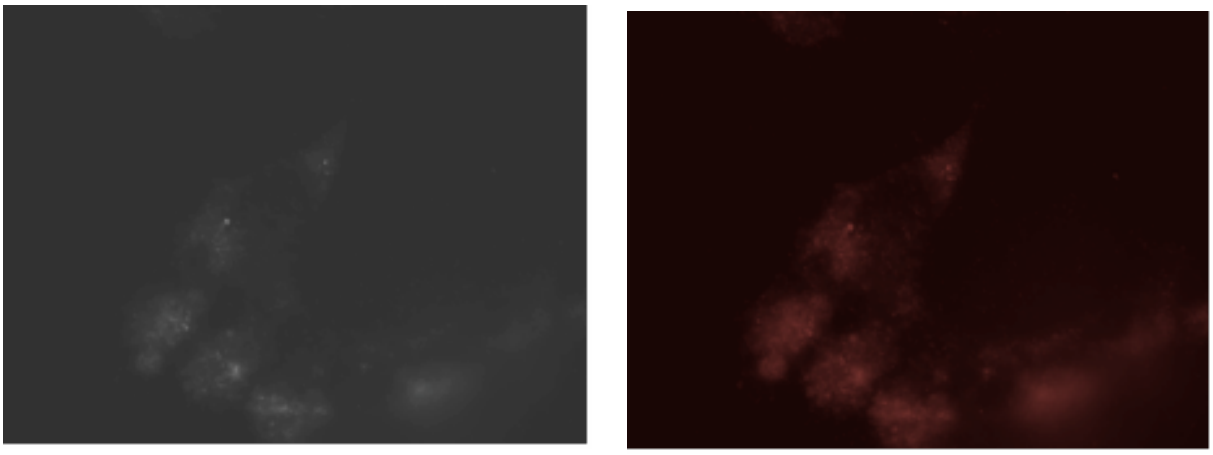


Figure 5. Indirect Immunofluorescence Assay, CV-1 cells infected with *T. gondii*. Normal Mouse Serum (1/50), 1/500 dilution of secondary antibody (Goat anti-mouse Ig – Quantum Dot conjugated). Left panel shown in black/white, Right panel shown in color.

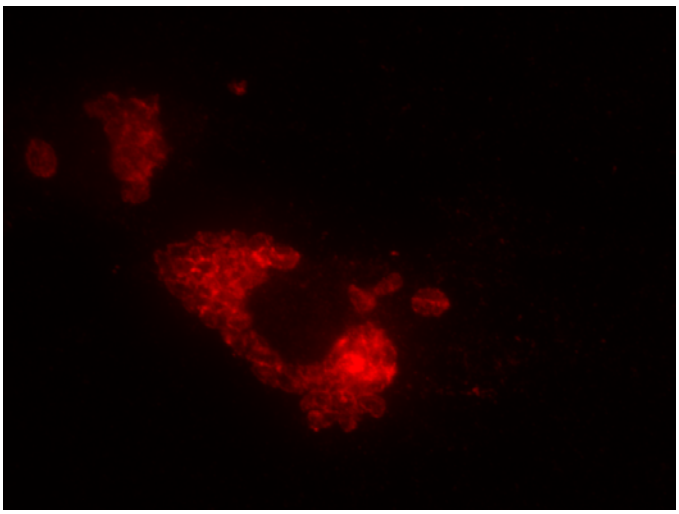


Figure 6. Indirect Immunofluorescence Assay, CV-1 cells infected with *T. gondii*. Mouse Anti-*T.gondii* (1/100 dilution), 1/500 dilution of secondary antibody (Goat anti-mouse Ig – Quantum Dot conjugated). This image depicts a cell heavily infected with tachyzoite stages of *T. gondii*. Note intense immuno-staining of tachyzoite stages located around the nucleus of the infected cell. Magnification 1000X.

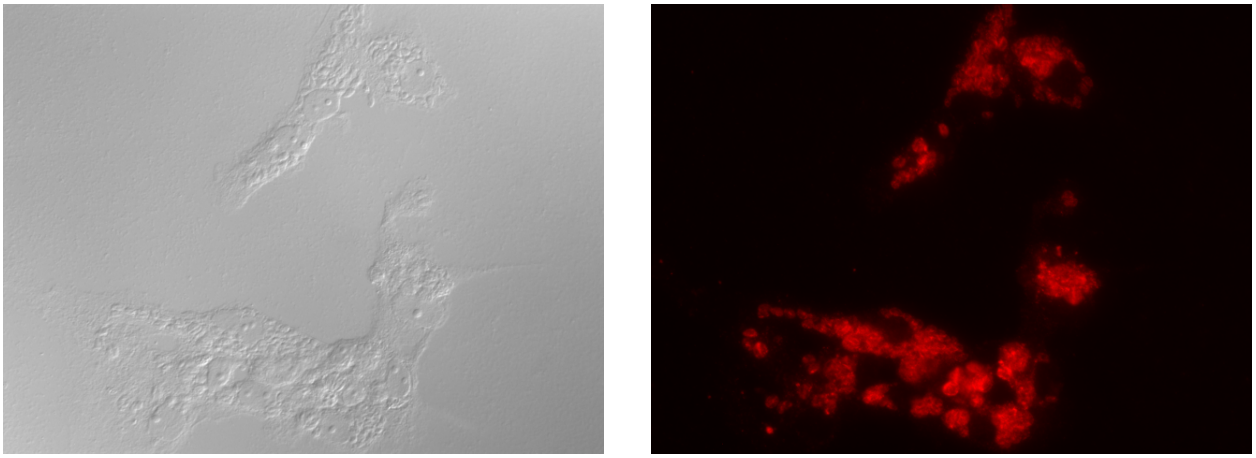


Figure 7. Indirect Immunofluorescence Assay, CV-1 cells infected with *T. gondii*. Mouse Anti-*T.gondii* (1/100 dilution), 1/500 dilution of secondary antibody (Goat anti-mouse Ig – Quantum Dot conjugated). Paired Nomarski (left) and fluorescent (right) images of heavily infected *T. gondii* –infected CV-1 cells. Cells shown in the same field of view**.** 400X magnification

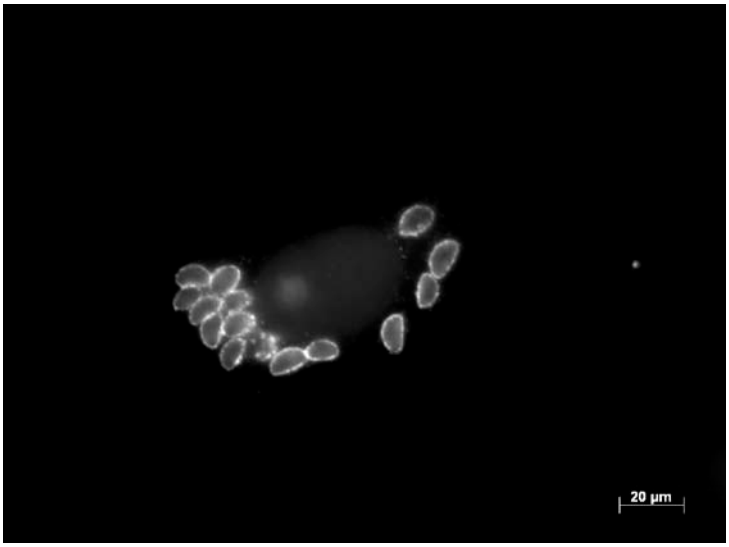


Figure 8. Indirect Immunofluorescence Assay, CV-1 cell infected with *T. gondii*. Mouse Anti-*T.gondii* (1/100 dilution), 1/500 dilution of secondary antibody (Goat anti-mouse Ig – Quantum Dot conjugated). This image depicts a CV-1 cell containing tachyzoite stages of *T. gondii*. Note intense, punctate, immuno-staining of tachyzoite stages located around the nucleus of the infected cell. Magnification 1000X.

DISCUSSION

In this study, we successfully adapted QD technology for the immunofluorescent detection of intracellular stages of *T. gondii* in CV-1 cells. Quantum Dots have been proven to be useful in immunofluorescence assays because of their photostability, lack of photo-bleaching, and ease of conjugation to immunoglobulins. In many applications, QDs have been shown to be far superior to conventional organic dyes. In the present study we confirmed that QDs are highly photo-stable and highly resistant to photo-bleaching. Strong fluorescent labeling was still visible for weeks and even months after the initial staining and fixing of the slides.

QDs also have been shown to be useful in a variety of other immuno-labeling studies. QD-conjugated antibodies were successfully used for the detection of *Cryptosporidium parvum* and *Giardia lamblia* in water samples (Zhu et al., 2004). Two strategies were used. In one of the strategies, the target cells were first bound to biotinylated antibodies before conjugation of QDs to the cell-bound antibodies. In the second strategy, the authors' used the manufacturer's recommended staining protocol and the QDs were linked to the antibodies prior to addition to the samples. The authors reported that QDs showed better photo-stability and higher brightness than the two most commonly used commercial staining kits: a A100DF AquaGlo Dual Fluorochrome Kit and a KR1 Crypto-Cel IF test kit. The cells stained with the organic dyes bleached rapidly under continuous UV exposure. In contrast, cells labeled with QDs remained photostable after 5 minutes of continuous exposure to UV light. QDs also facilitated a dual-color labeling for *C .parvum* and *G .lamblia* from various water sources (Zhu et al., 2004)

Quantum dots have also been used to label erythrocyte membranes and to identify modifications occurring in the plasma membrane at the time of infection with *Plasmodium spp*. A QD (QD 650) suspension was diluted to a final concentration of 10-15nM 10 minutes prior to use. Erythrocytes were incubated with the QD suspension for 30 minutes. In order to label the intracellular malarial stages, 0.1ug ml-1 of a Hoechst 33258 solution containing 10ug mL-1 of NaN_3 was added to the second wash in the assay. A direct comparison of Alexa 568 and QD 605 was performed. A 50% decrease in fluorescence was observed in the erythrocytes labeled with Alexa 568 after 10 seconds of continuous UV irradiation. In contrast, there was no measurable decrease in fluorescence intensity in the QD labeled erythrocytes, even after 20 minutes continuous irradiation. In the same study, merozoite stages of *P. falciparum* were also successfully labeled with QD conjugated antibody. This immunochemical study was important in furthering our understanding of the intra-erythrocyte cycle of malaria (Tokumasu and Dvorak, 2003)

Quantum Dots also have been used successfully for the labeling of *C. parvum* oocytes using QD605-streptavidin together with biotinylated anti-*Crypstosporidium* monoclonal antibody (MAb). The immunofluorescent detection of *C. parvum* oocytes using QD-conjugated antibodies was shown to be a highly specific, sensitive, and photostable (Lee et al., 2004).

In the present study, we have successfully developed and optimized an in vitro cell culture model of *T. gondii* infection that will allow future investigators to readily evaluate the infectivity and intracellular replication rate of the parasite. The CV-1 cell line was successfully grown in a complete RPMI-1640 medium, and proved to be an excellent host cell model for the analysis of the intracellular cycle of *T. gondii*. The large size of the cells,

and their extended and flattened morphology, allowed for the unambiguous visual detection of QD-labeled tachyzoite stages. Different concentrations of both primary and secondary antibodies were tested in indirect immunofluorescence assays (IFAs) to find the optimal conditions for staining intracellular stages. The optimal concentrations were found to be 1/50 and 1/100 dilution of the primary antibody (mouse anti-*T. gondii*), together with 1/500 or 1/1000 dilutions of secondary antibody (goat anti-mouse Ig – Quantum Dot conjugated).

The present study is the first to report of the use of QD technology for the immuno-fluorescent detection of intracellular tachyzoite stages of *T. gondii*. The development and optimization of this technique has successfully paved the way for future investigators in the laboratory to investigate the impact of selenium and vitamin E exposure on the intracellular cycle of *T. gondii*.

LITERATURE CITED

Drbohlavova, Jana., Adam, Vojtech., Hubalek, Jaromir. 2009. Quantum Dots — characterization, preparation and usage in biological systems. International Journal of Molecular Sciences. 10(2):656-673.

Dubey , J.P., Lindsay, D.S. and Speer, C.A. 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clinical Microbiology Reviews 11(2): 267–299.

Howarth, Mark, Takao, Lee, Hayashi, Yasunori, and Ting, Alice Y. 2005. Targeting quantum dots to surface proteins in living cells with biotin ligase. Proceedings of the National Academy of Sciences of the United States of America. 102(21): 7583-7588.

Lee, Lai Y., Ong, Say Leong, Hu, Jiang Yong, Ng, Wun Jern, Feng, Yaoyu, Tan, Xiaolan, Wong, Shih Wei. 2004. Use of semiconductor quantum dots for photostable immunofluorescence labeling of *Cryptosporidium parvum*. Applied and Environmental Microbiology. American Society of Microbiology. 70(10):5732-5736.

McCarthy, S.M., and Davis, C.D. 2003. Prooxidant diet provides protection during murine infection with *Toxoplasma gondii*. Journal of Parasitology. 89(5):886-894.

Pathak, Smita, Cao, Elizabeth, Davidson, Marie C, Jin, Sungho, silva, Gabriel A. 2005. Quantum dot applications to neuroscience: New tools for probing neurons and glia. The Journal of Neuroscience. 26(7):1893-1895.

Tokumasu, F., and Dvorak, J. 2003. Development and application of quantum dots for 2003. Immunocytochemistry of Human Erythrocytes. Journal of Microscopy. 211:256-261.

Toxoplasma gondii Life Cycle. 2009. Cambridge University Website.

http://journals.cambridge.org/fulltext_content/ERM/ERM3_01/S1462399401002204sup014.gif.

Zhu, Liang, Ang, Simon, Liu, Wen-Tso. 2004. Quantum dots as novel immunofluorescent detection system for *Cryptosporidium parvum* and *Giardia lamblia*." Applied and Environmental Microbiology. 70(1):597-598.