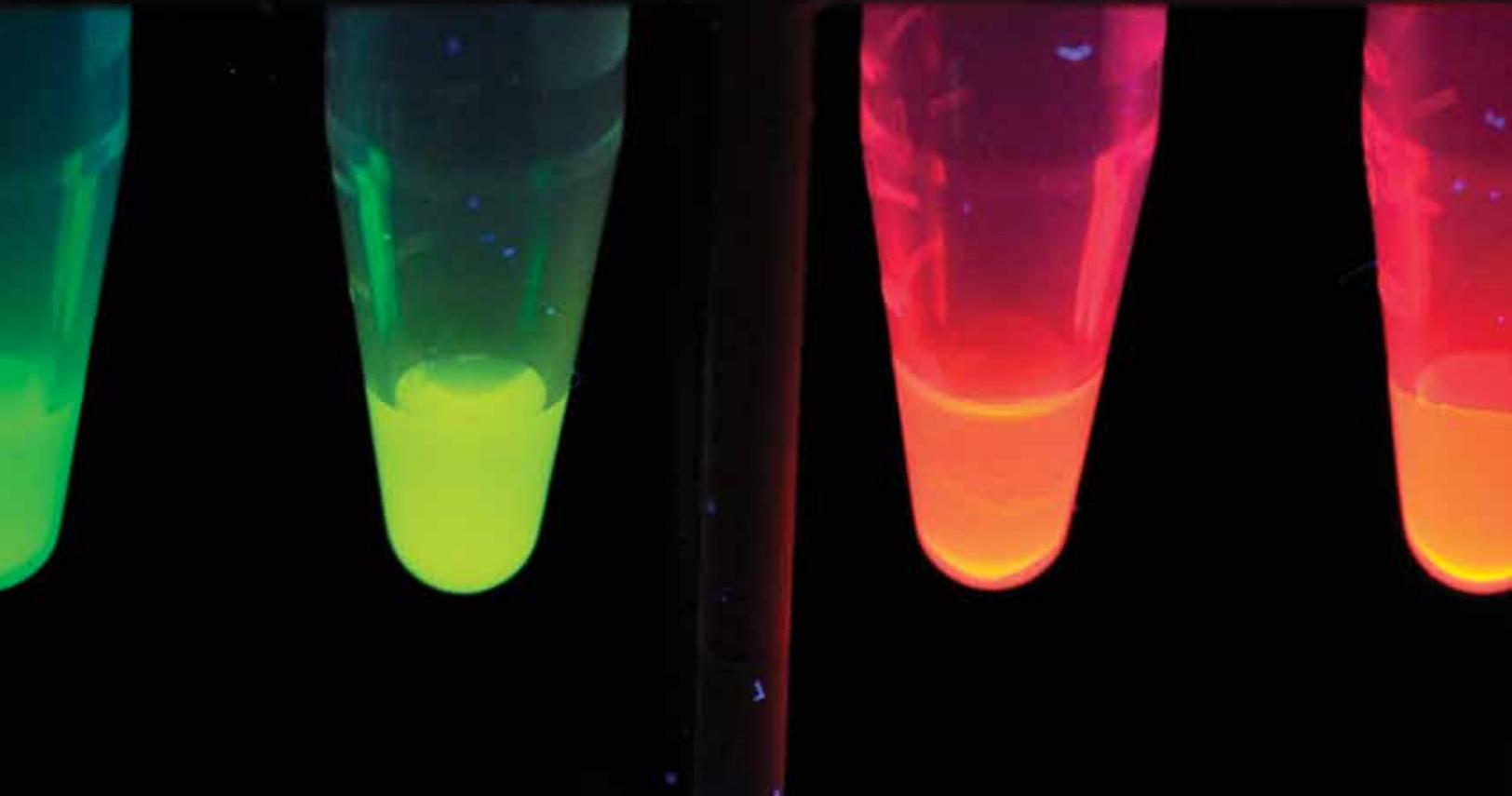


# Viewing Living Mammalian Cells and Life Processes — In Living Color, Courtesy of Quantum Dots

**T**he ability to see what is going on inside living bodies has been of immeasurable use in medicine and biology for many years. However, traditional CT scans and fluorescence-enhanced optical tomography do not easily allow for five-color spectral imaging, which would enable simultaneous labeling of multiple structures both inside and outside of cells. A novel nanoparticle-based approach under development at the Naval Research Laboratory uses semiconductor quantum dots (QDs) imported into live cells via peptides, polymers, and microinjection as bright probes to achieve multicolor fluorescent labeling. The unique physical, spectral, and chemical qualities of QDs make them applicable to labeling different parts of cells noninvasively and without killing the cells. NRL's approach seems destined to be a key player in both *in vitro* and *in vivo* research that should yield new treatments, tests, and therapies.





## Spatiotemporal Multicolor Labeling of Mammalian Cells: Quantum Dots Extend the Utility of Fluorescent Techniques in Biology

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The fluorescent labeling of mammalian cells is a fundamental yet very powerful technique in basic biological studies and applied medicine/imaging, as it can provide valuable insight into biochemical processes and cellular homeostasis. Multicolor fluorescent labeling, the simultaneous labeling of multiple, disparate intra- and extracellular structures, has long been a goal of fluorescent labeling, yet remains challenging as it requires (1) the combined use of numerous targeting molecules (e.g., antibodies), (2) multistep bioconjugation chemistries, (3) mixed delivery strategies (e.g., transfection reagents), (4) cellular fixation/permeabilization, and (5) complex spectral deconvolution. In this article, we detail a nanoparticle-based approach that uses semiconductor quantum dots (QDs) as extremely bright fluorescent probes delivered to various cellular targets in live cells using peptides, polymers, and microinjection. The unique photophysical attributes of the QDs allow for facile five-color spectral imaging that is not readily achievable with conventional fluorophores. This enabling work has important implications for *in vitro* cellular labeling/sensing and *in vivo* imaging applications.

### INTRODUCTION

The labeling of mammalian cells with fluorescent molecules (e.g., organic dyes, fluorescent proteins) is a fundamental technique in basic biology and medical diagnostics as it allows for the direct visualization of organelles/cellular structures and the real-time monitoring of biochemical processes *in vivo*.<sup>1</sup> Of particular

interest has been the development of facile ways to achieve the complex multicolor fluorescent labeling within the same cells, as this can ultimately allow for the spatiotemporal resolution of coordinated biological processes. Two major roadblocks have limited the successful achievement of this goal to date: (1) the physicochemical limitations of traditional fluorophores and (2) the development of robust strategies for specifically

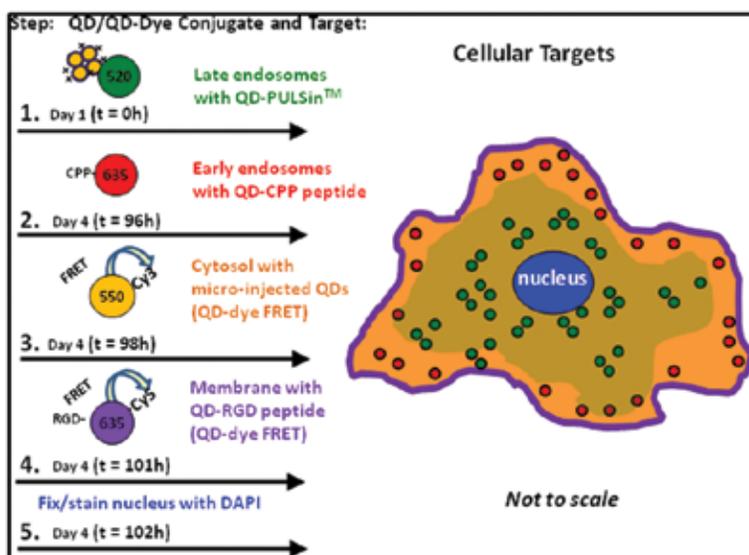
labeling subcellular structures. Organic dyes suffer from photoinstability, pH and ionic sensitivity, susceptibility to chemical degradation, low quantum yields, and solubility issues.<sup>2</sup> The approach most often taken for the multicolor labeling of disparate cellular structures is to use differentially labeled primary or secondary antibodies. However, the large size (>150 kDa) and cell impermeability of these antibodies necessitates cellular fixation and permeabilization. These processes preserve cellular structure at the cost of killing the cell; hence, this is not a viable technique for the multicolor labeling of live cells.

Recent years have seen the continued development of new fluorophores (e.g., long-lifetime chelates, metallic nanocrystals, and fluorescent proteins) with optical properties that can potentially circumvent some of the aforementioned issues. Semiconductor quantum dots (QDs) appear to hold exciting promise for use in multiplexing applications.<sup>3</sup> Among their advantageous photophysical properties are: (1) size-tunable narrow-symmetric photoluminescence, (2) small size (<25 nm hydrodynamic diameter), (3) excellent chemical/photostability, (4) very large two-photon action cross sections, (5) ability to excite multiple QD populations at one wavelength significantly blue-shifted from their emission, and (6) ability to serve as efficient Förster resonance energy transfer (FRET) donors.<sup>2</sup> Concomitant with the development of new QD materials has been the emergence of a range of delivery modalities (e.g., cell-penetrating peptides [CPPs] and polymer transfection reagents) aimed at the noninvasive and nontoxic delivery of a range of materials (including QDs and other nanoparticles) into live cells.<sup>4,5</sup>

This report presents an antibody-free, spatiotemporal methodology for the simultaneous multicolor labeling of distinct intra- and extracellular structures in living cells. We use a multipronged approach that exploits multivalent peptide display on QDs, FRET between QD donors and dye acceptors, innate cellular uptake processes (endocytosis and receptor-ligand binding), and physical microinjection (see the schematic in Fig. 1). Our results show the utility of QDs as the basis of a multicolor, live-cell labeling scheme without the need for conjugation to antibodies or cellular fixation/permeabilization. More generally, they demonstrate our fine control over materials at the nanoscale within the context of cells, with important implications for in vitro/ in vivo imaging and sensing.

### A STRATEGY FOR MULTICOLOR CELLULAR LABELING

In this study we used peptides, transfection reagents, and microinjection in a combinatorial fashion to target fluorescent QD materials to specific cellular locations and structures. The peptides used herein are shown in Table 1. The cell-penetrating peptide based on polyarginine (polyArg) is described elsewhere.<sup>6,7</sup> The RGD<sub>3</sub> (Arg-Gly-Asp) peptide contains a trimeric “RGD” repeat that is known to facilitate binding to integrin receptors on the cell membrane.<sup>8</sup> Each peptide contains a spacer domain that separates the functional domain from a polyhistidine (His<sub>6</sub>) tract for peptide assembly to the QD surface. The acceptor peptide bears a terminal cysteine residue for labeling with a dye and subsequent use as FRET acceptor when assembled onto the QD.



**FIGURE 1** Spatiotemporal strategy for the multicolor QD labeling of A549 cells. Peptides, transfection reagents, and microinjection delivered QDs to cellular structures. In some cases, QD-dye energy transfer (FRET) was used to create discrete “spectral windows.”

TABLE 1 — Peptide Sequences Used

Peptide:	Sequence:
CPP	(Arg) <sub>9</sub> GlyGlyLeuAlaAibSerGlyTrpLys(His) <sub>6</sub>
RGD	(ArgGlyAspSerGly) <sub>2</sub> ArgGlyAspGlyLeuAib(Ala) <sub>3</sub> TrpGlyGly(His) <sub>6</sub>
Acceptor	Cys*GlySerGly(Ala) <sub>3</sub> GlyLeuSer(His) <sub>6</sub>

Aib =  $\alpha$ -amino isobutyric acid (a synthetic amino acid derivative).

Functional peptide modules: cell uptake, membrane-binding, spacer, QD attachment, and \*dye-labeling-site.

The specific spectral windows or “channels” used to image the various fluorescent materials used here are represented graphically in Fig. 2. Here, a distinct “slice” or “window” was selected for each fluorophore so as to accommodate the imaging of the ensemble fluorescent materials within cells without the need for complicated spectral deconvolution. The resulting spectral ensemble consisted of both QDs alone and QD-sensitized proximal dye-labeled acceptors to give a series of five increasingly red-shifted emission windows that spanned from 510 to 730 nm. QDs emitting at 520 nm and 635 nm provided the first two discrete emission windows. When appended with proximal dye acceptors, two more windows were established. For example, 550 nm QDs with sensitized proximal Cy3 dyes and 635 nm QDs assembled with Cy5 acceptors each created two more spectral channels. These provided four discrete spectral channels that were collectively excited at 457 nm, thus requiring only one excitation source to excite the QD samples. The final channel corresponded to the nuclear dye, DAPI, excited at 405 nm and imaged at <450 nm.

## MULTISTEP, MULTICOLOR LABELING

To achieve multicolor labeling, we delivered QDs to A549 cells (a human adenocarcinomic alveolar basal epithelial cell line) over a 4-day period while maintaining cellular integrity and viability. We recently showed that PULSin™, a commercially available amphiphilic polymer, could mediate the efficient cellular uptake of QDs. After 4 days in culture, a modest portion of the QDs were released to the cytosol while the majority remained entrapped within late endocytic vesicles.<sup>6</sup> Our five-step delivery regime outlined in Fig. 1 began with the delivery of 520 nm QDs using the PULSin™ polymer. After 4 days in culture, peptide-mediated QD delivery was then used to deliver QDs to the early endosomal pathway. To achieve this, 635 nm QDs appended with 25 CPP peptides per QD were incubated with cells for 30 minutes. The next step used the direct microinjection of QDs into the cellular cytosol. Here, 550 nm QDs appended with Cy3-bearing acceptor peptides (for FRET configuration) or 580 nm QDs with no peptides (non-FRET configuration) were injected under microscopic examination over

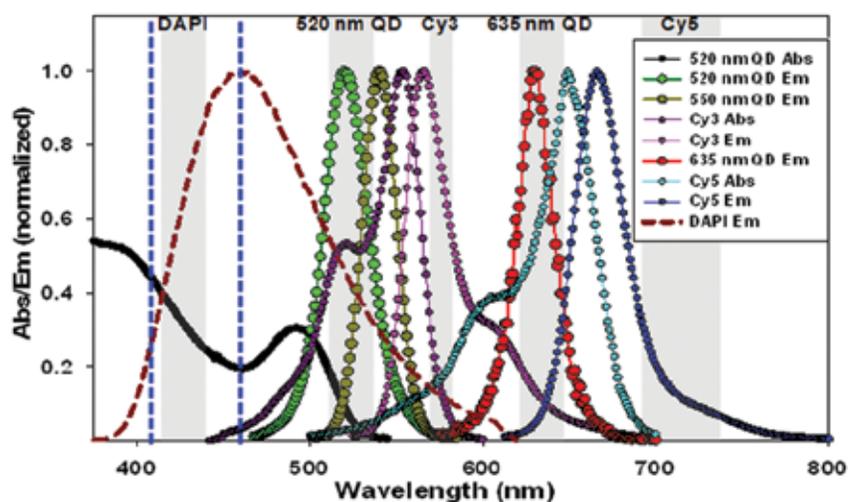


FIGURE 2

Spectral windows. Selected absorption and emission spectra of the QDs and dyes used in this study. DAPI, QD, and dye emission windows are in gray. DAPI (405 nm) and QD-dye (457 nm) excitation are represented by vertical blue dotted lines.

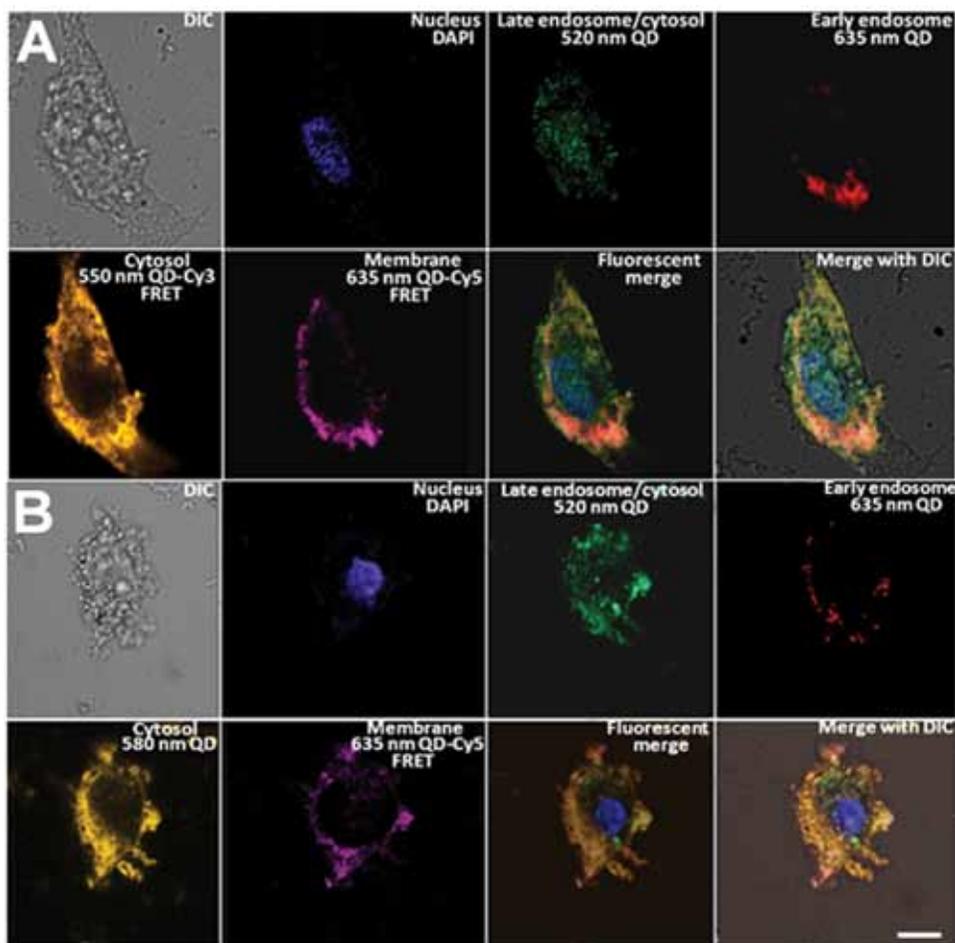
a 3-hour period. After microinjection, another QD-dye pair was used to label integrin receptors on the plasma membrane. For this step, 635 nm QDs were first decorated with four Cy5-containing acceptor peptides and 20 copies of the integrin-binding RGD peptide. After incubation for 20 minutes, the cells were washed, fixed to preserve cellular structure and labeling, and subsequently incubated with DAPI for nuclear staining.

As evidenced in Fig. 3 (panel set A), this delivery regime resulted in cells that were clearly labeled in a spatiotemporal fashion with five distinct fluorophores — four QDs (two engaged in FRET) and one nuclear dye — and these are imaged in six separate spectral windows including differential interference contrast (DIC). As shown in Fig. 3 (panel set B), we repeated this same delivery scheme using microinjected 580 nm QDs that were not engaged in FRET. As expected, a very similar staining pattern was observed, highlighting the flexibility available in selecting various emission

windows by simply altering the nature of the materials used.

## SUMMARY

In this report, we have detailed a facile method for the robust, multicolor labeling of live cells without the need for fixation or permeabilization. The scheme takes advantage of the unique spectral properties of QDs combined with various cellular delivery modalities to label cells in culture over a 4-day period. Further, it eliminates the reliance on elaborate imaging acquisition schemes and complicated deconvolution exercises to spectrally resolve the distinct spectral windows corresponding to each color. This approach demonstrates the facile nature by which correct materials selection can avail controlled, noninvasive labeling of live cells and shows the fine nature of control over materials at the nanoscale for biological applications. We envision



**FIGURE 3** Representative images of an A549 cell collected after sequential delivery of the indicated color QDs to specific cellular structures. Each color corresponds to a different QD or QD-dye-labeled cellular structure. The DIC image shows the cellular outline and contour. (A) Image set from cells microinjected (cytosol) with 550 nm QD-Cy3 conjugates engaged in FRET. (B) Panel showing 580 nm QDs that were injected into the cytosol yielding a non-FRET emission for that channel. Scale bar is 5  $\mu$ m.

our approach will have a profound impact on the use of QDs and other nanoparticle materials in Department of Defense applications such as vaccine and therapeutics development.

## ACKNOWLEDGMENT

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## References

- <sup>1</sup> B.N. Giepmans, S.R. Adams, M.H. Ellisman, and R.Y. Tsien, "The Fluorescent Toolbox for Assessing Protein Location and Function," *Science* **312**, 217–224 (2006).
- <sup>2</sup> K.E. Sapsford, L. Berti, and I.L. Medintz, "Materials for Fluorescence Resonance Energy Transfer Analysis: Beyond Traditional Donor-Acceptor Combinations," *Angew. Chem. Int. Ed. Engl.* **45**, 4562–4589 (2006).
- <sup>3</sup> I.L. Medintz and H. Mattoussi, "Quantum Dot-Based Resonance Energy Transfer and its Growing Application in Biology," *Phys. Chem. Chem. Phys.* **11**, 17–45 (2009).
- <sup>4</sup> J.B. Delehanty, K. Boeneman, C.E. Bradburne, G. Robertson, and I.L. Medintz, "Quantum Dots: A Powerful Tool for Understanding the Intricacies of Nanoparticle-Mediated Drug Delivery," *Expert Opin. Drug Del.* **6**, 1091–1112 (2009).
- <sup>5</sup> J.B. Delehanty, H. Mattoussi, and I.L. Medintz, "Delivering Quantum Dots Into Cells: Strategies, Progress and Remaining Issues," *Anal. Bioanal. Chem.* **393**, 1091–1105 (2009).
- <sup>6</sup> J.B. Delehanty, C.E. Bradburne, K. Boeneman, K. Susumu, D. Farrell, J.B. Blanco-Canosa, G. Dawson, P.E. Dawson, and I.L. Medintz, "Delivering Quantum Dot–Peptide Bioconjugates to the Cellular Cytosol: Escaping from the Endolysosomal System," *Integrat. Biol.* **2**, 265–277 (2010).
- <sup>7</sup> J.B. Delehanty, I.L. Medintz, T. Pons, F.M. Brunel, P.E. Dawson, and H. Mattoussi, "Self-Assembled Quantum Dot–Peptide Bioconjugates for Selective Intracellular Delivery," *Bioconj. Chem.* **17**, 920–927 (2006).
- <sup>8</sup> U. Hersel, C. Dahmen, and H. Kessler, "RGD Modified Polymers: Biomaterials for Stimulated Cell Adhesion and Beyond," *Biomaterials* **24**, 4385–4415 (2003).

## THE AUTHORS



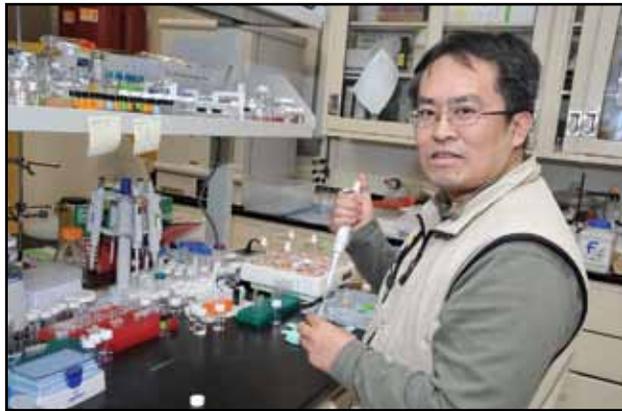
**JAMES B. DELEHANTY** received his Ph.D. in cellular and molecular biology from Tulane University Health Sciences Center in New Orleans, LA, in 2001 where his work focused on elucidating the mechanisms of antibody recognition of metal–chelate complexes. Since joining NRL, his research interests have included the development of portable sensors for biothreat agent detection, the investigation of novel high-throughput strategies for mammalian gene delivery, and the development of metal–chelate complexes as antimicrobial and antiviral agents. Currently his research is focused on expanding the biological labeling and sensing applications of semiconductor nanocrystals (quantum dots).



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