

Long-term multiple color imaging of live cells using quantum dot bioconjugates

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Luminescent quantum dots (QDs)—semiconductor nanocrystals—are a promising alternative to organic dyes for fluorescence-based applications. We have developed procedures for using QDs to label live cells and have demonstrated their use for long-term multicolor imaging of live cells. The two approaches presented are (i) endocytic uptake of QDs and (ii) selective labeling of cell surface proteins with QDs conjugated to antibodies. Live cells labeled using these approaches were used for long-term multicolor imaging. The cells remained stably labeled for over a week as they grew and developed. These approaches should permit the simultaneous study of multiple cells over long periods of time as they proceed through growth and development.

To understand the complexity and dynamics of biological interactions, it is desirable to monitor the interactions of multiple proteins or cells within an organism^{1,2}. Fluorescent tagging of cells and biomolecules with organic fluorophores has been used for these purposes³. Unfortunately, the use of organic fluorophores for live-cell applications is subject to certain limitations. QDs, such as CdSe-ZnS core-shell nanoparticles, are inorganic fluorophores that potentially circumvent these limitations and are thus a promising alternative to organic dyes⁴⁻⁶. Whereas organic fluorophores are restricted by their narrow excitation spectra, QDs can be excited by any wavelength from UV to red. This enables efficient excitation and collection of fluorescent emission^{5,7-9}. Another limitation of organic fluorophores is their broad emission spectra, which limits the number of fluorescent probes that can be simultaneously resolved. In contrast, QDs have narrow, tunable emission spectra, and thus emissions from many QDs can be resolved over the same spectral range. Moreover, in contrast to organic fluorophores, QDs are highly resistant to chemical and metabolic degradation and have a higher photobleaching threshold. Finally, whereas the organic fluorophores require customized chemistry for conjugation of biomolecules to each fluorophore, a universal approach can be used for conjugating biomolecules to all QDs.

The challenges to using QDs for biological studies include: (i) designing hydrophilic QDs that are luminescent, with surface chemistry adaptable to varied biological applications; (ii) developing versatile techniques for selectively and specifically labeling cells and biomolecules; and (iii) demonstrating that QDs do not interfere with normal physiology.

We have previously developed water-soluble CdSe/ZnS QDs using an electrostatic self-assembly approach to conjugate negatively charged QDs capped with dihydrolipoic acid (DHLA) ligands to positively charged proteins¹⁰. The bridging protein could be a naturally charged molecule, such as avidin, or a protein of interest fused to a positively charged basic leucine-zipper peptide (zb)^{10,11}. These QD bioconjugates have been used for numerous *in vitro* assays^{6,11}.

Silica-capped QDs conjugated to phalloidin and mercaptoacetic acid-capped QDs conjugated to human transferrin have been used in earlier attempts to label cells with QDs^{4,12}. Overnight incubation of the QD-transferrin conjugates with HeLa cells led to internalization of the QD conjugates, which was postulated to have occurred by receptor-mediated endocytosis. Although these studies suggest the potential of QDs for labeling cells, the inflexibility of the conjugation approaches limits the applications.

Electrostatic self-assembly has provided a flexible means for conjugating desired antibodies to any DHLA-capped QD¹⁰. Antibodies are conjugated to QDs via either avidin or a synthetically engineered protein G-zb (the leucine zipper-containing peptide fused to the B2 binding domain of streptococcal protein G). These QD bioconjugates show high specificity and stability in immunoassays¹³. In this study, we tested these QD bioconjugates for live cell labeling and developed approaches for their use in generalized and specific labeling of live cells. Whereas the QD-antibody bioconjugates selectively label only those cells and subcellular regions that express the protein of interest, the generalized labeling can be used to label any cell. Labeling with QDs did not interfere with the growth or differentiation of the cells. With these approaches we have imaged and tracked live cells for long periods as they developed in a mixture of cells labeled with different-colored QDs.

Results and Discussion

Generalized approach for labeling cells with QDs. It is possible to label cells with QDs by invasive techniques such as scrape loading, electroporation, or microinjection. We used two different approaches to non-invasively label cells with QDs. The first is based on the ability of cells to endocytose. Mammalian (HeLa) cells or *Dictyostelium discoideum* (AX2) cells were incubated with 400–600 nM DHLA-capped QDs for 2–3 hours and then washed to remove excess QDs. In both mammalian (Fig. 1A) and *D. discoideum* cells (Fig. 1B), the QDs were present in a large number of vesicles. The juxtannuclear distribution of the QDs in the HeLa cells was consistent with an endosomal location.

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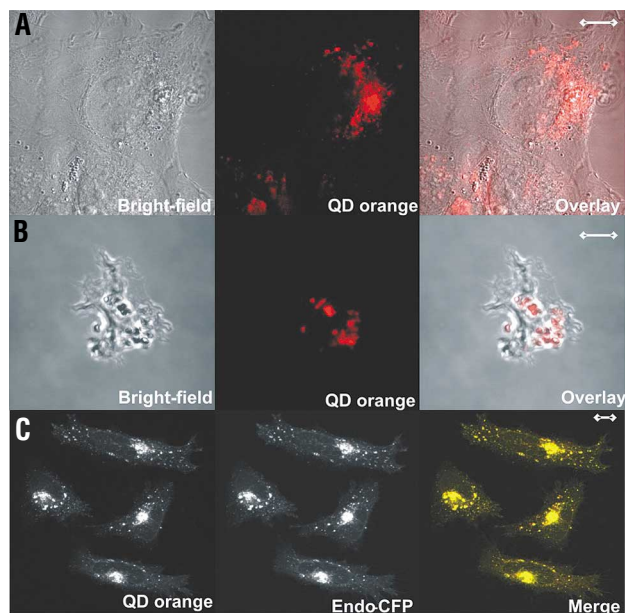
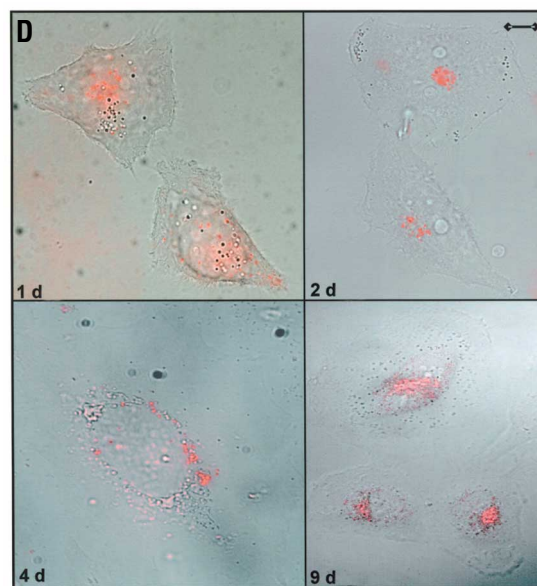


Figure 1. Generalized labeling of living cells using QDs. Cells were incubated for 2 h in orange QDs, after which the free QDs were washed away. (A, B) Confocal images of (A) HeLa and (B) *D. discoideum* cells. (C) Three-dimensional confocal projection of HeLa cells transiently transfected with the plasmid pECFP-Endo (encoding an endosome-specific reporter) and labeled with QD orange as above. (D) Overlay of bright-field and epifluorescence images of live HeLa cells labeled with orange QD and allowed to grow for the indicated period of time. Bar, 5 μm .

We tested this by transiently expressing an endosome-specific marker, pECFP-Endo (Clontech)¹⁴, in HeLa cells that were labeled with orange-emitting QDs. The vesicles containing QDs (Fig. 1C, left, pseudocolored red in the merged image) colocalized almost completely with those labeled by the endosomal marker (Fig. 1C, middle, pseudocolored green in the merged image). This further indicates that the QDs were internalized by endocytosis. Moreover, when endocytosis was blocked by incubation at 4 °C, neither mammalian nor *D. discoideum* cells were labeled by QDs even after extended (>6 hour) incubation. After endocytic uptake of QDs, HeLa cells were stably labeled for over a week with no detectable effects on cell morphology or physiology (Fig. 1D). Even after continuous growth for 12 days (beyond which the cells became too crowded to grow further), the cells were still labeled (data not shown).

The second approach used is independent of endocytosis. Here we biotinylated the cell surface using a membrane-impermeant, amino-directed coupling agent, sulfo-NHS-SS-biotin. The cells were then incubated for 10 minutes at 4 °C with 0.5–1 μM avidin-conjugated QDs and washed to remove unbound QDs. Initially, we observed the label only on the surface of cells (Fig. 2A). But when these cells were maintained for 2 hours at 37 °C, the label was predominantly in a juxtannuclear pool of vesicles (Fig. 2B). However, there was still some residual label on the membrane and many apparently endocytic,



labeled vesicles moving inward from the cell surface (Fig. 2B, arrows). This indicated that the QDs were internalized by endocytosis and not as a result of compromised permeability of the plasma membrane. As cells constitutively endocytose their cell surface proteins, these results indicate that QD labeling did not have any deleterious effects on the normal endocytic physiology. It is important to note, however, that this approach permits labeling of even those cells that do not undergo endocytosis.

Having determined that QD labeling had no detectable effect on cell growth, we tested whether it affected development. We examined the ability of QD-labeled *D. discoideum* cells to initiate and undergo development. cAMP signaling is the basis for aggregation in starving *D. discoideum* cells¹⁵. The starving cells respond to cAMP by a characteristic cringing and relaxation response that results in a net movement towards the source of cAMP¹⁶. We found that starved *Dictyostelium* cells labeled with green-emitting QDs were able to initiate and respond to cAMP signaling (Fig. 3; a time-lapse film is also available online (see URL in Experimental protocol)). Cringing and relaxation of cells, an indicator of the cAMP pulsing frequency, occurred once every 6 ± 2 minutes, which is comparable to the values reported for unlabeled AX2 cells¹⁷. Thus, labeling the *D. discoideum* cells with QDs had no deleterious effects on their cellular signaling and motility during development.

We therefore concluded that these approaches can be used to non-invasively label a variety of cells for long periods (over 12 days) without affecting cell growth or development. This opens up the

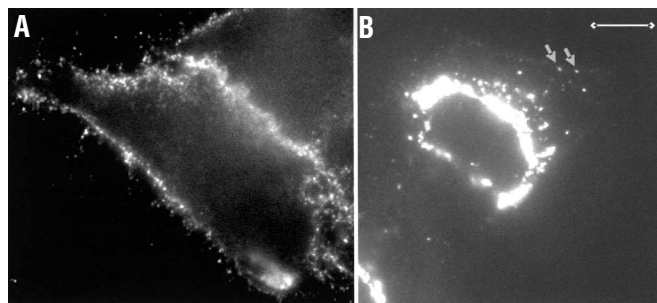


Figure 2. Generalized labeling of live cells using avidin-conjugated QDs. HeLa cells were biotinylated using sulfo-NHS-SS biotinylating reagent and then incubated for 15 min with the avidin-conjugated yellow-emitting QDs. (A) Epifluorescence image of cells immediately after the unbound QDs were removed; labeling is restricted to the cell surface. (B) Epifluorescence image of a cell that was allowed to grow for 2 h after washing out of unbound QDs. The QD-labeled vesicles are predominantly perinuclear; arrows point to the vesicles that are being formed by the endocytosis of the residual label at the plasma membrane. Bar, 10 μm .



Figure 3. Aggregation of QD-labeled starved *D. discoideum* amoebae. Growing AX2 cells were labeled with green-emitting QDs. After removal of the excess QDs, cells were plated in the starvation buffer and starved for 4 h. The image shows a field of aggregating cells after 4 h of starvation. Bar, 15 μm .

possibility of using QDs to study the behavior of cells that are difficult to genetically engineer to express fluorescent reporter proteins.

Specific labeling of cells with QDs. To tag specific proteins or specific cells, we assayed the utility of QD bioconjugates prepared by attaching an antibody directed towards a specific membrane protein⁶. In the present study, we used an antibody (clone 4E3) that is specific for the extracellular epitope of the multidrug transporter P-glycoprotein (Pgp). We used QD bioconjugates to label HeLa cells that were transiently transfected with a plasmid encoding Pgp fused to enhanced green fluorescent protein (EGFP). After transient transfection, only a subset of cells express Pgp-EGFP, with levels of expression varying over 1,000-fold¹⁸. As both expressing and non-expressing cells can be simultaneously visualized in a given microscopic field of view, the non-expressing cells serve as a good internal control for any non-specific labeling¹⁸. Specific labeling using QD bioconjugates should parallel the labeling by EGFP. Comparing the fluorescence signals of QD and EGFP thus provides a direct test of the specificity of labeling.

We used two approaches for specific labeling of live cells. In the first method, a biotinylated primary antibody was incubated with cells and then, after washing, an avidin-conjugated QD was used for labeling (Fig. 4A, C). In the second method, before incubation with cells, the primary antibody was conjugated with the QDs using either a recombinant protein G (QD-PG-zb) (Fig. 4B) or avidin^{6,11}.

Examples of the tests for the specificity of the QD labeling are shown (Fig. 4C, D). Each bright-field image shows multiple cells, but only two cells in each field are expressing Pgp-EGFP fusion protein. After a 1-hour incubation with biotinylated anti-Pgp antibody and a subsequent 15-minute incubation with QD-avidin conjugates, only cells expressing Pgp-EGFP were labeled with QDs (Fig. 4C). Thus, labeling with the QD-avidin conjugate was specific. Similar specificity was seen when the cells were incubated for 1 hour at 4 °C with preformed QD-antibody bioconjugates prepared via PG-zb (Fig. 4D). This correlation demonstrates the specific plasma membrane labeling by QD-antibody bioconjugates of only those cells that express the protein of interest. This approach can thus be extended to specifi-

cally label multiple different proteins or cell types simultaneously to visualize their interactions in live cells and *in situ* in whole organisms.

Use of QDs for long-term imaging of live cells. Unlike conventional fluorophores, QDs are highly photostable. Even after continuous illumination for 14 h by a 50 mW, 488 nm laser, there was no loss in the emission intensity of an ensemble of DHLA-capped QDs (Fig. 5A). The initial increase in emission intensity has been attributed to photo-induced healing of the surface states when the surrounding environment is equivalent to an insulator with a very high energy barrier, such as an organic matrix¹⁹. Thus the QDs not only permit multicolor tagging of live cells but also make it possible to track them for extended periods without any decline in the emission intensity of the label.

HeLa cells were stably labeled with QDs for over 1 week without harm to their growth (Fig. 1D). Similarly, QD labeling did not affect the initiation of development of *D. discoideum* cells (Fig. 3). We next tested if QDs could be used to continuously image *D. discoideum* through development. *D. discoideum* cells labeled with red-emitting QDs (emission maximum 613 nm) were mixed with a 100-fold excess of unlabeled cells and plated on non-nutrient agar plates for development. To track developing cells, we illuminated the cells once per minute (500 ms exposures) during the entire period of development (14 hours), using transmitted light from a halogen lamp and excitation light (470/40 nm) from a 75 W xenon lamp. QD labeled cells underwent aggregation and developed normally, participating in slug formation and showing no obvious cell-type preference in the slug (Fig. 6A; a time-lapse film is also available on the authors' website (see URL)).

A characteristic feature of the development of *D. discoideum* is that the number of cells capable of releasing cAMP autonomously, and thereby acting as centers of incipient aggregates, rises steadily

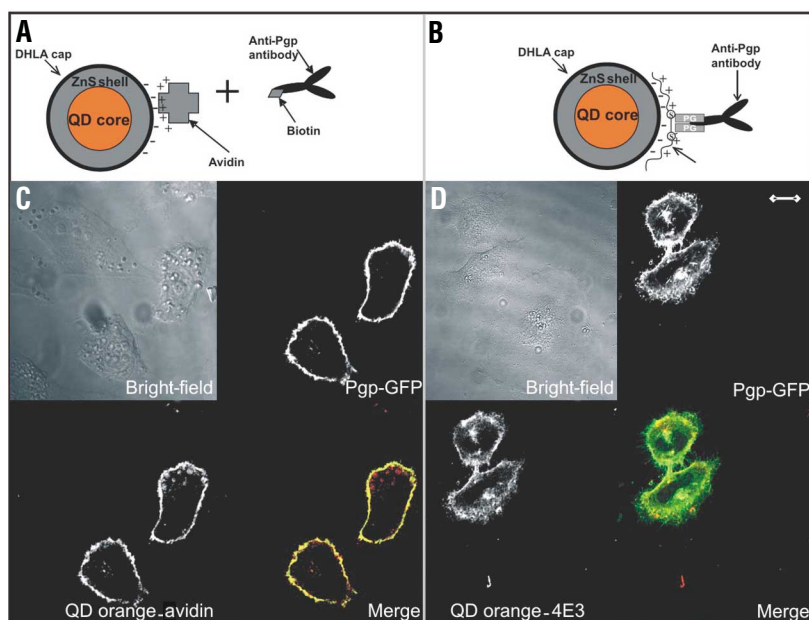


Figure 4. Specific labeling of live cells expressing Pgp-GFP using QD bioconjugates. (A–D) Orange-emitting QDs were conjugated to either (A) avidin or (B) anti-Pgp antibody, using the recombinant protein G–leucine zipper fusion protein (PG-zb). Confocal images of HeLa cells transiently transfected with Pgp-EGFP were taken after (C) pre-incubation with the biotinylated anti-Pgp antibody followed by a brief incubation with QD-avidin conjugates or (D) incubation with QDs conjugated to anti-Pgp antibody via PG-zb. In both cases, the QD bioconjugates labeled the cell membrane, and although many cells are visible in the bright-field image, only those that are expressing detectable levels of Pgp-EGFP are labeled with QDs. Bar, 10 μm .

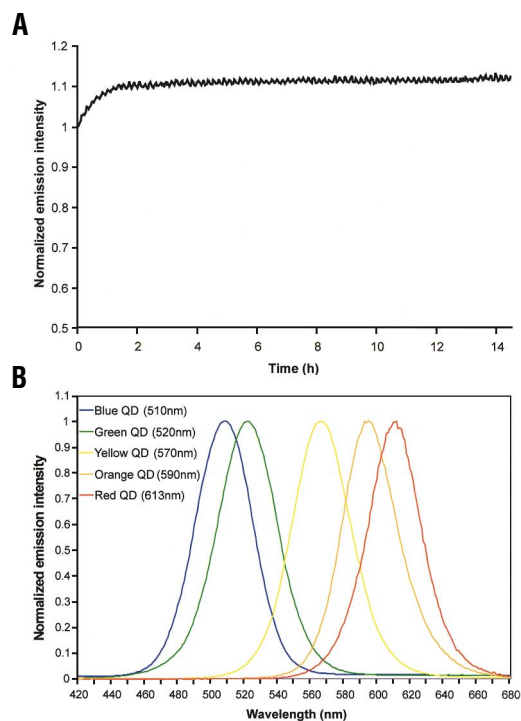


Figure 5. Photostability and emission spectra of QDs. (A) Photodecay of DHLA-capped CdSe-ZnS QDs. 500 μ l of ~ 1 μ M yellow-emitting QDs (emission max = 570 nm) dispersed in 10 mM sodium tetraborate buffer, pH 9, was illuminated with 50 mW of a 488 nm laser (beam diameter at the sample position was 1 mm). The peak fluorescence emission was continuously recorded once every 3 min. (B) Solutions of various QDs (<1 μ M in sodium tetraborate buffer, pH 9) were excited at 350 nm and fluorescence emission spectra recorded from 420 nm to 680 nm.

as a function of time after starvation^{20,21}. Thus, the starved cells are more likely to form aggregation centers when mixed with vegetatively growing cells. However, it is not known whether this ability increases continually with increased length of starvation or whether this is an on-and-off phenomenon whereby the cell either has or does not have the capability to form centers. The ability to label and track multiple cells for long periods using QD labeling provided us with an opportunity to address this question. Using the endocytic labeling approach, we labeled AX2 amoebae that had been starved for different periods of time with different QDs. Cells that were not pre-starved were labeled with green QD (emission maximum 520 nm; Fig. 5B, green line), cells starved for 3 hours were labeled with yellow QDs (emission maximum 570 nm; Fig. 5B, yellow line), and cells starved for 6 hours were labeled with red QDs (emission maximum 613 nm; Fig. 5B, red line). These cells were then mixed with a tenfold excess of unlabeled, non-starved cells (to allow easy resolution of the labeled cells in a mass of aggregating cells), and the mixture was placed on thin agar plates for development. We followed the QD labeled cells beginning 1 hour after plating. The cells were illuminated with 2 s pulses using a 470/40 nm excitation filter. Images corresponding to each of the different QDs were collected sequentially. Each image was taken once every 2 minutes for the entire period. The cells starved for 3 hours (pseudocolored green) and 6 hours (pseudocolored red) had equal propensity to form the aggregate centers (Fig. 6B, 2.5 and 3.5 hour), whereas the cells that were not pre-starved (pseudocolored blue, also indicated by arrows) were not part of the aggregate centers (Fig. 6B; a time-lapse film is also available on the authors' website). These cells responded to signaling by the aggregation centers formed by the pre-starved cells, by

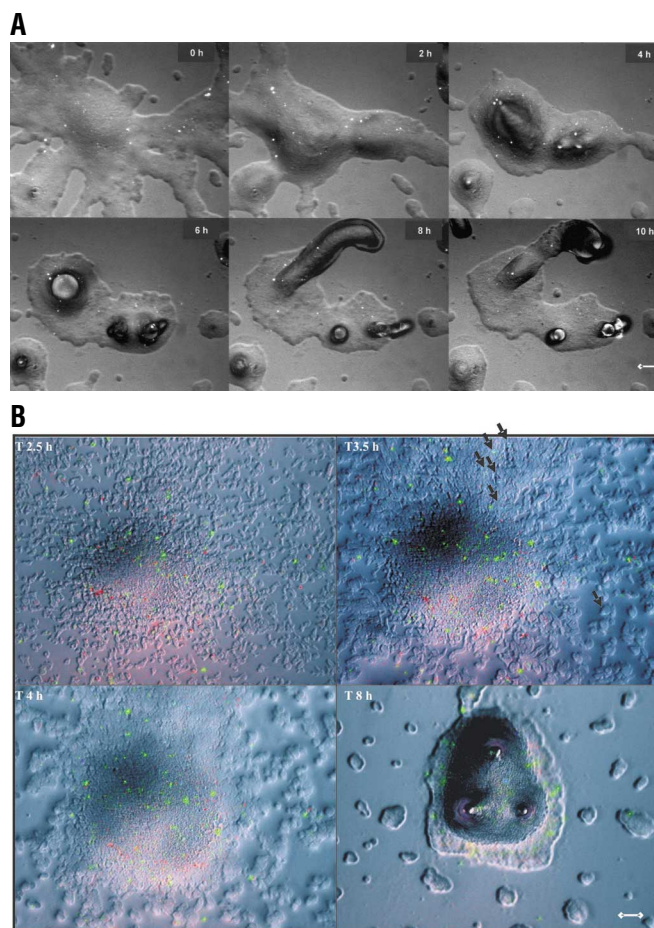


Figure 6. Long-term, multiple color imaging of developing cells of *D. discoideum*. Growing AX2 cells were labeled with (A) red- (613 nm) emitting QDs alone or (B) green- (525 nm), yellow- (570 nm), and red- (613 nm) emitting QDs. (A) Labeled cells were mixed with a 100-fold excess of unlabeled cells. The images show a developing aggregate of cells starting 1 h after plating. (B) Yellow and red QD-labeled cells (pseudocolored green and red) were starved for 3 and 6 h, respectively. Subsequently, green QD-labeled cells (pseudocolored blue) and unlabeled cells were resuspended in the starvation buffer, mixed with other QD-labeled cells, and plated on non-nutrient agarose plates. The bright-field and pseudocolored fluorescence images of a developing aggregate of cells were overlaid. The time indicates the period elapsed since the start of imaging—that is, 1 h after plating. Bar, 150 μ m.

chemotaxing towards them. Thus it appears that the ability to form centers in *D. discoideum* is an all-or-none response rather than a graded response. Notably, we saw no discernible fluorescence loss of the QD labels: brightly fluorescent cells were visible during the entire imaging sequence (Fig. 6B and time-lapse film online (see URL)).

The approaches presented here enable specific labeling of proteins for live cell imaging with QDs. The manipulations did not affect normal growth and development and had no obvious effect on cellular signaling. These approaches thus create the possibility of realizing the considerable potential of these inorganic fluorophores, such as resistance to photochemical, chemical, and metabolic damage and wide choice of excitation and emission wavelengths, in living cells. These features make QDs suitable for simultaneous tracking of multiple proteins and live cells for long periods and, therefore, for investigating a range of phenomena in cell and developmental biology that have been unexplored because of the lack of suitable fluorescent labels.

Experimental protocol

Preparation, protein conjugation, and spectral analysis of nanocrystal QDs. DHLA-coated QDs were conjugated to an antibody (4E3) specific to Pgp (Dako, Carpinteria, CA) via recombinant protein G- or avidin-coated QDs as described earlier^{11,13}. The emission and absorption spectra were collected using $1\ \mu\text{M}$ solutions of QDs. Emission spectra were collected using SPEX Fluorolog-3 spectrophotometer equipped with a 100 W xenon lamp source; all the solutions were excited at 350 nm. Absorption spectra were collected using a diode array UV-VIS spectrometer HP8453 (Hewlett-Packard). Photostability under continuous illumination was measured with QDs at an $\text{OD}_{488\text{nm}} = 0.02\text{--}0.03$ in a 4 mm optical path length cuvette with a 50 mW argon laser.

Cell growth and treatments. HeLa cells (ATCC CCL-2, American Type Culture Collection, Manassas, VA) were cultured as per ATCC recommendations. Cells were grown on coverslips (Fisher Scientific, Atlanta, GA) and imaged in medium containing 10 mM HBSS, 10 mM HEPES, and 5% FBS, pH 7.4. Cells were transiently transfected using Fugene 6 (Roche Diagnostics, Indianapolis, IN). The *D. discoideum* cell line AX2 was grown in HL5 medium²² at 23 °C. For development, cells were placed on non-nutrient agar plates. The plates were kept in the dark till they were ready for imaging. Cell-surface biotinylation was performed as previously described^{22,23}. Briefly, the cells were washed free of growth medium and incubated for 30 min at 4 °C in 1 mg/ml solution of sulfo-NHS-SS biotin (Pierce, Rockford, IL) in phosphate-buffered saline (PBS). Excess biotin reagent was quenched by washing with Tris-buffered saline (TBS).

Immunolabeling of cells using QD conjugates. For labeling with QD-antibody bioconjugates, cells were first washed with PBS, then incubated at 4 °C for 20 min in 1% BSA in PBS and for 45–60 min with the specific antibody–QD conjugates in 1% BSA. Unbound QDs were removed by washing with PBS. For labeling of cells using avidin-conjugated QDs, cells were incubated with 1% BSA as above, then incubated with biotinylated antibody for 45–60 min at 4 °C, and washed to remove unbound antibodies. Finally, the cells were incubated for 15 min with QD-avidin conjugate prepared in 1% BSA.

Laser scanning confocal microscopy. Images were collected with a Zeiss LSM 510 confocal microscope with 63X (Plan-Apochromat, NA 1.40) water-immersion objective. GFP was imaged using 488 nm laser light and a 505–530 nm BP emission filter. The QDs were imaged using 488 nm laser excitation and a 560 nm LP emission filter for orange and red QDs. Serial optical sections were taken using 0.4 μm optical slices.

Long-term and multicolor imaging of live cells. QD-labeled AX2 cells were imaged on an Olympus IX70 microscope with 75W xenon lamp and 470/40 nm band-pass excitation filter. The emission was collected using either a 590 nm LP or 525/50 nm, 580/30 nm, and 620/60 nm filters (Chroma Technologies, Brattleboro, VT). Images were acquired with an ORCA-ER camera (Hamamatsu Photonics, Hamamatsu, Japan). Image acquisition, processing, and analysis were done using MetaMorph (Universal Imaging, Downingtown, PA). Separate channels of a multicolored sequence were pseudocolor encoded and combined in an RGB sequence to generate the time-lapse films that are available on the author's website.

URL. For time-lapse films, see <http://www.rockefeller.edu/labheads/simon/movies/>.

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Competing interests statement

The authors declare that they have no competing financial interests.

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