Multiplexed Homogeneous Assays of Proteolytic Activity Using a Smartphone and Quantum Dots

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Supporting Information

ABSTRACT: Semiconductor quantum dot (QD) bioconjugates, with their unique and highly advantageous physicochemical and optical properties, have been extensively utilized as probes for bioanalysis and continue to generate widespread interest for these applications. An important consideration for expanding the utility of QDs and making their use routine is to make assays with QDs more accessible for laboratories that do not specialize in nanomaterials. Here, we show that digital color imaging of QD photoluminescence (PL) with a smartphone camera is a viable, easily accessible readout platform for quantitative, multiplexed, and real-time bioanalyses. Red-, green-, and blue-emitting CdSeS/ZnS QDs were conjugated with peptides that were labeled with a deep-red fluorescent dye, Alexa Fluor 647, and the dark quenchers, QSY9 and QSY35, respectively, to generate Förster resonance energy transfer (FRET) pairs sensitive to proteolytic activity. Changes in QD PL caused by the activity of picomolar to nanomolar concentrations of protease were detected as changes in the red-green-blue (RGB) channel intensities in digital color images. Importantly, measurements of replicate samples made with smartphone imaging and a sophisticated fluorescence plate reader yielded the same quantitative results, including initial proteolytic rates and specificity constants. Homogeneous two-plex and three-plex assays for the activity of trypsin, chymotrypsin, and enterokinase were demonstrated with RGB imaging. Given the ubiquity of smartphones, this work largely removes any instrumental impediments to the adoption of QDs as routine tools for bioanalysis in research laboratories and is a critical step toward the use of QDs for point-of-care diagnostics. This work also adds to the growing utility of smartphones in analytical methods by enabling multiplexed fluorimetric assays within a single sample volume and across multiple samples in parallel.

Semiconductor quantum dots (QDs) are one of many promising nanomaterials for bioanalytical applications.1−4 Their cumulatively unique properties include, but are not limited to, size- and composition-tunable photoluminescence (PL), broad absorption spectra with large molar absorption coefficients, and an inorganic interface that can be chemically derivatized and conjugated with biomolecules.1,2 QDs can therefore act as scaffolds for the assembly of bioprobes and biosensors and have been widely utilized for multiplexed assays and multicolor imaging, where their spectrally narrow PL and the ability to excite many colors of QD at a common wavelength are highly advantageous.5,4 Many of these applications utilize Förster resonance energy transfer (FRET) for the detection of biological targets such as nucleic acids,5 metal ions,6 drugs,7 nitric oxide,8 and antigens,9 as well as the activity of proteases,10 kinases,11 and nucleases.12 The optical properties of QDs are ideal for FRET, permitting optimization of spectral overlap integrals and FRET efficiencies while minimizing crosstalk and background signals. Increases and decreases in FRET efficiency can be coupled to biorecognition events to generate “turn off” and “turn on” sensing depending on the design. Such configurations have been the topic of several detailed reviews.13,14

Despite the capabilities and growing popularity of QDs, there are still impediments to their broader utilization in laboratories that do not specialize in nanomaterials. That is, laboratories in which researchers want to use QDs in a “kit”-like fashion for bioanalyses, but the QD itself is not a focus of the research. One impediment is the availability of biofunctional QD materials, which is being addressed through growing commercial availability, as well as refined synthetic, derivatization, and bioconjugation methods that are both simpler and greener.15−17 Another impediment to the broader utilization of QDs is the availability of instrumentation that can fully access the advantages of QDs, such as in optical multiplexing. For example, many laboratories may not have convenient access to fluorimetric equipment that can operate with excitation at ultraviolet/blue wavelengths and offer spectral acquisition or suitable filter-based color channels across the visible spectrum, particularly when real-time detection is desired. In such cases, there is no reason to switch from assay methods that are compatible with existing equipment, even if a multiplexed assay with QDs would be advantageous in principle. There is a clear need for analytical methods that can take full advantage of the optical properties of QDs and which have a minimal barrier for

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adoption in laboratories that have little or no prior experience with QDs or limited instrumentation available. Importantly, methods that can achieve this goal are also a critical step toward adapting QD-based bioanalyses for point-of-care (POC) diagnostics.

To address the above challenges, we have combined the spectrally narrow PL emission and strong, broad light absorption of QDs with red-green-blue (RGB) digital color imaging for quantitative, multiplexed homogeneous assays. A smartphone camera, which is ubiquitous in modern society, was used as the imaging platform, and multiplexed homogeneous assays of proteolytic activity were demonstrated as proof-of-concept. As shown in Figure 1, alloyed CdSeS/ZnS QDs with emission in the blue, green, and red region of the spectrum were each conjugated with a dye-labeled peptide substrate containing a recognition site that was hydrolyzed by one of three proteases (trypsin, chymotrypsin, or enterokinase). The dye labels were selected to be acceptors for the QDs, quenching their PL via FRET. In the absence of protease activity, the FRET acceptors remained in close proximity to their QDs, leading to efficient energy transfer. Proteolysis of the peptide substrates released the acceptor dyes from the QD and restored QD PL. Analogous FRET-based probes for sensing proteolytic activity have been reported previously but have required fluorimeters, or fluorescence plate readers, or fluorescence microscopes for readout. Most of these proteolytic assays have also been nonmultiplexed. Here, we show that quantitative, real-time detection of proteolytic activity is possible using a readout platform as simple and as accessible as a smartphone camera and a hand-held UV lamp (i.e., black light), where changes in QD PL were measured from RGB channel intensities in digital color images. The smartphone readout was first validated by comparison to assays done with a fluorescence plate reader; then, multiplexed smartphone assays were demonstrated, including a homogeneous three-plex assay of proteolytic activity with QDs. The results show that the unique optical properties of QDs can be combined with FRET and signal readout from smartphone camera images for multiplexed, quantitative bioanalyses that are feasible in almost any laboratory.

## EXPERIMENTAL SECTION

Detailed experimental methods can be found in the Supporting Information.

**Materials.** Alloyed CdSeS/ZnS core/shell QDs (CytoDiagnostics, Burlington, ON, Canada) with PL emission maxima at 450, 525, 540, and 625 nm (abbreviated QD450, QD525, QD540, and QD625, respectively) were made water-soluble by coating with glutathione (GSH) ligands. Peptides (Bio Synthesis Inc., Lewisville, TX) were labeled with QSY35-iodoacetamide, QSY9-maleimide, or Alexa Fluor 647 (A647)-maleimide (Life Technologies, Carlsbad, CA) as described previously (see Supporting Information for details).

The peptide sequences are shown in Table 1 and are designed to be substrates for either trypsin (TRP), chymotrypsin (ChT), or enterokinase (EK), with almost no cross-reactivity. The peptides were terminated with a hexahistidine sequence to permit spontaneous, high-affinity (Kd ~ 10^-9 M) self-assembly to the QDs. Analogous FRET-based assays were prepared by mixing the desired number of moles of QDs and peptides together, at the desired ratio, in the desired volume of buffer (see Supporting Information, Table S1).

**Enzyme Assays.** For protease assays, samples of QD–peptide conjugates were individually prepared with QD–peptide ratios of 1:12, 1:16, and 1:12 for QD450, QD540, and QD625, respectively, using the dye-labeled peptides specified in Table 1. Peptide conjugates of QD450 (100 pmol), QD540 (20 pmol), and/or QD625 (30 pmol) were mixed in a final volume of 50 μL of borate buffer (10 mM, pH 8.4, 50 mM NaCl). Solutions of trypsin (TRP), chymotrypsin (ChT), and enterokinase (EK) were each conjugated with a dye-labeled peptide substrate with a hexahistidine sequence to permit spontaneous, high-affinity (Kd ~ 10^-9 M) self-assembly to the QDs.
elements with overlaid red, green, and blue (RGB) images are interpolated from 2 similar) placed over CCD or CMOS detectors, where color produce color images using a built-in Bayer mosaic. The wavelength ranges are estimated to be 360–700 nm for the blue, green, and red channels, respectively. To test the suitability of the Bayer filter pattern for imaging multiple colors of QDs, samples of QDs with emission maxima within the transmission range of each RGB channel (QD625, QD525 or QD540, and QD450) were added to the clear-bottomed wells of a microtiter plate, obliquely trans-illuminated with a hand-held UV lamp, and RGB color images acquired using a smartphone camera. The relative crosstalk (650 nm cutoff) and a short-pass filter (400 nm cutoff) were placed in front of the camera lens to block excitation light and any A647 emission, respectively. PL spectra were acquired with a Tecan Infinite M1000 plate reader (Tecan Ltd., Morrisville, NC, USA). A detailed description of the data analysis methods is included in the Supporting Information.

Table 2. Properties of the QDs and FRET Pairs Used for RGB Imaging

<table>
<thead>
<tr>
<th>channel</th>
<th>QD PL (nm)</th>
<th>QY</th>
<th>BX</th>
<th>GX</th>
<th>RX</th>
<th>acceptor</th>
<th>R0 (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blue (B)</td>
<td>450</td>
<td>0.032</td>
<td>-</td>
<td>-</td>
<td>&lt;0.1%</td>
<td>QSY35</td>
<td>3.5</td>
</tr>
<tr>
<td>green (G)</td>
<td>540</td>
<td>0.16</td>
<td>9%</td>
<td>-</td>
<td>&lt;0.1%</td>
<td>QSY9</td>
<td>4.7</td>
</tr>
<tr>
<td>red (R)</td>
<td>625</td>
<td>0.10</td>
<td>7%</td>
<td>6%</td>
<td>-</td>
<td>A647</td>
<td>5.3</td>
</tr>
</tbody>
</table>

“QY = QD donor quantum yield; X = crosstalk; B = blue channel; G = green channel; R = red channel; R0 = Förster distance.

(RChT; 20 pM–40 nM), and/or enterokinase (EK; 1–9 nM) were prepared at twice the desired concentration, and 50 μL of the protease solution was added to the QD–peptide conjugates. Color images were acquired at 20 s intervals for 1 h. Protease activity was quantified from the initial rates of hydrolysis, measured as the slope of the initial linear region of progress curves (see Supporting Information for details).

RESULTS

RGB Color Imaging of Quantum Dots. Digital cameras produce color images using a built-in Bayer mosaic filter (or similar) placed over CCD or CMOS detectors, where color images are interpolated from 2 × 2 arrays of physical pixel elements with overlaid red, green, and blue (RGB) filters.25 The wavelength ranges are estimated to be 360–510 nm, 500–600 nm, and 560–700 nm for the blue, green, and red channels, respectively. To test the suitability of the Bayer filter pattern for imaging multiple colors of QDs, samples of QDs with emission maxima within the transmission range of each RGB channel (QD625, QD525 or QD540, and QD450) were added to the clear-bottomed wells of a microtiter plate, obliquely trans-illuminated with a hand-held UV lamp, and RGB color images acquired using a smartphone camera. The relative crosstalk (400 nm cutoff) and a short-pass filter (650 nm cutoff) were placed in front of the camera lens to block excitation light and any A647 emission, respectively. PL spectra were acquired with a Tecan Infinite M1000 plate reader (Tecan Ltd., Morrisville, NC, USA). A detailed description of the data analysis methods is included in the Supporting Information.

FRET Pairs and Their Fidelity. The three FRET pairs used in this work were QD450–QSY35, QD540–QSY9, and QD625–A647 (written as donor–acceptor). The absorption and emission spectra for each QD and each dye are shown in the Supporting Information (Figure S3). The dark quenchers, QSY35 and QSY9, were paired with QD450 and QD540 to maximize the multiplexing capacity of the homogeneous assay within the visible spectrum. Use of a fluorescent dye acceptor would have introduced broad FRET-sensitized emission, causing significant crosstalk in other color channels (e.g., a fluorescent acceptor for the QD540 would have had emission...
in the red channel, which was used to measure QD625 PL). A647 was used as the acceptor for the QD625 because it provided a large Förster distance and because its FRET-sensitized emission was easily blocked with a short-pass filter. The Förster distances for the FRET pairs are given in Table 2, and the spectral overlap integrals and other pertinent photophysical parameters are listed in the Supporting Information (Table S2). One of the advantages of using QDs as FRET donors is that energy transfer efficiencies can be incrementally improved by the progressive assembly of more acceptor-labeled peptides per QD. Figure 2B shows changes in QD PL intensity, measured with a fluorescence plate reader, as a function of the number of acceptor-labeled peptides per QD. Figure 2C shows the same calibration curves but instead plots the RGB color channel intensities measured from smartphone camera images. The QD PL and RGB channel intensities decreased as the number of acceptors increased. FRET efficiencies (see Supporting Information, Figure S4) were calculated from QD PL quenching and reached 77% for QD450−QSY35 (20 peptides), 73% for QD540−QSY9 (20 peptides), and 74% for QD625−A647 (12 peptides).

It should be noted that the final concentration of QD450 was 1.00 μM in all experiments (cf. 0.20 μM for QD540 and 0.30 μM for QD625) to achieve signal intensities in the blue channel that were comparable to those in the green and red channels of color images. This concentration was necessitated by the lower quantum yield of the QD450 (see Table 2). Although it was possible to acquire smartphone images of QDs down to concentrations as low as 10−50 nM by adjusting the excitation intensity from the UV lamp (i.e., proximity to the microtiter plate), a minimum QD concentration of 0.20 μM was used to ensure complete assembly with the labeled peptides. The coordination of the hexahistidine-terminated peptides to QDs is an equilibrium process governed by $K_d \approx 10^{-9}$ M and the overall concentrations of peptide and QD.23,24 The fidelity of the unique QD−FRET pair probes prepared for multiplexed assays depended on the off-rate ($k_{off}$) for the assembled peptides being slow relative to the duration of the assay. To confirm the stability of the prepared QD−peptide conjugates, RGB channel intensities were tracked over 1 h in the presence and absence of other QDs. Less than 4% increases in RGB channel intensities (see Supporting Information, Table S3) were observed for QD donors in mixtures with unconjugated QDs (cf. >150% change during proteolysis), suggesting that there was minimal dissociation of peptides from QDs. The fidelity of the initial peptide assembly was further confirmed by tracking mobility shifts between QDs and QD−peptide conjugates on an agarose gel, where a QD−peptide conjugate in a mixture retained an electrophoretic mobility that was lower than the nonconjugated QDs and consistent with that measured for the conjugate alone (see Supporting Information, Figure S5).

Figure 3. Proteolytic digestion of QD625−[Sub(TRP1)-QSY35]12, QD540−[Sub(ChT)-QSY9]16, and QD625−[Sub(TRP2)-A647]12 conjugates by TRP and comparison of data acquired with (A) a fluorescence plate reader and (B) a smartphone and RGB imaging: (i) raw PL data; (ii) normalized PL data; (iii) conversion of the normalized PL data to the average number of acceptors per QD and the bulk equivalent concentration of peptide substrate. (C) Comparison of initial proteolytic rates measured from the fluorescence plate reader data and smartphone RGB imaging data.
QD625−[Sub(TRP2)-A647]_{12} conjugates were exposed to various concentrations of either TRP or ChT. In each case, the number of peptides per QD was chosen so that there would be an approximately linear increase in the corresponding RGB channel intensity as the number of acceptors per QD decreased with proteolysis (see Figure 2C). Samples dispensed into the wells of a microtiter plate were illuminated with a UV lamp, and digital color images were acquired with a smartphone. A blank sample (i.e., no enzyme) was measured in parallel and used as a reference to account for drift in the UV lamp intensity (see Supporting Information, Figure S6) and potential photobrightening or photobleaching of the QDs.26 The response of the smartphone camera was relatively constant with estimated fluctuations <0.6% (see Supporting Information, Figure S7).

Figure 3 shows representative data for the activity of TRP with QD625−[Sub(TRP2)-A647]_{12} conjugates, where changes in QD625 PL were monitored in the red channel of digital images. Increases in protease concentration increased the rate of peptide digestion, resulting in faster increases in the red channel intensity. For data analysis, the measured red channel intensities of the acquired images were normalized to unity at the initial time point, thereby accounting for small variations in excitation intensity between different wells of the microtiter plate and between different experiments. To validate the smartphone imaging readout, proteolytic activity in replicate samples was measured with a fluorescence plate reader in parallel. For quantitative analysis of proteolysis kinetics, the relative QD PL signals were converted into the number of substrates per QD at each time point in an assay by comparison to calibration curves (see Supporting Information, Figure S8). These calibration curves needed to account for the nonspecific adsorption of digested peptide on the QDs, and we therefore prepared mixed digest/substrate calibration curves as described previously.27,28 Importantly, this calibration was also able to account for decreases (up to 18%) in QD PL intensity upon the assembly of peptides to the QDs (see Supporting Information, Figure S9). Initial rates of digestion were determined from the initial slope of the progress curves (see Supporting Information for details) and plotted as a function of protease concentration to determine the apparent specificity constant, $k_{cat}/K_m$ (see Supporting Information for details). Although proteolysis at the interface of nanoparticles does not necessarily follow standard Michaelis–Menten kinetics,27 this parameter was useful for comparing the data between the smartphone and fluorescence plate reader detection formats. As listed in Table 3, similar values of $k_{cat}/K_m$ were obtained from both the smartphone and plate reader formats, indicating good quantitative agreement between the two methods. A paired $t$ test comparison of the initial rates measured with the smartphone and plate reader readouts indicated that the null hypothesis could not be rejected at any reasonable level of confidence ($p = 0.47$ across all TRP concentrations, where $p$ is the probability that the observed variation will occur if the null hypothesis is true).

Similar proteolytic assays were done with QD540−[Sub(ChT)-QSY9]_{16} and QD450−[Sub(TRP1)-QSY35]_{12} conjugates using both smartphone digital imaging and the fluorescence plate reader (see Supporting Information, Figures S10 and S11). Again, the progress curves generated from the smartphone imaging and plate reader measurements were qualitatively similar and, in a statistical comparison of the initial rates, the null hypothesis could not be rejected at a reasonable level of confidence ($p = 0.21$ or 0.30 for QD540 across all ChT concentrations or $[\text{ChT}] > 0.6 \text{ nM}$, respectively; $p = 0.68$ for QD450 across all TRP concentrations). As shown in Table 3, there was also good agreement between the calculated specificity constants. Overall, the utility of the smartphone readout format for quantitative measurements of protease activity was confirmed by the close correlation between the data generated with a fluorescence plate reader and the data generated via RGB imaging.

**Multiplexed Proteolytic Assays with Digital Color Imaging.** We next evaluated the two-plex detection of TRP and ChT using the smartphone imaging readout. Mixtures of QD450−[Sub(TRP1)-QSY35]_{12} and QD540−[Sub(ChT)-QSY9]_{16}, or QD625−[Sub(TRP2)-A647]_{12} and QD540−[Sub(ChT)-QSY9]_{16} were exposed to different concentrations of TRP and ChT. Progress curves for three different combinations of ChT and TRP are shown in Figure 4. Representative raw data is shown in the Supporting Information (Figure S12). Analogous to the one-plex assays, the rates of change in the RGB channel intensities were proportional to the concentrations of the corresponding proteases. Interestingly, small decreases in the initial rates of digestion were observed in two-plex assays relative to one-plex assays with QD625−[Sub(TRP2)-A647]_{12} (30 pmol), QD540−[Sub(ChT)-QSY9]_{16} (20 pmol), and a significant decrease was observed for ChT activity in the system containing the QD450−[Sub(TRP1)-QSY35]_{12} (100 pmol) and QD540−[Sub(ChT)-QSY9]_{16} (20 pmol) conjugates. This effect was observed with both the smartphone imaging and the fluorescence plate reader detection formats. Nontrivial interactions between proteases (or other proteins) and nanoparticle interfaces have been reported previously.27,29,30 These results suggest that protease activity can be influenced by the total concentration of QDs in a sample and not just the concentration of the QDs carrying the substrate of interest. Our group is currently studying such interactions between QDs and proteases, but we limit our focus here to the smartphone readout strategy, which gave results analogous to the fluorescence plate reader. Another interesting observation in two-plex assays was that the increased optical density of QD mixtures decreased the overall signal intensities relative to samples with only one color of QD, in both the RGB imaging and plate reader formats (see Supporting Information, Figure S2). Quantitative multiplexed assays should therefore be calibrated as mixtures.

To demonstrate three-plex assays, a series of samples containing QD450−[Sub(TRP1)-QSY35]_{12} (100 pmol), QD540−[Sub(ChT)-QSY9]_{16} (20 pmol), and QD625−[Sub(EK)-A647]_{12} (30 pmol) were exposed to various concentrations of TRP, ChT, and EK. Mixtures were interrogated in microtiter plates using UV lamp illumination and smartphone digital imaging. Figure 5 shows progress curves and a time series of RGB color images obtained with three different
mixtures of TRP, ChT, and EK. Consistent with the expectations, increases in the concentration of one of the proteases in the mixture correlated with more rapid changes in the RGB channel intensity for the corresponding color of QD. The normalized initial rates of digestion of QD450−[Sub(TRP1)-QSY35]12 conjugates by TRP were in relatively good agreement with those in the one-plex assay; however, ChT activity in the three-plex assays decreased by ca. 50% in comparison to the one-plex assay, suggesting that there may be stronger nonproteolytic interactions between QDs and ChT than between QDs and TRP. Nonetheless, the rates of change in the RGB channel intensities in each three-plex assay were proportional to the concentration of the corresponding target protease.

**DISCUSSION**

The foregoing experiments have clearly demonstrated that RGB imaging of QD PL with a smartphone camera is suitable for quantitative, real-time, in vitro bioanalysis. Although we used a kinetic assay format to demonstrate the full capability of the method, single-point measurements are also possible. Suitable assay times may range from 10 min to upward of 1 h with longer times providing lower limits of detection. A kinetic analysis using initial rates offers a larger dynamic range. Proteolytic assays were an excellent model system because QD−FRET probes for protease activity are well-known and because assays of proteolytic activity have diagnostic and therapeutic value. Abnormal protease activity is associated with various diseases, including cancer,32 neurodegenerative diseases,33 and arthritis,34 among many others. Protease inhibitors are also an important class of drugs.35 The apparent effect of QD concentration on proteolytic rates is a biophysical phenomenon that remains to be further elucidated, but this effect is independent of the analytical readout platform, and QD-based assays of activity and inhibition remain possible. We expect that this smartphone readout methodology will be compatible with many other QD−FRET assays reported in the literature, including hybridization assays,5 carbohydrate assays,36 and immunoassays.37,38 The methodology should also be compatible with charge transfer-based quenching assays with QDs.39,40

There is a growing body of literature on the use of smartphones as detectors in various analytical methods. In particular, smartphones have been suggested to be amenable to POC diagnostics and telemedicine.41 Compared to traditional analytical instruments, smartphones are more portable, have lower cost, and have an inherent capacity for data storage, data processing, and data sharing/retrieval. Smartphones are also ubiquitous, with an estimated 56% of American and Canadian adults owning one in 2013.42,43 Martinez et al. were among the first to suggest the use of a smartphone camera for quantitative analysis of colorimetric assays.41 More recently, Wei et al. used a diode laser and smartphone camera to image single fluorescent dye-doped polystyrene nanoparticles and dye-labeled virus particles.44 Gallegos et al. used a smartphone camera in combination with a diffraction grating as a transducer for light transmission in bioassays on photonic crystal chips,45 and Ayas et al. integrated a smartphone camera into a confocal Raman system to measure single molecules on plasmonic substrates.46 Our work here adds to this literature, introducing for the first time the possibility of a homogeneous multiplexed analysis of a single sample volume through the unique PL properties of QDs and the color channels of a smartphone camera. In parallel, our method also provides for the simultaneous analysis of many samples in an array format through imaging. These capabilities are achieved with minimal expense, without custom or complex instrumentation, and would not have been feasible without QDs. Tunable, spectrally

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**Figure 4.** Progress curves for two-plex homogeneous assays of proteolytic activity. (A) RG two-plex assay with QD540−[Sub(ChT)-QSY9]16 (20 pmol) and QD625−[Sub(TRP2)-A647]12 (30 pmol). (B) GB two-plex assay with QD450−[Sub(TRP1)-QSY35]12 (100 pmol) and QD540−[Sub(ChT)-QSY9]16 (20 pmol). The conjugates were exposed to the indicated mixtures of TRP and ChT. The ordinate and abscissa scales are the same in each panel of (A) or (B).
narrow PL was needed to match the Bayer color filter channels of the smartphone camera, and a large effective Stokes shift was necessary to permit rejection of excitation light with simple optical filters. Moreover, the bright QD PL was needed to provide sufficient sensitivity.

Our work here is significant because it makes QDs a more accessible research tool for all laboratories and because it further suggests that smartphone imaging, particularly with QD probes, has long-term potential for POC diagnostics. In this vein, we have previously shown that emission from QD525−Alexa Fluor 555 FRET pairs immobilized on a paper substrate can be used for quantitative analysis of proteolytic activity using an R/G ratio and smartphone color digital imaging. This format was heterogeneous and relied on a spatial array format for multiplexing. The current homogeneous format with spectral multiplexing is similarly promising for POC applications but is more versatile and better suited for laboratory research. Our previous work also demonstrated that three different camera devices gave analogous results. We therefore expect that different models of smartphones can be used for assays similar to those described here with an iPhone.

■ CONCLUSIONS

We have shown that the combination of QDs, FRET, and imaging with a smartphone is a viable, easily accessible platform for quantitative, real-time bioanalyses. The optical properties of QDs also enhance the general utility of smartphone cameras as detectors in analytical methods, particularly in terms of multiplexing. Homogeneous assays of proteolytic activity were used as model systems for proof-of-concept, where a deep-red fluorescent dye and dark quencher labels on peptides were FRET acceptors for red-, green-, and blue-emitting QD donors. Changes in the PL emission from these QDs were detected in the RGB color channels of digital color images and could be correlated to the activity of picomolar to nanomolar concentrations of protease. Importantly, RGB imaging with a smartphone yielded the same quantitative results as replicate assays done with a sophisticated fluorescence plate reader. In addition to assays with a single color of QD, homogeneous two-plex and three-plex assays for the activity of TRP, ChT, and EK were also demonstrated. We expect that the methods and data analysis presented here can be extended to a wide range of established QD−FRET bioanalyses for targets other than proteases. Given the ubiquity of smartphones, this work largely removes any instrumental impediments to the adoption of QDs as routine bioanalytical tools in laboratories that do not specialize in luminescent nanomaterials. Additionally, this work represents an important preliminary step toward POC diagnostics based on QDs.

■ ASSOCIATED CONTENT

* Supporting Information
Detailed experimental methods including materials, instrumentation, data acquisition and analysis, additional results including analysis of crosstalk and QD mixtures, PL spectra and spectral overlap, tests of QD−peptide conjugate stability, additional results for RGB readout in one-plex assays, and representative non-normalized data for multiplexed smartphone camera assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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■ REFERENCES

SUPPORTING INFORMATION

Multiplexed Homogeneous Assays of Proteolytic Activity Using a Smartphone and Quantum Dots

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1. Detailed Experimental Methods

1.1 Materials and Reagents

Glutathione (GSH) and tetramethylammonium hydroxide (TMAH) in methanol (25% w/v) were from Sigma-Aldrich (Oakville, ON, Canada). All buffer salts were from Fisher Scientific (Ottawa, ON, Canada).

Trypsin (TRP) from bovine pancreas (#T1426, TPCK treated, ≥10,000 BAEE units/mg protein) and α-chymotrypsin (ChT) from bovine pancreas (#C3412, TLCK treated, Type VII, ≥40 units/mg protein) were from Sigma-Aldrich. Recombinant light chain bovine enterokinase (EK, P8070S) was from New England Biolabs (Whitby, ON, Canada).

CdSe1-xSx/ZnS core/shell nanocrystals (dispersed in toluene) with emission maxima at 450 nm (Blue), 525 nm and 540 nm (Green), and 625 nm (Red) were from CytoDiagnostics (Burlington, ON, Canada). Peptides were from Bio Synthesis Inc. (Lewisville, TX, USA). Alexa Fluor 647 (A647) C2 maleimide dye, QSY35 iodoacetamide, and QSY9 C5 maleimide were purchased from Life Technologies (Carlsbad, CA, USA). Buffers were prepared with water purified by a Barnstead Nanopure water purification system (Thermo Scientific, Ottawa, ON, Canada) and
sterilized by autoclaving prior to use. Buffers included borate buffer (50 mM, pH 9.2), borate buffered saline (BBS, 10 mM, 50 mM NaCl, pH 8.5), and phosphate buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4).

1.2 QD Ligand Exchange

GSH-QDs were prepared by diluting 100 µL of 10 µM QDs in toluene with 1 mL of CHCl₃ and mixing with a solution of GSH (80 mg) prepared in 300 µL of 25% TMAH in methanol. This mixture was vortexed and allowed to stand for 12 h. GSH-coated QDs were then extracted into 200 µL of borate buffer (pH 9.2, 50 mM, 250 mM NaCl), the organic layer discarded, and the QDs were washed by precipitation with ethanol and centrifugation (4800 rcf, 4 min). QDs were redispersed in 200 µL of buffer and washed twice more with ethanol. After the final wash, QDs were dissolved in BBS and stored at 4 °C.

1.3 Peptide Labeling

Peptides with a terminal cysteine residue were labeled with Alexa Fluor 647 C2 maleimide (A647) and QSY9 C5 maleimide dye according to previously published protocols with slight modifications. Briefly, 0.75 mg (0.22–0.27 µmol) of peptide was dissolved in 50 µL of 50% v/v MeCN (aq), diluted with 550 µL of HEPES buffer (pH 7.0, 100 mM, 50 mM NaCl), and mixed with 0.5 mg (0.4 µmol) of the A647 dye dissolved in 25 µL of DMSO. The reaction was placed on a mixer for 24 h at room temperature in the dark. The QSY35 iodoacetamide labeling reaction was done in DMF over 24 h at room temperature using 5–10 equivalents of dye per peptide and subsequently diluted with borate buffer. All labeled peptides were purified using a nickel(II)-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen Valencia, CA). Excess dye was removed by washing with 50% DMSO/BBS (10 mL), PBS (10 mL), 50% EtOH/PBS (10 mL), and PBS (3 × 10 mL). The labeled peptide was eluted with 1.5 mL of 300 mM imidazole in PBS (filtered through decolorizing carbon) per cartridge and desalted using an oligonucleotide purification cartridge (OPC, Life Technologies, Carlsbad, CA, USA). Labeled peptide was eluted using 70% acetonitrile in water and quantitated using UV-visible spectrophotometry (see Table S2 for molar extinction coefficients). The peptide was aliquoted into 20 nmol fractions, dried under vacuum, and stored at –20 °C until needed.
1.4 Instrumentation and Data Acquisition

Sample solutions were added to the wells of a 96-well microtiter plate (#3615; special optics, flat, clear bottom, black walls; Corning, Corning, NY, USA) and illuminated obliquely (40–50° angle of incidence) from underneath with a UV lamp. The UV excitation source was either a handheld (6 W, UVGL-58) or portable, AA battery-powered (4 W, UVL-4) UV lamp with emission at 365 nm (UVP, Upland, CA, USA). The microtiter plate was placed on top of an elevated glass pane with the UV lamp underneath. The distance between the lamp and the glass pane was adjusted to obtain QD PL intensities within the dynamic range of the smartphone camera. A longer distance was used with a more powerful lamp. The smartphone (iPhone) digital camera was placed above the microtiter plate at a distance of ca. 16 cm. The camera was set on a UV-blocking plastic plate (UVP 38-0013-01) with a long-pass filter (400 nm cut-off; FEL0400, Thorlabs, Newton, NJ, USA) and a short-pass filter (650 nm cut-off; FES0650, Thorlabs) placed prior to the camera lens on the other side of the plastic plate. Images were acquired using the TimeLapse software available for the iPhone. All image analysis was done using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Solution-phase PL spectra and absorbance spectra were acquired with an Infinite M1000 fluorescence plate reader (Tecan, Morrisville, NC, USA).

Note: The off-axis alignment of the UV light source was observed as a shadow at the edge of some wells. This artifact did not interfere with data analysis. In Figure 2A, the change in the position of the shadow for the QD540 images is a result of stitching together two separate fields of view to form a single image.

1.5 Conjugation of Peptides to QDs and Enzyme Assays

Stock solutions of QD-peptide conjugates were prepared by mixing QD450, QD540, and QD625 with 12, 16, and 12 equivalents of peptide, respectively, in BBS for 1 h in the dark. For multiplexed assays, the different QD-peptide conjugates were mixed together in the same final volume. The concentrations of the QD-peptide conjugates in the solutions were twice the desire concentration in the assays. Table S1 summarizes the stock solution recipes. Stock solutions of
TRP, ChT, and EK were prepared in BBS and diluted to twice the concentrations desired for assays. For multiplexed assays, TRP/ChT/EK were mixed together in the same final volume. Proteolytic assays were started by adding 50 µL of enzyme solution to 50 µL of QD-peptide conjugates in microtiter plate wells. Images were acquired every 20 s for 1 h under UV illumination.

Table S1. Preparation of stock solutions of QD peptide conjugates.

<table>
<thead>
<tr>
<th>Assay</th>
<th>QD Color</th>
<th>QD (nmol)</th>
<th>Peptide Substrate</th>
<th>Substrate (nmol)</th>
<th>[QD] (µM)</th>
<th>[Peptide Substrate] (µM)</th>
<th>Final volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-plex</td>
<td>QD625</td>
<td>0.49</td>
<td>Sub(TRA P2)-A647</td>
<td>5.9</td>
<td>0.60</td>
<td>7.2</td>
<td>820</td>
</tr>
<tr>
<td>1-plex</td>
<td>QD540</td>
<td>0.33</td>
<td>Sub(ChT)-QSY9</td>
<td>5.25</td>
<td>0.40</td>
<td>6.4</td>
<td>820</td>
</tr>
<tr>
<td>1-plex</td>
<td>QD450</td>
<td>1.04</td>
<td>Sub(TRP1)-QSY35</td>
<td>12.5</td>
<td>2.00</td>
<td>15.2</td>
<td>820</td>
</tr>
<tr>
<td>2-plex</td>
<td>QD625</td>
<td>0.30</td>
<td>Sub(TRP2)-A647</td>
<td>3.6</td>
<td>3.00</td>
<td>36.0</td>
<td>100</td>
</tr>
<tr>
<td>3-plex</td>
<td>QD625</td>
<td>0.30</td>
<td>Sub(EK)-A647</td>
<td>3.6</td>
<td>3.00</td>
<td>36.0</td>
<td>100</td>
</tr>
<tr>
<td>2- or 3-plex</td>
<td>QD540</td>
<td>0.28</td>
<td>Sub(ChT)-QSY9</td>
<td>4.48</td>
<td>2.00</td>
<td>32.0</td>
<td>140</td>
</tr>
<tr>
<td>2- or 3-plex</td>
<td>QD450</td>
<td>0.83</td>
<td>Sub(TRP1)-QSY35</td>
<td>9.9</td>
<td>3.33</td>
<td>39.6</td>
<td>250</td>
</tr>
</tbody>
</table>

1.6 Data Analysis

**FRET parameters.** The FRET pairs were characterized using the Förster formalism. The Förster distance, $R_0$ (units of cm), was calculated using Eqn. S1,

$$R_0^6 = 8.79 \times 10^{-28} \text{mol} \times (n^{-4} \kappa^2 \Phi_D J) \quad (S1)$$

where $n = 1.335$ is the refractive index of the surrounding medium, $\kappa^2 = 2/3$ (assumed) is the orientation factor, and $\Phi_D$ is the quantum yield of the donor. $J$ is the spectral overlap integral, which was calculated according to Eqn. S2,

$$J = \frac{\int F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda}{\int F_D(\lambda) d\lambda} \quad (S2)$$

where $F_D(\lambda)$ is the fluorescence intensity of the donor, and $\varepsilon_A(\lambda)$ is the molar absorption coefficient of the acceptor as a function of wavelength, $\lambda$. The values for $J$ and $\Phi_D$ were calculated from experimental measurements of QDs and QSY35/QSY9/A647-labeled peptide in bulk solution. Fluorescein was used as a standard for quantum yield measurements.5
The FRET efficiency, $E$, was calculated from measurements of QD PL using Eqn. S3. The terms $F_D$ and $F_{DA}$ are the fluorescence intensity of the QD donor ($D$) in the absence and presence of QSY35/QU9/A647 acceptor ($A$), respectively.

$$E = 1 - \frac{F_{DA}}{F_D}$$ (S3)

The RGB channel intensities in digital color images were calculated by splitting the images into corresponding RGB channels and determining the mean intensity ($I$) of a well of interest in blue (B), green (G), and red (R) channels. All of these operations were done in ImageJ. Next, eqns. S4-S6 were applied to account for crosstalk in the analysis of mixtures for multiplexed assays. The terms $I_R$, $I_G$, and $I_B$ denote measured, background-corrected intensities in the corresponding RGB channels of color images. The correction factors were determined experimentally (see section 2.1).

$$I_{QD625} = I_R$$ (S4)

$$I_{QD540} = I_G - (0.06)I_{QD625}$$ (S5)

$$I_{QD450} = I_B - (0.09)I_{QD540} - (0.07)I_{QD625}$$ (S6)

**Normalization of progress curves.** All experiments were done in parallel with at least one control sample with no added protease (i.e., blank) to account for any drift in the UV lamp intensity (see Figure S7) or other non-proteolytic sources of temporal variation, including potential photobrightening or photobleaching of the QDs. At each time point, $t$, the RGB channel intensity, $I_{t, [E]}$, was calculated. As shown in Eqn. S7, each of these values for a given enzyme concentration, $[E]$, were normalized to an initial value of unity by dividing by the intensity value at $t = 0$. All subsequent time points were then scaled to the control sample as a reference point.

$$\left( I_t \right)_{\text{normalized}} = \frac{\left(I_{t, [E]} / I_{t=0, [E]} \right)}{I_{t=0, [E]}}$$ (S7)

**Calculation of initial rates and specificity constants.** Initial rates of proteolysis were determined from the slope of the initial linear region of progress curves using a regression analysis in ProFit...
software (QuantumSoft, Uetikon am See, Switzerland). The progress curves with very fast digestion (the first data point corresponded to >50% digestion) were omitted from the initial rate analysis. Plots of initial proteolytic rate versus protease concentration were analyzed using a simplification of the Michaelis-Menten equation, eqn. S8,6 where \( v_0 \) is the initial reaction rate, \([S]\) is the substrate concentration, \(K_m\) is the Michaelis constant, \(V_{\text{max}} = k_{\text{cat}}[E]_0\), is the limiting reaction rate, \([E]_0\) is the protease concentration, and \(k_{\text{cat}}\) is the turnover number. Eqn. S9 is a good approximation of eqn. S8 when \([S] \ll K_m\). Initial rates measured for high protease concentrations were omitted from the regression analysis if they deviated from a linear trend (i.e., the rates began to slow as the limiting velocity started to be approached).

\[
v_0 = \frac{d[P]}{dt} = \frac{V_{\text{max}}[S]}{K_m+[S]} \tag{S8}
\]

\[
v_0 \approx \frac{k_{\text{cat}}[E]_0[S]_0}{K_m} \tag{S9}
\]

Only the specificity constant, \(k_{\text{cat}}/K_m\), was calculated because the protease concentrations used in experiments did not approach \(V_{\text{max}}\), and thus the data did not permit reliable determination of both \(k_{\text{cat}}\) and \(K_m\). It is important to note that the specificity constant was calculated for the purpose of comparison between the two readout formats (fluorescence plate reader versus smartphone imaging), and not to characterize the enzymes and their mechanism of action.
2. Additional Results and Discussion

2.1 Characterization of RGB Intensity of QDs

Four types of QDs with emission maxima at 450 nm, 525 nm, 540 nm, and 625 nm were evaluated with the RGB digital color imaging readout format. Figure S1 shows digital color images of these QDs after splitting into distinct red, green, and blue channels. QD525 showed the largest crosstalk with >30% crosstalk in the blue channel. In contrast, QD540 contributed only 9% of its green channel intensity to the blue channel. QD450 were exclusively observed in blue channel. QD625 were detected in all three RGB channels, with 6% crosstalk in the green channel and 7% crosstalk in the blue channel. Based on these results, QD450, QD540, and QD625 were used in all protease assays, and QD mixtures were analyzed according to eqns. S4–S6 to account for crosstalk.

Figure S1. False colored, RGB split digital images of QD samples of various concentrations. Each row in the figure is an RGB channel from a digital color image of a single microtiter plate row.
2.2 RGB analysis of QD mixtures

To assess QD mixtures, a range of samples with various concentrations of QD450, QD540, and QD625 were prepared. Concurrent analysis of PL spectra and RGB channel intensities for QD450, QD540, QD625, and their mixtures were well correlated, as shown in Figure S2. Increasing the amount of QDs in the mixtures resulted in an overall decrease in observed PL intensity and RGB channel intensity for each QD emitter, presumably due to the increased optical density of the samples and decreased excitation efficiency.

Figure S2. (A) QD PL spectra and (B) corresponding color images for the (a) QD mixture, (b) QD450, (c) QD540, and (d) QD625. (i) QD450 (30 pmol), QD540 (5 pmol), and QD625 (5 pmol). (ii) QD450 (50 pmol), QD540 (10 pmol), and QD625 (10 pmol). (iii) QD450 (75 pmol), QD540 (20 pmol), and QD625 (20 pmol). RGB values shown in panel (B) were calculated by accounting for the crosstalk using eqns. S4-S6. The dashed lines in panel (A) are spectra for mixtures, and the solid colored lines are for the individual QDs.
2.3 UV lamp Excitation and the FRET Pairs

Absorption and PL spectra for QDs and their corresponding FRET acceptors are shown in Figure S3. The QDs were excited with a UV lamp, the spectral output of which is shown in Figure S3A along with the approximate cutoff of the long-pass filter used to prevent excitation light from reaching the smartphone camera. The three distinct colors of QD PL emission, shown in Figure S3A, were detected with the built-in RGB filter of the smartphone digital camera, and emission from A647 (primarily from FRET-sensitization from the QD625) was filtered out using a 650 nm short pass filter with the cutoff also shown in Figure S3A. Table S2 summarizes the photophysical parameters of FRET pairs used in this study, and the pairwise absorption and emission spectra are shown in Figures S3B–D.

Figure S3. (A) Emission profile for the UV lamp excitation source; emission spectra of the QD450, QD520, QD625, and A647; and approximate cutoff wavelengths for the shortpass and longpass filters placed in front of the smartphone camera lens. Normalized absorption and emission spectra of (B) the QD450–QSY35 FRET pair, (C) the QD540–QSY9 FRET pair, and (D) the QD625–A647 FRET pair.
Table S2. Photophysical parameters of FRET pairs

<table>
<thead>
<tr>
<th>λ_{em, QD}^{a} (nm)</th>
<th>Φ_D</th>
<th>Acceptor</th>
<th>λ_{Abs, A}^{b} (nm)</th>
<th>ε_{max, A}^{c} (cm^{-1} M^{-1})</th>
<th>J_d^{d} (mol^{-1} cm^{6})</th>
<th>R_0 (nm)</th>
<th>r^{e} (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>450</td>
<td>0.03</td>
<td>QSY35</td>
<td>476</td>
<td>25400</td>
<td>8.6 × 10^{-11}</td>
<td>3.5</td>
<td>5.1</td>
</tr>
<tr>
<td>540</td>
<td>0.16</td>
<td>QSY9</td>
<td>561</td>
<td>98000</td>
<td>5.5 × 10^{-10}</td>
<td>4.7</td>
<td>5.1</td>
</tr>
<tr>
<td>625</td>
<td>0.09</td>
<td>A647</td>
<td>651</td>
<td>250000</td>
<td>1.9 × 10^{-9}</td>
<td>5.3</td>
<td>4.5</td>
</tr>
</tbody>
</table>

\(^{a}\)Wavelength of maximum donor PL; \(^{b}\)wavelength of maximum acceptor absorption; \(^{c}\)molar absorption coefficient at absorption maximum of the acceptor; \(^{d}\)spectral overlap integral; \(^{e}\)experimentally calculated donor-acceptor separation distance.

2.4 Characterization of QD-peptide conjugates

PL spectra were acquired for QD-peptide conjugates prepared with valences of 0–20 peptides per QD and the FRET efficiency was calculated according to eqn. S3. The data is shown in Figure S4. The corresponding RGB channel intensities are shown in Figure 2. The experimentally determined donor-acceptor separation, r, was calculated by fitting the FRET efficiency data to Eqn. S10, where \(n\) is the number of acceptors per QD.

\[
E = \frac{nR_0^6}{r^6 + nR_0^6}
\]  

(S10)
2.5 Evaluating stability of QD-peptide conjugates

The homogeneous multiplexed assays done with mixtures of pre-assembled QD–peptide substrate conjugates relied on strong affinity between the QDs and the hexahistidine tag on the peptides. Dissociation of peptide from the QDs or non-specific exchange of peptides between different QDs (i.e., “migration”) would cause decreases in FRET (due to the reduced number of acceptors), and concomitant increases in QD PL, that were unrelated to proteolytic activity.
Mixtures of QD-peptide conjugates and other unconjugated QDs were prepared and monitored in parallel with control samples for 1 h. Only marginal increases in RGB channel intensities (< 2.3 units over a 255 unit scale for an 8-bit image) were noted for the QD-peptide conjugates. Overall, QD PL was typically noted to decrease, particularly in the presence of a high concentration of QD450, similar to the trends observed with mixtures of QDs without peptides (Figure S2). Table S3 summarizes the results of an RGB image analysis of QD–peptide samples over a 1 h period.

**Table S3.** Relative changes in RGB channel intensity after 1 h.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ΔB&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ΔG&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ΔR&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>QD625–A647</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>QD450 + QD540</td>
<td>–0.2</td>
<td>–3.1</td>
<td>–</td>
</tr>
<tr>
<td>(QD625–A647) + QD450 + QD540</td>
<td>–2.3</td>
<td>–3.8</td>
<td>–13</td>
</tr>
<tr>
<td>QD540–QSY9</td>
<td>–</td>
<td>0.3</td>
<td>–</td>
</tr>
<tr>
<td>QD450 + QD625</td>
<td>1.4</td>
<td>–</td>
<td>–11</td>
</tr>
<tr>
<td>(QD540–QSY9) + QD450 + QD625</td>
<td>–3.9</td>
<td>2.3</td>
<td>–18</td>
</tr>
<tr>
<td>QD450–QSY35</td>
<td>–1.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>QD540 + QD625</td>
<td>–</td>
<td>–0.8</td>
<td>–10</td>
</tr>
<tr>
<td>(QD450–QSY35) + QD540 + QD625</td>
<td>–0.4</td>
<td>–22</td>
<td>–35</td>
</tr>
<tr>
<td>QD450</td>
<td>2.0</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>QD540</td>
<td>–</td>
<td>–3</td>
<td></td>
</tr>
<tr>
<td>QD625</td>
<td></td>
<td></td>
<td>–12</td>
</tr>
</tbody>
</table>

<sup>a</sup>QD625–A647 conjugates were prepared at a ratio 1:8; QD540–QSY9 and QD450–QSY35 conjugates were prepared at a ratio 1:10.  
<sup>b</sup>Change in the signal of the corresponding color channel: B = blue, G = green, and R = red.

Gel electrophoresis experiments were also done to confirm the stability of QD-peptide conjugates. QD-peptide conjugates have a lower electrophoretic mobility than unconjugated QDs, and thus can be resolved by agarose gel electrophoresis. Figure S5 shows an image of an agarose gel with a resolved band for QD450–[Sub(TRP1)–QSY35]<sub>20</sub> conjugates (10 pmol) and no apparent decrease in the mobility for QD540 (2 pmol) and QD625 (3 pmol) that were incubated with pre-prepared QD450–[Sub(TRP1)–QSY35]<sub>20</sub> conjugates prior to running the gel. This suggested stable binding, particularly since the QD450–[Sub(TRP1)–QSY35] conjugates were prepared with 20 equivalents of peptide (200 pmol), which translated into a 67–100 fold excess relative to
the amount of QD625 and QD540. Since clear band separation can be observed between QDs and conjugates with as little as 5 peptides per QD (data not shown), the alignment of the QD540 and QD625 bands between the mixture and control samples further suggested that the peptides did not migrate between QDs on the timescale of the assays.

2.6 UV lamp intensity output

Given the variation in the measured intensity levels of QD samples over 1 h, the time-dependent output of the UV lamp was measured using a spectrometer (Greenwave 16 VIS-50, StellarNet, Tampa, FL, USA). As shown in Figure S6, the relative intensity decreased by ca. 20% over 60 min. To account for this drift, all enzyme samples were normalized to a reference sample (i.e. no enzyme).
2.7 Stability of smartphone image intensity time series

The potential for temporal variation in RGB channel intensities from a time series of smartphone images was investigated using LEDs with blue and green emission (470 nm and 530 nm peak wavelength; Visual Communications Company, CA, USA). The LEDs were powered using data acquisition module (DAQ USB-6008, National Instruments, Austin, TX, USA). Following a 6 h warm-up period with operation at the manufacturer-specified voltage, LED emission spectra were collected every 20 s using a fiber-optic spectrometer (Greenwave16 VIS-50, StellarNet, Tampa, FL, USA) and a custom data acquisition program written in LabVIEW (National Instruments). The maximum emission intensity output by LEDs was measured at 468 nm for the blue LED and 523 nm for the green LED. The time traces are shown in Figure S7A, where the relative variation was ca. ±0.45 % for both LEDs. In parallel, color images were acquired with the iPhone using iLapse software and were split into RGB channels using Image J. Emission intensities from the blue and green LEDs were measured from the blue channel and green channel of the RGB images, respectively. These time courses are shown in Figure S7B. The saturated pixels observed at the tip of the LEDs (inset, Figure S7C) were omitted from the calculations. The relative variation associated with the RGB images was < 0.6%, indicating that the precision of the RGB image analysis was comparable to that of a research grade spectrometer.
Figure S7. Time traces for blue and green LED output measured with (A) a spectrometer and (B) a smartphone digital camera. (C) The blue and green LED emission spectrum and the corresponding color image of the LEDs.

2.8 Digest calibration curves

We identified three mechanisms through which dye-labeled peptides quenched QD PL: (1) energy transfer from the QD to acceptor-labeled peptide assembled through its terminal hexahistidine tag (important with native substrate); (2) energy transfer from the QD to acceptor-labeled peptide that was non-specifically adsorbed (important with protease-digested substrate); and (3) changes in the quantum yield of the QD as a result of binding of the hexahistidine tag to its surface (important with both native and digested substrate). Point (3) is not common to all QDs, but was found to be important for these commercial QDs. To account for (1), (2), and (3) concurrently, we prepared mixed digest/substrate calibration curves, mixing fractions of pre-digested and native substrate, as described previously. The calibration curves used to quantitatively analyze proteolytic activity in one-plex assays are shown in Figure S8 with analogous calibration curves for the fluorescence plate reader. Figure S9 shows the relative decrease in QD625 PL intensity upon the assembly of 4, 8, or 12 peptides per QD. Peptides with different amino acid sequences and modified with Alexa Fluor 488 (A488), Alexa Fluor 555 (A555), or a capped cysteine residue were used for these experiments. Neither A488 nor A555 is a FRET acceptor for the QD625.
Figure S8. Mixed digest/substrate calibration curves used for the analysis of progress curves in one-plex assays with (A) a fluorescence plate reader and (B) smartphone RGB imaging. The x-axis refers to the number of native peptide substrates per QD (with $N-x$ pre-digested peptide, where $N = 12$ or 16): (i) QD625–[Sub(TRP2)-A647]$_{12}$ conjugates, (ii) QD540–[Sub(ChT)-QSY9]$_{16}$ conjugates, (iii) QD450–[Sub(TRP1)-QSY35]$_{12}$ conjugates. The data points were fit with the simplest possible polynomial function (linear, quadratic, or cubic).

Figure S9. Relative decreases in QD625 PL intensity upon assembly of 4, 8, and 12 equivalents of peptide with various modifications of the C-terminal residue (A488 = Alexa Fluor 488, A555 = Alexa Fluor 555, Cys = cysteine). Note that neither A488 nor A555 is a FRET acceptor for QD625.
2.9 One-plex protease assays with TRP and ChT

Figures S8–S9 (and Figure 3, main text) show progress curves that track proteolytic activity in one-plex assays using the RGB imaging readout. QD540–[Sub(ChT)-QSY9]_{16} (20 pmol) and QD450–[Sub(TRP1)-QSY35]_{12} (100 pmol) were exposed to different concentrations of ChT and TRP, respectively, and the recovery of QD PL was tracked using digital imaging and compared to analogous measurements made with a fluorescence plate reader. Figures S8–S9 show both raw data and progress curves normalized to unit intensity at $t = 0$. Intensity data was converted into the amount of substrate remaining using the calibration curves described in Section 2.8. Initial rates of proteolysis were calculated from the slope of the initial linear region of the progress curves, and this data was fit with eq. S9 to determine the specificity constant, $k_{cat}/K_m$. Good correspondence was observed between the smartphone imaging and fluorescence plate reader results, validating the red and blue channels of the smartphone digital imaging readout platform, analogous to the red channel in Figure 3 of the main text.
Figure S10. Proteolytic digestion of QD540–[Sub(ChT)-QSY9]_{16} conjugates by ChT and comparison of data acquired with (A) a fluorescence plate reader and (B) a smartphone and RGB imaging: (i) raw PL data; (ii) normalized PL data; (iii) conversion of the normalized PL data to the average number of acceptors per QD and the bulk equivalent concentration of peptide substrate. (C) Comparison of initial proteolytic rates measured from the fluorescence plate reader data and smartphone RGB imaging data.
Figure S11. Proteolytic digestion of QD450-[Sub(TRP1)-QSY35]12 conjugates by TRP and comparison of data acquired with (A) a fluorescence plate reader and (B) a smartphone and RGB imaging: (i) raw PL data; (ii) normalized PL data; (iii) conversion of the normalized PL data to the average number of acceptors per QD and the bulk equivalent concentration of peptide substrate. (C) Comparison of initial proteolytic rates measured from the fluorescence plate reader data and smartphone RGB imaging data.
2.10 Representative raw data from multiplexed assays with RGB imaging

Figure S12 shows representative raw data acquired in two-plex assays with RGB imaging *via* a smartphone camera. Figure S13 shows representative raw images obtained using the smartphone camera during three-plex assays.

![Figure S12](image)

**Figure S12.** Representative unprocessed RGB intensity time traces for two-plex assays with (A) QD540–[Sub(ChT)-QSY9]_{16} and QD450–[Sub(TRP1)-QSY35]_{12} conjugates upon exposure to various concentrations of TRP and ChT, and (B) QD540–[Sub(ChT)-QSY9]_{16} and QD625–[Sub(TRP2)-A647]_{12} upon exposure to various concentrations of TRP and ChT.
Figure S13. (A) Brightness enhanced images shown in the main text (Figure 5) and (B) the raw images used for data analysis. Note that each column of the figure is an image, with the images from the indicated times concatenated to make the time series.

3. References