A Non-fluorescent, Broad Range Quencher Dye for FRET Assays

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ABSTRACT

We report here a novel, water soluble, non-fluorescent dye that efficiently quenches fluorescence from a broad range of visible and near-infrared (NIR) fluorophores in Förster resonance energy transfer (FRET) systems. A model FRET-based caspase-3 assay system was used to test the performance of the quencher dye. Fluorogenic caspase-3 substrates were prepared by conjugating the quencher, IRDye® QC-1, to a GDEVDGAK peptide in combination with fluorescein (emission maximum ~540 nm), Cy3 (~570 nm), Cy5 (~670 nm), IRDye 680 (~700 nm), IRDye 700DX (~690 nm) or IRDye 800CW (~790 nm). The Förster distance R_0 values are calculated as 41 to 65 Å for these dye/quencher pairs. The fluorescence quenching efficiencies of these peptides were determined by measuring the fluorescence change on complete cleavage by recombinant caspase-3, and ranged from 97.5% to 98.8%. The fold increase in fluorescence on caspase cleavage of the fluorogenic substrates ranged from 40 to 83 depending on the dye/quencher pair. Because IRDye QC-1 effectively quenches both the NIR fluorophores (e.g., IRDye 700DX, IRDye 680 and IRDye 800CW) and the visible fluorophores (e.g. fluorescein, Cy3, Cy5), it should find broad applicability in FRET assays using a wide variety of fluorescent dyes.

Keywords: *Non-fluorescent dye*
Quenching dye
Fluorogenic peptide substrate
Caspase-3 activity assay
Cell apoptosis
Near-infrared fluorescence
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Enzymatic assay

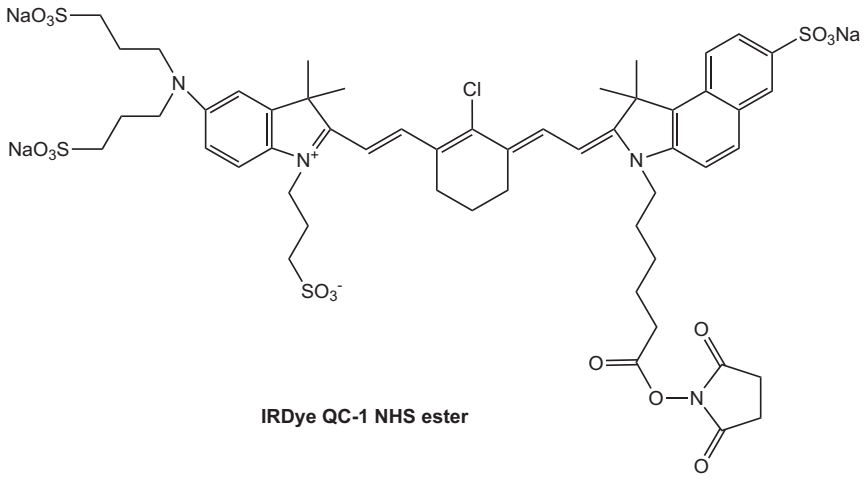

Figure 1. Chemical structure of IRDye® QC-1 NHS ester.

Non-fluorescent quenching dyes, also known as dark quenchers, have been commonly used in FRET based fluorogenic probes for protease activity detection[1-13], nucleic acid hybridization[14, 15], and real-time PCR [16-18]. In FRET-based systems, a specific quencher is normally able to quench the fluorescence only from those fluorophore donors that have significant overlap of their emission spectra with the absorption spectrum of the quencher when the donor and quencher are brought into proximity. To design a donor-quencher FRET system, the quenching range information and/or careful comparison of the donor's fluorescence spectrum with the quencher's absorption spectrum is required[1].

Although non-fluorescent dyes that efficiently quench visible fluorescent donors have been described, there is an unmet need for an efficient non-fluorescent quencher for near-infrared (NIR) dyes. In addition, it would be advantageous to have a non-fluorescent dye with broad capability to quench both visible and NIR donors. Broad quenching ability would simplify assay development, and applicability to NIR dyes would take advantage of the low assay background characteristics of this spectral region.

There are several advantages to working in the NIR region. While FRET based assays using red-shifted fluorophores such as rhodamine, Cy3, and Cy5 can reduce background compared to traditional assays using shorter wavelength donor/quencher pairs, longer wavelength NIR fluorescence assays can virtually eliminate background fluorescence due to the extremely low autofluorescence in the NIR [19, 20]. For *in vivo* imaging applications, NIR assays also benefit from the enhanced tissue penetration of light near 650-900 nm[21].

To develop NIR FRET assays, it is essential to have a well-matched NIR fluorophore and quencher. Considerable efforts in developing NIR FRET assays have been reported. Pham *et al.* reported a NIR fluorescence probe for sensing MMP-7 protease activity using a Cy 5.5 donor paired with a fluorescent NIRQ820 dye as the acceptor[21]. The probe showed a limited working range due to a maximum 7-fold fluorescence increase after complete proteolytic cleavage. Furthermore, a NIR caspase-3 assay using a non-fluorescent azulene dye and Alexa Fluor® 680 has also been reported, but showed only a 4-fold fluorescence increase[22].

We report here a novel, water soluble, monoreactive, nonfluorescent dye, IRDye® QC-1, that efficiently quenches fluorescence from a wide range of fluorophores spanning the visible to NIR spectrum (~500 - ~800 nm). We synthesized a series of fluorogenic caspase-3 peptide substrates using IRDye QC-1 paired with various fluorophore donors and measured the fluorescence quenching efficiencies by cleaving these substrates with human recombinant caspase-3. IRDye QC-1 showed efficient quenching with all dyes tested in the model system and showed fluorescent signal increases ranging between 40-fold and 83-fold upon complete cleavage of the fluorogenic substrates. The broad applicability of IRDye QC-1 should simplify FRET assay development and enable development of sensitive NIR assays.

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[1] *Abbreviations used:* FRET, Förster resonance energy transfer; PCR, polymerase chain reaction; NIR, near-infrared; MMP-7, matrix metalloproteinase 7; NHS, *N*-hydroxysuccinimide; TCEP•HC1, tris(2-carboxyethyl)phosphine hydrochloride; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; LC/MSD, liquid chromatography/mass selective detection; UV, ultraviolet; ICG, indocyanine green; DOTCI, 3,3′-diethyloxatricarbocyanine iodide; DMF, dimethylformamide; DIPEA, diisopropylethylamine; TFA, trifluoroacetic acid; TEAA, triethylammonium acetate; EDTA, ethylenediaminetetraacetic acid; *S/N*, signal-to-noise; FBS, fetal bovine serum; NaOAc, sodium acetate; BSA, bovine serum albumin LOD, limit of detection.

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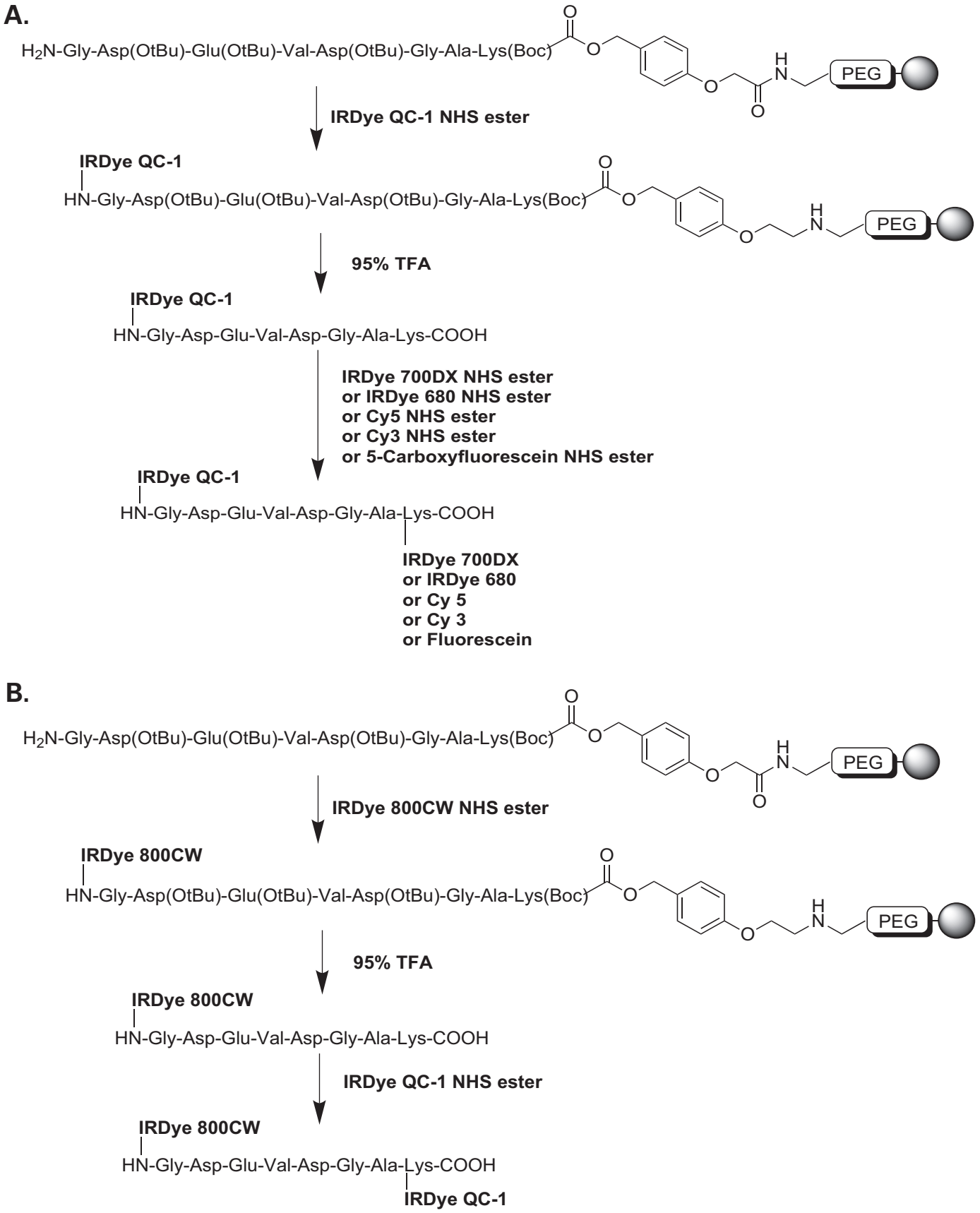

Figure 2. Synthesis of fluorophore/IRDye QC-1-conjugated GDEVDGAK peptide substrates. (A. On bead labeling with IRDye QC-1. B. On bead labeling with IRDye 800CW.)

Materials and Methods

General aspects

Unless otherwise noted, all general chemical reagents were purchased from commercial suppliers and used without further purification. Resin bound peptide H_2 N-Gly-Asp(OtBu)-Glu-Val-Asp(OtBu)-Gly-Ala-Lys(Boc)-NovaSyn TGA resin was custom synthesized by Pi Proteomics LLC (Huntsville, AL, USA). IRDye QC-1, IRDye 800CW, IRDye 680 and IRDye 700DX N-hydroxysuccinimide (NHS) ester dyes were obtained from LI-COR® Biosciences (Lincoln, NE, USA). Cy3 and Cy5 NHS ester dyes were purchased from Amersham Biosciences (Buckinghamshire, UK). 5-carboxyfluorescein succinimidyl ester dye was from Invitrogen/Molecular Probes (Eugene, OR, USA). Human recombinant caspase-3 was purchased from Upstate Biotechnology (Rochester, NY, USA) and contained 300 to 400 units per µg enzyme, where one unit equals 1 nmole of DEVD-pNA substrate cleavage per hour at 37 °C at saturated substrate concentrations. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP•HCl) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). The Phosphate-buffered saline (PBS) was purchased as the 10× concentrate from Sigma (St. Louis, MO, USA) and diluted 10-fold prior to use.

Reverse-phase high-performance liquid chromatography (HPLC) was conducted with an Agilent 1100 series HPLC system using a ZORBAX 300SB-C18 column (Agilent Technologies, Santa Clara, CA, USA). Mass spectra were obtained on an Agilent 1100 series liquid chromatography/mass selective detection (LC/MSD) ion trap mass spectrometer. Ultraviolet (UV)-visible spectra were measured using an Agilent 8453 spectrophotometer. Fluorescence spectra were measured using a PTI QuantaMaster luminescence spectrometer from Photon Technology International (Birmingham, NJ, USA). Microtiter plate images were obtained with Falcon 96-well plates from BD Biosciences (San Jose, CA, USA) using an Aerius® Infrared Imager (LI-COR Biosciences). The fluorescence quantum yield ϕ_{sample} was measured using indocyanine green (ICG, Aldrich, Milwaukee, WI) as the fluorescence standard in methanol solution (ϕ_{ST} =0.043)[23] and calculated according to equation (1):

$$\phi_{sample} = \frac{A_{ST}}{A_{sample}} \times \frac{F_{sample}}{F_{ST}} \times \frac{\eta^2_{sample}}{\eta^2_{ST}} \times \phi_{ST} \qquad (1)$$

where F_{sample} and F_{ST}, respectively, are the integrated fluorescence intensity of the full, corrected emission spectra of the sample and standard (solvent blank corrected), respectively; A_{sample} and A_{ST} are the absorbance of the sample and standard; and η_{sample} and η_{ST} are the refractive indices of the solvents for the sample and standard, respectively. We used 3,3′-diethyloxatricarbocyanine iodide (DOTCI; Eastman Kodak Company, Rochester, NY, USA) as a secondary standard to check the validity of the quantum yield measurements.

Fluorogenic caspase-3 substrate synthesis

The structure of IRDye QC-1 is shown schematically in Figure 1. The structure of the dye was verified by mass spectrometry and nuclear magnetic resonance. The preparation of the dual labeled peptides is shown in Figure 2. Resin-bound peptide, was mixed with either IRDye 800CW NHS ester or IRDye QC-1 NHS ester in dimethylformamide (DMF) and diisopropylethylamine (DIPEA) at room temperature overnight. The resulting IRDye 800CW or IRDye QC-1-conjugated peptide-tethered resin was washed with DMF and methanol and then dried. The resin was treated with a solution containing trifluoroacetic acid (TFA, 95%), water (2.5%) and triisopropylsilane (2.5%) to remove all the protecting groups on the side chains of the peptide and to cleave the peptide from the resin at the same time. The product was isolated by filtration and ethyl ether precipitation. Further purification by reverse-phase C18 preparative HPLC using an acetonitrile and 50 mM triethylammonium acetate (TEAA) buffer (pH ~5.5) gradient provided the intermediate product, IRDye 800CW-GDEVDGAK or IRDye QC-1-GDEVDGAK.

IRDye 800CW-GDEVDGAK peptide was reacted with IRDye QC-1 NHS ester in 0.4 M phosphate buffer (pH 8) at room temperature for 3 hours. Reporter-quencher-labeled peptide product was purified by reverse-phase C18 preparative HPLC using an acetonitrile and 50 mM TEAA buffer (pH 5.5) gradient. The product was subjected to ion-exchange chromatography (sodium form ion exchange resin) and dried to provide IRDye 800CW-GDEVDGAK-IRDye QC-1 (Qcsp3 IRDye 800CW). Peptides labeled with IRDye 680, IRDye 700DX, Cy5, Cy3, or fluorescein were prepared similarly to IRDye QC-1-labeled peptide (Figure 2).

Quenching efficiency measurements

Fluorescence quenching efficiencies of IRDye QC-1 for various fluorophores in the caspase-3 substrates were measured by monitoring the fluorescence intensity change of the corresponding fluorophore/IRDye QC-1 conjugated GDEVDGAK peptide upon cleavage by caspase-3. The fluorescence quenching efficiencies were calculated as follows: quenching efficiency (%) = 100 × [(end point fluorescence – starting point fluorescence)/(end point fluorescence – buffer blank background)]. Assay buffer for recombinant human caspase-3 was 100 mM HEPES (pH 7.0) with 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Chaps, 10% glycerol and 5 mM TCEP•HCl. The TCEP•HCl in the assay buffer was freshly added for each experiment.

Enzyme cleavage experiments were performed in a cuvette for Qcsp3-fluorescein and Qcsp3-Cy3 substrates, and the fluorescence spectra at different cleavage time points were recorded with the spectrometer. For all the other peptide substrates (Qcsp3-IRDye 800CW, Qcsp3-IRDye 680, Qcsp3-IRDye 700DX and

Qcsp3-Cy5), measurements were obtained using an Aerius Infrared Imager. The final concentrations of peptide and enzyme in the reaction solutions were 200 nM and 2.8 U/mL, respectively. The fluorescence spectra of the enzymatic reaction solutions were recorded immediately and at various time points after mixing. Fluorescence spectra of the enzymatic reaction solutions at time zero were obtained from reaction solutions without enzyme.

Caspase-3 activity assay using recombinant enzyme

Homogenous biochemical assays using recombinant caspase-3 were performed in 96-well plates in duplicate. The assay buffer was as described above. The final concentration of fluorogenic peptide was 200 nM, and the final concentrations of caspase-3 ranged from 2.8 to 5.5 x 10^{-6} U/mL. Negative control wells containing substrate but not enzyme were included for background determination. After incubation at room temperature on a plate shaker for 1 hour, the plate was imaged. The background was determined in substrate wells without caspase-3. Signal-to-noise (S/N) ratios were calculated as follows: S/N ratio = (mean signal – mean background) / (standard deviation of background)[24].

Cell-based caspase-3 activity assay

Anisomycin and camptothecin were obtained from Sigma (Saint Louis, MO, USA). Jurkat cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 + 10% fetal bovine serum (FBS) to reach 0.5 x 10^6/ml. Cells were centrifuged and resuspended in fresh media at 2 x 10^6/ml. Anisomycin or camptothecin were added to a final concentration of 1 µg/ml. Treated cells were immediately two-fold serially diluted with media containing either anisomycin or camptothecin (1 µg/ml) in 96-well plates in 100 µl volumes. Untreated cells were serially diluted in media without drug. One set of negative control wells contained media only. The plates were incubated at 37 °C in 5% CO_2 for 4 hours, followed by addition of 100 µL of 400 nM Qcsp3-IRDye 800CW or Qcsp3-IRDye 680 in cellular assay buffer (200 mM Hepes [pH 7.5], 2 mM EDTA, 0.2% Chaps, 0.2% Triton X-100, 20% glycerol, and freshly added 1 mM TCEP•HCl). After 1 hour at 37 °C, the fluorescence intensity was measured with an Aerius Infrared Imager.

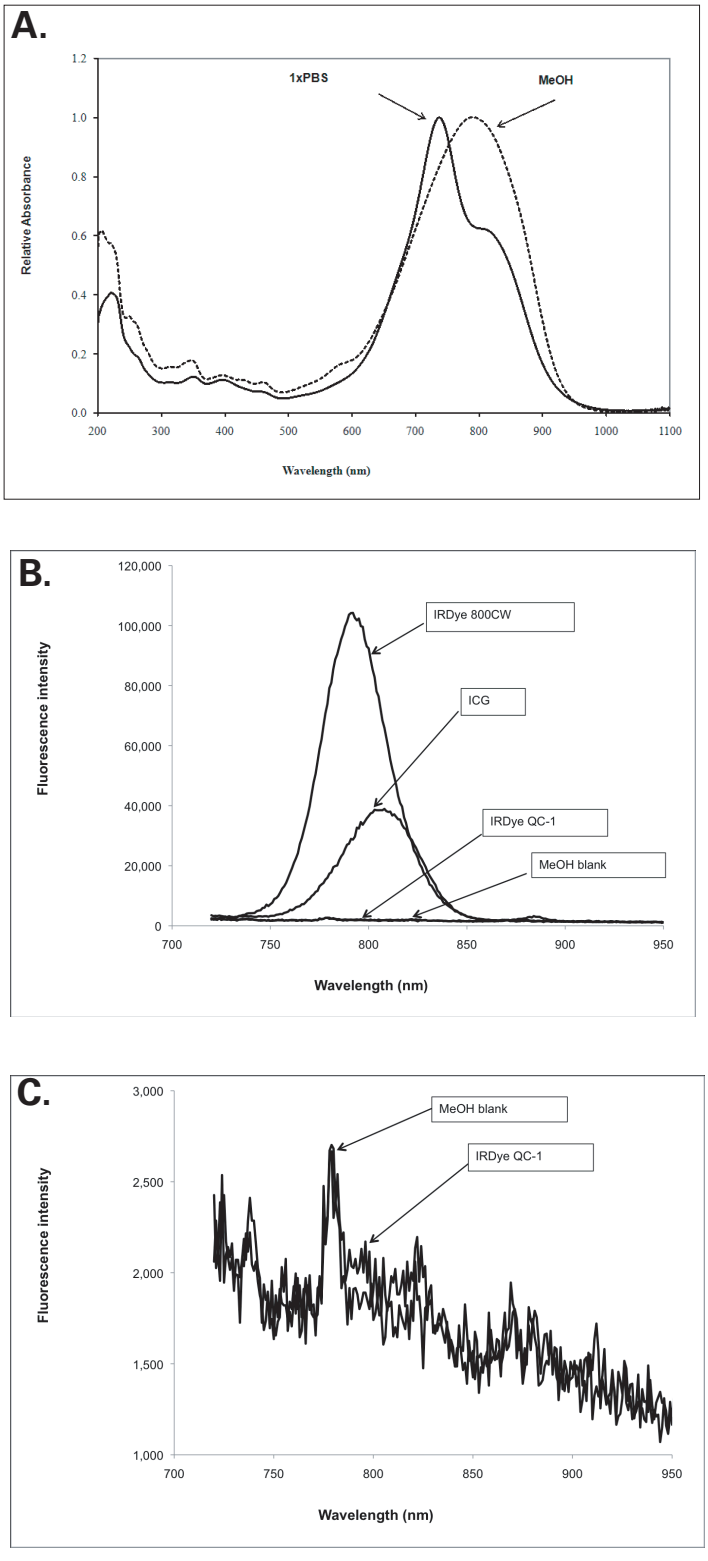

Figure 3. Spectral characteristics of IRDye QC-1. A.) Absorption spectra for IRDye QC-1 in methanol and PBS. B.) Fluorescence spectra of ICG, IRDye 800CW and IRDye QC-1 in methanol (each at 0.01 OD absorbance at 700 nm) following excitation by 700 nm light. Methanol alone was included as a control. C.) Expansion of the QC-1 and methanol absorbance spectra in B.

Results

Absorption and fluorescence of IRDye QC-1

The absorption spectrum of IRDye QC-1 showed a broad peak over the range of approximately 550 to 950 nm (Figure 3A). The extinction coefficient at the absorption maximum was determined to be 96,000 M^{-1} cm^{-1} in 1X PBS and 98,000 M^{-1} cm^{-1} in methanol. The peak shape and absorption maximum were distorted in high-salt buffers compared with organic solutions. In methanol, the absorption spectrum shows a symmetric peak with a maximum at 788 nm. In 1X PBS, the absorption spectrum of the IRDye QC-1 exhibited a shoulder at approximately 815 nm with the maximum blue-shifted to 737 nm.

Ideally, a dark quenching dye should have no intrinsic fluorescence. To determine the intrinsic fluorescence of

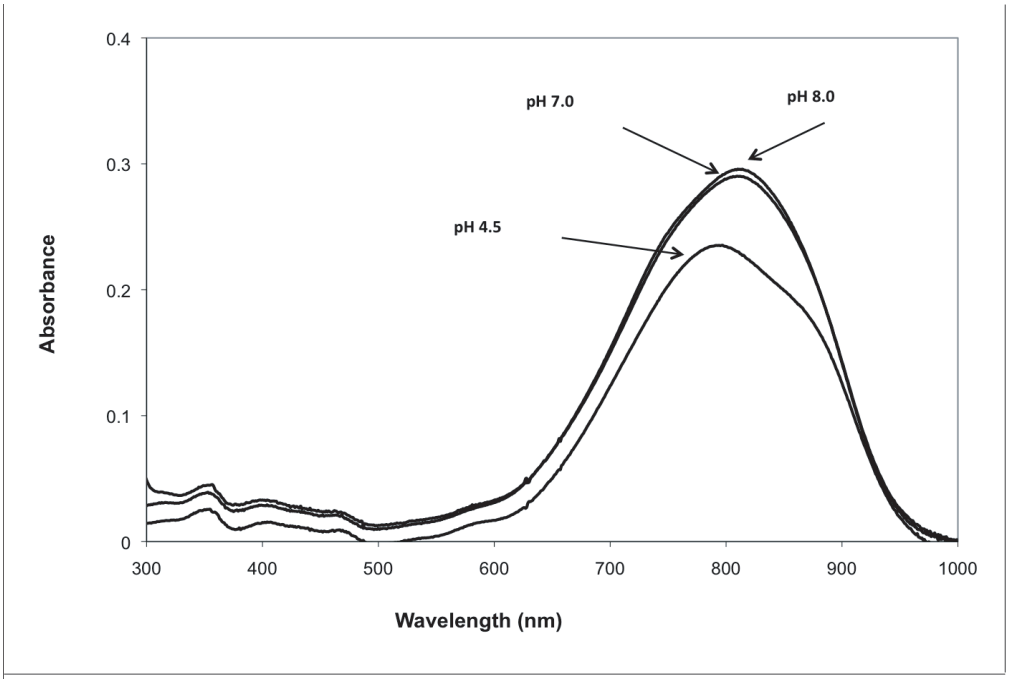

Figure 4. Absorption spectra for 5.0 µM of IRDye QC-1 in 100 mM NaOAc buffer containing 0.25 mg/mL BSA at pH 4.5, 7.0 or 8.0.

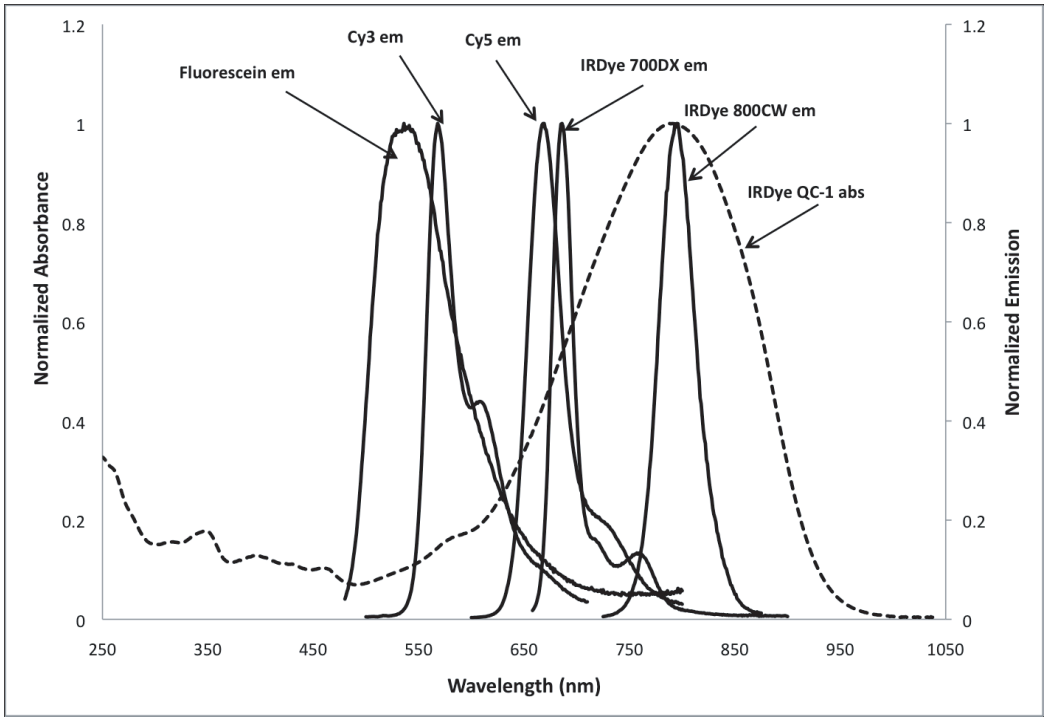

Figure 5. Spectral overlap between the absorption spectrum of IRDye QC-1 and the fluorescence spectra of fluorescein, Cy3, Cy5, IRDye 700DX, and IRDye 800CW.

IRDye QC-1, we measured the dye's fluorescence quantum yield using ICG in methanol as the fluorescence standard. The fluorescence quantum yield of ICG has been reported to be 0.043 in methanol[23]. To verify our methodology, we measured the quantum yield of ICG using DOTCI in methanol ($_{\varphi f}$ = 0.28 in methanol)[25] as the corresponding fluorescence standard. We obtained a value of $_{\varphi f}$ = 0.05 ± 0.01 for ICG in methanol, consistent with the reported value ($_{\varphi f}$ = 0.043). The quantum yield measurement for IRDye QC-1 gave a very small negative number, $_{\varphi f}$ = -0.00034 (±1.3 × 10^{-4}). A "negative" quantum yield is impossible, and the fluorescence of IRDye QC-1 is essentially the same as that of the blank (Figure 3C). This indicates that IRDye QC-1 is a nonfluorescent, dark quencher.

We also measured the fluorescence quantum yield of IRDye 800CW, an NIR fluorescent donor dye, as 0.10 ± 0.004 in methanol using the same method. The non-fluorescent nature of IRDye QC-1 compared with IRDye 800CW and ICG is shown in Figures 3B and 3C (all 0.01 OD at 700 nm, the excitation wavelength), along with the methanol blank. The IRDye QC-1 signal intensity is nearly identical to that of the blank (Figure 3C).

To examine the intrinsic fluorescence of IRDye QC-1 in aqueous solutions and the effect of pH, we compared the fluorescence signals of IRDye QC-1 and IRDye 800CW at the same concentration in acidic, neutral and basic buffer solutions (100 mM sodium acetate (NaOAc) buffer containing 0.25 mg/mL bovine serum albumin [BSA]). The fluorescence intensity of IRDye QC-1 (expressed as a percentage of the IRDye 800CW fluorescence) was slightly higher in acidic buffer (pH 4.5) than in neutral (pH 7.0) or basic (pH 8.0) buffer (data not shown). However, all solutions showed very weak fluorescence (0.05-0.1% of IRDye 800CW fluorescence intensity) at the same dye concentration, suggesting that IRDye QC-1 remains non-fluorescent across a wide pH range from 4.5 to 8.0.

To further understand the effects of pH on IRDye QC-1's lack of intrinsic fluorescence, we measured the absorption spectra of 5 µM IRDye QC-1 carboxylic acid solutions under the same buffer and pH conditions. The absorption in these solutions (Figure 4) shows a symmetric, broad peak without the blue-shift phenomenon seen in 1X PBS buffer (Figure 3A). The presence of BSA and low salt concentration in the NaOAc buffer appeared to minimize the dye aggregation that was observed in 1X PBS buffer. The absorption spectra were indistinguishable at pH 7.0 and pH 8.0, with maximum absorption at approximately 810 nm. At pH 4.5, the absorbance was slightly decreased and the absorption peak was slightly blue-shifted, from approximately 810 nm at pH 7.0/8.0 to 797 nm at pH 4.5.

Table 1
Förster distance R_0 values for IRDye QC-1 quencher with select fluorophores.

Donor (fluorophore)	Donor's fluorescence quantum yield and reference	R_0 for IRDye QC-1 and corresponding fluorophore pairs (Å)
Fluorescein	0.91[32]	58
Cy3	0.09[33]	41 Å
Cy5	0.30[33]	64 Å
IRDye 700DX	0.24[34]	65 Å
IRDye 800CW	0.10	65 Å

Förster distance for IRDye QC-1 quencher with various fluorophore donors

As shown in Figure 5, IRDye QC-1 has a broad absorption peak in approximately the 550- to 950 nm range and overlaps very well with the fluorescence spectra of the far-red to NIR fluorophores (e.g., Cy5, IRDye 700DX, IRDye 800CW). This quencher also shows some degree of spectral overlap with fluorophores in the visible region (e.g., fluorescein, Cy3). To assess the quenching potential of IRDye QC-1 for these fluorophores, we calculated the Förster distance (R_0, in Å) for various fluo-

Table 2
Fluorescence increase and quenching efficiency for IRDye QC-1 and various fluorophores conjugated to GDEVDGAK peptides in caspase-3 cleavage measurements.

Fluorogenic caspase-3 probe	Fluorophore/ quencher pair	Fluorescence fold increase after cleavage by caspase-3 (A)	Measured quenching efficiency (%)	R0 for donor quencher pair in probe	Calculated FRET quenching efficiency based on R0 donor-quencher distance of 29A) (%)
Qcsp3-Fluorescein	Fluorescein/IRDye QC-1	40	97.5	58	98.4
Qcsp3-Cy3	Cy3/IRDye QC-1	52	98.1	41	88.9
Qcsp3-Cy5	Cy5/IRDye QC-1	47	97.9	64	99.1
Qcsp3-IRDye 680	IRDye 680/IRDye QC-1	81	98.8	No data	No data
Qcsp3-IRDye 700DX	IRDye 700DX/IRDye QC-1	83	98.8	65	99.2
Qcsp3-IRDye 800CW	IRDye 800CW/IRDye QC-1	75	98.7	65	99.2

rophore/IRDye QC-1 pairs. Absorption curves, fluorescence curves, and quantum yield values for methanol solutions of the free dyes were used for the calculations. This allows all of the pairs to be compared on the same basis, although the actual R_0 values would differ in other solvent systems.

The R_0 values were calculated, as described by Lakowicz [26] according to equation (2):

$$R_0 = 0.211[\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \quad (2)$$

where

$$J(\lambda) = \frac{\int_0^\infty F_d(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_d(\lambda)d\lambda} \quad (\text{M}^{-1}\,\text{cm}^{-1}\,\text{nm}^4); \quad (3)$$

In equations (2) and (3), $F_d(\lambda)$ is the donor's fluorescence spectrum; $\epsilon_A(\lambda)$ is the acceptor's absorption spectrum; Q_D is the donor's fluorescence quantum yield; n is the refractive index of the medium; κ^2 is the "orientation factor," reflecting the effects of relative orientation of the transition dipoles of the donor and acceptor (assumed to be 2/3).

As shown in Table 1, the R_0 values are long with 64 to 65 Å for spectrally well-overlapped, far-red, NIR fluorophores and IRDye QC-1 pairs. For visible fluorophores/IRDye QC-1 pairs, the R_0 values are also good with 58 Å for fluorescein/IRDye QC-1 pairs and reasonably good with 41 Å for Cy3/IRDye QC-1 pairs. These calculated R_0 values indicate that the IRDye QC-1 can quench a wide range of fluorophores from visible to NIR spectrum through FRET in a system where the donor and quencher are in close proximity.

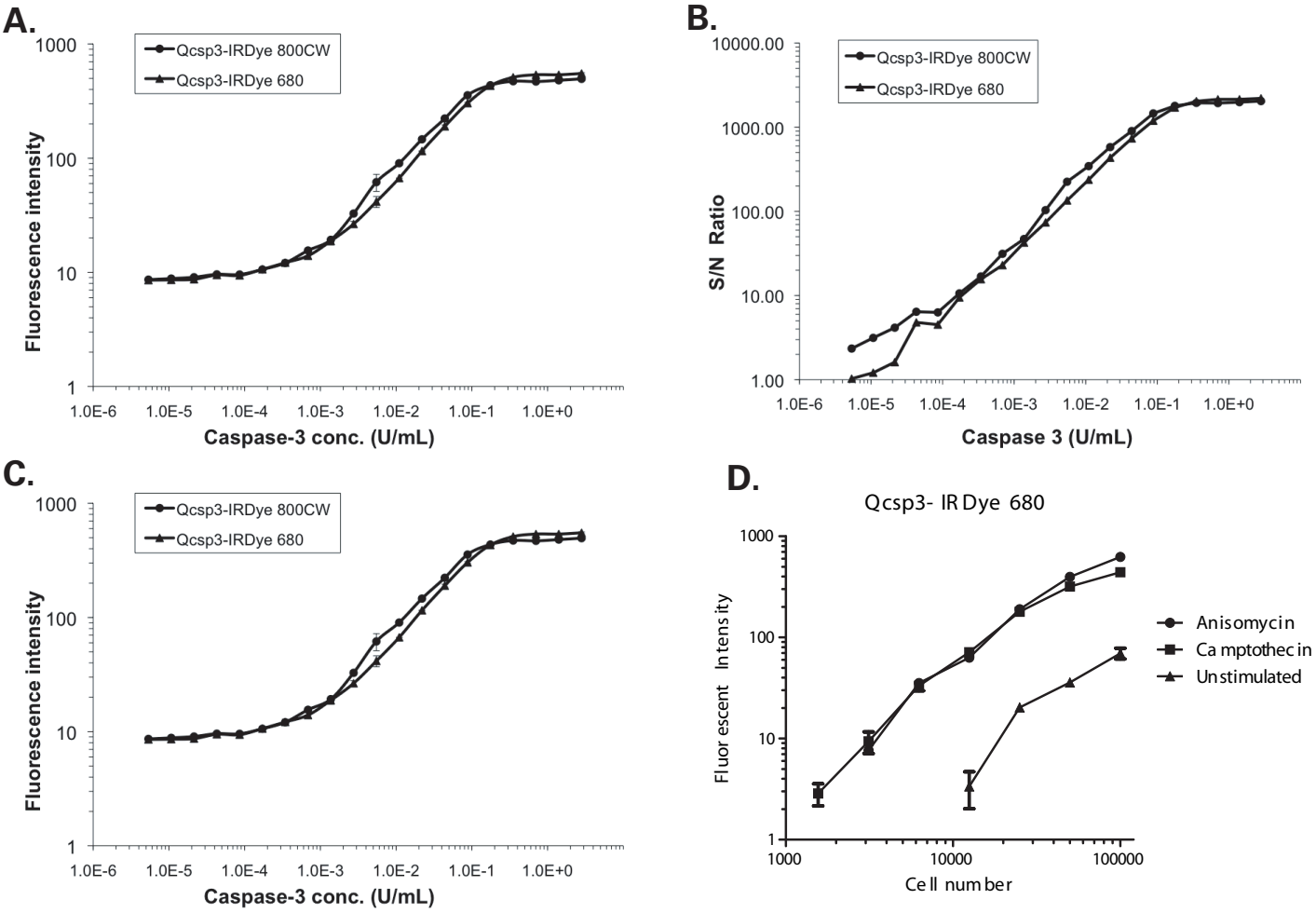

Figure 6. Performance of near-infrared fluorescent caspase-3 assays. A.) LOD for IRDye 800CW and IRDye 680 caspase assays. B.) S/N for IRDye 800CW and IRDye 680 caspase assays. C. & D.: Detection of endogenous caspase-3 activity in Jurkat cells untreated or treated with anisomycin (1 µg/ml) or camptothecin (1 µg/ml). C.) IRDye QC/IRDye 800CW. D.) IRDye QC/IRDye 680. Error bars are ± standard deviation.

Syntheses of caspase-3 FRET substrates and measurement of fluorescence quenching efficiencies

The GDEVDGAK peptide substrate used in this study contains a DEVD sequence that is a preferred substrate for caspase-3[27-29]. The syntheses and nomenclature of the conjugated peptide substrates are summarized in Figure 2. The first donor or quencher dye was covalently conjugated to the peptide through the terminal amine group using an on-bead labeling approach, whereas the second amine (on the lysine) was protected by a Boc group. The dye-peptide was cleaved from the beads with 95% TFA, and the unmasked lysine in the peptide was used to conjugate the second dye. The final products were obtained after preparative HPLC purification and sodium ion exchange. Purity was typically ≥98% by HPLC.

To measure the quenching efficiency of IRDye QC-1 for this group of fluorophores in a FRET system, we synthesized the series of IRDye QC-1/donor conjugated GDEVDGAK caspase-3 peptide substrates shown in Figure 2. These peptide substrates were incubated with 2.8 U/mL caspase-3, and the fluorescence intensity change was monitored. The relative increase in fluorescence (fold increase) after cleavage by caspase-3 and the corresponding fluorescence quenching efficiencies are summarized in Table 2. After complete cleavage of each substrate by caspase-3, the fluorescence in the reaction increased 40-fold to 83-fold, depending on the specific donor/quencher pair. The fluorescence quenching efficiencies ranged from 97.5% to 98.8%. These data demonstrate that IRDye QC-1 is able to efficiently quench fluorescence across the visible (fluorescein, Cy3), far-red (Cy5) and NIR (IRDye 680, IRDye 700DX and IRDye 800CW) regions of the light spectrum in the caspase-3 FRET system.

NIR Fluorescent Caspase-3 Activity Assays

Two caspase-3 substrates labeled with NIR fluorescent donors, Qcsp3-IRDye 800CW and Qcsp3-IRDye 680, were selected for further evaluation. The enzymatic sensitivity of this assay was measured by incubating either substrate at a final concentration of 200 nM with a dilution series of purified human recombinant caspase-3 from 5.36×10^{-6} to 2.8 U/mL. Using an *S/N* ratio ≥3 to define the limit of detection (LOD), the assay detected caspase-3 activity as low as approximately 1.1×10^{-5} U/mL using Qcsp3-IRDye 800CW as the substrate and 4.3×10^{-5} U/mL using Qcsp3-IRDye 680 (Figures 6A. and 6B.).

To demonstrate that the quenching capability of IRDye QC-1 in Qcsp3-IRDye 800CW or Qcsp3-IRDye 680 could result in sensitive fluorogenic substrates for endogenous caspase-3 activity detection, the substrates were used to assess apoptosis in Jurkat cells previously treated with either anisomycin or camptothecin. Figures 6C. and 6D. show the relative fluorescence signals obtained by incubating the substrates with serially diluted apoptotic Jurkat cells in 96-well plates. Similar characteristics were observed for the two substrates. The LOD was approximately 1500 camptothecin-treated cells, or 3000 anisomycin-treated cells. A linear relationship between fluorescence intensity and cell number was seen across a range of 1,500 to 100,000 camptothecin-treated cells or 3,000 to 100,000 anisomycin-treated cells. The level of caspase-3 activity observed in Jurkat cells treated with anisomycin or camptothecin was at least 8-fold higher than that in untreated cells. Therefore, IRDye QC-1 functions efficiently in a cellular assay format.

Discussion

Non-fluorescent quenchers have been commonly used in FRET-based assays. Normally, a quencher is only able to quench a narrow range of donors. In general, the quenchers described to date have been useful only in the UV and visible spectral regions. This article has described a new, nonfluorescent quencher, IRDye QC-1, that can efficiently quench fluorescence from a wide range of fluorophores emitting from the visible to NIR regions of the light spectrum.

IRDye QC-1 is an amino-substituted cyanine dye with a conjugated cyclic heptamethine structure. Cyanine dyes of this class typically have long wavelength absorption and fluorescence in the NIR region. Surprisingly, the amino substitution on the indole essentially eliminates the fluorescence of IRDye QC-1 dye. This structural modification also results in a much broader absorption peak for the dye (550 - 950 nm, Figure 3A). The lone pair of electrons on the amine is apparently the key element for the spectral changes.

Many cyanine dyes form dimeric associations ("π stacking") in solutions, particularly hydrophobic dyes in aqueous solutions[30]. The broad absorption curves of IRDye QC-1 in methanol and PBS suggest that stacking may be occurring. However, the dye dissolves readily in methanol and even in aqueous salt solutions (i.e., PBS). The extinction coefficient measurements were made on solutions from 0.3 to 10.0 μM. In methanol there is excellent agreement with Beer's Law ($R^2 > 0.9999$) and no evidence of curvature. In PBS, the linearity is still good ($R^2 = 0.998$ and 0.997), but the residuals reveal a small, upward curvature in the plot (data not shown). This is consistent with the absorption maximum at 737 nm in PBS arising from π-stacking, given that increasing concentration would enhance stacking.

The effect of BSA and lower ionic strength on the absorption spectrum (cf. Figures 4 and 3A.) is also consistent with some stacking. Because BSA is known to bind to many species, it could disrupt dye stacking here. However, the methanol data and overall agreement

with Beer's Law in both solvents indicate that the broadness of the curves arises primarily from other effects. To the extent that stacking of the quencher with the fluorophore occurs, this would provide an additional quenching mechanism.

When IRDye QC-1 is exposed to acid solutions such as methanol with 1% acetic acid, the amino group is protonated and the dye becomes fluorescent with a sharper absorption spectrum (data not shown). However, such protonation is negligible for IRDye QC-1 at pH levels commonly used in biological assays. The dye remains non-fluorescent and exhibits a broad absorption peak in pH 4.5, 7.0 and 8.0 buffers (Figure 4). The slight shift in the absorption spectrum of IRDye QC-1 at pH 4.5 may be due to a low degree of protonation of the amine and/or a pH dependence in binding of the dye to BSA.

To examine the utility of IRDye QC-1 to quench fluorophores, we conjugated an octapeptide that is a caspase-3 substrate to IRDye QC-1 in combination with a variety of fluorophores spanning the absorption spectrum from visible to NIR. All fluorophores tested were efficiently quenched in this system, indicating IRDye QC-1 is a broadly functional quencher.

Caspase-3 activity assays based on the NIR versions of these substrates perform well. The limits of detection for purified caspase-3 with these peptides using IRDye 800CW (1.7×10^{-3} pg) and IRDye 680 (6.6×10^{-3} pg) are slightly better than those reported for the Z-DEVD-aminoluciferin based bioluminescent Caspase-3 assay and approximately 1000 times lower than the Z-DEVD-AMC based fluorescence assay[29].

The quencher and fluorophore in both these systems are large compared to the peptide substrate, and steric effects could conceivably affect the acceptance of the substrate by enzyme. This is a general issue with peptide-based assays, even with smaller dyes, but previous work on related NIR assays with IRDye 800CW and IRDye QC-1[31] has demonstrated that known inhibitors give comparable IC_{50} results to other peptide assays. The LOD comparisons with Z DEVD assays just discussed also demonstrate Caspase-3 enzyme tolerates the presence of these large dyes.

To understand IRDye QC-1's broad quenching ability, we calculated the FRET efficiencies (E) for each donor/quencher pair in these octapeptides according to equation (4):

$$E = R_0^6/(R_0^6 + r^6) \quad (4)$$

where R_0 is the Förster distance for each donor/quencher pair and r is the donor-quencher distance in this FRET system (estimated at 29 Å). The results (Table 2) demonstrate that the FRET mechanism alone is sufficient to account for the observed quenching efficiency for each fluorophore in this system, with the possible exception of Cy3.

As shown in Figure 5, the degree of overlap of the various fluorophores with IRDye QC-1 ranges from very high (IRDye 800CW) to relatively low (fluorescein), yet all are quenched with high efficiency in the octapeptide system. The quenching efficiencies are high because all of the pairs have R_0 values larger than the expected distance between the dyes. Matayoshi and coworkers[1] estimated a 29 Å end-to-end distance for a fully extended octapeptide, and found highly efficient energy transfer for a FRET pair with an R_0 of just 33 Å. So, high quenching efficiency in the current system is not surprising, considering the large R_0 values.

As shown in equations (2) and (3), the value of R_0 for a pair of dyes is determined largely by the overlap integral, $J(\lambda)$, and the quantum yield of the donor. The overlap integral is affected strongly by the dye absorptivity (vs. wavelength) and by the fourth power of the wavelength. The extinction coefficient of IRDye QC-1 is high (98,000), and absorptivity is particularly strong at long wavelengths, so $J(\lambda)$values are generally large.

We have synthesized and characterized a new dark quencher with broad functionality across the visible and NIR spectral regions. The ability of IRDye QC-1 to quench a wide range of fluorophores may simplify the design of FRET-type assays, reducing the need to spectrally match fluorophore and quencher.

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