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July 2013

The final publication is available at:

http://dx.doi.org/10.1039/C3PP50118C

Comparison of quenching efficiencies of various dye-quencher pairs for the design of an optimal ‘’activatable’’ nanoprobe for optical imaging.

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Running Title: Optimal dye-quencher pairs for ‘’activatable’’ optical nanoprobes

Keywords: IR dye QC-1, BHQ-3, Nanoparticles (NPs), near infrared, quencher, activatable, turn-on fluorescence, optical imaging

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ABSTRACT

Optical imaging offer high sensitivity and resolution, and (potentially) portability at low cost. The design of ‘’activatable’’ optical imaging agents could greatly decrease the background and increase the specificity of the signal. Five different molecules have been used to quench basal fluorescence of an enzyme substrate labeled with Cy5, Cy5.5 or IR800 at a distance of 8 amino acids (32 Å): a 6 nm gold Nanoparticle (NP), a 20 nm and a 30 nm iron oxide NP, the black hole quencher BHQ-3 and the IR dye quencher QC-1. The quenching efficiencies were 99% for QC1-IR800, 98% for QC1-Cy5.5, 96% for 30nm IO NP-Cy5.5, 89% for BHQ3-Cy5, 84% for BHQ3-Cy5.5, 77-90% for 6nm gold NP-Cy5.5, depending on the number of dyes around the NP, 79% for 20nm IO NP-Cy5.5 and 77% for Cy5.5-Cy5. Signal activation upon cleavage by MMP9 was proportional to the quenching efficiencies, ranging from 3-fold with Cy5.5-Cy5 to 67-fold with QC1-IR800. This independent work will review the properties of the dyes and quenchers behind their relative performances.

INTRODUCTION

Optical imaging is a modality of choice for surgical guidance given the portability, low cost and great sensitivity of CCD (charge-coupled device) cameras. The design of ''activatable'' fluorescent probes allows to greatly decrease the background and increase the specificity of the signal. The transfer of energy between one donor (fluorescent dye) and one acceptor (chromophore) allows to turn off the signal of a probe until it reach its target and get ''activated'' by local enzyme[1] or pH[2] . The transfer of energy can occur by static interaction between the donor and the acceptor or by Förster Resonance Energy Transfer (FRET)[3,4]. The first can occur at distance (r) below 20 Å between two molecules, attracted to each other by electrostatic or hydrophobic interaction. It is very temperature dependent and proportional to e^{-R}. On its side, dynamic quenching occurs by FRET between the donor and acceptor at distances (r) of 40 to 100Å, proportionally to $1/r^6$. To be effective though, the acceptor must have a high molar extinction coefficient with a good spectral overlap with the donor's emission.

To be compatible with in vivo imaging, we selected a couple of dyes and quenchers emitting and absorbing at wavelength comprised between 650-800 nm where the absorbance of hemoglobin[6] and water[7] is low (in vivo optical window). The cyanine dyes Cy5 (Ex.: 650nm, Em.: 670nm) and Cy5.5 (Ex.: 675nm, Em.: 695nm) and the IRdye800 (Ex.: 775nm, Em.: 795nm) are among the most used fluorescent dyes used for in vivo imaging. They are now synthesized with several sulfonic acid groups to increase their aqueous solubility and decrease their stacking[8]. Among the dark quenchers absorbing in the 650-800nm range, the Black Hole Quencher 3 is an

analogue of Rhodamine, developed by Biosearch Technologies, with a maximal absorbance at 672nm. The IRdye quencher QC-1, developed by LiCOR, is an IRdye analogue with a maximal absorbance at 723nm. In contrast to FRET between single molecules, the plasmon coupling of metal nanoparticles (NP) decreases less with the distance ($1/d^4$ instead of $1/d^6$). Thus, small gold nanoparticles have been developed as quenchers, due to their huge and broad absorbance. For example, a 6nm gold NP will have an extinction coefficient of 1.8E7 M^{-1} cm^{-1} at 520nm and its coefficients could be increased to the power of 3.3 the size of the NPs[9] with a slight shift of its maximum toward the red by 1nm for every 3nm of diameter increase[10],[11]. Moreover, the diameter of the NP will also affect the distance at which it can absorb light[12]. Iron oxide NPs are mainly used has magnetic resonance imaging agent (MRI). Its absorbance is much lower than gold, but NPs of 20nm, or more, can also absorb light efficiently. To be activated, the donor and acceptor pair can be coupled to antibodies which disrupt the energy transfer upon binding to its target and internalization in cells [13,14]. They can also be conjugated on opposite ends of enzyme substrates[15,16]. In the present study, the peptide GPLG VRGK was chosen for its good cleavability by MMPs [19]. Various dyes were labeled on the amino group of the lysine in *C*-terminal and various quenchers were conjugated in *N*-terminal to compare their efficiencies.

MATERIAL AND METHODS

EDC and sulfo-NHS were purchased at Pierce. Octapeptide GPLG VRGK and decapeptide GGPRQ ITAGK were synthesized by Biomatik. Nanoparticles were from Ocean Nanotech. Cyanine dyes were from GE Lifesciences and IRdye800 and QC-1 quencher were from LiCOR. BHQ3 was from Biosearch Technologies.

Conjugation of Cy5, Cy5.5 or IR800 sulfo-NHS esters on peptide's lysine

2 mM of Cy5, Cy5.5 or IR800 sulfo-NHS esters were reacted with 5mM of peptides in 50mM Trihydroxylamine acetate pH 8.5 for 2h at RT. Excess peptides were separated from peptide conjugated in N-terminal or on Lysine's epsilon side chain using RP-HPLC.

Purification by RP-HPLC

The conjugates were purified by RP-HPLC using a Beckman Gold dual-pump gradient system mounted with a Supelcosil LC-18-DB column of 300x4mm with 5 µm beads of 12nm pores, using 10%Acetonitrile, 50mM Triethylamine acetate pH 5.8 as solvent A and 80%Acetonitrile, 50mM Triethylamine acetate pH 5.8 as solvant B. The use of a 500µl injection loop allowed dilution of sample in 4 volume of solvent A to decrease DMSO and salts concentration before loading, allowing the conjugates to stack in the column while DMSO and salts elute directly at a flow rate of 1ml of solvent A/min. Then a gradient of 20-60% B ran over 40min allowed elution of free peptides, hydrolysed dyes, Lysine-labeled peptides, N-terminal-labeled peptides and then sulfo-NHS-dyes. Samples were lyophilized at -80C under vacuum. Samples were dissolved in a small volume of deionised water and re-lyophilised to remove residual acetate.

Conjugation of BHQ3, QC1 or Cy5.5 sulfo-NHS esters on peptide's N-terminal

1 mM of peptide previously labeled with Cy5, Cy5.5 or IR800 on its Lysine's side chain were conjugated O.N. with 2mM of BHQ3, QC1 or Cy5.5 sulfo-NHS in 50mM phosphate buffer, pH7, at 4ºC.

Excess of BHQ3, QC1 or Cy5.5 (sulfo-NHS or hydrolysed) were separated from conjugates using the same RP-HPLC procedure and then lyophilised.

Conjugation of 6nm gold or 20-30nm IO NPs with Cy5.5-labeled peptide

The carboxylated PEGs surrounding the NPs (1mg) were activated with 2mM EDC and 5mM sulfo-NHS for 15min at RT. After washing with 5ml of 50mM phosphate buffer, pH7 on 100K Amicon filters and decreased the volume to its minimum (50µl), 2mM of peptides, labeled with Cy5.5 on their lysine, were conjugated on their N-terminal with the activated NPs, O.N. at 4ºC. Excess of Cy5.5-peptide were washed 5 times with 5ml of 10mM Tris-HCl buffer on 100K Amicon filters. To determine the number of dyes per NP, the exact same concentration of bare NPs and NP conjugates was prepared by adjusting their absorbance at 425nm to 0.400. The molar concentration and extinction coefficient of bare NPs can be found on Ocean Nanotech's website. Then the difference between the absorbance of the conjugate at 695 or 795nm (depending if Cy5.5 or IR800 was used) with the absorbance of the bare NP, was divided by the dye's extinction coefficient (2.0×10^5 and 2.4×10^5 $M^{-1}cm^{-1}$ for Cy5.5 and IR800, respectively).

Determination of quenching efficiency

Conjugate were dissolved at 0.1uM in 10mM TRIS-HCl buffer pH7 to measure the absorbance and emission spectrum of each conjugate using the UV-Vis spectrophotometer DU-530 from Beckman and the fluorescence spectrometer Lumina from ThermoScientific equipped with a photomultiplier tube (PMT). The emission of dye-peptide-quencher conjugates (F) was compare to the emission of the dye-peptide conjugate (F_0) to calculate the quenching efficiency (Q), according to $Q\ (\%) = (F_0\text{-}F) / F_0 \times 100$.

Cleavage by MMP9

Fluorescence of the conjugate and labeled peptide (unquenched) was measured at t_0. 12nM of active MMP9 (Calbiochem) or buffer (negative control) were added to the conjugate (0.1uM of dye) in 50mM Tris-HCl, 100mM NaCl, 10mM CaCl2, 20uM ZnCl2 and 0.007% Brij-35, pH 7.8 and incubated at 37°C on a rotator. Fluorescence was measured next day (F_t). Fluorescence levels measured on day 0 and day 1 were normalized according to the reference (unquenched labeled peptide). The signal activation (fold) was calculated by dividing the fluorescence of the conjugate incubated with MMP9 by the fluorescence of the conjugate incubated without the enzyme (buffer only).

RESULTS

The wavelengths of the measured maximal absorbance and emission of the various dye-quencher conjugates are shown in Table 1. Surprisingly, the maximal absorbance of free BHQ3 was found to be at 625nm instead of the advertised 672nm, thus not suitable for Cy5.5 which showed an emission peak at, 695nm. Cy5, however, had peak of emission at 668nm, which is closer to BHQ3's maximal absorbance (Figure1). The maximal absorbance of QC1 was, as advertised, around 723nm, but once conjugated to a peptide, shifted to 755nm.. The maximal emission of IRdye 800 was measured at 794nm and was the closest to QC1's peak of absorbance. Figure 1 presents the relative absorbance spectrum of 1uM of BHQ3 and QC1 and the relative emission spectrum of 1uM of Cy5, Cy5.5 and IRdye800 to see their overlap. Conjugation of BHQ3 with Cy5.5 didn't change the spectrum of absorbance (both peaks were distinct), while Cy5-BHQ3 conjugate didn't show the absorbance peak at 646nm characteristic of Cy5, but only one broad peak at 625nm. Similarly, the IR800-QC1 conjugate didn't show the absorbance peak at 789nm characteristic of IR800, but only one single peak at 720nm. These blue shifting were less pronounced with longer linker (decapeptide instead of octapeptide) or when methanol was added. The relative absorbance of the quenchers at dye's emission peak (668nm for Cy5, 695nm for Cy5.5 and 794nm for IR800) are presented in table 2. Quenching efficiencies of the different dye-quencher pairs are also reported in table 2. QC1-IR800, QC1-Cy5.5 and 30nm IO NP-Cy5.5 gave the best results with 99, 98 and 96% quenching respectively. The maximal distance (unfolded) between the dye and the quencher was 3.1nm when

conjugated both on the octapeptide or 3.9nm when conjugated both on the decapeptide and an additional 38.1nm was added by the length of the PEGs when conjugated around NPs (see Pierce's website for various lengths of PEGs). The hydrodynamic radius measured by Ocean Nanotech indicated however an average of only 4nm added by the folded PEGs. BHQ3-Cy5, 20nm IO NP-Cy5.5, QC1-Cy5.5 and BHQ3-Cy5.5 also gave acceptable quenching, ranging from 89-84%. The Cy5.5-Cy5 conjugates only gave quenching of 77%. The 6nm gold NP showed 70% quenching when 7 peptide-Cy5.5 were conjugated per NP and 90% with 15 peptide-Cy5.5 around it. The signal activation by 12nM MMP9 was proportional to the initial quenching efficiency, ranging from 3 to 67 fold increases for the Cy5.5-Cy5 and QC1-IR800 conjugates, respectively. Conjugation on a decapeptide instead of an octapeptide decreased the quenching efficiency of QC1-IR800 by 2% resulting in a signal activation 15% lower.

DISCUSSION

The need of improvement in signal background and specifity in the field of in vivo imaging led to the design of 'activatable probes', which can be made of a fluorophores and a chromophores conjugated on separate ends of an enzyme substrate. The work of Kridel [17] and Prudova [18] highlighted peptide sequences susceptible to be cleaved by MMPs expressed in ill tissue. In the present study, various small non-fluorescent dye analogues and 6-30nm metallic nanoparticles were chosen for their ability to absorb light between the 650 and 850nm, which is the optimal optical window for in vivo imaging. Cy5, Cy5.5 or IRdye800-labeled MMPs substrate were conjugated with BHQ3, IRdye QC1, 6nm Gold NPs and 20 or 30nm Iron Oxide NPs to quench their fluorescence. Non-fluorescent dye analogues (dark quenchers) were developed to improve the poor quenching obtained with the combination of two fluorescent dyes in spite of an excellent spectral overlap allowing efficient FRET. As example, TAMRA was found to absorb FAM emission, but its emission was leaking into the FAM's channel[5]. The group of Weissleder also developed a self-quenching probe, by conjugating approximately 13 labeled peptide on a 450kDa polylysine PEGylated backbone [19]. They reported an activation of 8.5-fold upon cleavage by MMP2 in vitro and 2.4 fold in vivo. In the present study, Cy5.5 apparently quenched Cy5 emission by only 77% and signal could be activated by only 2.9 fold with MMP9 in vitro. The combination of a dye and an appropriate dark quencher has the potential to perform better. However, the quenching of Cy5.5 by the black hole quencher BHQ3 was below expectation. Given that the experimental peak of absorbance of BHQ3 was measured at 625nm instead of 672nm, the suboptimal spectral overlap between Cy5.5 emission (695nm) and BHQ3's

absorbance could easily explain the moderate quenching. Accordingly, Cy5, which had an emission peak at 668nm, showed a better spectral overlap and was consequently quenched more efficiently by BHQ3. The shift of the absorbance spectrum suggests some static interactions. Pi-bonding (also called H-aggregation) between the hydrophobic rings of the cyanine dyes and BHQ3 is supposed to be decreased by the multiple negatively charged sulfonic acids added to the cyanine dye, but the absence of charges around BHQ3 could allow it, particularly when the dye's sulfonate groups are neutralized by positive ions, like Na+, found in phosphate buffers. The use of unsulfonated cyanine dyes could have increased the static interactions with BHQ3 and decreased their distance, resulting in a better quenching, but the aqueous solubility of the conjugate would have been compromised. QSY21 is another example of dark quencher with an optimal absorbance wavelength for Cy5's emission, which benefit from H-aggregation. Authors reported quenching of 96-99% depending on the level of binding[20]. Consequently, the activation could differ by 8-fold. They also noted that the quenching enhancement was not reliable in vivo given the amount of proteins in blood able to compete with the binding. In that study, the signal of the Cy5-QSY21 conjugate actually increased by 2-fold immediately after addition of plasma. Static interactions are also very temperature dependant and expected to decrease at 37°C compared to R.T. In spite of the relatively good quenching of Cy5 by BHQ3 or QSY21, their use is limited in vivo, because …… . Indeed, Cy5 emission can be partially scattered by the tissue and the tissue autofluorescence is also higher at shorter wavelenghts and therefore not optimal for in vivo optical imaging. Accordingly, the IRDye 800CW, which has an emission peak around 800nm showed reduced background from autofluorescence [21].

BHQ3 has been reported to efficiently quench IR800 despite the absence of spectral overlap, but this was at a distance of only 5 amino acids. In addition this study reported instability of BHQ3 in vivo related to its azo bond [22].

IRdye-QC1 is a dark quencher with a predicted broad absorbance around 723nm. However, when conjugated to a peptide, its maximum red shifted to 755nm. Thus, the spectral overlap of QC1 with the emission peak of IRdye800 (794nm) was superior to the overlap with Cy5.5's emission (695nm) (figure 2). The spectrum of IR800 and Cy5.5 also changed after conjugation with QC1, which could be indicative of static interactions occurring between the dye and the quencher, given their structural similarity, although the fact that they are both sulfonated decreases the probability. Increasing the distance by 8 Å or addition of methanol seemed to decrease the blue shifting. The impact on quenching efficiency and signal activation was significant for Cy5.5-QC1 conjugate but neglectable in the case of IR800-QC1 conjugate. However, the pH was found to be very important in both case, because QC1 can be protonated and become fluorescent at acidic pH[16]. The TFA used during purification by HPLC is volatile but may not have been removed completely by one lyophilization step. Thus, it is recommended to add a small volume of water and perform a second lyophilization to ensure complete TFA removal. The addition of a neutral buffer will also prevent any intrinsic fluorescence of QC1 in vitro. It also raises the question if internalization of QC1 conjugates in lysosomes could increase the background. The possibility to improve the quenching by decreasing the distance was also investigated. Calculation from the data published by LiCOR on their caspase3 activatable probes indicated that the quenching decreased to the power 5.2 of the distance[16] (theoretical value = 6). To preserve substrate affinity

and specificity for its enzyme however, it is recommended to design a peptide of at least 4 specific amino acids on each side of the cleavage site[23], which represent a minimum distance of 32Å.

The small size of these activatable probes has the advantage of allowing a better cell penetration. However, the charges added to the dyes and QC1 quencher, to increase their aqueous solubility, could actually decrease their passive transport across the hydrophobic cell membranes and hast their clearance. Consequently, they would probably benefit from being conjugated to an antibody to target them in the tissue of interest and extend their half-life.

Small gold nanoparticles (6nm) were also used because of its huge and broad absorbance and the capacity to bear multiple labeled peptides and antibodies. The weak quenching efficiency observed with the near infrared dye can be explained by the fact that its maximal absorbance is around 520nm and decreases to only 4% at 695nm. Second, the difference between the theoretical length of the PEG_{5000} chains (stretched) and the average length, suggested by the hydrodynamic size measured by the company, makes it difficult to determine the real distance between the NP and the dye, but it is nevertheless the most important factor, since the transfer of energy by plasmon resonance decreases by $1/R^4$. One advantage of NP is the possibility of conjugating multiple labeled peptides around it. Our results show that twice more labeled peptides conjugated around the 6nm gold NPs resulted in a better quenching, suggesting the occurrence of self-quenching when numerous dyes are in proximity. Note, however, that heavily conjugated NPs quickly precipitate in aqueous solution. Iron oxide NP of 20-30nm were initially developed for magnetic resonance imaging (MRI). Even if IO

NPs absorb 35 times less than gold NPs, the fact that the absorbance increases by a power of 3.5 its size, results in a 2 to 7.5-fold increase of absorbance when comparing a 20 or 30nm IO NP to a 6nm gold NP (calculated from data on Ocean Nanotech website). The increase in size also extends the reach of plasmon resonance, which solves the problem of distance added by the PEG chains. Accordingly, the quenching efficiency of Cy5.5 by IO NPs was proportional to its size.

The signal activation by MMP9 was found to by proportional to the quenching efficiencies, with the exception made for the biggest NPs. It is possible that the number of labeled peptides around the NPs has to be optimized to avoid steric hindrance of the enzyme. Nevertheless, nanoparticles offer great flexibility. Their hydrophobicity can be tuned by modifying the composition of the coating. Some functions can also be used to conjugate targeting antibodies. Given the potential of bi-modal imaging (optical + MRI), 30nm IO NPs could find great applications in clinic. Multiple substrates could also be conjugated to different dyes and be detected simultaneously with adequate filters mounted in an optical camera, providing a comprehensive profile of the disease.

TABLES AND LEGENDS

Dye	Excitation	Emission
	nm	nm
Cy5	646	668
Cy5.5	674	695
IR800	774	794
BHQ3	625	-
QC1	755	-
Gold NP	520	-

Table 1: Observed λ_{max} of excitation and emission for the dyes and quenchers studied (indicative of best couples for FRET).

Quencher/Dye pairs	Core size	Distance	Quencher's Ext coeff.*	number of dyes	Quenching	Activation by MMP9
	nm	nm	($M^{-1}cm^{-1}$)	per quencher	%	fold increase
Au NP---Cy5.5	6	7.2	8.0E+05	7	78	n.d.
				15	90	6.7
IO NP---Cy5.5	20		9.7E+05	3	79	3.0
	30		3.9E+06	109	96	5.4
Cy5.5---Cy5	-	3.2	2.0E+05	1	77	2.9
BHQ3---Cy5.5			2.0E+04		84	4.9
BHQ3---Cy5			3.5E+04		89	7.4
QC1---Cy5.5		4.0	7.0E+04		87	n.d.
		3.2			98	53.2
QC1---IR800		4.0	7.5E+04		97	57.3
		3.2			99	67.3

Table 2: **Quenching efficiencies of various dyes in function of quencher's extinction coefficient (*at dye's emission wavelength) and distance from the dye.** Core size of NPs was determined by TEM performed by Ocean Nanotech. A theoretical distance of 0.4 nm per amino acid was estimated for the peptides. The hydrodynamic sizes measured by the company using dynamic light scattering, indicate an additional distance of 4nm for the PEG_{5000} chains surrounding the NPs, although much longer theoretical distance would have been calculated for straight PEGs. The absorbance of

0.01-1µM of BHQ3, QC1, Gold NP and IO NP was measured at 668, 695 and 794nm to calculate the molar extinction coefficient applicable for FRET. To determine the number of dyes per NP, the exact same concentration of bare NPs and NP conjugates was prepared by adjusting their absorbance at 425nm to 0.400. The molar concentration and extinction coefficient of bare NPs can be found on Ocean Nanotech's website. Then the difference between the absorbance of the conjugate at 695 or 795nm (depending if Cy5.5 or IR800 was used) with the absorbance of the bare NP, was divided by the dye's extinction coefficient ($2.0x10^5$ and $2.4x10^5$ $M^{-1}cm^{-1}$ for Cy5.5 and IR800 respectively). The molar fluorescence coefficient of labeled peptide (F_0) and quenched conjugate (F) were measured and quenching efficiencies (Q) were determined by $Q = 100 (F_0 - F) / F_0$. Conjugates (0.1uM of dye) were incubated O.N. with 0 or 12nM of active MMP9 (Calbiochem) in 50mM Tris-HCl, 100mM NaCl, 10mM CaCl2, 20µM ZnCl2, 0.007%Brij35, pH 7.8 at 37°C on a rotator for cleavage and signal activation. The unquenched labeled peptide was used as reference to normalize fluorescence levels measured at 0 and 24h.

FIGURES

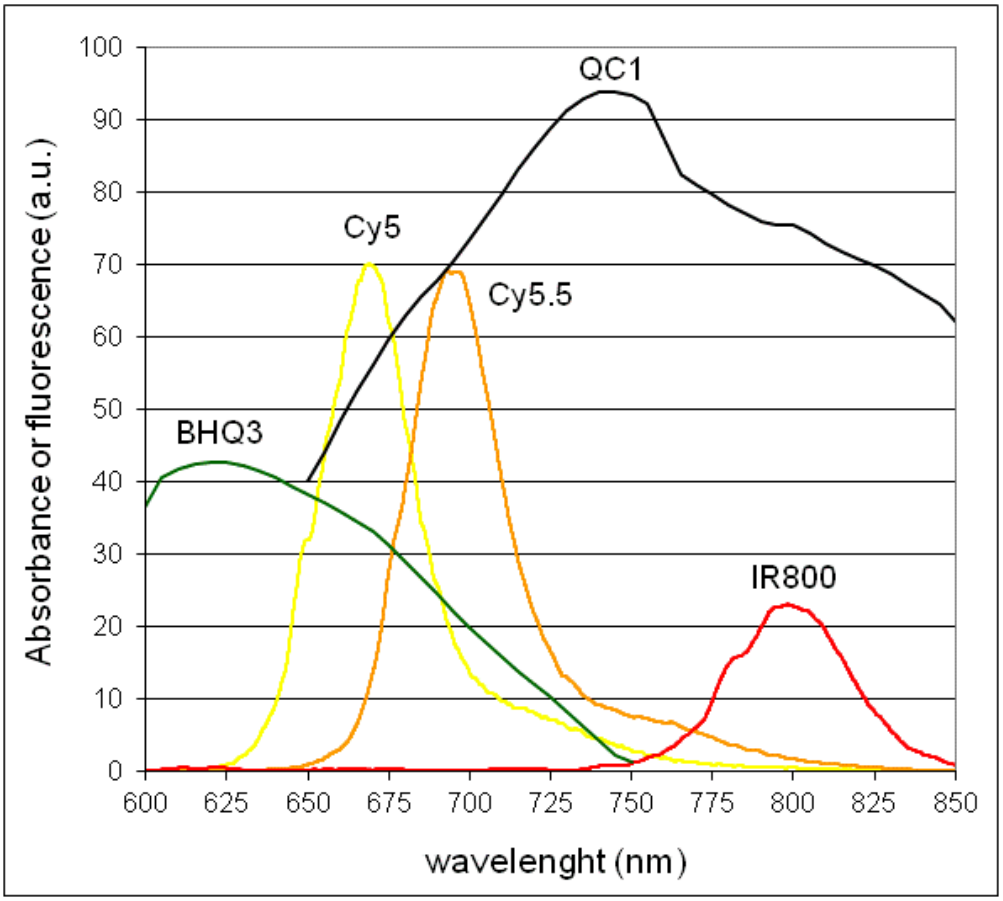

Figure 1: Spectral Overlap between Emission of Cy5, Cy5.5 and IR800 dyes and Absorbance of BHQ3 and QC1 quenchers. Cy5, Cy5.5 and IR800 (1uM) were excited at 646, 674 or 774 ± 5nm respectively and their emission intensities were scanned with a fluorescence spectrometer mounted with a photomultiplier tube (PMT). UV-Vis absorbance of 1uM of BHQ3 and QC1 was scanned with a spectrophotometer. QC1's extinction coefficient is twice BHQ3's. The spectrum of absorbance of BHQ3 (green) overlaps more with Cy5 emission curve (yellow), while QC1 absorbance (black) cover more Cy5.5 (orange) or even more IR800 emission curve (red).

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