

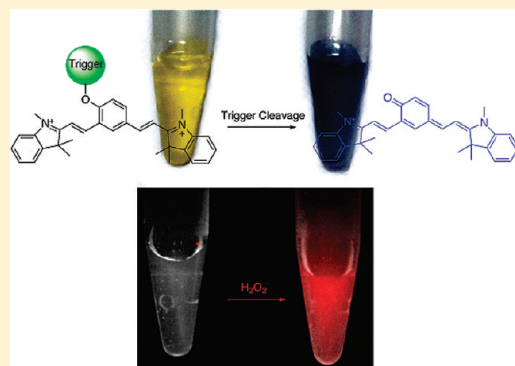
# A Unique Paradigm for a Turn-ON Near-Infrared Cyanine-Based Probe: Noninvasive Intravital Optical Imaging of Hydrogen Peroxide

Naama Karton-Lifshin,<sup>†</sup> Ehud Segal,<sup>‡</sup> Liora Omer,<sup>‡</sup> Moshe Portnoy,<sup>†</sup> Ronit Satchi-Fainaro,<sup>\*,‡</sup> and Doron Shabat<sup>\*,†</sup>

<sup>†</sup>School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences and <sup>‡</sup>Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

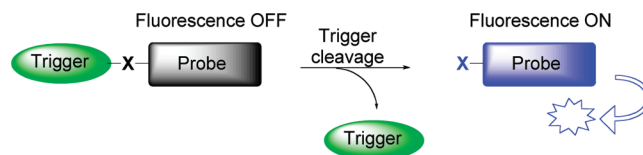
## S Supporting Information

**ABSTRACT:** The development of highly sensitive fluorescent probes in combination with innovative optical techniques is a promising strategy for intravital noninvasive quantitative imaging. Cyanine fluorochromes belong to a superfamily of dyes that have attracted substantial attention in probe design for molecular imaging. We have developed a novel paradigm to introduce a Turn-ON mechanism in cyanine molecules, based on a distinctive change in their  $\pi$ -electrons system. Our new cyanine fluorochrome is synthesized through a simple two-step procedure and has an unprecedented high fluorescence quantum yield of 16% and large extinction coefficient of  $52\,000\text{ M}^{-1}\text{cm}^{-1}$ . The synthetic strategy allows one to prepare probes for various analytes by introducing a specific triggering group on the probe molecule. The probe was equipped with a corresponding trigger and demonstrated efficient imaging of endogenous hydrogen peroxide, produced in an acute lipopolysaccharide-induced inflammation model in mice. This approach provides, for the first time, an available methodology to prepare modular molecular Turn-ON probes that can release an active cyanine fluorophore upon reaction with specific analyte.



## INTRODUCTION

Optical imaging in the near-infrared (NIR) range enables detection of molecular activity *in vivo* due to high penetration of NIR photons through organic tissues and low autofluorescence background.<sup>1–5</sup> Cyanine dyes are widely employed as fluorescence labels for NIR imaging, since they are compounds with large extinction coefficient and relatively high quantum yield.<sup>6</sup> These qualities allow their fluorescence to penetrate deep tissues, and thus, cyanines serve as attractive probes for intravital noninvasive imaging. To generate a Turn-ON system for a cyanine molecule, a fluorescence resonance energy transfer<sup>7</sup> (FRET) approach is usually applied.<sup>8–12</sup> To incorporate a cyanine molecule in a FRET-based probe, an additional dye must be included in the molecular system. An alternative simpler approach, to turn ON a fluorophore, could be achieved by applying changes in the pull-push conjugated  $\pi$ -electron system of the dye. Such concept was mostly demonstrated for dyes with UV–vis fluorescence.<sup>13,14</sup> Implementing reversible changes in the  $\pi$ -electron system of cyanine molecules is typically more complicated due to an absence of a conjugated functional group. Here, we report a novel simple strategy to introduce a Turn-ON mechanism in molecules with cyanine spectroscopic characteristics. The approach is based on a new design of a distinctive fluorochrome, which can be modularly derivatized to generate different imaging probes for biological research and clinical needs. We have applied our fluorochrome in a



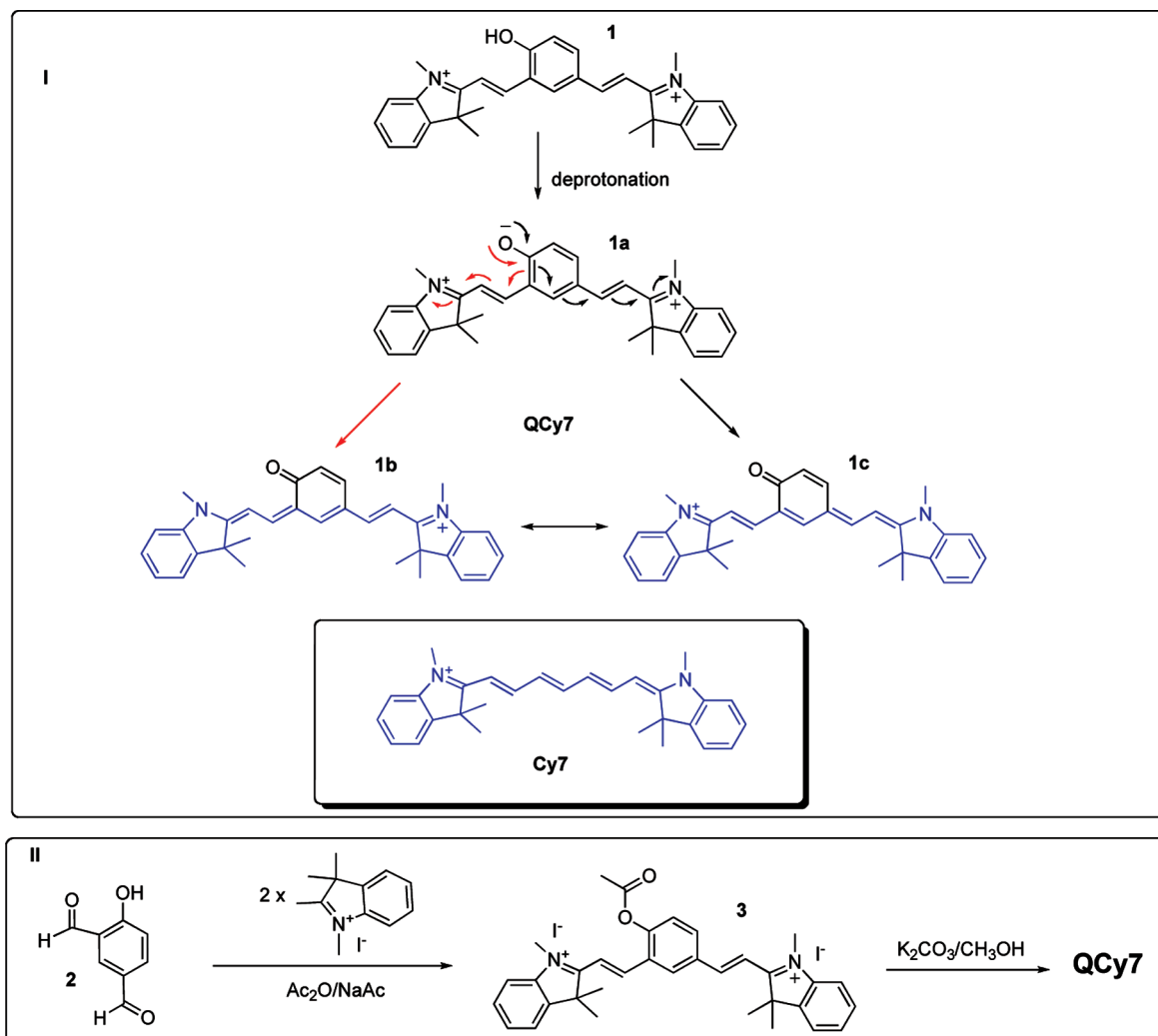
**Figure 1.** Classic mechanism for a modular Turn-ON fluorescence probe.

specific Turn-ON probe to demonstrate noninvasive *in vivo* imaging of endogenously produced hydrogen peroxide.

A classic modular Turn-ON fluorescence probe is constructed of a dye molecule with a conjugated functional group (X) linked to a trigger moiety (Figure 1). The masking of the functional group reduces the conjugated  $\pi$ -electron system of the dye and thus turns OFF the fluorescence of the probe. Removal of the trigger renews the conjugation of the functional group with the  $\pi$ -electrons of the dye and thereby turns ON the fluorescent signal of the probe.

Applying this Turn-ON mechanism in cyanine dyes is more difficult than for other common fluorescent dyes, such as coumarin and fluorescein, since cyanine molecules have a polymethine  $\pi$ -electron system in between two nitrogen atoms that cannot be masked by a trigger. To generate such a Turn-ON mechanism in cyanine molecules, a new approach should be considered.

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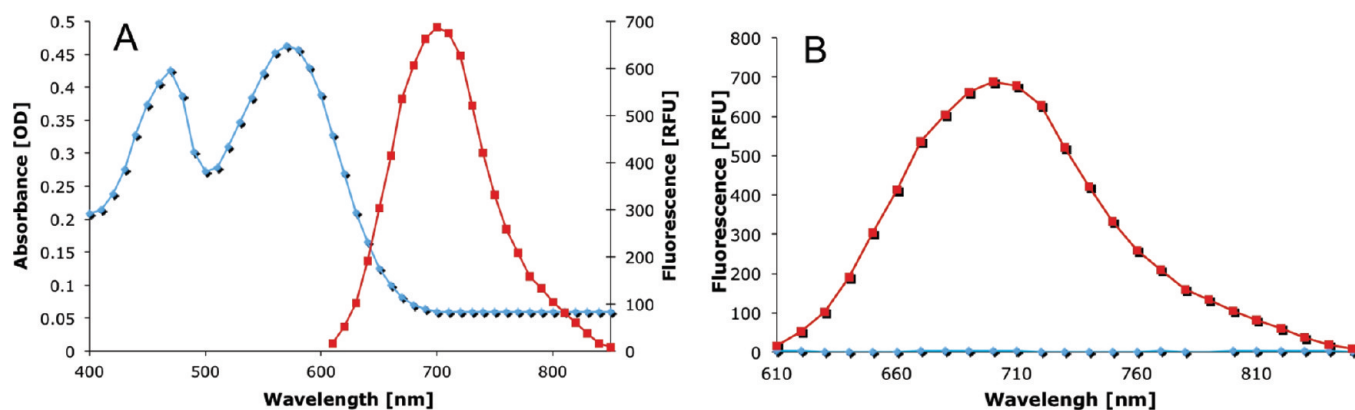
**Figure 2.** (I) Design of a cyanine-based Turn-ON probe with NIR fluorescence. (II) Synthesis of QCy7.

## RESULTS AND DISCUSSION

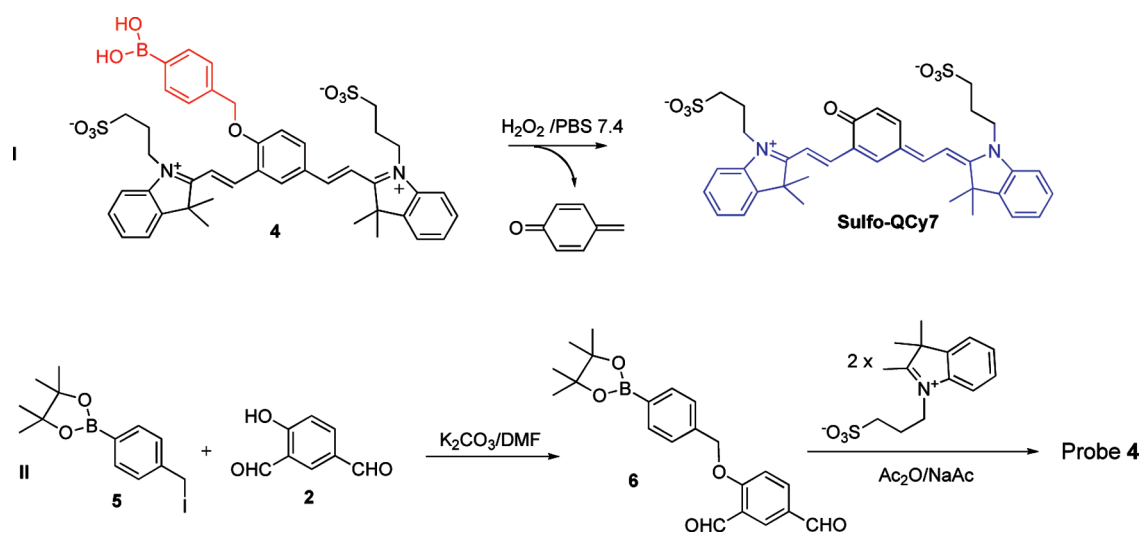
Our probe's design is based on a new Cy7-like molecule, with a conjugated functional group that can be masked, as presented in Figure 2. Cy7 is a known heptamethine cyanine dye, which is widely used for NIR imaging.<sup>15–17</sup> The conjugated  $\pi$ -electron system of Cy7 has a positive charge that is delocalized between the two nitrogen atoms. Unlike Cy7, in phenol **1**, the nitrogen atoms have two positive charges, (one on each) and thus, the conjugation pattern is significantly different. Deprotonation of phenol **1** results in generation of phenolate **1a**. The negative charge of the phenolate can be delocalized toward one of the nitrogen atoms to form quinones **1b** or **1c** and thereby eliminating one positive charge. The obtained quinone derivative **QCy7** (the resonance hybrid of species **1a**, **1b** and **1c**) has now similar conjugation pattern to that of Cy7 and thus is expected to emit NIR fluorescence. The incorporation of various protecting groups to phenol **1** should allow masking of the NIR fluorescence of **QCy7** by a specific trigger moiety by which, upon its removal, the free fluorophore will be obtained. **QCy7** was synthesized by a simple two-step procedure as presented in Figure 2. Commercially available dialdehyde **2** was condensed with 2 equiv. of indolium-iodide to give ester **3**. The acetate group of **3** was removed with potassium carbonate in methanol to afford **QCy7**.

The dye **QCy7** was obtained as an orange crystalline powder. The UV–vis spectrum exhibits two absorption peaks with maximum at 460 and 570 nm (Figure 3A). As expected, the fluorescence spectrum indeed shows emission peak in the NIR region at a wavelength of 715 nm. The obtained large Stokes shift between the excitation and the emission wavelengths of about 150 nm is a desirable feature for fluorescence probes, which assists in increasing the signal-to-noise ratio.<sup>18,19</sup> Next, we measured the fluorescence spectrum of acetate **3** in comparison with that of **QCy7**. Since acetate **3** is a masked derivative of **QCy7**, it can be used to model the fluorescence activity of a probe with a specific trigger. As predicted, the acetate derivative of **QCy7** did not show any fluorescence emission in the NIR region (Figure 3B, blue plot).

With these results in hand, we turn to develop a specific diagnostic probe based on our NIR fluorophore. It is well-known that inflammatory events lead to oxidative stress and overproduction of hydrogen peroxide, which can damage living tissues.<sup>5,20</sup> Thus, the development of optical probes that can image hydrogen peroxide *in vivo* is a major challenge in the field of molecular imaging. There are numerous reports describing the development and evaluation of such imaging probes.<sup>21–24</sup> These are commonly based on boronic acid/ester as a specific masking group<sup>25–28</sup> that



**Figure 3.** (A) Absorption and fluorescence spectra of QCy7 in PBS 7.4 [100  $\mu$ M]. (B) NIR fluorescence spectra of QCy7 (red) in comparison to that of Acetate 3 (blue) in PBS 7.4 [100  $\mu$ M] ( $\lambda_{\text{exc}}$  = 560 nm).



**Figure 4.** (I) Activation of probe 4 by hydrogen peroxide releases sulfo-QCy7. (II) Synthesis of probe 4.

can be removed upon reaction with hydrogen peroxide. Accordingly, we designed molecular probe 4, which has phenylboronic acid attached through an ether-linkage to a sulfonated derivative of QCy7 (Figure 4). The purpose of sulfonated alkyl-chains is merely to increase the aqueous solubility and to prevent aggregation of the relatively hydrophobic QCy7 molecules. Incubation of hydrogen peroxide with probe 4 under physiological conditions should result in oxidation of the phenylboronic acid, which is followed by hydrolysis and 1,6-elimination of *p*-quinone-methide to release the active fluorophore sulfo-QCy7.

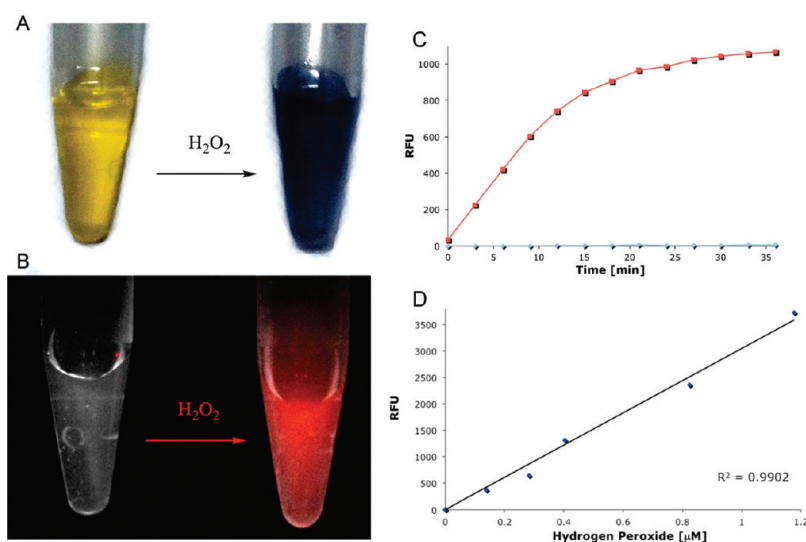
Probe 4 was synthesized as shown in Figure 4. Phenol 2 was alkylated with previously synthesized<sup>27</sup> benzyl-iodide 5 to generate ether 6. The aldehydes groups of 6 were condensed with indolium-propane-sulfonate to give probe 4. Sulfo-QCy7 was synthesized similarly as described for QCy7 (Figure 2) by using indolium-3-propyl-sulfonate instead of indolium-iodide.

Sulfo-QCy7 presented similar spectroscopic behavior to that of QCy7 with NIR fluorescence maxima at wavelength of 720 nm (see Supporting Information). Figure 5A shows the typical cyan color for solution of sulfo-QCy7 obtained in PBS 7.4. Expectedly, due to the 150 nm blue Stokes shift, the masked form of sulfo-QCy7, probe 4, had a yellow color in aqueous solution. When imaged using NIR imaging camera, the solution of probe 4 is

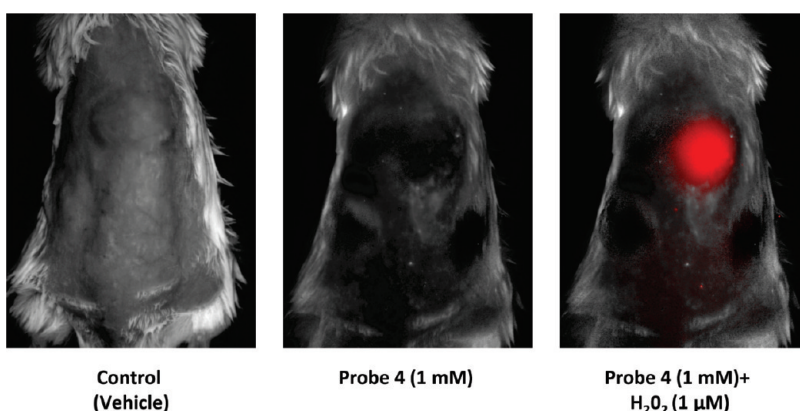
clearly transparent while the solution of sulfo-QCy7 exhibits a well-observed NIR fluorescence (Figure 5B). The fluorescence quantum yield (16%) and the extinction coefficient (52 000  $\text{mol}^{-1} \text{cm}^{-1}$ ) of sulfo-QCy7 were measured as high values that are adequate for *in vivo* measurements. The phenol's  $\text{pK}_a$  value of sulfo-QCy7 (4.5) indicates the possibility of gaining NIR fluorescence also under relatively acidic conditions (see Supporting Information).

To evaluate the NIR fluorescence obtained by probe 4 upon reaction with hydrogen peroxide under physiological conditions, the probe was incubated in PBS 7.4 at 37  $^{\circ}\text{C}$ , in the presence or absence of hydrogen peroxide, and the emitted NIR fluorescence was measured by a spectrofluorometer. The results, presented in Figure 5C, indicate full conversion of probe 4 to sulfo-QCy7 within 30 min (red plot). No NIR fluorescence is observed in the absence of hydrogen peroxide (blue plot). The sensitivity of probe 4 toward detection of hydrogen peroxide was then evaluated. Figure 5D shows that the probe can straightforwardly detect hydrogen peroxide concentrations below 1  $\mu\text{M}$ .

Since the overproduction of hydrogen peroxide *in vivo* is concerned with the development of numerous inflammatory diseases, we wanted to investigate the ability of our Turn-ON NIR-probe to image endogenously produced hydrogen peroxide



**Figure 5.** Photograph of probe 4 and sulfo-QCy7 in PBS 7.4 [ $50 \mu M$ ] solutions. (B) Photograph of probe 4 and sulfo-QCy7 in PBS 7.4 [ $50 \mu M$ ] solutions by NIR camera (excitation at wavelength 595 nm, cutting filter of  $>635$  nm). (C) NIR fluorescence ( $\lambda_{Ex} = 590$  nm,  $\lambda_{Em} = 720$  nm) emitted upon incubation of probe 4 [ $50 \mu M$ ] in the presence (red) or absence (blue) of hydrogen peroxide [ $50 \mu M$ ] in PBS 7.4 at  $37^\circ C$ . (D) NIR fluorescence emitted of probe 4 [ $100 \mu M$ ] upon incubation with the indicated concentrations of hydrogen peroxide.

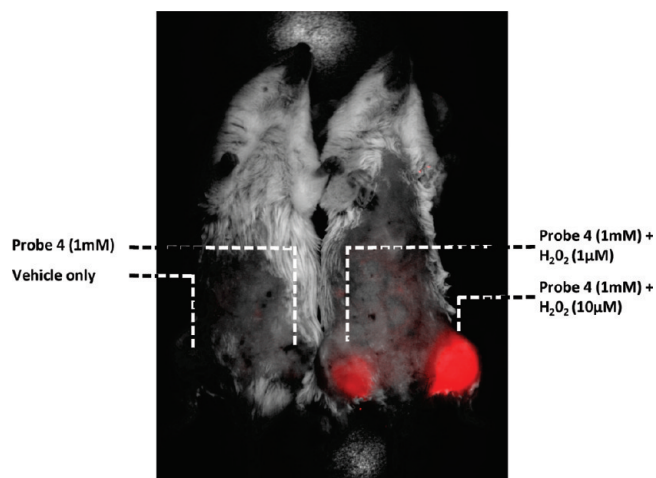


**Figure 6.** *In vivo* imaging of exogenous hydrogen peroxide by probe 4. CRI Maestro image. Excitation at 595 nm, emission cutoff filter of 635 nm.

in a mouse model. Initially, as a control experiment, we injected a premixed solution of probe 4 with hydrogen peroxide, into the peritoneal cavity, and imaged the mice using the CRI Maestro noninvasive fluorescence imaging system. Figure 6 clearly shows that exogenously activated probe 4 can generate NIR fluorescence *in vivo* image. Mice treated only with the probe or with the buffer did not show detectable NIR fluorescence at similar imaging conditions.

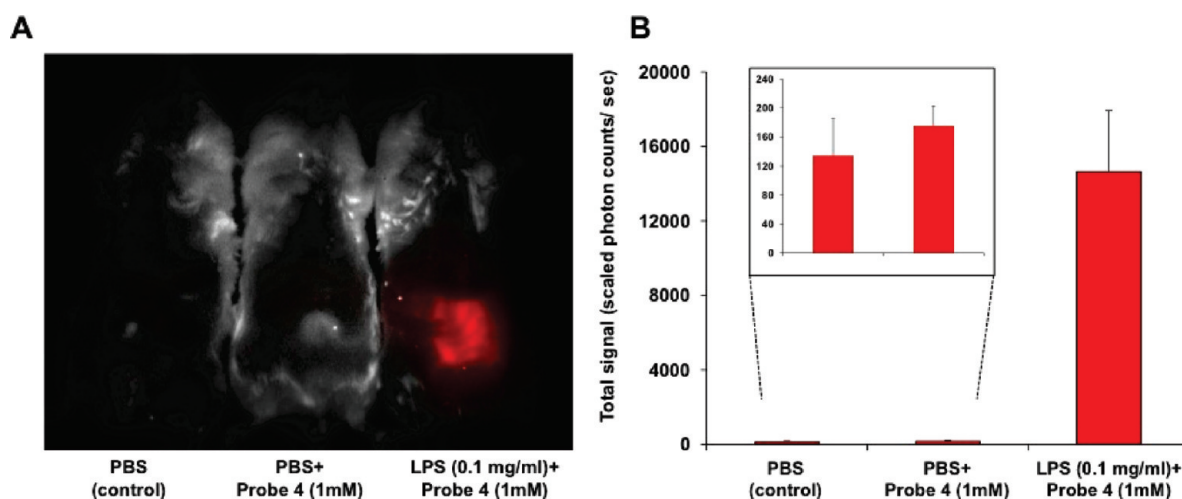
Next, we wanted to test whether probe 4 can be used to differentiate *in vivo* various concentrations of hydrogen peroxide. Therefore, two different concentrations of hydrogen peroxide were premixed with probe 4; immediately injected intramuscularly, into the leg; and then imaged by the CRI Maestro noninvasive fluorescence imaging system. Figure 7 shows that the intensity of the NIR emitted fluorescence from probe 4 was indeed dependent on the hydrogen peroxide initial concentration.

Finally, we tested the ability of our probe to visualize endogenously produced hydrogen peroxide using noninvasive imaging technique. We have used a previously described model of acute inflammation in mice that can be induced by lipopolysaccharide



**Figure 7.** Probe 4-fluorescence dependency on hydrogen peroxide concentration. CRI Maestro image. Excitation at 595 nm, emission cutoff filter of 635 nm.



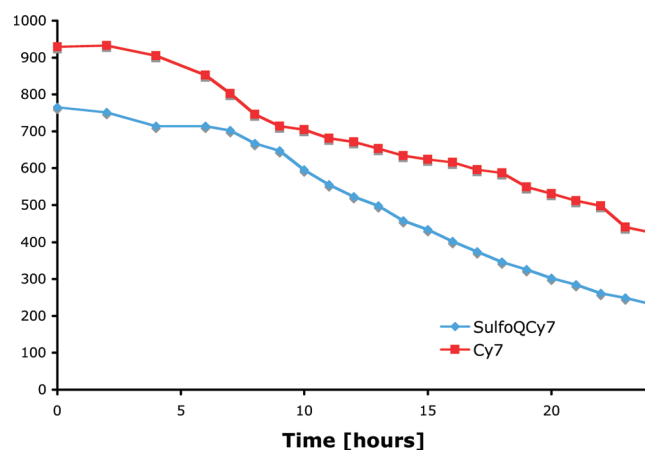


**Figure 8.** *In vivo* imaging of endogenous hydrogen peroxide in the peritoneal cavity of mice during an LPS-induced inflammatory response, using probe 4. (A) CRI Maestro image. Excitation at 595 nm, emission cutoff filter of 635 nm. Right mouse: 1 mL of 0.1 mg/mL LPS was injected into the peritoneal cavity of mice, followed 6 h later by an ip injection of probe 4. Middle mouse: 1 mL PBS was injected into the peritoneal cavity of mice, followed 6 h later by an ip injection of probe 4. Left mouse: negative control (PBS only). (B) Quantification of the NIR-fluorescence emission intensity from the mice injected with probe 4 in the presence or absence of LPS-induced inflammation (i.e.,  $\text{H}_2\text{O}_2$ ). The total number of photons from the entire peritoneal cavity was integrated and plotted compared with the control group ( $n = 5$ ). The inset focuses on the NIR-fluorescence emission intensity obtained from injections of PBS (control) or probe 4 in absence of LPS-induced inflammation.

(LPS).<sup>20</sup> Mice were injected intraperitoneally (ip) with LPS (1 mL of 0.1 mg/mL), followed 6 h later by an additional ip injection of 400  $\mu\text{L}$  probe 4 (1 mM), and thereafter imaged by CRI Maestro noninvasive fluorescence imaging system. Figure 8 shows that LPS-treated mice injected with probe 4 generated a significantly greater intensity of NIR fluorescence signal compared with non-LPS treated mice injected with probe 4 or PBS. The slight NIR fluorescence observed in mice treated only with probe 4 is attributed to basal levels of hydrogen peroxide produced in living animals (Figure 8, inset).<sup>24</sup> The signal-to-noise ratio of the NIR fluorescence intensity observed by the hydrogen peroxide imaging probe in mice was about 10-fold higher compared with the control group. Such ratio should be adequate to obtain strong contrast image. As far as we know, this is the highest ratio obtained of a Turn-ON probe ever observed for an *in vivo* hydrogen peroxide imaging probe.

To assess the photo and chemical stability of sulfo-QCy7 under physiological conditions, its fluorescence decrease over time was compared with that of the known dye Cy7. Both dyes were incubated in PBS 7.4 at 37  $^{\circ}\text{C}$  and their NIR fluorescence was monitored with spectrofluorometer. Figure 9 shows that sulfo-QCy7 loses fluorescence in a similar rate to that of Cy7. It should be noted that in the first few hours there is hardly any decrease in the fluorescence intensity. Thus, in the time-scale of the described *in vivo* imaging evaluations, sulfo-QCy7 exhibits good fluorescence stability.

The boronate triggering group is efficiently removed upon reaction with hydrogen peroxide and therefore can be introduced to mask various fluorophores. That approach has been comprehensively demonstrated by the Chang group in probes designed for detection and imaging of hydrogen peroxide.<sup>21</sup> They have also shown that the boronate-based probes are highly selective to hydrogen peroxide among several other reactive oxygen species. To demonstrate our probe's capability to image biological processes *in vivo*, we chose hydrogen peroxide as the analyte of interest. In this regard, two elegant examples of chemiluminescent probes for imaging hydrogen peroxide in mice were recently



**Figure 9.** NIR fluorescence emitted upon incubation of sulfo-QCy7 and Cy7 [50  $\mu\text{M}$ ] in PBS 7.4 at 37  $^{\circ}\text{C}$  vs time. Sulfo-QCy7:  $\lambda_{\text{Ex}} = 590$  nm,  $\lambda_{\text{Em}} = 720$  nm. Cy7:  $\lambda_{\text{Ex}} = 700$  nm,  $\lambda_{\text{Em}} = 780$  nm.

reported.<sup>20,24</sup> These probes exhibited high selectivity and sensitivity and were effective for imaging hydrogen peroxide produced by the inflammatory response to lipopolysaccharide or by testosterone-mediated elevation of cell-proliferation in tumor xenografts.

As previously indicated, to develop methods that allow direct and reliable assessment of analytes *in vivo*, a water-soluble and NIR fluorescence-based probe having emission maxima between 650 and 900 nm is preferred. Previous efforts to design such Turn-ON probes typically had limitations of low fluorescence quantum yield, low extinction coefficient and complex synthesis.<sup>29,30</sup> Because of their superior spectroscopic characteristics, cyanine dyes have received immense attention and can be considered as the main class of NIR fluorescent probes for biological applications. Therefore, there is an obvious need to develop effective Turn-ON probes, which are based on these dyes. Our new cyanine fluorochrome is synthesized through a simple two-step procedure and has a relative

high fluorescence quantum yield and large extinction coefficient. The probe's design allows formation of a cyanine dye through  $\pi$ -electrons relocation, upon removal of trigger by the appropriate analyte. While most of the cyanine Turn-ON probes are based on the FRET technique, there are only limited specific examples for cyanine probes that involve changes with the  $\pi$ -electrons conjugation.<sup>31,32</sup> We have presented a modular approach for cyanine-based probe, which suitably can be adjusted for imaging of other factors such as enzymes, by introducing a specific substrate as the trigger.

## CONCLUSIONS

In summary, we have developed a new paradigm for generation of novel class of Turn-ON NIR cyanine-based probes. For the first time, there is an available methodology to prepare modular molecular probes that can release an active cyanine fluorophore upon reaction with specific analyte. The probe is based on a new fluorochrome (QCy7), which is obtained upon removal of a trigger moiety by the analyte of interest. A distinctive change of  $\pi$ -electrons system leads to generation of a cyanine dye with strong NIR fluorescence. The probe was demonstrated to efficiently image endogenous hydrogen peroxide produced in an acute inflammation model in mice. The synthesis of the QCy7 is very simple and can be accustomed to prepare various probes for detection and imaging of other analytes or enzymes. We envision that QCy7-based probes will be commonly used as effective research tools for noninvasive imaging for a range of important biological factors in animals and, in the future, in humans.

## ASSOCIATED CONTENT

**S Supporting Information.** Full experimental details, characterization data of all new compounds, spectroscopic assay conditions and *in vivo* experimental conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Corresponding Authors

\*ronitsf@post.tau.ac.il; chdoron@post.tau.ac.il.

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