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# Squalene-based oxygen-scavenging system for enhancing fluorescent dye stability for biosensing applications

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Photobleaching impedes the application of fluorescent dyes in long-term imaging. While enzymatic oxygen scavenging enhances photostability, it induces progressive acidification of the solution. Here, we report a new oxygen-scavenging system comprising squalene (SQ) and Trolox that improves dye stability without perturbing pH. DPPH and ABTS radical cation scavenging assays verified the antioxidant activity of SQ, with 0.7–1% concentrations achieving 35% radical scavenging capacity. The system maintains pH stability for more than 18 min, in contrast to the rapid acidification observed with glucose oxidase/catalase. Total internal reflection fluorescence (TIRF) imaging of cyanine 3 (Cy3) and cyanine 5 (Cy5) revealed that SQ with Trolox performs comparably to enzymatic systems while permitting extended illumination, establishing a pH-neutral strategy for enhanced photostability.

### KEYWORDS

oxygen-scavenging system, photostability, fluorescent imaging time, bioimaging, squalene

## 1 Introduction

In fluorescence experiments, fluorophore stability is a critical factor that directly influences the accuracy and reliability of the measurements (Rasnik et al., 2006; Aintken et al., 2008). One of the major challenges in fluorescent imaging, particularly in fluorescence resonance energy transfer (FRET) and other techniques, is the photobleaching of fluorophores, which is the irreversible loss of fluorescence due to prolonged exposure to oxygen and light. This phenomenon can significantly reduce the observation time and hinder the ability to study transient molecular processes with high temporal resolution.

The use of an oxygen-scavenging system (OSS) is one promising strategy to mitigate photobleaching and extend the lifespan of fluorophores (Basu et al., 2018). Molecular oxygen is known to contribute to the oxidative degradation of fluorophores, accelerating photobleaching. By incorporating oxygen-scavenging reagents, which rapidly consume dissolved oxygen in the solution, it is possible to reduce the rate of photobleaching, thereby increasing the lifespan of fluorescent dyes. Oxygen-scavenging systems contain

glucose-coupled glucose oxidase/catalase (GOD) and protocatechuic acid-protocatechuate-3,4-dioxygenase (PCA/PCD). These approaches have shown particular promise in improving the performance of fluorophores used in TIRF microscopy, enabling more prolonged and precise observation of the interactions and behaviors in biomolecular experiments (Aintken et al., 2008; Lemke et al., 2009).

Although the oxygen-scavenging system can lead to oxygen depletion in single-molecule detection, it also causes acidification of the solution, which damages fluorescent dyes and exacerbates unintended oxygen-based reactions (Shi et al., 2010). For example, GOD produces gluconic acid as a by-product, which leads to a continuous decrease in pH if not buffered sufficiently. Therefore, the buffer conditions change during the experiment. However, most proteins are sensitive to pH, buffer composition, temperature, and experimental conditions. Therefore, an oxygen-scavenging system that maintains a constant pH without fluctuations is of high priority.

Moreover, compatibility with biological systems must also be carefully considered. From previous research, we found that nuclease contamination is controversial when using some commercial OSS enzymes, such as PCA/PCD (Gahlon et al., 2017). It shows that enzyme preparation is frequently contaminated with nuclease that damages nucleic acid substrates (Senavirathne et al., 2015). The enzyme must subsequently be purified using advanced methods. For example, the PCD/PCA system is beneficial to those specific experiments (Senavirathne et al., 2018). An alternative system should be considered for future experiments.

As we know, GOD can remove oxygen by oxidizing glucose, but it changes the pH value of the solution in experiments. The PCA/PCD preparation is frequently contaminated with nucleases that damage nucleic acid substrates. Therefore, an efficient oxygen-scavenging system that is non-blinking, pH-stable, and contamination-free is a high priority. SQ is a triterpenoid compound that is highly susceptible to oxidation (Yao et al., 2024; Paramasivan and Mutturi, 2022; Naziti et al., 2014; Zhang et al., 2023; Zhou et al., 2016; Lou-Bonafonte et al., 2018; Warleta et al., 2010). The photophysical parameter of SQ was found to be better than the conventional OSS system, and other physicochemical properties, such as temperature, pH value, and peroxide value, have shown satisfactory results.

Here, we propose a new oxygen-scavenging system to enhance the stability of the biomolecule of interest labeled with small fluorescent dyes, that is, squalene, in combination with a triplet quencher. Squalene is an unsaturated hydrocarbon in the terpenoid family containing six isoprene double bonds. These bonds give squalene the ability to act as a free radical scavenger by donating electrons to neutralize harmful free radicals, preventing oxidative stress (Zhang et al., 2023; Lou-Bonafonte et al., 2018; Swoboda et al., 2012; Schäfer et al., 2013; Heilemann et al., 2005; Ji et al., 2022). Additionally, squalene's antioxidant activity is often enhanced when combined with other antioxidants, as it can synergize to protect cells and tissues from oxidative damage. Therefore, we propose an alternative system to remove molecular oxygen: SQ in combination with a triplet quencher, such as  $\beta$ -mercaptoethanol, Trolox, ascorbic acid, cyclooctatetraene

(COT), or 4-nitrobenzylalcohol (NBA) (Schäfer et al., 2013; Heilemann et al., 2005; Ji et al., 2022; Xu et al., 2021; Yao et al., 2024; Yu et al., 2015). Trolox (a water-soluble vitamin E analog) or BME is used to eliminate the blinking of fluorophores. We use cyanine 3 (Cy3) and cyanine 5 (Cy5) as model dyes for fluorescence imaging experiments. These experiments compare the photostability of GOC, a common oxygen scavenger, with our squalene-based system. The photostability of our system was significantly improved.

## 2 Methods

### 2.1 Sample preparation

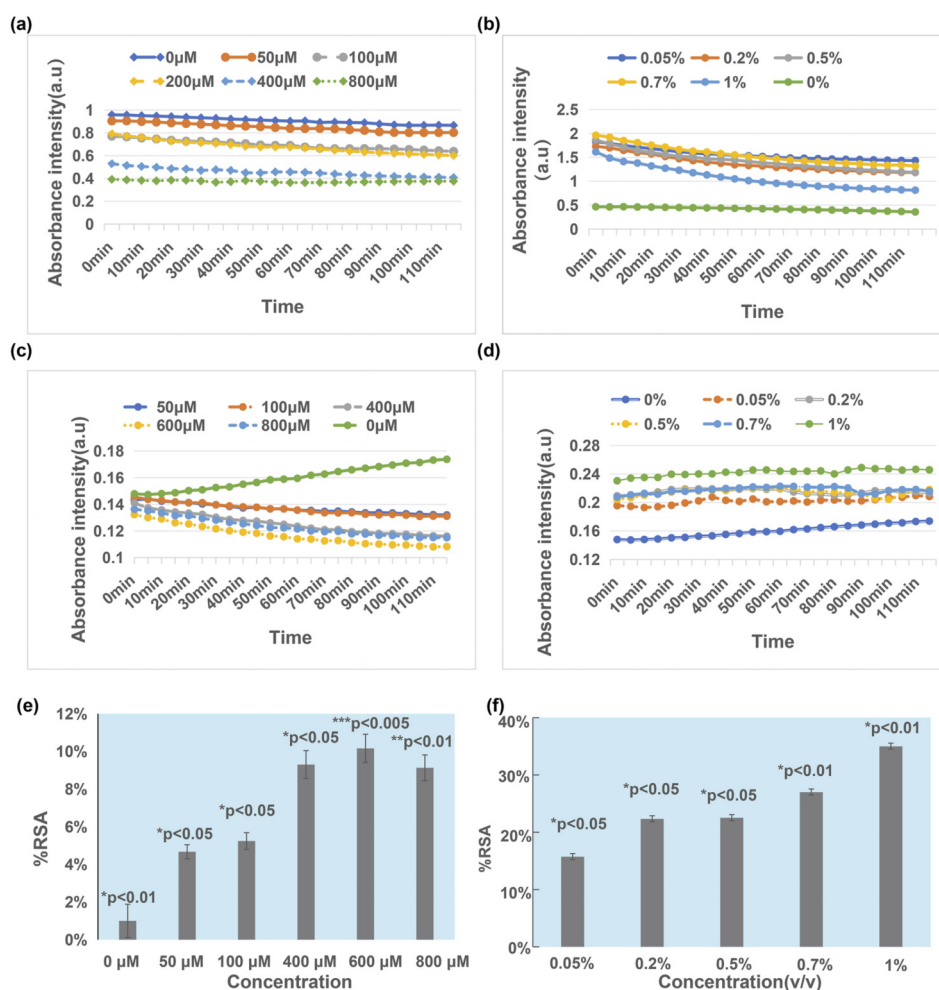
Squalene (99% purity) is an oily liquid at room temperature, so its concentration is expressed as a volume fraction. Dimethyl sulfoxide (DMSO) was used as the solvent for the squalene samples. These samples were dissolved in a mixture of 6% DMSO and 90% squalene to form a 4% stock solution. Before use, the stock solution was further diluted with squalene to achieve various concentrations of the test sample. The final concentrations prepared were 0%, 0.05%, 0.2%, 0.5%, 0.7%, and 1%.

### 2.2 Coverslip cleaning and sample immobilization

The surfaces of the glass coverslips (No. 1.5, 24 mm  $\times$  30 mm and 24 mm  $\times$  40 mm, VWR, Germany) and slides are modified following a previously established protocol (He et al., 2024; Yu et al., 2015). First, the glass pieces are cleaned by immersing them in acetone for 30 min and then etching them with 3 M KOH for 1 h. After these treatments, the glass pieces are rinsed with distilled water, dried with nitrogen gas, and then immersed in an amino-silane solution. This solution is composed of 100 mL methanol, 5 mL glacial acetic acid, and 1 mL N-(2-aminoethyl)-3-aminopropylmethyldimethoxysilane (BIOFOUNT, China). The glass pieces are kept in this solution for 10 min in the dark. Subsequently, the glass pieces are sonicated for 1–2 min, followed by another 10-minute incubation in the dark.

After rinsing and drying, 4 mg of mPEG-biotin (MW 5000, Aladdin, China) and 115 mg of mPEG-succinimidyl valerate (MW 5000, Seebio, China) are dissolved in 490  $\mu$ L sodium bicarbonate buffer and applied to the glass surfaces for overnight incubation. Finally, after rinsing and drying again, the glass pieces are placed in a container and stored at  $-80^{\circ}\text{C}$ .

The coverslip channel is constructed by placing a smaller coverslip (No. 1.5, 24 mm  $\times$  30 mm) on top of a larger slide (No. 1.5, 24 mm  $\times$  40 mm), with the modified surfaces facing each other. The two glass pieces are secured together using two double-sided adhesive strips. Next, 1 mg/mL of streptavidin (Merck, Germany) is pipetted into the channel and incubated for 10 min. After this incubation, any residual streptavidin is flushed away. For the reporter sample (5'-Biotin/TTATT/3'-Cy3 and 5'-Biotin/TTATT/3'-Cy5), the reaction system containing the TN50 buffer (20 mM Tris-HCl and 50 mM NaCl, pH 8) with 100 mM  $\text{MgCl}_2$  was assayed as described above. For the control group, the TN50 buffer is used for blank comparison.



**FIGURE 1**  
Scavenging activity was measured by the reduction of the DPPH radical (100  $\mu\text{mol}$ ) by ethanolic solutions of (a) Trolox and (b) squalene for a period of 120 min. ABTS radical cation antioxidant activity of (c) Trolox and (d) ethanolic solutions of squalene. (e) Reactive scavenging activity measured by ABTS assay. (f) Reactive scavenging activity measured by DPPH assay. Each bar represents the mean  $\pm$  S.D. for three different experiments performed in triplicate.

## 2.3 TIRFM imaging

The fluorescent signal is imaged using TIRFM (Ti2, Nikon, Japan) with an  $80^\circ$  incident angle and an exposure time of 300 ms. The laser power is set to 0.8 mW, the gamma parameter is adjusted to 1, and the LUT intensity range is from 100 to 10,000. The electron-multiplying charge-coupled device (EMCCD) camera used has a resolution of  $1,024 \times 1,024$  pixels. More than 20 images from different areas of the individual channel are captured.

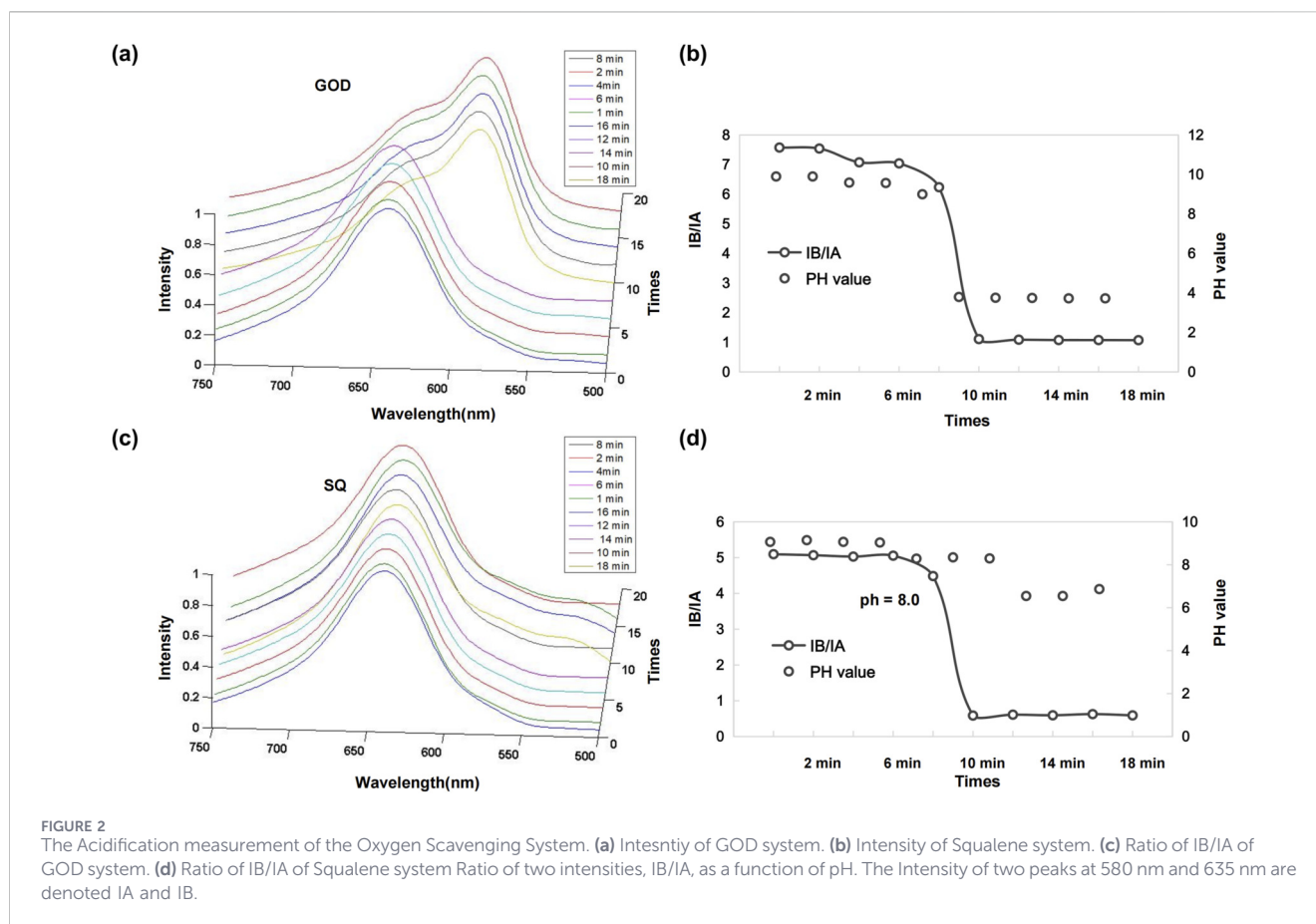
For the positive group, 10  $\mu\text{M}$  DNA, 0.7% squalene (Merck, Germany), and a reaction buffer (TN50) are flowed into the channel for 5–10 min to cleave the immobilized DNA reporters on the coverslip surface. In the control group, no OSS system is used for DNA detection at 10  $\mu\text{M}$ . The average fluorescence signal intensities of all regions of interest (ROIs) are measured, and all data are normalized by the negative control. The data are then integrated and analyzed using a two-sample t-test.

Data are displayed as mean  $\pm$  s.d., with replicates indicated in the figure legends. Student's two-sample unpaired t-test was used for statistical analysis. The statistical significance of the data is indicated as follows: \*p < 0.05, \*\*p < 0.005, and \*\*\*p < 0.0005. N.D. indicates no difference.

## 2.4 Acidification measurement

Immediately before the experiment, glucose oxidase and catalase were added to a buffer with an initial pH of 8.0. This buffer contained 10  $\mu\text{M}$  SNARF-1, 5 mM  $\text{MgCl}_2$ , 0.8% (w/v) dextrose, 2 mM Trolox, and the specified concentration of the buffering agent as described in the text. The resulting mixture was then quickly transferred into the flow chamber.

Alternatively, 0.7% squalene was added to a buffer with the same starting pH, which also contained 10  $\mu\text{M}$  SNARF-1, 5 mM  $\text{MgCl}_2$ , 2 mM Trolox, and 25 mM Tris. Fluorescence emission spectra of



SNARF-1 in these solutions were recorded inside the flow chamber at 2-min intervals for up to 20 min.

### 3 Results

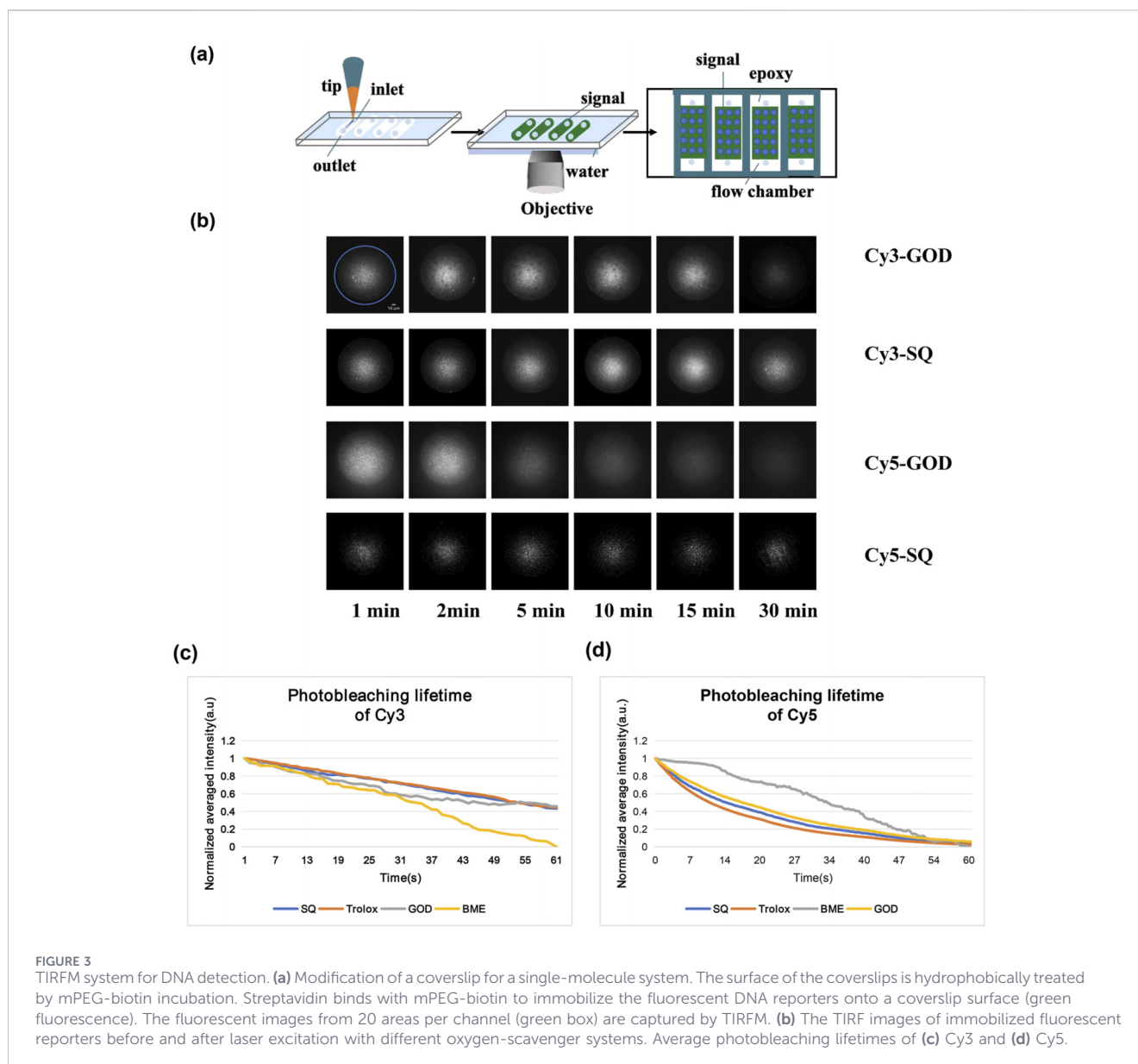
#### 3.1 Oxidative properties of squalene in DPPH/ABTS assays

The antioxidant properties of squalene were determined by measuring radical scavenging activity using DPPH and ABTS assays (Warleta et al., 2010). The antioxidant activity of squalene, as measured by scavenging activity in the DPPH radical assay (Figures 1a,b) or in the ABTS cationic radical assay (Figures 1c,d), indicates that squalene at up to 1% [mole antioxidant/mole DPPH] possesses antioxidant activity, and it possesses such activity at up to 800  $\mu$ M. Trolox is used as a standard solution, and distilled water was used as the blank control (Figures 1a,c). Relative radical scavenging activity (RSA) for all assayed concentrations of squalene at 30 min was <10%, while the RSA of Trolox reached 50% below 200  $\mu$ M. At the concentrations tested in the experiment, squalene's scavenging activity increased in a dose-dependent manner. The ABTS radical scavenging activity was highest at 600  $\mu$ M squalene, reaching 10% (Figure 1e). The DPPH radical scavenging activity was highest at 1% squalene, reaching 35% (Figure 1f). These results indicate that squalene exhibits significant DPPH free radical scavenging activity. Given the complexity of the component in

question when used for oxidation testing, both the DPPH and ABTS methods were employed for cross-validation, and the results confirmed that their trends of change were consistent.

#### 3.2 Effect of reaction conditions on acidification activity

The fluorescent dye photostability in imaging experiments is a key point, which is evidenced by photobleaching and blinking of the biomolecule of interest that was labeled with the dye. Next, solution acidification is evaluated through a commercial assay. A pH-sensitive fluorescent dye, 5-(and-6)-carboxy SNARF-1, is used in single-molecule studies. It has two fluorescent dyes to emit two signals at the same time. We only need to calculate the ratio of the dual signal according to the established relationship between the two. This dual probe has an advantage over a single-emission probe in avoiding any intensity-drift error caused by the photobleaching or excitation power. To use the SNARF-1, we measure the fluorescent emission spectra of the probe in a series of standard solutions with pH values ranging from 5.98 to 8.02 (data in Supplementary Figure S1). Thus, the mapping curve exhibits a sigmoidal relationship between the intensity ratio and pH. Note that we observed the acidification behavior of the SQ system together with Trolox or BME is significantly improved in the experiment in Figure 2. The data showed that, in the experiment with GOD, starting at a pH of 8.0, the pH decreased quickly below 7.0 and then continued to decrease more slowly (Figure 2b). In comparison, SQ maintained a stable



**FIGURE 3** TIRFM system for DNA detection. **(a)** Modification of a coverslip for a single-molecule system. The surface of the coverslips is hydrophobically treated by mPEG-biotin incubation. Streptavidin binds with mPEG-biotin to immobilize the fluorescent DNA reporters onto a coverslip surface (green fluorescence). The fluorescent images from 20 areas per channel (green box) are captured by TIRFM. **(b)** The TIRF images of immobilized fluorescent reporters before and after laser excitation with different oxygen-scavenger systems. Average photobleaching lifetimes of **(c)** Cy3 and **(d)** Cy5.

pH during oxygen scavenging for up to 18 min and showed a slight drop (Figure 2b). We further verify that the SQ system performs as well as the common oxygen-scavenging system, but the pH stability extends the experiment time.

### 3.3 Effect of the oxygen-scavenging system on photoblinking

The biomolecule of interest was labeled with an organic dye such as Cy3 or Cy5 by removing oxygen using an enzymatic oxygen-scavenging system. However, the photobleaching and blinking of the fluorescent dye pose a challenge in single-molecule studies. The enzymatic oxygen-scavenging system is a mixture of glucose oxidase and catalase that converts glucose and  $O_2$  into gluconic acid and water. This approach efficiently reduces the dissolved oxygen in the experimental solution and increases the photobleaching lifetime of Cy3, but it causes fluctuations in the fluorescence intensity of Cy5.

Here, we show that 0.7% squalene, in combination with a triplet quencher (such as 150  $\mu$ M BME and 2 mM Trolox), can eliminate radical scavenging and oxygen-related reactive species and improve the dye photostability of Cy5 and Cy3, as shown in Figures 3, 4. Figure 3b shows that the fluorescence intensity of the molecules exhibits a gradual decline over time. However, squalene shows significantly improved resistance to photobleaching compared to the system with glucose oxidase/catalase. We further plot the survival curves of Cy3 and Cy5 and extract the photobleaching lifetimes in the field of view. Our data indicate that BME enhances the fluctuating intensity of Cy5, but Trolox shows no increasing trend. Therefore, BME has a direct effect on the blinking of Cy5. In addition, we found that squalene, together with Trolox, enhances the performance of Cy3 and Cy5.

To quantify blinking caused by the products of the oxygen-scavenging system over an extended time scale, we calculated the ratio of non-emitting to emitting time (off/on ratio) by analyzing

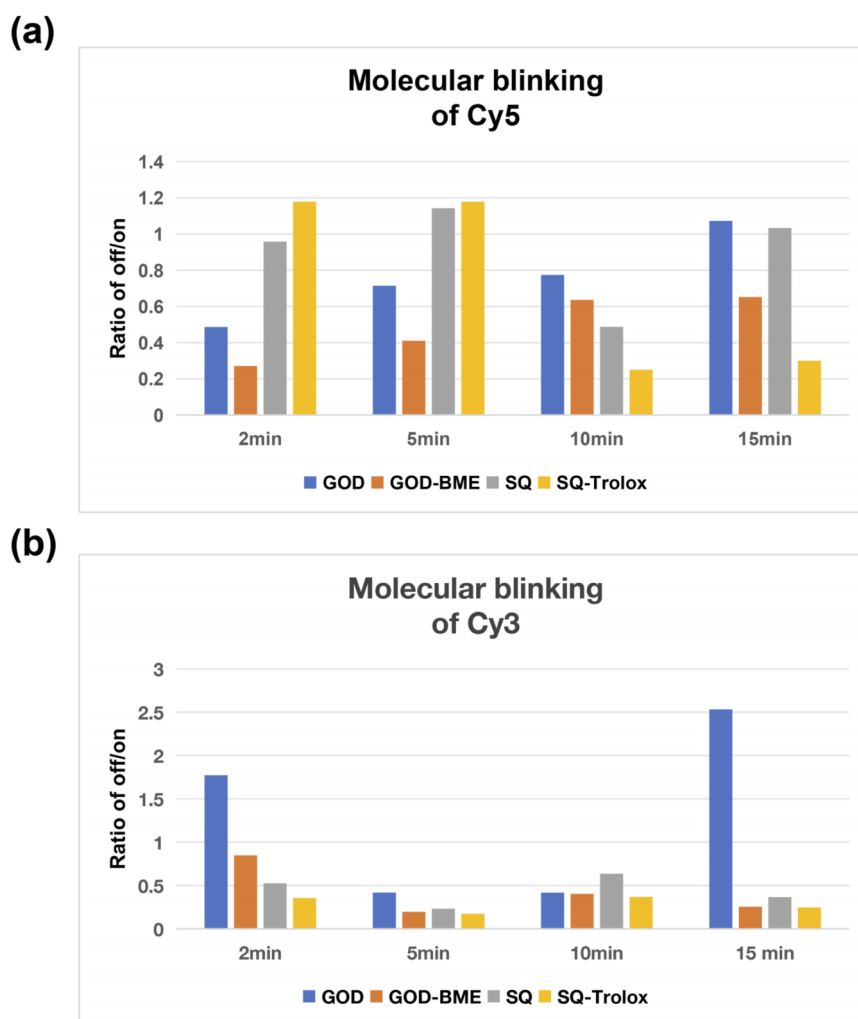


FIGURE 4 Ratio of off/on (a) for Cy5 and (b) Cy3 under different oxygen-scavenger systems.

individual molecules prior to photobleaching. To evaluate blinking, we analyzed the fluorescence intensity traces of single molecules. A molecule was classified as “off” in a frame if its fluorescence intensity in that frame was below 35% of the range between the maximum and minimum intensity of the entire trace,  $I_{\max}$  and  $I_{\min}$ , respectively, and this value was added to  $I$ . Therefore, the limit shows

$$I_{\text{off}} < 0.35 * (I_{\max} - I_{\min}) + I_{\min} < I_{\text{on}}$$

The average off/on ratio of Cy3 ranged from 0.2% to 6% within 1 min after buffer injection and remained nearly constant when using the SQ system. However, when using the GOD system, the off/on ratio increased rapidly and steadily. For Cy5, we observed similar behavior, with significantly increased blinking after prolonged incubation in the GOD imaging buffer.

We tested the effect of the triplet quencher to improve the image quantity under the same concentration and reaction buffer conditions of SQ. We use SQ as an oxygen scavenger in the TIRFM experiment, which measures the fluorescent changes of a single biomolecule. We compared the effect of different

combinations of BME and Trolox in single-molecule experiments. The results indicate that SQ, together with Trolox, has good performance.

## 4 Discussion

Fluorescent dyes have a direct impact on imaging quality in imaging experiments, particularly in techniques such as single-molecule fluorescence resonance energy transfer (smFRET). However, the photostability of these dyes remains a significant challenge, as photobleaching induced by molecular oxygen can severely limit the duration and quality of observations. To address this issue, oxygen-scavenging systems have been utilized to reduce oxygen levels in the experimental environment, thereby enhancing dye stability and extending the lifespan of fluorophores.

This study explores the influence of various oxygen-scavenging systems on the stability of fluorescent dyes employed in smFRET experiments. We systematically assess the effects of different

scavenger combinations, including enzymatic (e.g., glucose oxidase and catalase) and chemical-based systems, on the photobleaching rates of commonly used fluorophores. By quantifying the impact of these systems on fluorophore stability under a wide range of experimental conditions, we offer new insights into the optimization of oxygen-scavenging strategies for improved single-molecule fluorescence imaging. Our results indicate that novel oxygen-scavenging reagents can significantly prolong the observation time of individual molecules, increasing the accuracy and reliability of measurements. The photophysical parameters of SQ were found to be superior to those of the conventional oxygen-scavenger system, while other physicochemical properties, such as radical oxygen activity, pH value, and peroxide value, showed satisfactory results. These findings have significant implications for the design of more robust single-molecule experiments, facilitating more detailed investigations of molecular dynamics, interactions, and conformational changes with higher precision.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

LL: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. IG: Visualization, Writing – review and editing. XY: Visualization, Writing – review and editing. ZD: Writing – review and editing. ZC: Writing – review and editing. ZL: Writing – review and editing. SH: Writing – review and editing. LZ: Resources, Writing – review and editing. JJ: Resources, Writing – review and editing. DY: Supervision, Writing – review and editing. PQ: Conceptualization, Funding acquisition, Supervision, Writing – review and editing.

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## Conflict of interest

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchbi.2026.1749158/full#supplementary-material>

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