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Detection of Intracellular Selenol-Containing Molecules Using A Fluorescent Probe with Near-Zero Background Signal

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ABSTRACT

Selenocysteine (Sec), encoded as the 21st amino acid, is the predominant chemical form of selenium that is closely related to various human diseases. Thus, it is of high importance to identify novel probes for sensitive and selective recognition of Sec and Sec-containing proteins. Although a few probes were reported to detect artificially introduced selenols in cells or tissues, none of them has been shown to be sensitive enough to detect endogenous selenols. We report the characterization and application of a new fluorogenic molecular probe for the detection of intracellular selenols. This probe exhibits near-zero background fluorescence but produces remarkable fluorescence enhancement upon reacting with selenols in a fast chemical reaction. It is highly specific and sensitive for intracellular selenium-containing molecules such as Sec and selenoproteins. When combined with flow cytometry, this probe is able to detect endogenous selenols in various human cancer cells. It is also able to image endogenous selenol-containing molecules in zebrafish under fluorescence microscope. These results demonstrate that this molecular probe can function as a useful molecular tool for intracellular selenol sensing, which is valuable in the clinical diagnosis for human diseases associated with Sec-deficiency or overdose.

Keywords: Selenocysteine, fluorescent probes, benzoquinolizine coumarin, cellular imaging, sensors

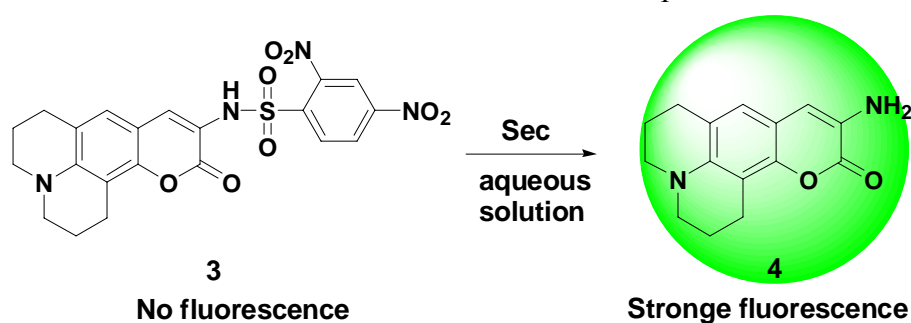
INTRODUCTION

Selenium, discovered in 1817, is a micronutrient known to be important to human health.¹⁻⁷ The year 2017 was announced to be the selenium year in recognition of the significance of selenium for 200 years. Intracellular selenium mainly takes the form of selenocysteine (Sec),^{8, 9} which is an essential building block for selenoproteins (SePs).¹⁰⁻¹⁶ SePs have been implicated in various cellular functions and linked to several human diseases, such as cancers, cardiovascular diseases, neurodegenerative diseases and inflammations.^{6, 17-21} Due to the importance of biological selenols, there is a growing interest in developing highly selective fluorescent molecular probes that can facilitate intracellular selenol-containing molecule detection and creation of molecular tools to diagnose human diseases associated with selenium deficiency and overdose.²²⁻²⁵ However, designing selenol-selective probes represents a significant challenge mostly due to the interference of biological thiols that share similar chemical properties to selenols but are much more abundant in cells and other biological samples. To date, only a few fluorescent probes have been designed to selectively detect biological selenols.^{22, 24, 26} Although these probes are able to detect artificially introduced selenols in cells or tissues (thus at significantly elevated levels), none of them has been shown to be sensitive enough to detect endogenous selenols, an important issue that we sought to solve in the current study.

The selenol pK_a value in Sec (~ 5.8) is lower than thiophenol (~ 6.5) and biological thiols (~ 8.3), which means Sec is more likely to exist as deprotonated state. So, the thiophenol probe may also react with Sec in acidic conditions due to the same mechanism of nucleophilic attack. We have previously reported a thiophenol probe, denoted **1**, that is only weakly fluorescent (quantum yield $\Phi = 0.0050$).²⁷ Probe **1** indeed reacted with selenol, such as Sec, resulting in the release of the free fluorophore, 7-*N*, *N*-diethylaminocoumarin (**2**). Because the quantum yield (Φ) of **2** is 0.14, this reaction leads to the generation of a strong fluorescence. Probe **1** shows excellent selectivity and sensitivity for Sec, with a limit of detection (LOD) is ~ 62 nM.²⁶ It is sensitive enough for quantifying the serum concentration of selenium in

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4 healthy adults (>500 nM), but not sufficient for accurate selenium determination in
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6 the selenium-deficient patients with Keshan disease or Kashin-Beck disease (~ 100
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8 nM).^{20, 28, 29} Therefore, further modification of probe **1** is necessary in order to further
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10 improve its detection sensitivity.

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12 Recently, we hypothesized that the blockage of internal twisting of the nitrogen
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14 atom at 7-position on coumarin ring of probe **1** can significantly enhance the quantum
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16 yield of the freed fluorophore, and then discovered a new thiophenol probe with
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18 improved the sensitivity through the strategy of blocking the internal twisting of the
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20 nitrogen atom at 7-position on coumarin ring (denoted **3** in Scheme 1).³⁰ Inspired by
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22 the good detection capability against selenol with thiophenol probe **1**, the newly
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24 developed probe **3** was applied for selenol-containing molecule detection in this study.
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26 After comprehensive characterization, the new probe shows much improved
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28 properties for selenol-containing molecules: (1) exhibits first-order rate constant (k) of
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30 2.15 min^{-1} , the best among all reported Sec probes to date; (2) about 250-fold
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32 fluorescence intensity enhancement in pure aqueous solution; and (3) sufficient LOD
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34 (~ 18 nM) for selenium determination in selenium-deficient patients. More importantly,
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36 probe **3** is able to detect intracellular selenols with flow cytometry, and discriminate
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38 the cancer cells from normal cells. To the best of our knowledge, this is the first report
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40 that cancer cells showed higher intracellular level of selenol-containing molecules
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42 compared to normal cells. In addition, the probe successfully imaged the endogenous
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44 selenol-containing molecule and exogenous Sec in zebrafish, demonstrating its
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46 potential for the biological function study of SePs, and the clinical diagnosis for the
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48 selenium-related diseases.
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Scheme 1. The mechanism of Sec detection with molecular probe **3**.

EXPERIMENTAL SECTION

Organic Synthesis

Unless otherwise noted, all chemical reagents were commercially available and treated with standard methods before use. Silica gel column chromatography (CC): silica gel (200-300 mesh); Qingdao Makall Group Co., Ltd (Qingdao; China). Solvents were dried in a routine way and redistilled. ^1H NMR was recorded in DMSO- d_6 or CDCl_3 on a Varian Mercury 400 MHz and 600 MHz spectrometer, while the ^{13}C NMR spectra were recorded at 100 MHz and 150 MHz spectrometer. The resonances (δ) are given in ppm relative to tetramethylsilane (TMS). The following abbreviations were used to designate chemical shift multiplicities: *s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet, *br* = broad. High resolution Mass Spectra (HRMS) were acquired in positive mode on a WATERS MALDI SYNAPT G2 HDMS (MA, USA). Melting points were taken on a Buchi B-545 melting point apparatus and uncorrected.

Synthesis of Probe 3

The major intermediates (**M** and **4**) and the final product (probe **3**) were synthesized according to reported methods.³¹⁻³³

(10-nitro-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinolin-11-one) (M): purple solid, yield: 77%, ^1H NMR (400 MHz, CDCl_3): δ 8.55 (s, 1H), 6.98 (s, 1H), 3.41 (q, J = 5.3 Hz, 4H), 2.76-2.86 (m, 4H), 1.99 (s, 4H).

(10-amino-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinolin-11-one) (4): yellow solid, yield: 61%, ^1H NMR (400 MHz, DMSO- d_6): δ 6.79 (s, 1H), 6.62 (s, 1H), 4.97 (s, 2H), 3.11-3.14 (m, 4H), 2.67-2.74 (m, 4H), 1.85-1.91 (m, 4H). HRMS calcd for $[\text{M}^+]$: 256.1206. Found: 256.1211.

(2,4-dinitro-N-(11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinolin-10-yl)benzenesulfonamide) (probe 3): black solid, yield: 86 %. m.p.: 206-207 °C. ^1H NMR (600 MHz, CDCl_3): δ 8.76 (d, J = 1.8 Hz, 1H), 8.41 (d, J = 6 Hz, 1H), 8.13 (d, J = 8.4 Hz, 1H), 7.78 (d, J = 3.6 Hz, 2H), 6.94 (s, 1H), 3.25-3.30 (m, 4H), 2.77 (q, J = 8.0 Hz, 4H), 1.92-1.98 (m, 4H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 164.32, 154.90, 154.65, 152.34, 150.94, 145.58, 142.95, 137.05, 131.84, 130.54, 124.59, 123.66, 118.07, 111.83, 110.16, 59.78, 54.22, 53.69, 31.72, 25.68, 24.65. HRMS calcd for $[\text{M}+\text{H}]^+$: 487.0918. Found: 487.0914. Anal. Calcd for $\text{C}_{21}\text{H}_{18}\text{N}_4\text{O}_8\text{S}$: C, 51.85; H, 3.73; N, 11.52; S, 6.59; Found: C, 51.82; H, 3.72; N, 11.53; S, 6.56.

Sec Sensing and Selectivity Evaluation

The sensing reaction of probe **3** (10 μM) with certain amount of Sec was carried out in 100 mM phosphate buffer at desired pH environment at 30 °C. The Sec was freshly prepared by the preincubation of equal molar of (Sec) $_2$ with DTT in the same phosphate buffer. The fluorescence intensity of the above system was monitored at the wavelength of 535 nm (λ_{ex} = 380 nm).

To study the interference that may be caused by small molecules or ions, probe **3** (10 μM) was mixed with various analytes, such as amino acids (Phe, Arg, His, Tyr, Ala, Trp, Lys and Met), metal ions (Ca^{2+} , Mg^{2+} , Na^+ and K^+) and biological thiols (Cys, Hcy and GSH), respectively. After 30 min incubation, the fluorescence intensity of each sample was measured at the wavelength of 535 nm (λ_{ex} = 380 nm). Similar selectivity study were performed against protein and enzymes (bovine serum albumin (BSA), human elastase, acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and trypsin, and their final concentrations were around 0.01 mg/mL, while the concentration of recombinant human thioredoxin reductase (hTR) is about 0.01

mg/mL. The fold of fluorescence increase (F/F_0) was normalized to the base fluorescence intensity of probe **3** for the above studies.

The recombinant hTR was generated by overexpression of hTR-pGEX plasmid that was purchased from Protech Company (Wuhan, China). The mutants U498C and U498S were obtained by site-directed mutagenesis.

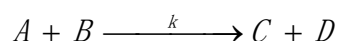
Determination of Quantum Yield

According to the reported method, the quantum yields for probe **3** and its fluorophore (compound **4**) were determined with rhodamine B as reference.^{27, 34, 35} More specifically, using rhodamine B ($\Phi = 0.88$, ethanol) as reference, compounds **3** and **4** were firstly diluted to suitable concentration by PBS (0.1 M, pH 7.0) buffer to make their absorption less than 0.05. Then, their absorbance and integral areas were studied at this concentration. Finally, the quantum yields of these two compounds were calculated by Abbe's refractometer and the following equation.

$$\phi_s = \frac{F_s \cdot A_c}{F_c \cdot A_s} \phi_c \quad (1)$$

Determination of Second Order Reaction Rate Constant

For a second order reaction, A and B are reactants, while the C and D are products.



If the starting concentrations of compound A and B are equal, such as **a**, the reaction rate can be calculated by the following equation. K is the second order reaction rate, X means the concentrations of products, such as C and D.

$$\frac{dx}{dt} = K \times (a - x)^2 \quad (2)$$

Then the equation (3) was converted from equation (2).

$$\frac{1}{a - x} = K \times t \quad (3)$$

For our system, the fluorescence intensity F and concentration of products are in the linear relationship at the early stage. So the concentration of the product can be

calculated by the equation (4)

$$x = \frac{F_t - F_0}{F_\infty - F_t} \times a \quad (4)$$

In the above equation, F_0 represent the start fluorescence intensity of reaction systems at $t = 0$. F_∞ represent the total fluorescence intensity of reaction systems when the reaction had been complete response. F_t means the fluorescence intensity of reaction systems after incubation time t . The fluorescence intensity of reaction systems was monitored at the wavelength of 535 nm ($\lambda_{\text{ex}} = 380$ nm).

The equation (4) was substituted into equation (3) to yield the equation (5). Based on this equation, the second order reaction rate K could be calculated.

$$\frac{F_t - F_0}{F_\infty - F_t} = K \times a \times t \quad (5)$$

Living Cell Imaging and Cytotoxicity Study

H460 cells were cultured in DMEM supplemented with 10% (V/V) fetal bovine serum, 1% (V/V) penicillin/streptomycin and maintained in the atmosphere of 5% CO₂ at 37 °C. The cells were seeded on 6-well plates at 1×10^5 cells per well in 2 mL culture medium and incubated overnight in 5% CO₂ at 37 °C. Then both (Sec)₂ and DTT (20 μM) were added into the cells and incubated for different times (0h, 6 h and 12 h). After washing the cells with PBS three times to move the remaining (Sec)₂ and DTT, the cells were further incubated with probe **3** (20 μM) for 20 min at 37 °C. After washing the cells with PBS three times, the treated cells were imaged by inverted fluorescence microscopy (Olympus IX71, Japan). In order to induce endogenous Sec, the H460 cells were treated with sodium selenite (20 μM) for different times (0 h, 6 h and 12 h). And then the cells were incubated with the probe **3** (20 μM) for 20 min at 37 °C. After the cells were washed three times to remove the remaining probe **3**, the fluorescence images were acquired with inverted fluorescence microscopy. In the mean time, the control group just incubated with the probe **3** (20 μM) for different times (0h, 6 h and 12 h) at 37 °C and then imaged by the microscopy.

To study the cytotoxicity, HEK293 cells were seeded at 1×10^5 cells per well in 96-well plates and incubated for 24 h before treatment, followed by exposure to different concentrations (0, 1, 5, 10, 20, 50 μM) of probe **3**, Sec, Na_2SeO_3 and compound **4** for additional 24 h. To determine the cell viability, the colorimetric assay with 3-(4, 5-dimethylthiazol-2-yl)-3, 5-diphenyltetrazolium bromide (MTT) was applied for evaluating the metabolic activity. Cell samples were incubated with MTT solution (containing 5 mg/mL MTT reagent in PBS) for 4 h, then DMSO was added to dissolve the formazan crystals after removal of the MTT solution. The absorbance was measured at 540 nm with a microplate reader (SpectraMax M5, Molecular Devices). Each experiment was performed at least three times. The cytotoxic effect was evaluated accordingly.

Flow Cytometry Analysis

Flow cytometry (C6, BD Biosciences) was employed to determine endogenous selenol with developed probe. Cancer and normal cell cultures were prepared for sample analysis. After removing media and rinsing with PBS buffer, the cells were treated with trypsin (0.25 %). The digested cells were centrifugated and further suspended at a density of $2 \times 10^5/\text{mL}$ with PBS in 1.5 mL eppendorf tubes. Following by treat with a range of concentrations (0, 0.1 μM , 1 μM , 10 μM , 100 μM) of probe **3** at room temperature for 10 min. the fluorescence signal were determined in FL1-A detector by flow cytometry. Analysis was conducted for a minimum of 10000 counting events and median values of histograms were used to quantify the Sec recognition with probe **3**.

In Vivo Imaging of Zebrafish

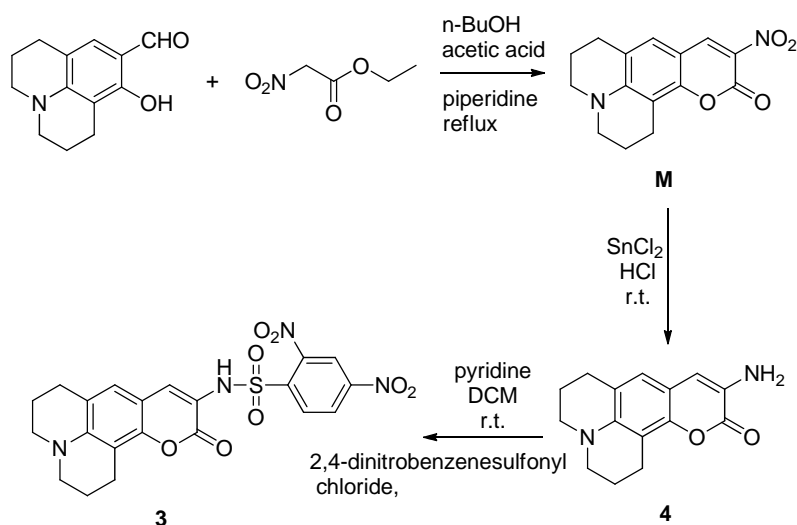
To explore the potential of probe **3** as an *in vivo* imaging tool, we applied it to detection in living zebrafish. The 3-7 days zebrafishes post-fertilization were purchased from Eze-Rinka Company (Nanjing, China). The zebrafishes were cultured in 5 mL embryo medium supplemented with 1-phenyl-2-thiourea (PTU) in 6-well plates for 24 h at 30 $^{\circ}\text{C}$. Then the embryo zebrafishes were washed three times to

remove the remaining (Sec)₂ and DTT, and further incubated with probe **3** (10 μM) for 24 h at 30 °C. After removing the embryo medium and washing the zebrafish with PBS for three times, the fluorescence images were acquired with stereo microscopy (Olympus SZX16, Japan). In order to detect endogenous selenol contents, the zebrafish was directly treated with embryo medium containing probe **3** (10 μM) for 24 h. After washing the zebrafish for three times, the zebrafishes were imaged by stereo microscopy. As the negative control, the zebrafishes without any treatment were imaged directly.

RESULTS AND DISCUSSION

Probe Synthesis

The new probe **3** was prepared in three steps and the detailed synthetic route is shown in Scheme 2, and the structures of probe **3** and intermediate molecules were fully characterized (see Figure S1-S6). The water solubility of probe **3** was investigated prior to the fluorescence property characterization. It revealed that and the maximum absorbance in 0.1 M phosphate buffer (pH 7.4) linearly increased with increasing the concentration of probe **3** (0-200 μM, see Figure S7), indicating the good water solubility of the newly synthesized probe. Thus probe **3** is readily soluble in phosphate buffer system, and there is no need for co-solvent for biological selenol-containing molecule detection.

Scheme 2 The synthetic route of probe **3**.

Fluorescence, pH dependence and Sensitivity of Probe **3**

Based on the primary characterization, probe **3** exhibits several very desirable properties in pure aqueous solution. First, the new probe has near zero fluorescence background ($\Phi = 0.0014$). Second, molecule **4**, the fluorophore of probe **3**, has an increased quantum yield (0.30; in comparison, Φ of **2** is 0.14) and a notable red shift ($\lambda_{\text{ex}} = 380 \text{ nm}$ and $\lambda_{\text{em}} = 535 \text{ nm}$; Figure S8). Third, probe **3** exhibits first-order rate constant (k) of 2.15 min^{-1} when reacting with ten times higher concentration of Sec at 30°C (Figure 2a), the best for all reported Sec probes to date (for comparison, k is $\sim 1.34 \text{ min}^{-1}$ for Sel-green at 30°C and $\sim 0.16 \text{ min}^{-1}$ for HD-Sec at 25°C).^{24, 26} To compare with the reported BESTthio probe²² with second-order rate constant of $740 \text{ M}^{-1}\text{s}^{-1}$ at 37°C , the second-order rate constant of probe **3** reacting with Sec was measured at 30°C (Figure 3). The second-order rate constant was calculated to be $\sim 700 \text{ M}^{-1}\text{s}^{-1}$ by equation 5, which should be even higher at 37°C . In addition, The Sec-reactivity of probe **3** has a bell-shaped pH-dependence (Figure S9), with the maximal value at pH 6.0. Based on this observation, pH 6.0 was chosen for the remaining experiments in this study. From the concentration titration experiment shown in Figure S10 and Figure 2b, Sec can produce up to 250-fold fluorescence enhancement. On the basis of the calibration curve of response of probe **3** to Sec in aqueous solution, its LOD for Sec is further determined to be about 18 nM (Figure

S11).

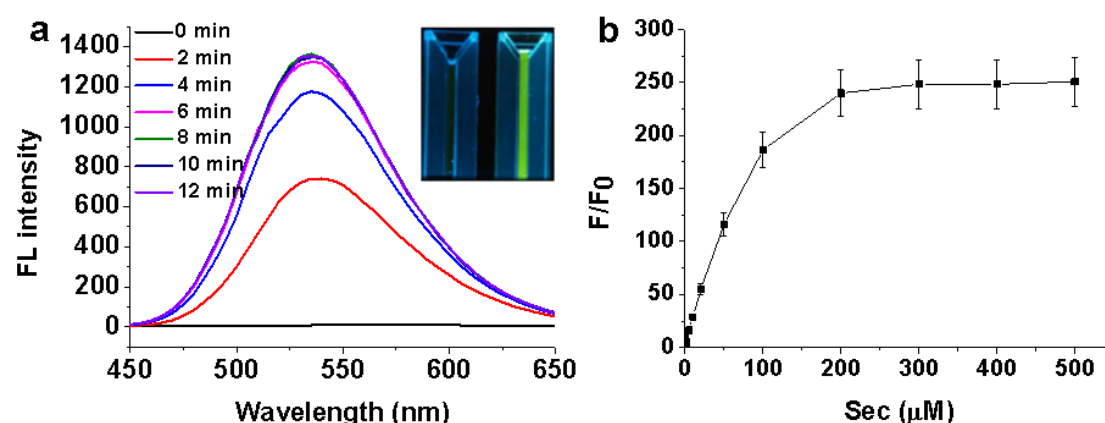


Figure 1. (a) Time dependence for the fluorescence spectra ($\lambda_{\text{ex}} = 380 \text{ nm}$) of probe **3** (10 μM) in the presence of Sec (50 μM) in 0.1 M phosphate buffer (pH 7.0) at 30 °C. Inset: photo of samples illuminated with 365 nm UV light with and without the addition of Sec (50 μM). (b) The change of the relative fluorescence intensity (F/F_0) at 535 nm of the probe **3** (10 μM) with increasing concentrations of Sec.

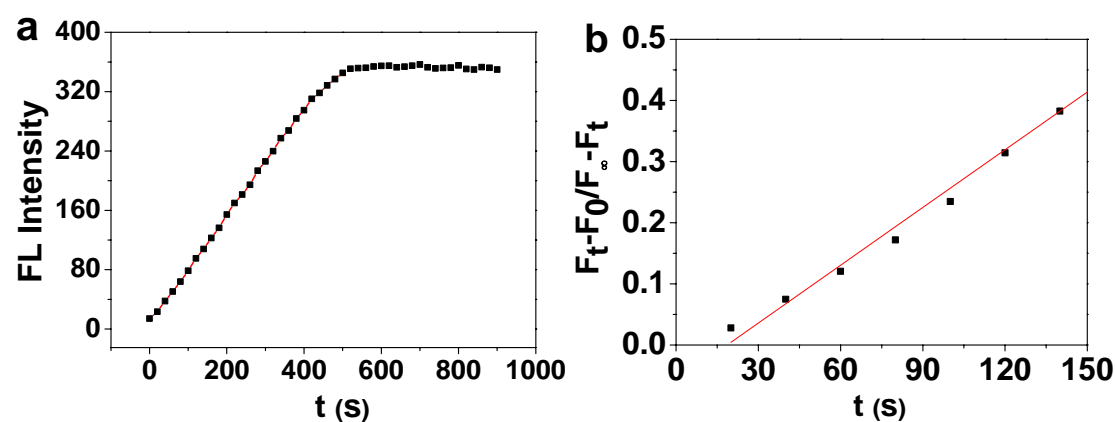


Figure 2. (a) Time dependence for the fluorescence spectra ($\lambda_{\text{em}} = 515 \text{ nm}$) of probe **3** (10 μM) in the presence of Sec (10 μM) in 0.1 M phosphate buffer (pH 7.0) at 30 °C. (b) The plot of the reaction progress ($(F_t - F_0)/(F_\infty - F_t)$) vs the reaction time. F_0 is the initial fluorescence intensity of reaction system, F_t is the fluorescence intensity of reaction system at the corresponding time, F_∞ is the total fluorescence intensity when 100% substrate converted, and t is incubation time in second.

It is noteworthy that the turn-on fluorescence sensing mechanism of probe **3** upon the recognition of Sec was validated by HRMS and HPLC analysis. As the reference, Figure 3a shows the HRMS peak of the probe $[3+H^+]$ (calcd. for 487.0918) before the addition of Sec. When monitoring the reaction system of probe **3** and Sec that are solved in dd-water containing 0.1% (V/V) DMSO (the final concentration are 20 μ M), the HRMS peak of compound **4** (calcd. for 256.1206) was also found in Figure 3b. To further validate this reaction mechanism, the reaction of probe **3** in the absence and presence of Sec was monitored by HPLC, with fluorophore (**4**) as reference (Fig. S12). All of them were solved in CH_3CN , the mobile phase (ACN:H₂O) is 60%: 40%, and the flow rate is 0.3 mL/min. It shows that the signal peak of reaction system between probe **3** and Sec correlates well with the signal peaks of the fluorophore (**4**). These results indicate that probe **3** actually reacted with Sec and gave out **4** as a product.

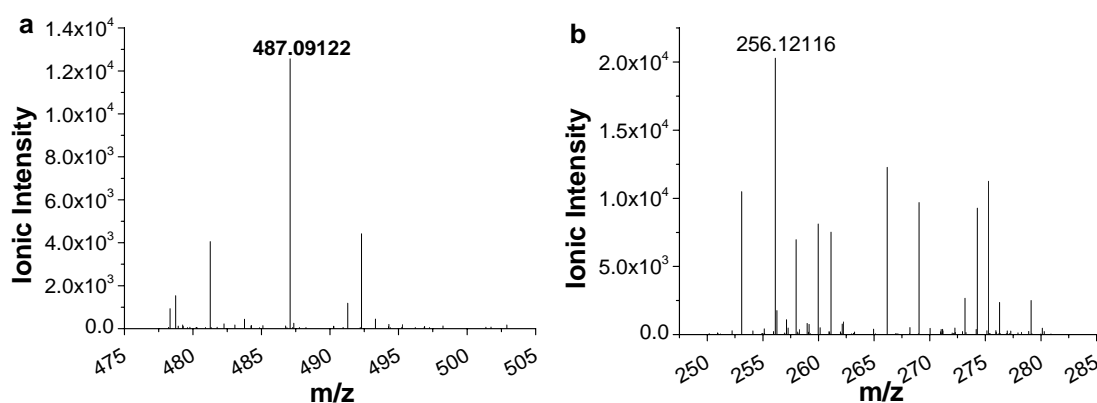


Figure 3. HPLC-MS spectrum of pure probe **3** (a), and the reaction system between probe **3** and Sec. Molecular ion mass peak of (a) $[3+H^+]$ (calcd. for 487.0918), (b) $[4]^+$ (calcd. for 256.1206).

Probe Selectivity

We tested the selectivity profile of probe **3**. The data presented in Figure 4a shows that this molecular probe is highly selective for selenols (represented by Sec) over competing amino acids (Phe, Arg, His, Tyr, Trp, Ala, Lys and Met), metal ions (Ca^{2+} , Mg^{2+} , Na^+ and K^+) and biothiols (Cys, Hcy and GSH). It displayed 120-fold fluorescence enhancement upon treatment with Sec, indicating that probe **3** is

particularly selective toward Sec. We also tested the reactivity of probe **3** with SePs. We chose human thioredoxin reductase (hTR), a well-characterized human SeP for this examination. It is known that hTR contains a single Sec residue locating at the C terminal. We prepared and tested the recombinant version of hTR (1 $\mu\text{g/mL}$), as well as two hTR mutants, U498C and U498S, in which Sec was substituted with cysteine and serine, respectively. In addition, we also tested five control proteins (10 $\mu\text{g/mL}$) – bovine serum albumin (BSA), human elastase, acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and trypsin – that do not contain Sec but commonly exist in serum. As shown in Figure 4b, only wild type hTR (wt hTR) was able to produce significant fluorescence enhancement, indicating our molecular probe is able to selectively detect SePs. In addition, the second-order reaction rate constant for the reaction between probe **3** and hTR was measured to be about $1200 \text{ M}^{-1}\text{s}^{-1}$. Thus the reaction between probe **3** and hTR was only slightly faster than that of reaction between probe **3** and Sec ($700 \text{ M}^{-1}\text{s}^{-1}$) at 30°C , which indicates that the probe **3** could not discriminate the hTR and Sec.

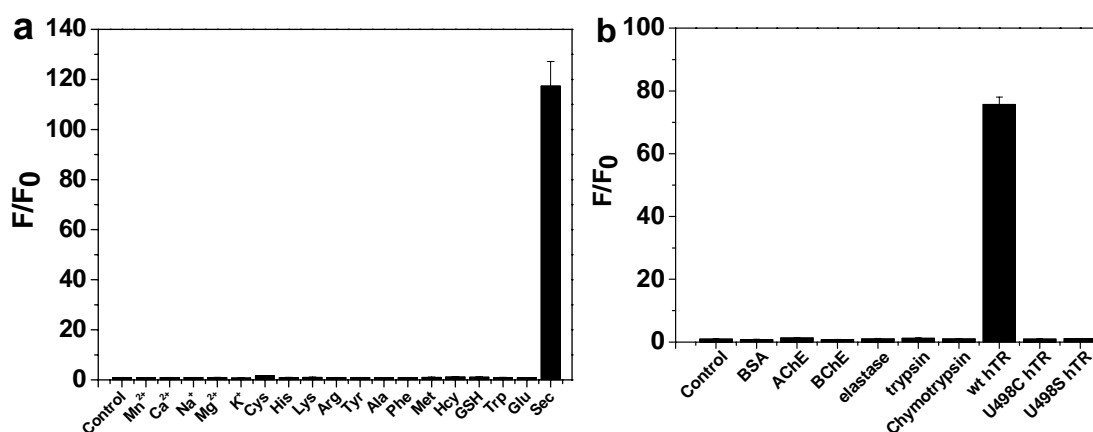


Figure 4. (a) The selectivity profile of probe **3** against metal ions, amino acids, biological thiols and Sec. F/F_0 represents the relative fluorescence response. The probe concentration was 10 μM , the Sec concentration was 100 μM and other analytes were added at 1000 μM . (b) The selectivity profile of probe **3** against hTR (1 $\mu\text{g/mL}$) and proteins (10 $\mu\text{g/mL}$) that do not have Sec residue. F/F_0 represents the relative fluorescence response. The probe concentration was 10 μM .

Cytotoxicity of Probe 3 and Its Application in Cell Imaging

We next carried out an imaging study using probe **3** against human lung cancer cells (A549). We first studied the response of probe **3** to the exogenous Sec in cells at different time points. After treated with Sec newly prepared by equimolar complex of (Sec)₂ and DTT to the cultured cells, a bright and strong green fluorescence could be observed after 6 h and the green fluorescence became stronger after 12 h, while no significant fluorescence signal in control cells all the time (Figure 5a and Figure 5c). We also examined the cells treated with sodium selenium (Na₂SeO₃), a classical precursor of Sec biosynthesis. It was expected that the cells treated with Na₂SeO₃ would produce increased level of reactive selenol species. This was confirmed by the data presented in Figure 5b, and the green fluorescence also increased with the extension of the incubation time. These results show that probe **3** is capable of detecting purposely elevated Sec in mammalian cells. In addition, the results obtained from MTT assay indicated that, probe **3** and its fluorophore did not cause significant cytotoxicity against HEK293 cells, whereas the representative selenocompounds, Sec and Na₂SeO₃, caused severe cytotoxicity (see Figure 6).

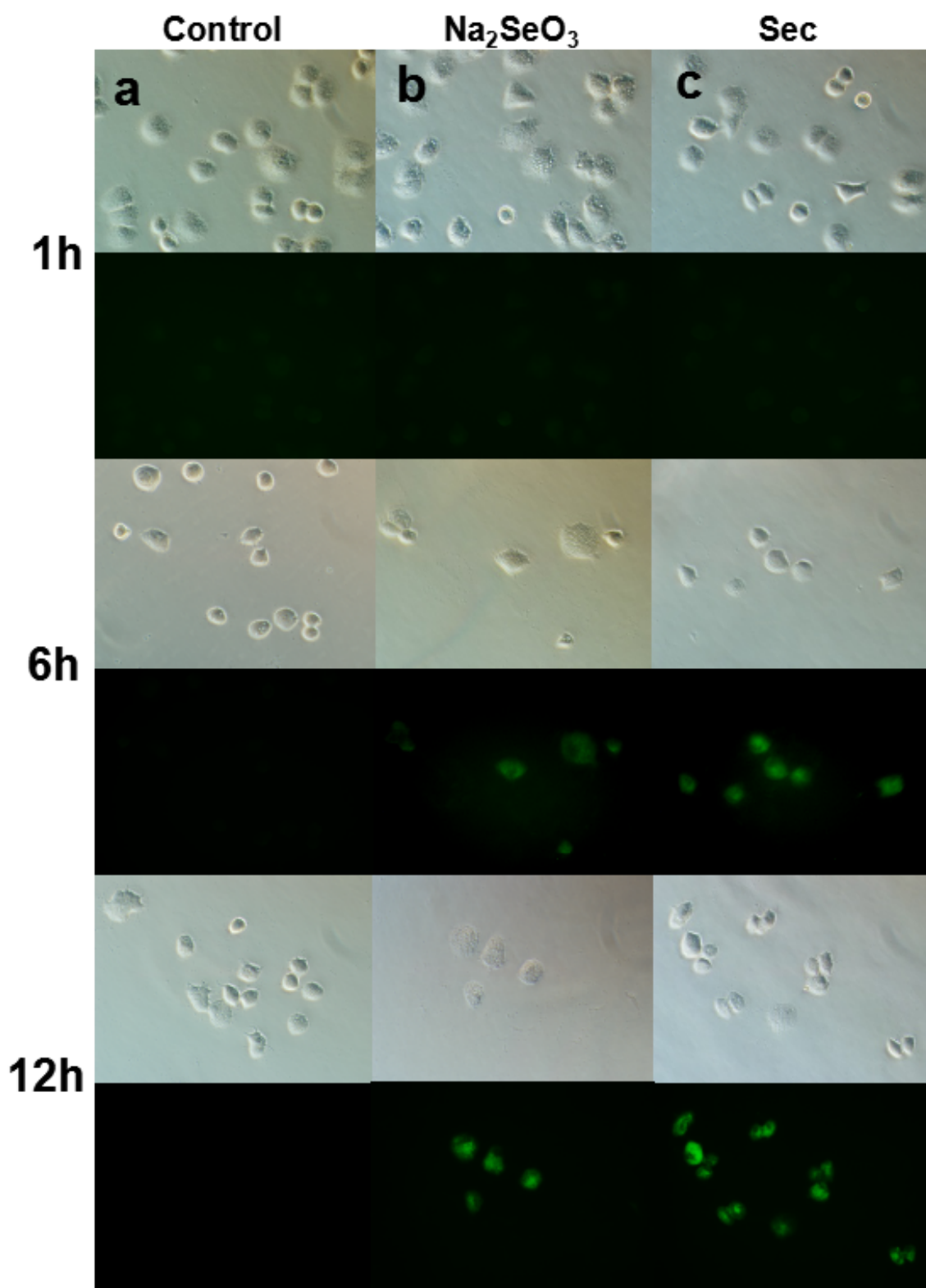


Figure 5. Fluorescence imaging in A549 cells at different incubation times (0 h, 6 h and 12 h). The cells were untreated (a), with 20 μM Na₂SeO₃ (b), and treated with 20 μM (Sec)₂ and 20 μM DTT(c), and the bright-field images (top panel of each time point), fluorescence images (bottom panel of each time point) were acquired.

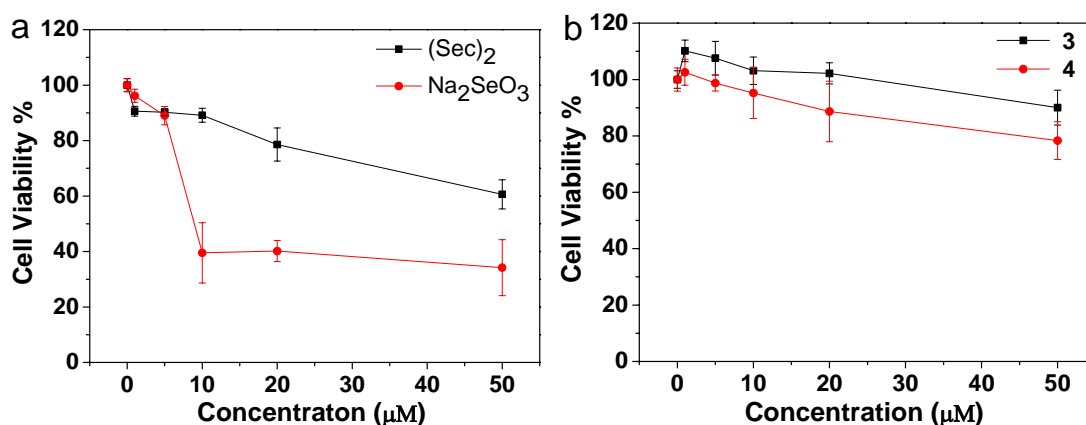


Figure 6. Cytotoxicity of representative selenocompounds (a) and new synthesized compounds (b) against HEK293 cells. The cell viability was determined by the MTT assay. Data represent average values of three independent experiments.

Endogenous Cellular Selenol/Sec Determination

We then examined whether probe **3** was useful for detecting endogenous selenols in single cells using flow cytometry. Probe **3** was tested at four different concentrations (0.1, 1, 10 and 100 μM) with A549, a lung cancer cell. The results depicted in Figure 7a (as well as Figure S13) and the resultant plot of the fluorescence intensity versus the concentration of probe **3** was shown in Figure 7b. Moreover, the time dependent profile of the fluorescence for A549 cells was investigated and the corresponding results were showed in Figure 7c and 7d. Notably, those data demonstrate that probe **3** can indeed detect intracellular selenols in individual cells, reaching the highest signal at 10 μM of probe and a cell-probe incubation time of 10 minutes.

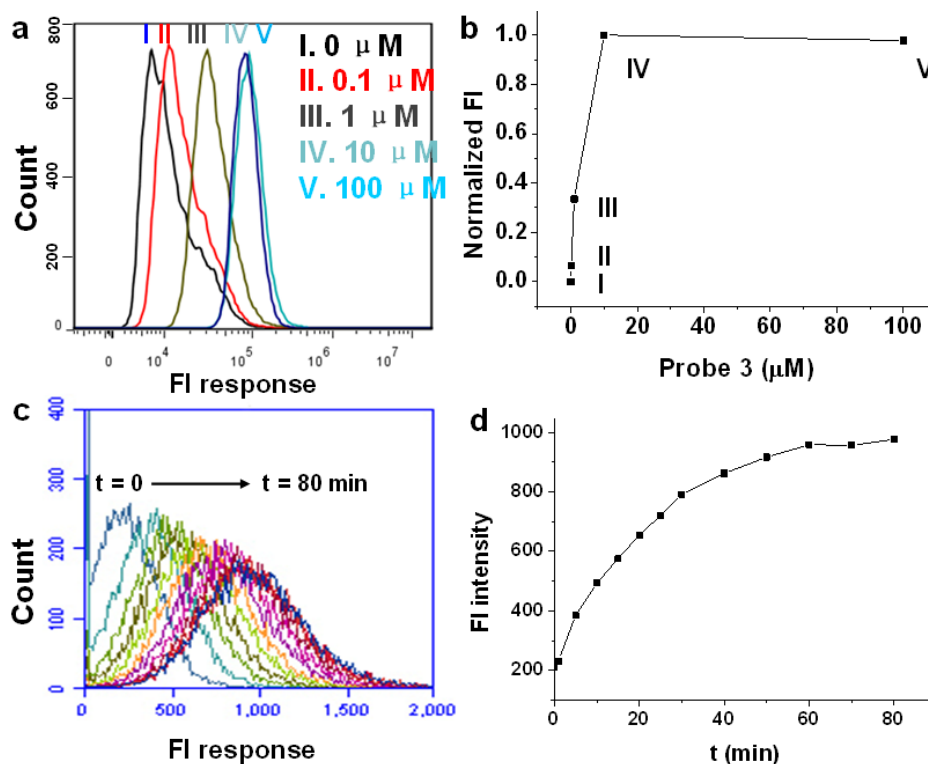


Figure 7. (a) The flow cytometric analysis of A549 cells exposed to different concentrations of probe 3 (I, 0 μM ; II, 0.1 μM ; III, 1 μM ; IV, 10 μM ; V, 100 μM) after 10 min incubation. (b) Plot of the fluorescence signal versus the probe concentration in flow cytometry. (c) The flow cytometric analysis of A549 cells exposed to probe 3 (10 μM) after different time of incubation. (d) Plot of the fluorescence signal versus the incubation time in flow cytometry.

The above flow cytometry-based method was further used to measure the relative selenol concentrations by probe 3 in both normal cells (HEK293 and COS) and other three cancer cells (A549, H460, and HeLa). Interestingly, the data presented in Figure 8 revealed that selenol levels in the chosen cancer cells are noticeably higher than that of normal cells, a novel observation that has never been described before.

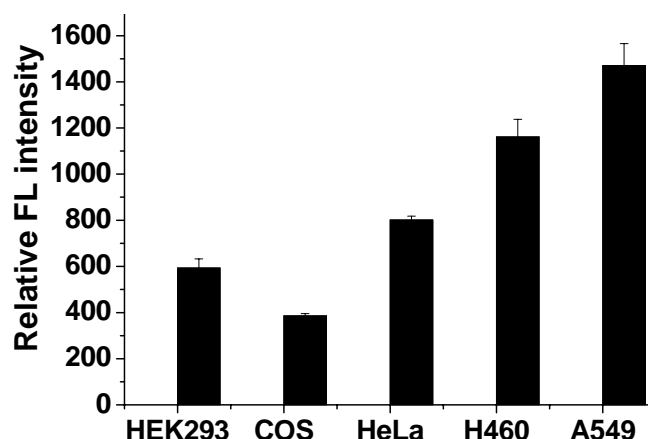


Figure 8. The flow cytometric analysis of normal cells (HEK293 and COS) and cancer cells with probe **3** (10 μ M).

***In Vivo* Selenol/Sec Imaging of Zebrafish with Probe 3**

We also investigated the suitability of probe **3** as *in vivo* imaging agent. Zebrafish were chosen for this investigation mainly for experimental simplicity (it can be conveniently imaged under fluorescence microscopy).^{36, 37} Zebrafish were divided into three groups: the control group (group a in Figure 9; no probe **3** added), intrinsic selenol test group (group b in Figure 9, treated with probe **3** alone), and chemically enhanced selenol test group (group c in Figure 9, treated with probe **3** as well as equimolar complex of (Sec)₂ and DTT to produce enhanced level of selenols). No significant fluorescence was observed in the control group. However, probe **3** was able to detect both intracellular selenol-containing molecules in the intrinsic selenol group b (strong fluorescence was observed in the head) and chemically enhanced selenol group c (strong fluorescence was seen in both the head and the venter). These results demonstrate that probe **3** can perform sensitive Sec imaging in the living organisms.

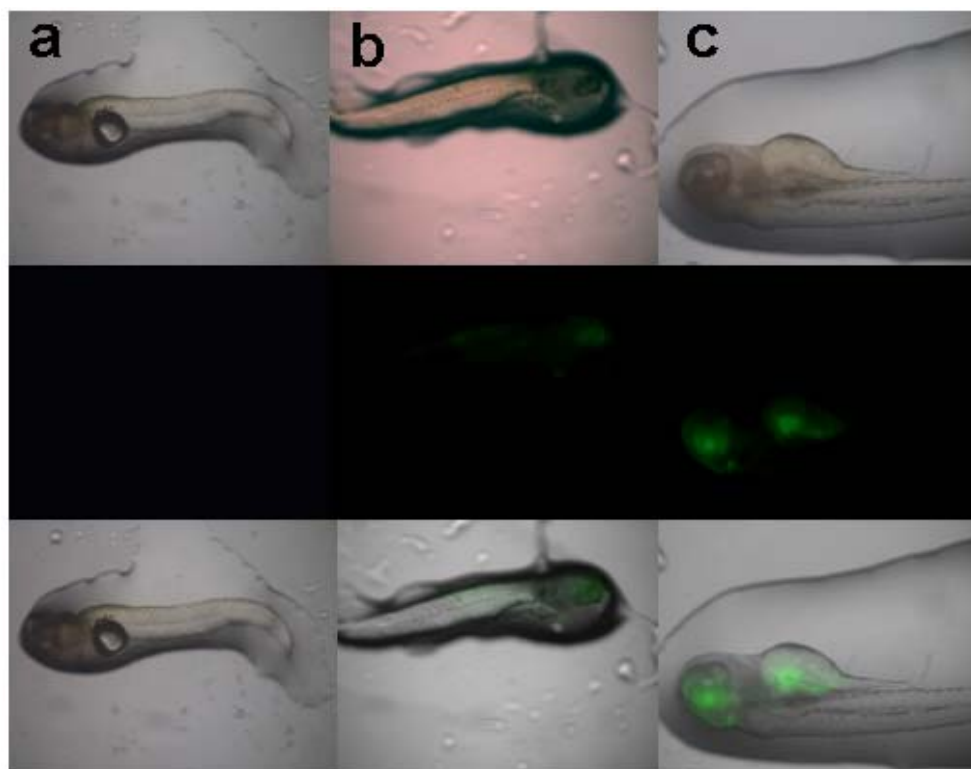


Figure 9. Confocal imaging of selenols in zebrafish embryos, 30 min after incubation of probe **3**: (a) Control group (no probe), (b) Probe group (only 10 μM probe **3** was added), (c) Sample group (10 μM probe **3**, 20 μM $(\text{Sec})_2$ and 20 μM DTT were added).

CONCLUSION

In conclusion, we have successfully identified a new molecular probe for selenol-containing molecule recognition. This probe exhibits several very desirable properties, including near-zero background fluorescence, large fluorescence enhancement upon reacting with Sec (~ 250 -fold), a low LOD (18 nM), and a large Sec-reacting rate constant ($\sim 2.15 \text{ min}^{-1}$). Furthermore, probe **3** can be used to carry out sensitive intracellular selenol-containing molecule detection using live cells and living organisms. We expect this molecular probe can find useful applications both in the study of biological function related to SePs and in the clinical diagnosis for human diseases associated with Se-deficiency or overdose.

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SUPPORTING INFORMATION

The NMR, LC-MS and HPLC spectra for the newly synthesized compounds, and some figures for probe **3** characterization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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