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Article

A Selective Fluorescent Probe for the Determination and Imaging of Fluoride in Living Cells

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Fluoride anions are indispensable trace elements required for sustaining life. To investigate the homeostasis and action of fluoride in the body, a new highly sensitive and selective fluorescence detection method was designed for fluoride in aqueous solutions. A fluorescent probe for fluoride (FP-F) was synthesized for imaging F^- in living cells. The design strategy for the probe was based on the specific reaction between fluoride and silica to mediate deprotection of this probe to fluorescein. Upon treatment with F^-, FP-F, a closed and weakly fluorescent lactone, was transformed into an open and strongly fluorescent product. Under the optimum conditions, the detection limit for fluoride was 0.526 nM. FP-F could detect micromolar changes in F^- concentrations in living cells by confocal microscopy.

Keywords: Fluoride determination. Indispensable trace element. Fluorescent probe. Fluorescence image. Macrophages.

INTRODUCTION

Anions play essential roles in many biological processes in the human body. Particularly, fluoride (F^-), the smallest anion, is an indispensable trace element in maintaining life. As an essential element in humans, F^- plays a dual role. Appropriate amounts of the fluoride compounds can be used in medical treatment (Aaseth *et al.,* 2004; Zhou *et al.,* 2016; Huysmans, Young, Ganss, 2014; Wang *et al.,* 2013; Göstemeyer *et al.,* 2017). However, excessive F^- results in fluorosis and renal, gastrointestinal and immunological toxicity, such as dental fluorosis, crippling fluorosis, impairment of the immune system, as well as the inhibition of crucial enzymes (Mahapatra *et al.,* 2015). Massive F^- levels may also alter gene expression and cause apoptosis (Barbier, Arreola-Mendoza, Del Razo, 2010). Although the structural formula of F^- is well understood, little is known about the fluoride homeostasis and action in the body. Thus, it is appealing to make F^- "visible" in tissues, even in living cells.

The quantification of F^- tracing within biological samples and the elucidation of complex physiological and pathological roles motivates investigations into dynamic fluoride chemistry in living systems. As materials, fluorescent probes allow for effective imaging and identification in vivo (Tang *et al.,* 2016; Jiao *et al.,* 2017; Ke *et al*., 2013). In this regard, live-cell fluorescent imaging with fluoride-responsive chemosensors is a potentially powerful approach to interrogate aspects of labile F^- accumulation, metabolism and trafficking in real time in living systems at the molecular level. Therefore, there is a necessity to develop highly sensitive and specific fluorescent probes that can be applied in the

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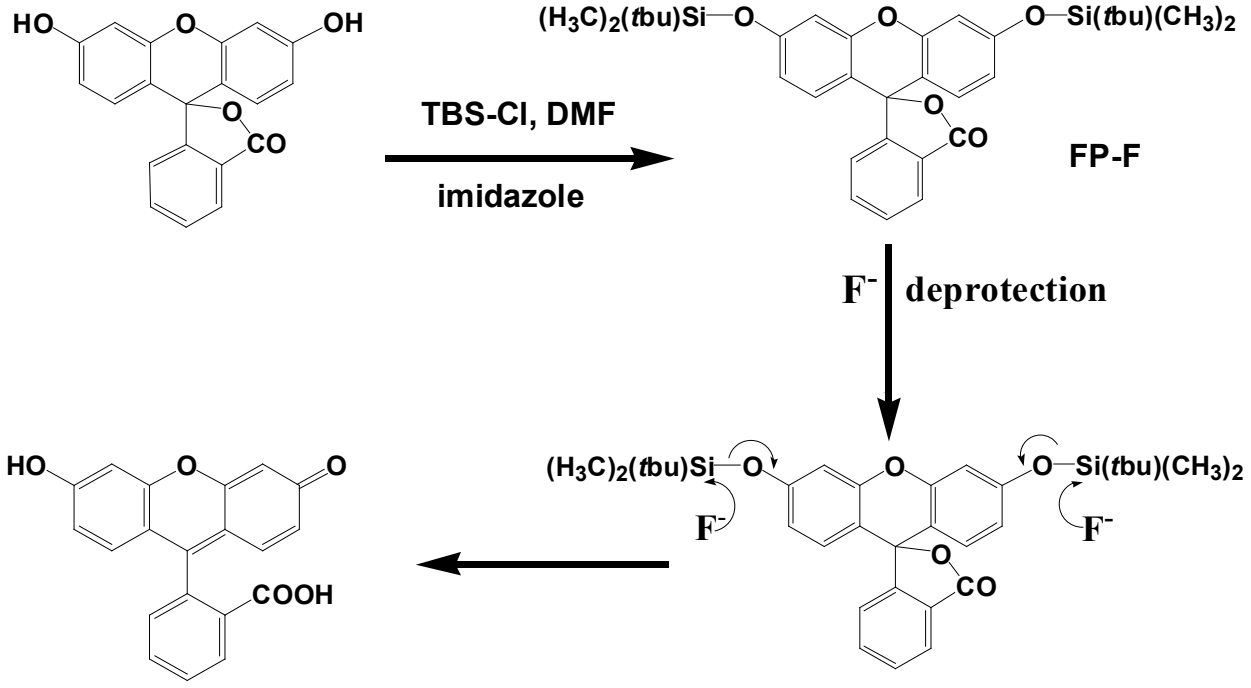
detection of cellular F^-. In previous studies involving molecular probes for F^- detection, hydrogen bonding between the N-H of a urea or pyrrole group and F^- was utilized for recognition (Zhao *et al.,* 2006; Ali *et al.,* 2016; Ashokkumar *et al.,* 2014; Li *et al.,* 2011). However, the solvent played crucial roles in controlling the binding strength and probe selectivity for F^-. Additionally, there have been a few reports regarding F^- detection utilizing a unique fluoride-boron interactions (Toni, Yvonne, Thomas, 2006; Swamy *et al.,* 2006; Kubo *et al.,* 2005; Arimori *et al.,* 2004; Liu *et al.,* 2005) and fluoride-silica interactions (Zhang *et al.,* 2017; Zhu *et al.,* 2005; Yang *et al.,* 2007). Kim and Swager (2003) reported a series of coumarin derivatives serving as fluoride selective receptors. In order to impart greater sensitivity to the fluoride-induced-lactonization detection scheme, they designed semiconductor polymer indicator conjugates that could amplify the response (Kim, Swager, 2003). Nevertheless, the polymer had poor membrane permeability, making it difficult to monitor intracellular F^- levels within living cells. To date, no small molecules are available for imaging F^- in living biological systems. We now present the synthesis, properties and live-cell imaging applications of a fluorescent probe for fluoride (FP-F), a new type of fluorescent probe that exhibits high selectivity and sensitivity for F^- in both aqueous solution and in living cells. In addition, confocal microscopy further indicated that FP-F was membrane-permeable and could be used to monitor intracellular F^- levels.

Our design strategy for fluorescence detection of F^- was inspired by the unique chemical reactivity of F^- with silicon (Gu, Mani, Huang, 2015). In this fluoride-sensing system, F^- attacks silica and triggers Si-O bond cleavage, which results in the release of the organic molecule, coupling with a spectra character-signaling event. Therefore, we focused on the fluoride ion-triggered formation of fluorescein based upon the fact that fluorescein is a fluorescent dye with a high radiative quantum yield. Along these lines, the synthesis of FP-F proceeded, as shown in Scheme 1. Coupling of fluorescein and *tert*-butyldimethylsilyl chloride (TBS-Cl) in imidazole/DMF led to the generation of FP-F that was separable by flash column chromatography with 55% yield.



Scheme 1 - Synthesis of FP-F and the reaction of FP-F with F^-

The FP-F probe based on fluorescein scaffold showed favorable compatibility with biological samples, including water solubility and membrane permeability, visible-wavelength excitation and emission profiles. Moreover, there was minimal sample damage and native cellular autofluorescence, along with a turn-on or ratiometric fluorescence response for mapping spatial resolution. In addition, FP-F featured good stability, low toxicity and resistance to oxidation. More promisingly, FP-F presented excellent selectivity toward F^- over abundant cellular ions. All these properties are required for an effective F^- probe in a biological environment. We hypothesized that weakly fluorescent FP-F, a closed lactone, is deprotected and transformed into an open and strongly fluorescent product upon reacting with F^-.

MATERIAL AND METHODS

Material

Silica gel (100-200 mesh, Haiyang Chemical, Qingdao, China) was used for column chromatography. Analytical thin layer chromatography was performed using GF254 silica gel (precoated sheets, 0.77 mm thick, Si-Jia Biochemical Plastic, Taizhou, China). Tert-butyldimethylsilyl chloride, imidazole and fluorescein (Sigma-Aldrich, CA, USA) were used without further purification. DMF was dried over 3 Å molecular sieves. The buffer was prepared by mixing aqueous solution of K_2HPO_4 (1 M) and KH_2PO_4 (1 M) (final pH 7.40) following dilution with distilled water. Water was purified using an Arium 611VF system with ultrafilter and UV lamp (Sartorius, Germany).

Sample preparation

A stock solution (0.12 mM) of FP-F was prepared by dissolving in DMSO. Solutions of NaF (1 mM), NaCl (1 mM), NaBr (1 mM), KI (1 mM), $NaNO_3$ (1 mM), $NaHCO_3$ (1 mM), Na_2HPO_4 (1 mM), Na_2SO_4 (1 mM) were prepared with doubly distilled water. The xanthine (XA) solution (1 mM) was prepared by dissolving an appropriate amount of XA in 1.00×10^{-2} M NaOH. Xanthine oxidase (XO) was purchased from Sigma. A stock solution of XO (1.00 U/mL) was prepared according to the manufacturer's instructions in 2.30 M $(NH4)_2SO_4$, 1.00×10^{-2} M sodium salicylate biology buffer, stored at 2-8ºC.

Preparation of FP-F

The fluorescein (3.00 g, 9.00 mmol) was combined with imidazole (3.40 g, 50.5 mmol) in DMF (300 mL) and stirred. To the resulting red slurry was added tert-butyldimethylsilyl chloride (6.92 g, 45.9 mmol). The reaction mixture was stirred for 12 h at room temperature. A portion of the DMF was removed (~250 mL), and the reaction mixture was diluted with saturated brine (~300 mL). The aqueous layer was extracted with ethyl acetate, and the combined organic extracts were dried over $MgSO_4$ to give a brown oil after filtration and solvent removal. The crude product was filtered through silica (7:2:1 hexanes/$C_6H_5CH_3$/ethyl acetate) and the solvents removed. Flash chromatography (9:1 hexanes/ethyl acetate) yielded a white solid (2.75 g, a yield of 55%). ^1H NMR spectra was recorded on a Bruker Avance 300 MHz. The IR spectrum was obtained on a Bruker Tensor-27. Elemental analysis was performed on Perkin Elmer Series II CHNS/O analyzer. Melting points were measured on a Yanaco micro-melting point apparatus (Yanagimoto MFG, Beijing, China).

Fluorescence analysis

For this section, 1 mL of a DMSO solution of FP-F was added into a 10 mL color comparison tube, and then various concentrations of sodium fluoride solution in turn. Then the mixture was equilibrated and set aside for 15 min before 1 mL of PBS buffer (0.10 M) was added. Then the mixture was diluted to 5.00 mL with doubly distilled water, before determination. The fluorescence intensity was measured at λ ex/em = 490/513 nm against a reagent blank at the same time. The excitation and emission slit were set to 0.5 nm and 0.5 nm, respectively. All pH measurements were made with a pH-3c digital pH-meter (Leici Device, Shanghai, China) with a combined glass-calomel

electrode. Fluorimetric spectra were obtained with FLS-920 Edinburgh fluorescence spectrometer with a xenon lamp and 1.0 cm quartz cells.

Preparation and staining of cell cultures

The concentration of RAW264.7 macrophages was adjusted to 1×10^6 cells/mL in DMEM containing 10% fetal bovine serum, 1% penicillin and 1% streptomycin and cells were added to glass coverslips in culture plates. After incubation at 37 °C for 2 hours in an MCO-15AC 5% CO_2/95% air incubator (Sanyo, Japan), non-adherent cells were removed by vigorous washing (three times) with warm serum-free medium, and the adherent cells were incubated overnight in complete medium to form macrophage monolayers. A set of cells were supplemented with NaF (4 μM $CaCl_2$ in serum-free DMEM medium, 2.5 mL) at 37 °C for 1 h. Then, some of the F^--supplemented cells were washed with serum-free DMEM, followed by adding a Ca^{2+} solution (0.4 mM in serum-free DMEM medium). After 30 min, cells were washed with DMEM, and a DMSO solution of FP-F (10 μM) was added to each well containing 2.5 mL serum-free DMEM medium, and then the mixture was incubated for 30 min. Each coverslip was washed with PBS (0.02 M, pH 7.40), followed by imaging.

Confocal fluorescence imaging

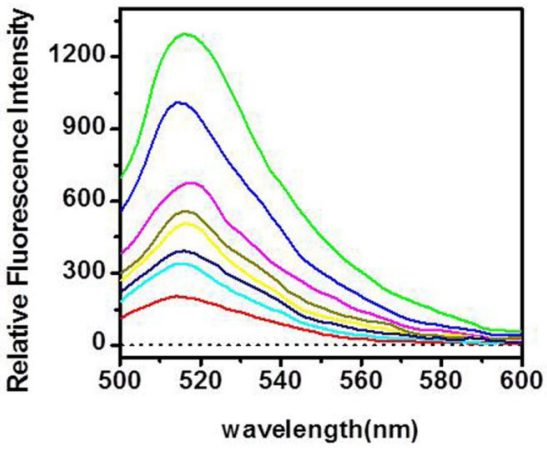
Confocal fluorescence imaging was performed with a Zeiss LSM510 laser scanning microscope containing an Axioplan 2 MOT upright microscope under a magnification of 40×. Excitation of probe-loaded cells at 488 nm was carried out with an argon ion laser, and emission was collected in a window from 505 to 550 nm using a META detection system.

RESULTS AND DISCUSSION

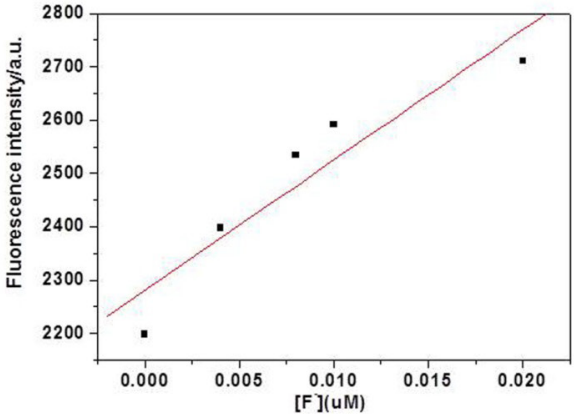
Preparation of FP-F and spectral properties

The data on the probe's molecular characterization were: 1H NMR (CDCl3, 300 MHz) δ 0.22 (12 H, s), 1.01 (18 H, s), 6.55 (2 H, s), 6.64 (2 H, d), 6.80 (2 H, d),7.30 (2 H, t), 7.78 (1 H, m), 8.02 (1 H, d). FTIR (thin film) 2954, 2931, 2859, 1769, 1611, 1466, 1421, 1281, 1254, 1219, 1181, 1084. Elemental analysis (%) calcd for $C_{32}H_{40}O_3Si_2$: C 72.73, H 7.58, Si 10.61; found: C 72.76, H 7.57, Si 10.58. M.p. 154-156 °C.

Initially, we investigated the reactivity of FP-F towards F^- in an abiotic system. The fluorescence responses of FP-F were characterized over a F^- concentration range of 0-4.0 μM with 10 μM FP-F using a fluorescence spectrometer. As indicated in Figure 1, the fluorescence intensity showed a gradual increase in a F^- concentration-dependent manner. Moreover, we observed two linear correlations between the emission intensity and the concentration of F^- (Figure 1, insert). The linear correlation range was from 1.75×10^{-9}~2.0×10^{-8} M with a correlation coefficient of 0.9889. The detection limit was 0.526 nM (relative standard deviation, n=11; 3.81%). The other linear calibration curve was from 1.0×10^{-7}~4×10^{-6} M with a correlation coefficient of 0.9892. These results demonstrated that FP-F could detect F^- in a qualitative and quantitative manner.



(a)



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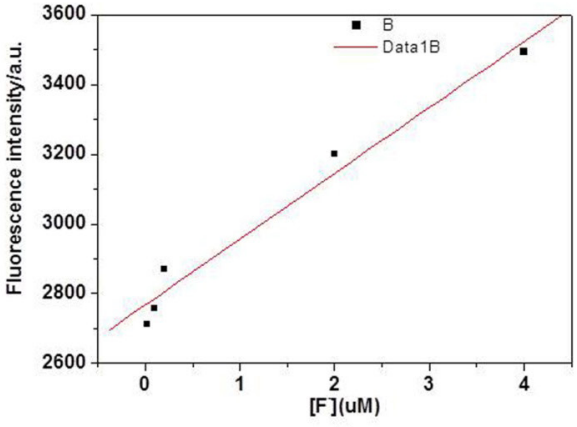


(c)

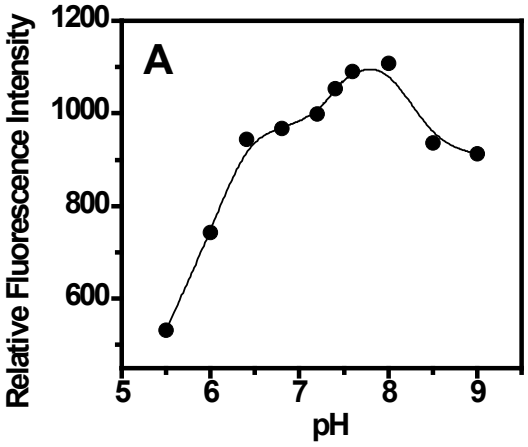
FIGURE 1 - (a) The relative fluorescence intensity of 10 μM FP-F to F^-. Spectra were acquired in 0.02 M PBS buffer at pH 7.40 (λex = 490 nm, λem =513 nm, slit width 1.0 nm). Spectra shown are for buffered [F^-] of 4, 8, 10 20 nM, and 0.1, 0.2, 2 and 4 μM. (b) The linear correlation between the emission intensity and the concentration of F^- (ranged from 1.75×10^{-9}~ 2.0×10^{-8} M). (c) The linear calibration curve between the emission intensity and the concentration of F^- (ranged from 1.0×10^{-7}~4×10^{-6} M).

Reaction conditions

To determine the optimum reaction conditions for the analysis of F^-, the effects of buffer solution and the concentration of fluorescent probe were investigated.

Effect of pH and buffer concentration

In the experiment, the pH of the medium and concentration of buffer solution had considerable effects on fluorescence intensity. The experiment showed that the optimal pH for detection F^- was in the range of 7.20-8.50 (Figure 2A). The relative fluorescence intensity of the system was high and stable in the buffer concentration range of 8-22 mM (Figure 2B). Thus 20 mM of PBS buffer solution (pH 7.40) was chosen throughout the experiment.



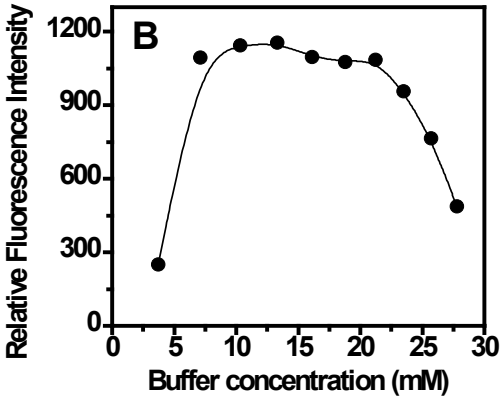


FIGURE 2 - (a). Effect of pH. (b) Effect of buffer concentration. FP-F (10 μM), NaF (4 μM), PBS (0.02 M).

Effect of the fluorescent probe concentration

The effects of probe concentration on the relative fluorescence intensity were studied under the selected conditions. The relative fluorescence intensity increased with the FP-F concentration and remained constant between 9.2 μM and 10.5 μM, then decreased with additional amounts. A suitable probe concentration was advantageous while superfluous FP-F could absorb its own fluorescence (Figure 3). Therefore, 10 μM of FP-F was selected for subsequent experiments.

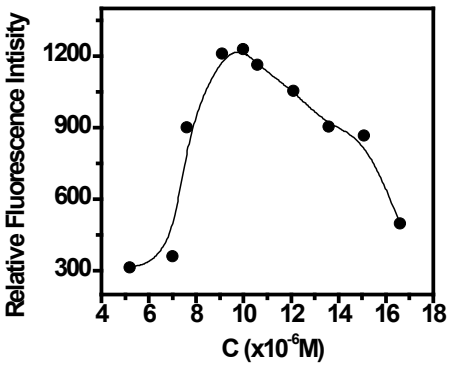


FIGURE 3 - Effect of the fluorescent probe concentration. NaF (4.0 μM), PBS (pH 7.40, 0.02 M).

Effect of reaction time

Different reaction times were tested. In a 37 °C water bath, the fluorescence intensity of the reaction system was measured at 5 min intervals. As shown in Figure 4, the fluorescence intensity of the reaction system increased with time up to 15 min, and then remained constant, indicating that the reaction was rapidly accomplished within 15 min.

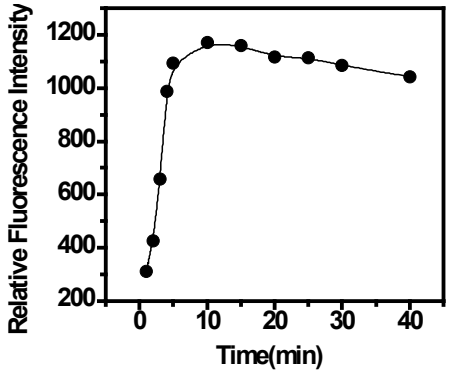


FIGURE 4 - Effects of reaction time. FP-F (10 μM), NaF (4 μM), PBS (pH 7.40, 0.02 M).

Interference from other anions and oxidative species

To investigate the specificity of FP-F towards F^-, we tested its response to various anions and oxidative species that are present in biological systems including Cl^-, Br^-, I^-, HCO_3^-, HPO_4^{2-}, SO_4^{2-}, NO_3^-, H_2O_2, HO^- and $O_2^{\cdot-}$. The observed fluorescence response is shown in Figure 5. The experimental results show that Cl^-, SO_4^{2-} and NO_3^- caused no remarkable differences in enhancing fluorescence even at a high concentration (1 mM). HCO_3^- and HPO_4^{2-} (20 μM) induced a slight enhancement of the fluorescence intensity, and Br^- and I^- quenched the fluorescence due to heavy atom effects. No interference was encountered from H_2O_2 and HO^- (4 μM). All the results indicated that FP-F is a highly selective fluorescent probe for the detection of F^-, inspired by the unique chemical reactivity of fluoride ions with silicon. The Si-F bond is a very stable, highly energetic bond of 590 kJ/mole, which is stronger than many double bonds. There is a similar specific reaction between other anions and oxidative species with silica. The fluoride attacked silica and triggered Si-O bond cleavage, which resulted in the release of the organic molecule, coupled with a spectra character-signaling event.

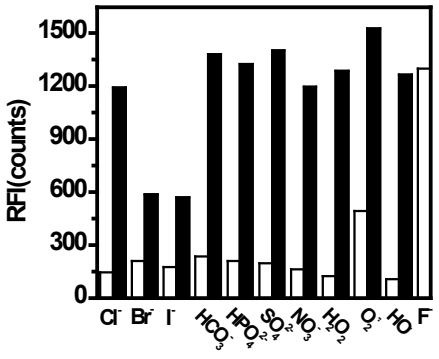


FIGURE 5 - Selectivity of FP-F for F^- at pH 7.40 (20 mM PBS). Fluorescence response of FP-F to F^-, other anions and oxidative species; bars represent the relative fluorescence intensity (RFI). Initial spectra were acquired in a 10 μM solution of FP-F. White bars represent the addition of an excess of the appropriate anion or oxidative species (1.0 mM for Cl^-, Br^-, I^-, SO_4^{2-}, and NO_3^-, 20 μM for HCO_3^- and HPO_4^{2-}, 4.0 μM for H_2O_2, HO^-, $O_2^{\cdot-}$, and F^-) to a 10 μM solution of FP-F. Black bars represented the subsequent addition of 4.0 μM F^- to the solution. The fluorescence intensity was determined at 513 nm with excitation at 490 nm (slit width 0.5 nm).

Confocal fluorescence imaging and detection of F^- in cells

RAW264.7 macrophages were seeded to evaluate the ability of FP-F to operate within living cells using confocal microscopy. As expected, macrophages incubated with 10 μM of FP-F in DMEM showed faint intracellular fluorescence (Figure 6a). In contrast, supplementing cells with 4 μM F^- in the growth medium and then staining with FP-F under the same loading conditions resulted in a marked increase in the observed intracellular fluorescence, as determined by scanning confocal microscopy on live samples (Figure 6b). Pretreatment of F^- supplemented cells with 0.4 mM $CaCl_2$ (a membrane-permeable scavenger of F^-) before FP-F staining also showed faint intracellular fluorescence (Figure 6c). Taken together, these data show that FP-F is membrane-permeable, and can respond to micromolar change in intracellular F^- levels within living cells.

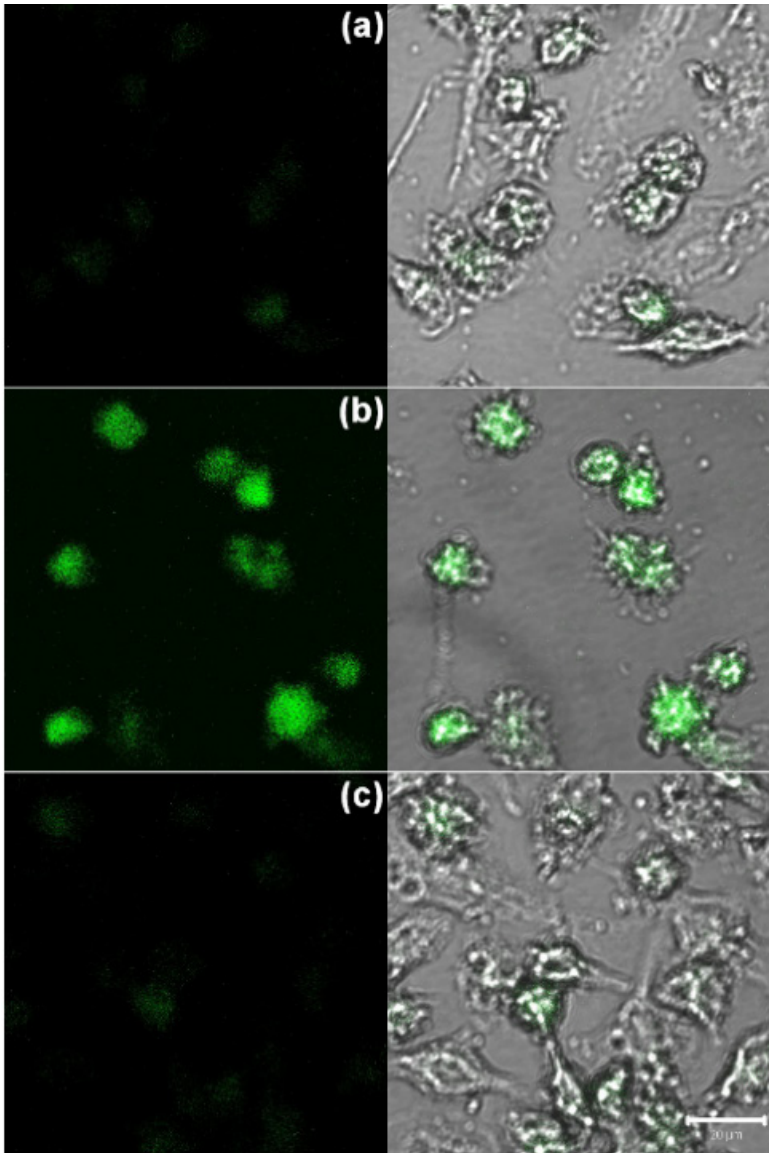


FIGURE 6 - Confocal fluorescence and brightfield images of live RAW264.7 cells. (a) Cells incubated with 10 μM FP-F for 30 min at 37 °C. (b) Cells supplemented with 4 μM F^- in the growth media for 1 h at 37 °C and stained with 10 μM FP-F for 30 min at 37 °C. (c) F^--supplemented cells pretreated with 0.4 mM of a F^- scavenger, $CaCl_2$, for 0.5 h at 37 °C before staining with 10 μM FP-F. Scale bar=20 μm.

CONCLUSION

In summary, we have described the synthesis, properties and live-cell imaging applications of FP-F, which is effective for the detection of F^- in living biological samples. In addition, desirable features of this fluorescein-based probe include good stability, favorable optical properties and a rapid reaction time with high selectivity. We anticipate that our simple, sensitive fluorescent probe will be of great benefit for biomedical researchers investigating the effects of F^- in biological systems.

AUTHOR CONTRIBUTIONS

Xuejun Cui contributed to the whole process of the preparation of the fluorescent probe, characterization, confocal fluorescence imaging tests, interpretation of the results and preparation of the manuscript. Yanchao Liang supervised the whole procedure and also contributed to the data interpretation and preparation of the manuscript. Xingong Wang and Xiaoyan Lin contributed significantly to data analysis and probe characterization.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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