CHAPTER TWO

Synthesis and application of a ratiometric probe for hydrogen peroxide

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Contents

Abstract

Molecular imaging of biological analytes provides detailed insights into signaling processes. Ratiometric probes are particularly attractive due to the ability to quantify analyte production. However, design strategies for ratiometric probes can be hindered by spectral overlap of the product and reactant species. In this chapter, we provide protocols for the synthesis and application of **RF_{620}**, a ratiometric probe for H_2O_2 displaying dramatic changes in both excitation and emission wavelengths, designed using an approach we term chemoselective alteration of fluorophore scaffolds. The probe contains a chemoselective functional group within a fluorescent xanthene scaffold, resulting in the in situ synthesis of a new fluorophore upon reaction with H_2O_2.

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Under physiological conditions, **RF_{620}** exhibits far-red to near-infrared excitation and emission, and upon reaction with H_2O_2, **RF_{620}** is chemically converted into tetramethylrhodamine, producing a significant (~66 nm) blue-shift in excitation and emission. **RF_{620}** can be used for ratiometric, molecular imaging of endogenous H_2O_2 production in living cells.

1. Introduction

Ratiometric fluorescent probes allow for the quantitative analysis of analytes in complex biological systems (Lee, Kim, & Sessler, 2015). Common design strategies for ratiometric probes rely on intramolecular charge transfer or fluorescence resonance energy transfer. Although powerful, these design approaches can suffer from spectral overlap of product and reactant species, making quantification difficult. Our lab was intrigued by the observations that: (1) reactions on functional groups placed at the bridging position of the xanthene scaffold could be utilized to tune both excitation and emission of the fluorophore (Zhou, Lai, Beck, Li, & Stains, 2016) and (2) the pioneering work of the Chang lab in the development of activity-based sensing (ABS) approaches for molecular imaging (Bruemmer, Crossley, & Chang, in press; Chan, Dodani, & Chang, 2012). Expanding upon this work, we envisioned an approach in which the chemical reactivity of a functional group within a fluorescent scaffold would be used to afford the in situ generation of a new fluorophore in the presence of a target analyte, producing a robust change in ratiometric signal (Fig. 1A) (Zhou et al., 2017). We termed this approach Chemoselective Alteration of Fluorophore Scaffolds (CAFS).

As our first target for design of a CAFS probe, we chose hydrogen peroxide (H_2O_2), now recognized as an important signaling molecule in living systems (D'Autreaux & Toledano, 2007). The physiological role of H_2O_2 has been illuminated by elegant efforts to generate ABS-based optical probes for H_2O_2 detection in living systems (Brewer, Garcia, Onak, Carroll, & Chang, 2015; Lin, Dickinson, & Chang, 2013; Lippert, De Bittner, & Chang, 2011). Indeed, this early ABS work demonstrated the selectivity of boronates for H_2O_2 in living systems. Building off of this work, we chose to incorporate a borinate functionality at the bridging position of tetramethylrhodamine (TMR). We hypothesized that the resulting TMR derivative, termed **RF_{620}**, would react selectively with H_2O_2 to produce TMR in situ (Fig. 1B). Indeed, reaction of H_2O_2 with **RF_{620}** produces

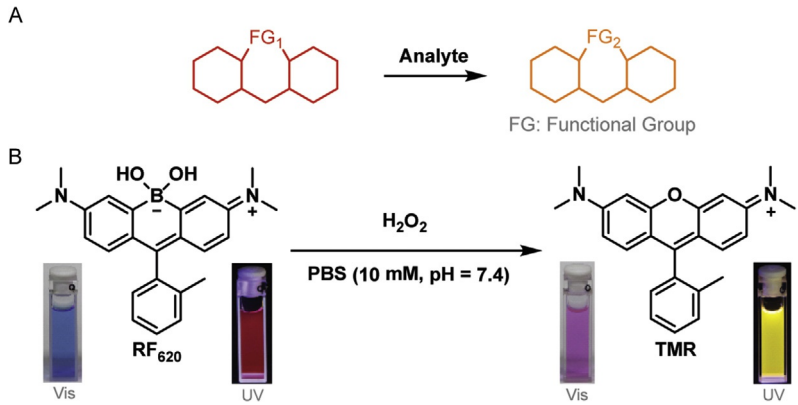


Fig. 1 The CAFS approach and its use to design a ratiometric probe for H_2O_2. (A) A schematic of the CAFS approach. Installation of a chemoselective functional group (FG) within a fluorescent scaffold results in in situ generation of a new fluorophore upon reaction with a target analyte. Production of a new fluorophore enables ratiometric detection of the target analyte. (B) **RF_{620}**, a CAFS-based probe for detection of H_2O_2. Oxidation by H_2O_2 under physiological conditions leads to formation of TMR and a clear change in absorbance and fluorescence. *Reprinted with permission from Zhou, X., Lesiak, L., Lai, R., Beck, J. R., Zhao, J., Elowsky, C. G., et al. (2017). Chemoselective alteration of fluorophore scaffolds as a strategy for the development of ratiometric chemodosimeters,* Angewandte Chemie International Edition, 56 *(15), 2017, 4197–4200, https://doi.org/10.1002/anie.201612628.*

an ~66 nm blue-shift in both excitation and emission, enabling sensitive detection of H_2O_2 with a 1720-fold change in ratiometric signal. Moreover, **RF_{620}** is selective for H_2O_2 and can be used to image endogenous H_2O_2 accumulation in living cells in response to biologically relevant stimulation. Below we provide our optimized protocols for synthesis of **RF_{620}** and its use for ratiometric imaging of H_2O_2 accumulation. These procedures can be modified to detect H_2O_2 production in applications defined by the user.

2. Synthesis of RF_{620}

In this section, we provide a detailed procedure for the synthesis of **RF_{620}**, a ratiometric probe for detection of H_2O_2 (Fig. 2). This procedure routinely yields ~128 mg of **RF_{620}** which can be used for ~17,000 downstream assays, using the conditions described below. This procedure assumes knowledge of standard organic chemistry techniques and the availability of basic organic synthesis laboratory equipment.

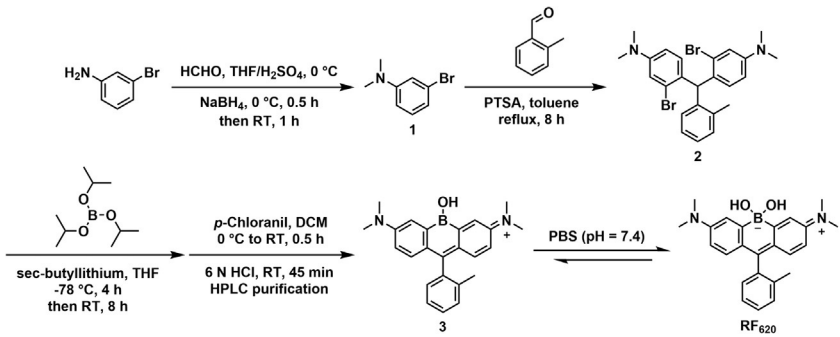


Fig. 2 Synthetic scheme for $\mathbf{RF_{620}}$.

2.1 General procedures and instrumentation

All reactions were performed in oven-dried glassware under nitrogen unless otherwise noted. All the reagents and solvents were used directly from commercial suppliers without further purification. Anhydrous THF was prepared using 3 Å molecular sieves (Bradley, Williams, & Lawton, 2010). Reaction progress was monitored by thin layer chromatography (TLC). Products were purified by flash chromatography using Merck silica gel 60 (230–400 mesh). Prep HPLC purification was done with a semi-prep column (YMC-Pack ODS-A, 5 μm, 250 × 20 mm).

2.2 Synthesis of 3-bromo-*N*,*N*-dimethylaniline (1)

1. Add aqueous formaldehyde (37%, 32.5 mL, 435.9 mmol) to a 1 L round bottom flask containing a magnetic stir bar.
2. Add tetrahydrofuran (390 mL) to the same flask.
3. Immerse the solution in an ice bath (0 °C) and stir continuously for 5 min.
4. Slowly add aqueous H_2SO_4 (3 M, 87.5 mL) using a graduated cylinder.
5. Slowly add 3-bromoaniline (25.0 g, 145.3 mmol) to the flask using a dropping funnel.
6. Add sodium borohydride (22.0 g, 581.3 mmol) to the flask over 30 min while maintaining the reaction temperature at 0 °C.
7. Allow the reaction mixture to warm up to room temperature, then stir at room temperature for an additional 1 h.
8. Immerse the reaction mixture into an ice bath (0 °C) for 10 min.
9. Slowly pour 300 mL of saturated sodium bicarbonate solution into the reaction mixture while maintaining its temperature at 0 °C.
10. Evaporate the tetrahydrofuran using a rotary evaporator.

11. Transfer the reaction mixture to a 1 L separatory funnel and extract with dichloromethane (200 mL, three times).
12. Combine the organic layers, transfer them into a 1 L separatory funnel and wash with saturated aqueous sodium chloride (200 mL).
13. Transfer the organic layer to a 1 L Erlenmeyer flask, dry the solution with a bed of sodium sulfate (Na_2SO_4), and filter the mixture.
14. Dry the filtrate using low-vacuum followed by high-vacuum to furnish a pale-yellow oil (**1**), which is used in the next step without further purification.

2.3 Synthesis of 4,4′-(*o*-tolylmethylene)bis(3-bromo-*N*,*N*-dimethylaniline) (2)

1. Sequentially add 3-bromo-*N*,*N*-dimethylaniline (**1**) (8 g, 40.2 mmol), 2-methylbenzaldehyde (1.2 mL, 10.0 mmol), and toluene (20 mL) to a 25 mL round bottom flask containing a magnetic stir bar.
2. Add p-Toluenesulfonic acid monohydrate (1.9 g, 10.0 mmol) to the reaction mixture.
3. Attach a Dean-Stark apparatus (10 mL scale) to the round bottom flask and add 10 mL of toluene to the trap of the Dean-Stark apparatus.
4. Reflux the reaction mixture at 130 °C for 8 h.
5. Cool down the reaction mixture then evaporate the toluene by rotovap.
6. Dissolve the resulting residue in 20 mL dichloromethane, transfer the solution to a 250 mL separatory funnel.
7. Add 100 mL dichloromethane into the separatory funnel.
8. Slowly pour a solution of saturated aqueous sodium bicarbonate (50 mL) into the separatory funnel and shake vigorously with frequent venting.
9. Wash the organic phase with saturated aqueous sodium chloride solution (50 mL).
10. Transfer the organic phase to a 250 mL Erlenmeyer flask and dry the solution with anhydrous sodium sulfate (Na_2SO_4).
11. Filter the mixture and dry the filtrate using low vacuum rotovap to furnish a brownish oil.
12. The resulting oil is further purified by flash chromatography (silica gel, 230–400 mesh, 15:1 hexanes/ethyl acetate) to afford a white solid (**2**) (2.7 g, 53%) after solvent evaporation.
13. The identity of the product should be confirmed using NMR and MS by referring to the published spectra (Zhou et al., 2016).

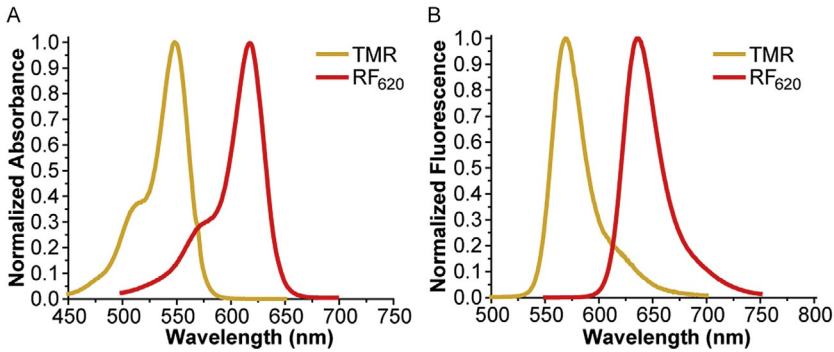
2.4 Synthesis of the borinic acid form of RF_{620} (3)

1. Flame dry a 50 mL round bottom flask for 10 min, add a dry stir bar, and cap the flask with a rubber septum.
2. Charge the flask with nitrogen.
3. Dissolve 4,4′-(*o*-tolylmethylene)bis(3-bromo-*N*,*N*-dimethylaniline) (**2**) (200 mg, 0.4 mmol) in anhydrous tetrahydrofuran (10 mL) and add the solution into the flask using a 10 mL syringe.
4. Immerse the reaction mixture in an acetone/dry ice bath for 10 min.
5. Add sec-butyllithium (1.4 M in cyclohexane, 626 μL) dropwise through a 1 mL syringe to the reaction mixture.
6. Stir the reaction in the acetone/dry ice bath for 1 h.
7. Add triisopropyl borate (101 μL, 0.44 mmol) to the reaction mixture dropwise using a 500 μL microscale syringe.
8. Stir the reaction in the acetone/dry ice bath for 3 h.
9. Transfer the reaction to an ice bath and let it warm up gradually to room temperature. Stir the reaction for another 8 h.
10. Add a solution of saturated aqueous ammonium chloride (10 mL) to the reaction mixture to quench the reaction.
11. Transfer the reaction to a 50 mL separatory funnel.
12. Add dichloromethane (20 mL) and shake vigorously with frequent venting.
13. Dry the organic phase using anhydrous sodium sulfate.
14. Filter the mixture and dry the filtrate using low-vacuum followed by high-vacuum rotovap to furnish a pale-yellow oil.
15. In a 25 mL round bottom flask, dissolve the pale-yellow oil in anhydrous dichloromethane (10 mL).
16. Immerse the solution into an ice bath for 5 min.
17. To the solution, add p-chloranil (294 mg, 1.2 mmol).
18. Transfer the reaction mixture to room temperature and let it proceed for 30 min.
19. Place the reaction mixture in an ice bath for 10 min.
20. Filter the reaction suspension with vacuum and wash the filter cake with cool dichloromethane.
21. Evaporate the dichloromethane using a rotovap.
22. Dissolve the resulting dark blue solid in 6 N hydrochloric acid (10 mL) and stir at room temperature for 45 min.
23. Filter the reaction mixture and dry the filtrate by rotovap.
24. Dissolve the resulting residue in 8 mL of HPLC solvent (50% acetonitrile in water with 0.1% trifluoroacetic acid).

25. Filter the solution through a 0.22 μm filter and then purify via HPLC using a gradient from 5% acetonitrile in water (containing 0.1% trifluoroacetic acid) to 95% acetonitrile in water (containing 0.1% trifluoroacetic acid) over 40 min. Monitor absorption at 280 and 365 nm.
26. Lyophilize fractions containing the product to yield a dark blue solid (128 mg, 67%).
27. Verify the structure and purity of the product by NMR and MS by referring to the published spectra (Zhou et al., 2017).

3. In vitro detection of hydrogen peroxide

With a purified sample of **RF_{620}** now in-hand, H_2O_2 can be detected in samples using a ratiometirc fluorescence assay (Zhou et al., 2017). Exposure of **RF_{620}** to H_2O_2 leads to formation of TMR with a rate constant of 0.15 M^{-1} s^{-1}. The resulting ~66 nm blue-shift in excitation and emission relative to **RF_{620}**, allows for facile spectroscopic monitoring of reactant and product (Fig. 3A and B). Additionally, previous work has also shown that



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Dyes	λ_{max} (nm)	λ_{em} (nm)	ε (M^{-1} cm^{-1})	ϕ	ε × ϕ (M^{-1} cm^{-1})
TMR	550	570	74,500	0.31	23,095
RF_{620}	620	636	109,000	0.36	39,000

Fig. 3 Comparison of the photophysical properties of **RF_{620}** and TMR. (A) Normalized absorption spectra. (B) Normalized emission spectra. (C) Summary of photophysical parameters. *Reprinted with permission from Zhou, X., Lesiak, L., Lai, R., Beck, J. R., Zhao, J., Elowsky, C. G., et al. (2017). Chemoselective alteration of fluorophore scaffolds as a strategy for the development of ratiometric chemodosimeters,* Angewandte Chemie International Edition, 56 *(15), 2017, 4197–4200, https://doi.org/10.1002/anie.201612628.*

RF$_{620}$ and TMR display similar brightness (Fig. 3C), allowing for sensitive detection of H_2O_2. Below we provide a protocol for validation of **RF$_{620}$** activity and selectivity towards H_2O_2. This can be used to confirm the activity of **RF$_{620}$** as well as analyze in vitro samples.

3.1 General reagents and instrumentation

DMSO (Sigma, D8418, for molecular biology)
PBS (Gibco, 10010023, 1×, pH 7.4)
H_2O_2 (Sigma, 516813, 50 wt% in H_2O)
Potential off-target reactive oxygen species (Srikun, Miller, Dornaille, & Chang, 2008; Sun et al., 2014)
Vortex mixer (VWR, 97043-562)
Synergy H1 Hybrid Multi-Mode Plate Reader (BioTek Instruments) or similar
Eppendorf tubes (1.5 mL, Fisher Scientific, 05-402-27)
Nontreated clear flat bottom 96-well assay plates (Corning 3370)

3.2 In vitro H_2O_2 assay

1. In a series of 1.5 mL eppendorf tubes; add 999 μL PBS (pH 7.4) containing H_2O_2 (200 μM), superoxide (20 μM), superoxide (20 μM) + superoxide dismutase (SOD, 15 U mL^{-1}), superoxide (20 μM) + catalase (15 U mL^{-1}), tert-butyl hydrogen peroxide (TBHP) (200 μM), HOCl (20 μM), NO (200 μM), ·O^{t}Bu (200 μM), ·OH (200 μM), or pure PBS (blank).

   Note: Superoxide dismutase and catalase are used to assess off-target signal from superoxide decomposition.
2. To each tube add 1 μL of a 10 mM **3** stock prepared in DMSO. Stock concentrations can be determined using the extinction coefficient of **RF$_{620}$** in PBS (Fig. 3C).

   Note: Previous experiments have shown that **3** equilibrates to **RF$_{620}$** at pH 7.4 (Zhou et al., 2017).
3. Vortex each sample.
4. Transfer 200 μL of each sample to one well of a 96-well plate. Repeat three times for a total of three assays per sample.
5. Overlay each well with 70 μL mineral oil to prevent evaporation.
6. Setup the fluorescence plate reader to detect the fluorescence of **RF$_{620}$** and TMR. **RF$_{620}$** signal is readout by 620 determined by excitation

at 610 nm and monitoring emission at 636 nm. For TMR, 544 nm excitation is used, and emission is monitored at 570 nm.

7. Record the fluorescence data at intervals of 5, 15, 30, 60, 120, and 180 min.
8. Determine the ratio of TMR/**RF_{620}** fluorescence by dividing the emission of TMR by the emission of **RF_{620}**.
9. The ratio of TMR/**RF_{620}** fluorescence is averaged for each sample and normalized to the 5 min time point. The normalized TMR/**RF_{620}** fluorescence ratio is then plotted for each sample over time (Fig. 4).

Note: Using this procedure we routinely obtain ~1700-fold increases in TMR/**RF_{620}** fluorescence in the presence of H_2O_2.

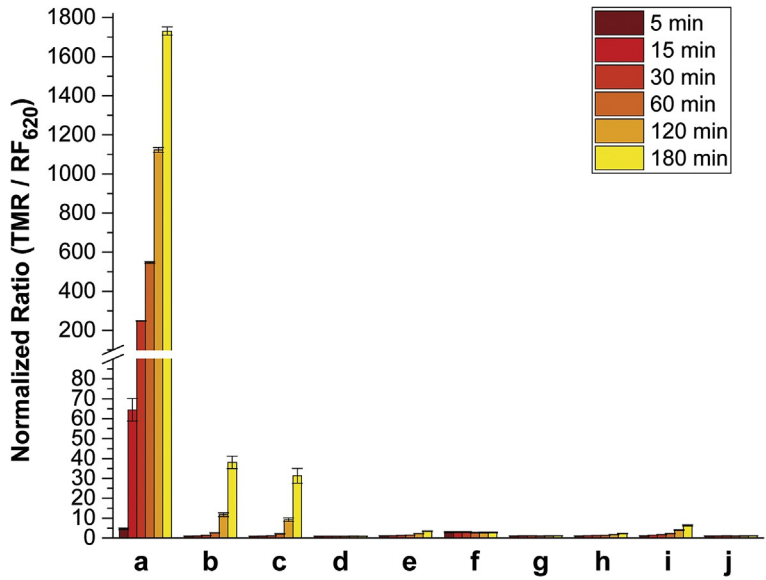

Fig. 4 Selectivity of **RF_{620}** (10 μM) toward different reactive oxygen species over time. (a) H_2O_2, (b) superoxide, (c) superoxide with superoxide dismutase (SOD, 15 U mL^{-1}), (d) superoxide with catalase (15 U mL^{-1}), (e) tert-Butyl hydrogen peroxide (TBHP), (f) HOCl, (g) NO, (h) ·O^{t}Bu, (i) ·OH, and (j) blank. *Reprinted with permission from Zhou, X., Lesiak, L., Lai, R., Beck, J. R., Zhao, J., Elowsky, C. G., et al. (2017). Chemoselective alteration of fluorophore scaffolds as a strategy for the development of ratiometric chemodosimeters,* Angewandte Chemie International Edition, 56 *(15), 2017, 4197–4200, https://doi.org/10.1002/anie.201612628.*

4. Ratiometric imaging of hydrogen peroxide accumulation in living cells

After validation, $\mathbf{RF_{620}}$ can be used to ratiometrically detect H_2O_2 accumulation in mammalian cells. Phorbol 12-myristate 13-acetate (PMA) (Xu, Zhang, Yu, Gao, & Shao, 2016) and human epidermal growth factor (EGF) (Ermakova et al., 2014) can be used as positive controls to stimulate production of endogenous H_2O_2. Ebselen is used as a H_2O_2 scavenger to verify the selectivity of $\mathbf{RF_{620}}$ signal production in cells (Muller, Cadenas, Graf, & Sies, 1984). This protocol can be adapted to investigate H_2O_2 production in response to user-defined conditions.

4.1 General reagents and instrumentation

HeLa cells (ATTC, CCL-2)
Dulbecco's Modified Eagle Medium (DMEM, high glucose, GlutaMAX Supplement, pyruvate, Gibco, 10569010)
Imaging Media (DMEM, high glucose, HEPES, no phenol red, Gibco, 21063029)
Fetal Bovine Serum (FBS, Gibco, 16000044)
Antibiotic-Antimycotic (Anti-Anti, 100 ×, Gibco, 15240062)
DPBS (with calcium and magnesium, Gibco, 14040133)
Phorbol 12-myristate 13-acetate (PMA, Sigma, P1585)
Ebselen (Sigma, E3520)
Human epidermal growth factor (EGF, Sigma, E9644)
Hoechst 33342 (Invitrogen, H1399)
35 mm tissue-culture treated culture dishes (Corning, CLS430165)
Confocal imaging system with the following lasers and emission filters: blue (excitation: 405 nm, emission filter: 425–475 nm), green (excitation: 490 nm, emission filter: 500–530 nm), red (excitation: 560 nm, emission filter: 560–617 nm), and deep red (excitation: 640 nm, emission filter: 663–738 nm)
ImageJ software (Schneider, Rasband, & Eliceiri, 2012)

4.2 Ratiometric detection of endogenous H_2O_2 production in living cells

1. Prepare cell culture media as follows. To 445 mL of DMEM (Gibco, 10569010) add 50 mL FBS and 5 mL Anti-Anti. Sterile filter using a

0.22 μm filter. This media contains 10% FBS and 1 × Anti-Anti, store at 4 °C and warm to 37 °C before use.

2. Prepare the indicated reagents as follows:
   a. PMA is dissolved in ethanol to make a 1 mg mL^{-1} stock, store at −20 °C.
   b. Ebselen is dissolved in DMSO to make a 1 mg mL^{-1} stock, store at −20 °C.
   c. EGF is dissolved in 10 mM acetic acid to make a 1 mg mL^{-1} stock, store at −20 °C.
   d. Hoechst 33342 is dissolved in DMSO to make a 10 mg mL^{-1} stock, store at −20 °C.
3. Seed five cultures of HeLa cells on 35 mm culture dishes and grow to 80% confluency in an incubator at 37 °C with a humidified, 5% CO_2 atmosphere.
4. Remove the cell culture medium and wash with prewarmed DPBS (37 °C).
5. Add 2 mL DPBS containing 0.5 μg mL^{-1} Hoechst 33342 and 10 μM **RF_{620}** to all the dishes.
6. Place the dishes back in the CO_2 incubator for 30 min.
7. Label the dishes as sample i, ii, iii, iv, and v.
8. Remove the DPBS solution and wash the cells with prewarmed DPBS three times.
9. Add reagent to each dish as follows:
   a. 2 mL DPBS to dish i.
   b. 2 mL DPBS containing 1 μg mL^{-1} PMA to dish ii.
   c. 2 mL DPBS containing 1 μg mL^{-1} PMA and 5 μM ebselen to dish iii.
   d. 2 mL DPBS containing 500 ng mL^{-1} EGF to dish iv.
   e. 2 mL DPBS containing 500 ng mL^{-1} EGF and 5 μM ebselen to dish v.
10. Put all five dishes back in the CO_2 incubator for 90 min.
11. Aspirate off the media and wash the cells with prewarmed DPBS three times.
12. Add 2 mL prewarmed imaging media and image.
13. If using a confocal imaging setup as described above, use the blue channel for Hoechst 33342, the red channel for TMR, and the deep red channel for **RF_{620}**. Use a 60 × objective to find three different places on the dish to take images. The images should be taken with the same parameters to allow for subsequent analysis.

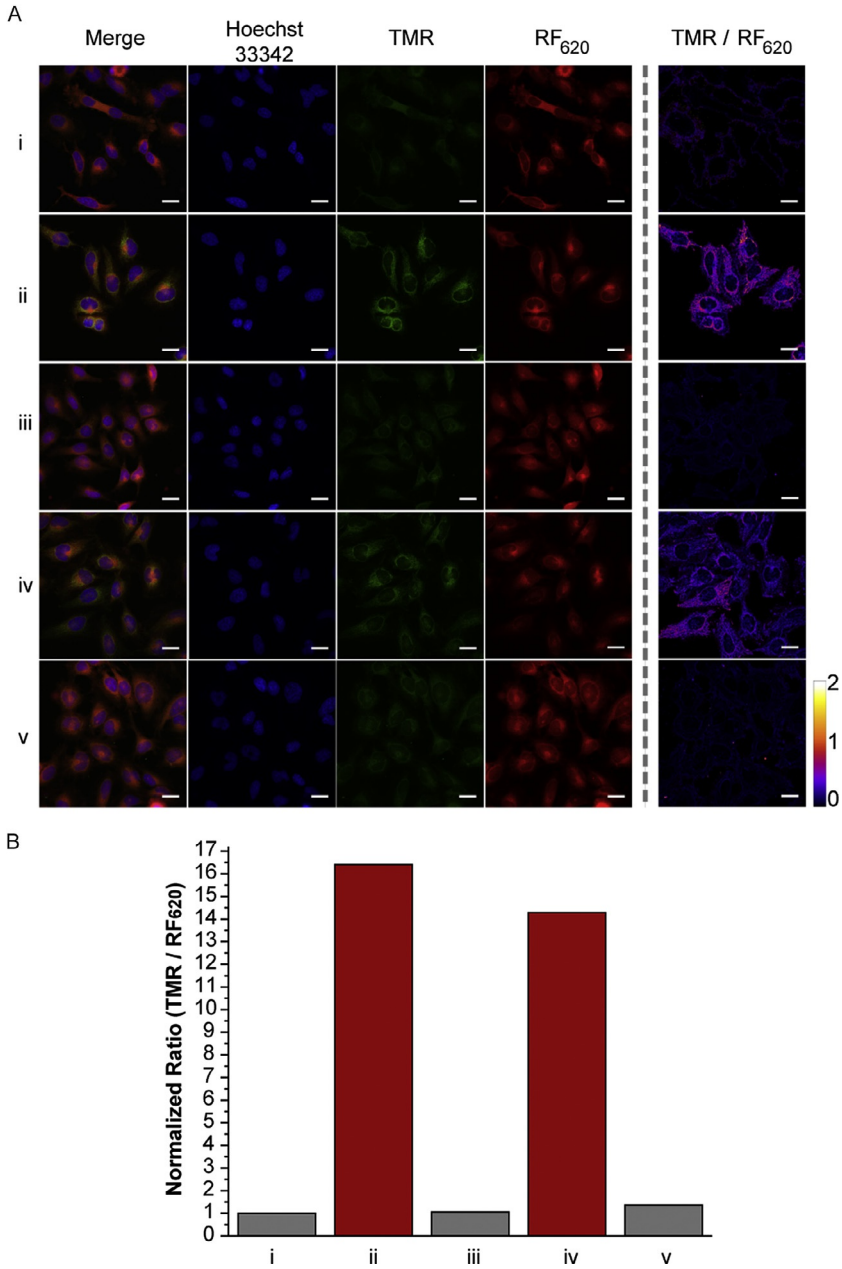

Fig. 5 Ratiometric detection of endogenous production of H_2O_2 in living cells by **RF_{620}**. (A) Confocal fluorescence microscopy imaging of living HeLa cells incubated with 10 μM **RF_{620}** for 30 min, washed (3 ×), and incubated with (i) blank, (ii) PMA (1 μg mL^{-1}), (iii) PMA (1 μg mL^{-1}) with ebselen (5 μM), (iv) EGF (500 ng mL^{-1}) or (v) EGF (500 ng mL^{-1}) with ebselen (5 μM) for 90 min. (B) Comparison of the normalized ratio between TMR and **RF_{620}** emission. Scale bar: 25 μm. *Adapted with permission from Zhou, X., Lesiak, L., Lai, R., Beck, J. R., Zhao, J., Elowsky, C. G., et al. (2017). Chemoselective alteration of fluorophore scaffolds as a strategy for the development of ratiometric chemodosimeters,* Angewandte Chemie International Edition, 56 *(15), 2017, 4197–4200, https://doi.org/10.1002/anie.201612628.*

14. Use ImageJ software to obtain quantified data for Hoechst 33342, TMR, and **RF$_{620}$** channels.
    a. Using these conditions we routinely obtain ~16- and ~14-fold increases in the TMR/**RF$_{620}$** fluorescence ratio with PMA and EGF stimulation, respectively (Fig. 5) (Zhou et al., 2017). Samples containing ebselen, should display a TMR/**RF$_{620}$** fluorescence ratio similar to the blank (i), validating the selectivity of the assay.
    b. Previous work has demonstrated that TMR formation is primarily observed in the mitochondria when using **RF$_{620}$** (Zhou et al., 2017).

5. Summary and conclusions

We have developed a new approach for the design of ratiometric probes, termed CAFS. Utilizing this approach, we designed **RF$_{620}$** a ratiometric fluorescent probe for H_2O_2. **RF$_{620}$** produces a ~66 nm blue-shift in excitation and emission upon reaction with H_2O_2, resulting in a 1720-fold increase in ratiometric fluorescence signal. In this chapter, we provide protocols for the synthesis of **RF$_{620}$** and its application to imaging endogenous H_2O_2 production. This probe enables quantitative imaging of H_2O_2 accumulation in living systems. We envision that **RF$_{620}$** will provide new insights into H_2O_2-mediated cellular signaling process.

Acknowledgments

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