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HKOCl-3: A fluorescent hypochlorous acid probe for live-cell and *in vivo* imaging and quantitative application in flow cytometry and 96-well microplate assay†

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Ultra-selective and ultra-sensitive probes for hypochlorous acid (HOCl), one of the poorly understood reactive oxygen species (ROS), are urgently needed to unravel HOCl functions in important biological processes such as development and innate immunity. Based on a selective oxidative *O*-dearylation reaction of 2,6-dichlorophenol toward HOCl over other reactive oxygen species, we have developed a novel fluorescent probe **HKOCl-3** for HOCl detection with ultra-selectivity, ultra-sensitivity and rapid turn-on response. The functional robustness of **HKOCl-3** for endogenous HOCl detection and imaging has been thoroughly scrutinized in multiple types of phagocytes and *in vivo* imaging in live intact zebrafish embryos. Furthermore, **HKOCl-3** has been successfully applied to detection of endogenous HOCl by 96-well microplate assay and flow cytometry. Therefore, **HKOCl-3** holds great promise as a versatile molecular tool that enables innovative investigation on HOCl biology and ROS-related diseases in multiple detection modalities.

Introduction

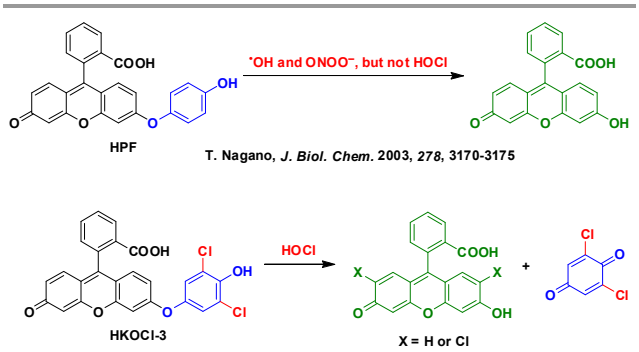
A wide spectrum of reactive oxygen/nitrogen species such as $O_2^{\bullet-}$, H_2O_2, $^{\bullet}NO$, HOCl and $ONOO^-$ form the chemical underpinnings of cellular life. Their actions are tied with diverse physiological and pathological conditions, e.g. development, aging, inflammation, autoimmunity, cancer and neurodegeneration.[1] In particular, HOCl, produced by the myeloperoxidase-catalyzed reaction of H_2O_2 with chloride ion (Cl^-), has been reputed to be a key microbicidal effector in innate immunity.[2-4] Additionally, aberrant accumulation of HOCl in phagocytes can trigger tissue damage or remodeling, with profound implications for many human diseases including cardiovascular diseases,[5] inflammatory diseases,[6] acute lung injury,[7] nephropathies,[8] cystic fibrosis,[9] neurodegenerative disorders,[10] and certain cancers.[11]

The past decade has witnessed tremendous efforts toward the development of fluorescent probes for HOCl imaging and detection based on distinct strategies.[12] However, a lack of suitable fluorescent probes for selective and sensitive HOCl detection in quantitative platforms such as flow cytometry and 96-well microplate assays has seriously impeded advances in understanding the roles of HOCl in various biological processes. To meet the demands of intricate biological inquiry, especially quantitative detection in cells, highly versatile and robust HOCl probes with ultra-selectivity (i.e. >50-fold more sensitive toward HOCl than other reactive oxygen/nitrogen species), ultra-sensitivity (i.e. >100-fold enhancement toward 1 equiv HOCl) and rapid turn-on response (i.e. <1 min) need to be developed.[13]

Here we report the development of a novel HOCl fluorescent probe **HKOCl-3** with ultra-selectivity, ultra-sensitivity and rapid turn-on response for live-cell and *in vivo* imaging and quantitative application in flow cytometry and 96-well microplate assay.

Results and discussion

Design and synthesis of fluorescent probe HKOCl-3



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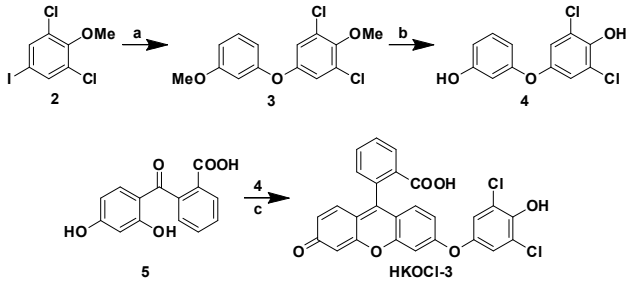
Scheme 1 Design of the new fluorescent probe **HKOCl-3**.



Scheme 2 Synthesis of fluorescent probe **HKOCl-3**. Reagents and conditions: (a) CuI, *N*,*N*-dimethylglycine hydrochloride, Cs_2CO_3, dioxane, 90 °C, 24 h, 81%; (b) HBr (48 wt. %), AcOH, reflux,12 h, 91%; (c) TFA, 100 °C, 12 h, 61%.

A widely used fluorescent probe HPF, reported by Nagano and coworkers,[12a] shows strong response toward $^{\bullet}$OH and $ONOO^-$, but not HOCl. As part of our continued efforts in developing fluorescent probes for detection of specific reactive oxygen species,[14] we noticed that, by incorporating *ortho* halogen substituents, the selectivity of resulting phenols toward HOCl over other reactive oxygen/nitrogen species can be dramtically improved. Therefore, we designed a new fluorescent probe **HKOCl-3** (Scheme 1) by incorporating two *ortho* chlorine substituents into HPF. Since the introduction of two chlorine atoms (2,6-dichlorophenol moiety) lowers the pK_a of the phenol by greater than 3 orders of magnitude (from 10.0 to 6.79), the election-rich phenoxide form of **HKOCl-3** will be dominant under physiological conditions (pH = 7.4), resulting in great enhancement of its nucleophilicity and reactivity toward HOCl. In addition, the phenoxide form of **HKOCl-3** can quench the fluorescence by a photoinduced electron transfer (PeT) process more efficiently, affording lower basal fluorescence. Herein, we report **HKOCl-3** is indeed an excellent fluorescent probe for HOCl detection with ultra-selectivity, ultra-sensitivity and rapid turn-on response in live cells and *in vivo*.

As shown in Scheme 2, the probe **HKOCl-3** was readily synthesized from diarylether **4** (obtained by Ullmann coupling of 3-methoxyphenol and aryl iodide **2**, followed by demethylation) and benzoic acid **5** (prepared from fluorescein) in good yield.

Reactivity and selectivity of HKOCl-3 for HOCl

For chemical characterization, we first investigated the spectroscopic properties of fluorescent probe **HKOCl-3** (10 μM) in potassium phosphate buffer (0.1 M, pH 7.4, 0.1% DMF) at 25 °C. **HKOCl-3** showed an obvious absorption peak at 455 nm (ε = 2.3 × 10^4 M^{-1} cm^{-1}; Fig. S1), and as expected, its fluorescence is completely quenched (Φ = 0.001). Upon exposure to 1 equiv HOCl, a >358-fold enhancement in fluorescence intensity was observed, demonstrating the ultra-sensitivity of this probe in aqueous solution (Fig. 1a). A linear relationship of fluorescence intensity of **HKOCl-3** at 527 nm with the concentrations of HOCl (0–10 μM) was observed (Fig. 1b). Moreover, the detection limit of **HKOCl-3** was calculated to be as low as 0.33

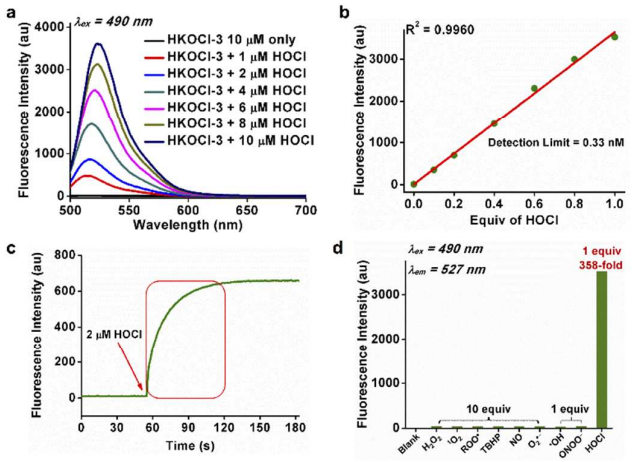


Fig. 1 Characterization of **HKOCl-3** performance in chemical systems. The fluorescent probe **HKOCl-3** was dissolved in 0.1 M potassium phosphate buffer at pH 7.4 to a final concentration of 10 μM (containing 0.1% DMF). (**a**) Fluorescence emission spectra of the fluorescent probe **HKOCl-3** upon treatment with different amounts of HOCl (0–10 μM); (**b**) fluorescence intensity of **HKOCl-3** (at 527 nm) as a function of HOCl (at 0–10 μM); (**c**) Time course of fluorescence intensity of **HKOCl-3** at 527 nm after treatment with 2 μM HOCl (time range 0–180 s); (**d**) Fluorescence responses of probe **HKOCl-3** toward various reactive oxygen/nitrogen species. The fluorescence spectra were recorded at 30 min with an excitation at 490 nm.

nM (3σ/k). This again confirms the ultrasensitivity of **HKOCl-3**, compared with previously reported HOCl probes. In particular, the reaction of **HKOCl-3** (10 μM) with HOCl caused a dramatic time-dependent fluorescence increase, which was complete within 1 min (Fig. 1c), suggesting a remarkably fast reaction between the probe and HOCl.

Interestingly, upon treatment with HOCl, **HKOCl-3** underwent a bathochromic shift in absorption peak (from 455 nm to 499 nm; Fig. S1, ESI†) and fluorescence emission peak (Fig. 1a and S2, ESI†). This indicates that the oxidation of **HKOCl-3** produced fluorescent products, such as fluorescein and its mono- or di-chlorinated derivatives (Scheme 1), which have been confirmed by ESI-MS analysis (ESI†). Moreover, the fluorescence signal remained unchanged for 30 min (Fig. S3, ESI†), indicating the striking chemo-stability of the fluorescent products toward highly reactive HOCl in the reaction mixture.

Next, the selectivity of **HKOCl-3** was examined by measuring its fluorescence response upon treatment with various analytes in potassium phosphate buffer (0.1 M, pH 7.4). As shown in Fig. 1d (ESI†) and Table S1 (ESI†), 1 equiv of HOCl gave a >358-fold enhancement in fluorescence intensity, while 10 equiv (100 μM) of other biologically relevant reactive oxygen/nitrogen species (H_2O_2, $^1O_2$, ROO$^{\bullet}$, TBHP, $^{\bullet}$NO and $O_2^{\bullet-}$) only triggered negligible fluorescence increase. Specifically, the probe exhibited >83-fold higher increase in fluorescence intensity toward HOCl over other potentially interfering highly reactive oxygen species ($^{\bullet}$OH and $ONOO^-$). Collectively, these results demonstrate the ultra-selectivity of **HKOCl-3** for HOCl.

Biocompatibilty of **HKOCl-3** was then examined. **HKOCl-3** exhibits excellent stability toward pH changes (3.0–10.8; Fig.

S4, ESI†) and its fluorescence response toward HOCl was significant across a wide range of pH (4–10) relevant to cellular processes (Fig. S5, ESI†). The interference from common coexisting biological substances (Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, glutathione, etc.) was found to be minimal (Fig. S6, ESI†). Collectively, these results suggest that probe **HKOCl-3** could be used to detect HOCl reliably in complex cellular milieux.

Evaluation of HKOCl-3 for endogenous HOCl detection in live cells and *in vivo*

As a first step to establish the biological applications of **HKOCl-3**, the cytotoxicity of **HKOCl-3** was assessed in RAW264.7 mouse macrophages. **HKOCl-3** was found to be virtually nontoxic when used at up to 20 μM after 24-h incubation (Fig. S7, ESI†). Then, we examined the performance of this probe in confocal imaging of endogenous HOCl in different types of activated phagocytes: RAW264.7 mouse macrophages, BV-2 mouse microglia, THP-1 human monocytic macrophages[15] and primary human polymorphonuclear neutrophils (PMNs). To induce endogenous HOCl, different types of phagocytes were co-incubated with **HKOCl-3** (1 μM) and the PKC activator PMA (phorbol myristate acetate: 500 ng/mL) for 30 min, followed by confocal imaging (Fig. 2, S8 and S9, ESI†). As expected, unstimulated phagocytes loaded with **HKOCl-3** showed barely detectable background fluorescence signals (Fig. 2). In the presence of PMA, the fluorescence signal was significantly enhanced in all four types of cells. This result suggests that endogenous HOCl production can be robustly visualized with **HKOCl-3** in activated phagocytes.

96-Well microplate fluorescence measurement is an indispensable platform for the development of low-cost, high-throughput screening assays, and is preferred over flow cytometry for its ability to preserve physical integrity of live adherent cells during tests. However, high background noise of 96-well microplate assays prohibits the successful application of numerous fluorescent probes despite their excellent performance in chemical systems. To test the applicability of **HKOCl-3** in this platform, RAW264.7 mouse macrophages were co-incubated with **HKOCl-3** (2 μM) and various concentrations of PMA (0–1000 ng/mL) for 30 min before fluorescence measurement. We found that **HKOCl-3** gave a dose-dependent response toward PMA stimulation in RAW264.7 cells in a sensitive manner (Fig. 3a), which is in agreement with our confocal imaging results (Fig. S10, ESI†).

Next, the selectivity of **HKOCl-3** toward HOCl was thoroughly scrutinized by examining the effects of a series of enzyme inhibitors. RAW264.7 cells were co-incubated with **HKOCl-3** (2 μM) and PMA (500 ng/mL) in the presence or absence of one of the following inhibitors: the PKC inhibitors Gö6983, Gö6976 and Ro32-0432, the NOX (NADPH oxidase) inhibitor DPI (diphenyleneiodonium chloride), and the MPO (myeloperoxidase) inhibitor ABAH (aminobenzoic acid hydrazide). The observation that the fluorescence signal was potently and dose-dependently reduced by these inhibitors confirms the selectivity of **HKOCl-3** toward HOCl detection, as PKC and NOX are known to be upstream of MPO on the pathway for endogenous HOCl production in activated RAW264.7 cells (Fig. 3b, ESI†).

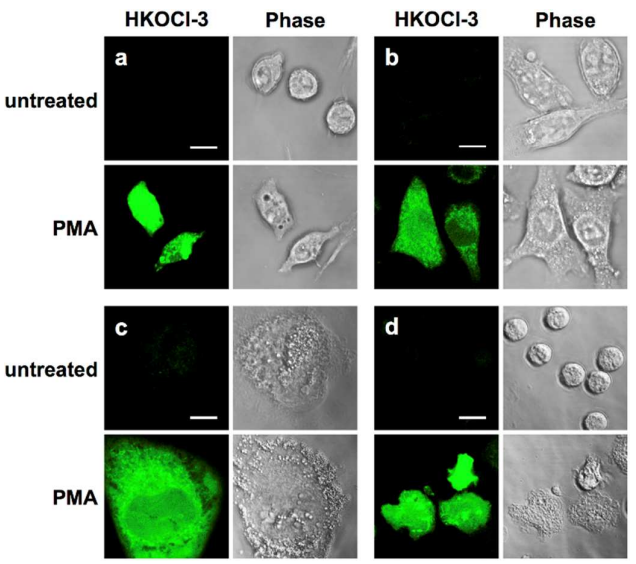


Fig. 2 Detection of endogenous HOCl by **HKOCl-3** in multiple cell types. Four types of phagocytes: RAW264.7 (**a**), BV-2 (**b**) and differentiated THP-1 (**c**), and primary human PMN (**d**) cells were co-incubated with **HKOCl-3** (1 μM) with or without PMA (phorbol myristate acetate: 500 ng/mL), before confocal imaging. Scale bars represent 10 μm.

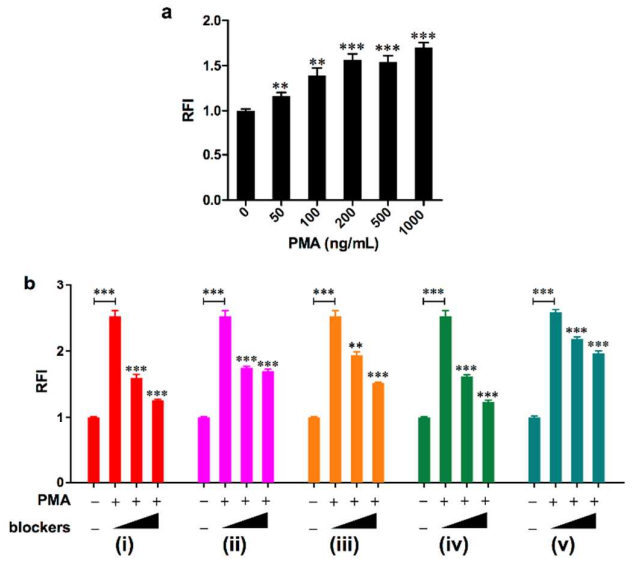


Fig. 3 Detection of endogenous HOCl with **HKOCl-3** in 96-well microplate assay. (**a**) Dose-dependent **HKOCl-3** fluorescence changes in response to PMA in RAW264.7 cells. Fluorescence intensity of **HKOCl-3** (2 μM) after 30-min PMA challenge was measured. (**b**) Validation of **HKOCl-3** selectivity in PMA-stimulated RAW264.7 cells in the presence of various enzyme inhibitors. Probe (2 μM) and inhibitors were co-incubated for 30 min before measurement. (i) Gö6983 (0, 25 and 100 nM); (ii) Gö6976 (0, 25 and 100 nM); (iii) Ro32-0432 (0, 25 and 100 nM); (iv) DPI (0, 10 and 50 nM); (v) ABAH (0, 50 and 200 μM). Results are representative of at least three independent experiments. RFI: relative fluorescence intensity.

The 96-well microplate platform has also been applied to evaluate the HOCl scavenging efficiency of five organosulfur compounds, namely: α-lipoic acid (which participates in

aerobic metabolism), NAC (*N*-acetylcysteine), L-methionine, L-cysteine, and L-cystine (Fig. 4 and S11, ESI†). Except for L-cystine, which is a dimeric form of oxidized L-cysteine with very poor aqueous solubility (200 μM in HBSS), all scavengers in mM range were able to remove HOCl generated in cells, with an order of scavenging efficiency as follows: L-cysteine < L-methionine < α- lipoic acid < NAC. Therefore, for the first time, we quantitatively and directly evaluated the HOCl removal effects of these scavengers, which support findings generated in cell-free systems or by indirect measurement.[16] To the best of our knowledge, **HKOCl-3** is the first small-molecule probe that can be successfully applied to 96-well microplate assay and screening for molecules that scavenge HOCl or modulate its production.

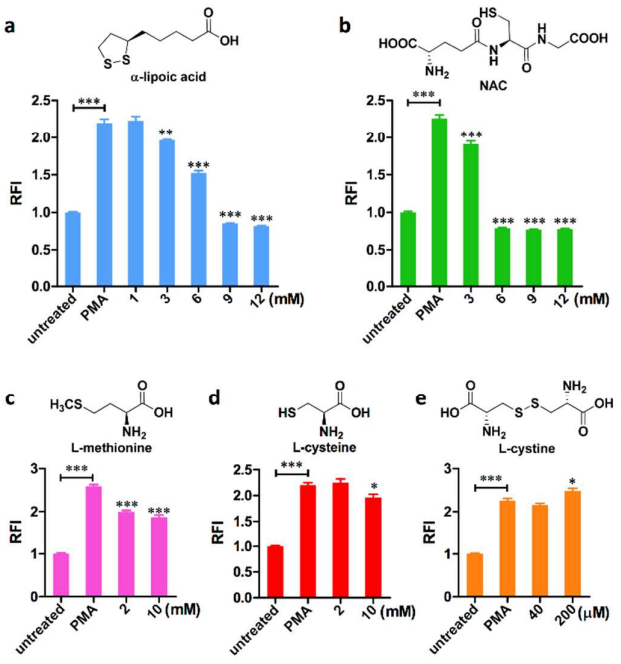


Fig. 4 Screening of HOCl scavengers with **HKOCl-3** in 96-well microplate assay. RAW264.7 cells were co-incubated with **HKOCl-3** (2 μM), PMA (500 ng/mL), in the absence or presence of HOCl scavengers: (**a**) α-lipoic acid, (**b**) NAC, (**c**) L-methionine, (**d**) L-cysteine, and (**e**) L-cystine (at indicated doses) for 30 min, before 96-well microplate analysis. Results are representative of at least three independent experiments.

Flow cytometry has become a definitive quantitative cellular analysis technique that rapidly integrates information about multifaceted characteristics of cell populations (e.g. 10^3 cells/second). Based on the superb probe performance seen in confocal imaging and 96-well microplate assay, we decided to explore the application of **HKOCl-3** in flow cytometry. RAW264.7 cells were co-incubated with **HKOCl-3** (2 μM) and PMA (500 ng/mL) in the presence or absence of NOX inhibitor DPI (100 nM) for 30 min, followed by flow cytometry analysis (Fig. 5). In the presence of PMA, the geometric mean of fluorescence intensity was significantly elevated. The appearance of two populations of cells upon PMA challenge is an interesting and highly reproducible phenomenon in this assay, which could be explained by the distinct excitability or responsiveness of cell subpopulations (possibly under different cellular redox status) toward PMA treatment. In addition, DPI blunted this fluorescence increase to a great extent. This result demonstrates that endogenous HOCl production in RAW264.7 cells can be readily detected in flow cytometry by using **HKOCl-3** as a fluorescent probe.

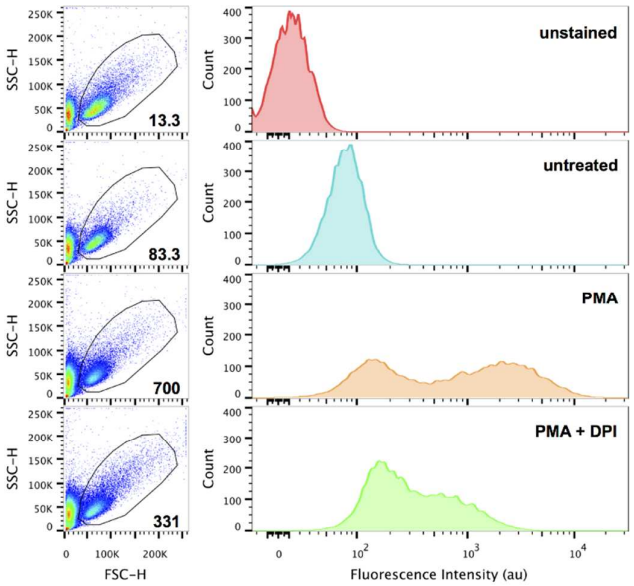


Fig. 5 Detection of endogenous HOCl in flow cytometry with **HKOCl-3**. RAW264.7 cells were co-incubated with **HKOCl-3** (2 μM) with or without PMA (500 ng/mL) and DPI (100 nM) for 30 min and analyzed by flow cytometry. The numbers in the dot plots represent the geometric means of fluorescence intensity. Results are representative of at least three independent experiments.

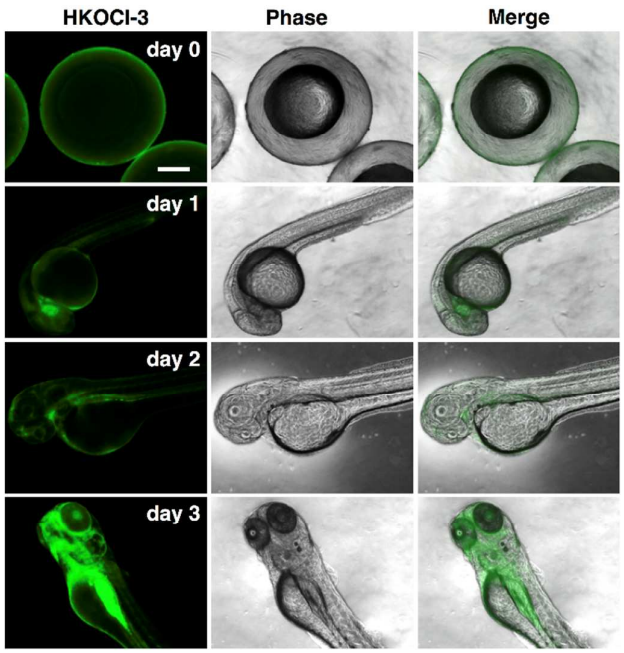


Fig. 6 Imaging of endogenous HOCl in live intact zebrafish embryos on different post-fertilization days. Zebrafish embryos (*n* = 6) at indicated time of harvest were incubated

with **HKOCl-3** (10 μM) in E3 buffer for 30 min, before imaging at low magnification (10×). Merge: fluorescence and phase images merged. Scale bar represents 250 μm.

For decades, MPO-related oxidative damage in diverse inflammatory, vascular and neurodengenerative diseases have been reported in numerous studies, which vividly illustrates the functional relevance of HOCl in cardiovascular and nervous systems and the dire consequences of its dysregulated production.[17] Despite well-documented pathological roles of HOCl in tissue damage, virtually nothing is known of HOCl function in normal tissues, such as that in developing fetuses. Strikingly, in zebrafish, an excellent *in vivo* model for studying hematopoiesis and maturation of the immune system, transcriptional expression of MPO has been known to occur in very early stages of development (e.g. post-fertilization day 1).[18] Yet, the nature and distribution of MPO activity (monitored through HOCl production) remains unclear. To this end, we sought to apply our probe **HKOCl-3** to visualize endogenous HOCl in live intact zebrafish embryos at different developmental stages. Briefly, wild-type zebrafish embryos were harvested on 1–3 post-fertilization days (pfd). The embryos were incubated with **HKOCl-3** (10 μM) for 30 min, and imaged under a fluorescence microscope. An intriguing pattern of progressively more intense and localized HOCl production in live intact zebrafish embryos was observed (Fig. 6), which parallels the evolving complexity of tissue differentiation during 1–3 pfd. Hence **HKOCl-3** can be readily applied to profile the distribution of MPO activity in delicate live tissues in an ultra-sensitive and noninvasive manner.

Conclusions

As a strong oxidant with promiscuous reactivity toward multiple biomolecules, HOCl has been among the most mysterious reactive oxygen species in biology. To provide powerful tools for investigation of HOCl biology, in this study, we have successfully developed a new ultra-selective, ultra-sensitive and rapid turn-on fluorescent probe **HKOCl-3** for molecular imaging and quantitative detection of HOCl. Specifically, the functional robustness of **HKOCl-3** has been established by visualizing endogenous HOCl in multiple living phagocytes in an oxidative burst model and in *in vivo* imaging of live zebrafish embryos at different developmental stages. In addition, **HKOCl-3** has shown superior sensitivity in quantitative detection of endogenous HOCl in flow cytometry and 96-well microplate assay. Collectively, our probe is expected to offer exciting glimpses into the unknown roles of HOCl in cellular processes and to help accelerate discoveries of drugs for the treatment of human diseases.

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13 A comparison of the performance of recently published fluorescent probes for HOCl imaging in terms of selectivity and sensitivity can be found in Table S2 of Electronic Supplementary Information.

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