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An Activity-Based Methionine Bioconjugation Approach To Developing Proximity-Activated Imaging Reporters

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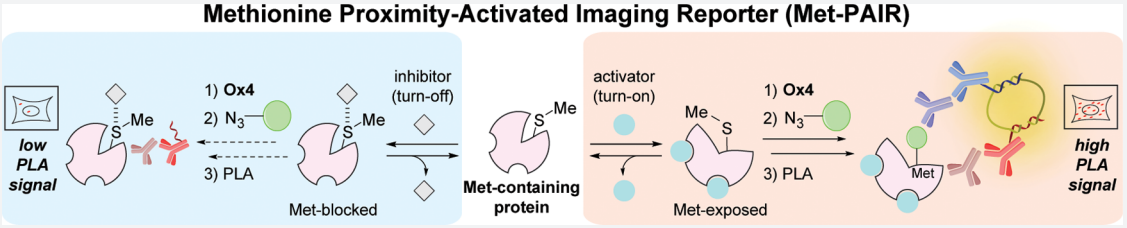
Supporting Information



ABSTRACT: Chemical probes that report on protein activity, rather than protein abundance, with spatial and temporal resolution can enable studies of their native function in biological contexts as well as provide opportunities for developing new types of biochemical reporters. Here we present a sensing platform, termed proximity-activated imaging reporter (PAIR), which combines activity-based methionine bioconjugation and antibody labeling with proximity-dependent oligonucleotide-based amplification to monitor dynamic changes of a given analyte in cells and animals through context-dependent methionine labeling of specific protein targets. We establish this PAIR method to develop sensors for imaging reactive oxygen species (ROS) and calcium ions through oxaziridine-directed labeling of reactive methionine residues on β-actin and calmodulin (CaM), respectively, where the extent of methionine bioconjugation on these protein targets can serve as an indicator of oxidative stress or calcium status. In particular, application of PAIR to activity-based CaM detection provides a method for imaging integrated calcium activity in both in vitro cell and in vivo zebrafish models. By relying on native protein biochemistry, PAIR enables redox and metal imaging without introduction of external small molecules or genetically encoded indicators that can potentially buffer the natural/existing pools. This approach can be potentially generalized to target a broader range of analytes by pairing appropriate activity-based protein probes with protein detection reagents in a proximity-driven manner, providing a starting point not only for designing new sensors but also for monitoring endogenous activity of specific protein targets in biological specimens with spatial and temporal fidelity.

INTRODUCTION

New methods for monitoring dynamic chemical analytes in native biological contexts and the protein targets that they regulate can help decipher their contributions to downstream signaling and stress pathways in healthy and disease states. In this regard, reactive oxygen species (ROS) and calcium ions exemplify two important carriers of chemical information for biological communication with a diverse array of physiological and pathological outcomes. Indeed, calcium is a canonical second messenger that can relay signals originating from primary events, such as changes in membrane potential and/or receptor activation, to intracellular targets, thereby enabling chemical responses to external biological stimuli.[1] One major regulatory protein for sensing and integrating calcium responses is calmodulin (CaM), where dynamic calcium binding triggers rapid conformational changes that mediate a host of downstream protein−protein interactions for information transfer.[2]

The multifaceted roles of these chemical messengers have motivated the development of fluorescence reporters for their study, where activity-based sensing of ROS[3,4] and binding-based sensing of calcium[1,5] represent some of the most common strategies for detection. However, regardless of sensing mechanism, the introduction of small-molecule and/or protein reporters can potentially perturb the target analyte of interest by its consumption or sequestration, particularly when high sensor concentrations are required to compensate for low signal-to-noise ratios or when sensors possess exceedingly high reactivity and/or tight-binding capacities. This buffering effect is a particular caveat for designing effective fluorescent calcium sensors[6−9] as well as probes for other analytes.[10−15]

Here, we report a generalizable sensing platform that operates via dual labeling of native regulatory proteins at endogenous levels with methionine-reactive bioconjugation probes for protein activity (chemical labeling) and antibody-based detection of the methionine-containing protein (target

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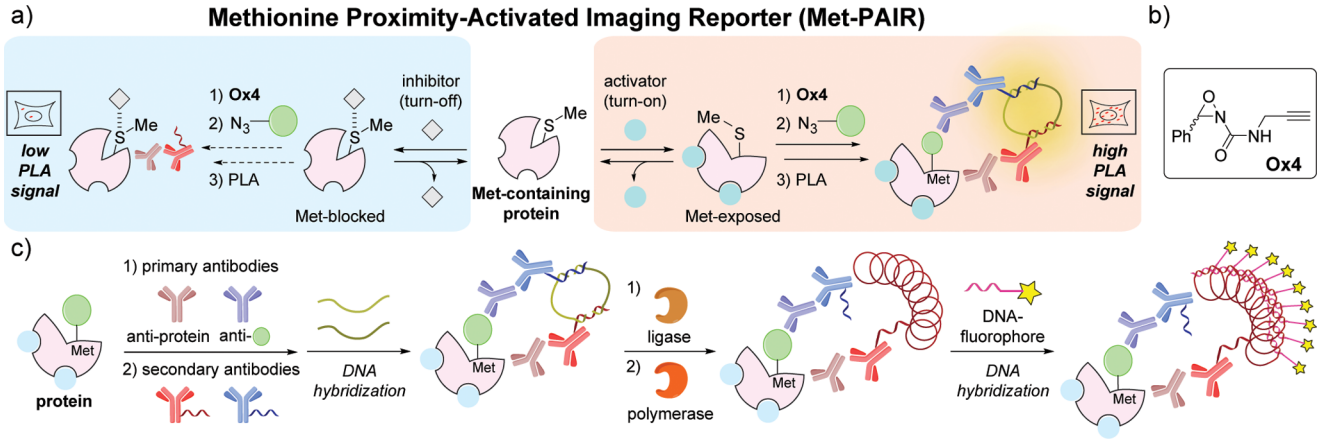

Figure 1. Methionine proximity-activated imaging reporter (Met-PAIR) through live-cell chemical modification of endogenous proteins and proximity-ligation assay (PLA). (a) General description of the PAIR method for "turn-off" sensing (left, blue shaded box) or "turn-on" sensing (right, red shaded box). Gray rectangle: analyte that deactivates methionine residues (e.g., H_2O_2 for actin). Blue ball: analyte that activates methionine residues (e.g., Ca for calmodulin). Green ball: an antibody-detectable chemical handle (e.g., Oregon Green and biotin). (b) Structure of alkyne-functionalized oxaziridine (**Ox4**) for the methionine labeling. (c) Schematic illustration of PLA for a protein with a methionine residue functionalized by the oxaziridine and chemical handle (green ball). Dark yellow strands: connector oligonucleotides that can be amplified by polymerase when transformed to circular oligonucleotides by ligase.

labeling) with proximity-dependent oligonucleotide amplification (Figure 1). Because analyte recognition relies on the native activity of proteins in the cell at endogenous levels, buffering effects would be minimized through this method. Signal is generated by an AND-type logic gate, where an amplified response will occur if and only if both the methionine activity label and methionine-sensing protein label bind to the same protein target, which minimizes background signal from off-target binding. We establish this approach, which we term proximity-activated imaging reporter (PAIR), by applying our recently reported redox-activated chemical tagging (ReACT) method for modification of methionine residues[16,17] to proteins that possess stimulus-responsive methionine sites. Specifically, an oxaziridine reagent bearing a bioorthogonal alkyne functional group can label functional methionine sites on endogenous proteins that are responsive to its native activity with ROS (β-actin) or calcium (calmodulin, CaM), where these chemical signals cause an increase or decrease in ReACT-based methionine labeling. Antibody labeling of the ROS- or calcium-responsive protein in conjunction with a proximity ligation assay (PLA) provides a proxy for the relative levels of the chosen analyte, as well as a method for imaging integrated snapshots of the activity of a specific protein target in biological specimens with spatial resolution. We demonstrate the utility of the Met-PAIR version of this platform for "turn-off" detection of ROS and "turn-on" detection of calcium, where the calcium-responsive sensor can image integrated calcium activity across in vitro cell to in vivo zebrafish models upon external stimulation. Taken together, this work provides a starting point for the design of a broader array of proximity-activated sensors for biochemical function based on native proteins at endogenous levels.

RESULTS AND DISCUSSION

Design of Proximity-Activated Imaging Reporters (PAIRs) Based on Activity-Dependent Methionine Bioconjugation. Our strategy for utilizing the native protein function at endogenous levels for creating new biochemical imaging reporters relies on proximity ligation assays (PLAs), which enable amplification of low signal-to-noise events through catalyzing rolling-circle DNA polymerization. Indeed, in situ PLA technology allows for visualization of two different "antigens" within 40 nm range by DNA hybridization and signal enhancement through the polymerization process (Figure 1c).[18−20] Innovative recent examples of PLA assays applied to chemical biology include ultrasensitive detection of post-translational modifications[19,21] (e.g., Glycoseek[22]) and antibodies,[23,24] as well as assessing hydrolytic[20] and kinase[25] enzyme activity (e.g., activity-dependent proximity ligation, ADPL).

In our proximity-activated imaging reporter (PAIR) approach, we exploit the sensitivity of methionine bioconjugation sites on endogenous proteins in an activity-dependent context, where the interaction of a protein with a given analyte can result in an increase or decrease in methionine labeling (Met-PAIR). In this manner, the endogenous protein activity serves as a proxy for the level of the target chemical analyte, without introducing exogenous recognition units that can buffer the analyte itself and can be expanded to other protein-labeling warheads. For chemoselective methionine modification, we chose to utilize our recently reported ReACT oxaziridine method, which labels methionine over cysteine and other amino acids in the proteome.[16,17] After methionine labeling and the copper-catalyzed azide−alkyne cycloaddition (CuAAC) reaction to append an affinity probe such as biotin or a fluorophore such as Oregon Green, antibodies against the protein of interest and against either biotin or Oregon Green can be applied. Subsequent addition of secondary antibodies conjugated to single-stranded DNA, followed by connector oligonucleotides causes hybridization only when the antibodies are in close proximity. This proximity-driven process eliminates false positives arising from protein labeling without the chemical tag and signals from other proteins with or without the chemical tag. The bound connector oligonucleotides can be ligated by a ligase to form a circular DNA template, which can subsequently undergo the polymerization process to produce multiple copies of the circular DNA (rolling-circle amplification) and can be visualized by complementary DNA−fluorophore conjugates.[18]

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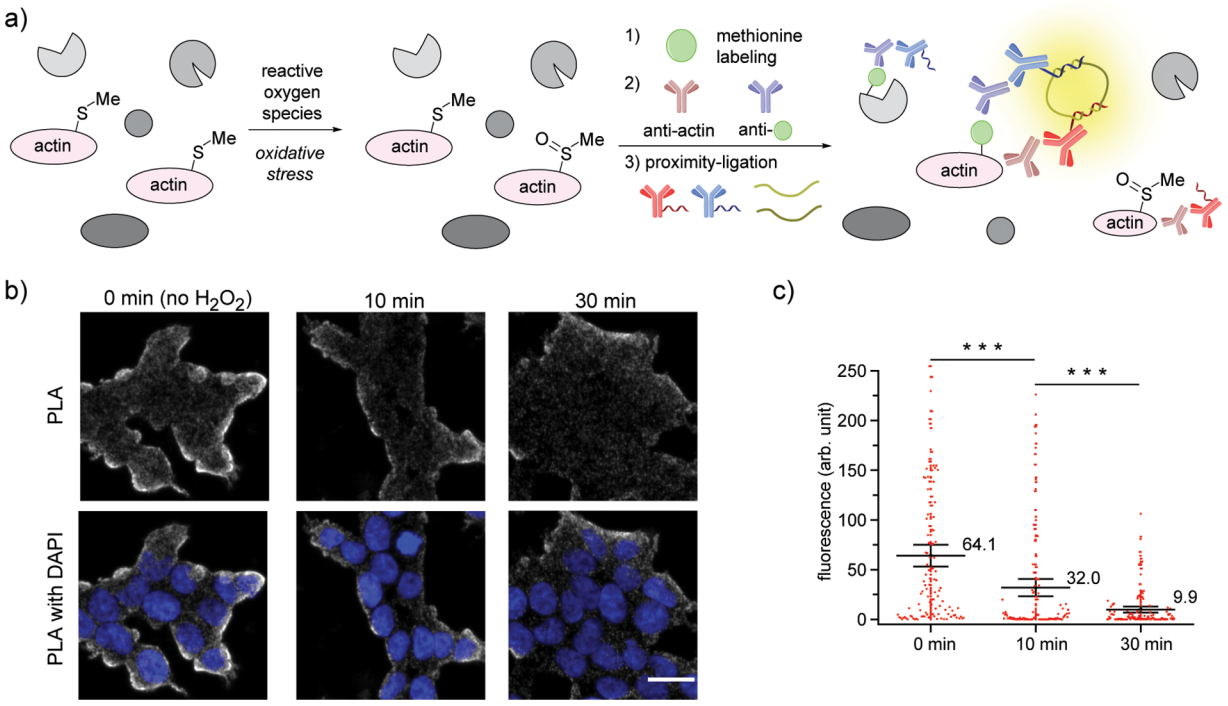

Figure 2. "Turn-off" detection of oxidative stress in cells via methionine proximity-activated imaging reporter (Met-PAIR) on β-actin. (a) Schematic for Met-PAIR on β-actin. (b) Confocal images of β-actin Met-PAIR on HEK293T cells preincubated with H_2O_2 (1 mM) for 0, 10, or 30 min, washed, and labeled with alkyne-tagged oxaziridine (**Ox4**, 20 μM) for 20 min. **Ox4**-labeled β-actin was further functionalized with Oregon Green-azide, and proximity-ligation assay (PLA) stain was performed with mouse anti-β-actin antibody and rabbit anti-Oregon Green antibody. Gray: PLA staining. Blue: DAPI nuclear staining. Scale bar: 20 μm. (c) Whisker plots for the confocal images in (b). Each dot represents fluorescence intensity of single cells. Whisker and center line represent 95% confidence interval and mean intensity, respectively. The mean value is shown near the center line. The quantification was conducted by imaging 4 regions in each of 2 independent biological replicates (total 8 cell images). The number of the quantified cells: 119 (0 min), 131 (10 min), and 157 (30 min). ***$P < 0.001$, Student's t test.

Reactive Oxygen Species Detection by Met-PAIR on β-Actin. As a starting point to establish the feasibility of the PAIR method, we chose β-actin as a model protein for detection of intracellular redox status. Actin is one of the most abundant cytoskeletal proteins, and oxidation of its two hyperreactive methionine residues (Met44 and Met47) under oxidative stress conditions plays important roles in its self-polymerization process.[26,27] We previously described that the hyperreactive methionine residues of β-actin are sensitive to labeling by oxaziridine reagents in cell lysate,[16] and we envisioned that these sites can serve as an indicator of intracellular oxidative stress in live cells through formation of methionine sulfoxide, as methionine oxidation would block oxaziridine labeling and decrease the PLA signal (Figure 2a). To test this hypothesis, live human embryonic kidney (HEK) 293T cells were treated with hydrogen peroxide (H_2O_2, 1 mM) for 10 or 30 min, washed with Hanks' balanced salt solution (HBSS), and incubated with the alkyne-tagged oxaziridine reagent **Ox4** at room temperature for 20 min. The oxaziridine-labeled cells were fixed, and an Oregon Green fluorophore was subsequently introduced via CuAAC. With anti-β-actin antibody and anti-Oregon Green antibody as primary antibodies, the labeled cells were subjected to PLA visualization. Indeed, we observed a substantial decrease in PLA signal from H_2O_2-treated cells compared to the untreated control cells (Figure 2b) with a ca. 6-fold turn-off response (Figure 2c). Notably, H_2O_2 treatment had no substantial effect on the Oregon Green signal as a proxy for the extent of oxaziridine labeling on all proteins in these cells, confirming that the hyperreactive methionine sites on β-actin are key for the selective PLA response to oxidation (Figure S1). The data establish the successful application of the Met-PAIR method on β-actin for the "turn-off" visualization of intracellular oxidative stress without introduction of exogenous synthetic or genetically encodable sensors that might buffer ROS pools.

Calcium-Dependent Oxaziridine Labeling of Calmodulin. In a second application of the PAIR method, we turned our attention to a native calcium-binding protein for visualization of intracellular calcium changes without introduction of chelation-based sensors. Calmodulin (CaM) is a ubiquitous calcium-binding protein expressed in all eukaryotic cells with a highly conserved amino acid sequence among species.[28] Upon binding to calcium, CaM undergoes a dynamic structural change which exposes hydrophobic pockets in the protein structure, thereby enabling a slew of subsequent protein−protein interactions to propagate signaling cascades.[29] In particular, human CaM possesses a total of 10 methionine residues, 8 of which are located in the two calcium-binding domains (4 each in N- and C-terminal domain) and become surface-exposed upon calcium binding (Figure 3a,b). This dynamic, calcium-dependent conformational change led us to pursue a calcium-sensing strategy in which the extent of methionine modification on CaM would be sensitive to calcium binding, where increased accessibility of these methionine sites upon metal coordination would lead to increased oxaziridine labeling. To this end, we first probed the reactivity of CaM to confirm that methionine labeling can be promoted by the calcium binding. Purified CaM was incubated with **Ox4** in the presence of varying amounts of calcium. Indeed, fluorescent gel imaging after incorporation of a

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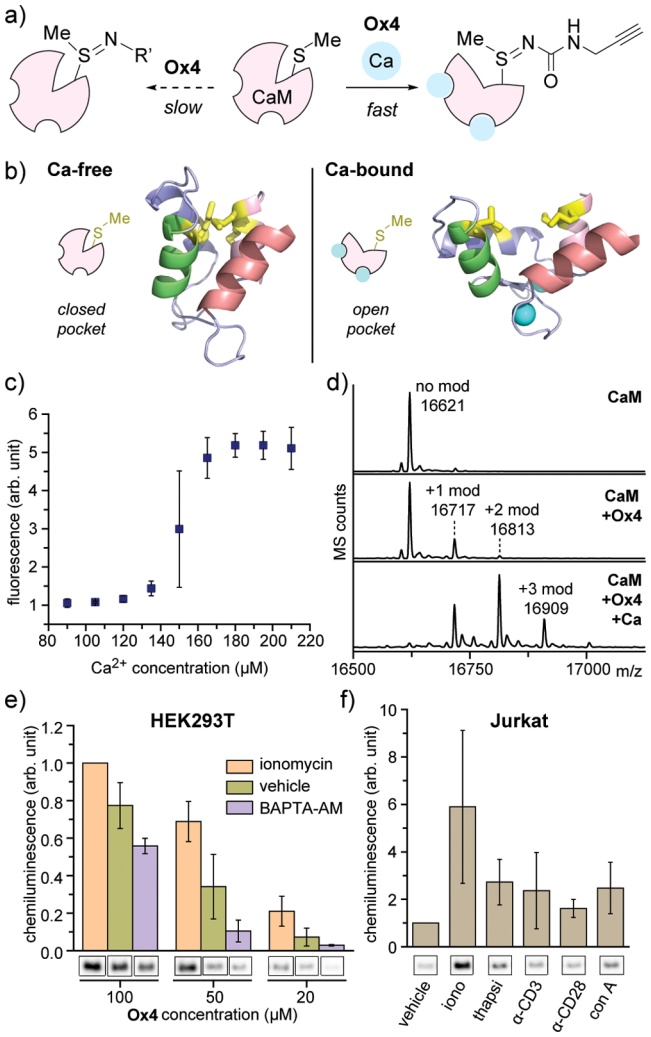

Figure 3. In vitro and live-cell labeling of calmodulin (CaM) with methionine-directed oxaziridine reagents show calcium-dependent increases in bioconjugation efficiency. (a) Schematic of the CaM labeling with oxaziridine probes promoted by calcium binding. (b) Structure of a C-terminal domain of Ca-free (left, PDB ID: 1CFD) and Ca-bound (right, PDB ID: 1CLL) CaM. The four methionine residues in the C-terminus are depicted in yellow. (c) Relative extent of in vitro labeling of CaM with **Ox4** under varying doses of calcium, analyzed by analyzed by in-gel fluorescence after copper-catalyzed azide-alkyne cycloaddition (CuAAC). Labeling conditions: CaM (2.75 μM), **Ox4** (15 μM), and $CaCl_2$ (indicated amount) in phosphate-buffered saline (PBS) solution at rt for 5 min (d) ESI-MS analysis of the in vitro labeling of CaM (2.75 μM) with **Ox4** (15 μM) in the absence or presence (180 μM) of $CaCl_2$. (e, f) Relative levels of CaM labeled with **Ox4** in live cells under various conditions, as assessed by streptavidin pull-down processes and analyzed by anti-CaM Western blot. Labeling conditions: **Ox4** (indicated amount for HEK293T and 20 μM for Jurkat) with various stimulants in PBS solution at rt for 20 min. Abbreviations: ionomycin (iono), thapsigargin (thapsi), anti-CD3 antibody (α-CD3), anti-CD28 antibody (α-CD28), and concanavalin A (con A). Error bars represent standard error of mean (n = 3). An individual band for the blot is shown for the sake of clarity. Full-width blot membrane images are shown in Figures S5 (HEK293T) and S8 (Jurkat).

rhodamine fluorophore via CuAAC reaction showed a calcium dose-dependent increase (Figure 3c and Figure S2). Notably, this calcium-dependent reactivity change was not observed for other model proteins that do not bind calcium such as lysozyme (Figure S2), confirming that calcium addition alone does not alter oxaziridine labeling of methionine residues on proteins but rather that the observed reactivity enhancement stems from calcium-specific binding and subsequent calcium-dependent conformational changes of CaM. The increase in oxaziridine-mediated methionine labeling of CaM with increasing concentrations of calcium was also identified by mass spectrometry analysis. Multiple modifications of CaM were observed in the presence of calcium, whereas reactions between CaM and **Ox4** without calcium showed minimal modification (Figure 3d). Moreover, analysis of methionine-modified CaM confirmed that the oxaziridine-derived moiety was introduced onto peptide fragments corresponding to the C-terminal domain (Figure S3), consistent with previous literature reporting that the C-terminal domain has a higher affinity to calcium compared with the N-terminal one.[30] Furthermore, we established that methionine labeling with micromolar amounts of Ox reagent can proceed even in the presence of 1000-fold excess of competing thiol groups at millimolar levels (Figure S4), demonstrating the potential utility of the Ox reagent in complex biological mixtures bearing high concentrations of these chemical functionalities (e.g., live cells and animals). Indeed, the success of Ox-mediated methionine labeling on proteins in live samples was demonstrated in six different cell lines: HEK293T, Jurkat, HCT-116, MCF-7, A-549, and PC-3 (Figures S5−S8, vide infra). These in vitro oxaziridine labeling experiments on CaM suggested that the calcium-binding status of the protein tracks with methionine modifications.

Calcium-Dependent Oxaziridine Labeling of Endogenous Calmodulin in Live Cells. We next confirmed calcium-dependent, oxaziridine-directed methionine labeling of endogenous CaM in live cells (Figure 3e,f). To assess the extent of methionine-directed CaM labeling in cellular environments, we utilized a CuAAC reaction with biotin-azide followed by streptavidin enrichment to capture proteins that were labeled by **Ox4**. After the enrichment, the relative level of modified CaM in the cells was assessed by anti-CaM Western blot analysis. Treatment of HEK293T cells with various doses of **Ox4** in the presence of ionomycin (calcium ionophore), DMSO vehicle, or BAPTA-AM (calcium chelator) was performed for 20 min at room temperature, and then cells were lysed and subjected to CuAAC and streptavidin enrichment. Anti-CaM Western blot analysis demonstrated enhanced signal upon increasing cellular calcium levels, indicating more efficient CaM modification in the calcium-enriched sample and less efficient CaM modification in the calcium-depleted sample compared to vehicle control (Figure 3e, Figure S5). Control blot analyses of other proteins, including β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), showed no substantial difference of chemiluminescence signal by changes in cellular calcium levels, consistent with the notion that the enhancement of the methionine labeling is specific to proteins that undergo Ca-dependent conformational changes (Figure S6). As similar trends were also observed for live-cell oxaziridine labeling and enrichment in colon, breast, lung, and prostate cancer cell lines (Figure S7), this approach is potentially generalizable to a broader set of biological models.

Live-cell experiments with activation of cellular pathways that utilize calcium as a second messenger also showed calcium-dependent labeling of CaM with oxaziridine reagents. Various stimulations are known to trigger increased calcium

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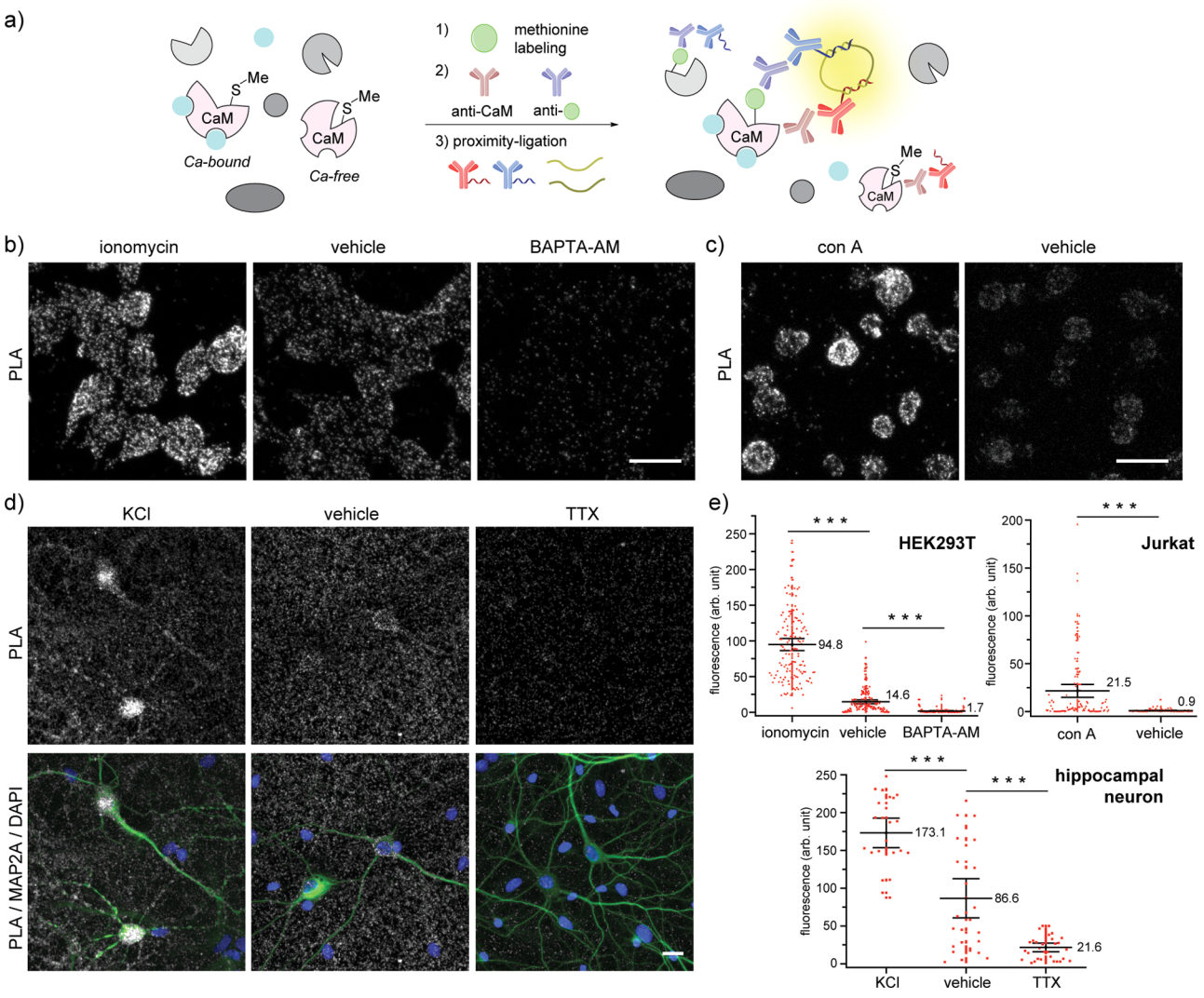

Figure 4. "Turn-on" detection of changes in calcium levels in cells by applying the methionine proximity-activated imaging reporter (Met-PAIR) method to the native calcium regulatory protein calmodulin (CaM) at endogenous levels. Oxaziridine labeling in live cells was conducted in the presence of various reagents in Hanks' balanced salt solution (HBSS) with alkyne-tagged oxaziridine (**Ox4**, 20 μM) at rt for 20 min (HEK293T and Jurkat) or with **Ox4** (0.5 μM) at 37 °C for 5 min (hippocampal neurons). Proximity-ligation assay (PLA) staining was performed with mouse anti-CaM antibody and rabbit anti-Oregon Green antibody (HEK293T and Jurkat) or rabbit anti-biotin antibody (neuron). Gray: PLA staining. Blue: DAPI nuclear staining. Scale bar: 20 μm. (a) A schematic illustration of Met-PAIR on CaM. Blue ball: calcium ions in live cells. (b) Confocal images of HEK293T cells stained by Met-PAIR with intracellular calcium increases (ionomycin: 10 μM) or decreases (BAPTA-AM: 30 μM). (c) Confocal images of Jurkat cells stained by Met-PAIR with concanavalin A (con A, 0.1 mg/mL) to trigger calcium increases over vehicle control. (d) Confocal images of rat hippocampal neuron stained by Met-PAIR with activation (KCl: 90 mM) or deactivation (tetrodotoxin, TTX: 10 μM) to increase or decrease calcium transients, respectively. Anti-MAP2A antibody (green) was used to identify neurons. (e) Whisker plots for the confocal images in (b) HEK293T, (c) Jurkat, and (d) neurons. Each dot represents the fluorescence intensity of single cells or neuronal cell bodies. Whisker and center line represent 95% confidence interval and mean intensity, respectively. The mean value is shown near the center line. The quantification was conducted by imaging 4 regions in each of 2 independent biological replicates for HEK293T and Jurkat (total 8 cell images), and 5 regions in each of 2 independent biological replicates (total 10 images) were used for the quantification of neuronal cell bodies identified by the MAP2A staining. The number of quantified cells: 139 (ionomycin), 164 (HEK293T vehicle), 170 (BAPTA-AM), 113 (con A), 105 (Jurkat vehicle), 24 (KCl), 30 (neuron vehicle), and 32 (TTX). ***$P < 0.001$, Student's t test.

levels in human T lymphocyte (Jurkat) cells,[31] and indeed we observed that the extent of CaM labeling upon addition of these external stimuli can be visualized (Figure 3f and Figure S8). Thapsigargin, a noncompetitive sarco/endoplasmic reticulum calcium ATPase inhibitor, is known to increase cytosolic calcium levels,[32] and more **Ox4**-labeled CaM was detected upon treatment of cells with this inhibitor. Agents that induce T-cell receptor activation, including anti-CD3/28 antibodies[33] or concanavalin A (con A),[34] also led to increased **Ox4**-labeled CaM signal, presumably through the increase of intracellular calcium levels that track labeling efficiency. Finally, as a key control experiment, we confirmed that **Ox4** treatment itself does not disrupt CaM-related physiological responses in these models. The amount of interleukin-2 (IL-2), a cytokine secreted from activated Jurkat cells,[35] was unaffected in the presence of **Ox4** (Figure S9), suggesting minimal effect of the oxaziridine labeling on CaM for its native calcium-binding activity.

Met-PAIR on Calmodulin for Integrated Calcium Imaging in Cells. Encouraged by the blot analysis data

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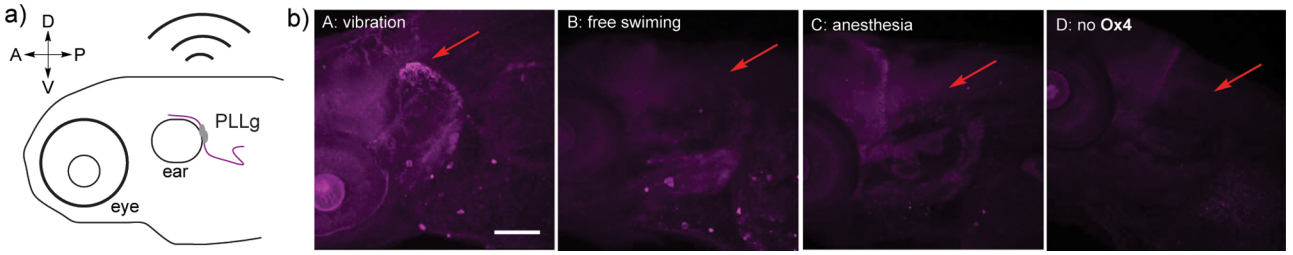

Figure 5. Methionine proximity-activated imaging reporter (Met-PAIR) staining on calmodulin (CaM) in zebrafish with external stimulation. Live zebrafish (3 day post-fertilization) were treated with **Ox4** (100 μM) at rt for 20 min, further functionalized with biotin-azide via copper-catalyzed azide alkyne cycloaddition (CuAAC), and stained by mouse anti-CaM antibody and rabbit anti-biotin antibody as primary antibody and proximity-ligation assay (PLA) reagents. (a) Schematic illustration of the head of zebrafish for stimulation of posterior lateralis ganglion (PLLg) response through physical vibration. A: Anterior. D: Dorsal. P: Posterior. V: Ventral. (b) Representative confocal images of zebrafish stained by Met-PAIR with increases (vibration) or decreases (anesthesia with 920 μM tricaine) in local calcium. The images are representative of six biological replicates for each condition. PLA signal is shown in magenta. PLLg is noted with a red arrow. DAPI counterstaining and merged images are shown in Figure S17. Scale bar: 100 μm.

showing activity-dependent labeling of CaM with **Ox4** in live cells as a function of calcium status, we next sought to advance the Met-PAIR platform to visualize changes in calcium levels by microscopy (Figure 4a). To this end, HEK293T cells were treated with **Ox4** at room temperature for 20 min in the presence of various stimulants (ionomycin, DMSO vehicle control, or BAPTA-AM), and after CuAAC with Oregon Green azide, the labeling status of CaM was visualized by PLA with anti-CaM (mouse) and anti-Oregon Green (rabbit) antibodies. Confocal fluorescence imaging confirmed increases and decreases in PLA signal correlating with increases and decreases in intracellular calcium upon treatment with the calcium ionophore ionomycin and calcium chelator BAPTA-AM, respectively. The data can be resolved to the single-cell level (Figure 4b,e). These results establish the feasibility of using Met-PAIR for displaying the extent of activity-dependent oxaziridine-CaM labeling as a proxy for the calcium status of live cells. For comparison, conventional calcium indicators, like Oregon Green BAPTA (OGB), can also track changes in calcium concentration in live cells (Figure S10a). However, unlike Met-PAIR, OGB keeps no record of calcium changes, and any dynamic range between calcium-treated cells collapses to zero upon fixation (Figure S10b). Indeed, in contrast to the PLA signal by Met-PAIR, as a key control experiment, the Oregon Green signal alone, which represents the extent of oxaziridine labeling in all proteins in the cell, was not calcium-dependent, corroborating the need for a specific calcium-sensitive protein as an endogenous recognition moiety to achieve metal detection (Figure S11). Moreover, the PAIR method is not limited to fluorophore payloads, as biotin can also resolve calcium-dependent changes (Figure S12) using different types of antibodies: rabbit anti-CaM and mouse anti-biotin (Figure S13). As expected, negligible PLA signal was observed in the absence of one of the primary antibodies (Figure S14), and siRNA-mediated knockdown of CaM levels led to a loss of PLA signal (Figure S15). Taken together, the data show that the Met-PAIR method can be employed to visualize calcium levels in cells using endogenous CaM.

The Met-PAIR method for the calcium imaging was also expanded to a broader range of cell models. For example, cytosolic calcium levels are known to increase during con A-mediated activation of Jurkat cells as a proxy for inflammatory immune response, and the increase was clearly visualized by Met-PAIR, with a ca. 20-fold increase in mean PLA signal intensity observed relative to vehicle control (Figure 4c,e). Met-PAIR can also be utilized to image calcium status of cultured rat hippocampal neurons, where neuronal activation is typically accompanied by an increase in intracellular calcium levels. Potassium chloride is known to activate calcium signaling through a depolarization mechanism,[36] and the Met-PAIR method visualized the calcium increase under these conditions as compared to vehicle control (Figure 4d,e). In turn, tetrodotoxin (TTX), a known sodium channel blocker that inhibits action potentials and consequently leads to decreases in intracellular calcium,[37] was successfully visualized by the Met-PAIR method as well. As a control, we utilized the voltage-sensitive fluorophore, BeRST 1,[38] to confirm that treatment of neurons with the **Ox4** reagent alone under these conditions does not affect spontaneous neuronal activity (Figure S16), indicating compatibility of the reagent even in this complex cell model. Overall, the data indicate that Met-PAIR is a suitable tool for calcium detection across various cell types.

Met-PAIR on Calmodulin for Detection of Integrated Calcium in Vivo. Finally, we applied the Met-PAIR method for tracking calcium status in vivo through integrated calcium activity in zebrafish in response to external stimuli. To evaluate the possibility of using the Met-PAIR method in live zebrafish, vibration stimulation was employed to trigger calcium transients in posterior lateralis ganglion (PLLg) region (Figure 5a).[39] Specifically, zebrafish larvae (3 day post-fertilization) were incubated with **Ox4** (0 or 100 μM) at 25 °C for 20 min, fixed with paraformaldehyde and processed in a similar way as noted for the cell experiments except with a longer PLA incubation time (see Supporting Information). Sensory stimulus results in a patent increase in PLA signal in the PLLg region (Figure 5b panel A, Figure S17−19). Unstimulated animals (panel B) and anesthetized animals (panel C) show minimal fluorescence intensity in the region, and animals without **Ox4** treatment did not display appreciable fluorescence intensity (panel D), indicating that the PLA signal observed stems from the methionine-directed oxaziridine labeling. The results show that PAIR can be employed for in vivo labeling and analyte detection.

CONCLUDING REMARKS

We have presented the design, construction, and evaluation of a proximity-activated imaging reporter (PAIR) platform as a generalizable sensing strategy that makes use of activity-dependent labeling of methionine residues on native proteins

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at endogenous levels coupled with antibody labeling and proximity-dependent ligation to amplify low signal-to-noise events. This method addresses a common limitation of exogenously added synthetic or genetically encodable sensors that can buffer the analyte of interest, as PAIR relies on native proteins without further genetic engineering as a recognition module. We put this concept into practice using our oxaziridine-based reagents for chemoselective methionine modification (Met-PAIR), noting that other warheads for reactive amino acid sites and related post-translational modifications are also possible. Met-PAIR can serve as an integrator to capture snapshots of changes in analyte concentrations with high spatial resolution, as these type of sensors are increasingly valued owing to their experimental simplicity, multiplex capabilities, and potential application for in vitro and in vivo imaging for small- or large-scale experiments.[40−43] Specifically, we applied the Met-PAIR method to β-actin bearing oxidatively sensitive methionine residues, which allowed for "turn-off" sensing of reactive oxygen species (ROS) through live-cell chemical modification with the oxaziridine reagent. The PAIR concept was further generalized into sensing of calcium through live-cell chemical modification of methionine residues on an endogenous calcium-binding protein calmodulin (CaM) that shows increased reactivity upon calcium binding. The "turn-on" type Met-PAIR for calcium was applied for several live-cell models, including Jurkat immune cells and mammalian neurons, as well as zebrafish through in vivo chemoselective methionine modification. In live zebrafish, the Met-PAIR method enables detection of integrated calcium activity within the posterior lateralis ganglion region in response to a physical vibration stimulus.

The calcium snapshots provided by the Met-PAIR method complement traditional calcium indicators, including Oregon Green-BAPTA (OGB) or genetically encoded calcium indicator GCaMP reporters, which require continuous monitoring. Activity integrators that enable retrospective analysis of neuronal activity directly coupled to molecular cues like calcium dynamics would be of use to the neurobiological community. However, traditional methods of retrospective analysis rely on expression of immediate early genes, like *c-fos*,[44] which are only weakly coupled to calcium flux. More recent methods for activity integration rely on molecular logic gates that couple high calcium concentrations with photoconversion,[40] light-induced transcription of reporter genes,[45] or light-induced protease activation.[41] Although the light activation confers precise gating of activity integration, the amount of photoconversion light and the stoichiometry of the multiple genetically encoded components must be carefully controlled. Additionally, all of these methods require overexpression of exogenously added calcium-binding proteins which can interfere with the native calcium buffering machinery.[46] Our new Met-PAIR approach avoids these limitations by targeting methionine residues on native proteins. Gating of activity integration is controlled by delivery of the **Ox4** reagent, which will provide sufficient temporal gaging of activity integration for most behaviors.

In contrast to genetically encoded sensors that can perturb biological models with long-term expression,[47] Met-PAIR detection requires a relatively short incubation time with the oxaziridine reagents (5−20 min), with minimal off-target physiological effects observed under conditions tested. Indeed, despite the widespread use of CaM for various calcium-sensing technologies, to the best of our knowledge, Met-PAIR is a unique method to exploit endogenous CaM for calcium detection through rapid and tunable chemical modification. We envision that the combination of native protein tagging using activity-based bioconjugation probes and proximity-ligation assay (PLA) can be further expanded to other labeling methods and other large and small biomolecule substrates to create new types of chemical tools to further our understanding of complex pathways of biological communication.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.9b01038.

Materials and methods, detailed experimental procedures and protocols, raw data for in vitro and biochemical analyses, fluorescence images for cellular and zebrafish experiments (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors have filed a patent application on the Met-PAIR method.

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