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Journal

Journal of the American Chemical Society, 139(32)

ISSN

0002-7863

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Publication Date

2017-08-16

DOI

10.1021/jacs.7b05002

Peer reviewed



Published in final edited form as:

J Am Chem Soc. 2017 August 16; 139(32): 10964–10967. doi:10.1021/jacs.7b05002.

Site-Selective Sensing of Histone Methylation Enzyme Activity via an Arrayed Supramolecular Tandem Assay

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Abstract

Arrayed deep cavitands can be coupled to a fluorescence-based supramolecular tandem assay that allows site-selective *in situ* monitoring of post-translational modifications catalyzed by the lysine methyltransferase PRDM9 or the lysine demethylase JMJD2E. An arrayed sensor system containing only three cavitand components can detect the specific substrates of enzyme modification, in the presence of other histone peptides in the enzyme assay, enabling investigation of cross-reactivity over multiple methylation sites and interference from non-substrate peptides.

Histone post-translational modifications (PTMs) play important roles in regulation of gene expression.¹ Methylations occur primarily at lysine and arginine residues on the histone backbone,² affecting transcriptional regulation,³ heterochromatin assembly, and cell cycle progression.⁴ Site-specific lysine methylations are created by lysine methyltransferases (KMT) and removed by lysine demethylases (KDM).⁵ As dynamic control of histone methylation regulates numerous cellular processes, mechanistic study of their function is essential for understanding epigenetic regulation and its effect on disease progression. This requires assays that are able to differentiate not only methylation states, but also the positions of methylation. Antibody-based assays are capable of this selectivity,⁶ but have certain limitations: specific antibodies need to be accessed for each substrate target, which increases the cost and limits the accessibility of the method. Supramolecular tandem assays

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b05002.

Experimental procedures, additional spectral data (PDF)

The authors declare no competing financial interest.

that employ small molecule synthetic receptors as host molecules for the targets of interest are an enticing alternative to antibody-based enzyme assays.⁷ Water-soluble hosts such as sulfonated calixarenes (CXs), cucurbiturils (CBs) or cyclophanes can recognize the substrates or byproducts of enzymatic reactions, and be paired with fluorescence displacement assays (FDAs) for selective, *in situ* monitoring of the process.⁸ This small molecule approach has many advantages, such as high sensitivity, simple, rapid detection, and low cost. A number of enzymatic targets have been monitored using small molecule FDAs,⁹ such as cholinesterases,^{9a} decarboxylases,^{9b-d} protease,^{9e,f} histone deacetylase,^{9f,g} and lysine methyltransferase.¹⁰

These advantages are offset by one specific weakness, especially when compared to antibody-based assays: site selectivity. Small molecule receptors are pan-specific, and generally only recognize a single type of functional group. Though excellent selectivities for a specific group type are possible, differentiating identical modifications at different positions is extremely challenging, especially under the conditions of an enzyme assay. CX¹¹ and cyclophane^{9f,g,12} sensors are capable of selectively recognizing lysine and arginine methylations, and array-based versions can distinguish methylations at different positions on modified peptide substrates.¹³ These techniques have not yet been coupled to an enzyme assay, though. While monitoring KMT activity is possible with a CB-based tandem assay,¹⁰ site-selective activity monitoring was not shown. Effective *in vitro* assays of methylation enzyme activity require both site-selective molecular recognition and an optical readout of the KMT or KDM activity. Here we show that an FDA sensing system based on deep cavitand synthetic receptors can be used to monitor the function of multiple histone methylation enzymes in a site-selective manner.

Water-soluble deep cavitands¹⁴ such as **1–3** (Figure 1) are useful for recognition and sensing of biomolecules. We have previously shown that the combination of cavitand **1** and a suitable fluorophore allows sensing of Kme₃ peptides via FDA.¹⁵ This concept can be extended to allow site-selective Kme₃ recognition by the application of cavitands **1–3** in an array-based format,¹⁶ followed by discrimination via principal component analysis (PCA).¹⁷ The sensor can discriminate Kme₃ residues at different positions on peptides, and even sense remote changes in peptide structure. The sensitivity of the system to methylation site¹⁶ and state¹⁵ suggests that it would be capable of site-selective monitoring of methylation enzyme activity (Figure 1).

Initially, we established the ability of the sensor system to discriminate between methylations in peptide mixtures (Figure 2, see Supporting Information for full peptide sequences). Three different histone H3 peptides with trimethylation (Kme₃) modifications at different positions (H3K4me₃, H3K9me₃, H3K27me₃) were combined and added to an array of hosts **1–3** and fluorophore **4**. The array was constructed by incorporating the three cavitands (**1–3**, 4 μ M) and their complexes with guest **4** (3 μ M) at two different pHs (pH 7.4 and 9.0), for a total of 6 variables. The peptides were mixed with each sensor solution per well in a 96-well plate at a fixed concentration of 2 μ M, and the relative fluorescence recovery detected for each well. Analysis of the array readouts (4 separate runs) was performed by PCA, and the scores plot is shown in Figure 2a. The selectivity was tested by switching a single Kme₃ peptide from the mixture with its nonmethylated control. The 6-

variable (1–3)·4 array can detect the absence of each methylation (K4me₃, K9me₃, K27me₃) in the peptide mixture. We also performed a mimic of the enzymatic demethylation process^{9f} by varying the proportion of each Kme₃ peptide versus the corresponding nonmethylated peptide (Figure 2b) in 20% increments. As the proportion of each Kme₃ peptide decreased, a continuous movement of the clusters toward each end product in the PCA plot was observed, indicating the suitability of the sensor for the analysis of the product mixtures during enzymatic reaction. The sensor is also functional in the presence of histone digests. H3(1–21), H3K4me₃, H3K9me₃, and H3K27me₃ can be well separated on the scores plot (see Figure S-10) after 2 μ M (~4 μ g/mL) of the peptide was spiked into the protease digest of bovine histone at 100 μ g/mL.

Of course, discrimination of the products and intermediates is not the only requirement for a successful enzyme assay. A simple array should provide optimal signal to minimize enzyme and substrate consumption, and must function in the presence of cofactors and a mixture of potential substrates. The (1–3)·4 sensor (at 4 and 3 μ M respectively) could detect as little as 22 nM of H3K9me₃ (see Figure S-1a) and was responsive to change in [peptide] up to 10 μ M at pH 7.4. The detection performance was not affected by the presence of a complex peptide mixture prepared by digesting bovine histones at a concentration of 5–250-fold of the H3K9me₃ peptide (see Figure S-1b). The sensor system was also tolerant to the presence of other chemicals required for JMJD2E catalyzed demethylation (2-oxoglutarate (2-OG), Fe²⁺, and ascorbate) and HMT methylation (S-adenosyl-methionine (SAM) and byproduct S-adenosyl-homocysteine (SAH)). Sodium ascorbate (0.5 mM) and Fe²⁺ (6 μ M) only slightly increased (~5%) quenching of the guest 4 fluorescence by the cavitand 1, and 2-OG (50 μ M) had no effect at all. Addition of SAM did not induce any change to sensor fluorescence within the concentration range (0–100 μ M) required for enzyme assays. None of these additives had any effect on the fluorescence recovery profiles upon displacement of 4 by target peptides, nor did up to 0.5% DMSO. Finally, the tolerance of the system to repeated exposure with UV light was tested. Figure S-5 shows that guest 4 is somewhat susceptible to photobleaching, with a 15% loss of signal observed after 225 min continuous exposure to UV light, cavitand 1 acts as a protecting agent and only <5% loss of signal was observed for the 1·4 complex. The fluorescence recovered by H3K9me₃ was also stable over 120 min under the same conditions.

The sensor was applied to monitor the reactions catalyzed by two histone methylation enzymes: the lysine demethylase JMJD2E, and the lysine methyltransferase PRDM9. Both of these enzymes specifically act on a single lysine residue: JMJD2E catalyzes the demethylation of histone H3 at lysine residue 9, and PRDM9 catalyzes the trimethylation of H3 at lysine residue 4 (Figure 3). By adding JMJD2E to a 2 μ M H3K9me₃ solution with 50 μ M 2-OG, 6 μ M Fe²⁺, and 0.5 mM ascorbate, the enzyme activity can be monitored over time by the fluorescence changes in the single component 1·4 sensor ([1] = 3 μ M, [4] = 4 μ M, Figure 3a,b). As the demethylation reaction proceeds, the concentration of H3K9me₃ decreases, leading to less displacement of the guest 4 from cavitand 1, and thus lower fluorescence. The simple 1·4 sensor was able to monitor the JMJD2E reactivity with as little as 200 nM enzyme, with a 10% fluorescence decrease observed after 20 min reaction. The reaction process was confirmed by MALDI-TOF MS analysis of the mixture at individual

time points (Figure 3b, same conditions as for the FDA analysis). At time zero, only H3K9me₃ ($m/z = 2297.05$) was detected. H3K9me₁ ($m/z = 2269.0$) and H3K9me₂ ($m/z = 2283.0$) appeared at $t = 10$ min, and complete conversion to the product H3K9me₁ occurred after 2 h.

The same concept can be applied to monitor methyltransferase activity. In this case, the Kme₃ product formed by trimethylation of the H3 peptide at K4 by PRDM9 displaces fluorophore **4** from host **1**, causing continuous fluorescence increase. Mixing 200 nM PRDM9 with 2 μ M H3K4, 100 μ M SAM, and 1 mM dithiothreitol (DTT), the sensor fluorescence increased rapidly within the first 15 min and leveled off afterward (Figure 3c,d). MALDI-TOF-MS also confirmed that the initial fluorescence recovery within 5 min of reaction was mainly caused by the production of H3K4me₁. The amount of H3K4me₂ and H3K4me₃ started to increase significantly after $t = 10$ min. With continuous conversion of H3K4me₁ and H3K4me₂ to H3K4me₃, only H3K4me₃ was detected at $t > 30$ min, at which time the fluorescence recovery of the **1-4** sensor reached a plateau.

The simple **1-4** assay allows single substrate analysis, but the more exciting challenge is to monitor the site-selective function of the methylation enzymes in a substrate mixture. The full six-component array composed of the three host:guest complexes (**1-3**)-**4**, at pH 7.4 or pH 9.0 was capable of distinguishing different methylation sites in histone peptide mixtures (Figure 2). Having separately shown enzyme monitoring and site-selective Kme₃ sensing, we combined the two systems to sense a specific peptide substrate for the methylation enzyme from a mixture of peptides. We prepared identical enzyme reaction mixtures as above, but used a mixture of peptides as the substrates instead of one. The reaction catalyzed by JMJD2E was supplied with 2 μ M of each peptide H3K9me₃, H3K4me₃, and H3K27me₃ (along with 50 μ M 2-OG, 6 μ M Fe²⁺, and 0.5 mM ascorbate), and PRDM9 was mixed with 2 μ M of H3(1–21), H3(23–34) (along with 100 μ M SAM and 1 mM DTT). The reactions were terminated at various time points by incubating in boiling water for 5 min to deactivate the enzyme, the individual components of the (**1-3**)-**4** sensor array (4 μ M host, 3 μ M **4**) added and the fluorescence analyzed. Though the initial Kme₃ sensor utilized 6 elements to achieve the selective detection of different methylation sites, we found that addition of the sensors present at pH 9.0 did not further enhance the separation of different peptides on the scores plot compared to using only the pH 7.4 sensors. We therefore only used three elements of the array for monitoring the enzyme reaction with the peptide mixtures, i.e., (**1-3**)-**4** at pH 7.4. For monitoring the site-selective JMJD2E demethylation process, four controls were prepared in the presence of all cofactors and deactivated JMJD2E: the peptide mixtures of H3(1–21) + K4me₃ + K27me₃, K9me₃ + K4me₃ + H3(23–34), and K9me₃ + H3(1–21) + H3K27me₃, as well as the product K4me₃ + K27me₃ + K9me₁, illustrating the scores plot position of the “correct” H3K9me₁ demethylated target, and incorrect demethylation at either K4 or K27. For monitoring the site-selective methylation process with PRDM9, the three controls were K4me₃ + H3(23–34), K9me₃ + H3(23–34), and H3(1–21) + H3K27me₃, validating that the sensor can track the site-selective enzymatic reaction.

The sensor responses terminated at various time points were subjected to PCA, together with the controls (Figure 4). As the JMJD2E-catalyzed demethylation reaction proceeds, the

reaction mixture moves toward the position where K9me₁ + K4me₃ + K27me₃ is located, and further away from the other two controls, indicating that the enzyme acts on only the K9 methylation site, but not K4 nor K27. The analysis is not only capable of distinguishing incorrect reaction products, but also the state of the product: fully demethylated K9 is distinguishable from K9me₁. Similarly, because PRDM9 only adds methyl groups to the K4 position, the reaction mixture catalyzed by PRDM9 moved toward the control containing K4me₃, and not the other controls with K9 or K27 methylation. Each mixture was separated from each other (at 95% confidence), except for the demethylation reaction mixture at *t* = 60 min and the product mixture of K9me₁ + K4me₃ + K27me₃. The significance in group separation was confirmed by hierarchical cluster analysis (HCA) and one-way MANOVA, respectively (see Supporting Information).

In conclusion, we have employed supramolecular receptors to site-selectively recognize specific peptide modifications from a mixture of peptides in real enzymatic reactions. The activity of both histone methyltransferase and demethylase on specific methylation sites can be monitored. The sensitive molecular recognition capabilities of tailored deep cavitands provides site-selective discrimination between K methylations at different positions on the peptide backbone in a complex reaction mixture, and in the presence of interfering peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the National Science Foundation (CHE-1151773 to R.J.H.) and the National Institutes of Health (R01 CA188991 to W.Z., R21 ES025392 to Y.W.) for support. Dr. Karen Xu at the UCR Statistical Consulting Collaboratory is acknowledged for providing statistical analysis support.

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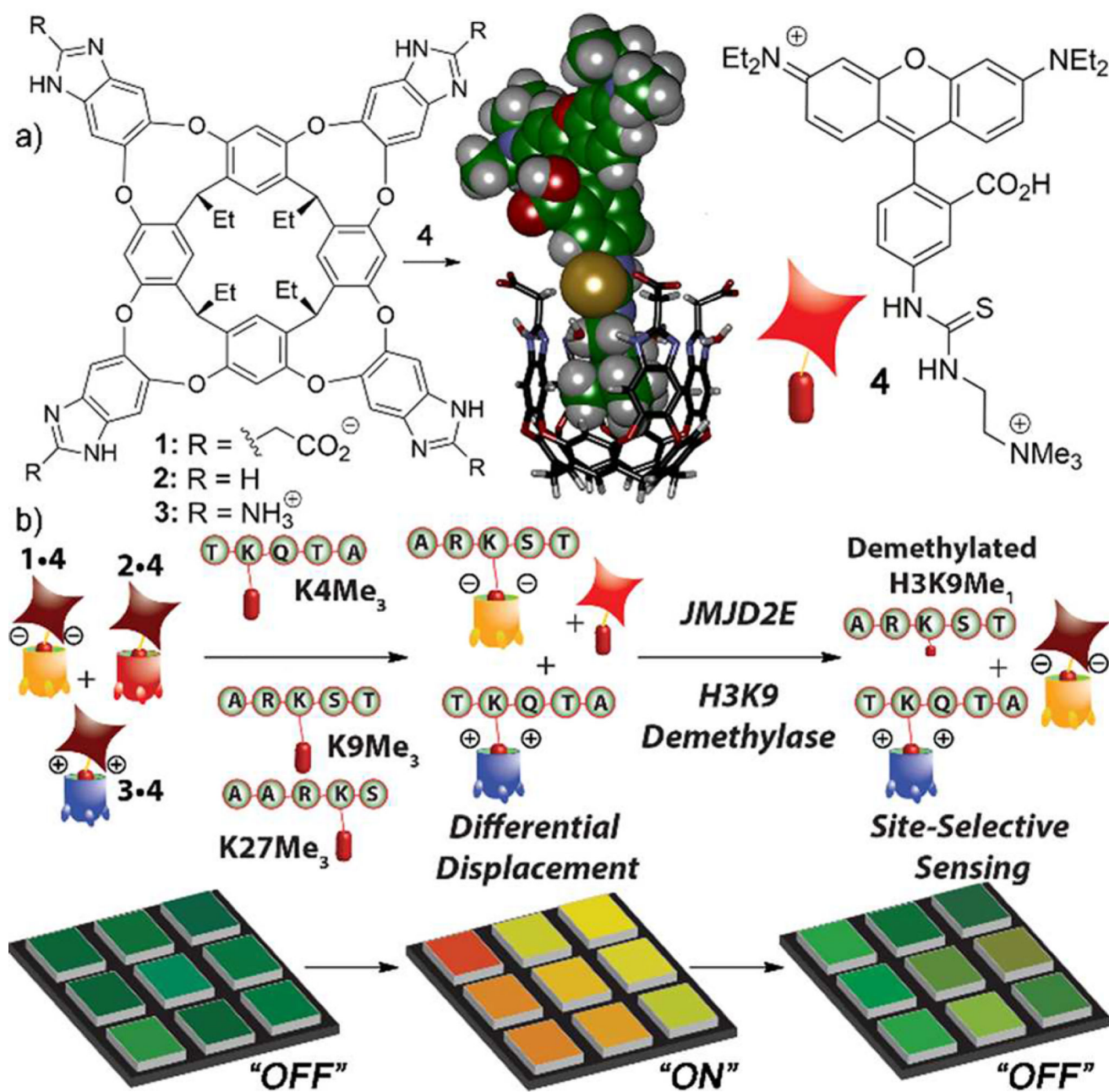


Figure 1.

(a) Structure of hosts **1–3**, guest **4** and a minimized model of the **1·4** host:guest complex (SPARTAN); (b) supramolecular tandem assay for a lysine demethylase enzyme.

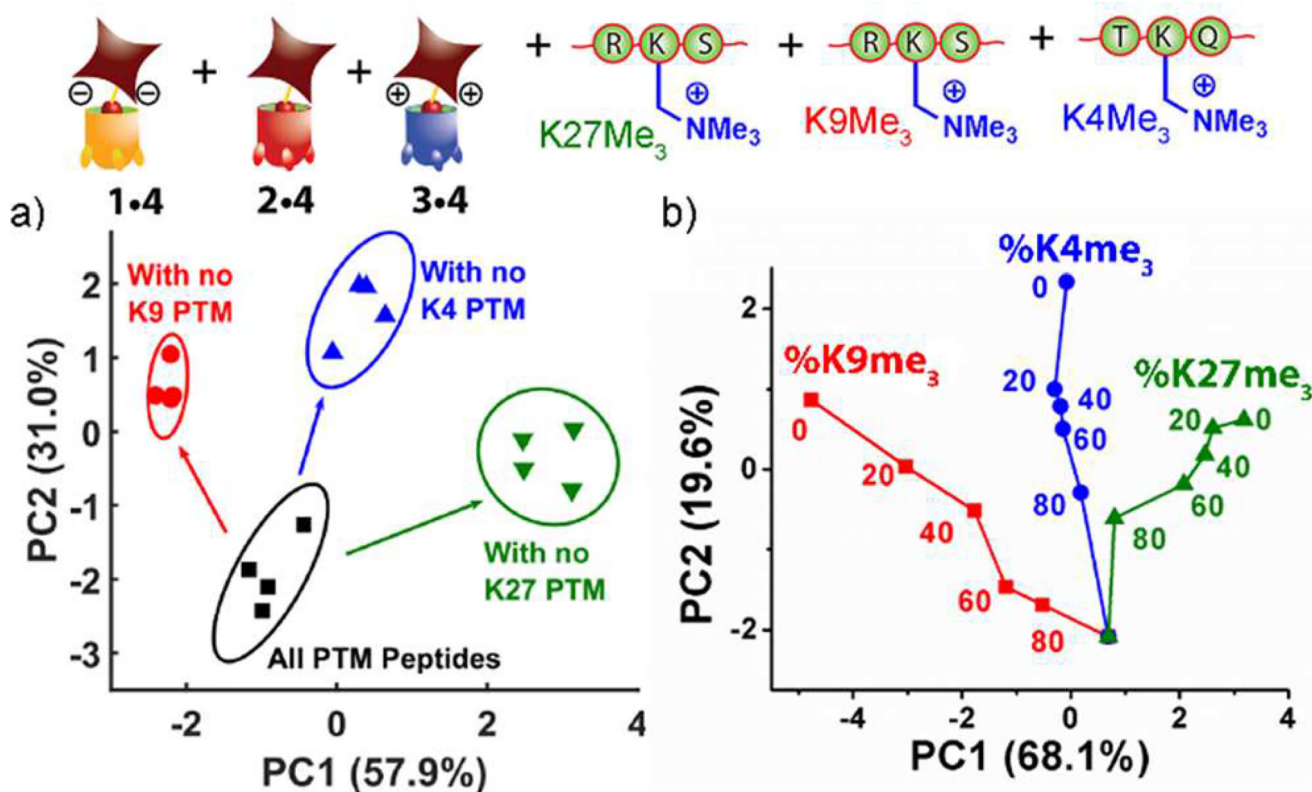


Figure 2.

Site-selective sensing in peptide mixtures. (a) Scores plot of the selective recognition of Kme₃ peptides in a peptide mixture with the (1-3)·4 sensor; (b) PCA scores plot of a lysine demethylase reaction mimic. [4] = 3 μ M, [1] = 4 μ M, [2,3] = 5 μ M, 50 mM phosphate buffer at pH = 7.4 or 50 mM carbonate buffer at pH = 9.0, [peptide] = 2 μ M. Ellipses drawn at 95% confidence.

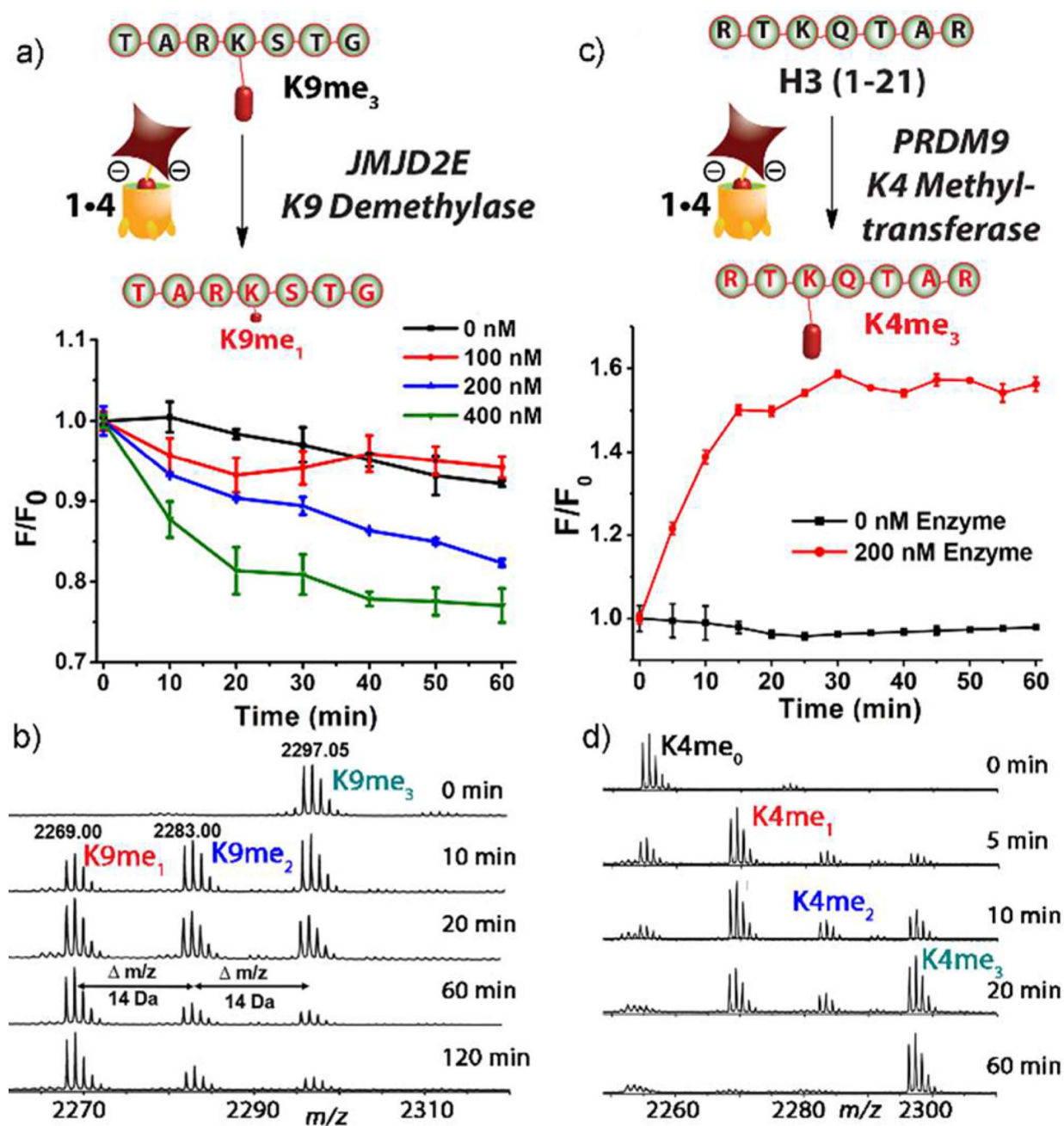
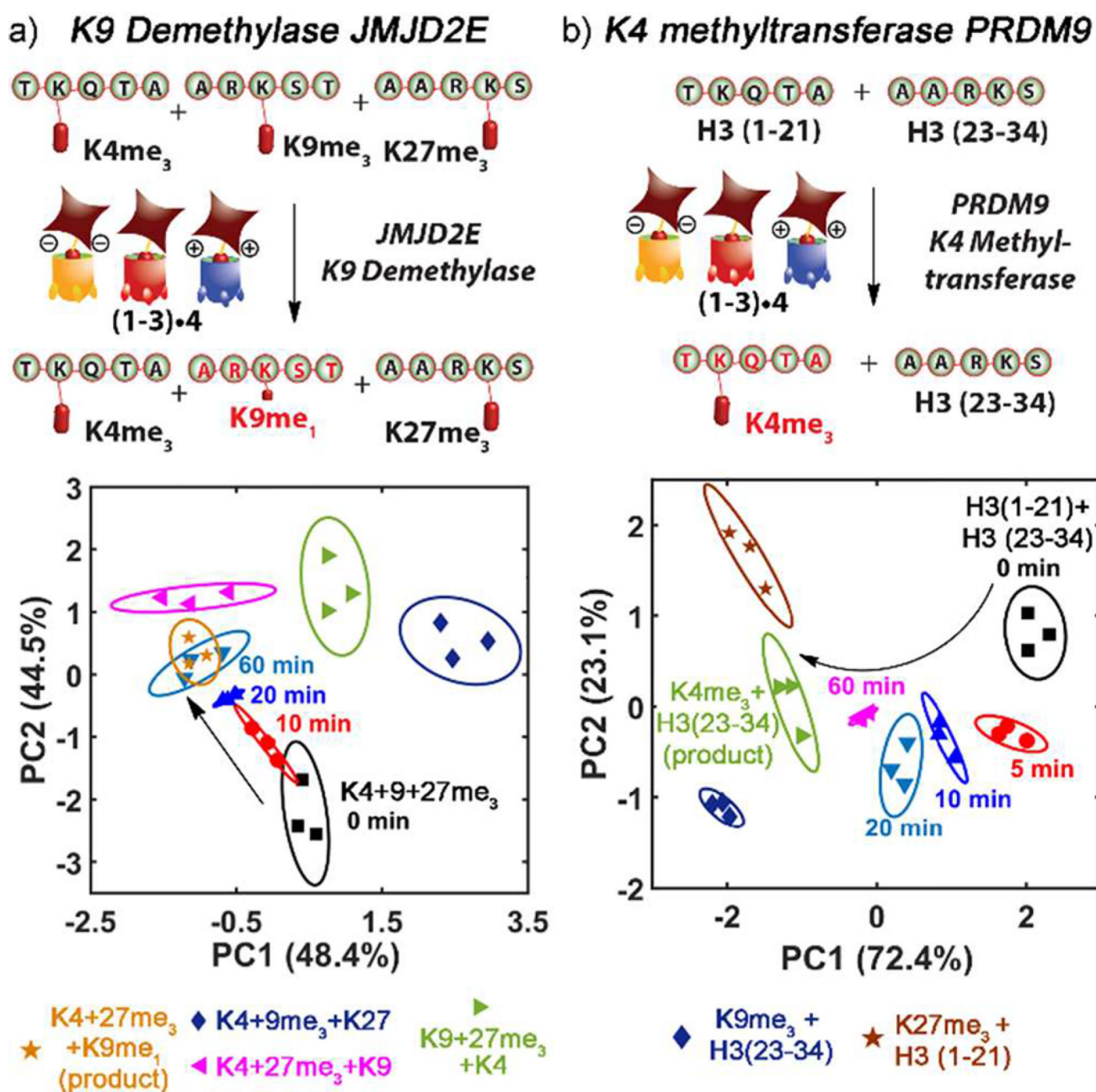


Figure 3.

Enzyme activity monitoring with a single component sensor. (a) [JMJD2E] dependence on fluorescence response; (b) MALDI-MS identification of the demethylation products over time, [4] = 3 μ M, [1] = 4 μ M, [peptide] = 2 μ M, [2-OG] = 50 μ M, [Fe²⁺] = 6 μ M; [ascorbate] = 0.5 mM; (c) [PRDM9] dependence on fluorescence response; (d) MALDI-MS identification of the methylation products over time, [4] = 3 μ M, [1] = 4 μ M, [peptide] = 2 μ M, [PRDM9] = 200 nM, [SAM] = 100 μ M [DTT] = 1 mM.

**Figure 4.**

Site-selective enzyme assays. (a) PCA scores plot of the selective demethylation of K9me₃ in a mixture of K4me₃, K9me₃, and K27me₃ peptides by JMJD2E with the (1-3)·4 sensor; (b) PCA scores plot of the selective methylation of K4me₃ in a mixture of H3 (1-21) and H3 (23-24) peptides by PRDM9 with the (1-3)·4 sensor. [4] = 3 μM, [1] = 4 μM, [2,3] = 5 μM, in 50 mM phosphate (pH 7.4). Ellipses drawn at 95% confidence.