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Antibody-Free Reading of the Histone Code Using a Simple Chemical Sensor Array

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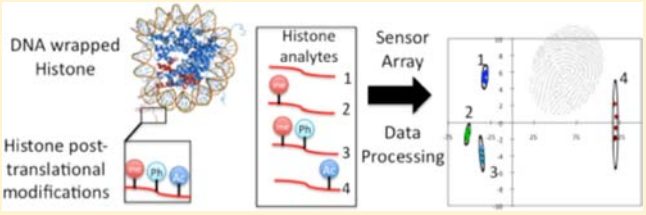
Supporting Information

ABSTRACT: The histone code refers to the complex network of histone post-translational modifications that control gene expression and are of high interest as drivers of a large number of human diseases. We report here on a mix-and-match toolkit of readily available dyes and calixarene host molecules that can be combined to form dye-displacement sensors that respond to a wide variety of cationic peptides. Using the data from only two or three such simple supramolecular sensors as a chemical sensor array produces fingerprints of data that discriminate robustly among many kinds of histone code elements. "Reads" that are accomplished include the discrimination of unmethylated, mono-, di-, and trimethylated lysines on a single histone tail sequence, identification of different modifications and combinations of modifications on a single histone tail sequence, identification of a single modification type in several different sequence contexts, and identification of isomeric dimethylarginine modifications. Reads that are sometimes troublesome for antibodies are achieved. We also report on the ability of the sensor array to report simultaneously on the concentrations and identities of histone modifications. This sensor array discriminates between post-translationally modified analytes without being limited to partners that contain a single, programmed binding interaction.



INTRODUCTION

The "histone code" refers to the numerous post-translational modifications present mainly on the N-terminal tails of the DNA-packaging proteins called histones.[1,2] The language of the code is written and erased by numerous enzymatic processes: phosphorylation of serine and threonine, acetylation or ubiquitination of lysine, multiple kinds of methylation of lysine and arginine, de-imination of arginine ("citrullination"), cis–trans isomerization of proline, and other modifications that continue to be discovered (Figure 1a).[3,4] The code is the control and communication mechanism for epigenetic signaling—post-translational modifications on histones control the expression of the genes coded in the associated DNA, often by signaling downstream DNA methylation (for silencing) or DNA demethylation (for expression) of the genes themselves.[5] Gene misregulation caused by aberrant histone code signaling is suggested to cause numerous cancers[6−8] and other human diseases,[9] and histone code pathways are increasingly being exploited as targets for novel therapeutics.

The histone code presents enormous complexity—a large variety of modification types and a large number of modification sites combine to produce millions of possible modification sets possible for a single histone tail.[11,12] Some individual modifications, and combinations of modifications, have attracted particular attention for their linkages to human disease. "Reads" that are required for various *in vitro* analyses of these biomedically important modifications include identifying the degree of methylation at a single site, the modification type at a single site, and multiple modifications at nearby sites, identifying one modification site from another, and identifying isomeric modification states such as asymmetric dimethylarginine (aDMA) and symmetric dimethylarginine (sDMA). Mass spectrometry can been used to monitor histone modifications, and aptamer-based approaches to recognition of histone modifications show promise.[10] However, antibodies remain the dominant tools used for identifying histone-code-related analytes. They are many and varied, and their power for discriminating analytes is considerable, yet the shortcomings of antibodies raised against histone-code targets are well known and are increasingly being reported in the literature. Known problems include high batch-to-batch variability and poor selectivity between similar analytes (e.g., histone 3, lysine 9 trimethylated = H3K9me3, and histone 3, lysine 27 trimethylated = H3K27me3, which are trimethyllysine marks on the same histone but flanked by highly similar amino acid sequences).[13−17] A recent study established standard performance benchmarks and tested hundreds of commercial polyclonal and monoclonal antibodies; over 20% failed specificity tests, leading the authors to establish a public database of antibody performance for use by researchers in this area.[13] One inherent problem that "quality control" efforts can do little about is epitope masking[18,19]—the mis-identification of analytes when an antibody misses its target residue because of a nearby residue that also bears a post-translational modification.

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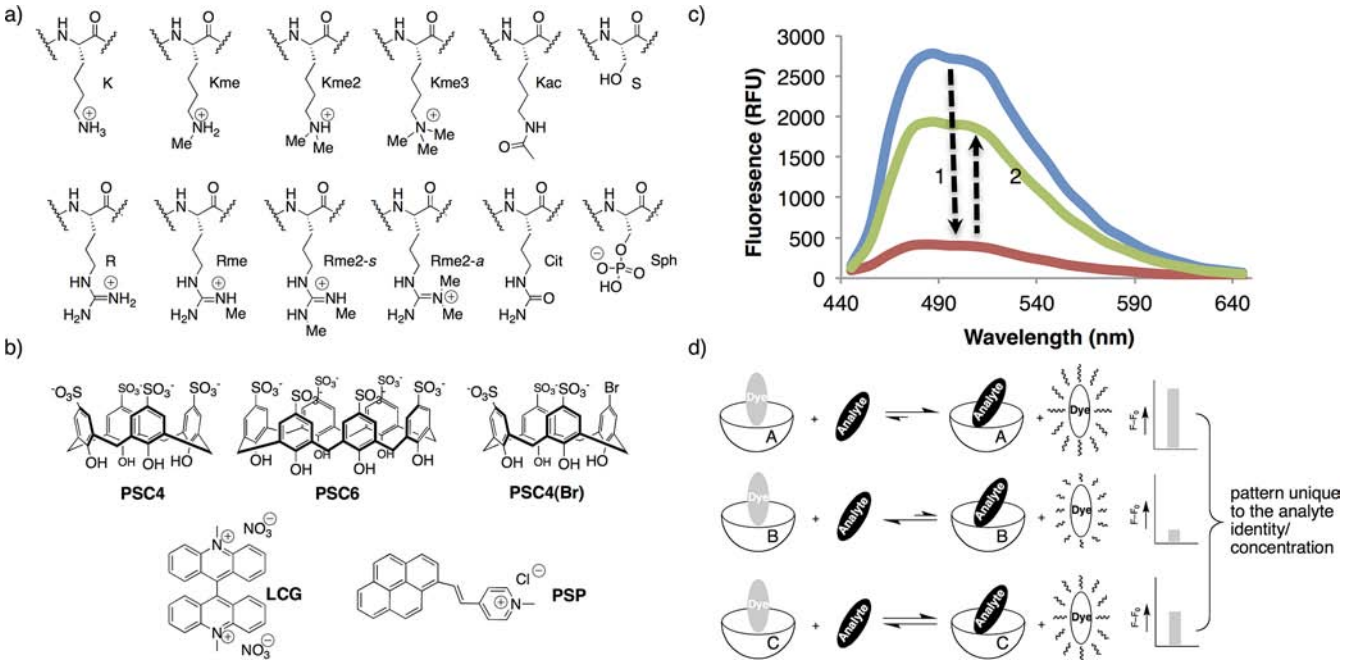

Figure 1. Structures of histone code analytes and basis for the performance of sensor components. (a) structures of post-translationally modified residues used in this study. (b) Hosts and fluorescent dyes used in the construction of the sensor arrays. (c) The principle of the fluorescent response is provided with representative data from a single sensor + analyte combination: the emission of a fluorescent dye (blue trace) is quenched upon addition of anionic host (red trace, step 1) and then restored to some extent upon addition of analyte (green trace, step 2) to produce a signal in the form of $F - F_o$. Conditions: phosphate buffer, 10 mM, pH 7.4; [**LCG**] = 0.5 μM; [**PSC4**] = 1.5 μM; [**Kme3**] = 200 μM. (d) Illustration of the principle of using patterns of data produced by chemical sensor arrays to identify and quantify analytes.

This problem is exacerbated for antibodies against the densely modified peptides that make up the histone code.

We wondered if a simple chemical sensor array approach might be able to provide a general solution for *in vitro* analysis of histone code analytes. The principle behind chemical sensor arrays is that they, while not being highly specific for any one analyte, can produce a *pattern* of signals that is unique to each analyte. The principle has been demonstrated for analyte sets of many types.[20−22] Examples of their use for biological analytes have included sensor arrays that can differentiate carbohydrates,[23] amino acids,[24] phosphorylated peptides,[25] small collections of proteins and glycoproteins,[26−29] and even cell subtypes.[30] Each of these systems is tuned to its analyte class and typically takes advantage of distinct programmed supramolecular interactions that are present in the whole class of analytes. But the chemistry of the histone code is remarkably diverse, involving modifications that neutralize cationic groups (acetylation), those that render neutral groups anionic (phosphorylation), and methylations that subtly change the size, shape, and hydrophobicity of cationic side chains without affecting their charge state (Figure 1a). All of this happens as part of signaling pathways where sequence context defines the biological outcomes of each modification. Despite the complexity of the targets, we report here on a new system that can, with a simple set of only three sensors, reliably distinguish a broad set of histone code analytes that encompasses several major forms of post-translational modification.

■ RESULTS AND DISCUSSION

Our sensors are based on an indicator displacement scheme in which a fluorescent dye is bound to and quenched by a suitable host molecule.[31,32] Addition of an analyte that can compete for the host's binding site causes release of some or all of the dye—a fluorescent turn-on response (Figure 1b−d). Our hosts of choice are sulfonated calixarenes (Figure 1b), which are readily available, promiscuous hosts that bind many cationic and neutral guests in polar solutions. We have previously shown that *p*-sulfonatocalix[4]arene (**PSC4**) and its simple analogues have a strong affinity for methylated lysines and moderate affinity for methylated arginines,[33,34] and others have measured affinities of **PSC4** and related hosts for many other peptides and proteins.[35−41]

We developed two distinct types of sensor arrays using these common building blocks: The first array, "Type 1", employs a single calixarene host (*p*-sulfonatocalix[6]arene, **PSC6**) in all sensor elements and uses the variation of pH and/or organic co-solvent conditions as a primary means of generating different fluorescent responses for different analytes (i.e., a "fingerprint"). In our hands, the dye **PSP**, first used in this context by Shinkai, is best suited to operating with co-solvents present.[19] Our "Type 2" array uses pure, buffered water and generates varied analyte-specific fingerprints by using different calixarene hosts (**PSC6**, **PSC4**, and **PSC4(Br)**) for each element of the sensor array. The dye **LCG**, reported in a related dye displacement application by Nau, was best suited for this type of array, as it has affinity for almost any sulfonated calixarene and does not require organic co-solvents.[20] In each case, we operate the arrays by mixing sensor array components (dye, calixarene, buffer, solvent) and analytes (see below) in a 96-well plate. The signal from each analyte/sensor element combination is the fluorescence emission ($F - F_o$) value at λ_{max}, determined using a fluorescence plate reader.

Initial proof-of-concept studies involved a set of closely related modified amino acids (R, K, Kac, Kme, Kme2, and Kme3; Figure 1a) that cannot be distinguished by conventional, single-sensor dye displacement due to their very similar

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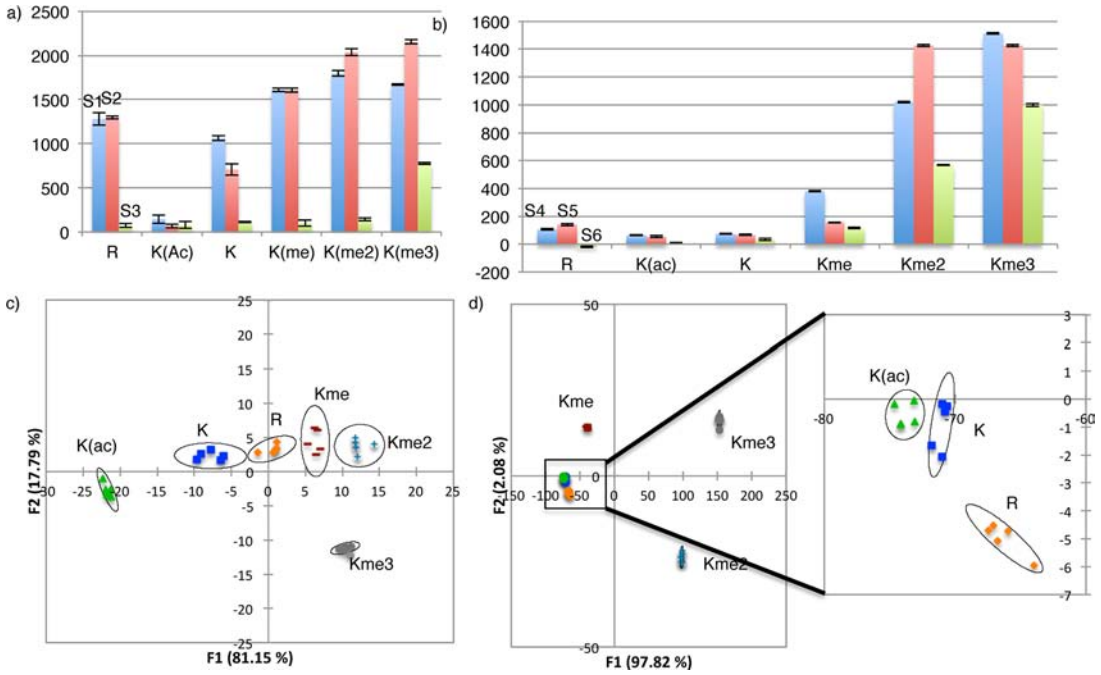

Figure 2. Fingerprints in raw and processed forms for a test set of modified amino acids. (a,c) Raw fingerprints ($F - F_o$) (top) and LDA (bottom) for analysis of amino acids by the Type 1 array in which the host and dye remain the same but pH and solvent composition vary. Error bars on bar graph are standard deviations of individual sensor responses. Ellipsoids on the scatter plot are drawn at 99% confidence. Conditions: [**PSP**] = 100 μM; [**PSC6**] = 100 μM; [analyte] = 4 mM. Sensor element 1 (S1): [$NH_4CH_3CO_2$] buffer] = 20 mM, pH 4.8, in 67% MeOH/H_2O. Sensor element 2 (S2): [Na_2HPO_4/NaH_2PO_4 buffer] = 20 mM, pH 7.4, in 67% MeOH/H_2O. Sensor element 3 (S3): [$Na_2CO_3/NaHCO_3$ buffer] = 20 mM, pH 10.8, in 67% MeOH/H_2O. (b,d) Raw fingerprints ($F - F_o$) (top), LDA (bottom left), and LDA of expanded K, Kac, R region (bottom right) for analysis of amino acids by the Type 2 array in which pH and solvent remain the same but different hosts are used together with the dye **LCG**. Error bars on the bar graph are standard deviations of individual sensor responses. Ellipsoids on the scatter plot are drawn at 90% confidence. Conditions: [**LCG**] = 0.5 μM; [Na_2HPO_4/NaH_2PO_4 buffer] = 10 mM, pH 7.4; [analyte] = 200 μM. Sensor element 4 (S4): [**PSC4**] = 1.5 μM. Sensor element 5 (S5): [**PSC6**] = 1.5 μM. Sensor element 6 (S6): [**PSC4(Br)**] = 1.5 μM. See SI Figures 1S and 2S for raw fluorescence data.

structures and/or charge states at neutral pH. The Type 1 array, which varies pH and organic co-solvent, generated unique sets of fluorescent responses for this set of analytes that arise from the analytes' different affinities for the host at different pH values. In all of these cases, the simple three-element "fingerprints" of each analyte are highly reproducible and easily discernible from each other by human inspection of the raw data (Figure 2a and SI). In order to put the analyte identification on a solid footing, we executed linear discriminant analysis (LDA), a statistical method that converts the multivariable raw fingerprint data into simple plots that express the multidimensional variance in the data sets as only two variables (Figure 2c).[42,43] The confidence level of discrimination (in the case of Figure 2c, 99%) can be determined and depicted graphically; inspection of the resulting ellipsoids at various confidence levels together with the set of 5−6 actual data points used to train the system provides a good understanding of a particular sensor array's ability to discriminate between a given set of analytes. Two additional types of controls were performed: an internal statistical control, wherein a single replicate is omitted from the LDA analysis and then re-entered as a naïve test point, and an external blind test, wherein analytes are prepared as fresh stock solutions by one researcher and provided blindly to another for identification.

While the Type 1 system using only **PSC6** proved the concept, we sought also a simple sensor array that could operate in pure, buffered water without needing an organic co-solvent. The Type 2 array, constructed from three different calixarenes, all at neutral pH, also achieved discrimination of a similar set of amino acid analytes with 90% confidence (Figure 2d). In this case the different fingerprints have nothing to do with the analytes' pK_a values but instead arise from differences in analyte affinities for each of the three different calixarene hosts used. Of note in this data set is that the method does not require high-affinity binding; even the very low fluorescent responses generated by R, K, and Kac in the Type 2 array (~5% of maximum signal intensity) are sufficient for discrimination of analytes with 90% confidence. We also found generally better reproducibility for the **LCG**-based sensors than the **PSP**-based sensors (see SI for raw data for all replicates, and for an extended discussion of the tests carried out to confirm reproducibility).

The vast majority of *in vitro* biochemical reads of the histone code use peptides bearing the specific modification(s) in question. The use of peptides is generally justified by the fact that the completely unstructured tails of whole histones are well represented by peptides.[44] We extended our analyses beyond simple amino acids by using synthetic modified peptides, first finding that that the pure-water Type 2 conditions proved adept at discriminating mono-, di-, and trimethylation states of lysine on histone 3, lysine 36 (H3K36) peptides at 99% confidence (Figure 3). An alternate set of analytes arises when considering the histone 3, lysine 9 (H3K9) site that is known to be methylated *or* acetylated, and whose biological functions are also controlled by phosphorylation of neighboring serine 10. These modifications operate as an interconnected set of gene up- and down-regulation signals that are important in human cancers.[45−47] The Type 2 array as previously optimized was

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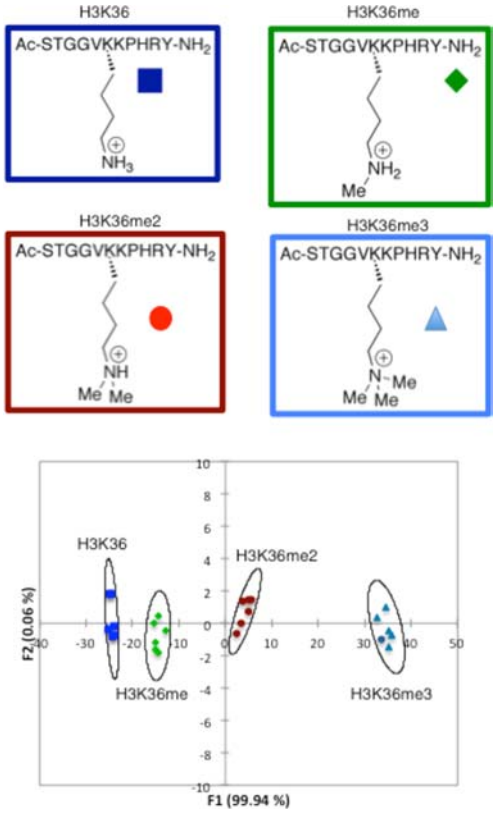

Figure 3. Reading lysine methylation states. LDA (bottom) of $F - F_o$ data arising from a Type 2 sensor array composed of S4 and S6 treated with analytes (top) at 5 μM. Ellipsoids drawn at 99% confidence. See SI Figure 3S for raw fluorescence data.

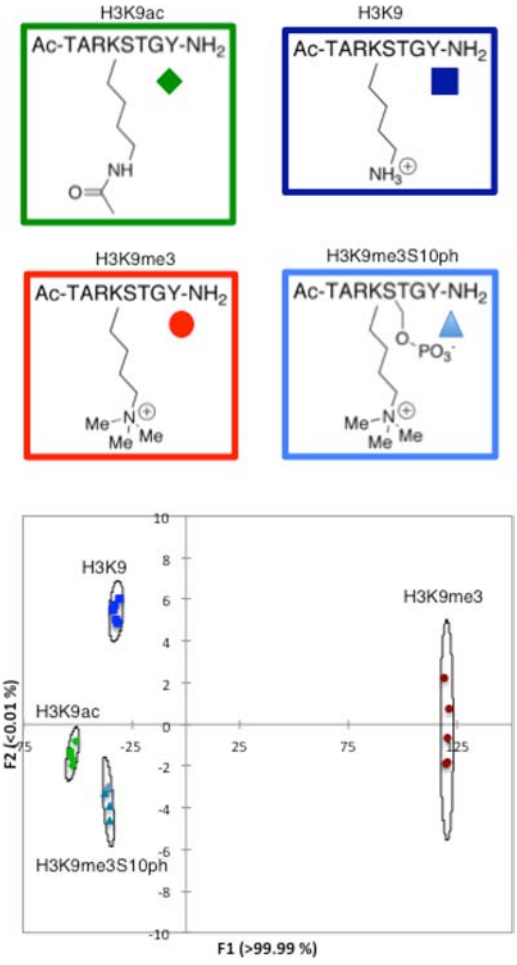

Figure 4. Reading H3K9-related histone code elements. LDA (bottom) of $F - F_o$ data arising from a Type 2 sensor array composed of S4, S5, and S7 treated with analytes (top) at 5 μM. Ellipsoids drawn at 99% confidence. Sensor element 7 (S7): [**LCG**] = 0.5 μM; [**PSC4**] = 1.5 μM; [$NH_4CH_3CO_2$ buffer] = 20 mM, pH 4.8. See SI Figure 4S for raw fluorescence data.

able to discriminate among the modified states of H3K9ac, H3K9, and H3K9me3 but was unable to discriminate H3K9me3S10ph (data not shown). The simple addition of an array element composed of a low-pH buffered condition with **PSC4** added an additional discriminating element to the fingerprint and allowed the global discrimination of all four analytes in this series using only three sensors at 99% confidence (Figure 4).

Next, we studied a set of histone peptides bearing known trimethyllysine marks on different peptide sequences (H3K4me3, H3K9me3, H3K27me3, H3K36me3, and H4K20me3). This challenge can be difficult for antibodies, and cross-reactivity is a known problem here, especially between the similar H3K9me3 and H3K27me3 sequences.[13,48,49] The Type 2 array as initially optimized succeeded at discriminating between all of these analytes. In fact, we found that only the responses of two sensors, **PSC4** and **PSC4(Br)**, were required to achieve the discrimination of all five trimethyllysine peptides to 99% confidence (Figure 5). The scatter in the H4K20me3 data can be attributed to the response from the sensor containing **PSC4**, as the sensor's response was near saturation for this analyte only. In spite of the scattered values, the array easily discriminated this analyte at high confidence.

Finally, we tackled one of the most difficult reads—discriminating between post-translational modification states of arginine—by analyzing the peptides related to the histone 4, arginine 3 signaling motif composed of H4R3 (the parent peptide), H4R(Cit)3, H4R3me2-*a*, and H4R3me2-*s*. Methylation levels of H4R3 are regulated by de-imination by PAD4 to make citrulline,[50] and H4R3 can also be transformed to asymmetric dimethylarginine (Rme2-*a*, or aDMA) or symmetric dimethylarginine (Rme2-*s*, or sDMA), which are isomeric marks specifically installed by different methyltransferases.[51,52] Despite the chemical similarity of these marks,[53] they encode biologically distinct signals that can completely oppose each other in the cell.[54] (The monomethylated derivative, H4R3me, was not included here as it is apparently unused in the biology of H4R3 signaling.) While using the Type 2 sensor array for these analytes, we found that the magnitudes of fluorescence responses were small (10-fold weaker than for Kme3-containing peptides, see SI for raw data) under our standard conditions. Nevertheless, the fingerprint data allowed discrimination of these four related analytes, including the separation of H4R3me2-*a* and H4R3me2-*s* peptides that differ only by isomeric transposition of a single methyl group from one nitrogen to another on the same arginine side chain (Figure 6).

Chemical pattern recognition-based analyses are well suited to these complex biological analytes, and long-term prospects for applications in epigenomics research and health diagnostics beckon. Shorter-term targets include the development of novel biochemical assays that can be used to characterize writer/

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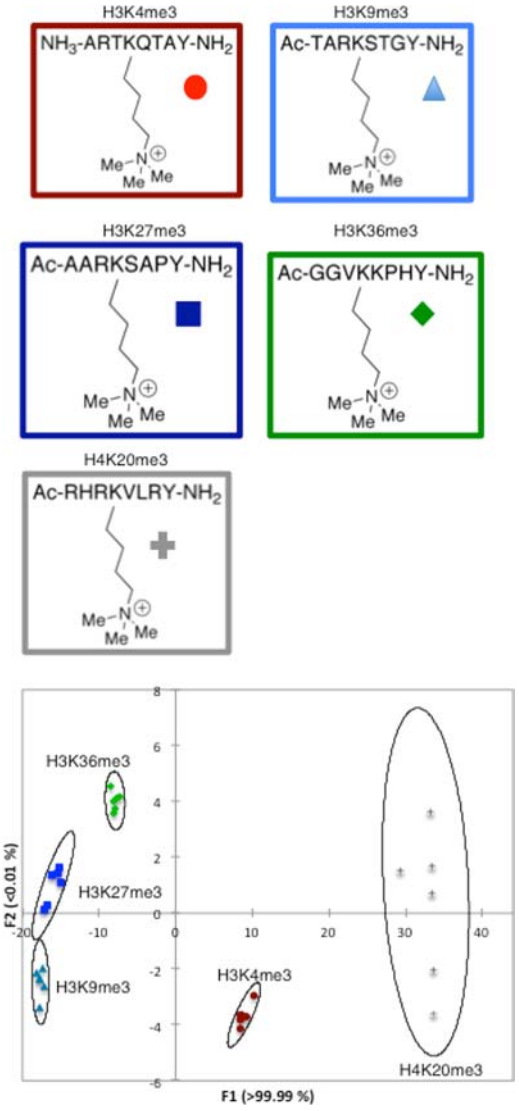

Figure 5. Reading different trimethyllysine sites. LDA (bottom) of $F - F_o$ data arising from a Type 2 sensor array composed of S4 and S6 treated with analytes (top) at 5 μM. Ellipsoids drawn at 99% confidence. See SI Figure 5S for raw fluorescence data.

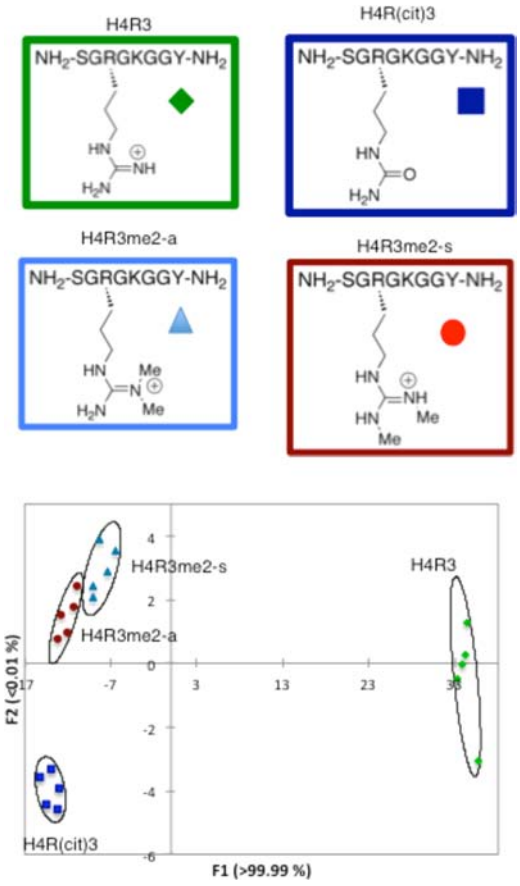

Figure 6. Reading arginine methylation states. LDA (bottom) of $F - F_o$ data arising from a Type 2 sensor array composed of S4, S5, and S6 treated with analytes (top) at 5 μM. Ellipsoids drawn at 85% confidence. See SI Figure 6S for raw fluorescence data.

eraser enzyme behavior and to discover inhibitors and/or modulators of the same enzymes. Our preliminary data suggest that a single sensor array might serve as an *in vitro* readout for dozens or hundreds of enzymes, without the need for antibodies specific to the products of the various enzymatic reactions. Indeed, a recent report by Nau has demonstrated that a single dye-displacement sensor made from **PSC4** and **LCG** can track lysine methylation on a single substrate, proving that this basic supramolecular setup is capable of monitoring enzymatic activities.[55] We tested the ability of our dye-displacement sensors to track the conversion of one common histone tail starting material (H3 tail, aa's 1−12) to two distinct end products representing methylation at two biomedically important sites (H3K4me3 and H3K9me3). The process involved preparing different individual sensors each composed of **LCG** mixed with a different calixarene at neutral pH, and treating each sensor with peptides H3 and H3K4me3 *or* H3 and H3K9me3 in ratios varying from 100:0 to 0:100 to reflect the conversion of H3 to either product (total peptide concentration was maintained at 500 nM). The performance of each dye-displacement sensor considered individually was relatively poor: overall signal changes for extent of conversion of H3 to either product were low, and in most cases little difference existed between the signal arising from the creation of each product (see Figure 7b,c for representative examples from two of the calixarenes). The processing of data from two different sensors as a simple, two-component sensor array, however, revealed the unique capabilities of this toolkit. Executing principal component analysis (PCA), an unsupervised statistical method that is complementary to LDA,[43] on the data sets arising from two simple sensors (**PSC6** and **PSC4(Br)**) produced data that easily resolved both the extent of conversion and the identities of the products from each other (Figure 7d). This type of analysis is simple to execute yet extremely information rich; such data represent a unique determination of both identities and concentrations of histone code elements that can be operated in a continuous and homogeneous manner.

■ CONCLUSION

Broadly, these sensor arrays have several novel features that distinguish them from the multitude of antibody-based reagents and assays that have been developed to serve this important field of research. They are made from simple chemicals purified to homogeneity. As such, they provide superb replicate-to-replicate reproducibility and can be expected to give better batch-to-batch and lab-to-lab reproducibility than any form of

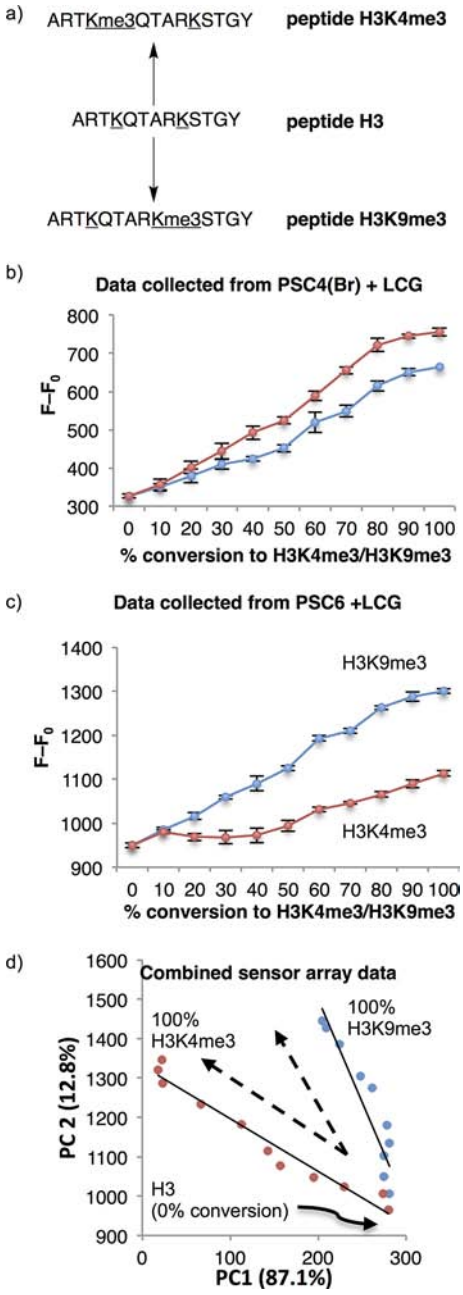

Figure 7. Identifying and quantifying alternate reaction products from a single starting material. (a) Histone 3 residues 1−12 (peptide H3) can be trimethylated at either K4 or K9. (b−d) Dye displacement data for mixtures of H3 and H3K4me3 or H3K9me3 in ratios varying from 100:0 to 0:100, representing conversion of starting material to either product at 500 nM peptide. Data points shown are averages of five determinations; error bars represent standard deviations: (b) data arising from a single dye displacement sensor based on **PSC4(Br)** (S6), and (c) data arising from a single dye displacement sensor based on **PSC6** (S5). (d) PCA treatment of data from (b) and (c) together as a two-component sensor array, providing simultaneous readout of extent of conversion and product identity.

antibody (even monoclonals). They are cheap and easy to set up (**PSC4** and **PSC6** are commercially available, and **PSC(Br)** is easily prepared).[34] Once toolkit members are in hand, they are optimized for each task through simple mix-and-match protocols that can be completed in approximately 1 h, including solution preparation, pipetting, data collection, and data processing steps. Despite the diversity of motifs studied, reads of all types reported in this paper were achieved using only two or three calixarenes. If needed, the toolkit will be easily expandable; there exists a large set of anionic calixarenes that bind to cationic peptides and proteins[34,56] and a diverse set of dyes that are quenched by such hosts.[32] Unlike antibody-based assays that rely on stopping reactions after set times and then adding antibodies and other reagents to "develop" a signal (normally by ELISA-like methods), these sensor arrays operate in homogeneous solution and can provide continuous data. They are obviously limited, in their current embodiment, to *in vitro* analyses. Antibodies are able to function at lower analyte concentrations, in physiologically relevant conditions, and in complex chemical mixtures and thus will continue to rule where analysis of heterogeneous samples from natural sources is required. But for the in vitro biochemical analyses that are the backbone of basic biochemical research on epigenetic pathway members and associated drug development efforts, these sensor arrays represent a single tool that can read histone code modifications of many types, likely including many that are yet undiscovered.

The ease with which these simple sensor arrays distinguish such varied motifs might also teach us something about nature. While most histone-modifying enzyme "writer" and "eraser" enzymes function with very high specificity on a single substrate, most studies on individual "reader" proteins that have evolved to bind to histone code elements show that they have broad and overlapping selectivities for their targets.[57] There is a growing understanding that the histone code is read out in the cell by a complex network of interdependent and parallel signals that are patterned throughout the chromatin, and *not* as simple serial on/off signals generated by individual marks on individual genes. So it is perhaps not surprising that a similar approach, using synthetic agents that are broadly selective, but not highly specific, recognition elements, is so adept at reading the histone code.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Complete description of synthesis of peptides, procedures for sensor array operation, and raw fluorescence data for all replicates of all determinations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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