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Single-Cell Quantification of Cytosine Modifications by Hyperspectral Dark-Field Imaging

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Abstract

Epigenetic modifications on DNA, especially on cytosine, play a critical role in regulating gene expression and genome stability. It is known that the levels of different cytosine derivatives are highly dynamic and are regulated by a variety of factors that act on the chromatin. Here we report an optical methodology based on hyperspectral dark-field imaging (HSDFI) using plasmonic nanoprobes to quantify the recently identified cytosine modifications on DNA in single cells. Gold (Au) and silver (Ag) nanoparticles (NPs) functionalized with specific antibodies were used as contrast-generating agents due to their strong Local Surface Plasmon Resonance (LSPR) properties. With this powerful platform we have revealed the spatial distribution and quantity of 5-carboxylcytosine (5caC) at the different stages in cell cycle, and demonstrated that 5caC was a stably inherited epigenetic mark. We have also shown that the regional density of 5caC on a single chromosome can be mapped due to the spectral sensitivity of the nanoprobes in relation to the inter-particle distance. Notably, HSDFI enables an efficient removal of the scattering noises from non-specifically aggregated nanoprobes, to improve accuracy in the quantification of different cytosine modifications in single cells. Further, by separating the LSPR fingerprints of AuNPs and AgNPs, multiplex detection of two cytosine modifications was also performed. Our results demonstrate HSDFI as a versatile platform for spatial and spectroscopic characterization of plasmonic nanoprobe-labeled nuclear targets at the single-cell level for quantitative epigenetic screening.

Keywords

quantitative imaging; epigenetics; cytosine modification; hyperspectral dark-field microscopy; plasmonic nanoprobes

Introduction

A growing body of research evidence has suggested that epigenetic regulation and aberration play pivotal roles in a wide range of physiological and pathological processes.¹

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As one of the dominant epigenetic modulators, DNA methylation, which involves the addition of a methyl-group to the 5-carbon of cytosine, serves to functionally switch on/off the downstream transcription.²⁻⁵ Since the discovery of the ten-eleven translocation (TET) family protein-mediated oxidization of 5mC into 5-hydroxymethylcytosine (5hmC)^{6, 7} and the very recent findings that TET proteins can further oxidize 5hmC to 5-formylcytosine (5fC) and eventually 5-carboxylcytosine (5caC),⁸ it is believed that these cytosine derivatives act in cohort to regulate gene expression. However, the distribution and relative abundance of those newly discovered cytosine modifications (*i.e.* 5fC and 5caC) in different cell types, as well as at different cell phases, are poorly characterized. Past work has extensively expounded on the effect of 5mC and 5hmC,⁹⁻¹² on cell state and disease. While our understanding of 5fC and 5caC is still in its infancy, some attempts utilizing ensemble biochemical approaches have been made to characterize the overall properties of these cytosine marks from average measurements in population of cells to provide a general estimate.^{11, 13, 14} Fluorescence microscopy has been one of the most widely used optical methods for *in situ* visualization of biological molecules at the cellular and subcellular levels,¹⁵ but quantification of cytosine modifications has been a grand challenge due to the inherently small quantum yield of available fluorophores and the trace amount of targets. Hence, quantitative assessment of epigenetic marks at the single-cell level has been impeded by the limits in spatiotemporal resolution and low signal-to-noise ratio (SNR) of the current imaging methodologies.

Hyperspectral imaging (HSI) is an approach that allows for a high-resolution spectrum to be acquired for each pixel in an image.^{16, 17} From the collected spectral signatures, the spatial distribution of the optically active probes can be accurately obtained. Dark-field microscopy can achieve a high SNR by excluding the unscattered incident beam to generate a clear background, which enhances the contrast when imaging unstained samples. Combining the dark-field illumination with an HSI module, a unique platform can be developed for identification of the location and composition of plasmonic nanomaterials in biological specimen with a better quantitative acuity. Compared with fluorescence microscopy, the HSDFI approach suffers minimally from auto-fluorescence, photobleaching, and phototoxicity. Table S1 provides a synopsis of the comparison between fluorescence and plasmonic imaging methods.

Noble metal nonmaterial has been the subject of intense research and proven to be photostable, yielding strong LSPR signals, which is applicable for intracellular single-particle detection.^{18, 19} Owing to the dipole resonance from the interaction with incident photons, the large scattering cross-section of metal NPs can generate a ten- to million-fold stronger signal than conventional fluorophores,^{20, 21} providing a high SNR without laser excitation. Besides, the LSPR spectrum can be fine-tuned, dependent on the NP size, shape, material, and surrounding environment.^{22, 23} Noble metal NPs exhibit their LSPR peaks over a wide range of wavelengths, covering from the visible to near-infrared regions.²⁰ The wide coverage and sharp bandwidth of LSPR spectra will potentially allow for a large number of distinct labels used for multiplex molecular imaging. Recently, several groups have achieved preliminary success in using the spectral shift of plasmonic nanoparticles to infer on the local density of nanoparticles as well as targeting key biomolecules of

interest.²⁴⁻²⁷ However, all of these works have focused on detection of cell surface markers, while achieving similar imaging sensitivity in the quantification of nuclear targets has been challenging because of the strong background noise from the cytoplasmic organelles and inefficient probe delivery.

In this study, we present an approach based on HSDFI with plasmonic nanoprobe, to detect key cytosine modifications on DNA at the single-cell level. Our method was successfully applied to characterize the low-level cytosine modifications under different conditions, such as in different cell lines, at different cell phases, and even on a single chromosome. The quantification accuracy of HSDFI was validated with an enhanced enzyme-linked immunosorbent assay (ELISA) developed in our laboratory.^{28, 29} Further, by using different NPs (Au and Ag), multiplex detection of two cytosine modifications along with information on their distribution and co-localization was demonstrated in single cells. These results evidently support the uniqueness of our strategy for single-cell quantitative epigenetic profiling not easily accessible by other methods.

Results

Plasmonic nanoprobe for detection of cytosine modifications

As depicted in Fig. 1(a), experiments in this study were performed with primary antibodies targeting specific DNA modifications and secondary antibodies conjugated with 30 nm AuNPs or 20 nm AgNPs to balance the trade-off between steric hindrance, binding efficiency and SNR. Fig. S1 provides the characterization of the probes by TEM imaging, UV-Vis spectroscopy and photostability test, which prove the excellent uniformity and superior photostability of the probes. Targeting these probes to a cytosine modification site will produce a strong signal due to the large LSPR scattering cross-section of plasmonic nanoprobe. The basic quantity and local density of plasmonic nanoprobe, as determined by the LSPR spectral signature and signal intensity, could be used to quantify the average number and regional distribution of a specific cytosine modification in a single cell with HSDFI microscopy (Fig. 1(b)). The optical layout and imaging procedure of HSDFI is depicted in Fig. 2 where *in vitro* detection of the AuNPs probes and AgNPs probes is demonstrated. As illustrated in Fig. 2(a), 1D hyperspectral images along the x-coordinate are obtained with a slit as an initial step, then the spectral data for each pixel in the image is acquired by slit-scanning along the y-axis. The characteristic spectra of AuNPs probes and AgNPs probes extracted from the obtained hyperspectral dataset are shown in Fig. 2(b-c). The LSPR peak of AuNPs in buffer is around 537 nm, while the peak of AgNPs is around 419 nm, corresponding to the perceived colors – green and blue, respectively. This set of *in vitro* characterization of the chosen nanoprobe demonstrates the optical validity of our method for the detection of specific cytosine modification sites on the DNA.

Single-cell quantification of cytosine modifications

To evaluate the quantification efficiency of our strategy, first we demonstrate the detection of one type of cytosine modification. Specifically, we probed a cell cycle-dependent abundance of 5caC. The heritability of epigenetic marks, including cytosine modifications, is elusive. Although in somatic cells the landscape of 5mC could be re-established in high

To demonstrate the quantification accuracy of HSDFI, we conducted experiments in two cell lines for different cytosine modifications – 5caC in HeLa cells and SF767 cells, as well as 5fC in HeLa cells, which can also provide valuable information about the cell type-dependent epigenetic heterogeneity. From our experience, plasmonic nanoprobe might aggregate to some extent for which a filter function is needed to exclude these abnormal spots to obtain precise quantification. The LSPR spectra of over-aggregated nanoprobe are usually distantly shifted from the spectra of non-aggregated ones. Based on the histograms of LSPR peaks of different nanoprobe (Fig. S3), wavelength filters can be set (*e.g.* 635 nm for AuNPs). Fig. 4(a) presents the process of filter-facilitated quantification. After excluding those outlier spots beyond the wavelength threshold, better quantification information can be obtained. Fig. 4(b) shows the representative post-processed binary images and the quantitative results. Our data suggest that the relative amount of 5caC in SF767 cells to that in HeLa cells is around 1.97, which is in excellent agreement with the ELISA quantification. In general, 5mC is the most abundant form of cytosine modifications while 5caC and 5fC accounts for less than 0.01%.^{8, 28} From the results presented in Fig. 4, the brain SF767 cells

have a higher level of 5caC than HeLa cells. In fact, this observation is consistent with previous research reported on 5hmC since brain tissue has the highest abundance of 5hmC compared to other human tissues. Considering the fact that 5caC is the oxidized derivative from 5hmC, the relative quantities of 5caC thus exhibit a similar pattern to 5hmC in our tested cell lines.³³ Fig. 4(b) also shows that the detected level of 5caC is higher than 5fC in SF767 cells, with a ratio of around 1.56, which indirectly supports the fact that 5caC may be more stable than 5fC in cells and agrees with the results obtained with ELISA. These results support the quantification capability of our strategy and thus can be extended to detect the terminal cytosine derivatives (substrates for the TDG enzyme-mediated base excision repair) to assess the turnover process of DNA methylation in various biological processes.

Multiplex detection of cytosine modifications

After demonstration of detecting individual cytosine modifications, we performed experiments to evaluate the effectiveness of HSDFI for multiplex detection. To obtain equivalent scattering intensities from plasmonic nanoprobe of different materials, 30 nm AuNPs and 20 nm AgNPs were chosen. *In vitro* calibration was performed to ensure that the LSPR spectra did not interfere with each other. A mixture of AuNPs and AgNPs nanoprobe was placed onto the glass slide to obtain the hyperspectral dark-field images. From Fig. 5, these two types of nanoprobe can be readily distinguished based on their distinct scattering colors and spectral signatures. The green color corresponds to single AuNPs, while the blue color corresponds to single AgNPs. Some cyan spots exist as circled in Fig. 5(a), indicating the co-localization of AuNPs and AgNPs. The extracted spectral information in Fig. 5(b) shows the superposed LSPR peaks of AgNPs and AuNPs without a wavelength shift, which implies that no LSPR coupling occurs for these two nanoprobe. These results indicate that the spectra at each spot could denote the localization of plasmonic nanoprobe and also implicate the possible interaction of the target markers at the single-particle resolution.

Multiplex detection was demonstrated to analyze 5ca and 5mC in MCF-7 cells (Fig. 6). First, the two modifications were respectively tagged by AgNPs and AuNPs nanoprobe as shown in Fig. S3. The image of multiplex labeling is shown in Fig. 6(a). Fig. 6(b) provides the spectra corresponding to three plasmonic patterns as observed in Fig. 6(a), which represents separate AgNPs, AuNPs, and LSPR-coupled AgNP-AuNP probes (the coupled resonance is characterized by the red curve and denotes a red-shift in the spectrum). The inter-particle distance was estimated to be ~ 1 nm from the theoretical FDTD simulation (Fig. 6(c)). The plasmonic wavelength peaks exhibit a small overlap between these three conditions with respect to their wavelength windows as illustrated with blue, green, and red columns in Fig. 6(c). By processing the image from this dual-labeled cell, binary images from the three channels are reconstructed as in Fig. 6(d-f), depicting the degree of 5caC-5mC co-localization and that the abundance of 5caC-enriched sites is much less than 5mC. The relative quantity, distribution, and co-localization of 5caC and 5mC can be easily perceived from the merged image in Fig. 6(g). These results indicate that HSDFI with targeting plasmonic probe can realize multiplex detection to assess the quantity and interaction of epigenetic modifications that can be potential biomarkers for single-cell identity profiling.

Discussion

Until today our understanding of epigenetic regulation has primarily come from ensemble measurements using population of cells or bulky samples. Although the current analytical methods such as mass spectrometry and next-generation sequencing could provide exceptional sensitivity and precision,^{14, 34} their readouts are average values from numerous cells that might be in distinct states. It has been recognized that the epigenetic profile of human cells stays in dynamic homeostasis. For instance, the landscape of DNA methylation can radically fluctuate even in a single cell cycle.³⁵⁻³⁷ To uncover epigenomic heterogeneity, modern optical microscopy provides elegant solutions for inspecting a single cell.^{18, 38, 39}

Our study demonstrates the feasibility of complementing HSDFI with plasmonic nanoprobe (as photostable contrast agent) for quantification, local density mapping, and multiplex detection of cytosine modifications in single cells. We applied this strategy to analyze the level of 5caC during cell cycle progression, the distribution of 5caC in intact nucleus and on single chromosomes. We also exploited our technique to study the levels of 5caC in different cell lines, to assess the ratio of 5fC and 5caC, and to determine the co-localization of 5caC and 5mC within a single cell. Based on the quantification of 5caC at different cell phases, a significant difference can be noted between the G1 and G2/M phases. In spite of some heterogeneous distribution of 5caC in different cells, the number of 5caC at the G2/M phase was found to be nearly double than that at the G1 phase, which can potentially establish 5caC as an independent epigenetic biomarker inherited across cell division. The local density mapping of 5caC on the chromosomes revealed a specific distribution pattern at the single-pixel level. The comparison between the fluorescence images and HSDFI images of 5caC on single chromosomes proves HSDFI with plasmonic probes to be an effective technology for evaluating the local density of targets. Data from quantifying 5caC in different cell lines as well as different cytosine modifications (*i.e.* 5fC and 5caC) in the same cell line validate the capability of our technology to detect different DNA modifications effectively at single-modification resolution. The consistency between HSDFI and ELISA consolidates the potential of our method. The dual-labeling experiments for 5caC and 5mC enabled profiling the distribution of these modifications in the same nucleus. Plasmonic coupling between nanoprobe leads to a red shift, which can shed light on the degree of co-localization of the modifications.

Our experiments point to the key advantages of HSDFI using plasmonic nanoprobe for high-resolution molecular imaging, local density mapping, and quantification compared to the prevalent fluorescence methodologies.^{37, 40, 41} The major advantage of our technique is the strong signal from single plasmonic probe bound to target sites due to the large LSPR cross-section, which makes the mapping and quantification of epigenetic marks with high SNR at the single-cell level a reality. As provided in Fig. S4, the SNR of HSDFI is much higher than that of the conventional fluorescence imaging (FI). Relative quantification with FI is possible, but it is difficult to quantify and compare rare targets with minute difference, such as 5fC and 5caC (Fig. S4). Second, HSDFI is a powerful technique for mapping the local density of targets, as shown by the comparison between the HSDFI and FI data of 5caC distribution on single chromosomes in Fig. 3. No other available optical methods can

conveniently reveal such information with a single label. Furthermore, the broad spectral coverage of the available plasmonic nanoprobe, the pixel-based spectrometric collection, and the customizable post-processing methods render multiplex detection possible. Significant red-shift in the spectrum is a sensitive measure of plasmonic coupling between closely aligned scattering nanoprobe, which can provide information on the molecular interaction and stoichiometry, similar to FRET techniques.^{42, 43} However, Considering the size difference between a cytosine nucleotide (~ 0.3 nm) and our nanoprobe complex (~ 50 nm), it is difficult to precisely estimate the number of target cytosine modifications in an image pixel, especially on a single chromosome where chromatin structures are highly condensed. In addition, the quantitative correlation between the plasmonic shift and higher-order configuration of nanoprobe coupling (*e.g.* polarization, orientation, and multi-probe coupling) requires more theoretical work. In the near future, we will improve our HSDFI methodology in obtaining more robust measurements, with consideration of polarization characterization, probe optimization, and biophysical simulation.

Conclusion

Overall, in this work our results demonstrate the capability of the proposed HSDFI method for quantification and mapping of multiple epigenetic marks at single-modification resolution in single cells. Taking advantage of the plasmonic nanoprobe with characteristic LSPR, we have successfully collected important *in situ* information, such as distribution, dynamic abundance, local density, and co-localization of the recently known cytosine modifications in a single nucleus. Our technique effectively circumvents the common limits that the conventional FI suffers from, and provides better SNR for high-resolution biological imaging using a simple instrumentation, not generally possible even with super-resolution imaging that requires complex sample preparation and image analysis. Our approach constituting one of the first attempts in the field of single-cell epigenetic quantification can be expanded for a more comprehensive cellular profiling and tissue evaluation in clinical diagnosis.

Materials and Methods

Sample preparation

Human breast adenocarcinoma cell line MCF-7, primary glioblastoma multiforme cell line SF767, and cervical cancer cell line HeLa were routinely cultured in appropriate medium (DMEM for MCF-7 and HeLa; IMDM containing 1% extra glutamate for SF767) supplemented with 10% fetal bovine serum, 1% antibiotics and 5% CO₂. Before targeting and imaging, cytoplasm was removed by treating with hypotonic 0.56% potassium chloride solution for 10 min at room temperature, followed by fixation with cold methanol:acetic acid mixture (1:1) for 5 min. In order to obtain G2/M phase-synchronized cells, 1 μ g/ml Colcemid (Life Technologies) was applied for 2 h prior to cell harvest. G1-enriched cell population was obtained by treating with 400 μ M L-mimosine (Sigma) for 24 h. The isolated nucleus or chromosome spreads were incubated with 0.25% Triton-X 100 for at least 30 min for full permeabilization to ensure the applied nanoprobe (~ 30 nm in size) could freely diffuse through, and then denaturated with 1 N hydrogen chloride for 30 min at 37°C. After

blocking with 5% goat serum, the sample was incubated with primary antibody (1 $\mu\text{g}/\text{ml}$) overnight at 4°C. In the next day, NP-conjugated secondary antibody (1:50 diluted) was applied and incubated for 1 h at room temperature. To avoid/remove non-specific binding, we prolonged the washing time, compared to immunofluorescence staining, after applying the nanoprobe: 6 $\times$ 10 min washing was performed with PBS buffer containing 0.05% Tween 20. Antibodies used in this study: Mouse anti-5mC-IgG (Eurogentec), Rabbit anti-5fC/5caC-IgG (Active Motif), anti-Mouse-20 nm Ag-conjugate, anti-Rabbit-30 nm Au-conjugate (Cytodiagnostics). ELISA was performed according to the established protocol.^{28, 29}

Instrumentation

A home-built HSDFI system was used for imaging cells labeled with AgNPs and AuNPs. The HSDFI platform was installed based on an Olympus BX51 microscope fitted to a vibration-reducing optical breadboard (Thorlabs). A tungsten halogen source (3900, Illumination technologies Inc.) with highly stable light output (intensity fluctuation < 0.1%) was coupled to a CytoViva dark-field condenser (NA 1.2-1.4) *via* a fiber optic light guide to minimize the thermal fluctuation of light intensity for accurate measurements. Pre-aligned Koehler illumination allowed the tungsten halogen source to focus precisely on the entrance slit of the condenser. Combining the pre-aligned Koehler, critical illumination and structured illumination into the dark-field condenser, high spatial resolution could be obtained. A motorized stepper Prior stage with a minimum step size of 40 nm (H101A, Prior Scientific Inc.) was used to obtain scanning hyperspectral data with high spatial resolution. The scattering light was collected with a 100 $\times$ oil immersion objective with an iris, and then split into two light paths, one for realtime optical dark-field imaging and the other for hyperspectral measurement. For hyperspectral imaging mode, the scattering signal goes through a narrow slit, then being dispersed by the gratings into the spectrograph and collected by the thermoelectrically cooled (air) CCD (PIXIS-400BR, Princeton Instruments). The spectrograph (SP2150, Princeton Instruments) includes dual indexable gratings (300 g/mm and 1200 g/mm) and a direct digital grating scanning mechanism with dual-grating imaging and full wavelength scanning capabilities. The fine grating of the spectrograph and the very low dark current of the CCD (0.03 at -75°C) contributes to the high spectral resolution and SNR of the hyperspectral image.

Hyperspectral image acquisition and processing

HSDFI generates a 3D dataset of spatial and spectral information at each pixel. Hyperspectral data can be obtained by stacking the 1D spectra image along the x-coordinate (slit) by scanning the sample-stage along the y-axis. First, dark-field images were obtained to determine the region of interest (ROI). The detector was uniformly illuminated to acquire a flatfield file to exclude small non-uniformities in gain pixel-by-pixel for correction. Then pre-measurement was performed with 1D spectral imaging to set the light illumination level, which was optimized to have a maximum signal without saturation, critical for signal normalization. With these preset parameters, 1D spectral image was obtained by projecting a strip of the image onto a 10- μm slit, followed by dispersing the slit image with 300 g/mm grating to obtain a high spectral resolution of 0.5 nm. With a line-scanning stage of 40-nm step size, the spectral information of the 2D spatial dimension was collected over the

wavelength range from 400 nm to 950 nm. Stabilized mounts guarantee the accurate position information for reconstruction of the images. Hyperspectral images spanning the entire ROI at each wavelength channel can then be constructed from collected 1D spectral images with a data analysis algorithm.

To obtain hyperspectral images of nanoprobe with high SNR, intensity and wavelength filters were applied for the removal of background noise, as well as noises from dusts, glassware defects, and non-specific aggregates of nanoprobe. By post-processing the HSDFI data, a hyperspectral image with a preset wavelength range can be generated. Based on this hyperspectral image, an intensity threshold filter was determined to exclude the background noise for the subsequent spectral mapping. By presetting intensity filters and stacking all 1D spectral images, the wavelength peak of each pixel was extracted to reconstruct a spectral map. Based on the spectral histogram from control experiments, wavelength filters can be established for the removal of signal outliers. Further details about the processing of hyperspectral images are described in Supporting Information Section 3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Illustration of quantification and mapping of cytosine modifications with plasmonic nanoprobe. (a) Schematic of targeting a modified cytosine site by nanomaterial-tagged antibodies. The differential structures of targeted cytosine modifications are provided. (b) The proposed HSDFI can not only provide quantitative information of the target cytosine modification in a single cell (upper panel), but also map the local density of the modification as reflected by the spectral red-shift of the NPs (middle panel). Finite difference time domain (FDTD) simulation (lower panel) shows the distinct scattering pattern when two NPs are closely located compared with a single NP.

Figure 2.

Demonstration of HSDFI and spectral characterization of AuNPs and AgNPs. (a) Optical layout of the HSDFI system. Hyperspectral image is reconstructed by stacking multiple 1D spectral images acquired from slit-scanning (upper panels in (b-c)). The spectral fingerprints of the antibody-conjugated AuNPs and AgNPs are presented (lower panels in (b-c)).

Figure 3.

Mapping and quantification of 5caC in MCF-7 cells at different cell phases. (a) Dark-field images of the representative cells at G2/M phase and G1 phase. (b) Quantities of 5caC at different cell phases. (Scale bar = 10 μ m) (c) Dark-field image of 5caC distribution on a single chromosome. (Scale bar = 5 μ m) (d) LSPR spectra of nanoprobe at the telomere and centromere. (e) Fluorescence image of the 5caC distribution on a single chromosome. (Scale bar = 5 μ m) (f) Reconstructed spectral maps of the 5caC-targeting nanoprobe in an intact nucleus and on a single chromosome.

Figure 4.

Precise quantification of cytosine modifications with a filter function enabled by HSDFI. (a) The process of applying a filter function for AuNPs. (b) The upper row shows the binary images of the centroids of AuNPs targeting 5caC in SF767 cells, 5caC in HeLa cells, and 5fC in SF767 cells, respectively. The lower row summarizes the counting results from HSDFI ($n = 10$ images), which is validated by ELISA quantification in parallel ($n = 3$ replicates). Data are presented as mean with standard deviation. (Scale bar = 10 μ m)

Figure 5. Characterization of a solution containing a mixture of 30 nm AuNPs and 20 nm AgNPs. (a) The representative dark-field image and (b) LSPR spectra of a spot containing uncoupled AgNP and AuNP are shown. (Scale bar = 5 μ m)

Figure 6.

Multiplex detection of 5caC and 5mC in single nucleus. (a) Dark-field image for dual labeling of 5caC and 5mC. (Scale bar = 10 μm) (b) The spectra of three spots circled in (a) are provided as blue, green, and red lines, respectively. (c) Theoretically simulated spectra that correspond to (b). Blue, green, and red bar represent three wavelength windows that correspond to separate AgNPs (5caC), AuNPs (5mC), and co-localized AgNP-AuNP (5caC-5mC) with an inter-particle distance of 1 nm. (d-g) Binary images based on the three wavelength windows are accordingly presented.