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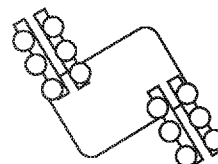
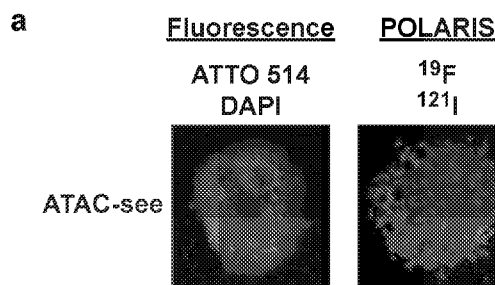
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(54) Title: LABELING OF MOLECULAR PROBES WITH NONMETAL AND METALLOID ISOTOPES FOR MULTIPLEXED
HIGH RESOLUTION ANALYSIS



(57) Abstract: Provided herein, among other things, is a labeling moiety for the analysis of biological samples. In some embodiments, the labeling moiety comprises multiple internal atoms of a non-biological stable nonmetal or metalloid isotope, wherein: i. the labeling moiety is or comprises a binding agent that specifically binds to a complementary site in or on a cell, or ii. the labeling moiety comprises a chemoselective group. In either case, the labeling moiety is not an anti-IgG antibody. Methods for using the labeling moiety, and multiplexed mixture of the same, are also provided.



**LABELING OF MOLECULAR PROBES WITH NONMETAL AND
METALLOID ISOTOPES FOR MULTIPLEXED HIGH RESOLUTION
ANALYSIS**

CROSS-REFERENCING

This application claims the benefit of U.S. provisional application serial no. 62/661,533, filed on April 23, 2018, which application is incorporated by reference herein.

**STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR
DEVELOPMENT**

This invention was made with Government support under contract HHSF223201610018C awarded by the The Food and Drug Administration. The Government has certain rights in the invention.

BACKGROUND

Single cell mass cytometry and multiplexed ion beam imaging (MIBI) facilitate high-dimensional, quantitative analysis of the effects of bioactive molecules on cell populations at single-cell resolution. In conventional implementations of these methods, datasets are generated with antibody or oligonucleotide probes panels in which each antibody or oligonucleotide is conjugated to a polymer that chelates stable metal isotopes, usually in the lanthanide series of the periodic table, through metal-chelating chemical moieties, e.g., diethylene triamine pentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). Typically, the current generation of isotope-chelating polymers can bear up to 120 lanthanide ions per antibody molecule with the detection limit of 300-1000 target protein copies and up to 50 simultaneously measureable parameters. Current implementations of MIBI that use antibodies that have a metal chelate have a resolution that is similar to that of a light microscope, i.e., of 200-300 nm.

SUMMARY

Provided herein, among other things, is a labeling moiety for the analysis of biological samples. In some embodiments, the labeling moiety may comprise multiple internal atoms of a non-biological stable nonmetal or metalloid isotope, wherein: i. the labeling moiety is or comprises a binding agent, e.g., an antibody, that specifically binds to a complementary site in or on a cell, or ii. the labeling moiety comprises a chemoselective group, e.g., may be a polymer such as an oligonucleotide that comprises a chemoselective group. In either case, the labeling moiety is not an anti-IgG antibody.

Methods for using the labeling moiety and multiplexed mixture of the same, are also provided. In some embodiments, the labeling moiety may be used for the multiplexed analysis of target biomolecules by elemental mass spectrometry, secondary ion mass spectrometry (SIMS), mass cytometry (CyTOF), laser-ablation inductively-coupled mass spectrometry (LA-ICP-MS), X-ray microscopy (XRM), X-ray fluorescence (XRF), laser-induced breakdown spectroscopy (LIBS), focused ion beam (FIB), energy-dispersive X-ray spectroscopy (EDX), electron microscopy, or nuclear magnetic resonance spectroscopy.

Labeling binding agents internally, using e.g., using a non-biological isotope of carbon, nitrogen, chalcogen, or a halogen, etc. not only expands the number of available isotopes, thereby exponentially increasing the measurable parameters, the present labeling moiety may be superior to chelate-labeled binding agents for a variety of reasons.

For example, depending on how the labeling moiety is made, the labeling moiety may contain a large number of atoms of the isotope (e.g., at least 5, at least 10, at least 100, at least 500, or at least 1,000), which should, in theory, make any assays that use the labeling moiety more sensitive. For example, monoclonal antibodies contain thousands of nonmetal atoms (for example Trastuzumab, is composed of $C_{6470}H_{10012}N_{1726}O_{2013}S_{42}$) and, in principle, synthesis of an antibody using amino acids that contain carbon 13 would greatly enhance the signal intensity by at least 50-fold compared to the current antibody reagents that are labeled by a metal chelate.

Moreover, and depending on how the labeling moiety is made, the labeling moiety may contain a defined number of isotopically labeled atoms. For example, the number of isotopically labeled atoms that are in a labeling moiety made by adding monomers that contain isotopically labeled atoms to a growing chain can be highly controlled by controlling the

number of labeled monomers used. If each labeling moiety has the same or similar number of labeled atoms, then the signals from each of those labeling moieties should be very similar. The number of labeled atoms in a labeling moiety can therefore be increased or decreased, as desired.

Further, because some embodiments of the present labeling moiety can be made in a controlled manner by adding monomers that contain isotopically labeled atoms to a growing chain, labeling moieties that contain defined ratios of two or more labeled atoms can be made. This provides more options for multiplexing because different labeling moieties that are labeled with different ratios of the labeled atoms are readily distinguishable in some analysis method (e.g., SIMS).

When used in SIMS, some embodiments of the present labeling moiety provide higher resolution (e.g., sub-30nm resolution) results. At this resolution single binding events can be detected.

Finally, the present labeling moiety do not require a chelator that can potentially interfere with binding. The absence of interference from the chelator allows for more accurate measurement of the biomolecules.

These and other advantages may become apparent in view of the description that follows below.

BRIEF DESCRIPTION OF THE FIGURES

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the
5 Office upon request and payment of the necessary fee.

The skilled artisan will understand that the drawings, described below, are for illustration purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

10 **Fig. 1** shows data obtained from various embodiments of the present labeling moiety. (a) open chromatin labeling (described in greater detail below), (b) an Iodine-modified

oligonucleotide was conjugated to an anti-H3K27Ac antibody provides a specific signal via secondary ion beam imaging. Such signal can be amplified by varying the length or the structure of the oligonucleotide. The oligonucleotide sequence is set forth in SEQ ID NO: 7.

(c) Specific fluorine-modified oligonucleotides are assembled into a Cas9 protein, which retains activity and renders visualization of pericentromeres. (d) shows a signal deriving from a $^{13}\text{C}/^{12}\text{C}$ modified anti-dsDNA antibody, which enables visualization of nuclear dsDNA by secondary ion beam imaging. This antibody was produced by culturing hybridoma cells in ^{13}C -rich media.

Fig. 2: (a) Schematic representation of ATAC-mass. Fluorine-labeled adaptors were loaded with Tn5 enzyme to specifically label open chromatin. Mass tags were detected directly by Ion Beam Imaging or through metal (^{156}Gd)-conjugated antibody by mass cytometry. (b,c) Validation of ATAC-mass by sequencing. Genome-wide comparison (b) and TSS enrichment (c) of ATAC-seq reads of GM12878 libraries (GM) prepared by using either Nextera Tn5 or Fluoro Tn5. (d) Validation of ATAC-mass by fluorescence imaging. Representative image of ATAC-mass in HeLa cells. Very limited ATAC-mass signal is observed in +EDTA negative control. Scale bars, 5 μm .

Fig. 3: (a) Representative image of DNA in HeLa cells. IdU labelled DNA was detected by high-resolution secondary ion mass spectrometry. (b) Serial scanning of IdU labeled, Fluoro-Tn5 tagmented K562 cells. (c) Line scans were performed on the nanoSIMS images in the areas indicated by the colored bars. The resolution, calculated using the 16–84% criterion, is shown as an average of the three line scans $\pm$ s.d. (d) Representative images of DNA together with ATAC-mass in K562 cells. In blue, ^{127}I to visualize IdU-labelled DNA. In red, ^{19}F to visualize ATAC-mass signal. Open arrow indicates an example of open chromatin intertwined with compacted DNA while closed arrow points to region where high ATAC-mass is clearly separated from high density of DNA. Scale bar: 2 μm (left) and 1 μm (right).

Fig. 4: (a) ATAC-mass as measured by mass-cytometry. The indicated cell-cycle phases of Jurkat cells were measured along with ATAC-mass signal. (b) Phosphorylation of

retinoblastoma protein was measured along with ATAC-mass signal. Highlighted area defines cells in G0 phase of cell-cycle. (c) Heat map depicting each signaling protein and ATAC-mass in control, IL12 and IL18 priming conditions. The color scale indicates changes in median intensity (arcsinh ratio) compared to control (no priming). (d) Spearman correlation matrix from control, IL-12 and IL18 priming conditions. The matrix was clustered and yellow represents a positive correlation while blue represents no correlation. (e) Validation of ATAC-mass finding by ATAC-seq. Heatmap shows cluster of different ATAC-seq accessible regions between without and IL-12 priming as well as without and IL-18 priming (FD > 2, FDR < 0.05); each group has one replicate.

Fig. 5: (a) Boolean gating analysis of polyfunctional NKL cells in response to PMA plus ionomycin after control, IL12 or IL18 priming. Color represents the indicated number of functional effectors coexpressed in a single cell. (b) Heat map depicting each effector functions in control, IL12 and IL18 priming conditions. The color scale indicates changes in median intensity (arcsinh ratio) compared to control (no priming). (c) Representative biaxial plots showing the polyfunctional NKL cells after IL18 priming. Number indicates the percentage of cells in each quadrat.

Fig. 6: Workflow of High-Resolution 3D MIBI for drug visualization. Cells are treated with the drug of study, stained with antibodies conjugated to mass-oligos (SEQ ID NO: 11) and processed for MIBI. The cells are rastered by a cesium primary ion beam, with a lateral resolution up to 50 nm and an axial resolution previously calculated to be 14.3 nm. Serial scans of the sputtered negatively charged secondary ions and secondary electrons are recorded. 3D rendering of the data summarizes sub-cellular location of the drug and markers of interest.

Fig. 7: Validation of High-Resolution MIBI. (a-d) A schematic of the antibody conjugation strategy, immunofluorescence confocal microscopy image and high-resolution MIBI image of (a) α -Nucleolin- ^{19}F /FITC, (b) α -CENP-A- ^{81}Br /Cy3, (c) α -H3K27Ac- ^{127}I /Cy5 and (d) α -SC 35 -biotin:Streptavidin- ^{197}Au /FITC. (e) Five-color high-resolution image of a HeLa cell nucleus. Scale bars: 4 μm . The sequence of (a) is set forth in SEQ ID NO: 11. The

sequence of (b) is set forth in SEQ ID NO: 11. The sequence of (c) is set forth in SEQ ID NO: 11.

Fig. 8: Sub-cellular visualization of cisplatin distribution by High-Resolution MIBI.

(a) Chemical structure of cisplatin. (b) Cisplatin distribution in TyKNU cells treated with 5 μM $^{194}\text{Cisplatin}$ or DMSO for 24 hours. (c) Cisplatin distribution of a TyKNU cell treated with 5 μM $^{194}\text{Cisplatin}$ for 24 hours. (d) Seven-color high-resolution composite of cell in (c). (e) Two-color images of cell in (c). Summation of 10 (b) or 50 (c-e) planes. Scale bars: 4 μm .

Fig. 9: Three dimension rendering of high-resolution MIBI data. (a) 3D rendering of a TyKNU cell treated with 5 μM $^{194}\text{Cisplatin}$ for 24 hours. (b) Single-color 3D rendered images of cell in (a).

Fig. 10: Validation of ATAC-mass. (a) ATAC DNA adaptors are modified to include fluorine-containing ATTO 514 at the 5' end and 5-fluoro-2'-deoxyuridine and 5-fluoro-2'-deoxycytidine. Tn5 transposase loaded *in vitro* with fluorine-labeled adaptors (Fluoro-Tn5) is used to specifically label open chromatin. (b) The adaptor can be detected by five different modalities: (1) ATAC-seq with sequencing adaptors, (2) high-resolution ion beam imaging due to the presence of fluorine, (3) mass cytometry through metal (^{156}Gd)-conjugated antibody against ATTO 514, and (4) fluorescence microscopy and (5) flow cytometry via detection of the intrinsic fluorescence of ATTO 514. (c) Genome-wide comparison of detection of ATAC-seq reads of GM12878 libraries prepared using Nextera-Tn5 or Fluoro-Tn5. Nextera-Tn5 data was from Buenrostro et al⁶. Two biological replicates for Fluoro-Tn5 were performed. Individual replicates were assessed for high reproducibility and then pooled for peak calling. The log2 reads within each peak per condition was calculated and plotted (Pearson Cor. = 0.81). (d) TSS enrichment of ATAC-seq reads of GM libraries prepared using Nextera-Tn5 or Fluoro-Tn5. Reads were normalized to tag counts per million mapped pair of reads, around +/- 2 kb of TSSs. (e) Genomic tracks for biological replicates of ATAC-seq data from Nextera-Tn5 and Fluoro-Tn5 on Chr18. X-axis, genomic coordinates; Y-axis, normalized ATAC-seq read counts. (f) Representative fluorescence microscopy image of ATAC-mass signal due to the presence of

ATTO 514 in the modified adaptor (red) in HeLa cells. EDTA treatment served as a negative control. Nuclei were stained with DAPI (blue). The boxed areas are enlarged on the right. Scale bars, 5 μ m. **(g)** Histogram of ATAC-mass signals at indicated cell cycle phases in Jurkat cells analyzed by mass cytometry. The color scale represents the median intensity of ATAC-mass. Y-axis, cell cycle phases. **(h)** Phosphorylated retinoblastoma protein (S807/811) and ATAC-mass signal measured in Jurkat cells. The highlighted area indicates low ATAC-mass signal from cells in the G0 phase of the cell cycle defined by low levels of phosphorylated retinoblastoma protein.

Fig. 11: ATAC-mass reveals increased genome accessibility and correlated activation of signaling pathways in IL-18-treated NK cells. **(a)** Heat map of signals due to antibodies to indicated signaling proteins and ATAC-mass in control cells and cells treated with IL-12 and IL-18 analyzed by mass cytometry. The color scale indicates changes in median intensity (arcsinh ratio) compared to control. **(b)** tSNE plots of mass cytometry results depicted in Figure 12 a from NKL cells treated as indicated. Numbers indicate the percentage of cells in the gate. **(c)** ATAC-mass signal intensity in the corresponding tSNE plot as in Figure 12 b. Color scale represents ATAC-mass intensity. **(d)** Spearman correlation matrix of control and IL-12- and IL-18-treated cells of mass cytometry results depicted in Figure 12 a. Yellow indicates a positive correlation; blue indicates no correlation.

Fig. 12: ATAC-seq reveals increased genome accessibility of quiescent enhancers in IL-18-treated NK cells. **(a)** Meta-analysis of ATAC-seq reads at peaks in IL-12-treated and IL-18-treated significantly different from control NKL cells. Differentially accessible ATAC-seq peaks from two biological replicates were identified using DESeq2. Four separate clusters were identified via *k*-means clustering, with distinctive patterns of accessibility. Representative motifs enriched within each cluster are shown on the right (p-value < 1e-5). **(b)** Peak distribution for each cluster annotated using the CD56 primary NK cells from the chromHMM database. **(c)** Density histograms of the distribution across the TSS of the most accessible regions in the four clusters.

Fig. 13: IL-18 treatment increases genome accessibility in uncompacted DNA and the frequency of polyfunctional NK cells. **(a)** Representative 3D reconstruction of a K562 cells obtained from high-resolution ion beam imaging of a cell labeled with IdU (green, ^{127}I) and ATAC-mass (purple, ^{19}F). A 3D reconstruction of 70 consecutive planes is shown. **(b)** Enlarged image of boxed region in panel a. Open arrows indicate open chromatin intertwined with compacted DNA (overlapping regions are in white). Closed arrows indicate regions where high ATAC-mass is clearly separated from compact DNA. Scale bar, 200 nm. **(c)** Stacked bar graph of the percentage of pixels in high-resolution ion beam imaging images across the four indicated categories in control, IL-12-treated, and IL-18 treated NKL cells. **(d)** Violin plot of the ATAC-mass (^{19}F) counts per pixel in high-resolution ion beam imaging images in ATAC-high and IdU-low regions from control, IL-12-treated, and IL-18 treated NKL cells. Boxplots were drawn following the Tukey method; p-values determined by one-way ANOVA with post-hoc Tukey HSD test. **(e)** The percentages of cells with the indicated number of functional effectors in control, IL-12-treated, and IL-18 treated NKL cells in response to PMA plus ionomycin. The number of functional effectors was calculated by summing the number of cytokines expressed per single cell based on mass cytometry analysis. **(f)** Heat map depicting each effector function in control, IL-12-treated, and IL-18-treated conditions. The color scale indicates changes in median intensity (arcsinh ratio) compared to control. **(g)** Representative biaxial plots showing the increased frequency of NKL cells with multiple cytokines produced after stimulation of IL-18-treated cells compared to control and IL-12-treated cells. Blue lines are the thresholds for multifunctional capacity. Numbers indicate the percentage of cells in each quadrant.

Fig. 14: Super-resolution visualization of nuclear structures using srIBI. **(A)** Workflow of super-resolution ion beam imaging (srIBI). (1) Cells are treated with the drug of study, fixed, and stained with MoC-Abs. Endogenous elemental masses can also be detected. (2) Cells are rasterized by a cesium primary ion beam, and the secondary ions are collected by a magnetic sector mass spectrometer. (3) Spatial information is serially recorded in up to 8 channels simultaneously. (4) A composite image of the cell is reconstructed by combining and pseudo-coloring the total number of channels at each depth. (5) srIBI yields a 3D rendering based on the depth profile that reveals the specific sub-cellular localization of endogenous and labeled

targets. (6) Ion count extraction on a pixel-by-pixel basis and application of dimensional reduction methods identifies nuclear neighborhoods for feature extraction. (B) Schematic of ^{19}F -based MoC-Ab. The oligonucleotide includes a FITC fluorophore at the 3' end and the modified base 2-F-Ac-C (green) in place of deoxycytidine. The oligonucleotide sequence is set forth in

5 SEQ ID NO: 11. (C) Representative τ_e and ^{19}F images of a HeLa cell stained with anti-nucleolin- ^{19}F /FITC. From left to right: (1) τ_e image of a HeLa cell; nucleoli are indicated with white arrows. (2) Image based on ^{19}F signals originating from anti-nucleolin- ^{19}F /FITC. (3) Overlay of images showing the co-localization of ^{19}F signal with the subnuclear structures predicted to be the nucleoli from the τ_e image. (4) An enlarged image of a nucleolus. (D)

10 Representative srIBI image of a HeLa cell stained with anti-H3K27Ac- ^{19}F /FITC. Overlay of ion images for ^{19}F (green) and ^{31}P (blue). The image is the sum of 10 consecutive planes. (E) HeLa cells were labeled for 24 hours with IdU (^{127}I) and stained with anti-nucleolin- ^{19}F /FITC. Using srIBI, 40 depths of a single cell were acquired with an increase in the current every 10 scans. Images of newly synthesized DNA (top) and nucleolin (middle) and their overlay (bottom) show

15 details of a region within the nucleus, at different currents. Details of DNA and nucleolus in these settings are increasing by lowering the current, at the cost of ion yield. Colored dots indicate the image used for the quantification shown in panel F. (F) Quantification of the relationship between srIBI resolution and secondary ion yield. The lateral resolution of ^{127}I (present in IdU) at different currents from panel E was calculated using the 84% to 16%

20 criterion. The R^2 for the relationship between the increase in resolution and decrease in ion yield was 0.91. See Fig. 32 for details.

Fig. 15: Multiparametric srIBI using MoC-Abs identifies unique subcellular features. (A-C) (Left) Representative srIBI images of a HeLa cell stained with A) anti-CENP-A- ^{81}Br /Cy3, B) anti-H3K27Ac- ^{127}Ir /Cy5, and C) anti-SC35-biotin recognized by streptavidin- ^{197}Au /FITC. Overlay of ion images for MoC-Ab (red) and phosphorus (^{31}P ; blue). (Right) A digital zoom of boxed area in the original image to show specificity of MoC-Ab signal. All images consist of the sums of 10 consecutive planes. (D) Composite srIBI image of nucleolin (cyan), H3K9me3 (magenta), H3K27Ac (green), and SC35 (red) in a HeLa cell. Cells were

25 stained with anti-nucleolin- ^{19}F /FITC, anti-H3K9me3- ^{81}Br /Cy3, anti-H3K27Ac- ^{127}Ir /Cy5, and

30

anti-SC35-biotin (recognized by streptavidin- ^{197}Au /FITC). The image consists of the sum of 10 consecutive planes. (E-G) Multiple enlarged images from boxed regions in panel D showing (E) a nuclear speckle, (F) a nucleolus, and (G) heterochromatin. (H) The largest nucleus in panel D was masked using the phosphorus signal. The ion counts for each acquired marker (nucleolin, ^{19}F ; DNA, ^{31}P ; H3K9me3, ^{81}Br ; H3K27Ac, ^{127}I ; and SC35, ^{197}Au) were extracted. (I) viSNE maps of the nucleus in the center of the image shown in panel D were created using the ion counts for each of the 84836 pixels from the mask. The ion count for each marker in each pixel was min-max normalized to scale the range to [0, 1]. Each point represents a pixel. Pixels grouped in distinct regions based on the expression of each marker. The color in each map represents the intensity of the indicated marker in each pixel. The areas marked A-C indicate three distinct groups of nucleolin-positive pixels identified manually in the nucleolin map. (J) Grouped pixels from the unsupervised viSNE map are differentially distributed in space as shown in srIBI images of (1) total nucleolin (^{19}F) from the HeLa cell shown in panel D, (2) ^{19}F signals from pixels within gate A, (3) gate B, (4) gate C, and (5) overlay of ^{19}F signals from gates A (red), B (green), and C (blue).

Fig. 16: 3D nanoscale imaging of the nucleus through iterative srIBI. (A) Representative single-plane srIBI images at different depths of a HeLa cell nucleolus. HeLa cells were stained with anti-nucleolin- ^{19}F /FITC, and 785 individual planes were acquired to obtain srIBI images of a nucleolus from its appearance to its disappearance. Single planes every 100 depths show a distinctive molecular distribution of nucleolin in the 3D space. See Fig. 42 for images of each individual plane. Blue, red, and green arrows indicate x-axis, y-axis, and z-axis, respectively. (B) (Left) 3D surface reconstruction of images of nucleolin staining of a nucleolus shown in panel A. (Right) Overviews of the same nucleolus along x-axis (blue arrow), y-axis (red arrow), and z-axis (green arrow) with the origin represented as a black dot. (C) Representative 3D surface reconstruction of nucleolus (green), centromeres (red), and nuclear speckles (cyan). HeLa cells were stained with anti-nucleolin- ^{19}F /FITC, anti-CENP-A- ^{81}Br /Cy3, and anti-SC35-biotin (recognized by streptavidin- ^{197}Au /FITC). The image consists of the 3D reconstruction of a stack of 40 consecutive planes. (D) Representative 3D reconstruction of nucleolin (cyan), phosphorus (blue), H3K9me3 (magenta), H3K27Ac (green), and SC35 (red) in a HeLa cell

stained with anti-nucleolin- ^{19}F /FITC, anti-H3K9me3- ^{81}Br /Cy3, anti-H3K27Ac- ^{127}I /Cy5, and anti-SC35-biotin (recognized by streptavidin- ^{197}Au /FITC). The image consists of the 3D reconstruction of a stack of 400 consecutive planes. Enlarged images from the boxed region show details of marker distribution. (E) (Left) Workflow of iterative srIBI: (1) Five to ten depths at high current are acquired in a cell of interest at $25 \times 25 \mu\text{m}$ to identify a ROI. (2) Iterative acquisition is performed by focusing the beam into the ROI at lower currents in a smaller area of $5 \times 5 \mu\text{m}$ or $10 \times 10 \mu\text{m}$. (Right) (Top) Representative region of the IdU signal (^{127}I) in a nucleus of a HeLa cell labeled for 24 hours with IdU (^{127}I). (Bottom) A $10 \times 10 \mu\text{m}$ ROI was acquired for super-resolution imaging of chromatin folding. Enlarged image of the boxed region shows fine detail of IdU labeled chromatin. (F) Quantification of the resolution of iterative srIBI imaging of a nucleolus. From left to right: (1) The image of a HeLa cell reveals subnuclear structures including the nucleoli chosen for iterative srIBI. (2) A $3 \times 3 \mu\text{m}$ ROI was acquired by iterative srIBI for nucleolin (^{19}F). (3) An enlarged view from the boxed region in image 2 showing the nanoscale organization of nucleolin (^{19}F). (4) Line scan along the red line in the boxed region in image 3 demonstrates identification of molecules spaced about 30 nm. (5) An enlarged view of the boxed region in image 3. See Fig. 43 for additional examples. (G) Iterative srIBI 3D reconstruction. HeLa cells were stained with anti-FBL- ^{81}Br /Cy3, anti-nucleolin- ^{127}I /Cy5, and anti-NPM1-biotin (detected with streptavidin- ^{197}Au /FITC). Iterative srIBI was performed on a site with three nucleolin. The The image confirmed that the ROI was acquired. Nucleolin (cyan), phosphorus (blue), FBL (green), and NPM1 (red) were used for 3D reconstruction from 40 consecutive planes. These images identify the granular component (GC, NPM1-positive), dense fibrillar component (FBL- and nucleolin-positive), and perinucleolar heterochromatin (PNC, phosphorus-high).

Fig. 17: Subcellular localization of cisplatin distribution. (A-B) TYK-nu ovarian cancer cells were treated with $5 \mu\text{M}$ cisplatin for 24 hours and stained with anti-nucleolin- ^{19}F /FITC, anti-H3K9me3- ^{81}Br /Cy3, anti-H3K27Ac- ^{127}I /Cy5, and anti-SC35-biotin (detected with streptavidin- ^{197}Au /FITC). We simultaneously acquired images of nucleolin (^{19}F), DNA (^{31}P), H3K9me3 (^{81}Br), H3K27Ac (^{127}I), cisplatin (^{194}Pt), and SC35 (^{197}Au), in addition to The. (A) Representative srIBI image of cisplatin (^{194}Pt) distribution within the cell. (B) Overlay of ion

images for cisplatin (^{194}Pt ; red) and a second marker (green). All images consist of the sums of 10 consecutive planes. (C) A schematic for the identification of nuclear neighborhoods. (1) SrIBI acquisition is performed on single TYK-nu cells treated with cisplatin and labeled with MoC-Abs as described in panel A. (2) A sliding window of $6 * 6 * 20$ pixels is used for feature
 5 extraction for all channels imaged. (3) Unsupervised clustering is performed on extracted features to identify nuclear neighborhoods. (4) Identified clusters are recolored back onto the cell to visualize nuclear neighborhoods spatially. (D) A heatmap of the 10 distinctive neighborhoods identified based on the five indicated nuclear markers. The intensity of each marker is denoted by the scale bar on the left. The identified clusters of interactions, termed
 10 nuclear neighborhoods, are colored and numbered. (E) Identified nuclear neighborhoods are used to recreate the cell, resulting in distinctive structures that resemble known features in the nucleus. Two representative TYK-nu nuclei are shown. (F) The cisplatin counts are plotted for each nuclear neighborhood identified in panel D for the two nuclei shown in panel E.

Fig. 18: An analytical framework to identify nuclear neighborhood interactions in
 15 multiplexed super-resolved data. (A) Representative srIBI image of cisplatin-treated TYK-nu cells. TYK-nu ovarian cancer cells were treated with $5 \mu\text{M}$ cisplatin for 24 hours and stained with anti-nucleolin- ^{19}F /FITC, anti-H3K9me3- ^{81}Br /Cy3, anti-H3K27Ac- ^{127}I /Cy5, and anti-SC35-biotin (detected with streptavidin- ^{197}Au /FITC). Images of nucleolin (^{19}F), DNA (^{31}P), H3K9me3
 20 (^{81}Br), H3K27Ac (^{127}I), cisplatin (^{194}Pt), and SC35 (^{197}Au) were simultaneously acquired. A $100\text{-}\mu\text{m}^2$ ROI in the nucleus was acquired by iterative srIBI. Displayed are nucleolin (cyan), phosphorus (blue), H3K9me3 (magenta), H3K27Ac (green), cisplatin (grey), and SC35 (red). Denoising was performed using a k nearest neighbor approach. An unfiltered image is shown in Fig. 48 C. Enlarged images from boxed regions exemplify the nuclear organization diversity.
 25 Images consist of the sums of 20 consecutive planes. (B) t-SNE map colored by the 11 identified nuclear neighborhoods. The t-SNE map is derived from 100000 voxels of dimension $(x, y, z) = (10, 10, 5)$ pixels (20000 voxels were randomly sampled across 5 different cells). Each point represents a voxel. Voxels grouped in distinct regions based on the expression of each marker. The 11 hierarchical clusters were identified by unsupervised hierarchically
 30 clustering, followed by manual annotation. (C) An expression profile of the mean marker

expression in each of the 11 distinctive nuclear neighborhoods. The scale intensity of each marker is denoted by the color bar on the top right (Z-score, normalized to each row). (D) Annotation of each neighborhood, based on their mean marker expression profile from panel C. (E) Identified nuclear neighborhoods described in panel D are used to recreate the cell, resulting in distinctive structures that resemble known features in the nucleus shown in panel A. (F) Pairwise interaction frequency calculations. (Left) A graphical schematic of the permutation test implemented to quantify the frequency of pairwise neighborhood interactions is shown. If two points (e.g., A and B) were within 5 pixels of each other, they were defined as being “attracted”. Point labels were shuffled 1000 times, and the mean interaction frequency noted. The final distribution of shuffled interaction frequencies was plotted, and the mean was taken as the expected interaction frequency. Real interaction frequencies below the expected were indicative of repulsion between the points, whereas those above were attraction. (Right) The neighborhood interaction frequency map was calculated using the log₂ enrichment of the real over expected number of pairwise interaction frequencies. (G-H) Nuclear neighborhoods are colored as shown in the legend of panel D to show (G) chromatin-specific neighborhoods, and (H) cisplatin-enriched neighborhoods. Enlarged images from boxed regions exemplify different neighborhood interactions: accessible euchromatin (purple box), heterochromatin near the nucleolus (green box), an inactive/active boundary of cisplatin containing nuclear speckles (orange box), and cisplatin-euchromatin adducts within the nucleolus (brown box).

Fig. 19: Sequences and validation of size and purify of mass adaptors. (a) Adaptor sequences for Fluoro-Tn5. The sequences from top to bottom are set forth in SEQ ID NOs:1-3. (b) Representative electrospray mass spectra of Tn5ME-A-ATTO514-5FdU, Tn5ME-B-ATTO514-5FdU, and Tn5MERev-5FdU-5FdC.

Fig. 20: Confirmation of purity of Tn5. Pre-induction (Pre) crude lysate, post-induction (Post) crude lysate, and DTT-eluted products were separated on SDS-PAGE, and the gel was stained with Coomassie blue. The expected sizes of the Tn5 fusion protein (open arrowhead) and cleaved Tn5 (arrow) are indicated. Marker molecular weights in kDa are listed to the left.

Fig. 21: Fluorescein/Oregon green antibody recognizes ATTO 514. **(a)** Chemical structures of fluorescein, Oregon green, and ATTO 514. **(b)** Flow cytometry analysis of beads loaded with ATTO 514 stained with isotype control (left) or anti-fluorescein/Oregon green antibody (right) followed by secondary antibody conjugated with Alexa647.

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Fig. 22: Cell-cycle phase gating strategy. Strategy used to define each cell cycle phase of Jurkat cells (related to Figure 10 g and h).

Fig. 23: Correlation of ATAC-mass signal and phosphorylation of various signaling pathway markers. **(a)** Representative biaxial plots of ATAC-mass and pNFkB (S536) signals in NKL cells treated as indicated. Numbers indicate the percentage of cells in each gate. **(b)** Representative biaxial plots of ATAC-mass and pc-Jun (S73) signals in NKL cells treated as indicated. Numbers indicate the percentage of cells in each gate. **(c)** Representative biaxial plots of ATAC-mass and pSLP-76 (Y128) signals in NKL cells treated as indicated. Numbers indicate the percentage of cells in each gate. **(d)** Representative biaxial plots of ATAC-mass and pStat4 (Y693) signals in NKL cells treated as indicated. Numbers indicate the percentage of cells in each gate.

Fig. 24: Principal component analysis of ATAC-seq open chromatin peaks from IL-12-treated, IL-18-treated, and control NKL cells. Points indicate data from the two biological replicates per condition.

Fig. 25: Ion beam imaging enables high-resolution visualization of the nucleus. **(a)** Representative secondary ion mass spectrometry image of a HeLa cell labeled with IdU. Left: Ion image for ^{31}P . Middle: Ion image for ^{127}I . Right: Overlay of ^{31}P (blue) and ^{127}I (green). Arrow points to region where high amounts of IdU were incorporated, indicative of a high density of DNA. Scale bars, 5 μm . **(b)** Line scan along the red line in the right image in panel a. Raw ion counts were scaled using mix-man normalization. IdU (green line) and phosphorus (blue line).

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Fig. 26: Tagmentation using Fluoro-Tn5 detected by ion beam imaging reveals the spatial organization of the accessible genome. **(a)** High-resolution ion beam images of a K562 cell labeled with the ATAC-mass tag (^{19}F) and IdU (^{127}I). Scale bars: 2 μm . **(b)** Lateral resolution of high-resolution ion beam imaging of ATAC-mass in a K562 cell. Line scans were performed on the high-resolution ion beam imaging ATAC-mass image in the areas indicated by the colored bars. The average resolution ($\pm$ standard deviations), calculated using the 16–84% criterion, is 98 ± 24 nm. Scale bar, 2 μm . **(c)** Left: High-resolution ion beam image of ATAC-mass and IdU-labeled DNA in a K562 cell. Scale bar: 2 μm . Right: ATAC-mass (red line) and IdU (blue line) signals along the white line in the left image. Open arrows indicate examples of open chromatin intertwined with compacted DNA. Closed arrows point to regions where high ATAC-mass is clearly separated from a high density of DNA.

Fig. 27: IL-18 treatment increases genome accessibility in uncompact DNA in NKL cells. **(a)** Bar graph of total fluorescence intensity of ATAC-mass signal per nucleus from serial z-sections of control NKL cells and cells treated with IL-12 or IL-18. Data are means ($\pm$ standard deviations); p-values determined by Student's t-test; n.s., non-significant. **(b)** Representative fluorescence microscopy images of ATAC-mass (red) in NKL cells treated as indicated. Nuclei were stained with DAPI (blue). Scale bars, 5 μm . **(c)** Representative high-resolution ion beam imaging of DNA (^{127}I , green) and ATAC-mass (^{19}F , purple) signals in an NKL cell treated with IL-18. The inset image is the whole cell. The enlargement is of the region boxed with a white dashed line in the inset. Open arrows indicate examples of open chromatin intertwined with compacted DNA (overlapping regions are in white). Filled arrows point to regions where high ATAC-mass signal is clearly separated from high density of DNA. Scale bars, 2 μm for whole cell image and 200 nm for high-magnification image.

Fig. 28: Strategy for the synthesis and conjugation of mass-oligonucleotides to antibodies. **(A)** Mass-oligonucleotides were synthesized with internal isotope-derivatized nucleotides and a maleimide cycloadduct at the 5' position. Dashed box shows chemical structures of isotope-derivatized cytidines and thymidines that are commercially available or that can be synthesized with previously reported protocols (48, 49). In this study, we

incorporated 11 fluorine, bromine, or iodine-derivatized cytidines (shown in red) into a 47-nucleotide long oligonucleotide. The DNA sequence was selected to minimize potential for binding to the human genome. (SEQ ID NO: 8) (B) Oligonucleotides were deprotected in toluene for 4 hours at 90 °C, washed in ethanol, lyophilized, and stored at -20 °C. (SEQ ID NO: 9) (C) The deprotected oligonucleotides were conjugated to partially reduced antibodies through thiol-maleimide click chemistry. Free oligonucleotides were removed via size-exclusion by centrifugation using a 50-KDa MWCO filter. (SEQ ID NO: 10).

Fig. 29: Protocol optimization for intracellular staining with MoC-Abs. HeLa cells were fixed and permeabilized as indicated in the second column. Cells were then blocked for 30 minutes at room temperature as indicated in the third column, stained for 1 hour at room temperature with unconjugated, lanthanide-polymer conjugated (^{162}Dy) or mass-oligonucleotide-conjugated (^{127}I) primary anti-double-stranded DNA (anti-dsDNA), washed three times in PBS, and stained for 1 hour at room temperature with secondary anti-mouse-Alexa 647. Cells were then washed and mounted with fluoromount containing DAPI for confocal microscopy analysis. In HeLa cells stained with anti-dsDNA (sample 1), specific nuclear staining co-localizing with DAPI was observed as expected. HeLa cells stained with anti-dsDNA- ^{162}Dy (sample 2) showed the same nuclear staining indicating that the conjugation process did not alter antibody specificity. The presence of high salt concentration and salmon sperm DNA in cells stained with anti-dsDNA- ^{162}Dy (sample 3) did not interfere with antibody staining. HeLa cells fixed and permeabilized with methanol (sample 4), acetone (sample 6), or fixed with 1.6% paraformaldehyde and permeabilized with methanol (sample 8) and stained with anti-dsDNA- ^{127}I showed nonspecific cytoplasmic staining. The addition of high salt concentration and salmon sperm DNA in the blocking buffer of HeLa cells fixed and permeabilized with methanol (sample 5) or acetone (sample 7) and stained with anti-dsDNA- ^{127}I were stained nonspecifically, although the nonspecific cytoplasmic staining was partially reduced in acetone-treated cells. Fixation with 1.6% paraformaldehyde, permeabilization with methanol, and blocking with a high salt concentration and salmon sperm DNA (sample 9) resulted in a highly specific and background-free visualization of dsDNA in the nucleus of HeLa cells stained with anti-dsDNA- ^{127}I . Permeabilization with other reagents such as Triton

X-100 or saponin was also compatible with specific MoC-Ab staining (data not shown). Scale bars, 20 μm .

Fig. 30: Validation of ^{19}F /FITC MoC-Abs for srIBI. (A) A schematic of the ^{19}F /FITC MoC-Ab. The antibody was conjugated to the 5' end of an oligonucleotide in which all cytidines were replaced with 2-F-Ac-C. The oligonucleotide also had a 3' FITC. (SEQ ID NO: 11) (B) Representative confocal microscopy image of a HeLa cell stained with unconjugated anti-nucleolin- ^{19}F /FITC and a secondary anti-mouse-Alexa488 (green) and DAPI (blue). Scale bar, 4 μm . (C) Representative confocal microscopy images of control HeLa cells (no antibody) or HeLa cells stained with anti-nucleolin- ^{19}F /FITC (green) at different concentrations. ^{31}P is shown in blue. Cells in red boxes in the composite image are shown enlarged in the right column. Scale bars, 20 μm in standard images and 4 μm in enlarged images. A 1:100 dilution of this antibody was used in subsequent experiments. (D) Representative srIBI images of a control HeLa cell (no antibody) and HeLa cells stained with anti-nucleolin- ^{19}F /FITC. Scale bar, 4 μm .

Fig. 31: High-phosphorus regions within the nucleus mainly originate from the DNA backbone. (A) Representative srIBI image of a HeLa cell treated with 5-iodo-2'-deoxyuridine (IdU) for 24 hours to label DNA. Ion images for phosphorus (^{31}P ; left panel) and newly synthesized DNA (^{127}I ; middle panel) are overlaid in the right panel. (B) Line scan along the dashed red line in the right image in panel A. Raw ion counts were scaled by max-min normalization. Newly replicated DNA (^{127}I ; green line) and phosphorus (^{31}P ; blue line) in the nucleus show a similar pattern, confirming that high-phosphorus regions mainly originate from the DNA backbone rather than from RNA or phosphorylated proteins.

Fig. 32: Regions of active transcription within the nucleus mainly originate from low-phosphorus regions. (A) Representative srIBI image of a HeLa cell stained with anti-H3K27Ac- ^{19}F /FITC. Ion images for H3K27Ac (^{19}F ; left panel) and phosphorus (^{31}P ; middle panel) are overlaid in the right panel. Scale bars, 4 μm . Cell in the composite image is also shown in Fig. 14 D. (B-C) Line scan along the lines in the boxes outlined in B) red and C)

yellow dashed lines in the image to the right in panel A. Raw ion counts were scaled by mix-man normalization. Black arrows point to regions of high H3K27Ac counts (green line) that are anti-correlated with phosphorus counts (blue line). Red arrows point to low H3K27Ac counts (green line) that are anti-correlated with phosphorus counts (blue line). Images below
 5 the line scans are magnified from regions boxed in panel A. Scale bars, 800 nm.

Fig. 33: Beam current scales with ion counts per pixel at the expense of resolution.

HeLa cells were labeled with IdU for 24 hours and stained with anti-nucleolin-¹⁹F/FITC. The images were acquired with a constant 25 * 25 μ m field of view, 1 ms dwell time per pixel, and
 10 512 * 512 pixels (xy). The beam current was increased every 10 planes by changing the D1 aperture width to obtain a beam diameter of ~50 nm (D1: 5), ~75 nm (D1: 4), ~100 nm (D1: 3) and ~150 nm (D1: 2). **(A)** Representative srIBI images of nucleolin staining in the same HeLa cell using different aperture widths. Different brightness intensities to normalize for variation in ion counts are shown for clarity. Scale bars, 4 μ m. **(B)** Enlarged images of colored boxes
 15 within images in panel A show an individual nucleolus. Details are increasingly lost as the beam current is elevated. Scale bars, 400 nm. **(C)** Ion count per pixel of all pixels in images shown in panel B. Each colored boxplot represents color-coded images in panel B. Boxplots at high current (D1: 2 and 3) show higher ion counts per pixel than boxplots at low current (D1: 4 and 5), highlighting loss of ion counts per pixel at lower current. **(D)** Line scans from lines
 20 within images shown in panel B. Line scans at high current (D1: 2 and 3) are smoother than line scans at low current (D1: 4 and 5), showing loss of resolution at higher current. **(E-H)** Quantification of lateral resolution using a beam diameter of **(E)** ~50 nm (D1: 5), **(F)** ~75 nm (D1: 4), **(G)** ~100 nm (D1: 3), and **(H)** ~150 nm (D1: 2). (Left) Representative srIBI images of newly replicated DNA (IdU) staining in the same HeLa cell. Scale bars, 4 μ m. (Right) Line
 25 scan for each colored bar in the images shown on the left. The lateral resolution calculated using the 16%-84% criterion is shown as an average of each analyzed line $\pm$ s.d. **(I)** Single channel and composite images of images shown in Fig. 14 E of ¹⁹F, nucleolin, and IdU.

Fig. 34: Validation of ⁸¹Br/Cy3 MoC-Abs for srIBI. **(A)** A schematic of the ⁸¹Br/Cy3
 30 MoC-Ab. The antibody was conjugated to the 5' end of an oligonucleotide in which all

cytidines were replaced by 5-Br-dC. The oligonucleotide also had a 3' Cy3. (SEQ ID NO:11)
 (B) Representative confocal microscopy image of a HeLa cell stained with unconjugated anti-CENP-A-⁸¹Br/Cy3 and a secondary anti-mouse-Alexa488 (green) and DAPI (blue). Scale bar, 4 μm. (C) Representative confocal microscopy images of control HeLa cells (no antibody) and HeLa cells stained with anti-CENP-A-⁸¹Br/Cy3 (orange) at three different concentrations. ³¹P is shown in blue. Cells in red boxes in the composite image are enlarged in the right column. Scale bars, 20 μm in standard images and 4 μm in enlarged images. A 1:500 dilution of this antibody was used in subsequent experiments. (D) Representative srIBI images of control HeLa cell (no antibody) and HeLa cell stained with anti-CENP-A-⁸¹Br/Cy3. Scale bar, 4 μm.

Fig. 35: Validation of ¹²⁷I/Cy5 MoC-Abs for srIBI. (A) A schematic of the ¹²⁷I/Cy5 MoC-Ab. The antibody was conjugated to the 5' end of an oligonucleotide in which all cytidines were replaced by 5-I-dC. The oligonucleotide also had a 3' Cy5. (SEQ ID NO: 11)
 (B) Representative confocal microscopy image of a HeLa cell stained with unconjugated anti-H3K27Ac and a secondary anti-mouse-Alexa488 (green) and DAPI (blue). Scale bar, 4 μm.
 (C) Representative confocal microscopy images of control HeLa cells (no antibody) and HeLa cells stained with anti-H3K27Ac-¹²⁷I/Cy5 (red) at three different concentrations. ³¹P is shown in blue. Cells in red boxes in the composite image are enlarged in the right column. Scale bars, 20 μm in standard images and 4 μm in enlarged images. A 1:100 dilution of this antibody was used in subsequent experiments. (D) Representative srIBI images of control HeLa cell (no antibody) and HeLa cell stained with anti-H3K27Ac-¹²⁷I/Cy5. Scale bar, 4 μm.

Fig. 36: Validation of Phalloidin-ATTO514 for srIBI. (A) Chemical structure of ATTO 514. Green arrows indicate the presence of the six ¹⁹F atoms in ATTO 514. (B) Representative confocal microscopy image of a HeLa cell stained with phalloidin-ATTO514. Scale bars, 4 μm. (C) Representative srIBI images of a HeLa cell stained with phalloidin-ATTO514. Scale bars, 4 μm.

Fig. 37: Validation of biotin-conjugated antibodies stained with ¹⁹⁷Au/FITC-conjugated streptavidin. (A) A schematic of the antibody conjugation strategy. Primary

antibodies were conjugated to biotin and subsequently stained with ^{197}Au /FITC-conjugated streptavidin. The ^{197}Au consists of 1.4 nm nanoparticles, a diameter below the theoretical srIBI resolution limit. **(B)** Representative confocal microscopy image of a HeLa cell stained with unconjugated anti-SC35 and a secondary anti-mouse-Alexa488 (green). DAPI (blue) was used to stain the nucleus. Scale bars, 4 μm . **(C)** Representative confocal microscopy images of HeLa cells stained with streptavidin- ^{197}Au /FITC and HeLa cells stained with anti-SC35-biotin followed by streptavidin- ^{197}Au /FITC at different concentrations of the primary antibody and 1:40 of streptavidin- ^{197}Au /FITC (green). DAPI (blue) was used to stain nuclei. Scale bars, 20 μm . Cells in red boxes in the composite image are magnified in the right column. Scale bars, 4 μm . A 1:100 dilution of this antibody was used on following experiments. **(D)** Representative srIBI image of a control HeLa cell (no antibody) and a HeLa cell stained with anti-SC35-biotin followed by streptavidin- ^{197}Au /FITC. Scale bar, 4 μm .

Fig. 38: Validation of antibody-based tools for srIBI by targeting other proteins. **(A)** Representative srIBI image of a HeLa cell stained with anti-H3K9me3- ^{81}Br /Cy3 (red). Phosphorus is shown in blue. Scale bar, 4 μm . **(B)** Representative srIBI image of a HeLa cell stained with anti-nucleolin- ^{127}I /Cy5 (red). Phosphorus is shown in blue. Scale bar, 4 μm . **(C)** Representative srIBI image of a HeLa cell stained with anti-NPM1-biotin followed by streptavidin- ^{197}Au /FITC (red). Phosphorus is shown in blue. Scale bars, 4 μm .

Fig. 39: Assessment of srIBI channel crosstalk. Representative srIBI images of a control HeLa cell control (no antibody) and HeLa cells stained with the indicated antibodies. Each row represents a staining condition and each column the ion channel extracted for the indicated isotope. Scale bars, 4 μm .

Fig. 40: Two-channel srIBI on HeLa cells using a beam diameter of ~ 50 nm (D1: 5). Representative confocal microscopy (left) and srIBI (right) images of HeLa cells stained with anti-nucleolin- ^{19}F /FITC and anti-CENP-A- ^{81}Br /Cy3 MoC-Ab. Scale bars, 4 μm (top 3 rows) and 1 μm (bottom row).

Fig. 41: Six-channel srIBI on HeLa cells using a beam diameter of ~50 nm (D1: 5). Representative srIBI image of a HeLa cell stained with anti-nucleolin- ^{19}F /FITC, anti-H3K9me3- ^{81}Br /Cy3, anti-H3K27Ac- ^{127}I /Cy5, and anti-SC35-biotin (recognized by streptavidin- ^{197}Au /FITC). Images of the e^- , nucleolin (^{19}F), DNA (^{31}P), H3K9me3 (^{81}Br), H3K27Ac (^{127}I), and SC35 (^{197}Au) were simultaneously acquired. A composite image is shown in Fig. 15 D. Scale bars, 5 μm .

Fig. 42: Sequential planes are similar in ion counts. (A) Representative srIBI image of a HeLa cell stained with anti-nucleolin- ^{19}F /FITC. Scale bars, 4 μm ; and 1 μm for enlarged images. (B) srIBI images of the 10 individual planes summed in panel A in consecutive order. The ^{19}F signal is similar in all individual images. Scale bars, 4 μm (C) Line scans on the nucleolus enlarged in panel A for each individual plane. Line scans were used to quantify ion counts per pixel in individual planes. Scale bars, 1 μm .

Fig. 43: 3D surface reconstruction of CENP-A signal at 5 nm Z-depth shows centromeres with a spherical shape. HeLa cells were stained with anti-CENP-A- ^{81}Br /Cy3, and 40 individual planes were acquired to obtain srIBI images of centromeres from its appearance to its disappearance. 3D surface reconstruction of images of CENP-A at 5 nm reveal centromeres with spherical shape.

Fig. 44: Individual planes of a whole nucleolus. (A) (Left) Representative srIBI image of a HeLa cell stained with anti-nucleolin- ^{19}F /FITC. Scale bar, 4 μm . (Right) Enlarged image of the nucleolus shown in Fig. 16 A-B. Scale bar, 400 nm. (B) Individual planes of a whole nucleolus, in order from left to right and from top to bottom; these 783 images were used for the volumetric reconstruction of the nucleolus shown in Fig. 16 B. Images have the same scale for intensity, and sequential planes have similar ^{19}F ion counts.

Fig. 45: Iterative srIBI is able to resolve MoC-Ab signals at the nanoscale. (Left) Representative enlarged image of a nucleolus of a HeLa cell stained with anti-nucleolin- ^{19}F /FITC. The same nucleolus is shown in Fig. 16 F. Scale bar, 300 nm. (Middle) Higher

magnification images of the area boxed in the image on the left. (Right) Line scans of the lines in the middle images demonstrate that srIBI can resolve signals spaced a few tens of nm.

Fig. 46: Iterative super-resolution imaging reveals nucleolar substructures. (A)

Representative secondary electron image of a HeLa cell. Scale bar, 5 μm . (B) (Left) High-resolution scan of the HeLa cell used to find a ROI. The HeLa cell shown in panel A was stained with anti-nucleolin- ^{19}F /FITC, anti-FIB-1- ^{81}Br /Cy3, and anti-NPM1-biotin (recognized by streptavidin- ^{197}Au /FITC). Scale bar, 5 μm . (Right) Iterative super-resolution imaging for the visualization of the nucleolar structure. NPM1, a marker of the granular component (GC), is distributed in regions distinct from those stained with FIB-1, a marker of the dense fibrillary component (DFC). Regions with high phosphorus surrounding the nucleolus indicate perinucleolar heterochromatin (PHC). Scale bar, 1 μm . (C) Individual images of the super-resolution image shown in panel B. Scale bars, 1 μm .

Fig. 47: Validation of cisplatin for srIBI. (A) Chemical structure of cisplatin. (B)

CyTOF analysis of cisplatin distribution in TYK-nu ovarian cancer cells. TYK-nu cells were treated with different concentrations of cisplatin for 24 hours, washed, and treated with 1 μM Rh-intercalator for 15 minutes (to discriminate dead from live cells) and analyzed by CyTOF. Maximal drug uptake with minimal cell death at 24 hours was observed with 5 μM . (C) The five naturally occurring stable isotopes of cisplatin. (D) Mean of ion count per pixel of all pixels in srIBI images from TYK-nu cells treated with cisplatin. TYK-nu cells were treated with DMSO (n=3) or 5 μM cisplatin (n=9) for 24 hours and analyzed by srIBI. Minimal signal was observed in DMSO-treated cells in the ^{194}Pt channel. Scale bars, 4 μm . (E) Cisplatin is observed in both the cytoplasm and nucleus. Representative srIBI images of a TYK-nu cell treated with DMSO (top) or 5 μM cisplatin for 24 hours without antibody staining (bottom). Scale bars, 4 μm . (F) Assessment of ^{194}Pt channel crosstalk. (Top) Representative srIBI image of a TYK-nu cell treated with DMSO and stained with anti-nucleolin- ^{19}F , anti-H3K9me3- ^{81}Br , anti-H3K27Ac- ^{127}I , and anti-SC35-biotin (recognized by streptavidin- ^{197}Au). (Bottom) Representative srIBI image of a TYK-nu cell treated with 5 μM cisplatin for 24 hours without

antibody staining. Each row represents a staining condition and each column the ion map for the indicated isotope. Scale bars, 4 μm .

Fig. 48: Six-channel srIBI images on TYK-nu cells treated with cisplatin. (A-C)

5 Representative srIBI images of TYK-nu cells treated with 5 μM cisplatin for 24 hours and stained with anti-nucleolin- ^{19}F /FITC, anti-H3K9me3- ^{81}Br /Cy3, anti-H3K27Ac- ^{127}I /Cy5, and anti-SC35-biotin (recognized by streptavidin- ^{197}Au). Images of nucleolin (^{19}F ; cyan), DNA (^{31}P ; blue), H3K9me3 (^{81}Br ; magenta), H3K27Ac (^{127}I ; green), cisplatin (^{194}Pt ; grey), and SC35 (^{197}Au ; red) were simultaneously acquired. (A) A composite image of a TYK-nu cell
10 nucleus. Scale bars, 2 μm (right image), 200 nm (middle image) and 67 nm (left images). (B) Individual images of the cell shown in panel A. Scale bars, 2 μm . (C) Composite images from three additional TYK-nu cell nuclei. Scale bars, 2 μm .

Fig. 49 Identification of nuclear neighborhoods by the iterative srIBI analysis

15 framework. (A) t-SNE was performed on 100000 voxels of dimensions (x, y, z) = (10, 10, 5) pixels (20000 voxels from each cell were randomly sampled across 5 different cells). Each point represents a voxel, consisting of the expression of six different parameters (phosphorus, nucleolin, H3K9me3, SC35, cisplatin, and H3K27Ac). Voxels grouped into distinct regions based on similar combinatory expression of each marker. The t-SNE map was recolored from
20 left to right to represent the scaled intensity (Z-score) of each indicated marker. (B) Unsupervised hierarchical clustering was performed on the voxels to separate them into 29 distinct groups; the t-SNE map from panel A is shown recolored based on these groups. (C) A heatmap representation of the 29 distinct clusters identified based on the average expression per cluster of SC35, H3K9me3, phosphorus, nucleolin, H3K27Ac, and cisplatin. The scale
25 intensity of each marker is denoted by the color bar on the top right (Z-score, normalized to each row). (D) Clusters were manually merged by tree distance, proximity on the t-SNE map and similar expression profiles. This resulted in a reduction of 29 clusters into 11 clusters, which were then annotated based on their final average expression profile. (E) t-SNE map from panel A colored by the cell of origin (from 5 different cells).

Fig. 50: The spatial distribution of nuclear neighborhoods across cells reveals order and diversity in nuclear organization. Nuclear neighborhoods were recolored by each cluster in each of five cells to allow a visual understanding of nuclear neighborhood interactions and conserved and divergent features. Cells 1, 2, 4, and 5 are shown here. Cell 3 is shown in Fig. 18 F.

Fig. 51: Pairwise neighborhood interaction frequency at different cutoffs. Pairwise voxels were defined as neighbors using a cutoff of n pixels in 3D Euclidean space (see Materials and Methods for details). We tested two distances here, using (A) 5 pixels and (B) 25 pixels as the cutoff. The neighborhood interaction frequency map was calculated using the log2 enrichment of the real over expected number of pairwise interaction frequencies (left). The adjusted p-values are plotted as significant ($p < 0.05$, cream) or not significant ($p \geq 0.05$, red) (right).

Fig. 52: Identification of a cisplatin gradient within SC35 nuclear speckles. (A) Neighborhoods 6 (Active SC35 and Cisplatin) and 7 (Inactive SC35 and Cisplatin) were plotted for all 5 cells, to represent the spatial distribution of these two neighborhoods relative to each other. (B) Scaled cisplatin levels (Z-score) were plotted on neighborhoods 6 and 7. An increasing gradient of cisplatin concentration is observed across the inactive-active SC35 transition boundary.

Fig. 53 schematically illustrates a labeling system that can be used to labeled nucleic acid in a cell *in situ*.

Fig. 54 shows results obtained using the labeling system illustrated in Fig. 53.

DEFINITIONS

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those

described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

5 Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

The headings provided herein are not limitations of the various aspects or embodiments of the invention. Accordingly, the terms defined immediately below are more fully defined by
10 reference to the specification as a whole.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Markham, THE
15 HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, N.Y. (1991) provide one of skill with the general meaning of many of the terms used herein. Still, certain terms are defined below for the sake of clarity and ease of reference.

The term “nucleotide” is intended to include those moieties that contain not only the natural purine and pyrimidine bases, but also other heterocyclic bases that have been modified.
20 Such modifications include methylated purines or pyrimidines, acylated purines or pyrimidines, alkylated riboses or other heterocycles. In addition, the term “nucleotide” includes those moieties that contain hapten or fluorescent labels and may contain not only conventional ribose and deoxyribose sugars, but other sugars as well. Modified nucleosides or nucleotides also include modifications on the sugar moiety, e.g., wherein one or more of the
25 hydroxyl groups are replaced with halogen atoms or aliphatic groups, or are functionalized as ethers, amines, or the like. In some embodiments, a nucleotide may be modified to contain a non-biological stable isotope. 2-F-Ac-C, 5-Br-dC, 5-I-dC are examples of such modified nucleotides.

The term “nucleic acid” and “polynucleotide” are used interchangeably herein to
30 describe a polymer of any length, e.g., greater than about 2 bases, greater than about 10 bases,

greater than about 100 bases, greater than about 500 bases, greater than 1000 bases, up to about 10,000 or more bases composed of nucleotides, e.g., deoxyribonucleotides or ribonucleotides, and may be produced enzymatically or synthetically (e.g., PNA as described in U.S. Patent No. 5,948,902 and the references cited therein) which can hybridize with

5 naturally occurring nucleic acids in a sequence specific manner analogous to that of two naturally occurring nucleic acids, e.g., can participate in Watson-Crick base pairing interactions. Naturally-occurring nucleotides include guanine, cytosine, adenine and thymine (G, C, A and T, respectively). DNA and RNA have a deoxyribose and ribose sugar backbone, respectively, whereas PNA's backbone is composed of repeating N-(2-aminoethyl)-glycine

10 units linked by peptide bonds. In PNA various purine and pyrimidine bases are linked to the backbone by methylene carbonyl bonds. A locked nucleic acid (LNA), often referred to as inaccessible RNA, is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo (North) conformation, which is often found in the A-form duplexes. LNA

15 nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired. The term "unstructured nucleic acid," or "UNA," is a nucleic acid containing non-natural nucleotides that bind to each other with reduced stability. For example, an unstructured nucleic acid may contain a G' residue and a C' residue, where these residues correspond to non-naturally occurring forms, i.e., analogs, of G and C that base pair with each other with reduced

20 stability, but retain an ability to base pair with naturally occurring C and G residues, respectively. Unstructured nucleic acid is described in US20050233340, which is incorporated by reference herein for disclosure of UNA. Also included in this definition are ZNAs, i.e., zip nucleic acids.

As used herein, the term "biological feature of interest" refers to any part of a cell that

25 can be indicated by binding to a capture agent. Exemplary biological features of interest include cell walls, nuclei, cytoplasm, membrane, keratin, muscle fibers, collagen, bone, proteins, nucleic acid (e.g., mRNA or genomic DNA, etc). fat, etc. A biological feature of interest can also be indicated by immunohistological methods, e.g., a capture agent that is linked to an oligonucleotide. In these embodiments, the capture agent binds to a site, e.g., a

30 protein epitope, in the sample. Exemplary epitopes include, but are not limited to

carcinoembryonic antigen (for identification of adenocarcinomas, cytokeratins (for identification of carcinomas but may also be expressed in some sarcomas) CD15 and CD30 (for Hodgkin's disease), alpha fetoprotein (for yolk sac tumors and hepatocellular carcinoma), CD117 (for gastrointestinal stromal tumors), CD10 (for renal cell carcinoma and acute lymphoblastic leukemia), prostate specific antigen (for prostate cancer), estrogens and progesterone (for tumour identification), CD20 (for identification of B-cell lymphomas), CD3 (for identification of T-cell lymphomas). Complementary nucleic acid molecules (e.g., DNA and/or RNA) in the sample provide binding complementary sites for oligonucleotide probes.

As used herein, the term "multiplexing" refers to using more than one distinguishably labeled reagent (e.g., at least 5, at least 10, at least 50, at least 100, up to 200 or 500 or more distinguishably labeled reagents), for the simultaneous or sequential detection and measurement of biologically active material.

As used herein, the terms "antibody" and "immunoglobulin" are used interchangeably herein and are well understood by those in the field. Those terms refer to a protein consisting of one or more polypeptides that specifically binds an antigen. One form of antibody constitutes the basic structural unit of an antibody. This form is a tetramer and consists of two identical pairs of antibody chains, each pair having one light and one heavy chain. In each pair, the light and heavy chain variable regions are together responsible for binding to an antigen, and the constant regions are responsible for the antibody effector functions.

The recognized immunoglobulin polypeptides include the kappa and lambda light chains and the alpha, gamma (IgG₁, IgG₂, IgG₃, IgG₄), delta, epsilon and mu heavy chains or equivalents in other species. Full-length immunoglobulin "light chains" (of about 25 kDa or about 214 amino acids) comprise a variable region of about 110 amino acids at the NH₂-terminus and a kappa or lambda constant region at the COOH-terminus. Full-length immunoglobulin "heavy chains" (of about 50 kDa or about 446 amino acids), similarly comprise a variable region (of about 116 amino acids) and one of the aforementioned heavy chain constant regions, e.g., gamma (of about 330 amino acids).

The terms "antibodies" and "immunoglobulin" include antibodies or immunoglobulins of any isotype, fragments of antibodies which retain specific binding to antigen, including, but not limited to, Fab, Fv, scFv, and Fd fragments, chimeric antibodies, humanized antibodies,

minibodies, single-chain antibodies, and fusion proteins comprising an antigen-binding portion of an antibody and a non-antibody protein. Also encompassed by the term are Fab', Fv, F(ab')₂, and or other antibody fragments that retain specific binding to antigen, and monoclonal antibodies. Antibodies may exist in a variety of other forms including, for
5 example, Fv, Fab, and (Fab')₂, as well as bi-functional (i.e. bi-specific) hybrid antibodies (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)) and in single chains (e. g., Huston et al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et al., Science, 242, 423-426 (1988), which are incorporated herein by reference). (See, generally, Hood et al., "Immunology", Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, Nature, 323, 15-
10 16 (1986),).

The term "specific binding" refers to the ability of a binding agent to preferentially bind to a particular analyte that is present in a homogeneous mixture of different analytes. In certain embodiments, a specific binding interaction will discriminate between desirable and undesirable analytes in a sample, in some embodiments more than about 10 to 100-fold or
15 more (e.g., more than about 1000- or 10,000-fold).

In certain embodiments, the affinity between a binding agent and analyte when they are specifically bound in a binding agent/analyte complex is characterized by a K_D (dissociation constant) of less than 10⁻⁶ M, less than 10⁻⁷ M, less than 10⁻⁸ M, less than 10⁻⁹ M, less than 10⁻⁹ M, less than 10⁻¹¹ M, or less than about 10⁻¹² M or less.

A "plurality" contains at least 2 members. In certain cases, a plurality may have at least
20 2, at least 5, at least 10, at least 100, at least 1000, at least 10,000, at least 100,000, at least 10⁶, at least 10⁷, at least 10⁸ or at least 10⁹ or more members.

As used herein, the term "labeling" refers to attaching a label to specific sites in a sample (e.g., sites containing an epitope for the antibody being used, for example) such that
25 the presence and/or abundance of the sites can be determined by evaluating the presence and/or abundance of the label.

As used herein, the term "biological sample" refers to a sample, which may be substantially planar, i.e., two dimensional, that contains cells. A biological sample can be made by, e.g., growing cells on a planar surface, depositing cells on a planar surface, e.g., by
30 centrifugation, by cutting a three dimensional object that contains cells into sections and

mounting the sections onto a planar surface, i.e., producing a tissue section, or any other means. The cells may be fixed using any number of reagents including formalin, methanol, paraformaldehyde, methanol:acetic acid etc. A planar sample has a thickness.

As used herein, the term “tissue section” refers to a piece of tissue that has been
5 obtained from a subject, and optionally fixed, sectioned, and mounted on a planar surface, e.g., a microscope slide.

As used herein, the term “formalin-fixed paraffin embedded (FFPE) tissue section” refers to a piece of tissue, e.g., a biopsy that has been obtained from a subject, fixed in formaldehyde (e.g., 3%-5% formaldehyde in phosphate buffered saline) or Bouin solution,
10 embedded in wax, and optionally cut into thin sections, and then mounted on a microscope slide.

As used herein, the term “spatially-addressable measurements” refers to a set of values that are each associated with a specific position on a surface. Spatially-addressable measurements can be mapped to a position in a sample and can be used to reconstruct a 2d or
15 3d image of the sample.

A “diagnostic marker” is a specific biochemical in the body which has a particular molecular feature that makes it useful for detecting a disease, measuring the progress of disease or the effects of treatment, or for measuring a process of interest.

A “pathoindicative” cell is a cell which, when present in a tissue, indicates that the
20 animal in which the tissue is located (or from which the tissue was obtained) is afflicted with a disease or disorder. By way of example, the presence of one or more breast cells in a lung tissue of an animal is an indication that the animal is afflicted with metastatic breast cancer.

The term “complementary site” is used to refer to an epitope for an antibody or aptamer, or a nucleic acid molecule if the capture agent is an oligonucleotide probe.
25 Specifically, if a binding agent is an antibody, then the complementary site for the binding agent is the epitope in the sample to which the antibody binds. If the capture agent is an oligonucleotide probe, then the complementary site for the capture agent is a complementary sequence in a DNA or RNA molecule in the sample.

The term “epitope” as used herein is defined as small chemical groups on the antigen
30 molecule that is bound to by an antibody. An antigen can have one or more epitopes. In many

cases, an epitope is roughly five amino acids or sugars in size. One skilled in the art understands that generally the overall three-dimensional structure or the specific linear sequence of the molecule can be the main criterion of antigenic specificity.

A “subject” of diagnosis or treatment is a plant or animal, including a human. Non-
5 human animals subject to diagnosis or treatment include, for example, livestock and pets.

As used herein, the term “incubating” refers to maintaining a biological sample and binding agent under conditions (which conditions include a period of time, a temperature, an appropriate binding buffer and a wash) that are suitable for specific binding of the binding agent to molecules (e.g., epitopes or complementary nucleic acid) in the biological sample.

10 As used herein, the term “binding agent” refers to an agent that can specifically bind to complementary sites in a biological sample. Exemplary capture agents include, e.g., an antibody, an aptamer, and an oligonucleotide probe (which may be DNA or RNA) that hybridizes to a binding site.

As used herein, the term “binding agent that is linked to a polymer” refers to a binding
15 agent, e.g., an antibody or an oligonucleotide probe covalently linked to a polymer, e.g., an oligonucleotide. The polymer and the capture agent may be linked via a number of different methods, including those that use maleimide or halogen-containing group, which are cysteine-reactive.

As used herein, the term “oligonucleotide” refers to a multimer of at least 10, e.g., at
20 least 15 or at least 30 nucleotides. In some embodiments, an oligonucleotide may be in the range of 15-200 nucleotides in length.

As used herein, the term “reading” in the context of reading a signal, refers to obtaining an image by scanning or by microscopy, where the image shows the pattern of fluorescence as well as the intensity of fluorescence in a field of view.

25 As used herein, the term “isotopically tagged” refers to a molecule that is tagged with either a single kind of stable isotope that is identifiable by its unique mass or mass profile or a combination of the same, where the combination of stable isotopes provides an identifier. Combinations of stable isotopes permit channel compression and/or barcoding. Examples of elements that are identifiable by their mass are listed below, although other elements may be

employed. The terms “mass tagged” and “elementally tagged” may be used interchangeably herein.

As used herein, the term “isotopically tag” means any isotope of any element that is identifiable by its mass, distinguishable from other mass tags, and used to tag a biologically active material or analyte. A mass tag has an atomic mass that is distinguishable from the atomic masses present in the analytical sample and in the particle of interest. The term “monoisotopic” means that a tag contains a single type of metal isotope (although any one tag may contain multiple metal atoms of the same type).

As used herein, the term “elemental analysis” refers to a method by which the presence and/or abundance of elements of a sample are evaluated.

A “sample comprising cells” is a sample of biological origin that contains intact, e.g., fixed, cells. In some embodiments, the sample may be substantially planar. Examples of such samples include tissue sections, samples that are made by depositing disassociated cells onto a planar surface, and samples that are made by growing a sheet of cells on a planar surface.

As used herein, the term “scanning” refers to a method by which a source of radiation (e.g., a laser) is zig-zagged or rastered over a surface until a substantial two dimensional area has been irradiated by the source of energy.

As used herein, the term “across an area”, in the context of spatially-addressable measurements of the abundance of an isotope tag across an area of a sample, refers to measurements of mass tags that are at or under (e.g., on or within cells that are proximal to) the surface of the sample. The depth of the area analyzed can vary depending on the energy of the ion beam.

As used herein, the term “mass cytometry” refers to a method in which cells are separated from one another by use of a flow cell and then subjected to elemental analysis.

As used herein, the term “substantially pure” indicates that a reagent has been isolated from other components by purification and is relatively pure form, e.g., at least 50% free, at least 70% free or at least 90% free of other components that were present prior to purification. Reagents that are made synthetically are substantially pure.

As used herein, the term “internal atoms” refers to atoms that are part of the molecule, not non-covalently attached the molecule. Atoms that are chelated to a molecule are not internal atoms.

As used herein, the term “non-biological” refers to isotopes that have low abundance in biological samples, e.g., human cells.

“Stable” isotopes are non-radioactive.

Other definitions of terms may appear throughout the specification.

DETAILED DESCRIPTION

As noted above, this disclosure provides a substantially pure labeling moiety, where the labeling moiety comprises multiple internal atoms of a non-biological stable nonmetal or metalloid isotope. In some embodiments, the labeling moiety may be a binding agent that specifically binds to a complementary site in or on a cell (in which case the isotopically-labeled atoms may be part of the binding agent), or the labeling moiety may comprise a binding agent that specifically binds to a complementary site in or on a cell (in which case the isotopically-labeled atoms may be part of an adjunct such as a polymer that is covalently linked to the binding agent). In other embodiments, the labeling moiety may comprise a chemoselective group, such that the labeling moiety can be readily covalently linked with a binding agent via any suitable reaction. In any embodiment, the labeling moiety is not antibody that binds to other antibodies. For example, in some embodiments, the labeling moiety is not or does not comprise an anti-IgG antibody (an antibody that binds to IgG antibodies).

The isotope used should fulfill two requirements: it should be efficiently ionized upon impact of a positively charged ion and it should have low abundance in cells. As such, in many cases, any nonmetal or metalloid isotope (except for ^1H , ^{12}C , ^{14}N , ^{16}O and ^{32}S , which are commonly found in cells) can be used.

In some embodiments, the labeling moiety comprises a non-biological stable nonmetal isotope of carbon (C), nitrogen (N), oxygen (O), fluorine (F), phosphorus (P), sulfur (S), chlorine (Cl), selenium (Se), bromine (Br), or iodine (I), with the exception of ^1H , ^{12}C , ^{14}N , ^{16}O and ^{32}S . In particular embodiments, the labeling moiety may comprise a stable isotope of a

halogen (e.g., fluorine, chlorine, bromine, or iodine). ^{19}F , ^{81}Br and ^{127}I are examples of halogens that can be used herein. In some embodiments, the labeling moiety may comprise a stable metalloid isotope of boron (B), silicon (Si), germanium (Ge), arsenic (As), antimony (Sb) or tellurium (Te). As would be apparent, the isotope used should be “non-biological” in
 5 that it is not found in abundance in biological samples, i.e., samples that contain cells.

In any embodiment, a nonmetal or metalloid isotope may be selected from the following table:

Element	Atomic Mass
Deuterium (D)	2
Boron (B)	10
Boron (B)	11
Carbon (C)	13
Nitrogen (N)	15
Oxygen (O)	17
Oxygen (O)	18
Fluorine (F)	19
Silicon (Si)	28
Silicon (Si)	29
Silicon (Si)	30
Sulphur (S)	33
Sulphur (S)	34
Chlorine (Cl)	35
Sulphur (S)	36
Chlorine (Cl)	37
Germanium (Ge)	70
Germanium (Ge)	72

Element	Atomic Mass
Germanium (Ge)	73
Germanium (Ge)	74
Selenium (Se)	74
Arsenic (As)	75
Selenium (Se)	76
Selenium (Se)	77
Selenium (Se)	78
Bromine (Br)	79
Selenium (Se)	80
Bromine (Br)	81
Tellurium (Te)	120
Antimony (Sb)	121
Tellurium (Te)	122
Antimony (Sb)	123
Tellurium (Te)	123
Tellurium (Te)	124
Tellurium (Te)	125
Tellurium (Te)	126
Iodine (I)	127

Exemplary types of labeling moiety are shown in Fig. 1.

In some embodiments (and as shown in Fig. 1, b and d) the labeling reagent may be (Fig. 1, d) or may comprise (Fig. 1, b) a binding agent (e.g., an antibody). As shown in Fig. 1, d, if the labeling reagent is a binding agent, the labeling reagent itself may comprise
5 internal atoms of the non-biological stable nonmetal or metalloid isotope. In some of these embodiments, the labeling reagent may be a protein, e.g., an antibody. In these embodiments, the labeling reagent may be made by growing cells comprising a nucleic acid encoding the labeling reagent on a medium that comprises isotopically labeled amino acids, then purifying the labeling reagent. In these embodiments, the labeling reagent may contain isotopically
10 labeled C, N or O (but not ^{12}C , ^{14}N , ^{16}O), because these atoms are abundant in proteins.

As shown in Fig. 1, b, in some embodiments the isotopically-labeled atoms may be internal to an adjunct that is covalently linked to a binding agent, e.g., an antibody. In these embodiments, the labeling moiety may be composed of: i. of a polymer and ii. a binding agent that specifically binds to a complementary site in or on a cell, wherein only the polymer
15 comprises the atoms of the stable nonmetal or metalloid isotope. In these embodiments, the polymer may be an oligonucleotide, although any polymer that can be built to a defined size, monomer-by-monomer, can be used. If the polymer is an oligonucleotide, then the isotope may be part of the nucleotides that make up the oligonucleotide. The monomers may be joined to one another using, e.g., phosphoramidite chemistry, which is well known. In these
20 embodiments, the oligonucleotide may contain non-abundant isotopes of C, N, O or P but not ^{12}C , ^{14}N , ^{16}O or ^{15}P , since these atoms are abundant in oligonucleotides) since these atoms are abundant in oligonucleotides, or one or more of the atoms of a nucleotide may be substitute with the stable isotope. 2-F-Ac-C, 5-Br-dC, 5-I-dC, as shown in Fig. 7, are examples of such modified nucleotides. In some embodiments, an oligonucleotide may be composed of
25 nucleotides that have a group attached thereto, where the group has the labeled isotope. For example, the group may have a fluorophore or another label that contains a halogen atom. These labeling moieties may be made by: i. making the binding agent part of the moiety (e.g., an antibody) in a cell, ii. making the isotopically-labeled polymer synthetically, and iii. linking the binding agent the isotopically-labeled polymer, thereby making the binding agent. Methods
30 for linking oligonucleotides to proteins, e.g., antibodies, are well known (see, e.g., Gong et al.,

Bioconjugate Chem. 2016 27: 217–225 and Kazane et al., Proc Natl Acad Sci 2012 109: 3731-3736). A variety of linkage methods are available. For example, an oligonucleotide may be linked to an antibody directly using any suitable chemical moiety on the antibody (e.g., a cysteine residue or via an engineered site).

5 One advantage of using polymers to label a binding agent is that the number of types of atoms of a non-biological stable nonmetal or metalloid isotope that are incorporated into the polymer can be highly controlled. For example, in the case of an oligonucleotide, the number, position and identity of the isotope can be accurately defined in the oligonucleotide and, as such, binding moieties that can be identified by a specific combination of isotopes (e.g., two
10 different halogens, for example) or by a specific ratios of isotopes (e.g., a 1:4, 1:2, 1:1, 2:1 or 4:1 ratio of different isotopes, for example) can be readily made. A polymer of up to, e.g., 500 monomers, such as a polymer of 10-400 or 20-300 monomers, may be used in some embodiments.

In another example shown in Fig. 1, a, the binding moiety may be a non-covalently
15 associated complex comprising at least two components, where one of the components is labeled with one or more isotopes, and the other component specifically binds to sites in or on a cell. By way of example (and as shown in Fig. 1), the binding moiety may be a transposase complex that comprises i. a transposase and ii. a pair of double stranded nucleic acids comprising transposon end sequences, where the nucleic acids are isotopically labeled, as
20 described above. In these embodiments, the transposase complex can be contacted with chromatin in the presence of a divalent cation, and the transposase inserts the labeled nucleic acids into the open chromatin, thereby allowing the open chromatin to be analyzed.

In another example shown in Fig. 1, c, the isotopically-labeled atoms may be part of a nucleic acid that specifically binds to protein. In the example shown, the nucleic acid is a guide
25 nucleic acid and the protein may be a nucleic acid guided endonuclease, e.g., Cas9 or the like.

In certain embodiments, the labeling moiety may be a polymer (e.g., an oligonucleotide) that has a chemoselective group. In some embodiments, the chemoselective group may be amine-reactive, thiol reactive or hydroxyl reactive. In some embodiments, the chemoselective group may be capable of participating in a 1,3 cycloaddition (or “click”) reaction. Such groups include azido and alkynyl (e.g., cyclooctyne) groups, but others are
30

known. In some embodiments, the polymer may comprises a biotin moiety, i.e., a tag that includes biotin or a biotin analogue such as desthiobiotin, oxybiotin, 2-iminobiotin, diaminobiotin, biotin sulfoxide, biocytin, etc. Biotin moieties bind to streptavidin with an affinity of at least 10^{-8} M. Such labeled polymers (e.g., oligonucleotides) are readily
5 conjugated to antibodies and, as such, they can used to make multiplexed populations of labeled antibodies relatively straightforwardly.

In some embodiments, a labeling moiety may comprise two or more (e.g., two, three, four, five or more) different stable nonmetal and/or metalloïd isotopes. In some embodiments, about 95% of the isotopically labeled atoms in the moiety may be of one isotope, and about
10 5% of the isotopically labeled atoms in the moiety may be of the other isotope, although the percentage of the different isotopes may be different, e.g., 90%/10%, 80%:20%, 50%:50%, 20%:80%, 10%:90%, or 5%:95% in other embodiments.

Also provided is set of substantially pure labeling moieties as described above, wherein the moieties are distinguishably labeled by their stable nonmetal isotopes. In these
15 embodiments, a set may comprise at least 5, at least 10, at least 20, at least 30, at least 50, or at least 100, at least 150, at least 200, up to 300 or 500 or more labeling moieties that binds to different complementary sites (e.g., epitopes) in or on a cell. Each labeling moiety may be labeled with a single isotope or multiple isotopes, and the different labeling moieties may be distinguishable by the identity of the isotope in a labeling moiety, the combination of isotopes
20 in a labeling moiety, and/or the ratio of isotopes in a labeling moiety. In some embodiments, the moieties may comprise multiple internal atoms of at least two stable nonmetal or metalloïd isotopes, wherein the moieties are distinguishable by the identity and/or ratio of the different isotopes. For example, in some embodiments a panel of at least 5, at least 10, at least 20, at least 30, at least 50, or at least 100, at least 150, at least 200, up to 300 or 500 or more
25 antibodies, each conjugated to a distinguishably labeled polymers, e.g., oligonucleotide is provided. In other embodiments, a panel of distinguishably labeled polymers, e.g., oligonucleotides, each containing the same chemoselective groups is provided.

These labeling moieties may be in separate tubes (particularly if they contain a chemoselective group) or they may be mixed together.

The labeling reagents described above may be employed in a variety of analysis methods, e.g., elemental mass spectrometry, secondary ion mass spectrometry (SIMS), mass cytometry (CyTOF), laser-ablation inductively-coupled mass spectrometry (LA-ICP-MS), X-ray microscopy (XRM), X-ray fluorescence (XRF), laser-induced breakdown spectroscopy (LIBS), focused ion beam (FIB), energy-dispersive X-ray spectroscopy (EDX), electron microscopy, or nuclear magnetic resonance spectroscopy, for example. In general, the method may comprise labeling biological sample with a plurality of distinguishably labeled binding moieties, where each moiety recognizes a different complementary site in the sample, and analyzing the sample.

In one example, the labeling moieties may be used to generate a high resolution image by secondary ion mass spectrometry (SIMS). This method may comprise incubating a sample with one or more labeling moieties, thereby producing a labeled sample in which a biological feature of interest is bound to the one or more labeling moieties, scanning the sample by secondary ion mass spectrometry (SIMS) using a positively charged ion beam (e.g., a Cs ion beam) to generate a data set that comprises spatially-addressable measurements of the abundance of said at least one stable nonmetal or metalloid isotope across said sample; and outputting the data set.

The general principles of MIBI, including methods by which samples may be made, methods by which cells can be labeled using, e.g., mass-tagged antibodies, methods for ionizing the tags, and methods for analyzing the data, as well as hardware that can be employed in MIBI, including, mass spectrometers and computer control systems are known and are reviewed in a variety of publications including, but not limited to Angelo et al. Nature Medicine 2014 20:436, Rost et al Lab. Invest. 2017 97: 992-1003, US patent 9,766,224, US patent 9,312,111 and US20150080233, among many others, which publications are incorporated by reference herein for disclosure of those methods and hardware.

In another embodiment, the labeling moieties may be used to analyze cells by mass cytometry. This method may comprise incubating the cells with one or more labeling moieties, thereby producing a labeled sample in which the labeling moieties are bound to the cells; analyzing the cells by mass cytometry, using a positively charged plasma beam to atomize the cells and generate a data set that comprises spatially-addressable measurements of the

abundance of the stable nonmetal or metalloid isotopes in or on the cells and outputting the data set. The general principles of mass cytometry, including methods by which single cell suspensions can be made, methods by which cells can be labeled using, e.g., mass-tagged antibodies, methods for atomizing particles and methods for performing elemental analysis on particles, as well as hardware that can be employed in mass cytometry, including flow cells, ionization chambers, reagents, mass spectrometers and computer control systems are known and are reviewed in a variety of publications including, but not limited to Bandura et al Analytical Chemistry 2009 81 6813-6822), Tanner et al (Pure Appl. Chem 2008 80: 2627-2641), U.S. Patent Nos. 7,479,630 (Method and apparatus for flow cytometry linked with elemental analysis) and 7,135,296 (Elemental analysis of tagged biologically active materials); and published U.S. patent application 20080046194, for example, which publications are incorporated by reference herein for disclosure of those methods and hardware.

In particular embodiments, the method described above may be employed in a multiplex assay in which a heterogeneous population of cells is labeled with a plurality of distinguishably mass tagged binding agents (e.g., a number of different antibodies). The population of cells may be labeled using at least 5, at least 10, at least 20, at least 30, at least 50, or at least 100, up to 150 or more different binding agents (that bind to, for example different cell surface markers) that are each tagged with a different isotope. After the population of cells is labeled, the cells are introduced into the flow cell, individually analyzed using the method described above, and the cells are separated based on the mass tags associated with each of the cells. In particular embodiments, a cell having a particular profile of mass tags is desired, and the machine performing the method may be programmed to sort cells having the profile away from other cells that do not have the profile.

In some embodiments, the nucleic acid of a cell may be stained *in situ* using: (a) a primary oligonucleotide and (b) a labeled oligonucleotide that contains one or more non-metal/metalloid isotopes, where primary oligonucleotide has (i) a first, target-specific, sequence, i.e., a sequence that is complementary to a sequence in a cell (e.g., to a sequence in the genome of a mammalian cell) and (b) a second sequence that is complementary to the labeled oligonucleotide. This embodiment is illustrated in Fig. 53.

In such methods, multiple (at least two, at least 3 at least 5, etc.) sets of primary

oligonucleotides may be used wherein, within each set, the oligonucleotides can have: i. different target-specific sequences so that they hybridize to different target sequences in the cell and ii. the same second sequence. In these embodiments, the different sets may have different second sequences, thereby allowing different nucleic acid sequences targeted by the oligonucleotides (e.g. different chromosomes or chromosomal regions) to be labeled with different labels. In some embodiments, this method may be used to stain different chromosomes, different chromosomal regions, or different structures (A/B compartments, etc.) with different labels, thereby allowing those regions to be analyzed at high resolution.

The above-described method can be used to analyze a cell from a subject to determine, for example, whether the cell is normal or not or to determine whether the cells are responding to a treatment. In one embodiment, the method may be employed to determine the degree of dysplasia in cancer cells. In these embodiments, the cells may be from a sample of from a multicellular organism or a microbe. A biological sample may be isolated from an individual, e.g., from a soft tissue or from a bodily fluid, or from a cell culture that is grown *in vitro*. A biological sample may be made from a soft tissue such as brain, adrenal gland, skin, lung, spleen, kidney, liver, spleen, lymph node, bone marrow, bladder stomach, small intestine, large intestine or muscle, etc. Bodily fluids include blood, plasma, saliva, mucous, phlegm, cerebral spinal fluid, pleural fluid, tears, lactal duct fluid, lymph, sputum, cerebrospinal fluid, synovial fluid, urine, amniotic fluid, and semen, etc. Biological samples also include cells grown in culture *in vitro*. A cell may be a cell of a tissue biopsy, scrape or lavage or cells. In particular embodiments, the cell may of a cell in a formalin fixed paraffin embedded (FFPE), or frozen sample. In particular cases, the method may be used to distinguish different types of cancer cells in FFPE samples.

In some embodiments, the method may be used to produce a signature that may be diagnostic (e.g., may provide a diagnosis of a disease or condition or the type or stage of a disease or condition, etc.), prognostic (e.g., indicating a clinical outcome, e.g., survival or death within a time frame) or theranostic (e.g., indicating which treatment would be the most effective).

In certain embodiments, the binding moieties specifically bind to biomarkers, including cancer biomarkers, that may be proteinaceous or a nucleic acid. Exemplary cancer biomarkers,

include, but are not limited to carcinoembryonic antigen (for identification of adenocarcinomas), cytokeratins (for identification of carcinomas but may also be expressed in some sarcomas), CD15 and CD30 (for Hodgkin's disease), alpha fetoprotein (for yolk sac tumors and hepatocellular carcinoma), CD117 (for gastrointestinal stromal tumors), CD10 (for renal cell carcinoma and acute lymphoblastic leukemia), prostate specific antigen (for prostate cancer), estrogens and progesterone (for tumour identification), CD20 (for identification of B-cell lymphomas) and CD3 (for identification of T-cell lymphomas).

In some embodiments, the binding moieties may bind to a drug, and the method may be used to examine the distribution of the drug in a cell after it has been administered to by a subject, e.g., a human. In another embodiment, the drug itself may be isotopically labeled, and the binding moieties may be used to identify cellular proteins.

The above-described method can be used to analyze cells from a subject to determine, for example, whether the cell is normal or not or to determine whether the cells are responding to a treatment. In one embodiment, the method may be employed to determine the degree of dysplasia in cancer cells. In these embodiments, the cells may be a sample from a multicellular organism. A biological sample may be isolated from an individual, e.g., from a soft tissue. In particular cases, the method may be used to distinguish different types of cancer cells in FFPE samples. In some embodiments, the analysis may be to determine an appropriate therapy.

The method described above finds particular utility in examining planar samples using a plurality of antibodies, each antibodies recognizing a different marker. Examples of cancers, and biomarkers that can be used to identify those cancers, are shown below. In these embodiments, one does not need to examine all of the markers listed below in order to make a diagnosis.

Acute Leukemia IHC Panel	CD3, CD7, CD20, CD34, CD45, CD56, CD117, MPO, PAX-5, and TdT.
Adenocarcinoma vs. Mesothelioma IHC Panel	Pan-CK, CEA, MOC-31, BerEP4, TTF1, calretinin, and WT-1.
Bladder vs. Prostate Carcinoma IHC Panel	CK7, CK20, PSA, CK 903, and p63.
Breast IHC Panel	ER, PR, Ki-67, and HER2. Reflex to HER2 FISH after HER2 IHC is available.

Burkitt vs. DLBC Lymphoma IHC panel	BCL-2, c-MYC, Ki-67.
Carcinoma Unknown Primary Site, Female (CUPS IHC Panel - Female)	CK7, CK20, mammaglobin, ER, TTF1, CEA, CA19-9, S100, synaptophysin, and WT-1.
Carcinoma Unknown Primary Site, Male (CUPS IHC Panel - Male)	CK7, CK20, TTF1, PSA, CEA, CA19-9, S100, and synaptophysin.
GIST IHC Panel	CD117, DOG-1, CD34, and desmin.
Hepatoma/Cholangio vs. Metastatic Carcinoma IHC Panel	HSA (HepPar 1), CDX2, CK7, CK20, CAM 5.2, TTF-1, and CEA (polyclonal).
Hodgkin vs. NHL IHC Panel	BOB-1, BCL-6, CD3, CD10, CD15, CD20, CD30, CD45 LCA, CD79a, MUM1, OCT-2, PAX-5, and EBER ISH.
Lung Cancer IHC Panel	chromogranin A, synaptophysin, CK7, p63, and TTF-1.
Lung vs. Metastatic Breast Carcinoma IHC Panel	TTF1, mammaglobin, GCDFP-15 (BRST-2), and ER.
Lymphoma Phenotype IHC Panel	BCL-2, BCL-6, CD3, CD4, CD5, CD7, CD8, CD10, CD15, CD20, CD30, CD79a, CD138, cyclin D1, Ki67, MUM1, PAX-5, TdT, and EBER ISH.
Lymphoma vs. Carcinoma IHC Panel	CD30, CD45, CD68, CD117, pan-keratin, MPO, S100, and synaptophysin.
Lymphoma vs. Reactive Hyperplasia IHC Panel	BCL-2, BCL-6, CD3, CD5, CD10, CD20, CD23, CD43, cyclin D1, and Ki-67.
Melanoma vs. Squamous Cell Carcinoma IHC Panel	CD68, Factor XIIIa, CEA (polyclonal), S-100, melanoma cocktail (HMB-45, MART-1/Melan-A, tyrosinase) and Pan-CK.
Mismatch Repair Proteins IHC Panel (MMR/Colon Cancer)	MLH1, MSH2, MSH6, and PMS2.
Neuroendocrine Neoplasm IHC Panel	CD56, synaptophysin, chromogranin A, TTF-1, Pan-CK, and CEA (polyclonal).
Plasma Cell Neoplasm IHC Panel	CD19, CD20, CD38, CD43, CD56, CD79a, CD138, cyclin D1, EMA, kappa, lambda, and MUM1.
Prostate vs. Colon Carcinoma IHC Panel	CDX2, CK 20, CEA (monoclonal), CA19-9, PLAP, CK 7, and PSA.
Soft Tissue Tumor IHC Panel	Pan-CK, SMA, desmin, S100, CD34, vimentin, and CD68.
T-Cell Lymphoma IHC panel	ALK1, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD20, CD21, CD30, CD56, TdT, and EBER ISH.
T-LGL Leukemia IHC panel	CD3, CD8, granzyme B, and TIA-1.

Undifferentiated Tumor IHC Panel	Pan-CK, S100, CD45, and vimentin.
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In some embodiments, the method may involve obtaining an image as described above (an electronic form of which may have been forwarded from a remote location) and may be analyzed by a doctor or other medical professional to determine whether a patient has
5 abnormal cells (e.g., cancerous cells) or which type of abnormal cells are present. The image may be used as a diagnostic to determine whether the subject has a disease or condition, e.g., a cancer. In certain embodiments, the method may be used to determine the stage of a cancer, to identify metastasized cells, or to monitor a patient's response to a treatment, for example.

In any embodiment, data can be forwarded to a "remote location", where "remote
10 location," means a location other than the location at which the image is examined. For example, a remote location could be another location (e.g., office, lab, etc.) in the same city, another location in a different city, another location in a different state, another location in a different country, etc. As such, when one item is indicated as being "remote" from another, what is meant is that the two items can be in the same room but separated, or at least in
15 different rooms or different buildings, and can be at least one mile, ten miles, or at least one hundred miles apart. "Communicating" information references transmitting the data representing that information as electrical signals over a suitable communication channel (e.g., a private or public network). "Forwarding" an item refers to any means of getting that item from one location to the next, whether by physically transporting that item or otherwise (where
20 that is possible) and includes, at least in the case of data, physically transporting a medium carrying the data or communicating the data. Examples of communicating media include radio or infra-red transmission channels as well as a network connection to another computer or networked device, and the internet or including email transmissions and information recorded on websites and the like. In certain embodiments, the image may be analyzed by an MD or
25 other qualified medical professional, and a report based on the results of the analysis of the image may be forwarded to the patient from which the sample was obtained.

In some cases, the method may be employed in a variety of diagnostic, drug discovery, and research applications that include, but are not limited to, diagnosis or monitoring of a disease or condition (where the image identifies a marker for the disease or condition),

discovery of drug targets (where the a marker in the image may be targeted for drug therapy), drug screening (where the effects of a drug are monitored by a marker shown in the image), determining drug susceptibility (where drug susceptibility is associated with a marker) and basic research (where is it desirable to measure the differences between cells in a sample).

5 In certain embodiments, two different samples may be compared using the above methods. The different samples may be composed of an “experimental” sample, i.e., a sample of interest, and a “control” sample to which the experimental sample may be compared. In many embodiments, the different samples are pairs of cell types or fractions thereof, one cell type being a cell type of interest, e.g., an abnormal cell, and the other a control, e.g., normal,
10 cell. If two fractions of cells are compared, the fractions are usually the same fraction from each of the two cells. In certain embodiments, however, two fractions of the same cell may be compared. Exemplary cell type pairs include, for example, cells isolated from a tissue biopsy (e.g., from a tissue having a disease such as colon, breast, prostate, lung, skin cancer, or infected with a pathogen etc.) and normal cells from the same tissue, usually from the same
15 patient; cells grown in tissue culture that are immortal (e.g., cells with a proliferative mutation or an immortalizing transgene), infected with a pathogen, or treated (e.g., with environmental or chemical agents such as peptides, hormones, altered temperature, growth condition, physical stress, cellular transformation, etc.), and a normal cell (e.g., a cell that is otherwise identical to the experimental cell except that it is not immortal, infected, or treated, etc.); a cell
20 isolated from a mammal with a cancer, a disease, a geriatric mammal, or a mammal exposed to a condition, and a cell from a mammal of the same species, preferably from the same family, that is healthy or young; and differentiated cells and non-differentiated cells from the same mammal (e.g., one cell being the progenitor of the other in a mammal, for example). In one embodiment, cells of different types, e.g., neuronal and non-neuronal cells, or cells of different
25 status (e.g., before and after a stimulus on the cells) may be employed. In another embodiment of the invention, the experimental material is cells susceptible to infection by a pathogen such as a virus, e.g., human immunodeficiency virus (HIV), etc., and the control material is cells resistant to infection by the pathogen. In another embodiment, the sample pair is represented by undifferentiated cells, e.g., stem cells, and differentiated cells.

The images produced by the method may be viewed side-by-side or, in some embodiments, the images may be superimposed, combined or staked to produce a 3-d image. In some cases, the images may be in color, where the colors used in the images may correspond to the labels used.

Cells any organism, e.g., from bacteria, yeast, plants and animals, such as fish, birds, reptiles, amphibians and mammals may be used in the subject methods. In certain embodiments, mammalian cells, i.e., cells from mice, rabbits, primates, or humans, or cultured derivatives thereof, may be used.

EXAMPLES

In order to further illustrate the present invention, the following specific examples are given with the understanding that they are being offered to illustrate the present invention and should not be construed in any way as limiting its scope.

EXAMPLE 1

Design and use of ATAC adaptors is compatible with ATAC-seq and ATAC-seq analysis

Methods

Cell culture: GM12878 were grown in RPMI 1640 (Life Technologies), 15% fetal bovine serum (FBS; Omega Scientific) and 2mM L-glutamine (Life Technologies). Jurkat and K562 cells were grown in RPMI 1640, 10% FBS and 2mM L-glutamine. HeLa cells were grown in DMEM (Life Technologies), 10% FBS and passaged with TrypLE Express (Gibco). Cells were maintained in 100 U/mL penicillin (Life Technologies) and 100 mg/mL streptomycin (Life Technologies) and kept at 37°C in 5% CO₂ conditions.

Adaptor synthesis and Electrospray Ionization Mass Spectrometry (ESI-MS): The Fluorine-labeled oligonucleotides for Tn5 transposome adaptor were synthesized at Biosynthesis Inc, and the LC-MS was run on a Thermo linear ion trap. A short 8 minute gradient using HFIP ion pairing buffer for A and methanol for B was used at a flow rate of 0.4 ml/min on a phenomenex clarity 2.1 mm column (00B-4746-AN) at 60°C. Spray voltage was

set at -1.5 kV. LC-MS was run in Negative Ion mode set at full scan. The fluorine-labeled oligonucleotides sequences are as follows (5' to 3'):

- Tn5ME-A-ATTO514-5FdU:

(ATTO514)(5FdU)CG(5FdU)CGGCAGCG(5FdU)CAGA(5FdU)G(5FdU)G(5FdU)A(5FdU)
 5 AAGAGACAG (SEQ ID NO: 1)

- Tn5ME-B-ATTO514-5FdU:

(ATTO514)G(5FdU)C(5FdU)CG(5FdU)GGGC(5FdU)CGGAGA(5FdU)G(5FdU)G(5FdU)A(5FdU)
 5FdU)AAGAGACAG (SEQ ID NO: 2)

- Tn5MERev-5FdU-5FdC:

10 [phos](5FdC)(5FdU)G(5FdU)(5FdC)(5FdU)(5FdC)(5FdU)(5FdU)A(5FdU)A(5FdC)A(5FdC)
 A(5FdU)(5FdC)(5FdU) (SEQ ID NO: 3)

Tn5 transposome assembly: The assembly of Tn5 transposome was performed as described². Briefly, fluorine-labeled oligonucleotides were each resuspended in Nuclease-Free water (Ambion) to 100 μ M. The Tn5MERev-5FdU-5FdC/Tn5ME-A-ATTO514-5FdU and
 15 Tn5MERev-5FdU-5FdC/Tn5ME-B-ATTO514-5FdU duplexes were created by mixing oligonucleotides at molar ratio of 1:1 (final concentration of 50 μ M) in sterile microcentrifuge tubes. The mixtures were heated at 95°C for 5 min and cooled in the thermocycler by turning it off. To assemble the Tn5 transposome, 0.1 vol SL-Tn5 (50 μ M) was mixed with 0.125 vol
 Tn5MERev-5FdU-5FdC/Tn5ME-A-ATTO514-5FdU and 0.125 vol Tn5MERev-5FdU-
 20 5FdC/Tn5ME-B-ATTO514-5FdU in 0.12 vol 2x dialysis buffer (100 mM HEPES–KOH, pH 7.2, 0.2 M NaCl, 0.2 mM EDTA, 2 mM DTT, 0.2% Triton X-100, 20% glycerol), 0.4 vol glycerol (100% solution) and 0.13 vol Nuclease-Free water. After gentle pipetting, the mixture was incubated at room temperature for 1 h protected from light to assemble the complex (Fluoro-Tn5) and finally stored at -20°C.

25 **Confocal microscopy of ATAC-mass:** HeLa cells were grown in 12 mm glass coverslips until 80-90% confluent, fixed with 1% paraformaldehyde for 10 min at room temperature and washed 3 times with PBS. Cells were permeabilized in lysis buffer (10 mM Tris–Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% Igepal CA-630) at room temperature for 10 min. Samples were washed three times with PBS for 5 minutes at room temperature and
 30 incubated for 1 h at 37°C in a humid chamber with transposase mixture solution (20 μ l 2x TD

buffer, final concentration of 100 nM Fluoro-Tn5, adding Nuclease-Free water up to 40 μ l). The reaction was terminated by washing three times for 15 min at 55°C with PBS containing 0.01% SDS and 50 mM EDTA. For confocal microscopy analysis of ATAC-mass, cells were washed three times with PBS, rinsed once with water and mounted using Vectashield with
5 DAPI (cat# H-1200, Vector Laboratories). All samples were imaged using a LSM 880 (Zeiss) with a 63x oil-immersion objective (Zeiss Plan-Apochromat 63x/1.4 Oil).

High Resolution Secondary Ion Mass Spectrometry of ATAC-mass: Silicon wafers (Silicon Valley Microelectronics) were rinsed twice with methanol and cleaned with a cotton-tipped applicator. Substrates were then immersed in acetone for 5 min, placed in Vectabond
10 reagent solution (cat# SP-1800, Vector Laboratories) for 30 min at room temperature, washed once in acetone, air-dried using compressed air and baked at 70°C for 1 h. K562 cells were incubated with 10 μ M 5-Iodo-2'-deoxyuridine (cat# I7125, Sigma-Aldrich) in complete RPMI 1640 media for 24 h to label DNA. 200,000 cells were washed once with PBS, centrifuged on an 18 mm² silicon wafer at 100 g for 5 min, fixed with 1% paraformaldehyde for 10 min at
15 room temperature and washed 3 times with PBS. For high resolution secondary ion mass spectrometry analysis of ATAC-mass, cells were treated as for confocal microscopy analysis, washed three times with PBS, fixed in PBS with 2% glutaraldehyde, rinsed five times with water and stored in a vacuum desiccator for at least 24 h before analysis. Samples were imaged with a NanoSIMS 50L mass spectrometer (Cameca) using a ¹³³Cs⁺ primary ion beam supplied
20 by a microbeam cesium source. The primary optics, secondary optics, mass spectrometer and detector trolleys were tuned before each experiment. Preliminary ¹³³Cs⁺ implantation was done by scanning the region of interest for 1 to 5 minutes without limiting the angular aperture of the primary beam (D1 aperture 0). High spatial resolution negative-ion maps were acquired with a ~2-4 pA ¹³³Cs⁺ primary beam focused to a ~100-200 nm diameter spot. Images over
25 areas of 15 μ m² containing 256 x 256 pixels were acquired with entrance slit 0 and aperture slit 0 and a pixel dwell time of 1 ms/pixel. Ion maps were obtained simultaneously for ¹⁹F⁻ and ¹²⁷I ion species in multicollection mode. Up to 70 sequential scans over a single area were collected for 3D image reconstruction.

Flow cytometry and mass cytometry of ATAC-mass: For flow cytometry analysis of
30 ATTO 514 loaded beads, Dynabeads® M-280 Streptavidin (cat# 11205D, Life Technologies)

were incubated with ATTO 514 biotin (cat# AD 514-71, ATTO-TEC GmbH) in PBS for 30 min at room temperature and washed twice with PBS. The resulting ATTO 514 Dynabeads were stained with either isotype control or anti-Fluorescein/Oregon green antibody (cat# A-889, Life Technologies) for 30 min at room temperature in cell staining media (CSM; PBS containing 0.5% BSA), followed by two washes with CSM, and staining with Alexa 647 conjugated anti-rabbit secondary antibody (cat# 4414S, Cell Signaling Technology). After two washes with CSM, beads were analyzed using a BD LSRII. For mass cytometry analysis of ATAC-mass, Jurkat cells were washed with PBS and incubated with 10 μ M IdU in complete RPMI 1640 media for 10 min at 37°C to label S-phase cells. Viability staining was performed by incubating the cells with 50 μ M cisplatin (cat# P4394, Sigma-Aldrich) in 1 ml of serum-free RPMI 1640 for 1 min at room temperature. An equal volume of complete RPMI 1640 medium was added and cells were centrifuged, followed by fixation with 1% paraformaldehyde in PBS for 10 min at room temperature. ATAC-mass was performed as described above and then staining with the following antibodies: cyclin B1 ^{164}Dy (cat# 3164010A, Fluidigm), p-pRb (S807/811) ^{165}Ho (clone J112-906, BD), p-Histone H3 (S28) ^{143}Nd (clone HTA28, Biolegend), Fluorescein/Oregon green ^{156}Gd (cat# A-889, Life Technologies), and cleaved caspase-3 ^{172}Yb (clone C92-605, BD) for 30 min at room temperature. Cells were washed once with CSM and then incubated at room temperature for 20 min with an iridium-containing DNA intercalator (cat# 201192B, Fluidigm) in PBS containing 1.5% paraformaldehyde. After intercalation/fixation, the cell samples were washed once with CSM and twice with water before measurement on a CyTOFII mass cytometer (Fluidigm). The individual FCS files were analyzed by first gating out doublets, debris, and dead cells based on cell length, DNA content, cisplatin staining and cleaved caspase-3. Data analysis was done in Cytobank.

Sequencing of ATAC-mass: ATAC-seq in GM12878 cells using with Illumina Tn5 transposase (Nextera kit; cat# FC-121-1030, Illumina) and Fluoro-Tn5 was performed as described¹. Briefly, 50,000 cells were centrifuged at 500 g for 5 min at 4°C and washed once in cold PBS. The cell pellet was gently resuspended in 50 μ l pre-chilled lysis buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% Igepal CA-630) and centrifuged immediately at 500 g for 10 min at 4 °C. The cell pellet was resuspended in 50 μ l transposase

mixture (25 µl 2× TD buffer (Nextera kit), 2.5 µl Illumina Tn5 transposase or final concentration of 100 nM Fluoro-Tn5, and Nuclease-Free water up to 40 µl) and incubated at 37 °C 30 min. After transposition, the mixture was purified with Qiagen Min-Elute PCR purification kit (cat# 28004, Qiagen) and eluted in 10 µl Qiagen buffer EB. Sequencing
5 libraries were prepared following the original ATAC-seq protocol¹. The sequencing was performed on Illumina NextSeq at the Stanford Functional Genomics Facility.

ATAC-seq library data preprocessing and analysis: ATAC-seq paired-end reads were trimmed for Illumina adaptor sequences and transposase sequences using an in-house script, and they were mapped to hg19 using Bowtie2 v2.1.0 with parameters – very-sensitive.
10 Over 50 million mapped reads were generated in each sequencing library and used for downstream data mining. Duplicate reads were removed with Picard v1.79. Peak calling was performed by MACS2 narrow peak mode with parameters –q 0.01 –nomodel –shift 0. Overlapping peaks from all samples were merged together to a consensus peak list, and number of unique-mapped and properly paired reads mapped to each peak for each individual
15 samples was quantified to calculate the Pearson correlation. For ATAC-seq signal intensity around TSS, a 2 kb window centered on TSS was divided into 40 equally sized bins of 50 bp. The number of unique-mapped and properly paired ATAC-seq tags overlapping each bin was counted.

Image analysis for ATAC-mass: Raw ion beam images were converted to TIFF files
20 by OpenMIMS ImageJ. Cell nuclei were identified and segmented by Gaussian filtering raw IdU image data for ion beam imaging. A custom MATLAB algorithm was then used to generate single cell maps of ATAC mass and nuclear DNA signals.

Results

To enable chromatin analysis through multiple platforms, modified ATAC DNA-
25 adaptors were synthesized. 5-fluoro-2'-deoxyuridine and 5-fluoro-2'-deoxycytidine nucleotides were incorporated into the adaptors sequence, while ATTO 514 was conjugated at the 5'-end of each of them (Fig. 2, a). Through these modifications, a fluorescent molecule (ATTO 514) and multiple atoms of fluorine per adapter were incorporated (please note that ATTO 514 also contains 6 fluorines *per* fluorophore; Fig. 2, a). The size and purity of these adaptors were
30 validated by electrospray ionization mass spectrometry. Recombinant Tn5 transposase was

purified as previously reported, loaded it with fluorine-labeled adaptors (Fluoro-Tn5; Fig. 2, a) and tested whether Fluoro-Tn5 retained the activity and selectivity for open chromatin sites. ATAC-seq of human B cells (GM 12878) performed using Fluoro-Tn5 showed similar genomic distribution of ATAC-seq peaks and transcriptional start site enrichment when
5 compared to results using Nextera-Tn5 (the commercially available adaptors and enzyme) (Fig. 1, b,c).

As demonstrated by fluorescence microscopy, accessible genome of HeLa cells displays similar pattern as previously reported ², with increasing of fluorescent signals from the peripheral to the interior of nucleus, which is anti-correlated with DAPI staining (Fig. 1, d).
10 As a control, 100 mM EDTA treatment of samples inhibited enzymatic activity thus impairing adaptors integration into genomic DNA. Therefore no apparent fluorescent signals were detected as expected (Fig. 1, d).

Overall, the modified adaptors allow genome accessibility to be analyzed by sequencing and fluorescence-microscopy, demonstrating retention of Tn5 enzymatic activity
15 and specificity.

EXAMPLE 2

Simultaneous high-resolution metabolic DNA labeling and ATAC-mass enables visualization of accessible genome with sub 100nm resolution by secondary ion 20 beam imaging

To enable high resolution nuclear studies by secondary ion beam imaging, cells were cultured with 5-iodo-2'-deoxyuridine (IdU). Bombardment of the biological sample with Cesium (¹³³Cs⁺) beam induces the release of negative secondary ions from the targeted area including I⁻ ions from IdU-labeled DNA (Fig. 3, b). The calculated Z-resolution of ~14 nm for
25 nanoSIMS instruments enabled detailed 3D-image reconstruction of nuclear DNA. As clearly demonstrated in Fig. 3, a, distinct areas of varying levels of DNA compaction were detected, defining subnuclear territories. The validity of I⁻ ions signals from DNA is also corroborated with its high correlation with endogenous phosphorus which revealed the combined signals from DNA and RNA molecules and phosphorylated proteins.

Having exploited the potential of nanoSIMS to resolve the nuclear structure of a cell at high resolution through Cesium ion beam, the accessible genome was examined. K562 cells metabolically labelled with IdU were tagged using Fluoro-Tn5 for ATAC-mass analysis. Serial scanning of the samples did show changes of the distribution of $^{19}\text{F}^-$ ions from labeled open chromatin as well as F^- ions from DNA (Fig. 3, b). The XY resolution of the ATAC-mass signals reached sub-100 nm range when calculated based on 16–84% criterion (Fig 3, c).

3D-reconstruction of ATAC-mass signals overlaying on the DNA signals from IdU led to an unprecedented view of open chromatin structure, with ATAC-mass signal finely interspersed into IdU-high compacted DNA regions. Accessible-DNA "territories", as revealed by secondary ion beam imaging, are assembled in far more complex structures than previously shown by fluorescence-microscopy. For example, some ATAC-mass high regions have clear boundary demarcations from DNA compact regions, while some chromatin open regions are intermingled with compacted DNA (Fig. 3, d). Chromatin analysis, as rendered by secondary ion mass spectrometry, therefore reached high resolution while opening new doors for multi-parameter imaging and metabolic labeling.

EXAMPLE 3

ATAC-mass by mass-cytometry

Leveraging upon recent advancements in mass spectrometry based cytometry, this platform was implemented on a mass cytometer (CyTOF), which thus enables cellular phenotyping, functional studies and, now, simultaneous analysis of chromatin states in millions of single cells. Given the structural resemblance between Fluorescein, Oregon Green and ATTO 514, it was demonstrated by flow cytometry that anti-Fluorescein/Oregon Green antibody can specifically bind to ATTO 514. Using CyTOF, when Jurkat cells were stained with Fluoro-Tn5 followed by metal-conjugated anti-FITC/Oregon Green and markers for cell cycle analysis, cell-cycle-specific genome accessibility profiles were revealed in agreement with results from ATAC-seq (Fig. 4, a). Interestingly, by detecting the phosphorylation level of retinoblastoma protein at serine 807 and 811 together with ATAC-mass signal, it was found that cells carrying low-ATAC-seq signal, as previously reported, reside in G0-phase of the cell cycle-- pinpointing to limited genome accessibility in non-cycling cells (Fig. 4, b).

EXAMPLE 4**ATAC-mass reveals increased chromatin accessibility in interleukin-18 primed NK cells`**

5 Synergistic interaction between cytokines is a common feature that immune cells may react in a non-linear way to challenges from the environment. For example, it is well established that during acute infection, interleukin-12(IL-12) and IL-18 secreted by the first line defenders, such as dendritic cells and macrophages, could synergistically activate natural killer cells(NK cells) and promote the secretion of IFN γ from NK cells. However, it is still not

10 clear how this synergistic effect is achieved and what the role of individual cytokines is. The ATAC-mass was applied together with multiple intracellular signaling pathways staining to interrogate the effect of IL-12 or IL-18 on epigenetic states as well as signaling networks. In the population level, increased ATAC-mass signals were observed in IL-18 primed NK cell line, NKL cells, in comparison to either without priming or IL-12 primed cells as shown in

15 Fig. 4, c indicating that IL-18 affect NKL cells at epigenetic level by opening up genome accessibility. By calculating the Spearmann correlation between individual markers at single cell level, it was noticed that ATAC-mass is highly correlated with NF κ B, c-Jun and CD3zeta-SLP76 pathway (Fig. 4, d). As a control of specificity, phospho-Stat4 which are downstream of IL-12 did increase after IL-12 priming but did not correlated with ATAC-mass signals (Fig.

20 4, c,d). To further validate these findings, ATAC-seq was performed on cells of these three conditions and also saw significant increase of ATAC-peak in IL-18 group but not IL-12 or without priming group (Fig. 4, e). Consistently, the upregulated promoter regions are enriched with NF κ B and AP-1 binding motif, which brings up the possibility that IL-18 promotes the binding of NF κ B and c-Jun to enhancers and subsequently facilitate the recruitment of

25 chromatin remodeling factors to open the promoter region similar to the mechanism of transcription factor synergism in the induction of human interferon- β (IFN β) gene.

Because IL-18 priming opens the genome accessibility, it was hypothesized that IL-18 could be a mediator of more potent effector functions in polyfunctional immune cells, which are commonly observed in the setting of effectively controlled viral infections. Indeed, when

30 NKL are primed with IL-18 first and then stimulated with PMA plus ionomycin, percentage of

polyfunctional NK cells are significantly increased (Fig. 5, a,c). In contrast, IL-12 only enhances the intensity of IFN γ but has no effect on the number of functional effectors (Fig. 5, a,b,c).

Collectively, these experiments demonstrate the feasibility of using ATAC-mass and mass cytometry to dissect cell-population heterogeneity in both signaling and epigenetic states at single cell level.

In study set forth above, it has been demonstrated that modification of ATAC adaptors through the addition of a 3'- ATTO 514 molecule and Fluorine-labeled nucleotides enables interrogation of chromatin states through four different technologies. Validation experiments demonstrate that the Tn5 transposase, when loaded with Fluorin-adaptors, retains its activity and specificity and is compatible with conventional ATAC-seq and ATAC-seq experiments.

In addition, chromatin states can now be interrogated by ATAC-mass through two more technologies.

For secondary ion beam imaging, it has been previously demonstrated how NanoSIMs instruments enable multiplexed imaging through an oxygen beam. In the present case the same instrument (NanoSIMs) is used, but with a different ion beam (i.e. Cesium), which enables high-resolution secondary ion beam imaging. To exploit the potential of Cesium beam an innovative labeling method of ATAC-adaptors with Fluorine is presented. Detection of $^{19}\text{F}^-$ secondary ions empowers imaging of accessible genome with higher resolution than previously reported (Fig. 3). Through co-detection of metabolic DNA labeling, the nuclear ultrastructure can be examined in a multiplexed fashion. Through the future development of additional molecular labeling toolkit, e.g., antibody labeling, this will also enable 3D multiparameter high resolution imaging. The multiplexing potential of NanoSIMs instruments is limited by the number of detectors installed in each specific machine. However, given the very high Z-resolution, higher order multiplexing can be achieved by rendering a different set of masses at each scan, thus de facto bringing the theoretical multiplexing capability up to the number of isotopes potentially detectable after bombardment with each specific

Finally, the technology is "portable" to more traditional mass cytometry approaches, allowing simultaneous detection of chromatin states with phenotypic and functional analysis in millions of single cells. As previously reported, single-cell analysis of intra-cellular molecules

is relevant to the understanding of the functional state of complex and diseased cellular populations. Here it has been demonstrated that layering on top of intracellular molecule analysis information of their chromatin state enables further characterization of cellular populations otherwise not detectable. The fact that IL-18 opens up genome accessibility in NK cells could shed light on different approach to develop new therapeutics to several IL-18 related diseases, which could not be easily achieved by targeting IL-18 directly due to the complexed interaction partners of IL-18.

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EXAMPLE 5

Methods

Cell culture

GM12878 cells were grown in RPMI 1640 (Life Technologies), 15% fetal bovine serum (FBS; Omega Scientific), and 2 mM L-glutamine (Life Technologies). Jurkat and K562 cells were grown in RPMI 1640, 10% FBS, and 2 mM L-glutamine. NKL cells were grown in RPMI 1640, 10% FBS, 2 mM L-glutamine, and 200 U/mL rhIL-2 (National Cancer Institute). HeLa cells were grown in DMEM (Life Technologies), 10% FBS and passaged with TrypLE Express (Gibco). Cells were maintained in 100 U/mL penicillin (Life Technologies) and 100 mg/mL streptomycin (Life Technologies) at 37 °C in 5% CO₂ conditions. For cytokine

treatment of NKL cells, cells were incubated with 200 U/mL rhIL-2 plus 20 ng/ml human IL-18 (R&D Systems) or 10 ng/ml human IL-12 (R&D Systems) for 24 h. For intracellular cytokine staining, NKL cells were stimulated with 1X PMA plus ionomycin (eBioscience) in the presence of 1X brefeldin A plus monensin (eBioscience) and 1:250 anti-CD107a ¹⁵¹Eu (Fluidigm) antibody for 4 h.

Adaptor synthesis and electrospray ionization mass spectrometry

The fluorine-labeled oligonucleotides for the Tn5 transposase adaptor were synthesized at Biosynthesis Inc., and a Thermo linear ion trap was used for LC-MS analyses of adaptors. A short 8-min gradient using HFIP ion pairing buffer for A and methanol for B was used at a flow rate of 0.4 ml/min on a Phenomenex clarity 2.1-mm column (00B-4746-AN) run at 60 °C. Spray voltage was set at -1.5 kV. LC-MS was run in negative ion mode set at full scan. The fluorine-labeled oligonucleotide sequences are as follows:

- Tn5ME-A-ATTO514-5FdU:

5'-(ATTO

514)(5FdU)CG(5FdU)CGGCAGCG(5FdU)CAGA(5FdU)G(5FdU)G(5FdU)A(5FdU)AAGAGACAG-3' (SEQ ID NO: 1)

- Tn5ME-B-ATTO514-5FdU:

5'-(ATTO

514)G(5FdU)C(5FdU)CG(5FdU)GGGC(5FdU)CGGAGA(5FdU)G(5FdU)G(5FdU)A(5FdU)AAGAGACAG-3' (SEQ ID NO: 2)

- Tn5MERev-5FdU-5FdC:

5'-

[phos](5FdC)(5FdU)G(5FdU)(5FdC)(5FdU)(5FdC)(5FdU)(5FdU)A(5FdU)A(5FdC)A(5FdC)A(5FdU)(5FdC)(5FdU)-3' (SEQ ID NO: 3)

Tn5 transposome assembly

The assembly of Tn5 transposome was performed as described². Briefly, fluorine-labeled oligonucleotides were each resuspended in nuclease-free water (Ambion) to 100 μM. The Tn5MERev-5FdU-5FdC/Tn5ME-A-ATTO514-5FdU and Tn5MERev-5FdU-5FdC/Tn5ME-B-ATTO514-5FdU duplexes were created by mixing oligonucleotides at molar ratio of 1:1 (final concentration of 50 μM) in sterile microcentrifuge tubes. The mixtures were

heated at 95 °C for 5 min and cooled in the thermocycler by turning it off. To assemble the Tn5 transposome, 0.1 volume SL-Tn5 (50 µM) was mixed with 0.125 volume Tn5MERev-5FdU-5FdC/Tn5ME-A-ATTO514-5FdU and 0.125 volume Tn5MERev-5FdU-5FdC/Tn5ME-B-ATTO514-5FdU in 0.12 volume 2x dialysis buffer (100 mM HEPES-KOH, pH 7.2, 0.2 M NaCl, 0.2 mM EDTA, 2 mM DTT, 0.2% Triton X-100, 20% glycerol), 0.4 volume glycerol (100% solution), and 0.13 volume nuclease-free water. After gentle pipetting, the mixture was incubated at room temperature for 1 h protected from light to assemble the complex and then was stored at -20 °C.

Tagmentation

Cells were grown in 12-mm glass coverslips until 80-90% confluent, fixed with 1% paraformaldehyde for 10 min at room temperature, and washed three times with PBS. Cells were permeabilized in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% Igepal CA-630) at room temperature for 10 min. Samples were washed three times with PBS for 5 min each wash at room temperature and incubated for 1 h at 37 °C in a humidified chamber with transposase mixture solution (20 µl 2x TD buffer, final concentration of 100 nM Fluoro-Tn5, and nuclease-free water to a final volume of 40 µl). The reaction was terminated by washing three times at 55 °C with PBS containing 0.01% SDS and 50 mM EDTA for 15 min each wash.

Confocal microscopy

For confocal microscopy analysis of ATAC-mass, cells were washed three times with PBS, rinsed once with water, and mounted using Vectashield with DAPI (Vector Laboratories). All samples were imaged using a LSM 880 (Zeiss) with a 63x oil-immersion objective (Zeiss Plan-Apochromat 63x/1.4 Oil).

High-resolution secondary ion mass spectrometry

Silicon wafers (Silicon Valley Microelectronics) were rinsed twice with methanol and cleaned with a cotton-tipped applicator. Substrates were then immersed in acetone for 5 min, placed in Vectabond reagent solution (Vector Laboratories) for 30 min at room temperature, washed once in acetone, air-dried using compressed air, and baked at 70 °C for 1 h. K562 cells were incubated with 10 µM 5-iodo-2'-deoxyuridine (Sigma-Aldrich) in complete RPMI 1640 for 24 h to label DNA. Approximately 200,000 cells were washed once with PBS, centrifuged

on an 18 x 18 mm silicon wafer at 100 g for 5 min, fixed with 1% paraformaldehyde for 10 min at room temperature, and washed three times with PBS. For high-resolution secondary ion mass spectrometry analysis of ATAC-mass, cells were treated as for confocal microscopy analysis, washed three times with PBS, fixed in PBS with 2% glutaraldehyde, rinsed five times
5 with water, and stored in a vacuum desiccator for at least 24 h before analysis. Samples were imaged with a NanoSIMS 50L mass spectrometer (Cameca) using a cesium primary ion beam supplied by a microbeam cesium source. The primary optics, secondary optics, mass spectrometer, and detector trolleys were tuned before each experiment. Preliminary cesium implantation was done by scanning the region of interest for 1 to 5 min without limiting the
10 angular aperture of the primary beam (D1 aperture 0). High spatial resolution negative-ion maps were acquired with a 2-4 pA cesium primary beam focused to a 100-200 nm diameter spot. Images over areas of 15 x 15 μm containing 256 x 256 pixels were acquired with entrance slit 0 and aperture slit 0 and a pixel dwell time of 1 ms/pixel. Ion maps were obtained simultaneously for ^{19}F and ^{127}I ion species in multicollection mode. Up to 70 sequential scans
15 over a single area were collected for 3D image reconstruction.

Flow cytometry and mass cytometry

For flow cytometry analysis, Dynabeads M-280 Streptavidin (Life Technologies) were incubated with ATTO 514-biotin (ATTO-TEC) in PBS for 30 min at room temperature and washed twice with PBS. The resulting ATTO 514 Dynabeads were stained with either isotype
20 control or anti-fluorescein/Oregon green antibody (cat# A-889, Life Technologies) for 30 min at room temperature in cell staining media (CSM; PBS containing 0.5% BSA), followed by two washes with CSM, and staining with Alexa 647-conjugated anti-rabbit secondary antibody (cat# 4414S, Cell Signaling Technology). After two washes with CSM, beads were analyzed using a BD LSRII. For mass cytometry analysis of ATAC-mass together with signaling
25 molecules, Jurkat or NKL cells were washed with PBS and incubated with 10 μM IdU in complete RPMI 1640 for 10 min at 37 °C to label S-phase cells. Viability staining was performed by incubating the cells with 50 μM cisplatin (Sigma-Aldrich) in 1 ml of serum-free RPMI 1640 for 1 min at room temperature. An equal volume of complete RPMI 1640 was added, and cells were centrifuged, followed by fixation with 1% paraformaldehyde in PBS for
30 10 min at room temperature.

Fluoro-Tn5 tagmentation was performed as described above, and then cells were permeabilized with ice-cold methanol for 10 min on ice and washed twice with CSM. Jurkat cells were stained with the following antibodies: cyclin B1 ¹⁶⁴Dy (cat# 3164010A, Fluidigm, 1:50), p-Rb (S807/811) ¹⁶⁵Ho (clone J112-906, BD Biosciences, 2 µg/mL), p-Histone H3 (S28) ¹⁴³Nd (clone HTA28, Biolegend, 1 µg/mL), Fluorescein/Oregon green ¹⁵⁶Gd (cat# A-889, Life Technologies, 2 µg/mL), and cleaved caspase-3 ¹⁷²Yb (clone C92-605, BD Biosciences, 0.1 µg/mL) for 30 min at room temperature. The antibodies used for experiments with NKL cells are listed in Tables 1 and 2. After staining, cells were washed once with CSM and then incubated at room temperature for 20 min with an iridium-containing DNA intercalator (cat# 201192B, Fluidigm, 1:4000) in PBS containing 1.5% paraformaldehyde. After intercalation/fixation, the cell samples were washed once with CSM and twice with water before measurement on a CyTOFII mass cytometer (Fluidigm). The individual FCS files were analyzed by first gating out doublets, debris, and dead cells based on cell length, DNA content, and cisplatin and cleaved caspase-3 staining. Data analysis was done in Cytobank (www.cytobank.org). For intracellular cytokine staining, cells were fixed with 1.5% paraformaldehyde in PBS and permeabilized using the Cytofix/Cytoperm kit (eBioscience) according to the manufacturer's protocol and subsequently stained with the indicated antibodies (Table 2).

Table 1. Antibodies used for mass cytometry analysis of signaling in NKL cells. The lanthanide, mass, target, clone, manufacturer and staining concentration are listed.

Lanthanide	Mass	Protein	Clone	Manufacturer	Conc. (µg/mL)
Nd	142	Cleaved caspase3	D3E9	Fluidigm	1 µl/ 100 µl
Nd	146	pStat3(S727)	49/p-Stat3	BD Biosciences	1
Nd	148	pStat4(Y693)	38/p-Stat4	BD Biosciences	2
Nd	150	pStat5(Y694)	47/p-Stat5	BD Biosciences	2
Sm	152	pAkt(S473)	D9E	Fluidigm	2 µl/ 100 µl
Eu	153	pStat1(Y701)	4a	BD Biosciences	2
Sm	154	p c-Jun(S73)	D47G9	Cell Signaling	4

Gd	155	pCD3zeta(Y142)	K25-407.69	BD Biosciences	2
Gd	156	Fluorescein/Oregon Green	Polyclonal	ThermoFisher	2
Gd	158	pTBK1(S172)	D52C2	Cell Signaling	2
Tb	159	pStat3(Y705)	4/p-Stat3	BD Biosciences	2
Dy	163	pAkt(T308)	J1-223.371	BD Biosciences	2
Dy	164	cyclinB	GNS-1	Fluidigm	2 µl/ 100 µl
Er	166	pRb(S807/S811)	J112-906	BD Biosciences	2
Er	167	pErk(T202/Y204)	D13.14.4E	Fluidigm	2 µl/ 100 µl
Tm	169	pNFkB(S536)	93H1	Cell Signaling	2
Yb	172	pS6(S235/S236)	N7-548	Fluidigm	1 µl/ 100 µl
Yb	173	cPARP	F21-852	BD Biosciences	0.5
Yb	174	pSlp76(Y128)	J141-668.36.58	BD Biosciences	2
Yb	176	pCreb(S133)	87G3	Cell Signaling	1

Table 2. Antibodies used for mass cytometry analysis of cytokines in NKL cells. The lanthanide, mass, target, clone, manufacturer and staining concentration used for NKL mass cytometry analysis are listed.

Lanthanide	Mass	Protein	Clone	Manufacturer	Conc. (µg/mL)
Y	89	CD45	HI30	Fluidigm	0.5 µl/ 100 µl
Nd	145	IFNγ	B27	Biologend	2
Nd	150	MIP-1b	D21-1351	Fluidigm	2 µl/ 100 µl
Eu	151	CD107a	H4A3	Fluidigm	0.4 µl/ 100 µl
Sm	152	TNFα	Mab11	Fluidigm	1 µl/ 100 µl
Eu	153	EBI-3	B032F6	Biologend	2
Dy	162	IL-8	G265-8	BD Biosciences	2
Dy	164	GM-CSF	BVD2-21C11	Biologend	4
Er	170	IL-32	KU32-52	Biologend	2

5

Sequencing of ATAC-mass

ATAC-seq in GM12878 and NKL cells was performed using Illumina Tn5 transposase (Nextera kit; cat# FC-121-1030, Illumina), and ATAC-seq was performed in GM12878 cells with Fluoro-Tn5 as described previously¹. Briefly, 50,000 cells were centrifuged at 500 g for 5

min at 4 °C and washed once in cold PBS. The cell pellet was gently resuspended in 50 µl pre-chilled lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% Igepal CA-630) and centrifuged immediately at 500 g for 10 min at 4 °C. The cell pellet was resuspended in 50 µl transposase mixture (25 µl 2× TD buffer (Nextera kit), 2.5 µl Illumina Tn5

5 transposase or final concentration of 100 nM Fluoro-Tn5, and nuclease-free water to a final volume of 40 µl), and incubated at 37 °C for 30 min. After purification with Qiagen Min-Elute PCR purification kit, DNA was eluted in 10 µl Qiagen buffer EB. Sequencing libraries were prepared following the original ATAC-seq protocol with slight modifications¹. A PCR library amplification step was performed with a final forward and reverse primer concentration of

10 0.625 µM. An AMPure XP (Beckman Coulter) purification step (1.8X volume) was performed in place of a PCR purification step after the PCR library amplification. Sequencing libraries were assessed using the 2100 Bioanalyzer (Agilent) for nucleosome profiles, and the library concentration was estimated by analysis of library fragments between 50 and 450 base pairs (bps). The sequencing was performed on Illumina NextSeq or HiSeq at the Stanford

15 Functional Genomics Facility.

ATAC-seq library data preprocessing and analysis

ATAC-seq paired-end reads were trimmed for Illumina adaptor sequences and transposase sequences using an in-house script and were mapped to hg19 using Bowtie2 v2.1.0 with the parameter `-very-sensitive`. Over 50 million mapped reads were generated in each

20 sequencing library and used for downstream data mining. Duplicate reads were removed with Picard v1.79. Peak calling was performed using MACS2 in narrow peak mode with parameters `-q 0.01, -nomodel, -shift 0`. Overlapping peaks from all samples were merged into a consensus peak list, and the number of uniquely mapped and properly paired reads mapped to each peak for each individual sample was quantified to calculate the Pearson correlation. For

25 ATAC-seq signal intensity around TSSs, a 2-kbp window centered on a TSS was divided into 40 equally sized bins of 50 bp. The number of uniquely mapped and properly paired ATAC-seq tags overlapping each bin was counted. The replicate samples were merged, and differential peak analysis was performed with DESeq2⁶⁵. The peaks of significantly different intensity between different conditions were selected based on $(\log_2(\text{fold change}) > 1 \text{ or } < -1$

and BH-adjusted p-value < 0.01 ⁶⁵. The motif enrichment analysis was performed with Homer using the whole genome as background⁶⁶.

Image analysis for ATAC-mass

Raw ion beam images were converted to TIFF files by OpenMIMS ImageJ, and the
5 signal to noise was computationally enhanced in each channel. Briefly, multiple depth ion
images (e.g., 3-5 slices) were combined to generate ion images with lower noise, followed by a
digital sharpening of the images using a custom deconvolution method⁴⁷. These improved ion
images were then imported to the 3D rendering software (Imaris, Bitplane). The surfaces
function in the rendering module was used to define ATAC-mass signal and DNA signal in the
10 3D visualization images (Figure 13 a) and 3D videos. Line-scan analyses of 2D images were
performed to plot pixel intensities for each isotope along straight lines crossing features of
interest.

Results for Example 5

15 Isotope-modified ATAC adaptors are compatible with multiple technology platforms

SIMS is a powerful tool for studying biological samples due to its high spatial
resolution and ability to detect multiple ion species simultaneously³³⁻³⁵. The primary cesium
ion beam in the NanoSIMS instrument currently achieves one of the highest lateral resolutions
in the SIMS arena^{31, 36, 37}. NanoSIMS operates by rastering the finely focused primary ion
20 beam over the sample surface and detecting the secondary ions released using a magnetic
sector mass spectrometer. The elemental information of the secondary ions is registered to the
position of the primary ion beam, and the ion map image is reconstructed pixel by pixel. Serial
plane acquisitions result in a stack of composite images that can be used to reconstruct cellular
features of interest.

25 In ATAC-seq, transposon insertion tags are used to label DNA. For analysis by ion
beam imaging, ATAC DNA adaptors were modified to contain 5-fluoro-2'-deoxyuridine and
5-fluoro-2'-deoxycytidine nucleotides and a 5' ATTO 514 (Figure 10 a and Figure 19 a). This
design facilitates five applications (Figure 10 b): ATAC-seq analysis, high-resolution ion
beam imaging (enabled by the fluorine atoms), fluorescence microscopy and flow cytometry

(by detection of the ATTO 514 tag), and mass cytometry (using a metal-conjugated antibody to the ATTO 514 tag).

The molecular weights and purities of the adaptors were validated by electrospray ionization mass spectrometry (Figure 19 b). Recombinant Tn5 transposase was purified as previously reported (Figure 20) and it was loaded with fluorine-labeled adaptor (Figure 10 a). To test whether the enzyme loaded with the fluorine-modified adaptor (Fluoro-Tn5) retained activity and selectivity, ATAC-seq of human B cells (GM 12878) was performed. The genomic distribution of Fluoro-Tn5 peaks correlated with results obtained using Nextera-Tn5, the commercially available adaptor and enzyme (Figure 10 c, Pearson correlation $R=0.81$). Transcriptional start site (TSS) enrichment (Figure 10 d) and ATAC-seq profiles (Figure 10 e) were similar, although there were minor differences in peak height in certain nuclear domains. When visualized by fluorescence microscopy, the Fluoro-Tn5-accessible regions of the HeLa cell genome were anti-correlated with DAPI staining (Figure 10 f, top) as expected based on previous reports⁷. Treatment with 100 mM EDTA treatment, which should inhibit transposase activity and thereby impair adaptor integration into genomic DNA, resulted in no fluorescent signal (Figure 10 f, bottom). Collectively, these experiments demonstrated that modified adaptors did not alter Tn5 enzymatic activity or specificity for chromatin domains in the nuclei of cells.

ATAC-mass enables multi-parametric single-cell mass cytometric accessible genome analysis

The CyTOF mass cytometer is a multi-parameter tool that exploits isotopic masses as tags. The inductively coupled plasma mass spectrometer deployed in the CyTOF is not able to evaluate masses below 75 atomic mass units due to a mass cutoff that filters out abundant low mass ions; thus, it is unable to directly detect the fluorine atoms in the ATAC-mass adaptors. To detect the ATAC adaptor by mass cytometry, the structural resemblance between fluorescein, Oregon green, and ATTO 514 was capitalized on (Figure 21 a) and an anti-fluorescein/Oregon green antibody, which specifically binds to ATTO 514 (Figure 21 b), was used to detect the adaptor by mass cytometry.

Jurkat cells were tagmented with Fluoro-Tn5, stained with metal-conjugated anti-ATTO 514 antibody and with metal-tagged antibodies against cell-cycle markers, and

analyzed by mass cytometry (Figure 22). In agreement with results from flow cytometry⁷, high ATAC-mass signal was observed in cycling cells (i.e., G1, S, and G2 cells; Figure 10 g), whereas non-cycling cells (G0) had low ATAC-mass signals (Figure 10 g and h). These data show that ATAC-mass can integrate genome accessibility quantification with analysis of
 5 protein. As demonstrated previously, mass cytometry enables analysis of protein²⁴⁻²⁹ and RNA expression³⁰, so ATAC-mass will allow single-cell detection of genome accessibility simultaneously with multi-parametric protein and transcript analyses.

ATAC-mass reveals increased genome accessibility in NK cells treated with interleukin-18

ATAC-mass was used to study the regulation of chromatin states in natural killer (NK)
 10 cells treated with the interleukins IL-12 and IL-18. IL-12 and IL-18 function cooperatively to enhance NK cell activity both *in vitro* and *in vivo*^{38, 39}. To explore the possibility that an epigenetic mechanism drives the effects of IL-12 and IL-18 on signaling states, NK cells from the NKL line were treated or not with IL-12 or IL-18, stained with a panel of previously validated metal-conjugated antibodies against key signaling components^{40, 41}, and subjected to
 15 ATAC-mass. As compared to untreated cells, IL-18-treated NKL cells showed increased genome accessibility (i.e., higher ATAC-mass signal) and increased levels of phosphorylation of TBK1, p65/RELA (NFkB), CD3zeta, SLP76, Stat3, Stat4, Stat5, c-Jun, and ribosomal protein S6 (Figure 11 a). In contrast, IL-12 treatment of NKL cells did not cause an increase in ATAC-mass signal as compared to untreated cells, although it did induce the expected patterns
 20 of signaling with phosphorylation of Stat3, Stat4, and S6 (Figure 11 a)^{42, 43}. A t-SNE visualization revealed that a subpopulation of cells with high ATAC-mass intensity was induced by treatment with IL-18 but not with IL-12 (Figure 11 b and c).

To better understand which pathways coordinate NK activation and chromatin remodeling upon IL-18 treatment, Spearman correlations were calculated between individual
 25 markers from mass cytometry in single cells (Figure 11 d). The ATAC-mass signal was correlated with activation of c-Jun and the axis TBK1-NFkB and CD3zeta-SLP76 (Figure 11 d and Figure 23 a-c) but not with activation of Stat proteins (Figure 2d and Figure 23 d). The induction of Stat4 by both cytokines is in agreement with previous reports⁴⁴. Taken together, these results indicate that treatment with IL-18, but not IL-12, increases total chromatin

accessibility in NK cells and that the increase in chromatin accessibility is associated with activation of the transcription factors NFkB and c-Jun.

Interleukin-18 treatment increases accessibility of quiescent genomic regions in NK cells

To further understand the effect of IL-18 on genome accessibility, ATAC-seq was performed on control, IL-12-, and IL-18-treated NKL cells. ATAC-seq experiments showed that the genome accessibility profile of IL-18 treated cells is distinct from those of control or IL-12-treated cells (Figure 24). *k*-means clustering ($k = 4$) revealed 572 regions with significantly higher genome accessibility in IL-18-treated cells (Figure 12 a, clusters 3 and 4) and 160 regions reflected increased accessibility after IL-12 treatment (Figure 12 a, cluster 2) compared to control cells. Another 89 regions showed minimal variability in accessibility (Figure 12 a, cluster 1). These results confirm the increase in genome accessibility in IL-18-treated NK cells detected by mass cytometry.

Among ATAC-seq peaks upregulated in IL-18-treated cells compared to control cells, it was identified that significantly enriched motifs corresponded to NFkB and AP-1 binding sites (Figure 12 a, right, clusters 3 and 4). In the chromHMM database⁴⁵, large fractions of the IL-18-upregulated ATAC-seq peaks were annotated as quiescent genomic regions in unstimulated primary NK cells (60% as quiescent regions for cluster 3 and 47% for cluster 4, Figure 12 b). The IL-18-upregulated ATAC-seq peaks were far from TSSs (Figure 12 c, clusters 3 and 4). In contrast, most ATAC-seq peaks upregulated by IL-12 were annotated as active TSS proximal promoter regions (42% flanking active TSS and 24% TSS, Figure 12 b) and were mainly located near TSSs (Figure 12 c, cluster 2). These results suggest that IL-12 and IL-18 target distinct genomic regions: IL-18 activates quiescent enhancers mainly via the transcription factors NF-kB and AP-1, and IL-12 activates promotor and gene-centric regions.

ATAC-mass enables high-resolution visualization of the accessible genomic regions by ion beam imaging

Architectural changes in DNA occur in NK cells in response IL-18 and IL-12. Fluorescence-based approaches such as super-resolution microscopy could be used to investigate DNA architecture, but these methods are time consuming and only a few parameters can be evaluated simultaneously. Using the NanoSIMS instrument, cellular features can be visualized at a resolution below the diffraction limit of light^{46, 47}. To

demonstrate that NanoSIMS can detect genomic structures at high resolution, HeLa cells were cultured for 24 hours in presence of 5-iodo-2'-deoxyuridine (IdU) to label newly synthesized DNA and used the cesium beam to induce the release of negative secondary ions from targeted areas of the nucleus. As expected, ^{127}I , from IdU-labeled DNA, colocalized with phosphorus ions (^{31}P), which mainly originate from DNA in the nucleus (Figure 25 a-b). Regions with varying ^{127}I densities corresponding to define nuclear sub-territories with various levels of DNA compaction were detected (Figure 25 a, closed arrows).

Next, K562 cells metabolically labeled with IdU were tagmented using Fluoro-Tn5 to visualize the spatial organization of the accessible genome in relation to DNA compactness. Serial scanning at different depth planes showed non-uniform distribution of fluorine ions (^{19}F) (Figure 26 a). The lateral resolution of the ATAC-mass signals was at about 100 nm, calculated using the 16-84% criterion³⁶ (Figure 26 b), enabling higher resolution visualization of the accessible genome than previously reported methods⁷. A 3D reconstruction of ATAC-mass signals overlaid with that of the ^{127}I signal (Figure 13 a) revealed that some foci with high ATAC-mass signals are clearly separated from compact DNA regions (Figure 13 b, closed arrows). At this level of resolving power, it was also detected that some regions of open chromatin are intermingled with compact DNA (Figure 13 b, open arrows). As shown in a line-scan analysis, ATAC and IdU labeling were often anti-correlated, although in a minority of regions signal overlap was detected (Figure 26 c). This is indicative of the previously reported complexity of chromosome organization^{48, 49}. These visual proof-of-concept experiments demonstrate that ATAC-mass reveals spatial features of the accessible genome at about 100-nm resolution.

High-resolution spatial ATAC-mass analysis reveals that interleukin-18 treatment increases chromatin accessibility of the uncompact DNA in NK cells

Having established the effects of IL-18 priming at the cell-population level by mass cytometry and at the genomic level by ATAC-seq, the spatial localization and structure of open chromatin in Fluoro-Tn5 tagmented control and IL-12- and IL-18-treated NKL cells were investigated by traditional fluorescence microscopy and high-resolution ion beam imaging. Cells counterstained with DAPI were first analyzed by fluorescence microscopy. Total nuclear fluorescence intensity from ATTO 514 of Fluoro-Tn5 in control and treated cells was

quantified by delineating the boundaries of individual nuclei using the DAPI signal. Consistent with the results from mass cytometry and ATAC-seq, a higher intensity ATTO 514 signal was observed in nuclei of IL-18-treated cells than in control or IL-12-treated cells (Figure 27 a and b).

High-resolution ion beam imaging was then used to determine the locations of ^{19}F labeling in the NKL cells. As observed in the K562 cell (Figure 13 a and b), some foci in NKL cells with high ATAC-mass high signals have clear boundary demarcations from compact DNA regions (Figure 27 c), whereas other regions with high ATAC-mass signals overlap with compact DNA (Figure 27 c). To analyze the chromatin structure reorganization upon IL-12 and IL-18 treatment, four sub-nuclear territories were defined based on their genome accessibility (low and high) and DNA compaction levels (high and low) and their abundances in each condition were quantified (Figure 13 c). Control and IL-18-treated NKL cells had similar proportions of the analyzed nuclear territories, whereas IL-12-treated cells had significantly more areas with ATAC-mass low regions associated with uncompact DNA as compared to control and IL-18 cells (77.6%, 54.2%, and 59.2%, respectively, Figure 13 c). The average ATAC-mass intensity in regions of low levels of DNA compaction and high genome accessibility was significantly higher in both IL-12- and IL-18-treated cells than in control cells (6.4 and 6.35 counts per pixel in IL-12- and IL-18-treated cells, respectively, vs. 4.41 counts per pixel in control cells, Figure 13 d). No significant differences were observed in the other sub-nuclear territories when untreated and treated cells were compared. These results suggest that the increase in ATAC-mass signal induced by IL-18 results from an increase of chromatin accessibility in non-compacted DNA. IL-12 treatment did not increase the total genome accessibility; however, a spatial nuclear reorganization was induced by IL-12 treatment as demonstrated by the increased proportions of regions showing low ATAC-mass associated with uncompact DNA compared to control cells.

Interleukin-18 induces NK polyfunctionality

Having identified an apparent epigenetic difference between IL-12-treated and IL-18-treated NK cells, the next step was to determine the functional effects of the observed nuclear remodeling. The frequency of polyfunctional immune cells, defined as cells that produce multiple cytokines, is known to be regulated at the epigenetic level⁵⁰⁻⁵². Therefore, it was

asked whether the observed effects of IL-18 on the genome accessibility of NK cells was a result of an increase of cytokine production. When NKL cells were treated with IL-18 and then stimulated with PMA and ionomycin, the frequency of polyfunctional NK cells significantly increased relative to cells treated only with PMA and ionomycin: 72.7% of IL-18-treated cells produced more than five cytokines per cell but only 43.8% of control cells did (Figure 13 e). There were also higher levels of EBI3, IL-32, IFN γ , MIP1 β , IL-8, and TNF α in the IL-18-treated cells than in control cells (Figure 13 f and g). In contrast, IL-12 treatment did not increase the frequency of polyfunctional cells (Figure 13 e), although it resulted in increased IFN γ levels (Figure 13 f and g). Collectively, these experiments demonstrate that IL-18, but not IL-12, induces polyfunctionality. Thus, chromatin changes are correlated with NK cell function.

Discussion for Example 5

The present study demonstrated that modification of ATAC adaptors through the conjugation of ATTO 514 and incorporation of fluorine-labeled nucleotides enables interrogation of genome accessibility by mass cytometry and high-resolution ion beam imaging, while retaining compatibility with sequencing- and fluorescence-based ATAC platforms. Coupling ATAC-mass with antibody labeling and a mass cytometry readout enabled analysis of genome accessibility in conjunction with single-cell resolution analysis of cell populations (i.e., identified based on cell-cycle stage or signaling status). For ATAC-mass the high-resolution capability of a cesium beam was relied on. Detection of $^{19}\text{F}^-$ in the ATAC DNA adaptor allowed imaging of accessible genome at approximately 100-nm resolution. These methods coupled with ATAC-mass should enable 3D imaging of the accessible genome in distinct nuclear territories at the nanoscale. Although the multiplexing potential of high-resolution ion beam imaging based on NanoSIMS is currently limited by the number of detectors, given the axial resolution, high-order multiplexing can be achieved by rendering different sets of masses in each scan²².

Due to the absence of auto-fluorescence in tissues analyzed by MIBI²², adaptation of ATAC-mass for *in situ* visualization of genome accessibility in tissues should facilitate application of single-cell epigenomics and proteomics to clinically relevant specimens. Stable isotopes such as ^{13}C and ^{15}N can be used as labels for metabolite visualization because of the

excellent mass resolution of high-resolution ion beam imaging^{31, 32, 53, 54}. Chromatin remodeling is a highly energy-consuming process⁵⁵, thus, it will be of interest to visualize genome accessibility in the presence of different metabolic labels to identify the metabolic determinants of chromatin remodeling at the nanoscale. In addition, ATAC-mass coupled with DNA-FISH will enable high-throughput quantification of accessibility at the single-locus level, which will be especially relevant for drug screens targeting chromatin remodeling using barcoding techniques already developed for mass cytometry⁵⁶.

ATAC-mass provides the opportunity to integrate epigenomics, proteomics, and high-resolution imaging at the single-cell level. In this study, the effect of IL-12 and IL-18 on NK cells was focused on. Synergistic interactions between cytokines allow immune cells to respond in a non-linear way to challenges from the environment⁵⁷. During acute infection, IL-12 and IL-18, secreted by first-line defenders such as dendritic cells and macrophages, synergistically activate and promote the secretion of IFN γ from NK cells⁵⁸⁻⁶⁰. Using ATAC-mass to study IL-12- or IL-18-treated NKL cells, it was found that IL-18 increased total chromatin accessibility and that the increase in chromatin accessibility correlated with an increase of accessible (non-compact) DNA subnuclear territories but not the compacted regions. ATAC-seq results confirmed this and showed increased accessibility at regions that are quiescent, with low accessibility and high DNA methylation under unstimulated conditions⁴⁵. In contrast, IL-12 treatment also increased the ATAC-mass signals in uncompact DNA but with a preference for promoter regions, which are highly accessible even in resting NK cells.

These findings suggest that IL-18 may function as the initiator of stepwise transcription activation, recruiting chromatin remodeling factors to the quiescent genomic regions, with subsequent synergistic interaction with promoter regions targeted by IL-12. Interestingly, this mechanism of transcription factor synergism might be an efficient regulatory principle for innate immune system as similarly ordered epigenetic regulation has been reported for human *interferon- β* expression in response to viral infection^{61, 62}.

In summary, ATAC-mass revealed that IL-18 is an epigenetic modulator and, in combination with other ATAC methods, pinpointed mechanisms of gene regulation that could be exploited for vaccine or infection-control therapy development. Elevated IL-18 levels have

been detected in the serum of patients suffering from several autoimmune disorders^{63, 64}. It will be useful to investigate the epigenetic states of different immune subsets from such patients by ATAC-mass, especially in patients with diseases associated with NK cell dysfunction.

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EXAMPLE 6

Here, the development and application of super-resolution MIBI using a positively charged cesium primary ion beam that allows visualization of subcellular structures with

5 lateral (XY) and axial (Z) resolutions down to approximately 30 nm and 5 nm, respectively, is reported. The development of a toolkit for specific protein imaging with this approach is highlighted. This method, which is called super-resolution ion beam imaging (srIBI), is capable of determining the precise subcellular locations of multiple small molecules, proteins, and/or nucleic acids at single-molecule resolution. The capabilities of srIBI are demonstrated

10 by simultaneously imaging five distinct subnuclear structures and the chemotherapeutic drug cisplatin. Cisplatin is enriched in nuclear speckles, suggesting that this drug influences pre-mRNA processing. Utilizing a framework incorporating dimensional reduction and clustering methods to analyze srIBI data, microenvironments within the nucleus, termed nuclear neighborhoods, are identified. Studying the interaction of these nuclear neighborhoods, the

15 results suggest a directionality of cisplatin action within nuclear speckles. srIBI joins the growing suite of techniques that image molecular component in situ. Use of these approaches

will allow study of the roles of small molecules in biological processes and the functions of distinct biomolecules in multi-component molecular pathways.

Materials and Methods

Cells

5 TYK-nu cells were grown in Eagle's minimal essential medium (American Tissue Culture Collection). HeLa cells were grown in DMEM (Gibco, Invitrogen). Media were supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin (Gibco, Invitrogen), and 100 mg/mL streptomycin (Gibco, Invitrogen). Cells were cultured in a humidified cell incubator at 37 °C with 5% CO₂ conditions and split with TrypLE Express
10 (Gibco, Invitrogen) every 2-3 days.

MoC-Ab preparation

Oligonucleotides (Table 3) were synthesized at the Stanford Protein and Nucleic Acid Facility with internal isotope-derivatized nucleotides, a fluorophore at the 3' position, and a maleimide cycloadduct at the 5' position. The maleimide was deprotected by a retro Diels-
15 Alder reaction. The lyophilized oligonucleotide was suspended in 1 mL anhydrous toluene (MTX07327, Millipore) for 4 hours at 90 °C, washed four times with anhydrous ethanol, and solubilized in buffer C (2 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.2). The oligonucleotide concentration was determined using a Nanodrop spectrophotometer. Aliquots of 8.5 nmol were prepared, lyophilized overnight, and stored in a desiccator at -20 °C. Antibodies (Table 4) in
20 carrier-free PBS were conjugated to the deprotected oligonucleotides. Briefly, 50 µg of antibody was loaded into a 50-KDa 0.5-mL centrifugal filter column with 400 µL PBS and reduced with 400 µL reduction buffer (PBS with 2.5 mM TCEP and 2.5 mM EDTA) for 30 minutes at room temperature. Antibodies were then washed with 400 µL of C buffer into a 50-KDa 0.5-mL centrifugal filter column and conjugated to 8.5 nmol of oligonucleotide in 400 µL
25 conjugation buffer (buffer C with 0.5 M NaCl) for 2 hours at room temperature. Antibodies were washed five times with 400 µL of high-salt PBS (PBS with 1 M NaCl), diluted into storage buffer (Candor PBS Antibody Stabilization Solution with 0.5 M NaCl and 5 mM EDTA) and stored at 4 °C. Each MoC-Ab was titrated by immunofluorescence using HeLa

cells, as exemplified in Fig. 30, and the staining pattern was compared to the staining pattern of the unconjugated antibody.

Table 3. Oligonucleotides.

Oligo Name	Sequence (5' → 3')	5'	3'	Z-Modification
Det.F.6-FAM	ATAGZAGTZZAGZZGAAZGGTAZ AGATGZZTAAAZAZTTGTZG (SEQ ID NO: 4)	Maleimide	6- FAM	2'-F-Ac-C
Det.Br.Cy3	ATAGZAGTZZAGZZGAAZGGTAZ AGATGZZTAAAZAZTTGTZG (SEQ ID NO: 5)	Maleimide	Cy3	5-Br-dC
Det.I.Cy5	ATAGZAGTZZAGZZGAAZGGTAZ AGATGZZTAAAZAZTTGTZG (SEQ ID NO: 6)	Maleimide	Cy5	5-I-dC

5 **Table 4. Primary antibodies.**

Antigen	Catalog number; Provider	Clone	Tag	Staining concentration
dsDNA	ab27156; Abcam	35I9	¹⁶² Dy	1:50
dsDNA	ab27156; Abcam	35I9	¹²⁷ I	1:50
nucleolin	ADI-KAM-CP100-E; Enzo	4E2	¹⁹ F / 6- FAM	1:100
H3K27Ac	ab4729; Abcam	pAb	¹⁹ F / 6- FAM	1:100

CENP-A	ab13939; Abcam	3-19	⁸¹ Br / Cy3	1:500
H3K27Ac	ab4729; Abcam	pAb	¹²⁷ I / Cy5	1:100
SC35	556363; BD Biosciences	α SC35	Biotin	1:100
H3K9me3	ab8898; Abcam	pAb	⁸¹ Br / Cy3	1:100
nucleolin	ADI-KAM-CP100-E; Enzo	4E2	¹²⁷ I / Cy5	1:100
NPM1	MABE937MI; EMD Millipore	28M1	Biotin	1:100
FBL	MABE1154; EMD Millipore	38F3	⁸¹ Br / Cy3	1:50

Lanthanide-conjugated and biotin-conjugated antibodies

Antibodies (Table 4) in carrier-free PBS were conjugated to metal-chelated polymers (MaxPAR Antibody Conjugation Kit, Fluidigm) or Sulfo-NHS-SS-Biotin (A39258, ThermoFisher) according to the manufacturer's protocol. Antibodies were diluted to 0.2 mg/mL in Candor PBS Antibody Stabilization Solution and stored at 4 °C.

Evaluation of cisplatin accumulation in cells by mass cytometry

TYK-nu cells were cultured in 0.5, 5, or 50 μ M of cisplatin (P4394, Sigma-Aldrich) for 24 hours, washed, and treated with 1 μ M Rh-intercalator (201103B, Fluidigm) for 15 minutes to discriminate dead from live cells. Cells were analyzed in a CyTOF2 instrument (Fluidigm) as previously described (63, 64).

Intracellular staining

Compositions of buffers used for staining are given in Table 5. Cells were fixed and permeabilized during 30 minutes at 4 °C in Fixation/Permeabilization buffer. Cells were then gently washed three times with Wash Buffer and blocked in Block Buffer 1 during 30 minutes at room temperature, washed three times with Wash Buffer, blocked in Block Buffer 2 during 30 minutes at room temperature, and washed three times with Wash Buffer. The cells were stained with a mixture of MoC-Abs in Reaction Buffer for 3 hours at room temperature.

Following staining with MoC-Abs, the Reaction Buffer was removed by gently touching the sample with a precision wipe (05511, Kimtech Science). Cells were then washed twice in Wash Buffer and incubated with Reaction Buffer containing FluoroNanogold Streptavidin (1:40 titer, 7016, Nanoprobes) for 30 minutes at room temperature. Cells were washed twice with Wash Buffer before preparing them for confocal microscopy or srIBI analysis. Cell fixation in PBS with 1.6% paraformaldehyde for 10 minutes at room temperature, permeabilization in methanol (10 minutes at -20 °C) or with Triton X-100 (0.5% in PBS, 10 minutes at room temperature) and staining produced comparable results for most of the antibodies tested.

Table 5. Buffers for intracellular staining.

Buffer	Composition	Catalog number; Provider
Fixation / Permeabilization	1X Fixation/Permeabilization concentrate in Fixation/Permeabilization diluent	00-5123; ThermoFisher 00-5223; ThermoFisher
Wash Buffer	1X Permeabilization buffer in water	00-8333; ThermoFisher
Block 1	1X Permeabilization buffer 0.5% BSA 0.5 M NaCl 200 µg/mL Salmon Sperm DNA 10 µg/ml Human FcX Block in avidin buffer	00-8333; ThermoFisher A3059; Sigma-Aldrich AM9760G; ThermoFisher AM9680; ThermoFisher 564765; BD Biosciences 927301; BioLegend
Block 2	1X Permeabilization buffer 0.5% BSA 0.5 M NaCl 200 µg/mL Salmon Sperm DNA	00-8333; ThermoFisher A3059; Sigma-Aldrich AM9760G; ThermoFisher AM9680; ThermoFisher

	10 µg/ml Human FcX Block in biotin buffer	564765; BD Biosciences 927301; BioLegend
Reaction	1X Permeabilization buffer 0.5% BSA 0.5 M NaCl 200 µg/mL Salmon Sperm DNA in water	00-8333; ThermoFisher A3059; Sigma-Aldrich AM9760G; ThermoFisher AM9680; ThermoFisher
Post-Fixation	2% Glutaraldehyde in 1X PBS	G7526; Sigma-Aldrich

Confocal microscopy

For confocal microscopy analysis, cells were grown in 12-mm diameter glass coverslips (72226-01, Electron Microscopy Sciences) until 80-90% confluent and stained as described. After staining, cells were washed three times with PBS, rinsed once with water, and mounted using Vectashield with DAPI (H-1200, Vector Laboratories). Anti-mouse-Alexa488 (1:2000 titer, 4408S, Molecular Probes) or anti-mouse-Alexa647 (1:2000 titer, 4410S, Molecular Probes) were the secondary antibodies used to detect unconjugated antibodies. Cells were analyzed in a LSM 880 (Zeiss) with a 63x oil-immersion objective (Zeiss Plan-Apochromat 63x/1.4 Oil).

Super-resolution Ion Beam Imaging

For srIBI analysis, cells were grown on silicon wafers (7 nm * 7 nm or 18 nm * 18 mm, Silicon Valley Microelectronics). Wafers were rinsed twice in methanol, air-dried with compressed air, washed with ethanol for 10 minutes, and rinsed three times with sterile PBS in a cell culture hood prior to cell seeding. When cells reached 80-90% confluence, they were stained as described above. After staining, cells were washed twice in PBS, fixed for 5 minutes in Post-Fixation buffer, and rinsed five times with water. Cells were dehydrated using a graded

ethanol series, air dried in a desiccator chamber, and stored at room temperature in a vacuum desiccator until analysis. srIBI images were acquired with the NanoSIMS 50L mass spectrometer (Cameca) at Stanford University using the CAMECA Microbeam Cesium Source. Before each experiment, tuning of the primary optics, secondary optics, and mass spectrometer was performed. The detectors were tuned by identifying each isotope of interest in an air-dried drop placed on a silicon wafer. The ion detectors were set as follows: detector 1, ^{12}C ; detector 2, ^{19}F ; detector 3, ^{31}P ; detector 4, ^{81}Br ; detector 5, ^{127}I ; detector 6, ^{194}Pt ; and detector 7, ^{197}Au . Secondary electrons were detected in parallel with ion information. The secondary ion peaks and EOS were tuned before the acquisition of each new cell to correct for magnetic drift. All images were collected with 1-ms dwell time per pixel. The raster size, number of pixels, repeat scans over the same area, and total scan time for each image are listed in Table 6. For each srIBI image, individual planes described in Table 6 were summed and gaussian blurred with a sigma of one using FIJI.

Table 6. Raster size, pixel number, plane number, and total scan time for each cell.

Cell in figure	Raster size (μm)	Pixel number	Plane number	Aperture (D1)	Total scan time (s)
1A	25	512*512	160	5	41966
1C	25	256*256	10	3	678
1D	25	256*256	10	3	678
1E (red dot)	25	512*512	10	2	2644
1E (orange dot)	25	512*512	10	3	2644
1E (yellow dot)	25	512*512	10	4	2644
1E (green dot)	25	512*512	10	5	2644
2A	25	256*256	10	3	678
2B	25	256*256	10	3	678

2C	25	256*256	50	3	3390
2D-H, J	25	512*512	10	5	2644
3A, B	25	256*256	785	3	53223
3C	25	256*256	40	3	2712
3D	25	256*256	400	3	27120
3E (top)	25	256*256	10	3	678
3E (bottom)	10	1024*1024	1	3	1048
3F	3	1024*1024	1	5	1048
3G	10	512*512	200	5	52451
4A, B	25	256*256	10	3	678
5A	10	1024*1024	20	5	20960
S3D (left)	25	256*256	10	3	678
S3D (right)	25	256*256	10	3	678
S4A	25	256*256	100	3	6780
S5A-C (related to 1D)	25	256*256	10	3	678
S6 (related to 1E)	25	512*512	10	2 / 3 / 4 / 5	2644
S7D (left)	25	256*256	10	3	678
S7D (right)	25	256*256	10	3	678
S8D (left)	25	256*256	10	3	678
S8D (right)	25	256*256	10	3	678
S9C	25	256*256	170	3	11526
S10D (left)	25	256*256	10	3	678

S10D (right)	25	256*256	10	3	678
S11A	25	256*256	10	3	678
S11B	25	256*256	20	3	1356
S11C	25	256*256	10	3	678
S12 (each cell)	25	256*256	10	3	678
S13 (right)	25	512*512	40	5	10576
S14	25	512*512	10	5	2644
S15	25	256*256	10	3	678
S16	25	256*256	10	3	678
S17 (related to 3A-B)	25	256*256	785	3	53223
S18 (related to 3F)	3	1024*1024	1	5	1048
S19	5	256*256	20	5	1356
S20E (top)	25	256*256	10	3	678
S20E (bottom)	25	256*256	10	3	678
S20F (top)	25	256*256	10	3	678
S20F (bottom)	25	256*256	10	3	678
S21A-B	10	1024*1024	21	5	22043
S21C (each cell; related to 5A)	10	1024*1024	20	5	20960
Movies S1 and S2 (related to 3A-B and S17)	25	256*256	785	3	53223
Movie S3 (related to 3D)	25	256*256	400	3	27120
Movie S4 (related to 1A)	25	512*512	160	5	41966

Nuclear neighborhood analysis

A mask for each nucleus was created using the phosphorus channel, and 20000 3D voxels, each of dimension $(x, y, z) = (6, 6, 20)$ pixels, were extracted from each of two cells by random sampling from the middle 40 Z-planes (for a total of 40000 voxels). Each channel was z-normalized across all pixels contained within the sampled image regions. Unsupervised hierarchical clustering was performed using the markers SC35, H3K9me3, phosphorus, nucleolin, and H3K27Ac to identify 10 clusters across the 20000 extracted voxels. The average expression of each marker from both cells was then plotted in a heatmap form. Each voxel was colored by its cluster, and replotted on a X-Y plane to recreate the cell. The count distribution of cisplatin within regions assigned to each cluster was computed for each cell.

Iterative srIBI data analysis

The low pixel signal intensity values of iterative srIBI due to the much lower current during data acquisition than in conventional srIBI is a barrier for robust data analysis. We adapted a filtering strategy described in Keren et al. to filter noise from signal using a k nearest neighbor (KNN) approach (14). The assumption for this approach is that in sparse data, the signal density offers more information than signal intensity. Thus, regions with dense signals are more likely to contain true signals, compared with regions with sparse signals. We first summed the image data (1024 x 1024 pixel) across 20 Z-planes, and then measured the average distance between positive pixels for the nearest 25 neighbors within each channel. We next applied a cutoff to distinguish between signal (regions of high signal density) and noise (regions with sparse signal density), to create a mask for each channel. The masks were applied to each individual Z-plane to create a denoised image for subsequent analysis. The 100 pixels around each border were discarded to avoid potential edge effects. A 3D sliding window of dimension $(x, y, z) = (10, 10, 5)$ pixels and step-size of $(x, y, z) = (5, 5, 3)$ was applied to the image to calculate the mean of each channel within the voxel. We randomly sampled 20000 voxels across each image (from 5 different cells) to obtain a final 100000 voxels. Counts were log2 transformed after adding a "0.0001" value to avoid zeros. Unsupervised hierarchical

clustering was performed on all voxels using the markers SC35, H3K9me3, phosphorus, nucleolin, H3K27Ac, and cisplatin. A t-Distributed Stochastic Neighbor Embedding (t-SNE) using a Barnes-Hut implementation was performed, and each identified cluster was differentially colored. The means of each cluster was also plotted and subsequently manually merged by similarity into 11 final clusters. The t-SNE plot was also colored by the cell of origin of each voxel to ensure minimal batch effects.

Nuclear neighborhood interaction frequency

To identify and test for the significance of how frequently clusters interacted (as defined by the number of pixels separating voxels from each other), we implemented a permutation test. First, the Euclidian distance in 3D space between voxels was calculated (Equation 1).

Equation 1:

$$Dist = \sqrt{(x_1 - x_0)^2 + (y_1 - y_0)^2 + (z_1 - z_0)^2}$$

Next, we defined interacting voxels as those less than or equal to 5 pixels away from each other (between the center of each voxel). After 1000 permutations, where the cluster annotation was shuffled, and the p-value calculated on either tail (Equation 2).

Equation 2:

$$Pvalue = \frac{(\sum Permutations > (<) RealData) + 1}{\# Permutations + 1}$$

Essentially, 1 plus the number of permutations with distances smaller than (or greater than) the real data was divided by 1 plus the total number of permutations performed. Finally, the fold enrichment of interactions for real data over the mean interactions from the permutation tests was calculated (Equation 3).

Equation 3:

$$\text{Log2 Fold Enrichment} = \log_2 \frac{\text{Real Data}}{\text{mean}(\# \text{ Permutations})}$$

Results for example 6

Overview of srIBI

srIBI uses a positively charged cesium primary ion beam with a small spot size to obtain 3D super-resolution multiparametric visualization of cellular features (**Fig. 14 A**), including the distribution of small molecules. Cells are cultured on a conductive substrate and treated with a small molecule that carries a stable isotope that can be efficiently ionized by the cesium beam. Cells are then simultaneously stained with multiple isotope-tagged antibodies using a protocol optimized for intracellular staining, dried under vacuum, and loaded into the instrument. A cell of interest is identified with an optical camera and then iteratively rasterized with the cesium primary ion beam. This process releases a cloud of negative secondary ions at the point of contact on the cell surface. The liberated secondary ions are collected and recorded, pixel-by-pixel, using a magnetic sector mass spectrometer. This results in a two-dimensional (2D) image for each analyzed isotope. Serial acquisition of hundreds of planes yields a stack of 2D images for each isotope that can be merged to obtain a multiparameter visualization of the cellular features of interest and volumetrically reconstructed to provide a 3D composite image of the analyzed cell at super-resolution.

Design and validation of novel antibody tagging protocol for srIBI

In this original MIBI approach, an oxygen duoplasmatron source was used to generate 2D images of tissue sections stained with lanthanide-tagged antibodies down to 260 nm resolution (13). Attaining resolutions beyond this limit requires a more finely focused beam. The cesium primary ion beam can achieve resolutions down to 30 nm in biological samples (17, 24). However, lanthanide-tagged antibodies cannot be used since lanthanides are not efficiently ionized with a cesium ion beam (25). To circumvent this issue, a new labeling strategy using isotopes that are efficiently ionized with a cesium beam is developed. DNA is a versatile polymer that has been extensively functionalized for imaging purposes (3, 26-28). It was reasoned that antibodies conjugated with single-stranded DNA (ssDNA) oligonucleotides

carrying stable isotopes that are present in low amounts in cells would enable super-resolution protein visualization by MIBI. To this end, a tool called a mass-oligonucleotide-conjugated antibody (MoC-Ab) was developed. Isotope-modified ssDNA oligonucleotides were conjugated to antibodies through thiol-maleimide click chemistry (**Fig. 28**) (29). MoC-Abs can be used for intracellular protein staining in an optimized protocol that involves fixation with 1.6% paraformaldehyde, permeabilization, and blocking with a high salt concentration in the presence of sheared salmon sperm DNA to avoid non-specific binding (**Fig. 29**).

In a proof-of-concept experiment, the single stable fluorine isotope (^{19}F) was incorporated by chemical synthesis into a FITC-labeled ssDNA by substituting all cytidines with the commercially available N4-acetyl-2'-fluoro-2'-deoxycytidine (2-F-Ac-C) (**Fig. 14 B**; ^{19}F /FITC MoC-Ab). The behavior of MoC-Abs is similar to that of unmodified antibodies as shown by confocal microscopy analyses of target specificity, staining intensity, and background levels of nucleolin staining in HeLa cells (**Fig. 30 A-C**). Next, the performance of MoC-Abs in srIBI by visualizing HeLa cells stained with anti-nucleolin- ^{19}F /FITC was assessed. Rastering a HeLa cell with the cesium ion beam revealed an enriched ^{19}F signal inside the nucleus akin to the fluorescence signal observed using confocal microscopy (**Fig. 30 D**, right images). Moreover, control experiments without anti-nucleolin- ^{19}F /FITC resulted in absence of ^{19}F signal (**Fig. 30 D**, left images), suggesting that the ^{19}F signal observed in the stained cell resulted from the MoC-Ab. The high ^{31}P content of the DNA phosphate backbone enabled visualization of the nucleus in the srIBI experiment in a manner analogous to use of DAPI in conventional fluorescent microscopy (**Fig. 31**).

In srIBI, secondary electron (e^-) and elemental ion information are simultaneously recorded for each plane. The e^- image, akin to that from a scanning electron microscope, enables the identification of certain subnuclear structures such as the nucleolus (**Fig. 14 C**, left image). To confirm the specificity of anti-nucleolin- ^{19}F /FITC, the ^{19}F and e^- signals were merged and a specific enrichment of the ^{19}F signal in nucleolar structures identified through the e^- image was observed (**Fig. 14 C**, overlay and right image). To demonstrate the versatility of srIBI, transcriptionally active chromatin was labeled using a ^{19}F /FITC MoC-Ab that binds to H3K27Ac (**Fig. 14 D**). The ^{19}F signal overlapped with nuclear regions low in ^{31}P confirming the specificity of anti-H3K27Ac- ^{19}F /FITC (**Fig. 32**). These results demonstrate that

detection of MoC-Abs labeled with ^{19}F by ion beam imaging can reveal subnuclear structures of known provenance.

In SIMS, the beam size dictates the lateral resolution and ion yield of the acquired image (30). The size of the beam is proportional to its current. The effect of beam current on the ion count per pixel in the samples was quantified by acquiring sequential images of the nucleolus of a HeLa cell. Raster size, pixel dwell time, and number of pixels acquired were maintained at constant levels, and the beam current was varied (**Fig. 33 A and B**). It was observed that the increase in current scaled with ion counts per pixel (**Fig. 33 C and D**) at the expense of resolution (**Fig. 33 B**). To quantify the lateral resolution, sequential images of a HeLa cell incubated for 24 hours with 5-iodo-2'-deoxyuridine (IdU) were acquired to specifically label newly replicated DNA. In the condition tested, reduction of the beam current improved the lateral resolution from 193 nm to 113 nm and lowered the total ion count 16 fold ($R^2 = 0.91$; **Fig. 14 E and F** and **Fig. 33 E-I**). These results show that srIBI resolves targeted subcellular structures with a resolution not attainable with current MSI methods that target specific proteins (13, 15).

Multiparametric imaging and unsupervised identification of subnuclear structures

The capabilities of mass spectrometry for simultaneous data acquisition was leveraged by expanding the number of elements used to label MoC-Abs. Bromine ($^{79/81}\text{Br}$) and iodine (^{127}I) are two elements used to produce synthetic nucleotides classically harnessed to study cell proliferation, DNA replication, and RNA synthesis. MoC-Abs containing 5-bromo-2'-deoxycytidine (5-Br-dC) and 5-iodo-2'-deoxycytidine (5-I-dC) were synthesized and validated for the labeling of centromeres (anti-CENP-A- $^{81}\text{Br}/\text{Cy3}$) (**Fig. 15 A** and **Fig. 34**) and transcriptionally active chromatin (anti-H3K27Ac- $^{127}\text{I}/\text{Cy5}$) (**Fig. 15 B** and **Fig. 35**), respectively.

In addition to nucleotide-based labeling methods, other reagents can be harnessed for srIBI (31, 32). HeLa cells were stained with phalloidin conjugated to ATTO514, a fluorophore that contains six ^{19}F atoms (**Fig. 36A**). The distribution of actin filaments observed in HeLa cells labeled with this reagent was comparable in confocal microscopy and srIBI images (**Fig. 36 B and C**). Nuclear speckles are nuclear domains containing inter-chromatin material

enriched in pre-mRNA splicing components (33). Detection of SC35, a nuclear speckle protein, was validated using anti-SC35-biotin with streptavidin-labeled 1.4 nm gold nanoparticles (streptavidin-¹⁹⁷Au/FITC) by srIBI (**Fig. 15 C** and **Fig. 37**). To further demonstrate the specificity of antibody-based tools for srIBI, transcriptionally silent chromatin (anti-H3K9me3-⁸¹Br/Cy3) and nucleoli (anti-nucleolin-¹²⁷I/Cy5 and anti-NPM1-biotin recognized by streptavidin-¹⁹⁷Au/FITC) were labeled in HeLa cells, obtaining the expected patterns for all (**Fig. 38**).

The crosstalk between channels by staining HeLa cells with individual MoC-Abs and assessing the signal in unstained channels were evaluated. Minimal crosstalk between channels was observed (**Fig. 39**), therefore multiplexed imaging, in a manner akin to fluorescence microscopy, can be performed using srIBI (**Fig. 40**). To demonstrate the multi-parameter capabilities of srIBI, nucleolin, H3K9me3, H3K27Ac, and SC35 in HeLa cells were simultaneously visualized (**Fig. 15 D**). Magnification of the composite image revealed a complexity not observed in single-channel images (**Fig. 15 E-G** and **Fig. 41**). The ion count per channel on each pixel of the multiplexed image was extracted (**Fig. 15 H**) and viSNE was used to identify subnuclear structures (**Fig. 15 I**) as previously described (34). Pixels with high expression of nucleolin were grouped in three distinct regions that had variable levels of H3K9me3 and low levels of SC35, H3K27Ac, and phosphorus (**Fig. 15 I**; gates A to C).

To gain insights into nucleolar organization, the pixels within nucleolin-rich regions were categorized in the three distinct subsets and generated a composite image of spatial distribution (**Fig. 15 J**). There was a different localization pattern of pixels with high H3K9me3 signal within the nucleolin-rich regions (**Fig. 15 J**, gate B) than in the regions with low amounts of H3K9me3 (**Fig. 15 J**, gate A); these patterns were consistent in all nucleoli of this cell (**Fig. 15 J**, gate A+B+C). Thus, srIBI can be used to visualize multiple isotopes simultaneously, and the application of dimensionality reduction techniques to the data enables identification of distinct subnuclear structures.

3D reconstruction at the nanoscale with srIBI

In srIBI, ion beam strength and residence time at the spot can yield secondary ion information for each plane that results from the ablation of a just a few nanometers of the

sample surface, and consecutive planes have similar ion counts (**Fig. 42**). It was reasoned that whole 3D nuclear reconstruction could be achieved with srIBI if data were acquired on many consecutive planes. Previous axial resolution quantifications in biological samples on the nanoSIMS was determined to be around 5 nm (35). In agreement with this measurement, reconstructions of centromeres from srIBI (**Fig. 43**) resembled those identified by recent super-resolution microscopy experiments (36).

To demonstrate the feasibility of whole organelle 3D reconstruction, 785 planes of a single nucleolus were acquired (**Fig. 16 A** and **Fig. 44**) and a volumetric reconstruction was performed (**Fig. 16 B**). srIBI is capable of resolving juxtaposed subnuclear structures as a 50-plane acquisition was sufficient to distinguish between the centromere, nucleolus, and nuclear speckles in a HeLa cell (**Fig. 16 C**). As further validation, 3D rendering of 400 planes from a single HeLa cell labeled with anti-nucleolin-¹⁹F, anti-H3K9me3-⁸¹Br, anti-H3K27Ac-¹²⁷I, and anti-SC35 (recognized by streptavidin-¹⁹⁷Au) was performed, creating a composite 3D visualization of the nucleus (**Fig. 16 D**). These results indicate that srIBI enables whole-cell reconstruction of the genome and epigenome. Combined with an analytical framework, as shown below, srIBI will allow intricate dissection of subcellular microenvironments in single cells.

Iterative srIBI resolves nanoscale structures

A lateral resolution down to 30 nm in biological samples has been previously reported using the cesium primary ion beam by focusing it to an area of interest measuring a few microns (17, 24). During each pass of the primary ion beam only the top material contacted is ablated. The majority of the sample is preserved due to the high axial resolution and thus is available for further imaging. It was reasoned that an initial acquisition of a target cell to define a region of interest (ROI) could be performed and that sub-cellular feature could be focused on for higher resolution profiling (**Fig. 16 E**, workflow). Indeed, re-probing a region of interest in an IdU-treated cell enabled iterative imaging at higher resolution (**Fig. 16 E**, IdU images).

To achieve the highest benchmarked resolution in MSI of specific proteins, iterative srIBI was performed in a single HeLa cell labeled with anti-nucleolin-¹⁹F. Using line scans

across the image to quantify the distance between ^{19}F signals as commonly done for fluorescence-based super-resolution images (37), nucleolin molecules spaced at about 30 nm apart were identified (**Fig. 16 F** and **Fig. 45**). The secondary electron image of a cell before and after an iterative srIBI acquisition showed a generally even ablation of the field of view, which focused on three nucleoli (**Fig. 16 G**, e^- image). A 3D reconstruction of a single nucleolus from this acquisition (**Fig. 16 G**) revealed the expected spatial distribution of nucleolar proteins within a single nucleolus (**Fig. 16 G** and **Fig. 46**). Hence, a first scan to define an area of interest and subsequent imaging to enhance the resolution revealed molecules spaced about 30 nm apart.

Differential drug distribution in nuclear neighborhoods

SIMS enables subcellular visualization of exogenously incorporated small molecules and has been previously used to study the cellular distribution of the metallo-drug cisplatin (38), which is used to treat various types of cancer (39). The platinum atom of cisplatin (**Fig. 47 A**) can be readily ionized using the cesium beam. TYK-nu cells, which are immortalized ovarian cancer cells, were incubated for 24 hours with a range of concentrations of cisplatin to identify a concentration at which drug was internalized but caused minimal cell death (**Fig. 47 B**). Naturally occurring platinum is composed of five stable isotopes (**Fig. 47 C**); the study took advantage of isotopically pure cisplatin (^{194}Pt) to maximize Pt counts. Cells treated with 5 μM cisplatin for 24 hours exhibited high ^{194}Pt counts (**Fig. 47 D**), and signal was observed in cytoplasm and nuclei (**Fig. 47 E**), consistent with published results (38). The crosstalk between ^{194}Pt and the channels used for srIBI were minimal (**Fig. 47 F**). MoC-Ab staining of cisplatin-treated cells was performed and images of ^-e , ^{12}C (indicative of overall cellular structure), ^{19}F (nucleolin), ^{31}P (DNA), ^{81}Br (H3K9me3), ^{127}I (H3K27Ac), ^{194}Pt (cisplatin), and ^{197}Au (SC35) were acquired to generate an eight-channel image at super-resolution. srIBI revealed a non-uniform distribution of cisplatin in TYK-nu nuclei (**Fig. 17 A**) and composite images enabled the visualization of the subnuclear distribution of cisplatin (**Fig. 17 B**).

Biomolecules in the nucleus are organized spatially to form pockets of interaction (40, 41), akin to tissue microenvironments (3, 14, 42). In such a nuclear neighborhood, the function of various biomolecules are dependent on their spatial localization and the context of other

nearby constituents. The multiplexed data generated from srIBI allowed the testing of the hypothesis that biomolecules in the nucleus have context-dependent functions. Whole cell srIBI acquisition was performed on cisplatin-treated cells (**Fig. 17 C**, top left). A sliding window, consisting of $6 * 6 * 20$ pixels, was applied to extract the average isotope counts per window for five channel (H3K9me3, H3K27Ac, SC35, nucleolin and phosphorus; **Fig. 17 C**, top right). Unsupervised clustering was then performed on these extracted features to group windows based on similarity (**Fig. 17 C**, bottom right). Individual voxels were then pseudo-colored based on these groups for visualization purposes (**Fig. 17 C**, bottom left). In total, 20,000 randomly sampled 3D voxels from the middle 40 Z-planes were extracted from each of the 2 cells, for a sum of 40,000 voxels.

Clustering of these 40,000 voxels based on the five channels revealed 10 distinct clusters, which represented 10 different nuclear neighborhoods (**Fig. 17 D**) at this scale of observation. The histone markers H3K9me3 and H3K27Ac were used as well-characterized markers for heterochromatin (higher DNA density) and euchromatin (low DNA density), respectively (43). That these markers were located in different clusters is supportive of the technique's capability (**Fig. 17 D**, Neighborhoods 5, 7, and 10). Regions that are high in H3K9me3 also have abundant phosphorus were observed, as expected because of the condensed nature of heterochromatin (**Fig. 17 D**, Neighborhoods 5). Each nucleus was then colored based on these groups and the 3D voxels were projected onto a 2D plane to better visualize the spatial distribution of the nuclear neighborhoods (**Fig. 17 E**). These images reveal distinctive nuclear structures, such as nucleolin-like structures (Neighborhood 1), nuclear speckles (Neighborhood 4), and chromatin that resembles lamin-associated domains (Neighborhood 8). Features that are difficult to distinguish visually in the srIBI images (**Fig. 17 B**) could now be appreciated (**Fig. 17 E**), such as peri nucleolar heterochromatin (Neighborhood 9), as well as heterochromatin-like regions (Neighborhood 10), and euchromatin (Neighborhoods 6 and 7). This is also able to distinguish features that appear to be edge effects (Neighborhoods 2 and 3).

Identification of subcellular microenvironments, combined with the spatial characterization of atomic components from small molecules, offers the ability to create subcellular maps for multiple types of biomolecules. To identify the distribution of cisplatin in

the nucleus, the study set out to quantify cisplatin levels in each nuclear neighborhood. It was observed that nuclear speckles contained high levels of cisplatin (**Fig. 17 F**, Neighborhood 4). Cisplatin was depleted from heterochromatin regions and lamin-associated domains (**Fig. 17 F**, Neighborhoods 5 and 8). To obtain a super-resolution image of cisplatin localization, iterative srIBI was used, to reveal at an unprecedented level, the spatial enrichment of cisplatin along the nuclear speckles and their depletion from closed chromatin (**Fig. 18 A** and **Fig. 48**). These results demonstrate a differential distribution of cisplatin in distinct nuclear neighborhoods, suggestive of different mechanisms of action.

An analytical framework to identify nuclear neighborhood interactions in multiplexed super-resolved data

Diversity of nuclear organization is readily appreciated upon inspection of iterative srIBI images (**Fig. 18 A**, right panels). To extract biological meaning from these images, the visual nature of dimensionality reduction methods was combined with the spatial information encoded within the data. 20000 voxels across five different cells were randomly selected for combined analysis. Dimensional reduction was then performed using t-Distributed Stochastic Neighbor Embedding (t-SNE) on the log transformed, mean isotope counts per voxel (**Fig. 49 A**). As expected, signals were grouped together based on levels of marker expression (**Fig. 49 A**). The study next applied unsupervised hierarchically clustering followed by manual annotation of the clusters to identify 11 unique nuclear neighborhoods (**Fig. 18 B-D** and **Fig. 49 B-D**). Each cell was relatively well represented in each neighborhood (**Fig. 49E**). The nuclear organization diversity observed in iterative srIBI images of cisplatin treated cells (**Fig. 18 A**) were able to be reconstructed using the neighborhood and spatial information alone (**Fig. 18 E** and **Fig. 50**), demonstrating that the inferred neighborhoods are an appropriate representation of nuclear organization.

If the nucleus is organized with intent, certain neighborhoods would interact with each other at higher frequency than random. To infer this, a pairwise interaction permutation test was devised and implemented (**Fig. 18 F**, see Materials and Methods for details). In addition to testing for the frequency of interactions, statistical significance for each pairwise interaction was also tested for (**Fig. 18 F** and **Fig. 51**). Certain nuclear neighborhoods tended to attract or

repel each other. For example, neighborhoods rich in both SC35 and cisplatin repelled neighborhoods rich in nucleolin (**Fig. 18 F**, Neighborhood 6 in A to Neighborhood 9 in B).

Focusing on neighborhoods representing chromatin (Neighborhoods 1 to 4), it was observed that heterochromatin self-aggregated, was tightly packed, and surrounded the nucleolus (**Fig. 18 G**, green box), whereas euchromatin was dispersed, as expected due to higher accessibility of the DNA (**Fig. 18 G**, violet box). In line with previous reports, this study corroborated that cisplatin accumulated in the nucleolus (**Fig. 18 H**) (38) and also observed that cisplatin within the nucleolus spatially segregated into two distinct neighborhoods (**Fig. 18 H**, brown box, Neighborhoods 5 and 10). In addition, there was a difference between two nuclear speckle neighborhoods enriched in cisplatin (**Fig. 18 H**, red box, Neighborhoods 6 and 7). It was observed that inactive SC35 neighborhoods (Neighborhood 7) tended to envelope the active SC35 neighborhoods (Neighborhood 6), suggesting directionality of cisplatin action (**Fig. 18 H**, orange box and **Fig. 52**). These results indicate that biologically relevant information can be obtained through the analysis of iterative srIBI images of subcellular entities.

Probes for detecting centromeres were designed in accordance with the illustration in Fig. 53 and hybridized in situ. The stained cells were analyzed. The results of this assay, as shown in Fig. 54, demonstrate that the probes can be used to label centromeres *in situ*.

Discussion for Example 6

srIBI is an MSI method enabled by a new set of reagents, MoC-Abs, that allow for the specific detection of proteins using MIBI coupled to a cesium primary ion beam. This method enables targeted super-resolution, 3D, multiplexed imaging of different biomolecule types in cells. srIBI detection of MoC-Abs yielded results akin to those obtained using standard confocal light microscopy but use of iterative srIBI imaging resulted in lateral resolutions of approximately 30 nm. Application of unsupervised dimensional reduction methods enabled the identification of subcellular structures and localization of the small-molecule drug cisplatin. As most drugs can be isotopically labeled with isotopes such as ^{13}C or ^{15}N , srIBI will allow for study of localization of a variety of agents. Drug metabolism in and out of cells might also be determined by such imaging. Understanding the subcellular localization of drugs may

maximize the success rates of drug candidates in clinical trials, holding promise for a more efficient and cost-effective drug discovery process (44).

Although srIBI has theoretical resolutions below the antibody size and potential for at least 50-plex imaging, it is currently hindered by low ion yields at high resolutions, the number of isotopes available for labeling, and the number of available detectors for higher multiplexing. Improvements to the ion yield can be linearly ameliorated through the attachment of more labeling isotopes per probe (which may result in slight loss of resolution). The MoC-Abs used in this work each contain 44 to 66 isotopic labels. An increase in labels per antibody can be readily achieved by increasing the number of substituted nucleotides, increasing the length of labeling oligonucleotide, or through nucleotide-based amplification methods (28, 45-47). Better estimations of the placement of the epitope at which the antibody interacts (through use of centroid estimations) might be obtained by directional labeling of the antibody: If one isotope was located at the 5' end of the mass-oligonucleotide and another isotope at the 3' end, it would provide a "pointer" to the epitope is located. The atomic elements used for srIBI can be expanded by synthesizing isotope-derivatized nucleotides with previously reported protocols (48, 49). Moreover, a recently developed cesium ion beam has attained currents as high as one pA at 25-nm beam size (50), which might enable more precise localizations. Physical expansion of biological samples, mediated by polymer swelling as previously described (51), would further increase the resolution of srIBI. Other primary beam sources, such as helium- or neon-based beams, which have beam sizes of 0.5 nm and 1.5 nm (52), respectively, have been recently adapted for SIMS (52). These improvements may enable 3D imaging of single antibodies, hence opening the door for such opportunities as isotope barcoding to increase the number of simultaneously measured channels. For example, with the seven-detector design presented here, a triple-barcoded labeling would theoretically enable deconvolution of 35 targets.

MIBI and IMC are orthogonal approaches to use of fluorescence for imaging the tissue microenvironment (13-15). Currently, these methods are focused on large fields of view and fast acquisition times, due to their focus on large-scale tissue cellular contexts. IMC is based on laser-ablation inductively coupled plasma mass spectrometry with a 1000-nm laser beam. In addition, the cutoff at 75 atomic mass units in the Helios system does not allow imaging of

naturally occurring organic isotopes. MIBI has a higher lateral resolution than IMC due to a smaller beam size (260 to 500 nm), but the negative charge of the oxygen duoplasmatron limits detection of intrinsic biomolecules. Thus, current protein-targeted MSI technologies cannot be used for the study of biomolecules or small molecules at the nanoscale. Super-resolution light microscopy methods are limited by the number of fluorophores that can be simultaneously recorded. While recent applications overcome this issue by using cycling of oligonucleotide probes (2, 3, 5, 8), these methods suffer from drawbacks such as sample drift between cycles, low axial resolution, photobleaching, and long acquisition times. Current methods such as the tetrapod PSF and DNA-PAINT are promising solutions to some of these limitations (53, 54).

Since srIBI acquires data on multiple biomolecules simultaneously, it is an alternative to current super-resolution light microscopy techniques. srIBI samples can be stored for extended periods as the isotopes detected are stable; this is not the case for fluorophores used in light imaging. Furthermore, srIBI enables specific protein staining compatible with direct imaging of exogenously added small molecules. Extension of fluorescent-based methods for specific nucleic acid staining, like CRISPR/Cas-FISH (55), Oligopaint (27, 56), and ATAC-seq (57), to srIBI will provide a unique opportunity for an integrative understanding of cellular processes at the nanoscale beyond current means.

Multiplexed imaging of an entire cell, at resolutions afforded by srIBI, introduces new challenges in how to interpret the results. To this end, an analytical framework that leverages on established methods to infer biological meaning was designed and implemented. For example, hierarchical clustering and t-SNE are able to simplify multiplexed srIBI data across multiple cells. Combined with neighborhood interaction maps, this study was able to reveal interactions within the subcellular domain not obvious to the naked eye. The ability to learn and infer information about other unlabeled but related biomolecules within the cell will provide a way to describe an entire cell with a limited number of parameters (58, 59). Further integrative analysis, particularly incorporation of biophysical and genomic information, will also allow more accurate modeling of nuclear structures (8, 60, 61). Here, srIBI was applied to study the localization of the cancer drug cisplatin. Cisplatin accumulation was observed in SC35-positive nuclear speckles, which are sites of mRNA processing and splicing. This study

was able to distinguish between cisplatin at active regions of transcription (H3K27Ac) and nuclear speckles (SC35), placing the drug functionally at sites of RNA splicing in addition to general sites of active transcription. Indeed, cisplatin has been shown to reduce pre-mRNA splicing in a dose-dependent manner which might be additive to its well-known role inducing adducts, single strand gaps post repair and other lesions in DNA (39, 62). These results suggest potential vulnerabilities of mRNA processing that can be targeted for combinatorial therapies to increase the effectiveness of cisplatin.

SrIBI is an imaging method that can be applied in single-cell studies of the diverse molecular interactions in subcellular microenvironments. Combination with other unbiased or population-based methods, such as small molecule screens and multiplexed proteomics, promises to reveal new understanding into metabolic pathways or mechanisms of drug resistance. This has the potential to drive new therapeutic discoveries and inform decision-making processes of small molecules.

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CLAIMS

What is claimed is:

1. A labeling moiety, wherein the labeling moiety comprises multiple internal atoms of a non-biological stable nonmetal or metalloid isotope, wherein:
 - i. the labeling moiety is or comprises a binding agent that specifically binds to or reacts with a complementary site in or on a cell, or
 - ii. the labeling moiety comprises a chemoselective group, wherein the labeling moiety is not an anti-IgG antibody.
2. The labeling moiety of claim 1, wherein the labeling moiety is or comprises a binding agent that specifically binds to a complementary site in or on a cell.
3. The labeling moiety of claim 2, wherein the binding agent is an antibody.
4. The labeling moiety of claim 1, wherein the labeling moiety is a polymer that has a chemoselective group.
5. The labeling moiety of claim 4, wherein the polymer is an oligonucleotide.
6. The labeling moiety of claim 5, wherein the chemoselective group is amine-reactive, thiol reactive or hydroxyl reactive.
7. The labeling moiety of claim 1, wherein the labeling moiety is composed of a polymer and a binding agent that specifically binds to a complementary site in or on a cell, wherein only the polymer comprises the atoms of the stable nonmetal or metalloid isotope.
8. The labeling moiety of claim 7, wherein the labeling moiety comprises an antibody conjugated to an oligonucleotide that comprises atoms of a stable nonmetal or metalloid isotope.

9. The labeling moiety of claim 1, wherein herein the labeling moiety is or comprises a nucleic acid that specifically binds to a protein in a sequence-specific manner.
10. The labeling moiety of claim 1, wherein herein the labeling moiety is a transposase complex.
11. The labeling moiety of any prior claim, wherein the labeling moiety comprises a stable nonmetal isotope of C, N, O, F, P, S, Cl, Se, Br, or I.
12. The labeling moiety of any prior claim, wherein the moiety comprises a stable isotope of a halogen.
13. The labeling moiety of any prior claim, wherein the labeling moiety comprises a stable metalloid isotope of B, Si, Ge, As, Sb or Te.
14. The labeling moiety of any of any prior claim, wherein the moiety comprises two or more different stable nonmetal or metalloid isotopes.
15. The labeling moiety of any prior claim, wherein the moiety comprises:
 - (a) a primary oligonucleotide and
 - (b) a labeled oligonucleotide that contains one or more contains non-metal/metalloid isotopes,where the primary oligonucleotide comprises:
 - (i) a first, target-specific, sequence that is complementary to a sequence in a cell, and
 - (ii) a second sequence that is complementary to the labeled oligonucleotide.
16. A set of labeling moieties of any of claims 1-15, wherein the moieties are distinguishably labeled by the stable nonmetal isotopes.

17. The set of labeling moieties of claim 15, wherein the moieties are in separate tubes.
18. The set of labeling moieties of claim 15, wherein the moieties are mixed.
19. The set of labeling moieties of any of claims 16-18, wherein the moieties comprises multiple internal atoms of at least two stable nonmetal or metalloid isotopes, wherein the moieties are distinguishable by the identity and/or ratio of the different isotopes.
20. A method of generating a high resolution image by secondary ion mass spectrometry (SIMS), comprising:
 - incubating the sample with one or more labeling moieties of claims 1-19, thereby producing a labeled sample in which a biological feature of interest is bound to the one or more labeling moieties;
 - scanning the sample by secondary ion mass spectrometry (SIMS) using a positively charged ion beam to generate a data set that comprises spatially-addressable measurements of the abundance of said at least one stable nonmetal or metalloid isotope across said sample; and
 - outputting the data set.
21. A method of analyzing cells by mass cytometry, comprising:
 - incubating the cells with one or more labeling moieties of claims 1-19, thereby producing a labeled sample in which the labeling moieties are bound to the cells;
 - analyzing the cells by mass cytometry, using a positively charged plasma beam to atomize the cells and generate a data set that comprises spatially-addressable measurements of the abundance of the stable nonmetal or metalloid isotopes in or on the cells; and
 - outputting the data set.

1 / 69

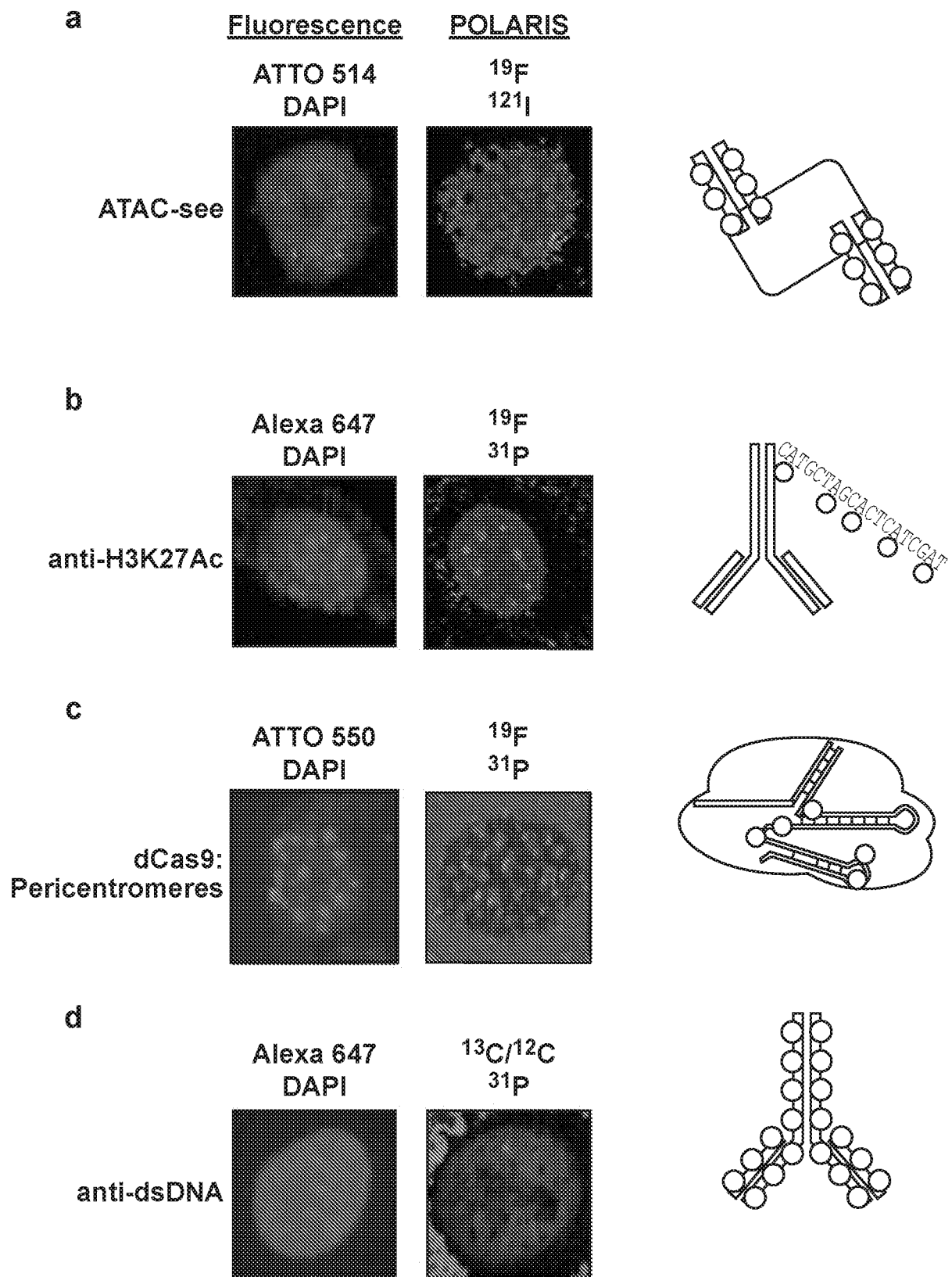


FIG. 1

2 / 69

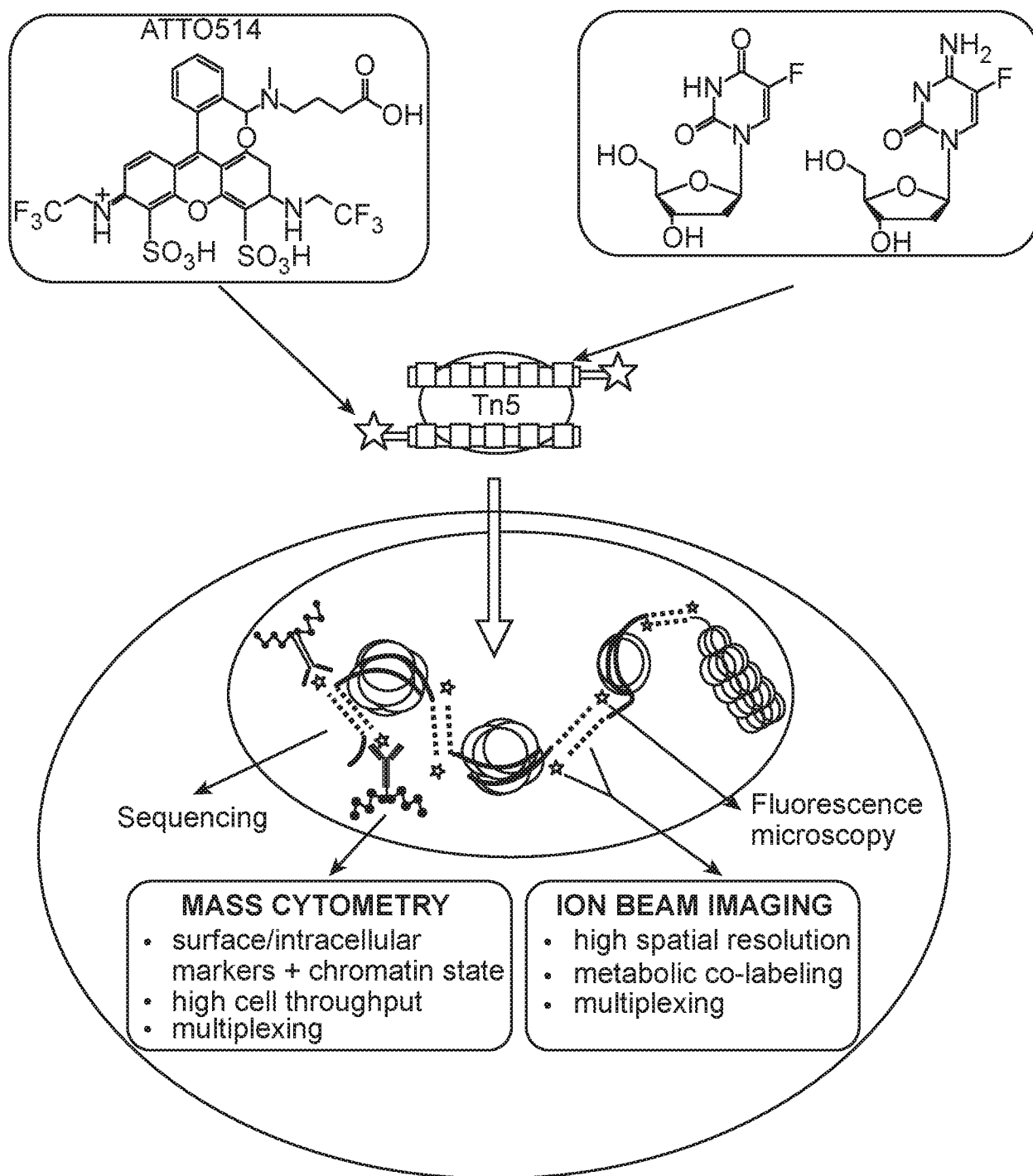
a

FIG. 2

3 / 69

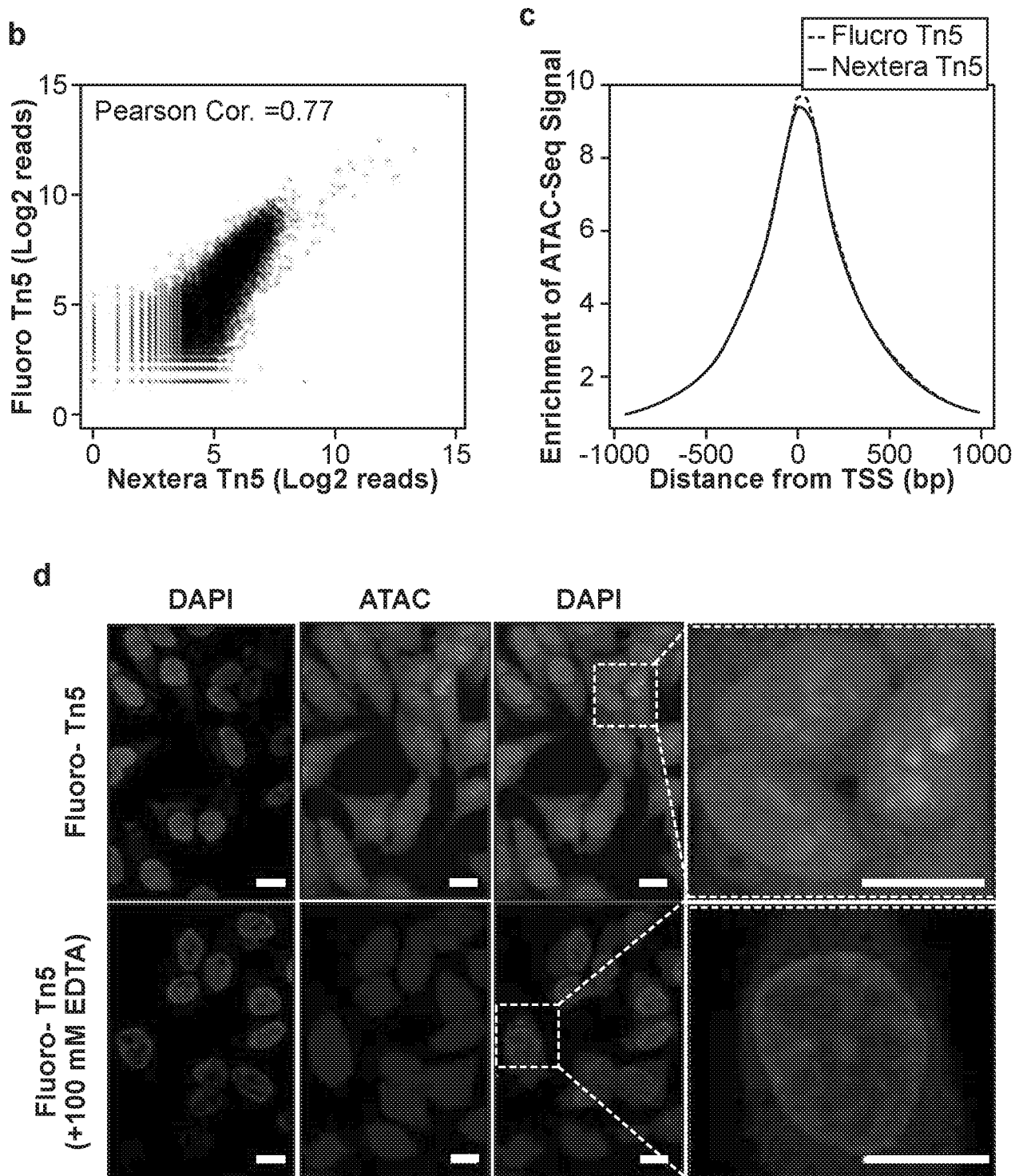


FIG. 2 (Cont.)

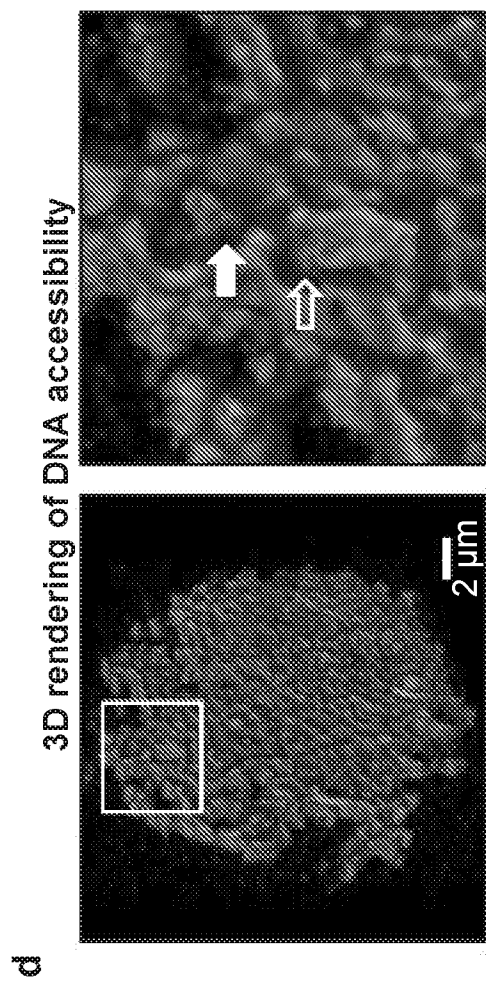
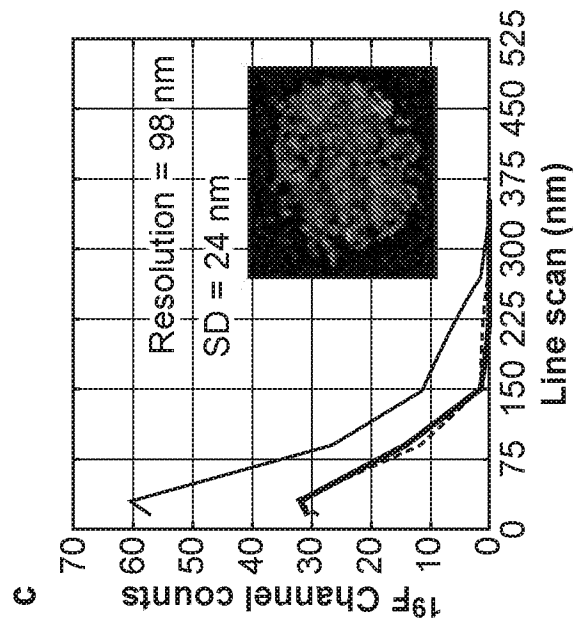
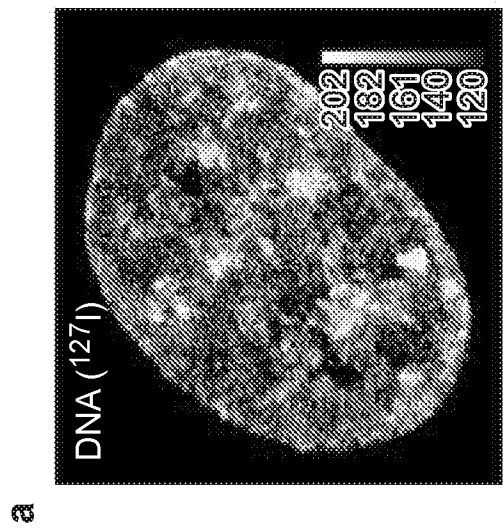
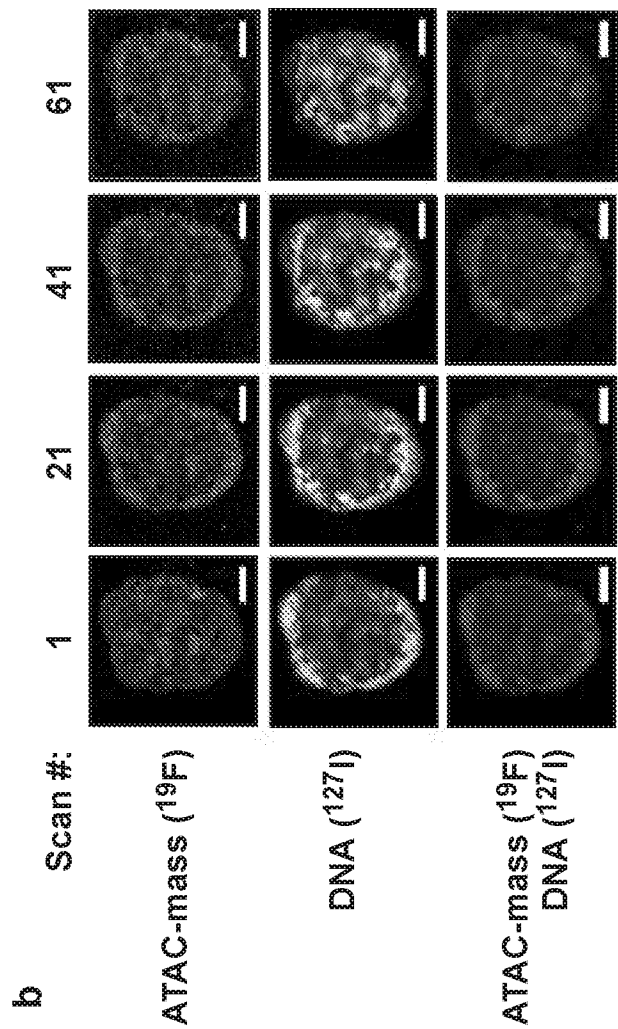


FIG. 3

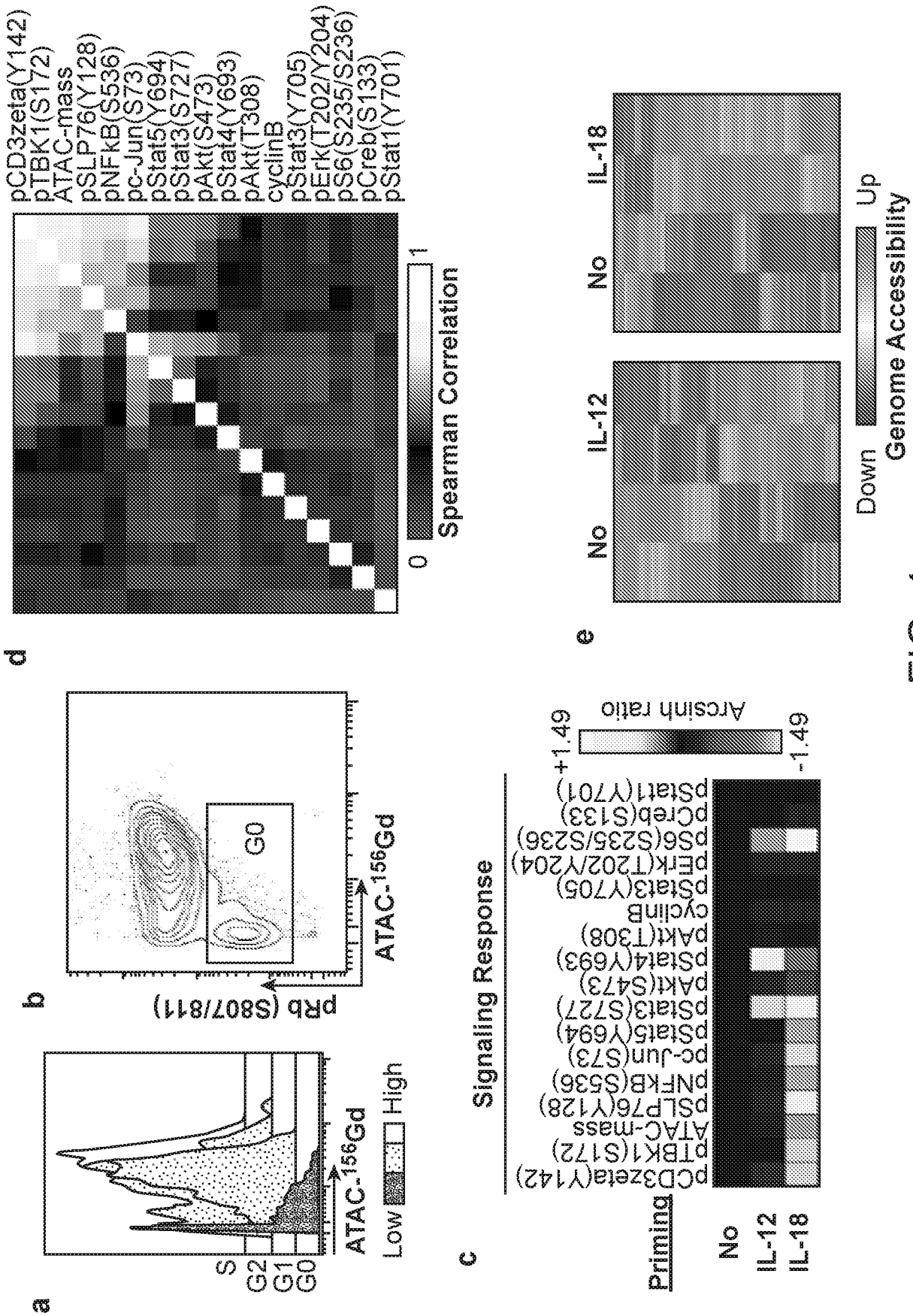


FIG. 4

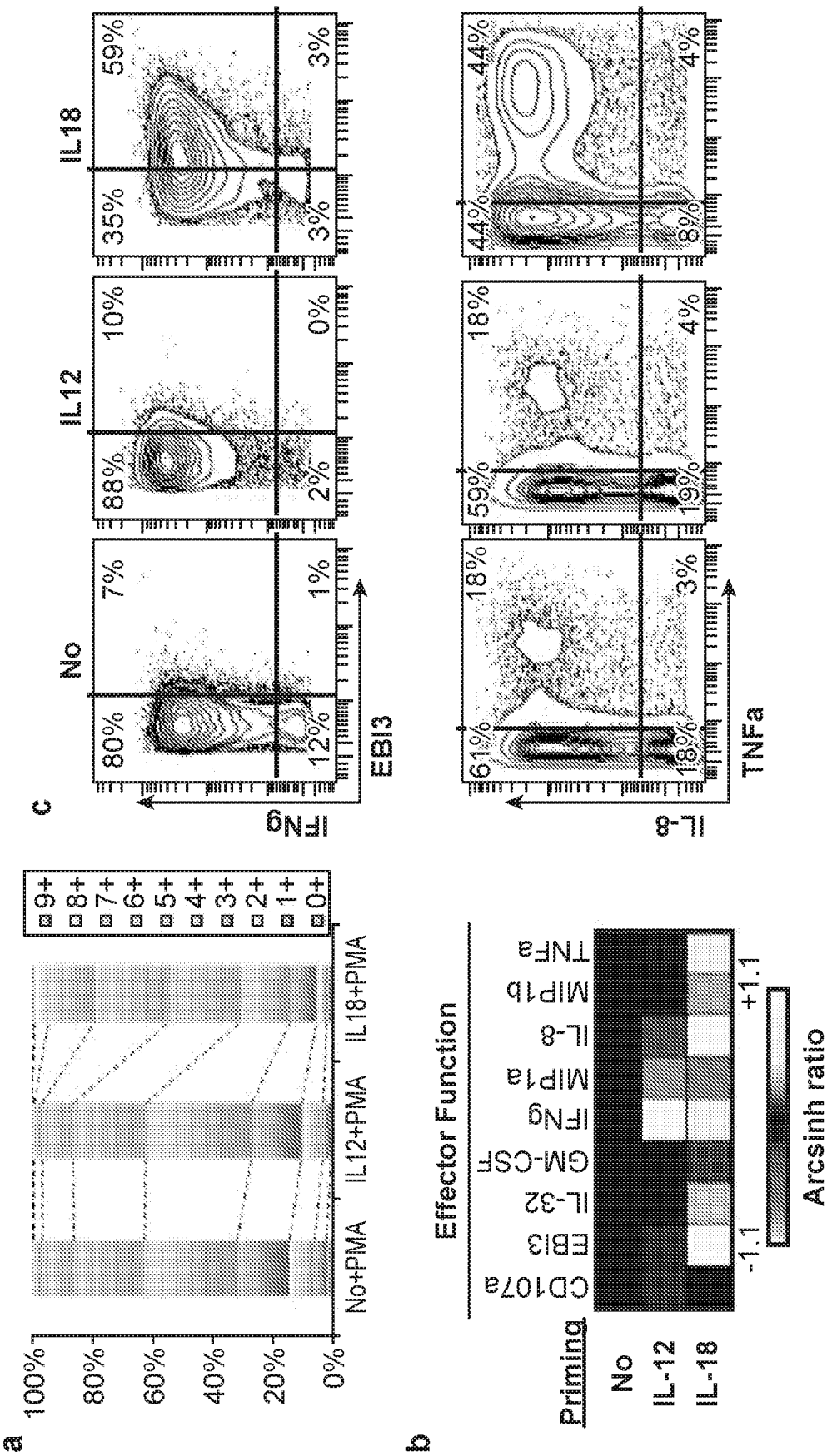


FIG. 5

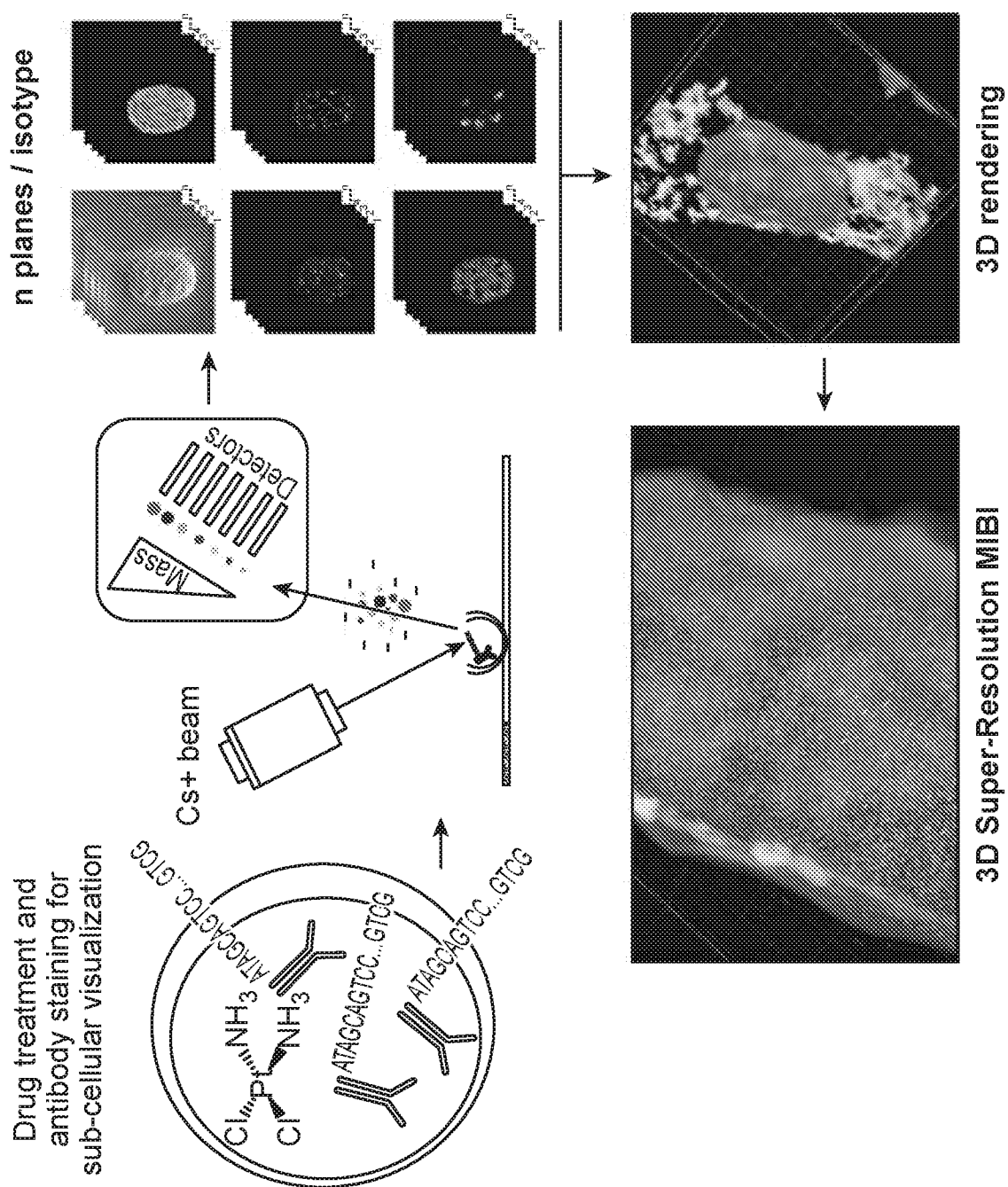


FIG. 6

8 / 69

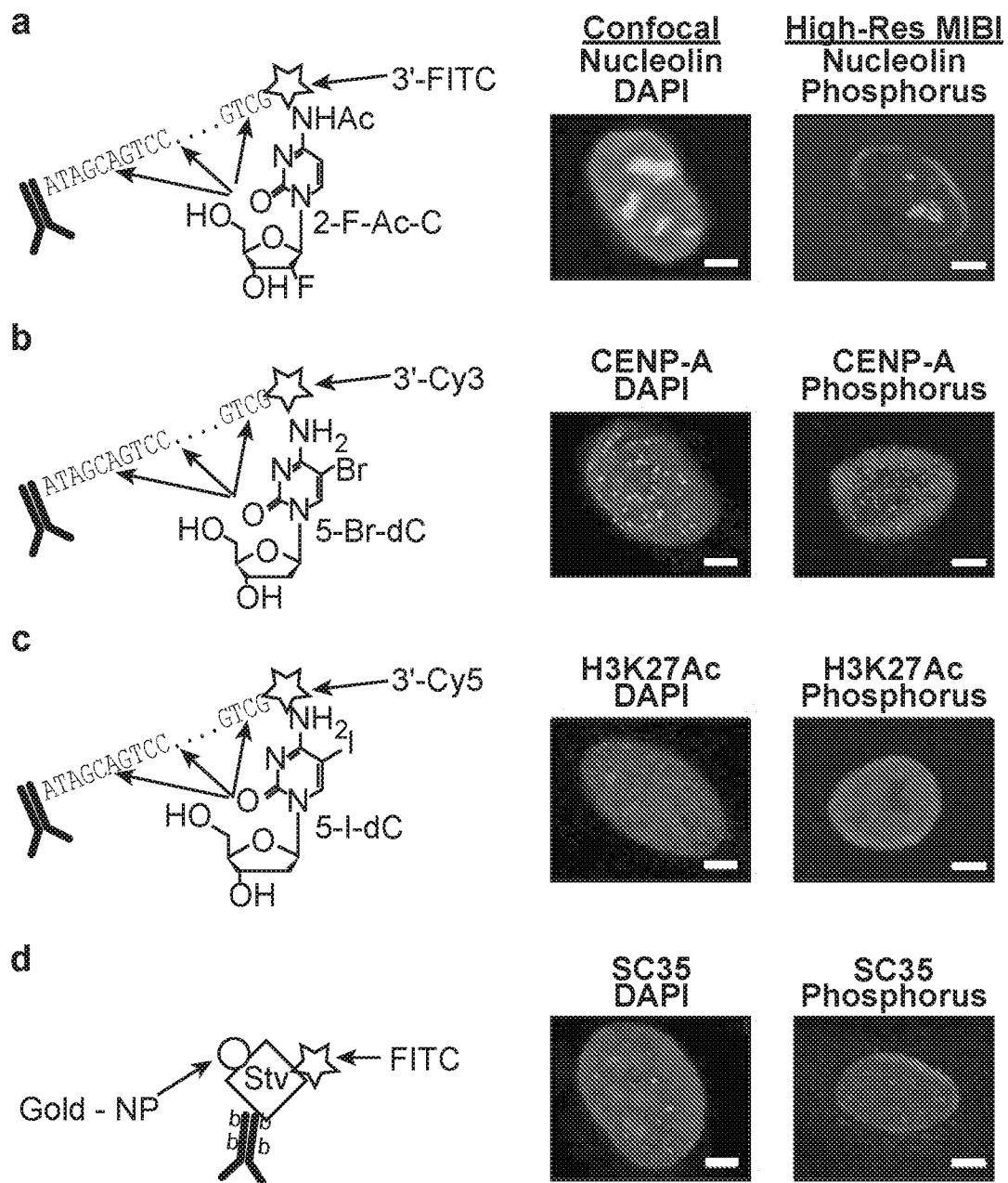


FIG. 7

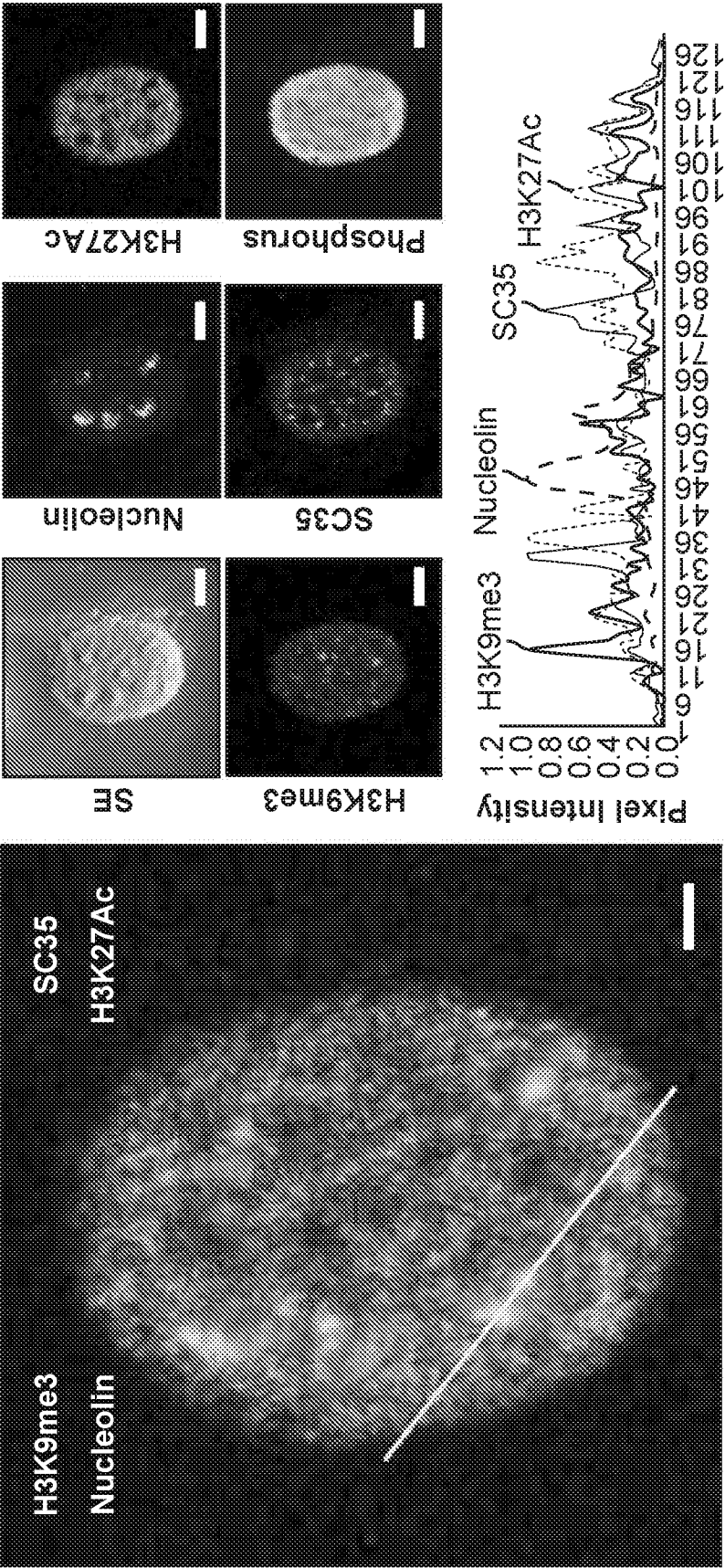


FIG. 7 (Cont.)

10 / 69

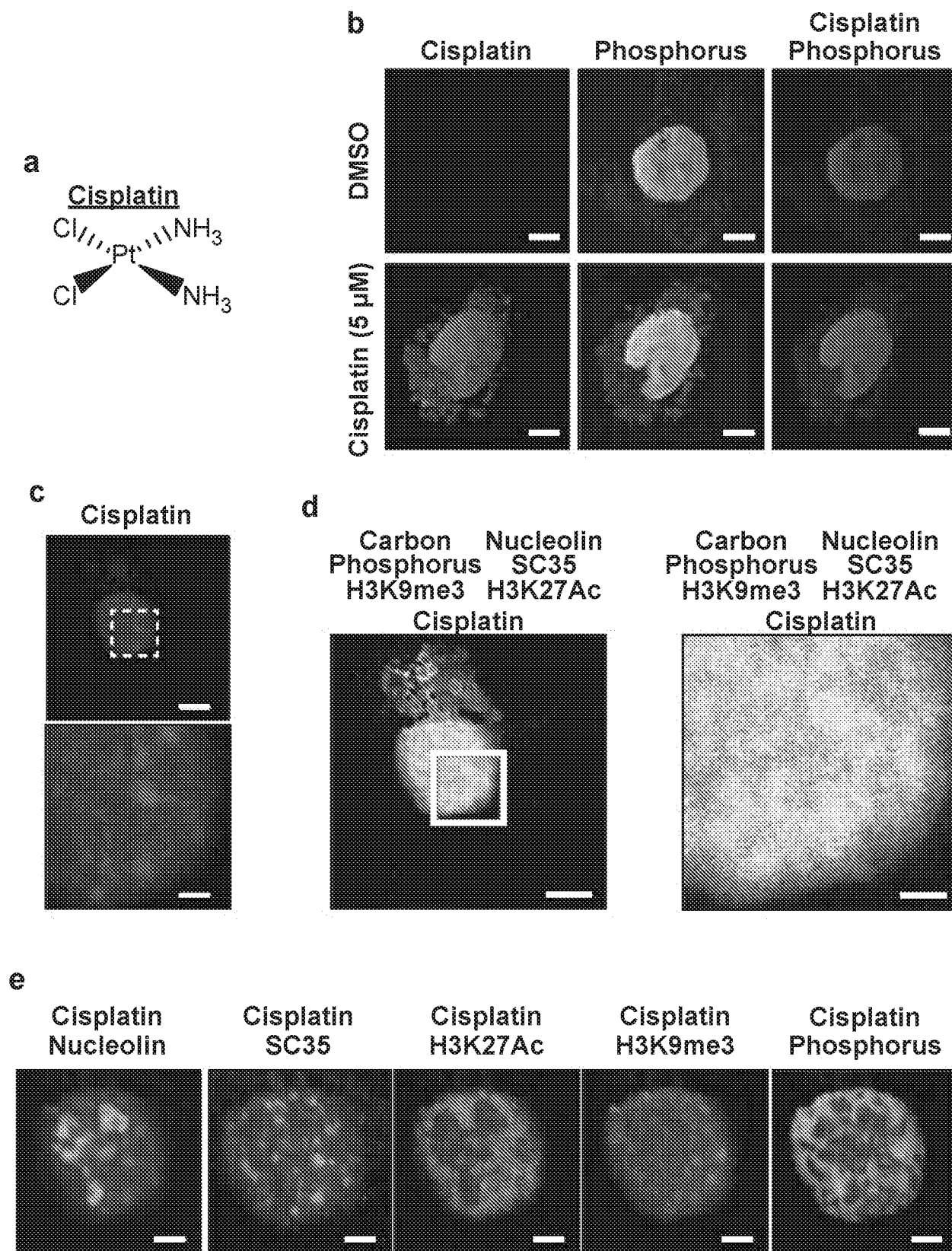
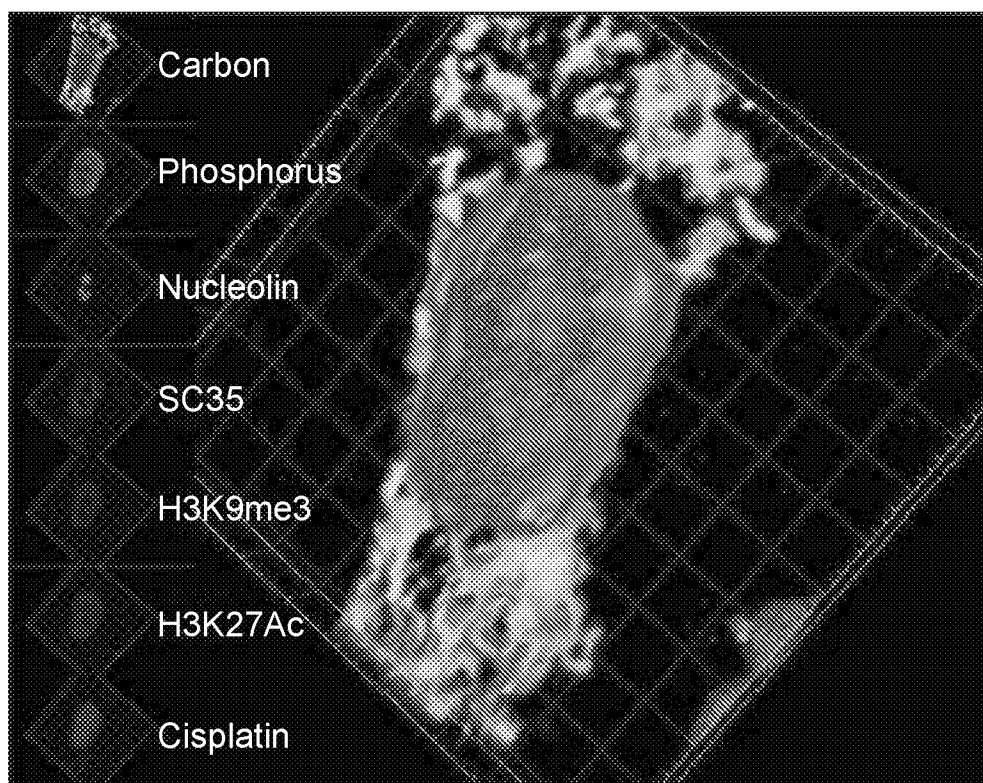


FIG. 8

11 / 69

a



b

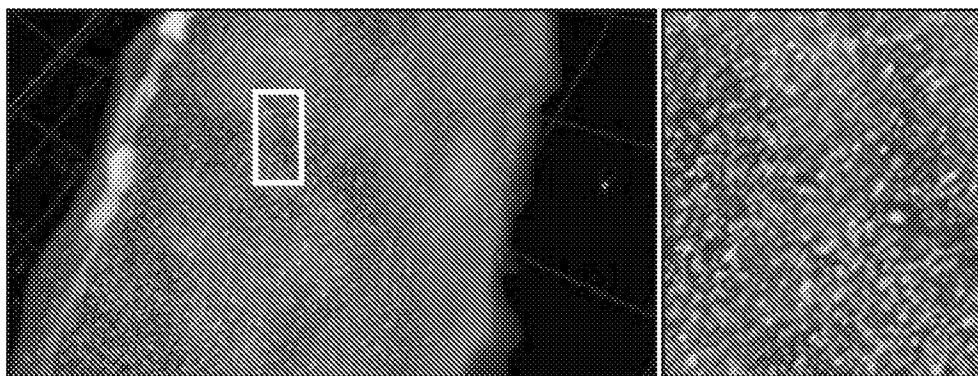


FIG. 9

12 / 69

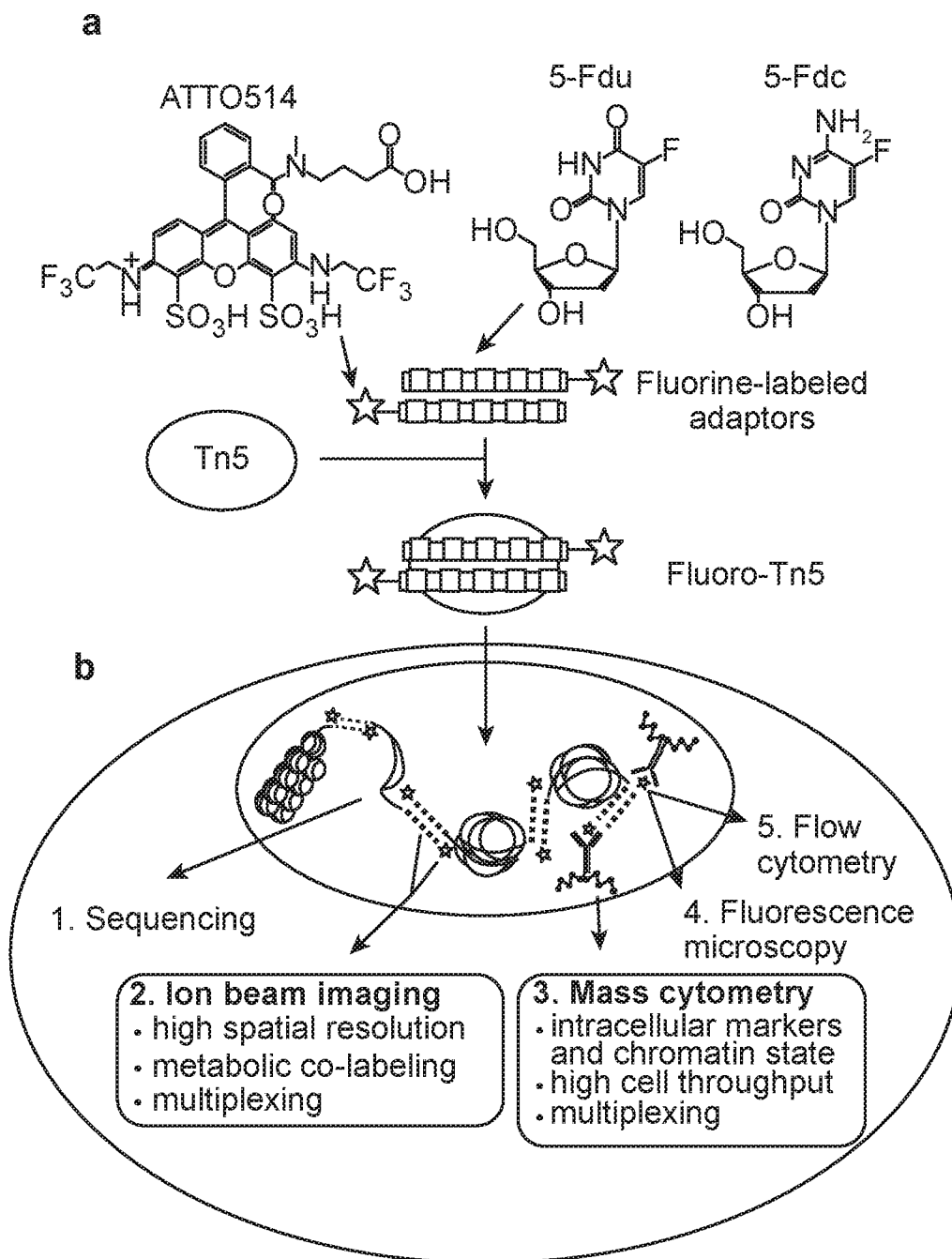


FIG. 10

13 / 69

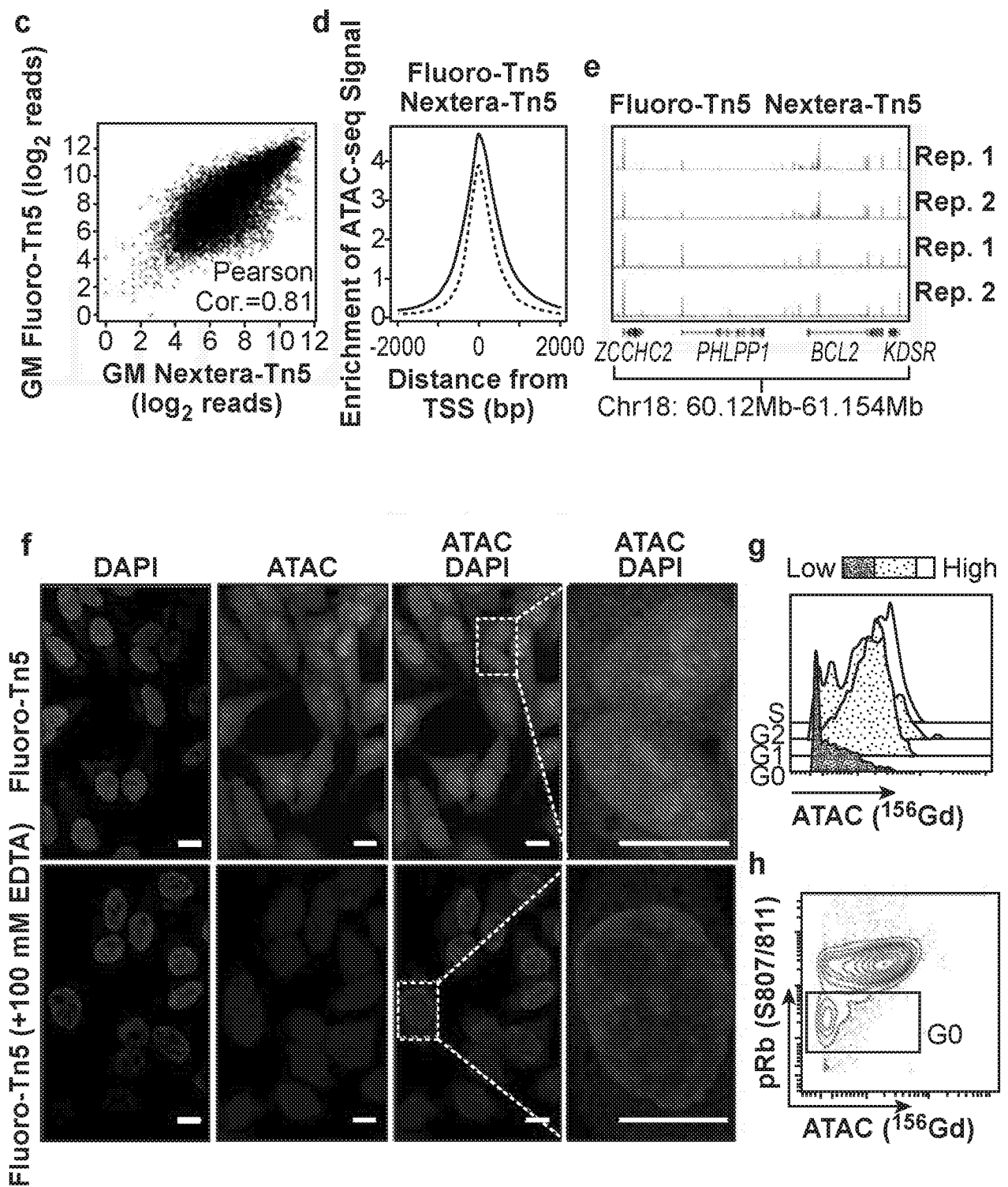


FIG. 10 (Cont.)

14 / 69

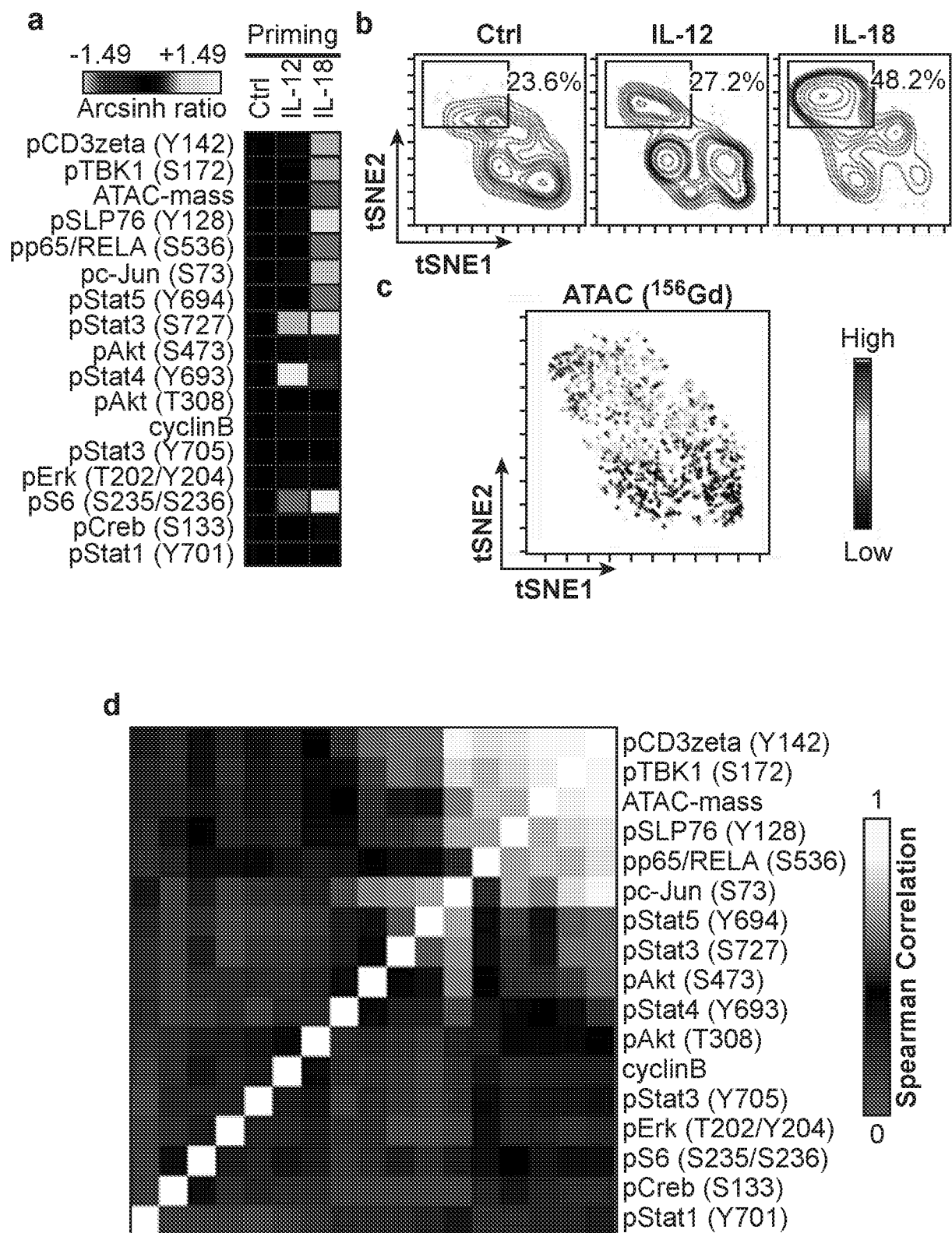


FIG. 11

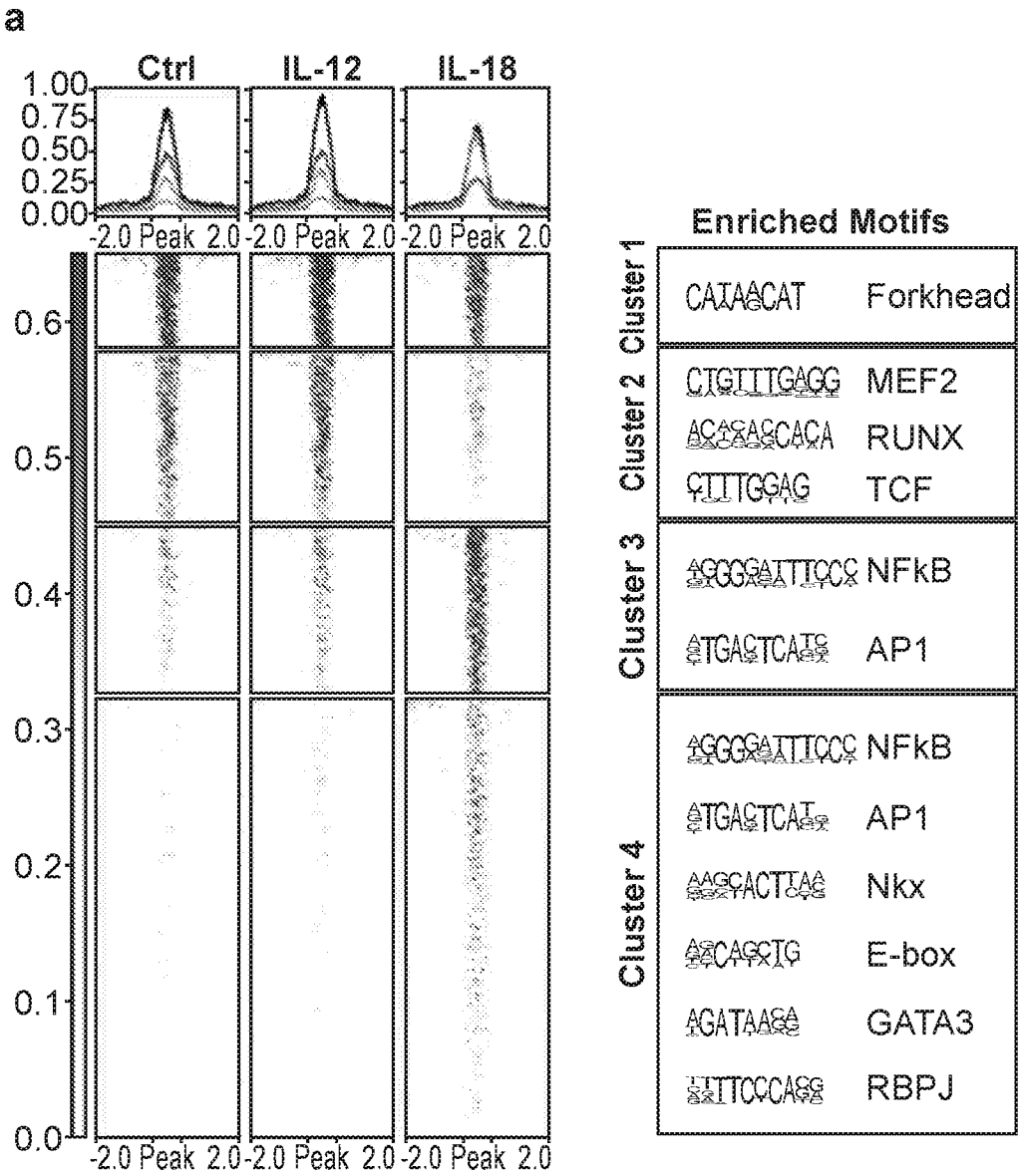


FIG. 12

16 / 69

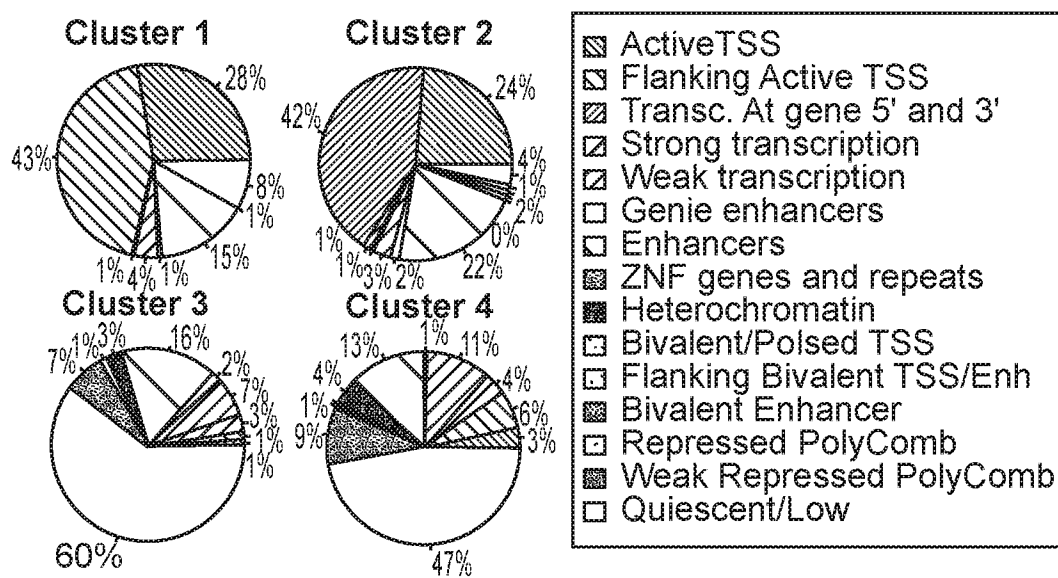
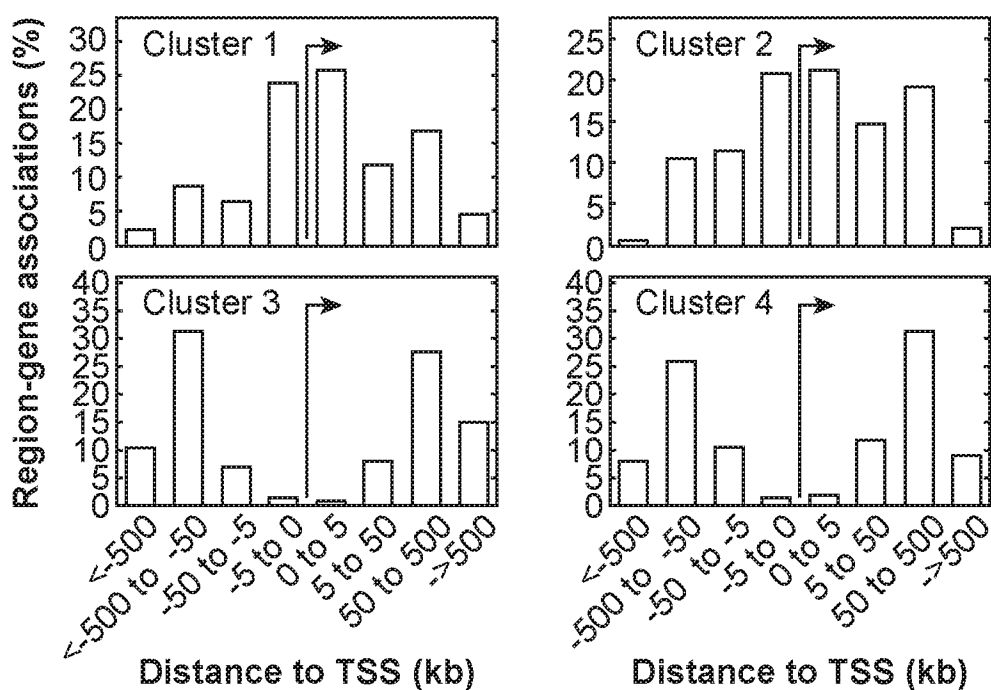
b**c**

FIG. 12 (Cont.)

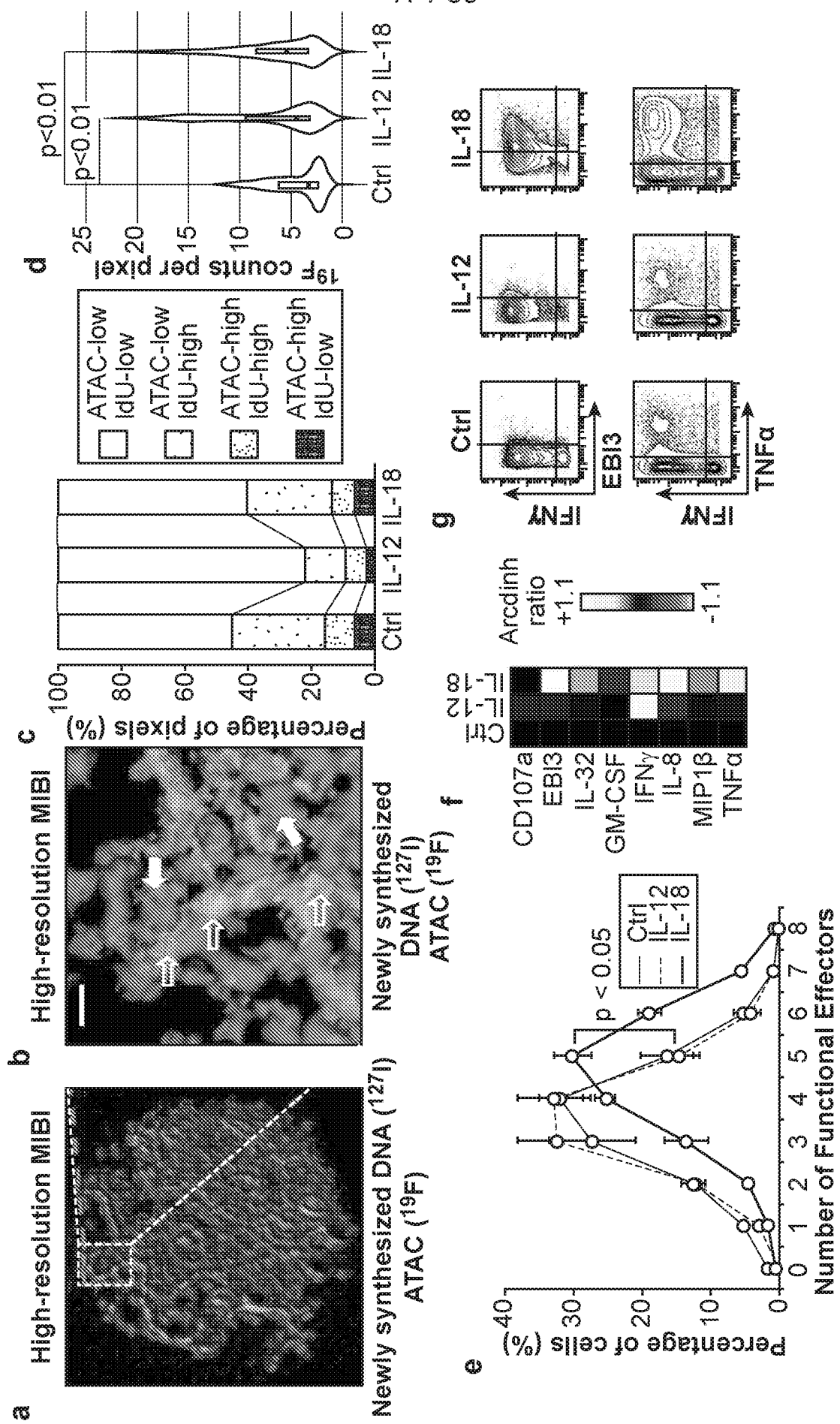


FIG. 13

18 / 69

A

1. Depth-wise MIBI acquisition of a single cell labeled with MoC-Abs or endogenous elemental masses. 2. Se

2. Secondary ions of various elemental isotopes are separated and collected on an array of detectors with X, Y and z spatial information.

Primary
Cs⁺ Beam

Secondary
ions

3. Pseudo-coloring of elemental isotopes into stacks of 2D images

5. 3D Super-Resolution Multiplexed Ion Beam Imaging

4. 2D composite images are created for each depth

6. Dimensional reduction and analysis

B

3'-FITC

ATAGCAGTCC...G

NHAc

OH

2-F-Ac-C

13F

C



Nucleolin (^{19}F)

Nucleolin (^{19}F)

Nucleolin (^{19}F)

D

H3K27Ac (^{19}F)
Phosphorus (^{31}P)

E

U.S. DEPARTMENT OF COMMERCE

~6 pA ~3 pA ~1.2 pA ~0.7 pA

Figure 1

Resolution (nm)

300
200
100
0

 $R^2 = 0.91$

FIG. 14

19 / 69

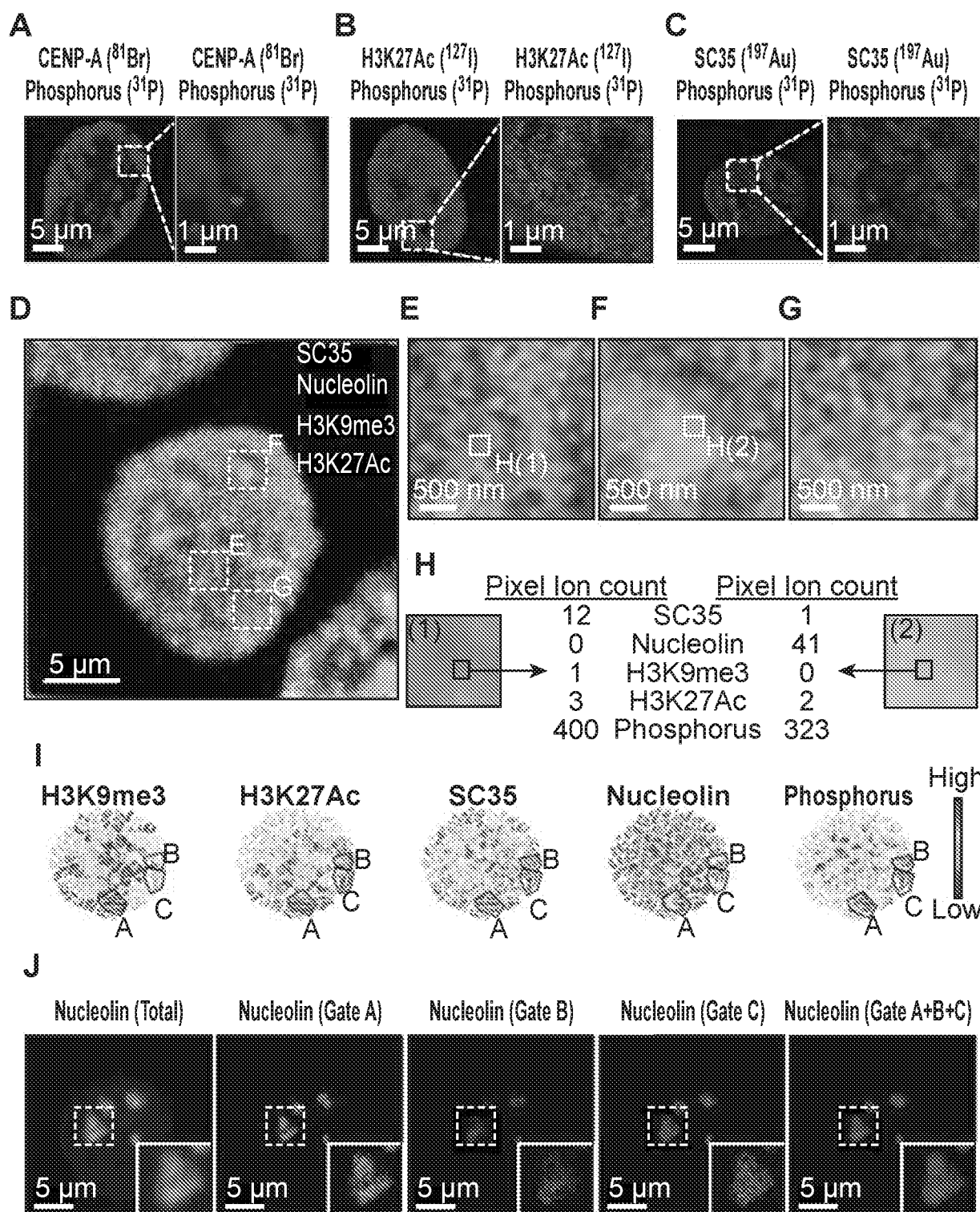


FIG. 15

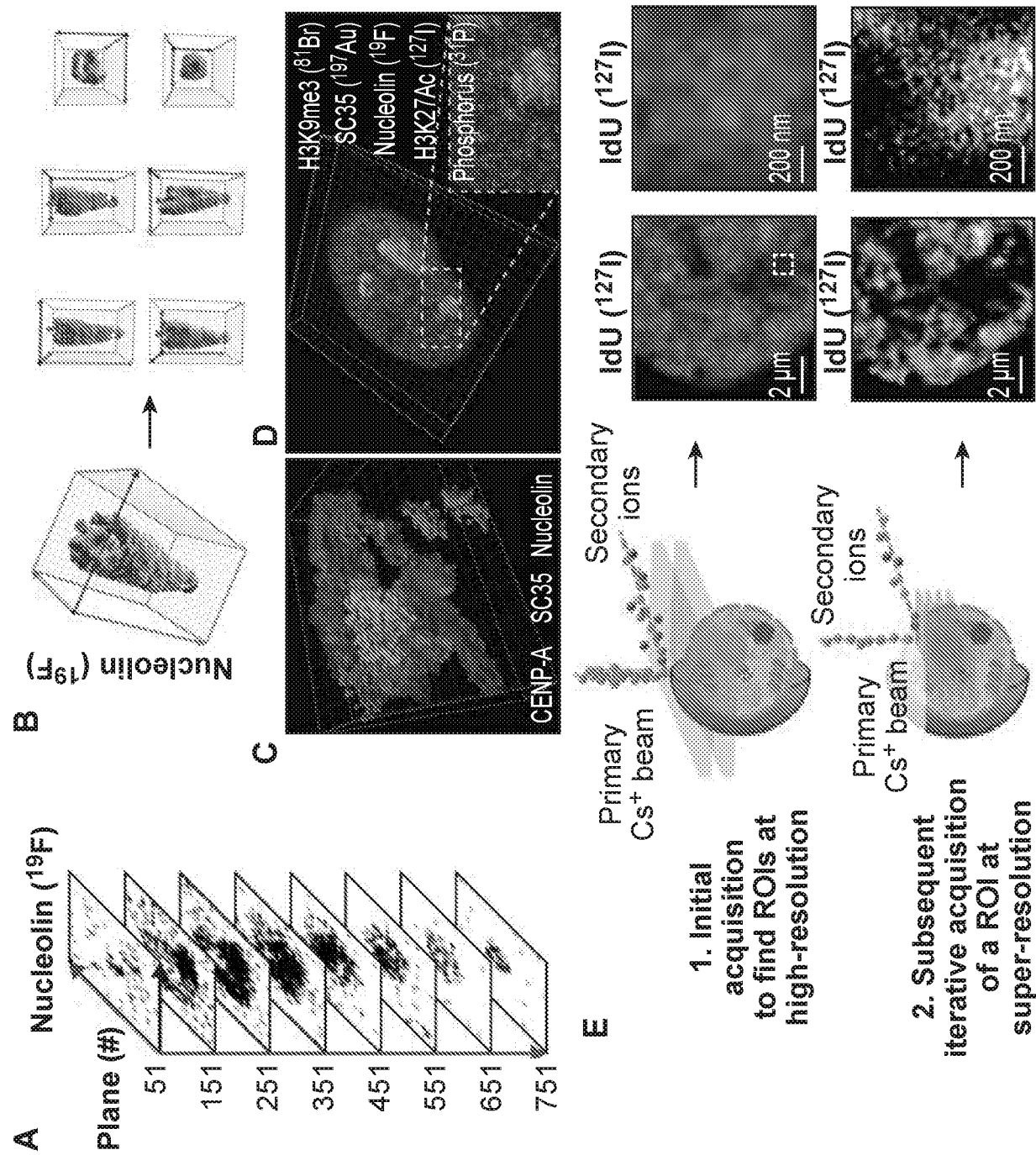
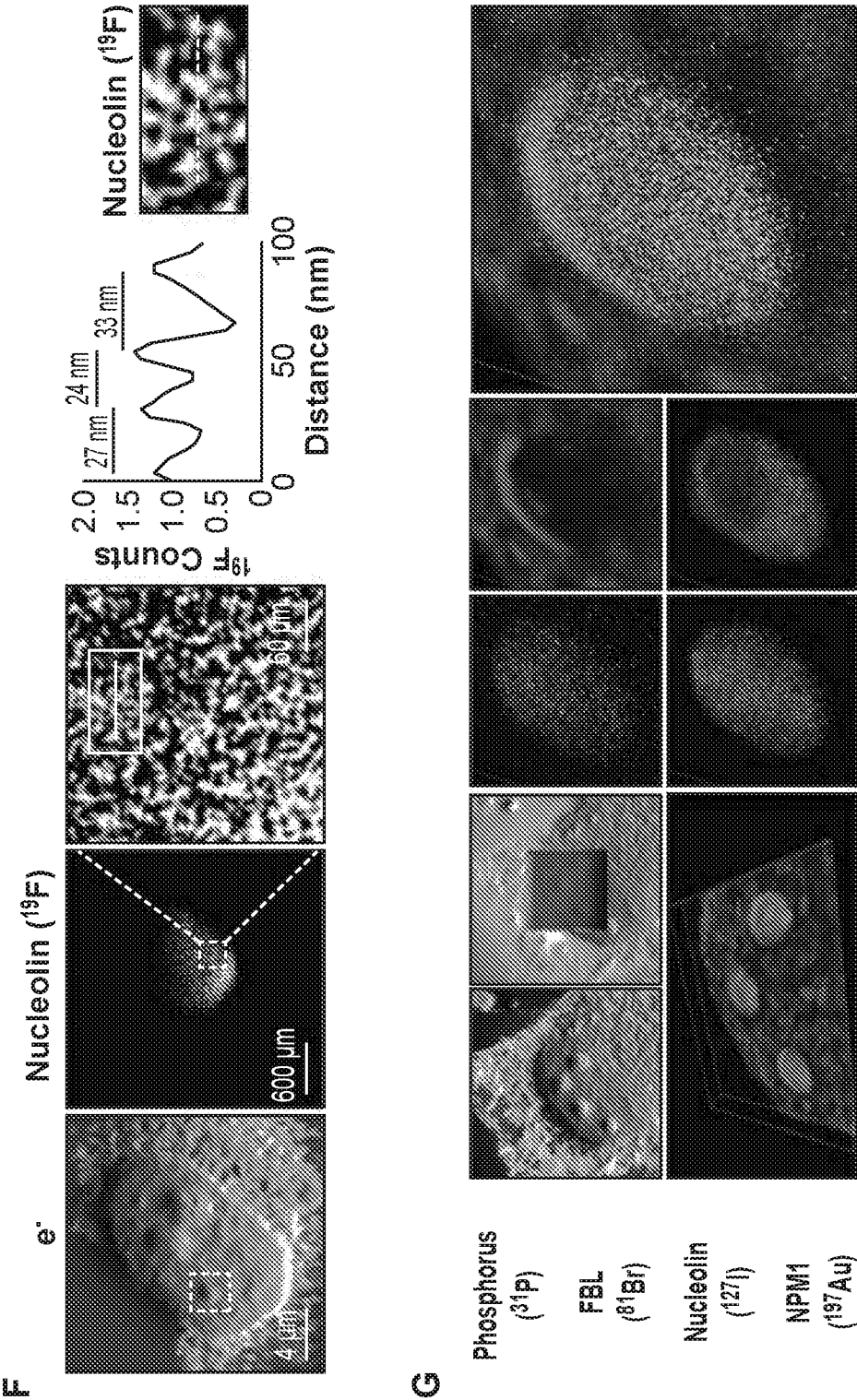


FIG. 16



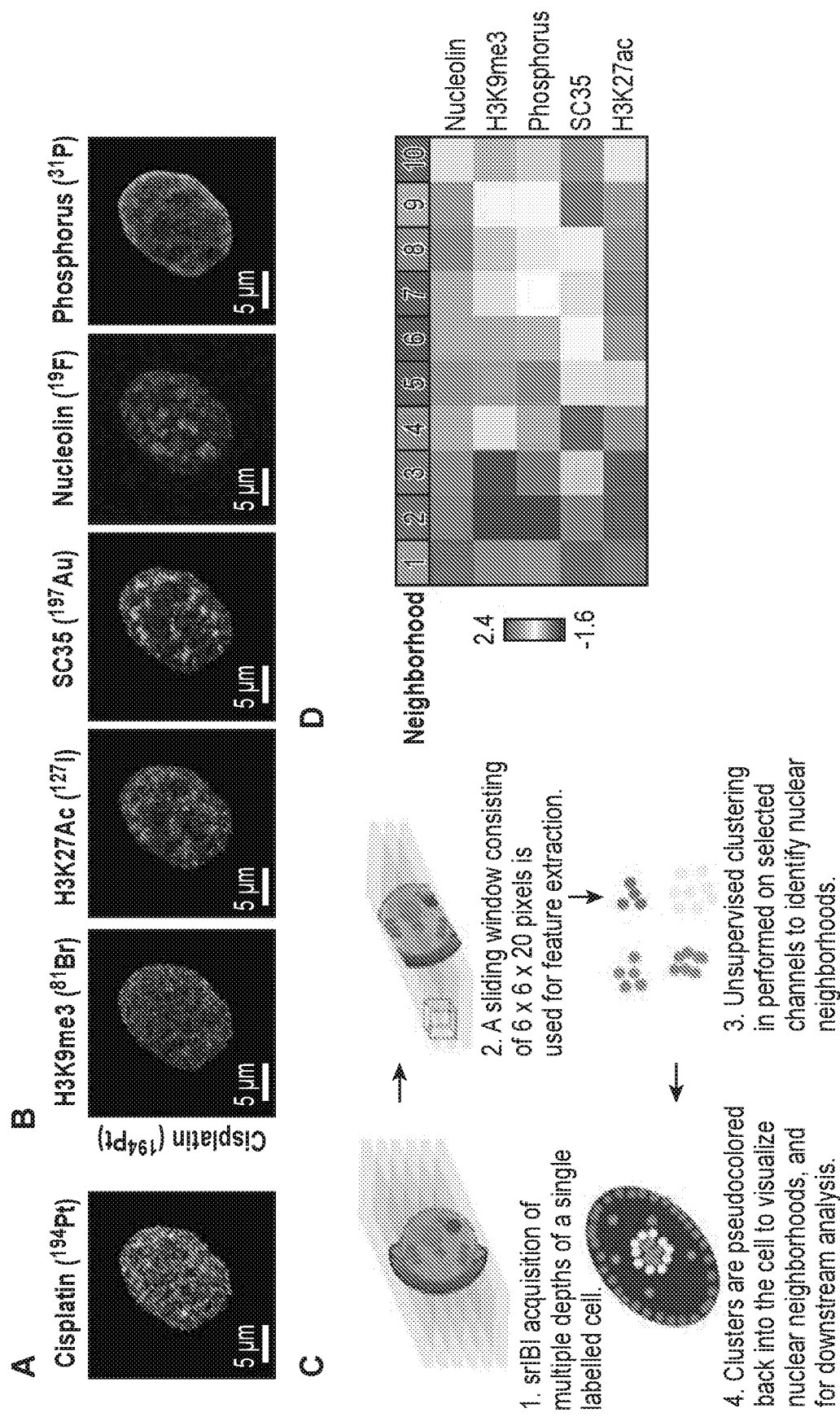


FIG. 17

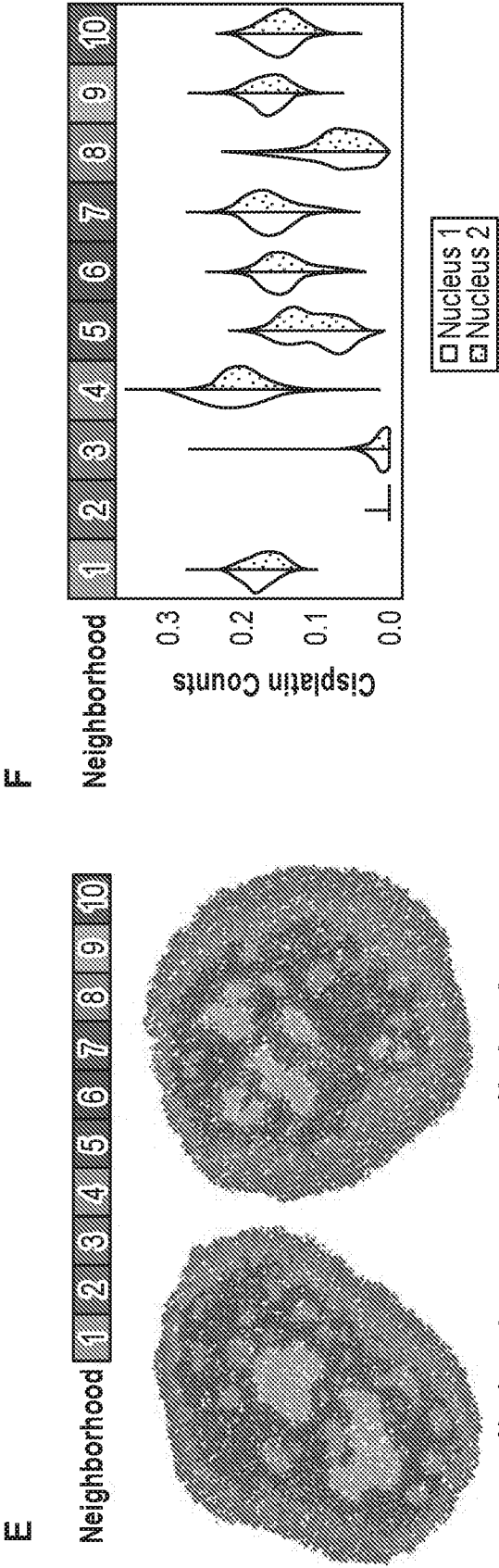


FIG. 17 (Cont.)

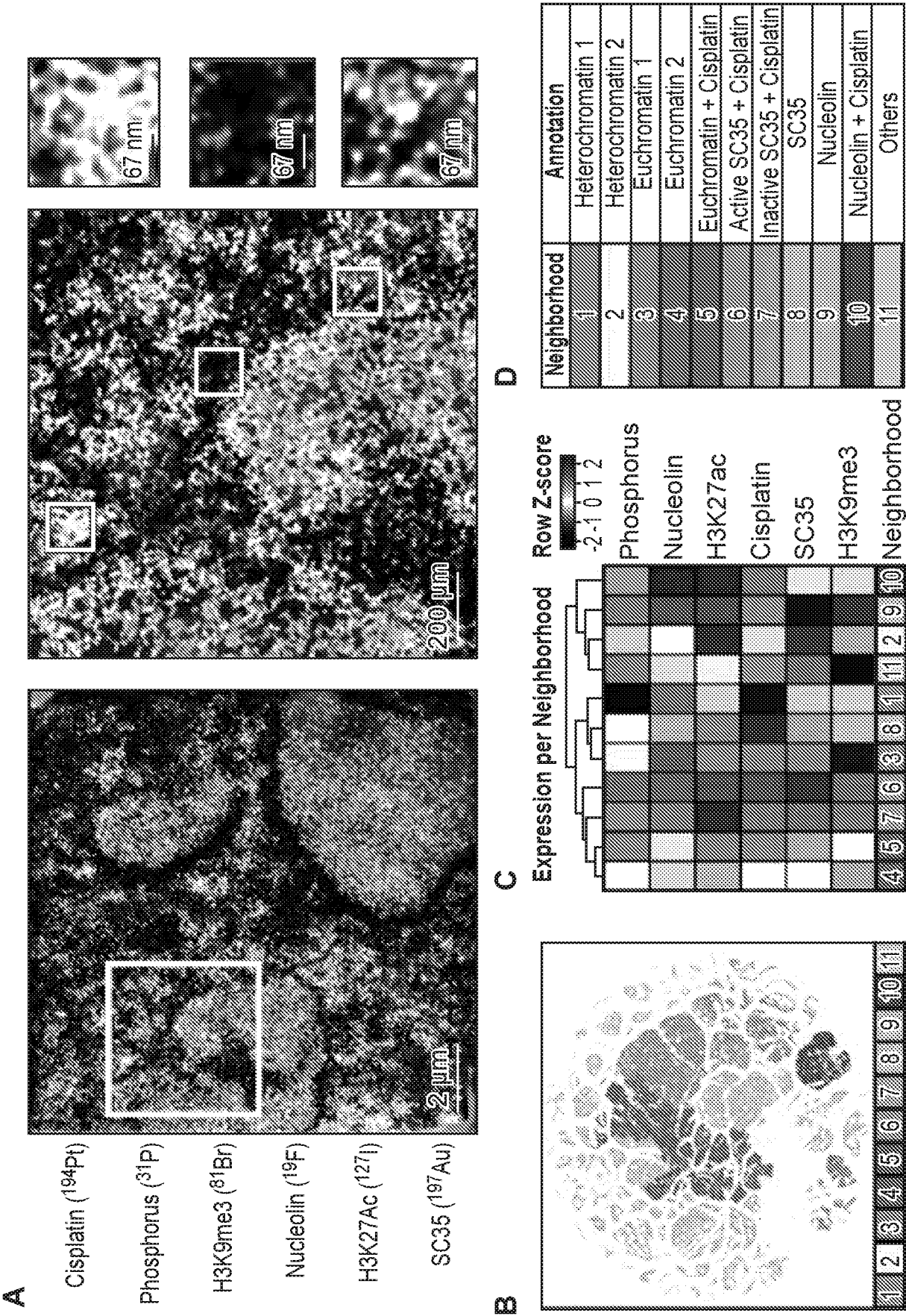


FIG. 18

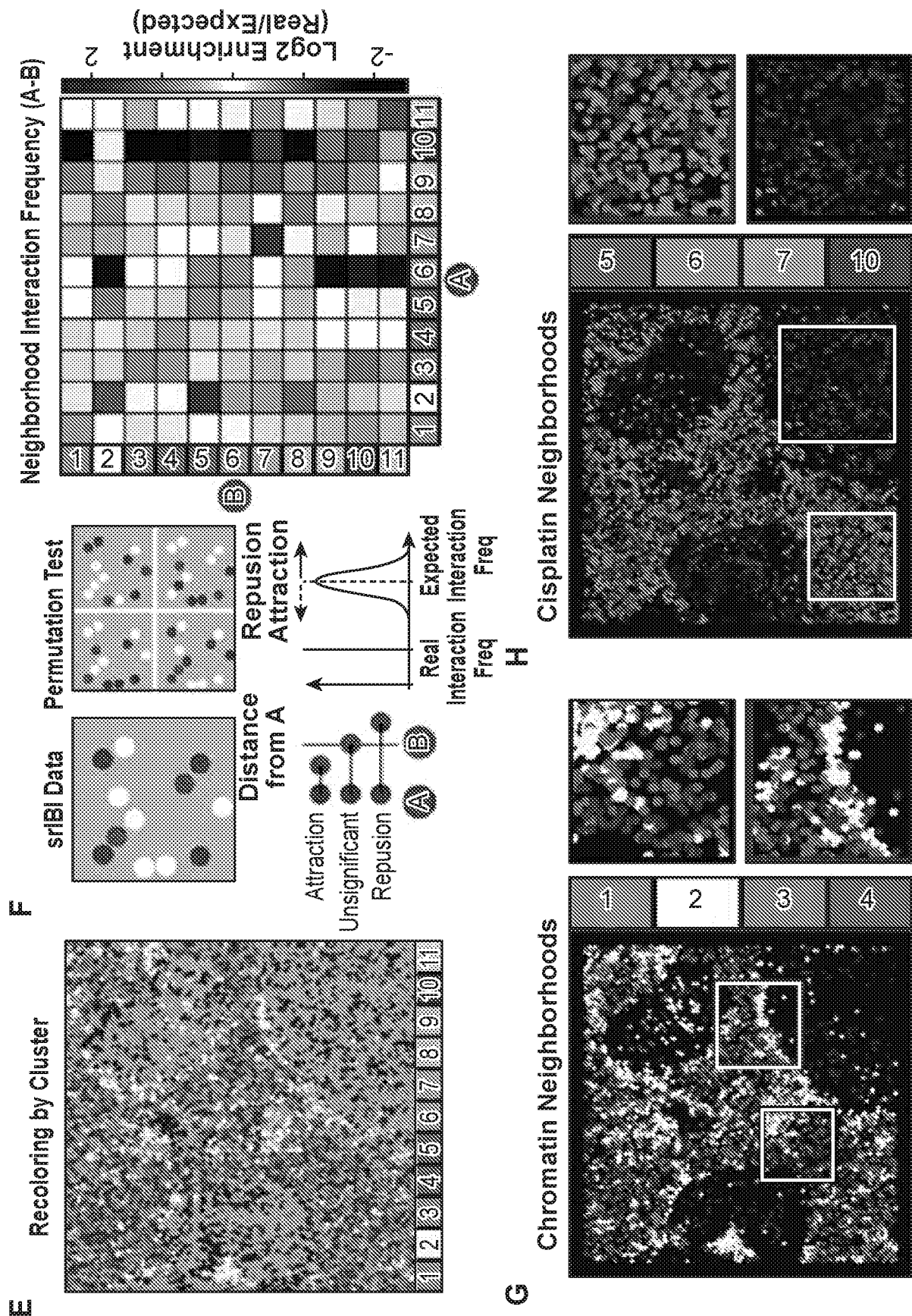


FIG. 18 (Cont.)

a

Tn5ME-A-ATTO514-5FdU → 5'- (ATTO514) (5FdU) CG (5FdU) CGGCAGCG (5FdU) CAGA (5FdU) G (5FdU) A (5FdU) AAGAGACAG-3'
Tn5ME-B-ATTO514-5FdU → 5'- (ATTO514) G (5FdU) C (5FdU) CG (5FdU) CGGACA (5FdU) G (5FdU) A (5FdU) AAGAGACAG-3'
Tn5ME-Rev-5FdU-5FdU → 5'- [phos] (5FdU) (5FdU) G (5FdU) (5FdC) (5FdU) A (5FdC) A (5FdU) (5FdC) (5FdU) -3'

b

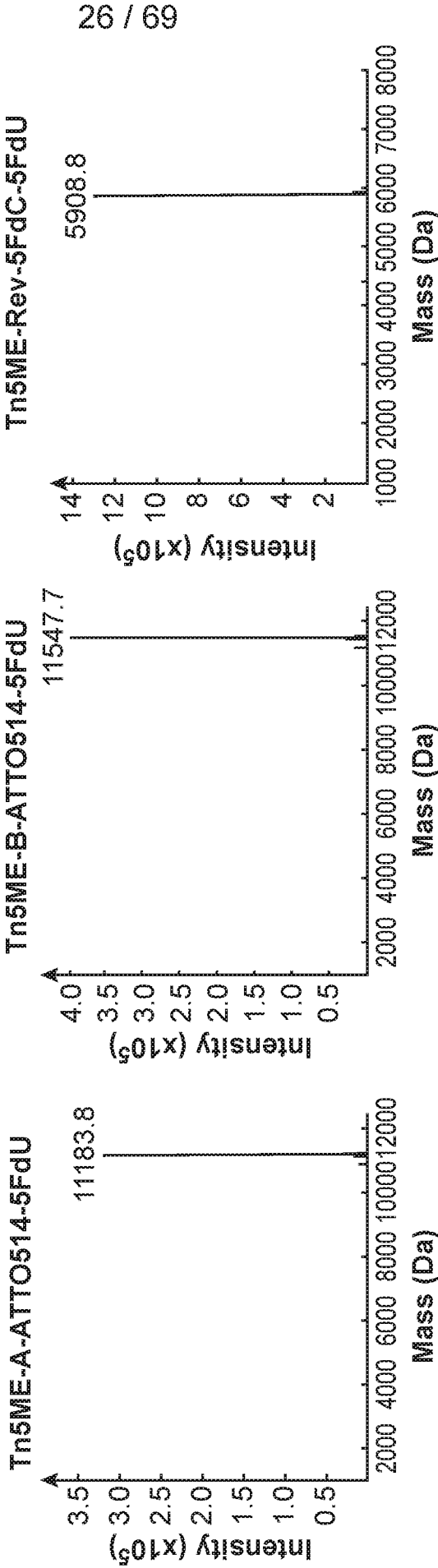


FIG. 19

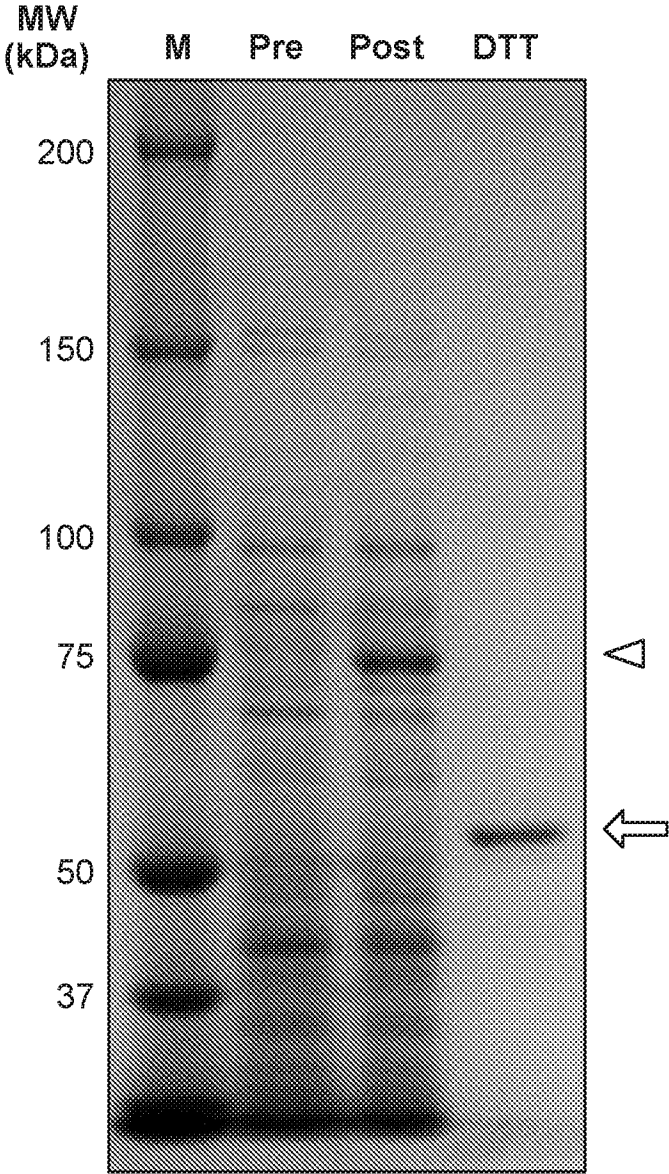
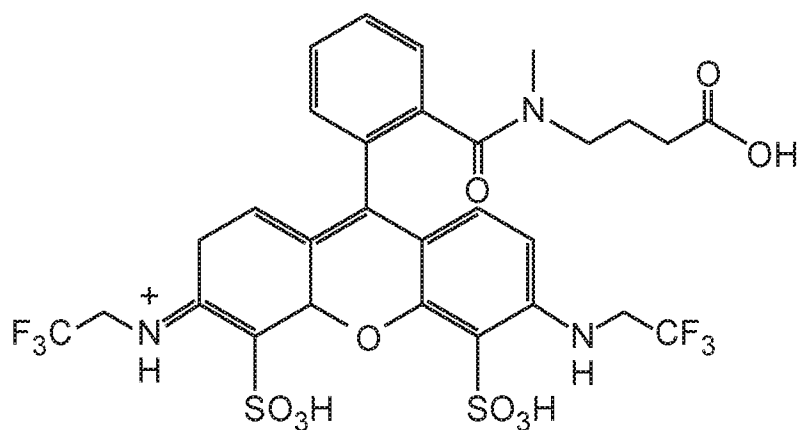
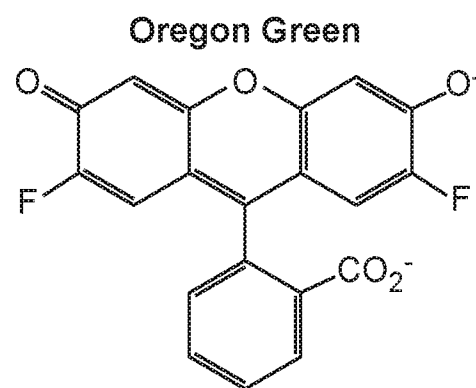
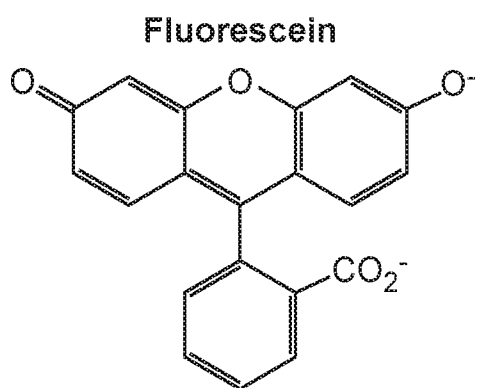


FIG. 20

28 / 69



b

Isotype Control

Anti-FITC/Oregon Green

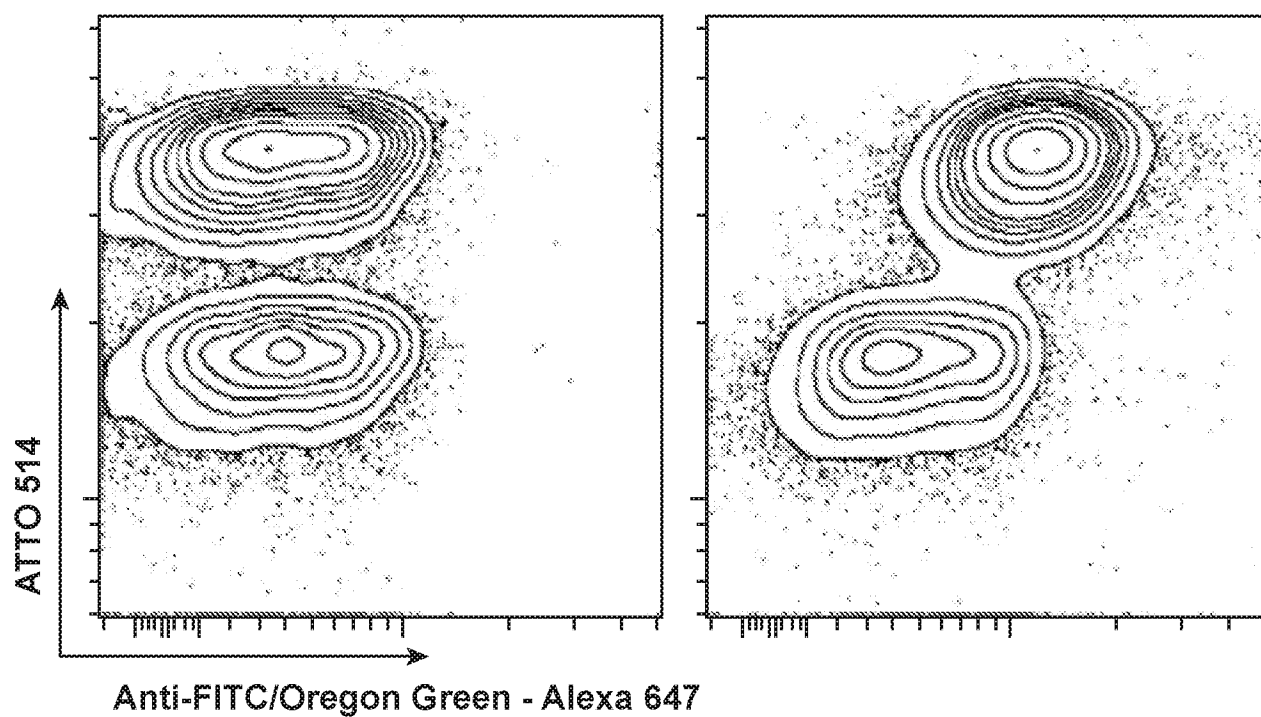


FIG. 21

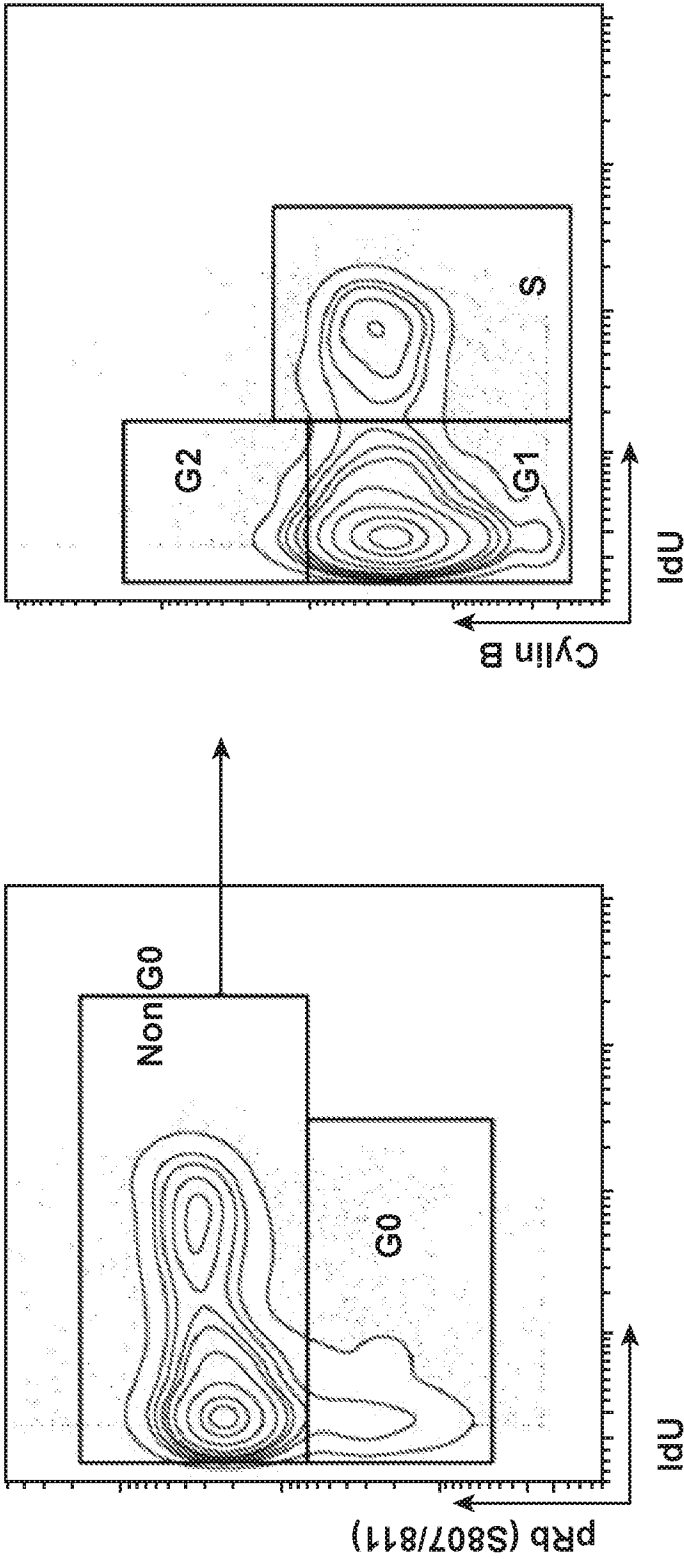


FIG. 22

30 / 69

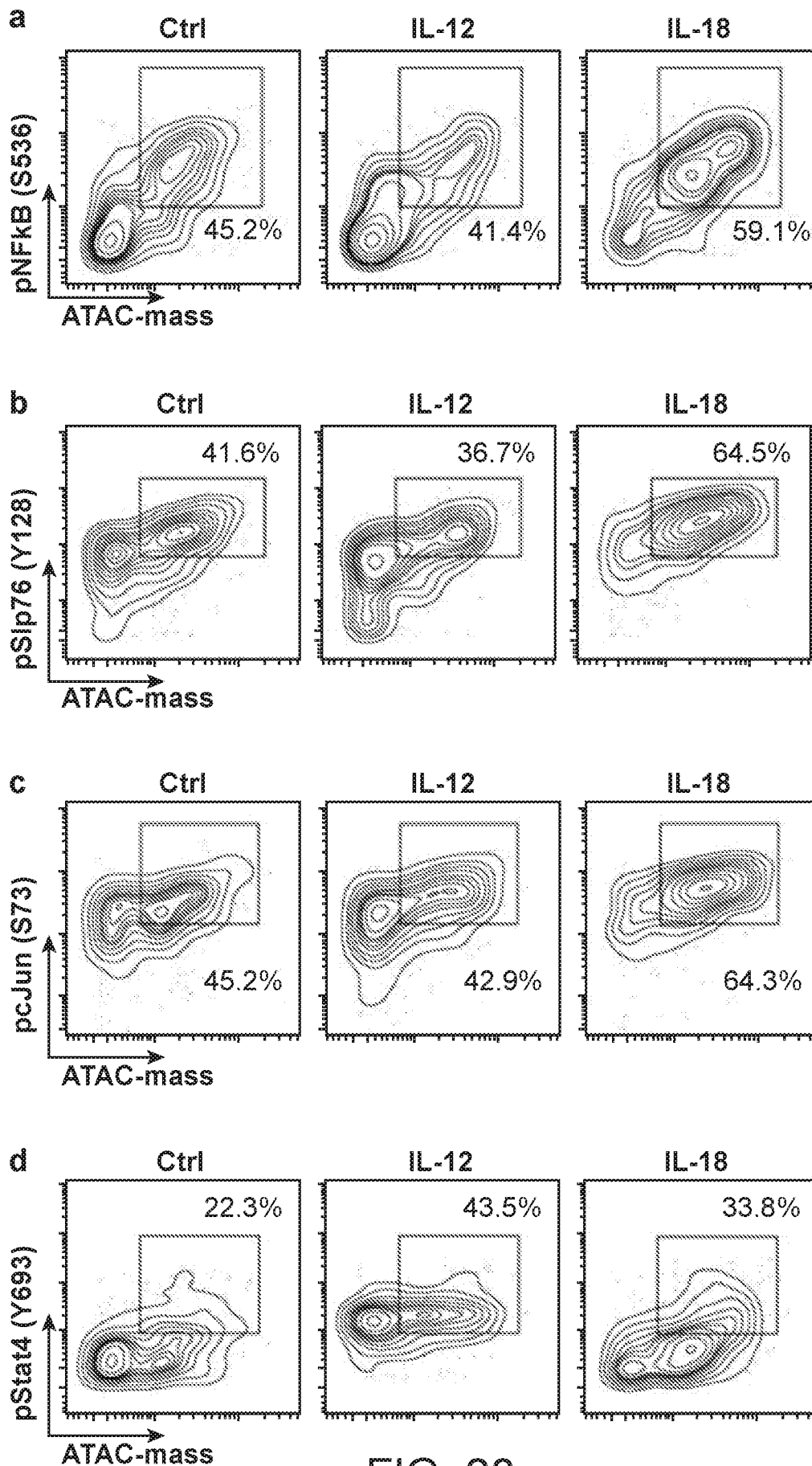


FIG. 23

31 / 69

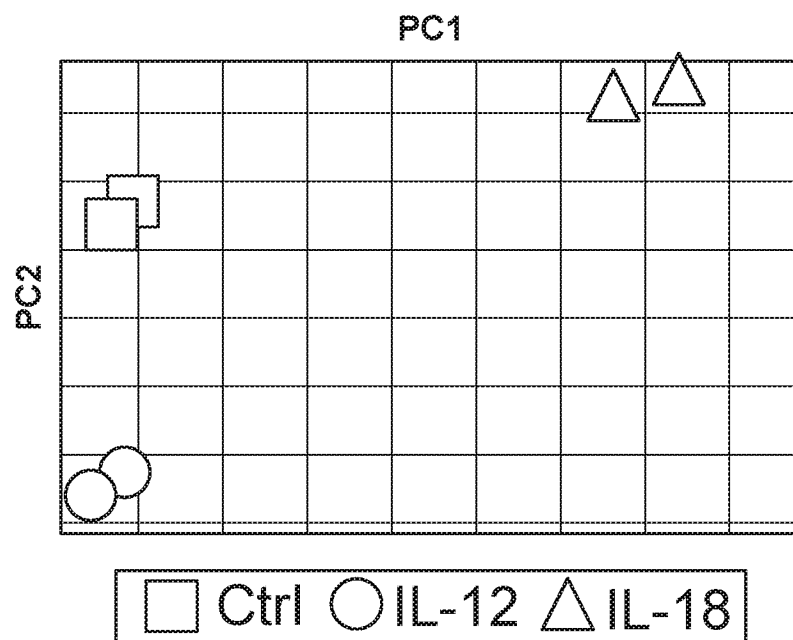


FIG. 24

32 / 69

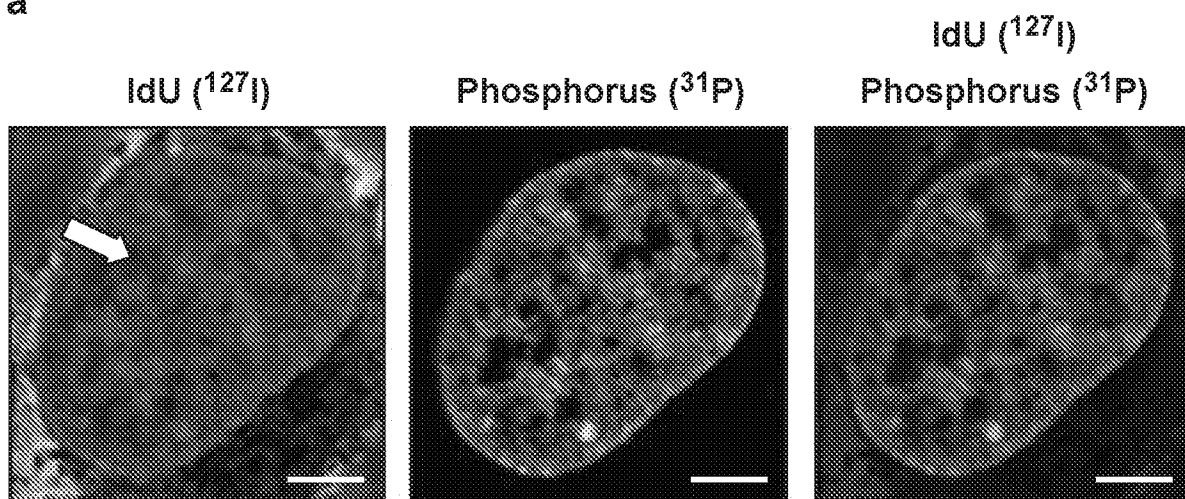
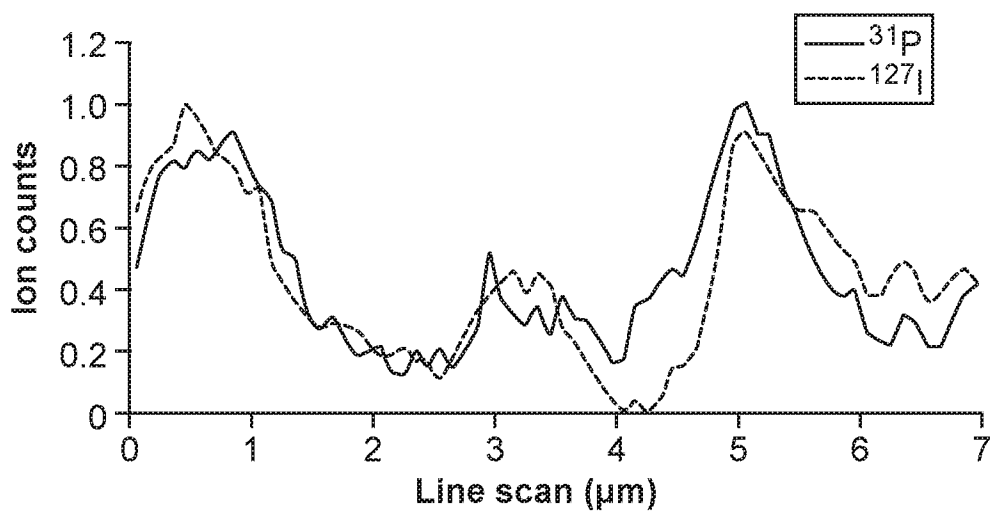
a**b**

FIG. 25

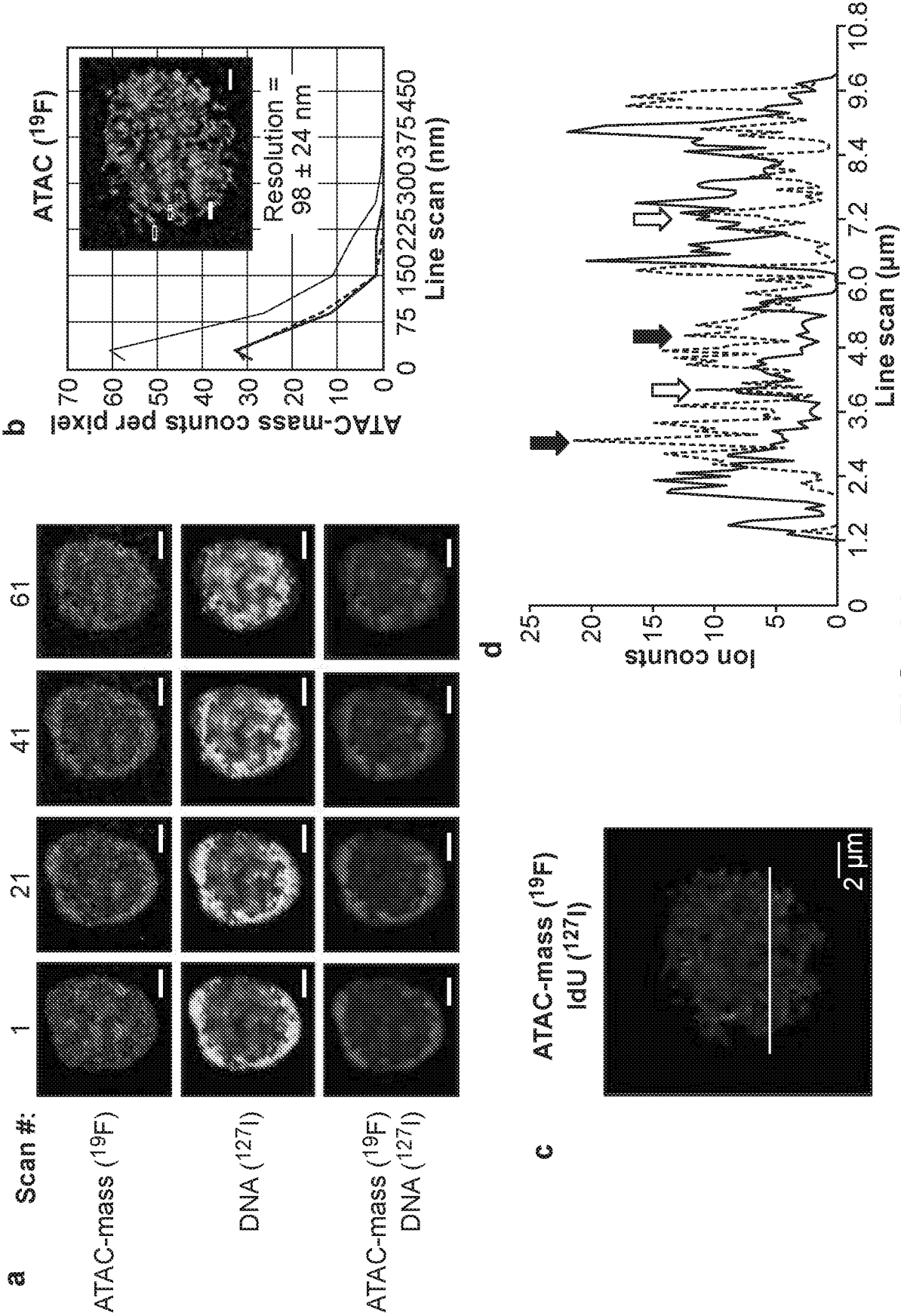


FIG. 26

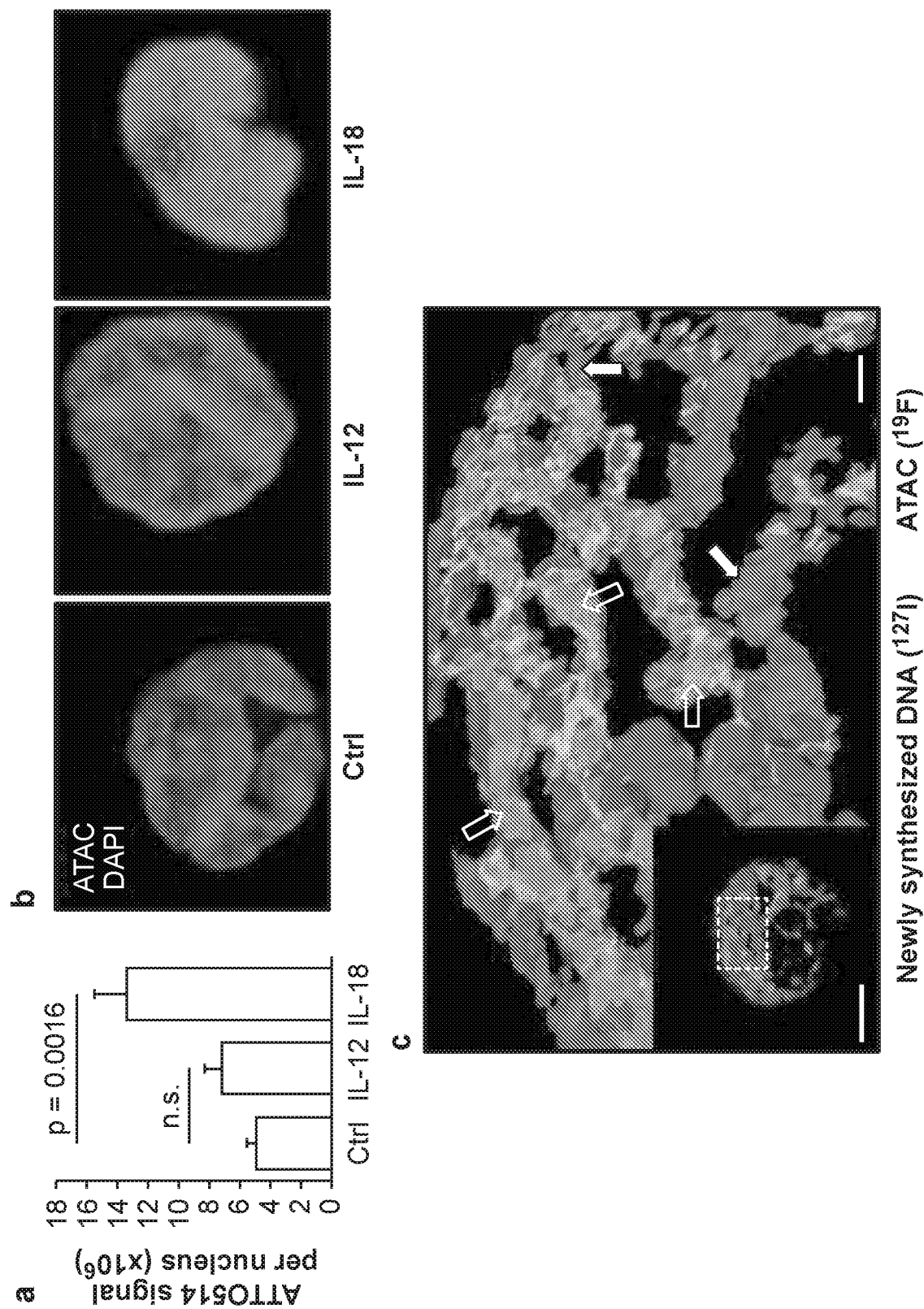
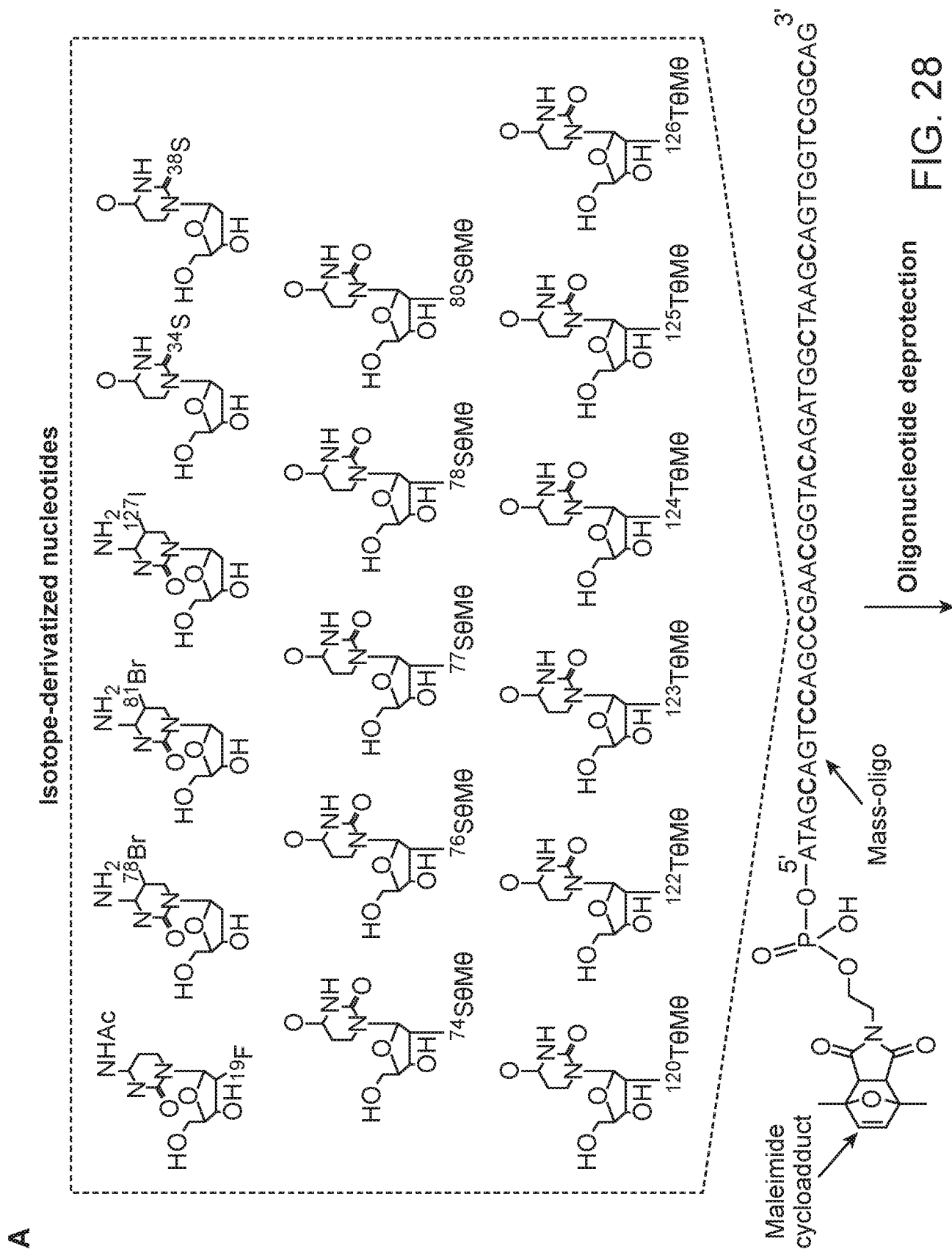


FIG. 27

35 / 69



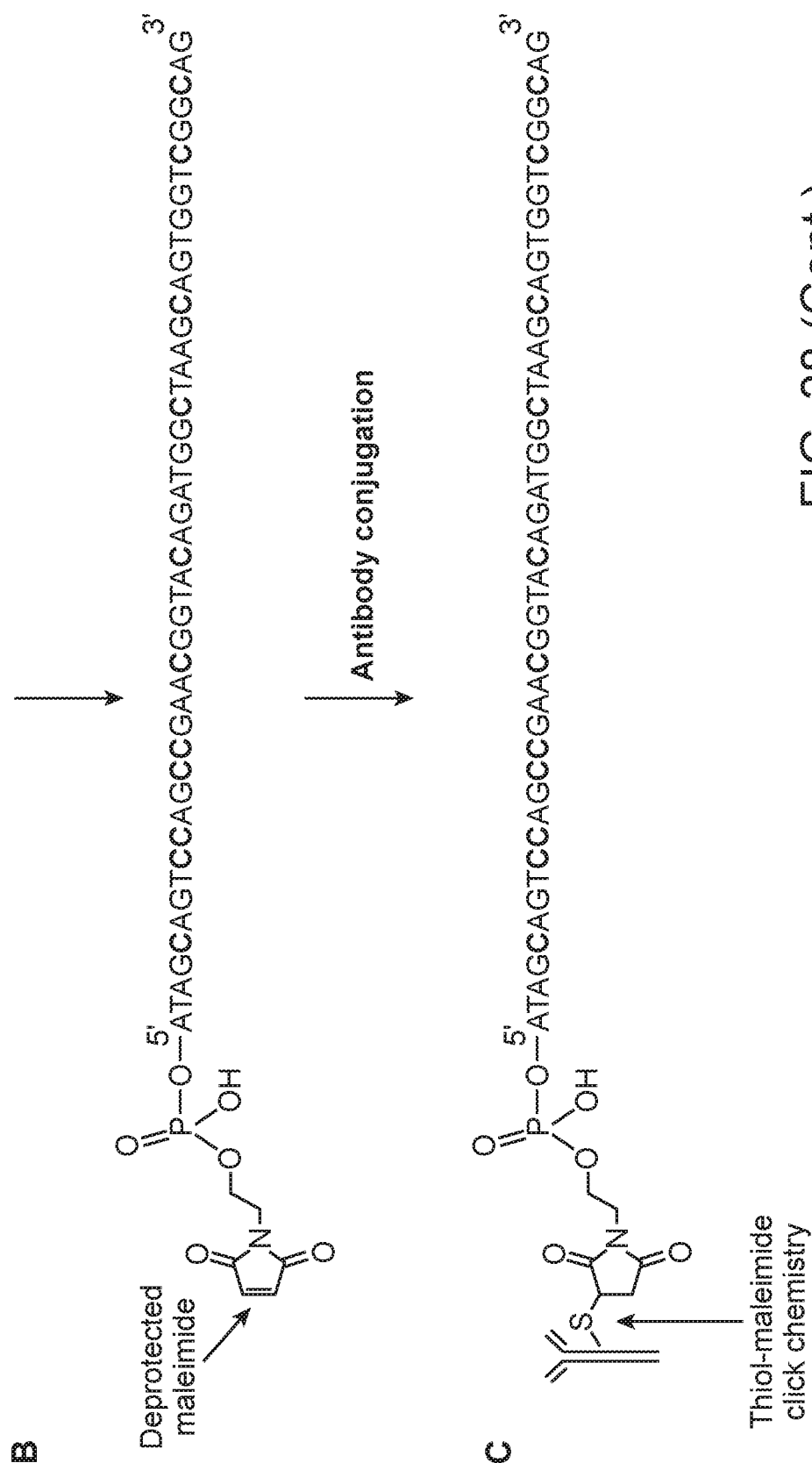


FIG. 28 (Cont.)

Sample	Fixation and Permeabilization	Blocking	Primary antibody	Secondary antibody	dsDNA	DAPI	dsDNA DAPI
1	Methanol / Acetic acid (1:1; 10 min; -20°C)	PBS (1X) Tween-20 (0.05%) FBS (5%)	anti-dsDNA (1:500)	anti-mouse-Ax657 (1:2000)			
2	Methanol / Acetic acid (1:1; 10 min; -20°C)	PBS (1X) Tween-20 (0.05%) FBS (5%)	anti-dsDNA- ¹⁶² Dy (1:50)	anti-mouse-Ax657 (1:2000)			
3	Methanol / Acetic acid (1:1; 10 min; -20°C)	PBS (1X) BSA (0.5%) EDTA (5 mM) NaCl (0.5 M) Salmon sperm DNA (200 µg/mL) Human TruStain FcX (20 µg/mL)	anti-dsDNA- ¹⁶² Dy (1:50)	anti-mouse-Ax657 (1:2000)			
4	Methanol / Acetic acid (1:1; 10 min; -20°C)	PBS (1X) Tween-20 (0.05%) FBS (5%)	anti-dsDNA- ¹²⁷ I (1:50)	anti-mouse-Ax657 (1:2000)			
5	Methanol / Acetic acid (1:1; 10 min; -20°C)	PBS (1X) BSA (0.5%) EDTA (5 mM) NaCl (0.5 M) Salmon sperm DNA (200 µg/mL) Human TruStain FcX (20 µg/mL)	anti-dsDNA- ¹²⁷ I (1:50)	anti-mouse-Ax657 (1:2000)			

FIG. 29

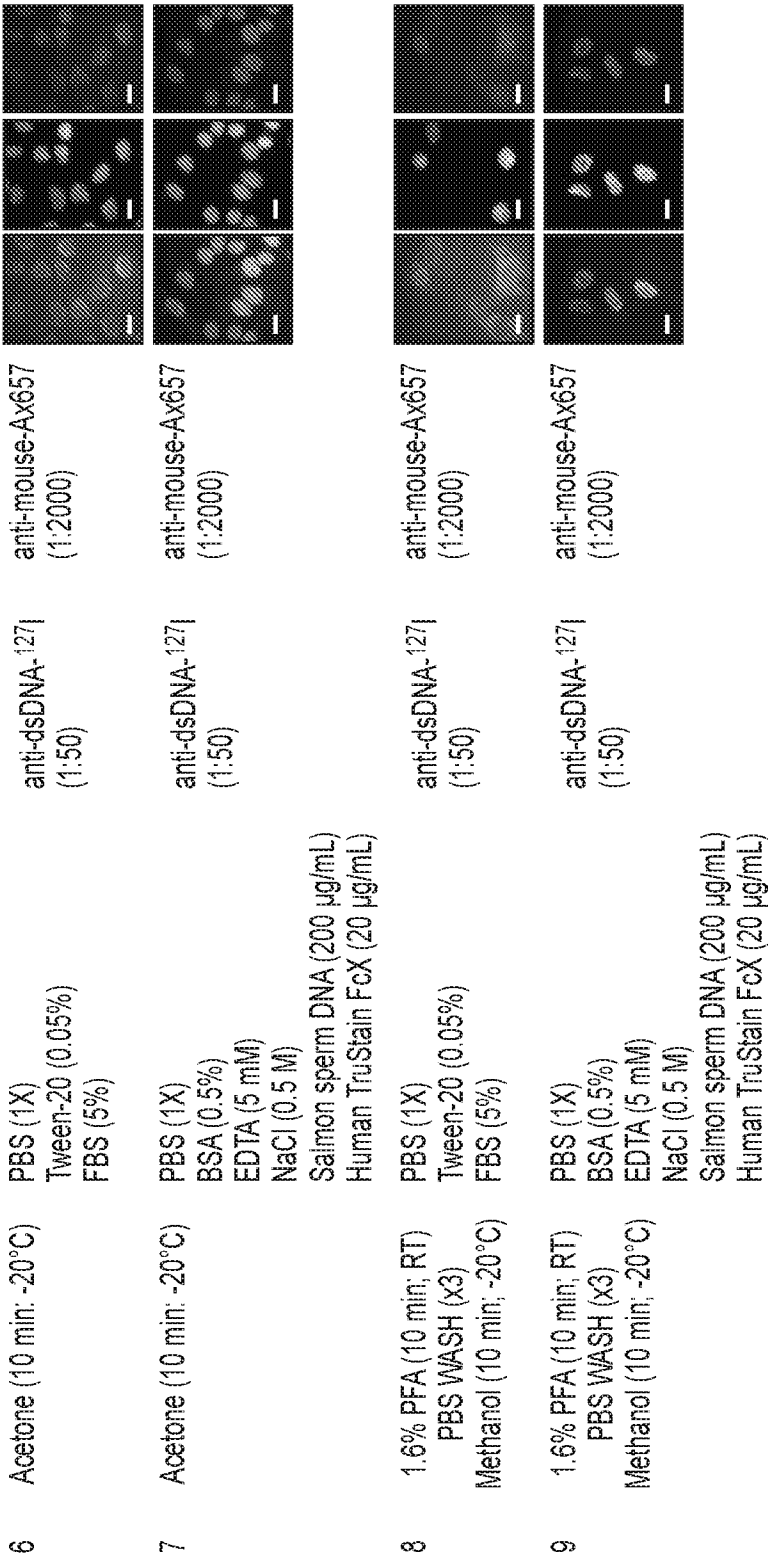


FIG. 29 (Cont.)

39 / 69

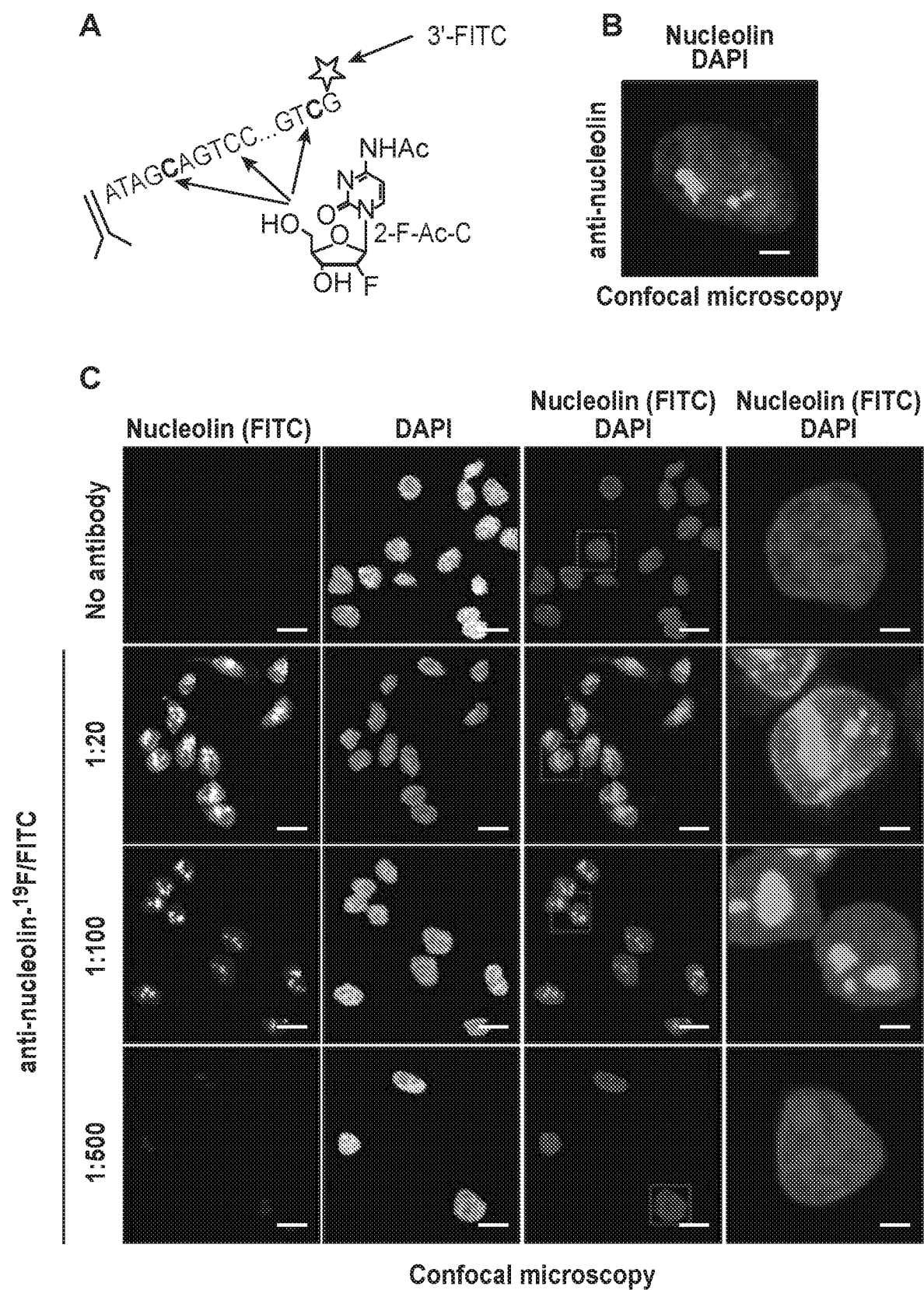


FIG. 30

40 / 69

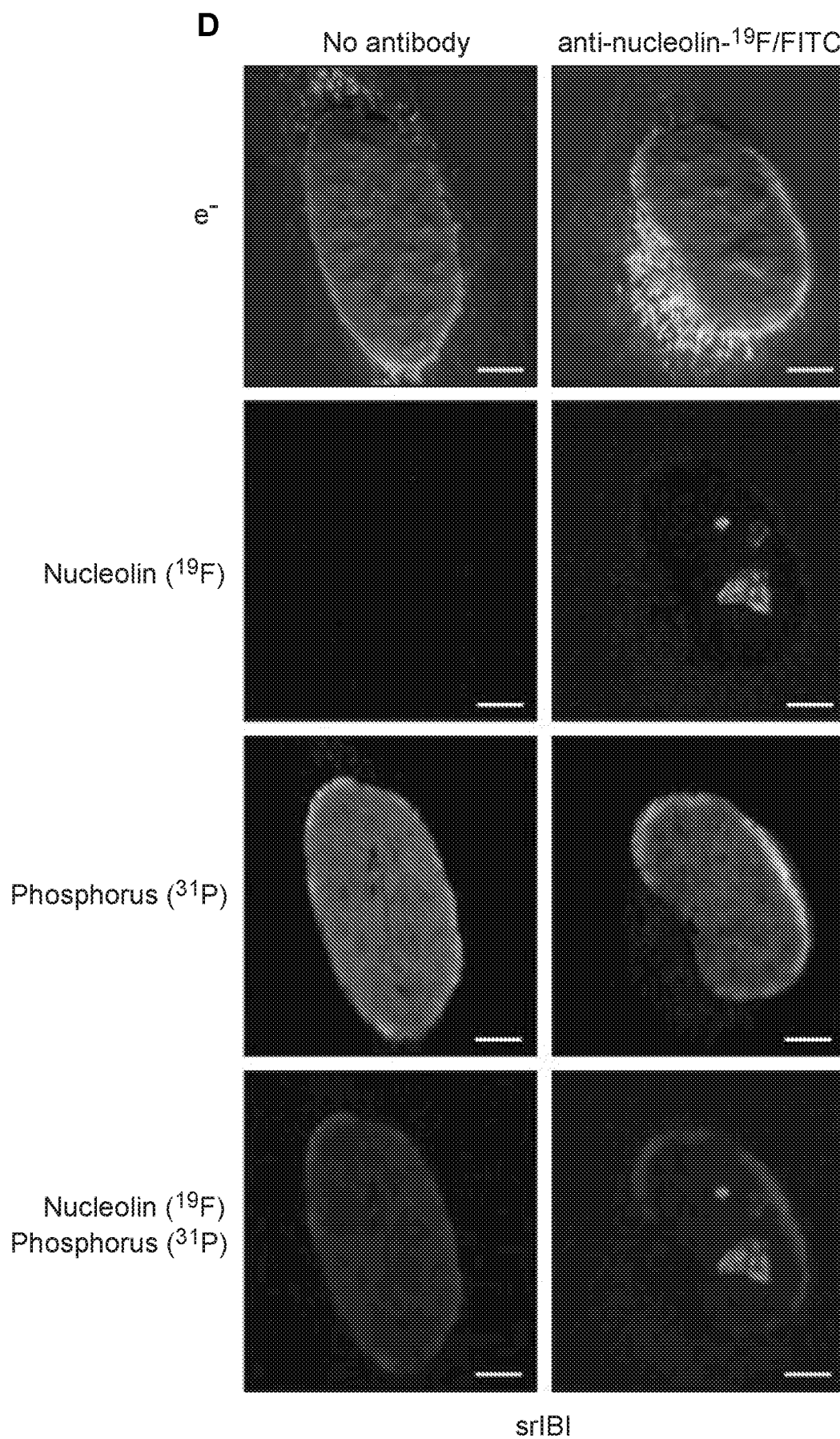


FIG. 30 (Cont.)

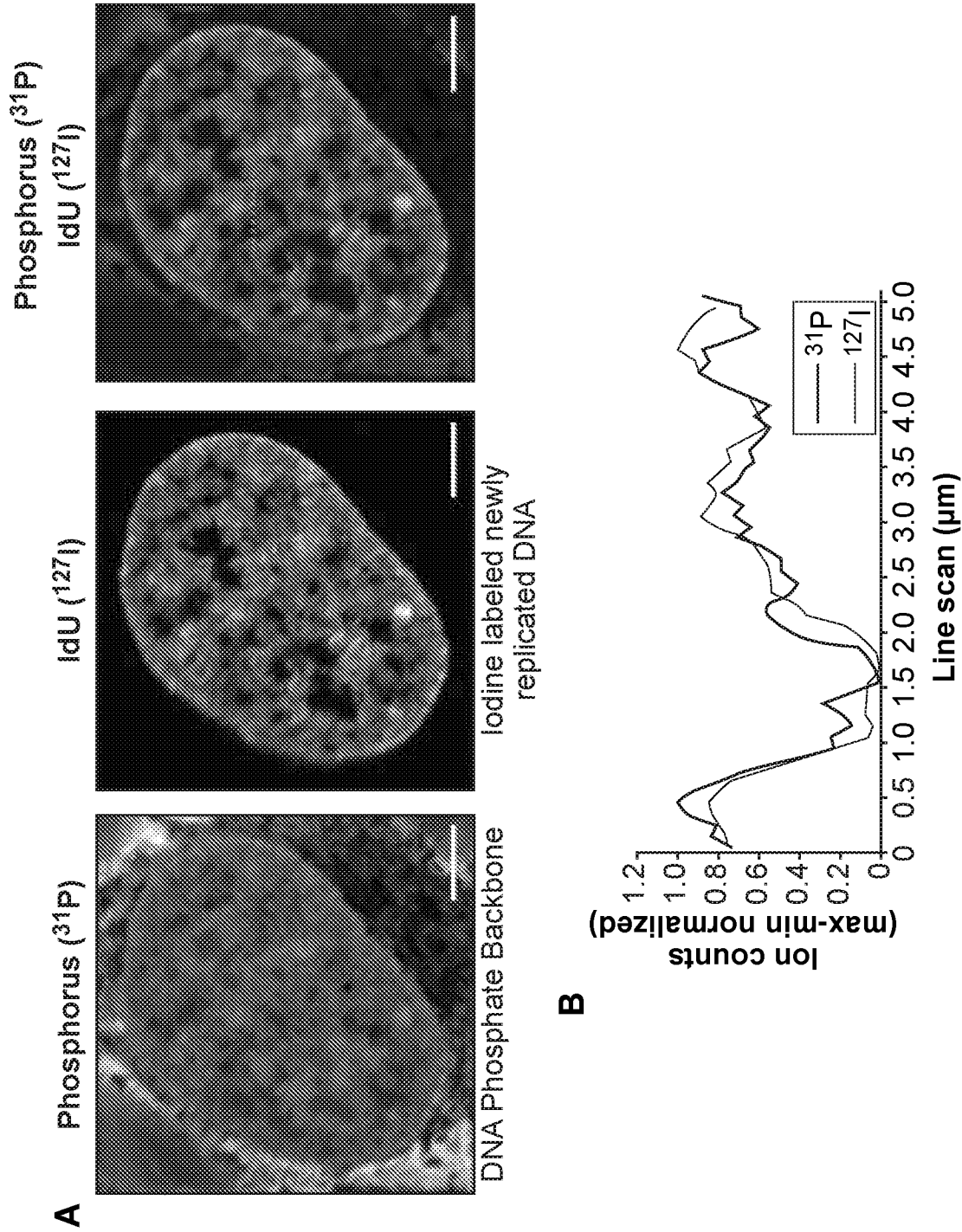


FIG. 31

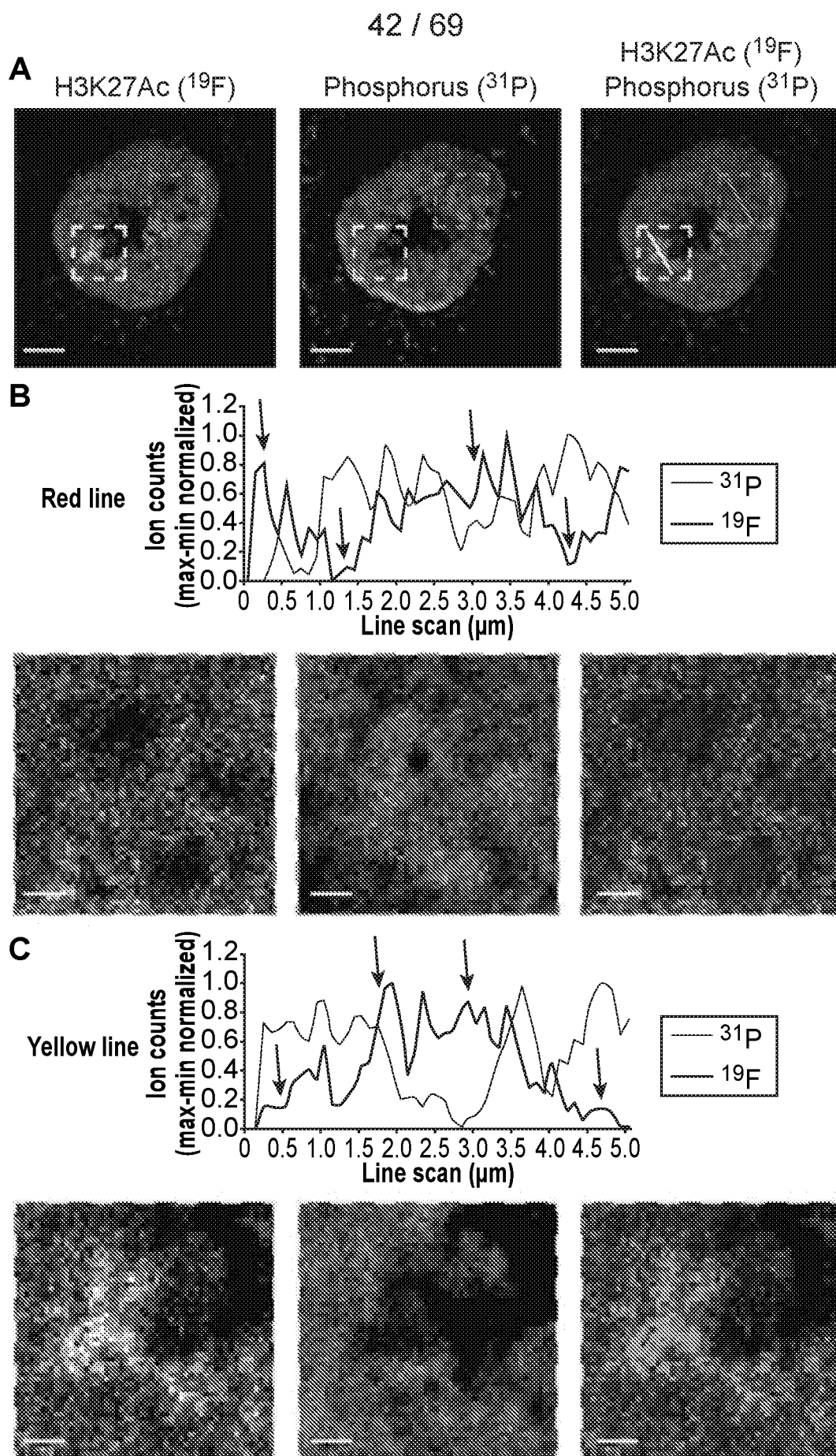


FIG. 32

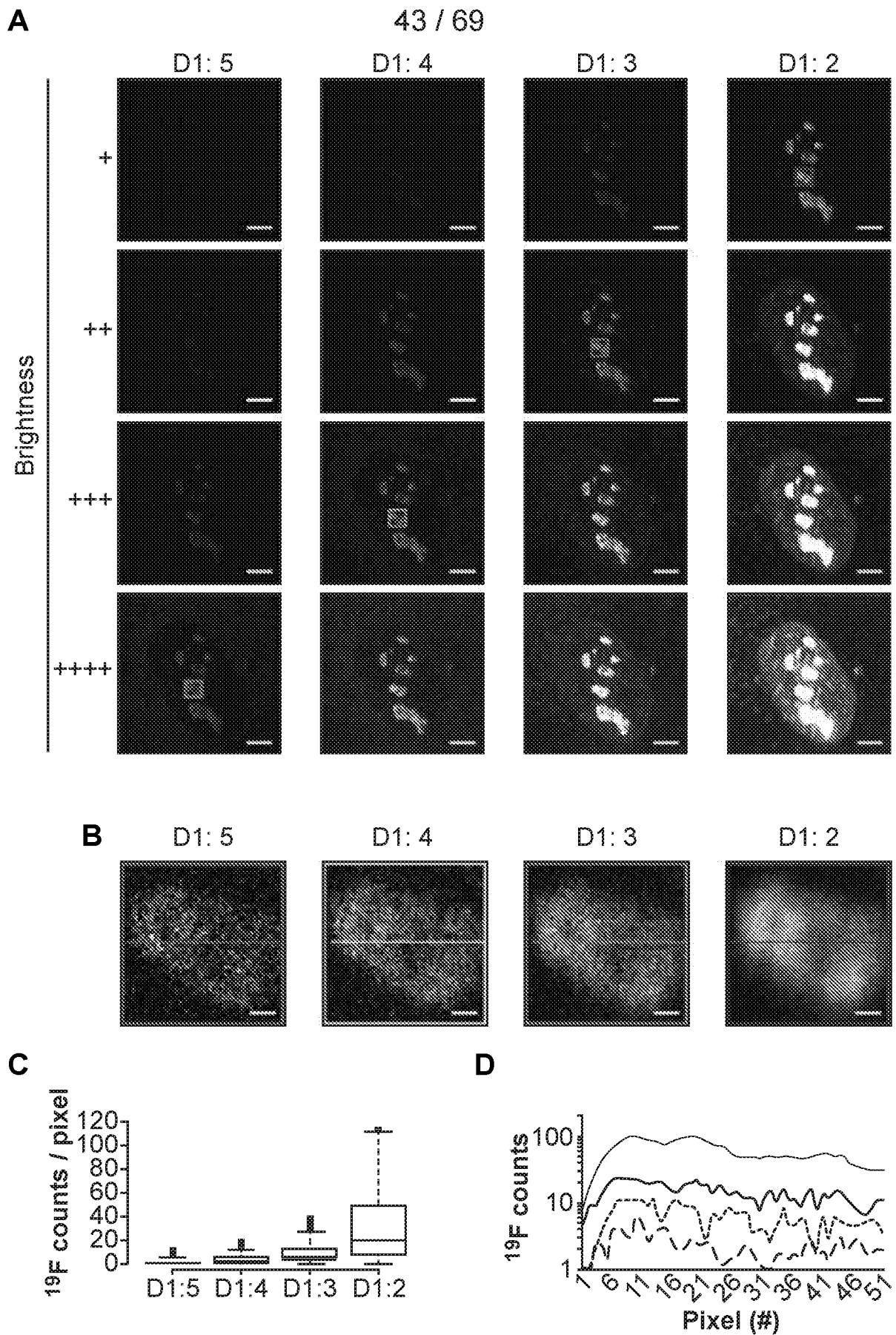


FIG. 33

44 / 69

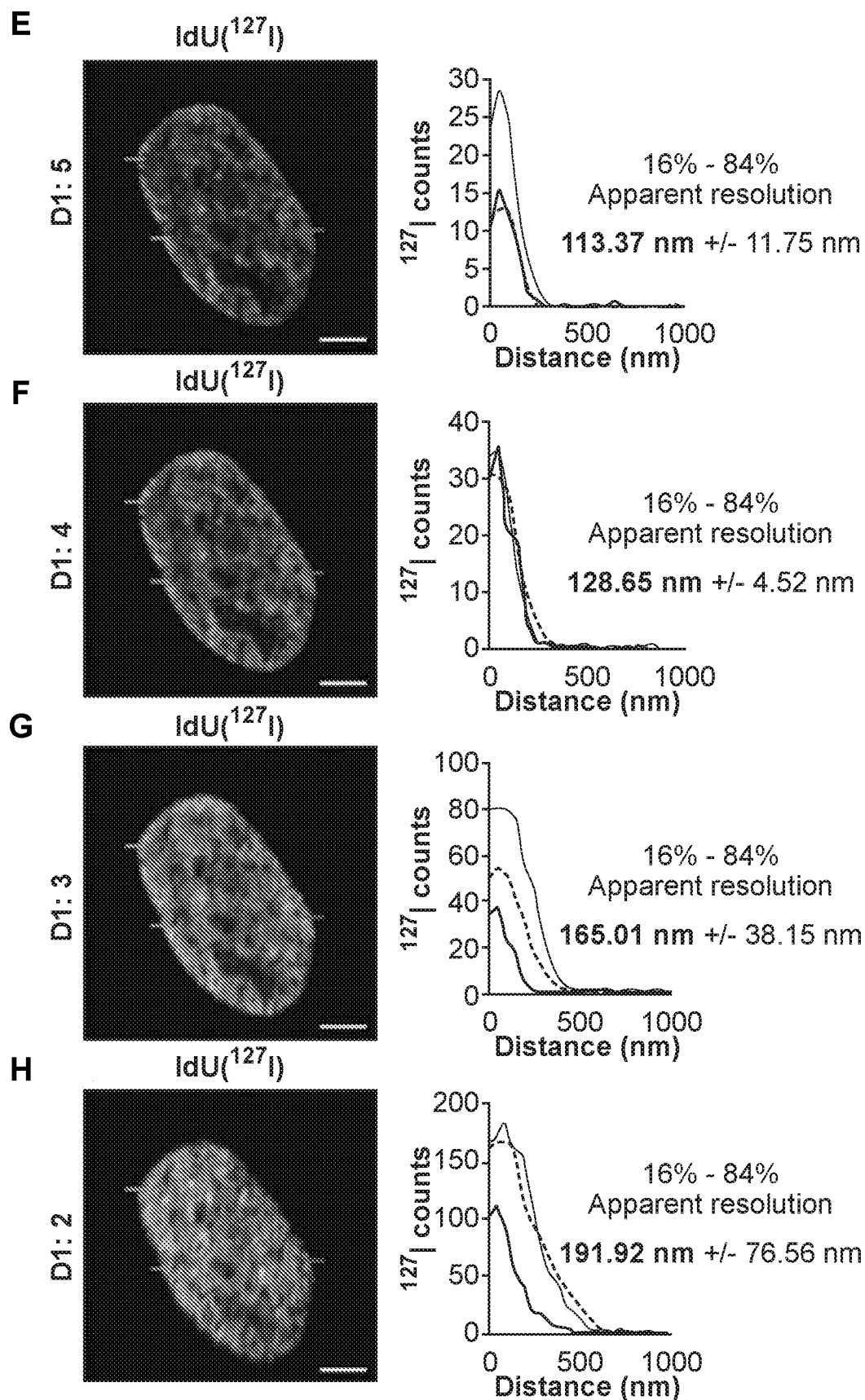


FIG. 33 (Cont.)

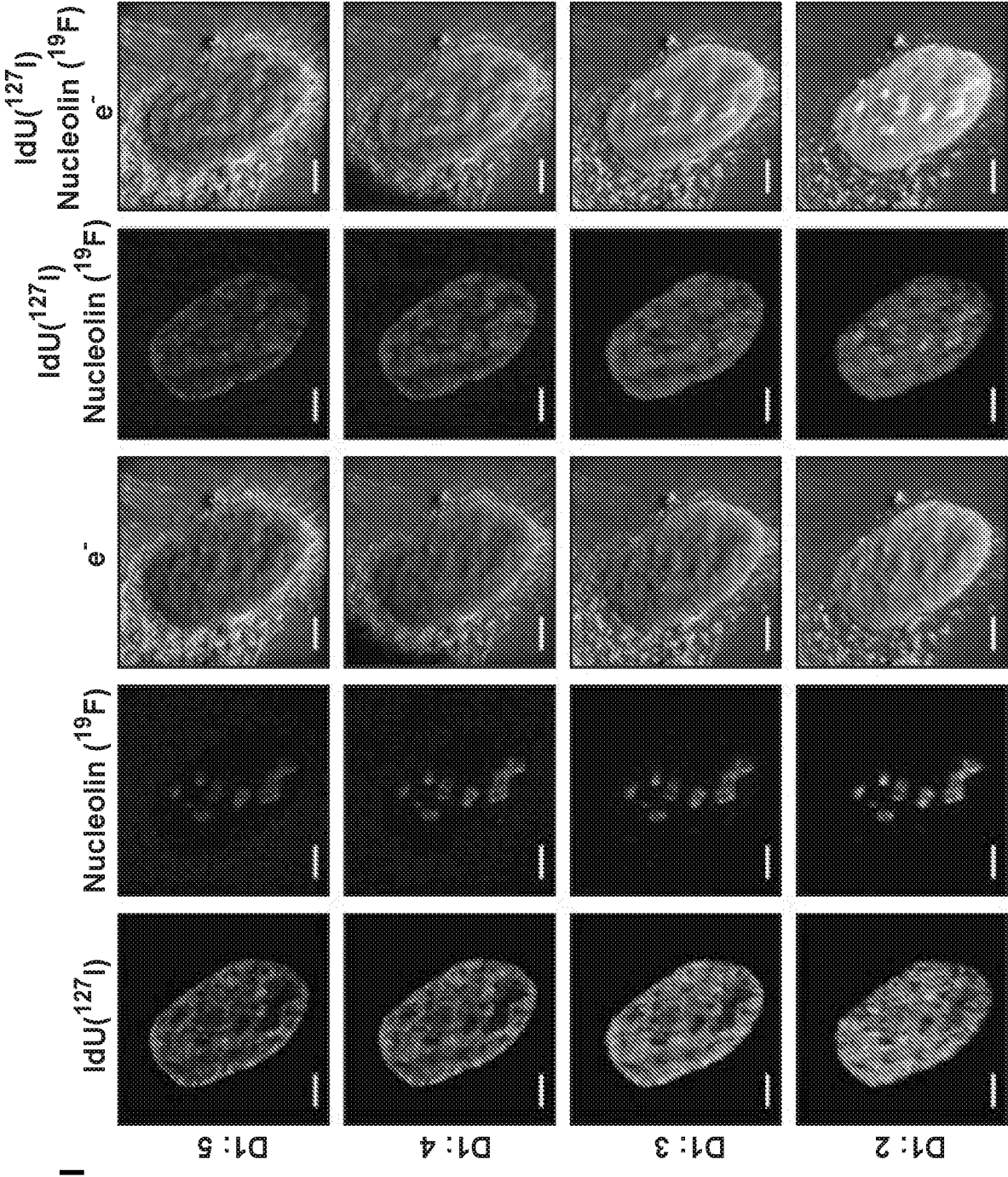


FIG. 33 (Cont.)

46 / 69

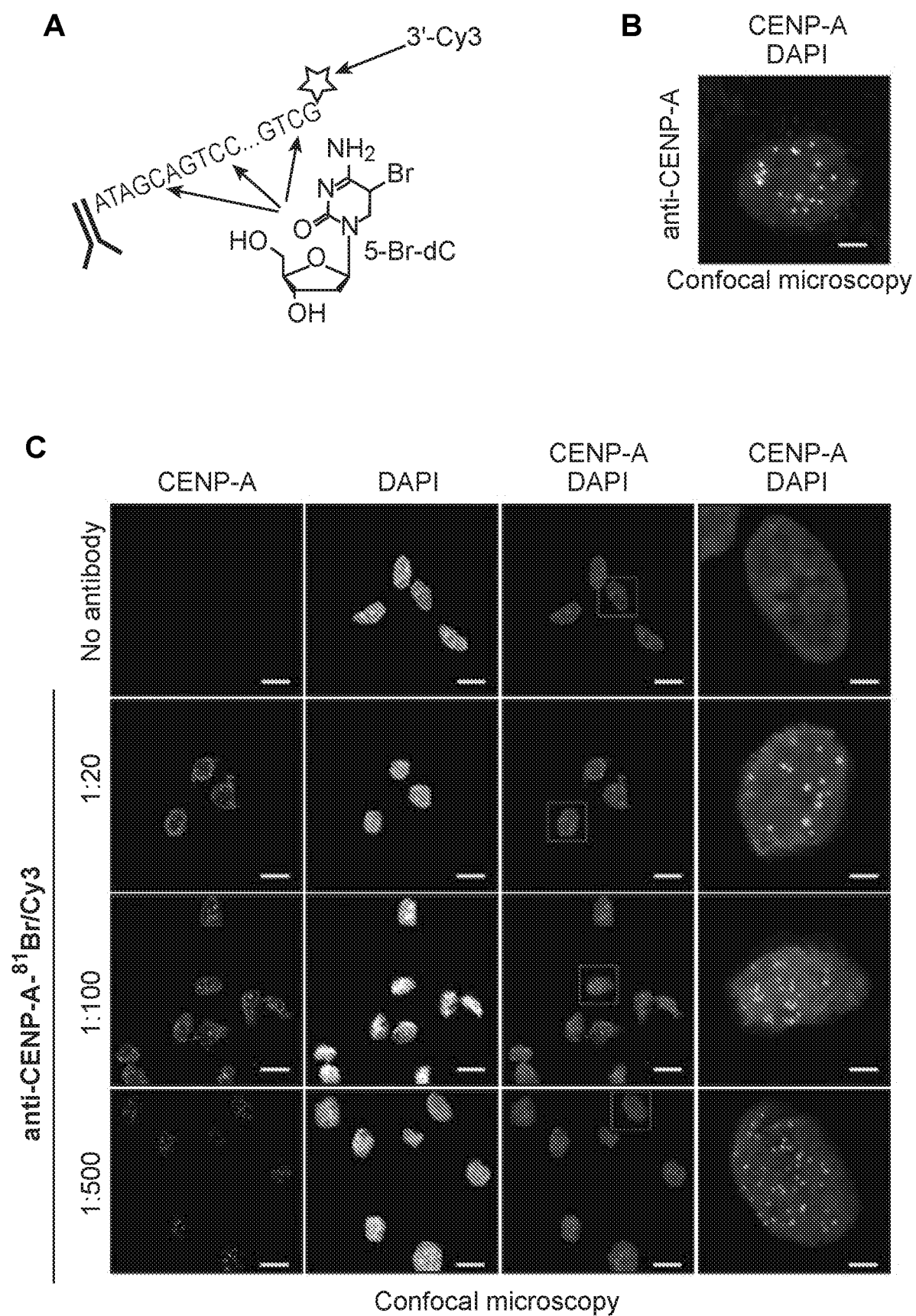


FIG. 34

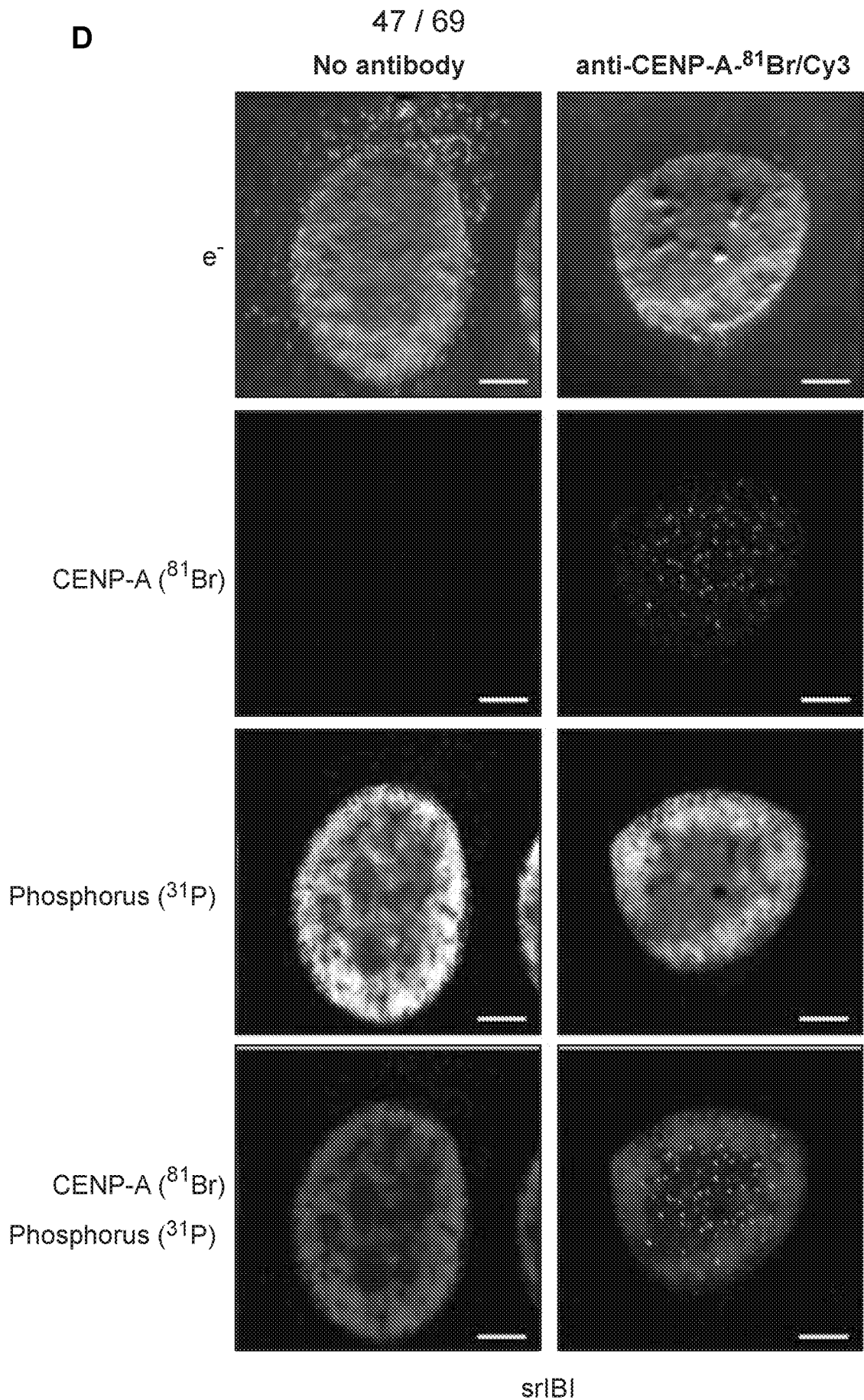


FIG. 34 (Cont.)

48 / 69

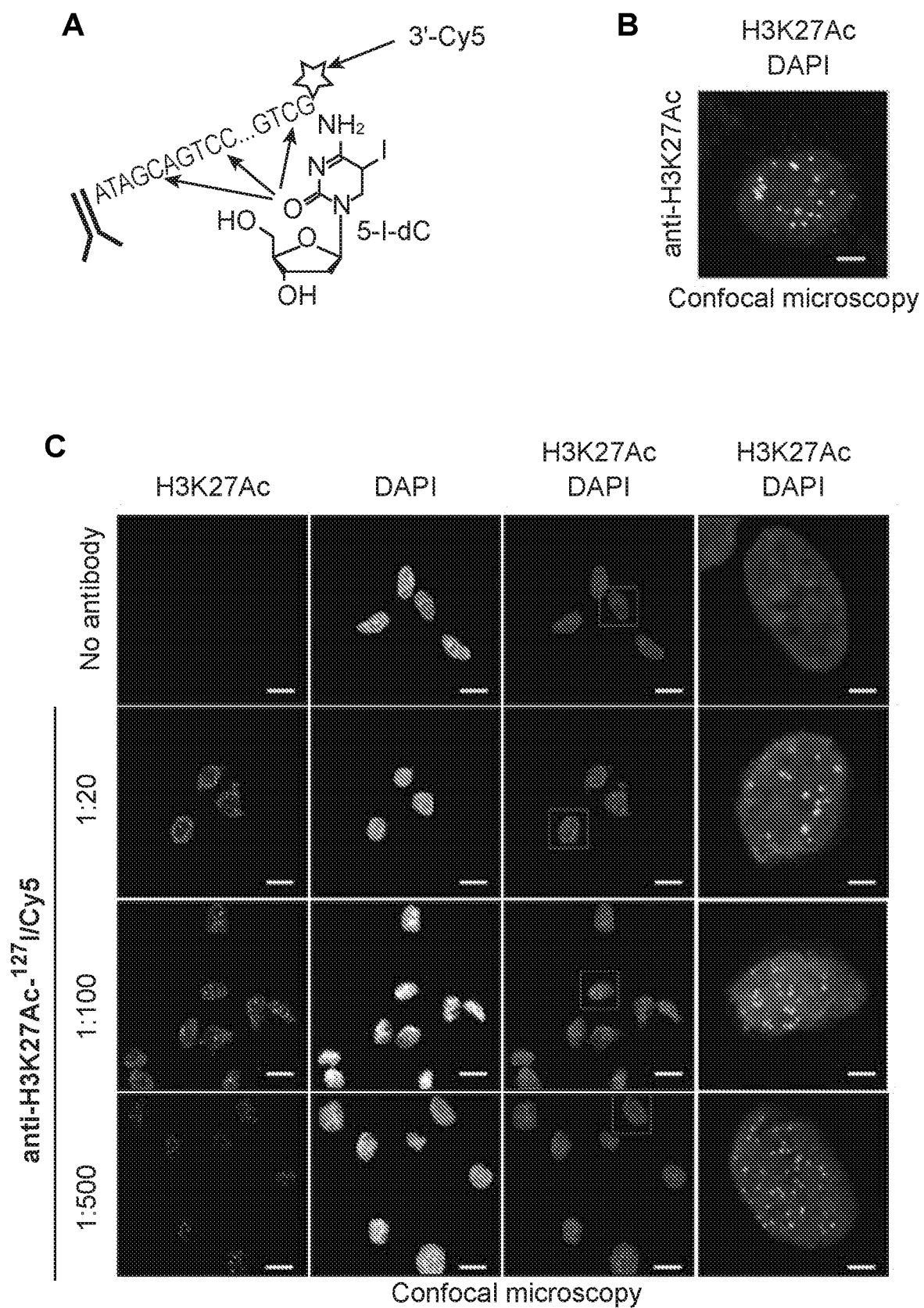


FIG. 35

49 / 69

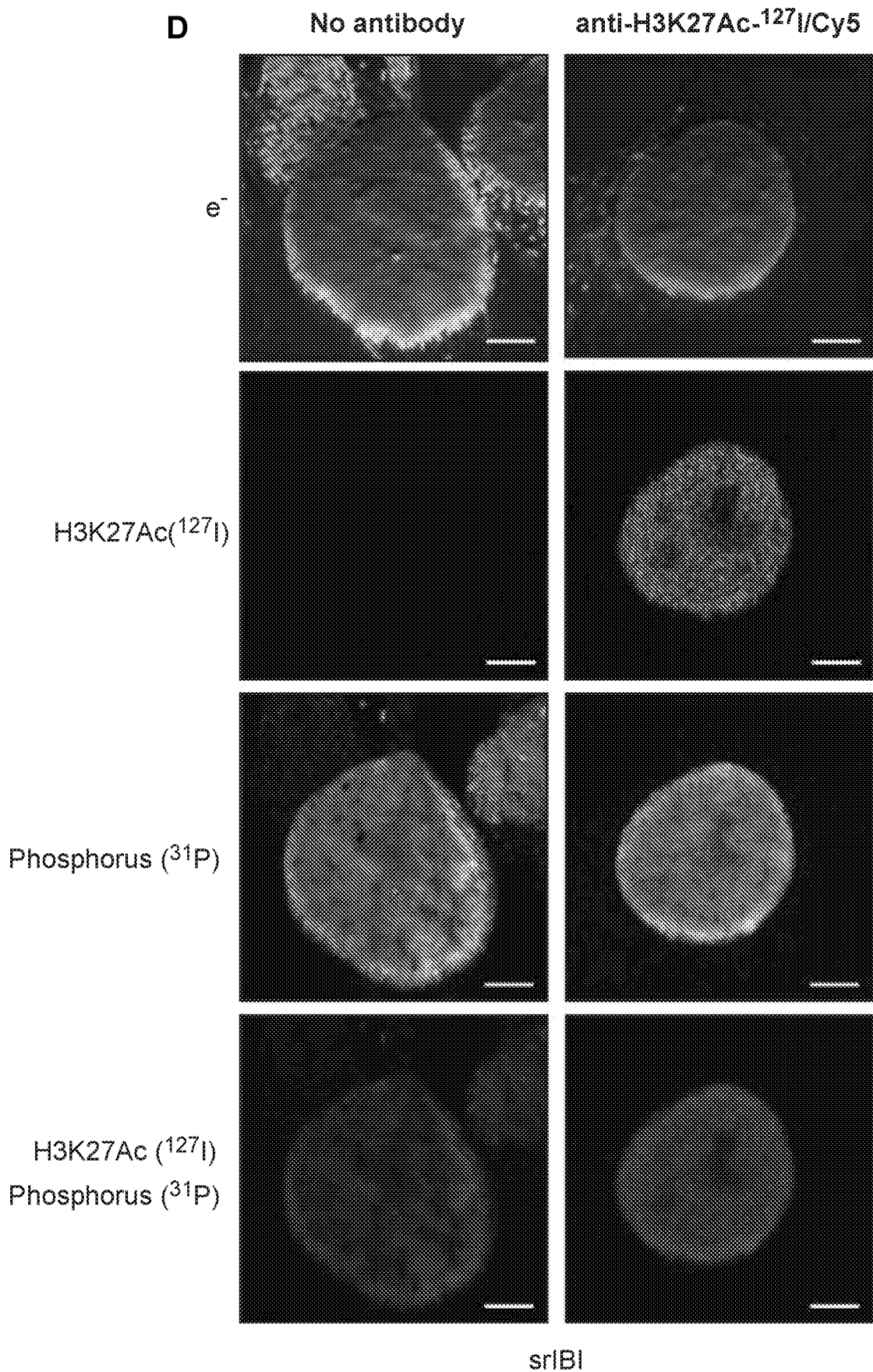


FIG. 35 (Cont.)

50 / 69

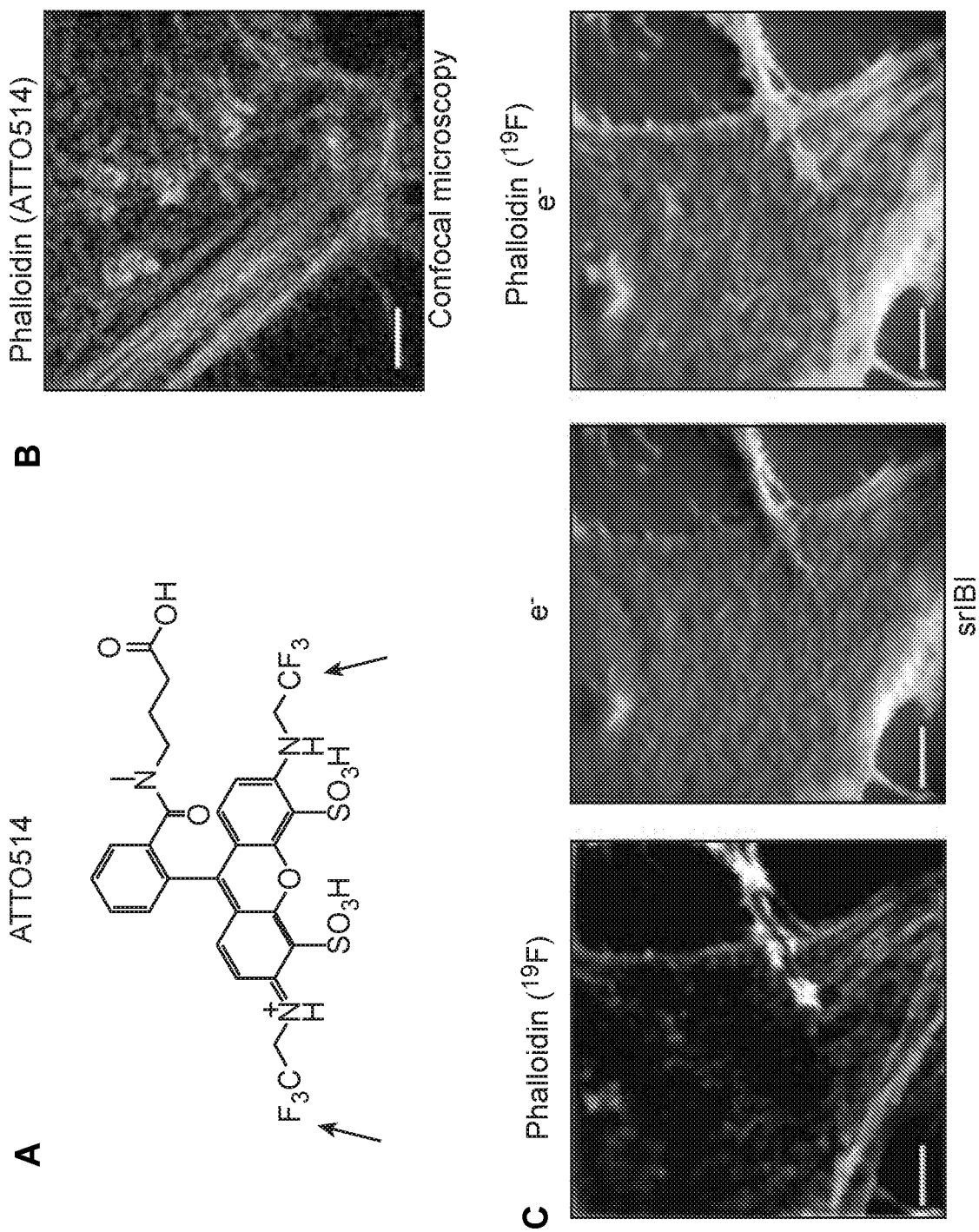


FIG. 36

51 / 69

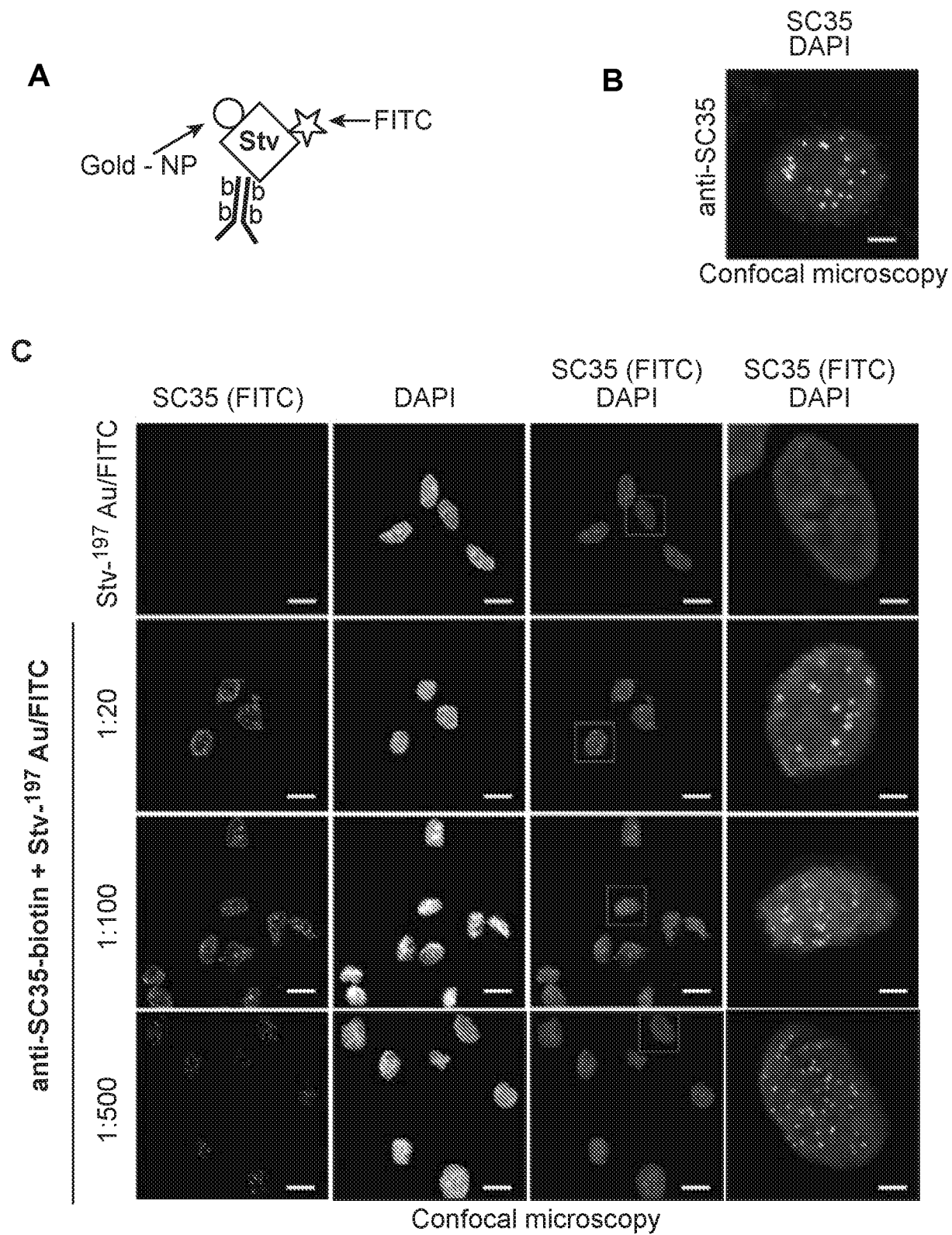


FIG. 37

52 / 69

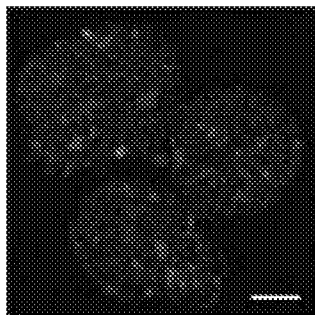
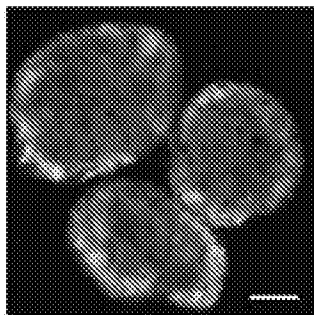
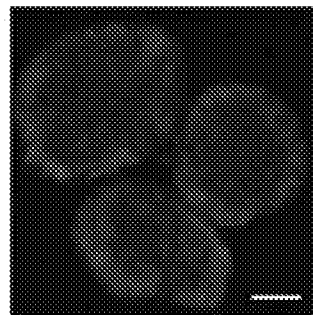
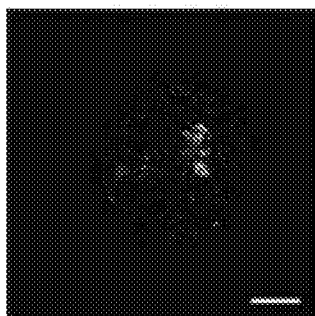
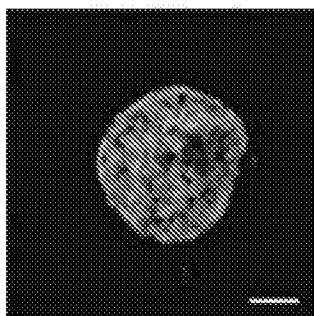
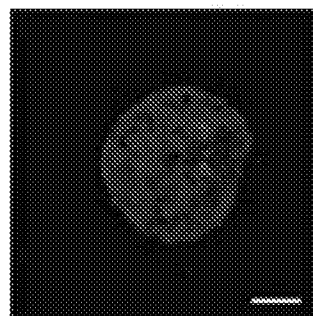
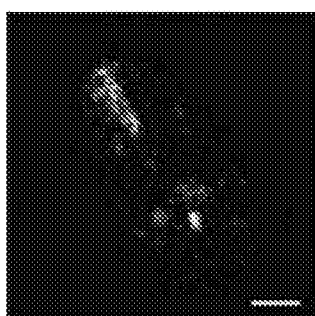
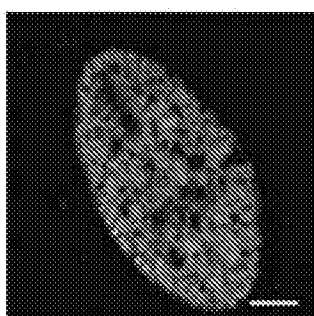
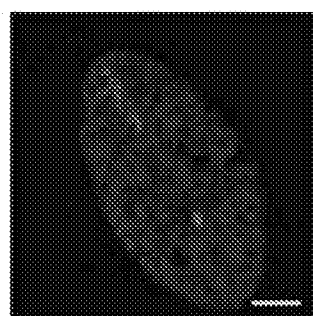
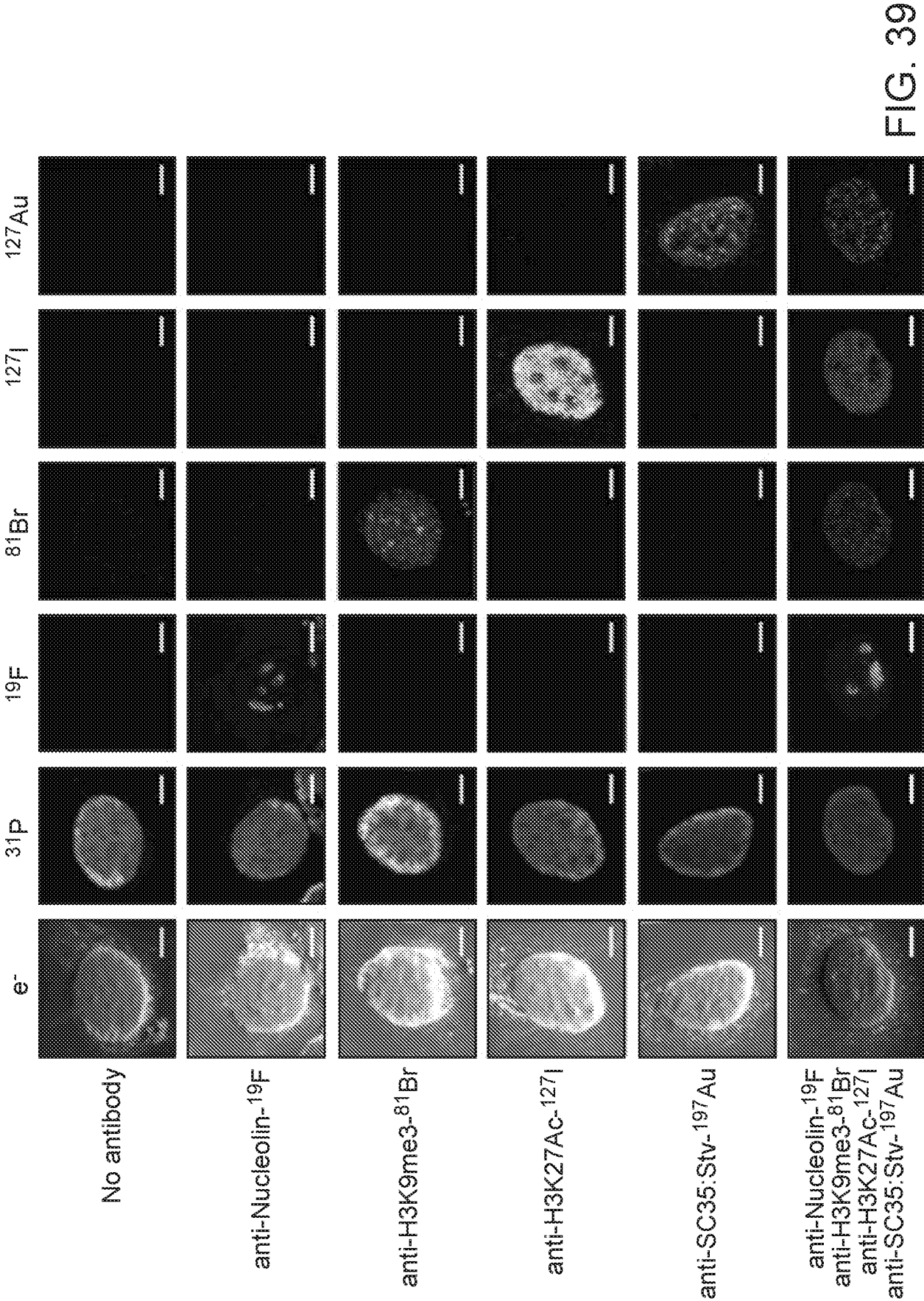
AH3K9me3 (^{81}Br)Phosphorus (^{31}P)H3K9me3 (^{81}Br)
Phosphorus (^{31}P)**B**Nucleolin (^{127}I)Phosphorus (^{31}P)Nucleolin (^{127}I)
Phosphorus (^{31}P)**C**NPM1 (^{197}Au)Phosphorus (^{31}P)NPM1 (^{197}Au)
Phosphorus (^{31}P)

FIG. 38



54 / 69

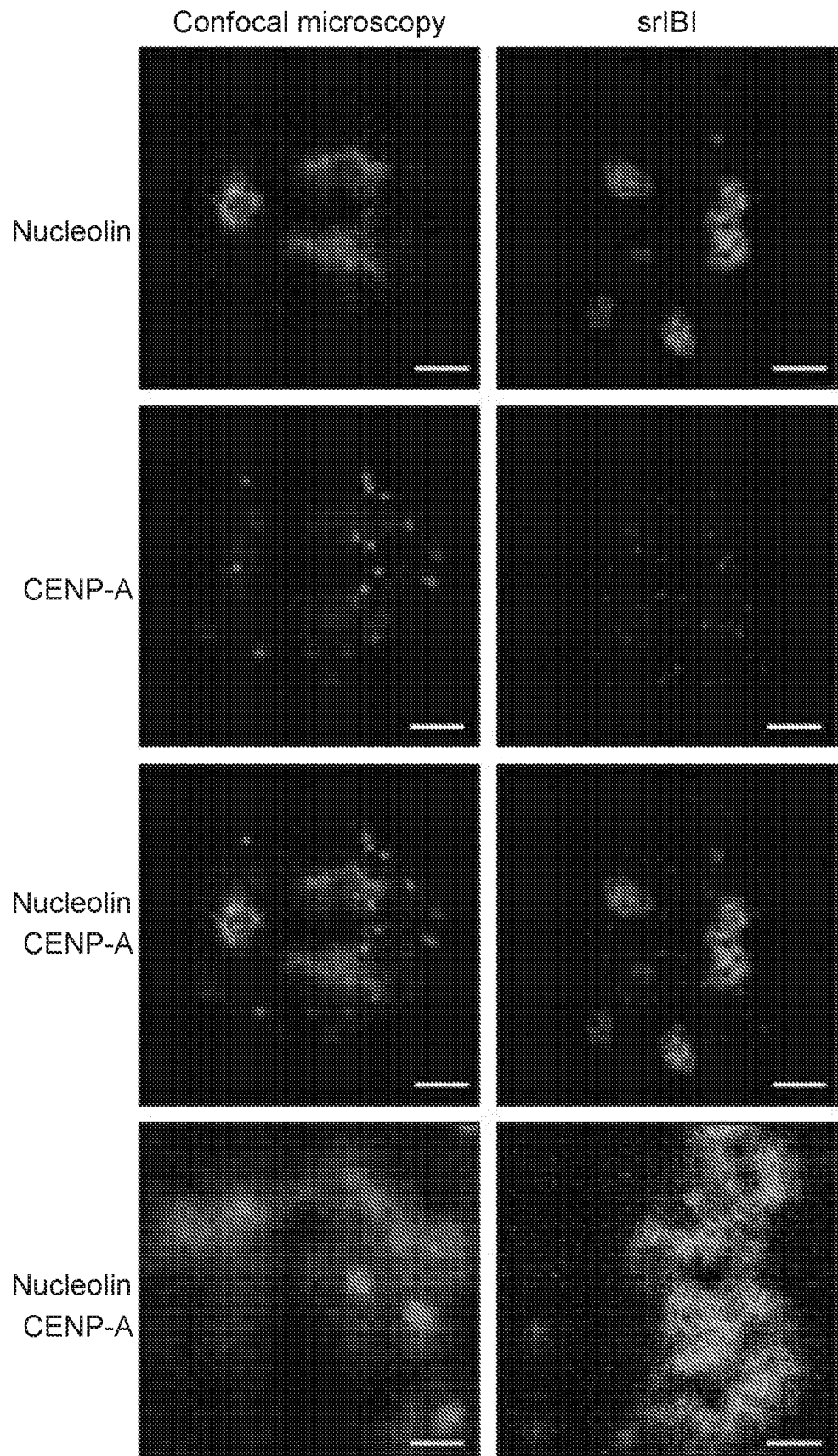


FIG. 40

55 / 69

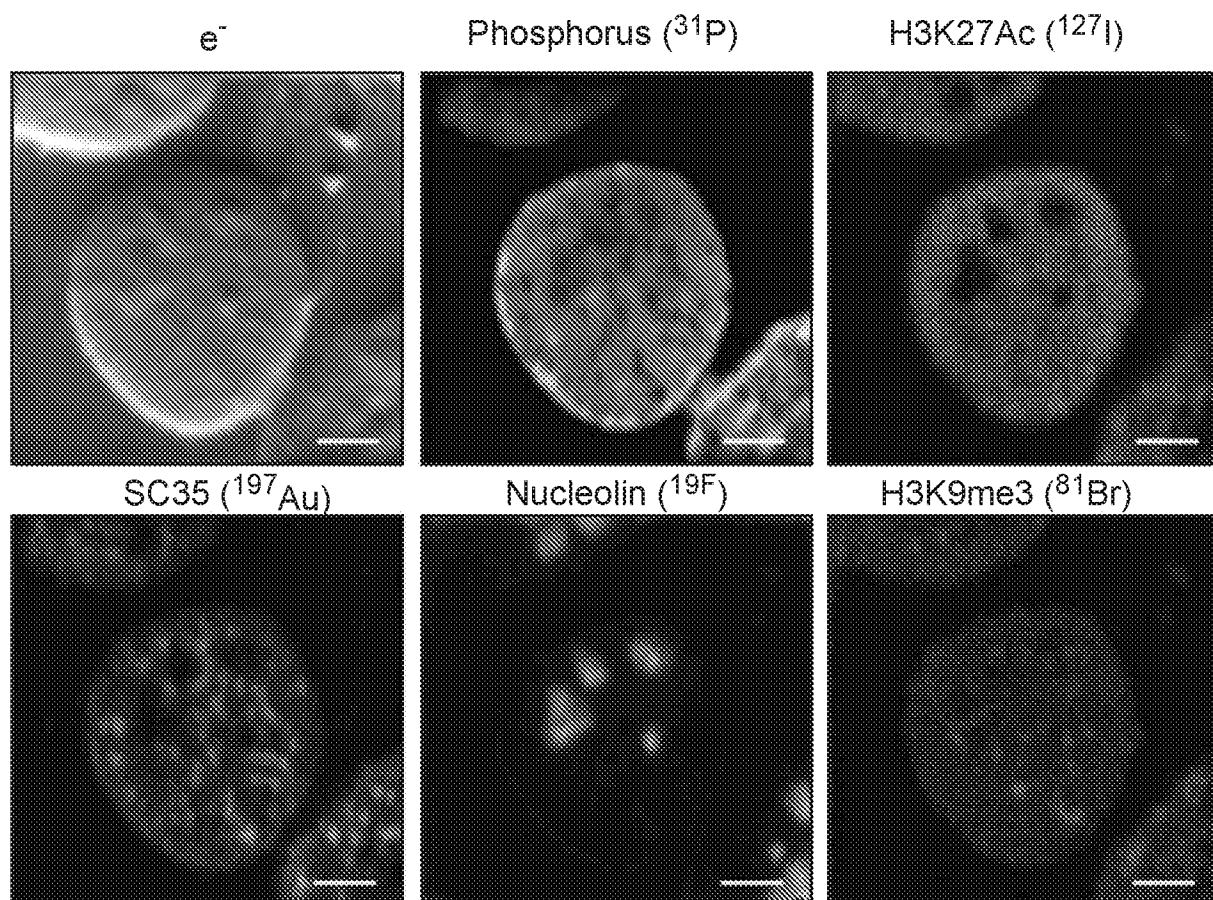


FIG. 41

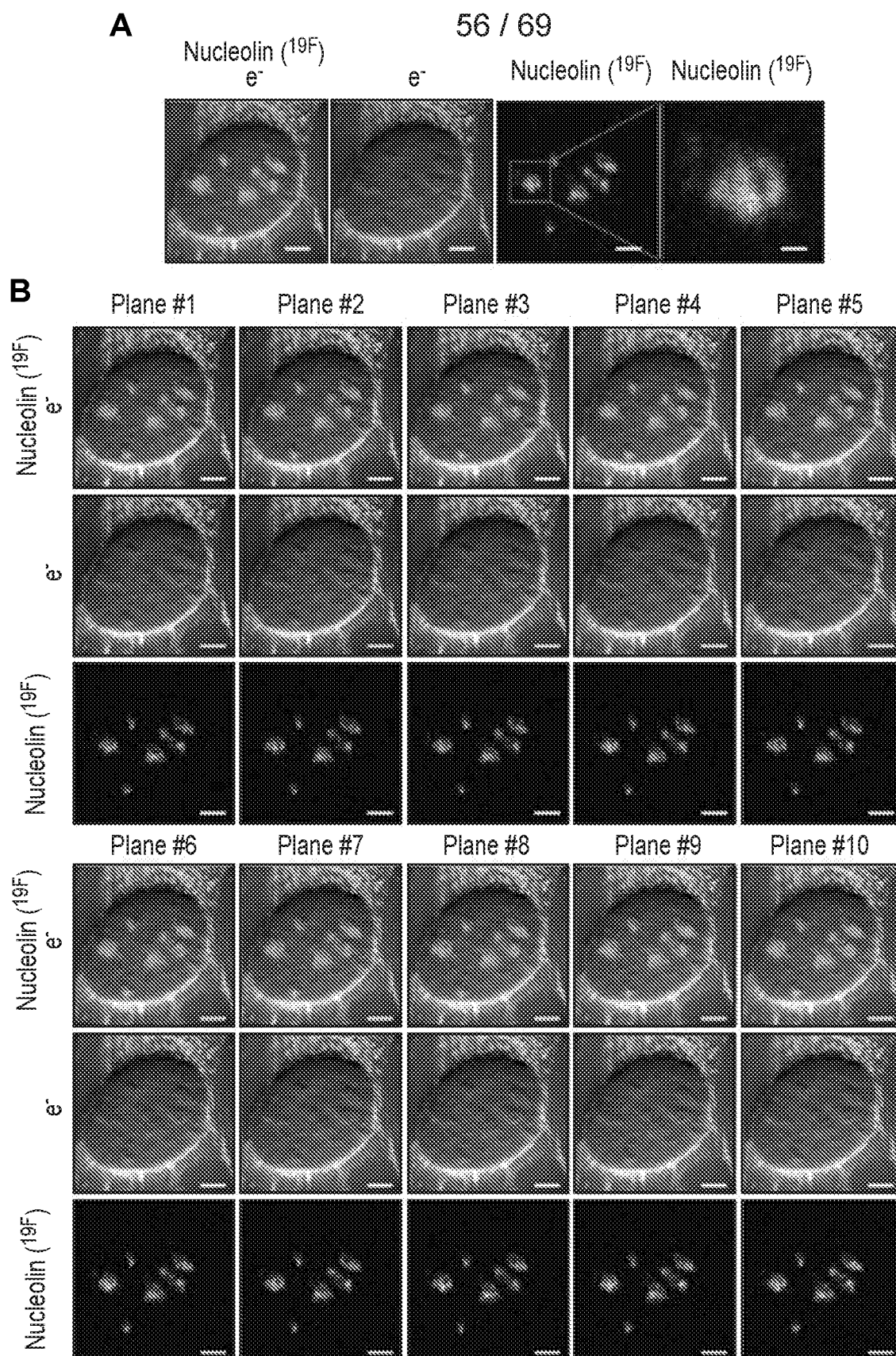


FIG. 42

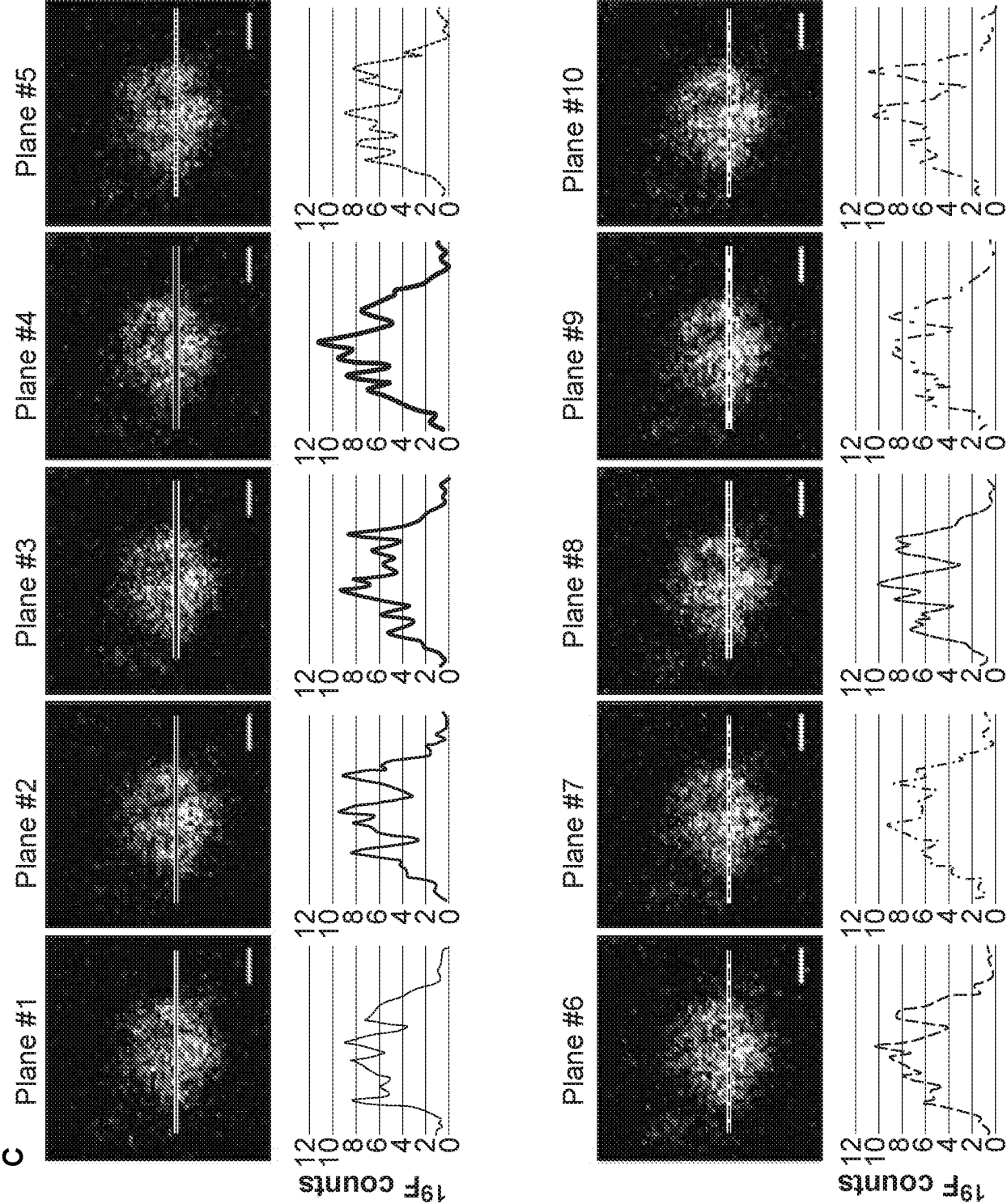


FIG. 42 (Cont.)

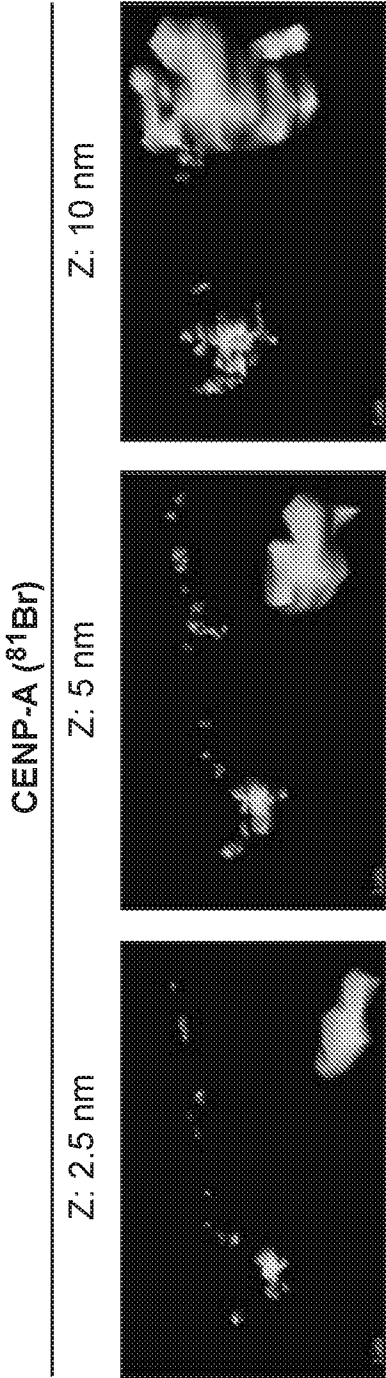
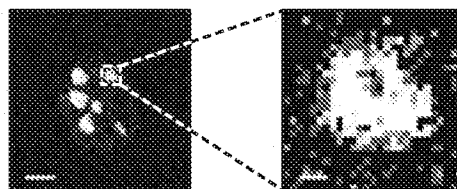


FIG. 43

59 / 69

A

Nucleolin (^{19}F) Nucleolin (^{19}F)

B

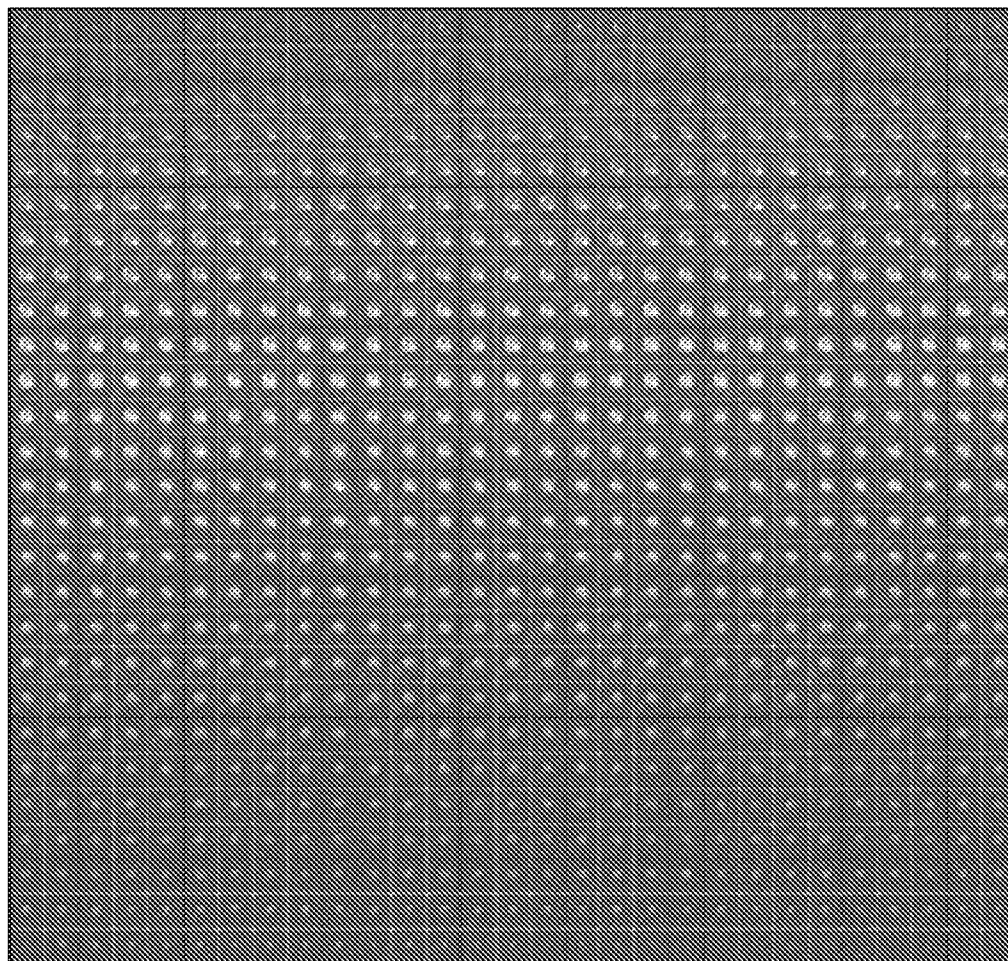


FIG. 44

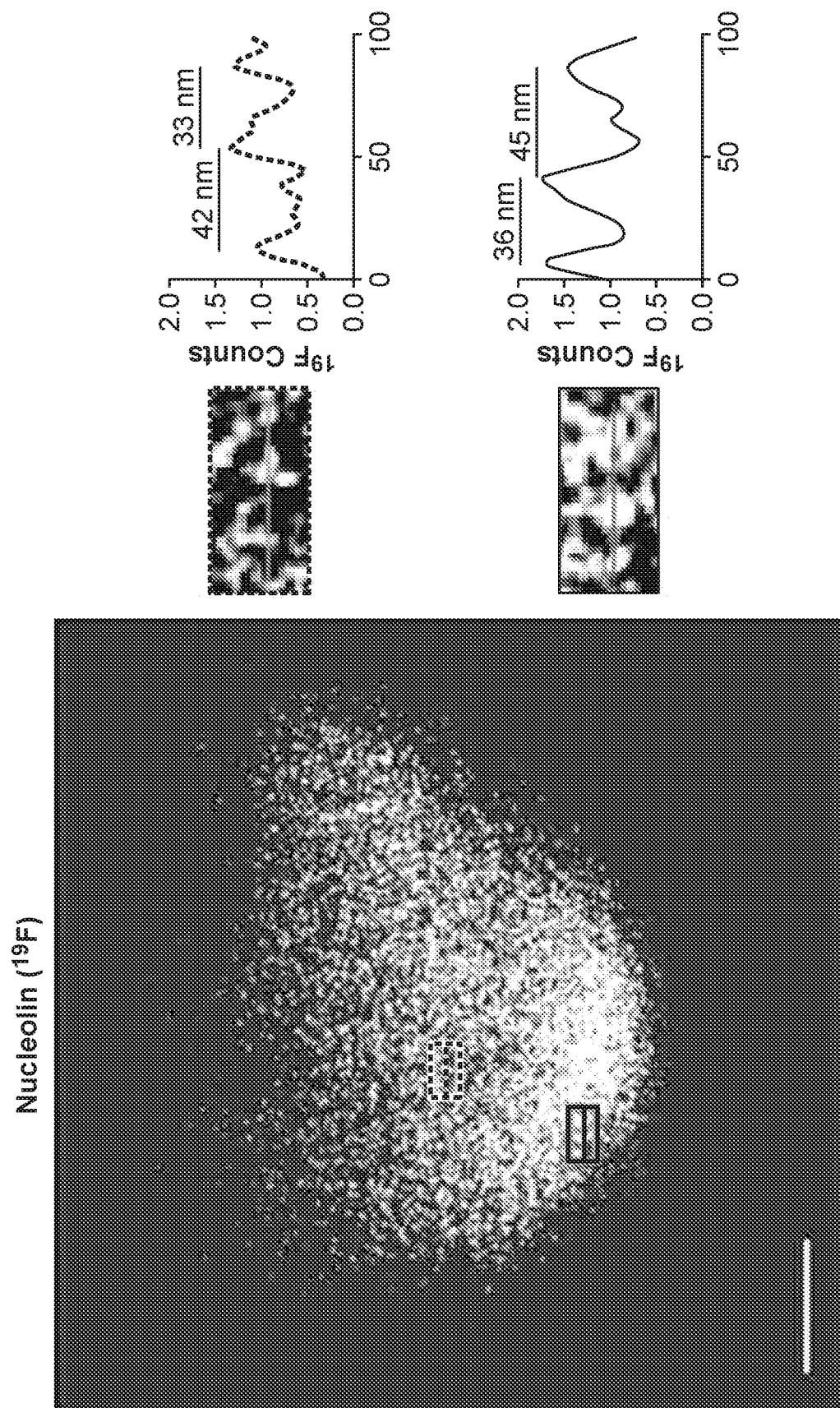


FIG. 45

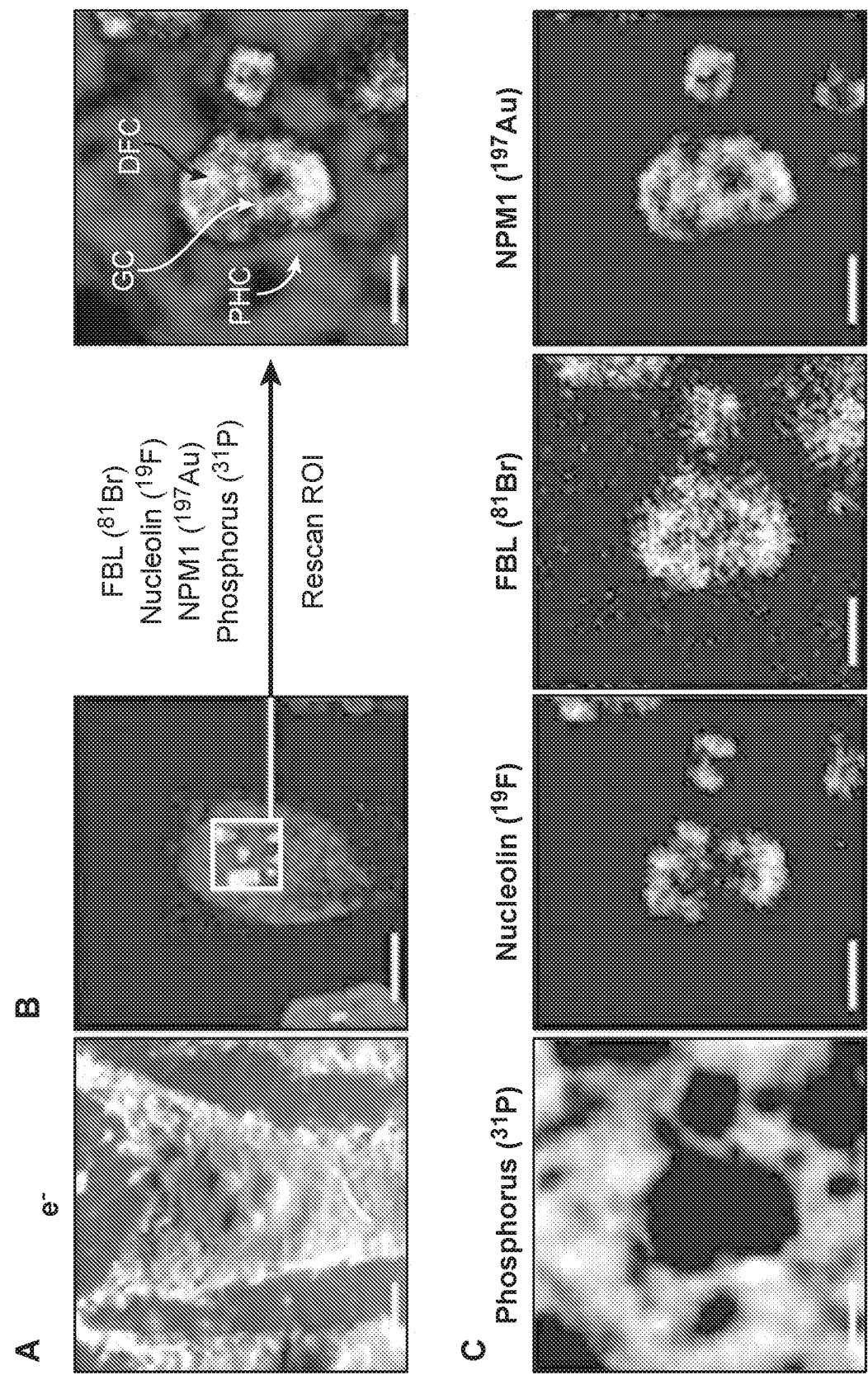


FIG. 46

62 / 69

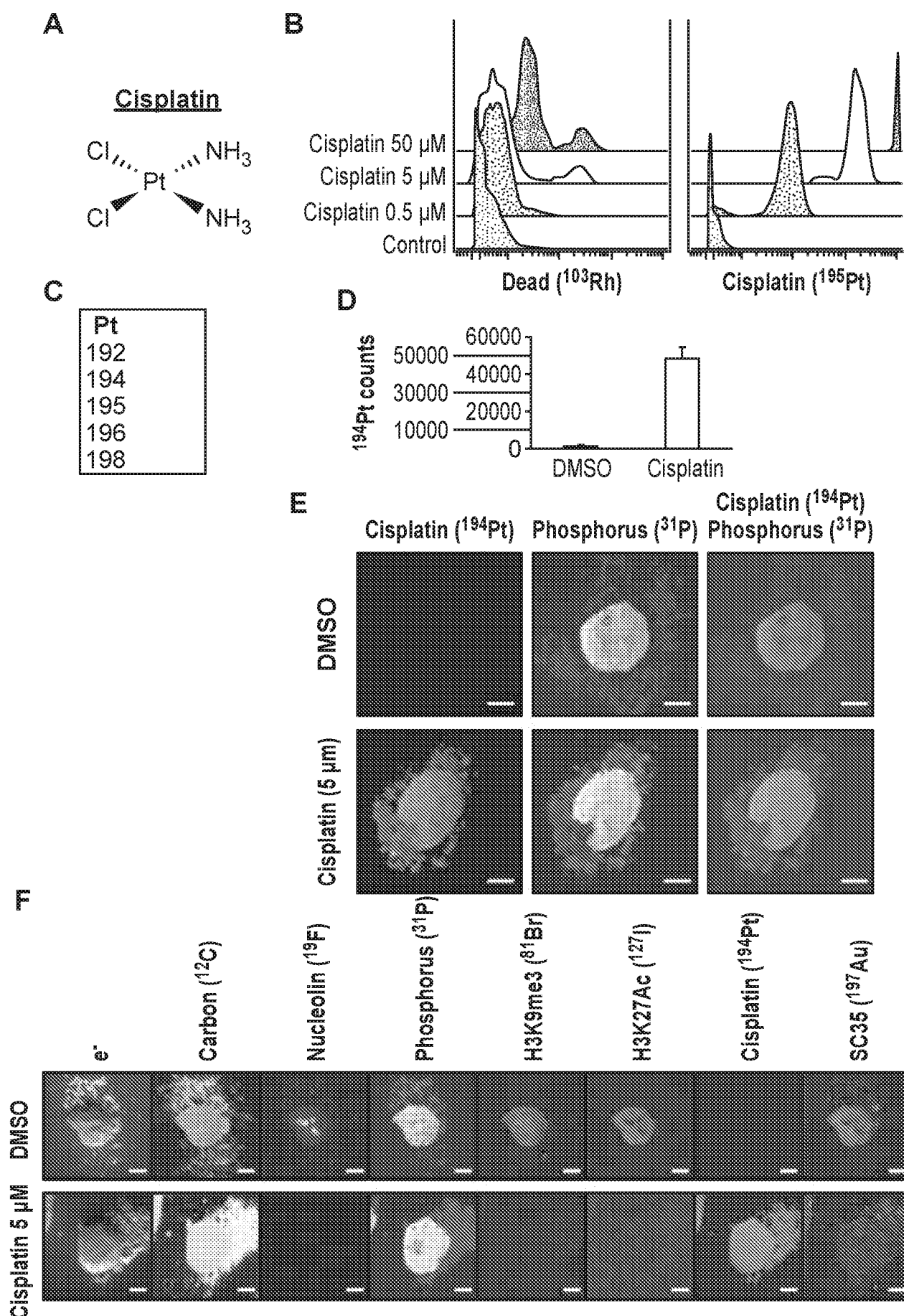


FIG. 47

63 / 69

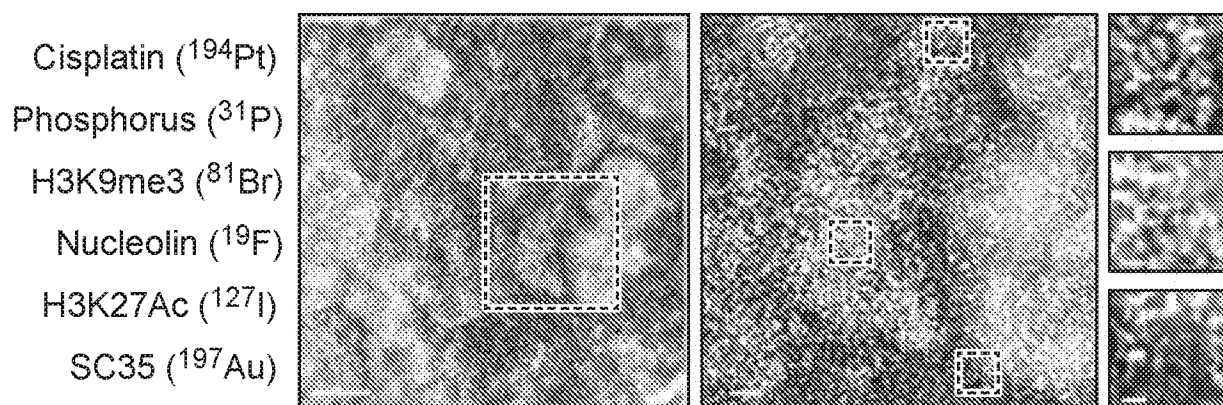
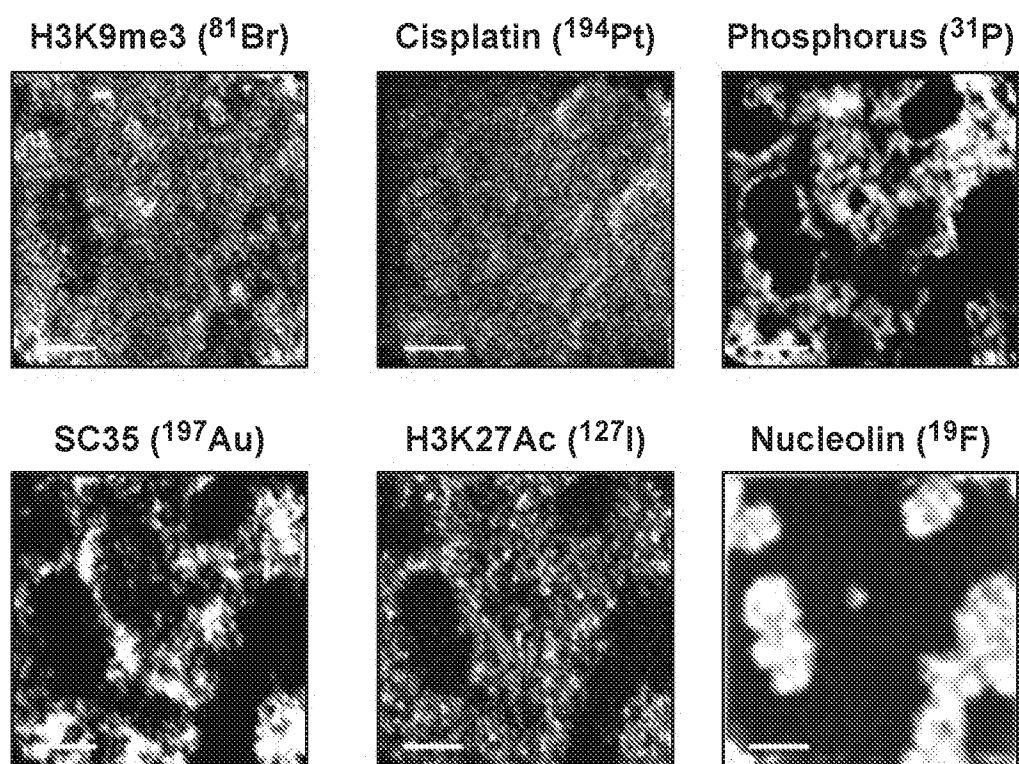
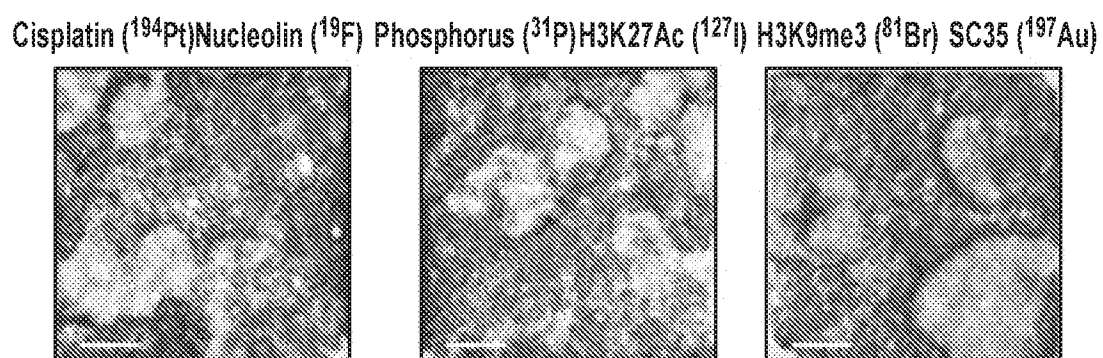
A**B****C**

FIG. 48

64 / 69

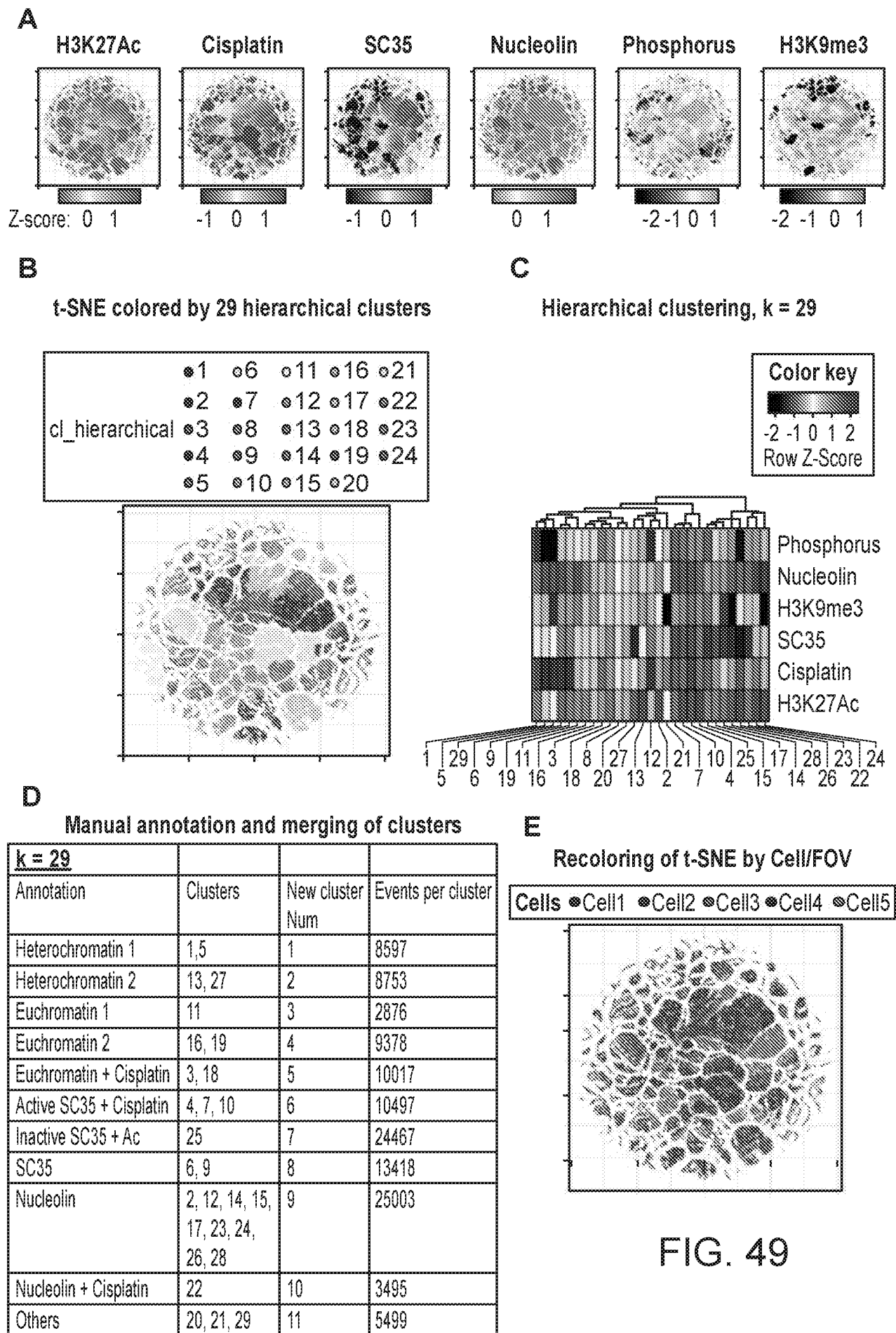


FIG. 49

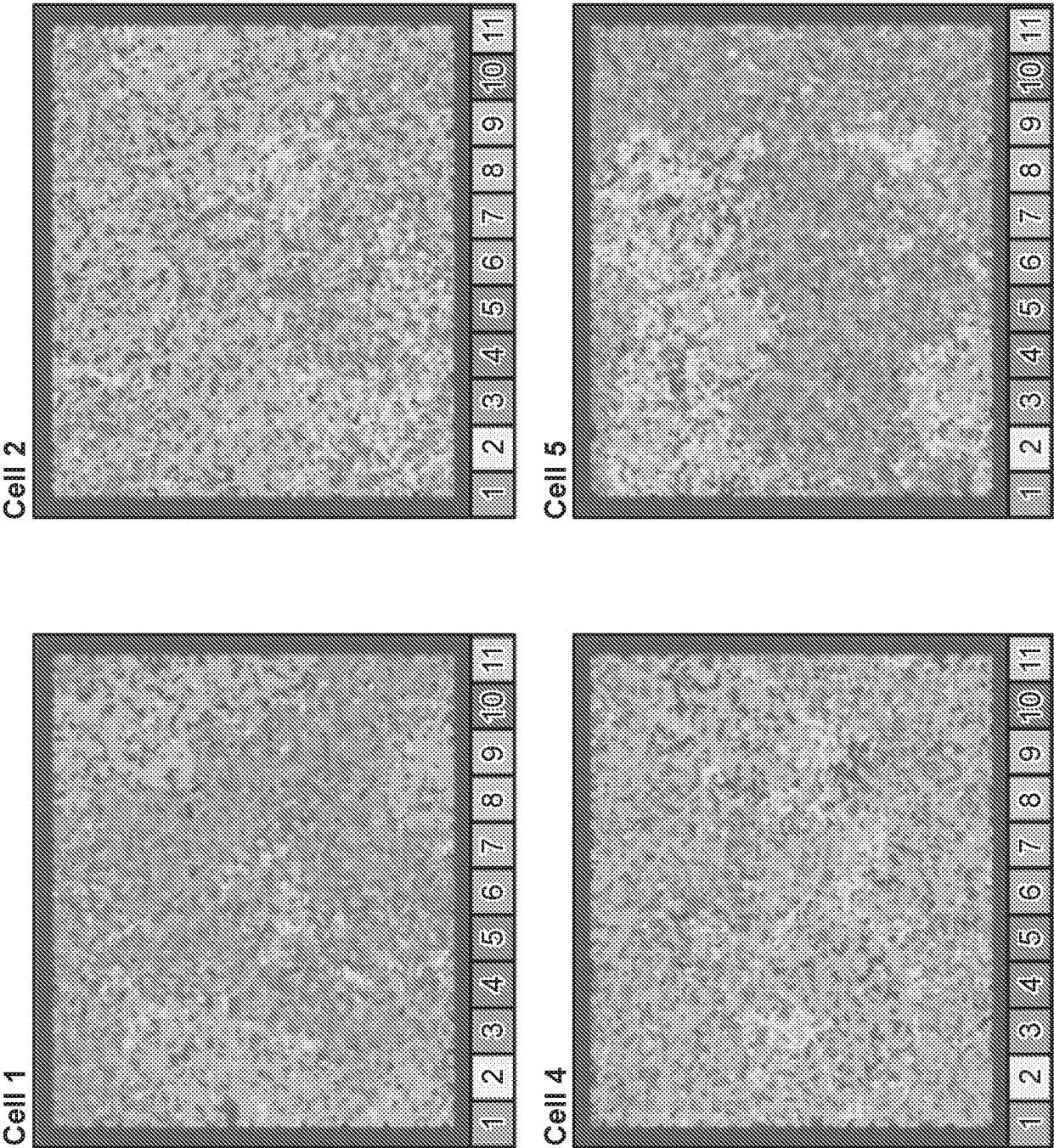


FIG. 50

66 / 69

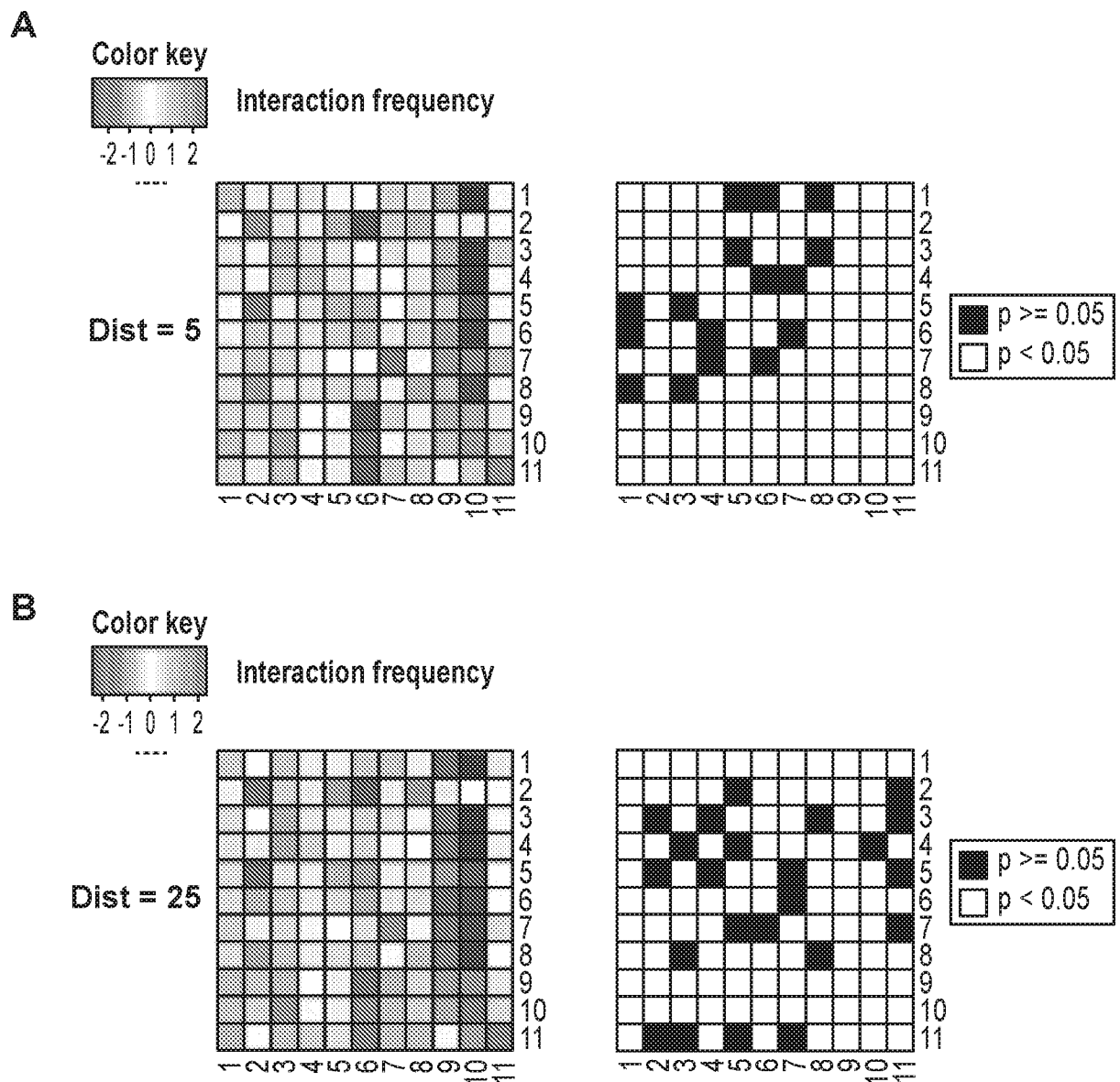


FIG. 51

67 / 69

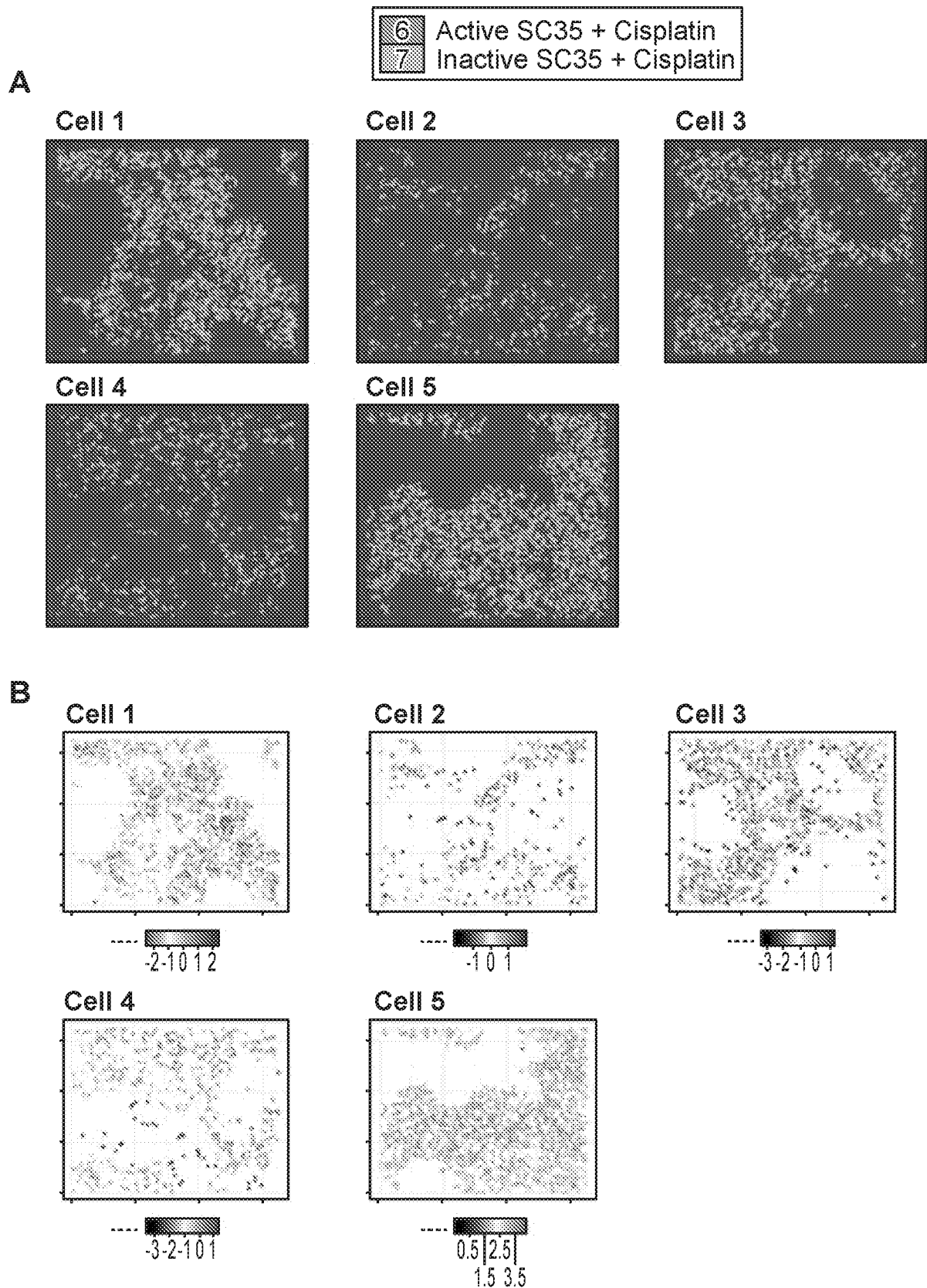


FIG. 52

68 / 69

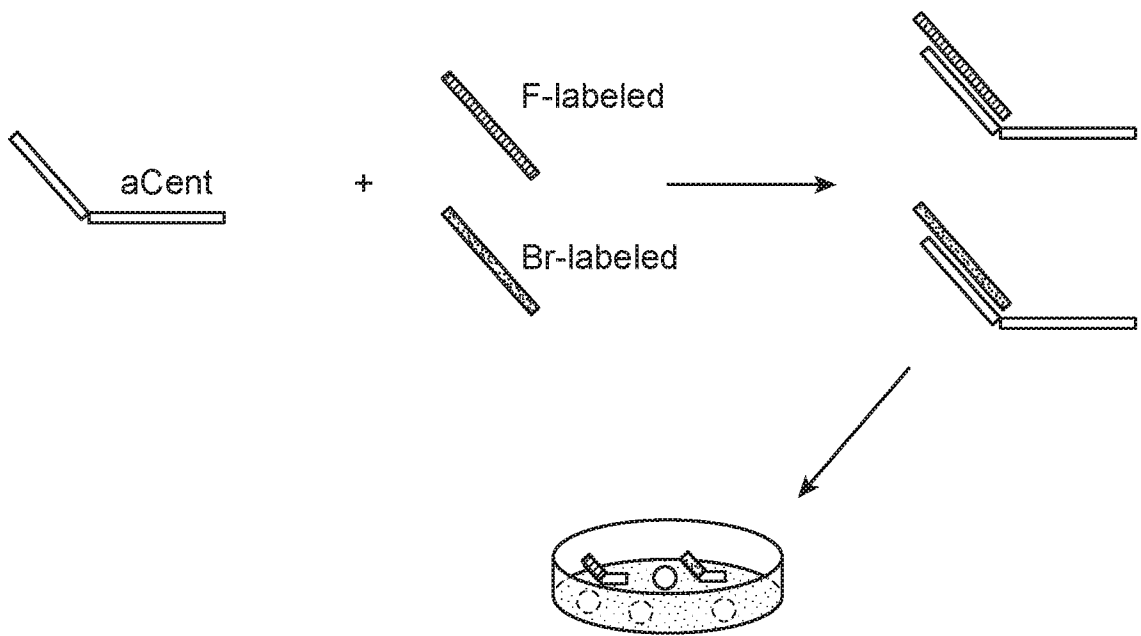


FIG. 53

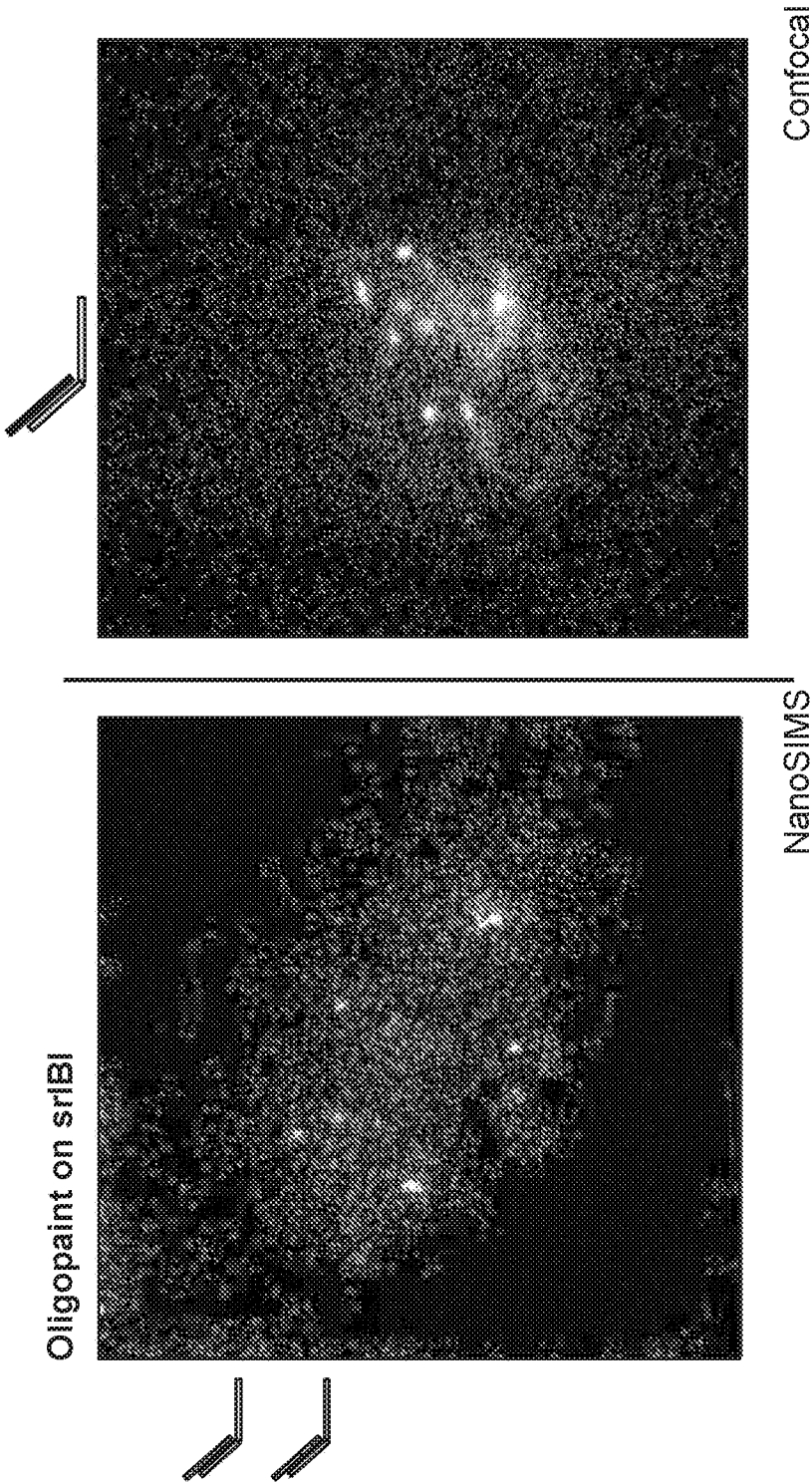


FIG. 54

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/028359

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/6872; G01N 33/532; G01N 33/68 (2019.01)

CPC - C12Q 1/6872; C12Q 2600/158; G01N 33/532; G01N 33/6848 (2019.05)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 514/44A; 530/391.3 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2016/0025731 A1 (FLUIDIGM CANADA INC.) 28 January 2016 (28.01.2016) entire document	1-9 ----- 10, 11
Y	WO 2017/156336 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 14 September 2017 (14.09.2017) entire document	10
Y	US 5,084,266 A (MCKENZIE et al) 28 January 1992 (28.01.1992) entire document	11
A	US 6,401,267 B1 (DRMANAC) 11 June 2002 (11.06.2002) entire document	1-11
A	US 2010/0273677 A1 (LUND-JOHANSEN) 28 October 2010 (28.10.2010) entire document	1-11

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 June 2019

Date of mailing of the international search report

05 JUL 2019

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, VA 22313-1450

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Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/028359

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 12-21
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.