Claims

1. A method for detecting different analytes in a sample simultaneously comprising the steps of:

(A) contacting the sample with at least four (4) different sets of analyte-specific probes for detecting of at least 4 different analytes, each set of analyte-specific probes interacting with a different analyte, wherein each analyte-specific probe is a complex comprising

(aa) a binding element (Y) that specifically interacts with one of the different analytes to be encoded, wherein each binding element (Y) of each set of analyte-specific probes comprises an identical identifier element (T), and

(bb) an identifier element (Z) that specifically interact and bind to the identifier element (T) of the binding element (Y), wherein the identifier element (Z) is coupled to an identifier fluorophore (f) or an identifier oligonucleotide (o) that is used to identify the identity of the identifier element (Z), wherein the identifier fluorophore (f) or the identifier oligonucleotide (o) of the identifier element (Z) in a particular set of analyte-specific probes differ from the identifier fluorophore (f) or the identifier oligonucleotide (o) of the identifier element (Z) of another set of analyte-specific probes,

wherein the binding element (Y) and the identifier element (Z) are premixed for each set of analyte-specific probes in a separate reaction tube to form complexes between the binding element (Y) and the identifier element (Z) before contacting the sample with the different sets of analyte-specific probes, wherein the identifier element (Z) interacts and binds to the identical identifier element (T) of the binding element (Y),

(B) detecting the signal caused by the identifier fluorophore (f) coupled to the identifier element (Z); or

(C) i) contacting the sample with different set of signal oligonucleotides, each signal oligonucleotide comprising:

(aa) a translator connector element (C) comprising a nucleotide sequence which is essentially complementary to at least a section of the nucleotide

sequence of an identifier oligonucleotide (o) comprised in an identifier element (Z), and

(bb) a signal element and/or an additional sequence that can be detected by hybridizing to one or multiple complementary oligonucleotides that are labeled or

ii) contacting the sample with at least one set of decoding oligonucleotides per analyte, wherein in each set of decoding oligonucleotides for an individual analyte each decoding oligonucleotide comprises:

(aa) an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the identifier oligonucleotide (o) of an identifier element (Z) of the corresponding analyte-specific probe set, and

(bb) a translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide;

wherein the decoding oligonucleotides of a set for an individual analyte differ from the decoding oligonucleotides of another set for a different analyte in the first connect element (t); and contacting the sample with at least a set of signal oligonucleotides, each signal oligonucleotide comprising:

(aa) a translator connector element (C) comprising a nucleotide sequence which is essentially complementary to at least a section of the nucleotide sequence of a translator element (c) comprised in a decoding oligonucleotide, and

(bb) a signal element and/or an additional sequence that can be detected by hybridizing to one or multiple complementary oligonucleotides that are labeled;

(D) Detecting the signal caused by the signal element of section C) i) and/or C) ii); and optionally

(E) Selectively removing of the signal oligonucleotides of section C) i) or the decoding oligonucleotides and signal oligonucleotides of section C) ii) from the sample,
thereby essentially maintaining the specific binding of the analyte-specific probes to the analytes to be encoded; and optionally

(F) Performing at least three (3) further cycles comprising steps C) to E) to generate an encoding scheme with a code word per analyte, wherein in particular the last cycle may stop with step (D).

2. The method according to claim 1, wherein the sample is a biological sample, preferably comprising biological tissue from human, animals, or plants, further preferably comprising biological cells and/or extracts and/or part of cells.

3. The method according to any one of claims 1 to 2, wherein the binding element (Y) is a protein and/or a nucleic acid that specifically recognizes and binds to the analyte.

4. The method according to any one of claims 1 to 3, wherein the binding element (Y) is an antibody, an antibody fragment, an aptamer or a nucleic acid that specifically interacts and binds to the analyte, wherein in particular the antibody fragment is selected from the group consisting of a Fab, scFv, single domain, bis scFv, Fab_2, Fab_3, minibody, diabody, triplebody, tetrabody and tandab.

5. The method according to any one of claims 1 to 4, wherein the identifier element (Z) is an antibody, an antibody fragment, an aptamer or a nucleic acid that specifically interacts and binds to the identifier element (Z), wherein in particular the antibody fragment is selected from the group consisting of a Fab, scFv, single domain, bis scFv, Fab_2, Fab_3, minibody, diabody, triplebody, tetrabody and tandab.

6. The method according to any one of claims 1 to 5, wherein the analyte is a protein comprised in a biological sample, the binding element (Y) is an antibody or an antibody fragment comprising a (Fc) region and the identifier element (Z) is a secondary antibody, a Fab, Fab_2 and/or a Fab_3 fragment.

7. The method according to any one of claims 1 to 6, wherein after step A) and before step B) the identifier elements (Z) are blocked to prevent cross reactivity, in particular by mixing with the binding element specific for the identifier element (Z).

8. The method according to any one of claims 1 to 7, wherein the analyte is protein, the identifier element (Y) is an antibody comprising a fragment crystallizable region (Fc region) as the identifier element (T), the identifier element (Z) is a secondary antibody, a Fab, Fab_2 and/or a Fab_3 fragment comprising an identifier oligonucleotide (o).

9. The method according to any one of claims 1 to 8, wherein the analyte is protein, the identifier element (Y) is an antibody comprising a fragment crystallizable region (Fc region) as the identifier element (T), the identifier element (Z) is a secondary antibody, a Fab, Fab_2 and/or a Fab_3 fragment comprising an identifier oligonucleotide (o), wherein the sample is contacted with at least one set of decoding oligonucleotides per analyte, wherein in each set of decoding oligonucleotides for an individual analyte each decoding oligonucleotide comprises:

    (aa) an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the identifier oligonucleotide (o) of an identifier element (Z) of the corresponding analyte-specific probe set, and

    (bb) a translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide;

    wherein the decoding oligonucleotides of a set for an individual analyte differ from the decoding oligonucleotides of another set for a different analyte in the first connect element (t); and contacting the sample with at least a set of signal oligonucleotides, each signal oligonucleotide comprising:

    (aa) a translator connector element (C) comprising a nucleotide sequence which is essentially complementary to at least a section of the nucleotide sequence of a translator element (c) comprised in a decoding oligonucleotide, and

    (bb) a signal element and/or an additional sequence that can be detected by hybridizing to one or multiple complementary oligonucleotides that are labeled.

10. The method according to any one of claims 1 to 9, wherein the premixing step is performed before step (A) performing at least 4 different complexes of individual Ys and individual Zs, wherein Z is coupled to a certain identifier oligonucleotide (o) to form 4 different combinations such as
    - Y1-Z(identfier-oligo1)
    - Y2-Z(identfier-oligo2)
    - Y3-Z(identfier-oligo3)
    - Y4-Z(identfier-oligo4)

11. The method according to any one of claims 1 to 10, wherein the sets of analyte-specific probes comprising the complexes between the binding element (Y) and the identifier

element (Z) are stored before contacting the sample with the different sets of analyte-specific probes.

12. The method according to any one of claims 1 to 11, wherein the complexes between the binding element (Y) and the identifier element (Z) are purified and/or filtered before contacting the sample with the complexes comprised in the different sets of analyte-specific probes.

13. The method according to any one of claims 1 to 12, wherein the complexes between the binding element (Y) and the identifier element (Z) are premixed in separate multiple tubes.

14. A kit for detecting different analytes in a sample simultaneously, wherein the kit comprises separately at least four (4) different sets of analyte-specific probes for encoding of at least 4 different analytes, each set of analyte-specific probes interacting with a different analyte, wherein each analyte-specific probe comprises complexes having

    (aa) a binding element (Y) that specifically interacts with one of the different analytes to be encoded, wherein each binding element (Y) of each set of analyte-specific probes comprises an identical identifier element (T), and

    (bb) an identifier element (Z) that specifically interact and bind to the identifier element (T) of the binding element (Y), wherein the identifier element (Z) is coupled to an identifier fluorophore (f) or an identifier oligonucleotide (o) that is used to identify the identity of the identifier element (Z), wherein the identifier fluorophore (f) or the identifier oligonucleotide (o) of the identifier element (Z) in a particular set of analyte-specific probes differ from the identifier fluorophore (f) or the identifier oligonucleotide (o) of the identifier element (Z) of another set of analyte-specific probes.

15. A method according to any of the proceeding claims for the use in an application selected from the group consisting of identify regions of interest, for image analysis, develop cell segmentation algorithms using immunofluorescence images, to aid the segmentation of cells in a sample, to establish AI-based image analysis algorithm using immunofluorescence images to identify disorders, feature extraction like nucleus morphology, signal homogeneity, signal intensity and/or centroids, to determine

expression of proteins and genes of interest via semi-quantitative approaches, diagnosis and prognosis applications, determination of the distribution of a target protein in the of health and disease organ, tissue and/or cell, to predict the prognosis of diseases by using disease-related prognostic and/or predictive biomarkers, to provide disease diagnosis e.g. tumor of unknow origin, neurodegenerative disorders, muscle disorders, brain trauma, infectious diseases, to predict a therapeutic response, subclassification of diseases, examine gene expression and to study gene regulation in normal and pathological tissues, morphological studies, recognition of tissue structures and/or cell type classifications, multiomics studies, quantitative correlation and spatial colocalization study between ISH and ICH, study epigenetic modifications and chromatin structures, identify spatial distribution, and cell-specific ISH signals (Cell segmentation) and determine the correlation of genetic aberrations and/or the copy number variations (CNV) to the corresponding proteins.