

COMPLEX FORMATION FOR PARALLEL DETECTION OF MULTIPLE ANALYTES IN MULTIPLE APPLICATIONS

5 FIELD OF THE DISCLOSURE

The technology provided herein relates to multiplex methods and kits for detecting different analytes in a sample simultaneously, in particular by using premixed macromolecule complexes like complexes having a primary and a secondary antibody, wherein the primary antibodies contain identical identifier elements.

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BACKGROUND

The analysis and detection of small quantities of analytes like RNA or proteins in biological and non-biological samples has become a routine practice in the clinical and analytical environment.

15 Numerous analytical methods have been established for this purpose. Some of them use encoding techniques assigning a particular readable code to a specific first analyte which differs from a code assigned to a specific second analyte.

One of the prior art techniques in this field is the so-called 'single molecule fluorescence in situ hybridization' (smFISH) essentially developed to detect mRNA molecules in a sample. In Lubeck et al. (2014), Single-cell in situ RNA profiling by sequential hybridization, Nat. Methods 11(4), p. 360-361, the mRNAs of interest are detected via specific directly labeled probe sets. After one round of hybridization and detection, the set of mRNA specific probes is eluted from the mRNAs and the same set of probes with other (or the same) fluorescent labels is used in the next round of hybridization and imaging to generate gene specific color-code schemes over several rounds. The technology needs several differently tagged probe sets per transcript and needs to denature these probe sets after every detection round.

A further development of this technology does not use directly labeled probe sets. Instead, the oligonucleotides of the probe sets provide nucleic acid sequences that serve as initiator for hybridization chain reactions (HCR), a technology that enables signal amplification; see Shah et al. (2016), In situ transcription profiling of single cells reveals spatial organization of cells in the mouse hippocampus, Neuron 92(2), p. 342-357.

Immunohistochemistry (IHC) refers to a processes of detecting, localizing, and/or quantifying antigens, such as a protein, in a biological sample using antibodies specific to the particular antigens. IHC provides the substantial advantage of identifying exactly where a particular protein is located within the tissue sample. It is also an effective way to examine the tissues themselves.

5 In situ hybridization (ISH) refers to the process of detecting, localizing, and quantifying nucleic acids. Both IHC and ISH can be performed on various biological samples, such as tissue (e.g. fresh frozen, formalin fixed, paraffin embedded) and cytological samples. Recognition of the targets can be detected using various labels (e.g., chromogenic, fluorescent, luminescent, radiometric), irrespective of whether the target is a nucleic acid or an antigen. To robustly detect, locate, and
10 quantify targets in a clinical setting, amplification of the recognition event is desirable as the ability to confidently detect cellular markers of low abundance becomes increasingly important for diagnostic purposes. For example, depositing at the marker's site hundreds or thousands of label molecules in response to a single antigen detection event enhances, through amplification, the ability to detect that recognition event.

15 However, conventional immunohistochemistry (IHC) methods do not necessarily provide adequate signal for detection, particularly for immunological targets that are weakly expressed or which are not efficiently targeted by existing IHC reagents. The foregoing immunohistochemical methods for target analyte labeling can e.g. be limited by the quantity of probe dye that can be delivered to specifically label each of the target analytes in the sample. Because probe dyes are
20 conjugated to specific antibodies, the amount of probe dye that can be applied to a specific location in a sample depends directly on the number of probe dye moieties that are directly attached to an antibody that binds to an analyte at that location. Certain immunohistochemical methods are therefore somewhat limited in their ability to amplify signals arising from particular target
25 analytes by selectively depositing larger quantities of probe dye at locations corresponding to those target analytes.

One technique based on Dako ARK™ is a chromogenic immunohistological staining. The system is designed for staining with mouse primary antibodies on formalin-fixed, paraffin-embedded tissues, cryostat tissues or cell preparations from any species, including mouse. The technique
30 used in this system are based on the avidin-biotin and peroxidase methodologies. The primary antibody and the biotinylation reagent are mixed in solution, resulting in binding of biotinylated secondary antibody to the primary antibody. The blocking reagent, containing normal serum, is then added to the mixture. The immunoglobulin present in the blocking reagent binds residual

biotinylation reagent not bound to the primary antibody. The biotin-labelled primary antibody is then applied to the specimen. The specimen is next incubated with streptavidin-peroxidase, followed by reaction with diaminobenzidine/hydrogen peroxide as substrate-chromogen.

5 The drawback of this technique are that the detection system, which is based on the enzymatic reaction of diaminobenzidine/hydrogen peroxide as substrate-chromogen, is not applicable for parallel protein detection (more than 2 proteins/ detection). Furthermore, streptavidin has a strong affinity to biotin, forming a biotin-avidin complex. Increasing number of parallel protein detection can cause cross interaction among the different primary antibody and Fab-Biotin
10 complex.

Therefore, the methods for detection of multiple analytes known in the art have numerous disadvantages. In particular, they are inflexible, expensive, complex, time consuming and quite often provide non-accurate results.

15 Against this background, it is an object underlying the present disclosure to provide a method by means of which the disadvantages of the prior art methods can be reduced or even avoided.

20 SUMMARY OF THE DISCLOSURE

The present disclosure pertains to novel multiplex methods and kits for detecting different analytes in a sample in parallel by using premixed complexes. In particular, the present disclosure relates to a parallel detection of analytes by primary decodeable complexes.

In a first aspect, the present disclosure pertains to methods 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
30 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:

5 (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 (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 of section C) ii) from the sample, thereby essentially maintaining the specific binding of the analyte-specific probes to the analytes to be

15 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).

In some embodiments, the present disclosure pertains to the use of complexes, wherein each
20 complex comprises an antigen(analyte)-specific primary antibody and a secondary antibody or an antibody fragment, wherein the secondary antibody or the antibody fragment is conjugated (tagged) with an oligonucleotide that is used to detect the antigens (analytes) of interest simultaneously. The complexes are formed before contacting to the sample in multiple reaction tubes and added to biological specimens. The presence of antigens (analytes) can be identified via
25 various options using complementary oligonucleotides. This allows a simultaneous detection of analytes.

In a second aspect, embodiments of the disclosure pertains to a method for detecting different analytes in a sample simultaneously, comprising: contacting the sample to a population of separately premixed different analyte-specific probe sets, wherein members of the population of
30 each analyte-specific probe set comprises complexes having

a) 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

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(b) 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 oligonucleotide (o) that is used to identify the identity of the identifier element (Z), wherein the identifier oligonucleotide (o) of the identifier element (Z) in a particular set of analyte-specific probes differ
10 from the identifier oligonucleotide (o) of the identifier element (Z) of another set of analyte-specific probes,

contacting the complexes bound to the analytes to a first decoder element population, the first decoder element population comprising probes having a region complementary to the identifier oligonucleotide (o) and a region having a first signal-element binding domain;
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contacting the first decoder element population bound to the complexes bound to the to the analytes with a first signal element probe comprising a first fluorophore; detecting a first fluorophore emission spectrum; removing the first decoder element population; contacting the complexes bound to the analytes to a second decoder element population, the second decoder element population comprising probes having a region complementary to the identifier oligonucleotide (o) element and a region having a second signal-element binding domain; contacting the identifier element bound analyte complex to a second signal element probe comprising a second fluorophore; detecting a second fluorophore emission spectrum; and
20 associating a pattern of first fluorophore emission spectra and second fluorophore emission spectra consistent with an order of addition of first decoder element populations and second decoder element populations.
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In a second aspect, embodiments of the disclosure pertains to a method for detecting different analytes in a sample simultaneously comprising: a) contacting a biological sample with at least
30 four (4) different sets of analyte-specific antibodies or antibody fragments for detecting at least four (4) different analytes, wherein the antibodies or antibody fragments of each set bind to different analytes compared to the antibodies or antibody fragments of another set, wherein the

antibodies or antibody fragments in all sets comprise the same (Fc) region, wherein the different antibodies or antibody fragments in the different sets are separately premixed in each set of analyte-specific probes with secondary antibodies or antibody fragments that binds to the (Fc) region of the analyte-specific antibodies or antibody fragments to form complexes between the

5 analyte-specific antibodies or antibody fragments and the secondary antibodies, wherein the secondary antibodies comprise each an identifier oligonucleotide (o), wherein the identifier oligonucleotide (o) comprised on the secondary antibodies in each set differs from the identifier oligonucleotide (o) in another set; b) contacting said complexes bound to the analytes with decoding oligonucleotides; c) contacting said decoding oligonucleotides bound to said complexes

10 with signal oligonucleotides; d) detection of the signal oligonucleotides bound to said decoding oligonucleotides comprised in said complexes bound to the analytes.

In a third aspect, embodiments of the disclosure pertains to a method for detecting different analytes in a sample simultaneously comprising the steps of:

15 (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 having

20 (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 interacts and binds to the identifier element (T) of the binding element (Y), wherein the identifier element (Z) is coupled to an identifier fluorophore (f) and/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

25 analyte-specific probes,

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wherein the binding element (Y) and the identifier element (Z) are premixed in each set of analyte-specific probes 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 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

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(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)

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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

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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

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(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

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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;

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

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

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

10 In a third aspect, embodiments of the disclosure pertains to a mixture for detecting different analytes in a sample simultaneously, wherein the mixture comprises 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

- 15 (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.

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In particular, the present description describes the combinatorial use of antibodies together with secondary antibodies, FABs or proteins that specially bind to an antibody or protein that recognize and bind specially another protein. Therefore, the present disclosure relates to a parallel detection of analytes by primary decodeable complexes. This refers to complexes of an antigen-specific of primary antibody and am secondary antibody or Fab conjugated oligonucleotide that are used to detect antigens of interest simultaneously. The complexes between the first antibodies and the secondary bantibodies are formed in multiple reaction tubes and added to biological specimens.

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The present of antigens can be identified via various options using complementary oligonucleotides. This allows a simultaneous detection of analytes, in particular of proteins.

In a further aspect, embodiments of the disclosure pertains to 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.

Before the disclosure is described in detail, it is to be understood that this disclosure is not limited to the particular component parts of the steps of the methods described. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include singular and/or plural referents unless the context clearly dictates otherwise. It is moreover to be understood that, in case parameter ranges are given which are delimited by numeric values, the ranges are deemed to include these limitation values.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows examples of 1) identifier element (Z) that specifically interact and bind to the identifier element (T) of a binding element (Y) and examples 2) of complexes comprising

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),
 5 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
 10 set of analyte-specific probes.

Fig. 2 are schematic diagrams showing methods for sample analysis according to the present disclosure.

15 Fig. 3 shows a cryosection of murine kidney was probed for nestin. Rabbit primary antibody against nestin was prelabelled with Alexa Flour 647 conjugated to goat anti-rabbit IgG (upper panel) or goat anti rabbit Fab (lower panel) with a ratio between primary antibody and secondary antibody of 1:0.2 and 1:2 (molecules/molecules), respectively. The prelabelling was performed for 30 minutes and residual secondary antibody or Fab not
 20 bound to primary antibody was blocked with 0.1% v/v normal rabbit serum for 5 min. The antibody complexes were added to tissues and incubated overnight. Images were captured with ZEISS Celldiscoverer 7 using a Apochromat 5X/0.35 objective and 2X Tubelens. (Exposure time: 1 second and 0.1 second for Alexa Flour 647 and DAPI, respectively).

25 Fig. 4 shows a cyrosection of murine kidney probed for Nestin. Detection of Nestin was done by precomplexed primary antibody (Abcam ab176571) with oligos conjugated secondary antibody followed by complementary oligos conjugated to fluorophores (A). The localization of the primary-secondary antibody complexes was confirmed by probing the tissue with fluorophores conjugated antibody complemented to the oligo conjugated
 30 secondary antibody (B).

DETAILED DESCRIPTION OF THE DISCLOSURE

The present disclosure features methods for performing multiplexed labeling, identification and quantification of target analytes in a biological sample. The methods can be used to perform multiple cycles of target analyte labeling, detection, and removal of certain agents involved in the labeling process, without disrupting antibody-analyte binding in the sample. Instead, removal of
5 agents involved in the labeling process is performed by dehybridizing the agents under relatively mild conditions, preserving sample integrity and ensuring that removal of the agents during each labeling and detection cycle is nearly complete. As a result, little or no cross-species labeling occurs. Instead, each target analyte in the sample can be selectively labeled with a different labeling species, and deposition of each labeling species is highly constrained to regions of the
10 sample that specifically correspond to the location of a specific target analyte.

With the methods of the present disclosure multiplexed labeling, identification, signal amplification, and quantification of target analytes in a biological sample may be performed. The methods of the present disclosure can be used to perform multiple cycles of target analyte labeling, detection, and removal of certain agents involved in the labeling process, without
15 disrupting antibody-analyte binding in the sample. Instead, removal of agents involved in the labeling process is performed by dehybridizing the agents under relatively mild conditions, preserving sample integrity and ensuring that removal of the agents during each labeling and detection cycle is nearly complete. As a result, little or no cross-species labeling occurs. Instead, each target analyte in the sample can be selectively labeled with a different labeling species, and
20 deposition of each labeling species is highly constrained to regions of the sample that specifically correspond to the location of a specific target analyte. The present disclosure describes the usage of a set of labeled and unlabeled nucleic acid sequences for specific quantitative and/or spatial detection of different analytes in parallel via specific hybridization. The technology allows the discrimination of more different analytes than different detection signals are available. The
25 discrimination may be realized via sequential signal-coding of the analytes achieved by several cycles of specific hybridization, detection of signals and selective elution of the hybridized nucleic acid sequences.

In contrast to other state-of-the-art methods, the oligonucleotides providing the detectable signal are not directly interacting with sample-specific analyte-specific probes but may be also mediated
30 by so called "decoding-oligonucleotides". This mechanism decouples the dependency between the analyte-specific probes and the signal oligonucleotides. The use of decoding-oligonucleotides allows a much higher flexibility while dramatically decreasing the number of different signal

oligonucleotides needed which in turn increases the coding capacity achieved with a certain number of detection rounds.

The utilization of decoding-oligonucleotides leads to a sequential signal-coding technology that is more flexible, cheaper, simpler, faster and/or more accurate than other methods.

5 In particular, the step of premixing the binding elements (Y) with the identifier elements (Z) in multiple reaction tubes (for each type of analyte-specific probes one separate tube) to form complexes before contacting the sample with these complexes has the advantage that:

- 1) Reduction of experimental complexity I:
Usually primary ABs generated in different species (goat, rabbit, chicken etc.) must be used
10 for multiplex protein detection (see table 1a). If a complex between primary antibody (primary AB) and secondary antibody (secondary AB) or secondary antibody FAB fragment is formed before applying primary AB to the sample, different primary ABs generated in the same species can be used (see table 1b below)

15 Table 1a:

Target Protein ¹⁾	Target organis m ²⁾	Primary AB	Primary AB derived from :	Secondary AB	Fluorescence	Secondary AB derived from :
T1	Human	Anti T1 AB	rabbit	specific for rabbit	Fluorophore 1	goat
T2	Human	Anti T2 AB	chicken	specific for chicken	Fluorophore 2	goat
T3	Human	Anti T3 AB	donkey	specific for donkey	Fluorophore 3	goat

Table 1b:

Target Protein ¹⁾	Target organis m ²⁾	Primary AB	Primary AB derived from :	Secondary AB	Fluorescence	Secondary AB derived from :
T1	Human	Anti T1 AB	rabbit	specific for rabbit	Fluorophore 1	goat

T2	Human	Anti T2	rabbit	specific for	Fluorophore 2	goat
		AB		rabbit		
T3	Human	Anti T3	rabbit	specific for	Fluorophore 3	goat
		AB		rabbit		

2) Reduction of experimental complexity II:

If the the detection is achieved by an oligonucleotide coupled to the AB, complex formation prior binding to the sample may be of advantage. In this case, a secondary AB (or FAB) can be used for the coupling with different oligonucleotides. The advantage here is that not all primary ABs must be linked to oligonucleotides but only a limited library of secondary ABs. Secondary ABs (or FABs) could be linked to oligos in an much greater set up than individual primary ABs.

3) Reduction of cost and time:

Labeling of primary ABs is very expensive an time consuming because the scale is small. On the other side, a labeling of secondary ABs (or FAB) can be done at a larger scale and more cost effective. The labelled secondary AB (orFAB) can be used in a versatile and ubiquitous way which is not possible with fluorophore labelled primary ABs. A complex pre-formation of primary AB and fluorophore labelled secondary AB (orFAB) can substitute for fluorophore labelled primary AB.

4) Increasing sensitivity:

In highly multiplexed protein detection systems, labeled primary ABs are used to avoid problems in compatibility with secondary ABs. For these assays, the use of labeled primary ABs limits the sensitivity by the number of labels coupled to the primary ABs. If complex pre-formation between primary ABs and labeled secondary ABs can be performed, the sensitivity can increase because multiple secondary ABs can bind to the primary AB and each of the secondary ABs are labeled multiple times.

5) Reduction of time:

Preformation of a complex between primary AB and labelled, secondary AB (or FAB) can be done in an highly parallel way. After complex pre-formation, multiple complexes can be

incubated with the sample simultaneously. Thereafter, a sequential detection can be performed.

6) Reducing background effects I:

- 5 Background effects can occur by primary and secondary ABs. The background effect by secondary ABs can be minimized by complex pre-formation between primary and secondary AB if the concentration of the secondary AB is lower than the concentration of the primary AB. In this case, the secondary AB is almost completely bound to the primary AB and no secondary AB is left over.

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A. Definitions

- According to the present disclosure an "analyte" is the subject to be specifically detected as being present or absent in a sample and, in case of its presence, to encode it. It can be any kind of entity, including a protein, polypeptide, protein or a nucleic acid molecule (e.g. RNA, PNA or DNA) of interest. The analyte provides at least one site for specific binding with analyte-specific probes. Sometimes herein the term "analyte" is replaced by "target". An "analyte" according to the disclosure includes a complex of subjects, e.g. at least two individual nucleic acid, protein or peptides molecules. In an embodiment of the disclosure an "analyte" excludes a chromosome. In another embodiment of the disclosure an "analyte" excludes DNA. In an advantageous embodiment, the analyte is a protein/polypeptide.
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- As used herein, the terms "contacts" and "contacting" mean that an agent, species, moiety, or other element is brought into association with a sample, or another agent, species, moiety, or element, such that the two interact with one another. For example, when the sample comprising the analytes is "contacted" with the different sets of analyte-specific probes, the analyte-specific probes are brought into close enough association with the sample that they interact with the analytes in the sample.
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- In some embodiments, an analyte may be a "coding sequence", "encoding sequence", "structural nucleotide sequence" or "structural nucleic acid molecule" which refers to a nucleotide sequence that is translated into a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding
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sequence can include, but is not limited to, genomic DNA, cDNA, EST and recombinant nucleotide sequences.

5 A "sample" as referred to herein is a composition in liquid or solid form suspected of comprising the analytes to be encoded. In particular, the sample is a biological sample, preferably comprising biological tissue, further preferably comprising biological cells and/or extracts and/or part of cells. For example, the cell is a prokaryotic cell or a eukaryotic cell, in particular a mammalian cell, in particular a human cell. In some embodiments, the biological tissue, biological cells, extracts and/or part of cells are fixed. In particular, the analytes are fixed in a permeabilized sample, such as a cell-containing sample.

10 As used in the present disclosure, "cell", "cell line", and "cell culture" can be used interchangeably and all such designations include progeny. Thus, the words "transformants" or "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included.

20 An "encoding scheme" may describe a set of code words that are associated with the analytes to be detected. Each code word refers to one of the analytes and can be distinguished from all other code words. A code word hereby is a sequence of signs provided by the detection cycles of the method. A sign within a code word is a detectable signal or the absence of a signal. A code word does not need to comprise of all different signals used in the method. The number of signs in a code word is defined by the number of detection cycles.

25 An "oligonucleotide" as used herein, refers to a short nucleic acid molecule, such as DNA, PNA, LNA or RNA. The length of the oligonucleotides is within the range 4-200 nucleotides (nt), preferably 6-80 nt, more preferably 8-60 nt, more preferably 10-50 nt, more preferably 12 to 35 depending on the number of consecutive sequence elements. The nucleic acid molecule can be fully or partially single-stranded. The oligonucleotides may be linear or may comprise hairpin or loop structures. The oligonucleotides may comprise modifications such as biotin, labeling moieties, blocking moieties, or other modifications.

The "analyte-specific probe" is a complex consisting of at least two elements, namely the so-called binding element (Y) which specifically interacts with one of the analytes, wherein each binding element (Y) of each set of analyte-specific probes comprises an identical identifier element (T), and a so-called identifier element (Z) that specifically interacts and bind to the identical identifier element (T) of the binding element (Y) and comprises an identifier fluorophore (f) or an identifier oligonucleotide (o). The binding element (Y) may be a nucleic acid such as a hybridization sequence or an aptamer, or a peptidic structure such as an antibody.

In particular, 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, Fab₂, Fab₃, minibody, diabody, triplebody, tetrabody and tandab.

In particular, the identifier element (Z) 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, Fab₂, Fab₃, minibody, diabody, triplebody, tetrabody and tandab.

In an advantageous embodiment of the present disclosure, the binding element (Y) is an antibody or an antibody fragment like a Fab, scFv, single domain, bis scFv, Fab₂, Fab₃ fragment and the identifier element (Z) is also a an antibody or an antibody fragment like a Fab, scFv, single domain, bis scFv, Fab₂, Fab₃ fragment. In an advantageous embodiment of the present disclosure, the binding element (Y) is an antibody and the identifier element (Z) is a secondary antibody.

In an advantageous embodiment of the present disclosure, the analyte is a protein/polypeptide 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, Fab₂ and/or a Fab₃ fragment.

In particular, in some embodiments the binding element (Y) comprises moieties which are affinity moieties from affinity substances or affinity substances in their entirety selected from the group consisting of antibodies, antibody fragments, receptor ligands, enzyme substrates, lectins, cytokines, lymphokines, interleukins, angiogenic or virulence factors, allergens, peptidic

allergens, recombinant allergens, allergen-idiotypal antibodies, autoimmune-provoking structures, tissue-rejection-inducing structures, immunoglobulin constant regions and their derivatives, mutants or combinations thereof. In further advantageous embodiments, the antibody fragment is a Fab, an scFv; a single domain, or a fragment thereof, a bis scFv, Fab2, Fab3, 5 minibody, maxibody, diabody, triabody, tetrabody or tandab, in particular a single-chain variable fragment (scFv).

The "identical identifier element (T)" as comprised in a binding element (Y) of an analyte-specific probe is identical in its sequence compared to other identical identifier elements (T) in other 10 binding element (Y) of another analyte-specific probe. In other words, the identical identifier elements (T) comprised in all binding elements (Y) in all sets of analyte-specific probes are identical. Or, in other words, there is only one identical identifier element (T) sequence for all binding elements (Y) used in a approach. An identical identifier element may be a sequence element of the binding element (Y), attached directly or by a linker, a covalent bond or high affinity 15 binding modes, e.g. antibody-antigen interaction, streptavidin-biotin interaction etc.

It is understood that the term "analyte specific probe" includes a plurality of complexes which may differ in their binding elements (Y) in a way that each probe binds to the same analyte but possibly to different parts thereof, for instance to different (e.g. neighboring) or overlapping 20 sections of the polypeptide sequence comprised by the protein to be detected. However, each of the plurality of the probes comprises the same identical identifier element (T).

A "decoding oligonucleotide" or an "adapter" or a /adapter segment" consists of at least two sequence elements. One sequence element that can specifically bind to a unique identifier 25 sequence, referred to as an "identifier connector element "(t) or "first connector element" (t), and a second sequence element specifically binding to a signal oligonucleotide, referred to as "translator element" (c). The length of the sequence elements is within the range 8-60 nt, preferably 12-40 nt, more preferably 14-20 nt, depending on the number of analytes to be encoded in parallel, the stability of interaction needed and the number of different signal 30 oligonucleotides used. The length of the two sequence elements may or may not be the same.

In some advantageous embodiments, the decoding oligonucleotide in the kits and/or methods of the present disclosure may be a "multi-decoder". A "multi-decoder" is a decoding oligonucleotide

that consists of at least three sequence elements. One sequence element (the identifier connector element (t)) can specifically bind to identifier oligonucleotide (o) of an identifier element (Z) of the corresponding analyte-specific probe set and at least two other sequence elements (translator elements (c)) specifically bind different signal oligonucleotides (each of these sequence elements specifically binds a signal oligonucleotide that differs to all other signal oligonucleotides recruited by other elements of the multi-decoder). The length of the sequence elements is within the range 8-60 nt, preferably 12-40 nt, more preferably 14-20 nt, depending on the number of analytes detected in parallel, the stability needed and the number of different signal oligonucleotides used. The length of the sequence elements may or may not be the same.

Therefore, in some advantageous embodiments, the decoding oligonucleotide is a multi-decoder comprising

- 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
- at least two translator elements (c) comprising each a nucleotide sequence allowing a specific hybridization of a different signal oligonucleotide.

Therefore, the first translator element binds a different signal oligonucleotide as the second translator element. In particular, the signal oligonucleotides differ in the signal element comprised in the signal oligonucleotide, e.g. in the kind of the fluorophore.

A "signal oligonucleotide" or a "reporter" as used herein comprises at least two elements, a so-called "translator connector element" (C) or "second connector element" (C) having a nucleotide sequence specifically hybridizable to at least a section of the nucleotide sequence of the identifier oligonucleotide (o) comprised in an identifier element (Z) and/or the translator element (c) of a decoding oligonucleotide, and a "signal element" which provides a detectable signal and/or an additional sequence that can be detected by hybridizing to one or multiple complementary oligonucleotides that are labeled. This element can either actively generate a detectable signal or provide such a signal via manipulation, e.g. fluorescent excitation. Typical signal elements are, for example, enzymes that catalyze a detectable reaction, fluorophores, radioactive elements or dyes.

A "set" refers to a plurality of moieties or subjects, e.g. analyte-specific probes, signal oligonucleotides or decoding oligonucleotides, whether the individual members of said plurality

are identical or different from each other. In all analyte specific probe sets, the identical identifier elements (T) of the binding elements (Y) of the analyte specific probes are identical.

Therefore, in an advantageous embodiment each set of analyte-specific probes comprises
5 premixed complexes, wherein the complexes of one analyte-specific probe set compared to the complexes of another analyte-specific probe set comprises a different binding element (Y) that specifically interacts with one of the different analytes to be encoded but comprises identical identifier elements (T) that interact with identifier elements (Z) comprising different identifier fluorophores (f) or identifier oligonucleotides (o) compared to the the identifier elements (Z) of
10 another set of analyte-specific probes.

"Selective denaturation" may be the process of eliminating bound decoding oligonucleotides and signal oligonucleotides with highest efficiency while at the same time the target specific probes have to stay hybridized with the highest efficiency. The total efficiency of these two combined
15 events may to be at least 0.22 for two detection cycles, 0.37 for three detection cycles, 0.47 for four detection cycles, 0.55 for five detection cycles, 0.61 for six detection cycles, 0.65 for seven detection cycles, 0.69 for eight detection cycles, 0.72 for nine detection cycles and 0.74 for 10 detection cycles, 0.76 for 11 detection cycles and 0.78 for 12 detection cycles.

20 In an embodiment of the disclosure a single set refers to a plurality of oligonucleotides.

An "analyte specific probe set" refers to a plurality of moieties or subjects, e.g. analyte-specific probes that are different from each other and bind to independent regions of the analyte. A single analyte specific probe set is further characterized by the same identifier element (Z).

25 A "decoding oligonucleotide set" refers to a plurality of decoding oligonucleotides specific for a certain unique identifier needed to realize the encoding independent of the length of the code word. Each and all of the decoding oligonucleotides included in a "decoding oligonucleotide set" bind to the same identifier oligonucleotide (o) of the analyte-specific probe.

30 In certain embodiments, this pattern of binding or hybridization of the decoding oligonucleotides may be converted into a "code word." For example, the code words could be also "101" and "110" for an analyte, where a value of 1 represents binding and a value of 0 represents no binding. The

code words may also have longer lengths in other embodiments (see Fig. 13). A code word can be directly related to a specific unique identifier sequence of an analyte-specific probe. Accordingly, different analyte-specific probes may match certain code words, which can then be used to identify the different analytes of the analyte-specific probe based on the binding patterns of the decoding oligonucleotide. However, if no binding is evident, then the code word would be "000" in this example.

The values in each code word can also be assigned in different fashions in some embodiments. For example, a value of 0 could represent binding while a value of 1 represents no binding. Similarly, a value of 1 could represent binding of a secondary nucleic acid probe with one type of signaling entity while a value of 0 could represent binding of a secondary nucleic acid probe with another type of distinguishable signaling entity. These signaling entities could be distinguished, for example, via different colors of fluorescence. In some cases, values in code words need not be confined to 0 and 1. The values could also be drawn from larger alphabets, such as ternary (e.g., 0, 1, and 2) or quaternary (e.g., 0, 1, 2, and 3) systems. Each different value could, for example, be represented by a different distinguishable signaling entity, including (in some cases) one value that may be represented by the absence of signal.

The code words for each analyte may be assigned sequentially, or may be assigned at random. For instance, a first analyte may be assigned to 101, while a second nucleic acid target may be assigned to 110. In addition, in some embodiments, the code words may be assigned using an error-detection system or an error-correcting system, such as a Hamming system, a Golay code, or an extended Hamming system (or a SECDED system, i.e., single error correction, double error detection). Generally speaking, such systems can be used to identify where errors have occurred, and in some cases, such systems can also be used to correct the errors and determine what the correct code word should have been. For example, a code word such as 001 may be detected as invalid and corrected using such a system to 101, e.g., if 001 is not previously assigned to a different target sequence. A variety of different error-correcting codes can be used, many of which have previously been developed for use within the computer industry; however, such error-correcting systems have not typically been used within biological systems. Additional examples of such error-correcting codes are discussed in more detail below.

"Essentially complementary" means, when referring to two nucleotide sequences, that both sequences can specifically hybridize to each other under stringent conditions, thereby forming a hybrid nucleic acid molecule with a sense and an antisense strand connected to each other via hydrogen bonds (Watson-and-Crick base pairs). "Essentially complementary" includes not only perfect base-pairing along the entire strands, i.e. perfect complementary sequences but also imperfect complementary sequences which, however, still have the capability to hybridize to each other under stringent conditions. Among experts it is well accepted that an "essentially complementary" sequence has at least 88% sequence identity to a fully or perfectly complementary sequence.

"Percent sequence identity" or "percent identity" in turn means that a sequence is compared to a claimed or described sequence after alignment of the sequence to be compared (the "Compared Sequence") with the described or claimed sequence (the "Reference Sequence"). The percent identity is then determined according to the following formula: percent identity = $100 [1 - (C/R)]$, wherein C is the number of differences between the Reference Sequence and the Compared Sequence over the length of alignment between the Reference Sequence and the Compared Sequence, wherein

(i) each base or amino acid in the Reference Sequence that does not have a corresponding aligned base or amino acid in the Compared Sequence and

(ii) each gap in the Reference Sequence and

(iii) each aligned base or amino acid in the Reference Sequence that is different from an aligned base or amino acid in the Compared Sequence, constitutes a difference and (iiii) the alignment has to start at position 1 of the aligned sequences;

and R is the number of bases or amino acids in the Reference Sequence over the length of the alignment with the Compared Sequence with any gap created in the Reference Sequence also being counted as a base or amino acid.

If an alignment exists between the Compared Sequence and the Reference Sequence for which the percent identity as calculated above is about equal to or greater than a specified minimum Percent Identity then the Compared Sequence has the specified minimum percent identity to the Reference Sequence even though alignments may exist in which the herein above calculated percent identity is less than the specified percent identity.

In the "incubation" steps as understood herein the respective moieties or subjects such as probes or oligonucleotide, are brought into contact with each other under conditions well known to the skilled person allowing a specific binding or hybridization reaction, e.g. pH, temperature, salt
5 conditions etc. Such steps may therefore, be preferably carried out in a liquid environment such as a buffer system which is well known in the art.

The "removing" steps according to the disclosure may include the washing away of the moieties or subjects to be removed such as the probes or oligonucleotides by certain conditions, e.g. pH,
10 temperature, salt conditions etc., as known in the art.

It is understood that in an embodiment of the method according to the present disclosure a plurality of analytes can be encoded simultaneously. This requires the use of different sets of analyte-specific probes in step (A). The analyte-specific probes of a particular set differ from the
15 analyte-specific probes of another set. This means that the analyte-specific probes of set 1 bind to analyte 1, the analyte-specific probes of set 2 bind to analyte 2, the analyte-specific probes of set 3 bind to analyte 3, etc. However, all analyte-specific probes comprises the same identical identifier element (T).

20 A "kit" is a combination of individual elements useful for carrying out the use and/or method of the disclosure, wherein the elements are optimized for use together in the methods. The kits may also contain additional reagents, chemicals, buffers, reaction vials etc. which may be useful for carrying out the method according to the disclosure. Such kits unify all essential elements required to work the method according to the disclosure, thus minimizing the risk of errors.
25 Therefore, such kits also allow semi-skilled laboratory staff to perform the method according to the present disclosure.

The method is particularly qualified to encode, identify, detect, count or quantify analytes or single analytes molecules in a biological sample, i.e. such as a sample which contains nucleic acids or
30 proteins as said analytes. It is understood that the biological sample may be in a form as it is in its natural environment (i.e. liquid, semi-liquid, solid etc.), or processed, e.g. as a dried film on the surface of a device which may be re-liquefied before the method is carried out.

- In another embodiment of the disclosure prior to step (A) the biological tissue and/or biological cells are fixed. For example, in some embodiments, the cell and/or the tissue is fixed prior to introducing the probes, e.g., to preserve the positions of the analytes like proteins or nucleic acids within the cell. Techniques for fixing cells are known to those of ordinary skill in the art. As non-limiting examples, a cell may be fixed using chemicals such as formaldehyde, paraformaldehyde, glutaraldehyde, ethanol, methanol, acetone, acetic acid, or the like. In one embodiment, a cell may be fixed using Hepes-glutamic acid buffer-mediated organic solvent (HOPE).
- 10 This measure has the advantage that the analytes to be encoded, e.g. the nuclei acids or proteins, are immobilized and cannot escape. In doing so, the analytes then prepared for a better detection or encoding by the method according to the disclosure.
- In yet a further embodiment within the set of analyte-specific probes the individual analyte-specific probes comprise binding elements (S1, S2, S3, S4, S5) which specifically interact with different sub-structures of one of the analytes to be encoded.
- 20 By this measure the method becomes even more robust and reliable because the signal intensity obtained at the end of the method or a cycle, respectively, is increased. It is understood, that the individual probes of a set while binding to the same analyte differ in their binding position or binding site at or on the analyte. The binding elements S1, S2, S3, S4, S5 etc. of the first, second, third fourth, fifth etc. analyte-specific probes therefore bind to or at a different position which, however, may or may not overlap.
- 25 In some advantageous embodiments according to the present disclosure at least 20, in particular at least 25, in particular at least 30 different analytes are detected and/or quantified in a sample in parallel. For example, there may be at least 5, at least 10, at least 20, at least 50, at least 75, at least 100, at least 300, at least 1,000, at least 3,000, at least 10,000, or at least 30,000 distinguishable analyte-specific probes that are applied to a sample, e.g., simultaneously or sequentially.
- 30

In some advantageous embodiments for the multiplexing twenty (20) or more different sets of analyte-specific probes for encoding of at least 20 different analytes or more are required, in particular more than 50, more than 100 or more than 200. In the multiplexing methods of the present disclosure, in particular at least 20 different groups of analytes (e.g. mRNA
5 molecules polypeptides/proteins) i.e. tags are targeted.

In some advantageous embodiments, at least 4 rounds to collect information for identification of the analyte are carried out, wherein multiple readout increases the accuracy of identification and avoids false positives. The unique tag can be identified by various techniques, including
10 hybridization, e.g. with labeled probes, directly or indirectly or by sequencing (by synthesis, ligation). In particular, the identity of the tag can be encoded with one single signal (binary code), two or more signals, wherein the signal can be a fluorescent label (e.g. attached to an oligonucleotide).

15 In some advantageous embodiments according to the present disclosure, the kit does not comprise sets of analyte-specific probes as defined under item A).

Preferably, if the analyte in the kits or methods according to the present disclosure is a nucleic acid, each set of analyte-specific probes comprises at least five (10) analyte-specific probes, in particular at least fifteen (15) analyte-specific probes, in particular at least twenty (20) analyte-specific probes which specifically interact with different sub-structures of the same analyte. Nucleic acid analyte includes specific DNA molecules, e.g. genomic DNA, nuclear DNA, mitochondrial DNA, viral DNA, bacterial DNA, extra- or intracellular DNA etc., and specific mRNA molecules, e.g. hnRNA, miRNA, viral RNA, bacterial RNA, extra- or intracellular RNA, etc.

25 Preferably, if the analyte in the kits or methods according to the present disclosure is a peptide, a polypeptide or a protein, each set of analyte-specific probes comprises at least two (2) analyte-specific probes, in particular at least three (3) analyte-specific probes, in particular at least four (4) analyte-specific probes which specifically interact with different sub-structures of the same
30 analyte.

In some advantageous embodiments according to the present disclosure the kit comprises at least two different sets of signal oligonucleotides, wherein the signal oligonucleotides in each set comprise a different signal element and comprise a different connector element (C).

5 In particular, the kit may comprise at least two different sets of decoding oligonucleotides per analyte, wherein the decoding oligonucleotides comprised in these different sets comprise the same identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of the corresponding analyte-specific probe set, and wherein the decoding oligonucleotides of the
10 different sets per analyte differ in the translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide.

In some embodiments the kit comprises at least two different sets of decoding oligonucleotides per analyte, wherein the decoding oligonucleotides comprised in these different sets comprise the
15 same identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of the corresponding analyte-specific probe set, and wherein the decoding oligonucleotides of the different sets for at least one analyte differ in the translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide.

20 In some advantageous embodiments, the number of different sets of decoding oligonucleotides per analyte comprising different translator elements (c) corresponds to the number of different sets of signal oligonucleotides comprising different connector elements (C). However, the decoding oligonucleotides in a particular set of decoding oligonucleotides may interact with
25 identical identifier oligonucleotide (o) of an identifier element (Z) which are unique to a particular analyte. In particular, all sets of decoding oligonucleotides for the different analytes may comprise the same type(s) of translator element(s) (c).

In another aspect, the present disclosure is generally directed to a method including acts of
30 exposing a sample to a plurality of analyte-specific probes; for each of the analyte-specific probes, determining binding of the analyte-specific probes within the sample; creating code words based on the binding of the analyte-specific probes, the decoding oligonucleotides and the signal oligonucleotides; and for at least some of the code words, matching the code word to a valid code

word. In certain embodiments, this pattern of binding or hybridization of the analyte-specific probes, the decoding oligonucleotides and the signal oligonucleotides may be converted into a "code word." For example, for instance, the code words may be "101" and "110" for a first analyte and a second analyte, respectively, where a value of 1 represents binding and a value of 0 represents no binding of decoding oligonucleotides and/or the binding of signal oligonucleotides without and/or quenched signal element. The analyte in the detection round/cycle is therefore not detectable during imaging.

To create such a zero (0) in a code word for an individual analyte the kit may comprise:

10 **(D)** at least a set of non-signal decoding oligonucleotides for binding to a particular identifier element (T) of analyte-specific probes, wherein the decoding oligonucleotides in the same set of non-signal decoding oligonucleotides interacting with the same different identifier element (T),

wherein each non-signal decoding oligonucleotide comprises an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of a unique identifier sequence, and does not comprise a translator element (c) comprising

15 a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide.

In some advantageous embodiments, all steps are automated, in particular by using a robotic system and/or an optical multiplexing system according to the present disclosure. In some

20 examples, the steps may be performed in a fluidic system.

As mentioned above, with the methods according to the present disclosure an encoding scheme with a code word per analyte is generated. Therefore, each analyte may be associated with a specific code word, wherein said code word comprise a number of positions, and wherein each position corresponds to one cycle resulting in a plurality of distinguishable encoding schemes with the plurality of code words. In particular, said encoding scheme may be predetermined and allocated to the analyte to be encoded.

25

As mentioned above, the analyte to be encoded may be a nucleic acid, preferably DNA, PNA, RNA, in particular mRNA, a peptide, polypeptide, a protein or combinations thereof. Therefore, the binding element (Y) may comprise an amino acid sequence allowing a specific binding to the analyte to be encoded. Examples for a binding element (Y) are moieties which are affinity moieties from affinity substances or affinity substances in their entirety selected from the group consisting of antibodies, antibody fragments, anticalin proteins, receptor ligands, enzyme substrates, lectins,

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cytokines, lymphokines, interleukins, angiogenic or virulence factors, allergens, peptidic allergens, recombinant allergens, allergen-idiotypal antibodies, autoimmune-provoking structures, tissue-rejection-inducing structures, immunoglobulin constant regions and combinations thereof. In particular, the binding element (Y is an antibody or an antibody fragment
5 selected from the group consisting of Fab, scFv; single domain, or a fragment thereof, bis scFv, Fab 2, Fab 3, minibody, diabody, triabody, tetrabody and tandab.

By this measure the method is further developed to such an extent that the encoded analytes can be detected by any means which is adapted to visualize the signal element. Examples of detectable
10 physical features include e.g. light, chemical reactions, molecular mass, radioactivity, etc.

In some advantageous embodiments, the signal caused by the signal element, therefore in particular the binding of the signal oligonucleotides to the decoding oligonucleotides, interacting with the corresponding analyte probes, bound to the respective analyte is determined by:

- 15 (a) Imaging at least a portion of the sample; and/or
(b) Using an optical imaging technique; and/or
(c) Using a fluorescence imaging technique; and/or
(d) Multi-color fluorescence imaging technique; and/or
(e) Super-resolution fluorescence imaging technique.

20

The kits and method according to the present disclosure may be used ideally for *in vitro* methods for diagnosis of a disease selected from the group comprising cancer, neuronal diseases, cardiovascular diseases, inflammatory diseases, autoimmune diseases, diseases due to a viral or bacterial infection, skin diseases, skeletal muscle diseases, dental diseases and prenatal diseases.

25 Further, the kits and method according to the present disclosure may be used also ideally for *in vitro* methods for diagnosis of a disease in plants selected from the group comprising: diseases caused by biotic stress, preferably by infectious and/or parasitic origin, or diseases caused by abiotic stress, preferably caused by nutritional deficiencies and/or unfavorable environment.

30

Further, the kits and method according to the present disclosure may be used also ideally for in vitro methods for screening, identifying and/or testing a substance and/or drug comprising:

- 5 (a) contacting a test sample comprising a sample with a substance and/or drug
- (b) detecting different analytes in a sample by sequential signal-encoding of said analytes with a method according to the present disclosure.

10 An optical multiplexing system suitable for the method according to the present disclosure, comprising at least:

- a reaction vessel for containing the kits or part of the kits according to the present disclosure;
- 15 - a detection unit comprising a microscope, in particular a fluorescence microscope;
- a camera;
- a liquid handling device.

20

In some embodiments, optical multiplexing system may comprises further a heat and cooling device and/or a robotic system.

25 In some advantageous embodiments, the method described herein is used for specific detection of many different analytes in parallel. The technology allows to distinguish a higher number of analytes than different signals are available. The process includes at least four consecutive rounds of specific binding, signal detection and selective denaturation (if a next round is required), eventually producing a signal code. To decouple the dependency between the analyte specific binding and the oligonucleotides providing the detectable signal, a so called "decoding"-
30 oligonucleotide is introduced. The decoding oligonucleotide transcribes the information of the analyte specific probe set to the signal oligonucleotides.

The disclosure is now further explained by means of embodiments resulting in additional features, characteristics and advantages of the disclosure. The embodiments are of pure illustrative nature and do not limit the scope or range of the disclosure. The features mentioned in the specific embodiments are general features of the disclosure which are not only applicable in the specific
5 embodiment but also in an isolated manner in the context of any embodiment of the disclosure.

The method disclosed herein is used for specific detection of many different analytes in parallel. The technology allows distinguishing a higher number of analytes than different signals are available. The process preferably includes at least two consecutive rounds of specific binding,
10 signal detection and selective denaturation (if a next round is required), eventually producing a signal code. To decouple the dependency between the analyte specific binding and the oligonucleotides providing the detectable signal, a so called "decoding" oligonucleotide is introduced. The decoding oligonucleotide transcribes the information of the analyte specific probe set to the signal oligonucleotides.

15

The present disclosure pertains further to methods of detecting an analyte, comprising:

- attaching a plurality of premixed analyte-specific probes to the analyte, wherein the analyte-specific probes independently attach to the analyte and wherein the analyte-specific probes in all sets share a identical identifier element (T) and comprises an
20 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
25 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;
- detecting the signal caused by the identifier fluorophore (f) coupled to the identifier
30 element (Z); or

- annealing signal oligonucleotides to to the identifier oligonucleotide (o) of the identifier element (Z) comprised in the analyte-specific probe complexes; hybridizing one or multiple complementary oligonucleotides to the signal oligonucleotides that are labeled
- 5 - Detecting the signals caused by the signal elements of the signal oligonucleotides or by the complementary oligonucleotides, or
- annealing a plurality of first decoding oligonucleotides to the analyte-specific probes;
- 10 - contacting the sample with at least a set of signal oligonucleotides
- detecting the signal oligonucleotides;
- removing the plurality of first decoding oligonucleotides;
- 15 - annealing a plurality of second decoding oligonucleotides to the analyte-specific probes;
- annealing a second signal oligonucleotide to at least one of the plurality of second decoding oligonucleotides.

20

In summary, in particular the present disclosure pertains to the combinatorial use of primary antibodies together with secondary antibodies, FABs or proteins that specially bind to the primary antibodies or proteins that recognize and bind specially another

25 protein.

The analyte is in particular a protein, a nucleic acid, or another molecule that specifically is recognized and bound by the binding element (Y). An analyte may be part of a eucaryotic, prokaryotic, animal, fungi, plant cell, an organelle, or an extracellular area

30 within an organism. It can be part of an intact or disintegrated organisms or cell. Disintegration of an organism: is defined as any type of surgery, extraction, lysis, purification or other methods that disintegrate the organism or cell.

The binding element (Y) is defined in general as a protein or nucleic acid that specifically recognizes and binds another molecule X. Y can be an antibody, a fragment of an antibody, e.g., FAB or F(ab)2, any other proteins, aptamers or nucleic acids that specifically interact and bind to X.

The identifier element (Z) is defined in general as a protein or nucleic acid that specifically recognizes and binds to Y. Z can be an antibody, a fragment of an antibody, e.g., FAB or F(ab)2, any other proteins, aptamers or nucleic acids that specifically interact and bind to Y. In addition, Z is coupled to an identifier fluorophore (f) or identifier (o) oligonucleotide that is used to identify the identity of Z. The same type of Z can be coupled to different oligonucleotides identifiers (T) to get a library of Zs coupled to different oligonucleotides. The library may have a size of at least 5, 10, 20, 40, 80 or more than 80 Zs coupled to different identifier oligonucleotides.

The process is divided in several steps:

- 1) Preforming at least 4 different complexes of individual Ys and individual Zs, wherein Z is coupled to a certain oligonucleotide to form 4 different combinations such as
 - a) Y1-Z(identifier-oligoT1)
 - b) Y2-Z(identifier-oligoT2)
 - c) Y3-Z(identifier-oligoT3)
 - d) Y4-Z(identifier-oligoT4)

Time, temperature, concentration, and other conditions may vary depending on the various Ys and Zs)

- 2) Bind the complexes to the analyte (time, temperature, concentration, and other conditions may vary depending on the application). Thereafter, washing steps may be required to reduce the number of unspecific binding events.

3) Detect the Y-Z complexes bound to the analyte by hybridizing to the oligonucleotide coupled to Z. Thereafter, washing steps may be required to reduce the number of unspecific binding events. Various options are possible to identify the
5 identifier:

- a) Identifier may hybridize to a labeled oligonucleotide that is complementary to the sequence of the identifier. The label may be a fluorophore, an enzyme, or another label that can be detected.
- 10 b) Identifier may hybridize to a non-labeled oligonucleotide that is complementary to the sequence of the identifier. This oligonucleotide has an addition sequence that can be detected by hybridizing to one or multiple complementary oligonucleotides that are labeled.
- c) The identification of the identifier may require several rounds to build up a code word.

15 The process may require additional steps for complex formation such as filtration, purification, absorption, size selection etc. to purify proper complexes from individual components or side products.

20 In particular, the methods of the present disclosure may be used to identify regions of interest, in particular in an organ, tissue or single cell.

In some advantageous embodiments, the methods of the present disclosure can be used for image analysis. In this context, the methods of the present disclosure are used to develop cell
25 segmentation algorithms using immunofluorescence images. The methods of the present disclosure could be used to aid the segmentation of cells in a sample, in particular in a tissue. Cell segmentation is a crucial step since errors in identifying cells and their boundaries have a direct impact on the ability to correctly quantify the expression levels of proteins in these cells. For example, information about the levels of proteins in single cells can also be used to answer
30 questions regarding the set of cell types in a sample, the spatial distribution of these types, and the interactions between different cells and cell types in a tissue.

A unique advantage of spatial proteomics is that, unlike prior methods, they do not require specific markers to be selected in advance. Since these studies profile tens of proteins, a marker can be selected post-experiment. Moreover, there is no need to select a single marker. Even if a single marker does not work for all cells, a combination of such markers may be useful for segmenting
5 all cells.

Furthermore, the methods of the present disclosure provide spatial information about the location of the cells being profiled enabling the analysis of cell-cell signaling and cell type organization.

By using the methods according to the present disclosure of detecting analytes like biomolecules
10 in a fixed biological sample, up to three analytes like proteins on the surface of cells could be detected thereby visualizing the boundaries of cells. The analyte may be bound by 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, Fab₂, Fab₃, minibody, diabody, triplebody, tetrabody and
15 tandab.

In particular, the analyte to be detected may be Sodium-potassium ATPase, plasma membrane calcium ATPase (PMCA), Cadherin, CD98, Caveolae, HER2, Beta-Catenin, CAH9, E-Cadherin, c-MYC, Vimentin, among others. The choice of the binding elements like antibodies will vary based
20 on the cell type to be detected. Each tissue has a different composition of cell types. By visualizing the boundaries of cells, a user could by eye or through a computational tool segment cell.

In some further advantageous embodiments, the methods of the present disclosure may be used to establish AI-based image analysis algorithm using immunofluorescence images to identify
25 disorders. Furthermore, the advantageous embodiments the methods of the present disclosure may be used for feature extraction like nucleus morphology, signal homogeneity, signal intensity and/or centroids. For example, the morphology of cells often reflects their tissue-specific function or state. Cells undergo morphological changes to become motile in response to various stimuli, either as part of normal physiology and development, or due to pathologic disorder. In some
30 cancers, epithelial cells lose their inter-cellular connections, and take on protrusive, exploratory morphologies as they transit from static to migratory phenotypes. Recognizing such abnormal cell shapes can aide in properly identifying malignant (as distinct from benign) tumors.

A further application for the methods of the present disclosure is to determine expression of proteins and genes of interest via semi-quantitative approaches. Semi-quantitative immunohistochemistry (IHC) for example is a powerful method for investigating protein expression and localization within tissues.

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In some further advantageous embodiments, the methods of the present disclosure may be used for diagnosis and prognosis applications. In particular, the methods of the present disclosure are used for the determination of the distribution of a target protein in the of health and disease organ, tissue and/or cell. Furthermore, the methods of the present disclosure may be used to predict the

10 prognosis of diseases by using disease-related prognostic and/or predictive biomarkers. A variety of factors influence a patient's clinical outcome, including intrinsic characteristics of the patient, disease, or medical condition, and the effects of any treatments that the patient receives. Some of the intrinsic characteristics may be reflected as prognostic biomarkers, i.e., biomarkers used to identify likelihood of a clinical event, disease recurrence or progression in patients who have the

15 disease or medical condition of interest, and others as predictive biomarkers, i.e., biomarkers used to identify individuals who are more likely than similar individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product or an environmental agent.

In some further advantageous embodiments, the methods of the present disclosure may be used

20 to provide disease diagnosis e.g. tumor of unknown origin, neurodegenerative disorders, muscle disorders, brain trauma, infectious diseases. In particular, the methods of the present disclosure are used to predict a therapeutic response. A further application of the methods of the present disclosure is the subclassification of diseases. Classifications of diseases become extremely important in the compilation of statistics on causes of illness (morbidity) and causes of death

25 (mortality).

Furthermore, the methods of the present disclosure may be used to examine gene expression and to study gene regulation in normal and pathological tissues.

30 In some further advantageous embodiments, the methods of the present disclosure may be used for morphological studies. In particular, the methods of the present disclosure are used for the recognition of tissue structures and/or cell type classifications. In this context, a cell type is a classification used to identify cells that share morphological or phenotypical features. A

multicellular organism may contain cells of a number of widely differing and specialized cell types, such as muscle cells and skin cells, that differ both in appearance and function yet have identical genomic sequences. Cells may have the same genotype, but belong to different cell types due to the differential regulation of the genes they contain. Classification of a specific cell type is often
5 done through the use of microscopy (such as those from the cluster of differentiation family that are commonly used for this purpose in immunology).

In some further advantageous embodiments, the methods of the present disclosure may be used for multiomics studies. Multiomics is a biological analysis approach in which the data sets are
10 multiple "omes", such as the genome, proteome, transcriptome, epigenome, metabolome, and microbiome. By combining these "omes", scientists can analyze complex biological big data to find novel associations between biological entities, pinpoint relevant biomarkers and build elaborate markers of disease and physiology. In doing so, multiomics integrates diverse omics data to find a coherently matching geno-pheno-envirotypes relationship or association. In particular, the
15 methods of the present disclosure are used for the quantitative correlation and spatial colocalization study between ISH and ICH. As mentioned above, immunohistochemistry (IHC) is an invaluable tool for the detection, localization, and quantification of antigens in preserved tissue for research and diagnostic purposes. In situ hybridization (ISH) is a unique molecular analysis method because it provides the precise microscopic localization of analytes like proteins, nucleic
20 acids such as DNA, mRNA and microRNA in metaphase spreads and cell and tissue preparations. In particular, the methods of the present disclosure are used for cell type classification based on such multi-omics data. In a further embodiment, the methods of the present disclosure are used to study epigenetic modifications and chromatin structures. In particular, the methods of the present disclosure are used to identify spatial distribution, and cell-specific ISH signals (Cell
25 segmentation).

In some further advantageous embodiments, the methods of the present disclosure may be used to determine the correlation of genetic aberrations and/or the copy number variations (CNV) to the corresponding proteins, wherein CNV is a phenomenon in which sections of the genome are
30 repeated and the number of repeats in the genome varies between individuals.

Therefore, the present disclosure pertains to the following items:

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
- 5 (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
- 10 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
- 15 (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
- 20 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
- 25 (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
- 30 optionally
- (E) Selectively removing of the signal oligonucleotides of section C) i) or the decoding oligonucleotides and signal oligonucleotides of 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

5 **(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).

10 2. The method according to item 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.

15 3. The method to any one of items 1 to 2, wherein the cell is a prokaryotic cell or a eukaryotic cell, an animal cell, a plant cell, in particular a cell from vertebrate cells, in particular a mammalian cell, in particular a human cell.

 4. The method according to item 2, wherein the biological tissues, biological cells, extracts and/or part of cells are fixed.

20 5. The method according to any one of items 1 to 4, wherein the analytes are fixed in a permeabilized sample, such as a cell-containing sample.

 6. The method according to any one of items 1 to 5, wherein the analyte is a nucleic acid, preferably DNA, PNA or RNA, in particular mRNA.

25 7. The method according to any one of items 1 to 5, wherein the analyte is a peptide, polypeptide or a protein.

30 8. The method according to any one of items 1 to 7, wherein the binding element (Y) is a protein and/or a nucleic acid that specifically recognizes and binds to the analyte.

 9. The method according to any one of items 1 to 8, 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, Fab₂, Fab₃, minibody, diabody, triplebody, tetrabody and tandab.

- 5 10. The method according to any one of items 1 to 9, 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, Fab₂, Fab₃, minibody, diabody, triplebody, tetrabody and tandab.
- 10 11. The method according to any one of items 1 to 10, wherein the identifier element (Z) is an antibody fragment selected from the group consisting of a Fab, scFv, single domain, bis scFv, Fab₂, Fab₃, minibody, diabody, triplebody, tetrabody and tandab, wherein in particular the antibody fragment is a Fab.
- 15 12. The method according to any one of items 1 to 11, 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, Fab₂ and/or a Fab₃ fragment.
- 20 13. The method according to any one of items 1 to 12, 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).
- 25 14. The method according to any one of items 1 to 13, wherein after step A) and before step B) the non-bound analyte-specific probes are removed, in particular by washing.
15. The method according to any one of items 1 to 14, wherein all steps are automated, in particular by using a robotic system.
- 30 16. The method according to any one of items 1 to 15, wherein all steps are performed in a fluidic system.

17. The method according to any one of items 1 to 16, wherein each analyte is associated with a specific code word, wherein said code word comprise a number of positions, and wherein each position corresponds to one cycle resulting in a plurality of distinguishable encoding schemes with the plurality of code words.
- 5
18. The method according to any one of items 1 to 17, wherein said encoding scheme is predetermined and allocated to the analyte to be encoded.
19. The method according to any one of items 1 to 18, wherein the code words obtained for the individual analytes in the performed cycles comprise the detected signals and additionally at least one element corresponding to no detected signal.
- 10
20. The method according to any one of claims 1 to 19, wherein no signal is detected for at least one analyte within at least one cycle.
- 15
21. The method according to any one of items 1 to 20, wherein for at least for one individual analyte a position of the code word is zero (0).
22. The method according to any one of items 1 to 21, wherein the code word zero (0_{CK1}) is generated by using no decoding oligonucleotides having an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of a corresponding analyte-specific probe for an individual analyte.
- 20
23. The method according to any one of items 1 to 21, wherein if at least for one individual analyte a position of the code word is zero (0) in this cycle no corresponding decoding oligonucleotides having an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of a corresponding analyte-specific probe for an individual analyte are used.
- 25
- 30
24. The method according to any one of items 1 to 23, wherein the sample is contacted with at least two different sets of signal oligonucleotides, wherein the signal oligonucleotides

in each set comprise a different signal element and comprise a different connector element (C).

25. The method according to any one of items 1 to 24, wherein the sample is contacted with
5 at least two different sets of decoding oligonucleotides per analyte,
wherein the decoding oligonucleotides comprised in these different sets comprise the same identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of the corresponding analyte-specific probe set, and
10 wherein the decoding oligonucleotides of the different sets per analyte differ in the translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide.
26. The method according to any one of items 1 to 25, wherein the sample is contacted with
15 at least two different sets of decoding oligonucleotides per analyte,
wherein the decoding oligonucleotides comprised in these different sets comprise the same identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of the corresponding analyte-specific probe set, and
20 wherein the decoding oligonucleotides of the different sets per analyte differ in the translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide;
wherein only one set of decoding oligonucleotides per analyte is used per cycle, and/or wherein different sets of decoding oligonucleotides are used in different cycles in
25 combination with the corresponding set of signal oligonucleotides in the same cycle.
27. The method according to of any one of items 1 to 26, wherein the number of different sets
of decoding oligonucleotides per analyte comprising different translator elements (c)
corresponds to the number of different sets of signal oligonucleotides comprising
30 different connector elements (C).

28. The method according to any one of items 1 to 27, wherein all sets of decoding oligonucleotides for the different analytes comprise the same type(s) of translator element(s) (c).
- 5 29. The method according to any one of items 1 to 28, wherein the different sets of decoding oligonucleotides may be comprised in a pre-mixture of different sets of decoding oligonucleotides or exist separately.
- 10 30. The method according to any one of items 1 to 29, wherein the different sets of analyte-specific probes may be comprised in a pre-mixture of different sets of analyte-specific probes or exist separately.
- 15 31. The method according to any one of items 1 to 30, wherein the different sets of signal oligonucleotides may be comprised in a pre-mixture of different sets of signal oligonucleotides or exist separately.
- 20 32. The method according to any one of items 1 to 31, wherein the sample is a biological sample, preferably comprising biological tissue, further preferably comprising biological cells and/or extracts and/or part of cells.
33. The method according to item 32, wherein the cell is a prokaryotic cell or a eukaryotic cell, in particular a mammalian cell, in particular a human cell.
- 25 34. The method according to item 33, wherein the biological tissue, biological cells, extracts and/or part of cells are fixed.
35. The method according to any one of items 1 to 34, wherein the analytes are fixed in a permeabilized sample, such as a cell-containing sample.
- 30 36. The method according to any one of items 1 to 33, wherein the non-bound identifier elements (Z) in the premixed set of analyte specific probes are removed, in particular by washing.

37. The method according to any one of items 1 to 35, wherein after step A) and before step B) the non-bound analyte-specific probes are removed, in particular by washing.
38. The method according to any one of items 1 to 37, wherein after step C) and before step D) the non-bound signal oligonucleotides and/or the non-bound decoding oligonucleotides are removed, in particular by washing.
39. The method according to any one of items 1 to 38, wherein the analyte specific probes are incubated with the sample, thereby allowing a specific binding of the analyte specific probes to the analytes to be encoded.
40. The method according to any one of items 1 to 39, wherein the decoding oligonucleotides are incubated with the sample, thereby allowing a specific hybridization of the decoding oligonucleotides to identifier elements (T) of the respective analyte-specific probes.
41. The method according to any of items 1 to 40, wherein the binding element (Y) comprise an amino acid sequence allowing a specific binding to the analyte to be encoded, in particular wherein the analyte is a protein.
42. The method according to any of items 1 to 41, wherein the binding element (Y) comprises moieties which are affinity moieties from affinity substances or affinity substances in their entirety selected from the group consisting of antibodies, antibody fragments, anticalin proteins, receptor ligands, enzyme substrates, lectins, cytokines, lymphokines, interleukins, angiogenic or virulence factors, allergens, peptidic allergens, recombinant allergens, allergen-idiotypal antibodies, autoimmune-provoking structures, tissue-rejection-inducing structures, immunoglobulin constant regions and combinations thereof.
43. The method according to any of items 1 to 42, wherein the binding element (S) is an antibody or an antibody fragment selected from the group consisting of Fab, scFv; single domain, or a fragment thereof, bis scFv, Fab₂, Fab₃, minibody, diabody, triabody, tetrabody and tandab.

44. The method according to any of items 1 to 43, wherein the signal caused by the identifier fluorophore (f) or the signal element is detected by:
- (a) Imaging at least a portion of the sample; and/or
 - (b) Using an optical imaging technique; and/or
 - 5 (c) Using a fluorescence imaging technique; and/or
 - (d) Multi-color fluorescence imaging technique; and/or
 - (e) Super-resolution fluorescence imaging technique.
- 10 45. The method according to any one of items 1 to 44, 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, Fab₂ and/or a Fab₃ fragment comprising an identifier oligonucleotide (o).
- 15 46. The method according to any one of items 1 to 45, 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, Fab₂ and/or a Fab₃ fragment comprising an identifier oligonucleotide (o), wherein the sample is contacted with at least one set of decoding oligonucleotides per analyte, wherein
- 20 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
 - 25 (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
- 30 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.

5

47. The method according to any one of items 1 to 46, 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

10

- Y1-Z(identifier-oligo1)
- Y2-Z(identifier-oligo2)
- Y3-Z(identifier-oligo3)
- Y4-Z(identifier-oligo4)

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48. The method according to any one of items 1 to 47, 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.

20

49. The method according to any one of items 1 to 48, 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.

25

50. The method according to any one of items 1 to 49, wherein the complexes between the binding element (Y) and the identifier element (Z) are premixed in separate multiple tubes.

30

51. 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

5 (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

10 (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
15 oligonucleotide (o) of the identifier element (Z) of another set of analyte-specific probes.

52. The kit according to item 51, wherein the different sets of analyte-specific probes are comprised in separate multiple tubes.

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53. A method according to any of the proceeding items 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
25 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
30 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,

5 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.

Experimental data

10 Cryosection of murine kidney was probed for nestin. Rabbit primary antibody against nestin was prelabelled with Alexa Flour 647 conjugated to goat anti-rabbit IgG (upper panel) or goat anti rabbit Fab (lower panel) with a ratio between primary antibody and secondary antibody of 1:0.2 and 1:2 (molecules/molecules), respectively. The prelabelling was performed for 30 minutes and residual secondary antibody or Fab not bound to primary antibody was blocked with 0.1% v/v normal rabbit serum for 5 min. The antibody complexes were added to tissues and incubated
15 overnight. Images were captured with ZEISS Celldiscoverer 7 using a Apochromat 5X/0.35 objective and 2X Tubelens. (Exposure time: 1 second and 0.1 second for Alexa Flour 647 and DAPI, respectively). The results are shown in Figure 3.

Cryosection of murine kidney were probed for Nestin. Detection of nestin was done by precomplexed primary antibody (Abcam ab176571) with oligos conjugated secondary antibody
20 followed by complementary oligos conjugated to fluorophores (A). The localization of the primary-secondary antibody complexes was confirmed by probing the tissue with fluorophores conjugated antibody complemented to the oligo conjugated secondary antibody (B). The results are shown in Figure 4.

25

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 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 to any one of claims 1 to 2, wherein the cell is a prokaryotic cell or a eukaryotic cell, an animal cell, a plant cell, in particular a cell from vertebrate cells, in particular a mammalian cell, in particular a human cell.
 4. The method according to claim 2, wherein the biological tissues, biological cells, extracts and/or part of cells are fixed.
 5. The method according to any one of claims 1 to 4, wherein the analytes are fixed in a permeabilized sample, such as a cell-containing sample.
 6. The method according to any one of claims 1 to 5, wherein the analyte is a nucleic acid, preferably DNA, PNA or RNA, in particular mRNA.
 7. The method according to any one of claims 1 to 5, wherein the analyte is a peptide, polypeptide or a protein.
 8. The method according to any one of claims 1 to 7, wherein the binding element (Y) is a protein and/or a nucleic acid that specifically recognizes and binds to the analyte.
 9. The method according to any one of claims 1 to 8, 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, Fab₂, Fab₃, minibody, diabody, triplebody, tetrabody and tandab.
 10. The method according to any one of claims 1 to 9, 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, Fab₂, Fab₃, minibody, diabody, triplebody, tetrabody and tandab.

11. The method according to any one of claims 1 to 10, wherein the identifier element (Z) is an antibody fragment selected from the group consisting of a Fab, scFv, single domain, bis scFv, Fab₂, Fab₃, minibody, diabody, triplebody, tetrabody and tandab, wherein in particular the antibody fragment is a Fab.
12. The method according to any one of claims 1 to 11, 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, Fab₂ and/or a Fab₃ fragment.
13. The method according to any one of claims 1 to 12, 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).
14. The method according to any one of claims 1 to 3 wherein after step A) and before step B) the non-bound analyte-specific probes are removed, in particular by washing.
15. The method according to any one of claims 1 to 14, wherein all steps are automated, in particular by using a robotic system.
16. The method according to any one of claims 1 to 15, wherein all steps are performed in a fluidic system.
17. The method according to any one of claims 1 to 16, wherein each analyte is associated with a specific code word, wherein said code word comprise a number of positions, and wherein each position corresponds to one cycle resulting in a plurality of distinguishable encoding schemes with the plurality of code words.
18. The method according to any one of claims 1 to 17, wherein said encoding scheme is predetermined and allocated to the analyte to be encoded.
19. The method according to any one of claims 1 to 18, wherein the code words obtained for the individual analytes in the performed cycles comprise the detected signals and additionally at least one element corresponding to no detected signal.

20. The method according to any one of claims 1 to 19, wherein no signal is detected for at least one analyte within at least one cycle.
21. The method according to any one of claims 1 to 20, wherein for at least for one individual analyte a position of the code word is zero (0).
22. The method according to any one of claims 1 to 21, wherein the code word zero (0) is generated by using no decoding oligonucleotides having an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of a corresponding analyte-specific probe for an individual analyte.
23. The method according to any one of claims 1 to 22, wherein if at least for one individual analyte a position of the code word is zero (0) in this cycle no corresponding decoding oligonucleotides having an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of a corresponding analyte-specific probe for an individual analyte are used.
24. The method according to any one of claims 1 to 23, wherein the sample is contacted with at least two different sets of signal oligonucleotides, wherein the signal oligonucleotides in each set comprise a different signal element and comprise a different connector element (C).
25. The method according to any one of claims 12 to 24, wherein the sample is contacted with at least two different sets of decoding oligonucleotides per analyte,
wherein the decoding oligonucleotides comprised in these different sets comprise the same identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of the corresponding analyte-specific probe set, and
wherein the decoding oligonucleotides of the different sets per analyte differ in the translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide.
26. The method according to any one of claims 1 to 25, wherein the sample is contacted with at least two different sets of decoding oligonucleotides per analyte,

wherein the decoding oligonucleotides comprised in these different sets comprise the same identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of the corresponding analyte-specific probe set, and

wherein the decoding oligonucleotides of the different sets per analyte differ in the translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide;

wherein only one set of decoding oligonucleotides per analyte is used per cycle, and/or wherein different sets of decoding oligonucleotides are used in different cycles in combination with the corresponding set of signal oligonucleotides in the same cycle.

27. The method according to any one of claims 1 to 26, wherein the number of different sets of decoding oligonucleotides per analyte comprising different translator elements (c) corresponds to the number of different sets of signal oligonucleotides comprising different connector elements (C).
28. The method according to any one of claims 1 to 27, wherein all sets of decoding oligonucleotides for the different analytes comprise the same type(s) of translator element(s) (c).
29. The method according to any one of claims 1 to 28, wherein the different sets of decoding oligonucleotides may be comprised in a pre-mixture of different sets of decoding oligonucleotides or exist separately.
30. The method according to any one of claims 1 to 29, wherein the different sets of analyte-specific probes may be comprised in a pre-mixture of different sets of analyte-specific probes or exist separately.
31. The method according to any one of claims 1 to 30, wherein the different sets of signal oligonucleotides may be comprised in a pre-mixture of different sets of signal oligonucleotides or exist separately.
32. The method according to any one of claims 1 to 31, wherein the sample is a biological sample, preferably comprising biological tissue, further preferably comprising biological cells and/or extracts and/or part of cells.

33. The method according to claim 32, wherein the cell is a prokaryotic cell or a eukaryotic cell, in particular a mammalian cell, in particular a human cell.
34. The method according to claim 32, wherein the biological tissue, biological cells, extracts and/or part of cells are fixed.
35. The method according to any one of claims 1 to 34, wherein the analytes are fixed in a permeabilized sample, such as a cell-containing sample.
36. The method according to any one of claims 1 to 35, wherein the non-bound identifier elements (Z) in the premixed set of analyte specific probes are removed, in particular by washing.
37. The method according to any one of claims 1 to 36, wherein after step A) and before step B) the non-bound analyte-specific probes are removed, in particular by washing.
38. The method according to any one of claims 1 to 37, wherein after step C) and before step D) the non-bound signal oligonucleotides and/or the non-bound decoding oligonucleotides are removed, in particular by washing.
39. The method according to any one of claims 1 to 38, wherein the analyte specific probes are incubated with the sample, thereby allowing a specific binding of the analyte specific probes to the analytes to be encoded.
40. The method according to any one of claims 1 to 39, wherein the decoding oligonucleotides are incubated with the sample, thereby allowing a specific hybridization of the decoding oligonucleotides to identifier elements (T) of the respective analyte-specific probes.
41. The method according to any of claims 1 to 40, wherein the binding element (Y) comprise an amino acid sequence allowing a specific binding to the analyte to be encoded, in particular wherein the analyte is a protein.
42. The method according to any of claims 1 to 41, wherein the binding element (Y) comprises moieties which are affinity moieties from affinity substances or affinity substances in their entirety selected from the group consisting of antibodies, antibody fragments, anticalin proteins, receptor ligands, enzyme substrates, lectins, cytokines, lymphokines, interleukins, angiogenic or virulence factors, allergens, peptidic allergens, recombinant

allergens, allergen-idiotypical antibodies, autoimmune-provoking structures, tissue-rejection-inducing structures, immunoglobulin constant regions and combinations thereof.

43. The method according to any of claims 1 to 42, wherein the binding element (S) is an antibody or an antibody fragment selected from the group consisting of Fab, scFv; single domain, or a fragment thereof, bis scFv, Fab₂, Fab₃, minibody, diabody, triabody, tetrabody and tandab.
44. The method according to any of claims 1 to 43, wherein the signal caused by the identifier fluorophore (f) or the signal element is detected by:
- (a) Imaging at least a portion of the sample; and/or
 - (b) Using an optical imaging technique; and/or
 - (c) Using a fluorescence imaging technique; and/or
 - (d) Multi-color fluorescence imaging technique; and/or
 - (e) Super-resolution fluorescence imaging technique.
45. The method according to any one of claims 1 to 44, 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, Fab₂ and/or a Fab₃ fragment comprising an identifier oligonucleotide (o).
46. The method according to any one of claims 1 to 45, 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, Fab₂ and/or a Fab₃ 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.

47. The method according to any one of claims 1 to 46, 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(identifier-oligo1)
- Y2-Z(identifier-oligo2)
- Y3-Z(identifier-oligo3)
- Y4-Z(identifier-oligo4)

48. The method according to any one of claims 1 to 47, 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.

49. The method according to any one of claims 1 to 48, 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.

50. The method according to any one of claims 1 to 49, wherein the complexes between the binding element (Y) and the identifier element (Z) are premixed in separate multiple tubes.

51. The method according to any one of claims 1 to 50, wherein the sample is contacted with at least four (4) additional sets of analyte specific probes for detecting of at least four

additional analytes, wherein each set of the additional analyte-specific probes comprises an identical identifier element (T) that is not identical with the identical identifier element (T) comprised in the complexes of the first four sets of analyte-specific probes.

52. 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.

53. The kit according to claim 52, wherein the different sets of analyte-specific probes are comprised in separate multiple tubes.

54. 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.