**[0013]** A further development of the technology uses so called split initiator probes that have to hybridize in close proximity to form the initiator sequence for HCR, similarly to the RNAscope technology, this reduces the number of false positive signals; see Choi et al. (2018), Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. Development 145(12).

**[0014]** Mateo et al. (2019), Visualizing DNA folding and RNA in embryos at single-cell resolution, Nature Vol, 568, p. 49ff., disclose a method called 'optical reconstruction of chromatin structure (ORCA). This method is intended to make the chromosome line visible.

**[0015]** The methods known in the art, however, have numerous disadvantages. In particular, they are inflexible, expensive, complex, time consuming and quite often provide non-accurate results. In particular, the encoding capacities of the existing methods are low and do not meet the requirements of modern molecular biology and medicine.

**[0016]** Against this background, it is an object underlying the present invention to provide a method by means of which the disadvantages of the prior art methods can be reduced or even avoided.

**[0017]** The present invention satisfies these and other needs.

## SUMMARY ~~OF THE INVENTION~~

**[0018]** The present ~~invention~~disclosure provides a method of sequential signal-encoding of analytes in a sample, the method comprising the steps:

(1) providing a set of analyte-specific probes, each analyte-specific probe comprising:

- a binding element (S) that specifically interacts with one of the analytes to be encoded, and

(8) incubating the set of signal oligonucleotides with the sample, thereby allowing a specific hybridization of the signal oligonucleotides to the translator element (c).

**[0019]** The inventors have realized that this novel method provides the essential steps required to set up a process allowing the specific quantitative and/or spatial detection or counting of different analytes or different single analyte molecules in a sample in parallel via specific hybridization. The technology allows distinguishing a higher number of analytes than different signals available. In contrast to other state-of-the-art methods the oligonucleotides providing the detectable signal are not directly interacting with sample-specific nucleic acid sequences but are mediated by so called 'decoding-oligonucleotides'. This mechanism decouples the dependency between the analyte-specific oligonucleotides and the signal oligonucleotides and, therefore, results in a dramatical increase of the encoding capacity.

**[0020]** Another subject-matter of the ~~invention~~disclosure is the use of a set of decoding oligonucleotides to sequentially signal-encode analytes in a sample, each decoding oligonucleotide comprising:

- a first connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of a nucleotide sequence which is unique to a set of analyte-specific probes (unique identifier sequence), and

- a translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide.

**[0020a]** In one aspect, the present disclosure refers to a kit for sequentially signal-encoding of analytes in a sample, comprising
a set of analyte-specific probes, each analyte-specific probe comprising:
- a binding element (S) that specifically interacts with one of the analytes to be encoded, and
- an identifier element (T) comprising a nucleotide sequence which is

unique to said set of analyte-specific probes (unique identifier sequence); and
a set of decoding oligonucleotides, each decoding oligonucleotide comprising:
- a first connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence, and
- a translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide

**[0021]** A still further subject-matter of the present ~~invention~~disclosure is a kit for sequentially signal-encoding of analytes in a sample, comprising

- a set of analyte-specific probes, each analyte-specific probe comprising:

- a binding element (S) that specifically interacts with one of the analytes to be encoded, and

- an identifier element (T) comprising a nucleotide sequence which is unique to said set of analyte-specific probes (unique identifier sequence); and

- a set of decoding oligonucleotides, each decoding oligonucleotide comprising:

- a first connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence, and

- a translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide; and, preferably,

- a set of signal oligonucleotides, each signal oligonucleotide comprising:

- a second connector element (C) comprising a nucleotide sequence which is essentially complementary to at least a section of the nucleotide sequence of the translator element (c), and

- a signal element.

**[0022]** 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 encoding capacity achieved. 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. In particular, the ~~invention~~disclosure results in a significant increase of the encoding capacity in comparison to the prior art methods.

**[0023]** The use of decoding oligonucleotides breaks the dependencies between the target specific probes and the signal oligonucleotides. Without decoupling target specific probes and signal generation as in the methods of the state of the art, two different signals can only be generated for a certain target if using two different molecular tags. Each of these molecular tags can only be used once. Multiple readouts of the same molecular tag do not increase the information about the target. In order to create an encoding scheme, a change of the target specific probe set after each round is required (SeqFISH) or multiple molecular tags must be present on the same probe set (like merFISH, intronSeqFISH). These restrictions in the art are very relevant and reduce the flexibility, coding capacity, accuracy, reproducibility and increase the costs of the experiment.

**[0024]** According to the ~~invention~~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 or a nucleic acid molecule (RNA 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 ~~invention~~disclosure incudes a complex of subjects, e.g. at least two individual nucleic acid, protein or peptides molecules. In an ~~embodiment~~example of the ~~invention~~disclosure an "analyte" excludes a chromosome. In another ~~embodiment~~example of the ~~invention~~disclosure an "analyte" excludes DNA.

**[0025]** A "sample" as referred to herein is a composition in liquid or solid form suspected of comprising the analytes to be encoded.

**[0026]** An "oligonucleotide" as used herein, refers to s 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.

tion needed and the number of different signal oligonucleotides used. The length of the two sequence elements may or may not be the same.

**[0029]** A "signal oligonucleotide" as used herein comprises at least two elements, a so-called "second connector element" (C) having a nucleotide sequence specifically hybridizable to at least a section of the nucleotide sequence of the translator element (c) of the decoding oligonucleotide, and a "signal element" which provides a detectable signal. 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.

**[0030]** A "set" refers to a plurality of moieties or subjects, e.g. analyte-specific probes or decoding oligonucleotides, whether the individual members of said plurality are identical or different from each other. In an ~~embodiment~~example of the ~~invention~~disclosure a single set refers to a plurality of oligonucleotides

**[0031]** 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 unique identifier.

**[0032]** 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 unique identifier element (T) of the analyte-specific probe.

**[0033]** "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).

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.

**[0036]** 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 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.

**[0037]** The "removing" steps according to the ~~invention~~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, temperature, salt conditions etc., as known in the art.

**[0038]** It is understood that in an ~~embodiment~~example of the method according to the ~~invention~~disclosure a plurality of analytes can be encoded in parallel. This requires the use of different sets of analyte-specific probes in step (1). The analyte-specific probes of a particular set differ from the 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. In this ~~embodiment~~example also the use of different sets of decoding oligonucleotides is required in step (4). The decoding oligonucleotides of a particular set differ from the decoding oligonucleotides of another set. This means, the decoding oligonucleotides of set 1 bind to the analyte-specific probes of above set 1 of analyte-specific probes, the decoding oligonucleotides of set 2 bind to the analyte-specific probes of above set 2 of analyte-specific probes, the decoding oligonucleotides of set 3 bind to the analyte-specific probes of above set 3 of analyte-specific probes, etc. In this ~~embodiment~~example where a plurality of analytes is to be encoded in parallel the different sets of analyte-specific probes may be provided in step (1) as a premixture of different sets of analyte-specific probes and/or the different sets of decoding oligonucleotides may be provided in step (4) as a premixture of

different sets of decoding oligonucleotides. Each mixture may be contained in a single vial. Alternatively, the different sets of analyte-specific probes and/or the different sets of decoding oligonucleotides may be provided in steps (1) and/or (4) singularly.

**[0039]** A "kit" is a combination of individual elements useful for carrying out the use and/or method of the ~~invention~~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 ~~invention~~disclosure. Such kits unify all essential elements required to work the method according to the ~~invention~~disclosure, thus minimizing the risk of errors. Therefore, such kits also allow semi-skilled laboratory staff to perform the method according to the ~~invention~~disclosure.

**[0040]** The features, characteristics, advantages and ~~embodiment~~examples specified herein apply to the method, use, and kit according to the ~~invention~~disclosure, even if not specifically indicated.

**[0041]** In an ~~embodiment~~example of the ~~invention~~disclosure the sample is a biological sample, preferably comprising biological tissue, further preferably comprising biological cells. A biological sample may be derived from an organ, organoids, cell cultures, stem cells, cell suspensions, primary cells, samples infected by viruses, bacteria or fungi, eukaryotic or prokaryotic samples, smears, disease samples, a tissue section.

**[0042]** 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 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.

**[0043]** In another ~~embodiment~~example of the ~~invention~~disclosure prior to step (2) the biological tissue and/or biological cells are fixed.

**[0044]** 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 ~~invention~~disclosure. The fixation of the sample can be, e.g., carried out by means of formaline, ethanol, methanol or other components well known to the skilled person.

**[0045]** In yet a further ~~embodiment~~example 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.

**[0046]** 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.

**[0047]** in another ~~embodiment~~example of the method according to the ~~invention~~disclosure it comprises the following additional steps:

(9) removing non-bound signal oligonucleotides from the sample; and

(10) detecting the signal.

**[0048]** 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 physical features include e.g. light, chemical reactions, molecular mass, radioactivity, etc.

**[0049]** In a further ~~embodiment~~example of the method according to the ~~invention~~disclosure the following additional step is carried out:

(11) selectively removing the decoding oligonucleotides and signal oligonucleotides from the sample, thereby essentially maintaining the specific binding of the analyte-specific probes to the analyte to be encoded.

**[0050]** By this measure the requirements for another round of binding further decoding oligonucleotides to the same analyte-specific probes are established, thus finally resulting in a code or encoding scheme comprising more than one signal. This step is realized by applying conditions and factors well known to the skilled person, e.g. pH, temperature, salt conditions, oligonucleotide concentration, polymers etc.

**[0051]** In another ~~embodiment~~example of the ~~invention~~disclosure, the method comprises the following step:

(12) repeating steps (4)-(11) at least once [$(4_2)$-$(11_2)$] to generate an encoding scheme consisting of at least two signals.

**[0052]** With this measure a code of more than one signal is set up, i.e. of two, three, four, five etc. signals in case of two [$(4_2)$-$(11_2)$], three [$(4_3)$-$(11_3)$], four [$(4_4)$-$(11_4)$], five [$(4_5)$-$(11_5)$], etc. [$(4_n)$-$(11_n)$] rounds which are carried out by the user, where 'n' is an integer representing the number of rounds. The encoding capacity of the method according to the ~~invention~~disclosure is herewith increased depending on the nature of the analyte and the needs of the operator.

**[0053]** In an ~~embodiment~~example of the ~~invention~~disclosure said encoding scheme is predetermined and allocated to the analyte to be encoded.

**[0054]** This measure enables a precise experimental set-up by providing the appropriate sequential order of the employed decoding and signal oligonucleotides and, therefore, allows the correct allocation of a specific analyte to a respective encoding scheme.

**[0055]** The decoding oligonucleotides which are used in repeated steps ($4_2$)-($11_2$) may comprise a translator element (c2) which is identical with the translator element (c1) of the decoding oligonucleotides used in previous steps (4)-(11). In another ~~embodiment~~example of the ~~invention~~disclosure decoding oligonucleotides are used in repeated steps ($4_2$)-($11_2$) comprising a translator element (c2) which differs from the translator element (c1) of the decoding oligonucleotides used in previous steps (4)-(11).

**[0056]** It is understood that the decoding elements may or may not be changed from round to round, i.e. in the second round ($4_2$)-($11_2$) comprising the translator element c2, in the third round ($4_3$)-($11_3$) comprising the translator element c3, in the fourth round ($4_4$)-($11_4$) comprising the translator element c4, in the fifths round comprising the translator element c5, in the 'n' round ($4_n$)-($11_n$) comprising the translator element cn, etc., wherein 'n' is an integer representing the number of rounds.

**[0057]** The signal oligonucleotides which are used in repeated steps ($4_2$)-($11_2$) may comprise a signal element which is identical with the signal element of the decoding oligonucleotides used in previous steps (4)-(11). In a further ~~embodiment~~example of the ~~invention~~disclosure signal oligonucleotides are used in repeated steps ($4_2$)-($11_2$) comprising a signal element which differs from the signal element of the decoding oligonucleotides used in previous steps (4)-(11).

**[0058]** By this measure each round the same or a different signal is provided resulting in an encoding scheme characterized by a signal sequence consisting of numerous different signals. This measure allows the creation of a unique code or code word which differs from all other code words of the encoding scheme.

**[0059]** In another ~~embodiment~~example of the ~~invention~~disclosure the binding element (S) of the analyte-specific probe comprises a nucleic acid comprising a nucleotide sequence allowing a specific binding to the analyte to be encoded, preferably a specific hybridization to the analyte to be encoded.

**[0060]** This measure creates the condition for encoding a nucleic acid analyte, such as specific DNA molecules, e.g. genomic DNA, nuclear DNA, mitochondrial DNA,

viral DNA, bacterial DNA, extra- or intracellular DNA etc., or specific mRNA molecules, e.g. hnRNA, miRNA, viral RNA, bacterial RNA, extra- or intracellular RNA, etc.

**[0061]** In an alternative ~~embodiment~~example of the ~~invention~~disclosure the binding element (S) of the analyte-specific probe comprises an amino acid sequence allowing a specific binding to the analyte to be encoded, preferably the binding element is an antibody.

**[0062]** This measure creates the condition for encoding a nucleic acid analyte, such as an mRNA, e.g. such an mRNA coding for a particular protein.

**[0063]** In another ~~embodiment~~example the analyte to be encoded or detected is a nucleic acid, preferably DNA or RNA, further preferably mRNA, and/or, alternatively the analyte to be decoded is a peptide or a protein.

**[0064]** By this measure the ~~invention~~disclosure is adapted to the detection of such kinds of analytes which are of upmost importance in the clinical routine or the focus of biological questions.

**[0065]** It is to be understood that the before-mentioned features and those to be mentioned in the following cannot only be used in the combination indicated in the respective case, but also in other combinations or in an isolated manner without departing from the scope of the ~~invention~~disclosure.

**[0066]** The ~~invention~~disclosure is now further explained by means of ~~embodiment~~examples resulting in additional features, characteristics and advantages of the ~~invention~~disclosure. The ~~embodiment~~examples are of pure illustrative nature and do not limit the scope or range of the ~~invention~~disclosure. The features mentioned in the specific ~~embodiment~~examples are general features of the ~~invention~~disclosure which are not only applicable in the specific ~~embodiment~~example but also in an isolated manner in the context of any ~~embodiment~~example of the ~~invention~~disclosure.

**[0067]** The ~~invention~~disclosure is now described and explained in further detail by referring to the following non-limiting examples and figures.

Fig. 1: ~~Embodiment~~Example where the analyte is a nucleic acid and the probe set comprises oligonucleotides specifically binding to the analyte. The probes comprise a unique identifier sequence allowing hybridization of decoding oligonucleotides;

Fig. 2: ~~Embodiment~~Example where the analyte is a protein and the probe set comprises proteins (here: antibodies) specifically binding to the analyte. The probes comprise a unique identifier sequence allowing hybridization of decoding oligonucleotides;

Fig. 3: Flowchart of the method according to the ~~invention~~disclosure;

Fig. 4: Alternative options for the application of decoding and signal oligonucleotides.

Fig. 5: Example for signal encoding of three different nucleic acid sequences by two different signal types and three detection rounds; in this example, the encoding scheme includes error detection;

Fig. 6: Number of generated code words (logarithmic scale) against number of detection cycles;

Fig. 7: Calculated total efficiency of a 5-round encoding scheme based on single step efficiencies;

Fig. 8: Comparison of relative transcript abundances between different experiments;

Fig. 9: Correlation of relative transcript abundances between different experiments;

Fig. 10 Comparison of intercellular distribution of signals;