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(71) Applicant: TECHNISCHE UNIVERSITEIT EINDHOVEN [NL/NL]; Groene Loper 3, 5612 AE EINDHOVEN (NL).

(72) Inventors: ALBERTAZZI, Lorenzo; Groene Loper 3, 5612 AE EINDHOVEN (NL). RIERA BRILLAS, Roger; Groene Loper 3, 5612 AE EINDHOVEN (NL).

(74) Agent: ALGEMEEN OCTROOI- EN MERKENBUREAU B.V.; P.O. Box 645, 5600 AP EINDHOVEN (NL).

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(54) Title: CELL RECEPTOR SINGLE-MOLECULE TRACKING WITH PAINT IMAGING FOR DIAGNOSTICS AND PRECISION MEDICINE

(57) Abstract: The present invention relates to a method for obtaining diagnostic predictive patterns from the mobility of cell membrane compounds such as proteins embedded in the cell membrane, or associated to the said membrane. The invention also provides for a method for classifying subjects for a therapy.

Title: Cell receptor single-molecule tracking with PAINT imaging for diagnostics and precision medicine

This application claims the benefit of the U.S. provisional application No. 63/444,641
5 filed on February 10, 2023.

FIELD OF THE INVENTION

[001] This invention pertains in general to the field of Cancer diagnosis, stratifying cancer patients and precision medicine.

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BACKGROUND OF THE INVENTION

[002] The background description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any
15 publication specifically or implicitly referenced is prior art.

[003] Cancer is widespread and diverse disease. Proper diagnosis is needed to stratify patients and determine the best treatment for each one (precision medicine). Current clinical methods used for cancer diagnostics do not fully predict response to treatment leading to patient not receiving the most adequate treatment and hospitals
20 wasting resources in ineffective treatments. This invention is aimed to improve cancer diagnostics for better therapy effectiveness prediction and clinical decision-making. This also applies to other diseases where the therapeutic decision making is difficult.

[004] Current diagnostic methods as based on determining the presence of oncogenic driver mutations or quantifying the expression levels of biomarker proteins.
25 These include oligonucleotide sequencing (DNA/RNAseq), PCR and in situ hybridization (ISH) for the detection of genetic mutations, and immunohistochemistry (IHC) for the evaluation of biomarkers expression levels.

[005] Diagnostic and prognostic testing in diseases such as cancer are aimed at understanding the disease mechanism and classifying the patients to better assign
30 treatment options. Although tumor imaging techniques (e.g. PET, MRI, X-ray, CT scans) are performed in situ, the molecular diagnostic techniques that provide a deeper understanding of the disease at the cellular or molecular level are done ex vivo. These are based in either identifying genetic alterations that explain the cause

of the disease, measuring the presence and abundance of protein/metabolic biomarkers, or quantifying the abundance of certain types of cells. There are various methodologies used in clinical diagnostic as said (e.g. immunohistochemistry, flow cytometry, next-generation sequencing, PCR), however, they generally fall within one of the previous categories. This results in limited capacity biomarkers that obviate the information that can be obtained from live cells and the multitude of molecular interactions that occur in them. This is becoming more relevant since therapies are becoming more complex and evolving towards personalized medicine (e.g. cancer immunotherapy). These new therapies require a more comprehensive understanding of the state of the disease in each individual patient for the more patient-tailored approaches, and such requirements are not fully meet with current diagnostic approaches.

[006] An example of this are the immune checkpoint inhibitors (ICIs), which are a type of cancer immunotherapy that reactivates the immune system's surveillance that some tumors block. These have shown incredible results in incrementing the progression-free survival in comparison to standard chemotherapy. Despite the efficacy of these therapies, they are only effective on a subset of patients with polarized response effectiveness. This kind of therapies are quite expensive (up to hundreds of thousands of dollars per patient) and although the side effects are not as severe as chemotherapy, they are still a treat. However, with current diagnostic practices there is no clear predictor of the effectiveness of these techniques, since the complexity of the cellular and molecular pathways involved is quite high. Moreover, high cancer heterogeneity within a patient may promote resistance to the therapy or may require a change in treatment. This is commonly overseen by current diagnostic tests.

[007] Therefore, a more comprehensive information of the state of the disease from living cells is needed, which are not possible from a static image of molecules in a sample. In contrast to ex vivo measurements, where a static image of molecules just gives information about a precise instant, measurements in live cells show the complex network of molecular interactions occurring in real time. In other words, current diagnosis is based on measuring the genome, proteome or metabolome, whereas the interactome (i.e. network of molecular interactions) is unexplored in terms of diagnosis and prognosis.

[008] Techniques that study the interactions of individual molecules require of single-molecule sensitivity. Microscopists have developed techniques to achieve single-molecule imaging of biomolecules in live cells, however, these generally require some sort of labelling of the desired target. This is achieved either through genetically engineering a tag onto the target, or labelling it with a bulky, non-native probe such as an antibody. These approaches work well for scientific research, but are not fitted for the clinical setting since both would alter the physiology of the cells, therefore influencing the validity of the diagnostic results.

[009] In light of this, new products, compositions, methods and uses for in the treatment of cancer would be highly desirable but are not yet readily available. In particular, there is a clear need in the art for reliable, efficient, and reproducible products, compositions, methods and uses that allow to be used in the treatment of cancer. Accordingly, the technical problem underlying the present invention can be seen in the provision of such products, compositions, methods and uses for complying with any of the aforementioned needs, or at least providing the public with a useful choice. The technical problem is solved by the embodiments characterized in the claims and herein below.

SUMMARY OF THE INVENTION

[010] As embodied and broadly described herein, the present invention is directed to the surprising finding that with the transient interaction of a labelled probe with a compound directly or indirectly bound to or embedded in or localized in the membrane of a cell (i.e., to the lipidic bilayer) a behavior of a protein in the cell membrane can be established that is informative of disease (e.g., cancer) and of treatment options for a subject. More particularly, the point accumulation imaging in nanotopography (PAINT) microscopy and the single-particle allow for tracking the behavior of a protein in the cell membrane, and this is informative of disease (i.e., cancer) and of treatment options for a subject.

[011] The super-resolution single-molecule technique PAINT is used to track individual molecules in live cells without altering its physiology. PAINT is a single-molecule localization microscopy technique based on the transient interaction between a labelled molecule (i.e. probe) and a target molecule (i.e. target). The labelled probe is localized under the microscope while bound to the target molecule, and its position

is tracked until the probe detaches. After the probe unbinds from the target, another one from the pool of free floating probes in solution would be able to interact with the target again and this process is repeated over and over to obtain multiple trajectories.

[012] As will be illustrated below, the invention is thus capable of extracting or providing diagnostic predictive patterns from the mobility of cell membrane receptors with PAINT microscopy. As said, PAINT is a single-molecule imaging technique based on the reversible binding of a labelled probe to the target molecule of interest. The current invention enables imaging cell membrane proteins in a non-invasive way, which allows its applicability in diagnostics on unmodified patients cells. By tracking the number, distribution and mobility of molecules on cell surface, variability is observed due to changes in the interactions of the target molecule between different disease subtypes. The invention can be used in the field of cancer as well as applied to other diseases.

[013] Thus, in a first aspect the invention relates to an in vitro method of extracting diagnostic predictive patterns from the mobility of cell membrane molecules, preferably cell membrane proteins, such as cell membrane receptors or any ligand bound to it, the method comprising the steps of:

- a) to add to an isolated cell a labelled probe, preferably a fluorophore-conjugated probe, that targets a molecule on the cell membrane, preferably a protein on the cell membrane, of the isolated cell, preferably an isolated cancer cell of a patient;
- b) to localize the position of the molecule on the cell membrane of the isolated cell, preferably a protein on the cell membrane of the isolated cell, bonded to the labelled probe at multiple frames of time (i.e., at multiple points of time in which a frame is taken);
- c) to track the number, and/or distribution, and/or mobility of the molecule, preferably a protein, bound to the labelled probe;
- d) to determine one or more of the binding kinetics and/or the diffusion and/or the speed of the protein (R_c) from the tracking of step (c) to obtain a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule on the cell membrane, preferably of the protein on the cell membrane, bonded to the labelled probe.

[014] With the extracting of diagnostic predictive patterns from the interactome of a compound directly or indirectly bound to the cell membrane, it derives that a diagnostic

can be made with high precision, because not only the presence of a compound is determined, but also how this compound behaves due to the interaction with other molecules above or below (in case of a transmembrane protein) the cell membrane. Differences in the interactome (i.e., in the set of interactions a protein or compound in the cell membrane can do) may be due to differing disease states or disease subtypes.

[015] Therefore, another aspect of the invention is an in vitro method for the diagnosis of a disease in a subject, wherein the method comprises performing the method of extracting diagnostic predictive patterns from the mobility of cell membrane molecules, preferably cell membrane proteins, such as cell membrane receptors or any ligand bound to it, in an isolated cell of the subject, and as defined above in the first aspect, and further comparing the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the cell membrane molecule, preferably of a protein on the cell membrane, bonded to the labelled probe with a reference, (herewith also referred as control or control reference), wherein the reference is selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a cell membrane molecules, preferably a cell membrane protein in a membrane cell of a subject suffering from the disease; and/or the reference is selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a cell membrane molecule, preferably a cell membrane protein in a membrane cell of a subject not suffering from the disease; and wherein:

- if the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the isolated cell is equal or within a range defined by the reference of a subject suffering from the disease, the subject is diagnosed of suffering the disease; or
- if the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the isolated cell is equal or within a range defined by the reference of a subject not suffering from the disease, the subject is diagnosed of not suffering the disease.

[016] The present invention provides, thus, a diagnostic method based on the mobility and diffusion of cell membrane molecules, such as cell membrane receptors. The receptors change their diffusive patterns based on interactions with neighboring molecules, providing with information about their interactome. The present invention also provides with a measurement of the affinity between a labelled probe and a molecule, such as receptor or other molecule that directly or indirectly (e.g. through interaction with other compounds) is associated (bound, embedded or localized) with the cell membrane, as a further diagnostic biomarker.

[017] Yet another aspect of the invention is an in vitro method to stratify (that is, to classify) subjects suffering from a disease in strata of that disease (i.e., in types or subtypes within that disease), preferably to stratify patients suffering from cancer, the method comprising performing the in vitro method for the diagnosis of a disease as defined in the previous aspects in an isolated cell of the subject, and further compare the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule (i.e., targeted molecule) on the cell membrane, preferably of a protein on the cell membrane, bonded to the labelled probe, with one or more references, wherein the reference is selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a molecule on the cell membrane, preferably of a protein on the cell membrane, of one or more of a subject suffering from a stratum (i.e., subtype) of the disease, wherein:

- the subject is stratified (i.e., classified) as suffering from a disease stratum/subtype when one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states determined in the isolated cell is equal or within a range defined by the reference of a subject suffering from the disease stratum (i.e., disease subtype).

[018] In another aspect, the invention relates to an in vitro method to determine (select) a treatment for a subject suffering from a disease, preferably for a subject suffering from cancer, the method comprising performing the in vitro method for the diagnosis of a disease as defined in any one of the previous aspects in an isolated cell of the subject, and further compare the one or more of the diffusion pattern (or diffusive

state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule, preferably of a protein, on the cell membrane bonded to the labelled probe, with one or more control references, wherein the reference(s) is(are) selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a molecule on a cell membrane, preferably of a protein on a cell membrane, of a subject resistant or responsive to a treatment for that disease, and wherein:

- the subject is determined for a treatment for the disease, or alternatively is ruled out for a treatment, when one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states determined in the isolated cell is equal or within a range defined by the reference of a subject as responsive to a treatment, or resistant to a treatment, respectively.

[019] The invention also relates to the use of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a molecule on a cell membrane bonded to a labelled probe, preferably of a protein on a cell membrane bonded to a labelled probe, in in vitro diagnostic methods.

[020] Thus, the invention also provides the use of a diffusion pattern (or diffusive state), and/or of a transition probability between diffusive states, and/or of a proportion of diffusive states of a molecule on a cell membrane bonded to a labelled probe, preferably of a protein on a cell membrane bonded to a labelled probe, as biomarker.

[021] The invention also relates to a diffusion pattern (or diffusive state), and/or to a transition probability between diffusive states, and/or to a proportion of diffusive states of a molecule on a cell membrane bonded to a labelled probe, preferably of a protein on a cell membrane bonded to a labelled probe, for use in diagnostics.

[022] The invention also provides a method of determining or obtaining one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a molecule on a cell membrane bonded to a labelled probe, preferably of a protein on the cell membrane, bonded to a labelled probe, the method comprising to track, from the localization at one or more time points of said labelled probe, one or more of the number, and/or the distribution, and/or the mobility of the labelled probe on a cell membrane surface. Preferably, the localization of the molecule on a cell membrane bonded to the labelled

probe comprises the localization at multiple frames of time, preferably two or more frames of time. This realization refers, thus, to the obtention of one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a molecule on a cell membrane, for example from the analysis of a sequence of images or from a sequence of outputs of the (labelled) probe. These sequence of outputs can be analyzed at a stage or moment when any in vitro assay on an isolated cell was performed.

[023] The invention relates in an embodiment to an in vitro method of obtaining or determining a (living) cell condition from the mobility of one or more molecules associated to a cell membrane (i.e., embedded or attached to the lipidic bilayer; or associated by any means to a molecule embedded or attached to the lipidic bilayer, including for example complex formation with molecules in the membrane outside or inside the cell), preferably a protein associated to a cell membrane, the method comprising the steps of:

- a) to add to an isolated cell a compound capable to target, preferably by means of covalent or electrostatic interactions, a molecule on the cell membrane, preferably a compound capable to target a protein on the cell membrane of the isolated cell, preferably an isolated cancer cell of a patient;
- b) to localize the position of the targeted molecule on the cell membrane of the isolated cell, preferably a protein on the cell membrane of the isolated cell, bonded to or interacting with the compound capable to target, at multiple points of time, to preferably obtain outputs (e.g., images) of the localized targeted molecule;
- c) to track the number, and/or distribution, and/or mobility of the localized targeted molecule on cell surface;
- d) to determine one or more of the binding kinetics and/or the diffusion and/or the speed of the protein (R_c) from the tracking of step (c) to obtain a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule on the cell membrane, preferably of the protein on the cell membrane, bonded to or interacting with the compound capable to target the said molecule on the membrane. In an embodiment, the compound capable to target a molecule on the cell membrane is a labelled, preferably a fluorophore-labelled compound (i.e., a (labelled) probe).

BRIEF DESCRIPTION OF THE DRAWINGS

[024] Embodiments of the invention are further described hereinafter with reference to the accompanying drawings, in which:

[025] Figure 1: EGF-PAINT imaging of EGFR single-molecules in live cells. a) Schematic representation of PAINT experiment. b) Representation of acquired resistance process to a therapeutic drug. c) Individual point-spread functions (PSF's) of labelled EGF on the apical membrane of MDA-468 cells. d) Reconstruction of a PAINT image showing the position of individual EGFR molecules. e) Single-particle tracking trajectories from the inset in d). Scale bars, 5 μm (c-d) and 1 μm (e).

[026] Figure 2. Panel of TKI resistant cell lines. a) Drug treatment process of erlotinib resistant MDA-468 cells at different passages and concentrations. b-g) Sensitivity curves of parental (black) and resistant (red) MDA-468 cells to every drug. Individual data points show mean $\pm$ SD from triplicates. Sigmoidal fitting curves and IC50 points are also shown. Scale bar, 50 μm .

[027] Figure 3. EGFR expression quantification. a-g) Reconstructed PAINT images of the parental [a] and resistant cell lines at the apical membrane under the same conditions. h) Quantification of the density of binding events (i.e. trajectories) of 50 cells per cell line. Scale bar, 5 μm .

[028] Figure 4. Diffusive states of EGFR. a) Representation of the four-state diffusive model of EGFR on MDA-468 cells. The diffusion coefficient and the frequency of the most relevant transitions are shown. b) Representative EGFR trajectories on MDA-468 cells, color-coded by the diffusive states. Scale bar, 500 nm. c) Diffusive state frequencies of the resistant cell line panel. d) State transition frequencies in descending order of abundance ($3 \leftrightarrow 4$, $2 \leftrightarrow 3$, $1 \leftrightarrow 2$, $1 \leftrightarrow 4$, $1 \leftrightarrow 3$, $2 \leftrightarrow 4$).

[029] Figure 5. Transition probabilities sorted by diffuse state pairs.

[030] Figure 6. Schematic view of a targeted molecule (e.g. cell membrane receptor) transiently interacting with a labelled probe. (a) interaction (K_{on}). (b) diffusion with the labelled probe bound to the target. (c) release of the probe.

[031] Figure 7. Schematic view of an individual point spread as illustrated in Figure 1 (c).

[032] Figure 8. Example of three different trajectories and their corresponding Mean square displacement (MSD) graph, MSD in μm^2 vs Delay (number of frames) (a). Proportions of diffusive states (4 diffusive states) of EGFR molecules on different cell lines (b). Confusion matrix of a classification algorithm (based on a gradient boosting

machine) that classifies cells into their corresponding cell lines using the diffusion states of EGFR (c). The assays were done in the following cell lines (all commercially available): MDA-468, MDA-231, MCF-7; and A-431.

[033] Figure 9. Schematic view of a lectin molecule in a cell membrane targeted with a glycan labelled with a fluorophore according to the method of the invention (a). Diffraction-limited image of single ligand–receptor binding and the reconstructed image of a mannose receptor on a group of cells derived from the accumulation of multiple binding events over time (b). An example of a set of trajectories obtained with the sugar labelled probe (c).

[034] Figure 10. Schematic view of a glycan molecule in a cell membrane targeted with a lectin labelled with a fluorophore according to the method of the invention (a). An example of a set of trajectories obtained with the lectin labelled probe (b).

[035] Figure 11. Example of EGFR trajectories obtained according to the method of the invention on a circulating tumor cell isolated from a lung cancer patient.

DESCRIPTION

Definitions

[036] A portion of this disclosure contains material that is subject to copyright protection (such as, but not limited to, diagrams, device photographs, or any other aspects of this submission for which copyright protection is or may be available in any jurisdiction.). The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or patent disclosure, as it appears in the Patent Office patent file or records, but otherwise reserves all copyright rights whatsoever.

[037] Various terms relating to the methods, compositions, uses and other aspects of the present invention are used throughout the specification and claims. Such terms are to be given their ordinary meaning in the art to which the invention pertains, unless otherwise indicated. Other specifically defined terms are to be construed in a manner consistent with the definition provided herein. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein.

[038] For purposes of the present invention, the following terms are defined below.

[039] As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. For example, a protein on a cell

membrane includes a plurality of molecules (e.g. 10's, 100's, 1000's, 10's of thousands, 100's of thousands, millions, or more molecules) in that cell membrane or in the cell membranes of different cells.

[040] As used herein, the term “and/or” indicates that one or more of the stated cases may occur, alone or in combination with at least one of the stated cases, up to with all of the stated cases.

[041] As used herein, the term “at least” a particular value means that particular value or more. For example, “at least 2” is understood to be the same as “2 or more” i.e., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, ..., etc.

[042] As used herein, the terms “cancer” and “tumor” (used interchangeably) refer to or describe the physiological condition in a host organism (e.g. a human) that is typically characterized by unregulated cell growth. The terms “cancer” and “tumor” also refer to cells that have undergone a cancerous/malignant transformation that makes them pathological to the host organism. Cancerous cells can be distinguished from non-cancerous cells by techniques known to the skilled person.

[043] As used herein, the term “subject” refers to any vertebrate animal, but will typically pertain to a mammal, for example a human, a domesticated animal (such as dog or cat), a farm animal (such as horse, cow, or sheep) or a laboratory animal (such as rat, mouse, non-human primate or guinea pig). In preferred examples, the subject is human and includes males, females, adult, elderly, children or infants, suffering from or expected to be suffering from a tumor, regardless of the stage or state of the tumor.

[044] As used herein, the terms “treatment” and “treating” refer to therapeutic treatment. The object of the treatment is to at least slow down the disease condition. Those in need of the treatment include those already with the disease condition.

[045] As used herein, the expression “extracting a diagnostic predictive pattern” refers to the action of “concluding”, “obtaining” or “determining” information that can be associated to a certain condition of an isolated cell. As used herein a “diagnostic predictive pattern” is a retrievable information, regularly obtainable from an event or entity (i.e., a molecule) in the same form or output and that has a diagnostic value. It provides information about a fact, in this case of condition of a cell. The term “diagnostic predictive pattern” is used in this description as a synonym of a “behavior of a molecule on or associated with a cell membrane”, the behavior encompassing the

presence or not of the molecule and, if present, the mobility through the membrane surface.

[046] As used herein, the term “diffusive state” or “diffusion pattern”, both used as synonyms, is to be understood as the trajectory done or described by a targeted compound directly or indirectly associated with the cell membrane, thus, with the lipidic bilayer. The trajectory is determined by the mobility within an area of the cell membrane surface in 2D or 3D, considering the cell curvature.

[047] As used herein the term “(targeted) protein”, or “(targeted) receptor” or “(targeted) molecule/compound” is to be understood as the element associated with the cell membrane that is contacted (interacted) with a probe, and which mobility is desired to be determined to obtain one or more of a diffusion pattern, and/or of a transition probability between diffusive states, and/or of a proportion of diffusive states of the protein. The protein/compound/receptor is a compound embedded in the cell membrane, or associated to the cell membrane either above (extracellular) or below (cytoplasm side) directly or indirectly. Directly means that the compound is in direct contact with the lipidic bilayer of the cell membrane. Indirectly encompasses the association by means of interaction with another compound that is in contact with the lipidic bilayer. The membrane proteins may include either channels, transporters or other proteins usually found in or associated to the cell membrane.

[048] As used herein, a “probe” is to be understood as any molecule useful to study the properties of other molecules or structures, due to a measurable property of the molecular probe. This measurable property in the probe may derive from the molecule itself, or due to a label, wherein a label is any compound that is attached (e.g., covalently) or associated to the probe (e.g., coordinated) and provides that measurable property. An example of measurable property is the emission of light at certain wavelengths.

[049] As used herein, the expression “transition probability between diffusive states”, refers to the probability a targeted molecule (e.g. protein) that moves through the membrane by means of, or according, a diffusive state (i.e., follows a kind of trajectory or trajectory type) changes to another possible diffusive state. This transition probability can be determined by the amount of times a molecule changes states compared to the total number of times this molecule is localized. As will be illustrated in the examples, the probability of transition from one to another possible diffusive

state of a compound that moves through a cell membrane provides also patterns with diagnostic value (i.e., diagnostic predictive patterns). Thus, it provides for biomarkers, which are signals of a biological condition.

[050] The terms “total internal reflection fluorescence” and “total internal reflection fluorescence microscope (TIRFM)”, refer, respectively, to the technology using total internal reflection of a fluorescent light. The TIRFM is a type of microscope with which a thin region of a specimen, usually less than 200 nanometers can be observed. TIRFM is an imaging modality which uses the excitation of fluorescent cells in a thin optical specimen section that is supported on a glass slide. The technique is based on the principle that when excitation light is totally internally reflected in a transparent solid coverglass at its interface with a liquid medium, an electromagnetic field, also known as an evanescent wave, is generated at the solid-liquid interface with the same frequency as the excitation light. The intensity of the evanescent wave exponentially decays with distance from the surface of the solid so that only fluorescent molecules within a few hundred nanometers of the solid are efficiently excited. Two-dimensional images of the fluorescence can then be obtained, although there are also mechanisms in which three-dimensional information on the location of vesicles or structures in cells can be obtained.

[051] As used herein, the term “single-molecule or single-particle tracking (SMT/SPT)” is the observation of the motion of individual particles (e.g., a protein) within a medium (e.g., a cell membrane- lipidic bilayer). The coordinates time series, which can be either in two dimensions (x, y) or in three dimensions (x, y, z), is referred to as a trajectory. The trajectory is typically analyzed using statistical methods (i.e., mathematical stochastic models) to extract information about the underlying dynamics of the particle. These dynamics can reveal information about the type of transport being observed (e.g., thermal or active), the medium where the particle is moving, and interactions with other particles. In the case of random motion, trajectory analysis can be used to measure the diffusion pattern. In life sciences, single-particle tracking is broadly used to quantify the dynamics of molecules/proteins in live cells.

Detailed description

[052] The invention is defined herein, and in particular in the accompanying claims. Subject-matter which is not encompassed by the scope of the claims does not form part of the present claimed invention.

[053] It is contemplated that any method, use, or composition described herein can be implemented with respect to any other method, use or composition described herein. Embodiments discussed in the context of methods, use and/or compositions of the invention may be employed with respect to any other method, use or composition described herein. Thus, an embodiment pertaining to one method, use or composition may be applied to other methods, uses and compositions of the invention as well.

[054] Any references in the description to methods of treatment refer to the compounds, pharmaceutical compositions, and medicaments of the present invention for use in a method for treatment of the human (or animal) body by therapy.

[055] As embodied and broadly described herein, the present invention is directed to the surprising finding that the behavior, in terms of mobility, of a compound directly or indirectly associated with the cell membrane, allows for the provision of a diagnostic of a disease in relation to a non-disease cell. That is, can be used as a biomarker. Moreover, this behavior also allows to classify the cells between sub-diagnostics or subtypes of a disease, which is relevant for the adequate selection of a treatment. Thus, the invention provides a solution for individual therapy and precision medicine.

[056] In other words, the aspects and corresponding embodiments of the present invention are aimed at helping clinicians make more informed decisions in disease treatment by using single-molecule tracking information of cell membrane receptors (or other molecules) using transit interacting probes as a diagnostic tool.

[057] As previously indicated, a first aspect of the invention is an in vitro method of extracting diagnostic predictive patterns from the mobility of cell membrane molecules, preferably cell membrane proteins, such as cell membrane receptors or any ligand bound to it, the method comprising the steps of:

- a) to add to an isolated cell a labelled probe, preferably a fluorophore-conjugated probe, that targets a molecule on the cell membrane, preferably a protein on the cell membrane, of the isolated cell, preferably an isolated cancer cell of a patient;
- b) to localize the position of the molecule on the cell membrane of the isolated cell, preferably a protein on the cell membrane of the isolated cell, bonded to the

labelled probe at multiple frames of time (i.e., at multiple time points a frame is taken/recorded and the molecule is localized);

c) to track the number, and/or distribution, and/or mobility of the molecule, preferably a protein, bound to the labelled probe;

- 5 d) to determine one or more of the binding kinetics and/or the diffusion and/or the speed of the protein (R_c) from the tracking of step (c) to obtain a diffusion pattern (also herewith referred as diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule on the cell membrane, preferably of the protein on the cell membrane, bonded to the labelled probe.

10 [058] In an embodiment of the invention, step (a) is carried out by Point accumulation imaging in nanotopography (PAINT).

[059] In another embodiment of the method of extracting diagnostic predictive patterns, the concentration of labelled probes added in step (a) is that providing a density of point spread functions (PSFs) wherein the signals of two molecules are not
15 mixed together. This can be determined by conventional techniques. The point spread function (PSF) describes the response of a focused optical imaging system to a point source or point object (point accumulation).

[060] As the skilled person will commonly understand, the step (a) is carried out at the cell culture conditions that allow the labelled probe to interact, at least transiently,
20 with the targeted molecule. These conditions comprise the culturing of the cells at the conventional cell culturing temperatures, preferably from 25 to 37 Celsius degrees, and at the conventional cell culturing relative humidity, preferably from 4 to 6 % of relative humidity.

[061] In another embodiment of the first aspect, the step (b) of localizing the position
25 of the molecule, for example of a protein, on the cell membrane bonded to the labelled probe (e.g. a fluorophore-conjugated probe) at multiple frames of time (i.e., at multiple points of time where a frame is taken) is performed by imaging or visualizing the interaction by any means. In an embodiment the localizing is performed by imaging through a microscope, preferably in a total internal reflection fluorescent (TIRF)
30 microscope. Multiple frames of time means to localize at two or more time points. At each time point a frame is taken.

[062] The localization, or which is the same, the spatial coordinate parameter, may, in some embodiments, be measured by illuminating a light emitting molecule, such as

a fluorescent molecule or particle attached to the probe, and determining its position from the emitted radiation at any time point, preferably using a TIRF or Highly Inclined and Laminated Optica sheet (HILO) illumination system.

[063] In a preferred embodiment, a total internal reflection (TIR) optical setup is used to excite and capture the emitted light of labelled probes, for example the fluorescence of fluorescent probes, onto an active-pixel sensor (CMOS) or an electron-multiplying charge-coupled device (EM-CCD). The settings for an optimal image comprise a trade-off between illumination intensity and exposure time. Both settings affect the signal-to-noise ratio, and are adjusted to get the lowest time steps, while not increasing the illumination intensity in excess to avoid phototoxicity and bleaching. This are parameters the skilled person in the art know how to adjust following conventional techniques.

[064] In an embodiment of this aspect of the invention, the coordinate-based data obtained from the measurement of probe-target interaction (either 2D or 3D) is transformed into trajectory data by single-molecule or single-particle tracking (SMT/SPT). The trajectories resulting from SMT analysis can then be analyzed by mathematical stochastic models, for example, using hidden Markov models (HMM) to obtain patterns and quantify types of trajectories to serve as a biomarkers in a diagnostic setting.

[065] In a preferred embodiment, the spatial coordinates of individual molecules are retrieved by fitting a 2D gaussian function to the fluorescence pattern of each PSF to identify the center position. This is done for each PSF on each frame of the recording, and spatial (x,y) as well as temporal (t) coordinates are noted.

[066] Another embodiment of the first aspect is an in vitro method in which the step (c) to track the number, and/or distribution, and/or mobility of the labelled (fluorophore-conjugated) probe on cell surface (i.e., the labelled probe interacting with the targeted molecule/protein) is carried out by linking the position of individual molecules over time using a single-particle (or single-molecule) tracking algorithm (SPT/SMT).

[067] According to this embodiment, the number, and/or distribution, and/or mobility of the labelled probe (such as a fluorophore-conjugated probe) on cell surface is tracked from the localization at different time points, at least at 2, 3, 4, 5 or more time points, preferably from 5000 to 10000 frames (i.e., time points), and then this allows

(see below) to obtain a trajectory of the labelled probe on the targeted protein (i.e., interacting with the protein).

[068] With 5.000-10.000 frames (step b), which corresponds generally to a timing of 2.5 to 5 minutes of positioning, enough data may be retrieved for the tracking, in particular for one or more targeted molecules on a cell a membrane. The timing of each frame is of about 30 milliseconds, preferably below 30 milliseconds, and most preferably as low as the instrumentation used for the taking a frame allows. Preferably the timing a molecule is positioned and tracked with the transient interaction with the (labelled) probe is that longer as possible.

[069] In an embodiment, the isolated cells subjected to the method, including the localization of any labelled probe bound to a targeted molecule (for example by imaging)) and the tracking, are immobilized onto a surface. This embodiment avoids any movement of the cells within the duration of any localization (e.g. imaging) recording time, and prevent tracking aberrations. This has to be done in such a way that preserves the viability and physiology of cells; one example of this is letting the cells attach naturally onto a surface before imaging. In a typical experiment, this surface consists of a cover glass.

[070] In another embodiment of the first aspect, in step (d), one or more of the diffusion pattern (or diffusive state), and/or the transition probability between diffusive states, and/or the proportion of diffusive states of the protein on the cell membrane bonded to the labelled probe, is determined by one or more of a mathematical model, preferably selected from one or more of Bayesian statistics and Hidden Markov Models (HMMs). These mathematical probability or stochastic models are commonly used and under the expertise of the skilled person in the art.

[071] The binding kinetics and/or the diffusion pattern, and/or the transition probability between diffusive states, and/or the proportion of diffusive states of the molecule, for example a protein, on the cell membrane bonded to the labelled probe, allows to determine (or establish) the behavior of the molecule, for example a protein, on a cell membrane. As said, this behavior is the result of the interactions that the molecule does with other compounds in the cell in, above or below the cell membrane, and it is indicative of a cell state or condition which may then be correlated or associated, for example, with a disease condition or even with a disease subtype.

[072] In another embodiment of the method of the first aspect, in step (d) the binding kinetics preferably include determining binding events and/or time of binding. Binding events relates to the number a labelled probe is bound to a target. The time of binding is defined by the time the probe and the molecule (protein) are in contact, thus interacting. This can be determined by the length of the trajectory obtained when connection individual localizations.

[073] In also another embodiment of the method of extracting diagnostic predictive patterns of the invention, the in vitro method further comprises an step (e) of comparing the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the protein on the cell membrane bonded to the labelled probe with a (control) reference, wherein the reference is preferably a diagnostic predictive pattern reference.

[074] With this embodiment a test sample can be compared with a control with a defined or known diagnostic predictive patterns extracted from the mobility of a protein bound to a labelled probe. The comparison provides then information about the sample matching or not with that diagnostic predictive pattern. That is, it provides information of a diagnostic in a test.

[075] In another embodiment of the in vitro method according to the first aspect, the isolated cell is a cell from a biological sample selected from one or more of a tumor biopsy, blood, serum, plasma, and urine, saliva and tears. The skilled person will understand that any cell isolated from any kind of sample of a subject can be used to carry on the method of extracting a diagnostic predictive pattern. Preferred samples are cells isolated from tumor biopsies, as well as from any fluid, such as blood, tumor interstitial fluid, plasma, etc., where tumor cells can be commonly found. The term "isolated", such as "isolated sample" or "isolated cell", means that any method performed with the same is done outside the body of the subject.

[076] As possible labelled probes there are included, in an embodiment of the invention, those with an affinity constant (K_{off}) for the targeted molecule providing a binding time that is below the average lifetime of the labelling molecule (i.e., label), preferably between few hundred millisecond and few seconds. For example, organic fluorophores are often used to label probes, but would bleach under long laser exposures. Preferably, the labelled probes have to bind to the target molecule with a

low affinity to achieve a short binding time (e.g. transient interaction), and allow the replacement with a new probe.

[077] Thus, for the imaging of the probes and the probes interacting with the target, this probe is to be specific for the desired target (e.g. protein/receptor/ligand of a receptor, etc.). Although more broad-binding probes that have multiple targets can be used, the most important is to avoid non-specific interactions of the probe with other elements, such as the cell membrane or the surface where the cells are anchored to. In relation to the specificity, these probes also require a certain affinity, as previously disclosed.

[078] In another embodiment, the probes (and labelled probes) are compounds which have such a low affinity for the target molecule on the cell membrane that results in a transient interaction and the constant replacement of probes, within the lifespan of the labelling molecule. The probe has, in another embodiment, a minimal influence on the target molecule (i.e., protein, receptor) mobility, or represent a naïve interaction relevant to the phenomena that is desired to be determined, for example for the diagnostic of a disease.

[079] The probe that binds to the target molecule, in some embodiments, is either from a natural or synthetic source, and it may have diverse nature. Examples of probes according to the invention are selected from proteins, hormones, vitamins, sugars (e.g., carbohydrates), and lipids.

[080] In a particular embodiment of the first aspect, the labelled probe is a labelled growth factor, more in particular is a labelled epidermal growth factor (EGF), thus a protein.

[081] In another embodiment, the probe is a glycan (i.e., a polysaccharide). In another embodiment, the probe is a lectin. Lectins are carbohydrate-binding proteins that are highly specific for sugar groups.

[082] All kind of labels (label molecules) may be employed among the commonly used for labelling cell compounds and widely known in the art.

[083] The labelling approach used in the probes, that is, the label molecules or molecules used to visualize the interaction between the probe and the target are, preferably, those that provide with enough signal-to-noise ratio to be detectable under viable conditions for prolonged live cell imaging. In a preferred embodiment, a

fluorophore with a high quantum yield that provides a good signal-to-noise ratio with minimal phototoxicity and bleaching is used.

[084] In an embodiment of the first aspect, the labelled probe is a fluorophore-labelled probe, and the fluorophore is preferably selected from one or more of the fluorophores of the group consisting of an ATTO compound (from ATTO Tech GmbH) or a cyanine, such as ATTO643, ATTO655, ATTO532 or Cy3B fluorophores, and combinations thereof. Preferably is the fluorophore ATTO643.

[085] The ATTO compounds and their esters, ATTO NHS-esters, offer a large variety of high-quality amine-reactive dyes for labeling proteins and other amine containing substrates. The dyes cover the spectral region from 350 nm in the UV to 750 nm in the NIR.

[086] In another embodiment, the probe and in particular the labelled probe, is non-toxic for the cells. This embodiment provides for a live cell positioning (i.e., imaging) and tracking conditions of the targeted molecules, since live cells are those performing as in the body from where they were isolated. Preferred probes are those that interact with the target molecule with minimal effects of toxicity on the cells, and avoid undesired non-naïve changes to the mobility of target molecules.

[087] In another embodiment of the first aspect of the invention, the targeted protein or molecule on the cell membrane of an isolated cell is a cell membrane receptor, preferably a receptor tyrosine kinase involved in carcinogenic processes, preferably is an epidermal growth factor receptor, preferably selected from one or more of: the epidermal growth factor receptor 1, also known as EGFR or ERBB1; the epidermal growth factor receptor 2, also known as ERBB2 or ER2; the epidermal growth factor receptor 3, also known as ERBB3 or HER3; and the epidermal growth factor receptor 4, also known as ERBB4 or HER4.

[088] Summarizing, the embodiments of the method of extracting diagnostic predictive patterns from the mobility of a protein (or cell membrane associated compound/molecule) bound to a labelled probe of the invention, allow at least for three interpretations of the tracking data. First, the amount of trajectories recorded over an area of a cell (i.e. density) is directly proportional to the amount of target molecules on the membrane of the cells. An important advantage of the proposed method is that the quantification of targeted molecules is done at the single-cell level with single-molecule sensitivity. For example, with this method a classification of low expressing

molecules (e.g. receptor) can be created instead of a single negative category. Moreover, by using naïve probes the quantitative analysis of the targeted compound refers to the biologically available amount of that target, rather than a blunt count of all molecules in a cell regardless of their bioactivity.

5 [089] In another disclosed instance, the affinity (i.e. the kinetic constant K_{off}) can be calculated from the tracking data. The mean lifetime of the single-molecule trajectories (i.e. τ) is inversely proportional to the kinetic constant K_{off} . As said, embodiments may include natural binding probes to the target molecules that represent the naïve interaction that occurs in the body. Changes in the affinity between the probe and the
10 target molecule define different behavior of cells in relation to disease. An advantage of the proposed method is the quantification of biochemical interactions at the single-molecule level in undisturbed living cells, which allows to obtain more precise information about the disease mechanics.

[090] In another disclosed instance, the spatial coordinates of the trajectories are
15 analyzed to obtain the mobility profiles of the target molecule (see for example figures 4(B) or Fig. 8(A)). Embodiments may include a feature extraction analysis, such as diffusion coefficient ($\mu\text{m}^2/\text{s}$), confinement ratios or trajectory speeds ($\mu\text{m}/\text{s}$), in order to differentiate between different diffusive states of the target molecule. Alternatively, embodiments may also include more complex trajectory classification analysis such
20 as Hidden Markov Models, in order to obtain the different diffusive states of the target molecules.

[091] In a preferred embodiment, cells are classified based on the proportion of diffusive states that a targeted molecule has (Figure 8B-C). Diffusive states (i.e. types of trajectories) among different cell types may be similar, but the proportion at which
25 those are represented in each cell type is different. This in itself constitutes a biomarker that can be used to classify cells and patients in a diagnostic setting.

[092] A second aspect of the invention is an in vitro method for the diagnosis of a disease in a subject, wherein the method comprises performing the method of extracting diagnostic predictive patterns from the mobility of cell membrane molecules,
30 preferably proteins (e.g., receptors) as defined in the first aspect, in an isolated cell of the subject, and further comparing the one or more of the diffusion pattern, and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the cell membrane molecule, preferably of a protein on the cell membrane bonded

to the labelled probe, preferably bonded a fluorophore-conjugated probe, with a reference, wherein the reference is selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a cell membrane molecule, preferably a cell membrane protein in a membrane cell of a subject suffering from the disease; and/or the reference is selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a cell membrane molecule, preferably a cell membrane protein in a membrane cell of a subject not suffering from the disease; and wherein:

- if the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states determined in the isolated cell is equal or within a range defined by the reference of a subject suffering from the disease, the subject is diagnosed of suffering the disease; or

- if the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states determined in the isolated cell is equal or within a range defined by the reference of a subject not suffering from the disease, the subject is diagnosed of not suffering the disease.

[093] In an embodiment of the in vitro method for the diagnosis of the second aspect of the invention, the disease is a membrane receptor mediated disease. For “membrane receptor mediated disease” is to be understood a disease that occurs at least because of the presence of a receptor in the cell membrane that for any cause, including mutation of the receptor, absence of the receptor, or non-functioning of the receptor, results in a cell condition acknowledged as pathological.

[094] In another embodiment, it is a disease selected from one or more of cancer, autoimmune disease, and combinations thereof. Preferably, the disease is cancer.

[095] In yet another preferred embodiment of the second aspect, the disease is cancer and is selected from one or more of breast cancer, skin cancer, pulmonary cancer, genitourinary tract cancer, bone cancer, head cancer, neck cancer, meroblastic cancer, gastrointestinal cancer, colorectal cancer, pancreatic cancer, hematopoietic cancer and lymphoid tissue cancer, preferably is breast cancer.

[096] The inventors have surprisingly found that the behavior in terms of mobility of a compound directly or indirectly associated with the cell membrane, not only allows for the provision of a diagnostic of a disease in relation to a non-disease cell, but also allows to stratify (i.e., classify) between sub-diagnostics or subtypes of a disease.

5 These subtypes of a disease may encompass differing clinical outcomes, as well as differing responses to treatment. Thus, they require different types of treatment.

[097] Therefore, in another aspect, the third aspect, the invention relates to an in vitro method to stratify subjects suffering from a disease in strata of that disease, that is, in subtypes of the disease, preferably to stratify patients suffering from cancer, the method comprising performing the in vitro method for the diagnosis of a disease as defined in the second aspect, in an isolated cell of the subject, and further compare the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the protein on the cell membrane bonded to the labelled probe, with one or more (control) references, wherein the reference is selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of one or more of a subject suffering from a stratum (i.e., subtype) of the disease, wherein:

- 20 - the subject is classified as suffering from a particular disease subtype or is determined at a particular disease stratum, when one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the isolated cell is equal or within a range defined by the reference of a subject suffering from the disease stratum.
- 25

[098] This aspect can also be reworded as an in vitro method for classifying a subject suffering from a disease, in a subgroup of that disease. For example, the method is for classifying a subject suffering from cancer in a tissue in a particular subtype of cancer in that tissue, and which subtype may be linked to a differing clinical due to, for example, mutations in a receptor in the cell membrane that is involved is that cancer.

30

[099] The particular embodiments defined for the in vitro method of diagnosis of a disease of the second aspect of the invention do also apply as embodiments of this third aspect.

[100] In a fourth aspect the invention also encompasses an in vitro method to determine a treatment for a subject suffering from a disease, that is, to select a treatment for a subject, preferably for a subject suffering from cancer, the method comprising performing the in vitro method for the diagnosis of a disease, or for the stratifying of a subject as defined in any one of the previous aspects, in an isolated cell of the subject, and further compare the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule, preferably of a protein, on the cell membrane bonded to the labelled probe, with one or more (control) references, wherein the reference(s) is(are) selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a subject resistant or responsive to a treatment for that disease, wherein:

- the subject is determined/selected for a treatment for the disease, or alternatively is ruled out for a treatment, when one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states determined in the isolated cell is equal or within a range defined by the reference of a subject as responsive to a treatment, or resistant to a treatment, respectively.

[101] This aspect can also be formulated as an in vitro method for selecting a subject suffering from a disease for a therapy for that disease, the method comprising the previously disclosed comparison of the comparison of the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule, such as of a protein, on the cell membrane bonded to the labelled probe, with one or more (control) references, wherein the reference(s) is(are) selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a subject resistant or responsive to a treatment for that disease

[102] The particular embodiments defined for the in vitro method of diagnosis of a disease of the second aspect of the invention do also apply as embodiments of this fourth aspect.

[103] In an embodiment of the in vitro method according the fourth aspect, the subject is determined (selected) for a treatment for cancer comprising the administering of a chemotherapeutic drug, an immunotherapy, and/or ionizing radiation.

5 [104] In another embodiment, the drug selected from the group consisting of pelitinib, erlotinib, gefitinib, sapitinib, osimertinib, tivozanib, lapatinib, trastuzumab, and combinations thereof.

[105] In yet another embodiment of the fourth aspect, it further comprises the step of administering to the subject the treatment for which the subject is responsive.

10 [106] Thus, herewith disclosed is also a method of treating a subject suffering a disease, preferably a subject suffering from a cancer or an autoimmune disease, wherein the method comprises to perform any one of the in vitro methods of the second and third aspects of the invention, and further to administer a treatment to the subject in need thereof, preferably a treatment for cancer as previously disclosed, or a treatment for an autoimmune disease.

15 [107] The treatment may encompass the administering of a therapeutically effective amount of a drug to the subject.

[108] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or
20 adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein.

25 [109] All references cited herein, including journal articles or abstracts, published or corresponding patent applications, patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by references.

30 [110] It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the

teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

[111] It will be understood that all details, embodiments, and preferences discussed with respect to one aspect of embodiment of the invention is likewise applicable to any other aspect or embodiment of the invention and that there is therefore not need to detail all such details, embodiments, and preferences for all aspect separately.

[112] Having now generally described the invention, the same will be more readily understood through reference to the following examples which is provided by way of illustration and is not intended to be limiting of the present invention. Further aspects and embodiments will be apparent to those skilled in the art.

EXAMPLES

[113] Current diagnostic approaches for cancer patient stratification, mostly based on immunohistochemistry and fluorescence in situ hybridization, are currently falling short in effectively predict the suitable patients for each treatment. This may be related with the low multiplexing (monitoring one biomarker at a time), limited sensitivity and the inability to capture the complexity of cancer proteins signalling. This invention uses PAINT single-molecule imaging to obtain novel predictive features based on the whole interactome of cell membrane proteins. Fluorophore-conjugated probes are added to patients' cancer cells and interact with the target protein on the cell membrane. Cell membrane molecules are important biomarkers in cancer (e.g. EGFR, HER2) immunotherapy (e.g. PD-1, PD-L1) and anti-angiogenic therapies (e.g. VEGFR2) among others.

[114] Using a super-resolution microscope, we can detect this binding and localize the position of these proteins. While the probe remains bound, we observe the movement of the receptor on the cell membrane, creating a trajectory that can be analysed by single particle tracking (SPT). These trajectories are valuable since they encode multiple information from binding kinetics and diffusion of the receptor. First, the number of binding events has a direct correlation with the expression level of the target protein on the cell membrane, the more proteins available, the higher amount of binding there would be. Moreover, the time of binding will provide information about the probe-target affinity. This allows to detect if a mutation reduces or increase the binding affinity of certain biomarkers. This is important since signal transduction is dependent on this affinity and times and mutations affecting the strength of the

interaction could determine the type of the patient response to treatment. These kinetic features cannot be measured with standard diagnostic procedures and comprehend in situ information about proteins behaviour. Lastly, the diffusion pattern and speed of target proteins on the cell membrane can be analysed from these trajectories.

5 Interactions with other biomolecules affect their diffusion by trapping/releasing their movement or actively modifying their velocity. All these interactions create characteristic patterns in the diffusion based on what is occurring to the receptor. Artificial intelligence is then used to identify and classify the underlying patterns in different diffusional states. Changes in these states would indicate changes in the interactome of target proteins, which can be used as a predictive feature to identify
10 different cancer cell responses.

[115] Example 1. EGFR diffusive model as a predictive marker for drug resistance with live-cell PAINT.

[116] The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase
15 involved in many carcinogenic processes. Tyrosine kinase inhibitors against EGFR have become the standard of care for many patients, but resistance to these drugs hinder their efficacy. Resistance mechanism are often complex and involve many interlinked cell signaling pathways. Current predictive biomarkers for drug sensitivity focus mainly on a few genetic alterations, but fail to cope with the high complexity of
20 the cellular environment. Live-cell PAINT single-molecule imaging is a non-invasive approach to obtain diffusion information about endogenous cell membrane receptors. We demonstrated the potential use of the diffusive behavior of the EGFR to predict drug sensitivity on MDA-468 cells.

[117] Introduction

25 [118] Receptor tyrosine kinases (RTK) are a family of signaling receptors involved in a multitude of cellular processes like proliferation, differentiation and migration.¹ Upon binding of a ligand (i.e. growth factors) conformational changes promote oligomerization^{2,3} and the phosphorylation of binding sites for the binding of adaptor proteins.^{4,5} This triggers a signaling cascade in the cytoplasm that results in the
30 activation of specific transcription factors and the consequent alteration of gene expression. It is not surprising that given RTKs pivotal role in cell proliferation and metabolism, they are strongly linked with cancer.^{6,7} RTKs compress a multitude of oncogenes^{6,8} and they are commonly the target of anticancer drugs, such as small-

molecule tyrosine kinase inhibitors (TKI)⁹⁻¹¹ and immunotherapies (i.e. monoclonal antibodies).¹²

[119] The RTK sub-family named ErbB is probably the most studied for its strong involvement in cancer. It comprises four different receptors (HER1 [EGFR], HER2, HER3 and HER4) with a multitude of ligands that modulate the response of their signaling pathways.¹³ The activation of these receptors lead to the formation of homo- and hetero- dimers,^{14,15} although they have been found forming bigger oligomeric complexes.^{16,17} The binding of different adaptors proteins to their cytoplasmatic domain would determine which signaling pathway is activated, and therefore, the response of the cell to that stimuli. Many oncogenes are found among their adaptor proteins and signaling pathways, such as Ras,¹⁸ AKT,¹⁹ TP53²⁰ or the MAPK pathway.²¹

[120] There are two particular cases where ErbB family receptors are important targets in cancer: HER2 in breast cancer^{22,23} and EGFR in non-small cell lung cancer (NSCLC).²⁴ HER2 is found amplified or overexpressed in 10-25% of breast carcinomas²⁵. The presence of HER2 is generally evaluated by an immunohistochemistry (IHC) score or an increased gene copy number measured by in-situ hybridization (ISH).²² These are treated with tyrosine kinase inhibitors (e.g. Lapatinib) or monoclonal antibodies (e.g. trastuzumab), and there are other possible biomarkers that predict resistance to those therapies.²⁵ Similarly, a study in 2016 revealed that EGFR mutations are present in as many as 32% of non-small cell lung cancer patients.²⁶ Some of those are associated with drug sensitivity (EGFR Gly719X, exon 19 deletion, Leu858Arg or Leu861Gln) and others with primary (EGFR exon 20 insertions) or acquired (EGFR Thr790M, Asp761Tyr, Leu747Ser or Thr854Ala) drug resistance.²⁷ FDA-approved therapies for NSCLC include first-generation TKIs such as afatinib, erlotinib or gefitinib, as well as the third-generation TKI osimertinib that overcomes the Thr790M acquired resistance to previous drugs.^{11,28}

[121] However, drug sensitivity prediction still constitutes one of the main challenges in personalized medicine. As described above, current approaches are based on mutation screening or protein overexpression, but the sensitivity of cancer cells to treatment depends on a large number of biological features. The interactome (i.e. set of molecular interactions that a protein makes) of RTKs is vast and comprises multiple heterodimeric forms and transactivation processes.^{5,29} For instance, it is known that a

mechanism of resistance to EGFR inhibitors is the transactivation of signaling pathways through MET or HGF overexpression and HER3 transactivation.^{30–32} Here we propose to analyze changes in EGFR interactome by fingerprinting its mobility on the cell membrane. Interaction with ligands, other receptors or adaptor proteins would lead to alterations to EGFR diffusion (e.g. confined diffusion or Brownian motion), which could be fingerprinted as a predictive feature for drug resistance. Diffusion fingerprinting of receptors has been used to gain biological insights since it combines the information of multiple interactions (interactome) and provides a measurement of the heterogeneity of receptor behavior.^{33–36}

[122] We measured the diffusion of biologically active EGFR with single-molecule PAINT (Point Accumulation in the Nanoscale Topography) imaging using EGF as a probe as described by Winckler et. al.³⁷ PAINT is a single-molecule imaging technique based on the transient interaction of a labelled probe in solution (i.e. fluorophore-conjugated EGF) and its target (i.e. EGFR), as shown in Figure 1a. It allows to non-invasively extract diffusion information of endogenous receptors through a single-particle tracking (SPT) analysis, compared to classical SPT approaches that require the use of bulky antibodies or genetically encoded proteins.^{38,39} We created a panel of resistance cells to a variety of TKIs (Figure 1b) and measured the changes in the diffusion fingerprint. Results showed that diffusion is altered in some cases, independently of the degree of resistance acquired.

[123] Results and discussion

[124] EGF-PAINT live-cell imaging on MDA-468 cells

[125] The human epidermal growth factor binds to EGFR on the membrane of cells with high specificity. A recombinant human EGF protein is labelled with ATTO643 though its lysine residues using an NHS-ester reaction. The labelled EGF is added to the medium of live MDA-468 cells at low concentrations. Using a total internal reflection fluorescent (TIRF) microscope, individual EGF-EGFR complexes are observed as fluorescent puncta in the apical membrane of the cells (Figure 1c).

[126] The low concentration of labelled EGF in solution (pM-nM) allows that just a few sparse dots are visible in every frame, so the position of these EGFR molecules can be localized with high precision. The reconstruction of multiple frames of time yield a position/density map of EGFR molecules in the membrane of the cells (Figure 1b). The mobility of EGFR molecules can be reconstructed by linking the position of

individual molecules over time using single-particle tracking algorithms. This results in trajectories that define the diffusion of the EGFR molecules.

[127] Panel of TKI resistant cell lines

[128] Six tyrosine kinase inhibitors were selected for this project (Table 1): two first-generation EGFR inhibitors (Erlotinib and Gefitinib), one third-generation EGFR inhibitor effective against Thr790M mutation (Osimertinib), two broader ErbB inhibitors (Sapitinib and Pelitinib) and one VEGFR inhibitor (Tivozanib). We created a panel of MDA-468 cells (EGFR overexpression)⁴⁰ resistant to these tyrosine kinase inhibitors. Briefly, cells were grown to a cell confluency of 20-30%, before incubation with the drugs for 72h. Then, cells were transferred to a new plate to start the process again with an increased drug concentration. In doing this, cells that have a higher resistance for the drug would be selected and enriched in the next culture. An example of erlotinib-treated cells at 1 μ M, 8 μ M and 12 μ M is shown in Figure 2a. Note that there are more dead cells in the first image (P0) than in the last one (P13), despite the concentration being 10-fold higher. The detailed process of each of the cell lines can be found in Table 2.

Table 1. Tyrosine kinase inhibitors and IC₅₀ values.

Name	Target	IC₅₀ Parental (μM)	IC₅₀ Resistant (μM)	Increase
<i>Pelitinib</i>	EGFR (HER2)	0.36	0.54	1.5
<i>Erlotinib</i>	EGFR	9.88	28.13	2.9
<i>Gefitinib</i>	EGFR	6.33	6.21	1.0
<i>Sapitinib</i>	EGFR, HER2, HER3	6.76	25.42	3.8
<i>Osimertinib</i>	EGFR (<i>Thr790M</i>)	2.44	4.86	2.0
<i>Tivozanib</i>	VEGFR	3.48	2.81	0.8

Table 2. Drug concentrations (μ M) in resistant cell lines development

Passage	<i>Pelitinib</i>	<i>Erlotinib</i>	<i>Gefitinib</i>	<i>Sapitinib</i>	<i>Osimertinib</i>	<i>Tivozanib</i>
<i>P0</i>	0.1	1	1	1	0.5	0.5
<i>P1</i>	0.2	2	1	1	1	1
<i>P2</i>	0.2	2	2	1	1	2
<i>P3</i>	0.2	4	4	2	1	4
<i>P4</i>	0.3	6	4	2	1	6
<i>P5</i>	0.3	8	6	2	1	8
<i>P6</i>	0.3	8	6	2	1	8
<i>P7</i>	0.4	8	8	2	2	8

Passage	Pelitinib	Erlotinib	Gefitinib	Sapitinib	Osimertinib	Tivozanib
<i>P8</i>	0.4	8	8	4	2	8
<i>P9</i>	0.6	10	10	6	4	10
<i>P10</i>	0.6	10	10	6	4	10
<i>P11</i>	0.6	10	10	6	4	10
<i>P12</i>	0.8	12	10	8	6	10
<i>P13</i>	0.8	12	10	8	6	10
<i>P14</i>	0.8	12	10	8	6	10
<i>P15</i>	0.8	12	10	8	6	10

[129] Sensitivity curves comparing the parental (MDA-468) with the drug-resistant cell lines were performed to obtain IC₅₀ values and evaluate the resistance acquired (Figure 2b-g). Drug-treated cell lines had a negligible to moderate improvement in their resistance to the tyrosine kinase inhibitors. Firstly, erlotinib and sapitinib resistant cell lines displayed the greatest increase in IC₅₀ (2.9-fold and 3.8-fold). However, pelitinib and osimertinib treated cells had a lower increase, and gefitinib and tivozanib treated cells did not show any improvement.

[130] EGF-PAINT single-molecule imaging of resistant cell panel

[131] The panel of MDA-468 resistant cells was imaged with the labelled EGF probe to obtain single-molecule binding events to the EGFR. Using a super-resolution localization algorithm, the position of EGFRs on the surface of the cells was localized with a high precision (~18 nm). Figure 3a-g shows the reconstruction of a representative PAINT image of one cell from each cell line. We observe that despite being small, there is some variability in terms of EGFR expression among the drug treated cells. To have a more representative view on the receptor expression, single-particle tracking was used to generate trajectories from the receptor positions, so binding events could be quantified (Figure 3h).

[132] Changes of receptor expression of resistant cell lines are small. We observe that in some cases (pelitinib, erlotinib, gefitinib and sapitinib) the average density of trajectories is lower than the parental, but it is slightly higher for Osimertinib and tivozanib resistance cells. Although receptor density does not seem to be a key characteristic in the resistance mechanism of these cells, they show an increased variability compared to the parental cell line. This is consistent with the increased intratumoral heterogeneity found in tumors after therapy resistance.⁴¹

[133] EGFR diffusion fingerprinting

[134] Single-particle tracking produces trajectories that contain information about the receptor mobility and reflect the biochemical interactions in the system.⁴² The classical diffusive model for freely diffusing molecules is Brownian motion,⁴³ although in a complex cellular environment there may be existing multiple diffusive states with specific diffusion characteristics.^{33,36,44–46} Bayesian statistics and Hidden Markov Models (HMMs) have been used to analyze SPT trajectories without assuming a predetermined number of diffusive states for those systems where there is no prior knowledge of the inherent diffusive sub-populations.^{33,45,47,48} These have a clear advantage over artificial intelligence powered methods to study diffusive states,^{49,50} since they have the ability to infer populations in single trajectories (i.e. they do not treat each trajectory as an individual unit, but rather assume it may jump between states), therefore they provide transition probabilities between states.

[135] We have used vbSPT, published by Persson et.al,³³ to extract the EGFR diffusive states of the SPT trajectories, which uses a maximum-evidence criterion to select between models with different numbers of diffusive states. The combined data from 50 cells from each cell line resulted in a 4-state model shown in Figure 4a. These four states are defined by distinct average diffusion coefficients and have specific transition probabilities between those states. The transitions are defined by the probability of a receptor to change from one state to another, which can occur multiple times in the duration of a trajectory (Figure 4b). These four diffusive states are related to different subsets of interaction, which could range from dimerization, binding of adaptor proteins or internalization. For instance, conformational fluctuations of EGFR dimers have been observed by smFRET, which could promote consecutive transitions between two states.⁵¹

[136] In our previous work (Chapter 4 in this thesis) we observed that multiple cancer cell lines do not have distinct diffusive states, but rather different proportions of these. Therefore, in order to fingerprint resistance to TKIs using EGFR diffusive states, we have to compare the proportion at which these states are present. In Figure 4c we can observe the frequency of the four diffusive states in each of the resistance cell lines compared to the parental MDA-468 cells. At a first glance, there are three cell lines that did not show a notable shift in diffusive populations percentages: pelitinib, erlotinib and gefitinib treated cells. On the other hand, sapitinib, osimertinib and tivozanib did

display a more notable change in population frequency, with some states having a 3-fold difference. Surprisingly, this does not correlate with acquired cell resistance to the different drugs. This implies two things: on one hand, EGFR diffusional fingerprinting does discriminate between cell lines with resistance to certain drugs.

5 Even the treatment of cells with Tivozanib did not alter the resistance to the drug, however, it was sufficient to promote changes in EGFR dynamics. On the other hand, certain resistance mechanisms, such as in the erlotinib resistant cells, do not alter the diffusional behavior of EGFR.

[137] The diffusive state frequencies are not the only information that can be derived
10 from these analysis. The transition probabilities can also hold information about the heterogeneity of interactions of the EGFR. Figure 4d shows the frequency of each state-pair transition by each cell line, which creates an extra fingerprint in addition to state frequencies. In order to determine the most relevant transitions, Supplementary Figure 1 shows the frequencies sorted by each diffuse state pair. This shows that the
15 transitions between state 1-3, 1-4 and 2-4 hold the most variability. Interestingly, transitions between state 2 and 4 only occur in the parental cell line and seem to disappear in all drug resistance cells. Moreover, the transitions between states 1 and 3 do not occur in gefitinib, sapitinib and tivozanib resistant cells and its frequency is quite variable in the other cell lines.

20 [138] The case of tivozanib resistance cell line is worth special attention. This tyrosine kinase inhibitor targets the VEGFR family of growth factors receptors. Although VEGFR and ErbB receptors should not directly interact together, their signaling pathways are interconnected and RTK cross-talk is a common resistance mechanism to TKIs.⁵²⁻⁵⁴ We observed a strong alteration of the EGFR dynamics on
25 this cell line in comparison with the parental MDA-468 cell line, which highlights the complexity and interconnectivity of these signaling pathways. The dynamics information that this approach holds comprehends a multitude of molecular interactions (even indirect effects) and cannot be obtained by the common diagnostics assays on fixed cells.

30 [139] Conclusion

[140] Single-molecule EGF-PAINT imaging allows to extract diffusive states of active EGFR receptors in unaltered cells. The combination of diffusive states and transition frequencies create a fingerprint based on the change in the biochemical interactions

of EGFR. The drug resistance predictiveness of these new features is drug specific and does not seem to correlate with the level of acquired resistance. However, it is sensitive to a multitude of molecular alterations, even suggesting unknown complex feedback mechanisms. This is a clear advantage to other biomarker assessment approaches, where knowing the exact alterations is a prior requisite (e.g. genomics). The information that this fingerprinting holds is unique (cannot be extracted from common diagnostic assays done in fixed cells), is quantitative and it is sensitive to multiple molecular changes.

[141] Experimental Section

10 [142] Cell culture and drug resistance cell lines

[143] MDA-468 cells (ATCC HTB-132) were purchased from the American Type Collection (ATCC, Manassas, USA). They were cultured in Leibovitz supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin and 100 µg/mL Streptomycin, in a 37°C incubator.

15 [144] To induce drug resistance, MDA-468 cells were culture in 6-well plates until a 20-30% confluency was reached. Then, cells were washed with PBS and incubated with the drugs in fresh medium for 72h. After the incubation with drugs cells were washed with PBS, transferred to a new plate and grown until the desired confluency is reached to start the process again. Increasing concentrations of drugs were added at each passage (see Table 2, supplementary Table 1 in priority document). After the last passage cells were transferred to a bigger flask before freezing for long term storage with 5% DMSO in complete medium. All drugs were purchased from Selleck Chemicals LLC (Houston, USA).

[145] Sensitivity curves of resistant cell lines

25 [146] To determine the acquired resistance to the drugs by the resistant cell lines, cells were seeded in a 96-well plate at a density of 20,000 cells per well. For each concentration triplicates were used, and the outermost wells were filled with PBS. Cells were grown for 24h before incubated with the drugs in fresh medium for 72h. After, medium was substituted by fresh medium with 10% AlamarBlue HS Cell Viability reagent (ThermoFisher, Waltham, USA) and incubated for 4 hours at 37°C. Fluorescent emission at 590 nm was measured in a Varioskan LUX microplate reader with 560 nm excitation. Triplicate measurements were averaged are plotted (mean ± SD) and fitted with a sigmoidal curve in OriginLab to obtain the IC₅₀ values.

[147] EGF labelling with ATTO643

[148] Recombinant human epidermal growth factor (rhEGF) was purchased from R&DSYSTEMS (Minneapolis, USA) and ATTO643-NHS-ester fluorophore was purchased from ATTO-TEC GmbH (Martinsried, Germany). rhEGF was conjugated with ATTO643-NHS-ester by mixing 50 µg of EGF (2 mg/mL) with 5 µL of ATTO643-NHS-ester (10 mM) in presence of NaHCO₃ (0.1 M) at pH 8.3. The mixture is incubated for 2 hours at room temperature. Purification of rhEGF from unconjugated dye was performed in size-exclusion columns (PD Spintrap G-25, Merck) followed by 24h dialysis at 4°C (Slide-A-Lyzer MINI 3.5K MWCO, Fisher Scientific).

[149] Sample preparation and optical setup for live-cell PAINT imaging

[150] MDA-468 cells were seeded in a µ-Slide 8 well glass bottom (Ibidi GmbH, Germany) and grown for 24h. Cells were then washed with PBS and fresh medium containing 0.25 nM of ATTO643-EGF was added. Single-molecule imaging was performed in a ONI microscope (Oxford nanoimaging, UK) prewarmed at 37°C. The sample was illuminated using a HILO alignment system and fluorescence was recorded using a ×100/1.4-numerical aperture oil immersion objective, passed through a beam splitter. Images were acquired on a 420 × 500-pixel region (pixel size, 0.117 µm) of a sCMOS camera at 30 ms integration time. ATTO643-labelled EGF was imaged with a 640-nm laser at 40 mW. Each cell was recorded for 9,000 frames, with a prior 200 frames at 180 mW to bleach EGF molecules that have been internalized in the cell.

[151] Single-molecule localization and tracking

[152] Super-resolution image reconstruction was performed in ONI software Nimos 1.18, by fitting a two-dimensional Gaussian to individual fluorescence spots to identify single molecules. Single-particle tracking to obtain EGFR trajectories was performed with the same software and the following parameters set: maximum frame gap, 3; maximum distance between frames, 0.1 µm; exclusion radius, 1.0 µm; and minimum trajectory length was set to 5 steps.

[153] Diffusive state analysis was performed using variational Bayes single particle tracking (vbSPT) as per Persson et.al³³ on pooled data from 50 cells per cell line. Transition probabilities are then calculated dividing the amount of steps that changed from one state to another by the total number of steps of that state (not taking into account steps at the end of a track since they cannot be linked to a new state).

[154] Example 2. EGFR diffusive model as a predictive marker with live-cell PAINT in several cell lines.

[155] Following the same protocol as in Example 1, and as illustrated in Figure 8, the diffusive states and proportions of diffusion states were analyzed by targeting the EGFR molecules on different cell lines (i.e., MDA-468, MDA-231, MCF-7; and A-431). In Figure 8 (a) there is an example of three different trajectories and their corresponding MSD graph (MSD μm^2 in function of the frame number). The proportions of 4 diffusive states determined of EGFR molecules on the different cell lines is depicted in Figure 8 (b). The confusion matrix of a classification algorithm (based on a gradient boosting machine) classifies cells into their corresponding cell lines using the diffusion states of EGFR (Figure 8 (c)).

[156] This example illustrates the effectivity of the method of the invention in characterizing the cells by particular diffusive states of a cell membrane receptor (i.e., EGFR) and the proportion between these states. These two parameters extracted from the mobility of a targeted EGFR on the cell membrane provides an fingerprint (marker) of each of the cell lines. These cell lines are models of different cancers or subtypes of the same cancer, thus confirming the utility of the method of the invention also for the diagnosis of cancer in patients, as well as for the classification (stratification) of the patients.

[157] Example 3. EGFR diffusive model as a predictive marker with live-cell PAINT of lung cancer.

[158] In Figure 11 there are the EGFR trajectories obtained according to the method of the invention on a circulating tumor cell isolated from a lung cancer patient. The images were obtained as indicated in Example 1. This example proves the applicability of the method in a test sample isolated from a patient suffering from lung cancer. The trajectories and any analysis derived from the same can be compared with a reference from a lung cancer. On the other hand, this example serves as a source of a possible reference for another test sample.

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CLAIMS

1. An in vitro method of extracting diagnostic predictive patterns from the mobility of cell membrane molecules, preferably from the mobility of cell membrane proteins, the method comprising the steps of:
 - (a) to add to an isolated cell a labelled probe, preferably a fluorophore-conjugated probe, that targets a molecule on the cell membrane, preferably a protein on the cell membrane, of the isolated cell, preferably an isolated cancer cell of a patient;
 - (b) to localize the position of the molecule on the cell membrane of the isolated cell, preferably of a protein on the cell membrane of the isolated cell, bonded to the labelled probe at multiple frames of time;
 - (c) to track the number, and/or distribution, and/or mobility of the molecule, preferably a protein, bound to the labelled probe;
 - (d) to determine one or more of the binding kinetics and/or the diffusion and/or the speed of the protein (R_c) from the tracking of step (c) to obtain a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule on the cell membrane, preferably of the protein on the cell membrane, bonded to the labelled probe.
2. The in vitro method according to claim 1, wherein step (a) is carried out by point accumulation imaging in nanotopography (PAINT).
3. The in vitro method according to any one of claims 1-2, wherein the step (b) of localizing the position of a protein on the cell membrane bonded to the labelled probe at multiple frames of time is performed in a microscope, preferably in a total internal reflection fluorescent (TIRF) microscope.
4. The in vitro method according to any one of claims 1-3, wherein the step (c) to track the number, and/or distribution, and/or mobility of the molecule bound to the labelled probe on the cell surface is carried out by linking the position of individual molecules over time using a single-particle tracking algorithm.

5. The in vitro method according to any one of claims 1-4, wherein in step (d), one or more of the diffusion pattern (or diffusive state), and/or the transition probability between diffusive states, and/or the proportion of diffusive states of the protein on the cell membrane bonded to the labelled probe is determined by one or more of a mathematical model, preferably selected from one or more of Bayesian statistics and Hidden Markov Models (HMMs).
6. The in vitro method according to any one of claims 1-5, wherein in step (d), the binding kinetics preferably include determining binding events and/or time of binding.
7. The in vitro method according to any one of claims 1-6, which further comprises an step (e) of comparing the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the protein on the cell membrane bonded to the labelled probe with a (control) reference, wherein the reference is preferably a diagnostic predictive pattern reference.
8. The in vitro method according to any one of claims 1-7, wherein the isolated cell is a cell from a biological sample selected from one or more of a tumor biopsy, blood, serum, plasma, and urine.
9. The in vitro method according to any one of claims 1-8, wherein the labelled probe is a fluorophore-labelled probe, and the fluorophore is preferably selected from one or more of the fluorophores of the group consisting of ATTO643, ATTO655, ATTO532, Cy3B, and combinations thereof, preferably is ATTO643.
10. The in vitro method according to any one of claims 1-9, wherein the targeted molecule, preferably the targeted protein, on the cell membrane of an isolated cell is a cell membrane receptor, preferably a receptor tyrosine kinase involved in carcinogenic processes, preferably is an epidermal growth factor

receptor, preferably selected from one or more of the epidermal growth factor receptor 1, the epidermal growth factor receptor 2, the epidermal growth factor receptor 3, and the epidermal growth factor receptor 4.

- 5 11. An in vitro method for the diagnosis of a disease in a subject, wherein the method comprises performing the method of extracting diagnostic predictive patterns from the mobility of cell membrane molecules, preferably from the mobility of cell membrane proteins as defined in any one of claims 1-10, in an isolated cell of the subject, and further comparing the one or more of the
- 10 diffusion pattern (or diffusive state), and/or the transition probability between diffusive states, and/or the proportion of diffusive states of the cell membrane molecule bonded to the labelled probe, preferably of a protein on the cell membrane bonded to the labelled probe, with a reference, wherein the reference is selected from one or more of a diffusion pattern (or diffusive
- 15 state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a cell membrane molecule, preferably a cell membrane protein in a membrane cell of a subject suffering from the disease; and/or the reference is selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or
- 20 a proportion of diffusive states of a cell membrane molecule, preferably a cell membrane protein in a membrane cell of a subject not suffering from the disease; and wherein:
- if the one or more of the diffusion pattern (or diffusive state), and/or a
 - 25 transition probability between diffusive states, and/or a proportion of diffusive states of the cell membrane molecule, preferably of the cell membrane protein in the isolated cell is equal or within a range defined by the reference of a subject suffering from the disease, the subject is diagnosed of suffering the disease; or
 - 30 - if the one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the cell membrane molecule, preferably of the cell membrane protein in the isolated cell is equal or within a range defined by the reference

of a subject not suffering from the disease, the subject is diagnosed of not suffering the disease.

5 12. The in vitro method according to claim 11, wherein the disease is selected from cancer, autoimmune disease, and combinations thereof, preferably the disease is cancer.

10 13. The in vitro method according to any one of claims 11-12, wherein the disease is cancer and is selected from one or more of breast cancer, skin cancer, pulmonary cancer, genitourinary tract cancer, bone cancer, head cancer, neck cancer, meroblastic cancer, gastrointestinal cancer, colorectal cancer, pancreatic cancer, hematopoietic cancer and lymphoid tissue cancer, preferably is breast cancer.

15 14. An in vitro method to stratify subjects suffering from a disease in strata of that disease, preferably to stratify patients suffering from cancer, the method comprising performing the in vitro method for the diagnosis of a disease as defined in any one of claims 11-13, in an isolated cell of the subject, and further compare the one or more of the diffusion pattern (or diffusive state),
20 and/or the transition probability between diffusive states, and/or the proportion of diffusive states of the protein on the cell membrane bonded to the labelled probe in the isolated cell, with one or more references, wherein the reference is selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a
25 proportion of diffusive states of a molecule on the cell membrane, preferably of a protein on the cell membrane of a cell of a subject suffering from a stratum of the disease, wherein:

- 30 - the subject is stratified as suffering from a disease stratum when one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule on the cell membrane, preferably of a protein on the cell membrane

the isolated cell is equal or within a range defined by the reference of a subject suffering from the a disease stratum.

5 15. An in vitro method to determine a treatment for a subject suffering from a disease, preferably for a subject suffering from cancer, the method comprising performing the in vitro method for the diagnosis of a disease as defined in any one of claims 11-14, in an isolated cell of the subject, and further compare the one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule on the cell membrane, preferably of a protein on the cell membrane, bonded to the labelled probe, with one or more references, wherein the reference(s) is(are) selected from one or more of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a molecule on the cell membrane, preferably of a protein on the cell membrane, of a subject resistant or responsive to a treatment for that disease, wherein:

- the subject is determined for a treatment for the disease, or alternatively is ruled out for a treatment, when one or more of the diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of the molecule on the cell membrane, preferably of a protein on the cell membrane in the isolated cell, is equal or within a range defined by the reference of a subject as responsive to a treatment, or resistant to a treatment, respectively.

16. The in vitro method according to claim 15, wherein the subject is determined for a treatment for cancer comprising the administering of a drug selected from the group consisting of pelitinib, erlotinib, gefitinib, sapitinib, osimertinib, tivozanib, lapatinib, trastuzumab, and combinations thereof.

17. Use of a diffusion pattern (or diffusive state), and/or a transition probability between diffusive states, and/or a proportion of diffusive states of a molecule

on a cell membrane bonded to a labelled probe, preferably of a protein on a cell membrane bonded to a labelled probe in in vitro diagnostic methods.

- 5 18. A method of determining or obtaining one or more of a diffusion pattern, a transition probability between diffusive states, and a proportion of diffusive states of a molecule on a cell membrane bonded to a labelled probe, preferably of a protein on the cell membrane bonded to a labelled probe, the method comprising to track, from the localization at one or more time points of said labelled probe, one or more of the number, and/or the distribution, and/or the mobility of the labelled probe on a cell membrane surface.
- 10

Fig. 1

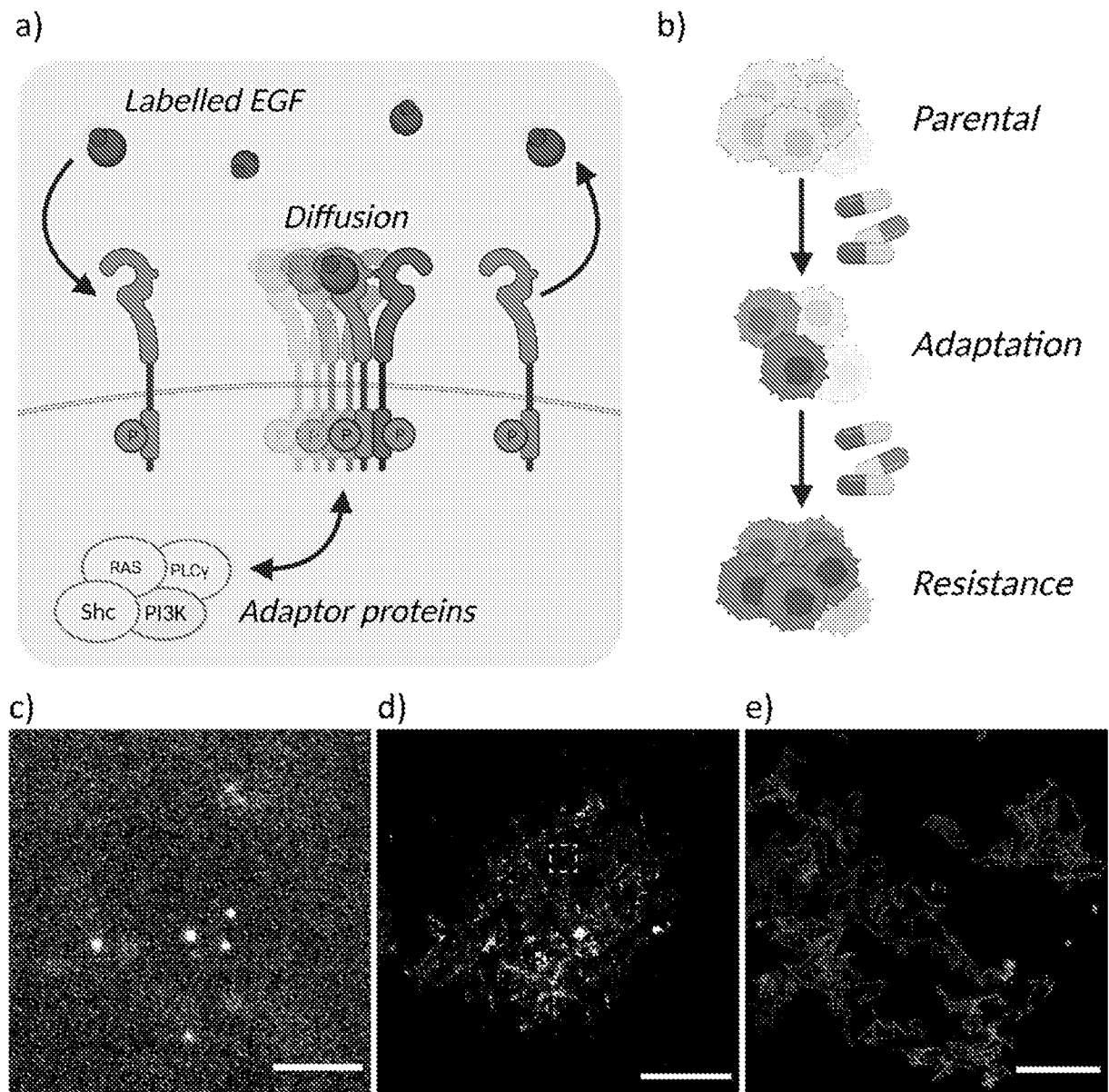
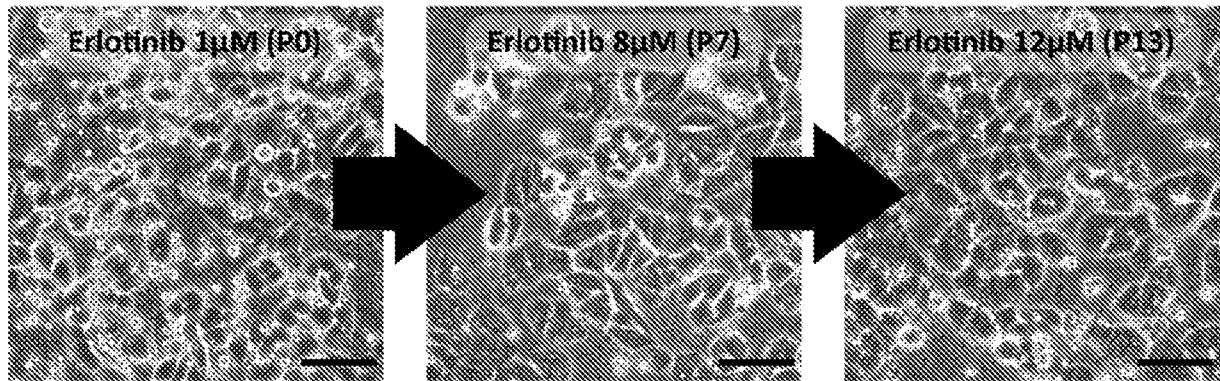
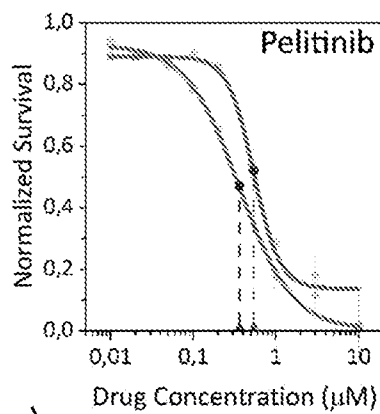


Fig. 2

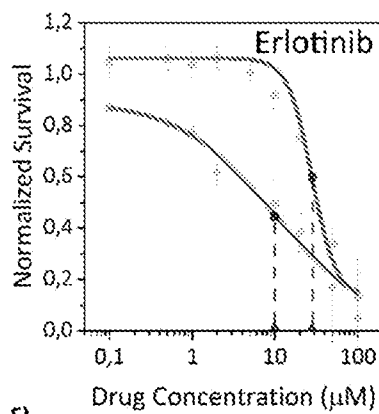
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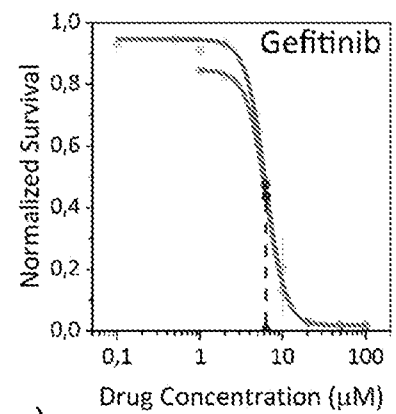
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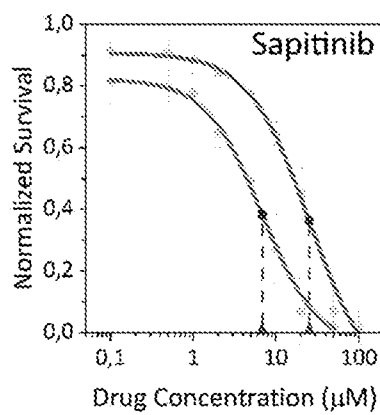
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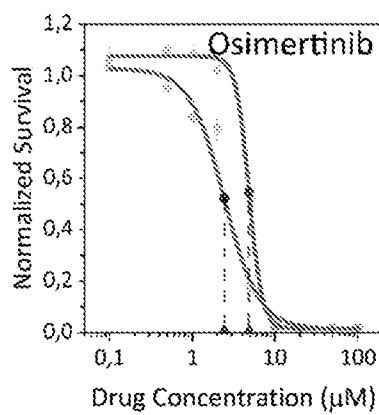
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e)



f)



g)

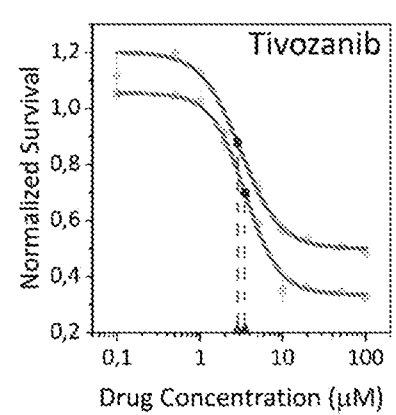


Fig. 3

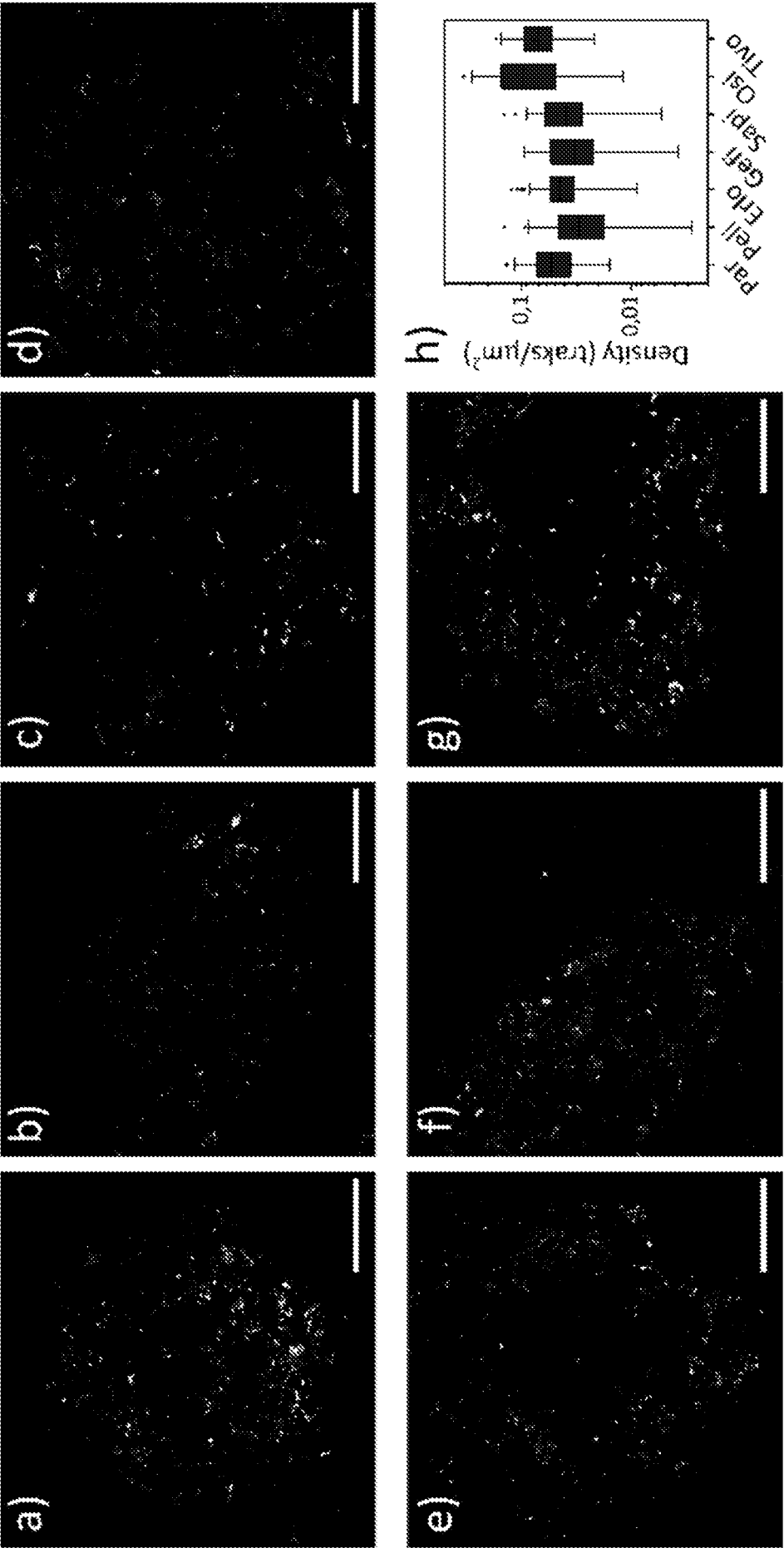


Fig. 4

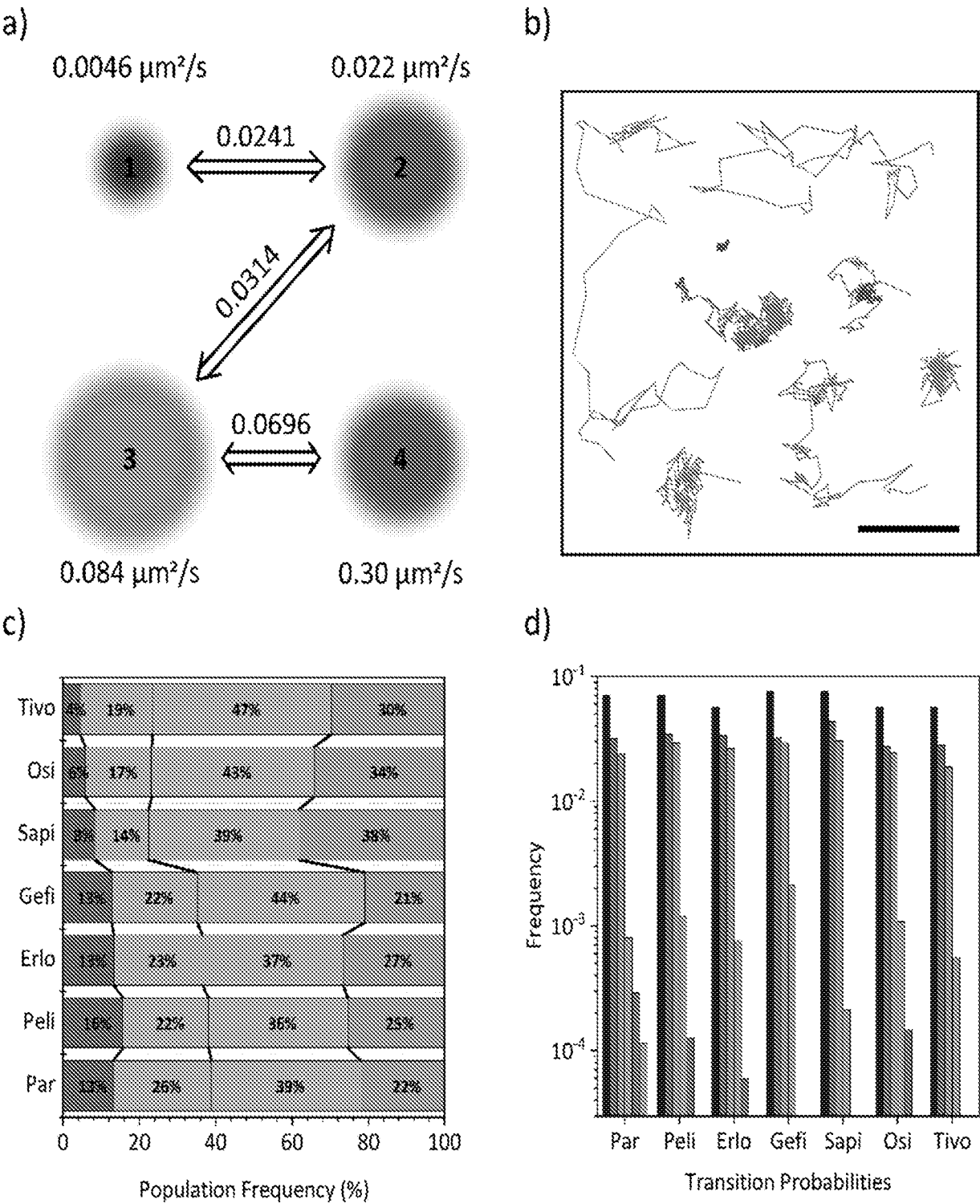


Fig. 5

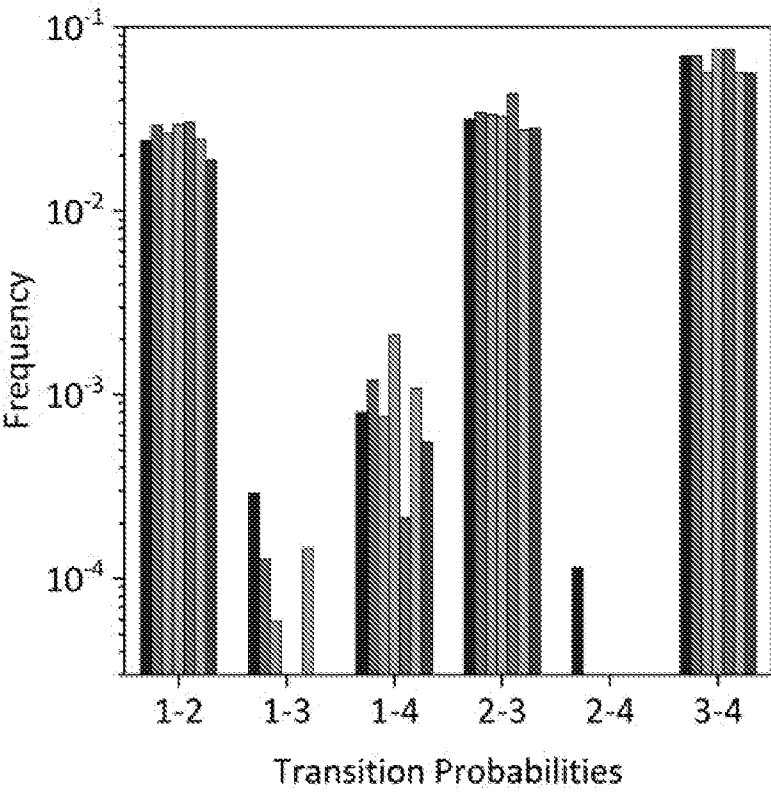
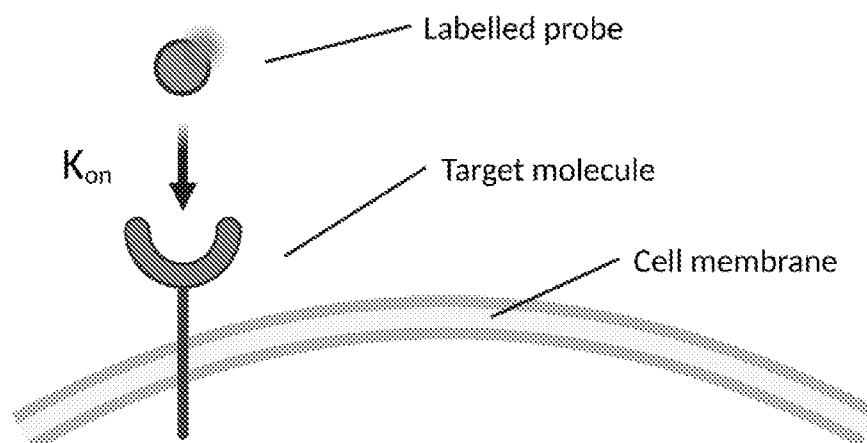
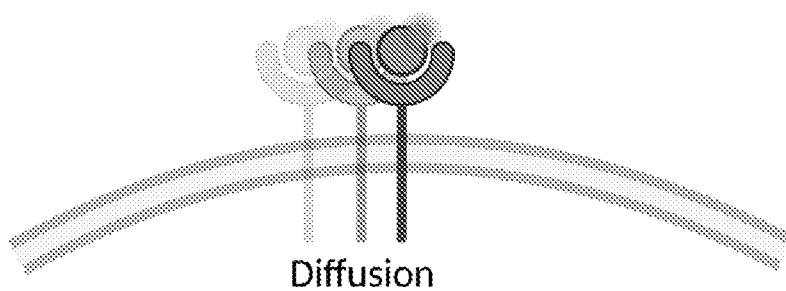


Fig. 6

(A)



(B)



(C)

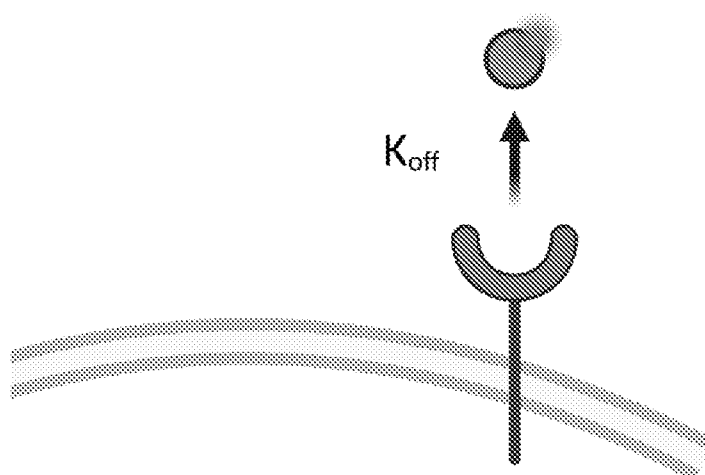


Fig. 7

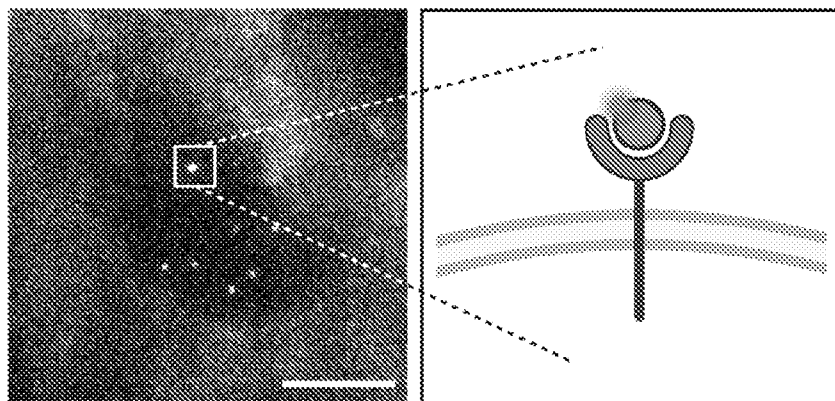


Fig. 8

(A)

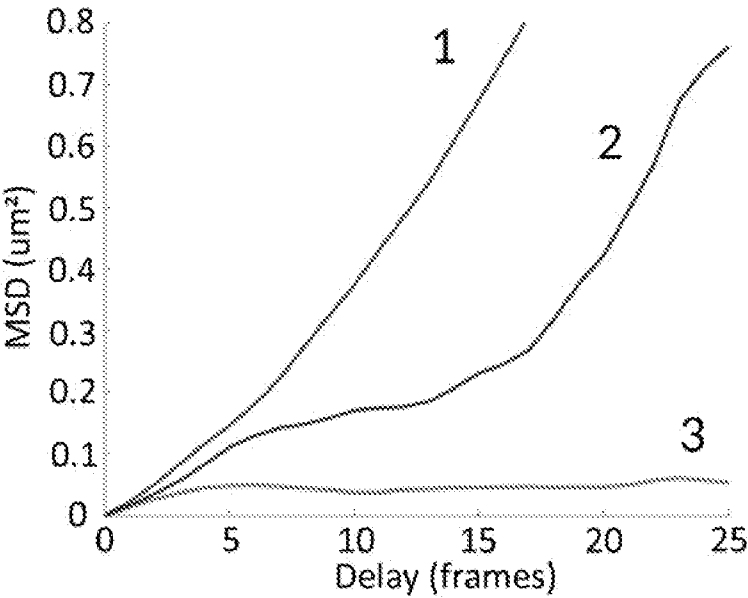
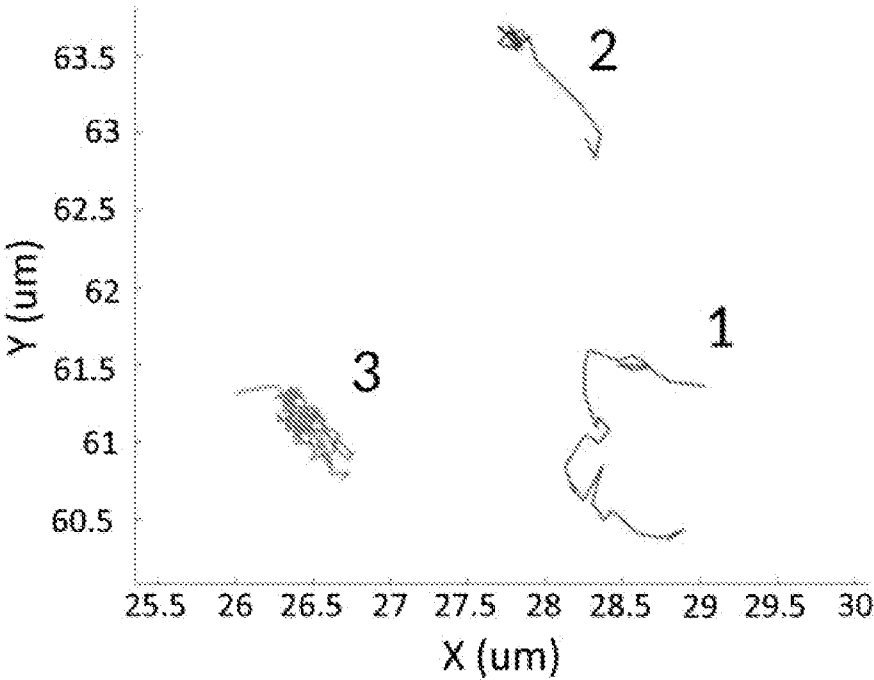
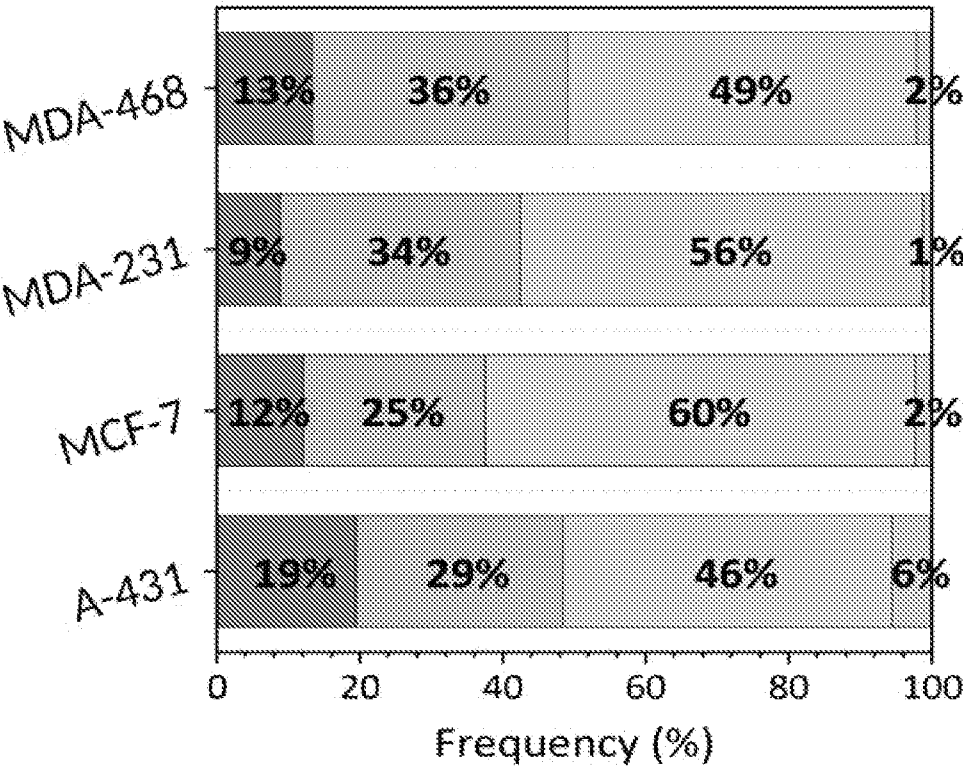


Fig. 8 – cont.

(B)

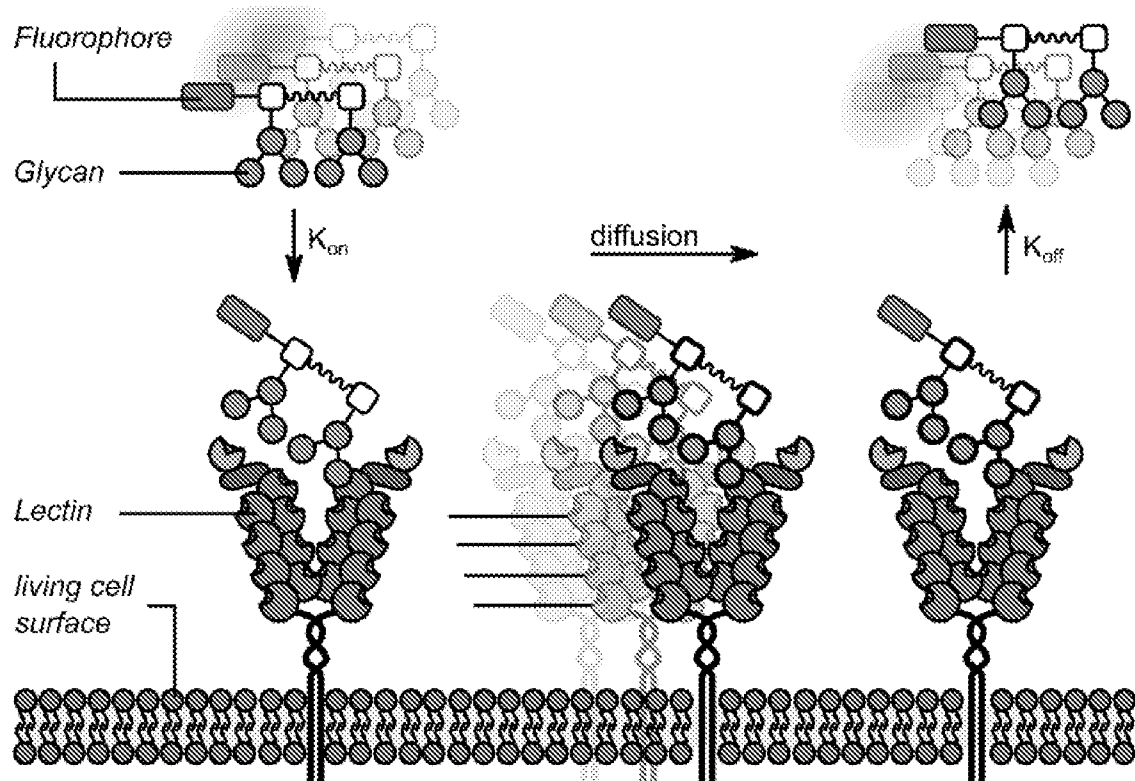


(C)

Gradient Boosting Machine					
Predicted Class	A-431	MDA-468	MDA-231	MCF-7	
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	MDA-468		15		
	MDA-231		1	10	
	MCF-7			1	12
		True Class			
		A-431	MDA-468	MDA-231	MCF-7

Fig. 9

(A)



(B)

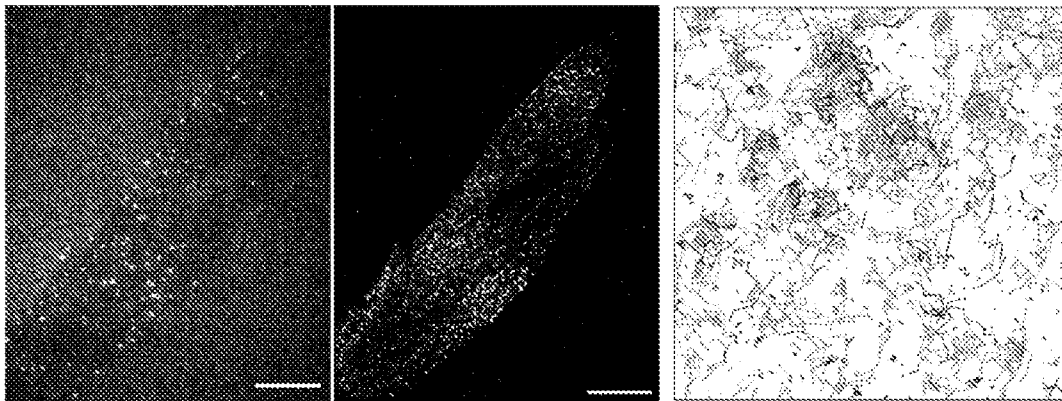
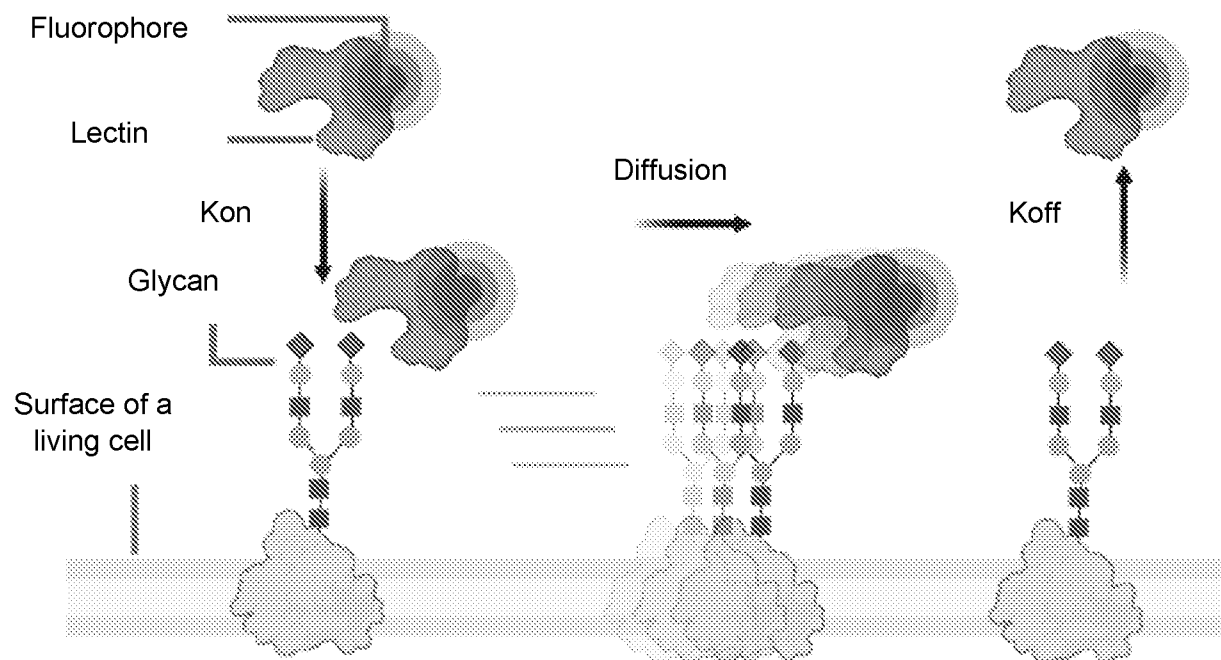


Fig. 10

(A)



(B)

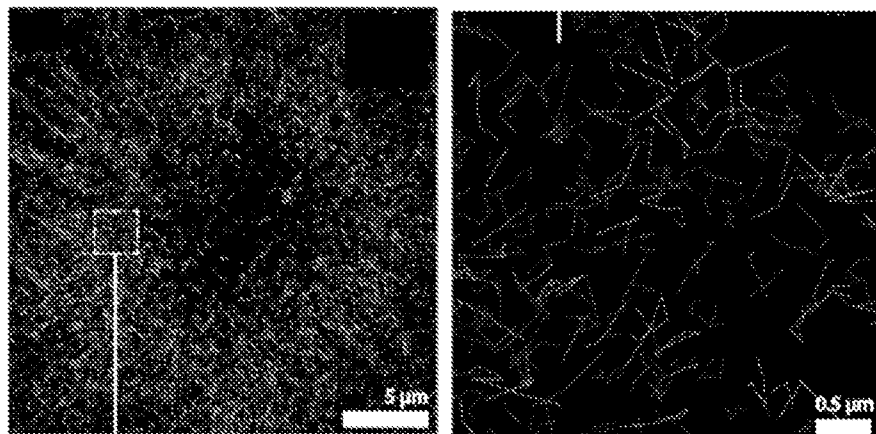
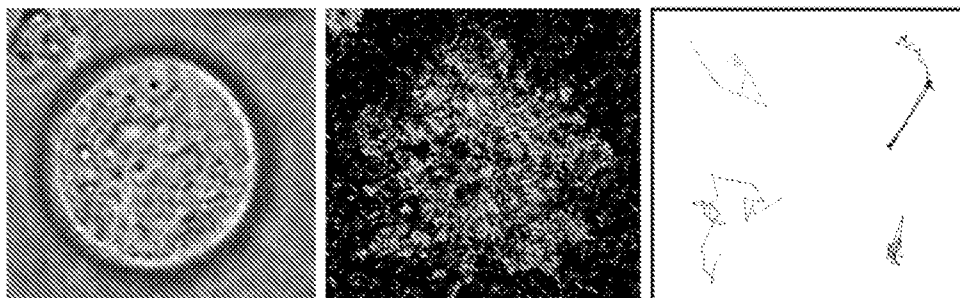


Fig. 11



INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2024/050067

A. CLASSIFICATION OF SUBJECT MATTER INV. G01N33/574 G01N21/64 G02B21/16 G02B27/58 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) G01N G02B		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 3 789 769 A1 (RES & INNOVATION UK [GB]) 10 March 2021 (2021-03-10) paragraphs [0009] - [0016], [0099] - [0104] -----	1-17
X	CN 106 661 603 B (KOREA ADVANCED INST SCI & TECH) 18 January 2022 (2022-01-18) paragraphs [0008] - [0011], [0041] ----- -/--	1-17
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 4 April 2024		Date of mailing of the international search report 18/04/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Lunter, Pim

INTERNATIONAL SEARCH REPORT

International application No

PCT/NL2024/050067

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	US 2019/271592 A1 (GRAY JOE [US] ET AL) 5 September 2019 (2019-09-05) paragraphs [0007], [0030], [0037]; figures 5b, 10b -----	18
X	PASCALE WINCKLER ET AL: "Identification and super-resolution imaging of ligand-activated receptor dimers in live cells", SCIENTIFIC REPORTS, vol. 3, 8 August 2013 (2013-08-08), XP055154089, DOI: 10.1038/srep02387 pages 2-3 abstract -----	18
X	US 11 002 728 B2 (RIKEN [JP]) 11 May 2021 (2021-05-11) columns 1-2 -----	18
X	RIERA ROGER ET AL: "Single-molecule imaging of glycan-lectin interactions on cells with Glyco-PAINT", NATURE CHEMICAL BIOLOGY, NATURE PUBLISHING GROUP US, NEW YORK, vol. 17, no. 12, 11 November 2021 (2021-11-11), pages 1281-1288, XP037624181, ISSN: 1552-4450, DOI: 10.1038/S41589-021-00896-2 [retrieved on 2021-11-11] page 1283 - page 1284 -----	18
X	ASHER WESLEY B ET AL: "Single-molecule FRET imaging of GPCR dimers in living cells", NATURE METHODS, vol. 18, no. 4, 8 March 2021 (2021-03-08), pages 397-405, XP037417646, ISSN: 1548-7091, DOI: 10.1038/S41592-021-01081-Y page 400 abstract -----	18

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Information on patent family members

International application No

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