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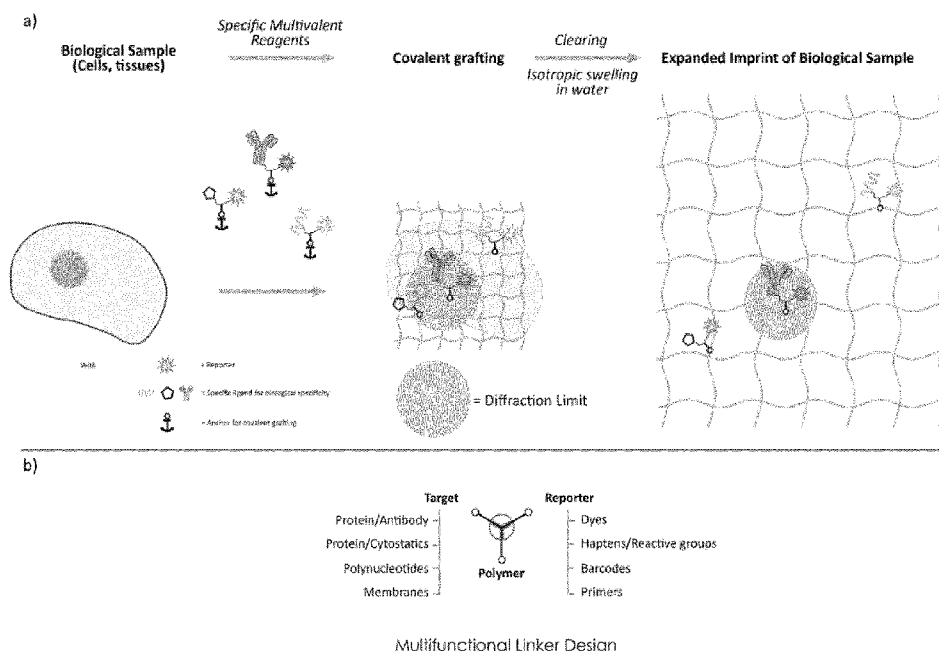


Figure 1

(57) Abstract: The invention relates to methods for identifying and locating a biomolecule in a sample, using a multifunctional reagent with moieties, R₁, R₂, R₃ and Y wherein R₁ is a specific ligand for a biological structure, R₂ is a reactive group capable of binding covalently to a polymeric matrix, R₃ is a reporter moiety, and Y is a moiety covalently connecting R₁, R₂ and R₃.

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MULTIFUNCTIONAL COMPOUNDS FOR POLYMER MATRIX GRAFTING

Technical field of the invention

The invention relates generally to the field of detection and quantification of analytes
5 (e.g. targets).

Background of the invention

Microscopy is a cornerstone of current research. Especially in scientific branches
investigating biological phenomena, this technique has helped unravel key questions
10 in the quest for understanding of disease and life in all its aspects.

Here, resolution of the images obtained is key. While specialized techniques, such as
Cryo-Electron-Microscopy, can provide access to high resolution images of biological
structures, their technical requirements prohibit large scale adoption. Hence, optical
microscopy techniques are currently most used for uncovering this information.

15 However, (near)-visible light is bound by the physical limits of their imaging
approach. The diffraction of light is a restraint to the theoretical resolution obtained.
To circumvent these limitations, optical microscopy has recently seen several clever
approaches to make singular signals stand out from signals within their vicinity, such
as Photoactivated localization microscopy (PALM), stochastic optical reconstruction
20 microscopy (STORM) or Super-resolution optical fluctuation imaging (SOFI). All these
approaches use specialized materials and/or optical techniques to pass beyond the
diffraction limit imposed by physics.

In a recent disclosure [Chen et al. (2015) *Science* **347**, 543–548]. Expansion
microscopy (ExM) enables imaging of thick preserved specimens with <70 nm lateral
25 resolution. In ExM the optical diffraction limit is circumvented by physically expanding
a biological specimen before imaging, thus bringing sub-diffraction limited structures
into the size range viewable by a conventional diffraction-limited microscope. ExM
can image biological specimens at the voxel rates of a diffraction limited microscope,
but with the voxel sizes of a super-resolution microscope. Expanded samples are
30 transparent, and index-matched to water, as the expanded material is >99% water.
The original ExM protocol worked by labelling biomolecules of interest with a gel-
anchorable fluorophore. Then, a swellable poly electrolyte gel was synthesized in the
sample, so that it incorporated the labels. Finally, the sample was treated with a
nonspecific protease to homogenize its mechanical properties, followed by dialysis in
35 water to mediate uniform physical expansion of the polymer-specimen composite. All
of the chemicals required for ExM can be purchased except for the gel-anchorable

label, which requires custom synthesis and raises the barrier for adopting the method.

The combination of ExM with more established super resolution techniques has also been explored, offering an additional 4–5-fold improvement in resolution with these modalities. For example, in combination with stimulated emission depletion microscopy (STED) or structured illumination microscopy (SIM), lateral imaging resolution of <10 and 30nm, respectively, can be achieved. To this end, novel polymer formulations and 'iterative expansion microscopy' allow similar image resolution with standard optical microscopies.

However, not all standard staining approaches used for super-resolution imaging are compatible with ExM. One example of such a labelling strategy is the widespread use of small molecule stains in cellular imaging, such as the actin-binding peptide phalloidin, as small labelling molecules for targeting the cytoskeleton. Such small labels cannot be linked to the gel matrix and are washed out of the sample during the expansion process. [Park et al. (2019) *Nat. Biotechnol.* **37**, 73–83]. The combination of ExM with more established super resolution techniques has also been explored, offering an additional 4–5-fold improvement in resolution with these modalities. For example, in combination with stimulated emission depletion microscopy (STED) or structured illumination microscopy (SIM), lateral imaging resolution of <10 and 30nm, respectively, can be achieved. To this end, novel polymer formulations and 'iterative expansion microscopy' allow similar image resolution with standard optical microscopies [Gao, et al. (2018) *ACS Nano* **12**, 4178–4185; Halpern et al. (2017) *ACS Nano* **11**, 12677–12686].

Furthermore, labelling density in ExM remains a challenge due to fluorescence signal loss during both the polymerization and digestion steps. Many fluorophores are prone to degradation during the radical polymerization process, with some being entirely destroyed (e.g., cyanine dyes).

Another current drawback of the ExM protocol is that genetically encoded fluorophores cannot be imaged without antibody labelling. Additionally, ExM was unable to retain native proteins in the gel and used custom made reagents not widely available.

Furthermore, ExM labels are preserved to a differing degree through the sample preparation, thus biasing the final result, as for example native proteins are not retained in the gel or small oligonucleotides are not covalently bound to the matrix. Furthermore, important features of the cell currently cannot be addressed, as they

cannot react with gel-anchorable fluorophores. Hence, a major drawback of ExM lies in the labelling protocols.

Thus, it would be desirable to devise new methods for in situ retention and imaging multiple biomolecules within a sample.

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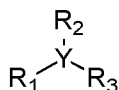
Summary of the invention

The invention provides compounds and methods for the covalent grafting of reporter molecules to a polymeric gel, using a cross-linking molecule that combines moieties for specific recognition of the targeted biomolecules, a monomer and a permanent
10 reporter. This strategy can be used to perform nanoscale imaging of immunostained cells and tissues, high resolution polynucleotide hybridization assays and provide insight on overall biological structure.

Disclosed herein are a new generation of specific reagents and methods for their use. These reagents are able to recognize specific elements within a biological structure,
15 and provide more favourable information on the location and identity of said biological entity in high resolution microscopy than current labelling techniques. Among these favourable properties, are permanent grafting of the reporter, improved sensitivity, multiplexing capacity and minimum number of intermediate steps.

20 The present invention provides, inter alia, methods and compositions for detecting, imaging and/or quantitating targets (e.g., biomolecules) of interest. Some of the methods provided herein involve (1) contacting a sample to be analysed (e.g., a sample suspected of containing one or more targets of interest) with moieties that bind specifically to the targets (each moiety being a binding partner of a given
25 target), wherein each moiety is conjugated to a reactive capable of binding covalently to a polymeric matrix while at the same time being conjugated to a reporter moiety. (2) homogenizing and clearing the sample (3) optionally swelling the polymerix sample (4) optionally contacting the sample with additional labelled reporters (e.g., labelled antibodies, antibody fragments or fluorescently labelled nucleic acids having
30 a nucleotide sequence that is complementary to and thus specific for one polymer bound target (5) imaging the sample in whole or in part to detect the location and number of bound imager strands, (6) and optionally repeating steps (4)-(5) each time with additional labelled reporters having a unique polymer bound target.

One particular aspect of the present disclosure relates to a compound represented
35 by formula (I):



wherein

R1 a specific ligand for biological structures.

R2 is a reactive group capable of binding covalently to the polymeric matrix.

5 R3 is a reporter moiety.

Y is a covalent linker attaching R1, R2 and R3.

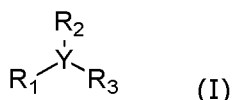
One particular aspect of the present disclosure relates to compounds where one or more of specific ligands for biological structures are covalently linked to one or more reactive groups capable of binding covalently to a polymeric matrix and
10 further comprising a labelling moiety.

In another aspect, R1, R2 and R3 are sequentially linked.

The invention is further summarised in the following statements:

1. A method for identifying and locating a biomolecule in a sample, the method
15 comprising the steps of:

a) conjugating the biomolecules within the sample with a multifunctional reagent according to the formula (I)



wherein R₁ is a specific ligand for a biological structure, R₂ is a reactive group capable of binding covalently to a polymeric matrix, R₃ is a reporter moiety, Y is a
20 moiety covalently connecting R₁, R₂ and R₃,

b) embedding the sample in a polymeric material wherein the reporter moiety R₃ reacts with the polymeric material thereby becoming anchored to the polymeric material,

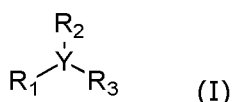
25 c) subjecting the sample to digestion or clearing,

d) optionally swelling the material to form an expanded sample,

e) optionally adding one or more additional stains, and

f) imaging the sample.

2. The method according to statement 1 wherein R_1 is a polypeptide, a polynucleotide or a small-molecule ligand.
3. The method according to statement 1 or 2, wherein the biomolecule is selected from the group consisting of DNA, RNA, proteins and lipids.
- 5 4. The method according to any one of statements 1 to 3, wherein the polymeric material is a swellable material.
5. The method according to any one of statements 1 to 4, wherein step b) of embedding comprises the step of permeating the biological sample with a composition comprising precursors of a swellable polymer and forming a swellable
10 polymer in situ.
6. The method according to an one of statements 1 to 5, wherein multiple biomolecules are targeted in the same sample.
7. The method according to any one of statements 1 to 6, wherein steps (e) to (f) are repeated one or more times .
- 15 8. The method according to any one of statements 1 to 7, wherein R_3 is a dye, a hapten or an oligonucleotide.
9. The method according to any one of statements 1 to 8, where the additional stain in e) is an antibody or antibody fragment specifically binding R_3 .
10. The method according to any one of statements 1 to 8, where the additional
20 stain in step e) is a dye labeled oligonucleotide specifically binding R_3 .
11. The method according to any one of statements 1 to 10, wherein the sample in step d) is expanded isotropically by adding water to swell the swellable material.
12. The method according to statement 11, wherein the volume of the sample is expanded by a factor of at least 3.
- 25 13. The method according to any one of statements 1 to 12, wherein in step c) the sample is subjected to enzymatic proteolysis.
14. The method according to any one of statements 1 to 14, wherein the imaging is performed by expansion microscopy.
15. The method according to statement 14, wherein the imaging is performed on
30 an expanded sample.
16. The method according to statement any one of statements 1 to 15, wherein different multifunctional reagents are used for the detection of different biomolecules.
17. The method according to statement 16, wherein the two of different molecules are at a distance of less than 500, 200, 100, or 50 nm in the biological sample.
- 35 18. A multifunctional reagent according to the formula (I)



wherein

R₁ is a specific ligand for a biological structure,

R₂ is a reactive group capable of binding covalently to a polymeric matrix,

5 R₃ is a reporter moiety,

Y is a moiety covalently connecting R₁, R₂ and R₃ connecting R₁, R₂ and R₃,

19. Use of a multifunctional reagent as defined in statement 18, for identifying and locating a biomolecule in a sample.

20. The use according to statement 19 in expansion microscopy.

10

Brief description of the drawings

Figure 1 is a drawing that outlines of certain embodiments of the invention, where following biomolecule target binding, the multifunctional reagent is bound to a polymeric matrix and subjected to expansion and imaging for high resolution
15 multiplexed analysis

Figure 2 is an example of biomolecule position grafting with specific antibody recognition, followed by expansion and imaging. Pre-expansion (Panel B, D) and Post-expansion (Panel A, C) stained HeLa cells (alpha-tubulin)

Figure 3 is an example of biomolecule position grafting with specific small molecule recognition (phalloidin), followed by expansion and imaging. Pre-ExM (Panel A, B) and Post-ExM (Panel C) Phalloidin stained HeLa cells (F-actin).
20

Figure 4 is an example of biomolecule position grafting with oligo-dye reporting after expansion, thus allowing the use of previously unattainable dyes.

Figure 5 is an example of biomolecule position grafting with a lipid target, before and
25 after expansion, highlighting previously unattainable biomolecules in Expansion Microscopy.

Detailed description of the invention

30 In one embodiment, the invention provides a method for converting a sample of interest comprising the steps conjugating biomolecules within the sample with multifunctional crosslinkers; embedding the sample in a swellable material wherein the crosslinkers within the sample are anchored to the swellable material; subjecting

the sample to digestion; swelling the swellable material to form an expanded sample; and imaging the sample of interest.

The invention provides a variant of ExM, in which biomolecule identity and position are translated into a permanent label, which is anchored to the swellable gel, using
5 a specific multivalent cross-linking molecule. As such, the signals are preserved even when the original biomolecule is lost or displaced, by for example nonspecific proteolytic digestion used in current ExM protocols. This is an extension of standard histological methods used to prepare samples for imaging. This strategy can be used to perform nanoscale imaging of immunostained cells and tissues as as support post-
10 expansion read-out schemes and multiplexed analysis.

In a particular embodiment, the invention provides a method for the retention and imaging of the structure of a biological sample of interest comprising the steps of:

- 15 (a) Complexing biomolecules within the sample with one or more multifunctional crosslinker;
- (b) embedding the sample in a polymeric material wherein the crosslinkers are anchored to the polymeric material;
- (c) subjecting the sample to digestion;
- (d) optionally swelling the polymeric material to form an expanded sample; and
- 20 (e) imaging the sample of interest.

In one embodiment, the multifunctional reagent is a hetero-multifunctional reagent. Hetero-multifunctional reagents possess different reactive groups at spaced locations within the compound, thus separating the reactive groups. These reagents not only
25 allow for single-step conjugation of molecules that have the respective target functional group, but they also allow for sequential conjugations that minimize undesirable side-reactions.

In one embodiment, the multifunctional reagent comprises selective groups for
30 biomolecules (R1 in formula I). The use of such a multifunctional reagent allows for the location and/or identity of the protein of choice to be grafted to the swellable material. The biomolecules targeted can be selected independently from oligonucleotides (e.g. DNA, RNA), proteins, lipids or cellular structures or a combination of two or more of the listed items. The selective groups are preferentially
35 selected from oligonucleotides, polynucleotides, oligopeptides, polypeptides (including antibodies and antibody fragments) and small-molecule ligands or a

combination of two or more of the listed items. A person skilled in the art will appreciate that virtually any specific biological molecule or structure can be addressed in this manner. Advantageously, through the compounds and methods presented herein, biomolecular structures that were previously unable to address in
5 Expansion Microscopy can now be analysed.

In one embodiment, the multifunctional crosslinker or multifunctional reagent comprises a protein-reactive chemical moiety and a gel-reactive chemical moiety (i.e., a monomeric chemical moiety) and a signal moiety. The protein-reactive
10 chemical group includes, but is not limited to, N- hydroxysuccinimide (NHS) ester, thiol, amine, maleimide, imidoester, pyridyldithiol, hydrazide, phthalimide, diazirine, aryl azide, isocyanate, or carboxylic acid, which, for example, can be reacted with amino or carboxylic acid groups on proteins or peptides. In one embodiment, the protein-reactive groups include, but are not limited to, N-succinimidyl ester,
15 pentafluorophenyl ester, carboxylic acid, or thiol. The monomers include, but are not limited to, vinyl or vinyl monomers such as styrene and its derivatives (e.g., divinyl benzene), acrylamide and its derivatives, butadiene, acrylonitrile, vinyl acetate, maleimides, aldehydes or acrylates and acrylic acid derivatives.

20 In another embodiment, the multifunctional crosslinker or multifunctional reagent comprises a nucleotide-reactive chemical moiety and a gel-reactive chemical moiety (i.e., a monomeric chemical moiety) and a signal moiety. The nucleotide-reactive chemical group includes, but is not limited to, N- hydroxysuccinimide (NHS) ester, thiol, amine, maleimide, imidoester, pyridyldithiol, hydrazide, phthalimide, diazirine,
25 aryl azide, platinum complex, psoralen derivative, isocyanate, or carboxylic acid, which, for example, can be reacted with the nucleobases, the sugar moieties or the phosphate backbone of the polynucleotide, or alternatively with reactive groups introduced on the polynucleotide. The gel-reactive groups include, but are not limited to, vinyl or vinyl monomers such as styrene and its derivatives (e.g., divinyl
30 benzene), acrylamide and its derivatives, butadiene, acrylonitrile, vinyl acetate, maleimides, aldehydes or acrylates and acrylic acid derivatives.

In a further embodiment, the invention provides a method that combines the convenience of direct protein anchoring with strong enzymatic digestion, for example
35 through proteinase K.

Having observed how following gelation and digestion specimens labelled with secondary antibodies bearing a variety of small-molecule fluorophores lose 50% or more of their initial brightness, some of the multifunctional reagents have been shown to retain close to a 100% of their initial brightness.

5

In contrast to the previously described ExM methods, the present invention also allows signal staining to be carried out in the expanded state, with the position and identity of the biomolecules transferred to the quasi-in vitro environment of the expanded gel. By converting the biological environment into a swollen matrix where
10 the biomolecules are no longer imperative to obtaining signal, it is believed that this simplified chemical environment alleviates many issues that place limitations on biological staining methods including steric hindrance and diffusional access, and potentially also autofluorescence and non-specific binding. Thus, rapid staining of thick tissue specimens, higher staining intensity, and potentially better staining of
15 challenging targets is provided with less optimization than is required with current staining methods, and this readily combined with multiplexed and repeated read-out. The present invention also enables the use of probes that would not be compatible with the native tissue environment or the sample preparation conditions, among other potential applications. (See Example 3)

20 In a further embodiment, the invention provides a method that is amenable to the tissue disruption that is designed to allow uniform expansion of the tissue-gel composite while minimally disturbing the tissue at the molecular level, in essence fragmenting and expanding the tissue rather than strongly dissolving it. In one embodiment, the invention provides the use of detergents and high temperature
25 without enzymes, enzymes that cleave biomolecules other than proteins, enzymes that cleave proteins with greater specificity or lesser extent than proteinase K, non-aqueous solvents used in lipid extraction, and controlled chemical cleavage of proteins and other biomolecules including nucleotides, polysaccharides, and lipids, separately and in combination. This also includes strong enzymatic digestion in the
30 case where the proteins under study are robust against this treatment.

In one embodiment the reporter can be, for example, a fluorophore, a quantum dot, a dendrimer, a nanowire, a bead, a hapten, a streptavidin, an avidin, a neutravidin,
35 a biotin, a reactive group, a peptide, a protein, a magnetic bead, a radiolabel, a non-optical label, or a combination of two or more of the listed items.

In yet another embodiment, the reporter moiety comprises multiple distinct signals.

In one embodiment, the reporter moiety is fluorescent. In accordance with the present invention, Alexa, BODIPY, ATTO, coumarin, Cascade blue, dansyl, dapoxyl, fluorescein, mansyl, MANT, Oregon green, pyrene, rhodamine, Texas red, TNS, fluorescent nanocrystals (quantum dots), a cyanine fluorophore and derivatives thereof are particularly preferred labels.

In one embodiment, the reporter is an oligonucleotide. Through specific hybridization with complementary oligonucleotides, this reporter can serve as a barcode for the biomolecule originally present at said location. The complementary oligonucleotide may be fluorescently labelled (i.e., they are conjugated to a fluorophore). Fluorophores conjugated to imager strands of different nucleotide sequence may be identical to each other, or they may have an emission profile that overlaps or that doesn't overlap with that of other fluorophores. The fluorescently labelled imager strand may comprise at least one fluorophore. Both the reporter oligonucleotides and the signalling oligonucleotide can comprise a hairpin secondary structure. The term "oligonucleotide", as used throughout the invention, refers to a short nucleic acid molecule from about 8 to about 50, eventually to about 90 nucleotides in length, whether natural or synthetic, capable of acting as a point of initiation of complementary nucleic acid hybridization. By itself, the oligonucleotide can also be labelled, if desired, by incorporating a compound detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include, but are not limited to, fluorescent dyes, electron-dense reagents, biotin, or small peptides for which antisera or monoclonal antibodies are available. Some of the preferred labels include fluorochromes, e.g. xanthene dyes, cyanine dyes, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-4I,5'-dichloro-6-carboxyfluorescein (JOE), 5-carboxyfluorescein (5-FAM) or N.N.N'.N'-tetramethyl- θ -carboxyrhodamine (TAMRA), radioactive labels, e.g. ^{32}P , ^{35}S , ^3H ; etc. In one embodiment the reporter can be, for example, a fluorophore, a quantum dot, a dendrimer, a nanowire, a bead, a hapten, a streptavidin, an avidin, a neutravidin, a biotin, a reactive group, a peptide, a protein, a magnetic bead, a radiolabel, a non-optical label, or a combination of two or more of the listed items.

In yet another embodiment, the reporter moiety comprises multiple distinct signals

In one embodiment, the complementary oligonucleotide hybridization is transient in nature, with binding times in a sufficient time-scale to provide a 'blinking' event.

In another embodiment, the reporter moiety is a hapten for antibody or antibody
5 fragment recognition. Some well-known examples of such haptens are biotin, digoxigenin, fluorescein or dinitrophenol.

All moieties of the multifunction reagent are connected, preferentially through
10 linkers. Such linkers can be branched, or linear. In addition, these linkers can impart beneficial properties to the compounds herein described, improving properties such as but not limited to improved water solubility, reduced aggregation or improved stability. Particular examples are linkers comprising ether functionalities or oligomers thereof, or spacers comprising charges.

15

In one embodiment, the reactive group capable of binding to the polymeric matrix can comprise one or more substituted or unsubstituted methacrylates, acrylates, acrylamides, methacrylamides, vinylalcohols, vinylamines, allylamines, allylalcohols, including divinyllic crosslinkers thereof (e.g., N, N-alkylene bisacrylamides) or a
20 combination of two or more of the listed items.

In yet another embodiment, the reporter moiety comprises multiple distinct signals.

In another embodiment, multiple moieties are included, independently selected from
25 the reporting moiety, specific ligand for biological structures and reactive group capable of binding to the polymeric matrix. It is obvious to a person skilled in the art that judicious selection of such combinations can provide specific benefits, with such examples as, but not limited to, multichannel labelling, multicolour labelling and improved matrix binding by optimized polymerization

30

In another embodiment, the sample is simultaneously infused with multiple multifunctional reagents, where each carries a distinct reporter.

The invention is of special use for detecting, image and/or quantitate multiple targets
35 in a sample, regardless of their location in the sample, including regardless of whether their location in the sample is so close together to be indistinguishable if

signal from the two or more targets was observed simultaneously. Thus, the distance between two or more targets may be below the resolution distance of the imaging system used to detect the targets, and still using the methods provided herein it would be possible to distinguish the two or more targets from each other, thereby
5 facilitating a more accurate and robust detection and quantitation of such targets. In some instances, the resolution distance may be about 50 nm, as an example.

In one embodiment, the invention provides a method for staining and other biochemical characterization of tissue in the expanded state.

10 As used herein, the term "sample of interest" generally refers to, but is not limited to, a biological, chemical or biochemical sample. In one embodiment, the sample of interest is a biological. A biological sample includes, but is not limited to: a tissue, a cell or any components thereof, a tumour, or all or a part of any organ including, but not limited to brain, heart, lung, liver, kidney, stomach, colon, bones, muscle, skin,
15 glands, lymph nodes, genitals, breasts, pancreas, prostate, bladder, thyroid, and eyes.

In an embodiment, the sample of interest can be labelled or tagged preferably with a detectable label. Typically, the label or tag will bind chemically (e.g., covalently, hydrogen bonding or ionic bonding) to the sample, or a component thereof, for
20 example, one or more proteins. The detectable label can be selective for a specific target (e.g., a biomarker or class of molecule), as can be accomplished with an antibody or other target specific binder. The detectable label preferably comprises a visible component, as is typical of a dye or fluorescent molecule; however, any signalling means used by the label is also contemplated. Contacting the sample of
25 interest with a detectable label results in a "labelled sample of interest."

A fluorescently labelled sample of interest, for example, is a sample of interest labelled through techniques such as, but not limited to, immunofluorescence, immunohistochemical or immunocytochemical staining to assist in microscopic analysis. Thus, the detectable label is preferably chemically attached to the sample
30 of interest, or a targeted component thereof. In one embodiment, the detectable label is an antibody and/or fluorescent dye. The antibody and/or fluorescent dye, further comprises a physical, biological, or chemical anchor or moiety that attaches or crosslinks the sample to the swellable material, such as a hydrogel.

The labelled sample may furthermore include more than one label. For example, each
35 label can have a particular or distinguishable fluorescent property, e.g., distinguishable excitation and emission wavelengths. Further, each label can have a

different target specific binder that is selective for a specific and distinguishable target in, or component of, the sample.

As used herein, the term "gel" or "swellable material" are used interchangeably to generally refer to a material that expands when contacted with a liquid, such as water or other solvent. In one embodiment, the swellable material uniformly expands in three dimensions. Additionally or alternatively, the material is transparent such that, upon expansion, light can pass through the sample. In one embodiment, the swellable material is a swellable polymer or hydrogel. In one embodiment, the swellable material is formed in situ from precursors thereof. For example, one or more polymerizable materials, monomers or oligomers can be used, such as monomers selected from the group consisting of water soluble groups containing a polymerizable ethylenically unsaturated group. Monomers or oligomers can comprise one or more substituted or unsubstituted methacrylates, acrylates, acrylamides, methacrylamides, vinylalcohols, vinylamines, allylamines, allylalcohols, including divinyllic crosslinkers thereof (e.g., N, N-alkylene bisacrylamides). Precursors can also comprise polymerization initiators, accelerators, inhibitors, buffers, salts, and crosslinkers.

In an embodiment, the polymer is polyacrylate and copolymers or crosslinked copolymers thereof. Alternatively or additionally, the material can be formed in situ by chemically crosslinking water soluble oligomers or polymers.

As used herein, the terms "gelation" or "embedding" the sample in a swellable material are used interchangeably to refer to permeating (such as, perfusing, infusing, soaking, adding or other intermixing) the sample with the swellable material, preferably by adding precursors thereof. Alternatively or additionally, embedding the sample in a swellable material comprises permeating one or more monomers or other precursors throughout the sample and polymerizing and/or crosslinking the monomers or precursors to form the swellable material or polymer in situ. In this manner the sample of interest is embedded in the swellable material. In one embodiment, a sample of interest, or a labelled sample, is permeated with a composition comprising water soluble precursors of a water swellable material and reacting the precursors to form the water swellable material in situ.

In certain embodiments, the sample of interest, or a labelled sample, can, optionally, be treated with a detergent prior to being contacted with the one or more swellable material precursors. The use of a detergent can improve the wettability of the sample or disrupt the sample to allow the one or more swellable monomer precursors to permeate throughout sample.

In one embodiment, the sample of interest is permeated with one or more monomers or a solution comprising one or more monomers or precursors which are then reacted to form a swellable or non-swallowable polymerized gel depending on what step of the method is being performed. For example, if the sample of interest is to be embedded
5 in sodium polyacrylate, a solution comprising the monomers sodium acrylate and acrylamide, and a crosslinker selected from N,N-methylenebisacrylamide (BIS), N,N'-(1,2-Dihydroxyethylene)bisacrylamide), and (DHEBA) N,N'-Bis(acryloyl)cystamine (BAC), are perfused throughout the sample. Once the sample, or labelled sample, is permeated, the solution is activated to form sodium polyacrylate or copolymer thereof. In one embodiment, the solution comprising the monomers is aqueous.

In one embodiment, one or more biomolecules of the sample (e.g., a labelled sample) are anchored or crosslinked to the swellable material before expansion. This can preferably be accomplished by chemically crosslinking a detectable label with the swellable material, such as during or after the polymerization or in situ formation of
15 the swellable material.

In one embodiment, after the labelled sample has been anchored to the swellable material, the sample is, optionally, subjected to an enzymatic, chemical and/or physical disruption of the endogenous biological molecules (or the physical structure of the sample of interest, where the sample is other than a biological material),
20 leaving the detectable labels such as fluorescent dye molecules or oligonucleotides intact and anchored to the swellable material. In this way, the mechanical properties of the sample material complex are rendered more spatially uniform, allowing isotropic expansion with minimal artefacts. Alternatively or additionally, a clearing of the sample is conducted to remove biomolecules and their residues, which could
25 cause background fluorescence or provide aspecific signal.

As used herein, the terms "digestion" or "disruption of the endogenous physical structure of the sample" or the term "disruption of the endogenous biological molecules" of the sample of interest are used interchangeably and generally refer to the physical, chemical, or enzymatic digestion, disruption or break up of the sample
30 so that it will not resist expansion.

In one embodiment, a protease enzyme is used to digest the sample-swallowable material complex. It is preferable that the disruption does not impact the structure of the swellable material but disrupts the structure of the sample. Thus, the sample disruption should be substantially inert to the swellable material. The degree of
35 digestion can be sufficient to compromise the integrity of the mechanical structure of

the sample or it can be complete to the extent that the sample-swellable material complex is rendered substantially free of the sample.

In one embodiment, the physical disruption of the sample is accomplished by a more mild disruption treatment that minimizes damage to the individual proteins, allowing staining and other treatments on the proteins to be carried out after expansion. In some embodiments, such milder treatment is performed by using LyC. In some
5 embodiments, such milder treatment is performed by autoclaving the sample.

The sample-swellable material complex is then isotropically expanded. In one embodiment, a solvent or liquid is added to the complex which is then absorbed by the swellable material and causes swelling. In one embodiment, the liquid is water.
10 Where the swellable material is water swellable, an aqueous solution can be used.

In one embodiment, the addition of water allows for the embedded sample to expand at least 3 times, preferably 4 times, preferably 5 times, or more its original size in three- dimensions. Thus, the sample can be increased, 10, 20, 40, 60, 80 100-fold
15 or more in volume. This is because the polymer is embedded throughout the sample, therefore, as the polymer swells (grows) it expands the tissue as well. Thus, the tissue sample itself becomes bigger. As the multifunctional reagent is covalently bound to the matrix, as the material swells isotropically, the anchored tags maintain their relative spatial relationship.

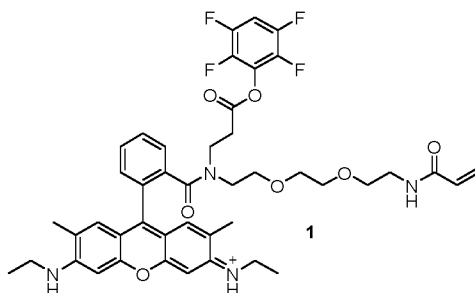
The swollen material with the embedded sample of interest can be imaged on any
20 optical microscope, allowing effective imaging of features below the classical diffraction limit. Since the resultant specimen is preferably transparent, custom microscopes capable of large volume, Widefield of view, 3D scanning may also be used in conjunction with the expanded sample.

In a particular embodiment, the invention provides methods for multiplexed and repeated measurements of the labelled sample. This can be combined with amplification of the detectable label, with for example Hybridization Chain Reactions or Rolling Circle amplification. Depending on the type of amplified signal, this amplification can be performed before or after the swelling. Optionally, clearing of
25 the sample can be conducted between repeated rounds of amplification to increase the number of biological targets that can be addressed.
30

As used herein, the term "ExM workflow" refers to the process of infusing a sample of interest with swellable material, which undergoes in situ polymerization (i.e., gelation), digestion of the sample-polymer composite, and expansion of the sample-
35 polymer composite.

As used herein, the term "labelling workflow" refers to the process of infusing a sample of interest with multifunctional reagents, followed by fixing the reagents to a swellable material at the location of their biological target, followed by gelation, digestion, expansion, and imaging.

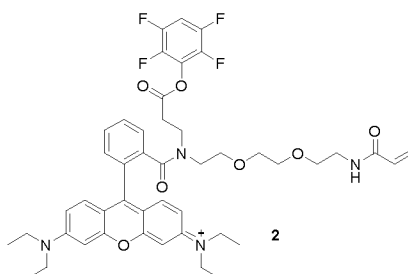
Examples

Example 1: Fluorescent trivalent linker

- 5 To Rhodamine 6G acid (1.407 g; 3.12 mmol) was added 30 mL of acetonitrile, under stirring and inert atmosphere (N₂). To the resulting dark purple solution were added HBTU (1.307 g, 3.45 mmol, ~1.1 equivalents) of HBTU, 2.6 mL (18.7 mmol, ~6 equivalents) of triethylamine, and 1.292 g (3.43 mmol, ~1.1 equivalents) of amine diPEG linker. The reagents were left to react for 90 minutes, with reaction progress
- 10 followed by TLC (Silica, DCM:MeOH (9:1 v/v)). The solvent was evaporated at 50°C under reduced atmosphere, giving a dark purple oil. After chromatographic purification (Silica), 1.995 g (2.46 mmol, 79% yield) of pure compound obtained after silica purification. The compound was dissolved in minimal DCM (approx.. 5.6 ml). The resulting dark-purple solution were put under stirring and inert atmosphere
- 15 (N₂) in an ice bath. Trifluoroacetic acid (1 ml) were added dropwise. The reaction was followed with TLC (Silica, DCM:MeOH (9:1 v/v)). Once all the starting material was converted, all solvents were evaporated under reduced atmosphere giving a dark orange film, that was subsequently purified chromatographically, yielding 1.470 g of the amino acid (2.25 mmol, 91% yield). The compound was dissolved in minimal
- 20 MeOH (4 mL, ~0.6 mL per mmol) giving a dark purple solution. Tetrahydrofuran was added, bringing the total concentration to 0.25 M. Reaction solution was stirred under inert atmosphere (N₂) and sodium carbonate (0.507 g , 4.79 mmol, ~2 equivalents) in distilled water (1.5 mL per Na₂CO₃ mmol) are added. The mixture was cooled to 0°C, followed by dropwise addition of acryloyl chloride (0.179 ml, 2.21 mmol, ~1
- 25 equivalent) in 1,4-dioxane (0.5 mL per acryloyl chloride mmol), The reaction was stirred at room temperature for 20 minutes, and reaction's extent was followed with TLC (Silica, DCM:MeOH (9:1 v/v)). The reaction solution was evaporated under reduced atmosphere at 50°C, and then purified on silica, giving a dark purple thin-crystalline film (1.390 g, 1.97 mmol, yield = 87%), The compound (0.212 g, 0.30
- 30 mmol) was dissolved in acetonitrile (~1 mL per mmol). The solution was stirred under inert atmosphere (N₂) in the dark. N,N'-dicyclohexyl carbodiimide (DCC, 0.062 g

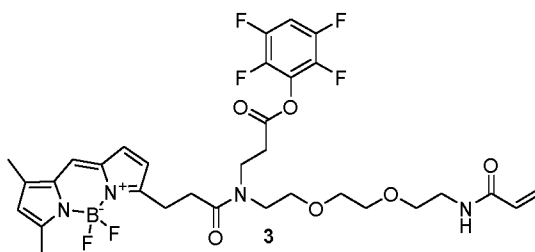
(0.30 mmol, ~1 equivalent)) was added to the reaction together with 2,3,5,6-tetrafluorophenol (0.051 g, 0.30 mmol, ~1 equivalent) was added. Reaction extent was followed with TLC (Silica, DCM:MeOH (9:1 v/v)). After complete reaction, the solution was filtered, concentrated and purified through chromatography yielding the
5 desired compound as a dark-red/purple film (0.147 g, 0.17 mmol, yield = 57%).

Example 2: Fluorescent trivalent linker 2

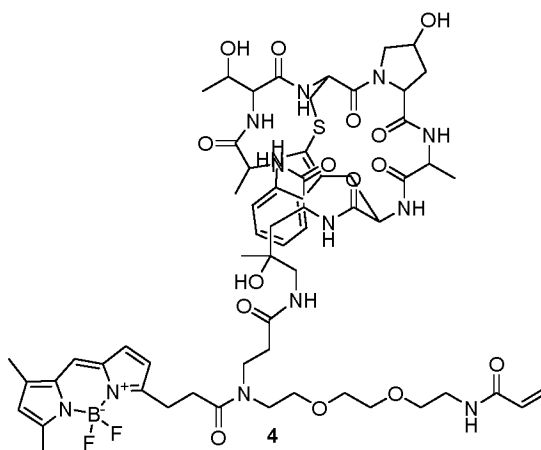


10 Product was obtained according to the general procedure outlined in example one, starting from Rhodamine B.

Example 3: Fluorescent trivalent linker 3



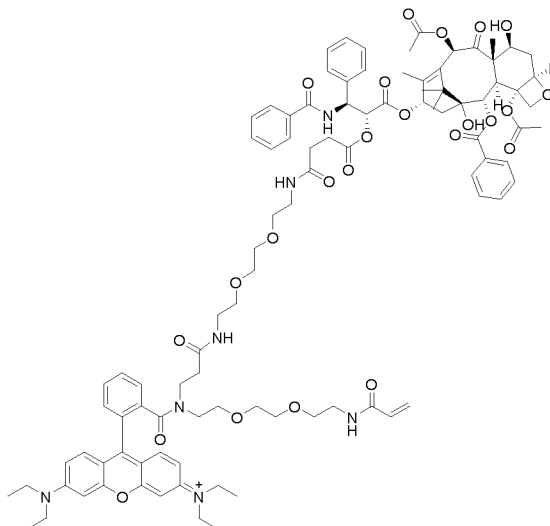
15 Product was obtained according to the general procedure outlined in example one, starting from BODIPY FL.

Example 4: Phalloidin trivalent linker 4

Phalloidin Amine solution (5 μ L from a 10 mM solution) was mixed with one equivalent
 5 of compound **3** (as a 12.46 mM stock solution in DMSO), and stirred in 11.4 μ L of
 Borate buffer (pH = 8). Reaction progress was monitored by LC-MS analysis.
 Following complete coupling, the compound was used for immediate reaction.

Example 5: Paclitaxel trivalent linker 5

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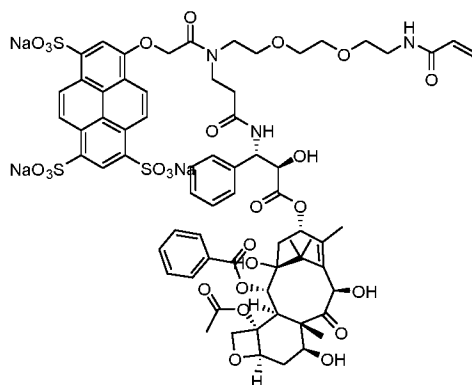
To a solution of Taxol (1 g, 1.18 mmol) in dry pyridine (24 mL) was added succinic
 anhydride (1.8 g, 18.0 mmol). The reaction mixture was stirred at room temperature
 for 3 h. After complete reaction, pyridine was removed under reduced pressure and
 15 the residue was dissolved in DCM (100 mL). The resulting mixture was then washed
 with water (100 mL) two times and brine (100 mL) one time, successively. The
 organic phase was dried over MgSO_4 and evaporated to get the crude product. The

crude product was purified by column chromatography to get the intermediate as a white solid (85%).

To a solution of the intermediate (0.83 g, 0.87 mmol) in DCM (70 mL) was added DCC (0.22 g, 1.07 mmol) and N-Hydroxysuccinimide (0.11 g, 0.96 mmol),
5 successively. The reaction mixture was stirred at room temperature overnight. The reaction was putted into the freezer and the solid was removed by filtration. The filtrate was evaporated under the reduced pressure to get the intermediate without further purification.

To a solution of the intermediate (0.3 g, 0.29 mmol) in DMF (10 mL) was added
10 trimethylamine (0.12 μ L, 0.86 mmol) and 1,2-bis(2-aminoethoxy)ethane (0.51 g, 3.42 mmol). The reaction mixture was stirred at room temperature for 3 h. Ethyl acetate (50 mL) was added the resulting mixture was washed with water (50 mL) two times and brine (50 mL) one time, successively. The organic phase was dried
15 over MgSO_4 , and then the solvent was removed under reduced pressure and the residue was purified by column chromatography to get the intermediate as a yellow solid (33%).

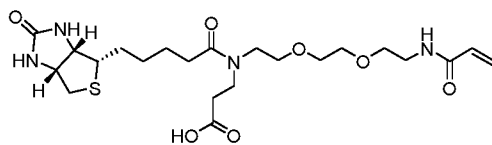
To a solution of the modified paclitaxel (44 mg, 0.041 mmol) in THF (3 mL) was added trimethylamine (6 μ L, 0.043 mmol) and Fluorescent trivalent linker 2 (42 mg, 0.048 mmol) under the protection of N_2 . The reaction mixture was stirred overnight
20 at room temperature. And then the solvent was removed under reduced pressure and the residue was purified by column chromatography to get the product (red solid, 45%).

Example 6: Docetaxel trivalent linker 6

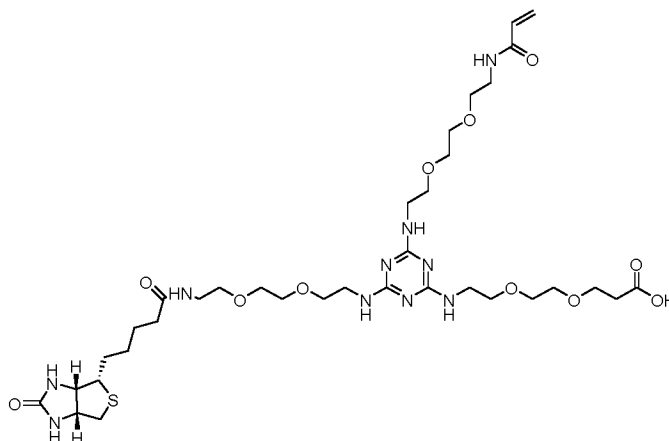
Docetaxel (50 mg, 0.062 mmol) and formic acid (0.25 mL) were added to the flask
 5 (5 mL), and the reaction mixture was stirred at room temperature for 3 h. The solvent
 was removed under reduced pressure to get the product without further purification
 (white solid, 100%).

Product was obtained according to the general procedure outlined in example one
 and five, starting from Alexa 405.

10

Example 7: Biotin trivalent linker 7

15 Product was obtained according to the general procedure outlined in example one,
 starting from Biotin.

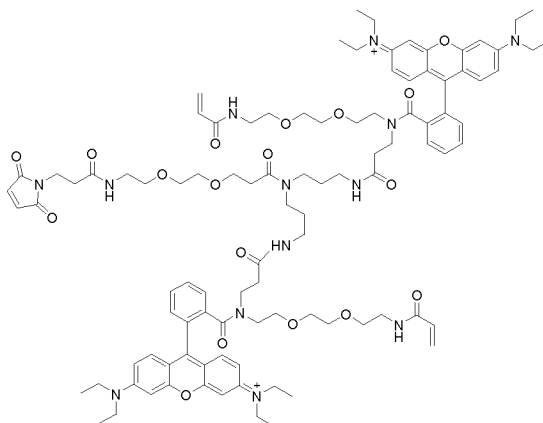
Example 8: Biotin trivalent linker 8

- 5 To a solution of cyanuric chloride (1.23 g, 6.71 mmol) in anhydrous THF (50 mL) was added N-Boc- 2,2'-(ethylenedioxy)diethylamine (1.78 g, 7.18 mmol) at 0 °C with an ice bath. After 5 min, N,N-Diisopropylethylamine (2.46 mL, 14.12 mmol) was added to the reaction solution slowly at 0 °C under the protection of N₂, and the reaction mixture was stirred at 0 °C for another 1.5 h. After complete reaction, the white solid
- 10 was removed by filtration and the filtrate was evaporated. The residue was then purified by column chromatography to yield the intermediate as a yellow oil (32%). To a solution of the intermediate (0.358 g, 0.906 mmol) in ACN (22 mL) was added a solution of 3-(2-(2-Aminoethoxy)ethoxy)propanoic acid t-butyl ester (0.254 g, 1.09 mmol) in ACN (7 mL) and DIPEA (237 µL, 1.36 mmol) in two portions with a 30 min
- 15 interval at 0 °C under the protection of N₂. And then the reaction was heated to 55 °C for 3 h. After complete reaction, all solvents were evaporated and the residue was purified by column chromatography to yield the intermediate as a yellow oil (59%). To a solution of the intermediate (300 mg, 507 µmol) in ACN (4 mL) was added 2-(2-(2-aminoethoxy)ethoxy)-N,N-dibenzylethan-1-amine (825 mg, 1.48 mmol) and
- 20 DIPEA (614 µL, 3.55 mmol). And then the reaction was heated to 140 °C for 1.5 h under microwave. After complete reaction, all solvents were evaporated and the residue was purified by column chromatography to yield the intermediate as a yellow oil (47%). To a solution of the intermediate (210 mg) in MeOH: THF (v:v = 0.5 mL: 0.5 mL) was added Pd/C (21 mg) and the reaction mixture was stirred overnight at
- 25 room temperature. After the reaction finished, the black solid was removed by filtration and the filtrate was evaporated to get the intermediate (90%). To a solution of the intermediate (71 mg, 101 µmol) in DMF (3 mL) was added triethylamine (42 µL, 302 µmol) and HBTU (42 mg, 111 µmol). And then the modified biotin(27.1 mg,

111 μmol) was added and the reaction mixture was stirred at room temperature for 2 h. After complete reaction, 30 mL ethyl acetate was added into the reaction flask, followed by washing with water (30 mL, 2 x) and brine (30 mL). The organic layer was dried over MgSO_4 and then evaporated under reduced pressure. The residue was purified by column chromatography to yield the intermediate as a yellow oil (43%).

To a solution of the intermediate (40 mg) in DCM (0.2 mL) was added TFA (0.2 mL) and the reaction mixture was stirred overnight at room temperature. After the reaction finished, evaporated all solvents to get the intermediate as a yellow oil, of sufficient purity for further use. To a solution of the intermediate (41 mg, 45 μmol) in MeOH: THF (v:v = 0.2 mL: 0.3 mL) was added a solution of Na_2CO_3 (9.6 mg, 90 μmol) in water (150 μL) under the protection of N_2 , followed by cooling the reaction flask to 0 $^\circ\text{C}$ with an ice bath. A solution of acryloyl chloride (4.5 μL , 55 μmol) in dry dioxane (100 μL) was added dropwise to the reaction flask, and the reaction mixture was allowed to come to room temperature over 20 min. After complete reaction, all solvents were evaporated and the residue was purified by column chromatography to yield the product as yellow oil (45%).

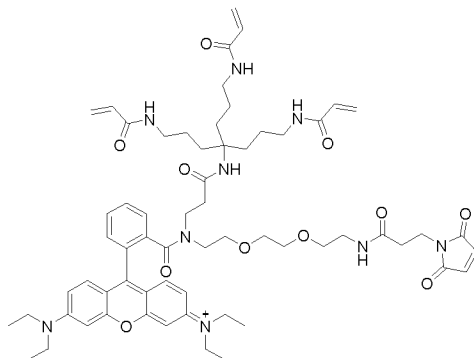
Example 9: Fluorescent Multivalent Fluorescent linker 9



To a solution of 3-(2-(2-Aminoethoxy)ethoxy)propanoic acid t-butyl ester (1.55 g, 6.65 mmol) in DCM (20 mL) was added N-methylmorpholine (0.91 mL, 9.97 mmol) and N-Succinimidyl 3-maleimidopropionate (1.48 g, 5.56 mmol). And then the reaction mixture was stirred at room temperature for 2 h. After complete reaction, all solvents were evaporated and the residue was purified by column chromatography to yield the intermediate as a yellow oil (76%). To a solution of the intermediate (1.2 g) in DCM (2 mL) was added TFA (2 mL) and the reaction mixture was stirred overnight at room temperature. After the reaction finished, evaporated all solvents to get the intermediate as a yellow oil, which was used without further purification.

To a solution of the intermediate (95 mg, 290 μ mol) in DMF (3 mL) was added triethylamine (100 μ L, 724 μ mol) and HBTU (110 mg, 290 μ mol). And then 1,9-Bis-Boc-1,5,9-triazanonane (80 mg, 241 μ mol) was added and the reaction mixture was stirred at room temperature for 2 h. After complete reaction, 30 mL ethyl acetate was added into the reaction flask, followed by washing with water (30 mL, 2 x) and brine (30 mL). The organic layer was dried over MgSO_4 and then evaporated under reduced pressure. The residue was purified by column chromatography to yield the intermediate as a yellow oil (60%). To a solution of Rhodamine B (1 g, 2.09 mmol) in DMF (10 mL) was added triethylamine (0.87 mL, 6.27 mmol) and HBTU (0.87 g, 2.30 mmol). After 10 minutes, linker (0.87 g, 2.30 mmol) was added and the reaction mixture was stirred at room temperature for 2 h. After complete reaction, 50 mL ethyl acetate was added into the reaction flask, followed by washing with water (50 mL, 2 x) and brine (50 mL). The organic layer was dried over MgSO_4 and evaporated to get the intermediate, of sufficient purity for further use. To a solution of the intermediate in DCM (2 mL) was added TFA (2 mL) and the reaction mixture was stirred overnight at room temperature. After the reaction finished, evaporated all solvents to get the intermediate as a red solid (60%).

To a solution of the intermediate (0.757 g, 1.11 mmol) in MeOH: THF (v:v = 2 mL: 3 mL) was added a solution of Na_2CO_3 (0.239 g, 2.25 mmol) in water (3.5 mL) under the protection of N_2 , followed by cooling the reaction flask to 0°C with an ice bath. A solution of acryloyl chloride (90 μ L, 1.11 mmol) in dry dioxane (0.55 mL) was added dropwise to the reaction flask, and the reaction mixture was allowed to come to room temperature over 20 min. After complete reaction, all solvents were evaporated and the residue was purified by column chromatography to yield the intermediate as a red solid (40%). To a solution of the intermediate (1.2 g) in DCM (2 mL) was added TFA (2 mL) and the reaction mixture was stirred overnight at room temperature. After the reaction finished, evaporated all solvents to get the intermediate as a yellow oil, which was used without further purification. To a solution of the intermediate (modified rhodamine B, 52 mg, 74 μ mol) in DMF (3 mL) was added triethylamine (39 μ L, 279 μ mol) and HBTU (42 mg, 111 μ mol), followed by the modified maleimide intermediate (20.7 mg, 31 μ mol). The reaction mixture was stirred at room temperature for 2 h. After complete reaction, all solvents were evaporated and the residue was purified by column chromatography to yield the product as a red solid (20%).

Example 10: Multivalent Fluorescent trivalent linker 10

To a solution of the intermediate (0.313 g, 0.413 mmol) in DMF (3 mL) was added triethylamine (0.115 mL, 0.827 mmol) and N-Succinimidyl 3-maleimidopropionate (0.143 g, 0.538 mmol). And then the reaction mixture was stirred at room temperature for 2 h. After complete reaction, all solvents were evaporated and the residue was purified by column chromatography to yield the intermediate as a red solid (55%).

To a solution of the intermediate (26 mg, 33 μ mol) in DMF (2 mL) was added triethylamine (14 μ L, 98 μ mol) and HBTU (15 mg, 39 μ mol). And then Tris(N-Boc-3-aminopropyl)aminomethane (18 mg, 36 μ mol) was added and the reaction mixture was stirred at room temperature for 2 h. After complete reaction, 30 mL ethyl acetate was added into the reaction flask, followed by washing with water (30 mL, 2 x) and brine (30 mL). The organic layer was dried over MgSO_4 and then evaporated under reduced pressure. The residue was purified by column chromatography to yield the intermediate as a red solid (75%).

To a solution of the intermediate in DCM (0.3 mL) was added TFA (0.3 mL) and the reaction mixture was stirred overnight at room temperature. After the reaction finished, evaporated all solvents to get the intermediate as a red solid, which was used without further purification.

To a solution of the intermediate (30 mg, 23 μ mol) in MeOH: THF (v:v = 1 mL: 1.5 mL) was added triethylamine (16 μ L, 113 μ mol) under the protection of N_2 , followed by cooling the reaction flask to 0 $^\circ\text{C}$ with an ice bath. A solution of acryloyl chloride (11 μ L, 137 μ mol) in dry dioxane (0.5 mL) was added dropwise to the reaction flask, and the reaction mixture was allowed to come to room temperature over 20 min. After complete reaction, all solvents were evaporated and the residue was purified by column chromatography to yield the product as a red solid (35 %).

Example 11: Antibody functionalization, conjugate immunostaining, expansion and imaging

250 μ L of a Goat Anti Mouse antibody solution (2 mg/ml) was concentrated in sodium tetraborate buffer (STB, yielding 50 μ L 15.6 μ M). A linker stock solution (example 2, 0.31 μ L) were added in the reaction eppendorf in order to get a stoichiometric ration of 4/1 reagent/antibody. The reaction solution was left to react for 3 hours at 37°C, and 350 rpm. The conjugate was a PD 10 Desalting Column, using PBS as eluent, to yield 20 μ L of GAM conjugate.

- 10 Fixed and permeabilized HeLa Cells were incubated for 15 minutes in 150 μ L of blocking buffer (BB; 1% of 20% Tween, 10% of FBS in PBS), to inhibit aspecific binding of the primary antibody. Cells were incubated with 150 μ L of primary antibody (Anti-alpha Tubulin antibody [DM1A] - Loading Control) in BB overnight in the fridge. Cells were washed 4 times for 5 minutes with 150 μ L of 1xPBS to remove the primary antibody. Then they were then incubated with 150 μ L of 20 μ g/mL secondary antibody (GAM-conjugates mentioned above) in BB for 4 hours at room temperature. Cells were washed 4 times for 5 minutes with 100 μ L of BB, and once again with 100 μ L PBS, followed by polymerization, expansion and imaging according to literature examples
- 20 From the example, it is clear the conjugations neither impair antibody recognition nor give rise to aspecific staining. Brightness is very high and expanded samples fluorescence is retained for more than three months if the samples are kept at 4°C and in Milli-Q and covered from light.

25 Example 12: Primary Antibody conjugate immunostaining, expansion and imaging

Primary antibody conjugate was prepared and image in line with procedures outlined in Example 11. Anti-alpha tubulin antibody was used, followed by polymerization, expansion and imaging according to literature examples.

- 30 From the example, it is clear the conjugations neither impair antibody recognition nor give rise to aspecific staining. Brightness is very high and expanded samples fluorescence is retained for more than three months if the samples are kept at 4°C and in Milli-Q and covered from light.

Example 13: Cytoskeleton staining, expansion and imaging

Labelling solution was prepared putting 0.66 μL from the reaction (stock solution, example 4) in 1000 μL of PBS and the diluting it 10 times. Cells were covered with 200 μL of labelling solution and incubated at room temperature for 1 hour. After that

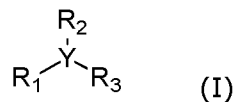
5 time, the wells were washed 3 times with PBS to remove dye excess, followed by polymerization, expansion and imaging according to literature examples.

From the example, it is clear that the herein disclosed compounds and methods enable previously undetectable biomolecules in expansion microscopy protocols.

Claims

1. A method for identifying and locating a biomolecule in a sample, the method comprising the steps of:

5 a) conjugating the biomolecules within the sample with a multifunctional reagent according to the formula (I)



wherein

- 10 R_1 is a specific ligand for a biological structure,
 R_2 is a reactive group capable of binding covalently to a polymeric matrix,
 R_3 is a reporter moiety,
 Y is a moiety covalently connecting R_1 , R_2 and R_3 ,
- 15 b) embedding the sample in a polymeric material wherein the reporter moiety R_3 reacts with the polymeric material thereby becoming anchored to the polymeric material,
- c) subjecting the sample to digestion or clearing,
- d) optionally swelling the material to form an expanded sample,
- e) optionally adding one or more additional stains, and
- 20 f) imaging the sample.

2. The method according to claim 1 wherein R_1 is a polypeptide, a polynucleotide or a small-molecule ligand.

- 25 3. The method according to claim 1 or 2, wherein the biomolecule is selected from the group consisting of DNA, RNA, proteins and lipids.

4. The method according to any one of claims 1 to 3, wherein the polymeric material is a swellable material.

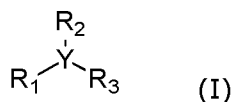
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5. The method according to any one of claims 1 to 4, wherein step b) of embedding comprises the step of permeating the biological sample with a composition comprising precursors of a swellable polymer and forming a swellable polymer in situ.

35

6. The method according to an one of claims 1 to 5, wherein multiple biomolecules are targeted in the same sample.
7. The method according to any one of claims 1 to 6, wherein steps (e) to (f) are repeated one or more times.
8. The method according to any one of claims 1 to 7, wherein R_3 is a dye, a hapten or an oligonucleotide.
9. The method according to any one of claims 1 to 8, where the additional stain in e) is an antibody or antibody fragment specifically binding R_3 .
10. The method according to any one of claims 1 to 8, where the additional stain in step e) is a dye labeled oligonucleotide specifically binding R_3 .
11. The method according to any one of claims 1 to 10, wherein the sample in step d) is expanded isotropically by adding water to swell the swellable material.
12. The method according to claim 11, wherein the volume of the sample is expanded by a factor of at least 3.
13. The method according to any one of claims 1 to 12, wherein in step c) the sample is subjected to enzymatic proteolysis.
14. The method according to any one of claims 1 to 14, wherein the imaging is performed by expansion microscopy.
15. The method according to claim 14, wherein the imaging is performed on an expanded sample.
16. The method according to claim any one of claims 1 to 15, wherein different multifunctional reagents are used for the detection of different biomolecules.
17. The method according to claim 16, wherein the two of different molecules are at a distance of less than 500, 200, 100, or 50 nm in the biological sample.

18. A multifunctional reagent according to the formula (I)



5 wherein

R₁ is a specific ligand for a biological structure,

R₂ is a reactive group capable of binding covalently to a polymeric matrix,

R₃ is a reporter moiety,

Y is a moiety covalently connecting R₁, R₂ and R₃ connecting R₁, R₂ and R₃,

10

19. Use of a multifunctional reagent as defined in claim 18, for identifying and locating a biomolecule in a sample.

20. The use according to claim 19 in expansion microscopy.

15

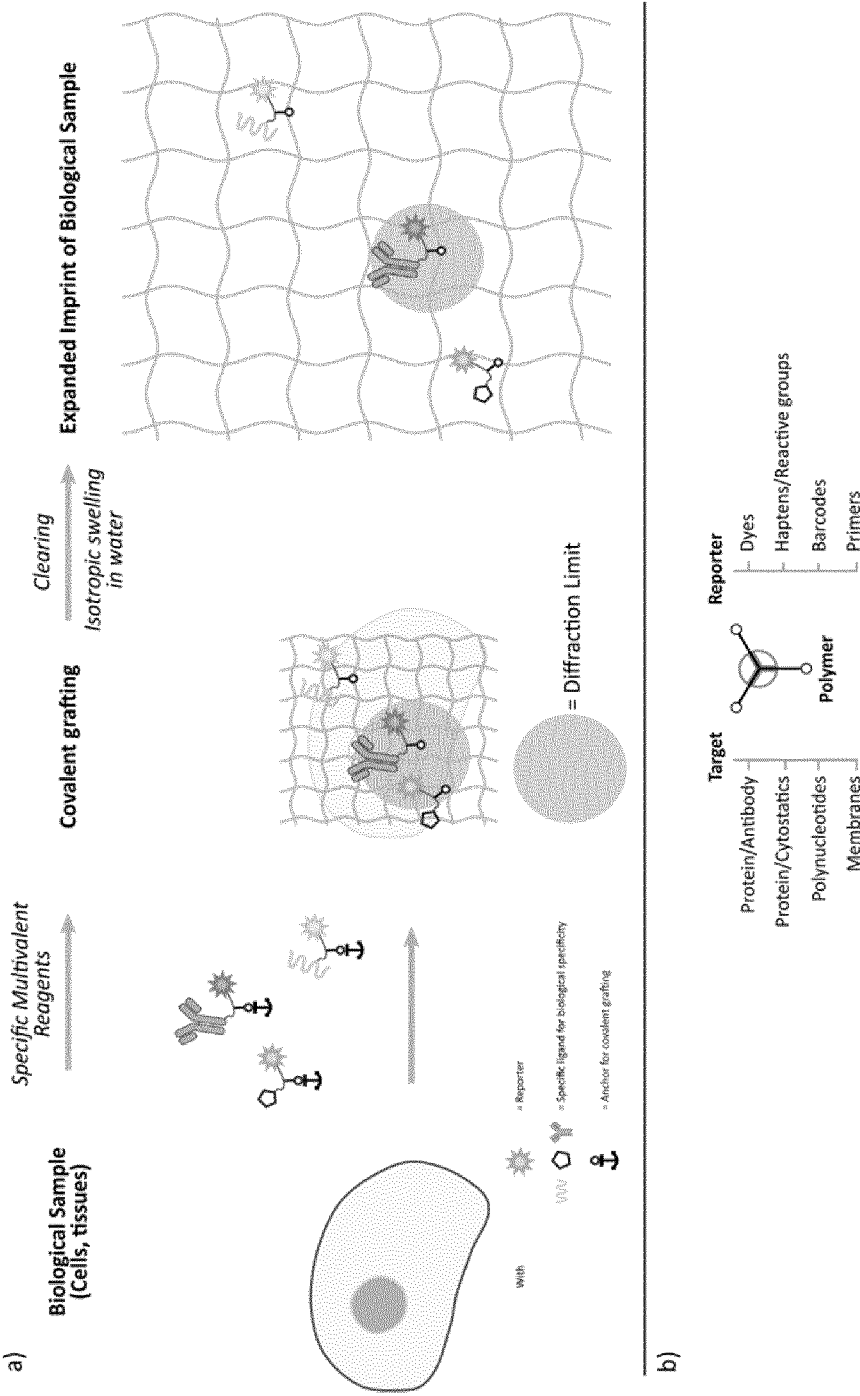


Figure 1

Multifunctional Linker Design

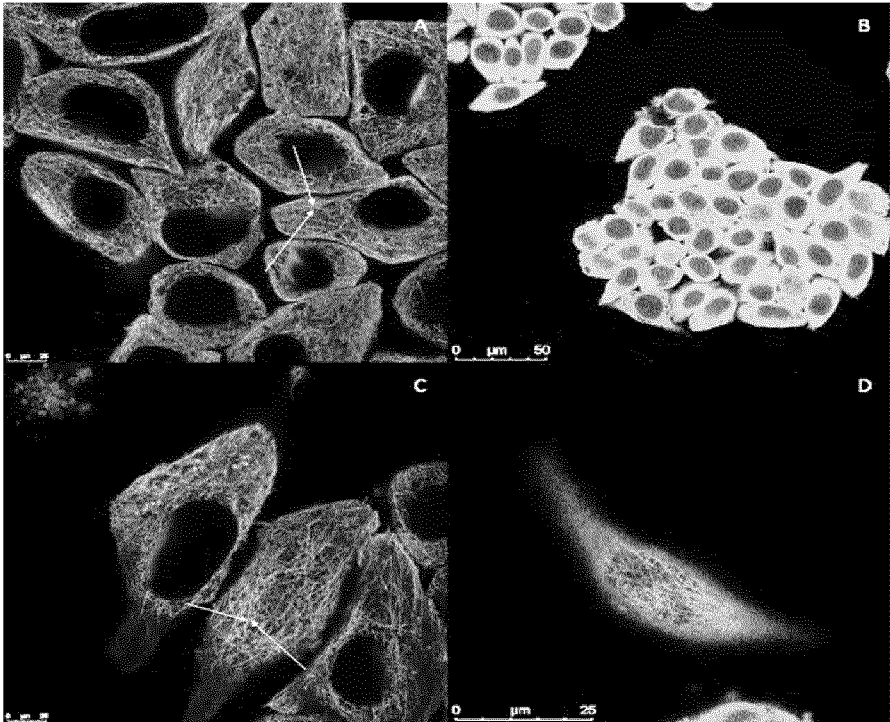


Figure 2

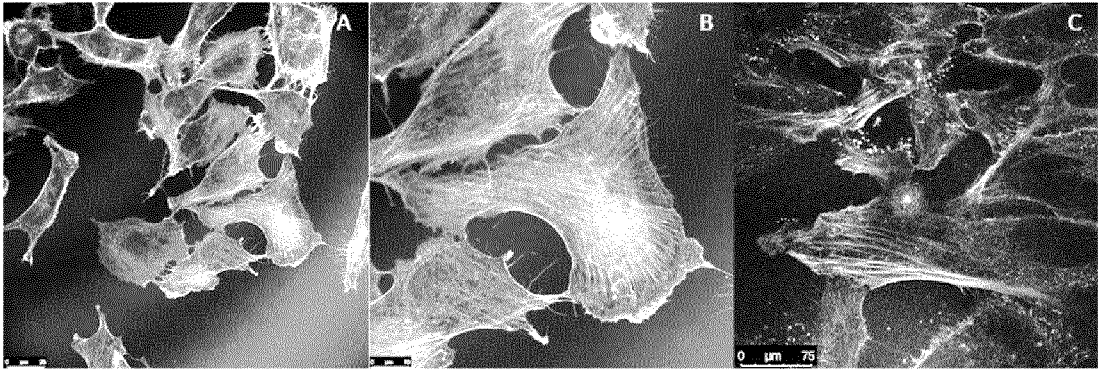


Figure 3

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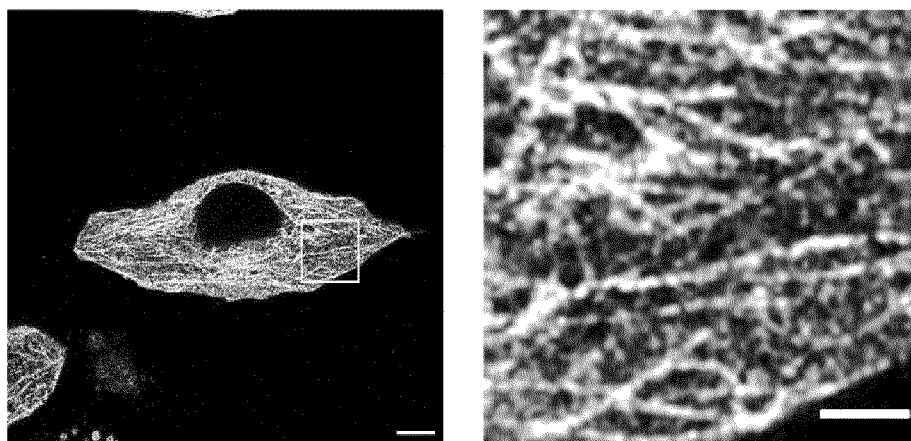


Figure 4

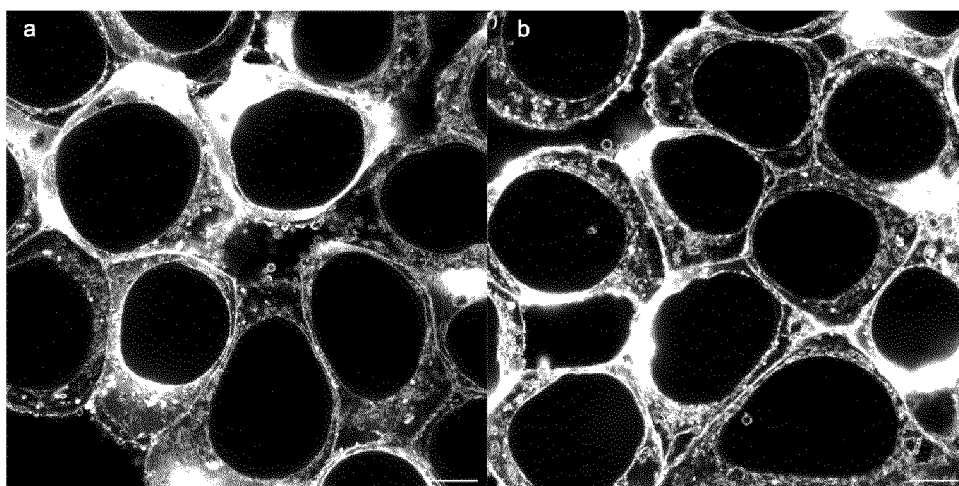


Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/059604

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/58 C12Q1/6841 G01N33/68
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 C12Q

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, 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	PAUL W TILLBERG ET AL: "Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies", NATURE BIOTECHNOLOGY, vol. 34, no. 9, 4 July 2016 (2016-07-04), pages 987-992, XP055486308, US ISSN: 1087-0156, DOI: 10.1038/nbt.3625 Abstract; Fig. 4 ----- -/--	1-20



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

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Date of the actual completion of the international search

15 June 2020

Date of mailing of the international search report

23/06/2020

Name and mailing address of the ISA/

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Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2020/059604

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AARON R. HALPERN ET AL: "Hybrid Structured Illumination Expansion Microscopy Reveals Microbial Cytoskeleton Organization", ACS NANO, vol. 11, no. 12, 30 November 2017 (2017-11-30), pages 12677-12686, XP055603959, US ISSN: 1936-0851, DOI: 10.1021/acsnano.7b07200 cited in the application Abstract; p. 12678, right hand column, l. 12-20	1-20
X	----- WO 2016/030546 A1 (UNIV LEUVEN KATH [BE]) 3 March 2016 (2016-03-03) Abstract; claim 3	18
X,P	----- Xiaoyu Shi ET AL: "Label-retention expansion microscopy", bioRxiv, 2 July 2019 (2019-07-02), pages 1-21, XP055702227, DOI: 10.1101/687954 Retrieved from the Internet: URL:https://www.biorxiv.org/content/10.1101/687954v2.full.pdf [retrieved on 2020-06-08] the whole document	1-20
X,P	----- GANG WEN ET AL: "Evaluation of Direct Grafting Strategies via Trivalent Anchoring for Enabling Lipid Membrane and Cytoskeleton Staining in Expansion Microscopy", ACS NANO, 16 March 2020 (2020-03-16), XP055702127, US ISSN: 1936-0851, DOI: 10.1021/acsnano.9b09259 the whole document	1-20
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2020/059604

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016030546 A1	03-03-2016	EP 3186266 A1	05-07-2017
		PL 3186266 T3	01-06-2020
		US 2017283453 A1	05-10-2017
		WO 2016030546 A1	03-03-2016
