when large amounts of the fluorescent dyes are confined to a small subcellular area; (c) they can only be used in very diluted solutions and have very poor photostability; (d) they have small Stokes shifts (usually less than 30 nm), which is difficult to distinguish from the background signals and require precise emission filters; (e) their synthesis or preparation is usually difficult, which makes them hard to adjust the emission spectrum and introduce reactive sites for enzymes or bioactive molecules.

Therefore there is a need for fluorescent probes that target organelles, do not easily diffuse away from the enzyme reactive sites due to their aqueous solubility, show higher Stokes shifts and at the same time are less difficult to synthesize and functionalize. The probes should show less background noise as the known AIE based probes. The previous fluorescent probes based solely on an AIE turn-on mechanism must have good water solubility to ensure low background noise, which restricts their applications for detection of enzymes with hydrophobic ligand-binding sites.

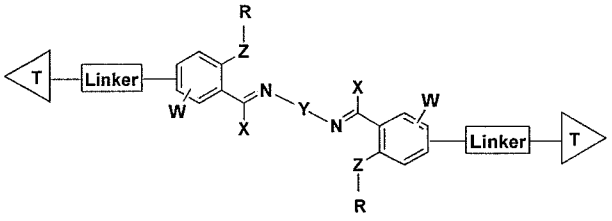
Thus, there is a need for improved imaging methods and new fluorescent probes for organelle-specific enzyme and bioactive molecule detection.

Summary

According to a first aspect of the invention, there is provided a method for imaging the activity of enzymes or bioactive molecules in cells using fluorescent probes with aggregation-induced emission ("AIE") properties and excited-state intramolecular proton transfer ("ESIPT") properties, the method comprises the steps of (a) incubating the cells with a fluorescent probe precursor and (b) allowing the enzymes or bioactive molecules to interact with the fluorescent probe precursor by a

cleavage of a group at a reactive site of the fluorescent probe precursor to form the fluorescent probe and (c) measuring the fluorescence response,

wherein the fluorescent probe precursor has the formula (I),



(I)

wherein

X represents hydrogen, alkyl, alkynyl or heteroaryl,

Y represents a direct bond between the two nitrogen atoms or represents an aryl or heteroaryl group which are optionally substituted by one or more substituents selected from halogen, cyano, alkyl, perfluoroalkyl, alkoxy, aryl-oxy, alkenyl, alkynyl, cycloalkyl, alkylthio, amido, amino, monoalkylamino, dialkylamino, carboxamide, hydroxy, mercapto, aryl or heteroaryl,

Z represents O, NH or NR',

R' represents alkyl, aryl or heteroaryl,

R represents the reactive site of the fluorescent probe precursor for reaction with enzymes or biomolecules,

W represents no substituent or one or more substituents selected from halogen, cyano, alkyl, perfluoroalkyl, alkoxy, aryl-oxy, alkenyl, alkynyl, cycloalkyl, alkylthio, amido, amino, monoalkylamino, dialkylamino, carboxamide, hydroxy, mercapto, aryl and heteroaryl; or represents a fused aromatic ring to the phenyl moiety that it is linked to and is optionally substituted by one or more substituents selected from halogen, cyano, alkyl, perfluoroalkyl, alkoxy, aryl-oxy, alkenyl, alkynyl, cycloalkyl, alkylthio, amido, amino,

negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Detailed Disclosure of Optional Embodiments

Exemplary, non-limiting embodiments of the method for imaging the activity of enzymes or bioactive molecules in cells using fluorescent probes with aggregation-induced emission properties and excited-state intramolecular proton transfer properties are described.

The enzymes and bioactive molecules include all substances that can interact with the fluorescent probe precursor to form the fluorescent probe. Preferably the interaction is characterized by having a non-emitting or little emitting probe before the action of the enzyme/bioactive molecule which changes into an emitting probe ("turn-on mechanism"). For instance an enzyme can cleave certain part off the probe after which it aggregates and shows the AIE and ESIPT effect. Figure 1 shows the schematic principle of such turn on mechanism.

The fluorescent probe precursor is preferably a probe that has an organelle specific target group T and a reactive site R for reaction with enzymes or biomolecules. The reactive site interacts with the enzyme or bioactive molecule while the T moiety links the probe to the desired cell part which is an organelle (see Figure 1 for the cleavage of an R group by the enzyme and the linkage to the organelle by the T group). The method uses at least one fluorescent probe, but can also utilize a combination of two to four or more probes.

The activity of enzymes that can be detected in the inventive method include for instance esterases (acting on acetoxy groups), sulfatases (acting on sulfonate groups), β-galactosidases (acting on β-galactoside groups), β-

glucosidases (acting on β-glucoside groups), α-glucosidases (acting on α-glucoside groups), hexosaminidases (acting on N-acetyl-D-hexosamines), monoamine oxidases (acting on alkylamine groups), Cytochrome P450 (acting on ethyl groups), and nitroreductases (acting on 2-ethyl-5-nitrothiophene groups).

Bioactive molecules that can be used and the activity of which can be detected in the inventive method include for instance hydrogen peroxide (H_2O_2) (acting on arylboronate groups), hypochlorous acid (HOCl) (acting on p-aminophenol groups), ozone (O_3) (acting on homoallyl ether groups), superoxides ($O_2\bullet-$) (acting on diphenylphosphinyl groups), thiols (RSH) (acting on 2,4-dinitrobenzenesulfonyl groups); hydrogen sulfide H_2S (acting the pendant disulfide and ester groups), sulfites (SO_3^{2-}) (acting on 1,4-diketone groups), and fluoride ions (acting on diphenyl(tert-butyl)silyl groups).

The method according to the invention can be used for in situ imaging of biological activities of the enzymes and bioactive molecules in the cells. The cells are therefore incubated with the fluorescent probe or its precursor. It can be run directly with the cells. Solvents that can be used are those in which the cells are kept, e.g. water or PBS buffer. Preferably the probe is dissolved in serum free solution. The concentration of the fluorescent probe is µM or nM level, such as 1 nM to 100 µM, preferably 100 nM to 20 µM. The temperature can be varied, but is usually around 20 to 40 °C, preferably 25 to 38 °C. The emission of the probes can be measured in real time. A preferred observation time is 3 min to 2 h after incubation with the probe, most preferably 5 to 12 min after incubation. A preferred incubation time at about

homoallyl ether, diphenylphosphinyl, 2,4-dinitrobenzenesulfonyl, 1,4-diketone, carbohydrate, amino acid or peptide group.

W = a fused aromatic ring that is optionally and independently substituted once or more, at any position, by halogen, cyano, alkyl, perfluoroalkyl, alkoxy, aryloxy, alkenyl, alkynyl, cycloalkyl, alkylthio, amido, amino, monoalkylamino, dialkylamino, carboxamide, hydroxy, mercapto, aryl, heteroaryl;

Linker = The LINK groups are composed of any combination of chemical bonds, including ether, thioether, amine, ester, carboxamide, sulfonamide, and single, double, triple carbon-carbon bonds. Selected examples of LINK optionally include oligomethylenes, carboxamides, and sulfonamides;

T = Targeting groups for specific organelles, including pyrrolidine, piperidine, piperazine, morpholine, imidazole, diazepine, triazepine, oxazepine, triphenylphosphonium, pyridinium, peptide, carbohydrate, and other ligands or antibodies for organelles.

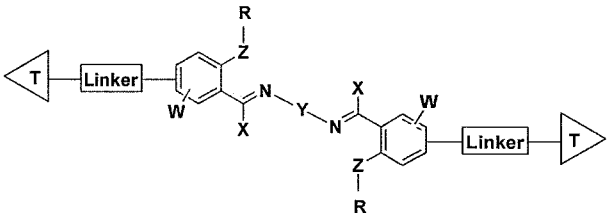
(2) The method of (1), wherein "**T**" for targeting lysosome is selected from the group consisting of pyrrolidine, piperidine, piperazine, morpholine, imidazole, diazepine, triazepine, and oxazepine. The reactive groups for specific detection of lysosomal enzymes are consisting of acetoxy group for esterase, sulfonate group for sulfatase, β-galactoside group for β-galactosidase, β-glucoside group for β-glucosidase, α-glucoside group for α-glucosidase, and *N*-acetyl-D-hexosamine for hexosaminidase.

(3) The method of (1), wherein "**T**" for targeting mitochondria is selected from the group consisting of triphenylphosphonium, and pyridinium groups. The reactive groups are consisting of alkylamine group for monoamine oxidase, ethyl group for Cytochrome P450, 2-ethyl-5-

CLAIMS

1. A method for imaging the activity of enzymes or bioactive molecules in cells using fluorescent probes with aggregation-induced emission properties and excited-state intramolecular proton transfer properties, the method comprises the steps of (a) incubating the cells with a fluorescent probe precursor and (b) allowing the enzymes or bioactive molecules to interact with the fluorescent probe precursor by a cleavage of a group at a reactive site of the fluorescent probe precursor to form the fluorescent probe and (c) measuring the fluorescence response,

wherein the fluorescent probe precursor has the formula (I),



(I)

wherein

X represents hydrogen, alkyl, alkynyl or heteroaryl,

Y represents a direct bond between the two nitrogen atoms or represents an aryl or heteroaryl group which are optionally substituted by one or more substituents selected from halogen, cyano, alkyl, perfluoroalkyl, alkoxy, aryl-oxy, alkenyl, alkynyl, cycloalkyl, alkylthio, amido, amino, monoalkylamino, dialkylamino, carboxamide, hydroxy, mercapto, aryl or heteroaryl,

Z represents O, NH or NR',

R' represents alkyl, aryl or heteroaryl,

bridge having 1 to 16 carbon atoms and which is satureated or partly saturated.

3. The method according to claim 1 or 2, wherein in formula (I),

X represents hydrogen,

Y represents a direct bond between the two nitrogen atoms or represents phenyl,

Z represents O or NH,

W represents no substituent,

R represents an acetyl or a galactosidyl group,

Linker represents an oligomethyleneoxy group having 1 to 16 carbon atoms, and

T represents represents a pyrrolidine, piperidine, piperazine, morpholine, imidazole, diazepine, triazepine, oxazepine, triphenylphosphonium, pyridinium, peptide or carbohydrate moiety.

4. The method according to any one of claims 1 to 3, wherein in formula (I),

T represents pyrrolidine, piperidine, piperazine, morpholine, imidazole, diazepine, triazepine, or oxazepine, and

R represents an acetoxy, sulfonate, β-galactoside, β-glucoside, α-glucoside, or N-acetyl-D-hexosamine group.

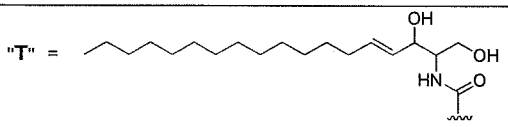
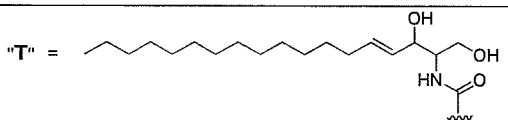
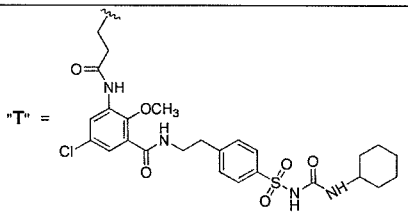
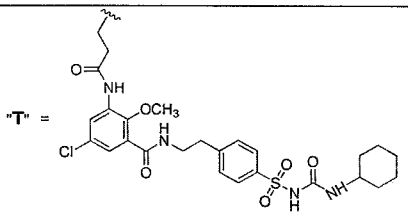
5. The method according to any one of claims 1 to 4, wherein in formula (I)

T represents a triphenylphosphonium or pyridinium group, and

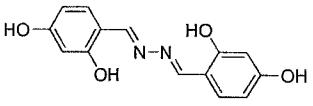
R represents an alkylamine, ethyl, 2-ethyl-5-nitrothiophene, arylboronate, p-aminophenol, homoallyl ether, diphenylphosphinyl or 2,4-dinitrobenzenesulfonyl

group; or represents a pendant disulfide, a pendant ester, a 1,4-diketone, or a diphenyl(tert-butyl)silyl group.

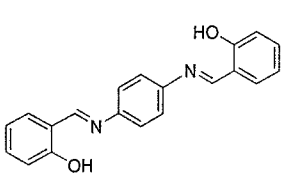
6. The method according to any one of claims 1 to 5, wherein in formula (I), R and T have the meaning as listed in the following table:

R	
Peptide (Asp-Glu-Val-Asp) (= DEVD)	
α-1,2-mannoside	
Peptide (Ac-Leu-Glu-Val-Asp-)	
Peptide (Ac-Ala-Thr-Ala-Asp-)	

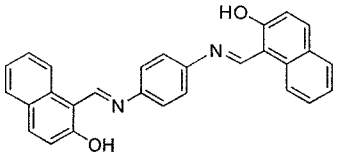
7. The method according to any one of claims 1 to 6, wherein the fluorescent probe comprises one of the following fluorophores or derivative thereof:



Green-2

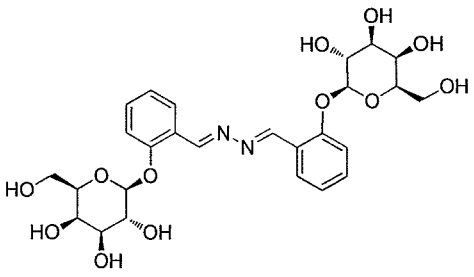


Yellow-3



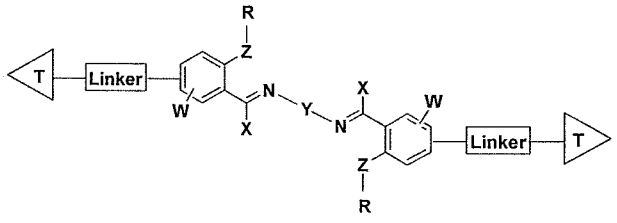
Red-4

8. The method according to any one of claims 1 to 7, wherein the fluorescent probe is the compound of formula (IV) or a derivative thereof



(IV).

9. A probe or precursor of a probe of the formula (I)',



(I)'

wherein

X represents hydrogen, alkyl, alkynyl or heteroaryl,

Y represents a direct bond between the two nitrogen atoms or represents an aryl or heteroaryl group which are optionally substituted by one or more substituents