

**METHODS AND SYSTEMS FOR DETECTING AN ANALYTE OR
CLASSIFYING A SAMPLE**

FIELD OF THE INVENTION

- 5 The present invention relates to methods and systems for detecting one or more analytes in a sample and/or for classifying a sample. In particular, the present invention relates to methods and systems which can be used to detect the analytes in real time and which rely on flowing through a microfluidic device one or more types of sensor molecule each comprising a domain that binds one or more analytes, a
10 chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance.

BACKGROUND OF THE INVENTION

- 15 Bioluminescence resonance energy transfer (BRET) occurs naturally in marine organisms such as *Aequorea victoria* and *Renilla reniformis* (Morin and Hastings, 1971). BRET is a form of Förster resonance energy transfer (RET), which is the non-radiative transfer of energy from an excited state donor to a ground state acceptor. There are two commonly used forms of the BRET principle, i.e., BRET¹ and BRET².
20 Both use *Renilla luciferase* (RLuc) as the energy donor. In BRET¹, the substrate is native coelenterazine (CLZ) or coelenterazine h (CLZh). RLuc and a yellow fluorescent protein (YFP) are the energy donor and acceptor, respectively, giving peak donor emission at 475 nm and peak acceptor emission at 535 nm. In BRET², YFP is replaced with GFP² and a modified CLZ substrate, i.e. coelenterazine-400a or
25 (CLZ400a) is used. The peak donor emission and acceptor emission are shifted to 395 nm and 515 nm, respectively (Dacres et al., 2009a, b; Pflieger and Eidne, 2006). A third form of BRET, i.e., BRET³, has recently been developed. It used CLZh as the substrate and RLuc8 as the energy donor and mOrange as the acceptor, resulting in improved spectral resolution (De et al., 2009).
30 RET is a ratiometric technique which can eliminate data variability caused by fluctuations in light output due to variations in assay volume, assay conditions and signal decay across different wells in a plate. RET-based reactions are homogeneous, generally occurring in solution without solid-phase attachment. This allows for detection of analytes in different forms such as liquid, gas and even particulates without
35 separation. The avoidance of solid-phase attachment eliminates the process of surface regeneration used in many surface-based techniques such as Surface Plasmon

Resonance (SPR) (Fang et al., 2005) and, in conjunction with the fast reaction rate, allows it to be used for on-line monitoring.

So far, however, uses of BRET have been restricted to research laboratories using sophisticated detection equipment. Microfluidic technologies are attracting
5 interest in many fields, including chemistry, biology, medicine, sensing and materials. Their advantages over conventional technologies include reduced reagent consumption, fast reaction rate, short analysis time, and amenability to automation and mass production (Holden and Cremer, 2005).

There have been substantial research and development in microfluidic
10 technologies. Examples include an integrated biochip design with fluorescence light collection (EP 2221606), on-chip biosensing using Raman spectroscopy (WO 2009/020479), a biosensing device (WO 2009/018467) for detecting GPCR-ligand binding using surface plasmon resonance techniques, a light detection chip (US 2011/0037077 and US 2008085552) with mirrors as light reflectors, an assay device
15 with a cartridge format (WO 2009/044088), a chemiluminescence-based microfluidic biochip (US 2002/0123059) and so on. Many of these device have the disadvantages of high cost per chip due to integration of multiple components, inability to perform real-time monitoring due to the requirement for surface regeneration and slow reaction of reagents, limited detection sensitivity, or signal drift.

20 Furthermore, there is considerable background art in the fields of electronic noses and electronic tongues, which contact a gaseous or liquid sample with an array of solid state sensors in order to detect analytes and/or classify the samples. Electronic noses and tongues have been bedevilled by poor performance due to limited selectivity of the sensors, poor sensitivity, sensor saturation and slow regeneration and sensor drift
25 over time.

There is therefore a need for further methods of detecting analytes and classifying samples based on the analytes they contain, particularly methods that can be performed in real time and with increased sensitivity and that do not suffer from downtime due to the need to regenerate the sensing surface and that resist the
30 confounding effects of sensor drift. Multiple channel microfluidic systems deploying an array of biologically derived sensors electro-optically coupled to a detection system offer a novel solution to these problems.

SUMMARY OF THE INVENTION

The present inventors have identified an improved method of detecting an analyte in a sample.

In one aspect, the present invention provides a method of detecting an analyte in
5 a sample, the method comprising

- i) flowing through a microfluidic device comprising one or more microchannels,
 - a) the sample,
 - b) a sensor molecule comprising a domain that binds the analyte, a chemiluminescent donor domain and an acceptor domain, wherein the separation and
10 relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance,
 - c) a substrate of the chemiluminescent donor,
- ii) mixing the sensor molecule, sample and substrate in the device, and
- iii) detecting modification of the substrate by the chemiluminescent donor using
15 an electro-optical sensing device,
wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the analyte binds the sensor molecule.

In a preferred embodiment, the sensor molecule is not fixed to the device.

20 In a further preferred embodiment, the method can be used to detect the analyte in real time.

In another preferred embodiment, the sensor molecule and substrate enter the device through different microchannels. In an alternate embodiment, the sensor molecule and substrate enter the device through the same microchannel, however, in
25 this embodiment it is preferred that the sensor molecule and substrate are mixed shortly before (for example 10 seconds, more preferably 1 second, or less) before entering the microchannel.

In a preferred embodiment, the Förster distance of the chemiluminescent donor domain and the acceptor domain is at least 5.6 nm, or at least 6 nm. In another
30 preferred embodiment, the Förster distance of the chemiluminescent donor domain and the acceptor domain is between about 5.6 nm and about 10 nm, or is between about 6 nm and about 10 nm.

In a further preferred embodiment, the analyte binding or releasing from the sensor molecule results in a change in BRET ratio which is $\geq 15\%$, $\geq 20\%$, $\geq 30\%$, $\geq 35\%$,
35 about 15% to about 50%, or about 15% to about 40%, of the maximum observed BRET ratio. A change in the BRET ratio of 15% or more increases the signal to noise ratio of

analyte detection. This results in a superior limit of detection for any given sampling time and more precise coding of the level of concentration of analyte. Alternatively, at a fixed limit of detection, the greater change in BRET ratio facilitates shorter signal integration times and therefore more rapid detection.

- 5 In a further preferred embodiment, the quantum yield detected by the electro-optical sensing device is less than about 8%, or less than about 5%, or less than about 2%.

 In another preferred embodiment, the acceptor domain has a Stokes Shift of between about 50nm and about 150nm. In an embodiment, the acceptor domain has a
10 Stokes Shift of about 100nm.

 An advantage of the method of the present invention is that it is highly time resolved. Thus, in a preferred embodiment, the method is performed within about 1s to about 100s.

- The sample can be in any form that is capable of being flowed through a
15 microfluidic device. Examples include, but are not necessarily limited to, a liquid, gas, emulsion or suspension. In an embodiment, the sample is a liquid which has been pre-equilibrated with a gas.

 In one embodiment, the suspension is, or comprises, a cell-free composition. In an alternate embodiment, the suspension comprises cells.

- 20 In an embodiment, the flow rate through the microfluidic device is between about 1 μ l/hour to about 10ml/hour, or 1 μ l/hour to about 1ml/hour, or 1 μ l/hour to about 1.5ml/hour, or about 20 μ l/hour to about 0.5ml/hour, and the preferred flow rate is between about 200 μ l/hour to about 1ml/hour.

- In a preferred embodiment, the flow rate and length of the section of the
25 microchannel comprising the sample, sensor molecule and substrate is such that the sample, sensor molecule and substrate are in the section for at least about 5sec, at least about 10sec, at least about 15sec, at least about 20sec, about 5sec to about 50sec, or about 10sec to about 30sec.

- In one embodiment, for instance when the sensor molecule comprises a protein
30 receptor such as a G coupled protein receptor, the concentration of the sensor molecule following step ii) is between about 1nM to about 10 μ M or between about 1nM to about 1 μ M. In another embodiment, for instance when the sensor molecule comprises a cleavable peptide-derived or periplasmic binding protein, the concentration of the sensor molecule following step ii) is between about 0.1 μ M to about 10 μ M.

- 35 In an embodiment, the flow through the microfluidic device is continuous flow, batch flow or stop flow.

The sample, sensor molecule and substrate may be actively mixed using mechanical, electrokinetic, acoustical or other suitable means. In a preferred embodiment, the mixing is achieved by diffusion over dimensions perpendicular to the direction of flow through a microchannel comprising the sample, sensor molecule and substrate. For example, efficient mixing ($\geq 20\%$) of sample, sensor molecule and substrate can be conveniently achieved by predominantly passive diffusional (non-turbulent) processes. The typical conditions include, flow rates of approximately no more than 1,000 microlitres per hour, common microchannel lengths of approximately 10 mm or more and that the summed height of the stacked inputs when they are flowing in contact with each other in the common channel is approximately 200 micrometres or less (measured perpendicular to the direction of flow).

The sample, sensor molecule and substrate can be flowed through the microfluidic device by any suitable means such as, but are not necessarily limited to, one or more of pumping, vacuum, hydraulics, suction, electrokinesis, chemiosmosis, capillary force, acoustics, electromagnetics, piezoelectrics. Pumping mechanisms can be realised in compact, miniaturised and micron-size pumps. In a preferred embodiment, the sample, sensor molecule and substrate is flowed through the microfluidic device by suction (negative pressure), for example using a syringe pump in withdrawal mode.

In an embodiment, each microchannel has a cross-sectional area of about $1\mu\text{m}^2$ to about 1mm^2 .

In a further embodiment, the microchannel for the sample, sensor molecule and substrate each have a width of $\geq 300\mu\text{m}$ and height $\geq 60\mu\text{m}$, width of $\geq 600\mu\text{m}$ and height $\geq 30\mu\text{m}$ or width of $\geq 1200\mu\text{m}$ and height $\geq 15\mu\text{m}$. In an embodiment, the height is no greater than about 1mm or about 0.5mm. In another embodiment, the height is about $15\mu\text{m}$ to about 1mm or about $15\mu\text{m}$ to about 0.5mm. In a further embodiment, the width is no greater than about 1.5mm. In yet a further embodiment, the width is about $300\mu\text{m}$ to about 1.5mm or about $300\mu\text{m}$ to about 1.2mm.

The lengths of the input microchannels for substrate, sensor molecule and sample are as short as possible, preferably less than 10 mm, and more preferably less than 5 mm.

The length of the common microchannel where the substrate, sensor molecule and sample are allowed to mix and react may be between 5 mm and 100 mm, or between 10 mm and 100 mm, preferably between 20 mm and 50 mm and may be linear, serpentine or any suitable combination of straight curved geometries. In an alternate embodiment, the common microchannel may be dispensed with entirely and

the sensor molecule, substrate and sample may be introduced directly into the reaction chamber with or without active mixing.

In a further embodiment, step iii) is performed in a reaction chamber with a volume of about 1 pL (i.e. picoliter or a trillionth of a liter) to about 200 μ L and the preferred volume is 0.5 μ L to about 8 μ L, or 0.5 μ L to about 2 μ L.

In yet another embodiment, step iii) comprises processing at least one signal from the electro-optical sensing device to determine whether the analyte is absent or present in the sample, and if present optionally determining the concentration of the analyte in the sample.

10 In an embodiment, the domain that binds the analyte is a protein (which may be a peptide) or a nucleic acid. In a preferred embodiment, the domain is a protein. In an embodiment, the protein is a naturally occurring protein, which binds one or more analytes (ligand), or a variant of the protein which retains analyte (ligand) binding activity. Examples include, but are not necessarily limited to, a receptor, odorant
15 binding protein, pheromone-binding protein, enzyme, ligand carrier or bacterial periplasmic binding protein. In an embodiment, the receptor is a G protein coupled receptor such as an odorant receptor or a taste receptor. In a further embodiment, the odorant receptor or taste receptor is from a nematode or vertebrate or is a mutant thereof.

20 In an embodiment, the chemiluminescent donor domain is a bioluminescent protein. Examples include, but are not necessarily limited to, a luciferase, a β -galactosidase, a lactamase, a horseradish peroxidase, an alkaline phosphatase, a β -glucuronidase or a β -glucosidase. Examples of luciferases include, but are not necessarily limited to, a *Renilla* luciferase, a Firefly luciferase, a Coelenterate
25 luciferase, a North American glow worm luciferase, a click beetle luciferase, a railroad worm luciferase, a bacterial luciferase, a *Gaussia* luciferase, Aequorin, an *Arachnocampa* luciferase, or a biologically active variant or fragment of any one, or chimera of two or more, thereof. In a preferred embodiment, the *Renilla* luciferase variant is RLuc2 or RLuc8.

30 In an embodiment, the substrate is luciferin (such as a beetle luciferin), calcium, coelenterazine, or a derivative or analogue of coelenterazine.

In a preferred embodiment, the acceptor domain is a fluorescent acceptor domain.

In a further embodiment, the fluorescent acceptor domain is a protein.
35 Examples include, but are not necessarily limited to, green fluorescent protein (GFP), blue fluorescent variant of GFP (BFP), cyan fluorescent variant of GFP (CFP), yellow

fluorescent variant of GFP (YFP), enhanced GFP (EGFP), enhanced CFP (ECFP), enhanced YFP (EYFP), GFPS65T, Emerald, Venus, mOrange, Topaz, GFPuv, destabilised EGFP (dEGFP), destabilised ECFP (dECFP), destabilised EYFP (dEYFP), HcRed, t-HcRed, DsRed, DsRed2, t-dimer2, t-dimer2(12), mRFP1, pocilloporin,
 5 Renilla GFP, Monster GFP, paGFP, Kaede protein or a Phycobiliprotein, or a biologically active variant or fragment of any one thereof.

In an alternate embodiment, the fluorescent acceptor domain is a non-protein. Examples include, but are not necessarily limited to, an Alexa Fluor dye, Bodipy dye, Cy dye, fluorescein, dansyl, umbelliferone, fluorescent microsphere, luminescent
 10 microsphere, fluorescent nanocrystal, Marina Blue, Cascade Blue, Cascade Yellow, Pacific Blue, Oregon Green, Tetramethylrhodamine, Rhodamine, Texas Red, rare earth element chelates, or any combination or derivatives thereof.

In an embodiment, the method further comprises providing a co-factor of the bioluminescent protein. Examples of co-factors include, but are not necessarily limited
 15 to, ATP, magnesium, oxygen, FMNH₂, calcium, or a combination of any two or more thereof.

In a preferred embodiment,

- i) the bioluminescent protein is a luciferase or a biologically active variant or fragment, and/or
- 20 ii) the substrate is luciferin, coelenterazine, or a derivative or analogue of coelenterazine, and/or
- iii) the acceptor domain is green fluorescent protein (GFP), Venus, mOrange, or a biologically active variant or fragment of any one thereof.

In a further preferred embodiment,

- 25 i) the luciferase is a *Renilla* luciferase, the acceptor domain is GFP², and the substrate is coelenterazine 400a,
- ii) the luciferase is a *Renilla* luciferase 2, the acceptor domain is GFP², and the substrate is coelenterazine 400a,
- iii) the luciferase is a *Renilla* luciferase 8, the acceptor domain is GFP², and the
 30 substrate is coelenterazine 400a,
- iv) the luciferase is a *Renilla* luciferase 2, the acceptor domain is Venus, and the substrate is coelenterazine,
- v) the luciferase is a *Renilla* luciferase 8, the acceptor domain is Venus, and the substrate is coelenterazine,
- 35 vi) the luciferase is a *Renilla* luciferase 8.6-535, the acceptor domain is mOrange, and the substrate is coelenterazine, or

vii) the luciferase is a *Renilla* luciferase 8, the acceptor domain is mOrange, and the substrate is coelenterazine.

More preferably,

i) the luciferase is a *Renilla* luciferase, the acceptor domain is GFP², and the substrate is Coelenterazine 400a,

ii) the luciferase is a *Renilla* luciferase 2, the acceptor domain is GFP², and the substrate is Coelenterazine 400a,

iii) the luciferase is a *Renilla* luciferase 8, the acceptor domain is GFP², and the substrate is Coelenterazine 400a,

iv) the luciferase is a *Renilla* luciferase 8.6-535, the acceptor domain is mOrange, and the substrate is Coelenterazine, or

v) the luciferase is a *Renilla* luciferase 8, the acceptor domain is mOrange, and the substrate is Coelenterazine.

Even more preferably,

i) the luciferase is a *Renilla* luciferase, the acceptor domain is GFP², and the substrate is Coelenterazine 400a,

ii) the luciferase is a *Renilla* luciferase 2, the acceptor domain is GFP², and the substrate is Coelenterazine 400a, or

iii) the luciferase is a *Renilla* luciferase 8, the acceptor domain is GFP², and the substrate is Coelenterazine 400a.

In an embodiment, the method comprises simultaneously or sequentially detecting two or more different analytes using the same microfluidic device, for example using a device as shown in Figures 14b and 14c.

In an embodiment, the microfluidic device comprises one or more sets of

a) three input microchannels, one each for the sensor molecule, substrate and sample, or

b) two input microchannels, one for the substrate and the other for a pre-mixture of the sensor molecule and sample, or

c) two input microchannels, one for the sensor molecule and the other for a pre-mixture of the substrate and sample.

In a further embodiment, at least one microchannel comprises a reaction chamber which has a different volume to at least one other microchannel.

In another embodiment, at least one microchannel comprises two or more reaction chambers of the same or different volume.

In a preferred embodiment, the electro-optical sensing device has at least two different wavelength channels, which may detect overlapping or non-overlapping

wavelengths. In an alternate embodiment, the electro-optical sensing device has a single wavelength channel, wherein in this embodiment the donor quenches emission from the acceptor.

In an embodiment, the electro-optical sensing device comprises fibre bundle or liquid light guides. In an embodiment, diameter of the fibre bundle or liquid light guide is between about 1mm and about 10mm, or about 1mm and about 6mm. In an embodiment, the electro-optical sensing device further comprises a shutter box.

In an embodiment, the electro-optical sensing device comprises a bifurcated light guide, and no dichroic block.

In a preferred embodiment, the sensor molecule is present in a cell-free extract. In an alternate embodiment, the sensor molecule is expressed by cells (for example present on the surface of the cells or secreted by the cells) and provided as a cell suspension where the cells are intact.

The method can be used to sort cells. Thus, in an embodiment, the analyte is exposed on the surface of a cell and the method further comprises diverting cells comprising the analyte through a different microchannel than cells in the sample lacking the analyte, and collecting the cells comprising the analyte and/or collecting the cells lacking the analyte, wherein if both cell types are collected they are collected in separate containers.

In another aspect, the present invention provides a microfluidic system for detecting an analyte in a sample, the system comprising

i) at least one reservoir suitable for containing (or comprising) a sensor molecule comprising a domain that binds the analyte, a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance,

ii) a microfluidic device comprising one or more microchannels,

iii) means for mixing the sensor molecule, the sample and a substrate of the chemiluminescent donor domain in the device,

iv) a reaction chamber for detecting binding of the analyte to the sensor molecule, and

v) an electro-optical sensing device,

wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the analyte binds the sensor

molecule.

In a preferred embodiment, the sensor molecule is not fixed to the microfluidic device.

In a further preferred embodiment, the system can be used to detect the analyte in real time.

5 In another preferred embodiment, the sensor molecule and substrate enter the device through different microchannels.

In a further preferred embodiment, the microfluidic device comprises at least two input microchannels, wherein one of the input microchannels is for flowing the sensor molecule into the device.

10 As the skilled addressee will appreciate, each of the preferred embodiments relating to the method of the invention also relate to the system of the invention and/or how the system can operate.

In an embodiment, the electro-optical sensing device comprises at least two different wavelength channels.

15 In a particularly preferred embodiment, the electro-optical sensing device is capable of simultaneously, or in rapid succession, detecting two different wavelength channels. For example, the electro-optical sensing device is capable of detecting two different wavelength channels in less than 1 second.

In a further embodiment, the microfluidic device is designed to enable the
20 detection of two or more analytes. In an embodiment, the device comprises a separate microchannel for flowing each different sensor molecule into the device.

In an embodiment, the mixing occurs in the reaction chamber.

The present invention can also be used to classify a sample. For this purpose it is not essential that it already be known which analyte(s) in a sample actually bind(s)
25 one or more sensor molecules. Thus, in another aspect the present invention provides a method of classifying a sample, the method comprising

i) flowing through a microfluidic device comprising one or more microchannels,

a) the sample,

b) a sensor molecule comprising a domain that binds one or more analytes,

30 a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte(s), is within $\pm 50\%$ of the Forster distance,

c) a substrate of the chemiluminescent donor,

ii) mixing the sensor molecule, sample and substrate in the device,

35 iii) detecting modification of the substrate by the chemiluminescent donor using an electro-optical sensing device,

iv) processing at least one signal from the electro-optical sensing device and correlating the pattern of electro-optical responses with one or more pre-determined characteristics of one or more samples of interest, and

- 5 v) classifying the sample based on the correlation of the pattern of responses, wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the one or more analytes binds the sensor molecule.

In a preferred embodiment, the above method comprises two or more different sensor molecules each of which binds a different analyte (which may be a different set of analytes) or range of analytes, and step v) comprises classifying the sample based on the presence, absence or concentration of each of the analytes or range of analytes.

In an embodiment, one or more of the analytes are unknown.

In a further embodiment, the method can be used to classify the sample in real time.

- 15 In another embodiment, the sensor molecule is not fixed to the device.

In yet a further embodiment, the sensor molecule and substrate enter the device through different microchannels.

Also provided is a microfluidic system for classifying a sample, the system comprising

- 20 i) at least one reservoir suitable for containing (or comprising) a sensor molecule comprising a domain that binds one or more analytes, a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance,

- 25 ii) a microfluidic device comprising one or more microchannels,

iii) means for mixing the sensor molecule, the sample and a substrate of the chemiluminescent donor domain in the device,

iv) a reaction chamber for detecting binding of the analyte to the sensor molecule, and

- 30 v) an electro-optical sensing device,

wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the one or more analytes binds the sensor molecule.

- In an embodiment, the system comprises two or more different sensor molecules each of which binds a different analyte, which may be a different set of analytes, or range of analytes.

In an embodiment, the system comprises two or more different sensor molecules each of which binds the same analyte at a different site and/or with a different level of affinity.

In another embodiment, one or more of the analytes, or range of analytes, are
5 unknown.

In a further embodiment, the system can be used to classify samples in real time.

In an embodiment, the sensor molecule is not fixed to the device.

In a further embodiment, the sensor molecule and substrate enter the device through different microchannels.

10 In another embodiment, the microfluidic device comprises at least two input microchannels, wherein one of the input microchannels is for flowing the sensor molecule into the device.

In a further aspect, the present invention provides a method of screening for a compound that binds a molecule of interest, the method comprising

- 15 i) flowing through a microfluidic device comprising one or more microchannels,
a) a candidate compound,
b) a sensor molecule comprising the molecule of interest, a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in
20 the presence and/or the absence the candidate compound, is within $\pm 50\%$ of the Forster distance,
c) a substrate of the chemiluminescent donor,
ii) mixing the sensor molecule, the candidate compound and substrate in the device,
25 iv) detecting modification of the substrate by the chemiluminescent donor using an electro-optical sensing device,
v) processing at least one signal from the electro-optical sensing device to determine whether the candidate compound binds the sensor molecule, and
vi) selecting the compound if it binds the sensor molecule,
30 wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the candidate compound binds the sensor molecule.

In a preferred embodiment, the method can be used to detect binding of the candidate compound to the sensor molecule in real time.

35 In a further preferred embodiment, the sensor molecule is not fixed to the device.

In another preferred embodiment, the sensor molecule and substrate enter the device through different microchannels.

In a preferred embodiment, the method further comprises confirming that the candidate compound binds the binding domain of the molecule of interest and not other domains of the sensor molecule. As the skilled person would appreciate, this can be performed using any one of a wide variety of techniques in the art such as using the molecule of interest on a column to capture the candidate compound, competitive binding assays, determining whether following incubation of the candidate compound with the molecule of interest modifies of the migration of the molecule of interest using gel chromatography and so on.

The candidate compound and the molecule of interest can be the same type of substance, for example, both could be nucleic acids, proteins (including peptides) or small molecules. In one embodiment, the molecule of interest is a protein such as, but not limited to, a receptor, odorant binding protein, pheromone-binding protein, enzyme, ligand carrier or bacterial periplasmic binding protein. The molecule of interest may be naturally occurring or a mutant/variant thereof.

In a further aspect, the present invention provides a microfluidic system for screening for a compound that binds a molecule of interest, the system comprising

- i) at least one reservoir suitable for containing (or comprising) a sensor molecule comprising the molecule of interest, a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of a candidate compound, is within $\pm 50\%$ of the Forster distance,
 - ii) a microfluidic device comprising one or more microchannels,
 - iii) means for mixing the sensor molecule, the candidate compound, and a substrate of the chemiluminescent donor domain in the device,
 - iv) a reaction chamber for detecting binding of the candidate compound to the sensor molecule, and
 - v) an electro-optical sensing device,
- wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the candidate compound binds the sensor molecule.

In a preferred embodiment, the system can be used to detect binding of the candidate compound to the sensor molecule in real time.

In a further preferred embodiment, the sensor molecule is not fixed to the device.

In another preferred embodiment, the sensor molecule and substrate enter the device through different microchannels.

In a further embodiment, the microfluidic device comprises at least two input microchannels, wherein one of the input microchannels is for flowing the sensor molecule into the device

The present inventors have also identified a hybrid BRET (BRET^H) detection system that does not suffer from the low luminescence trait of BRET2. Thus, in a further aspect, the present invention provides a method of detecting an analyte in a sample, the method comprising

i) contacting the sample, in the presence of coelenterazine, with a sensor molecule comprising

- a) a domain that binds the analyte,
- b) *Renilla* luciferase, and
- c) green fluorescent protein 2, and

ii) determining whether bioluminescent resonance energy transfer (BRET) between the bioluminescent protein and the acceptor molecule is modified, wherein the spatial location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule is altered when the analyte binds the domain.

Naturally, the above method can readily be used in a method of the invention using a microfluidic device.

In a further aspect, the present invention provides an isolated sensor molecule comprising a domain that binds one or more analytes, *Renilla* luciferase, and green fluorescent protein 2.

The present inventors have identified polypeptides which bind 2-pentanone, and hence these polypeptides can be used to detect this compound.

Accordingly, in a further aspect the present invention provides a method of detecting 2-pentanone in a sample, the method comprising

- i) contacting the sample with a polypeptide which is *C. elegans* str-112 (SEQ ID 41) or str-113 (SEQ ID NO:42), or a variant thereof which binds 2-pentanone, and
- ii) detecting whether any of the polypeptide is bound to 2-pentanone.

As the skilled person would appreciate, there is an enormous array of different assays that can be configured once a new ligand/polypeptide binding pair has been identified. In one embodiment, the methods of the invention are used to detect 2-pentanone in a sample.

In an embodiment, the variant of str-113 is a str-114/str-113 fusion (SEQ ID NO:43).

In an embodiment, the polypeptide is detectably labelled. Examples of such detectably labelled polypeptides include, but are not limited to, those provided as SEQ ID NOs 13, 14, 18, 27, 28 and 30.

2-pentanone is produced by bacteria, and hence the above method can be used to
5 detect, for example, bacterial infections or contaminations.

Thus, is a further aspect the present invention provides a method of detecting bacteria in a sample comprising detecting 2-pentanone using the method of the invention.

In an embodiment, the bacteria is *Escherichia sp.* such as *E. coli*.

10 Any embodiment herein shall be taken to apply *mutatis mutandis* to any other embodiment unless specifically stated otherwise.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the
15 scope of the invention, as described herein.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of
20 compositions of matter.

The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

25 **Figure 1** - Generic arrangement for performing the claimed method, showing the microfluidic chip (microchip) and a BRET detection system. DM = dichroic mirror, BP = band pass filter (wavelength centre in nm, width in nm).

Figure 2 - Thrombin cleavage of GFP²-RG-RLuc fusion protein sensor molecule monitored by (A) Spectral change of the hybrid BRET system upon addition 5 µM of native coelenterazine to GFP²-RG-RLuc fusion protein with and without the addition of 2 units of thrombin and (B) SDS-PAGE analysis of purified His-tagged BRET proteins. 2.5 µg protein loaded per lane. From left to right; Molecular markers (KDa), RLuc, GFP², GFP²-RG-RLuc, GFP²-RG-RLuc following incubation with 54 nM thrombin for
30 90 minutes at 30 °C; GFP²-RG-RLuc same conditions as previous lane except that thrombin has been pre-incubated with 2 units of hirudin for 10 minutes at room temp.

Figure 3 - Change in normalised BRET^H ratio following thrombin cleavage (mean $\pm$ S.D., n=3) of 1 μ M of fusion proteins upon addition of 5 μ M of native coelenterazine; GFP²-RG-RLuc following treatment (90 minutes, 30 °C) with 54 nM thrombin or 54 nM of thrombin following pre-treatment (10 minutes, room temperature) with 2 units of hirudin. Controls consist of 1 μ M of RLuc and GFP² proteins. $p \leq 0.001$ indicates a highly significant difference, $p = 0.33$ indicates the changes are not significant.

Figure 4 - Experimental set-up for on-chip detection. (a) A schematic drawing of the hybrid BRET reaction, and (b and c) the microfluidic chip system for BRET signal detection (Clz = Native coelenterazine, DM = dichroic mirror, BP = band pass, PMT = photomultiplier tube).

Figure 5 - Bioluminescence intensity (AU) of GFP²-RG-RLuc thrombin sensor protein upon the addition of coelenterazine substrate and the BRET^H ratio as a function of distance x from the Y-junction as labelled in Figure 4b. The symbols $+$ and ∇ represent bioluminescence background of RLuc and GFP² channel, respectively. $\circ$ and $\times$ represent bioluminescence intensity of RLuc and GFP² channel, respectively. $\blacklozenge$ represents the BRET ratio. The fusion protein concentration was 3.0 μ M. Native coelenterazine concentration was 58.6 μ M; each aqueous flow rate was 20 μ l/h; a 20 $\times$ objective was used; filter band pass for GFP² and RLuc channel are 515 nm – 555 nm and 430 nm – 455 nm respectively; an internal gate time of 200 ms was used for data acquisition.

Figure 6 - BRET^H ratios as a function of total flow rate of the aqueous streams (a) and the thrombin sensor concentration (b). (a) Thrombin sensor protein concentration was 3.0 μ M and native coelenterazine concentration was 58.6 μ M; (b) Native coelenterazine concentration was 58.6 μ M; each aqueous flow rate was 20 μ l/h; a 20 $\times$ objective was used; filter band pass for GFP² and RLuc channel are 515 nm – 555 nm and 430 nm – 455 nm respectively; an internal gate time of 200 ms was used for data acquisition.

Figure 7 - Calibration curves for the BRET^H thrombin sensors in microfluidic and microplate formats (mean $\pm$ SD, n=5). All microfluidic measurements were obtained at $x = 2.1$ mm. $\circ$ and $\square$ represent data for microchip and microplate measurements, respectively. The main graph shows the data at low thrombin concentrations while the

insets show the corresponding full-range measurements. The lines are the linear regressions, which are $y=0.835x+1.019$ ($R^2=0.996$) for the microchip data and $y=0.1797x+1.001$ ($R^2=0.995$) for the microplate data. For the microchip method, the fusion protein concentration was $3.0\ \mu\text{M}$ and native coelenterazine concentration was $58.6\ \mu\text{M}$, each aqueous flow rate was $20\ \mu\text{l/h}$. For the microplate method, the fusion protein and native coelenterazine concentrations were $1\ \mu\text{M}$ and $5\ \mu\text{M}$, respectively

Figure 8 - Examples of passive mixing designs (a) Y-shape with linear contact region (b) narrow serpentine, (c) wide serpentine, (d) spiral, (e) Y-shape channel with size variations and baffles, and (f) three inlet with narrow serpentine contact region.

Figure 9 – Schematics of a particular example of a chip and on-chip optical detection system of the invention. PMT - photomultiplier tube. PDMS - polydimethylsiloxane chip matrix.

Figure 10 – Sample data indicates detection of thrombin by the change in BRET^2 ratio of a $\text{GFP}^2\text{-RG-RLuc}$ thrombin sensor. a. GFP (green – top) and *Renilla luciferase* (blue – bottom) channel emission intensities with no thrombin present. b. GFP (green – bottom) and *Renilla luciferase* (blue – top) channel emission intensities following incubation of the sensor with $270\ \text{pM}$ thrombin. c. BRET^2 ratio for the no thrombin and $270\ \text{pM}$ thrombin conditions.

Figure 11 - Direct comparison of the sensitivity of thrombin detection by BRET^2 measurement, using a $\text{GFP}^2\text{-RG-RLuc}$ thrombin sensor in the microfluidic device of Figure 9 (blue line) compared with the results from a commercially available plate reader instrument (red line).

Figure 12 - BRET^2 ratio measured with a two inlet microfluidic device upon mixing thrombin biosensor ($1\ \mu\text{M}$) with a preparation containing thrombin ($540\ \text{nM}$) and coelenterazine 400a substrate ($12.5\ \mu\text{M}$).

Figure 13 - Example of the subsystems of a system of the invention.

Figure 14 – Example of single and multiple sensor sensor chip designs.

Figure 15 – Example of the zone of passive or diffusion-based reagent mixing when three microfluidic flows come into contact.

Figure 16 – Example of electro-optical detection system.

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Figure 17 - Designs of two BRET optical detection elements (a) a double-convex microlens serves at the bottom of the reaction chamber to collect fluorescence from many BRET point sources inside the reaction chamber and focus onto a multimode optical fiber (core 200 μm) (b) a plano-convex microlens also serve as the bottom of the reaction chamber. However an aspherical micro mirror on top (micro-machined on the ferrule) will collect fluorescence from the top of the BRET point sources and collimate onto the microlens. Similar to (a), the plano-convex microlens will then focus the fluorescence into the core of the optical fiber.

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15 **Figure 18** - Principle of resonance energy transfer in ODR-10 receptor constructs fused to RLuc2 and GFP². GFP² is inserted in the third intracellular loop of ODR-10 and RLuc2 at the C-terminus (OGOR2). Diacetyl binding causes a conformational change in the OGOR2 biosensor resulting in an increase in distance, or a change in the orientation of dipole moments, between the BRET² components. Clz400a =
20 Coelenterazine 400a substrate.

Figure 19 - Bioluminescence intensity of OGOR and OGOR2 sensors upon addition of 5 μM Clz400a to 20 nM of the sensor.

25 **Figure 20** - Bioluminescence intensity of OGOR and OGOR2 sensors upon on-chip mixing of 12.5 μM Clz400a to 1 μM of the sensor.

Figure 21 - BRET² signal from OGOR2 in the wells of a 96-well plate following incubation with 1 μM diacetyl in water, or a water only control (mean $\pm$ SD, n=3).

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Figure 22 - Diacetyl concentration response curve for BRET² signal of OGOR2 in a microwell plate format.

Figure 23 - Change in BRET² signal detected with on-chip microfluidic assay of OGOR2
35 signal following incubation with 10 fM diacetyl in PBS or a PBS only as control (mean $\pm$ SD, n=3).

Figure 24 - Dose response of OGOR2 with on-chip (microfluidic) assay measurements.

5 **Figure 25** - Averaged change in BRET² signal detected using real-time microfluidic measurement upon on-chip contact of OGOR2 over a range of flow rates. 1 fM diacetyl in PBS or PBS only and 12.5μM Clz400a substrate (mean ± SD of BRET² ratio at four different flow rates).

10 **Figure 26** - BRET² ratio measured at different common flow rates with a three-inlet microfluidic device upon contacting OGOR2 (290nM) with 1femtomolar diacetyl solution in PBS and with 12.5μM coelenterazine 400a.

Figure 27 - Principle of BRET in MBP receptor constructs fused to BRET² components GFP² and RLuc2. Maltose binding causes a conformational change in the BRET² tagged MBP receptor bringing the BRET² components in closer proximity causing an increase in the efficiency of energy transfer from RLuc2 to GFP². Clz400a = Coelenterazine 400a substrate.

20 **Figure 28** - Effect of 0.1 mM of various sugars on the BRET² ratio of the GFP²-MBP-RLuc2 sensor. BRET² ratio (Mean ± SD, n=3) was recorded following addition of 16.7 μM coelenterazine 400a to 1 μM GFP²-MBP-RLuc2 or W140A mutant (hatched bar) following incubation with water (grey bar) or 0.1 mM of the stated sugars for 30 minutes at 28 ° C. BRET² ratios were normalized by the water response. ** P < 0.01 and P * < 0.05.

Figure 29 - Response time (minutes, mean ± S.D., n=3) of 1 μM GFP²-MBP-RLuc2 to 0.1 mM Maltose upon addition of 16.67 μM coelenterazine 400a. The BRET² response following incubation with maltose for any time period was normalized by the BRET² response following incubation with water for the same time period.

Figure 30 - (A) FRET vs BRET². Maltose concentration dependence of the BRET² response (mean ± SD, n=11) of 1 μM GFP²-MBP-RLuc2 fusion protein upon addition of 16.67 μM coelenterazine 400a compared to the FRET response (mean ± SD, n=3) of FLIPmal-2μ (530/485-nm ratio). The latter dose-response curve was re-plotted from data presented by Fehr et al. (2002). Data was fitted to a log [Agonist] vs response.

BRET² EC₅₀ = 0.4 μ M and FRET EC₅₀ = 3.2 μ M. (B) Comparison of BRET²-based MBP assay for maltose on a microfluidic chip versus using a microplate assay.

Figure 31 - Experimental setup for collecting data from a microfluidic chip system without (A) and with (B) an in-line optical fibre switch. BP - band pass, PMT - photomultiplier; NA - numerical aperture.

Figure 32 - Real-time Rluc/Clz400a bioluminescent signal collected without (A) and with optical switch (B) for three runs.

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Figure 33 - Mean bioluminescent signal comparison for Rluc/Clz400a and GFP channels without and with optical switch.

Figure 34 - Example showing arrangement of increased diameter BRET reaction chamber ($\varnothing$ = 4mm, h = 2 mm) coupled through a liquid light guide to the photodetector.

Figure 35 - Comparison of performance of narrow vs wide bore BRET detection chamber/optical system. In both cases BRET emissions are measured from a 1/100 dilution of an OGOR2 sensor flowing through the microfluidic channel. (a) narrow bore system (chamber size $\varnothing$ = 2mm, h = 2mm; fiber core diameter = 1 mm, NA = 0.48). (b) wide bore system (chamber size $\varnothing$ = 4mm, h = 2mm, light guide core diameter = 5 mm, NA = 0.59).

Figure 36 - Demonstration of detection of 1 μ M diacetyl using highly diluted OGOR2 sensors, using a more efficient light capture system based on a wider diameter BRET reaction chamber (4 mm) and a wide bore liquid light guide. (a) 100 times sensor dilution. Diacetyl-dependent reduction in BRET² ratio = 13.7% and (b) 50 times sensor dilution, Diacetyl-dependent reduction in BRET² ratio = 14.7%. Error bars represent the standard deviation for 3 experiments (N = 3).

Figure 37 - Example of a single microfluidic channel and BRET light collection system using bifurcated light guide and without dichroic block. Additional sets of bifurcated light guides with filters and pairs of photodetectors can be added to accommodate one or more additional microfluidic channels and/or BRET reaction chambers.

Figure 38 - Optical architecture with a shutter box facilitating multichannel measurements

- 5 **Figure 39** - Comparison of the strength of BRET signals detected with a bifurcated light guide or a dichroic block. a, b: bifurcated light guide, reaction chamber dimensions $\varnothing = 4\text{mm}$; $h = 1\text{mm}$, with and without reflective lid. c,d: single light guide with dichroic block, reaction chamber dimensions $\varnothing = 4\text{mm}$; $h = 2\text{mm}$ (i.e. double the volume of a, b). All panels show the increase in signal with time, following initiation
10 of flow at $t = 0$. Panels a & c blue channel, panels b & d green channel

Figure 40 - Multifurcated light guide arrangement suitable for measuring BRET outputs from a shutterbox.

- 15 **Figure 41** - Comparison of multifurcated light guide converging on a single dichroic block against bifurcated light guides diverging to two separate colour filters. Relative light intensity collected in the blue (RLuc; 1, 3) and green (GFP; 2, 4) channels. 1, 2: Multifurcated light guide with inputs allocated to different microfluidic channels and output directed to a pair of PMTs via an optimised dichroic filter as per Figures 38 &
20 40. 3, 4: Bifurcated light guide with output allocated to different spectral channels, as per Figure 37.

Figure 42 - Example of an ultra low level light photodetector using vacuum photomultiplier technology. Source Hamamatsu

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Figure 43 - Example of an ultra low light level photodetector implemented using solid state technology. Dimensions in mm. Source: Hamamatsu

- Figure 44** - Direct visualisation of laminar flow and diffusional mixing in a three-inlet
30 microfluidic device operating at two different flow rates. Two of the three aqueous inputs are colored with blue or red food colouring. The device was operated with a single syringe pump working in withdrawal mode. Direction of flow: right to left.

- Figure 45** - Passive mixing example design based on vertically stacked streamsThe
35 width of the channel is starting from $600\mu\text{m}$ up to 2mm , the thickness is $20\text{--}60\mu\text{m}$. The length is flexible starting from 20mm to 100mm , the angle is 45 degrees (which may be

varied over a wide range, from 0° to close to approximately 170° or indeed at an angle to the plane of the microfluidic chip).

Figure 46 - Comparison of BRET ratio measurements. Error bars reflect the standard deviation (n=3).

Figure 47 - Microfluidic chip design example for multiplexed detection. The inlet at the top is designated for substrate while the three inlets at the bottom are used for introducing sensor 1, sample and sensor 2.

Figure 48 - Microfluidic chip design example for parallel detection. Flow direction is bottom to top. Flow rates were 150 µl/hr and 1500 µl/hr. Red food dye was introduced from the substrate inlet and blue dye was introduced from the sensor inlet. No food coloring was used for the sample inlet.

Figure 49 - Three inlet microfluidic device operated by a single suction pump in withdrawal mode. A pump in suction mode creates negative pressure at the device outlet. Sample, sensor and substrate streams are sucked into the common channel (shown as a serpentine arrangement) and are passively mixed. A BRET reaction chamber is situated just before the outlet. The pump used here is a syringe pump, a wide variety of pumping methods could also be used.

Figure 50 - Architecture for parallel independent operation using syringe pumps working in withdrawal mode. An example in which four sensor channels are operated independently, in parallel, by using four separate syringes. This enables the operation of the sensor channels at different flow rates to meet a range of different requirements.

Figure 51 - a. RLuc and GFP signals from on chip thrombin sensor operating in suction mode with multifurcated light guide and optimised dichroic block. Reaction chamber Ø = 2mm, H = 1 mm. b. BRET² signal from "a". c. Demonstration of expected signal for chambers of the same diameter and varying heights based on the equivalent residence times.

Figure 52 - Specific maltose detection achieved in a microfluidic format. Comparison of the BRET² responses of a BRET²-MBP sensor to 0.1 mM maltose, glucose and sucrose.

Figure 53 - BRET² thrombin sensor with GFP² at the N-terminus and RLuc2 at the C-terminus has been modified to mimic plasmin's κ -casein target sequence: XKZX, where Z = K, Y, V or E.

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Figure 54 - BRET² signal intensity and ratio generated in various dilutions of PBS, milk c) and d) or orange juice (a and b) using the GFP²-FL₁-RLuc2 sensor in the presence of 5 μ M coelenterazine

10 **Figure 55** - Time course of a BRET² signal generated in undiluted whole milk using the GFP²-FL₁-RLuc2 sensor in the presence of 5 μ M coelenterazine A. a. Intensity. b. BRET² ratio.

15 **Figure 56** - Bioluminescent intensity (GFP² channel - 515 nm bandpass 30nm) for GTR2 protein in thrombin cleavage buffer or various dilutions of (a) serum (b) orange or (c) milk.

Figure 57 - BRET² ratio GTR2 protein in thrombin cleavage buffer or various dilutions of (a) serum (b) orange or (c) milk.

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Figure 58 - Detection of thrombin protease activity (2 units) in thrombin cleavage buffer, diluted milk, orange juice or serum using GTR2. Results are presented as normalised BRET² ratios because the absolute BRET² ratios vary according to the sample and its dilution. Note that the absence of an effect in the 1/10 dilution of serum is possibly due to the inactivation of added thrombin by residual activated antithrombin III generated by the heparin used in serum preparation.

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Figure 59 - Specimen holder for gas-liquid partition experiments

30 **Figure 60** - Timecourse of uptake of oxygen into deoxygenated SASS2400 sample fluid following fan start up at time = 0.

Figure 61 - Timecourse of phenol uptake into SASS2400 sample fluid following fan start up at time = 0. Relative phenol concentration indicated using arbitrary units.

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Figure 62 - 3-way L port pneumatically operated ball valve. This allows rapid (≤ 1 s) switching of the volatile headspace into the SASS2400 air inflow during active sampling.

- 5 **Figure 63** - Schematic showing set up for SASS2400 testing with three way valve for rapid switching of air intake

Figure 64 - Selectivity of Str 112 (A), Str114/113 (B) and Str113 (C). BRET2 response of Str112 (mean $\pm$ SD, n=3), Str114/113(mean $\pm$ SD, n=14) and Str113 (mean $\pm$ SD, n=6) to 1 μ M of odorant or water (grey bar),. **** P < 0.0001, ** P < 0.001 and * P < 0.05.

Figure 65 - BRET responses of BRET tagged SGSR-112 (A) and SGSR-114/113 (B) nematode odorant receptors to 2-pentanone.

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Figure 66 - BRET response of BRET tagged (A) Str114/113 nematode odorant receptor to diacetyl and 2-pentanone and (B) Str-113 nematode odorant receptor to 1-hexanol.

- 20 **Figure 67** - Real time, continuous, on-chip detection of 1 μ M maltose using GMR BRET²-based sensor. A) Channel luminance changing with time at 100 μ L/hour input rate B) BRET² ratio changing with time at 100 μ L/hour input. C) Channel luminance changing with time at 200 μ L/hour input rate D) BRET² ratio changing with time at 200 μ L/hour input rate. E) Comparison of BRET² ratio between water control and 1 μ M maltose averaged from 200-250 seconds at 100 μ L/hour. F) Comparison of BRET² ratio between water control and 1 μ M maltose averaged from 200-250 seconds at 200 μ L/hour.

KEY TO SEQUENCE LISTING

- 30 SEQ ID NO:1 – Nucleotide sequence encoding OGOR2 fusion protein.
 SEQ ID NO:2 - OGOR2 fusion protein.
 SEQ ID NO:3 - Nucleotide sequence encoding GFP²-MBP-RLuc2 fusion protein.
 SEQ ID NO:4 - GFP²-MBP-RLuc2 fusion protein.
 SEQ ID NO:5 - Nucleotide sequence encoding GFP²-MBP-RLuc2 W140A fusion
 35 protein.
 SEQ ID NO:6 - GFP²-MBP-RLuc2 W140A fusion protein.

- SEQ ID NOs:7 to 12 – Oligonucleotide primers.
- SEQ ID NO:13 - GFP²-str-112 SGSR-RLuc fusion protein.
- SEQ ID NO:14 - GFP²-str-113 SGSR-RLuc fusion protein.
- SEQ ID NO:15 – GFP²-str-114 SGSR-RLuc fusion protein.
- 5 SEQ ID NO:16 - GFP²-str-115 SGSR-RLuc fusion protein.
- SEQ ID NO:17 - GFP²-str-116 SGSR-RLuc fusion protein.
- SEQ ID NO:18 - GFP²-str-114/113 SGSR-RLuc fusion protein.
- SEQ ID NO:19 - Nucleotide sequence encoding GFP²-str-112 SGSR-RLuc fusion protein.
- 10 SEQ ID NO:20 - Nucleotide sequence encoding GFP²-str-113 SGSR-RLuc fusion protein.
- SEQ ID NO:21 - Nucleotide sequence encoding GFP²-str-114 SGSR-RLuc fusion protein.
- SEQ ID NO:22 - Nucleotide sequence encoding GFP²-str-115 SGSR-RLuc fusion
- 15 protein.
- SEQ ID NO:23 - Nucleotide sequence encoding GFP²-str-116 SGSR-RLuc fusion protein.
- SEQ ID NO:24 - Nucleotide sequence encoding GFP²-str-114/113 SGSR-RLuc fusion protein.
- 20 SEQ ID NO:25 - GFP²-OGOR-RLuc2 fusion protein.
- SEQ ID NO:26 - GFP²-OGOR mutant-RLuc2 fusion protein.
- SEQ ID NO:27 - GFP²-str-112 SGSR-RLuc2 fusion protein.
- SEQ ID NO:28 - GFP²-str-113 SGSR-RLuc2 fusion protein.
- SEQ ID NO:29 - GFP²-str-114 SGSR-RLuc2 fusion protein.
- 25 SEQ ID NO:30 - GFP²-str-114/113 SGSR-RLuc2 fusion protein.
- SEQ ID NO:31 - GFP²-str-115 SGSR-RLuc2 fusion protein.
- SEQ ID NO:32 - GFP²-str-116 SGSR-RLuc2 fusion protein.
- SEQ ID NO:33 - Nucleotide sequence encoding GFP²-OGOR-RLuc2 fusion protein.
- SEQ ID NO:34 - Nucleotide sequence encoding GFP²-OGOR mutant-RLuc2 fusion
- 30 protein.
- SEQ ID NO:35 - Nucleotide sequence encoding GFP²-str-112 SGSR-RLuc2 fusion protein.
- SEQ ID NO:36 - Nucleotide sequence encoding GFP²-str-113 SGSR-RLuc2 fusion protein.
- 35 SEQ ID NO:37 - Nucleotide sequence encoding GFP²-str-114 SGSR-RLuc2 fusion protein.

SEQ ID NO:38 - Nucleotide sequence encoding GFP²-str-114/113 SGSR-RLuc2 fusion protein.

SEQ ID NO:39 - Nucleotide sequence encoding GFP²-str-115 SGSR-RLuc2 fusion protein.

- 5 SEQ ID NO:40 - Nucleotide sequence encoding GFP²-str-116 SGSR-RLuc2 fusion protein.

SEQ ID NO:41 - *C. elegans* str-112.

SEQ ID NO:42 - *C. elegans* str-113.

SEQ ID NO:43 - *C. elegans* str-114/113 chimeric protein.

- 10 SEQ ID NO:44 - Open reading frame encoding *C. elegans* str-112.

SEQ ID NO:45 - Open reading frame encoding *C. elegans* str-113.

SEQ ID NO:46 - Open reading frame encoding *C. elegans* str-114/113 chimeric protein.

15 **DETAILED DESCRIPTION OF THE INVENTION**

General Techniques and Definitions

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, immunology, immunohistochemistry, protein chemistry, and biochemistry).

- 20 Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors),
- 25 Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan *et al.* (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).
- 30

The term "and/or", e.g., "X and/or Y" shall be understood to mean either "X and Y" or "X or Y" and shall be taken to provide explicit support for both meanings or for either meaning.

Unless the context suggests otherwise, the mention of a term in singular such as
5 sensor molecule and substrate clearly means the plural as well. For instance, logically many individual sensor molecules will be flowed through the device rather than a single molecule.

As used herein, the term about, unless stated to the contrary, refers to +/- 20%, more preferably +/- 10%, even more preferably +/- 5%, of the designated value.

10 Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

15 Detection/Classification/Screening System

The present invention relates to a method of detecting an analyte in a sample, the method comprising

- i) flowing through a microfluidic device comprising one or more microchannels,
 - a) the sample,
 - 20 b) a sensor molecule comprising a domain that binds the analyte, a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance,
 - c) a substrate of the chemiluminescent donor,
- 25 ii) mixing the sensor molecule, sample and substrate in the device, and
- iii) detecting modification of the substrate by the chemiluminescent donor using an electro-optical sensing device,
wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the analyte binds the
30 sensor molecule.

The present invention also relates to a microfluidic system for performing the method of the invention, the system comprising

- i) at least one reservoir suitable for containing (or comprising) a sensor molecule comprising a domain that binds the analyte, a chemiluminescent donor domain and an
35 acceptor domain, wherein the separation and relative orientation of the

chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance,

ii) a microfluidic device comprising one or more microchannels,

iii) means for mixing the sensor molecule, the sample and a substrate of the chemiluminescent donor domain in the device,

iv) a reaction chamber for detecting binding of the analyte to the sensor molecule, and

v) an electro-optical sensing device,

wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the analyte binds the sensor molecule.

As the skilled person would appreciate, the methods and systems of the invention can be used to detect the presence or absence of an analyte in a sample, and if present may also be used to determine the concentration of the analyte.

The present invention has numerous advantages over the prior art, particularly when compared to methods and systems where the sensor molecule is fixed to the device. First, there is no need to regenerate (re-set) the device. Second, there is less drift in signal in the methods and systems of the invention. Third, costs are reduced because the device can be re-used many more times than when the sensor molecule is fixed. Fourth, the invention avoids the problem of low signal with fixed configurations due to surface area and density of sensor molecule. Fifth, the current technique is a volume-based detection technique, not a surface-based technique such as surface plasmon resonance in which the sensor molecules need to be attached to the surface. The sensor-analyte reaction happens much more rapidly, thereby reducing analysis time even without active control.

Furthermore, BRET has several advantages over fluorescence based technologies because it does not require excitation of the donor with an external light source. BRET does not suffer from autofluorescence, light scattering, photobleaching and/or photoisomerization of the donor moiety or photodamage to cells. The absence of an external light source in BRET assays results in a very low background and consequently increased detection sensitivity. For example, BRET is 50 times more sensitive than FRET for monitoring thrombin-catalysed proteolytic cleavage (Dacres et al., 2009b).

With regard to the use of a method of the invention for classifying a sample, a sensor molecule, more typically a set of sensor molecules, can be used to detect patterns of substances (analytes) that are representative for a specific sub-population.

As an example, the method can be used to classify different types, age, quality etc of beer, wine, cheese or other consumables. The method can also be used broadly with samples of foods, beverages, perfumes, fragrances and the like to classify or quantify their organoleptic properties such as sweetness, bitterness, umami character, "heat" for example in relation to capsaicin or hydroxy- α -sanshool, "coolness" for example in relation to menthol and/or any olfactory notes for which suitable sensor molecules can be isolated or engineered. The method can also be used to classify a wide range of other samples based on chemical signatures, for example the health, nutritional or disease status of humans, animals or plants based on samples of headspace, breath, sweat, urine, other biological fluids. Another use of the method is to classify samples based on their toxicity or noxiousness, such as the presence of explosives or explosive-associated components, toxic industrial chemicals, chemical or biological warfare agents or pathogenic microbes. The method can also be used for monitoring of industrial processes including conformity to specifications or the presence, absence of levels of any group or groups of chemicals. The method can also be used to classify environmental samples either in real time or in batch mode, for example to determine air quality, presence or level of unpleasant odours or toxic chemicals or, similarly, the quality of natural or reticulated water systems, sewerage systems or ground water or to classify fluids in contact with soils or rocks.

Classification of samples is usually performed by generating a discriminating function or classifier based on the pattern of electro-optical responses to a training set of samples representing or encompassing all the classes of samples that one wishes to discriminate. This may be achieved routinely using multivariate statistical approaches, such as principal components analysis, linear discriminant analysis, stepwise discrimination analysis and the like. Alternately, a wide variety of machine learning approaches may be used, one example being support vector machines. A similar approach is to use Bayesian networks or train an artificial neural network to make such discriminations among samples of the test set. One viable approach is then to capture a pattern of electro-optical responses from the test or unknown sample(s) process them in real time and compare them with saved patterns, obtained with the training set of samples, assigning them to known classes according to the best matches or assigning them to a novel class or classes if a similar pattern has not previously been observed. For classification methods it is not essential that the actual analyte(s) be known, simply that a sensor molecule (or group of sensor molecules) reproducibly produce a different pattern of signals with different classes of sample, which enables the user to classify the sample(s) being analysed.

With regard to sensitivity, concentrations of analyte as low as micromolar, nanomolar, femtomolar, attomolar or even lower can be detected. In an embodiment, the method of the invention is at least 5 fold, or at least 10 fold, or a 5 fold to 1,000 fold, or 5 fold to 100 fold, or 5 fold to 50 fold, or 5 fold to 20 fold, or and in some
5 circumstances up to 100 to 1,000 fold more sensitive than if the method was performed on a microwell plate with the same concentration of reagents.

The present invention is particularly useful for detecting an analyte in real time. As used herein, the term "real time" means that a certain state is substantially simultaneously displayed in another form (e.g., as an image on a display or a graph
10 with processed data). In such a case, the "real time" lags behind an actual event by the time required for data processing. Such a time lag is included in the scope of "real time" if it is substantially negligible. Such a time lag may be typically within 10 seconds, and preferably within 1 second, without limitation. In a preferred embodiment, the method of the invention is performed within about 1s to about 100s.

15 As used herein, the "Förster distance" is the distance between the donor and acceptor at which the energy transfer is (on average) 50% efficient. Förster distance (R_0) is dependent on a number of factors, including the quantum yield of the donor in the absence of acceptor, the refractive index of the solution, the dipole angular orientation of each domain, and the spectral overlap integral of the donor and acceptor.

20 As used herein, "quantum yield" refers to a measure of final emission of original energy donation.

As used herein, "Stokes shift" is the difference in wavelength between positions of the band maxima of the absorption and emission spectra of the same electronic transition.

25 In an embodiment, the invention is used to analyse, on the device, an increasing (for example, through synthesis of the analyte on the chip) or decreasing (for example, degradation or modification of the analyte on the chip) concentration of the analyte. Typically, this will require detecting modification of the substrate at two different points on the device, for instance in a first and second reaction chamber through which
30 the analyte flows. Thus, in an embodiment, the analyte releasing from the sensor molecule results in a change in BRET ratio which is $\geq 15\%$, $\geq 20\%$, $\geq 30\%$, $\geq 35\%$, about 15% to about 50%, or about 15% to about 40%, of the maximum observed BRET ratio.

In a further embodiment, the sensor molecule may enter the input microchannel bound to a ligand, and the analyte to be detected (for example catalytic enzyme)
35 cleaves and/or modifies the ligand such that the modified ligand releases from (is no longer bound) to the sensor molecule.

The BRET sensing in the methods and systems of the invention is realized in a microfluidic device. In one configuration, the system comprises several modules which include (1) sample delivery, (2) reagent storage and handling, (3) microfluidic chip and loading system, (4) optionally temperature control, (5) electro-optical system for light collection, (6) electro-optical detection system, (7) data acquisition and processing, and (8) software and embedded control system (Figure 13).

Sample delivery

The "sample" can be any substance or composition known or suspected of comprising the analyte to be detected or from which it is expected or required that a particular substance, set of substances or composition is absent. Examples of samples include air, liquid, and biological material. The sample may be obtained directly from the environment or source, or may be at least partially purified by a suitable procedure before a method of the invention is performed.

The sample can be in any form that is capable of being flowed through a microfluidic device. Examples include, but are not necessarily limited to, a liquid, gas, emulsion or suspension. In an embodiment, the sample is a liquid, which has been pre-equilibrated with a gas. Examples of suspensions include, but are not necessarily limited to, water-in-oil, oil-in-water and gas in liquid.

In a more specific embodiment, ambient air or other gases from a location of interest or the headspace from any object or sample of interest is brought into close contact with water or an aqueous solution so that rapid mass transfer of analytes may occur from the gas phase to the liquid phase based on the gaseous concentration, solubility and partition coefficients of the analytes and the composition of the liquid phase. Any method that generates a gas-liquid interface with a large area, relative to the volume of the liquid, is potentially suitable. Example methods include wetted wall cyclones, misting or bubbling systems. The SASS2400 wetted wall cyclone is a specific example of a suitable device for accelerating the partition of volatiles from air into an aqueous phase. Preferably, the method allows a large volume of air to come into contact with a smaller volume of liquid, thereby permitting large volumes of the gas phase to be sampled and providing a concentration step. Based on the published specifications of the SASS2400 it is possible to contact 1 volume of water or aqueous solution with 40,000 volumes of gas at standard temperature and pressure per minute. Depending on the dimensions and operational characteristics of equipment used, much lower or higher gas-liquid ratios may be sampled.

In one embodiment, the suspension is, or comprises, a cell-free extract. In an alternate embodiment, the suspension comprises cells.

The sample (and the sensor molecule and substrate) can be flowed through the microfluidic device by any suitable means such as, but are not necessarily limited to, one or more of pumping, vacuum, hydraulics, suction, electrokinesis, chemiosmosis, capillary force, acoustics, electromagnetics, piezoelectrics and so on. Pumping mechanisms can be realised in compact, miniaturised and micron-size pumps for the applications. There may be a pre-conditioning device to filter out debris such as particles, organic droplets and so on from the sample, and/or a condition monitoring device which measures some basic parameters of the samples such as temperature, humidity, flow rate and volume of samples.

Reagent storage and handling

A disposable and retractable liquid storage system for multiple reagents (BRET reagent, cleaning DI water, substrate etc.) storage can be used. The device can be pre-loaded in the laboratory and be inserted into the sensing device during operation.

Microfluidic device and loading system

Microfluidic devices (also referred to in the art as a chip or “lab-on-a-chip”) perform chemical or biochemical reactions or analyses by manipulating fluid reagents in chambers and passages which are generally sized in cross-section from approximately 10 to 50 μm (micrometers) up to approximately 100 to 1000 μm , and which are formed on or in a usually flat substrate having linear dimensions from approximately 1 mm to approximately 20 cm. A microfluidic device may manipulate fluid reactants as they flow through the passages and chambers of the device, either as continuous flows from input reservoirs through the device to outlet ports, or as semi-continuous flows of fluid aliquots substantially filling the passages and chambers of the device during operation. Alternatively, microfluidic devices may manipulate fluid reagents as separate and discrete micro-droplets that are characterized by having lengths that are approximately an order of magnitude or more smaller than the dimensions of the device.

The microfluidic device may maintain connection with all sample and reagent delivery outlet once it is inserted into a chip loading system. This loading system can form part of the temperature control and optical components. Single to multiple reactors can be integrated into a chip (Figure 14). Three reagent (sample, sensor molecule and substrate) flows can be pumped into the chip and mixed in the mixing

region. In an alternate configuration, there are two input microchannels, one for the substrate and the other for a pre-mixture of the sensor molecule and sample. In a further configuration, there are two input microchannels, one for the sensor molecule and the other for a pre-mixture of the substrate and sample.

- 5 The mixed reagents will undergo BRET reactions and the products can be continuously pumped through the detection chamber before being collected from the waste outlets (Figure 15).

As used herein, the term “mixing” or variations thereof mean that the analyte(s), sensor molecule and substrate come into contact through any kind of means whether it
10 be diffusion (for example resulting from linear (laminar) flow) and/or through some sort of active mixing means. Thus, in one embodiment the “means for mixing” can be passive diffusion in a linear (laminar) flow. In this embodiment, although complete mixing is not achieved, the present inventors have found that a sufficient amount of mixing occurs for the methods of the invention to function properly. In an
15 embodiment, the diffusion mixing results in at least 20% of the microchannel comprising the sample, sensor molecule and substrate having a homogeneous mixture of these components.

The angle at least two microchannels converge to form the common microchannel can vary from 0° to close to approximately 170° or indeed at an angle to
20 the plane of the microfluidic chip (device).

As used herein, the term “common microchannel” or variations thereof refers to a microchannel, or section thereof, comprising the sample, sensor molecule and the substrate.

As used herein, the term “input microchannel” or variations thereof refers to the
25 microchannel through which a particular reagent such as the sample, sensor molecule or the substrate, or combination of reagents, enters the microfluidic device.

In at least some embodiments, due to the existence of a laminar flow region, a mixing means is preferably implemented in the mixing region for enhancing the contact of the reactants. The mixing means may include passive mixing (Figure 8 and Figure
30 44) and/or active mixing such as with an acoustic mixer (for example as described in WO 2006/105616). Other mixing techniques can also be implemented for such a purpose such as that described in WO 2003/015923.

As used herein, the term “mixing the sensor molecule, sample and substrate in the device” and variations thereof encompasses mixing the sensor molecule, sample
35 and substrate in a reservoir of the device, mixing the sensor molecule, sample and substrate in tubes which flow into the microchannels of the device, mixing the sensor

molecule, sample and substrate in the microchannels of the device or mixing the sensor molecule, sample and substrate in a reaction chamber, or a combination or two or more thereof. In a preferred embodiment, the sensor molecule, sample and substrate are mixed in a microchannel. Preferably, if the sensor molecule and sample are not mixed
5 in the microchannels they are mixed shortly before (for example 10 seconds, more preferably 1 second, or less) before entering the microchannels.

In an embodiment, the mixing step results in the sensor molecule, substrate and analyte forming a mixture which is at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%,
10 homogeneous. As indicated above, active mixing will result in greater levels of homogeneity for any given flow rate and channel architecture, but this is not necessarily required to perform the invention.

The methods of the invention can be used to simultaneously or sequentially detect two or more different analytes using the same microfluidic device, for example
15 using a device as shown in Figures 14b and 14c. In an embodiment, different sensor molecules are flowed into the device using different microchannels. For convenience, if the sample to be analysed is the same for each of the analytes there may be a single flow of sample into the device which then branches and joins with other channels comprising different sensor molecules (see Figures 14b and 14c). The same applies if
20 the substrate for each of the sensor molecules is the same. The skilled person can readily design a suitable configuration for the microchannels depending on the number of samples to be analysed, the number of sensor molecules required to detect each target analyte, and the number of corresponding substrates required in light of the different sensor molecules being used.

25 Microfluidic devices can be fabricated from any material that has the necessary characteristics of chemical compatibility and mechanical strength. Examples of such substances include, but are not necessarily limited to, silicon, glass (e.g. fused silica, fused quartz, boro-silicate, or any type of glass with different additives), polydimethylsiloxane, polyimide, polyethylene terephthalate, polymethylmethacrylate,
30 polyurethane, polyvinylchloride, polystyrene polysulfone, polycarbonate, polymethylpentene, polypropylene, a polyvinylidene fluoride, polysilicon, polytetrafluoroethylene, polysulfone, acrylonitrile butadiene styrene, polyacrylonitrile, polybutadiene, poly(butylene terephthalate), poly(ether sulfone), poly(ether ether ketones), poly(ethylene glycol), styrene-acrylonitrile resin, poly(trimethylene
35 terephthalate), polyvinyl butyral, polyvinylidenedifluoride, poly(vinyl pyrrolidone), cyclic olefin-copolymer and any combination thereof.

The device (chip) can be constructed using standard techniques in the art such as single and multilayer soft lithography (MSL) techniques and/or sacrificial-layer encapsulation methods (see, e.g., Unger et al. (2000); WO 01/01025). Further methods of fabricating microfluidic devices include micromachining, micromilling, laser-based machining, chemical etching, (deep) reactive ion etching, imprinting techniques. These techniques can be used in conjunction with hot embossing and/or injection moulding techniques for mass production of the microdevices. There is a large body of prior art in fabrication techniques. Some of these are also described in US 5,858,195, US 5,126,022, US 4,891,120, US 4,908,112, US 5,750,015, US 5,580,523, US 5,571,410, US 5,885,470 and US 6,793,753. Freestanding structures can be made to have very thin or very thick walls in relation to the channel width and height. The walls, as well as the top and bottom of a channel can all be of different thickness and can be made of the same material or of different materials or a combination of materials such as a combination of glass, silicon, and a biologically-compatible material such as PDMS. Sealed channels or chambers can be made entirely from biologically-compatible material such as PDMS.

Devices useful for the invention can have one or more channels and/or reaction chambers. For example, the device can comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more channels. Furthermore, the device can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more reactions chambers.

The reaction chamber and microchannels can be any suitable shape known in the art such as, but are not limited to, cylindrical, rectangular, semi-spherical or trapezoidal.

In an embodiment, where the device is capable of performing more than one reaction the channel design is such that each component flows through channels with the same length, size and configuration such as the bilaterally symmetrical parallel channel layout provided in Figure 47.

In another embodiment, the function of the microchannels and the functions of the reaction chamber may be fulfilled by a single combined microfluidic element in which mixing occurs and from which light is collected.

The microfluidic device will typically have one or more reaction chamber volumes of about 1 pL (i.e. picoliter or a trillionth of a liter) to about 200 μ L. However, reaction chamber volumes of between about 0.01 nL (i.e. nanoliter or a billionth of a liter) to about 100 nL, or between about 0.01 nL and 10 nL may be advantageous in certain applications. Some embodiments may optimally perform when the volume of each reaction chambers is between about 0.20 nL to about 5 nL. An additional

embodiment is where the volume of each reaction chambers is between about 0.25 nl to about 2 nl. In a further embodiment, the reaction chamber(s) have a volume of about 1 μ l to about 12 μ l. Other possible reaction chamber volumes may be used where appropriate.

- 5 In a preferred embodiment, the reaction chamber is wider than it is short. In one example, the reaction chamber has a cross-sectional area of about 1mm² to 1cm² and a height of no more than about 5 mm.

In some circumstances it may be desirable to use reaction chambers of more than one size and shape. For example, the present inventors have observed that larger
10 reaction chambers generate more light and allow the detection of more weakly emitting sensor molecules, often at the expense of a slower response time (time to peak change in BRET ratio) upon presentation of a sample containing a target analyte and a longer off-time when the analyte is removed. Different channels may therefore be equipped with reaction chambers of different sizes in order to match the sensitivity, precision and
15 time dynamics of particular combinations of sensor molecules and analytes or sets of analytes. It would also be possible, and may in some cases be desirable, to implement more than one reaction chamber per sensor channel. This would allow near simultaneous detection of samples with higher luminance and quantitative precision (large chamber) and higher time resolution (smaller chamber). This could easily be
20 achieved by fitting the large and smaller chambers with their own light path to the detector via an optical switch or equivalent, or fitting each with separate solid state light detectors.

Dimensions of the microchannels can be chosen based on the specific application of the device. Accordingly, width of the microchannel can range from, for
25 example, about 0.1mm to about 10mm. In some embodiments, the width of the microchannel is from about 0.5mm to about 5mm. In some embodiments, the width of the microchannel is from about 1mm to about 4mm. In some embodiments, the width of the microchannel is about 2.5mm. Depth or height of the microchannel can also be chosen based on the specific application of the device. Accordingly, the depth of the
30 microchannel can range from, for example, about 5 μ m to about 2000 μ m. In some embodiments, the depth of the microchannel is from about 100 μ m to about 1000 μ m. In some embodiments, the depth of the microchannel is from about 250 μ m to about 750 μ m. In some embodiments, the depth of the microchannel is about 560 μ m. In another embodiment, each microchannel has a cross-sectional area of about 1 μ m² to
35 about 1mm².

As the skilled person will understand, the device will have suitable inlet ports and outlet ports to enable the relevant components to flow through the device.

The skilled artisan is well aware that the flow through a microfluidic device is dependent on various factors including, but not limited to, dimensions of the microchannels, viscosity of the fluid, and the detection and method employed. Accordingly, the sample (sensor molecule and substrate) can flow through the chip microchannel at a rate of about 1 μ l/hr to about 10 ml/hr. In some embodiments, the sample (sensor molecule and substrate) can flow through the device microchannel at a rate of about 1 μ l/hr to about 100 μ l/hr, about 5 μ l/hr to about 200 μ l/hr, from about 7.5 μ l/hr to about 500 μ l/hr, or about 10 μ l/hr to about 1 ml/hr.

In one embodiment, the device comprises multiple reaction chambers for sensing, for example, different analytes in the same source (sample). Due to the likely need to use different sensor molecules to detect different analytes, in some instances it may be desirable to modify the flow rate in different, preferably parallel, channels. In this regard, each individual flow rate can be set to optimize the sensitivity for each individual sensor molecule.

In one embodiment, in a device comprising multiple reaction chambers the flow rate to and from each reaction chamber is controlled by separate means, for instance a separate suction pump controls the flow rate to and from each reaction chamber. This configuration allows the simultaneous operation of multiple sensor channels independently of each other with potentially different flow rates and consequently different balances between speed and sensitivity.

The surface of the microfluidic channels may be passivated by exposure to a solution of a suitable reagent, such as a 0.1-5% (w/v) aqueous solution of bovine serum albumin, diluted mammalian serum, fish-skin gelatin, fat free milk proteins and/or using a solution of a non-ionic detergent, such as Tween-20 or by using a suspension of yeast microsomes.

After the reagents (sample, sensor molecule, substrate) have passed through the device, the device can be washed by flowing an appropriate fluid, e.g., a washing fluid such as a buffer, through the microchannels. This enables the device to be re-used. According, in some embodiments, the method further comprises the step of flowing a fluid, such as a buffer, through the microdevice after the analyte has been detected. The amount of fluid to be flown through the microdevice can be any amount and can be based on the volume of the chip. In some embodiments, the amount of the washing fluid is from about 0.5x to about 10x total volume of the microchannels in the device.

In one embodiment, the amount of the washing fluid is from about 1.5x to about 2.5x total volume of the microchannels in the device.

Temperature control

- 5 If present, this module is used to maintain desired temperatures for reagent storage, 1-8°C, preferably 2-4°C and BRET reactions, 20-37°C, preferably 25-28°C. The BRET temperature control can be integrated with the loading mechanism and the electro-optical collection device. In particular they are constructed according to a technology that uses local resistive heating or Peltier-device cooling for control
- 10 functions. For example, a thermally-controlled processor can be maintained at baseline temperature by a temperature-controlled heat sink or a cooling element, such as a Peltier device, with actuators controlled by localized heating above the baseline. Alternatively, cooling may be provided using a miniature heat pump. Localized heating may preferably be provided by low power resistive heaters of less than
- 15 approximately 1 to 2 W, advantageously controlled by low voltages, for example, less than 50, 25, 15 or 10 V.

Electro-optical system for light collection

- The term "signal" as used herein refers to luminescence measured as a change in
- 20 absorbance. In some embodiments, the signal will be "emitted light", wherein the step of detecting the signal will be the detection of photons of specific wavelengths of light by one or more photodetectors. Example photodetectors include photomultiplier tubes (PMTs), photodiodes, avalanche photodiodes, silicon or other solid state photomultipliers (http://en.wikipedia.org/wiki/Silicon_photomultiplier) or CCD
- 25 cameras, which may be cooled. Preferably, the photodetector has a photon detecting efficiency of $\geq 10\%$, more preferably $\geq 30\%$ and most preferably $\geq 50\%$. Preferably these efficiencies operate in the blue and green bands of the optical spectrum. The detector further comprises a means of restricting the detected light to specific wavelength(s) or specific ranges of wavelengths. This can be, for example, suitable
- 30 filters optionally mounted to a filter wheel or a filter slide or a monochromator or a dichroic mirror or a combination of two or more of these devices.

- The electro-optical system may mainly consist of optical fibres and an optical switch (Figure 16). The optical fibre can be replaced by fibre bundles or liquid light guides. The fibres with core diameters from about 10 μm to about 3000 μm can be
- 35 fixed into the loading system. For fibre bundles or liquid light guides, the core diameter can be in the range from 0.5 mm to 10 mm. The flat ends of the fibres may be

located right below the reaction chambers, each fibre collecting light from a particular chamber. Two BRET electro-optical detection elements can be designed which can be integrated with the reaction chamber (see Figure 17). A spherical microlens might be incorporated into the reaction chamber to help focus BRET light into the core of the optical fiber.

In another embodiment, the optical fibre/liquid light guide system can be replaced by a set of lenses and mirrors so that light from each chamber is relayed to the detectors. The switching for multiple channel system can be realised by a mechanical chopper or other switching mechanisms.

To further increase the BRET signal collected, a flat or aspherical mirror might be placed on top of the reaction chamber (Figure 17b). In this case, most of the BRET light emitted to the top will be reflected back into the optical fibers. For multi-channel detection, an optical switch can be used to collect lights from all channels. The material for this element can be in glass or polymeric materials, which have excellent optical, chemical properties such as polydimethylsiloxane, cyclic olefin co-polymer (COC) and so on. The BRET reaction chamber will be connected to the microfluidic network designed above for sample delivery and mixing.

Digital Photon Integration

Ultra low level light detection requires a highly sensitive photomultiplier tube (PMT) and digital signal processing unit to eliminate the dark current. Figure 42 illustrates an example system for use in the invention composed of three units: PMT, Photon counting unit, USB counting unit. H10721P-210 is a current output PMT with ultrabialkali photocathode providing high sensitivity in visible wavelengths. The photosensitive area is round shape with a diameter of 8mm. When a photon reaches to the ultrabialkali photocathode, photoelectrons are generated. The photoelectrons are accelerated towards a series of cascaded electrode structures at which the number of electrons increased exponentially at each stage. At the final stage the PMT outputs the sum of the generated electrons as a photoelectron pulse or current.

The dark current is defined as the current output appearing from a PMT in the absence of incident light. By identifying and eliminating the so-called the "dark pulses" it is possible to minimize the dark current. In this example, a signal processing unit (C9744 Photon counting unit) is used to eliminate the dark pulses. The unit allows implementation of a user set threshold value such that only the pulses with amplitude higher than the threshold value are sent to output. The remaining pulses are filtered out

of the output signal. In this unit, the pulses which pass the discrimination criteria are converted to 5 V digital signal pulses and sent to the output terminal.

C8855-01 is an USB interface counting unit designed to count digital signal pulses without dead time. The signal generated in C9744 Photon counting unit is input
5 to the counting unit and results are sent to a PC with a USB connection.

Optical detection system

Detection can be achieved using detectors that are incorporated into the device or that are separate from the device but aligned with the region of the device to be
10 detected.

The optical detection system samples the light output from each microfluidic channel, including each BRET reaction chamber. Each microfluidic channel may be equipped with a dedicated photodetector. For example, a fraction of the optical output of a single BRET reaction chamber may be channelled through a blue band-pass filter,
15 while the remainder may be channelled through a green band-pass filter (or other suitable band-pass characteristics depending on the type of BRET in use). Light from these filters may be directed to separate photomultipliers using optical fibres, bundles of optical fibres or liquid light guides. Alternatively, silicon or other highly sensitive solid-state photomultipliers may be placed in close proximity to the band pass filters so
20 as to sample the light emissions directly from each microfluidic channel and BRET reaction chamber. In an other embodiment, an aspherical lens or set of aspherical lenses might be placed at the end of the optical fibre, bundles of optical fibre or liquid light guide to collimate the output beam before entering the PMTs, thus reducing the optical loss due to rays diverging outside the sensitive area of the PMTs. In a preferred
25 embodiment, the bandpass filters are placed on opposite sides of the microfluidic chip and in close contact with it and solid state photomultipliers or other photodetectors are placed in close contact with both of the bandpass filters on each of the microfluidic channels. The advantages of providing a dedicated detection system for each microfluidic channel are that it minimises the complexity of the optical system and
30 minimise potential photon losses, including those due to the switching dead time, where each microfluidic channel is optically silent for the majority of the polling cycle.

Alternatively, each microfluidic channel may be polled sequentially by one or more shared photodetectors.

In one embodiment, the detection system may consist of an optical or
35 optomechanical switching device, which receives light via an optical fiber or light guide from each of the microfluidic channels and sequentially outputs the optical signal

of each of these inputs via a single optical fiber or light guide. The switching time from chamber to chamber could be in the range of nanoseconds to a few seconds (for example 2 or 3), preferably in the range of a 10-500 milliseconds or less. The output of the optical switching device impinges on and a photodetector such as a dichroic mirror
5 to split the light into two wavelength ranges corresponding to the emissions of BRET donor and acceptor, respectively and two photomultiplier tubes for simultaneously detecting the light in each of these wavelength ranges (Figure 16). Optionally, the dichroic block may be augmented by band pass filters tuned to the BRET donor and acceptor emission spectra.

10 In an alternative embodiment, a shutter box may be used instead of the optical switch. In such an arrangement, the output of the shutter box may be a many-to-one multifurcated light guide, which constrains the output of all optical channels into a single light guide that directs light to the photodetector. In this arrangement the shutters are operated so that the light from each single microfluidic channel is passed to
15 the photodetector sequentially. The potential advantage of a switching system is that it allows a smaller number of photodetectors to sample the optical output of a larger number of microfluidic channels with cost, weight and power savings.

In another embodiment, the operating characteristics of the paired solid state photodetectors may be chosen so that their peak photon detection efficiencies (PDEs)
20 are selective or semi-selective for the peak emissions of the BRET donor and acceptor. In this case, it is possible to dispense with the spectral filters and dichroic block and rely on the inherent differential spectral sensitivities different types of solid state photodetector to generate a BRET ratio.

25 *Data acquisition and processing*

The BRET signal of donors and acceptors in terms of counts/gate versus time will be collected by suitable software. A BRET ratio is calculated based on the ratio of light collected from the acceptors channel to the light collected from the donor channel. This BRET ratio should be constant if the ratio of flows of the sensor molecule and
30 substrate remains constant and without the analyte to be detected in the BRET chamber. However, in the presence of the analyte, the BRET ratio will change corresponding to the amount of reagent in the reaction chambers.

Software and embedded control system

35 Preferably, the system comprises a trainable data processing and output software algorithm that can learn and discriminate the response patterns characteristic of

different chemical samples. The software will capture salient features of the signal from each microfluidic channel, such as the baseline BRET ratio, the steady state BRET ratio when exposed to a sample and various features of the time course of changes in BRET ratio. These features from each channel will be input into a variety of discriminating algorithms, such as principle components analysis, linear discriminant analysis, stepwise discriminant analysis, machine learning algorithms such as support vector machines, Bayesian network analysis or neural network algorithms and compared with the results of previously learned sample classifications. Tentative sample analysis or classification is provided to the operator, preferably through a GUI and/or acoustical output. Alternatively, the signal strength in each channel may be output visually and/or acoustically so that the operator may match response patterns with those they have previously been trained to recognise.

In one embodiment, GUI software is used for controlling all in-device compositions such as the speed and concentration ratio of the sampling subsystem, the rate of flow of the microfluidic channels, the timing of flushing and purging cycles, pumps, the rate and intensity of any active mixing, the sensitivity and integration time of the photodetector systems, the temperature of the reagent reservoirs and the reaction chamber and all aspects of data acquisition and processing. Optionally many of these functions may be carried out through an embedded microcontroller. Some or all of the functions may be carried out on a laptop or tablet computer or equivalent device.

Chemiluminescence Resonance Energy Transfer

Chemiluminescence is the emission of energy with limited emission of heat (luminescence), as the result of a chemical reaction. The term "chemiluminescence" is used herein to encompass bioluminescence, which relies upon the activity of an enzyme.

As used herein, bioluminescent resonance energy transfer (BRET) is a proximity assay based on the non-radioactive transfer of energy between the bioluminescent protein donor and the acceptor molecule.

As used herein, the term "spatial location" refers to the three dimensional positioning of the donor relative to the acceptor molecule which changes as a result of the analyte binding or releasing from the sensor molecule.

As used herein, the term "dipole orientation" refers to the direction in three-dimensional space of the dipole moment associated either with the donor and/or the acceptor molecule relative their orientation in three-dimensional space. The dipole moment is a consequence of a variation in electrical charge over a molecule.

Using BRET as an example, in an embodiment the energy transfer occurring between the bioluminescent protein and acceptor molecule is presented as calculated ratios from the emissions measured using optical filters (one for the acceptor molecule emission and the other for the bioluminescent protein emission) that select specific wavelengths (see equation 1).

$$E_a/E_d = \text{BRET ratio} \quad (1)$$

where E_a is defined as the acceptor molecule emission intensity (emission light is selected using a specific filter adapted for the emission of the acceptor) and E_d is defined as the bioluminescent protein emission intensity (emission light is selected using a specific filter adapted for the emission of the bioluminescent protein).

It should be readily appreciated by those skilled in the art that the optical filters may be any type of filter that permits wavelength discrimination suitable for BRET. For example, optical filters used in accordance with the present invention can be interference filters, long pass filters, short pass filters, etc. Intensities (usually in counts per second (CPS) or relative luminescence units (RLU)) of the wavelengths passing through filters can be quantified using either a photo-multiplier tube (PMT), photodiode, including a cascade photodiode, photodiode array or a sensitive camera such as a charge coupled device (CCD) camera. The quantified signals are subsequently used to calculate BRET ratios and represent energy transfer efficiency. The BRET ratio increases with increasing intensity of the acceptor emission.

Generally, a ratio of the acceptor emission intensity over the donor emission intensity is determined (see equation 1), which is a number expressed in arbitrary units that reflects energy transfer efficiency. The ratio increases with an increase of energy transfer efficiency (see Xu et al., 1999).

Energy transfer efficiencies can also be represented using the inverse ratio of donor emission intensity over acceptor emission intensity (see equation 2). In this case, ratios decrease with increasing energy transfer efficiency. Prior to performing this calculation the emission intensities are corrected for the presence of background light and auto-luminescence of the substrate. This correction is generally made by subtracting the emission intensity, measured at the appropriate wavelength, from a control sample containing the substrate but no bioluminescent protein, acceptor molecule or polypeptide of the invention.

$$E_d/E_a = \text{BRET ratio} \quad (2)$$

where E_a and E_d are as defined above.

The light intensity of the bioluminescent protein and acceptor molecule emission can also be quantified using a monochromator-based instrument such as a

spectrofluorometer, a charged coupled device (CCD) camera or a diode array detector. Using a spectrofluorometer, the emission scan is performed such that both bioluminescent protein and acceptor molecule emission peaks are detected upon addition of the substrate. The areas under the peaks represent the relative light intensities and are used to calculate the ratios, as outlined above. Any instrument capable of measuring lights for the bioluminescent protein and acceptor molecule from the same sample, can be used to monitor the BRET system of the present invention.

In an alternative embodiment, the acceptor molecule emission alone is suitable for effective detection and/or quantification of BRET. In this case, the energy transfer efficiency is represented using only the acceptor emission intensity. It would be readily apparent to one skilled in the art that in order to measure energy transfer, one can use the acceptor emission intensity without making any ratio calculation. This is due to the fact that ideally the acceptor molecule will emit light only if it absorbs the light transferred from the bioluminescent protein. In this case only one light filter is necessary.

In a related embodiment, the bioluminescent protein emission alone is suitable for effective detection and/or quantification of BRET. In this case, the energy transfer efficiency is calculated using only the bioluminescent protein emission intensity. It would be readily apparent to one skilled in the art that in order to measure energy transfer, one can use the donor emission intensity without making any ratio calculation. This is due to the fact that as the acceptor molecule absorbs the light transferred from the bioluminescent protein there is a corresponding decrease in detectable emission from the bioluminescent protein. In this case only one light filter is necessary.

In an alternative embodiment, the energy transfer efficiency is represented using a ratiometric measurement which only requires one optical filter for the measurement. In this case, light intensity for the donor or the acceptor is determined using the appropriate optical filter and another measurement of the samples is made without the use of any filter (intensity of the open spectrum). In this latter measurement, total light output (for all wavelengths) is quantified. Ratio calculations are then made using either equation 3 or 4. For the equation 3, only the optical filter for the acceptor is required. For the equation 4, only the optical filter for the donor is required.

$$E_a/E_o - E_a = \text{BRET ratio or } = E_o - E_a/E_a \quad (3)$$

$$E_o - E_d/E_d = \text{BRET ratio or } = E_d/E_o - E_d \quad (4)$$

where E_a and E_d are as defined above and E_o is defined as the emission intensity for all wavelengths combined (open spectrum).

It should be readily apparent to one skilled in the art that further equations can be derived from equations 1 through 4. For example, one such derivative involves correcting for background light present at the emission wavelength for bioluminescent protein and/or acceptor molecule.

5 In performing a BRET assay, light emissions can be determined from each well using the BRETCount. The BRETCount instrument is a modified TopCount, wherein the TopCount is a microtiterplate scintillation and luminescence counter sold by Packard Instrument (Meriden, CT). Unlike classical counters which utilise two photomultiplier tubes (PMTs) in coincidence to eliminate background noise, TopCount
10 employs single- PMT technology and time-resolved pulse counting for noise reduction to allow counting in standard opaque microtiter plates. The use of opaque microtiterplates can reduce optical crosstalk to negligible level. TopCount comes in various formats, including 1, 2, 6 and 12 detectors (PMTs), which allow simultaneous reading of 1, 2, 6 or 12 samples, respectively. Beside the BRETCount, other
15 commercially available instruments are capable of performing BRET: the Victor 2 (Wallac, Finland (Perkin Elmer Life Sciences)) and the Fusion (Packard Instrument, Meriden). BRET can be performed using readers that can detect at least the acceptor molecule emission and preferably two wavelengths (for the acceptor molecule and the bioluminescent protein) or more.

20

Chemiluminescence

Non-enzymatic chemiluminescence is the result of chemical reactions between an organic dye and an oxidizing agent in the presence of a catalyst. Chemiluminescence emission occurs as the energy from the excited states of organic dyes, which are
25 chemically induced, decays to ground state. The duration and the intensity of the chemiluminescence emission are mostly dependent on the extent of the chemical reagents present in the reaction solution.

As used herein, the term "bioluminescent protein" refers to any protein capable of acting on a suitable substrate to generate luminescence.

30 It is understood in the art that a bioluminescent protein is an enzyme which converts a substrate into an activated product which then releases energy as it relaxes. The activated product (generated by the activity of the bioluminescent protein on the substrate) is the source of the bioluminescent protein-generated luminescence that is transferred to the acceptor molecule.

35 There are a number of different bioluminescent proteins that can be employed in this invention (see, for example, Table 1). Light-emitting systems have been known

and isolated from many luminescent organisms including bacteria, protozoa, coelenterates, molluscs, fish, millipedes, flies, fungi, worms, crustaceans, and beetles, particularly click beetles of genus *Pyrophorus* and the fireflies of the genera *Photinus*, *Photuris*, and *Luciola*. Additional organisms displaying bioluminescence are listed in
5 WO 00/024878, WO 99/049019 and Viviani (2002).

One very well known example is the class of proteins known as luciferases which catalyze an energy-yielding chemical reaction in which a specific biochemical substance, a luciferin (a naturally occurring fluorophore), is oxidized by an enzyme having a luciferase activity (Hastings, 1996). A great diversity of organisms, both
10 prokaryotic and eukaryotic, including species of bacteria, algae, fungi, insects, fish and other marine forms can emit light energy in this manner and each has specific luciferase activities and luciferins which are chemically distinct from those of other organisms. Luciferin/luciferase systems are very diverse in form, chemistry and function. Bioluminescent proteins with luciferase activity are thus available from a
15 variety of sources or by a variety of means. Examples of bioluminescent proteins with luciferase activity may be found in US 5,229,285, 5,219,737, 5,843,746, 5,196,524, and 5,670,356. Two of the most widely used luciferases are: (i) *Renilla* luciferase (from *R. reniformis*), a 35 kDa protein, which uses coelenterazine as a substrate and emits light at 480 nm (Lorenz et al., 1991); and (ii) Firefly luciferase (from *Photinus pyralis*), a 61
20 kDa protein, which uses luciferin as a substrate and emits light at 560 nm (de Wet et al., 1987).

Gaussia luciferase (from *Gaussia princeps*) has been used in biochemical assays (Verhaegen et al., 2002). *Gaussia* luciferase is a 20 kDa protein that oxidises coelenterazine in a rapid reaction resulting in a bright light emission at 470 nm.

25 Luciferases useful for the present invention have also been characterized from *Anachnocalampa* sp (WO 2007/019634). These enzymes are about 59 kDa in size and are ATP-dependent luciferases that catalyze luminescence reactions with emission spectra within the blue portion of the spectrum.

Biologically active variants or fragments of naturally occurring bioluminescent
30 protein can readily be produced by those skilled in the art. Three examples of such variants useful for the invention are Rluc2 (Loening et al., 2006), Rluc8 (Loening et al., 2006) and Rluc8.6-535 (Loening et al., 2007) which are each variants of *Renilla* luciferase. In a further preferred embodiment, the sequence of the BRET chemiluminescent donor is chosen to have greater thermal stability than sensor
35 molecules incorporating native *Renilla* luciferase sensors. RLuc2 or RLuc8 are convenient examples of suitable choices, which consequently exhibit $\geq 5x$ or $\geq 10x$

higher luminance than sensors incorporating the native *Renilla* luciferase sequence. Such enhanced luminance has significant benefits as it permits the use of lower detection chamber volumes and/or faster on-chip flow rates with concomitant improvement in time resolution at any given combination of detection chamber volume and flow rate. Alternatively, it permits more economical use of reagents for any given time resolution.

Table 1. Exemplary bioluminescent proteins.

<u>Species</u>	<u>Name</u>	<u>Organism</u>	<u>MW</u> <u>kDa x 10⁻³</u>	<u>Emission</u> <u>(nm)</u>	<u>Example of</u> <u>Substrate</u>
Insect	FFluc	Photinus pyralis (North American Firefly)	~61	560	D-(-)-2-(6'-hydroxybenzothiazolyl)- Δ^2 -thiazoline-4-carboxylic acid, HBTTCa (C ₁₁ H ₈ N ₂ O ₃ S ₂) (luciferin)
Insect	FF'luc	Luciola cruciata (Japanese Firefly)		560-590 (many mutants)	Luciferin
Insect		Phengodid beetles (railroad worms)			
Insect		Arachnocampa sp.			Luciferin
Insect		Orphelia fultoni (North American glow worm)			
Insect	Cluc	Pyrophorus plagiophthalmus (click beetle)		546, 560, 578 and 593	Luciferin
Jellyfish	Aequorin	Aequorea	44.9	460-470	Coelenterazine
Sea pansy	Rluc	Renilla reniformis	36	480	Coelenterazine
Sea pansy (modified)	Rluc8	Renilla reniformis (modified)	36	487 (peak)	Coelenterazine /Deep Blue C
Sea pansy	Rluc2	Renilla reniformis	36	480	Coelenterazine

(modified)		(modified M185V/Q235A)			
Sea pansy (modified)	RLuc8.6 -535	Renilla reniformis (modified)	36	535	Coelenterazine
Sea pansy	Rmluc	Renilla mullerei	36.1	~480	Coelenterazine
Sea pansy		Renilla kollikeri			
Crustacea (shrimp)	Vluc	Vargula hilgendorffii	~62	~460	coelenterazine *
Crustacea		Cypridina (sea firefly)	75	460	coelenterazine **
Dinoflagellate (marine alga)		Gonyaulax polyedra	130	~475	Tetrapyrrole
Mollusc		Latia (fresh water limpet)	170	500	Enol formate, terpene, aldehyde
Hydroid		Obelia bicuspidata	~20	~470	Coelenterazine
Shrimp		Oplophorus graciliorostris	31	462	Coelenterazine
Others	Ptluc	Ptilosarcus		~490	Coelenterazine
	Gluc	Gaussia	~20	~475	Coelenterazine
	Plluc	Pleuromamma	22.6	~475	Coelenterazine

As used herein, a "biologically active fragment" is a portion of a polypeptide as described herein which maintains a defined activity of the full-length polypeptide. As used herein, a "biologically active variant" is a molecule which differs from a naturally occurring and/or defined molecule by one or more amino acids but maintains a defined activity, such as defined above for biologically active fragments. Biologically active variants are typically least 50%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97%, and even more preferably at least 99% identical to the naturally occurring and/or defined molecule.

Alternative, non-luciferase, bioluminescent proteins that can be employed in this invention are any enzymes which can act on suitable substrates to generate a luminescent signal. Specific examples of such enzymes are β -galactosidase, lactamase, horseradish peroxidase, alkaline phosphatase, β -glucuronidase and β -glucosidase.

Synthetic luminescent substrates for these enzymes are well known in the art and are commercially available from companies, such as Tropix Inc. (Bedford, MA, USA).

An example of a peroxidase useful for the present invention is described by Hushpulian et al. (2007).

- 5 In a preferred embodiment, a bioluminescent protein with a small molecular weight is used to prevent an inhibition of the interaction due to steric hindrance. The bioluminescent protein preferably consists of a single polypeptide chain. Also the bioluminescent proteins preferably do not form oligomers or aggregates. The bioluminescent proteins *Renilla* luciferase, *Gaussia* luciferase and Firefly luciferase
10 meet all or most of these criteria.

Substrates

- As used herein, the term "substrate" refers to any molecule that can be used in conjunction with a chemiluminescent donor to generate or absorb luminescence. The
15 choice of the substrate can impact on the wavelength and the intensity of the light generated by the chemiluminescent donor.

- A widely known substrate is coelenterazine which occurs in cnidarians, copepods, chaetognaths, ctenophores, decapod shrimps, mysid shrimps, radiolarians and some fish taxa (Greer and Szalay, 2002). For *Renilla* luciferase for example,
20 coelenterazine analogues/derivatives are available that result in light emission between 418 and 512 nm (Inouye et al., 1997). A coelenterazine analogue/derivative (400A, DeepBlueC) has been described emitting light at 400 nm with *Renilla* luciferase (WO 01/46691). Other examples of coelenterazine analogues/derivatives are EnduRen and ViviRen.

- 25 As used herein, the term "luciferin" refers to a class of light-emitting biological pigments found in organisms capable of bioluminescence, which are oxidised in the presence of the enzyme luciferase to produce oxyluciferin and energy in the form of light. Luciferin, or 2-(6-hydroxybenzothiazol-2-yl)-2-thiazoline-4-carboxylic acid, was first isolated from the firefly *Photinus pyralis*. Since then, various forms of luciferin
30 have been discovered and studied from various different organisms, mainly from the ocean, for example fish and squid, however, many have been identified in land dwelling organisms, for example, worms, beetles and various other insects (Day et al., 2004; Viviani, 2002).

- There are at least five general types of luciferin, which are each chemically
35 different and catalysed by chemically and structurally different luciferases that employ a wide range of different cofactors. First, is firefly luciferin, the substrate of firefly

luciferase, which requires ATP for catalysis (EC 1.13.12.7). Second, is bacterial luciferin, also found in some squid and fish, that consists of a long chain aldehyde and a reduced riboflavin phosphate. Bacterial luciferase is FMNH-dependent. Third, is dinoflagellate luciferin, a tetrapyrrolic chlorophyll derivative found in dinoflagellates (marine plankton), the organisms responsible for night-time ocean phosphorescence. Dinoflagellate luciferase catalyses the oxidation of dinoflagellate luciferin and consists of three identical and catalytically active domains. Fourth, is the imidazolopyrazine vargulin, which is found in certain ostracods and deep-sea fish, for example, *Porichthys*. Last, is coelenterazine (an imidazolopyrazine), the light-emitter of the protein aequorin, found in radiolarians, ctenophores, cnidarians, squid, copepods, chaetognaths, fish and shrimp.

Acceptor Molecules

As used herein, the term "fluorescent acceptor domain" (also referred herein to as "acceptor molecule") refers to any compound which can accept energy emitted as a result of the activity of a chemiluminescent donor, and re-emit it as light energy. There are a number of different acceptor molecules that can be employed in this invention. The acceptor molecules may be a protein or non-proteinaceous. Examples of acceptor molecules that are protein include, but are not limited to, green fluorescent protein (GFP), blue fluorescent variant of GFP (BFP), cyan fluorescent variant of GFP (CFP), yellow fluorescent variant of GFP (YFP), enhanced GFP (EGFP), enhanced CFP (ECFP), enhanced YFP (EYFP), GFPS65T, Emerald, Venus, mOrange, Topaz, GFPuv, destabilised EGFP (dEGFP), destabilised ECFP (dECFP), destabilised EYFP (dEYFP), HcRed, t-HcRed, DsRed, DsRed2, t-dimer2, t-dimer2(12), mRFP1, pocilloporin, *Renilla* GFP, Monster GFP, paGFP, Kaede protein or a Phycobiliprotein, or a biologically active variant or fragment of any one thereof. Examples of acceptor molecules that are not proteins include, but are not limited to, Alexa Fluor dye, Bodipy dye, Cy dye, fluorescein, dansyl, umbelliferone, fluorescent microsphere, luminescent microsphere, fluorescent nanocrystal, Marina Blue, Cascade Blue, Cascade Yellow, Pacific Blue, Oregon Green, Tetramethylrhodamine, Rhodamine, Texas Red, rare earth element chelates, or any combination or derivatives thereof.

One very well known example is the group of fluorophores that includes the green fluorescent protein from the jellyfish *Aequorea victoria* and numerous other variants (GFPs) arising from the application of molecular biology, for example mutagenesis and chimeric protein technologies (Tsien, 1998). GFPs are classified based on the distinctive component of their chromophores, each class having distinct

excitation and emission wavelengths: class 1, wild-type mixture of neutral phenol and anionic phenolate: class 2, phenolate anion : class 3, neutral phenol : class 4, phenolate anion with stacked s-electron system: class 5, indole : class 6, imidazole : and class 7, phenyl.

5 A naturally occurring acceptor molecule which has been mutated (variants) can also be useful for the present invention. One example of an engineered system which is suitable for BRET is a *Renilla* luciferase and enhanced yellow mutant of GFP (EYFP) pairing which do not directly interact to a significant degree with one another alone in the absence of a mediating protein(s) (in this case, the G protein coupled receptor) (Xu
10 et al., 1999).

In another embodiment, the acceptor molecule is a fluorescent nanocrystal. Nanocrystals, or “quantum dots”, have several advantages over organic molecules as fluorescent labels, including resistance to photodegradation, improved brightness, non-toxicity, and size dependent, narrow emission spectra that enables the monitoring of
15 several processes simultaneously. Additionally, the absorption spectrum of nanocrystals is continuous above the first peak, enabling all sizes, and hence all colors, to be excited with a single excitation wavelength.

Fluorescent nanocrystals may be attached, or “bioconjugated”, to proteins in a variety of ways. For example, the surface cap of a “quantum dot” may be negatively
20 charged with carboxylate groups from either dihydrolipoic acid (DHLA) or an amphiphilic polymer. Proteins can be conjugated to the DHLA–nanocrystals electrostatically, either directly or via a bridge consisting of a positively charged leucine zipper peptide fused to recombinant protein. The latter binds to a primary antibody with specificity for the intended target. Alternatively, antibodies, streptavidin,
25 or other proteins are coupled covalently to the polyacrylate cap of the nanocrystal with conventional carbodiimide chemistry.

There are colloidal methods to produce nanocrystals, including cadmium selenide, cadmium sulfide, indium arsenide, and indium phosphide. These quantum dots can contain as few as 100 to 100,000 atoms within the quantum dot volume, with a
30 diameter of 10 to 50 atoms. Some quantum dots are small regions of one material buried in another with a larger band gap. These can be so-called core-shell structures, for example, with CdSe in the core and ZnS in the shell or from special forms of silica called ormosil. The larger the dot, the redder (lower energy) its fluorescence spectrum. Conversely, smaller dots emit bluer (higher energy) light. The coloration is directly
35 related to the energy levels of the quantum dot. Quantitatively speaking, the bandgap energy that determines the energy (and hence color) of the fluoresced light is inversely

proportional to the square of the size of the quantum dot. Larger quantum dots have more energy levels which are more closely spaced. This allows the quantum dot to absorb photons containing less energy, i.e. those closer to the red end of the spectrum.

In an alternate embodiment, the acceptor molecule is a fluorescent microsphere.

- 5 These are typically made from polymers, and contain fluorescent molecules (for example fluorescein GFP or YFP) incorporated into the polymer matrix, which can be conjugated to a variety of reagents. Fluorescent microspheres may be labelled internally or on the surface. Internal labelling produces very bright and stable particles with typically narrow fluorescent emission spectra. With internal labelling, surface
10 groups remain available for conjugating ligands (for example, proteins) to the surface of the bead. Internally-labelled beads are used extensively in imaging applications, as they display a greater resistance to photobleaching.

Carboxylate-modified fluorescent microspheres are suitable for covalent coupling of proteins using water-soluble carbodiimide reagents such as 1-ethyl-3-(3-
15 dimethylaminopropyl) carbodiimide hydrochloride (EDAC). Sulfate fluorescent microspheres are relatively hydrophobic and will passively and nearly irreversibly adsorb almost any protein. Aldehyde-sulfate fluorescent microspheres are sulfate microspheres that have been modified to add surface aldehyde groups, and react with proteins.

- 20 In another embodiment, the acceptor molecule is a luminescent microsphere. These are typically made from polymers, which contain luminescent molecules (for example complexes of europium or platinum) incorporated into the polymer matrix, which can be conjugated to a variety of reagents.

Examples of non-fluorescent acceptor domains useful for the invention include
25 quenchers such as DABCYL [4-((4-(Dimethylamino) phenyl)azo)benzoic acid], DABSYL (Dimethylaminoazosulfonic acid), metal nanoparticles such as gold and silver, lack hole quenchers (BHQ) and QXL quenchers.

Chemiluminescent Donor Domain and Acceptor Domain Pairs

- 30 As used herein, the term "the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance" refers to the steady state RET measurements which can be carried out within a range of $\pm 50\%$ of R_0 . This phrase encompasses an efficiency of luminescence energy transfer from the
35 chemiluminescent donor domain to the acceptor domain in the range of 10-90%.

Outside of these distance limits it is still possible to estimate distance but the uncertainty is increased.

A criterion which should be considered in determining suitable pairings is the relative emission/fluorescence spectrum of the acceptor molecule compared to that of the donor. The emission spectrum of the donor should overlap with the absorbance spectrum of the acceptor molecule such that the light energy from the donor luminescence emission is at a wavelength that is able to excite the acceptor molecule and thereby promote acceptor molecule fluorescence when the two molecules are in a proper proximity and orientation with respect to one another. For example, it has been demonstrated that an *Renilla* luciferase/EGFP pairing is not as good as an *Renilla* luciferase/EYEF pairing based on observable emission spectral peaks (Xu, 1999; Wang, et al. (1997) in *Bioluminescence and Chemiluminescence : Molecular Reporting with Photons*, eds. Hastings et al. (Wiley, New York), pp. 419-422). To study potential pairing, protein fusions (for example) are prepared containing the selected bioluminescent protein and acceptor molecule and are tested, in the presence of an appropriate substrate.

It should also be confirmed that the donor and acceptor molecule do not spuriously associate with each other. This can be accomplished by, for example, separate co-expression of a bioluminescent protein and acceptor molecule in the same cells and then monitoring the luminescence spectrum in order to determine if BRET occurs. This may be achieved, for example, using the method of Xu et al. (1999). The selected bioluminescent protein and acceptor molecule form a suitable BRET pair if little or no BRET is observed.

The donor emission can be manipulated by modifications to the substrate. In the case of luciferases the substrate is coelenterazine. The rationale behind altering the donor emission is to improve the resolution between donor emission and acceptor emissions. The original BRET system uses the *Renilla* luciferase as donor, EYFP (or Topaz) as the acceptor and coelenterazine h derivative as the substrate. These components when combined in a BRET assay, generate light in the 475-480 nm range for the bioluminescent protein and the 525-530 nm range for the acceptor molecule, giving a spectral resolution of 45-55 nm.

Unfortunately, *Renilla* luciferase generates a broad emission peak overlapping substantially the GFP emission, which in turn contributes to decrease the signal to noise of the system. One BRET system of the present invention, using coel400a as the *Renilla* luciferase substrate, provides broad spectral resolution between donor and acceptor emission wavelengths (-105nm). *Renilla* luciferase with coel400a generates

light between 390-400 nm and a GFP was prepared which absorbs light in this range and re-emits light at 505-508 nm. Because of this increase in spectral resolution between *Renilla* luciferase and GFP emissions, this BRET system provides an excellent biological tool to monitor small changes in conformation of a polypeptide of the invention. This is a significant improvement over the system described previously using the coelenterazine h derivative and EYFP, which has a wavelength difference between donor and acceptor of approximately 51 nm.

Various coelenterazine derivatives are known in the art, including coel400a, that generate light at various wavelengths (distinct from that generated by the wild type coelenterazine) as a result of *Renilla* luciferase activity. A worker skilled in the art would appreciate that because the light emission peak of the donor has changed, it is necessary to select an acceptor molecule which will absorb light at this wavelength and thereby permit efficient energy transfer. This can be done, for example by altering a GFP class 4 such that it becomes a class 3 or 1 GFP. Spectral overlapping between light emission of the donor and the light absorption peak of the acceptor is one condition among others for an efficient energy transfer. Class 3 and 1 GFPs are known to absorb light at 400 nm and re-emit between 505-511 nm. This results in a wavelength difference between donor and acceptor emissions of approximately 111 nm.

Examples of further bioluminescent protein and acceptor molecule pairs are provided in Table 2.

Sensor Molecule

As used herein, the term "sensor molecule" refers to any molecule, complex of two or more covalently or non-covalently associated molecules, or two or more molecules which can be at least at some stage closely associated to enable RET between the donor and acceptor. Furthermore, if present, the two or more separate molecules which form the domain can be associated via an intermediate molecule. In one example, the sensor molecule can be a protein complex, where each subunit of the complex is non-covalently associated and the acceptor and domain may be on the same or different subunits of the protein complex. In another example, the sensor molecule is two separate nucleic acid strands, one labelled with the acceptor and the other labelled with the donor, such that hybridization to a target (analyte) results in the donor and acceptor being sufficiently close to result in RET. In this example, it can be considered that the sensor molecule is formed when it binds the analyte.

Table 2. Exemplary BRET bioluminescent proteins and acceptor molecule pairs.

<u>BDP</u>	<u>Substrate</u>	<u>Substrate wavelength (peak)</u>	<u>Fluorescence acceptor molecule</u>	<u>Wavelength of acceptor (Ex/Em)</u>
Rluc2 Rluc8	Native coelenterazine	470 nm	Venus	515/528 nm
Rluc2 Rluc8	Native coelenterazine	470 nm	mOrange	548/562 nm
Rluc2 Rluc8	Native Coelenterazine	470 nm	EYFP/Topaz	514/527 nm
Rluc2 Rluc8	Native Coelenterazine	470 nm	mCitrine	516/529 nm
Rluc Rluc2 Rluc8	Native Coelenterazine	470 nm	YPet	517/530 nm
Rluc2 Rluc8	Native Coelenterazine	470 nm	Fluorescein	495/519 nm
Rluc2 Rluc8	Native Coelenterazine	470 nm	Acridine yellow	470/550 nm
Rluc2 Rluc8	Native Coelenterazine	470 nm	Nile red	485/525 nm
Rluc2 Rluc8	Native Coelenterazine	470 nm	R-Phycoerythrin	480/578
Rluc2 Rluc8	Native Coelenterazine	470 nm	Red 613	480/613
Rluc2	Native	470 nm	TruRed	490/695

Rluc8	Coelenterazine			
RLuc8.6-5.35	Native Coelenterazine	535 nm	mOrange	548/562 nm
Rluc2 Rluc8	Coelenterazine <i>h</i>	470 nm	Venus	515/528 nm
Rluc2 Rluc8	Coelenterazine <i>h</i>	470 nm	mOrange	548/528 nm
Rluc2 Rluc8	Coelenterazine <i>h</i>	470 nm	EYFP/Topaz	514/527 nm
Rluc2 Rluc8	Coelenterazine <i>h</i>	470 nm	mCitrine	516/529 nm
Rluc2 Rluc8	Native Coelenterazine	470 nm	YPet	517/530 nm
Rluc Rluc2 Rluc8	Coelenterazine <i>h</i>	470 nm	Fluorescein	490/525nm
Rluc Rluc2 Rluc8	Coelenterazine <i>h</i>	470 nm	Acridine yellow	470/550 nm
Rluc Rluc2 Rluc8	Coelenterazine <i>h</i>	470 nm	Nile red	485/525 nm
Rluc Rluc2 Rluc8	Coelenterazine <i>h</i>	470 nm	R-Phycoerythrin	480/578
Rluc	Coelenterazine	470 nm	Red 613	480/613

Rluc2 Rluc8	<i>h</i>			
Rluc Rluc2 Rluc8	Coelenterazine <i>h</i>	470 nm	TruRed	490/695
RLuc8.6-5.35	Coelenterazine <i>h</i>	535 nm	mOrange	548/562 nm
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	GFP2	396/508 nm
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	GFP10	400/510 nm
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	Wild type GFP	396 (475)/508 nm
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	TagBFP	402/457 nm
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	Cerulean/mCFP	433/475 nm
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	ECFP/CyPet	434/477 nm
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	Y66W	436/485 nm
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	dKeima-Red	440/616 nm
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	mKeima-Red	440/620 nm
Rluc	Coelenterazine	400 nm	Quin-2	365/490 nm

Rluc2 Rluc8	400a			
Rluc Rluc2 Rluc8	Coelenterazine 400a	400 nm	Pacific blue	403/551 nm
Rluc Rluc2 Rluc8	Coelenterazine 400	400 nm	Dansychloride	380/475 nm
Firefly luciferase	Luciferin	560 nm	Cyanine Cy3	575/605 nm
Firefly luciferase	Luciferin	560nm	Texas red	590/615
Firefly luciferase	Luciferin	560 nm	TurboRed	553/574 nm
Firefly luciferase	Luciferin	560 nm	tdTomato	554/581 nm
Firefly luciferase	Luciferin	560 nm	TagRFP	555/584 nm
Firefly luciferase	Luciferin	560 nm	DsRed	557/592 nm
Firefly luciferase	Luciferin	560 nm	mRFP1	584/607 nm
Firefly luciferase	Luciferin	560 nm	mCherry	587/610 nm

The domain which binds the analyte (or candidate compound) may be any molecule as long as it can be appropriately associated with the donor and acceptor.

In an embodiment, the domain that binds the analyte is a protein or a nucleic acid. In a preferred embodiment, the domain is a protein. In an embodiment, the protein is a naturally occurring protein which binds one or more analytes (ligand), or a variant of the protein which retains analyte (ligand) binding activity. Examples include, but are not necessarily limited to, a receptor, odorant binding protein, pheromone-binding protein, enzyme (for example a protease, an oxidase, a phytase, a chitinase, an invertase, a lipase, a cellulase, a xylanase, a kinase, a phosphatase, an elongase, a transferase, a desaturase), ligand carrier or bacterial periplasmic binding protein. In an embodiment, the receptor is a G protein coupled receptor such as an

odorant receptor or a taste receptor (for example a sweet, bitter or umami taste receptor, such as those described in Doty, 2012). In a further embodiment, the odorant receptor or taste receptor is from a nematode or vertebrate or is a mutant thereof.

- In one embodiment, the sensor molecule is provided as a cell-free composition.
- 5 As used herein, the term "cell free composition" refers to an isolated composition which contains few, if any, intact cells and which comprises the sensor molecule. Examples of cell free compositions include cell (such as yeast cell) extracts and compositions containing an isolated and/or recombinant sensor molecules (such as proteins). Methods for preparing cell-free compositions from cells are well-known in
- 10 the art and are described in WO 2010/085844. In certain embodiments, the sensor molecule is embedded in a lipid bilayer such as of a liposome preparation, in cell or cell-free extract.

G protein coupled receptors

- 15 As used herein, unless specified otherwise, the term "G protein coupled receptor" refers to a seven transmembrane receptor which signals through G proteins. The receptor may be a single subunit, or two or more receptor subunits. When two or more receptor subunits are present they may be the same, different, or a combination thereof (for example, two of one subunit and a single of another subunit). Furthermore,
- 20 unless specified or implied otherwise the terms "G protein coupled receptor" and "subunit of a G protein coupled receptor", or variations thereof, are used interchangeably.

- As used herein, the term "odorant receptor", "olfactory receptor", "OR" or variations thereof refers to a polypeptide which, when present in a cell of an organism,
- 25 is involved in chemosensory perception. In an embodiment, the cell is a neuron. Furthermore, the term "odorant receptor" or "olfactory receptor" refers to a polypeptide which binds an odorant ligand, or forms part of a protein complex that binds to an odorant ligand, resulting in a physiologic response.

- As used herein, the term "forms part of" refers to the bioluminescent protein or
- 30 acceptor molecule being located within the specified region of the G protein coupled receptor, or subunit thereof. This term also includes the possibility that the bioluminescent protein and/or acceptor molecule is attached to or binds the G protein coupled receptor but does not form a continuous chain of amino acids. In one embodiment, the bioluminescent protein or acceptor molecule completely replaces the
- 35 specified region of the G protein coupled receptor. In another embodiment, some, but not all, of the specified region of the G protein coupled receptor is replaced. In yet

another embodiment, none of the specified region of the G protein coupled receptor is replaced. As the skilled addressee will appreciate, the bioluminescent protein or acceptor molecule will not be inserted such that it makes the G protein coupled receptor portion of a polypeptide incapable of binding the analyte to result in a spatial change to the location and/or dipole orientation of the bioluminescent protein relative to the acceptor molecule.

G protein-coupled receptors (GPCRs) are also known as seven transmembrane receptors, 7TM receptors, serpentine receptors, heptahelical receptors, and G protein linked receptors (GPLR). GPCRs are a large protein family of transmembrane receptors that sense molecules outside the cell and activate inside signal transduction pathways and, ultimately, cellular responses. The ligands that bind and activate these receptors include light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters, and vary in size from small molecules to peptides to large proteins. GPCRs are involved in many diseases, but are also the target of around half of all modern medicinal drugs. GPCRs can be grouped into at least 5 classes based on sequence homology and functional similarity:

- Class A rhodopsin-like,
- Class B secretin-like,
- Class C metabotropic/pheromone,
- Class D fungal pheromone, and
- Class E cAMP receptors.

Class A Rhodopsin like receptors include: Amine receptors: Acetylcholine, Alpha Adrenoceptors, Beta Adrenoceptors, Dopamine, Histamine, Serotonin, Octopamine, and Trace amine; Peptide receptors: Angiotensin, Bombesin, Bradykinin, C5a anaphylatoxin, Fmet-leu-phe, APJ like, Interleukin-8, Chemokine receptors (C-C Chemokine, C-X-C Chemokine, BONZO receptors (CXC6R), C-X3-C Chemokine, and XC Chemokine), CCK receptors, Endothelin receptors, Melanocortin receptors, Neuropeptide Y receptors, Neurotensin receptors, Opioid receptors, Somatostatin receptors, Tachykinin receptors, (Substance P (NK1), Substance K (NK2), Neuromedin K (NK3), Tachykinin like 1, and Tachykinin like 2), Vasopressin-like receptors (Vasopressin, Oxytocin, and Conopressin), Galanin like receptors (Galanin, Allatostatin, and GPCR 54), Proteinase-activated like receptors (e.g., Thrombin), Orexin & neuropeptide FF, Urotensin II receptors, Adrenomedullin (G10D) receptors, GPR37/endothelin B-like receptors, Chemokine receptor-like receptors, and Neuromedin U receptors; Hormone protein receptors: Follicle stimulating hormone, Lutropin-choriogonadotropic hormone, Thyrotropin, and Gonadotropin; (Rhod)opsin

receptors; Olfactory receptors; Prostanoid receptors: Prostaglandin, Prostacyclin, and Thromboxane; Nucleotide-like receptors: Adenosine and Purinoceptors; Cannabis receptors; Platelet activating factor receptors; Gonadotropin-releasing hormone receptors; Thyrotropin-releasing hormone & Secretagogue receptors: Thyrotropin-releasing hormone, Growth hormone secretagogue, and Growth hormone secretagogue like; Melatonin receptors; Viral receptors; Lysosphingolipid & LPA (EDG) receptors; Leukotriene B4 receptor: Leukotriene B4 receptor BLT1 and Leukotriene B4 receptor BLT2; and Class A Orphan/other receptors: Platelet ADP & KI01 receptors, SREB, Mas proto-oncogene, RDC1, ORPH, LGR like (hormone receptors), GPR, GPR45 like, Cysteinyl leukotriene, Mas-related receptors (MRGs), and GP40 like receptors.

Class B (the secretin-receptor family) of the GPCRs includes receptors for polypeptide hormones (Calcitonin, Corticotropin releasing factor, Gastric inhibitory peptide, Glucagon, Glucagon-like peptide-1,-2, Growth hormone-releasing hormone, Parathyroid hormone, PACAP, Secretin, Vasoactive intestinal polypeptide, Diuretic hormone, EMR1, Latrophilin), molecules thought to mediate intercellular interactions at the plasma membrane (Brain-specific angiogenesis inhibitor (BAI)) and a group of *Drosophila* proteins (Methuselah-like proteins) that regulate stress responses and longevity.

Class C Metabotropic glutamate/pheromone receptors include Metabotropic glutamate, Metabotropic glutamate group I, Metabotropic glutamate group II, Metabotropic glutamate group III, Metabotropic glutamate other, Extracellular calcium-sensing, Putative pheromone Receptors, GABA-B, GABA-B subtype 1, GABA-B subtype 2, and Orphan GPRC5 receptors.

Sensor molecules useful for the invention may comprise G protein coupled receptors which, when expressed in a cell the N-terminus of the receptor is outside the cell and the C-terminus is inside the cell. The person skilled in the art is aware of suitable techniques for detecting the orientation of a transmembrane protein. Such techniques comprise but are not limited to crystallography, NMR-studies, modeling studies as well as microscopy techniques, like immunolabeling combined with detergent permeabilisation controls for light or electron microscopy preparation, fragment complementation tagging of two polypeptides and the like.

In a preferred embodiment, the G protein coupled receptor is a Class A GPCR. In a further preferred embodiment, the class A (rhodopsin-like) GPCR is an odorant receptor, dopamine receptor, muscarinic receptor or an adrenergic receptor, more preferably an odorant receptor. The odorant receptor can be from any source as long as when expressed in a cell the N-terminus of the receptor is outside the cell and the C-

terminus is inside the cell. Examples include, but are not limited to, a chordate receptor, a nematode receptor, or a biologically active variant or fragment of any one thereof. Examples of chordate receptors include, but are not limited to mammalian receptors, avian receptors and fish receptors. In a preferred embodiment, the odorant
5 receptor is a nematode receptor or biologically active variant or fragment thereof. In an embodiment, the nematode receptor is a *Caenorhabditis elegans* receptor, or biologically active variant or fragment thereof. Examples of odorant receptors that can be used to produce polypeptides of the invention and/or used in the methods of the invention are described in Buck and Axel (1991), Robertson (1998 and 2001), Aloni et al. (2006), Feldmesser (2006), Olender et al. (2004a and b), Glusman et al. (2000a,
10 2000b and 2001), Fuchs et al. (2001), Pilpel and Lancet (1999), Sharon et al. (1998), Zozulya et al. (2001), Niimura and Nei (2003), Lander et al. (2001), Zhang and Firestein (2002), Young et al. (2002), and Fredriksson and Schioth (2005). Furthermore, a comprehensive list of odorant receptors are available from the SenseLab
15 website (<http://senselab.med.yale.edu>).

In other embodiments, the GPCR is a Class B or Class C receptor, with Class C being more preferred of these two embodiments.

In a particularly preferred embodiment, the G protein coupled receptor comprises seven transmembrane domains.

20 The bioluminescent protein can form part of the first, third, fifth non-transmembrane loops (domains) or the C-terminus of the G protein coupled receptor (or polypeptide of the invention). The acceptor molecule also can form part of the first, third, fifth non-transmembrane loops (domains) or the C-terminus of the G protein coupled receptor (or polypeptide of the invention). Each of these regions is
25 intracellular when the G protein coupled receptor is expressed and present in a cell.

The acceptor molecule cannot be in the same region as the bioluminescent protein when part of the same molecule (namely, the same single polypeptide chain), however, the acceptor molecule can be in the equivalent region as the bioluminescent protein when the G protein coupled receptor is present as a dimer or higher multimer.
30 For example, the bioluminescent protein can form part of the C-terminus of one subunit of the receptor, and the acceptor molecule can form part of the C-terminus of another subunit of the receptor. In this example, the subunit to which the label is associated can be the same or different, for instance the two subunits can be identical apart from one labelled with the bioluminescent protein and the other labelled with the acceptor
35 molecule.

In one embodiment, the bioluminescent protein forms part of the third non-transmembrane loop of the GPCR subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop. In an alternate embodiment, the acceptor molecule forms part of the third non-transmembrane loop of the GPCR subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop.

In another embodiment, the bioluminescent protein forms part of the first non-transmembrane loop of the GPCR subunit, and the acceptor molecule forms part of the third non-transmembrane loop. In another embodiment, the acceptor molecule forms part of the first non-transmembrane loop of the GPCR subunit, and the bioluminescent protein forms part of the third non-transmembrane loop.

In a preferred embodiment, the bioluminescent protein forms part of the fifth non-transmembrane loop of the GPCR subunit, and the acceptor molecule forms part of the C-terminus. In an alternate embodiment, the acceptor molecule forms part of the fifth non-transmembrane loop of the GPCR subunit, and the bioluminescent protein forms part of the C-terminus.

In another embodiment, the G protein coupled receptor comprises at least two subunits, where the bioluminescent protein forms part of the third non-transmembrane loop of a first subunit, and the acceptor molecule forms part of the fifth non-transmembrane loop of a second subunit. In an alternate embodiment, the acceptor molecule forms part of the third non-transmembrane loop of a first subunit, and the bioluminescent protein forms part of the fifth non-transmembrane loop of a second subunit.

In another embodiment, the G protein coupled receptor comprises at least two subunits, where the bioluminescent protein forms part of the first non-transmembrane loop of a first subunit, and the acceptor molecule forms part of the third non-transmembrane loop of a second subunit. In another embodiment, the acceptor molecule forms part of the first non-transmembrane loop of a first subunit, and the bioluminescent protein forms part of the third non-transmembrane loop of a second subunit.

In another embodiment, the G protein coupled receptor comprises at least two subunits, where the bioluminescent protein forms part of the fifth non-transmembrane loop of a first subunit, and the acceptor molecule forms part of the C-terminus of a second subunit. In an alternate embodiment, the acceptor molecule forms part of the fifth non-transmembrane loop of a first subunit, and the bioluminescent protein forms part of the C-terminus of a second subunit.

In another embodiment, the G protein coupled receptor comprises at least two subunits and the donor and acceptor molecule are in the same site of the first and second subunits respectively.

In an embodiment, the bioluminescent protein or acceptor molecule is located
5 after the second amino acid of the fifth transmembrane domain and before the second amino acid before the beginning of sixth transmembrane domain. In another embodiment, the bioluminescent protein or acceptor molecule is located after about amino acid 8 after the fifth transmembrane domain or after about amino acid 22 after the fifth transmembrane domain. In a further embodiment, the bioluminescent protein
10 or acceptor molecule is inserted about 10 or 12 amino acids before the sixth transmembrane domain. Most preferably, the bioluminescent protein or acceptor molecule is located in the middle of the third non-transmembrane loop (domain).

With regard to the C-terminus, it is preferred that about 5 to 25 amino acids of the natural C-terminus remain at the end of seventh transmembrane domain.
15 Preferably, the bioluminescent protein or acceptor molecule is inserted after about the 16 or 20 amino acids after the seventh transmembrane.

Turning to the location of the bioluminescent protein or acceptor molecule in the first non-transmembrane loop (domain), it is preferred that said label is inserted about two amino acids after the end of first transmembrane domain and about two amino
20 acids before the beginning of the second transmembrane domain. Most preferably, the bioluminescent protein or acceptor molecule is located in the middle of the first non-transmembrane loop (domain).

In a further embodiment, the bioluminescent protein can form part of the N-terminus, second, fourth, or sixth non-transmembrane loops (domains) of the G protein
25 coupled receptor (or polypeptide of the invention). The acceptor molecule also can form part of the N-terminus, second, fourth, or sixth non-transmembrane loops (domains) of the G protein coupled receptor (or polypeptide of the invention), however, it cannot be in the same region as the bioluminescent protein when part of the same molecule. Each of these regions is extracellular when the G protein coupled receptor is
30 expressed and present in a cell.

The GPCR may be a non-naturally occurring chimera of two or more different GPCRs. In particular, this enables a transduction cassette to be produced where portions of one receptor are always present in the chimera into which other portions of a wide variety of GPCRs are inserted depending on the compound to be detected.

35 In one embodiment, the subunit comprises the N-terminus and at least a majority of the first transmembrane domain of a first G protein coupled receptor subunit, at least

a majority of the first non-transmembrane loop through to at least a majority of the fifth transmembrane domain of a second G protein coupled receptor subunit, and at least a majority of the fifth non-transmembrane loop through to the C-terminal end of the first G protein coupled receptor subunit.

- 5 In another embodiment, the subunit comprises the N-terminus through to at least a majority of the fifth transmembrane domain of a first G protein coupled receptor subunit, and at least a majority of the fifth non-transmembrane loop through to the C-terminal end of a second G protein coupled receptor subunit.

As used herein, the term "at least a majority" of a specified portion (domain) of
10 a G protein coupled receptor, refers to at least 51%, more preferably at least 75% and even more preferably at least 90% of the specified region.

The skilled person can readily determine the N-terminal end, transmembrane domains, non-transmembrane loops (domains) and C-terminus of a G protein coupled. For example, a variety of bioinformatics approaches may be used to determine the
15 location and topology of transmembrane domains in a protein, based on its amino acid sequence and similarity with known transmembrane domain of G protein coupled receptors. Alignments and amino acid sequence comparisons are routinely performed in the art, for example, by using the BLAST program or the CLUSTAL W program. Based on alignments with known transmembrane domain-containing proteins, it is
20 possible for one skilled in the art to predict the location of transmembrane domains. Furthermore, the 3 dimensional structures of some membrane-spanning proteins are known, for example, the seven transmembrane G-protein coupled rhodopsin photoreceptor structure has been solved by x-ray crystallography. Based on analyses and comparisons with such 3D structures, it may be possible to predict the location and
25 topology of transmembrane domains in other membrane proteins. There are also many programs available for predicting the location and topology of transmembrane domains in proteins. For example, one may use one or a combination of the TMpred (Hofmann and Stoffel, 1993), which predicts membrane spanning proteins segments; TopPred (von Heijne et al., 1992) which predicts the topology of membrane proteins;
30 PREDATOR (Frishman and Argos, 1997), which predicts secondary structure from single and multiple sequences; TMAP (Persson and Argos, 1994), which predicts transmembrane regions of proteins from multiply aligned sequences; and ALOM2 (Klien et al., 1984), which predicts transmembrane regions from single sequences.

In accordance with standard nomenclature, the numbering of the transmembrane
35 domains and non-transmembrane loops (domains) is relative to the N-terminus of the polypeptide.

5 Variants of *C. elegans* str-112 (SEQ ID NO:41) and/or str-113 (SEQ ID NO:42) which bind 2-pentanone include, but are not limited to, molecules which are at least 90% identical to str-112 (SEQ ID NO:41) and/or str-113 (SEQ ID NO:42), biologically active fragments which are at least 90% identical to str-112 (SEQ ID NO:41) and/or str-113 (SEQ ID NO:42), and fusion proteins thereof such as str-114/113 (SEQ ID NO:43). As the skilled person would appreciate, when determining the % identity, sections of the proteins comprising labels, such as the acceptor and donor in SEQ ID NOs 13, 14, 18, 27, 28 and 30, are preferably ignored.

10 *Nucleic Acids*

In one embodiment, the domain (or molecule of interest) is a nucleic acid. As the skilled addressee would be aware, there are many detection systems which rely on nucleic acid binding which would be adapted for use in the methods of the invention.

15 Molecular beacons (MBs) have been extensively researched in the construction of probes useful for detecting specific nucleic acids in homogenous solutions. MBs consist of a single stranded nucleic acid sequence that possesses a stem and loop structure and is labelled with BRET components at the 5' and 3' ends. The close proximity of the 5' and 3' ends cause energy transfer to occur. The target DNA sequence hybridises with the probe nucleic acid sequence forcing the BRET
20 components to move apart and causing the BRET ratio to decrease. Although a combination of fluorophore-acceptor pairs have been investigated the method is flawed by requiring an excitation source which could cause autofluorescence of the nucleic acids. Replacing the fluorophore with a bioluminescent protein overcomes this problem.

25 In another example, an acceptor and donor can be conjugated to two different antisense oligonucleotides, each complementary to different portions of the same target nucleic acid sequence. The different portions of the target sequences are located in closely within the target nucleic acid sequence. BRET components are brought into close proximity upon hybridisation to the target nucleic acid resulting in an increase in
30 the BRET ratio.

In a further example, in the absence of target nucleic acid, acceptor and donor labelled complementary oligonucleotide probes hybridise causing energy transfer. In the presence of target nucleic acid, the target and donor labelled compete to hybridise with the acceptor protein thus lowering the BRET ratio. The decrease in BRET ratio
35 can be correlated with the amount of total nucleic acid present in the sample.

Uses

The present invention can be used to detect the presence or absence or concentration of a wide variety of analytes including small volatile and non-volatile organic molecules, macromolecules, and biological particles and cells. The invention is compatible with almost any biological recognition element that can be functionally coupled to a chemiluminescence transduction system, including G-protein coupled and other receptors, binding proteins, enzymes, peptides and nucleic acid molecules. Examples of uses of microfluidic methods and systems of the invention are described in Li and Lin (2009), Mark et al. (2010), Theberge et al. (2010), Mohammed and Desmulliez (2011), Esch et al. (2011), Yeo et al. (2011), Noh et al. (2011) and Godin et al. (2008).

In a particularly preferred embodiment, the analyte is an odorant. Typically, the odorant will be a volatile organic or inorganic compound or inorganic gas that may be detected by chemosensory odorant receptors of at least one organism. These may include amine- and/or sulphydryl-containing compounds, carboxylic acids, alcohols, aldehydes, alkanes, alkenes, aromatic compounds, esters, terpenes or terpene-derivatives, ethers, CO₂ etc. as well as compounds bearing combinations of these features.

Odorants may be indicative of some biological or chemical state of value or of interest to humans. Such indications may include:

- The sensory appeal, quality or safety of food and beverages, pharmaceuticals or related materials.
- The health, nutritional or exercise status of humans or animals.
- The presence or absence of hazardous substances, including pathogens.
- The progress or status of industrial processes.
- An environmental contamination or state.
- The sensory appeal, quality or safety of perfumes, fragrances or other cosmetics.

In a particularly preferred embodiment, the analyte does not bind the donor or acceptor domain.

In another embodiment, the method may be used for screening for a compound which binds the sensor molecule. As the skilled person would appreciate this allows the methods to be use in, for example, drug discovery and/or development. More specifically, the domain to which the analyte binds is a target for potential therapeutics. Thus, in this embodiment it is preferred that the domain bound by the analyte is a clinically important molecule such as, but are not limited to, an adrenergic receptor, a

serotonin receptor, a dopamine receptor, metabotropic/glutamate receptor, a GABA receptor, a vomeronasal receptor, a taste receptor, or a secretin-like receptor.

As another example, a method of the invention could be used to detect spoilage of milk such as ultra-high temperature (UHT) processed milk. In this example, the
5 sensor molecule can be a molecule cleaved by a bacterial protease that causes, at least in part, milk spoilage. For instance, the sensor molecule may comprise a region of a milk protein, such as κ -casein, which is cleaved by the protease(s) labelled with the chemiluminescent donor domain and an acceptor domain.

As the skilled person would be aware, the present invention can also be
10 multiplexed. In this system, two or more different sensor molecules are provided which bind different compounds. Each different sensor molecule includes a different donor and/or acceptor molecule such that they emit at different wavelengths to enable the detection and quantification of different target compounds.

15 **EXAMPLES**

EXAMPLE 1 – Performance of a hybrid BRET system in a microfluidic system for thrombin cleavage assay

Materials and Methods

BRET System

20 A combination of BRET¹ and BRET² techniques was used and referred to herein as hybrid BRET. Specifically, RLuc with native coelenterazine substrate was used as the bioluminescent donor and GFP² as the acceptor molecule. The donor and acceptor were linked by a peptide sequence containing the thrombin cleavage site (LQGSLVPR↓GSLQ (RG)) (GFP²-RG-RLuc) and expressed in *E. coli*. Thrombin
25 cleavage of the cleavage site resulted in a change in the hybrid BRET signal. The mechanism of BRET system is shown in Figure 1A.

Materials

GFP²-RG-RLuc biosensor was expressed and purified as reported previously
30 (Dacres et al. 2009a). The purified fusion protein was in thrombin cleavage buffer (10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA). The final concentration of the native coelenterazine substrate (Biosynth) used for microfluidic based assays was 58.6 μ M and 5 μ M for plate-reader based assays. 1 unit (U)/ μ l thrombin protease (Amersham Biosciences) solution was prepared in 1 $\times$ phosphate buffer saline (PBS).

35

Microfluidic Chip Fabrication and Experimental Set-Up

Simultaneous dual emission hybrid BRET measurements were carried out both in a microplate using a SpectraMax M2 spectrofluorometer (Molecular Devices) and in the microfluidics apparatus described below. Spectral scans of BRET constructs were recorded using the luminescence scan mode between 400 and 650 nm on addition of 5 μ M native coelenterazine substrate to 1 μ M of biosensor.

For the microchip BRET measurements, a simple Y-shape microchannel microchip (Figure 1B), 70 μ m wide and 50 μ m high was fabricated from polydimethylsiloxane (PDMS) using standard photolithography. The chip design was completed in a commercial drawing package (Adobe Illustrator CS4) and the design pattern was printed on a transparency mask (5,080 dpi, Allardice). Master patterns of the microfluidic devices were fabricated using a laminar dry film resist (Shipley 5038). Multiple layers of resist were laminated at 113°C onto a substrate of polished stainless steel. The channels were lithographically patterned using a collimated UV source (λ = 350-450 nm) operated at 20 mJ/cm² and a transparency film mask. After exposure, the test pattern was developed in a 20% Na₂CO₃ solution.

The pattern in resist was subsequently replicated as a Nickel shim using an initial sputter deposition of 100 nm Ni followed by electroplating to a thickness of 150 μ m. Then a 10/1 (w/w) ratio of PDMS and curing agent was poured over the shim, degassed and baked overnight at 75°C. The device was cut and peeled off the shim and then exposed to air plasma for 10 minutes. The PDMS was then immediately sealed with a glass slide; after baking for three hours at 75°C, the PDMS adhered strongly to the surface of the glass and the PDMS glass microchip was ready to use.

A schematic of the set-up for microfluidic measurement is shown in Figure 1C. A neMESYS high pressure pump system (Cetoni, Germany) was used to pump the fluids from two SGE syringes (Supelco) with 50 μ l capacity onto the microchip. The flow rates of both streams were 20 μ l/h. The microchip was placed on a microscope (Nikon Eclipse TE2000-U) stage for visualization and measurement. A sapphire laser (488 nm, Coherent) was used to locate the detection spot. Emitted bioluminescence was collected with a 20 $\times$ objective (Plan Fluor, Nikon). Bandpass filters (Nikon) of 515 nm – 555 nm for GFP² and 430 nm – 455 nm for RLuc were used for the two channels. De-magnification lenses (Nikon C-0.45 $\times$) were used to focus light emitted from each channel onto the photomultiplier tube (Hamamatsu H7421). Integration time was 200 ms for data acquisition for each channel of light. The measurement position was varied along the main channel starting at the first confluence of the input channels ($x = 0$) to the end of microchip.

Thrombin Assay

Various concentrations of thrombin were added to purified GFP²-RG-RLuc biosensor and incubated at 30°C for 90 minutes. To measure the extent of thrombin cleavage the sample mixture following incubation and the native CLZ solution were pumped from separate syringes into the two inlet channels and allowed to flow through the main channel. Diffusion between the two streams induced a BRET reaction at the interface. Recombinant hirudin from yeast (Sigma) was incubated with thrombin at room temperature for ten minutes prior to the protease assay.

10

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To confirm complete thrombin cleavage of the GFP²-RG-RLuc biosensor, SDS-PAGE analysis was carried out. Proteins (2.5 µg) were diluted in 1 × sample loading buffer (Invitrogen) for SDS-gel electrophoresis in a 12 % Bis-Tris gel with MOPS running buffer (NuPAGE, Invitrogen). Bands were stained with Fast StainTM (Fisher) and then visualised.

Data Analysis

Using the microplate spectrometer, hybrid BRET ratios were calculated as the ratio of bioluminescence emissions measured at 500 nm and 470 nm. Using the microchip system, the hybrid BRET ratio was calculated as ratio of the long wavelength emission (515 nm-555 nm) to the short wavelength emission (430 nm-455 nm) (Pfleger and Eidne, 2006). To allow comparison between the two different detection systems, the Hybrid BRET ratios were normalized by expressing them as a multiple of the BRET ratio without added thrombin, in the same measurement system. All data are reported as means ± standard deviation (SD). Two-tailed unpaired t-tests were performed using Graphpad prism (version 5.00 for Windows, Graphpad Software, San Diego, California, USA). Statistical significance is defined as $p < 0.05$.

30 Results

Effect of Thrombin on BRET Spectra and Ratio

The bioluminescent spectrum of the thrombin biosensor before thrombin treatment was bimodal with a peak at 470 nm representing RLuc emission and a second peak at 500 nm representing GFP² emission (Figure 2A). This indicates energy transfer from the excited state of native coelenterazine to GFP². Upon thrombin cleavage the green component of the spectrum was reduced, demonstrating that thrombin cleavage

35

of the thrombin biosensor had reduced the efficiency of energy transfer between donor and acceptor.

SDS-PAGE (Figure 2B) confirmed that following thrombin treatment, the fusion protein was cleaved into two components with molecular weights of 32.4 KDa and 36.4 KDa (Lane 5, Figure 2B) corresponding to His-tagged GFP² and untagged RLuc. Pre-incubation of the BRET biosensor with hirudin inhibited the formation of the two components demonstrating thrombin specificity (Lane 6).

The effect of thrombin on the biosensor cleavage was quantified using the change in BRET^H ratio. Following thrombin cleavage, the BRET^H ratio decreased significantly ($P = 0.0009$), by approximately 32 %, from 1.11 ± 0.06 to 0.75 ± 0.04 . The hybrid BRET ratio of 0.79 ± 0.05 following thrombin cleavage was not significantly different ($P = 0.3309$) from those obtained by mixing 1 μ M each of RLuc and GFP² (Figure 3, control). Pre-addition of hirudin prevented the thrombin induced reduction in the BRET^H ratio of 1.08 ± 0.14 . This was not significantly different to the ratio measured without thrombin ($P = 0.7663$).

On-chip BRET Measurement

To optimize the flow conditions for detecting the BRET biosensor, a series of experiments were carried out to image and quantify the BRET luminance at different locations, flow rates and biosensor concentrations (Figure 5). In the initial stage of contact of the two fluid streams of fusion protein and substrate, the diffusion layer was narrow and only a small volume of the liquid emitted bioluminescence (data not shown). The intensity of the bioluminescence was also low (~ 457) but significantly higher than the background (~ 2.3). From $x=1$ to 5 mm, the bioluminescence intensities remained almost constant but there was a significant increase at $x = 7$ mm. This increase may reflect increased mixing in the region. Regardless of the intensity of the bioluminescence, the BRET^H ratio remained almost constant (~ 5.2) throughout the entire measurement region. To benchmark the on-chip measurements, the BRET ratios were compared with the microplate data measured using a commercial BRET detection instrument. The relative changes of BRET ratio, i.e. BRET ratio measured with thrombin vs that measured without, are very consistent between microchip and microplate measurements (to within $\pm 4\%$).

The effect of varying flow rate and biosensor concentration on the BRET^H ratio was also investigated (Figure 6). The flow rate dependence was measured at two locations, $x = 0$ and 4.9 mm (Figure 6a). At $x = 0$, the BRET^H ratio was also constant (to within $\pm 1.1\%$) for the range of flow rates studied, i.e. 20 – 60 μ l/h. At $x = 4.9$ mm,

there was a slightly larger variation (to within $\pm 2\%$) of the BRET ratio due to the change in flow conditions. However, the overall uncertainty between the two measurement locations was within $\pm 5\%$. Figure 6b shows hybrid BRET ratio as a function of the biosensor concentration. Even though the uncertainty for low protein concentration was relatively high (e.g. standard deviation = 7.7% at 1.49 μM biosensor concentration), the overall variation in mean BRET^H ratios varied less than with biosensor concentration, i.e. a standard deviation of 2.7%. The small variation in the BRET^H ratio under different measurement conditions is an important finding since it means that the completeness of the BRET^H reaction is not crucial for quantification as long as bioluminescence can be measured. Although more complete mixing would be predicted to increase the luminosity of the system our data imply that it would have little effect in BRET^H ratio. This potentially simplifies the design requirements of microfluidic devices for BRET-based detection, at least for the levels of thrombin concentration considered in the study.

15

Effect of Thrombin Concentration

Using a flow rate of 20 $\mu\text{l/h}$ and biosensor concentration 2.972 μM (Figure 6) with the measurement fixed at $x = 2.1$ mm (Figure 5) we compared the microchip and the microplate systems for measuring thrombin using a range of thrombin concentrations (Figure 7). The BRET^H ratio changed linearly with increasing concentrations of thrombin up to 0.24 nM for microfluidic measurements and 2.7 nM for microplate measurements. At higher concentrations, the change of BRET ratio is much less pronounced due to the saturation of thrombin. In the low thrombin concentration regions, calibrations were linear with R^2 values exceeding 0.995. Comparison of the gradients of the calibrations revealed that the microfluidic method is 4.7 times more sensitive to changing thrombin concentrations than the microplate method. The detection limits for thrombin are 27 pM for the microchip-based technique compared to 310 pM using the microplate-based technique. The microchip-based BRET^H system has a detection limit intermediate between the values calculated for the BRET² and BRET¹ microplate-based assays of 15 pM and 53 pM, respectively (Dacres et al. 2009a).

Conclusion

Bioluminescence resonance energy transfer method has been demonstrated for the first time in a flow format using a fluid phase thrombin-sensitive biosensor. The BRET^H technique used is a combination of BRET¹ and BRET² which allows testing of

the BRET² components with measurable luminosity. The BRET reaction and detection were carried out in a Y-shape microchannel network in a microchip. Experiments quantified the effects of measurement location, flow rate and biosensor concentration. These factors affected the bioluminescence intensities in both optical channels but not the BRET^H ratio. The microchip-based technique showed an improved sensitivity for detecting thrombin compared to an equivalent microplate-based technique measured with a commercial instrument. The detection limits for thrombin were 27 pM for the microchip-based technique compared to 310 pM using the microplate-based technique.

10 **EXAMPLE 2 – Performance of BRET² in a microfluidic system**

In a BRET² system, *Renilla luciferase* (RLuc) with coelenterazine 400a (CLZ400A) substrate was used as the photon donor and GFP² was used as the acceptor molecule. As the luminescence of BRET² is 100-fold smaller than that of the BRET^{1,5}, the BRET² reaction requires efficient mixing at optimal temperature, detection chamber size, flow rates and concentrations to produce highest bioluminescence signal. Thus, this system is used to evaluate different mixing mechanisms (Figure 8), reaction chamber designs and reaction conditions.

PDMS chips with a Y-shaped microchannel with three mixing elements (Figure 8), with a rectangular cross section in the microchannels (200 µm in width and 30 µm in height) was used to monitor BRET² assays. The detection chambers with different diameter and height were located at the end of the microchannels. The emitting bioluminescence was collected by a multimode optical fiber located underneath the detection chamber. The emissions will be split by dichroic block and going through two band pass filters corresponding to emission band of the donor (430 nm-455 nm) and the emission band of the acceptor (515 nm-555 nm) before going into two corresponding photomultiplier tubes (Hamamatsu H7421).

The method involves flowing the protein solution in one the inlet of the Y-shaped channel and flowing coelenterazine 400a substrate in another inlet of the channel.

30 The method requires efficient mixing of the protein flow and the substrate flow by the passive mixing elements at a suitable flow rate.

The method aims to collect as high bioluminescence signal as possible by varying mixing elements, chamber size, protein and substrate concentration, flow rate, temperature etc. As a result, the optimum microfluidic mixing chip design and reaction conditions will be obtained and be translated into other BRET assays.

In order to enhance the efficiency, a fluidic chip integrated with a large optical detection chamber is used. By locating an optical fibre under the chamber the light emitted from BRET² reaction is collected and transmitted to the detection sub-system (Figure 9). This approach ensures minimal losses and therefore high sensitivity and
5 allows simultaneous capture of minute changes in emission levels at two wavelengths.

The inventors tested the detection sensitivity using a sensor protein capable of detecting a model protease (thrombin). The results indicated five fold improvement in BRET² detection sensitivity in comparison to commercially available microplate readers. In these tests we also confirmed that the detection limit was less than 20 pM.

10 Figure 10 shows sample data from the sensitivity tests. Emission counts for GFP and Rluc are indicated with green (top line in the first panel, bottom line in the second panel) and blue lines (bottom line in the first panel, top line in the second panel) respectively. The raw data are shown for the no thrombin blank and the condition with 270pM thrombin. The ratio between emission levels (BRET² ratio: GFP/RLuc)
15 indicates an approximately tenfold change in response to digestion of the sensor with 270pM of thrombin.

Figure 11 shows the response of the sensor when the thrombin concentration is varied from 0 to 270pM. The figure also indicates results when the same experiment was repeated with a commercially available instrument. The sensitivity (slope) is five
20 fold higher in the microfluidic system. The calculated limit of detection is less than 20 pM.

The BRET² ratio was measured with a two-inlet microfluidic device upon mixing sensor protein (1μM) with a preparation involving thrombin (540nM) and substrate (12.5μM). Control experiment was carried out by mixing sensor protein
25 (1μM) with substrate (12.5μM). Approximately 75% decrease in BRET² signal was measured for on-chip reaction at input flow rate of 50μL/hr (Figure 12).

Example 3 - Performance of a BRET² based odorant sensor in a microfluidic system

30 The BRET² system is more suitable for measuring ligand-induced molecular rearrangements in GPCRs compared to FRET (WO 2010/085844) or standard BRET. This is because the 6.8 nm separation of the BRET pair in preferred GPCR constructs (Dacres et al., 2010 and 2011) is well matched to the Förster distance of the BRET² donor and acceptor combination. However, one tradeoff is the low quantum yield for
35 the RLuc donor when using BRET² chemistry. This results in fewer photons being available for detection. The use of RLuc2 and 8 mutations has been shown to improve

the quantum yield (De et al., 2007) of the BRET² system whilst only having minimal effects on the Förster distance of the BRET² system (Figure 1). The present inventors replaced RLuc with RLuc2 or RLuc8 in a OGOR sensor in an attempt to increase photon yield without detriment to the sensitivity of the odorant assay. Figure 18 shows the transduction scheme for diacetyl detection using OGOR2 incorporating RLuc2.

Materials and Methods

Construction of BRET²-GPCR sensors incorporating tagged C. elegans odorant receptors

Chimaeric BRET² tagged odorant receptors have the BRET² components inserted into the third intracellular loop (IC3) and at the C-terminus of the *C. elegans* odorant receptor with green fluorescent protein, GFP² at IC3 and *Renilla* luciferase, RLuc at the C-terminus of the protein (OGOR). Using site-directed mutagenesis the RLuc2 mutations were introduced into the pYES-DEST-52 OGOR sequence. Primers 1 and 2 (Table 3) were used to introduce the mutation C124A and primer pair 3 and 4 was used to introduce the M185 V mutation to make the construct named OGOR2 (Figure 18, SEQ ID NO:1, SEQ ID NO:2).

Table 3. Primers for introducing the RLuc2 mutations. C124A and M185V, into the pYES-DEST52-OGOR sequence.

	Primer name	Sequence
1	C124A	CACGACTGGGGCGCCGCCCTGGCCTTCCACTAC (SEQ ID NO:7)
2	C124A Antisense	GTAGTGGAAGGCCAGGGCGGCGCCCCAGTCGTG (SEQ ID NO:8)
3	M185V	CTTCTTCGTGGAGACCGTGCTGCCCAGCAAGATC (SEQ ID NO:9)
4	M185V Antisense	GATCTTGCTGGGCAGCACGGTCTCCACGAAGAAG (SEQ ID NO:10)

OGOR2 Sample Preparation

Yeast colonies were inoculated in 10 mL SCMM-U (*S. cerevisiae* minimal media, composition per 200 mL: 1.34 g yeast extract without amino acids and 0.38 g yeast supplementation media without uracil) supplemented with 2 % glucose and incubated overnight at 28 °C. An aliquot of the overnight culture was used to inoculate

SCMM-U supplemented with 2 % raffinose and 2% galactose to a final O.D.₆₀₀ of 0.4 and incubated for an additional 72 h at 15 °C with shaking at 200 rpm.

Cell cultures were centrifuged at 1500 x g for 5 minutes at 4°C. Cells were resuspended in 1 mL of sterile water and centrifuged for 1 minute at 10,000 x g. Cells
5 were resuspended in 4 mL phosphate buffer solution (PBS). The cells were lysed by French press (~ 18000 psi) and cellular debris was removed by centrifugation at 15000 x g (4°C) for 15 minutes. Following this the supernatant fraction was centrifuged at 40,000 rpm (Beckman Coulter L-80 ultra-centrifuge) for 1 hour at 4°C. The supernatant was decanted and the membrane pellet was resuspended in 1 mL of PBS
10 and stored at 4°C for 48 hrs.

Diacetyl Assay

All ligand solutions were prepared directly in water. The OGOR concentration was normalized using GFP² intensity at 510 nm. Assays were carried out in 96-well
15 plates (Perkin-Elmer) in a total volume of 100 µL in phosphate buffered saline. OGOR was incubated with each ligand for 45 minutes at 28 °C in wells sealed with Topseal-ATM (Packard).

Plate-Reader Measurements

20 Following the incubation, Coelenterazine 400a substrate (Biosynth) was added to a final concentration of 5 µM. Simultaneous dual emission BRET² measurements were recorded with a POLARstar OPTIMA microplate reader (BMG LabTech) using the BRET² emission filter set, comprising an RLuc/Clz400a emission filter (410nm bandpass 80 nm) and a GFP² emission filter (515 nm bandpass 30nm), with gains set
25 set to 3300 and 4095, respectively, for the two channels, with an integration time of 0.5s.

Endpoint On-Chip Microfluidic Measurements

Endpoint microfluidic assays were carried out on-chip in two inlet microfluidic
30 mixer devices integrated with an optical detection chamber. Coelenterazine 400a substrate (Biosynth) was prepared to a final concentration 12.5 µM and introduced in first inlet. OGOR and OGOR2 membrane pellets were resuspended in 1 mL of PBS, diluted as required and incubated at 28°C for 45 minutes with diacetyl solution at concentrations ranging from 1aM – 1µM. The preparation was introduced in the
35 second inlet. On-chip mixing was initiated with an input flow rate of 400 µl/hr for each inlet. BRET² measurements were recorded using two photomultiplier tubes, one

equipped with an RLuc/Clz400a emission filter (410nm bandpass 80 nm) and the other with GFP² emission filter (515 nm bandpass 30nm). The optical output was collected using an optical fiber with 1 mm core diameter aligned with an on-chip optical detection chamber.

5

Real-Time On-Chip Microfluidic Measurements

On-chip real-time measurements were carried out in a three-inlet microfluidic mixer device integrated with an optical detection chamber. The first inlet was used to introduce Coelenterazine 400a substrate (Biosynth), which was prepared to give a final concentration of 12.5 μ M in PBS. The second inlet contained diacetyl (2,3-butanedione) diluted to give a final concentration of 1 fM in PBS, or PBS only as a control. The third inlet was used to introduce sensor protein suspension, which was prepared by resuspending the membrane pellet describe above in 1 mL of PBS and further diluting as required. The on-chip mixing was initiated by using input flow rates ranging from 50 - 400 μ L/hr. BRET² measurements were recorded using two photomultiplier tubes, one equipped with an RLuc/Clz400a emission filter (410nm bandpass 80 nm) and the other with GFP² emission filter (515 nm bandpass 30nm). The optical output was collected using an optical fiber with 1 mm core diameter aligned with an on-chip optical detection chamber.

20

Analysis

BRET² signals were calculated as the ratio of emission intensity at 515 nm to that at 410 nm. All data are reported as the mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM) as described in the text. Curves were fitted with log [agonist] vs response curves with variable slopes following normalization of data, using Graphpad Prism version 5.03 for Windows. Two-tailed unpaired t-tests were carried out in Graphpad prism. Statistical significance was defined as $p < 0.05$.

25

Results

30 *Intensity*

Introducing the RLuc2 mutation into OGOR increased the bioluminescence intensity by a factor of approximately ~ 150 from 1766 ± 125 RLU for OGOR to 272886 ± 26754 RLU for OGOR2 (Figure 19). This mutant version of OGOR therefore showed potential for facilitating BRET² detection of odorant binding by OGOR2 on a microfluidic chip. This prediction was confirmed by on chip

35

measurements. The RLuc2 mutation increased the bioluminescence intensity by a factor of approximately ~126 from 6.45 ± 2.9 RLU to 809.74 ± 116 RLU (Figure 20).

Demonstration of odorant binding by OGOR2 in a multiwell plate

5 There was a 21.4 % decrease in BRET² signal upon addition of 1 μ M diacetyl to membrane preparations containing OGOR2 (Figure 21) in the wells of a microplate. The diacetyl-induced change in the BRET² signal is significantly different ($P=0.0136$) from the control response to water. The percentage change in the signal in response to diacetyl is smaller than seen with the original OGOR sensor (32 %) incorporating
10 native RLuc. However, there was less variance in the blank measurements for OGOR2, 1.0 ± 7.0 % ($n=3$) compared with 1.0 ± 13.5 % ($n=4$) for OGOR. Therefore the OGOR2 sensor is potentially capable of detecting lower concentrations of diacetyl than the OGOR sensor because detection limit is calculated as the blank signal $\pm 3 \times$ S.D.

15 The OGOR2 response to diacetyl (Figure 22) is dose-dependent, with a linear range (spanning six log units, from 10^{-18} to 10^{-12} M (Figure 22). The calculated EC₅₀ value is 11.4 aM diacetyl. This is two orders of magnitude lower than for the OGOR response in a microwell, suggesting improved sensitivity for diacetyl.

20 *Detection of diacetyl binding by OGOR2 using a microfluidic endpoint assay*

Following incubation of 10 fM diacetyl with membrane suspensions containing OGOR2, microfluidic on-chip measurements showed a 36.9 % decrease in BRET² signal (Figure 23). The decrease in the BRET² signal was 1.5 fold greater than equivalent measurements made using a plate reader (Figure 21). This indicates that
25 microfluidic measurements using the OGOR family of biosensors are potentially more sensitive, *sensu stricto*, than plate reader measurements.

The concentration-dependent response of OGOR2, spans 2 log units from 10^{-18} to 10^{-16} M (Figure 24). The calculated EC₅₀ value is approximately ~ 10 aM diacetyl. This level is in good agreement with plate-reader measurements (Figure 22).

30

Real-time on-chip detection of odorant binding by OGOR2 with a microfluidic device

Real-time on-chip measurements showed a 27.4% decrease in the BRET² ratio following on-chip mixing of 1fM diacetyl with 290 nM of protein and 12.5 μ M substrate at input flow rates of 50, 100, 200 and 300 μ L/hr (Figures 25 and 26).

35

Example 4 - Performance of a BRET² based periplasmic binding protein sensor in a microfluidic system

Periplasmic binding proteins (PBPs) form a large and diverse family of soluble proteins found in bacteria. PBPs bind a diverse range of chemically disparate species including carbohydrates, amino acids, and neurotransmitters, metals and ions to name a few (Medintz et al., 2006). Although PBPs are unrelated at the primary sequence they all undergo a large ligand-induced conformational rearrangement commonly referred to as the 'venus-fly-trap' mechanism (Sharff et al., 1992 and 1993; Spurlino et al., 1991).

The measured distance between a FRET tagged N and C terminus of MBP of 6.93 nm (Park et al., 2009) is of a similar scale to the measured distance within the GPCR suggesting that BRET may be a better option compared to FRET for measuring distance in this range. Measurement of ligand binding by a PBP on a microfluidic chip could lead to a generic transduction platform with a wide range of applications areas including security, food and drink quality control, environmental and health-care. The inventors chose MBP as the initial test of this concept because it is a well-characterised member of the PBP superfamily and potentially representative of all PBPs. The BRET² transduction mechanism for maltose binding by periplasmic protein MBP is shown in Figure 27. This sensor is a proof-of-concept for all PBPs with a similar structure and/or ligand binding mechanism to MBP. It is well known that the affinity of MBP-based biosensors for maltose can be altered by targeted mutations of the MBP domain. A similar approach is applicable to other PBPs.

Materials and Methods

Construction of BRET proteins

RLuc2 was amplified by polymerase chain reaction (PCR) and cloned into pGEM[®]-T Easy vector (Promega, Australia). This resulted in a *Bst*BI site being introduced downstream of the amplified gene and a *Xho*I restriction site directly upstream from the amplified gene. DNA sequencing confirmed the correct amplicon sequence. The amplicon was inserted into the *Bst*BI and *Xho*I sites of pRSET GFP²-FL1-RLuc (Dacres et al., 2010) replacing RLuc to give pRSET GFP²-FL1-RLuc2.

MBP was amplified and ligated into the pGEM[®]-T Easy vector. During this process, a *Bst*BI site was inserted upstream of the amplified gene and a *Pst*I site downstream. MBP was restriction cloned into the *Pst*I and *Bst*BI sites of the pRSET GFP²-FL1-RLuc2 replacing the FL1 sequence with MBP to generate pRSET GFP²-MBP-RLuc2.

The W140 mutation was introduced into pRSET GFP²-MBP-RLuc2 using site-directed mutagenesis (Stratagene) using primers C1 (CAGATGTCCGCTTTCGCGTATGCCGTGCGTAC) (SEQ ID NO:11) and C2 (GTACGCACGGCATAACGCGAAAGCGGACATCTG) (SEQ ID NO:12). Nucleotide and amino acid sequences for BRET² tagged MBP receptor provided as SEQ ID NOs: 3 to 6).

Expression and Purification of BRET² proteins

Proteins were expressed in *E.coli* strain BL21 DE3 (Novagen). An overnight culture was grown from a single colony in LB (10 g tryptone, 5 g yeast extract, 5 g NaCl (pH 7.4)) containing 100 µg/mL ampicillin and 2 % glucose at 37 °C, 200 rpm. Expression was induced by inoculating 500 mL LB containing 100 µg/mL ampicillin to an A₆₀₀ of 0.1 and incubating at 37 °C (200 rpm) for 3.5 hours followed by overnight incubation at 22 °C (200 rpm). Cells were harvested 24 hr after inoculation.

For protein purification, cells were harvested by centrifugation at 4335 × g (4 °C) for 15 minutes and resuspended in equilibration buffer (50 mM sodium phosphate buffer, 300 mM NaCl, pH 7.0). The cells suspension was passed through a homogeniser (Avestin emulsiflex C3 (ATA Scientific, Australia)) at a pressure of ~ 22000 psi and the soluble protein fractions were isolated by centrifugation at 15000 × g (4 °C) for 15 minutes. Proteins were purified using cobalt affinity chromatography according to the supplied instructions (BD Talon (BD Biosciences, Clontech, Australia)). Following elution of the purified protein with 150 mM imidazole, the sample was dialysed against 50 mM Tris (pH 8), 100 mM NaCl, and 1 mM EDTA using a cellulose membrane (12,000 molecular weight cut off (Sigma)). Aliquots of 500 µL protein were snap frozen on dry ice and stored at -80 °C. Protein concentrations were determined by absorbance at 280 nm and calculated according to the method of Gill and von Hippel (1989).

Spectral Scans

All spectral scans were recorded with a SpectraMax M2 plate-reading spectrofluorometer (Molecular Devices, Australia). The reactions were carried out in 96-well plates (Perkin-Elmer, Australia). Bioluminescence scans of BRET² constructs were recorded using the luminescence scan mode scanning between 360 and 650 nm with 20 nm intervals.

End-Point On-Chip Microfluidic Measurements

On-chip measurements were carried out with microfluidic mixers with two inlets, a passive micromixer and an integrated optical detection chamber. BRET² measurements were recorded by using two photo multiplier tubes one equipped with an RLuc/Clz400a emission filter (410nm bandpass 80 nm) and the other with a GFP² emission filter (515 nm bandpass 30nm). The optical emission was collected by using an optical fiber with 1 mm core diameter aligned with on-chip optical detection chamber through a dichroic mirror.

Real-Time On-Chip Microfluidic Measurements

Real-time on-chip measurements were carried out with microfluidic mixers with three inlets, a passive micromixer and an integrated optical detection chamber. BRET² measurements were recorded by using two photo multiplier tubes one equipped with an RLuc/Clz400a emission filter (410nm bandpass 80 nm) and the other with a GFP² emission filter (515 nm bandpass 30nm). The optical emission was collected using an optical fiber with 1 mm core diameter aligned with on-chip optical detection chamber through a dichroic mirror.

BRET Protein Assays

1 μ M purified protein was used for all the energy transfer assays (final volume of 100 μ L). 1 μ M purified protein was prepared by diluting the protein in phosphate buffer solution (PBS, 0.058M Na₂H₂PO₄, 0.017 NaH₂PO₄, 0.068 M NaCl (pH 7.4)). Purified protein was incubated with the sugar dissolved in double deionised water or water for 30 minutes at 28 °C. Following incubation 16.67 μ M coelenterazine 400a was added and the signal was recorded immediately.

On-chip BRET Protein Assays

For on-chip assays 1 μ M purified protein was incubated with 1mM maltose solution at 28 °C for 40 minutes. The preparation was then mixed on-chip with 5 μ M coelenterazine 400a and the optical signal was recorded from the detection chamber. The control experiments was carried out by incubating protein preparations with water and mixing on the chip under the same conditions. The results were compared to determine the percentage change in BRET² signal upon addition of maltose and extended to determine the maltose sensitivity.

Real-time On-chip BRET Protein Assays

For real-time assays the protein preparation, maltose and the substrate were mixed on the chip simultaneously. 1 μ M purified protein was mixed with 1mM maltose solution and 5 μ M coelenterazine 400a substrate solution. The optical signal was recorded from the detection chamber. The on-chip reaction time was controlled by varying the flow rate. The change in the BRET² signal was measured for different reaction times and results were compared with a control experiment with water.

BRET Ratio Determinations

BRET ratios were calculated as the ratio of maximum acceptor emission intensity to maximum donor emission intensity.

Real-time On-chip Detection of Maltose

A y-shaped chip with two input channels and a serpentine common channel of length 18 mm was used. The cross-sectional dimensions of the common channel were 0.2 mm x 0.035 mm. The BRET reaction chamber was $\varnothing = 4$ mm and H = 1 mm. There was no mirror on the upper surface of the chamber. Light was captured and transferred to the standard dichroic detector using a bifurcated light guide with input (trunk diameter of 6 mm and NA = 0.59). PMT gate time was 500 milliseconds. Two different flow rates were tested: 200 μ L per hour and 400 μ L in the common channel.

Input A was prepared to contain 1 μ M maltose and 31.25 μ M Clz400a substrate or in the case of the negative control, 31.25 μ M Clz400a substrate only. Input B contained 1 μ M GMR sensor. A and B were pumped into separate arms of the Y-shaped microfluidic chip at input flow rates of 100 μ L/hour or 200 μ L/hour to give common channel flow rates of 200 μ L/hour or 400 μ L/hour, respectively. Total residence times were estimated at approximately 230 seconds in the first case and 115 seconds in the latter case. BRET² ratios were determined on the average data collected from 200-250 seconds after flow was commenced.

Data Analysis

All data analysis was carried out using GraphPad Prism (version 5 for Windows, Graphpad Software, San Diego, California, USA). All data will be reported as means $\pm$ standard deviation (SD) unless otherwise stated in the text. Two-tailed unpaired t-tests will be performed using Graphpad prism. Statistical significance is defined as $p < 0.05$.

Results

Maltose Detection by MBP BRET Ratio - Plate Reader Assay

The selectivity of the BRET tagged MBP protein was determined by testing the response to a range of sugars including monosaccharides, disaccharides and trisaccharides (Figure 28). Only maltose ($P = .001$) and maltotriose ($P = 0.02$) produced significant ($P < 0.05$) changes in the BRET signal from the BRET tagged MBP. The BRET biosensor did not respond to glucose, fructose, sucrose or raffinose. Fehr et al. (2002) demonstrated that the FRET biosensor was able to detect maltose and a range of maltose oligosaccharides but did not specifically recognise any pentoses, hexoses, sugar alcohols, disaccharides or trisaccharides that do not contain the α -1,4-glucosidic link. The amplitude of the change in BRET ratio decreased from 29.65 % to 17.03 % with increasing length of the maltose chain from two units (maltose) to three (maltotriose) (Figure 28). This is in agreement with reduced closing movement in the presence of larger α 1,4-oligomaltoside chains as demonstrated by electroparamagnetic resonance (EPR) studies (Hall et al., 1997) and FRET measurements (Fehr et al., 2002). Comparison of the relative size of the BRET² response to that of the FRET response reported in the literature (Fehr et al., 2002) demonstrated that substitution of BRET components for FRET components can increase the dynamic range of the biosensor resulting in signal changes of 29.65 ± 1.11 % for BRET compared to ~ 12 % for FRET (Fehr et al., 2002). The inventors expect the signal change for classic BRET¹ would be of the same order as that for FRET, based on the similarities in Forster distance.

Introduction of the W140A mutant into GFP²-MBP-RLuc2 abolished the BRET² response to maltose (Figure 28). No significant difference ($P = 0.63$) was observed between the BRET² response to either water or maltose. The W140A mutant has a dissociation constant higher than 100 mM for maltose and was previously used as a control for the FRET tagged MBP when applied to monitoring the uptake of maltose into yeast (Fehr et al., 2002). The lack of response of the W140A mutant to maltose indicates that the effect of maltose on the BRET² ratio of GFP²-MBP-RLuc2 is not due to a direct interaction between maltose and the BRET components themselves. These results confirm the potential suitability of the GFP²-MBP-RLUC2 (GMR) and similar sensors in a BRET² based microfluidic chip assay.

Response Time

The BRET² response increased with increasing incubation time until 30 minutes when the response reached a maximum (Figure 29). Thirty minutes was used for

further assays. In the present microplate assay format it is not possible to record the BRET² response in real-time but real-time maltose assay could be carried out using a microfluidic chip format.

5 Sensitivity

The BRET² tagged MBP biosensor was capable of quantifying different concentrations of maltose spanning three log units ranging from 1×10^{-8} M to 3.16×10^{-6} M with an EC₅₀ of 3.71×10^{-7} M (Figure 30a). The response of the FRET tagged MBP receptor is linear only over two log units ranging from 0.26 – 21.12 μ M with an EC₅₀ of 3.24 μ M.

On-chip Assay Measurements

Sensitivity assays were carried out on a two input microfluidic mixing device (Figure 8b) with a serpentine common channel of $L \approx 18$ mm. The BRET reaction chamber was $\varnothing = 4$ mm and $H = 1$ mm with no mirror. A bifurcated light guide was used with a 6 mm trunk and $NA = 0.59$. 1 μ M GMR sensor was incubated with maltose at concentrations ranging from 10^{-9} to 10^{-3} M for 40 min at 28 °C. The incubated sample and 31.25 μ M Clz400a substrate were each pumped onto the chip at input flow rates of 400 μ l/hr. The trunk end of the light guide was used to collect signal from the BRET reaction chamber. Two branches of the light guide were directed toward to two sets of filter blocks (410/80 for blue, 515/30 for green) in front of two PMTs. The BRET² signal was measured at each concentration. The experiment was repeated on each of three days, using the same batch of GMR sensor. Nine chips were used, one for each concentration tested, across all three days. The log concentration response curve for on-chip detection was effectively identical to that of the previous plate based measurements as was the EC₅₀ = 2.2×10^{-7} M (Figure 30b).

The specificity of the sensor for maltose over the saccharides glucose and sucrose was investigated on chip. The amplitudes of the emissions were high with Rluc/Clz400a signals in the range of 10000 to 25000 counts/gate (500 ms) and GFP2 signal in the range of 2000 to 10000 counts/gate (500 ms). In the absence of analyte, the BRET² ratio was 0.225. Binding of maltose (Figure 52) resulted in a large change in BRET² ratio with a mean BRET² ratio of 0.35 for 0.1 mM maltose, an increase over the no sugar control of 52%. Reactions with glucose and sucrose resulted in 7 and 15% increases in BRET² ratio, respectively, confirming that the selectivity of the BRET²-based sensor for maltose is maintained in a microfluidic format.

Real-time On-chip Detection of Maltose

As the inventors have previously shown for protease and volatile detection, the invention is capable of mixing sample with sensor and substrate on chip, so that the entire detection reaction can be performed in a continuous flow format on the chip.

- 5 The inventors demonstrated that this works with three inputs: sample, sensor and the coelenterazine 400A substrate using OGOR2 sensor and diacetyl (Figure 26) and using two inputs for thrombin (see, for example, Figure 51). The inventors extended this work to show that the same process works with the BRET-based maltose sensor. In this case, for convenience, the inventors also used a two input microfluidic chip. The
- 10 coelenterazine substrate and a sample solution containing maltose (or a negative control without maltose) were premixed and pumped into one input and the GMR sensor solution was pumped into the other input.

- Signals were detected approximately 40 seconds after initiating flow at both flow rates but stabilised more quickly (60 seconds vs approximately 130 seconds) in
- 15 the case of the faster flow rate (200 μ L per hour inputs) (Figure 67). Total luminance was strong and easily detected 1000-1500 counts/gate for Rluc/Clz400a and 6000-7000 counts/gate for GFP² at 100 μ L/hr or 2000-3000 counts/gate for Rluc/Clz400a and 7000-9000 counts/gate for GFP² at 200 μ L/hr.

- Once stabilised, 1 μ M maltose was easily distinguished from control at both
- 20 flow rates (Figure 64), with the slower flow rate giving a change in BRET² ratio of $\approx$ 20% and the faster flow rate showing a change of $\approx$ 27%.

Example 5 – Comparison of bioluminescent signal collection with and without fiber optical switch

- 25 One of the highly preferred requirements of the instrument for use in the invention is multiplexing, meaning the instrument must be able to detect several analytes nearly at the same time (i.e. with very small time delay between one analyte to the next one). To reduce the cost and space as well as weight of the instrument, optical fiber switch could be used to enable multiplexing. In an embodiment, several input
- 30 optical fibers will concurrently collect bioluminescent signal from at least six detection chambers. The optical switch will then connect one particular input fiber for a short period of time (few hundred milliseconds) to the single output fiber which connects to the optical blocks for splitting and band-passing the signal into two photomultiplier tubes (PMT). In the same fashion, the switch will turn on the next input fiber. In the
- 35 current example, the switch takes 50 ms to change between input fibers. Optical switching inevitably introduces optical losses in the collected signal. Thus, this

experiment was done to confirm if it is possible to collect signal through the optical switch and calculate the amount of signal loss due to optical switching.

Experimental Setup

5 The experimental setup is shown in Figure 31. Figure 31A shows the set-up without the optical switch. A single fiber was manipulated to align below the optical detection chamber of the microfluidic chip. The fiber is then connected directly the optical blocks. Figure 31B shows the set-up with the optical switch. A Leoni 1x9 mol fiber optical switch was used. The 9 inputs are terminated by the sub miniature A
10 (SMA) connectors while the single output fiber is terminated by ferrule connector (FC). A stainless plate was machined to contain an array of SMA receptacles allowing connection of optical fibers from the bottom. On the top of the plate sat the microfluidic chip with optical chamber aligned at the tip of the fiber. For this experiment one input fiber was used. The output fiber was then connected to the optical
15 blocks.

Materials and Methods

Endpoint microfluidic assays were carried out on-chip in two inlet microfluidic mixer devices integrated with an optical detection chamber. Coelenterazine 400a
20 substrate (Biosynth) was prepared to a final concentration 12.5 μ M TE buffer (10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA) and introduced in first inlet. GTR membrane protein was diluted in TE buffer (10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA) at concentration of 1 μ M. The prepared GTR solution was introduced in the second inlet. On-chip mixing was initiated with an input flow rate of 400 μ l/hr for
25 each inlet. BRET2 measurements were recorded using two photomultiplier tubes, one equipped with an RLuc/Clz400a emission filter (410nm bandpass 80 nm) and the other with GFP2 emission filter (515 nm bandpass 30nm). The optical output was collected using one single input fiber for both cases in Figure 31. The gate time was 500 ms.

Results

Figure 32 shows the real-time bioluminescent signal in the RLuc/Clz400 channel collected without and with optical switching for three runs. The GFP also shows similar behaviour (data not shown). From these results, it is confirmed that bioluminescent signal has been successful passed through the optical switch. In term of loss, Figure 33
35 compares the luminescent signals collected without and with optical switch in both

RLuc/Clz400a and GFP channels. The loss due to the optical switch for the RLuc/Clz400a channel was 40% while for the GFP channel it was 28%.

Example 6 –Optimisation of overall system luminance and photon detection efficiency

One of the important features of the invention is its ability to detect very low levels of analyte in real time with economical use of reagents. One of the keys to achieving this is to generate the maximum number of photons per unit volume of sensor solution. Incorporation of improvements like the RLuc2 mutation assist with this. It is also vital to minimise loss of photons in the optical detection system. High photon detection efficiency permits a desirable combination of response time, reagent economy and signal to noise ratio. Increasing the volume of the BRET reaction chamber and the common channels tends to increase the available light signal, which improves the signal to noise ratio but reduces the time resolution of the system. Increasing the flow rate in the device, other things being equal, improves the time resolution of the device but reduces its reagent economy and the signal to noise ratio. It is also vital to minimise losses in the optical detection system. A number of alternative experimental set ups were developed to improve the photon detection efficiency.

Experimental set up

On-chip measurements were carried out with a microfluidic chip similar to that shown in Figure 31A with two inlets, a passive mixing element and a detection chamber. An aluminium mirror was placed on top of the reaction chamber to enhance the BRET signal. BRET emissions were recorded using two photo multiplier tubes one equipped with an RLuc/Clz400a emission filter (410nm bandpass 80 nm) and the other with a GFP² emission filter (515 nm bandpass 30nm).

A number of variations were compared with the original set up (Figure 31A). The diameter of the reaction chamber was increased to 4 mm (Figure 34A) from an original diameter of 2 mm. The height of the reaction chamber was varied between 1 and 2 mm. A single optical fiber of diameter 0.4-1.0 mm (Figure 31A) was replaced with a liquid light guide or fibre-optic bundle of 5 mm core diameter NA = 0.59, feeding into a dichroic block (Figure 34A), or a bifurcated light guide of trunk core diameter 6 mm branch core diameter of 4 mm (Figure 37) or a multifurcated light guide of trunk core diameter 8 mm and branch core diameter 4mm (Figures 38 and 40).

Materials and Methods

OGOR2 sensor solution was prepared at day 4 in PBS at high (100 and 50 times) dilution ratios from stock solution. Clz400a was prepared in PBS at 31.25 μ M. When diacetyl was included, it was added to the OGOR2 sensor solution at a final concentration of 1 μ M. For the control experiment, only DI water was added. The sensor tubes without and with diacetyl were both incubated at 28°C in 30 min.

Results

Light collection was compared between the original system with fiber diameter 1.0 mm and the modified system using a single 5 mm core liquid light guide (Figure 35). At a 100 fold dilution of sensor, the original system had high noise levels and neither the RLuc2 nor the GFP² signals could be discriminated from noise. In contrast, using the liquid light guide, background noise levels were substantially lower and both emission channels could be clearly discriminated from noise by approximately 50 seconds after switching the system on. Maximum signal levels were achieved by approximately 200 seconds after reagent pumping commenced. It should be noted that because the newer configuration has a BRET reaction chamber volume 4 fold greater than the original one, it takes up to 100 seconds longer for the signal to saturate in this condition. Nevertheless, discrimination from baseline can be detected by 50 seconds. Similar results were observed for the 50 times dilution ratio (results not shown). With the improved system, it was possible to detect analyte using OGOR2 at 50x and 100x dilutions (Figure 36).

If each microfluidic channel has a dedicated photodetector then a scheme such as that shown in (Figure 37), in which each microfluidic channel is interfaced with a bifurcated light guide that channels light into a pair of photomultipliers or equivalent detectors, is a suitable arrangement. Comparison of this arrangement with a dichroic block shows that it may have better photon detection efficiency and signal to noise characteristics under otherwise comparable conditions (Figure 39).

If light guides (core diameter $\geq$ 1.0 mm) are used instead of optical fibres (core diameter $\leq$ 1.0) it is difficult to obtain a suitable optical switch for time domain switching of the optical detection system between different microfluidic channels. In this case, a shutter box may be used. An example of a suitable optical architecture is shown in Figure 38. Light collected from the multichannel chip is directed to the shutter box and the output is channelled to the optical detector via a multifurcated fiber bundle. The shutters are located in the input side of the multifurcated fiber bundle allowing software selection of the sequence of channels and the duration of monitoring.

A suitable multifurcated light guide arrangement is shown in Figure 40. Results obtained comparing this arrangement with channel-specific bifurcated light guides (Figure 37) indicate that the dichroic filter gives an 8x higher signal in the GFP channel and a 6.6x higher signal in the Rluc channel compared with bifurcated light guides (Figure 41). The BRET² ratio was 4.67 ± 0.07 with the dichroic filter and 3.85 ± 0.25 for the bifurcated arrangement.

Example 7 - Examples of suitable valve and solid state-based photodetectors

A very wide variety of vacuum based and solid state sensors are commercially available, which can be interfaced with the microfluidic chip in order to measure the light produced by the BRET reaction. Figure 42 illustrates a high counting efficiency, high gain, low dark-noise assembly using traditional vacuum tubes. Figure 43 illustrates a high photon detection efficiency, high gain, low dark-noise assembly implemented using solid-state technology. Many variants on these two approaches are available.

Example 8 - Experimental and theoretical optimisation of diffusional mixing in the microfluidic chip

Laminar flow conditions may pertain in a microfluidic chip of typical dimensions used in this invention. In this case mixing occurs principally by diffusion and may require slow flow rates and long residence times to approach completion. Slow flow rates are undesirable because they result in a slower time to first detection of analyte or analytes than would otherwise be the case. This limitation may be overcome by forcing turbulent mixing, using for example more complex microfluidic geometries and/or pulsatile flow and/or micromechanical mixing and/or acoustic and/or electrokinetic means. All of these methods, whilst feasible, potentially involve additional engineering complexity and cost. The inventors therefore investigated simple passive design features that can enhance diffusive mixing in a laminar flow environment.

Experimental setup

For investigations with dyes, the inventors used a three inlet microfluidic network (Figure 44). Thrombin detection experiments used a two-inlet microfluidic network, with Y-shaped geometry (Figure 31a). The BRET reaction chamber (not shown) was either 2 mm or 4 mm in diameter. The dimensions of all microchannels were 200µm wide by 35 µm deep. In the original (side-by-side) set up, the channels

contacted each other along their vertical (35 μm) sides and were fabricated with a serpentine mixing region approximately 28 mm long (Figure 44). In a modified (pancake stack) set up (Figure 45), the two input channels were 30 μm deep and 600 μm wide and were stacked on top of each other, in contact via their horizontal (600 μm) sides to form a linear common channel 600 μm wide and 60 μm deep. The length of the common channel was 20 mm.

For dye experiments, solutions of food dyes were drawn from three input microchannels using a single pump in withdrawal mode at flow rates of 30-300 μL per hour. For thrombin sensing experiments we used 12.5 μM coelenterazine A substrate, which was premixed with the test concentration of thrombin and pumped into one arm of the Y-shaped microchannel. 1 μM of a BRET² based thrombin biosensor, prepared as previously described (Dacres et al., 2009b) was pumped into the other arm of the microchannel. Input channel flow rates were varied from 50-400 μL per hour. The limits of detection were estimated informally as the lowest concentration of thrombin for which the operator could discern a change in the BRET² ratio at a input reagent flow rate of 50 μL per hour.

Results

As shown in Figure 44, using a side-by-side configuration, flow is laminar at high flow rates (common channel flow rate = 300 μL hour), corresponding to residence times of 2.35 seconds, and there is little or no observable mixing. Flow remains laminar at lower flow rates (common channel flow rate = 30 μL per hour) corresponding to a residence time of 23.5 seconds, but significant diffusional mixing can be detected over this period.

Calculations of residence times

i) For withdrawal flow rates of 300 μL per hour and the three input network:

Volume of the serpentine region is $0.2 \text{ mm} \times 0.035 \text{ mm} \times 28 \text{ mm} \Rightarrow 0.196 \mu\text{L}$.

Flow rate in the common channel is 300 μL per hour.

$0.196/300 = 6.53 \times 10^{-4} \text{ hours} = 2.352 \text{ seconds}$.

30

ii) For input flow rates of 30 μL per hour:

The residence time is 10 x longer, i.e. 23.52 seconds.

iii) For the side by side stack:

35 Volume of the *serpentine region* is $0.2 \text{ mm} \times 0.035 \text{ mm} \times 28 \text{ mm} \Rightarrow 0.196 \mu\text{L}$. Flow rate in the common channel is 100 μL per hour.

$0.196/100 = 1.96 \times 10^{-3}$ hours = 7.056 seconds.

Volume of the *BRET reaction chamber* ($\pi 1^2 \times 1 \text{ mm}^3$) = 3.14 μL

Flow rate is 100 μL per hour. $3.14/100 = 0.0314$ per hour = 113 seconds

Total residence time is 120 seconds.

5

iv) For the pancake stack:

Volume of channel is $0.6 \text{ mm} \times 0.06 \times 20 \text{ mm} \Rightarrow 0.72 \mu\text{L}$. Flow rate in the channel is 100 μL per hour

$0.72/100 = 7.2 \times 10^{-3}$ per hour = 25.92 seconds

10 Volume of the *BRET reaction chamber* ($\pi 2^2 \times 1 \text{ mm}^3$) = 12.6 μL

Flow rate is 100 μL per hour. $12.6/100 = 0.126$ per hour = 452 seconds.

Total residence time is 478 seconds or 7 minutes 58 seconds.

15 The thrombin assay is very sensitive to sub-optimal mixing because both the analyte and the sensor are macromolecules with slow diffusion coefficients and also because thrombin ($k_{\text{cat}} \approx 85 \text{ s}^{-1}$) has to process a large number of sensors by proteolytic cleavage before a signal can be detected and this takes time.

20 Using a traditional side-by-side network, with a flow rate of 50 μl per hour in each input arm, the lowest concentration of thrombin observable in real time (120 seconds) was 540 nM. Using a pancake stack network at the same flow rate (478 second residence time) 27 nM thrombin could be detected easily (Figure 46). Thrombin was detected down to 14 nM, the lowest concentration tested. After adjusting for the fourfold difference in residence times there is at least a tenfold benefit in detecting a lower concentration of thrombin for the pancake stack.

25 The inventors attribute this improvement to improved diffusional mixing in the pancake stack architecture. They therefore compared the length of microfluidic channels (28 mm in the side-by-side example and 20 mm in the pancake stack example) with the theoretical distance required for complete mixing in these different configurations.

30 Calculation of distance required for complete diffusional mixing

L: Channel length (mm)

Q: Volumetric flow rate ($\mu\text{l/hr}$) or (mm^3/s)

$$Q = 200 \mu\text{l/hr} = 200 \text{ mm}^3/\text{hr} = 0.055 \text{ mm}^3/\text{s}$$

D : Diffusion coefficient (for thrombin) $D = 4.16 \times 10^{-5} \text{ mm}^2/\text{s}$

35 U: average velocity in channel (mm/s) = $Q/\text{width} \times \text{height}$ (channel cross sectional area)

X: Diffusional distance travelled in period t is estimated by $X^2 = t D$.

The X value for complete mixing depends on the channel configuration. Assuming a two-input channel architecture, for the side-by-side design, X is half the common channel width and, for the pancake stack design, X is half the channel height.

5

Residence time for complete mixing = $L / U = X^2 / D \Rightarrow QX^2 / \text{width} \times \text{height} \times D$

Therefore:

10 1. Current (side-by-side) design where:

H (Channel Height) = $34 \mu\text{m} = 0.034 \text{ mm}$ and

W (Channel Width) = $200 \mu\text{m} = 0.2 \text{ mm}$

$L \approx QW^2/4WHD = 0.055 \text{ mm}^3/\text{s} \times 0.04 \text{ mm}^2 / 4 \times 0.2 \text{ mm} \times 0.034 \text{ mm} \times 4.16 \times 10^{-5}$
 15 $\text{mm}^2/\text{s} = 1944 \text{ mm}$

2. Current (pancake stack) design where:

H (Channel Height) = $60 \mu\text{m} = 0.060 \text{ mm}$ and

W (Channel Width) = $600 \mu\text{m} = 0.6 \text{ mm}$

20

$L \approx QH^2/4WHD = 0.055 \text{ mm}^3/\text{s} \times 3.6 \times 10^{-3} \text{ mm}^2 / 4 \times 0.6 \text{ mm} \times 0.06 \text{ mm} \times 4.16 \times 10^{-5}$
 $\text{mm}^2/\text{s} = 33 \text{ mm}$

3. Optimised (pancake stack) design where:

H (Channel Height) = $14 \mu\text{m} = 14 \times 10^{-3} \text{ mm}$ and

25 W (Channel Width) = $1200 \mu\text{m} = 1.2 \text{ mm}$

$L \approx QH^2/4WHD = 0.055 \text{ mm}^3/\text{s} \times 196 \times 10^{-6} \text{ mm}^2 / 4 \times 1.2 \text{ mm} \times 14 \times 10^{-3} \text{ mm} \times 4.16 \times 10^{-5}$
 $\text{mm}^2/\text{s} = 3.9 \text{ mm}$

30 Therefore the side-by-side stack provides only 28 mm (i.e. 1.4%) of the 1944 mm required for complete mixing whereas the pancake stack arrangement tested provided 20 (i.e 61%) of the 33 mm required for complete mixing. Calculations demonstrate that with minor additional changes it would be feasible to arrange for diffusive mixing to be complete within 7.7 mm i.e. less than 40% of the length
 35 available in the current design.

Example 9 - Improvements to microfluidic network designs and pumping arrangements

Microfluidic networks where the lengths of multiple reagent input channels connected to a common port vary as, for example in Figure 14, had poor reliability (results not shown) because of the tendency for the differences in back pressure to prevent flow in longer channels, routing all flow through shorter channels. These designs are also very susceptible to blocking by bubbles, for similar reasons. The inventors therefore tested a number of different design features and pumping arrangements to improve the reliability of the multichannel device.

Experimental Setup for Paired Symmetrical Microfluidic Sensors

In one example (Figure 47), the inventors designed a network with a bilaterally symmetrical parallel channel layout to obtain two simultaneous reactions using two different sensors. This arrangement can be replicated to obtain any even number of sensor channels.

Results

The inventors demonstrated fluid flow on the chip using food-colouring dye at two flow rates (Figure 48). Diffusive mixing was largely complete at a flow rate of 150 $\mu\text{l/hr}$, whereas at 1500 $\mu\text{l/hr}$ the input streams remained largely separate.

Flow was continuous and even along both arms of the network and blockage occurred less frequently than with asymmetric designs. Nevertheless, this design is still susceptible to blockage or uneven flow if a bubble or other obstruction lodges in one of the two parallel arms. The inventors therefore investigated other approaches for driving the sample and reagents through the microfluidic network.

It is desirable that each common channel has its own dedicated pressure source(s) that is/are not shared with any other common channel. This means that, should there be variation in the backpressures in the network for any reason, then flow cannot be diverted to a different common channel. Unfortunately, when operating in positive pressure mode, observation of this principle would mean that each common channel requires three dedicated pumps: one each for the sensor, substrate and sample, resulting in a system with complex and potentially expensive engineering requirements. An elegant and superior alternative is to drive the reagents through individual common channels using a single dedicated pump operating in suction (negative) mode. This only requires a single pump per common channel, as shown in Figure 49. The quality of laminar flow in a network driven by suction pressure is even and reliable as shown

in Figure 44. In the worst case, blockage or partial obstruction in one common channel or the microfluidic channels leading into it only affects that sensor channel.

An ancillary benefit of providing a dedicated source(s) of pressure for each common microfluidic channel (and therefore for each different sensor) is that it allows
5 the simultaneous operation of multiple sensor channels independently of each other with potentially different flow rates and consequently different balances between speed and sensitivity (Figure 50). This might for example, allow two or more channels to use the same sensor at different flow rates in order to give a range of different limits of detection and time constants. Another option is to operate several channels with
10 different sensors, at different flow rates optimised for each sensor. An extreme example of this would be to run completely different sensor types such as a GPCR-based volatile sensor and a protease sensor in parallel on the same chip, with the same or different samples, and tailor the flow rates for the very different reaction kinetics of the two reaction classes. It would also be possible to use suction pumping, as described
15 here, to support the use of different types or dilutions of sample or substrate chemistry on the same chip at the same time.

Use of suction mode is fully compatible with the concept of providing reagents in simple disposable cartridges on the inlet side of the network. Changing cartridges would allow simple and rapid switching of applications or targets, using the same basic
20 hardware. An additional advantage is that the need to decontaminate the pumping device, between samples or after analyte detection is minimised in the suction mode.

The inventors performed an additional experiment incorporating the preferred light collection and detection setup from Example 6 together with the preferred suction mode pumping of this Example and a BRET²-based thrombin sensor from Example 2.
25 In this experiment, we used a simple Y-shaped microfluidic network with a serpentine common channel having dimensions of 0.2 mm x 0.035 mm x 28 mm. The BRET reaction chamber was $\varnothing = 2\text{mm}$ and height 1mm, giving a volume of 3.14 μL . Light from the reaction chamber was fed into one branch of a four-branch fiberoptic bundle and thence into a 25.4 mm (1 inch) diameter optical block for simultaneous dual
30 wavelength measurement with a dedicated dichroic filter and two PMTs. The chip was primed with buffer and 50 μL input reservoirs were loaded with 50 μL of 1 μM GTR thrombin sensor and 50 μL of 12.5 μM coelenterazine 400a substrate. Flow was started using negative pressure (suction mode) at the outlet with a common channel flow rate of 200 $\mu\text{L/hr} = 0.055\mu\text{L/sec}$. Under these conditions, the BRET reaction chamber
35 residence time was 57 seconds.

As shown in Figure 51, after startup was complete, the system generated very strong signals in both optical channels with excellent signal to noise ratios (compare with Figure 10a). Based on the rate at which signal develops and the BRET reaction chamber residence time, for a chamber with diameter 2mm, we estimate that a minimum chamber height of 300µm would still give measurable signals above background (Figure 51c). At the specified flow rate, this would correspond to a reaction mixture integration time of approximately 20 seconds. A chamber diameter of 4 mm, would potentially allow the chamber height to be reduced to 75 µm, whilst retaining the same signal strength and reaction mixture integration time.

10

Example 10 - Example of application of the invention to beverages and other fluids, including prophetic example of predicting plasmin spoilage of UHT milk

Background

The invention is readily applicable to any analytes that will dissolve in water or an aqueous solution, including volatile chemicals that will partition into an aqueous solution. Analytes that are already present in an aqueous liquid, including milk, fruit juices, other beverages and bodily fluids including blood serum are especially amenable to detection because there is no need for a preliminary gas-liquid partition prior to analyte measurement.

A simple example of an application in this area includes prediction of spoilage of UHT milk. Proteases from bacteria that have been killed by UHT treatment may cause increased viscosity, gelation, and bitterness in whole and skimmed UHT milk during storage, thereby leading to loss of shelf-life. Specifically, it is proteolysis of casein by plasmin that causes these problems in UHT milk. Commonly used assays for detecting proteases are slow and are insufficiently sensitive to easily detect the very low levels of protease that can result in spoilage of UHT milk after 6 – 9 months or more of storage at ambient temperature. The invention could measure such very low levels of plasmin in UHT milk with great sensitivity and in real time. It would be applicable to an in-line monitoring in a commercial setting. This will allow estimation of product shelf life and identify any need for additional processing prior to packaging. Based on our previous BRET² sensors for thrombin and caspase proteases, we would construct a biosensor for detection of plasmin activity in milk, by incorporating the target peptide sequence, preferably Lysine-X (where X = Lysine, Tyrosine, Valine or Glutamic acid), into the linker between BRET donor and acceptor (Figure 53). This sensor would be incorporated into a version of the invention suitable for on-farm and in-factory use. However, because we did not have such a sensor readily to hand, we

used some existing sensors, including one that detects thrombin, to demonstrate the feasibility of using our method to detect proteases in milk or indeed other commercially or medically important fluids such as orange juice and mammalian serum.

5 Method

In one experiment, we used the GFP²-FL₁-RLuc2 construct described by Dacres et al. (2012) diluted 1/125 (i.e. $\approx 0.5\mu\text{M}$) with 5 μM coelenterazine A in PBS or in various dilutions of full fat “Canberra Milk” brand milk, “Just Juice” reconstituted orange juice and mammalian blood or serum.

- 10 In another experiment, the inventors used the GTR BRET²-based thrombin sensor described in Examples 1 and 2 with RLuc replaced by RLuc2 (GFP²-RG-RLuc2 (GTR2)), construct described by Dacres et al. (2012) diluted 1/100 ($\approx 0.5\mu\text{M}$) with 5 μM coelenterazine A in thrombin cleavage buffer or various dilutions of milk, orange juice or serum. Thrombin cleavage of GTR2 in various dilutions of milk, orange juice
15 or serum was assessed by spiking the samples with 2 units of exogenous thrombin to simulate an endogenous protease. Serum was prepared from mammalian heparinized (250 IU/ml) blood samples. To prepare serum blood sample were left undisturbed at room temperature for 30 minutes and then centrifuged at 1,000-2,000 x g for 15 minutes. The supernatant is designated serum. The serum samples were maintained at
20 2-8°C while handling. All experiments were performed in 100 μL final volume in a microwell plate and BRET² signals were read in a Polarstar Optima microplate reader (BMG Labtech) as described previously.

Results

- 25 Preliminary results, using GFP²-FL₁-RLuc2, demonstrated that the BRET² chemistry functions well in the aqueous environment of whole milk and orange juice, diluted 1/10 with PBS (Figure 54). Figure 55 shows the time dependent decay of the BRET² signal of GFP²-FL₁-RLuc2 in whole milk.

- Results using GTR2 demonstrate that the BRET² chemistry also functions in
30 serum when diluted 1/10 in thrombin cleavage buffer (Figures 56 and 57). Bioluminescence activity was completely recovered when diluted 1/500 for serum, 1/50 for orange and 1/10 for milk in thrombin assay buffer compared to resuspension in thrombin assay buffer alone (Figure 56). The BRET² ratio was higher for all serum dilutions compared to buffer except for 1/1000 dilutions in buffer (Figure 57a). When
35 resuspended in orange the BRET² ratio was higher when diluted 1/10 in buffer compared to buffer alone but was a consistent value for all other dilutions (Figure 57

b). Only GTR2 resuspended in undiluted milk resulted in a BRET² ratio higher than when resuspended in buffer alone compared to all other milk dilutions (Figure 57c).

Thrombin activity was detected in 1/10 dilutions of milk and orange juice (Figure 58). In serum a 1/100 dilution of the serum sample in buffer resulted in
5 thrombin activity. All dilutions resulting in thrombin activity produced significant changes ($P < 0.0001$) in the BRET² ratio compared to samples without addition of thrombin. Thrombin activity in serum diluted 1/1000, orange diluted 1/100 and milk diluted 1/100 in buffer resulted in BRET² signal changes not significantly different ($P > 0.25$) from those generated in thrombin cleavage buffer.

10

Example 11 - Demonstration of gas liquid transfer of volatiles using a wetted wall cyclone

One of the advantages of the invention is that it can be applied to detection of volatile analytes. However, because the sensors are necessarily dissolved or suspended
15 in aqueous solution, volatile analytes must partition from the gas phase into aqueous solution before they are available to contact the sensors. The inventors set out to demonstrate the feasibility of transferring volatile chemicals from air to liquid in a format compatible with the invention. There are a number of methods that could be used rapidly to equilibrate ambient air or target headspace with an aqueous based
20 sample liquid, including gas in liquid bubbling or misting. However, the inventors selected a wetted-wall cyclone to demonstrate the concept because suitable equipment is available commercially.

Experimental set up for initial tests

25 Initial tests were completed using the SASS2400 wetted-wall cyclone (Research International). The internal fan draws air at 40 L/min and equilibrates it with 1 mL water. To compensate for evaporation, the sample level is monitored and replenished from a 1300 mL reservoir of de-ionised water.

The sample chemical was placed in a 1.7-2 mL eppendorf tube. A hole was
30 drilled in an 80mm section of aluminium tubing to mount the tube in the inlet port of the SASS2400 (similar to Figure 59). Sampling time for these tests was inclusive of fan start-up and sample liquid filling times.

Method

35 Two consecutive runs were done for each test, to give a total volume of 2 mL of sample. Tests were done following the time kinetics of re-absorption of oxygen into

de-ionised water that had been de-oxygenated by sparging with nitrogen and with acetaldehyde and phenol. Oxygen concentration was measured with an oxygen electrode. Volatile concentrations in the SASS2400 sample fluid were estimated by liquid or gas chromatography, relative to standards.

5

Results

The wetted wall action of the sampler exposes a large area of the sample fluid to the sampled air flow and was very efficient in re-oxygenating the nitrogen sparged sample (Figure 60). At the earliest time point that could be measured with this equipment, 80% saturation with oxygen was achieved within about 7 seconds and the process was complete in under 60 seconds.

Phenol was detected in the SASS2400 sample at the earliest time point measured (Figure 61) and continued to accumulate in an approximately linear fashion with time up to the last sample point at 60 seconds.

Using this protocol, results obtained with acetaldehyde were unreliable with the concentration decreasing with using longer sampling times. The inventors attributed this to the very volatile nature of acetaldehyde and its propensity to degas rapidly from the sample reservoir.

20 Modified equipment setup

In order to improve the time resolution of experiments at early time points, the inventors modified the equipment to allow rapid re-direction of the input airflow after the SASS2400 fan was up to speed and the sample chamber had been filled, which takes up to about 12 seconds, usually about 9 seconds.

25 The fan in the SASS2400 is designed to draw in air from ambient around the device. Restricting the airflow through narrow or excessive lengths of plumbing could affect the designed airflow of 40 L/min and switching of the air path needs to be fast so the sampling times are consistent (Figure 63). Solenoid valves or butterfly valves were considered less than ideal because of their effects on air-flow. A 3-way L port pneumatically operated ball valve (BLS3L6B) was chosen to allow rapid switching of air intake from room air to room air plus the volatile specimen (Figure 60). The valve was connected with 1" BSP fittings for minimal constriction for the air flow and as it is a 3-way valve there was minimal additional plumbing required and the valve could be mounted very close to the SASS2400 air inlet. The valve was driven through a double acting solenoid and a 12 solenoid (ENS1275) connected to a 6 Bar air supply.

A microcontroller circuit controlled the operation of the solenoid valve. The operating time is programmable and was set to 15 seconds. No digital output was available from the SAS2400 to synchronise the microcontroller so it was triggered by a manual push button activated at the start of the SASS2400 test cycle. The response of the 3-way valve was below one second.

All testing was done in a fume hood because of the nature of the volatile samples. Specimens could not simply be placed near the inlet of the 3-way valve as specimen concentration drawn into the SASS2400 may have varied with any air-flow change in the fume hood. A 1¼ inch pvc barb fitting was attached to the specimen inlet side of the solenoid (Figure 62). A hole to mount a 2ml safe-lock conical tube was drilled on the lower side of the PVC fitting 20mm in from the end furthest from the solenoid. The air-flow at this point should be constant at 40 L/s due to the fan in the SASS2400. Specimens to be tested were placed in 1.7-2ml safe-lock conical tubes with the lids removed. The conical tubes were filled to the upper rim at the start of each test. The constant surface area exposed to the air-flow should ensure a constant rate of evaporation within each test cycle.

Modified test procedure

All experiments were conducted in a fume hood. Personal protection equipment including gowns, gloves, shoe covers and a full face mask were used.

After powering up and establishing a computer connection, the SASS2400 was set to run for 20 seconds, drawing only from room air with no sample exposure, to flush the system. The solenoid and valves were not active at this stage. The solenoid and its control circuitry were powered up and several operations of the solenoid valve were run to ensure that air pressure was adequate for quick operation of the valve.

The SASS was programmed for the duration of the test run. This was the desired exposure time for sampling plus an addition fifteen seconds for initial fan start-up during which the 3-way air intake valve was switched away from the specimen.

A clean 8 mL sample bottle was placed in the collection position on the front of the sampler. 0.6 mL of de-ionised water was placed in a new 2 ml sample vial ready to take the sample after the test. Temperature, humidity and atmospheric pressure were recorded.

The lid was removed from a clean 2 mL safe-lock tube. Phenol specimens were placed in the safe lock tube, filling it as close as possible to the rim, before placing the tube in the mounting hole. For liquid specimens the tube was placed in its mount and then filled with specimen. Care was taken to ensure that the liquid was filled to the rim

of the specimen tube so that a consistent surface area of the liquid was exposed to the air-flow.

The SASS2400 and the delay trigger for the solenoid were activated simultaneously. After fifteen seconds the specimen was switched into the SASS2400
5 air intake. At the end of the sampling time, the fan was switched off and the sample was pumped to a 8ml collection bottle on the front of the SASS2400 for 20 seconds to ensure that all of the sample was transferred to the sample bottle.

At the end of each test cycle the SASS2400 was programmed to run an internal peristaltic pump for 20 seconds to transfer the 1mL test sample into an 8ml bottle fitted
10 to the front of the SASS2400. 0.8 mL of the sample was transferred from the collection bottle to the analysis vial and a further 0.6 mL of de-ionised water was used to fill the vial before it was sealed and sent for analysis. Tests were run for acetaldehyde and phenol for sampling times from 5 seconds to 600 seconds and sent for chromatographic analysis.

15

Results using new procedure

Mean phenol concentration was 7.7 $\mu\text{g/mL}$ (i.e. $\approx 8\text{ppm w/v}$) in the SASS2400 sample after only 15 seconds exposure to ≈ 1 gram sample of phenol with a $\varnothing = 6\text{mm}$ surface area. Phenol is an example of a highly volatile compound with a vapour
20 pressure = 0.474×10^{-4} atmospheres at 20°C . Even after only a few seconds exposure, acetaldehyde concentrations in the sample vial were off-scale (i.e. $\gg \text{mg/L}$) demonstrating very rapid partition of acetaldehyde into the water. Rapid uptake and equilibration of these volatile organic compounds demonstrate the feasibility of the wetted wall cyclone as a gas-liquid transfer module prior to on-chip microfluidic
25 detection.

Example 12 - Additional GPCR volatile sensors developed

The inventors previously described the construction of a BRET²-based diacetyl sensor by inserting RLuc or RLuc2 and GFP² domains into the sequence of the odr-10
30 diacetyl receptor from *C. elegans*. This sensor was expressed in *S. cerevisiae* and a crude membrane suspension was prepared and demonstrated to respond with exquisite sensitivity and selectivity for diacetyl in a plate based assay and also in the microfluidic format described in Example 3 above. As noted however, one of the advantages of the current invention is that multiple microfluidic sensor channels can be operated
35 simultaneously in order to detect multiple individual analytes or, with appropriate selection of sensors, to provide a chemical fingerprint of a complex sample. To enable

this it is necessary to derive a number of compatible volatile sensors with distinct specificities. The inventors therefore selected five additional putative chemoreceptor cDNAs from *C. elegans* as starting points for the engineering of novel BRET²-based sensors. The inventors also constructed a chimaera between str-113 and str-114 in order to demonstrate the feasibility of deriving sensors that are synthetic molecular hybrids of naturally occurring receptors, with potentially novel ligand specificity that is not available readily from naturally occurring sequences.

SGSRs are chimaeras of *C. elegans* str-112, str-113, str-114, str-115, str-116 and str-114/113 with the BRET² tags GFP² and RLuc or RLuc2 inserted in the third intracellular loop and at the C-terminus, respectively. The positions of the third intracellular loops of STR proteins were predicted using "TMAP" an algorithm from "The Biology Workbench" (a web-based tool for prediction of transmembrane segments <http://seqtool.sdsc.edu>). These were named SGSR-112, SGSR-113, SGSR-114/113, SGSR-114, SGSR-115 and SGSR-116.

15

Method for design and construction of BRET² tagged *C. elegans* str odorant receptors

Except for SGSR-112, which was commercially synthesised, SGSR expression cassettes were designed and made by introducing multiple restriction sites into the relevant gene-specific PCR primers. PCR products containing those sites were cloned into TOPO PCR vectors (Invitrogen) and then digested with the corresponding restriction enzymes (REs) and ligated into the expression cassettes. Some alterations were made to suit particular genes if they possessed one or more RE sites used by the cassettes.

RE sites for str fragment 1 were NcoI (5') and BspEI (3'), for GFP2 are BspEI and SalI, for str fragment 2 are SalI and KpnI/ EcoRI as EcoRI cuts Str116 fragment2, and, for Renilla luciferase, KpnI/EcoRI and NotI.

The SGSR 114/113 chimera was constructed by modifying *C. elegans* SGSR-113 (by replacing the first fragment of *str-113*, 113-1 using the restriction sites NcoI and BspEI in the cassette with the corresponding fragment of *str-114*, str-114-1). Str114-1 contains the first 720 *str-114* nucleotides of, corresponding to its first 240 amino acids and was amplified by high fidelity PCR using primers incorporating the restriction sites, NcoI at the 5' end and BspEI at the 3' end.

All constructs were confirmed to be error free by restriction digestion and DNA sequencing.

The amino acid sequences of GFP2 RLuc labelled SGSR-112, SGSR-113, SGSR-114, SGSR-115, SGSR-116 and SGSR-114/113 receptors are provided as SEQ

ID NOs 13 to 18 respectively, whereas the corresponding open reading frames are provided as SEQ ID NOs 19 to 24 respectively.

Results with additional six sensors

5 All SGSR yeast membrane preps had strong GFP² and BRET² signals after induction by galactose at 15°C for 72 hours and all of them showed changes in BRET² ratio when exposed to a medium conditioned with OP50 *E. coli* bacteria (a food source for *C. elegans*) compared to LB medium alone. The inventors selected a number of specific volatiles (including 1-hexanol, 1 butanol, butane-2,3-dione, 3-
10 hydroxybutanone, 2-pentanone and 2 nonanone) for further testing based on GC-MS analysis of the headspace of OP50 bacteria grown on LB. The volatile ligand, 2-pentanone, tested positive for three of these sensors SGSR-112, SGSR-113 and SGSR-114/113 (Figure 64). This is the first time that a volatile ligand (or indeed any ligand) has been identified for a BRET-based GPCR sensor, in the absence of prior knowledge
15 of the ligand based on research with the unmodified parental GPCR. It is a demonstration of the utility of the BRET system for de-orphaning receptors generally and *C. elegans* chemoreceptors in particular.

Concentration-response characteristics (Figure 65) indicate an EC₅₀ likely to be in the picomolar range. Not only does this reduce to practice the process of
20 engineering, de-orphaning and characterising novel volatile sensors, it also demonstrates, at least in the case of SGSR-112 a viable method for de-orphaning the parental native receptors, the first time this has been achieved for almost 20 years.

The BRET² tagged SGSR-114/113 and SGSR-113 sensors responded to a range of volatile ligands including alcohols and ketones (Figure 64). The inventors identified
25 six volatile ligands for SGSR-114/113 and four for SGSR-113. The SGSR-112 response to 2-pentanone was linear over 9 log units from 1×10^{-14} M to 1×10^{-5} M with an EC₅₀ of 1.5×10^{-10} M (1.3 ppt) (Figure 65). This broad concentration-dependency is consistent with the response of the BRET² tagged ODR-10 which was also linear over 9 log units and also the response of the whole organism.

30 The inventors quantified the sensitivity of, SGSR-114/113 *in vitro*, for two of its ligands, 2-pentanone and diacetyl and of SGSR-113 to 1-hexanol (Figure 66). The BRET² tagged Str114/113 receptor can detect parts per quadrillion (sub pM) levels of diacetyl and parts per billion levels (nM) levels of 2-pentanone and the Str-113 receptor can detect parts per billion (nM) levels of 1-hexanol. These would be particularly
35 useful semi-broad sensors for use in the invention.

Construction of BRET² tagged *C. elegans* odorant receptors with RLuc2

Following de-orphaning of the five additional natural and one chimaeric BRET² tagged sensors, which was accomplished in a plate based assay, the inventors incorporated the RLuc2 mutations into each of them. This was required because, as
5 described above, the RLuc2 variant has been shown to be much brighter and therefore essential for practical use at a microfluidic scale.

The BRET² components were inserted into the third intracellular loop (IC3) and at the C-terminus of the *C. elegans* odorant receptor with green fluorescent protein, GFP² at IC3 and *Renilla* luciferase, RLuc at the C-terminus of the protein. Using site-
10 directed mutagenesis the RLuc2 mutations were introduced into the pYES-DEST-52 BRET2 tagged odorant receptor sequence. Primers 1 and 2 (Table 3) were used to introduce the mutation C124A and primer pair 3 and 4 were used to introduce the M185 V mutation. The RLuc2 mutations were introduced into all of the sensors based on OGOR, Str-112, Str-113, Str-114, Str-114/113, Str-115 and Str-116 as well as an
15 additional model receptor called OGOR mutant which contains the original odr-10 mutation (H110Y) identified by Sengupta et al. (1996) that has previously been shown previously to be unresponsive to diacetyl.

The amino acid sequence of GFP2 RLuc2 labelled OGOR, OGOR mutant, SGSR-112, SGSR-113, SGSR-114, SGSR-114/113 SGSR-115 and SGSR-116 and
20 receptors are provided as SEQ ID NOs 25 to 32 respectively, whereas the corresponding open reading frames are provided as SEQ ID NOs 33 to 40 respectively.

It will be appreciated by persons skilled in the art that numerous variations
25 and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

The present application claims priority from US 61/624,899 filed 16 April 2012,
30 and AU 2013204332 filed 12 April 2013, the entire contents of both of which are incorporated herein by reference.

All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which
35 has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of

these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

REFERENCES

- Aloni et al. (2006) *Genome Biol.* 7:R88.
- Buck and Axel *Cell* (1991) 65: 175-187.
- 5 Dacres et al. (2009a) *Anal. Biochem.* 385:194-202.
- Dacres et al. (2009b) *Biosensors and Bioelectronics* 24:1164-1170.
- Dacres et al. (2010) *Anal. Chem.* 82: 432-435.
- Dacres et al. (2011) *Biosens. Bioelectron* 29: 119-124.
- Dacres et al. (2012) *Biochem. Biophys. Res. Commun.* 425:625-629.
- 10 Day et al. (2004) *Luminescence* 19:8-20.
- De et al. (2007) *Cancer Res.* 67: 7175-7183.
- De et al. (2009) *FASEB Journal* 23:2702-2709.
- de Wet et al. (1987) *Mol. Cell. Biol.* 29:725-737.
- Doty (2012) *Gustation. Wiley Interdisciplinary Reviews-Cognitive Science*, 3:29-46.
- 15 Esch et al. (2011) *Annu. Rev. Biomed. Eng.* 13:55-72.
- Fang et al. (2005) *Anal. Chem.* 77:6528-6534.
- Fehr et al. (2002) *PNAS* 99: 9846-9851.
- Feldmesser et al. (2006) *BMC Genomics* 7:121.
- Fredriksson and Schioth (2005) *Mol. Pharmacol.*, 67, 1414-1425.
- 20 Frishman and Argos (1997) *Proteins* 27:329-335.
- Fuchs et al. (2001) *Human Genetics* 108:1-13.
- Gill and von Hippel (1989) *Anal. Biochem.* 182: 319-326.
- Glusman et al. (2000a) *Mammalian Genome* 11: 1016-1023
- Glusman et al. (2000b) *Genomics* 63: 227-245.
- 25 Glusman et al. (2001) *Genome Res.* 11:685-702.
- Godin et al. (2008) *J. Biophotonics* 1:355-376.
- Greer and Szalay (2002) *Luminescence* 17:43-74.
- Hall et al. (1997) *J. Biol. Chem.* 272: 17610-17614.
- Hastings (1996) *Gene* 173:5-11.
- 30 Hofmann and Stoffel (1993) *Biol. Chem.* 374:166.
- Holden and Cremer (2005) *Annu. Rev. Phys. Chem.* 56:369-387.
- Hushpulia et al. (2007) *Biotransformation* 25:2-4.
- Inouye et al. (1997) *Biochem. J.* 233:349-353.
- Klein et al. (1984) *Biochim. Biophys. Acta* 787:221-226
- 35 Lander et al. (2001) *Nature* 409:860-921.
- Li and Lin (2008) *Anal Bioanal. Chem.* 555-567.

- Loening et al. (2006) *Protein Eng. Des. Sel.* 19:391-400.
- Loening et al. (2007) *Nature Methods* 4:641-643.
- Lorenz et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:4438-4442.
- Mark et al. (2010) *Chem. Soc. Rev.* 39:1153-1182.
- 5 Medintz et al. (2006) *Curr. Opin. Biotech.* 17: 17-27.
- Mohammed and Desmulliez (2011) *Lab. Chip.* 11:569-595.
- Morin and Hastings (1971) *J. Cell. Physiol.* 77:313-318.
- Niimura and Nei (2003) *Proc. Natl. Acad. Sci. USA.* 100:12235-12240.
- Noh et al. (2011) *Top. Curr. Chem.* 304:117-52.
- 10 Olender et al. (2004a) *Genet Mol Res.* 3:545-53.
- Olender et al. (2004b) *Genomics.* 83:361-72.
- Park et al. (2009) *Biochem. Biophys. Res. Commun.* 388: 560-564.
- Persson and Argos (1994) *J. Mol. Biol.* 237:182-192.
- Pflegler and Eidne (2006) *Nature Methods* 3:165-174.
- 15 Pilpel and Lancet (1999) *Protein Science* 8: 969-77.
- Robertson (1998) *Genome Research* 8:449-463.
- Robertson (2001) *Chem Senses* 26:151-159.
- Sengupta et al. (1996) *Cell* 84:899-909.
- Sharff et al. (1992) *Biochemistry* 31: 10657-10663.
- 20 Sharff et al. (1993) *Biochemistry* 32: 10553-10559.
- Sharon et al. (1998) *Ann N Y Acad Sci.* 30:855:182-93
- Spurlino et al. (1991) *J. Biol. Chem.* 266: 5202-5219.
- Theberge et al. (2010) *Angew. Chem Int. Ed* 49:5846-5868.
- Tsien (1998) *Ann. Rev. Biochem.* 63:509-544.
- 25 Unger et al. (2000) *Science* 288:113-116.
- Verhaegen et al. (2002) *Anal. Chem.* 74: 4378-4385
- Viviani (2002) *Cell. Mol. Life Sci.* 59:1833-1850.
- von Heijne (1992) *J.Mol.Biol.* 225:487-494.
- Xu et al. (1999) *Proc. Natl. Acad. Sci. USA.* 96:151-156.
- 30 Yeo et al. (2011) *Small* 1: 12-48.
- Young et al. (2002) *J. Human Mol. Genet.* 11:535-4.
- Zhang and Firestein (2002) *Nat. Neurosci.* 5:81.
- Zozulya et al. (2001) *Genome Biol.* 2:0018.1-0018.12.

CLAIMS

1. A method of detecting an analyte in a sample, the method comprising
 - i) flowing through a microfluidic device comprising one or more microchannels,
 - a) the sample,
 - b) a sensor molecule comprising a domain that binds the analyte, a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance, and
 - wherein the sensor molecule is not fixed to the device,
 - c) a substrate of the chemiluminescent donor,
 - ii) mixing the sensor molecule, sample and substrate in the device, and
 - iii) detecting modification of the substrate by the chemiluminescent donor using an electro-optical sensing device and calculating as a ratio the energy transfer occurring between the chemiluminescent donor domain and the acceptor domain,
 - wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the analyte binds the sensor molecule.

2. A method of classifying a sample, the method comprising
 - i) flowing through a microfluidic device comprising one or more microchannels,
 - a) the sample,
 - b) a sensor molecule comprising a domain that binds one or more analytes, a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte(s), is within $\pm 50\%$ of the Forster distance, and wherein the sensor molecule is not fixed to the device,
 - c) a substrate of the chemiluminescent donor,
 - ii) mixing the sensor molecule, sample and substrate in the device,
 - iii) detecting modification of the substrate by the chemiluminescent donor using an electro-optical sensing device and calculating as a ratio the energy transfer occurring between the chemiluminescent donor domain and the acceptor domain,
 - iv) processing at least one signal from the electro-optical sensing device and correlating the pattern of electro-optical responses with one or more pre-determined characteristics of one or more samples of interest, and
 - v) classifying the sample based on the correlation of the pattern of responses,

wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the one or more analytes binds the sensor molecule.

5 3. The method of claim 2, wherein one or more of the analytes are unknown.

4. The method according to any one of claims 1 to 3, wherein the method can be used to detect the analyte in real time and/or which is performed within about 1s to about 100s.

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5. The method according to any one of claims 1 to 4, wherein the sensor molecule and substrate enter the device through different microchannels.

6. The method according to any one of claims 1 to 5, wherein one or more or all of the following features apply;

15

i) the Förster distance of the chemiluminescent donor domain and the acceptor domain is at least 5.6nm,

ii) the analyte binding or releasing from the sensor molecule results in a change in BRET ratio which is $\geq 15\%$ of the maximum observed BRET ratio, or

20

iii) the acceptor domain has a Stokes Shift of between about 50nm and about 150nm.

7. The method according to any one of claims 1 to 6, wherein the quantum yield detected by the electro-optical sensing device is less than about 8%, or less than about 5%, or less than about 2%.

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8. The method according to any one of claims 1 to 7, wherein the sample is a liquid, gas, liquid which has been pre-equilibrated with a gas, emulsion or suspension.

9. The method according to any one of claims 1 to 8, wherein

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i) the flow rate through the microfluidic device is between about 1 μ l/hour to about 1.5ml/hour, and/or

ii) flowing through the microfluidic device is continuous flow, batch flow or stop flow.

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10. The method according to any one of claims 1 to 9, wherein step iii) is performed in a reaction chamber with a volume of about 1 pL to about 200 μ L.

11. The method according to any one of claims 1 to 10, wherein the domain that
5 binds the analyte is a protein or a nucleic acid.

12. The method of claim 11, wherein the protein is a receptor, odorant binding protein, pheromone-binding protein, enzyme, ligand carrier, bacterial periplasmic binding protein or a G protein coupled receptor.

10

13. The method according to any one of claims 1 to 12, wherein one or more or all of the following features apply;

i) the chemiluminescent donor domain is a bioluminescent protein,

ii) the substrate is luciferin, calcium, coelenterazine, or a derivative or analogue

15 of coelenterazine, or

iii) the acceptor domain is a fluorescent acceptor domain.

14. The method of claim 13, wherein

i) the bioluminescent protein is a luciferase or a biologically active variant or
20 fragment, and/or

ii) the substrate is luciferin, coelenterazine, or a derivative or analogue of coelenterazine, and/or

iii) the acceptor domain is green fluorescent protein (GFP), Venus, mOrange, or a biologically active variant or fragment of any one thereof.

25

15. The method according to any one of claims 1 to 14 which comprises simultaneously or sequentially detecting two or more different analytes using the same microfluidic device.

30 16. The method according to any one of claims 1 to 15, wherein the microfluidic device comprises one or more sets of

a) three input microchannels, one each for the sensor molecule, substrate and sample, or

b) two input microchannels, one for the substrate and the other for a pre-mixture
35 of the sensor molecule and sample, or

c) two input microchannels, one for the sensor molecule and the other for a pre-mixture of the substrate and sample.

17. The method according to any one of claims 1 to 16, wherein one or more or all of the following features apply;

i) at least one microchannel comprises a reaction chamber which has a different volume to at least one other microchannel,

ii) at least one microchannel comprises two or more reaction chambers of the same or different volume, or

10 iii) the electro-optical sensing device has at least two different wavelength channels.

18. A microfluidic system for detecting an analyte in a sample, the system comprising

15 i) at least one reservoir suitable for containing a sensor molecule comprising a domain that binds the analyte, a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance, and wherein the sensor molecule is not fixed to the device,

ii) a microfluidic device comprising one or more microchannels,

iii) means for mixing the sensor molecule, the sample and a substrate of the chemiluminescent donor domain in the device,

20 iv) a reaction chamber for detecting binding of the analyte to the sensor molecule, and

v) an electro-optical sensing device,

wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the analyte binds the sensor molecule.

30

19. The system of claim 18

i) which can be used to detect the analyte in real time, and/or

ii) wherein the microfluidic device is designed to enable the detection of two or more analytes.

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20. A microfluidic system for classifying a sample, the system comprising

- i) at least one reservoir suitable for containing a sensor molecule comprising a domain that binds one or more analytes, a chemiluminescent donor domain and an acceptor domain, wherein the separation and relative orientation of the chemiluminescent donor domain and the acceptor domain, in the presence and/or the absence of analyte, is within $\pm 50\%$ of the Forster distance, and wherein the sensor molecule is not fixed to the device,
- ii) a microfluidic device comprising one or more microchannels,
- iii) means for mixing the sensor molecule, the sample and a substrate of the chemiluminescent donor domain in the device,
- iv) a reaction chamber for detecting binding of the analyte to the sensor molecule, and
- v) an electro-optical sensing device,
- wherein the spatial location and/or dipole orientation of the chemiluminescent donor domain relative to the acceptor domain is altered when the one or more analytes binds the sensor molecule.