Title: PROTEIN KINASE ASSAY

Abstract: An assay for the activity of a kinase enzyme contained in a sample, which comprises: i) contacting the sample with a pair of protein constructs, one of which comprises a donor fluorescent protein and the other of which comprises an acceptor fluorescent protein moiety, the donor protein moiety and the acceptor protein moiety being selected such that, when the said fluorescent protein moieties are in sufficiently close proximity, they exhibit resonance energy transfer when the donor moiety is excited, wherein a first one of said constructs comprises a target peptide moiety having an amino acid sequence which is a target for phosphorylation by the kinase enzyme, and the other (the second) of which comprises a binding protein moiety which either: a) selectively binds to the target peptide moiety of the first construct only in its phosphorylated form; or b) selectively binds to the target peptide moiety of the first construct only in its non-phosphorylated form; to form a complex in which the donor and acceptor protein moieties are brought into sufficiently close proximity that resonance energy transfer is exhibited upon excitation of the donor moiety; ii) exciting the donor fluorescent protein moiety; iii) determining the degree of fluorescence resonance energy transfer in the sample.

CFP-14-3-3

YFP-substrate
PROTEIN KINASE ASSAY

The present invention relates to an assay, which can be used in vitro or in vivo, for the activity of a protein kinase enzyme, which uses resonance energy transfer to analyse the interaction between a peptide substrate for the kinase enzyme and a protein which specifically binds the phosphorylated form of the peptide. There is also provided a screen (particularly a high throughput screen) for a compound capable of influencing the activity of the kinase enzyme, using such an assay. There are also provided protein constructs, such as fusion proteins, suitable for use in the assay, nucleic acid sequences capable of encoding such fusion proteins, expression vectors comprising such nucleic acid sequences and cell lines transfected with such expression vectors.

BACKGROUND

The functions of cells are controlled by external signals which stimulate or inhibit intracellular events. The process by which stimulatory or inhibitory signals are transmitted into and within a cell to elicit an intracellular response is referred to as signal transduction. Defects in various components of signal transduction pathways, from cell receptors to activators of gene transcription, account for a vast number of diseases, including numerous forms of cancer, diabetes, vascular diseases and neuronal diseases.

Protein kinases are enzymes involved in signal transduction which phosphorylate other proteins and/or themselves (autophosphorylation). Protein kinases involved in signal transduction in eukaryotic cells can be divided into three major groups based upon their substrate utilisation: protein-tyrosine specific kinases (which phosphorylate substrates on tyrosine residues), protein serine/threonine specific kinases
(which phosphorylate substrates on serine and/or threonine residues) and dual-specificity kinases (which phosphorylate substrates on tyrosine, serine and/or threonine residues).

An example of a protein kinase which is currently of considerable commercial interest is protein kinase B (PKB). The activation of protein kinase B (also known as Akt or Rac1) is central to the ability of insulin to stimulate glucose uptake and for several growth factors to suppress programmed cell death (apoptosis). As such, protein kinase B represents an important target molecule for the development of drugs that could activate or inhibit the enzyme, and thus can be used for therapeutic intervention in diseases such as diabetes and cancer.

At present, potential agonists and antagonists of protein kinase B are analysed using in vitro systems such as enzyme-linked immunosorbent assays (ELISAs). There are also other ways to measure the activity of PKB such as by measuring the incorporation of $^{32}$P from [γ-$^{32}$P]ATP into a substrate peptide or protein, or by measuring the state of phosphorylation of threonine 308 and serine 473 that are responsible for the activation of the enzyme - the latter can be achieved using phosphospecific antibodies and in theory could be used as a cell based assay, but it would be necessary to lyse the cells and prepare them for an immunoassay. A drawback with in vitro assays is that they do not select for compounds which are membrane permeant.

Therefore, time and effort may be expended investigating a compound which would be ineffective for treatment because of its inability to cross cell membranes. Since in vitro assays involve examining the activity of an enzyme in an artificial environment, it can be difficult to apply the results obtained to an in
vivo system.

Another group of important protein kinases are the tyrosine kinases which are known to phosphorylate substrates having a variety of sequences which then bind to different SH2 (Src homology-2) or PTB (phosphotyrosine binding) domains.

Fluorescent molecules are attractive as reporter molecules in many assay systems because of their high sensitivity and ease of quantification. Recently, fluorescent proteins have been the focus of much attention because they can be produced in vivo by biological systems, and can be used to trace intracellular events without the need to be introduced into the cell through microinjection or permeabilization. The green fluorescent protein of Aequorea victoria is particularly interesting as a fluorescent indicator protein. A cDNA for the protein has been cloned. Not only can the primary amino acid sequence of the protein be expressed from the cDNA, but the expressed protein can fluoresce. This indicates that the protein can undergo the cyclization and oxidation believed to be necessary for fluorescence.

Fluorescent proteins have been used as markers of gene expression, tracers of cell lineage and as fusion tags to monitor protein localization within living cells. (M. Chalfie et al., "Green fluorescent protein as a marker for gene expression," Science 263:802-805; A. B. Cubitt et al., "Understanding, improving and using green fluorescent proteins," TIBS 20, November 1995, pp. 448-455. U.S. Pat. No. 5,491,084, M. Chalfie and D. Prasher.) Furthermore, mutant versions of green fluorescent protein have been identified that exhibit altered fluorescence characteristics, including altered excitation and emission maxima, as well as excitation and emission spectra of different shapes. (R. Heim et

Luminescence is produced in certain organisms as a result of luciferase-mediated oxidation reactions. Currently, luciferase genes from a wide variety of vastly different species, particularly the luciferase genes of Photinus pyralis (the common firefly of North America), Pyrophorus plagiophthalmus (the Jamaican click beetle), Renilla reniformis (the sea pansy), and several bacteria (e.g., Xenorhabdus luminescens and Vibrio spp), are extremely popular luminescence reporter genes. Reference is made to Bronstein, I., et al. (1994) Anal-Biochem:219, 169-181, for a review of luminescence reporter gene assays. Firefly luciferase is also a popular reporter for ATP concentrations, and in that role is widely used to detect biomass. Various other reporter applications of luciferases have been described in the scientific literature. Luminescence may be produced by other enzymes when mixed with certain synthetic substrates, such as alkaline phosphatase mixed with adamantyl dioxetanes, or horseradish peroxidase mixed with luminol.

Luciferase genes are widely used as genetic reporters due to the non-radioactive nature, sensitivity, and extreme linear range of luminescence assays. For instance, as few as 10^-20 moles of the firefly luciferase can be detected. Consequently, luciferase assays of gene activity are used in virtually every experimental biological system, including both prokaryotic and eukaryotic cell cultures, transgenic plants and animals, and cell-free
expression systems. Similarly, luciferase assays of ATP are highly sensitive, enabling detection to below \(10^{-16}\) moles.

Luciferases generate light via the oxidation of enzyme-specific substrates, called luciferins. For firefly luciferase and all other beetle luciferases, this is done in the presence of magnesium ions, oxygen, and ATP. For anthozoan luciferases, including Renilla luciferase, only oxygen is required along with the luciferin which, in the case of Renilla, is coelenterazine. Generally, in luminescence assays of genetic activity, reaction substrates and other luminescence-activating reagents are introduced into a biological system suspected of expressing a reporter enzyme. Resultant luminescence, if any, is then measured using a luminometer or any suitable radiant energy-measuring device.

**DESCRIPTION OF THE PRIOR ART**

US-A-5925558 discloses assays for protein kinase activity using fluorescent proteins engineered to include sequences that can be phosphorylated by protein kinases; the proteins exhibit different fluorescent properties in the non-phosphorylated and phosphorylated states.

US-A-5981200 provides a tandem fluorescent protein construct including a donor fluorescent protein moiety, an acceptor protein moiety and a linker moiety that couples the donor and acceptor moieties. The donor and acceptor moieties exhibit fluorescence resonance energy transfer which is eliminated on cleavage. The constructs are stated to be useful in enzymatic assays.

**BRIEF DESCRIPTION OF THE INVENTION**

This invention provides protein constructs and methods for using them in enzymatic assays for protein kinase enzymes, both in vitro and in vivo. A first
protein construct used in the invention comprises a luminescence or fluorescent protein moiety linked to a peptide moiety which is a target for phosphorylation by a kinase enzyme. A second protein construct used in the invention comprises a luminescent or fluorescent protein moiety linked to a binding protein moiety; the binding protein moiety selectively binds to the peptide moiety of the first construct when that peptide moiety is its phosphorylated state. When used in the assay aspects of this invention, one of the said protein moieties is a donor fluorescent protein moiety or a luminescence protein capable of mediating a luminescence reaction, and the other is an acceptor fluorescent protein moiety. It is presently preferred that the protein moiety of the first protein construct is an acceptor fluorescent protein moiety and the protein moiety of the second protein construct is a donor fluorescent protein moiety or a bioluminescence protein moiety, preferably a donor fluorescent protein moiety. The said protein moieties of the respective protein constructs cause resonance energy transfer to occur when the donor moiety is excited (or bioluminescence is initiated) and the said moieties are in sufficiently close proximity to allow radiationless transfer of energy from the donor moiety to the acceptor moiety. The fluorescent protein moieties can be Aequorea-related fluorescent protein moieties, such as green fluorescent protein and blue fluorescent protein. The protein constructs may be fusion proteins in which the fluorescent (or luminescence) protein moiety and the target peptide moiety or the binding protein moiety are each part of a single polypeptide.

In a first assay aspect of the invention, there is provided an assay for the activity of a kinase enzyme contained in a sample, which comprises
(i) contacting the sample with a pair of protein constructs, one of which comprises a donor fluorescent protein and the other of which comprises an acceptor fluorescent protein moiety, the donor protein moiety and the acceptor protein moiety being selected such that, when the said fluorescent protein moieties are in sufficiently close proximity, they exhibit resonance energy transfer when the donor moiety is excited,

wherein a first one of said constructs comprises a target peptide moiety having an amino acid sequence which is a target for phosphorylation by the kinase enzyme, and the other (the second) of which comprises a binding protein moiety which either:

(a) selectively binds to the target peptide moiety of the first construct only in its phosphorylated form; or

(b) selectively binds to the target peptide moiety of the first construct only in its non-phosphorylated form;

to form a complex in which the donor and acceptor protein moieties are brought into sufficiently close proximity that resonance energy transfer is exhibited upon excitation of the donor moiety;

(ii) exciting the donor fluorescent protein moiety;

(iii) determining the degree of fluorescence resonance energy transfer in the sample.

In a second aspect of the invention, there is provided an assay for the activity of a kinase enzyme contained in a sample, which comprises

(i) contacting the sample with a pair of protein constructs, one of which comprises a luminescence protein moiety capable of mediating a luminescence reaction and the other of which comprises an acceptor fluorescent protein moiety, the luminescence protein
moiety and the acceptor protein moiety being selected such that, when in sufficiently close proximity, resonance energy transfer is exhibited upon mediation of a luminescence reaction by the said luminescence protein moiety,

wherein a first one of said constructs comprises a target peptide moiety having an amino acid sequence which is a target for phosphorylation by the kinase enzyme, and the other (the second) of which comprises a binding protein moiety which either:

(a) selectively binds to the target peptide moiety of the first construct only in its phosphorylated form; or

(b) selectively binds to the target peptide moiety of the first construct only in its non-phosphorylated form;

to form a complex in which the luminescence protein moiety and the acceptor fluorescence protein moiety are brought into sufficiently close proximity that resonance energy transfer is exhibited upon mediation of a luminescence reaction by the said luminescence protein moiety;

(ii) causing a luminescence reaction to be mediated by the said luminescence protein moiety;

(iii) determining the degree of resonance energy transfer in the sample.

In the second assay aspect of the invention, the luminescence reaction mediated by the luminescence protein moiety involves a substrate specific to the luminescence protein moiety. The substrate undergoes a chemical reaction, mediated by the luminescence protein moiety, to generate photons. Where the luminescence protein moiety (and therefore the location of the generation of the photons in the luminescence reaction) and the acceptor fluorescence protein moiety are in
sufficiently close proximity, resonance energy transfer from the site of the luminescence reaction to the acceptor fluorescent protein moiety may occur.

In both assay aspects of the present invention, the binding protein selectively binds either to the target peptide moiety of the first construct only in its phosphorylated form or to the target peptide moiety of the first construct only in its non-phosphorylated form to form a complex in which the donor fluorescent protein moiety (or luminescence protein moiety) and the acceptor fluorescence protein moiety are in sufficiently close proximity that resonance energy transfer may occur. In the embodiment in which the binding protein selectively binds to the target peptide moiety of the first construct only in its phosphorylated form, an increase in resonance energy transfer indicates the presence of protein kinase activity. This results from the active protein kinase phosphorylating the target peptide of the first construct to which the binding protein moiety of the second construct binds thereby bringing the donor fluorescent protein moiety (or luminescence protein moiety) and the acceptor fluorescence protein moiety into sufficiently close proximity such that resonance energy transfer may occur. This embodiment is presently preferred. In the embodiment in which the binding protein selectively binds to the target peptide moiety of the first construct only in its non-phosphorylated form, a decrease in resonance energy transfer indicates the presence of protein kinase activity. This results from the active protein kinase phosphorylating the target peptide of the first construct thereby disrupting possible binding of the second construct to the target peptide and separating the donor fluorescent protein moiety (or luminescence
protein moiety) and the acceptor fluorescence protein moiety such that resonance energy transfer can not occur.

According to another aspect of the invention, there is provided a first fusion protein construct, suitable for use in the assay aspects of the invention, comprising a target peptide moiety, which is a target for phosphorylation by a kinase enzyme, linked to a luminescence or a fluorescent protein moiety. This aspect of the present invention also provides a second fusion protein construct, suitable for use in the assay aspects of the invention, comprising a binding protein moiety, which selectively binds to the target peptide moiety of the first protein construct only in its phosphorylated form, linked to a luminescence or fluorescent protein moiety.

As another aspect, this invention also provides recombinant nucleic acids coding for expression of the first and second protein constructs each of which is encoded in or by a single polypeptide. The invention also provides expression vectors comprising expression control sequences operatively linked to a recombinant nucleic acid coding for the expression of the protein constructs, as well as host cells and cell lines (which may be mammalian or non-mammalian - such as insect Sf9 cells or bacterial cells) transfected with those expression vectors.

The constructs of this invention are useful in the assay aspects of the invention for determining whether a sample contains an active protein kinase enzyme. In the presently preferred embodiment, each of the first and second protein constructs comprises a fluorescent protein moiety and the target peptide and indicator protein are chosen such that the binding protein moiety selectively binds to the target peptide moiety only...
when the target peptide moiety is in its phosphorylated form. The sample is contacted with the first and second fluorescent protein constructs. The donor moiety is excited. Then the degree of fluorescence resonance energy transfer in the sample (to which the reagents have been added) is determined. A degree of fluorescence resonance energy transfer that is higher than an expected amount indicates that the target peptide moiety has been phosphorylated and formed a complex with the second fluorescent protein construct via the binding protein moiety, and so indicates the presence of the active kinase enzyme. The degree of fluorescence resonance energy transfer in the sample can be determined as a function of the amount of fluorescence from the donor moiety, the amount of fluorescence from the acceptor donor moiety, the ratio of the amount of fluorescence from the donor moiety to the amount of fluorescence from the acceptor moiety or the excitation state lifetime of the donor moiety.

In the assay in which a luminescence protein moiety takes the place of the donor fluorescent protein moiety, the sample is contacted with the first and second constructs and, at a suitable stage, the substrate for the luminescence protein moiety is introduced. The luminescence protein moiety mediates a luminescence reaction resulting in the emission of photons. This process is analogous to the generation of photons by excitation of a donor fluorescent protein moiety in the first assay aspect of the invention.

The assays of the invention are also useful for determining the amount of enzyme in a sample by determining the degree of resonance energy transfer at a first and second time after contact between the enzyme and the constructs, and determining the difference in the degree of resonance energy transfer.
The difference in the degree of resonance energy transfer reflects the amount of enzyme in the sample. The invention also provides methods for determining the amount of activity of an enzyme in a cell. The methods preferably involve providing a cell that expresses the first and second protein constructs, for example by transfecting the cell with appropriate expression vectors. In the first assay aspect of the invention, the cell is exposed to light in order to excite the donor moiety. In the second aspect of the invention, a substrate for the luminescence protein moiety is added to the cell in order to stimulate photon emission. Then the degree of resonance energy transfer in the cell is determined. The degree of resonance energy transfer reflects the amount of enzyme activity in the cell. As an alternative but presently less preferred method, the first and second constructs may be introduced into a cell by microinjection. Relevant techniques are also known for introducing proteins into permeabilised cells using electroporation or using toxins such as streptolysin O; peptides may be introduced into cells by fusing them with a peptide from the Antenepedia protein of Drosophila melanogaster (called "penetratin").

The assay methods also can be used to determine whether a compound alters the activity of an enzyme, i.e., screening assays. The methods involve contacting a sample containing an amount of the protein kinase enzyme with the compound and with the first and second protein constructs; exciting the donor moiety (or adding a substrate for the luminescence protein moiety to the sample); determining the amount of enzyme activity in the sample as a function of the degree of resonance energy transfer in the sample; and comparing the amount of activity in the sample with a standard
activity for the same amount of the enzyme. A difference between the amount of enzyme activity in the sample and the standard activity indicates that the compound alters the activity of the enzyme.

Similar methods are useful for determining whether a compound (such as a potential agonist or antagonist) alters the activity of a protein kinase enzyme in a cell. The methods involve providing first and second cells that each express both the first and second protein constructs; contacting the first cell with an amount of the compound; contacting the second cell with a different amount of the compound; exciting the donor moiety (or stimulating luminescence) in the first and second cell; determining the degree of resonance energy transfer in the first and second cells; and comparing the degree of resonance energy transfer in the first and second cells. A difference in the degree of resonance energy transfer indicates that the compound alters the activity of the enzyme.

When the assay of the present invention is used in a cell-based system, it inherently screens against compounds which are not membrane permeant. The assay of the invention is able to detect compounds that inhibit/activate the kinases directly as well as indirectly. For example, compounds that inhibit/inactivate upstream regulators, or protein phosphatases, specific for the kinase in question may also be identified. Furthermore, in addition to primary compound screening, the assay of the invention may be used for compound and target validation. Compound validation is used to evaluate compounds discovered by a more traditional route. Target validation is an assay to confirm the properties of an enzyme as determined in vivo being similar to those of the same target or enzyme in vitro.
DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, organic synthetic chemistry, and pharmaceutical formulation described below are those well known and commonly employed in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical formulation and delivery, and treatment of patients. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

The term "construct" means that the target peptide moiety/binding protein moiety and their respective fluorescent protein moiety are associated such that
they behave as a single entity. The target peptide moiety/binding protein moiety and the fluorescent protein moiety may be physically linked, for example by a linker molecule, a peptide or a peptide bond.

Alternatively, the target peptide moiety/binding protein and the fluorescent protein moiety may be non-covalently associated by, for example hydrogen bonds, electrostatic interactions or van der Waals forces.

"Moiety" refers to the radical of a molecule that is attached to another moiety. Thus, a "fluorescent protein moiety" is the radical of a fluorescent protein coupled to the target peptide moiety. By the same token, the term "target peptide moiety" refers to the radical of a target peptide for the kinase enzyme that is coupled to the fluorescent protein moiety.

"Fluorescent protein" refers to any protein capable of fluorescence when excited with appropriate electromagnetic radiation. This includes fluorescent proteins whose amino acid sequences are either natural or engineered.

"Sufficiently close proximity " means that the respective moieties are sufficiently close to allow radiationless transfer of energy from the donor fluorescent protein moiety to the acceptor fluorescent protein moiety (in the case of the first assay aspect of the invention) or from the location of generation of luminescence on the luminescence protein moiety to the acceptor fluorescence protein moiety (in the case of the second assay aspect of the invention).

"Target for phosphorylation" means an amino acid sequence recognised by a kinase enzyme which phosphorylates at least one of the amino acids in the sequence.

"Luminescence protein moiety" refers to a protein moiety which is capable of mediating a luminescence
reaction, under suitable conditions and in the presence of the necessary reagents.

"Target peptide moiety" refers to a peptide having an amino acid sequence which is recognised by a protein kinase which, under suitable conditions and in the presence of the necessary reagents (such as ATP), phosphorylates at least one of the amino acid residues in the sequence.

"Peptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. The conventional nomenclature for representing amino acid sequences is used herein.

"Naturally-occurring" as used herein, as applied to an object, refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding and non-coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences
include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

"Polynucleotide" refers to a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

The term "modulator" refers to a chemical compound (naturally occurring or non-naturally occurring), such as a biological macromolecule (e.g. nucleic acid, protein, non-peptide, or organic molecule), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Modulators are evaluated for potential activity as inhibitors or activators (directly or indirectly) of a biological process or processes (e.g., agonist, partial antagonist, partial agonist, antagonist, antineoplastic agents, cytotoxic agents, inhibitors of neoplastic transformation or cell proliferation, cell proliferation-promoting agents, and the like) by inclusion in screening assays described herein. The activities (or activity) of a modulator may be known, unknown or partial known. Such modulators can be screened using the methods described herein.

The term "test compound" refers to a compound to be tested by one or more screening method(s) of the invention as a putative modulator. Usually, various predetermined concentrations are used for screening such as 0.01 uM, 0.1 uM, 1.0 uM, and 10.0 uM. Test compound controls can include the measurement of a
signal in the absence of the test compound or comparison to a compound known to modulate the target.

**DETAILED DESCRIPTION OF THE INVENTION**

It has previously been discovered that fluorescent proteins having the proper emission and excitation spectra that are brought into physically close proximity with one another can exhibit fluorescence resonance energy transfer ("FRET"). Resonance energy transfer between a luminescence protein and a fluorescent protein is also known (see US-A-5976796). This invention takes advantage of that discovery to provide a pair of protein constructs which, when brought together in a complex (as herein described), are able to exhibit resonance energy transfer under the appropriate conditions. In the assay of the invention in which both the donor protein moiety and the acceptor protein moiety are fluorescent protein moieties, they are chosen such that the excitation spectrum of one of the moieties (the acceptor moiety) overlaps with the emission spectrum of the excited protein moiety (the donor moiety). The donor moiety is excited by light of appropriate intensity within the donor's excitation spectrum. The donor then emits the absorbed energy as fluorescent light. When the pair of constructs are in close proximity (as when a complex is formed between them on phosphorylation of the target peptide of the first construct), the fluorescent energy produced by the donor moiety is quenched by the acceptor fluorescent protein moiety. FRET can be manifested as a reduction in the intensity of the fluorescent signal from the donor, reduction in the lifetime of its excited state, and re-emission of fluorescent light at the longer wavelengths (lower energies) characteristic of the acceptor.

In the assay of the invention using a protein
construct comprising a luminescent protein moiety, similar considerations apply, with the exception that the emission is mediated by the luminescence protein moiety as opposed to being caused by an excitation.

One can take advantage of the resonance energy transfer exhibited by the pair of protein constructs of the invention in performing enzymatic assays as described in more detail below, with particular reference to the use of protein constructs both of which contain a fluorescent protein moiety.

The protein constructs of this invention are useful as substrates to study kinase enzymes which phosphorylate the target peptide of the first construct. In particular, this invention contemplates a pair of constructs in which the target peptide moiety contains an amino acid sequence that is a phosphorylation site for a protein kinase of interest. The amount of the protein kinase in a sample is determined by contacting the sample with the pair of protein constructs and measuring changes in fluorescence of the donor moiety, the acceptor moiety or the relative fluorescence of both. In one embodiment, the first construct is a recombinant fusion protein produced by expression of a nucleic acid that encodes a single polypeptide containing a fluorescent protein moiety (either donor or acceptor) and the target peptide moiety, and the second construct is a recombinant fusion protein produced by expression of a nucleic acid that encodes a single polypeptide containing a fluorescent protein moiety (either acceptor or donor) and the binding protein moiety. Fusion proteins can be used for, among other things, monitoring the activity of a protein kinase inside the cell that expresses the pair of recombinant constructs.

Protein kinases
In one embodiment, the assay of the invention may be used to assay the activity of protein kinase B in a sample. Protein kinase B phosphorylates a number of intracellular proteins on serine and threonine residues, more specifically within the consensus sequence RXRXXS/TΨ (in which Ψ may be any amino acid, but preferably is a hydrophobic residue). Many of the proteins phosphorylated by protein kinase B then bind, via the phosphorylated residue, to members of the 14-3-3 protein family. 14-3-3 protein family members bind to an overlapping consensus namely RXRXXpS/pTLP (and sequences such as RSXpSXP and RXY/FXpSXP), where pS and pT are phosphoserine and phosphothreonine respectively. Thus, in an embodiment of the invention which is an assay for the activity of protein kinase B, the target peptide moiety of the first construct includes the sequence RXRXXS/TLP, and the binding protein moiety of the second construct comprises a 14-3-3 protein moiety or binding fragment thereof, capable of recognising and binding to the phosphorylated target peptide sequence.

In some embodiments of this embodiment of the invention, it may be desirable to inhibit the activity of other protein kinases which phosphorylate the target protein sequence. One such enzyme is p70S6 kinase which is involved in the same signalling pathway as PKB. p70S6 kinase may be effectively inactivated by the use of rapamycin which blocks the pathway by which p70S6 kinase is activated.

In another embodiment, the assay of the invention may be used to assay the activity of a tyrosine kinase enzyme in a sample. Tyrosine kinases are known to phosphorylate substrates having a variety of sequences which then bind to different SH2 (Src homology-2) or PTB (phosphotyrosine binding) domains. Different SH2 domains have different substrate specificities (for
example SH2 domain from p85 subunit of PI3 kinase binds
peptides with a pYXXM motif; Grb2 SH2 domain binds
pYXN). PTB domains have specificities for NPXYpY
sequences (e.g. PTB domain of Shc binds the insulin
receptor at tyrosine 960 within an NpEypY sequence).
Thus, in one embodiment of the invention which is an
assay for the activity of a tyrosine kinase, the target
peptide moiety of the first construct has a sequence
YXXM and the binding protein moiety of the second
construct is SH2 domain from p85 subunit of PI3 kinase.
In another embodiment of the invention which is an
assay for the activity of a tyrosine kinase, the target
peptide moiety of the first construct has a sequence
YXN and the binding protein moiety of the second
construct is Grb2 SH2 domain. In yet another
embodiment of the invention which is an assay for the
activity of a tyrosine kinase, the target peptide
moiety of the first construct has a sequence NpXY and
the binding protein moiety of the second construct is a
Shc PTB domain or a PTB domain from insulin receptor
substrate-1.

The recent discovery of so-called FHA and WW
domains which bind phosphothreonine/phosphoserine
residues within specific motifs can also be used in the
present invention in assays for determining the
activity of the serine or threonine kinase which
generate these motifs. In such embodiments of the
invention, the binding protein moiety comprises the FHA
or WW domain and the target peptide moiety contains a
specific amino acid sequence (including a serine or
threonine residue) recognized by the FHA or WW domain
when in its phosphorylated form. In the case of the WW
domain, reference is made to Lu et al., Science, 283,
pages 1325-1328 (1999). In the case of the FHA domain,
reference is made to Yaffe et al, Nature, 402, pages
The fluorescent protein moiety

As described in greater detail herein, the donor fluorescent protein moiety should be selected with an emission spectrum that overlaps with the excitation spectrum of an acceptor fluorescent protein moiety.

Green fluorescent proteins ("GFPs") of cnidarians, which act as their energy-transfer acceptors in bioluminescence, can be used in the invention. A green fluorescent protein, as used herein, is a protein that fluoresces green light, and a blue fluorescent protein is a protein that fluoresces blue light. GFPs have been isolated from the Pacific Northwest jellyfish, Aequorea victoria, the sea pansy, Renilla reniformis, and Phialidium gregarium. W. W. Ward et al., Photochem. Photobiol., 35:803-808 (1982); L. D. Levine et al., Comp. Biochem. Physiol., 72B:77-85 (1982).

A variety of Aequorea-related GFPs having useful excitation and emission spectra have been engineered by modifying the amino acid sequence of a naturally occurring GFP from Aequorea victoria. (D. C. Prasher et al., Gene, 111:229-233 (1992); R. Heim et al., Proc. Natl. Acad. Sci., USA, 91:12501-04 (1994)). As used herein, a fluorescent protein is an Aequorea-related fluorescent protein if any contiguous sequence of 150 amino acids of the fluorescent protein has at least 85% sequence identity with an amino acid sequence, either contiguous or non-contiguous, from the wild type Aequorea green fluorescent protein of SEQ ID NO:2 of US-A-5981200. More preferably, a fluorescent protein is an Aequorea-related fluorescent protein if any contiguous sequence of 200 amino acids of the fluorescent protein has at least 95% sequence identity.
with an amino acid sequence, either contiguous or non-contiguous, from the wild type Aequorea green fluorescent protein of SEQ ID NO:2 of US-A-5981200. Similarly, the fluorescent protein may be related to Renilla or Phialidium wild-type fluorescent proteins using the same standards.

This invention contemplates the use of other fluorescent proteins in the constructs of the invention. The cloning and expression of yellow fluorescent protein from Vibrio fischeri strain Y-1 has been described by T. O. Baldwin et al., Biochemistry. (1990) 29:5509-15. This protein requires flavins as fluorescent co-factors. The cloning of Peridinin-chlorophyll a binding protein from the dinoflagellate Symbiodinium sp. was described by B. J. Morris et al., Plant Molecular Biology, (1994) 24:673:77. The cloning of phycobiliproteins from marine cyanobacteria such as Synechococcus, e.g., phycoerythrin and phycocyanin, is described in S. M. Wilbanks et al., J. Biol. Chem. (1993) 268:1226-35. These proteins require phycobilins as fluorescent co-factors, whose insertion into the proteins involves auxiliary enzymes. The proteins fluoresce at yellow to red wavelengths.

Another fluorescent protein which may be used in the invention is red fluorescent protein isolated from the IndoPacific sea anemone relative Discosoma striata and available from Clontech Laboratories, Inc., Palo Alto, Ca., USA.

**Fluorescence Resonance Energy Transfer**

For FRET, the donor fluorescent protein moiety and the acceptor fluorescent protein moiety are selected so that the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited. In order for the proximity of the first and second fusion proteins to be analysed using
fluorescence resonance energy transfer (FRET), it is necessary for the first fluorescent protein moiety to have an emission spectrum that overlaps with the excitation spectrum of the second fluorescent protein moiety and that the excitation spectrum of the first fluorescent protein moiety should overlap minimally with the excitation and emission spectra of the second fluorescent protein moiety and that the emission spectrum of the first fluorescent protein should overlap minimally with the emission spectrum of the second fluorescent protein. For example, one of the fluorescent proteins may be CFP (λ<sub>ex</sub> = 440nm, λ<sub>em</sub> = 480nm) and the other may be YFP (λ<sub>ex</sub> = 515nm, λ<sub>em</sub> = 530nm). Thus, the target peptide moiety may be associated with CFP in the first construct, and the binding protein moiety may be associated with YFP in the second construct. In this case, if an interaction between the peptide moiety and the binding protein moiety occurs, FRET will cause an excitation at 440nm to lead to an emission at 530nm. If, however, the interaction between the peptide moiety and the binding protein moiety does not occur, FRET decreases and a 480nm emission will predominate from a 440nm excitation.

One factor to be considered in choosing the fluorescent protein moiety pair is the efficiency of fluorescence resonance energy transfer between them. Preferably, the efficiency of FRET between the donor and acceptor moieties is at least 10%, more preferably at least 50% and even more preferably at least 80%. The efficiency of FRET can easily be empirically tested using the methods described herein and known in the art, particularly, using the conditions set forth in the Examples.

The efficiency of FRET is dependent on the
separation distance and the orientation of the donor and acceptor moieties, as described by the Forster equation, the fluorescent quantum yield of the donor moiety and the energetic overlap with the acceptor moiety. Forster derived the relationship:

\[ E = \frac{F^0 - F}{F^0} = R_0^6 \left( R^6 + R_0^6 \right) \]

where \( E \) is the efficiency of FRET, \( F \) and \( F^0 \) are the fluorescence intensities of the donor in the presence and absence of the acceptor, respectively, and \( R \) is the distance between the donor and the acceptor. \( R_0 \), the distance at which the energy transfer efficiency is 50%, is given (in Å) by

\[ R_0 = 9.79 \times 10^3 \left( K^2 Q J n^4 \right)^{1/6} \]

where \( K^2 \) is an orientation factor having an average value close to 0.67 for freely mobile donors and acceptors, \( Q \) is the quantum yield of the unquenched fluorescent donor, \( n \) is the refractive index of the intervening medium, and \( J \) is the overlap integral, which expresses in quantitative terms the degree of spectral overlap,

\[ J = \int_0^\infty e_\lambda F_\lambda \lambda^4 d\lambda \left/ \int_0^\infty F_\lambda d\lambda \right. \]

where \( e_\lambda \) is the molar absorptivity of the acceptor in \( \text{M}^{-1}\text{cm}^{-1} \) and \( F_\lambda \) is the donor fluorescence at wavelength \( \lambda \) measured in cm (Forster, T. (1948) Ann. Physik 2:55-75. Tables of spectral overlap integrals are readily available to those working in the field (for example, Berlman, I. B. Energy transfer parameters of aromatic compounds, Academic Press, New York and London (1973)).

The characteristic distance \( R_0 \) at which FRET is 50% efficient depends on the quantum yield of the donor i.e., the shorter-wavelength fluorophore, the extinction coefficient of the acceptor, i.e., the longer-wavelength fluorophore, and the overlap between the donor's emission spectrum and the acceptor's excitation spectrum. Calculated values of \( R_0 \) for P4-3
to S65T and S65C are both 4.03 nm because the slightly higher extinction coefficient of S65T compensates for its slightly longer emission wavelength. R. Heim et al., "Improved green fluorescence," Nature (1995) 373:663-664.

To optimize the efficiency and detectability of FRET, several factors need to be balanced. The emission spectrum of the donor moiety should overlap as much as possible with the excitation spectrum of the acceptor moiety to maximize the overlap integral J. Also, the quantum yield of the donor moiety and the extinction coefficient of the acceptor should likewise be as high as possible to maximize $R_o$. However, the excitation spectra of the donor and acceptor moieties should overlap as little as possible so that a wavelength region can be found at which the donor can be excited efficiently without directly exciting the acceptor. Fluorescence arising from direct excitation of the acceptor is difficult to distinguish from fluorescence arising from FRET. Similarly, the emission spectra of the donor and acceptor moieties should overlap as little as possible so that the two emissions can be clearly distinguished. High fluorescence quantum yield of the acceptor moiety is desirable if the emission from the acceptor is to be measured either as the sole readout or as part of an emission ratio. In one embodiment, the donor moiety is excited by ultraviolet (<400 nm) and emits blue light (<500 nm), whereas the acceptor is efficiently excited by blue but not by ultraviolet light and emits green light (>500 nm), for example, P4-3 and S65C. Another embodiment uses CFP (cyan fluorescent protein) which has an excitation frequency at 458 nm and a YFP (yellow fluorescent protein) having an emission frequency at 535 nm.
This invention contemplates fluorescent protein constructs produced in the form of a fusion protein by recombinant DNA technology as well as constructs produced by chemically coupling fluorescent proteins to the target peptide. In either case, the fluorescent proteins for use as donor or acceptor moieties in the constructs of the invention preferably are produced recombinantly.

Recombinant production of fluorescent proteins involves expressing nucleic acids having sequences that encode the proteins. Nucleic acids encoding fluorescent proteins can be obtained by methods known in the art. For example, a nucleic acid encoding the protein can be isolated by polymerase chain reaction of cDNA from A. victoria using primers based on the DNA sequence of A. victoria green fluorescent protein, as presented in FIG. 1. PCR methods are described in, for example, U.S. Pat. No. 4,683,195; Mullis et al. (1987) Cold Spring Harbor Symp. Quant. Biol. 51:263; and Erlich, ed., PCR Technology, (Stockton Press, New York, 1989). Mutant versions of fluorescent proteins can be made by site-specific mutagenesis of other nucleic acids encoding fluorescent proteins, or by random mutagenesis caused by increasing the error rate of PCR of the original polymonucleotide with 0.1 mM MnCl₂ and unbalanced nucleotide concentrations. See, e.g. WO-A-96/23810 (PCT/US95/14692).

Publishing Associates, Inc. and John Wiley & Sons, Inc., (most recent Supplement)).

Nucleic acids used to transfect cells with sequences coding for expression of the polypeptide of interest generally will be in the form of an expression vector including expression control sequences operatively linked to a nucleotide sequence coding for expression of the polypeptide. As used, the term "nucleotide sequence coding for expression of" a polypeptide refers to a sequence that, upon transcription and translation of mRNA, produces the polypeptide. This can include sequences containing, e.g., introns. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are "operatively linked" to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of the correct reading frame of that gene to permit proper translation of the mRNA, and stop codons. Since GFP and its derivatives may be toxic to cells, it is convenient if the expression of the or each construct is under the control of an inducible promoter, such as tetracycline responsive promoters (e.g. the Clontech T-Rex system), Bodysome-based systems and the well known metallothionein promoter.

Recombinant fluorescent protein can be produced by expression of nucleic acid encoding the protein in E. coli. The fluorophore of Aequorea-related fluorescent
proteins results from cyclization and oxidation of residues 65-67. Aequorea-related fluorescent proteins are best expressed by cells cultured between about 20°C and 30°C. After synthesis, these enzymes are stable at higher temperatures (e.g. 37°C) and can be used in assays at those temperatures.

The construct can also contain a tag to simplify isolation of the constructs. For example, a polyhistidine tag of, e.g., six histidine residues, can be incorporated at the amino terminal end of the fluorescent protein. The polyhistidine tag allows convenient isolation of the protein in a single step by nickel-chelate chromatography.

In a preferred embodiment, each of the pair of constructs is a fusion protein produced by recombinant DNA technology in which a single polypeptide includes, in the case of the first construct, the fluorescent protein moiety linked to the target peptidie moiety and, in the case of the second construct, the fluorescent protein moiety linked to the binding protein moiety. The fluorescent protein moiety can be positioned at the amino-terminus relative to the target peptide moiety or binding protein moiety in the polypeptide. Alternatively, and presently preferred, the fluorescent protein moiety can be positioned at the carboxy-terminus relative to the target peptide moiety or binding protein moiety in the polypeptide. The invention also envisions fusion proteins that contain extra amino acid sequences at the amino and/or carboxy termini, for example, polyhistidine tags.

The recombinant nucleic acid can be incorporated into an expression vector comprising expression control sequences operatively linked to the recombinant nucleic acid. The expression vector can be adapted for function in prokaryotes or eukaryotes by inclusion of
appropriate promoters, replication sequences, markers, etc.

The expression vector can be transfected into a host cell for expression of the recombinant nucleic acid. Host cells can be selected for high levels of expression in order to purify the construct fusion protein. E. coli is useful for this purpose. Alternatively, the host cell can be a prokaryotic or eukaryotic cell selected to study the activity of an enzyme produced by the cell.

The constructs can be expressed in E. coli in large scale for in vitro assays. Purification from bacteria is simplified when the sequences include polyhistidine tags for one-step purification by nickel-chelate chromatography. Alternatively, the substrates can be expressed directly in a desired host cell for assays in situ, which is particularly advantageous if the protein kinase of interest is membrane-bound or regulated in a complex fashion or not yet abundant as purified stable enzymes.

Fluorescent proteins can be attached through non-recombinant means. In one embodiment, the moieties are attached to the target peptide moiety or the binding protein moiety by chemical means. Chemical methods for specifically linking molecules to the amino- or carboxy-terminus of a protein are reviewed by R. E. Offord, "Chemical Approaches to Protein Engineering," in Protein Engineering--A Practical Approach, (1992) A. R. Rees, M. Sternberg and R. Wetzel, eds., Oxford University Press.

When the protein moieties are to be chemically coupled, fluorescent proteins can be isolated from natural sources by means known in the art. One method involves purifying the proteins to electrophoretic homogeneity. Also, J. R. Deschamps et al. describe

In another embodiment, the moieties are coupled by attaching each to a polypeptide pair capable of bonding through dimerization. For example, the peptide can include sequences that form a leucine zipper, shown to enable dimerization of a protein to which it was attached. See A. Blondel et al., "Engineering the quaternary structure of an exported protein with a leucine zipper," Protein Engineering (1991) 4:457-461. The linker containing the leucine zipper in the Blondel et al. article had the sequence: IQRKQLED KVEELLSKNY HLNEVARLK KLVGER. In another embodiment, a peptide linker moiety can comprise the sequence SKVILF, which also is capable of dimerization (see WO 94/28173).

Luminescence protein moiety

As described above, the place of the donor protein moiety may be taken by a luminescence protein moiety. This aspect of the invention is directly analogous to the aspect in which a donor protein moiety is employed, with the exception that the luminescence protein moiety is capable of mediating a luminescence reaction in the presence of the appropriate substrate, thus generating the necessary photons for resonance energy transfer.

Construction of protein constructs comprising a luminescence protein is carried out in analogous fashion to the construction of fluorescent protein constructs as described above. Similarly, preparation of suitable nucleic acid, expression vectors and transfected cell lines proceeds in an analogous manner. Furthermore, resonance energy transfer may be detected and interpreted in an analogous manner to where resonance energy transfer is FRET.

A protein-mediated luminescence reaction is any chemical reaction which yields photons as a consequence
of the reaction, and uses a protein, normally an enzyme to effectively enable the reaction. Examples are luciferases isolated from a variety of luminous organisms, such as the firefly luciferase of Photinus pyralis or the Renilla luciferase of Renilla reniformis. Luciferases are enzymes found in luminous organisms which catalyze luminescence reactions. They are organized into groups based on commonalities of their luminescence reactions. All luciferases within a group are derived from related luminous organisms, and all catalyze the same chemical reaction. Examples are beetle luciferases, which all catalyze ATP-mediated oxidation of the beetle luciferin; and anthozoan luciferases which all catalyze oxidation of coelenterazine. With the technical capabilities of molecular biology, it is possible to alter the structure of a luciferase found in nature to yield a functional equivalent thereof. A functional equivalent is an enzyme that maintains the ability to catalyze the same luminescence reaction, and thus it remains in the same group of enzymes. Other enzymes that are not luciferases can mediate a luminescence reaction using synthetic substrates. Examples are horseradish peroxidase, which catalyzes a reaction involving luminol (Thorp et al., (1986) Methods in Enzymology: 133, 331-353); and alkaline phosphatase, which catalyzes a reaction with adamantyl 1,2-dioxetane phosphate (Schaap et al., (1989), Clinical Chemistry: 35, 1863-1864).

A luminescence reporter is a molecule which mediates a luminescence reaction, and by doing so yields information about the state of a chemical or biochemical system. Examples are genetic reporters, immunoassay reporters and ATP reporters. Enzymes are proteins which catalyze a chemical transformation, and
thus are not changed by that transformation. Because the enzyme is regenerated at the conclusion of the transformation, it is available for additional cycles of transformation; enzymes thus have the capacity for substrate turnover. This property allows the capacity for continuous luminescence in enzyme-mediated luminescence reactions. Some proteins, such as aequorin do not allow substrate turnover, and this do not support continuous luminescence. However, these proteins may still be used as luminescence proteins within the scope of this invention.

The enzymatic assays of the invention will be described with particular reference to the use of a pair of fluorescent protein constructs. This description applies, mutatis mutandis, to assays which make use of a bioluminescence protein construct and an acceptor fluorescent protein construct.

**Enzymatic Assays**

The fluorescent protein constructs of the invention are useful in enzymatic assays. These assays take advantage of the fact that binding of the pair of constructs (on phosphorylation of the target peptide) brings the donor fluorescent protein moiety and the acceptor protein moiety into close proximity resulting in a measurable change in FRET. Methods for determining whether a sample has activity of a protein kinase enzyme involve contacting the sample with the pair of fluorescent protein constructs in which the target peptide moiety of the first construct has a phosphorylation sequence which is specifically recognized by the protein kinase. Then the donor moiety is excited with light in its excitation spectrum. If the target moiety has been phosphorylated, the phosphorylated site is recognized by the binding protein moiety of the second construct
and a complex formed in which the first and second constructs are in close proximity. Then, the degree of FRET in the sample is determined. A degree of FRET that is higher than the amount expected in a sample in which the pair of constructs do not associate indicates that active protein kinase enzyme is present. The amount of activity of a protein kinase enzyme in a sample can be determined by determining the degree of FRET in the sample at a first and second time after contact between the sample and the pair of constructs, determining the difference in the degree of FRET. The amount of enzyme in the sample can be calculated as a function of the difference in the degree of FRET using appropriate standards. The faster or larger the increase of FRET, the more enzyme activity must have been present in the sample.

Assays in which a luminescence protein moiety is used are carried out in an analogous fashion to those in which a donor fluorescent protein moiety is employed, with the exception that, in order to initiate resonance energy transfer, the necessary substrate for the luminescence protein moiety must be introduced into the sample. Upon introduction of the substrate, luminescence occurs and, assuming that the acceptor fluorescent protein moiety is in sufficiently close proximity to the luminescence protein moiety, resonance energy transfer will take place and can be detected as described above.

The degree of resonance energy transfer, specifically FRET in the embodiment in which both donor and acceptor protein moieties are employed, can be determined by any spectral or fluorescence lifetime characteristic of the excited construct, for example, by determining the intensity of the fluorescent signal from the donor, the intensity of fluorescent signal
from the acceptor, the ratio of the fluorescence amplitudes near the acceptor's emission maxima to the fluorescence amplitudes near the donor's emission maximum, or the excited state lifetime of the donor.

Preferably, changes in the degree of FRET are determined as a function of the change in the ratio of the amount of fluorescence from the donor and acceptor moieties, a process referred to as "ratioing." Changes in the absolute amount of substrate, excitation intensity, and turbidity or other background absorbances in the sample at the excitation wavelength affect the intensities of fluorescence from both the donor and acceptor approximately in parallel. Therefore the ratio of the two emission intensities is a more robust and preferred measure of binding of the pair of constructs than either intensity alone.

The excitation state lifetime of the donor moiety is, likewise, independent of the absolute amount of substrate, excitation intensity, or turbidity or other background absorbances. Its measurement requires equipment with nanosecond time resolution.

Fluorescence in a sample is measured using a fluorimeter. In general, excitation radiation, from an excitation source such as a laser having a first wavelength, passes through excitation optics. The excitation optics cause the excitation radiation to excite the sample. In response, fluorescent proteins in the sample emit radiation which has a wavelength that is different from the excitation wavelength. Collection optics then collect the emission from the sample. The device can include a temperature controller to maintain the sample at a specific temperature while it is being scanned. According to one embodiment, a multi-axis translation stage moves a microtiter plate holding a plurality of samples in
order to position different wells to be exposed. The multi-axis translation stage, temperature controller, auto-focusing feature, and electronics associated with imaging and data collection can be managed by an appropriately programmed digital computer. The computer also can transform the data collected during the assay into another format for presentation.


Enzymatic assays also can be performed on living cells in vivo, or from samples derived from organisms transfected to express the pair of constructs. Because the fusion proteins constructs can be expressed recombinantly inside a cell, the amount of enzyme activity in the cell or organism of which it is a part can be determined by determining changes in fluorescence of cells or samples from the organism.

In one embodiment, a cell is transiently or stably transfected with an expression vector encoding the pair of fluorescent protein constructs of the invention. This expression vector optionally includes controlling nucleotide sequences such as promoter or enhancing elements. The enzyme to be assayed may either be intrinsic to the cell or may be introduced by stable transfection or transient co-transfection with another expression vector encoding the protein kinase enzyme
and optionally including controlling nucleotide sequences such as promoter or enhancer elements. The fluorescent protein construct and the enzyme preferably are expressed in the same cellular compartment so that they have more opportunity to come into contact.

In some embodiments of the invention, it may be desirable to target the constructs to specific subcellular compartments. This may be achieved by including on the or each construct a C-terminal CAAX motif (which allows isoprenylation) or an N-terminal myristoylation signal. Both of these approaches allow targeting to the plasma membrane. Alternatively, it may be desired to target the constructs to the nucleus specifically (using a nuclear localisation signal) or the endoplasmic reticulum (for example using a KDEL targeting motif).

If the cell does not possess protein kinase enzyme activity, the efficiency of FRET in the cell is low, and the fluorescence characteristics of the cell reflect this efficiency. If the cell possesses a high degree of protein kinase enzyme activity, the target peptide moiety of the first construct will be phosphorylated and will complex with the second construct by way of the binding protein moiety of the second construct which selectively binds to the target peptide when in the phosphorylated state. In this case, the efficiency of FRET is high.

A contemplated variation of the above assay is to use the controlling nucleotide sequences to produce a sudden increase in the expression of the pair of fluorescent protein constructs, e.g., by inducing expression of the construct. The efficiency of FRET is monitored at one or more time intervals after the onset of increased expression. A low efficiency of FRET reflects a low amount or low efficiency of the protein
kinase enzyme. This kinetic determination has the advantage of minimizing any dependency of the assay on the rates of degradation or loss of the fluorescent protein moieties.

The enzymatic assays of the invention can be used in drug screening assays to identify compounds that alter the activity of an enzyme. In one embodiment, the assay is performed on a sample in vitro containing the protein kinase enzyme. A sample containing a known amount of enzyme is mixed with the pair of constructs of the invention and with a test compound. The amount of the enzyme activity in the sample is then determined as above, e.g., by determining the degree of fluorescence at a first and second time after contact between the sample, the constructs and the compound. Then the amount of activity per mole of enzyme in the presence of the test compound is compared with the activity per mole of enzyme in the absence of the test compound. A difference indicates that the test compound alters the activity of the enzyme.

In another embodiment, the ability of a compound to alter protein kinase enzyme activity in vivo is determined. In an in vivo assay, cells transfected with a expression vector encoding the pair of constructs of the invention are exposed to different amounts of the test compound, and the effect on fluorescence in each cell can be determined. Typically, the difference is calibrated against standard measurements to yield an absolute amount of enzyme activity. A test compound that inhibits or blocks the expression of the protein kinase enzyme can be detected by decreased FRET in treated cells compared to untreated controls.

Screening assays to examine the effect of a compound on kinase activity may be conducted in vivo or
in vitro. In such in vitro assays, various orders of addition may be employed. One approach is to pre-incubate the sample containing the kinase in the presence of test compounds plus the first and second protein constructs, and then to add ATP to start the reaction. Another approach is to pre-incubate the sample containing the kinase in the presence of the test compound, and then add the first and second protein constructs and ATP. Yet another approach is to pre-incubate the sample containing the kinase in the presence of ATP to first activate the kinase (for example where the kinase is one - such as a tyrosine kinase - which is activated by phosphorylation), and then add the test compound; finally, the reaction is started by adding the first and second constructs.

Where the screen is carried out in vitro, the sample kinase enzyme could be pre-treated with the compound, and then added to a solution containing the first and second constructs. The degree of phosphorylation of the peptide (and therefore the kinase activity) could then be determined by examining any changes in emission wavelength caused by FRET.

Alternatively, the screen may be cell-based. For example, intracellular kinase activity could be analysed following treatment of the cells with the compound. The first and/or second constructs may be introduced into the cells, or expressed within the cells.

It may be desirable, in the assay of the invention to induce phosphorylation of the target peptide. For example, this may be achieved by stimulating the cells with a ligand such as a growth factor or insulin or, alternatively, by over-expressing the kinase responsible for direct phosphorylation of the peptide. The latter approach has the advantage that any other
upstream effects such as activation of upstream effectors are excluded.

In a preferred embodiment of an in vivo assay, the cells are from a stable cell line which express the first and second constructs as fusion proteins, under the control of an inducible promoter. In this embodiment, the screen may involve the following steps:

(i) inducing the expression of the first and second constructs within the cells;
(ii) incubating the induced cells with a test compound;
(iii) detecting the presence or absence of an interaction between the peptide of the first construct and the binding protein of the second construct by detecting the presence or absence of fluorescence resonance energy transfer between the first and second proteins.

The assay of the present invention is particularly suited for use in a high throughput screening method. There are available on the market a number of standard fluorescence based microtitre plate readers which would be suitable for such a method.

In a preferred embodiment, the assays of the present invention may be used in a high throughput screen to analyse simultaneously the capacity of a plurality of compounds to influence the activity of the kinase enzyme. For example, if a cell line were used which expressed both of the fusion proteins of the second aspect of the invention, a high throughput screen may comprise the following steps:

(i) applying the cells to a receptacle having a plurality of compartments;
(ii) incubating the cells in a particular compartment with a particular compound;
(iii) analysing the presence or absence of FRET in
each compartment.

A suitable receptacle would be a multi-well plate, for example, a 16-, 24-, 48 or 96-well plate.

The emission wavelengths from a plurality of wells may be analysed by known techniques, as described above.

The present invention will now be described, by way of example only, and with reference to Figs. 1 to 3, in which:

Fig. 1 is a diagrammatic representation of two tagged protein constructs;

Fig. 2 is a western blot of several cell lysates blotted using an anti-HA antibody; and

Fig. 3 is a graph showing the emission ratios from four cells co-transfected with plasmids encoding CFP-14-3-3 and YFP-substrate tagged proteins.

Cyan fluorescent protein (CFP) was tagged at the C-terminus with a human 14-3-3ζ isoform followed by a haemagglutinin epitope tag, which is indicated by a shaded area in the construct shown in Fig. 1.

Yellow fluorescent protein (YFP) was tagged at the C-terminus with a 36 amino acid sequence containing a protein kinase B substrate sequence, RGRSRS, as shown in Fig. 1. RGRSRS is homologous to the protein kinase B consensus sequence RXRXXS/TY. The serine in this construct, which is phosphorylated by protein kinase B, is underlined in Fig. 1.

CHO cells were then transfected with either (a) YFP-substrate, (b) CFP-14-3-3, (c) CFP-14-3-3 and YFP-substrate and a constitutively active myristoylated
protein kinase B (myrPKB) or (d) CFP-14-3-3 and YFP-substrate.

After 24 hours the cells were extracted and the CFP-14-3-3 construct was immunoprecipitated from resulting cell lysates using an anti-HA antibody.

**Extraction method**

Cells in 60mm dishes were gently washed twice in PBS (phosphate buffered saline) before the addition of 250 ml ice-cold cell extraction buffer (50mM beta-glycerophosphate, 1.5mM EDTA (Ethylene glycol-bis (b-aminoethyl ether) N,N,N,N-tetraacetic acid), 1mM benzamidine, 10mM NaF (Sodium Fluoride), 1mM Na,VO₄ (Sodium Orthovanadate), 1mM DTT (Dithiothreitol), 1%(v/v) Triton X-100, 1mg/ml PAL (pepstatin, antipain, leupeptin), 0.1mM PMSF (Phenylmethylsulfonylfluoride)). The cells were scraped into the extraction buffer and then transferred to 1.5 ml Eppendorf tubes. These were then left on ice for 10 min. Extracts were then clarified by centrifugation at 4 °C for 5 min at 12000 rpm. The supernatants were transferred to fresh 1.5 ml Eppendorf tubes on ice.

**Immunoprecipitation**

2.5 mg Protein A-Sepharose beads per immunoprecipitation (IP) reaction were weighed into a 1.5 ml Eppendorf tube and pre-swelled by incubation in 1ml PBS at room temperature for 5 min. The supernatant was removed and the beads washed twice in 1ml cell extraction buffer (50mM beta-glycerophosphate, 1.5mM EDTA, 1mM benzamidine, 10mM NaF, 1mM Na,VO₄, 1mM DTT, 1%(v/v) Triton X-100, 1mg/ml PAL, 0.1mM PMSF). The
beads were then resuspended in 50 ml of extraction buffer per IP. 50 ml pre-swelled beads and an appropriate dilution of antibody (50 mg/ml final concentration of the monoclonal anti-haemagglutinin (anti-HA) antibody HA11 (obtained from Babco, Richmond, CA, USA) were then added to cell lysates prepared as described above in a total final volume of 500ml cell extraction buffer. The cell lysate/antibody/protein A-Sepharose mixture was then tumbled at 4°C for 2 h. Subsequently, cell lysate/antibody/protein A-Sepharose conjugates were isolated by brief centrifugation in order to pellet the Sepharose beads. Beads were washed three times in cell extraction buffer then immunopurified proteins were eluted by boiling in 50ml 1x SDS-PAGE sample buffer (50mM Tris-HCl pH 6.8, 0.5mM DTT, 10% (w/v) SDS (sodium dodecyl sulphate), 0.5% (w/v) bromophenol blue, 50% (v/v) glycerol).

The immunoprecipitates were then western blotted using an anti-GFP antibody to detect YFP.

Western Blotting

Protein samples of equal protein concentration were solubilised by boiling in SDS-PAGE sample buffer and separated by electrophoresis using 10% acrylamide separating gels with 4% stacking gels in a BioRad Mini Protean II SDS-PAGE apparatus. Gels were run at a constant voltage of 150 V in 1x SDS-PAGE gel running buffer (192mM glycine, 25mM Tris-HCl, 0.1% SDS) until proteins were sufficiently separated as judged by the migration of pre-stained size markers (BioRad) run alongside the protein samples.
Proteins separated by SDS-PAGE were transferred to Immobilon P membranes using a BioRad Mini Protean II blotting apparatus. Prior to Western blotting, Immobilon P membranes were activated by immersion in methanol for 15 s, H₂O for 2 min, then Western transfer buffer (20mM Tris-base, 150mM glycine, 20% (v/v) methanol) for at least 5 min. Proteins were transferred for 1 h at a constant voltage of 100 V. Non-specific binding sites were blocked by incubating the membranes in TBS-T (20mM Tris-HCl, 136mM NaCl, 0.05% Tween-20, pH 7.6) containing 10 % (w/v) BSA (Bovine serum albumin).

Membranes were then incubated at room temperature in 0.4mg/ml anti GFP antibody (obtained from Roche Molecular Biochemicals, East Sussex, U.K.) diluted in TBS-T for 1 h with shaking. Membranes were then washed three times for 5 min in TBS-T before incubating for 1 h at room temperature in a 1/10,000 dilution of the appropriate HRP-conjugated secondary antibody (Amersham Pharmacia Biotech) diluted in TBS-T as required. Subsequently, membranes were washed three times for 5 min in TBS-T and finally washed twice for 5 min in TBS. Antibody bound to the membrane was detected using ECL™ Western Blotting Reagent (Amersham Pharmacia Biotech). Membranes were incubated in a 1:1 mixture of the two ECL reagents for 90 s then exposed to Amersham Hyperfilm for sufficient time to obtain a clear result (typically 1-5 min).

The resulting western blot is shown in Fig 2, and the migration positions of CFP-14-3-3 and YFP-substrate are indicated. Fig.2 demonstrates that the constitutively-active protein kinase B (lane c) selectively induces the phosphorylation of the YFP-substrate protein in the cells, thus promoting co-precipitation of the YFP-substrate when the CFP-14-3-3
protein is immunoprecipitated with anti-HA antibodies.

Six hours after transfection with plasmids encoding CFP-14-3-3 and YFP-substrate, H4IIE cells were serum starved overnight prior to being subjected to imaging using a Leica SP2 inverted confocal imaging spectrophotometer. FRET was determined by excitation using a 458nm laser line. Fluorescence emission was monitored at 1 frame per minute, after the addition of 100 nM insulin, by collection across windows of 470-490nm (CFP emission) and 520-540nm (YFP emission). A CFP/YFP emission ratio was calculated using the Leica confocal software physiology package. Fig.3 is a graph showing the emission ratios from four cells in the field of view. Two of the cells showed no change in emission ratio in response to insulin (solid lines). However, the remaining two cells exhibited a decrease in emission ratio (dashed lines), which is indicative of an increase in FRET, and thus interaction between CFP-14-3-3 and YFP-substrate after phosphorylation by insulin-activated protein kinase B in the cells.
CLAIMS

1. An assay for the activity of a kinase enzyme contained in a sample, which comprises
   (i) contacting the sample with a pair of protein constructs, one of which comprises a donor fluorescent protein and the other of which comprises an acceptor fluorescent protein moiety, the donor protein moiety and the acceptor protein moiety being selected such that, when the said fluorescent protein moieties are in sufficiently close proximity, they exhibit resonance energy transfer when the donor moiety is excited,

   wherein a first one of said constructs comprises a target peptide moiety having an amino acid sequence which is a target for phosphorylation by the kinase enzyme, and the other (the second) of which comprises a binding protein moiety which either:

   (a) selectively binds to the target peptide moiety of the first construct only in its phosphorylated form; or

   (b) selectively binds to the target peptide moiety of the first construct only in its non-phosphorylated form;

   to form a complex in which the donor and acceptor protein moieties are brought into sufficiently close proximity that resonance energy transfer is exhibited upon excitation of the donor moiety;

   (ii) exciting the donor fluorescent protein moiety;

   (iii) determining the degree of fluorescence resonance energy transfer in the sample.

2. An assay according to claim 1, wherein the binding protein moiety selectively binds to the target peptide moiety of the first construct only in its phosphorylated form.

3. An assay according to claim 1 or claim 2 wherein the first construct is a recombinant fusion protein produced
by expression of a nucleic acid that encodes a single polypeptide containing a fluorescent protein moiety and the target peptide moiety, and the second construct is a recombinant fusion protein produced by expression of a nucleic acid that encodes a single polypeptide containing a fluorescent protein moiety and the binding protein moiety.

4. An assay according to any preceding claim wherein the assay is for the activity of protein kinase B contained in the sample.

5. An assay according to claim 4 wherein the target protein moiety of the first construct comprises the sequence RXRXXS/TLP, and the binding protein moiety of the second construct comprises a 14-3-3 protein moiety or binding fragment thereof, capable of recognising and binding to the phosphorylated target peptide sequence.

6. An assay according to any one of claims 1 to 3 wherein the assay is for the activity of a tyrosine kinase contained in the sample.

7. An assay according to claim 6 wherein the target peptide moiety of the first construct has a sequence YXXM and the binding protein moiety of the second construct is an SH2 domain from the p85 subunit of PI3 kinase.

8. An assay according to claim 6 wherein the target peptide moiety of the first construct has a sequence YXXN and the binding protein moiety of the second construct is Grb2 SH2 domain.

9. An assay according to claim 6 wherein the target peptide moiety of the first construct has a sequence NPXY and the binding protein moiety of the second construct is a Shc PTB domain or a PTB domain from insulin receptor substrate-1.

10. An assay according to any one of claims 1 to 3 wherein the assay is for the activity of a serine or threonine kinase contained in the sample.
11. An assay according to claim 10 wherein the binding protein moiety comprises an FHA or WW domain and the target protein moiety contains a specific amino acid sequence recognised by the FHA or WW domain when in its phosphorylated form.

12. A first fusion protein construct for use in an assay according to any preceding claim, comprising a target peptide moiety, which is a target for phosphorylation by a kinase enzyme, linked to a fluorescent protein moiety.

13. A second fusion protein construct for use in an assay according to any one of claims 1 to 11 comprising a binding protein moiety, which selectively binds to the target peptide sequence moiety of the first protein construct only in its phosphorylated form, linked to a fluorescent moiety.
Figure 1

CFP-14-3-3

YFP-substrate
Figure 3

Insulin

Ratio (CFP/YFP) vs. Time (min)