

[DESCRIPTION]

[Title of Invention] METHOD FOR DETERMINING A
PROTEIN-PROTEIN INTERACTION

[Technical Field]

5 The present invention relates to a method for
determining a protein-protein interaction, as well as a
vector and a kit for use in the method.

[Background Art]

10 Methods for determining a protein-protein
interaction can be roughly categorized into two groups.
One is a method characterized by using a protein having
been separated from living cells. Examples of such a method
include surface plasmon resonance, protein mass
spectroscopy, and anisotropy measurements. However,
15 these methods have difficulty determining an interaction
in an environment similar to an actual intracellular
environment.

20 Then, as the second method, a method has been developed,
in which a protein-protein interaction is determined using
living cells. Typical methods thereof are a yeast two
hybrid system which detects a transcriptional activity of
a reporter, and modified methods thereof. Besides,
another method has been also developed which utilizes
reconstitution of enzymes such as β -galactosidase and
25 dihydrofolate reductase (DHFR).

Nevertheless, these methods have problems that they

are incapable of determining a position where a protein-protein interaction has taken place (positional information on the protein-protein interaction), as well as a period until a protein-protein interaction takes place, a period until the interaction ends, a duration of the interaction, and the like (temporal information on the protein-protein interaction).

Meanwhile, the method for determining a protein-protein interaction using living cells also includes a method utilizing reconstitution of a fluorescent protein. Nevertheless, once reconstituted, the fluorescent protein does not dissociate. Accordingly, this method has a problem that it is incapable of determining a period until a protein-protein interaction ends, a duration of the interaction, and the like. Further, there is another problem that a period until a protein-protein interaction takes place and the like cannot be determined because emission of fluorescence requires a certain time after a protein-protein interaction takes place.

On the other hand, as a method for determining a protein-protein interaction in living cells, fluorescence resonance energy transfer (FRET) has been developed which detects energy transfer dependent on a distance between molecules. This method has an advantage of obtaining positional information and temporal information on where and when a protein-protein interaction takes place.

Nevertheless, since a positional relation between a donor fluorescent protein and an acceptor fluorescent protein used in the method is important to determine the protein-protein interaction, the method involves a complicated step of investigating the optimization of a linker (spacer) connecting these fluorescent proteins to a detection-target protein, so that such a system has been difficult to construct. Further, it has also been difficult to analyze the result due to cross excitation by which an acceptor fluorescent protein is excited, and due to bleed-through in which fluorescence of a donor fluorescent protein bleeds through a filter (absorption filter) set for detecting fluorescence of an acceptor fluorescent protein. Moreover, use of fluorescent proteins of two colors (donor fluorescent protein and acceptor fluorescent protein) also brings about a problem that only a limited number of fluorescent proteins are usable in order to obtain information other than a detection-target protein.

Recently, Tobias Meyer et al. have reported a method for determining a protein-protein interaction by utilizing intracellular localization (PTL 1). In this method, one of proteins subjected to interaction determination is fused to a protein that specifically binds to a particular site in a cell, while the other of the proteins subjected to interaction determination is fused to a fluorescent protein

or the like. Then, these fusion proteins are expressed in a cell, and the protein-protein interaction is determined on the basis of a signal of the fluorescent protein or the like at the particular site in the cell.

5 In addition, Nibert et al. have reported a method for determining a protein-protein interaction by using a fusion protein in which one of proteins subjected to interaction determination is fused to a protein for forming a viral inclusion body, and using, as an indicator,
10 accumulation of the other of the proteins subjected to interaction determination in the viral inclusion body (PTL 2).

 However, in these methods for determining a protein-protein interaction by utilizing intracellular
15 localization, one of proteins subjected to interaction determination is forcibly (artificially) translocated and confined at a particular site in a cell. Accordingly, the determination is impossible at a site where a protein-protein interaction naturally takes place, that
20 is, in an intracellular environment unique to the protein-protein interaction, which brings about a problem that positional information on the protein-protein interaction cannot be obtained, and other similar problems. Moreover, it is also impossible to determine the
25 interaction between proteins localized in a natural state at the same site as the site of the intracellular

localization.

Against this problem, Sara Peterson Bjorn et al. have reported a method for determining a protein-protein interaction (redistribution-trap method) in which proteins are allowed to interact with each other in an intracellular environment where the proteins naturally function, and then the cells are stimulated with a drug or the like to thereby induce aggregate formation from the interacting proteins, the aggregate formation being indicative of the interaction (PTL 3).

However, this method needs to stimulate cells at certain time so that the aggregate formation can be induced, and also needs to remove the drug or the like used for the stimulation to determine the presence or absence of an interaction subsequently after the stimulation. Hence, the method has problems such as being incapable of obtaining temporal information on when the protein-protein interaction takes place, and incapable of determining a protein-protein interaction that changes (takes place, ends, takes place again, and so forth) for a certain period and at a certain position.

[Citation List]

[Patent Literature]

[PTL 1] International Publication No. W02000/017221

[PTL 2] International Publication No. W02006/099486

[PTL 3] United States Patent No. 7282347

[PTL 4] International Publication No. W02013/084950

[Summary of Invention]

[Technical Problem]

5 The present invention has been made in consideration
of the above-described problems in the conventional
techniques. An object of the present invention is to
provide a method capable of determining a protein-protein
interaction in a cell in an intracellular environment
unique to the protein-protein interaction, and also capable
10 of obtaining positional information and temporal
information on the protein-protein interaction.

[Solution to Problem]

The present inventors have conducted earnest studies
in order to achieve the above object. As a result, the
15 inventors have come up with an idea of utilizing, in
determining an interaction between two proteins (a first
protein and a second protein): a first fusion protein
comprising the first protein, a multimerizable protein,
and a fluorescent protein; and a second fusion protein
20 comprising the second protein and a multimerizable protein
(see Figs. 1 and 2). To be more specific, the inventors
have come up with a system as follows. When these two fusion
proteins are expressed in a cell, an interaction if any
between the first protein and the second protein induces
25 multimer formation between the multimerizable protein and
another multimerizable protein. Thereby, the fusion

proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

Then, the inventors actually constructed this system, and expressed these fusion proteins in cells. The result revealed that determination of certain protein-protein interactions is possible on the basis of fluorescent foci detected in the cells. Moreover, it was also revealed that the system enables determination of a position where a protein-protein interaction takes place (positional information on the protein-protein interaction), as well as a period until a protein-protein interaction takes place, a period until the interaction ends, and the like (temporal information on the protein-protein interaction). Further, the result of comparing this method with another method for determining a protein-protein interaction on the basis of a fluorescent focus (PTL 4) previously invented by the present inventors revealed that the method of the present invention is superior to the method of PTL 4 in the efficiency of determining homomultimer formation; here, at least the first protein and the second protein are the same molecules, and these molecules form a dimer or a higher multimer as the homomultimer.

In addition, after verifying the effectiveness of the systems for detecting a fluorescent focus formed by assembly formation between the first fusion protein and

the second fusion protein shown in Figs. 1 and 2, the inventors have come up with other detection systems for determining a protein-protein interaction (see Figs. 3 to 6). Specifically, the inventors have come up with detection systems for determining a protein-protein interaction by expressing in a cell or introducing into a cell the first fusion protein and the second fusion protein separately for reconstitution of the fusion proteins in the cell.

To be more specific, as shown in Fig. 3, in determining an interaction between a first protein and a second protein, when a third fusion protein comprising the first protein and a multimerizable protein, a fourth fusion protein comprising the second protein and a multimerizable protein, and fifth fusion proteins each comprising a multimerizable protein and a fluorescent protein are expressed in a cell or introduced into a cell, the multimerizable proteins associate with each other. Thereby, the third fusion protein binds to the fifth fusion protein while the fourth fusion protein binds to the fifth fusion protein, so that proteins respectively corresponding to the first fusion protein and the second fusion protein shown in Figs. 1 and 2 are presumably expressed in the cell. Moreover, if such a presumption is correct, as in the case of the systems for detecting a fluorescent focus formed by assembly formation between the first fusion protein and the second

fusion protein shown in Figs. 1 and 2, the following can be expected also from a system for detecting a fluorescent focus formed by assembly formation among the third fusion protein, the fourth fusion protein, and the fifth fusion protein shown in Fig. 3: an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and another multimerizable protein; thereby, the fusion proteins autonomously form an assembly, and the fluorescent proteins contained in the fusion proteins are detected as fluorescent foci.

Moreover, as shown in Fig. 4, when a first labeled protein comprising an affinity tag and the first protein, a second labeled protein comprising an affinity tag and the second protein, and sixth fusion proteins each comprising a multimerizable protein, a fluorescent protein, and a binding partner having an affinity for any of the affinity tags and bound to the sixth fusion protein are expressed in a cell or introduced into a cell, the affinity tags bind to the binding partners. Thereby, the first labeled protein binds to the sixth fusion protein comprising the binding partner bound thereto while the second labeled protein binds to the sixth fusion protein comprising the binding partner bound thereto, so that protein complexes respectively corresponding to the first fusion protein and the second fusion protein shown in Fig.

2 are presumably expressed in the cell. Specifically, it is presumed that the complex composed of the fluorescent protein, the multimerizable protein, the binding partner, the affinity tag, and the first protein functions as the first fusion protein shown in Fig. 2, while the complex composed of the fluorescent protein, the multimerizable protein, the binding partner, the affinity tag, and the second protein functions as the second fusion protein shown in Fig. 2. Moreover, as in the case of the system shown in Fig. 2, when these protein complexes are expressed, an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and another multimerizable protein; thereby, the fusion proteins autonomously form an assembly, and the fluorescent proteins contained in the fusion proteins are detected as fluorescent foci.

Further, as shown in Fig. 5, when a seventh fusion protein comprising the first protein and a first partial peptide constituting a fluorescent protein, an eighth fusion protein comprising the second protein and a first partial peptide constituting a fluorescent protein, and ninth fusion proteins each comprising a multimerizable protein and a second partial peptide capable of reconstituting any of the fluorescent proteins by binding to the first partial peptide are expressed in a cell or introduced into a cell, the fluorescent proteins are

expressed in the cell by the reconstitution. Moreover,
as in the case of the system shown in Fig. 2, an interaction
if any between the first protein and the second protein
induces multimer formation between the multimerizable
protein and another multimerizable protein; thereby, the
fusion proteins autonomously form an assembly, and the
fluorescent proteins contained in the fusion proteins are
detected as fluorescent foci.

Furthermore, as shown in Fig. 6, when a tenth fusion
protein comprising the first protein and a third partial
peptide constituting a multimerizable protein, an eleventh
fusion protein comprising the second protein and a third
partial peptide constituting a multimerizable protein, and
twelfth fusion proteins each comprising a fluorescent
protein and a fourth partial peptide capable of
reconstituting any of the multimerizable proteins by
binding to the third partial peptide are expressed in a
cell or introduced into a cell, the multimerizable proteins
are expressed in the cell by the reconstitution. Moreover,
as in the case of the system shown in Fig. 2, an interaction
if any between the first protein and the second protein
induces multimer formation between the multimerizable
protein and another multimerizable protein; thereby, the
fusion proteins autonomously form an assembly, and the
fluorescent proteins contained in the fusion proteins are
detected as fluorescent foci.

Thus, the inventors actually constructed the detection system shown in Fig. 3, too, and expressed the above-described fusion proteins in cells. The result revealed that the system for detecting a fluorescent focus formed by assembly formation among the third fusion protein, the fourth fusion protein, and the fifth fusion protein also enables determination of certain protein-protein interactions on the basis of fluorescent foci detected in the cells. Moreover, the inventors constructed the detection system shown in Fig. 4, too, and expressed the above-described fusion proteins in cells. The result revealed that the system for detecting a fluorescent focus formed by an association among the first labeled protein, the second labeled protein, and the sixth fusion protein comprising the binding partner bound thereto also enables determination of certain protein-protein interactions on the basis of fluorescent foci detected in the cells.

Further, based on the systems shown in Fig. 1 and so forth, the present inventors have come up with a system shown in Fig. 7 as an embodiment for detecting an interaction among three or more proteins. Specifically, when a thirteenth fusion protein comprising the first protein and a multimerizable protein, a fourteenth fusion protein comprising the second protein and a multimerizable protein, and a fifteenth fusion protein comprising a third protein and a fluorescent protein are expressed in a cell or

introduced into a cell, an interaction if any among the first protein, the second protein, and the third protein, as in the cases of the systems shown in Fig. 1 and so forth, induces multimer formation between the multimerizable protein and another multimerizable protein; thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

Hence, the inventors actually constructed the detection system shown in Fig. 7, too, and expressed the above fusion proteins in cells. The result revealed that the system for detecting a fluorescent focus formed by an association among the thirteenth fusion protein, the fourteenth fusion protein, and the fifteenth fusion protein, that is, a fluorescent focus formed by the assembly formation, also enables determination of certain protein-protein interactions on the basis of fluorescent foci detected in the cells.

Further, based on the above results, the present inventors have come up with a system shown in Fig. 8 as a system for determining a protein-protein interaction. Specifically, the inventors have come up with a construction of a system in which a sixteenth fusion protein comprising the first protein, a first multimerizable protein, and a fluorescent protein, and a seventeenth fusion protein comprising the second protein and a second

multimerizable protein are expressed in a cell or introduced into a cell. In a case where the first multimerizable protein is a different protein from the second multimerizable protein, the first protein and the second protein interact with each other, further inducing multimer formation between the multimerizable protein and another multimerizable protein; thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

Then, the inventors actually constructed the detection system shown in Fig. 8, too, and expressed the above fusion proteins in cells. The result revealed that the system for detecting a fluorescent focus formed by an association between the sixteenth fusion protein and the seventeenth fusion protein, that is, a fluorescent focus formed by the assembly formation, also enables determination of certain protein-protein interactions on the basis of fluorescent foci detected in the cells.

Thus, the present invention relates to a method for determining a protein-protein interaction, as well as a vector and a kit for use in the method. More specifically, the present invention provides the following inventions.

<1> A method for determining an interaction between a first protein and a second protein, the method comprising the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell
a first fusion protein comprising the first
protein, a multimerizable protein, and a fluorescent
protein, and

5 a second fusion protein comprising the second
protein and a multimerizable protein;

(2) detecting a fluorescent focus formed by an
association between the first fusion protein and the second
fusion protein in the cell; and

10 (3) determining an interaction between the first
protein and the second protein according to the detection
of the fluorescent focus.

<2> The method according to <1>, wherein the second fusion
protein further comprises a fluorescent protein.

15 <3> The method according to <1> or <2>, wherein the
fluorescent protein is a monomeric fluorescent protein.

<4> A method for detecting an interaction between a first
protein and a second protein, the method comprising the
following steps (1) to (3):

20 (1) expressing in a cell or introducing into a cell
a third fusion protein comprising the first
protein and a multimerizable protein,

a fourth fusion protein comprising the second
protein and a multimerizable protein, and

25 a fifth fusion protein comprising a
multimerizable protein and a fluorescent protein;

(2) detecting a fluorescent focus formed by an association among the third fusion protein, the fourth fusion protein, and the fifth fusion protein in the cell; and

5 (3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

<5> The method according to <4>, wherein the fluorescent protein is a monomeric fluorescent protein.

10 <6> A vector comprising a DNA encoding a multimerizable protein, a DNA encoding a fluorescent protein, and a cloning site, wherein

the vector is capable of expressing a fusion protein comprising the multimerizable protein, the fluorescent protein, and a certain protein when a DNA encoding the

15 certain protein is inserted in the cloning site.

<7> A kit for use in the method according to any one of <1> to <3>, the kit comprising an instruction and at least one substance selected from the group consisting of the

20 following (a) to (h):

- (a) the vector according to <6>;
- (b) a vector encoding the first fusion protein;
- (c) a vector encoding the second fusion protein;
- (d) a transformed cell comprising a vector encoding
- 25 the first fusion protein;
- (e) a transformed cell comprising a vector encoding

the second fusion protein;

(f) a transformed cell comprising a vector encoding the first fusion protein and a vector encoding the second fusion protein;

5 (g) the first fusion protein; and

(h) the second fusion protein.

<8> A kit for use in the method according to <4> or <5>, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

10 (a) a vector comprising a DNA encoding the multimerizable protein and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the multimerizable protein and a certain protein when a DNA encoding the certain protein is inserted in the cloning site;

(b) a vector encoding the third fusion protein;

(c) a vector encoding the fourth fusion protein;

(d) a vector encoding the fifth fusion protein;

20 (e) a transformed cell comprising a vector encoding the fifth fusion protein;

(f) the third fusion protein;

(g) the fourth fusion protein; and

(h) the fifth fusion protein.

25 <9> A method for detecting an interaction between a first protein and a second protein, the method comprising the

following steps (1) to (3):

(1) expressing in a cell or introducing into a cell
a first labeled protein comprising an affinity
tag and the first protein,

5 a second labeled protein comprising an affinity
tag and the second protein, and

a sixth fusion protein comprising a
multimerizable protein, a fluorescent protein, and a
binding partner having an affinity for any of the affinity
10 tags and bound to the sixth fusion protein;

(2) detecting a fluorescent focus formed by an
association among the first labeled protein, the second
labeled protein, and the sixth fusion protein comprising
the binding partner bound thereto in the cell; and

15 (3) determining an interaction between the first
protein and the second protein according to the detection
of the fluorescent focus.

<10> A kit for use in the method according to <9>, the
kit comprising an instruction and at least one substance
20 selected from the group consisting of the following (a)
to (h):

(a) a vector comprising a DNA encoding the affinity
tag and a cloning site, wherein the vector is capable of
expressing a fusion protein comprising the affinity tag
25 when a DNA encoding a certain protein is inserted in the
cloning site;

(b) a vector encoding the first labeled protein;
(c) a vector encoding the second labeled protein;
(d) a vector encoding the sixth fusion protein
comprising the binding partner bound thereto;

5 (e) a transformed cell comprising a vector encoding
the sixth fusion protein comprising the binding partner
bound thereto;

(f) the first labeled protein;

(g) the second labeled protein; and

10 (h) the sixth fusion protein comprising the binding
partner bound thereto.

<11> A method for detecting an interaction between a first
protein and a second protein, the method comprising the
following steps (1) to (3):

15 (1) expressing in a cell or introducing into a cell
a seventh fusion protein comprising the first
protein and a first partial peptide constituting a
fluorescent protein,

20 an eighth fusion protein comprising the second
protein and a first partial peptide constituting a
fluorescent protein, and

a ninth fusion protein comprising a
multimerizable protein and a second partial peptide capable
of reconstituting any of the fluorescent proteins by
25 binding to the first partial peptide;

(2) detecting a fluorescent focus formed by an

association among the seventh fusion protein, the eighth fusion protein, and the ninth fusion protein in the cell; and

(3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

<12> A kit for use in the method according to <11>, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

(a) a vector comprising a DNA encoding the first partial peptide and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the first partial peptide when a DNA encoding a certain protein is inserted in the cloning site;

(b) a vector encoding the seventh fusion protein;

(c) a vector encoding the eighth fusion protein;

(d) a vector encoding the ninth fusion protein;

(e) a transformed cell comprising a vector encoding the ninth fusion protein;

(f) the seventh fusion protein;

(g) the eighth fusion protein; and

(h) the ninth fusion protein.

<13> A method for detecting an interaction between a first protein and a second protein, the method comprising the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell
a tenth fusion protein comprising the first
protein and a third partial peptide constituting a
multimerizable protein,

5 an eleventh fusion protein comprising the
second protein and a third partial peptide constituting
a multimerizable protein, and

a twelfth fusion protein comprising a
fluorescent protein and a fourth partial peptide capable
10 of reconstituting any of the multimerizable proteins by
binding to the third partial peptide;

(2) detecting a fluorescent focus formed by an
association among the tenth fusion protein, the eleventh
fusion protein, and the twelfth fusion protein in the cell;
15 and

(3) determining an interaction between the first
protein and the second protein according to the detection
of the fluorescent focus.

<14> A kit for use in the method according to <13>, the
20 kit comprising an instruction and at least one substance
selected from the group consisting of the following (a)
to (h):

(a) a vector comprising a DNA encoding the third
partial peptide and a cloning site, wherein the vector is
25 capable of expressing a fusion protein comprising the third
partial peptide when a DNA encoding a certain protein is

inserted in the cloning site;

(b) a vector encoding the tenth fusion protein;

(c) a vector encoding the eleventh fusion protein;

(d) a vector encoding the twelfth fusion protein;

5 (e) a transformed cell comprising a vector encoding the twelfth fusion protein;

(f) the tenth fusion protein;

(g) the eleventh fusion protein; and

(h) the twelfth fusion protein.

10 <15> A method for detecting an interaction among a first protein, a second protein, and a third protein, the method comprising the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell

15 a thirteenth fusion protein comprising the first protein and a multimerizable protein,

a fourteenth fusion protein comprising the second protein and a multimerizable protein, and

a fifteenth fusion protein comprising the third protein and a fluorescent protein;

20 (2) detecting a fluorescent focus formed by an association among the thirteenth fusion protein, the fourteenth fusion protein, and the fifteenth fusion protein in the cell; and

25 (3) determining an interaction among the first protein, the second protein, and the third protein according to the detection of the fluorescent focus.

<16> A kit for use in the method according to <15>, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

5 (a) a vector comprising a DNA encoding the multimerizable protein and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the multimerizable protein when a DNA encoding a certain protein is inserted in the cloning site;

10 (b) a vector comprising a DNA encoding the fluorescent protein and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the fluorescent protein when a DNA encoding a certain protein is inserted in the cloning site;

15 (c) a vector encoding the thirteenth fusion protein;

 (d) a vector encoding the fourteenth fusion protein;

 (e) a vector encoding the fifteenth fusion protein;

 (f) the thirteenth fusion protein;

 (g) the fourteenth fusion protein; and

20 (h) the fifteenth fusion protein.

<17> A method for determining an interaction between a first protein and a second protein, wherein

 a first multimerizable protein is a different protein from a second multimerizable protein, and

25 the method comprises the following steps (1) to (3):

 (1) expressing in a cell or introducing into a cell

a sixteenth fusion protein comprising the first protein, the first multimerizable protein, and a fluorescent protein, and

a seventeenth fusion protein comprising the second protein and the second multimerizable protein;

(2) detecting a fluorescent focus formed by an association between the sixteenth fusion protein and the seventeenth fusion protein in the cell; and

(3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

<18> A kit for use in the method according to <17>, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (f):

(a) a vector comprising a DNA encoding the first multimerizable protein, a DNA encoding the fluorescent protein, and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the first multimerizable protein, the fluorescent protein, and a certain protein when a DNA encoding the certain protein is inserted in the cloning site;

(b) a vector comprising a DNA encoding the second multimerizable protein, a DNA encoding the fluorescent protein, and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the second

multimerizable protein, the fluorescent protein, and a certain protein when a DNA encoding the certain protein is inserted in the cloning site;

(c) a vector encoding the sixteenth fusion protein;

5 (d) a vector encoding the seventeenth fusion protein;

(e) the sixteenth fusion protein; and

(f) the seventeenth fusion protein.

[Advantageous Effects of Invention]

10 The present invention makes it possible to determine a protein-protein interaction in an intracellular environment unique thereto, and to obtain positional information and temporal information on the protein-protein interaction based on the determination result. Particularly, the present invention makes it possible to efficiently determine homomultimer formation in an intracellular environment unique to the homomultimer formation, and also to efficiently obtain positional information and temporal information on the homomultimer formation based on the determination result.

20 [Brief Description of Drawings]

[Fig. 1] Fig. 1 is a conceptual diagram for illustrating one embodiment of a first method for determining a protein-protein interaction of the present invention. Specifically, the diagram illustrates that when a first fusion protein comprising a first protein (in the figure, A), a multimerizable protein (in the figure, C) and a

fluorescent protein (in the figure, D), and a second fusion protein comprising a second protein (in the figure, B) and a multimerizable protein (in the figure, C) are expressed in a cell or introduced into a cell, an interaction between the first protein and the second protein can be determined according to the detection of a fluorescent focus formed by assembly formation between the first fusion protein and the second fusion protein in the cell.

[Fig. 2] Fig. 2 is a conceptual diagram for illustrating one embodiment of the first method for determining a protein-protein interaction of the present invention. Specifically, the diagram illustrates that when the first fusion protein comprising the first protein (in the figure, A), the multimerizable protein (in the figure, C) and the fluorescent protein (in the figure, D), and the second fusion protein comprising the second protein (in the figure, B) and multimerizable protein (in the figure, C) and further comprising a fluorescent protein (in the figure, D) are expressed in a cell or introduced into a cell, an interaction between the first protein and the second protein can be determined according to the detection of a fluorescent focus formed by assembly formation between the first fusion protein and the second fusion protein in the cell.

[Fig. 3] Fig. 3 is a conceptual diagram for illustrating one embodiment of a second method for determining a protein-protein interaction of the present invention.

Specifically, the diagram illustrates that when a third fusion protein comprising a first protein (in the figure, A) and a multimerizable protein (in the figure, C), a fourth fusion protein comprising a second protein (in the figure, B) and a multimerizable protein (in the figure, C), and a fifth fusion protein comprising a multimerizable protein (in the figure, C) and a fluorescent protein (in the figure, D) are expressed in a cell or introduced into a cell, an interaction between the first protein and the second protein can be determined according to the detection of a fluorescent focus formed by assembly formation among the third fusion protein, the fourth fusion protein, and the fifth fusion protein in the cell.

[Fig. 4] Fig. 4 is a conceptual diagram for illustrating one embodiment of a third method for determining a protein-protein interaction of the present invention. Specifically, the diagram illustrates that when a first labeled protein comprising a first protein (in the figure, A) and an affinity tag (in the figure, E1), a second labeled protein comprising a second protein (in the figure, B) and an affinity tag (in the figure, E1), and a sixth fusion protein comprising a multimerizable protein (in the figure, C), a fluorescent protein (in the figure, D), and a binding partner (in the figure, E2) having an affinity for any of the affinity tags and bound to the sixth fusion protein are expressed in a cell or introduced into a cell, an

interaction between the first protein and the second protein can be determined according to the detection of a fluorescent focus formed by assembly formation among the first labeled protein, the second labeled protein, and the sixth fusion protein comprising the binding partner bound thereto in the cell.

[Fig. 5] Fig. 5 is a conceptual diagram for illustrating one embodiment of a fourth method for determining a protein-protein interaction of the present invention. Specifically, the diagram illustrates that when a seventh fusion protein comprising a first protein (in the figure, A) and a first partial peptide (in the figure, d1) constituting a fluorescent protein, an eighth fusion protein comprising a second protein (in the figure, B) and a first partial peptide (in the figure, d1) constituting a fluorescent protein, and a ninth fusion protein comprising a multimerizable protein (in the figure, C) and a second partial peptide (in the figure, d2) capable of reconstituting any of the fluorescent proteins by binding to the first partial peptide are expressed in a cell or introduced into a cell, an interaction between the first protein and the second protein can be determined according to the detection of a fluorescent focus formed by assembly formation among the seventh fusion protein, the eighth fusion protein, and the ninth fusion protein in the cell.

[Fig. 6] Fig. 6 is a conceptual diagram for illustrating

one embodiment of a fifth method for determining a protein-protein interaction of the present invention. Specifically, the diagram illustrates that when a tenth fusion protein comprising a first protein (in the figure, A) and a third partial peptide (in the figure, c1) constituting a multimerizable protein, an eleventh fusion protein comprising a second protein (in the figure, B) and a third partial peptide (in the figure, c1) constituting a multimerizable protein, and a twelfth fusion protein comprising a fluorescent protein (in the figure, D) and a fourth partial peptide (in the figure, c2) capable of reconstituting any of the multimerizable proteins by binding to the third partial peptide are expressed in a cell or introduced into a cell, an interaction between the first protein and the second protein can be determined according to the detection of a fluorescent focus formed by assembly formation among the tenth fusion protein, the eleventh fusion protein, and the twelfth fusion protein in the cell.

[Fig. 7] Fig. 7 is a conceptual diagram for illustrating one embodiment of a sixth method for determining a protein-protein interaction of the present invention. Specifically, the diagram illustrates that when a thirteenth fusion protein comprising a first protein (in the figure, A) and a multimerizable protein (in the figure, C), a fourteenth fusion protein comprising a second protein

(in the figure, B) and a multimerizable protein (in the figure, C), and a fifteenth fusion protein comprising a third protein (in the figure, F) and a fluorescent protein (in the figure, D) are expressed in a cell or introduced into a cell, an interaction among the first protein, the second protein, and the third protein can be determined according to the detection of a fluorescent focus formed by assembly formation among the thirteenth fusion protein, the fourteenth fusion protein, and the fifteenth fusion protein in the cell.

[Fig. 8] Fig. 8 is a conceptual diagram for illustrating one embodiment of a seventh method for determining a protein-protein interaction of the present invention. Specifically, the diagram illustrates that a first multimerizable protein (in the figure, C) is a different protein from a second multimerizable protein (in the figure, G), and that when a sixteenth fusion protein comprising a first protein (in the figure, A), the first multimerizable protein, and a fluorescent protein (in the figure, D), and a seventeenth fusion protein comprising a second protein (in the figure, B) and the second multimerizable protein are expressed in a cell or introduced into a cell, an interaction between the first protein and the second protein can be determined according to the detection of a fluorescent focus formed by assembly formation between the sixteenth fusion protein and the seventeenth fusion

protein in the cell.

[Fig. 9] Fig. 9 shows micrographs for illustrating the result of expressing in cells a fusion protein according to the present invention (PB1-mAG1-FKBP12 mutant) composed of a PB1 domain of a p62 protein as a multimerizable protein and mAG1 as a fluorescent protein fused to an analysis target FKBP12 mutant, and then adding a drug B/B Homodimerizer to the cells to analyze whether or not it is possible to determine, on the basis of a fluorescent focus, homodimer formation of the FKBP12 mutant attributable to the drug addition. The figure shows the result of analyzing the cells before the drug addition, the cells 38 minutes after the drug addition, and the cells 76 minutes after the drug addition, in this order from the left. Moreover, in the figure, the scale bars represent 20 μ m.

[Fig. 10] Fig. 10 shows micrographs for illustrating the result of detecting fluorescent foci indicating the homodimer formation of the FKBP12 mutant attributable to the drug addition according to the method described in International Publication No. W02013/084950. The figure shows the result of analyzing the cells before the drug addition, the cells 58 minutes after the drug addition, and the cells 66 minutes after the drug addition, in this order from the left. Moreover, in the figure, the scale bars represent 20 μ m.

[Fig. 11] Fig. 11 shows micrographs for illustrating the

result of analyzing whether or not it is possible to determine, on the basis of a fluorescent focus, that the homodimer formation of the FKBP12 mutant attributable to the addition of the B/B Homodimerizer to cells constitutively expressing the PB1-mAG1-FKBP12 mutant is dissociated (disrupted) by adding B/B Washout Ligand. The figure shows the result of analyzing the cells before the drug addition, the cells 90 minutes after the B/B Homodimerizer addition, and the cells 90 minutes after the B/B Homodimerizer was washed away and then the B/B Washout Ligand was added, in this order from the left. Moreover, in the figure, the scale bars represent 20 μ m.

[Fig. 12] Fig. 12 is a graph for illustrating the result of analyzing whether or not it is possible to determine, on the basis of a fluorescent focus, that the homodimer formation of the FKBP12 mutant attributable to the addition of the B/B Homodimerizer to the cells constitutively expressing the PB1-mAG1-FKBP12 mutant is dissociated by adding the B/B Washout Ligand. In the figure, A shows the elapsed time after the B/B Homodimerizer was added to the cells. B shows the time of washing away the B/B Homodimerizer having been added to the cells. C shows the elapsed time after the washing followed by the B/B Washout Ligand addition to the cells.

[Fig. 13] Fig. 13 shows micrographs for illustrating the result of expressing in cells a fusion protein according

to the present invention (mAG1-PB1-p53 or p53-mAG1-PB1) composed of a PB1 domain of a p62 protein as a multimerizable protein and mAG1 as a fluorescent protein fused to an analysis target p53 to analyze whether or not it is possible to determine homomultimer formation of p53 on the basis of a fluorescent focus. Note that, in the figure, "mAG1-PB1-p53" shows the result of detecting fluorescent signals derived from the protein composed of a protein tag according to the present invention (the fusion protein comprising the multimerizable protein and the fluorescent protein) fused to the N-terminus of p53. The panel on the right of "mAG1-PB1-p53" shows the result of detecting nuclei by treating the cells with Hoechst 33342. Moreover, "p53-mAG1-PB1" shows the result of detecting fluorescent signals derived from the protein composed of the protein tag according to the present invention fused to the C-terminus of p53. The panel on the right of "p53-mAG1-PB1" shows the result of detecting nuclei by treating the cells with Hoechst 33342. In the figure, the scale bars represent 20 μ m.

[Fig. 14] Fig. 14 shows micrographs for illustrating the result of expressing in cells a fusion protein (mAG1-p53 or p53-mAG1) composed of only mAG1 as a fluorescent protein fused to an analysis target p53 to analyze whether or not it is possible to determine homomultimer formation of p53 on the basis of a fluorescent focus. Note that, in the

figure, "mAG1-p53" shows the result of detecting fluorescent signals derived from the protein composed of the fluorescent protein fused to the N-terminus of p53. The panel on the right of "mAG1-p53" shows the result of detecting nuclei by treating the cells with Hoechst 33342. Moreover, "p53-mAG1" shows the result of detecting fluorescent signals derived from the protein composed of the fluorescent protein fused to the C-terminus of p53. The panel on the right of "p53-mAG1" shows the result of detecting nuclei by treating the cells with Hoechst 33342. In the figure, the scale bars represent 20 μ m.

[Fig. 15] Fig. 15 shows micrographs for illustrating the result of expressing in cells a protein (PB1-p53(70)) composed of a PB1 domain of a p62 protein fused to the N-terminus of a partial peptide of an analysis target p53, and (PB1-mAG1-MDM2) composed of a PB1 domain of a p62 protein and mAG1 fused to the N-terminus of an analysis target MDM2 to analyze whether or not it is possible to determine a protein-protein interaction between p53(70) and MDM2 on the basis of a fluorescent focus. Note that, in the figure, "Nutlin-3 (-)" shows the result of observing the cells before the addition of Nutlin-3, which is an inhibitor against the protein-protein interaction. "Nutlin-3 (+), 30min" shows the result of observing the cells when 30 minutes elapsed after the Nutlin-3 addition. In the figure, the scale bar represents 20 μ m.

[Fig. 16] Fig. 16 shows micrographs for illustrating the result of expressing in cells a protein (p53(70)-mAG1-PB1) composed of mAG1 and a PB1 domain of a p62 protein fused to the C-terminus of a partial peptide of an analysis target p53, and (PB1-MDM2) composed of a PB1 domain of a p62 protein fused to the N-terminus of an analysis target MDM2 to analyze whether or not it is possible to determine a protein-protein interaction between p53(70) and MDM2 on the basis of a fluorescent focus. Note that, in the figure, "Nutlin-3 (-)" shows the result of observing the cells before the addition of Nutlin-3, which is an inhibitor against the protein-protein interaction. "Nutlin-3 (+), 30min" shows the result of observing the cells when 30 minutes elapsed after the Nutlin-3 addition. In the figure, the scale bars represent 20 μ m.

[Fig. 17] Fig. 17 shows micrographs for illustrating the result of expressing in cells a protein (PB1-mAG1-FKBP12) composed of a PB1 domain of a p62 protein and mAG1 fused to the N-terminus of an analysis target FKBP12, and a protein (mTOR(FRB domain)-PB1) composed of a PB1 domain of a p62 protein fused to the C-terminus of an analysis target FRB domain of a mTOR protein to analyze whether or not it is possible to determine a protein-protein interaction between FKBP12 and mTOR(FRB domain) on the basis of a fluorescent focus. Note that, in the figure, "Rapamycin (-)" shows the result of observing the cells before the

addition of rapamycin, which is an inducer for the protein-protein interaction. "Rapamycin (+), 30min" shows the result of observing the cells when 30 minutes elapsed after the rapamycin addition. In the figure, the scale bars represent 100 μ m.

[Fig. 18] Fig. 18 shows micrographs for illustrating the result of expressing in cells a protein (p53(70)-mAG1-PB1) composed of mAG1 and a PB1 domain of a p62 protein fused to the N-terminus of a partial peptide of an analysis target p53, and (PB1-mAG1-MDM2) composed of a PB1 domain of a p62 protein and mAG1 fused to the N-terminus of an analysis target MDM2 to analyze whether or not it is possible to determine a protein-protein interaction between p53(70) and MDM2 on the basis of a fluorescent focus. Note that, in the figure, "Nutlin-3 (-)" shows the result of observing the cells before the addition of Nutlin-3, which is an inhibitor against the protein-protein interaction. "Nutlin-3 (+), 30min" shows the result of observing the cells when 30 minutes elapsed after the Nutlin-3 addition. In the figure, the scale bars represent 20 μ m.

[Fig. 19] Fig. 19 shows micrographs for illustrating the result of expressing in cells a protein (p53(70)-PB1-mAG1) composed of a PB1 domain of a p62 protein and mAG1 fused to the C-terminus of a partial peptide of an analysis target p53, and (PB1-mAG1-MDM2) composed of a PB1 domain of a p62 protein and mAG1 fused to the N-terminus of an analysis

target MDM2 to analyze whether or not it is possible to determine a protein-protein interaction between p53(70) and MDM2 on the basis of a fluorescent focus. Note that, in the figure, "Nutlin-3 (-)" shows the result of observing the cells before the addition of Nutlin-3, which is an inhibitor against the protein-protein interaction. "Nutlin-3 (+), 30min" shows the result of observing the cells when 30 minutes elapsed after the Nutlin-3 addition. In the figure, the scale bars represent 20 μ m.

[Fig. 20] Fig. 20 shows micrographs for illustrating the result of expressing in cells a protein (mTOR(FRB domain)-mAG1-PB1) composed of mAG1 and a PB1 domain of a p62 protein fused to the C-terminus of an analysis target FRB domain of a mTOR protein, and a protein (PB1-mAG1-FKBP12) composed of a PB1 domain of a p62 protein and mAG1 fused to the N-terminus of an analysis target FKBP12 to analyze whether or not it is possible to determine a protein-protein interaction between FKBP12 and mTOR(FRB domain) on the basis of a fluorescent focus. Note that, in the figure, "Rapamycin (-)" shows the result of observing the cells before the addition of rapamycin, which is an inducer for the protein-protein interaction. "Rapamycin (+), 30min" shows the result of observing the cells when 30 minutes elapsed after the rapamycin addition. In the figure, the scale bars represent 100 μ m.

[Fig. 21] Fig. 21 shows photographs obtained by partially

enlarging the micrographs shown in Fig. 20. In the figure, the scale bars represent 20 μ m.

[Fig. 22] Fig. 22 shows micrographs for illustrating the result of expressing in cells a protein (PB1-DG1-FKBP12 mutant, i.e., PB1-DG1-FKBP12 (F36V)) composed of a PB1 domain of a p62 protein and a fluorescent protein DG1 fused to the N-terminus of an analysis target FKBP12 mutant, and then adding a drug B/B Homodimerizer to the cells to analyze whether or not it is possible to determine, on the basis of a fluorescent focus, homodimer formation of the FKBP12 mutant attributable to the drug addition. The figure shows the result of analyzing the cells before the drug addition, the cells 1 hour after the drug addition, the cells 2 hours after the drug addition, and the cells 3 hours after the drug addition, in this order from the left. Moreover, in the figure, the scale bars represent 20 μ m.

[Fig. 23] Fig. 23 shows micrographs for illustrating the result of expressing in cells a protein (PB1-mCherry-FKBP12 mutant, i.e., PB1-mCherry-FKBP12 (F36V)) composed of a PB1 domain of a p62 protein and a fluorescent protein mCherry fused to the N-terminus of an analysis target FKBP12 mutant, and then adding a drug B/B Homodimerizer to the cells to analyze whether or not it is possible to determine, on the basis of a fluorescent focus, homodimer formation of the FKBP12 mutant attributable to the drug addition. The figure shows the result of analyzing the cells before the

drug addition, the cells 1 hour after the drug addition, the cells 2 hours after the drug addition, and the cells 3 hours after the drug addition, in this order from the left. Moreover, in the figure, the scale bars represent
5 20 μm .

[Fig. 24] Fig. 24 shows micrographs for illustrating the result of expressing in cells a protein (Tankyrase-mAG1-FKBP12 mutant, Tankyrase-mAG1-FKBP12 (F36V)) composed of a SAM domain of a Tankyrase 1 protein
10 as a multimerizable protein and mAG1 fused to the N-terminus of an analysis target FKBP12 mutant, and then adding a drug B/B Homodimerizer to the cells to analyze whether or not it is possible to determine, on the basis of a fluorescent focus, homodimer formation of the FKBP12 mutant
15 attributable to the drug addition. The figure shows the result of analyzing the cells before the drug addition and the cells 3 hours after the drug addition, in this order from the left. Moreover, in the figure, the scale bar represents 20 μm .

[Fig. 25] Fig. 25 shows micrographs for illustrating the result of expressing in cells a protein (Tankyrase-mAG1-p53, PKCi-mAG1-p53, TEL-mAG1-p53, or DGKd-mAG1-p53) composed
20 of a SAM domain of a Tankyrase 1 protein, a PB1 domain of a PKCiota protein, a SAM domain of a TEL protein, or a SAM domain of a DGK delta protein, as a multimerizable protein, fused to the N-terminus of p53 to analyze whether or not
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it is possible to determine homomultimer formation of p53 on the basis of a fluorescent focus. In the figure, the scale bar represents 20 μm .

[Fig. 26] Fig. 26 shows micrographs for illustrating the result of expressing in cells a protein (ERK2-mAG1-PB1) composed of mAG1 and a PB1 domain of a p62 protein fused to the C-terminus of ERK2, and then adding EGF to the cells to analyze whether or not it is possible to determine, on the basis of a fluorescent focus, homodimer formation of ERK2 attributable to the addition. Note that, in the figure, "before EGF addition" shows the result of observing the cells before the EGF addition. "EGF (50 ng/ml), 8 min" shows the result of observing the cells when 8 minutes elapsed after the EGF addition. In the figure, the scale bars represent 20 μm .

[Fig. 27] Fig. 27 is a micrograph for illustrating the result of observing, 8 minutes after EGF addition, cells constitutively expressing a protein (ERK2-PB1-mAG1) composed of a PB1 domain of a p62 protein and mAG1 fused to the C-terminus of ERK2 to analyze whether or not it is possible to determine, on the basis of a fluorescent focus, homodimer formation of ERK2 attributable to the addition. In the figure, the scale bar represents 20 μm .

[Fig. 28] Fig. 28 shows micrographs for illustrating the result of expressing in cells a protein (PB1-mAG1-STAT3) composed of a PB1 domain of a p62 protein and mAG1 fused

to the N-terminus of STAT3, and then adding IL-6 to the cells to analyze whether or not it is possible to determine, on the basis of a fluorescent focus, homodimer formation of STAT3 attributable to the addition. Note that, in the figure, "before" shows the result of observing the cells before the IL-6 addition. "IL6 (100 ng/ml), 50 min" shows the result of observing the cells when 50 minutes elapsed after the IL-6 addition. In the figure, the scale bar represents 20 μ m.

[Fig. 29] Fig. 29 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus by transiently expressing p53-PB1 and PB1-MDM2, PB1-MDM2 and PB1, or p53-PB1 and PB1 in cells constitutively expressing a fusion protein (PB1-mAG1) comprising a PB1 domain of a p62 protein and mAG1. Note that "p53-PB1" means a protein composed of a PB1 domain of a p62 protein fused to the C-terminus of a portion of a p53 protein. "PB1-MDM2" means a protein composed of a PB1 domain of a p62 protein fused to the N-terminus of MDM2. "PB1" means a PB1 domain of a p62 protein. Moreover, in the figure, the scale bars represent 20 μ m, and arrows indicate detected fluorescent foci.

[Fig. 30] Fig. 30 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine, on the basis of a fluorescent focus, a

termination of the protein-protein interaction between p53 and MDM2 by adding Nutlin-3 to the cells constitutively expressing PB1-mAG1 and further transiently expressing p53(70)-PB1 and PB1-MDM2. In the figure, the photograph on the left shows the result of analyzing the cells before the Nutlin-3 addition, and the photograph on the right shows the result of analyzing the cells after the Nutlin-3 addition. Moreover, in the figure, the scale bars represent 10 μ m.

[Fig. 31] Fig. 31 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between FKBP12 and mTOR(FRB domain) on the basis of a fluorescent focus by expressing in cells: a protein (PB1-mTOR(FRB domain) or mTOR(FRB domain)-PB1) composed of a PB1 domain of a p62 protein fused to the N-terminus or the C-terminus of mTOR(FRB domain); a protein (PB1-FKBP12 or FKBP12-PB1) composed of a PB1 domain of a p62 protein fused to the N-terminus or the C-terminus of FKBP12; and mAG1 fused to the N-terminus of a PB1 domain of a p62 protein (mAG1-PB1), in combinations of the following (A) to (D). Note that, in the figure, "before rapamycin addition" shows the result of observing the cells before the addition of rapamycin, which is an inducer for the protein-protein interaction. "Rapamycin (500 nM), room temperature, 60 min" shows the result of observing the cells when 60 minutes elapsed after

the rapamycin addition. In the figure, the scale bar represents 20 μ m. (A) PB1-mTOR(FRB domain), PB1-FKBP12, and mAG1-PB1; (B) PB1-mTOR(FRB domain), FKBP12-PB1, and mAG1-PB1; (C) mTOR(FRB domain)-PB1, PB1-FKBP12, and mAG1-PB1; and (D) mTOR(FRB domain)-PB1, FKBP12-PB1, and mAG1-PB1.

[Fig. 32] Fig. 32 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine homodimer formation of CD80 on the basis of a fluorescent focus after rapamycin for inducing the binding between FKBP12 and a FRB domain of a mTOR protein was added to cells expressing: a protein (CD80-FRB) composed of a FRB domain of a mTOR protein as an affinity tag fused to the N-terminus of the analysis target CD80; and a protein (PB1-mAG1-FKBP12) composed of FKBP12 as a binding partner of the affinity tag, a PB1 domain of a p62 protein as a multimerizable protein, and mAG1 as a fluorescent protein fused to each other. In the figure, the upper panel shows the result of analyzing the above-described CD80, and the lower panel shows the result of analyzing CD2 used as a negative control thereof in place of CD80. In the figure, the scale bar represents 20 μ m.

[Fig. 33] Fig. 33 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine trimer formation among p50, p65, and I κ B α on the basis of a fluorescent focus in cells expressing: a protein

(PB1-p50) composed of a PB1 domain of a p62 protein fused to the N-terminus of the analysis target p50; a protein (p65-PB1) composed of a PB1 domain of a p62 protein fused to the C-terminus of the analysis target p65; and a protein (mKO2-I κ B α) composed of mKO2 as a fluorescent protein fused to the N-terminus of I κ B α . In the figure, the left panel shows the result. The right panel shows the result of analyzing a negative control in which the p65-PB1 was not expressed. In the figure, the scale bars represent 20 μ m.

[Fig. 34] Fig. 34 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine trimer formation among Cyclin D1, p21, and CDK4 on the basis of a fluorescent focus in cells expressing: a protein (mAG1-Cyclin D1) composed of mAG1 fused to the N-terminus of the analysis target Cyclin D1; a protein (p21-PB1) composed of a PB1 domain of a p62 protein fused to the C-terminus of the analysis target p21; and a protein (CDK4-PB1) composed of a PB1 domain of a p62 protein fused to the C-terminus of the analysis target CDK4. In the figure, the left panel shows the result. The panel in the middle shows the result of analyzing a negative control in which only PB1 was expressed in place of the CDK4-PB1. The right panel shows the result of analyzing a negative control in which CDK4 not fused to other proteins was expressed in place of the CDK4-PB1. In the figure, the scale bars represent 20 μ m.

[Fig. 35] Fig. 35 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine, on the basis of a fluorescent focus, heterotetramer formation from calmodulin and an M13 peptide by adding histamine, which induces the tetramer formation, to cells expressing: a protein (Calmodulin-mAG1) composed of mAG1 fused to the C-terminus of the analysis target calmodulin; and a protein (M13peptide-PB1) composed of a PB1 domain of a p62 protein fused to the C-terminus of the analysis target M13 peptide. In the figure, the left panel shows the result of analyzing the cells before the histamine addition. The panel in the middle shows the result of analyzing the cells 60 seconds after the histamine addition. The right panel shows the result of analyzing the cells 300 seconds after the histamine addition. In the figure, the scale bar represents 20 μ m.

[Fig. 36] Fig. 36 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine, on the basis of a fluorescent focus, heterotetramer formation from calmodulin and an M13 peptide by adding histamine, which induces the tetramer formation, to cells expressing: a protein (Calmodulin-mUkG1) composed of mUkG1 as a fluorescent protein fused to the C-terminus of the analysis target calmodulin; and the protein (M13peptide-PB1) composed of a PB1 domain of a p62 protein fused to the C-terminus of the analysis target M13 peptide.

In the figure, the left panel shows the result of analyzing the cells before the histamine addition. The right panel shows the result of analyzing the cells 60 seconds after the histamine addition. In the figure, the scale bar represents 20 μm .

[Fig. 37] Fig. 37 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus in cells expressing:
a protein (PKCi-mAG1-p53) composed of a PB1 domain of a PKC δ protein as a multimerizable protein and mAG1 fused to the N-terminus of p53; and a protein (DGKd-mAG1-MDM2) composed of a SAM domain of a DGK δ protein as a multimerizable protein and mAG1 fused to the N-terminus of MDM2. In the figure, the left panel shows the result of analyzing the cells before the Nutlin-3 addition, and the right panel shows the result of analyzing the cells 60 minutes after the Nutlin-3 addition. In the figure, the scale bar represents 10 μm (hereinafter, regarding the representations in the drawings, the same shall apply to Figs. 38 to 45).

[Fig. 38] Fig. 38 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus in cells expressing:
the PKCi-mAG1-p53; and a protein (TEL-mAG1-MDM2) composed

of a SAM domain of a TEL protein as a multimerizable protein and mAG1 fused to the N-terminus of MDM2.

[Fig. 39] Fig. 39 is shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus in cells expressing: a protein (Tankyrase-mAG1-p53) composed of a SAM domain of a Tankyrase 1 protein as a multimerizable protein and mAG1 fused to the N-terminus of p53; and a protein (PKCi-mAG1-MDM2) composed of a PB1 domain of a PKCiota protein as a multimerizable protein and mAG1 fused to the N-terminus of MDM2.

[Fig. 40] Fig. 40 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus in cells expressing: the Tankyrase-mAG1-p53; and the DGKd-mAG1-MDM2.

[Fig. 41] Fig. 41 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus in cells expressing: the Tankyrase-mAG1-p53; and the TEL-mAG1-MDM2.

[Fig. 42] Fig. 42 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus in cells expressing:

a protein (DGKd-mAG1-p53) composed of a SAM domain of a DGK delta protein as a multimerizable protein and mAG1 fused to the N-terminus of p53; and the PKCi-mAG1-MDM2.

[Fig. 43] Fig. 43 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus in cells expressing: the DGKd-mAG1-p53; and the TEL-mAG1-MDM2.

[Fig. 44] Fig. 44 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus in cells expressing: a protein (TEL-mAG1-p53) composed of a SAM domain of a TEL protein as a multimerizable protein and mAG1 fused to the N-terminus of p53; and a protein (Tankyrase-mAG1-MDM2) composed of a SAM domain of a Tankyrase 1 protein as a multimerizable protein and mAG1 fused to the N-terminus of MDM2.

[Fig. 45] Fig. 45 shows micrographs for illustrating the result of analyzing whether or not it is possible to determine a protein-protein interaction between p53 and MDM2 on the basis of a fluorescent focus in cells expressing: the TEL-mAG1-p53; and the DGKd-mAG1-MDM2.

[Description of Embodiments]

Hereinafter, the present invention will be described in detail based on preferred embodiments thereof.

Moreover, as a matter of course, unless otherwise specified, those described in the following sections should be construed in the same manner in other sections and so forth also throughout the present invention.

5 <Method 1 for Detecting Protein-Protein Interaction>

A first embodiment of a method for determining a protein-protein interaction of the present invention is a method comprising the steps (1) to (3):

10 (1) expressing in a cell or introducing into a cell
a first fusion protein comprising a first protein, a multimerizable protein, and a fluorescent protein, and

a second fusion protein comprising a second protein and a multimerizable protein;

15 (2) detecting a fluorescent focus formed by an association between the first fusion protein and the second fusion protein in the cell; and

20 (3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

According to the method, as described in Examples 1 to 12 later, when the first fusion protein comprising the first protein, the multimerizable protein, and the fluorescent protein, and the second fusion protein comprising the second protein and the multimerizable protein are expressed in a cell or introduced into a cell,

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an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and the other multimerizable protein. Thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus (see Figs. 1 and 2).

In the present invention, the term "protein" means a molecule in which 2 or more amino acids are linked by a peptide bond(s), and modified products thereof. Thus, the term is a concept including not only full-length proteins, but also so-called oligopeptides and polypeptides. Examples of the modification of the protein include phosphorylation, glycosylation, palmitoylation, prenylation (for example, geranylgeranylation), methylation, acetylation, ubiquitination, SUMOylation, hydroxylation, and amidation.

As the "first protein" and the "second protein" according to the present invention, it is possible to use desired proteins intended for detection of interaction. Moreover, the first protein may be different from or the same as the second protein. Nevertheless, as described in Examples 1 to 12 and Comparative Example 1 later, the method of the present invention is capable of efficiently determining homomultimer formation and dissociation (disruption); accordingly, the method of the present

invention is more useful when the first protein is the same as the second protein.

The "interaction between the first protein and the second protein" according to the present invention includes not only direct interactions, but also indirect interactions such as an interaction for forming a complex in which another molecule (protein, nucleic acid, sugar, lipid, low-molecular-weight compound, or the like) is interposed between the first protein and the second protein.

The "multimerizable protein" according to the present invention is not particularly limited, as long as the protein is capable of forming a multimer. Nevertheless, from the viewpoint of facilitating detection of a fluorescent focus in the method of the present invention, the protein is preferably capable of forming a trimer or a higher multimer. Examples of such a multimerizable protein include a PB1 domain of p62, a PB1 domain of TFG, a PB1 domain of PKCιota, a SAM domain of TEL, a SAM domain of DGK delta, and a SAM domain of Tankyrase-1. Among these, from the viewpoint of facilitating detection of a fluorescent focus in the method of the present invention to form homotetramer to homooctamer, a PB1 domain of p62 is more preferable.

Note that, typically, a PB1 domain of p62, a PB1 domain of TFG, a PB1 domain of PKCιota, a SAM domain of TEL, a

SAM domain of DGK delta, and a SAM domain of Tankyrase-1 are respectively a protein having the amino acid sequence specified under SEQ ID NO: 2, a protein having the amino acid sequence specified under SEQ ID NO: 4, a protein having the amino acid sequence specified under SEQ ID NO: 6, a protein having the amino acid sequence specified under SEQ ID NO: 8, a protein having the amino acid sequence specified under SEQ ID NO: 10, and a protein having the amino acid sequence specified under SEQ ID NO: 12.

The amino acid sequences of these "multimerizable proteins" may be mutated naturally (i.e., non-artificially). Moreover, a mutation can also be introduced artificially. Such a mutant can also be used in the present invention, as long as it has a multimerization ability and a nature of forming an assembly (fluorescent focus) when the fused first protein and second protein interact with each other.

The "fluorescent protein" according to the present invention should be a protein capable of emitting fluorescence. Examples thereof include monomeric Azami Green 1 (mAG1), monomeric Umikinoko-Green (mUKG), monomeric Kusabira-Orange 2 (mK02), monomeric Keima-Red (mKeima, mKeima-Red), monomeric Midoriishi-Cyan1 (mMiCy), monomeric Kusabira-Orange 1 (mK01), monomeric Cherry (mCherry), FusionRed, Dronpa-Green1 (DG1), Midoriishi-Cyan1 (MiCy1), Kusabira-Cyan1 (KCy1), dimeric

Azami-Green (AB) (dAG (AB)), Kusabira-Orange 1 (K01), dimeric Keima-Red (dKeima, dKeima-Red), and TGuv. In a case where the fluorescent protein is a protein capable of forming a multimer, preferable are monomeric fluorescent proteins (such as mAG1, mUkG, mK02, mKeima, mMiCy, mK01, mCherry, FusionRed, DG1), more preferable are mAG1 and FusionRed, and furthermore preferable is mAG1, from the viewpoint of facilitating formation of an assembly (fluorescent focus) regardless of the interaction between the first protein and the second protein when the fluorescent protein is fused to the above-described multimerizable protein.

Note that, typically, mAG1, mUkG, mK02, mKeima, mMiCy, mK01, MiCy1, KCy1, dAG (AB), K01, dKeima, TGuv, mCherry, FusionRed, and DG1 are respectively a protein having the amino acid sequence of SEQ ID NO: 14, a protein having the amino acid sequence of SEQ ID NO: 16, a protein having the amino acid sequence of SEQ ID NO: 18, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB209969, a protein having the amino acid sequence of SEQ ID NO: 20, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB128821, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB128822, a protein having the amino acid sequence of SEQ ID NO: 22, a protein having the amino acid sequence of SEQ ID NO: 24, a protein having the amino acid sequence specified

under Genbank ACCESSION No: AB128820, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB209968, a protein having the amino acid sequence of SEQ ID NO: 26, a protein having the amino acid sequence specified under GenBank ACCESSION No: AAV52164, a protein having the amino acid sequence of SEQ ID NO: 40, and a protein having the amino acid sequence specified under GenBank ACCESSION No: BAD72874.

The amino acid sequences of these fluorescent proteins may be mutated naturally (i.e., non-artificially). Moreover, a mutation can also be introduced artificially. Such a mutant can also be used in the present invention, as long as it can emit fluorescence.

Alternatively, a chemiluminescent protein such as luciferases can also be used in place of the above-described fluorescent protein in the present invention. A fluorescent protein emits an electromagnetic wave (light) released when electrons of the protein having been excited by electromagnetic wave radiation return to the ground state (photo luminescence). On the other hand, in the case of a chemiluminescent protein, an electromagnetic wave (light) is emitted when electrons of a light emitting substrate are excited through a chemical reaction catalyzed by the protein. As described above, a fluorescent protein and a chemiluminescent protein are similar to each other in the light emitting mechanisms in principle. Hence, a

chemiluminescent protein is also usable in the present invention.

Such a chemiluminescent protein is not particularly limited. Examples thereof include luciferases, and
5 preferable examples thereof include Promega NanoLuc(registered trademark, Nluc) luciferase (manufactured by Promega Corporation) and Gaussia luciferase (see Chem. Sci., 2013, vol. 4, pp. 4395 to 4400).

Moreover, in the present invention, it is also
10 possible to use, in place of the above-described fluorescent protein, a fusion protein between a fluorescent protein and a luciferase in which the fluorescent protein emits an electromagnetic wave (light) by utilizing an energy of the chemical reaction (Nano-lantern, see Nat
15 Commun., 2012, vol. 3, p. 1262).

Further, a protein capable of specifically binding to a fluorescent dye can be used in place of the fluorescent protein. This is because by expressing such a protein in a cell and then introducing the fluorescent dye into the
20 cell, the resultant can be used as a fluorescent protein. An example of such a protein capable of specifically binding to a fluorescent dye includes HaloTag (manufactured by Promega Corporation).

The "first fusion protein" according to the present
25 invention comprises the first protein, the multimerizable protein, and the fluorescent protein. As long as the

formation of an assembly (fluorescent focus) with the second fusion protein is not inhibited, the first protein, the multimerizable protein, and the fluorescent protein may be fused in this order from the N-terminus side, or
5 in the following orders: the first protein, the fluorescent protein, and the multimerizable protein; the multimerizable protein, the first protein, and the fluorescent protein; the multimerizable protein, the fluorescent protein, and the first protein; the fluorescent
10 protein, the first protein, and the multimerizable protein; and the fluorescent protein, the multimerizable protein, and the first protein .

The "second fusion protein" according to the present invention comprises the second protein and the
15 multimerizable protein. As long as the formation of an assembly (fluorescent focus) with the first fusion protein is not inhibited, the multimerizable protein may be fused to either the N-terminus or the C-terminus of the second protein. Moreover, from the viewpoint of facilitating
20 detection of a fluorescent focus, the second fusion protein preferably further comprises a fluorescent protein (see Fig. 2). In addition, the fluorescent protein may be fused to one or both of the N-terminus and the C-terminus of the fusion protein, or may be fused between the second protein
25 and the multimerizable protein. The fluorescent protein to be contained in the second fusion protein is as described

above, and may be the same as or different from the fluorescent protein contained in the first fusion protein. Nevertheless, from the viewpoint of enabling tracing of a change in intracellular localization of each of the first protein and the second protein in accordance with when a protein-protein interaction takes place and terminates and so forth, the fluorescent protein contained in the first fusion protein and the fluorescent protein contained in the second fusion protein are preferably different from each other. Further, the use of such different fluorescent proteins, which enable separate spectrometries, makes it possible to measure amounts of the first protein and the second protein expressed, too. Furthermore, since such different fluorescent proteins exist at high concentrations inside an assembly (fluorescent focus) and very close to each other, FRET or fluorescence reabsorption may occur. Then, in such a case, without performing imaging or the like, measuring the intensity of fluorescence emitted from the fluorescent focus as a result of the FRET or fluorescence reabsorption makes it possible to determine a protein-protein interaction (as to the fluorescence reabsorption, see "Fluorescent imaging revolution (27th); To understand FRET in distinction from reabsorption" in Cell Technology, 25 (1), 67-69, 2006-01).

The proteins in the "first fusion protein" and the "second fusion protein" may be fused to each other directly,

or may be fused indirectly via a linker or a spacer protein. Additionally, as long as the formation of an assembly (fluorescent focus) is not inhibited, the "first fusion protein" and the "second fusion protein" may comprise
5 another functional protein. In this case, the other functional protein may be fused to one or both of the N-terminus and the C-terminus of each fusion protein, or may be fused directly or indirectly among the first protein or second protein, the multimerizable protein, and
10 fluorescent protein. The other functional protein is not particularly limited, and selected as appropriate depending on a function desirably provided to the fusion proteins according to the present invention. Examples of a functional protein used to facilitate purification of
15 the fusion protein include a Myc-tag protein, a His-tag protein, a hemagglutinin (HA)-tag protein, a FLAG-tag protein (registered trademark, Sigma-Aldrich Co.), and a glutathione S-transferase (GST) protein.

The "cell" according to the present invention is not
20 particularly limited, and may be a eukaryotic cell, or may be a prokaryotic cell. Examples of the "cell" include an animal cell (HEK293 cell, U2OS cell, Hela cell, and the like), an insect cell (Sf9 cell, and the like), a plant cell, yeast, and *Escherichia coli*. Moreover, such cells
25 may be in a state of being cultured *in vitro* (for example, cells grown in a medium or on a medium), or in a state of

being present *in vivo* (for example, cells in a transgenic animal in which a DNA encoding the first fusion protein and a DNA encoding the second fusion protein are introduced). Further, the fusion proteins and so forth according to the present invention are normally introduced into or expressed in the same cell, but may be introduced into or expressed in different cells. Then, in such a case, it is also possible to determine protein-protein interactions between different cells such as an interaction between a cytokine and a receptor and an interaction between receptors.

The expression of the fusion proteins in the cell may be a transient expression or a constitutive expression, depending on the purpose. The fusion proteins in the cell can be expressed by introducing into the cell a vector according to the present invention, which will be described later. Examples of known techniques for introducing the vector into the cell include, in the case of an animal cell, a lipofection method, an electroporation method, a phosphate calcium method, a DEAE-dextran method, and methods utilizing a virus (adenovirus, lentivirus, adeno-associated virus, or the like) and a microinjection method. Moreover, in the case of an insect cell, the examples include methods utilizing a baculovirus. Further, in the case of a plant cell, the examples include an *Agrobacterium* method, an electroporation method, a

particle gun method, and the like. In addition, in the case of yeasts, the examples include a lithium acetate method, an electroporation method, and a spheroplast method. Furthermore, in the case of *Escherichia coli*, the examples include a heat shock method (for example, a calcium chloride method, a rubidium chloride method), an electroporation method, and the like.

Meanwhile, in the present invention, those skilled in the art can introduce the fusion proteins into the cell by selecting known techniques as appropriate in accordance with the type of the cell. Examples of known techniques for introducing the proteins into the cell include a method using a reagent for introducing the proteins, an electroporation method, and a microinjection method.

A "fluorescent focus" to be detected in the present invention is formed by high-density localization of the fluorescent protein contained in an assembly formed between the fusion proteins and so forth as a result of a protein-protein interaction according to the present invention. Typically, in a two-dimensional observation with a microscope, the "fluorescent focus" has a fluorescence intensity, in a region whose size diameter is 0.2 to 5 μm (0.03 to 20 μm^2), higher than a fluorescence intensity of the fluorescent protein present in a dispersed state.

The "detection of the fluorescent focus" can be

carried out, for example, through observation using a fluorescence microscope including an excitation filter and an absorption filter corresponding to a fluorescent protein, and analysis using an imaging cytometer such as IN Cell Analyzer (manufactured by GE Healthcare). Moreover, a fluorescent focus can be detected also by processing the obtained image with an image analysis program (for example, an icy spot detection program to be described below). Note that, in the detection of the fluorescent focus, those skilled in the art can select and set as appropriate a filter, a detector, various parameters, and so forth in accordance with the characteristics of a fluorescent focus to be detected (such as the wavelength and the intensity of a fluorescence emitted by the fluorescent focus), as well as the device, the program, and so on.

For example, as described in Example 2 later, in a spot detection analysis program Icy.5.4.2 (see J.-C.Olivo-Marin "Extraction of spots in biological images using multiscale products" Pattern recognition, vol. 35-9, pp. 1989 to 1996, 2002, <http://icy.bioimageanalysis.com>), if a "fluorescence intensity value per unit calculated by the analysis program," which is detectable with set values of Spot Detector plugin (under conditions when all of Scale 1, Scale 2, and Scale 3 are checked and all sensitivities thereof are set to 100), is at least 2.0 times or more, more preferably 2.5 times or more, furthermore preferably

5.0 times or more, and particularly preferably 10 times or more, as large as the background (for example, the aforementioned fluorescence intensity value in a cell serving as a negative control), it can be determined that a fluorescent focus is detected.

In the method of the present invention, if the fluorescent focus is detected in the cell, it can be determined that the first protein and the second protein interact with each other; meanwhile, if the fluorescent focus is not detected, it can be determined that the first protein and the second protein do not interact with each other.

Hereinabove, one embodiment of the method for determining a protein-protein interaction of the present invention has been described. However, the method for determining a protein-protein interaction of the present invention is not limited to the above-described embodiment. For example, the present invention also makes it possible to determine a protein-protein interaction by expressing in a cell or introducing into a cell the first fusion protein and the second fusion protein separately, and then reconstituting the fusion proteins in the cell. To be more specific, the present invention can also encompass second to fifth embodiments described below.

<Method 2 for Detecting Protein-Protein Interaction>

A second embodiment of the method for determining

a protein-protein interaction of the present invention is a method for detecting an interaction between a first protein and a second protein, the method comprising the following steps (1) to (3):

5 (1) expressing in a cell or introducing into a cell
 a third fusion protein comprising the first
 protein and a multimerizable protein,

 a fourth fusion protein comprising the second
 protein and a multimerizable protein, and

10 a fifth fusion protein comprising a
 multimerizable protein and a fluorescent protein;

 (2) detecting a fluorescent focus formed by an
 association among the third fusion protein, the fourth
 fusion protein, and the fifth fusion protein in the cell;

15 and

 (3) determining an interaction between the first
 protein and the second protein according to the detection
 of the fluorescent focus.

 According to the method, as described in Examples
20 later 13 and 14, when the third fusion protein comprising
 the first protein and the multimerizable protein, the
 fourth fusion protein comprising the second protein and
 the multimerizable protein, and the fifth fusion protein
 comprising the multimerizable protein and the fluorescent
25 protein are expressed in a cell or introduced into a cell,
 the multimerizable proteins associates with each other.

Thereby, the third fusion protein binds to the fifth fusion protein while the fourth fusion protein binds to the fifth fusion protein, so that proteins respectively corresponding to the first fusion protein and the second fusion protein described above are expressed in the cell (see Fig. 3). Moreover, as in the case of the above-described method utilizing the first fusion protein and the second fusion protein, an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and another multimerizable protein. Thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

The "third fusion protein" according to the present invention should comprise the first protein and the multimerizable protein, while the "fourth fusion protein" according to the present invention should comprise the second protein and the multimerizable protein. As long as the formation of an assembly (fluorescent focus) is not inhibited, the multimerizable protein may be fused to either the N-terminus or the C-terminus of the first protein or the second protein. Moreover, the proteins in the "third fusion protein" and the "fourth fusion protein" may be fused to each other directly, or may be fused indirectly via a linker or a spacer protein.

Further, as long as the formation of an assembly (fluorescent focus) is not inhibited, each of the "third fusion protein" and the "fourth fusion protein" may comprise another functional protein. In this case, the other functional protein may be fused to one or both of the N-terminus and the C-terminus of each fusion protein, or may be fused directly or indirectly between the first protein or second protein and the multimerizable protein. The other functional protein is not particularly limited, and selected as appropriate depending on a function desirably provided to the fusion proteins according to the present invention as in the cases of the above-described first and second fusion proteins.

The "fifth fusion protein" according to the present invention should comprise the multimerizable protein and the fluorescent protein. As long as the formation of an assembly (fluorescent focus) is not inhibited, the multimerizable protein may be fused to either the N-terminus or the C-terminus of the fluorescent protein. Moreover, the proteins may be fused to each other directly, or may be fused indirectly via a linker or a spacer protein.

Further, as long as the formation of an assembly (fluorescent focus) is not inhibited, the "fifth fusion protein" may comprise another functional protein. In this case, the other functional protein may be fused to one or both of the N-terminus and the C-terminus of the fusion

protein, or may be fused directly or indirectly between the multimerizable protein and the fluorescent protein. The other functional protein is not particularly limited, and selected as appropriate depending on a function desirably provided to the fusion protein according to the present invention as in the cases of the above-described first and second fusion proteins.

<Method 3 for Detecting Protein-Protein Interaction>

A third embodiment of the method for determining a protein-protein interaction of the present invention is a method comprising the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell

a first labeled protein comprising an affinity tag and the first protein,

a second labeled protein comprising an affinity tag and the second protein, and

a sixth fusion protein comprising a multimerizable protein, a fluorescent protein, and a binding partner having an affinity for any of the affinity tags and bound to the sixth fusion protein;

(2) detecting a fluorescent focus formed by an association among the first labeled protein, the second labeled protein, and the sixth fusion protein comprising the binding partner bound thereto in the cell; and

(3) determining an interaction between the first protein and the second protein according to the detection

of the fluorescent focus.

According to the method, as described in Example 15 later, when the first labeled protein comprising the affinity tag and the first protein, the second labeled protein comprising the affinity tag and the second protein, and the sixth fusion protein comprising the multimerizable protein, the fluorescent protein, and the binding partner having an affinity for any of the affinity tags and bound to the sixth fusion protein are expressed in a cell or introduced into a cell, each affinity tag binds to the binding partner. Thereby, the first labeled protein binds to the sixth fusion protein comprising the binding partner bound thereto while the second labeled protein binds to the sixth fusion protein comprising the binding partner bound thereto, so that proteins respectively corresponding to the first fusion protein and the second fusion protein shown in Figs. 1 and 2 are expressed in the cell (see Fig. 4). Moreover, as in the case of the above-described method utilizing the first fusion protein and the second fusion protein, an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and another multimerizable protein. Thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

The "affinity tag" and the "binding partner"

according to the present invention are not particularly limited, as long as the two have such a relation that they can bind to each other in a cell, and the two compounds are capable of binding to the proteins. For example, it is possible to use combinations of proteins known to interact. As such combinations, FKBP12 and a FRB domain of a mTOR protein and other combinations are suitably used as described in Examples later. In addition, phytochrome B (PHYB) and phytochrome-interacting factor (PIF), cryptochrome 2 (CRY2) and cryptochrome-interacting bHLH1 (CIB1), LOV and ePDZ, Dronpa145K-CAAX and mNeptune, and others which interact with each other depending on light irradiation can also be suitably used (as to combinations of proteins which interact with each other depending on light irradiation, see Nat Rev Mol Cell Biol., 2014, vol. 15, no. 8, pp. 551 to 558). Further, it is also possible to suitably use subunits constituting enzymes such as dihydrofolate reductase, β -galactosidase, β -lactamase, firefly luciferase, and Gaussia luciferase. Moreover, as the "affinity tag" and the "binding partner" according to the present invention, it is also possible to utilize not only known protein-protein interactions as described above, but also to utilize, for example, biotin and avidin, a lectin and a sugar, Protein A(or G) and an immunoglobulin constant region, an antigen and an antibody, and other similar combinations.

The affinity tag may bind to the binding partner autonomously as in antigen-antibody reactions, or the binding may be induced in response to a stimulus. The stimulus for inducing the binding is not particularly limited. Examples thereof include low-molecular-weight compounds such as rapamycin which induces the binding between FKBP12 and a FRB domain of a mTOR protein, and light irradiation which can induce the binding between PHYB and PIF, and so on. In addition, in the case of using the affinity tag and the binding partner whose binding is induced by such a stimulus, it is necessary to apply the stimulus to the cell in the step (1) or between the steps (1) and (2).

In the "first labeled protein" and the "second labeled protein" according to the present invention, the first protein and the second protein should be labeled with the affinity tags, respectively. As long as the formation of an assembly (fluorescent focus) is not inhibited, the affinity tag may be bound to any of the N-terminus, the C-terminus, and a region at a middle portion of the first protein or the second protein.

The "sixth fusion protein" according to the present invention should comprise the multimerizable protein, the fluorescent protein, and the binding partner bound to the sixth fusion protein. As long as the formation of an assembly (fluorescent focus) is not inhibited, the binding

partner may be bound to any of the N-terminus, the C-terminus, and a region at a middle portion of the sixth fusion protein. Moreover, the proteins in the "sixth fusion protein" may be fused to each other directly, or may be fused indirectly via a linker or a spacer protein.

The "binding" of the affinity tag or the binding partner to the corresponding protein is not particularly limited. The binding may be accomplished at a gene level, or may be chemical bonding. The binding at a gene level is so-called "fusion" as described in Examples above or later, and accomplished by adding or inserting a DNA encoding the affinity tag or the binding partner to or into a DNA encoding the first protein, the second protein, or the sixth fusion protein, in accordance with the reading frame. Meanwhile, the chemical bonding may be covalent bonding or may be non-covalent bonding. The "covalent bonding" is not particularly limited. Examples thereof include an amide bond between an amino group and a carboxyl group, an alkylamine bond between an amino group and an alkyl halide group, a disulfide bond between thiols, and a thioester bond between a thiol group and a maleimide group or an alkyl halide group.

Further, all of the first labeled protein, the second labeled protein, and the sixth fusion protein may comprise another functional protein, as long as the formation of an assembly (fluorescent focus) is not inhibited. In this

case, the other functional protein may be fused to one or both of the N-terminus and the C-terminus of each protein, or may be fused directly or indirectly between the constituent proteins (for example, the multimerizable protein and the fluorescent protein). The other functional protein is not particularly limited, and selected as appropriate depending on a function desirably provided to the fusion protein according to the present invention as in the cases of the above-described first and second fusion proteins.

<Method 4 for Detecting Protein-Protein Interaction>

A fourth embodiment of the method for determining a protein-protein interaction of the present invention is a method comprising the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell a seventh fusion protein comprising the first protein and a first partial peptide constituting a fluorescent protein,

an eighth fusion protein comprising the second protein and a first partial peptide constituting a fluorescent protein, and

a ninth fusion protein comprising a multimerizable protein and a second partial peptide capable of reconstituting any of the fluorescent proteins by binding to the first partial peptide;

(2) detecting a fluorescent focus formed by an

association among the seventh fusion protein, the eighth fusion protein, and the ninth fusion protein in the cell; and

(3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

According to the method, when the seventh fusion protein comprising the first protein and the first partial peptide constituting a fluorescent protein, the eighth fusion protein comprising the second protein and the first partial peptide constituting a fluorescent protein, and the ninth fusion protein comprising the multimerizable protein and the second partial peptide capable of reconstituting any of the fluorescent proteins by binding to the first partial peptide are expressed in a cell or introduced into a cell, the fluorescent proteins are expressed in the cell by the reconstitution. Eventually, proteins respectively corresponding to the first fusion protein and the second fusion protein shown in Figs. 1 and 2 are expressed in the cell (see Fig. 5). Moreover, as in the case of the above-described method utilizing the first fusion protein and the second fusion protein, an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and another multimerizable protein. Thereby, the fusion proteins autonomously form an assembly,

and the fluorescent proteins contained in the fusion proteins are detected as fluorescent foci.

The "first partial peptide" and the "second partial peptide" according to the present invention should be capable of reconstituting a fluorescent protein in a cell as these peptides are linked to each other. Note that, in the present invention, "reconstitution" and related terms mean that multiple partial peptides are returned to a state where a protein the partial peptides having originally constituted can function (for example, in the case of a fluorescent protein, a state where fluorescence can be emitted; in a case of a multimerizable protein, a state where a multimer can be formed) by linking the partial peptides as described above.

Additionally, those skilled in the art can design and prepare the partial peptides capable of such reconstitution as appropriate with reference to the descriptions of, for example, Cabantous S et al., Nature Biotechnology, 2005, vol. 23, pp. 102 to 107 and so forth.

Note that, as described in Cabantous S et al., Sci Rep., October 2013, 4; 3: 2854 and so forth, it has been revealed that even when a fluorescent protein is split into three or more parts, the fluorescent protein can be reconstituted. Thus, the first partial peptide and the second partial peptide may be selected from such three or more fragments. Further, in this case, the fragment(s)

not used as the first and second partial peptides need to be expressed in the cell or introduced into the cell in the step (1) or between the steps (1) and (2).

In addition, the first partial peptide may be fused to either the N-terminus or the C-terminus of the first or second protein, while the second partial peptide may be fused to either the N-terminus or the C-terminus of the multimerizable protein. Further, the proteins in all of the seventh fusion protein, the eighth fusion protein, and the ninth fusion protein may be fused to each other directly, or may be fused indirectly via a linker or a spacer protein.

Furthermore, all of the seventh fusion protein, the eighth fusion protein, and the ninth fusion protein may comprise another functional protein, as long as the formation of an assembly (fluorescent focus) is not inhibited. In this case, the other functional protein may be fused to one or both of the N-terminus and the C-terminus of each fusion protein, or may be fused directly or indirectly between the constituent proteins (for example, between the first partial peptide and the first or second protein, between the second partial peptide and the multimerizable protein). The other functional protein is not particularly limited, and selected as appropriate depending on a function desirably provided to the fusion proteins according to the present invention as in the cases of the above-described first and second fusion proteins.

<Method 5 for Detecting Protein-Protein Interaction>

A fifth embodiment of the method for determining a protein-protein interaction of the present invention is a method comprising the following steps (1) to (3):

- 5 (1) expressing in a cell or introducing into a cell
 a tenth fusion protein comprising the first
 protein and a third partial peptide constituting a
 multimerizable protein,
 an eleventh fusion protein comprising the
10 second protein and a third partial peptide constituting
 a multimerizable protein, and
 a twelfth fusion protein comprising a
 fluorescent protein and a fourth partial peptide capable
 of reconstituting any of the multimerizable proteins by
15 binding to the third partial peptide;
 (2) detecting a fluorescent focus formed by an
 association among the tenth fusion protein, the eleventh
 fusion protein, and the twelfth fusion protein in the cell;
 and
20 (3) determining an interaction between the first
 protein and the second protein according to the detection
 of the fluorescent focus.

 According to the method, when the tenth fusion protein
 comprising the first protein and the third partial peptide
25 constituting a multimerizable protein, the eleventh fusion
 protein comprising the second protein and the third partial

peptide constituting a multimerizable protein, and the twelfth fusion protein comprising the fluorescent protein and the fourth partial peptide capable of reconstituting any of the multimerizable proteins by binding to the third partial peptide are expressed in a cell or introduced into a cell, the multimerizable proteins are expressed in the cell by the reconstitution. Eventually, proteins respectively corresponding to the first fusion protein and the second fusion protein shown in Figs. 1 and 2 are expressed in the cell (see Fig. 6). Moreover, as in the case of the above-described method utilizing the first fusion protein and the second fusion protein, an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and another multimerizable protein thus reconstituted. Thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

The "third partial peptide" and the "fourth partial peptide" according to the present invention should be capable of reconstituting a multimerizable protein in a cell as these peptides are linked to each other. Those skilled in the art can design and prepare the partial peptides as appropriate as described above with reference to the descriptions of Ohashi K et al., *Biotechniques.*, 2012, vol. 52, no. 1, pp. 45 to 50 and so forth.

In addition, the third partial peptide may be fused to either the N-terminus or the C-terminus of the first or second protein, while the fourth partial peptide may be fused to either the N-terminus or the C-terminus of the multimerizable protein. Moreover, the proteins in all of the tenth fusion protein, the eleventh fusion protein, and the twelfth fusion protein may be fused to each other directly, or may be fused indirectly via a linker or a spacer protein.

Further, all of the tenth fusion protein, the eleventh fusion protein, and the twelfth fusion protein may comprise another functional protein, as long as the formation of an assembly (fluorescent focus) is not inhibited. In this case, the other functional protein may be fused to one or both of the N-terminus and the C-terminus of each fusion protein, or may be fused directly or indirectly between the constituent proteins (for example, between the third partial peptide and the first or second protein, between the fourth partial peptide and the fluorescent protein). The other functional protein is not particularly limited, and selected as appropriate depending on a function desirably provided to the fusion proteins according to the present invention as in the cases of the above-described first and second fusion proteins.

Hereinabove, the description has been given of the second to fifth embodiments of the method for determining

a protein-protein interaction of the present invention. In these methods, since the first fusion protein and the second fusion protein are expressed in a cell or introduced into a cell separately, this allows a reduction in the molecular weight of tags such as the proteins fused to the first or second protein to be analyzed. Therefore, in the determination and the like of a protein-protein interaction, the functions of proteins to be analyzed, change in the localization, and so forth are hardly affected.

Additionally, proteins to be analyzed can be kept in a cell while being fused to a tag having a low molecular weight in this manner. This makes it possible to set a state where a protein-protein interaction can be determined at any timing by constructing a system in which the fusion protein(s) according to the present invention are reconstituted in response to a stimulus such as the addition of a compound, light irradiation, or the like, as described above.

Moreover, preparing cells constitutively expressing a fusion protein(s) comprising a fluorescent protein in advance reduces the influence of a variation in the fluorescent signal among the cells, even if fusion proteins comprising proteins to be analyzed are transiently expressed in these cells. This makes it also possible to more stably determine a protein-protein interaction.

<Method 6 for Detecting Protein-Protein Interaction>

For a case where analysis targets are three or more proteins, the present invention can also apply the above-described first embodiment to provide a method comprising the following steps (1) to (3) as a sixth
5 embodiment of the method for determining a protein-protein interaction:

(1) expressing in a cell or introducing into a cell
a thirteenth fusion protein comprising the first protein and a multimerizable protein,
10 a fourteenth fusion protein comprising the second protein and a multimerizable protein, and
a fifteenth fusion protein comprising the third protein and a fluorescent protein;

(2) detecting a fluorescent focus formed by an
15 association among the thirteenth fusion protein, the fourteenth fusion protein, and the fifteenth fusion protein in the cell; and

(3) determining an interaction among the first protein, the second protein, and the third protein
20 according to the detection of the fluorescent focus.

According to the method, as described in Examples 16 to 18 later, when the thirteenth fusion protein comprising the first protein and the multimerizable protein, the fourteenth fusion protein comprising the second protein and the multimerizable protein, and the fifteenth fusion
25 protein comprising the third protein and the fluorescent

protein are expressed in a cell or introduced into a cell,
an interaction if any among the first protein, the second
protein, and the third protein induces multimer formation
between the multimerizable protein and another
5 multimerizable protein, as in the cases of the systems shown
in Fig. 1 and so forth. Thereby, the fusion proteins
autonomously form an assembly, and the fluorescent protein
contained in the fusion protein is detected as a fluorescent
focus (see Fig. 7).

10 As the "third protein" according to the present
invention, it is possible to use desired proteins intended
for detection of interaction as in the cases of the
above-described first and second proteins. Moreover, the
first protein, the second protein, and the third protein
15 may be different from each other. Alternatively, two of
them may be the same, or all the three may be the same.

The "interaction among the first protein, the second
protein, and the third protein" according to the present
invention includes not only direct interactions, but also
20 indirect interactions such as an interaction for forming
a complex in which another molecule (protein, nucleic acid,
sugar, lipid, low-molecular-weight compound, or the like)
is interposed among the proteins.

The "thirteenth fusion protein" according to the
25 present invention should be a protein comprising the first
protein and the multimerizable protein. Moreover, the

"fourteenth fusion protein" should be a protein comprising the second protein and the multimerizable protein. In each of these fusion proteins, the multimerizable protein may be fused to either the N-terminus or the C-terminus of the first or second protein, as long as the formation of an assembly (fluorescent focus) is not inhibited. In addition, the proteins may be fused to each other directly, or may be fused indirectly via a linker or a spacer protein.

The "fifteenth fusion protein" according to the present invention should be a protein comprising the third protein and the fluorescent protein. As long as the formation of an assembly (fluorescent focus) is not inhibited, the fluorescent protein may be fused to either the N-terminus or the C-terminus of the third protein. Moreover, the proteins may be fused to each other directly, or may be fused indirectly via a linker or a spacer protein.

<Method 7 for Detecting Protein-Protein Interaction>

The present invention can also provide the following method as a seventh embodiment for determining a protein-protein interaction.

A method for determining an interaction between a first protein and a second protein, wherein

a first multimerizable protein is a different protein from a second multimerizable protein, and

the method comprises the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell

a sixteenth fusion protein comprising the first protein, the first multimerizable protein, and a fluorescent protein, and

a seventeenth fusion protein comprising the second protein and the second multimerizable protein;

(2) detecting a fluorescent focus formed by an association between the sixteenth fusion protein and the seventeenth fusion protein in the cell; and

(3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

The "sixteenth fusion protein" and the "seventeenth fusion protein" according to the present invention are proteins respectively similar to the above-described first fusion protein and second fusion protein, except that the multimerizable proteins (the first multimerizable protein and the second multimerizable protein) are different. Note that the fusion proteins are the same regarding the first protein and the second protein, too, so that these proteins may be different from each other, or may be the same. As described in Examples later, the seventh embodiment for determining a protein-protein interaction of the present invention is more useful when the first protein is different from the second protein.

Hereinabove, preferred embodiments for determining a protein-protein interaction have been described.

Additionally, the present invention can also utilize these methods to provide methods described below.

<Method for Obtaining Temporal Information and the like on Protein-Protein Interaction>

5 As described in Examples such as Examples 2 and 11 to 13 later, the method of the present invention is capable of determining not only a protein-protein interaction taking place, but also a protein-protein interaction ending, on the basis of the present or absence of a fluorescent focus. Moreover, it is also possible to trace occurrence or the like of such a protein-protein interaction over time. Further, the present invention is also capable of determining a protein-protein interaction in any region in a cell without being influenced by localization of a multimerizable protein and a fluorescent protein, and so forth.

10 Thus, the present invention can provide a method, wherein a fluorescent focus is detected to determine a protein-protein interaction taking place or ending, a period until the interaction takes place or ends, or a duration of the interaction.

15 In determining the "protein-protein interaction taking place or ending" in the manner described above, the present invention is also capable of specifying an intracellular region where the protein-protein interaction takes place. Additionally, according to the

present invention, determining the "protein-protein interaction taking place or ending" makes it possible to determine a signal transduction occurring and ending, in which the protein-protein interaction is involved, a period
5 until the signal transduction occurs or ends, and a duration of the signal transduction, and also to specify an intracellular region where the signal transduction occurs. Moreover, the present invention is capable of determining the interaction between the first protein and the second
10 protein (or the interaction among the first protein, the second protein, and the third protein) even if the interaction takes place or ends in response to a particular stimulus. Thus, the present invention can also provide a method for detecting the fluorescent focus according to
15 the present invention, wherein the fluorescent focus is detected to determine the protein-protein interaction taking place or ending in response to a particular stimulus, a period until the interaction takes place or ends, or a duration of the interaction.

20 It is only necessary that the "particular stimulus" according to the present invention be a stimulus capable of directly or indirectly inducing or inhibiting a protein-protein interaction. Moreover, the "particular stimulus" may be a stimulus attributable to an endogenous
25 factor produced in a cell (for example, increase or decrease in intracellular calcium ion concentration, activation or

inactivation of an enzyme), or may be a stimulus applied to a cell from the outside (for example, administration of a ligand (agonist or antagonist) to a receptor in a cell).

Further, the method of the present invention is also capable of determining a particular stimulation starting or ending, a period until the stimulation starts or ends, or a duration of the stimulation, by detecting a fluorescent focus.

Furthermore, the method of the present invention is also capable of determining an increase or decrease of a protein-protein interaction in accordance with a degree of the particular stimulus (for example, in a case where the particular stimulus is a drug, its concentration). Thus, in the case where the particular stimulus is a drug, the 50% effective concentration (EC50) and the 50% inhibitory concentration (IC50) of the drug against a protein-protein interaction can be determined by the present invention.

In addition, the method of the present invention is capable of determining, in a single cell, multiple types of protein-protein interactions, multiple types of protein-protein interactions dependent respectively on particular stimuli, and eventually a signal transduction in which these protein-protein interactions are involved.

<Screening Method for Protein Interacting with Particular Protein>

The present invention makes it possible to determine any protein-protein interaction. Thus, the present invention can provide a method for screening for a protein interacting with a particular protein, wherein one of the first protein and the second protein is the particular protein, while the other is a test protein, and a protein interacting with the particular protein is selected according to the detection of the fluorescent focus according to the present invention. Moreover, for a case of targeting an interaction among three or more proteins, the present invention can provide a method for screening for a protein interacting with particular proteins, wherein two proteins of the first to third proteins are the particular proteins, while the remaining one is a test protein, and a protein interacting with the particular proteins is selected according to the detection of the fluorescent focus according to the present invention.

The "test protein" according to the present invention is not particularly limited. Protein groups encoded by cDNA libraries can be suitably used from the viewpoint that it is possible to comprehensively and efficiently select proteins interacting with particular proteins.

<Method for Identifying Amino Acid Residue Involved in Protein-Protein Interaction>

As described in Examples such as Example 2 later, the present invention is also capable of quantitatively

analyzing a strength of a protein-protein interaction by utilizing a fluorescence intensity of a fluorescent focus. Thus, the present invention can provide a method for identifying any one of an amino acid residue in the first protein and an amino acid residue in the second protein (or amino acid residues in the first to third proteins), which are involved in the protein-protein interaction, wherein in a case where a protein in which a mutation is introduced is used as any one of the first protein and the second protein (or a mutation is introduced in any one of the first to third proteins), if an intensity of the fluorescent focus is reduced in comparison with a case of using a protein in which no mutation is introduced, the amino acid residue in which the mutation is introduced is determined to be involved in the interaction.

The "fluorescence intensity of the fluorescent focus" according to the present invention includes not only a fluorescence intensity of a single fluorescent focus, but also a total fluorescence intensity of fluorescent foci present in a certain region (for example, in one cell, in one field of view and in one fluorescence image observed with a fluorescence microscope).

Those skilled in the art can prepare the "protein obtained by introducing a mutation into the first protein and the like" by selecting known techniques as appropriate. An example of such known techniques includes site-directed

mutagenesis.

<Screening Method for Substance Capable of Modulating Protein-Protein Interaction>

As described above, in the method of the present invention, a strength of a protein-protein interaction can be grasped on the basis of the fluorescence intensity of the fluorescent focus. Thus, the present invention can provide a method comprising the steps of:

expressing in a cell or introducing into a cell a first fusion protein and a second fusion protein in presence of a test compound;

detecting a fluorescent focus formed by an association between the first fusion protein and the second fusion protein in the cell; and

selecting the test compound as a substance inducing the interaction if an intensity of the fluorescent focus is higher than an intensity of a fluorescent focus formed in absence of the test compound, or selecting the test compound as a substance suppressing the interaction if the intensity of the fluorescent focus is lower than the intensity of the fluorescent focus formed in the absence of the test compound.

The test compound used in the screening method of the present invention is not particularly limited. Examples thereof include an expression product from a gene library, a synthetic low-molecular-weight compound

library, a peptide library, an antibody, a substance released by a bacterium, a liquid extract and a culture supernatant of cells (microorganisms, plant cells, animal cells), a purified or partially purified polypeptide, an
5 extract derived from a marine organism, plant, or animal, soil, and a random phage peptide display library.

Moreover, examples of a state of being in the presence of the test compound include a state where the cell according to the present invention is in contact with the test compound
10 by addition or the like of the test compound to a medium, and a state where the test compound is introduced in the cell according to the present invention.

Note that, the screening method of the present invention is not limited to the above-described embodiment,
15 as long as the method is based on the intensity of the fluorescent focus. Thus, the method can also encompass embodiments described below as other embodiments.

A method comprising the steps of:

expressing in a cell or introducing into a cell a
20 third fusion protein, a fourth fusion protein, and a fifth fusion protein in presence of a test compound;

detecting a fluorescent focus formed by an association among the third fusion protein, the fourth fusion protein, and the fifth fusion protein in the cell;
25 and

selecting the test compound as a substance inducing

the interaction if an intensity of the fluorescent focus is higher than an intensity of a fluorescent focus formed in absence of the test compound, or selecting the test compound as a substance suppressing the interaction if the intensity of the fluorescent focus is lower than the intensity of the fluorescent focus formed in the absence of the test compound.

A method comprising the steps of:

expressing in a cell or introducing into a cell a first labeled protein, a second labeled protein, and a sixth fusion protein comprising a binding partner bound thereto in presence of a test compound;

detecting a fluorescent focus formed by an association among the first labeled protein, the second labeled protein, and the sixth fusion protein comprising the binding partner bound thereto in the cell; and

selecting the test compound as a substance inducing the interaction if an intensity of the fluorescent focus is higher than an intensity of a fluorescent focus formed in absence of the test compound, or selecting the test compound as a substance suppressing the interaction if the intensity of the fluorescent focus is lower than the intensity of the fluorescent focus formed in the absence of the test compound.

A method comprising the steps of:

expressing in a cell or introducing into a cell a

seventh fusion protein, an eighth fusion protein, and a ninth fusion protein in presence of a test compound;

detecting a fluorescent focus formed by an association among the seventh fusion protein, the eighth fusion protein, and the ninth fusion protein in the cell; and

selecting the test compound as a substance inducing the interaction if an intensity of the fluorescent focus is higher than an intensity of a fluorescent focus formed in absence of the test compound, or selecting the test compound as a substance suppressing the interaction if the intensity of the fluorescent focus is lower than the intensity of the fluorescent focus formed in the absence of the test compound.

A method comprising the steps of:

expressing in a cell or introducing into a cell a tenth fusion protein, an eleventh fusion protein, and a twelfth fusion protein in presence of a test compound;

detecting a fluorescent focus formed by an association among the tenth fusion protein, the eleventh fusion protein, and the twelfth fusion protein in the cell; and

selecting the test compound as a substance inducing the interaction if an intensity of the fluorescent focus is higher than an intensity of a fluorescent focus formed in absence of the test compound, or selecting the test

compound as a substance suppressing the interaction if the intensity of the fluorescent focus is lower than the intensity of the fluorescent focus formed in the absence of the test compound.

5 A method comprising the steps of:

 expressing in a cell or introducing into a cell a thirteenth fusion protein, a fourteenth fusion protein, and a fifteenth fusion protein in presence of a test compound;

10 detecting a fluorescent focus formed by an association among the thirteenth fusion protein, the fourteenth fusion protein, and the fifteenth fusion protein in the cell; and

 selecting the test compound as a substance inducing
15 the interaction if an intensity of the fluorescent focus is higher than an intensity of a fluorescent focus formed in absence of the test compound, or selecting the test compound as a substance suppressing the interaction if the
20 intensity of the fluorescent focus is lower than the intensity of the fluorescent focus formed in the absence of the test compound.

 A method comprising the steps of:

 expressing in a cell or introducing into a cell a
 sixteenth fusion protein and a seventeenth fusion protein
25 in presence of a test compound;

 detecting a fluorescent focus formed by an

association between the sixteenth fusion protein and the seventeenth fusion protein in the cell; and

selecting the test compound as a substance inducing the interaction if an intensity of the fluorescent focus is higher than an intensity of a fluorescent focus formed in absence of the test compound, or selecting the test compound as a substance suppressing the interaction if the intensity of the fluorescent focus is lower than the intensity of the fluorescent focus formed in the absence of the test compound.

<Kit for Use in Methods of the present invention>

The present invention can provide a kit for use in the above-described methods. The kit of the present invention is a kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

(a) a vector comprising a DNA encoding a multimerizable protein, a DNA encoding a fluorescent protein, and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the multimerizable protein, the fluorescent protein, and a certain protein when a DNA encoding the certain protein is inserted in the cloning site;

(b) a vector encoding the first fusion protein;

(c) a vector encoding the second fusion protein;

(d) a transformed cell comprising a vector encoding

the first fusion protein;

(e) a transformed cell comprising a vector encoding the second fusion protein;

(f) a transformed cell comprising a vector encoding the first fusion protein and a vector encoding the second fusion protein;

(g) the first fusion protein; and

(h) the second fusion protein.

It is only necessary that the vectors according to the present invention comprise a regulatory sequence necessary for an expression (transcription and translation) of the inserted DNA in the above-described cell. Examples of such a regulatory sequence include a promoter, an enhancer, a silencer, a terminator, a poly(A) tail, and a ribosomal binding site (Shine-Dalgarno (SD) sequence). Further, the vectors according to the present invention may comprise a selection marker (such as a drug resistance gene), and a reporter gene (such as a luciferase gene, a β -galactosidase gene, a chloramphenicol acetyltransferase (CAT) gene). Moreover, examples of a type of such vectors according to the present invention include a plasmid vector, an episomal vector, and a viral vector.

The proteins encoded by the vectors according to the present invention are the above-described fusion proteins and the like. From the viewpoint of further improving the

efficiency of expressing such proteins, a DNA having codons optimized in accordance with the species of a cell expressing the proteins (for example, humanized-codon DNA) may be inserted in the vectors according to the present invention.

Additionally, those skilled in the art can prepare the vectors according to the present invention as appropriate by utilizing known techniques such as DNA chemical synthesis methods and genetic recombination techniques as described in Examples later.

Examples of the "cloning site allowing an insertion of a DNA encoding a certain protein" in (a) above include a multiple cloning site containing one or more restriction-enzyme recognition sites, a TA cloning site, and a GATEWAY(registered trademark) cloning site.

Those skilled in the art can synthesize and prepare the fusion proteins according to the present invention based on the nucleotide sequences of these fusion protein by genetic techniques using a cell-free protein synthesis system (for example, reticulocyte extract, wheat germ extract), *Escherichia coli*, animal cells, insect cells, plant cells, or the like. Alternatively, the fusion proteins according to the present invention can be chemically synthesized and prepared based on the amino acid sequences by using a commercially-available polypeptide synthesizer.

To preparations of the vectors or the fusion proteins according to the present invention, other components such as a buffer, a stabilizer, a preservative, and an antiseptic may be added.

5 The transformed cell according to the present invention can be prepared, as described above, by introducing the vectors according to the present invention into a cell. Moreover, to a preparation of the transformed cell according to the present invention, a medium necessary
10 for storage and culturing of the cell and other components such as a stabilizer, a preservative, and an antiseptic may be added or attached.

 The "instruction" according to the present invention is an instruction for utilizing the vectors or the
15 transformed cell in the methods of the present invention. The instruction may comprise, for example, experimental techniques and experimental conditions for the methods of the present invention, and information on the preparations of the present invention (for example, information such
20 as a vector map indicating the base sequence, cloning site, and the like of the vectors, information on the origin and nature of the transformed cell, culture conditions of the cell, and so forth).

 Note that the kit of the present invention is not
25 limited to the above-described embodiment and can also encompass embodiments as follows in accordance with the

above-described embodiments for determining a protein-protein interaction.

A kit for use in the second method for determining a protein-protein interaction, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

(a) a vector comprising a DNA encoding the multimerizable protein and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the multimerizable protein and a certain protein when a DNA encoding the certain protein is inserted in the cloning site;

(b) a vector encoding the third fusion protein;

(c) a vector encoding the fourth fusion protein;

(d) a vector encoding the fifth fusion protein;

(e) a transformed cell comprising a vector encoding the fifth fusion protein;

(f) the third fusion protein;

(g) the fourth fusion protein; and

(h) the fifth fusion protein.

A kit for use in the third method for determining a protein-protein interaction, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

(a) a vector comprising a DNA encoding the affinity tag and a cloning site, wherein the vector is capable of

expressing a fusion protein comprising the affinity tag when a DNA encoding a certain protein is inserted in the cloning site;

(b) a vector encoding the first labeled protein;

5 (c) a vector encoding the second labeled protein;

(d) a vector encoding the sixth fusion protein comprising the binding partner bound thereto;

10 (e) a transformed cell comprising a vector encoding the sixth fusion protein comprising the binding partner bound thereto;

(f) the first labeled protein;

(g) the second labeled protein; and

(h) the sixth fusion protein comprising the binding partner bound thereto.

15 A kit for use in the fourth method for determining a protein-protein interaction, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

20 (a) a vector comprising a DNA encoding the first partial peptide and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the first partial peptide when a DNA encoding a certain protein is inserted in the cloning site;

(b) a vector encoding the seventh fusion protein;

25 (c) a vector encoding the eighth fusion protein;

(d) a vector encoding the ninth fusion protein;

(e) a transformed cell comprising a vector encoding the ninth fusion protein;

(f) the seventh fusion protein;

(g) the eighth fusion protein; and

5 (h) the ninth fusion protein.

A kit for use in the fifth method for determining a protein-protein interaction, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

10 (a) a vector comprising a DNA encoding the third partial peptide and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the third partial peptide when a DNA encoding a certain protein is inserted in the cloning site;

15 (b) a vector encoding the tenth fusion protein;

(c) a vector encoding the eleventh fusion protein;

(d) a vector encoding the twelfth fusion protein;

(e) a transformed cell comprising a vector encoding the twelfth fusion protein;

20 (f) the tenth fusion protein;

(g) the eleventh fusion protein; and

(h) the twelfth fusion protein.

A kit for use in the sixth method for determining a protein-protein interaction, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

25

(a) a vector comprising a DNA encoding the multimerizable protein and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the multimerizable protein when a DNA encoding a certain protein is inserted in the cloning site;

(b) a vector comprising a DNA encoding the fluorescent protein and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the fluorescent protein when a DNA encoding a certain protein is inserted in the cloning site;

(c) a vector encoding the thirteenth fusion protein;

(d) a vector encoding the fourteenth fusion protein;

(e) a vector encoding the fifteenth fusion protein;

(f) the thirteenth fusion protein;

(g) the fourteenth fusion protein; and

(h) the fifteenth fusion protein.

A kit for use in the seventh method for determining a protein-protein interaction, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (f):

(a) a vector comprising a DNA encoding the first multimerizable protein, a DNA encoding the fluorescent protein, and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the first multimerizable protein, the fluorescent protein, and a certain protein when a DNA encoding the certain protein

is inserted in the cloning site;

(b) a vector comprising a DNA encoding the second multimerizable protein, a DNA encoding the fluorescent protein, and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the second multimerizable protein, the fluorescent protein, and a certain protein when a DNA encoding the certain protein is inserted in the cloning site;

(c) a vector encoding the sixteenth fusion protein;

(d) a vector encoding the seventeenth fusion protein;

(e) the sixteenth fusion protein; and

(f) the seventeenth fusion protein.

[Examples]

Hereinafter, the present invention will be described more specifically based on Examples and Comparative Example. However, the present invention is not limited to the following Examples.

(Example 1)

The present inventors came up with an idea of utilizing, in determining an interaction between two proteins (a first protein and a second protein): a first fusion protein comprising the first protein, a multimerizable protein, and a fluorescent protein; and a second fusion protein comprising the second protein and a multimerizable protein (see Fig. 1). To be more specific, the inventors came up with a construction of a system as follows. When these

two fusion proteins are expressed in a cell, an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and another multimerizable protein. Thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

Then, the effectiveness of this system was actually examined by selecting a PB1 domain of a p62 protein as the multimerizable protein and selecting monomeric Azami Green 1 (mAG1) as the fluorescent protein. Further, this examination was conducted on an embodiment in which both of the first fusion protein and the second fusion protein comprised mAG1 in order to facilitate detection of a fluorescent focus (see Fig. 2). Additionally, a humanized-codon DNA encoding the fluorescent protein (humanized-codon monomeric Azami Green 1, hmAG1) was used for the expression of mAG1.

Moreover, in this examination, a FKBP12 protein mutant was used which had been known to form a homodimer by an addition of a drug (AP1903 or AP20187). Specifically, in the examination this time, the first protein was the same protein as the second protein, and the first fusion protein was the same protein as the second fusion protein. Note that, as to the homodimer formation of the FKBP12 protein mutant controlled by AP1903 or the like, see TIM

CLACKSON et al., Proc. Natl. Acad. Sci. USA, 1998, vol. 95, pp. 10437 to 10442. Hereinafter, the materials and methods for constructing this detection system will be described.

5 <Preparation of pPB1-hmAG1-MCLinker and phmAG1-PB1-MCLinker>

In this detection system, prepared were expression vectors (pPB1-hmAG1-MCLinker and phmAG1-PB1-MCLinker) respectively encoding as the fusion proteins composed of
10 the multimerizable protein and the fluorescent protein: a protein tag (PB1-mAG1) composed of mAG1 fused to the C-terminus of the PB1 domain of the p62 protein; and a protein tag (mAG1-PB1) composed of mAG1 fused to the N-terminus of the PB1 domain of the p62 protein. Specifically, a DNA
15 having the nucleotide sequence of SEQ ID NO: 27 was artificially synthesized which contained restriction enzyme sites at either end of a DNA having a nucleotide sequence encoding the PB1-mAG1 (the DNA encoding a protein having the amino acid sequence of SEQ ID NO: 28). Moreover,
20 a DNA having the nucleotide sequence of SEQ ID NO: 29 was artificially synthesized which contained restriction enzyme sites at either end of a DNA having a nucleotide sequence encoding the mAG1-PB1 (the DNA encoding a protein having the amino acid sequence of SEQ ID NO: 30). Then,
25 the synthetic DNAs obtained in this manner were cleaved with NheI and AgeI, and inserted into phmAG1-MCLinker

(manufactured by limited company Amalgaam Co., Ltd.) having been treated with a combination of the same restriction enzymes. Thus, pPB1-hmAG1-MCLinker and phmAG1-PB1-MCLinker were constructed.

5 <Preparation of pPB1-hmAG1-FKBP12 Mutant>

Next, the pPB1-hmAG1-MCLinker prepared as described above was used to prepare a vector for expressing the fusion protein according to the present invention (FKBP12 mutant fused to the PB1-mAG1). Specifically, a DNA having the
10 nucleotide sequence of SEQ ID NO: 31 was artificially synthesized which contained restriction enzyme sites at either end of a DNA having a nucleotide sequence encoding the FKBP12 mutant (the DNA encoding a protein having the amino acid sequence of SEQ ID NO: 32). Then, the obtained
15 synthetic DNA was cleaved with EcoRI and XhoI, and inserted into the pPB1-hmAG1-MCLinker having been treated with a combination of the same restriction enzymes. Thus, a pPB1-hmAG1-FKBP12 mutant was constructed.

<Cell Culturing and Transfection>

20 Next, the pPB1-hmAG1-FKBP12 mutant prepared as described above was introduced into HEK293 cells, and the fusion protein according to the present invention was expressed in the cells. Specifically, first, HEK293 cells were cultured in DMEM (high glucose, manufactured by SIGMA
25 ALDRICH CO.) supplemented with 10% FBS (manufactured by Equitech-Bio Inc.) and 1% penicillin-streptomycin

(manufactured by Life Technologies Corporation). Then, on the day before the transfection, the HEK293 cells cultured in this manner were seeded onto an 8-well chamber slide (manufactured by Nunc A/S), and cultured in 200 μ L of the culture solution per well. Subsequently, 100 ng of the pPB1-hmAG1-FKBP12 mutant was diluted with 10 μ L of OptiMEM (manufactured by Life Technologies Corporation), and 0.4 μ L of Turbofect Transfection Reagent (manufactured by Thermo Fisher Scientific Inc.) was added thereto and then stirred. Thereafter, the resultant was further mixed with 100 μ L of the culture solution, then added to the HEK293 cells, and cultured for 20 hours.

<Observation of Cells>

After the transfection treatment, the HEK293 cells were observed using an IX-71 inverted fluorescence microscope (manufactured by Olympus Corporation, the magnification of the objective lens: 20 $\times$), a U-MGFPHQ filter (manufactured by Olympus Corporation), and an ORCA-ER digital camera (manufactured by Hamamatsu Photonics K. K.). The homodimer formation of the FKBP12 mutant was induced by adding 500 nM B/B Homodimerizer (AP20187, manufactured by Takara Bio Inc.) to the medium. In addition, after the drug was added, images were obtained over time. Fig. 9 shows the obtained result.

As apparent from the result shown in Fig. 9, no fluorescent focus was detected before the drug was added,

and fluorescent signals were dispersedly observed in the cytoplasms (see the image on the left in Fig. 9). Then, 38 minutes after the drug addition, fluorescent foci were observed in multiple cells (see the image in the middle of Fig. 9). As the observation was further continued, many cells which exhibited distinctive fluorescent foci were observed 76 minutes after the addition (see the image on the right in Fig. 9). Thus, the result of this examination revealed that the use of the system utilizing the protein tag composed of the multimerizable protein and the fluorescent protein shown in Fig. 1 or 2 enabled determination of homodimer formation, eventually determination of a protein-protein interaction. Further, as shown in Fig. 9, it was also revealed that the present invention made it possible to determine the homodimer formation of the FKBP12 mutant in the cytoplasm, that is, an intracellular environment unique to the homodimer formation.

(Comparative Example 1)

The present inventors have invented another system for determining a protein-protein interaction, that is, a system in which a first fusion protein comprising a first protein and an association-inducing protein, and a second fusion protein comprising a second protein and a fluorescent multimerizable protein are expressed in a cell, and a fluorescent focus formed by an association between

the first fusion protein and the second fusion protein is detected to determine an interaction between the first protein and the second protein (see PTL 4).

Hence, using this system, the homodimer formation of the FKBP12 protein mutant described above was determined and compared with the result described in Example 1 above to evaluate the effectiveness of the present invention. Hereinafter, the materials and methods for constructing the detection system described in PTL 4 will be described.

<Preparation of pPB1-FKBP12 Mutant and phAG-FKBP12 Mutant>

In the detection system described in PTL 4, the PB1 domain of the p62 protein was selected as the association-inducing protein. Moreover, AG was selected as the fluorescent multimerizable protein, and a DNA encoding humanized-codon AG (hAG) was used.

Further, an expression vector (pPB1-FKBP12 mutant) encoding a fusion protein composed of the FKBP12 mutant fused to the C-terminus of the PB1 domain of the p62 protein was prepared as follows. Specifically, a DNA having the nucleotide sequence of SEQ ID NO: 31 was artificially synthesized which contained restriction enzyme sites at either end of a DNA having a nucleotide sequence encoding the FKBP12 mutant (the DNA encoding a protein having the amino acid sequence of SEQ ID NO: 32). Then, the obtained synthetic DNA was cleaved with EcoRI and XhoI, and inserted

into pAsh-MCLinker (manufactured by MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.) having been treated with a combination of the same restriction enzymes. Thus, a pPB1-FKBP12 mutant was constructed.

5 Moreover, an expression vector (phAG-FKBP12 mutant) encoding a fusion protein composed of the FKBP12 mutant fused to the C-terminus of the AG protein was constructed by inserting the synthetic DNA encoding the FKBP12 mutant into phAG-MCLinker (manufactured by MEDICAL & BIOLOGICAL
10 LABORATORIES CO., LTD.) according to the same method as that described above.

 Further, 100 ng of each of the expression vectors thus constructed was mixed, that is, 200 ng of these were mixed in total, and fluorescent foci in cells before and
15 after the drug addition were detected according to the same methods as <Cell Culturing and Transfection> and <Observation of Cells> described in Example 1. Fig. 10 shows the obtained result.

 As shown in Fig. 10, it was confirmed that the method
20 described in PTL 4 was also capable of detecting a fluorescent focus indicating the homodimer formation of the FKBP12 protein mutant. However, in this method, even at the time point when 66 minutes elapsed after the drug addition, fluorescent foci were formed only in a few cells.
25 This revealed that the method of the present invention was superior to the method described in W02013/084950 in at

least the efficiency of the homodimer formation determination.

(Example 2)

Next, an examination was conducted on whether or not the method of the present invention was capable of determining reversible homodimer formation, in other words, whether or not fluorescent foci having been formed were extinguished as the homodimer dissociated.

It has been known that a homodimer of the FKBP12 protein mutant induced and formed by the above-described B/B Homodimerizer (AP20187) is dissociated by B/B Washout Ligand (manufactured by Takara Bio Inc.). Hence, the examination was conducted using materials and methods described below.

<Preparation of Cells Constitutively Expressing PB1-hmAG1-FKBP12 Mutant>

The pPB1-hmAG1-FKBP12 mutant was introduced into HEK293 cells, and cells constitutively expressing the fusion protein according to the present invention (PB1-hmAG1-FKBP12 mutant) were prepared. Specifically, on the day before the introduction of the expression vector, HEK293 cells were seeded onto a 35-mm dish (manufactured by BD Falcon), and cultured in 1.5 mL of the culture solution. Then, 1 µg of the pPB1-hmAG1-FKBP12 mutant was diluted with 100 µL of OptiMEM (manufactured by Life Technologies Corporation), and 10 µL of PolyFect (registered trademark)

Transfection Reagent (manufactured by QIAGEN N.V.) was added thereto and then stirred. Subsequently, the resultant was further mixed with 600 μ L of the culture solution, then added to the HEK293 cells, and cultured for 20 hours. Thereafter, G418 was added thereto at a concentration of 500 μ g/mL, and further cultured for one week. After that, viable cells were detached using a trypsin solution, diluted with a culture solution containing G418 (500 μ g/ml), and then cultured in a 96-well plate at 0.5 cells/well. Subsequently, cells which formed single colonies were cultured to expand.

<Observation of Cells>

The cells constitutively expressing the PB1-hmAG1-FKBP12 mutant established above were observed by the same method as that described in Example 1. Moreover, after no fluorescent focus was confirmed in the steady state, 500 nM B/B Homodimerizer was added to the culture solution, and the cells were photographed over time for 150 minutes. Note that, in this observation, the formation of fluorescent foci indicating the homodimer formation of the FKBP12 mutant were observed as in Example 1 above. Next, the cells were washed by replacing the culture solution containing the B/B Homodimerizer with a culture solution normally used for culturing. Then, 500 nM B/B Washout Ligand was added, and images were obtained over time for 100 minutes. Fig. 11 shows the obtained result.

<Image Analysis>

The images captured over time by the above method were analyzed using spot detection (spot detector) of an icy image analysis program (http://icy.bioimageanalysis.org/). Moreover, from the images, a graph was created in which the X axis represents the operation time (minutes), and the Y axis represents a numerical value of a total fluorescence intensity in regions of the fluorescent foci within boxes drawn around the regions by lines. Note that the analysis with the spot detector was performed by setting parameters (processing paths) as follows.

Pre Processing: null,

Detector: UDWTWaveletDetector, Detect bright spot over dark background,

Scale enabled: scale 3, Threshold: 50.

Fig. 12 shows the obtained result.

As apparent from the result shown in Fig. 11, similarly to the result described in Example 1, no fluorescent focus was detected before the drug addition, and fluorescent signals were dispersedly observed in the cytoplasms (see the image on the left in Fig. 11). Then, 90 minutes after the B/B Homodimerizer addition, distinctive fluorescent foci were observed in multiple cells (see the image in the middle in Fig. 11).

Moreover, it was revealed that as a result of adding

the B/B Washout Ligand, most of the fluorescent foci were extinguished (see the image on the right in Fig. 11). Further, it can be seen from the graph shown in Fig. 12, the fluorescent foci were formed and extinguished in a stepwise manner. These revealed that the present invention was capable of grasping, on the basis of the formation and extinction of the fluorescent foci, a change over time in the protein-protein interaction attributable to the drugs added. Furthermore, as shown in Fig. 11, it was also revealed that the present invention made it possible to determine the homodimer formation and disruption of the FKBP12 mutant in the cytoplasm, that is, an intracellular environment unique to the homodimer formation and disruption. Additionally, when the formation of the fluorescent foci started to be observed under the microscope observation immediately after the B/B Homodimerizer addition, the value of a fluorescence intensity was 1.9 times as large as that before the addition. This revealed that it was possible to determine formation of a fluorescent focus indicating the protein-protein interaction when a detected value was at least twice as large as a fluorescence intensity in a negative control (for example, cells before a protein-protein interaction took place).

As described above, it was also revealed that the present invention was capable of determining a reversible

change in a protein-protein interaction on the basis of formation and extinction of fluorescent foci to be detected. Moreover, since the fluorescent foci increased or decreased in a stepwise manner as time elapsed, this revealed that the present invention was also capable of quantitatively analyzing a protein-protein interaction. Further, the cells constitutively expressing the fusion protein according to the present invention were successfully prepared by introducing the fusion protein into the cells, thereby confirming that the protein tag contained in the fusion protein exhibited no cytotoxicity and the like.

(Example 3)

Whether or not the present invention was also capable of determining homomultimer formation was examined using materials and methods described below. Note that this examination was conducted by targeting homomultimer formation of a p53 protein (as to the homomultimer formation, see Yoko Itahana et al., J Biol Chem., 2009, vol. 284, no. 8, pp. 5158 to 5164).

<Preparation of phmAG1-PB1-p53 and phmAG1-p53>

A nucleotide sequence was designed in which a BamHI recognition sequence was added to the 5' end of a nucleotide sequence encoding p53 (the nucleotide sequence encoding a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_000537) while the stop codon and a NotI recognition sequence were the 3' end. Then, a DNA having

the resulting nucleotide sequence was artificially synthesized, cleaved with BamHI and NotI, and subsequently inserted into phmAG1-PB1-MCLinker and phmAG1-MCLinker (manufactured by limited company Amalgaam Co., Ltd.) having been treated with a combination of the same restriction enzymes. Thus, phmAG1-PB1-p53 and phmAG1-p53 were prepared.

<Preparation of pp53-hmAG1-PB1 and pp53-hmAG1>

In addition to the expression vectors encoding the fusion protein having the protein tag at the N-terminus of the p53 protein, expression vectors encoding a fusion protein having the protein tag at the C-terminus were also prepared by a method described below.

Specifically, the stop codon was removed from the synthetic DNA encoding p53. The resulting synthetic DNA was cleaved with BamHI and NotI, and then inserted into phmAG1-PB1-MNLinker and phmAG1-MNLinker (manufactured by limited company Amalgaam Co., Ltd.) having been treated with a combination of the same restriction enzymes. Thus, pp53-hmAG1-PB1 and pp53-hmAG1 were prepared.

<Cell Culturing and Transfection>

U2OS cells were selected as cells into which the expression vectors prepared above were introduced. The cells were first cultured in DMEM (high glucose, manufactured by SIGMA ALDRICH CO.) supplemented with 10% FBS (manufactured by Equitech-Bio Inc.) and 1%

penicillin-streptomycin (manufactured by Life Technologies Corporation). Then, on the day before the introduction of the expression vectors, these cells were seeded onto a 35-mm dish (manufactured by BD Falcon), and cultured in 1.5 ml of the culture solution. Subsequently, 1 µg of the expression vector (phmAG1-PB1-p53, phmAG1-p53, pp53-hmAG1-PB1, or pp53-hmAG1) was diluted with 100 µL of OptiMEM (manufactured by Life Technologies Corporation), and 10 µL of PolyFect(registered trademark) Transfection Reagent (manufactured by QIAGEN N.V.) was added thereto and then stirred. The resultant was further mixed with 600 µL of the culture solution. Thereafter, each mixture solution of the expression vectors was added to the cultured cells, and further cultured for 20 hours.

<Observation of Cells>

The cells transfected above were observed in a buffer at pH of 7.4 containing 20 mM HEPES (manufactured by Dojindo Laboratories) and Hanks' Balanced Salt Solutions (manufactured by Life Technologies Corporation) having been diluted with Hoechst 33342 (manufactured by Dojindo Laboratories) to 1 µg/ml. Figs. 13 and 14 show the obtained result. Note that micrographs shown in Figs. 13 and 14 are results of the observation with a 40× magnification of the objective lens.

As apparent from the result shown in Fig. 13, distinctive fluorescent focus formations were observed in

cells expressing the mAG1-PB1-p53 fusion protein and cells expressing the p53-mAG1-PB1 fusion protein, that is, cells expressing the fusion proteins according to the present invention.

5 On the other hand, as shown in Fig. 14, no fluorescent focus was observed in cells expressing the mAG1-p53 fusion protein and cells expressing the p53-mAG1 fusion protein, and fluorescent signals were observed in a dispersed state.

10 Thus, it was revealed that the present invention was capable of determining not only the homodimer formation but also the homomultimer formation. Further, as shown in Fig. 13, it was also revealed that the present invention was capable of uniformly determining the p53 homomultimer formation in the nuclei and the cytoplasms in the cells
15 by utilizing uniform fluorescent focus formation. Moreover, it was also confirmed that the protein tag according to the present invention functioned even when fused to any of the N-terminus and the C-terminus of the analysis-target protein.

20 (Example 4)

 It was confirmed that the system shown in Fig. 1 was effective in determining not only the above-described homomultimer formation but also heteromultimer formation. Note that the detection target in this examination was an
25 interaction between a p53 protein and an MDM2 protein, and that Nutlin-3 known as an inhibitor against the interaction

was also used in this confirmation experiment (see Vassilev
LT et al., Science, February 6, 2004, vol. 303, no. 5659,
pp. 844 to 848). Further, in this confirmation experiment,
a PB1 domain of a p62 protein was used as the multimerizable
protein, and a mAG1 protein was used as the fluorescent
protein.

<Preparation of pPB1-p53(70)>

To express a partial peptide of p53 whose N-terminus
was fused to the PB1 domain of the p62 protein (PB1-p53(70))
as a second fusion protein in cells, a pPB1-p53(70) vector
was prepared as follows.

In preparing pPB1-p53(70), first, a nucleotide
sequence was designed in which a BamHI recognition sequence
was added to the 5' end of a DNA encoding a portion of p53
(a region (p53(70)) having the 1st to 70th amino acids of
the p53 protein, the region had the amino acid sequence
of SEQ ID NO: 34) while the stop codon and a NotI recognition
sequence were added to the 3' end of the DNA. Then, a DNA
having the nucleotide sequence was artificially
synthesized, cleaved with BamHI and NotI, and subsequently
inserted into pAsh-MCLinker having been treated with a
combination of the same restriction enzymes. Thus,
pPB1-p53(70) was prepared.

<Preparation of pPB1-hmAG1-MDM2>

To express MDM2 whose N-terminus was fused to the
PB1 domain of the p62 protein and mAG1 (PB1-mAG1-MDM2) as

a first fusion protein in cells, a pPB1-hmAG1-MDM2 vector was prepared as follows.

pPB1-MDM2 was cleaved with BamHI and NotI, and then inserted into pPB1-hmAG1-MCLinker having been treated with
5 a combination of the same restriction enzymes. Thus, pPB1-hmAG1-MDM2 was prepared.

Note that the pPB1-MDM2 is a plasmid vector for expressing, as a second fusion protein, an MDM2 protein whose N-terminus was fused to the PB1 domain of the p62
10 protein (PB1-MDM2). As to the preparation method and so forth of the pPB1-MDM2, see Example 13 described later.

<Preparation of pp53(70)-hmAG1-PB1>

To express a partial peptide of p53 whose C-terminus was fused to the PB1 domain of the p62 protein and mAG1
15 (p53(70)-mAG1-PB1)) as a first fusion protein in cells, a pp53(70)-hmAG1-PB1 vector was prepared as follows.

In preparing pp53(70)-hmAG1-PB1, first, a nucleotide sequence was designed in which a BamHI recognition sequence and a NotI recognition sequence were respectively added
20 to the 5' end and the 3' end of the DNA encoding p53(70). Then, a DNA having the nucleotide sequence was artificially synthesized, cleaved with BamHI and NotI, and subsequently inserted into phmAG1-PB1-MNLinker having been treated with a combination of the same restriction enzymes. Thus,
25 pp53(70)-hmAG1-PB1 was prepared.

<Cell Culturing and Transfection>

To express the fusion proteins in a combination according to the following (A) or (B) in cells, the vectors encoding these fusion proteins were introduced into HEK293 cells by a method described below:

- 5 (A) PB1-p53(70) and PB1-mAG1-MDM2; and
(B) p53(70)-mAG1-PB1 and PB1-MDM2.

First, HEK293 cells were cultured in a culture solution (DMEM high glucose (manufactured by SIGMA ALDRICH CO.), 10% FBS (manufactured by Equitech-Bio Inc.), 1%
10 penicillin-streptomycin (manufactured by Life Technologies Corporation)). On the day before the transfection, the cells were seeded onto an 8-well chamber slide (manufactured by Nunc A/S), and cultured in 200 μ L of the culture solution per well. Then, a DNA solution
15 in which 100 ng of each of the vectors were mixed was diluted with 10 μ L of OptiMEM (manufactured by Life Technologies Corporation), and 0.8 μ L of Eugene HD (manufactured by Promega Corporation) was added thereto and then stirred. The resultant was further mixed with 100 μ L of the culture
20 solution, then added to the cultured cells, and cultured for 20 hours.

<Observation of Cells>

After the transfection treatment, the HEK293 cells were observed using an IX-81 inverted microscope, a
25 U-MNIBA3 filter (manufactured by Olympus Corporation), and an ORCA-Flash 4.0 digital camera (manufactured by Hamamatsu

Photonics K. K.). As an inhibitor against the protein-protein interaction between p53 and MDM2, Nutlin-3 was added to the culture solution to the final concentration of 40 μ M. After the drug addition, the resultant was left standing at room temperature for 30 minutes, and the cells were observed. Figs. 15 and 16 show images before the Nutlin-3 addition and 30 minutes after the addition.

As apparent from the result shown in Figs. 15 and 16, distinctive fluorescent foci were observed in cells expressing the PB1-p53(70) and the PB1-mAG1-MDM2 and in cells expressing the p53(70)-mAG1-PB1 and the PB1-MDM2. Further, these fluorescent foci were extinguished by the Nutlin-3 addition, and the fluorescent signals were dispersed. As apparent from these, it was confirmed that the fluorescent foci were formed dependently on the protein-protein interaction between the p53 protein and the MDM2 protein.

Thus, it was confirmed that the present invention was capable of determining not only the homomultimer formation but also the heteromultimer formation. As verified in Example 3 also, Example 4 confirmed that the protein tags according to the present invention functioned even when fused to any of the N-terminus and the C-terminus of the analysis-target protein.

(Example 5)

The effectiveness of the system shown in Fig. 1 in

determining a protein-protein interaction was confirmed by a method described below. Note that the detection targets in this examination were a FRB domain of a mTOR protein (hereinafter also referred to as "mTOR(FRB domain)") and a FKBP12 protein. These proteins have been known to interact with each other in the presence of rapamycin (see Chen J et al., Proc Natl Acad Sci U S A., May 23, 1995, vol. 92, no. 11, pp. 4947 to 4951). Additionally, in this confirmation experiment also, a PB1 domain of a p62 protein was used as the multimerizable protein, and a mAG1 protein was used as the fluorescent protein, as in Example 5.

<Preparation of pPB1-hmAG1-FKBP12>

In constructing a vector (pPB1-hmAG1-FKBP12) for expressing a protein (PB1-mAG1-FKBP12) composed of the PB1 domain of the p62 protein and the mAG1 protein fused to the N-terminus of the FKBP12 protein, first, a pPB1-FKBP12 vector was prepared as follows.

A DNA was artificially synthesized which contained recognition sites of restriction enzymes (EcoRI and XhoI) and the stop codon at either end of a nucleotide sequence encoding the FKBP12 protein (the protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_463460.1). Then, the obtained synthetic DNA was cleaved with EcoRI and XhoI, and inserted into pAsh-MCLinker having been treated with a combination of the same restriction enzymes.

Thus, an expression vector (pPB1-FKBP12) encoding PB1-FKBP12 was constructed.

Next, the pPB1-FKBP12 was cleaved with BamHI and NotI, and then inserted into pPB1-hmAG1-MCLinker having been
5 treated with a combination of the same restriction enzymes. Thus, pPB1-hmAG1-FKBP12 was prepared.

<Preparation of pmTOR(FRB domain)-PB1>

A vector (pmTOR(FRB domain)-PB1) for expressing a protein (mTOR(FRB domain)-PB1) composed of the PB1 domain
10 of the p62 protein fused to the C-terminus of the FRB domain of the mTOR protein was prepared as follows. A DNA was artificially synthesized which contained EcoRI and XhoI recognition sites respectively at both ends of a nucleotide sequence encoding a portion of the mTOR protein (a region
15 having the amino acids from positions 2025 to 2114 of the mTOR protein; a polypeptide having the amino acid sequence of SEQ ID NO: 41). Then, the obtained synthetic DNA was cleaved with EcoRI and XhoI, and inserted into pAsh-MNLinker having been treated with a combination of
20 the same restriction enzymes. Thus, an expression vector (pmTOR(FRB domain)-PB1) encoding mTOR(FRB domain)-PB1 was prepared.

<Cell Culturing and Transfection>

To express the fusion proteins in a combination
25 according to the following (C) in the cells, the vectors encoding these fusion proteins were introduced into HEK293

cells by the same method as that described in Example 4.

(C) PB1-mAG1-FKBP12 and mTOR(FRB domain)-PB1

<Observation of Cells>

After the transfection treatment, the HEK293 cells
5 were observed by the same method as that described in Example
4. Moreover, as an inducer for the protein-protein
interaction between mTOR(FRB domain) and FKBP12, rapamycin
was added to the culture solution to the final concentration
of 500 nM. After the drug addition, the resultant was left
10 standing at room temperature for 30 minutes, and the cells
were observed. Fig. 17 shows images before the drug
addition and 30 minutes after the addition.

As apparent from the result shown in Fig. 17, no
fluorescent focus was observed in cells expressing the
15 PB1-mAG1-FKBP12 and the mTOR(FRB domain)-PB1 before
rapamycin was added. However, 30 minutes after the
rapamycin addition, fluorescent foci were observed. As
described above, since the fluorescent foci were formed
in a manner dependent on rapamycin, it was confirmed that
20 these fluorescent foci were formed dependently on the
protein-protein interaction between FKBP12 and mTOR(FRB
domain).

Thus, as verified in Example 4 also, it was confirmed
that the present invention is capable of detecting not only
25 the homomultimer formation as described in Examples 1 to
3, but also an interaction between different proteins.

(Example 6)

The effectiveness of the system shown in Fig. 2 in determining a protein-protein interaction was confirmed by a method described below. Note that the detection target in this examination was an interaction between a p53 protein and an MDM2 protein, and that Nutlin-3 known as an inhibitor against the interaction was also used in this confirmation experiment. Further, in this confirmation experiment, a PB1 domain of a p62 protein was used as the multimerizable protein. Moreover, a mAG1 protein was used as the fluorescent protein. In fusion proteins used, both of the p53 protein and the MDM2 protein were directly or indirectly bound to the fluorescent protein.

<Preparation of pPB1hmAG1-MNLinker>

A DNA (the DNA having the nucleotide sequence of SEQ ID NO: 27) was artificially synthesized which contained NheI and AgeI recognition sites respectively at both ends of a DNA fragment encoding a protein composed of mAG fused to the C-terminus of the PB1 domain of the PB1p62 protein. The DNA was cleaved with NheI and AgeI, and inserted into pAsh-MNLinker from which the DNA encoding the PB1 domain of the PB1p62 protein had been removed. Thus, a pPB1hmAG1-MNLinker plasmid vector was prepared.

<Preparation of pp53(70)-PB1-hmAG1>

To express a partial peptide of p53 whose C-terminus was fused to the PB1 domain of the p62 protein and mAG1

(p53(70)-PB1-mAG1) as a first fusion protein in cells, a pp53(70)-PB1-hmAG1 vector was prepared as follows.

In preparing pp53(70)-PB1-hmAG1, first, a nucleotide sequence was designed in which a BamHI recognition sequence and a NotI recognition sequence were respectively added to the 5' end and the 3' end of the DNA encoding p53(70). Then, a DNA having the nucleotide sequence was artificially synthesized, cleaved with BamHI and NotI, and subsequently inserted into pPB1-hmAG1-MNLinker having been treated with a combination of the same restriction enzymes. Thus, pp53(70)-PB1-hmAG1 was prepared.

<Cell Culturing and Transfection>

To express the fusion proteins in a combination according to the following (A) or (B) in cells, the vectors encoding these fusion proteins were introduced into HEK293 cells by the same method as that described in Example 5; (A) p53(70)-mAG1-PB1 and PB1-mAG1-MDM2; and (B) p53(70)-PB1-mAG1 and PB1-mAG1-MDM2.

<Observation of Cells>

After the transfection treatment, the HEK293 cells were observed by the same method as that described in Example 4. Specifically, as an inhibitor against the protein-protein interaction between p53 and MDM2, Nutlin-3 was added to the culture solution to the final concentration of 40 μ M. After the drug addition, the resultant was left standing at room temperature for 30 minutes, and the cells

were observed. Figs. 18 and 19 show images before the Nutlin-3 addition and 30 minutes after the addition.

As apparent from the result shown in Figs. 18 and 19, distinctive fluorescent foci were observed in all cells. Further, these fluorescent foci were extinguished by the Nutlin-3 addition, and the fluorescent signals were dispersed. As apparent from these, it was confirmed that the fluorescent foci were formed dependently on the protein-protein interaction between the p53 protein and the MDM2 protein. Moreover, it was also confirmed that the method of the present invention was capable of determining the protein-protein interaction regardless of whether the protein directly fused to p53(70) was either mAG1 (in the case of (A) above) or the PB1 domain (in the case of (B) above).

(Example 7)

The effectiveness of the system shown in Fig. 2 in determining a protein-protein interaction was confirmed by a method described below. Note that the detection target in this examination was a protein-protein interaction between mTOR(FRB domain) and a FKBP12 protein. Moreover, to induce the interaction, rapamycin was also used in this system. Additionally, in this confirmation experiment also, a PB1 domain of a p62 protein was used as the multimerizable protein, and a mAG1 protein was used as the fluorescent protein, as in Example 7.

<Preparation of pmTOR(FRB domain)-hmAG1-PB1>

A vector (pmTOR(FRB domain)-hmAG1-PB1) for expressing a protein (mTOR(FRB domain)-mAG1-PB1) composed of mAG1 and the PB1 domain of the p62 protein fused to the C-terminus of the FRB domain of the mTOR protein was prepared as follows.

The pmTOR(FRB domain)-PB1 was cleaved with BamHI and NotI, and the mTOR(FRB domain) fragment was purified. Then, the fragment was inserted into phmAG1-PB1-MNLinker having been treated with a combination of the same restriction enzymes. Thus, pmTOR(FRB domain)-hmAG1-PB1 was prepared.

<Cell Culturing and Transfection>

To express the fusion proteins in a combination according to the following (C) in the cells, the vectors encoding these fusion proteins were introduced into HEK293 cells by the same method as that described in Example 5. (C) mTOR(FRB domain)-mAG1-PB1 and PB1-mAG1-FKBP12.

<Observation of Cells>

After the transfection treatment, the HEK293 cells were observed by the same method as that described in Example 5. Specifically, as an inducer for the protein-protein interaction between mTOR(FRB domain) and FKBP12, rapamycin was added to the culture solution to the final concentration of 500 nM. After the drug addition, the resultant was left standing at room temperature for 30 minutes, and the cells were observed. Figs. 20 and 21 show images before the drug

addition and 30 minutes after the addition.

As apparent from the result shown in Figs. 20 and 21, no fluorescent focus was observed in cells expressing the mTOR(FRB domain)-mAG1-PB1 and the PB1-mAG1-FKBP12 before rapamycin was added. However, 30 minutes after the rapamycin addition, fluorescent foci were observed. As described above, since the fluorescent foci were formed in a manner dependent on rapamycin, it was confirmed that these fluorescent foci were formed dependently on the protein-protein interaction between FKBP12 and mTOR(FRB domain).

As verified in Examples 1 to 3 also, the effectiveness of the system for detecting a fluorescent focus formed by assembly formation between a first fusion protein and a second fusion protein shown in Fig. 2 was confirmed in Examples 6 and 7, too. Particularly, it was confirmed that the system shown in Fig. 2 according to the present invention was capable of determining not only the homomultimer formations as described in Examples 1 to 3, but also the interactions between different proteins.

(Example 8)

It was confirmed that the fluorescent protein used in the present invention was not limited to mAG1 by a method described below in which the fluorescent protein in Example 1 was changed from mAG1 to DG1 or mCherry.

<Preparation of pPB1-hDG1-FKBP12 Mutant>

A vector (pPB1-hDG1-FKBP12 mutant) for expressing a protein (PB1-DG1-FKBP12 mutant) composed of the PB1 domain of the p62 protein and the Dronpa-Green1 fluorescent protein fused to the N-terminus of the FKBP12 mutant was prepared as follows.

A DNA sequence was artificially synthesized which encoded DG1 (a protein having the amino acid sequence of Genbank ACCESSION No: BAD72874), and which had a SpeI recognition sequence at the 5' end and an AgeI recognition sequence at the 3' end. Then, the obtained DNA was cleaved with these restriction enzymes, and inserted into the pPB1-hmAG1-FKBP12 mutant which had been treated with a combination of the same restriction enzymes, and from which the hmAG1 portion had been removed. Thus, a pPB1-hDG1-FKBP12 mutant was prepared.

<Preparation of pPB1-mCherry-FKBP12 Mutant>

A vector (pPB1-mCherry-FKBP12 mutant) for expressing a protein (PB1-mCherry-FKBP12 mutant) composed of the PB1 domain of the p62 protein and the mCherry fluorescent protein fused to the N-terminus of the FKBP12 mutant was prepared as follows.

In preparing pPB1-mCherry-FKBP12, first, a nucleotide sequence was designed in which a SpeI recognition sequence and an AgeI recognition sequence were respectively added to the 5' end and the 3' end of a DNA encoding mCherry (a protein having the amino acid sequence

of Genbank ACCESSION No: AAV52164). Then, a DNA having the nucleotide sequence was artificially synthesized, cleaved with SpeI and AgeI, and subsequently inserted into the pPB1-hmAG1-FKBP12 mutant which had been treated with
5 a combination of the same restriction enzymes, and from which the hmAG1 portion had been removed. Thus, a pPB1-mCherry-FKBP12 mutant was prepared.

<Cell Culturing and Transfection>

To express the PB1-hDG1-FKBP12 mutant or the
10 PB1-mCherry-FKBP12 mutant in cells, the vectors encoding these fusion proteins were introduced into HEK293 cells by the same method as that described in Example 4.

<Observation of Cells>

After the transfection treatment, the HEK293 cells
15 were observed using an IX-81 inverted microscope and an ORCA-Flash 4.0 digital camera. A U-MNIBA3 filter (manufactured by Olympus Corporation) and a U-MWIG3 filter (manufactured by Olympus Corporation) were used respectively for the DG1 and mCherry observations. The
20 homodimer formation of the FKBP12 mutant was induced by adding 500 nM B/B Homodimerizer. After the drug addition, the resultant was left standing at room temperature. The cells were observed after 1, 2, and 3 hours elapsed. Figs.
22 and 23 show images before the drug addition and 1, 2,
25 and 3 hours after the addition.

As apparent from the result shown in Figs. 22 and

23, drug-addition dependent increases in fluorescent foci were observed in both of cells expressing the PB1-DG1-FKBP12 mutant and cells expressing the PB1-mCherry-FKBP12 mutant. Thus, it was confirmed that the fluorescent protein used in the present invention was not limited to mAG1.

(Example 9)

It was confirmed that a protein other than the PB1 domain of the p62 protein was also utilizable as a multimerizable protein in the present invention. Specifically, a SAM domain of a Tankyrase 1 protein was used in place of the PB1 domain in the method described in Example 1, and the homodimer formation of the FKBP12 protein mutant was detected using materials and methods described below.

<Preparation of pTankyrase-hmAG1-FKBP12 Mutant>

A vector (pTankyrase-hmAG1-FKBP12 mutant) for expressing a protein (Tankyrase-mAG1-FKBP12 mutant) composed of the SAM domain of the Tankyrase 1 protein and mAG1 fused to the N-terminus of the FKBP12 mutant was prepared as follows.

A DNA sequence was artificially synthesized which encoded a protein (Tankyrase-mAG1, the protein had the amino acid sequence of SEQ ID NO: 43) composed of mAG1 fused to the C-terminus of the SAM domain of the Tankyrase 1 protein, and which had a NheI recognition sequence at the 5' end

and an AgeI recognition sequence at the 3' end. The DNA was cleaved with NheI and AgeI, and then inserted into the pPB1-hmAG1-FKBP12 mutant which had been treated with the same restriction enzymes, and from which the PB1-hmAG1 portion had been removed. Thus, a pTankyrase-hmAG1-FKBP12 mutant vector was prepared.

<Cell Culturing and Transfection>

HEK293 cells were cultured in DMEM (high glucose) supplemented with 10% FBS and 1% penicillin-streptomycin. Then, on the day before the transfection, the cells were seeded onto an 8-well chamber slide. The cells were cultured in 200 μ L of the culture solution per well. Subsequently, a DNA solution was prepared by mixing 200 ng of the plasmid with 10 μ L of OptiMEM, and 0.8 μ L of Eugene HD (manufactured by Promega Corporation) was added thereto and then stirred. The resultant was further mixed with 100 μ L of the culture solution, then added to the HEK293 cells, and cultured for 20 hours.

<Observation of Cells>

After the transfection treatment, the HEK293 cells were observed using an IX-81 inverted microscope and an ORCA-Flash 4.0 digital camera. A U-MNIBA3 filter was used for the observation. The homodimer formation of the FKBP12 mutant was induced by adding 500 nM B/B Homodimerizer to the medium. In addition, after the drug was added, the resultant was left standing at room temperature. The cells

were observed after 3 hours elapsed. Fig. 24 shows images obtained before the drug addition and 3 hours after the addition.

As apparent from the result shown in Fig. 24, fluorescent focus formations attributable to the addition of the drug for inducing the homodimer formation of the Tankyrase-mAG1-FKBP12 mutant were observed in cells expressing this mutant. Thus, it was confirmed that if a protein was capable of forming a multimer, the protein was utilizable in the present invention.

(Example 10)

As in Example 9, it was confirmed that proteins other than the PB1 domain of the p62 protein were also utilizable as a multimerizable protein in the present invention. Specifically, a SAM domain of a Tankyrase 1 protein, a PB1 domain of a PKCιota protein, a SAM domain of a TEL protein, or a SAM domain of a DGK delta protein was used in place of the PB1 domain in the method described in Example 3, and the homomultimer formation of the p53 protein was detected using materials and methods described below.

<Preparation of pTankyrase-hmAG1-p53>

A vector (pTankyrase-hmAG1-p53) for expressing a protein (Tankyrase-mAG1-p53) composed of the SAM domain of the Tankyrase 1 protein and mAG1 fused to the N-terminus of p53 was prepared as follows.

A DNA sequence was artificially synthesized which

encoded a protein (Tankyrase-mAG1, the protein had the amino acid sequence of SEQ ID NO: 43) composed of mAG1 fused to the C-terminus of the SAM domain of the Tankyrase 1 protein, and which had a NheI recognition sequence at the 5' end and an AgeI recognition sequence at the 3' end. The DNA was cleaved with NheI and AgeI, and then inserted into the phmAG1PB1-p53 which had been treated with the same restriction enzymes, and from which the hmAG1PB1 portion had been removed. Thus, a pTankyrase-hmAG1-p53 vector was prepared.

<Preparation of pPKCi-hmAG1-p53>

A vector (pTankyrase-hmAG1-p53) for expressing a protein (PKCi-mAG1-p53) composed of the PB1 domain of the PKCιota protein and mAG1 fused to the N-terminus of p53 was prepared as follows.

A DNA sequence was artificially synthesized which encoded a protein (PKCi-mAG1, the protein had the amino acid sequence of SEQ ID NO: 45) composed of mAG1 fused to the C-terminus of the PB1 domain of the PKCιota protein, and which had a NheI recognition sequence at the 5' end and an AgeI recognition sequence at the 3' end. The DNA was cleaved with NheI and AgeI, and then inserted into the phmAG1PB1-p53 which had been treated with the same restriction enzymes, and from which the hmAG1-PB1 portion had been removed. Thus, a pPKCi-hmAG1-p53 vector was prepared.

<Preparation of pTEL-hmAG1-p53>

A vector (pTEL-hmAG1-p53) for expressing a protein (TEL-mAG1-p53) composed of the SAM domain of the TEL protein and mAG1 fused to the N-terminus of p53 was prepared as follows.

A DNA sequence was artificially synthesized which encoded a protein (TEL-mAG1, the protein had the amino acid sequence of SEQ ID NO: 47) composed of mAG1 fused to the C-terminus of the SAM domain of the TEL protein, and which had a NheI recognition sequence at the 5' end and an AgeI recognition sequence at the 3' end. The DNA was cleaved with NheI and AgeI, and then inserted into the phmAG1-PB1-p53 which had been treated with the same restriction enzymes, and from which the hmAG1-PB1 portion had been removed. Thus, a pTEL-hmAG1-p53 vector was prepared.

<Preparation of pDGKd-hmAG1-p53>

A vector (pDGKd-hmAG1-p53) for expressing a protein (DGKd-mAG1-p53) composed of the SAM domain of the DGK delta protein and mAG1 fused to the N-terminus of p53 was prepared as follows.

A DNA sequence was artificially synthesized which encoded a protein (DGKd-mAG1, the protein had the amino acid sequence of SEQ ID NO: 49) composed of mAG1 fused to the C-terminus of the SAM domain of the DGK delta protein, and which had a NheI recognition sequence at the 5' end

and an AgeI recognition sequence at the 3' end. The DNA was cleaved with NheI and AgeI, and then inserted into the phmAG1PB1-p53 which had been treated with the same restriction enzymes, and from which the hmAG1-PB1 portion had been removed. Thus, a pDGKd-hmAG1-p53 vector was prepared.

<Cell Culturing and Transfection>

HEK293 cells were cultured in DMEM (high glucose) supplemented with 10% FBS and 1% penicillin-streptomycin. Then, on the day before the transfection, the cells were seeded onto an 8-well chamber slide, and cultured in 200 μ L of the culture solution per well. Subsequently, a DNA solution was prepared by mixing 200 ng of the plasmid with 10 μ L of OptiMEM, and 0.8 μ L of Eugene HD was added thereto and then stirred. The resultant was further mixed with 100 μ L of the culture solution, then added to the HEK293 cells, and cultured for 48 hours.

<Observation of Cells>

After the transfection treatment, the HEK293 cells were observed using an IX-81 inverted microscope and an ORCA-Flash 4.0 digital camera. A U-MNIBA3 filter was used for the observation. Fig. 25 shows the obtained result.

As apparent from the result shown in Fig. 25, fluorescent focus formations indicating the homomultimer formation of p53 were observed in all of cells expressing the Tankyrase-mAG1-p53, cells expressing the

PKCi-mAG1-p53, cells expressing the TEL-mAG1-p53, and cells expressing the DGKd-mAG1-p53. Thus, it was confirmed that any protein capable of forming a multimer was utilizable in the present invention.

5 (Example 11)

It was confirmed by a method described below that the present invention was capable of detecting a homodimer formed in a manner dependent on a signal transduction. When a signal transduction pathway involving EGF, GPCR, and so forth is activated, ERK2 located downstream thereof is phosphorylated and activated. In addition, it has been known that ERK2 forms a homodimer after the activation (see Harvey CD et al., Proc Natl Acad Sci USA, 2008, vol. 105, no. 49, pp. 19264 to 19269, Khokhlatchev AV et al., Cell, 10 1998, vol. 93, iss. 4, pp. 605 to 615). Hence, the detection of this homodimer formation was tested according to the present invention. Note that, in this confirmation experiment, a PB1 domain of a p62 protein was used as the multimerizable protein, and a mAG1 protein was used as the fluorescent protein. Additionally, it has also been revealed that the ERK2 activation takes place in the cytoplasm and the nucleus (see Ebisuya M et al., J Cell Sci., 2005, 118 (Pt 14), pp. 2997 to 3002).

25 <Preparation of pERK2-hmAG1-PB1 and pERK2-PB1-hmAG1>

Vectors (pERK2-hmAG1-PB1 and pERK2-PB1-hmAG1) for

expressing proteins (ERK2-mAG1-PB1 and ERK2-PB1-mAG1) each composed of mAG1 and the PB1 domain of the p62 protein fused to the C-terminus of ERK2 were prepared as follows.

A nucleotide sequence was designed in which an XhoI recognition sequence and a NotI recognition sequence were respectively added to the 5' end and the 3' end of a nucleotide sequence encoding ERK2 (a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_002736.3). Then, a DNA having the resulting nucleotide sequence was artificially synthesized, and cleaved with XhoI and NotI. Meanwhile, phmAG1-PB1-MNLinker and pPB1-hmAG1-MNLinker were treated with a combination of the same restriction enzymes. Subsequently, the synthetic DNA fragment was inserted into these vectors. Thus, vectors (pERK2-hmAG1-PB1 and pERK2-PB1-hmAG1) encoding ERK2-mAG1-PB1 and ERK2-PB1-mAG1 were prepared.

<Cell Culturing and Transfection>

HEK293 cells were selected as cells into which the expression vectors prepared above were introduced. The cells were first cultured in DMEM (high glucose) supplemented with 10% FBS (manufactured by Equitech-Bio Inc.) and 1% penicillin-streptomycin. Then, on the day before the introduction of the expression vectors, these cells were seeded onto a 35-mm dish, and cultured in 1.5 ml of the culture solution. Subsequently, 1 µg of the expression vector (pERK2-hmAG1-PB1 or pERK2-PB1-hmAG1)

was diluted with 100 μ L of OptiMEM, and 10 μ L of PolyFect(registered trademark) Transfection Reagent was added thereto and then stirred. The resultant was further mixed with 600 μ L of the culture solution. Thereafter, each solution of the expression vectors was added to the cultured cells. The pERK2-hmAG1PB1 transfected cells were observed 20 hours after the vector introduction. To the pERK2-PB1-hmAG1 transfected cells, G418 was added at a concentration of 500 μ g/mL, and further cultured for one week. After that, viable cells were detached using a trypsin solution, diluted with a culture solution containing G418 (500 μ g/ml), and then cultured in a 96-well plate at 0.5 cells/well. Subsequently, cells which formed single colonies were cultured to expand.

<Observation of Cells>

After the transfection treatment, the HEK293 cells were cultured for 4 hours in a buffer at pH of 7.4 containing Hanks' Balanced Salt Solutions and 20 mM HEPES, so that the cells were under a serum-starved condition. Then, the cells were observed using an IX-71 inverted fluorescence microscope (the magnification of the objective lens: 20 $\times$), a U-MGFPHQ filter, and an ORCA-ER digital camera. The homodimer formation of ERK2 was induced by adding EGF (epidermal growth factor, manufactured by Sigma-Aldrich Co.) to the buffer to the final concentration of 50 ng/ml. Figs. 26 and 27 show images before the EGF addition and

8 minutes after the addition.

As apparent from the result shown in Fig. 26, no fluorescent focus was detected before EGF was added, and fluorescent signals were dispersedly observed in the cytoplasms. Then, 8 minutes after the EGF addition, fluorescent foci were observed in multiple cells.

Moreover, as apparent from the result shown in Fig. 27, fluorescent foci indicating the homodimer formation of ERK2 were detected throughout HEK293 cells constitutively expressing the ERK2-PB1-hmAG1.

Thus, it was confirmed that the present invention was capable of detecting the homodimer formation of ERK2 attributable to the activation of the intracellular signal transduction pathway. Moreover, the present invention confirmed that detecting a fluorescent focus enabled detection of an activation attributable to the phosphorylation of ERK2, and also eventually an activation of a signal transduction pathway involving EGF and so forth located upstream of ERK2.

(Example 12)

It was confirmed by a method described below that the present invention was capable of detecting a homodimer formed in a manner dependent on a signal transduction. Specifically, when a signal transduction pathway involving IL-6 and so forth is activated, STAT3 located downstream thereof is phosphorylated and activated. In addition, it

has been known that STAT3 forms a homodimer after the activation and transmits the extracellular signal into the nucleus (see Becker S et al., Nature, 1998, vol. 394, nol. 6689, pp. 145 to 151, Johnston PA et al., Mol Interv., 2011, 5 vol. 11, no. 1, pp.18 to 26). Hence, the detection of the STAT3 homodimer formation was tested according to the present invention. Note that, in this confirmation experiment, a PB1 domain of a p62 protein was used as the multimerizable protein, and a mAG1 protein was used as the 10 fluorescent protein.

<Preparation of pPB1-hmAG1-STAT3>

A vector (pPB1-hmAG1-STAT3) for expressing a protein (PB1-mAG1-STAT3) composed of the PB1 domain of the p62 protein and mAG1 fused to the N-terminus of STAT3 was 15 prepared as follows.

A nucleotide sequence was designed in which an XhoI recognition sequence was added to the 5' end of a nucleotide sequence encoding STAT3 (a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_644805.1) while 20 the stop codon and a NotI recognition sequence were added to the 3' end of the nucleotide sequence. Then, a DNA having the resulting nucleotide sequence was artificially synthesized, cleaved with XhoI and NotI, and then inserted into pPB1-hmAG1-MCLinker having been treated with a 25 combination of the same restriction enzymes. Thus, pPB1-hmAG1-STAT3 was prepared.

<Cell Culturing and Transfection>

HEK293 cells were selected as cells into which the expression vector prepared above was introduced. The cells were first cultured in DMEM (high glucose) supplemented with 10% FBS and 1% penicillin-streptomycin. Then, on the day before the introduction of the expression vector, these cells were seeded onto a 35-mm dish, and cultured in 1.5 ml of the culture solution. Subsequently, 1 µg of the expression vector (pPB1-hmAG1-STAT3) was diluted with 100 µL of OptiMEM, and 10 µL of PolyFect(registered trademark) Transfection Reagent was added thereto and then stirred. The resultant was further mixed with 600 µL of the culture solution. Thereafter, the mixture solution of the expression vector was added to the cultured cells. After that, G418 was added thereto at a concentration of 500 µg/mL, and further cultured for one week. Next, viable cells were detached using a trypsin solution, diluted with a culture solution containing G418 (500 µg/ml), and then cultured in a 96-well plate at 0.5 cells/well. Subsequently, cells which formed single colonies were cultured to expand.

<Observation of Cells>

The HEK293 cells constitutively expressing the PB1-mAG1-STAT3 were cultured for 4 hours in a buffer at pH of 7.4 containing Hanks' Balanced Salt Solutions and 20 mM HEPES, so that the cells were under a serum-starved

condition. Then, the cells were observed using an IX-71 inverted fluorescence microscope (the magnification of the objective lens: 20×), a U-MGFPHQ filter, and an ORCA-ER digital camera. The homodimer formation of STAT3 was induced by adding Human Recombinant IL-6 (manufactured by R&D SYSTEMS, INC.) to the buffer to the final concentration of 100 ng/ml. Fig. 28 shows images before the IL-6 addition and 50 minutes after the addition.

As apparent from the result shown in Fig. 28, no fluorescent focus was detected before IL-6 was added, and fluorescent signals were dispersedly observed in the cytoplasms. Then, 50 minutes after the IL-6 addition, fluorescent foci were observed in multiple cells.

Thus, it was confirmed that the present invention was capable of detecting the homodimer formation of STAT3 attributable to the activation of the intracellular signal transduction pathway. Moreover, the present invention confirmed that detecting a fluorescent focus enabled detection of an activation attributable to the phosphorylation of STAT3, and also eventually an activation of a signal transduction pathway involving IL-6 and so forth located upstream of STAT3.

(Example 13)

As described in Examples 1 to 12, it was revealed that the systems shown in Figs. 1 and 2 were effective in determining a protein-protein interaction. Next, the

present inventors came up with a system shown in Fig. 3 as another embodiment of such systems.

Specifically, when a third fusion protein comprising a first protein and a multimerizable protein, a fourth fusion protein comprising a second protein and a multimerizable protein, and a fifth fusion protein comprising a multimerizable protein and a fluorescent protein are expressed in a cell or introduced into a cell, the multimerizable proteins associates with each other. Thereby, the third fusion protein binds to the fifth fusion protein while the fourth fusion protein binds to the fifth fusion protein, so that proteins respectively corresponding to the first fusion protein and the second fusion protein shown in Figs. 1 and 2 are presumably expressed in the cell. Moreover, if such a presumption is correct, as in the cases of the systems shown in Figs. 1 and 2, the following is presumed also from the system shown in Fig. 3: an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and another multimerizable protein; thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

Hence, the effectiveness of the system shown in Fig. 3 in determining a protein-protein interaction was examined

using materials and methods described below. Note that the detection target in this examination was an interaction between a p53 protein and an MDM2 protein, and that Nutlin-3 known as an inhibitor against the interaction was also used in this examination (see Vassilev LT et al., Science, February 6, 2004, vol. 303, no. 5659, pp. 844 to 848). Further, in this examination, a PB1 domain of a p62 protein was used as the multimerizable protein, and a hmAG1 protein was used as the fluorescent protein.

<Preparation of pp53-PB1 and pPB1-MDM2>

To express in cells the third and fourth fusion proteins according to the present invention, that is, a fusion protein (p53-PB1) comprising the p53 protein and the PB1 domain of the p62 protein as well as a fusion protein (PB1-MDM2) comprising the MDM2 protein and the PB1 domain of the p62 protein, expression vectors encoding the fusion proteins were prepared by methods described below.

A DNA (the DNA having the nucleotide sequence of SEQ ID NO: 33) was artificially synthesized which contained restriction enzyme sites at either end of a nucleotide sequence encoding a portion of the p53 protein (a region having the amino acids from positions 1 to 70 of the p53 protein, the region had the amino acid sequence of SEQ ID NO: 34, hereinafter also referred to as "p53(70)"). Then, the obtained synthetic DNA was cleaved with EcoRI and XhoI, and inserted into pAsh-MNLinker (manufactured by MEDICAL

& BIOLOGICAL LABORATORIES CO., LTD.) having been treated with a combination of the same restriction enzymes. Thus, an expression vector (pp53(70)-PB1) encoding p53(70)-PB1 was constructed. Note that inserting a DNA encoding a certain protein into a multiple cloning site of the pAsh-MNLinker in accordance with the reading frame allows an expression, in cells, of a protein having an Ash protein tag (the PB1 domain of the p62 protein) fused to the C-terminus of the certain protein via a linker peptide.

Moreover, a DNA (the DNA having the nucleotide sequence of SEQ ID NO: 35) was artificially synthesized which contained restriction enzyme sites at either end of a nucleotide sequence encoding a portion of MDM2 (a region having the amino acids from positions 1 to 119 of the MDM2 protein, the region had the amino acid sequence of SEQ ID NO: 36). Then, the obtained synthetic DNA was cleaved with EcoRI and XhoI, and inserted into pAsh-MCLinker (manufactured by MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.) having been treated with a combination of the same restriction enzymes. Thus, an expression vector (pPB1-MDM2) encoding PB1-MDM2 was constructed. Note that inserting a DNA encoding a certain protein into a multiple cloning site of the pAsh-MCLinker in accordance with the reading frame allows an expression, in cells, of a protein having an Ash protein tag (the PB1 domain of the p62 protein) fused to the N-terminus of the certain protein via a linker

peptide.

<Preparation of phmAG1PB1>

To express the fifth fusion protein in cells, an expression vector was prepared by a method described below.

5 Specifically, first, a DNA having the nucleotide sequence of SEQ ID NO: 37 was artificially synthesized which contained restriction enzyme sites at either end of a DNA having a nucleotide sequence encoding the PB1 domain of the p62 protein (the DNA encoding a protein having the amino
10 acid sequence of SEQ ID NO: 38). Then, the obtained synthetic DNA was cleaved with EcoRI and NotI, and inserted into phmAG1-MCLinker (manufactured by limited company Amalgaam Co., Ltd.) having been treated with a combination of the same restriction enzymes. Thus, an expression
15 vector (phmAG1-PB1) encoding a fusion protein (mAG1-PB1) composed of mAG1 and the PB1 domain of the p62 protein was constructed.

<Preparation of Cells Constitutively Expressing mAG1-PB1>

20 Next, the phmAG1-PB1 prepared above was introduced into HeLaS3 cells to establish cells constitutively expressing the fifth fusion protein (mAG1-PB1) according to the present invention. Specifically, first, HeLaS3 cells were cultured in DMEM (low glucose, manufactured by
25 SIGMA ALDRICH CO.) supplemented with 10% FBS (manufactured by Equitech-Bio Inc.). Then, on the day before the

transfection, the HeLaS3 cells were seeded onto a 35-mm dish (manufactured by BD Falcon) and cultured in 1.5 mL of the culture solution. Subsequently, 1 µg of the phmAG1-PB1 was diluted with 100 µL of OptiMEM (manufactured by Life Technologies Corporation), and 10 µL of PolyFect(registered trademark) Transfection Reagent (manufactured by QIAGEN N.V.) was added thereto and then stirred. The resultant was further mixed with 600 µL of the culture solution, thereafter added to the HeLaS3 cells, and cultured for 20 hours. After that, G418 was added thereto at a concentration of 500 µg/mL, and further cultured for one week. Next, viable cells were detached using a trypsin solution, diluted with a culture solution containing G418 (500 µg/ml), and then cultured in a 96-well plate at 0.5 cells/well. Subsequently, cells which formed single colonies were cultured to expand.

<Cell Culturing and Transfection>

To additionally express the p53(70)-PB1 and the PB1-MDM2 in the cells constitutively expressing the mAG1-PB1, the pp53(70)-PB1 and the pPB1-MDM2 were introduced into the cells by a method described below.

Specifically, first, the cells constitutively expressing the mAG1-PB1 established above were cultured in DMEM (high glucose, manufactured by SIGMA ALDRICH CO.) supplemented with 10% FBS (Equitech-Bio Inc.) and 1% penicillin-streptomycin (Life Technologies Corporation).

Then, on the day before the transfection, the cells were seeded onto an 8-well chamber slide (manufactured by Nunc A/S), and cultured in 200 μ L of the culture solution per well. Subsequently, a DNA solution prepared by mixing 100
5 ng of each of the pp53(70)-PB1 and the pPB1-MDM2 was diluted with 10 μ L of OptiMEM (manufactured by Life Technologies Corporation). Thereafter, 0.4 μ L of Turbofect (manufactured by Thermo Fisher Scientific Inc.) was added thereto and then stirred. The resultant was further mixed
10 with 100 μ L of the culture solution. After that, the obtained mixture solution was added to the cultured cells and cultured for 20 hours.

Meanwhile, as negative controls, a DNA solution prepared by mixing 100 ng of each of the pp53(70)-PB1 and
15 pAsh-MNLinker, and a DNA solution prepared by mixing 100 ng of each of the pPB1-MDM and pAsh-MNLinker were respectively introduced into the cells constitutively expressing the mAG1-PB1 by the same method as that described above.

20 <Observation of Cells>

The three types of cells prepared above and described below were observed by the method described in Example 1:

cells constitutively expressing the mAG1-PB1 and further expressing the p53(70)-PB1 and the PB1-MDM2;

25 cells constitutively expressing the mAG1-PB1 and further expressing the p53(70)-PB1 and the PB1 domain of

the p62 protein (negative control); and

cells constitutively expressing the mAG1-PB1 and further expressing the PB1-MDM and the PB1 domain of the p62 protein (negative control).

5 Fig. 29 shows the obtained result.

Moreover, to the cells expressing the p53(70)-PB1 and the PB1-MDM2, Nutlin-3 was added to the final concentration of 20 μ M. Then, the cells were also observed under a condition of inhibiting the interaction between
10 p53 and MDM2. Fig. 30 shows the obtained result. Note that Fig. 29 shows the result of the observation with a 20 $\times$ magnification of the objective lens as in Example 1, while Fig. 30 shows the result of the observation with a 40 $\times$ magnification of the objective lens.

15 As apparent from the result shown in Fig. 29, fluorescent foci were detected in the cells expressing the mAG1-PB1, the p53(70)-PB1, and the PB1-MDM2 (see the image on the left in Fig. 29). On the other hand, no fluorescent focus was detected in the cells expressing the mAG1-PB1,
20 the p53(70)-PB1, and the PB1 domain of the p62 protein, and in the cells expressing the mAG1-PB1, the PB1-MDM2, and the PB1 domain of the p62 protein.

Further, Nutlin-3 was added to the cells from which the fluorescent foci were detected, and observed 15 minutes
25 thereafter. As a result, the fluorescent foci were extinguished as shown in Fig. 30. Thus, it was confirmed

that the fluorescent foci were formed dependently on the interaction between p53 and MDM2.

(Example 14)

As in Example 13, the effectiveness of the system shown in Fig. 3 in determining a protein-protein interaction was confirmed by a method described below. Note that the detection target in this examination was a protein-protein interaction between mTOR(FRB domain) and a FKBP12 protein. Moreover, to induce the interaction, rapamycin was also used in this system. Further, in this confirmation experiment, a PB1 domain of a p62 protein was used as the multimerizable protein, and a mAG1 protein was used as the fluorescent protein.

<Preparation of pmTOR(FRB domain)-PB1>

A vector for expressing a protein (mTOR(FRB domain)-PB1) composed of the PB1 domain of the p62 protein fused to the C-terminus of mTOR(FRB domain) as the third fusion protein according to the present invention was prepared as described in Example 5.

<Preparation of pPB1-mTOR(FRB domain)>

A vector (pPB1-mTOR(FRB domain)) for expressing a protein (PB1-mTOR(FRB domain)) composed of the PB1 domain of the p62 protein fused to the N-terminus of mTOR(FRB domain) as the third fusion protein according to the present invention was prepared as follows.

A DNA was artificially synthesized which contained

recognition sites of restriction enzymes (EcoRI and XhoI) and the stop codon at either end of a nucleotide sequence encoding mTOR(FRB domain). Then, the obtained synthetic DNA was cleaved with EcoRI and XhoI, and inserted into
5 pAsh-MCLinker having been treated with a combination of the same restriction enzymes. Thus, an expression vector (pPB1-mTOR(FRB domain)) encoding PB1-mTOR(FRB domain) was constructed.

<Preparation of pFKBP12-PB1>

10 A vector (pFKBP12-PB1) for expressing a protein (FKBP12-PB1) composed of the PB1 domain of the p62 protein fused to the C-terminus of the FKBP12 protein as the fourth fusion protein according to the present invention was prepared as follows.

15 A DNA was artificially synthesized which contained EcoRI and XhoI recognition sites respectively at both ends of a nucleotide sequence encoding the FKBP12 protein (the protein having the amino acid sequence of NCBI RefSeq
20 ACCESSION No: NP_463460.1). Then, the obtained synthetic DNA was cleaved with EcoRI and XhoI, and inserted into pAsh-MNLinker having been treated with a combination of the same restriction enzymes. Thus, an expression vector (pFKBP12-PB1) encoding FKBP12-PB1 was constructed.

<Preparation of pPB1-FKBP12>

25 A plasmid vector (pPB1-FKBP12) for expressing a protein (PB1-FKBP12) composed of the PB1 domain of the p62

protein fused to the N-terminus of FKBP12 protein as the fourth fusion protein according to the present invention was prepared as described in Example 5.

<Cell Culturing and Transfection>

5 (A) PB1-mTOR(FRB domain), PB1-FKBP12, and mAG1PB1

(B) PB1-mTOR(FRB domain), FKBP12-PB1, and mAG1PB1

(C) mTOR(FRB domain)-PB1, PB1-FKBP12, and mAG1PB1

(D) mTOR(FRB domain)-PB1, FKBP12-PB1, and mAG1PB1

10 To express the fusion proteins in combinations according to the above (A) to (D), each vector was introduced into HEK293 cells by a method described below.

Specifically, first, HEK293 cells were cultured in DMEM (high glucose) supplemented with 10% FBS and 1% penicillin-streptomycin. Then, on the day before the
15 transfection, the cells were seeded onto a 96-well multiwell plate, and cultured in 140 μ L of the culture solution per well. Subsequently, a DNA solution prepared by mixing 133 ng of each of the plasmid vectors was diluted with 10 μ L of OptiMEM. Thereafter, 0.8 μ L of Eugene HD
20 was added thereto and then stirred. The resultant was further mixed with 90 μ L of the culture solution. After that, the obtained mixture solution was added to the cultured cells and cultured for 20 hours.

<Observation of Cells>

25 The culture solution of the cells prepared above was discarded, and 100 μ L of a buffer at pH 7.4 containing Hanks'

Balanced Salt Solutions and 20 mM HEPES was added. Then, the cells were observed using IN Cell Analyzer 1000. Moreover, rapamycin was added to each well to the final concentration of 500 nM. After left standing at room temperature for 1 hour, the cells were observed. Fig. 31 shows the obtained result.

As apparent from the result shown in Fig. 31, no fluorescent focus was detected in any cells before rapamycin was added. However, as a result of adding rapamycin, fluorescent foci were detected. As described above, since the fluorescent foci were detected in a manner dependent on rapamycin, it was confirmed that the fluorescent foci were formed dependently on the interaction between mTOR(FRB domain) and FKBP12. Moreover, this Example also confirmed that the protein tag according to the present invention (the protein comprising the multimerizable protein or the fluorescent protein) functioned even when fused to any of the N-terminus and the C-terminus of the analysis-target protein.

Hereinabove, the present invention revealed as described in Examples 4 and 5 that it was possible to determine a protein-protein interaction also by utilizing an association between the multimerizable protein and the fluorescent protein in a cell as a result of an autonomous-association between the multimerizable protein and another multimerizable protein as shown in Fig. 3.

Further, since the cells constitutively expressing the fifth fusion protein according to the present invention were successfully established, it was confirmed that the protein exhibited no cytotoxicity and the like.

5 Moreover, in the method shown in Fig. 3, the protein fused to proteins to be analyzed (first and second proteins) was a multimerizable protein. On the other hand, in the methods shown in Figs. 1 and 2, the protein fused to proteins to be analyzed includes a fluorescent protein in addition
10 to a multimerizable protein. Thus, the method shown in Fig. 3 allows a reduction in the molecular weight of the protein tag fused to the proteins to be analyzed, in comparison with the methods shown in Figs. 1 and 2. Therefore, the functions of the proteins to be analyzed
15 are hardly affected.

 Furthermore, in the method shown in Fig. 3, cells constitutively expressing the fusion protein comprising the fluorescent protein are prepared in advance as described in Example 4. This reduces the influence of a
20 variation in the fluorescent signals among the cells, even if the fusion proteins comprising the proteins to be analyzed are transiently expressed in these cells. This makes it possible to more stably determine a protein-protein interaction.

25 (Example 15)

 As described above, it was revealed that the systems

shown in Figs. 1 to 3 were effective in determining a protein-protein interaction. Next, the present inventors came up with a system shown in Fig. 4 as another embodiment of these systems.

5 Specifically, when a first labeled protein comprising an affinity tag and a first protein, a second labeled protein comprising an affinity tag and a second protein, and a sixth fusion protein comprising a multimerizable protein, a fluorescent protein, and a binding partner having an
10 affinity for any of the affinity tags are expressed in a cell or introduced into a cell, each affinity tag binds to the binding partner. Thereby, the first labeled protein binds to the sixth fusion protein comprising the binding partner bound thereto while the second labeled protein
15 binds to the sixth fusion protein comprising the binding partner bound thereto, so that proteins respectively corresponding to the first fusion protein and the second fusion protein shown in Figs. 1 and 2 are expressed in the cell. Moreover, as in the cases of the systems shown in
20 Figs. 1 and 2, when these proteins are expressed, an interaction if any between the first protein and the second protein induces multimer formation between the multimerizable protein and another multimerizable protein; thereby, the fusion proteins autonomously form
25 an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

Then, the effectiveness of the system shown in Fig. 4 in determining a protein-protein interaction was confirmed using materials and methods described below. Note that the detection target in this confirmation experiment was homodimer formation of a cell transmembrane protein CD80. In addition, a cell transmembrane protein CD2 known to be present in the form of monomer in cells was also used as the negative control (as to the protein-protein interactions of these proteins, see James JR et al., Nat Methods., 2006, vol. 3, nol. 12, pp. 1001 to 1006).

<Preparation of pCD80-FRB>

The mTOR(FRB domain) was selected as the affinity tag. To express a CD80 protein (CD80-FRB) having this tag fused thereto as the first and second labeled proteins in cells, a pCD80-FRB vector was prepared as follows.

First, a DNA was artificially synthesized which contained BamHI and EcoRI recognition sequences respectively at both ends of a nucleotide sequence encoding CD80 (a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_005182.1). Then, the obtained synthetic DNA was cleaved with BamHI and EcoRI, and inserted into phmAG1-MNLinker having been treated with a combination of the same restriction enzymes. Thus, a pCD80-hmAG1 vector was constructed.

Next, a DNA was artificially synthesized which

contained AgeI and XbaI recognition sequences and the stop codon at either end of a nucleotide sequence encoding mTOR(FRB domain). Then, the obtained synthetic DNA was cleaved with AgeI and XbaI, and inserted into the pCD80-hmAG1 which had been treated with a combination of the same restriction enzymes, and from which the hmAG1 region had been removed. Thus, an expression vector (pCD80-FRB) encoding CD80-FRB was constructed.

<Preparation of pCD2-FRB>

As described above, to express a CD2 protein (CD2-FRB) having the affinity tag mTOR(FRB domain) fused thereto as the first and second labeled proteins in cells, a vector was prepared as follows.

First, a DNA was artificially synthesized which contained BamHI and EcoRI recognition sequences respectively at both ends of a nucleotide sequence encoding a CD2 protein (the protein having the amino acid sequence of Genbank ACCESSION No: AAA51946.1). Then, the obtained synthetic DNA was cleaved with BamHI and EcoRI, and inserted into phmAG1-MNLinker having been treated with a combination of the same restriction enzymes. Thus, pCD2-hmAG1 was constructed.

Next, a DNA was artificially synthesized which contained AgeI and XbaI recognition sequences and the stop codon at either end of a nucleotide sequence encoding mTOR(FRB domain). Then, the obtained synthetic DNA was

cleaved with AgeI and XbaI, and inserted into the pCD2-hmAG1 which had been treated with a combination of the same restriction enzymes, and from which the hmAG1 region had been removed. Thus, an expression vector (pCD2-FRB) encoding CD2-FRB was constructed.

<Preparation of pPB1-hmAG1-FKBP12>

FKBP12 was selected as the binding partner having an affinity for the affinity tag, a PB1 domain of p62 was selected as the multimerizable protein, and mAG1 was selected as the fluorescent protein. Moreover, to express PB1-hmAG1-FKBP12 composed of these proteins fused to each other as the sixth fusion protein in cells, a pPB1-hmAG1-FKBP12 vector was prepared as described in Example 5.

<Cell Culturing and Transfection>

To express the fusion proteins in a combination according to the following (A) or (B), the plasmid vectors prepared above were introduced into HEK293 cells by a method described below;

- (A) CD80-FRB and PB1-mAG1-FKBP12; and
- (B) CD2-FRB and PB1-mAG1-FKBP12.

Specifically, first, HEK293 cells were cultured in a culture solution (DMEM high glucose, 10% FBS, 1% penicillin-streptomycin). Then, on the day before the transfection, the cells were seeded onto an 8-well chamber, and cultured in 100 μ L of the culture solution per well.

Subsequently, a DNA solution prepared by mixing 200 ng of each of the plasmids was diluted with 10 μ L of OptiMEM. Thereafter, 0.8 μ L of Fugene HD was added thereto and then stirred. The resultant was further mixed with 90 μ L of the culture solution. After that, the obtained mixture solution was added to the cultured cells and cultured for 20 hours.

<Observation of Cells>

After the transfection treatment but before the observation, the culture solution of the HEK293 cells was replaced with an observation buffer (buffer at pH of 7.4 containing Hanks' Balanced Salt Solutions and 20 mM HEPES) containing 500 nM rapamycin. After the replacement with the drug-supplemented buffer, the resultant was left standing at room temperature for 30 minutes. Then, the cells were observed using an IX-71 inverted fluorescence microscope, a U-MGFPHQ filter, and an ORCA-ER digital camera. Fig. 32 shows the obtained result.

As shown in Fig. 32, when the CD80 was used as the detection target, fluorescent foci were observed at the cell membrane. On the other hand, when the CD2 was used as the detection target, no fluorescent focus was observed. Thus, the effectiveness of the system shown in Fig. 4 was confirmed.

Moreover, in the third method for determining a protein-protein interaction of the present invention shown

in Fig. 4, as in the case of the method shown in Fig. 3, it is not necessary to directly or indirectly fuse a fluorescent protein to the proteins to be analyzed. This allows a reduction in the molecular weight of the protein tag fused to the proteins to be analyzed, in comparison with the methods shown in Figs. 1 and 2. Therefore, the functions of the proteins to be analyzed are hardly affected.

Further, since the proteins to be analyzed can be kept in a cell while being fused to the tag having a low molecular weight in this manner, this makes it possible to set a state where a protein-protein interaction can be determined at any timing by constructing a system in which the fusion proteins according to the present invention are reconstituted in response to a stimulus such as rapamycin addition as described above.

Furthermore, preparing cells constitutively expressing the sixth fusion protein comprising the fluorescent protein in advance reduces the influence of a variation in the fluorescent signals among the cells, even if the fusion proteins comprising the proteins to be analyzed are transiently expressed in these cells. This makes it also possible to more stably determine a protein-protein interaction.

Meanwhile, cell transmembrane proteins such as CD80 pass through organelles such as Golgi body and are localized

at the cell membrane. For this reason, when such a protein fused to a tag is expressed, this makes it difficult for the protein to pass through the organelle and reach the cell membrane. Therefore, it is generally said that it is difficult to detect an interaction of a cell membrane protein unlike those of cytoplasmic proteins and the like.

On the other hand, according to the third method for determining a protein-protein interaction of the present invention, since the tag to be fused can be relatively small, this makes it easy for cell transmembrane proteins to reach the cell membrane. Moreover, expressing the tag-fused cell transmembrane proteins such as CD80 on the membrane and then binding the multimerizable protein and the fluorescent protein expressed in the cytoplasm to the tag make it possible to easily detect protein-protein interactions of the cell transmembrane proteins, too, as apparent from the result shown in Fig. 26.

Further, the use of this third determination method also makes it possible to determine protein-protein interactions between separate cells (for example, a protein-protein interaction between PD1 and PDL1), which cannot be detected by techniques such as FRET/BRET/BiFC utilizing the proximity of tags.

(Example 16)

Next, based on the systems shown in Fig. 1 and so forth, the present inventors came up with a system shown

in Fig. 7 as one embodiment for detecting an interaction among three or more proteins.

Specifically, when a thirteenth fusion protein comprising a first protein and a multimerizable protein, a fourteenth fusion protein comprising a second protein and a multimerizable protein, and a fifteenth fusion protein comprising a third protein and a fluorescent protein are expressed in a cell or introduced into a cell, an interaction if any among the first protein, the second protein, and the third protein, as in the cases of the systems shown in Fig. 1 and so forth, induces multimer formation between the multimerizable protein and another multimerizable protein; thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus.

Then, the effectiveness of the system shown in Fig. 7 in determining a protein-protein interaction was confirmed using materials and methods described below. Note that the detection target in this confirmation experiment was heterotrimer formation among p50, p60, and I κ B α .

Note that it has been known that p50 and p65 form a heterodimer, constituting NF κ B. Further, NF κ B functions in the nucleus as a transcription factor playing a role in modulating inflammatory cytokine expression. However,

it has been known that a further interaction with I κ B α retains NF κ B in the cytoplasm, suppressing the transcription function (see Marc D. Jacobs et al., Cell, December 11, 1998, vol. 95, pp. 749 to 758). Thus, in this Example, multimerizable proteins were respectively fused to p50 and p65, while a fluorescent protein was fused to I κ B α . These proteins were expressed in cells. Thereby, p50 and p65 interacted with each other and formed an assembly, and further the I κ B α fused to the fluorescent protein accumulated on the assembly, forming a fluorescent focus. Thus, it was confirmed that the detection of a trimer complex was possible.

<Preparation of pPB1-p50>

To express, as the thirteenth fusion protein, a p50 protein whose N-terminus was fused to a PB1 domain of p62 as the multimerizable protein (PB1-p50) in cells, a pPB1-p50 vector was constructed as described below.

A DNA was artificially synthesized which contained EcoRI and XhoI recognition sequences respectively at both ends of a nucleotide sequence encoding p50 (a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_003989.2). Then, the obtained synthetic DNA was treated with EcoRI and XhoI, and inserted into pAsh-MCLinker having been treated with the same restriction enzymes. Thus, a pPB1-p50 was prepared.

<Preparation of pp65-PB1>

To express, as the fourteenth fusion protein, a p65 protein whose C-terminus was fused to a PB1 domain of p62 as the multimerizable protein (p65-PB1) in cells, a pp65-PB1 vector was constructed as described below.

5 A DNA was artificially synthesized which contained BamHI and XhoI recognition sequences respectively at both ends of a nucleotide sequence encoding p65 (a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_068810.3). Then, the obtained synthetic DNA was
10 treated with BamHI and XhoI, and inserted into pAsh-MNLinker having been treated with the same restriction enzymes. Thus, a pp65-PB1 vector was prepared.

<Preparation of phmKO2-I κ B α >

To express, as the fifteenth fusion protein, an I κ B α
15 protein whose N-terminus was fused to mKO2 as the fluorescent protein (mKO2-I κ B α) in cells, a phmKO2-I κ B α vector was constructed as described below.

A DNA was artificially synthesized which contained BamHI and EcoRI recognition sequences respectively at both
20 ends of a nucleotide sequence encoding I κ B α (a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_065390.1). Then, the obtained synthetic DNA was treated with BamHI and EcoRI, and inserted into phmKO2-MCLinker (manufactured by MEDICAL & BIOLOGICAL
25 LABORATORIES CO., LTD.) having been treated with the same restriction enzymes. Thus, a phmKO2-I κ B α vector was

prepared.

<Cell Culturing and Transfection>

To express the fusion proteins in a combination of the following (A) or (B), the plasmid vectors prepared above were introduced into HEK293 cells by the same method as that described in Example 12:

(A) PB1-p50, p65-PB1, and mKO2-I κ B α ; and

(B) PB1-p50 and mKO2-I κ B α (negative control).

<Observation of Cells>

After the transfection treatment, the HEK293 cells were observed using an IX-71 inverted fluorescence microscope, a U-MGFPHQ filter, and an ORCA-Flash 4.0 digital camera. Fig. 33 shows the obtained result.

As apparent from the result shown in Fig. 33, when the PB1-p50, the p65-PB1, and the mKO2-I κ B α were expressed in the cells, fluorescent foci were detected. On the other hand, when the PB1-p50 and the mKO2-I κ B α were expressed in the cells, no fluorescent focus was detected. Thus, it was confirmed that, as shown in Fig. 7, the interaction between p50 and p65 formed an assembly by utilizing the multimerizable proteins, and further that the I κ B α fused to the fluorescent protein accumulated on the assembly, thereby forming the fluorescent foci. To put it differently, it was confirmed that detecting the fluorescent foci enabled determination of an interaction among three types of proteins.

(Example 17)

As in Example 16, the effectiveness of the system shown in Fig. 7 in determining a protein-protein interaction was confirmed using materials and methods described below. Note that the detection target in this confirmation experiment was heterotrimer formation among CDK4, Cyclin D1, and p21. It has been known that, in the nucleus of a cell, p21 recognizes and interacts with a complex composed of CDK4 and Cyclin D1 (see LaBaer J et al., Genes Dev., April 1, 1997, vol. 11, no. 7, pp. 847 to 862). Moreover, it has also been revealed that such heterotrimer formation inhibits cell-cycle progression (transition from a G1 phase to an S phase) otherwise promoted by a complex composed of CDK4 and Cyclin D1.

<Preparation of pp21-PB1>

To express, as the thirteenth fusion protein, a p21 protein whose C-terminus was fused to a PB1 domain of p62 as the multimerizable protein (p21-PB1) in cells, a pp21-PB1 vector was constructed as described below.

A DNA was artificially synthesized which contained BamHI and NotI recognition sequences respectively at both ends of a nucleotide sequence encoding p21 (a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_510867.1). Then, the obtained synthetic DNA was treated with BamHI and NotI, and inserted into pAsh-MNLinker having been treated with the same restriction

enzymes. Thus, a pp21-PB1 vector was prepared.

<Preparation of pCDK4-PB1>

To express, as the fourteenth fusion protein, a CDK4 protein whose C-terminus was fused to a PB1 domain of p62 as the multimerizable protein (CDK4-PB1), in cells, a pCDK4-PB1 vector was constructed as described below.

A DNA was artificially synthesized which contained BamHI and NotI recognition sequences respectively at both ends of a nucleotide sequence encoding CDK4 (a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_000066.1). Then, the obtained synthetic DNA was treated with BamHI and NotI, and inserted into pAsh-MNLinker having been treated with the same restriction enzymes. Thus, a pCDK4-PB1 vector was prepared.

<Preparation of pCDK4>

To express, as a negative control, a CDK4 protein (CDK4) not fused to other proteins, a pCDK4 vector was constructed as described below.

A DNA was artificially synthesized which contained a BamHI recognition sequence at one end of the nucleotide sequence encoding CDK4 while the stop codon and a NotI recognition sequence at the other end. Then, the obtained synthetic DNA was treated with BamHI and NotI, and inserted into pAsh-MNLinker having been treated with the same restriction enzymes. Thus, a pCDK4 vector was prepared.

<phmAG1-Cyclin D1>

To express, as the fifteenth fusion protein, a Cyclin D1 protein whose N-terminus was fused to mAG1 as the fluorescent protein (mAG1-Cyclin D1) in cells, a phmAG1-CyclinD1 vector was constructed as described below.

5 A DNA was artificially synthesized which contained a BamHI recognition sequence at one end of a nucleotide sequence encoding Cyclin D1 (a protein having the amino acid sequence of NCBI RefSeq ACCESSION No: NP_444284.1) while the stop codon and a NotI recognition sequence at
10 the other end. Then, the obtained synthetic DNA was treated with BamHI and NotI, and inserted into phmAG1-MCLinker having been treated with the same restriction enzymes. Thus, a phmAG1-Cyclin D1 vector was prepared.

<Cell Culturing and Transfection>

15 To express the fusion proteins in a combination of the following (A), (B), or (C), the plasmid vectors prepared above were introduced into HEK293 cells by the same method as that described in Example 14:

(A) mAG1-Cyclin D1, p21-PB1, and CDK4-PB1;

20 (B) mAG1-Cyclin D1, p21-PB1, and PB1 (negative control);
and

(C) mAG1-Cyclin D1, p21-PB1, and CDK4 (negative control).

Note that, in (B), to express PB1 (a PB1 domain of a p62 protein), pAsh-MNLinker was introduced into the
25 cells.

<Observation of Cells>

After the transfection treatment, the HEK293 cells were observed by the same method as that described in Example 14. Fig. 34 shows the obtained result.

As apparent from the result shown in Fig. 34, fluorescent foci were detected in cells expressing the mAG1-Cyclin D1, the p21-PB1, and the CDK4-PB1. On the other hand, when no CDK4 was expressed in the cells, and when the CDK4 not fused to any multimerizable protein was expressed in the cells, no fluorescent focus was detected. Thus, it was confirmed that, as shown in Fig. 7, the interaction between CDK4 and Cyclin D1 formed a complex having the multimerizable protein and the fluorescent protein, and that the p21 fused to the multimerizable protein interacted with the complex, thereby forming the fluorescent foci. To put it differently, it was confirmed that detecting the fluorescent foci enabled determination of an interaction among three types of proteins.

(Example 18)

As in Examples 16 and 17, the effectiveness of the system shown in Fig. 7 in determining a protein-protein interaction was confirmed. Note that the detection target in this confirmation experiment was tetramer formation from calmodulin and a partial sequence (M13 peptide) of myosin light chain kinase 2. It has been revealed that the interaction between calmodulin and an M13 peptide takes place in response to a transient increase in intracellular

calcium ion concentration (second messenger) that occurs when a G protein-coupled receptor (GPCR) receives a ligand (see Miyawaki A et al., Nature, August 28, 1997, vol. 388, no. 6645, pp. 882 to 887). In addition, it has also been
5 revealed that an interaction between calmodulin and a calmodulin-binding peptide such as M13 peptide forms a tetramer composed of two sets of these molecules (see Ye Q et al., Biochemistry, January 24, 2006, vol. 45, no. 3, pp. 738 to 745). Then, it was confirmed using materials
10 and methods described below that the system shown in Fig. 7 was capable of detecting such tetramer formation. Further, it was also confirmed that detecting a fluorescent focus enabled detection of a change in intracellular calcium ion concentration over time.

15 <Preparation of pM13peptide-PB1>

To express, as the thirteenth and fourteenth fusion proteins, an M13 peptide whose C-terminus was fused to a PB1 domain of p62 as the multimerizable protein (M13peptide-PB1) in cells, a pM13peptide-PB1 vector was
20 constructed as described below.

A DNA was artificially synthesized which contained BamHI and EcoRI recognition sequences respectively at both ends of a nucleotide sequence encoding an M13 peptide (the nucleotide sequence of SEQ ID NO: 50). Then, the obtained
25 synthetic DNA was treated with BamHI and EcoRI, and inserted into pAsh-MNLinker having been treated with the same

restriction enzymes. Thus, a pM13peptide-PB1 vector was prepared.

<Preparation of pCalumodulin-hmAG1 and pCalumodulin-hmUkG1>

5 To express, as the fifteenth fusion protein, a calmodulin protein whose C-terminus was fused to mAG1 as the fluorescent protein (Calumodulin-mAG1), or to express, as the fifteenth fusion protein, a calmodulin protein whose C-terminus was fused to mUkG1 as the fluorescent protein
10 (Calumodulin-mUkG1) in cells, a pCalumodulin-hmAG1 vector and a pCalumodulin-hmUkG1 vector were constructed as described below.

A DNA was artificially synthesized which contained BamHI and EcoRI recognition sequences respectively at both
15 ends of a nucleotide sequence encoding calmodulin (the nucleotide sequence of SEQ ID NO: 52). Then, the obtained synthetic DNA was treated with BamHI and EcoRI, and inserted into phmAG1-MNLinker and phmUkG1-MNLinker (both manufactured by MEDICAL & BIOLOGICAL LABORATORIES CO.,
20 LTD.) having been treated with the same restriction enzymes. Thus, a pCalumodulin-hmAG1 vector and a pCalumodulin-hmUkG1 vector were prepared.

<Cell Culturing and Transfection>

25 To express the fusion proteins in a combination of the following (A) or (B), the plasmid vectors prepared above were introduced into HeLaS3 cells by a method described

below:

(A) Calumodulin-mAG1 and M13peptide-PB1; and

(B) Calumodulin-mUkG1 and M13peptide-PB1.

First, HeLaS3 cells were cultured in DMEM (low
5 glucose) containing 10% FBS (manufactured by Equitech-Bio
Inc.). Then, on the day before the transfection, the HeLaS3
cells were seeded onto a 35-mm glass base dish (manufactured
by Asahi Glass Co., Ltd.). Subsequently, at the time of
the transfection, the plasmids (1 μ g + 1 μ g) were diluted
10 with OptiMEM, and 10 μ l of PolyFect(registered trademark)
Transfection Reagent was added thereto and stirred. The
resultant was further mixed with 600 μ l of the culture
solution, thereafter added to the HeLaS3 cells, and
cultured for 22 hours.

15 <Observation of Cells>

Using an IX-71 inverted fluorescence microscope, a
U-MGFPHQ filter, and an ORCA-ER digital camera, the
transfected HeLaS3 cells cultured for 22 hours above were
observed in a buffer at pH of 7.4 containing Hanks' Balanced
20 Salt Solutions and 20 mM HEPES. Then, 100 μ M histamine
(manufactured by Wako Pure Chemical Industries, Ltd.) was
added thereto, and fluorescence images were captured over
time. Note that it has been revealed that histamine
functions as a ligand of an H1 receptor, one of GPCRs, which
25 is expressed also in HeLaS3 cells. Figs. 35 and 36 show
the obtained result.

As apparent from the result shown in Figs. 35 and 36, before the ligand (histamine) was added, the Calmodulin-mAG1 and the Calmodulin-mUkG1 were present in a dispersed manner in the cells. Meanwhile, 60 seconds after the ligand was added, fluorescent focus formation was detected, confirming the assembly formation containing the M13peptide-PB1. Moreover, as a result of the subsequent continuous observation, 300 seconds after the ligand addition, the fluorescent foci (assemblies) were extinguished as shown in Fig. 35 because the cytoplasm calcium ion concentration transiently increased by adding the ligand was decreased.

Thus, it was confirmed that the present invention was also capable of detecting such heterotetramer formation. Further, it was also revealed that the use of the method for detecting a protein-protein interaction of the present invention enabled real-time measurement of the calcium ion concentration transiently increased by the signal transduction from the H1 receptor. Moreover, the result of this Example also demonstrated that the application was eventually possible to detection of and screening for: an endogenous factor such as a second messenger causing a protein-protein interaction; a signal transduction to which the second messenger or the like contributed; and a stimulus from the outside such as an extracellular ligand eliciting the signal transduction.

(Example 19)

Based on the above results, the present inventors came up with a system shown in Fig. 8 as a system for determining a protein-protein interaction.

5 Specifically, the inventors came up with a construction of a system in which a sixteenth fusion protein comprising a first protein, a first multimerizable protein, and a fluorescent protein, and a seventeenth fusion protein comprising a second protein and a second multimerizable
10 protein are expressed in a cell or introduced into a cell. In a case where the first protein is a different protein from the second protein and the first multimerizable protein is a different protein from the second multimerizable protein, the first protein and the second
15 protein interact with each other, further inducing multimer formation between the multimerizable protein and another multimerizable protein; thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent
20 focus.

Then, the effectiveness of the system shown in Fig. 8 in determining a protein-protein interaction was confirmed using materials and methods described below. Note that the detection target in this examination was an
25 interaction between a p53 protein and an MDM2 protein, and an inhibitor Nutlin-3 against the interaction was also used

in this examination. Further, in this examination, a SAM domain of a Tankyrase 1 protein, a PB1 domain of a PKC α protein, a SAM domain of a TEL protein, and a SAM domain of a DGK delta protein were used as the multimerizable proteins in appropriate combinations. Moreover, a mAG1 protein was used as the fluorescent protein. Note that, in the system shown in Fig. 8, the fluorescent protein is fused to only one of the proteins to be analyzed for the protein interaction. Nevertheless, in this Example, the fluorescent protein was fused to both of the proteins for the examination.

<Preparation of Plasmid Vectors for Expressing Various Fusion Proteins>

pTankyrase-hmAG1-p53, pPKC α -hmAG1-p53, pTEL-hmAG1-p53, and pDGKd-hmAG1-p53 prepared as described above were treated with restriction enzymes NheI and AgeI. Thereby, nucleotide fragments Tankyrase-hmAG1, PKC α -hmAG1, TEL-hmAG1, and DGKd-hmAG1 were prepared. Then, these were inserted into pPB1-p53(70) which had been treated with the same restriction enzymes, and from which the PB1 region had been removed. Thus, the following plasmid vectors were prepared:

- (a) pTankyrase-hmAG1-p53(70),
- (b) pPKC α -hmAG1-p53(70),
- (c) pTEL-hmAG1-p53(70), and
- (d) pDGKd-hmAG1-p53(70).

In addition, the pPB1-MDM2 prepared in Example 4 was treated with NheI and AgeI to remove the PB1 region. Then, the above nucleotide fragments were inserted therein. Thus, the following plasmid vectors were also prepared:

- (e) pTankyrase-hmAG1-MDM2,
- (f) pPKCi-hmAG1-MDM2,
- (g) pTEL-hmAG1-MDM2, and
- (h) pDGKd-hmAG1-MDM2.

<Cell Culturing and Transfection>

The plasmid vectors (a) to (h) prepared as described above were introduced into HEK293 cells by a method described below in the following combinations:

- combination 1: plasmid vectors (b) and (h),
- combination 2: plasmid vectors (b) and (g),
- combination 3: plasmid vectors (a) and (f),
- combination 4: plasmid vectors (a) and (h),
- combination 5: plasmid vectors (a) and (g),
- combination 6: plasmid vectors (d) and (f),
- combination 7: plasmid vectors (d) and (g),
- combination 8: plasmid vectors (c) and (e), and
- combination 9: plasmid vectors (c) and (h).

HEK293 cells were cultured in DMEM (high glucose) supplemented with 10% FBS and 1% penicillin-streptomycin. Then, on the day before the transfection, the cells were seeded onto a 96-well multiwell plate (manufactured by Greiner Bio-One International GmbH), and cultured in 100

μL of the culture solution per well. Subsequently, a DNA solution prepared by mixing 200 ng of each of the plasmids was diluted with 10 μL of OptiMEM. Thereafter, 0.8 μL of Eugene HD was added thereto and then stirred. The resultant
5 was further mixed with 90 μL of the culture solution. After that, the obtained mixture solution was added to the cultured cells and cultured for 20 hours.

<Observation of Cells>

After the culturing, the culture solution of the cells
10 was discarded, and 100 μL of a buffer at pH 7.4 containing Hanks' Balanced Salt Solutions and 20 mM HEPES was added. Then, the cells were observed using IN Cell Analyzer 1000. Next, Nutlin-3 was added to each well to the final concentration of 40 μM. After left standing at room
15 temperature for 1 hour, the cells were observed. Figs. 37 to 45 show the obtained result.

As apparent from the result shown in Figs. 37 to 45, distinctive fluorescent foci were observed in all the combinations before Nutlin-3 was added. On the other hand,
20 as a result of the re-observation 30 minutes after the Nutlin-3 addition, the fluorescent foci were extinguished, and the fluorescent signals were dispersed.

In this manner, the system shown in Fig. 8 was also capable of detecting fluorescent foci formed dependently
25 on the interaction between p53 and MDM2, and the effectiveness of the system was demonstrated.

[Industrial Applicability]

As has been described above, the present invention makes it possible to determine a protein-protein interaction in an intracellular environment unique thereto, and to obtain positional information and temporal information on the protein-protein interaction. Moreover, in the present invention, a strength of a protein-protein interaction correlates with the fluorescence intensity of a fluorescent focus. Accordingly, the method is utilizable in identifying an amino acid residue involved in a protein-protein interaction, and also in screening for a substance modulating a protein-protein interaction, on the basis of the fluorescence intensity.

Thus, the method for determining a protein-protein interaction and so forth of the present invention and a vector or a kit for use in these methods are useful in the development of pharmaceutical products and so on through elucidations of various signal transductions *in vivo*, various biological reaction controls, and the like, and eventually, through elucidations of disease mechanisms.

[Sequence Listing Free Text]

SEQ ID NOs: 1 and 2

<223> PB1 domain of p62

SEQ ID NOs: 3 and 4

<223> PB1 domain of TFG

SEQ ID NOs: 5 and 6

<223> PB1 domain of PKCιota
 SEQ ID NOs: 7 and 8
 <223> SAM domain of TEL
 SEQ ID NOs: 9 and 10
 5 <223> SAM domain of DGK delta
 SEQ ID NOs: 11 and 12
 <223> SAM domain of Tankyrase-1
 SEQ ID NOs: 13 and 14
 <223> humanized-codon monomeric Azami Green 1 (mAG1)
 10 SEQ ID NOs: 15 and 16
 <223> monomeric Umikinoko-Green (mUKG)
 SEQ ID NOs: 17 and 18
 <223> monomeric Kusabira-Orange 2 (mK02)
 SEQ ID NOs: 19 and 20
 15 <223> monomeric Midoriishi-Cyan1 (mMiCy)
 SEQ ID NOs: 21 and 22
 <223> Kusabira-Cyan1 (KCy1)
 SEQ ID NOs: 23 and 24
 <223> dimeric Azami-Green (AB) (dAG (AB))
 20 SEQ ID NOs: 25 and 26
 <223> TGuv
 SEQ ID NOs: 27 and 28
 <223> PB1hmAG1
 SEQ ID NOs: 29 and 30
 25 <223> hmAG1PB1
 SEQ ID NOs: 31 and 32

<223> FKBP12 mutant
SEQ ID NOs: 33 and 34
<223> p53
SEQ ID NOs: 35 and 36
5 <223> MDM2
SEQ ID NOs: 37 and 38
<223> PB1
SEQ ID NOs: 39 and 40
<223> FusionRed
10 SEQ ID NOs: 42 and 43
<223> Tankyrase-hmAG1
SEQ ID NOs: 44 and 45
<223> PKCιota-hmAG1
SEQ ID NOs: 46 and 47
15 <223> TEL-hmAG1
SEQ ID NOs: 48 and 49
<223> DGK delt-hmAG1
SEQ ID NOs: 50 and 51
<223> M13 peptide
20 SEQ ID NOs: 52 and 53
<223> calmodulin

[CLAIMS]

[Claim 1]

A method for determining an interaction between a first protein and a second protein, the method comprising the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell

a first fusion protein comprising the first protein, a multimerizable protein, and a fluorescent protein, and

a second fusion protein comprising the second protein and a multimerizable protein;

(2) detecting a fluorescent focus formed by an association between the first fusion protein and the second fusion protein in the cell; and

(3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

[Claim 2]

The method according to claim 1, wherein the second fusion protein further comprises a fluorescent protein.

[Claim 3]

The method according to claim 1 or 2, wherein the fluorescent protein is a monomeric fluorescent protein.

[Claim 4]

A method for detecting an interaction between a first protein and a second protein, the method comprising the

following steps (1) to (3):

(1) expressing in a cell or introducing into a cell
a third fusion protein comprising the first
protein and a multimerizable protein,

5 a fourth fusion protein comprising the second
protein and a multimerizable protein, and

a fifth fusion protein comprising a
multimerizable protein and a fluorescent protein;

(2) detecting a fluorescent focus formed by an
10 association among the third fusion protein, the fourth
fusion protein, and the fifth fusion protein in the cell;
and

(3) determining an interaction between the first
protein and the second protein according to the detection
15 of the fluorescent focus.

[Claim 5]

The method according to claim 4, wherein the
fluorescent protein is a monomeric fluorescent protein.

[Claim 6]

20 A vector comprising a DNA encoding a multimerizable
protein, a DNA encoding a fluorescent protein, and a cloning
site, wherein

the vector is capable of expressing a fusion protein
comprising the multimerizable protein, the fluorescent
25 protein, and a certain protein when a DNA encoding the
certain protein is inserted in the cloning site.

[Claim 7]

A kit for use in the method according to any one of claims 1 to 3, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

(a) the vector according to claim 6;

(b) a vector encoding the first fusion protein;

(c) a vector encoding the second fusion protein;

(d) a transformed cell comprising a vector encoding the first fusion protein;

(e) a transformed cell comprising a vector encoding the second fusion protein;

(f) a transformed cell comprising a vector encoding the first fusion protein and a vector encoding the second fusion protein;

(g) the first fusion protein; and

(h) the second fusion protein.

[Claim 8]

A kit for use in the method according to claim 4 or 5, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

(a) a vector comprising a DNA encoding the multimerizable protein and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the multimerizable protein and a certain protein when a

DNA encoding the certain protein is inserted in the cloning site;

(b) a vector encoding the third fusion protein;

(c) a vector encoding the fourth fusion protein;

5 (d) a vector encoding the fifth fusion protein;

(e) a transformed cell comprising a vector encoding the fifth fusion protein;

(f) the third fusion protein;

(g) the fourth fusion protein; and

10 (h) the fifth fusion protein.

[Claim 9]

A method for detecting an interaction between a first protein and a second protein, the method comprising the following steps (1) to (3):

15 (1) expressing in a cell or introducing into a cell a first labeled protein comprising an affinity tag and the first protein,

a second labeled protein comprising an affinity tag and the second protein, and

20 a sixth fusion protein comprising a multimerizable protein, a fluorescent protein, and a binding partner having an affinity for any of the affinity tags and bound to the sixth fusion protein;

(2) detecting a fluorescent focus formed by an association among the first labeled protein, the second labeled protein, and the sixth fusion protein comprising

25

the binding partner bound thereto in the cell; and

(3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

5 [Claim 10]

A kit for use in the method according to claim 9, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

10 (a) a vector comprising a DNA encoding the affinity tag and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the affinity tag when a DNA encoding a certain protein is inserted in the cloning site;

15 (b) a vector encoding the first labeled protein;

(c) a vector encoding the second labeled protein;

(d) a vector encoding the sixth fusion protein comprising the binding partner bound thereto;

20 (e) a transformed cell comprising a vector encoding the sixth fusion protein comprising the binding partner bound thereto;

(f) the first labeled protein;

(g) the second labeled protein; and

25 (h) the sixth fusion protein comprising the binding partner bound thereto.

[Claim 11]

A method for detecting an interaction between a first protein and a second protein, the method comprising the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell
5 a seventh fusion protein comprising the first protein and a first partial peptide constituting a fluorescent protein,

an eighth fusion protein comprising the second protein and a first partial peptide constituting a
10 fluorescent protein, and

a ninth fusion protein comprising a multimerizable protein and a second partial peptide capable of reconstituting any of the fluorescent proteins by binding to the first partial peptide;

15 (2) detecting a fluorescent focus formed by an association among the seventh fusion protein, the eighth fusion protein, and the ninth fusion protein in the cell; and

(3) determining an interaction between the first
20 protein and the second protein according to the detection of the fluorescent focus.

[Claim 12]

A kit for use in the method according to claim 11, the kit comprising an instruction and at least one substance
25 selected from the group consisting of the following (a) to (h):

(a) a vector comprising a DNA encoding the first partial peptide and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the first partial peptide when a DNA encoding a certain protein is inserted in the cloning site;

(b) a vector encoding the seventh fusion protein;

(c) a vector encoding the eighth fusion protein;

(d) a vector encoding the ninth fusion protein;

(e) a transformed cell comprising a vector encoding the ninth fusion protein;

(f) the seventh fusion protein;

(g) the eighth fusion protein; and

(h) the ninth fusion protein.

[Claim 13]

A method for detecting an interaction between a first protein and a second protein, the method comprising the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell a tenth fusion protein comprising the first protein and a third partial peptide constituting a multimerizable protein,

an eleventh fusion protein comprising the second protein and a third partial peptide constituting a multimerizable protein, and

a twelfth fusion protein comprising a fluorescent protein and a fourth partial peptide capable

of reconstituting any of the multimerizable proteins by binding to the third partial peptide;

(2) detecting a fluorescent focus formed by an association among the tenth fusion protein, the eleventh fusion protein, and the twelfth fusion protein in the cell; and

(3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

[Claim 14]

A kit for use in the method according to claim 13, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

(a) a vector comprising a DNA encoding the third partial peptide and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the third partial peptide when a DNA encoding a certain protein is inserted in the cloning site;

(b) a vector encoding the tenth fusion protein;
(c) a vector encoding the eleventh fusion protein;
(d) a vector encoding the twelfth fusion protein;
(e) a transformed cell comprising a vector encoding the twelfth fusion protein;

(f) the tenth fusion protein;
(g) the eleventh fusion protein; and

(h) the twelfth fusion protein.

[Claim 15]

A method for detecting an interaction among a first protein, a second protein, and a third protein, the method comprising the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell

a thirteenth fusion protein comprising the first protein and a multimerizable protein,

a fourteenth fusion protein comprising the second protein and a multimerizable protein, and

a fifteenth fusion protein comprising the third protein and a fluorescent protein;

(2) detecting a fluorescent focus formed by an association among the thirteenth fusion protein, the fourteenth fusion protein, and the fifteenth fusion protein in the cell; and

(3) determining an interaction among the first protein, the second protein, and the third protein according to the detection of the fluorescent focus.

[Claim 16]

A kit for use in the method according to claim 15, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (h):

(a) a vector comprising a DNA encoding the multimerizable protein and a cloning site, wherein the

vector is capable of expressing a fusion protein comprising the multimerizable protein when a DNA encoding a certain protein is inserted in the cloning site;

(b) a vector comprising a DNA encoding the fluorescent protein and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the fluorescent protein when a DNA encoding a certain protein is inserted in the cloning site;

(c) a vector encoding the thirteenth fusion protein;

(d) a vector encoding the fourteenth fusion protein;

(e) a vector encoding the fifteenth fusion protein;

(f) the thirteenth fusion protein;

(g) the fourteenth fusion protein; and

(h) the fifteenth fusion protein.

[Claim 17]

A method for determining an interaction between a first protein and a second protein, wherein

a first multimerizable protein is a different protein from a second multimerizable protein, and

the method comprises the following steps (1) to (3):

(1) expressing in a cell or introducing into a cell

a sixteenth fusion protein comprising the first protein, the first multimerizable protein, and a fluorescent protein, and

a seventeenth fusion protein comprising the second protein and the second multimerizable protein;

(2) detecting a fluorescent focus formed by an association between the sixteenth fusion protein and the seventeenth fusion protein in the cell; and

(3) determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

[Claim 18]

A kit for use in the method according to claim 17, the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (f):

(a) a vector comprising a DNA encoding the first multimerizable protein, a DNA encoding the fluorescent protein, and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the first multimerizable protein, the fluorescent protein, and a certain protein when a DNA encoding the certain protein is inserted in the cloning site;

(b) a vector comprising a DNA encoding the second multimerizable protein, a DNA encoding the fluorescent protein, and a cloning site, wherein the vector is capable of expressing a fusion protein comprising the second multimerizable protein, the fluorescent protein, and a certain protein when a DNA encoding the certain protein is inserted in the cloning site;

(c) a vector encoding the sixteenth fusion protein;

- (d) a vector encoding the seventeenth fusion protein;
- (e) the sixteenth fusion protein; and
- (f) the seventeenth fusion protein.