Abstract:

Isolated protein complexes having long fluorescent lifetimes that can be 4.0 ns or greater.

(54) Title: GENETICALLY ENCODED SENSORS FOR IMAGING PROTEINS AND THEIR COMPLEXES

(57) Abstract: Isolated truncated and mutated sensor proteins derived from flavoproteins that are 12-20KDa or less, genetically encoded for detection and imaging of protein complexes having long fluorescent lifetimes that can be 4.0 ns or greater.
— with sequence listing part of description (Rule 5.2(a))

(88) Date of publication of the international search report:
16 April 2015

(15) Information about Correction.

Previous Correction:
see N-te of 26 February 2015
A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07D 475/00; C11C 5/02; C12P 21/06; C12N 15/00; C07K 1/00 (2014.01 )
CPC - C07D 475/02; G01N 21/314; A61K 38/00; C12N 15/86
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
CPC: C07D 475/02; G01N 21/314; A61K 38/00; C12N 15/86
USPC: 544/257; 356/51 ; 435/69.1 , 320.1 ; 530/350 (text search)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: C07D 475/02; G01N 21/314; A61K 38/00; C12N 15/86 (text search)
USPC: 544/257; 356/51 ; 435/69.1 , 320.1 ; 530/350 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data base: PatBase; Google Scholar; Google Patents; GenCore sequence search
Search terms: LUMP protein (lumazine encoding protein), fluorescence anisotropy, genetically-encoded sensor, fusion protein, target molecule imaging

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>LIN et al. The lumazine protein-encoding gene in Photobacterium leiognathi is linked to the Lux operon. Gene 15 April 1993 Vol 126 No 1 Pages 153-154. Especially pg 154 fig 1.</td>
<td>2</td>
</tr>
<tr>
<td>Y</td>
<td>VISSER et al. Determination of rotational correlation times from deconvolved fluorescence anisotropy decay curves. Demonstration with 6,7-dimethyl-8-ribityllumazine and lumazine protein from Photobacterium leiognathi as a fluorescent indicator. Biochemistry 12 March 1985 Vol 24 No 6 pages 1489-1496. Especially pg 1492 Table 1.</td>
<td>3, 6-11</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C.

- Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "&" document member of the same patent family

Date of the actual completion of the international search
02 December 2014 (02.12.2014)

Date of mailing of the international search report
22 Dec 2014

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
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Authorized officer:
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PCT OSP: 571-272-7774
### Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.:  
   because they relate to subject matter not required to be searched by this Authority, namely:

2. □ Claims Nos.:  
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. □ Claims Nos.:  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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go to Extra Sheet for continuation
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1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   Claims 1-8 and 9-11 (in part)

**Remark on Protest**

- □ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- □ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- □ No protest accompanied the payment of additional search fees.
<table>
<thead>
<tr>
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Stickand et al. &quot;Light-activated DNA binding in a designed allosteric protein&quot; Proc Nat Acad Sci 5 August 2008 Vol 105 No 31 Pages 10709-14; pg 10711 col 2 para 2-3, Fig. 1D, Fig. 2A-C</td>
<td>1, 9</td>
</tr>
</tbody>
</table>
Continuation of Box III (Lack of Unity of Invention)

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-8 and 9-11 (in part), drawn to an isolated truncated and mutated sensor protein derived from LUMP that is 12KDa or less, genetically encoded for detection and imaging of protein complexes, wherein the sensor protein has a sequence comprising SEQ ID NO: 8 or 9, be 10KDa in size or less, have a fluorescent anisotropic lifetime that is greater than 4.0 ns, and be covalently linked to a targeting protein. In some inventions, the fluorescent anisotropy sensor based protein comprises isolated and truncated N-terminal riboflavin synthase (N-RFS, SEQ ID NO: 11).

Group II: Clairiris 9-11 (in part) and 16-24, drawn to an isolated fluorescent variant of LOV2, wherein the variant is truncated such that the variant is 12KDa or less in size, wherein the variant has a sequence that is at least 90% identical to SEQ ID NO 4, and wherein the variant has a fluorescent anisotropic lifetime that is greater than 4 ns. In some inventions, the LOV2 protein of Group II may also have sequence identity of at least 75% to SEQ ID NO: 4, be 10KDa in size or less, and be covalently linked to a targeting protein. In some inventions, the fluorescent anisotropy based sensor comprises isolated and truncated N-terminal riboflavin synthase (N-RFS, SEQ ID NO: 11).

Group III: Claims 12-15, drawn to a method of detecting a target molecule, the method comprising:
- providing an amino acid based fluorescent molecule, wherein the fluorescent molecule is covalently linked to a targeting molecule that binds to the target molecule, and wherein the fluorescent molecule has a fluorescent anisotropic lifetime that is greater than 4.0 ns;
- adding the fluorescent molecule to a sample; and detecting if there is a change in fluorescent anisotropy of the fluorescent molecule.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I requires the special technical feature of an isolated truncated and mutated sensor protein derived from LUMP, not required by Groups II and III.

Group II requires the special technical feature of an isolated and truncated fluorescent variant of LOV2, not required by Groups I and III.

Group III has the special technical feature of a method of detecting a target molecule using an amino acid based fluorescent molecule, not required by Groups I or II.

Common Technical Feature:

Groups I, II and III share the common technical feature of a fluorescent anisotropy based sensor, the sensor comprising: a targeting protein; and a fluorescent molecule that is covalently linked to the targeting molecule, wherein the fluorescent molecule is a truncated variant of a protein wherein the truncated variant is no greater in size than about 10 KDa, and wherein the truncated variant has an anisotropic lifetime of greater than 4 ns.

However, said common technical feature does not represent a contribution over the prior art, and is obvious over the publication titled "Light-activated DNA binding in a designed allosteric protein" by Strickland et al. (hereinafter "Strickland") [Proc Nat Acad Sci 5 August 2008 Vol 105 No 31 Pages 10709-14], in view of the publication titled "Purification and Characterization of the Amino-Terminal Domain of Lumazine Protein from Photobacterium leiognathi" by Kim et al. (hereinafter "Kim") [Bull Korean Chem Soc 2010, Vol. 31, No. 4 Pages 1017-1020], in final view of the publication titled "Determination of rotational correlation times from deconvoluted fluorescence anisotropy decay curves. Demonstration with 6,7-dimethyl-8-ribityllumazine and lumazine protein from Photobacterium leiognathi as fluorescent indicators" by Visser et al. (hereinafter "Visser") [Biochemistry 12 March 1995 Vol 24 No 6 pages 1489-1496].

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Strickland teaches a targeting protein [trpR] and a fluorescent molecule [Lov2 domain] that is covalently linked to the targeting (protein), wherein the fluorescent molecule is a truncated variant of a protein (pg 1071 col 2 para 2-3; We ligated AsoLOV2 (residues 404-543) via its carboxyl-terminal Jalpha-helix to a succession of 13 amino-terminal truncations of TrpR (residues 11-108) (Fig. 1D). One construct, which we refer to as the LOV- and tryptophan activated protein (LovTAP), preferentially protects cognate DNA when illuminated (Fig. 2 A-C). This construct joins the carboxyl terminus of the J-alpha helix of the LOV domain to the middle of the amino-terminal helix of TrpR at Phe 22 (Fig. 1D). At saturating photoexcitation (20 mWxcmexp-2 irradiance at 470 nm) and micromolar LovTAP concentration, the apparent rate of Real digestion of cognate DNA is decreased compared with the rate in the dark. DNA binding is specific for the trp operator and requires free L-tryptophan (data not shown), suggesting that LovTAP binds DNA in a manner that is characteristic of the TrpR domain. Strickland does not teach truncated variant is no greater in size than about 10 KDa, or that the truncated variant has an anisotropic lifetime of greater than 4 ns. However, truncated variants of fluorescent protein LUMP of about 10KDa were well known in the art, as taught by Kim (pg 1018 col 1 para 1-2; The translated amino acid sequences of the protein coded by N-LumP gene, produced by PCR, is shown in Figure 1A (N-LP) and 1B (upper amino acid sequences) [...] A recombinant E. coli strain carrying the plasmid pPHL36-N produced N-LumP to a level of 5 - 10% of cell protein following induction with IPTG. The apparent mass as observed by SDS/PAGE was about 11 kDa in agreement with the predicted mass of the recombinant protein of 10,550 Da (Figure 2)*: pg 1018 fig 4* Relative fluorescence intensity with increasing concentrations of fluorescent ligand 6,7-dimethyl-8-ribityllumazine*). Additionally, Visser teaches that the LUMP protein has a fluorescent anisotropic lifetime >4ns, [i.e. 15ns] (pg 1492 Table i: Parameters Describing Fluorescence and Anisotropy Decay of Free and Bound 6,7-Dimethyl-8-ribityllumazine. 7.7 uM lumazine protein, 2C T=15 nanoseconds*). It would have been obvious for an artisan of ordinary skill to construct a fluorescent N-terminal domain of LUMP (10.5KDa), as taught by Kim covalently fused to the (targeting protein) trpR taught by Strickland to generate a fluorescent based anisotropic sensor, where the fluorescent N-terminal LUMP domain would have a predicted a fluorescent anisotropic lifetime [i.e. tau] of about 15ns.

Groups I and II further share the common technical feature of a isolated and truncated N-terminal riboflavin synthase which is taught by Kim (Fig. 1A and Fig. 2A, Purification of amino- terminal domain of riboflavin synthase (N-RS) from E. Coli). As the common technical feature was known in the art at the time of the invention, this cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I-III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.