Screening combinatorial libraries to aid in total defense : developing sensors for chemical weapons and explosives

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2007

Chen, M. W. (2007, March). Screening combinatorial libraries to aid in total defense: developing sensors for chemical weapons and explosives. Presented at Discover URECA @ NTU poster exhibition and competition, Nanyang Technological University, Singapore.

https://hdl.handle.net/10356/95501

NANYANG TECHNOLOGICAL UNIVERSITY

URECA *Undergraduate Research Experience on CAmpus*

Category: 6
Project ID: SPMS06020

Screening Combinatorial Libraries to Aid in Total Defense: Developing Sensors for Chemical Weapons and Explosives

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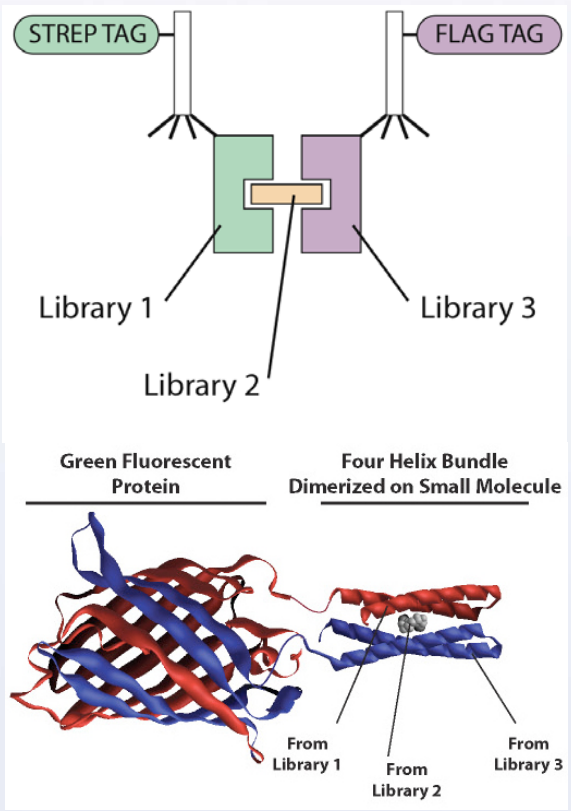
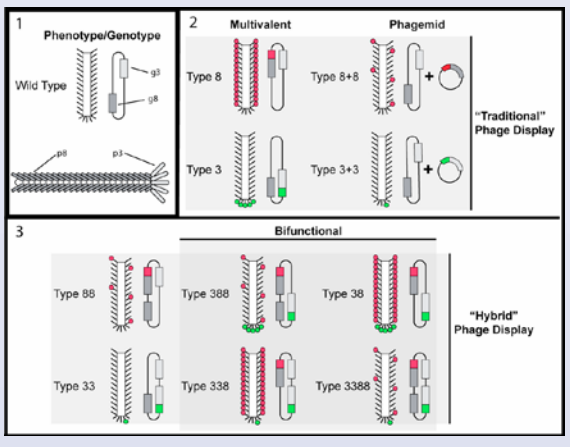
Development of biosensors as fusion of green fluorescent protein (GFP) fragments and Rop protein dimers evolved through a novel phage display technique

Proteins which fluoresce when target molecules (e.g. those associated with chemical weapons or explosives) are present will be developed. These sensors could provide an alternative to dangerous mine-detection methods and help to ensure public safety in the face of bioterrorism. A novel M13 phage display vector is designed for *in vitro* selection of Rop varients, which dimerize only in the presence of target small molecules. Then, the Rop monomers will be fused to GFP fragments to generate the biosensors.



1. Novel "338" M13 phage display vector

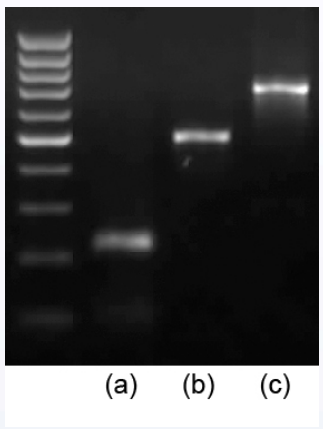
- The commercially available (New England Biolabs) phage display cloning vector, M13KE, will be modified to monovalently display libraries of small proteins, without phagemid or helper phage-based technology.
- A second copy of gene III under the control of a *lac* promoter, and containing SacII and SpeI cloning sites, will be engineered into M13KE. To this second gene III will be fused genes for library proteins. Two protein products of gene III will be produced – one wildtype and one as a fusion. Both of these proteins will be assembled into the M13 capsid thereby ensuring a low copy number of the library protein on the surface of the phage.
- Wildtype gene VIII in M13KE will be engineered to multivalently display either FLAG- and Strep-tags on major coat protein VIII for each of the two phage libraries. Engineering of BbsI and StyI sites will allow insertion and optimization of tag sequences.
- Mutagenic PCR and modified OE-PCR (overlap-extension PCR) gene synthesis will be used to engineer two libraries of Rop protein (see section 2 for design details), which will be ligated into the SacII and SpeI cloning sites to fuse the *rop* gene into M13KE gene III.



Assembly of engineered gene III.

(Product *a*) *lac* promoter-gIII signal sequence assembled using overlapping oligonucleotides:

F1: GCCGCAGTGAGCGCAACGCAATTAATGT
R1: CAATTAATGTGAGTTAGCTCACTCA
F2: AGCTCACTCATTAGGCACCCCAGGC
R2: CACCCCAGGCTTTACACTTTATGCT
F3: ACTTTATGCTTCCGGCTCGTATGTT
R3: CTCGTATGTTGTGTGGAATTGTGAG
F4: GAATTGTGAGCGGATAACAATTTCA
R4: AACAATTTCACACAGGAAACAGCTG
F5: GAAACAGCTGTGAAAAAATTATTAT
R5: AAATTATTATTCGCAATTCCTTTAG
F6: ATTCCTTTAGTGGTACCTTTCTATT
R6: CCTTTCTATTCTCACTCGCCGCGGT
F7: TCGCCGCGGTCCTGATTTCGGACTA
R7: TTTCGGACTAGTGGTGGCTCTGGTT



(Product *b*) Truncated gIII (product b, encoding aa 269 – 424) was acquired by PCR:

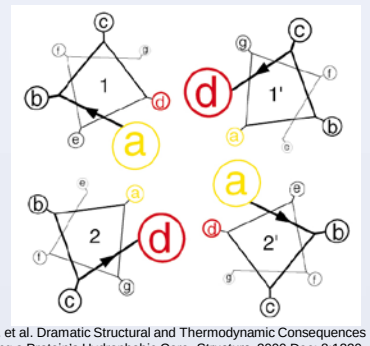
M13F3: ACTAGTGGTGGCTCTGGTTCCGGTGATTTTG
M13R3: CCGGAATTCGCGCACTGCTTATTAAGACTCCTTATTAC

(Product *c*) Fragments *a* and *b* were then joined, first by primerless PCR to fill gaps, then primered PCR for amplification.

2. Randomization of Rop core residues and hence hydrophobic core "holes"

- Rop is a helix-turn-helix protein which can dimerize to form four-helix bundles due to its hydrophobic interface. Native Rop dimers are positioned anti-parallel to each other.
- Varient Ala_2Ile_2-6 dimerizes in a parallel orientation due to reengineered hydrophobic core.
- Randomizing hydrophobic residues *a*, *d*, *e* and *g* potentially creates "holes" into which "knobs" (small molecules of interest) can fit, facilitating dimerization of Rop monomers. Thus engineered Rop will serve as the sensor domain in our biosensor proteins.

Residues *a* and *d* are hydrophobic while residues *g* and *e* are polar. These will be randomized using oligonucleotides randomized at fixed positions. The resultant *rop* genes will then be transformed into two phage display libraries.



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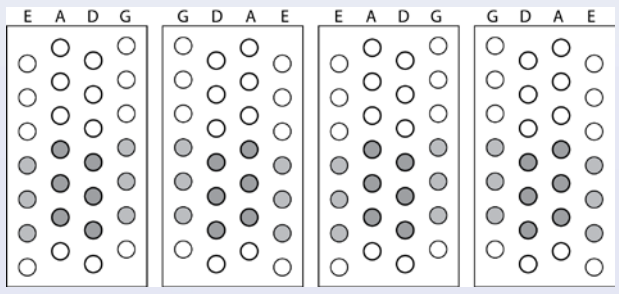
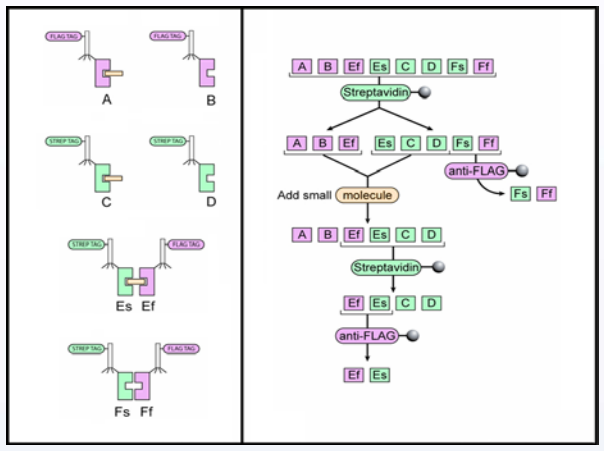


3. Library vs. library vs. library panning procedure selects for target-dependent dimerizing pairs of Rop

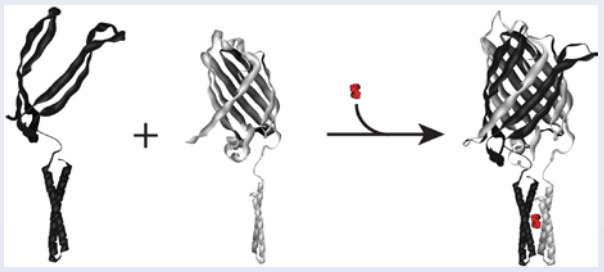
- Library 1: Phage displaying monovalent Rop varient on protein III; multivalent FLAG-tag on protein VIII.
- Library 2: Small molecules of interest i.e. safe varients of those associated with chemical weapons or explosives, e.g. 2,4-DNT, PMPA, etc.
- Library 3: Phage displaying monovalent Rop varient on protein III; multivalent Strep-tag on protein VIII.
- Selection is based on affinities of the orthogonally tagged protein VIII for agarose beads coated with anti-FLAG antibody and streptavidin.
- Two phage which dimerize only in the presence of the small molecule will be retained finally.
- The varient *rop* genes are packaged within the phage. Repeated panning enriches phage which carry our desired *rop* genes.
- This methodology can be used to screen for conditional dimerizing partners for various purposes in homeland security, biomedicine, etc.

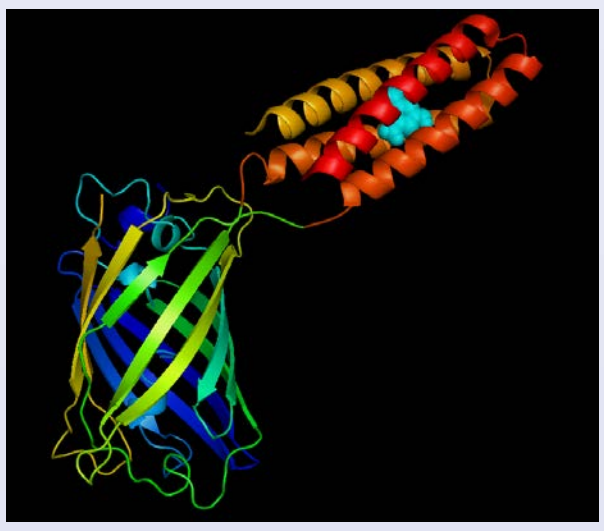


The proposed panning procedure removes false positive dimerizing partners (Fs-Ff) and retains true positive partners (Es-Ef) which dimerize only when the small molecule of interest is present.

4. Fusion of Rop varients to GFP fragments generates fluorescent biosensor molecules

- GFP is a unique fluorescent protein which does not require exogenous substrates, can be expressed in most cell-types and subcellular compartments, and highly stable once formed.
- When dissected and separately expressed, GFP fragments are insoluble. The fluorophore does not mature, hence no fluorescence.
- NGFP (aa 1 – 157) and CGFP (aa 158 – 238) can be fused to soluble dimerizing partners. When dimerization occurs, reassembly of GFP fragments follows irreversibly and effectively.
- The two fragments of GFP will be fused to Rop varient dimerizing partners generated through phage display. The fusion protein should reassemble in the presence of the small target molecule, allowing fluorophore maturation. Hence, the small molecule will set off a fluorescent signal, either in living cells or on protein chips coated with the fusion GFP segments.
- Modeled upon Regan et al., we will assemble expression vectors which carry *NGFP-Rop1* and *Rop2-CGFP* genes under the control of *T7* and *araBAD* promoters respectively.





Rop varient Ala_2Ile_2-6 fused to GFP at position 157-158, with small target molecule (cyan) in Rop dimer hydrophobic core. Created with PyMOL (http://www.pymol.org) from PDB entries 1EMA and 1F4N.

Conclusions and future directions

An alternative to computational design, this strategy should prove universal in creating biosensors for homeland defense and biomedical applications. We will probe further the application of resultant biosensor molecules in microbes, ornamental plants, protein chips etc.

School of Physical and Mathematical Sciences
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