Biotechnology Letters (2006) 28: 471–475
DOI 10.1007/s10529-006-0007-6


# Creation of circularly permutated yellow fluorescent proteins using fluorescence screening and a tandem fusion template

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Received 6 October 2005; Revisions requested 27 October 2005; Revisions received 23 December 2005; Accepted 2 January 2006

*Key words:* circular permutation, fluorescent protein, protein engineering

**Abstract**

By experimenting with many different circularly permutated yellow fluorescent protein (cpYFP) variants as acceptors in fluorescence resonance energy transfer based biosensors, the optimal dynamic range can be discovered by sampling the possibilities of relative fluorophore orientations before and after bioactivity. Hence, to facilitate the sampling process, we introduced a new approach to construct a library of cpYFP variants using fluorescence screening and a tandem fusion template. This new approach is rapid because it does not require creating intermediate *N*- and *C*-terminal fragments and it allows quick screening for positive colonies by fluorescence. As a demonstration, eleven cpYFP variants were created and eight showed fluorescence. The emission and excitation spectra of these cpYFP variants showed strong similarity to YFP and therefore can be used in replacement.

## Introduction

Circularly permutated yellow fluorescent proteins (cpYFP) are important in creating improved fluorescent protein biosensors based on the principle of fluorescence resonance energy transfer (FRET) (Truong & Ikura 2001). In these biosensors, when a change in bioactivity occurs (such as phosphorylation, protease activity and $Ca^{2+}$ concentration) (Zhang *et al.* 2002), a change in FRET from the biosensor will result between a cyan fluorescent protein (CFP) donor and yellow fluorescent protein (YFP) acceptor. This change in FRET, before and after the bioactivity, is determined by the relative distance and orientation of the fluorophores. Since the change in the FRET signal (called dynamic range) is often weak, efforts have focused on optimizing the distance factor (Truong *et al.* 2001). Recently, however, using a library of cpYFP acceptors, it is possible to optimize the dynamic range by the orientation factor (Nagai *et al.* 2004). A circularly permutated protein is created by first choosing a splice site, then producing the corresponding *N*- and *C*-terminal fragments, and then finally ligating the fragments such that the C-terminal fragment precedes the *N*-terminal fragment. As the choice of splice site in cpYFP affects the relative orientation of the fused fluorophores, it is necessary to experiment with many cpYFP acceptors to optimize the dynamic range.

Here, we present an approach to rapidly create a library of cpYFP using fluorescence screening and a tandem fusion template. The common approach involves subcloning the *N*- and *C*-terminal fragments and then reversing the order of fragments by DNA ligation. This approach involves at least three steps of screening

intermediate and final products using traditional techniques such as fragment size assays or DNA sequencing. Alternatively, our approach involves first subcloning a tandem fusion of two YFP sequences [in our case, the Venus (Nagai *et al.* 2002) variant of YFP] and then selecting PCR primer pairs for DNA amplication such that each primer anneals to a different YFP. The PCR fragment is then inserted into a host vector and screened for fluorescence directly on the bacterial culture plate. This process is quick because it avoids the creation of intermediate *N*- and *C*-terminal fragments and it decreases the time for screening positive colonies. Using this approach, we created eleven new cpYFP variants and eight showed fluorescence properties with similar emission and excitation spectra. Lastly, this method can be adapted to circularly permutate other proteins of interest.

## Materials and methods

### *Construction of the base plasmids*

The plasmids for expressing the cpYFP variants were created using two different base plasmids: pYFPtx3 and pRFPtx3. To create pYFPtx3, the pEYFP-1 (Clontech) plasmid was amplified with the following primers: forward, 5′-CAT GCCATGGGCCTGACTAGT GTGAGCAAGGGCGAGGAGCTG-3′; reverse, 5′-CCGCTCGAG**TTA**GCCGCTAGCGGCGGCGGTCACGAACTCCA-3′. Similarly, to create pRFPtx3, a plasmid containing mRFP1 (Campbell *et al.* 2002) was amplified with the primers: forward, 5′-CATGCCATGGGCCTGACT AGTATGGCCTCCTCCGAGGACGTC-3′ reverse, 5′- CCGCTCGAG**TTA**GCCGCTAGCGGCGCCGGTGGAGTGGCGGC-3′. The forward primer contained *Nco*I and *Spe*I sites (underlined), while reverse primer contained *Xho*I and *Nhe*I sites (underlined) flanking a stop codon (bolded). These resulting PCR fragments were digested with *Nco*I–*Xho*I and ligated into the *Nco*I–*Xho*I site of pTriEx-3 (Novagen) to create pYFPtx3 and pRFPtx3, respectively.

### *Construction of plasmids for cpYFP variants*

The pRYYtx3 plasmid consisting of a tandem fusion for RFP, YFP, and YFP was created as described in the Results and discussion. To create the plasmid for the cpYFP33 variant, the pRYYtx3 plasmid was amplified with the following primers: forward, 5′-CATGCCATGGGCCTGACTAGTGGCGAGGGCGATGCCACCTA-3′; reverse, 5′-CCGCTCGAG**TTA**GCCGCTAGCCTCGCCGGACACGCTGAACT-3′. The resulting PCR fragment was digested with *Nco*I–*Xho*I and ligated into the *Nco*I–*Xho*I site of pTriEx-3 to create pcpYFP33tx3. Similarly, plasmids containing the other cpYFP variants can be created by keeping the same overhanging residues and choosing appropriate annealing residues.

### *Protein expression*

*E. coli* cell strain Rosetta (Novagen) were transformed with each cpYFP plasmid and plated. A single colony was selected from each culture medium plate and grown overnight at 37 °C. Proteins were created by leak expression from the cells. Next, the cells were centrifuged, resuspended and sonicated in the buffer (50 mM Tris-HCl, pH 7.9 and 100 mM NaCl).

### *Spectroscopy*

The emission spectra were recorded using a spectrofluorimeter with excitation at 480 nm and emission at 560 nm.

## Results and discussion

The first part in this approach involved the creation of a host plasmid with two tandem YFP sequences. Note that this host plasmid encodes all possible cpYFP variants. Note also that all subcloning was performed using Truong's cassette methodology (Truong *et al.* 2003). Truong's cassette structure uses a subtle property of compatible cohesive end restriction enzymes that allows domains to be fused in any order and number of times. Using this cassette method, two base plasmids were created containing YFP and monomeric red fluorescent protein (RFP) (Figure 1a) (Campbell *et al.* 2002), named pYFPtx3 and pRFPtx3, respectively. Since the emission spectrum of RFP significantly differs from YFP, a fusion of RFP to any YFP-only protein can be screened by red fluorescence or *vice versa*. To

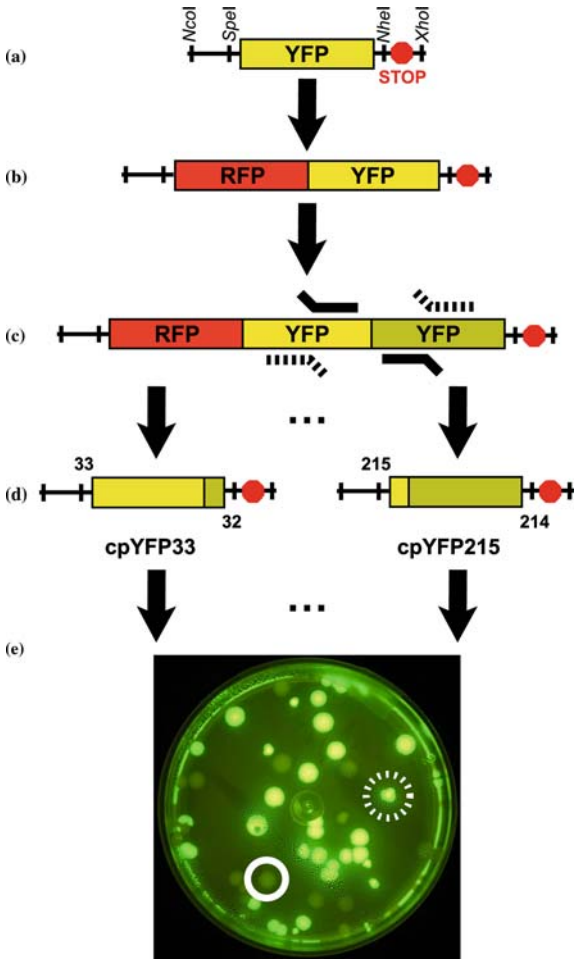


*Fig. 1.* Methodology for the construction of the cpYFP variant library. Schematic diagram of (a) the original YFP sequence (pYFPtx3) as well as with the locations of the enzyme cutting sites and the stop codon; (b) tandem fusion of RFP and YFP (pRYtx3); (c) tandem fusion of RFP, YFP and YFP (pRYYtx3). The forward and reverse primer pairs can anneal to both YFP sequences, however, the desired fragment is amplified by only one primer pair (solid line). (d) Schematic diagram of cpYFP variants created with the corresponding primer pairs. (e) Fluorescence screening in bacterial culture plates. A non-fluorescent colony is identified by a solid white circle; a fluorescent colony, by a dotted white circle.

create the RFP-YFP cassette (named pRYtx3), the pRFPtx3 plasmid was digested with *Nco*I and *Nhe*I sites and ligated into the *Nco*I and *Spe*I site of pYFPtx3 plasmid (Figure 1b). *E. coli* DH$\alpha$ cell strain were then transformed with the ligation mixture and plated. A positive colony was selected by the presence of red fluorescence on the bacterial culture plate excited with a wavelength of $535 \pm 5$ nm. Fluorescence screening allows for the fast selection of positive colonies on the plate. Similarly to create the RFP-YFP-YFP cassette (named pRYYtx3), the pRYtx3 was digested with *Nco*I and *Nhe*I sites and ligated into the *Nco*I and *Spe*I site of pYFPtx3 plasmid (Figure 1c). Again, red fluorescent colonies were screened. This plasmid, pRYYtx3, is the desired host plasmid as it contains two tandem YFP sequences.

The second part in the approach was to subclone a library of cpYFP variants from pRYYtx3 (Figure 1d). This was accomplished by selecting splice sites for the cpYFP variants and then designing the corresponding PCR primer pairs such that the forward primers anneal to the YFP sequence downstream to RFP and the reverse primers to the other YFP. Note that both forward and reverse primers can anneal to either YFP sequence of the pRYYtx3, however, any product with its primer pair acting on the same YFP sequence will not be amplified as the PCR primer pair extends away from each other (Figure 1c). Accordingly, we then created eleven customized cpYFP variants named cpYFP33, cpYFP53, cpYFP75, cpYFP95, cpYFP115, cpY FP135, cpYFP145, cpYFP155, cpYFP174, cpY FP195 and cpYFP215. The number following the cpYFP variant corresponds to the residue of new *N*-termini. For example, the cpYFP33 variant was created with corresponding primer pairs such that the new *N*- and *C*-termini residues are Gly-33 and Glu-32, respectively. Among these cpYFP variants, four splice sites were located within the $\beta$-sheets (cpYFP33, cpYFP95, cpYFP115 and cpYFP155), while the rest at either the turns or loops (Figure 2). The cpYFP variant plasmids were constructed using the cassette methodology and a positive colony was selected on the bacterial culture plate by the presence of yellow fluorescence excited with a wavelength of $488 \pm 5$ nm (Figure 1e). Note that there are two reasons for a non-fluorescent colony: the ligation failed or the ligation succeeded but the expressed protein has lost fluorescence. Both are undesirable outcomes.

The excitation and emission spectra of the eight flourescent cpYFP variants were similar to YFP (Figure 3) and therefore they can be used as its replacement. Surprisingly, of the eight fluorescent cpYFP variants, there was no correlation in the splice site location (i.e. within $\beta$-sheets, turns or loops) to the retention of fluorescence

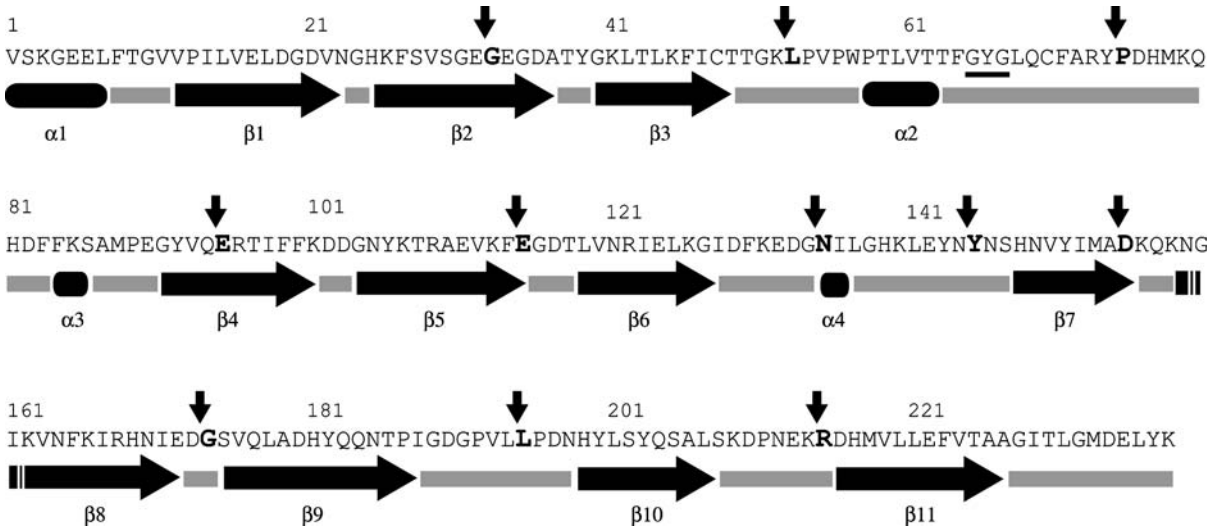


*Fig. 2.* Schematic topology of cpYFP variants. $\beta$-strands are shown as extended arrows, while $\alpha$-helices as boxes with rounded corners. The glycine-tyrosine-glycine residues comprising the chromophore are underlined. The splice sites of the cpYFP variants are indicated by the downward arrows. The boldfaced residue to the right of the downward arrow is the new *N*-termini for the cpYFP variant, while the residue to the left is the new *C*-termini.

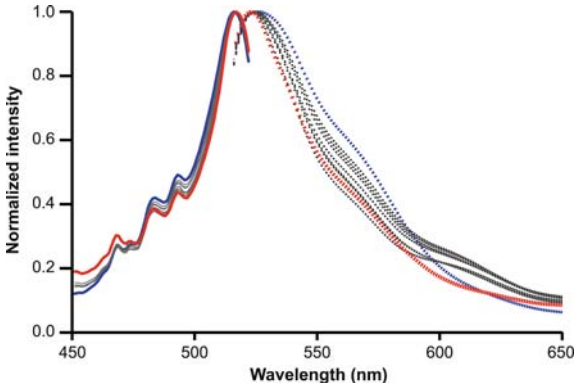


*Fig. 3.* The excitation spectra (solid line) and emission spectra (dotted line) of control YFP (red), cpYFP145 (blue), and the rest of the cpYFP variants (black).

property. From the emission and excitation spectra, all the cpYFP variants have similar excitation and emission peaks of $517 \pm 1$ nm and $525 \pm 1$ nm, respectively. In comparison, most of cpYFP variants have broader emission spectra than YFP. This is expected since the protein sequences have changed and therefore, should have slightly different stability and folding properties. In particular, cpYFP145 has the broadest emission spectrum and incidentally, this cpYFP variant has been used in many $Ca^{2+}$ sensors designs (Baird *et al.* 1999, Nagai *et al.* 2001, Nakai *et al.* 2001). However, all the spectra of cpYFP have a strong linear relationship with YFP as statistically calculated by their Pearson correlation coefficients that were all greater than 0.97 (where 1 is identical and $-1$ is disjoint).

## Conclusions

Circularly permutated yellow fluorescent proteins are used as acceptors to improve the dynamic range of FRET-based biosensors. Here, we introduced a new approach to construct a library of cpYFP using fluorescence screening and a tandem fusion template. As a demonstration, eleven cpYFP variants were created among which eight showed fluorescence similar to YFP. Thus, these cpYFP variants can be used in replacement of YFP. Furthermore, these cpYFP variants can be used to optimize single fluorescent protein biosensor designs such as pericam (Nagai *et al.* 2001), G-CaMP (Nakai *et al.* 2001), and camgaroo (Baird *et al.* 1999).

## Acknowledgements

This work was supported by grants from the Banting Foundation and the National Science and Engineering Research Council (NSERC).

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