Fluorescent protein (FP)-based biosensors have revolutionized the ability of researchers to monitor enzyme activities in live cells. While the basic design principles for FP-based biosensors are well established, first-generation biosensor constructs typically suffer from relatively low fluorescence responses that limit their general applicability. The protein engineering efforts required to substantially improve the biosensor responses are often both labour and time intensive. Here we report the application of a high throughput bacterial colony screen for improving the response of kinase biosensors. This effort led to the development of a second-generation cyclin B1-CDK1 biosensor with a 4.5-fold greater response than the first-generation biosensor.

Since the first report of using engineered FPs to construct Förster resonance energy transfer (FRET)-based biosensors for imaging of intracellular calcium ion dynamics, a this versatile strategy has been applied to the detection of a diverse selection of biological molecules and events. Nonetheless, development and optimization of a biosensor remains a difficult and laborious undertaking, owing to the fact that even when guided by high resolution structural information, the specific properties of the resulting biosensor are generally difficult to predict. Accordingly, essentially all efforts to improve the performance of such biosensors depend on empirical screens of large numbers of variants, though some generally applicable improvement strategies have been reported. One promising strategy for accelerating the optimization of a FP FRET-based biosensor is to transplant the mammalian enzyme activity of interest into Escherichia coli, and perform high throughput image-based screening in the context of bacterial colonies. We now report the application of this screening strategy to the optimization of FRET-based biosensors for protein kinase B (PKB)/Akt and cyclin dependent kinase 1 (CDK1) in complex with cyclin B1.

Construction of a FP FRET-based biosensor requires genetic fusion of a conformationally labile protein domain, with specific molecular recognition properties, between a FRET pair of FPs. The conformational change that accompanies a binding or post-translational modification (PTM) event modifies the distance and/or dipole orientation between the FPs and the FRET efficiency is changed. For biosensors of PTMs, the conformationally labile domain is typically a peptide substrate fused to a PTM-binding domain. When the enzyme activity of interest modifies the substrate domain, the modified peptide will bind to the binding domain, causing a large conformational change and a change in FRET efficiency.

Our lab has previously reported a strategy for screening biosensor constructs for improved emission ratio change in colonies of E. coli. The key to this strategy is a dual expression plasmid (pUADE) for expression of two proteins under different promoters (Fig. 1). Briefly, biosensor variants are expressed under the P_tac promoter and a constitutively active enzyme, which catalyzes the post-translational modification of the FRET-based biosensor, is expressed under the P_BAD promoter. The P_BAD promoter provides tight control of enzyme expression, and can be

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**Fig. 1** A protein expression system for high-throughput function-based screening of libraries of kinase biosensor variants. The plasmid encodes a constitutively active kinase downstream of the l-arabinose-inducible promoter P_BAD and a kinase biosensor downstream of IPTG-inducible promoter P_tac. Activation of P_BAD promoter in the context of E. coli colonies leads to the expression of the kinase and phosphorylation of the biosensor.
induced with l-arabinose and repressed with d-glucose.11 Inducing kinase expression leads to the post-translational modification of the FRET construct and, for potentially useful biosensors, a change in FRET efficiency and emission ratio. By imaging a plate of bacterial colonies before and after induction of enzyme expression, the biosensor variants that exhibit the largest changes in emission ratio can be identified (Fig. 2). In this way, screening of large libraries for improved biosensors is feasible. A similar approach has been used to screen a library of FRET biosensors for RNA detection.12

We previously used this system for optimization of a biosensor for histone lysine methylation, though we reasoned that this system could also be used for optimization of biosensors for kinase activity. Such biosensors are composed of a FRET pair of fluorescent proteins joined by a kinase-specific substrate sequence bound by a linker to an appropriate phosphoaminoacid-binding domain. For example, Kunkel et al. have reported the development of a PKB/Akt activity FRET-based biosensor designated B-kinase activity reporter (BKAR).10 BKAR was constructed by genetically fusing the consensus substrate sequence RKKRDRLG between the cyan FP and the binding domain or between the substrate and yellow FP. We have previously shown that optimization of these other linkers can lead to substantial improvements in biosensor performance.7 The linker library was designed to include both unstructured (i.e., GGSGG repeats) sequences as well as sequences that were expected to be highly α-helical (i.e., EAAAK repeats).17 In addition, the original BKAR linker,18 the linkers used in our previous work,7 and the proteolytically stable “218” linker,18 were included. To maintain a reasonable library size, and to prevent library construction from becoming intractable, we did not explore linker diversity between the cyan FP and the binding domain or between the substrate and yellow FP. We have previously shown that optimization of these other linkers can lead to substantial improvements in biosensor performance.7 The gene library in pUADE was used to transform E. coli which was then plated on LB-agar with 0.04% ampicillin, 1 mM IPTG and 20 mM d-glucose.

The gene library was screened using a modified version of our previously reported procedure.7 In our previous work single colonies were picked and then resptotted onto both d-glucose and l-arabinose agar plates in ordered arrays. In our current work we omitted the resptotting step, and plates of colonies were simply imaged, sprayed with l-arabinose, and then imaged a second time. Pre-spray imaging was performed using a custom built imaging system that acquired fluorescence images of both donor emission (excitation 420–440 nm; emission 460–500 nm) and acceptor intensities for each colony pre- and post-spray. The data was exported to Microsoft Excel where the ratio of emission intensity of acceptor to donor emission (I_{acceptor}/I_{donor}) was calculated for each colony pre- and post-spray. The emission ratio change was calculated as \( \Delta R/R_{\text{max}} \) % and colonies showing the highest ratio becomes active following translocation to the plasma membrane and phosphorylation of Thr308 and Ser473.14 However, mutation of both PKB phosphorylation sites to aspartic acid has been shown to render PKB constitutively active.15 Accordingly, we cloned the gene for the double aspartic acid mutant of bovine PKB14 downstream of F_{BAD} promoter of pUADE. Our library of BKAR variants was composed of 5 different cyan FPs, 22 different binding domain to substrate linkers, and 4 different yellow FPs (Table 1), giving a total diversity of 440 variants. The linker library was designed to include both unstructured (i.e., GGSGG repeats) sequences as well as sequences that were expected to be highly α-helical (i.e., EAAAK repeats).17 In addition, the original BKAR linker,18 the linkers used in our previous work,7 and the proteolytically stable “218” linker,18 were included. To maintain a reasonable library size, and to prevent library construction from becoming intractable, we did not explore linker diversity between the cyan FP and the binding domain or between the substrate and yellow FP. We have previously shown that optimization of these other linkers can lead to substantial improvements in biosensor performance.7

| Table 1 Library design | Diversity included in library |
|------------------------|-----------------------------|
| Donor FP | CFP, mTFP4,19 CyPet,20 cpmTFP193a, cpmTFP207 |
| Acceptor FP | YFP, YPet,20 cpmTFP193a, cpmTFP207 |
| Binding domain | IR; INSR; INSRIR; INSRIRNR; INSRIRNR; (EAAAK) |
| Linker | (n = 1–10); (GGSGG), (n = 1–10); GSTSGSGKPQGESGTKG (218 linker) |

*Previously unreported. Spectral characterization revealed that both variants have absorbance \( \lambda_{\text{max}} = 465 \text{ nm} \), emission \( \lambda_{\text{max}} = 491 \text{ nm} \), extinction coefficient = 55 000 M⁻¹ cm⁻¹, and quantum yield = 0.59.*
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Fig. 3 Results of screening for improved BKAR variants. (A) Candidates with the highest emission ratio changes were grown under both kinase inducing and repressing conditions, and \( \Delta R/R_{\text{min}} \) % was determined for the purified proteins. Error bars represent standard deviations of 4 independent experiments. (B) Coomassie blue stained SDS-PAGE gel (left side) of molecular weight (MW) ladder, of the ‘218’ variant expressed under enzyme repressing (glucose) and inducing (arabinose) conditions. The * indicates the biosensor and ** indicates the enzyme band. On the right side of the panel is an anti-phosphothreonine Western Blot for biosensor expressed under repressing and inducing conditions. (C) Emission spectra of the BKAR variant with the ‘218’ linker expressed under inducing (arabinose) and repressing (glucose) conditions. All spectra are taken at \( \lambda_{\text{excitation}} = 430 \) nm and normalized with respect to maximum donor intensity. (D) The (GGSGG)\(_2\) variant represented as in (C).

Having demonstrated that our screening strategy could be used for the optimization of an existing kinase biosensor, we applied it to the development of a new biosensor for CDK1 in complex with cyclin B1. The activity of the cyclin B1-CDK1 complex is well understood to determine the onset of mitosis. A FRET-based biosensor for cyclin B1-CDK1, composed of a substrate from human cyclin B1 (PEPILVDTSPPSME) fused to a polo-box phosphoamino acid binding domain and the mCerulean–YPet FRET pair, has previously been reported by Gavet and Pines. Despite having a relatively small emission ratio change of ~10–15% upon cyclin B1-CDK1 activation, this biosensor has proven very useful for correlating activation of cyclin B1-CDK1 as well as with nuclear import and nuclear envelope breakdown. As expression of the previously reported biosensor gene in E. coli did not produce any soluble protein, we created a new biosensor in which the polo-box domain was replaced with the FHA2 domain. The substrate from the earlier cyclin B1-CDK1 biosensor was retained. To apply our colony based screening protocol, we also required a recombinant constitutively active kinase that could phosphorylate this substrate when expressed in E. coli. Accordingly, we cloned a polycistronic unit (CDC28–CAK1–CKS1–CLB5) that encodes for active Saccharomyces cerevisiae rCdc28–Clb5 (a cyclin B1-CDK1 homologue), downstream of the PBAD promoter in pUADE. In addition, a library of potential cyclin B1-CDK1 biosensors with the diversity indicated in Table 1 was inserted downstream of the P\(_{\text{lac}}\) promoter.

Fig. 4 Results from screening a library of cyclin B1-CDK1 biosensors. (A) Each top clone was grown individually under both kinase inducing and repressing conditions and \( \Delta R/R_{\text{min}} \) % was determined for the purified proteins of 4 independent experiments. (B) The purified (GGSGG)\(_2\) variant cultured either with or without arabinose migrated identically on SDS-PAGE gel. Anti-phosphoserine Western blot confirmed phosphorylation under kinase inducing conditions. (C) Emission spectra of the purified (GGSGG)\(_2\) variant cultured under kinase inducing (arabinose) and repressing (glucose) conditions.
Application of the colony based screening procedure (16 Petri dishes, each with hundreds of colonies) led to the identification of seven colonies that showed large emission ratio changes. Each of these colonies was picked, propagated, and their respective biosensor variants were expressed under both enzyme inducing and repressing conditions. Fluorescence spectra of the purified proteins provided the percent ratio changes associated with phosphorylation (Fig. 4A). As with the BKAR-type biosensor, a Western blot confirmed biosensor phosphorylation under inducing conditions (Fig. 4B). DNA sequencing of the biosensor with the highest ratio change (67.0 ± 2.1%; Fig. 4C) revealed that it was composed of the mTFP1-YFP FRET pair with (GGSGG)2 as the middle linker between FHA2 and substrate sequence.

Encouraged by the improved ratio change of our cyclin B1-CDK1 biosensor, we expressed it in HeLa cells and imaged emission ratio changes in single cells as they progressed through mitosis (Fig. 5). These single cell imaging experiments revealed a pattern of activation very similar to the average activity for multiple mitosis (Fig. 5). These single cell imaging studies aimed at understanding the dynamics of cyclin B1-CDK1 activation during cell cycle progression. In particular, its improved performance should diminish the need for averaging fluorescence intensities from multiple cells in order to obtain reliable data, potentially providing a new window into cell-to-cell heterogeneity in cyclin B1-CDK1 activation.

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