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Optical tracking of a stress-responsive gene amplifier applied to cell-based biosensing and the study of synthetic architectures

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ABSTRACT

A synthetic regulatory construct based on a two-stage amplifying promoter cascade is applied to whole-cell biosensing. Green fluorescent protein (GFP) and red fluorescent protein (RFP) enable two-component tracking of the response event, enabling the system to exhibit increased sensitivity, a lower limit of detection, and a unique optical density-free assessment mode. Specifically, the *recA* and *tac* promoters are linked by the LacI repressor in *Escherichia coli*, where DNA-damage activates the *recA* promoter and the up-regulation of GFP and LacI proteins. LacI represses the *tac* promoter, down-regulating the otherwise constitutive *mCherry* transcription. The response of the construct was compared with two singly tagged, conventional *recA* promoter-reporter constructs: *recA::gfpmut3.1* and *recA::mCherry*. Using a miniature LED-based flow-through optical detector developed for remote sensing applications, limits of detection for the dual reporter construct reached as low as 0.1 nM MMC. By comparison, single-ended reporters *recA::mCherry* and *recA::gfpmut3.1* achieved best limits of detection of 0.25 nM and 2.0 nM, respectively. An approach to three-component optical analysis, based on a system of detectors with coupled calibration equations enables accurate assessments of the red fluorescence, green fluorescence, and biomass of sensor cell suspensions. The system approach is effective at overcoming interferences caused by optically dense samples and overlapping fluorescence spectra. Such a technique may be useful in studying the biological mechanisms which underlie the synthetic regulatory device of this work and others.

1. Introduction

Whole-cell biosensors have been developed for a host of applications, ranging from drug screening to environmental quality assessments. A popular approach is based on tracking promoter activity in response to cell–analyte interactions using endogenously produced reporter molecules (Daunert et al., 2000). Particular analytes of interest include mutagens, heavy metals, phenols, naphthalene, toluene, ammonia, methane, and various biologicals (Chalova et al., 2008; Ron, 2007; Yagi, 2007). Applications that stand to gain from improved whole-cell biosensing technology include air and water quality assessment, bio-warfare defense, drug screening, chemical mutagenicity assessment, and space biology and hazard assessment. Many of these applications require systems that are suited to remote or distributed sensing.

Recent work in synthetic biology has resulted in significant advances toward engineered genetic regulation. Successes include bistable toggle switches (Atkinson et al., 2003; Gardner et al., 2000), logical operators (Hasty et al., 2002), and signal amplifiers (Karig and Weiss, 2005). Despite these successes, it is difficult to find examples where whole-cell biosensors have been improved through gene circuit engineering (Antunes et al., 2006). This work draws from advances in synthetic regulatory networks to augment whole-cell detection capabilities. Specifically, fluorescent reporter proteins under synthetic regulatory mechanisms are shown to enable high-sensitivity measurements using relatively unsophisticated optical instrumentation suitable for remote, miniaturized, and potentially distributable systems.

1.1. Fluorescent reporting

Techniques for monitoring the activity of promoters via fluorescent reporter proteins have been well-developed but have been limited in their practical application by both an unacceptably slow response time and generally poor sensitivity (Daunert et al., 2000; Hakkila et al., 2002; Justus and Thomas, 1999; Kostrzynska et al., 2002; Norman et al., 2005; Sagi et al., 2003). Nevertheless, certain other properties of fluorescent reporters make them attractive

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candidates for miniature or remotely deployable assays. Namely, high reporter stability, signal accumulation, absence of a requirement for accessory substrates, and the non-invasiveness of the optical probing enable remote or miniature sensing applications (Baumstark-Khan et al., 2001, 2003; Belkin, 2003; Knight et al., 1999; Kostov et al., 2000; Kostrzynska et al., 2002; Kuang et al., 2004; Lichtenberg-Fraté et al., 2003; Norman et al., 2005; Rabbow et al., 2003; Sagi et al., 2003; Zanzotto et al., 2006).

1.2. Dual reporter fluorescence

A few cases in the literature report the use of multiple, simultaneous luminescent reporters applied to whole-cell environmental biosensing. Some of these approaches are based on mixed-mode reporting, employing both bioluminescent and fluorescent reporters. A few other cases demonstrate the possibility of using single-mode, multiplexed reporting including dual colored bioluminescence (Shapiro et al., 2005; Wood and Gruber, 1996) and dual colored fluorescence (Belkin, 2003; Mirasoli et al., 2002; Mitchell and Gu, 2004; Shrestha et al., 2001).

The use of multiple fluorescent reporters in simultaneous, multiplexed fashion has been encouraged by advances in protein engineering which have made available a full-spectrum palette of reporter species. Of particular note has been the isolation, characterization, and manipulation of red fluorescent proteins for reporter applications (Andersen et al., 1998; Bevis and Glick, 2002; Campbell et al., 2002; Gurskaya et al., 2001; Matz et al., 1999; Patterson et al., 2001; Shaner et al., 2004; Wiedenmann et al., 2002, 2005; Zhang et al., 2002). Only recently have rapidly maturing and bright red fluorescent proteins come available.

There appear to have been two goals of investigations involving multiple-reporter systems: (1) to track multiple promoters, and (2) to create internal reference signals to stabilize reporter readings. In this work, we employ a two-reporter system to attack the second goal above. Specifically, we use a two-reporter system to probe the activity of a single promoter in an OD-independent fashion, whereby confounding factors such as growth rate and stage, culture purity, and hardware instabilities can be mitigated.

1.3. Synthetic regulatory networks and biosensing

The architecture of a two-reporter gene cascade, as developed in this work, is presented below in Fig. 1. Functionally, the RFP MCherry (Shaner et al., 2004) is down-regulated in response to DNA damage, while Gfpmut3.1 (Cormack et al., 1996) is up-regulated. The ratio of the two fluorescence signals can be used to estimate the presence of DNA damaging stimuli. Alternatively, the fluorescence channels can be measured independently. An important feature of the cascade is the amplification stage that takes place due to LacI inhibition of the *tac* promoter. This amplification has been harnessed to improve the biosensor's limit of detection, addressing one of the weaknesses of fluorescent proteins as reporters.

2. Materials and methods

2.1. Plasmid construction

Routine genetic work was done using DH5α cells (Invitrogen). All final plasmid constructs were transformed into DJ480 cells (Majdalani et al., 2005). DJ480 cells are based on *Escherichia coli* MG1655 with Δ(argF-*lac*)U169. Phenotypically, DJ480 cells are *lacI*$^-$ but *recA*$^+$.

All plasmids in this work are based on the plasmid pTrc99a (Amann et al., 1988). pTrc99a is an expression vector characterized by an ampicillin resistance gene, a medium copy origin of replication, a *tac* promoter with a convenient multiple cloning site,

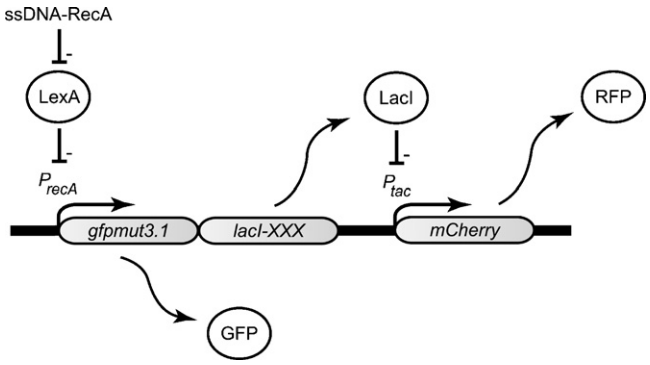

Fig. 1. Dual reporter cascade. When wild-type *E. coli* cells undergo DNA damage, native repair mechanisms create and accumulate ssDNA in the cell. ssDNA reacts with RecA and LexA in a manner that causes LexA cleavage, and since LexA represses *recA* promoter activity, the presence of DNA damage increases *recA* promoter activity. In the synthetic construct depicted above, the activation of the *recA* promoter expresses both GFP and a degradation tag-destabilized repressor to the *tac* promoter, LacI-XXX, where XXX represents any generic degradation tag. High levels of LacI-XXX cause repression of the *tac* promoter. Thus, the absence of DNA damage results in a low expression of GFP and a high expression of RFP. Conversely, sensed DNA damage creates a high level of GFP expression and a low level of RFP expression.

an overexpressed *tac* repressor (LacI), and a strong transcription terminator. pTrc99a contains approximately 4.2 kbp.

Standard molecular techniques were employed to create the various components required to both assemble and characterize the dual reporter construct of this work (see supplementary material). In brief, constructs were created for: constitutive GFP production (*pMB99a::gfpmut3.1*), constitutive RFP production (*pMB99a::mcherry*), background-matched dark cells (*pMB99a*), MMC-inducible GFP production (pRM124, with *recA::gfpmut3.1*), MMC-inducible RFP (pRM125, with *recA::mcherry*), and three dual reporter constructs with degradation tag-destabilized *lacI* variants: pRM2451 (*recA::gfp-LacI(ASV)/tac::mCherry*), pRM2452 (*recA::gfp-LacI(LVA)/tac::mCherry*), and pRM2453 (*recA::gfp-LacI(AAV)/tac::mCherry*).

2.2. Cell culture and treatments

For all routine DNA work, cells were cultured in LB medium supplemented with appropriate antibiotics at standard concentrations. Neidhardt EZ-Rich Medium (Teknova, CA) was used for all experiments. Neidhardt medium enables a very fast growth rate while preserving optical clarity.

Cells were, in general, first grown from frozen stock overnight on Neidhardt medium with agar, after which a single colony was grown in liquid Neidhardt overnight. Three aliquots of the overnight culture were generally taken and resuspended into 3 mL each of fresh Neidhardt EZ-Rich medium at a 1:100 dilution factor. When cells were incubated with treatment chemicals, the mixtures were pre-equilibrated and taken to temperature before cells were added.

2.2.1. DNA-damage induction

As a test system to demonstrate promoter activity tracking, the DNA-damage responsive *recA* promoter was selected for these studies. *recA* is a key component of the SOS regulon, and *recA* promoter activity increases as a result of accumulated single-strand DNA (ssDNA) in the intracellular space. ssDNA accumulates when DNA repair mechanisms act on damaged DNA.

Mitomycin C (MMC) has been extensively studied and used as a model DNA damage inducing agent. MMC was employed in this work to facilitate comparisons with previous studies. A 15 mM mitomycin C stock solution was prepared by adding 1 mL of a 90% propylene glycol solution to 5 mg mitomycin C powder