Characterization of a rationally engineered nitric oxide, nitrate and nitrite biosensor linked to a hybrid bacterial-mammalian promoter

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Author Contributions

Authors are equal first author, each of whom were equally involved in the design of the iGEM project and the molecular and microbial work carried out. R. K. and R. B. supervised and coordinated the study.

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Conflict of interest:

The authors declare no conflict of interest and no competing financial interests.

Abstract

Synthetic biology is principally concerned with the rational design and engineering of biological systems that serve useful applied purposes. Biosensors are of particular interest to the field since they serve a broad array of applications, such as medical devices, environmental sensors for the detection of contaminants, toxins or pathogens or in metabolic engineering, to monitor product formation. In this study, we describe the characterization of a family of four nitric oxide, nitrate and nitrite whole-cell biosensors that are based upon a hybrid bacterial-mammalian promoter design. The hybrid-design of the synthetic promoter has been engineered for the detection of these nitrogenous species across both bacterial (*Escherichia coli*) and mammalian systems (MCF-7). As such, these biosensors may be useful across applications as diverse as cancer therapeutics and the agricultural monitoring of nitrates and nitrites in fertiliser-treated soil. Qualitative and quantitative analysis of these biosensors in *E. coli* confirmed that all four biosensor designs (termed B-M_eCFP, B-M_mRFP, M-B_eCFP and M-B_mRFP) were able to quantitatively detect 5-20 mM of potassium nitrate. In summary, these pilot data suggest that, with further characterisation, this family of biosensors will be able to assess nitrogenous species present within both bacterial (*E. coli*) and mammalian systems (MCF-7).

Key Words: Nitric oxide; biosensor; hybrid promoter; iGEM

Introduction

Synthetic biology is principally concerned with the rational design and engineering of biological systems that serve useful applied purposes. Biosensors are of particular interest to the field since they serve a broad array of applications as medical devices, environmental sensors for the detection of contaminants, toxins or pathogens or in metabolic engineering, to monitor product formation (Khalil and Collins, 2010). The detection of nitrogenous species - particularly nitric oxide, nitrates and nitrites - are of interest for a broad set of applications from cancer therapeutics to the agricultural monitoring of nitrates and nitrites in fertiliser-treated soil (Madasamy *et al.,* 2014; Worthington *et al.,* 2005). Yet, current detection methods that measure accurate levels of these nitrogenous species from soil-samples require expensive equipment and specialist knowledge. In the case of nitric oxide (NO), detection is useful in a cancer therapeutic context since basal expression of nitric oxide synthases (NOS), enzymes which generate NO, is common in tumour cells (Worthington *et al.,* 2005). Yet, acute high-level expression of NO is known to induce tumour cell apoptosis and thus may be an effective cancer therapeutic (Worthington *et al.,* 2005). However, NO is very difficult to detect since NO has a very short half-life and the delivery of robust levels of NO that induce pro- rather than anti-apoptotic signals within tumour cells is difficult. In order to begin to address these diverse applications we set out to engineer a family of robust nitric oxide, nitrate and nitrite biosensors. We designed four whole-cell biosensors that are based upon a hybrid bacterial-mammalian promoter design (Figures 1, 2).

In this study we wished to develop a hybrid-design of synthetic promoter that has been engineered for the detection of these nitrogenous species across both bacterial (*Escherichia coli*) and mammalian (MCF-7) systems. However, the detection mechanisms markedly differ between the two systems. The bacterial component of the hybrid promoter, PyeaR, is found in the yeaR/yoaG operon of *E. coli* and is associated with induction by nitric oxide, nitrates and nitrites (Lin *et al.,* 2007). PyeaR is repressed by two main repressors: Nar, which is regulated by nitrates and nitrites; and NsrR, which is regulated by nitric oxide. PyeaR is not repressed in aerobic conditions, and is thus suitable as a transcriptional biosensor in a range of conditions (Lin *et al.,* 2007). In our hybrid promoter designs, the PyeaR component of the hybrid promoter is referred to as B (for “Bacterial”).

The mammalian component of the hybrid promoter consists of nine CArG elements (repeated elements of CC(A/T)(6)GG), and is originally derived from a repeat sequence in the EGR1 gene, which encodes for early growth response protein 1 (Scott *et al.,* 2002). The CArG component of the hybrid promoter is referred to as M (for “Mammalian”).

These two different promoter sequences were combined together in two orientations: B upstream of M (B-M) and M upstream of B (M-B) (Figure 2). To separate the B and M sequences an insulator sequence, containing a BamH1 restriction site, was encoded between them (Figure 1). Hybrid promoters, B-M and M-B, were cloned according to the Biobrick^{tm} assembly standard, upstream of cyan fluorescent protein (eCFP; BBa_E0420) and monomeric red fluorescent protein (mRFP; BBa_K081014) reporter constructs. Here we describe the qualitative and quantitative characterization of the resultant biosensor constructs, B-M_eCFP, B-M_mRFP, M-B_eCFP and M-B_mRFP. These biosensor constructs were developed by NRP-UEA-Norwich, a team of seven undergraduate students as part of the 2012 International Genetically Engineered Machine (iGEM) competition. The full project details can be found at http://2012.igem.org/Team:NRP-UEA-Norwich.

Results and Discussion

E. coli transformed with a plasmid containing B-M-eCFP (BBa_K774004), M-B_eCFP (BBa_K774006), B-M_mRFP (BBa_K774005) or M-B_mRFP (BBa_K774007) biosensor sequence were cultured overnight at 37 ^{o}C in lysogeny broth (LB) media containing 10 mM potassium nitrate. Cultures were centrifuged to pellet the cells and were qualitatively analysed under a blue light box, to assess induction of fluorescent protein expression. Qualitative analysis of these biosensors in *E. coli* confirmed that all four biosensor designs, B-M_eCFP, B-M_mRFP, M-B_eCFP and M-B_mRFP, were inducible with 10 mM potassium nitrate (Figure 3). Additionally, qualitative assessment of M-B_eCFP induction with 0-100 mM potassium nitrate (Figure 3) is indicative that these biosensors may be able to detect nitrates at levels that are relevant to agricultural biosensor applications. In order to provide a more robust characterisation of these biosensor designs, quantitative assessment of these biosensors were undertaken via flow cytometry (Figure 4) and fluorometry (Figure 5).

Flow cytometric characterisation of *E. coli* transformed with either M-B_eCFP biosensor (BBa_K774006) or B-M_mRFP (BBa_K774005), revealed that these biosensors were able to detect potassium nitrate at 0 mM, 1 mM or 10 mM in a dose response-like manner (Figure 4). M-B_eCFP was the more sensitive biosensor, detecting potassium nitrate concentrations as low as 1 mM. Indeed, fluorometry (Figure 5) characterisation of these biosensors confirmed that all four designs were capable of detecting potassium nitrate at a concentration of >5 mM. However, again M-B_eCFP was the most sensitive design. Interestingly, 20 mM concentrations of potassium nitrate inhibited biosensor-reporter expression in all four designs, which suggests that at high concentrations potassium nitrate may be toxic to *E. coli* and/or high levels of biosensor expression led to chassis burden, thus perturbing protein expression. Yet, conversely, qualitative analysis of these biosensors induced with 100 mM potassium nitrate were functional and their expressing *E. coli* were viable and, thus, the reason for inhibition at 20 mM is not fully apparent. Additional characterisation is needed to confirm that these biosensor designs detect nitrites and nitric oxide across both bacterial and mammalian systems. However, these pilot data provide a set of promising results and suggest that, with further characterisation, these biosensors represent a family that are able to function to detect various nitrogenous species across both bacterial (*E. coli*) and mammalian systems (MCF-7) (Supplementary MCF7 transfection data).

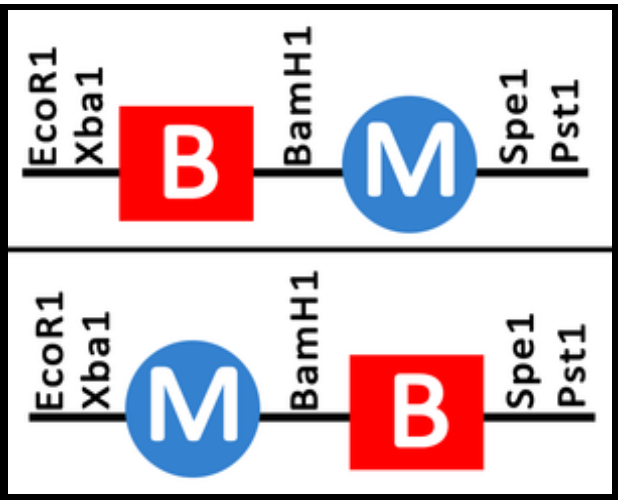
Figure 1. Hybrid promoter designs

BBaK216005 + BHN + CArG promoter sequence E9-ns2 (B-M)

5′-
GAATTCGCGGCCGCTTCTAGAGttcccatctataatcctccctgattcttcgctgatatg
gtgctaaaaagtaaccaataaatggtatttaaaatgcaaattatcaggcgtaccctgaaa
cgGGATCCAAGCTTCATATGccatataaggccatataaggccatataaggccatataagg
ccatataaggccatataaggccatataaggccatataaggTACTAGTAGC
GGCCGCTGCAG -3′

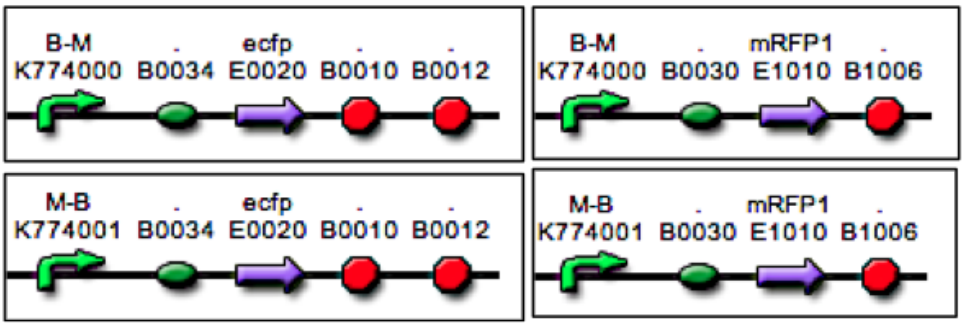
E9-ns2 CArG promoter sequence + BHN + BBaK216005 (M-B)

5′-
GAATTCGCGGCCGCTTCTAGAGccatataaggccatataaggccatataaggccatataa
ggccatataaggccatataaggccatataaggccatataaggccatataaggGGATCCAA
GCTTCATATGttcccatctataatcctccctgattcttcgctgatatggtgctaaaaagt
aaccaataaatggtatttaaaatgcaaattatcaggcgtaccctgaaacgTACTAGTAGC
GGCCGCTGCAG -3′



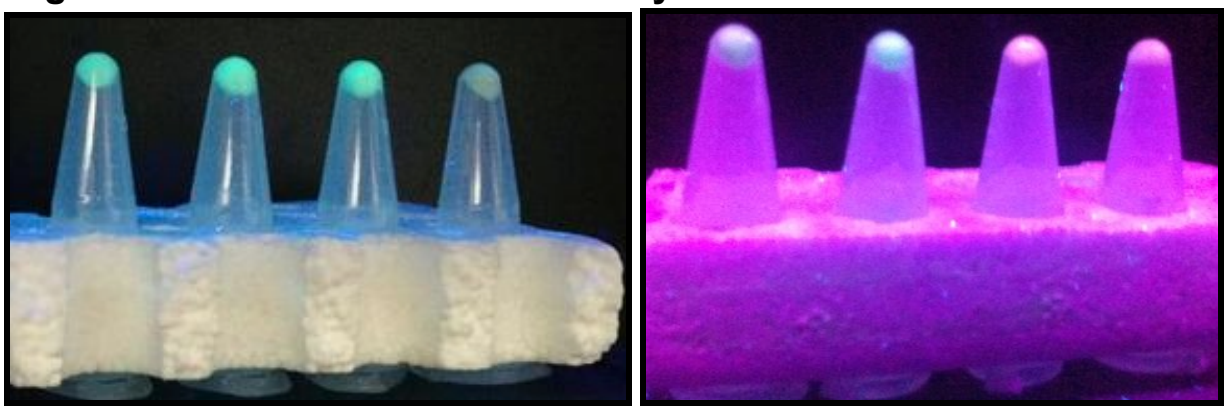
Graphical and sequence representation of the hybrid promoter in its two orientations; [top] Bacterial-Mammalian configuration (B-M) **[bottom]** Mammalian-Bacterial (M-B) configuration. The bacterial promoter PyeaR sequence originates in the *yeaR/yoaG* operon of *Escherichia coli* (Part BBa_K216005). The mammalian promoter sequence is derived from nine repeated elements of CC(A/T)(6)GG - CArG, as previously described (Scott *et al.* 2002; Worthington *et al.* 2005). Several restriction sites are also indicated within the coloured sequence regions: **{Red}** Indicates iGEM restriction site prefix sequence; **{Green]** Indicates a restriction site (BamHI, HindIII, NdeI) linker sequence that may act as an insulator between the two promoters; **{Blue}** Indicates iGEM restriction site suffix sequence. The full sequences are provided as supplementary data and are also available from the iGEM Registry of Standard Biological Parts (http://parts.igem.org/).

Figure 2. Nitric oxide, nitrate and nitrite biosensor designs



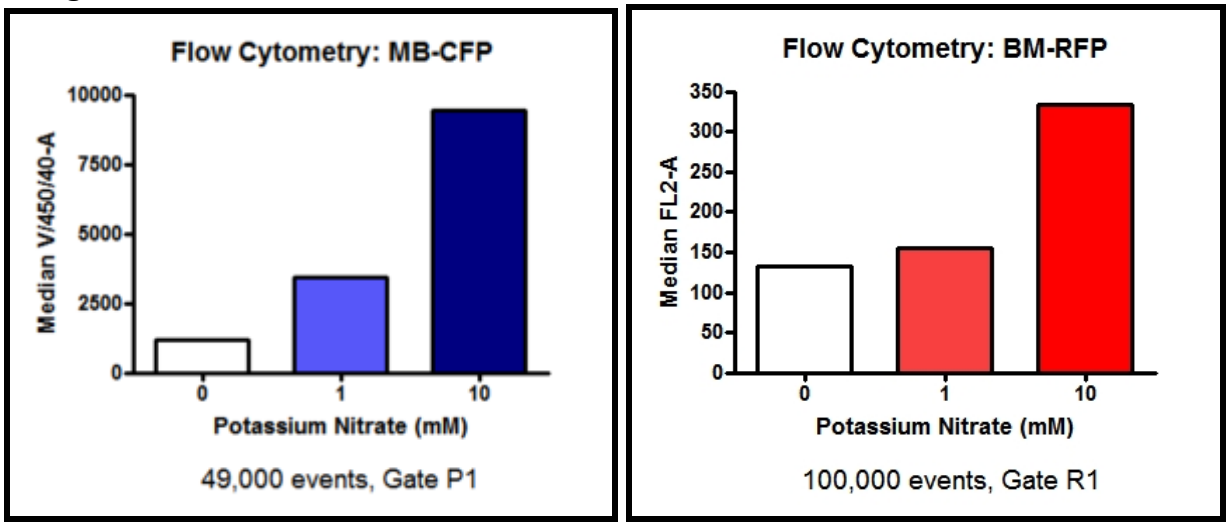
Graphical representation of four nitric oxide, nitrate and nitrite biosensors [top] B-M biosensors; B-M-eCFP (BBa_K774004) and B-M_mRFP (BBa_K774005) **[bottom]** M-B biosensors; M-B_eCFP (BBa_K774006), and M-B_mRFP (BBa_K774007). Biobrick numbers for Individual parts are also indicated.

Figure 3. Qualitative biosensor analysis



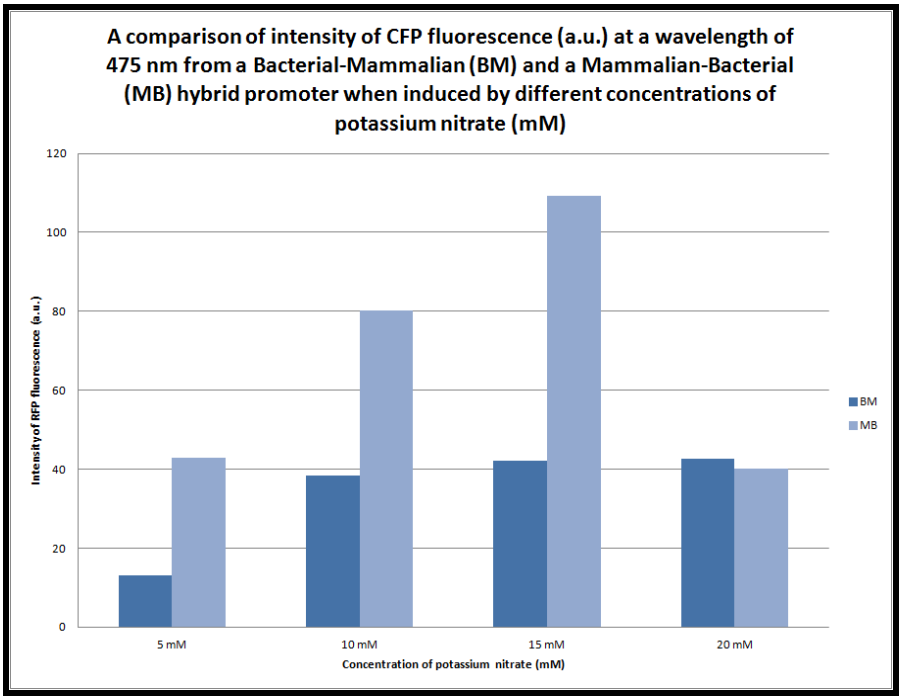
Qualitative biosensor analysis [left] *E. coli* transformed with a plasmid containing M-B_eCFP biosensor (BBa_K774006) sequence were cultured overnight at 37 ºC in LB media containing potassium nitrate as follows (left to right): 100 mM, 50 mM, 10 mM, 0 mM. **[right]** *E. coli* transformed with a plasmid containing either {left to right} B-M-eCFP (BBa_K774004), M-B_eCFP (BBa_K774006), B-M_mRFP (BBa_K774005) or M-B_mRFP (BBa_K774007) biosensor sequences were cultured overnight at 37 ºC in LB media containing 10 mM potassium nitrate. These cultures were centrifuged and the resultant pellets were placed under a blue light box and imaged.

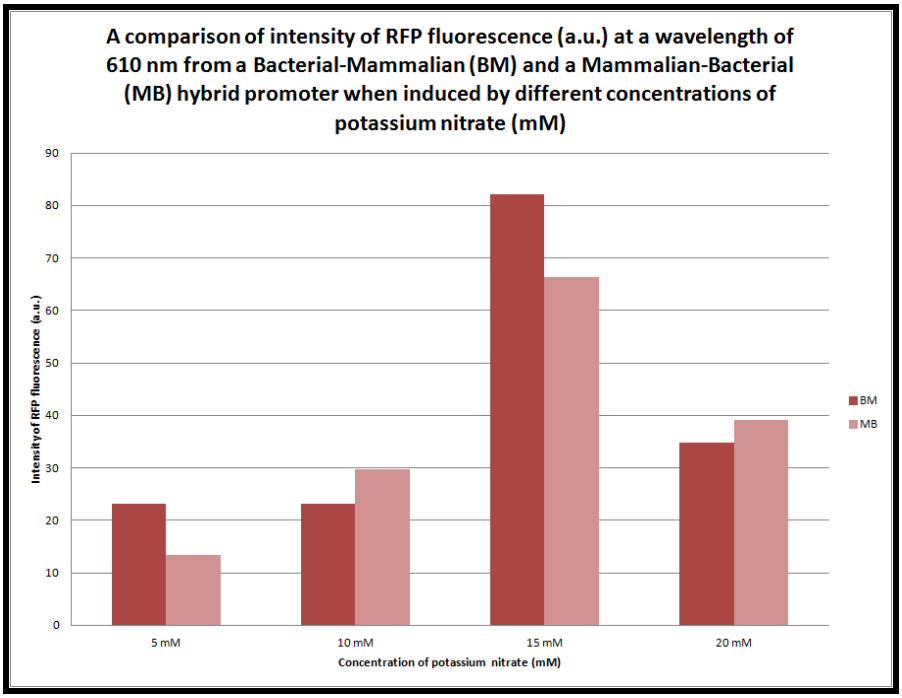
Figure 4. Flow cytometry characterisation of B-M_mRFP and M-B_eCFP biosensor designs



Flow cytometry characterisation of *E. coli* transformed with [left] M-B_eCFP biosensor (BBa_K774006) or **[right]** B-M_mRFP (BBa_K774005), were cultured overnight at 37 ºC in LB media containing potassium nitrate (0mM, 1mM or 10 mM). Cells were washed and resuspended in phosphate buffered saline (PBS) and analysed via flow cytometry. Data represent 49,000 or 100,000 individually measured cells per group. See methods and flow cytometry supplementary data for more details.

Figure 5. Fluorometric biosensor characterisation





Fluorometric characterisation of E. coli transformed with [top] B-M_eCFP (BBa_K774004), M-B_eCFP biosensor (BBa_K774006) or **[bottom]** B-M_mRFP (BBa_K774005), M-B_mRFP (BBa_K774007) were cultured overnight at 37 ºC in LB media with potassium nitrate (5 mM - 20 mM). Cells were washed and sonication-lysed to release protein for fluorometric analysis. Cultures without added nitrate were utilised to determine background fluorescence levels, which were subtracted from all other data points.

Methods

Construct Assembly

Hybrid-promoter constructs B-M (BBa_K774000) and M-B (BBa_K774001) were commercially synthesised (Genscript, NJ USA). B-M_eCFP (BBa_K774004), M-B_eCFP (BBa_K774006), B-M_mRFP (BBa_K774005) and M-B_mRFP (BBa_K774007) were generated using Biobrick^{tm} standard assembly. B-M (BBa_K774000) and M-B (BBa_K774001) were ligated upstream of eCFP (BBa_E0420) and mRFP (BBa_K081014), respectively, to create four nitric oxide, nitrate and nitrite biosensor constructs. These part numbers represent biopart chracterisation entries on the Registry of Standard Biological Parts (http://parts.igem.org/Main_Page). All constructs were cloned into the standard iGEM plasmid backbone, pSB1C3, for selection with 36 µg/ml chloramphenicol. DH5-alpha (Invitrogen, CA USA) chemically competent *E. coli* were utilised in all cloning steps and characterisation assays. Visit http://2012.igem.org/Team:NRP-UEA-Norwich for additional details.

Growth conditions

DH5-alpha (Invitrogen, CA USA) electrocompetent *E. coli* cells were routinely cultured in LB media at 37 ºC, 200 rpm. In several experiments 0-20 mM potassium nitrate was added to the culture media as indicated.

Flow Cytometry

E. coli transformed with either M-B_eCFP biosensor (BBa_K774006) or B-M_mRFP (BBa_K774005), were cultured from glycerol stocks in 5 ml LB media containing potassium nitrate (0 mM, 1 mM or 10 mM) at 37 ºC overnight (16 hours). The next day, 1 ml of cell culture were centrifuged (4000 rpm, 10 minutes at 4 ºC), washed in PBS and then fixed in 500 µl 4% paraformaldehyde (PFA) (vol:vol, PBS). Cells were washed again and and analysed via flow cytometry. BM-RFP transformed *E. coli* were analysed using a BD Accuri C6 flow cytometer, with Laser set at 488 nm / Filter FL2-585. MB-CFP samples were analysed using a BD FACSAria II Cell Sorter, with the laser set at 405 nm and detection settings at V/450/40-A. See flow cytometry supplementary data for additional details about the gating strategy.

Acknowledgements:

This project was developed by NRP-UEA-Norwich, a team of seven undergraduate students as part of the 2012 International Genetically Engineered Machine (iGEM) competition. The full project details can be found at http://2012.igem.org/Team:NRP-UEA-Norwich

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Madasamy, T., Pandiaraj, M., Balamurugan, M., Bhargava, K., Sethy, N. K., and Karunakaran, C. (2014) Copper, zinc superoxide dismutase and nitrate reductase co immobilized bienzymatic biosensor for the simultaneous determination of nitrite and nitrate, *Biosensors & bioelectronics*, **52**: 209-215.

Scott S.D., Joiner M.C., Marples B. (2002) Optimizing radiation-responsive gene promoters for radiogenetic cancer therapy, *Gene Therapy*, **9**: 1396-1402

Worthington J., Robson T., Scott S., Hirst, D. (2005) Evaluation of a synthetic CArG promoter for nitric oxide synthase gene therapy of cancer, *Gene Therapy*, **12**: 1417–1423

Supplementary construct sequences

PyeaR + CArG nitric oxide, nitrate & nitrite-sensing hybrid 'Bacterial-Mammalian' promoter

>BBa_K774000 Part-only sequence (208 bp)

```
ttcccatctataatcctccctgattcttcgctgatatggtgctaaaaagtaaccaataaatg
gtatttaaaatgcaaattatcaggcgtaccctgaaacgggatccaagcttcatatgccatat
aaggccatataaggccatataaggccatataaggccatataaggccatataaggccatataa
ggccatataaggccatataagg
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CArG + PyeaR nitric oxide, nitrate & nitrite-sensing hybrid 'Mammalian-Bacterial' promoter

>BBa_K774001 Part-only sequence (208 bp)

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ccatataaggccatataaggccatataaggccatataaggccatataaggccatataaggcc
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tccctgattcttcgctgatatggtgctaaaaagtaaccaataaatggtatttaaaatgcaaa
ttatcaggcgtaccctgaaacg
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Bacterial-Mammalian promoter with eCFP reporter: BBa_K216005 + CArG promoter sequence + BBa_E0420

>BBa_K774004 Part-only sequence (1094 bp)

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agtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagt
cacactggctcaccttcgggtgggcctttctgcgtttata

Bacterial-Mammalian promoter with RFP reporter: BBa_K216005 + CArG promoter sequence + BBa_K08101

>BBa_K774005 Part-only sequence (990 bp)
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tagtgtagatcgctactagagaaaaaaaaaccccgcccctgacagggcggggttttttt

Mammalian-Bacterial promoter with eCFP reporter: CArG promoter sequence + BBa_K216005 + BBa_E0420

>BBa_K774006 Part-only sequence (1094 bp)
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Mammalian-Bacterial promoter with RFP reporter: CArG promoter sequence + BBa_K216005 + BBa_K08101

>BBa_K774007 Part-only sequence (990 bp)

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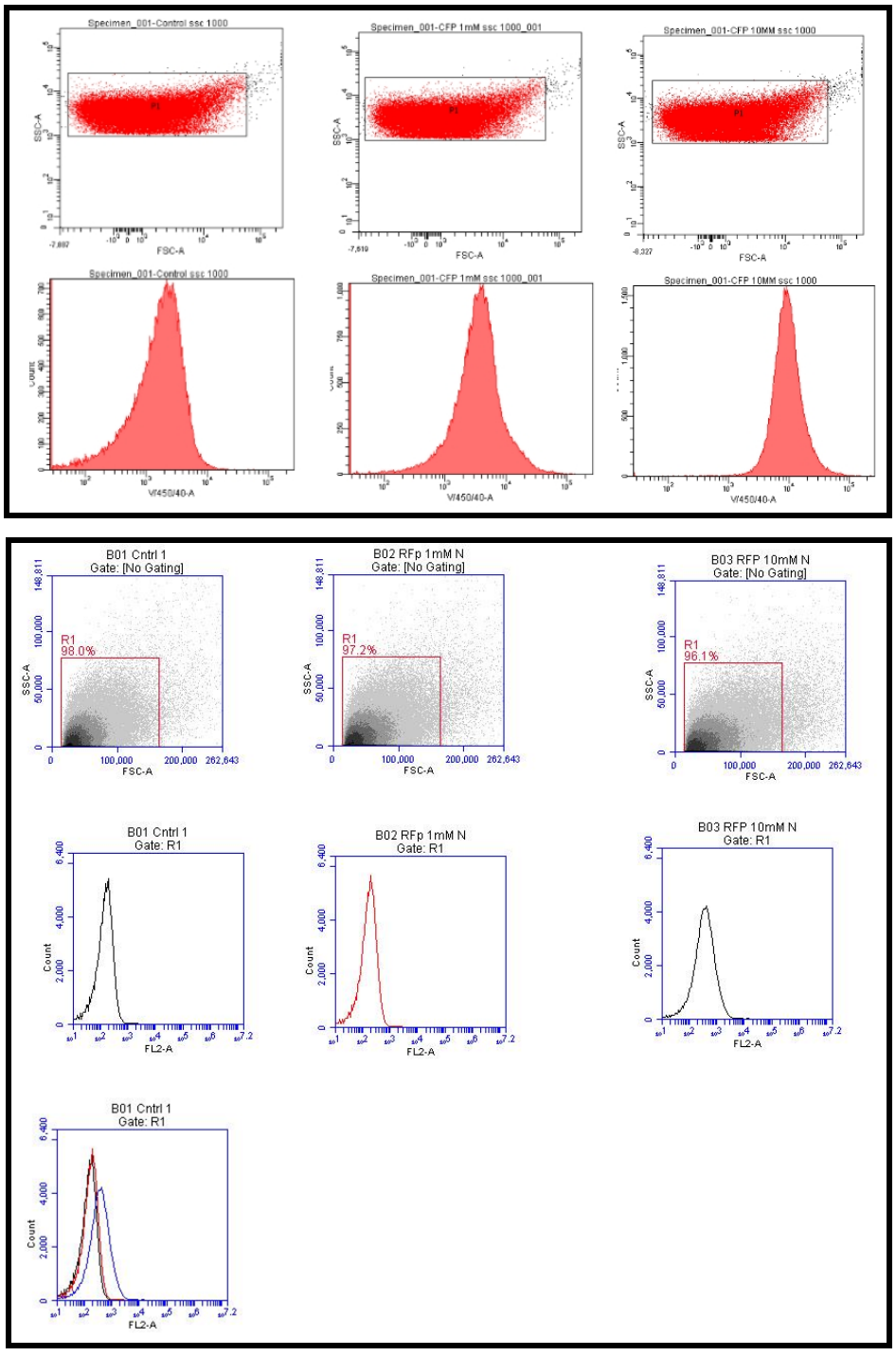
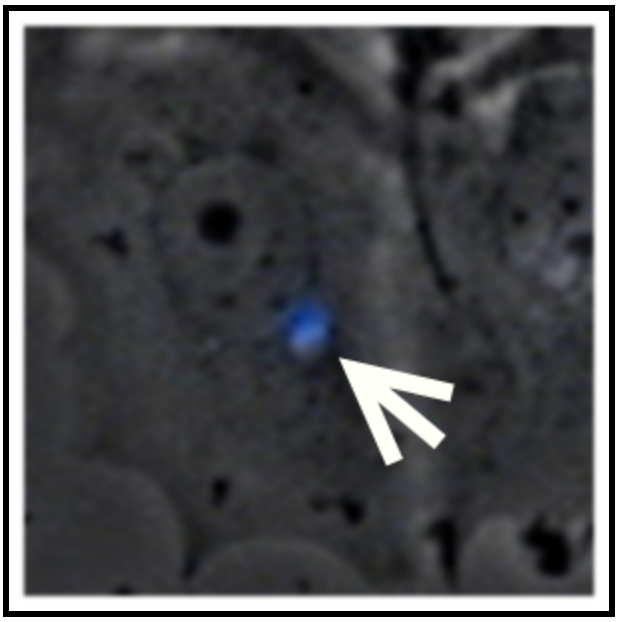
Supplementary flow cytometry data



[top] Gating strategy for M-B_eCFP flow cytometry data as shown in figure 4. Data captured with BD Aria II flow cytometer and software. **[bottom]** Gating strategy for B-M_mRFP flow cytometry data as shown in figure 4. Data captured with Accuri C6 flow cytometer and software.

Supplementary MCF7 transfection data



MCF-7, breast tumour cells, were transfected with M-B_eCFP biosensor (BBa_K774006) and, 24h post transfection, cells were imaged via Zeiss CCD2 inverted microscope to detect fluorescence. Image is representative of several cells, though the transfection efficiency was low (<30%) and requires optimisation.